

A History of Cellular Senescence
and Its Relation to Stem Cells in the Twentieth and Twenty-First Centuries

by

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ABSTRACT

Researchers in the twentieth and twenty-first centuries identify the study of the intrinsic and external factors that influence human aging as senescence. A commonly held belief in the year 2015 is that at least some kinds of cells can replicate over long periods or even indefinitely, thereby meaning the cell does not undergo senescence (also known as replicative senescence) and is considered immortal. This study aims to provide information to answer the following question: While some scientists claim they can indefinitely culture a stem cell line in vitro, what are the consequences of those culturing practices? An analysis of a cluster of articles from the Embryo Project Encyclopedia provides information to suggest possible solutions to some potential problems in cell culturing, recognition of benefits for existing or historical culturing practices, and identification of gaps in scientific knowledge that warrant further research. Recent research suggests that hESCs, and immortalized cell lines in general, do not escape the effects of senescence. While there exists a constant change in the practices of cell culturing, a large portion of scientists still rely on practices established before modern senescence research: research that seems to suggest that cultured hESCs, among other immortal cell lines, are not truly immortal.

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CHAPTER 1

INTRODUCTION

One objective of medical science is to prolong human life. In order to understand how science can extend a human life, it is helpful to understand what scientists commonly think are causes of human aging, and subsequently death. Researchers in the twentieth and twenty-first centuries identify the study of the intrinsic and external factors that influence human aging as senescence. Much of the scientific research of senescence focuses on the intrinsic and external factors of the cell in particular, as it is the basic unit of biological life forms. Therefore, cellular senescence (also known as cytoogerontology during the 1970s and early 80s), refers to factors that cause cells to age, particularly relating to the capacity of a cell to replicate (Campisi).

A commonly held belief in the year 2015 is that at least some kinds of cells can replicate over long periods or even indefinitely, thereby meaning the cell does not undergo senescence (also known as replicative senescence) and is considered immortal. A cell line that does not undergo senescence is called an immortalized cell line. Immortalized cell lines can include stem cells, as well as cancer cells. Immortalized cells that acquire replicative immortality through various mutations are called cancer cells, whereas immortalized cells that are a normal part of development are called stem cells. Scientists define stem cells based on the following criteria (NIH):

1. Stem cells can renew themselves for prolonged periods through cellular division (self-renewal)
2. Stem cells give rise to other cell types in the body (differentiation)

Stem cells are very alluring to researchers because of their potential applications in medicine to regenerate damaged tissues and slow the human aging process.

Scientists classify stem cells into two categories: adult and embryonic. The most sought after stem cells are human embryonic stem cells (hESC), or cells from the inner cell mass of a blastocyst of a human pre-implantation embryo. Adult stem cells are typically limited to becoming only a few cell types whereas embryonic stem cells from the blastocyst are capable of becoming all cell types, except for extra-embryonic structures such as the placenta. Extra-embryonic structures are derived from cells as part of the morula, which scientists consider “totipotent”, or capable of becoming all cell types. Researchers call hESCs “pluripotent”. Some scientists claim that hESCs have “basically indefinite proliferative capacity, [and] they lend themselves as practically inexhaustible resource” (Zachar).

While scientists claim they can indefinitely culture a stem cell line *in vitro*, what are the consequences of those culturing practices? To understand such a question, and the modern definition of senescence and how it applies to immortal cell lines, it is useful to examine what scientists thought about cells and aging in decades past. For the purposes of this research, the historical background will be limited within the timeframe of the late 19th century to the year 2015. A series of articles published in The Embryo Project Encyclopedia can serve as a tool to help understand what scientists thought about aging and cells in the past.

The Embryo Project Encyclopedia is a part of the Embryo Project at Arizona State University. The Embryo Project Encyclopedia houses a collection of research “related to the sciences of embryology, development, and reproductive medicine, their

legal, ethical and social contexts, and histories of those sciences and contexts” (The Embryo Project). Though the Embryo Project Encyclopedia is a collection of individual, independent articles, inquiring minds can examine clusters of topically related articles to provide insights into why scientists might think the way they do, or why they might conduct certain research. This method of examining clusters of articles seems particularly useful to determine how modern scientists could view research related to stem cells and aging. With the aid of Embryo Project Encyclopedia articles, this thesis aims to provide information to help answer the following question: What are some of the common techniques used to acquire stem cells and what happens to immortal cell lines (particularly embryonic stem cell lines) when scientists culture them indefinitely *in vitro*?

Chapters 2 through 5 highlight multiple methods of acquiring cell populations, including immortal cell populations. The research is grouped both chronologically and contextually, with related articles clustered together in the same chapter. Each chapter begins with a brief synopsis of related Embryo Project Encyclopedia articles, as well as relevant historical information. Embryo Project Encyclopedia article titles are followed by citations to the corresponding article. For convenience, the full articles are included in this thesis. More specifically, chapter 2 examines general senescence history and information, chapter 3 examines research on telomeres and telomerase, chapter 4 examines research on cloning, and chapter 5 examines a modern cell culturing practice.

Chapter 6 attempts to give answers to the driving question of the thesis. The answers can come in part from examining the work of scientists from the 19th to 21st centuries. Such an examination will provide recognition of possible benefits and detriments for existing or historical culturing practices, identification of potential gaps in

scientific knowledge that warrant further research, and the formulation of possible solutions to potential problems.

CHAPTER 2

IMMORTAL, MORTAL, OR BOTH? – GENERAL SENESENCE HISTORY AND INFORMATION

Historians often accredit August Weismann as one of the earliest to identify a possible link between cell biology and aging in the late nineteenth century. In search of an evolutionary explanation for aging, Weismann theorized that an organism's lifespan was determined by the number of generations a cell could produce. However, Weismann was unable to provide substantial empirical evidence to support his claims (Kirkwood).

Weismann was not the only researcher in the early 1900s to explore the idea of aging in cells. Charles Sedgewick Minot also studied senescence in his work with embryos and human anatomy. In his 1908 book, Minot defined senescence as a decrease of power of cell growth, which occurs every time a cell divides.

In 1912, the Nobel Laureate Alexis Carrel published an experiment that gave evidence to suggest that the power of cell growth does not diminish with time when cultivated *in vitro*, or outside of the body. Immortality, according to Carrel, was an intrinsic characteristic of all cells. With further research, Carrel claimed that "permanent life" *in vitro* was possible if researchers paid special attention to maintain glassware, and if correct nutrients were given to the cultured cells (Carrel, Jiang, Landecker). Though Carrel's hypothesis suggested immortality as an internal factor in cells, the causes of aging were external. Once Carrel published and propagated his research, Weismann and Minot's theories were quickly overshadowed. For many years, the majority of biologists considered Carrel's experiments and his interpretations as fact, despite repeated failures in attempts to reproduce Carrel's results.

As a result of Carrel's work, other scientists attempted to establish "immortal" cell lines, with hopes of achieving immortality for human cells. Among the eager scientists was George Gey, at John's Hopkins University. In 1951, Gey obtained and cultured cells from Henrietta Lacks, a woman suffering from cervical cancer. Scientists named the cells HeLa, after the patient's name. His team dubbed the resulting cell cultures immortal in the same way that Alexis Carrel's cell lines were immortal, as they were able to propagate the human cell line for over a year without any signs that the cells were subject to senescence (Scherer). HeLa cells continue to be widely used in research to develop vaccines and study human disease.

In 1961, a scientist named Leonard Hayflick observed a phenomenon in human cells he cultured in vitro. He noted that the cells ceased replication after forty to sixty divisions, or cell population doublings (Hayflick). Such an observation was contradictory to Carrel's commonly accepted notion of cell biology, and cell cultures such as HeLa. Hayflick experimented further in 1965, and concluded that Carrel's theory of senescence was incorrect, as it appeared that some cells were not capable of replicating indefinitely. He also hypothesized that there was a factor within cells that caused the cessation in replication. Hayflick rigorously researched and reported his findings, and soon Carrel's hypothesis of universal cell immortality was disregarded. As a result of Hayflick's experiments, Sir Macfarlane Burnet named the limited capacity for cellular division the Hayflick Limit in 1974.

During the 1950s, scientists were also researching the causes of cell death. In 1951, Alfred Glücksmann observed a phenomenon of cell death in embryos, which he claimed was necessary for tissue and organ formation. It was a phenomenon that John

Kerr and his colleagues at the University of Aberdeen, in Aberdeen, Scotland would call apoptosis in 1972.

To begin understanding the mechanics of life, scientists often asked why or how life ended. The following articles from the Embryo Project Encyclopedia are glimpses into how scientists in the 19th and 20th centuries viewed death, particularly in terms of the death and lifespan of cells.

Apoptosis and its Role in Embryonic Development

Bartlett, Zane. "Apoptosis and its Role in Embryonic Development". (Citation pending publication in the Embryo Project Encyclopedia)

Apoptosis is an essential mechanism in embryonic development and it is a form of programmed cell death, which occurs naturally in organisms. The process of apoptosis is different from cell necrosis, which usually occurs uncontrolled after infection or specific trauma. As rapid cell proliferation occurs during development, certain cells undergo apoptosis, which is necessary for many stages in development, including neural development, reduction in oocytes at birth, as well as the shaping of digits and vestigial organs in humans and other animals (Barres, Jacobson, Reynaud). Karl Vogt first observed the phenomenon of apoptosis in 1842 while studying the midwife toad (*Alytes obstetricans*) in Neuchâtel, Switzerland, but little effort occurred to understand it for about a century. Alfred Glücksmann researched the phenomenon in 1951 while working at the Strangeways Research Laboratory in Cambridge, England, and published a review, claiming it was important to embryological development. In 1972, John Kerr and his colleagues at the University of Aberdeen, in Aberdeen, Scotland, claimed that apoptosis

was the same process in body cells, called somatic cells, as it was during development of embryological cells. Kerr and his colleagues coined the term apoptosis, after the Greek words *apo* and *ptosis*, which translates to falling, or dropping off. Sydney Brenner, H. Robert Horvitz and John E. Sulston received the Nobel Prize in Physiology or Medicine in 2002 for their work on the genetic regulation of organ development and programmed cell death. As researchers continue to map out the processes of cell lineages before and after embryonic development, they may also learn new ways to reduce or promote cell death, which can be important in preventing diseases such as Alzheimer's or cancer.

The phenomenon of apoptosis was first observed by Karl Vogt in in Neuchâtel, Switzerland, in 1842, however Vogt did not call the phenomenon apoptosis. Vogt noticed in midwife toad embryos that cells in the notochord, a cartilaginous skeletal structure, disappeared and were replaced by cells of the vertebrae (Clark). Though Vogt documented the disappearance of cells during development, he did not focus his research on the disappearances (Vogt). Because of that lack of focus, the phenomenon was not given very much attention until 1885 when Walther Flemming, who worked at the University of Kiel, Kiel, Germany, used more advanced staining techniques of the cell nucleus to observe what he called chromatolysis, the distinct diminishing of nuclear material in dying cells (Flemming). Chromatolysis is part of the process of apoptosis, but Flemming's research was forgotten until the developmental biologist, Alfred Glücksmann, published a review on cell death literature in 1951 while working at the Strangeways Research Laboratory in Cambridge, England (Peter).

Glücksmann's embryological review on cell death influenced general acceptance of apoptosis as an essential part of embryonic development. He hypothesized that for an

organism to grow and develop, cell death must occur. At the time of Glücksmann's review, many scientists mistook dead cells observed under a microscope as metabolic byproducts of cells undergoing mitosis, or cellular replication. Glücksmann attempted to dispel these beliefs by presenting evidence from past embryological research that pointed to planned cell death as an aspect of normal development. Glücksmann's findings went largely unnoticed for a period of over twenty years. However, John Kerr, Andrew Wyllie, and Alastair Currie, pathologists working at the University of Aberdeen, Aberdeen, Scotland, referenced Glücksmann's review as motivation to develop their own research in 1972.

Kerr had first studied cell death in 1965 when he noticed atrophy, or shrinkage, in rat liver cells under an electron microscope. Kerr noticed that the shrinkage was distinct from necrosis due to trauma, which normally causes the cell to rupture and release its contents, so Kerr decided to study it further with Wyllie and Currie. They noticed common patterns involved in cell death, as recorded in previous experiments and reviews, including Glücksmann's work. Kerr and his team felt there should be a specific name for what they considered to be programmed cell death, a concept they understood from research conducted by Richard Lockshin and Carroll Williams at St. John's University, New York City, New York, in 1964. Kerr and his colleagues coined the term for the new form of programmed cell death, apoptosis. They claimed that cell death from apoptosis was not accidental, and that it followed the same pattern in developing cells as it did in developed cells. Kerr and his team's work unified years of embryological research conducted by scientists like Vogt, Flemming, and Glücksmann. Kerr and

colleagues brought the idea of apoptosis to greater scientific attention when they published their research in 1972.

After Kerr, Wyllie, and Currie's research, scientists understood that apoptosis followed specific steps, which scientists independently verified. First, cells undergoing apoptosis begin to shrink in size and lose physical connections with neighboring cells. Second, the chromatin, which is the combination of DNA and protein within the cell nucleus, condenses and enzymes begin to fragment the chromatin within the cell. Third, the cell membrane bulges irregularly, or blebs. Fourth, the nucleus collapses and breaks into fragments containing pieces of chromatin, while the cell continues to bleb. Fifth, the cell breaks into several smaller membrane bodies that contain various cellular fragments, called apoptotic bodies. Lastly, white blood cells (phagocytes) or neighboring cells engulf the apoptotic bodies and break them down. The organism suffers no major injury as a result.

After Kerr, Wyllie, and Currie's findings, scientists accepted apoptosis as a mechanism in cellular development and began to study its significance in development and disease. Some of those scientists were Sydney Brenner, Robert Horvitz, and John Sulston, who researched at Berkeley, California, Cambridge, Massachusetts, and Cambridge, England, respectively. They conducted much of their early research on the nematode, *Caenorhabditis elegans* (*C. elegans*) in the 1970s, and continued through the late twentieth century. Through diagrams of cell lineages and careful documentation, Brenner, Horvitz and Sulston predicted when cell death would occur, and they identified some of the genes involved in the regulation of cell death. Horvitz, in particular, noted in his research that *C. elegans* neurological development included a large amount of

apoptosis, with 105 of the 131 programmed cell deaths occurring in neural cells. Brenner, Horvitz, and Sulston received the Nobel Prize in Physiology or Medicine in 2002 for their work in genetic regulation of organ development and programmed cell death.

Research conducted since Brenner, Horvitz, and Sulston published their findings on *C. elegans* continues to reinforce the idea that programmed cell death through apoptosis is essential for development in animals. In 1993, scientists working with Horvitz found that a gene in mice was very similar to a gene in *C. elegans*, which coded for an enzyme that caused cell death during development. The research provided evidence to believe that the process involved with apoptosis in *C. elegans* is the same in mammals.

In 1997, Michael Jacobson and researchers at the MRC Lab of Molecular Biology in Cambridge, England, outlined the importance of cell death in animals in the article "Programmed cell death in animal Development". They claimed that the primary functions of apoptosis are to sculpt the organism by deleting unwanted structures, controlling cell numbers, and eliminating nonfunctional, harmful, abnormal, or misplaced cells. Absence of apoptosis can include malformations of digits, decreased neurological function, malformations of the heart, or even cancer. For example, soft tissue cells between the fingers and toes undergo apoptosis in order to separate the digits from each other during development. The proper formation of heart loops also relies on the process of apoptosis to correctly shape the heart.

Gary Wessel, in his article "The apoptotic oocyte", provides research about the role of apoptosis in human females. Human female oocytes undergo apoptosis during development and after birth. Scientists estimate there are seven to eight million oocytes

formed in the fetus, which are reduced to about 100,000 oocytes at birth, and then only a few hundred at the onset of menopause (Wessel).

Jacobson and his colleagues claimed that apoptosis occurs not only during embryonic development, but also after birth (Jacobson). In humans for example, brain cells undergo apoptosis prior to and following birth to eliminate excess brain cells and streamline nerve impulses. Apoptosis also occurs in some cells that the body identifies as cancerous in order to prevent the spread of the cancer and safely kill the cancerous cell. However, unregulated apoptosis can cause disorders, such as amyotrophic lateral sclerosis, which is a motor neuron disease, and Alzheimer's disease, which affects the brain, due to cell death in crucial memory areas of the brain (Honig).

Barres, Ben A., and Martin C. Raff. "Axonal control of oligodendrocyte development." *The Journal of cell biology* 147 (1999): 1123–1128.

Brenner, Sydney. "The genetics of *Caenorhabditis elegans*." *Genetics* 77 (1974): 71–94.

Clarke, Peter GH, and Stephanie Clarke. "Nineteenth century research on naturally occurring cell death and related phenomena." *Anatomy and embryology* 193 (1996): 81–99.

Flemming, Walther. *Über die bildung von richtungsfiguren in säugethiereiern beim untergang graafscher follikel*. 1885.

Glücksmann, Alfred. "Cell deaths in normal vertebrate ontogeny." *Biological Reviews* 26, no. 1 (1951): 59–86.

Honig, Lawrence S., and Roger N. Rosenberg. "Apoptosis and neurologic disease." *The American journal of medicine* 108 (2000): 317–330.

Kerr, John FR. "A histochemical study of hypertrophy and ischaemic injury of rat liver with special reference to changes in lysosomes." *The Journal of pathology and bacteriology* 90 (1965): 419–435.

Kerr, John F., Wyllie, Andrew H., and Currie, Alastair R. "Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics." *Br. J. Cancer*, 26 (1972): 239–257.

Jacobson, Michael D., Miguel Weil, and Martin C. Raff. "Programmed cell death in animal development." *Cell* 88 (1997): 347–354.

Lockshin, Richard A., and Carroll M. Williams. "Programmed cell death—II. Endocrine potentiation of the breakdown of the intersegmental muscles of silkmoths." *Journal of Insect Physiology* 10 (1964): 643–649.

Nobel Media. "The Nobel Prize in Physiology or Medicine 2002." Nobelprize.org. http://www.nobelprize.org/nobel_prizes/medicine/laureates/2002/ (Accessed March 15, 2014).

Peter, Marcus E., Armin E. Heufelder, and Michael O. Hengartner. "Advances in apoptosis research." *Proceedings of the National Academy of Sciences* 94 (1997): 12736–12737.

Reynaud, K., and M. A. Driancourt. "Oocyte attrition." *Molecular and cellular endocrinology* 163 (2000): 101–108.

Sulston, John, and Sydney Brenner. "The DNA of *Caenorhabditis elegans*." *Genetics* 77 (1974): 95–104.

Vogt, Karl Christoph. *Untersuchungen über die Entwicklungsgeschichte der Geburtshelferkröte (Alytes obstetricans)*. Jent & Gassmann, 1842.

Wessel, Gary M. "The apoptotic oocyte." *Molecular reproduction and development* 77: (2010).

Yuan, J., Shaham, S., Ledoux, S., Ellis, H. and Horvitz, Robert H. "The *C. elegans* cell death gene *ced-3* encodes a protein similar to mammalian interleukin-1 β -converting enzyme." *Cell* 75 (1993): 641–652.

Leonard Hayflick (1928-)

Bartlett, Zane, "Leonard Hayflick (1928-)". Embryo Project Encyclopedia (2014-07-20). ISSN: 1940-5030
<http://embryo.asu.edu/handle/10776/8042>.

During the twentieth and twenty-first centuries in the United States, Leonard Hayflick studied the processes by which cells age. In 1961 at the Wistar Institute in the US, Hayflick researched a phenomenon later called the Hayflick Limit, or the claim that normal human cells can only divide forty to sixty times before they cannot divide any further. Researchers later found that the cause of the Hayflick Limit is the shortening of telomeres, or portions of DNA at the ends of chromosomes that slowly degrade as cells replicate. Hayflick used his research on normal embryonic cells to develop a vaccine for polio, and from Hayflick's published directions, scientists developed vaccines for rubella, rabies, adenovirus, measles, chickenpox and shingles.

Hayflick was born on 20 May 1928 in Philadelphia, Pennsylvania, to Edna Hayflick and Nathan Hayflick. His father designed prosthetics for dental patients. When Hayflick was nine, one of his uncles bought him a chemistry set. Hayflick's parents allowed him to build a laboratory in the basement of their home, where he performed biology and chemistry experiments. Hayflick attended John Bartram High School in Philadelphia. During some of his first chemistry lectures, Hayflick corrected his teacher, who then allowed him to help in the chemical stockroom, where Hayflick acquired more chemicals for his studies.

Hayflick enrolled in the University of Pennsylvania in Philadelphia in January 1946 but postponed his studies for military service. After he served in the military for

eighteen months from 1946 to 1948, Hayflick received a four-year scholarship through the GI Bill, a US federal government initiative to help veterans return to civilian life after World War II. Hayflick studied microbiology at the University of Pennsylvania and graduated in 1951. After graduating, Hayflick took a job as a research assistant in bacteriology in 1951 with Merck, Sharp, and Dohme laboratories in North Wales, Pennsylvania. According to Hayflick, he felt that graduate school would have been too difficult for him until he met a friend who studied bacteria species in the genus *Mycoplasma*, which are bacteria often involved in pneumonia and other diseases. Those studies convinced him to return to the University of Pennsylvania to receive a Master's degree for his study of *Mycoplasma*. While at the University of Pennsylvania, Hayflick also met Ruth Heckler, whom he married and with whom he had five children.

As a Master's student, Hayflick's mentor Warren Stinebring sent him to the Wistar Institute to research an infection in a rat colony. Hayflick showed that the infection was due to *Mycoplasmas*. Hayflick received a Master's degree in medical microbiology in 1953. He won a fellowship to a PhD program, in which he continued his research. Hayflick received a PhD in medical microbiology and chemistry in 1956 from the University of Pennsylvania.

In 1956, Hayflick moved to the department of microbiology at the University of Texas in Galveston, Texas. He worked with cell culturist Charles Pomerat. With Pomerat, Hayflick learned techniques to produce cell cultures, or populations of cells living in controlled conditions. When the fellowship at the University of Texas expired, Hayflick applied for work elsewhere. In 1958, the new director of the Wistar Institute, Hilary Koprowski, offered Hayflick a job to culture cells for the other scientists at the institute.

Hayflick accepted the position and moved back to Philadelphia to work at the Wistar Institute.

Since the early 1900s, a predominant theory of cells in culture assumed that cells could multiply and grow forever. This assumption relied on the early twentieth century studies of Alexis Carrel, a physician in France, whose work with chick hearts had convinced many that all cells were immortal. If cells failed to grow in the lab, researchers attributed the failure to unknown nutritional conditions, to technicians incorrectly handling the cells, or to contaminations in the glassware.

At the Wistar Institute in 1958, Hayflick began to study whether or not viruses could cause cancers in humans. Hayflick cultured human cancer cells. He aimed to extract from them viruses, hypothesized to cause cancer, that he could isolate by introducing them into normal cells in culture. For this study, he needed to grow normal human cells not containing any viruses in a lab. Hayflick used fetal human lung cells because, compared to adult cells, they had a lower chance of containing viruses, and lung tissue allowed for the formation of fibroblasts, or structural cells in the body.

While he cultured twenty-five strains of fetal cells, Hayflick noticed that some of the cultures stopped dividing after about 50 cell population doublings. The cells were not dead, as they continued metabolizing, but the cells did not divide. Hayflick published an article in 1961 detailing the results of these cell cultures titled "The serial cultivation of human diploid cell strains."

In 1965, Hayflick published "The limited *in vitro* lifetime of human diploid cell strains." In this article, Hayflick notes that adult cells divide fewer times than do fetal cells, and that the cause of this limited cell division isn't caused by a virus, bacteria, or

accounted for by error. Hayflick hypothesized in his article that cellular aging could result from one or many targets or substances of cellular damage and that the cells could hold intrinsic factors that determine aging.

At the time of the 1965 experiment, Hayflick had established a cell culture, the WI-38 strain, with fetal cells that Hayflick had acquired from Sweden. Hayflick shipped these WI-38 cultures to many other scientists, including Stanley Plotkin at the Wistar Institute, where the cells were later used for rubella vaccines. WI-38 cells began to be in high demand during the 1960s.

Despite Hayflick's recognition for the WI-38 cultures, he remained in the same associate position at the Wistar Institute, which was partially funded by the US National Institutes of Health (NIH) headquartered in Bethesda, Maryland. Koprowski had also created a guaranteed deal with SmithKline Beckman in the US and the Burroughs Wellcome Company in the UK to purchase WI-38 cultures, as well as the cell-culture technology that Hayflick created to produce a polio vaccine with the live, attenuated virus. Hayflick later said that he was unhappy with Koprowski's decision to sell the cells for profit.

In 1968, Hayflick accepted a position as a professor of medical microbiology at Stanford University in Stanford, California. In January of 1968, Hayflick met with Koprowski and representatives of the NIH and American Type Culture Collection (ATCC), based in Rockville, Maryland, to discuss what would occur with the remaining containers of WI-38 cells, as Hayflick would soon be changing jobs. Those present at the meeting agreed to allow Hayflick ten containers of the WI-38 cells, with ten of the remaining WI-38 cultures placed at the Wistar Institute and the rest in the care of the

ATCC as property of the NIH. However, the NIH claimed that it owned the cells and that only it could distribute and sell the cells through the ATCC. Hayflick claimed to have title to own the cells, and that the estate of the fetus also had an interest in the cells.

Hayflick reported that he felt a sense of injustice, and he went to the Wistar Institute and took all of the WI-38 samples before leaving for California. He sent these cultures to scientists for the same price that the ATCC would have charged researchers for shipping the cells, and he saved that money into a special account until a further decision on ownership of the cells could be determined. In the spring of 1975, Hayflick brought the issue of ownership to the NIH in hopes of settling who owned the cells. Later that year, NIH accountants and some US Department of Health, Education, and Welfare (DHEW) employees investigated Hayflick. Under the 1966 US Freedom of Information Act, the NIH gave the accountant's report to journalists who published it in March of 1976. The report claimed that Hayflick had stolen and sold property of the United States Government, and that he had mishandled the cells.

Hayflick sued the NIH in 1975 to prevent the release of the report and to gain the title to the WI-38 cells, arguing that the report violated the 1974 US Privacy Act. A California court denied a motion for preliminary injunction of the reports, and Hayflick followed with an appeal in 1978, after the reports were released. The US Ninth District Court of Appeals affirmed the previous decision, and they dismissed the appeal. With tension growing from the release of the reports, Hayflick left his job at Stanford in what he called a protest to the behavior of the university leadership, and the NIH confiscated his WI-38 cell cultures while Hayflick attended a conference. The issue as to who had title to the WI-38 cells remained unresolved.

In late 1976, Hayflick accepted a position as a senior researcher at Children's Hospital Medical Center in Oakland, California. In 1977, the NIH accepted Hayflick's application for a grant for some money as well as for WI-38 cells. Hayflick did not receive that grant or any WI-38 cells until January of 1981. Hayflick continued to ask for the title of the WI-38 cells, and an out-of court settlement was offered by the US Justice Department, the US Food and Drug Administration (FDA), NIH, and the DHEW in the summer of 1981. After some modifications, Hayflick settled with the FDA, the NIH, and the DHEW, and he received title to six of the original containers of WI-38 cells. He also received the ability to use the money, plus interest, from an escrow account. He had opened the account at the start of the suit and had deposited in it the money he'd received from distributing the WI-38 cells while at Stanford. Hayflick later said that all of that money paid litigation fees. After the settlement, eighty-five scientists filed a letter in favor of Hayflick in the journal, *Science*. Hayflick and others claimed that it was through his efforts in asking for the title to the WI-38 cells that helped US policy to evolve, and to allow research institutions and scientists to receive title to new life forms, even when the research is federally funded.

In his 1974 work, *Intrinsic Mutagenesis: a Genetic Approach to Ageing*, the Nobel Prize Laureate Frank MacFarlane Burnet called Hayflick's account of cellular aging The Hayflick Limit. In 1981, Hayflick became director of the Center for Gerontological Studies in Gainesville, Florida, as well as professor at the University of Florida in Gainesville. In 1982, Hayflick became president of the Gerontological Society of America in Washington D.C.

In 1988, Hayflick became a professor at the University of California in San Francisco, California. In 1994, he published a book titled *How and Why We Age*. Hayflick received greater than twenty-five awards, authored greater than two-hundred and fifty scientific works, and has participated in various editing, government, and medical boards and councils. As of 2014, Hayflick serves on various scientific committees and is writing an autobiography while living in California.

Boffey, Philip M. "The Fall and Rise of Leonard Hayflick, Biologist Whose Fight With U.S. Seems Over." *New York Times*, January 19, 1982. <http://www.nytimes.com/1982/01/19/science/the-falland-rise-of-leonard-hayflick-biologist-whose-fight-with-us.html> (Accessed June 2, 2014).

Burnet, Macfarlane. *Intrinsic Mutagenesis*. Houten: Springer, 1974.
Carrel, Alexis. "On the permanent life of tissues outside of the organism." *The Journal of Experimental Medicine* 15 (1912): 516–28. <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2124948/pdf/516.pdf> (Accessed June 2, 2014).

Daar, Abdallah, and Heather L. Greenwood. "A proposed definition of regenerative medicine." *Journal of Tissue Engineering and Regenerative Medicine* 1 (2007): 179–84.

Cell Associates Inc v. National Institute of Health Department of Health. 579 F.2d 1155 (App. Div. 1978). <https://law.resource.org/pub/us/case/reporter/F2/579/579.F2d.1155.76-1978.html> (Accessed June 11, 2014).

Hayflick, Leonard, and Paul S. Moorhead. "The serial cultivation of human diploid cell strains." *Experimental Cell Research* 25 (1961): 585–621.

Hayflick, Leonard. "The limited in vitro lifetime of human diploid cell strains." *Experimental Cell Research* 37 (1965): 614–36.

Hayflick, Leonard. "Hayflick's Reply (To Wade)." *Science* 202 (1978): 128–135.

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

Hayflick, Leonard. "A Novel Technique for Transforming the Theft of Mortal Human Cells into Praiseworthy Federal Policy." *Experimental Gerontology* 33: 191–207.

- Hayflick, Leonard. "The future of ageing." *Nature* 408 (2000): 267–269.
- Hayflick, Leonard. Web of Stories. July, 2011. <http://www.webofstories.com/play/leonard.hayflick/1> (Accessed September 7, 2013).
- Hayflick, Leonard. Email Communications with *Embryo Project Encyclopedia* Editors. June– July, 2014.
- Jiang, Lijing. "Degeneration in Miniature History of Cell Death and Aging Research in the Twentieth Century" PhD diss., Arizona State University, 2013. <http://hdl.handle.net/2286/R.A.114558> (Accessed June 18, 2014).
- "Life's Limit." *Radiolab*. June 14, 2007. <http://www.radiolab.org/story/91563-lifes-limit/> (Accessed July 14, 2014).
- Shay, Jerry W., and Woodring E. Wright. "Hayflick, his limit, and cellular ageing." *Nature Reviews Molecular Biology* 1 (2000): 72–76.
- Stevenson, Robert. "Dr. Leonard Hayflick's Oral History." <http://history.nih.gov/archives/downloads/hayflicktranscript.pdf> (Accessed June 11, 2014).
- Strehler, Bernard L., et al. "Hayflick-NIH settlement." *Science* 215 (1982): 240–242.
- U.S. Freedom of Information Act of 1966. Public Law 89-487. <http://www.gpo.gov/fdsys/pkg/STATUTE-80/pdf/STATUTE-80-Pg250.pdf> (Accessed July 14, 2014).
- U.S. Privacy Act of 1974, 5 U.S.C. Section 522a (1974). <http://www.gpo.gov/fdsys/pkg/STATUTE-88/pdf/STATUTE-88-Pg1896.pdf> (Accessed July 14, 2014).
- Wade, Nicholas. "Hayflick's Tragedy: The Rise and Fall of a Human Cell Line." *Science* 192 (1976): 125–127.
- Wadman, Meredith. "Medical Research: Cell division." *Nature* 498 (2013): 422–6.
- Watts, Geoff. "Profile: Leonard Hayflick and the Limits of Ageing." *Lancet* 377 (2011): 2075.

The Hayflick Limit

Bartlett, Zane, "The Hayflick Limit". Embryo Project Encyclopedia (2014-11-14). ISSN: 1940-5030
<http://embryo.asu.edu/handle/10776/8237>.

The Hayflick Limit is a concept that helps to explain the mechanisms behind cellular aging. The concept states that a normal human cell can only replicate and divide forty to sixty times before it cannot divide anymore, and will break down by programmed cell death or apoptosis. The concept of the Hayflick Limit revised Alexis Carrel's earlier theory, which stated that cells can replicate themselves infinitely. Leonard Hayflick developed the concept while at the Wistar Institute in Philadelphia, Pennsylvania, in 1965. In his 1974 book *Intrinsic Mutagenesis*, Frank Macfarlane Burnet named the concept after Hayflick. The concept of the Hayflick Limit helped scientists study the effects of cellular aging on human populations from embryonic development to death, including the discovery of the effects of shortening repetitive sequences of DNA, called telomeres, on the ends of chromosomes. Elizabeth Blackburn, Jack Szostak and Carol Greider received the Nobel Prize in Physiology or Medicine in 2009 for their work on genetic structures related to the Hayflick Limit.

Carrel, a surgeon in the early twentieth century France working on cultures of chick heart tissue, argued that cells can infinitely replicate. Carrel claimed that he had been able to have those heart cells replicate in culture for greater than twenty years. His experiments on chick heart tissue supported the theory of infinite replication. Scientists tried to replicate Carrel's work many times, but these repeated experiments never confirmed Carrel's findings.

Hayflick worked for the Wistar Institute in 1961 where he observed that human cells do not replicate infinitely. Hayflick and Paul Moorhead described the phenomenon in a paper titled "The serial cultivation of human diploid cell strains." Hayflick's job at the Wistar Institute was to provide cell cultures to scientists who conducted experiments at the Institute, but Hayflick pursued his own research on the effects of viruses in cells. In 1965, Hayflick further detailed the concept of the Hayflick Limit in cells in a paper titled "The limited *in vitro* lifetime of human diploid cell strains."

In that article, Hayflick concluded that a cell could complete mitosis, or cellular duplication and division, only forty to sixty times before undergoing apoptosis and subsequent death. The conclusion held for many cell types, whether they were adult cells or fetal cells. Hayflick hypothesized that the limited replicative capability of the cell related to aging in cells and, consequently, to human aging.

The publication of Hayflick's experiments disconfirmed Carrel's theory about indefinite cellular replication. Some, such as Harry Rubin at the University of California at Berkeley in Berkeley, California, argued in the 1990s that the Hayflick Limit pertained only to damaged cells. Rubin suggested that cellular damage could result from the cells being in an environment that differed from their original environment in the body, or when researchers subjected the cells to laboratory practices.

Regardless of the criticism, other scientists used Hayflick's theory in support of further studies about cellular aging, especially with research in telomeres, which are repetitive sequences of DNA at the ends of chromosomes. Telomeres protect the chromosome from folding in on itself, and they decrease mutations in the DNA. In 1973, Alexey Olovnikov, in Russia, applied Hayflick's theories of cell death to his studies of

the ends of chromosomes that did not replicate themselves during mitosis. He said that the process of cell division ends once the cell cannot replicate the ends of their chromosomes.

Although Olovnikov applied Hayflick's theory to his experiments, Olovnikov did not name Hayflick's theory. One year later in 1974, Burnet coined the term Hayflick Limit in his work, *Intrinsic Mutagenesis*. Burnet's work focused on the claim that age was intrinsic to the cells in each species and that they followed the Hayflick Limit, thus establishing a programmed age in which an organism would die. Elizabeth H. Blackburn at the University of California San Francisco in San Francisco, California, and Jack W. Szostak at Harvard Medical School in Boston, Massachusetts, also applied Hayflick's theory of cellular aging to their research on the structures of telomeres in 1982, when they cloned and isolated telomeres. In 1989, Greider, and Blackburn further developed the theory of cellular aging to discover the enzyme that replicates telomeres, called telomerase. Greider and Blackburn found that the presence of telomerase helps cells escape programmed cell death.

With theories about the biological mechanisms behind aging, scientists expected that they could create a cure for aging. Hayflick helped found the National Institute on Aging in Bethesda, Maryland, in 1974, a branch of the National Institutes of Health in the United States. In 1982, Hayflick also became the president of the Gerontological Society of America, founded in 1945 in New York, New York. Hayflick role helped to spread the theory of the Hayflick Limit and to further counter the theory of cellular immortality as established by Carrel.

In 2009, Blackburn and Szostak received the Nobel Prize in Physiology or Medicine for their work on telomerase, in which the Hayflick Limit played an essential role.

Burnet, Frank Macfarlane. *Intrinsic Mutagenesis*. Houten: Springer Netherlands, 1974.

Carrel, Alexis. "On the permanent life of tissues outside the organism." *Journal of Experimental Medicine* 15 (1912): 516–28. <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2124948/pdf/516.pdf> (Accessed November 11, 2014).

Greider, Carol W., and Elizabeth H. Blackburn. "A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis." *Nature* 337 (1989): 331–7.

Hayflick, Leonard. "C.V." Aging Intervention Foundation. <http://www.agelessanimals.org/hayflickcv.htm> (Accessed September 7, 2013).

Hayflick, Leonard. "The limited in vitro lifetime of human diploid cell strains." *Experimental Cell Research* 37 (1965): 614–36.

Hayflick, Leonard, and Paul S. Moorhead. "The serial cultivation of human diploid cell strains." *Experimental Cell Research* 25 (1961): 585–621.

Hayflick, Leonard. Web of Stories. July, 2011. <http://www.webofstories.com/play/leonard.hayflick/1> (Accessed October 5, 2013).

Jiang, Lijing. "Degeneration in Miniature History of Cell Death and Aging Research in the Twentieth Century." PhD diss., Arizona State University, 2013. <http://hdl.handle.net/2286/R.A.114558> (Accessed May 21, 2014).

Nobel Prize. "The Nobel Prize in Physiology or Medicine 2009" Nobel Media. http://www.nobelprize.org/nobel_prizes/medicine/laureates/2009/ (Accessed October 5, 2013).

Olovnikov, Alexey M. "A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon." *Journal of Theoretical Biology* 41 (1973): 181–90.

Rubin, Harry. "Telomerase and cellular lifespan: ending the debate?" *Nature Biotechnology* 16 (1998): 396–7.

Shay, Jerry W., and Woodring E. Wright "Hayflick, his limit, and cellular ageing". *Nature Reviews Molecular Cell Biology* 1 (2000): 72–6.

Szostak, Jack W. and Elizabeth H. Blackburn. "Cloning yeast telomeres on linear plasmid vectors." *Cell* 29 (1982): 245–55.

Leonard Hayflick’s 1964 Experiment, “The Limited In Vitro Lifetime of Human Diploid Cell Strains”

Bartlett, Zane. “Leonard Hayflick’s 1964 Experiment, ‘The limited In Vitro Lifetime of Human Diploid Cell Strains’”. (Citation pending publication in the Embryo Project Encyclopedia)

“The Limited In Vitro Lifetime of Human Diploid Cell Strains” reports on a scientific experiment published in *Experimental Cell Research* on 4 May 1964. The experiment details information on the cell’s limited ability for cellular division. Leonard Hayflick performed the experiment with WI-38 fetal lung cells, named after the Wistar Institute, in Philadelphia, Pennsylvania, where Hayflick worked. The Nobel Prize Laureate, Sir Frank MacFarlane Burnet, later called the limit in capacity for cellular division the Hayflick Limit in his work, *Intrinsic Mutagenesis: a Genetic Approach to Ageing*, published in 1974 (Web of Stories, Burnet). In the experiment, Hayflick refutes Alexis Carrel’s hypothesis that cells could be transplanted and multiplied indefinitely (by a process called sub-culturing) from a single parent cell line. The experiment helped scientists gain greater insight into cellular and human aging, as well as the implications of this form of cellular aging for understanding embryonic stem cells.

Leonard Hayflick is the sole experimenter of the “Limited In Vitro Lifetime” experiment. Hayflick received a PhD in Medical Microbiology and Chemistry in 1956

from the University of Pennsylvania in Philadelphia, Pennsylvania, (Hayflick) and worked at the Wistar Institute in 1964, where he conducted the experiment. His primary role at the Wistar Institute was to provide cell cultures to the scientists there who were performing research in various areas of microbiology (Jiang). While providing cell cultures to other researchers, Hayflick worked on his own research on viruses and how they can cause cancer in human cells.

Hayflick's motivation for his 1964 experiment arose in response to phenomena observed in a previous experiment, "The serial cultivation of human diploid cell strains," conducted with Paul Moorhead in 1961 (Hayflick). They were trying to find better and more effective ways to cultivate cell populations for future experiments. In the process of culturing twenty-five strains of fetal lung cells for his experiment with Moorhead, Hayflick noticed that some of the cultures had completely stopped dividing, although they were still metabolizing. Hayflick found it odd that the cells stopped dividing in older cell populations, despite the fact that the same technician conducted the procedure using the same culture media and the same glassware (Web of Stories). Hayflick's observations contradicted Carrel's theory that cells would continue to replicate indefinitely. At the time of the experiment's publication in 1961, Hayflick hypothesized that cells had intrinsic factors that determined aging. However, he and Moorhead were not able to rule out the possibility that viruses interfered with the functioning of the cells and caused the cells to stop dividing.

In his 1964 experiment, Hayflick decided to further examine the mechanisms that could cause the fetal cells to stop dividing. He created subcultures from fetal lung cell lines and adult lung tissue and named the cultures after the Wistar Institute (Hayflick) and

by number order. Hayflick cultured fetal lung tissue received from male and female fetuses and he named the male derived cells WI-26 and the female derived cells WI-38 and WI-44. He extracted the fetal lung tissue from aborted fetuses at about three months' gestation (Hayflick), and he generated the adult strains from lung tissue of dead adults, and froze both of them in a container with liquid nitrogen in order to preserve them. Each subculture split in a two-to-one ratio, which means that each cell in the samples divided once, producing two more cells.

Hayflick's results pointed to the fact that in a two-to-one split ratio, the average number of times a fetal cell could divide was between forty to sixty times. In adult cells, the average number of times a cell would multiply was twenty after cultivation. That data suggested that numerous cell duplications had already occurred in adult cells, and that the number of duplications in the lab setting were less in comparison to the fetal cell strains. In the experiment, he called the phase when the cell ceases to divide phase III, with phase I and II being periods where a cell rapidly proliferates (Hayflick).

Hayflick was curious to see whether fifty was the average number of divisions for every fetal cell within the same population or if it was just a randomly occurring number. To test his hypothesis, Hayflick randomly selected and cloned three samples of the fetal cell line, WI-38, and recorded when each population stopped dividing. The resulting range for each clone was from fifty to fifty-four. That data lead Hayflick to believe the amount of times a cell can double is determined by an internal factor within the cell, which Hayflick thought was some kind of cellular memory (Web of stories). From the results, Hayflick concluded that cellular aging is based on the amount of times a cell doubles (Hayflick).

To test whether or not there were cell-signaling factors involved in this cessation of duplication, as opposed to internal factors, Hayflick paired adult cells and fetal cells together in culture. The results did not vary from previous data. The younger cells were unaffected by any factor involved in the older cells and continued to divide an average of about fifty times (Hayflick). Hayflick also found that change in a cell's duplication potential was not caused by freezing the cell samples in liquid nitrogen because the unfrozen samples obtained similar results. Researchers who used WI-38 strains in their experiments also replicated the results of Hayflick's experiment and found that the cells stopped dividing at about fifty replications.

Hayflick's conclusion drawn from his experimental results presented counterargument against Carrel's theory that cellular replication is infinite. The data supported the idea that there is a cellular theory of aging, refuting Carrel's hypothesis based on experiments with chick heart tissue in 1912 (Carrel). Before Hayflick's discovery, scientists attributed the failure in infinite replication of human cells to incorrect laboratory practices or interference of a virus or other microorganism (web of stories).

As a cause for cellular aging, Hayflick hypothesized that it can be a result of one or many targets of cellular damage (Hayflick). In particular, he claimed that this damage is likely associated with chromosomes. He called that damage hits, or errors in DNA replication that accumulate over time, which cause a more rapid onset of phase III in a cell and results in cellular aging. The hits on the DNA were associated with telomeres in the 1970s.

Hayflick's work generated considerable interest due to his extensive data as well as the data from other researchers who replicated his experiments (Web of Stories). Cellular aging, however, was not confirmed until its association with telomeres in DNA replication in the late 1970s, when Alexey Olovnikov published his work on telomeres (Olovnikov). Elizabeth Blackburn, Carol Greider, and Jack Szostak later received the Nobel Prize in Physiology or Medicine in 2009 for their work telomeres, or repetitive sequences of DNA on the ends of chromosomes, and telomerase, the enzyme responsible for repairing telomeres. The scientists related telomeres and telomerase to cellular aging, as inspired by the Hayflick Limit.

Hayflick's experiment, published in "The Limited In Vitro Lifetime of Human Diploid Cell Strains," contributed to the idea that cells undergo an aging process that is influenced by telomeres. The Hayflick Limit is applicable to adult cells as well as cells found in human embryos. Nature claims that Hayflick's experiment is one of the most cited articles in all of biology (Wadman). As of 2013, the WI-38 fetal cell cultures established in Hayflick's experiment are still used by scientists in biological research.

Burnet, Sir Macfarlane. *Intrinsic Mutagenesis*. Houten: Springer Netherlands, 1974.

Carrel, Alexis. "On the permanent life of tissues outside the organism" *Journal of Experimental Medicine* 15 (1912): 516–28.

Greider, Carol W., and Elizabeth H. Blackburn "A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis." *Nature* 337 (1989): 331–7.

Hayflick, Leonard. "C.V." *Aging Intervention Foundation*. <http://www.agelessanimals.org/hayflickcv.htm> (Accessed September 21, 2013).

Hayflick, Leonard. "The limited in vitro lifetime of human diploid cell strains." *Experimental Cell Research* 37 (1965):614–36.

Hayflick, Leonard, and Paul S. Moorhead. "The serial cultivation of human diploid cell strains." *Experimental Cell Research* 25 (1961): 585–621.

Hayflick, Leonard. *Web of Stories*. July, 2011
<http://www.webofstories.com/play/leonard.hayflick/1> (Accessed September 21, 2013).

Jiang, Lijing. "Degeneration in Miniature: History of Cell Death and Aging Research in the Twentieth Century." PhD diss., Arizona State University, 2013.

Olovnikov, Alexey M. "A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon". *Journal of Theoretical Biology* 41 (1973): 181–90.

Szostak, Jack W., and Elizabeth H. Blackburn. "Cloning yeast telomeres on linear plasmid vectors". *Cell* 29 (1982): 245–55.

Wadman, Meredith "Medical Research: Cell division." *Nature* 498 (2013): 422–6.

CHAPTER 3

TELOMERES/TELOMERASE AND SENESCENCE

Once scientists began to understand the different ways that cells experience death, or that some cells have a limited lifespan, there were questions as to what kept cells alive. In the 1970s and 1980s, Elizabeth Blackburn, Jack Szostak, and Carol Greider validated Hayflick's hypothesis that cells contained intrinsic factors of aging. The scientist Alexey Olovnikov also hypothesized about intrinsic factors in aging, though Blackburn, Szostak and Greider were more recognized for their research in the nematode, *Caenorhabditis elegans*. They found that telomeres, or protective sequences of DNA on the end of chromosomes, shorten each time the cell replicates its DNA.

Telomeres provide protection to cellular DNA by preventing DNA tangling, and some DNA mutations. Over time, telomeres reach what researchers call a critical length, where a cell is unable to replicate the DNA anymore, and the cell cannot repair serious damage to the DNA. Blackburn and others hypothesized that telomeres affect cellular aging, and in 1989, Vicki Lundblad and Szostak validated the scientists' hypotheses with research on yeast (Lundblad, Nobel). The critical length of telomeres also was found to correspond directly with the Hayflick limit.

Blackburn, Szostak and Greider's collaborations eventually led to the discovery and isolation of the enzyme that repairs telomeres, called telomerase. Telomerase repairs the ends of the chromosome, the telomere, during DNA replication as the cell prepares for division. The importance of telomerase is evident in an examination of immortalized cell lines such as hESCs.

HESCs and other immortalized cell lines such as HeLa cells are able to renew themselves and replicate in part because they produce the enzyme telomerase. As long as there is enough active telomerase within a cell, the cell appears to avoid the effects of the Hayflick Limit. In non-immortal cells such as those studied by Hayflick, the telomeres of chromosomes decrease little by little with each cellular division because they do not produce telomerase.

After the discoveries of telomeres and telomerase, researchers had a method of quantifying senescence. With time, scientists found telomerase expression in many human cells. In 1992, it was found that human sperm cell telomere lengths do not decrease with age. Some studies demonstrated that telomere length was a heritable characteristic passed from parent to offspring, as was the case for sperm telomere length.

The following Embryo Project articles give extended information on telomere and telomerase research. It is appropriate to start by examining the contributions of Carol Greider, who is one of the leading scientists involved in telomere and telomerase research.

Carol Widney Greider (1961-)

Bartlett, Zane, "Carol Widney Greider (1961-)". Embryo Project Encyclopedia (2015-01-26). ISSN: 1940-5030 <http://embryo.asu.edu/handle/10776/8283>.

Carol Widney Greider studied telomeres and telomerase in the US at the turn of the twenty-first century. She worked primarily at the University of California, Berkeley in Berkeley, California. She received the Nobel Prize in Physiology or Medicine in 2009, along with Elizabeth Blackburn and Jack Szostak, for their research on telomeres and

telomerase. Telomeres are repetitive sequences of DNA at the ends of chromosomes that protect chromosomes from tangling, and they provide some protection from mutations. Greider also studied telomerase, an enzyme that repairs telomeres. Without telomeres, chromosomes are subject to mutations that can lead to cell death, and without telomerase, cells might not reproduce fast enough during embryonic development. Greider's research on telomeres helped scientists explain how chromosomes function within cells.

Carol Greider was born 15 April 1961 in San Diego, California, to Jean Foley Greider and Kenneth Greider. Greider also had a brother named Mark, one year older than she. Greider's parents both received their PhDs from the University of California, Berkeley, her mother in botany, and her father in physics. During the early 1960s, the Greider family moved often as Greider's parents accepted different academic teaching positions. In 1965, the family moved to Davis, California. In 1967, when Greider was in first grade, her mother died. Greider later reported that her mother's death played a role in learning how to take responsibility to do things on her own. In her statement for the Nobel Prize, Greider also notes that the death of her mother contributed to difficulties Greider had in school, especially after learning she had dyslexia.

In 1971, Greider's father took a sabbatical from his position at the University of California, Davis, and moved to Heidelberg, Germany, with the family. As Greider states in her Nobel Prize biography, the foreign environment enabled her to make new friends and to recognize and enjoy differences between people. In 1972, the family left Germany and returned to Davis, where Greider attended junior high schools. Greider reported discovering an aptitude for biology in her junior year of high school. In 1979, she started

an undergraduate program to study marine ecology at the University of California, Santa Barbara in Santa Barbara, California.

In 1982, Greider participated in a study abroad program at the University of Göttingen, in Göttingen, Germany, where she worked at the Max Planck Institute for Biophysical Chemistry. During her second semester, Greider attended a course on chromosomes taught by Ulrich Grossbach. Greider later said that she was entranced by the work being done on chromosomes in *Chironomus* flies. She finished her work on microtubules and switched to chromosomal work with Grossbac and others. Greider returned to Santa Barbara for her senior year, and she received her undergraduate degree in Biology in 1983.

Greider then applied to graduate schools, and the University of California, Berkeley accepted her as a graduate student in molecular biology. Greider met Elizabeth Blackburn, a faculty member in the Department of Molecular Biology. After hearing about Blackburn's work on telomeres Greider planned to work with Blackburn.

As a first-year graduate student, Greider worked in different labs and did not immediately work with Blackburn. She first worked with Richard Calendar, who studied the interactions between bacteriophages, or viruses that infect bacteria, to learn more about the properties of DNA. Next, Greider worked with Blackburn. Greider cloned telomeres to try to determine why those chromosomal ends did not shorten when the cell replicated. Her work was based on research conducted by Blackburn and Jack Szostak, who in 1982 were the first to identify telomeres in *Tetrahymena*, a ciliated single-celled protozoan. For that research, Szostak worked at Harvard Medical School in Boston, Massachusetts. In 1984, Greider joined Blackburn's lab to work on a telomere elongation

project. Greider began work on making extracts of the enzymes from *Tetrahymena*, the same organism that Blackburn and Szostak had used to identify telomeres. Greider made extracts of the enzymes from the cells in *Tetrahymenato* identify the enzyme responsible for telomere elongation.

At the end of December 1984, Greider and Blackburn identified telomere terminal transferase as the enzyme that replicated telomeres in *Tetrahymena*, and in 1985 they published their results. Shortly after publishing their article, Greider and Blackburn shortened the name of the enzyme to telomerase, after Greider received feedback from a fellow student that the name was too long. In 1987, Greider and Blackburn began studying how genes produce the telomerase enzyme. After conducting experiments, Greider and Blackburn found that telomerase contains an RNA component, which is a template that enables telomerase to repair telomeres on chromosomes. Repaired telomeres enable organisms to replicate cells without potentially harmful mutations or without apoptosis. Greider and Blackburn published their results in 1987, the same year that Greider received her PhD in molecular biology.

In 1987, Greider was hired as an independent researcher at Cold Spring Harbor Laboratory (CSHL) in Cold Spring Harbor, New York by CSHL president Bruce Stillman. Greider studied the sequence of nucleotides that comprise telomerase RNA, and she cloned and isolated the RNA sequence of telomerase. She published her results in 1989. Then the CSHL hired her as a faculty member. She continued to research telomeres and telomerase activity in cells and their roles in the cell cycle through the late 1980s and early 1990s.

While at CSHL, Greider met Nathaniel C. Comfort, a historian of biology, who was working in the public affairs office at CSHL. The two married in 1993. In 1996, when Greider was pregnant with their son she suggested that CSHL should have a childcare facility. Officials of the laboratory agreed to her request, and Greider participated in the ground breaking of the facility while pregnant. Once her son Charles was born, Greider enrolled him in the childcare facility, and she often brought him to meetings and to her office. She also encouraged other women with children at CSHL to do the same.

In 1997, Greider was offered a faculty position at Johns Hopkins University in Baltimore, Maryland. Her husband was concurrently offered a faculty position in George Washington University, in Washington, D.C. Greider and Comfort accepted their positions and the family moved to Baltimore, Maryland. At Johns Hopkins, while working with mice and yeast, Greider studied how cells function without telomerase. Greider and Comfort's second child was born in Baltimore in 1999. According to Greider, her job as a mother came before her work in the laboratory and the flexible work environment of her lab helped ensure that priority. In 2003, she became director of molecular biology and genetics, where she continued to work as of 2014.

Greider received numerous awards, including the Albert Lasker Award for Basic Medical Research in 2006, and the Nobel Prize in Physiology or Medicine, which she received in 2009 along with Blackburn and Szostak. Greider received the awards in recognition for her accomplishments in telomere and telomerase research. She and Comfort divorced around the same time she received the Nobel Prize.

As of 2014, Greider was the youngest woman to receive the Nobel Prize in Physiology or Medicine, at the age of forty-eight. Some also recognize Greider as a leading woman scientist at a time when there were relatively few. Into the first decades of the twenty-first century, Greider researched telomeres in relation to cellular death, regenerative disease, DNA stability, and the relationship between stem cells and telomerase.

Greider, Carol W., and Elizabeth H. Blackburn. "A telomeric sequence in the RNA of *Tetrahymena* telomerase required for telomere repeat synthesis." *Nature* 337 (1989): 331–7.

Greider, Carol W., and Elizabeth H. Blackburn. "Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts." *Cell* 43 (1985): 405–13.

Greider, Carol W., and Elizabeth H. Blackburn. "The telomere terminal transferase of *Tetrahymena* is a ribonucleoprotein enzyme with two kinds of primer specificity." *Cell* 51 (1987): 887–98.

Harley, Calvin B., A. Bruce Futcher, and Carol W. Greider. "Telomeres shorten during ageing of human fibroblasts." *Nature* 345 (1990): 458–60.

Hemann, Michael T., Margaret A. Strong, Ling-Yang Hao, and Carol W. Greider. "The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability." *Cell* 107 (2001): 67–77.

Mundy, Liza. "Profile of Nobel Prize Winner Carol Greider, a Johns Hopkins Molecular Biologist." *The Washington Post* October 20, 2009, Arts & Living section. <http://www.washingtonpost.com/wp-dyn/content/article/2009/10/19/AR2009101903328.html> (Accessed January 22, 2015).

Nobel Media. "Carol W. Greider-Biographical." Nobel Media. http://www.nobelprize.org/nobel_prizes/medicine/laureates/2009/greider-bio.html (Accessed January 25, 2014).

Nobel Prize. "The Nobel Prize in Physiology or Medicine 2009" Nobel Media. http://www.nobelprize.org/nobel_prizes/medicine/laureates/2009/ (Accessed October 5, 2013).

Steedman, Emily J. "Carol W. Greider, Ph.D." Archives of Maryland (Biographical Series). <http://msa.maryland.gov/megafile/msa/speccol/sc3500/sc3520/015500/015533/html/15533bio.html> (Accessed January 25, 2014).

Szostak, Jack W., and Elizabeth H. Blackburn. "Cloning yeast telomeres on linear plasmid vectors." *Cell* 29 (1982): 245–55.

Trucks, Rob. "The 49ers: Oral Histories of Americans Facing 50. #134: Carol Greider." *McSweeney's*. <http://www.mcsweeneys.net/articles/134-carol-greider> (Accessed February 11, 2014).

Elizabeth Blackburn, Carol Greider and Jack Szostak's Telomere and Telomerase Experiments (1982-1989)

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Experiments conducted by Elizabeth Blackburn, Carol Greider, and Jack Szostak from 1982 to 1989 provided theories of how the ends of chromosomes, called telomeres, and the enzyme that repairs telomeres, called telomerase, worked. The experiments took place at the Sidney Farber Cancer Institute and at Harvard Medical School in Boston, Massachusetts, and at the University of California in Berkeley, California. For their research on telomeres and telomerase, Blackburn, Greider, and Szostak received the Nobel Prize in Physiology or Medicine in 2009. Telomeres and telomerase affect the lifespan of mammalian cells and ensure that cells rapidly develop within developing embryos.

The scientists involved in the experiments with telomeres and telomerase came from a variety of disciplines. Blackburn worked at the University of California in Berkeley (UC Berkeley) from 1982 to 1989. In 1975, she had received her PhD in

molecular and cellular biology from the University of Cambridge in Cambridge, England, after which she did postdoctoral work with Joseph Gall at Yale University in New Haven, Connecticut from 1975 to 1977.

As Blackburn's graduate student, Greider studied telomeres and telomerase at UC Berkeley from 1984 to 1987. Greider received her PhD in molecular biology in 1987 from UC Berkeley and continued her research with Blackburn through 1989. Szostak had received his PhD in biochemistry from Cornell University in Ithaca, New York, in 1977, where he specialized in cloning yeast and in manipulating genes. Szostak began to study telomeres and telomerase after hearing a conference presentation given by Blackburn in 1980, during which she explained her work on telomeres in *Tetrahymena*, a single-celled freshwater organism. After meeting Blackburn and discussing her work, Szostak accepted a position at Harvard Medical School in 1982, where he and Blackburn collaborated to investigate the functions of the telomeres of *Tetrahymena* in yeast.

Blackburn and Szostak's 1982 experiment addressed an issue with how DNA replicates copies of itself within a cell. The issue was that after replication, one of the two DNA strands remains incomplete. When a cell replicates itself, the end of a strand of chromosomal DNA, the telomere, shortens. The telomere shortens because the enzyme that replicates DNA, DNA polymerase, only works in one direction on DNA. This process creates what scientists call a leading and a lagging strand during DNA duplication. The leading strand is named such because DNA polymerase moves in one direction across the nucleotide sequence, and replicates the DNA without any breaks in the genetic material. The lagging strand is composed of individual fragments of DNA formed by DNA polymerase (Okazaki fragments) that are later sealed together by the

enzyme DNA ligase to create one continuous strand. This strand is called the lagging strand because it can take longer to seal together the individual DNA fragments than the leading strand takes to continuously replicate a strand. The DNA polymerase detaches at the end of the lagging strand and leaves a space that measures a few nucleotides in length. The identity of those nucleotides remained unknown until Szostak and Blackburn published their results in 1982.

To identify the nucleotides, Szostak and Blackburn removed what Blackburn hypothesized were the telomeres in *Tetrahymena*. The hypothesized telomeres were highly repetitive nucleotide segments of DNA at the ends of chromosomes. The researchers placed the telomeres in circular genetic material, called linearized plasmids, from yeast species. Blackburn and Szostak used yeast and *Tetrahymena* because of their distant evolutionary relationship from each other, and to see if the telomeres were similar across different species of eukaryotes. They found that the yeast added new DNA to the *Tetrahymena* telomeres, which led the researchers to conclude that telomeres were highly conserved evolutionarily, or similar across distantly related species, across yeast and *Tetrahymena*, and hypothetically across other species. Additionally, Blackburn and Szostak observed that the telomeres functioned similarly to each other in yeast and *Tetrahymena*. Blackburn and Szostak cut out pieces of the similar telomeres from each species and identified them by describing their sequences of nucleotides (DNA sequencing). The experiment confirmed the description of the telomere as a highly repetitive nucleotide segment, particularly rich in the nucleotide guanine, which accumulates at one end of chromosomal DNA.

In 1985, Greider and Blackburn further investigated the mechanism by which DNA was added to the ends of telomeres. Blackburn and Greider noted the composition of telomere ends, but they could not explain what was adding the guanine-rich ends to the DNA. In different species, the lengths of telomeres differ, and with the sequencing techniques available in the 1980s, scientists couldn't determine how DNA was added to the ends of telomeres. Telomeres also appeared to grow over time in *Tetrahymena* and yeast. Blackburn and Szostak hypothesized that a specific, not yet identified, enzyme added new DNA to the ends of telomeres.

In 1984 when Greider joined Blackburn's lab, they formulated a procedure to discover that unidentified enzyme. They took extracts of *Tetrahymena* chromosomes, cut them into small pieces and added radioactively labeled nucleotides to help identify parts of the original DNA sequence. They observed which nucleotides were added to the sequence and also examined the changes in length. The radiolabeled nucleotides, which Greider prepared, indicated that there was an enzyme responsible for telomere additions. Greider and Blackburn labeled this enzyme as telomere terminal transferase, a name later shortened to telomerase. In 1985, Greider and Blackburn published the results of their experiment. The evidence that telomerase existed signified that cells contain a mechanism to repair the gap resulting from the lagging strand of DNA and polymerase detachment. Telomerase enabled cells to replicate themselves rapidly and without hindrance in developing organisms.

Greider and Blackburn continued to investigate the structure and function of telomerase by studying the origin of the guanine-rich repeat sequences of DNA. Greider hypothesized that RNA provided the necessary template for guanine-rich repeat

sequences to be added to telomeres. Greider added RNase, an enzyme that breaks down RNA, and DNase, an enzyme that breaks down DNA, to the telomere extracts. She found that the RNase stopped the telomeres from getting longer. She and Blackburn published the results in 1987. Their results concluded that telomerase had an RNA component and could serve as a template for the process of DNA replication.

After further experimentation from 1987 to 1989, Greider sequenced the RNA component of telomerase. Greider created multiple RNA probes that could bind to the partial RNA fragments that she had obtained from the 1987 experiment. Greider diagrammed what she hypothesized was the mechanism by which telomerase added nucleotides to telomeres. She published her experiment and model on the RNA component of telomerase in 1989, with Blackburn as a coauthor.

Along with the work of Blackburn, Greider, and Szostak, others have showed that mammals have telomeres. In 1978, Blackburn had hypothesized that telomeres affect cellular aging, in relation to the Hayflick Limit, or the cell's limited capacity to divide from forty to sixty times before it can no longer replicate. Scientists such as Geraldine Aubert and Peter M. Langsdorp at the University of British Columbia in Vancouver, British Columbia validated Blackburn's original hypothesis. In 2009, Blackburn, Greider, and Szostak received the Nobel Prize in Physiology or Medicine for their research on telomeres and telomerase. The results of their work have influenced research related to cellular aging.

Aten, David, Kuo Anna, and Questell Adam. "What are telomeres and telomerase?" Shay/Wright laboratory. http://www4.utsouthwestern.edu/cellbio/shay-wright/intro/facts/sw_facts.html (Accessed November 2, 2013).

Aubert, Geraldine, and Peter M. Langsdorp "Telomeres and aging." *Physiological Reviews* 88 (2008): 557–79. <http://physrev.physiology.org/content/88/2/557.short> (Accessed February 5, 2015).

Blackburn, Elizabeth H., and Joseph G. Gall. "A tandemly repeated sequence at the termini of the extrachromosomal ribosomal RNA genes in *Tetrahymena*." *Journal of Molecular Biology* 120 (1978): 33–53. http://nobel.ucsf.edu/pdf/nobel/blackburn_and_gall_1978.pdf (Accessed February 5, 2015).

Greider, Carol W., and Elizabeth H. Blackburn. "Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts." *Cell* 43 (1985): 405–13. [http://www.cell.com/cell/pdf/0092-8674\(85\)90170-9.pdf](http://www.cell.com/cell/pdf/0092-8674(85)90170-9.pdf) (Accessed February 5, 2015).

Greider, Carol W., and Elizabeth H. Blackburn. "The telomere terminal transferase of *Tetrahymena* is a ribonucleoprotein enzyme with two kinds of primer specificity." *Cell* 51 (1987): 887–98.

Greider, Carol W., and Elizabeth H. Blackburn. "A telomeric sequence in the RNA of *Tetrahymena* telomerase required for telomere repeat synthesis." *Nature* 337 (1989): 331–7.

Nobel Media. "Carol W. Greider–Biographical." http://www.nobelprize.org/nobel_prizes/medicine/laureates/2009/greider-bio.html (Accessed January 25, 2014).

Nobel Prize. "The Nobel Prize in Physiology or Medicine 2009." http://www.nobelprize.org/nobel_prizes/medicine/laureates/2009/ (Accessed October 5, 2013).

Szostak, Jack W., and Elizabeth H. Blackburn. "Cloning yeast telomeres on linear plasmid vectors." *Cell* 29 (1982): 245–55.

Telomerase in Human Development

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Telomerase is an enzyme that regulates the lengths of telomeres in the cells of many organisms, and in humans it begins to function in the early stages of embryonic

development. Telomeres are repetitive sequences of DNA on the ends of chromosomes that protect chromosomes from sticking to each other or tangling. In 1989, Gregg Morin found that telomerase was present in human cells. In 1996, Woodring Wright and his team examined human embryonic cells and found that telomerase was active in them. Scientists manipulate telomerase in cells to give cells the capacity to replicate infinitely. Telomerase is also necessary for stem cells to replicate themselves and to develop into more specialized cells in embryos and fetuses.

Carol Greider and Elizabeth Blackburn discovered telomeres in 1978. Greider was Blackburn's student at the University of California in Berkeley, California. Blackburn had used the protozoan *Tetrahymena thermophila* to study telomeres in the process of cellular replication. In 1985 Greider and Blackburn discovered in *Tetrahymena* terminal transferase, which later was called telomerase. Greider and Blackburn found that telomerase was a type of enzyme that organizes DNA at the end of the strand in a reverse fashion from normal transcription. Scientists label any enzyme that follows the reverse pattern as a reverse transcriptase. Telomerase consists of protein and RNA that add thymine and guanine (TTGGGG) repeated nucleotide sequences on the ends of chromosomes. Telomerase fills a gap on the chromosome that exists due to imperfect DNA replication. DNA replication is imperfect because there is a space where the enzyme DNA polymerase detaches from the DNA strand.

In 1994 at the Cold Spring Harbor Laboratory in Cold Spring Harbor, New York, Lin Mantell and Greider showed that telomerase was necessary in germline and embryonic cells of developing frogs *Xenopus laevis*. For their work with telomeres and

telomerase, Greider and Blackburn received the Nobel Prize in Physiology or Medicine in 2009, along with Jack Szostak.

Building upon the finding of telomerase in *Tetrahymena*, Gregg Morin studied if telomerase was present in human cells. In 1989, while working at Yale University in New Haven, Connecticut, Morin isolated telomerase from human HeLa cells. HeLa cells are named after Henrietta Lacks, a cervical cancer patient from whom the cells were taken. HeLa cells are cancerous cells that proliferate indefinitely. The telomerase that Morin found in the HeLa cells differed from the telomerase found in *Tetrahymena*. HeLa cells' telomerase had a six-nucleotide sequence of thymine, adenine and guanine (TTAGGG). Morin hypothesized that totipotent cells, or cells capable of becoming any types of cells such as embryonic stem cells, produced telomerase to retain the infinite replicative properties needed for an organism to develop.

Mantell and Greider verified Morin's hypothesis in *Xenopus* in 1994. Mantell and Greider found that during early development, telomerase is highly active. Telomeres were active throughout embryogenesis and oogenesis in *Xenopus* egg cells. The researchers also found that germline cells, such as cells found in the ovaries and testes of *Xenopus*, continued to produce telomerase. Mantell and Greider further suggested that telomerase functions in germline cells to preserve telomeres for future generations.

In 1996, scientists verified Morin's hypothesis in humans. Woodring Wright, Piatyszek Mieczyslaw, William Rainey, William Byrd, and Jerry Shay at the University of Texas Southwestern Medical Center in Dallas, Texas, performed an experiment that detected high amounts of telomerase activity in human blastocysts, and in tissues at

sixteen to twenty weeks after fertilization. The activity rapidly declined and became undetectable after the neonatal period, or the first twenty-eight days of a child's life post-birth. Wright and his team concluded that the human body regulates and represses telomerase activity after birth except in some tissues. Particularly, Wright's team found that telomerase was expressed in fetal, newborn, and adult testes and ovaries, but not in mature sperm or eggs, which differed from the results found in *Xenopus*. Wright and his team hypothesized that the difference was due to the fact that human sperm telomeres do not shorten with age, and human zygotes produce telomerase after the first cell division, therefore egg cells need not carry telomerase.

In 2001, researchers including Diane L. Wright at the Eastern Virginia Medical School in Norfolk, Virginia, found that telomerase is necessary for cells in human embryos to rapidly proliferate. Cells need telomerase during embryogenesis because as they replicate, their telomeres shorten. Without the presence of telomerase, the first few cells in the zygote would be unable to replicate and develop into an embryo and eventually a fetus. The scientists reported that telomerase was active in every stage of development of the embryo. They also observed that while telomerase activity was necessary for an embryo to develop, the amount of telomerase present did not predict the potential for embryonic growth.

Scientists found that genetic variation between humans influences telomerase activity during development and adult life. In 2010, researchers including Gil Atzmon at the Albert Einstein College of Medicine in Bronx, New York, published a study that explains the effects of telomerase in centenarians of Ashkenazi Jewish descent. That research showed that the centenarians and their offspring showed increased production of

telomerase. Due to that increase, those studied had fewer than normal age-related diseases such as cardiovascular disease and diabetes mellitus, as well as other diseases caused by genetic mutations. The researchers named the genes associated with the increase in telomerase activity hTERT and hTERC. They further hypothesized that hTERT and hTERC became active during development and that their repression was not as drastic after birth for those studied as it was for those in a more general population.

Although scientists researched the link between telomerase activity and the process of how cells replicate, some criticized the way the data was produced. Critics of the research, such as Harry Rubin at the University of California, Berkeley, California, argued that the data collected on telomerase could be skewed by experimental techniques. Rubin argues that incorrect data of telomerase activity can result from tumor cells within the population or the result of a virus that causes overproduction of telomerase. Others argued that scientists over emphasized research into telomerase and telomeres and that other elements of development and the cell cycle are overlooked due to the attention given to telomeres.

Atzmon, Gil, Miok Cho, Richard M. Cawthon, Temuri Budagov, Micol Katz, Xioman Yang, Glenn Siegel, Aviv Bergman, Derek M. Huffman, Clyde B. Schechter, Woodring E. Wright, Jerry W. Shay, Nir Barzilai, Diddahally R. Govindaraju, and Yousin Suh. "Genetic variation in human telomerase is associated with telomere length in Ashkenazi centenarians." *Proceedings of the National Academy of Sciences* 107 (2010): 1710–7. http://www.pnas.org/content/107/suppl_1/1710.full (Accessed March 11, 2015).

Aubert, Geraldine, and Peter M. Langsdorp. "Telomeres and aging" *Physiological Reviews* 88 (2008): 557–79. <http://physrev.physiology.org/content/88/2/557.short> (Accessed March 11, 2015).

Blackburn, Elizabeth H., and Joseph G. Gall. "A tandemly repeated sequence at the termini of the extrachromosomal ribosomal RNA genes in *Tetrahymena*." *Journal of Molecular Biology* 120 (1978): 33–53.

Greider, Carol W., and Elizabeth H. Blackburn. "A telomeric sequence in the RNA of *Tetrahymena* telomerase required for telomere repeat synthesis." *Nature* 337 (1989): 331–7.

Greider, Carol W., and Elizabeth H. Blackburn. "Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts." *Cell* 43 (1985): 405–13. [http://www.cell.com/cell/pdf/0092-8674\(85\)90170-9.pdf](http://www.cell.com/cell/pdf/0092-8674(85)90170-9.pdf) (Accessed March 11, 2015).

Greider, Carol W., and Elizabeth H. Blackburn. "The telomere terminal transferase of *Tetrahymena* is a ribonucleoprotein enzyme with two kinds of primer specificity." *Cell* 51 (1987): 887–98.

Hayflick, Leonard. "The limited *in vitro* lifetime of human diploid cell strains." *Experimental Cell Research* 37 (1965): 614–36.

Hayflick, Leonard, and Paul S. Moorhead. "The serial cultivation of human diploid cell strains." *Experimental Cell Research* 25 (1961): 585–621.

Jiang, Lijing. "Degeneration in Miniature: History of Cell Death and Aging Research in the Twentieth Century." PhD diss., Arizona State University, 2013. http://repository.asu.edu/attachments/114558/content/Jiang_asu_0010E_13292.pdf (Accessed June 6, 2014).

Lin, Jue, Elissas Epel, and Elizabeth H. Blackburn. "Telomeres and lifestyle factors: roles in cellular aging." *Mutation Research* 730 (2012): 85–9.

Mantell, Lin L., and Carol W. Greider. "Telomerase activity in germline and embryonic cells of *Xenopus*." *EMBO Journal* 13 (1994): 3211–7. <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC395213/pdf/emboj00061-0269.pdf> (Accessed March 11, 2015).

Morin, Gregg B. "The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats." *Cell* 59 (1989): 521–9.

Nobel Prize. "The Nobel Prize in Physiology or Medicine 2009." *Nobel Media*. http://www.nobelprize.org/nobel_prizes/medicine/laureates/2009/ (Accessed March 11, 2015).

Olovnikov, Alexey M. "A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon." *Journal of Theoretical Biology* 41 (1973): 181–90.

Rubin, Harry. "Telomerase and cellular lifespan: ending the debate?" *Nature Biotechnology* 16 (1998): 396–7.

Szostak, Jack W., and Elizabeth H. Blackburn. "Cloning yeast telomeres on linear plasmid vectors." *Cell* 29 (1982): 245–55.

Wright, Diane L., Estella L. Jones, Jacob F. Mayer, Sergio Oehninger, William E. Gibbons, and Susan E. Lanzendorf. "Characterization of telomerase activity in the human oocyte and preimplantation embryo." *Molecular Human Reproduction* 7 (2001): 947–55. <http://molehr.oxfordjournals.org/content/7/10/947.long> (Accessed March 11, 2015).

Wright, Woodring E., Piatyszek A. Mieczyslaw, William E. Rainey, William Byrd, and Jerry W. Shay. "Telomerase activity in human germline and embryonic tissues and cells." *Developmental Genetics* 18 (1996): 173–9.

Telomeres and Telomerase in Cellular Aging (Senescence)

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Telomeres are bits of DNA on the ends of chromosomes that protect chromosomes from sticking to each other or tangling, which could cause DNA to abnormally function. As cells replicate, telomeres shorten at the end of chromosomes, and this process correlates to senescence or cellular aging. Integral to this process is telomerase, which is an enzyme that repairs telomeres and is present in various cells in the human body, especially during human growth and development. Telomeres and telomerase are required for normal animal development because they protect DNA as it duplicates copies of itself.

In 1965, Leonard Hayflick's research at the Wistar Institute in Philadelphia, Pennsylvania, showed the limit to which cells duplicate themselves before aging. Hayflick established what became called the Hayflick limit, which states that a cell can divide forty to sixty times before it cannot divide further and begins to age. In the 1970s, scientists researched telomeres. Elizabeth Blackburn studied telomeres while she worked at Yale University in New Haven, Connecticut. Alexey Olovnikov in Russia related telomeres to cellular aging and to the Hayflick Limit. Although Blackburn had helped discover telomeres in 1975, two years before, in 1973, Olovnikov had hypothesized the existence of telomerase, the length of telomeres, and their connections to cellular aging in his study on the Hayflick Limit. Unaware of Olovnikov's research, Blackburn and Joseph G. Gall independently found a repetitive sequence of DNA at the end of chromosomes of the yeast, *Tetrahymena thermophila*. Blackburn and Gall published the results of their research in 1978. In 1982 Blackburn, then at the University of California in Berkeley, California, collaborated with Jack W. Szostak at the Harvard Medical School in Boston, Massachusetts. The pair isolated and cloned telomeres in *Tetrahymena* DNA. Blackburn, with the help of her student Carol Greider, then identified telomerase in 1984 and isolated it from *Tetrahymena* in 1989. Blackburn, Jack Szostak, and Carol Greider received the Nobel Prize in Physiology or Medicine in 2009 for their work to identify and isolate telomeres and telomerase.

Blackburn and others found that cells age when the length of the telomeres in the cells shortens. Each time a cell replicates itself, the end of a strand of DNA or the telomere shrinks in length. The telomeres shrink across replications because the enzyme that replicates DNA, DNA polymerase, only works in one specific direction on

the DNA strand. It creates what is called a leading and a lagging strand of duplication. The leading strand receives its name because DNA polymerase constantly moves in one direction and replicates the DNA until it completes the strand of DNA without any breaks. The lagging strand is composed of individual fragments of DNA created by DNA polymerase, called Okazaki fragments, which are later sealed together by the enzyme DNA ligase to create one continuous strand. The name lagging strand derives from the fact that it lags behind the leading strand since lagging or leading strand can take longer to seal together the individual DNA fragments. The DNA polymerase detaches from DNA at the end of a lagging strand and leaves a space that measures a few nucleotides in length. Telomerase normally fills in the gap at the end of the DNA after the polymerase detaches from it.

Telomerase, the enzyme that repairs telomeres, exists in high quantities in developing organisms and in embryonic stem cells. Some cells have higher amounts of telomerase activity than do others. As a human develops and cells replicate with greater frequency, excess telomerase is used and is later replenished only in minute quantities. At the end of the process of DNA replication, without telomerase to fill in the gaps of a new DNA strand, telomeres shorten with each cellular division. According to Blackburn, the Hayflick Limit is a result of decreased telomere length. Decreased telomere length also leads to chromosomal abnormalities that result in mutations in the genetic code, mutations that can cause cancer and further aging in humans. However, too much telomerase can also lead to cancer by helping cells to become functionally immortal by avoiding the Hayflick Limit. Immortal cells can carry mutations in unrepaired areas of the DNA, and they pass the cancerous mutations to other cells through replication.

Scientists continued researching telomerase, telomere activity, and cellular aging after Blackburn's experiments in the 1980s. Scientists studied how environmental factors could affect the length of telomeres and, consequently, cellular aging. In 2008, Geraldine Aubert and Peter Langsdorp at the Terry Fox Laboratory in Vancouver, Canada, published research showing that cells replicate in response to mutations in the genetic code or in response to stress. Aubert and Langsdorp showed that a cell will replicate to attempt to repair damaged DNA and, in turn, will shorten the telomeres.

In 2012, Blackburn, Jue Lin, and Elissa Epel at the University of California in San Francisco, California, showed the influence of lifestyle on the length of telomeres and cellular aging in humans. They found that stress, nutrition, and personality influence the length of telomeres and telomerase enzyme activity. They defined stress as adverse life events such as death in the family or chronically sick children, and they found that personality also influences how a person perceives stressful events. The authors noted that those who perceived events as less stressful than what the researchers expected had longer telomere lengths compared to individuals who perceived events as more stressful than what researchers expected. Behaviors such as smoking or eating processed meats also correlated with shorter than normal telomere lengths. Also, those who took vitamin C or E supplements had longer than normal telomere lengths. The results of Blackburn and her team's experiment verified that environmental factors affect the length of telomeres.

Some people have criticized telomere research. Harry Rubin, at the University of California in Berkeley argued that researchers who studied telomeres in relation to cancer

sometimes produce suspect data. Others argued that scientists pay too much attention on telomere research when they should also study other factors involved in the cell cycle.

Aubert, Geraldine, and Peter M. Langsdorp "Telomeres and aging." *Physiological Reviews* 88 (2008): 557–79. <http://physrev.physiology.org/content/physrev/88/2/557.full.pdf> (Accessed February 17, 2015).

Blackburn, Elizabeth H., and Joseph G. Gall. "A tandemly repeated sequence at the termini of the extrachromosomal ribosomal RNA genes in *Tetrahymena*." *Journal of Molecular Biology* 120 (1978): 33–53.

Greider, Carol W., and Elizabeth H. Blackburn. "A telomeric sequence in the RNA of *Tetrahymena* telomerase required for telomere repeat synthesis." *Nature* 337 (1989): 331–7.

Hayflick, Leonard. "The limited in vitro lifetime of human diploid cell strains." *Experimental Cell Research* 37 (1965): 614–36.

Hayflick, Leonard, and Paul S. Moorhead. "The serial cultivation of human diploid cell strains." *Experimental Cell Research* 25 (1961): 585–621.

Jiang, Lijing. "Degeneration in Miniature: History of Cell Death and Aging Research in the Twentieth Century" PhD diss., *Arizona State University*, 2013. http://repository.asu.edu/attachments/114558/content/Jiang_asu_0010E_13292.pdf (Accessed June 6, 2014).

Lin, Jue, Elissas Epel, and Elizabeth Blackburn. "Telomeres and lifestyle factors: roles in cellular Aging." *Mutation Research* 730 (2012): 85–9.

Nobel Prize. "The Nobel Prize in Physiology or Medicine 2009." *Nobel Media*. http://www.nobelprize.org/nobel_prizes/medicine/laureates/2009/ (Accessed October 5, 2013).

Olovnikov, Alexey M. "A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon." *Journal of Theoretical Biology* 41 (1973): 181–90.

Rubin, Harry. "Telomerase and cellular lifespan: ending the debate?" *Nature Biotechnology* 16 (1998): 396–7.

Strauss, Evelyn. "Albert Lasker Award for Special Achievement in Medical Science." *Lasker Foundation*. http://www.laskerfoundation.org/awards/2006_s_description.htm (Accessed October 20, 2013).

Szostak, Jack W., and Elizabeth H. Blackburn. "Cloning yeast telomeres on linear plasmid vectors." *Cell* 29 (1982): 245–55.

Paternal Sperm Telomere Elongation and its Impact on Offspring Fitness

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Telomeres are the protective caps at the end of DNA strands that preserve chromosomal integrity and contribute to DNA length and stability. Because telomeres shorten with each cell division due to incomplete replication, the enzyme telomerase is present in certain cell lines that undergo repetitive divisions to replenish any lost length and to prevent degradation. Cells, and therefore organisms, with short telomeres are more susceptible to mutations and genetic disease. Research by Elizabeth Blackburn, Carol Greider, and Jack Szostak in the 1980s laid the foundation for telomere research in a wide array of fields, ranging from senescence, or aging, to cancer studies and prenatal development. That research earned them the 2009 Nobel Prize in Physiology or Medicine. In 1992, scientists found that telomere length (TL) in sperm increases with age as opposed to most other cells, where telomeres actually shorten. Strong TL correlations between father and child passed on in sperm imply that there are paternally heritable biological traits that can affect offspring's fitness. The offspring's fitness can affect the longevity and health of future generations that continue to carry the heritable trait originally passed by the father.

Since the research of telomeres and telomerase in the 1980s by Blackburn and her team, scientists have used embryonic and prenatal experimentation to provide insight on how telomeres preserve chromosomal integrity. Telomeres shorten with each cell replication due to DNA's method of synthesis, which requires short strands of nucleic acids called RNA primers. Enzymes lengthen the RNA primers to form Okazaki fragments, or short strands of DNA, which enzymes combine to become one continuous strand. The RNA primers, however, occupy a space on the DNA template and once removed, there is a remaining gap at the end of the DNA. This phenomenon, known as the end-replication problem, creates loss of genetic material that cells counteract by producing telomerase to repair telomere ends.

Scientists link TL's heritability, demonstrated initially through studies on twins, and its implications for predicting longevity and genetic stability, to telomerases' key role in counteracting the end-replication problem of DNA. Researchers demonstrate that Leukocyte, or white blood cell telomere length (LTL) in particular is heritable, although mechanisms behind the extreme diversity of LTL length in humans are unknown as of 2014. While researchers in the late twentieth and early twenty-first centuries studied maternal influence on developing oocytes extensively, paternal effects of sperm were less understood. Scientists attribute the lack of understanding to the idea that there is a stronger perceived maternal influence on children. For example, scientists found that mRNA, or protein, comes from the egg, and not from the sperm. Some scientists challenge the idea that maternal factors alone influence children with research that shows how oocyte viability is dependent on both mother and father.

In 1992, Allsopp and his research team found that sperm telomeres do not

decrease with age like their somatic (body) cell counterparts. That research indicated that a mechanism, through telomerase or some other method, is in place to protect germ-line cells. With longer TL associated with more potential cell divisions, researchers became interested in studying sperm interactions with eggs. Scientists state that TL can be influenced by heredity, age, and gender, but significant variability in telomere length across the human population indicates that some other, more potent, determinant is at hand. A 2005 study by Brad Unryn and colleagues at the University of Calgary, in Calgary, Canada, further demonstrated that there is a positive correlation between TL and increased paternal age. They hypothesized that this may account for the large range of TL observed throughout the human population, especially if paternal contribution to TL is cumulative over generations.

As a genetic mechanism, the specifics behind sperm TL elongation remain elusive as of 2014, even if its implications in development are well established. Scientific understanding of how sperm TL contributes to offspring TL, however, has changed drastically. In 2007, Tim De Meyer and his colleagues in Ghent, Belgium, further corroborated the results of Unryn and his team through analysis of 2433 volunteers, about half of whom were male and the other half were female. De Meyer and his team compared information on the volunteers' telomere lengths to statistical information of their parents' ages at conception and the current ages of the volunteers. De Meyer showed that older fathers' ages at conception were positively associated with longer TL in their children. The study provided a quantitative gain of seventeen additional base pairs in TL for each additional year of age in the father. Furthermore, differences between daughters and sons were insignificant, contradicting earlier thoughts on gender-specific

variability. The results added evidence to substantiate the sperm's role in contributing to TL variance and added to a growing body of work demonstrating how TL is not completely reset during embryonic development in the zygote, but rather can be inherited from father to child.

De Meyer and his colleagues' work was verified through further studies by Masayuki Kimura and his team by demonstrating how a father's age positively correlates with his children's leukocyte telomere lengths (LTL). Kimura's study was conducted in four distinct populations and they found that children's LTL increased anywhere from half-fold to twofold with each additional year of their fathers' age. Sperm telomere elongation in older men and its potential effect on offspring LTL holds implications for potentially increased longevity and lessened age-related disorders in offspring of older fathers.

In 2010, a study by Katarina Nordfjäll and her colleagues at Umeå University in Umeå, Sweden, further demonstrated that a child's TL correlated more with his or her father's TL than with his or her mother's TL. There was no statistical difference between father-to-son or father-to-daughter, and there was even an observed correlation between grandfather and grandchild. That association over the course of multiple generations lends credence to the idea that sperm TL contributes to population-wide TL variability due to paternal inheritance. Tracing TL over multiple generations down the paternal family line suggests a concentration upon the father's lineage when looking to examine potential TL in children. The 962 individuals examined in Nordfjäll's study also demonstrated diminishing father-child correlations over time, which shows that

environmental (non-genetic) factors such as oxidative stress can influence telomere length fluctuations throughout a lifetime.

While the correlation between a father's sperm TL and his child's TL is largely accepted by scientists as of 2014, the implications of a longer TL for biological fitness of the child are not so clear. Longer TL, when viewed in itself, is a positive trait in most cells. However, aging can negatively affect genomic stability when mutation accumulations and other lifestyle factors counteract the positive benefits of having longer telomeres. A 2009 study by Silvia García-Palomares and colleagues in mice found that older paternal ages at conception have negative effects on offspring viability. The scientists individually housed male mice at a variety of different ages with same-aged female mice. They monitored the resulting offspring for two generations (F1 and F2). F1 females from older fathers had longer intervals between births indicating reduced fertility, and F2 generation mice had lower weaning weights overall. F1 mice also displayed shorter lifespans accompanied by a lower incidence of tumor development due to early death, and decreased body weight at death.

A 2010 literature review by Gideon Sartorius and Eberhard Nieschlag at the University of Domagkstrasse, in Muenster, Germany, concluded that older paternal age at conception negatively influences offspring fitness overall. While TL increases in a subset of sperm cells and longer telomeres may prevent early disintegration of DNA, it may also prevent natural mechanisms of apoptosis, or cell death, from occurring in unhealthy sperm because a longer TL can reduce incidences of cellular disease. Beyond telomere interactions, increased age in males correlates with decreased sexual activity, infertility, increasing miscarriage rates, and deflated male hormones, such as testosterone, in the

body. Sartorius and Nieschlag state that if paternal TL's effect on offspring TL does contribute somehow to offspring fitness, researchers must consider other factors to provide a comprehensive understanding of how age at conception has a positive or negative impact. According to the authors of the study, isolating the specifics of whatever positives there are may also prove to increase longevity for future generations and provide a better quality of life in a society that is gradually having children at an older age.

While the mechanisms behind sperm telomere elongation remained elusive in the beginning of the twenty-first century, scientists have established its heritability. Researchers claim that whether or not older paternal age at conception contributes to increased offspring fitness overall is debatable. The potential for the longer telomeres of paternal sperm to transfer their properties of longer telomere length in offspring may contribute to a better understanding of just how telomeres contribute to embryonic development and subsequent longevity.

Allsopp, Richard C., Homayoun Vaziri, Christopher Patterson, Samuel Goldstein, Edward V. Younglai, A. Bruce Futcher, Carol W. Greider, and Calvin B. Harvey. "Telomere Length Predicts Replicative Capacity of Human Fibroblasts." *Proceedings of the National Academy of Sciences of the United States of America* 89 (1992): 10114–8.

Blackburn, Elizabeth H., and Joseph G. Gall. "A tandemly repeated sequence at the termini of the extrachromosomal ribosomal RNA genes in *Tetrahymena*" *Journal of Molecular Biology* 120 (1978): 33–53.

De Meyer, Tim, Ernst R. Rietzschel, Marc L. De Buyzere, Dirk De Bacquer, Wim Van Criekinge, Guy G. De Backer, Thierry C. Gillebert, Patrick Van Oostveldt, and Sofie Bekaert. "Paternal Age at Birth is an Important Determinant of Offspring Telomere Length." *Human Molecular Genetics* 15 (2007): 3097–102.

García-Palomares, Silvia, Samuel Navarro, José F. Pertusa, Carlos Hermenegildo, Miguel A. García-Pérez, Francisco Rausell, Antonio Cano, and Juan J. Tarín. “Delayed Fatherhood in Mice Decreases Reproductive Fitness and Longevity of Offspring.” *Biology of Reproduction* 80 (2009): 343–9.

Greider, Carol W., and Elizabeth H. Blackburn. “A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis”. *Nature* 337 (1989): 331–7.

Greider Carol W., and Elizabeth H. Blackburn. “Identification of a specific telomere terminal transferase activity in Tetrahymena extracts”. *Cell* 43 (1985): 405–13.

Greider Carol W., and Elizabeth H. Blackburn. “The telomere terminal transferase of Tetrahymena is a ribonucleoprotein enzyme with two kinds of primer specificity”. *Cell* 51 (1987): 887–98.

Kimura, Masayuki, Lynn F. Cherkas, Bernet S. Kato, Serkalem Demissie, Jacob B. Hjelmborg, Michael Brimacombe, Adrienne Cupples, Janice L. Hunkin, Jeffrey P. Gardner, Xiaobin Lu, Xiaojian Cao, Malinee Sastrasin, Michael A. Province, Steven C. Hunt, Kaare Christensen, Daniel Levy, Tim D. Spector, and Abraham Aviv. “Offspring’s Leukocyte Telomere Length, Paternal Age, and Telomere Elongation in Sperm.” *Public Library of Science Genetics* 4 (2008): 1–9.

Nobel Prize. “The Nobel Prize in Physiology or Medicine 2009” Nobel Media. http://www.nobelprize.org/nobel_prizes/medicine/laureates/2009/ (Accessed October 5, 2013).

Nordfjäll, Katarina, Ulrika Svenson, Karl-Fredrik Norrback, Rolf Adolfsson, and Göran Roos. “Large-Scale Parent-Child Comparison Confirms a Strong Paternal Influence on Telomere Length.” *European Journal of Human Genetics* 18 (2010): 385–9.

Sartorius, Gideon A., and Eberhard Nieschlag. “Paternal Age and Reproduction.” *Human Reproduction Update* 16 (2010): 65–79.

Szostak, Jack W., Elizabeth H. Blackburn. “Cloning yeast telomeres on linear plasmid vectors”. *Cell* 29 (1982): 245–55.

Tong, Zhi-Bin, Lyn Gold, Karl Pfeifer, Heidi Dorward, Eric Lee, Carolyn A. Bondy, Jurrien Dean, and Lawrence M. Nelson. “Mater, a Maternal Effect Gene Required for Early Embryonic Development in Mice.” *Nature Genetics* 26 (2000): 267–8.

Unryn, Brad M., Linda S. Cook, and Karl T. Riabowol. "Paternal Age is Positively Linked to Telomere Length of Children." *Aging Cell* 4 (2005): 97–101.

CHAPTER 4

SHEEP, SENESCENCE, AND STEM CELLS – CLONING AND SCNT TO "TURN BACK THE CLOCK"

Scientists heavily studied telomeres and telomerase through the late 20th century in hopes of finding a way to activate or deactivate immortality in cell populations. However, some scientists were looking for a way to acquire immortalized cell lines through a different method: cloning. Cloning, as defined by the NIH, “describes the processes used to create an exact genetic replica of another cell, tissue or organism. The copied material, which has the same genetic makeup as the original, is referred to as a clone” (MedlinePlus).

In 1952, Robert King and Thomas Briggs conducted experiments that involved transplantation of cell nuclei. They designed a protocol to remove the nucleus from an unfertilized frog egg, which they then replaced with the nucleus of a cell from a different frog blastocyst. Briggs and King observed that the resulting frog embryo developed normally. Their work was quickly recognized in the scientific community, and scientists were eager to try a similar technique in other animal species.

From the 1950s onward, scientists used transplantation techniques similar to Briggs and King’s technique to produce a variety of clones. However, mammalian embryos proved difficult to clone. Though it was possible to transfer the nucleus of a mammalian cell into a vacant egg cell, resulting embryos failed to develop past the morula. Many hypothesized that the fault lies in the methods for transferring nuclei, as the transfer required researchers to puncture the cell membrane of the egg cell, thus

resulting in cellular damage. In 1984, Steen Willadsen modified Briggs and King's methods and successfully cloned a sheep embryo. Though Willadsen's additions allowed cloned mammalian embryos to divide past the morula stage, the cloned embryos never grew into adult organisms.

Perhaps one of the most famous examples of cloning occurred in 1997, when scientists at the Roslin institute in Edinburgh, Scotland, successfully cloned a sheep, which they named Dolly. Dolly was the first mammal cloned from an adult somatic cell. Previous experiments in mammalian cloning relied on using cells from the blastocyst, rather than fully differentiated adult cells. The new technique became known as Somatic Cell Nuclear Transfer (SCNT).

With the modifications that the Roslin Institute Scientists made in cloning techniques, it had become possible to take adults cells and revert them into embryonic cells capable of becoming a complete organism. Fueled in part by the publicity of Dolly the sheep, scientists eagerly sought ways to apply the same SCNT techniques to human cells. To understand the historical context of SCNT, it seems appropriate to examine Embryo Project Encyclopedia articles related to Dolly the Sheep, beginning with the history of Keith Campbell, one of the key researchers who contributed to SCNT research.

Keith Henry Stockman Campbell (1954-2012)

Bartlett, Zane, "Keith Henry Stockman Campbell (1954-2012)". Embryo Project Encyclopedia (2014-06-27). ISSN: 1940-5030 <http://embryo.asu.edu/handle/10776/8013>.

Keith Henry Stockman Campbell studied embryo growth and cell differentiation during the twentieth and twenty-first centuries in the UK. In 1995,

Campbell and his scientific team used cells grown and differentiated in a laboratory to clone sheep for the first time. They named these two sheep Megan and Morag. Campbell and his team also cloned a sheep from adult cells in 1996, which they named Dolly. Dolly was the first mammal cloned from specialized adult (somatic) cells with the technique of somatic cell nuclear transfer (SCNT). Campbell helped develop cloning techniques that used a common form of connective tissue cells (fibroblasts). Besides working at the Roslin Institute, in Edinburgh, Scotland, for most of his career, Campbell also taught at the University of Nottingham in Nottingham, England.

Campbell was born 23 May 1954 in Birmingham, England, to Marjorie Regina Smith Campbell and Henry Stockman Campbell. Campbell had one younger sibling, a sister. At age three, Campbell and his family moved to Perth, Scotland, where Campbell began his formal education. His mother noted that he was a curious and adventurous boy who loved the outdoors and she often had to sweep frogs out of the family kitchen after Campbell had brought them home. Campbell returned to Birmingham with his family when he was eight, and he remained in Birmingham until he was twenty-one. Campbell attended school at the King Edward VI Grammar School for boys on a scholarship, but he received no outstanding grades.

Campbell said he did not like the atmosphere of that school, so he took a vocational exam and that qualified him as a medical laboratory technician at the age of nineteen, in 1973. Campbell worked at the Selly Oak Hospital in Birmingham, England. Campbell later said that the work as a technician did not intellectually satisfy him because he could not perform his own research. He enrolled at Queen Elizabeth College in London, England, to study microbiology. He quit his job at the hospital the same day

he received his national certificate in medical technology. The certificate qualified Campbell as a medical technologist, but he later said that he did not want to continue with this career.

At Queen Elizabeth College Campbell studied the mechanisms of life cycles for cells. He graduated in 1978 with a Bachelor of Science in microbiology. After receiving his degree, Campbell worked as a medical technologist in southern Yemen until he returned to England a year later. He was in England from 1979 to 1980 as part of a program that aimed to eradicate a fungus that attacks trees, Dutch elm disease, from parts of southern England. Campbell said that he was also unsatisfied with this work.

Campbell began his PhD in 1980 at the Marie Curie Institute in Oxted, England. He joined a group led by Nutan Bishun that studied chromosome structures in cells. Bishun left due to illness shortly after Campbell joined, which left Campbell without a supervisor. Campbell worked alone on his PhD for six months until 1983 when the Marie Curie Institute awarded him a scholarship to study and finish his PhD at the University of Sussex in Sussex, England. There, Campbell studied the cell cycle with the direction of Chris Ford. Campbell learned of a technique to mature egg cells (oocyte) *in vitro*, a technique he later used at the Roslin Institute.

At the University of Sussex, Campbell studied the embryos and oocytes of South African clawed frogs (*Xenopus laevis*). He read the works of John Gurdon, at the University of Oxford in Oxford, UK. Gurdon researched cloning and nuclear transplantation, a technique of transplanting the nucleus of one cell to a host egg cell that had had its nucleus removed. Campbell met another biologist who studied nuclear transplantation, Karl Illmensee from the University of Geneva in Geneva, Switzerland,

who further fueled Campbell's interest in cloning and the cell cycle. During his time at Sussex, Campbell worked in the lab with Chris Hutchinson and collaborated with Ron Laskey and Julian Blow at Cambridge University in Cambridge, England. In 1986, Campbell received a PhD for his dissertation titled "Aspects of Cell Cycle Control in Yeast and *Xenopus*."

After receiving his PhD, Campbell returned to Scotland. Campbell said he enjoyed mountain biking, hiking, and the outdoors, and he felt that Scotland was where he could enjoy these activities and have a career of his choice. He became a postdoctoral research fellow in the zoology department at Edinburgh University in Edinburgh, Scotland. Campbell joined a group led by Peter Fantes. The group worked to control the cell cycle of *Schizosaccharomyces pombe*, a species of yeast that reproduces by fission. At Edinburgh, Campbell met Murdoch Mitchison, who worked in cellular biology and developed the fission yeast *S. pombe* as a model system for studying the cell cycle.

In 1989, Campbell left his fellowship in Edinburgh and began another post-doctoral fellowship at the University of Dundee in Dundee, Scotland, where he worked again with Hutchinson on frog embryonic development. In his studies of frog embryos, Campbell studied cell nuclei, and he began to hypothesize about the ability to reprogram an already determined cell. In 1991, Campbell saw a job posting for a post-doctoral position at the Roslin Institute. Later he said that he saw the opportunity as a better way to provide for his romantic partner, Angela, and for their first daughter Clair, and to pursue cloning. Campbell applied for and received the job offer. Campbell and Angela later had another daughter named Lauren.

At the Roslin Institute, Campbell met and became friends with Ian Wilmut, who led a research group that would eventually clone Dolly. Campbell studied the effects of cloning by transferring the nucleus of one cell to another (nuclear transplantation). According to Wilmut, Campbell felt that the key to cloning was to transplant the nucleus to the egg during the second stage of meiosis. Campbell also altered the amounts of Maturation Promoting Factor, or MPF, a protein activity modifier (kinase) that regulates the cell cycle in many animal cells. Campbell and Wilmut first experimented on cattle embryos for this research.

Using the techniques developed with the cattle embryo experiments, Campbell and Wilmut tried cloning sheep because they were cheaper than cattle and because the Roslin Institute already had grown sheep cells in the laboratory. Campbell first tried to clone sheep stem cells, but he was unsuccessful because sheep stem cells were difficult to grow in the laboratory.

Two years later, in 1995, Campbell successfully cloned two Welsh Mountain lambs using nuclei from sheep embryo cells grown in the laboratory. Campbell started with a multicelled sheep embryo. He used techniques of mechanical and enzymatic digestion to disassociate the cells apart, and he suspended the individual cells in a liquid. He then placed the cells into cell culture medium and incubated them at thirty-seven degrees centigrade. Those cells were called the primary culture. After several days Campbell removed a portion of the cells from the primary culture and placed them into fresh cell culture media, a process called the first passage. Campbell repeatedly passed cells to new cultures from six to thirteen times, and he used the nuclei from the

transferred embryo cells, instead of nuclei from stem cells or primary culture embryo cells, to clone the two lambs.

The researchers transplanted nuclei from the transferred cells into oocytes, each of which had had its original nucleus removed, to form embryos, and then they implanted those embryos into surrogate ewes. When the time was near for the lambs to be born, Campbell, and other members of the team took turns watching the ewes from midnight to five in the morning each day to ensure that the pregnant ewes had no complications. The names of the lambs born in 1996 were Megan and Morag and they were the first cloned sheep. Campbell successfully transplanted nuclei from differentiated embryonic cells because he had discovered how to place these cells into a state called quiescence, which is a state when cell activity is limited. Quiescence more commonly called G0. This resting state is normally unattainable in stem cells, but is attainable in differentiated embryonic cells. Wilmut attributed the success of the experiment to Campbell's hypothesis that if you removed nuclei from differentiated embryonic cells at the G0 stage and transplanted them to oocytes with high MPF, then you could clone differentiated embryonic cells. With the success of Megan and Morag, Campbell insisted that researchers could clone sheep with nuclei from fully differentiated adult cells as well as from differentiated embryonic cells. This claim conflicted with the long-standing theory that once embryonic cells differentiated into specific kinds of adult cells, those adult cells could not return to a totipotent state, or retain the ability to reinitiate the development of a complete organism.

Campbell continued his research with the techniques developed from the Megan and Morag experiment and funding from a new partnership with PPL Therapeutics in

Roslin, Scotland. Campbell and his team extracted adult mammary cells from a six-year-old sheep and performed nuclear transplantation from these adult mammary cells into oocytes that had had their original nuclei removed. The team then implanted two hundred seventy-seven embryos, each of which had a nucleus and DNA from an adult mammary cell, into surrogate ewes. One of these attempts resulted in a pregnancy and successful birth of a female sheep in 1997. They named the sheep Dolly, after the singer Dolly Parton. They chose the name because Dolly was cloned from a mammary gland cell and, according to Wilmut, Parton offered an excellent example. Dolly the sheep's existence helped correct theories about the impossibility of cloning new organisms with differentiated adult cells. Wilmut later said that Campbell was the main contributor to the Dolly project. In a retrospective article, Wilmut claimed that Campbell deserves two thirds of the credit for cloning Dolly.

After Dolly was born, many people discussed the implications for human cloning as well as the authenticity of the experiment. However, genetic tests proved Dolly to be a clone, and accusations of fraud faded. Campbell defended his experiments, but opposed human cloning. At the same time as the Dolly experiment, Campbell cloned two more sheep named Taffy and Tweed from a nuclei of fetal fibroblast cells. By using fetal fibroblasts, Campbell then helped genetically modify and create a cloned sheep named Polly. Campbell left the Roslin Institute in 1997 during the Polly experiment to become the head of the embryology department for PPL Therapeutics, and he finished the Polly experiment with PPL Therapeutics.

Polly was the first mammal that scientists made to have genes from another species (transgenic) through genetic engineering techniques. Researchers altered

Polly's genes to express Factor IX, which is a human protein that doctors use to treat a type of hemophilia, a blood clotting disorder. Campbell's team incorporated the human Factor IX gene by transfection into embryonic fibroblast cells of sheep. Next they transplanted the fibroblast nuclei into unfertilized sheep oocytes to produce embryos, which they implanted in ewes, one of which yielded Polly. Campbell continued to work with and clone livestock, including sheep, pigs, and cattle. In 1999, Campbell helped clone the first gene-targeted mammal. These mammals were sheep named Cupid and Diana. Gene targeting is the process of placing genes on the chromosome exactly where they will be best expressed and controlled. Campbell's experiments with Cupid and Diana in 1999 led to the first piglets cloned from somatic cells, which were born in the year 2000. Campbell planned to use gene-targeted cloned pigs to transplant tissues from one species of animal to another.

In 1999, Campbell left PPL Therapeutics and became a professor of animal development at the University of Nottingham in Nottingham, England, where he continued his work on cloning. He also tried to make stem cells out of already differentiated cells. According to Wilmut, Campbell did not like bureaucracy and he enjoyed his time as a professor because he was better able to pursue his own research. During his time at Nottingham, Campbell served on scientific advisory boards for various academic organizations and companies. In 2008, Keith Campbell, Ian Wilmut, and Shinya Yamanaka in Japan received the Shaw Prize for Medicine and Life Sciences. Campbell died on 5 October 2012 at the age of fifty-eight.

Campbell, Keith. "Autobiography of Keith H S Campbell." *The Shaw Prize Foundation* 2008. <http://www.shawprize.org/en/shaw.php?tmp=3&twoid=49&theid=56&fourid=72&fiveid=15>. (Accessed January 25, 2013).

Campbell, Keith Henry Stockman, "Aspects of Cell Cycle Control in Yeast and *Xenopus*." PhD diss., University of Sussex, 1988.

Campbell, Keith Henry Stockman, Ian Wilmut, William A. Ritchie, and Jim McWhir. "Sheep Cloned by Nuclear Transfer from a Cultured Cell Line." *Nature* 380 (1996): 64–6.

Cramb, Auslan. "I Didn't Clone Dolly the Sheep, Says Prof." *Telegraph Media Group Limited*, March 8 2006, UK News. <http://www.telegraph.co.uk/news/uknews/1512377/I-didnt-clone-Dolly-the-sheep-says-prof.html>. (Accessed January 26, 2013).

Creanor, James, and John Murdoch Mitchison. "Protein Synthesis and Its relation to the DNA-Division Cycles in the Fission Yeast *Schizosaccharomyces Pombe*." *Journal of Cell Science* 69 (1984): 199–200. <http://jcs.biologists.org/content/69/1/199.full.pdf+html> (Accessed April 3, 2014).

Gurdon, John B. "The Transplantation of Nuclei between Two Species of *Xenopus*." *Developmental Biology* 5 (1962): 68–83.

Hardie, David Grahame. "How I Became a Biochemist." *International Union of Biochemistry and Molecular Biology Life* 59 (2007): 793–6. <http://onlinelibrary.wiley.com/doi/10.1080/15216540701556873/abstract> (Accessed April 1, 2014).

Mitchison, John Murdoch. *Sequences, Pathways and Timers in the Cell Cycle*. Academic Press, New York, 1974.

Polejaeva, Irina A., Shu-Hung Chen, Todd D. Vaught, Raymond L. Page, June Mullins, Suyapa Ball, Yifan Dai, Jeremy Boone, Shawn Walker, David L. Ayares, Alan Colman and Keith H. S. Campbell. "Cloned pigs produced by nuclear transfer from adult somatic cells." *Nature* 407 (2000): 86–90. <http://www.nature.com/nature/journal/v407/n6800/full/407086a0.html> (Accessed April 3, 2014).

Schnieke, Angelika E., Alexander J. Kind, William A. Ritchie, Karen Mycock, Angela R. Scott, Marjorie Ritchie, Ian Wilmut, Alan Colman, and Keith Henry Stockman Campbell. "Human Factor IX Transgenic Sheep Produced by Transfer of Nuclei from Transfected Fetal Fibroblasts." *Science* 278 (1997): 2130–3.

Trounson, Alan. "Keith Henry Campbell (1954–2012)." *Nature* 491 (2012): 193.

Wilmut, Ian. "Retrospective Keith Campbell (1954–2012)". *Science* 338 (2012): 1553.

Wilmut, Ian, and Roger Highfield. *After Dolly: The Uses and Misuses of Human Cloning*. New York: W.W. Norton & Company Ltd., 2006.

Wilmut, Ian, Keith Campbell, and Colin Tudge. *The Second Creation: Dolly and the Age of Biological Control*. Cambridge, Massachusetts: The Harvard University Press, 2000.

Wilmut, Ian, William A. Ritchie, Keith Henry Stockman Campbell. "Nuclear-cytoplasmic Interactions during the First Cell Cycle of Nuclear Transfer Reconstructed Bovine Embryos: Implications for Deoxyribonucleic Acid Replication and Development." *Biology of Reproduction* 49 (1993): 933–42. <http://www.biolreprod.org/content/49/5/933.long> (Accessed April 21, 2014).

Wilmut, Ian, Angelika E. Schnieke, Jim McWhir, Alexander J. Kind, and Keith Henry Stockman Campbell. "Viable Offspring Derived from Fetal and Adult Mammalian Cells." *Nature* 385 (1997): 810–3.

"Sheep Cloned by Nuclear Transfer from a Cultured Cell Line" (1996), by Keith Campbell, Jim McWhir, William Ritchie, and Ian Wilmut

Bartlett, Zane, ""Sheep Cloned by Nuclear Transfer from a Cultured Cell Line" (1996), by Keith Campbell, Jim McWhir, William Ritchie, and Ian Wilmut". Embryo Project Encyclopedia (2014-09-19). ISSN: 1940-5030 <http://embryo.asu.edu/handle/10776/8203>.

In 1995 and 1996, researchers at the Roslin Institute in Edinburgh, Scotland, cloned mammals for the first time. Keith Campbell, Jim McWhir, William Ritchie, and Ian Wilmut cloned two sheep, Megan and Morag, using sheep embryo cells. The experiments indicated how to reprogram nuclei from differentiated cells to produce live offspring, and that a single population of differentiated cells could produce multiple offspring. They reported their results in the article "Sheep Cloned by Nuclear Transfer

from a Cultured Cell Line" in March 1996. This experiment led the Roslin team to later clone mammals from adult body cells and to genetically engineer mammals.

Campbell developed techniques to reprogram cell nuclei. Campbell had used techniques of nuclear transplantation from earlier work with frog embryos at the University of Sussex in Sussex, UK. McWhir worked primarily with embryonic stem cells in livestock at the University of Cambridge in Cambridge, UK, before working at the Roslin Institute. At the Roslin Institute, Ritchie worked with large and small animal embryology and specialized in the ability to work with small tools and specimens under a microscope, called micromanipulation. Wilmut studied cow embryos and researched possible implications for cloning them.

The foundation for the 1995 through 1996 cloning experiment came from experiments performed on cow embryos in 1993 by Campbell, Wilmut, and Ritchie. In the cow experiments, Campbell, Wilmut, and Ritchie removed an egg cell's (oocyte) nucleus and replaced it with the nucleus of another cell. They identified a protein that regulates the cell cycle of an activity modifier called maturation promoting factor (MPF). MPF influences how a recipient oocyte accepts a donor nucleus. A low concentration of MPF in the oocyte means that the oocyte can receive a nucleus without chromosomal damage, called a universal recipient. Oocytes with a high MPF concentration can be used only if the nucleus has two viable copies of each chromosome or if the nucleus is placed while the cell is in a resting state called quiescence.

The scientists tested two hypotheses in the sheep experiments from 1994 to 1995. The first hypothesis was Jim McWhir's, who said that to have embryos created from nuclear transfer, it was necessary to have nuclei that could produce cells that could

develop into any cell type, called totipotent cells. McWhir hypothesized that researchers could culture totipotent sheep cells. The second hypothesis, presented by Campbell, was the claim that reprogramed differentiated cells could be totipotent. Campbell suggested that to use nuclei from differentiated cells for cloning, scientists only had to ensure that the cytoplasm from the receiving egg cell and the donor nucleus were in quiescence.

The scientists selected four groups of female sheep (ewes). Using two distinct breeds of sheep ensured that the offspring produced were the result of cloning and not the result of eggs fertilized within the oviduct. Scottish Blackface sheep provided the oocytes, which had nuclei removed (enucleated) and which became the receiving enucleated oocytes. These sheep constituted the first of the four groups used. The scientists obtained the oocytes by injecting ewes with a follicle-stimulating hormone, called gonadotropin-releasing hormone, which facilitates the release of oocytes from the ewes' ovarian follicles. The scientists flushed out the oocytes from the ewes' fallopian tubes with a saline solution then collected the oocytes.

Ritchie removed the nucleus from each oocyte under a microscope by extracting the nucleus with a glass pipet. Scientists confirmed enucleation by placing donor oocytes in calcium-free media containing a chemical that fragments DNA, Cytochalasin B, and a fluorescent dye that specifically stains DNA, Hoechst 33342, and looked at the glass pipet under ultraviolet light. If the pipet glowed due to the fluorescent dyes present in the DNA, and if the cytoplasm, or inner part of the cell, did not glow, then enucleation was successful. In 1994, the cytoplasts used were all low in MPF protein, thus ensuring the cytoplasts could accept nuclei. In 1995, the scientists divided the cytoplasts into three different MPF protein groups. Cytoplasts in the first group had low amounts of MPF

protein, the second group had high amounts of MPF protein at first, but declined to low MPF after embryo fusion, thus emulating natural fertilization, and the third group had high amounts of MPF protein both pre- and post-embryo fusion.

The scientists used two processes to obtain nuclei (karyoplasts) to implant into the enucleated oocytes. The first used McWhir's theory of deriving totipotent cells from nine-day-old embryos, which had formed into an embryo disc. The researchers placed these cells in a medium that contained nutrients as well as a protein that discourages cell differentiation but not cell multiplication, the protein leukemia inhibition factor (LIF). The researchers placed the embryo disc in an incubator for a few days then removed the cells from the medium and divided them into four new cultures. This process is called a passage, meaning the cells were passed into a new medium. McWhir and his colleagues continued for thirteen passages. By the fourth passage, the cells began to differentiate, despite the presence of LIF proteins in the medium. The scientists named the cells that maintained totipotency after thirteen passages totipotent for nuclear transfer, or TNT4. Accordingly, they called this portion of the experiment the TNT4 section.

Next, the scientists tested Campbell's hypothesis called the cell-cycle step of the procedure. Campbell's hypothesis was that they could reprogram diploid cells nuclei that were in a state of quiescence by placing them into a cytoplasm with high concentrations of MPF protein. Campbell said that cells deprived of growth factors entered quiescence. Welsh Mountain sheep provided the karyoplasts for the testing of Campbell's hypothesis. The experimenters held cytoplasts in place with a glass pipet. Using another glass pipet, Ritchie inserted the karyoplasts into cytoplasts through the hole created from enucleated cytoplasts. The researchers then placed combinations of cytoplasts and karyoplasts

between two electrodes and fused them together. To do so, they used electrical fusion methods developed by Steen Willadsen at the British Agricultural Research Council's Institute of Animal Physiology at Cambridge, UK, in the 1980s. A gelatinous solution obtained through boiling algae (agar) coated over the fused embryos protected them from environmental factors between transfers.

The scientists injected a third group of ewes with gonadotropin releasing hormone to simulate pregnancy. They surgically implanted the altered embryos into the ewes. These sheep were temporary holders of the embryos. The ewes incubated the embryos in their oviducts until the embryos began to differentiate and contain an inner cell mass, called blastocysts. The scientists then removed the blastocysts and examined them under a microscope to ensure development had begun.

The scientist's implanted two blastocysts, one into each uterine horn, of the surrogate Scottish Blackface ewes of the fourth group. Those ewes gave birth after about one hundred and forty-seven days. The researchers monitored the ewes to ensure there were no complications. Of the forty-seven embryos from TNT4 blastocysts, six were born. Two resulted from a sixteen-cell blastocyst, one resulted from a first passage blastocyst, one from a second passage blastocyst, and two from a third passage blastocyst. There was one pregnancy established from a sixth passage blastocyst, but it was lost at about seventy to eighty days. McWhir said that the lack of births from higher passage embryos was due to other factors, such as infections. The claim was disproved in the cell-cycle portion of the experiment.

In the cell-cycle portion, the researchers found no significant difference between variations in concentrations of MPF proteins among the blastocysts whose karyoplasts

were in the quiescent state. There were thirty-four embryos from the cell-cycle experiment, which developed from passages six to thirteen karyoplasts and transferred into ewes. The thirty-four embryos produced eight fetuses and resulted in five live births, all exhibiting characteristics of the same female Welsh Mountain lamb. Two of the lambs died within a few minutes of birth and a third one died ten days after birth due to unknown factors. The remaining two lambs, named Megan and Morag, were healthy and normal after nine months and could reproduce normally. Morag became pregnant by a ram when she was eighteen months old and had a lamb of her own. Of the two, Megan lived to at least age ten in 2005.

The results of the second half of the experiment enabled the scientists to show that the quiescent phase is the period of time when chromatin, or a mix of DNA and protein, undergoes differentiation and modification resulting in successful blastocysts past the sixth passage. The quiescent phase enables researchers a greater window of time to work with DNA. The window enabled the Roslin team to genetically engineer the sheep Polly, as reported in the 1997 article "Human Factor IX Transgenic Sheep Produced by Transfer of Nuclei from Transfected Fetal Fibroblasts." The 1995 and 1996 experiments also opened the possibility of cloning a sheep from an already differentiated adult somatic cell, which led to the creation of Dolly the Sheep in 1997.

"Sheep Cloned by Nuclear Transfer from a Cultured Cell Line" is a precursor to many other scientific experiments involving cloning. Campbell and his colleagues showed that differentiated cell's DNA had the ability to become totipotent. Totipotent cells could create many offspring from cell line, thus producing identical offspring. In the

early twenty-first century, embryology and cloning methods use the techniques and theories developed from these experiments.

Campbell, Keith Henry Stockman. "Aspects of Cell Cycle Control in Yeast and Xenopus." PhD diss., University of Sussex, 1988.

Campbell, Keith Henry Stockman, Ian Wilmut, William A. Ritchie, and Jim McWhir. "Sheep Cloned by Nuclear Transfer from a Cultured Cell Line." *Nature* 380 (1996): 64–6.

Highfield, Roger. "Megan, Friend of Dolly, Turns 10." *The Age*, June 17, 2005, Science section, Australia. <http://www.theage.com.au/news/Science/Megan-friend-of-Dolly-turns-10/2005/06/16/1118869038992.html> (Accessed February 9, 2013).

McGrath, John A., Davor Solter. "Inability of Mouse Blastomere Nuclei Transferred to Enucleated Zygotes to Support Development In Vitro." *Science* 220 (1984): 1300–2.

Ritchie, William. Roslin Embryology Limited, "About Us." <http://www.roslinembryology.co.uk/page2.html> (Accessed February 9, 2013).

Schnieke, Angelika E., Alexander J. Kind, William A. Ritchie, Karen Mycock, Angela R. Scott, Marjorie Ritchie, Ian Wilmut, Alan Colman, and Keith H. S. Campbell. "Human Factor IX Transgenic Sheep Produced by Transfer of Nuclei from Transfected Fetal Fibroblasts." *Science* 278 (1997): 2130–3.

Walladsen, Steen Malte, "Nuclear Transplantation in Sheep Embryos." *Nature* 320 (1986): 63–5.

Wilmut, Ian, and Roger Highfield. *After Dolly: The Uses and Misuses of Human Cloning*. New York: W.W. Norton & Company Ltd., 2006.

Wilmut, Ian, Keith Campbell, and Colin Tudge. *The Second Creation: Dolly and the Age of Biological Control*. Cambridge, Massachusetts: The Harvard University Press, 2000.

Wilmut, Ian, William A. Ritchie, Keith Henry Stockman Campbell. "Nuclear-cytoplasmic Interactions during the First Cell Cycle of Nuclear Transfer Reconstructed Bovine Embryos: Implications for Deoxyribonucleic Acid Replication and Development." *Biology of Reproduction* 49 (1993): 933–42. <http://www.biolreprod.org/content/49/5/933.long> (Accessed April 21, 2014).

Wilmut, Ian, Angelika E. Schnieke, Jim McWhir, Alexander J. Kind, and Keith H.S Campbell. "Viable Offspring Derived from Fetal and Adult Mammalian Cells." *Nature* 385 (1997): 810–3.

"Viable Offspring Derived from Fetal and Adult Mammalian Cells" (1997), by Ian Wilmut et al.

Bartlett, Zane, ""Viable Offspring Derived from Fetal and Adult Mammalian Cells" (1997), by Ian Wilmut et al." Embryo Project Encyclopedia (2014-10-10). ISSN: 1940-5030
<http://embryo.asu.edu/handle/10776/8224>.

In the 1990s, Ian Wilmut, Jim McWhir, and Keith Campbell performed experiments while working at the Roslin Institute in Roslin, Scotland. Wilmut, McWhir, and Campbell collaborated with Angelica Schnieke and Alex J. Kind at PPL Therapeutics in Roslin, a company researching cloning and genetic manipulation for livestock. Their experiments resulted in several sheep being born in July 1996, one of which was a sheep named Dolly born 5 July 1996. Dolly was the first sheep cloned and developed from the nuclei of fully differentiated adult cells, rather than from the nuclei of early embryonic cells. They published their results in "Viable Offspring Derived from Fetal and Adult Mammalian Cells" (abbreviated Viable Offspring) on 27 February 1997.

In 1993, Wilmut, Campbell, and collaborators experimented on cow embryos at the Roslin Institute. These experiments helped develop the theory that differentiated cells in an appropriate environment could develop similar to embryonic cells. For the process of cloning organisms without defects, the cow embryo experiments revealed the optimal cell cycle stage and the need to avoid chromosomal damage when transferring nuclei.

Further information came from experiments described in the 1996 article titled "Sheep Cloned by Nuclear Transfer from a Cultured Cell Line." Campbell, McWhir, Ritchie, and Wilmut conducted experiments in which they cloned sheep from an established embryonic cell line experiments that produced the sheep named Megan and Morag. The scientists developed techniques and concepts from the sheep experiments, including how transferring nuclei during the resting state in the cell cycle called quiescence improves success rates. In the experiments, the scientist removed the nucleus from an egg cell and inserted a donor egg nucleus to replace the removed nucleus, a modified version of the technique called somatic cell nuclear transfer.

Wilmut was part of various animal embryo experiments at the Roslin Institute. Schnieke studied bioengineering at the Heinrich-Pette Institute in Hamburg, Germany, and subsequently at the Whitehead Institute of the Massachusetts Institute of Technology in Cambridge, Massachusetts. Before coming to the Roslin Institute, she researched modes to transmit genetic material into cells using RNA viruses of the *Retroviridae* family called retroviral vectors to incorporate therapeutic genes into host cells. She also worked on the production of cross-species genetic modified or transgenic animal models, and preventing specific gene expression in a process called gene knockout.

McWhir worked with embryonic stem cells in livestock at the University of Cambridge in Cambridge, United Kingdom, before working at the Roslin Institute. Kind was Schnieke's husband and worked at PPL Therapeutics. Keith Campbell had previously studied the reprogramming of cell nuclei as well as the transplantation of frog nuclear material. Campbell used an experimental method developed by Robert Briggs and

Thomas Joseph King in 1952 called nuclear transplantation at the Institute for Cancer Research in Fox Chase, Pennsylvania, which in 1974 became the Fox Chase Cancer Center in Philadelphia, Pennsylvania.

Alan Coleman directed research at the Roslin Institute at the time of the Dolly experiments. Coleman received his PhD in 1974 while studying with John Gurdon at Cambridge University, he worked on theories of cloning, and he often used frogs for his research. Gurdon's 1975 experiments did not show that adult cell nuclei could create fully developed adult frogs. However, after the sheep experiments in 1996 showed the success of transplanting cells in quiescence, the scientists at the Roslin Institute hypothesized that they could clone a mammal from adult cells, despite Gurdon's results that indicated otherwise. Wilmut later said that Coleman was somewhat skeptical, due to his experience with Gurdon, of what the scientists could accomplish.

The researchers tested their hypothesis with embryonic, fetal, and adult sheep cell samples. The embryonic cells came from a day-nine embryo, the fetal cells came from a day-twenty-six fetus, and the adult cells came from a mammary gland of a six-year-old female sheep (ewe) that was in her last trimester of pregnancy. Schnieke suggested using the mammary cells because they appeared similar to the embryo cells used to clone the first sheep Megan and Morag in the 1995 sheep experiment. The scientists used the method derived from 1995 experiments "Sheep Cloned by Nuclear Transfer from a Cultured Cell Line" to implant the cells of different ages into recipient egg cells.

The cells began embryo formation and the scientists then transferred the embryos that developed into a thin-walled early embryo, called a blastocyst, into awaiting ewes, and they used ultrasound to check on prenatal development. The results of

the ultrasound showed twenty-one fetuses developing normally in fifty to sixty days into the experiment. After the fifty to sixty day interval, the scientists continued to perform ultrasounds in fourteen-day intervals. Sixty-two percent of the fetuses were lost during the intervals after the fifty to sixty days, which, they suggested in the experiment was much greater than the natural prenatal loss of six percent.

The scientists dissected ewes whose fetuses did not survive to investigate why the fetuses did not develop. They found that four of the dead fetuses were specimens from embryo-derived cells. Two of these fetuses had abnormal liver development, but other than this defect, there was no sign of infection or other irregularity in development from the ewes or the fetuses.

Eight ewes gave birth to live lambs and the offspring represented each of the three cell types, four lambs were born from embryo-derived cells, three were born from fetal cells, and one was born from adult mammary-derived cells. There was one lamb born from fetal cells that died a few minutes after birth. After conducting a post-mortem analysis, the researchers found no abnormalities or signs of infection in this lamb. The researchers concluded that of all of the lambs born, twelve and a half percent died soon after birth. They considered the loss as similar to the eight percent perinatal loss of sheep born naturally. Each of the lambs showed characteristics of the breed that donated the nuclei and not the characteristics of the breed that donated the cytoplasm. These characteristics indicated that the lambs born were clones of the cell that donated their nuclei and not from the originating egg (oocyte) donor. DNA analysis also indicated that each of the lambs' DNA came from their nuclear donors.

The researchers recommended further experimentation to determine exactly what stage in the cell cycle is optimum for deriving nuclear and oocyte donors. The results of their experiment point to using cells that are in the quiescent state, but the experiment itself did not use the optimum cell cycle stage to retrieve donors' oocytes.

In the 1997 article, the scientists reflected on the sheep born from mammary cells, Dolly. According to the scientists, she was the first mammal to develop as a clone from an adult cell. They also said that there is a possibility that among the cell culture there could have been an undifferentiated stem cell present. An undifferentiated stem cell could have been responsible for allowing Dolly to develop to term. Regardless of this small probability, the birth of Dolly showed that once cells mature, control of gene expression is by the cell nucleus as well as changes in the cytoplasm. According to Campbell, this experiment helped to explain the reprogramming of cell DNA and consequently, the process of differentiation of stem cells into specific types of cells.

After the results of the experiment were published, Dolly and the scientists responsible for cloning her received much publicity. Dolly had her photo taken by sixteen film crews and sixty photographers within the first few weeks of the announcement. Wilmut later said that Dolly, amidst all of the attention, only became more tame and spoiled than she was before. Reactions from the media were at first critical and negative. According to Campbell, the negative attitudes toward Dolly and cloning came largely from implications that the same techniques would enable scientists to clone humans.

Wilmut later mentioned that Gurdon reported being shocked that Dolly existed because Gurdon could not produce similar results in his own experiments with frogs using adult nuclei. The Vatican and other religious groups voiced fears

about cloning humans. The President of the United States in 1997, Bill Clinton, called for a worldwide moratorium on similar experiments and asked a bioethics commission to report on the implications of the Dolly experiment and the ethical questions in relation to human cloning. Some scientists claimed that Dolly was not a clone of an adult cell at all, but that she developed from a stem cell present in the mammary tissue. Another paper published in 1998 that showed Dolly indeed developed from an adult differentiated cell and refuted these claims.

The controversies behind the Dolly experiment diminished with time as other experiments verified the results of those at the Roslin Institute. Other scientists soon cloned other livestock, and scientists in Japan cloned offspring from a Japanese Black cattle bull named Kamitakafuku in 1998. The Dolly experiment also enabled a later experiment, "Human Factor IX Transgenic Sheep Produced by Transfer of Nuclei from Transfected Fetal Fibroblasts" in which researchers cloned sheep that had human genes.

Dolly developed as normal sheep do. Dolly bred with a male sheep and gave birth to six lambs, the first born in 1998. In autumn of 2001, Dolly developed arthritis. The cause of the arthritis was unknown, but it was not attributed to her cloned status. In 2000, one of the cloned sheep in the experiment died of a pulmonary virus that causes incurable tumors in sheep. Dolly died of the same disease on 14 February 2003, likely due to exposure to this virus in 2000.

"A Life of Dolly." University of Edinburgh. <http://www.roslin.ed.ac.uk/public-interest/dolly-the-sheep/a-life-of-dolly/> (Accessed February 23, 2013).

Bauman, David. "Scientists Use Long-term Cultured Cells for Cloning." *University of Connecticut News* January 2000. <http://news.uconn.edu/2000/January/rel00001.htm> (Accessed July 25, 2014).

Briggs, Robert, and Thomas Joseph King. "Transplantation of Living Nuclei from Blastula Cells into Enucleated Frogs' Eggs." *PNAS* 38 (1952): 455–63. <http://www.pnas.org/content/38/5/455.full.pdf> (Accessed July 25, 2014).

Campbell, Keith Henry Stockman, Ian Wilmut, and William A. Ritchie. "Nuclear-Cytoplasmic Interactions during the First Cell Cycle of Nuclear Transfer Reconstructed Bovine Embryos: Implications for Deoxyribonucleic Acid Replication and Development." *Biology of Reproduction* 49 (1993): 933–42. <http://www.biolreprod.org/content/49/5/933.long> (Accessed April 21, 2014).

Campbell, Keith Henry Stockman, Ian Wilmut, William A. Ritchie, and Jim McWhir. "Sheep Cloned by Nuclear Transfer from a Cultured Cell Line." *Nature* 380 (1996): 64–6.

Gurdon, John B., Ronald A. Laskey, and O. Raymond Reeves. "The Developmental Capacity of Nuclei Transplanted from Keratinized Skin Cells of Adult Frogs." *Journal of Embryology and Experimental Morphology* 34 (1975): 93–112.

"Prof. Angelika Schnieke." Technische Universität München. <http://btn.wzw.tum.de/index.php?id=44> (Accessed February 23, 2013).
Schnieke, Angelika E., Alexander J. Kind, William A. Ritchie, Karen Mycock, Angela R. Scott, Marjorie Ritchie, Ian Wilmut, Alan Colman, and Keith H. S. Campbell. "Human Factor IX Transgenic Sheep Produced by Transfer of Nuclei from Transfected Fetal Fibroblasts." *Science* 278 (1997):2130–3.

Singer, Ester N., Yuri E. Dubrova, Alex J. Jeffreys, Colin Wilde, Lynn M.B. Finch, Michelle Wells, and Malcom Peaker. "DNA Fingerprinting Dolly." *Nature* 394 (1998): 329–30.

"The Importance of Dolly." University of Edinburgh. <http://www.roslin.ed.ac.uk/public-interest/dolly-the-sheep/the-importance-of-dolly/> (Accessed February 23, 2013).

Wilmut, Ian, and Roger Highfield. *After Dolly: The Uses and Misuses of Human Cloning*. New York: W.W. Norton & Company Ltd., 2006.

Wilmut, Ian, Keith Campbell, and Colin Tudge. *The Second Creation: Dolly and the Age of Biological Control*. Cambridge, Massachusetts: The Harvard University Press, 2000.

Wilmut, Ian, William A. Ritchie, Keith Henry Stockman Campbell. "Nuclear-cytoplasmic Interactions during the First Cell Cycle of Nuclear Transfer Reconstructed Bovine Embryos: Implications for Deoxyribonucleic Acid Replication and Development." *Biology of Reproduction* 49 (1993): 933–42. <http://www.biolreprod.org/content/49/5/933.long> (Accessed April 21, 2014).

Wilmut, Ian, Angelika E. Schnieke, Jim Mcwhir, Alexander J. Kind, and Keith Henry Stockman Campbell. "Viable Offspring Derived from Fetal and Adult Mammalian Cells." *Nature* 385 (1997): 810–3.

"Human Factor IX Transgenic Sheep Produced by Transfer of Nuclei from Transfected Fetal Fibroblasts" (1997), by Angelika E. Schnieke, et al.

Bartlett, Zane, "'Human Factor IX Transgenic Sheep Produced by Transfer of Nuclei from Transfected Fetal Fibroblasts" (1997), by Angelika E. Schnieke, et al.'" Embryo Project Encyclopedia (2014-08-19). ISSN: 1940-5030 <http://embryo.asu.edu/handle/10776/8150>.

In the 1990s, researchers working at the Roslin Institute in Edinburgh, Scotland, performed cloning experiments in collaboration with PPL Therapeutics in Roslin, Scotland, on human coagulation factor IX, a protein. The team of scientists used the methods identified during the Dolly experiments to produce transgenic livestock capable of producing milk containing human blood clotting factor IX, which helps to treat a type of hemophilia. By using a cell's resting state, called quiescence, or G₀, and transferring modified nuclear material from one cell to an egg cell that had had its nuclear material removed, the researchers developed a method to produce genetically modified mammals, including humans. Angelika E. Schnieke, Alexander J. Kind, William A. Ritchie, Karen Mycock, Angela R. Scott, Marjorie Ritchie, Ian Wilmut, Alan Colman, and Keith H. S. Campbell published the results of their experiments as "Human Factor IX Transgenic

Sheep Produced by Transfer of Nuclei from Transfected Fetal Fibroblasts" (hereafter called "Human Factor IX"). The article details the methods that produced the cloned sheep named Polly, as well as other cloned and genetically altered sheep.

In 1985, Robert E. Hammer's group at University of Pennsylvania in Philadelphia, Pennsylvania, produced animals that expressed genes from other species, called transgenic animals. Scientists genetically manipulated the animals in a process called pronuclear microinjection. In this technique, scientists inject DNA containing the desired gene into the nucleus of the egg, called a pronucleus, just before the pronuclei of the egg and sperm combine during fertilization. Some of the resulting animals are born with the injected genes expressed, but the success rate is low. Genetic material can also recombine in such a way that it becomes a detriment to the organism, or in such a way that not all of the cells in the organism have the gene.

In 1996 research on sheep embryos at the Roslin Institute, prior to the "Human Factor IX" experiment, established the claim that genetic alterations on adult body or somatic cell nuclei were possible. The researchers found that cloned sheep could express genes from another species by transferring altered nuclei into embryonic cells. The 1997 article "Viable Offspring Derived from Fetal and Adult Mammalian Cells" by Wilmut, Schnieke, and Jim McWhir, Kind, and Campbell reported the earlier sheep embryo experiments.

Other information for "Human Factor IX" came from the 1996 experiment, "Sheep Cloned by Nuclear Transfer from a Cultured Cell Line." Campbell, McWhir, Ritchie, and Wilmut cloned sheep from embryonic cells grown in a laboratory in 1996. In

that experiment, the researchers established that if they transferred genetic material into a cell that was in quiescence, then they could more efficiently clone the cells.

Before coming to the Roslin Institute, Schnieke studied the modes of transmission for RNA viruses, the production of cross-species genetic modification, or transgenic animal models, and the deactivation of specific genes from producing proteins. Kind was Schnieke's husband and worked at PPL Therapeutics. Ritchie specialized in micromanipulation procedures, or the ability to work with small tools and specimens under a microscope. He had previously performed procedures in other sheep embryo experiments, and he had helped with the same techniques that enabled the cloning of Dolly the sheep in 1996. Mycock was a technician who helped with embryological manipulation procedures in the lab with Ritchie. Scott was a technician working for PPL Therapeutics. Scott multiplied the cell cultures needed for the experiment. Marjorie Ritchie, William Ritchie's wife, organized the surgeries needed to implant the sheep embryos. Lastly, Wilmut researched animal genetics and was part of various animal embryo experiments at the Roslin Institute. Coleman was research director of the Roslin Institute at the time of the Dolly and Polly experiments. Campbell studied microbiology and had worked previously with reprogramming cell nuclei as well as transplantation of nuclear material in other experiments at the Roslin Institute.

The team of scientists used the methods identified during the Dolly experiments to produce transgenic livestock capable of producing milk containing human blood clotting factor IX, which helps to treat a type of hemophilia. Scientists had produced transgenic livestock since 1985, but only about five percent of these animals expressed a transgenic DNA, or transgene, in their own genomes. Among these animals, few of the

transgenes passed naturally to offspring. The researchers claimed that it is simpler to incorporate foreign DNA into adult cell nuclei as opposed to juvenile nuclei, a process called transfection. The scientists, first transfected adult cells and then used the methods introduced from cloning Dolly to transfer the modified nuclei into awaiting enucleated oocytes, and then they cloned a transgenic sheep.

The scientists used donor nuclei from cells, which the scientists said were fetal fibroblast cells, of thirty-five-day-old fetuses of a Poll-Dorset sheep. The donor nuclei, modified to be immune to the antibiotic neomycin, enabled the scientists to check that the cells in fetal sheep were from the implanted embryo. Immunity to neomycin would also identify the cells modified to have the transgenes. The researchers used a method called lipofection, which uses the chemical lipofectamine to penetrate the outer membrane of cells, to insert the DNA into these cells. Some fetal fibroblasts were non-transfected and used as controls.

The scientists deprived the cells of growth factors and induced them into a state of quiescence. The scientists then found that even though the cells had been transfected, they could withstand the lack of growth factors and resumed the cell cycle without damage after the reintroduction of the growth factors. The researchers transplanted four types of nuclei into enucleated egg cells: male nuclei without transfection, female nuclei without transfection, female nuclei with transfection of the FIX gene at less than five copies, and female nuclei with the transfection of the FIX gene with more than ten copies.

The researchers then transplanted the embryos into Scottish Blackface sheep, examined the pregnancies, and recorded the resulting births. Of the lambs born, there

were one non-transfected male, three non-transfected females, two transfected females with more than ten copies of the gene, and one transfected female with less than five copies of the gene. One of the female transfected lambs expressed the gene products at a particularly high level. The lamb was cloned from the Poll Dorset breed of lamb and was therefore name Polly. Each of the transgenic lambs was completely transgenic and not partially transgenic. A partially transgenic lamb would contain only a few cells that express the gene. Sheep born from microinjection techniques often are partially transgenic. Cloning produced one transgenic lamb per 20.8 sheep, as opposed to one transgenic lamb produced per 51.4 sheep by microinjection. The experiment indicated that cloning procedures produce a greater number of transgenic lambs than do microinjection techniques.

The scientists found some of the births problematic. The gestations of all of the lambs were longer than the normal gestational term for this particular breed of sheep. There were no increases in mortality rate or disease, in the ewes however, a result that Wilmut indicated was important in understanding that genetic modification does not increase death rate.

Outside the research team, scientists questioned whether transgenic animals were capable of reproducing and having offspring that contained the same genes. Eventually the transgenic sheep reproduced and had offspring with the same FIX protein present in the cells.

According to Wilmut, animal activists advocated against and misunderstood this experiment. Some activists claimed that Polly contained human characteristics. Wilmut

explained that while Polly's genome contains a human gene, it is only one gene out of thousands of normal sheep genes.

The "Human Factor IX Transgenic Sheep Produced by Transfer of Nuclei from Transfected Fetal Fibroblasts" experiment successfully produced a sheep that can express a human gene resulting in a human protein. Quiescence and nuclear transfer improved the ability to produce transgenic animals.

Campbell, Keith H. S., Jim McWhir, William A. Ritchie, and Ian Wilmut. "Sheep Cloned by Nuclear Transfer from a Cultured Cell Line." *Nature* 380 (1996): 64–6.

Hammer, Robert E., Vernon G. Pursel, Caird E. Rexroad Jr., Robert J. Wall, Douglas J. Bolt, Karl M. Ebert, Richard D. Palmiter, and Ralph L. Brinster. "Production of Transgenic Rabbits, Sheep, and Pigs by Microinjection." *Nature* 315 (1985): 680–3.

Ritchie, William. Roslin Embryology Limited, "About Us." <http://www.roslinembryology.co.uk/page2.html> (Accessed February 9, 2013).

Schnieke, Angelika E., Alexander J. Kind, William A. Ritchie, Karen Mycock, Angela R. Scott, Marjorie Ritchie, Ian Wilmut, Alan Colman, and Keith H. S. Campbell. "Human Factor IX Transgenic Sheep Produced by Transfer of Nuclei from Transfected Fetal Fibroblasts." *Science* 278 (1997): 2130–3.

Wilmut, Ian, Angelika E. Schnieke, Jim Mcwhir, Alexander J. Kind, and Keith H.S Campbell. "Viable Offspring Derived from Fetal and Adult Mammalian Cells." *Nature* 385 (1997): 810–3.

Wilmut, Ian, Keith Campbell, and Colin Tudge. *The Second Creation: Dolly and the Age of Biological Control*. Cambridge, Massachusetts: The Harvard University Press, 2000.

Wilmut, Ian, and Roger Highfield. *After Dolly: The Uses and Misuses of Human Cloning*. New York: W.W. Norton & Company Ltd., 2006.

The Roslin Institute (1993-)

Bartlett, Zane, "The Roslin Institute (1993-)". Embryo Project Encyclopedia (2014-09-29). ISSN: 1940-5030 <http://embryo.asu.edu/handle/10776/8207>.

The Roslin Institute was established in 1993 in the village of Roslin, Scotland, as an independent research center by the Biotechnology and Biological Sciences Research Council (BBSRC), and as of 2014 is part of the University of Edinburgh in Edinburgh, Scotland. Researchers at the Roslin Institute cloned the Dolly the sheep in 1996. According to the Roslin Institute, Dolly was the first mammal to develop into an adult from the transfer of the nucleus of an adult sheep cell into an ovum with the nucleus removed. The Roslin Institute performs genetic and medical based animal studies to help investigate human physiology and medicine and to improve agricultural research. The Roslin Institute studies embryology, cloning, hormones, and genetic alterations in animals and techniques such as somatic cell nuclear transfer.

Prior to the establishment and naming of the Roslin Institute in 1993, the University of Edinburgh created the Institute of Animal Genetics in 1919, the precursor to the Roslin Institute. In 1947 the Agricultural Research Council, later called the Agriculture and Food Research Council (AFRC), created the Poultry Research Centre (PRC) and the Animal Breeding Research Organisation (ABRO) as part of the Institute of Animal Genetics to help farmers find efficient ways to produce more food and livestock. The AFRC changed the name of the Institute of Animal Genetics to the Unit of Animal Genetics (UAG) and the University of Edinburgh maintained its presence at the UAG. In 1985, the AFRC reviewed greater than thirty Institutes and Units, among them were the UAG, PRC, and ARBO. The review contributed to the closure of the UAG in the same

year and the combining of PRC and ABRO with the Institute of Animal Physiology, located at the Babraham Institute in Cambridge, United Kingdom. The name of the new institute was the Institute of Animal Physiology and Genetics Research. In 1985, the staff relocated from ABRO to the PRC site located in Roslin, Scotland, and they formed the Edinburgh Research Station of the Institute of Animal Physiology and Genetics Research.

In 1992, the AFRC planned to separate the Roslin and Babraham Institutes. The AFRC provided each institute with unique objectives. The objective for the Roslin Institute was to focus on livestock improvements. On 1 April 1993, the officially named Roslin Institute formed as an independent institution owned by the BBSRC. In 1995, the Institute became a company limited by guarantee, which meant that it was a non-profit organization. The Institute was sponsored by a Scottish Charity and funded by the BBSRC. In 2008, the Roslin Institute became part of the College of Medicine and Veterinary Medicine of the University of Edinburgh, and the old legal entity continued as the Roslin Foundation. In 2011, the main research center for the Roslin Institute moved from Roslin to a new building located in Easter Bush, Scotland, on the University of Edinburgh Veterinary campus. As of 2014, the Roslin Institute remained affiliated with the University of Edinburgh and received funds from the BBSRC and the Roslin Foundation.

Various committees and groups run the Roslin Institute. All of the committees report to the Roslin Institute Executive Committee. The committees include the Science Management Group, which leads research and sets scientific goals, and the Business Finance Group, which handles everything else relating to business and finances of the institute. A director or chairperson leads the institute's executive committee and the

director reports to the University of Edinburgh. The director of the Roslin Institute in 2014 is David Hume.

The Roslin Institute was founded on six principles with the collaboration of the BBSRC. The first principle was to enhance animal health and welfare through knowledge of genetic factors affecting resistance to disease. The second principle was to enhance sustainability and productivity of livestock systems and food supply chains through understanding of reproductive and developmental biology. The third principle was to enhance food safety by understanding interactions between disease-causing organisms and animals. The fourth principle was to enhance human health through an understanding of basic mechanisms of health and disease and comparative biology of animal species. The fifth was to identify new and emerging process of infectious disease transmission between species, called zoonoses, and to understand how pathogens might cross from animals to humans. The sixth principle was to enhance the quality of life for animals by studying the mechanisms and behaviors associated with optimizing their environment and life experiences. As of 2014, these goals organized the Roslin Institute.

Researchers have pursued those goals through experiments at the Roslin Institute. These include experiments performed on cow embryos in 1993 by Ian Wilmut, William Ritchie, and Keith Campbell. These preliminary experiments led researchers to clone sheep from differentiated cells in the experiment entitled "Sheep Cloned by Nuclear Transfer from a Cultured Cell Line" by Campbell, Wilmut, Ritchie, and Jim McWhir in 1996. The scientists developed the techniques used in this sheep experiment that researchers applied to many future Roslin Institute experiments, including the cloning of Dolly the sheep. Campbell also formulated the plan to place cells in the

resting state, called quiescence, or induced cellular stasis, necessary for transfer and cloning of adult cells during the sheep experiments. According to Wilmut, placing cells in quiescence was an important insight needed to understand the mechanics of cloning and the cell cycle that are universal in mammals, including humans.

Experiments conducted by the organization included the "Viable Offspring Derived from Fetal and Adult Mammalian Cells" experiments in 1996. These experiments cloned sheep from adult sheep body or somatic cells. The scientists named the cloned sheep Dolly. She was the first mammal cloned from an adult differentiated cell. Dolly became famous internationally and the Roslin Institute received much recognition from the scientific community. The director of the institute at the time of the experiment was Graham Bulfield. Among the scientists in the experiment were Wilmut, McWhir, and Campbell, who had also contributed to previous sheep and cow embryo experiments at the Roslin Institute.

The work performed at the Roslin Institute spurred debates on the issue of cloning human beings. Some people argued against the practice of cloning animals because they said it would lead to cloning humans. Others claimed that cloning would bring great advances. Many of the scientists who worked at the Roslin Institute in 1996 received phone calls requesting clones of deceased loved ones.

After the Dolly experiment, the Roslin Institute continued to clone animals. The Institute aimed to engineer animals to produce proteins that could act as medicines in large quantities. The animals could produce cheaper, purer, and non-contaminated pharmaceuticals. In 1997, the Roslin Institute developed a sheep that produced a human protein in its milk in the experiment titled "Human Factor IX Transgenic Sheep Produced

by Transfer of Nuclei from Transfected Fetal Fibroblasts." The Roslin Institute worked to transfer human genes into animal cells (transgenic animals). For example, in 2000, Roslin researchers modified pigs to reduce the potential rejection rate of transplanted organs from other animal species (xenotransplanted) into humans. However, a few scientists argued that altering and researching human embryos as opposed to animals could make more progress.

In the year 2001, the Roslin Institute contacted the licensing agency for clinics and research involving human embryos in the UK, the Human Fertilisation and Embryology Authority (HFEA), to receive a license to work with cloning human embryos. The license was delayed for a variety of reasons. According to Wilmut, these reasons involved heavy amounts of regulatory paperwork as well as meetings with many committees and ethical assessments involving the HFEA. Furthermore, controversies were also present over the request to the HFEA to work on human embryos. For example, anti-abortion parties criticized the Roslin Institute in 2002 for requesting permission to experiment on human embryos. In 2004, a research group led by Woo-Suk Hwang in Seoul, South Korea, reported to have cloned human embryos using somatic cell nuclear transfer. The Journal retracted the paper after learning that Hwang's group falsified much of the data. The political reaction from the Hwang scandal further delayed the Roslin Institute's HFEA license, which it received in February of 2005. The Institute studied motor neuron disease in humans and how early development can affect this disease, but the Institute used donated embryos as opposed to cloned embryos. By 2013, the Roslin Institute no longer cloned animals, Wilmut left in 2005 to become director of the Centre

for Regenerative Medicine at the University of Edinburgh and most of the group members had moved on to other organizations.

Roslin researchers have further experimented with transgenic animals and genetic manipulation in animals. An example involves a type of chicken that the Institute calls a genetically modified chicken. Scientists have genetically altered these chickens so they do not transmit avian bird flu. Roslin researchers have also investigated animal behavior, for example, scientists at the Institute have investigated the role of the hormone vasopressin in animal behavior and mating. According to Wilmut, the Institute also frequently receives correspondence in regards to animal cruelty and testing.

Anonymous. "GM Chicken Could Fight H5N1." *Poultry World*, 165 February 2011, News section, Sutton, United Kingdom.

Campbell, Keith Henry Stockman, Ian Wilmut, William A. Ritchie, and Jim McWhir. "Sheep Cloned by Nuclear Transfer from a Cultured Cell Line." *Nature* 380 (1996): 64–6.

Campbell, Keith Henry Stockman, William A. Ritchie, and Ian Wilmut. "Nuclear-cytoplasmic Interactions during the First Cell Cycle of Nuclear Transfer Reconstructed Bovine Embryos: Implications for Deoxyribonucleic Acid Replication and Development." *Biology of Reproduction* 49 (1993): 933–42. <http://www.biolreprod.org/content/49/5/933.short> (Accessed April 9, 2014).

Edinburgh Science Triangle. "History of Roslin BioCentre." Roslin BioCentre. <http://www.roslinbiocentre.com/about/history> (Accessed March 30, 2013).

Hwang, Woo Suk, Young June Ryu, Jong Hyuk Park, Eul Soon Park, Eu Gene Lee, Ja Min Koo, Hyun Yong Jeon, Byeong Chun Lee, Sung Keun Kang, Sun Jong Kim, Curie Ahn, ung Hye Hwang, Ky Young Park, Jose B. Cibelli, Shin Yong Moon. "Evidence of a Pluripotent Human Embryonic Stem Cell Line Derived from a Cloned Blastocyst." *Science* 303 (2004): 1669–74. (Retracted January 12, 2006). <http://www.sciencemag.org/content/303/5664/1669.full> (Accessed May 9, 2014).

Kennedy, Donald. "Retraction of Hwang et al." *Science* 311 (2006): 335.

Schnieke, Angelika E., Alexander J. Kind, William A. Ritchie, Karen Mycock, Angela R. Scott, Marjorie Ritchie, Ian Wilmut, Alan Colman, and Keith Henry Stockman Campbell. "Human Factor IX Transgenic Sheep Produced by Transfer of Nuclei from Transfected Fetal Fibroblasts." *Science* 278 (1997): 2130–3.

Tobin, Vicky A., Hirofumi Hashimoto, Douglas W. Wacker, Yuki Takayanagi, Kristina Langnaese, Celine Caquineau, Julia Noack et al. "An Intrinsic Vasopressin System in the Olfactory Bulb is Involved in Social Recognition." *Nature* 464 (2010): 413–7.

University of Edinburgh. "History of The Institute."
Roslin. <http://www.roslin.ed.ac.uk/about-roslin/history-of-the-institute/> (Accessed March 30, 2013).

University of Edinburgh. "Project List."
Roslin. <http://www.roslin.ed.ac.uk/simone-meddle/project-list/> (Accessed March 30, 2013).

Walsh, Brian. "A Re-Engineered Chicken and the War on Bird Flu." *Time* 13 January 2011. <http://content.time.com/time/health/article/0,8599,2042381,00.html> (Accessed May 12, 2014).

Wilmut, Ian and Roger Highfield. *After Dolly: The Uses and Misuses of Human Cloning*. New York: W.W. Norton & Company Ltd., 2006.

Wilmut, Ian, Keith Campbell, and Colin Tudge. *The Second Creation: Dolly and the Age of Biological Control*. Cambridge, Massachusetts: The Harvard, 2000.

Wilmut, Ian, Angelika E. Schnieke, Jim Mcwhir, Alexander J. Kind, and Keith Henry Stockman Campbell. "Viable Offspring Derived from Fetal and Adult Mammalian Cells." *Nature* 385 (1997): 810–3.

Somatic Cell Nuclear Transfer in Mammals (1938-2013)

Bartlett, Zane, "Somatic Cell Nuclear Transfer in Mammals (1938-2013)". Embryo Project Encyclopedia (2014-11-04). ISSN: 1940-5030 <http://embryo.asu.edu/handle/10776/8231>.

In the second half of the twentieth century, scientists learned how to clone some species of mammals. Scientists have applied somatic cell nuclear transfer to clone human and mammalian embryos as a means to produce stem cells for laboratory and medical use. Somatic cell nuclear transfer (SCNT) is a technology applied in cloning, stem cell research, and regenerative medicine. Somatic cells are cells that have gone through the differentiation process and are not germ cells. Somatic cells donate their nuclei, which scientists transplant into eggs after removing their nucleuses (enucleated eggs). Therefore, in SCNT, scientists replace the nucleus in an egg cell with the nucleus from a somatic cell.

Although Karl Illmensee first cloned a mammal in 1981, other scientists had theorized and developed the techniques needed for SCNT in the form of nuclear transfer. Hans Spemann, who taught zoology at the University of Freiburg in Freiburg, Germany, theorized about SCNT in his 1938 book *Embryonic Development and Induction*. Spemann proposed to transplant a nucleus from an already differentiated cell from an embryo into an egg after removing the egg's nucleus. However, the technology required for this kind of experiment was not available to Spemann at that time, so he could not test his theory of nuclear transfer or SCNT. Robert King and Thomas Briggs developed the necessary protocol to conduct preliminary nuclear transfer at the Institute

for Cancer Research and Lankenau Hospital Research Institute in Philadelphia, Pennsylvania, in 1952. The same nuclear transfer techniques serve as the basis for SCNT.

While researching how embryos differentiate in 1952, Briggs and King transplanted the nucleus from an early embryonic blastula cell of a *Rana pipiens* frog embryo to an unfertilized egg after removing its nucleus. To enucleate the eggs, Briggs and King used a small glass needle to puncture the cell membrane, enter the cytoplasm, and suck out the nucleus of the egg cell. Briggs and King then transplanted the donor nucleus from a separate blastula cell to replace the nucleus that they removed from the egg cell. Briggs and King observed that the embryo developed normally.

Researchers struggled to clone mammals using the same procedure that Briggs and King used on frogs. In 1975, Derek Bromhall in Oxford, UK, conducted experiments using rabbit embryos and showed that, after a certain stage in development called the morula stage, embryos produced from nuclear transfer died. Bromhall hypothesized that they died as the result of complications from the punctures made in the cell membrane during the transfer.

Scientists performed nuclear transfer only on amphibians until 1981, when Illmensee in Geneva, Switzerland, claimed to have cloned mice using nuclear transfer technique. His work resulted in the birth of three live mice. Illmensee's experiments came under scrutiny and an investigation occurred concerning the veracity of his claims. Although the investigators never found conclusive evidence against Illmensee, the investigation cast doubts as to whether or not he had used nuclear transfer to clone the mice.

Scientists struggled to perform nuclear transfer on mammals larger than mice. Steen Willadsen at the Institute of Animal Physiology in Babraham Institute in Babraham, United Kingdom, was the first to clone a sheep embryo in 1984. Willadsen modified the technique of Briggs and King. After transferring the nucleus, Willadsen fused the embryo together using an electrofusion apparatus that has small electrodes that produce an electrical current. Willadsen coated the embryo with an agar jelly made from algae to reduce the damage caused by entry of the glass needle into the cell membrane. Once he had coated the embryos with agar jelly, Willadsen placed the embryos into the tied oviducts of a sheep, and he observed that the embryos were growing. From this experiment, Willadsen made viable mammalian embryos using his modified techniques, but they didn't grow into adult organisms.

In 1996, Keith Campbell, Jim McWhir, William Ritchie, and Ian Wilmut at the Roslin Institute in Edinburgh, UK, used nuclear transfer techniques to clone a sheep that was born and grew into an adult. The team manipulated a stage in the cell cycle called quiescence, when the cell undergoes a period of supposed hibernation and ceases to develop. Campbell induced quiescence in the donor blastocyst nuclei before transferring them to recipient egg cells by depriving the cells of proteins called growth factors. The change in the state of the donor nuclei before entering the receiving egg cells enabled embryos to develop to term in surrogate ewes.

According to Wilmut, the next experiment applied the same procedure to the nucleus of a fully differentiated adult cell as opposed to a blastocyst cell. The Roslin team hypothesized that the nuclear transfer procedure started by Briggs and King could be applied to somatic cells, thus becoming somatic cell nuclear transfer as opposed to just

nuclear transfer. The Roslin Institute performed this step in 1997. The result of the experiment was Dolly the sheep.

Dolly was the first mammal cloned from a fully differentiated adult cell. The main difference in the techniques producing Dolly was that the scientists used adult cell nuclei as opposed to the embryonic cell nuclei used in previous sheep experiments. After Dolly was born, the scientists applied these techniques in genetically modified mammalian embryos. Quiescence enabled the scientists to perform genetic modifications on the nucleus of the cell because growth factors were not altering the inserted DNA. In 1997, a Roslin Institute team used similar techniques to genetically modify Polly the sheep to express a human protein. After the success of Dolly and Polly, some scientists worked to clone human embryos using SCNT, however there were social, ethical and legal controversies over the practice. Many disagreed with the claims that scientists could or should clone, or perhaps genetically modify, humans using SCNT.

Scientists sought ways to clone human embryos without causing controversy. In 2011, Scott Noggle and his team at the New York Stem Cell Foundation in New York, New York, used SCNT to retrieve human embryonic stem cells. Although, Noggle's team did not perform SCNT using the same methods that produced Dolly. In fact, Noggle and his colleagues aimed to avoid the social and ethical implications of working with human embryos. Instead of removing the nucleus of the receiving egg cell before transfer, the scientists kept the egg nucleus and inserted the donor nucleus into the egg cell. As a result, the embryo developed into the blastocyst stage where scientists could extract stem cells. The chromosome count, however, was sixty-nine as opposed to the normal forty-six, because it contained the chromosomes from the full nucleus as well as

the egg nucleus, which only contains half, or twenty-three of the chromosomes in a zygote. This result meant that the blastocyst could not result in a pregnancy leading to birth because the cells would not progress to a further developmental state.

Embryonic stem cells are derived from these blastocyst cells.

Scientists report that SCNT is a plausible technique for creating human embryonic stem cells without extra chromosomes. In 2013, scientists in Oregon succeeded in using SCNT to reprogram somatic cells to become embryonic stem cells. After examining Noggle's research, Masahito Tachibana and his team at the Oregon National Primate Research Center in Hillsboro, Oregon, used the same methods that Campbell and his team had used to clone Dolly, but they also added a few extra procedures. The key differences were that they removed the spindle apparatus, which is responsible for movement of chromosomes in cellular mitosis and meiosis, from the donor egg cell before removing the egg cell nucleus. They reinserted the spindle apparatus into the cell when they inserted the donor nucleus. After removing the spindle apparatus, they also added caffeine, which inhibits the enzyme protein phosphatase, which activates proteins that begin cellular replication in the cytoplasm. Because the spindle apparatus was removed and the cytoplasm inactivated, the scientists could perform their procedures without risk of premature activation of the cell resulting in cellular damage and death. The results of the experiment showed that the cells altered with SCNT reached the blastocyst stage and produced viable embryonic stem cell lines of normal chromosome count. As of 2014, doctors use this version of SCNT for medical therapies and treatment, described as therapeutic cloning.

Controversies due to SCNT largely arise from the possibility of cloning humans. In 2003, a private company called Clonaid headquartered in Las Vegas, Nevada, claimed to have cloned the first human baby, called Eve, using SCNT. However, Clonaid did not allow scientists to perform a DNA test on Eve to confirm that she was indeed a clone, and therefore many in the scientific community doubted their claims. As of 2014, controversies arose over the possibility of human clones from SCNT. Some criticized the scientists who used SCNT to clone human stem cells in Oregon. The Oregon scientists justified their research by claiming that their only goal was to produce embryonic stem cells and not to produce a fully developed human being.

Associated Press. "Clonaid Baby: A Clone or a Fake?" *Wired Magazine*. <http://archive.wired.com/medtech/health/news/2002/12/57001> (Accessed April 13, 2013).

Briggs, Robert, and Thomas J. King. "Transplantation of Living Nuclei from Blastula Cells into Enucleated Frogs Eggs." *Proceedings of the National Academy of Sciences of the United States of America* 38 (1952): 455–63. <http://www.pnas.org/content/38/5/455.long> (Accessed November 3, 2014).

Bromhall, Derek J. "Nuclear transplantation in the rabbit egg." *Nature* 258 (1975): 719–22.

Campbell, Keith. H. S., Jim McWhir, William A. Ritchie, and Ian Wilmut. "Sheep cloned by nuclear transfer from a cultured cell line." *Nature* 380 (1996): 64–6.

Huxley, Aldous. *Brave New World*. Cutchogue: HarperCollins Publishers, Inc., 1932.

Illmensee, Karl, and Peter C. Hoppe. "Nuclear transplantation in mus musculus: Developmental potential of nuclei from preimplantation embryos." *Cell* 23 (1981): 9–18.

Lederberg, Joshua. "Experimental Genetics and Human Evolution". *The American Naturalist* 100 (1966): 519–31. <http://www.jstor.org/stable/2459206> (Accessed November 3, 2014).

Nobel Lectures, Physiology or Medicine 1922–1941. Amsterdam: Elsevier Publishing Company, 1965.

Noggle, Scott, Ho-Lim Fung, Athurva Gore, Hector Martinez, Kathleen Crumm Satriani, Robert Prosser, Kiboong Oum, Daniel Paull, Sarah Druckenmiller, Matthew Freeby, Ellen Greenberg, Kun Zhang, Robin Goland, Mark V. Sauer, Rudolph L. Leibel, and Dieter Egli. "Human oocytes reprogram somatic cells to a pluripotent state." *Nature* 478 (2011): 70–5.

Schmeck, Harold M., Jr. "Review Casts Some Doubt on Cloning Data." *The New York Times*. February 26, 1984. <http://www.nytimes.com/1984/02/26/us/review-casts-some-doubt-on-cloning-data.html> (Accessed June 18, 2014).

Schnieke Angelika E., Alexander J. Kind, William A. Ritchie, Karen Mycock, Angela R. Scott, Marjorie Ritchie, Ian Wilmut, Alan Colman, and Keith H. Campbell. "Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts." *Science* 278 (1997): 2130–3.

Spemann, Hans. *Embryonic Development and Induction*. New York: Hafner Publishing Company, 1962.

Tachibana, Masahito, Paula Amato, Michelle Sparman, Nuria M. Gutierrez, Rebecca Tippner-Hedges, Hong Ma, Eunju Kang, Alimujiang Fulati, Hyo-Sang Lee, Hathaitip Sritanaudomchai, Keith Masterson, Janine Larson, Deborah Eaton, Karen Sadler-Fredd, David Battaglia, David Lee, Diana Wu, Jeffrey Jensen, Phillip Patton, Sumita Gokhale, Richard L. Souffer, Don Wolf, and Shoukhrat Mitalipov. "Human Embryonic Stem Cells Derived by Somatic Cell Nuclear Transfer." *Cell* 153 (2013): 1228–38.

Weiss, Rick. "Human Cloning's 'Numbers Game'." *Washington Post*. October 10, 2000.

Willadsen, Steen M. "Nuclear Transplantation in Sheep Embryos." *Nature* 320 (1986): 63–5.

Wilmut, Ian, Angela E. Schnieke, Jim Mcwhir, Alexander J. Kind, and Keith H.S. Campbell. "Viable offspring derived from fetal and adult mammalian cells." *Nature* 385 (1997): 810–3.

Wilmut, Ian, and Roger Highfield. *After Dolly: The Uses and Misuses of Human Cloning*. New York: W.W. Norton & Company, Ltd., 20

CHAPTER 5 STEM CELLS AND SENESCENCE - MODERN RESEARCH

Moving into the late 20th and early 21st centuries, there is a growing body of research related to senescence and immortalized cell lines. With the successful application of SCNT, the scientists at the Roslin Institute introduced a method of acquiring human embryonic stem cells more readily. They also showed that the nucleus of a fully differentiated cell was capable of becoming a viable embryonic stem cell. However, SCNT is not a perfect technique. Though scientists attempt to mitigate the negative effects of the initial removal of the cell nucleus, it is difficult to eliminate all risk factors.

In 2006, researchers in Kyoto, Japan, found an alternative method of acquiring pluripotent human stem cells. Rather than transplanting cell nuclei, this new method involved incorporation of viral DNA into a fully functional cell, thus bypassing the potential negative effects of nucleus removal. The viral DNA caused adult cells to become pluripotent stem cells. They called the cells induced pluripotent stem cells, or iPSCs. This novel technique provided a way for production of immortalized cell lines without the negative effects that could result from techniques such as SCNT.

However, it was noted that there were differences between natural stem cells and the iPSCs. The scientists from Japan attributed those changes to intrinsic factors that were already present in the adult cells before the viral DNA was added. The following Embryo Project Encyclopedia article details the iPSC experiments.

Induced Pluripotent Stem Cell Experiments by Kazutoshi Takahashi and Shinya Yamanaka in 2006 and 2007

Bartlett, Zane, "Induced Pluripotent Stem Cell Experiments by Kazutoshi Takahashi and Shinya Yamanaka in 2006 and 2007". Embryo Project Encyclopedia (2015-06-01). ISSN: 1940-5030
<http://embryo.asu.edu/handle/10776/8325>.

In 2006, Kazutoshi Takahashi and Shinya Yamanaka reprogrammed mice fibroblast cells, which can produce only other fibroblast cells, to become pluripotent stem cells, which have the capacity to produce many different types of cells. Takahashi and Yamanaka also experimented with human cell cultures in 2007. Each worked at Kyoto University in Kyoto, Japan. They called the pluripotent stem cells that they produced induced pluripotent stem cells (iPSCs) because they had induced the adult cells, called differentiated cells, to become pluripotent stem cells through genetic manipulation. Yamanaka received the Nobel Prize in Physiology or Medicine in 2012, along with John Gurdon, as their work showed scientists how to reprogram mature cells to become pluripotent. Takahashi and Yamanaka's 2006 and 2007 experiments showed that scientists can prompt adult body cells to dedifferentiate, or lose specialized characteristics, and behave similarly to embryonic stem cells (ESCs).

Takahashi and Yamanaka worked together at Kyoto University. Takahashi was a post-doctoral researcher who had earned a graduate degree in biology at the Nara Institute of Science and Technology in Ikoma, Japan. Yamanaka had earned an MD from Kobe University in Kobe, Japan in 1987. In 2004, Yamanaka began working at Kyoto University as a professor, where he studied factors that help an organism fend off retroviruses, which are single-stranded RNA viruses that can incorporate their genetic

material into the DNA of a host cell. Yamanaka and others hypothesized that retroviruses could influence somatic cells to become stem cells. Yamanaka worked to find new ways to acquire embryonic stem cells to avoid the social and ethical controversies surrounding the use of human embryos in stem cell research during the late twentieth and early twenty-first centuries. Yamanaka studied the work of John Gurdon, a researcher who had experimented with *Xenopus* frogs at the University of Oxford in Oxford, United Kingdom. Yamanaka claimed that Gurdon's work in reprogramming mature cells in frogs (*Xenopus*) in 1962 influenced his own work in reprogramming differentiated cells.

Yamanaka also noted that experiments in cloning Dolly the sheep in 1996, conducted by Ian Wilmut, Angelica Schnieke, Jim McWhir, Alex Kind, and Keith Campbell at the Roslin Institute in Roslin, Scotland, influenced his work. The Dolly experiment showed that scientists could reprogram the nucleus of somatic cells by transferring the contents of the nucleus into oocytes that have had their nuclei removed, a technique called somatic cell nuclear transfer (SCNT). Other research groups such as Masako Tada's group in Japan in 2001 and Chad Cowan's group in Massachusetts in 2005 combined embryonic stem cells with somatic cells to produce pluripotent cells. After these experiments with somatic cells, Takahashi and Yamanaka hypothesized that there were common factors, genes in particular, which caused somatic cells to become pluripotent stem cells.

In 2006, Takahashi and Yamanaka selected twenty-four candidate genes as factors that they hypothesized could possibly induce somatic cells to become pluripotent, and they began to test them one at a time. They used retroviruses to insert each of the twenty-four genes into the chromosomes of differentiated mouse embryonic fibroblasts.

Each gene was inserted near the mouse *Fbx15* gene, a gene that embryonic stem cells express during development in mice. The newly inserted gene endowed mice with resistance to an antibiotic named G418. The researchers labeled the resulting retroviruses mixed with host DNA as retroviral factors. Takahashi and Yamanaka placed the retrovirus-infected cells into cell culture with G418 antibiotic and cells to provide nourishment, called feeder cells. If one of the infected cells showed G418 resistance, then the scientists would know that one of the twenty-four genes influenced the cell to become an embryonic stem cell-like cell. However, none of the cells showed a resistance to G418, so Takahashi and Yamanaka reworked their approach.

Next, Takahashi tried to insert into a fibroblast cell multiple retroviral factors instead of one at a time. The researchers added all of the twenty-four retroviral factors at the same time into mouse fibroblast cells. This time, there were twenty-two cell colonies that showed a resistance to G418, meaning that there were colonies in which the cells exhibited embryonic stem cell properties. After examination, Takahashi and Yamanaka concluded that the cells were similar to embryonic stem cells and duplicated themselves in similar periods of as embryonic stem cells. They named the cells iPS-MEF24, signifying pluripotent stem cells induced from mouse embryonic fibroblasts by twenty-four factors.

The next experiments aimed to identify specific factors responsible for the generation of iPS cells. To isolate these specific factors, the researchers removed retroviral factors one at a time from the original twenty-four, and each time they removed a factor, they repeated their cell colony procedures. If the researchers removed a factor and the resultant cell colony wasn't resistant to antibiotics, they knew that the

missing factor somehow influenced the generation of iPS cells. Takahashi and Yamanaka repeated their procedure until they found ten genes that, when combined together in cells, yielded colonies of cells with G418 resistance. They named those cells with the ten genes as iPS-MEF10 cells. Takahashi and Yamanaka found that of the ten genes, when they combined four genes in particular (*Oct3/4*, *Klf4*, *Sox2*, and *c-Myc*), they produced the most cells that were like embryonic stem cells. The scientists named the cells iPS-MEF4. Takahashi and Yamanaka deemed those four genes important in the role of iPS cell generation. They concluded that iPSCs are similar, but not identical to embryonic stem cells.

To determine how embryonic stem cells were different from iPSCs, Takahashi and Yamanaka used primers, or strands of nucleic acid that help to start the process of DNA synthesis, to promote replication of genes found in normal embryonic stem cells. If an iPSC had a normal embryonic stem cell gene, the primer would prompt the normal gene to replicate, and the scientists could then see that the iPSC had a normal gene.

Takahashi and Yamanaka continued their experiments and injected the iPSC samples into mice that had no body hair. These nude mice were a variation of the common mouse (*Mus musculus*), but they had an inhibited immune system and lacked the *Fox1* gene. When the researchers injected iPSCs into the mice, teratomas, which are tumors with germ layer components, formed. The teratomas resulting from iPS-MEF4 injections differentiated into all three germ layers (ectoderm, endoderm, and mesoderm), including neural and muscular tissues, cartilage, and epithelium. These tissue types formed aggregates of pluripotent stem cells called embryoid bodies. From the teratomas,

Takahashi and Yamanaka took some cell samples and cloned them. They inserted the cloned cells into blastocysts by microinjection and obtained four different embryos.

After analysis, Yamanaka and Takahashi found that the four embryos contained iPS cells that contributed to all three germ layers, providing further evidence that the four genes (*Oct3/4*, *Klf4*, *Sox2*, and *c-Myc*) helped produce cells that were the most like embryonic stem cells. Takahashi and Yamanaka observed that the iPS-MEF4 cells continued to be more similar to embryonic stem cells than to other iPS cells. After further experimentation, they concluded that the iPS cells they generated were pluripotent in mice, and therefore provided the possibility of repeating a similar experiment in humans. Takashi and Yamanaka published the results of their experiment in 2006.

After their mouse experiments, in 2007 Takahashi and Yamanaka published the results of another experiment that detailed methods and results used to produce iPS cells with human cells. They used the same four genes from humans that were used in mice. Another group led by James Thomson at the University of Wisconsin in Madison, Wisconsin, published their findings on iPSC in humans. They found that four genes—*Oct4*, *Sox2*, *NanoG*, and *Lin28*—were sufficient to reprogram human somatic cells into pluripotent stem cells. Independent confirmation of Takahashi and Yamanaka's previous experiments with mice supported the hypothesis that scientists can generate and use induced pluripotent stem cells in a similar manner as embryonic stem cells. Scientists later used iPSCs in regenerative medicine to research treatments for various human diseases such as Parkinson's disease, platelet deficiency, spinal cord injury, and macular degeneration.

In June 2012, Yamanaka reported that experiments showed many epigenetic differences, as well as gene expression differences, between iPSCs and embryonic stem cells. Yamanaka cited as an example the chemical addition of methyl groups (methylation) of the gene that makes bone morphogenic protein 3 (BMP3), which partly causes bone and cartilage development in humans. He also mentioned that there are other examples of high amounts of variation and mutations in iPSCs, which alarmed some scientists. Yamanaka said that many of the variations in the genes and epigenetics are likely from the original somatic cell from which the iPSCs are descended, and that the negative reports on iPSCs were often overstated.

In 2012, Yamanaka and John Gurdon received the Nobel Prize in Physiology or Medicine for their discovery that differentiated cells can be reprogrammed to become pluripotent. The Nobel Prize committee stated that Takahashi and Yamanaka's experiments with mice iPSCs contributed to Yamanaka's award. Scientists in the second decade of the twenty-first century experimented with iPSCs to generate cells similar to embryonic stem cells without destroying or manipulating embryos.

Cowan, Chad A., Jocelyn Atienza, Douglas A. Melton, and Kevin Eggan. "Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells." *Science* 309 (2005): 1369–73.

Fackler, Martin. "Risk taking is in his genes." *New York Times*, December 11, 2007, Science Section. <http://www.nytimes.com/2007/12/11/science/11prof.html> (Accessed April 6, 2014).

Gurdon, John B. "The transplantation of nuclei Between two species of *Xenopus*." *Developmental Biology* 5 (1962): 68–83.

Gurdon, John B. "Adult frogs derived from the nuclei of single somatic cells." *Developmental Biology* 4 (1962): 256–73.

Maienschein, Jane. *Embryos Under the Microscope: The Diverging Meanings of Life*. Cambridge, MA: Harvard University Press, 2014.

Nobel Media. "The Nobel Prize in Physiology or Medicine 2012". Nobelprize.org. http://www.nobelprize.org/nobel_prizes/medicine/laureates/2012/ (Accessed April 6, 2014).

Tada, Masako, Yousuke Takahama, Kuniya Abe, Norio Nakatsuji, and Takashi Tada. "Nuclear reprogramming of somatic cells by in vitro hybridization with ES cells." *Current Biology* 11 (2001): 1553–8. [http://dx.doi.org/10.1016/S0960-9822\(01\)00459-6](http://dx.doi.org/10.1016/S0960-9822(01)00459-6) (Accessed May 5, 2015).

Takahashi, Kazutoshi, Koji Tanabe, Mari Ohnuki, Megumi Narita, Tomoko Ichisaka, Kiichiro Tomoda, and Shinya Yamanaka. "Induction of pluripotent stem cells from adult human fibroblasts by defined factors." *Cell* 131 (2007): 861–72. <http://dx.doi.org/10.1016/j.cell.2007.11.019> (Accessed May 5, 2015).

Takahashi, Kazutoshi, and Shinya Yamanaka. "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors." *Cell* 126 (2006): 663–76. <http://dx.doi.org/10.1016/j.cell.2006.07.024> (Accessed May 5, 2015).

Wilmut, Ian, Angelica E. Schnieke, Jim McWhir, Alex J. Kind, and Keith H. S. Campbell. "Viable offspring derived from fetal and adult mammalian cells." *Nature* 385 (1997): 810–3.

Yamanaka, Shinya. "Induced pluripotent stem cells: past, present, and future." *Cell Stem Cell* 10 (2012): 678–84. <http://dx.doi.org/10.1016/j.stem.2012.05.005> (Accessed May 5, 2015).

Yu, Junying, Maxim A. Vodyanik, Kim Smuga-Otto, Jessica Antosiewicz-Bourget, Jennifer L. Frane, Shulan Tian, Jeff Nie, Gudrun A. Jonsdottir, Victor Ruotti, Ron Stewart, Igor I. Slukvin, and James A. Thomson. "Induced pluripotent stem cell lines derived from human somatic cells." *Science* 318 (2007): 1917–20.

CHAPTER 6

CONCLUSION

This cluster of articles from the Embryo Project Encyclopedia makes clear that there are a wide variety of methods of obtaining and culturing immortal cell lines. Modern methods borrow from practices and theories of scientists in the past, which was particularly evident in chapter 2 of this thesis. For example, scientists culture hESCs in a strikingly similar fashion as Alexis Carrel cultured the cells in his chick heart experiments: with the use of proper nutrients, skillful technicians, and meticulous selection of petri dishes. For the proper nutrients, scientists place embryonic cells of mice in petri dishes for hESCs to feed on. It is rare for hESCs to retain their pluripotency by using any other feeder cells. Though some researchers use human foreskin cells as feeder cells, the most common practice currently is to use mouse embryonic cells. If hESCs fail to retain their pluripotency through prolonged culturing, scientists often attribute the resulting senescence to external factors such as a lack of proper feeder cells, the shape of the petri dish, or incorrect laboratory methods.

Recent research suggests that hESCs, and immortalized cell lines in general, do not escape the effects of senescence. As was discussed in the Embryo Project Encyclopedia articles of chapter 3, much of the research on immortalized cell lines and senescence examines the function of telomeres and telomerase, which the majority of scientists use as the deciding factor to determine whether a cell is viable and will continue to replicate.

However, there is conflicting research among scientists over other factors besides telomeres and telomerase that contribute to senescence. For example, the human cell

organelle, the mitochondria, has DNA apart from the chromosome of the cell. Mutations can occur in human mitochondrial DNA (mtDNA), independently of nuclear DNA, which leads to dysfunctional cell metabolism and cessation of indefinite cellular replication, or a loss of immortality. Some suggest that hESCs do not appear to have mitochondrial defects, though other scientists claim there are defects (Bernardo).

SCNT could provide a possible method of testing whether mitochondrial defects influence senescence in immortal cell lines. A nucleus from an immortalized cell with notably lengthy telomeres, and high expression of telomerase could be transferred over to an enucleated cell that contains normal or mutated mitochondria. Scientists could also take existing immortal cell lines and perform SCNT to potentially mitigate mitochondrial defects in future cell generations. Conversely, articles from the Embryo Project Encyclopedia as described in chapters 4 and 5 suggest that SCNT is not a perfect technique. An alternative method such as the method used to produce iPSCs could be used to determine if there is a relationship between mitochondrial defects and senescence.

As was discussed in chapter 5, iPSCs have epigenetic differences when compared to natural pluripotent stem cells. Scientists also claim epigenetics can influence senescence. Epigenetics refer to heritable characteristics of genes, which do not directly involve changes to the DNA of an organism. An example of an epigenetic effect includes methylation of protein structures that coat DNA strands. An increase or decrease in methylation can cause a cell to produce more or less of a protein. In the case of iPSCs, Yamanaka claims epigenetic differences exist because of factors already present in the adult cells (Yamanaka). Further research is needed to understand the relationship between

selection of adult cells and the resulting epigenetic changes in iPSCs derived from those adult cells, as well as hESCs and other immortal cell lines.

Aside from epigenetic changes, there is evidence to suggest there are also direct genetic changes that occur in immortal cell lines over prolonged periods of cell culturing. In the journal *Nature biotechnology* in 2007, researchers in the United Kingdom reported that they observed chromosomal abnormalities in hESCs that had been cultured using mass production techniques in as little as 23 passages. In hESC cultures that were produced manually, they noted chromosomal abnormalities after about 105 passages. As the most common changes, they noted gains in chromosomes 1, 7, 12, 17, and x, with the gain in chromosome 17 as the most prominent (Baker). The researchers also reported chromosomal loss in chromosome 13. They suggest that those chromosomal changes lead to increasingly malignant cell cultures, and they attribute the cause of the chromosomal changes to selective pressures that occur when cells move from one environment to another, or from culture dish to culture dish. The scientists propose that more research is needed to find methods to adapt hESCs to culturing.

As a final inclusion to the contributing factors of senescence already mentioned, there are some studies that point to the idea that cells go through senescence independent of telomere shortening due to the generally vague term, “stress” (Campisi). Such stressors are of unknown causes, though the result of those stressors can be replicative senescence. As was the case in Leonard Hayflick’s day, further research is needed to properly address the issue of senescence in cell lines that were once thought of and cultured as immortal.

In addition to gleaning from past practices, researchers continue to conduct experiments with cell populations established before modern understanding of

senescence and cell biology. The most prominent immortal cell line being HeLa cells, which have been in use since 1951. HeLa cells also account for contamination in 27 of 93 cell culture suppliers, and are therefore contributing to studies that were not originally designed to use HeLa cells (Lucey). Questions arise as to the efficacy of repeatedly culturing an immortalized cell line such as HeLa. What might be some of the changes that occur in a cell line in the course of 64 years? If there are changes in those cell lines, are researchers giving proper attention to said changes?

In the case of hESCs, due to recent regulatory changes in the United States, only a select number of hESC lines are available for experimentation (NIH). Those limitations could be encouraging repeated use of already approved immortal cell lines that contain abnormalities. Some scientists may not even realize they are conducting experiments with cell lines that are distinctly different from natural cells. With the help of modern understanding of cell biology and senescence, scientists should investigate currently approved immortal cell lines and screen those lines for existing abnormalities.

While there exists a constant change in the practices of cell culturing, a large portion of scientists still rely on practices established before modern senescence research: research that seems to suggest that cultured hESCs, among other immortal cell lines, are not truly immortal. Though scientists are encouraged to present many solutions to the challenges of culturing immortal cell lines, this thesis proposes the following solutions:

- 1) Examine all known facets of senescence. Telomeres and telomerase are only one of many contributing causes of aging in cells. There is a need for more research

pertaining to those other contributing factors, particularly unknown causes of stressors and nutrients needed for immortal cell lines.

- 2) Renew populations. Though currently ethically perilous in the United States (which can be a thesis entirely in itself), scientists could retrieve fresh stem cells, as opposed to perpetually culturing aged cells. Such a practice would reduce chromosomal abnormalities. The length of time required before cell renewal warrants further research.
- 3) Select for healthy populations of immortalized cells. Continued selection may produce an immortalized cell line that is adapted to prolonged cell culturing. Selection techniques would need to be developed.
- 4) Reduce factors of senescence in the cell. Scientists could use SCNT to replace damaged mtDNA. iPSCs may reduce the cytoplasmic damage caused from SCNT, though iPSCs have epigenetic differences. There are likely many other ways to reduce other factors of senescence that need scientific attention.

REFERENCES

- Baker, Duncan EC, Neil J. Harrison, Edna Maltby, Kath Smith, Harry D. Moore, Pamela J. Shaw, Paul R. Heath, Hazel Holden, and Peter W. Andrews. "Adaptation to culture of human embryonic stem cells and oncogenesis in vivo." *Nature biotechnology* 25, no. 2 (2007): 207-215.
- Bernardo, Maria Ester, Nadia Zaffaroni, Francesca Novara, Angela Maria Cometa, Maria Antonietta Avanzini, Antonia Moretta, Daniela Montagna et al. "Human bone marrow-derived mesenchymal stem cells do not undergo transformation after long-term in vitro culture and do not exhibit telomere maintenance mechanisms." *Cancer research* 67, no. 19 (2007): 9142-9149.
- Blackburn, Elizabeth H., and Joseph G. Gall. "A tandemly repeated sequence at the termini of the extrachromosomal ribosomal RNA genes in *Tetrahymena*." *Journal of Molecular Biology* 120 (1978): 33–53.
- Campisi, Judith, and Fabrizio d'Adda di Fagagna. "Cellular senescence: when bad things happen to good cells." *Nature reviews Molecular cell biology* 8 (2007): 729-740.
- Carrel, Alexis. "On the permanent life of tissues outside the organism" *Journal of Experimental Medicine* 15 (1912): 516–28.
- Greider, Carol W., and Elizabeth H. Blackburn. "A telomeric sequence in the RNA of *Tetrahymena* telomerase required for telomere repeat synthesis." *Nature* 337 (1989): 331–7.
- Greider, Carol W., and Elizabeth H. Blackburn. "Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts." *Cell* 43 (1985): 405–13.
- Greider, Carol W., and Elizabeth H. Blackburn. "The telomere terminal transferase of *Tetrahymena* is a ribonucleoprotein enzyme with two kinds of primer specificity." *Cell* 51 (1987): 887–98.
- Hayflick, Leonard. "The limited in vitro lifetime of human diploid cell strains." *Experimental Cell Research* 37 (1965):614–36.
- Hayflick, Leonard, and Paul S. Moorhead. "The serial cultivation of human diploid cell strains." *Experimental Cell Research* 25 (1961): 585–621.
- Irfan Maqsood, Muhammad, Maryam M. Matin, Ahmad Reza Bahrami, and Mohammad M. Ghasroldasht. "Immortality of cell lines: challenges and advantages of establishment." *Cell biology international* 37, no. 10 (2013): 1038-1045.

- Jiang, Lijing. "Degeneration in Miniature History of Cell Death and Aging Research in the Twentieth Century" PhD diss., Arizona State University, 2013.
- Kirkwood, Thomas B.L. and Thomas Cremer. "Cytogerontology Since 1881: A reappraisal of August Weistmann and a Review of Modern Progress." *Human Genetics* 60 (1982): 101-121.
- Landecker, Hannah. *Culturing life*: Harvard University Press, 2007.
- Lucey, Brendan P., Walter A. Nelson-Rees, and Grover M. Hutchins. "Henrietta Lacks, HeLa cells, and cell culture contamination." *Archives of pathology & laboratory medicine* 133, (2009): 1463-1467.
- Lundblad, V. and Szostak, J.W., "A mutant with a defect in telomere elongation leads to senescence in yeast," *Cell* 57, 633–643 (1989).
- Maienschein, Jane. *Embryos Under the Microscope: the diverging meanings of life*: Harvard University Press, 2007.
- Maienschein, Jane and Manfred Laubichler, "The Embryo Project: An Integrated Approach to History, Practices, and Social Contexts of Embryo Research." *Journal of the History of Biology* 43 (2010): 1-16.
- Maitra, Anirban, Dan E. Arking, Narayan Shivapurkar, Morna Ikeda, Victor Stastny, Keyaunoosh Kassaei, Guoping Sui et al. "Genomic alterations in cultured human embryonic stem cells." *Nature genetics* 37, no. 10 (2005): 1099-1103.
- MedlinePlus. "Cloning". NIH U.S. National Library of Medicine. <https://www.nlm.nih.gov/medlineplus/cloning.html> (Accessed September 26, 2015).
- Minot, Charles Sedgwick. 1908. *The Problem of Age, Growth, and Death: A Study of Cytomorphosis*: The Rnickerbocker Press.
- National Institutes of Health. "2009 Guidelines on Human Stem Cell Research. In *Stem Cell Information* [World Wide Web site]". Bethesda, MD: National Institutes of Health, U.S. Department of Health and Human Services, 2015. <http://stemcells.nih.gov/policy/pages/2009guidelines.aspx> (Accessed October 8, 2015).
- National Institutes of Health. "Stem Cell Basics: Introduction. In *Stem Cell Information* [World Wide Web site]". Bethesda, MD: National Institutes of Health, U.S. Department of Health and Human Services, 2015. <http://stemcells.nih.gov/info/basics/pages/basics1.aspx> (Accessed January 17, 2015)

- National Institutes of Health. "What are the unique properties of all stem cells?. In *Stem Cell Information* [World Wide Web site]". Bethesda, MD: National Institutes of Health, U.S. Department of Health and Human Services, 2015.
<http://stemcells.nih.gov/info/basics/pages/basics2.aspx> (Accessed January 17, 2015)
- Nobel Media. "Carol W. Greider–Biographical." Nobel Media.
http://www.nobelprize.org/nobel_prizes/medicine/laureates/2009/greider-bio.html
 (Accessed January 25, 2014).
- Nobel Prize. "The Nobel Prize in Physiology or Medicine 2009" Nobel Media.
http://www.nobelprize.org/nobel_prizes/medicine/laureates/2009/ (Accessed October 5, 2013).
- Ogrunc, Müge, and Fabrizio d'Adda di Fagagna. "Never-ageing cellular senescence." *European Journal of Cancer* 47, no. 11 (2011): 1616-1622.
- Rando, Thomas A. "Stem cells, ageing and the quest for immortality." *Nature* 441, no. 7097 (2006): 1080-1086.
- Scherer, William F., Jerome T. Syverton, and George O. Gey. "Studies on the propagation in vitro of poliomyelitis viruses IV. Viral multiplication in a stable strain of human malignant epithelial cells (strain HeLa) derived from an epidermoid carcinoma of the cervix." *The Journal of experimental medicine* 97, no. 5 (1953): 695-710.
- Smith, Austin G. "Embryo-derived stem cells: of mice and men." *Annual review of cell and developmental biology* 17, no. 1 (2001): 435-462.
- Szostak, Jack W., and Elizabeth H. Blackburn. "Cloning yeast telomeres on linear plasmid vectors." *Cell* 29 (1982): 245–55.
- Weismann, A. (1892) *Über Leben und Tod*. Verlag von Gustav Fisher, Jena, Germany.
- Yamanaka, Shinya. "Induced pluripotent stem cells: past, present, and future." *Cell Stem Cell* 10 (2012): 678–84. <http://dx.doi.org/10.1016/j.stem.2012.05.005> (Accessed May 5, 2015).
- Zachar, Vladimir, Sinha M. Prasad, Simon C. Weli, Anette Gabrielsen, Karsten Petersen, Michael B. Petersen, and Trine Fink. "The effect of human embryonic stem cells (hESCs) long-term normoxic and hypoxic cultures on the maintenance of pluripotency." *In Vitro Cellular & Developmental Biology-Animal* 46, no. 3-4 (2010): 276-283.

Zeng, X., and M. S. Rao. "Human embryonic stem cells: long term stability, absence of senescence and a potential cell source for neural replacement." *Neuroscience* 145, no. 4 (2007): 1348-1358.