Chapter 2
Experimental Foundations of the Hayflick System

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The birth of modern cytogerontology is often, and rightly so, credited to Leonard Hayflick for demonstrating that normal diploid cells have a limited proliferative potential. However, in my view, it is Vincent Cristofalo's consistent and rigorous experimental testing of the Hayflick system that has nurtured it, established its scientific credibility, and has paved the way for major breakthroughs in the field.

2.1 Coming in Picture

SR: I have always considered you and your group as the people who actually helped develop the field of cytogerontology from a basic cell culture method to a mature science of cellular aging, but how and when did you come into the picture?

VC: I was doing hepatoma biochemistry with Sydney Weinhouse as a post doc at Temple University Medical School in the early 1960s when I read about the Hayflick and Moorhead findings. One of my frustrations with working with liver and liver tumors was the complexity of the tissue. The experiments were done by grinding up the tissue, fractionating the cell extract and then trying to interpret findings from the extracts that could be related to the intact tissue. Suddenly I was struck with the

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(*With kind permission from Springer Science+Business Media:* "I no longer believe that cell death is programmed …", an interview with Vincent Cristofalo*, by: Suresh I.S. Rattan; Biogerontology 12: 283–290, 2001).*

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power of the tissue culture system where you could have single cells in an environment that you could control, and you could ask questions of the cell in its normal state. I also read the papers which showed that you could also transform those cells to an immortalized cancer-like cell line, and I thought this is perfect to study cancer. Then David Kritchevsky, who was the deputy Director of the Wistar Institute came to see me and he said that they had made a decision to bring a biochemist to Wistar to expand the efforts of the Hayflick group. I was thrilled to be asked to join the group at Wistar and accepted their offer. When I arrived at Wistar, Len Hayflick had a laboratory with a desk in the middle of the lab. The lab was crowded with scientists from all over the world, learning to culture human diploid fibroblasts. Paul Moorhead, Len's chief collaborator, had space on another floor and was also a frequent visitor to Len's lab. I was assigned sterile room space in Len's lab while my space for biochemical studies was elsewhere in the building. For all of us these were less than ideal conditions but biology was the focus and we made it all work.

SR: When was it, 1965?

VC: No, no, it was 1963. I then learned cell culture technology from Len and his people. It was very interesting laboratory in those days, because people were coming from all over the world to learn about how to grow human cells. It was a very exciting lab to be in. My assignment was to bring biochemistry to cell aging because Len and Paul Moorhead were doing the biology/cytology and Girardi and Jenson were doing transformation studies.

The biggest concern of Len at that time was getting the human diploid fibroblasts accepted for anti-viral vaccine production. That was very controversial at that time. The aging side was interesting to him but perhaps less so. I began to do experiments on glycolysis and respiration as a function of age. Then I became interested in lysosomes. It was very obvious to me that one of the things that we could see under the microscope was that the senescent cells accumulated secondary lysosomes. We worked on the lysosomal enzymes for a while, and we also did electron microscopy with Jacques Lipetz. We did extensive morphologic studies: size, shape, ultrastructure and so forth. In the meantime, Hayflick moved to Stanford in 1968 or 1969. I had gotten an RO1 grant from the National Institute of Child Health and Human Development for aging studies in 1966. Originally, I had research money from the Army Chemical Warfare Center, but I was not doing any chemical warfare. They had some money for just basic research. So, I applied and I got a contract.

Then there were the riots in the US over the Vietnam War. The provost of the University of Pennsylvania required that anybody who had funding from Army Chemical Warfare Center, Dow Chemical Company, or a whole list of other sources had to give the money back. I was then a senior postdoc and the Army grant paid my salary. So, Hilary Koprowski came to me and said: "If you could write a grant application tonight, I am going to London tomorrow, and I will bring it to the Welcome Trust. Maybe we can get you some money." So, I worked all night, produced the short grant application, and I eventually became a Welcome Cancer Fellow with salary support. Then I saw an ad in Science magazine that said that the

aging program of the National Heart Institute was moving to the National Institute of Child Health and Human Development. I took a chance and I called them up, and told them what I was doing. They said that they were very interested in that and that I should write an RO1 grant application. I received that grant and that really established for me a long career in aging.

SR: When did you finally realize that you would like to focus more on the issues of aging than the cancer? When did that shift occur?

VC: Probably in the late 1960s. What happened was that there was a very high level meeting at Gatlinburg, Tenn. by invitation only. I wasn't invited there, but Koprowski and Hayflick were invited. Koprowski could not go, so he told me that I had to go in his place. When I arrived, the organizer of the meeting sent for me. The program was set up so that Hayflick was going to speak and then Harry Eagle, (of Eagle's medium fame) a very prominent scientist, was going to be the discussant. The organizer, whom I had never met before, said to me: "We just got a telegram that Harry Eagle cannot come. So you have to be the discussant." So, I was called into a situation where Hayflick, on whom I was dependent for lab space, was talking about his work, and I had to give a bit of a controversial talk, which of course I had not even prepared. I couldn't get out of it, so I did it. I managed to avoid antagonizing Hayflick while at the same time being reasonably critical. So I survived. Very significant though was that I met Bernie Strehler, who was at that time editing a series called 'Advances in Gerontological Research'.

SR: Did you speak in favor of Hayflick or ...

VC: The idea was to be provocative. There were many, many criticisms of the Hayflick system. I felt that some of these arguments were good arguments. I tried to prepare a very balanced talk, to analyze both the pros and cons ...

SR: So, that was an historic meeting!

VC: Yes, that was a historical meeting. I also met David Gershon at that meeting, and we have been friends since then. This year he spent 8 months of his sabbatical in my labs.

SR: Was he also doing cell culture at that time?

VC: No, no. He was absolutely opposed to cell culture as a model for aging and he criticized me after my talk, pointing out that I had not been as aggressive attacking Hayflick as I should have been. But, I didn't think all the attacks were fair. I felt there were both pros and cons. Anyway, as a result of that meeting Bernard Strehler asked me to write a chapter for his book, which I did. By the way, that chapter from 1969 I still give to students; and if I can be a little bit immodest, I laid out all the crucial questions, at that time, most of which are the same today.

SR: I remember that I was also given that chapter to read when I first started in Robin Holliday's lab in London in 1979.

VC: It was an important opportunity for me as a junior person, and I found two things. I found first of all that the field of aging was much more interesting than I

had thought. I had only seen it through the narrow window of the Hayflick system. When I listened to people talk about plants, *Drosophila* and other models with the broad menu of tantalizing questions, I became very intrigued. Secondly, I found myself thrust into a position from which I could not escape. Hayflick was completely convinced that what he was saying was absolutely correct. I felt that I was in a position to give credibility to the model of cellular aging in vitro by providing a lot of experimental data. I guess that it was basically at that point that I really began to examine aging as a field in a broader sense than the cell culture aging.

2.2 Program or Stochastic?

SR: How were you visualizing aging at that time? Have your views developed or changed over the years?

VC: My views have developed and turned around 180°! I originally thought of cell aging as a programmed phenomenon.

SR: Why was that?

VC: Basically because of my observations in cell culture and the regularity of the sequence of the cellular changes, I thought of it as a kind of a programmed phenomenon. In fact, I suggested it was kind of a differentiation sequence. I thought I was the first but Bernie Strehler had suggested that before me. Then of course many investigators later, notably Klaus Bayreuther also suggested differentiation. As I learned more and more, I realized that cell senescence is a stochastic phenomenon from the point of view of the individual cell, but it is programmed from the point of view of the population. I think that human aging is similar in that which one of us dies when, is dependent on a series of stochastic events.

SR: I think that is a very important point, and this issue of program versus *stochastic is a point of confusion in many people's minds. Would you like to elaborate on this a little bit?*

VC: Yes. The origins of that confusion are understandable. For example, if you look at Drosophila, there is a clock-like regularity of events at all stages of development. So, it was easy and convenient to believe that there was a program controlling the whole life history. I suppose that the most important influence on my thinking came as techniques of molecular biology were incorporated into aging research. Initially, it was very hard to find differences between young and old cells. But as the techniques and the resolving power improved, I began to see that an entire series of changes takes place during cellular aging. Almost everything one looks at is different between young and old cells, but the differences are more subtle; accumulation of post-translationally modified proteins, uncoupling of the cell cycle, loss of precision of the integration of the various cell functions give us a general picture for aging. What happens in aging is that as the precision of integrated

cell function begins to fail, the ability to survive becomes compromised. Then it becomes a matter of 'luck' in a sense and a random event for cell death.

SR: Do you mean to say that the process of aging and its consequences are purely a random and a chance event?

VC: I think dying is a chance event. Individual lifespan is a chance event. I think the changes that occur are due to a kind of 'molecular friction' that gradually destroys function. Evolution has selected a combination of factors that ensure highly integrated and precise functioning of the cell machinery, but it is only 'designed' to last so long. Long enough to allow reproduction, but not significantly longer.

SR: Longer than what?

VC: Longer than the Darwinian purpose of life. Evolutionary biologists have pointed out that the force of natural selection diminishes with age. What natural selection works on is survival for successful reproduction. So, what we have is the machinery to assure that we live that long. Beyond that the precise function of the machinery deteriorates. I don't know why it deteriorates. That's the central question of gerontology. The example I frequently use for this is the late Alex Comfort's example of designing a space ship for going to Mars to send back pictures to earth. The engineer designs redundancy in the systems so that successful travel to Mars is virtually assured. But there is no reason to provide additional redundancy.

SR: Do you then need to put in the systems to destroy it?

VC: No, whatever happens to it happens. It is random. That is why I like the approach that many people are taking now that, rather than looking at the damage, they are looking for the functional assurance mechanisms, like chaperones, antioxidant mechanisms, damage prevention mechanisms etc.

SR: Several people have said that the important question is not why we age and die, but why do we live as long as we do.

VC: Yes, that is right. I think the scientific question is what determines the rate of aging. Why do mice age 30 times faster than we do? What are the specific mechanisms that determine the rate of aging in different species? Those mechanisms must be at the very core of the mechanism of aging. The details of what happens during the aging process have important health implications but how it works is the key.

SR: But these 30-fold differences between mice and men are seen when we draw a graph of age-related changes on the same physical time scale. But if we draw the scale in terms of percent lifespan completed, the differences in the rates of aging disappear.

VC: Yes, that is true. We can normalize to lifespan. But still the question is that both these species live in a similar environment on the same planet. Yet the 'clock' runs very differently.

2.3 Cristofalo Index

SR: Coming back to the system of cell culture for studying aging. Although this system has been in use for more than 45 years now, still there are questions raised about its validity and usefulness even in terms of the kinetics of age-related changes. There has been some controversy about the so-called Cristofalo Index. What's that?

VC: That continues to be controversial with some people who still are not happy with the idea. The history of that is that Alvaro Macieira-Coelho and I were both in Hayflick's lab at the same time. We were both senior post-docs. He went on to Uppsala University in Sweden and did some studies on labeling cells with radioactive thymidine, and determining the lengths of the periods of the cell replicative cycle. He reported that there was a decline in both G1 and G2 periods of the cell cycle during aging. We wanted to find out where in the cell cycle senescent cells were arrested. At that time I had a graduate student, Roz Yanishevsky. Her PhD project was to periodically label different age cells with tritiated-thymidine to determine when DNA synthesis occurred and how long the G1 and G2 periods were by autoradiography. In the very same cells she determined the DNA content chemically by using dyes that bound to DNA and measuring the binding. She determined that the senescent cells were blocked in G1 and that cells with twice as much DNA were tetraploid G1and not diploid G2. At the same time, George Merz and John Ross at the Wistar Institute tackled the question of whether all the cells were declining gradually in proliferative capacity or whether cells were dropping out of the proliferative pool in an 'all or none' fashion. They did this by clonal analysis. I became interested in it because they could not really tell if cells were both slowing down and dropping out. We then did further studies on the timing of the cell cycle, and out of that came the discovery that if we labeled the cells with thymidine for a very specific time period and under a specific set of conditions, we got a log-linear relationship between the percent unlabelled cells (i.e. not synthesizing DNA) and the percent lifespan completed. The coordinates of any given point became known as the Cristofalo Index.

SR: Who used or coined this term for the first time?

VC: Don Murphy. He worked at the NIH, and when I showed him the data, he coined the term. The usefulness of this was to develop a set of guidelines to find out how old the cells were independently of chronological time or passage number. After all, Hayflick's original report gave a range for WI-38 cell lifespan of 38–59 doublings. The variation in techniques among laboratories made the designation of 'old cells' or 'young cells' quite imprecise. It provided a working method for assessing cell age according to a normalized measurement of function, i.e. proliferation. But still, there are some people who do not agree that such a measure is necessary. I think the main reason for variation is the difference in the way cell culturing is done in different labs. We always seeded the same number of cells at each subculturing, and when I hear people talking about 1:2 or 1:4 splits, I have reservations about their data because there are different numbers of cells introduced at each split and a lot of uncertainty gets introduced.

2.4 Paul Phillips

SR: In your labs you had a colleague Phillips who has many papers with you, often as the first author. What happened to him?

VC: Yes, Paul Phillips came to me as a postdoc from Penn State University, and then he was with me for a number of years. He did lots of work, and he was a sort of my chief associate. There were other people too, for example Robin Charpentier, Bob Pignolo, Gary Grove, Cathy Finlay and many others.

SR: I was still curious to know what happened to Phillips ...

VC: That's a hard story to tell, because he sort of lost interest in research. I will tell you exactly what happened. He did author a lot of papers. He even got his own grants. But then he came to me one day and he said, "I want to return to teaching." It was his personal decision, and he is now teaching at West Chester University in Pennsylvania. He teaches physiology and general biology etc. It was a big loss to me, because when you have somebody in your lab who is senior enough, doesn't need supervision, and works with you, it is a very nice situation. I miss him.

2.5 Aging as a Science

SR: About the aging field, there has been so much negative feeling about this field. People used to shy away from calling themselves biogerontologists ...

VC: That has always been true. The history of aging research has been full of charlatans. Some years ago, the Joseph Macy Foundation gave aging research a boost by giving a grant to have a series of conferences which were published as 'The Macy Foundation Conferences on Aging'. Many of the leading biologists in the USA were part of that group. The other book that really put aging on a scientific basis was a book called 'Cowdry's Problems on Aging'. Cowdry was a pathologist, and he published on age-related pathology in great detail. Later, others provided updates of this volume. In many ways we have not come too much further since these studies, although a great deal of work has been done.

In the early 1970s there was a strong surge of interest for establishing a National Institute on Aging at the NIH. This interest was not shared by everyone. Many agreed that all of the issues of aging (cancer, cardiovascular disease, neurological diseases, etc.) were already in existing National Institutes. The gerontological community argued back that the rationale for a National Institute on Aging was to provide an emphasis for support for studies of the aging process itself. Interestingly, in 1974, Congress established the NIA. Len Hayflick was the leading candidate to be the first Director. But then withdrew because of the controversy over ownership of WI-38 cells. Robert Butler then became the first director of the NIA. Beginning with Butler and even until today, the trend at the NIA, to some extent, has been the

reverse of the original rationale for the Institute, and justifies itself to Congress by looking at diseases of aging as well as mechanisms. The biology of aging program at the NIA has undergone a steady, relative contraction in size. Some of the biology programs are highly specific like the program on longevity assurance genes.

About the negative feelings toward aging research, especially since 1975 when the NIA started giving grants, a lot of marginal applications were submitted in an attempt to get new money. The proposals were descriptive, were often based on faulty logic or faulty biology. For a period of about 10 years, the view of aging as a marginal science was reinforced. Aging got a bad reputation because there were very few aging applications that could get through the NIH study sections. There were two reasons: one reason was the bad science; there was a lot of bad science. The other was that almost all proposals were descriptive. There is nothing wrong with a descriptive study if it is well done and if it needs to be done. But the study section had the mentality that unless it is a penetrating mechanistic experiment, it is not a good experiment. Charles Darwin could never get through a modern study section.

SR: But the so-called bad science will be there in any field. It can't be that aging specially attracts bad scientists!

VC: No, what happens is that people have a sense that perhaps the competition in aging is less. So, more of the fringe scientists try aging for support. That is my personal feeling.

SR: That sounds like a circular trap that since aging does not have a high profile, it does not attract better people; and it will not have a high profile unless better people work on aging.

VC: Exactly!

2.6 Cosmetics and Aging

SR: What is the role of the cosmetic industry and biotechnology revolution on aging research?

VC: I don't know of any good example where research done by any of the cosmetic companies has actually helped our understanding the fundamental biology of aging.

SR: But most of the senior scientists in the field of aging are associated with one or more of such companies. Are they not able to influence their research and approach?

VC: I have been invited to give talks from time to time at Johnson and Johnson for example. Then there is an annual symposium organized by the cosmetic companies. I spoke at the latter three times but then they stopped inviting me, I guess because I never talked about cosmetics and their effects. Some people do talk about testing this and that chemical as anti-aging therapies, but I have never done so. It is not

unreasonable that the cosmetic companies should search for products. It's just not my approach to aging research.

SR: But don't we need those kinds of products too? After all there is a multimillion dollar market for that?

VC: There are two kinds of approaches to science in the interest of public health. One kind says that if we understand how it works, then we can use it more effectively. The other kind says that if it works, we do not need to care how it works. I have never been involved in that second kind of approach. My interest is to understand how things work.

SR: So, aging research is purely an intellectual fascination for you?

VC: Yes, I think so.

SR: Why is that? Is that due to your cultural background? I know so little about your personal background except that you have some Italian connection.

VC: My parents came from Italy, but I was born and brought up in the US. Culturally, I don't come from a long line of scientists. I just became interested in science because I liked to solve problems.

2.7 Dilemma and Cellular Aging In Vitro

SR: Have you ever faced a situation where you may have felt a dilemma between your scientific principles and social pressures, or where you had to take some strong decisions for science?

VC: Our recent observations about the relationship between donor age and the proliferative lifespan of fibroblasts was a kind of a crisis. We set out doing those experiments believing fully that the answer would be as it was in the literature that there is a loss of about 0.2 population doublings per year of donor age. However, we did not find any correlation. I called George Martin and told him that we were unable to see a negative relationship between donor age and replicate lifespan. He asked me how big our sample size was. I told him that at the moment it was 42. Actually, we did 124 samples, and there was no correlation. I had enormous pressure from colleagues not to publish these data. Klaus Bayreuther wanted me to speak about our findings at a Gordon conference, but the invitation was withdrawn, to both Klaus and me. Several of my own collaborators suggested to me that it would not be a good idea, politically to publish it, and other scientists also suggested to me that I should not publish these results. I also realized that I was jeopardizing my own success, but I was so sure of the results, and I felt that it was important to report the results even if they cast some doubt on existing dogma. To allow people to continue to believe in something when I knew it was not true, was dishonest. That was the conclusion that I reached. To do anything else but publish would be completely dishonest and contrary to my standards of personal and scientific integrity.

SR: Where did you then present these data for the first time?

VC: I talked to Tom Kirkwood at one point and he was organizing a Gordon conference, and he indicated that he wanted me to talk about these new data. He then had a satellite meeting to the Gordon Conference in Italy to examine this general question of replicative lifespan. By that time the paper was in press in PNAS. The symposium that Tom organized was very interesting. I was the first speaker and I presented the detailed data. They are pretty hard to argue with. After my talk was over, George Martin spoke and said that when he had read my manuscript, he went back and recalculated his data, taking out all the data for which the samples were from cadavers. He did not find any correlation with donor age and the proliferative lifespan of fibroblasts in culture. Then Jim Smith spoke up and said that they had known for years that there was no strong relationship. Since then, I have had to deal with serious criticisms from a number of people who argued that I should not continue to work on cells in culture if I believed my own data. That is nonsense!

SR: But do these data have serious implications for the validity of the model system to study cellular aging in vitro*?*

VC: That's what I want to get to. When I really thought about all this, I certainly realized something that has bothered me for many years. That is, when you make a cell culture, the cells that grow out from an explant are those which are selected for doing exactly that, growing. Even very old people have some high performing cells because they never run out of them. So, there should not be any surprise if no relationship is found, and it is not a necessary issue for the use of this model for aging research. There is no question that replicative senescence occurs. The question is whether the changes that occur in fibroblasts during aging in vitro follow the same pathway of changes that occur in vivo. At the same time, we must remember that human cells in culture are not little humans. They represent one differentiated cell type, which undergoes a spontaneous and predictable deteriorative process. We can use them because the power of the model is in controlling the environment, which you can't do with humans. We can find out about how the cell is regulated and how it fails. Then our findings have to be verified in the intact organism. Just like when you do experiments on *C. elegans.* For those experiments to have a meaning for human aging, they have to be demonstrated in humans. I think that the cell culture model is much more powerful and useful than other models simply because these cells regulate their processes with human genetic information.

SR: Do you feel bad about this incident and all those negative feelings of others that you had to face?

VC: I feel disappointed in the sense that some of the criticism has come from people whose integrity I believed was better than that. But, I would not hesitate to do the same thing again.

SR: Is it because of the pressure of their profession that they reacted the way they did?

VC: Yes, there is a lot pressure. In some ways it's just like one feels when one's job is at stake; then it becomes more difficult to do the right thing. But if I had to do it again and again, I would do it exactly the same way. I am convinced that not publishing things you know are true is just as dishonest as publishing things you know are not true.

2.8 The Future

SR: For experimental gerontologists of the younger generation, what advice or suggestions you have?

VC: That's a tough question. I think what we have to do first is to confront the seductive idea that aging is controlled by a single switch mechanism. I don't think that the 'one switch' approach is going to provide anymore information in the future than that it already has. As we were discussing earlier, the important biological question to ask is what are the mechanisms that provide us with the ability to live as long as we do. From a biological point of view, that is the key question. Looking at the mechanisms by which life ends is not going to provide us information about how life is maintained. Of course, the important health question is about the changes that occur with aging. These are the changes that cause disease, pain and suffering I would like to understand more about that process. But the intellectual challenge is about why the species that survive on this planet, survive long enough to assure the reproduction of their offspring and then simply deteriorate through a cascade of different mechanisms that eventually end in death. Is it simply a matter of redundancy differences in the mechanisms that defend us from stress and damage that accounts for the differences in lifespan or are there additional mechanism, as yet undiscovered, which will need to be unveiled?

SR: From program to stochastic, that has been your journey for 40 years!

VC: Yes, I no longer believe that cell death is programmed. I believe that cell population replicative lifespan is somehow genetically controlled, but not individual cell death. The changes that lead to death happen stochastically, but the interesting question is why don't they happen at a constant rate? Genetically determined protective mechanisms determine the impact of those damages. If we could find out what determines the rate of aging, we could perhaps maintain the integrity of those mechanisms. Aging is not a disease, but it provides vulnerability to every kind of insult. We need to understand aging to understand mechanisms that make people vulnerable to diseases. This is a health issue.

SR: Personally, have these 40 years in the field of cellular aging been satisfactory to you?

VC: I think so.

SR: Did you get the credit you deserve?

VC: I think I probably got more credit than I deserved. It has been a fun ride. I learned a lot, I had good colleagues, and it has been exciting. I would like to be around when the answers finally fall out.

SR: Do you think that one-day we will have the ultimate answer?

VC: I don't know. I think the answers are going to come a piece at a time. For example we may find out that age-related vulnerability to cancer is due to this or that factor. But that will be for cancer. Then there will be other factors for atherosclerosis or something for something else. In the end, we will realize that our trajectory on this planet is determined by the equipment our genes supply. When that equipment wears out, then we become vulnerable to any insult.

SR: How do you perceive your contribution to this field?

VC: I did not plan it this way, but it turned out that I was providing the data that both validated and critically assessed the cell system that Hayflick had established. I think I have been, in some way, instrumental in gaining recognition and understanding about cellular gerontological research and the role it can play in helping us understanding aging.

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