Healthy Ageing and Longevity 4
Series Editor: Suresh I.S. Rattan

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Leonard Hayflick *Editors*

Cellular Ageing and Replicative Senescence

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Healthy Ageing and Longevity

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Suresh I.S. Rattan, Aarhus, Denmark

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Preface

We are delighted to present this book in which our contributors revisit more than 55 years of research based on the discovery that cultured normal cells are mortal and the interpretation that this phenomenon is associated with the origins of ageing. At the same time, and in the same laboratory, it was also discovered that the only immortal cells in vitro were those with the properties of cancer cells. The mortality of normal cells and the immortality of cancer cells were also reported to have in vivo counterparts.

Until these finding occurred the focus of research on ageing was mostly descriptive and the cause of ageing was thought to be driven by extracellular events like wear and tear, radiation and stress. With the discoveries described above the focus on the cause of ageing for the next decades was redirected toward intracellular events. Thus began the field of cytogerontology.

The origin of age changes has more recently evolved to include fundamental events that occur at the molecular level and that involve the flow of energy. The serial replication of normal cells in vitro is accompanied by progressive changes which eventually reach an irreversible state of growth and proliferative arrest, also known as replicative senescence.

This book covers the origins and subsequent history of research results in which attempts have been made to clarify issues related to cellular ageing, senescence, and age-related pathologies including cancer. The book is organized into five sections: (1) history and origins; (2) serial passaging and progressive aging; (3) cell cycle arrest and senescence; (4) system modulation; and (5) recapitulation and future expectations. These issues are discussed by leading thinkers and researchers in biogerontology and cytogerontology. We are confident that this collection of articles provides state-of-the-art information.

We hope that this book will encourage students, teachers, health care professionals and others interested in the biology of ageing to explore the fascinating and challenging question of why and how our cells age, and what can and cannot be done about it.

Aarhus, Denmark — Suresh I.S. Rattan
The Sea Ranch, CA, USA — Leonard Hayflick

Contents

Part I
History and Origins

Chapter 1
Origins of the Hayflick System, the Phenomenon and the Limit

Suresh I.S. Rattan

Keywords Hayflick phenomenon • Hayflick limit • Senescence • Evolution • Longevity • Serial passaging • Gerontogenes • Cancer • Telomere

SR: Let us start from the beginning: as far as I know, before 1960 you were very much in the forefront of mycoplasma research in relation to arthritis, so how did this shift from mycoplasma to cellular aging occur?

LH: I attended the University of Pennsylvania first as an undergraduate, then as a graduate student pursuing a Master's degree at first. My major subject in the university was microbiology, and I had a fundamental interest in bacteriology. After I graduated with my bachelor's degree, I went to work at what was then called Sharp and Dohme, which was then taken over by Merck, and Merck, Sharp and Dohme developed out of that. I then realized, having not appreciated this fact earlier, that I did have the intellect to obtain a PhD. I decided to go back to Penn and pursue a Masters and PhD degree. One of my friends at that time, who was in the Department of Medical Microbiology at Penn, which is the department that I chose to apply to, was working with the mycoplasmas. These are very interesting microorganisms – the smallest free-living microorganisms – and I decided that I would like to work with them. So I gained admission to the department and pursued a research project using those organisms when my chief, who was a very young fellow, returned from one of the first courses in cell culture given in the United States. He returned full of enthusiasm about cell culture, and tried to convince me to pursue a research problem having to do with cell culture ....

This chapter is a slightly edited version of an interview made with Leonard Hayflick (LH) by Suresh Rattan (SR), which was published in the inaugural issue of the journal BIOGERONTOLOGY (volume 1: pp. 79–87, 2000).

(*With kind permission from Springer Science+Business Media: 'Just a fellow who did his job . . . ', an interview with Leonard Hayflick, by: Suresh I.S. Rattan; Biogerontology 1: 79–87, 2000).*

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SR: Who was this . . . ?

LH: His name was Warren Stinebring who, unfortunately, is now dead. Warren was such a young Professor that my responsibility on paper was not to him, it was to Stuart Mudd, a very well known microbiologist and Chairman of the Department of Medical Microbiology. So it was my young boss returning from this course full of enthusiasm who tried to induce me to become interested in cell culture. I obtained my Master's degree working at the Wistar Institute, which is affiliated with Penn, and studied the mycoplasma contamination of cell cultures with mycoplasma. So my interest in cell culture resulted from gentle persuasion. But I began to enjoy using the technology and then it became fascinating. I ultimately received my degree, and accepted a post-doctoral position at the University of Texas, where I worked with one of the leading personalities in cell culture in the world, Charles M. Pomerat.

SR: So, you got your PhD from the University of Pennsylvania, in which year?

LH: In 1956, in Medical Microbiology. In any case, I was awarded a post-doctoral fellowship to study under Charlie Pomerat at the University of Texas, Galveston. He was a remarkable person, and the most unusual personality that I have ever met. Unusual in the sense that he had a prolific intellect that included expertise in anatomy, neurobiology, cell culture, marine biology, architecture, art, food preparation, and he was the best lecturer I ever met. After 2 years, I was contacted by Hilary Koprowski who by then had taken over the directorship of the Wistar Institute in Philadelphia, and induced me to return to Philadelphia to join the Wistar Institute at its new re-birth. It is the oldest biological research institute in the US, and enjoyed a renaissance once he took over. I was hired to run a cell culture laboratory. In addition to running that laboratory, my responsibilities included a research project directed toward investigating whether or not human cancers have a viral etiology. I was working on that project while simultaneously working with mycoplasmas which were still of great interest to me. Through a series of events that I won't relate in detail now, I also discovered, in the same year that I discovered the finite replicative capacity of normal human cells, the etiology of a very common human disease which in the US is called 'walking pneumonia'. It is properly called 'primary atypical pneumonia'. This is a common ailment, which had been thought to be caused by a virus, but I discovered that the etiological agent was in fact a mycoplasma that ultimately I named *Mycoplasma pneumoniae*. This is the only mycoplasma proven definitely to be the etiological agent of a human disease.

As I indicated before, I had also begun to work on the cancer research project, which necessitated the growth in culture of human cancer cells in order to make extractions, in the very primitive way in which this was done in the late 1950s and early 1960s. My plan was to put the cancer cell extracts on to normal human cells to see if normal cells changed in any way. We were searching after all for viruses. We wanted to use as our normal cells, the cells from human fetuses because at that time it was very well known that the tissue from adults frequently contained garden-variety viruses like adenoviruses, herpes and others. But, these were rarely found in human fetal tissues. We obtained human fetal tissues from a

local hospital and from Sweden where surgical abortions were legal, and where we had connections with some scientists in Stockholm. So, we started to grow cells from human cancers and from normal human fetuses.

After about a year or so of doing this I realized that despite the fact that human fetal tissue arrived in the laboratory at random times, all of the cultures were dying after about 50 subcultivations. This was itself not unexpected because I had been taught that cells will die in culture because we do not understand how to grow them properly. But, what struck me about my cultures was that those that were dying were the ones that were in culture longest despite the fact that I had one technician using one pool of media and one pool of glassware on all of the cultures. Nutritional inadequacy was inconsistent with my observation that only the cultures that were growing longest had stopped dividing. That was the inconsistency that tipped me off to what followed.

1.1 The Paradigm Shift

LH: I began to pursue this observation because it was very strange, and I pursued it to the point that it involved my full time attention. In fact, the original project on cancer biology was dropped, and fortunately my results with the normal human cells were so striking that my boss had no objection to my continuing to work on this observation. That was very fortunate for me because at that time there was a great deal of money available in the US for medical research, and following unusual results not written into grant proposals was easy to do. Today, if you do that you would be flirting with a jail sentence for using government funds for unapproved purposes. I did a number of experiments that proved to myself and to my colleague Paul Moorhead that my observation was not an error. I had worked with Paul initially at the University of Texas, and managed to bring him to the Wistar Institute. He was one of the first cytogeneticists, and he and I worked together for several years.

One of the things we wanted to prove was that these cells were normal. By this time the normal human chromosome number had been discovered. So, Paul did the cytogenetics and we discovered, as we had expected, that these cells were normal cytogenetically. We did many other studies that were reported in our first paper, and determined, at least to our satisfaction, that the cells had stopped dividing not because of any accident or ignorance about the culture media, but because of some internal clock. That is the long answer to your short question.

SR: But you insisted on pushing this interpretation that there were intrinsic reasons, which was going against the dominant paradigm of Alexis Carrel at that time!

LH: Exactly!

SR: How did you dare to do that? About a year before you, Swim and Parker had published almost similar curves but they reached the conclusion that there was something wrong with their methods that prevented cells from dividing forever.

Although Dr. Peyton Rous first rejected your paper from publication in the Journal of Experimental Medicine, and then later it was published in Experimental Cell Research, what made you stand by your observations? Where did this conviction come from? Can you look back into your bringing up?

LH: I would not have the conviction to stand by my interpretation of the results had it not been for the fact that the experimental results were very definitive. I won't go through all the experiments because there were many that were done. But, one experiment that I usually emphasize is the one in which Paul and I mixed young female cells with old male cells. By young I mean low population doubling level. We mixed equal numbers of female cells at the 20th population doubling with male cells at the 40th population doubling level. The limit for both was 50 doublings. We maintained these mixed cultures and found that the mixture stopped dividing when the youngest component stopped dividing. Therefore, the argument that media or viruses or some artifact of culture could explain this phenomenon is untenable. Another experiment that what we did and that does not appear in the published paper because of its unorthodoxy is this: I decided to challenge the grey eminences in the field who told me that my culture conditions were inadequate, by sending to them a culture of my human diploid cell strain WI-26 at that time, and telling them that the luxurious culture of human fibroblasts that I am sending to you will stop dividing in 6 months time. And so, in 6 months the telephone rang, and they said that their cultures had stopped dividing. I felt that if I had made a mistake then I would go down in flames with the best people in the field. So Paul and I decided to write the paper describing our observations. However, we were still left with the question of interpreting the results. I knew that for the previous 60 years (cell culture began in 1900) it was believed that cells put in culture always have the capacity for immortality. This had profound implications in the field of aging, because if what was believed for the first 60 years of the twentieth century was true then aging has nothing to do with intracellular events. It has only to do with extracellular phenomena. Cosmic rays were invoked at that time as causes of aging, and changes in the ground substance, or anything you can think of that is extracellular. So, this was a major shift in understanding with respect to aging that I was suggesting. I was now focusing attention on the behavior of the cells themselves, and on an intracellular counting mechanism specifically. And I was very well aware of this. We had eliminated other potential explanations. One was simple dilution of some nutritional factor that could not be synthesized in vitro but only in vivo, which we put to rest simply on mathematical grounds.

1.2 Human Vaccines in WI-38 Cells

LH: Our paper contained two other very important elements: one was our demonstration that these normal human diploid cells had the most exquisite sensitivity to human viruses known. They still do. By this time the poliomyelitis vaccine was

becoming accepted and it was also becoming apparent that the primary monkey kidney cells used for the production of poliomyelitis vaccines were contaminated with unwanted viruses whose behavior in human beings was not understood. This was a real threat. By this time we had established that cell strains, for example WI-38, were absolutely free of indigenous viruses, and therefore were much safer than monkey kidney. Not only that, monkey kidney was permitted for use only as a primary culture. I pointed out that human diploid cells could be frozen at low passage level, studied for years to prove that they are safe, and then used for vaccine production. You cannot do this with primary cells. For the first time we introduced the concept of characterization and standardization into cell substrates used for the manufacture of human biologicals.

It took 10 years of struggling to have these concepts accepted. Now these concepts are the foundation of the biotechnology industry. This in itself was an enormous practical advance but it took 10 years for that to be accepted by the FDA where it was mostly delayed for political reasons. In fact, the first licensed vaccine produced in my human diploid cell strain WI-38 was produced in Yugoslavia and later in the UK, Germany and Russia, and finally in the USA in 1972. The problem of having normal human cells accepted for use as substrates for human biologicals has as much a history of antagonism and emotional grief on my part as does the acceptance by the scientific community of the finite capacity of normal cells to divide. People were dying during that period of time because there were several monkey viruses that caused deaths in handlers of monkeys and their tissue cultures. About 20 people had died handling monkeys and their kidney cultures during this period of time, even when there was continued terrific resistance to the use of normal human cells for vaccine production. The reasons for that involve such things as personality clashes, ego, politics and economic concerns. In any case, the outcome of that is that today virtually every vaccine in the world is produced in my WI-38 cell strain or in similar cells. Hundreds of millions of humans have been vaccinated with products made in WI-38 or similar strains with no evidence of a safety issue traceable to the cell substrate.

SR: That is perhaps a much bigger practical application and contribution to science than is the aging story . . . !

LH: That is absolutely true. Almost one billion people worldwide have received and still do receive vaccines produced in my cells which are totally safe and efficacious.

1.3 Strength of Character and the Lawsuit

SR: One aspect of your character as a person that comes out of both your work regarding the limited proliferative capacity of cells, and later on with your struggle with NIH about the ownership of cells, is that you have a very strong personality. What makes you so strong?

LH: Well, I can't tell you specifically. I can only tell you that my upbringing is probably the key. As I indicated earlier, the only reason that I ever take a strong position is when I am absolutely confident that the scientific data and the reasoning behind the interpretation are sound. In all these cases that is how I felt even when I faced the NIH and brought a lawsuit against the US government. I felt absolutely certain that I was correct, and in fact as events have unfolded, my position has been shown to be correct and 10 years ahead of its time. The NIH accused me of having stolen government property and selling it for personal gain, which is, of course, a criminal offense. Today if you don't do what I had done, which was to claim intellectual property rights in WI-38, or some other biological entity, you are a failure in microbiology or cell biology. You are a total failure if you don't own a patent, if you don't have a commercial relationship or if you have not exploited something that has come out of your laboratory as a result of support from taxpayers' grant funds. My lawsuit against the US government was so successful, that in 10 years even NIH employees can now enjoy a maximum of 100,000 dollars over their salary per year for something they have discovered in a government laboratory, funded by taxpayers. That would have been unthinkable before my legal victory. People have made this observation, and I think it is correct, that had we not won our lawsuit, the biotechnology industry would be much different than it is today. That is because the biotechnology industry is based upon the principle that biologists have intellectual property rights. Prior to my lawsuit that principle was understood and exploited by physicists, electronic engineers, and software writers, but academic biologists were the last group in science in the US to turn 180° from having their skirts soiled by financial interests to becoming entrepreneurs. Now that behaviour by biologists is applauded and celebrated. When I became the first one in the US to claim intellectual property rights, I was branded a criminal. I was brought up to be independent and to have confidence in whatever I found that was demonstrably correct and true.

1.4 Humble Beginnings

SR: That is not a typical Jewish family bringing up where you are taught to respect tradition, to accept ....

LH: I don't think religion has anything to do with it. My mother and father were very liberal. They left me to do pretty much whatever I wanted to do because they had imbued in me the difference between right and wrong. I had the usual childhood exploits that all of us have, but nothing particularly striking that would immediately come to mind. But, my commitment to truth and justice was foremost. I take enjoyment in challenging dogma. If there is anything that I challenge, it is orthodoxy.

SR: Is it a reaction to parents or is it due to encouragement from them?

LH: Oh, it is encouragement, absolutely. My parents encouraged freedom. My father was not a formally educated man because he was forced to work all his life. When he was 7 or 8 years old he drove a horse and wagon and delivered dairy products from his mother's store in Philadelphia. He didn't go farther than the 7th or 8th grade. He then worked in a dental laboratory and became an expert on the design of most complex prosthetic devices. He eventually went into business for himself, but he never made a lot of money. It was in his later years that he felt a little bit more comfortable, but by no means wealthy. My mother came from a struggling family as well. So struggle was always a part of our background. I grew up in the depression and can recall when my father walked to work from suburban Philadelphia to the center of the city in order to save 15 cents trolley fare. So, being brought up in the depression has a lot to do with my work ethic, my belief in myself, and that I should have confidence in what I think is true and correct as long as it is demonstrably so. I have always had those traits.

1.5 Biogerontology as a Mature Science

SR: You are most well known for your contributions in the field of aging; and aging research is one field where an immediate response from the public is "...wow lot of money ...". What do you think of the present trend?.

LH: I never had that impression. Of course there is a hugely successful exploitation of the public's ignorance of scientific truths, which around the field of aging goes back centuries. One could forgive the lunacy practiced 3 or 400 years ago when not as much was known about science and about seeking the truth using the scientific method as we know today. But the lunatic fringe is certainly more present in the field of aging than it is in any other biological discipline, which is a perennial problem. When I entered this field, to declare publicly or even to your colleagues that you were working in the field of biogerontology or aging research was tantamount to committing professional suicide. So you called yourself something else, like a cell biologist or physiologist or whatever. I was doubly damned because cell culture in which I worked was considered to be a black art, and gerontology was considered to be a black art, and the combination was tantamount to having two strikes against you from the start.

SR: Has that situation changed for aging research now?

LH: It certainly has changed for the field of aging. There is no question about that. Aging research is now strongly rooted in the scientific main stream, and there are a number of interesting reasons why the field has become acceptable. It is part of main stream biology today, partly initiated by the changing demographics of populations in developed countries. We now have an increasingly greater proportion of our population who are elderly, and that is a very potent political and economic force. And potent economic and political forces ultimately meet with science, as they did

in this case, and promoted the field. The most important event in the US was the establishment of the National Institute on Aging, in which I had some direct role, and indeed was offered the first directorship. As a matter of fact I did not accept it for two reasons. First, the salary was too low to support a university education for my five children, and second, I asked the NIH to send an attorney to my lab so that I could talk to him about the ownership of my WI38 cell strain, which was an unique question then in not only science but also in law. Who owns the rights to a self-duplicating system? At that time the patent office refused to patent WI-38 cells because living things were unpatentable in 1962. So, I lost the opportunity to patent WI-38 cells which are now used to manufacture products that reap in the multiples of billions of dollars annually for many of the world's pharmaceutical companies. I have gotten only two good dinners. This could not happen today because living things like WI-38 can be patented. But in 1975, when the NIH sent to me an accountant, and not a lawyer, this person concluded that I had stolen WI-38, which triggered the lawsuit that I described earlier.

1.6 The Hayflick Phenomenon and Cancer

SR: Most of the experimental gerontology research that has given scientific credibility to aging research is based on the Hayflick system and the Hayflick phenomenon. How do you feel about naming this whole phenomenon of cellular aging in vitro as the Hayflick phenomenon?

LH: I am happy that the observation has finally been almost universally accepted. It was Sir Macfarlane Burnett, Nobel Laureate from Australia, who coined the phrase 'Hayflick Limit' for the first time. He was writing a book on aging, and was aware of my work, and he coined that phrase. I have never used the phrase and have always called it the Phase III phenomenon as stated in my original paper. It is a mixed blessing. I am not a seeker of self aggrandizement, but if my colleagues choose to use that term, fine, I have no objection to that, but I don't encourage it.

SR: What has this phenomenon taught us about the basic aging process?

LH: I don't think that it is fully appreciated that our demonstration that normal cells are mortal provided the essential information that made the concept of 'immortalization' possible. One of the major areas of interest in cancer biology and now in aging research is the change from a mortal cell population to an immortal cell population. That was the key part of our original paper, because you cannot talk about immortalization of cells unless there is such a thing as a mortal cell. Since much of cancer biology today is based on an understanding how a mortal cell becomes an immortal cell, that was the main thrust of our original paper. As to what this has taught us about aging research, it is this. The finite replicative capacity of normal cells, now known to be governed by telomere shortening, is probably telling us more about longevity determination than it is about aging. The hundreds

of published physiological decrements that herald the approach of Phase III is the aging process because aging is simply an increase in unrepaired molecular disorder.

SR: Yet it took cancer researchers more than 30 years to realize that!

LH: Absolutely! So our contribution to this fundamental aspect of modern cancer biology is at least as important, if not more important, than the aging interpretation. If we had not pointed out that normal cells are mortal, then immortality would have been assumed to be the property of not only cancer cells but of normal cells too as it had been for the first 60 years of the twentieth century. My thinking about aging has evolved, and some of my positions about aging have changed 180° because of new data. When I first entered the field of aging, it was in the Stone Age. So my views had to change, and I am sure they will change again. In our experiments we showed that normal mortal cells do exist and that there is a counting mechanism. We also showed that the replicometer was located in the nucleus. That is not well known today. My then doctoral student Woodring Wright and I demonstrated that the replicometer was located in the nucleus. I coined the term replicometer because it is really not a clock. A clock measures the passage of time. Telomere shortening is telling us about the number of rounds of DNA replication. A meter is an event counter, so I am calling this a replicometer. I never thought I would live long enough to see a molecular biological explanation of my finding. Today we have it, or we have a substantial part of it. It is telomere shortening. So, the past 10 years of research on telomeres and telomere shortening have just been very satisfying for me.

1.7 Aging In Vivo

SR: But all these explanations are for the ultimate phenotype of replicative senescence in vitro, whereas in the body, as it has been argued repeatedly, this phenotype may never be attained for most of the dividing cell types. However, most of the current research is focused on this final phenotype, but what about these 50 or so doublings that normal cells have to undergo before they stop dividing? After all, the process of aging is the whole lifespan curve of cells in culture and not merely the end stage. There is progressive accumulation of damage that finally is a kind of signal for cell cycle regulators to become active. How do you think the Hayflick system can be utilized experimentally to understand how and why damage accumulates in mortal cell populations but not in immortal cell populations?

LH: In the years since the publication of our original paper there have been hundreds, if not thousands of publications showing an equivalent number of incremental and decrimental changes in the biology of normal human cells as they get closer and closer to Phase III. Assuming that those changes are going on in vivo, and there are data for that happening, then those changes, I would argue, are aging changes. Those changes which precede the loss of replicative capacity in fact fit perfectly the definition of aging, namely increase in molecular disorder. If that happens in vivo, then you have an absolute counterpart *in vitro*.

People fail to appreciate the likelihood that the decrements that occur in dividing cell populations as they approach Phase III in vivo could have profound effects on non-dividing cell populations. Telomere loss, on the other hand, is telling us more about longevity determination than it is about aging. We do not know enough yet about the telomere system to argue against the possibility that as telomeres shorten they trigger losses in physiological capacity. What we know today is that telomere length is a measurement of permissible rounds of DNA replication and hence a longevity determinator of a dividing cell's capacity to replicate. I see this as a phenomenon distinct from aging. This distinction is not well understood in the biogerontological community. People talk glibly about aging genes when those genes govern longevity determination and not aging. I don't think there are genes that govern aging processes.

SR: Perhaps one can use a buzz word such as 'gerontogenes' to draw attention to the fact that when one is talking about any genes in relation to the progress of aging, without actually meaning that there are real aging genes. They are there only virtually!

LH: Longevity assurance genes, for example, or some other name. Yes, we need some better terms.

1.8 The Aim of Biogerontology

SR: Until now we have described the phenomenon of aging in many systems, and we still keep on describing it. However, almost all research grant applications are written containing a statement 'the aim of aging research is to improve the quality of life'. But one rarely sees any proper scientific experiments being performed to fulfill this aim. How do we go about intervening in the aging process to achieve better quality of life in old age?

LH: I am worried about a more fundamental question that is behind the one you asked, and that is why do it? What is the advantage for humans to have the power to manipulate the aging process or longevity determination? Why is that a desirable goal? The answer to that question is not clear to me. It is not necessarily good news today, for example, to have a pill taken each morning that would either arrest the aging process or permit us to live X number of years longer. Let us pursue that magic pill, because that is behind your question. Let us assume that we have now arrived at an understanding of the basic longevity determining processes to the extent that we have a therapy. The probability, first of all, is that that therapy is going to be available first to the rich and powerful. I am not sure at all that I would prefer to see the rich and powerful have a greater capacity for longevity than those who are not rich and powerful. I am worried also that tyrants, serial killers and other socially undesirable characters would have equal access to greater longevity. Of course, there

is an opposite argument that all the good people and contributors to our civilization will also have the same fate instead of simply dying at an earlier age.

But it becomes an even trickier question. People who want to live longer are arguing from a base of perceived life satisfaction. That is, they assess themselves to be happy at a particular moment in time. Significant numbers of happy people are a very recent phenomenon. Life satisfaction occurs less in poorer countries and in relatively greater numbers in developed countries. People will be faced with this problem: when in your life do you want your aging process to be either arrested totally or slowed down. That will vary from person to person depending on their life satisfaction at the particular time when this opportunity presents itself. The probability is that many people who are sufficiently knowledgeable will realize that there are many people in their seventies or later who will tell you that this is the happiest time of their lives. Their kids are married, they have no family responsibilities, they have bought themselves caravans, or recreational vehicles, and follow the sun from Florida in winter to Canada in summer and the reverse twice a year. This is the happiest time of their life; they are not wealthy but they have enough money to live comfortably. Had they decided to arrest their aging process at the age of 30, 40 or 50, they would have either delayed or eliminated the likelihood of experiencing the best time of their lives. So the decision to arrest the aging process or to prolong it is a very serious question. Not only that; not everyone will take this pill. I might choose to take the pill, but my kids might not, which means that my kids are going to become older than me! Human's ability to tamper with the aging process will produce societal dislocations and effects on human institutions that will be monumental.

SR: Of course that is a very important issue about longevity. But what about this question of healthy old age or disease-free old age?

LH: That is a completely different issue. There is no argument that there is a need to understand the etiology of the leading causes of death in order to intervene. But the resolution of diseases causing death in old age is not aging research. It is geriatric medicine. The failure to appreciate this critical distinction is a fundamental problem in the field of biogerontology.

SR: So, how do we go about it?

LH: That represents an area in biology that I think suffers from a fundamental loss in appreciating the role of biogerontology. For example, cancer, cardiovascular disease and stroke are more likely to occur in older cells than in younger cells. The question not asked, and that I think needs to be asked is: Why are old cells more vulnerable to pathology than are younger cells? I think that is the job of the biogerontologist. We wrongly assume that aging is a disease. It is not for several important reasons. Age changes simply increase the vulnerability to diseases. We are also not addressing another important question that was first raised by George Sacher. His perspective was that the key question in aging is not why we age, but why do we live as long as we do. That is a very important insight.

SR: What are the experimental approaches for that?

LH: There are dozens and dozens of approaches, but we must understand the nature of the problem. The nature of the problem is that age changes are stochastic, that is increasing disorder occurs at every level – from the molecular to the whole organ. So you cannot exclude any biological discipline as a potential contributor to our understanding of longevity determination and the aging process.

1.9 The Future

SR: In your view, what are the hot areas in aging research these days?

LH: Well, there are hot areas of emphasis today but that doesn't imply importance. Calorie restriction research and antioxidant and free radical research are popular now. But many other areas have been neglected, too. First of all I am not sure that aging is reversible or manipulatable. I think what is manipulatable is longevity determination. My reasons for that belief are based on the indisputable fact that everything in this universe ages. Automobiles are a good general analogy. When you buy a Mercedes or a Ford, you have an innate expectation of its longevity. In the former case you expect it to live for 8–10 years and in the other 3 or 4 years. Why? Because the design and the construction material used differ in quality, in efficiency and in many other attributes.

That is a perfect analogy with biological material. Also, there is no part of the blueprint for an automobile that dictates the aging process. I don't think, for the same reason, that there is in the genetic blueprint a gene for aging. Longevity can be engineered into an automobile and into an animal, but not aging. Disorder from order is the universal aging process. One can delay the appearance of the aging process only by tampering with the process of longevity determination.

1.10 And Finally . . .

SR: How would you like to be remembered – as a breakthrough scientist, a rebel, a thinker?

LH: Just a fellow who did his job, I suppose, that's all!! My job is to challenge authority. My career has been that of a rebel, and the things that I rebelled against turned out to be accepted, ultimately, by most scientists and most of society. So I guess I am guilty of being the arch typical iconoclast.

Chapter 2
Experimental Foundations of the Hayflick System

Suresh I.S. Rattan

Keywords Hayflick phenomenon • Hayflick limit • Senescence • Cell cycle • Mortality • Immortality • Growth factors • Telomere • Methylation

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.

Part II
Serial Passaging and Progressive Changes

Chapter 3
Slowing Down of the Cell Cycle During Fibroblast Proliferation

Alvaro Macieira-Coelho

Abstract During serial proliferation, the cell division cycle of normal human fibroblasts slows down due to several modifications in the cycle. It is now accepted that these modifications are associated with the cell ageing that occurs in vivo, which can also be reproduced in vitro. The slow down is due to different cycling characteristics, of each of the four fibroblast types that become progressively apparent during cell replication. Attempts were made to identify an event that could explain it all. However, among the plethora of functional changes that can be observed at the cellular, sub-cellular and molecular levels, none are likely triggers for the slow down of proliferation. There is, however, a basic phenomenon that is associated with the changes of the cell cycle, and can be identified to a great extent with the decreased probability of initiating and transiting the division cycle. It is the evolution of cell volume that is different for each of the four fibroblast types, and concerns the biology of conformation – a subject that has been neglected in the field of cell ageing, and is explained herein.

Keywords Cell ageing • Biology of conformation • Contact inhibition of division • G2 period • Telomeres • Asymmetric DNA synthesis

3.1 Evolution of Cell Types During Slowing Down of the Cell Cycle

It is now well accepted that four human fibroblast types of different sizes exist and can be observed in vitro and in vivo. This confirms the analogy observed in findings from experiments performed in vitro and in vivo. Type I fibroblasts decrease and types II and III increase during serial divisions (Franks and Cooper 1972; Russell

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and Witt 1976; Hennis et al. 1981; Steinhardt 1985); at the end of the replicative cell population's life span one can observe in vitro a final fourth type fibroblast with a significant different morphology and a reorganization of the cytoskeleton (Raes et al. 1983) and of the 30 nm chromatin fibers (Macieira-Coelho 1991). Human fibroblast evolution, during serial proliferation, results in slowing down of the cell cycle due to varying cycling times of each fibroblast type. The post mitotic type IV cell observed in vitro is not found in vivo in normal human donors. However, a pre-terminal type IV cell can be seen in normal tissues in vivo independently of donor's age (Macieira-Coelho 1995). It has the modifications of chromatin fibers characteristic of the terminal cells observed in vitro, but without the nucleolar changes. The unfolding of four different cell types during serial divisions, favors the suggestion that this constitutes a developmental program towards terminal differentiation (Martin et al. 1974).

Several markers of the type IV fibroblast were used attempting to evaluate its presence in vivo: modifications of heterochromatin and of the 30 nm chromatin fibers (Macieira-Coelho 1991), telomere length (Allsop et al. 1992), expression of genes involved in cell cycling (Grassilli et al. 1996), expression of the EPC-1 (early population doubling cDNA-1) quiescence-specific gene (Pignolo et al. 1993; Tresini et al. 1999), gene profile determined by cDNA microarrays (Park et al. 2001), none could detect the cells in normal tissues of human donors. The belief that the terminal fibroblast observed in vitro, is present in vivo, was the result of the utilization of the lysosomal enzyme beta-galactosidase to detect post-mitotic cells (Dimri et al. 1995). Lysosomal enzymes increase during a prolonged resting phase regardless of whether the resting is reversible or irreversible (Macieira-Coelho et al. 1971), therefore they are not suitable to distinguish between a long quiescence and a terminal post-mitotic state. However, the type IV fibroblasts observed in vitro were found in the tissues of human donors with different pathologies. Indeed, they were detected in a 30 year-old patient with diabetes and hypertension and in a patient with Werner's syndrome (Puvion-Dutilleul and Macieira-Coelho 1983), in patients with xeroderma pigmentosum, Cockayne syndrome (Puvion-Dutilleul and Sarrasin 1989), and with diabetes and kidney disease (Morocutti et al. 1996; Uziel et al. 2007), in venous ulcers and venous hypertension (Mendez et al. 1998), in post-radiation fibrosis (Herskind and Rodemann 2000), and in liver cirrhosis (Wiemann et al. 2002). So the type IV fibroblast must be normally eliminated, its persistence is part of the pathological process. This also favors the idea that the evolution of the fibroblast life span is actually a differentiation program (Martin et al. 1974). The terminal post-mitotic cell is normally eliminated but the pathologic process would create a deviation of the normal developmental process with accumulation of the terminal cell.

As explained below, the shift in cell volumes of the different types of fibroblasts, are associated with structural changes coupled with functional modifications causing the slow down of the cell division cycle.

3.2 Kinetics of Proliferation During Slowing Down of the Cell Cycle

From the beginning, at early population doubling levels (PDL) there is an entire spectrum of cells with different cycling probabilities, which evolve with an increasing heterogeneity (Macieira-Coelho et al. 1966a, b; Macieira-Coelho 1967, 1974; Macieira-Coelho and Berumen 1973; Absher et al. 1974; Absher and Absher 1976; Macieira-Coelho and Azzarone 1982). The first parameter that starts evolving through serial divisions is a steady delay in the commitment to divide expressed by the constant decline in the rate of entrance into the cell division cycle (prolongation of the G1 period) after plating the cells (Macieira-Coelho and Azzarone 1982). The second parameter that manifests early, is the decline in the maximal cell densities (Hayflick and Moorhead 1961), it is the expression of an increased sensitivity to the various mechanisms that arrest division due to cell crowding. It leads to a prolongation of the G2 period and the accumulation of cells, which can not finish the cell cycle during one passage (Macieira-Coelho et al. 1966b; Macieira-Coelho and Berumen 1973). The prolongation of the G2 period can be measured indirectly by grain counts on interphases checking if the counts have halved through division after sub-cultivation of the cell monolayer (Macieira-Coelho et al. 1966b), or directly checking the time the curve of labeled metaphases reaches 100 %. Measurement of the areas before the curve, reveals the size of the fraction of cells in the G2 period and the velocity through the cycle (Macieira-Coelho and Berumen 1973). Most of the cells that were first thought to be blocked in G2 (Macieira-Coelho et al. 1966b) were later found to be delayed, although some with very long delays in this period of the division cycle (Macieira-Coelho and Berumen 1973). A significant number of cells, though, are blocked in G2 (Kapp and Klevecz 1976). Gelfant and Smith (1972) studied extensively the problem of the time spent by cells in vivo in G2. They found that in the tissues there are cells that start cycling from the G2 period and that this fraction of cells is increased in old animals, showing an analogy between ageing in vitro and in vivo.

Other parameters characterizing the slow down of the cell cycle are a prolonged cell population doubling time (Hayflick and Moorhead 1961) and prolonged mean generation time (Macieira-Coelho et al. 1966b). Engaging in and completing the cell division cycle become more erratic, while in young cultures between sub-cultivation and resting phase the number of cells synthesizing DNA increases progressively to a maximum and then decreases to very low levels, in old cultures there is a burst of cells entering the S period, then the percentage goes down, and up again before it decreases when the cell monolayer becomes confluent. This erratic behavior was observed both with radio active thymidine labeling (Macieira-Coelho et al. 1966b) and direct examination with time lapse microscopy (Absher and Absher 1976). Although most of the cells are able to cycle through the proliferative life span, there is a constant increase of the percentage of slow dividers (Macieira-Coelho and Azzarone 1982). However, a fraction of rapidly dividing cells are present throughout

most of the cell population's proliferative life span although their number varies (Absher et al. 1974; Kapp and Klevecz 1976; Macieira-Coelho and Azzarone 1982). The number of eventual non-dividing cells during one passage is small during most of the cell life span. In terms of the maximal number of cells capable of engaging the cycle between subcultivation and reaching a confluent monolayer, measured with tritiated thymidine, the lifespan of diploid human lung embryonic fibroblasts can be divided into three periods (Macieira-Coelho and Azzarone 1982): (1) a first period where non-labeled cells vary between 0 % and 2 %, with about a third of the lifespan completed, (2) another lasting most of the second two thirds of life span, where there is a subtle increase in non-labeled cells whose percent varies between 5 % and 14 %; the non-labeled cells are slow dividers since cells that do not divide during one passage will do it during the following one (Macieira-Coelho 1967,1974; Absher and Absher 1976); (3) finally a period corresponding to the last two to three doublings, where the percent of non-labeled cells in the presence of tritiated thymidine increases abruptly.

Absher et al. (1974) and Absher and Absher (1976) analyzed the descendants of single cells without suppressing the interactions with the whole population. They isolated cells within small ponds in a Petri dish, so that the supernatant was still the same for the whole population in the dish, and followed with time-lapse microscopy the genealogy of individual cells. They could thus compare clones obtained from different population doubling levels, taking into account inter-division times, number of generations and clonal size up to cell division arrest through cell density. They found at higher PDL prolonged generation times and an increased heterogeneity of inter-division times. The division potential of individual cells within the same PDL was different since each cell could produce a different number of descendants due to variation of inter-division times and the appearance of cells that did not divide. The number of non-dividing cells was around 10 % until the 40th doubling and increased only afterwards. Absher's observations, like those of Macieira-Coelho, favored the concept of heterogeneity and uncertainty of the probability of cycling and of the non-dividing state. One of the interesting results of Absher's investigations was the finding that the correlation coefficient of inter-division times of sister pairs decreased with age and that cells with a preceding long generation time and large size could give rise to small daughter cells with shorter generation times. Smith and Whitney (1980) also found a difference in the ability to proliferate between two cells arising from a single mitosis. They could differ by as much as eight population doublings, i.e., 256-fold in the number of cells produced. They also observed the rapid development during serial divisions, of intra-clonal variability in doubling potential. Absher's experiments revealed fluctuations between cycling and resting stages suggesting a periodicity in the behavior of cells. Reversion was also suggested in the work of Mitsui and Schneider (1976a) who separated the large, slowly dividing cells from the small, rapidly dividing ones and found a shift between the two populations; a finding supported by other experiments (Macieira-Coelho 1967, 1974; Matsumura et al. 1979a). Reversion is also apparent in the experiments of Dell'Orco et al. (1974); these investigators were able to prolong the doubling potential of human fibroblasts by

keeping them for a long period of time in a near non-mitotic state. These results are compatible with the hypothesis of a regeneration taking place during resting.

The progressive changes in the cell cycle accumulating through serial divisions terminate with a final and rapid collapse of the probability to divide within approximately two to three doublings, leading to a practically post mitotic state with distinct metabolic properties. Even at this stage, if the nutrient medium is renewed regularly, DNA synthesis progresses slowly. Indeed a continuous label with tritiated thymidine shows that the number of labeled cells increases progressively to close to 100 % (Macieira-Coelho 1974; Schneider and Fowlkes 1976; Matsumura et al. 1979b; Ryan 1979); as the radioactivity eventually eliminates the labeled cells, the decline of non-labeled cells must be due to the recruitment of new cells rather than to dilution. Furthermore, labeling with bromodeoxyuridine, which eliminates dividing cells, does not completely eradicates the cells that initiate DNA synthesis even after several days of labeling (Burmer and Norwood 1980). Moreover changing the nutrient medium repeatedly to terminal cell cultures induces a cell doubling although it can take as long as 4 months (Pereira-Smith and Smith 1981). This type of evolution is found both in embryonic and postnatal human fibroblasts, although the latter seem to have evolved already during the life span in vivo, resulting in higher heterogeneity and a higher fraction of slow dividing cells (Macieira-Coelho and Pontén 1969; Vincent and Huang 1976). Terminal cells continue to metabolize, are resistant to death, and can be maintained in that state for at least several months (Sasaki et al. 2001). Macieira-Coelho et al. (1966a) and Matsumura et al. (1979b) observed towards the end of the life span the presence of multi-nucleated cells, which was attributed to a slow DNA synthesis without cell division. This would fit the results of Sisken and Bonner (1979) who found a disturbance of cytokinesis with ageing of the cultures.

Macieira-Coelho (1973) analyzed the putative effect of slowly dividing large cells on small fast dividers, and observed that with increasing proportions of the large ones there was a decrease in the final density of the mixed populations. The results were interpreted in terms of a competition for surface area of attachment in the culture vessel and favored the hypothesis that old cells may exert a more complex inhibiting influence on neighboring cells. The relative amount of both cell types was checked comparing the volume distributions in mixed and unmixed populations. Phase II cells (first third of life span) have a diameter distribution with a peak between 13.4 and 15.4 μm while phase III cells (second third of life span) have a peak between 16.9 and 19 μm. Kapp and Klevecz (1976) have also approached this problem, collecting mitotic cells from young and old cultures and following the cells during the first division. Generation times and the rate of entrance into the S-period were significantly reduced in the mixed cultures compared to that of the mass cultures.

The time of DNA synthesis (S period) varies only towards the end (70 % of life span completed) from 6 to 8 h (Macieira-Coelho et al. 1966a).

Summarizing one can say that proliferation slows down evolving through an equilibrium between rapid dividers, slow dividers, and reversion between the two

states, towards an abrupt decline in the probability of cycling. Each division modifies the cells structurally and metabolically with changes that are cumulative and terminate with a new state with different metabolic characteristics (terminal differentiation?).

The original division (Hayflick and Moorhead 1961) of the life span of human fibroblast populations into three periods is reasonable, although the evolution of four fibroblast types and recent knowledge concerning terminal events suggests a very late fourth phase with striking different cell characteristics. However a schematic division is always an oversimplification in such a complex biological system. The existence of phase I is suggested from the initial rise of the saturation densities, from the observations showing the appearance of a more homogeneous population, and from chromosome studies (Miller et al. 1977) that detect initial changes which disappear during the first passages. Although not all parameters analyzed remain steady during the following sub-cultivations, phase II can be defined as the period where the fraction of non-dividing cells recorded by autoradiography is relatively constant at each passage and no larger than 5–10 %; this figure has also been reported by Harley and Goldstein (1980). One can distinguish three states in phase II populations: a quiescent state in which cells periodically enter, a second state characterized by high proliferative activity and containing a progressively decreasing number of cells; a third state composed of cells with more heterogeneous behavior. Phase II evolves through an equilibrium between the two latter states, with a progressive predominance of the more heterogeneous one. The heterogeneity is manifested mainly at the start of the cell cycle and in the mechanisms, which arrest cell division during contact inhibition of growth. Phase III is characterized by an increase in the number of cells which do not divide during a passage, and is probably responsible first for the prolongation of the population doubling time, initially, and for a decline in the maximal cell densities, later on. In phase III reversion from the quiescent to the proliferating state appears to be more difficult. The existence of a final phase IV corresponding to the last two to three passages, is characterized by the rapid fall of the maximal cell densities, by the striking morphological changes, and by several events in the nucleus such as, decondensation of heterochromatin showing that the terminal cell is not in apoptosis (Brock and Hay 1971), presence of extrachromosomal circular DNA (Icard-Liepkalns et al. 1986), disorganization of 30 nm chromatin fibers (Macieira-Coelho 1991), shortening of telomeres to a critical length (Harley 1991), chaotic partition of DNA between daughter cells (Macieira-Coelho 1996, 2007), telomere-associated DNA damage (Herbig et al. 2004), and a multitude of other events previously reviewed (Macieira-Coelho 1988).

Thus, there is no simple explanation for the increasing difficulty of the cells to respond to growth signals and to transit through the division cycle. It is impossible to determine the modifications that play a bigger role in the commitment to divide – probably all are responsible. I will describe what I believe is the basic trigger for the slow down of the cell cycle, and how it can influence the cell cycle parameters. I also provide a few examples of events that are coupled with the changes in the kinetics of proliferation, reported above, to give an idea of the variety of the modifications taking place.

3.3 Changes Coupled with the Kinetics of Proliferation During Slowing Down of the Cell Cycle

3.3.1 Structure-Dependent Functional Changes

To understand the coupling of cell enlargement and decline of cell proliferation, a reminder of the structural organization of the cell is necessary. Mammalian DNA is a molecule 1 m long, confined in a sphere, the nucleus, with a diameter of about 5 μm. This implies an elaborate folding, which is regulated inter alia by the structure of DNA, by DNA-bound proteins, by enzymes, and by the anchorage of this make-up to a protein matrix. In addition there are glycoproteins present, which since they are highly charged, probably also function as regulators of DNA conformation. The scaffold upon which DNA is anchored plays a crucial role in its high order structure. There is indeed a protein framework called the nuclear protein matrix with which DNA is associated, and DNA synthesis-initiating sites are preferentially located at the nuclear periphery (Berezney and Coffey 1975). This shows the important role of the nuclear matrix, in particular the peripheral nuclear region, in the initiation of the replication of DNA. The anchorage of DNA is crucial not only for replication, but also for transcription, since nascent RNA is associated with the nuclear cage (Jackson et al. 1984). The nuclear lamina, a filamentous protein meshwork lining the nuclear envelope, provides an anchorage site at the nuclear periphery for interphase DNA (Gerace 1985). When the nuclear shell is isolated, it contains chromatin structures made of packed nucleosomes 28–32 nm thick that are associated with the three nuclear lamins (Bouvier et al. 1985). The lamina is composed of proteins called lamins, which are intermediary structures between DNA-binding proteins and the cytoskeleton. The lamina is tightly bound to chromatin since it can be dissociated from chromatin only by a high salt solution (Bouvier et al. 1985). The lamins have a striking sequence homology with intermediate filaments, a component of the cytoskeleton (Gerace 1985). Hence, the anchorage of chromatin seems to be fulfilled with the preservation of the continuity with the cytoplasmic scaffold. This way DNA is linked to the cytoskeleton through its anchorage to the nuclear cage and via the cytoskeleton to the cell membrane and the extracellular matrix. This whole structure has to be seen as a tridimensional manifold where the information flows to a great extent through topological constraints. The cytoskeleton has to be seen not only as an integrator of space, but also of function. It has been proposed that membrane movements are transmitted through the cytoskeleton to the nuclear cage and help to create the conformation of DNA favorable for gene expression and to give to initiating sites the right steric configuration for DNA synthesis (Macieira-Coelho 1983). This mechanism is impaired during proliferative senescence because of the alteration of the cellular scaffold. This is a problem in conformation biology (Ivanov et al. 1973), a fundamental area of cell biology, which is neglected in the field of cell senescence.

The relationship between changes in cell volume and cell division was observed with different methodologies. Tritiated thymidine labeling (Bowman and Daniel

1975), direct analysis by cinematography (Absher and Absher 1976), and comparison of cell volume with population doubling time (Mitsui and Schneider 1976b), all led to the conclusion that larger cells have a decreased probability of entering the cycle. With the increase in cell volume, higher growth factor concentration and larger substratum areas become necessary for proliferation (Collins et al. 1979). An increased cell volume and protein content both in vitro and in vivo (Simons and van den Broek 1970; Shevitz et al. 1986) is a constant finding of cell ageing. In chicken fibroblasts the first increase in size is preceded by a decline in protein synthesis on a per cell basis and coincides with a prolongation of the time needed to reach maximal DNA synthetic activity; it is followed by a prolongation of the doubling time (Macieira-Coelho and Lima 1973). Since protein content increases by a factor of 1.8 and cell volume by a factor of 4.7, it means that protein concentration is considerably diluted. The dilution of cellular constituents must create new topological constraints, which induce variations in the association between molecules due to conformational modifications at the molecular and supramolecular levels. As described above, at the end of the proliferative life span cell size increases dramatically accentuating all these effects. The patterns of increase in cell, nuclear, and nucleolar dry masses and areas are similar for all measures (Mitsui and Schneider 1976b; Bemiller and Miller 1979).

The enlargements of the nucleus and nucleolus coincide with progressive modifications in the 30 nm chromatin fibers detected under electron microscopy coupled with the modifications of the cell cycle (Macieira-Coelho 1991). Electron microscopic pictures of the chromatin fibers at the nuclear periphery of cells at different PDL were screened with an image processor. Two measurements were made, one expressing the ratio between the dark and light areas was called the density of the fibers; the other, which was called the spacing, was obtained with a sieve-like procedure that calculated the areas between the fibers. The density of the fibers, mainly at the level of their anchorage to the lamina densa, was found to progressively decrease in a similar manner as seen during the initiation of DNA synthesis after cell plating. On the other hand, the spacing of the fibers varied very little during most of the cell population's life span and increased abruptly at the end with the fall in the maximal number of cells capable of synthesizing DNA when the cells entered the terminal stage. The measurements performed with the image processor were also made on fibroblasts cultivated from skin biopsies of normal donors of different ages (Macieira-Coelho 1991). It was found that the density decreased in an inverse relationship with the age of the donor. The spacing increased in a direct relationship with the age of the donor. These findings buttressed the relevance of the in vitro studies for the understanding of ageing of this cell compartment in vivo.

The modifications of the cytoskeleton and of the membrane explain the decline in cell migration and its influence on the capacity to proliferate (Soukupova and Holeckova 1964). One of the early effects of growth factors is the initiation of membrane movements (Baker and Humphreys 1971). It is well known that commitment to divide depends on cell movements, a feature that has been observed at the early stages of the science of cell culture. The first signal in growth modulation

by membrane-substratum interactions is of a physical nature (Macieira-Coelho et al. 1974) and the flow of information inside the cell depends on structural flexibility. Larger cells are less motile, lack prominent bundles of microfilaments, and have a decreased ability to make cell contacts (Bowman and Daniel 1975). The structural reorganization at the cellular and supramolecular levels decreases the conformational flexibility and lowers the probability of activating energy barriers, this is a fundamental feature of cellular senescence.

There is an interesting correlation between cell contractility and the percentage of cells synthesizing DNA during a 24 h period between sub-cultivation and resting phase (Macieira-Coelho and Azzarone 1990). Contractility was determined by the capacity to retract a fibrin clot, it decreased coinciding with the decline of DNA synthesis, with a pattern analogous to that of the different phases identifiable by the kinetics of proliferation. Decreased retractile activity was also observed in postnatal cells as compared with embryonic cells. The results showed a correlation between the initiation of DNA synthesis and the turnover of cytoskeletal elements supporting our results that the earliest proliferative disturbance consists in a decline in the probability of initiating the division cycle linked to impaired cell attachment and spreading. The cytoskeleton must be the link through which flows this growth-regulatory information from the membrane to the nucleus (Puck 1977). The decrease of the hybridization signal with the beta-actin gene probe with loss of sequences into extra-chromosomal small circular DNA, are probably related with the alterations of the actin cytoskeletal proteins and the functional decline of the cytoskeletal elements (Macieira-Coelho 1990).

Changes in protein phosphorylation are particularly relevant for the decline in conformational flexibility. Phosphorylation and dephosphorylation are fundamental tools to activate energy barriers through modifications of molecular conformation. These concern the transduction of energy – another subject largely neglected in the study of the mechanisms of cellular senescence. ATP content decreases following exposure to metabolic poisons, showing a defect at the point of origin of inorganic phosphate (Muggleton-Harris and Defuria 1985). The pathways of the phosphorylation cascade initiated at the cell periphery are impaired. There is a progressive appearance of a new 3:5-cyclic AMP-independent histone kinase, raising the possibility that new genes become activated; qualitative modifications of the phosphorylation of proteins have been reported with reinforcement of the phosphorylation of some proteins and a relative decrease of others (Kahn et al. 1982). These authors remarked that these new regulations are progressive as is senescence itself, and observed the appearance of new isozymic forms of protein kinases and new phosphoproteins. The deregulation of phosphorylation becomes more marked at the end during the last mitoses.

In summary, motion and function are interdependent in a cell and result from the tension in a network of structures that extend from the membrane to the nuclear cage, which constitute the cellular scaffolding. The function of this network is regulated through the synthesis of molecules with the right steric configuration and through energy turnover, adhesion to a substratum is the trigger to built up tension. Cell behavior is determined by the way this network of structures is connected,

i.e. by its topology. Motility is closely linked with cell division because it can create new topological constraints; membrane movements transmitted through the cytoskeleton to the nuclear cage help to give DNA the right conformation for gene expression during the G1 period, and to initiating sites the right steric configuration for the synthesis of DNA. Too much stretching of the cell leads to a decreased probability of initiating DNA synthesis due to the loss of conformational flexibility inducing a shift from division to other functions such as a state of terminal differentiation. Investigators have been looking for "The Event" that could explain it all. I believe that this cannot be achieved. Additional functional changes that coincide with modifications of proliferation kinetics are described below, to illustrate the multiple modifications that accompany the slow down of the cell cycle.

3.3.2 Metabolic Events

An interesting metabolic finding is the decreased ratio between the activities of two enzymes involved in the purine salvage pathway, which also follows a constant decrease (Paz et al. 1981) like that of the initiation of DNA synthesis after plating the cells. One is an X-linked enzyme, hypoxanthine-guanine phosphoribosyltransferase, and the other a biochemically related autosomal-linked enzyme, adenine phosphoribosyltransferase. A decline in the purine salvage pathway could leave the de novo synthesis as the main source of DNA precursors and render the entrance into the S period more difficult, contributing to the decline in the commencement of DNA synthesis reported above. This finding is reinforced by the decline in ribonucleotide reductase (Dick and Wright 1985), and a progressive increase in the levels of the second enzyme of the pathway for de novo purine biosynthesis, glycinamide ribonucleotide synthetase (Hards and Patterson 1986).

Changes in glycolysis coincide with the changes in the kinetics of proliferation. Between population doublings 20 and 40, glucose uptake and lactate production increase (Bittles and Harper 1984); the critical change in the biphasic curve of glucose uptake/lactate production corresponds to the transition from phase II to phase III as defined above. The same investigators also observed an increase in the specific activity of piruvate kinase, a regulatory enzyme of the glycolytic pathway. The responsiveness to EGF decreases also during the transition from phase II to phase III (Kaji and Matsuo 1983).

3.3.3 Gene Related Events

The activity of p53, a positive transactivator of p21 gene expression, was found to increase in a stepwise fashion through the different phases of the human fibroblast life span (Bond et al. 1996). This is one of the mechanisms at the molecular level underlying contact inhibition of growth during serial proliferation. The G2/M transition is regulated inter alia by p53 which acts as a check point in the presence

of DNA damage, hence the increased activity of this protein must be responsible for the prolongation of the G2 period associated with the increased sensitivity to contact inhibition of growth that characterizes the slow down of the cycle during serial proliferation. It is interesting that in plants extra-chromosomal DNA replicates in cells that differentiate from G2. It has been suggested that this is due to the failure of nascent replicons to join when cells reach G2, leaving gaps that serve as recognition sites for the initiation of DNA amplification (Van'T Hof and Bjerknes 1982). The production of extra-chromosomal DNA is also a feature of the terminal phase IV fibroblast (Icard-Liepkalns et al. 1986); hence the same mechanism could be operative in the terminal fibroblast since a prolongation of the G2 period (Macieira-Coelho et al. 1966b; Macieira-Coelho and Berumen 1973; Kapp and Klevecz 1976) and a defect in the gap-filling step (Macieira-Coelho 1991) also take place in the terminal stage of the human fibroblast life span. These data would also favor the hypothesis that the fibroblast population evolves towards a terminal differentiation (Martin et al. 1974). Emergence of extra-chromosomal DNA circles also accompanies cellular differentiation in the early development of mouse embryos (Yamagishi et al. 1983).

The expression of the EPC-1 gene declines gradually during the fibroblast proliferation life span (Pignolo et al. 1993). Several other genes are also affected during the proliferation life span of human fibroblasts, however these have been reviewed elsewhere (Macieira-Coelho 1988, 2003).

3.3.4 Shortening of Telomeres

Shortening of telomeres was considered the critical event in the arrest of cell division (Harley 1991). The hybridization of the terminal restriction fragments (TRF) of DNA from serially dividing human fibroblasts with the appropriate probe showed a reduction of the hybridization signal with increasing population doublings, which was interpreted as a shortening of the telomeres. The erosion of telomeres through division is not universal. In humans the division potential in vitro of normal keratinocytes (Kang et al. 1998; Rheinwald et al. 2002), cardiomyocites (Ball and Levine 2005), and astrocytes (Evans et al. 2003) is independent of telomere size. Moreover, telomere lengths did not show a clear correlation with tissue renewal times in vivo, lengths are characteristic of each human individual (Takubo et al. 2002), and the rate of telomere loss slows throughout the human life span (Unryn et al. 2005). The implications of telomere dynamics in the biology of the organism are complex and have been previously reviewed in regard to ageing, longevity, and development (Macieira-Coelho 2011). They are far from elucidated. Telomere-promoted recombination can lead to degeneration of the telomeric sequence and subsequent loss of the hybridization ability without shortening of telomeres (Ashley and Ward 1993). Indeed, several examples of apparent terminal deletions are actually subtelomeric translocations (Meltzer et al. 1993). Blackburn (2000) has suggested that the structure of telomeres is more important than their length.

3.3.5 Asymmetric Cell Division

There is a contribution of the investigations concerning ageing of proliferative cells, which has been largely ignored. Due to the experiments of Meselson and Stahl performed on mass cell populations showing that DNA synthesis is semi-conservative, it was thought that after division daughter cells are identical. However, when one analyses the phenomenon at the level of individual cells it can be observed that DNA synthesis and the distribution of DNA between daughter cells are asymmetric (Macieira-Coelho et al. 1982). The DNA contents of pairs of anaphases and telophases were measured through serial population doubling of human fibroblasts in vitro after staining with either ethidium bromide or Feulgen (Macieira-Coelho et al. 1982). The data showed that at each cell population doubling, in a significant fraction of cells, the distribution of DNA content between daughter cells is asymmetric. The fraction of cells with significant differences was constant throughout the proliferative life span of the fibroblast population, increasing only at the end during the last mitoses. The distribution of DNA contents between sister cells followed a normal Gaussian curve through the whole cell population's life span giving a straight line on probit paper. When cells entered the last divisions, a breaking point in the slope was observed suggesting a phase transition (Macieira-Coelho 1996). Labeling with tritiated thymidine showed a significant asymmetric distribution of newly synthesized DNA between daughter cells (Macieira-Coelho et al. 1982). The differences were constant during serial population doublings and increased only during the last divisions. These experiments are a direct illustration of modifications occurring at each division, and of how cellular senescence is a slow cumulative process.

3.4 Other Cell Systems

The functional changes originated during proliferation explain several modifications occurring in the organism during ageing. The role of fibroblasts is to regulate the homeostasis of the microenvironment. Other mitotic cell systems that are substratum-dependent also have a limited division potential and their function changes as they replicate towards the limit. Their cell cycle has not been studied in such detail as with fibroblasts but the functional evolution and the relationship with ageing of the organism have been analyzed. That is the case of glial cells, which evolve like the fibroblasts with longer division cycles (Fedoroff et al. 1990); this evolution reflects the metabolic changes they incur, which alter their multiple interactions with the neuron and are responsible for many aspects of ageing of the brain. Keratinocytes proliferate in vivo and in vitro to a terminally differentiated cell (Rheinwald and Green 1977) with cytoplasmic enlargement, development of detergent-insoluble cytoplasmic filaments, formation of a cornified cell envelope, and destruction of the cell nucleus (Green 1977). The increase in size is accompanied by the appearance of new proteins such as keratin, involucrin,

and other specific proteins (Simon and Green 1984). The role of this cell system is the production of the external layer protecting the skin. Chondrocytes represent another differentiated cell system whose rate of division declines with senescence with a division potential in vitro inversely correlated with the age of the donor (Evans and Georgescu 1983). It is not a terminal differentiating system since the cells are already differentiated and their function does not evolve towards a more differentiated state. Their role is the formation of the layer that covers the joints, the modifications occurring through proliferation contribute to the inadequate function of the joints during senescence. Lens cells constitute an example of a terminal differentiation that evolves with formation of elongated enucleated fibers with synthesis of specific proteins called crystallins differently distributed in the pre-elongation fibers and in the fiber cells (Simonneau et al. 1983). Lens cells are derived from the ectoderm and like the hair or the nails continue growth throughout life. The proliferation potential of lens cells declines with ageing of the organism, senescence of this cell system is the result of the accumulation of fibers with a progressive loss of focusing power. Aortic endothelial cells have kinetics of proliferation during their in vitro life span very similar to those of fibroblasts (Rosen et al. 1981). These cells maintained in vitro as monolayers retain throughout their life span a specialized function of the tissue of origin – factor VIII antigen expression. They also retain the ability to take up intercellular silver nitrate stain, which is another characteristic of endothelial cells (Rosen et al. 1981). When they enter the terminal post-mitotic stage they enlarge but maintain cell contacts and a monolayer typical of endothelial cells. Cultivated vascular smooth muscle cells were extensively studied. They have a finite ability to proliferate and their proliferation rate at each passage in vitro declines significantly with increased age of the donor (Ruiz-Torres et al. 1999). In adrenocortical cells serially dividing in vitro, some changes in the differentiated function occur during the cells' life span without major changes towards the post-mitotic state (Hornsby and Gill 1981). In this cell system fully differentiated from the start there are different regulations that become apparent but one cannot speak of dedifferentiation or further differentiation. Another cell type, brain derived cells have also been used to study ageing in vitro (Pontén et al. 1983). They were later shown to be of lepto-meningeal origin (Rutka et al. 1986). Their division cycle slows down with proliferation with final enlargement when the post-mitotic stage is reached. They have been used to demonstrate how the division cycle depends on nutrients, surface area, and cell cooperation (Blomquist et al. 1993). These different cell types have in common a finite division potential, but their role in the remodeling of the organism, taking place through the animal life span, is different.

Lymphocytes, a non-substratum dependent cell system, are difficult to study in vitro because they cannot be maintained serially proliferating without acquiring an immortalized status. A decreased proliferative response in vitro to mitogens, related with donor's age, has been reported (Hori et al. 1973; Price and Makinodan 1972). When serially transplanted they have a limited division potential (Siminovitch et al. 1964; Cudkowicz et al. 1964), which is not affected by donor's age (Harrison et al. 1978; Schofield et al. 1986). A finite proliferative potential has also been reported for an antibody-forming clone after serial transplantation (Williamson and Askonas

1972). The number of potential transplants increases with longer intervals between transplants (Vos and Dolmans 1972), which suggests a regeneration taking place in the division potential during resting phase as reported above for fibroblasts. The same result was reported with serial transplantation of mammary glands (Daniel and Young 1971).

3.5 Conclusions

What is described herein shows that the deregulations caused by the proliferation of normal cells are complex, as should be expected. The ascertainment that there is a finite capacity to divide implicates that cells change at each division tending towards the limit. Ageing of dividing cells is a question of deregulation of the commitment to divide due to multiple events, rather than the attainment of the limit of the potential to divide due to a single cause. The connotation of the finite proliferation for ageing of the organism is the drift in cell function due to changes caused by replication rather than the accumulation of non-dividing cells. Before the concept of ageing of mitotic cells was introduced by Hayflick and Moorhead (1961) and before the multiple attempts to understand the mechanisms involved were performed, the idea about the control of cell division was straight forward based on experiments performed on malignant cell lines. It was thought that the commencement of the division cycle depended on a trigger, once initiated it would be completed without interruption. The experiments reported above were important not only from the point of view of ageing – they were the first description of the regulation of cell division in normal cells. A Manichean reasoning has prevailed, the discussion being all about dividers and non-dividers and of reaching a non-dividing state. Fifty-four years later a different interpretation is finally settling down emphasizing the progressive slow nature of cell senescence, where no single mechanism can explain it all.

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Chapter 4
Influence of Donor Age and Species Longevity on Replicative Cellular Senescence

Antonello Lorenzini and Andrea B. Maier

Abstract The replicative life span of cell strains obtained from multiple explants from the same individual is highly variable, additional variability is added when strains are obtained from different individuals. This variability is probably due to both technical issues and heterogeneity inside the tissues. Notwithstanding these limitations, many scientists searched for an inverse relationship of proliferative potential and donor age. Reviewing this literature, we conclude that this inverse correlation is likely more dependent to developmental stages than to aging per se; i.e. cells taken from a developing organism have higher replicative capacity than cells taken from an adult.

Replicative senescence has been studied also across species searching for a positive relationship with longevity. Recently it has been show that when specific culture conditions (mainly low oxygen tension) are applied, strains from several species appear immortals. Moreover, for species that do present cellular senescence, it seems that replicative capacity relates primarily to species adult body mass more than to longevity.

Keywords Replicative senescence • Donor age • Colony forming ability • Comparative biology • Species longevity • Species body mass

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4.1 Introduction

4.1.1 Senescent Arrests

In this chapter we will focus on mitotic competent cells, without entering the field of stem cells aging, to which Chap. 5 is dedicated, and on senescence observed as a consequence of serial passaging of cells in culture, including colony forming assay and the supposed capacity of this technique to predict senescence.

The term replicative cellular senescence describes a phenomenon primarily observed in culture where mitotic competent cells cease to proliferate and enter an apparently irreversible state characterized visually by an enlarged and spreaded morphology (Hayflick 1965). In itself, the sequential passaging of cells in culture could carry multiple potential inducers of senescence: intrinsic, i.e. related to the physiological functioning of cells, and extrinsic, i.e. depending from the protocol used to propagate the culture (media, serum or growth factors, temperature and atmospheric settings and others, for these aspects see also Chaps. 10 and 13). Recently it has been observed that also in vitro imposed quiescence is able to induce the senescent phenotype in human fibroblasts (Marthandan et al. 2014). Quiescence induced senescence seems to associate with DNA damage and failure to undergo apoptosis as does replicative senescence. These findings may have important implications if we consider that this "senescent like" condition was obtained with a quiescence of only 5 months and that the maximum life span of our species is over 120 years. Other factors that can potentially influence cellular replicative senescence are the way the culture has been established, the tissue of origin, the age of the donor and the species of the donor organism. In this chapter, we will focus on these last two aspects: donor age and species. In addition to serial passaging, senescence can be induced by genotoxic and oxidative stresses and by oncogene activation; these aspects are discussed in Chaps. 10, 11 and 13.

It is opportune to mention that in the field of replicative senescence some have considered the appearance of senescence cells as a differentiation phenomenon. In this view all proliferative compartments are not composed of an omegenous multitude of similarly differentiated cells but are a complex systems that include both stem cells and cells differentiated at different stages, similarly to what is better understood for the hematopoietic system. Senescent cells are thus considered the result of a terminal differentiation (Bayreuther et al. 1992). This is not only a question of terminology since it may influence the selection of the experimental approaches chosen to study cellular senescence in vivo. One way to choose between the "senescence" and the "differentiation" options is to consider the concepts of loss or gain of functions. If cells are losing their physiological functions, we can say they are senescing while if they are gaining specific functions we can say they are differentiating. In this line of thoughts it is noteworthy that senescent fibroblasts have a superior capacity to attach to type I collagen (Stephens et al. 2003) and the demonstration of their essential positive role in wound healing (Demaria et al. 2014).

Finally, a recent characterization of their metabolism concluded that they enter a state of low oxidative stress (James et al. 2015).

4.1.2 Cell Replication Capacity as "Anti-aging" Mechanism

It is generally accepted that replicative senescence contribute to in vivo aging; for additional discussion on this point see Chaps. 1 and 2 and the literature by van Deursen (2014). In this chapter we discuss data concerning two expected consequences of this idea: (1) that older organism should have more senescent cells and, (2) that cells of shorter lived species should reach senescence sooner. The hypothesis that the unlimited proliferative capacity of tissues could be the key of immortality in some animals species like some sponges and some cnidarians is quite accepted, reviewed in (Petralia et al. 2014). If the capacity to replicate is somehow counteracting the aging process, as a proof of principle we are expecting to observe absence of aging in clones of symmetrically dividing single cell organisms, where it is virtually impossible to make the classical distinction between germline and soma proposed by August Weismann (1893). The majority of microbiologist will probably agree with this statement and they will refer to the possible disappearance of a unicellular species as "selected-against" by the force of natural selection instead of "having aged". Opening a parenthesis on the concept of symmetry, it is interesting to mention that in Escherichia coli, that reproduce with a morphological symmetry, cells that inherit the old pole (i.e. inherit, indeed, a molecular asymmetry) exhibits a diminished growth rate, decreased offspring production, and an increased incidence of death (Stewart et al. 2005). Going back to the above stated "principle", if then cell replication, with its inevitably associated new molecular synthesis, is "anti-aging", on the opposite end, we can suppose that the processes of aging should me more evident in non-replicating tissues or in less replicating tissues. Several studies have documented an increasing numbers of senescent cells in tissue from older donors (Herbig et al. 2006; Jeyapalan et al. 2007; Wang et al. 2009). Here, instead, we will review specifically in vitro studies, or more appropriately *in culture* studies, both in human of different age as well as in species with different longevity.

4.2 Technique: Mass Culture Maximum Replicative Potential Versus Colony Forming Ability

Two methods have been mostly used to study in vitro proliferative capacity of cells. One is mass culture proliferative potential, which is the method used by Hayflick and Moorhead in their seminal work (Hayflick and Moorhead 1961) where cells from a tissue explant are cumulatively subcultured until they reach senescence. The other is the measure of the ability to form colonies, where cells are seeded at very low density and left undisturbed for a fixed amount of time, then, the formed colonies are

scored in their number and dimensions. This approach was proposed by Puck and colleagues and is usually referred to as the colony formation assay (CFA) (Puck et al. 1956). In the field of cellular aging authors often refer to this assay as the colony size distribution, terminology proposed by Smith and colleagues who suggested to score the percentage of cells able to form colonies of 16 or more cells in a period of 14 days (Smith et al. 1977). Smith and colleagues have produced proofs that the CFA performed on cells from the early passages of a mass culture can be used to predict the maximum proliferative potential of the same mass culture (Smith et al. 1978, 2002). These two reports contain data collected respectively from 17 and 83 different cultures that demonstrated a proliferative capacity as mass culture ranging from less than 20 population doublings (PD) to almost 80 PD and that originate from donors ranging in age between 19 and 89 years. Maier et al. have failed to show this correlation examining 23 cultures derived from a cohort of 90 year old donors that showed a proliferative potential, when examined as mass culture, ranging between 51 PD and 108 PD (Maier et al. 2008a). A possible explanation is that CFA is predictive of mass culture proliferative potential only for culture with a low proliferative potential while for culture with high proliferative potential this relationship is lost. In 14 days even the largest clones have no more than 1500 cells which is about 10,5 PD and which may represent only a small fraction of the mass culture proliferative potential. Another issue to consider when evaluating CFA is that low density represents a highly stressful condition for cells, see (Wright and Shay 2002). This is has been elegantly demonstrated utilizing hTERT immortalized human fibroblasts. If these cells are seeded at low density, only 50 % of them will form colonies similarly with not immortalized fibroblasts, alternatively, if they are seeded at the same low density but over a feeder layer, 98 % of them will proliferate (Moldaver and Yegorov 2009). Cristofalo and Allen, together with other colleagues have also reported significant differences between replicative life span estimated by CFA and actual life span (Cristofalo et al. 1998). In light of the above considerations, the statement published in 2002 by these two authors may still be true: "*In view of the differences in initial growth rates and intraclonal variations in the proliferative potential of single cells, it seems probable that the clone-size distribution method of estimating proliferative life span and an actual determination of replicative life span measure different things*" (Cristofalo and Allen 2002).

4.3 Donor Age

4.3.1 Donor Age and Mass Culture Proliferative Life Span

The existence of a relationship between donor age and proliferative potential of mass culture would represent a clear support for using the model of replicative senescence for aging studies; consequently, many studies have been performed searching for this relationship. After the above considerations that the maximum proliferative potential of mass culture may not be assessed by CFA, we will distinguish between

the data collected using these two different approaches. The main cellular type used for such a study has been dermal fibroblasts. Maier and Westendorp have reviewed the literature and organized the data to evaluate the effect of accelerated aging syndromes, age related disease and donor age on fibroblasts replicative capacity (Maier and Westendorp 2009). While fibroblasts from patients with accelerated aging syndromes appear consistently to possess a lower proliferative capacity, fibroblasts from patients with age related disease did not show a lower capacity when compared to undiseased controls. More importantly, reviewing 23 studies where the impact of donor age was determined, they have noted two important facts: firstly, the higher the number of subjects included in each study, the lower was the effect of donor age on replicative capacity; second, performing a statistical test to evaluate the possibility of publication bias (Egger et al. 1997) they found that, publication bias was at play (Fig. 4.1). Smaller studies with few subjects were probably more often submitted and accepted by journal editors if reporting data that supported the expected negative correlation with donor age. The largest study performed, excluding the study by Smith et al. (2002) that was performed using the CFA approach, was performed by Cristofalo and colleagues that analyzed the data from 112 strains and found no significant correlation (Fig. 4.2) (Cristofalo et al. 1998). They also analyzed the effect of donor age comparing proliferative life span of fibroblasts from biopsies taken at different moment during the life span of the same individual. This longitudinal approach also failed to reveal a relationship with donor age since for four out of six donors the culture established from the older biopsy had a higher proliferate potential than that established from the biopsy obtained at younger age. Considering that the vast majority of the studies reviewed by Maier and Westendorp measured proliferative capacity of dermal fibroblasts is worthwhile citing a recent analysis of telomere length of these cells. Waldera-Lupa et al. isolated dermal fibroblasts from 15 females donors of different age and passaged them less than five times before measuring telomere length. They failed to find any correlation of telomere length with donor age (Waldera-Lupa et al. 2015). For additional consideration on telomeres and cellular senescence, the reader is referred to Chap. 7. In vivo support for the lack of correlation between replicative life span and donor age may derive from the work by Stephens et al. who have demonstrated no evidence of senescence in fibroblasts from chronic wounds (Stephens et al. 2003). Chronic wounds are in fact, pathological instances where we would expect to observe an accumulation of replicatively senescent fibroblasts.

Replicative senescence has been studied extensively also in other cells types. Karavassilis and Faragher reviewed 12 studies evaluating the relationship between disease status, donor age and proliferative potential of endothelial and vascular smooth muscle cells; respectively 8 and 4 studies for the two cell types (Karavassilis and Faragher 2013). For endothelial cells the correlation linking replicative capacity with donor age was moderate for donors without cardiovascular disease (coefficients of determinations = 0.50) and absent for donors with cardiovascular disease (Fig. 4.3a), for vascular smooth muscle cells the correlation was weak both for donors without or with cardiovascular diseases (Fig. 4.3b). Although the authors alert the readers that "*a quantitative relationship between proliferative capacity and*

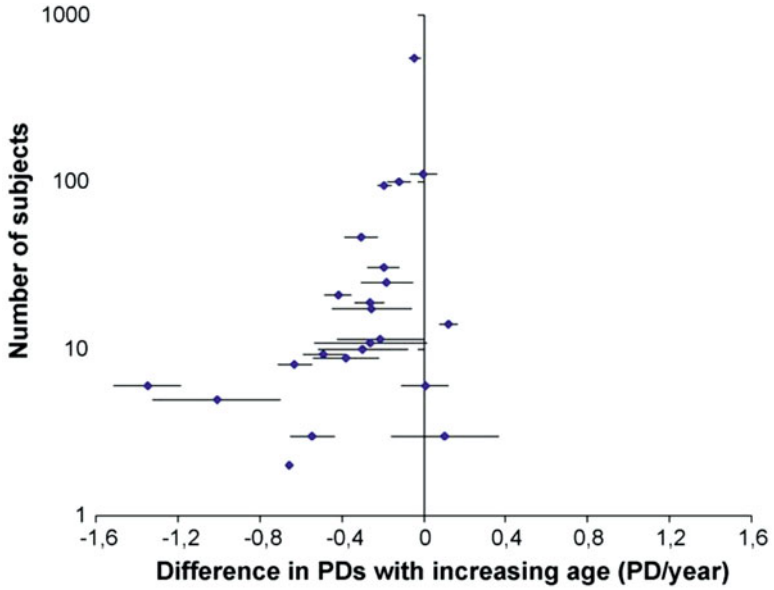

Fig. 4.1 Decrease in population doublings (PDs) per calendar age dependent on the number of subjects included in each study. The symbols indicate the change in the number of PDs ± S.D. per year increase in donor age (Reproduced from (Maier and Westendorp 2009) with permission from Elsevier to Antonello Lorenzini (license 3635901021474))

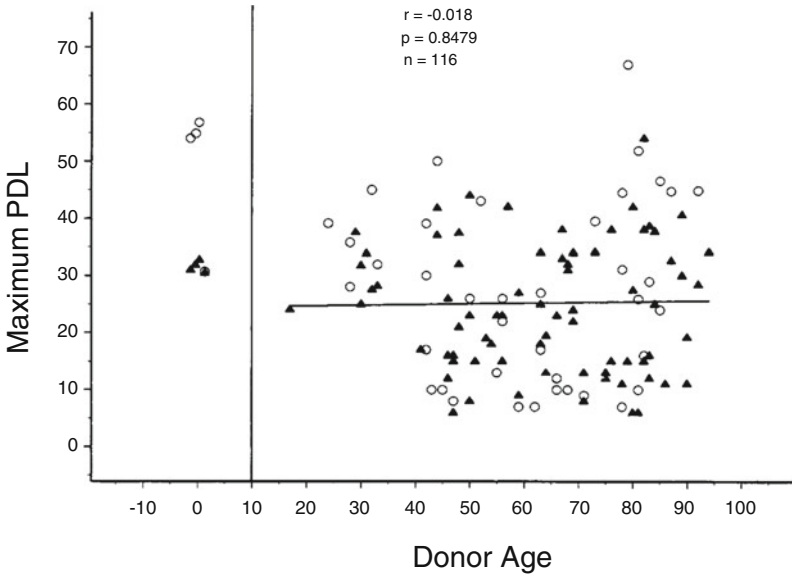

Fig. 4.2 Relationship between in vitro proliferative capacity of postnatal skin fibroblast cell lines and human donor age (in years). A regression line ($r = -0.018$, $P = 0.85$, and $n = 116$) is shown. Males are represented by *closed triangles* and females by *open circles* (Reproduced from (Cristofalo et al. 1998) with permission from the National Academy of Sciences, U.S.A. Copyright (1998) to Antonello Lorenzini and Andrea Maier)

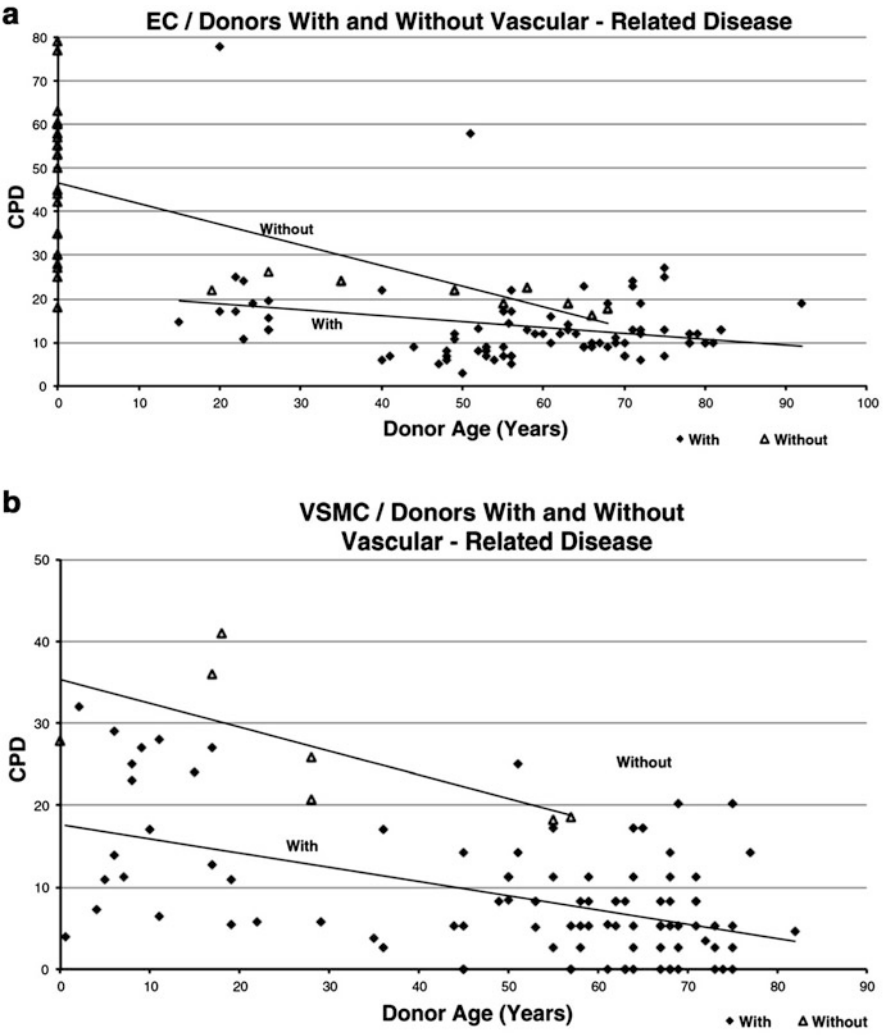

Fig. 4.3 Effect of age and disease status on replicative capacity of endothelial and vascular smooth muscle cells. (**a**) Relationship between cumulative population doublings (CPDs) and donor age in endothelial cells, showing donors with and without cardiovascular-related diseases. (**b**) Relationship between CPDs and donor age in vascular smooth muscle cells, showing donors with and without cardiovascular-related diseases (Reproduced without modification from (Karavassilis and Faragher 2013) under Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0/)

age cannot be made with absolute certainty", their opinion is for the existence of relationship between proliferative capacity with age and with cardiovascular related pathologies for both cell types. The presence of diseases, in any case, appears to affect replicative capacity more profoundly than donor age. It is necessary to note

Table 4.1 Average proliferative capacity of dermal fibroblasts with increasing age at biopsy in Syrian hamsters

Age at biopsy (months)	Number of animals analyzed	Average maximum PDL
1	12	13.9 ± 1.1[a]
6	12	8.5 ± 1.1
12	10	5.5 ± 0.6
18	8	5.3 ± 0.7
24	3	4.5 ± 0.4
30	2	5.0 ± 0.1
36	2	4.0 ± 0.1
24–36[b]	7	4.8 ± 0.3

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[a] ± Standard deviation

[b] Data from 24-, 30-, and 36 month time points pooled together

here that the 2 largest studies, among the 12 reviewed, do not support a correlation with donor age in the opinion of their respective authors. These two studies, by themselves alone and respectively for the two cell types, comprised more than half of the total number of donors from all the 12 considered studies. Specifically the largest studies with endothelial cells reported no correlation between the age of 58 donors and the number of days in culture to reach 50 % of senescence-associated b-galactosidase staining that was used as a substitute of maximum proliferative potential to determine propensity to senescence. Similarly the largest study on vascular smooth muscle cells, that involved an analysis of 76 biopsies concluded that "*proliferative potential did not vary with age or sex, … or with whether the cells were plaque derived or not*" (Eskin et al. 1981).

Other cell types that have been studies are lens epithelial cells (Tassin et al. 1979; Power et al. 1993) and osteoblasts (Koshihara et al. 1991) where an inverse correlation of proliferative potential with donor age has been reported. Bruce and Deamond, measured replicative capacity of dermal fibroblasts on 18 Syrian hamsters, taking multiple biopsies at different ages and searching for a possible correlation also with the ultimate lifespan of the donor (Bruce and Deamond 1991). They observed a correlation of proliferative potential with age at biopsy only for cells taken at ages younger than the average life span of this spices while they did not observe a correlation for higher ages (Table 4.1 and Fig. 4.4). Additionally they did not find the searched correlation of proliferative life span with the ultimate life span of the individual animal (Fig. 4.4).

Finally is worth mentioning the consideration of Holliday on the very large heterogeneity of replicative life span even in multiple population from the same embryo strain such WI-38, IMR-90 and MRC-5 (Holliday 2014). Holliday suggested that it would be more informative to evaluate multiple parallel cultures (5 or even better 10) from the initial outgrowth from the biopsy of a single donor even if this came at the cost of evaluating less donors. The reason of the variability remains an important question that the Commitment theory of Kirkwood and Holliday is proposing to explain (Kirkwood and Holliday 1975). Nevertheless,

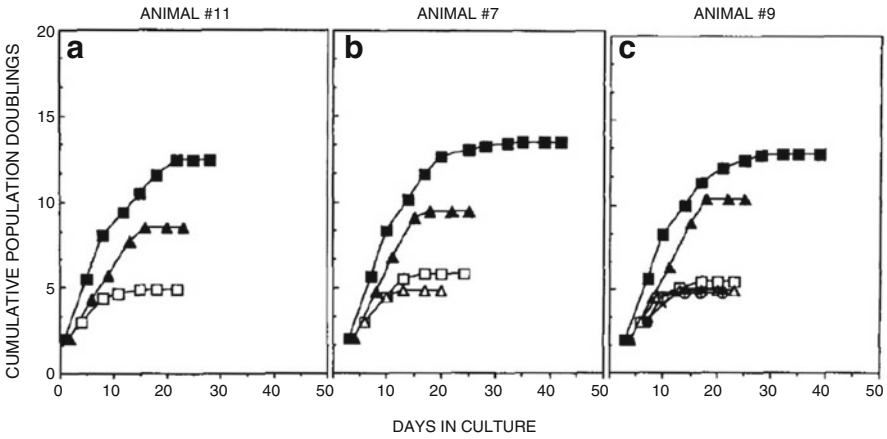

Fig. 4.4 Growth history of dermal fibroblast cultures derived from representative short-lived (panel **a**), average-lived (panel **b**), and long lived (panel **c**) Syrian hamsters. Age of death of animals #11, #7, and #9 was 15, 21, and 36 months, respectively. 1 month (■); 6 months (▲); 12 months (□); 18 months (Δ); 24 months (○): 30 months (*); 36 months (+) (Reproduced from (Bruce and Deamond 1991) with permission from Elsevier to Antonello Lorenzini (license 3635901110367))

regarding the present discussion on donor age, two facts should be considered. First, that Maier et al. have reported that 57 strains of dermal fibroblasts, each derived from a different nonagenarians, were able to proliferate in average up to 73 PD ranging from 51 to 108 (Fig. 4.5) (Maier et al. 2007). Second, that the vast literature on the proliferative potential of dermal fibroblasts from much younger and even newborn donors predominantly does not report higher values than the ones obtained from nonagenarians by Maier and colleagues. These last two facts together make it improbable that even increasing the number of replicates will give a substantial reduction of proliferative potential with higher age, this at least for dermal fibroblasts.

4.3.2 Donor Age and Colony Forming Ability

Concerning the colony formation assay, we may still await the final verdict regarding its capacity to predict donor age. In fact, Simith and colleagues were able to find an age effect on CFA only within subjects in the third decade of life in a large study involving 552 subjects ranging in age from 20 to almost 100 years old. No age effect was found for all ages above (Fig. 4.6) (Smith et al. 2002). In line with this finding, Ng et al. reported no age effect on the growth rate of dermal fibroblasts and keratinocytes on 12 donors, where 6 donors were below 40 years, and 6 donors above 40 years old (Ng et al. 2009). Dekker et al. instead, were able to observe a significant difference comparing a group of ten young individuals (averaging 25 years of age) with a group of 9 nonagenarians as well as comparing 37 individuals

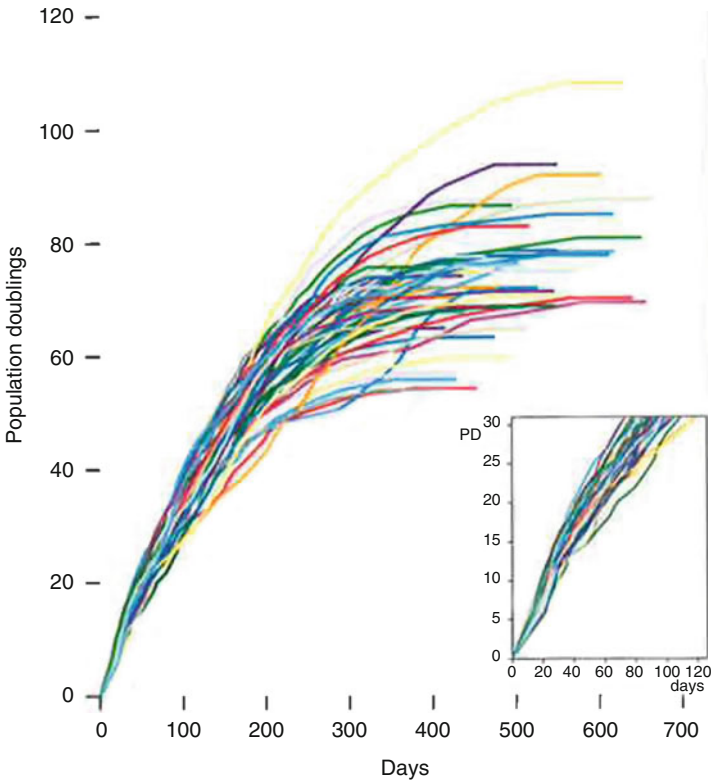

Fig. 4.5 Observed growth kinetics of 57 fibroblasts strains from 90 year old human donors (Reproduced from (Maier et al. 2007) with permission from John Wiley and Sons to Andrea Maier and Antonello Lorenzini (License 3635300200978))

with the propensity of familiar longevity with 37 age-matched controls. In both settings the number of colonies was higher at chronologically and biologically younger ages (Dekker et al. 2009).

Cloning efficiencies have been reported to decline with increasing age also in several experiments with other species and with different cell types: in dermal fibroblasts from rhesus monkeys (Pendergrass et al. 1999); in mouse, in kidney and lens epithelial cells, in bone marrow and spleen fibroblasts and in bone marrow endothelium, reviewed in (Wolf and Pendergrass 1999) and in (Wolf 2010); in rat myoblasts (Schultz and Lipton 1982) and in dog fibroblasts (Li et al. 1996).

Finally, in retinal pigmental epithelial cells Flood and colleagues reported an inverse correlation between donor age and days to reach confluency (200 cells/mm^2) after the initial plating following tissue trypsinization (Flood et al. 1980).

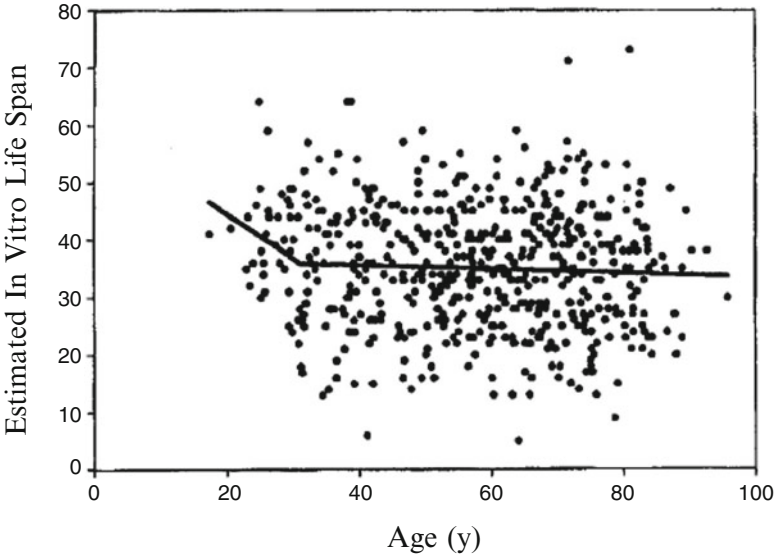

Fig. 4.6 Relationship between estimated in vitro life span and human age. A piecewise linear regression was fitted to the data with the following model: estimated lifespan span = a + b1 × age + b2 × (age − *k*) × (age > *k*) where *k* is an age where there is a change in the slope of the line; *k* was found to be 31.1 years, and the slope above that age did not differ from zero (Reproduced from (Smith et al. 2002) with permission from Oxford University Press to Antonello Lorenzini (License Number 3641250362676))

4.4 Species Longevity

As mentioned above, another consequence that logically follows the idea that senescence is somehow related to organismal aging is the expectation that cells from short-lived species should show a precocious senescent state in culture. Initial studies that measured replicative potential in only few species have obtained encouraging results that supported the expected correlation. For example a report on skin fibroblasts from Galapagos tortoises, a species that live much longer than ours, showed a superior proliferative capacity than that reported for humans (Goldstein 1974). Two larger studies tried specifically and systematically to address this issue and showed completely opposite results. In the first, Stanley and colleagues cultured ten cells strains each from a different mammalian species finding no association between proliferative capacity and maximum life span (Fig. 4.7) (Stanley et al. 1975). In the second study, instead, Röhme, using eight mammalian species succeeded in finding a strong positive correlation with a coefficient of determination of 0.90 (Röhme 1981). Subsequently, the study by Röhme, most probably because it was by far larger, containing data from a total of 36 strains and because the result was expected by the readership, ended up to be routinely cited at the expenses of the former. Next to that, studies utilizing the colony forming assay, obtained result in agreement with Röhme findings, for instance studies comparing dog breeds of

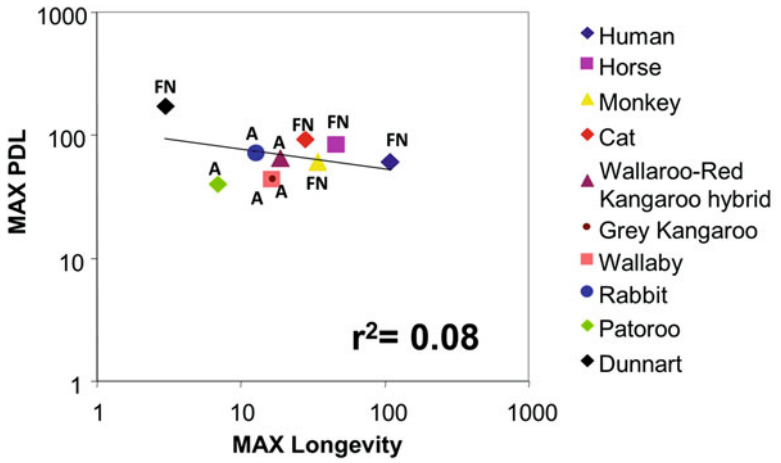

Fig. 4.7 Replicative capacity of fibroblasts from different tissues vs maximum longevity of mammalian species. Correlation between maximum longevity and proliferative capacity of fibroblast like diploid cells derived from different tissue types (lung: human, macaque and cat; kidney: horse; skin: wallaroo x red kangaroo hybrid, grey kangaroo, rabbit, and fat-tailed dunnart; heart: pretty-faced wallaby and potoroo). Ten strains each from a different species were analyzed. FN, fetal or newborn tissue derived, A adult derived (Drawn from data reported in Nature (Stanley et al. 1975) with permission from Macmillan Publishers Ltd, copyright (1975) to Antonello Lorenzini (license 3634810896648))

different longevity and mice in ad libitum or calorically restricted diets (Li et al. 1996; Pendergrass et al. 1995).

An important requirement, in studies of comparative biology, is to address the possible correlation of the measure of interest with species body mass since body mass is known to influence several aspect of physiology. Another important aspect is controlling for phylogenetic relations among species since all species are more or less evolutively related and consequently are not truly independent values as requested by the statistic of correlations (Speakman 2005). Since both of these aspects were neglected in the two initial studies, Lorenzini and collaborators conducted a third investigation in 11 mammalian species who differed greatly for both their longevity and their body mass (Lorenzini et al. 2005). Compared with the two previous, this study was larger (with data from 59 strains) and had two important methodological differences. First, only adult derived cells were used. The two previous in fact, mixed data of cells from embryo, newborn and adult biopsies that may have different proliferative potential. Another important difference was that the tissue of origin was always skin while in the previous studies different tissues or whole embryo were used (about the possible importance of these two aspects see related discussion in the last paragraph of this chapter). The data reported a weak correlation of replicative capacity with maximum longevity ($r^2 = 0.36$, and $p = 0.051$, Fig. 4.8a) and a strong correlation with adult body mass ($r^2 = 0.86$, and $p < 0.001$, Fig. 4.8b). More clearly, controlling for body mass, no significant correlation was found between replicative capacity and longevity

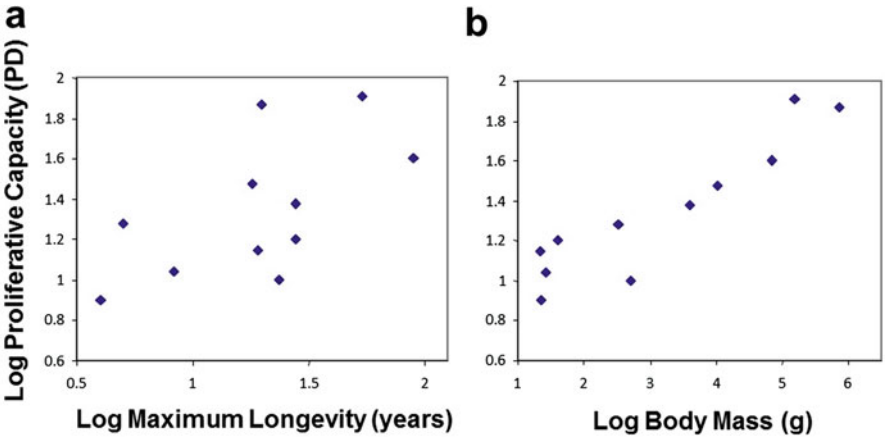

Fig. 4.8 Skin fibroblast replicative capacity vs maximum longevity and vs body mass of mammals. Plots are derived from the analysis of a total of 59 strains from 26 adults of 11 mammalian species. **a** Correlation between maximum longevity and proliferative capacity ($r^2 = 0.36$, and $p = 0.051$). **b** Correlation between adult body mass and proliferative capacity $r^2 = 0.86$, and $p < 0.001$) (Drawn from data reported in (Lorenzini et al. 2005) with permission from Elsevier to Antonello Lorenzini (license 3635900983725))

($r^2 = 0.02$, $p = 0.71$, Fig. 4.9a), and controlling for longevity, replicative capacity remained highly correlated with body mass ($r^2 = 0.79$, $p = 0.001$, Fig. 4.9b). This partial correlation analysis together with the phylogenetically independent pairwise comparisons between species brought to the conclusion that proliferative capacity is primarily related to adult body mass rather than life span.

More recently Gillooly and colleagues have proposed a general model to explain the differences in lifespan and replicative capacity of cells in comparative studies (Gillooly et al. 2012). Their model assumes the existance of a positive relationship between cell mortality and metabolic rate, based on the assumption which follows Pearl's rate of living hypothesis (Pearl 1928). Another assumption is that the replicative age of a cell strain, defined as the total time from birth to senescence of cells in culture, scales with body mass and temperature. They tested the prediction of their model using published data on average lifespan of red blood cell from 49 species. Regarding replicative capacity they used published data of mainly muscle cells from 18 species. In both cases the species included both endotherms that ectotherms. For the chronological lifespan of cells their conclusion was that cell chronological lifespan decreases about 2.5-fold for every 10 °C increase in body temperature. Regarding replicative life span, they concluded, as Lorenzini and colleagues, for the existance of a relation with body mass with a coefficient of determination of 0.52 (Fig. 4.10). Opening a parenthesis on Pearl's rate of living hypothesis, we should mention that in a comparative analysis that used the AnAge database, basal metabolic rate does correlate negatively with marsupial longevity but does not correlated with longevity of eutherians or birds (Magalhães et al. 2007).

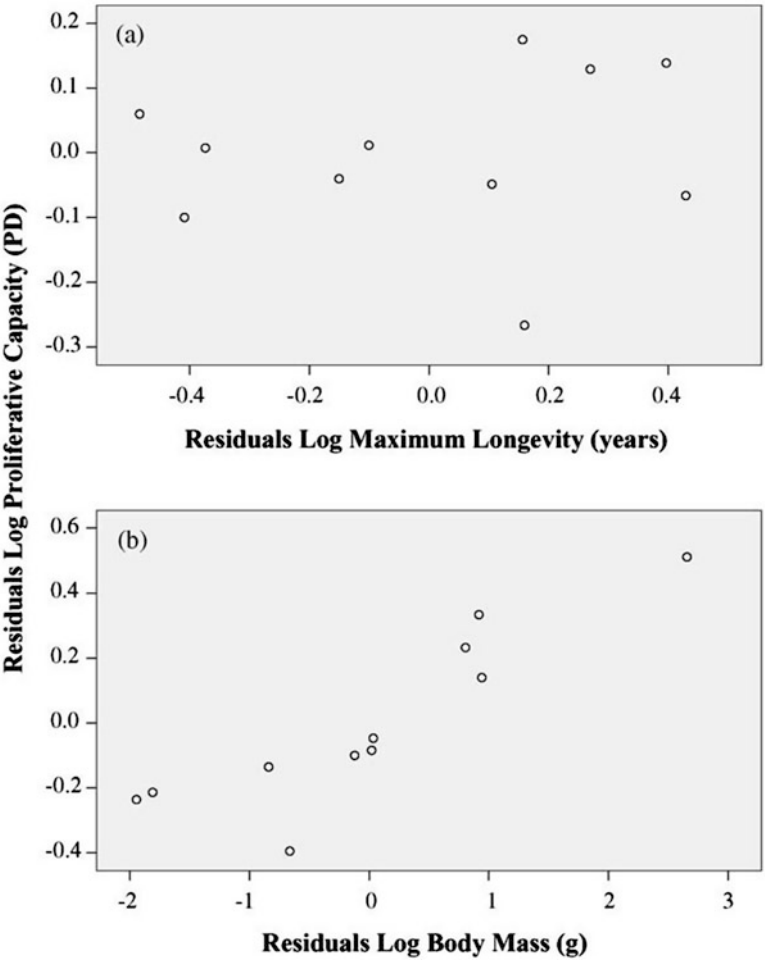

Fig. 4.9 Partial correlation plots between proliferative capacity, maximum longevity and adult body mass of 11 mammalian species. (**a**) Controlling for body mass, no significant correlation was found between replicative capacity and longevity ($r^2 = 0.02$, $p = 0.71$, $n = 11$), (**b**) Controlling for longevity, replicative capacity remained highly correlated with body mass ($r^2 = 0.79$, $p = 0.001$, $n = 11$) (Reproduced from (Lorenzini et al. 2005) with permission from Elsevier to Antonello Lorenzini (license 3635900983725))

Regarding the proposed link between proliferative capacity and body mass the observation is noteworthy that taller nonagenarians tend to have skin fibroblasts with less residual proliferative capacity compared to their shorter nonagenarian pears (Maier et al. 2008b) These data, together with the above reviewed data on donor age, suggest that the replication needed during development to cover large body surfaces, more than life-long cell turnover, use up more of the allocated replicative potential of our species leavening less of it for in vitro propagation. Finally, it is

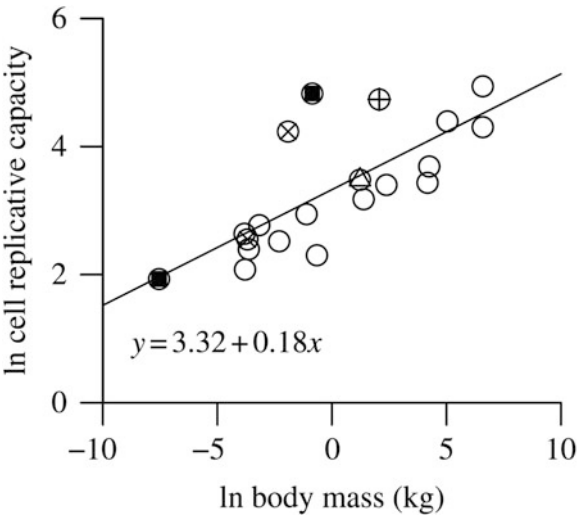

Fig. 4.10 The natural logarithm of cell replicative capacity in culture as a function of the natural logarithm of body mass in animals. *Circles*, mammals; *triangles*, birds; plus symbols, reptiles; *cross symbols*, amphibians; *squares*, fishes (Reproduced from (Gillooly et al. 2012) with permission from The Royal Society to Marleen Moore (License Number 3658790853900))

relevant to mention that telomerase activity correlates inversingly with adult body mass but does not correlate with life span (Seluanov et al. 2007; Gomes et al. 2011). Telomere length, however, seems to be inversely related to species life span; this trend was observed as not statistically significant in previous observations of 15 rodent species (Seluanov et al. 2007) and of 10 mammalian species (Lorenzini et al. 2009) but later found to be statistically significant in a larger study of 61 mammalian species (Gomes et al. 2011). For more discussion of the role of telomeres in cellular senescence we refer to Chap. 7.

4.5 Replicative Senescence Appears as a Not Universal Phenomenon

It has been reported by multiple investigators that cellular stress resistance is correlated with species life span, reviewed in (Stuart et al. 2013). Establishing and maintaining a culture may expose cells to multiple stressors. Consequently it is important to expect different levels of tolerability among species for a given culture condition. A parameter influencing the culture condition is for example oxygen tension. Cells in vivo are supposedly exposed to much lower concentration of oxygen than cells cultivated with the most frequent incubator settings, i.e. 20 % O_2 (Carreau et al. 2011). Pickering and colleagues, in fact, have recently observed that in skin-derived fibroblasts the resistance to protein oxidation is associated with the maximal life span of donor species (Pickering et al. 2014) (for a more in depth discussion on oxidative stress and cellular ageing we remand the reader to Chap. 10).

Parrinello et al. have reported that under 3 % O_2 mouse cells do not show the frequently observed premature proliferative arrest but keep on proliferating well over 60 PD (Parrinello et al. 2003). This finding suggest that the shorter

proliferative life span observed under high oxygen condition is more stress induced senescence than replicative senescence and that mouse cells, in a more physiological culture environment could be immortal. Another, probably underappreciated paper, stated a similar conclusion implicating a serum component as a possible inducer of senescence (Loo et al. 1987). Successively, absence of senescence was observed in rodent glia (Tang et al. 2001; Mathon et al. 2001). Seluanov and colleagues have studied 15 rodent species cultivating their fibroblasts in 3 % O_2 to prevent stress induced senescence. They have observed three different proliferative strategies: species displaying proliferative senescence (characterized by body mass above 8 kg), species displaying indefinite low-rate proliferation (characterized by small body mass and long live span) and species with indefinite normal-rate proliferation (characterized by small body mass and short life span) (Seluanov et al. 2008).

Gomes and colleagues, also cultivating cells under more physiological oxygen tension (3 % O_2), concluded that replicative senescence is present in the majority of species larger than 1 kg, that usually have telomeres shorter then 20 kb and repress telomerase expression in adult tissue, but it is not a universal occurrence (Gomes et al. 2011). They report that several species seems to proliferate indefinitely reaching high population doubling without signs of senescence or "stasis", acronym for stress or aberrant signaling induced senescence. For example, cells from the following species reached high PD before being frozen away without showing a senescent arrest: Dromedary Camel 143 PD, Little Brown Bat 100 PD, Mexican Free-tailed Bat 105 PD, European White rabbit≈180 PD, Pika≈90 PD (Gomes et al. 2011; Forsyth et al. 2005). Finally, an investigation in the extremely short-lived killifish Nothobranchius furzeri, whose maximum life span varies between about 1 year and few months depending from the strain, shows absence of replicative senescence (Graf et al. 2013).

As defined by Hayflick, finite growth should be a property of all normal diploid cells while immortality should be a consequence of cellular alterations, being a property of heteroploid cells such as cancer cells (Hayflick 1965). All the above species where immortality appears in cultures that never show a senescent phase suggest that a third condition could exist: a priori immortality without cellular alterations. The issue of distinguishing between a priori immortal supposedly-normal culture and immortalized culture, i.e. the appearance of a forever-proliferating culture from a senescing one, is not trivial. Spontaneously immortalized cells can outgrowth senescing normal cells before or after the senescent arrest is becoming evident in a growth chart. If immortalization happens before the senescent plateau is reached, the culture's growth trend will be indistinguishable from that of a priori immortal one. A priori immortal culture should retain a normal karyotype, but even normal diploid human cells show some variation of their ploidy during their life span in culture (see (Matsuo et al. 1982) and reference therein). Embryonic stem cells, additionally, which should be the prototype of a priori immortality of normal cells, show increased genomic instability with prolonged subculturing (see (Peterson and Loring 2014) and reference therein). On the other side, a not negligible numbers of tumors are also classified as normal diploid (Gagos and Irminger-Finger 2005). Theoretically, the ultimate test for a priori immortality of a culture will be organism

cloning. The majority of nuclei in such a culture should be able to generate healthy organisms through somatic cell nuclear transfer. The success rate of cloning is still too low, though, to make such a test feasible. On the other hand HeLa cells, for example, are probably among that most widely used immortalized cell lines but nobody will believe it possible, omitting for a second ethical issues, to use one of their nuclei to bring back an healthy Henrietta Lacks by cloning. In any case, the issue of distinguishing between a priori immortal culture and immortalized culture will become more and more relevant with the increasing demand for stem cells therapy since these cells, once obtained, will need to be expanded significantly before being used.

4.6 Summary and Conclusions

We are summarizing the major points discussed in this chapter, referencing only key publications while proposing few general considerations:

- Colony forming ability may not represent the cellular replicative potential of the mass culture (Maier et al. 2008a). The low density seeding typical of cloning represents a stressful condition that is adding his burden to the intrinsic stress of cell culture environment, consequently the capacity to form colonies may be interpreted as a stress resistance capacity. This capacity has been observed to decline with age by several authors both in human and in other species. Nevertheless, the by far largest study including 669 cell cultures derived from 552 donors, showed a correlation with donor age only up to the third decade of life, after that, no further decline was observed.
- No strong evidence for a correlation between maximum proliferative potential and donor age has been found, although conflicting reports have been published (Maier and Westendorp 2009). For this lack of correlation, we suggest the following not exclusive explanations. Firstly, in human adult tissues, senescent cells accumulate both because of progressive telomere shortening that because of stress induced senescence. Their numerosity, although possibly relevant in compromising the normal functioning of old tissues, is not usually high enough to affect the maximum population doubling achievable by mass culturing with the techniques routinely used to establish and maintain cell cultures. In other words, the selection imposed by the process of establishing a cell culture (obtained either by enzymatic digestions or by simple outgrowth from minced tissue) together with the multiple sub culturing of a small fraction of the cells obtained at each passage may cancel out the influence of senescent cells present in the initial biopsy. A second possible explanation is that skin as a tissue is evolutionarily equipped with large proliferative capacity, or with higher number or stem cells, since it is more exposed to external insults in comparison with internal tissues. As already mentioned, the vast majority of studies have been performed on dermal fibroblasts and are in fact the studies on this cell type that do not show a correlation. The effect of aging could be negligible in a cell type predisposed

Table 4.2 A comparison of the passage levels at which Phase III occurred in human diploid cell strains of adult and fetal origin

Fetal lung		Adult lung			
Strain	Passage level at which Phase III occurred (cell doublings)	Strain	Passage level at which Phase III occurred (cell doublings)	Age of donor	Cause of death
WI-1	51	WI-1000	29	87	Heart failure
WI-3	35	WI-1001	18	80	Cerebral vascular accident
WI-11	57	WI-1002	21	69	Bronchial pneumonia
WI-16	44	WI-1003	24	67	Dissecting aneurysm
WI-18	53	WI-1004	22	61	Renal failure
WI-19	50	WI-1005	16	58	Rheumatoid arthritis
WI-23	55	WI-1006	14	58	Pulmonary embolus
WI-24	39	WI-1007	20	26	Auto accident
WI-25	41				
WI-26	50				
WI-27	41				
WI-38	48				
WI-44	63				
Average	48		20		
Range	35–63		14–29		

All strains cultivated at a 2:1 split ratio. Fetal strains derived from donors of 3–4 months' gestation obtained by surgical abortion. Adult and fetal strains derived from both male and female tissue
Reproduced from Hayflick (1965) with permission from Elsevier to Antonello Lorenzini (license 3635901147200)

to answer to high proliferative demand. The less numerous studies on other cells type here reviewed in many instances show a weak but significant decrease in proliferative capacity with increasing age and generally these other cells types show an equal or lower proliferative potential than that of dermal fibroblasts even considering only cultures established from young donors. A third hypothesis is that the sometimes-observed difference in proliferative capacity is due mainly to development and not to aging per se. In other words, in this view, cells from developing tissues have higher proliferative potential than cells from adult tissues. Since these studies not always include samples taken at ages when development is still ongoing; this could possibly explain the contradictory result obtained so far. The original Hayflick data on lung fibroblasts from embryos and adult of different ages (Table 4.2) (Hayflick 1965), the data by Cristofalo et al. on embryo and adult donors (Fig. 4.2) (Cristofalo et al. 1998), the data reviewed by Karavassilis and Faragher that include cells derived from umbilical cord of newborns (for endothelial cells, Fig. 4.3a) and from children and teenagers (for vascular smooth muscle cells, Fig. 4.3b) (Karavassilis and Faragher 2013) as well as the data from Bruce and Deamond on hamster fibroblasts taken from the same

animal at different ages can be easily interpreted in light of this last hypothesis (Fig. 4.4) (Bruce and Deamond 1991).

– Maximum replicative capacity of mass culture seem to be related mainly to species adult body mass more than to species maximum longevity (Lorenzini et al. 2005). The weak relationship of replicative capacity with species longevity is probably due to the known relationship between species longevity and body mass. In support to the relationship between replicative capacity and body mass, a relationship with body mass is observable also for telomerase activity (Seluanov et al. 2007) that is controlling telomere length, the mitotic clock of replicative senescence. These observations suggest that replicative senescence represents a strategy to prevent cancer that has been adopted multiple times during the evolution of large-frame species. Large species, in fact, having more cells are potentially more exposed to the risk of oncogenesis.
– The observation of cellular immortality in different species, when proper cultured conditions are provided (Seluanov et al. 2008; Gomes et al. 2011), has profound theoretical implication re-actualizing the concept of Alexis Carrel of aging been the consequence of multi-cellularity (Carrel 1912).

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Chapter 5
Ageing of the Stem Cells: The Conjoined Twosome Growing Old: Stem Cell and Its Niche

Günter Lepperdinger

Abstract Stem cells are considered distinctly localized within tissues because of specific anatomical structures called the stem cell niche. Intimately connected, the stem cell and the niche are subject to aging. Potent mechanisms of biological aging are control of cellular metabolism, the action of aging genes, epigenetic drift and inflammation, which also modulate the cellular age of stem cells. A common feature of aged stem cells is however hyperactivation.

Keywords Replicative senescence • Differentiation • Molecular damage • Repair • Mortality • Immortality • Ageing • Anti-ageing • Longevity

5.1 Background

In the adult, perpetuated integrity of cellular ensembles is essential to ensure tissue and organ homeostasis. In cycling tissues it is assumed that a small subset of somatic cells is causally involved in maintenance and repair. These cells are better known as adult stem cells. This particular type of cell is thought to exist in most mammalian organs and tissues. As a well-known example given here, the skeletal muscle relies on life-long maintenance by endogenous stem cells, often called satellite cells. Another prominent and well-studied example is hematopoiesis in the adult, which is dependent on a stem cell. Hematopoietic stem cells mainly reside in the bone marrow, yet are also found in circulation.

A commonly appreciated concept that describes the life-long ability to maintain somatic tissues builds on the stem cell's ability to self-renew. This consideration is however mutually exclusive to tissue aging. Strictly speaking, self-renewal implies that a cell would remain in flawless naivety and youthfulness while continuously bringing forth off-springs. Hence, stem cells are thought to undergo asymmetric cell division: while the cellular offspring of the dividing stem cell that is destined

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to constitute tissue-specific precursors, adopts symmetric cell division. Following this fashion progenitor cell pools rapidly grow to respective numbers before commencing with differentiation into tissue-specific precursors. At that point, these cells functionally integrate into previously existing micro-anatomical structures. Terminally differentiated, functional effector cells are considered post-mitotic. As a widely accepted perception, the manifestation of fate asymmetry in a stem cell primarily accounts for cell-intrinsic controls.

Fate asymmetry may however also be accomplished by extrinsic or environmental cues. The local environment of stem cells is termed the stem cell niche. In there, stem cells are believed to reside in a quiescent mode. In its own interest the quiescent cell places emphasis on cellular maintenance to protect its integrity, but also lies in waiting of appropriately responding to stressful events in a minimum time. It is unquestionable that for the remodeling or regeneration of adult tissues, self-renewal of the stem cell pool, transient amplification of the progenitor population and terminal differentiation of precursors need to be accurately balanced. The purpose of this specialized maintenance system is to provide robust means to accomplish life-long functional integrity and homeostasis of tissues and organs. In the young, coordination of stem cell function and molecular mechanisms controlling self-renewal, differentiation and niche integrity is precisely tuned. This situation may change during aging as the cells themselves undergo chronological aging, also in case balanced coordination of stemness deteriorates due to systemic aging. As a consequence thereof, adult stem cells in the aged organism show decreased capacities in remodeling, regenerating and repairing tissues, which have been damaged by stress, injury or attrition.

5.2 Current Distinctions of What an Adult Stem Cells Is

More often than not, the stem cell has been imagined being a single cell exhibiting very specific distinctions. The notion is that it shall bear the potential to self-renew and that it exhibits the capacity of bringing forth progenitor cells which eventually differentiate into mature and functionally effective tissue-specific cells. A stem cell should thus exhibit specific biomarkers. In due course of the emergence of the first daughter generations phenotypic changes should be distinguishable. Not only within the transiently amplifying cells, even more distinct should be the differentiating cell population. It is further believed that changes along this hierarchy are irreversible. In order to substantiate this concept, biomarkers capable of singling out stem cell in vivo have been chased. Working along these lines, marker-based assays in combination with transcriptional profiling of fixed samples allowed to define key regulatory networks and signaling pathways, which control stem cell activity and fate behavior (Bhaskar et al. 2014; Blank and Karlsson 2015; Buckingham and Montarras 2008; Campbell and Copland 2015; Crane and Cao 2014; Janesick et al. 2015; Niessen et al. 2012; Paridaen and Huttner 2014; Shigdar et al. 2014; Shimomura and Christiano 2010; Van Camp et al. 2014).

Another very simple approach had been to discriminate stem cells in vivo because cells incorporate and retain labels in their DNA over long periods of time. Initially introduced for lineage-tracing, later on in order to study stem cells, this experimental approach was pioneered by Charles Philippe Leblond already in the 1940s (Belanger and Leblond 1946). Further along these lines, John Cairns and Christopher Potten hypothesized that somatic stem cells segregate their genomic DNA asymmetrically, retaining an "immortal" DNA template while passing on newly formed chromatids to daughter cells (Potten et al. 1978). Regulated mitotic quiescence together with asymmetric DNA segregation may thus offer stem cells a distinct mode allowing them to reduce accumulation of mutations as well as maintaining a defined epigenetic memory as they divide. Early experiments employed [H^3]-thymidine in developing mice to show that label-retaining cells persist within intestinal crypts and hair follicles over an extended period of time (Potten et al. 1974). More sophisticated strategies have been established using nucleotide analogues containing fluorine, brome or iodine containing nucleotide analogs to identify label-retaining cells and to track asymmetric DNA distribution in a variety of tissues (Mull and Asakura 2012). Both growth and injury models have been employed to study non-random DNA strand segregation. To date, evidences in support or opposition of asymmetric DNA segregation are still mixed, particularly also because the theory is difficult to reconcile with our current understanding of genome organization, DNA repair, and stem cell turnover (Lansdorp 2007). Hence drawing a definite conclusion about the asymmetric segregation of genomic DNA appears difficult. Only the unambiguous identification of a specific subpopulation of true stem cells through the use of suitable markers and functional tests may eventually allow confirming or refuting this hypothesis. Notably in this context are recently established methods which allow tracing asymmetric apportioning of organelles to two daughters. Organelles could be specifically labeled with tags which could be activated at exact times. In this way newly synthesized organelles could be tracked before during and after cell division. Working along this line, mitochondria showed differential segregation, such that the stem-cell daughter received most of the newly synthesized mitochondria, whereas the tissue-progenitor daughter received more of the old mitochondria (Katajisto et al. 2015). Apparently an exciting question now is whether the mechanism described is a general feature of stem cells, and more than that, whether newly synthesized mitochondria are segregated together with the immortal genomic DNA strand.

The method of label retention has been greatly appreciated as it potentially enables distinguishing putative stem cells from differentiated progeny. Applied in this context, it facilitated to study static versus dynamic cell pools. The latter is important because the environment of an activated stem cell is considered highly dynamic. As pointed out before, methods employing radioactive tracers in vivo have provided first insights into stem cell behaviors. These observations have now been further developed in transgenic animal models. Diligently designed genetic elements can be placed between recombination sites. In combination with controlled cell-specific expression of recombinases they can be turned into transposable elements. In this way the expression of fluorescent proteins can be switched on

or off in a spatial and/or temporal manner (Kretzschmar and Watt 2012). This particular technique has been also employed to prove the widely persistent view of a stem cell constituting the foundation of a linear hierarchy of cells. The clear-cut scheme includes one or more types of transiently-amplifying progenitors, eventually discharging terminally differentiating cells. Provided recent experimental observations, there is now good reasons to believe that functional stem cells exhibit dynamic changes, which are by and large modulated by niche-specific properties and soluble factors. It is further highly likely that, stem cells comprise a pool of heterogeneous dynamic cells rather than being strictly persistent in their phenotype and appearance. They are capable of displaying enhanced proliferative capacity and lineage commitment while also being capable of stalling mitosis thereby turning into a quiescent state. In stark contrast to the classic view, especially in the situation of a crisis or after injury, potentially determined progenitors may fall back into a naïve ground state and reacquire self-renewal potential. Very probable this behavior is largely triggered and modulated by exogenous factors provided by the local microenvironment.

This wide range of properties, modes and appearances described for various tissue-borne adult stem cells can be subject to aging at the molecular, cellular and organismic level.

5.3 Somatic Stem Cell Aging and Loss of Tissue Regenerative Potential

Theories have been proposed to explain aging. The ‘disposable soma theory’ circumscribes how resource utilization between somatic maintenance and reproduction accounts for selective pressures in order to maximize reproductive success. By sensing environmental conditions, reproduction is favored when resources are affluent. However when resources are scarce, survival pathways and maintenance of bodily structures should be promoted pulling back energy from reproduction (Kirkwood 1977). These general assumptions could be also proven in human societies (Westendorp and Kirkwood 1998). This implies that aging sets in after development as a result of the decline in homeostatic remodeling of tissues and blemished controls of bodily defense mechanisms. Tissue-borne stem cells are generally believed to spur somatic homeostasis. While somatic stem cells face the same selection pressures as the rest of the soma, it is indeed conceivable that distant similarities between stem cells and germline cells exist. For instance, in drosophila, stem cells and germline stem cells utilize the same nutritional information via insulin. Yet they process the signal in a manner distinctive to their niche (Shim et al. 2013). In the female fly germline, insulin-like peptides secreted from the neuroendocrine cells maintain niche integrity. In this way insulin indirectly affects germline cell behavior. However insulin also directly promotes the proliferation of germline stem cells (Hsu and Drummond-Barbosa 2009; LaFever and Drummond-Barbosa 2005). Also in the male germline, insulin acts directly and

indirectly via the hub cells (Ueishi et al. 2009). In the male, one of the cellular mechanisms involved in this response is the control of centrosome orientation, which attenuates germline stem cell proliferation under poor nutrient conditions (Roth et al. 2012). Similarly, neuroblasts require local insulin secreted from niche cells (Chell and Brand 2010; Sousa-Nunes et al. 2011), and nutrition promotes the proliferation and maintenance of blood stem/progenitor population (Shim et al. 2012), while poor diet and low insulin reduces the proliferation of intestinal stem cells (McLeod et al. 2010). Many tissue types and signals are conserved between fly and vertebrates. It is therefore conceivable that stem cell populations in mammals also employ comparable regulatory circuits in response to nutrient signals. In mammals, another aspect needs to be added here: influences of diet and metabolic pathway regulate adult stem cells, however, it is widely believed that this may also have considerable implications regarding cancer initiation (Jung and Brack 2014).

Provided these assumptions, a second well perceived aging theory is applicable for explaining stem cell aging: the 'antagonistic pleiotropy' theory (Williams 1957). It assumes that particular genes exhibit not only one feature but impact on several traits of an organism (pleiotropy). They are selected during evolution for being distinctively supportive for organismic fitness early in life, while later in life they actually unfold their harmful side. Many of the antagonistic pleiotropy genes are tumor suppressors. They are beneficial by suppressing cancer during any stage in life. In turn they drive aging by the same mechanisms by which they suppress cancer. p53 is a well-known example of an antagonistic pleiotropy tumor suppressor gene. It prominently mediates DNA damage response pathways. p53 is a centerpiece in controlling cell-intrinsic responses to genome instability, including a transient cell cycle arrest, but also senescence and apoptosis. Senescence and apoptosis are preventing the uncontrolled proliferation of potentially transformed cells. The same mechanisms can deplete stem and progenitor cell pools efficiently, and as a consequence thereof can drive tissue degeneration and organ failure (Reinhardt and Schumacher 2012). Another antagonistic pleiotropy gene is mammalian/mechanistic target of rapamycin (mTOR), which is a highly conserved cytoplasmic serine/threonine kinase. mTOR responds to environmental determinants, nutrient availability, energy sufficiency and stress. mTOR regulates cell growth and metabolism in response to mitogens, amino acids, glucose and fatty acids, and hormones including insulin. Constitutive mTOR signaling converts cellular quiescence into senescence (Halicka et al. 2012). Notably in the context of stem cell aging, pharmaceutical inhibition of mTOR by rapamycin has been reported to restore the self-renewal and hematopoietic potential of aged HSCs (Chen et al. 2009). Rapamycin also increases intestinal stem cell self-renewal via inhibition of mTORC1 in the adjacent Paneth cells (Yilmaz et al. 2012). Rapamycin also depletes leukemia-initiating cells (Yilmaz et al. 2006). It further inhibits the self-renewal as well as the differentiation capacities of stem cells derived from infantile hemangioma, suggesting a protective effect against cancer stem cells (Greenberger et al. 2011). So taken together mTOR activities counteract "stemness" and may turn into a driver of aging in adult stem cells types (Lamming et al. 2013).

The study of such genes with regard to age-related changes in stem cell function has fostered our current understanding of how stem cell fates, particularly malignant transformations or senescence influence tissue ageing and healthy longevity. When persisting in a quiescent state during most of the lifespan, stem cells may have experienced long exposure times to genotoxic stressors, and thus accumulated damages of biomolecules. Consistent with this notion, stem cells are thought to be better safeguarded by enhanced protective measures, such as expression of high levels of ABC transporters conferring xenobiotic efflux (Krishnamurthy et al. 2004) or controlling reactive oxygen species at a low level (Kocabas et al. 2012). Persistent DNA damage is of major concern in this context, as progeny will inherit changes, and mutations are thus being broadly incorporated in tissues. Dormancy of stem cells may decrease chances of mutations which otherwise remained unresolved by DNA repair. Interestingly, an increased number of DNA double-strand breaks could be revealed in aged stem cells when staining for nuclear foci containing the phosphorylated form of the variant histone H2A.X (Rossi et al. 2007; Sinha et al. 2014), indicating that stem cells appear to be not comprehensively genoprotected during aging. In HSC, it could be further shown that DNA damage accumulation comes along with attenuation of DNA repair although the cells were in a quiescent state. Interestingly however, exit from G_0 and entry into cycle led to global up-regulation of essentially all DNA repair and response pathways. Moreover DNA repair was found enhanced in downstream progenitors, regardless of age (Beerman et al. 2014). With a grain of salt, DNA damage should not be considered deleterious at any instance, as for instance DNA strand breaks were found to be induced during differentiation (Larsen et al. 2010), and as pointed out above, could be asymmetrically passed on to daughter cells. Whether this is common in most stem cell types remains to be shown? Conceivably however, damage being accumulated in quiescent stem cells over long time may eventually exceed a threshold which triggers stem cell dysfunction, or turn them senescent. In contrast to differentiated tissue, there is hardly any data showing that senescence occurs to stem cells in vivo. Along the same line, the rate of apoptosis occurring in stem cells in vivo is difficult to assess. Indeed, low levels of apoptosis could occur in a stem cell pool during normal ageing and could thus be a distinct feature of normal ageing.

Lifelong autonomous capacitation of individual stem cells borne within tissues will primarily shape functionally heterogeneous subsets of adult stem cells. Consequently functional changes of a stem cell can result in selective loss or expansion of discrete subsets during stress, turnover, and aging. Clonal expansion of a stem cell that is intrinsically superior or has acquired increased cellular fitness will under selective pressure eventually dominate the aged stem cell pool (Jung and Brack 2014). Determining the consequence of clonal expansion or loss in specific subsets will certainly require comprehensively studying stem cell heterogeneity in the context of those corresponding molecular signals that balance stemness and cellular fitness. Besides cell-autonomous variations, any age-dependent changes of the environment, be it systemic or be it contiguous, definitely molds the epigenetic state of stem cells.

The epigenetic code enables cells to receive and remember environmentally induced signals in order to create a more stable state. Epigenetic marks such as DNA methylation and histone modifications shape a specific epigenetic landscape (Waddington 1942). Although all cells of the soma bear the very same genetic information, epigenetic modifications allow cells developing distinctly different features and carrying out clearly defined programs. Acetylation of histones plays a role in acquiring longevity. Sirtuins, a family of histone deacetylases regulate organismal lifespan as well as responses towards oxidative stress and DNA damage (Imai and Guarente 2014). Genome-wide studies in aging cells and tissues have uncovered stochastic DNA methylation drift (gradual increases or decreases at specific loci) that reflects imperfect maintenance of epigenetic marks. In contrast to immortal cells, normal fibroblasts progressively lose 5-methylcytosine when undergoing replicative aging in vitro (Wilson and Jones 1983). Although the changes are quite small, similar changes are detectable in aging tissues and cells (Ahuja and Issa 2000; Fuke et al. 2004). Drift creates epigenetic mosaicism in aging stem cells that could potentially restrict their plasticity and worsen phenotypes such as stem cell exhaustion (Issa 2014). Intestinal stem cells are residing in the epithelial crypts of the colon. Microdissection and analysis of individual crypts revealed age-related methylation variations suggesting that age-related hypermethylation might be a property of stem cells (Shibata 2009). Given that every stem cell replication event results in changing DNA methylation at a certain chance, quantitative assessment of methylation should actually enable to reveal mitotic activity over time similar to telomere shortening. Replication-dependent changes were indeed found in hematopoietic stem cells (Beerman et al. 2013). As progenitor cell differentiation was also found to be associated with methylation changes, a general assignment of this finding becoming valid cellular aging biomarker for any type of stem cell is therefore difficult (Lister et al. 2009). Consistent with these data aged stem cells appear to be epigenetic mosaics. Differences of CpG island methylation patterns especially in promoters are thought to account for largely varying gene expression between stem cells albeit residing in the same tissue. As a consequence of epigenetic mosaicism the function of aged stem cells should be diverse. Aged hematopoietic progenitors show skewed differentiation bringing forth more myeloid than lymphoid progeny in older age. It is by now generally accepted that epigenetic plasticity is contributing to this defect as particular targets in specific regions of the genome which are critically important for lineage-specific gene expression could be identified (Beerman et al. 2013; Oshima and Iwama 2014). More severe effects are associated with hypermethylation mediating silencing of genes, which are regulating self-renewal potential and capacity. As a consequence thereof, a stem cell becomes gradually exhausted over time leading to age-related depletion of the stem cell pool (Issa 2014). In this context also perturbations of histone modifications (for example, H3K4me3) were identified which can greatly alter the expression of HSC self-renewal genes (Sun et al. 2014).

Overall, compromised stem cell function is a likely consequence of an age-related epigenetic drift, and there is some evidence that stem cell aging is a key mechanism regulating life span (Issa 2014). The epigenetic memory is considered a

key element of stem cell aging, and means and measures have been searched how to preserve, or once it is lost, how to reset it. Somatic nuclear transfer (Gurdon 1962; Simonsson and Gurdon 2004) indicated that fate distinction and developmental memory of somatic cells can be reversed (Holliday 1987; Surani 2001). More recently by means of enforced expression of a few embryonic transcription factors somatic cells could be reprogrammed into induced pluripotent stem cells (iPS) (Hochedlinger and Jaenisch 2006; Stadtfeld and Hochedlinger 2010; Takahashi and Yamanaka 2006). iPS greatly resemble embryonic stem cells, which normally can solely be derived from primordial cells of the inner cell mass of a blastocyst. Provided this evidence, it was thought that the epigenetic memory not only from aged, terminally differentiated somatic cells but also from stem and progenitor cells can be reset. Indeed this possibility was tested by experimentally showing that aged hematopoietic progenitors when reprogrammed into iPSC can be used to make a chimeric embryo which now contained rejuvenated HSC (Wahlestedt et al. 2013). It is unlikely that this special, currently broadly appreciated technology of rejuvenating stem cells can be applied clinically as such. However provided a plethora of convincing experimental results, it appears likely that epigenetic rejuvenation can be reached by treatment with exogenous agents, meaning that it may become feasible to distinctly shift epigenetic signatures into a desired state. Apparently however the question of how and whether stem cells can be rejuvenated in situ may become a future emerging therapeutic technology is currently a matter of debate (Anjamrooz 2015; Nguyen and Sussman 2015; Nurkovic et al. 2015; Scudellari 2015; Sousa-Victor et al. 2015).

5.4 Aging of Stem Cell Niches and Extrinsic Aging of Stem Cells

It is generally believed that tissue-specific stem cells reside in, and intimately correspond with their niches. Stem cells depend upon other cells. They continuously pursue cell–cell contacts and partake in paracrine signaling. The term '*niche*' was first coined by Schofield in 1978 (Schofield 1978). By now stem cell niches have been operationally defined for many stem cell types also in mammalia (Morrison and Spradling 2008). Pertinent components of a stem niche are the vasculature and interstitium, as well as matrix proteins and constituents. The traditional view of a niche was maintaining a stem cell and promoting self-renewal. This vista has been by and large replaced by a more complex situation which comprises diverse regulations that enhance dormancy, promote transient activation while preserving the blank state of a stem cell (Hsu and Fuchs 2012).

For sure stem cell function and stem cell fates are influenced by soluble factors. For instance, the hematopoietic niche in bone marrow is characteristic for stem-cell-factor, SCF, CXCL12, osteopontin, angiopoietin 1, and thrombopoietin (Kiel and Morrison 2008). Paneth cells are found in close association with intestinal stem cells. They secrete epidermal growth factor, WNT3A, TGF-α and Notch ligands

(Sato et al. 2011). In skeletal muscle, the niche comprises immune cells (Schnoor et al. 2008) and fibro/adipogenic progenitors that facilitate muscle regeneration (Murphy et al. 2011). Control of stem cell dormancy by the niche is considered relevant for stem cell aging. As the niche is part of the soma, niche aging occurs as the organism ages. It could be shown that the combination of the four factors IL-1α, IL-13, TNF-α, and IFN-γ exogenously given to satellite cells were sufficient to promote proliferation. This is a convincing indication of inflammation playing a major role in stem cell activation, however it also indicates that deterioration of the niche by 'inflamm-aging' may eventually drive stem cell aging (Franceschi et al. 2000). Along this line there is more growing evidence that a common feature of an aged stem cell niche is dysregulation by chronic inflammation, which in particular results in the increased activation of stem cells, and perturbing quiescence control (Chakkalakal et al. 2012; Vas et al. 2012). Conclusively, systemic inflammation appears to be a driver of age-related changes in stem cell niches. It also contributes to an age-related decline in stem cell function (Font de Mora and Diez Juan 2013; Geiger et al. 2013, 2014; Hsu et al. 2014; Nakamura-Ishizu and Suda 2014; Raveh-Amit et al. 2013). Activation of the NF-kB pathway is a known transcriptional signature that mediates chronic inflammation in aged skin, skeletal muscle, bone and nervous system, although the direct effects of NF-kB activation on stem function is not clear. Accumulation of senescent cells in tissues of advanced age can be another driver of chronic inflammation, as these cells secrete inflammatory factors, growth regulators, proteases and other signaling molecules, affecting neighboring cells in the local environment and promoting senescence and inflammation (Coppe et al. 2010).

5.5 Perspective

Stem and progenitor cell niches regulate the naïve ground state of stem cells and their imperative propensity to differentiate according to tissue-specific anatomical requirements. Local signals act on a stem cell in order to regulate dormancy, yet also to control the specific modes of activation and to guide asymmetric division, while at the same time accounting homeostatic proliferation of tissue progenitors and targeting differentiated cells to sites of integration. Stem cells when quiescently residing in their corresponding niches, yet also the niches on their own steadily sense external signals. Notably, stem cell activation is the sole requirement for continuously monitoring the environment. It is seemingly important returning into a dormant state. Pathways mediating the latter are scarcely investigated, neither in young nor in advanced ages. This particular question is yet becoming more tantalizing as the concept of cancer stem cells has emerged. Working along these lines more molecular, cellular and organismic links and interrelations will be elucidated. It however remains a largely unanswered question how the twosome of a stem cell and its niche processes signals and interprets physiological demands to ensure an appropriate response.

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Chapter 6
Ageing and Senescence in Immune Cells In Vitro and In Vivo

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Abstract Since the discovery of T cell growth factor, now known as interleukin 2 (IL 2), it has been possible to serially culture normal human T lymphocytes, something that cannot be done with other immune cells such as B cells or monocytes/macrophages. This chapter will focus on cloning efficiencies of T cells from younger or older subjects, their longevity in culture under different conditions and the progressive changes occurring over their in vitro lifespan.

Keywords Immunology • T-cell • Interleukins • Cytokines • Infection • Stress • Hyperoxia

6.1 Introduction

Following the development of techniques for maintaining human T lymphocytes in long-term culture, which depended on the discovery of "T cell growth factor", TCGF (Smith and Ruscetti 1981), it came as a surprise to most immunologists trying to clone these cells that they were not immortal, but had finite lifespans. This should of course have been anticipated, given the work on fibroblasts two decades earlier (Hayflick 1965), but such was the lack of interdisciplinarity in biology that history repeated itself and many immunologists were skeptical about limited lifespans. The same main argument was used as had been in the 1960s concerning the fibroblast work – i.e. you only have to get the culture conditions right, and T cells will live for ever. One does have to wonder why there was such resistance, for no real reason, to the idea of a "Hayflick Limit" for T lymphocytes, which are, after

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all, also normal somatic cells (Effros and Pawelec 1997). With the emergence of the powerful, but imperfect, idea that replicative senescence is a tumor suppressor mechanism (Campisi 1997), this is even more difficult to understand. However, one has to consider the prevailing technology and degree of immunological knowledge at the time: TCGF as originally employed was a "soup" of factors, with its main component Interleukin 2 (IL 2) not yet available in pure recombinant form (or even named IL 2 yet); and the requirements for T cell activation and, importantly, intermittent reactivation, were not yet clear. For this reason, some of the data indicating a very short lifespan for human T lymphocytes were in a way indeed due to suboptimal culture conditions. It must also be borne in mind that T cells and fibroblasts are very different types of cells, and even within the three major mature T cell subsets (CD4+ T cells, CD8+ T cells and CD4,8-double negative gamma/delta T cells), there is immense heterogeneity. In attempts to reduce this heterogeneity to manageable proportions, at least in model systems, we exerted much effort in generating monoclonal human T cell populations and longitudinally following them over their (finite) lifespans. Although limited to representing only a small fraction of all T cells likely to be present in an individual, we examined the properties of T cell clones (TCC) derived from different sources (young donors, old donors, centenarians, cancer patients). This chapter reviews our experience with these putative in vitro models for T lymphocyte ageing, and attempts to make some comparisons with what we know about human T cell ageing in vivo.

6.2 Culture and Cloning of Human T Lymphocytes

Methods for culturing human peripheral blood T cells derived from early studies using mitogens such as phytohemagglutinin to activate the cells, which otherwise remain quiescent in vitro. These stimuli cause a burst of vigorous cell proliferation, after which cultures rapidly die out. Unlike fibroblasts, where a source of serum in culture medium is sufficient to maintain growth, human T cells require activation by stimulation through their antigen receptor (so-called signal 1) and simultaneous ligation of "costimulatory" receptors, the best known but not only one of which is CD28 (so-called signal 2). These two signals trigger a multitude of responses in the T cell, culminating in extensive clonal expansion and acquisition of differentiated effector functions (Bour-Jordan et al. 2011). In complete culture medium containing all necessary nutrients and serum, sufficient to maintain fibroblasts long-term, human T cells stop proliferating after relatively few population doublings (PD). This is because their autocrine production of required growth factors ceases. It can be substituted by TCGF, enabling several more rounds of cell division, but this also ceases quite rapidly because growth factor receptors are downregulated in the absence of repetitive activation via signal 1. For extended proliferation, T cells require both growth factors and intermittent restimulation via the T cell receptor (TCR) and costimulatory receptors such as CD28. Hence, earlier studies supplying one but not the other of these necessary conditions resulted in cultures

with very limited proliferative capacity (Perillo et al. 1989). However, peripheral blood T cells from both young and old donors can be cultured longer-term in vitro provided that they are not only supplied with growth factors but also intermittently stimulated via several surface receptors. Restimulation via the antigen receptor or, under certain circumstances, other surface receptors, is required for re-upregulation of growth factor receptors and continued growth. Restimulation too soon after antigen exposure, on the other hand, may lead to activation-induced cell death by apoptosis. Hence, T cells must be maintained in culture by balancing provision of sufficient antigen to avoid growth cessation and excess antigen, which increases apoptosis. Under appropriate culture conditions, all major subsets of T cells can be cultured (ie. CD4+ TCR2, CD8+ TCR2, CD4,8-negative TCR1, and NKT cells). CD4+ T cells lend themselves to these types of experiments (under these culture conditions at least) more readily than CD8+ T cells; the latter two types tend to be much more difficult to maintain.

Early assessments of longevity of human T cell cells in culture, even if restimulation conditions were appropriate, commonly measured culture duration of mixed populations of cells, i.e. they measured only the longevity of the longest-lived clone, in the same manner as most experiments with uncloned fibroblasts had been performed. Assessments of clonal longevity have remained rare in the literature, possibly because of the laborious nature of the experiments, but over the years, we attempted to obtain quantitative data on TCC from different sources generated and maintained under diverse conditions. Our earliest investigations sought to optimize cloning efficiency and clonal expansion by comparing different sources of "filler" or "feeder" cells (required to support single cell cloning), different sources of serum, and different sources of TCGF.

6.2.1 Cloning Conditions and Cloning Efficiencies

Our initial studies compared sources of IL 2 (TCGF, highly-purified natural human IL 2 and recombinant human IL 2). Cloning efficiencies (CE) of activated peripheral blood T cells from healthy young donors were high (up to 50 %) when TCGF ("soup" supernatant of activated pooled T cells from several donors) was used together with irradiated pooled PBMC as filler cells (Kahle et al. 1981). Other tested conditions did not yield such impressive results, and later studies showed that this natural purified IL 2 source was superior to recombinant IL 2 for both CD4+ and CD8+ T cell cloning, but that CD8+ TCC were less likely to persist long-term (Pawelec et al. 1987). Thus, a CE of 50 % for a mixture of CD4 and CD8 cells implies that almost all of the former are capable of clonal outgrowth in culture (with a clone being defined as the accumulation of ca. 1,000 cells from 1 original cell). We can therefore conclude that the CD4 T cell repertoire is present more or less in its entirety in the starting clonal populations under these conditions.

6.2.2 Clonal Expansion

Maintaining all derived TCC from each cloning experiment and recording the longevity of the expanding clones indicated that "life expectancy" of these cells was very heterogeneous. We assessed clonal expansion as the proportion of clones (defined as accumulating at least 10^3 cells) reaching 20 PD (10^6 cells), then 30 PD, 40 PD and so on. For TCC from young donors, only half of derived clones achieved even 20 PD, another half was lost before 30 PD, and another half by 40 PD. Thus, only around 15 % of the originally clonable cells had a proliferative life expectancy of 40 PD. Nonetheless, this represents theoretically 10^{12} cells per clone if all survived, which would be getting close to the size of a leukemia clone. In many experiments carried out on numerous donors, we found that the average clonal life expectancy was 30 PD, but that the longest-lived single clone from each experiment commonly achieved 50–70 PD. Without cloning, one might have concluded that the Hayflick Limit for T cells in general is 50–70 PD, very similar to fibroblasts. A similar sort of clonal selection might be taking place in fibroblast cultures too. These results therefore suggest that the great majority of somatic cells possess in vitro lifespans far lower than the Hayflick Limit indicates (Pawelec 2003).

6.2.3 Human TCC from Different Sources of Lymphocytes

As with fibroblast cultures, early data on human T cell cultures suggested that their longevity was inversely proportional to the age of the donor. We therefore undertook cloning experiments using cells from the oldest old (over 85, up to centenarian). We found that CE of cells from very old donors were indeed lower than in the young. However, around that time, we started to use serum-free culture medium instead of medium supplemented with pooled human serum, and were surprised to find that CE were no longer lower in the elderly than in the young (Pawelec et al. 2000). Bearing in mind that the culture system selected for CD4 cells, and that the CE are high, we concluded that CE of the T cells from older donors are not markedly different from the young and that human serum blocks the proper functioning of the cells from older but not younger subjects. These early results are reminiscent of recent data from heterochronic parabiosis experiments, suggesting the presence of inhibitory factors in sera of older animals (Conboy et al. 2013).

Having investigated CE of T cells from young and old subjects, we then asked whether their clonal longevities in culture were different. We reasoned that old cells with a more extensive proliferative history in vivo might have achieved the ca. 10 PD needed to be counted as a clone in culture, but not the 20, 30 or more as an established TCC. However, all our quantitative data (from cultures in serum-free media) suggest that this is not the case. Very similar proportions of TCC achieved 20, 30 and 40 PD regardless of their origin, and maximum longevity of the longest-lived clone was also similar and very close to the Hayflick Limit of 50–60 PD. Overall, it seems

that CE and longevities of T cells in culture are essentially identical regardless of whether the cells to be cloned are derived from young or old donors, provided that the use of pooled human serum supplements is avoided (Pawelec et al. 2000).

6.2.4 *Culturing at Lower Oxygen Levels and Use of Anti-oxidants*

Culture conditions as generally employed for these types of experiments almost always represent a hyperoxic state, because cells are maintained in air (20 % oxygen, as opposed to the 2–5 % equivalent oxygen tension thought to reflect the in vivo situation). This artefactual circumstance may contribute to DNA damage accumulation. Our experiments showed that increased levels of reactive oxygen species (ROS)-induced DNA damage with increasing PD were indeed present in the TCC (see Sect. 6.3.3). Nonetheless, there were no consistent changes in the expression of cyclin-dependent kinase inhibitors (CKI) p16, p21 or p27, often associated (in fibroblasts) with a replicative senescent state (Hyland et al. 2001). Thus, at the end of their lifespans, these TCC did not have a CKI phenotype characteristic of senescent fibroblasts (i.e. increased p16 and p21 levels). This oxidative DNA damage may contribute to TCC ageing in vitro in other ways because of hyperoxic culture conditions. We established that DNA damage levels over the TCC lifespan were significantly decreased when culturing at 6 % oxygen, but that total PD achieved and proliferative capacity were reduced, not increased (Duggan et al. 2004). This could be because ROS production is required for delivering the required intermittent T cell activating signals to maintain up-regulated growth factor receptor expression by the cells. Arguing against this, however, are findings that culturing cells in air, but with the addition of anti-oxidants to the culture medium, did result in extending the lifespan of these TCC, but only if present for most of the clones' lifespan (Hyland et al. 2000; Marthandan et al. 2013). Thus, there may be a delicate trade-off balance between the damaging effects of oxygen and the requirement for free radical production to maintain signaling (Larbi et al. 2007). This interpretation is consistent with our later findings that primary cultures of T cells are better activated at higher oxygen concentrations (Larbi et al. 2010).

Again, unlike in cultured fibroblasts, we have shown that pro-apoptotic pathways become activated in TCCs grown in air as the cells "age" in vitro (activation of JNK, p38 and c-Jun, irrespective of originating subject age). Ebselen and NAC supplementation significantly decreased phosphorylation of JNK and c-Jun in late PD TCCs. The latter also decreased p38 phosphorylation in these late PD TCCs. However, neither Ebselen nor NAC could alter the activation of p38, JNK and c-Jun in TCCs derived from an 80 year-old subject, and thus failed to affect the time to onset of apoptosis. This is essentially the only evidence to date suggesting that there may be intrinsic alterations to intracellular processes, which have accumulated during the presumed prolonged existence of T cells from elderly subjects (Marthandan et al. 2014). There may be some parallels with the situation

in vivo in this case, because earlier work from our group on blood samples from nonagenarians participating in the Swedish NONA Immune Study revealed levels of DNA damage in PBMCs similar to those found in middle-aged donors, in those elderly with an enhanced in vivo plasma antioxidant capacity (Hyland et al. 2002).

6.3 Progressive Changes to TCC with Accumulating PD

6.3.1 Surface Phenotype

Because by definition clones derive from a single cell, "young" TCC have already undergone at least ca. 22, 23 PD before the first analysis can be performed, but as discussed above they do still seem to represent a high proportion of the original (CD4+) starting population. Nonetheless, those clones that can be studied over an extended time do clearly represent only the small fraction surviving up to the Hayflick limit (and occasionally beyond, our record being ca. 170 PD). Using this selected subset, one can ask whether the expression of molecules at the cell surface changes with increasing PD in culture. Our many studies on a large range of markers found relatively few changing robustly with culture age. The most reproducible pattern of age-associated alterations involved a reduction of the level of expression of the costimulatory receptor CD28, as well as other costimulatory receptors such as CD134 and CD154 (Pawelec et al. 1997). These cells have a memory effector phenotype (CD45RA-negative, CCR7-negative, CD45RO+) as would be expected from chronically-stimulated memory effector cells. The level of the TCR also remains stable, suggesting that these cells would retain the ability to recognize and respond to antigen. This is a typical pattern but is not universally observed. In some clones, CD134 and/or CD154 expression was also retained by late passage cells. However, the most consistent pattern is a quantitative reduction in the levels of expression of CD28, which is essentially universal in the ageing TCC. Nonetheless, in certain clones, although they show this age-associated decrease at first, CD28 re-expression can occur later in the lifespan. We have correlated this CD28 re-expression with a decreased ability of the TCC themselves to secrete TNF, consistent with the observation that TNF downregulates CD28 expression and with our observations that TNF can directly inhibit some TCC (Pawelec et al. 1989; Bryl et al. 2001).

6.3.2 Functional Changes

A decreased level of expression of costimulatory receptors would be expected to have major functional consequences for the cell, and this is what we observed. The most dramatic changes were in the patterns of cytokines secreted, commonly resulting in decreased levels of IL 2 and increased levels of IL 10 (Pawelec et al. 1997). Because the expression of the TCR is maintained and antigen-specific signaling still occurs, it is likely that these differences are caused by differences

in the delivery of costimuli to the T cells, ever-increasing numbers of which are now being defined. The balance of these, and their function, will determine the consequences of TCR ligation, apparently more so than this antigen-specific "signal one" itself, although this may itself also be changed with aging (Larbi et al. 2011). We have also gathered evidence for the emergence of functional variants within clonal populations, which can be visualized in subcloning experiments. The finding that cumulative PD of the some of the subclones exceeded that of the parental clone suggested the development of negative interactions between cells in these monoclonal populations. As far as we were able to ascertain, such results were not attributable to outgrowth of irradiated filler cells escaping radio blockade, nor to microbiological contamination, and also not to the starting population not being truly monoclonal (Pawelec et al. 1986). The development of these "suppressive" variants remains mysterious, but suggests a hitherto unsuspected degree of T cell functional flexibility. That similar phenomena occur in vivo has been suggested by others (Vukmanovic-Stejic et al. 2006).

6.3.3 Telomere Lengths, DNA Damage Levels, Mitochondrial Damage, miRNA

Telomere lengths as assessed by flow-FISH in early-passage TCC are in the region of 10–12 kb, generally decreasing to below 5 kb at the end of their lifespan, as one would expect for extensively divided cells. Short telomeres may trigger apoptosis, consistent with our findings (Sect. 6.2.4) that despite increased ROS-induced DNA damage it was unlikely that apoptosis was triggered via the p53/p21 pathway. At least according to the results of an assay to analyze accumulated point mutations in mitochondrial DNA, these were not increased in an age-related manner (Ross et al. 2002). Further investigations on TCC DNA damage focused on repair mediated by the so-called mismatch repair system (MMR), the main post-replicative pathway for the correction of replication errors. When we assessed DNA mismatch frequency In TCCs from donors aged 26, 45 or 80 years old we found that unchallenged, dividing TCCs have variable levels of DNA mismatches throughout their lifespan, not affecting proliferation capacity. However, when challenged with acridine mutagen ICR-191 to induce supra-physiological levels of mismatches, deficiencies in MMR were found in most late passage TCCs. We conclude that MMR has an important role in the maintenance of the relative levels of genomic stability in lymphocytes (Annett et al. 2005). These findings on TCC from donors of various ages including very old subjects are consistent with the results of studies examining microsatellite instability at five different loci interspersed in the genome (CD4, VWA31, Tpox, Fes/FPS and p53). In total DNA from TCC, these showed increasing instability accumulating with increasing PD (Neri et al. 2004; Krichevsky et al. 2004). Levels of mismatch repair proteins were shown to be related to the development of microsatellite instability in TCC. Thus, Msh2, Msh3, Msh6, Pms1, and Pms2 protein expression was compromised in TCC showing microsatellite instability (Neri et al. 2008).

We have been interested in examining whether this accumulation of DNA damage in TCCs might contribute to their decreased proliferative capacity and/or increased cell death via apoptosis. The results of our investigations have demonstrated that for TCCs grown in air, an accumulation of oxidative DNA damage occurs during the replicative lifespan vitro, with a sharp rise in damage levels just ahead of the self-deletion of these clones via apoptosis (Hyland et al. 2001). Alongside of this increase in levels of DNA damage we also discovered that DNA excision repair capacity declined with increasing in vitro age in TCCs from younger subjects. Intriguingly, this was in contrast to the DNA excision repair pathways in TCCs from a very healthy ("Senieur") subject, which did not decline with increasing age in vitro, despite the fact that DNA damage levels did increase with increased in vitro age of these TCCs (Annet et al. 2004). Our studies on mutation levels and DNA repair capacity in ex vivo PBMCs from healthy free-living humans have also shown a similar age-related increase in mutation frequency and a decline in DNA repair capacity (Barnett and King 1995).

6.3.4 Changes in Gene Expression

cDNA array technology was employed to investigate differences in gene expression in early, middle and late passage TCC under standard culture conditions. Differentially-expressed genes were confirmed by real-time PCR, and relationships between these assessed using Ingenuity Systems evidence-based association analysis. Several genes and chemokines related to induction of apoptosis and signal transduction pathways regulated by transforming growth factor β (TGFβ), epidermal growth factor (EGF), fos and β-catenin were altered in late compared to early passage cells (Mazzatti et al. 2007b). These pathways and affected genes warrant further investigation as potential biomarkers of aging and senescence. Recently, we have also examined regulatory mechanisms and found a borderline significant increase in miR-21 levels with increasing PD in these TCC (Teteloshvili et al. 2015), which may be interesting because this miRNA targets TGF-βR2 (one of the nodes changed with accumulating PD in the above study by Mazzatti et al. 2007b) and has been implicated as a marker of "inflammageing" in vivo (Olivieri et al. 2012).

6.3.5 Proteomic Changes

Using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) proteins from TCC of different ages were analyzed on ProteinChip arrays. Discriminant analysis identified several protein or peptide peaks that accumulated with culture age. Human profilin-1, a ubiquitous protein associated with actin remodelling and cellular motility, was unambiguously identified as the most prominent potential age-associated factor progressively changing in

these clones. Its altered expression was confirmed by Western blotting (Mazzatti et al. 2007a). Profilin-1 is involved in cellular survival, cytoskeleton remodelling, motility, and proliferation, and is another important candidate for functionally-relevant age-associated changes in these TCC. Profilin 1 seems not yet to have been implicated in other cellular senescence studies, but has been identified as a protein showing increased tyrosine nitration in an age-dependent manner (Starr et al. 2011).

6.4 Conclusions

A wealth of data on human CD4+ T cell clones suggests that many of the progressive changes over their finite lifespans in culture do parallel the differences seen ex vivo in younger and older humans and therefore can be informative for immune ageing. This is important because although age-associated *changes* to immune parameters are often referred to in the literature, in most cases, due to a lack of longitudinal design, these *differences* cannot be formally declared "changes". Human TCC show progressive alterations to cell surface phenotype, functional activity, DNA damage and repair, telomere length, gene expression patterns and the proteome, all of which could be informative to validate changes in immune status in human populations

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Chapter 7
Telomeres Shortening: A Mere Replicometer?

Stella Victorelli and João F. Passos

Abstract Telomeres are protective structures at the ends of linear chromosomes that play an important role in maintaining genomic stability. Telomere shortening, which occurs with each round of cell division, leads to a permanent proliferation arrest, also known as replicative senescence. This process has been shown to have important implications in vivo as an increase in the frequency of senescent cells occurs in mammalian tissue with age and in a variety of age-related diseases. Telomeres possess unique features, namely the presence of telomere-binding proteins collectively known as "shelterin", that equally prevent telomere end-to-end fusions and the repair of damage induced by extrinsic and intrinsic stress. This inability to repair damage contributes to the activation of a persistent DNA damage response, which has been shown to be important in the establishment of cellular senescence. Evidence suggests that telomeres not only limit the proliferative potential of cells, but also act as highly sensitive sensors of stress in cells. This may be a protective mechanism against cancer, but also contribute to tissue dysfunction observed during ageing.

Keywords Replicative senescence • Telomerase • DNA damage • Repair • Mortality • Immortality • Ageing • Anti-ageing • Longevity

7.1 Telomeres and Senescence

7.1.1 Cellular Senescence

The irreversible proliferation arrest of somatic cells, also known as cellular senescence, was first described by Leonard Hayflick in 1961, who showed that cells in vitro were only capable of undergoing a limited number of population doublings, after which they entered a state of terminal arrest (Hayflick and Moorhead 1961).

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This was later suggested to be related to the shortening of telomeres, protective caps present at the ends of linear chromosomes that decrease in length with each cell division, inducing cells to become senescent upon reaching a critical length (Hastie et al. 1990; Harley 1991; Counter et al. 1992; Chang and Harley 1995). Maintenance of telomeres is vital for genomic stability and thus cell viability; therefore, it is not surprising that cells have evolved mechanisms to ensure that these structures remain well preserved. For example, telomere length regulation can be achieved by telomerase, an enzyme that contains reverse transcriptase activity and can elongate the ends of chromosomes with the aid of an RNA template (Blackburn et al. 1989). However, this enzyme is only present in certain cell types such as those of the germline and stem cells, where genomic integrity is presumably most important (Collins and Mitchell 2002). Cells lacking telomerase activity, such as the majority of somatic cells, are subject to telomere attrition with each cell division due to the so-called "end-replication problem", which occurs due to the inability of conventional DNA polymerases to completely replicate the lagging-strand, and was first predicted to occur by Olovnikov and Watson in the early 1970s (Olovnikov 1971; Watson 1972). During the process of lagging-strand synthesis, the DNA replication machinery is required to insert RNA primers from which polymerases can initiate replication, which means that upon removal of the last primer at the 3′ end, the newly synthesised strand is shorter than the template by a few nucleotides. Moreover, the final primer required for replication is not necessarily inserted at the very 3′ end of the parent DNA, further contributing to the loss of telomeric sequences with each round of replication. Indeed, later studies demonstrated experimentally that telomere shortening occurs in human fibroblasts with age, suggesting that attrition of chromosome ends limits the replicative lifespan of proliferating cells (Harley et al. 1990; Hastie et al. 1990; Counter et al. 1992).

The discovery of cellular senescence prompted scientists to question whether this phenomenon was merely a cell culture artefact or did this permanent arrest really have a role in vivo? Indeed, since the Hayflick's discovery, a multitude of reports have shown an age-dependent increase in the frequency of senescent cells in a variety of mammalian tissues, suggesting that senescence may play a role in organismal ageing (van Deursen 2014). More recently, evidence suggests that accumulation of senescent cells with age may be a major contributing factor to the loss of tissue regeneration and function during the ageing process. This was elegantly demonstrated by the Jan van Deursen group who showed that inducible elimination of p16Ink4a-positive senescent cells was able to delay the acquisition of age-related pathologies in the BubR1 progeroid mouse (Baker et al. 2011). By eliminating senescent cells from these mice, the authors were able to not only delay age-related degeneration but also to slow down the progression of already established conditions, suggesting a causal role for cellular senescence in age-associated tissue dysfunction (Baker et al. 2011). Accumulation of senescent cells has been associated with many age-related pathologies such as type 2 diabetes (Sone and Kagawa 2005), atherosclerosis and osteoarthritis (van Deursen 2014).

Consistent with a role for telomeres in the ageing process, telomere shortening is observed in telomerase knock-out mice in a generation-dependent manner (Blasco

et al. 1997), which results in both cell senescence and apoptosis (Lee et al. 1998). These mice not only display a bone phenotype that resembles age-related osteoporosis (Brennan et al. 2014), but accelerated telomere shortening in this model also has been shown to compromise stem cell function, regeneration, organ homeostasis, and decrease lifespan (Wong et al. 2003).

7.1.2 What Are Telomeres?

Telomeres are tandem TTAGGG repeats present at the ends of linear chromosomes, ranging from 5 to 15 kb in humans and can reach up to 100 kb in rodents (O'Sullivan and Karlseder 2010). They contain a C-rich lagging strand and a G-rich leading strand, where the latter comprises 3′ single-stranded nucleotide repeats giving rise to the G-overhang (McElligott and Wellinger 1997; O'Sullivan and Karlseder 2010). Early studies by Griffith et al. suggest that this 3′ -overhang is sequestered into the upstream double-stranded telomeric region and binds to one of the DNA strands, forming a structure known as the telomere-loop (T-loop), which is thought to contribute to chromosome stability by preventing the ends of chromosomes from being recognised as double-strand breaks (DSB) (Griffith et al. 1999; Lin et al. 2014). Telomeres are also associated with a specialised set of proteins termed the shelterin complex, which play extensive roles in controlling molecular signalling from the ends of chromosomes (O'Sullivan and Karlseder 2010). In mammals, this complex is comprised of six main proteins: telomeric repeat binding factor 1 (TRF1), telomeric repeat binding factor 2 (TRF2), TRF2 interacting protein (RAP1), TRF1-interacting nuclear factor 2 (TIN2), adrenocortical dysplasia protein homolog (TPP1, also known as ACD) and protection of telomeres 1 (POT1) (O'Sullivan and Karlseder 2010; Sfeir 2012). TRF1 and TRF2 bind to double-stranded telomeric sequences, whereas the single-stranded G-overhang is coated by POT1 (Zhong et al. 1992; Bianchi et al. 1997; Bilaud et al. 1997; Baumann and Cech 2001) (Fig. 7.1).

Each of these proteins plays essential functions that help maintain telomere homeostasis within the cell. For example, studies have shown that overexpression of TRF1 or TRF2 leads to telomere shortening, thus implicating these shelterin components as negative regulators of telomere length (van Steensel and de Lange 1997; Smogorzewska et al. 2000). Moreover, TRF1 has also been shown to play roles in DNA remodelling as well as being important for efficient replication of telomeres (Bianchi et al. 1997; Griffith et al. 1998; Sfeir et al. 2009). TRF2, on the other hand, is important in telomere protection as it is involved in T-loop assembly by promoting topological changes in DNA that facilitate the formation of such structure (Stansel et al. 2001; Amiard et al. 2007). This protein also inhibits ATM-dependent DNA damage response (DDR) and DNA ligase IV-dependent non-homologous end joining (NHEJ), thus playing a critical role in preventing chromosome end-to-end fusions and chromosomal instability (van Steensel et al. 1998; Smogorzewska et al. 2002; Karlseder et al. 2004; Denchi and de Lange 2007).

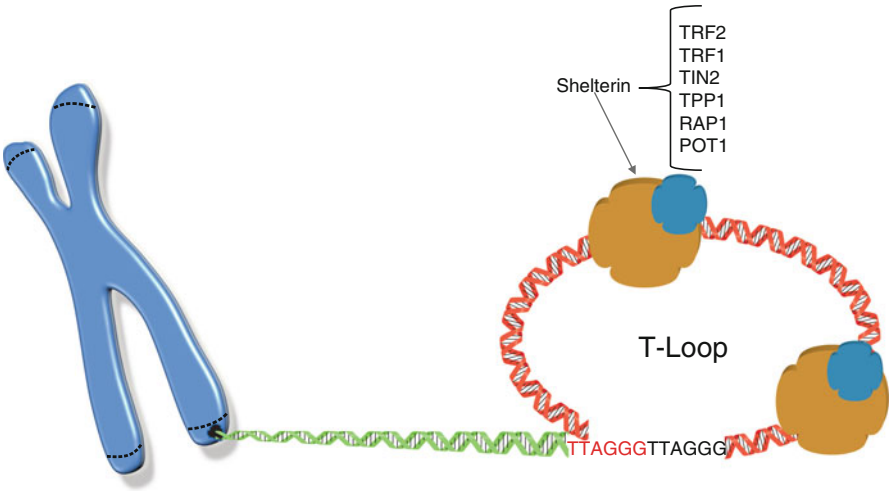

Fig. 7.1 Structure of telomeres. Telomeres are tandem TTAGGG repeats found at the ends of linear chromosomes. The 3′ end is guanidine-rich and consists of single-stranded nucleotide repeats, forming the 3′- or G-overhang, which is thought to contribute to T-loop formation by binding to upstream double-stranded telomeric regions. Telomeres are also associated with a specialized set of proteins called the shelterin complex, which are important for maintaining telomere structure and function. In mammals, this complex is composed of six main proteins, namely TRF1 and TRF2, components that bind to double-stranded telomeric regions, POT1, which coats the single-stranded G-overhang, TIN2, TPP1, and RAP1

In contrast to its homologue in *S. cerevisiae*, mammalian RAP1 lacks the ability to bind to telomeric repeats and relies on interactions with TRF2 for its localisation to telomeres (Li et al. 2000). It has been suggested that TRF2 inhibits NHEJ partly by recruiting RAP1, and the latter has been directly implicated in protecting telomeres from this DNA damage repair mechanism (Bae and Baumann 2007; Sarthy et al. 2009). POT1 contributes to genomic stability by inhibiting ATR-dependent DDR signalling, and it has also been shown to be an important regulator of telomere length through interactions with TRF1 (Loayza and De Lange 2003; Denchi and de Lange 2007). Studies have demonstrated that telomere length is also controlled by TPP1, which recruits telomerase to chromosome ends by directly interacting with this enzyme responsible for telomere elongation (Xin et al. 2007). Moreover, TPP1 directly interacts with POT1, increasing the affinity of the latter for single-stranded telomeric sequences, and it has been shown that a TPP1-POT1 complex increases the processivity of telomerase during the elongation process (Wang et al. 2007; Lu et al. 2013). TIN2 plays a crucial role in the assembly of the shelterin complex by bridging TRF1 and TRF2; furthermore, TPP1 was shown to stabilise this interaction and contribute to the formation of the six-protein complex that is essential for telomere protection (O'Connor et al. 2006). Additionally, TIN2 was demonstrated to be essential in stabilising TPP1/POT1 complexes on single-stranded telomeric

DNA, thus contributing to the effective inhibition of ATR signalling at telomeres (Takai et al. 2011).

These six core components of the shelterin complex also interact with other proteins to form a higher order nucleoprotein complex (Blackburn 2001). For example, protein-protein interactions have been shown to occur between TRF1 and Ku, a protein that plays a role in NHEJ (Hsu et al. 2000). However, the authors showed that at telomeres, Ku performs a capping function, since Ku80-deficient primary mouse embryonic fibroblasts (MEFs) accumulated large amounts of telomere fusions (Hsu et al. 2000). Moreover, recent studies involving genome-wide YFP fluorescence complementation screens have revealed that shelterin proteins associate with over 300 proteins with functions ranging from chromatin organisation to apoptosis and stress response sensors, reiterating the complexity underlying telomere maintenance and signalling (Lee et al. 2011).

7.1.3 The Role of Dysfunctional Telomeres in Replicative Senescence

The idea that telomere shortening is a major driver of cellular senescence was corroborated by studies demonstrating that the lifespan of normal human cells could be extended by expression of telomerase in otherwise telomerase-negative cells (Bodnar et al. 1998). These cells were not only able to undergo a higher number of population doublings but also had reduced β-galactosidase activity, a marker of senescence (Bodnar et al. 1998).

It is believed that telomere attrition may lead to loss of shelterin components, consequently compromising T-loop conformation such that the ends of chromosomes become exposed and are recognised as double-strand breaks by the DNA repair machinery (O'Sullivan and Karlseder 2010). Initial recognition of DNA damage is mediated by factors such as replication factor A (RPA) and replication factor C (RFC)-like complexes, and also by the MRN complex (MRE-RAD50-NBS1). Subsequently, upstream kinases such as ATM in the case of double-strand breaks, become activated, and trigger chromatin modifications in the vicinity of the break, namely the phosphorylation of histone H2A variant H2AX (γH2A.X being the phosphorylated form). This allows the recruitment of other proteins that may function to amplify the signal or are involved in damage repair. Adaptor proteins such as 53BP1, BRCA1, MDC1 and claspin are also involved in this response and are responsible for recruiting the downstream kinases CHK1 and CHK2, which transduce the signal to effector molecules such as p53, inducing a G1 and G2-M phase cell-cycle arrest (Campisi and d'Adda di Fagagna 2007; O'Sullivan and Karlseder 2010). Data supporting the idea that uncapping of telomeres leads to the activation of a DNA damage response (DDR) came from studies where a dominant-negative TRF2 allele was expressed in human fibroblasts, preventing the accumulation of endogenous TRF2 on chromosome ends and thus triggering

uncapping . It was shown that telomeres from these cells became associated with DNA damage response factors such as 53BP1, Mre11 complex, and phosphorylated forms of ATM, H2AX and Rad17, indicating that uncapped telomeres resemble DNA lesions (Takai et al. 2003). Moreover, treatment with PI3 kinase inhibitors that affect ATM was able to reduce the accumulation of 53BP1 at telomeres, showing that DDR proteins play a major role in the cellular response to telomere dysfunction (Takai et al. 2003). Another group showed that conditional deletion of Pot1a in mice caused telomere deprotection and consequently activation of a DDR at telomeres (Wu et al. 2006). This was also accompanied by a senescent phenotype in these cells comparable to replicative senescence and by the occurrence of aberrant homologous recombination (HR) at telomeres, supporting the idea that telomere uncapping may lead to genomic instability and acts through a DDR to induce a permanent cell-cycle arrest (Wu et al. 2006). In fact, during replicative senescence, it has been shown that human fibroblasts accumulate markers of double-strand breaks such as γH2A.X and a range of DDR proteins, including 53BP1, MC1 and NBS1 at telomeric regions (d'Adda di Fagagna et al. 2003).

Ultimately, telomere dysfunction leads to activation of the effector molecule p53, which may trigger cell-cycle arrest or apoptosis (Karlseder et al. 1999; Herbig et al. 2004; Campisi and d'Adda di Fagagna 2007). The transcription factor p53 is a positive regulator of p21 expression, a cyclin-dependent kinase (CDK) inhibitor that initiates growth arrest by inhibiting the action of kinases important for cell cycle progression (Campisi and d'Adda di Fagagna 2007). The importance of the p53 pathway during telomere-induced senescence has been reinforced by studies where deletion of p21 improved the regenerative capacity of intestinal crypts and hematopoietic stem cells in late-generation telomerase-deficient mice, which contain dysfunctional telomeres (Choudhury et al. 2007). Moreover, deletion of Chk2, a protein which has been shown to stabilise p53, in mice with Trf1 deficiency was shown to rescue survival and the frequency and severity of epithelial pathologies associated with telomere dysfunction (Garcia-Beccaria et al. 2014). A rescue in proliferation in the skin associated with a decrease in p53 and p21 induction was also observed (Garcia-Beccaria et al. 2014). Although the p16-pRb pathway is also important in establishing and maintaining senescence, studies have provided conflicting data on its involvement in telomere-induced senescence. It has been shown that p16 can be activated independently of telomere dysfunction in human fibroblasts (Herbig et al. 2004). However, another study demonstrated that deletion of p16 in Wrn-deficient mice, which lack a protein needed for efficient telomere replication and thus have dysfunctional telomeres, rescued proliferation of mouse embryonic fibroblasts (MEFs) when compared to Wrn-deficient MEFs (Zhang et al. 2012). Moreover, loss of p16 function either alone or in Wrn-deficient MEFs prevented these cells from undergoing senescence in response to telomere damage, implicating p16 as an important contributor to growth arrest in the presence of telomere dysfunction (Zhang et al. 2012). Induction of p16 in response to telomere damage has also been observed upon expression of a dominant negative allele of TRF2 (Jacobs and de Lange 2004). Although p16 deficiency in these cells only partially rescued growth arrest induced by telomere damage, proliferation

was almost completely restored when both p16 and p53 were inhibited, suggesting that p16 may act as a second barrier to inhibit entry into the next cell cycle following damage in chromosome ends (Jacobs and de Lange 2004). In accordance to this, another study showed that growth arrest induced by telomere dysfunction is primarily maintained by p53 and can be rescued by inhibition of this transcription factor (Beausejour et al. 2003). However, p16 was shown to provide a second barrier to cell cycle progression in some human cells, which could not be reversed even upon pRB inactivation (Beausejour et al. 2003). These studies reinforce the importance of the p53 and p16 pathways in senescence and also allude to the fact that the extent of the contribution of each pathway to the senescent phenotype may vary amongst different cell types.

Indeed, several studies have indicated that telomere-induced senescence may play an important role during organismal ageing. Telomere dysfunction, measured by the association of DDR proteins with telomeres, has been show to increase with age in vivo in the skin of primates (Herbig et al. 2006; Jeyapalan et al. 2007) and in liver and gut of mice (Hewitt et al. 2012). Moreover, it has been shown that reintroducing telomerase activity in telomerase-deficient mice was able to revert premature ageing phenotypes in tissues such as spleen, intestine and testes, suggesting that telomere attrition is an important factor affecting decline of organ function with age (Jaskelioff et al. 2011). Telomere dysfunction is also related to human diseases such as dyskeratosis congenita (DC). This condition is associated with mutations in the genes encoding telomerase components, and fibroblasts and keratinocytes derived from DC patients have very short telomeres (Buckingham and Klingelhutz 2011). DC is accompanied by a range of epidermal defects such as poor nail growth, early hair loss and skin atrophy, suggesting that telomere length maintenance is essential for epidermal homeostasis (Buckingham and Klingelhutz 2011).

7.1.4 Stress and Telomere Shortening

Although telomere attrition can occur as a result of cellular replication, other factors can influence the rate at which telomeres shorten. Indeed, many studies have shown that mild oxidative stress causes telomere shortening and reduces the replicative lifespan of cells, resulting in a phenotype similar to replicative senescence (von Zglinicki et al. 1995; Xu et al. 2000; von Zglinicki 2002; Saretzki et al. 2003). The role of reactive oxygen species (ROS) in telomere-dependent senescence has been reinforced by studies where human fibroblasts overexpressing the antioxidant enzyme superoxide dismutase (SOD3) or treated with antioxidants showed reduced levels of intracellular peroxide, a slower rate of telomere shortening and prolonged replicative lifespan (von Zglinicki et al. 2000; Serra et al. 2003). In fact, a direct relationship between telomere shortening and the levels of cellular oxidative stress has been previously proposed following observations that cells with a low antioxidant capacity and shorter lifespan displayed a higher rate of

telomere shortening than strains with a higher antioxidant capacity (Richter and von Zglinicki 2007). The involvement of ROS in telomere dysfunction is not only limited to in vitro studies. Indeed, oxidative stress has been suggested to mediate demyelination and axonal damage in multiple sclerosis (MS) patients (Gilgun-Sherki et al. 2004), who have been recently reported to have increased markers of oxidative stress and shorter telomeres when compared to healthy controls (Guan et al. 2015). Although it is difficult to delineate the major source of ROS responsible for telomere attrition, studies have suggested that oxidative stress originating from mitochondria is an important contributor to telomere shortening. For example, when fibroblasts were treated with MitoQ, an antioxidant which is targeted directly to the mitochondria, the rate of telomere shortening was significantly reduced, and this was also sufficient to extend replicative lifespan under conditions of hyperoxia (Saretzki et al. 2003). The importance of mitochondrial-derived ROS has also been reiterated by studies showing that mild uncoupling of mitochondria, which reduces the accumulation of superoxide in the latter, extended the lifespan of human fibroblasts and also decreased the rate of telomere shortening in these cells (Passos et al. 2007). Moreover, inducing mitochondrial dysfunction by severe depolarization using FCCP was shown to increase ROS production and lead to telomere attrition, telomere loss and chromosome fusions in mouse embryos (Liu et al. 2002). The suggestion that mitochondrial dysfunction can accelerate telomere shortening is further supported by studies showing that patients with mitochondrial diseases such as MELAS and LHON, which are characterised by respiratory chain dysfunction, contain on average shorter telomeres in white blood cells when compared to healthy subjects (Oexle and Zwirner 1997). Importantly, increased ROS production as a result of mitochondrial dysfunction has been reported in many age-related disorders such as Alzheimer's and cardiovascular disease (Dai et al. 2014), both of which have also been associated with telomere shortening (Fyhrquist and Saijonmaa 2012; Cai et al. 2013).

Telomeres are thought to be particularly susceptible to oxidative stress relative to the rest of the genome due to their high content of guanine triplets, which are highly sensitive to oxidative modifications (Oikawa and Kawanishi 1999). In fact, mild oxidative stress has been shown to cause single-stranded breaks to preferentially accumulate at telomeres, and it has been suggested that such lesions contribute to accelerated telomere shortening by stalling the replication fork, leading to incomplete replication of chromosome ends (Petersen et al. 1998; von Zglinicki 2000) (Fig. 7.2). Further studies have also shown that oxidative damage at telomeres disrupts the binding of TRF1 and TRF2, possibly offering another mechanism by which oxidative stress may contribute to telomere dysfunction (Opresko et al. 2005). However, a more recent report has provided evidence that stress-induced telomere damage can still occur in the presence of TRF2, suggesting that telomere dysfunction may also occur via alternative mechanisms other than loss of shelterin components (Fumagalli et al. 2012). In addition, telomeric damage has been shown to be less efficiently repaired when compared to non-telomeric lesions, a characteristic mainly owed to the presence of shelterin proteins (Kruk et al. 1995). Components such as TRF2 and RAP1 have been shown to prevent

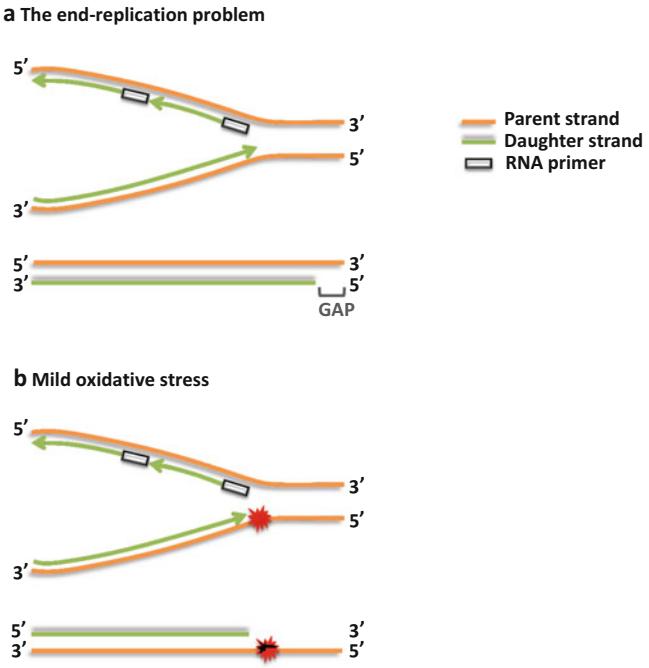

Fig. 7.2 Mechanisms that contribute to telomere shortening. **a** The end replication problem. Replication of the lagging-strand (*top strand*) occurs in short fragments, known as Okazaki fragments, each starting from a different RNA primer (shown in *grey*). When DNA replication is complete, the primers are removed, and the gaps between Okazaki fragments are filled; however, the gap left from the removal of the primer at the most distal position cannot be filled, leading to shortening of the newly-synthesised strand (*bottom*). **b** Mild oxidative stress. Oxidative damage has been shown to preferentially accumulate at telomeres. It is suggested that damage occurring in telomeric regions (*bottom*) causes stalling of the DNA polymerase on that strand, whilst not necessarily affecting replication of the opposite strand. This may cause the replication fork to collapse before replication is fully completed, resulting in a shorter daughter strand

NHEJ at telomeres by inhibiting DNA-PK, a double-stranded break repair complex protein, and also by preventing ligase-IV-mediated end joining (Bombarde et al. 2010). In accordance to this, inducing DNA double-stranded breaks adjacent to telomeric repeats in budding yeast results in impaired recruitment of ligase IV to the site of damage (Fumagalli et al. 2012). Moreover, expression of TRF2 in the vicinity of a DSB in mammalian cells leads to persistent DNA damage, reinforcing the irreparability of telomeres (Fumagalli et al. 2012). ROS are not only involved in the induction of senescence via telomere dysfunction, but also are believed to act as an effector mechanism during senescence. Our group has shown that mitochondrial ROS can be induced downstream of a DNA damage response and is partially dependent on the p53 and p21 pathways (Passos et al. 2010). Consistently, mice with dysfunctional telomeres (late generation $TERC^{-/-}$ mice) show increased markers of oxidative damage in tissues which can be significantly decreased by deletion of p21

(Passos et al. 2010). Moreover, a separate study has shown that short telomeres activate p53 which in turn binds and represses PGC-1α and PGC-1β promoters inducing mitochondrial dysfunction (Sahin et al. 2011). Evidence suggests that ROS emanating from dysfunctional mitochondria may contribute to the stabilisation of the senescent phenotype by further increasing DNA damage and maintaining the DDR (Passos et al. 2010).

Recently, studies have pointed towards an association between psychological stress and reduced telomere length. It was shown that both chronic stress, such as that experienced by mothers caring for critically ill children, and perceived stress were linked to shorter telomeres, decreased telomerase activity and increased oxidative stress in leukocytes of healthy women (Epel et al. 2004). A relationship between chronic psychological stress and reduced telomere length has also been observed in caregivers of Alzheimer's disease patients who also displayed higher levels of depressive symptoms when compared to controls (Damjanovic et al. 2007). Furthermore, many reports have now suggested that early life stresses may have an impact on the rate of telomere shortening. Indeed, it has been shown that adults with a history of childhood maltreatment, including physical and emotional neglect, had shorter telomeres in leukocytes when compared to participants who did not report maltreatment (Tyrka et al. 2010). Further support is given by a recent longitudinal study where children exposed to two or more types of violence displayed accelerated rate of telomere shortening in buccal cells between the ages of 5 and 10 years (Shalev et al. 2013). These associations raise questions about how early life stresses that affect telomere length may be related to the risk of disease development in later life. Although further research is necessary to establish the mechanisms involved, one possibility is that inflammation may mediate the relationship between early life stresses and telomere shortening (Shalev 2012). Whilst the link between stress and increased inflammation is not mechanistically clear, studies have shown that a history of childhood stress is associated with enhanced inflammation in adulthood (Danese et al. 2007). Indeed, increased inflammation has been linked to the development of conditions such as cardiovascular diseases (Pearson et al. 2003) and depression (Dantzer et al. 2008). Moreover, inflammation has been shown to stimulate proliferation of immune cells, thus leading to accelerated telomere shortening in circulating leukocytes (Goronzy et al. 2006). Consistent with a link between inflammation and telomere damage, chronic inflammation in mice has been shown to lead to telomere dysfunction in a variety of tissues, such as gut and liver, and also impair tissue regenerative capacity, a phenotype ameliorated by anti-inflammatory ibuprofen treatment (Jurk et al. 2014). Another plausible mediator may be cortisol, the main stress hormone. It has been shown that cortisol increases the susceptibility of neurons to oxidative stress (Behl et al. 1997), and it also reduces telomerase activity in human T lymphocytes (Choi et al. 2008). Therefore, it is possible that increased oxidative stress and decreased telomerase activity triggered by stress-induced cortisol release may contribute to accelerated telomere shortening, although further studies are necessary to confirm these associations (Shalev 2012).

Telomere shortening has also been associated with increased risk of morbidity and mortality. For example, a recent study has suggested that longer telomeres

in early life strongly correlates with a longer lifespan in birds (Heidinger et al. 2012). A relationship between decreased telomere length and higher mortality risk has also been observed in humans, where individuals over 60 years old who had shorter telomeres in blood cells were more likely to die from conditions such as heart disease and infectious diseases (Cawthon et al. 2003). Following these observations, researchers in the field began to question whether telomere length may be used as a good biomarker of ageing, in other words, can telomeres be used as diagnostic tools to predict lifespan and age-related diseases? This hypothesis is contradicted by other reports, such as a study involving a large cohort of healthy participants that failed to identify any correlations between telomere length and lifespan or increased mortality risk, although they showed that individuals with longer telomeres generally had more years of healthy life (Njajou et al. 2009). These disparities can be partly explained by issues involved in different methods used to measure telomere length by different laboratories (Martin-Ruiz et al. 2014). A recent study revealed a large inter-laboratory variation of telomere length estimates (Martin-Ruiz et al. 2014), challenging the possibility of using telomere length measurements as a prognostic tool (von Zglinicki 2012). Moreover, normative reference values for telomere length are not available, limiting its use as a predictor of morbidity and mortality (von Zglinicki 2012).

7.2 Persistent DNA Damage Is Important for Stabilising Senescence

Normally, DNA damage acquired during the lifespan of a cell becomes repaired relatively rapidly; however, research now indicates that telomeric lesions persist for several months both in vitro and in vivo (Fumagalli et al. 2012; Hewitt et al. 2012). In fact, live-cell imaging experiments have shown that in stress-induced senescence, the majority of long-lived foci are associated with telomeres (Hewitt et al. 2012). Moreover, persistent DDR signalling at telomeres has also been observed in vivo in mouse hippocampal neurons even 3 months after exposure to genotoxic stress (Fumagalli et al. 2012). Telomere dysfunction has also been shown to be important in oncogene-induced senescence and play a role in preventing human cancer progression. Recent evidence indicates that oncogene activation in human cells leads to persistent telomeric DDR foci, stalling at telomeric replication forks and DNA replication stress (Suram et al. 2012). Stable growth arrest caused by oncogene activation was not achieved in cells overexpressing telomerase, which prevents telomeric DDR foci formation in response to oncogene-induced replication stress, indicating that telomere dysfunction is important to reinforce oncogene-induced senescence (Suram et al. 2012). Moreover, cancer precursor lesions, such as human melanocytic nevi, ductal breast hyperplasias, and colonic adenomas display features of telomere dysfunction-induced senescence, such as the accumulation of damage at telomeres, suggesting that this is also important in vivo as a tumour-suppressor mechanism. It has been suggested that oncogene activation causes

cells to undergo replication stress, leading to telomere erosion and dysfunction in telomerase-negative cells. This in turn induces senescence in normal somatic cells, and prevents formation of malignant cancers (Suram et al. 2012).

Given that activation of a DDR at telomeres is a key player in the initiation of replicative senescence, and that persistent telomeric DNA damage is observed in both stress- and oncogene-induced senescence, it has been proposed that persistent DDR signalling at unrepaired telomeres may be a major contributor to the establishment of senescence (Fumagalli et al. 2012). However, non-telomeric damage has also been shown to play a role in the senescent phenotype. In replicative and stress-induced senescence, half of the DNA damage foci have been shown to be located in non-telomeric regions, and although these are short-lived, they are constantly renewed during senescence (Passos et al. 2010; Hewitt et al. 2012). This occurs possibly as a result of increased ROS production during senescence, as inhibition of ROS has been shown to rescue the proliferation arrest in a proportion of cells (Passos et al. 2010). Therefore, DNA damage signalling occurring at both telomeric and non-telomeric regions plays important roles in the senescent phenotype, although distinguishing how each of these contributes to senescence may prove experimentally difficult.

7.3 Dysfunction in Telomeres Irrespective of Length

So far, emphasis of numerous studies has been given to senescence induced by telomere shortening. However, recent amounting evidence now indicates that telomere dysfunction can also occur independently of shortening (Fig. 7.3). Studies have reported that genotoxic stress leads to persistent DDR signalling at telomeres irrespectively of length in human fibroblasts in vitro and in mouse neurons in vivo (Fumagalli et al. 2012). DNA damage proteins have also been shown to associate with longer telomeres in vivo during the ageing process. Early observations showing that mouse embryonic fibroblasts could replicate indefinitely when cultured at low oxygen conditions, coupled with the fact that mice have extremely long telomeres in comparison to humans and express telomerase ubiquitously, led to the belief murine cell senescence occurred in a telomere-independent manner, and was generally mediated by oxidative damage (Parrinello et al. 2003). However, more recently, evidence suggests that this is not the case, as an age-dependent increase in telomeric DNA damage has been shown in the gut and liver of mice, which occurred independently of length (Hewitt et al. 2012; Jurk et al. 2014). Length-independent telomere dysfunction has also been reported in hippocampal neurons and liver of baboons with age, suggesting that this possibly contributes to tissue dysfunction during ageing by leading to cellular senescence (Fumagalli et al. 2012). Furthermore, in benign lesions such as melanocytic nevi, telomeres signalling a DDR were not shorter compared to functional ones, showing that this phenomenon is also a feature of oncogene-induced senescence (Suram et al. 2012). In fact, the authors showed that a fraction of dysfunctional telomeres still

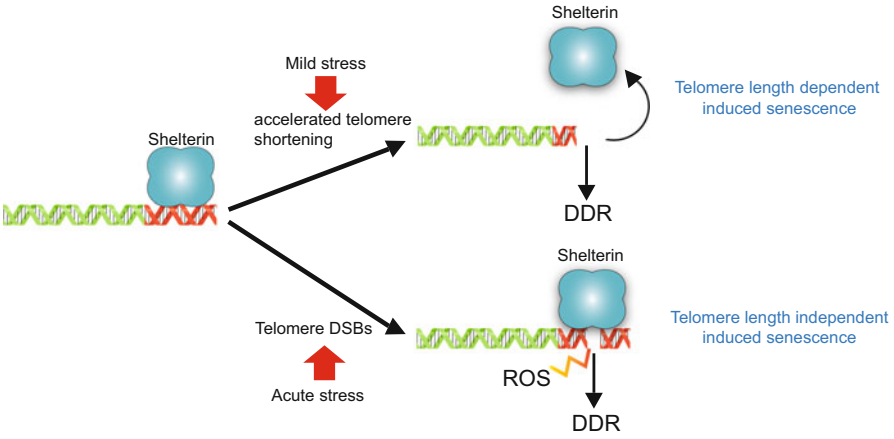

Fig. 7.3 Telomeres as sensors of stress. Recent evidence suggests that telomeres play an intricate role within cells, not only limiting their replicative lifespan by "counting" the number of cell divisions, but they may also serve as sensors of both extrinsic and intrinsic sources of stress. It is possible that cells that acquire telomere damage (*double and single-stranded breaks*) caused from mild stress are still able to replicate; however, this may result in accelerated telomere shortening, leading cells to a premature proliferative arrest, or "telomere length dependent induced senescence". However, acute stresses may result in double-strand breaks occurring at telomere regions which cannot be efficiently repaired by cells, preventing them from undergoing any further rounds of replication regardless of telomere length status (as shown in Hewitt et al. 2012; Fumagalli et al. 2012), which can also be termed "telomere length independent induced senescence"

retained TRF2, suggesting that a DDR may be activated due to factors other than shortening and uncapping (i.e. loss of shelterin proteins) (Suram et al. 2012). In support of this, dysfunction independent of telomere length has also been reported in cells undergoing replicative senescence, where some DDR-positive telomeres were still associated with TRF2 and RAP1, suggesting that displacement of shelterin components does not necessarily precede DDR activation at telomeres (Kaul et al. 2012).

It has been suggested that long telomeres represent a more abundant target for DNA damage to occur, making them more susceptible to damage and explaining the occurrence of DDR activation independently of length in replicative-, stress-, and oncogene-induced senescence (Fumagalli et al. 2012). However, so far evidence does not unequivocally indicate that longer telomeres are particularly susceptible to damage when compared to shorter ones. In fact, analysis of individual telomere length in melanocytic nevi and in mice revealed no significant length difference between damaged and undamaged telomeres (Hewitt et al. 2012; Suram et al. 2012). It is possible that the current methods to analyse individual telomeres in tissues may not be good enough to detect the shortest of telomeres, masking any significant differences in length that may exist between dysfunctional and functional telomeres.

In order to explain how telomeres can signal a DDR in the presence of shelterin components Roger Reddel's lab has proposed that telomeres may exist in three

different states. Firstly, telomeres may maintain an intact t-loop conformation in a length-dependent manner, thus preventing DDR activation, and inhibiting NHEJ through the presence of shelterin proteins. This is also known as the fully capped or closed state. However, if telomeres lose the T-loop conformation they may adopt an intermediate state, where a DDR is activated but shelterin proteins such as TRF2 are maintained at sufficient levels such that prevention of end-to-end fusions still occurs. This may occur in a length-dependent and – independent manner, possibly offering an explanation to the occurrence of damage at telomeres that are not critically short and still contain shelterin proteins. Lastly, the fully uncapped state occurs when extreme telomere erosion leads to loss of a protective structure, activating a DDR. Extensive loss of telomeric repeats also means that shelterin components can no longer bind to chromosome ends and inhibition of NHEJ is compromised, causing end-to-end fusions to occur (Cesare et al. 2009). Based on recent data from our lab and others we propose that telomeres may also exist in a fourth state. Telomeres may still maintain the T-loop conformation and shelterin components, but activate a persistent DDR within telomere repeats.

Overall, it appears that telomeres play a much more intricate role in modulating DNA damage responses in senescence, and do not only simply act as a replicometers, limiting the number of DNA replication cycles cells are capable of undergoing. Instead, evidence suggests that telomeres act as sensors of stress and damage, and prevent genomic instability from occurring by inhibiting division of cells that have accumulated genomic damage.

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Chapter 8
Modeling Cellular Aging: An Introduction – Mathematical and Computational Approaches

Tarynn M. Witten

Abstract In this chapter we examine a variety of modeling approaches that have been historically used to understand the sub-cellular and cellular biology of aging. We find that there are a large array of methods from discrete to continuous and from deterministic to stochastic. This chapter is not meant to be a comprehensive coverage of all of the modeling efforts but rather a buffet introduction to what has been done in the field over the last 50–60 years.

Keywords Replicative senescence • Cell cycle • Serial passaging • Mathematics • Modelling • Repair • Longevity • Cancer

8.1 Models of Cellular and Subcellular Aging

> "Each particular discipline contains only as much science as it contains mathematics... Immanual Kant, in Metaphysical Foundations Of Science"

Living systems are ubiquitous. Moreover, most of them are so complex that it is difficult to understand their behavior. Experimental science works from the obvious historical perspective of reductionism. Break the organism apart and hope (1) that you can understand how the pieces work and (2) that if you are lucky, you can put the pieces back together again and perhaps understand more about how the whole organism works. However, sometimes it is impossible to test an experimental hypothesis. Perhaps the equipment doesn't exist or it's too expensive. Sometimes we just don't quite know how the pieces should fit back together and we would like to examine a number of different hypotheses. We might want to determine the most important genes in a very large known network. However, knocking them out – one by one – would be time-consuming and expensive. One way to handle problems of such a complex nature is to make use of mathematical and computational

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models. The literature in mathematical modeling and computer simulation, like living systems, is also ubiquitous. New books and papers appear regularly. It is beyond the scope of this short chapter to cover this literature. Some excellent starting texts can be found in references (Hannon and Ruth 1997; Murray 1989; Meerschaert 1993; Jacquez 1985; Godfrey 1983; Tautu 1990; Goel and Richter-Dyn 1974).

8.1.1 Thinking About, Building and Analyzing Mathematical Models and Computer Simulations

Modeling in aging has been around for as long as demography and survival theory have been disciplines; certainly since the famous Benjamin Gompertz and subsequent mortality theorists (Carnes et al. 2006). Circa the 1960s, mathematical and computational methods began to be applied to other areas of aging dynamics. Brain/body-mass and metabolic rate vs. maximum lifespan relationships (Cutler 1982) were two of the earliest of the non-demographic applications of simple mathematical modeling. In complexity theory, these are called scaling relationships (West and Bergman 2009). More recently, there has been a growing interest in graph theoretic/network methods to understand how longevity-related genes and proteins are linked together in networks and how those networks behave (Witten 2014; Wimble and Witten 2014). Graph theoretic methods lead to systems biological approaches that apply differential equation modeling (Jones and Sleeman 1983; Dalle Pezze et al. 2014) and simulation to various pathways in order to better understand their dynamics (Glass 1975). Today, mathematical modeling and computer simulation have been applied to a far larger variety of aging-related problems across all scales of the aging hierarchy, from molecular through population levels.

One area of great interest, in part because of the early ability to make experimental measurements and now to be able to obtain additional data through the use of "omic" methods, is the modeling of sub-cellular, single cell and cell population dynamics. In this chapter we will focus on applications of aging-related modeling in these areas.

8.1.2 Constructing a Model

Mathematical and computer modeling might be seen as the comprehensive processes of representing real world phenomena in terms of mathematical equations or computer equations and subsequently extracting from those frameworks potentially useful information that could further the understanding of the system of interest. The process of mathematical and computer modeling does not have a specific set of rules. It is still as much of an art as it is a science. This isn't to say that there are

not useful guiding principles and approaches to constructing models. An excellent discussion of the process of constructing a mathematical/computer representation of a living system is presented in Kirkwood et al. (2006).

Thinking About the Time Variable

Time, one of the major means by which aging is measured, discussed and by which dynamical systems are studied, is fundamental to any analysis of aging processes. The following citations address the concept of time from various perspectives (Denbigh 1981; Featherman and Peterson 1986; Witten 1984; Vrobel 2011). When we consider modeling cellular aging, we are typically constructing some sort of relationship between a collection of dependent variables such as $N(t)$ the number of individuals at time t or the amount of waste $w(t)$ in a cell at time t. Time is usually expressed as a continuous variable t or as a discrete variable t_n where the subscript indicates the nth timepoint. The timepoints t_n could be the population doubling times or the generation number of yeast buds. It is also possible to describe time t as a random or stochastic variable (Witten 1984, 1994) such as time to failure of a pathway or intermitotic times and even as a fractal (Vrobel 2011). In this chapter I will focus on just discrete and continuous time models of aging.

Thinking About the Dependent Variable(s)

For the most part, a dependent variable will be a variable of interest that depends upon time or age in some way. It will also take on either a discrete, continuous or probabilistic/stochastic demeanor depending upon what you choose to describe. For example, a simple model of cell growth in an unlimited resource could be modeled by a continuous time – continuous dependent variable differential equation of the form

$$\frac{dN(t)}{dt} = bN(t) \tag{8.1}$$

$$N(0) = N_0 \tag{8.2}$$

where $N(t)$ is the number of individuals at time t, b is the per capita net growth rate, and N_0 is the initial population size. We could choose to model the same system using a discrete time – continuous independent variable model as follows

$$N_{n+1} = bN_n \tag{8.3}$$

$$N_0 = \textit{given as initial information} \tag{8.4}$$

where N_n is the number of cells at population doubling point n, b is the per capita net doubling rate, and N_0 is a known initial amount of cells in the population. We can

also have models where one variable, say time t is continuous and the other variable, say $R(t)$ is discrete. For example, we might like to look at how many receptor sites are occupied on a cell at time t. Here, clearly t is continuous but the number of receptor sites $R(t)$ is discrete.

Alternatively, we might also ask for the probability that there are N divisions in a given time interval. We choose to examine probabilities because we recognize that if we were to repeat the experiment over and over, we would not get the same exact numbers of doublings at the timepoints that we measure. Defining $P_N(t)$ to be the probability that there will be N doublings at a given timepoint, it can be shown that the doubling equation (under certain assumptions of course) can be expressed as follows

$$\frac{dP_N(t)}{dt} = bP_{N-1}(t) - bP_N(t) \tag{8.5}$$

$$P_N(0) = 0 \tag{8.6}$$

which is an equation that describes a continuous time, discrete state (N), continuous random variable $P_N(t)$ model. The solution to Eq. (8.5) is the Poisson function $P_N(t)$ given by Eq. (8.7)

$$P_N(t) = \frac{(bt)^N e^{-bt}}{N!} \tag{8.7}$$

All of these models are deterministic models because they have no randomness in them. We could, however, easily introduce randomness in a number of ways. For example, randomness may be directly included as a *noise* term in our model system. For example, we don't expect the net reproductive rate to be exactly the same at each timepoint t, so we may then suppose that the growth rate b in Eq. (8.1) was not constant but rather varied about some mean value b_0, we might assume $\omega(t)$ where $\omega(t)$ was a mean zero variance σ^2 noise process. Under this assumption, we would re-express Eq. (8.1) as follows

$$\frac{dN(t)}{dt} = b_0 N(t) - \omega(t)N(t) \tag{8.8}$$

$$N(0) = N_0 \tag{8.9}$$

In this case, while we wish to solve for $N(t)$ and we have expressed our equation in that form, it is not possible to solve for $N(t)$ exactly. Instead, we must solve for a probability of a particular value of $N(t)$ at time t. Consequently, $N(t)$ is a stochastic variable (Witten 1994). Obviously, we can do the same type of alteration in our discrete model as well. It should be noted that one has to be more careful about constructing discrete time models because they can exhibit behaviors that differential equation models cannot. Consider the following two very simple differential equation models for cellular growth in a limited food environment (density-dependent cell growth). Model 1, the continuous model would be expressed as

$$\frac{dN(t)}{dt} = bN(t)\left[1 - \frac{N(t)}{K}\right] \tag{8.10}$$

$$N(0) = N_0 \tag{8.11}$$

where K is the maximum number of cells that the food resource can support. Similarly, the discrete model would look as follows

$$N_{n+1} = bN_n\left[1 - \frac{N_n}{K}\right] \tag{8.12}$$

$$N_0 = \textit{given as initial information} \tag{8.13}$$

It can be shown that there is no possible way that Eq. (8.10) can oscillate. However, Eq. (8.12) can. Consequently, part of the modeling process is understanding the types of behaviors that the experimental system can exhibit and making sure that those dynamics are demonstrated by the model. Once that is done, then we can examine any unknown behaviors displayed by the model and see if they exist in the experimental system.

More Than One Dependent Variable

Obviously, biological systems are more complex than one independent variable. For example, if we were interested in the total amount of waste in a cell as a function of time, we would be interested in a set of equations; one that describes the waste rate of change and the other that describes the number of cells at a given time. This would be easily described by a system of differential equations as discussed in Hirsch (1978, 1986) and Hirsch et al. (1989). We call this a system of coupled differential equations because the dependent variable of one equation can be found in the other equation or equations. For example, Zheng (1991) discusses a mathematical model that describes the proliferative senescence of cells in a cell culture. The model is based upon the DNA damage hypothesis of cellular aging and is able to account for both the limited and unlimited in vitro proliferative potential of normal and transformed cells. In this model the author uses a system of coupled differential equations (a matrix differential equation system) that describe the transition of a cell population vector through the cell cycle.

More Than One Independent Variable

The dynamics of growing systems of cells (and of people) has been of interest for decades and many mathematical models have been created to describe various aspects of such systems. One of the most famous of these is the Volterra-Lotka equation describing $n(t, a)$ the number of organisms (in this case it was originally people), having age a at time t in the population. Demographers of aging have been using this system for over 100 years (Carnes et al. 2006). I will not go through the

derivation of the equation system. One of the first researchers to use this system as a means of explaining cellular dynamics was Trucco (1965a,b,c). Instead, I will show the Von Foerster system and discuss its general meaning. The system is given by

$$\frac{\partial n(t,a)}{\partial t} + \frac{\partial n(t,a)}{\partial a} = -\mu(t,a,\ldots)n(t,a) \tag{8.14}$$

$$n(t,0) = \int_0^\infty \lambda(t,a,\ldots)n(t,a)da \tag{8.15}$$

$$n(0,a) = n_0(a) \tag{8.16}$$

where $n(t,a)$ is the number (or density) of individuals of age a at time t, $\mu(t,a,\ldots)$ is the per capita mortality rate, $\lambda(t,a,\ldots)$ is the per capita birth rate, and $n_0(a)$ is the initial population distribution and is a given. This type of equation is called a partial differential equation because it has partial derivatives instead of the derivatives you learned in Calculus 1 and we note that there is a derivative related, in the same equation, to each of the independent variables a and t. Demographers have been using this form of equation for decades. The first equation (the partial differential part) describes how individuals of age a at time t exit the population through death; note the mortality rate term on the right hand side of the equation. The second equation describes how newborns arrive in the population. It calculates the total number of newborns by integrating over the whole age distribution in the population. Obviously, this isn't entirely realistic, but its a start. And one thing about modeling is that you have to start very simple otherwise it is very easy to get lost in the model and never really be able to come up with believable results. The third equation simply describes the starting distribution; how many individuals there were of age a at time $t = 0$. Again, note that this is a deterministic equation system as there is no randomness in the model. However, you can include it. But that makes it very hard to solve. As you probably thought while you were reading this, these must be impossible to solve unless you are a brilliant mathematician. Well, not quite but they are hard and require some serious mathematical background if the modeler is going to go this route. A recent application of equation system (8.14) may be found in Stukalin et al. (2013).

The problem with the previous modeling formulation is that both age a and time t are related to each other in demographic models. This is problematic for cellular models because chronological age of a cell is not necessarily related to biological age. Rubinow (1968) and Lebowitz and Rubinow (1974), in a now classic series of articles, introduced the maturity μ of a cell as a possible variable of interest. The Rubinow model is generally difficult to implement in that it is challenging, if not impossible, to assign a biological meaning to the *cellular maturity* of a cell, it has allowed for the introduction of the concept of maturation or change in a *cellular variable*. The partial differential equation portion takes on the form

$$\frac{\partial n}{\partial t} + \frac{\partial (vn)}{\partial \mu} = -\phi(\ldots)n(\mu,t) \tag{8.17}$$

where v is the individual cellular growth rate, ϕ represents the death rate and μ is the maturity variable. Many authors have looked at a variety of formulations of this basic model.

The sum and substance of our discussion is that mathematical and computer simulation models come in a wide variety of types and there is not necessarily a correct or unique approach for modeling or simulating a given physical system. So how then do we go about the actual modeling effort given all of these ambiguities and philosophical difficulties? Start with a simple model and see what kind of behaviors you get. Interact with the experimentalists regularly and read the literature. Understand and be able to explain the limits of your model. Identify the most important dependent and independent variables first. Draw lots of pictures describing the known experimental results. Now we can talk about some of the modeling.

8.2 Senescence at the Sub-cellular and the Single Cellular Levels

As technological progress invades the biosciences, scientific innovation allows us to ask increasingly more precise and detailed questions about the complex workings of cells. These rapid technological advances have made it possible to address issues of aging at the molecular level.

As early as 1967, Strehler addressed the issue of irreplaceable components; components which allowed "the adult organism to persist only as long as the irreplaceable elements continued to function in a manner commensurate with life." He related these elements to molecular/genetic mechanisms in living systems. These issues were further addressed in Strehler and Freeman (1980) and Strehler (1986). We cannot hope to address the myriad of molecular aging theories, their experimental validity, and their mathematical treatment. In this chapter, we will address only some of the historical hypotheses that had mathematical or computational models associated with them.

- **Somatic Mutation Theory**: Aging is due to changes (of various types) in the DNA of somatic cells.
- **Error Catastrophe Theory**: Aging is due to a progressive accumulation of DNA errors leading to protein errors, etc.
- **Differentiation Theory**: Aging is due to changes in gene regulation which control differentiation. Altered gene expression might subsequently lead to aging processes.
- **Mitochondrial Mutation Theory**: Aging results from the accumulation of mutations in the mitochondrial genome.
- **Codon Restriction Theory**: Aging is a result of increasing inability to decode the genetic material.

- **General Theories**: Aging is the result of the gradual *wear and tear* of subcellular components or of the **accumulation and/or depletion** of some necessary material, component, product, or operation.

8.2.1 Mathematics of Somatic Mutations

In an excellent review paper on the subject, Hirsch (1978) points out that somatic mutations have been a major focus of aging research for a number of years. The initial arguments for a somatic mutation theory of aging grew out of the view that aging in mammals was due to a gradual accumulation of somatic mutations; mutations due to radiation in the environment. The theory proposes that aging is due to additions to, losses from, or other changes in the DNA base sequence of somatic cells.

The mathematical modeling methods used in somatic mutation models hinge upon concepts involved in *target theory*. Namely, one asks for the probability that a certain number of sites (of some type) are knocked out by a radiation or other event such as a chemo-mutagenic event. This probability is then related, via some sequence of mathematical arguments, to the probability that the whole system under investigation will survive. The survival of the system is assumed to depend upon the fact that a certain number of sites must receive a certain number of *hits*, in order for the system to *fail* or be unable to survive. Let us now briefly examine the target theory approach and some of its extensions.

Let $P(n,t)$ be the probability that the target area receives n hits at time t. Assume that the system survives as long as it does not receive the environment. We will not specify the nature of these hits for the moment. Let us call this critical number of hits n_c. Hence, the probability that the system

$$P_{SURV}(t) = P(0,t) + P(1,t) + P(2,t) + \ldots + P(n_c - 1, t) \tag{8.18}$$

We can see that Eq. (8.18) is true as the probability of any hit number n occurring is independent from the occurrence of any other hit 2 hits and 3 hits as that is equivalent hits. Hence, from basic probability theory, the probability that the system survives $P_{SURV}(t)$ is equal to the sum of all of the individual hitting probabilities $P(n,t)$ whose number of hits n is strictly less than n_c. Since Eq. (8.18) describes the probability that the system survives, we can – from basic probability theory – find the failure probability. This is given by

$$P_{FAILS}(t) = 1 - P_{SURV}(t) = 1 - \sum_{n=0}^{n_c} P(n,t) \tag{8.19}$$

Suppose, however, that it is required that not only must the system receive a critical number of hits, but also a certain critical number of hits must be received by each of

p target sites in the system. That is, in order for the system to fail, each of the p sites in the system must receive the required critical number n_c hits. Since, within reason, it is justifiable to assume that failure of one target site does not induce failure in another target site, we may write the probability of system failure as follows

$$P_{FAILS}(t) = [1 - P_{SURV}(t)]^p \tag{8.20}$$

Equation (8.20) represents the culmination of a general discussion of what a simple mathematical model might be like, if we required that a transformation to senescence would necessitate that a system of the form specified by Eq. (8.18) have p targets each requiring n_c hits. This is a very simple model. It assumes that the p targets are all homogeneous in their behavior. That is, they all require the same minimum number of hits before they fail. It might be that different targets require a different number of hits before they fail. It might be that a certain fraction of the target population requires $n_c^{(1)}$ while the other fraction of the population requires $n_c^{(2)}$. We might require that the total accumulated number of hits not exceed η by time t. In other words, Eq. (8.20) describes an extremely simplified target model for consider a more complex version of this model. In order to further analyze Eq. (8.20), it next becomes necessary to give a mathematical form to the probability of receiving n hits at time t; $P(n, t)$. There are two ways of looking at the form of $P(n, t)$. If we consider the hitting agent as radiation related, then the natural choice for the hitting probability is the Poisson probability distribution. The particular reason for this choice has to do with the methodology which one uses to create a mathematical model for radiation emission. However, the general form for the probability that n hits are received at time t, given a radiation argument is

$$P(n, t) = \frac{(IAt)^n \exp(-IAt)}{n!} \tag{8.21}$$

where A is the target area, I is the average number of ionizing events per unit time, and t is the given time. In this case, combining Eqs. (8.19), (8.20), and (8.21) yields a general radiation model hitting model. In the simple case where $n_c = 1$ the model is very simple and can be written as

$$P_{FAILS}(t) = 1 - \exp(-IAt) \tag{8.22}$$

For the p target model we would have a probability of failure given by

$$P_{FAILS}(t) = [1 - \exp(-IAt)]^p \tag{8.23}$$

as the probability that the system fails. Consequently, for the p target model, the probability of survival is given by

$$P_{SURV}(t) = 1 - [1 - \exp(-IAt)]^p \tag{8.24}$$

Biological systems, even at the molecular level, are considerably more complex systems than the simple system that we have just described.

Such a model is extremely complex and depends upon knowledge of a number of pieces of information about the structure of the biological system to which it will be applied. For example, how does one define the biological equivalent of sites and components? One might conceivably argue that the target sites are the DNA bases themselves, while the components are the genes. However, one might also argue that the genes are the target sites and the chromosomes are the components. Then, within the framework of the application, one would need to estimate how many sites and components are relevant to the model. Let us now consider an alternative approach to the mathematical modeling of somatic mutations.

The arguments that we will now discuss arise out of the modeling of carcinogenic processes – or more generally – the development of disease. Suppose that, in order to initiate a given disease, r distinct mutations must occur. These mutations give rise to a clone of cells whose subsequent growth gives rise to the disease. If we assume that attaining the disease state is equivalent to system failure, then a cell is said to fail when it receives r mutations. Or, if we wish, when r of the subsystems are said to fail.

Suppose that we assume that λ_j is the mutation rate at the jth locus/cell/unit time. We may then show that the probability of a mutation at the jth locus of a given cell, in the time interval $(0, t)$ is just

$$Prob[j\text{th locus mutation in } (0,t)] = \lambda_j t \tag{8.25}$$

If $\lambda_j t$ is small then, for any given cell, the probability that there will be r mutations before time t (that is, in the interval $(0, t)$) is just

$$Prob[\text{r mutations in } (0,t)] = (\lambda_1 t)(\lambda_2 t)\ldots(\lambda_r t) \tag{8.26}$$

or, more compactly,

$$Prob[\text{r mutations in } (0,t)] = \left(\prod_{k=1}^{r} \lambda_k\right) t^r \tag{8.27}$$

Equation (8.27) gives us the probability that a single cell (or target subsystem) will receive r mutating or damaging events before time t. If we now assume that the given system has m_c of these subsystems, then the *average number of damaged clones* (if we think of the systems as cells) at time t is given by

$$\eta = m_c t^r \left(\prod_{k=1}^{r} \lambda_k\right) = \beta t^r \tag{8.28}$$

As we know the average occurrence rate of the senescence inducing clones (Eq. (8.28)), it is natural to assume that they are distributed in a Poisson-like manner.

That is, the probability that there are n senescence inducing clones in the time interval $(0, t)$ is Poisson with mean η. We may then ask the question of how long will it take for the first occurrence of the disease? In our case, how long will it take for the first occurrence of "senescence"? Such a question is answered by knowledge of the cumulative distribution function for the waiting time to the first occurrence of interest. Under our given assumptions, this is approximated by

$$Prob[\text{first occurrence is in } (0, t)] = 1 - \exp(-\beta t^r) \tag{8.29}$$

As before, we assume that r mutations are necessary to damage the target. However, we also assume that m_c targets must fail before senescence may be initiated. That is, each of m_c targets must receive r mutation events in order that the senescence process may be initiated. In this case, Eq. (8.29) may be generalized to

$$Prob[\text{first occurrence is in } (0, t)] = [1 - \exp(-\beta t^r)]^{m_c} \tag{8.30}$$

A closing alternative to the target theory approach to modeling senescence is the so-called *forbidden clone* theory (Hirsch 1974). In this modeling approach, cells are assumed to transition through a sequence of states, in an irreversible manner. As the cells transition through these states, they are assumed to be transitioning towards a state of senescence. These models are often called Markov models (See also Kirkwood and Proctor 2003). In the next section we will discuss the concepts of error catastrophe and error propagation and how they have been modeled.

8.2.2 Mathematics of Error Catastrophe

Mistakes in the translation of genetic information provide a theoretical mechanism/argument for the variety of age related changes seen in the experimental literature. While *error catastrophe* and *error propagation* are accumulation theories of aging (that is they are based upon the accumulation of a toxic effect upon the cell), the large body of mathematical modeling in this particular area warrants the separation of these two theories under a separate heading. In a subsequent section, discuss more general accumulation/depletion theories. Mistakes in the translation apparatus can lead to two distinct types of *error theory*: (1) *Error Catastrophe* and (2) *Error Propagation*.

Orgel (1963, 1970) proposed that errors in the translation of genetic information contain an element of positive incidents of mistakes and therefore increase the probability of subsequent mistakes. This mechanism, is based upon the assumption that the initial presence of errors in enzymes involved in the transcription/translation process may lead to further to cell death.

The issue of stability of the proof-reading apparatus, the fidelity of cellular translation and DNA synthesis, and the possible variations in proteins as related

to aging processes have been discussed by a number of authors. With this in mind, let us begin to make some simple models of error catastrophe.

As a preliminary approach to error catastrophe modeling, let us denote the number of errors at time t as $\mathscr{E}(t)$. Let us denote the rate of error accumulation as $r(t)$. Then, if Δt is small, and if the errors are not depleted, the number of errors at time $t + \Delta t$ is just

$$\mathscr{E}(t + \Delta t) = \mathscr{E}(t) + r(t)\Delta t \tag{8.31}$$

If we allow Δt go to zero, we obtain the following differential equation for the number of errors $\mathscr{E}(t)$.

$$\frac{d\mathscr{E}(t)}{dt} = r(t)\mathscr{E}(t) \tag{8.32}$$

While Eq. (8.32) may be solved for the general case where $r(t)$ is arbitrary, let us consider a specific choice for $r(t)$ as motivated by biological considerations. The Orgelian hypothesis is that errors create more errors. Therefore, let us assume that the error accumulation rate $r(t)$ is proportional to the number of errors $\mathscr{E}(t)$. Consequently, we would expect the rate of accumulation of errors to be proportional to the current amount of errors. If we wish, we might assume, more generally, that $r(t)$ is some function of $\mathscr{E}(t)$ and then we can examine the system's behavior. For example, we might assume that when there are no errors, the system grows at a constant exponential rate r_0 however, as the errors increase, we can assume that it eventually stops growing.

While the theory of error catastrophe has fallen by the biological wayside, a number of more complex mathematical models were developed to test the various hypotheses involved in the error catastrophe theory of aging. These models attempted to be much more rigorous as well as biologically faithful in their construction. And, as such, are worthy of discussion. Further, they introduce the concept of *error propagation* rather than *error catastrophe* as a possible model for a sub-cellular theory of aging.

The major "biologically faithful" models are the models found in Hoffman (1974), Kirkwood and Holliday (1975a,b), Goel and Yças (1975) and Goel and Islam (1980). Briefly, Hoffman (1974) argues that the fidelity of translation, denoted q_{n+1}, of the $(n + 1)$st generation is related to the fidelity of the nth generation by the formula

$$q_{n+1} = \frac{(S-1)\, q_n^m + 1}{(S-1)\, q_n^m + \lambda} \tag{8.33}$$

where S is a dimensionless specificity coefficient for a perfect translation apparatus, λ is the number of amino acids from which the translation apparatus chooses its assignments, and m is the critical number of sites in the translation apparatus where substitution of any of the $\lambda - 1$ incorrect amino acids is assumed to reduce the

value of S to one (Gallant and Prothero 1980). In Kirkwood and Holliday (1975b), the authors extend the previous model to include another parameter R, the relative overall activity of a translation apparatus that has been rendered non-specific by amino acid substitution in one of the m critical sites. This new model analysis leads to the more complex fidelity equation given by

$$q_{n+1} = \frac{q_n^m [\lambda S - R(S - \lambda - 1)] + R(S + \lambda - 1)}{q_n^m [\lambda(1 - R)(S + \lambda - 1)] + R(S + \lambda - 1)} \tag{8.34}$$

The formulations of these two models assume that a single mistake in the apparatus can produce a non-specific translation apparatus in one fell swoop (Gallant and Prothero 1980). The work of Goel and Yças (1975) and Goel and Islam (1980) relaxes this constraint by assuming that y of the m sites must be required for a loss of specificity. For the purposes of understanding these models, let us briefly cover the construction of this model. A given synthetase model is allowed to remain functional only as long as a certain number of amino acid sites on that molecule remain unchanged. Functionality is defined to be the ability to attach the correct amino acid to the correct t-RNA molecule. The activity of a synthetase molecule is defined to be the rate at which it attaches the correct amino acid to the correct t-RNA molecule. The sites of attachment may be assumed to be different for each of the different synthetases. Let i be the subscript which of the indexes which synthetases that we are discussing $i = 1, \ldots, N$. Let j index the sites of attachment. Next, define x_{ij} to be the number of such sites of an amino acid a_j in the ith synthetase.

Let q_i, q_i', q_i'' denote the fractions of normal, erroneous, and inactive ith synthetase molecules. Observing that the actions of an erroneous molecule are not site specific, the normal fraction $q_1(t + 1)$ of normal synthetase for amino acid a_1 is given by

$$\left(\frac{q_1}{q_1 + q_1' + q_1''}\right)_{t+1} = \mathcal{Q}(1)_t^{x_{11}} \mathcal{Q}(2)_t^{x_{12}} \cdots \mathcal{Q}(N)_t^{x_{1N}} \tag{8.35}$$

where

$$\mathcal{Q}(j) = \frac{q_j}{q_j + q_j'} \tag{8.36}$$

The authors argue that Eqs. (8.35) and (8.36) follow from the fact that the errors will be distributed in a binomial fashion. Therefore, the fraction of molecules with amino acid a_1 at the specified x_{11} locations is given by the first term on the r.h.s. of Eqs. (8.35) and (8.36). The fraction of molecules with amino acid a_2 at specified x_{12} locations is given by the second term, etc.

If we now assume that there are y_{ij} sites which are occupied by incorrect amino acids. Suppose that we now wish to obtain an equation for the fraction of erroneous synthetases. The authors further argue that, if one assumes that the occupation of y_{ij} sites by any of the incorrect amino acids produces an erroneous synthetase, then the

fraction of erroneous synthetases in generation $t+1$ is given by $q_1'(t+1)$ and may be expressed by the following equation

$$\left(\frac{q_1'}{q_1+q_1'+q_1''}\right)_{t+1} = \mathcal{Q}(1)_t^{z11}\,\mathcal{Q}(1)_t'^{y11}\,\mathcal{Q}(2)_t^{z12}\,\mathcal{Q}(2)_t'^{y12}\cdots\mathcal{Q}(N)_t^{z1N}\,\mathcal{Q}(N)_t'^{y1N} \tag{8.37}$$

where

$$\mathcal{Q}(j)' = \frac{q_j'}{q_j+q_j'} \tag{8.38}$$

and $z_{ij} = x_{ij} - y_{ij}$.

Finally, Goel and Yças (1975) introduce the following change of variables which allows them to simplify their system of equations. Letting

$$Q_i = \ln\left(\frac{q_i(t)}{q_i'(t)}\right) \tag{8.39}$$

they are able to reduce the complex system of N equations to a simple linear matrix system of the form

$$Q_i(t+1) = \sum_{j=1}^{N} y_{ij}Q_j(t) \qquad i = 1,\ldots,N \tag{8.40}$$

The models of Goel and Yças and Goel and Islam predict a variety of dynamical behaviors. In particular, they predict that *error catastrophe* is one of a number of possible outcomes in a model of this sort. Of great importance is the fact that *error propagation* is also a possible outcome. The search for the *error catastrophe* has, however, lead to the search for errors in the more general sense. In the next section of this chapter, we will continue our discussion of mathematical models of errors from the viewpoint of *error propagation*. And, in doing so, we will return to the error catastrophe papers to see which of the models hold true, in light of the known experimental data.

8.2.3 Mathematics of Error Propagation

An elegant review of the various mathematical models of error propagation may be found in Gallant and Prothero (1980). The elegance of this paper derives from the mathematical simplicity of the model and its subsequent predictive power when applied to a specific biological system; error-promoting drugs in a bacterial system. Let us briefly review the formulation, which grew out of the paper of Orgel (1970).

In this paper the author proposed that a given generation n produces the next generation's $n+1$ proteins. Letting e_n denote the aggregate error frequency in generation n, and letting E be the residual error frequency inherent in the translation machinery. The authors argue that the aggregate error at generation $n+1$ is given by the finite difference equation

$$e_{n+1} = E + \alpha e_n \tag{8.41}$$

where α is a proportionality constant. If we know the initial aggregate error in the system, denoted e_0,

$$e_n = E\frac{1-\alpha^n}{1-\alpha} + \alpha^n e_0 \tag{8.42}$$

As we are interested in the time course of the aggregate errors, it is natural to inquire as to what happens to the frequency when we examine the system after a large (infinite) number of generations have past. Without that the system error equilibrium given by

$$e_{eq} = \frac{E}{1-\alpha} \tag{8.43}$$

if the value of α satisfies $\alpha < 1$. Otherwise, the system will suffer an error catastrophe; the aggregate error $e_n \to \infty$ as $n \to \infty$.

Next, the authors demonstrate how this simple model of error propagation may be used to discuss mistranslation of a specific UUA codon in *E. coli*. They demonstrate that their data fits the model when $\alpha \approx 0.5$. They further demonstrate that changes in the dose of streptomycin change E; thus raising the eventual error equilibrium but not changing the eventual dynamics of the system. Their conclusion is that, for *E. coli*, the translation system functions at a safe distance ($\alpha \approx 0.5$) from the region of instability ($\alpha \geq 1$). Therefore, there is no error catastrophe. Rather, there is a propagation of errors leading to an eventual equilibrium error level. reject the error catastrophe hypothesis for somatic cells.

8.2.4 Mathematics of Recombination

The concept of tandem gene strings as an evolutionary strategy is an old one. The argument for tandem gene string involvement in an evolutionary theory follows along the line of thought that a newly arisen tandem gene sequence will be, more or less, physiologically superfluous. Hence, mutations in the tandem regions would be less likely to be disastrous to an organism than if they had occurred in a non-tandem region. Thus, duplications may be looked upon as resource material for evolution of new gene sequences; new organismic biological complexity.

In order to discuss how tandem genes might be involved in aging/evolutionary strategies, it becomes necessary to have a mathematical description of how tandem gene repeats would be dispersed/diluted/amplified in an evolving cellular system. We begin by letting m be the tandem repeat number of a hypothetical gene. Using a deterministic approach, we would model $N(m,t)$; the number of cells having a tandem gene sequence of length m at time t. The simplest way to construct such a model is to consider time t to be in MPDT's or Mean Population Doubling Times. Further, we assume that the cells are dividing synchronously. Hence, we wish to relate the number of cells with various repeat sequences at time $t = n$ to the number of cells, one MPDT later or $t = n + 1$, with various repeat sequences. Let us briefly examine how we might construct such a relationship.

We follow the discussion in Witten (1980) begin by assuming that we are given some initial gene distribution , which we shall denote as $N(m, 0)$. This is the number of cells having tandem gene sequences of length m at time $t = 0$. Further, assume that the longest initial tandem gene sequence is of length m^*. If we assume that a gene sequence of length m may undergo a recombination event which can lead to a new sequence of length 0 to $2m$, then we may define $P_m(n,t)$ to be the associated probability that a gene sequence of length m will give rise to a recombinant gene sequence of length n where $0 \leq n \leq 2m$. Since cells with 0 or 1 gene cannot recombine, it is important to realize that we must keep track of these portions of the gene population separately. Cells with a 0 gene sequence are assumed to be dead. The number of cells containing an n-gene sequence, which results from a recombination, is obtained in two steps: First, we assume that there is a population of cells which will undergo a recombination event, and second, we observe that not all cells in the recombining portion of the population will yield daughters of the required n-gene sequence length. If we let $R(n,t)$ be the fraction of m-gene sequence cells that undergo a recombination event at time t, then we may obtain the following equation for $N(m,n,0)$

$$N(m,n,0) = P_m(n,0)R(m,0)N(m,0) \quad 2 \leq m \leq m^* \quad ; 0 \leq n \leq 2m \tag{8.44}$$

where $N(m,n,0)$ represents the number of cells with a repeat length n arising from a parent of repeat length m which divided at time $t = 0$. Remember, however, that the total number of daughter n-gene sequences resulting from the recombination is arrived at by totaling the production of n-gene sequences from all possible m-gene parent sequences of length $m = 2, 3, 4, \ldots, m^*$ in that portion of the cell population undergoing recombination events. Hence, the total number of new n-gene daughter sequences is given by the following expression

$$N(n,0) = \sum_{m=2}^{m^*} N(m,n,0) \quad 0 \leq n \leq 2m \tag{8.45}$$

This results in a recombinant daughter distribution which describes the distribution of tandem genes of length n as generated from all of the dividing cells which

were allowed to undergo a recombination event. It is important to realize that the daughter distribution must be combined with the distribution of parent cells that did not undergo a recombination event, in order to obtain the final and complete distribution of new n-gene sequences.

Before we close our discussion of this type of model, we must realize that tandem genes may confer on the cell containing them

- A growth advantage which allows them to replicate in a shorter timespan than cells with less genes and,
- A survival advantage which allows them to better compete for available resources.

That is, a cell with three genes may double three times in the time that it would take a cell with one gene to double. Details of the mathematical development and analysis of this formalism may be found in Witten (1980).

The results of these models support a variety of dynamical outcomes. Briefly, when there is no recombination, gene sequences compete only through survival and growth advantage. Thus, if the overall advantage is for cells containing the longer gene repeats(tandem repeats), then the population will tend to a final population distribution containing only cells with the longest possible repeat length. This result is independent of the initial distribution of repeat lengths in the population. Likewise, if the short repeat lengths are to be considered advantageous, then the population will tend to a final distribution containing only the shortest repeat length. This result is also independent of the initial distribution of repeat lengths in the population.

In the event we choose to include recombination effects, the complexity of possible behaviors becomes increasingly great. The inclusion of recombination can slow trends to a limiting distribution or it can allow a system to sustain distributions of genes over to multi-model or unimodal distributions. Or they may not tend to a final equilibrium at all. Let us now discuss some of the different probabilistic models of recombination. A similar model with stochastic components was proposed by Lumpkin and Smith (1980).

Probabilistic models of recombination seek to describe the probability $P_{n_i}(t)$ that there are n_i copies of the ith gene in a cell. If we consider having n_i copies of the ith as equivalent to being in state s_{n_i}, then we may make use of a class of mathematical model known as a Markov model. Perelson and Bell (1977) make use of a Markov model to describe transitions between various states. To construct their model, Perelson and Bell make use of the following series of arguments. Suppose that the ith gene can exist in any one of a number of states denoted s_{n_i} where s_{n_i} is the state in which the ith gene has n_i copies of itself in the cell. Further, they assume that n_i may take on the values $n_i = 0, 1, 2, \ldots$. They next assume that they are looking at a time interval $(t, t + dt)$ small enough so that the only way to reach state s_{n_i} is to be in state s_{n_i-1} (meaning that there are $n_i - 1$ copies of the ith gene) and a recombination event occurs, adding an additional copy of the copy of the ith gene. Or, they may be in state s_{n_i+1} and a copy of the ith gene is deleted by a recombination event. They then define $\lambda_{n_i}(t)$ to be the probability that an addition recombination

occurs per unit time, and $\mu_{n_i}(t)$ to be the probability that a subtraction recombination occurs per unit time. They show that the following system of differential equations describes the probabilities $P_{n_i}(t)$.

$$\frac{dP_{n_i}(t)}{dt} = -\left[\lambda_{n_i}(t) + \mu_{n_i}(t)\right] P_{n_i}(t) \tag{8.46}$$

$$+ \lambda_{n_i-1} P_{n_i-1}(t) + \mu_{n_i+i}(t) P_{n_i+1}(t) \quad n_i \geq 1 \quad i = 1, 2, \ldots, N_0 \tag{8.47}$$

$$\frac{dP_0(t)}{dt} = -\lambda_0(t) P_0(t) + \mu_1(t) P_1(t) \tag{8.48}$$

$$n_i = 0 \quad i = 1, 2, \ldots, N_0 \tag{8.49}$$

where the initial conditions are specified as $P_{n_i}(0) = 1$ if $n_i = 1$ otherwise $P_{n_i}(0) = 0$. To make the model tractable for analysis, assume that, at $t = 0$ there is only one copy of each gene in the population. The solution to the model depends, intimately, upon the form one chooses for the functions $\lambda_{n_i}(t)$ and $\mu_{n_i}(t)$.

8.2.5 *Mathematics of Accumulation/Depletion*

The variety of aging theories leads to a variety of models for aging processes in mammalian systems. One major class of model is the accumulation/depletion model which argues that senescence is the result of some gradual accumulation or depletion of various mysterious (or not so mysterious) cell functions, cell products, cellular debris (waste), or other cellular activities and more recently discussed in Grűning and Vinayak (2011).

One of the earliest of the "waste" papers was Hirsch (1978). This paper discusses the dilution of "cellular waste" due to symmetric or asymmetric cell division. A discussion of the modeling of asymmetric cell division may be found in Hirsch (1977). In Hirsch (1978), the author makes use of a differential equations approach to the modeling of the dilution of a cellular waste product. He assumes (1) that waste is created at a rate which is either constant or proportional to the waste already formed, (2) that waste is neither destroyed nor transported across cell walls, and (3) that the rate of cell division at large values of time is inversely proportional to some power of the waste per cell. A review of the literature justifying these assumptions may be found in the aforementioned paper.

The growth of our cell population is governed, as Hirsch (1978) points out, by the cell division rate which may be a function of cell density, waste level, and time. Letting $w(t)$ be the total waste at time t, and letting $n(t)$ be the total number of cells at time t, and assuming that the cells are undergoing density dependent growth, we may write the cell division rate as

$$\frac{1}{n(t)} \frac{dn(t)}{dt} = b(t) k(w) \left(1 - \frac{n(t)}{E}\right) \tag{8.50}$$

where $b(t)$ is the time dependent reproductive rate, $k(w)$ is the waste-cell interaction function, and E is the environmental carrying capacity. More accurately, $b(t)$ is the net per capita birth rate minus death rate. Hirsch (1977) demonstrates that it is more natural to assume that the cell division rate is an explicit function of the waste per cell, denoted $w'(t)$, rather that the overall waste $w(t)$. That is, as $w'(t)$ increases, the cell division rate should decrease. Further, for the sake of simplicity, we assume that the cell division rate is a power function of the waste per cell. That is, the waste-cell interaction function $k(w)$ is of the form

$$k(w) = \frac{1}{w'(t)} \tag{8.51}$$

and the waste per cell is given by

$$w'(t) = \frac{w(t)}{n(t)} \tag{8.52}$$

Combining this with our Eq. (8.50) we obtain the following equation for the cell division rate.

$$\frac{1}{n(t)}\frac{dn(t)}{dt} = b(t)\left(\frac{n(t)}{w(t)}\right)^j\left(1 - \frac{n(t)}{E}\right) \tag{8.53}$$

Equation (8.53) may be rewritten as follows.

$$\frac{dn(t)}{dt} = b(t)n(t)\left(\frac{n(t)}{w(t)}\right)^j\left(1 - \frac{n(t)}{E}\right) \tag{8.54}$$

While Eq. (8.54) is sufficiently general, it does not allow for any obvious biological insights. Let us begin by making the simplification that $b(t) = b_0$ a constant. Further, let us assume that there is no waste effect on the cells. This would correspond to the case where $j = 0$. Replacing these assumptions into Eq. (8.54) we obtain the very familiar equation

$$\frac{dn(t)}{dt} = b_0 n\left(1 - \frac{n}{E}\right) \tag{8.55}$$

the logistic growth equation; the standard mathematical model for density-dependent cell growth in a cell culture environment. This equation has a solution given by

$$n(t) = \frac{E}{1 + \left(\frac{E}{n_0} - 1\right)\exp\left(-b_0 t\right)} \tag{8.56}$$

where n_0 is the initial number of cells at time $t = 0$. Hence, on the basis of our simple assumptions, we see that our model describes known cellular growth phenomena. Let us now examine the possible effects of waste in the model.

We begin this investigation by considering how to describe the production of the waste material. That is, we wish to write an equation for $w(t)$. As an initial assumption, it is reasonable to assume that the waste production is simply proportional to the number of cells at any given time t. That is,

$$\frac{dw(t)}{dt} = r_0 n(t) \tag{8.57}$$

Equations (8.53), (8.54), and (8.57) constitute a simple model for waste production and its interaction with a population of cells which are growing in a density-dependent environment such as a cell culture dish.

Suppose that we wish to determine whether or not our model makes any biological sense. One easy way to do this is to assume that the waste does not affect cell growth (Eq. (8.55)) and see what happens to the waste production over time. If we let our equations be Eqs. (8.55) and (8.57) then, after much algebra, one can show that the solution to the waste equation is given by

$$w(t) = w(0) + E\left(\frac{r_0}{b_0}\right) \ln\left[\frac{n_0}{E}\left(\exp\left(b_0 t\right) - 1\right) + 1\right] \tag{8.58}$$

where $w(0)$ is the initial amount of waste in the system. Observe that we may obtain this solution by replacing $n(t)$ in Eq. (8.57) with the solution for $n(t)$ as given in Eq. (8.56).

We then simply integrate the resultant differential equation to obtain Eq. (8.58). We may simplify Eq. (8.58) as follows. If we assume that t is large enough, then Eq. (8.58) may be approximated by

$$w(t) \approx w(0) + E\left(\frac{r_0}{b_0}\right)\left[\ln\left(\frac{n_0}{E}\right) + b_0 t\right] \tag{8.59}$$

Rearranging Eq. (8.59) leads to

$$w(t) \approx w^*(0) + E r_0 t \tag{8.60}$$

where $w^*(0)$ is given by

$$w^*(0) = w(0) + E\left(\frac{r_0}{b_0}\right) \ln\left(\frac{n_0}{E}\right) \tag{8.61}$$

Notice that Eq. (8.60) says that if cells are growing logistically (in a density-dependent manner), and waste does not affect their growth, then the total waste in the cell system must increase without bound; even though the number of cells

in the cell culture plateaus out to the value of the carrying capacity E. Hence, the asymptotic waste per cell can be shown to be given by

$$w'(t) = \frac{w(t)}{n(t)} \approx \frac{w^*(0)}{E} + r_0 t \tag{8.62}$$

which also grows without bound. Clearly, this cannot be the case. Hence, we must assume that there is a waste-cell interaction or else our model is incorrectly formulated.

Sheldrake (1974) has suggested that the rate of waste production might be proportional to the amount of waste per cell already accumulated. This would require that we utilize a waste equation of the form

$$\frac{dw(t)}{dt} = a_0 w(t) \tag{8.63}$$

where a_0 is a proportionality constant. Considering this waste production model, along with our logistic growth model (8.56), we find that the waste per cell is given by

$$w'(t) = \frac{w(0)}{E} \exp(a_0 t) + \frac{w(0)}{E}\left(\frac{E}{n_0} - 1\right) \exp[(a_0 - b_0)t] \tag{8.64}$$

As $a_0 > 0$ we see that $w'(t)$ grows without bound. Whether $a_0 > b_0$ is irrelevant to the large time dynamics of the waste per cell. Again, in the this model, we see that it is biologically unreasonable to assume that there is no waste interaction. In both cases, under this assumption of no interaction, the waste per cell accumulates without limit.

Let us now take a look at the case where $j \neq 0$. That is, we wish to examine the case where there exists a waste-cell interaction. As $w'(t)$ is given by

$$w'(t) = \frac{w(t)}{n(t)} \tag{8.65}$$

then taking the derivative of both sides of this equation leads to the differential equation

$$\frac{dw'(t)}{dt} + b_0 (w'(t))^{1-j}\left(1 - \frac{n(t)}{E}\right) = \frac{1}{n(t)}\frac{dw(t)}{dt} \tag{8.66}$$

In the case of exponential growth ($E \to \infty$), Eq. (8.66) reduces to the simpler equation

$$\frac{dw'(t)}{dt} + b_0 (w'(t))^{1-j} = \frac{1}{n(t)}\frac{dw(t)}{dt} \tag{8.67}$$

which is discussed in Hirsch (1978). The author demonstrates that for a waste equation where the waste production is proportional to the number of cells (Eq. (8.57)), a variety of population dynamics may occur; governed by the value of j. Of interest is the fact that the finite lifespan of WI-38's can be described by waste models of this type only if $j > 1$; though $j = 1$ will work for appropriately chosen values of b_0 and r_0 satisfying $r_0 > b_0$. Hirsch (1978) also shows that a Sheldrake model for waste production (Eq. (8.63)) can lead to models displaying a senescence-like behavior.

This work is further extended in Hirsch et al. (1989) which discusses how the waste production may be affected by the requirement for an underlying resource which is a precursor for the waste product. Preliminary results show that by controlling the resource, it is possible to control waste levels; and thereby control cell growth. This type of model may have applications in the study of dietary restriction.

Other accumulation/depletion models may be found in the work of Strehler et al. (1971). This work argues that clonal aging processes are explicable as the consequences of irreversible and reversible repressor accumulation on plasma membranes. The authors propose a simple equation describing the kinetics of accumulation. Letting X_n be the fraction of repressors which are irreversibly bound to the membrane/generation, they show that the ratio of repressors/cell between the nth generation and the first generation is given by the following equation

$$\rho_n = \frac{2^n - X_n}{2^{n-1}(2 - X_n)} \tag{8.68}$$

They then go on to demonstrate how this model is consistent with a variety of known experimental results in clonal aging processes.

8.3 Concluding Chapter Thoughts

The dynamics of aging offers a wealth of potential mathematical and computational modeling challenges. This review has touched upon just a small portion of them. The modeling of the cellular dynamics of aging and its interface with tumorigenic processes is an open question with only a few papers written about it. Mathematical models of cellular population dynamics abound in the literature. Historically, this literature does not describe the propagation of a vector of general properties through a population of cells and how those properties might affect the propagation of the cell line. This extension has important ramifications in studying the dynamics of numerous cell system such as aging, cellular co-culture, embryogenesis, and feeder layer dynamics. As such, it represents the next natural level of cellular modeling.

In this chapter, I focused upon the historical literature in the mathematical modeling of cellular level processes. Starting with some of the basic theories of sub-cellular aging processes, we discussed various methods which one can use to

create models designed to study those same aging processes. We observed that some approaches used simple iterative (recursive) models while others used differential equation models; both single and multi-equation. Other forms of model utilized deterministic probability calculations while others used matrix approaches. We also saw that some models used partial differential equations. More recently, systems biological approaches have been used Kowald and Klipp (2013) and Dalle Pezze et al. (2014). These models involve understanding the actual biological pathways (graph theoretic structures) and turning them into differential equation models. Others have modeled focused cellular systems such as aging in the bone (Mehr et al. 1993), the hematopoetic system (Marciniak-Czochra et al. 2009) or yeast (Gillespie et al. 2004). Others have focused on modeling the dynamics of sub-cellular components such as the chaperones (Proctor et al. 2005) or the mitochondria (Kowald and Klipp 2013) and yeast. In summary, we have seen a variety of modeling approaches applied to a diverse collection of biological aging processes. We see that there are numerous mathematical and computational approaches to creating models of aging and the cellular and cell-population levels and we find that there is no one correct way to build one of these models. However, it is essential that we understand the biology of our system to the best of our ability and that we carefully formulate our questions so that they can be turned into models.

Acknowledgements In 1974 I wrote my very first mathematical modeling of aging manuscript. It was my masters dissertation while I was a student at SUNY Buffalo in the Center for Theoretical Biology. That paper was subsequently published in 1980. In the now 41 years since my initial foray into this field, my career has been touched by many individuals who have provided support and guidance to a then young graduate student trying to find her way. I simply cannot acknowledge all of you. Nevertheless, I would like to acknowledge on singular individual and dedicate this paper to him; Professor Bernard Strehler, my mentor and friend. He took an unknown young mathematical physicist who knew nothing about the biology of aging and guided her into a 44 year long career in this fascinating field of Gerontology by supporting many of her research efforts as publications in the journal Mechanisms of Aging and Development. I cannot thank him enough.

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Part III
Ageing, Cancer and Senescence

Chapter 9
Cell Cycle Checkpoints and Senescence

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Abstract Cellular senescence, an outcome of finite proliferative, limited repair and defence capacity of normal cells, is a widely accepted in vitro model for ageing studies. In a sharp contrast to cancer cells, it is firmly regulated by cell cycle checkpoints that ensure evasion of stressed and genetically modified cells, limiting their expansion and serve as an innate check to carcinogenesis. Tumour suppressors and their regulatory proteins play key roles, both as molecular sensors and regulators in this process. Aim of the present chapter is to sketch a brief understanding on how cellular senescence is regulated by major tumour suppressor and cell cycle checkpoint proteins as well as by some emerging molecules.

Keywords Stress • Cellular senescence • Cell cycle • Tumour suppressors • DNA damage signalling • Checkpoint proteins

9.1 Cellular Senescence

Senescence (derived from Latin: senescere, meaning "to grow old") is a property of all living organisms. It is a process that leads to functional decline, and an increase in vulnerability to a spectrum of diseases eventually leading to death of an organism.

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From evolutionary perspectives, decline in reproductive capacity and increase in mortality rate with age, set up conditions which eradicate exhaustive competition for resources and favour continual survival of a population versus the individuals (Kirkwood 2008). Senescence is considered to be an outcome of limited maintenance and repair capacity of living organisms leading to accumulation of damage that occurs as a consequence of gene activities or functions essential for survival. In other words, it is an indispensable outcome of life; further complicated by the environmental factors without which life cannot exist or function (Kirkwood 2008). A large number of model systems have been used for studying senescence in vitro. It has been established that a cell, the smallest functional unit of life, mimics highly complex organismal ageing phenomenon, and was first demonstrated by its limited capacity to divide despite the availability of sufficient nutrients, growth factors and space (Hayflick 2007; Hayflick and Moorhead 1961). The total number of population doublings (PDs) that normal cell cultures can attain before senescence depends on the cell type and the age of the donor, and not the chronological age of the culture (Hayflick and Moorhead 1961; Maier and Westendorp 2009). The present chapter provides a simple sketch on the current understanding on the role of cell cycle checkpoint proteins in regulation of proliferation in normal and cancer cells.

Senescent cell can be identified in cell culture by virtue of its phenotypic characteristics including increased cell size, flattened and irregular shape, multinucleation and cytoplasmic vacuolation (Mitsui and Schneider 1976; Robbins et al. 1970; Sikora et al. 2011). Young fibroblasts have an organized fusiform appearance in culture. On the other hand, senescent fibroblasts appear flattened, disorganized and are randomly oriented in culture dish. They show fragmented and distorted subcellular structures including nucleus, mitochondria and endoplasmic reticulum, and have a high rate of autophagy that is associated with an increase in lysosomal mass (Gerland et al. 2003; Goligorsky et al. 2009; Kurz et al. 2000). Young cultures are heterogeneous and contain a mixture of dividing, growth-arrested and senescent cells. The proportion of senescent cells increases progressively until the whole culture has entered senescence, a state in which they can remain metabolically active for long periods of time (Cristofalo and Sharf 1973; Smith et al. 1980; Wadhwa et al. 1991). Senescent culture has a lower cell density at confluence than a confluent young culture suggesting that the senescent cells are more sensitive to cell-cell contact inhibition. Although these phenotypes of senescent cells are firmly established, the underlying molecular mechanism(s) remain obscure. A few molecular events have been allied to senescent cell morphology and replicative senescence. Caveolin-1, an integral membrane protein and the principal component of caveolae, was shown to play an important role in senescence-associated morphological changes by regulating focal adhesion kinase activity and actin stress fibre formation in the senescent cells (Cho et al. 2004). Reduction in the levels of caveolin-1 caused resumption of DNA synthesis in senescent cells (Cho et al. 2003) suggesting that it is required for maintaining the state of replicative senescence. Senescence-associated β-galactosidase (SA β-gal) activity was associated with the unusual behaviour of the enzyme β-galactosidase, a lysosomal hydrolase. β-galactosidase is normally active at pH 4, but in senescent cells it becomes active at pH 6. Cells positive for SA β-gal

increase with cell culture passage and age, both in vitro and in vivo, respectively (Bandyopadhyay et al. 2005; Carnero 2013; Dimri and Campisi 1994; Dimri et al. 1995). Early studies showed that the lysosomes increase in number and size in senescent cells. SA β-gal appeared to be a result of increased lysosomal activity at a suboptimal pH, which becomes detectable in senescent cells due to an increase in lysosomal content (Kurz et al. 2000).

Functionally, senescent cells can be distinguished from pre-senescent cells by their increased resistance to apoptotic death, refractoriness to various growth factors and mitogens and increased sensitivity to toxins, antibiotics, irradiation, oxidation and heat shock (Aggarwal et al. 1995; Blake et al. 1991; Fargnoli et al. 1990). Several studies have demonstrated that the rate of protein, DNA and RNA synthesis is reduced in senescent cells and is accompanied by substantial alterations in gene expression involved in processes including cell cycle control, stress response, signal transduction and synthesis of extracellular matrix, mitochondrial, and cytoskeletal proteins (Carnero 2013; Cristofalo et al. 1998; Duncan and Reddel 1997; Falandry et al. 2013; Goldstein 1990; Holliday 1990; Kuilman et al. 2010; Rattan 1996). Some of the most commonly used senescent cell specific biomarkers are: osteonectin, fibronectin, apolipoprotein J, smooth muscle cells 22 (SM22) protein, and type II (1)-pro-collagen (Gonos et al. 1998; Kumazaki et al. 1991). Senescent cells also display an increased activity of metalloproteinases, which degrade the extracellular matrix (Campisi 2000). Such senescent cell specific markers provide hints for elucidating the underlying molecular mechanism(s).

A large number of studies have confirmed that the replicatively senescent cells make a permanent exit from cell cycle and arrest at the G1/S or G2/M boundary representing exhaustion of their division capacity (Campisi 2000; Cheung et al. 2010; Goldstein 1990; Herbig et al. 2004; Marcotte and Wang 2002; Pignolo et al. 1998; Rayess et al. 2012; Stein and Dulic 1995; Vargas et al. 2012). They have been detected in a variety of tissues and in a number of different organisms including mouse, primate and human (Dimri et al. 1995; Jeyapalan et al. 2007; Krishnamurthy et al. 2004; Krtolica and Campisi 2002; Michaloglou et al. 2005; Molofsky et al. 2006; Prieur and Peeper 2008; Satyanarayana et al. 2004; Campisi and Robert 2014; Demaria et al. 2015). In old baboons, over 15 % of dermal fibroblasts showed a senescent phenotype as determined by damaged telomeres, increased $p16^{INK4A}$ expression and an activation of ATM kinase (Jeyapalan et al. 2007). Michaloglou and colleagues showed that the melanocytic naevi (benign skin moles, that may be precursors of malignant melanoma) have increased levels of senescent markers and do not seem to proliferate, yet can persist for many years (Michaloglou et al. 2005). However, evidence regarding the role of senescent cells in vivo ageing and pathologies of old age is only limited. Apparently, in contrast to replicative senescence of cells in vitro, tissue and organismal ageing is multifactorial and is more complex because of the existence of heterogeneous populations of cell types that function and senesce in different ways influenced by individual genetic and environmental factors. Knockout mouse models have been generated to recapitulate human genetic diseases associated with premature ageing and cancer predisposition. Individuals with premature ageing disorders such as

Werner Syndrome, Down Syndrome and Hutchison-Gilford Progeria have a shorter in vitro life span supporting the use of replicative senescence as a valuable model to understand the ageing in vivo (Chun et al. 2011; Mackenzie and MacRae 2011; Raghu et al. 2001; Thweatt and Goldstein 1993; Vaziri et al. 1993).

9.2 Triggers of Senescence

Over the last three decades, there have been several studies to understand the mechanisms responsible for the limited replicative potential of normal human fibroblasts. The most consistent view probably is that the normal biological functions required for life are important triggers of senescence. Although distinguishing between the causes and consequences of senescence may be difficult, some of the most consistent manifestations of cellular senescence appear to be (i) upregulation of specific tumour suppressor activities (Atadja et al. 1995; Brown et al. 1997; Gire 2005; Gire and Wynford-Thomas 1998; Liu et al. 2015), (ii) accumulation of DNA damage response proteins at telomeres due to telomere shortening (d'Adda di Fagagna et al. 2003; Kaul et al. 2012; Mengual Gomez et al. 2014), (iii) an increase in inadequately repaired single stranded (ss) or double stranded (ds) DNA breaks (Campisi and Robert 2014; Klement and Goodarzi 2014; Wang et al. 2015) (iv) increase in intrinsic stress including mitochondrial dysfunction and accumulation of reactive oxygen species (ROS) (Passos and Von Zglinicki 2006; Passos et al. 2006; Burhans and Heintz 2009; Vurusaner et al. 2012; Yan et al. 2014), and (v) increase in secreted growth factors, matrix remodelling enzymes, and inflammatory cytokines that contribute to age-related pathologies including cancers (Campisi 2005a, b; Krtolica et al. 2001; Tchkonia et al. 2013; Velarde et al. 2013).

9.3 Stress Induced Premature Senescence (SIPS)

Any form of sub-cytotoxic stress that can accelerate the appearance of the senescent phenotype in cells is regarded as a trigger of SIPS. Although replicative- and stress-induced senescence initiate from different origins, both processes demonstrate strong similarities with regard to activation of DNA damage response, upregulation of tumour suppressor functions and permanent growth arrest at G1/S or G2/M checkpoints (Halazonetis et al. 2008; Horn and Vousden 2007), and hence both have been used as convenient model systems for understanding the molecular basis of senescence. Sub-lethal stresses such as oxidative stress or gamma irradiation, chromatin remodelling, oncogenic stress, DNA damage and strong mitogenic responses have been shown to result in SIPS (Campisi and Robert 2014; Di Leonardo et al. 1994; Martien and Abbadie 2007; Passos et al. 2006; Saretzki et al. 1998; Suzuki and Boothman 2008; Toussaint et al. 2000). Nuclear and mitochondrial DNA damage (mtDNA) induced by physiological levels of ROS has been shown to

have a significant impact on cellular senescence. Many studies have shown that the telomere shortening is stress dependent and mtDNA damage is closely related to ROS production (Passos et al. 2007). Improvement in mitochondrial function resulted in less telomeric damage and slower telomere shortening. Moreover, telomerase, an enzyme complex that re-elongates shortened telomeres, was shown to protect against oxidative stress, suggesting a strong link between mitochondrial and telomeric DNA damage leading to cellular senescence (Ale-Agha et al. 2014). Examples of oxidative stress causing senescence include treatment with sub-lethal levels of hydrogen peroxide (Chen 2000), UV (Ma et al. 2002; Wlaschek et al. 2003) and interferon-γ (Weyand et al. 2003) that were shown to induce reactive oxygen species and DNA damage (Ale-Agha et al. 2014; Klement and Goodarzi 2014; Passos et al. 2007; Vurusaner et al. 2012). Whereas lifespan of primary human fibroblasts cultured in 3 % O_2 was extended by 20 PDs (Chen et al. 1995; Gelvan et al. 1995) as compared to the ones cultures in 20 % O_2 by Hayflick and Moorhead (1961), the cells cultured in >20 % O_2 displayed reduced growth rate and underwent fewer PDs (Horikoshi et al. 1986; Indran et al. 2010; Passos et al. 2006; Przybylska and Mosieniak 2014; von Zglinicki et al. 1995).

Oncogene-induced premature senescence (OIPS) is a subset of SIPS that occurs in response to excessive mitogenic signals. Inappropriate mitogenic signaling such as inhibition of phosphatidylinositol 3-kinase or constitutive MAP kinase signaling via overexpression of oncogenic Ras, Raf or MEK induces premature senescence in human diploid fibroblasts (Lin et al. 1998; Tresini et al. 1998; Zhu et al. 1998). It was shown that the oncogenic Ras-induced SIPS was mediated by an increase in ROS (Lee et al. 1999; Wei and Sedivy 1999; Sikora et al. 2011). It was shown that the ROS acts as both an upstream signal that triggers p53 activation as well as a downstream effector that mediate apoptosis. Low levels of p53 induce expression of antioxidant enzymes, and its high levels promote the expression of genes that contribute to ROS formation (Liu et al. 2008). Upon continuous exposure to over-expression of the oncogene, normal cells stop proliferating long before their telomeres become short. The RAS proto-oncogenes encode small GTPase proteins that are involved in cellular signal transduction pathways including induction of cell growth and survival, as well as differentiation. A mutation in codon 12, 13 or 61 in any of the three RAS genes (N-RAS, K-RAS and H-RAS) transforms them into active oncogenes but, paradoxically, overexpression of mutant RAS oncogenes in normal cells causes senescence rather than malignant transformation. Studies described in 1997 provided a partial explanation on this finding by showing that the activated H-RAS triggered an initial wave of proliferation in vitro, followed by an irreversible growth arrest and a concomitant accumulation of p53 and $p16^{INK4A}$ proteins (Serrano et al. 1997). Several reports have now demonstrated that the oncogene-induced senescence occurs in vivo in mouse tumor models and in human tumors. A mouse strain containing a knocked-in conditional oncogenic mutant H-RAS allele developed lung adenomas that were characterized by a low proliferative index and increased SA β-gal activity and other senescence markers including senescence-associated heterochromatin foci and elevated levels of $p16^{INK4a}$ and $p15^{INK4a}$ (Collado et al. 2007). Eμ-N-RAS transgenic mice harboring targeted

heterozygous lesions in the gene encoding Suv39h (histone methyltransferase) developed mouse T cell lymphomas that entered senescence after drug therapy. Intriguingly, these studies show that tumor cells are still capable of activating the senescence program when triggered by exogenous stimuli such as DNA-damaging anticancer drugs (Braig et al. 2005). Oncogene-induced senescence has been studied largely in vitro, which has evoked debate whether this type of senescence has physiological relevance. Studies have shown that the stress and oncogene-induced senescence occur in vivo in response to mutations in Ras (Serrano et al. 1997), Raf (Dankort et al. 2007; Michaloglou et al. 2005), NF1 (Courtois-Cox et al. 2006) and PTEN (Chen et al. 2005; Courtois-Cox et al. 2006) genes in mouse as well as human tumors. Sarkisian and colleagues have postulated a triple step model for dose-dependent oncogene induced senescence in vivo (Sarkisian et al. 2007). They generated doxycycline-inducible transgenic mice that allowed titrated Ras activation. Initial activation of *Ras* mutation resulted in low levels of oncogene activation that stimulated proliferation; high level of ras activation activated tumor suppressor pathways and caused induction of senescence; inactivation of the latter was indispensible for tumor progression (Sarkisian et al. 2007). Feng and colleagues demonstrated the relationship between oxidative stress and senescence in vivo using mouse oxidative stress model (Feng et al. 2001). Several tissues from the ozone inhalated mice showed decline in anti-oxidative capacity, increased production of ROS and senescence-related alterations in physiological and physical strength parameters.

9.4 Cellular Senescence and Cancer

Cellular senescence is largely accepted as an in-built anticancer mechanism. Whereas cancer cells show a state that has escaped senescence, reimposition of senescence is considered to be a promising anticancer strategy. Pereira-Smith and Smith illustrated that the hybrids obtained from fusion of normal human diploid fibroblasts with immortal human cell lines exhibited limited division potential suggesting that the cellular senescence is dominant over immortalization (Pereira-Smith and Smith 1983). The latter has been widely accepted as an early prerequisite towards tumorigenesis (Campisi 2013; Duncan and Reddel 1997; Reddel 1998; Shay and Wright 2005; Wadhwa et al. 1994, 2000a, 2002b) and is achieved by overriding multiple proliferative checkpoints by events mediated by genetic, epigenetic, intracellular and extracellular environmental factors (Campisi and Robert 2014; Serrano and Blasco 2007; Shay and Roninson 2004; Moore et al. 2003; Vallejo et al. 2004). It was shown that the introduction of transforming genes of DNA tumour virus, such as SV40, papilloma and adenovirus, prevents cells from entering senescence and confers a finite extension of proliferative lifespan, of approximately 20–60 PD, which eventually ends in culture crisis (Girardi et al. 1965). Only a small number of cells (at a frequency of 10^{-5} to $10^{-9)}$ were able to escape from crisis (Girardi et al. 1965; Huschtscha and Holliday 1983; Shay and Wright 1989) by inactivation of cell cycle checkpoints and activation of a telomere maintenance

mechanisms (Campisi 2002, 2005b, 2008; Duncan and Reddel 1997; Reddel 1998; Smith and Pereira-Smith 1996). Reversal of these signalings has been shown to trigger cells to senescence and has been considered to be a promising anticancer strategy. Indeed several anticancer drugs have been shown to induce premature age-related pathologies like visual deterioration, musculoskeletal decline, osteoporosis, skin changes, chronic fatigue, sexual dysfunction and cardiovascular complications (Lee and Lee 2014; Maccormick 2006; Meinardi et al. 2000; Ventura et al. 2007).

9.5 Cell Cycle Checkpoints and Senescence

The cell cycle checkpoints provide important regulatory machinery for ensuring integrity of genetic information in normal cells. In response to genotoxic stress, they block cell cycle progression, thus allowing DNA repair systems to correct replication errors. Whereas upon correction of the DNA errors, checkpoint signals are attenuated resulting in cell cycle renewal, failure of repair triggers senescence/apoptosis, failure of the latter results in carcinogenesis. Recent studies have shown that the checkpoints-mediated DNA damage response (DDR) signalling acts as "a double-edged sword" in cancer prevention and cancer therapy (Tian et al. 2015). On one hand, it safeguards genomic stability and prevent from tumorigenesis, and on the other, it contributes to the resistance of cancer cells to chemo- and radiotherapy.

The DNA damage checkpoint control is constituted of sensors (MRN complex and RPA), transducers (Ataxia telangiectasia mutated, ATM; Ataxia telangiectasia and Rad3-related, ATR and DNA-PK) and their effector proteins (Chk1, Chk2, p53, Cdc25A, Cdk1, Cdk2 and several others) (Broustas and Lieberman 2014; Elias et al. 2014; Stracker et al. 2013; Zannini et al. 2014; Zhang and Hunter 2014). The ATM/ATR-Chk1/Chk2-p53-p21 axis is a primary regulator of DNA damage response (Stracker et al. 2013). Each of these proteins plays a specific role in regulation of cell cycle and DNA damage response. ATM and DNA-PK respond to double strand DNA breaks, ATR is involved in single-strand DNA breaks. In response to genotoxic, oncogenic and environmental stresses, it is activated by phosphorylation on specific Ser or Thr residues and causes G1/S and G2/M cell cycle arrest. Furthermore, they exhibit stress specific activation. Whereas ATM and DNA-PK respond mainly to DSBs, ATR is activated by single-strand DNA and stalled DNA replication forks (Sperka et al. 2012). Chk1, Chk2, p53 and its downstream regulators execute cell cycle arrest and are most frequently inactivated in cancer cells. On the other hand, they are activated in DDR and OIPS. Furthermore, whereas precancerous cells possess active DDR and OIS, aggressive and advanced cancers show their inactivation suggesting that DDR and checkpoint barriers are overridden during the process of carcinogenesis (Broustas and Lieberman 2014; Sperka et al. 2012; Wang et al. 2015). p53 is activated in response to a variety of stresses and inactivated in large majority of cancers (Blagosklonny 2002; Wynford-Thomas 1996; Xue et al. 2007). Although, mechanisms of functional inactivation

of this axis at various checkpoints are not well understood, recent data show that many checkpoint recovery proteins are overexpressed in various cancer tissues suggesting that they function not only in the cell cycle control, but also in the process of cancer development. On the other hand, several proteins, such as Wee 1, Claspin, Plk1, Wip1, Gwl, FoxM1, Cdh1/APC, and PP2A have been shown to inactivate checkpoint regulators by ubiquitin mediated degradation or other mechanisms and promote cell transformation (Wang et al. 2015; Zannini et al. 2014; Zhang and Hunter 2014). The following discussion is limited to the major cell cycle checkpoints involved in senescence.

9.6 p53 Checkpoint

Involvement of multifunctional p53 tumour suppressor protein in senescence has been firmly established in last two to three decades. The two main activities of p53, DNA binding and transcriptional activation, have been shown to increase as cells approach senescence (Atadja et al. 1995; Bond et al. 1996; Kulju and Lehman 1995) or undergo SIPS in response to oncogenic or environmental stimuli (Chen et al. 1998). It activates transcription of a large variety of genes including $p21^{WAF1}$, GADD45, MDM2, Bax, thrombospondin 1, cyclin G, IGF-BP3, TGFα, 14-3-3 s and MDM2 (el-Deiry 1998; Elias et al. 2014; Fang et al. 1999; Liu et al. 2015; Menon and Povirk 2014; Mirzayans et al. 2012; Zhang et al. 2014) and in turn regulated by HDM2, predominantly, by proteasome mediated degradation (Courtois-Cox et al. 2006). Several studies have shown that p53 must be transcriptionally active in order to induce senescence through its downstream effector cyclin dependent kinase inhibitor $p21^{WAF1}$ that causes arrest at G1/S or G2/M stage of cell cycle. Whereas overexpression of $p21^{WAF1}$ in p53 compromised cells resulted in their senescence (Wang et al. 1999; Fang et al. 1999), $p21^{WAF1}$ compromised cells were refractory to this effect (Brown et al. 1997). Microinjection of anti-p53 antibodies rescued cells from senescence and this effect was accompanied by a decrease in $p21^{WAF1}$ expression (Bond et al. 1994; Gire and Wynford-Thomas 1998; Shay et al. 1993). p21 expression is upregulated in a p53-dependent manner as cells approach senescence (Alcorta et al. 1996; Dulic et al. 1994; Harper et al. 1993; Noda et al. 1994; Stein et al. 1999; Vaziri et al. 1993). Exogenous expression of $p21^{WAF1}$ induced senescence in early passage human diploid fibroblasts (Fang et al. 1999; McConnell et al. 1998; Vogt et al. 1998). Disruption of both p21 alleles conferred an extended lifespan (Brown et al. 1997). However, a high level of p21 expression was neither maintained in human senescent cells nor was necessary for acquisition of senescence in mouse cells (Medcalf et al. 1996; Pantoja and Serrano 1999). Thus it was proposed that $p21^{WAF1}$ may initiate senescence but may not be involved in its maintenance. On the other hand, p53 is inactivated in about 60 % of human cancers (Sharpless and DePinho 2002) by mechanisms involving (i) mutations, (ii) inactivation by either DNA tumour virus oncoproteins or cellular partners/antagonists. Cells from individuals with Li-Fraumeni syndrome (an inherited mutation in one TP53

allele) were shown to exhibit lifespan extension when their wtTP53 was inactivated spontaneously (Maclean et al. 1994; Rogan et al. 1995). And, introduction of wtp53 into immortalized cells resulted in their growth arrest or apoptosis.

For its role as a guardian of the genome, p53 has been shown to induce a variety of genes that promote cell death or apoptosis in response to stress (DNA damage, hyperoxia, hypoxia, activated oncogenes, heat shock, cytokines and growth factors) and evade expansion of cells with genomic anomalies. These include BAX, APAF1, PUMA, p53AIP1, NOXA, Wip1 and Gadd45 (Elias et al. 2014; Liu et al. 2014, 2015; Menon and Povirk 2014). During apoptosis, several of these proteins are found in mitochondria and involved in triggering the caspase cascade. It is still unclear what regulates p53 activities to induce either cell cycle arrest or apoptosis.

Site-specific phosphorylation of p53 in response to DNA damage and other stresses has been used as a reliable indicator of stressed state of cells. For example, ionising radiation induces phosphorylation of p53 at serine 15, and this requires Checkpoint kinase 2 (Chk2) and ATM kinase (Banin et al. 1998; Canman and Lim 1998; Knippschild et al. 1996). UV induces phosphorylation at serines 15 and 37, which is dependent on Chk1 and ATR kinase (Chehab et al. 1999; Hirao et al. 2000) and Chk1 also specifically phosphorylates p53 at serine 20 (Chehab et al. 1999; Shieh et al. 2000). UV has also been found to induce phosphorylation at five other N-terminal serines and two threonine residues and, in the C-terminus, at serine 392 (Appella 2001; Appella and Anderson 2001). Phosphorylation of p53 has been shown to prevent its binding to antagonist HDM2, resulting in its stability and upregulation in stressed cells.

Due to critical role of p53 in cell cycle arrest, and its frequent inactivation in human cancers, it has been established as a key regulator of ageing and carcinogenesis. Mouse models with enforced increase in p53 activity have provided contrasting results due to differences in the transgenic p53 allele (Garcia-Cao et al. 2002; Lavigueur et al. 1989; Maier et al. 2004; Tyner et al. 2002). These studies showed that (i) mice with truncated or mutated p53 allele have accelerated ageing and shorter lifespan, and (ii) the mice with multiple copies of the entire p53 locus (super p53) exhibit decreased cancer incidence. Furthermore, the transgenic animals with supernumerary copies of both p53 and its associated regulator $p19^{ARF}$ (super $p19^{ARF}$/p53) exhibited a high degree of tumour resistance and delay in ageing (Matheu et al. 2007). These studies have highlighted the importance of regulation of p53 activity and not just its expression (Liu et al. 2015; Papazoglu and Mills 2007). Furthermore, It has recently been shown to regulate microRNAs that mediate its spectrum of activities in cell cycle arrest, apoptosis and metabolic regulation (Zhang et al. 2014) (Liao et al. 2014; Musilova and Mraz 2015; Penna et al. 2015).

Just as p53 regulates a large number of proteins, it is affected and regulated by a enormous number of factors. One of its strong upstream regulators of p53 is ARF (Alternate Reading Frame) protein coded by INK4a locus on human chromosome 9p21 that also encodes $p16^{INK4A}$, an upstream regulator of pRB (Quelle et al. 1995). Both these proteins have been shown to act as key regulators of replicative senescence, SIPS, OIPS and immortalization of human cells (Kamijo et al. 1997; Quelle et al. 1995; Serrano et al. 1996). ARF was shown to inactivate ubiquitin

ligase HDM2, responsible for degradation of p53 and pRB, resulting in increased level of expression and activation of these proteins. It also functions independent of HDM2 and involves several other interacting proteins including E2F family members, spinophilin, topoisomerase I, Pex19p, cyclin G1, p120 (E4F), WRN helicase c-myc and CARF (Menendez et al. 2003; Martelli et al. 2001; Vivo et al. 2001; Karayan et al. 2001; Sugihara et al. 2001; Zhao et al. 2003; Rizos et al. 2003; Woods et al. 2004; Hasan et al. 2002, 2004, 2008; Qi et al. 2004). It has been established that compared to human cells, mouse cells possess milder tumour suppressor mechanisms and hence undergo spontaneous immortalization in culture. Explanation to such difference in the activity of mouse and human p53 was provided by the study that isolated Pex19p as an ARF interacting partner in mouse cells. It was shown that Pex19p interacts with mouse ARF ($p19^{ARF}$), but not human ARF ($p14^{ARF}$), and inactivate its p53-activating function accounting for weaker p53 activity in mouse cells (Wadhwa et al. 2002b). ARF was also found to interact with a novel 61-kDa serine-rich ubiquitous unique protein coded by human chromosome 4q35. It was named CARF (collaborator of ARF) due to its interaction and collaboration with ARF for activation of p53 function. Targeted siRNA mediated knockdown of CARF resulted in downregulation of ARF expression and its activity that was also translated to downregulation of p53 and $p21^{WAF1}$ expression and activities (Hasan et al. 2002, 2004). The data suggested that CARF is required for ARF function. Furthermore, CARF interacted with p53 causing its stability and activation (Hasan et al. 2004), and HDM2 (Hasan et al. 2008) resulting in its degradation. In a feedback regulation, CARF acts as a transcriptional suppressor of HDM2 and protects itself from HDM2-mediated proteasomal degradation (Cheung et al. 2010; Hasan et al. 2008). It was shown that CARF regulates senescence and carcinogenesis by its dose dependent two-way regulation of DNA damage response. Whereas high level of CARF activated DNA damage response and p53 pathway, its super high levels were shown to inactivate these pathways and lead to malignant transformation of cells (Cheung et al. 2014). Knockdown of CARF on the other hand caused apoptosis depicting that it is an essential protein for cell survival (Cheung et al. 2011, 2014).

p53 has been shown to be regulated by stress chaperone mortalin that is enriched in cancer cells (Deocaris et al. 2013; Wadhwa et al. 2006). Amino-terminus region of mortalin binds to the carboxy-terminus region of p53 (Kaul et al. 2001, 2005; Wadhwa et al. 1998). The small molecules and peptides that bind to mortalin were able to act as binding antagonists resulting in translocation and reactivation of wild type p53 (Deocaris et al. 2007; Grover et al. 2012; Kaul et al. 2005; Wadhwa et al. 2000b, 2002a). Furthermore, an activation of p53 was observed in cells compromised for mortalin expression (Wadhwa et al. 2003; Yoo et al. 2010). It included not only the activation of transcriptional activation function but also control of centrosomal duplication (Kanai et al. 2007; Ma et al. 2006) and apoptotic functions (Lu et al. 2011a, b). Based on these findings, a model on stress-regulation of mortalin-p53 interaction was proposed. Unstressed normal, immortalized and non-malignant cancer cells possess low level of p53 expression and does not interact with mortalin. Genotoxic or environmental stress induces mortalin-p53 interaction leading to inhibition of the apoptotic ability of p53. Physiologically stressed and

malignant cancer cells accumulate p53 (mutant) that is highly phosphorylated and have mortalin-p53 interaction (Lu et al. 2011a, b).

An allelic form of mouse mortalin (mot-1) that differs by two amino acids, M618V and G624R, in the carboxy-terminus substrate-binding domain was earlier shown to induce senescence in mouse immortal cells. By genome sequencing of human mortalin (hmot-2) from Parkinson disease (PD) patients two missense mutants, R126W and P509S, were identified. In comparative functional analysis mouse mot-1 and human PD mutants, R126W and P509S, it was shown that these lack mot-2 functions involved in carcinogenesis. These included p53 inactivation, hTERT/hnRNP-K activation. Of note, mot-1 and PD mutants caused increased level of endogenous oxidative stress, and resulted in decreased tolerance of cells to exogenous oxidative stress. Growth characteristics of hmot-2 and PD mutant revealed that whereas hmot-2 promotes cell cycle progression, PD mots caused cell cycle retardation (Wadhwa et al. 2015). By functional and biochemical assays on protein-protein interactions, it was found that they possess differential chaperoning activities and binding to proteins including RPL-7 and EF-1α proteins. These factors were predicted to mediate the transformation of longevity/pro-proliferative function of hmot-2 to the premature aging/anti-proliferative effect of PD mutants, that operates through their impact on cell cycle checkpoints involved in regulation of cellular senescence and carcinogenesis.

9.7 pRB Checkpoint

Retinoblastoma protein (pRb) is a negative regulator of cell cycle. In its un-phosphorylated form, it binds to E2F family of transcription factors and inactivate their function for cell cycle progression. Phosphorylation of pRB abrogates its interaction with E2F proteins and activate cell cycle progression through G1 to S phase (Nevins 1992; Benevolenskaya and Frolov 2015; Dyson 1994). Several studies have shown that pRB is under-phosphorylated in senescent cells causing them to arrest at the G1 stage of cell cycle (Futreal and Barrett 1991; Stein et al. 1990). Similar to p53, pRB is a target of DNA tumour virus transforming proteins (Ludlow et al. 1989) and is inactivated in large majority of tumours (Shay et al. 1993; Cipressa and Cenci 2013; Jarrard et al. 1999). Introduction of pRB gene into p53/pRB deficient immortal tumour cells induced senescence (Xu et al. 1997). Downstream target of the pRB, the family of E2F transcription factors, is the key regulator of cell cycle progression, apoptosis and a number of other biologic processes. Most recently, it is implicated in regulation of mitochondria-associated genes (Benevolenskaya and Frolov 2015).

Phosphorylation of pRB is regulated by $p16^{INK4a}$ (an inhibitor of the cyclin D-dependent kinase) coded by the CDKN2A locus on chromosome 9p21 that also encodes ARF (Kamijo et al. 1998; Stott et al. 1998). It has been shown to maintain hypo-phosphorylated pRB in senescent human cells. Unlike $p21^{WAF1}$, $p16^{INK4A}$ remains high in late senescent cells (Alcorta et al. 1996; Hara et al. 1991; Reznikoff

et al. 1996; Jarrard et al. 1999; Stein et al. 1990). Introduction of exogenous $p16^{INK4A}$ into normal or immortal human cells resulted in their growth arrest, and induction of premature senescence by ectopic expression of activated Ras or Raf was mediated by $p16^{INK4A}$ (Kato et al. 1998; Serrano et al. 1997; Lin et al. 1998; Zhu et al. 1998). Whereas elevated level of $p16^{INK4A}$ is also responsible for maintenance of a senescent-like state in cells treated with DNA damaging agents (Robles and Adami 1998), its spontaneous loss was associated with lifespan extension in mammary epithelial cells (Brenner et al. 1998; Huschtscha et al. 1998). In Li-Fraumeni syndrome fibroblasts, loss of wild type p53 or $p16^{INK4A}$ caused lifespan extension and, the effects of losing both the p53 and the pRb/$p16^{INK4A}$ pathways were additive (Huschtscha and Reddel 1999). Consistent with the role of p53 and pRb in cellular senescence in vitro, mice with mutations in p53, and pRb or $p16^{INK4A}$ were prone to tumour formation (Donehower et al. 1992; Sharpless et al. 2001), suggesting the role of these proteins in organismal ageing. The level of $p16^{INK4A}$/ARF was elevated when cells were accelerated to age with the exogenous stress (Halvorsen et al. 2000; Krishnamurthy et al. 2004). On the other hand, caloric restriction, known to retard ageing, caused marked reduction (2–16-fold) in age-induced $p16^{INK4A}$/ARF (Krishnamurthy et al. 2004). Induction of ARF expression has been shown to stabilize and increase the activity of p53, resulting in upregulation of $p21^{WAF1}$, which in turn inhibits CDKs and pRB phosphorylation. ARF and $p16^{INK4A}$ are proposed as biomarkers of ageing through their tumour suppression and senescence-inducing functions. Krishnamurthy and colleagues demonstrated a significant increase in expression of the $p16^{INK4A}$ and ARF in most of the tissues in aged mice and rats (Krishnamurthy et al. 2004). They were associated with upregulation of SA-β-gal activity in several tissues. In calorie-restricted animals, increase in lifespan and reduction in age-associated pathologies was correlated with decrease in both $p19^{ARF}$ and $p16^{INK4A}$ expression (Krishnamurthy et al. 2004). Sharpless and colleagues showed that the animals deficient in $p16^{INK4a}$ and/or p53 are developmentally normal, but showed increased frequency of cancer; notably, $p16^{INK4a}$ and p53 double knockout mice have severely shortened lifespan (Sharpless 2004). In contrast, super Ink4A/ARF mice, carrying its extra locus in addition to the endogenous alleles, are more resistant to the development of a variety of chemically-induced tumorigenesis, and have a lower incidence of spontaneous tumours without affecting normal viability or ageing (Matheu et al. 2004, 2007).

Stress induced senescence in human cells is associated with increase in the expression of $p16^{INK4A}$ (Toussaint et al. 2000; Suzuki and Boothman 2008; Serrano and Blasco 2007; Mirzayans et al. 2012). On the other hand, it was shown that ARF is not directly induced by acute DNA damage (Zindy et al. 2003). It mediates the DNA damage response through its effects on HDM2, p53, ATM and ATR (Pauklin et al. 2005). It may interact directly with ATM and/or ATR kinases or may regulate them through TIP60 (Kim and Sharpless 2006). Although the two major checkpoint pathways, p53 and pRB, show overlapping activities to trigger and maintain senescence through activities of $p21^{WAF1}$ and $p16^{INK4A}$, they also work independent to each other and have a vital role in senescence related checkpoint controls, and their loss during cancer development and progression.

Research in last two to three decades has resolved several questions on functional intricacies of in-built cell cycle checkpoints and tumour suppressor mechanisms that regulate limited proliferative capacity of cells and safeguard them against cancer. Further research on feed-back and feed-forward regulation of these cell cycle checkpoints in normal and stressed physiological conditions, their crosstalk with intra- and extra-cellular regulators will be helpful in designing novel strategies for extending functional lifespan of normal, and therapy of cancer cells.

Conflict of Interest The authors declare that they have no competing interests to disclose.

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Chapter 10
Mitochondrial Reactive Oxygen Species in Cellular Senescence

Timothy Nacarelli, Claudio Torres, and Christian Sell

Abstract Mitochondria are central for the maintenance of cellular homeostasis and both cellular dysfunction and aging are linked to mitochondrial dysfunction. Mitochondrial dysfunction is the principle cause of increased levels of reactive oxygen species (ROS) and oxidative stress, which is a key mediator of aging. The cell responds to this stressful stimulus by the induction of the cellular aging-stress response, cellular senescence. Here, we discuss the mechanisms through which mitochondrial ROS promotes senescence. In this context, we will highlight how mitochondrial ROS serves an initiating upstream, or sustaining downstream, role in the induction of senescence. We will also discuss potential interventions to alleviate mitochondrial ROS and delay cellular senescence.

Keywords Aging • Cellular senescence • Mitochondria • ROS

10.1 Introduction: Mitochondria and Cellular Senescence

Mitochondria are essential for normal cellular processes including aerobic metabolism for the production of ATP and critical metabolic intermediates, calcium homeostasis, apoptotic signaling, beta oxidation, and regulations of redox status. Because mitochondria are central to energy metabolism and affect signaling pathways, they also play an important regulatory role in the cell. Specifically, mitochondria respond to cellular signals or altered status by communicating to the nucleus to alter gene expression through retrograde signaling. This is vital not only for adapting cellular energy status, but also for maintaining mitochondrial quality control. Mitochondrial defects are an acknowledged feature of cellular dysfunction in the aging process where the most prominent aspects of mitochondrial dysfunction include reduced function, structural disorganization, and increased production of reactive oxygen species (ROS). These alterations are common in aged tissues

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and are associated with age-related pathologies, yet the mechanisms by which mitochondrial dysfunction promotes cellular aging and tissue deterioration are unclear (Bratic and Larsson 2013; Lee and Wei 2012; Cagin and Enriquez 2015). One possibility is that mitochondrial dysfunction promotes the cellular aging-stress response, cellular senescence. Although senescent cells are metabolically active and viable (Campisi 2013), they exhibit alterations in mitochondrial morphology, function, metabolism, and redox state (Passos et al. 2013; Hutter et al. 2004; Hwang et al. 2009; Ahmed et al. 2010).

Since mitochondria are central to cellular metabolism, it is compelling to speculate the requirement of mitochondrial alterations in senescence. A major detrimental aspect of mitochondria dysfunction is the generation of ROS, which not only leads to cellular damage but also reinforces mitochondrial dysfunction by damaging mitochondrial components, altering mitochondrial metabolism and dynamics, and depleting antioxidant defenses (Bratic and Larsson 2013; Seo et al. 2010). As a result of amplified mitochondrial dysfunction, this stressed cellular state might then signal to promote senescence. In this review, we will focus on how mitochondrial ROS might serve as an effector of cellular senescence. We will also discuss potential therapeutic approaches to improve mitochondrial homeostasis and prevent the pathologic generation of ROS.

10.2 The Generation of Mitochondrial ROS

One of the first theories of aging proposed that free radical-generated ROS insidiously impaired cellular homeostasis and caused aging (Harman 1956). However, demonstration that antioxidants failed to slow the aging process in mammals led to revision of this theory, with mitochondria identified as being the principal endogenous source and target of ROS responsible for cellular aging (Harman 1972). Mitochondria produce ROS as a byproduct of oxidative phosphorylation when electrons, leaked mainly from complexes I and III of the electron transport chain (ETC), reduce oxygen into toxic superoxide anion. The ETC establishes a membrane potential across the inner mitochondrial membrane to control oxidative phosphorylation. Alteration of this membrane potential through hyperpolarization or depolarization accelerates the formation of superoxide anion (Korshunov et al. 1997; Nicholls 2004; Suski et al. 2012; Zorov et al. 2006). ETC-generated superoxide anion can be converted through redox reactions into intermediates and other harmful ROS, such as hydrogen peroxide, peroxynitrite anion, and hydroxyl radical (Turrens 2003; Brand 2010). The mitochondria maintain ROS levels and redox status using an endogenous antioxidant defense system consisting of the enzymes manganese superoxide dismutase (MnSOD), glutathione peroxidase, and thioredoxin 2 (Li et al. 2013b). High levels of ROS damage macromolecules, including mitochondrial DNA (mtDNA), proteins, and lipids; developing a model of vicious cycle where ROS produced in the mitochondria propagates further damage within the cell. The

mitochondrial genome is susceptible to ROS-induced mutagenesis because of its close proximity to ROS, absence of protective histones, and lower fidelity DNA repair system. ROS-induced mutagenesis compromises oxidative phosphorylation by incorporation of defective mtDNA-encoded subunits or altering complex stoichiometry within the ETC. mtDNA encodes 24 mitochondrial translation-related RNAs and 13 proteins that comprise subunits of the ETC complexes and ATP synthase. Also vulnerable to ROS is the tricarboxylic acid (TCA) cycle enzyme aconitase, which attempts to compensate for the production of ROS by slowing TCA cycle and ETC activity. However, slowing TCA cycle activity may not counteract, but worsen the production of ROS by malfunctioning ETC complexes (Bandy and Davison 1990; Shokolenko et al. 2014; Alexeyev 2009). The aforementioned actions form a feed-forward progression of ROS-damaged mitochondria, amplifying the production of ROS and damaging neighboring mitochondria.

10.3 Mitochondrial ROS and Longevity

Despite the general acceptance that mitochondrial dysfunction accelerates the aging process, there is not a clear consensus in the literature that this acceleration is mediated by increased mitochondrial ROS. The most compelling evidence supporting the notion that mitochondrial ROS regulates lifespan, as well as healthspan, comes from mice overexpressing a ROS-scavenging mitochondrial-targeted catalase. These mice, with reduced mitochondrial oxidative damage and mtDNA mutations and deletions, exhibit increased mean and maximal lifespan, as well as resistance to a number of age-related pathologies (Schriner et al. 2005; Dai et al. 2009; Treuting et al. 2008; Dai et al. 2014). Additionally, a noninvasive approach using the mitochondrial-targeted antioxidant SkQ1, has been shown to suppress cardiomyopathy and traits of aging and increase lifespan in mice (Anisimov et al. 2008, 2011; Dai et al. 2014; Manskikh et al. 2015; Skulachev et al. 2009). However, evidence against the pathologic role of mitochondrial ROS is provided by some early studies in 'mutator mice' harboring an exonuclease proofreading-deficient mitochondrial DNA polymerase gamma. Although these mice exhibited an accelerated aging phenotype driven by mtDNA mutations and deletions, they did not exhibit increased levels of ROS (Kujoth et al. 2005; Vermulst et al. 2008; Trifunovic et al. 2005; Hiona et al. 2010). However, recent studies using more sensitive approaches did detect increased mitochondrial ROS and oxidative damage in 'mutator mice' (Kolesar et al. 2014; Logan et al. 2014). In support of mitochondrial ROS as a driver of pathologic effects in this model, overexpression of mitochondrial-targeted catalase in 'mutator mice' alleviated mtDNA deletions, mitochondrial oxidative damage, and age-dependent cardiomyopathy (Dai et al. 2010, 2014). Additionally, antioxidant treatment attenuated somatic progenitor cell mtDNA mutagenesis and loss of self-renewal capacity in 'mutator mice' (Ahlqvist et al. 2012). These studies support the role of mitochondrial ROS as a driver of mitochondrial dysfunction and aging.

10.4 Mitochondrial ROS as an Inducer of Senescence

Although it is currently debated whether and how mitochondrial-derived ROS serves as an inducer of senescence, senescence induction by ROS is clear. ROS has been identified as a key player in establishing replicative, genotoxic stress and oncogene-induced senescence (Lu and Finkel 2008; Nair et al. 2015; Colavitti and Finkel 2005). In addition, exposure to exogenous hydrogen peroxide induces senescence through mechanisms involving genomic DNA damage (Duan et al. 2005; von Zglinicki et al. 2005; Borodkina et al. 2014), transforming growth factor-β (TGF-β) (Frippiat et al. 2001; Yu et al. 2009; Hassona et al. 2013), and $p38^{MAPK}$ signaling (Frippiat et al. 2002; Zdanov et al. 2006; Barascu et al. 2012; Iwasa et al. 2003). The generation of intracellular ROS appears to be a much earlier event than the onset of other senescent phenotypes as shown in a time-progression analysis of replicative senescence in human fibroblast (Kim et al. 2013). A significant increase in mitochondrial superoxide anion has been reported with increasing population doublings and replicative senescence in human fibroblasts, mesenchymal stem cells and vascular smooth muscle cells (Passos et al. 2007, 2013; Bielak-Zmijewska et al. 2014; Estrada et al. 2013; Nacarelli et al. 2015; Lerner et al. 2013). The mitochondria undergo distinct changes that may support the production of ROS during the induction of senescence. For instance, increased mitochondrial respiration has been detected in replicative, oxidative stress, and oncogene-induced senescence (Hutter et al. 2004; Kaplon et al. 2013; Quijano et al. 2012; Nacarelli et al. 2015). Not only are mitochondria the suspected culprits in the generation of ROS in promoting senescence, but also the immediate target. In replicative senescence of human fibroblasts, the most evidence of ROS-induced oxidative damage was detected within the mitochondria (Ahmed et al. 2010). This preferential accumulation of oxidative damage not only suggests that the mitochondria are a ROS source and target, but also that mitochondria are subject to reduced quality control in senescence.

Several studies have highlighted mitochondrial ROS in altering susceptibility to senescence. HIV highly active antiretroviral therapy nucleoside reverse transcriptase inhibitors (NRTIs), which are known to insult the mitochondria as an off-target effect and increase susceptibility to the development of age-related pathologies, increase ROS and induce senescence in human fibroblasts (Caron et al. 2008; Nacarelli et al. 2015). Mice deficient for the mitochondrial superoxide anion-scavenger MnSOD are more susceptible to oxidative stress and a range of age-related pathologies, and exhibit an abbreviated lifespan. In vivo and in vitro studies in these mice show evidence of increased vulnerability to oxidative stress-induced senescence (Velarde et al. 2012; Treiber et al. 2011). Contrary to the idea that MnSOD promotes longevity, overexpression of MnSOD fails to increase lifespan in mice (Jang and Van Remmen 2009). This is not surprising since overexpressing MnSOD may generate more hydrogen peroxide that will serve as a precursor for more harmful ROS (MacMillan-Crow and Crow 2011). Interestingly, 'mutator mice' exhibit increased p16 expression in their hearts. Remarkably, overexpressing

the mitochondrial-targeted catalase in these mice reduces expression of p16 in their heart, attenuating their age-dependent cardiomyopathy (Dai et al. 2010). It is possible that the mitochondrial-targeted catalase alleviates mitochondrial oxidative stress that induces senescence and contributes to the pathologic phenotype of cardiomyopathy.

10.5 ETC Dysfunction in Generating Mitochondria ROS

Given a severe insult, mitochondria can produce enough ROS to cause genomic instability and telomere attrition, providing a setting conducive for senescence (Liu et al. 2002). Disrupting the mitochondrial ETC and oxidative phosphorylation by severely uncoupling the proton gradient using *p*-trifluoromethoxyphenylhydrazone (FCCP) to produce high levels of mitochondrial ROS accelerates telomere attrition and induces oxidative stress-induced senescence in human fibroblasts (Stockl et al. 2007). Supporting a role for mitochondrial-generated ROS in replicative senescence, mild uncoupling to lower mitochondrial ROS reduced the rate of telomere shortening and extended replicative lifespan in human fibroblasts. The fact that the mitochondrial-targeted antioxidant mitoquinone (mitoQ) yielded the same effect in human fibroblasts pinpoints mitochondrial ROS as a factor in replicative senescence (Saretzki et al. 2003; Passos et al. 2007). Mild uncoupling of the mitochondria to reduce mitochondrial ROS was able to protect against hydrogen peroxide-mediated oxidative stress-induced senescence in human fibroblasts (Cho et al. 2014). This approach appears to be therapeutic at the organismal level, as mildly uncoupling mitochondria reduced mitochondrial ROS and oxidative damage in various tissues and extended lifespan in mice (Caldeira da Silva et al. 2008). One may speculate whether senescence was alleviated within the tissues of these mice during physiologic aging.

Besides uncoupling, direct damage to the ETC can lead to the production of mitochondrial superoxide anion from complexes I and III. Indeed, inhibiting complex I using rotenone or complex III using antimycin A increased mitochondrial ROS and induced senescence in human fibroblasts (Velarde et al. 2012; Moiseeva et al. 2009; Stockl et al. 2006). Additionally, disrupting complex I by silencing its assembly factor NDUFAF1 increased mitochondrial ROS and induced senescence in human fibroblasts. Supporting a role in longevity, efficient assembly of complex I is associated with lower ROS levels and a feature of young, rapamycin-fed, and long-lived mice (Miwa et al. 2014). The ETC can also be altered in response to signaling to provoke the generation of mitochondrial ROS that induces senescence. For instance, the senescence-promoting proinflammatory cytokine transforming growth factor-β1 inactivates glycogen synthase kinase 3 to suppress complex IV activity of the ETC that results in the generation of mitochondrial ROS and induction of senescence (Byun et al. 2012). Another cascade in mitochondrial ROS-induced senescence is in response to angiotensin II-mediated NADPH oxidase activation in vascular smooth muscle cells. Activation of NADPH oxidase by angiotensin

II elicits a feed-forward mechanism where the production of superoxide anion from the mitochondria further activates NADPH oxidase and causes senescence. Suppressing mitochondrial ROS in this setting by mild inhibition of complex I using rotenone or a mitochondrial-target antioxidant prevented this cross-talk and senescence (Mistry et al. 2013).

10.6 Impaired Mitochondrial Dynamics in Generating Mitochondrial ROS

Mitochondria are dynamic organelles that continually undergo fusion and fission events to alter their morphology and organization. These processes, which are required for maintenance and quality control of the mitochondria, facilitate the degradation of dysfunctional mitochondria through autophagy, a process termed mitophagy. Altering mitochondrial fission has been shown to impact ROS levels and senescence (Seo et al. 2010). Inhibiting mitochondrial fission by knocking down fission protein 1 (Fis1) caused mitochondrial elongation, increased ROS levels, DNA damage, and senescence (Lee et al. 2007). Similar effects were observed when mitochondrial fusion and enlargement were aberrantly stimulated using deferoxamine (Yoon et al. 2006). Likewise, enlarged mitochondrial morphology has been observed in replicative senescence (Hwang et al. 2009). Senescence has also been studied in response to mitochondrial insults that impair dynamics. Disruption of mitochondrial dynamics and mitochondrial fragmentation induced by cigarette smoke extract increased mitochondrial ROS and induced senescence. Mitochondrial ROS appeared to be required for the induction of senescence in this setting, as these responses were prevented in the presence of a mitochondrial-targeted antioxidant (Hara et al. 2013). Mitochondrial ROS-induced senescence in response to cigarette smoke extract was also ameliorated when mitophagy was stimulated to remove dysfunctional mitochondria (Ito et al. 2015). These studies highlight the importance of quality control to eliminate aberrant mitochondria with the potential to increase ROS and put the cells at risk for senescence.

10.7 Metabolic Disruption in Promoting Mitochondrial ROS

Increased mitochondrial ROS in the induction of senescence has been studied in regards to glucose metabolism. Although senescent cells are metabolically active, the glycolytic status within senescent cells is unclear. Increased glycolysis has been reported in replicative and radiation-induced senescent cells (Bittles and Harper 1984; Goldstein et al. 1982; Liao et al. 2014; James et al. 2015). However, oncogene-induced senescence is characterized by a metabolic shift from glycolysis to the TCA cycle due to decreased expression of glycolytic proteins and increased

pyruvate oxidation (Kaplon et al. 2013; Li et al. 2013a). Exposing cells to a high concentration of glucose increased mitochondrial ROS and caused oxidative stress-induced senescence (Ksiazek et al. 2008; Park et al. 2014). Unfortunately, these studies did not address whether high glucose aberrantly stimulated glycolysis or had a direct effect on the mitochondria. Similarly, supplying the cell with excessive TCA cycle intermediates by overexpressing sodium-dependent dicarboxylate cotransporter 3, disrupted mitochondrial ETC activity, increased ROS levels, and caused oxidative stress-induced senescence in human fibroblasts (Ma et al. 2014). Interestingly, high levels of the TCA cycle intermediate fumarate lowered antioxidant defenses by inactivating glutathione and caused oxidative stress-induced senescence (Zheng et al. 2015). These results support the idea that an altered metabolism or altered levels of metabolites are capable of damaging mitochondria and increasing ROS to induce senescence. Of course it is possible that mitochondrial dysfunction alters glucose metabolism in a way that promotes the generation of mitochondrial ROS. For instance, it is suspected that mitochondrial dysfunction accounts for transcriptional and metabolic changes in the TCA cycle during replicative senescence in *Saccharomyces cerevisiae* (Kamei et al. 2014).

10.8 Senescence Induction of Mitochondrial ROS: p53 Effector Response

Although discussed up to this point as an upstream effector of senescence, elevated mitochondrial ROS might also represent a downstream mechanism to maintain the senescence phenotype. Senescence induction by the p53/p21 pathway as part of the DNA damage response increased and sustained ROS production from the mitochondria, forming a positive feedback mechanism, whereby further DNA damage and p53/p21 pathway activation served to maintain senescence (Passos et al. 2010). p53 can serve as a major effector in establishing high levels of ROS following senescence induction, by altering mitochondria redox status. Elevated p53 can transcriptionally downregulate mitochondrial MnSOD, the mitochondrial antioxidant that dismutates mitochondrial superoxide anion into hydrogen peroxide. Also, MnSOD ROS-scavenging activity can be impaired by physical interaction with p53 following localization of p53 to the mitochondria (Lebedeva et al. 2009; Pani and Galeotti 2011). p53 also serves a mitochondrial pro-oxidant role by promoting p66Shc, which translocates to the mitochondria and generates ROS by transferring electrons from cytochrome c to molecular oxygen (Pani and Galeotti 2011; Galimov et al. 2014; Giorgio et al. 2005). p66Shc also is responsive to ROS and has been shown to increase at the mRNA and protein level during oxidative stress-induced senescence in fetal bovine fibroblasts (Favetta et al. 2004). Suggesting a role in longevity, mice deficient for p66Shc are less susceptible to oxidative stress and age-related pathologies (Migliaccio et al. 1999; Berry and Cirulli 2013). These mice accumulate fewer senescent cells in their thymus during physiological aging,

and embryonic fibroblasts from these mice are resistant to oxidative stress-induced senescence, further supporting the notion that these mice are protected against oxidative stress (Gambino et al. 2013). Mitochondrial autophagy and dynamics can also be impaired by p53, leading to the generation of mitochondrial ROS. For instance, in replicatively senescent mouse embryonic fibroblasts, cytosolic p53 prevents mitochondrial localization and action of parkin, an E3 ubiquitin ligase that facilitates autophagic clearance of dysfunctional mitochondria (Hoshino et al. 2013).

10.9 Senescence Induction of Mitochondrial ROS: Metabolic Disruption

Oncogene-induced senescence is also triggered by a p53 response that promotes the production of mitochondrial ROS. For example, ras-mediated oncogene-induced senescence entails p53-dependent mitochondrial dysfunction that is defined by increased mitochondrial mass and production of superoxide anion in human fibroblasts (Moiseeva et al. 2009; Lee et al. 1999). Supporting the notion of altered mitochondrial function, ras-mediated senescence increases the rate of mitochondrial respiration and the proteins that support this, particularly mitochondrial pyruvate dehydrogenase (Quijano et al. 2012; Li et al. 2013a). Pyruvate dehydrogenase has also been identified as a key factor in mediating increased mitochondrial respiration and ROS in oncogenic $BRAF^{V600E}$-induced senescence. This senescent phenotype was maintained by elevated pyruvate dehydrogenase activity, which increased pyruvate oxidation and sustained a high level of mitochondrial respiration (Kaplon et al. 2013). Interestingly, pyruvate dehydrogenase, itself, is capable of generating ROS from the mitochondria (Quinlan et al. 2014). It is currently unknown whether pyruvate dehydrogenase activity is increased in replicative and oxidative stress-induced senescence. Mitochondrial metabolism might also become dysregulated in such a way that increases ROS in senescent cells through feedback between p53 and the mitochondrial $NAD(P)^+$-dependent malic enzyme (ME2). Through oxidative decarboxylation of malic acid into pyruvate within the mitochondria, ME2 replenishes the mitochondrial NADPH pool that is required by endogenous mitochondrial antioxidants. ME2 can be transcriptionally repressed by p53, leading to elevated levels of ROS that activate p53 in an AMPK-dependent manner. Since ME2 reciprocally represses p53, knocking down ME2 induces p53-dependent senescence (Jiang et al. 2013; Korge et al. 2015). It is unclear whether increased mitochondrial ROS in these settings serves as a driver in sustaining the senescent phenotype, rather than an effect. Nonetheless, it is evident that an altered mitochondrial function that supports the production of ROS is a part of the metabolic phenotype of senescent cells.

10.10 Mitochondrial ROS Interventions for Suppressing Cellular Senescence

Given its impact on senescence, mitochondrial ROS could be targeted as a strategy to provide longevity assurance. The most direct strategy might make use of the aforementioned mitochondrial-targeted antioxidants. However, the most effective and long-term intervention would be an intervention that improves mitochondrial homeostasis and provides resistance against mitochondrial stress. Our laboratory has shown that senescence is delayed and replicative lifespan is increased in human fibroblasts treated long term with 1 nM rapamycin, a pharmacologic inhibitor of mTORC1. Notable features of these rapamycin-treated cells included improved mitochondrial homeostasis and diminished ROS (Lerner et al. 2013). Other studies have also highlighted the suppression of senescence by rapamycin (Demidenko et al. 2009; Pospelova et al. 2012). Rapamycin treatment also provides resistance to mitochondrial insults, such as ethidium bromide or NRTI treatment, which increase ROS levels and susceptibility to senescence (Nacarelli et al. 2014, 2015). These results may help to explain the benefits of rapamycin in slowing aging and extending organismal lifespan among various species, and protecting against age-related pathologies in disease mouse models (Johnson et al. 2013; Ehninger et al. 2014). Rapamycin is thought to converge on similar longevity-extending pathways as caloric restriction, a well-known intervention that also improves mitochondrial function, and protects against age-related diseases (Colman et al. 2009; Bratic and Larsson 2013).

Interestingly, at least some aspects of the longevity features and benefits of caloric restriction can be obtained through restricting the amino acid L-methionine in rodents. A targeted reduction of methionine synthase to reduce methionine levels has also been shown to extend lifespan and increase stress resistance in *S. cerevisiae* and human fibroblasts (Johnson and Johnson 2014). A major effect of L-methionine restriction appears to be the reduction of mitochondrial ROS generation and oxidative stress (Sanchez-Roman and Barja 2013). Remarkably, restricting L-methionine in cell culture media suppressed the generation of mitochondrial ROS and oxidative damage, delayed senescence, and extended replicative lifespan in human fibroblasts (Koziel et al. 2014). These interventions provide mechanistic insight into how improving mitochondrial homeostasis and sustaining low levels of mitochondrial ROS could support longevity pathways and delay senescence.

10.11 Conclusion

Mitochondria maintain cellular homeostasis and are critical determinants of cellular longevity. One detrimental effect of mitochondrial dysfunction is the generation of superoxide anion, which gives rise to high levels of ROS. High levels of ROS

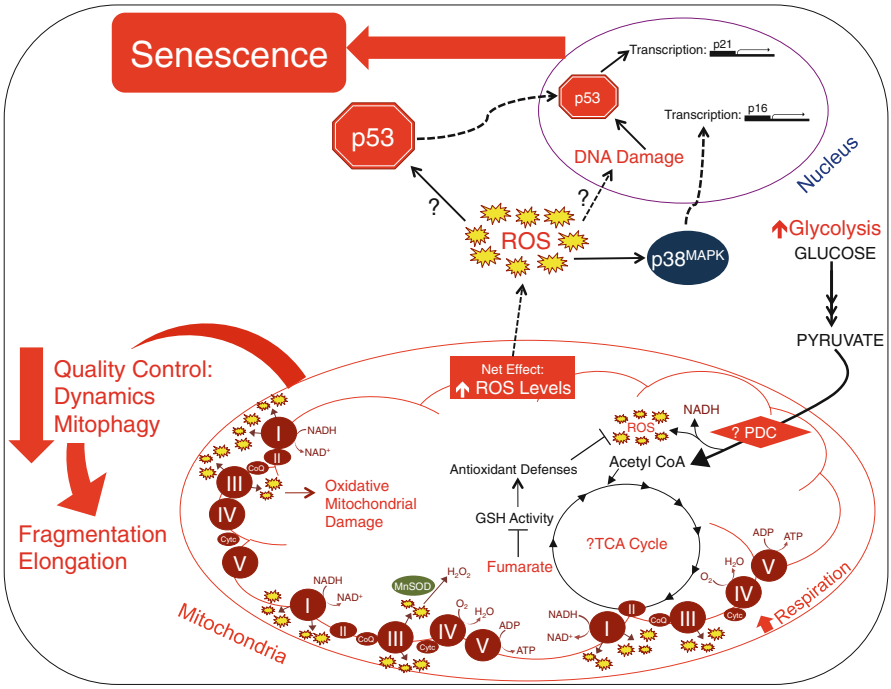

Fig. 10.1 Mitochondrial ROS as an inducer of senescence. Synthesis of events that increase mitochondrial ROS in the induction of cellular senescence

damages macromolecules and establish a stressful cellular environment. This stress stimulus may induce senescence, promoting aging. In Figs. 10.1 and 10.2, we put forth a synthesis of the mechanisms by which mitochondrial ROS initiates or helps maintain senescence. Alterations in the mitochondria that support the generation stress-inducing ROS include ETC dysfunction, altered dynamics that impair quality control, and metabolic disruption. Given a non-mitochondrial stimulus, senescent cells can also generate signals as a mechanism to maintain the senescence phenotype to induce mitochondrial ROS. This encompasses effector responses of p53 and changes in metabolism. Various interventions that alleviate increased mitochondrial ROS levels, such as rapamycin treatment or methionine restriction, might be effective in preventing senescence and extending longevity. These methods might succeed in preventing cascades initiated, amplified, or maintained by mitochondrial ROS in promoting senescence. Although senescence is irreversible, these interventions may also assist in confining the pathological effects of senescent cells through a reduction in SASP, the senescence-associated secretory phenotype that acts to

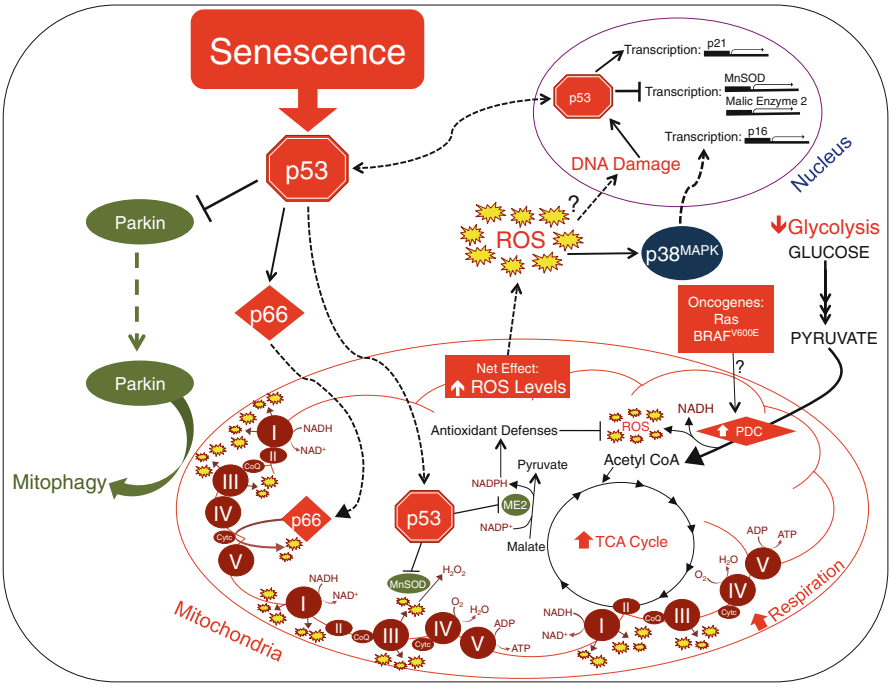

Fig. 10.2 Senescence induction of mitochondrial ROS. Synthesis of events mediating increased levels of mitochondrial ROS following the induction of cellular senescence

promote a pro-inflammatory state. With mitochondrial ROS being a common target, these therapeutic approaches are important in understanding age-promoting stress signaling and ways to decelerate senescence.

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Chapter 11
Cellular Aging and Tumor Regulation

Andreas Simm, Barbara Seliger, and Lars-Oliver Klotz

Abstract Aging is the basis of most of the degenerative diseases in the elderly. These diseases can be defined as loss of function diseases. On the level of a cell, senescence can be one driving force of such diseases. In contrast, cancer is, on the one hand, associated with age as well, but can be seen as a gain of function disease. Cellular senescence can act to prevent cell proliferation and is believed to inhibit cancer growth. Interestingly, this tumor defense system can stimulate tumor formation at old age, if senescent cells start to accumulate. The connection between cellular senescence is described on the level of intracellular cell signaling as well as on the level of the immune system.

Keywords Replicative senescence • Cell cycle • Brain • Age-related diseases • Neurodegeneration • Cancer

11.1 History

The history of the discovery of cellular senescence is connected with the problem of tumor formation. The German biologist, August Weismann, proposed in 1881 the hypothesis that aging in somatic cells of higher metazoans is based on the limited abilities of these cells to divide and to repair damage. Only cells of the germ line retain an unlimited replicative potential. Interestingly, it was mentioned in 1908 that cancer cells differ from other epithelial cells in respect to the power of indefinitely continued division (Calkins 1908). Experimental proof of these hypotheses using

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isolated cells was not possible at that time due to uncontrolled contaminations. The first primary cell experiments of the American biologist Ross Harrison (1907) all ended with bacterial infections after some days. It was the French surgeon and noble prize winner Alexis Carrel, who introduced aseptic methods into cell biology. On the other hand, based on long-term experiments with chicken cells he also set up a dogma that cells by themselves – e.g. outside of the body – do not age. Rather, cellular aging may only take place in the context of the organism, i.e. within the body. This dogma was further supported by early established long-lived cell lines from short-lived mice indicating that isolated cells by themselves do not age. This phenomenon can now be explained by the high frequency of spontaneous transformation of primary rodent cells.

It was the work of Leonard Hayflick and Paul Moorhead, who analyzed primary embryonal human lung fibroblasts, which disproved this dogma. They could clearly show that primary human fibroblast undergo an aging process after about 50 population doublings, which is now known as replicative senescence (Hayflick and Moorhead 1961). This was indeed the real starting point of cellular biogerontology. During the following years, a great deal of discussion was going on about the molecular mechanisms of this aging process. Whereas one group of people believed in a genetic program (an aging program), the others believed in the accumulation of molecular damage as the driving force of cellular senescence (aging as a stochastic process). During this time, aging on the level of a cell was mostly believed to be a major pathophysiological process. Cells age, die and thereby limit the lifespan of the individual. Parallel in time, many people investigated cellular death. Whereas cell necrosis was shown to be a major pathophysiological process, the detection of cellular apoptosis clearly indicated a physiological role of cellular death within a multicellular organism. Analogously, a physiological role of cellular senescence was seen as a possibility as well. Cellular senescence as a defense mechanism against tumor formation was the most popular example for this.

11.2 Cell Senescence and Tumor Formation

In a remarkable article published in 1983, Hartmut Land, Luis F. Parada and Robert Weinberg could show that transformation of primary rat embryonic fibroblasts into tumor cells needs at least two steps: immortalization of cells and cellular transformation. In their work they demonstrated that these fibroblasts become only tumorigenic if two oncogenes, a myc gene together with the ras gene, are introduced in parallel (Land et al. 1983). Transfection with oncogenic Ras alone will trigger senescence. In normal cells, Ras triggers proliferation, but these cells expressing oncogenic Ras enter into a permanent cell-cycle arrest, accompanied by overexpression of the senescence-associated genes $p16^{INK4a}$ and $p19^{ARF}$ (Serrano et al. 1997). C-Myc is a transcription factor and targets the catalytic subunit of telomerase, which can be responsible for the immortalizing effects (see below). Interestingly, in cooperation with $p19^{ARF}$ and p53, c-myc can induce apoptosis as well (Bringold and Serrano

2000). These data indicate that oncogenic transformation (by oncogenes like myc and ras) and cell senescence are more closely connected than previously believed.

Replicative senescence itself can be induced by several mechanisms with the telomere shortening process as the best investigated one. Telomeres are long repetitive nucleotide sequences (multimers of TTAGGG in vertebrates), which cap the ends of linear chromosomes. In combination with associated proteins, this structure protects chromosome ends from damage. Due to the DNA replication process, telomeres are shortened after every cell division. Reaching a critical length, a DNA damage response is triggered and replicative senescence is induced. Stem cells as well as germ cells have the possibility to extend the length of the telomeres through the activity of an enzyme, telomerase. Besides this function, this enzyme may also have a protective role in mitochondria (Ale-Agha et al. 2014). Interestingly, telomerase is re-expressed in tumor cells as well. Many stimuli that induce tumor formation are known to induce cellular aging, too. Oxidative stress, oncogene induction and DNA damage can both trigger tumor growth and cellular aging. In light of this view, cellular senescence is thought to be a tumor defense system. Loss of the senescence induction, for example by knocking down the $p16^{INK4A}/p19^{ARF}$ tumor suppressors, which are required for cellular senescence, significantly exacerbated the tumor formation rate in mice (Sherr 2001).

But how can cellular senescence inhibit tumor formation? The underlying idea is simple: cells which display prolonged proliferation (and can maybe not be regulated any more) will lose their telomeres fast, so that the inhibition of cell proliferation by replicative senescence can take place. Therefore, this process prevents cancer by arresting the growth of potentially oncogenic cells. To confirm this and to avoid cell culture artefacts, it is important to detect such cells in vivo as well. The detection of a senescence associated beta-galactosidase in vitro and in vivo was the basis of such an assay (Dimri et al. 1995). Indeed, in liver samples from pathology, an increasing percentage of replicative senescent liver cells in vivo from normal liver to chronic hepatitis and hepatocellular carcinoma was observed (Paradis et al. 2001). In a mouse model of colorectal cancer, cell senescence using the senescence-associated β-galactosidase activity assay was detected in vivo as well (Liu et al. 2013). Unfortunately, replicative senescence not only inhibits but can also induce tumor growth. This was first shown using senescent fibroblasts in an epithelial cell growth model in vivo. Preneoplastic epithelial cells injected into nude mice alone or together with young fibroblasts did not form tumors. In contrast, these cells did form large lethal tumors in the presence of senescent fibroblasts. This effect could be shown with preneoplastic human keratinocytes as well (Krtolica et al. 2001). It was demonstrated later that the deleterious effects of senescent cells is based on the interaction with the tissue microenvironment.

Senescent fibroblasts gain a senescence-associated secretory phenotype (SASP). A set of SASP factors were identified, like soluble signaling factors (mostly proinflammatory cytokines and growth factors), secreted proteases (inducing tissue remodeling), and secreted extracellular matrix components (e.g. fibronectin) (Coppe et al. 2010). The SASP promotes cell proliferation in different tumors (breast cancer, prostate cancer, melanoma), induces cell mobility (invasion, migration, metastasis)

and affects leukocyte infiltration (Coppe et al. 2010). In summary, the SASP can induce a change in the tissue microenvironment promoting tumor progression. Cellular senescence clearly shows a Yin and Yang principle, also known as antagonistic pleiotropy. Whereas it can block tumor growth at the beginning, the accumulation of senescent cells in the tissue may promote tumor growth later in life. This interaction of tumor growth and cellular senescence can also be seen on the level of intracellular signaling.

11.3 Cellular Signaling in Tumorigenesis and Senescence

Several stimuli inducing a transition of cells into a state of senescence may also support cell proliferation and trigger processes contributing to carcinogenesis. Such stimuli include DNA damage and the expression of oncogenes. Whereas DNA damage is a prerequisite for tumor initiation, and whereas an impaired regulation of signaling proteins that are protooncogene products is involved in tumor promotion and progression, both can contribute to cell cycle arrest and senescence (see Fig. 11.1). The tumor suppressor p53 appears to be at the crossroads of DNA damage-induced and oncogene-induced senescence (Itahana et al. 2001).

11.3.1 DNA Damage, Telomere Shortening and Senescence

DNA damage, via stimulation of p53 activity and transcriptional upregulation of the cell cycle-inhibitory protein $p21^{Waf1}$, may cause cell cycle arrest – be it to provide time for DNA repair or the induction of apoptosis. An alternative consequence is the induction of senescence, i.e. a permanent cell cycle arrest. $P21^{Waf1}$ is an inhibitor of cyclin-dependent kinases (CDK) and interrupts CDK-dependent phosphorylation of the retinoblastoma protein, RB, causing the cell cycle to stop in G1 phase due to RB complexing E2F transcription factors, whose activity would be required to initiate the transcription of genes coding for proteins involved in later steps of the cell cycle (Sherr 1996). Interestingly, a DNA damage response resulting in p53 activation may also be elicited by telomere shortening occurring as a result of DNA replication in mammalian somatic cells devoid of telomerase. Extensive telomere shortening will thus render cells permanently arrested and unresponsive to mitogen treatment, i.e. senescent, as a result of "replicative senescence" (Itahana et al. 2001).

Next to the detection of "senescence-associated" (SA) β-galactosidase (βgal) activity in senescent cells (see above), the identification of elevated levels of CDK inhibitors such as $p15^{Ink4b}$, $p16^{Ink4a}$, $p21^{Waf1}$ and $p27^{Kip1}$ is commonly accepted as a marker of cellular senescence (Carnero 2013). While SA-βgal activity is detectable owing to a higher lysosomal content of affected cells, the induction of the above CDK inhibitors is due to signaling processes stimulated in cells prior to their entering the senescent state. These signaling cascades are not specific to

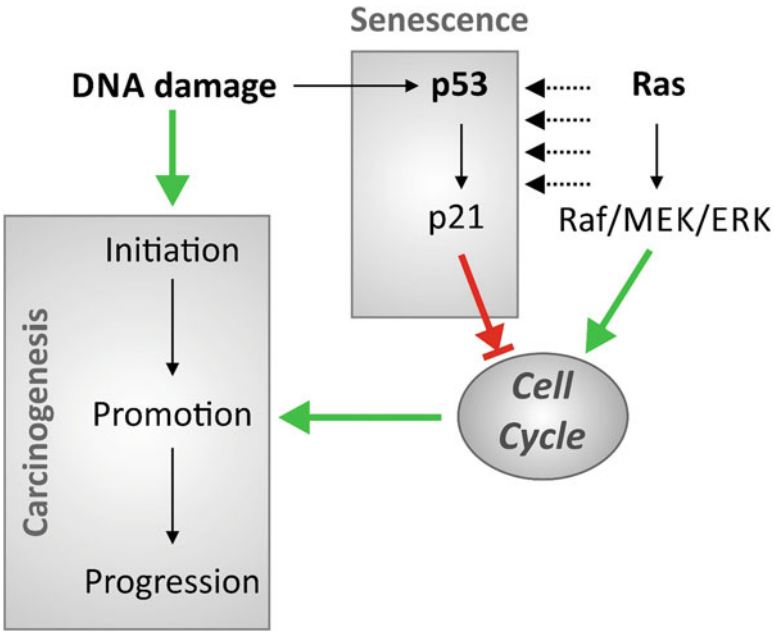

Fig. 11.1 Carcinogenesis vs. Senescence. DNA damage may initiate carcinogenesis, unless DNA damage-induced upregulation of p53 attenuates this process through induction of repair processes. Moreover, p53 will inhibit cell cycle progression, e.g. by upregulating the expression of the cyclin-dependent kinase inhibitor, p21. Permanent cell cycle arrest constitutes a landmark in senescence. Activation or overexpression of oncogenic proteins such as constitutively active Ras will promote cell cycle progression and contribute to carcinogenesis at the level of tumor promotion and progression. In addition, constitutive oncogene activation stimulates p53-dependent senescence (oncogene-induced senescence, *dashed arrows*)

senescence, as already shown for the p53-p21^{Waf1} axis – but they are multiple rather common stress-responsive pathways that are stimulated in parallel and converge in the observed senescence-associated induction of the expression of cell cycle arrest-inducing proteins (Munoz-Espin and Serrano 2014).

11.3.2 Oncogene-Induced Senescence

Permanent cell cycle arrest can also be induced by overexpression of oncogenes, resulting in "oncogene-induced senescence" (OIS). The overexpression of oncogenic variants of Ras (Serrano et al. 1997), Raf (Zhu et al. 1998) and other proteins was observed to cause cell cycle arrest. Again, the stimulation of CDK inhibitor synthesis is believed to mediate these effects (Munoz-Espin and Serrano 2014), and some of the signaling cascades from oncogene activation to CDK inhibitor upregulation will be mentioned below.

Stimulation of a mitogenic signaling cascade by overexpression of an oncogene such as Ras will, at first, cause enhanced proliferation through the classical cascade known to be triggered upon Ras activation, which leads to a stimulation of transcription via activation of Raf kinases, MEK (MAPK/ERK kinases), ERK (extracellular signal-regulated kinases) and ERK-dependent phosphorylation of transcriptional regulators (see Whitmarsh and Davis 1996; Kyriakis and Avruch 2012 for review). As a result of ERK activation, the transcriptional upregulation of cyclin production to stimulate cell cycle progression will be initiated (Meloche and Pouyssegur 2007). Moreover, ERK activation sensitizes metabolic enzymes such as carbamoyl phosphate synthetase II to prepare for an upcoming elevated nucleotide requirement (Graves et al. 2000), or the loss of gap junctional intercellular communication in order to allow for autonomous cell duplication (Warn-Cramer et al. 1998; Melchheier et al. 2005). *Constitutive* or *intense* stimulation of Ras (Serrano et al. 1997) or MEK (Lin et al. 1998) signaling, however, leads to an upregulation of cell cycle-inhibitory factors, such as p53 and $p16^{Ink4a}$. These will accumulate and bring the cell cycle to a halt, in part permanently, thereby inducing senescence. In fact, deletion of p53 or CDK inhibitors will prevent cell cycle arrest and allow the cells to undergo transformation (Serrano et al. 1997; Lin et al. 1998).

Ras overactivation not only stimulates Raf-MEK-ERK-signaling to result in transcriptional regulation, but also enhances the activation of other mitogen-activated protein kinases (MAPK), such as $p38^{MAPK}$, or of phosphoinositide 3′-kinase (PI3K)-dependent signaling (Karnoub and Weinberg 2008). Nevertheless, ERK2 was recently demonstrated to be essential for Ras-induced senescence and upregulation of CDK inhibitors in murine embryonic fibroblasts (Shin et al. 2013): the authors demonstrated that ERK2 mediates (i) Ras-induced $p38^{MAPK}$ activation and phosphorylation/activation of Ets transcription factors, which are known to be activated by ERKs as well as $p38^{MAPK}$ (Yordy and Muise-Helmericks 2000), and to stimulate the expression of $p16^{Ink4a}$ (Ohtani et al. 2001). (ii) They further demonstrated that ERK2 mediates the activation of transcription factors c-Jun and Fra under these conditions, resulting in an upregulation of $p14/19^{Arf}$, an inhibitor of Mdm2, which is a negative regulator of p53 (Harris and Levine 2005). Upregulation of $p14/19^{Arf}$ then results in stabilization of p53 and p53-dependent upregulation of $p21^{Waf1}$. (iii) The authors also suggest a role of mammalian target of rapamycin (mTOR) in mediating Ras-ERK2-induced senescence.

11.3.3 Stress-Induced Premature Senescence

As demonstrated above, DNA damage (also in the form of telomere erosion) and oncogene overactivation may induce cellular senescence. Interestingly, both DNA damage and oncogenic signaling may be elicited in cells exposed to stressful stimuli, such as reactive oxygen species (ROS), ultraviolet radiation, or xenobiotics. For example, various ROS (Klotz 2014), ultraviolet radiation (Klotz et al. 2001; Schieke et al. 2004), xenobiotics such as quinones (Klaus et al. 2010; Klotz

et al. 2014), transition metal ions (Eckers and Klotz 2009; Eckers et al. 2009; Hamann et al. 2014a) or metalloid compounds such as arsenite (Hamann and Klotz 2013; Hamann et al. 2014b) all stimulate receptor tyrosine kinase-like signaling, leading to modulation of MAPK and/or of PI3K-dependent cascades. Therefore, an exposure of cells to such stimuli should be capable of imitating both DNA damage or oncogene-induced senescence and cause stress-induced premature senescence (SIPS). This was indeed demonstrated to be the case for a repeated exposure of cells to hydrogen peroxide (Dumont et al. 2000; Toussaint et al. 2000), to ultraviolet-B radiation (Chainiaux et al. 2002) or to copper ions (Boilan et al. 2013).

11.3.4 Interfering with Senescence Signaling: A Therapeutic Approach?

Senescence may, like apoptosis, be regarded as an endogenous anti-tumorigenic protective mechanism. Accordingly, therapeutic approaches are being evaluated to induce senescence in cancer therapy. Owing to the similarities in signaling between senescence and carcinogenesis outlined above, this is quite a bold approach, and the line between induction of oncogenesis and senescence may be thin. Nevertheless, testing of potential pharmacological strategies is underway, and approaches include a pharmacological stabilization of p53, p53 reactivation, the use of low molecular weight CDK inhibitors (all imitating p53 induction and CDK inhibitor upregulation in senescent cells), telomerase inhibition or inhibition of the lipid phosphatase, PTEN, to stimulate PI3K signaling (Nardella et al. 2011).

11.4 Cellular Senescence in the Immune System and Tumor Growth

An interesting model of senescence affecting tumor growth is the immune system. Especially in patients with immune deficiency, as association can be seen with cancer risk especially with infection-related cancers (Grulich and Vajdic 2015). The aging of the immune system, e.g. cellular senescence within the T-cell population is discussed to play an important role within the increase of cancer patients with age.

11.4.1 Definition and Features of Immunosenescence

It is generally accepted that an age-associated decline in the immune competence exists, which is characterized by functional and phenotypic alterations of the immune system and termed immunosenescence. This process is characterized in the elderly human population with an increased susceptibility to infectious diseases

and cancer, a reduced efficacy of vaccinations, an increased frequency of autoimmunity and damage to various organ systems through deregulated inflammatory processes (Deeks et al. 2012). These various changes are often accompanied by an increased morbidity and mortality of patients. Despite immunosenescence represents an ubiquitous process it is highly variable from individual to individual due genetic and environmental factors. It is driven by a systematic remodeling of the immune system over life time due to antigenic exposures and responses mediated by inflammation. Essentially, all components of the innate and adaptive immunity could be affected and altered by aging, but to a different extent. However, not only immune cells themselves, but also changes in the macro- and microenvironment affecting the immune responses were found during aging and thus were essential for the development of an immunosenescence status. This might be associated with low levels of chronic inflammation, known to be involved in the development of various diseases including cancer (De la Fuente and Miquel 2009).

11.4.2 Involvement of the Innate and Adaptive Immune System in Immunosenescence

Despite the immune system is altered during the aging process, there exist differences between the components of the innate and adaptive immunity. Cells of the innate immune system demonstrate alterations with aging, but the most important alterations involve T cells concomitant with the presence of low grade inflammation (Fulop et al. 2013). An association of aging with innate immune cells has been shown for NK cells. Aged mice exhibit a decreased NK cell maturation in the blood, spleen, lymph nodes and bone marrow, which results in an impaired NK cell migration (Fang et al. 2010; Beli et al. 2011, 2014). In addition, activation, cytotoxicity and IFN-γ secretion of NK cells are reduced in aged humans and mice (Albright and Albright 1983; Fang et al. 2010; Hazeldine et al. 2012; Guo et al. 2014). The NK cell dysfunction of aged mice has been shown to be due to defects in the bone marrow stroma, and could not be restored by IL-15/IL-15Rα despite a massive expansion of NK cells, but not of the functionally relevant $CD27^{-}$ $CD11b^{+}$ compartment (Nair et al. 2015). In addition, a mature subset of aged NK cells has an impaired capacity to home to draining lymph node upon virus infection (Fang et al. 2010). Recently extrinsic and intrinsic factors of the non-hematopoietic microenvironment have been shown to be responsible for the impaired maturation and function of T cells (Shehata et al. 2015).

Upon proper activation of T cells in vitro via the interaction of HLA antigens with the respective TCR and exposure to IL-2 human T cells undergo 25–40 population doublings before senescence is reached. Repeated cycles of activation and cell division in vitro could lead to a replicative senescence of $CD8^{+}$ T cells, which is accompanied by a telomere (TEL) shortening from 10 to 11 kb of "young" T cells to approximately 5–7 kb in senescent T cells and an activation of a senescent phenotype. In contrast, $CD4^{+}$ T cells retain their telomere activity (Valenzuela and

Effros 2002). Long-term cultures restrict the amount of cells available for T cell-based therapies, which are currently developed for the treatment of cancer pathways, e.g. adoptive T cell. Therefore the maintenance of the biological properties of T cells during long-term culture is crucial. This is associated with very short telomeres found in the peripheral blood lymphocytes of tumor patients. The loss of telomerase activity is further associated with a diminished CD28 expression in the presence of CD57 (Perillo et al. 1989; Fagnoni et al. 1996; Effros and Pawelec 1997; Weng et al. 1997). This replicative senescence of T cells represents the end-stage of memory T cell differentiation and proliferation associated with an irreversible cell cycle arrest.

In elderly persons increased levels of pro – inflammatory cytokines were found in the general circulation, but also in tissue-resident macrophages. The secretion of pro-inflammatory cytokines and chemokines as well as adhesion molecules define a senescence associated secretory phenotype (SASP Sagiv and Krizhanovsky 2013), which can recruit and activate different subpopulations of the innate and adaptive immune system such as NK cells, monocytes and T cells and immune suppressive cells. Significant changes have been in particular demonstrated regarding in the repertoire diversity of immune cells that is dramatically declined with age (Johnson et al. 2014). Other immune alterations associated with immunosenescence include a reduced frequency and function of hematopoietic stem cells, thymus involution, reduced circulating naive T cells, decreased ratio of $CD4^+$ and $CD8^+$ T cells, increased levels of pro-inflammatory cytokines, like IL-6 and tumor necrosis factor (TNF)-α, an accumulation of late differentiated memory B and $CD8^+$ T cells mediated by significant phenotypic and functional changes, with features of cellular immunosenescence (Sasaki et al. 2011; Herndler-Brandstetter et al. 2012; Buffa et al. 2013). The activation of the p38 MAPK pathway is involved in the senescence of $CD4^+$ effector memory cells, which is reversible by inhibition of p38 signaling (Di Mitri et al. 2011). Furthermore, there exists an age-associated shrinkage of the compartment size and a decline in the T cell receptor (TCR) repertoire/diversity. The shift from naive to terminally differentiated T cells is associated with a reduced proliferative capacity (replicative senescence) and/or function of these cells (Akbar and Henson 2011). In addition, multiple maturation defects have been described in immune cells, which are associated with a reduced proliferation, high level of immaturity, altered expression of activity and inhibitory receptors as well as integrins (Nair et al. 2015). Furthermore, age-related variations in DNA methylation have been identified in T cells and monocytes, which have the functional relevance (Reynolds et al. 2014).

11.4.3 Immunosenescence and Cancer

Many changes in the immune system are associated with its capacity to compete with the initiation as well as progression of tumors and decrease the immunosurveillance of tumor cells due to a host/tumor balance leading to the development and progression of cancer. Most important were the alterations in T cell functions and

phenotypes, which are associated with the presence of low grade inflammation. At the clinical level the impaired immune responses in elderly explain decreased efficacy of immune cell activation and the induction of immune tolerance leading to an increase in the frequency during age.

Accumulation of such senescent $CD8^+$ T cells has been found in persons during normal aging, in younger persons with chronic viral infections, and also in patients with certain types of cancers (Newgard and Sharpless 2013). It could be also correlated with tumor progression and predict poor patients' prognosis. In vivo the presence of oligoclonally expanded populations of late differentiated T cells play not only an important role in the development of immunosenescence, but could also predict prognosis of patients.

Furthermore, it has been shown that naturally occurring human $CD4^+CD25^+$ Treg cells, which are increased in frequency in tumor patients, can induce responder T lymphocyte senescence (Fessler et al. 2013). Immune-mediated clearance of senescence cells in vivo represents a critical mechanism, which limits the development of cancer (Kang et al. 2012; Baker and Sedivy 2013). These senescent T cells are characterized by phenotypic and functional alterations, such as loss of CD28 expression, cell cycle arrest accompanied by an up-regulation of cell cycle-related genes such as p53, p21 and p16 (Mondal et al. 2013) as well as reduced cytotoxicity and the development of potent negative regulatory functions (Ye et al. 2012). This could at least partially explain the reduced or loss of T cell-based elimination of tumor cells. In addition, other suppressive mechanism(s) on cells of the adaptive and innate immune system could be mediated by tumor-derived γδ Treg cells (Ye et al. 2013). These γδ Treg cells are able to induce both T cell and DC senescence resulting in their impaired phenotypic and functional features and further amplification of immune suppression mediated by Treg cells. It is noteworthy that the number of MDSC and Treg increased during progression of tumors, but also during aging, which further contributes to unresponsiveness of T cell-based immunotherapies at older age.

For in vivo studies mouse models have been employed to study immunosenescence. For example TEL-deficient mice display a reduced proliferative capacity of lymphocytes as well as reduced antigen reactivity upon immunization. However, the dynamics of the immunosenescence processes in mice and humans differ due to their distinct lifespans, the variety of encountered antigens and the competition for space by lymphocytes (Aspinall 1999; Rufer et al. 2001). Therefore, murine tumor models were not helpful to elucidate the role of immunosenescence in tumor patients. An accumulation of senescent, late differentiated $CD8^+$ T cells ($CD28^-$, $CD27^-$) has been demonstrated, which directly correlated with disease progression and reduced survival of tumor patients (Hase et al. 2011).

The hematopoietic stem cell (HSC) compartment is also negatively modulated by aging. This leads to a skewing of the HSC pool characterized by a shift from the lymphoid to the myeloid lineage, the latter leading to myelo-proliferative disorders. Furthermore, age is associated with chemotherapy-induced hematotoxicities compared to younger patients. Therefore biomarkers are urgently needed to predict tumor growth, immunosurveillance as well as chemotherapy-induced hematologic toxicities.

Signaling pathways could also undergo age-related alterations thereby also inhibiting anti-tumoral immune responses. These could occur at the level of TCR signaling, co-stimulation and cytokine receptor signaling and lead to a functional decline of immune responses in cancer patients. For example there exists an altered expression level of the co-inhibitory receptors between younger and aged populations and tumor patients (Cao et al. 2010; Goronzy et al. 2012). Higher levels of CTLA4 on $CD8^+$ T cells in elderly caused a reduced T cell response. In addition, $CD57^+$ T cells also accumulate with age, but also in tumor patients (Ouyang et al. 2003). Since these inhibitory molecules were also accompanied by a reduced T cell response in tumor patients is currently successfully used for therapy of tumor patients and aging faintly, treatment with checkpoint inhibitors block the negative signal enhance T cell response of tumor patients.

11.4.4 Strategies to Revert Immunosenescence

With the increased knowledge of immunosenescence and their correlation with immune responses different strategies could be developed to reverse this process. For example, inhibition of the γδ Treg cell suppression could be performed by manipulation of TLR8 signaling in γδ Treg cells, which then block γδ Treg-induced conversion of T cells and DCs into senescent cells both in vitro and in vivo in animal models. Furthermore, immune suppressive cells should be inhibited by different antibodies or targeted therapies. Thus not only insights into the development of immunosenescence in tumor patients are given, but also this information is relevant for the development of strong and innovative approaches for improved tumor immunotherapy. Since chronic inflammation is often found in cancer patients and has been correlated with oxidative stress and immunosenescence one could also consider short-term therapies decreasing the oxidative stress. This might improve different immune functions in particular of the short lived macrophages, granulocytes, T cells and DCs, like chemotaxis, lymphoproliferative responses, IL-2 production and cytotoxicity, features, which are essential for proper anti-tumoral immune responses (Wu and Meydani 2008).

11.5 Conclusions

Cellular senescence as well as immune senescence affect the cancer risk of patients. Therefore a number of questions arise, which have to be addressed in the future: (i) should we monitor tumor patients regarding their senescent properties and define commonly used markers of senescence in these malignancies; (ii) should we categorize age-related changes of immune cell functions?; (iii) could the cellular-/immune-senescence pattern of tumor patients predict their clinical outcome independent of other markers?; (iv) could we determine senescence markers earlier

in life and could we use these for the prediction of long-term outcome?; and (v) could the knowledge about the processes be used for therapeutic intervention in patients?

In order to follow up on these questions, prospective studies with large cohorts and defined clinical end-points are urgently required and will help to elucidate the complexity of the cellular as well as immune-senescence, their clinical relevance and possibilities for therapeutic strategies to modify this process.

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Chapter 12
Biomarkers of Replicative Senescence Revisited

Jan O. Nehlin

Abstract Biomarkers of replicative senescence can be defined as those ultrastructural and physiological variations as well as molecules whose changes in expression, activity or function correlate with aging, as a result of the gradual exhaustion of replicative potential and a state of permanent cell cycle arrest. The biomarkers that characterize the path to an irreversible state of cell cycle arrest due to proliferative exhaustion may also be shared by other forms of senescence-inducing mechanisms. Validation of senescence markers is crucial in circumstances where quiescence or temporary growth arrest may be triggered or is thought to be induced. Pre-senescence biomarkers are also important to consider as their presence indicate that induction of aging processes is taking place. The bona fide pathway leading to replicative senescence that has been extensively characterized is a consequence of gradual reduction of telomere length and associated damage, and the accompanying changes that take place elicit signals that have an impact on a number of molecules and downstream events. Precise measurements of replicative senescence biomarkers in biological samples from individuals could be clinically associated with their chronological age and present health status, help define their current rate of aging and contribute to establish personalized therapy plans to reduce, counteract or even avoid the appearance of aging biomarkers.

Keywords Replicative senescence • Cell cycle • Biomarkers • Health • Longevity • Mortality • Immortality

12.1 Introduction

Biomarkers of aging comprise any number of physiological processes that become affected during the aging process. Complete understanding of these biomarkers needs to take into account what the meaning is of a disease-free, fully-healthy body. Optimal health is based not alone on self-perception but mainly on a variety of

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medical parameters that confirm whether an individual is absolutely healthy, with those parameters varying according to gender, epigenetics and genetic variability. It is very complex to estimate or quantitate the degree of change of a given biomarker in a large population unless one focuses on cohort studies over an extended period of time, ideally starting as soon as physiological development ends, and perhaps even earlier. A vast amount of studies have resulted in the choice of various biomarkers of aging that typically are used when assessing a senescent phenotype. The search for clinically targetable biomarkers that are uniquely expressed, ideally on the surface of senescent cells, and that are completely absent in younger cells, remains a major undertaking.

Analyses of cells undergoing replicative senescence in culture have rendered a better understanding of the pathways induced and their corresponding signaling components. A summary of some of the most important biomarkers of replicative senescence to date will be hereby presented, some of which have also been accounted for in cases of non-replicative senescence, and lastly, a number of clinical biomarkers of aging will be briefly explained that could possibly have an association with replicative senescence *in vivo*.

12.2 Criteria to Define Biomarkers of Aging

The search for aging biomarkers has been attempted for many years and it has resulted in very interesting findings. The criteria set by the American Federation for Aging Research as for the definition of a biomarker of aging was that (a) it must help predict the rate of aging, being a better predictor of life span than of chronological age; (b) it must monitor a basic process that underlies the aging process, not the effects of disease; (c) it must be able to be tested repeatedly in a non-invasive manner e.g. body fluids; and (d) it must be something that applies to both humans and also in laboratory animals, for validation purposes (Johnson 2006). A set of potential biomarkers of aging was compiled whose underlying biological process were either oxidative stress, inflammation, protein glycation, telomere shortening, replicative senescence or hormones (Simm et al. 2008). A more comprehensive set of criteria for the definition of a biomarker of aging was compiled by Richard Sprott (2010). The definition and classification of ubiquitous hallmarks of aging (Lopez-Otin et al. 2013) and of fibroblast hallmarks of aging (Tigges et al. 2014) were recently presented. In general, the hallmarks are considered as genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication (Lopez-Otin et al. 2013). All these hallmarks need to be reconciled with the accurate presence or absence of a given senescence biomarker or combination of biomarkers thereof as a way to define the type of senescent phenotype/pathway being examined.

It is complex to define biomarkers of aging especially when the underlying causes of the process can be not one, but many, especially when dealing with aging

in parallel within different tissues and organs of the body. Cellular senescence is characterized by a series of morphological and physiological changes, and can be attained by many different ways, that ultimately leads to an irreversible state of proliferative arrest. Depending on the type of stimulus, one can in general describe two main types of senescence, replicative senescence which results from a gradual reduction in telomere length and/or loss of telomere function or telomerase inactivation (Martinez and Blasco 2015; Wright and Shay 2002) and a more accelerated type known as stress-induced premature senescence (SIPS), which is generally caused by exposure of cells to genotoxic, oncogenic or oxidative stress that cause sudden damage that cells cannot repair in time (Chen et al. 1995; Sikora et al. 2014; Toussaint et al. 2002). Both types of senescence possess unique and common characteristics (Sect. 12.3).

Replicative senescence can be defined as a physiological process of proliferative exhaustion leading to a state of irreversible cell cycle arrest, during which a gradual increase in cell size and an increase in the size of organelles such as lysosomes, mitochondria, nucleus occurs. Senescent cells acquire a distinct flat and enlarged morphology manifesting a number of changes that will be described in Sect. 12.3 (Hayflick 1965; Lipetz and Cristofalo 1972; Robbins et al. 1970).

As a result of an increase in cell size or organelle size due to biogenesis processes, the number of molecules that are expressed within each compartment increase concomitantly with the size of the compartment giving the impression that the levels are unique to the senescent phenotype. However, upon adjustment or normalization to cell size, it is possible to observe that the levels of a given putative biomarker may not necessarily have changed from a youthful state to a senescent state, that is, there is no statistical significance between the two states (Nehlin et al. unpublished). This type of observations were not always fully addressed when analyzing changes in expression of gene transcripts by various methods such as real-time PCR, Northern blotting, transcriptional profiling using microarrays, differential display or transcriptome RNA sequencing, or changes in protein expression using immunoblotting (Western blotting), proteomics or conventional immunocytochemical or histochemical techniques, where adjustments are done with regards to cell numbers or amount of biological material between young and senescent samples (Doggett et al. 1992; Linskens et al. 1995). Often, associations have been reported whereby a given molecule showed an increase in its expression as a function of replicative aging, and this association correlated with a concomitant increase in cell size (Kumazaki et al. 1991).

With the advent of more sophisticated ways of analysis using e.g. high-content cell imaging or laser scanning imaging cytometry (Wylie et al. 2015), it is now possible to characterize at a much greater level of detail the features that accompany replicative aging, and even to validate putative biomarkers. Advanced algorithms have been developed that can define the area of a cell or any of its contents by using various stains that identify the shape of the cell surface membrane, and inner compartments. After exhaustive analysis of cells undergoing senescence *in vitro*, several observations arise as they reach an irreversible state of cell cycle arrest (Nehlin et al. unpublished). Single-cell metabolic, proteomic, transcriptomic

profiling will enable to understand the causes by which each of the cells has reached senescence. Therefore, it is worthwhile to recapitulate some of the findings that have led to the definition of biomarkers and examine their usefulness in a clinical context and in the whole living body.

Most replicative senescence biomarkers, in one way or another, are related to the pathways that are activated as a result of reduction of telomere length as a function of the number of cellular divisions. Once a critical telomere length has been reached, cells do show the highest possible levels of expression of certain biomarkers. However, in cell culture, some cells reach senescence much earlier than reaching their critical telomere length (Martin-Ruiz et al. 2004; Passos et al. 2007), suggesting that other mechanisms are responsible for triggering senescence, as will be presented in detail below.

12.3 Biomarkers of Replicative Senescence

Cellular senescence represents the gradual deterioration of cell function over time in culture. It is considered a tumor suppression mechanism that evolved to establish a limit to somatic cell division to escape from deleterious events leading to immortalization, whether it is due to natural replicative boundaries or stress-induced signals (Campisi 2005). Cellular senescence might participate in up to four complex biological processes including tumor suppression, tumor promotion, aging, and tissue repair, some of which have apparently opposing effects (Rodier and Campisi 2011). Replicative senescent cells display shrinkage of the homeodynamic space with altered stress response profiles (Demirovic and Rattan 2013) and exhibit distinct phenotypic and molecular characteristics both *in vitro* or *in vivo* (Carnero 2013; Sikora et al. 2014) with compromised telomere-associated functions (Rubin 2002; Wright and Shay 2002) (Sect. 12.3.3).

Many biomarkers of replicative senescence also appear in stress-induced premature senescence (SIPS) of human diploid fibroblasts (Toussaint et al. 2002) and half of the DNA damage foci re-located at telomeres irrespective of telomerase activity (Hewitt et al. 2012). Mitochondrial reactive oxygen species (ROS) inducing DNA damage were shown to be a major determinant of telomere-dependent senescence at the single-cell level that is responsible for cell-to-cell variation in replicative lifespan (Passos et al. 2007) suggesting that additional biomarkers stemming from mitochondria metabolism influencing telomere function remain to be defined. These and many other findings suggest that mitochondria dysfunction can influence directly the senescent program (Correia-Melo and Passos 2015; Ziegler et al. 2015). In the unified field theory of aging, it was proposed that telomere dysfunction activates the tumor suppressor protein p53 which binds and represses PGC-1a and PGC-1b promoters, resulting in impaired mitochondrial biogenesis and function, decreased gluconeogenesis, cardiomyopathy, and increased reactive oxygen species (Sahin et al. 2011).

Some features of cellular senescence are considered universal for many types of cells and include an altered cell morphology, increased activity of lysosomal senescence associated-β-galactosidase (SA-β-gal) and lipofuscin, accumulation of DNA damage foci and increased level of cyclin-dependent kinase inhibitors p21, a transcriptional target of p53, and p16, an upstream activator of Rb, belonging to tumor suppressor pathways (Sikora et al. 2014; van Deursen 2014). Phosphorylation of p53 followed by expression of p21 is a consequence of activation of the DNA damage response pathway (DDR). This signaling pathway can be induced by DNA double strand breaks in telomeric regions during replicative senescence as a consequence of telomere shortening or during SIPS (Jackson and Bartek 2009).

Several attempts have investigated the usefulness of available cellular senescence biomarkers to try to establish quantitative measurements to define the length of replicative lifespan for cell population studies. The candidate senescence markers Ki67, p21 (CDKN1A), γH2AX, SAHF and Senescent-β-Gal (explained in detail below) either alone or in combination were used to determine senescence at various population doublings and compared with those derived from growth curves. This comparison allowed ranking Senescent-β-Gal, SAHFs and the combination of Ki67 negativity with high (>5 per nucleus) γH2A.X foci density in human MRC5 fibroblasts with the highest scores (Lawless et al. 2010). Interestingly, oxygen concentrations during cell culture determined species-specific differences in the expression of some markers of the senescent phenotype between mouse and human cells (Coppe et al. 2010). These initiatives contributed to define the potential role of cellular biomarkers in assessing whole organ aging (Bernardes de Jesus and Blasco 2012). A brief summary of the most conventional biomarkers of replicative senescence and aging-related processes will be hereby presented.

12.3.1 Replication Potential

Fifty years have passed since the finite lifespan of human diploid cells was described (Hayflick 1965). The cumulative number of mitotic events and not the total length of time that human fibroblasts were maintained in culture was described as the main reason for their *in vitro* lifespan (Dell'Orco et al. 1974).

A major hallmark of senescence is an irreversible cell cycle arrest, but cell cycle arrest per se cannot be considered senescence, even though cell cycle arrest and senescence can be triggered by similar effectors such as DNA-damaging agents, suboptimal cell culture conditions or genes (Blagosklonny 2011). During quiescence, induced by anti-mitogenic signals, cells exit the cell cycle entering a G_0 state, giving them the opportunity to overcome life-threatening challenges, including repair of damage, until a point where the cells have the ability to re-enter the cell cycle (Coller 2011; Coller et al. 2006). Long-term quiescent, confluent fibroblast cultures, can reach senescence by mechanism(s) independent of proliferative exhaustion and telomere dynamics. It is therefore crucial to differentiate quiescence from cellular senescence since both share expression of some

biomarkers e.g. quiescent cells show high expression of CDKIs p21, p27, p53 and p57. Therefore, in the case of adherent replicating cells, in the presence of optimal nutrient and culture conditions, in the absence of cell-cell contact inhibition, one can define a senescent cell as a cell that is unable to proliferate or re-enter the cell cycle to complete a new round of replication, if at least 3 weeks have passed (Marthandan et al. 2014). The absence of incorporation of labelled-DNA by any means, in cells in culture is one feature characteristic of senescent cells (Bowman and Daniel 1975; Bowman et al. 1976), although some few cells may still have individual replication foci (Matsumura et al. 1979).

12.3.2 Morphological Biomarkers: Cell Size and Ultrastructural Changes

Shape and Size

Senescent cells are characterized by an enlarged, flat, multinucleated and vacuole-rich morphology (Fig. 12.1). A useful common marker of cell senescence is a large cell morphology also known as hypertrophy. Cellular hypertrophy is usually measured as a cell diameter but also cell area is considered. Among the first studies of cells undergoing senescence in culture was the description of the cellular and ultrastructural changes occurring in non-dividing senescent cells in comparison with actively proliferating cells (Robbins et al. 1970). A more detailed characterization of young and senescent WI-38 fibroblast cultures showed that there was a noticeable age-related increase in size. The average cell volume at 90 % lifespan completed ($\sim$6,000 μ^3), was almost three times greater than the volume exhibited by cells at early passage ($\sim$2,000 μ^3), and in some extreme cases some senescent cells showed up to 15,000 μ^3 in volume. Complete failure to synthesize DNA as a result of aging was irreversible except by SV-40 transformation (Bowman and Daniel 1975). Many other works have confirmed these early observations.

Lysosomes

The number and size of lysosomes was reported to increase progressively during *in vitro* human fibroblast aging, especially after passage 15. The lysosomes of serially propagated human fibroblasts gradually transformed to residual bodies which increased in number and size, and showed progressive degenerative changes (Robbins et al. 1970). In old cells this organelle is much more prominent, has swollen cisternae, and is surrounded by numerous vacuoles. Old cells have statistically significant increases in both lysosomes and autophagic vacuoles as compared to young cells (Brandes et al. 1972; Lipetz and Cristofalo 1972). Even glia cells aging in culture had increasing accumulation of these organelles resulting in heavy loading of almost all cells. Interestingly, individual cells which did not longer divide

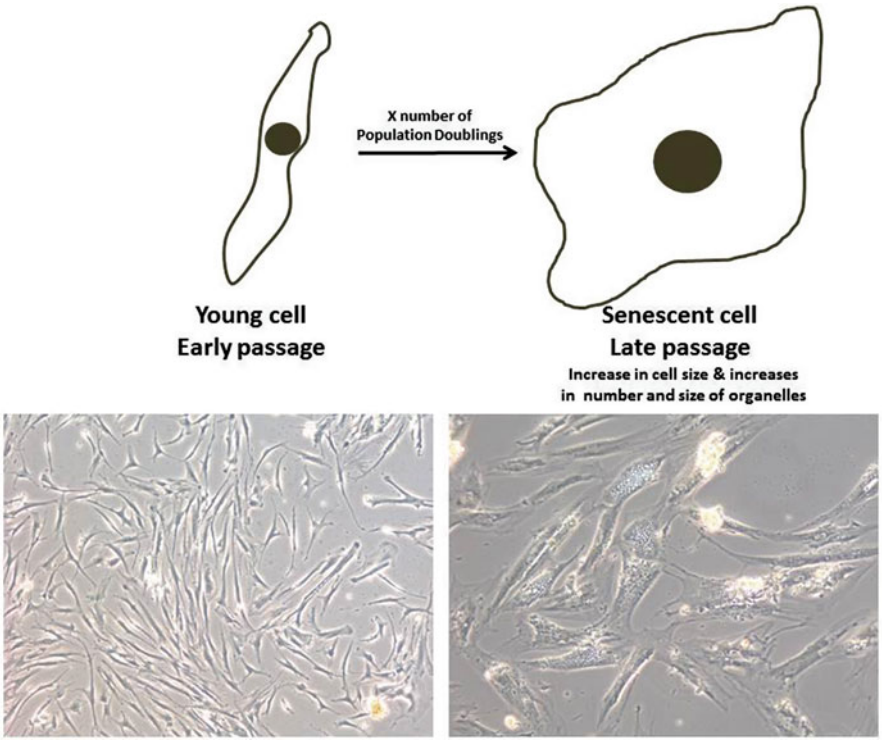

Fig. 12.1 Scheme depicting the transition from a young to a senescent cell during replicative senescence along with phase contrast images of young and senescent human bone marrow-derived stromal cells at 20× magnification

appeared to accumulate large and irregularly shaped residual bodies long before the population as a whole (Brunk et al. 1973).

Mitochondria

Early studies showed that the mitochondria increase in size with age, especially after 60 years of age, in human hepatic cells from biopsies (Tauchi and Sato 1968). No statistically significant change in mitochondrial number was observed in human fibroblasts as they aged in culture. However, the proportion of mitochondria with completely transverse cristae decreased with the age as well as an the appearance of increasing numbers of bizarre-shaped mitochondria having few cristae, some of them in tubular conformation (Lipetz and Cristofalo 1972). Morphometric analysis of mitochondria in human fibroblasts revealed that mitochondrial mass including mean number, weight, and total length of mitochondria per cell weight tended to increase in old and progeria cells, with great heterogeneity between cells. The mitochondria width decreased and cystic blebs were more apparent in old cells

(Goldstein et al. 1984). Lowered mitochondrial membrane potential measured by DASPMI staining has been proposed as a mitochondrial biomarker of senescence (Unterluggauer et al. 2007).

Nucleus and Nucleolus

The area/volume of a senescent nucleus is often larger, deeply lobed and irregularly shaped, compared to the nucleus of a cycling cell and is therefore considered a marker of senescence. (Lipetz and Cristofalo 1972). A cytological analysis of human WI-38 fibroblasts undergoing senescence revealed that the cell, nuclear, nucleolar dry masses and areas increased with cell divisions. There were passage to passage differences in the relative rates of-dry mass increases which were not apparent over long periods of growth. The increases in all nuclear and nucleolar areas were interdependent until the last doubling where they increased independently. The standard deviation for all six parameters increased as a function of *in vitro* senescence indicating that there was an increase in the heterogeneity of cells relative to these parameters (Bemiller and Miller 1979).

Using laser scanning cytometry on human WI-38 fibroblasts undergoing senescence it was possible to quantify an increase in nucleus size/area, but the decrease in ratio of maximal pixel to nuclear area was even a more sensitive senescence biomarker than the change in maximal pixel or nuclear area, each alone. A decrease in the saturation cell density at plateau phase of growth was also used as an additional marker. The characteristic "flattening" of senescent cells was reflected by the decline in the density of DAPI-staining of DNA-associated fluorescence (Zhao et al. 2010).

Endoplasmic Reticulum-Polysomes

The endoplasmic reticulum (ER) of young fibroblasts appeared to be dilated and swollen into large cisternae, whereas in old cells it appeared to be condensed or constricted and "empty" (Lipetz and Cristofalo 1972). Polysomes or polyribosomes are complexes of mRNA molecules and two or more ribosomes formed during active translation. A 30–60 % decrease in the number of free polysomes compared to early passage cells and a persistent accumulation of glycogen was observed at passage 15 in fibroblasts undergoing senescence. Cytoplasmic polyribosomes had largely disappeared by passage 22 (Robbins et al. 1970).

Golgi and Vacuoles

Old cells are also characterized by prominent and active Golgi-vacuolar apparati. Senescent cells had many more autophagic vacuoles as compared to young cells (Lipetz and Cristofalo 1972).

12.3.3 Telomere Length and Telomerase

Telomeres are the structures that cap the ends of chromosomes. Human telomeres are composed of tandem repeats of hexameric DNA sequence TTAGGG. The replicative capacity of fibroblasts from different human donors was found to be directly proportional to the mean telomere length, with telomere loss occurring during replicative aging but not chronological aging (Harley et al. 1990). Once a critical average telomere length was reached, whereby telomeres of <13 pure TTAGGG repeats distal to the telomere variant repeat region become fusogenic, cellular senescence would follow (Allsopp and Harley 1995; Capper et al. 2007). Relevant to these observations is that there is a great degree of interclonal variability, suggesting that not all cells might have reached a critical telomere length upon reaching senescence. A great deal of heterogeneity was found among fibroblasts undergoing senescence, with cells already senescent at early passages, showing significant stochastic cell-to-cell variation in telomere shortening (Martin-Ruiz et al. 2004).

Telomere erosion occurs at a rate of ~60–120 bp per cell division (Harley et al. 1990). The absence of telomerase in cultured human cells and the shortening of telomeres at each population doubling have suggested that telomere length acts as a mitotic clock that accounts for their limited lifespan (Rubin 2002). Telomere maintenance mechanisms counteract the telomere loss accompanying linear chromosome replication by synthesizing new telomeric DNA from either an RNA template (telomerase-mediated reverse transcription) or a DNA template by Alternative Lengthening of Telomeres (ALT) (Bryan et al. 1995). The enzyme telomerase helps to counteract this erosion by catalyzing the de novo addition of telomere repeats, but its activity is absent in most somatic cells except in embryonic stem cells, certain multipotent stem cells and cancer cells, with signs of negligible or no senescence (Harley et al. 1990).

As a consequence of critically short telomere length, a DNA damage response pathway was activated that resulted in poly(ADP-ribose) polymerase associated post-translational activation of p53 and induction of p21 (see below) (Vaziri et al. 1997).

A review confronting the evidence from clinical studies, that telomere length is a biomarker of aging, concluded that it was equivocal, requesting many more longitudinal studies (Mather et al. 2011). Several attempts confirmed that telomere length reduction was associated with age-related diseases such as cardiovascular disease, type 2 diabetes, dementia, and cancer (Needham et al. 2015). However, sample size, environmental factors, gender, ethnicity, type of cells analyzed, may represent confounding factors, which may require sorting of pre-selected cells showing signs of senescence with further analysis and quantification.

The expression of serum biomarkers of DNA damage such as CRAMP, EF-1alpha, stathmin, n-acetyl-glucosaminidase and chitinase, that are induced by telomere dysfunction and DNA damage (von Figura et al. 2009), showed an increased expression in blood during human aging, correlated positively with

p16(INK4a) expression but negatively with telomere length in peripheral blood T-lymphocytes. Smoking and increased body mass indices were associated with elevated levels of biomarkers of DNA damage independent of the age of the individuals (Song et al. 2010) but these lifestyle factors did not associate with a reduction of telomere length (Needham et al. 2015).

Post-mortem cellular analyses of a 115-year old supercentenarian revealed that the telomere lengths of her white blood cells were significantly shorter than telomere lengths from any other of her tissues. Severe hematopoietic stem cell exhaustion led to oligoclonality of the stem cell pool. This is probably the first human clinical case suggesting a link between critical telomere length and stem cell demise (Holstege et al. 2014). Thus, the continuous replenishment of blood cells throughout life influences dramatically aging of the hematopoietic compartment.

12.3.4 Resistance to Apoptosis

Senescent fibroblasts were found to be resistant to the initiation of the programmed cell death program (Wang et al. 1994). Replicative senescence in CD8+ T cells is also correlated with significant resistance to apoptosis (Spaulding et al. 1999). Because impaired T-cell apoptosis *in vivo* has been reported during both normal aging and autoimmune disease, it may be indicative of an increased proportion of putatively senescent T cells in human blood, as evidenced from many studies (Chou and Effros 2013). Despite the finding that the anti-apoptosis and anti-necrotic factor Bcl-2 was found to be highly suppressed in normal senescent fibroblast strains, senescent fibroblasts appeared to be highly resistant to apoptosis in comparison with young cells (Sasaki et al. 2001). Programmed cell death dysfunction is increasingly implicated in aging and aging-related diseases and mitochondrial malfunction is a key underlying factor (Tower 2015).

12.3.5 Cell Cycle-Dependent Kinase Inhibitors

Proliferative arrest during replicative senescence is exerted by two main effector pathways, the $p16^{INK4a}$/pRB pathway and the $p19^{ARF}$/p53/$p21^{CIP1/WAF1}$ pathways, that represent the core of many of the biomarkers of aging known to date, as they ultimately define the state of permanent cell cycle arrest (Fig. 12.2). Cell cycle progression is under the control of negative regulators, the cell cycle-dependent kinase (CDK) inhibitors (CKIs), which belong to either the $p16^{Ink4}$ or Cip/Kip families. Members of the Ink4 family such as $p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4c}$, and $p19^{Ink4d}$ are inhibitors specific for CDK4 or CDK6, whereas those of the Cip/Kip family, including $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$, mainly inhibit CDK2 and CDK4 (and sometimes CDK1) inducing cell cycle and growth arrest (Sherr and Roberts 1999). The tumor suppressor proteins retinoblastoma (pRB) and p53 prevent aberrant cell renewal by inducing the senescent phenotype. Inhibition of their function by various viral proteins contributes to induce an immortal phenotype (Shay et al. 1991).

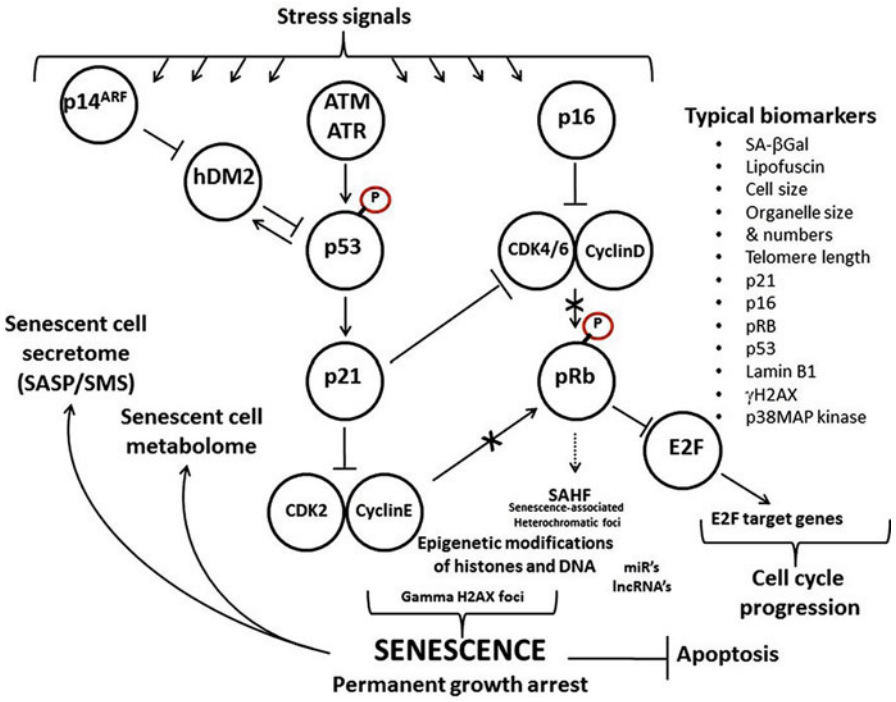

Fig. 12.2 Simplified schematic overview of the $p16^{INK4a}$/pRB and the $p19^{ARF}$/p53/$p21^{CIP1/WAF1}$ pathways inducing replicative senescence, including some of the currently used biomarkers of senescence

The expression of many genes implicated in cell cycle regulation were characterized in aging human fibroblasts concluding that $p16^{Ink4a}$, cyclin D and $p21^{Cip1}$ were the most up-regulated in comparison to early passage cells (Wong and Riabowol 1996). Expression of cyclin D1 increased markedly in senescent human fibroblasts *in vitro* inhibiting cell proliferation through binding to PCNA and Cdk2 (Fukami-Kobayashi and Mitsui 1999).

The INK4a/ARF tumor suppressor gene locus regulates both the pRB and the p53 tumor suppressor pathways by expressing $p14^{ARF}$ and p16. $p14^{ARF}$ is not a CDK inhibitor, but instead induces the CDK inhibitor $p21^{CIP1}$, activating the p53 pathway (Campisi and d'Adda di Fagagna 2007; Sherr 2012).

p16

p16 is a commonly used biomarker of replicative senescence (Krishnamurthy et al. 2004). The p16 gene, also known as CDKN2A and INK4a, was initially cloned as an inhibitor of cyclin D/cdk4 complexes (Serrano et al. 1993) and was subsequently shown to be highly expressed in senescent cells (Hara et al. 1996; Wong and

Riabowol 1996). p16 mRNA and protein levels gradually rose with protein levels as human fibroblasts became senescent, reaching nearly 40-fold higher amounts than early passage cells. p16 was found to be the major CDK inhibitor for both CDK4 and CDK6 kinases and its up-regulation is considered a key event in the terminal stages of growth arrest in senescence, which may explain why p16 but not p21 was found commonly mutated in immortal cells and human tumors (Alcorta et al. 1996). p16 expression in cancer cells induced senescence (Uhrbom et al. 1997). p16 is therefore defined as a tumor suppressor protein.

IMR90 human fibroblasts deficient in p53 only partially abrogated the telomere damaged-induced cell cycle arrest, but when both p53 and p16 were inhibited, a complete bypass of telomere-induced senescence took place, indicating that p16 was required for the p53-independent response to telomere damage (Jacobs and de Lange 2004). $p16^{Ink4a}$-deficient fibroblasts have an appreciable lifespan extension (60–88 PD's) compared to controls (median of 35–40 PD's) but still undergo senescence with the usual phenotypes. This indicated that p16 itself contributes but is not essential to replicative senescence of fibroblasts. Interestingly, a high variation of p16 levels was found in different strains of fibroblasts at comparable passages, and curiously, even though there was a general accumulation of p16 at senescence, this was not true for all cells at the end of the proliferative life span of the culture, suggesting that different senescence-inducing mechanisms are acting within different cells (Brookes et al. 2004). p16 accumulates in parallel with the increases in senescence-associated beta-galactosidase activity (section "Senescence-associated beta galactosidase") and cell volume that characterize the senescent phenotype (Stein et al. 1999).

p21 (CIP1/WAF1/SDI1)

p21 is considered a biomarker of replicative senescence because increased levels of p21 have been described in senescent fibroblasts with a 10- to -20-fold expression difference between early- and late-passage cells, although not adjusted for cell size (Noda et al. 1994; Wong and Riabowol 1996). The CDKN1A gene encodes p21, a protein that belongs to the family of cyclin-dependent kinase inhibitors (CKI) and was identified independently by various laboratories (el-Deiry et al. 1993; Harper et al. 1993; Noda et al. 1994; Xiong et al. 1993). p21 (CIP1/WAF1/SDI1) protein binds to and inhibits the activity of cyclin-CDK2, -CDK1, and -CDK4/6 complexes, inducing growth arrest, and its expression is induced by p53. Senescence is a multistep process that requires the expression of not only p16 but also p21. As human fibroblasts approached the final stages of senescence, p21 protein expression increased but declined at the last passage, indicating that it can mediate senescence (Alcorta et al. 1996). This has been confirmed by many labs, but in a comparison of human fibroblasts strains it was possible to measure, not only heterogeneity in p21 levels between them at similar passages, but also that there were substantial increases of $p21^{CIP1}$ and $p16^{INK4a}$ levels relatively early in their passage history, and the levels remained constant or declined during the remainder of the life span

(Brookes et al. 2004). p21 promoter activity in senescent cells was dependent on both p53 and the transcriptional co-activator p300 (Vaziri et al. 1997) and its disruption in fibroblasts prevented senescence (Brown et al. 1997). Its ectopic expression in early passage human fibroblasts induced a state reminiscent of senescence (McConnell et al. 1998). It was later found that phenotypic and replicative senescence were uncoupled in the absence of normal p21 levels suggesting that p21-dependent Cdk inactivation is important for G1 arrest in senescence, while other aspects of the senescent phenotype appeared to occur independently of p21 (Dulic et al. 2000).

12.3.6 Tumor Suppressor Proteins

The retinoblastoma protein (pRb) pathway and the p53 pathway represent key components in the regulation of the cell cycle and tumour suppression (Berkers et al. 2013; Munro et al. 2012). The role of tumor suppressor p53 and pRb as biomarkers of aging is very much dependent on the senescence-inducing context.

pRb

Failure to phosphorylate pRB in senescent cells is another potential biomarker of replicative senescence. This event prevented human fibroblasts from entering S phase of the cell cycle, whereas quiescent cells retained the ability to phosphorylate pRB and entered S phase (Stein et al. 1990). The hypo-phosphorylated pRB prevents the transcription factor E2F from inducing S phase target genes essential for cell cycle progression. A kinase screen revealed that LATS2 kinase, a component of the Hippo pathway, cooperates with pRB to promote the silencing of E2F target genes (Tschop et al. 2011). pRb upregulates the CKI p27Kip1 (Cdkn1b) expression leading to a post-transcriptional accumulation of $p27^{KIP1}$ levels, binding to cyclin E and a persistent inhibition of cyclin E-cdk2 kinase activity that triggers senescence (Alexander and Hinds 2001). It is unclear whether these events apply to all cells reaching a senescent state, and decisive evidence that can fulfill the criteria of a biomarker role is missing.

A repertoire of post-translational modifications that occur on pRb together with its key effector E2F-1 endows pRb with the ability to function in diverse physiological settings (Munro et al. 2012).

p53

p53 mRNA and protein were expressed at similar levels in low-passage (young) and high-passage (old) cells but both the DNA binding activity *in vitro* and transcriptional activity of p53 *in vivo* were increased several-fold in high-passage

cells (Atadja et al. 1995). The DNA binding and transcriptional activity of p53 protein increases with cell age in the absence of any marked increase in the level of p53 protein (Vaziri et al. 1997).

Replicative senescence in human fibroblasts is dependent on the function of the phosphoprotein p53 and correlates with activation of p53-dependent transcription. Increased phosphorylation of p53 in senescent fibroblasts occurred at serine-15, threonine-18, and serine-376, while decreased phosphorylation took place at serine-392. Phosphorylation at serine-15 was generally a common theme across several senescence-inducing stressors. Thus, p53 fulfills an important role in signaling replicative senescence and the DNA damage response linked to telomere erosion with age (Webley et al. 2000).

p53 isoforms regulate aging- and tumor-associated replicative senescence in T lymphocytes (Mondal et al. 2013). The underlying mechanisms by which p53 regulates aging are not completely understood, but it is becoming evident that apart from being crucial in the response to genotoxic stress it also regulates metabolic homeostasis including mitochondria function (Berkers et al. 2013; Sahin et al. 2011).

12.3.7 Lamin B

Lamin B1 (LMNB1) expression within the nuclear lamina is lost from primary human and murine cell strains when they are induced to senesce by DNA damage, replicative exhaustion, or oncogene expression, suggesting that its absence can serve as a biomarker of aging *in vitro* and *in vivo* (Freund et al. 2012) (Sects. 12.3.9 and 12.3.11).

12.3.8 Promyelocytic Leukemia Nuclear Bodies – PML NB's

The promyelocytic leukemia tumor suppressor (PML), initially identified as a component of the PML-RARa oncoprotein of acute promyelocytic leukemia (APL) plays a critical role in oncogenic K-RAS-induced Oncogene-induced senescence (OIS), being highly upregulated (Munch et al. 2014). PML accumulates in nuclear bodies (PML bodies) upon senescence both in fibroblasts an cancer cell lines after being redistributed intracellularly, (Jiang and Ringertz 1997). This change in phenotype represented a biomarker of damage accompanying senescence. However, PML associated in a non-random fashion with persistent DNA damage foci in unperturbed human skin and in high-dose irradiated cell culture systems. The PML bodies did not associate with transient gammaH2AX foci (section "Gamma-histone A2X and DNA damage") after low-dose gamma irradiation. Even though PML bodies specifically accumulated at Rad51/RPA-containing lesions and senescence-derived persistent DNA damage foci, they are not essential for DNA damage-induced and replicative senescence of human and murine fibroblasts (Munch et al. 2014).

12.3.9 Chromatin-Associated Biomarkers

As cells become senescent, there is a progressive shift that accompanies the loss of cell division leading to chromatin reorganization (Macieira-Coelho 1991). Heterochromatin consists of transcriptionally silent chromatin that can promote the stable repression of many genes. Constitutive heterochromatin occurs at repetitive DNA sequences and is typically characterized by histone hypoacetylation, methylation of lysine 9 of histone H3 (Me-K^9-H3), and binding of Heterochromatin Proteins 1 (HP1α, β, γ) (Maison and Almouzni 2004). On the other hand, facultative heterochromatin is inducible heterochromatin that is most prominent at genes and genomic regions that are repressed in a developmental and cell-type specific such as the single X chromosome of female cells which is silenced during embryogenesis (X inactivation) and contains the characteristic histone H2A variant known as macroH2A (Trojer and Reinberg 2007). A comprehensive analysis of senescence-associated chromatin reorganization was recently presented (Swanson et al. 2015).

Senescence-Associated Heterochromatic Foci (SAHF)

SAHF has been used as a biomarker of aging (Lawless et al. 2010) but upon deeper scrutiny in recent times, it does not longer satisfy the absolute requirements of a senescence biomarker (Kosar et al. 2011; Swanson et al. 2015).

Once cells approach senescence, proliferation-promoting genes such as E2F target genes cyclin A, DHFR, and Mcm3 are incorporated into transcriptionally silent heterochromatin, called senescence-associated heterochromatin foci (SAHF). Once SAHF is formed, cells exit the cell cycle and enter senescence. Formation of SAHF depends on the pRB tumor suppressor pathway (Narita et al. 2003). As cells approach senescence, a known chromatin regulator, HIRA, enters PML nuclear bodies (see Sect. 12.3.8), where it transiently co-localizes with HP1 proteins, prior to incorporation of HP1 proteins into SAHF. A physical complex containing HIRA and another chromatin regulator, ASF1a, is rate limiting for the formation of SAHF and onset of senescence, and ASF1a is required for formation of SAHF and efficient senescence-associated cell cycle exit.

Formation of SAHF enriched in macroH2A- and HP1 contributes to cell cycle exit in senescent cells (Zhang et al. 2005). High-Mobility Group A (HMGA) proteins accumulate on the chromatin of senescent fibroblasts and are enriched in SAHF. HMGA proteins cooperate with the p16INK4a tumor suppressor to promote SAHF formation and proliferative arrest and stabilize senescence by contributing to the repression of proliferation-associated genes (Narita et al. 2006).

However, SAHF formation (Narita et al. 2003) occurs relatively late during the onset of senescence, does not form in every senescent cell or cell type (e.g. not observed in mouse or Hutchinson-Gilford Progeria Syndrome cells), and has not been observed *in vivo*. Its failure to occur early and consistently in response to senescence brings into question the importance of SAHF in the senescence

process (Kennedy et al. 2010; Kosar et al. 2011; Kreiling et al. 2011; Swanson et al. 2015). Senescence-associated heterochromatin foci are dispensable for cellular senescence, occur in a cell type- and insult-dependent manner and follow expression of p16 (ink4a) (Kosar et al. 2011). SAHF formation is preceded by the unravelling of constitutive heterochromatin into visibly extended structures called Senescent Associated Distension of Satellites or SADS, occurring within 48 h of the last cell cycle. Lamin B1 loss was evident before SADS formation in over half of the cells, indicating that Lamin B1 depletion maybe one of the factors facilitating the repackaging of satellite DNA during senescence. The consistent loss of higher order chromatin packaging during SADS formation, the frequent reorganization of chromatin into SAHF, and the reduction of Lamin B1 are fundamental for senescence (Swanson et al. 2015). Further work will be necessary to address whether potential senescence-associated chromatin biomarkers can be used in a reliable and faithful manner.

Gamma-Histone A2X and DNA Damage

Gamma-H2AX foci or gamma-foci, which reveal DNA double-strand breaks (DSB's), accumulate in senescing human cell cultures and in aging mice, indicating that the ability of cells to sense and repair DNA damage declines with age (Sedelnikova et al. 2004). The gamma-H2AX foci consist of DNA-damage induced foci of phosphorylated histone H2AX and co-localize with DSB-repair factors, and also to a certain extent at shortened telomeres. The rates of recruitment of DSB repair proteins to gamma-H2AX foci correlated inversely with age and it was therefore proposed that accumulation of DNA damage contributes to the genome instability associated with cellular senescence and body aging (Sedelnikova et al. 2008). DNA mutations are not only found at chromosomal sites as a function of age but also in mitochondrial DNA which is thought to be caused by replication errors and failure of mitochondrial DNA repair mechanisms, and the accumulation of these mutations as observed in aged organisms seems to occur by clonal expansion and not caused by a reactive oxygen species-dependent vicious cycle (Pinto and Moraes 2015). A review of the literature revealed that organs with limited cell proliferation such as liver, kidney, brain, heart, pancreas, and muscle, tended to show accumulation of DNA damage with age, whereas organs with highly proliferating cells, such as intestine, spleen, and testis, showed more equivocal or no effect of age (Moller et al. 2010). Apart from DSB, there are also many other types of DNA damage that are associated with aging but their precise role as biomarkers contributing to replicative senescence requires further studies as well as the role of multiple DNA-repair pathways that can protect against replicative decline (Vermeij et al. 2014). The most common DNA damage biomarkers of senescence associated with age are accumulation of DNA damage foci, single-stranded and double-stranded breaks and oxidative damage (Sect. 12.3.10) (Jacob et al. 2013).

12.3.10 DNA, Lipid and Protein Oxidative Damage

A summary of the markers of oxidative stress on DNA, lipid and protein molecules used in the field as biomarkers of aging and age-related diseases was presented (Jacob et al. 2013). Oxidative modifications are associated with a number of bi-products or end-products that are linked to the oxidatively-modified macromolecules (DNA adducts and strand breaks as markers of DNA damage, heme degradation products and protein carbonyls as markers of protein damage and F2 isoprostanes as markers of lipid peroxidation). Such modifications may alone or in association with other biological factors lead to epigenetic changes, changes in gene expression and/or DNA repair capacity or mitochondrial and membrane dysfunction that after a cumulative increment with time depending on a lifetime of exposures lead to aging and age-related diseases. The most common studied biomarkers of oxidative DNA damage are 8-Oxo-7,8-dihydro-guanine (8-oxoGua) and 8-oxo-7,8-dihydro-2′deoxyguanosine (8-oxodG) (Jacob et al. 2013). A higher content of oxidation products is found in post-mitotic cells compared to actively dividing cells (Moller et al. 2010). In replicating human WI-38 fibroblasts, oxidative DNA damage is proportional to the age of cells and the DNA damage in senescent cells reflects both an increased susceptibility to oxidative stress and a reduced efficiency of repair mechanisms. Mild chronic oxidative stress induced by prolonged exposure to 5 μM H_2O_2 accelerated senescence, increasing 8-oxodG levels, SA-β-galactosidase activity and causing cell cycle arrest (Wolf et al. 2002). The level of 8-oxo-2′-deoxyguanosine in the DNA of IMR-90 human fibroblast cells was found to be approximately 35 % higher in senescent cells than in young cells, and it was proposed that oxidative DNA damage contributes to replicative cessation in human diploid fibroblast cells (Chen et al. 1995). Increased oxidatively modified proteins with age in culture have been observed (Unterluggauer et al. 2007). In a population-based study, oxidative stress markers d-ROM representing derivatives of reactive oxygen metabolites as a proxy for the reactive oxygen species concentration, and total thiol levels (TTL) as a proxy for the redox control status were associated with mortality at older age (Schottker et al. 2015). While damage is evidently associated with replicative senescence judging by the increase in the presence of markers of oxidative damage, their role as being unique to senescence is limited.

12.3.11 Sirtuins and NAD+

Mammals contain seven sirtuins (SIRT1-7) that modulate distinct metabolic and stress response pathways, e.g. linking nutrient signals with the cellular responses to energy demands. Sirtuins are a phylogenetically conserved family of nicotinamide adenine dinucleotide (NAD+)-dependent deacetylases/ADP-ribosyltransferases that regulate various metabolic pathways and have emerged as important sensors of energy status in mammals (Chalkiadaki and Guarente 2012; Imai and Guarente 2014). Lower circulating SIRT1 and SIRT3 levels can be a distinctive marker of

aging as they were significantly associated with frailty after adjusting for age, gender, diabetes, hypertension, cognitive status and number of comorbidities (Kumar et al. 2014). SIRT3 knock-out mice develop several diseases of aging at an accelerated pace, such as cancer, metabolic syndrome, cardiovascular disease, and neurodegenerative diseases, and, thus, might be a valuable model of accelerated aging (McDonnell et al. 2015). Depletion of SIRT6 by RNA interference in human endothelial cells reduced cell proliferation and increased the fraction of senescence-associated-β-galactosidase-positive cells (section "Senescence-associated beta galactosidase") indicating that SIRT6 may have a protective role against DNA damage, telomere dysfunction and senescence (Cardus et al. 2013). Very little is known about the expression and activity of various sirtuins in human senescent fibroblasts including the SIRT3 and SIRT4 mitochondrial tumor suppressors that signal changes in cellular nutrient status to targets that help maintain energy production (Zhu et al. 2014). Also NAD^+ levels decline during the aging process causing defects in nuclear and mitochondrial functions and resulting in many age-associated pathologies. Since sirtuins require NAD+ to function efficiently, NAD^+ supplementation leads to sirtuin activation (Imai and Guarente 2014). The intracellular NAD^+ levels and NAD:NADH ratio in all middle-aged rat organs were significantly reduced compared to young rats. An age-dependent increase in lipid peroxidation and protein carbonyls formation, an increase in DNA damage (phosphorylated H2AX), a decline in total antioxidant capacity, a decreased Sirt1 activity and increased acetylated p53 were observed in organ tissues in parallel with the drop in NAD^+ and moderate overexpression of Sirt1 protein. Reduced mitochondrial activity of complex I–IV was also observed in aging animals, impacting both redox status and ATP production (Braidy et al. 2011). Adequate levels of NAD^+ may represent a marker of optimal metabolic function and longevity.

12.3.12 Lysosomal Markers

Senescence-Associated Beta Galactosidase

A biomarker of aging that has reached its 20th anniversary since its discovery is the increase in activity of the senescence-associated beta-galactosidase (SAβGal) at acidic pH 6, based on the presence of a higher chromogenic cellular staining (Dimri et al. 1995) (Fig. 12.3). SAβGal activity is expressed from GLB1, the gene encoding lysosomal β-D-galactosidase (Lee et al. 2006). This marker is at present one of the most common markers in the Biogerontology field and contributed to identify replicatively senescent cells in culture (Debacq-Chainiaux et al. 2009; Dimri et al. 1995). Coincidentally, it was the first marker that enabled detection of senescent cells *in vivo*, suggesting a link between cellular replicative exhaustion and aging-associated phenotypes (Dimri et al. 1995). However, it has been concluded that no relationship between fibroblast aging *in vivo* and SAβ-gal staining was observed

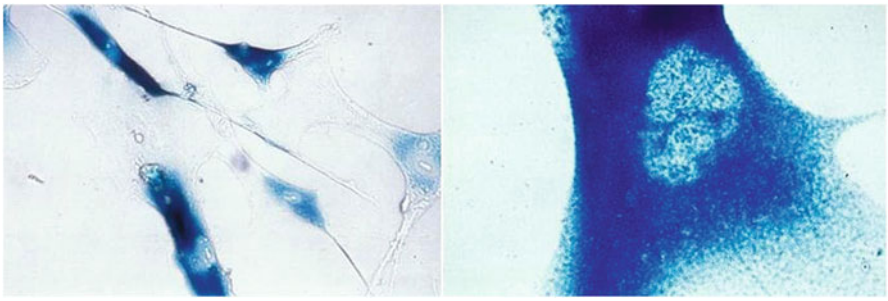

Fig. 12.3 A typical SA-βGal chromogenic staining of human diploid fibroblasts at senescence is shown at low and high magnification

either in fibroblast cultures established from donors of different ages or in tissue sections obtained from donors of different ages. Cultures established from donors of different ages stained for SAβ-gal activity as a function of *in vitro* replicative age, not donor age (Severino et al. 2000). p16 accumulates in parallel with the increases in SAβGal activity and cell volume that characterize the senescent phenotype (Stein et al. 1999).

Lipofuscin

Lipofuscin are complexes of pigmented yellow-brown granules or aggregates derived from lipid-containing residues of lysosomal digestion. It has been considered an aging pigment because with increasing age, persistent accumulation of lipofuscin has been documented in the lysosomal compartment. It was first described in 1842 and associated with age in 1886 (Brunk and Terman 2002). Already in the early 1940s there was evidence for the existence of a ceroid pigment, initially visualized in the cirrhotic livers of rats, categorized as one of many lipofuscins (Casselman 1951) but the pigmented bodies were also found to be abundant on neuron sections, and were correlated with the age of the individual (Keefe and Ordy 1964). A broad spectrum autofluorescence is characteristic of lipofuscin (Terman and Brunk 2004). These age-related pigments were not exclusive of post-mitotic cells but were also detectable in replicatively senescent WI-38 fibroblasts (Deamer and Gonzales 1974; Rattan et al. 1982) and in premature-aged cells, as autofluorescent bodies whose signals could be quantitated to yield an index of the rate of aging (Rattan et al. 1982). Numerous investigations throughout the years yielded important insights into the origins of the lipofuscin pigments and their consequence for cell homeodynamics. Lipofuscin consists of oxidatively modified proteins, lipid degradation residues, represented by free fatty acids, cholesterol and phospholipids, some carbohydrates and less than 2 % metals such as Fe, Cu, Al, Zn, Ca, and Mn. This intralysosomal polymeric material is undegradable and cannot be exocytosed, and it originates from autophagocytosed cellular components that have

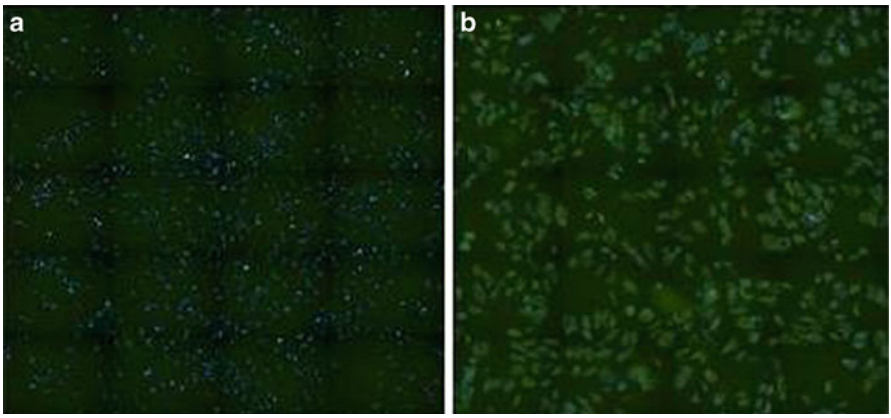

Fig. 12.4 Autofluorescence of young (**a**) and senescent (**b**) human bone-marrow derived stromal cells at 488 nm excitation wavelength

become oxidized outside or inside the lysosomal compartment (Terman and Brunk 2004). Because of autophagy of iron-containing macromolecules, such as ferritin and mitochondrial complexes, the lysosomal compartment is rich in partly redox active iron making lysosomes sensitive to oxidative stress through Fenton-type reactions. The hydroxyl radicals that form may give rise to peroxidation of material under degradation, resulting in lipofuscin formation. The lysosomal compartment is crucial for cell maintenance and has a variety of important functions, including endocytic uptake of materials from the outside and autophagic degradation of damaged mitochondria and other organelles, such as ribosomes, endoplasmic reticulum, and the proteasome, as well as numerous, mostly long-lived, proteins. The balance between digestion of damaged mitochondria and functional lysosomes with gradual accumulation of lipofuscin with age leading eventually to dysfunction was termed the mitochondria-lysosomal axis of aging, one of several leading theories of the causes of aging (Brunk and Terman 2002; Nowotny et al. 2014; Terman et al. 2010). More recently, the use of lipofuscin chromogenic staining as a trustworthy biomarker of aging of replicative and stress-induced senescence was reported (Georgakopoulou et al. 2013). The autofluorescence properties of lipofuscin aggregates of oxidized proteins, lipids and metals are elevated in replicative senescence cells as compared to early-passage cells (Fig. 12.4).

12.3.13 Mitochondrial Activities

Although cytochrome oxidase activity was not reduced in human fibroblasts in an age-dependent manner (Hakami and Pious 1968), the mitochondrial enzyme activities, mitochondrial nitric oxide synthase (mtNOS), NADH dehydrogenase and cytochrome oxidase, behaved as markers of brain aging. The decrease in enzyme

activities was directly related to the content of oxidation products and to the loss of neurological function in aged mice (Navarro 2004). Reduced TCA cycle flux is a marker of aging-associated diabetes type 2 (Gaster et al. 2012). Mitochondria dysfunction is intimately associated with other cellular events, and its impact on metabolism results eventually in senescence (Berkers et al. 2013; Correia-Melo and Passos 2015; Sahin et al. 2011; Ziegler et al. 2015).

12.4 Senescent Cell Plasma-Membrane Associated Biomarkers

Recently it has been shown that senescence can arise during early developmental stages, contributing to embryonic growth and patterning (Chuprin et al. 2013; Munoz-Espin et al. 2013; Storer et al. 2013). Evidence for the selective attack of senescent cells by Natural Killer (NK) cells was indicative of the presence of particular cues arising from the senescent cells and by the presence of selective surface markers (Sagiv et al. 2013; Sagiv and Krizhanovsky 2013). Cytosine phosphate guanine (CpG), an agonist of TLR9 receptor among others, activates hepatic stellate cells inducing fibrosis. Increased senescence, increased serum alanine transferase and attenuated fibrosis was found in TLR9-/- livers. Since TLR9 activation mediated stimulation of NK cells, it was proposed that TLR9 triggering would generate an NK-mediated immune anti-fibrotic response (Abu-Tair et al. 2013). Nuclear DNA and circulating mitochondrial DNA (mtDNA) are also agonists of TLR9 (Brenner et al. 2013) and coincidentally plasma levels of total and unmethylated plasma cell-free DNA and the mtDNA copy number have been proposed as biomarkers of frailty (Jylhava et al. 2013).

Senescent cells communicate with NK cells by multiple mechanisms. Senescent cells influence NK cells by (i) senescent associated secretory phenotype (SASP) (see Sect. 12.7 below); (ii) specific receptor-ligand interactions; (iii) cytoplasmic bridges. These diverse mechanisms trigger PI3K and p-AKT pathways, resulting in activation of the granule exocytosis in the NK cells and elimination of the senescent target cells by activation of apoptosis (Biran and Krizhanovsky 2015). Earlier works predicted an association between level of NK cell activity and progression of senescence, indicating that biomarkers of senescence are being expressed on relevant target cells that are eliminated efficiently as long as aging-preventive NK cell functions are preserved (Solana and Mariani 2000). The precise cell surface markers on pre-senescent/senescent NK target cells await further characterization.

A recent mass spectrometry screening for plasma membrane-associated proteins that were preferentially expressed in a bladder cancer cell line EJ expressing tetracycline-inducible p16 or p21, that induced senescence, helped to identify 132 and 107 potential protein markers, respectively, 10 of which were validated as having an increased expression on p21- and p16-induced senescent cells. Among the new confirmed senescence-associated biomarkers were DEP1, NTAL, EBP50, STX4, VAMP3 and ARMX3 that may well be correlated with replicative senescence

biomarkers, but it remains to be analyzed. Other expressed proteins that also were identified included membrane proteins DCR2, NOTCH3 and ICAM1 previously known as being highly expressed in senescent cells (Althubiti et al. 2014). However, even if the proteins are highly expressed in senescent cells, they are also expressed in non-senescent cells although at much lower levels. It would therefore be difficult to call them unique for the senescent state and be used in clinical targeting and eradication approaches.

$CD8^+$ T cells are crucial to the recognition and clearance of virus-infected cells. The lack of CD28 expression on T lymphocytes was regarded as a marker of replicative immunosenescence (Effros 1997). In another study, the phenotype associated with replicatively senescent $CD8^+$ T cells was correlated with increased CD57 expression, irrespective of the CD28 or CCR7 expression status, and this marker can render the cells susceptible to antigen-induced apoptosis (Brenchley et al. 2003). Another putative biomarker of immunosenescence was identified through proteome profiling of T-cell clones as human profilin-I, an abundant cytoplasmic protein (Mazzatti et al. 2007). Another immunologically relevant protein, marker of frailty, is beta 2 microglobulin (B2M), whose elevated plasma concentrations were associated with frailty among geriatric patients (Annweiler et al. 2011). B2M, a component of major histocompatibility complex class 1 molecules was described as a circulating factor that negatively regulates cognitive and regenerative function in the adult hippocampus in an age-dependent manner, and is elevated in the blood of aging humans and mice (Smith et al. 2015).

12.5 Epigenetic Biomarkers

Several epigenetic biomarkers displaying a good correlation with age have been described.

A cytosine next to a guanine in DNA constitutes a CpG island, and the cytosine can be methylated. The hypermethylation of tens of thousands of CpG sites during aging is well documented and represent a highly accurate biological clock across most cell types, referred to as epigenetic clock or DNA methylation age (DNAm age) that could be used as an age predictor or a biomarker (Horvath 2013). An association study between whole-blood DNA methylation patterns in 172 female twins aged 32–80 with age and age-related phenotypes resulted in the mapping of differentially methylated regions for 16 age-related phenotypes and chronological age. Out of hundreds of predominantly hyper-methylated age regions only one, TBX20, was also associated with an age-related phenotype. The study concluded that the majority of age-related changes in DNA methylation are not associated with phenotypic measures of healthy ageing in later life (Bell et al. 2012). Six CpG sites were identified which revealed either linear hyper-methylation or hypo-methylation with respect to the number of cumulative population doublings of human fibroblasts and mesenchymal stem cells providing an epigenetic biomarker to determine the state of senescence in cell preparations (Koch and Wagner 2013). The degree of

cytosine methylation at 88 sites in or near 80 genes was significantly correlated with age in saliva of 34 male identical twin pairs between 21 and 55 years of age. In particular, the methylation state in the promoters of the EDARADD, TOM1L1, and NPTX2 genes was found to be linear with age over a range of five decades among 31 males and 29 females between 18 and 70 years of age. Using just two cytosines from these loci, a regression model could explain 73 % of the variance in age and predict the age of an individual with an average accuracy of 5.2 years (Bocklandt et al. 2011). A whole blood DNA methylation profile (methylome) of 32 mother-offspring couples in the CpG islands within the promoters of the ELOVL2, FHL2, and PENK genes showed that their methylation level strongly correlated with age, especially the ELOVL2 methylation pattern that exhibited a progressive increase from the very first years of life. ELOVL2 encodes for a transmembrane protein involved in the synthesis of long (C22 and C24) ×3 and ×6 polyunsaturated fatty acids (PUFA). Thus, ELOVL2 methylation is a promising biomarker of aging (Garagnani et al. 2012). Also, methylation profiles from 102 age-related CpG sites in blood rendered three CpG sites located in the genes ITGA2B, ASPA and PDE4C, allowing the prediction of chronological age with an accuracy of less than 5 years (Weidner et al. 2014). As mentioned previously (Sect. 12.3.7), LMNB1 is depleted during senescence, preferentially from the central regions of lamina-associated domains, which are enriched for Lys9 tri-methylation on histone H3 (H3K9me3 marker) promoting SAHF formation. Despite the decrease in LMNB1 expression in senescence, LMNB1 binding increases during senescence in a small subset of gene-rich regions where H3K27me3 also increases and gene expression becomes repressed (Sadaie et al. 2013). During long-term culture of human fibroblasts and mesenchymal stromal cells it was found that senescence-associated DNA hyper-methylation occurred in regions associated with H3K27me3, H3K4me3, and H3K4me1 histone marks, whereas hypo-methylation was associated with chromatin containing H3K9me3 and lamina-associated domains. DNA hyper-methylation was significantly enriched in the vicinity of genes that are either up- or down-regulated at later passages. Furthermore, specific transcription factor binding motifs located in the EGR1, TFAP2A, and ETS1 genes were significantly enriched in differentially methylated regions and in the promoters of differentially expressed genes (Hanzelmann et al. 2015). Future works will highlight which hyper-methylated site is most reliable as an aging biomarker across cell types and classes of senescence-inducing pathways, not only associated with chronological age but also with age-related phenotypes.

12.6 Small and Long Non-coding RNAs

Post-transcriptional regulators of senescence comprise (i) RNA-binding proteins (RBP), such as human antigen R (HuR), AU-binding factor 1 (AUF1), and tristetraprolin (TTP), which associate with target mRNAs that encode senescence factors and influence cellular senescence (ii) microRNAs (miR's) a large group of small

(~22 nt), non-coding RNAs that are differentially expressed during senescence and (iii) long non-coding RNA's (lncRNA).

Small non-coding RNA molecules (sncRNA's) consist mainly of miR's, 5′ transfer RNA (tRNA) halves, and YRNA fragments (Dhahbi 2014). Whilst many of these molecules have important roles in the regulation of protein expression and are associated with signal-mediated disease pathways or with organ-specific aging, there is limited knowledge about a universal sncRNA in all cell types undergoing replicative senescence.

Circulating miR's are found in secreted microvesicles or exosomes present in body fluids, or in complex with other factors such as RNA-binding proteins and high-density lipoprotein particles. miR's were proposed as novel markers of cell aging in humans; specifically miR-17, -19b, -20a, and 106a were down-regulated in different models of cellular aging, targeting mainly p21/CDKN1a (Hackl et al. 2010). A number of works followed up and a review of aging-associated sncRNA's was recently presented (Dhahbi 2014). A summary of mouse and human circulating miR's were found in individuals with aging-related diseases such as type 2 diabetes, Alzheimer's disease and myocardial disease (Jung and Suh 2014). Plasma levels of miR-20a-3p, miR-30b-5p, miR106b, miR191 and miR-301a were confirmed to show significant age-related decreases, but were not associated with the fitness or frailty status (Hatse et al. 2014). An example of miR's associated with cardiac aging was recently reviewed, explaining how up-regulation of various miR's targeting specific genes could affect tissue function, such as miR-18, -19, -22, -29, -34, -146, and -217 (Dimmeler and Nicotera 2013). In human endothelial cells undergoing replicative senescence, miR-146a, -9, -204 and -367 showed the highest up-regulation in senescent cells, with miR-146a as a promising marker of a senescence-associated pro-inflammatory status in vascular remodeling cells (Olivieri et al. 2013). In another similar study, miR-23a, -23b, -24, -27a, -29a, -31, -100, -193a, -221, -222 and let-7i were consistently up-regulated in human replicatively senescent endothelial cells. Surprisingly, also miR-21 was found up-regulated by replicative and stress-induced senescence, despite being described as oncogenic (Dellago et al. 2013). Among miR's, miR-638 and miR-663 are strongly up-regulated in replicative senescence of human WI-38 fibroblasts (Maes et al. 2009). Among the miRNAs down-regulated with senescence in cultured human fibroblasts were members of the let-7 family, while upregulated miRNAs included miR-1204, miR-663 and miR-519 (Marasa et al. 2010). A set of senescence-associated miR's in human fibroblasts were identified that could either facilitate DNA damage or regulate the expression of several senescence mediators to sustain the senescent phenotype. miR-210, -376a*, -486-5p, -494, and -542-5p induced double-strand DNA breaks and reactive oxygen species accumulation in transfected cells (Faraonio et al. 2012). In human mammary epithelial cells, miR's 26b, 181a, 210 and 424 actions convey on various targets leading to p16 activation (Overhoff et al. 2014). In several cases, miR's have been shown to actually induce senescence when experimentally up-regulated, such as miR-21 (Dellago et al. 2013), miR-22 (Dimmeler and Nicotera 2013), miR-34a (Rokavec et al. 2014), miR-205 (Dar et al. 2011) or miR-519 (Marasa et al. 2010), with accompanying p16/p21 expression,

that could further be validated as aging biomarkers. miR-21 could be considered a new biomarker of "inflammaging" since its expression was higher in patients with cardiovascular disease, and it was correlated with C-reactive protein and fibrinogen levels. Also, TGF-bR2 mRNA, a validated miR-21 target, showed the highest expression in the leukocytes from a subset of the octogenarians (Olivieri et al. 2012). Even disruption of miR biogenesis can cause senescence (Gomez-Cabello et al. 2013). AGO2, RB1 and miR's such as let-7, physically and functionally interact to repress RB1/E2F-target genes in senescence, a process that was called senescence-associated transcriptional gene silencing (SA-TGS) (Benhamed et al. 2012). Finally, associations of miR's with human longevity have also been uncovered (ElSharawy et al. 2012).

Other post-transcriptional regulators in senescent cells have also been characterized. For example, the RNA-binding protein HuR is strongly down-regulated in senescence, affecting the expression of diverse proliferative genes (Wang et al. 2001). A profiling comparison between young and senescent human WI-38 fibroblasts rendered senescence-associated lncRNA's (SAL-RNA's) whose expression patterns could be correlated with the senescent phenotype (Abdelmohsen et al. 2013) but their potential as biomarkers of aging awaits further *in vivo* characterization. lncRNA's controlling telomere function, epigenetic changes, proteostasis, stem cell function, proliferation and senescence, intercellular communication were described. Several molecules such as ANRIL, SRA, HEIH, HULC and UCA1 inhibit senescence by targeting different CKI's (Grammatikakis et al. 2014).

Not all targets of the above-mentioned miR's are fully characterized; it is therefore premature to conclude what functional implications they have on the aging rate, and whether their amounts have been normalized to cell size. Their targets are usually p53 or CKI's that, in some cases, could result in inhibition of senescence.

12.7 Senescence Secretome and Metabolome, Frailty and Whole-Body Physiological Biomarkers

A major feature that has become characteristic of the senescent phenotype is the release of secreted factors by replicatively senescent cells that elicit various responses including disruption of tissue structure and function, growth promotion of neighbor pre-malignant cells, mitogenic stimuli, induction of a pro-inflammatory response and associated "inflammaging" (Laberge et al. 2015). These factors have collectively been called Senescence-messaging secretome (SMS) factors (Kuilman and Peeper 2009) or Senescence-associated secretory phenotype (SASP) factors (Freund et al. 2010). Senescence-inducing signals activate the stress-inducible kinase p38MAPK and a SASP response in normal human fibroblasts and its inhibition markedly reduced the secretion of most SASP factors (Freund et al. 2011). This showed that the SASP response could be a therapeutic target of novel anti-aging compounds (Tchkonia et al. 2013).

Ongoing detailed analyses of the functional consequences of the presence of these factors with regards to their effects in eliciting age-related pathologies and chronic disease is currently being investigated (Lopez-Otin et al. 2013; Tchkonia et al. 2013; van Deursen 2014). Not all the secreted factors seem to be detrimental: during tissue repair, it was shown that senescent fibroblasts and endothelial cells appear very early in response to a cutaneous wound, where they accelerate wound closure by inducing myofibroblast differentiation through the secretion of platelet-derived growth factor AA (PDGF-AA) (Demaria et al. 2014).

Metabolic comparisons between young and old mice rendered the first known aging metabolome, representing metabolites that can be considered biomarkers of age and healthspan. A human aging metabolome is being characterized as well (Houtkooper et al. 2011). The metabolomic signature associated with natural mouse aging accurately predicted aging as a result of telomere shortening, or the presence of short telomeres (Tomas-Loba et al. 2013).

Several attempts have been launched at estimating the degree of biological aging based on whole-body physiological parameters. Biological markers were found to be helpful to predict future disability, morbidity, and mortality outcomes in older adult populations, and included markers of neuroendocrine functioning (epinephrine, norepinephrine, cortisol, and dehydroepiandrosterone), immune activity (C-reactive protein, fibrinogen, IL-6, and albumin), cardiovascular functioning (systolic and diastolic blood pressure), and metabolic activity [high-density lipoprotein (HDL) cholesterol, total to HDL cholesterol ratio, and glycosylated hemoglobin] (Gruenewald et al. 2006). Similarly, higher levels of transferrin, fibrinogen, and interleukin-6 were associated with frailty status and frailty score in community-dwelling older adults (Darvin et al. 2014).

Liver, kidney, spleen, lung and brain were sampled at regular time intervals during the entire C57BL/6 J murine lifespan from a controlled *in vivo* aging study and characterized using 18 different age-related pathological parameters and transcriptomic profiles. The study revealed that lipofuscin-correlated transcripts were overrepresented in many more functional pathways than the 1000 False Discovery Rate-ranked chronological genes, resulting in an increase in the number of functional (mostly immune-related) pathways. Besides existing overlap between chronological and pathological aging processes (e.g. mitochondrial processes and lipid metabolism), many divergent functional responses were revealed often using a tissue-specific pathological scale (Melis et al. 2013).

In another study involving human subjects, 43 common clinical biomarkers were measured longitudinally in 3694 people from three longitudinal cohort studies on two continents (Women's Health and Aging I and II, InCHIANTI, and the Baltimore Longitudinal Study on Aging). Increasing age was associated with anemia, inflammation, and low levels of calcium and albumin (albunemia). Integrated albunemia increased and accelerated with age in all populations predicting mortality and frailty (Cohen et al. 2015).

Senescence biomarkers are analogous to frailty factors, which are events reflecting the body's functional degree. Using e.g. a FI34 frailty index based on 34 health

variables, it was found that the index increases non-linearly with advancing age and is a better indicator of biological aging than chronological age (Kim and Jazwinski 2015).

Multiple physiological integrity biomarkers monitoring the cardiovascular, metabolic, and immune systems, kidneys, livers, and lungs, dental health, and DNA were tracked in 954 young chronic disease free-participants of the Dunedin Study birth cohort, tracking multiple biomarkers across three time points spanning their third and fourth decades of life. Already, before midlife, individuals who were aging more rapidly were less physically able, showed cognitive decline and brain aging, reported a worsened self-perceived health status, and looked older. Thus, quantification of biological aging in young adults can be used to identify the causes of aging and evaluate rejuvenation therapies (Belsky et al. 2015).

Expression of skin-specific aging biomarkers such as pro-collagen I and VII, elastin, fibrillin-1, and SIRT1 and SIRT6, were down-regulated during human fibroblast replicative senescence. The level of procollagen Type I was significantly associated with early aging, while a change in the level of fibrillin-1 was associated with late aging. All biomarkers except elastin showed a strong correlation with the cellular stiffness of human fibroblasts with increasing passage (Kim et al. 2015).

Finally, the MARK-AGE (European Study to Establish Biomarkers of Human Ageing) program has sought novel early biomarkers of biological vs. chronological age in plasma and serum in a cohort of approximately 3200 individuals in the age range 34–75 years, that could help predict the rate of aging later in life (Burkle et al. 2015; Griffiths et al. 2015).

12.8 Concluding Remarks

While it is apparent that at the cell culture level many biomarkers of replicative senescence do indeed show an increase in their levels, independently of cell size, at the individual cell level there are many individual biomarkers that do not fulfill their role, even though the cells are no longer proliferating. Heterogeneity amongst cells regarding biomarker expression is evident (Brookes et al. 2004; Martin-Ruiz et al. 2004; Passos et al. 2007) (Nehlin et al. unpublished). This heterogeneity was already documented statistically early on, when cytological analyses clearly showed that the morphological changes observed were not the same amongst all fibroblasts in the culture, indicated by a widening standard deviation for each parameter measured (Bemiller and Miller 1979). This represents a hurdle as regards the intent to characterize senescent cells *in vivo*. Thus, quantitative correlations of all suggested biomarkers for each single cell in a culture compared with their proliferative capacity need to be explored.

In addition, much more work remains to be done to define the similarities and differences between cells that become senescent in tissue culture and aged cells in the intact organism.

Estimation of the real biological age of an individual could probably predict mortality better than chronological age (Levine 2013), but this would require many

more studies that would examine in-depth the reason or causes by which individual cells become senescent *in vivo*.

One approach to estimate a person's age is based on facial appearance. In fact, how old one looks for one's age (perceived age) has been proposed as a biomarker of ageing (Christensen et al. 2009; Gunn et al. 2013; van Drielen et al. 2015). A youthful appearance was correlated with delayed senescence and seems to better reflect the general health status than chronological age (Chen et al. 2015).

Biomarkers of gradual senescence should be accompanied by control biomarkers of optimal health, where cell metabolism is at best, and stress signal profiles could help with such endeavor (Demirovic and Rattan 2013).

The epigenetic aging signature has provided the possibility to make age predictions with a mean absolute deviation from chronological age of approximately 3, 3–5 years (Garagnani et al. 2012; Weidner et al. 2014). If hyper-methylation of given promoters in genes can reflect the environmental/epigenetic insults that cells and the whole body are subject to, and that can be reliably analyzed in blood samples, they would be very suitable as aging biomarkers.

More validation studies are required to compare the existence of biomarkers of replicative exhausted senescent cells adjusted for cell size with the same biomarkers analyzed in patient samples. Moreover, there seems to be a trend towards organ or tissue-specific biomarkers of aging taking in consideration their functional specialization, suggesting the need for validation of biomarkers of replicative senescence for each of the cell types in the body to compare similarities and differences.

The recent discovery that senescent cells can be surveyed by the immune system and be eliminated, suggests why it has been complicated to demonstrate the presence of fully-senescent cells *in vivo*. It is therefore important to concentrate efforts in better defining and validating biomarkers of pre-senescence e.g. epigenetic marks, while cells are still active proliferating, as early predictors of the rate of senescence. The cell culture biomarkers of replicative senescence will be useful in assessing the rate of aging of highly proliferating cells in the body, and possibly other cell types as well, but they will need to be complemented with other types of markers that represent the type of senescence-inducing pathway being activated. The final executors of permanent cell cycle arrest in senescent cells within the nuclei of cells, may be the most reliable biomarkers after all, but will stop short of completely fulfilling the criteria of ideal biomarkers, as stated early on.

Based on a number of recent works, it is becoming increasingly evident that a combination of events acting in parallel influence the chronological accumulation of pre-senescent and senescent cells in the aging body due to either replicative exhaustion, increase in damage and waste products, or failure in the immune surveillance mechanisms that regulate the presence of senescent cells in the body. The relationships between these events and the outcome measured as biomarkers will undoubtedly help to tackle the aging process.

The present trend is a global pursuit of quantitative markers that can disclose the rate of aging and the actual health status. With the advent of recent technological advances, we will not need to wait another 50 years to define which, how many and

under what circumstances given biomarkers will be needed to predict the degree of aging and eventually help to achieve the long-term goal of better healthy aging.

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Part IV
Ageing Modulators

Chapter 13
Stress-Induced (Premature) Senescence

Florence Debacq-Chainiaux, Randa Ben Ameur, Emilie Bauwens, Elise Dumortier, Marie Toutfaire, and Olivier Toussaint

Abstract Three main roads lead to senescence: telomere-dependent replicative senescence, oncogene-induced senescence and stress-induced (premature) senescence. This latter type of senescence appears after exposure of normal, immortalized or transformed cells to stress of chemical or physical nature inducing oxidative stress and/or DNA damage. After these exposures, chronic or acute, single or multiple, stressed cells developed a "senescence-like" phenotype. This "senescence-like" phenotype presents several biomarkers of cellular senescence such as irreversible growth arrest, morphological change, senescence-associated ß-galactosidase (SA-ßgal) activity and senescence-associated secretory phenotype (SASP). However, large-scale studies of transcriptome and proteome of cells in replicative senescence or in stress-induced senescence show that although they share similarities, the two phenotypes are not identical. Different signaling pathways involved in the development of stress-induced senescence are presented as those dependent on TGF-ß1, $p38^{MAPK}$, IGF-R1 and DNA damage. The possible induction of this type of senescence *in vivo* and in cancer treatment is discussed.

Keywords Replicative senescence • Cell cycle • Stress • SIPS • Oxidation • DNA damage • Telomeres

13.1 Introduction

Ageing is characterized by a progressive functional decline, leading to increased risk of developing major human pathologies, such as cardiovascular disorders, cancer, diabetes and neurodegenerative diseases. Nine hallmarks of ageing have been established, including cellular senescence (Lopez-Otin et al. 2013). Cellular senescence, first described *in vitro* in normal human diploid fibroblasts (HDFs)

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by L. Hayflick in the early 1960s, is defined as an irreversible arrest of the cellular divisions and for this reason also called “replicative senescence” (RS) (Hayflick and Moorhead 1961). Almost 30 years later, it was shown that this type of senescence is associated with a « critical » shortening of the telomeres after extensive proliferation in the absence of endogenous telomerase activity (Harley et al. 1990).

After the discovery of cellular senescence, studies were performed to characterize the senescent phenotype and to identify specific biomarkers that allow to distinguish senescent cells from other non-dividing cells (such as quiescent or terminally differentiated cells). Senescence-associated biomarkers are among others typical morphology (Bayreuther et al. 1988), irreversible growth arrest with overexpression of $p21^{WAF-1}$ and $p16^{INK-4a}$ (Alcorta et al. 1996; Sherr and Roberts 1999), senescence-associated beta-galactosidase activity (SA-ßgal) (Dimri et al. 1995), altered gene expression (Dumont et al. 2000b), senescence-associated heterochromatin foci (SAHFs) and senescence-associated DNA-damage foci (SDFs) (d’Adda di Fagagna et al. 2003) and lamin B1 loss (Freund et al. 2012). Using some of these biomarkers such as SA-ßgal and $p16^{INK-4a}$ overexpression, it was demonstrated that senescent cells accumulate in tissues with age (Dimri et al. 1995; Ressler et al. 2006).

The role of senescent cells *in vivo* is still unclear, but from their discovery, it has been speculated that they may be involved in the functional decline associated with ageing. In 2011, Baker et al. (2011) demonstrated that clearance of senescent cells in mice delays ageing-associated disorders as cataract and muscular mass loss, demonstrating the impact of senescent cells on their cellular and matrix environment and on tissue function. This impact is more than probably related to their ability to secrete a variety of factors such as inflammatory cytokines, metalloproteinases and growth factors, identified as the senescence-associated secretory phenotype (SASP) (Coppe et al. 2008).

13.2 Different Types of Senescence

Many studies have shown that multiple ways can lead to senescence or at least to a “senescence-like” phenotype. Senescence can be induced by multiple stimuli such as shortened or dysfunctional telomeres, excessive mitogenic signals (including those produced by oncogenes) and stress. Three major types of senescence may thus be highlighted: replicative senescence or telomere-dependent senescence, oncogene-induced senescence and stress-induced (premature) senescence. These three types of senescence share a common senescent phenotype, as highlighted by the appearance of senescence-associated biomarkers (Fig. 13.1).

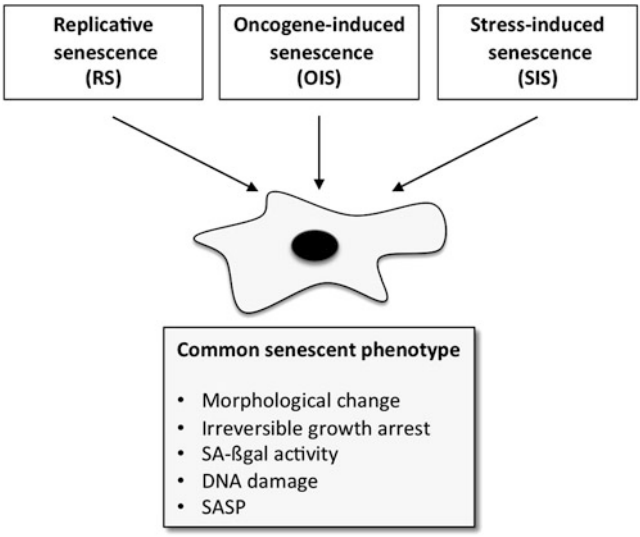

Fig. 13.1 Replicative senescence, oncogene-induced senescence and stress-induced senescence share a common senescent phenotype presenting several of the so-called senescence-associated biomarkers

13.2.1 Replicative Senescence or Telomere-Dependent Senescence

Replicative senescence is intimately linked to telomere shortening. Telomeres are sequences of repetitive DNA (5′-TTAGGG-3′ in vertebrates) located at the extremities of linear chromosomes and associated to telomere-specific protein complex, also known as shelterin/telosome, including TRF1, TRF2, TIN2, POT1, TPP1 and hRAP1 (for a review: Palm and de Lange 2008). Length of telomeres are shortened after each cellular division, this is inherently linked to the activity of the DNA polymerase which is not able to completely replicate one strand of DNA, also referred as the « end-replication problem » (Harley et al. 1990). This leads to critically short and dysfunctional telomeres, which are sensed by the cells as double-strand breaks (DSBs). There follows activation of a classical DNA-damage response (DDR) inducing activation of upstream kinases as ataxia telangectasia mutated (ATM) and ATM and Rad-3 related (ATR), DNA-damage adaptator proteins as MDC1 (mediator of DNA-damage checkpoint protein-1), 53BP1 (p53-binding protein-1), BRCA1 (breast cancer type-1 susceptibility protein) and claspin, downstream kinases such as checkpoint-1 and -2 (CHK1 and CHK2) and finally effectors proteins as p53, CDC25 and SMC1 (structural maintenance of chromosomes protein-1). This activated pathway induces cell cycle arrest, mainly by phosphorylating p53, activating the expression of $p21^{WAF-1}$ inhibiting CDK-cyclin complexes and triggering DNA repair (Campisi and d'Adda di Fagagna 2007; d'Adda di Fagagna et al. 2003).

13.2.2 Oncogene-Induced Senescence

Oncogenes are mutant version of normal genes that have the potential to transform cells in combination with additional mutations, notably leading to inactivation of tumor-suppressor genes such as p53 or $p16^{ink\text{-}4a}$. Oncogenes activation was shown to induce senescence in normal cells. This response was presented as a way to prevent oncogenic transformation. This was first observed by the expression of oncogenic *ras* ($H\text{-}RAS^{G\ V12}$) form in normal fibroblasts leading to growth arrest and SA-ßgal activity (Serrano et al. 1997), independently of the telomere shortening (Wei and Sedivy 1999). Oncogene-induced senescence (OIS) has been displayed by many oncogenes *in vitro* and *in vivo* including members of Ras signalling pathway as *N-Ras*, *Raf*, $BRAF^{E600}$ (for a review: Gorgoulis and Halazonetis 2010). Senescence can also be induced by the expression of mutated form of tumor suppressor genes like *PTEN*, *VHL*, *RB1*, and *NF-1* leading to their inactivation (Nardella et al. 2011).

13.2.3 Stress-Induced Senescence: Premature or Not?

Senescence can finally appear after exposure of normal, immortalized or transformed cells to stress from physical or chemical agents inducing oxidative stress and/or DNA damage. This was called « Stress Induced Premature Senescence » or SIPS (Toussaint et al. 2000). "Premature" refers that senescence following stress occurs at earlier population doublings (PDs) than the maximum number of PDs at which appears usually replicative senescence. "Premature" emphasizes the accelerated nature of the process. However, the use of the "premature" term may be questionable.

First because the *in vitro* cell culture conditions are still not ideal and that the definition of a "classical" number of passages at which cells enter in senescence is dependent of many variables of cell culture conditions. For instance current cell culture conditions consist in maintaining cells at atmospheric oxygen concentration (21 %). However, *in vivo*, cells are exposed to a reduced oxygen concentration ranging between tissues from 3 to 4 % in the brain (Dings et al. 1998), 3–7 % in the muscles (Vedsted et al. 2006), 5–7 % in the dermis and 16–19 % in the epidermis (Stucker et al. 2002). HDFs maintained in culture at a more physiological/reduced oxygen pressure, are able to achieve more passages before undergoing replicative senescence than cells maintained at atmospheric partial oxygen pressure (Packer and Fuehr 1977). From then on, one may wonder what the standard is and if the cells maintained at 21 % oxygen are not themselves subject to some oxidative stress (Toussaint et al. 2011). We understand here the difficulty of setting a classical or a premature onset of senescence. However, the culture conditions are identical between stressed and control cells, "premature" insists on the quickening of the process between the two conditions.

Second, transformed cells are also able to undergo senescence after stress. The mechanisms of growth arrest might be extremely different between cancer cells,

whose cycle is by definition deregulated for many possible reasons, and normal cells exposed to stressful agents. In that case, can we really speak of "premature" senescence, since these cells, by nature, do not enter in senescence?

These examples, beyond the debate on whether the use of the term "premature" is adequate, demonstrate the multiplicity of senescence-like phenotypes. To encompass all forms of senescence induced by stress, it therefore seems more prudent in this review to talk about "stress-induced senescence" or SIS (Dierick et al. 2003; Hornsby 2010).

13.3 Models of Stress-Induced Senescence

In vivo, cells are continuously exposed to different types of stress. Depending on the nature of the stress, its intensity and the cell type, stressed cells can select one of the three main tracks: repair, die or senesce. If the intensity of the stress is low, the cell can repair damage and after a transient cell cycle arrest, resumes its growth. If the stress is intense, apoptosis will be privileged. There is therefore a "moderate" stress zone for which cells exposed to high stress, but still in the subcytotoxic range, will not be able to repair all damage caused by the stress. With reference to the thermodynamic theory of far from equilibrium open systems, cells will drop to a lower steady state of internal entropy (with lower metabolic activity) and if exceeded, its cell cycle will be irreversibly arrested and cells will enter in premature senescence (Toussaint et al. 2002).

Potentially almost any stress from chemical or physical nature can induce cells to undergo senescence by provoking increased oxidative stress and/or DNA damage. Per se, the types of stress that can induce senescence seem infinite and many models of SIS exist which will not be possible to list here. However, we can describe the main classes of stress models that can induce stress-induced senescence: physical stress (UV and X-irradiation), oxidizing agents and hyperoxia, cytokines/adipokines, chemotherapeutic drugs and copper (Fig. 13.2). In the different models, the conditions used reduce the growth rate and the maximum obtainable cumulative population doublings (CPDs) of cells and induce the appearance of senescence-associated biomarkers.

Cellular senescence can be induced by both chronic or acute stress protocols in different cell types such as normal HDFs, endothelial cells, melanocytes and transformed cells. Both protocols use sublethal concentrations of stressors, in accordance with theoretical studies based on the stability of cellular systems (Toussaint et al. 1991).

Chronic stress protocols consist of treating different human cell types with prolonged exposure (several weeks) to a stressor like mild hyperoxia in HDFs (with oxygen partial concentration around 40 %) (von Zglinicki et al. 1995, 2000), chronic exposure of endothelial cells to homocysteine (Xu et al. 2000), prolonged culture of human umbilical vein endothelial cells on glycated collagen (Chen et al. 2002) and chronic exposure of colon fibroblasts to selenite (Rudolf et al. 2014).

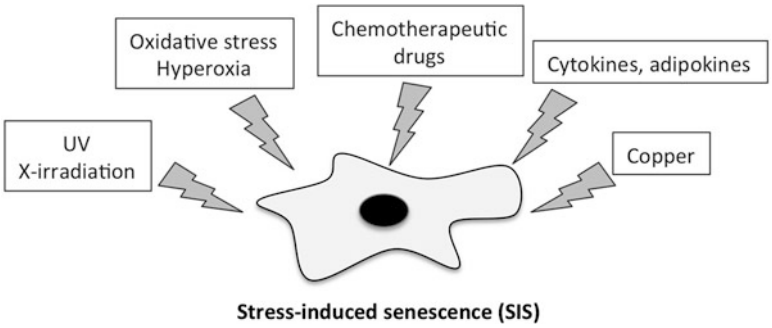

Fig. 13.2 Several types of stress can induce stress-induced senescence (SIS)

In the second type of protocol, cells, such as HDFs, at early CPDs are exposed once or several times to acute sublethal stress such as a unique H_2O_2 exposure (Chen and Ames 1994) or a repeated exposure to *tert*-butylhydroperoxide (*t*-BHP) (Dumont et al. 2000b). The use of repeated sublethal stresses allow reducing the concentration of the stressor necessary to trigger stress-induced senescence. The major advantage of this type of protocol is that the induction of senescence can be studied relatively independently from purely adaptive responses, if the cells are allowed to recover for at least 3 days after the stress before analysis of biomarkers of senescence and if the repetition of stresses increases the fraction of cells undergoing stress-induced senescence (Toussaint et al. 2000).

Among the different models of existing SIS, some are based on physical stress such as ionizing radiation of endothelial cells (ECs) (Igarashi et al. 2007; Panganiban et al. 2013), exposure of HDFs or melanocytes to UV light (Rodemann 1989; Debacq-Chainiaux et al. 2005; Medrano et al. 1995), to psoralen pre-treatment and UVA exposure (pUVA) (Ma et al. 2002) and to electromagnetic fields (Rodemann et al. 1989b) and exposure of human mesenchymal stem cells to sublethal heat shock (Alekseenko et al. 2014).

Other models induce oxidative stress as in HDFs exposed to chronic hyperoxia or to acute subcytotoxic exposure to oxidizing agents such as H_2O_2 or *t*-BHP (Chen and Ames 1994; Dumont et al. 2000a; von Zglinicki et al. 1995).

Stress-induced senescence can also be provoked by stimulating cells with cytokines or adipokines like repeated stimulation of lung HDFs or endothelial cells with interleukin-1α (IL-1α), tumor-necrosis factor-α (TNF-α) (Dumont et al. 2000a), transforming growth factor-beta 1 (TGF-ß1) (Debacq-Chainiaux et al. 2005, 2008; Frippiat et al. 2001) and visfatin/Nampt (Villalobos et al. 2014).

Senescence can also be induced by subcytotoxic doses of drugs and in particular chemotherapeutic drugs such as mitomycin C (Rodemann 1989), cisplatin (Berndtsson et al. 2007), etoposide (Chiu et al. 2005) and doxorubicin (Maejima et al. 2008; Piegari et al. 2013).

Finally, premature senescence appears by incubating HDFs with copper (Cu), which itself accumulates in cells during replicative senescence. Incubation of HDFs with copper sulphate ($CuSO_4$) for 16 h induces the appearance of several senescence-associated biomarkers (Boilan et al. 2013; Matos et al. 2012). Conversely, iron-chelation using desferroxamine mesylate induces growth arrest and the appearance of senescence-associated biomarkers in hepatocyte cell lines (Yoon et al. 2002).

13.4 Damage Induced by SIS

13.4.1 DNA Damage

DNA is a favourite target of stress, directly or indirectly. Many types of damage can be produced on DNA, but the most critical for the induction of senescence is double strand breaks (DSBs). Shortened and dysfunctional telomeres form similar structures of DSBs, inducing DDR pathway (Takai et al. 2003).

Several models of stress-induced senescence induced in normal or immortal cells by chemotherapeutic drugs (mitomycin C, bleomycin, actinomycin D) (Robles and Adami 1998) or ionizing radiation (X-irradiation) (Kim et al. 2014) generate DNA damage, including DSBs leading to senescence. Once DNA repair machinery detects DSBs, large protein complexes including in particular γH2Ax and 53BP1 are recruited at the cleavage site and help to stabilize the damaged strands and to activate the repair process. If the damage is too important for repair, DDR pathway is activated and induces activation of p53 transcription factor and expression of $p21^{WAF\text{-}1}$. The overexpression of $p21^{WAF\text{-}1}$ is transient and followed by a delayed induction of $p16^{INK\text{-}4a}$, as it has been demonstrated in RS. The pathway leading to $p16^{INK\text{-}4a}$ overexpression after DNA damage is still unclear (Robles and Adami 1998) but could be linked to oxidative stress.

13.4.2 Oxidative Stress

Oxidative stress is probably the most often used inducer of stress-induced senescence. The link between replicative senescence and oxidative stress was clearly demonstrated by comparing the number of passages in culture performed under conditions of hypoxia or hyperoxia, and by the use of antioxidants and/or free radical scavengers as, for instance *N-tert*-butyl-alpha-phenylnitrone (PBN). PBN is a biphasic antioxidant that is soluble in lipid and water, and is stable in cell culture media. Presenescent HDFs incubated with PBN are able to achieve between four and seven additional CPDs than untreated cells (von Zglinicki et al. 2000).

An increase of radical oxygen species (ROS) is highlighted in HDFs exposed to H_2O_2, UVB and copper (Boilan et al. 2013; Borlon et al. 2007; Zdanov et al. 2006b). The use of antioxidants as mannitol or trolox significantly reduces the appearance of senescence-associated biomarkers following copper incubation of HDFs (Boilan et al. 2013). Nrf-2 transcription factor is also activated in HDFs incubated with copper (Boilan et al. 2013).

An hypothesis to explain the expression of $p16^{INK-4a}$ after DNA damage is the induction of a second phase of stress after direct DNA damage via oxidative stress inducing accumulation of ROS, activation of $p38^{MAPK}$ and $p16^{INK-4a}$ overexpression (Hornsby 2010).

13.5 Cellular and Molecular Characteristics of SIS

13.5.1 Biomarkers of Senescence

Cells in stress-induced senescence share common features with replicative senescence as it was highlighted by the study of biomarkers of senescence.

Senescent cells are characterized by morphological change, they generally enlarge, often doubling in volume, and, if adherent, adopt a flattened morphology. Using the morphotypes classification of HDFs (Rodemann et al. 1989a), it was shown that after sublethal stress under H_2O_2, *t*-BHP, UV light, mitomycin C, etc., the treated HDFs acquired the morphological features of senescent HDFs (Chen and Ames 1994; Debacq-Chainiaux et al. 2005; Dumont et al. 2000b; Rodemann et al. 1989a).

Their growth is irreversibly arrested at the G1/S phase of the cell cycle due to the overexpression of several cyclin-dependant kinase inhibitors as $p21^{waf-1}$ and $p16^{ink-4a}$ and hypophosphorylation of the retinoblastoma protein (pRb) (Chen et al. 1998).

Senescence-associated ß-galactosidase (SA-ßgal) is a commonly used marker of senescent cells allowing to identify easily senescent cells both *in vitro* and *in vivo* (Dimri et al. 1995). This activity derives from the lysosomal ß-galactosidase, overexpressed in senescent cells, and then detectable at a suboptimal pH (Kurz et al. 2000). After exposure to stress inducing senescence, proportion of cells positive for SA-ßgal activity increases.

Mitochondrial DNA undergoes many changes during replicative senescence among which a 4977 bp « common » mitochondrial DNA deletion. This deletion is clearly detected in HDFs exposed to sublethal stresses with *t*-BHP or UVB (Debacq-Chainiaux et al. 2005; Dumont et al. 2000b).

HDFs in RS and in SIS induced by X-irradiation or by bleomycin display a highly correlated SASP including increased expression of IL-1ß, IL-8, GROα and MMP-1 (Coppe et al. 2008; Bavik et al. 2006).

13.5.2 Gene and Protein Expression: Are RS and SIS Identical Phenotypes?

Senescent cells show striking changes in gene expression, including gene expression changes of cell-cycle effectors. Two cyclin-dependent kinase inhibitors (CDKIs) are often overexpressed in senescent cells: $p21^{waf\text{-}1}$ (*CDKN1a*) and $p16^{ink\text{-}4a}$ (*CDKN2a*). On the contrary, senescent cells can also repress genes that encode for cell-cycle activators as *c-fos*, *cyclin B* and *PCNA* (*proliferating cell nuclear antigen*).

But many changes in gene expression are unrelated to growth arrest. Senescent cells overexpress genes encoding secreted proteins that can alter the tissue microenvironment, for instance to remodel extracellular matrix or to mediate local inflammation (Campisi et al. 1996).

If similar gene expression change is observed in stress-induced senescence as overexpression of $p21^{waf\text{-}1}$, $p16^{ink\text{-}4a}$, *apolipoprotein J*, *osteonectin* and *fibronectin* (Debacq-Chainiaux et al. 2005; Dumont et al. 2000b; Pascal et al. 2005), large-scale studies also show specific gene expression change associated either to replicative senescence or to stress-induced senescence (Pascal et al. 2005).

The study of the different biomarkers tends to show the similarities between the two phenotypes of RS and SIS. But are they identical phenotypes? To answer this question, the simple study of the presence of replicative senescence biomarkers after SIS was not enough. It was therefore necessary to achieve a more global comparison between these two phenotypes, through larger studies of gene and protein expression in both phenotypes.

Studies on global gene expression using differential display or cDNA microarray firstly confirmed the presence of genes whose expression is identically modified between RS and SIS, but also demonstrated that specific changes were associated either to RS or to SIS induced by *t*-BHP, ethanol, H_2O_2 or bleomycin in HDFs (Pascal et al. 2005; Debacq-Chainiaux et al. 2008; Bavik et al. 2006). In parallel, a proteomic identification by mass spectrometry after two-dimensional gel electrophoresis (2DGE) came to the same conclusion on the comparison of the same models (Dierick et al. 2002). This observation was confirmed by a proteomic analysis of HDFs in RS and in H_2O_2-induced senescence (Aan et al. 2013). These results reinforced that despite displaying the so-called common biomarkers of senescence, RS and SIS are not alike.

These studies demonstrated that gene and protein expression changes of HDFs in SIS can be classified in three groups: firstly, the changes common with RS, secondly, the changes specific to each kind of stressor and thirdly, changes common to SIS induced by different stressors.

The specific changes related to one of several SIS models were presented as long-term effects of the stress and named “molecular scars”. These “molecular scars” might occur from a few days after the stress and be maintained at long term, after many types of stress (oxidative stress and/or DNA damage, inflammation) *in vitro* and *in vivo* (Dierick et al. 2003).

13.6 SIS, Telomeres and Telomerase

Telomere shortening, intimately linked to replicative senescence, is not always related to the induction of senescence after stress.

After chronic exposure to hyperoxia, telomeres are shortened five to ten times faster than normal (von Zglinicki et al. 1995). This increased shortening is due to accumulation of single-strand breaks in telomeres with hyperoxia, leading to faster telomere loss during DNA replication (von Zglinicki et al. 2000). Surprisingly, the presence of telomerase does not prevent telomere shortening due to oxidative stress, as shown in human endothelial cells with ectopically overexpressed telomerase (Kurz et al. 2004). This is linked to the export of *TERT*, the catalytic subunit of human telomerase, from the nucleus to mitochondria. *TERT*, once localized in mitochondria, protects mtDNA integrity (Ahmed et al. 2008).

In other models of SIS induced by H_2O_2, *t*-BHP or ionizing radiation, no increased telomere loss was brought out after stress whereas senescence biomarkers were well detected (Chen et al. 2001; Dumont et al. 2001; Suzuki et al. 2001).

Moreover, several HDFs strains expressing telomerase and exposed to subcytotoxic doses of H_2O_2, UV, UVB or γ-irradiation in conditions inducing senescence, displayed biomarkers of senescence (de Magalhaes et al. 2002; Gorbunova et al. 2002). This shows that telomere shortening is not detected systematically in all models of SIS and that SIS could be induced independently of telomere erosion.

13.7 Molecular Pathways

13.7.1 TGF-ß1 Pathway

TGF-ß1 (Transforming Growth Factor-ß1) is overexpressed in skin and lung fœtal HDFs after a single exposure to H_2O_2 (Frippiat et al. 2001) and after a series of exposures to UVB (Debacq-Chainiaux et al. 2005), *t*-BHP or ethanol (Pascal et al. 2005). TGF-ß1 overexpression is necessary for the overexpression of *apolipoprotein J*, *osteonectin*, *fibronectin* and *TGF-ß1* itself, namely via a positive feedback on the activation of $p38^{MAPK}$ (Frippiat et al. 2002). Incubation of lung or skin HDFs with TGF-ß1 for 3 days induces the same phenotype. If neutralizing antibodies directed against TGF-ß1 or its receptor II (TßRII) are incubated with cells after the last stress inducing senescence, this blocks the appearance of the biomarkers of senescence (Frippiat et al. 2001). This has been confirmed in lung or skin HDFs in various models of SIS (*t*-BHP, ethanol (Debacq-Chainiaux et al. 2008) and UVB (Debacq-Chainiaux et al. 2005)), reinforcing the major role played by TGF-ß1 activation in the SIS phenotype.

P38 Mitogen-activated protein kinase (MAPK) is activated in RS, in OIS induced by oncogenic Ras and in SIS induced by H_2O_2, UVB and X-irradiation (Debacq-Chainiaux et al. 2010; Wang et al. 2002; Freund et al. 2011). Activation of $p38^{MAPK}$ by phosphorylation is rapidly detected after the stress. Inhibition of $p38^{MAPK}$

activity attenuates the increase of SA-ßgal positive cells and modifies the profiles of senescence-associated gene expression in H_2O_2-induced senescence (Zdanov et al. 2006a) and reduces the secretion of SASP factors in X-irradiation-induced senescence (Freund et al. 2011).

Once activated, $p38^{MAPK}$ phosphorylates and activates ATF-2 transcription factor. ATF-2 is then responsible of *TGF-ß1* overexpression and interacts with pRb (Frippiat et al. 2002). TGF-ß1 protein is overexpressed both in latent and active forms and induces several biomarkers of senescence such as senescent morphology, SA-ßgal and senescence-associated gene expression (Debacq-Chainiaux et al. 2005; Frippiat et al. 2001), probably via downstream proteins as IGFBP-3 (Debacq-Chainiaux et al. 2008) (Fig. 13.3).

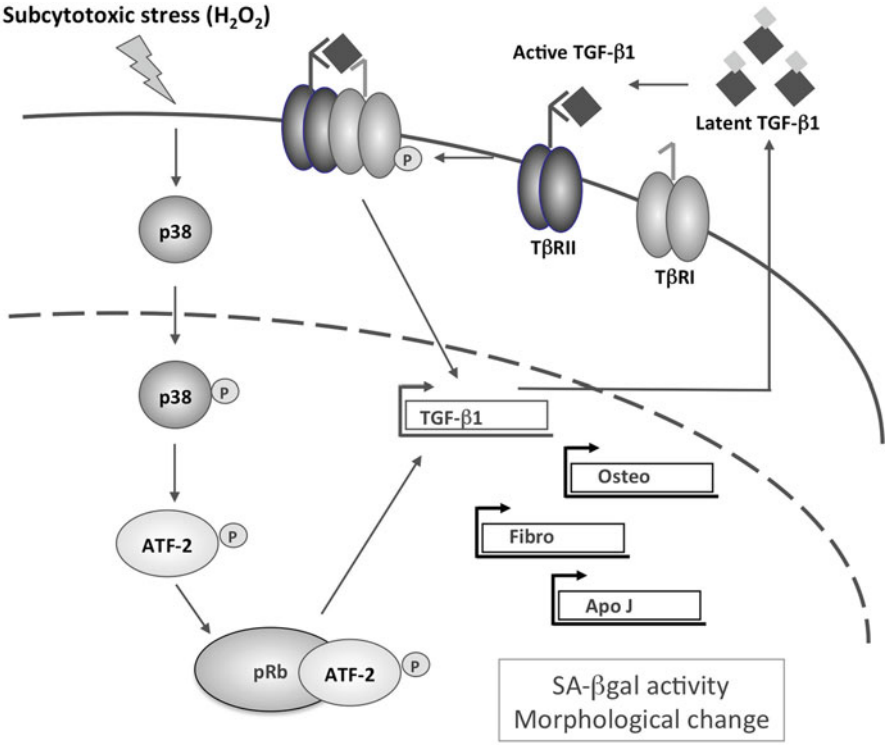

Fig. 13.3 Activation of $p38^{MAPK}$, ATF-2 and TGF-ß1 following H_2O_2-induced senescence. H_2O_2 stress immediately activates $p38^{MAPK}$ which is responsible of ATF-2 activation. Phosphorylated ATF-2 binds to hypophosphorylated pRb, which activates its transcription factor function, and is responsible for the overexpression of TGF-ß1, increasing the secretion of its latent and active protein forms. Active TGF-ß1 is able to bind to its receptors TßRI and TßRII (TGF-ß receptor I and II), leading to their interaction and phosphorylation. Activated TGF-ß1 pathway is responsible for the overexpression of senescence-associated genes as *osteonectin* (*osteo*), *fibronectin* (*fibro*) and *apolipoprotein J* (*apo J*), of morphological change and SA-ßgal activity as demonstrated by using neutralizing antibodies against TGF-ß1 or TßRII

13.7.2 IGF-1R

Several studies have highlighted the role of insulin like growth factor-1 receptor (IGF-1R) in the induction of stress-induced senescence. IGF-1R is a transmembrane tyrosine kinase receptor activated by binding with its ligands IGF-1 and IGF-2, leading to the autophosphorylation of its cytoplasmic domain. This pathway is responsible for the activation of PI3K/Akt, MAPK and mTOR (for a review: Riedemann and Macaulay 2006). Ionizing radiation of human pulmonary artery endothelial cells (HPAEC) induces senescence-associated biomarkers, IGF-1 and IGF-2 overexpression and IGF-1R activation. Treatment of HPAEC cells with an IGF-1R inhibitor (AG1024) protects from ionizing radiation-induced senescence (Panganiban and Day 2013). It was also demonstrated that the presence of a functional IGF-1R receptor was required for the activation of the UVB-induced senescence in normal human keratinocytes (Lewis et al. 2008).

13.8 Stress-Induced Senescence *In Vivo*

It is well established that senescent cells accumulate in tissues with age, as shown by studying SA-ßgal activity and DNA damage (Dimri et al. 1995). These senescent cells could be involved in the functional decline associated with ageing as postulated by Hayflick at the time of the discovery of replicative senescence. Indeed, a link between senescent cells accumulating in tissues and ageing-associated disorders such as cataract and muscular mass loss has been shown in mice by Baker et al. (2011).

How do these senescent cells appear *in vivo* and accumulate with age? Do they appear by telomere-dependent senescence or by another mechanism? By performing a simple calculation of the number of cells that could generate the two first telomerase-negative cells, appearing during the *in vivo* differentiation, based on the number of generations that HDFs can achieve before reaching replicative senescence under physiological low O_2 partial pressure (80 PDs), 2^{80} cells ($>10^{24}$ cells) must be produced before the first telomere-dependent replicatively senescent HDFs appear. Of course, to be correct, this extrapolation should also take account of the cellular turnover and of asymmetric divisions, but should also consider that more than two telomerase-negative cells will be generated by *in vivo* differentiation (Dierick et al. 2003).

In vivo, cells are exposed to a multitude of stresses whose nature depends on their location (pneumocytes facing air pollution and tobacco smoke; melanocytes, keratinocytes and skin HDFs facing UV; enterocytes facing food oxidants), particular conditions (endothelial cells facing inflammation, ischaemia/reperfusion, hypertension, shear stresses or diseases, etc.) and pathologies such as atherosclerosis, diabetes and age-related neurodegenerative diseases (for a review: Toussaint et al. 2002).

Chronic inflammation is associated to ageing and age-related diseases (Chung et al. 2009). This chronic inflammation could increase ROS accumulation and could induce appearance of senescent cells as detected by many studies in which premature appearance of cells presenting certain senescence-associated biomarkers in disease sites, including diseases subjected to chronic inflammation such as venous ulcers (Mendez et al. 1998), arteries subjected from balloon angioplasty (Fenton et al. 2001), emphysema (Muller et al. 2006), chronic hepatitis C and hepatocellular carcinoma (Paradis et al. 2001), prostatic hyperplasia (Choi et al. 2000) and intestinal metaplasia from the stomach (Going et al. 2002).

The premature appearance of these senescent cells could reinforce the inflammation by their SASP phenotype. This could lead to amplification of the phenomenon, worsen the inflammation and accelerate ageing. This hypothesis was confirmed recently *in vivo* in mice, in which chronic inflammation provoked by knockout of the *nfkb1* subunit of the transcription factor NF-κB induces telomere dysfunction and accelerates ageing (Jurk et al. 2014).

Many studies showed the implication of metals (Cu and Fe) in the development of age-related diseases and more precisely in age-related neurodegenerative diseases. These metals are able to generate ROS through the Fenton and Haber-Weiss reactions (Brewer 2007) and to induce accumulation of DNA damage, by their ability to inhibit a family of DNA glycosylases by oxidation, changing their structure and preventing their binding to downstream repair proteins (Hegde et al. 2011). Interestingly, these metals accumulate in HDFs with RS (Boilan et al. 2013; Killilea et al. 2004). The accumulation of Cu during RS of HDFs was detected by specific fluorescence-probes (CS1) or cytochemistry (rubeanic acid), but the mechanisms and the kinetics of this accumulation is still unknown. Incubation of HDFs at young CPDs with $CuSO_4$ induces senescence, this suggests that a transient increase of copper concentration is sufficient to launch the senescence process.

13.9 SIS in Cancer Treatment

Several studies have shown that in addition to induce apoptosis of cancer cells, some anti-cancer treatments such as chemotherapy and ionizing radiation were also able to induce senescence, generally using lower concentrations than those necessary to provoke apoptosis.

Chemotherapeutic agents able to induce senescence *in vitro* include among others cisplatin (Berndtsson et al. 2007), etoposide (Chiu et al. 2005), doxorubicin (Sliwinska et al. 2009), bleomycin (Aoshiba et al. 2003), vinblastine (Duan et al. 2007), etc (for a review: Bilsland and Keith 2010). An analysis of tumor tissues from patients treated with chemotherapeutic agents show a greater proportion of senescent cells (SA-ßgal positive, $p16^{ink\text{-}4a}$ overexpression) compared to healthy tissues in patients suffering from breast cancer, non-small-cell lung carcinoma or prostate cancer (Coppe et al. 2008; Roberson et al. 2005; te Poele et al. 2002). *In vivo*, first clinical data show that senescence markers ($p16^{ink\text{-}4a}$ overexpression,

VEGFA and MCP1 secretion) are more expressed in T lymphocytes from patients suffering for breast cancer treated with adjuvant chemotherapy (anthracyclin-based) than controls (Sanoff et al. 2014). This therefore accelerates senescence of hematopoietic tissues.

Furthermore ionizing radiation induces senescence in normal and tumor cells *in vitro* (Suzuki and Boothman 2008). So we should be careful about the possible induction of senescence by radiotherapy on the tumor being treated and its (micro)environment.

Induction of senescence-like phenotype in transformed cells and particularly of irreversible cell cycle arrest is seen as an interesting concept in the treatment of cancer. Indeed cancer cells manage to escape cellular senescence. A promising strategy is to successfully redirect cancer cells to cellular senescence by developing senescence-targeted drug. This pro-senescence therapy is presented as a new promising approach in cancer treatment (Nardella et al. 2011). The main pro-senescence strategies are to inhibit the activity of telomerase, to reactivate or stabilize p53, to induce Pten loss induced senescence (PICS) and to inhibit CDKs and MYC (Acosta and Gil 2012).

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Chapter 14
Implications of Cellular Senescence on Aging and Disease in the Brain

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Abstract Senescence is an irreversible mitotic arrest of the cell that can result from replicative aging or stressors. It can be beneficial by conferring resistance to apoptosis, or detrimental by inducing pro-inflammatory signaling in the microenvironment. Senescent cells have been observed in both aged and diseased tissue, including the brain. The aging brain undergoes changes such as cortical atrophy and increases in inflammatory and oxidative factors, with decreases in synaptic plasticity and mitochondrial function. Significant neuronal loss is observed and thought to drive the atrophy in the corresponding areas of the brain in neurodegenerative diseases (ND). Despite being terminally differentiated, a senescence-like phenotype is observed in neurons upon stress *in vitro* and also in neurocognitive disorders like HIV-associated dementia and Alzheimer's disease *in vivo*. Aging is also associated with lower regenerative capacity of neural stem and progenitor cells (NSPC). *In vivo,* their neurogenerative capacity is modulated by a variety of external factors, including growth factors, diet, and inflammation. NSPC have been observed to undergo stress-induced senescence *in vitro.* Deregulation of other CNS cell types, including oligodendrocytes and microglia occur in aging and ND. Microglia, which are not post-mitotic, senesce in culture in response to replicative or inflammatory stress. Astrocytes, which make up half of all cells in the CNS, maintain and protect neurons. In response to insult or injury however, astrocytes undergo phenotypic changes collectively termed reactive astrogliosis. This response can be both detrimental and beneficial to the neurons, and its downregulation improves disease parameters in a mouse model of AD. We have observed astrocyte senescence *in vitro* in response to replicative and oxidative stress and Aβ peptides, along with accumulation of senescent astrocytes in aged and AD brain. Given that astrocytes perform a myriad of complex functions in the CNS in order to maintain homeostasis, the loss of astrocyte function or the gain of neuroinflammatory function as a result of senescence could have profound implications for aging brain and neurodegenerative disorders.

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14.1 Introduction

Although the phenomenon of cellular senescence *in vitro* was originally observed more than 50 years ago (Hayflick and Moorhead 1961; Hayflick 1965), much of our understanding of the underlying mechanisms and physiologic relevance of cellular senescence to aging and disease has only been elucidated within the last decade. Normal human cells undergo a finite number of cell divisions *in vitro* before their growth is irreversibly arrested. This finite replicative lifespan, known as replicative senescence, was thought to reflect aging at the level of the individual cell (Hayflick 1965). Replicative senescence occurs as a result of telomere attrition from progressive rounds of DNA replication (Stewart et al. 2003); however, the senescence arrest can also be induced by a variety of stressors including oncogene activation, oxidative stress and proteasome inhibition, and is termed stress-induced senescence (Torres et al. 2006; Serrano et al. 1997; Chen and Ames 1994). Overall, the phenotypes elicited by replicative senescence and stress-induced senescence are collectively known as cellular senescence.

In addition to irreversible cell cycle arrest, the senescence phenotype is characterized by several biomarkers that serve to identify senescent cells. Senescent cells are metabolically active, resistant to apoptosis (Wang 1995), and undergo widespread changes in gene expression (Shelton et al. 1999). Alterations in gene expression are thought to increase the secretion of pro-inflammatory mediators and proteases that act on the microenvironment and potentially contribute to age-related declines in organ function (Campisi et al. 2011); this process has been termed the senescence-associated secretory phenotype (SASP) (Coppe et al. 2008). Perhaps one of the most relevant reports indicating the physiologic relevance of cellular senescence to aging is that the selective clearance of cells expressing the senescence biomarker $p16^{INK4a}$ in a mouse model of premature aging delayed and alleviated certain age-related pathologies in several organ systems (Baker et al. 2011). These findings implicate senescent cells in age-related tissue dysfunction and suggest that interventions to prevent the initiation of the senescence phenotype or to target the removal of senescent cells would be both relevant and beneficial.

Replicative senescence of normal human cells *in vitro* was considered a counterpart to aging *in vivo*; however, a landmark study by Cristofalo (Cristofalo et al. 1998) failed to establish a correlation between donor age and the proliferative potential of fibroblasts in culture. This finding seemingly challenged the prevailing concept at the time that all cells in an organism undergo senescence. However, the results did not preclude the possibility that there is an accumulation of a subpopulation of senescent cells in aged individuals. In fact, there is ample evidence that cells displaying biomarkers of senescence accumulate in tissues of aged animals and humans, suggesting that at least *in vivo*, cells undergo senescence prior to their

telomeres becoming critically short (Dimri et al. 1995; Jeyapalan and Sedivy 2008). Cells displaying biomarkers of senescence have been identified in tissue biopsies of aged animals and humans (Kreiling et al. 2011; Jeyapalan et al. 2006; Ressler et al. 2006). In addition, cells demonstrating biomarkers of senescence are localized to sites of common aging-related degenerative pathologies including atherosclerotic plaques (Vasile et al. 2001), osteoarthritic joints (Price et al. 2002), hypertensive kidney (Westhoff et al. 2008), pulmonary arteries from patients with chronic obstructive pulmonary disease (COPD) (Noureddine et al. 2011), and are found in cultured fibroblasts isolated from chronic non-healing venous ulcers (Stanley and Osler 2001), and from the lungs of emphysema patients (Müller et al. 2006). These studies associate the appearance of senescent cells with aging and age-related degenerative pathology, but additional work is required to test the more difficult question of causality. Interestingly, much less is known about the role of senescent cells in the brain. In this chapter we will review aging-related changes in the brain, including changes in physiology and the role of senescence-like phenotypes in CNS cell types and their potential impact on brain associated pathology.

14.2 The Aging Brain

Like many other organs throughout the body, the brain is susceptible to aging-related changes and functional decline. Often these aging-related alterations vary greatly among individuals or are specific to certain brain regions. Here, tissue-wide general features of aging brain and neurodegenerative disease are discussed, while cell-type specific changes will be described in more detail in a subsequent section.

At the most basic structural level, the normal aging brain is associated with cortical atrophy characterized by thinning of the gyri, widening of the sulci, and an expansion of the ventricles that contribute to an overall decrease in brain weight (Magnotta et al. 1999; Apostolova et al. 2012). Age-related structural changes can be evaluated in the brains of living subjects through the use of magnetic resonance imaging (MRI) based neuroimaging techniques (Walhovd et al. 2011). In longitudinal MRI studies, neuroanatomical volume loss follows a unique trajectory in individuals diagnosed with mild cognitive impairment (MCI) compared with normal aging (Driscoll et al. 2009). Brain regions that classically exhibit pathologic features of Alzheimer's disease (AD) early in the course of disease, such as the hippocampus and temporal gray matter, show accelerated volume loss in individuals with MCI, suggesting that volume loss may be a surrogate biomarker for disease progression (Driscoll et al. 2009).

Aging is the greatest risk factor for cognitive decline; however, not all aspects of cognitive function decline during normal aging (Yankner et al. 2008). Certain aspects of cognitive function such as processing speed and formation of new memories are compromised with normal aging, whereas other aspects including long-term memory and verbal knowledge remain stable (Salthouse 2010). An

age-related decrease in functional connectivity between different brain regions is thought to contribute to a decline in cognitive functions related to the hippocampus and pre-frontal cortex (Samson and Barnes 2013).

Several studies have examined the association between systemic inflammatory factors and cognitive performance during aging (Wright et al. 2006; Weaver et al. 2002). Evidence of systemic low-grade inflammation and endothelial dysfunction was associated with impaired attention and processing speed in older adults (Heringa et al. 2014). In addition, higher baseline levels of inflammatory mediators C-reactive protein (CRP) and IL-6 were associated with an increased risk of developing dementia (Yaffe et al. 2003; Dziedzic 2006). Compared with similar-aged controls, patients with AD demonstrated significantly higher levels of plasma inflammatory cytokines including IL-1β and TNF-α (Zuliani et al. 2007). In addition to systemic evidence of inflammation, higher levels of inflammatory mediators are also present in the cerebrospinal fluid (CSF) of individuals with MCI (Galimberti et al. 2006). The CSF inflammatory profile of individuals who are in the pre-clinical stage of AD is similar to that of patients with the prototypical neuroinflammatory disease multiple sclerosis, suggesting that neuroinflammation is an early event in AD pathogenesis (Monson et al. 2014). Studies of post-mortem tissues from AD patients have also overwhelmingly demonstrated evidence of neuroinflammation (Akiyama et al. 2000). Glial cells, including microglia and astrocytes, are likely sources of inflammatory mediators within the CNS during aging and neurodegeneration (Salminen et al. 2011; Jensen et al. 2013). Persistent neuroinflammation by sustained overexpression of IL-1β also exacerbates features of AD pathology in the triple transgenic mouse model of AD (3x-Tg-AD) (Ghosh et al. 2013).

In addition to neuroinflammation, oxidative stress is another prominent feature of the aging brain and plays an important role in the pathogenesis of several neurodegenerative diseases. Although low levels of reactive oxygen species (ROS) have physiologic signaling function in the CNS, an imbalance between their generation and detoxification leads to oxidative damage (Wang and Michaelis 2010). Evidence of oxidative damage to macromolecules including protein, nucleic acid, and lipids in the CNS occurs during normal aging and is an early feature of Alzheimer's disease (Bradley-Whitman et al. 2014; Bradley et al. 2010; Nunomura et al. 2001). The CNS in general and neurons in particular are thought to be vulnerable to oxidative stress because of the high metabolic rate and the propensity for mitochondrial dysfunction (Chen and Zhong 2014). The sources of oxidative stress and the mechanisms surrounding neuronal death and dysfunction during aging and neurodegeneration are active areas of investigation; however, dysfunctional mitochondria in neurons are likely key mediators (Mattson et al. 2008). The blood-brain barrier (BBB) which sits at the interface between the CNS and the periphery is another likely target of inflammatory and oxidative insult (Enciu et al. 2013). Disruption of this barrier is one potential mechanism by which oxidative stress and inflammation contribute to neurodegenerative disease. Changes in BBB permeability positively correlated with disease progression in patients with the Parkinson-like neurodegenerative disorder multiple system atrophy (MSA) (Lee et al. 2013).

Despite the evidence for the role of oxidative stress in the pathogenesis of aging related neurodegenerative disease, the clinical use of antioxidants as therapeutics has been inconclusive in large randomized clinical trials in humans (Kamat et al. 2008). Trials of vitamin E (alpha-tocopherol) failed to improve cognitive function in individuals with MCI, whereas in moderate to severe AD, long-term alpha-tocopherol treatment slowed disease progression (Evans et al. 2014; Sano et al. 1997). In a randomized, placebo-controlled clinical trial by Galasko et al. (2012) in subjects with mild to moderate AD, short-term combination treatment with antioxidants alpha-tocopherol, vitamin C, and α-lipoic acid had no impact on CSF biomarkers of AD pathology; however, they did observe a decrease in the level of F-2 isoprostane, which is a CSF biomarker of oxidative injury *in vivo* (Paolo et al. 2004). In addition, the effect of alpha-tocopherol alone or in combination with memantine, which is an FDA-approved drug for the treatment of AD, was examined in a recent trial in subjects with mild to moderate AD (Dysken et al. 2014). Subjects receiving alpha-tocopherol alone had slower rates of functional decline compared with placebo or alpha-tocopherol plus memantine (Dysken et al. 2014). There is also evidence that some ROS scavengers may be effective in mitigating cognitive decline in mice if treatment is started early enough. Chronic treatment with a superoxide dismutase/catalase mimetic (EUK-207) decreased oxidative damage to macromolecules and improved cognitive function in aged mice (Clausen et al. 2010), whereas EUK-207 treatment decreased oxidative damage, alleviated features of AD neuropathology, and improved cognitive function in 3x-Tg-AD mice (Clausen et al. 2012).

At the transcriptome level, the aging brain is associated with broad changes in gene expression. Analysis of the gene expression profile of aged mouse (30 months) neocortex revealed increased mRNA levels of genes involved in the inflammatory response and oxidative stress, while genes involved in protein-turnover and trophic support were decreased (Lee et al. 2000). A similar profile is noted in human frontal cortex; beginning in middle age there is a decrease in the expression of genes involved in synaptic plasticity, vesicular transport and mitochondrial function, while the expression of genes involved in the stress response and inflammation was increased (Lu et al. 2004). Interestingly, many of these age down-regulated genes demonstrated oxidative damage to their promoters (Lu et al. 2004). Furthermore, changes in the brain transcriptome are thought to precede the onset of neuropathology in AD (Bossers et al. 2010).

14.3 Senescence-Related Changes in CNS Cell Types

14.3.1 Neurons

With very few notable exceptions including the dentate gyrus (DG) and the subventricular zone (SVZ), new neurons are not generated throughout life in the

adult brain; therefore the neurons generated early in development must survive and remain functional for the lifetime of the organism (Yankner et al. 2008).

In most brain areas, normal aging is not associated with profound neuronal loss (Rapp and Gallagher 1996; Gazzaley et al. 1997; West et al. 1994); however, the aging brain is characterized by subtle morphological and functional alterations in neurons that contribute to cognitive decline (Burke and Barnes 2006) and may be brain region-specific (Morrison and Baxter 2012). Aged rats with spatial learning deficits related to hippocampal function exhibited a decline in levels of synaptophysin, which is a marker of pre-synaptic vesicles (Smith et al. 2000). Compared with similarly aged cognitively normal controls, subjects with Alzheimer's disease and to a lesser extent subjects with mild cognitive impairment (MCI) exhibited synaptic loss in the hippocampus suggesting that synaptic loss in this region is associated with cognitive ability (Scheff et al. 2006). In addition to changes in synapses, there is also a dramatic reduction in the number of thin dendritic spines, which are important for neuronal plasticity and learning (Kasai et al. 2003), on pyramidal neurons of the aged non-human primate brain (Dumitriu et al. 2010).

In contrast to normal aging, neurodegenerative disease is associated with profound neuronal loss in specific brain regions. Selective populations of neurons are vulnerable to degeneration in different disorders including dopaminergic neurons in the substantia nigra pars compacta in Parkinson's disease (PD), medium spiny neurons in the striatum in Huntington's disease (HD), motor neurons in amyotrophic lateral sclerosis (ALS), and hippocampal and frontal lobe pyramidal neurons in AD (Mattson and Magnus 2006). One of the greatest risk factors for the development of sporadic forms of any of these disorders is aging. General aging-associated changes are qualitatively similar, but often more severe in regions undergoing degeneration (Mattson and Magnus 2006).

Recent evidence suggests that although mature post-mitotic neurons fail to undergo replicative aging, these cells are subject to a stress-induced senescence-like phenotype. Purkinje, hippocampal, and cortical neurons from aged mice (32 months) in situ exhibit features of a p21-dependent senescence-like phenotype (Jurk et al. 2012). This phenotype is characterized by increases in SA β-gal activity, p38 MAPK activation, IL-6 production, lipid peroxidation and DNA damage, and could be mitigated by caloric restriction or exacerbated by telomere dysfunction in the aged mice (Jurk et al. 2012). Interestingly, p21 overexpression protects primary cultured neurons from apoptosis in response to treatment with the neurotoxin ethylcholine aziridinium (AF64A) (Harms et al. 2007).

A senescence-like phenotype is also evident in the neurons of aging rat (24 months) in the CA3 region of the hippocampus or upon prolonged culturing of primary hippocampal neurons by staining for SA β-gal activity, a senescence biomarker that differentiates between senescent and terminally-differentiated cells (Geng et al. 2010; Dimri et al. 1995). Finally, human and rodent neural cell lines undergo ROS-dependent senescence in response to treatment with the environmental toxin TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) (Wan et al. 2014). A likely trigger of stress-induced senescence in neurons is DNA damage. DNA damage in the form of double strand breaks in neurons is a consequence of normal

brain activity, but is increased in aged brain and can be exacerbated by the presence of amyloid-β (Suberbielle et al. 2013; Bhaskar and Rao 1994).

Even though neurons have undergone a permanent exit from the cell cycle, the cyclin-dependent kinase inhibitor $p16^{INK4a}$ is not normally detectable in adult brain (Robertson and Jones 1999), while p21 is not typically expressed in mature neurons (Pechnick et al. 2008). However, immunohistochemical analyses of post-mortem frontal cortex from subjects with HIV-associated dementia (HAD) revealed that p21 is increased in neurons and subcortical glia compared uninfected controls (Jayadev et al. 2007), whereas $p16^{INK4a}$ immunoreactivity was detectable in neurons containing neurofibrillary tangles in AD hippocampus and temporal cortex (McShea et al. 1997; Arendt et al. 1998).

14.4 Neural Stem/Progenitor Cells

In the mammalian adult brain, two major zones of neurogenesis persist: the subgranular zone of the dentate gyrus (DG) of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles (Alvarez-Buylla and Lim 2004). These regions harbor neural stem and progenitor cells (NSPC). NSPC are capable of self-renewal and differentiation into the major CNS types including astrocytes, oligodendrocytes, and neurons. The first evidence for new neurogenesis in adult human brain came from a landmark study by Eriksson et al. (1998) in post-mortem tissues from cancer patients, who were administered bromodeoxyuridine (BrdU) for diagnostic purposes while alive. They showed that new neurons arise from dividing progenitor cells in the DG. Aging is associated with a major decline in the proliferation and differentiation of NSPC. The steepest decline in neurogenesis occurs from young to middle age and is thought to underlie an aging-related decline in cognition (Hamilton et al. 2013).

One potential reason for the decline in neurogenesis with aging is the senescence of NSPC. The reduced regenerative capacity and frequency of neural stem cells in the SVZ correlates with increased $p16^{INK4a}$ expression in aged mice (Molofsky et al. 2006). Cultured NSPC undergo senescence in response to diverse stimuli. These include DNA-damage-induced senescence upon treatment with hydroxyurea (HU) (Dong et al. 2014) or ionizing radiation (Schneider et al. 2013), ROS-induced senescence in response to treatment with $A\beta_{1-42}$ oligomers (He et al. 2013), and oncogene-induced senescence with $BRAF^{V600E}$ in a model of pilocytic astrocytoma (Raabe et al. 2011). In order to maintain stem cell function, a proper balance of factors that promote stem cell self-renewal yet contribute to neoplasia relative to factors that reduce stem cell regenerative potential, decreasing the propensity for cancer but contributing to aging, is required (Molofsky et al. 2006).

NSPC self-renewal and differentiation are regulated by external cues from the specialized microenvironment or "niche" in which they reside (Alvarez-Buylla and Lim 2004). In contrast to the cell-intrinsic impact of stem cell senescence, age-related changes in the stem cell microenvironment and organismal systemic milieu

also impair stem cell function and neurogenesis (Villeda et al. 2011). These non-cell autonomous challenges to the neurogenic niche can be classified into two main categories: the loss of neurogenesis-promoting cues or gain of neurogenesis-inhibitory signals (Hamilton et al. 2013). For example, brain-derived neurotrophic factor (BDNF) is an important neurogenesis-promoting factor in the adult brain (Lee et al. 2002). Physical exercise, an intervention that improves overall health and increases hippocampal neurogenesis in aged mice also increases the level of BDNF, presumably contributing to the enhanced neurogenesis (van Praag et al. 2005). Like BDNF, insulin-like growth factor-I (IGF-I) is another growth factor that mediates the effect of exercise on neurogenesis in the adult brain (Cotman et al. 2007; Ding et al. 2006).

IGF-I increases the proliferation and differentiation of NSPC (Anderson et al. 2002), and circulating levels of IGF-I are increased in response to exercise (Trejo et al. 2001), but are decreased during aging (Gong et al. 2014). In contrast, a high-fat diet decreases hippocampal neurogenesis through an increase in lipid peroxidation and a decrease in BDNF (Park et al. 2010). Similarly, chronic inflammation inhibits neurogenesis in the adult brain although the precise mechanism is not yet clear (Ekdahl et al. 2003).

14.5 Oligodendrocytes

The major function of oligodendrocytes is the production of myelin, which ensheathes axons and is a major component of white matter in the vertebrate central nervous system (CNS). The myelin sheath facilitates conduction of signals along axons and provides metabolic support for axons in form of lactate (Fünfschilling et al. 2012). The process of myelination occurs in distinct regions of the human brain throughout life and reaches a pinnacle at middle-age. Demyelination occurs when axons lose their myelin sheath as a result of injury to the oligodendrocyte and results in a functional deficit (Franklin and Ffrench-Constant 2008). An age-related loss in the amount of myelin, but not the protein composition of myelin was observed in post-mortem analyses of human frontal white matter and corpus callosum (Berlet and Volk 1980). Increased degeneration of white matter has also been observed histologically in the frontal lobe of AD and dementia with Lewy bodies (DLB) compared with similar aged-controls (Ihara et al. 2010).

Mature oligodendrocytes are generated from the proliferation and differentiation of oligodendrocyte precursor cells (OP) in the early post-natal period and throughout adulthood (Kang et al. 2010; Young et al. 2013). While OP retain the ability to proliferate indefinitely and fail to undergo replicative senescence in culture (Tang et al. 2001), mature oligodendrocytes are post-mitotic.

Oligodendrocytes demonstrate morphological changes in aged brain which include the swelling of cell processes, and the presence of inclusions in the cell body and cell processes (Peters 2002), while a reduction in oligodendrocyte nuclear diameter is a feature of AD and DLB (Gagyi et al. 2012). These morphological

alterations correlate with a decline in myelination rate by oligodendrocytes in aged brain and neurodegenerative disease. Neuroimaging studies with magnetic resonance imaging (MRI) permit an *in vivo* assessment of white matter and confirm the loss of white matter volume and structural integrity accompanying aging and neurodegenerative disease (Bartzokis et al. 2004). In neuroimaging studies of healthy subjects, individuals carrying the ApoE4+ risk allele for the development of AD showed increased evidence of myelin breakdown (Bartzokis et al. 2006). In addition, myelination abnormalities are evident prior to the appearance of amyloid and tau pathology in the brains of a triple-transgenic AD mouse model (3x-Tg-AD) (Desai et al. 2009). In contrast, white matter integrity is preserved in the offspring of nonagenarians compared with age-matched controls, suggesting that familial longevity has a role in maintaining white matter health (Altmann-Schneider et al. 2013).

Like many repair processes during aging, remyelination efficiency following injury declines (Shields et al. 1999). The decline in remyelination in chronic multiple sclerosis lesions and following toxin-induced demyelination in rats was attributed to impaired recruitment and differentiation of OP (Sim et al. 2002; Kuhlmann et al. 2008). The aging-associated defect in remyelination can be rescued by over-expression of the anti-aging protein Klotho (Chen et al. 2013) or exposure to a more youthful systemic milieu via heterochronic parabioisis (Ruckh et al. 2012).

14.6 Microglia

In contrast to other major cell types in the CNS with neuroectodermal origins, microglia are derived from an erythromyeloid progenitor of mesodermal origin and migrate into the CNS very early in development (Alliot et al. 1999). Local proliferation of CNS resident parenchymal microglia is the sole source of microglia in adult CNS (Ajami et al. 2007). As the resident immune cells of the brain, microglial cells actively survey the microenvironment with their processes, and perform housekeeping functions through the phagocytosis of debris, clearance of apoptotic cells, and maintenance of synapses (Nimmerjahn et al. 2005; Wake et al. 2009). In response to CNS injury or to damage signaling with ATP, microglia migrate to the site of damage and proliferate. Although data from numerous *in vitro* studies support a neurotoxic role for microglia (Boje and Arora 1992; Burguillos et al. 2011) and microglia are found at the sites of infectious and sterile inflammatory CNS lesions, the role of microglia as mediators of neurotoxicity has been recently re-evaluated (Biber et al. 2014). The microglial response to injury, termed "activation", was once thought to be primarily detrimental and a major cause of neurodegeneration; however, recent findings have challenged this concept and highlighted beneficial roles of microglia (Biber et al. 2014).

Compared with young microglia, aged microglia function very differently under normal resting conditions and in response to injury. For example, microglia isolated from aged brains and cultured ex vivo constitutively secrete excess cytokines

including IL-6 and TNF-α and resist stimulation with LPS (Njie et al. 2012). Aged microglia demonstrate a blunted surveillance program and a delayed, but sustained response to injury in situ in aged mice (Damani et al. 2011; Wynne et al. 2010). The combination of constitutive inflammation and deregulated function in aged microglia has profound implications for both aged brain and neurodegenerative disease. Interestingly, transcriptome profiling studies of microglia isolated from healthy aged mice demonstrate increased expression of genes involved in neuroprotection relative to young adult mice (Hickman et al. 2013).

In human tissues, microglia exhibit "dystrophic" morphological changes in both aged brain and in association with tau pathology in AD brain (Streit et al. 2004, 2009). These dystrophic changes are characterized by a fragmented cytoplasm, gnarled cell processes, and the absence of features of microglial activation (Streit et al. 2004). The altered morphology seen in microglial cells with aging and neurodegenerative disease is thought to occur as these cells become senescent.

Microglial cells initiate a senescence program in response to replicative stress following overstimulation with mitogen (Flanary and Streit 2004) or repeated inflammatory activation with lipopolysaccharide (LPS) in culture (Yu et al. 2012). Reduced telomere length and telomerase activity were also observed in microglia purified from aged rat cortex, while telomere length was reduced in microglia isolated from post-mortem tissue from AD brain tissue (Flanary et al. 2007). In contrast, in response to acute nerve injury, microglia increase telomerase activity by upregulating the expression of telomerase protein up to 7 days post-injury (Flanary and Streit 2005). These findings suggest that the degree and timing of insult as well as the microglial proliferative response affect the ability of these cells to maintain telomere length.

14.7 Astrocytes

During mid- to late embryonic development, astrocytes are generated from NSC in the ventricular zone (VZ) (Namihira and Nakashima 2013) via a differentiation process that involves epigenetic de-repression of astrocyte-related genes (Namihira et al. 2004; Takizawa et al. 2001) and activation of several signaling pathways including JAK/STAT-3 (Bonni et al. 1997). In contrast, the majority of astrocytes in the postnatal CNS arise from the local symmetric division of differentiated, cortical astrocytes rather than from the differentiation and migration of progenitors (Ge et al. 2012).

Astrocytes are the most abundant population of cells within the human brain, accounting for about half of the total CNS cell number (Azevedo et al. 2009). The population of astrocytes within the human brain is more structurally complex and diverse compared with astrocytes from other mammalian species (Oberheim et al. 2006). This unique structural complexity of astrocytes is thought to underlie cognitive abilities that are uniquely human (Oberheim et al. 2009).

Astrocytes perform a diverse array of complex functions within the CNS to maintain homeostasis: regulation of ions, water, and neurotransmitter homeostasis (Simard and Nedergaard 2004; Schousboe et al. 2013), maintenance of the blood-brain barrier (BBB) (Abbott et al. 2006), contribution to CNS metabolism and participation in synaptic transmission as part of the tripartite synapse (Perea et al. 2009; Sofroniew and Vinters 2010). In addition to the pleiotropic array of functions they perform in normal brain, astrocytes acquire additional functions and release mediators that are known to result in neuronal toxicity (Bi et al. 2013).

Astrocytes undergo a spectrum of molecular and functional changes in order to respond to all forms of CNS insult and injury, which is collectively referred to as reactive astrogliosis (Sofroniew 2009). Recent studies have underscored the heterogeneity of reactive astrogliosis in response to different forms of insult (Zamanian et al. 2012) or distance from the site of injury (Wanner et al. 2013); however, typical features of this phenotype include increased expression of the intermediate filament proteins GFAP and vimentin, and variable cell hypertrophy in mild to moderate stages, while astrocyte proliferation, glial scar formation, and tissue reorganization are indicative of later stages (Sofroniew and Vinters 2010). Often this spectrum of changes is reversible upon removal of the insult, while glial scar formation is a terminal endpoint (Sofroniew 2009). While some forms of reactive astrogliosis are detrimental in some contexts, this response can also be beneficial (Hamby and Sofroniew 2010). For example, glial scar formation inhibits axon regeneration following injury (Iseki et al. 2012); however, the glial scar also surrounds and insulates damaged tissue to promote neuroprotection and repair (Bush et al. 1999). Several pathways including STAT3 (Herrmann et al. 2008), NF-κB (Brambilla et al. 2005), and p38MAPK (Roy Choudhury et al. 2014) have been implicated in reactive astrogliosis. The outcome of inhibition of these signalling pathways in astrocytes is often context dependent.

The interplay between changes that occur with reactive astrogliosis and changes that occur with astrocytes during aging is not entirely clear. Often, an increase in GFAP expression is used as a marker for evidence of reactive astrogliosis in tissues (Sofroniew and Vinters 2010). Astrocytes demonstrating evidence of this reactive phenotype can be found with increasing frequency at sites of virtually every CNS pathologic lesion regardless of etiology (Sofroniew 2009). Although several studies have demonstrated an increase in GFAP expression in aged brain especially in the hippocampus, there is also support for a decrease in GFAP expression during aging (Rodríguez et al. 2014). In contrast to increases in GFAP and hypertrophy, astroglial atrophy is an alternate pathologic feature of AD brain that has been observed in an AD mouse model (Olabarria et al. 2010; Kulijewicz-Nawrot et al. 2012; Yeh et al. 2011).

In contrast to neurons which are post-mitotic, glial cells are capable of undergoing cell division *in vitro* and in rodent brain tissues (Pontén and Macintyre 1968; Smart and Leblond 1961). Recent studies have also confirmed the proliferative potential of mature astrocytes in adult human brain tissue (Colodner et al. 2005). Remarkably, prolonged culture of glial cells results in a decline in their proliferative

capacity (Blomquist et al. 1980), which in normal human astrocytes could not be rescued by expression of hTERT (Evans et al. 2003). This form of *in vitro* aging by prolonged culture of astrocytes results in a decline in their functional properties including a loss of neuroprotective capacity (Pertusa et al. 2007), impaired glutamate uptake in response to oxidative stress (Gottfried et al. 2002), and impaired synaptic transmission in neurons in a co-culture system (Kawano et al. 2012). Astrocytes isolated from adult rodent cortex are also capable of undergoing replication in culture and are subject to dysfunction from oxidative stress (Souza et al. 2013).

Recently there has been a paradigm shift toward understanding the integral role of astrocyte function and dysfunction in the initiation and progression of neurodegenerative disease and cognitive decline with aging (Verkhratsky et al. 2012). The presence of astrocytes enhances tau phosphorylation and accelerates Aβ induced neurotoxicity in primary neuronal cultures (Garwood et al. 2011). This effect was mediated by secreted factors from astrocytes and could be abrogated upon treatment of the astrocytes with the anti-inflammatory agent minocycline (Garwood et al. 2011). In addition, selective targeting of calcineurin/NFAT (nuclear factor of activated T-cells) signalling in astrocytes improved disease parameters *in vivo* as evidenced by reduced astrocyte activation, lower Aβ levels, and improved cognitive function in a mouse model of AD (APP/PS1) (Furman et al. 2012). Aging-related changes in secreted factors from astrocytes impair NSPC proliferation in the neurogenic niche leading to a decline in neurogenesis with aging (Miranda et al. 2012).

We have demonstrated that in response to oxidative stress and exhaustive replication, human astrocytes activate a senescence program accompanied by the expression of p16, p21, p53, 53BP1; G1 cell cycle arrest; a reduction in telomere length; and increased co-localization of the histone chaperone HIRA and the promyelocytic leukemia PML proteins, a requirement for the formation of senescence-associated heterochromatin foci (Bitto et al. 2010). The importance of senescent astrocytes in age-related dementia has been the subject of recent discussion (Salminen et al. 2011), but to date, there is little evidence to suggest that senescent astrocytes accumulate in the brain. By examining brain tissue we have observed an increase in the number of astrocytes expressing the senescence marker p16 in aged brains, and in AD patient brains (Bhat et al. 2012). Furthermore, since Aβ peptides induce mitochondrial dysfunction, oxidative stress, and alterations in the metabolic phenotype of astrocytes (Abramov et al. 2004; Rhein et al. 2009; Allaman et al. 2010) we examined whether Aβ peptides initiate the senescence response in these cells. *In vitro*, we found that exposure of astrocytes to $A\beta_{1-42}$ triggers senescence and that senescent astrocytes produce high quantities of interleukin-6 (IL-6), which seems to be regulated by p38MAPK. IL-6 is a cytokine known to be increased in the CNS of AD patients (Glass et al. 2010). Based on this evidence, we have proposed that accumulation of senescent astrocytes may be one age-related risk factor for sporadic AD.

14.8 Discussion

Despite the realization that senescent cells play a causal role in many aging-related phenotypes (Baker et al. 2011), senescence has been largely understudied in the brain both *in vitro* and in tissues (Yeoman et al. 2012). A major challenge for the study of senescence in CNS-derived cell types in the adult has been the availability of tissue and the lack of a source for adult human CNS-derived cells including astrocytes, while studies of cellular senescence and its physiologic relevance to aging and disease in the periphery have been facilitated by tissue biopsies from living human donors. Therefore, the majority of tissues obtained for the study of CNS pathologic processes rely on tissues obtained post-mortem. In addition, a major conceptual challenge in the study of CNS senescence is the relationship between terminal differentiation and cellular senescence, which had been considered mutually exclusive but appear to have a much more complex relationship (Campisi and d'Adda di Fagagna 2007). Astrocytes have the potential to impact CNS function at multiple levels and senescence in this cell population has implications well beyond the simple loss of parenchyma. A model of the impact of astrocyte senescence is presented in Fig. 14.1.

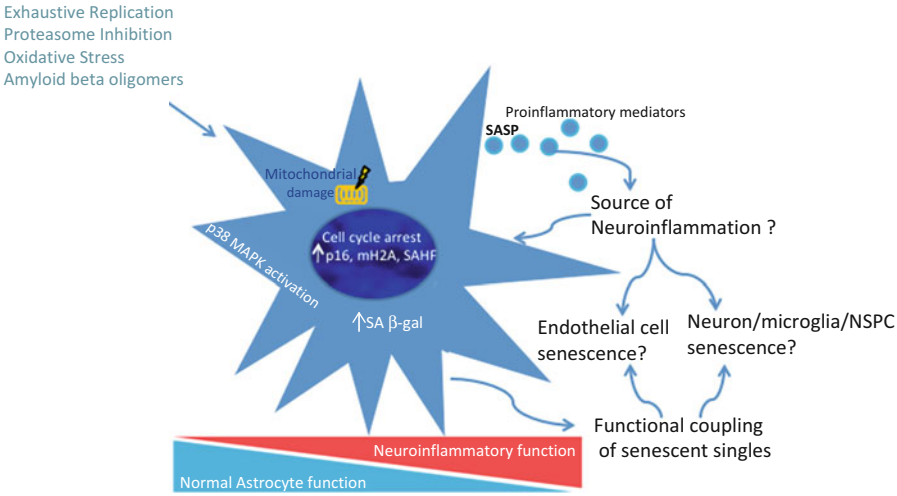

Fig. 14.1 Model. Astrocytes interact with a variety of cell types in the CNS and perform critical functions in order to maintain homeostasis. In response to classic stressors and stressors relevant to the aging brain, human astrocytes exhibit prototypical biomarkers of the senescent phenotype *in vitro*. Astrocyte senescence is also accompanied by profound changes in the transcriptome including the loss of brain-expressed transcripts, which suggests the loss of differentiated function in senescent astrocytes. The loss of differentiated function in senescent astrocytes and/or the gain of neuroinflammatory function as a result of the SASP have profound implications for the brain tissue microenvironment and the potential to impact virtually every CNS cell type (e.g. neurons, oligodendrocytes, microglia, brain microvascular endothelial cells, and neuronal stem and precursor cells (*NSPC*)) and every facet of normal CNS function

Our findings demonstrate that human astrocytes undergo cellular senescence in response to a variety of stressors including replicative stress, oxidative stress, and small oligomers of amyloid- beta ($A\beta_{1–42}$) *in vitro*. Consistent with our studies in human astrocytes, others have characterized the senescent phenotype in response to treatment with Aβ *in vitro* in endothelial cells (Donnini et al. 2010) as well as neuronal stem/precursor cells (NSPC) from model organisms (He et al. 2013). Further support for Aβ-induced senescence comes from studies performed in human retinal pigment epithelium (RPE), in which treatment with a low concentration of oligomeric Aβ (0.3 μM) causes RPE senescence and increased secretion of IL-8 and matrix metalloproteinase-9 (MMP-9) (Cao et al. 2013).

The timing of the appearance of senescent cells relative to the development of dysfunction and disease needs to be more thoroughly investigated especially in the context of the aging human CNS. Increased inflammation, oxidative damage, and transcriptional changes precede the onset of CNS dysfunction in AD (Monson et al. 2014; Bradley et al. 2010; Bradley-Whitman et al. 2014; Bossers et al. 2010); however, whether cellular senescence is a cause or a consequence of these insults remains to be determined. Overall, this suggests that in addition to being mediators and drivers of CNS dysfunction and disease during aging, senescent astrocytes could accumulate and persist in very old individuals with AD. By comparing brain regions that are selectively vulnerable to dysfunction and/or degeneration with areas that are relatively spared within the same subject, we can begin to establish whether CNS cellular senescence is a general feature of aged brain or localized to specific to sites of pathology. These studies could be expanded to tissues from other neurodegenerative disorders that increase with age including Parkinson's disease, ALS, and fronto-temporal lobar degeneration (FTLD) and HIV-associated neurocognitive disorders (HAND).

In addition to astrocytes, the possibility exists that other CNS cell types including brain microvascular endothelial cells, microglia, NSPC, and possibly neurons elicit certain aspects of the senescent phenotype (see model) (Streit et al. 2009; Dong et al. 2014; Molofsky et al. 2006; Jurk et al. 2012). While it was once thought that cellular senescence and terminal differentiation were mutually exclusive (Campisi and d'Adda di Fagagna 2007), recent evidence for a senescence-like phenotype in post-mitotic cells including megakaryocytes (Besancenot et al. 2010), and neurons (Jurk et al. 2012) has challenged this viewpoint. In response to damage, the direction of cell fate toward senescence and away from apoptosis may be a better strategy for potentially irreplaceable cells in order to maintain tissue function, although the senescent phenotype is associated with dysfunction (Naylor et al. 2013). Senescence could also be viewed as compensatory mechanism to avoid cell-cycle reentry-mediated cell death in neurons (Herrup and Yang 2007).

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Chapter 15
Small Noncoding RNAs in Senescence and Aging

Joseph M. Dhahbi

Abstract Small noncoding RNAs (sncRNAs) of length shorter than 400 nucleotides mediate fundamental cellular functions in animals and plants. Some sncRNAs are complexed with proteins that direct them to nucleic acids with sequence complementarity, where they may cleave or otherwise alter the target thereby controlling gene expression, chromatin remodeling, and genome stability. Though microRNAs are the most characterized species, next generation sequencing technology has allowed the discovery of new types of sncRNAs that are generated by processing of known sncRNAs into smaller but functional RNA molecules. The recent discovery of stable sncRNAs in the extracellular space suggests that they may serve as signaling molecules that can enter target cells and regulate cellular functions. Organismal aging has been linked to cellular senescence and both involve extensive changes in gene expression. Calorie restriction (CR), a reduced caloric intake without malnutrition, protects against senescence and ameliorates the age-related dysfunctions. Since sncRNAs regulate gene expression and control genome stability, it is conceivable that they may contribute to the biological and functional changes that accompany the development of cellular senescence and the progression of organismal aging. Furthermore sncRNAs may very well mediate the beneficial effects of CR on senescence and aging. In this chapter, I will give an overview on the involvement of intracellular and extracellular microRNAs and sncRNAs derived from tRNA and YRNA in the regulation of cellular senescence and organismal aging and their potential role in mediating the beneficial actions of CR.

Keywords Replicative senescence • Cell cycle • Transcription • Translation • Health • Longevity

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15.1 Introduction

Small and long noncoding RNAs are functional and structural RNAs that are not translated into protein; they form the majority of transcripts in a cell. Small noncoding RNAs (sncRNAs) are a diverse group of RNAs that are shorter than 400 nucleotides (nt) in length. Some sncRNAs [*i.e.*, microRNAs (miRNAs), small interfering RNAs (siRNAs), and Piwi-interacting RNAs (piRNAs)] are bound by Argonaute proteins, and have the common property of directing proteins complexes to nucleic acids with sequence complementarity, where they may cleave or otherwise alter the target (Joshua-Tor and Hannon 2011). Through interaction with target genes, sncRNAs control a wide variety of key cellular functions and biological processes including gene expression regulation, chromatin remodeling, genome stability, and development (Amaral and Mattick 2008; Carthew and Sontheimer 2009; Zhang 2009; Krude 2010; Wery et al. 2011; Okamura 2012; Li 2013). Disruption of sncRNAs expression and functions, especially miRNAs, has been associated with cellular senescence, aging, cancer, and non-neoplastic disorders including cardiovascular, neurological, autoimmune, and developmental diseases (reviewed in Grillari and Grillari-Voglauer 2010; Taft et al. 2010; Esteller 2011).

Recent improvements in the throughput of deep sequencing (also called next generation sequencing) have allowed the discovery of new types of small RNAs that are different from the well-established classes of sncRNAs. Particularly, well-known functional sncRNAs such as transfer RNA (tRNA), ribosomal RNA (rRNA), small nucleolar RNA (snoRNA) and YRNA can undergo processing into smaller RNA molecules (Rutjes et al. 1999; Rother and Meister 2011; Sobala and Hutvagner 2011). Although initially regarded as degradation products, accumulating evidence suggests that these RNA fragments, while derived from pre-existing sncRNAs, are themselves functional in normal biology and in pathologic conditions (Rutjes et al. 1999; Zhao et al. 1999; Fu et al. 2009; Lee et al. 2009; Haussecker et al. 2010; Wang et al. 2012; Martens-Uzunova et al. 2013). These new sncRNAs have only recently emerged from small RNA data because the experiments that produced the sequence datasets were designed to focus on miRNAs only. Sequences whose length exceeded the size of mature miRNAs (18–24 nt) were systematically discarded. In addition, sequencing reads that align with tRNAs, rRNAs, and snoRNAs were considered degradation products and excluded from further analysis.

SncRNAs and their derivatives can be released into the extracellular environment and thereby may be involved in paracrine and even endocrine signaling (Hoy and Buck 2012; Dhahbi et al. 2013a, b, c). There is evidence that extracellular miRNAs can enter cells and alter gene expression of the recipient cell (Zernecke et al. 2009; Zhang et al. 2010; Vickers et al. 2011; Hergenreider et al. 2012), which suggests a function in cell-to-cell communication and signaling both in normal biology and in disease states (Cortez et al. 2011; Shah and Calin 2012; Turchinovich et al. 2012; Kosaka et al. 2013). Our deep sequencing studies have consistently detected three major classes of extracellular small RNAs: miRNAs of size 20–24 nt, tRNA-derived RNAs of size 30–33 nt, and YRNA-derived RNAs of sizes 27 nt and 30–33 nt. Other

types of sncRNAs including rRNAs, snoRNAs, and small nuclear RNAs (snRNAs) are present in the bloodstream at very low levels, in aggregate less than 1 % of all small RNAs (Dhahbi et al. 2013a, b, c, 2014; Dhahbi 2014). This chapter will focus on these three major types of sncRNAs, but it should be kept in mind that the low levels of the other types do not necessarily imply that they lack biological significance.

The link between cellular senescence and organismal aging is becoming increasingly accepted; cellular senescence may contribute to a variety of age-associated diseases (Jeyapalan and Sedivy 2008; van Deursen 2014). Cellular senescence and organismal aging are characterized by extensive changes in gene expression linked to age-related pathologies specific to tissue types (Cao et al. 2001; Weindruch et al. 2001; Dhahbi et al. 2004; Spindler and Dhahbi 2007; Fridman and Tainsky 2008; Pawlikowski et al. 2013). Calorie restriction (CR), a decreased caloric intake without malnutrition, protects against senescence, delays the age-induced alterations in gene expression and ameliorates the age-associated dysfunctions (McCay et al. 1989; Lo et al. 2011; Ning et al. 2013). Given the pervasive role of sncRNAs in the regulation of gene expression and the pathogenesis of disease, it is plausible that sncRNAs mediate, at least in part, the changes in gene expression that accompany the development of cellular senescence and the progression of aging and the development of their deleterious effects on cellular function. Furthermore sncRNAs may very well mediate the beneficial effects of CR against the biological and functional alterations that are associated with senescence and aging. Consistent with this view, cellular senescence, and organismal aging and its related diseases, are associated with alterations in miRNA expression that could have multifarious physiological effects (Lafferty-Whyte et al. 2009; Grillari and Grillari-Voglauer 2010; Hackl et al. 2010; ElSharawy et al. 2012; Gombar et al. 2012; Liu et al. 2012a, b; Serna et al. 2012; Boon et al. 2013; Dimmeler and Nicotera 2013; Mercken et al. 2013). I will focus this chapter on intracellular and extracellular miRNAs and the sncRNAs derived from tRNA and YRNA, the most abundant classes identified by deep sequencing in the bloodstream, and highlight their relation to cellular senescence, organismal aging and CR.

15.2 miRNAs

miRNAs are ~22-nt regulatory small non-coding RNAs that inhibit gene expression, thereby controlling important cellular processes including development, cell growth, differentiation, cell proliferation, and apoptosis (Huang et al. 2011; Shukla et al. 2011; Stroynowska-Czerwinska et al. 2014; Tufekci et al. 2014). They direct Argonaute protein complexes to messenger RNAs (mRNAs) to initiate translational repression or mRNA degradation, leading to decreased protein synthesis (Fabian et al. 2010; Thomas et al. 2010). More than 2580 humans miRNAs have been catalogued in version 21 of miRBase (Kozomara and Griffiths-Jones 2011, 2014). Through targeting a vast number of distinct mRNAs, they regulate

critical physiological and pathological processes including cell signaling, immune responses, tumorigenesis, and the pathogenesis of non-neoplastic diseases (da Costa Martins et al. 2010; Ivey and Srivastava 2010; Saugstad 2010; Townley-Tilson et al. 2010; Farazi et al. 2011; Ichimura et al. 2011; Koturbash et al. 2011; Avraham and Yarden 2012; Heinrich and Dimmeler 2012; Iorio and Croce 2012). As potent regulators of gene expression and modulators of fundamental physiological and pathological processes, miRNAs are more than likely to play a role in the biology of cellular senescence, organismal aging and the deleterious changes that occur in cells as we get older.

15.2.1 miRNAs and the Advent of Deep Sequencing

Deep sequencing has become the technology of choice for analysis of miRNA expression and discovery of novel miRNAs (Wittmann and Jack 2010; McCormick et al. 2011). The steady improvement of bioinformatics analysis tools has facilitated accurate annotation of miRNA sequence datasets, consistent expression measurement with rigorous statistics, prediction of the targeted mRNAs, and functional annotation of the pathways regulated by specific miRNAs. The new miRNAs currently being discovered by deep sequencing have been missed by traditional techniques because they tend to be located within poorly annotated regions of the genome, and are expressed at low levels; such limitations are easily overcome by deep sequencing technology. The more abundant miRNAs have largely already been discovered; now, because of its higher sensitivity, deep sequencing is discovering the less abundant ones.

15.2.2 Intracellular miRNAs in Senescence, Aging, and Calorie Restriction

miRNAs are mostly known for their significant involvement in cancer, which is frequently associated with aging, and may result from failure of oncogene-induced cellular senescence in limiting tumorigenesis (Kuilman et al. 2008; Chandeck and Mooi 2010). miRNAs have been implicated in all stages of tumorigenesis, including proliferation, apoptosis, initiation, progression, invasion, chemo/radiotherapy resistance, metastasis, and relapse (reviewed in Cheng et al. 2013; Di Leva and Croce 2013; Farazi et al. 2013; Iuliano et al. 2013; Pencheva and Tavazoie 2013; Shen et al. 2013; Uchino et al. 2013). This intimate involvement in tumorigenesis reflects the pervasive roles of miRNAs in cellular pathophysiology, which also makes them attractive candidates for diagnostic markers and therapeutic targets in cancer (Uchino et al. 2013; Yang et al. 2013). It has been proposed that cellular senescence may play a role in balancing cancer and aging (Collado et al. 2007).

Senescence protects against tumorigenesis since aging disorders commence when the rate of generation of senescent cells surpasses the renewal capacity of the stem cells; miRNAs may participate in this balance since they play significant roles in both aging and cellular senescence (Liu et al. 2012a, b; Dimmeler and Nicotera 2013).

There is ample evidence pointing to miRNAs as senescence regulators (Liu et al. 2012a, b). miR-34a, miR-22, and miR-24 modulate senescence signaling pathways including the p53-p21-pRb and the p16-pRb tumor suppression pathways (Lal et al. 2008; Park et al. 2009). MiRNAs target important senescence-associated genes including the regulation of hTERT by miR-138, the direct targeting of Bmi-1 oncogene by miR-128a which increases p16 expression and promote cellular senescence, and the prevention of Ras-induced senescence through the down-regulation of tumor suppressor LATS2 by miR-372 and miR-373 (Voorhoeve et al. 2006; Mitomo et al. 2008). Other miRNAs contribute to various aspects of senescence phenotypes including the senescence-associated secretory phenotype and adhesion functions of senescent cells (Liu et al. 2012a, b). Expression of miRNAs is altered during cellular senescence, in the course of normal and healthy organismal aging, and in age-related diseases (He et al. 2007; Lafferty-Whyte et al. 2009; Maes et al. 2009; Grillari and Grillari-Voglauer 2010; Hackl et al. 2010; ElSharawy et al. 2012; Gombar et al. 2012; Liu et al. 2012a, b; Serna et al. 2012; Boon et al. 2013; Dimmeler and Nicotera 2013; Mercken et al. 2013; Gupta et al. 2014). Moreover, miRNAs that display age-dependent expression patterns tend to change expression in age-related diseases, and their potential targets are usually enriched in disease-associated pathways (Hackl et al. 2010; Noren Hooten et al. 2010; ElSharawy et al. 2012).

Application of deep sequencing to the study of miRNAs in cellular senescence and organismal aging has only recently begun. Using deep sequencing, we have found that senescence in fibroblasts is associated with changes in the expression of a group of miRNAs (miR-1246, miR-584 and miR-323) (Dhahbi et al. 2011) known to play a role in cancer (Dixon-McIver et al. 2008; Wang et al. 2010b; Witten et al. 2010). Other miRNAs whose levels change during senescence include miR-432, which has previously been reported only as an abundant miRNA in the earliest stage of fetal development (McDaneld et al. 2009) and may regulate endothelial cell-specific genes (Bhasin et al. 2010), and miR-145, whose involvement in breast and colon cancers suggests that it can function as a tumor suppressor (Gotte et al. 2010; Zhang et al. 2011b). We found that let-7 expression is increased during senescence, as previously reported in microarray miRNA profiling studies (Marasa et al. 2010). Let-7 is a tumor suppressor (Zhang et al. 2007; Ortholan et al. 2009), which supports the view that senescence evolved as a tumor-suppressive mechanism to diminish the risk that cancer poses to longevity (Campisi and d'Adda di Fagagna 2007; Ortholan et al. 2009). In addition to the abnormal expression of Let-7 miRNA in numerous cancer types (Jiang et al. 2009; Boyerinas et al. 2010), its overexpression was found to be associated with senescence in fibroids (Laser et al. 2010) and in skeletal muscle of aged humans, and was proposed to contribute to decreased muscle cell renewal and regeneration (Drummond et al. 2010). Senescence highly

induced members of the miR-449 family (Dhahbi et al. 2011). The induction of miR-449 may play a role in cell cycle arrest during senescence since miR-449 has been shown to inhibit cell cycle progression at G1 phase by targeting CDK6 and CDC25A, which are pivotal to G1/S-phase transition (Yang et al. 2009). In addition, senescence induced miR-499 expression in human mesenchymal stem cells, with the potential to modulate senescence induction by telomere attrition, oxidative stress, oncogene expression and DNA damage signaling (Lafferty-Whyte et al. 2009). Expression of all 6 members of the miR-17-92 cluster (miR-17, −20a, 18a, −19a, −19b-1 and -92a-1) was suppressed by senescence (Dhahbi et al. 2011). MiR-17-92 promote tumorigenesis by inhibiting oncogene-induced senescence and are overexpressed in human cancers (Mendell 2008; Hong et al. 2010). Given the anti-senescence activity of miR-17-92, our finding that all miR-17-92 members are sharply repressed with senescence suggests that their very low levels may initiate and/or sustain the senescence program. Furthermore, miR-19b was found to be downregulated in several human replicative and organismal aging models (Hackl et al. 2010), and has been identified as an oncogene that activates the AKT/mTOR pathway, which modulates organismal life spans (Grillari et al. 2010). These newly identified senescence-regulated miRNAs further emphasize the involvement of miRNAs in cellular senescence. Pathway analysis of the biological processes targeted by miRNA expression changes during senescence revealed that regulation of cell proliferation is the most enriched pathway, which is consistent with a fundamental role of miRNAs in cell proliferation regulation since inhibition of proliferation is the hallmark of senescence (Dhahbi et al. 2011). Other pathways that are potentially regulated by these miRNAs include regulation of cell adhesion and cytoskeleton remodeling, suggesting that miRNAs may influence the morphological changes characteristic of the senescence phenotype (Dhahbi et al. 2011).

Evidence implicating a miRNA in aging was first observed in *Caenorhabditis elegans*: the miRNA lin-4 influences lifespan, and regulates insulin, insulin-like growth factor-1 signaling, and cell cycle checkpoints for DNA damage pathways, all of which are known to participate in the aging process (Boehm and Slack 2005). Subsequent studies have established that miRNA levels are altered during organismal aging (Liang et al. 2009; Grillari and Grillari-Voglauer 2010; Hackl et al. 2010; Noren Hooten et al. 2010; ElSharawy et al. 2012; Liu et al. 2012a, b; Smith-Vikos and Slack 2012; Xu and Tahara 2012). The mRNAs targets of miRNAs whose expression changes with age are involved in tissue-specific age-related functions, including oxidative stress defense and mitochondrial maintenance in the liver, apoptosis in the brain, and cell cycle regulation and proliferation in skeletal muscle (reviewed in Smith-Vikos and Slack 2012). Despite the increasing evidence that miRNAs play a role in aging, there is at present little evidence that CR exerts any positive effects on the age-induced alterations in miRNAs or the pathophysiological consequences of those alterations. However, CR may exert its anti-aging effects, e.g., decreasing tumorigenesis, through modulation of miRNA expression (Olivo-Marston et al. 2014). One study of aged mouse brain showed that CR prevented increases of miR-181a-1, miR-30e and miR-34a, along with the

reciprocal up-regulation of their target Bcl-2 gene (Khanna et al. 2011). The study proposed that CR might decrease apoptosis and induce a gain in neuronal survival by modulating miRNA levels.

15.2.3 Extracellular miRNAs

Recently, miRNAs were discovered in the extracellular space as nuclease-resistant molecules, which raises the question of whether they carry biological functions similar to hormones (Valadi et al. 2007; Shah and Calin 2012). They were found in body fluids including serum, plasma, urine, saliva and milk (Weber et al. 2010; Lasser et al. 2011). There is accumulating evidence that these extracellular miRNAs participate in a novel intercellular genetic exchange that may modulate normal and pathologic processes (Lehmann et al. 2012; Salic and De Windt 2012; Dhahbi et al. 2013a, b, c; Dhahbi 2014). miRNAs may be present in various bound forms in extracellular space; while some are packaged in lipid vesicles, others are complexed with RNA-binding proteins. In the bloodstream, miRNAs circulate either as cargo of plasma exosomes or complexed with protein or lipoprotein factors (Arroyo et al. 2011; Vickers et al. 2011; Turchinovich and Burwinkel 2012; Vickers and Remaley 2012). Exosomes protect miRNAs against RNase activity and carry them to recipient tissues (Lasser et al. 2011; Pant et al. 2012; Zampetaki et al. 2012). However, miRNAs can be stable outside exosomes, indicating the existence of two transport systems of miRNAs, exosomal and extra-exosomal (Arroyo et al. 2011; Turchinovich and Burwinkel 2012). High-density lipoprotein (HDL) has been reported to carry and deliver miRNAs to recipient cells (Arroyo et al. 2011; Vickers et al. 2011; Turchinovich and Burwinkel 2012). Stable Argonaute2-miRNA complexes, independent of exosomes or microvesicles, are present in plasma and serum (Arroyo et al. 2011). In addition to HDL and Argonaute proteins, circulating miRNAs can be complexed to nucleophosmin-1 or ribosomal proteins L10a and L5 (Zernecke et al. 2009; Wang et al. 2010a; Arroyo et al. 2011; Turchinovich et al. 2011; Vickers et al. 2011; Turchinovich and Burwinkel 2012). These packaging components may confer target specificity on the exported miRNAs, through interaction with receptors on the recipient cells (Turchinovich et al. 2012).

There is currently little information about the tissues/cells that produce extracellular miRNAs, the mechanisms by which they are delivered, and their functions once inside recipient cells. Because of their contact with plasma, blood cells were at first thought to be the predominant source of extracellular miRNAs (Chen et al. 2008). However, the discovery in plasma of miRNAs specific to the liver, muscle, heart, and brain indicates a multi-tissue origin of extracellular miRNAs (Turchinovich et al. 2012). Furthermore, tumors can release miRNAs into the bloodstream (Healy et al. 2012; Ma et al. 2012; Zen and Zhang 2012). Vickers and colleagues were able to deliver exogenous HDL-miRNA complexes into hepatocytes, with subsequent alterations in the expression of genes involved in lipid metabolism, inflammation, and atherosclerosis (Vickers et al. 2011). In another study, extracellular miR-126

secreted by endothelial cells triggered the production of the chemokine CXCL12 in recipient vascular cells (Zernecke et al. 2009), and miR-143/145 altered gene expression in co-cultured smooth muscle cells to reduce atherosclerotic lesion formation in the aortas of ApoE(−/−) mice (Hergenreider et al. 2012). Similarly, miR-150 secreted by human blood cells and cultured monocytic THP-1 cells reduced c-Myb expression and enhanced cell migration after delivery into HMEC-1 cells (Zhang et al. 2010). Thus extracellular miRNAs can enter target cells and alter their gene expression, with functional consequences.

15.2.4 Extracellular miRNAs in Senescence, Aging and Calorie Restriction

Cultured cells release miRNAs in the surrounding medium either in exosomes or as membrane-free RNA/protein complexes (Skog et al. 2008; Nolte-'t Hoen et al. 2012). The composition of the released miRNAs is different from the parental cell indicating selectivity in the miRNA types secreted into the extracellular space. Extracellular miRNAs were successfully delivered to the cytoplasm of recipient cells (Montecalvo et al. 2012; Nolte-'t Hoen et al. 2012) where they remain active and modulate host cellular functions (van der Grein and Nolte-'t Hoen 2014). For example, various cancer cell lines release miRNAs that were shown to target immune cells and attenuate inflammatory response in macrophages (Li et al. 2012), induce pro-metastatic inflammatory responses by triggering TLRs (Fabbri et al. 2012; Mobergslien and Sioud 2014), and regulate T-cell functions (Ye et al. 2014). Also, tumor-associated macrophages secrete exosomes loaded with oncogenic miRNAs that promote metastasis upon delivery to breast cancer cells (Yang et al. 2011).

Extracellular miRNAs have not yet been implicated in cellular senescence. However, the well-established roles of exosomes in cell-to-cell communication in physiological and pathological conditions suggests that the miRNA cargo of exosomes shed by senescent cells (Lehmann et al. 2008) could be transferred to target cells and contribute to senescence signaling pathways in an autocrine, paracrine and even endocrine manner. The role of secretory factors in cellular senescence has been well studied; the senescence-associated secretory phenotype (SASP) is characterized by the secretion of various cytokines, chemokines, and growth factors associated with inflammation and malignancy as well as cell adhesion molecules and chemokines that attract phagocytic immune cells to remove senescent and potentially carcinogenic cells (Coppe et al. 2008). The adhesion molecule ICAM-1, an invariable SASP component that recruits leukocytes from the blood into tissues, is secreted by senescent cells via microvesicles, most likely exosomes (Effenberger et al. 2014). Considering the consistent presence of miRNAs in exosomes and their importance in regulating gene expression during senescence (Xu and Tahara 2012),

it is plausible that extracellular miRNAs may take part in implementing the complex effects of SASP on the senescent cell and its environment.

MiRNAs circulate in a stable form in plasma and serum shielded from ribonucleases by inclusion into exosomes or binding to protein or lipid factors, and may take part in cell-to-cell communication in normal biology and in pathophysiology (Cortez et al. 2011; Shah and Calin 2012; Turchinovich et al. 2012; Kosaka et al. 2013). Changes in the circulating levels of miRNAs are closely associated with various types of cancer (Reid et al. 2011; Healy et al. 2012; Ma et al. 2012; Zen and Zhang 2012). Non-neoplastic disorders can also exhibit alterations of circulating miRNA levels, which change during inflammatory, cardiovascular, and neurological disorders, including sepsis, rheumatoid arthritis, myocardial infarction, and Alzheimer's disease (Reid et al. 2011; Filkova et al. 2012; Lehmann et al. 2012; Salic and De Windt 2012; Xu et al. 2012; Zampetaki et al. 2012). Given the well-established regulatory role of miRNAs in carcinogenesis (Lages et al. 2012) and common diseases (Li and Kowdley 2012; Mo 2012), together with their stability and accessibility in the bloodstream, miRNAs hold great promise as systemic markers for diagnosis and prognosis of cancer and other diseases (Reid et al. 2011; Redova et al. 2013).

The first observations of altered circulating miRNA levels during aging was an increase in miR-34a in the plasma of old mice (Li et al. 2011). MiR-34a was also increased in PBMCs and brains of the old mice, with a reciprocal decrease of its target SIRT1 mRNA, suggesting that circulating miR-34a can be used as biomarker of brain aging. In flies, loss of miR-34 accelerates brain ageing and decreases survival, while miR-34 upregulation extends median lifespan and mitigates neurodegeneration (Liu et al. 2012a, b). Assessment of the plasma levels of 365 miRNAs in healthy young and old humans including centenarians, and in older patients with cardiovascular disease, revealed that transforming growth factor-beta signaling is the main pathway potentially regulated by the differentially abundant circulating miRNAs; the study proposed circulating miR-21 as an inflammatory biomarker linking the aging process to cardiovascular diseases (Olivieri et al. 2012). MiR-151a-3p, miR-181a-5p and miR-1248 were significantly decreased with age in human serum (Noren Hooten et al. 2013). These miRNAs are involved in development and organismal survival, and are predicted to mediate inflammation, suggesting that they may play a role in the age-associated inflammatory processes.

We found that levels of many miRNAs circulating in mouse serum are increased with age, and that CR antagonizes the age-induced increases (Dhahbi et al. 2013c). Functional annotation with Gene Ontology revealed the biological processes targeted by this group of miRNA. The most highly represented and enriched processes included 'positive regulation of macromolecule biosynthetic process', which represents the anabolic pathways that use ATP to synthesize polysaccharides, lipids, nucleic acids, and proteins. Both aging and CR are potent modulators of metabolism. Aging decreases macromolecular turnover which may underlie the age-related accumulation of oxidative damage, while CR is thought to extend lifespan by reducing metabolic rate and lowering the production of toxic by-products of

metabolism (Spindler 2010). We and others have shown that CR modulates the expression of genes involved in energy metabolism, and alleviates age-induced alterations of the activity of key metabolic enzymes (Lee et al. 1999; Cao et al. 2001; Weindruch et al. 2001; Dhahbi et al. 2004). Gene expression profiling studies suggested that CR might retard aging by causing a metabolic shift toward increased protein turnover and decreased macromolecular damage (Lee et al. 1999; Cao et al. 2001; Dhahbi et al. 2004). Thus, the changes in systemic miRNAs may contribute to establishing and maintaining this beneficial metabolic shift caused by CR.

The biological processes 'Negative regulation of apoptosis' and 'Wnt signaling' were also enriched by Gene Ontology functional analysis of age- and CR-regulated circulating miRNAs. Aging increases apoptosis in postmitotic tissues, including brain, skeletal and cardiac muscle, and germ cells, impeding the homeostasis of somatic organs and stem cell self-renewal (Wang et al. 1999; Higami and Shimokawa 2000; Pollack et al. 2002). In contrast, the suppression of apoptosis during tumorigenesis may underlie the age-associated increased prevalence of cancers (Hursting et al. 2003). CR increases apoptosis and decreases cellular proliferation in mitotic tissues, where it selectively eliminates preneoplastic and neoplastic cells, which are more sensitive to apoptosis than normal cells (reviewed in Spindler 2010). These complex effects of aging and CR on apoptosis may be mediated by the age- and CR-associated changes in the circulating levels of miRNAs if these extracellular miRNAs are indeed delivered to and directly regulate gene expression in recipient cells. Among the processes controlled by Wnt signaling are cell proliferation and differentiation, apoptosis, and stem cell renewal (Arthur and Cooley 2012). It also plays a complex role in aging involving the association of ß-catenin with the transcription factor FOXO to induce senescence, or with TCF/LEF to stimulate stem cell renewal. Since FOXO transcription factors regulate the rate of aging and may mediate the antineoplastic effects of CR (Lin et al. 1997; Yamaza et al. 2010), it is tempting to speculate that the CR-associated changes in the circulating levels of miRNAs may contribute to fine tuning of Wnt signaling in peripheral tissues, to preclude the age-induced senescence of stem and proliferating cells and delay the onset of age-related disorders.

Mining of miR2Disease and HMDD databases (Lu et al. 2008; Jiang et al. 2009) for associations between specific diseases and the changes in circulating miRNAs during aging and CR revealed cancer, and neurodegenerative, cardiovascular, and inflammatory disorders as the most significantly associated diseases (Dhahbi et al. 2013a, b, c). All of these pathologies are linked to old age. This association implies that circulating miRNAs whose serum levels are increased with age and decreased by CR may participate in the pathogenesis of age-induced diseases, and that their modulation by CR may underlie the anti-aging effects of CR.

Whether the changes in circulating miRNAs are of etiological origin, or merely consequences of deleterious age-induced dysfunctions, remains to be established. Moreover, the changes can be causal of pathophysiological alterations of aging and senescence only if the circulating miRNAs enter peripheral tissues, retain their functional mRNA targeting capabilities, and modulate gene expression. Zhang and

coworkers showed that miR-150 was transferred from THP-1 to HMEC-1 cells, where its target c-Myb was silenced thereby enhancing migration of the recipient HMEC-1 cells (Zhang et al. 2010). Also, Vickers and colleagues (Vickers et al. 2011) demonstrated that HDL-miRNA complexes are delivered into hepatocytes where they alter the expression of genes involved in lipid metabolism, inflammation, and atherosclerosis. This evidence that circulating miRNAs are taken up by targeted cells, and repress the translation of target genes, strongly supports the potential for functional significance of age- and CR-associated changes in the circulating levels of miRNAs.

15.3 tRNA- and YRNA-Derived Small RNAs

tRNAs, the adapter molecules that translate the genetic information in the mRNA to the amino acid sequence of protein, are 73–93 nt sncRNAs transcribed by RNA polymerase III. In addition to their function in translation, tRNAs can regulate gene expression (Li and Zhou 2009). In bacteria, yeast, and human cells, tRNAs have been shown to act as sensors of nutritional stress; they play a role in the amino acid starvation response during which gene expression is adjusted to promote cell survival (Shaheen et al. 2007; Green et al. 2010; Murguia and Serrano 2012).

YRNAs are 84–112 nt sncRNAs transcribed by RNA polymerase III from four genes in man (hY1, hY3, hY4 and hY5), and two genes in mice (mY1 and mY3) (Wolin and Steitz 1983). YRNAs fold into stem-loop structures and complex with Ro60 protein and other proteins to form ribonucleoproteins (RoRNPs). The Ro60 component of cellular RoRNPs is recognized by autoantibodies found in the serum of patients with inflammatory connective tissue diseases such as Sjogren's syndrome and systemic lupus erythematosus (Lerner et al. 1981; Bouffard et al. 1996). The YRNA moiety in RoRNPs seems to contribute to the tissue injury mediated by the anti-Ro60 autoantibodies. YRNAs were the first example of a sncRNA shown to regulate DNA replication (Krude 2010). The function of YRNAs in DNA replication does not require Ro protein (Langley et al. 2010); other factors may complex with YRNAs in DNA replication, since naked YRNAs are unstable.

Interestingly, a recent study revealed that some bacterial YRNAs are substrates for enzymes that recognize tRNAs and contain a domain resembling tRNA with nucleotide modifications characteristic of tRNAs (Chen et al. 2014). However, it remains unclear if these YRNAs carry tRNA-related functions, e.g., interaction with ribosomes, translation factors, or other tRNA-interacting proteins to participate in cellular processes. Interestingly, both tRNAs and YRNAs can be processed into stable smaller noncoding RNA molecules that were shown to be functional in normal biology and in disease (Haussecker et al. 2010; Sobala and Hutvagner 2011).

15.3.1 Intracellular tRNA- and YRNA-Derived Small RNAs

tRNA-derived small RNAs are classified into two types based on their size (Sobala and Hutvagner 2011; Martens-Uzunova et al. 2013): tRNA halves are 30–40 nt long produced by cleavage of full length mature tRNAs at the anticodon loop, and shorter tRNA-derived fragments (tRFs) are 18–22 nt in length produced from both mature and pre-tRNAs by Dicer or RNase Z (Thompson et al. 2008; Cole et al. 2009; Fu et al. 2009; Lee et al. 2009; Thompson and Parker 2009a, b; Pederson 2010; Sobala and Hutvagner 2011). The tRNA halves class includes 5′- and 3′-tRNA halves of 30–40 nt in length. These tRNA halves were first observed in stressed cultured cells where they are produced by cleavage near or at the anti-codon loop with the ribonuclease Rny1 in *S. cerevisiae* (Thompson and Parker 2009a) and Angiogenin in higher eukaryotes (Fu et al. 2009; Yamasaki et al. 2009). They were later observed in unstressed human cells (Kawaji et al. 2008; Fu et al. 2009); however, the levels of tRNA halves are low and often increase during stress conditions (Saikia et al. 2012), and they may reflect tRNA splicing intermediates of similar size (Schutz et al. 2010).

Much less is known about YRNA-derived small RNAs. They were also first observed in cells exposed to stress (Rutjes et al. 1999; Nicolas et al. 2012) and later detected in unstressed cultured cells, the brain and other tissues, and in tumors (Schotte et al. 2009; Meiri et al. 2010; Verhagen and Pruijn 2011; Nicolas et al. 2012; Chen and Heard 2013).

15.3.2 Extracellular tRNA- and YRNA-Derived Small RNAs

Various sncRNA species including tRNA- and YRNA-derived small RNAs have been observed in cell culture media (Nolte-'t Hoen et al. 2012). However, more is known about tRNA- and YRNA-derived small RNAs that circulate in the bloodstream. Using deep sequencing of serum small RNAs, our group and others detected tRNA- and YRNA-derived small RNAs circulating in mouse and human bloodstream (Meiri et al. 2010; Dhahbi et al. 2013a, b, 2014; Dhahbi 2014); they were later found in rat and monkey serum at levels higher than miRNAs (Zhang et al. 2014). Earlier work reported tRNA- and YRNA-derived small RNAs primarily in the cytoplasm of stressed cell lines (Lee and Collins 2005; Thompson et al. 2008; Fu et al. 2009; Yamasaki et al. 2009). By deep sequencing of serum small RNAs, we have repeatedly detected tRNA- and YRNA-derived small RNAs in addition to miRNA. These novel circulating small RNAs have been also reported in another biological fluid, human semen (Vojtech et al. 2014), and in plant phloem sap (Zhang et al. 2009). The tRNA- and YRNA-derived small RNAs found in serum/plasma originate mostly from the 5′ end of distinct subsets of tRNAs and YRNAs (*5′ tRNA* and *5′ YRNA halves*), and are as abundant as miRNAs (Dhahbi et al. 2013a, b; Dhahbi 2014). The preponderance of 5′- over 3′-end fragments may

reflect functional differences in addition to differences in stability. These 5′ tRNA and 5′ YRNA halves circulate outside exosomes as 100–300 kDa complexes; the chelating agent EDTA destabilizes the tRNA- but not the YRNA-derived complexes indicating differences in the binding proteins or other properties of these complexes (Dhahbi et al. 2013a, b). The shorter tRFs and similarly shorter YRNA-derived fragments were not detected at the sequencing depths we used in our studies of circulating sncRNAs.

15.3.3 Functions of tRNA- and YRNA-Derived Small RNAs

Some tRFs associate with Argonaute proteins and silence reporter transgenes suggesting that they regulate gene expression in a miRNA-like fashion (Yeung et al. 2009; Haussecker et al. 2010; Burroughs et al. 2011). A tRF derived from the 3′ end of a Ser-TGA tRNA precursor was found to be highly expressed in cancer cell lines, and its depletion impaired cell proliferation (Lee et al. 2009). More recently, 5′ tRFs were shown to inhibit translation at the elongation step (Sobala and Hutvagner 2013). These observations strongly suggest that tRFs may be effectors in new mechanisms of gene expression regulation by sncRNAs.

The 5′ tRNA halves found in cultured cells are produced by cleavage of mature tRNA in response to stresses such as arsenite, heat shock, hypoxia, amino acids depletion, or ultraviolet irradiation (Lee and Collins 2005; Thompson et al. 2008; Fu et al. 2009; Yamasaki et al. 2009). However, some stresses do not induce tRNA cleavage; amino acid or glucose starvation or UV irradiation failed to produce tRNA halves (Thompson et al. 2008; Fu et al. 2009; Yamasaki et al. 2009). The stress-induced 5′ tRNA halves promote assembly of stress granules and suppress protein synthesis as a strategy to divert energy toward damage repair during stress (Yamasaki et al. 2009; Emara et al. 2010; Ivanov et al. 2011). They inhibit translation by associating with the translational repressor YB-1 and displacing eIF4G/eIF4A from the translation initiation complex (Ivanov et al. 2011). Tissue distribution analysis revealed that 5′ tRNA halves are highly expressed in hematopoietic and lymphoid tissues of the mouse and in human leukocytes in the absence of any kind of stress (Dhahbi et al. 2013b). This suggests that 5′ tRNA halves may be involved in hematopoietic and immune processes and that circulating 5′ tRNA halves may act as secreted signals mediating these processes.

YRNA-derived small RNAs were first observed in cells exposed to apoptotic stimuli (Rutjes et al. 1999), and the stressor poly(I:C), a double-stranded RNA mimic immunostimulant chemical (Nicolas et al. 2012). The cleavage of YRNAs and production of YRNA-derived fragments during apoptosis are caspase-dependent (Rutjes et al. 1999). YRNA fragments are also detected in normal tissues such as brain, and in unstressed cultured cells and in tumors (Schotte et al. 2009; Meiri et al. 2010; Verhagen and Pruijn 2011; Nicolas et al. 2012; Chen and Heard 2013). Some YRNA-derived fragments, initially misannotated as miRNAs (Meiri et al. 2010; Verhagen and Pruijn 2011), were later identified in human and rhesus

macaque brain tissues (Shao et al. 2010), in renal cell carcinoma subtypes (Youssef et al. 2011), liver cancer tissues (Lin et al. 2013), and during viral infections (Cui et al. 2010; Qi et al. 2010). However, their role during stress and cancer, and in normal tissues, is at present unknown.

15.3.4 Potential Role of tRNA- and YRNA-Derived Small RNAs in Senescence, Aging and Calorie Restriction

Some of the functions attributed so far to tRNA- and YRNA-derived small RNAs are very closely related to senescence. Both tRNA- and YRNA-derived small RNAs have been shown to respond to stress and associate with cell proliferation and apoptosis, which are processes rigorously regulated during senescence. Recently, tRNA-derived small RNAs (Saikia et al. 2014) and full length tRNAs (Mei et al. 2010a, b, c; Suryanarayana et al. 2012; Hou and Yang 2013; Raina and Ibba 2014) have been shown to inhibit apoptosis by sequestering cytochrome c. Also, blocking the formation of tRNA-derived small RNAs by inhibiting tRNA cleavage slows tumor development (Olson et al. 1995). Inhibition of proliferation and resistance to apoptosis are hallmarks of senescence (Campisi and d'Adda di Fagagna 2007; Ryu and Park 2009). The tRNA-derived stress-induced small RNAs, which suppress global protein translation, seem to reprogram protein translation in response to stress, thereby promoting cell survival when cells are exposed to unfavorable conditions (Yamasaki et al. 2009; Emara et al. 2010; Ivanov et al. 2011). Moreover, Angiogenin, the ribonuclease that produces tRNA-derived stress-induced RNA to suppress protein translation, has been suggested to play a role in neuron survival, and its deficiency is a risk factor of neurodegenerative diseases (Wu et al. 2007; Li and Hu 2010). Promotion of cell survival by 5′ tRNA halves through reprogramming of protein translation may be part of the mechanisms that underlie the commitment of injured cells to survival instead of apoptosis during senescence. The cell commitment to senescence instead of apoptosis is pathologically relevant because, while apoptosis eliminates damaged or stressed cells, senescence arrests their growth and allows damaged cells to persist and acquire abnormalities that alter tissue microenvironment and promote aging and cancer. Evidently, these suggestions regarding a possible role of tRNA- and YRNA-derived small RNAs in senescence require more work to demonstrate direct targeting of common senescence pathways.

We have found that aging changes the circulating levels of 5′ tRNA halves derived from specific tRNA isoacceptors (Dhahbi et al. 2013b). For example, aging decreased the levels of 5′ tRNA halves derived from tRNA-Cys(GCA), which was also earlier reported to suppress translation more potently than other tRNA-derivatives (Ivanov et al. 2011). Whether the age-induced changes in the circulating levels of 5′ tRNA halves are linked to alterations in translation and/or other cellular processes during aging remains to be explored. The underlying mechanisms by

which age-associated changes in the levels of circulating 5′ tRNA halves may affect biological processes in potential target tissues has yet to be determined. A causal relationship between the changes of circulating 5′ tRNA halves and the manifestations of aging needs to be established. More remarkably, CR mitigated some of the age-related changes in circulating levels of 5′ tRNA halves (Dhahbi et al. 2013b). This reversal of the age-associated changes by CR is of particular significance because it validates the age-associated alterations in the levels of circulating 5′ tRNA halves, and provides further evidence that circulating 5′ tRNA halves are physiologically regulated. CR delays molecular and biological alterations that occur during aging, especially age-associated changes in gene expression (Dhahbi et al. 1998, 1999, 2004, 2006, 2012, 2013a, b, c; Lee et al. 1999; Weindruch et al. 2001; Spindler and Dhahbi 2007). It is tempting to speculate that circulating tRNA halves mediate the beneficial effects of CR against the biological and functional alterations that are associated with senescence and aging; however, a direct role of tRNA halves remains to be determined.

The predominant presence of 5′ tRNA halves in hematopoietic and lymphoid tissues suggests that circulating 5′ tRNA halves may be signaling molecules involved in immune-related processes such as inflammation. This is consistent with the strong link between aging and inflammation, and in line with our findings that circulating levels of 5′ tRNA halves are modulated by aging and respond to CR. Inflammation is one of the major driving forces in the development of age-related disorders, including immunosenescence (Martorana et al. 2012; Michaud et al. 2013). These interpretations point toward a role of tRNA-derived small RNAs in cellular senescence and organismal aging and the pathogenesis of their associated disorders.

Nothing is currently known about the effects of senescence, aging or CR on cellular and extracellular levels of YRNA-derived small RNAs. However, full length YRNAs or their derivatives are linked to processes relevant to senescence and aging including inflammatory responses (Clancy et al. 2010; Meredith et al. 2013; Reed et al. 2013), DNA replication and cell proliferation (Christov et al. 2006; Krude et al. 2009; Zhang et al. 2011a), stress responses (Nicolas et al. 2012), apoptosis (Rutjes et al. 1999), viral infections (Cui et al. 2010; Qi et al. 2010), and several types of cancer (Schotte et al. 2009; Meiri et al. 2010). We have recently used small RNA sequencing and found that changes in serum levels of specific types of both YRNA- and tRNA-derived small RNAs are associated with breast cancer and its clinicopathological characteristics (Dhahbi et al. 2014). These close associations of YRNA-derived small RNAs with relevant pathophysiological processes, in addition to their release in extracellular space, strongly implicate them in cellular senescence and organismal aging and the pathogenesis of their associated disorders, similarly to tRNA-derived small RNAs. However, more work is needed to elucidate the secretion pathways of extracellular YRNA- and tRNA-derived small RNAs, the cells that produce and release them in the extracellular environment, their packaging inside cells, their transport and delivery to their destination, and the functions they may exert once inside recipient cells to contribute to cellular senescence and organismal aging.

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Chapter 16
Targeting Senescent Cells to Improve Human Health

Tobias Wijshake and Jan M.A. van Deursen

Abstract The number of people reaching old age is expected to increase dramatically, and concomitantly age-related diseases, such as diabetes, chronic kidney disease, cardiovascular disease, cancer and neurodegenerative disorders. To improve health and quality of life at more advanced ages, it will be necessary to identify and characterize the molecular pathways and events that drive aging and aging-associated diseases. It was proposed that cellular senescence contributes to age-related pathologies, but definitive evidence has long been lacking. However, recent studies on BubR1 hypomorphic mice, which model a human progeroid syndrome referred mosaic variegated aneuploidy (MVA) syndrome, provide strong in vivo evidence senescent cells are causally implicated in aging-associated phenotypes and demonstrate that selective elimination of senescent cells can delay age-related tissue deterioration. These studies identify senescent cells and the senescence-associated secretory phenotype (SASP) they produce, as therapeutic targets for treatment of age-related disease and tissue/organ dysfunction. Here, we describe the formation and features of senescent cells, the evidence that cellular senescence drives age-related dysfunction, the accumulation of senescent cells at sites of pathology in chronic diseases, and potential therapeutic approaches specifically directed against senescent cells to improve human health.

Keywords Cellular senescence • Senescence-associated secretory phenotype (SASP) • Aging • Age-related diseases • Senotherapeutics • Healthspan • Longevity

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16.1 Cellular Senescence and the Senescence-Associated Secretory Phenotype

Cellular senescence is typically defined as stable, difficult-to-reverse arrest of cell proliferation in response to stressors, accompanied by distinctive phenotypic changes, including the production of a secretome that profoundly alters the tissue microenvironment of neighboring cells (Kuilman et al. 2010; Campisi 2013; van Deursen 2014). Cellular senescence is regarded as the main source of senescent cells with aging. Cellular senescence can be provoked by a wide variety of stressors, including genomic DNA damage, oncogenic activation, strong mitogenic signals, tumor suppressor loss, epigenetic changes, spindle stress, and skipping of mitosis (Campisi 2013; van Deursen 2014; Johmura et al. 2014; Krenning et al. 2014). The particular senescence-inducing stressor and its intensity determine whether the senescence program engages the p53/p21 or the $p16^{Ink4a}$/RB tumor suppressor pathway, or both (Campisi and d'Adda di Fagagna 2007; Rodier et al. 2009; Levine and Oren 2009; Adams 2009). Both pathways are highly complex and regulate each other (Adams 2009; Passos et al. 2010; Freund et al. 2011; Campisi 2013).

Senescent cells have several additional features and molecular markers, none of which is truly unique for the senescent state. Furthermore, senescence is heterogeneous meaning that not all senescent cells express the same genes and comprise the same set of traits (Campisi 2013). Cellular senescence requires prolonged inhibition of Cdk-cyclin activity by p21 or $p16^{Ink4a}$, or both, which has identified these cell-cycle inhibitors as biological markers of senescence. Currently, $p16^{Ink4a}$ is regarded as the leading indicator for the presence of senescent cells and its expression is up-regulated upon exposure to various senescence-inducing stresses and with aging (Collins and Sedivy 2003; Ohtani et al. 2004; Krishnamurthy et al. 2004). Additional characteristics of senescent cells include overexpression of senescence-associated β-galactosidase (SA-β-Gal), increased cell size and altered morphology, resistance to apoptosis, changes in chromatin organization and gene expression, and an increase in DNA damage-related foci (Campisi 2013; van Deursen 2014). However, one of the most intriguing characteristics of senescent cells is the SASP, which entails the expression and secretion of numerous pro-inflammatory cytokines and chemokines, growth factors and proteases (Coppé et al. 2008; Rodier et al. 2009; van Deursen 2014). Secretion of SASP factors occurs in a wide variety of cells and also takes place in vivo in mice and humans (Campisi et al. 2011; Baker et al. 2011a). The SASP shows great variability in the secretion of components depending on the cell types, the strength of and the kind of senescence-inducing stimuli (Kuilman et al. 2008; Coppé et al. 2008; Fumagalli et al. 2012; Campisi 2013). In many cases, SASP activation requires persistent DDR signaling via the DDR proteins ATM and checkpoint kinase 2 (CHK2) and is a slow and gradual process (Coppé et al. 2008; Rodier et al. 2009). How signaling via the DDR pathway promotes the expression of various SASP components remains to be elucidated, but a possible mechanism involves chromatin remodeling which leads to profound changes in

transcriptional regulation (Zhang et al. 2003; Shah et al. 2013). However, recent studies have demonstrated that several senescence-inducing stimuli can produce SASP factors without causing DNA damage, indicating that there are also DDR-independent mechanisms (Kaplon et al. 2013; Muñoz-Espín et al. 2013; Storer et al. 2013).

Due to the great diversity of the SASP, it was obvious that senescent cells are implicated in various biological processes that engage paracrine signaling, such as cell proliferation, angiogenesis, epithelial-to-mesenchymal transition, inflammation, stem cell renewal and differentiation, wound healing and tissue repair (Conboy et al. 2005; Coppé et al. 2006; Brack et al. 2007; Krizhanovsky et al. 2008; Freund et al. 2010; Jun and Lau 2010; Laberge et al. 2012). Several studies have suggested that the nature of processes in which senescence has been implicated may entail different courses of action that can be divided in acute versus chronic senescence (Fig. 16.1) (van Deursen 2014). For example, acute senescence occurs to limit fibrosis upon acute tissue damage. CCl_4-induced liver fibrosis in mice initially stimulates the activation and proliferation of hepatic stellate cells to produce the extracellular matrix deposited in fibrotic scars. These hepatic stellate cells will undergo senescence and display enhanced secretion of extracellular matrix-degrading enzymes and immune modulators. Subsequently, natural killer cells selectively eliminate these senescent stellate cells and hereby facilitate the resolution of liver fibrosis (Krizhanovsky et al. 2008). Similarly, the senescence program has been implicated in the process of wound healing. The cellular matrix protein CCN1 is dynamically expressed at sites of wound repair and induces senescence of myofibroblasts through integrin-dependent ROS generation by activation of NOX1 and the $p16^{Ink4a}$/RB pathway. CCN1-induced senescence of myofibroblasts limits excessive fibrosis in cutaneous wound healing by induction of anti-fibrotic gene expression (Jun and Lau 2010). In another study it was demonstrated that senescent fibroblasts and endothelial cells accumulate rapidly in response to a cutaneous wound, where they accelerate wound healing by activating myofibroblast differentiation through secretion of platelet-derived growth factor AA (PDGF-AA) (Demaria et al. 2014). Acute senescence also occurs during mammalian embryonic development at multiple locations to allow tissue growth and patterning. This is a highly programmed developmental response that is dependent on the induction of p21 and activation of downstream signaling pathways followed by macrophage infiltration, removal of senescent cells by apoptosis and immune-mediated clearance, and tissue remodeling (Muñoz-Espín et al. 2013; Storer et al. 2013).

Whereas acute senescence seems a scheduled or programmed process induced by specific stimuli targeting particular cell types, aging-associated chronic senescence involves a transition from temporal to persistent cell-cycle arrest that is more unscheduled and stochastic in nature, probably due to the combined effects of various senescence-inducing stresses acting simultaneously on a cell (van Deursen 2014). Chronic senescence seemingly plays a role in age-related tissue dysfunction and age-related diseases (Fig. 16.1 and see below).

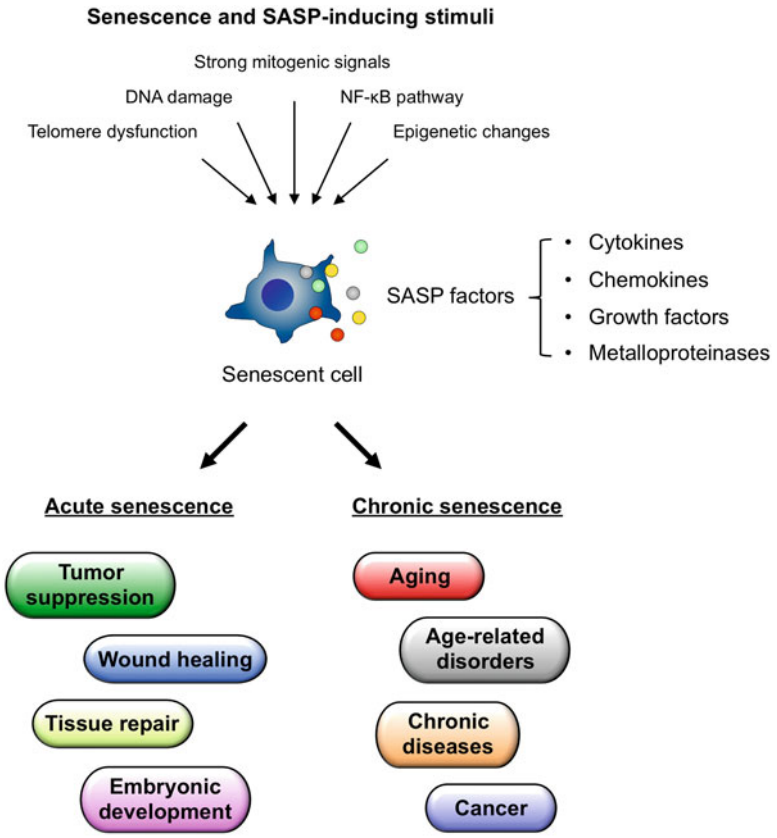

Fig. 16.1 Senescence-associated secretory phenotype is implicated in many biological processes. Numerous stressors and stimuli can induce senescence and SASP. This SASP can show great variety in the secretion of components, including cytokines, chemokines, growth factors and metalloproteinases. Due to the great diversity of the secreted SASP components, SASP has been implicated in a large number of biological processes, such as tumor suppression, wound healing, tissue repair, and embryonic development, but also in tumor promotion, chronic diseases, aging and age-related disorders

16.2 Senescence Drives Age-Related Phenotypes

Hayflick and Moorhead already interpreted the phenomenon of replicative senescence as one of the fundamental mechanisms underlying the process of organismal aging (Hayflick and Moorhead 1961). Consistent with this, it was demonstrated that senescent cells accumulate with passage in vitro, and in a number of tissues in aged mice and aged human skin (Dimri et al. 1995; Krishnamurthy et al. 2004). By now it has been widely established that senescent cells increase with age in tissues of humans, primates, and rodents (Herbig et al. 2006; Krishnamurthy et al. 2006; Jeyapalan et al. 2007; Wang et al. 2009; Lawless et al. 2010). Furthermore,

accumulation of senescent cells has been observed at sites of various age-related diseases including, cancer, cardiovascular disease, osteoporosis, arthritis, and neurodegenerative disorders (Campisi 2013; Naylor et al. 2013). This increase in senescent cells over time can be a cause of accumulation of DNA damage and/or increase of other senescence-inducing stresses. Senescing human cells and aging mice show increased numbers of DNA damage lesions with repairable double-strand breaks (DSBs) (Sedelnikova et al. 2004; Wang et al. 2009). Another possibility is that clearance of senescent cells by the immune system is less efficient with aging. Elimination of senescent cells by the immune system was elegantly demonstrated in mouse models of liver fibrosis and liver carcinoma (Xue et al. 2007; Krizhanovsky et al. 2008). Senescent hepatic stellate cells augment the expression of immune modulators encoding cytokines or receptors that activate natural killer cells (Krizhanovsky et al. 2008). This suggests that senescent cells have a self-elimination program, referred to as "senescence surveillance", that acts via attracting and activating various immune cells by the secretion of inflammatory cytokines and chemokines (Xue et al. 2007; Krizhanovsky et al. 2008; Kang et al. 2011; van Deursen 2014). With aging, efficient elimination of senescent cells is thought to be reduced as a consequence of aging-associated immune deficiency (Dorshkind et al. 2009). The function of hematopoietic stem cells declines with aging, resulting in decreased lymphopoiesis and increased myelopoiesis, an consequently impaired immune function (Wang et al. 2011). Furthermore, the immune system is unable to maintain a balanced T-cell repertoire due to changes in T-cell production and consumption in the later stages of mammalian lifespan (Nikolich-Zugich 2008).

The importance of in vivo senescence in the etiology of aging and age-related diseases has initially focused on the *Cdkn2a* locus, which encodes the tumor suppressors $p16^{Ink4a}$ and $p19^{Arf}$. Both $p16^{Ink4a}$ and $p19^{Arf}$ can induce senescence in cultured cells and the level of both proteins increases with aging in many tissues (Krishnamurthy et al. 2004; Kim and Sharpless 2006; Baker et al. 2008). Testing whether the induction of $p16^{Ink4a}$ and $p19^{Arf}$ causes in vivo senescence and organismal aging was hampered because mice deficient for $p16^{Ink4a}$ and $p19^{Arf}$ die early from cancer far before they reach the age that normal mice start to develop age-related phenotypes (Collado et al. 2007; Campisi and d'Adda di Fagagna 2007). However, the generation of the BubR1 hypomorphic mouse model offered an alternative approach to investigate the contribution of in vivo senescence in the aging process. Mutant mice carrying *BubR1* hypomorphic alleles ($BubR1^{H/H}$ mice) that produce low amounts of the mitotic checkpoint protein BubR1 have a fivefold reduced lifespan and develop several progeroid and age-related phenotypes at an early age, including growth retardation, facial dysmorphisms, cataracts, muscle wasting, lordokyphosis (increased curvature of the spine), fat loss, impaired wound healing, infertility, gliosis, arterial wall stiffening and cardiac arrhythmias (Baker et al. 2004, 2008; Matsumoto et al. 2007; Hartman et al. 2007). Importantly, skeletal muscle, fat and eye, tissues that develop early aging-associated phenotypes due to BubR1 insufficiency, have high levels of the senescence markers $p16^{Ink4a}$ and $p19^{Arf}$. Skeletal muscle of BubR1 hypomorphic mice also expressed high levels of other senescence-associated genes, including *Igfbp2*, *Mmp13* and *PAI-1*. In addition,

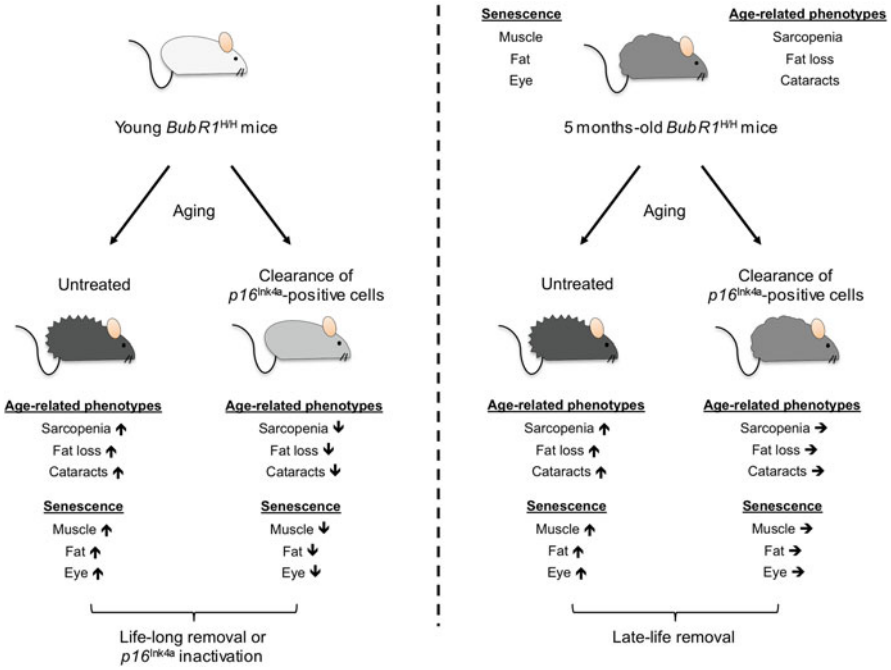

Fig. 16.2 Senescence and age-related phenotypes in the various BubR1 progeroid mouse models. Overview of the life-long clearance of p16^{Ink4a}-positive senescent cells and *p16*^{Ink4a} inactivation studies (*left*), and late-life clearance of p16^{Ink4a}-positive senescent cells (*right*) in *BubR1*$^{H/H}$ mice, indicating that senescence levels in tissues correlates with the severity of the age-related phenotypes. In addition, these studies identified senescent cells as a therapeutic target for treatment of aging and age-related disorders

inguinal adipose tissue of *BubR1*$^{H/H}$ mice stained highly positive for SA-β-Gal and in vivo 5-bromo-2-deoxyuridine (BrdU) labeling, which are both hallmarks of senescence, revealed reduced numbers of dividing cells in abdominal muscle and adipose tissue compared to wild-type mice. Altogether, this indicates that skeletal muscle, fat and eye have high levels of in vivo senescence in *BubR1*$^{H/H}$ mice (Baker et al. 2008).

Genetic inactivation of *p16*^{Ink4a} in *BubR1*$^{H/H}$ mice significantly delayed the onset of lordokyphosis and cataract formation, and significantly reduced loss of subcutaneous adipose tissue compared to *BubR1*$^{H/H}$ mice (Fig. 16.2). However, several other progeroid phenotypes observed in *BubR1*$^{H/H}$ mice were not improved upon *p16*^{Ink4a} inactivation, including dwarfism, arterial wall stiffening and infertility (Baker et al. 2008). The selective correction by *p16*^{Ink4a} disruption on specific progeroid phenotypes seems to be dependent on the engagement of *p16*^{Ink4a} and in vivo senescence in *BubR1*$^{H/H}$ mice. This was supported with the observation that *BubR1*$^{H/H}$; *p16*$^{Ink4a-/-}$ mice have significantly reduced levels of senescence in skeletal muscle, eye and adipose tissue compared to *BubR1*$^{H/H}$ mice. Altogether,

p16^{Ink4a} inactivation diminished both senescence and aging phenotypes in these tissues, demonstrating for the first time that senescence is causally implicated in the generation of age-related phenotypes (Baker et al. 2008).

16.3 Clearance of Senescent Cells Delays Aging-Associated Phenotypes

Disruption of *p16*^{Ink4a} in *BubR1*$^{H/H}$ mice identified senescent cells as a therapeutic target for treatment of aging and age-related diseases, but genetic inactivation of *p16*^{Ink4a} in humans would be an unfeasible approach as this would eliminate a key tumor-suppressive program, resulting in cancer (Naylor et al. 2013). To further examine the potential of eliminating senescent cells as a therapeutic target for aging, a transgenic mouse model was generated, termed *INK-ATTAC,* to enable the drug-inducible removal of p16^{Ink4a}-positive senescent cells in the BubR1 progeroid mouse model. The *INK-ATTAC* transgene was modified from a previous mouse model, named *FAT-ATTAC* (fat apoptosis through targeted activation of caspase) in which adipocytes are selectively killed by apoptosis upon administration of AP20187, a synthetic drug that activates the dimerization of a membrane-bound myristoylated FK506-binding-protein-caspase 8 (FKBP-Casp8) fusion protein that is under the transcriptional control of the *Fabp4* promoter and specifically expressed in adipocytes (Pajvani et al. 2005). For *INK-ATTAC* the *Fabp4* promoter was replaced with a 2,617-bp fragment of the *p16*^{Ink4a} gene promoter that is expressed in senescent, but in non-senescent cells (Wang et al. 2001). To examine whether removal of p16^{Ink4a}-positive senescent cells was able to attenuate age-associated deficits in mice, transgenic *INK-ATTAC* founder lines were bred onto a BubR1 hypomorphic background. These transgenic strains demonstrated that *INK-ATTAC* is specifically expressed in p16^{Ink4a}-positive senescent cells and that these cells were efficiently eliminated upon AP20187 treatment (Baker et al. 2011a).

Life-long treatment with AP20187 twice a week starting from weaning in *BubR1*$^{H/H}$; *INK-ATTAC* mice selectively suspended the acquisition of p16^{Ink4a}-mediated age-related pathologies: skeletal muscle deterioration, fat loss, and cataract formation (Fig. 16.2). The delayed onset of age-related phenotypes coincided with a reduction in the number of senescent cells in these tissues, indicating that senescent cells were efficiently removed and that this postpones the development of age-related dysfunction in BubR1 progeroid mice (Baker et al. 2011a). A second experiment, termed late-life treatment, started when *BubR1*$^{H/H}$ mice have already developed aging-associated phenotypes and was designed to investigate whether elimination of senescent cells was able to rejuvenate aged tissues. Late-life clearance of p16^{Ink4a}-positive senescent cells attenuated the progression of age-associated decline in skeletal muscle and adipose tissue in BubR1 hypomorphic mice (Figs. 16.2). However, removal of senescent cells later in life was unable to reverse established age-related phenotypes in *BubR1*$^{H/H}$

mice (Baker et al. 2011a). It should be noted that neither life-long nor late-life treatment was able to significantly prolong lifespan in these mice, most likely because cardiovascular defects of BubR1 hypomorphic mice (a $p16^{Ink4a}$-independent phenotype) were not ameliorated upon removal of senescent cells. Although the cardiovascular anomalies of BubR1 progeroid mice were senescence independent, this does not rule out that senescent cells contribute to cardiovascular disease in the elderly (Baker et al. 2011a; Naylor et al. 2013). For instance, cells with senescent properties accumulate in human heart tissue with aging and senescent vascular smooth muscle cells have been observed in human atherosclerotic plaques (Krishnamurthy et al. 2004; Erusalimsky and Kurz 2005; Gorenne et al. 2006).

The above senescent cell clearance experiments indicate that cellular senescence is causally implicated in the development of age-related phenotypes and that elimination of senescent cells can prevent tissue failure and extend healthspan. Furthermore, continuous treatment with AP20187 in *WT*;*INK-ATTAC* mice until 8 months of age did not reveal any overtly negative side effects and further strengthen the conclusion that removal of senescent cells is a new promising therapeutic target for treatment of age-associated pathologies (Baker et al. 2011a).

The fact that elimination of senescent was only able to delay rather than to revert the onset of age-related tissue deterioration, suggests that senescence causes permanent damage to tissues and/or organs, most likely through cell-autonomous, non-cell-autonomous mechanisms, or a combination of both. One possible cell-intrinsic mechanism could be that senescence specifically occurs in stem and/or progenitor cells thereby decreasing regenerative potential, a key hallmark of aging (Rossi et al. 2007; Sharpless and DePinho 2007). Indeed, progenitor cells in adipose tissue and gastrocnemius muscle of 2 month-old BubR1 progeroid mice have increased levels of senescence (Baker et al. 2013). In addition, fat and skeletal muscle of these *BubR1*$^{H/H}$ mice had dramatically decreased numbers of progenitor cells compared to wild-type controls. Genetic inactivation of *p21* in *BubR1*$^{H/H}$ mice resulted in accelerated onset of lordokyphosis and age-related fat loss, which was associated with increased levels of progenitor cell senescence and a decline in the total number of progenitor cells in these tissues. These results uncover a strong link between senescence of the progenitor cell compartment and age-related dysfunction in response to BubR1 insufficiency (Baker et al. 2013). This was further supported by a number of recent studies demonstrating that various senescence-inducing stresses, including DNA damage and chronic low grade inflammation, induces progenitor cell senescence or dysfunction and causes premature aging in transgenic mice (Chen et al. 2013; Jurk et al. 2014).

Next to cell-intrinsic functional decline, senescent cells can potentially also contribute to age-related tissue deterioration via the secretion of SASP factors and its various cell-non-autonomous functions on the tissue microenvironment, which we will shortly discuss here (Campisi et al. 2011; Campisi 2013; van Deursen 2014). First, disruption of the stem cell niche and stem cell function; secretion of soluble factors and extracellular matrix by the aged microenvironment and serum from aged mice can result in disruption of the local stem cell niche and

loss of self-renewal capacity (Krtolica et al. 2001; Brack et al. 2007; Chakkalakal et al. 2012). Exposure to young systemic factors by parabiosis or restoration of systemic GDF11 levels was able to reverse age-related tissue and stem cell dysfunction in heart, brain and skeletal muscle of aged mice (Conboy and Rando 2005; Loffredo et al. 2013; Katsimpardi et al. 2014; Sinha et al. 2014), further strengthening the idea that secreted SASP factors can have detrimental effects on tissue and stem cells. Second, disruption of the tissue architecture; senescent cells can produce and secrete matrix metalloproteinases (MMPs) that can cleave membrane-bound receptors, extracellular matrix proteins or components of the tissue microenvironment and hereby disrupt normal tissue structure, organization and function (Parrinello et al. 2005; Tsai et al. 2005; van Deursen 2014). Other SASP factors, such as IL-6 and IL-8, can induce proliferation and migration of epithelial cells and thus alter the tissue microenvironment (Coppé et al. 2008). Third, stimulation of tissue inflammation; chronic low grade tissue inflammation has been associated with aging and causally implicated in various age-related disorders such as atherosclerosis, diabetes and cancer (Tabas 2010; Freund et al. 2010; Baker et al. 2011b; Barzilai et al. 2012). It seems plausible that accumulation of senescent cells with aging and at sites of age-related pathologies contribute to the observed inflamed microenvironment through the SASP, which comprises a number of pro-inflammatory cytokines and chemokines (Campisi 2013; van Deursen 2014). Fourth, induction of senescence in neighboring cells (paracrine senescence); age-related tissue dysfunction can be exacerbated by senescent cell secretion of TGF-β, VEGF, CCL2, CCL20, IL-1α and ROS that are capable to provoke senescence in healthy neighboring cells and hereby spread senescence to the environment (Nelson et al. 2012; Acosta et al. 2013). Altogether, these data suggest that production and secretion of SASP components by senescent cells may well contribute to age-related deficits and that removal of these factors could improve the microenvironment, thus enhancing the performance of remaining cells.

16.4 Clearance of Senescent Cells for Therapeutic Purposes

Aging is characterized by a gradual decline of physiological function over time that affects most living organisms. This degenerative process is driven by a number of molecular, biochemical and metabolic alterations that occur at the cellular level (Newgard and Sharpless 2013). Traditional symptoms of natural aging in mammals include loss of skeletal muscle mass and strength, increased curvature of the spine, lipodystrophy, hair greying and hair loss, decreased eyesight, hearing and cognition, immune system failure, infertility, osteoporosis, and many others (Newgard and Sharpless 2013). Furthermore, aging is the main risk factor for major human pathologies, including cardiovascular diseases, type 2 diabetes, COPD, pulmonary fibrosis, osteoarthritis, cancer, and neurodegenerative disorders (DePinho 2000; Price et al. 2002; Ito and Barnes 2009; Niccoli and Partridge 2012; López-Otín et al. 2013). This in combination with the fact that $p16^{Ink4a}$-positive senescent cells

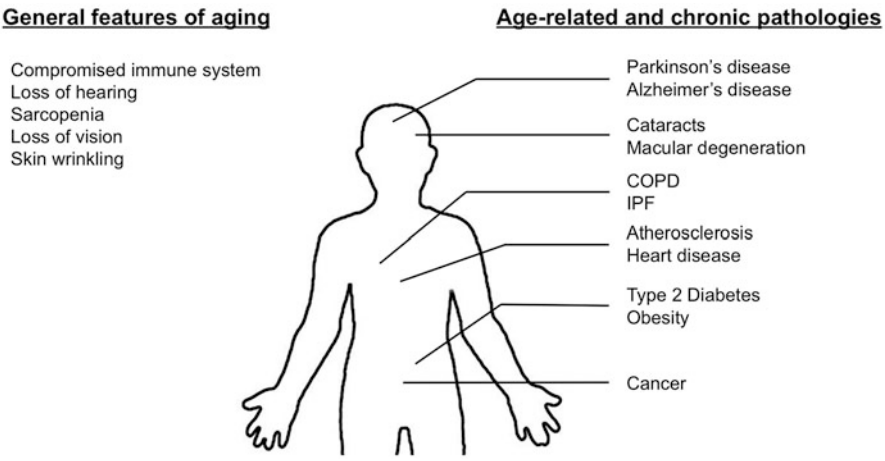

Fig. 16.3 Senescence has been associated with general features of aging and major human pathologies

accumulate with aging in many tissues of both humans and mice (Krishnamurthy et al. 2004; Baker et al. 2011a; Demaria et al. 2014), makes it of great interest to assess whether selective removal of senescent cells can delay aging and age-related deficits in chronologically aged mice.

The potential role and therapeutic implications for removal of senescent cells in the treatment of universal characteristics of natural aging will be discussed here (Fig. 16.3). It should be kept in mind that senescent cell clearance in the elderly may not act to rejuvenate tissues and organs but only attenuate the rate of further decline, Thus, intervention in the elderly population would imply a preventive strategy rather than a treatment per se.

Sarcopenia is the loss of muscle mass and strength that occurs in geriatric individuals and in patients with progeria syndromes (Burtner and Kennedy 2010). Sarcopenia contributes to frailty, reduced capability to handle disease-associated stress and to mortality in the elderly population. The etiology of sarcopenia is not well understood, but a number of factors are believed to participate in this process, including the loss of motor neurons, impaired muscle cell contractility and a reduction of stem cell numbers (Roubenoff 2000; Verdijk et al. 2012). A number of studies have demonstrated that muscle stem cells undergo senescence with aging and that reversal of senescence rescues their regenerative potential (Du et al. 2014; Bernet et al. 2014; Cosgrove et al. 2014). For instance, satellite cells in geriatric mice and humans lose reversible quiescence by switching to an irreversible pre-senescent state, which is caused by derepression of $p16^{Ink4a}$ and results in impaired myogenic function. Silencing of $p16^{Ink4a}$ in geriatric satellite cells was able to restore quiescence and muscle regenerative potential. These results indicate that maintenance of quiescence in adult satellite cells depends on the active repression of senescence (Sousa-Victor et al. 2014). Consistent with this, BubR1 progeroid

mice with high numbers of senescent cells in the skeletal muscle are predisposed to sarcopenia and removal of these senescent cells was able to attenuate muscle atrophy (Baker et al. 2011a).

Another hallmark of aging is the decline of the immune system, which leads to an impaired ability to cope with vaccinations and to fight infections. Elderly individuals also suffer from auto-immunity disorders more often, which further indicates the dysregulation of the immune system with age (Dorshkind et al. 2009). Although studies regarding the effects of aging on the innate immune system are inconclusive, there is ample evidence that the adaptive immune system is declining during aging, also known as immunosenescence, and includes the impaired development of B-cell and T-cell lymphocytes, and lymphocyte function (Franceschi et al. 2007; Dorshkind et al. 2009). In addition, centenarians, avoid immunosenescence and maintain a high T-cell CD4/CD8 ratio and low numbers of $CD8^{+}CD28^{-}$ cells (Strindhall et al. 2007). Conversely, the aging-associated decline of the immune system may also contribute to the accumulation of senescent cells with aging as natural killer cells have been implicated in the removal of senescent cells in the process of liver fibrosis and during embryonic development (Krizhanovsky et al. 2008; Muñoz-Espín et al. 2013; Storer et al. 2013). Therefore, it seems plausible that immune-mediated clearance of senescent cells also occurs during chronological aging. Taken together, this suggests that the removal of senescent immune cells in the elderly might be beneficial for functionality of the immune system as well as improve the quality of life.

Age-associated loss of hearing (presbycusis) and vision are common disabling conditions, but the underlying mechanisms are still not well understood. Presbycusis is characterized by increased higher hearing thresholds, especially at higher frequencies, and a progressive reduction of distortion-product otoacoustic emissions, as a result of a lifetime of insults to the auditory system (Gates and Mills 2005; Uchida et al. 2008; Spoor et al. 2012). Loss of vision with aging typically includes a decline of visual acuity and (spatial) contrast sensitivity. Age-related optic alterations are the impaired accommodation ability due to hardening of the lens, decrease in pupil size, increase in density in the lens, and alterations within the pupil (Spear 1993; Spoor et al. 2012). Studies have suggested that ROS-induced DNA damage is one of the driving forces of age-related hearing and vision loss (Darrat et al. 2007; Fletcher 2010). In support of this, patients suffering from the rare inherited DNA repair disorders xeroderma pigmentosum (XP) or Cockayne syndrome (CS) display features of presbycusis and vision loss at an early age (Kenyon et al. 1985; Rapin et al. 2006). Furthermore, DNA-repair deficient $Ercc1^{\Delta/-}$ mice have accelerated loss of hearing and vision. $Ercc1^{\Delta/-}$ mutants displayed progressive, accelerated increase of hearing threshold levels over time, probably due to deteriorating coachlear function. $Ercc1^{\Delta/-}$ mice also showed a progressive reduction in contrast sensitivity followed by a thinning of outer nuclear layer of the eye. These features show great resemblance with normal aging and suggest that DNA damage can provoke an age-related decline of the auditory and visual system (Spoor et al. 2012). As previously mentioned, DNA damage is a potent inducer of cellular senescence and it is possible that senescent cells contribute to the age-related loss of hearing and vision. Human

corneal endothelial cells derived from human donors demonstrated an increase of senescence with aging as determined by *p16*^{Ink4a} and SA-β-Gal activity, but whether cellular senescence is causally implicated in presbycusis and vision loss is unknown (Mimura and Joyce 2006; Song et al. 2008).

Skin aging is caused by both intrinsic and extrinsic factors. Intrinsic aging in the skin is associated with a marked thinning and loss of undulation in the epidermal layer, next to loss of subcutaneous fat and dermal thickness. While, extrinsic aging, mainly caused by sun exposure, significantly alters both the epidermis and dermis (Demaria et al. 2015). The number of senescent cells accumulates with aging equally in the epidermal and dermal layers, verified by elevated levels of p16^{Ink4a} and SA-β-Gal activity (Dimri et al. 1995; Krishnamurthy et al. 2004; Ressler et al. 2006). Furthermore, using an in vitro living skin equivalent (LSE) model with epidermal keratinocytes from human donors of varying age, it was demonstrated that increased expression of p16^{Ink4a} in keratinocytes from young donors generates a thinner epidermal layer similar to that of one made by keratinocytes from elderly donors. Along this line, silencing of p16^{Ink4a} expression in old keratinocytes was able to transform the aged skin phenotype into a thicker epidermis, which is comparable to skin formed by keratinocytes from younger donors (Adamus et al. 2014). These results were in accordance with the experiments in the BubR1 progeroid mice, where genetic inactivation of p16^{Ink4a} and clearance of p16^{Ink4a}-positive senescent cells resulted in an attenuation of p16-dependent skin aging (Baker et al. 2008, 2011a). Although clearance of senescent cells looks appealing as a therapeutic strategy in the treatment of skin aging, recent studies have indicated that senescent cells also have a beneficial effect in the process of cutaneous wound healing (Jun and Lau 2010; Demaria et al. 2014). It remains to be determined whether removal of senescent cells would compromise wound healing, as the induction of senescence in myofibroblasts appears to be transient and non-essential.

16.5 Clearance of Senescent Cells in Chronic Age-Related Diseases

Next to the accumulation of senescent cells with aging in various tissues, senescent cells are also present at sites of chronic diseases (Fig. 16.3). In addition, it is established that senescent cells arise as a consequence of certain treatments, including radiation and chemotherapy (Naylor et al. 2013). It is currently unclear if these senescent cells are causally involved in the pathogenesis of age-related disorders, but the development of the *INK-ATTAC* mouse model now enables researchers to investigate the contribution of senescent cells in chronic diseases and to assess the preventive and therapeutic outcomes of the removal of senescent cells (Baker et al. 2011a; Naylor et al. 2013).

16.5.1 Cancer

Aging is the main risk factor for cancer. In humans, the incidence of cancer increases exponentially in the last decades of life. However, the underlying mechanism and cellular foundation responsible for this increase are poorly understood. It has long been postulated that the accumulation of somatic mutations in certain genes over time might be sufficient to drive tumorigenesis, but recent studies have demonstrated that malignant transformation requires additional alterations in the tissue microenvironment (DePinho 2000; Naylor et al. 2013; Lopez-Otin et al. 2013).

Although cellular senescence is a tumor-suppressive program that prevents the proliferation of damaged cells, there is mounting evidence that senescent cells can also drive tumor progression. For example, xenograft studies have demonstrated that co-injection of senescent cells in immunocompromised mice can promote proliferation of epithelial tumor cells, stimulate tumor invasion and tumor angiogenesis, at least partially due to secretion of SASP components (Krtolica et al. 2001; Coppé et al. 2006; Liu and Hornsby 2007). Furthermore, senescent fibroblasts promote epithelial-to-mesenchymal transition in premalignant epithelial cells, a critical step in the development towards a metastatic cancer, through production of SASP factors IL-6 and IL-8 (Parrinello et al. 2005; Coppé et al. 2008; Laberge et al. 2012). The overall picture is that accumulation of senescent cells and its SASP components may create a microenvironment that facilitates the development and the progression of cancer. However, this process is not straightforward as the same SASP factors may promote tumor progression in one context, but can be essential in tumor suppression in the other (Campisi 2013; Pérez-Mancera et al. 2014). One disadvantage of these studies, which use either in vitro or xenograft mouse models, is that they do not accurately represent physiological conditions of cancers in mice or humans. One question that remains is whether senescent cells and their SASP provoke tumorigenesis under physiological circumstances, which can now be addressed with the existing *INK-ATTAC* mouse model.

16.5.2 Side Effects of Cancer Therapy

Cancer treatments can be toxic and typically have long-lasting effects due to collateral damage to normal cells health. Short-term side effects of chemotherapy and radiation therapy are probably caused by the destruction of dividing cells and affect the gastrointestinal tract, bone marrow or hair follicles, resulting in amongst others appetite loss, nausea, bone marrow alterations, and hair loss. Long-term effects of toxic cancer therapies can diminish the quality of life of cancer survivors and predisposes them to disabilities and comorbidities that strongly resemble features of accelerated aging (National Cancer Institute; Naylor et al. 2013; van Deursen 2014). An example, a proportion of breast cancer survivors experience a

disproportionate decline in the physical function, such as functional mobility, upper extreme function, and the basic and instrumental activities of daily living compared to individuals living without cancer for the same period (Schmitz et al. 2007). The association between cancer treatment and accelerated aging is further supported by the observation that the elevated levels of inflammatory cytokines are correlated with decreased physical function in cancer survivors as well as in the elderly population (Bower et al. 2002; Cesari et al. 2004). The mechanisms underlying these cancer-therapy associated disabilities are not well understood, but it seems likely that the induction of senescent cells due to DNA-damaging therapies is contributing to the long-term complications after cancer therapy (Roninson 2003; Le et al. 2010).

Another important concern of cancer therapy is the induction of paracrine factors in the tumor microenvironment that can modulate tumor survival. For instance, administration of chemotherapy in a mouse model of Burkitt's lymphoma causes genotoxic damage that induces the release of IL-6 and tissue inhibitor of metalloproteinases-1 (Timp-1) from the thymus creating a "chemo-resistant niche" that promotes the survival of the residual tumor cells and serves as a reservoir for tumor relapse (Gilbert and Hemann 2010). Similarly, cancer therapeutics stimulates the secretion of Wnt16B in the tumor microenvironment via DNA damage-induced activation of NF-κB. The expression of Wnt16B in the prostate tumor microenvironment diminished the effects of cytotoxic chemotherapy in vivo, promoting tumor cell survival and cancer progression (Sun et al. 2012). In contrast, inhibition of SASP components (through NF-κB) in a mouse lymphoma model avoided chemotherapy-induced senescence, producing drug resistance, early relapse, and reduced survival (Chien et al. 2011). The effect of senescent cells on the microenvironment in the setting of DNA-damaging cancer treatment suggests that it may be important to consider adjuvant therapies that eliminate normal and tumor-derived senescent cells. These therapies may result in more effective eradication of tumor cells by chemotherapy and radiation therapy and prevent the development of a senescence-provoked "chemo-resistant niche" (Campisi 2013). Potentially, removal of senescent cells can also attenuate the long-term complications and/or disabilities associated with toxic cancer therapy.

16.5.3 Atherosclerosis

Atherosclerosis and the subsequent cardiovascular complications, such as myocardial infarction, ischemic heart failure, and stroke is the major cause of death and morbidity in the Western world. Risk factors for atherosclerosis include hypertension, diabetes, high serum levels of total and low-density lipoprotein (LDL) cholesterol, and smoking. In addition, aging has been emerging as one of the main risk factors (Weber and Noels 2011; Wang and Bennett 2012). Atherogenesis has been associated with (premature) aging and this may be caused through the induction of cellular senescence. The presence of senescent cells at sites of atherosclerosis has been reported in several studies. For example, atherosclerotic plaques contain

SA-β-Gal-positive vascular smooth muscle cells and endothelial cells, and high levels of $p16^{Ink4a}$ expression (Minamino et al. 2002; Matthews et al. 2006; Gorenne et al. 2006; Holdt et al. 2011). Furthermore, plaque vascular smooth muscle cells, endothelial cells, and macrophages display telomere shortening, DNA damage, and various epigenetic alterations, which are all features of senescence. Senescent cells may contribute to the proinflammatory environment during artherogenesis through the production of cytokines and chemokines, and hereby provoke the infiltration of macrophages into the subendothelial layer (Wang and Bennett 2012). Other SASP factors may also enhance atherosclerosis by promoting the dysfunction of endothelial cells and vascular smooth muscle cells. Finally, senescence-associated adipose tissue dysfunction can impair proper lipid metabolism and storage and cause accumulation of lipids in the arterial wall, a key step in the process of atherogenesis (Naylor et al. 2013; Tchkonia et al. 2013).

16.5.4 Obesity

Excess consumption of nutrients and/or low energy expenditure leads to storage of energy in the adipose tissue. However, the storage capacity of adipocytes eventually reaches a limit that provokes a stress response and facilitates the recruitment of macrophages. This inflammatory reaction of the fat tissue induces a cascade of events with systemic pathological consequences, including liver steatosis (fatty liver) and insulin resistance, which are hallmarks of the metabolic syndrome (Gregor and Hotamisligil 2011; Muñoz-Espín and Serrano 2014). Interestingly, preadipocytes and fat cells from obese humans and mice display enhanced levels of senescence, which was characterized by higher SA-β-Gal activity, presence of SASP components, and upregulation of p21 and p53 (Minamino et al. 2009; Tchkonia et al. 2010; Markowski et al. 2013). In addition, the BubR1 progeroid mouse model showed high levels of senescence in the adipose tissue. Genetic inactivation of $p16^{Ink4a}$ or clearance of $p16^{Ink4a}$-positive senescent cells in BubR1 hypomorphic mice counteracted senescent cell accumulation in fat tissue and attenuate lipodystrophy (Baker et al. 2008, 2011a). Altogether, this suggests that fat tissue senescence is associated with obesity and may contribute to its pathological outcomes.

16.5.5 Type 2 Diabetes

Insulin resistance, a decrease in insulin-mediated glucose uptake, has been linked with obesity, aging and an increasing sedentary lifestyle. This process is initially compensated by enhanced pancreatic β-cell mass and insulin secretion. However, failure of functional expansion of islets β-cells to counteract the degree of insulin resistance results in insulin deficiency and ultimately in the development of type

2 diabetes mellitus (D2M). D2M is quickly becoming a global epidemic. D2M is also a great risk factor for a number of diseases, including cardiovascular disorders, stroke, chronic kidney failure, retinopathy, neuropathy and many other conditions (Donath and Shoelson 2011; Naylor et al. 2013). The role of senescent cells in the pathogenesis of D2M has been demonstrated in several studies. For instance, long-term high-fat diet feeding of mice results in reduced β-cell mass and reduced proliferation rates. After 12 months on the diet, atrophic β-cells stain strongly positive for SA-β-Gal activity (Sone and Kagawa 2005). Along this line, various transgenic mouse models that induce D2M showed a strong high levels of SA-β-Gal activity in β-cells (Muñoz-Espín and Serrano 2014). Senescent cells in fat tissue may promote D2M through secretion of proinflammatory SASP factors (Donath and Shoelson 2011). Inhibition of p53 activity in adipose tissue markedly reduced senescence and proinflammatory cytokine levels, and improved insulin resistance in mice with D2M, indicating the potential of senescent cell clearance as a therapeutic target in the context of diabetes (Minamino et al. 2009).

16.5.6 Neurodegenerative Disorders

Neurodegeneration is characterized by the progressive loss of structure and function of neurons, including the death of neurons. These neurodegenerative processes can result in numerous neurodegenerative disorders such as Alzheimer's and Parkinson's disease, which alone affect millions of individuals in the US alone (Chinta et al. 2013; Hebert et al. 2013).

Alzheimer's disease (AD) is the most common cause of dementia, accounting for 60–80 % of all cases. The disease is characterized by brain atrophy, extracellular deposition of amyloid (Aβ) peptide, accumulation of phosphorylated tau in neurons, inflammation, and neuronal and synaptic loss (Alzheimer's Association 2011; Bhat et al. 2012). Early clinical symptoms of AD can comprise of mild cognitive impairment (difficulty in remembering of recent life events), depression and apathy. As the disease progresses AD patients may experience impaired judgment, behavioral changes, confusion, and disorientation. In the final stages of the disorder, individuals need assistance with the basic activities of living, lose the ability to communicate, fail to recognize loved ones, and later become bed-bound. Currently, there is no available treatment for AD, which is ultimately fatal (Alzheimer's Association 2011; Naylor et al. 2013). Aging is the main risk factor for AD, but the features of the aging process that predispose the brain to the development of AD are largely unknown. Recent studies from several laboratories have identified senescent cells in the aging brain, where they could contribute to neurodegenerative processes by the secretion of proinflammatory SASP components and/or disrupting cell-to-cell contacts. Senescence in the aging brain most likely occurs in replication-competent glial cells (astrocytes, microglia, and oligodendrocytes), which are essential in maintaining structural, metabolic and trophic support to neurons (Chinta et al. 2014). Astrocytes are the most abundant

cell type in the brain and the primary response mechanism to insults against the central nervous system, such as trauma and neurodegeneration. Dysfunctional astrocytes have been implicated in neuropathology associated with both normal aging and neurodegenerative disorders (Chen and Swanson 2003). Along this line, external stressors hydrogen peroxide and γ-irradiation can induce various features of senescence in human and mouse astrocyte cultures (Bitto et al. 2010; Zou et al. 2012). In addition, aged rat astrocytes displayed high levels of SA-β-Gal activity in conjunction with reduced ability to maintain neuronal survival (Pertusa et al. 2007). Two independent GWAS studies found significant linkage between AD and polymorphic variants in the vicinity of the *CDKN2A-CDKN2B* locus (Hamshere et al. 2007; Züchner et al. 2008). In support of this, the frontal cortex of AD patients harbored a significantly higher burden of $p16^{Ink4a}$-positive astrocytes compared with non-AD adult control individuals of similar ages. Senescent $p16^{Ink4a}$-positive astrocytes were associated with increased levels of MMP-1 and the production of a number of inflammatory cytokines, including IL-6. In vitro, beta-amyloid 1-42 ($A\beta_{1\text{-}42}$)-induced neurotoxicity triggered senescence in human astrocytes, driving the expression of $p16^{Ink4a}$ and SA-β-Gal (Bhat et al. 2012). Summarized, these findings suggest a strong link between the accumulation of senescent $p16^{Ink4a}$-positive astrocytes and an increased risk for AD.

Parkinson's disease (PD) is a neurodegenerative movement disorder characterized by progressive loss of dopaminergic neurons in the substantia nigra, which controls voluntary movement (Thomas and Beal 2011). Another pathological feature of PD is the presence of cytoplasmic protein aggregates, known as Lewy bodies, in the dopaminergic nerve cells. Clinical characteristics of PD can include slowness of movement, tremors while at rest, rigidity, and poor balance, but patients can also display autonomic, cognitive, and psychiatric disturbances. The precise etiology of PD remains poorly understood and effective therapeutic options remain to be established despite intensive research for many years. Although, rare genetically linked cases of PD have been reported, most PD cases are sporadic. Late-onset, idiopathic PD is believed to be the result of the combined effects of aging, genetic risk factors, and the environmental exposure to toxins (Thomas and Beal 2011; Chinta et al. 2013). As with AD, dysfunctional astrocytes have also been associated with PD (Chen and Swanson 2003; Chinta et al. 2013). Another important type of glial cells, microglia, function as resident macrophages of the CNS and provide immune surveillance and facilitate innate immune response to pathogens or injury through a number of pathways, including the secretion of cytokines and stimulation of phagocytosis (Doorn et al. 2012; Chinta et al. 2013). Chronic activation of microglia, which secrete a variety of proinflammatory components and other potentially neurotoxic factors, has been implicated in neuronal death concomitant to neurodegenerative disorders, such as AD and PD (Perry et al. 2010; Cunningham 2013). In addition, functional abnormalities occur in the microglia with aging that impair their response to injury or other stimuli (Streit 2006; Chinta et al. 2013). Furthermore, rat microglia showed telomere shortening with aging both in vitro and in vivo, which is a potent inducer of cellular senescence (Flanary and Streit 2003, 2004). Next to aging, environmental stresses can stimulate senescence in

glial cells, such as repeated lipopolysaccharide administration (Yu et al. 2012). Epidemiological studies and similar work in rodents have implicated exposure to environmental toxins and pesticides, including paraquat and MPTP (1-methyl-4-phenyl-pyridinium ion), in the development of PD neuropathology at least in part due to chronic overactivation of microglia and its release of ROS and inflammatory cytokines (Block et al. 2007; Peng et al. 2009; Litteljohn et al. 2010). Altogether, these results indicate that aging and environmental toxins can provoke the cellular senescence response in the non-neuronal glial cells and this may in turn have detrimental effects on neighboring cells, including neurons, resulting in PD.

16.5.7 Chronic Obstructive Pulmonary Disease

Chronic obstructive pulmonary disease (COPD) is a form of pulmonary emphysema, which is caused by exposure to a noxious agent and is characterized by detrimental changes in small airways and alveolar spaces that result in loss of respiratory capacity. Major risk factors for COPD are tobacco smoke and aging (Shapiro and Ingenito 2005). Both aging and cigarette smoke result in higher expression of senescence markers in lung (alveolar) tissue, which suggests that senescent cells are possibly causally involved in the pathogenesis of COPD (Krishnamurthy et al. 2004; Tsuji et al. 2004). This is supported by the observation that cigarette smoke induced a dose- and time-dependent increase in SA-β-Gal activity, elevated expression of p21 protein, and growth arrest in cultured human alveolar epithelial cells (Tsuji et al. 2004). In addition, immunofluorescence on lung tissue samples from COPD patients showed enhanced numbers of DNA DSB foci, which was accompanied with increased expression of $p16^{Ink4a}$, phosphorylated NF-κB, and IL-6 compared to asymptomatic smokers and non-smokers (Aoshiba et al. 2012). This raises the interesting hypothesis that cigarette smoke and aging causes senescence in alveolar and airway cells and hereby contribute to the destruction of alveolar cells through impaired tissue repair and stimulation of chronic inflammation, which are both typical features of COPD (Aoshiba and Nagai 2009).

16.5.8 Idiopathic Pulmonary Fibrosis

Cellular senescence has been associated with idiopathic pulmonary fibrosis (IPF), a chronic and ultimately fatal respiratory disorder that is characterized by the progressive loss of alveolar cells, interstitial fibrosis and thickening of the wall with fibrous tissue in the bronchial cells resulting in loss of function (Chilosi et al. 2013; Muñoz-Espín and Serrano 2014). The annual incidence of IPF is about 70 per million Americans. Patients usually die within 3–5 years after diagnosis. Although the etiology is unknown, major risk factors for the development of IPF are aging and the inhalation of external noxious agents, similar to COPD

(Raghu et al. 2006). A small percentage of IPF patients have short telomeres, in some cases due to mutations in telomerase or telomerase RNA (Armanios et al. 2007; Alder et al. 2008). Treatment with the DNA-damaging compound bleomycin induces lung fibrosis and is a widely used, yet imperfect model for human IPF. Indeed, a subpopulation of DNA-damaged cells collected from bleomycin treated-lung tissue exhibited elevated levels of Il-6, Tnfα, Mmp-2 and Mmp-9, indicating that senescence is implicated in IPF (Aoshiba et al. 2013). Interestingly, human and mouse lungs displayed SA-β-Gal activity in three cell types, namely alveolar, epithelial and bronchial cells, all three of which are involved in IPF (Aoshiba et al. 2003; Minagawa et al. 2011; Hecker et al. 2014). Expression of $p16^{Ink4a}$ was upregulated in the lung myofibroblasts of aged mice with persistent fibrosis (Hecker et al. 2014). Along this line, IPF lung tissue showed higher TGF-β-induced p21 expression in epithelial cells with mild-to-moderate fibrosis and fibroblastic foci. Senescent human bronchial epithelial cells secrete IL-1β and thus contributing to myofibroblast differentiation and epithelial-mesenchymal aberrancies (Minagawa et al. 2011). Several studies have speculated on the harmful contribution of senescent cells in the development of IPF. For instance, *Caveolin-1* deficient mice are protected from bleomycin-induced pulmonary fibrosis through downregulation of cellular senescence in the epithelial cells (Shivshankar et al. 2012). Similarly, persistent fibrosis in aged mice was associated with the emergence of senescent myofibroblasts. The senescent phenotype seemed at least partly mediated by elevated expression of the ROS-generating enzyme Nox4 (NAPDH oxidase-4) and impaired induction of the Nrf2 (NFE2-related factor 2) antioxidant response. Genetic and pharmacological inhibition of Nox4 in aged mice with established fibrosis diminished the senescent myofibroblast phenotype and resulted in the reversal of persistent fibrosis (Hecker et al. 2014). Finally, rupatadine, an antagonist against platelet-activation factor (PAF), attenuated bleomycin- and silica-induced pulmonary fibrosis in rodents, through protection against the in vivo and in vitro activation of the p53/p21-dependent senescence pathway (Lv et al. 2013).

16.5.9 Ocular Diseases

Several diseases of the eye are associated with increased aging. One of the most common ocular disorders is a cataract, which is characterized by a clouding of the lens in the eye that affects vision. Cataracts are very common in the elderly and it is estimated that by the age of 80 more than half of the Americans either have a cataract or have had cataract surgery (National Eye Institute). The lens is a clear part of the eye that helps focus light, or an image, on the retina in order to receive a sharp image. However, with aging, the proteins that compose the lens lose their organization, form aggregates, and impair normal transmission of light. Fortunately, surgery to remove cataracts and replace the lens is one of the most common operations, and it is very safe and effective (National Eye Institute). Senescence has not been implicated in cataract formation in humans, but the BubR1 progeroid mouse model

has suggested that accelerated senescence of the eye tissue is positively correlated with bilateral cataract formation in mice. In addition, genetic inactivation of $p16^{Ink4a}$ in BubR1 hypomophic mice, to prevent the establishment of senescence in these mice, significantly delayed the onset of cataract formation (Baker et al. 2008). Furthermore, clearance of $p16^{Ink4a}$-positive senescent cells in BubR1 hypomorphic mice starting at early age was also able to attenuate the development of cataracts, further indicating that senescence plays a key role in cataract formation (Baker et al. 2011a).

A number of other ocular disorders increase in incidence with aging, including glaucoma and macular degeneration. Glaucoma is a group of diseases that damage the eye's optic nerve and can result in loss of vision and blindness. Glaucoma is projected to affect almost 80 million people worldwide by the year 2020 (Quigley and Broman 2006). Several studies have demonstrated that eye pressure is a major risk factor for optic nerve damage. Normally, a clear fluid continuously flows in and out a space in the front of the eye, termed the anterior chamber, and nourishes nearby tissues. In open-angle glaucoma, the fluid passes too slowly through the drain, leading to increased pressure within the eye. As the fluid builds up, the pressure rises to a level that can potentially damage the optic nerve and can lead to vision loss and ultimately complete blindness. A fourfold increase in senescence was observed in the outflow tissue pathway in primary open-angle glaucoma patients compared to age-matched controls (Liton et al. 2005). Another study demonstrated that increased senescence in primary human trabecular meshwork cells with passaging was accompanied with increased intrinsic stiffness in these cells, a feature that has been implicated in the etiology of glaucoma (Morgan et al. 2015).

Age-related macular degeneration (AMD) is the leading cause of visual impairment and blindness in older adults. It is estimated that more than two million individuals >50 years of age in the United States have advanced AMD. The macula, a central spot near the center of the retina that is required for the sharp, central vision and allows you to see fine detail, is gradually destroyed, and hereby affects simple everyday activities such as recognition of faces, reading, and driving. There are two forms of AMD: dry and wet. Dry AMD, the more common and untreatable form, is characterized by the accumulation of cellular debris, called drusen, between the retina and the choroid, causing atrophy and scarring to the retina. In wet AMD, the blood vessels behind the retina grow, which can lead to leakage of blood and fluid and cause extensive hemorrhages. Wet AMD can be treated with laser coagulation and medication that stops or reverses the growth of blood vessels (de Jong 2006). Senescent macrophages have been demonstrated to differentiate into a proangiogenic phenotype and promote the development of abnormal blood vessels underneath the retina (Kelly et al. 2007). Similarly, abnormal polarization in older macrophages is caused by programmatic changes that impair the ability of macrophages to effectively efflux intracellular cholesterol and results in higher levels of free cholesterol within senescent macrophages. Elevated intracellular lipid polarizes older (senescent) macrophages to an abnormal, alternatively activated state that promotes pathologic vascular proliferation and AMD. Importantly, restoration

of cholesterol efflux in macrophages improved the ability to inhibit vascular endothelial cell proliferation (Sene et al. 2013).

16.5.10 Other Diseases

Senescence has also been associated with pathology in a number of other human diseases, but this requires additional investigation to elucidate whether senescence contributes to the pathology or whether it serves to restrict the extent of the pathological process. Several senescence markers, including $p16^{Ink4a}$, were elevated in airway epithelial cells derived from patients with cystic fibrosis, a chronic lung disease, compared to healthy control samples (Fischer et al. 2013). Senescence has been associated with human aneurysms in both the brain and the aorta (Fukazawa et al. 2007; Wei et al. 2011). Consistent with this, GWAS have linked the *CDKN2A-CDKN2B* locus with intracranial and aortic aneurysms (Yasuno et al. 2010; Golledge and Kuivaniemi 2013). Osteoarthritis is a complex degenerative joint disease and is the most common cause of chronic disability in the elderly population. Chronic inflammation is believed to be the main underlying cause of osteoarthritis. In support of this, expression of many inflammatory cytokines and various matrix metalloproteinases are upregulated in arthritic joint tissues and chondrocytes, which are all components of the SASP. In addition, accumulation of senescent cells with SA-β-Gal activity and shorter telomeres were observed in osteoarthritic cartilage (Price et al. 2002; Martin et al. 2004; Freund et al. 2010). Other human diseases have also been associated with senescence, including intervertebral disc degeneration and inflammatory bowel disease (Roberts et al. 2006; Le Maitre et al. 2007; Sohn et al. 2012). Finally, the presence of senescent cells in human kidney pre-transplantation biopsies was correlated with a poor outcome in renal transplantation. Moreover, allografts of failed renal transplantation showed elevated expression of p16 (Naesens 2011). Interestingly, mice that received kidney transplants from $INK4a/ARF^{-/-}$ donors had a significantly better survival after life-supporting kidney transplantation and were protected from the development of interstitial fibrosis and tubular atrophy (Braun et al. 2012).

Altogether, the list of human diseases associated with senescence keeps expanding and it is anticipated that senescence will be important for even more pathologies.

16.5.11 Open Questions and Therapeutic Strategies

The use of the INK-ATTAC transgene and genetic inactivation of $p16^{Ink4a}$ in the BubR1 progeroid mouse model have provided proof-of-principle that preventing the formation and/or clearance of senescent cells can attenuate age-associated degenerative pathologies (Baker et al. 2008, 2011a). This, in combination with the

lack of overt detrimental side effects associated with long-term removal of senescent cells in BubR1 hypomorphic mice, implies that senescent cell clearance might be an attractive therapeutic strategy for treatment of age-related disorders or improvement of healthspan. Interestingly, these studies initiated screens for drugs that specific eradication of senescent cells, termed senolytic agents. A recent study implied that desatinib and quercetin have selective senolytic activity but whether this holds true remains to be seen because both compounds modulate a myriad of biological pathways and processes other than senescence (Zhu et al. 2015).

Although senescent cell clearance represents an attractive therapeutic opportunity, there are many unknowns and potential pitfalls along this approach. Our current knowledge about the induction and development of senescent cells over time in both humans and animal models during normal aging and in age-related disorders is still very limited. This is hampered by the fact that like arrested cell growth, many of the other features and (molecular) markers that are used to identify a senescent cell are not unique. In addition, senescent cells are heterogeneous meaning that not all cells express the same genes and comprise the same set of characteristics. For example, it seems likely that there is heterogeneity between the SASP composition in senescent cells that arise during acute senescence and chronic senescence, but also within these two categories. Furthermore, it will be of great importance to assess the power of senescent cell clearance on the lifespan and healthspan of chronologically aged mice, also in the context of recent evidence that senescence is beneficial for a number of biological processes, including embryonic development and tissue repair (van Deursen 2014).

Another potential caveat is whether the use of mouse models is actually recapitulating the physiological circumstances of senescent cell accumulation and removal that takes place in humans. For instance, telomere attrition, one prominent inducer of senescence, is specific to humans and may account for higher baseline levels of senescence in humans. It is possible that similar to telomere attrition, other senescence-inducing factors are also more prevalent in humans compared to mice, which would imply that clearance of senescent cells would have a more profound effect on health in humans. On the other side, removal of a higher percentage of senescent cells in humans may also have unwanted outcomes, such as loss of tissue architecture or tissue dysfunction. Along this line, senescent cell clearance experiments that demonstrated the greatest beneficial effect on health in mice, made use of *INK-ATTAC* transgene that enables the destruction of $p16^{Ink4a}$-positive senescent cells. However, this probably represents only a subpopulation of the total number of senescent cells (van Deursen 2014). The effects of removing $p16^{Ink4a}$-negative senescent cells or all senescent cells remains to be carefully assessed, but an initial study using senolytics showed that removal of all senescent cells might be more complicated than anticipated due to tissue-specific efficacies and suggest that a combination of therapies might be necessary to clear all senescent cells in vivo (Zhu et al. 2015). Conversely, the presence of multiple subtypes of senescent cell also offers opportunities, and targeting each of these subpopulations might either have beneficial or detrimental outcomes on health or more specific biological processes (van Deursen 2014). Current available evidence suggest that

pro-senescent approaches may be preferred depending on the therapeutic context. The promotion of senescence may be beneficial by limiting the fibrotic response during acute tissue damage and hereby promote tissue repair, or in cancer therapy by exploiting its tumor suppressive program (Nardella et al. 2011; Muñoz-Espín and Serrano 2014).

Our understanding of the causes, the cell-type and tissue-specific characteristics of cellular senescence, and its associations with diseases is gradually increasing, which include features that can be exploited in therapeutic strategies. Therapies can be developed to target and eliminate senescent cells directly by the use of small molecules or alternative approach would be to activate or reinforce the immune system against senescent cells (van Deursen 2014; Palmer et al. 2015). A number of studies have reported the removal of senescent cells by the immune system in the context of distinct biological process, such as tissue repair, embryonic development, and tumor growth (Xue et al. 2007; Krizhanovsky et al. 2008; Kang et al. 2011; Iannello et al. 2013; Muñoz-Espín et al. 2013; Storer et al. 2013). However, this approach requires greater understanding of molecular and cellular processes that underlie the clearance of senescent cells by the immune system. Potential limitations of this method are that age-associated immune dysfunction is not the cause of the accumulation of senescent cells with aging, or that chronically senescent cells secrete various SASP components creating a microenvironment that impairs immune clearance (Bhaumik et al. 2009; van Deursen 2014). Furthermore, the current lack of senescent-cell-specific antigens may limit the feasibility of exploiting the adaptive immune system in mounting an effective immune response against senescent cells (van Deursen 2014).

Another option would be to target senescence more indirectly by alleviating local and systemic effects of the SASP. For instance, the anti-diabetic drug metformin inhibits the senescence-associated secretory phenotype by interfering with IKK/NF-κB activation and was able to alleviate diabetes, diabetes complications, and the metabolic syndrome (Moiseeva et al. 2013). It remains to be determined whether inhibition of the SASP is as effective in preventing or delaying age-related disease progression compared to senescent cell clearance. This is complicated by the fact that SASP composition differs on the cellular, tissue, and organismal level and can have various consequences on various processes like inflammation, immunity and tumorigenesis. Targeting of individual SASP factors is a more feasible method and this may prevent paracrine senescence or interfere with senescence viability. In addition, individual SASP components may be responsible for specific pathologies and can be targeted directly in certain disorders. A disadvantage of targeting the SASP would be the potential unwanted side effects on other cellular processes involving those SASP factors (Muñoz-Espín and Serrano 2014; Demaria et al. 2014; Palmer et al. 2015).

In the coming years we will likely see a tremendous expansion of data on the mechanisms, features and functions of in vivo senescence, as well as an increase in our ability to use this knowledge and develop therapies against human age-related disorders and prolong healthy lifespan.

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Part V
Recapitulation and Future Expectations

Chapter 17
Unlike the Stochastic Events That Determine Ageing, Sex Determines Longevity

Leonard Hayflick

Abstract Contrary to popular belief, very little research is done on the fundamental etiology of biological ageing. Most research is done on longevity determinants or on age associated diseases, neither of which will provide insights into the fundamental cause of ageing. Although my research did not intend to answer questions in biogerontology the accidental discoveries that we made did. The phenomenological finding we made in 1961 that normal human cells have a finite replicative capacity torpedoed a dogma held since the invention of cell culture technology in 1907. The belief since then that cultured cells were immortal, mislead researchers to believe that ageing was caused by extracellular phenomena. Our findings focused attention on intracellular events as the origin of ageing. Twenty years later the discovery of telomere attrition and the enzyme telomerase explained the molecular basis for our findings and in 2009 their discoverers were awarded a Nobel Prize in Medicine or Physiology. The most likely cause of ageing in both animate and inanimate objects is based on the 2nd Law of Thermodynamics which underlies all other theories of ageing. For decades the failure to define key words and fundamental concepts in the field of biogerontology has thwarted progress in understanding the basic cause of ageing and this failure shows little sign of change.

Keywords Ageing • Longevity determinants • Age associated diseases • 2nd law of thermodynamics • Etiology of ageing • Intellectual property rights • Telomeres • Telomerase

17.1 Introduction

As in past centuries, most research on ageing today is predominantly descriptive at all levels of complexity. Studies on causation are a trivial part of what is called research on ageing. Efforts to slow, stop, or reverse the ageing process concerns a

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few biogerontologists but it is mostly the provenance of the huge lunatic fringe that, at least in the United States, has historically bedeviled the field. For centuries this fringe has exploited the ignorance of the general public by offering nostrums and life style changes that claimed, without evidence, to modify human ageing. These efforts are further confounded by the lack of biological markers to indicate the rate of aging. Birthdays are useless.

Even in today's era of scientific enlightenment main stream biogerontologists have collectively endorsed the fact that we know of no intervention that will perturb the fundamental process of ageing (Olshansky et al. 2002). The huge industries that market products to cover up the clinical manifestations of ageing depend on the differences in how much we value young people more than we value old people. These industries are a major economic force in many nations.

It has been argued that there are as many theories of the cause of ageing as there are biogerontologists but this cynical observation is now fading with the increasing understanding that, like all matter, – both animate and inanimate, the etiology can be found at the molecular level or even below. I will return to this notion later.

My experimental results in which we found that normal human cells have a finite capacity to replicate (Hayflick and Moorhead 1961) led us to three general conclusions, one of the most important of which was that the limited replicative capacity of normal human cells might be telling us something about ageing and the determinants of longevity. Second, that there are two classes of cultured cells, – mortal cell strains and immortal cell lines. I realized that having discovered that normal cells are mortal it was now possible for me to observe that only cancer cells were immortal (Hayflick 1965). These properties are also found in vivo where most cancer cells can be shown to be immortal and, of course, our normal cells are mortal. Later others have found senescent cells in vivo where considerable research is now ongoing (Jeyapalan and Sedivy 2008; Muller 2009; van Deursen 2014).

The third conclusion that my work revealed was that normal human cells were exquisitely sensitive to replicate virtually all of the then known human viruses, and were free of contaminating viruses. Consequently, I suggested that they would make an excellent alternative to the use of virus contaminated primary monkey kidney cells that were then used in the manufacture of the Sabin and Salk poliomyelitis vaccines and in other human virus vaccines (Hayflick and Moorhead 1961; Hayflick 1965). This will be discussed below.

17.2 Thanks for the Memories

Other discoveries followed. After a few months of frozen storage I was stunned to discover that, upon thawing, the cells had a memory. When they were thawed months after freezing and cultured they remembered at what doubling level they were frozen and then underwent only that number of doublings remaining from the total of 50 that were possible. Clearly, the cells had a system for counting the number of times that they could divide. One of the normal human fetal cell strains

that I developed, called WI-38, has been stored frozen for 53 years and the memory of the cells is as good today as it was in 1962. This is the longest time that normal human cells have ever been frozen in the living state.

17.3 The Importance of Being Normal

I realized that it was essential to prove that my cell strains were normal in order to make the novel claim that immortal cultured cell lines differed by having abnormal or cancer cell properties. For example, at this time the HeLa cancer cell line had been cultured continuously since 1952 (Gey et al. 1952) and after a decade of continuous culturing could be considered to be immortal. A few other immortal cancer cell lines also existed at this time. All were chromosomally abnormal, replicated when inoculated into laboratory animals, and were abnormal in other ways. The fact that my cultured cells were chromosomally normal, did not replicate in laboratory animals and were normal in all other respects demonstrated the critical insight that cell immortality is a property only of abnormal cancer cells (Hayflick 1965).

My additional finding in 1965 that the normal cells from adults replicated fewer times than those from fetuses seemed to support our earlier suggestion that my discovery might bear directly upon problems of ageing, or more precisely, "senescence" (Hayflick and Moorhead 1961). These observations compelled me to abandon my cancer virus research plans and motivated me to make an excursion into the question of why normal human cells stopped dividing after a specific number of population doublings.

What I thought would be a brief expedition lasted for more than 40 years. I never did return to the cancer virus project.

17.4 How Cultured Normal Human Cells Benefitted Billions

In the 1960s one of the major research areas at the Wistar Institute was the development of human virus vaccines. Many scientists, including the director, were engaged in these studies so I was immersed in knowing about this work because of the usual interactions with colleagues who worked in this area. This resulted in my efforts to determine whether my normal human cell strains would grow human viruses. I found that they grew all of the major human viruses then known (Hayflick and Moorhead 1961).

Of equal importance was my finding that the normal human cell strain WI-38, on which I decided to focus, did not contain any contaminating viruses. This was contrary to the many new and dangerous viruses found in primary monkey kidney cells then used for the manufacture of the widely used Salk and Sabin poliomyelitis vaccines.

WI-38 soon became a standard cell culture in virus diagnostic laboratories worldwide for the detection of viruses from human clinical specimens. We also isolated a new common cold virus using these cells (Tyrrell et al. 1962). This resulted in our suggestion that normal human cells would be a better and safer substrate for human virus vaccine preparation than the then existing and dangerous primary monkey kidney cells (Hayflick and Moorhead 1961). Two years later we reported that a safe and efficacious poliomyelitis vaccine had been produced in these cells (Hayflick et al. 1962).

After a 10-year struggle over objections made by the Division of Biologics Standards (DBS, NIH), now a part of the FDA, WI-38 became the first subcultivated culture and the first normal human cell strain to be used for human virus vaccine production (Hayflick 1989, 2001). Today, more than one billion people have received virus vaccines produced in WI-38 or similar normal human cell strains developed later by others. These include vaccines against poliomyelitis, adenovirus types 4 and 7, rubella, measles, varicella, mumps, cytomegalovirus, hepatitis A and rabies (Fletcher et al. 1998; Olshansky and Hayflick in preparation).

There is no other cell substrate, including the HeLa cell line that has benefited so many people. These benefits occurred without any of the putative dangerous side effects predicted for the use of these cells that had been feared by early detractors from both within and without of the U.S. Control Authority. These baseless fears caused a 10-year delay in the use of WI-38 as a human virus vaccine substrate in the United States until, in 1972, Pfizer Laboratories received U.S. approval for their Sabin poliomyelitis vaccine grown in WI-38. During the preceding decade WI-38 was widely used for vaccine manufacture in many other countries including Yugoslavia, the USSR, Poland, Germany, the UK, and France. In the same decade several people in the U.S. and elsewhere either died or became permanently comatose from working with virus contaminated primary monkey kidney cells. Also, prior to 1972, millions of people received poliomyelitis vaccines grown in primary monkey kidney cells and later found to be contaminated with the S.V.40 virus. This virus can transform normal human cultured cells into cancer cells (Koprowski et al. 1962; Shein and Enders 1962) and has been suspected to be associated with some human cancers (Hayflick 1984, 1989, 2001; Bookchin and Schumacher 2004).

17.5 Is It Ageing?

Our suggestion that we found ageing to have its origins within the cell was revolutionary because the central dogma claimed that all cultured cells were potentially immortal, so that those researchers on ageing, who preceded us, logically concluded that the ultimate cause of ageing did not have an intracellular origin. This was clear to them because if cultured normal human cells are immortal in the absence of the body's normal control mechanisms, then ageing could not be the result of intracellular events. It was for this reason that the focus of attention on

what little fundamental work was done in biogerontology during the 60 years prior to our work, was directed to extra-cellular causes of age changes like radiation, changes in the extra-cellular matrix, stress, and many other putative non intracellular causes.

Of the tens of thousands of papers published in this field in the last 42 years (which I subsequently named "cyto-gerontology", none have disproved our suggestion. In fact, most independent studies made during these years have added significant weight to our suggestion that the cessation of normal cell replication is telling us something about one or both aspects of the finitude of life. That is, ageing and longevity determination, each of which will be discussed subsequently.

The dogma that we thought we had overturned was so well entrenched that our manuscript was rejected in 1960 by *The Journal of Experimental Medicine* chosen because it had previously published most of the work by Alexis Carrel who reported that his chick cell culture grew continuously for 40 years until it was voluntarily terminated. We showed that his conclusion was erroneous (Hayflick and Moorhead 1961). In later years this journal published several articles by authors who had worked with cultured human cells but did not realize that their cells were mortal (Marcus et al. 1956).

The letter of rejection from *The Journal of Experimental Medicine* read, in part, "The inference that death of the cells . . . is due to 'senescence at the cellular level' seems notably rash. The largest fact to have come out from tissue culture in the last 50 years is that cells inherently capable of multiplying will do so indefinitely if supplied with the right milieu in vitro." The letter was signed by Peyton Rous, discoverer of the Rous sarcoma virus, the use of trypsin in cell culture, and soon to be awarded a Nobel Prize in Medicine or Physiology.

Paul Moorhead and I were crushed because we both thought that our 3 years of work was a significant contribution. The paper was then sent to Experimental Cell Research and within 2 months it was accepted for publication without change (Hayflick and Moorhead 1961). The paper has been cited more than 6,000 times (Hayflick 1978; Garfield 1980) and was one of the 200 most cited papers in the world for the 21-year period from 1961 to 1982 when the total number of citations reached 1,560 (Garfield 1984). Its sister paper, published in 1965, has been cited more than 4,000 times (Hayflick 1990). Only 0.4 % of all scientific papers receive more than 100 citations (Pendlebury 1999, Institute for scientific information, personal communication). Today, citations to the articles number above 10,000 (www.researchGate.net).

Despite the mounting citations to our publications and confirmation by others of our work, overturning the dogma took a decade or more. Full acceptance of my phenomenological finding did not occur until the molecular mechanism was discovered 20 years later.

It is a well-known phenomenon in science that the length of time necessary to accept a new discovery is directly proportional to how much that discovery is thought to defy received knowledge.

17.6 The Telomere Replicometer

Two observations led me to the notion that normal, mortal, human cells must contain a replication counting mechanism. First, was the reproducibility of our finding that normal human fibroblasts from different embryonic donors underwent a finite number of population doublings that spanned a narrow range between 40 and 60. Second, cells frozen at any population doubling level from 1 to 50 retained "memory" of that level until reconstitution so that the total number of population doublings traversed, both before and after freezing, totaled 50 (Hayflick and Moorhead 1961; Hayflick 1965).

The replication counting mechanism should not be called a clock or chronometer because time is not measured but cell doublings, or more precisely DNA replications, are. I named the unknown mechanism that I predicted a "replicometer" because it counts replication events.

In 1975 we made the first effort to determine the location of the putative replication counter. By employing enucleation and fusion techniques in which nuclei removed from old and young cultured cells were fused to opposite aged enucleated cytoplasts, we concluded that the counter was located in the nucleus (Wright and Hayflick 1975; Muggleton-Harris and Hayflick 1976).

17.7 The Coming of Age of Ageing Research

It is only within the past 40 years or so that the field of research on the biology of ageing has emerged as a legitimate area for scientific inquiry. Today, the science of biogerontology flourishes but it still has far to go before it escapes completely from what is analogous to alchemy in the middle-ages. The popular belief that the goal of most biogerontologists is to stop or reverse the ageing process or to make us all immortal is equivalent to the belief that the goal of modern chemistry is to turn base metals into gold.

Adding to the stigmatization of the field is the belief by a gullible public, at least in the United States, that some nostrum or life style will soon be found to slow or stop the ageing process in humans. The fact is that we know of no intervention that has been proven to alter the ageing process in humans nor is one likely to be found (Hayflick 1996, 2000; Olshansky et al. 2002). The goal of biogerontology research is not different from the goals of research in, for example, embryology and childhood development. That is, to understand the processes with no intention of reversing, slowing or stopping them. Curiosity itself is a legitimate goal in scientific research.

17.8 Old Things Considered

There are several impediments to our understanding of the ageing process, the most important of which is the belief that our present understanding of fundamental biological mechanisms is sufficient to understand its cause and to interfere in the

process. This same belief, which has been held during many previous decades, also did not result in an understanding. Research on the etiology of ageing in the nineteenth century or later, and prior to our understanding of the structure of complex biomolecules and pathways failed, although researchers had the chutzpah to believe that the state of knowledge at those times was sufficient to succeed. Those who pursue the etiology of ageing today are likely to fall into the same trap again because we have no biomarkers of ageing and still fail to understand that many major discoveries bearing on the biology of ageing have yet to be made. Worse, is the present lack of support to conduct research on the fundamental biology of ageing.

A second impediment to understanding the ageing process is the failure to distinguish ageing from the determinants of longevity and from age-associated diseases.

Finally, the terminology used in this field has resulted in the misdirection of most of the funds that could be available for research on the etiology of ageing into other fields. This aspect of research on biological ageing will be discussed later.

There are four aspects of the finitude of life, – ageing, longevity determinants, age-associated diseases and death. All but the latter will be discussed here.

17.9 Ageing and the Determinants of Longevity

Biological ageing can be defined at many levels of organization from population ageing to ageing at the molecular level or below.

Age changes can occur in only two fundamental ways, – either by a purposeful program driven by genes or by stochastic or random events.

It is a cornerstone of modern biology that a purposeful genetic program drives all biological processes that occur from conception to reproductive maturation. But, once reproductive maturation is reached, thought is divided in respect to whether the ageing process results from a continuation of the genetic program or whether it occurs by the accumulation of dysfunctional molecules. Yet, there is no direct evidence that genes drive age changes – a claim made because of the failure to distinguish age changes from longevity determinants.

The ageing phenotype is expressed after reproductive maturation and is driven by random events in animals that reach a fixed size in adulthood. No gene that codes for a universal biomarker of ageing has been found. Analogously, inanimate objects also require no instructions to age. Evidence for the belief that ageing is a random or stochastic process is that, (1) everything in the universe changes or ages in space-time without being driven by a purposeful program, (2) there is no direct evidence that age changes are governed by a genetic program and, (3) there is a huge body of knowledge indicating that all age changes are characterized by the expression and accumulation of dysfunctional molecules.

The common denominator that underlies all causes of ageing is change in molecular structure and hence, in function. It is caused by the intrinsic thermo-

dynamic instability of complex biomolecules, or the manifestations of the Second Law of Thermodynamics. Entropy increase in non-equilibrium systems was, until recently, dismissed as a cause of biological ageing because biological systems are open systems. The recent re-interpretation of the Second Law applies to biological systems and states that:

> Entropy is the tendency for concentrated energy to disperse when unhindered regardless of whether the system is open or closed. The 'hindrance' is the relative strength of chemical bonds. (See www.secondlawcom/six.html and www.entropysimple.com)

The prevention of chemical bond breakage until reproductive maturation is the *sine qua non* for the maintenance of life and species continuity. This is the role of longevity determinants or maintenance, repair and synthesis systems. All ultimately suffer the same effects of the Second Law as do their substrate molecules.

Thus, biological ageing can be defined as the random, systemic accumulation of dysfunctional molecules that exceeds repair or replacement capacity. This occurs throughout life, but in youth the balance favors the bodies' enormous capacity for repair, turnover and synthesis, – otherwise individuals would not live long enough to reproduce and the species would vanish. After reproductive maturation the balance shifts to slowly favor the accumulation of irreparable, dysfunctional molecules, including those that compose the maintenance and disposal systems themselves. Natural selection causes the balance to shift because in animals that reach a fixed size in adulthood favoring life to be extended beyond reproductive maturation is unnecessary for species survival. The repair shops also age. Then the myriad decrements that produce the ageing phenotype become slowly revealed. Most significantly, the accumulation of dysfunctional molecules increases vulnerability to age-associated diseases.

Blueprints contain no information to instruct a car, or other inanimate object, how to age. Yet, in the absence of blueprints, molecules composing these objects also obey the 2nd Law as their molecules dissipate energy (increasing entropy) and incur structural and functional losses over lengths of time that vary from picoseconds to light years. Analogously, the genome also does not contain instructions that determine age changes because, like the car and everything else in the universe, instructions are unnecessary to drive a spontaneous process.

17.10 Genes Do Not Govern Ageing

Because ageing is not a programmed process it is not governed directly by genes. On the contrary, ageing is a stochastic process. The many studies in recent years where invertebrates have been used have led to the view that genes are involved in ageing. Yet, none of these experiments has shown a reversal or arrest of the inexorable expression of molecular dysfunction that is the hallmark of ageing. These studies are more accurately interpreted to have increased our understanding of longevity determination (discussed below). Most of the experimental results

using invertebrates, and allegedly thought to modify age changes, alter physiological capacity well before the ageing process begins. Furthermore, most experiments with invertebrates use an "all-cause mortality" end point that is falsely interpreted to be exclusively caused by ageing. It erroneously excludes possible causes of death attributable to disease, pathology, predation, toxicity, accidents, etc. Finally, because there are no generally accepted biomarkers for ageing in these, or any other animals, the effects of experimental manipulations on their fundamental ageing process can only be speculated upon.

Another argument against the direct role of genes in programming the ageing process is that animals do not age at the same rate nor are the patterns of age changes identical. This results in the great variations found in the location and timing of the acquisition of pathology and the subsequent differences in the chronological age of death. When these random events, which characterize the ageing process, are compared with the orderly, virtually lock-step, changes that occur during genetically driven embryogenesis and development, that orderliness and precision stands out in stark contrast to the quantitative and qualitative disorder of age changes. The variability in the manifestations of ageing differs greatly from animal to animal but the variability in normal developmental changes from animal to animal differs trivially. Humans from conception to adulthood are virtually identical in respect to the stages and timing of biological development but from about age 20 on, age changes make humans much more heterogeneous.

That heterogeneity is often mollified by the reduction in pathological processes and causes of death that have occurred in recent decades in developed countries. This phenomenon is typical of the causes of loss in function that occurs in inanimate objects like automobiles. There is a striking similarity in the loss of function in identical systems in the same make, model and year of automobiles. Yet to argue that these are programmed similarities is indefensible. A weakest link always occurs in inanimate mechanical or electronic objects.

17.11 The Determinants of Longevity

The second aspect of the finitude of life is longevity determination – a completely different process from ageing.

Longevity is determined by the length of time that the synthesis, turnover, disposal and repair processes can maintain the biologically active state of molecules. These processes are governed by the genome.

Unlike the stochastic process that characterizes ageing, longevity determination is not a random process. It is governed by the enormous excess of physiological reserve produced prior to, and during, the time of reproductive maturation and evolved through natural selection to better guarantee survival to that age.

Life does not end immediately after reproductive maturation in most species because it does not benefit species survival. Also, the energy necessary to produce a mechanism that would cause death immediately after reproductive maturation in

higher animals is too costly. Exceptions are semelparous "big bang animals" like the Pacific salmon, and some insects. But, it is rare in vertebrates other than some bony fish.

Thus, the determination of longevity is incidental to the main function of the genome, which is to reach reproductive maturity.

After reproductive success feral animals soon die as the result of predation, disease or accidents. But humans have learned how to substantially eliminate or slow many of these causes of death allowing us and the animals we choose to protect to experience increased life expectancy. Ageing in its extreme manifestations is unique to humans and our protected animals.

Longevity determination is an entirely different process from ageing and is independent of it. One might think of longevity determination as the energy state of molecules before they incur age changes. This energy state is part of the answer to the question: "Why do we live as long as we do?"

One might think of ageing as the state of molecules after they have incurred irreparable damage that leads to the ageing phenotype. This condition answers the question: "Why do things eventually change or go wrong?"

Ageing is a catabolic (destructive) process that is chance driven. Longevity determination is an anabolic (constructive) process that, indirectly, is genome driven. They are opposing forces.

The genome directs events until reproductive maturation after which the ageing process dominates. Thus, the genome only indirectly determines potential longevity by governing the levels of excess physiological capacity, repair, synthesis, waste disposal and turnover. No specific genes determine longevity but, collectively, they all govern aspects of biological processes that increase the likelihood of survival to reproductive maturity. The quantitative variation in physiological capacity, repair, and turnover, accounts for the differences in longevity both within and between species.

Because longevity is indirectly governed by the genome it is sexually determined. Because ageing is a stochastic process it is not.

17.12 Age-Associated Diseases

Absent any discussion of death, the third and last of the four aspects of the finitude of life to be discussed are age-associated diseases. The distinction between the ageing process and age-associated disease is critical and it is rooted in several practical observations:

Unlike any disease, age changes: (1) occur in every animal that reaches a fixed size in adulthood; (2) cross virtually all species barriers; (3) occur in all members of a species only after the age of reproductive maturation; (4) occur in all animals protected by humans even when that species probably has not experienced ageing for thousands or even millions of years; (5) occur in most animate and all inanimate objects; and (6) have the same universal molecular etiology, that is, thermodynamic instability or increase in entropy.

There is no disease or pathology that has all of these properties.

Age-associated disease is the research and care provenance of geriatric medicine. The fundamental biology of ageing is the research provenance of biogerontology.

17.13 The Alzheimerization of Ageing

Since the establishment of the National Institute on Ageing in the USA in 1974, support for research on Alzheimer's disease has increased dramatically. One consequence of this has been the phenomenon in which, at almost every meeting or conference on ageing that has been held in the last 40 years, a session on Alzheimer's disease has been virtually mandatory. The phenomenon has been called "The Alzheimerization of Ageing" (Adelman 1998).

The resolution of Alzheimer's disease as a cause of death would add about 20 months onto human life expectancy (Arias et al. 2013). In the last 5 years $200,000,000 has been added to the Alzheimer's disease research budget. The budget for research on the biology of ageing has remained static at orders of magnitude less.

It is remarkable that this pathology has become so inseparable from research on ageing that its importance has eclipsed that of the major causes of death, – cardiovascular disease, stroke and cancer. These rarely appear as a separate part of conferences on the biology of ageing. Yet, these causes of death require as much attention, or more, from care givers, physicians and from researchers as does Alzheimer's disease.

17.14 What Would Life Expectancy Be if All Causes of Death Were Resolved?

In 2001 life expectancy at birth was 77 years (Arias et al. 2013). If cardiovascular diseases would be resolved life expectancy would increase by about 5.48 years, stroke 0.65 years and cancer 3.2 years. If all of the causes of death legally allowed on death certificates (ICD-10 or International Classification of Disease Version 10) were resolved, average human life expectancy could not increase more than about 12 years. Or, age 89 years would be the maximum life expectancy for humans if all of the present causes of death would be resolved (Olshansky et al. 1990; Hayflick 2003; Arias et al. 2013). Curiously, and contrary to what frequently appears in the media, it is illegal for anyone to die from either "natural causes" or "old age" in the United States or in other developed countries who have adopted the ICD-10.

For age associated diseases the fundamental question is: "Why are old cells or those near the end of a lineage more vulnerable to pathology than are young cells?" Regrettably, little research is, or has been, done in an effort to answer this important question.

17.15 Discovery of Telomere Attrition and the Enzyme Telomerase

In 1989, Calvin Harley, who had worked for several years with my system of senescent human cells, had a fortuitous discussion with Carol Greider that resulted in a collaborative experiment in which it was found that chromosome ends (telomeres) decreased in length at each round of normal human cell division (Harley et al 1990).

The remaining critical question was: "How does that class of cells that we identified as immortal avoid telomere shortening that, if it occurs, would lead to their loss of replicative capacity?"

In 1985 Greider and Blackburn discovered the enzyme "telomerase" that, in cancer cells, adds the missing molecules onto the telomeres at each division. Thus, the telomeres of cancer cells do not shorten to some critical length and therefore provide them with the property of immortality that I conjectured they had (Hayflick 1965). The Nobel Prize in Medicine or Physiology was awarded to Blackburn, Greider and Szostak in 2009 for their discovery that telomeres protected chromosome ends and for discovering the existence of telomerase (Gilson and Ségal-Bendirdjian 2010). This had the effect of eliminating all of the doubt about my phenomenological discoveries which were now explicable by their findings at the molecular level. It also provided enormous interest in my interpretation in 1961 that these findings might be associated with ageing when the Nobel Committee announcements associated the prize with my original suggestion that the phenomenon illuminated our knowledge of the biology of ageing.

17.16 Telomeres as Longevity Determinators

The suggestion that telomere attrition in cultured normal human cells is associated with biological ageing was a conclusion quickly reached by many. However, that conclusion is spurious because biological ageing as described above is a stochastic process that is not governed by the genome. The attrition of telomeres and the subsequent downstream chromosomal events that trigger the cessation of cell division is more likely to be associated with longevity determination than it is with the stochastic process of ageing.

17.17 Biologists Have Intellectual Property Rights

Other interesting events in the history of my discovery of the replicative limit of normal cells includes the confiscation of WI-38 from my Stanford University laboratory by NIH, FDA and DHEW zealots in 1975 (Hayflick 1984, 1990, 1998)

who believed that the government was the sole owner of the cells. Their belief was held despite the fact that the government did not support my discovery of the cell strains or of my discovery of the limited replicative capacity of cultured normal human cells. Overhead funds supported my laboratory which was a central supplier of cultures to other institute members. I used surplus materials from the cultures I prepared for others that would have ordinarily been discarded. I sued the NIH, FDA and DHEW in which I maintained that there were three other stakeholders that included the institute where the work was done, the estate of the donor and the scientists who gave value to the cells.

During the 6 years of litigation several significant events occurred that forced the government to ask me for an out-of-court settlement. First, amicus briefs were offered by the nascent biotechnology industry founded on the use of materials directly supported by government grants made to academic laboratories. Second, the Supreme Court ruled that living cells could be patented. Third, a presidential executive order declared that federally supported research resulting in cells or microorganisms with new features could be commercially exploited. Finally, the passage of the Bayh-Dole Act made the executive order law (35 USC 200-212). Critically, the settlement of my lawsuit established for the first time that biologists have intellectual property rights.

Eighty three scientists published a letter in Science in support of my position (Hayflick 1978, 1998; Strehler et al. 1975; Wadman 2013).

The history of WI-38 also includes the actions of the anti-choice people who picketed Cape Kennedy in an unsuccessful effort to thwart NASA's launch of Skylab 2 which contained an elaborate experiment designed to determine whether the normal WI-38 human chromosomes would be affected by zero G. WI-38 was chosen because it was the most well characterized human cell strain in the world. Objectors to orbiting WI-38 believed that it was wrong to undertake an experiment on cells obtained from an aborted fetus. This objection was held despite the fact that the abortion was a choice made by the mother and the fact that it would have otherwise been incinerated. However, after the launch director spoke with me and learned that the legal voluntary abortion occurred in Sweden, the protesters ultimately dispersed and the launch was successful (Montgomery et al. 1978).

17.18 The Present Status of Research on Ageing

Fifty or 60 years ago a review of the status of research on the etiology of biological ageing could have been done in a few pages because most work was descriptive. I do not intend to review the present state of what is commonly called "Research on Ageing" because the misleading use of this undefined and vague term has seriously compromised the field for the last half century.

Research on ageing is rarely defined to mean the study of the biology of the fundamental cause of ageing. Many would assume that the rubric "Research on Ageing" would be defined this way. It is not. And, it has resulted in a "One Billion Dollar Misunderstanding" (Hayflick 2003).

Research on ageing does not apply to geriatric medicine because that is the provenance of research on, or treatment of, age-associated pathologies and the decrements of old age in humans.

One example of the abuse of the term is how the word "ageing" is used in the titles of institutes, centers, departments and similar organizations. Rarely do any members of these organizations conduct research on the etiology of ageing. Research is either focused on the geriatric aspects of ageing or descriptive events that occur during the ageing process. When questioned, most organization leaders will reply that appeals for funding research on the fundamental biology of ageing rarely produces results. But, an appeal for the support of research on age-associated diseases is significantly more productive because most decision makers have had direct, or indirect, experience with at least one of these pathologies. The importance of the highly probable link between the biology of old cells, which increases vulnerability to all of these pathologies, goes unappreciated.

The rubric "Research on Ageing" could involve research on virtually any aspect of human, animal, microbial or plant life. It could also reasonably include the ageing of inanimate objects. These enormous areas would also be increased if we incorporate research from the molecular level up to the whole animal, object, and groups of each.

This universal embrace is one of the most serious past and present problems in the field called "Research on Ageing." It is also one of the least understood or appreciated problems. Yet, the impact that this language failure has had on the field, and will continue to have, is extraordinary.

17.19 The Tyranny of Words

Decision makers who direct and or fund "Research on Ageing" usually have little understanding of the imprecision of terms used in the field. In respect to biology, the term usually means research on longevity determinants or age-associated diseases. It rarely means research on understanding the cause of biological ageing which should be its only meaning. Biologists have attempted to distinguish themselves from geriatricians and non-biologists in the field of "Research on Ageing" by characterizing themselves as biogerontologist or cyto-gerontologists. But, these labels are not universally used. Calling those who do research on the non-biological aspects of ageing as "Researchers on Ageing" is misleading because it includes everything from economics, sociology, psychology and architecture to geriatric medicine and anything old.

Research funds that may be appropriated under the rubric "Research on Ageing" are largely expended for research on longevity determinants because of the failure to understand that increasing the longevity of animals by manipulating constructive, synthesizing or anabolic processes will tell us little about the dysfunctional molecules that characterize the destructive or catabolic process of ageing.

Further evidence for this misunderstanding is that the availability of funds for research on age-associated diseases is several orders of magnitude greater than what is available for research on the fundamental biology of ageing. What is far more meaningful is that most decision makers believe that the resolution of age-associated diseases will tell us something about the fundamental biology of ageing. It will not.

This spurious belief is comparable to the notion that resolving childhood diseases will enlighten us about the fundamental biology of embryogenesis or childhood development. The resolution of childhood diseases, like poliomyelitis, Wilms' tumors and iron deficiency anemia, added nothing to our fund of knowledge about embryogenesis or the biology of human development. Likewise, the resolution of age-associated diseases has not in the past, nor will it in the future, add to our understanding of the fundamental biology of ageing. A century ago, the leading cause of death in old age was pneumonia, often called "the old man's friend" (with its sexist overtones). Pneumonia is no longer one of the leading causes of death in old age but its resolution did not advance our knowledge of the biology of ageing. Nor will the resolution of any other age-associated cause of death or pathology. If the goal of research on ageing is to understand the fundamental process at the molecular level little, if any, progress has been made in the last 50 years.

The irony of these observations is that the common mantra uttered and published by most geriatricians, and also by some biogerontologists, is that "The greatest risk factor for cancer, Alzheimer's disease, cardiovascular disease, or stroke is ageing."

It does not take a great leap of intellect to conclude: "Then why are we not doing research on the fundamental biology of ageing."

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Index

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