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BLOOD SAMPLE PROCESSING FOR THE STUDY OF AGING, AND CHARACTERIZATION OF CASPASE mRNA EXPRESSION IN PERIPHERAL BLOOD MONONUCLEAR CELLS

by

Chantale Lacelle

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Department of Anatomy and Cell Biology

McGill University

Montréal, Canada

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ABSTRACT

By the year 2026, it is projected that one fifth of the Canadian population will be aged 65 and over. However, despite the growth of our elderly population and an ever-increasing average human lifespan, many elderly still spend several years of their life incapacitated by one or more age-associated diseases such as arthritis, cancer, diabetes, cardiovascular diseases or neurodegenerative disorders, which significantly decrease their ability to remain self-sufficient. To enable our elderly population (and eventually ourselves) to maintain good health until our death, it becomes necessary to better define the normal aging process, document the etiology of age-associated diseases, and identify the factors contributing to successful aging as defined by healthy aging.

Centenarian population studies are one of several approaches currently used to study the aging process and characterize successful aging. However, for any population study in aging research, the amount and type of biological material obtained from each individual within the population often remains an experimental limiting factor, leaving results to be concluded from fragmentary data generated from only one or a few types of biological material. Thus I have described a methodology permitting the simultaneous generation of RNA, DNA, protein, and plasma samples, as well as fixed peripheral blood mononuclear cells (PBMC) and frozen blood aliquots, from a single 10- to 30-ml sample of peripheral blood obtained from donors of any age, and showed that although extremely old individuals are somewhat anemic, it is possible to obtain enough biological material from their blood to conduct aging studies.

However, strict limitations on the amount of available biological material does not permit thorough investigation of the aging process, and impedes our ability to optimize procedures, perform functional studies, and subsequently generate large amounts of data. Thus I investigated the possibility of immortalizing B-lymphocytes from extremely old individuals, using the Epstein-Barr virus (EBV), and found that although extremely old individuals (90+ years) possess low levels of circulating B-lymphocytes, it is possible to immortalize B cells present in less than one milliliter of their blood using EBV. This permits the development of functional studies in culture conditions, as well as the regeneration of biological material extracted from important and irreplaceable samples.

Using biological material obtained from blood samples of individuals of all ages by the method for blood sample processing I have described, I studied the mRNA expression of cell death (specifically caspase) genes in nonagenarians and centenarians, successful models of aging who have survived or avoided age-associated diseases, as well as in their younger counterparts, to determine whether apoptotic genes may be part of the genetic determinants of longevity. I found that a population of extremely old individuals (90+) shows a unique pattern of caspase mRNA expression, characterized by high levels of caspase-1 and –3, and low levels of caspase-8, mRNA, while slightly less aged individuals (70-89) are characterized by high levels of caspase-8 mRNA expression. Furthermore, I showed that these changes in caspase mRNA do not appear to result from age-related changes in PBMC composition, such as decreases in CD24. Therefore, I suggest that unique patterns of caspase mRNA result from the regulation of message abundance on a per cell basis, *via* a putative regulation of caspase genes at the transcription or RNA processing level, rather than age-associated changes in immune profiles.

RÉSUMÉ

D'ici l'an 2026, un cinquième de la population canadienne sera âgée de plus de soixante-cinq ans. Parcontre, malgré l'augmentation du nombre de personnes agées au pays ainsi que de l'augmentation continuelle de l'espérance de vie moyenne, l'espérance de vie en bonne santé ne cesse de diminuer. Donc les personnes âgées passent une grande partie de leur vie affligée de maladies associées au vieillissement, tel l'arthrite, le cancer, le diabète, les maladies cardio-vasculaires et neurodégénératives. Ces maladies diminuent la capacité des personnes âgées à demeurer autonome et diminuent grandement leur qualité de vie. Afin de permettre aux personnes âgées de demeurer en bonne santé jusqu'à leur mort et de préserver leur dignité, il devient impératif de définir le processus de vieillissement normal, l'étiologie des maladies associées aux vieillissement, et d'identifier les facteurs permettant de vieillir sainement.

Les études de populations de centennaires sont un des moyens courament utilisés afin d'étudier le vieillissement et de charactériser les facteurs contribuant au vieillissement sain. Parcontre, le type de materiel biologique acquis de chaque individus d'une population est souvent le facteur limitatif des études de population. Ainsi donc, plusieurs résultats d'études de populations sont basés sur des résultats fragmentés originant d'un seul type de material biologique. Dans cette thèse, je decris une méthode permettant l'extraction simultanée de l'ARN, l'ADN, des protéines, du plasma sanguin et la génération de lymphocytes fixés à partir d'un seul échantillon de sang de 10 à 30 ml acquis de gens de tout âges. Je démontre que malgré la présence d'anémie chez les gens d'âge avancé, il est possible d'obtenir suffisament de matériel biologique de ces gens pour effectuer des recherches sur le vieillissement. Parcontre, le peu de matériel disponible ne permet pas une étude approfondie du mecanisme de vieillissement et ne permet pas la mise au point de certaines méthodes de recherche. Ainsi donc, cette limitation ne permet pas l'accomplissement d'études fonctionnelles et la génération de plusieurs résultats. Afin de pallier à cette situation, j'ai étudié la possibilité d'immortalisé les lymphocytes B de gens très ages (90 ans+) avec le virus Epstein-Barr. J'ai trouvé que malgré un bas niveau de lymphocytes B présent dans le sang périphérique, il est possible d'immortaliser les lymphocytes B présents dans moins de un millillitre de sang acquis de

gens très vieux en utilisant le virus Epstein-Barr. Ceci permettra éventuellement d'éffectuer des études fonctionnelles en culture *in vitro* et permettra la régéneration de matériel biologique extrait d'échantillons précieux.

Grace au matériel biologique acquis du sang de gens de tout âges en utilisant la methode que j'ai dévelloppée, j'ai étudié l'expression d'ARNm de gènes impliqués dans la mort cellulaire, les caspases, dans le sangs de centenaires, de nonagaires et de jeunes individus. Les gens trés vieux sont des modèles de vieillissement sain car ils ont survécu ou échappé aux maladies associées au veillissement. Le but de cette étude était de déterminer si les gènes impliqués dans la mort cellulaire font partie des determinants génétiques de la longévité. J'ai trouvé qu'une population composée de gens très vieux (90+) possède une expression unique d'ARNm de caspases caracterizé par un haut niveau d'expression de caspase-1 et –3 et un bas niveau d'expression de caspase-8. Parcontre, une populations des gens âgés (70-89 ans) se caracterize par un haut niveau d'ARNm pour caspases-8. Je démontre que ces changements au niveau de l'ARNm pour les caspases sont indépendant des changements de la composition du sang périphérique s'effectuant avec le vieillissement tel le décroissement du marqueur CD24. Donc, je propose que les profiles d'expressions de caspases unique à certaines sous-population déterminées par l'âge, découllent de la régulation de la transcription des gènes de caspases au niveau cellulaire.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

CHAPTER 1: LITERATURE REVIEW

CHAPTER 2: BLOOD SAMPLE PROCESSING FOR THE SUTDY OF AGE-DEPENDENT GENE EXPRESSION IN PERIPHERAL BLOOD MONONUCLEAR CELLS

CHAPTER 3: IDENTIFICATION OF HIGH CASPASE-3 mRNA EXPRESSION AS A UNIQUE SIGNATURE PROFILE FOR EXTREMELY OLD INDIVIDUALS

CHAPTER 4: ESTABLISHING LYMPHOBLASTOID CELL LINES FROM FROZEN BLOOD OF EXTREMELY OLD INDIVIDUALS

APPENDICES

PREFACE TO THE THESIS

This thesis is presented in the form of original papers. The faculty of Graduate Studies and Research of McGill University has established the following thesis guidelines: "Candidates have the option of including, as part of the thesis, the text of one of more papers submitted, or to be submitted, for publication, or the clearly-duplicated text of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes, and must be bound together as an integral part of the thesis. The thesis must be more than a mere collection of manuscripts. All components must be integrated into a cohesive unit, with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges preceding and following each manuscript are mandatory. The thesis must include a table of contents, an abstract, an introduction, a comprehensive review of the literature, and a conclusion."

Three original published papers are presented in this thesis. Each manuscript included in this thesis appears as published in the literature. Moreover, as strict space limitations exist for every journal, herein you will find the full version of the manuscript as accepted, but before editorial policies required shortening for publication. The shortened versions as published may be found in the appendices at the end of this thesis. As stipulated in the McGill University thesis guidelines, connecting texts have been inserted between chapters to provide logical bridges. This thesis also includes a table of content, a general abstract in both French and English, an introduction, a comprehensive literature review and a general conclusion.

The work described in chapters 2, 3 and 4 have been published as follows:

Chapter 2: Lacelle, C., H. Riol, S. Xu, Y.-J. Tang, Y.-S. Wang, Y.-L. Chuang, H.-S. Lin, M.-C. Chang, J. Liang, and E. Wang. 2002. Blood-sample processing for the study of age-dependent gene expression in peripheral blood mononuclear cells. *J. Gerontol. A Biol. Sci. Med. Sci.* 57A: B1-B3.

Chapter 3: Lacelle, C., S. Xu, and E. Wang. 2002. Identification of high caspase-3 mRNA expression as a unique signature profile for extremely old individuals. *Mech. Ageing Dev.* **123**: 1133-1144

Chapter 4: Lacelle, C., and E. Wang. 2002. Establishing lymphoblastoid cell lines from frozen blood of extremely old individuals. *Mech. Ageing Dev.* **123**: 1415-1418.

The work presented in the above manuscripts and herein is mine in conception, technical realization and presentation. All of the manuscripts have been written and drawn by myself. Co-authors of the manuscript presented in chapter 2 contributed to the recruitment of subjects, on-site (Taiwan) logistical organization, and/or medical supervision during blood collection. Dr. Suying Xu contributed some of her time to help with the extraction of RNA and DNA from the numerous blood samples.

At the end of this thesis (in appendices) you will find the following two papers:

1. Semov, A., N. Semova, C. Lacelle, R. Marcotte, E. Petroulakis, G. Proestou, and E. Wang. 2002. Alterations in TNF- and IL-related gene expression in space-flown WI38 human fibroblasts. *FASEB J.* **8**: 899-901

2. Wang, E., C. Lacelle, S. Xu, X. Zhao, and M. Hou. 2002. Designer microarrays: from soup to nuts. *J. Gerontol. A Biol. Sci. Med. Sci.* **Article in press**.

I worked on these papers along with the work presented in this thesis. You will also find the published versions of chapter 2 and 4. Chapter 3 is presented in the thesis in its published format.

ACKNOWLEDGMENTS

I would like to express my gratitude to my supervisor Dr. Eugenia Wang, for her continuing support during my Ph. D. training. Following her mentorship, I have learned to develop ideas, elaborate and realize projects, write manuscripts, and acquire a general sense of how to lead a laboratory.

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Finally, I would like to thank my parents for their support and moral encouragement throughout my studies, as well as my husband Bruno, for his love and friendship.

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

Work presented in this thesis focuses on blood sample processing for the study of aging and the identification of caspase mRNA profiles in the PBMC of individuals of all ages. Following is a list of contributions to original knowledge presented in this thesis and listed according to chapters.

CHAPTER 2: Blood Sample Processing for the Study of Age-Dependent Gene Expression in Peripheral Blood Mononuclear Cells

I have developed a unified protocol for the processing of blood samples extracted from young, intermediate, old and extreme old individuals. This protocol is unique in that it enables researchers to obtain several different types of biological materials from a single 10 ml sample of blood. Using this newly described protocol to process blood samples from 246 subjects from Chinese origin living in Taiwan, I have shown that this protocol yields similar amounts of RNA, DNA, proteins and fixed PBMC per milliliter of blood regardless of the age of the donor. Using the fixed PBMC obtained from our 246 subjects, I showed that CD24 expression, a B cell marker, decreases in extremely old individuals and thus could possibly be used as a biomarker for extreme elderliness. The protocol presented in this chapter is important as it renders population studies more cost effective and will enable biogerontologists conducting population studies to investigate the aging process more thoroughly.

CHAPTER 3: Identification of High Caspase-3 mRNA Expression as a Unique Signature Profile For Extremely Old Individuals

I studied the expression of caspase mRNA in PBMC of individuals of all age and found that sub-populations composed of extremely old individuals uniquely express a caspase mRNA expression profile characterized by high levels of caspase-1 and –3 and low level of caspase-8 mRNA. Furthermore, I have shown that caspase mRNA expression profiles appear to be independent of immune profiles. As such, changes in

PBMC composition occurring with age in our population such as decrease CD24 positive and naïve T cells, do not appear to be responsible for changes in caspase mRNA expression levels. This is the first study of this magnitude to investigate caspase mRNA expression in human population and attempt to determine whether unique caspase mRNA profile can be used as biomarkers for successful aging.

CHAPTER 4: Establishing Lymphoblastoid Cell Lines from Frozen Blood of Extremely Old Individuals

I showed that it is possible to generate lymphoblastoid cell lines using 1 ml of frozen blood obtained from extremely old individuals despite the fact that they possess much less circulating B lymphocytes than younger individuals. The fact that cell lines can be generated from small amounts of blood from extremely old individuals is important for aging research as biological materials obtained from nonagenarians and centenarians are usually not replenishable. The development of this protocol will also allow to perform functional studies.

LIST OF ABBREVIATIONS

ACE	angiotensin converting enzyme
AICD	activation-induced cell death
bHLH	basic helix-loop-helix
CAD	caspase activated DNAse
CARD	caspase recruitment domain
CDK	cyclin dependent kinase
clk	clock mutant
CSA	cyclosporine A
Daf or daf	dauer formation
DED	death effector domain
DMSO	dymethyl sulfoxide
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
ER	endoplasmic reticulum
ERC	extrachromosomal rDNA circles
FADD	Fas associated death domain
FKHR	forkhead
FSC	forward side scatter
GPI	glycosylphosphatidylinositol
HSA	heat stable antigen (CD24)
HSP	heat shock protein
Ig	immunoglobulin
IGF	insulin growth factor
IL	interleukins
INDY	I'm not dead yet (drosophila gene)
InR	drosophila's insulin-like receptor
LAG	longevity assurance gene
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid

mth	methuselah
NAD	nicotinamide adenine dinucleotide
PARP	poly (ADP-ribose) polymerase
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffured saline
PCR	polymerase chain reaction
PI3K	phosphatidylinositol 3-kinase
Pit1	pituitary-specific transcription factor-1
PKA	protein kinase A
PKB	protein kinase B
RBC	red blood cells
rDNA	ribosomal DNA
RNA	ribonucleic acid
ROS	reactive oxygen specie
SAM	senescence accelerated mouse
SD	standard deviation
SEM	standard error mean
SIR	silent information regulator
SOD	superoxide dismutase
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TRADD	TNF receptor associated death domain
UV	ultraviolet
Zip	leucine zipper protein

INTRODUCTION

By the year 2026, it is predicted that over one fifth (20%) of the Canadian population will be aged 65 and over (Statistics Canada), doubling the size of our current elderly population. While this growth, along with the ever increasing human average lifespan, will put economic pressures on our society and demand the re-organization of several "social institutions", such as the health care system, a more urgent problem is facing us: we are living longer, but not better. Despite a life expectancy at birth now reaching 80.7 years for women and 74.3 years for men, life expectancy without moderate or severe incapacities has regressed in the last few years to 70.5 and 67.7 years respectively (Statistics Canada). It is those last years of life with incapacities that often prevent elderly from remaining self-sufficient and keeping their dignity. Significant advances in aging research are now needed to bridge the gap between life expectancy at birth and life expectancy without incapacities.

During the last century, the average human lifespan has greatly increased as we were able to decrease infant mortality, discover new drugs, develop vaccines, and implement nutritional and hygiene standards. However, while continuously pushing the boundary of average lifespan, we fail to gain "disease-free" years. As such, since the last two decades, aging research has gained in popularity and several centers devoted to the study of aging and age-associated diseases have been created. Studies conducted at these centers and elsewhere, contributed to our understanding of age-associated diseases such as Alzheimer's disease, and helped characterized some of the changes in physiological parameters accompanying the aging process. However, these findings solely represent the tip of the iceberg, as biogerontologists are faced with the challenge of characterizing a biological process lasting a lifespan, for which no clear stages of progression have been identified.

Given the long lifespan of our specie and the impossibility to collect certain tissues from living humans, organisms having shorter lifespan, such as worms and flies, are routinely used to study the aging process. Studies of the aging process in those

organisms have permitted the identification of several genes regulating, as well as, environmental and nutritional factors influencing the lifespan of these species. However, their applicability to human aging remains unknown. The intrinsic problem with human aging is to differentiate between physiological changes occurring as a consequence of aging versus those arising from the development and progression of age-associated diseases. Consequently, it becomes arduous to define the parameters of successful aging. As such, in order to characterize successful human aging and identify the factors involved in healthy aging, few groups of scientist began investigating the biological characteristics of extremely old individuals. Extremely old individuals such as nonagenarians and centenarians, are believed to be successful model of aging as they have reached extreme old age by surviving, escaping or combating age-associated diseases. Interestingly, most nonagenarians and centenarians, unlike octogenarians, are not afflicted by severe form of age-associated diseases. What permits those individuals to develop only mild form of age-associated diseases or altogether avoid them? The answer to this question remains unknown however, it is likely that the answer to this question will allow us to develop better treatment for age-associated diseases and hopefully enable all of us to die with dignity.

Studies conducted on human nonagenarian and centenarian populations have already shown that certain genes are more prevalent in populations composed of extremely old individuals than in those composed of younger subjects. Thus at first glance, it appears that populations of extremely old individuals are genetically different from those composed of younger subjects. As several age-associated diseases result from excessive or inefficient cell death, it is valid to question whether extremely old individuals escape or survive age-associated diseases because they possess better apoptotic programs. For instance, neoplasms can arise from inefficient removal of damage or functionless cells while autoimmune diseases result from the non-deletion of certain immune cells. As such, the aim of my project is to determine whether extreme longevity is associated with a beneficial apoptotic program and whether apoptotic genes may be part of the genetic determinants of longevity.

However, before such questions can be addressed, one fundamental problem must be solved. How can we obtain enough biological material from extremely old individuals

to permit such investigations? The main problem with nonagenarian and centenarian population studies is that they are very expensive and yield minimal amount of data as very little biological material can be obtained from extremely old individuals without significantly compromising their health. As such, the first task of my research project was to develop methods rendering populations studies more effective by maximizing the amount of biological material obtained from extremely old individuals.

In this thesis, I will present the work I have accomplished over the last few years and the contributions I have made to aging studies, particularly to population studies. The results of my work were to characterize a method permitting the simultaneous extraction of RNA, DNA, protein, fixed peripheral blood mononuclear cells, plasma and serum from a single small amount of blood extracted from individuals of any age; demonstrate that although extremely old individuals possess low level of circulating B-cells it is possible to immortalize B-cells from less than one milliliter of their blood using the Epstein Barr virus; and lastly to characterize a unique pattern of caspase mRNA expression in the peripheral blood mononuclear cells and show that this pattern is likely to be independent of age-associated changes in immune profile. Caspases are important genes involved in the control of cell death and hopefully with more research, it will be possible to determine their role in healthy aging.

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION TO THE LITERATURE REVIEW

This section, titled Literature Review, provides the scientific conceptual framework from which emerges the central theme of my research project: Is there a genetic basis for human longevity? More specifically, are caspase gene expressions key determinants of human lifespan? Where applicable, historical perspectives are provided, along with hallmark experiments which have significantly contributed to the advancement of their respective fields of research, as well as the development of this research project. The objective of this chapter is not to provide an exhaustive review of the literature, but rather to provide background information relevant to the work presented in this thesis. The last sections of this review provide information specifically related to the thesis, mainly the immune system and caspases in aging. Each main section of this review is sub-divided, and bears sub-section headings to facilitate the reading of the thesis, as well as to provide a logical flow of information.

1.2 AGING

Webster's dictionary defines aging as "the process of becoming old or mature"[1], while it defines senescent as "to be old"[2]. However, what it fails to define, and what constitutes the cornerstone of aging research, is the process leading to senescence. This failure results in the interchangeable use of the term 'aging' with the term 'senescence', and the usage of both terms to define "the passage of chronological time eventually associated with a generalized impairment of physiological functions, a decreased ability to respond to a wide range of stresses, an increased risk of age-associated disease, and an increased likelihood of death"[3]. Aging is a dynamic process, constituting much more than a definition; thus this section introduces the basic science of biogerontology, the biology of aging.

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1. Webster's II *New college* dictionary. Houghton Mifflin. Boston, New York, 2001. p. 22.
2. Ibid., p. 1005
3. Kirkwood, T.B.L. 1996. Human senescence. *BioEssays.* **18**: 1009.

Theories of aging, models used to study the aging process, results obtained from studies conducted in these models, and general concepts and ideas about the aging process, are described in the following pages.

IMPORTANT DEFINITIONS AND CONCEPTS

Throughout this literature review, several terms such as maximum lifespan and average lifespan are used, and several concepts related to lifespan modulation are discussed. Thus it is important to provide an accurate definition of those terms, as well as general concepts about aging and longevity.

When conducting aging studies, lifespans of subjects, human or animals, are often compared and analyzed in terms of maximum and average lifespan. Each species has a unique characteristic maximum lifespan (Carey and Judge, 2000); that is the maximum length of time from birth until death observed or estimated for the longest-lived individual of that species. Most organisms of a species die before they reach the maximum lifespan; thus very few subjects are extremely long-lived, approaching or attaining maximum lifespan. On the other hand, the average lifespan is computed by taking the average mathematical age at death of all or a subset of individuals randomly chosen in an unbiased population. For instance, while the maximum human lifespan is about 122 years old, the average human lifespan is around 78 years. Likewise, while the maximum lifespan of F-344 rats is around 160 weeks (3 years), their average lifespan is about 110 weeks (2 years) (Sprott and Austad, 1995). However, lifespan can be modulated. As described further in this literature review, some genes can extend lifespan, while others act to reduce it. Thus gaining or losing certain genes functions can affect lifespan.

Benjamin Gompertz, a mathematician of the nineteenth century, was the first to mathematically model the relationship between age and mortality rate (1825). According to his model, the mortality rate increases exponentially as a function of age. However, in recent decades, it was shown that this exponential increase does not apply to extremely old age: in extreme old age, the mortality curve loses its exponential increase, flattens and

forms a plateau (Vaupel *et al.* 1998). Thus extremely old age individuals have a unique mortality rate that does not vary exponentially as a function of their age.

1.3 THEORIES OF AGING

1.3.1 Theories of Aging: An Historical Perspective

Changes accompanying the aging process have long been known to mankind, and documented as far back as the prehistoric age in the form of paintings on cave walls. However, while ancient texts including the Bible recognized the ephemeral nature of our species, and poets and philosophers conversed about "growing old" during the Middle Ages and Renaissance periods, it wasn't until the nineteenth century that gerontological reasoning emerged (attributed to Quetelet, 1842; reviewed in Hendricks and Achenbaum, 1999). Recognizing that elderly patients presented diseases and illnesses generally not prevalent in younger individuals, Jean-Martin Charcot, a French physician, began to address the pathological nature of aging in his 1867 compendium "*Diseases of the Elders and their Chronic Illnesses*". Charcot's compendium, as well as other documents, led to the formulation of rudimental theories of aging (reviewed in Hendricks and Achenbaum, 1999).

Russian Nobel Prize laureate Elie Metchnikoff, a reputable bacteriologist and immunologist of the early 1900's, was one of the first aging theoreticians. Metchnikoff envisioned a theory whereby the "morbid nature of old age" was caused by intestinal microbes. Several scientists following in Metchnikoff's footsteps thereafter proposed their own theories of aging. By the mid-twentieth century theories of aging became subjects of debate around the world, resulting in the rise of leading theories amongst which were the rate of living theory (Pearl, 1928) and the free-radical theory (Harman, 1956). More than 300 theories of aging can now be found in the published literature (Medvedev, 1990). Prevailing theories of aging, as well as those most pertinent to this thesis, are briefly described in the following section.

1.3.2 Prevailing Theories of Aging

Despite the likelihood of providing investigational biases, the importance of theorizing has long been accepted as theories enable the integration of knowledge, provide explanation for experimental data, and allow prediction of the unknown. It is the quest for knowledge that drives the development of theories and thus, given the curiosity of mankind and its need to understand the most fundamental processes, it is not surprising that myriad theories of aging exist; so many questions remain unanswered. Why do we age? How long can we live? *etc*... Experimental data obtained during the last century have partly quenched our thirst for knowledge by gathering information about the aging process and providing insights into the mechanisms of aging. However, despite significant advances in aging research, no unified theory of aging has yet emerged. While several theories have been disproven, others have been strengthened or remain in the realm of possibility.

Current prevailing theories of aging can be broadly classified as being either stochastic or programmed. The stochastic theories of aging attribute the causes of aging to various extrinsic factors causing mutations, resulting in oxidative stress damage or modifying metabolic rate as a consequence of exposure to these external factors. On the other hand, the programmed theories of aging propose that intrinsic factors such as genetic makeup are the causes of aging. Here is an overview of some of the most prevailing theories of aging and those pertaining to this thesis.

RATE OF LIVING THEORY OF AGING

The rate of living theory postulated by Pearl in 1928 (Pearl, 1928) proposes that metabolic rate and lifespan are inversely related. Organisms having high metabolic rate are thus predicted to have shorter lifespans. The observations made by Lyman *et al.* (1981) that hibernating hamsters exhibited increased longevity compared to the non-hibernating control group, as well as other experiments made on houseflies (Balin and Allen, 1986), tend to support this theory. However, the downfall is that given its early origin, this theory fails to propose a mechanism by which metabolic rate affects lifespan,

and experimental data show that not all reductions in metabolic rate have an effect on lifespan. For instance, the longevity of rats remains unchanged following exposure to cold temperatures (Holloszy and Smith, 1986).

FREE RADICAL THEORY OF AGING

Oxidative metabolism entails the production of reactive oxygen metabolites (also known as reactive oxygen species (ROS)), which can cause extensive damage, including DNA and protein damage (Berlett and Stadtman, 1997), inside cells. The free radical theory of aging proposes that cellular damage produced by ROS, such as superoxide, is the cause of aging. Furthermore, the fact that several organisms possess antioxidants such as superoxide dismutase (SOD) and catalase to counteract ROS implies that over-expression of antioxidants can increase lifespan. Experimental data gathered in the fruit fly *Drosophila melanogaster* support this notion, as over-expression of both SOD and catalase result in increased lifespan in these flies (Arking, 2000; Parkes, 1998). Other evidence supporting or challenging this theory will be provided in detail later in the text. As metabolic rate influences the production of ROS, the free radical theory may be perceived as a more specialized rate of living theory.

EVOLUTIONARY THEORIES OF AGING

The evolutionary theories of aging include the pleiotropic theory of aging proposed by Williams (1957), and the disposable soma theory of aging proposed by Kirkwood (1977). These theories attribute the cause of aging to the long-term selection for reproductive ability rather than healthy later life. In other words, in order to avoid extinction, we have evolved a series of genes favoring early life survivorship and the maintenance of good biological functions throughout reproductive age, rather than longevity genes. The disposable soma theory goes further and stipulates that genes favoring reproductivity might become harmful in later life. Fruit flies selected for longevity show decreased early life fertility (Rose and Charlesworth, 1981a, b). Partridge *et al.* also showed that in both male and female flies, reproduction results in

decreased lifespan (Cordt and Partridge, 1996; Partridge *et al.*, 1987). In a very controversial paper Westendrop and Kirkwood (1998), using data obtained from aristocratic families of Europe, identified as supporting evidence for the evolutionary theory of aging in humans a negative correlation between lifespan beyond 60 years and the number of children.

NETWORK THEORY OF AGING

This relatively recent theory proposed by Franceschi (1989) is an integrated theory of aging proposing that a "network" of defense functions controls the aging process. Cells are continuously being exposed to stressors (both internal and external) of biological (bacteria and viruses), chemical (ROS and others), and/or physical (radiation) nature, which can cause cellular and molecular damage. To counteract the effect of these stressors, cells possess a variety of "defense mechanisms" including DNA repair enzymes and antioxidants, while organisms have evolved an immune system. Accordingly, the network theory blames the failure of these defense mechanisms to maintain cellular homeostasis as the cause for aging (Franceschi, 1989; Kirkwood and Franceschi, 1992; Franceschi *et al.*, 2000). This theory is highly integrative, and one of the first to propose a multifactorial cause for aging.

REMODELING THEORY OF AGING

The remodeling theory of aging is one of the only theories conceptualized to account for data obtained from studies in humans. Attempting to reconcile the observation that some immune responses are extremely well conserved in centenarians with the fact that the aging process does not result in the systematic change of all immune parameters, Franceschi *et al.* developed the remodeling theory of aging (Francheschi *et al.*, 1995a; Franceschi and Cossarizza, 1995b). This theory defines immunosenescence as a highly adaptive and dynamic process, and suggests that centenarians and extremely old individuals genetically possess increased adaptability to stressors, particularly immunological stressors.

1.3.3 Conclusion (Theory of Aging)

While several other theories of aging exist, they are largely variations on a theme. For instance, the wear and tear theory of aging proposes that the accumulation of damage incurred as a consequence of daily living causes a decrease in cellular and organismal efficiency resulting in aging, and closely overlaps with the free radical theory of aging. The same is true for the somatic mutation theory, which attributes the causes of aging to the accumulation of somatic mutations and was disproved by Clark and Rubin's (1961) early experiment demonstrating that the lifespan of wasps exposed to ionizing radiation is not significantly different from that of a control group.

While the theories of aging are diverse, ranging from evolution to cellular damage, current research tends to indicate that human aging is multifactorial, and results from the interplay between unique sets of genes and diverging environments (Clare and Luckinbill, 1985). So far, no single theory is able to fully explain the aging process. However, despite the partial correctness of some of these theories, they are often used as working hypotheses, and thus enable the advancement of aging research. Nevertheless, one must realize that proving or disproving theories of aging is, as should be, by far the first concern of biogerontologists attempting to characterize the aging process using different models. In the next section, I will discuss the various models currently in use to study the aging process, and the advances made in aging research resulting from the use of these models.

1.4 MODELS OF AGING

Models of aging have been used for several decades in hope of providing insight into the mechanisms of aging. The use of models is indispensable for three main reasons: First, our species is long-lived; therefore it would be almost impossible to follow the aging process in a single individual (conduct a longitudinal study). Second, it is impossible to collect certain tissues from living humans; and third, it is difficult to discern whether the "phenotype" attributed to aging results from genetic or environmental causes. These reasons, as well as experimental data, have led to the

development of several models for studying the aging process. Cellular senescence, as well as the study of aging in shorter-lived species, will be described in the following sub-sections.

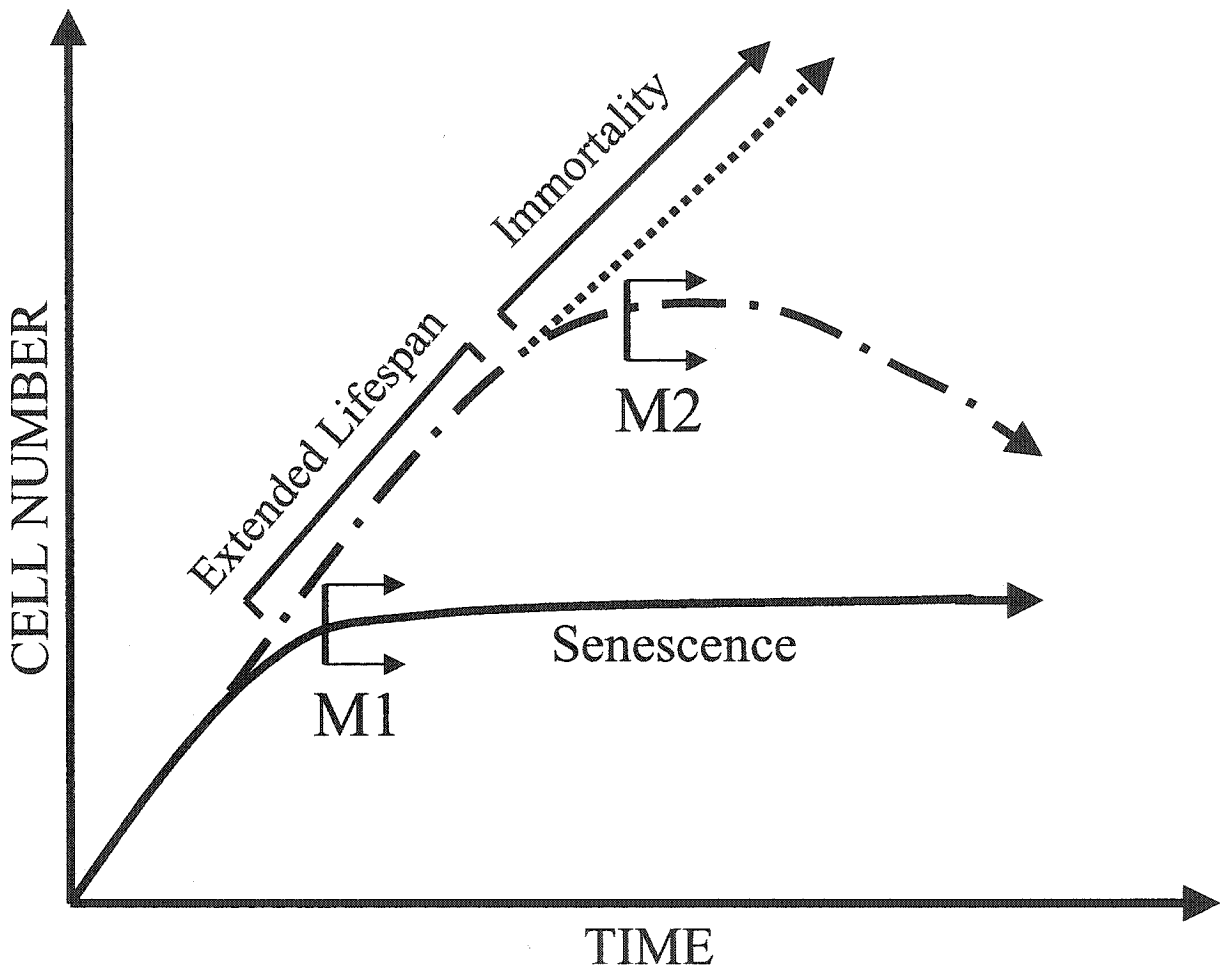
1.4.1 Cellular Senescence

Pioneer work by Hayflick in the early 1960's demonstrated that fibroblast cells in culture could only divide for a finite number of times, after which they stopped proliferating (Hayflick and Moorhead, 1961; Hayflick, 1965). This phenomenon, now termed "replicative" or "cellular" senescence, has since the '60's been shown to occur in most mammalian cell types grown *in vitro,* with the exception of tumor cells and certain stem cells (reviewed in Campisi, 1997). The maximum lifespan of cells is species-dependent, and varies according to the donor's age and cellular genetic background (reviewed in Stanulis-Praeger, 1987). Unlike quiescent cells, growth-arrested cells capable of re-entering the cell cycle if stimulated, senescent cells are irreversibly growth-arrested. Very few human cells acquire an extended lifespan, allowing them to bypass cellular senescence and forego more rounds of cellular replication (Yang *et al.*, 1999). The introduction of the SV40 large T antigen, infections with human papilloma virus E6 or E7, and the suppression of negative cell cycle effectors such as p21, are some of the few conditions enabling human cells to enter extended lifespan (Yang *et al.*, 1999; Hara *et al.*, 1991; Shay *et al.*, 1993; reviewed in Drayton and Peters, 2002). At the end of their extended lifespan, cells reach a crisis point where they can either become immortalized, if proper conditions exist, or reduce their rate of population doubling and slowly die (reviewed in Sedivy, 1998). Unlike senescent cells, cells in crisis cannot remain viable for extended time in culture. To differentiate between extended and normal lifespan, senescence is defined as mortality stage I (MI or M1), while cells having reached the crisis point are in mortality stage II (MII or M2) (Wright, 1989). Only immortalized cells live beyond MII, as they possess infinite replicative capabilities (Figure 1).

FIGURE 1: REPLICATIVE LIFESPAN OF CELLS IN CULTURE

Cultured mammalian cells usually divide for a finite number of times after which they stop proliferating and become long-lived non-replicating senescent cells (Hayflick and Moorhead, 1961; Hayflick, 1965). The moment at which cells enter senescence is define as mortality stage I (M1). However, under certain conditions such as infections with human papilloma virus or the suppression of inhibitors of the cell cycle such as p21, cells can by-pass senescence and as such acquire extended lifespan which permits them to undergo a few more rounds of cellular replication. (Yang *et al.*, 1999; Hara *et al.*, 1991; Shay *et al.*, 1993; reviewed in Drayton and Peters, 2002). At the end of their extended lifespan cells reach a crisis point, mortality stage II (M2). At this point immortal cell which are confer infinite lifespan will continue replicating while others will slow their rate of division and slowly die. Cells in M2 do not become senescent.

REPLICATIVE LIFESPAN OF CELLS IN CULTURE



TRIGGERS OF REPLICATIVE SENESCENCE

Cellular senescence can be achieved by extensively passing cells in culture (Hayflick and Moorhead, 1961), exposing cells to chemicals capable of inducing oxidative damage (Chen *et al.*, 1995; von Zglinicki *et al.*, 1995) or by overexpressing certain oncogenes (Serrano *et al.*, 1997; Zhu *et al.*, 1998). At the molecular level, telomere shortening and the p53 and pRb pathways are the main instigators of cellular senescence known to date (reviewed in Itahana *et al.*, 2001; Sedivy, 1998; Campisi, 2001).

Characterized in the ciliated protozoan *Tetrahymena thermophila* by Blackburn in the '70's, telomeres consist of a repetitive series of nucleic acid located at the end of chromosomes (Blackburn, 1978). In humans, telomeres consist of several TTAGGG repeats (Moyzis *et al.*, 1988) having an initial length of 10-15 kb in germ cells (Allshire *et al.*, 1989). With each cellular division, telomerase-negative cells reduce the length of their telomeres by 50 to 200 bp (Levy *et al.*, 1992; Allsopp *et al.*, 1992). Since telomerase, the enzyme capable of *de novo* telomere synthesis, is absent in most human cells, human cells extensively passaged in culture possess much smaller telomeres than "younger" cells. Upon reaching "critically" short length (estimated at about 4-6 kb in human (Allsopp *et al.*, 1992)), telomeres are believed to induce cellular senescence. The induction of senescence by telomere shortening is proposed by many scientists to act as a tumor suppressor mechanism (reviewed by Campisi, 2001) since critically short or absent telomeres permit chromosomal end-to-end fusion, thereby compromising chromosomal integrity.

While the presence of "critically" short telomere can cause cellular senescence, other methods can push cells having telomeric length far exceeding this "critically" short range, including those expressing hTERT, the catalytic subunit of telomerase (Gorbunova *et al.*, 2002; de Magalhaes *et al.*, 2002) to enter cellular senescence. For instance, increased activation of Ras (Newbold and Overell, 1983; Serrano *et al.*, 1997) or exposure to agents causing oxidative damage can induce "premature" cellular senescence. These stimuli modulate the p53 and pRb pathways which regulate the cell

cycle, thereby driving cells into an irreversible growth arrest stage (senescence). Again it appears that senescence functions to prevent tumorigenesis.

PATHWAYS TO SENESCENCE: A BRIEF OVERVIEW

Cell cycle progression is controlled by the sequential activation of complexes formed between cyclins and cyclin-dependent kinases (CDK) (reviewed in Sherr and Roberts, 1999). Thus entry into the cell cycle requires interaction between CDK4 and 6 with cyclin D, DNA replication requires the activation of CDK2/cyclin E and CDK2/cyclin A complexes, while termination of DNA replication and mitosis requires cyclin B/CDK1 complexes. As senescent cells are irreversibly growth-arrested, control must be exerted over cyclin/CDK complexes to prevent cells from cycling. Two families of CDK inhibitors, INK4 and Cip/Kip, exert some control over cell cycle progression. The INK4 family is composed of four members, $p15^{INK4a}$, $p16^{INK4b}$, $p18^{INK4c}$ and $p19^{ARF}$ ($p14^{ARF}$ when referring to humans), which serve to inhibit CDK4 and 6; the Cip/Kip family contains three members, $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$, which serve to inhibit the function of CDK2/cyclinA and CDK2/cyclin E (reviewed in Sherr and Roberts, 1999; Vidal and Koff, 2001). These inhibitors are players in one of the two main pathways controlling cellular senescence, the p53 and pRb pathways (Figure 2 and 3).

Cellular senescence is often associated with increased p53 DNA binding and transcriptional activity (Atadja *et al.,* 1995; Itahana *et al.,* 2001). p53 is a tumor suppressor gene found to be mutated in several human cancers, as its inactivation can lead to uncontrolled cellular growth. In replicating cells, MDM2 interacts with p53 and targets it for degradation (Haupt *et al.,* 1997; Kubbutat *et al.,* 1997). However, in senescent cells, increased expression of $p14^{ARF}$ promotes cell cycle arrest by sequestering MDM2 away from p53, thereby enabling the transcription of genes necessary for cell cycle arrest (Pomerantz *et al.,* 2000; Zhang *et al.,* 1998). $p21^{Cip1}$, a CDK inhibitor, is one of the genes under p53 transcriptional control (Itahana *et al.,* 2001). It was recently found that the promyelocytic leukemic protein (PML, a tumor suppressor) can also

stimulate the activity of p53 by interacting with CBP and p300, and induce the acetylation of p53 (Pearson *et al.*, 2000) (Figure 2).

Cyclin-dependent kinase inhibitors, such as p21 and p16, can repress the functions of CDK and prevent the phosphorylation of pRb, which is necessary for its function and the maintenance of cellular replication (Alcorta *et al.*, 1996). In the absence of CDK inhibitors, active cyclinD/CDK4 or 6 complexes are highly efficient at phosphorylating pRb, which induces the expression of genes involved in cell growth by favoring the transcription of genes under the control of the E2F family of transcription factors, including cyclins E and A (reviewed in Kaelin, 1999). In senescent cells, E2F cannot engage in transcriptional activity as it interacts with hypophosphorylated pRb (reviewed in Harbour *et al.*, 2000) (Figure 3).

Several pathways are involved in the control of cellular senescence; however, the pRb and p53 pathways are the best characterized among them.

SENESCENT PHENOTYPE

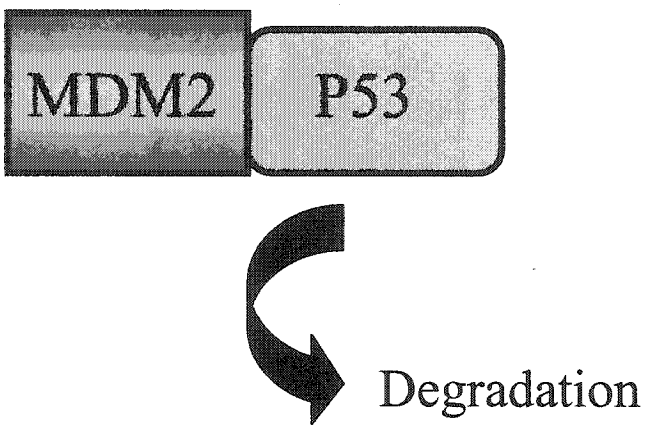
The process of cellular senescence causes cells to acquire distinctive characteristics. Senescent cells are irreversibly growth-arrested, exhibit morphological changes including flattening, become resistant to apoptosis (Wang, 1995), display different surface antigens, and stain positively for senescence-associated β-galactosidase (Dimri *et al.*, 1995). Furthermore, the gene expression profile of senescent cells is drastically different from that of younger cells. While the levels of expression of genes acting as cell cycle regulators are modified in senescent cells to permit the maintenance of the growth arrest stage, other changes in gene expression are cell-specific. For instance, senescent endothelial cells overexpress I-CAM, an adhesion molecule (Maier *et al.*, 1993), while dermal fibroblasts overexpress metalloproteinase (Millis *et al.*, 1992).

To determine whether senescence is a dominant phenotype, Pereira-Smith performed a series of cell fusion experiments (Pereira-Smith and Smith, 1983; Pereira-Smith *et al.*, 1990). When young and old cells are fused together, the resulting hybrid exhibits a lifespan comparable to that of old cells, while fusion of immortal and "normal"

FIGURE 2: p53 IN REPLICATING AND SENESCENT CELLS

In replicating cells, MDM2 interacts with p53 and targets it for degradation (Haupt *et al.,* 1997; Kubbutat *et al.,* 1997). Thus, p53 cannot promote the expression of genes involved in cell cycle arrest and as such, cell growth and division is favored. However, when $p14^{ARF}$ is present in cells, it interacts with MDM2 and sequesters it away from p53 (Pomerantz *et al.,* 2000; Zhang *et al.,* 1998). This permits p53 to favor the transcription of genes inhibiting cell cycling thus enabling cellular senescence. A complex form between PML, CBP and p300 can increase the activity of p53 by acetylating it (Pearson *et al.,* 2000).

P53 IN REPLICATING CELLS



P53 IN SENESCENT CELLS

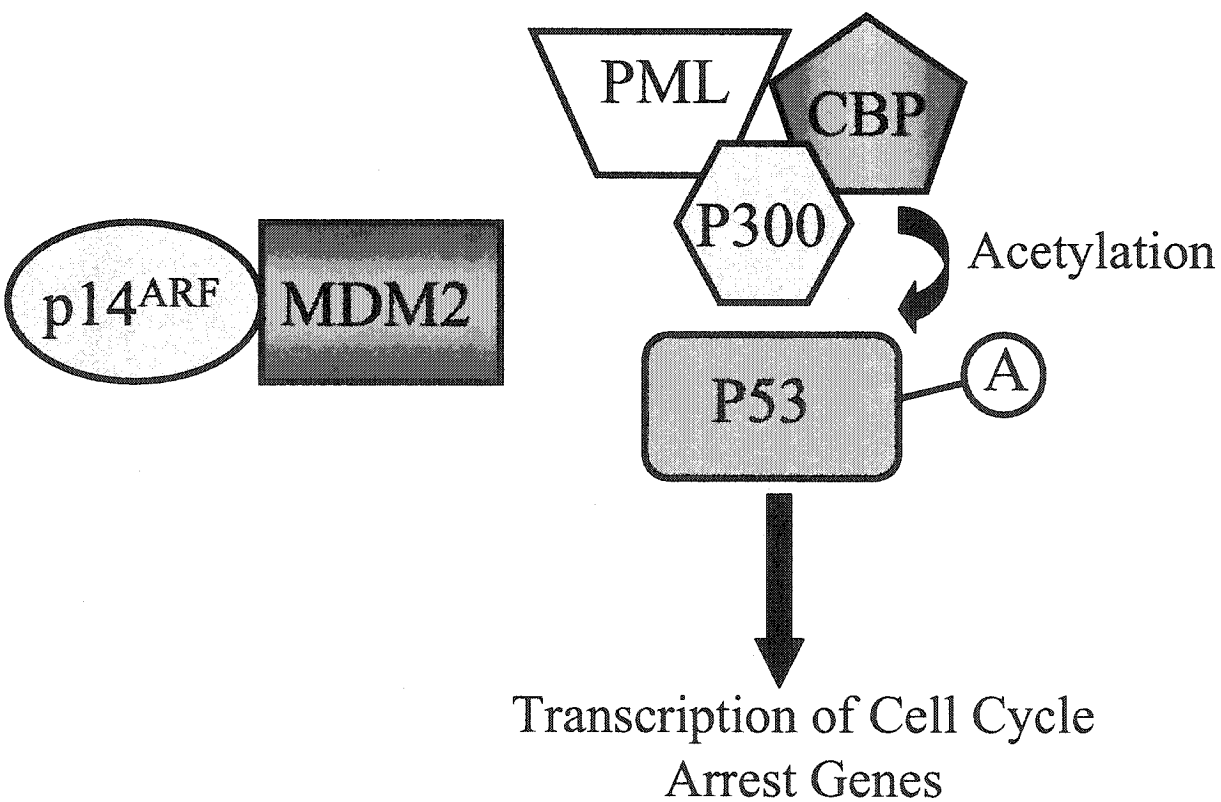
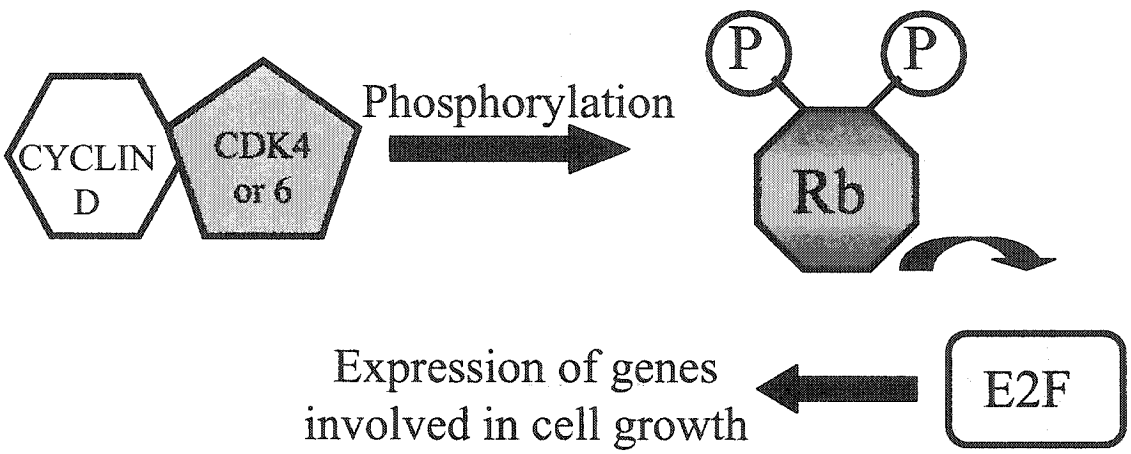


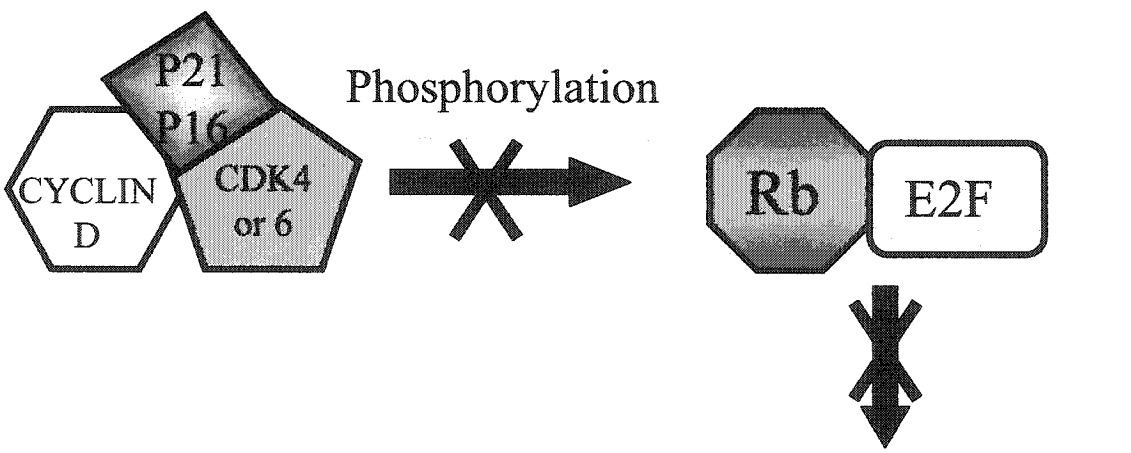
FIGURE 3: pRb IN REPLICATING AND SENESCENT CELLS

In replicating cells interaction between cyclin D and CDK4 or CDK6 serve to phosphorylate Rb. When phosphorylated Rb cannot bind to E2F, a transcription factor, and thus, E2F can promote the expression of genes involved in cell growth (reviewed in Kaelin, 1999). However, in senescent cells cell cycle inhibitor such as p21 and p16 can inactivate the cyclin D/CDK4 or 6 complexes and thus prevent the phosphorylation of Rb (Alcorta *et al.*, 1996). Hypophosphorylated Rb binds to E2F and prevents it from participating in the translation of genes involved in cell growth (reviewed in Harbour *et al.*, 2000).

Rb IN REPLICATING CELLS



Rb IN SENESCENT CELLS



finite lifespan cells gives rise to a hybrid with a finite lifespan. Such experiments support the dominant nature of the senescent phenotype.

REPLICATIVE SENESCENCE AND ORGANISMAL AGING

The study of lifespan and aging at the cellular level mostly revolves around the study of cellular senescence *in vitro*. Like our bodies, most human somatic cells are "born", replicate, then become old or "senescent". Do senescent cells contribute to organismal aging? Several data lead us to believe that they might. First, an inverse relationship generally exists between the maximum number of population doublings a cell can undergo before reaching senescence and the donor's age (reviewed in Stanulis-Praeger, 1987). Second, cells obtained from organisms with longer lifespan can generally undergo more population doublings before reaching senescence than those from shorter-lived species (Rohme, 1981). Third, cells collected from humans having premature aging diseases become senescent before those obtained from age-matched controls (Martin, 1970); and finally, more cells bearing senescence-associated markers are present in tissues extracted from older individuals than in tissues from younger individuals (Dimri *et al.*, 1995). These data (reviewed in Smith, 1996) suggest that the number of senescent cells throughout the body increases with age. As the gene expression profile of senescent cells is drastically different from that of younger cells, senescent cells may be partially responsible for the decreased functionality of tissues seen in aging humans. At this moment the exact contribution to organismal aging made by senescent cells, if any, remains unknown. However, data suggest that the accumulation of senescent cells is deleterious to organisms, as they support tumorigenesis in surrounding tissues. Krtolica and colleagues (2001) have shown that senescent fibroblasts do not give rise to cancer cells, but favor the development of pre-existing tumor cells.

As previously mentioned, senescence can be induced in response to oxidative stress, by increased activation of oncogenes, and by the presence of critically short telomeres. Thus, the induction of senescence may serve as a tumor suppressor mechanism, to avoid cancer-prone situations. However, this mechanism is likely to benefit only individuals of young reproductive age, as the accumulation of senescent cells

occurring with age promotes the development of pre-cancerous cells (Krtolica *et al.*, 2001); cancer is one of the leading causes of death in the elderly population. This leads us to question whether cellular senescence is an evidence for the pleiotropic (evolutionary) theory of aging.

1.4.2 Yeast as a Model of Aging

The budding yeast *Saccharomyces cerevisiae*, simply referred to as yeast throughout the subsequent text, is a unicellular eukaryote, first proposed as a possible model for aging studies following the publication of its finite lifespan by Mortimer and Johnson in 1959. The short lifespan of yeast, the presence of several yeast-human orthologue genes, the ability to easily modify its growth conditions, its small genome, and the ease of creating mutant yeast cells, all render it an attractive model in which to study aging. This section briefly describes yeast's life cycle, and provides an overview of data obtained from aging research in this species.

YEAST'S LIFE CYCLE

Under favorable growth conditions, where plenty of nutrients are present, yeast proliferates by asymmetrical division, in which a "mother" budding yeast cell gives rise to a small "daughter cell". The lifespan of such replicating yeast is finite, and can be assessed by the number of progeny issuing from a "mother cell". Mother cells generally divide 20-25 times before dying, and as they age, slowly display an age-associated phenotype including increased cell size, altered cell shape and accumulation of bud scars (Egilmez *et al.*, 1990; Mortimer and Johnston, 1959; reviewed in Jazwinski, 2001). Aging in yeast, as in other species, is associated with an age-dependent, exponentially increasing mortality rate, which plateaus at older ages (Jazwinski *et al.*, 1998). The finite budding lifespan, called "replicative yeast aging", is the one most often used to study aging, while "chronological yeast aging", the study of lifespan in stationary, non-replicating yeast, is rarely used as a model. Yeast populations become stationary in

response to various conditions including overcrowding, and remain in this phase until either proper growth conditions are restored or death occurs.

FREE RADICALS

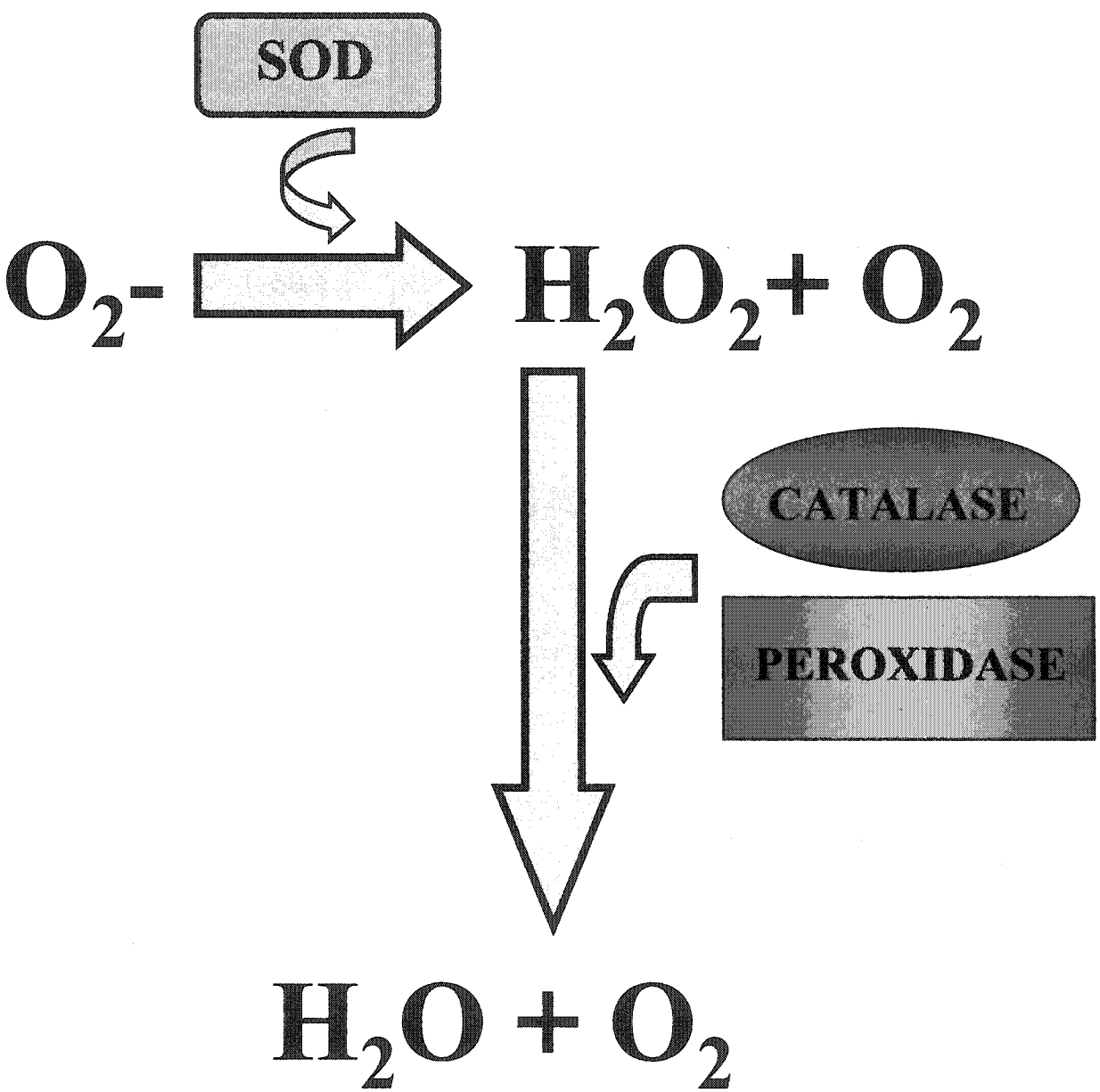
The free radical theory of aging proposes oxidative damage as one of the causes of aging, and thus implies that the presence of radical scavenger antioxidants may enable organisms to increase their lifespan (Harman, 1956). In living organisms such as yeast, superoxide radicals are converted to hydrogen peroxide and oxygen by the catalytic action of superoxide dismutase, while the resulting hydrogen peroxide molecules are further converted to oxygen and water by enzymes including catalases and peroxidases, as well as non-enzymatic glutathione (reviewed in Jamieson, 1998), thus neutralizing reactive oxygen species (ROS) (Figure 4). Yeast, like many other species, possesses two superoxide dismutase enzymes, encoded by genes SOD1 and SOD2. Copper-Zinc SOD (Cu,Zn-SOD), encoded by the SOD1 gene, is the most abundant and located in the cytoplasm, while Manganese SOD (Mn-SOD) is encoded by SOD2 and located in mitochondria (Bermingham-McDonogh *et al.,* 1988; Gralla and Kosman, 1992: Westerbeek-Marres *et al.,* 1988). Similarly two genes, CTA1 and CTT1, encode for catalases in yeast (Cohen *et al.,* 1988; Hartig and Ruis, 1986). The product of CTA1 is a peroxisomal protein (catalase A), while that of CTT1 is a cytosolic protein (CTT1).

In yeast, the expression of superoxide dismutase and catalase can modulate lifespan under certain conditions. For instance, SOD1 mutant yeasts grown on glucose, a fermentable carbon source, show a 50% reduction in mean lifespan compared to wild-type yeasts. However the addition of sub-cytotoxic level of manganese (Mn2+), a free radical scavenger, partly compensates for the lack of SOD1 (Barker *et al.,* 1999), indicating that the shortened lifespan as a result of SOD1 mutation partly results from inappropriate defense against free radicals. SOD1 mutant yeasts also have shorter lifespan than wild-type when grown on non-fermentable carbon sources such as ethanol or glycerol (Barker *et al.,* 1999).

When grown on either glucose or ethanol, acatalasaemic mutant yeasts, double mutants in CTA1 and CTT1 producing no catalases, show decreased mean lifespan

FIGURE 4: FREE RADICALS NEUTRALIZATION

In living organisms, superoxide radicals (O_2^-) can be partly neutralized by antioxidants such as catalases and SODs. It is important for biological systems to neutralize these radicals as they can cause extensive damages inside cells, including DNA damages. Superoxide radicals are converted to hydrogen peroxide (H_2O_2) and oxygen (O_2) by the catalytic action of superoxide dismutase. Hydrogen peroxide (H_2O_2) is then converted in oxygen (O_2) and water (H_2O) by enzymes such as catalases and peroxidases.


SOD
O_2-
H_2O_2+ O_2
CATALASE
PEROXIDASE
H_2O + O_2

compared to wild-type yeasts. On ethanol this decrease is greater than 50%, while on glucose it is 20% (Van Zandycke *et al.*, 2002). When single mutants are studied, the absence of catalase T is responsible for the shortening of lifespan seen in double mutants grown on glucose, while the absence of either catalase T or A decreases the lifespan of yeasts grown on ethanol (Van Zandycke *et al.*, 2002). Although both superoxide dismutase and catalase influence lifespan, the relationship between these enzymes and yeast aging remains obscure.

YEAST LONGEVITY GENES: AN HISTORICAL PERSPECTIVE

Egilmez and colleagues (1989), seeking to determine whether age-associated changes in mRNA expression exist in yeast, identified six genes differentially expressed in young and old yeast cells. Of these six genes, five are characteristic of young cells, and only one old-cell-specific. Further studies on two of these genes (one each characteristic of old and of young cells) established the cell-cycling independence of the differential expression, and confirmed the changes as being age-associated. These results led the scientific community to envision the possibility of identifying a "yeast longevity gene", a gene whose expression affects lifespan. Five years later, D'mello and colleagues (1994) cloned and characterized LAG1, one of the genes characteristic of young cells identified by Egilmez and colleagues, and the first yeast longevity gene. Soon after, other genes showing age-differential expression were characterized (Sun *et al.*, 1994). Currently, more than 15 genes are known to influence yeast's lifespan (see table in Jazwinski, 2000), some of which are discussed below.

LONGEVITY ASSURANCE GENE -1

The yeast longevity-assurance gene-1 (LAG1) was the first yeast gene shown to influence lifespan (D'mello *et al.*, 1994). LAG1 encodes for an ER protein with several transmembrane domains (Barz and Walter, 1999) which, when moderately expressed, increases the lifespan of yeast cells by more than 50% (D'mello *et al.*, 1994). In young cells, high levels of LAG1 expression generally induce senescence; however, individual yeast cells capable of escaping senescence despite high levels of LAG1 expression

generally attain extended lifespan (D'mello *et al.*, 1994). On the other hand, deletion of LAG1 almost irrevocably shortens lifespan by as much as 40% (D'mello *et al.*, 1994). Although LAG1 orthologues exist in several species including humans (Jiang *et al.*, 1998; Brandwagt *et al.*, 2000; Pan, 2001), the mechanism by which they extend lifespan remains unknown. It is postulated that LAG1 involvement in ceramide synthesis (Guillas *et al.*, 2001) serves to modulate lifespan, as ceramides are important messengers implicated in cell growth, death and survival (Hannun *et al.*, 2001; Mathias *et al.*, 1998).

SGS1, A DNA HELICASE ENCODING GENE

The SGS1 gene encodes a RecQ DNA helicase capable of binding enzymes topoisomerase I and II (Gangloff *et al.*, 1994; Watt *et al.*, 1995). DNA helicases are present in several species including man, and serve to unwind DNA during cellular replication and DNA repair. Hence, mutational loss of function of the SGS1 gene decreases yeast lifespan by more than 50%, and leads to the early onset of the age-associated phenotype (Sinclair *et al.*, 1997). This premature aging phenotype associated with mutation in DNA helicase is also seen in man, giving rise to Werner's, Bloom's and Rothmund Thompson's syndromes (to be discussed later) (van Brabant *et al.*, 2000). In the absence of SGS1 gene product, yeast cells are genomically unstable and possess low DNA repair capabilities, entailing increased unrestrained recombination, leading rapidly to the appearance of the age-associated phenotype and death (Gangloff *et al.*, 2000).

RAS1, RAS2, AND THE RESPONSE TO STRESS

Yeasts possess two Ras genes, RAS1 and RAS2, which exert opposing effects on lifespan; deleting RAS2 results in lifespan reduction, while deleting RAS1 markedly increases lifespan (Sun *et al.*, 1994). However, while overexpression of RAS2 in the absence of stress extends yeast's lifespan, overexpression of RAS1 has no effect on longevity. Since Ras normally functions to stimulate the activity of adenylate cyclase, Sun and colleagues (1994) investigated whether RAS2 exerts its effect on lifespan by modulating the cAMP pathway. When they overexpressed in yeast a mutant form of

RAS2, unable to stimulate adenylate cyclase and thus to signal *via* the cAMP pathway, the effect of RAS2 on lifespan extension was maintained, indicating that RAS2 exerts its effect on lifespan *via* a cAMP-independent signaling pathway (Sun *et al.,* 1994).

In yeast, resistance to UV parallels the expression of RAS2; it increases until midlife, and then declines (Kale and Jazwinski, 1996). This result is not surprising, as Engelbert and colleagues (1994) showed that in yeast, Ras activates AP-1 factors in response to UV in a DNA damage-independent manner. In yeast Gcn4, an AP-1 transcriptional activator, is activated in response to UV radiation, amino acid starvation, or growth stimulation by glucose. However, RAS2 and cAMP only participate in the increased Gcn4 activity mediated by UV stress and growth stimulation by glucose, not by amino acid starvation (Marbach *et al.,* 2001). Interestingly, yeasts selected for their ability to withstand starvation and cold stress have longer lifespan than non-selected unstressed (Kennedy *et al.,* 1995), indicating that genes conferring stress resistance may also play a role in longevity.

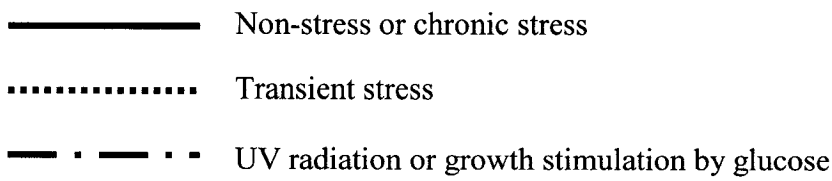
Decreased lifespan caused by chronic stress, induced by repetitive exposure to sublethal heat stress, can be abrogated in yeast by overexpressing RAS2 but not RAS1 (Shama *et al.,* 1998). This effect is cAMP-dependent, and results from the ability of RAS2 to downregulate stress genes and upregulate growth-promoting genes following exposure to chronic stress. In the absence of RAS2, yeast cells are capable of activating stress genes and downregulating growth-promoting genes when chronically stressed, but cannot recover rapidly as they are unable to upregulate growth-promoting genes (Shama *et al.,* 1998). In fact, overexpression of RAS2 in chronically stressed yeast results in extended lifespan, as it does in unstressed yeasts; however, under chronic stress, cAMP is involved (Figure 5).

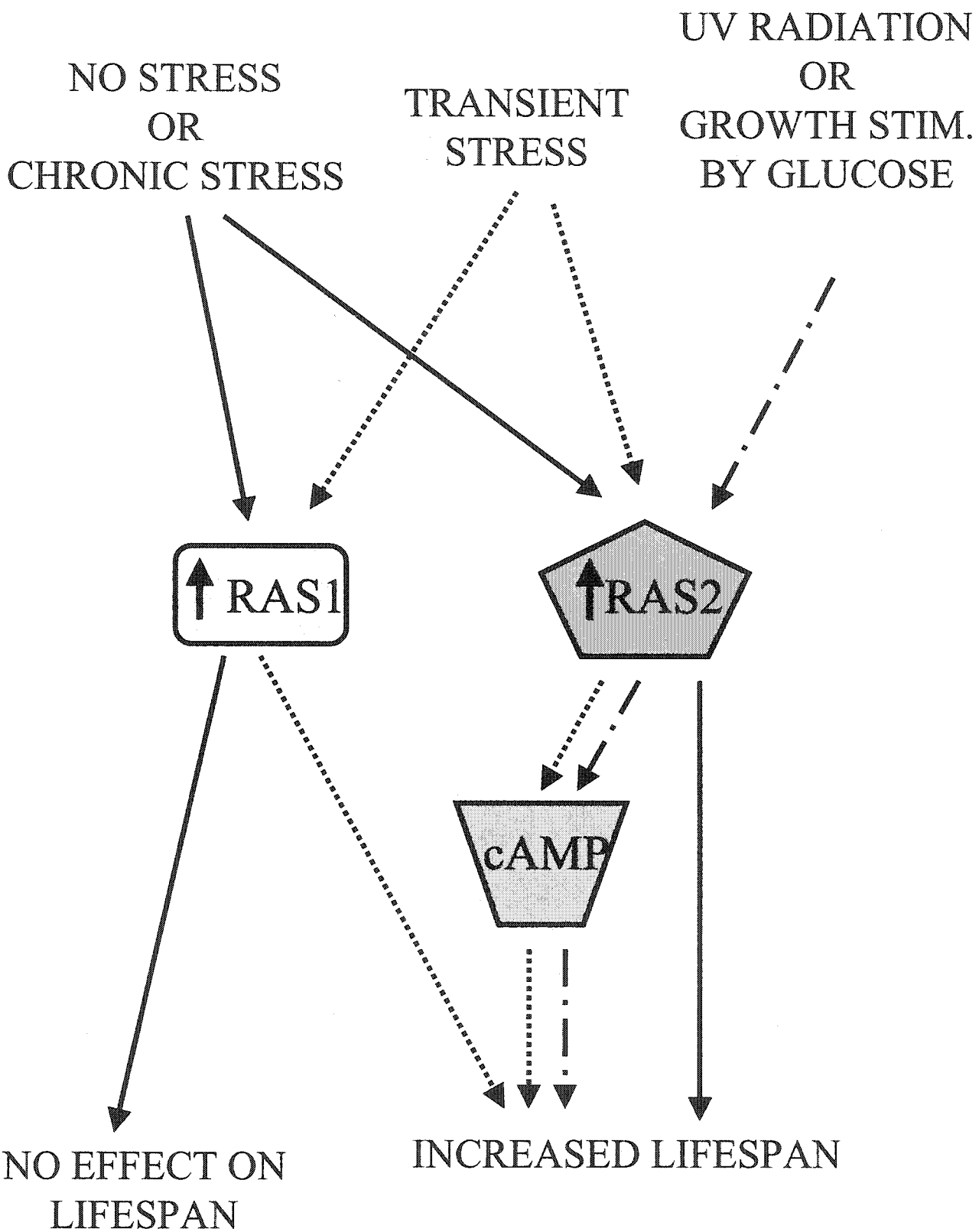
Under transient stress, such as that induced by sublethal heat stress delivered in early life, RAS1, RAS2, HSP104 (heat shock protein 104), and the presence of functional mitochondria are necessary to confer increased lifespan (Shama *et al.,* 1994). Once again, following transient stress, as for chronic stress, RAS2 permits prompt recovery by rapidly upregulating growth-promoting genes. The mechanism by which RAS1 acts to confer life extension in this situation remains unknown. However, Jazwinski (1999a)

FIGURE 5: MODULATION OF LIFESPAN BY RAS

In unstressed or chronically stressed yeasts, overexpression of RAS2 can increase lifespan in a cAMP-independent manner. However, under those conditions, overexpression of RAS1 has no effect on lifespan. Overexpression of RAS2 in transiently stress yeast can also extend lifespan however; this extension is cAMP dependent and further requires the presence functional mitochondria. Under transient stress, RAS1 by a yet unknown mechanism can also increase lifespan. Similarly UV-irradiated or glucose growth-stimulated yeasts can acquire extend lifespan by a cAMP-dependent pathway if RAS2 is overexpress.

In this diagram, arrow leading from each conditions (unstressed, transient stress, UV radiation) to RAS1 and 2 does not indicate that these conditions causes overexpression of RAS1 or 2 but indicate the effect on lifespan that would arise if each conditions is accompanied by overexpression of either RAS1 or 2.




NO STRESS
OR
CHRONIC STRESS
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STRESS
UV RADIATION
OR
GROWTH STIM.
BY GLUCOSE
RAS1
RAS2
cAMP
NO EFFECT ON
LIFESPAN
INCREASED LIFESPAN

proposed that the stimulation of inositolphospholipid turnover promoted by Ras1p may underlie a mechanism whereby RAS1 participates in life extension.

THE RETROGRADE RESPONSE

A study conducted by Kirchman and colleagues (1999) in petite yeasts, lacking functional mitochondria and hence non-respiring, revealed that some petite strains have lifespan greater than those of their grande counterparts with fully functional mitochondria. Upon further investigation, an interorganelle signaling pathway between mitochondria and the nucleus, the "retrograde response", was found to be responsible for the lifespan extension observed in some petites (Kirchman *et al.,* 1999). Currently, three main genes are known to participate in the retrograde response: RTG1, RTG2, and RTG3. Rtg1 and Rtg3 proteins are basic helix-loop-helix/leucine zipper (bHLH/Zip) transcription factors which heterodimerize to bind DNA regions containing an R box site (5'-GTCAC-3') (Jia *et al.,* 1997), and thus induce the transcription of genes encoding peroxisomal, mitochondrial and cytoplasmic proteins (Chelstowska *et al.,* 1995; Rothermel *et al.,* 1997). Rtg2 is a 68kDa protein possessing an N-terminal ATP binding domain, which acts upstream of Rtg1 and 3 (Koonin, 1994; Jia *et al.,* 1997; Rothermel *et al.,* 1997). Although the function of gene product Rtg2p is not fully characterized, mutation or deletion of the RTG2 gene abrogates the life extension conferred by the retrograde response (Kirchman *et al.,* 1999). As pointed out by Jazwinski (1999b), the net effect of the retrograde pathway is to change energy producing mode from the Krebs cycle to the glyoxylate cycle, a mechanism resembling the mobilization of fat stores seen in other species.

Interestingly, RAS2 interacts with the retrograde response signal, and once again serves to modulate lifespan (Kirchman *et al.,* 1999). However, the role of RAS2 in the retrograde response remains to be fully characterized.

SIR: THE SILENCERS OF TRANSCRIPTION

In yeast four SIR (silent information regulator) genes, SIR1, SIR2, SIR3 and SIR4, are involved in silencing the transcription of certain chromosomal regions (Ivy *et al.,* 1986). While SIR3 and 4 are found in the telomeric region, and also serve to silence the transcription of the chromosomal regions encoding for mating type, SIR2, a nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylase, serves to silence rDNA genes in addition to the mating type, but is also found in the telomere region (Rine and Herskowitz, 1987; Gottschling *et al.,* 1990; Bryk *et al.,* 1997).

Histone deacetylases such as SIR2 permit the maintenance of condensed DNA structures, and thus prevent interaction between DNA and the transcriptional machinery (reviewed in Chang and Min, 2002). In yeasts, null mutation of SIR2 results in shorter lifespan, while the presence of an extra copy of SIR2 increases lifespan and decreases the rate of extrachromosomal rDNA circle (ERC) accumulation (Kaeberlein *et al.,* 1999). This is not surprising, since aging in yeast is normally associated with a decrease in gene silencing. For instance, sterility observed in old yeast can result from the loss of silencing at the HM (mating) loci (Gottshling *et al.,* 1990) while silencing of subtelomeric regions normally decreases with age (Kim *et al.,* 1996). Loss of silencing at the mating loci results in sterility, as it permits the expression of both **a** and α mating types in a single yeast cell. Several hypotheses have been brought forward to explain the mechanisms by which SIR2 modulates lifespan; however at this time, none are truly proven.

One of the ideas leading to the development of a hypothetical mechanism by which SIR2 may act is the formation of ERC. Gottlieb and Esposito (1989) have shown that silencing the rDNA region encoding rRNA can repress the recombination of rDNA, and thus prevent the formation of ERC. Several data indicate that accumulation of ERC may cause aging in yeast. For instance, blocking rDNA replication increases lifespan, while introducing artificial ERC into yeast cells decreases it (Defossez *et al.,* 1999; Sinclair and Guarente, 1997). However, other evidence tends to disprove the ascription of a role for ERC in yeast aging. Notably, long-lived petite yeasts possess several ERC (Kirchman *et al.,* 1999), while short-lived SGS1 mutants do not show high levels of ERC

accumulation (Heo *et al.*, 1999). Similarly, while other hypotheses have been formulated, further research will be needed to clearly establish the role played by SIR2 in lifespan modulation.

WHAT HAVE WE LEARNED FROM THE YEAST MODEL?

Studies conducted in yeast have permitted us to uncover several longevity genes, and clearly demonstrated that genes are, at least in part, determinants of longevity which, even in a simple unicellular organism, can exert their effect on lifespan *via* several different molecular pathways. Thus mutations in genes encoding for proteins belonging to different classes can yield similar effects on lifespan. For instance, mutations producing loss of function in SGS1 and SIR2 both reduce lifespan, although the former encodes for a DNA helicase, while the latter encodes an NAD-dependent histone deacetylase (reviewed above). To increase the complexity of their actions, some genes encode proteins which participate in more than one lifespan-modulating pathway. RAS2 is a good example of this, as it confers life extension via both camp-dependent and -independent pathways (reviewed above).

The yeast model has also clearly established that environments influence longevity, especially in the context of gene/environment interaction. Thus the nutritional status of an organism can influence genes modulating lifespan. For instance, RAS 2 functions to mediate the response to growth stimulation by glucose (Marbach *et al.*, 2001). However, although yeast as a model to study aging has revealed that longevity has both genetic and environmental components, the pathways modulating lifespan remain obscure. So far, we know that the ability to repair DNA damage is important, as mutation in DNA helicase reduces lifespan, and likewise that the expression of genes whose products are involved in gene silencing, or participate in the retrograde response pathway, are capable of modulating lifespan. In light of these results, it is likely that lifespan determination relies on multiple pathways, modulated by both extrinsic and intrinsic factors. Current data indicate that yeast aging revolves around four main themes: metabolic control, maintenance of genetic stability (by permitting repair and silencing), resistance to stress, and gene dysregulation (reviewed in Jazwinski, 2001).

Can the information we have learned from yeast be applicable to other species? Several lines of evidence tend to indicate that to some extent it is. For instance, SIR2 homologues are found in several species, including man. In the worm *C. elegans*, increased dosage of SIR2 increases lifespan, as in yeast (Tissenbaum and Guarente, 2001). Oxidative stress also seems to influence the aging of several species. In chronologically aging yeast, deletion of SOD results in decreased lifespan, while its overexpression increases lifespan (Longo *et al.,* 1996); while in replicating yeast, deletion of both SOD and catalase decreases lifespan (Barker *et al.,* 1999; Van Zandycke *et al.,* 2002). This parallels the results obtained in the nematode *C. elegans* and the fruitfly *D. melanogaster* (Barker *et al.,* 1999; Parkes *et al.,* 1998; Melov *et al.,* 2000). However, it is likely that although the use of yeast as a model of aging can help in identifying evolutionarily conserved pathways, not all of the pathways or mechanisms modulating yeast's lifespan influence the aging of multicellular organisms possessing differentiated and specialized cells and organs. For instance, one can hardly conceptualize how ERC might play a role in human aging. Nevertheless, given their short lifespan and their small genome, yeasts are a valuable source of information, and have contributed to the advancement of aging research.

1.4.3 *Drosophila* as a Model of Aging

Drosophila melanogaster is a species of fruit flies widely used as a model for aging, since its lifespan is relatively short and several of its physiological systems are similar to those of mammals (Smith, 1968). For instance, they possess specialized cells such as neurons and a muscular and nervous system. However, unlike mice or humans, all somatic cells present in *Drosophila* are postmitotic; they do not replicate (Bozuck, 1972). In humans, only long-lived terminally differentiated neurons, caridomyocyteds and skeletal muscle myotubes are similarly postmitotic. Therefore, the applicability of results obtained from fruitfly aging studies to human aging, has to account for this fact. Data obtained from aging studies conducted in fruit flies are briefly described in the following sub-sections.

REPRODUCTION *VERSUS* LONGEVITY

The evolutionary theory of aging proposes that a trade-off exists between reproduction and longevity. Early studies conducted by Rose and Charlesworth (1981b) on twelve hundred female flies tend to support this idea, as they identified a negative correlation between early fecundity and lifespan, and between mean egg-laying rate and longevity. These data were further substantiated by studies showing that egg production and exposure to males could curtail the longevity of female flies (Partridge *et al.,* 1987; Partridge and Fowler, 1990). However, as all of those experiments were conducted using female flies, one could argue that reproduction might not affect the lifespan of male flies, and thus that this trade-off is sex-linked. Partridge and Farquhar (1981, 1983) clearly demonstrated that this is not the case, as in male flies courtship as well as reproduction curtails longevity. To further investigate the nature of this trade-off between reproduction and lifespan, a series of experiments was conducted independently by several groups of investigators to determine whether selecting for late reproduction, decreased fertility, or other factors reflecting decreased reproductive capacities or functions, could give rise to long-lived flies.

THE GENERATION OF LONG-LIVED FLIES FROM NORMAL–LIVED STRAINS

Rose and Charlesworth (1981a) were the first group to start selecting flies for late reproduction, and to assess whether this selection process could give rise to long-lived flies. The results of their experiments were striking. A report by Nusbaum and Rose (1994) indicates that the long-lived flies selected for late reproduction by Rose show a gain in lifespan by as much as 100% compared to the control group, and exhibited increased resistant to ethanol vapor (Service *et al.,* 1985), indicating that a link might exist between lifespan and resistance to starvation or stress. While Rose was conducting his experiments two other scientists, L.S. Luckinbill and R. Arking, also began selecting flies based on reproductive parameters (Luckinbill *et al.,* 1984; Arking, 1987). In their experiments, Luckinbill and Arking divided a pool of non-selected normal-lived *Drosophila*, the R strain, into four groups, Ra, Rb, Rc and Rd. By selecting each group

over several generations for late reproduction, they obtained four long-lived strains of flies, La, Lb, Lc and Ld (Luckinbill *et al.,* 1984; Arking, 1987). Each long-lived strain showed similar extension in lifespan compared to the control groups, once again suggesting a trade-off between lifespan and reproduction in flies. However, further studies are needed to determine the exact nature of this relationship, and if confirmed, to identify the factors and molecular pathways responsible for this trade-off.

The existence of this possible trade-off appears to be linked to factors which by themselves might account for decreasing lifespans. For instance, flies selected for increased fecundity are less resistant to starvation (Chippindale *et al.,* 1993) and oxidative stress (Wang *et al.,* 2001). Several experiments have been conducted in fruit flies to determine whether resistance to starvation and stress influences longevity, and to what extent.

OXIDATIVE STRESS

Several approaches, including *in vivo* overexpression and inactivation of antioxidant enzymes, were used to test the free radical theory of aging, and determine whether or not free radicals affect *Drosophila*'s lifespan (reviewed in Le Bourg, 2001).

Arking and colleagues (1991) investigated the possibility of creating long-lived strains of *Drosophila* by selecting normal-lived (Ra) flies for resistance to Paraquat, a xenobiotic agent capable of producing ROS. After more than 24 generations of continuous selection, the resulting insects showed a four-fold increase in their mean lethal time, thus exhibiting increased lifespan compared to the Ra normal-lived stain. However, when long-lived flies derived from Ra flies, selected for resistance to paraquat over several generations, are compared with the La strain (naturally resistant to paraquat), their level of antioxidants is lower, while their level of P450 enzymes is higher (Vettraino *et al.,* 2001), indicating that similar longevity phenotypes can be obtained though different gene expressions and enzymatic activity. Studies conducted by Arking and colleagues support this notion (Arking *et al.,* 2000b). For instance, while both La and Lb flies are resistant to paraquat, La flies over-express SOD (Arking *et al.,* 2000), while paraquat resistance in Lb flies seems to be dependent upon catalase expression (Arking *et*

al., 2000b). While the expression of antioxidant enzymes differs between long-lived strains, as well as between each long-lived strain and their normal-lived sisters (Arking *et al.*, 2000b), might other factors also contribute to the difference in lifespan observed between normal-lived (R) and long-lived (L) strains?

To identify whether changes in the mitochondrial genome could explain the increased longevity of La flies over Ra flies, mutant flies having the Ra nuclear genome and the La mitochondrial genome were generated (Driver and Tawadros, 2000). These mutant Ra flies, known as La-mt mutants, showed a significant increase in longevity compared to the Ra strain. This result, along with that of Ross (2000) showing that La mitochondria produce lower level of ROS leakage than Ra mitochondria, is further evidence that free radicals can play a role in abrogating life.

In an attempt to better document the role of SOD and catalase in modulating *Drosophila*'s lifespan, the effects of overexpressing and abrogating antioxidant enzymes were studied in flies (Phillips *et al.*, 1989; Orr and Sohal, 1994; Mockett *et al.*, 2002). CuZnSOD null mutant flies were found to have a significantly shorter lifespan than wild type flies (Phillips *et al.*, 1989), while on the other hand overexpression of CuZnSOD and catalase extends lifespan (Orr and Sohal, 1994, Sun *et al.* 2002). Overexpression of human SOD1 in *Drosophila* motor neurons is sufficient to increase lifespan (Parkes *et al.*, 1998).

While selection for paraquat resistance produces long-lived flies, and overexpression of antioxidant enzymes as well as limited mitochondrial leakage extends lifespan, can exogenous sources of free radical scavengers also modulate lifespan? To address this issue, Bonilla and colleagues (2002) supplemented the food supply of normal-lived flies with melatonin, a potent hydroxyl radical scavenger (Tan *et al.*, 1993), and found that daily food supplementation with melatonin increases the maximum lifespan of flies by 33.2%, as well as conferring increased resistance to paraquat.

METHUSELAH

In an attempt to identify genes involved in lifespan modulation in *Drosophila*, Lin and colleagues (1998) generated several lines of mutant flies with P-elements inserted

randomly in their genome. One of the resulting mutant fly strains was found to have a lifespan 35% longer than its parental strain. Upon characterization, these flies were found to be homozygous for P-element insertion in the *methuselah* gene (*mth* mutant). Along with increased lifespan, *mth* flies are more resistant to stress, including paraquat starvation and heat shock. However, when the P-element inserted in the *mth* gene is excised, flies revert to their shorter-lived phenotype, indicating that the insertion is sufficient to increase lifespan. On the other hand, when DNA regions flanking the P-element are excised along with it, the resulting flies exhibit lifespan shorter than that of the parental strain. Thus it is proposed that high levels as well as absence of *mth* gene expression negatively impact lifespan, while intermediate expression, caused by insertion of P-element, extends it. Methuselah was recently characterized as a G protein-coupled receptor belonging to the secretin family (West *et al.,* 2001). The mechanism by which *mth* expression modulates lifespan, and the tissues in which it is expressed, remain unknown.

INDY

By inserting P-elements randomly into the genome, as Lin and colleagues (1998) had done, Rogina and colleagues (2000) found that the insertion of five P-elements within a gene which they named INDY (for "I'm not dead yet") gave rise to flies with lifespan almost double that of the parental stain. Upon sequencing, Indy was found to bear significant homology to human and rat sodium dicarboxylate cotransporters (Rogina *et al.,* 2000). In mammals, such cotransporters are responsible for the uptake and reuptake of Krebs cycle intermediates (Chen *et al.,* 1998; Pajor, 1996). Unlike methuselah, the tissue distribution of Indy is known, and coincides with the primary sites of intermediate metabolism, absorption, and metabolic storage in flies: the fat body, midgut and oenocytes (Rogina *et al.,* 2000). Lifespan extension conferred by P-element insertion in the Indy genes is somewhat dose dependent , as flies heterozygous for the insertion, as previously stated, have lifespan almost double that of the parental strain, while homozygous flies only exhibit 20% life extension. Excision of the P-elements reverts the lifespan of flies to the length of their parental strain (Rogina *et al.,* 2000).

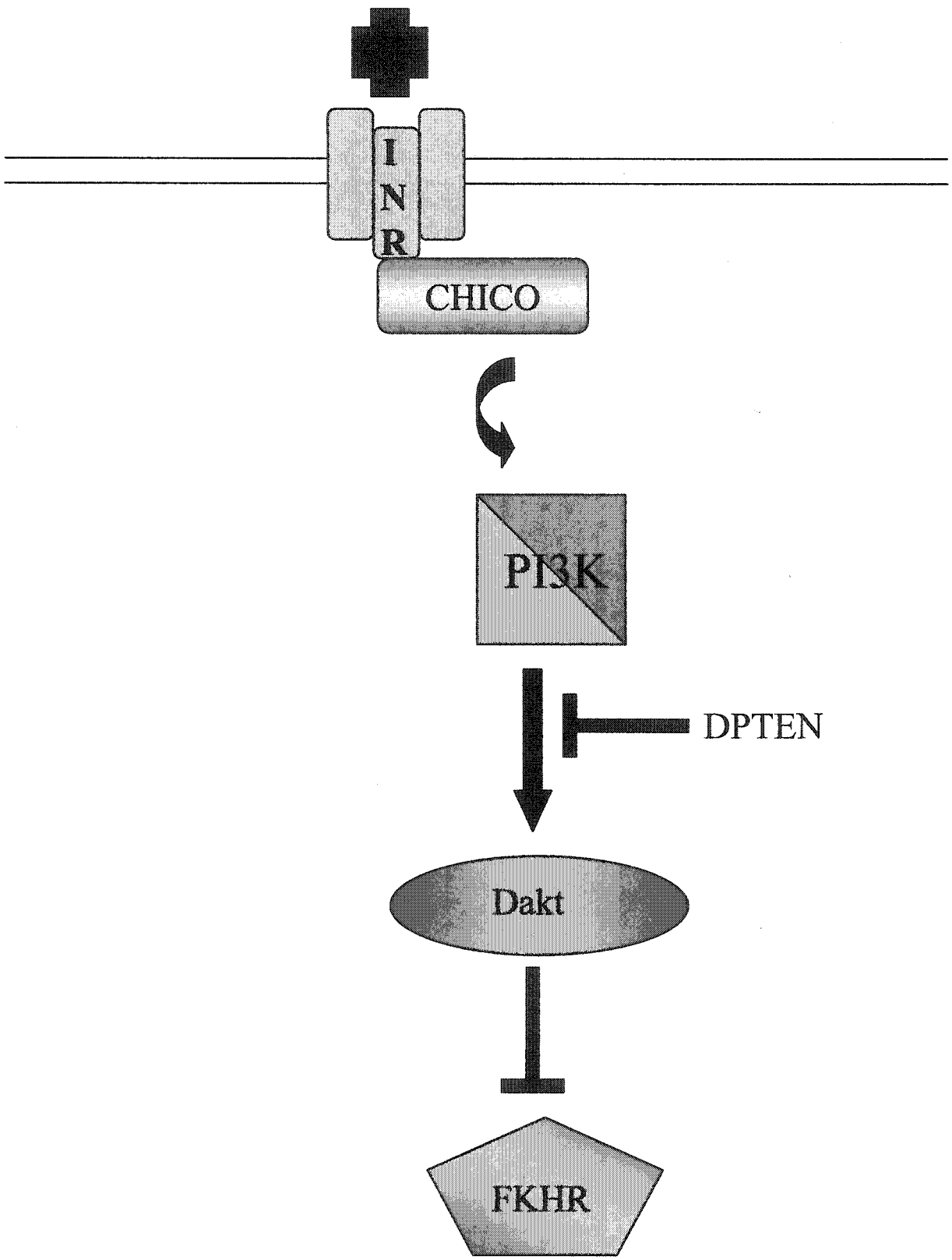
INSULIN SIGNALING PATHWAY

Drosophila possess an insulin-like receptor (InR) homologous to the *C. elegans* daf-2 and mammalian insulin receptor (Ruan *et al.,* 1995). Since daf-2 can modulate the lifespan of worms (Kimura *et al.,* 1997; Tissenbaum and Ruvkun, 1998), studies were conducted to determine whether InR could similarly affect the lifespan of flies. Mutations were introduced in the InR gene, and mutant flies created. While a homozygous genotype for InR mutation is lethal, some heterozygous flies are viable and live much longer than the parental stain (Tatar *et al.,* 2001). For instance, InR^{P5545}/InR^{E19} female flies have 85% greater lifespan than wild-type control (Tatar *et al.,* 2001). However, these flies are dwarf, sterile, and when stimulated by insulin exhibit kinase activity only minimally in excess of their basal level, indicating that they are unable to properly respond to stimulation by insulin (Tatar *et al.,* 2001). Not all heteroallelic flies for InR exhibit extended lifespan, as some InR mutations cause developmental defects that affect the adult (Tatar *et al.,* 2001). The lifespan of InR^{P5545}/InR^{E19} males is also extended compared to wild-type flies, but not as much as that of females (Tatar *et al.,* 2001). This may be attributed to the high mortality rate seen in males at an early age. Unlike females, the males are not sterile, but show a substantial decrease in fertility. Sterility or decreased fertility associated with decreased signal transduction from InR are believed to result from the down-regulation of juvenile hormone synthesis in mutant flies, which results in neuroendocrine deficiency (Tatar *et al.,* 2001).

In *Drosophila*, the insulin signaling pathway which serves to regulate the growth of flies is composed of several players, including CHICO, phosphatidylinositol 3-kinase (PI3K) Dp110/p60 and protein kinase B (PKB), also known as DAkt1 (Figure 6). Clancy and colleagues (2001) mutated CHICO, an InR substrate, in order to better characterize the role of the insulin pathway in modulating lifespan. Mutation of CHICO extends the mean lifespan of homozygous female flies by 48%, and that of heterozygotes by 36% (Clancy *et al.,* 2001). However, in males heterozygosity only extends lifespan by 13%, while homozygous males show a slight decrease in lifespan compare to wild-type flies (Clancy *et al.,* 2001). It is believed that unlike homozygous InR, homozygous CHICO is not lethal, as InR can signal to PI3K directly without the need for CHICO (Bohni *et al.,*

FIGURE 6: INSULIN-LIKE SIGNALING PATHWAY IN FLIES

Drosophila possess an insulin-like receptor INR to which an insulin-like ligand (DILP1-7) can bind. Binding of a ligand to insulin-like receptor results in the phosphorylation of CHICO, and insulin-like receptor substrate. CHICO then activates PI3K. The activation of PI3K results in the phosphorylation of Dakt (drosophila akt). The latter can repress the activity of the forkhead and hence releases the translational block that the forkhead is believed to exert on some genes. DPTEN can prevent the phosphorylation of Dakt and thus the repression of the forkhead.


INR
CHICO
PI3K
DPTEN
Dakt
FKHR

1999). As with several mutations in the insulin pathways, heterozygous CHICO females show decreased fertility, while homozygous CHICO females are sterile (Bohni *et al.*, 1999; Clancy *et al.*, 2001). The differential lifespan modulation conferred by mutation in CHICO in males *versus* females may indicate that lifespan extension by CHICO is mostly mediated by effects on oogenesis.

WHAT HAVE WE LEARNED FROM THE FLY MODEL?

Studies conducted in *Drosophila* have demonstrated that in flies, reduced fertility, resistance to free radicals, as well as decreased signaling *via* the insulin pathway, can all extend lifespan. Data further indicate that as seen in yeast, longevity in flies results from regulation of metabolism, control over the production of ROS, and defense against oxidative stress, and indicate that mitochondrial as well as genomic genes influence lifespan. Data emerging from these studies clearly show that reproduction and longevity somehow influence each other. Could energy expenditure for reproduction negatively impact longevity? More research is necessary to clearly answer this question; however, *a priori*, the notion seems valid. Perhaps the most fundamental principle *Drosophila* has revealed about aging is that different genetic profiles can lead to similar phenotypes in terms of lifespan, strongly suggesting that several pathways are involved in the modulation of lifespan, and that aging is multifactorial

Furthermore, population studies conducted on Mediterranean fruitflies revealed that in normal population of flies, mortality rate follow the Gompertz model and as such, increases exponentially as a function of age. However, as for humans, in extreme old age, the mortality rate of flies levels off (Carey *et al.* 1995).

1.4.4 The Nematode *C. elegans* as a Model of Aging

The soil-living nematode *C. elegans* has been widely used in aging studies for the last two decades, as its short lifespan, as well as the ability to easily modify its growth conditions and the ease of creating mutants, renders them good subjects. In the following

subsection, the life cycle of *C. elegans,* as well as data obtained from aging research in this species, is described.

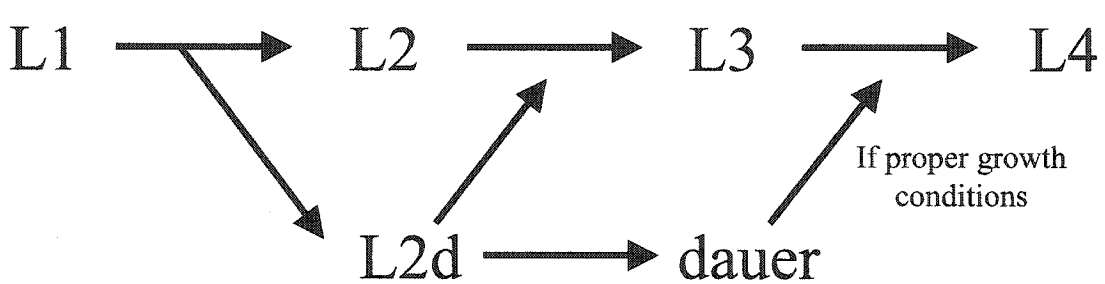
THE LIFE CYCLE OF THE NEMATODE *C. ELEGANS*

Under favorable growth conditions, the soil-living nematode *C. elegans* reaches adulthood in three days, by continuous development through four larval stages (L1-L4) (Byerly *et al.*, 1976). However, environmental cues reflecting unfavorable growth conditions can induce the molting of larva at the first larval stage (L1) into predauer larva, a modified second larval stage (L2d), which develops along an alternate developmental pathway by forming dauer larva, unless favorable growth conditions are restored to permit the molting of L2d larva into L3 larva (Cassada and Russell, 1975) (Figure 7).

Dauer is a state of dormancy, in which pharyngeal pumping is suppressed (Cassada and Russell, 1975) and the animals stand motionless on their tails (Croll and Matthews, 1977). Morphologically, dauer larva are thinner and denser than other larva, have an occluded buccal cavity, a smaller intestinal lumen (Cassada and Russell, 1975; Popham and Webster, 1979), exhibit unique changes in their cuticle including thickening of the outer cortex, which permits them to resist detergent treatments (Cassada and Russell, 1975; Popham and Webster, 1979; Swanson and Riddle, 1981), and have no secretory granules in their exocrine gland (Nelson *et al.*, 1983). Dauer larva are more thermotolerant (Anderson, 1978), but metabolically less active, than adults (Wadsworth and Riddle, 1989; O'Riordan and Burnell, 1989; 1990) and, although they have fifteen times more heat shock protein mRNA (Hsp90) than adults, their overall transcriptional activity is lower (Snutch and Baillie, 1983; Dalley and Golomb, 1982). Both superoxide dismutase (Anderson, 1982; Larsen, 1993) and catalase (Vanfleteren and DeVreese, 1995), two enzymes involved in protecting cells against oxidative damage, are elevated in dauer larva.

FIGURE 7: *C. ELEGANS* LIFE CYCLE

C. elegans reaches adulthood by continuous development through four larval stages known as L1 to L4. Under unfavorable growth conditions, animals at the L1 stage become predauer larva (L2d), which will form dauer larva if proper growth conditions are not restored to permit the molting of L2d larva into L3 larva. Dauer larva re-enter the life cycle at the fourth larval stage if proper growth conditions exists.


L1
L2
L3
L4
If proper growth conditions
L2d
dauer

Once food and proper growth conditions are restored, developmentally arrested dauer larva resume normal development within 50 to 60 minutes, by re-entering the life cycle at the fourth larval stage (L4) (Cassada and Russell, 1975). While the life span of dauer larva is 8 to 10 times longer than the mean 2 to 3 week adult life span, the duration of the dauer stage has no effect on the post-dauer life span (Klass and Hirsh, 1976). Sexually, adult *C. elegans* can be either hermaphroditic or male; however, while germ cells proliferate in reproductive adults and atrophy later in life, all somatic cells found in reproductive or postreproductive adults are postmitotic. As adults get older, a marked decrease in their swimming ability, pharyngeal pumping and defecation is observed (Bolanowski *et al.*, 1981; Duhon *et al.*, 1995), along with increased amounts of lysosomal hydrolases, and accumulation of lipofuscin-like granules (Klass, 1977; Bolanowski *et al.*, 1983). A few days before death, postreproductive adult *C. elegans* stop moving.

ENTRY INTO DAUER STAGE

During larval development, environmental conditions integrated at the first and second larval stages serve to determine whether nematodes will reach adulthood following normal or alternate (dauer-forming) developmental pathways (Golden and Riddle, 1982; 1984a,b,c). By monitoring the concentration of dauer-promoting pheromones and food signals, L1 larvae can assess population density, temperature and food supply. Both pheromones and food signals are sensed by the amphids, a pair of chemosensory organs located on the head of *C. elegans*. Each amphid is composed of two support cells and twelve neurons, eight of which are exposed to the environment *via* a pore in the cuticle (Ward *et al.* 1975). The fatty acid-like pheromone is constitutively released by the nematodes, and chemically very stable, while the hydrophilic carbohydrate-like food signal produced by yeast and bacteria is chemically unstable. When the population density of nematodes is high, the concentration of pheromones in the environment is elevated; thus, in overcrowded situations, the alternate life pathway (dauer-forming) is favored. Similarly, high temperature also favors dauer formation, as the response to pheromone is temperature-dependent, with higher temperatures favoring dauer formation. The food signal act as a pheromone antagonist; thus, a low food-to-

pheromone ratio favors dauer formation, while increased food signals promote normal development (Golden and Riddle, 1982; 1984a,b,c).

The formation of dauer larva requires the action of several genes, most of which belonging to the daf (dauer formation) gene family. Three main pathways involved in dauer formation have been identified:

1. TGF-β signaling pathway
2. Insulin receptor/PI3K signaling pathway
3. Guanydyl cyclase pathway

These three pathways have mostly been characterized by studying mutations in daf genes. Dauer-defective mutants (Daf-d) are unable to form dauer larvae, even under overcrowding or low food conditions. Dauer-constitutive (Daf-c) mutants are termed unconditional dauer mutants if they form dauer larvae independently of environmental cues, while temperature-sensitive mutants form dauer larvae at restrictive temperatures only (Table 1). Temperature-sensitive mutants do not form dauer larvae if they are grown at restrictive temperatures once they have reach adulthood.

Studies on dauer mutants reveal that daf genes belonging to the insulin receptor/PI3K pathway serve not only to promote entry into dauer, but also modulate the lifespan of the nematode *C. elegans*. In the next subsections, the insulin signaling pathway and its effect on worm lifespan are discussed.

INSULIN RECEPTOR/PI3K PATHWAY IN *C. ELEGANS*

Insulin and insulin-like signaling pathways are present in most species, including man and *C. elegans*. In the latter, the daf-2 gene encodes an insulin-like receptor orthologous to the mammalian insulin/IGF receptor (Kimura *et al.*, 1997). However, until recently no ligands for daf-2 were known. Studies conducted by Pierce and colleagues (2001) identified in *C. elegans* an insulin superfamily composed of several *ins* members, most of which are found in the amphids, and thus could potentially serve to control dauer formation. Ins-1 and –18 have been studied in detail, and found to interact with daf-2. However, unlike in mammals, these insulin-like ligands do not agonize but

TABLE 1. List of Dauer-Constitutive and Dauer-Defective Genes

Daf-c	-1, -2, -4, -7, -8, -11, -14, -21, -23 (age-1)
Daf-d	-3, -5, -6, -10, -12, -16, -22

This table shows the genes which when mutated, cause dauer-constitutive (Daf-c) and dauer-defective (Daf-d) phenotypes.

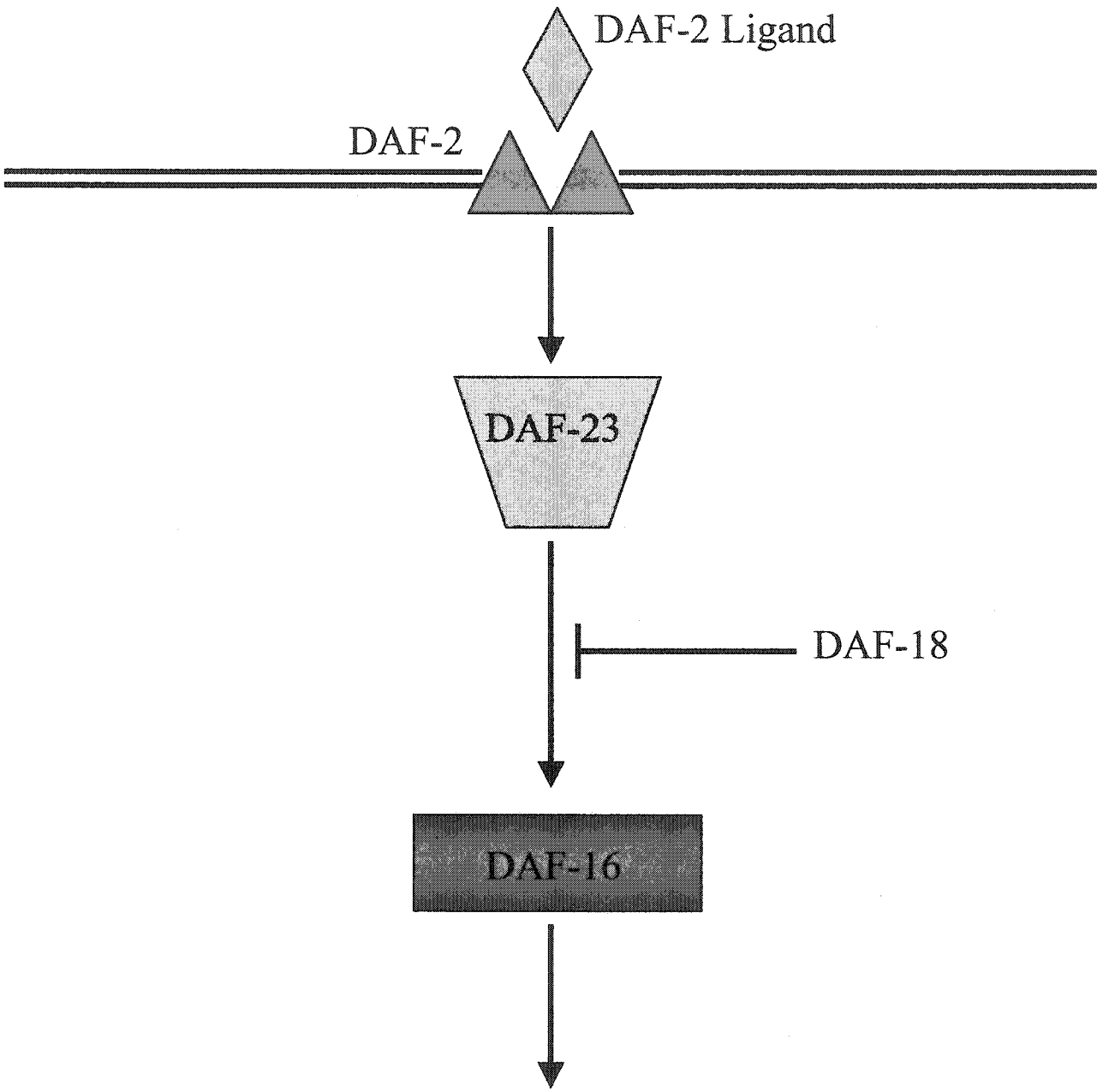
rather antagonize the daf-2 signaling pathway. Similarly, overexpression of human insulin in *C. elegans* also antagonizes daf-2. The net effect of this antagonistic function brings about the formation of dauer larva, which can be abrogated by mutation in daf-16 (Pierce *et al.,* 2001). Further study will be required in order to fully characterize the effect exerted by each *ins* member on the daf-2 pathway, and determine whether some of them act as agonists.

In mammals, the downstream signaling cascade resulting from stimulation of the insulin receptor includes the activation of PI3K, phosphorylation of Akt/PKB, a serine/threonine kinase, and phosphorylation of the winged-helix family of transcription factors, *forkhead* (FKHR) (Robinson-White and Stratakis, 2001). However, excess PTEN prevents the phosphorylation of Akt, and thus negatively influences the insulin-signaling pathway (Waite and Eng, 2002). Similarly, in *C. elegans*, signaling from the insulin-like receptor daf-2 results in the activation of *age-1* (daf-23), an orthologue of PI3K, phosphorylation of Akt/PBK kinase, and ultimately phosphorylation of daf-16, an FKHR transcription factor. The phosphorylation of daf-16 prevents dauer formation; however, the exact mechanisms for this action are unknown. It is believed that hypophosphorylated daf-16 act to repress growth genes, thereby permitting dauer formation, and thus phosphorylation causes daf-16 to lose its function, enabling expression of growth genes. In nematodes, excess daf-18, an ortholog of PTEN, prevents phosphorylation of Akt, thus aborting daf-2 signaling (reviewed in Vanfleteren and Braeckman, 1999)(Figure 8).

As in *Drosophila melanogaster*, mutations of *C. elegans* genes encoding protein involved in the insulin-like signaling pathway can modulate lifespan. Daf-2 mutants are conditional daf-c mutants; thus they form dauer larvae at restrictive temperatures only. However, if grown at a non-restrictive temperature until the end of their third larval stage, and then allowed to grow at 25°C, the adult daf-2 mutant nematodes exhibit twice the lifespan of wild-type counterparts (Kenyon *et al.,* 1993). Similarly, mutations in daf-23 also give rise to long-lived adults (Larsen *et al.,* 1995). To confer extended lifespan, mutations in daf-2 and –23 must be accompanied by functional daf-16 and –18 (Kenyon

FIGURE 8: INSULIN-LIKE SIGNALING PATHWAY IN *C. ELEGANS*

Stimulation of the worm insulin-like receptor, daf2, results in the activation of *age-1* (daf-23), an orthologue of mammalian PI3K. Following its activation, *age-1* phosphorylates Akt. This reaction can be prevented by daf-18. In the absence of daf-18 or when low amounts of daf-18 proteins are present in cells, daf-16 gets phosphorylated following the phosphorylation of Akt. It is believed that the phosphorylation of daf-16 enables the expression of growth genes by inhibiting the transcriptional repression exerted by hypophosphorylated daf-16 on those genes.



Promotes the expression
of growth genes.

et al., 1993; Dorman *et al.*, 1995; Larsen *et al.*, 1995). Mutations in daf-16 cause a small decrease in lifespan (Larsen *et al.*, 1995), while mutations in daf-18 cause premature death due to internal hatching and misshapen body (Dorman *et al.*, 1995; Larsen *et al.*, 1995). Daf-2 and –23 mutants possess body sizes, metabolic and pumping rates similar to those of wild-type animals; however, hermaphrodites show decreased self-fertility (Kenyon *et al.* 1993).

What largely differentiates daf-2 and age-1 (daf-23) mutants from wild-type animals is their level of antioxidant enzymes, as well as their thermotolerance and UV resistance. Both mutants show high levels of catalase and superoxide dismutase (SOD) expression (Larsen, 1993; Vanfleteren, 1993). Increased paraquat and hydrogen peroxide resistance are observed in age-1 mutants. Both daf-2 and age-1 mutants' resistance to UV and thermotolerance is suppressed by mutation in daf-16 (Lithgow *et al.*, 1995; Murakami and Johnson, 1996). As daf-16 is necessary for the expression of dauer genes, it can be presumed that increased resistance to UV and thermotolerance in adult mutants result from genes which also confer such resistance to dauer larva. This is substantiated by the fact that daf –c mutations of daf-7 and –4, two players in the TGF-β pathway necessary to permit dauer larva formation, are also thermotolerant, although they do not show increased lifespan (Lithgow *et al.*, 1995).

CLOCK MUTANTS

Isolated and characterized by Hekimi and colleagues (1995), clock mutants (*clk*) have a slow rate of living, showing an overall increase in the amount of time needed to accomplish each embryonic and post-embryonic developmental stage, compared to wild-type worms. Clk mutations affect the rate of growth to adulthood, and exhibit decreased metabolism, increased cell cycle length, and decreased rates of pharyngeal pumping, defecating, moving and egg laying. Currently 10 *clk* genes have been identified; all mutants show increased lifespan, although no selection on lifespan was conducted to identify the *clk* mutants (Bénard *et al.*, 2002). Lifespan extensions conferred by clock mutations can be increased by daf-2 mutations. The clk-1: daf-2 double mutants are the longest-lived *C. elegans* known (Lakosski and Hekimi, 1996; Wong *et al.*, 1995). The

mechanisms by which clk genes act remains unknown; however, it has been proposed that the slower rate of aging seen in clk mutants results in s slower rate of cellular damage incurred by the organism, and hence results in increased lifespan (Hekimi *et al.*, 1998; 2001). Data also indicate that the molecular pathway by which *clk* extends lifespan is independent of the daf-2 (insulin-like) pathway (Lakowski and Hekimi, 1996; Murakami and Johnson, 1996).

RESISTANCE TO STRESS

The genome of the nematode *C. elegans* possesses four genes encoding SODs, and two encoding catalases. *Sod-1* encodes a cytosolic Cu/Zn Sod; *sod-2, -3* encode two mitochondrial Mn SODs; and *sod-4* has two splicing isoforms, one encoding an extracellular Cu/Zn SOD, the other a membrane-bound Cu/Zn SOD. *ctl-1* encodes a cytosolic catalase, and *ctl-2* a peroxisomal catalase (Giglio *et al.*, 1994a, b; Fujii *et al.*, 1998; Hunter *et al.*, 1997; Taub *et al.*, 1999). Mutations producing a loss of function in the *ctl-1* gene result in decreased lifespan in wild-type animals as well as daf-c and clk-1 mutants (Taub *et al.*, 1999). The role of antioxidants in lifespan extension is evident, as treatment of wild-type worms with SOD/catalase synthetic compounds can increase their mean lifespan by 44% (Melov *et al.*, 2000). Furthermore, both daf-2 and age-1 mutants possess increased levels of catalase and superoxide dismutase, indicating that resistance to oxidative damage may contribute to lifespan extension in these animals (Larsen, 1993; Vanfleteren, 1993).

Stress resistance is a feature of long-lived daf-2 and age-1 mutants. For instance, long-lived age-1 (daf-23) mutants are resistant to hydrogen peroxide and paraquat, possessing increased levels of antioxidant enzymes (Larsen, 1993; Vanfleteren, 1993). Both daf-2 and –23 mutants are also more resistant to heavy metals such as cadmium (Cd) and copper (Cu) than wild-type animals, showing increased ability to synthesize metallothionein in response to some heavy metals (Barsyte *et al.*, 2001).

CALORIC RESTRICTION

To test whether caloric restriction may influence the lifespan of the nematode *C. elegans*, the lifespan of *eat* mutants was studied. *Eat* mutant worms have defective pharyngeal muscle, resulting in inefficient pharyngeal pumping, thus decreased food uptake (Avery, 1993). *Eat* mutants show increased lifespan, corresponding to the degree of food uptake impairment caused by their mutations; the more severe the pharyngeal pumping defect is, the longer the worm lives. In particular, *eat-2* mutants show lifespan 50% greater than wild-type worms (Lakowski and Hekimi, 1998). However, while *eat-2/daf-2* double mutants live longer than the single mutants, *eat-2/clk-1* double mutants do not show lifespan extension beyond that conferred by a single mutation. This indicates that *eat* and *clk* are probably part of the same pathway, or that their lifespan extension is conferred by the same downstream effector (Lakowski and Hekimi, 1998).

SEX AND LIFESPAN

In *C. elegans*, mating can modulate lifespan. However, while mating of male worms with hermaphrodites slightly increases their lifespan, the lifespan of the hermaphrodite is reduced by this mating action (Gems and Riddle, 1996). On the other hand, homosexual interactions between males greatly decrease their lifespan (Gems and Riddle, 2000).

WHAT HAVE WE LEARNED FROM *C. ELEGANS*?

Aging studies conducted in the nematode *C. elegans* have shown that, as observed in *Drosophila*, the overexpression or deletion of some genes can modulate lifespan. It is clear that the regulation of the insulin signaling pathway can greatly impact lifespan, and that the effect appears to be ultimately mediated by control exerted over the forkhead transcription factor. Furthermore, studies in worms have confirmed that oxidative stress can impact lifespan, and resistance to various stressors increases lifespan.

The novelty in nematode aging studies is the discovery of clock genes, and their ability to modulate lifespan. From studies conducted in clock mutant animals, increased lifespan can result from slower developmental and aging rates. Thus the study of nematode aging has revealed that both overexpression of certain genes, and loss of functions in others, may extend lifespan. Furthermore, the study of *eat* mutants reveals that caloric intake can also modulate lifespan.

1.4.5 Rodents as Model of Aging

Rodents are mammalians having much shorter lifespan than humans. As such, rats and mice can easily be used in aging studies. One of the advantages of using rodents in aging studies is that their diet can easily be controlled and modified, enabling us to study the effect of food and chemicals on lifespan. Furthermore, studies conducted on rodents allow us to test whether lifespan-modulating genes identified in unicellular eukaryotes and postmitotic multicellular organisms can also modulate the lifespan of mammalians. Another advantage of using rodents as a model organism is that like humans, they suffer from age-associated diseases which can curtail their lifespan. In this section, some of the factors affecting mice and/or rats lifespan will be briefly introduced.

THE ROLE OF p53 IN AGING

As described earlier in this literature review in the sub-section entitled "pathways to senescence: a brief overview", p53 is a tumor suppressor gene found mutated in several cancers as its inactivation can lead to uncontrolled cellular growth. In senescent cells, p53 is activated and controls the transcription of genes contributing to cell cycle arrest (Pomerantz *et al.* 2000; Zhang *et al.* 1998). Furthermore, p53 can induce apoptosis, also known as programmed cell death, in response to certain stressors (Vousden and Lu, 2002).

To investigate the role of p53 in aging, genetically engineered mice expressing a truncated form of p53 were generated (Tyner *et al.* 2002). This truncated form of p53 augments the activity of wild-type p53 and consistent with p53 function as a tumor

suppressor, mice carrying a single mutant p53 allele are more resistant to tumors than wild type mice. However, this resistance is dependent on the presence of one wild-type p53 allele and one mutant allele. Mutant mice also display early onset of age-associated phenotypes including osteoporosis, reduced body weight, loss of organ mass, decreased wound healing and muscle atrophy. Given this increase in p53 activity, it was anticipated that the mutant mice would live longer than the wild type animal as p53 prevents the spontaneous formations of cancerous cells however, this is not the case. It appears that the presence of excessive p53 activity prevents tissue homeostasis as more senescent cells are present in organs throughout the body. As such, when cell dies, they cannot be replace since senescent cells are unable to replicate and thus tissue mass is lost. Likewise, excessive expression of p53 causes stem cells to undergo premature replicative senescence (Tyner *et al.* 2002).

AMES AND SNELL DWARF MICE

Ames dwarf mice carry a mutation known as *Prop-1*^{df}. Mice homozygous for the *Prop-1* mutation (df/df mice) have a lifespan more than 50% longer than wild type mice. The mutation results in improper embryonic development of the anterior pituitary and as such results in the absence of growth hormone, thyroid-stimulating hormone and prolactin production from the pituitary gland. Ames dwarf mice as their name implies, possess small body size (Brown-Borg *et al.*, 1996).

Snell dwarf mice carry a mutation in the pituitary-specific transcription factor-1 (*pit1*) gene. Pit1 is expressed in mice from 14 days of embryonic life and is necessary for the development of the anterior pituitary. Pit1 and Prop1 are players in the same pathway, however the expression and activation of Prop-1 precedes that of Pit1. As the product of both genes lies within the same pathway, Ames and Snell dwarf mice are phenotypically very similar; both are long-lived, infertile, have small bodies and show signs of hypothyroidism (Gage *et al.* 1996; Sornson *et al.* 1996).

In mice, activation of the insulin receptor (InRβ) results in receptor autophosphorylation and downstream signalling via IRS-1 and –2 (insulin receptor signaling molecule-1 and –2), two molecules interacting with the insulin receptor. These

IRS then signal through PI3K and ultimately regulate the FKRH transcription factor. Upon examination, it was found that the circulating levels of glucose and insulin were much lower in young and aged Snell dwarf mice than in their wild-type counterparts and shown that decreasing amounts of IRS-2 and loss of PI3K activity were present in Snell mice (Hsieh *et al.*, 2002 a, b). As such, it is believed that the lifespan extension seen in dwarf mice is caused by the downregulation of the insulin pathway similar to that observed in long-lived flies and worm.

TWO MURINE MODELS FOR HUMAN AGING

Nine strains of senescence accelerated mice (SAM) were created by selective breading of mice showing early signs of aging. All strains of SAM show signs of aging in early life, some even as early as four months of age. However, not all SAM strains show the same signs of aging and the degree of each sign varies between strains. Signs of aging include hair loss, decreased motility, deficiencies in memory and learning, brain atrophy, hearing impairment, development of cataracts and osteoporosis (Takeda *et al.* 1997). SAM usually die between 12 and 15 months of age while wild type control die between 24 and 30 months of age. Senescence accelerated mice possess low level of Cu/Zn SOD and produce more reactive oxygen species (ROS) than control animals (Park *et al.* 1996; Yuneva *et al.* 2000). They also show an increase in chromosomal aberrations beginning at 3 months of age and have a much higher incidence of lymphomas and other malignancies than control mice. The genetics of SAM mice is not yet fully characterized.

Klotho mutant mice carry an autosomal recessive mutation resulting in a loss of function mutation in the single-pass membrane protein klotho. These mice exhibit phenotypes similar to those observed in human aging (Kuro-o *et al.*, 1997). However, unlike SAM, only one strain of *klotho* mice exist and animals show signs of aging including decreased body weight, arteriosclerosis, neoplasms, cataracts, diabetes, brain atrophy and osteoporosis. The average lifespan of these mutant mice is nine weeks. They are hypersensitive to insulin, indicating that increased signaling through the insulin

signaling pathway could contribute to the modulation of their lifespan (Utsugi *et al.*, 2000).

OVEREXPRESSION OF ANTIOXIDANT ENZYMES

As previously reported, overexpression of catalase and SOD in fly and worm is associated with increasing lifespan. To determine whether overexpression of antioxidant enzymes could yield similar effect in mice, transgenic mice for the hSOD-1 gene were generated (Nabarra *et al.*, 1997). Upon examination, it was found that these transgenic mice showed premature involution of the thymus but decreased age-related accumulation of oxidative DNA damages in the brainstem compared to wild type mice (Cardozo Pelaez *et al.*, 1998). In wild type animals, thymic involution occurs as a consequence of aging. When the lifespan of animals overexpressing hSOD-1 were compared to wild type mice, no significant differences in lifespan were observed (Gallagher *et al.*, 2000). It thus appears that overexpression of SOD in mice cannot extend lifespan as it does in flies and worms (Huang *et al.*, 2000). However, further studies will be needed to fully characterize the role of catalase and SOD, if any, in mice aging.

CALORIC RESTRICTION

Rodents were the first animals used to study the effect of caloric restriction on mammalian organisms. As early as 1935, McCay and colleagues published a paper showing that caloric restriction could increase the lifespan of rodents. However, recent studies show that in order for caloric restriction to extend lifespan, the diet must be undertaken before animal reach middle age (Lipman *et al.*, 1998). Prior to this age, the earlier animals go on the diet the longer they will live (Weindruch and Walford, 1982; Yu *et al.*, 1985). However, caloric restriction decreases fertility and if too severe, results in infertility (Weindruch *et al.* 1986). Life extension conferred by caloric restriction appears to result from retarded aging as Gompertz analysis of mortality rate show that the mortality doubling rate of calorie restricted rat ranges between 187-210 days while that of wild type animals range between 99-104 days (Holehan and Merry, 1986). Furthermore,

evaluation of physiological parameters indicates that most physiological processes in caloric restricted rodents correspond to those of much younger control animals. Increase apoptosis in several tissues including the liver, and of preneoplastic cells, occur as a consequence of caloric restriction and permit the elimination of damaged or potentially dangerous cells (Muskhelishvilli *et al.*, 1995; Grasl-Daraup *et al.*, 1996). While deterioration of the immune system is a well-documented age-associated phenotype in both mouse and humans, caloric restriction does prevent and slow down part of this deterioration. Consistent with a general increase in apoptosis, calorie restricted animals show increased apoptosis of nonfunctional T-cells in older individuals (Spaulding *et al.*, 1997). Caloric restriction also prevents or delays the appearance of age-associated diseases in rodents. Decreased incidences of neoplasms, degenerative disorders and autoimmune disorders are also seen in calorie restricted animals and to a great extent, result from increased apoptosis (Keenan *et al.*, 1995 a, b; Warner *et al.*, 1995). Several hypotheses have been brought forward to try explaining why caloric restriction increases lifespan, however, none have been proven thus far. Warner and colleagues (1995) have suggested that increase apoptosis occurring as a result of caloric restriction permits these animals to live longer as they have better defense against age-associated diseases such as cancers and autoimmune diseases. Other theories attribute this increase in lifespan to decrease in oxidative stress or increased defense against oxidative stress, and signaling *via* the insulin pathway. More research will be needed in order to fully comprehend how calorie restriction extends lifespan.

$p66^{Shc}$ KNOCKOUT MICE

$p66^{Shc}$ is one of the three proteins encoded by the SHC locus and has an src-homolog 2 (SH2) domain, a phosphotyrosine binding domain (PTB) and a unique amino-terminal sequence (Migliaccio *et al.*, 1997; Pelicci *et al.*, 1992). Unlike other Shc which, when a growth factor receptor is activated, are phosphorylated and form complexes with Grb2 in order to subsequently permit Sos (Son of sevenless) to activate Ras by catalyzing GDP/GTP exchange, p66 does not regulate signaling throught Ras.

Instead, p66 regulates by a yet uncharacterized pathway, forkhead proteins (Bonfini *et al.* 1996, Nemoto and Finkel, 2002).

In *C. elegans*, reduction in daf-16 forkhead transcriptional activity results in lifespan extension. In vertebrate, three homologues of daf-16 are present, FKHRL1, FKHR and AFX. Phosphorylation of those homologues prevents them from entering the nucleus and hence causes a decrease in forkhead-dependent gene expression. To investigate whether decrease forkhead activity could also increase the lifespan of mice, $p66^{Shc}$ knockout mice were generated. Homozygous p66 -/- mice live on average 973 days while heterozygous (+/-) live 815 days and wild-type control (+/+) 761 days (Migliaccio *et al.* 1999. Interestingly, p66 knockout mutants are more resistant to agents causing oxidative damage thus supporting the idea that p66 represses oxidative damage repair and participate in the induction of cell death following excessive cellular damage (reviewed in Guarente, 1999).

WILD TYPE DERIVED MICE

Mice used in the laboratory are usually obtained from commercial sources that specialize in breeding mice in captivity. Over several generations, it is possible that the genetic backgrounds of these mice were favored for reproductive capacities rather than for longevity and do not represent genetic backgrounds that would be favorable for these animals in their natural environments. As such, Miller and colleagues (2002) investigated whether wild type derived mice showed differences in lifespan compared to laboratory bred mice. To do so, they generated a DC mice line. The genetic makeup of the DC animals is one quarter of each BALB/c, C57BL/6, DBA/2 and C3H/He genes. When wild-caught Idaho mice were compared to the DC mice, it was found that their median lifespan was 24% longer than DC mice and their maximal lifespan 10% longer. Upon closer examination, it was found that the Idaho mice had lower level of circulating insulin-like growth factor 1 than the DC mice, indicating that these wild type derived mice probably had greater lifespan than laboratory mice because of decreased insulin signaling (Miller *et al.*, 2002).

Ku80 MUTANT MICE

Ku80 is a protein that normally functions to repair double-strand DNA breaks. To investigate whether loss of function mutation in the Ku80 genes would impede lifespan, Ku80 mutant mice (*ku80*-/-) were generated. When compared to wild-type control, the *ku80*-/- mice display premature aging phenotype including osteopenia, atrophic skin and hair follicles, hepatocellular degeneration and changes in mortality rates (Vogel *et al.* 1999). The phenotype of these mice resembles that of humans with Werner's syndrome. When double mutant *ku80*-/-, p53-/- were created, it was found that the cancer incidences rise drastically in those mice and that the animals develop pro-B-cell lymphomas (Lim *et al.* 2000, indicating that DNA repair is an important mechanisms capable of influencing lifespan.

WHAT HAVE WE LEARNED FROM RODENTS?

Rodent studies have traditionally focus on caloric restrictions. Using rodents, it was found that caloric restriction could significantly increase lifespan if the diet was started at an early age. However, the exact mechanism by which caloric restriction extend lifespan remains unknown. Two possibilities have gained popularity: first, that calorie restricted animals are victim of less oxidative stress damage than wild-type animals, and second that caloric restriction confers extended lifespan because it decreases signaling via the insulin or insulin-like pathway. As seen in worms and flies decreased signaling by this pathway can extend lifespan. This probably holds true in mice also as Snell dwarf mice possess low level of PI3K activity and life extension in p66shc mutant mice is likely to be FKHR dependent. However, a third possibility exists. Calorie restricted animals show enhanced apoptotic capacities and as such, it is possible that these animals are better equipped to fight against cancers and autoimmune diseases as they can destroy damaged cells.

The use of rodents in aging studies permits us to study age-associated diseases. Several rodent models such as the SAM and *klotho* mice have been genetically created. Studies conducted on these mice, could in the near future, help us to cure or treat age-

associated disease and enable us to live longer in better health. The study of age-associated disease had been proven limited with other models of aging.

1.5 HUMAN LONGEVITY AND AGING

While the life expectancy at birth in Canada now reach 80.7 years for women and 74.3 years for men, life expectancy without moderate or severe incapacities has regressed in the last few years to 70.5 and 67.7 years respectively (Statistics Canada). We live longer, but not better. In order to enable elderly to remain self-sufficient until their death, it becomes necessary to characterize physiological changes arising as a consequence of normal human aging, better understand the etiology of age-associated diseases, and identify the factors contributing to successful human aging as defined by healthy aging. Studies conducted on models of aging have determined that aging is multifactorial, and is affected by genetic and environmental factors (including life habits). Genetic studies conducted in those models have also shown that lifespan extension can result from the modulation of gene expression. As such, overexpression or on the contrary, decrease in the expression of some genes, can extend lifespan. However, the applicability of data obtained from aging studies conducted in model organisms, to that of human aging remains to be determined. For instance, flies and worms are postmitotic animals, meaning that all of their somatic cells are postmitotic therefore, do not divide. Only few cells, including cardiomyocytes, neurons and myotubes are terminally differentiated postmitotic cells in humans. Likewise, except for rodents, organisms used as models of aging do not appear to suffer from age-associated diseases as we do. Age-associated diseases are probably the main reason why the average lifespan of our species is far from the maximum human lifespan of 122.45 years set by the French centenarian Jeanne Calment in August 1997 (Robine and Allard, 1999). As such, it becomes imperative to conduct human aging studies and determine what affects the lifespan of our specie as well as how can one maintain health until death. There are two main problems one must face when trying to study the aging process, first our specie is long-lived and second how does one differentiate between the normal aging phenotype and that arising from age-

associated diseases? In the next few sub-sections various methods used to study human aging as well as data obtained from those studies will be briefly introduced.

RECQ DEFICIENCIES YIELDS PREMATURE AGING SYNDROMES

In humans, several autosomal recessive disorders give rise in young individuals to phenotypes similar to those observed in old age. Werner's syndrome is the most documented human syndrome associated with an early onset of age-associated phenotype, and results from mutations in a *RecQ* helicase (*wrn*). Individuals with Werner's syndrome age prematurely and develop several diseases common to old age such as osteoporosis, artherosclerosis, cataracts and diabetes type II, are more prone to cancers, and usually die by the age of 50 (Kipling and Faragher, 1997; Bohr, 2002). The mechanism by which *wrn* causes accelerated aging remains unknown however, it is believed that part of the phenotype results from genetic hyper-recombinations, defective DNA repair and improper interaction between the wrn protein and its several known partners, including Ku80, p53 and topoisomerase I (Cooper *et al.*, 2000; Li and Comai, 2000; Lebel *et al.*, 1999; Brosh *et al.*, 2001 a, b). Syndromes similar to Werner's include Bloom's syndrome and Rothmund-Thomson syndromes; both of which are also caused by mutation in RecQ helicases and results in premature aging phenotypes (Mohaghegh and Hickson, 2002). Although it is interesting that mutations in only one of the RecQ helicases can yield many of the phenotypes observed in normal aging, it is unlikely that normal aging results from mutations or deficiencies in only one gene.

HUMAN POPULATION STUDIES

Population studies is one of the approaches used to study human aging in an attempt to characterize the physiological changes accompanying the aging process and identify the factors contributing to successful aging. Two broad classes of population studies exist: longitudinal and cross-sectional studies. Longitudinal studies are design to study one or several parameters in a population by taking several measurements over time from each subject, while cross-sectional studies take only one measurement at a

given time for each subject without any follow-ups. Longitudinal studies are not commonly used to study the human aging process as our specie is long-lived and as such it would be hard to monitor each subject from birth to death to determine which parameters change with age in a given subject. Furthermore, these experiments would be so costly that most researches could not be conducted.

Since the eighties, research efforts have been devoted to the identification of genes and the characterization of gene expression associated with human longevity as it was realized that extremely old individuals such as nonagenarians and centenarians are good examples of successful aging. Extremely old individuals form a unique population as they have reach extreme old age by surviving or escaping age-associated diseases, infectious diseases and common illnesses. Furthermore, extreme old age flies, worms and humans have a decelerating mortality rate compared to the exponentially increasing mortality rate of younger population (Carey *et al.* 1992; Brooks *et al.* 1994; Thatcher *et al.* 1995). Thomas Perls (1995) proposed that this unique mortality rate results from the death of frailer individuals at younger age, leaving a more robust cohort to reach extreme old age. In the following sub-sections, I will introduce some of the results obtained from studies conducted using extremely old and younger age control populations.

GENETIC FACTORS

Population studies are often used in order to identify longevity candidate genes. By studying the prevalence of certain genes or polymorphisms in the extremely old age population versus the prevalence in younger age groups, it is possible to identify three types of genes: frail, neutral and robust genes (Yashin *et al.*, 1999). Frail genes are more prevalent in the younger age groups, neutral genes are present at the same frequency in young and extremely old individuals and robust genes are more prevalent in extremely old age populations. One of the first genes associated with lifespan identified in humans was the apolipoprotein E ε-2 allele (ApoE2)(Schächter *et al.*, 1994). Apolipoproteins are a family of proteins present on plasma lipoproteins, which serve as cholesterol, triglycerides and phospholipids carriers (Smith, 2002). ApoE, one of the members of this family is synthesize in various places in the body including the brain and liver. The liver

mostly contributes to the presence of ApoE in the plasma while the ApoE synthesized in the brain is mostly retained locally (Boyles *et al.*, 1985; Kraft *et al.* 1989). The human ApoE gene has different allelic isoforms called E2, E3 and E4 and their respective products can be distinguished from each other by single amino acid substitutions. The ApoE alleles coding for these different isoforms are referred as ε2, ε3, and ε4. As each individual inherit their genetic background from both of their parents, six different combinations of ApoE can be found ε2/ε2, ε2/ε3, ε2/ε4, ε3/ε3, ε3/ε4 and ε4/ε4. People carrying ε4 alleles have increased chances of developing Alzheimer's and cardiovascular diseases and as such, the ε4 allele is found at much lower frequencies in extremely old populations than in young populations (Mahley and Huang, 1999). While the ε4 allele is a frailty gene, the ε2 allele could be considered as a robustness gene. However, as stated by Perls (2001), to possess a good gene does not necessarily means that you will live old as a person's mortality is also influenced by environments and is likely to be dependent on much more than the presence of a single robust gene.

Several other polymorphisms including the D/D genotype in the gene coding for the angiotensin converting enzyme (ACE) are found at greater prevalence in populations composed of extremely old individuals than in those composed of younger individuals. However, for some genes, these differences are population dependent. For instance, in the Italian population, studies of the association between longevity and the 3'ApoB-Vntr polymorphisms revealed that sub-populations composed of extremely old individuals have a greater incidence of long variant of the gene while sub-populations composed of younger subjects possessed more individuals having the small variant of the gene (De Benedictis *et al.* 1998). When this same study was conducted in the Danish population, no differences were found between sub-populations composed of extremely old and young individuals. This suggests that some gene/longevity associations are population-specific and are influenced by the environmental exposure of each population as well as population-specific genetic (Varcasia *et al.* 2001).

Nevertheless, the genetic influence on one's ability to reach extreme old age cannot be disregarded. In the last decades the Danes have conducted extensive studies in population composed of monozygotic and dizygotic twins and have estimated the heritability of life expectancy to be about 25% (McGue *et al.* 1993; Herskind *et al.*

1996). In more recent years, the New England Centenarian Study found that the siblings of centenarians had ten times more chance to attain the age of 95 years than individuals whose parents died around the average lifespan (Perls *et al.* 1998). Furthermore, this study found that extreme longevity tend to cluster in some families indicating that genetic influences can be involved in achieving extreme old age. Linkage analysis of 137 sibships indicate that it is likely that a locus on chromosome 4 is involved in one's ability to attain extreme old age (Puca *et al.*, 2001).

AGE-ASSOCIATED DISEASES

Age-associated diseases including cardiovascular diseases, diabetes type II, arthritis, neurodegenerative disorders, autoimmune disorders and cancers are plaguing people as early as their fifties and are partly responsible for the gap existing between human average lifespan and maximum lifespan. Age-associated diseases are diseases that are not prevalent in populations composed of young individuals and increase in incidence with the increasing age of the population. It is believed that 20% of age-associated diseases have a strong genetic component (Schultz-Aellen, 1997). The remaining late-onset diseases develop due to a combination of both genetic and environmental factors (Ruse and Parker, 2001). For instance, smoking could trigger the onset of lung cancer in an individual that would, without exposure to smoke, not develop it. Likewise, individuals having robust genetic composition can tolerate life-long smoking without developing any form of cancers. In fact, cancer as a cause of death markedly decreases after 95 yrs of age (Bonafe *et al.* 2001). Furthermore, the incidence and prevalence of other age-associated diseases also decreases in extremely old individuals, partly explaining the leveling off of mortality rates in extreme old age.

While the causes for age-associated diseases are both genetic and environmental, at the molecular level, dysregulation of the balance between cell death and growth is at the center of several age-associated diseases. For instance, cancers results from uncontrolled cell growth caused by an inability of the body to destroy potentially tumorigenic cells before they start their unregulated proliferation (Strasser *et al.* 1997). Likewise, rheumatoid arthritis characterized by the presence of joint swelling is believed

to result from decreased B-cell apoptosis (Hayashida *et al.* 2000; Shimaoka *et al.* 1998). As such, it is likely that the ability to maintain proper balance between cell death and growth is important in attaining extreme old age.

1.6 CELL DEATH

Two modes of cell death are currently known, necrosis and apoptosis. Necrosis is a passive process occurring in cells following severe damage and is characterized by cell swelling, lost of membrane integrity, cell burst and finally cell death. While this process is relatively rapid, it can cause severe tissue damage as necrosis is generally accompanied with inflammation. Unlike necrosis, apoptosis, also known as programmed cell death, is an active process requiring energy, first characterized by Kerr, Wyllie and Currie in 1972. Apoptosis is morphologically and biochemically different from necrosis and is characterized by plasma membrane blebbing, cell shrinkage, nuclear chromatin condensation and in most instances, the formation of non-random DNA fragmentation (Wyllie *et al.* 1980). The DNA fragmentation pattern can be visualized on a gel as a ladder having fragments length of multiples of 180-200 base pairs (Arens *et al.* 1990). Apoptosis is much less damaging to tissue than necrosis as apoptotic cells do not burst or cause tissue inflammation, instead, they are phagocytosed, engulfed by neighboring cells (Savill and Fadok, 2000). *In vivo,* apoptosis is the method of choice to remove non-functional or damaged cells, autoreactive lymphocytes and to accomplish organ sculpting during embryogenesis (Jacobson *et al.* 1997).

CASPASES

Apoptosis can be triggered by several different pathological and physiological stimuli. However, whatever the stimuli, two main families of proteins, the caspases and the Bcl-2 family, serve to control the apoptotic process. While caspases are the executioners of apoptosis, Bcl-2 family members are the regulators of apoptosis (Cohen, 1997; Gross, 2001).

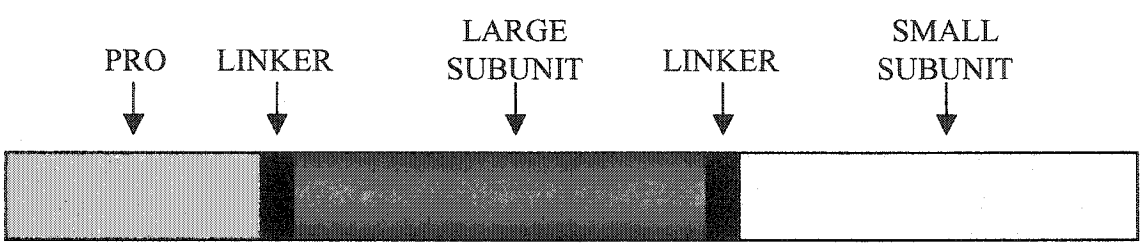
Caspases are cysteine-dependent aspartate-directed proteases, as such, they are proteins having cysteine domains capable of cleaving proteins after aspartate residues. Currently, 14 mammalian caspases are known and named caspases-1 to –14 in the order they were discovered (Van de Craen *et al.* 1998). All caspases are synthesized as zymogens, a form of inactive protein requiring processing before being able to perform their functions. At their amino terminal (N-terminal), they have a prodomain, which is followed by a sequence encoding a large protein subunit then by one encoding a small protein subunit. In addition, some caspases have a linking region between the large and small subunits. In order for caspases to be processed into their active form, they must be cleaved at aspartate residues (Cohen, 1997). However, since the only known proteins capable of accomplishing this task are caspases themselves and granzyme B, caspases processing mostly occurs by auto-catalysis (Greenberg, 1996; Pham and Ley, 1997; Thornberry *et al.* 1997). Some caspases such as caspase-1, have an intrinsic low level of catalytic activity even when they are in their proform and as such, can cleave other caspases and render them active (Ramage *et al.* 1995). Once cleaved, caspases assemble to form a tetramer composed of 2 large subunits and 2 small subunits. This tetramer forms an active enzyme having active sites at opposite ends (Reviewed in Cohen, 1997) (Figure 9).

The prodomain region of caspases varies in size and can be use to define sub-groups within the caspase family. As such, caspases having long prodomains such as caspase-8 and -10 are defined as initiator caspases while those having short prodomains, such as caspase-3 are defined as effector caspases (Reviewed in Earnshaw *et al.* 1999). The long prodomain of caspase-8 and -10 possesses a death effector domain (DED) which permits interaction between initiator caspases and adaptor signaling protein such as MORT1/FADD (Fas associated death domain) and TRADD (TNF receptor associated death domain) (Chinnaiyan *et al.* 1995; Hsu *et al.* 1995). Some of these adaptor proteins possess two DED, one to interact with the receptor and one to recruit caspases, while others possess a caspase recruitment domain (CARD) which can recruit caspases having similar CARD domains and serve to initiate a caspase cascade (Hofmann *et al.* 1997). Caspases are also sub-divided in families according to phylogenic analysis. Two principal caspase families exist: the caspase-1 and the caspase-3 family. The caspase-1

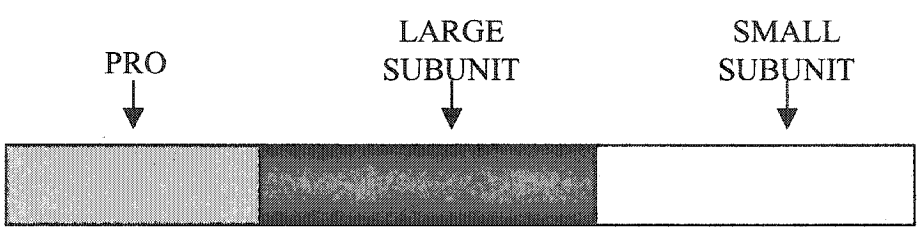
FIGURE 9: CASPASE-1 AND –3 PROFORMS

Caspases are synthesized an inactive protein known as zymogens. At their amino-terminal inactive caspases possess a prodomain region that gets cleaved when caspases are processed into their active forms. Following this prodomain region is a large subunit and then a small subunit. Some caspases such as caspase-1 have linker regions between their subunits. Both the prodomain and the linker regions are not retain in the mature forms of the protein. Active caspase enzymes are tetrameric, they are composed of two small and two large subunits which assemble together.

CASPASE-1 (ICE)



CASPASE-3 (CPP32)



family includes caspase-1, -4, -5 and –13 while the caspase-3 family include the caspase –3, -6, -7, -8, -9, and –10 (Reviewed in Earnshaw *et al.* 1999). The caspase-1 family is mostly involved in cytokine processing and inflammation while the caspase-3 family is the main apoptotic family. Another family centered on caspase-2 is also sometime defined, however this family as well as its functions remain poorly defined.

APOPTOSIS TRIGGERED BY SURFACE RECEPTORS

Several cells exhibit surface receptors that once activated by the binding of a specific ligand, can induce cells to undergo apoptosis. The most characterized of these pathways include the CD95/Fas/Apo-1 and the tumor necrosis factor receptor pathway (TNFR1).

CD95/Fas is a surface receptor capable of binding Fas ligand (FasL) and which upon ligation is activated as a trimeric receptor. Fas associated death domain protein (FADD/MORT1), an adaptor molecule, is recruited to the intracellular tail of the trimeric receptor where interactions between death domains (DD) enable physical contact between the receptor and the adaptor molecules (Chinnaiyan *et al.* 1995). FADD, which possesses a DED at its amino-terminal, can recruit caspase-8, an initiator caspase also possessing a DED, and facilitates the activation of caspase-8 such that the latter will become an active enzymes capable of cleaving caspase -3, -4, -7 and -9 into their active enzymatic forms (Boldin *et al.*, 1996; Murio *et al.* 1996; Srinivasula *et al.* 1996; Muzio *et al.* 1997). Activated caspases then cleave substrate proteins essential for cellular survival, and bring about the process of programmed cell death.

Ligation of other receptors such as the tumor necrosis factor receptor-1 (TNFR1) can also initiate apoptosis. When tumor necrosis factor alpha (TNFα) binds TNFR1, the receptor gets activated and thus causes the release of inhibitory proteins such as silencer of death domains (SODD), and enables the binding of the adaptor molecule TRADD (reviewed in Chen and Goeddel 2002). TRADD can in turn bind FADD, leading to the caspase cascade describe above. TNFR1 can also recruit the adaptor molecule receptor interacting protein (RIP) which can recruit RAIDD and CRADD. The latter possess a

CARD domain enabling them to recruit procaspase-2 (Duan and Dixit, 1997; Ahmad *et al.* 1997; Chou *et al.* 1998) (Figure 10).

Both CD95/Fas and TNFR1 receptor can also bind RIP2/CARDIAK and promote the activation of procaspase-1 (Thome *et al.* 1998; McCarthy *et al.* 1998). Procaspase-1 is then converted into its active form and generates a caspase cascade. This apoptotic pathway involving a kinase known as CARDIAK, is unique to caspase-1, and one of the only pathway in which caspase-1 plays a major role as the natural function of caspase-1 formerly known as ICE for interleukin-1β converting enzyme, is to cleave the proform of interleukin-1β, -1α, interleukin-18 and γ-interferon, and process them into mature cytokines (Thome *et al.* 1998; Miura *et al.* 1993; Kuida *et al.* 1995; Gu *et al.* 1997) (Figure 11).

APOPTOSIS VIA THE MITOCHONDRIA

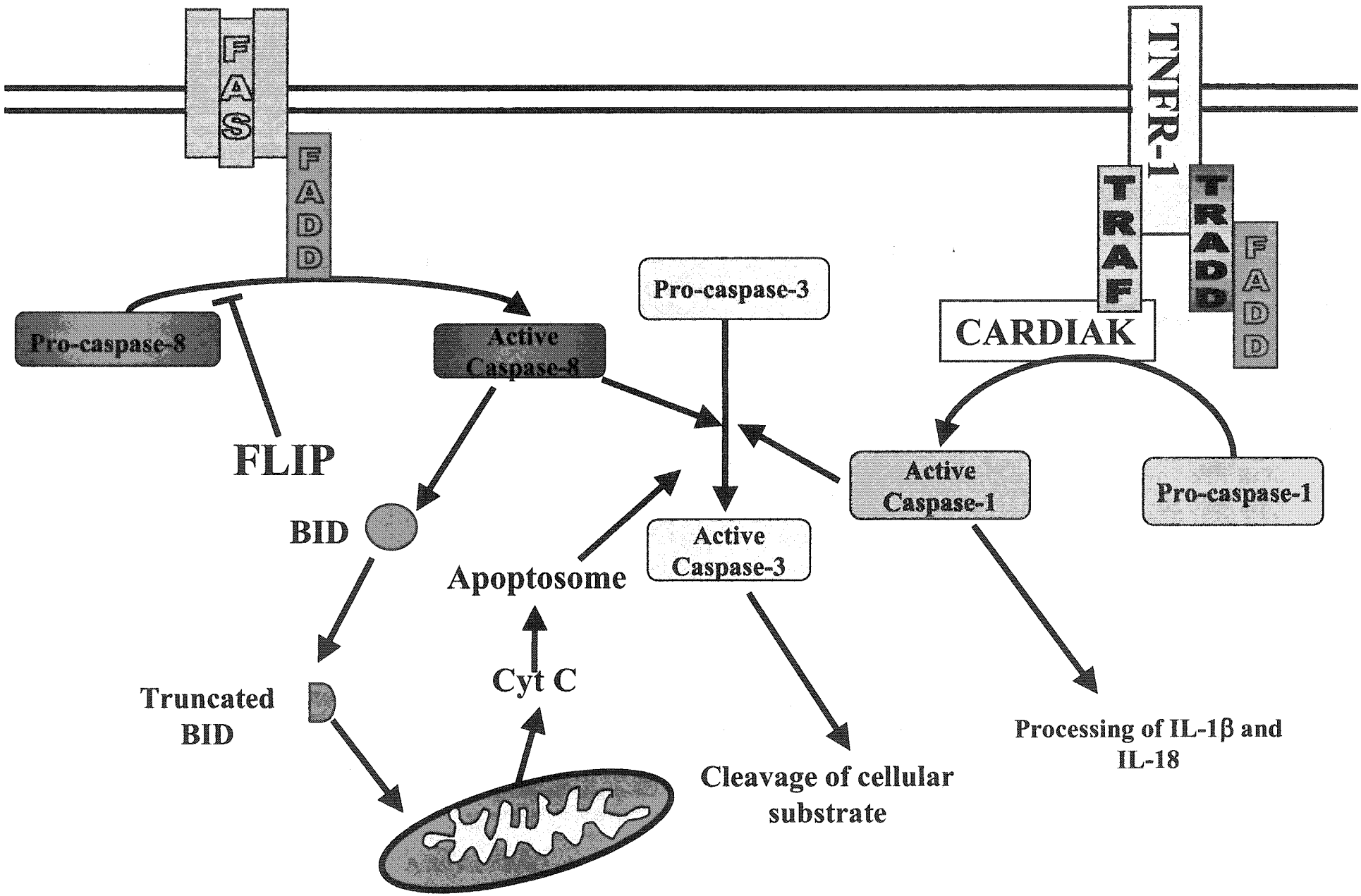
In response to oxidative damage and other forms of chemical damage, cells can trigger apoptosis independently of surface receptor by inducing the release of cytochrome-c from mitochondria (Eiscehn *et al.* 1997; Yang *et al.* 1997; Kluck *et al.* 1997; Kharbanda *et al.* 1997). The mechanism by which cytochrome-c is released from mitochondria remains unknown however, it is believed that the formation of a pore by pro-apoptotic Bcl-2 family members, including Bax, serves to bring about this process (Antonsson *et al.* 1997; Schlesinger *et al.* 1997). Upon cytochrome-c release, Apaf1, a cytosolic protein, binds to cytochrome-c causing Apaf1 to undergo a conformational change enabling its CARD domain to bind the CARD domain of caspase-9 which once activated, can cleave effector caspases such as caspase-3 and -7 and begin a caspase cascade (Li *et al.* 1997; Srinivasula *et al.* 1998).

BCL-2 FAMILY MEMBERS

The Bcl-2 family includes a wide range of pro-apoptotic as well as anti-apoptotic proteins, which serve to regulate the apoptotic process (reviewed in Gross, 2001). Their most characterized role is in the regulation of cytochrome-c associated apoptosis. For

FIGURE 10: PATHWAYS TO APOPTOSIS

CD95/Fas and TNFR1 are surface receptors capable of inducing cell death following ligation by their specific ligand. FADD, an adaptor molecule, is recruited to the intracellular tail of Fas receptor where interactions between death domains (DD) enable physical contact between the receptor and the adaptor molecules FADD, which possesses a DED at its amino-terminal, can recruit caspase-8, an initiator caspase also possessing a DED, and facilitates the activation of caspase-8 such that the latter will become an active enzyme capable of cleaving caspase -3, into its active enzymatic form. Activated caspases-3 can cleave substrate proteins essential for cellular survival, and bring about the process of programmed cell death. TNFR 1 can bind both TRADD and TRAF. TRAF can bind CARDIAK, a kinase, and promote the activation of caspase-1. Active caspase-1 can cleave pro-caspase-3 in its active form as well as process cytokines into their mature form. Activated caspase-8 can also cleave BID, which then translocates to the mitochondrial membrane where it causes the release of cytochrome-c. Upon cytochrome-c release, Apaf1, a cytosolic protein, binds to cytochrome-c causing Apaf1 to undergo a conformational change enabling it to bind caspase-9 and activate it. Once activated, caspase-9 can cleave caspase-3 and begin a caspase cascade. In the diagram, the apoptosome refers to the cytochrome-c/Apaf-1/caspase-9 complex.


FAS
FADD
TNFR-1
TRAF
TRADD
FADD
Pro-caspase-8
Active Caspase-8
Pro-caspase-3
CARDIAK
FLIP
BID
Active Caspase-1
Pro-caspase-1
Active Caspase-3
Apoptosome
Truncated BID
Cyt C
Cleavage of cellular substrate
Processing of IL-1β and IL-18

instance, it is known that cleavage and activation of Bid, a member of the Bcl-2 family causes it to translocate from the cytosol to the mitochondrial membrane where it induces the oligomerization of Bak and Bax which results in the release of cytochrome-c from mitochondria (Eskes *et al.* 2000; Wei *et al.* 2000). However, the exact method by which Bcl-2 family members participate in the release of cytochrome-c from mitochondria, remain unknown. Anti-apoptotic Bcl-2 family member such as Bcl-X_L, can inhibit the formation of the Apaf-1/caspase-9 complex and thus prevent cellular apoptosis (Hu *et al.* 1998). Bcl-2 family members posses conserved domains known as BH domains (Bcl-2 homology domain) that allow them to attach to the mitochondrial membrane, and often carry at their carboxy-terminal a hydrophobic domain (Adams and Cory, 2001). Bcl-2 members can form homo and heterodimers amongst themselves and as such, anti-apoptotic Bcl-2 family members can neutralize pro-apoptotic family members (reviewed in Gross, 2001).

CLEAVAGE OF CELLULAR COMPONENTS DURING APOPTOSIS

Upon activation, caspases act as a pair of scissors and cleave proteins throughout the cells. Among caspase preferred targets are key components of the cytoskeleton, the nucleus and important signaling pathways. Cytoplasmic proteins cleaved by caspases include actin and intermediate filament proteins while nuclear targets include lamins and RNA-binding and associated proteins (Mashima *et al.* 1997; Lazebnik *et al.* 1993; Oberhammer *et al.* 1994). Important signaling molecules affected include MAPK, and STAT (Widman *et al.* 1998; King and Goodbourne, 1998). Caspases also cleave proteins that are directly involved in the control of apoptosis, notably, they cleave procaspases into their active form, as well as the survival factor Bcl-2 in order to inactivate it (Cheng *et al.* 1997). Caspases also cleave ICAD (Inhibitor of CAD), a family of DNAse CAD (caspase activated DNAse) inhibitors, thereby enabling CAD to cleave nuclear DNA, thus resulting in general chromosomal degeneration observed as a ladder on a gel (Sakahira *et al.* 1998).

THE EMERGING PICTURE

As several different stimuli can trigger apoptotic cell death, not all caspases are involved in all pathways leading to apoptotic cell death. However, the result of all of those pathways is similar in that caspases serve to cleave essential cellular substrates thereby rendering the cells enable to perform their functions and rendering them suitable for phagocytosis and thus, permit their easy removal from tissues. Very little is currently known about the transcriptional regulation of apoptosis and until recently caspases were believed to be constitutively expressed. Several more years of research will be necessary to fully understand how apoptotic cell death occurs under different conditions and to determine the functions of all caspases.

1.7 THE IMMUNE SYSTEM

From birth to death, our immune system is constantly being modified in order to better protect our bodies against foreign organisms as well as in response to the natural aging process. In this section, I will briefly introduce basic immunobiology, most specifically related to B cells and T cells, and provide a global view of the changes that occur in the immune system with aging. In the last sub-section, I will briefly review the literature pertaining to apoptosis in the immune system.

THE IMMUNE SYSTEM: AN OVERVIEW

The immune system is composed of several specialized cells collaborating together to provide a line of defense against foreign organisms and substances. The immune system is traditionally divided into two main modes of defense, the innate immune system and the adaptive immune system. The innate immune system is the first line of defense and serves to fight many common microorganisms. This mode of defense does not require previous exposure to a pathogen however, its ability is limited as several pathogens cannot be recognized by the innate immune system. On the other hand, the adaptive immune system as its name implies, requires a minimum of 4 to 7 days exposure

to a pathogen before it can act against it unless it has previously encountered this pathogen. The adaptive immune system relies on antibodies and is highly specialized. Lymphocytes are the main effectors of the adaptive immune system. (reviewed in Janeway *et al.* 1999).

B CELL DEVELOPMENT

Three models have traditionally been used to describe the process of B cell development, one proposed by Osmond, one by Hardy and colleagues and one by Melchers-Rolink and colleagues (Osmond, 1990; Hardy *et al.* 1991; Rolink and Melchers, 1993). As in the last decade attempts to unify these models have been made, elements of these three models will be used here to provide a general overview of B lymphopoiesis (Lu *et al.* 1998; Osmond *et al.* 1998).

In adult, B cell development mostly takes place in the bone marrow and can be monitored by assessing the expression of various well-characterized cell surface and intracellular markers. It is currently believed that B cell are derived from a common lymphoid progenitor cell found in the bone marrow. This common lymphoid progenitor is derived from the pluripotent hematopoietic stem cell also found in the bone marrow. However, although pluripotent hematopoietic stem cells have been widely documented, the existence of a lymphoid progenitor cell, giving rise to both B and T cells, remains questionable.

Three main stages of B cell development have been characterized by Osmond, pro-B cells, pre-B cells and B cells (reviewed in Osmond *et al.* 1998). The pro-B cell stage is further subdivided into three groups, early, intermediate and late. Early pro-B cells express the enzyme TdT, an intracellular enzymes necessary for B cells μ-chain gene rearrangement. As mature B cells produce antibodies, this gene rearrangement serves to diversify the repertoire of antibodies and enables different B cells to recognize different pathogens. Intermediate pro-B cells express the surface antigen B220 also known as CD45 (RA isoform), a marker for hematopoietic derived cells, as well as CD19, a B cell marker, in addition to the intracellular enzyme TdT also found in early pro-B cells. In the last stage of pro-B cells development, cells known as late pro-B cells

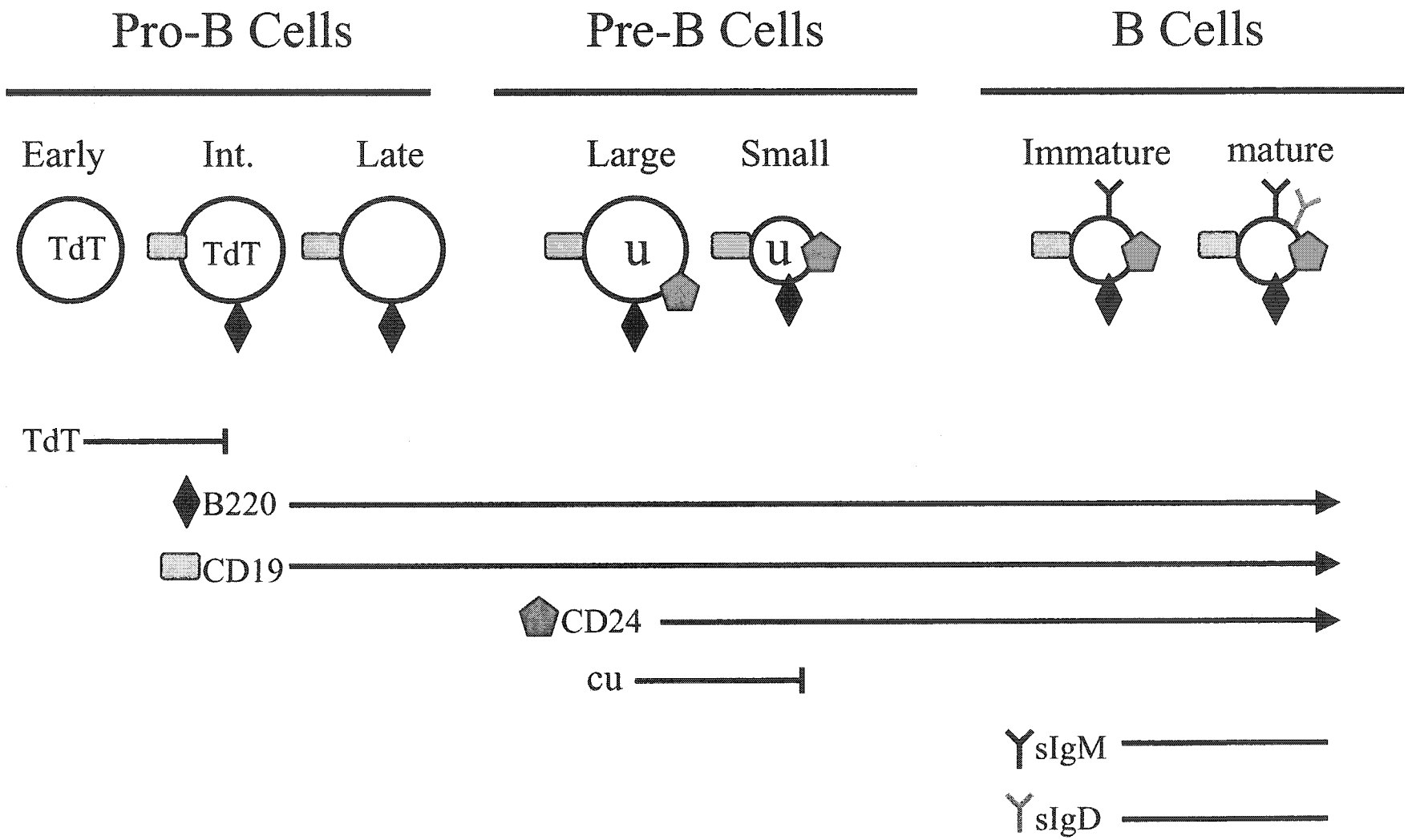
retain surface expression of B220 but stop expressing the intracellular enzyme TdT as the gene rearrangement necessitating this enzyme is completed. Late pro-B cells then become pre-B cells. The pre-B cell stage can be sub-divided in two categories based on cell size: large pre-B cell and small pre-B cell. Large pre-B cells become small pre-B cells and both express CD19, B220, the intracellular μ-chain, as well as the heat-stable antigen (HSA), also known as CD24. Small pre-B cells then become immature B cells expressing surface immunoglobulin M (IgM), CD19, CD24, and B220. Immature B cells migrate to the spleen where they become mature B cells following the appearance of IgD at their cell surface. Mature B cells express both IgM and IgD (Figure 111) These mature B cells are known as naïve B cells, meaning that they have not encountered the antigen against which their antibody will react and as such, have not undergone clonal proliferation. When mature naïve B cells encounter an antigen with whom to react, and after proper signaling, they become lymphoblasts. At this stage, they will rearrange their immunoglobulin gene by alternative splicing and start producing immunoglobulin G (IgG), a very specific type of antibody characteristic of memory B cells. If memory B cells re-encounter the antigen that triggered this Ig switching, they will become terminally differentiated as plasma cells and secrete their antibodies.

T CELL DEVELOPMENT

As for B cells, it is believed that T cells are derived from the common lymphoid progenitor cells, themselves a product of the pluripotent hematopoietic stem cells. Although T cells originate in the bone marrow, at a very early stage, they migrate to the thymus where their development will take place and can be assess by monitoring the expression of surface and intracellular markers. Precursor T cells entering the thymus do not express any common T cell surface molecules, and have not undergone T cell receptor gene rearrangement. These cells are known as double negative thymocytes as they do not express the surface antigens CD4 and CD8. In fact, they are also negative for the mature T cell marker CD3. Slowly, as double negative thymocytes start to assemble their pre-T cell receptors (pre-TCR), they begin expressing the surface molecule CD3, a T cells marker, as well as CD4 and CD8. As such, double negative thymocytes

FIGURE 11: B CELL DEVELOPMENT

This figure gives a schematic view of B cell development. B cell development can be divided in three main stages: pro-B cell, pre-B cell and B cell. The pro-B cell stage can be further subdivided in three stages: early, intermediate and late pro-B cell. Early pro-B cells express TdT. Intermediate pro-B cells express TdT as well as B220 and CD19. Late pro-B cell express B220 and CD19 but not TdT. The pre-B cell stage can be subdivided into two stages: large and small pre-B cells. Both large and small pre-B cells express the same markers, B220, CD19, CD24 and intracellular μ chain. However they can be differentiated by their size. B cells are either immature or mature. Immature B cells express B220, CD19, CD24 and surface IgM. Immature B cells migrate to the spleen where they will become mature B cells characterized by the appearance of IgD at their cell surface.


Pro-B Cells
Pre-B Cells
B Cells
Early
Int.
Late
Large
Small
Immature
mature
TdT
TdT
u
u
TdT
B220
CD19
CD24
cu
sIgM
sIgD

differentiate into double positive T cells. Double positive thymocytes expressing self recognizing MHC (major histocompatibility complex), then mature and become single CD4 or CD8 positive T cells. While naïve T cells are CD45RA positive, memory T cells become CD45RO positive (reviewed in Janeway *et al.* 1999).

T-CELL AND B-CELL FUNCTIONS: AN OVERVIEW

Two types of T cells exist, cytotoxic T cells characterized by the surface expression of CD8, and helper T cells characterized by the surface expression of CD4. Cytotoxic T cells serve to destroy cells that are infected with foreign pathogens such as viruses, while helper T cells serve to activate B cells and prompt them to release their antibodies. Antibodies released by B cells can participate in the host defense in three main ways: first they can neutralize bacterial toxins, second they can opsonize foreign pathogens such as bacteria, and third, they can activate the complement system.

Cytotoxic T cells can recognize infected cells, as cells throughout the body possess major histocompatibility complex (MHC). Two types of MHC exists, MHC type I, and MHC type II. MHC type I is found on most cells of the body and serves to collect peptides derived from proteins present in the cytosol and present them to the cell surface. As such, when a cell gets infected by viruses, MHC type I pickup pieces of viral particles in the cytosol of infected cells and display them at the cell surface. Cytotoxic T cells, which recognize the combination of self MHC containing foreign peptides in their groove, can then act to kill the cell. To ensure that cytotoxic T cells only recognize the combination of self MHC with foreign peptide and do not kill cells expressing at their surface self MHC containing self peptide, during their development, T cells are selected. As such T cells recognizing self peptides undergo apoptosis in the thymus.

Helper T cells serve to activate B cells as well as macrophages. Specialized type of cells known as antigen presenting cells (APC) including B cells, macrophages and dendritic cells, possess MHC type II. MHC type II collect peptides from intracellular membrane bound vesicles such as those created by phagocytosis, and display them at the cell surface. When helper T cells recognize the combination of self MHC type II and

foreign peptides on the surface of B cells, they signal and activate to B cells Similarly, they can also activate macrophages.

B cells unlike T cells do not serve to recognize internalized foreign peptide. Their main task is to recognize foreign peptide in the extracellular environment via their surface antibodies so foreign toxins and organisms can be phagocytosed by macrophages or other scavenging cells (reviewed in Janeway *et al.* 1999).

AGING, APOPTOSIS AND THE IMMUNE SYSTEM

The aging process brings about several physiological changes throughout the body including the immune system. For T cells, aging implies decrease output of naïve lymphocytes from the thymus, decrease T-cell response, non-specific activation, altered cytokine expression and the accumulation of memory T lymphocytes (Timm and Thoman, 1999; Li and Miller, 1993; reviewed in Miller, 1996). For B cells, aging mainly results in a decrease B cell production from the bone marrow and an increase production of low-affinity antibodies (Stephan *et al.*, 1996; Zharhary, 1988; Doria *et al.*, 1978). These changes in the immune system render elderly more vulnerable to infections, is associated with a rise in autoimmunity, and lead to immunosenescence characterized by the shortening of telomeres and the presence of replicative senescent immune cells (Adidzadeh *et al.*, 1996; Vaziri *et al.*, 1993).

Apoptosis is vital for the immune system as its permits the elimination of self-reactive immune cells as well as non-functional, defective B and T cells during the process of lymphocyte development (Lu and Osmond. 1997; Murphy *et al.* 1990; Surh and Sprent, 1994). If cells reacting against self are not deleted autoimmune disease can arise. Furthermore, following T cell activation and clonal expansion in response to antigens, in order to maintain a constant amount of circulating immune cells, the expanded population of T cell must be eliminated (Akbar and Salmon, 1997). This elimination is accomplished by apoptosis and is term activation-induced cell death (AICD). Failure to undergo AICD can lead to overstimulation and have pathological consequences (Abbas, 1996).

Apoptotic capabilities are modified as a consequence of aging. As such, senescent T cells accumulate in age mice as a result of decrease Fas-mediated apoptosis (Zhou *et al.* 1995). However in humans, some investigators have reported increased expression of Fas on lymphocytes from elderly donors, while others have shown that human T cells having reached replicative senescence show decrease Fas-mediated apoptosis (Gupta, 2000; Spaulding *et al.* 1999). Upon closer examination, Potestio and colleagues (1999) found that the expression of Fas on T cells increases up to the age of 75 and decline in the very old donors however, no correlation exist between increase Fas surface expression and increased apoptosis (Phelouzat *et al.,* 1997; Aggarwal and Gupta, 1998; Potestio *et al.*, 1998). As the expression of Fas appears to be a poor method to analyze the apoptotic susceptibility of T cells, investigators turned their attention to the study of caspases in aging immune cells. Analysis of caspase-3 activity was shown to be reduced in senescent T cells (Spaulding *et al.* 1999). More studies will be needed in order to fully document the changes in apoptosis accompanying the aging process.

1.8 MY PROJECT

Given the importance of apoptosis as a mechanism to maintain the homeostasis of the immune system, as well as to ensure the proper deletion of damaged and non-functional immune cells which would otherwise lead to the development of pathological conditions, it is likely that proper apoptotic functions contribute to healthy aging. As extremely old individuals such as nonagenarians and centenarians are successful model of aging, I hypothesize that extremely old individuals possess a robust immune system capable of adaptation and having beneficial apoptotic regulation. As such, in order to determine whether apoptosis in the immune system, could be part of the determinant of longevity the specific aims of my project were as follow:

1. To develop a method to maximize the recovery of biological sample from blood samples obtained from individuals of all ages including extremely old individuals.

2. To determine whether patterns of caspases mRNA expression change amongst sub-populations composed of individuals of different age.

3. To determine whether pattern of caspases mRNA expression are dependent on given immune profile.

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PREFACE TO CHAPTER 2

In order to test my hypothesis that apoptosis is one of the determinants of longevity, my first task was to obtain enough biological materials to conduct my study. As such, during the fall of 1998 and the summer of 1999, I went to Taiwan with some of my colleagues to collect the blood of 246 individuals of Chinese origin. As very limited amount of blood could be obtained from extremely old individuals, it became essential to develop a methodology to process the blood samples in order to generate as much biological material as possible. In the next chapter, I present the methodology that I developed to process the blood samples in a more efficient way. Since the laboratory we had in Taiwan was very rudimentary, as it consisted in an hospital room that we transformed into a laboratory, the protocol was also developed to ensure that the blood samples could be processed without the need for expensive, large fancy equipment.

CHAPTER 2

BLOOD SAMPLE PROCESSING FOR THE STUDY OF AGE-DEPENDENT GENE EXPRESSION IN PERIPHERAL BLOOD MONONUCLEAR CELLS

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2.1 ABSTRACT

Although previous population studies involving nonagenarians and centenarians have identified several candidate longevity genes, and described some age-related physiological changes, further research will be needed in order to fully comprehend the normal aging process and identify the factors involved in successful human aging. However, while most of these new biogerontological studies will be population-based, and likely to involve the collection of blood from extremely old individuals, to our knowledge, no unified protocols have yet been published to describe a methodology permitting the simultaneous generation of different kinds of biological specimens derived from a single source of a very small volume of peripheral blood. The restriction on volume of blood is due to the fact that extremely old individuals, such as nonagenarians and centenarians, cannot afford to donate large quantities of blood without significantly compromising their health. Here we describe a method permitting the generation of plasma, RNA, DNA, protein samples, fixed lymphocytes and frozen blood aliquots from a single 10 to 30 ml blood sample, and discuss the yield and quality of each kind of extracted biological material. Moreover, using fixed lymphocytes extracted following this protocol, we show that there is an age-dependent decrease in the expression of CD24 in peripheral blood mononuclear cells (PBMC). This assay shows the powerful use of these samples as vital biological materials for investigating the genetic mechanism for human longevity, and defining age-associated biological changes.

2.2 INTRODUCTION

Population studies involving nonagenarians and centenarians have long been used in aging research, and have permitted the identification of several genetic factors associated with life-span determination, and served to define age-related physiological changes. For instance, it was found that while HLA-DR1 is present at greater frequency in populations composed of extremely old individuals, thus found in association with longevity (Takata *et al.*, 1987), both the e4 allele of apolipoprotein E and HLA-DR9w are present at much lower frequency in populations composed of centenarians (Takata *et al.*, 1987; Schachter *et al.*, 1994; Louhija *et al.*, 1994). Furthermore, the use of peripheral blood samples extracted from individuals within populations have enabled us to describe some age-related changes occurring within the immune system, such as the decrease in naïve, and increase in memory, T lymphocytes with age (Miller, 1996). However, despite the identification of several factors associated with normal aging or favoring the attainment of extreme old age, the emerging picture shows that successful aging is regulated by complex traits involving genes, and their interactions with environmental factors. Investigation of these genes and their expression requires studies to be conducted at the DNA, RNA, protein and cellular levels concurrently. Therefore, for any population study in aging research, the amount and type of biological material obtained from each individual within the population often remains an experimental limiting factor, leaving results to be concluded from fragmented data generated from only one or a few of the above types of biological material.

In this paper, we describe a methodology permitting the simultaneous generation of RNA, DNA, protein and plasma samples, as well as fixed PBMC and frozen blood aliquots, from a single 10-30 ml sample of peripheral blood, and discuss the yield and quality of each derived biological material. Moreover, from samples extracted using this method, we have documented in peripheral blood mononuclear cells (PBMC) an age-related decrease in expression of CD24, a small, heavily glycosylated cell surface protein, present in abundance on unprimed B cells as well as on those differentiating into antibody producing cells, present in the PBMC (Alterman *et al.*, 1990; Allman *et al.*, 1992). The characterization of decreased CD24 expression with age is important, as it

can serve as an immunological marker for the normal aging process. The protocol reported here provides gerontologists a systematic method for generating banks of biological materials, suitable for analysis at the molecular and biochemical levels; the identification of an age-dependent decrease in CD24 expression is merely a foretaste of numerous studies to come.

2.3 MATERIALS AND METHODS

CHINESE POPULATION FROM TAIWAN

In order to study the factors involved in successful aging, we established a new research population composed of Chinese individuals from Taiwan. Over a three-year period, we recruited over 246 healthy individuals, aged 10 to 102 yrs, whose ancestors settled in Taiwan over 300 years ago, and who have inhabited the central part of the island since their birth. A description of the population can be found in Table 1. Prior to the beginning of our study, potential subjects were interviewed and explained the purpose of the study, how the study would be conducted and the risks involved in participating in the study. All retained subjects voluntarily signed the Institutional Review Board (IRB) consent form, and were allowed to withdraw from the study at any time. As part of the study, subjects were ask to provide information relating to their lifestyle, hobbies, daily activities and diets. Furthermore, each participant was asked to perform a MMSE (mini-mental status exam), and was medically examined by a qualified physician. This study was approved by the institutional review boards of Taichung Veterans General Hospital (Taichung, Taiwan), and the Sir Mortimer B. Davis Jewish General Hospital, a teaching hospital of McGill University (Montreal, Canada).

COLLECTION OF BLOOD SAMPLES

Between 10 to 30 ml of blood samples was collected from each subject in 5 ml ethylenediaminetetraacetate (EDTA) Vacutainers (Becton Dickinson). Blood-filled vacutainers were agitated to allow the mixing of the anticoagulant with the blood, and

stored on ice for a maximum of four hours transit before reaching the laboratory. Upon reaching the laboratory, blood samples were immediately processed or stored in a 4° refrigerator for no more than two hours.

PROCESSING OF BLOOD SAMPLES

For each subject, two to six 5 ml blood-filled vacutainers were collected at various field sites, and forwarded to the laboratory for processing. One tube was set aside to generate frozen blood aliquots, while the others were used for PBMC (peripheral blood mononuclear cell) extraction.

A. FROZEN BLOOD ALIQUOTS

One blood filled vacutainer was carefully open under sterile conditions, and aliquoted in 1 or 2 ml samples into 3 ml sterile cryovials. Each aliquot was supplemented with dimethyl sulfoxide (DMSO) (Sigma) to a 10% final concentration. Cryovials containing the aliquots were agitated to homogeneously mix the DMSO with the blood, and then put on ice. Once several samples were aliquoted, cryovials were transferred into a –80°C freezer for 1 to 12 months, and subsequently transferred to a –150°C freezer for long-term storage.

B. PLASMA-CELL SEPARATION

The remaining blood-filled vacutainers were centrifuged at room temperature for 15 min at 1100 rpm in a tissue culture centrifuge, to allow the separation of blood constituents. Following centrifugation, the plasma-containing layer in the top compartment of the tube was removed from each tube without disrupting the buffy coat, and stored in several 3 ml cryovials. Once more, cryovials were stored at -80°C for 1 to 12 months, and subsequently transferred to a -150°C freezer for long-term storage.

C. EXTRACTION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC)

After the removal of plasma, blood samples were reconstituted to their original volume using 1X phosphate-buffered saline (PBS), and further diluted in the same buffer to double the original blood volume. Diluted blood samples were gently loaded onto several 3 ml Ficoll-Paque (Amersham-Pharmacia) cushions, set up in 14 ml round bottom tubes (Becton Dickinson), and then centrifuged at room temperature for 45 minutes at 2200 rpm. Following centrifugation, the peripheral blood mononuclear cell (PBMC) "rings" were collected in empty 14 ml round-bottom tubes, which were then filled with PBS and centrifuged for 10 min at 1100 rpm. The supernatant was discarded, and the pellets containing PBMC were rinsed with PBS. Once more, after the second rinse, the supernatant was discarded and PBMC pellets were either resuspended in Trizol (Gibco) or fixed with 1% paraformaldehyde (Sigma). As more than one tube of PBMC was obtained for each individual, we ensured that one of those would be used to generate fixed cells, and the rest used for RNA, DNA and protein isolation.

D. FIXED CELLS

As described above, some of the isolated PBMC were resuspended in 1% paraformaldehyde (Sigma) and stored overnight at 4°C. The following morning, samples were centrifuged at 1100 rpm for 10 min. at 4°C. After centrifugation, the supernant was removed and pelleted cells were washed once more with PBS and re-centrifuged. Pellets were resuspended in 1X PBS, and samples were kept at 4°C.

E. TRIZOL SAMPLES

PBMC were resuspended in 1 ml Trizol, and stored at -20°C until RNA, DNA and protein extraction was accomplished, as described by Chomzynski (1993) and modified by Riol and colleagues (1999).

Briefly, following the addition of 0.2 ml chloroform to each 1 ml Trizol sample, phase separation was accomplished by centrifugation at 12,000 rpm for 15 min in a 4°C

refrigerated centrifuge (Heraeus biofuge, USA). After separation, the upper aqueous phase containing the RNA was removed from each sample, and collected into 2 ml microtubes. The interphase containing the DNA and the lower phenol (red) layer containing the protein were further processed for DNA and protein extraction.

RNA was precipitated from each collected aqueous phase sample by the addition of an equal volume of isopropyl alcohol (volume of aqueous phase = volume of added isopropyl alcohol) to each sample, and centrifuged at 12,000 rpm for 45 min in a refrigerated centrifuge (Heraeus biofuge). Following centrifugation, the RNA pellets were washed with 1 ml of 70% ethanol (EtOH), and centrifuged again as above but for 15 min. The ethanol portion was removed from each sample by inversion, and the RNA pellets were air-dried. Each pellet was dissolved in 60 µl of diethyl pyrocarbonate (DEPC) (ICN) treated water (treatment of water with DEPC renders permits the elimination of RNAse) and incubated in a 55°C water bath for 10 min. RNA samples were quantified by reading the optical density at a wavelength of 260 nm. RNA was stored at -150°C to prevent degradation.

For DNA extraction, 0.3 ml of 95% EtOH was added to the interphase and lower organic phase layer, and samples were mixed by repeated inversion. Following centrifugation at 5,000 rpm for 15 minutes in a refrigerated centrifuge, the supernatant was remove from each sample and set aside for protein extraction, while the DNA pellets were washed two times with 1 ml of 0.1M sodium citrate in 10% EtOH, incubated at room temperature for 30 minutes, and centrifuged at 5,000 rpm for 15 min. in a refrigerated centrifuge. Following the last centrifugation, DNA pellets were resuspended in 1 ml of 70% EtOH and incubated at room temperature for 20 min. Following incubation, samples were centrifuged at 5,000 rpm for 15 min. in a refrigerated centrifuge. DNA pellets were air-dried and dissolved in 300 µl of an 8mM sodium hydroxide (NaOH) solution. Samples were incubated in a 50°C water bath for 1 hour, following which DNA was precipitated using 0.3 volume of 10M ammonium acetate and 2.5 volume of 95% EtOH. Following centrifugation at 5,000 rpm for 15 min. in a refrigerated centrifuge, pellets were washed with 1 ml of 70% EtOH and resuspended in a solution of 10mM Tris EDTA at pH 8. Samples were then incubated in a 50°C water

bath for 1 hour. DNA samples were quantified by reading the optical density at a wavelength of 260 nm using a spectrophotometer.

Proteins were extracted by dialysis in SDS solution, as described by Riol and colleagues (1999). Proteins were quantified using either modified Lowry or Bradford methods, using BioRad reagents.

FLOW CYTOMETRY

The percentage of CD24-positive cells present in the PBMC of each subject was determined using flow cytometry (Becton Dickinson FACS). Briefly, $5x10^5$ fixed cells were incubated for 30 minutes on ice in the dark, with 100 µl of PBS buffer containing 7 µl of FITC conjugated CD24 antibodies (Zymed). Following incubation, cells were washed with 1 ml of PBS, and samples were centrifuged at 1100 rpm in a refrigerated centrifuge for 10 min. Supernatants were discarded, and pelleted cells resuspended in 400 µl of PBS. At least 10,000 events in cell-gated conditions were counted for each sample. Unstained and isotype (IgG-FITC antibody, Pharmingen) single-stained samples were used to set up flow cytometry parameters. Final graphs were drawn using WinMDI software (J. Trotter, in the public domain).

DATA ANALYSIS

Statistical analysis was done using MINITAB software, and age groups were defined as follows: very young or group pre-A (10-19 yrs.), young or group A (20-39 yrs.), intermediate or group B (40-69) yrs., old or group C (70-89 yrs.), and extremely old or group D (90+ yrs.). Statistical analyses performed included ANOVA, t-test, average and standard deviations where appropriate. Significant differences were defined as p □0.05.

2.4 RESULTS

As shown on the flow chart in Figure 1 and described in the Material and Methods section, we report here the development of a novel protocol permitting the

generation of several different kinds of biological material derived from a single sample of 10-30 ml of peripheral blood. Furthermore, using materials extracted by this method, from blood samples from a Chinese population from localized regions of Taiwan, we assessed the quality and quantity of each biological material, and find that they are remarkably useful in generating biologically meaningful data.

FROZEN BLOOD ALIQUOTS

Using our protocol, the preparation of blood samples for long term cryopreservation can rapidly be accomplished by supplementing blood samples with 10% DMSO, a cryopreservant, and aliquoting them into cryovials. However, to be sure good cellular viability is obtained, samples must be stored at temperatures lower than -80°C, perhaps even -150°C, and when needed rapidly thawed using a 37°C water bath. Blood aliquots from our population stored at -150°C for two years still retain good cellular viability and high recovery rate, when rapidly thawed and placed in culture (data not shown).

PERCENTAGE OF PLASMA WITHIN THE BLOOD

As shown in Table 3 and Figure 2, extremely old individuals (90+) exhibit a two-fold increase in the percentage of plasma present in their blood, compared to very young individuals (10-19 yrs old). While the very young population blood samples had significantly lower proportions of plasma than those of young, intermediate and old age groups, no significant difference was observed between the latter three age groups. However, extremely old individuals showed a significantly higher proportion of plasma in their blood samples than all other age groups. Thus, the plasma composition of blood changes from very young to the young age group, but then stabilizes until extreme old age. However, despite the lower amount of plasma present in very young individuals, more than enough plasma can be obtained from all individuals to permit several laboratory tests, including ELISA assays. Nevertheless, we recommend that plasma

samples be stored in cryovials and kept at temperatures lower than –80°C, to avoid plasma protein degradation.

RNA, DNA, PROTEINS AND FIXED PBMC SAMPLES

The simultaneous extraction of RNA, DNA and protein was performed as described by Chomzynski (1993) and modified by Riol and colleagues (1999). This methodology produces very high quality as well as good quantities of biological material. As shown in Figure 3A, RNA extracted using this procedure was not degraded, and both 18S and 28S RNA bands were distinct and easily identifiable. To further test the RNA, RT-PCR was performed using RNA isolated from our population sample, and as shown in Figure 3B, the co-amplification of aldolase C and 18S was very successful when 24 cycles were used. Similarly, the DNA obtained was free of degradation, and thus could be used for PCR reactions as well as DNA polymorphism studies. Figure 3C shows the DNA, while Figure 3D shows a sequence obtained from PCR amplification of trinucleotide repeats, generated by amplifying the genomic DNA extracted by this method. Proteins were run on 12% SDS polyacrylamide gels and stain using Ponceau (Sigma) stain to reveal the electrophoretic mobility pattern of the extracted proteins (Figure 3E). Figure 3F shows an example Western blot for biochemical characterization of beta-actin presence.

When the total amount of RNA, DNA and proteins obtained per ml of blood extracted was calculated, it was found that the quantity of each species obtained from the different age groups did not change significantly (Table 2). Thus the yield of RNA, DNA and proteins obtained per ml of blood does not vary among age groups. As shown in Table 2, old individuals appear to have less protein in their samples; however, t-test reveals no significant difference from the other age groups. The lower average amount of protein present in older individuals results from the fact that most of the samples we have for this age group could not be quantified due to damage incurred by the protein samples during one of the shipments from Taiwan to Canada.

All RNA and protein samples were kept at temperature lower than -135°C, while the DNA samples were kept at -20°C. Samples from our population have been used

more than two years after extraction, and still show no signs of degradation. Degradation was further prevented by generating aliquots of working solutions, thus preventing our stock solutions from undergoing several rounds of freeze-thaw cycles.

Paraformaldehyde-fixed PBMC were kept at 4°C until being used in flow cytometry experiments; and as observed for the RNA, DNA and protein, the quantity of fixed PBMC obtained per ml of blood sample did not significantly change with age. Thus, except for the yield of plasma, the quantity of biological materials extracted per ml of blood remains constant with age. As many commercially available antibodies do not work with paraformaldehyde-fixed PBMC, it is important to verify antibodies before conducting flow cytometry experiments, or use alternative method of fixation to avoid the loss of antigenicity, albeit tolerating the less than ideal morphological preservation.

CD24 EXPRESSION IN PBMC WITH AGE

Using fixed PBMC, we determined the expression of CD24 within PBMC using flow cytometry. Figure 4 shows the expression of CD24 within each age group, and flow profiles from two representative individuals. We found that the percentage of CD24 present in the PBMC of individuals changed greatly with age. While the PBMC of individuals within the young and intermediate age groups contain about 11% CD24-positive cells, the presence of CD24-positive cells decreases to about 8% in old individuals, and 6% in extremely old individuals. The reduction in CD24 expression is especially significant when the young age group is compared with both the old and extremely old age groups, or when the intermediate age group is compared with the extremely old age group. To the best of our knowledge, this is the first paper to document an age-dependent decrease in CD24 expression.

2.5 DISCUSSION

The use of population studies in aging research has contributed to our understanding of age-related physiological changes, and permitted the identification of several factors involved in successful aging. However, population studies are very

expensive, since the quantity of biological material obtained from each individual is very limited, being restricted by the inability to collect large amount of blood samples from extremely old individuals without significantly compromising their health. For this reason, most population studies currently underway are conducted using only one type of biological material, such as DNA from finger-pricked samples for genetic profiling. In this paper, we describe a methodology permitting the simultaneous extraction of several different kinds of biological specimens from a single source of relatively small amounts of peripheral blood. This protocol not only renders population studies more cost effective, but also allows for in-depth biological investigation, as demonstrated by one pilot study using one type of biological material, defining an age-specific CD24 gene expression profile.

As part of our study, Chinese individuals living in the central part of the island of Taiwan, whose ancestors settled on the island more than 300 years ago, were recruited to form the Chinese population from Taiwan. We chose these people because their ancestry could be traced back to a common origin, they live in an isolated geographical area, and age verification could easily be conducted using three methods: household registration, birthday records and neighbor verification. In general, less than 30 ml of peripheral blood was collected per subject, and used to generate frozen blood aliquots, plasma, RNA, DNA, protein and fixed PBMC samples. We found that the amounts of RNA, DNA, proteins and fixed PBMC obtained per ml of blood were similar for all four age groups, thus suggesting that the amount of PBMC remains constant in all age groups. However, when plasma was collected, it was found that the blood of extremely old individuals has twice as much plasma as that of very young individuals. Since our data indicate that the amount of PBMC per ml of blood does not significantly change with age, it is likely that other cellular blood components, such as red blood cells and multinuclear cells, decrease in extremely old individuals, resulting in changes in extremely old individuals' blood composition. Decrease in hematocrit levels has previously been report in old and extremely old individuals (Coppola *et al.,* 2000; Caprari *et al.* 1999) .

All biological materials extracted using the present described methodology were of excellent quality, as it is evident that both 18S and 28S RNA were not degraded, and

likewise the DNA and protein samples were intact and showed no detectable variation in their quality. To further verify the quality of the RNA, RT-PCR was performed to co-amplify 18S and aldolase C. Similarly, PCR amplifying trinucleotide repeats was conducted using the DNA sample, and products were sequenced. Western blotting and immunolabeling using beta-actin antibody (Oncogene) was used to verify the quality of the proteins. It is important to obtain different kinds of biological material from each individual, since the use of each material contributes to different aspects of aging studies. For instance, DNA can be used to identify single nucleotide polymorphisms involved in the development of age-associated diseases, RNA and proteins can help us identify gene products important in successful aging, frozen blood aliquots can be used in eventual functional studies using cultures derived from them, and fixed lymphocytes can help us document age-related changes in immune profile.

Using our fixed PBMC, we found that the percentage of CD24 cells present in the PBMC of individuals decreases with age. Extremely old individuals show a two-fold diminution in the percentage of CD24-positive cells among their PBMC, compared with their young counterparts. As CD24 cells present in the peripheral blood are mostly B cells that have not undergone Ig type-switching (Allman *et al.*, 1992), it is likely that the diminution of CD24-positive cells in older individuals is due to a decreased output of naïve B lymphocytes from the bone marrow, and an accumulation of memory B cells within the peripheral blood. It was previously documented that B cell production decreases with aging; however, further investigation is needed to document the cause of CD24 diminution with age (Ogawa *et al.*, 2000).

In this paper, we report the development of a new protocol for the simultaneous generation of RNA, DNA, protein, plasma and fixed PBMC samples as well as frozen blood aliquots from a single 10-30 ml source of peripheral blood, and discuss the yield and quality of each of the above biological materials. Furthermore, using samples from our population, we show that blood composition is not identical among different age groups, as the blood of extremely old individuals contains more plasma than that of younger individuals. The development of this new protocol will facilitate the identification of novel factors involved in successful aging, and render population study more cost-effective. We further demonstrate the powerful use of this protocol by

identifying an age-dependent reduction in CD24-positive cells. Altogether, the present report describes a standardized method to extract DNA, RNA, protein, plasma and cells from a small volume blood sample from each individual, thus allowing for studies of genetic profiling at the level of single nucleotide polymorphism and gene products as well as regulation of functions, and provides a comprehensive approach to study factors involved in aging.

2.6 ACKNOWLEDGEMENTS

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Table 1. Age Composition of the Selected Chinese Cohort from Taiwan

Age Groups (yrs)	Number of Individuals	Male (%)	Female (%)
10-19	15	46.6	53.4
20-29	23	34.8	65.2
30-39	32	40.6	59.4
40-49	36	47.2	52.8
50-59	21	23.8	76.2
60-69	22	40.9	59.1
70-79	18	55.5	44.5
80-89	21	61.9	38.1
90-99	52	48.1	51.9
100+	6	16.6	83.4
Total	246		

Table 2. Percentage of plasma in the blood

	Very Young (10-19)	Young (20-39)	Intermediate (40-69)	Old (70-89)	Extreme Old (90+)
% Plasma in blood	26.94 ± 5.76*	39.13 ± 8.86	40.25 ± 8.82	43.07 ± 8.97	50.17 ± 6.07*

Notes: The percentage of plasma within the blood is significantly different when extreme old age group is compared to all other age group. A significant difference also exists between the percentage of plasma present within the blood of very young individuals and all other age group. * indicates a significant difference with all other age groups.

Table 3. Amounts of Fixed Cells, RNA, DNA, and Protein Obtained per ml of Blood.

	Young (20-39)	Intermediate (40-69)	Old (70-89)	Extreme Old (90+)
Fixed cells/ml of blood ($x10^6$)	1.02 ± 0.62	1.55 ± 0.59	1.11 ± 0.63	1.25 ± 0.57
RNA (μg/ml of blood)	1.73 ± 0.68	1.72 ± 0.55	1.57 ± 0.54	1.44 ± 0.65
DNA (μg/ml of blood)	8.16 ± 3.53	6.96 ± 3.13	7.66 ± 1.40	8.91 ± 1.99
Proteins (μg/ml of blood)	64.73 ± 32.98	57.13 ± 35.85	32.34 ± 29.7	76.75 ± 68.02

Notes: The amount of fixed cells, RNA, DNA and protein obtained per ml of blood processed is similar for all age groups.

FIGURE1: PROTOCOL FOR BLOOD SAMPLE PROCESSING

Stepwise description of the protocol for the generation of different kinds of biological materials from a single sample of peripheral blood. The blood is first divided into two aliquots; the first is used to generate frozen blood samples for future development of tissue cultured cell samples, while the second is subjected to plasma-cell separation. Following plasma-cell separation, plasma is collected and PBMC are extracted from the remaining blood components. The extracted PBMC are used to generate fixed cells and DNA, RNA and protein samples.

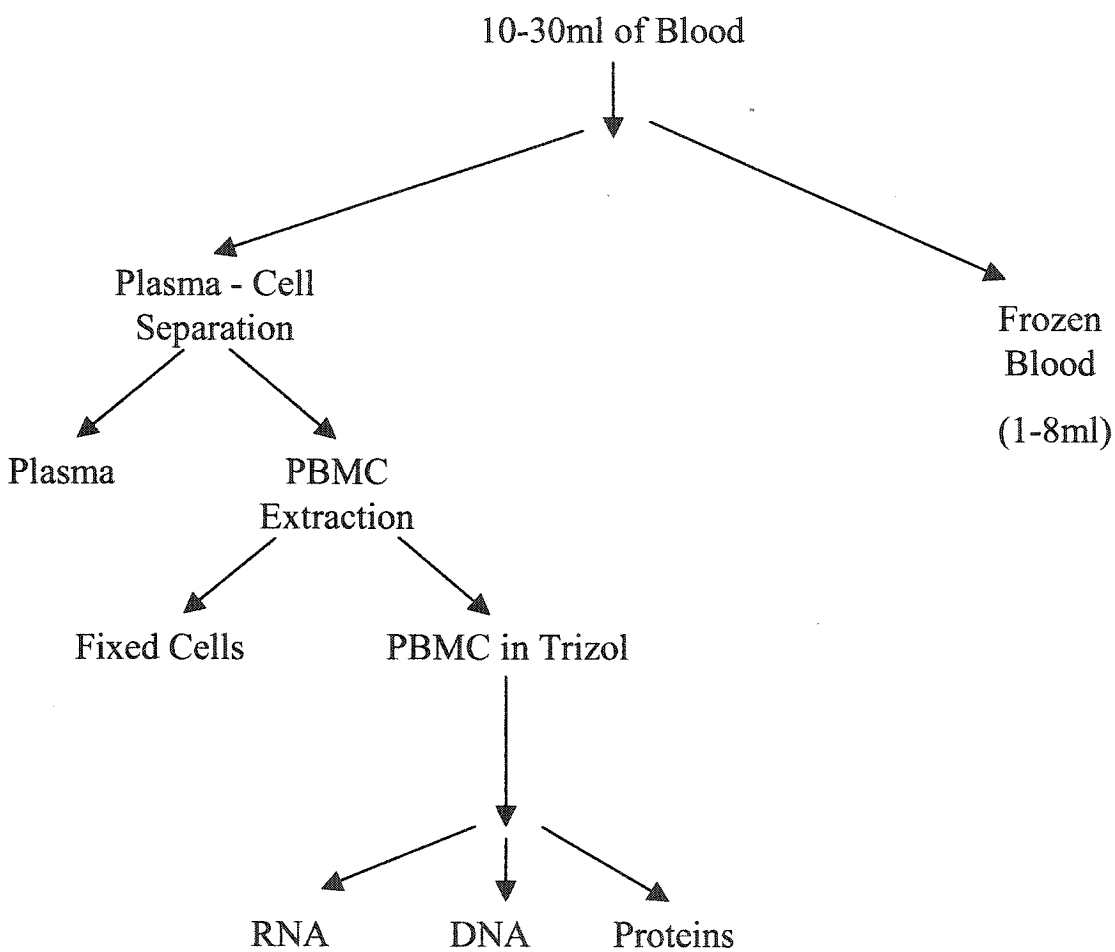

10-30ml of Blood
Plasma - Cell Separation
Frozen Blood
(1-8ml)
Plasma
PBMC Extraction
Fixed Cells
PBMC in Trizol
RNA
DNA
Proteins

FIGURE 2: PERCENTAGE OF PLASMA

Percentage of plasma within each blood sample according to age groups. Individuals belonging to the Pre-A (very young) group have significantly less plasma within their blood than individuals of groups A (young), B (intermediate), C (old) or D (extreme old). Similarly, individuals within group D have significantly more plasma within their blood than individuals of other age groups. Panel A shows averages and standard deviations for all age groups, while the second diagram shows the individual donors' plasma content in relation to their age. Cubic regression was used to model the relationship.

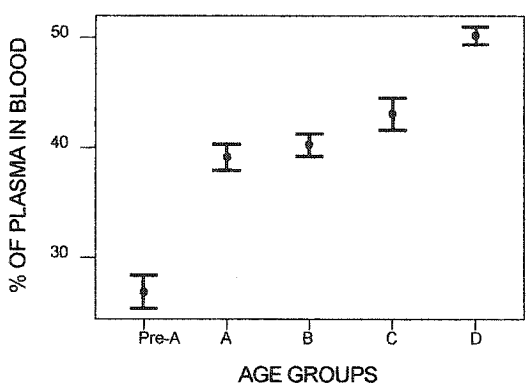

% OF PLASMA IN BLOOD
50
40
30
Pre-A
A
B
C
D
AGE GROUPS

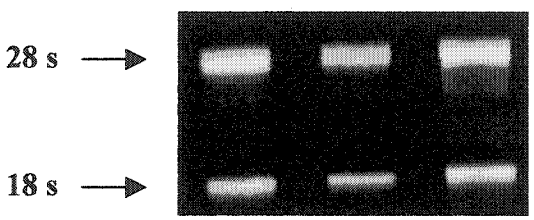
FIGURE 3: RNA, DNA AND PROTEIN SAMPLES

RNA, DNA and protein samples. Fig 3A: Agarose gel showing the 28S and 18S RNA extracted from three selected Taiwan samples. Fig 3B: The RNA was used in an RT-PCR reaction and co-amplified using 18S (Ambion) and Aldolase C primers. Lane 1: 100 bp ladder; lane 2: Negative control (no RT reaction was performed before PCR reaction); lane 3: Negative control (amplified DNA instead of RNA); lanes 4, 5 and 6: 18S and aldolase C co-amplification using three selected Taiwan samples. Fig 3C: Agarose gel showing the DNA extracted from three Taiwan samples. Fig 3D: Trinucleotide repeats of the DNA were amplified in a PCR reaction, and the obtained fragment was sequenced. Fig 3E: Ponceau red staining of nitrocellulose membrane showing the protein patterns of Jurkat cells in lane 1, and of selected Taiwan samples in lane 2 and 3. Fig 3F: Western blot using beta-actin antibody (Oncogene) was performed using three of our protein samples.

***FIGURE 3D was generated by Dr Suying Xu.

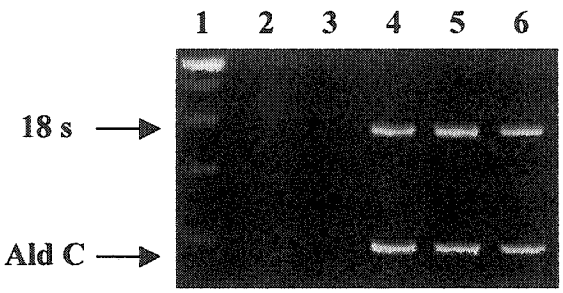
A

RNA



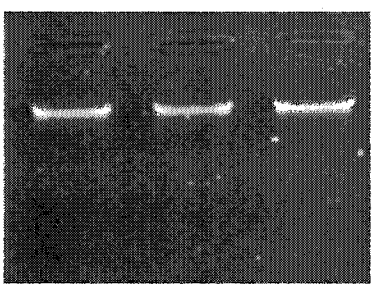
B

RT-PCR



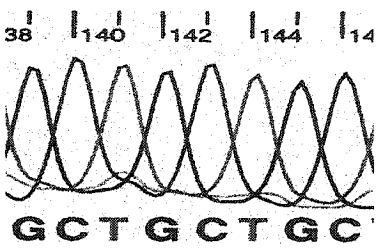
C

DNA



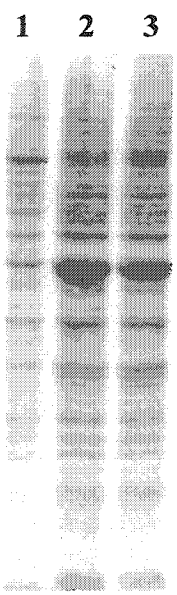
D

PORTION OF A TRINUCLEOTIDE REPEATS SEQUENCE



E

PROTEINS



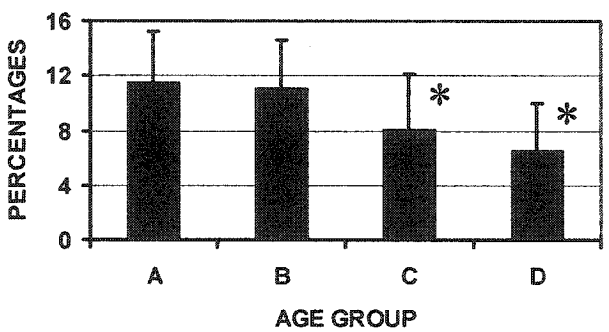
F

WESTERN BLOT

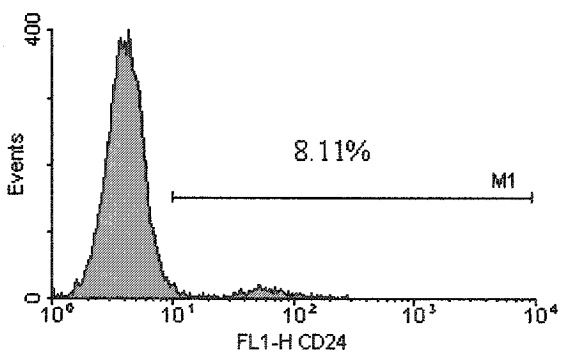
FIGURE 4: EXPRESSION OF CD24 IN PBMC

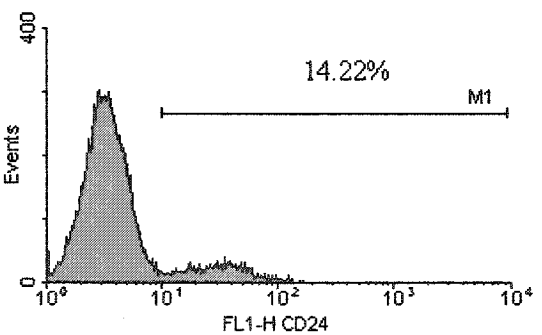
Expression of CD24 in PBMC. Fig 4A: The incidence of CD24-positive cells in PBMC by age group. Averages ± standard deviations are represented. * Indicates a significant difference compare to group A. Group D is also significantly different from group B. Fig 4B: CD24 flow cytometry profile of two selected Taiwan samples.

A


16
12
8
4
0
PERCENTAGES
*
*
A
B
C
D
AGE GROUP

B


400
0
Events
8.11%
M1
10⁰
10¹
10²
10³
10⁴
FL1-H CD24


400
0
Events
14.22%
M1
10⁰
10¹
10²
10³
10⁴
FL1-H CD24

PREFACE TO CHAPTER 3

Having developed a method to process blood samples in a way to maximize the recovery of biological material, and following the processing of 246 blood samples from individuals from Chinese origin living in Taiwan, I proceeded to test my hypothesis that apoptosis is one of the determinants of longevity. Using RNA extracted from the blood samples, I used quantitative RT-PCR to determine the levels of caspase-1, -3, -8 and –10 mRNA expression in each of the 246 samples and identified a unique pattern of caspase mRNA expression in the PBMC of extremely old individuals. Then, using flow cytometry, I characterized the immune profile of all 246 individuals using the fixed PBMC and found that the amount of CD24 positive and naïve CD4 cells is lower in PBMC extracted from extremely old individuals than in younger subjects. Having characterized changes in immune profiles, I proceeded to test whether those changes in immune profile were responsible for changes in caspase mRNA expression levels. As you will see, my results show that caspases mRNA expression levels appear to be independent of the immune profiles.

CHAPTER 3

IDENTIFICATION OF HIGH CASPASE-3 mRNA EXPRESSION AS A UNIQUE SIGNATURE PROFILE FOR EXTREMELY OLD INDIVIDUALS

This paper has been published in *Mech. Ageing Dev.* (2002) **123**: 1133-1144.

3.1. ABSTRACT

Apoptosis, or programmed cell death, is important for maintaining tissue homeostasis, as it permits the elimination of damaged, functionless or unwanted cells. As we age, our immune system undergoes constant remodeling, during which age-associated changes in immune parameters, including decreased naïve and increased memory T cells, have been reported. However, excessive immune cell loss, rendering the elderly more vulnerable to infections, and inappropriate deletion of damaged or functionless lymphocytes, can contribute to the development of age-associated diseases. As such, we studied the mRNA expression of cell death (specifically caspase) genes in nonagenarians and centenarians, successful models of ageing who have survived or avoided age-associated diseases, as well as in their younger counterparts and found that a population composed of extremely old individuals shows a unique pattern of caspase mRNA expression, characterized by high levels of caspase-1 and –3, and low levels of caspase-8, mRNA while those composed of old individuals are characterize by high level of caspase-8 mRNA expression. Furthermore, we show that the described changes in caspases mRNA do not appear to results from age-related changes in PBMC composition, such as decreases in CD24. Therefore, we suggest that unique patterns of caspase mRNA results from the regulation of message abundance on a per cell basis, *via* a putative regulation of caspase genes at the transcription or RNA processing level, rather than changes in immune profiles.

3.2. INTRODUCTION

Ageing is often associated with a progressive decline in the number of circulating B lymphocytes, a decrease in both B and T cell functions, changes in immunological profiles including increased memory and decreased naïve cells, and defects in T cell signaling pathways (Proust *et al.*, 1987; Philosophe and Miller, 1990; Miller, 1996; Globerson and Effros, 2000). The changes in immune parameters occurring during ageing are responsible for the increased mortality rate due to infections seen in the elderly (Globerson and Effros, 2000), and may also contribute to the development of age-associated diseases such as autoimmune diseases and cancers, resulting from diminished immune surveillance throughout the body, or from the inappropriate deletion of damaged lymphocytes from the immune system.

Apoptosis, or programmed cell death, is a highly regulated process involved in tissue sculpting during embryogenesis, maintenance of tissue homeostasis, and deletion of unwanted or damaged cells (Jacobson *et al.*, 1997). During lymphopoiesis, autoreactive or functionless B and T cells are deleted by apoptosis (Murphy *et al.*, 1990; Hartley *et al.*, 1993; Lu and Osmond, 1997); the same process prevents the accumulation of activated lymphocytes in the immune system (Akbar and Salmon, 1997). This process is controlled by genes involved in promoting or inhibiting cell death, such as caspases and Bcl-2 homologues, pertaining to the two major families of proteins functioning in the cell death program *via* a tightly controlled biochemical pathway (Reed, 1997; Earnshaw *et al.*, 1999; Gross *et al.*, 1999; Antonsson *et al.*, 2000).

Caspases, the key executioners of apoptosis, are a family of cystein proteases capable of cleaving essential cellular substrates after aspartic residues (Earnshaw *et al.*, 1999). Caspase-3, the enzyme capable of cleaving poly (ADP-ribose) polymerase (PARP), a nuclear enzyme involved in DNA repair, is the most prominent of all effector caspases (Tewari *et al.*, 1995). Caspase-3 can be activated by Granzyme B release from cytotoxic T cells, and thus contribute to the elimination of infected cells (Edwards *et al.*, 1999; Trapani *et al.*, 1999; Trapani *et al.*, 2000). Furthermore, it plays an important role in the immune system by deleting autoreactive or functionless lymphocytes during the negative selection process, as well as damaged lymphocytes (Hartley *et al.*, 1993; Lu and

Osmond; 1997). Caspase-1, the enzyme naturally responsible for the cleavage of pro-inflammatory cytokines interleukin-1β and –18 into their active forms, is also a potent transducer of cell death, as it can cleave caspase-3 into its active form, as well as other essential cellular substrates (Earnshaw *et al.*, 1999; Fantuzzi and Dinarello, 1999).

The mortality rate of extremely old individuals (90+ years) grows more slowly than that of the general population, with a flattened slope; this population attains such an extreme life span by escaping the mortality associated with common infections and age-associated diseases (Smith, 1997). Since both excessive cell loss and inefficient removal of defective or damaged immune cells have been proposed as major contributors to elderly mortality, we hypothesize that extremely old individuals uniquely express in their immune systems genes involved in apoptosis, permitting them to maintain a more robust immune system and thus experience successful aging. Our data show that the peripheral blood mononuclear cells (PBMC) of extremely old individuals express relatively high levels of caspase-1 and –3, and low level of caspase-8, mRNA. While high levels of caspase-1 mRNA are also seen in moderately old individuals, high levels of caspase-3 in association with low levels of caspase-8 mRNA are exclusively observed in extremely old individuals. Caspase-8 mRNA is expressed at low levels in younger subjects, but not in conjunction with high levels of caspase-3 mRNA. Thus, high levels of caspase-1 and -3 and low levels of caspase-8 gene expression constitute a biosignature for nonagenarians and centenarians in our study cohort while high levels of caspase-8 gene expression constitute a biosignature for old individuals in our study cohort. We also demonstrate that the changes in caspase mRNA expression, observed in extremely old individuals appear to be independent of age-dependent decreases in CD24 and naïve CD4 lymphocytes. Our results suggest that this unique expression of caspase mRNA in nonagenarians and centenarians results from the regulation of caspase genes at the transcription or RNA processing level.

3.3. MATERIALS AND METHODS

SUBJECTS

Between 10 and 30 ml of peripheral blood were obtained from each of 246 healthy Chinese subjects (aged 10 to 102 years old) living in Taiwan, following written consent (Table 1). Donors' age was verified using both a government-issued document and one or more other sources, such as governmental data bank, historical documents or relative references. Each collected sample was randomly assigned a sample number, to avoid easy identification of the donor's age during the experimental procedures, and to ensure the confidentiality of the donor's identity. This protocol was approved by the Institutional Review Boards of the Taichung Veterans General Hospital (Taichung, Taiwan) and the Sir Mortimer B. Davis Jewish General Hospital, McGill University (Montreal, Canada).

PROCESSING OF BLOOD SAMPLES

Each blood sample was divided into 2 aliquots. Blood from the first aliquot was supplemented with 5% dimethyl sulfoxide (DMSO) (Sigma) and stored at -80°C for future use. The second aliquot was centrifuged at 1100 RPM (Sorvall RT 7 plus) for 15 minutes, and serum was removed. Serum-depleted blood samples were reconstituted and further diluted with phosphate-buffered saline (PBS). Diluted blood samples were loaded onto Ficoll-Paque cushions (Pharmacia), and peripheral blood mononuclear cells (PBMC) were extracted according to the manufacturer's instructions. About one third of the PBMC were fixed overnight at 4°C using 1% paraformaldehyde (Sigma), while the remaining cells were homogenized in Trizol reagent (Gibco BRL) for subsequent extraction of RNA.

EXTRACTION OF RNA

Extraction of RNA from the Trizol homogenates was achieved as described by Chomczynski (Chomczynski, 1993) and modified by Riol and colleagues (1999). RNA samples were quantified by reading the optical density at a wavelength of 260 nm on a spectrophotometer (Beckman).

PCR PRIMERS

Caspase-1 primers (5'GATTGACTCCGTTATTCCGA and 5'TCATGCCTG-TGATGTCAACC) amplified the alpha isoform of caspase-1 to yield a fragment of 397 bp. Caspase-3 primers (5'AGAGGGGATCGTTGTAGAAG and 5'GTTGCCACCTT-TCGGTTAAC) were designed to generate a single fragment of 304 bp. For caspase-8, the selected primers (5'GCATTAGGGACAGGAATGGA and 5'CCCCTGACAAGCC-TGAATAA) generated more than one fragment. The major fragment of 319 bp was quantified, since it corresponded to the amplification of caspase-8 mRNA responsible for the generation of functional caspase-8 protein. Caspase-10 primers (5'GATAATCTGACATGCCTGGAG and 5'GAGGTAAAGCTGTGGTTGTTGA) generated 3 fragment sizes. Fragments amplifying at 394 bp were the most important, since they corresponded to the active delta and beta isoforms of caspase-10. Primers for Aldolase C (5'TGCAGAGGGTGTACGCTCACTG and 5'ATAATGGTGTTCCC-TTCGTCCGA) gave rise to a 217 bp fragment. For all sets of primers, the genomic DNA amplification generated larger fragments as primers were picked in different exons. Sequences of all mRNA and genes were found in GenBank, and primers were purchased from Alpha DNA (Montreal, Canada).

QUANTITATIVE RT-PCR

After denaturing the RNA samples at 65°C for 10 min., followed by cooling on ice, reverse transcription reagents were added to each sample to give a final volume of 20

μl. Thus, all samples had a final concentration of 1 mM dNTP (Amersham) and 0.3 μg of random nonamers (Alpha DNA), 15U RNAse inhibitor (RNAse Guard, Amersham), 200U M-MLV reverse transcriptase (Gibco BRL) and 1X PCR buffer (2 mM Tris pH 8.4, 5 mM KCl, 0.2 mM $MgCl_2$, 0.01% Triton-X). After the addition of reverse transcription reagents, samples were incubated for 10 min at room temperature to allow the annealing of primers, and reverse transcription was performed for 1 hr at 37°C. Newly generated cDNA samples were stored at -20°C until PCR reactions were performed.

Having optimized the conditions to ensure a linear range of amplification for caspases-1, -3, -8, and -10 and Aldolase C, we co-amplified each individual caspase with Aldolase C for every sample (Appendix 2). Aldolase C was chosen as an endogenous standard since its expression in PBMC remains constant with age (Riol; 1996). All PCR reactions were performed in a final volume of 100 μl with 1X PCR buffer, 0.2 mM dNTP (Amersham), 200 ng of each sense and antisense primer, Taq polymerase (laboratory clone) and 2 μCi of P^{32} radiolabelled dCTP (NEN Lifescience). PCR products were loaded onto 8% polyacrylamide gels to allow separation of the amplified fragments. Bands were revealed on photographic film (Kodak) and quantified using densitometry (Molecular Dynamics). The ratio of each caspase to Aldolase C was calculated, to allow comparison between samples.

FLOW CYTOMETRY

Fixed PBMC ($1x10^5$) were stained with fluorescence-conjugated monoclonal antibodies (mAb) in 100μl of 1X PBS for 45 min. on ice. The following FITC- or cy-chrome-conjugated mAb were used: CD4, CD8, CD45RA (Pharmingen) and CD24 (Zymed). Stained cells were washed twice with PBS, and samples were analyzed by FACScan (Becton Dickinson) using CellQuest software. At least 10,000 events in cell gated conditions, corrected for background using isotype IgG controls (Pharmingen), were counted for each sample. Unstained and single stained samples were used to set up the flow cytometer and as controls. Final graphs were drawn using WinMDI software (J. Trotter, in the public domain).

DATA ANALYSIS

Data analysis was conducted by dividing the population into four age groups: young (20-39 yrs), middle (40-69 yrs), old (70-89 yrs) and extremely old (90-102 yrs). Statistics (ANOVA and regression) were calculated using Minitab software. Significant differences were defined as $p \leq 0.05$. Linear, quadratic and cubic regressions were performed for each caspase. The best-fit regression was retained to model the relationship of each caspase with age.

3.4. RESULTS

CASPASES mRNA EXPRESSION IN PBMC

Previously it was found that T cells from aged humans (65-92 yrs) are more susceptible to Fas- and TNF-mediated cell death than those from younger subjects (Aggarwal and Gupta; 1998; Aggarwal et al.; 1999). As caspases are involved in most mechanisms leading to apoptotic cell death, including the Fas and TNF pathways, we first sought to investigate whether the constitutive expression of caspase mRNA in PBMC differs amongst various age groups. Given the limited amount of RNA available for some samples, such as those of extremely old individuals, we limited our study to the investigation of caspases-1,-3, -8 and -10. We examined the mRNA expression level of each of these caspases in the PBMC of every subject using quantitative RT-PCR, by co-amplifying every caspase with aldolase C, an age- independent internal control. Results obtained were expressed as the ratio of each specific caspase mRNA expression level to that of Aldolase C. The acquired ratios were subsequently analyzed using ANOVA, and statistical analysis was carried out on four age groups: young (20-39), middle (40-69), old (70-89) and extremely old (90+). Significant differences were defined as $p \leq 0.05$.

As shown in Table 2 and Figure 1A, the level of caspase-3 mRNA expression remains fairly constant amongst young, middle, and old age individuals; however, extremely old individuals exhibit significantly higher caspase-3 mRNA expression than the other age groups. In an attempt to better characterize the relationship between caspase-3 mRNA expression and age, statistical linear, quadratic and cubic regressions

were performed. We found that the relationship is best modeled using quadratic regression (Fig. 1D), defined by the following equation: Caspase-3 mRNA expression = 0.984215 - 0.0065668 (age) + 0.0000890 (age)^2. The fact that a second order equation was retained as the best-fit model indicates that at a given age, the linear trend describing the relationship between caspase-3 mRNA expression and age suddenly changes as a subset of individuals (in our case, the extremely old) express a significantly different level of caspase-3 mRNA. This can be visualized by the curvature in the regression line.

The presence of caspase-8 is essential for transduction through the Fas signaling pathway (Earnshaw *et al.*, 1999). The expression of caspase-8 mRNA is similar in young, middle and extremely old individuals; however, a significant increase in the level of caspase-8 mRNA expression is seen in the old age group (70-89 yrs). As shown in Table 2 and Figure 1B, older individuals almost double their level of caspase-8 mRNA, compared to young and extremely old subjects. Given the particular relationship between the expression of caspase-8 mRNA and age (Fig 1E), results were analyzed using MINITAB and the following cubic equation used to define the relationship: Caspase-8 mRNA expression = 5.34984 - 0.27297(age) + 0.0057704(age)^2 - 0.0000344(age)^3 Like caspase-8, caspase-10 is also an initiating caspase (Earnshaw *et al.*, 1999) however, its expression remains fairly constant amongst the four age groups (Table 2). Caspase-1 mRNA expression is elevated in both old and extremely old individuals, compared to young and middle age subjects (Table 2, Fig 1C). The following cubic regression was used to model the relationship between caspase-1 mRNA expression and age: Caspase-1 mRNA expression = 7.15309 - 0.124961(age) + 0.0025389(age)^2 - 0.0000130(age)^3.

In summary, these results show that caspase-3 mRNA expression level is significantly increased in extremely old individuals only, while high levels of caspase-8 mRNA expression are exclusively found in moderately old individuals. Unlike caspase-8, caspase-10 mRNA expression remains constant in all four age groups, while caspase-1 is increased in both old and extremely old age populations.

POPULATION DISTRIBUTION ACCORDING TO CASPASE mRNA EXPRESSION

To further examine the relationship of these caspase gene expressions with age, we investigated whether or not the observed changes in caspase mRNA expression were due to a slight increase in caspase mRNA expression in every individual, or correspond to a large increase in a subgroup of individuals. Since each of the young, middle, old and extremely old age groups are heterogeneous, reflected by the fact that not all individuals within each age group express the same level of a given caspase mRNA, we divided the range of each caspase mRNA expression into four arbitrary sub-groups, labeled A through D (Fig 2A, example for caspase-3), and for each age group generated a caspase population profile. Arbitrary sub-group A represents a low, B an intermediate, C a high, and D an extremely high level of caspase mRNA expression. We then calculated the percentage of individuals within each age-defined population expressing low (group A), intermediate (group B), high (group C) or extremely high (group D) levels of each caspase mRNA (Fig. 2C). Examples of arbitrary sub-groups for caspase-3 RT-PCR can be seen in Figure 2B.

The caspase-3 population profile (Fig. 2C) clearly shows that the young age group is predominantly composed of individuals expressing low and intermediate levels of caspase-3 mRNA (group A and B), while in contrast the extremely old age group is almost solely composed of individuals expressing high or extremely high levels of caspase-3 mRNA.

Population profiles for caspase-8 show a similar pattern for young, middle and extremely old age groups; however; the old age population is mostly composed of individuals expressing extremely high levels of caspase-8 mRNA (Fig 2C). From these profiles, it becomes apparent that extremely old individuals express levels of caspase-8 mRNA similar to younger subjects.

Two distinct types of population profile are seen for caspase-1 (Fig 2C). Whereas the young and middle age groups are mostly composed of individuals expressing intermediate levels of caspase-1 mRNA, both the old and extremely old populations show increasing numbers of individuals expressing high levels of caspase-1.

From these results, it is apparent that the increase in caspase-3 mRNA expression seen in the extremely old group (Fig 1A) is due to the lack of individuals within this age group expressing low levels of caspase-3 mRNA in their PBMC (Fig. 2C), and results from a progressive population shift from low to high levels of caspase-3 mRNA expression. A similar population shift is observed for caspase-1 mRNA expression, and also results from an increased number of individuals expressing high levels of caspase-1 mRNA amongst the old and extremely old age groups.

PBMC COMPOSITION AND CASPASES mRNA EXPRESSION

As the PBMC composition of individuals may change with age, we went on to investigate whether the differences seen in caspase mRNA expression between age groups are dependent on age-related changes in PBMC composition (Table 3, Fig 3). Consistent with previously published results documenting a decrease in B cell production with age (Klinman and Kline, 1997), we found *via* flow cytometry a significant decrease in the incidence of CD24-positive cells with age. Thus, extremely old individuals have many fewer CD24-positive cells in their PBMC than do younger subjects, despite expressing higher levels of caspase-1 and –3 mRNA. When young individuals expressing intermediate levels of caspase-3 or -1 mRNA (arbitrary sub-group B) are compared with extremely old individuals also expressing intermediate levels of caspase-3 or -1 mRNA, a significantly lower percentage of CD24-positive cells is found to be present in the PBMC of extremely old individuals (Fig. 4A). However, when young individuals expressing different levels of caspase-3 mRNA, or extremely old individuals expressing different levels of caspase-1 mRNA, are compared amongst themselves, the proportion of CD24-positive cells present within the PBMC does not significantly change (Fig 4 B, C). Thus, despite being able to express the same level of caspase-1 or -3 mRNA, young and extremely old individuals have different proportions of CD24-positive cells within their PBMC.

Using flow cytometry, we also found a significant decrease in the percentage of CD4-positive cells expressing CD45RA in the PBMC of older individuals (Table 4 and Fig. 3B) and, once more, we found that young and extremely old individuals expressing

the same level of caspase-3 mRNA have different percentages of CD4/CD45RA-double positive cells within their PBMC (Fig. 4D). This indicates that despite having similar level of caspase mRNA expression, individuals can have different immunological profiles.

In the light of these results, it appears that the differences in immunological profile between young and extremely old individuals are not responsible for the changes in caspase mRNA expression observed between those two age groups. However, further study involving cell sorting and the direct assessment of caspases mRNA expression in subsets of cells should be conducted to clearly establish this.

3.5. DISCUSSION

Our population is composed of subjects of Chinese origin whose ancestors have lived on the island of Taiwan for more than 300 years, and who have themselves inhabited the central part of the island since their birth. Thus, our population is isolated in a defined geographical area, and our subjects have shared and continue to share the same environment. Whether or not our findings can be applied to other populations, when both the ethnic background and environmental conditions are considered, remains to be established. However, evolutionarily conserved pathways such as those involving apoptosis should be applicable to every population, unless strong environmental factors such as infectious epidemics interfere with the pathway.

Many studies have shown the importance of cell death regulation. Both inhibition and promotion of cell death are intrinsically linked with age-associated diseases (Christensen and Vaupel, 1996; Cotman, 1998; Kaufmann and Gores, 2000; Vaux and Flavell, 2000). Caspases, the key executioners of apoptosis (Cohen, 1997), function in cell death mechanisms; thus defining their role in successful aging becomes a necessity. Using quantitative RT-PCR, we quantified the mRNA expression of caspase-1, -3, -8 and -10 in PBMC of young, middle, old and extremely old subjects, and showed that a population composed of extremely old individuals expresses higher level of caspase–3 mRNA than do younger subjects, and unlike all other age groups, a population composed of old individuals expresses extremely high levels of caspase-8 mRNA. Despite changes

in caspase-1, -3 and –8 mRNA expression amongst the four age groups, caspase-10 mRNA expression remains constant with age, and is not found useful as a biomarker. Thus, in our cohort, a high level of caspase-8 is the biosignature for old individuals aged 70 to 89, while high levels of caspase-3 and low levels of caspase-8 mRNA expressions are the hallmark biosignature for nonagenarians and centenarians.

With regard to both caspase-1 and –3, population profiles reveal a slow shift with age toward higher levels of caspase mRNA expression. While younger populations are mostly composed of individuals expressing low levels of caspase-1 and –3, progressively in the intermediate and old population, more and more individuals express higher levels of caspase-1 and –3 mRNA; thus, the extremely old population is mainly composed of individuals expressing high levels of caspase-1 and –3 mRNA. These age-related shifts are important, as they may indicate that a subgroup of individuals within the younger population, expressing high levels of caspase–3 mRNA in their PBMC, are favored to attain longevity. However, other factors such as immunosenescence in extremely old individuals, menopause in middle age woman or drug against certain diseases could also explain some, but not all variation seen in caspases mRNA expression with age. Unlike caspase-1 and –3 mRNA expressions, such an age-related shift is not observed for caspase-8; thus populations of extremely old individuals show a caspase-8 population profile similar to young and middle-aged individuals. However, a significant increase in the number of individuals expressing extremely high levels of caspase-8 mRNA is found within the old age population.

Although we found fewer CD24-positive (mostly B) cells and double-positive CD4/CD45RA cells (mostly naïve helper T cells) in the PBMC of older individuals, we show that neither of these changes in immune profile is responsible for the changes in caspase mRNA expression seen amongst the four age groups. Thus, our results indicate that caspase mRNA expressions are independent of the immunological profile, and may be molecularly defined by the regulation of caspase gene expressions. In an attempt to better characterize caspase mRNA expressions and their role in successful ageing, it now becomes necessary to establish cultures derived from blood samples obtained from individuals within our cohort, and conduct functional studies. Future experiments will

thus attempt to determine why caspase-3 is highly expressed in extremely old individuals, by investigating the functional regulation of apoptosis in culture.

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TABLE 1. TAIWAN POPULATION

Age Groups (yrs)	Male (#)	Female (#)	Total
10-19	7	8	15
20-29	8	15	23
30-39	13	19	32
40-49	17	19	36
50-59	5	16	21
60-69	9	13	22
70-79	10	8	18
80-89	13	8	21
90-99	25	27	52
100+	1	5	6
Total	108	138	246

TABLE 2. CASPASE mRNA EXPRESSION IN PBMC

	Young (20-39 yrs) (N=47)	Middle (40-69 yrs) (N= 71)	Old (70-89 yrs) (N=28)	Extremely old (90+ yrs) (N=50)
Caspase-1	5.56 ± 1.77	5.57 ± 1.90	7.20 ± 2.26*	6.90 ± 1.99*
Caspase-3	0.85 ± 0.41	0.93 ± 0.40	0.93 ± 0.48	1.19 ± 0.40*
Caspase-8	1.60 ±0.41	1.83 ± 0.68	3.38 ± 1.74*	1.74 ± 0.44
Caspase-10	0.87 ± 0.11	0.87 ± 0.07	0.89 ± 0.08	0.90 ± 0.06

Data represent the mean ± SD of the ratio of each caspase/aldolase C. * Indicates a significant difference of $p \leq 0.05$ compared to all other unmarked age groups. N indicates the minimum number of individuals within each age group for which each given caspase mRNA expression was quantified.

TABLE 3. PHENOTYPE OF CELLS IN THE PBMC EXTRACTS

	Percent of PBMC Composition			
	Young	Middle	Old	Extreme Old
$CD4^+$	33.11 ± 7.82	30.73 ± 9.35	33.30 ± 10.30	31.39 ± 11.60
$CD8^+$	33.85 ± 7.14	28.35 ± 7.33	28.28 ± 6.96	34.65 ± 7.23
$CD24^+$	11.54 ± 3.17	10.76 ± 3.23	8.20 ± 4.04	6.12 ± 3.00*
$CD45RA^+$	72.39 ± 4.59	66.40 ± 8.25	63.61 ± 1.63	67.07 ± 9.86

Data represent average ± SD. * Indicate a significant difference of $p \leq 0.05$ when extreme old is compared to young.

TABLE 4. EXPRESSION OF CD45RA IN T CELL SUBSETS

	Percent CD45RA-Positive Cells	
	$CD4^+$	$CD8^+$
Young	42.22 ± 10.26	79.72 ± 8.60
Extreme Old	17.51 ± 6.01*	79.46 ± 8.06

Data represent the mean ± SD percent $CD4^+$ or $CD8^+$ cells that are also CD45RA positive.

* Indicates a significant difference, $p \leq 0.05$.

FIGURE 1: CASPASES mRNA EXPRESSION

Caspase mRNA expression in PBMC of young, middle, old and extremely old individuals, determined by quantitative RT-PCR. **A, B, C** show the average and standard error mean (SEM) for each caspase mRNA expression according to age group. **D, E, F** show the best-fit regression analysis for each caspase. * Indicates a significant difference of $p \leq 0.05$ compared to all other unmarked age groups. Equations for regressions are given in the text.

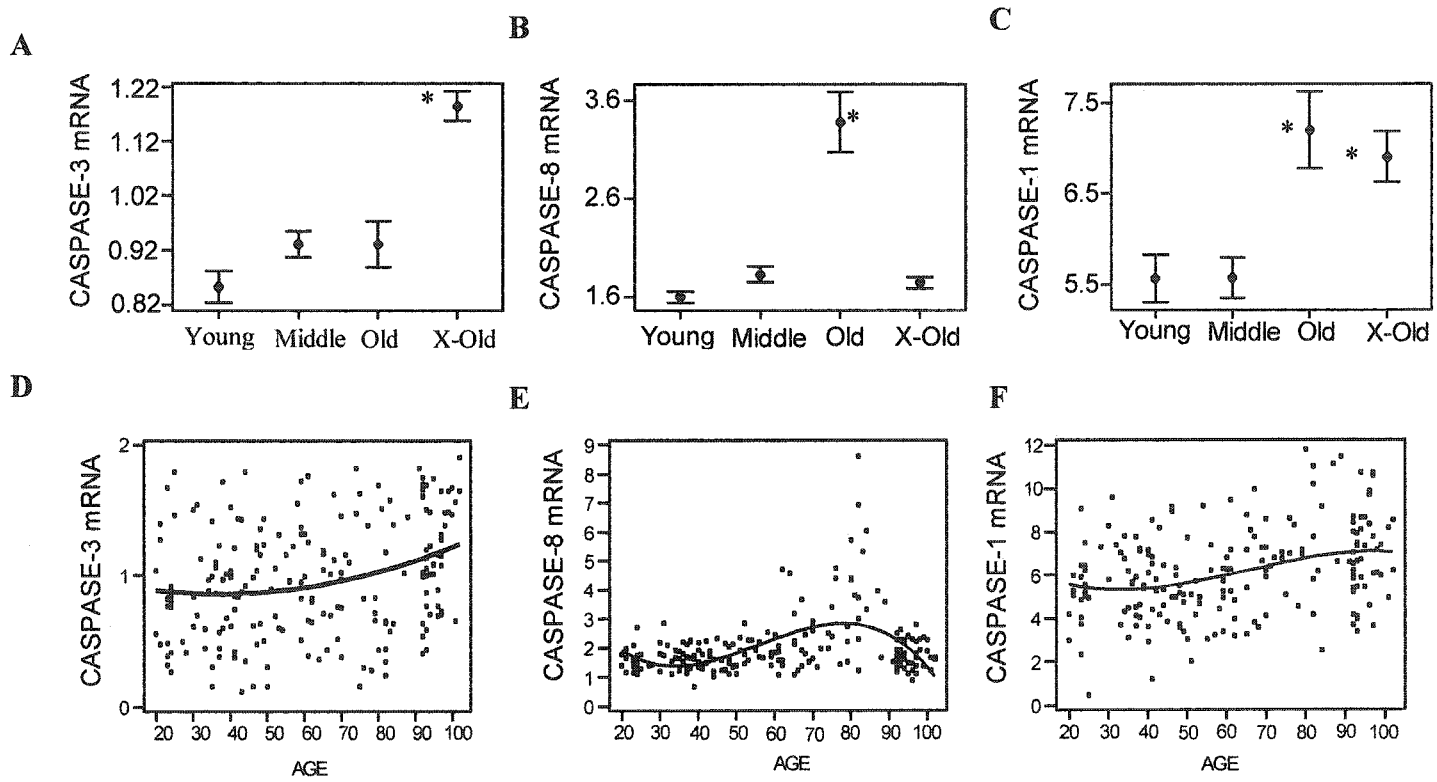
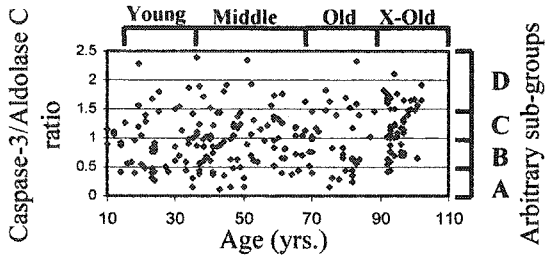

A
B
C
CASPASE-3 mRNA
1.22
1.12
1.02
0.92
0.82
Young
Middle
Old
X-Old
CASPASE-8 mRNA
3.6
2.6
1.6
CASPASE-1 mRNA
7.5
6.5
5.5
D
E
F
AGE
20 30 40 50 60 70 80 90 100

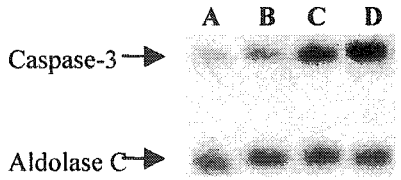
FIGURE 2: POPULATION DISTRIBUTION

Population distribution according to caspase mRNA expression. **A** shows the arbitrary sub-groups used to define low (group A), intermediate (group B), high (group C) and extremely high (group D) levels of caspase-3 mRNA expression. **B.** Example of RT-PCR showing the arbitrary sub-groups for caspase-3 mRNA expression. **C** shows the incidence (in percentage) within each age group, of individuals expressing low, intermediate, high and extremely high levels of caspase-1, -3 or -8 mRNA expression.

A


Young
Middle
Old
X-Old
Caspase-3/Aldolase C ratio
2.5
2
1.5
1
0.5
0
10
30
50
70
90
110
Age (yrs.)
D
C
B
A
Arbitrary sub-groups

B


A
B
C
D
Caspase-3
Aldolase C

C

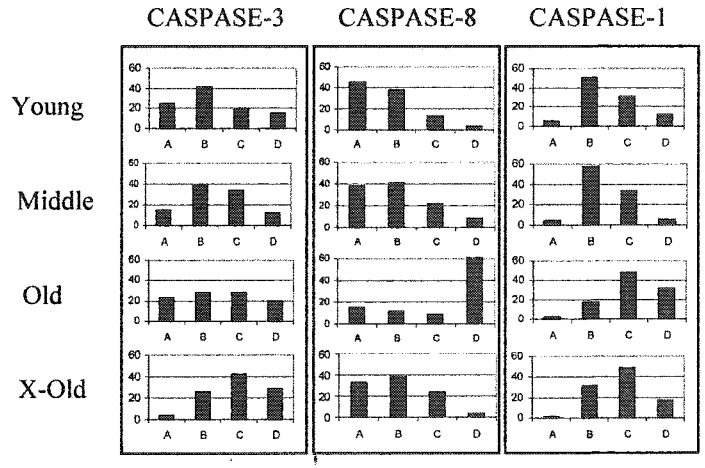
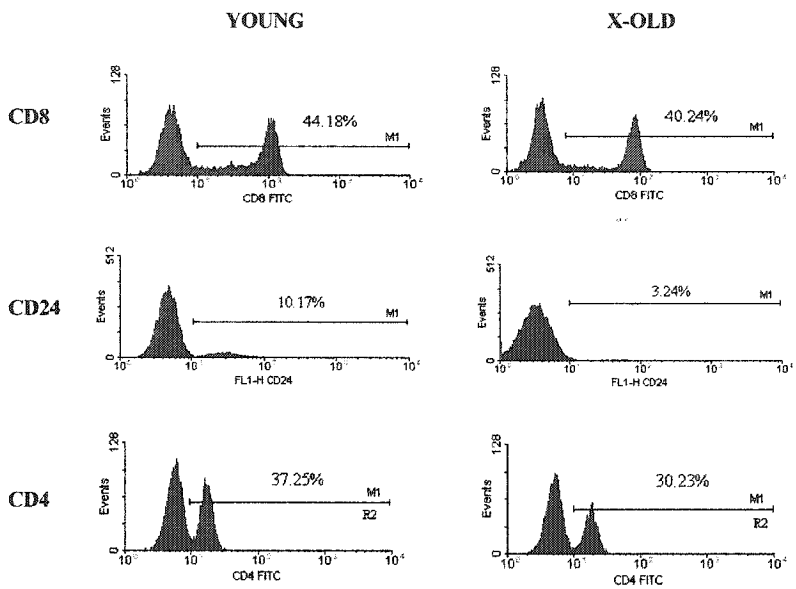

CASPASE-3
CASPASE-8
CASPASE-1
Young
Middle
Old
X-Old
60
40
20
0
A
B
C
D

FIGURE 3: FLOW CYTOMETRY PROFILE

Flow cytometry profile. A. Flow cytometry profile of representative young and extremely old individuals. **B.** Double labeling for CD4 and CD45RA.

A


YOUNG
X-OLD
CD8
CD24
CD4
44.18%
40.24%
10.17%
3.24%
37.25%
30.23%
M1
R2
Events
CD8 FITC
FL1-H CD24
CD4 FITC

B

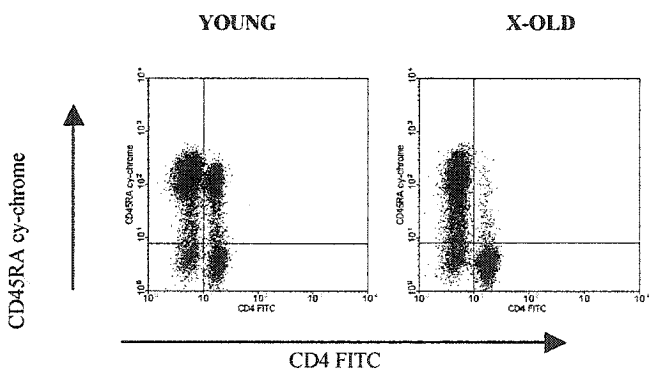
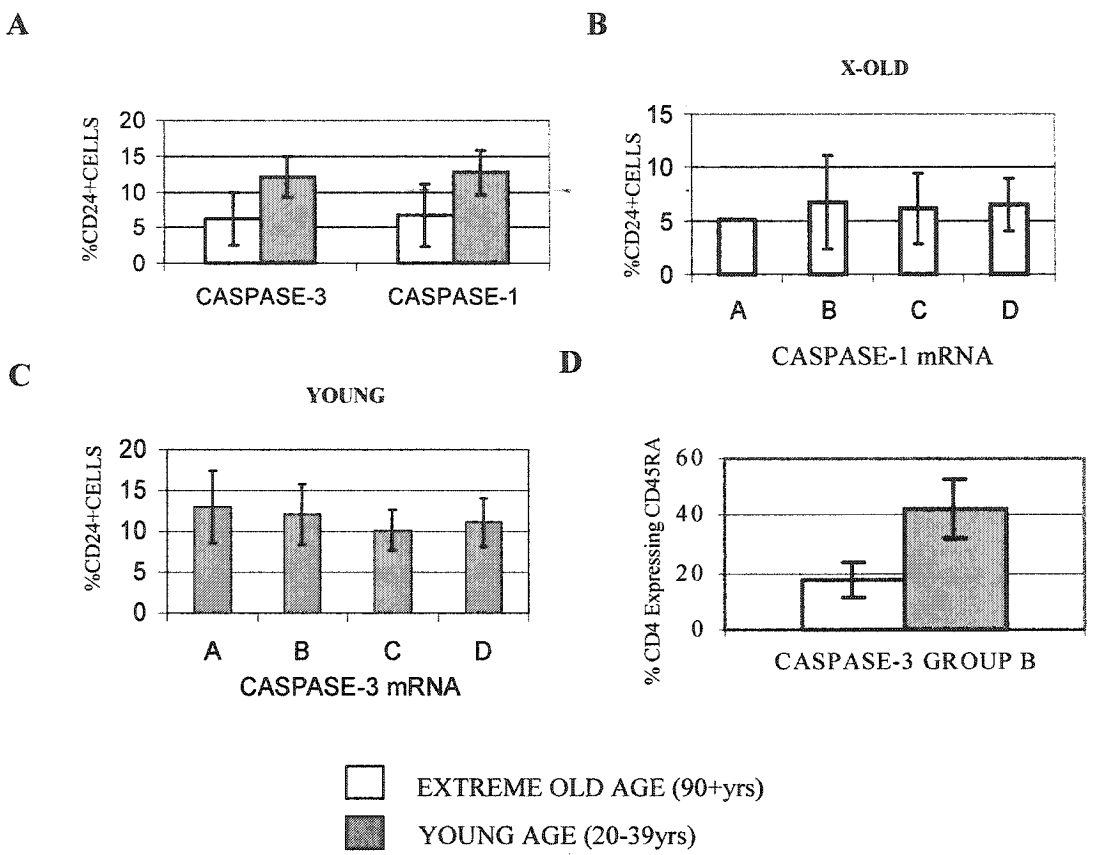

YOUNG
X-OLD
CD45RA cy-chrome
CD4 FITC

FIGURE 4: CASPASE mRNA EXPRESSION AND IMMUNOLOGICAL PROFILE

Caspase mRNA expressions and immunological profile. **A.** Percentage of CD24-positive cells in the PBMC of young (gray) and extremely old (white) individuals expressing intermediate (sub-group B) levels of either caspase –3 or –1 mRNA. **B.** Percentage of CD24-positive cells within the PBMC of extremely old individuals according to their level of caspase-1 mRNA expression. **C.** Percentage of CD24-positive cells within the PBMC of young individuals, according to their level of caspase-3 mRNA expression. **D.** Comparison between the percentage of CD4-positive cells also expressing CD45RA, in the PBMC of young and extremely old individuals expressing intermediate levels of caspase-3 mRNA (sub-group B).


A
B
X-OLD
%CD24+CELLS
20
15
10
5
0
CASPASE-3
CASPASE-1
%CD24+CELLS
15
10
5
0
A
B
C
D
CASPASE-1 mRNA
C
YOUNG
D
%CD24+CELLS
20
15
10
5
0
A
B
C
D
CASPASE-3 mRNA
% CD4 Expressing CD45RA
60
40
20
0
CASPASE-3 GROUP B
EXTREME OLD AGE (90+yrs)
YOUNG AGE (20-39yrs)

PREFACE TO CHAPTER 4

Realizing the need for a functional study in order to substantiate the data from my study and provide a renewable source of biological material derived from the blood of extremely old individuals, I investigated the possibility of immortalizing B lymphocytes from frozen blood aliquots. After several attempts, I found that it is possible to immortalize B lymphocytes using only 1 ml of frozen blood from individuals of any age, including extremely old individuals having low level of circulating B lymphocytes, using the Epstein-Barr virus. The next chapter, provides an account of the methodology used to establish cell lines from frozen blood aliquots of extremely old individuals.

CHAPTER 4

ESTABLISHING LYMPHOBLASTOID CELL LINES FROM FROZEN BLOOD OF EXTREMELY OLD INDIVIDUALS

Shorten version of this chapter has been published in *Mech. Ageing Dev.* (2002) **123**: 1415-1418.

4.1 ABSTRACT

When conducting a population study involving the use of biological material derived from peripheral blood obtained from donors of various ages and medical conditions, the amount of biological material available for research is, in some cases, restricted to very small amount. In order to maximize the resource of biological material available for research and permit us to perform more experiments, we investigated whether a minimal amount of total frozen blood from extremely old donors (90+) could be used to establish cell lines using the Epstein-Barr virus. Here, we show that although there is a constant decrease in the amount of circulating B cells with age, it is possible to established lymphoblastoid cell lines using very minute (one to two millilitres) amount of frozen blood aliquots obtained from donors of all ages, and thus permit functional study in culture conditions as well as the regeneration of biological material extracted from important and irreplaceable samples from donors of extreme old age.

4.2 INTRODUCTION

The quest for longevity candidate genes, as well as the identification of gene expressions involved in either successful aging or the development of age-associated diseases, all require large quantities of DNA, RNA, proteins and cells from young, middle, old and extremely old individuals (90+). However, due to their advancing age, extremely old individuals cannot, without significantly compromising their health, afford to donate large quantities of blood for biological studies. As a result, aging studies must be carefully designed, and the use of every microgram of biological material carefully thought out. This strict limitation on the amount of available biological material does not permit thorough investigation of the aging process, and impedes our ability to perform optimization procedures and subsequently generate large amounts of data. Thus, it becomes essential to develop a method permitting the regeneration of biological material obtained from individuals of all age groups, to facilitate aging as well as other population studies.

Currently, throughout the world, tissue culture has become the method of choice to generate large amounts of biological materials for biomedical research. Most notably, for several years, blood obtained from donor individuals has been cultured and successfully transformed using the Epstein-Barr virus (EBV) (3). EBV is capable of transforming human B-lymphocytes, and thus can be used to generate stable human lymphoblastoid cell lines (LCL). Previous studies have documented the use of EBV for transforming both fresh blood and purified frozen lymphocytes extracted from a variety of donors (2, 7). However, the standard protocol currently available for EBV transformation requires the use of several millilitres (10 ml or more) of fresh blood, or sufficient amounts of blood to obtain enough purified lymphocytes, and thus cannot be readily performed in aging studies (2, 5, 7). Large volumes of blood are needed to establish LCL using EBV, as the virus almost solely transforms B-lymphocytes.

With age, the bone marrow constantly diminishes its production of naïve B-lymphocytes, and thus the percentage of B-lymphocytes present in the PBMC of extremely old individuals may be lower than that of younger subjects (1). Thus, although one study has reported the use of smaller volumes of blood in EBV transformation (8), it

becomes necessary to evaluate the possibility of transforming cells from extremely old individuals using the Epstein-Barr virus, and to develop a protocol permitting the generation of stable LCL from individuals with low levels of circulating B-lymphocytes.

4.3 MATERIALS AND METHODS

SUBJECTS

Peripheral blood was obtained from healthy Chinese donors aged 20 to 102 years old, living in Taiwan, following written consent. This protocol was approved by the Institutional Review Boards of Taichung Veterans General Hospital (Taichung, Taiwan), and the Sir Mortimer B. Davis Jewish General Hospital, a teaching hospital of McGill University (Montreal, Canada).

BLOOD COLLECTION, PROCESSING, AND STORAGE

Between 10 and 30 ml of peripheral blood from each subject was collected into Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) containing ethylenediaminetetraacetic acid (EDTA). Blood-filled tubes were kept on ice for a maximum of four hours before processing.

One of the collected Vacutainers was open under sterile conditions and aliquoted into 2 ml samples in 3 ml cryovials. Each aliquoted blood sample was supplemented with dimethyl sulfoxide (DMSO) (Sigma, St-Louis, MO) to a final concentration of 10%. Vials supplemented with DMSO were agitated to permit the uniform mixing of DMSO with the blood, and subsequently stored at -80°C. Samples were kept at -80°C for a maximum of two months before being transferred to a -150°C freezer for long-term storage.

Remaining Vacutainers were centrifuged at 12,000 rpm for 10 min. Following centrifugation, the upper phase containing the plasma was removed using a pipette. Plasma-free samples were then diluted using 1X phosphate buffered saline (PBS) so as to double the original blood volume. Following dilution, blood samples were loaded onto 3

ml Ficoll-Paque (Amersham Pharmacia Biotech, Piscataway, NJ), cushions set up in round-bottom 14 ml tubes, and peripheral blood mononuclear cells (PBMC) were extracted according to the manufacturer's instructions (Amersham Pharmacia Biotech). Extracted PBMC were resuspended in 1X PBS and counted using a haemocytometer.

Counted PBMC samples were centrifuged at 1100 rpm for 10 min., and pellets were resuspended in 0.5 ml of a solution containing a final concentration of 1% paraformaldehyde. Samples were allowed to fix overnight at 4°C. After 12 to 16 hours, fixed samples were centrifuged at 1100 rpm for 10 min., washed once with 1X PBS, and following a second round of centrifugation, suspended in 0.5 ml of 1X PBS. Samples were kept at 4°C until flow cytometry experiments were conducted.

FLOW CYTOMETRY

To assess the quantity of B cells present in each sample, $1X10^5$ paraformaldehyde-fixed cells from each subject were incubated on ice, in the dark, for 45 minutes with 100 µl of 1X PBS containing 7 µl of PE-conjugated CD19 antibody (Pharmingen, Mississauga, ON). Following incubation, samples were washed with 1 ml of 1X PBS, centrifuged at 1100 rpm for 10 min., and resuspended in 400µl of PBS. Samples were analyzed with a FACScan (Becton Dickinson), and results were analysed using Cell Quest software. Final graphs were drawn using winMDI (J. Trotter, in the public domain).

EBV TRANSFORMATION

For each subject, one cryovial containing 2ml of DMSO-supplemented blood was rapidly thawed in a 37°C water bath. Each thawed blood sample was transferred to a 15 ml conical tube and diluted with growth medium (10 ml RPMI-1640 (Gibco BRL, Rockville, MD) supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin (both from Mediatech, Herndon, VA) and 20% fetal bovine serum (FBS) (Wisent, St-Bruno, Quebec). Diluted blood samples were centrifuged at 12,000 rpm for 10 min. Following centrifugation, the medium was removed with a pipette and pelleted cells were

resuspended as previously, in 10 ml of growth medium. Samples were washed a total of 4 times with growth medium. Following the last centrifugation, as the blood samples were still greatly contaminated with red blood cells (RBC), once the medium was removed using a pipette, cells were resuspended in 3 ml of growth medium and loaded onto 2.5 ml Ficoll-Paque cushions (Amersham Pharmacia Biotech) set up in 5 ml round-bottom tubes. Samples were centrifuged at 12,000 rpm for 20 minutes. Following centrifugation, each sample was transferred using a pipette to a 15 ml conical tube, leaving the pelleted red blood cells at the bottom of the 5 ml tube. Samples were washed three times with 10 ml of growth medium. After centrifugation at 12,000 rpm for 10 min., following the third wash, and removal of the medium, cells pelleted at the bottom of each tube were resuspended in 300 µl of Epstein-Barr virus (EBV) strain B95-8 for B cell immortalisation (ATCC, Manassas, VA). Resuspended cells were transferred in a single well of a 48 well tissue culture plate and place in a 37°C, 5% CO_2 tissue culture incubator for two hours. After incubation, 800 µl of growth medium and 2X cyclosporine A (CSA) were added to each well. As previously reported, CSA is needed to prevent the suppression of EBV transformation caused by T suppressor and cytotoxic cells (4, 6). Following the addition of medium, half of the total volume of one well was transferred into the neighbouring well. Three days latter, 1X CSA was added to each well.

Cultures were left untouched for about one month (no change of medium) or until nice "colony" clumps of growing cells were observed. Once growing cells were observed, 300 µl growth medium was added to every well, and cells were put in the incubator for an additional week or until densely grown. Cultures were conservatively expanded about once a week using 48, 24, 12 and 6 well plates. Once the 6 well plates were dense, cells were transferred to a 25 ml culture flask, and culture expanded until enough cells were produced. In general, a three months period was required to establish stable cell lines.

FLUORESCENT MICROSCOPY

To determine the phenotype of the transformed cells, 1 ml of culture cells were put into a 5 ml round-bottom tube and washed with 3 ml of 1X PBS. Following

centrifugation at 12,000 RPM for 10 min, cells were resuspended in 100ul of 1X PBS containing 10 ul of FITC-conjugated anti-CD19 antibody (Pharmingen). Following incubation, cells were rinsed with 1 ml of 1X PBS and centrifuged for 10 min at 1200 RPM, then resuspended in 20 ul of 1X PBS. The total volume was dropped on a slide, a cover slip was put on top and slides were viewed using a fluorescent microscope (Nikon Labophot, Nikon, Japan). Pictures were taken using a digital Spot Rt camera (Diagnostic Instruments, Sterling Heights, MI). Jurkat cells (transformed T lymphocytes) were used as negative controls (they are CD19 negative). Jurkat cells are commercially available from ATCC (Rockville, Maryland).

4.4 RESULTS

PERCENTAGE OF B-LYMPHOCYTES PRESENT IN PBMC WITH AGE

As the Epstein Barr virus (EBV) almost exclusively transforms B-lymphocytes, we first sought to investigate whether the number of B-lymphocytes present in our samples varies with the age of our subjects. As shown in Table 1, only 6.76% of cells present in the PBMC of extremely old individuals (aged 90-102 yrs old) are positive for CD19, a B cell marker. On the other hand, we found that 13.33% of the PBMC present in young individuals (19-29 yrs old) are CD19 positive. Extremely old individuals thus have two-fold less B cells in their PBMC than their young counterparts. Flow profiles for both a young and an extremely old individual can be seen in Figure 1.

RED BLOOD CELL CONTAMINATION

Once the count of CD19-positive cells present in each subject's PBMC was determined, we attempted to transform blood samples from a few subjects using 1 ml of cryopreserved blood samples, by EBV. From these preliminary experiments we found that red blood cell (RBC) contamination could not successfully be removed using several rounds of washing with PBS. We wanted to get rid of the RBC contamination to permit a better visualization of the cells when they were placed in culture, and thus allow us to

better monitor the presence of clumps indicating successful EBV transformation. When too much RBC are present in the culture, they obscure the field of vision, and given their clumpy appearance, can easily be confused with transformed EBV cells. To diminish the RBC contamination, we decided to include a mini-Ficoll Paque extraction in our protocol. We found that the use of Ficoll Paque greatly diminished our RBC contamination level.

USE OF CYCLOSPORINE –A

In order to prevent regression of LCL, as caused by cytotoxic T cell, cyclosporine A was added to our growth medium. After the first round of preliminary experiments, we noticed that if cyclosporine A was added to each culture three days after cells were put initially in culture, in growth medium also containing 2X CSA, cells lines were established much sooner. Thus, we added this extra step in our protocol.

GROWTH OF LCL AND CELL PHENOTYPE

Throughout their period of transformation and growth, cells were closely monitored by inverted microscopy. As shown in figure 2A, growth was first identified and confirmed by the presence of small clumps in the culture. After a few more weeks in culture, large regions of dividing cells were seen in the plates (Fig. 2B and C). At this stage, a small aliquot of the culture was removed and labelled with human anti-CD19 FITC-conjugated antibody, to determine whether the transformed cells had a B cell phenotype. As shown in Figure 3 (C, D), all of the cells obtained from our growing cultures were CD19-positive, thus indicating that they have a B cell phenotype. Jurkat cells, which are T cells are CD19-negative, and thus used as a negative control (Figure 3 A, B).

4.5 DISCUSSION

When conducting population studies, the amount and availability of biological material is limited, and often dictates which experiments can or cannot be performed. These limitations are attributable to two main factors: first, only minimal amounts of biological samples can be obtained from a donor at any given time, and second, many donors (such as very old or sick donors) will not be able to provide samples on more than one occasion. Thus, the availability of biological material greatly slows down aging research and basic research using human blood samples as the primary source of biological material. The sole means of preserving important precious samples is to develop a method permitting the renewal of biological material. With this in mind, we chose to attempt the transformation of our limited amount of frozen blood samples using the Epstein-Barr virus.

As the ages of the individuals present in our study cohort varied from young to extremely old, and given that the production of B lymphocytes changes with age, using flow cytometry we first documented the expression of CD19 in the peripheral blood mononuclear cells (PBMC) of all our samples. Our experiments revealed a two-fold decrease in the amount of B-cells present in PBMC of extremely old individuals compared to younger individuals. The identification of this decrease in B cells with age was important, as EBV almost exclusively transforms B-lymphocytes.

Having found a significant decrease in the amount of circulating B-lymphocytes with age, we performed a series of transformations to determine which conditions support production of LCL from 1 to 2 ml of frozen blood, from both young and extremely old individuals. We found that using a "mini" Ficoll Paque extraction was most effective in reducing RBC contamination, and thus enabled us to better monitor the growth of our cultures. Failure to remove RBC results in obstruction of the visual field, and prevents us from assessing transformation. Furthermore, we found that administering CSA three days after cells are put in culture, as well as its presence in the growth medium, greatly speeds up the growth of our cultures by preventing regression. As previously reported, regression of LCL can occur if cytotoxic T cells are not suppressed, as they react against

transformed B lymphocytes (4, 6). Currently, our transformed cell lines have all been in culture for more than four months. They are all stable, but grow very slowly.

Using this newly described procedure, we were able to successfully establish LCL from minimal amounts of frozen blood aliquots from extremely old individuals having low levels of circulating B-cells. The use of this protocol, we hope, will permit us to better investigate the mechanisms of aging by providing a source of biological samples that are renewable, and a tool for others who rely on the blood of subjects from all ages to obtain biological data and contribute to the advancement of their respective fields of research.

4.6 ACKNOWLEDGMENTS

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Table 1. Percentage of B-Cells present in PBMC with Age

Age Group (Years)	% of CD19 positive cells in extracted PBMC
19-29	13.33 ± 4.02*
30-59	10.01 ± 3.22
60-89	9.15 ± 3.05
90-102	6.76 ± 3.38*

* Indicates a significant difference of $p \leq 0.05$ with all other age groups

FIGURE 1: FLOW CYTOMETRY PROFILE FOR CD19

Flow cytometry profile for CD19. This figure shows by density plots the count of CD19-positive cells present in the PBMC of a young and an extremely old individual. CD19- positive cells (CD19 is a B cell marker) are found in the top right corner, while CD19 negative cells are found in the bottom right corner.

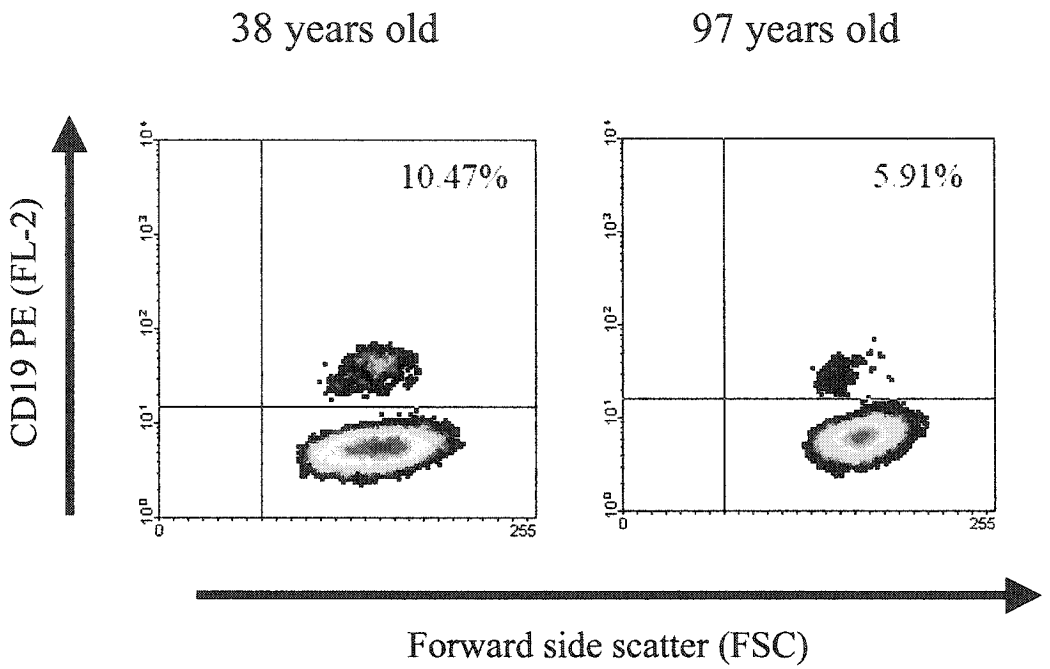
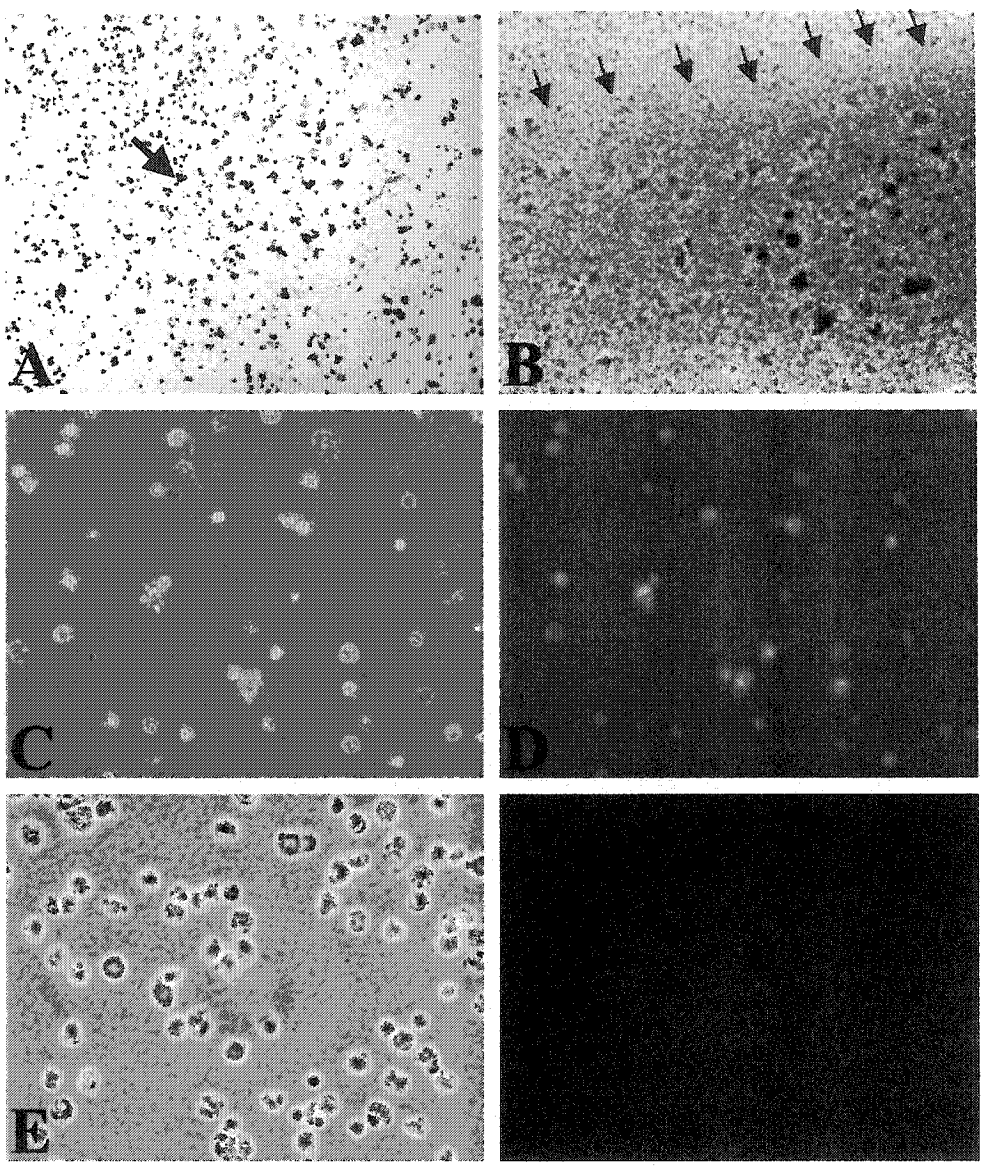

38 years old
97 years old
CD19 PE (FL-2)
10.47%
5.91%
0
255
Forward side scatter (FSC)

FIGURE 2: STAGES OF EBV TRANSFORMATION AND PHENOTYPE OF EBV IMMORTALIZED CELLS

Stages of EBV transformation and phenotype of EBV immortalized cells. **A.** Phase contrast picture of cells cultured with EBV for three weeks. In this panel a few clumps of proliferating cells can be seen (arrow). **B**. Phase contrast picture of cells cultured with EBV for five weeks. As indicated by the series of arrows, the cells have proliferated, and show a large region of growth. **C**. Phase contrast image of EBV transformed cells. **D**. Fluorescent staining of EBV transformed cells from an extremely old donor (90+) with CD19 FITC-conjugated antibody **E**. Phase contrast of Jurkat cells. **F**. Fluorescent staining of Jurkat T cells with CD19 FITC-conjugated antibody (negative control).


A
B
C
D
E

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSION

GENERAL DISCUSSION AND CONCLUSION

The applicability of aging studies conducted in model organisms to human aging is yet undetermined but likely to be limited as, unlike seen in most model organisms, human aging is closely associated with the onset and progression of age-associated diseases which greatly affect human lifespan. As such, it becomes primordial to conduct aging studies in our specie and identify the factors contributing to successful aging as defined by the ability to retain good health until death. In the last two decades, the study of human aging has focused on two main areas: first the study of age-associated diseases and second, the study of extreme elderliness.

By understanding the etiology and factors contributing to the development and progression of age-associated diseases, we have developed methods to retard the onset of severe, debilitating forms of these diseases and developed ways to improve the quality of life of elderly suffering from them. For instance, knee and hip replacements enable elderly suffering from osteoarthritis to retain their mobility and allow them to remain self-sufficient for more years. However, despite all the researches, current medical interventions can only delay the inevitable, the loss in quality of life.

On the other hand, studies of extreme elderliness have revealed that nonagenarians and centenarians are unique individuals in the sense that in general, they do not suffer from severe debilitating forms of age-associated diseases (Perls *et al.* 2002). In fact, a great majority of extremely old individuals (90+) only have mild forms of age-associated diseases or avoid them altogether, thus enabling them to remain self-sufficient until extreme old age. Interestingly, the mortality rate of populations composed of extreme old individuals is different from that of cohorts composed of younger age subjects, instead of increasing exponentially as a function of age, their mortality rate slows down and plateaus (Thatcher *et al.*, 1996). This is believed to result from the absence of frail individuals in cohorts composed of extremely old "robust" individuals. As such, it is believed that identifying the genetic, environmental and sociological factors enabling individuals to attain extreme old age and hence successfully age, will enable us to develop means to prevent, retard, and treat age-associated diseases that significantly decrease the quality of life of elderly. As the regulation of cell death is intrinsically

linked with age-associated diseases and plays an important role in the immune system (Christensen and Vaupel, 1996; Cotman, 1998; Kaufmann and Gores, 2000; Vaux and Flavell, 2000), I hypothesized that extremely old individuals, successful models of aging possess a more robust immune system capable of adaptation and having a beneficial apoptotic regulation.

However, investigating the process of human aging and determining the factors favoring healthy aging are not easy tasks as our specie is long lived, and only limited amount of biological materials can be obtained from living humans, especially extremely old individuals, without significantly compromising their health. Several other considerations must also be taken before embarking on a human population study. These studies are extremely costly, very few granting agencies will provide financial means to support them, and human subject's rights and protections must be a foremost concern. As such, investigators need to obtain permission from an internal review board before beginning any human study and obtain the consent from all subjects. For all of these reasons, current human population studies are extremely limited in terms of the amount of data they can generate. As, such, while previous population studies have mostly been limited to studies conducted with only one type of biological material, for example only with RNA or DNA or cells, I needed to develop a method by which it would be possible to obtain several different types of biological materials from very limited amount of blood extracted from individuals of all ages in order to test my hypothesis. The method that I developed provides the means to accomplish a more thorough investigation of the human aging process and will be of great use to biogerontologists (Lacelle *et* al., 2002a). Using this method, it is possible to obtain RNA, DNA, proteins, fixed PBMC, plasma and frozen blood aliquots from a single 10ml of blood obtained from individuals of any age and thus, conduct parallel studies on more than one biological species (ie: RNA, DNA...) derived from a single individual.

During the process of aging, the blood composition changes. For instance, elderly are often anemic hence, their hematocrit is often low (Coppola *et al.*, 2000; Caprari *et al.*, 1999). As such, following the development of this new methodology enabling the simultaneously extraction of different biological materials from a single blood sample, the first task was to determine whether changes in the recovery of biological sub-specie

(ie: RNA, DNA, proteins...) varied with the age of the subjects from whom the blood was obtained. Such variations could limit the availability of biological material for studies and impede on our ability to thoroughly study the aging process. I found that the methodology used did not result in significant changes in the amount of RNA, DNA, proteins and fixed cells recovered from 1ml of blood, between young and extremely old donors. However, the yield of plasma was greater in extremely old individuals than in younger subject. This correlates with the previous observation that the hematocrit of older individuals is lower than that of younger subjects (Coppola *et al.*, 2000; Caprari *et al.*, 1999). Consequently, since the blood of older individuals contains fewer red blood cells, the proportion of plasma in the blood is greater for the volume of blood. Thus, I concluded that the recovery of all biological materials, except for the plasma, was similar between young and old individuals because either no differences existed between the blood of young and elderly subject in term of the amount of biological sub-species present, or that the method use did not permit to differentiate between minimum variations in sample composition.

Although this method of processing blood sample can generate more biological materials than conventional methods such as finger pricked for DNA studies, the sample processing time is longer than would be if each blood sample was used to extract only one type of biological specie such as RNA, DNA, proteins or fixed cells. Furthermore the method does not permit to maintain enzymatic activity and hence caspase assays as well as other enzymatic assays cannot be performed using sample extracted by this method. As such, investigators wishing to study enzymatic activities should use a methodology enabling them to extract proteins while maintaining enzymatic activity despite the fact that such methods do not enable them to extract RNA and DNA from the same blood samples used to extract the proteins. Likewise, transcriptional and translation efficiencies cannot be assessed by the methodology I described, unless frozen blood samples are thawed and cells cultured. However, it is generally more suitable to perform those studies on fresh samples, as cellular viability is greater. Despite these disadvantages, the methodology I developed enables researchers to conduct a more thorough investigation of the aging process and renders population studies more cost effective.

As I hypothesized that extremely old individuals possess a more robust immune system capable of adaptation and having a beneficial apoptotic program and hence, to determine whether apoptosis is part of the determinants of longevity, I used biological materials that I extracted from the blood of 246 healthy Chinese donors aged 10 to 102, living in Taiwan and obtained following the newly described method, to study the expression of caspase genes in PBMC (Lacelle *et al.*, 2002b).

Characterization of caspases mRNA expression in PBMC revealed a unique pattern of caspases mRNA expression in extremely old individuals (90+) characterized by a high expression of caspase-1 and -3 and a low expression of caspase-8 mRNA. While high expression of caspase-1 mRNA is also seen in old individuals (70-89), high expressions of caspase-3, in conjuncture with a low expressions of caspase-8 mRNA, are only seen in extremely old individuals. The old age group is characterized by high levels of caspase-8 mRNA expression while the middle and young age groups express low levels of caspase-1, -3 and -8 mRNAs.

When caspases mRNA expression patterns are more closely studied, one can see that most extremely old individuals possess high or extremely high levels of caspase-3 mRNA while most young individuals possess low levels of caspase-3 mRNA within their PBMC. Given that our population study was cross-sectional, three possibilities exists: first that caspases-3 mRNA expression increases with age, second, that individuals having high levels of caspase-3 mRNA expression are favored to attain extreme old age, and third, that individuals capable of adapting to their environment by upregulating their caspase-3 mRNA expression are more successful in reaching extreme old age.

Unlike that seen for caspase-3 mRNA expression, sub-populations composed of young, middle and extremely old individuals present with similar patterns of caspase-8 mRNA expression: all of them express low levels. On the other hand, the sub-population composed of old individuals is drastically different from other age group sus-populations and express extremely high levels of caspase-8 mRNA. This observation clearly reveals that changes in caspase-8 mRNA expressions are not age-associated. Interestingly, most individuals afflicted by severe debilitating forms of age-associated diseases are found within the old age group, the only group expressing high level of caspase-8 mRNA. Could one's ability to maintain low levels of caspase-8 mRNA

expression in old age indicate that they might reach extreme old age and be less affected by age-associated diseases? Does increase caspase-8 mRNA expression in old age favors the degeneration of the immune system? The results obtained from the study of caspase-8 mRNA expression in PBMC raises many questions however, significant amounts of research will be needed before we can clearly identify why most old individuals, while very few extremely old individuals express extremely high levels of caspase-8 mRNA.

Caspase-1 mRNA profile reveals that sub-populations composed of both old and extremely old individuals possess higher number of subjects expressing high levels of caspase-1 mRNA than the young and middle age groups. Given that caspase-1 is involved in the processing of pro-inflammatory cytokines into their mature forms (Earnshaw *et al.*, 1999; Fantuzzi and Dinarello, 1999), changes in caspase-1 mRNA expression could arise either from changes in apoptotic program or likewise, in an increase demand for cytokine processing.

While it is interesting to consider the expression of each caspase mRNA as a separate entity and speculate on their role in successful aging, it is likely that beneficial apoptotic program enabling one to reach extreme elderliness results from beneficial expression in more than one caspase mRNA. As such, it becomes important to also consider the overall pattern of caspase mRNA expression. After all, caspases act in synergy to bring about the process of programmed cell death, better known as apoptosis.

As changes in PBMC composition could account for the changes in caspases mRNA profile between various age sub-populations, I used flow cytometry to profile each subject's PBMC and determined whether some changes in PBMC composition existed within age groups. Although limited amount of immunological markers where used, I found in our population, extreme old age individuals had lower amounts of circulating CD24 positive and naïve T cells in their PBMC than younger subjects. As such, it is likely that extremely old individuals possess more memory T cells, generally characterized by the surface expression of CD45RO. Unfortunately, this was not tested as CD45RO antibodies cannot be use on paraformaldehyde fixed lymphocytes. Lower levels of CD24 positive cells in extremely old individuals indicate that nonagenarians and centenarians have lower amount of circulating B cells than younger subjects.

Following the characterization of caspases mRNA expression in PBMC and immune profiling, I studied the relation between caspases mRNA expression and immunological profiles and found that in our population, changes in immune profiles do not appear to be responsible for changes in caspase mRNA expression levels. As such, I found that individuals having different PBMC compositions can have the same caspases mRNA expression profile. As this is the first study investigating the relationship between immune profiles and caspase mRNA profiles, more studies will be needed in order to fully dissociate changes in caspase mRNA expression profiles from changes in PBMC composition. I am currently unable to perform these studies, as the amount and type of biological materials that I possess do not permit such studies. Ideally, it would be interesting to study the mRNA expression as well as the protein abundance and the caspase activity directly within each sub-type of lymphocytes however, this would require more than 10ml of blood per subject. Furthermore, blood samples would need to be process following a different protocol. This ideal protocol would rely on cell sorting by flow cytometer at the site of blood collection. This was not available in Taiwan where the blood from our subjects was drawn.

While I identified a unique profile of caspase mRNA expression in extremely old individuals and showed that changes in caspase mRNA expression do not appear to be associated with changes in PBMC composition occurring as a consequence of aging, it remains unclear whether or not the unique caspase profile identified in extremely old individuals represents that of a robust immune system and is indicative of successful aging. Several factors will need to be examined before such conclusion can be drawn. For instance, what are the influences of circulating levels of cytokines and hormones on caspase mRNA expression and what is the impact of the subjects' nutritional status on that expression? It will also be necessary to determine whether the observed caspases mRNA expression levels correspond to similar levels of caspase activity. While I was unable perform this study as the biological samples did not retain their enzymatic activity due to the method used to process them, I am unable to tell whether the caspases mRNA expression level of my samples correspond to similar caspase activity levels. However a study carried by Aggarwal and Gupta (1999) using 15 blood samples from young individuals (18-22yrs) and 15 from older individuals (65-92 yrs), showed that in

peripheral T cells, increase in caspase-3 mRNA expression between young and old subject, corresponded to increase in caspase-3 protein abundances and caspase-3 activities. Data generated in our laboratory (unpublished data on Danish population), also reveal that samples having higher level of caspase-3 mRNA expression have higher amount of caspase-3 proteins. I started analyzing caspase-3 protein abundances in samples from the Taiwan population however, due to a problem during the shipping of one batch of samples from Taiwan to Canada, some samples are unfortunately unusable and as such the study could not be properly completed. However primary results revealed the same trend (data not shown).

Studies conducted in calorie-restricted rodents indicate that calorie restriction, which significantly extends lifespan, can slow down the deterioration of the immune system seen with age. This is consistent with the observation that most physiological processes in caloric restricted rodents correspond to those of much younger control animals. In calorie restricted animals, increase apoptosis of nonfunctional T-cells is seen in older animals (Spaulding *et al.*, 1997). Decrease incidences of neoplasms, degenerative disorders and autoimmune disorders are also seen in those animals and to a great extent were shown to result from increase apoptosis (Keenan *et al.*, 1995 a, b; Warner *et al.*, 1995). Warner and colleagues (1995) have suggested that increase apoptosis occurring as a result of caloric restriction permits animals to live longer as they have better defense against age-associated diseases such as cancers and autoimmune diseases. Thus results obtained from studies conducted in long-lived calorie restricted animals tend to support the idea that a more robust immune system with beneficial apoptotic program favors longevity. However, a full study of caspases mRNA expression, protein abundance and caspase activity remains to be performed in long-lived calorie restricted animals.

Results from my study indicating that extremely old individuals express higher level of caspase-3 mRNA along with those of Aggarwal and Gupta (1995) showing that increase caspase-3 mRNA expression yields increase amounts of caspase-3 proteins and results in higher caspase-3 activity, as those obtained from calorie restricted rodents, strongly suggest that it is possible that a beneficial apoptotic program enables some individuals to escape age-associated diseases and reach extreme old age. More studies

will however need to be conducted to clearly establish whether indeed this is the case and whether such beneficial apoptotic program is genetically programmed or rather results from an increase adaptability of the immune system in certain individuals.

While my study was focused on caspases and as such, on the effectors of apoptosis, it would be beneficial in future studies to also analyze the regulators of apoptosis by studying the Bcl-2 family. However, the study of Bcl-2 family members should be conducted at the protein rather than the RNA levels as it is the ratio between the amount of proteins from anti-apoptotic and pro-apoptotic Bcl-2 family members that serve to regulate apoptosis.

Realizing the limits of my system and the need to perform functional studies, I investigated the possibility of immortalizing B lymphocytes using the Epstein-Barr virus and found that with proper conditions, it is possible to immortalize B lymphocytes from individuals of any age, including extremely old individuals having low levels of circulating B cells, using only 1 ml of total frozen blood The cell lines thus obtained have been grown in culture for several months and are stable however, their replication rate is slow and as such, only limited amount of lymphocytes can be produced in short period of times. Despite this downside, immortalization of B lymphocytes from the peripheral blood of extremely old individuals is a replenishable source of biological material. This is of great importance as all the biological material we possess from our human population is non-replenishable, thus once we use it, we lose it. In the future, I hope to be able to perform functional studies in the established cell lines and assess whether cells retained their levels of caspase mRNA expression after immortalization and, if this is the case, perform apoptotic induction and caspase assays. This is however a long-termed goal and as with any immortalized or transformed cells, the impact of the virus on cells cannot be neglected. Although valuable information can be obtained from those studies, one will eventually need to use native lymphocytes to validate the results. However, the main advantage of an *in vitro* system is that all cell lines are grown under the same conditions and as such, environmental and nutritional status are similar for all samples. This is not the case *in vivo* where it is hard to determine whether observed changes result from the host environment or from genetic programming.

As previously mentioned, the cost of population study, the limited amount of biological material, and the need for internal review board and subject consent before beginning experimentation, all limit the amount of results than can be generate using population studies. Although not much can be done to decrease the cost of population studies at this moment, and the fact that subject consent and internal review board are important processes which can not be disregarded, as shown in this thesis, it is possible to maximize the amount of biological material available for research. Having developed a method to maximize the amount of material obtained from the blood samples of our subjects, I was able to identify a unique caspase mRNA expression profile in extremely old individuals and show that the expression of caspase mRNA in PBMC appears to be independent of immune profiles. I have also shown that it is possible to immortalized B lymphocytes from the blood of individuals of all ages, including extremely old individuals having low level of circulating B lymphocytes, using the EBV. The research presented in this thesis supports the possibility that apoptosis could be part of the determinant of longevity. However, further research is needed to substantiate the data presented herein. The use of the EBV immortalized B-lymphocytes in apoptotic assays, could be one of the methods used to provide support for the research presented however, this is a long-term goal. While at this moment it is not feasible to conduct longitudinal population studies to study caspases in the immune system or to perform caspase assays in the biological samples obtained from our cross-sectional population study, those studies might be feasible in the future and along with others might shed light on a beneficial apoptotic program contributing to successful aging.

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APPENDIX 1

IRB APPROVAL

HÔPITAL GÉNÉRAL JUIF
SIR MORTIMER B. DAVIS
JEWISH GENERAL HOSPITAL

UNIVERSITÉ
MCGILL
UNIVERSITY

BUREAU D'ÉTHIQUE DE LA RECHERCHE
RESEARCH ETHICS OFFICE

JACK MENDELSON, M.D., HEAD
BUREAU/ROOM C-142

FRANCA CANTINI, B.Sc.N., COORDINATOR
BUREAU/ROOM A-702

December 1, 2000

Dr. Eugenia Wang
Lady Davis Institute
SMBD-Jewish General Hospital

SUBJECT: **Protocol # 98-006 entitled "Functional Genomic Studies of Centenarians"**

Dear Dr. Wang:

Thank you for submitting the above-mentioned protocol to the Research Ethics Office for review. Please be advised that the protocol (dated November 14, 2000), and consent form have been granted expedited re-approval.

This expedited re-approval is for the period of one year, at which point you must submit for re-approval via the "Continuing Review Form". Should the study be complete before this time, you must submit a "Completion Form" to the Research Ethics Office A-702.

Expedited Re-Approval:	**December 2000**
Expiration of Expedited Re-Approval:	**December 2001**

Please note that the Continuing Review Package must be submitted to the Research Ethics Office no later than one month before the expiration date above to ensure sufficient time for review.

Sincerely,

Jack Mendelson, MD
Chairman, Research and Ethics Committee

JM/sl
98-006ContRevExpApp.doc

DR. RICHARD L. MILLER
CHAIRMAN

HUMAN STUDIES COMMITTEES

UNIVERSITY of LOUISVILLE
dare to be great

June 29, 2001

****EXEMPT****

Dr. Eugenia Wang
Biochemistry and Molecular Biology
Baxter Building, Room 304

RE: 404-01-A Functional Genomic Studies of Centenarians-Associated Chinese Families

Dear Dr. Wang:

The above study has been received by the HSC. It has been determined that the study is exempt according to 45 CFR 46.101(b) 4. We understand that you will use archived tissues from a study that was previously approved by the Institutional Review Board.

Sincerely,

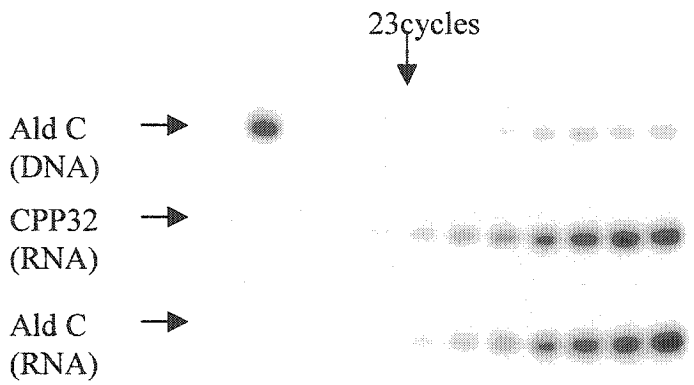
Richard L. Miller, D.D.S., Ph.D.
Chair, Human Studies Committee

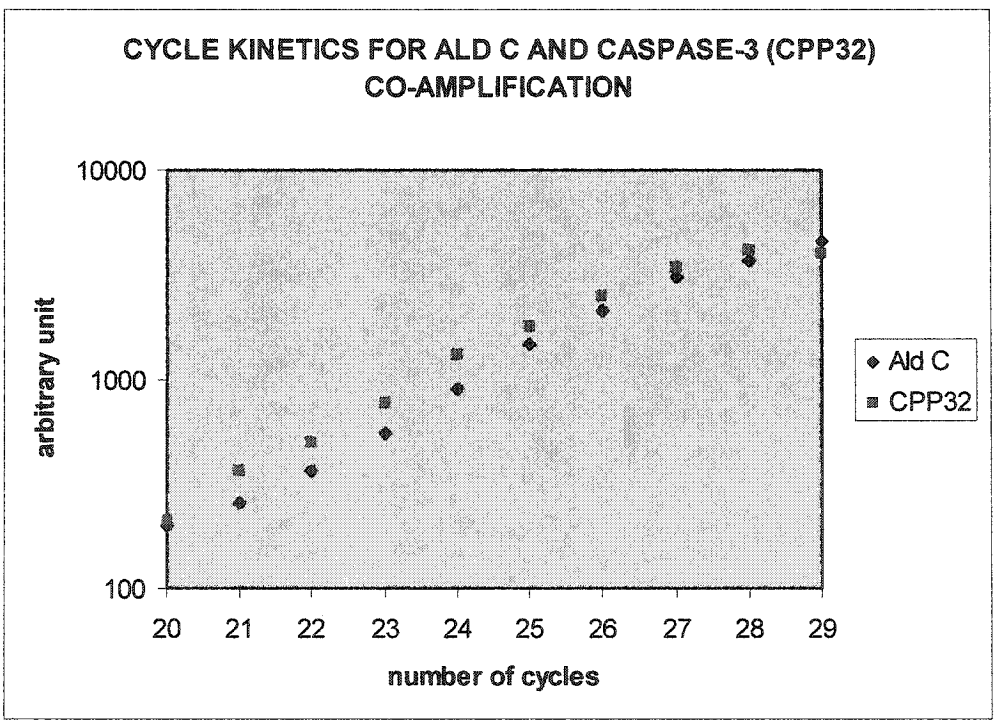
RLM:rsh

APPENDIX 2

CASPASE-3 AND ALDOLASE C LINEARITY

CYCLE KINETICS FOR CASPASE-3AND ALD C CO-AMPLIFICATION





APPENDIX 3

PUBLISHED VERSION OF CHAPTER 2

Journal of Gerontology: BIOLOGICAL SCIENCES
2002, Vol. 57A, No. 7, B285–B287

Blood-Sample Processing for the Study of Age-Dependent Gene Expression in Peripheral Blood Mononuclear Cells

Chantale Lacelle,[1,2,3] Hélène Riol,[3] Suying Xu,[2,3] Yih-Jing Tang,[4] Yuh-Shyun Wang,[4] Yi-Li Chuang,[5] Hui-Sheng Lin,[5] Ming-Cheng Chang,[6] Jersey Liang,[7] and Eugenia Wang[2,3]

[1]Department of Anatomy and Cell Biology, McGill University, Montréal, Québec, Canada.
[2]Department of Biochemistry and Molecular Biology, University of Louisville School of Medicine, Kentucky.
[3]Bloomfield Center for Research in Aging, Lady Davis Institute for Medical Research, Sir Mortimer B. Davis Jewish General Hospital, Montréal, Québec, Canada.
[4]Department of Family Medicine, Taichung Veterans General Hospital, Taiwan, Republic of China.
[5]Center for Population and Health Survey Research, [6]Bureau of Health Promotion, Department of Health, Taichung, Taiwan, Republic of China.
[7]School of Public Health and Institute of Gerontology, University of Michigan, Ann Arbor.

Although most new biogerontological studies seeking to identify longevity candidate genes and factors involved in successful human aging are population based, and likely to involve the collection of blood from extremely old individuals, to our knowledge no unified protocols have yet been published to describe a methodology permitting the simultaneous generation of different kinds of biological specimens derived from a single source of a very small volume of peripheral blood. Here we describe a method permitting the simultaneous generation of plasma, RNA, DNA, protein, fixed lymphocytes, and frozen blood aliquots from a single 10- to 30-ml blood sample obtained from donors of any age (10–102 years old), and we show that the quality and quantity of DNA, RNA, protein, and fixed lymphocytes obtained do not vary significantly with age. As is frequently observed, the older individuals have higher plasma proportions.

POPULATION studies involving nonagenarians and centenarians have long been used in aging research; they have permitted the identification of several genetic factors associated with life-span determination, and they have served to define age-related physiological changes (1–4). However, despite the identification of several factors associated with normal aging or favoring the attainment of extreme old age, the emerging picture shows that successful aging is regulated by complex traits involving numerous genes, and their interactions with environmental factors. Investigation of these genes and their expression requires studies conducted concurrently at the DNA, RNA, protein, and cellular levels. Therefore, for any population study in aging research, the amount and type of biological material obtained from each individual within the population often remain an experimental limiting factor, leaving results to be concluded from fragmented data generated only from one or a few of the above types of biological material. Here, we describe a methodology permitting the simultaneous generation of RNA, DNA, protein, and plasma samples, as well as fixed peripheral blood mononuclear cells (PBMCs) and frozen blood aliquots, from a single 10- to 30-ml sample of peripheral blood obtained from donors of any age (Figure 1).

Methods

Between 10 and 30 ml of blood sample is collected from donors of any age in several 5-ml ethylenediamine tetraacetic acid Vacutainers (Becton Dickinson, San Diego, CA). For each subject, one tube is set aside to generate frozen blood aliquots, whereas the others are used for PBMC extraction. The reserved Vacutainer is aliquoted into 1- or 2-ml samples under sterile conditions, supplemented with dimethyl sulfoxide to a 10% final concentration, and stored in a −150°C freezer.

The remaining blood-filled Vacutainers are centrifuged at room temperature for 15 minutes at 1100 rpm in a tissue-culture centrifuge to allow the separation of blood constituents. Following centrifugation, the plasma-containing layer is removed from each tube without disrupting the buffy coat, and it is stored in several 3-ml cryovials. After the removal of plasma, blood samples are diluted in 1× phosphate-buffered saline to double the original blood volume, and they are gently loaded onto several 3-ml Ficoll-Paque (Amersham–Pharmacia, Baie d'Urfé, Québec, Canada) cushions set up in 14-ml round-bottom tubes; PBMCs are extracted according to the manufacturer's instructions (Amersham–Pharmacia). Following extraction, some of the PBMCs are resuspended in Trizol (Gibco, Rockville, MD), whereas

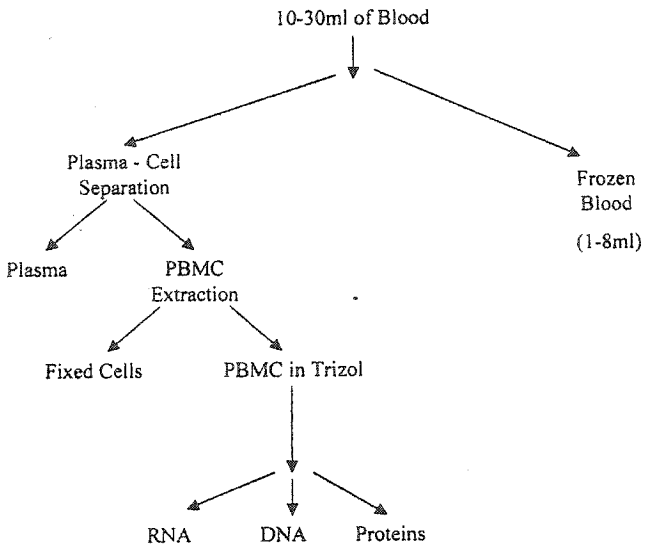


Figure 1. Stepwise description of the protocol for generating different kinds of biological materials from a single sample of peripheral blood. The blood is first divided into two aliquots; the first is used to generate frozen blood samples for the future development of tissue-cultured cell samples, whereas the second is subjected to plasma–cell separation. Following plasma–cell separation, plasma is collected and peripheral blood mononuclear cells (PBMCs) are extracted from the remaining blood components. The extracted PBMCs are used to generate fixed cells and DNA, RNA, and protein samples.

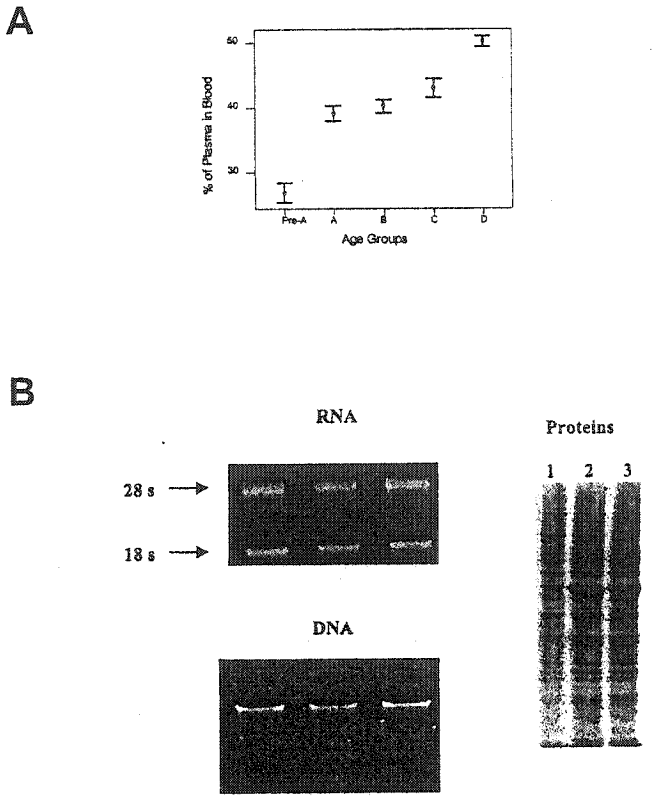


Figure 2. A: Percentage of plasma present in blood. Individuals belonging to the Pre-A group (very young individuals, aged 10–19 years) have significantly less plasma in their blood than individuals within groups A (young, 20–39 years), B (intermediate, 40–69 years), C (old, 70–89 years) and D (extremely old, 90+ years). Similarly, individuals within group D have significantly more plasma in their blood than individuals of other age groups. The figure shows averages and the standard error of the mean for all age groups. B: RNA, DNA, and protein samples extracted by using the described protocol. Agarose gel shows RNA and DNA, whereas Ponceau red staining of nitrocellulose membranes shows the protein pattern of Jurkat cells in lane 1 and of selected samples in lanes 2 and 3.

others are fixed with 1% paraformaldehyde. As more than one tube of PBMCs is obtained for each individual, it is necessary to ensure that one of these tubes is used to generate fixed cells and the rest are used for RNA, DNA, and protein isolation. RNA, DNA, and protein are extracted from PBMCs resuspended in Trizol, as described by Chomczynski (5) and as modified by Riol and colleagues (6).

Results

Using the following protocol, we processed blood samples obtained from 246 Chinese individuals aged 10 to 102 years, and we found that blood-plasma composition changes with age (Table 1 and Figure 2A). Thus, compared with very young individuals (10–19 years old), extremely old individuals (90+ years) exhibit a twofold increase in the percentage of plasma present in their blood. However, despite the lower amount of plasma present in very young individuals, more than enough plasma can be obtained from all individuals to permit several laboratory tests, including enzyme-linked immunosorbent assays. When the total amounts of RNA, DNA, and proteins obtained per milliliter of extracted blood were calculated, it was found that the quantity of each species obtained from the different age groups does not change significantly (Table 2); the yield of RNA, DNA, and proteins obtained per milliliter of blood does not vary among age groups. As shown in Table 2, old individuals appear to have less protein in their samples; however, a *t* test reveals no significant difference from the other age groups. The lower average amount of protein present in older individuals results from the fact that most of the samples we have for this age group could not be quantified, because of damage incurred during shipment from Taiwan to Canada (samples were allowed to thaw, and they were left at room temperature for several days as a result of an in-transit delay of the shipment). No damage occurred to

Table 1. Percentage of Plasma in the Blood

Parameter	Very Young (10–19 y)	Young (20–39 y)	Intermediate (40–69 y)	Old (70–89 y)	Extreme Old (90+ y)
% Plasma in blood	26.94 ± 5.76*	39.13 ± 8.86	40.25 ± 8.82	43.07 ± 8.82	50.17 ± 6.07*

Notes: The percentage of plasma within the blood is significantly different when the extreme old age group is compared with all other age groups. A significant difference also exists between the percentage of plasma present within the blood of very young individuals and all other age groups.

*Significant difference with all other age groups.

Table 2. Amounts of Fixed Cells, RNA, DNA, and Protein Obtained per Milliliter of Blood

Parameter	Young (20–39 y)	Intermediate (40–69 y)	Old (70–89 y)	Extreme Old (90+ y)
Fixed cells ($\times 10^6$)	1.02 ± 0.62	1.55 ± 0.59	1.11 ± 0.63	1.25 ± 0.57
RNA (μg)	1.73 ± 0.68	1.72 ± 0.55	1.57 ± 0.54	1.44 ± 0.65
DNA (μg)	8.16 ± 3.53	6.96 ± 3.13	7.66 ± 1.40	8.91 ± 1.99
Proteins (μg)	64.73 ± 32.98	57.13 ± 35.85	32.34 ± 29.7	76.75 ± 68.02

Note: The amount of fixed cells, RNA, DNA, and protein obtained per milliliter of blood processed is similar for all age groups.

the other samples, as samples from other age groups were shipped separately. Samples from our population have been studied more than 3 years after extraction and still show no signs of degradation. We find that degradation can be largely prevented by generating aliquots of working samples and thawing them as needed, thus preventing samples from undergoing several rounds of freeze–thaw cycles. Our RNA was used to conduct a quantitative reverse transcriptase chain reaction, whereas proteins were used for Western blotting; DNA was used for polymorphism studies. All biological species were of good quality (Figure 2B).

Paraformaldehyde-fixed PBMCs were kept at 4°C until they were used in flow cytometry experiments; as observed for the RNA, DNA, and protein, the quantity of fixed PBMCs obtained per milliliter of blood sample does not change significantly with age. Thus, except for the yield of plasma, the quantity of biological materials extracted per milliliter of blood remains constant with age. As many commercially available antibodies do not work with paraformaldehyde-fixed PBMCs, it is important to verify antibodies before conducting flow cytometry experiments, or to use alternative methods of fixation to avoid loss of antigenicity, while tolerating the less than ideal morphological preservation.

Discussion

The use of population studies in aging research has contributed to our understanding of age-related physiological changes and has permitted the identification of several factors involved in successful aging. However, population studies are very expensive, because the quantity of biological materials obtained from each individual is very limited; we cannot collect large volumes of blood from extremely old individuals without significantly compromising their health. For this reason, most population studies currently underway are conducted with only one type of biological material, such as DNA from finger-pricked samples for genetic profiling. In this paper, we describe a methodology permitting the simultaneous extraction of several different kinds of biological specimens from a single source of 10–30 ml of peripheral blood. This protocol not only renders population studies more cost effective, but also allows for in-depth biological investigation. It is important to obtain different kinds of biological material from each individual, because the analysis of each type contributes to different aspects of aging studies. Furthermore, our population confirms the common observation that blood composition is not identical among different age groups, as the blood of extremely old individuals contains proportionately more plasma than that of younger individuals. Because our data indicate that the volume of PBMCs per milliliter of blood does not significantly change with age, it seems that other cellular blood components, specifically red blood cells, decrease in extremely old individuals, resulting in decreased hematocrit levels, as is often reported (7,8). The implementation of this protocol will facilitate the identification of novel factors involved in successful aging, and it will render population studies more cost effective. The present report describes a standardized method for extracting DNA, RNA, protein, plasma, and cells from modest blood samples, thus allowing for studies of genetic profiling at the level of single nucleotide polymorphism and gene products, as well as regulation of functions, and it provides a comprehensive approach to studying factors involved in aging.

Acknowledgments

This research was supported by grants to E. Wang from the National Institute on Aging of the National Institutes of Health (Grant R37AG07444) and the Defense Advanced Research Project Agency of the U.S. Department of Defense.

We are grateful to Alan N. Bloch for proofreading the text.

Address correspondence to Dr. Eugenia Wang, Department of Biochemistry and Molecular Biology, University of Louisville School of Medicine, 570 South Preston Street, Baxter Building, Room 304, Louisville, KY 40209. E-mail: eugenia.wang@louisville.edu

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APPENDIX 4

PUBLISHED VERSION OF CHAPTER 4

ELSEVIER

Mechanisms of Ageing and Development 123 (2002) 1415–1418

mechanisms of ageing and development

www.elsevier.com/locate/mechagedev

Short communication

Establishing lymphoblastoid cell lines from frozen blood of extremely old individuals

Chantale Lacelle [a,b], Eugenia Wang [b,*]

[a] *Department of Anatomy and Cell Biology, McGill University, 3640 University Street, Montreal, Que., Canada*
[b] *Department of Biochemistry and Molecular Biology, University of Louisville School of Medicine, 570 South Preston Street, Baxter Building, Room 304, Louisville, KY 40292, USA*

Received 20 February 2002; received in revised form 3 June 2002; accepted 3 June 2002

Abstract

In population studies involving peripheral blood samples from nonagenarian and centenarian donors, the amount of biological material available for research is restricted, as only a few millilitres of blood can be obtained from extremely old donors without significantly compromising their health. Here we describe a protocol to immortalize small amounts of total frozen blood from extremely old donors (90+) using the Epstein-Barr virus, despite the low level of circulating B cells present in nonagenarian and centenarian blood samples. This methodology provides a unique way to maximize resources of biological material available for research.

Keywords: EBV; Extremely old; Longevity; Cell lines

Seeking candidate human longevity genes, or gene expressions involved in either successful aging or the development of age-associated diseases, requires large quantities of DNA, RNA, proteins and cells from extremely old individuals (90+). Due to their advancing age, however, nonagenarians and centenarians cannot, without significantly compromising their health, afford to donate large quantities of blood for biological studies. This strict limitation on the amount of available biological material challenges thorough investigation of the aging process, and impedes our ability to perform optimization procedures and subsequently generate large amounts of data. As the Epstein-Barr virus (EBV) has been successfully used to establish lymphoblastoid cell lines (LCL) (Sugden and Mark, 1977) from young, middle and old age donors, using fresh blood or purified frozen lymphocytes (Neitzel, 1986; Tremblay and Khandjian, 1998), we wondered whether a similar protocol could be used to immortalize B-lymphocytes from extremely old individuals, permitting functional study in culture conditions, as well as regenerating biological materials extracted from important and irreplaceable samples. Here we show that using 1 or 2 ml blood aliquots frozen supplemented with dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO) to a final concentration of

* Corresponding author. Tel.:

e.edu (E. Wang).

0047-6374/02/$ - see front matter
PII: S0047-6374(02)00074-X

Table 1
Percentage of B-cells present in PBMC with age

Age group (years)	Percentage of CD19 positive cells in extracted PBMC
19–29	13.33±4.02[a]
30–59	10.01±3.22
60–89	9.15±3.05
90–102	6.76±3.38[a]

[a] Indicates a significant difference of $P \leq 0.05$ with all other age groups.

nuclear cells (PBMC) of extremely old individuals (90–102 yrs old) are positive for CD19, a B-cell marker, whereas 13.33% of PBMC from young individuals (19–29 yrs old) are CD19-positive. Thus extremely old individuals have half as many B-cells in their PBMC as their younger counterparts. Flow profiles for both a young and an extremely old individual are shown in Fig. 1.

Once the count of CD19-positive cells in each subject's PBMC was determined, we attempted to immortalize with EBV B-lymphocytes from a few

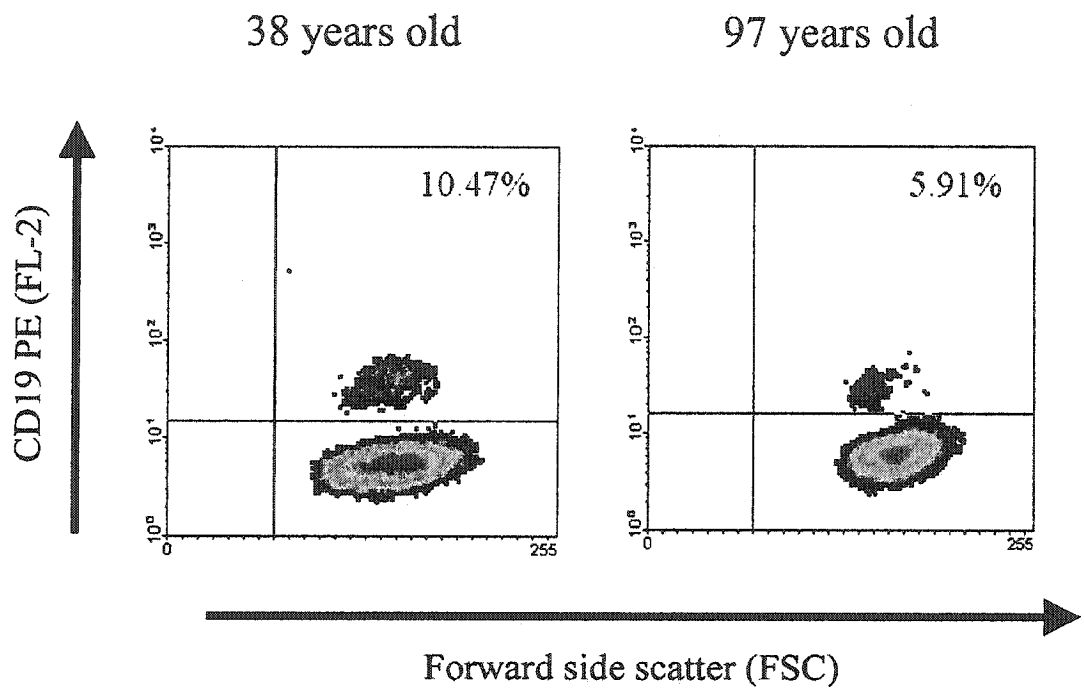

Fig. 1. Flow cytometry profile for CD19. This figure shows by density plots the count of CD19-positive cells present in the PBMC of a young and an extremely old individual. CD19- positive cells (CD19 is a B cell marker) are found in the top right corner, while CD19 negative cells are found in the bottom right corner.

10%, kept for up to 2 years in a −150°C freezer, it is possible to generate stable LCL from the blood of extremely old individuals.

As EBV immortalizes almost exclusively B-lymphocytes, and low levels of circulating B-lymphocytes might impede our ability to establish lymphoblastoid cell lines (LCL), we used flow cytometry to determine the extent of age-dependent decrease in circulating B-lymphocytes in nonagenarians and centenarians. As shown in Table 1, only 6.76% of peripheral blood mono-

subjects, using 1 ml of cryopreserved blood samples. From these preliminary experiments we found that several rounds of washing with PBS fails to remove red blood cell (RBC) contamination. As such, to permit a better visualization of the cells when they are placed in culture, and thus allow us to better monitor the presence of clumps indicating successful EBV immortalization we found that a small-scale Ficoll-Paque extraction in 5ml round-bottom tubes succeeds in removing the RBC contamination.

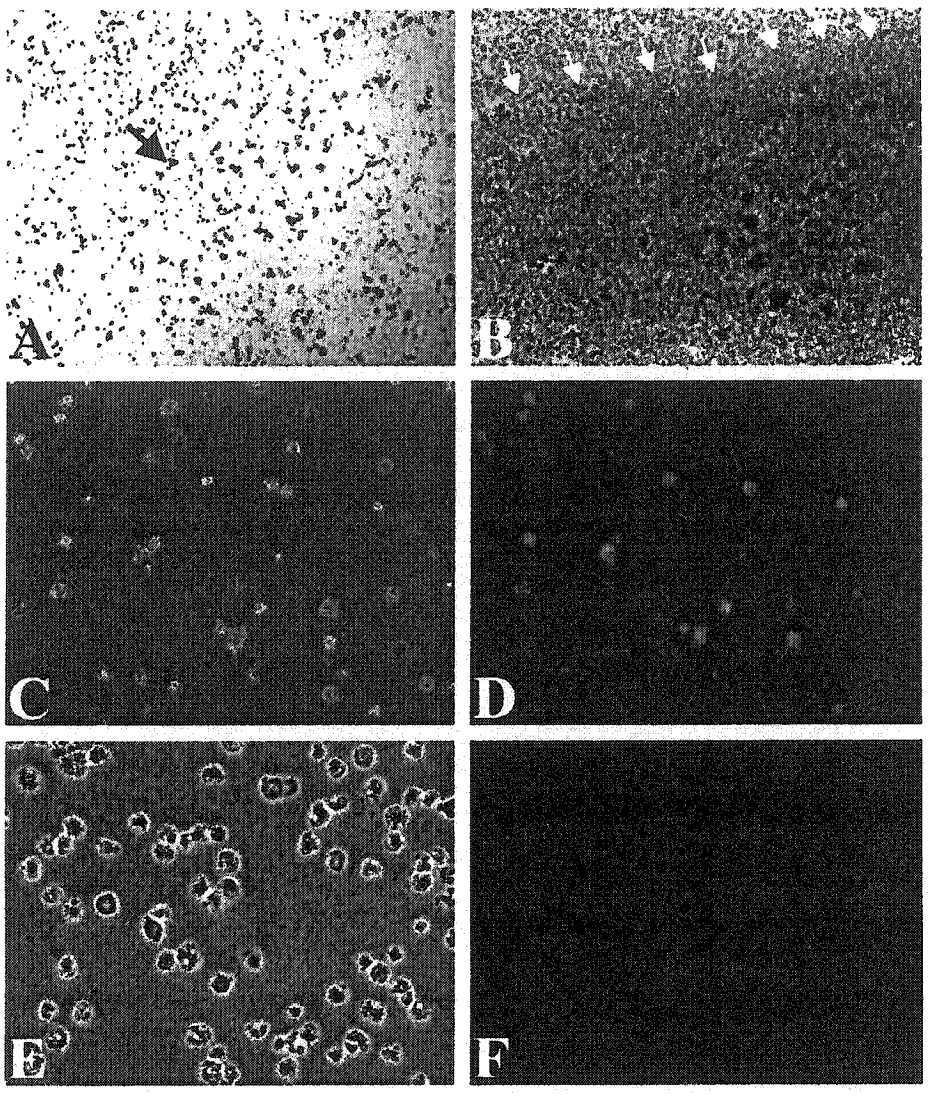

Fig. 2. Stages of EBV transformation and phenotype of EBV immortalized cells. A. Phase contrast picture of cells cultured with EBV for three weeks. In this panel a few clumps of proliferating cells can be seen (arrow). B. Phase contrast picture of cells cultured with EBV for five weeks. As indicated by the series of arrows, the cells have proliferated, and show a large region of growth. C. Phase contrast image of EBV transformed cells. D. Fluorescent staining of EBV transformed cells from an extremely old donor (90+) with CD19 FITC-conjugated antibody E. Phase contrast of Jurkat cells. F. Fluorescent staining of Jurkat T cells with CD19 FITC-conjugated antibody (negative control).

Following RBC removal, lymphocytes extracted using small-scale Ficoll-Paque extraction were resuspended in 300 μl of Epstein-Barr virus (EBV), strain B95-8 (ATCC, Manassas, VA), to immortalize the B-cells (minimum titer verified by ATCC). Resuspended cells were transferred into wells of a 48-well tissue culture plate, and placed in a 37°C, 5% CO_2 tissue culture incubator for two hours. After incubation, 800 μl of growth medium (RPMI1640 +10% FBS) and cyclosporine A (CSA) to a final concentration of 3 μg/ml, were added to each well. As previously reported, CSA is needed to prevent the suppression of EBV transformation caused by T suppressor and cytotoxic cells (Thornley-Lawson, et al., 1977; Tosato, et al., 1982). Following the addition of growth medium,

half of the total volume of one well was transferred into the neighbouring well. Three days latter, 1 μg/ml CSA was added to each well, as we noticed after the first round of preliminary experiments that this permits more rapid establishment of the cell lines. Cultures were left untouched for about one month (no change of medium), or until nice 'colony' clumps of growing cells were observed. Once growing cells were observed, 300 μl of growth medium was added to every well, and cells were kept in the incubator for an additional week or until densely grown. Cultures were conservatively expanded using 48, 24, 12 and 6 well plates. Once the 6 well plates were dense, cells were transferred to a 25 ml culture flask, and cultures expanded until enough cells were produced. In general, stable cell lines were established within three months.

Throughout their period of transformation and growth, cells were closely monitored by inverted microscopy. As shown in Fig. 2A, growth was first identified and confirmed by the presence of small clumps in the culture. After a few more weeks in culture, large regions of dividing cells were seen in the plates (Fig. 2B). At this stage, small aliquots of the cultures were removed and labelled with human anti-CD19 FITC-conjugated antibody, to determine whether the transformed cells had a B-cell phenotype. As shown in Fig. 2 (C, D), all of the cells obtained from our growing cultures were CD19-positive, indicating a B-cell phenotype. Jurkat CD19-negative T cells were used as a negative control (Fig. 2 E, F). Currently, some of our cell lines have been in culture for more than nine months; despite slow growth, all established cell lines are stable. So far, we have established cell lines for individuals of both genders aged 21 to 100 yrs; including 12 lines from individuals aged 90–100 yrs.

Here we show that despite the age-related decrease in circulating B-lymphocytes, although most standard protocols currently available for EBV transformation require the use of 10 ml or more of fresh blood (Tosato, 1991), it is possible to establish LCL from minimal amounts of blood from extremely old individuals, which has been frozen for years. This revised protocol for EBV immortalization permits us to develop cell-based models to investigate the mechanisms of aging by providing a renewable source of biological samples; it also provides a tool for other investigators who rely on the blood of extremely old subjects, to obtain biological data contributing to the advancement of the study of human longevity.

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APPENDIX 5

ALTERATIONS IN TNF- AND IL- RELATED GENE EXPRESSION IN SPACE-FLOWN WI38 HUMAN FIBROBLASTS

Alterations in TNF- and IL-related gene expression in space-flown WI38 human fibroblasts[1]

ALEXANDRE SEMOV, NATHALIA SEMOVA, CHANTALE LACELLE,*,† RICHARD MARCOTTE, EMMANUEL PETROULAKIS, GREGORY PROESTOU, AND EUGENIA WANG†,[2]

Bloomfield Center for Research in Aging, Lady Davis Institute for Medical Research, Sir Mortimer B. Davis Jewish General Hospital, Montréal, Québec, Canada; *Department of Anatomy and Cell Biology, McGill University, Montréal, Québec, Canada; and †Department of Biochemistry and Molecular Biology, University of Louisville School of Medicine, Louisville, Kentucky, USA

SPECIFIC AIM

As a consequence of spaceflight, the expression of several muscle- and bone-specific genes is decreased, leading to musculoskeletal changes such as loss of muscle mass and decreased bone density. As these changes greatly impede our ability to conduct long-term manned space missions, we developed and implemented a new approach to microarrays to study the change in the gene expression of E-box binding transcription factor, interleukin (IL), and tumor necrosis factor (TNF) -related genes in normal WI38 human fibroblasts exposed to microgravity during the STS-93 Space Shuttle mission.

PRINCIPAL FINDINGS

1. Development of microarrays

To investigate changes in mRNA expression as a result of spaceflight, we developed and implemented a novel approach to microarray technology consisting of digoxigenin (DIG) labeling of cDNA using gene-specific primers and arrays printed on Hybond nylon membranes. After careful evaluation of several different publicly available databases, we chose 202 genes belonging to either the TNF, IL, or E-box binding gene families, as well as 9 housekeeping genes and a series of negative controls for arraying. Once genes of interest were identified, sense and antisense primers were designed for each gene with the help of Primer3 software by maintaining constant selection parameters for all genes. The average annealing temperature of all primers is 60.1 ± 0.09°C, the average size of PCR product for arraying is 441 ± 58 bp, and the average melting temperature is 80 ± 3°C. Selecting these parameters allowed us to conduct hybridization and posthybridization washing under stringent conditions, thereby decreasing the probability of cross-hybridization and reducing the background. Amplicons generated using these primers were arrayed onto Hybond membranes attached to glass slides.

2. Expression profiling of WI38 cells from ground and spaceflight samples using microarrays

Microarrays arrayed with either E-box-, TNF-, or IL-related gene amplicons were hybridized with DIG-labeled spaceflight or ground control cDNA samples. Three independent hybridizations were done for spaceflight and ground control samples for each type of microarray. **Figure 1A** shows the average normalized expression levels for each type of array. Gene quantification and normalization was accomplished by averaging the intensities of the three replicated dots for each gene present on a single microarray, then normalizing each membrane according to the intensities of the housekeeping genes. Normalized intensities for each gene were then averaged for the three membranes.

After microarray analysis, we identified 10 genes belonging to the TNF- or IL-related gene families whose expression was modified as a consequence of spaceflight. Genes for which changes were identified show reproducible results in all three hybridization experiments and include two genes from the IL array: interleukin-1 receptor antagonist (IL1RN) and interleukin-15 alpha chain (IL15RA); and eight genes from the TNF array, including two ligands from the TNF superfamily, TNF-related weak inducer of apoptosis (TWEAK), and TNF superfamily member 15 (TNFSF15); two TNF receptor associated proteins, protein-tyrosine phosphatase nonreceptor type 13 (PTPN13), and neutral sphingomyelinase activation-associated factor (NSMAF); three TNF-inducible genes, pentraxin 3 (PTX3), small inducible cytokine subfamily A member 13 (SCYA13), and ATP binding cassette member 50 (ABC50); and one modulator of TNF activity: TNF-α converting enzyme (TACE). No

1 To read the full text of this article, go to http://www.fasebj.org/cgi/doi/10.1096/fj.01-1002fje; to cite this article, use *FASEB J.* (April 23, 2002) 10.1096/fj.01-1002fje.

[2] Correspondence: Department of Biochemistry and Molecular Biology, University of Louisville School of Medicine, 570 South Preston St., Baxter Building, Room 304, Louisville, Kentucky 40202, USA. E-mail: Eugenia.Wang@Louisville.edu

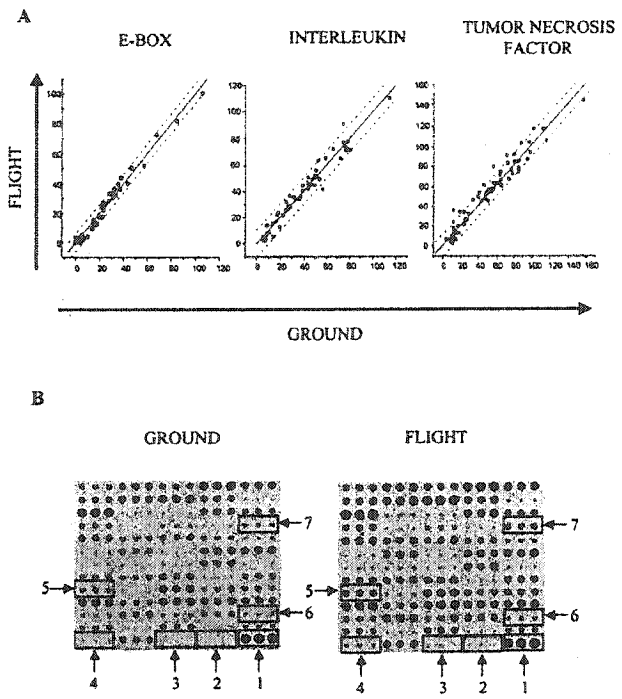

Figure 1. Comparison of gene expression between ground and spaceflight WI38 cells. *A)* Correlation between ground (x axis) and flight (y axis) gene expression for each gene included on either the E-box, interleukins, or TNF factor array. 95% confidence bands are shown as dashed lines. Genes significantly up-regulated as a consequence of spaceflight are found above the upper dashed line; those significantly down-regulated are found below the lower dashed line. *B)* A fragment of TNF microarray hybridized to digoxigenin-labeled cDNA from ground control and spaceflight fibroblasts. Box 1 = the cDNA positive control; 2, the λ phage DNA negative control; 3, the 2×SSC negative controls, 4, TWEAK; 5, NSMAF; 6, PTPN13; 7, PTX3.

significant changes were detected in genes on the E-box chip. Figure 1*B* shows a portion of a TNF array hybridized with WI38 fibroblast DIG-labeled cDNA from ground and spaceflight samples.

3. RT-PCR analysis of genes found to be differentially expressed by microarrays

After reverse transcription reaction, cDNA obtained from spaceflight and ground control samples were aliquoted in microtubes by fivefold serial dilutions and amplified by PCR in order to validate the results obtained by microarray techniques. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as control for the quantity of RNA as well as for gel loading. **Figure 2***A* shows representative gels for each PCR reaction and Fig. 2*B* shows the quantification for each gene expression for ground and spaceflight samples. Using RT-PCR, we confirmed the up-regulation of TWEAK, TNFSF15, PTPN13, PTX3, SCYA13, ABC50, NSMAF, TACE, and IL1RN, and down-regulation of IL15RA gene expression in spaceflight samples compared with ground controls. These alterations in mRNA levels correlate with those registered by microarray techniques. The average correlation coefficient between RT-PCR and microarray data is 0.89.

CONCLUSION

Although many methods exist to study multiple changes in gene expression, microarrays seem to be the best method available, as they can promptly characterize changes in many genes simultaneously. Despite the fast progress in the field of microarray technology, a major obstacle to their broader implementation is the requirement for large amounts of mRNA. Since we had only limited quantities of spaceflight WI38 mRNA, we developed and implemented microarrays enabling us to study gene changes using minimal amounts of biological material. To decrease the demand for RNA, we used gene-specific antisense primers to produce DIG-labeled cDNA and printed our arrays on nylon membranes.

Using our microarrays, we studied the expression of genes from three functional classes of genes (TNF, IL,

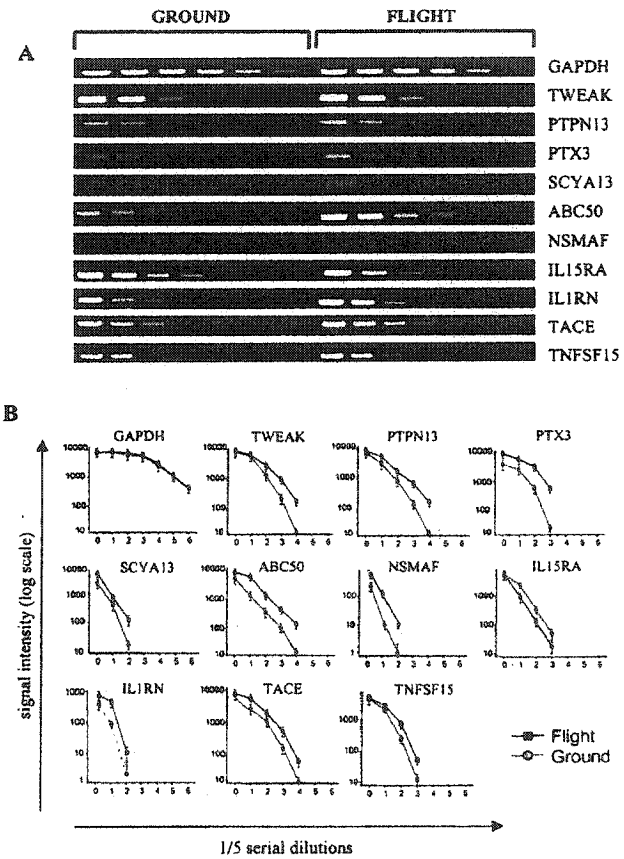

Figure 2. Semiquantitative RT-PCR of microarray-identified differentially expressed genes. *A)* PCR products obtained when fivefold serial dilutions of cDNA from ground and spaceflight samples are amplified for 30 PCR cycles. GAPDH was used as control. *B)* Quantitative analysis of band intensities for three independent quantitative RT-PCR experiments.

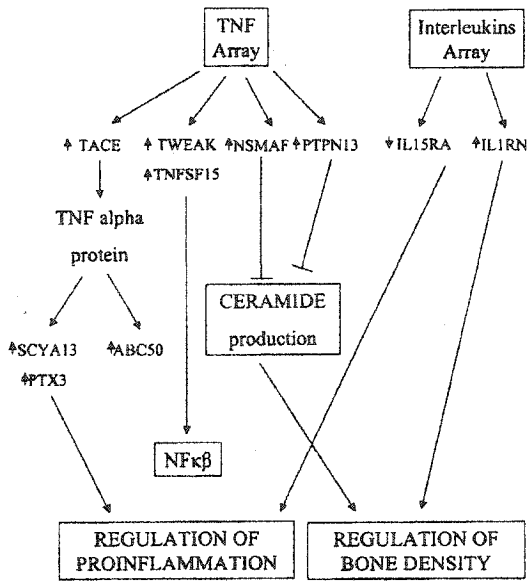

Figure 3. Summary of data obtained from microarray analysis of E-box, TNF, and IL-related gene expressions in normal WI38 cells exposed to microgravity compared to ground controls.

and E-box binding), which we believed to be important in adaptation to spaceflight and aging. For instance, variations in the expression of crucial transcription factors could be important for driving global gene expression whereas modulation of TNF and IL genes may represent inflammatory or general stress responses, resulting in the musculoskeletal changes seen in spaceflight and aging. Consistent with the published literature, our array failed to revealed any differential expression of E-box genes. However, we identified 10 genes belonging to the TNF- and IL-related gene families that are differentially expressed as a consequence of spaceflight (**Fig. 3**). Although TNF-α mRNA expression remains constant, up-regulation of TACE mRNA is detected in spaceflight samples. As TACE is necessary to produce mature TNF-α, its up-regulation may result in an augmentation of TNF-α secretion without changes in the level of TNF-α mRNA; this may explain why TNF secretion is increased in space flown macrophages. Two newly identified TNF superfamily members (TWEAK and TNFSF15) are also up-regulated as a consequence of spaceflight. Like TNF, TWEAK has apoptotic ability; however, its main function is believed to be to induce the NFκβ pathway rather than apoptosis. TNFSF15 is also capable of activating NFκβ, but in normal human fibroblasts it induces cell division.

Although TNF ligands mediate their cellular responses by first interacting with TNF surface receptor, none of the 20 TNF receptors represented on our TNF array was up- or down-regulated as a consequence of spaceflight. However, probably as a result of TACE up-regulation, increased expression of three TNF-inducible genes (PTX 3, SCYA13, and ABC50) is observed in spaceflight samples. Changes in the expression of these three genes may reflect stress or proinflammatory responses of fibroblasts to microgravity conditions, as PTX3 and SCYA13 participate in inflammatory reactions. On the other hand, down-regulation of IL15RA may indicate an attempt by cells to counteract this developing proinflammatory situation, as IL15, its ligand, is a regulator of macrophage proinflammatory cytokine.

Using our microarray, we have identified an up-regulation in IL1RN mRNA under spaceflight conditions. IL1RN is a cytokine receptor antagonist functioning to neutralize the biological activity of IL1α and IL1β by competing for binding to the IL1 receptor. It is possible that the up-regulation of IL1RN observed in spaceflight samples is part of a protective mechanism attempting to counteract the effect of IL1β, a potent stimulator of bone resorption involved in the pathogenesis of osteoporosis, whose biological effect depends on the IL1β/IL1RN ratio, to diminish the magnitude of bone loss during spaceflight. Increased mRNA expression of two TNF receptor-associated proteins (NSMAF and PTPN13) also occurs as a consequence of spaceflight. Whereas NSMAF regulates ceramide production, PTPN13 negatively regulates Fas-induced apoptosis. As Fas was recently shown to block calcium influx in lymphocytes through the activation of acidic sphingomyelinase and ceramide release, it is possible this up-regulation of PTPN13 and NSMAF may also function to counteract factors contributing to bone loss in space.

By comparing the level of expression of E-box binding, TNF-, and IL-related genes between spaceflight and ground control WI38 human fibroblasts, we identified a complex series of changes in response to microgravity. These changes reveal that various signaling pathways involved in the regulation of apoptosis and proinflammation are modified in fibroblasts as a consequence of spaceflight. Changes in TNF- and IL-related genes may underlie a mechanism attempting to counteract the bone loss occurring as a result of spaceflight. However, it remains to be determined whether these changes indeed contribute to the regulation of apoptosis, and thereby bone mass during spaceflight, and, most important, whether some of the changes in gene expression detected by microarray occur during normal aging. FJ

APPENDIX 6

DESIGNER MICROARRAYS: FROM SOUP TO NUTS

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Designer Microarrays: From Soup To Nuts

Eugenia Wang,[1] Chantale Lacelle,[1,2] Suying Xu,[1] Xuechun Zhao,[1] and Michael Hou[1]

[1]Department of Biochemistry and Molecular Biology, University of Louisville School of Medicine, Kentucky.
[2]Department of Anatomy and Cell Biology, McGill University, Montréal, Canada.

The recognition that multigene mechanisms control the pathways determining the aging process renders gene screening a necessary skill for biogerontologists. In the past few years, this task has become much more accessible, with the advent of DNA chip technology. Most commercially available microarrays are designed with prefixed templates of genes of general interest, allowing investigators little freedom of choice in attempting to focus gene screening on a particular thematic pathway of interest. This report describes our "designer microarray" approach as a next generation of DNA chips, allowing individual investigators to engage in gene screening with a user friendly, do-it-yourself approach, from designing the probe templates to data mining. The end result is the ability to use microarrays as a platform for versatile gene discovery.

OVER the past century, as a result of increasing medical knowledge, better sanitation, and improved nutrition, developed countries are experiencing an unprecedented increase in average human life span, along with a higher incidence of multifactorial diseases such as cardiovascular diseases, neurodegenerative disorders, type 2 diabetes, and cancers (1). These age-dependent diseases, plaguing people as young as their mid-50s, are products of the combined influences of genetics and environment (2). Nature and nurture together provide predispositions to cancer, cardiovascular disease, diabetes, and neurodegenerative disorders, presenting a complex picture for the development of these perils in the fast-growing middle- and old-age subpopulations of our society.

Although recent advances in medical research have enabled us to diagnose several age-associated diseases, alleviate pain associated with them, and retard the onset of their acute stages, we remain largely incapable of identifying at an early stage those individuals bearing genetic predispositions to these diseases, and thus of administering preventive medicine or treatment. Because the human genome contains some 30,000 genes (3), and modern industrialized society yields increasing environmental complexity, it is an ever-greater challenge to perceive how the integration of our genes and surrounding environment creates disease-predisposed states. For example, why do certain individuals suffer lung cancer at an early age, after a few years of cigarette smoking, whereas some centenarians tolerate lifelong smoking without dying of the same disease? Such questions led to the idea of the need to identify "genetic signatures." Once genetic signatures are secured, one may develop means to prevent and/or treat diseases in an individual manner, creating individualized medicine for prognostic, diagnostic, and therapeutic purposes.

In general, large-sample gene chips, bearing perhaps 10,000 genes, are applicable only to early-stage screening and may yield voluminous lists of potential positive results; here we describe ~~here~~ a next-generation, medium-density microarray approach embodying considerable quality control in both chip design and analysis, which results in fewer hits of enhanced accuracy and pertinence.

Genetic Signatures and Microarray Technology

During the 1980s and early 1990s, biologists were busy dissecting the functions of single genes, by using a reductionist approach, which, although thorough in its exact methodological analysis of genetic impact, was restricted in explaining how each particular single gene functions in the context of many homologous genes or partners to accomplish a biological task. In an attempt to shed light on these biological tasks, "biochips" were introduced in the mid-1990s (4) as a new tool for molecular medicine; they now constitute a rapidly developing field of biomedical research, which combines molecular medicine, nanotechnology, computer science, and engineering. Biochip technology was developed for high-throughput gene screening, capable of simultaneously identifying changes in the expression of hundreds or thousands of genes, and thus genetic signatures defining particular physiological states. Consequently, as a result of its power, the young field of DNA microarray technology has rapidly gathered speed and popularity within the biomedical research community. However, as universities across North America have started to establish microarray core facilities, they are realizing that the next generation of microarrays must be more versatile, user friendly, and inexpensive to ensure that these facilities will meet the divergent needs of their researchers, and ultimately provide practical answers to fundamental biological problems. Thus, 4 years ago, parallel to the development of commercial microarrays, we began devoting significant time and effort to developing pathway-specific "designer biochips"; here we provide an account of our quest for designer arrays, and we discuss some of the challenges that lie ahead for those seeking the perfect array.

The Theory Behind Microarrays

Unlike Northern blots, in microarrays the probes, not the targets, are immobilized to a physical platform. Microarrays consist of a platform to which are independently attached numerous nucleic acid sequences known as probes, to screen targets of prelabeled nucleic acids obtained from donors (human or animal). The quantification of probe–target complexes formed during hybridization permits measuring gene distribution and intensity, as complementary probe-labeled target sequences bind together.

Although the principle behind microarrays is simple, creating and implementing microarray technology is difficult, as several parameters (discussed in the paragraphs that follow) can drastically affect the validity of the results obtained from microarrays. Furthermore, because the number of probes included on each microarray platform is great, the magnitude of results obtained from microarrays is huge, and thus requires powerful computerized image processing and statistical software to classify and analyze the data; without these, little significant gain can be obtained from using microarrays. Thus, microarray core facilities must integrate expertise in biology, computer science, engineering, and statistics. It is with this in mind that we started our quest for designer biochips.

Platform and Printing Robots

The first consideration when developing microarray technology is the type of platform, commonly either membrane or glass slide (5–9). Membrane-based microarrays use nylon or nitrocellulose membranes, and they are generally used when radioactive or colorimetric tags are used to label the targets, whereas glass slides are more suitable for microarrays using fluorescent tagging; we chose to use a membrane-based platform for our microarrays. Unlike glass slides, which must be chemically treated to permit the attachment of probes and decrease background fluorescence before use as a platform for microarrays (5,8–13), most membranes (whether bearing positive, negative, or neutral charge) require no such pretreatment. Because nucleic acids are negatively charged, the use of positive or neutral membranes greatly improves the signal; however, neutral membranes are generally more suitable, as positive-charged membranes yield higher background readings as well. Negatively charged membranes, although providing very little background, are usually not suitable because they yield poor signal. Thus we chose to use neutral-charged membranes for our arrays.

Once a microarray platform has been chosen, probes must be attached to the platform. Two obvious methods exist: synthesis of probes directly on the platform (14–18), and probe-spotting by use of a contact or noncontact printing robot (4,19–22). Although leading biochip companies often synthesize oligos directly on their microarrays by using techniques such as photolithography, this method is not easily mastered, nor accessible to most laboratories. In contrast, probe-spotting can be accomplished by use of any of several commercially available printing robots (22). Because we use membranes attached to glass slides as our platform instead of glass slides directly, we encountered several problems, including skipped dots and uneven printing, when we first attempted to print arrays. We had to substantially modify the printing heads of the first robot we purchased, and we had to build an enclosure over it that permitted maintenance of constant humidity, to ensure even printing of the probes.

Probes

Almost any type of nucleic acid can be printed with a printing robot; nucleic acids obtained from complementary DNA (cDNA) libraries, as well as polymerase chain reaction (PCR) products generated by reverse transcription PCR (RT-PCR), are commonly used as probes for microarrays. The use of cDNA libraries is of considerable value for screening previously unknown genes or a great many genes with no predefined preferences for certain gene families. However, mistakes arising from mislabeling of clones or contamination can cause problems (23). We spot PCR products on our chips to generate thematic arrays of particular known genes of interest. Commercially available biochips are often restricted to specific sets of genes contained on each biochip, posing user-unfriendly conditions. In the gene discovery task, users are generally conditioned to screen according to the preset configuration of genes, without the possibility of generating thematic or pathway-specific microarrays covering genes known to belong to a specific family, as demanded by a particular research program. The use of designer biochips can circumvent this problem. The following sections describe the strategy we used to fabricate thematic arrays.

Thematic Microarray Design

Gene Selection and Primer Design

To generate thematic microarrays bearing genes from a particular family or the same pathway, we use several public databases, including Unigene and GenBank (http://www.ncbi.nlm.nih.gov/GenBank, http://www.ncbi.nlm.nih.gov/Unigene), and conduct an extensive literature search (http://www.ncbi.nlm.hih.gov/PubMed) to obtain a repertoire of genes. Once a list of potential genes is constructed, we use GenBank and Unigene to obtain the sequences of all candidate genes. Primers for each gene sequence are designed by using Primer3 software (http://www.genome.wi.mit.edu/genome_software/other/primer3.html) to generate a PCR product, or "amplicon," with a length between 300 and 700 bp and a melting constant that ranges from 75°C to 89°C. For each gene we choose a pair of sense and antisense primers with an annealing temperature of approximately 60°C and an average length of 23 bp, for amplicon production using 96-well PCR plates. These clustered amplicon sizes and melting constants support standardized hybridization conditions for all probes across the microarray platform, including stringent washing, thus decreasing nonspecific signals while maximizing a specific signal. The length of the PCR product, as documented by Stillman and Tonkinson (6), is particularly important in maximizing a specific signal.

Primer design is perhaps the most time-consuming step in our microarray production, because once a primer pair is selected, an analysis must be performed with Blast (proprietary software available on the National Center for Biotechnol-

ogy Information website) to ensure that each primer pair amplifies only the gene of interest. This is crucial, because results obtained from the microarray are dependent on the specificity of the amplicons. However, in some instances, the specificity of the primer may not guarantee the specificity of the generated amplicon, when a conserved or shared domain lurks somewhere within the sequence. It is therefore highly recommended that the entire amplicon sequences themselves be Blasted to identify homologous regions, which can cause nonspecific binding. With probes obtained from cDNA libraries this may become a pitfall, especially when the spotted nucleic acid sequence is unknown; often highly homogenous sequences may result in nonspecific binding between genes of high homology. Stringent hybridization conditions and washing can generally eliminate this nonspecific binding, if the homologous region is not too long.

Controls

As in any biological experiment, and most importantly for microarrays, controls must be carefully selected. It is important to spot on all microarrays negative and positive controls as well as "housekeeping genes," used in more traditional experiments such as quantitative RT-PCR, which show little or no physiological change in expression among the subjects or conditions being studied. The inclusion of housekeeping genes is useful for data normalization; for our designer microarrays targeted to mouse models, we selected six mouse genes (glyceraldehyde phosphate dehydrogenase, ribosomal S6, beta-actin, hypoxanthine-guanine phosphoribosyltransferase, phospholipase A2, and ubiquitin) commonly used in the literature as controls. In general, the validity of these controls must be determined a priori by using independent tests, such as Northern blotting assays or quantitative RT-PCR (24). For instance, EF-1α would be a poor choice for a housekeeping gene if the target nucleic acids were obtained from skeletal muscle, as it is not expressed in adult muscle cells; it would, however, be a good control when cDNA from liver was used as a target (25). The use of housekeeping genes permits the measuring of changes in gene expression against a gene whose expression does not vary significantly; in some cases this can be of great value. Negative controls should include buffer, bacterial, and viral DNA, as well as amplicons from genes known not to be expressed in target tissues. Negative controls are used to assess the level of background noise arising from nonspecific nucleic acid binding during probe–target hybridization. Positive controls such as total cDNA or genomic DNA permit the detection of suboptimal conditions of hybridization and staining, which may obscure appropriate signal intensity.

Quality Control for Amplicon Production

In order to avoid producing the wrong amplicon for printing as a result of contaminated PCR reactions, the use of dedicated equipment and reagents in the PCR setup and reaction areas is recommended. For each PCR reaction with a unique amplification primer pair, a negative control should be used to ensure the absence of reagent contamination, often caused by the presence of exogenous nucleic acids. This control reaction is identical to the regular reaction, except that no template is present. Agarose gel electrophoresis is used to verify the amplicons and ensure that they are of expected size. In instances where multiple bands result from the PCR, products can be resolved on an agarose gel, and the fragment of expected size excised; these amplicons can then be sequenced to confirm their identity. It is our experience that, when a primer pair is well chosen, multiple bands seldom result from the PCR reaction.

Printing the Arrays

Once amplicons have been produced for all genes of interest as well as housekeeping genes, arrays can be printed. To avoid positional bias, print arrays in a scattered fashion, with several repeats of the same amplicon located in different regions of the chip. It is important to avoid positional bias, as uneven distribution of charges on the membrane can result in regions of increased background. A typical microarray manufactured in this fashion carries arrayed triplicates or quadruplets of amplicons from selected genes, positioned on the array among many control spots. The rationale for triplicate printing is to provide three data points for statistical analysis of significance; ideally, the three could be expanded to four or five repeats, to yield more data points for statistical analysis. This approach of scattered array printing requires considerable careful analytical software design, to enable tracking of amplicon repeats across the platform; however, it approaches an ultimate solution to resolving positional bias.

Although the spots of microarrays printed onto membranes affixed to glass slides are usually colorless, it is possible to monitor quality to detect gross errors in printing, such as missing, smeared, or non-uniform spots; immediately before a batch of microarrays are printed, a colored dye can be used to print a test array of dots onto a membrane. Microscopic visual inspection of the spots enables any necessary adjustments to be made to the robot before sample printing begins. While large batches of chips are being printed, quality can be monitored by inserting poly-L-lysine-coated glass slides among the membrane platforms. Unlike membranes, the clear surface of glass slides permits the researcher to see of printed spots by breathing on the slide and viewing it through a transmitted light source.

Once the probes are printed on the membranes, they are cross-linked to the microarray to permit better attachment of the nucleic acids to the substratum; probes are denatured by boiling the membranes before hybridization.

Target Labeling

Donor nucleic acids can be labeled by adding a labeled base to the RT reaction used to generate target cDNA. Whereas most commercial arrays use fluorescence-conjugated or radiolabeled bases, we developed our microarrays based on a nonradioactive colorimetric method. The bulkiness of some of the fluorescent tags, fluorescent quenching over time, and the need of specialized scanners to read fluorescent signals dissuaded us from using the fluorescent approach (26). Similarly, we wanted to avoid using radioactive labels for safety reasons, because they often give saturated signals, and to save the time required for exposure of the labeled arrays to x-ray film.

Using commercially available digoxigenin (DIG)-labeled

dUTP (Roche, Palo Alto, CA) and alkaline phosphatase (AP)-conjugated anti-DIG antibody (27), we have developed a new application for DIG in microarrays (28). In our method, the cDNA to each donor RNA is synthesized with a DIG-labeled base. Following hybridization of the DIG-labeled target with the probes, positive reactions are revealed by incubating with anti-DIG antibody conjugated to AP, and subsequent staining with Nitro-blue-tetrazolium/5-Bromo-4-chloro-3-indolyl phosphate (NBT/BCIP, Roche) to detect AP (29). Taking advantage of the fact that two complementary nucleotide strands can hybridize with each other, we generate microarray results by quantifying the signal obtained from the labeled targets bound to the immobilized probes. Thus the positive loci are visible as bluish spots, easily identified as round deposits for each positive locus. The final detection is revealed as a matrix of many round dots of varying intensity of staining.

Microarray Inventory

Extensive records should be maintained on the microarray printing process, including logistical parameters of the physical status of the microarrayer (e.g., relative humidity of the chamber during printing, or how often the printing pins are cleaned during the run), the specifics of printing, including the printing sequence, the identity of each amplicon-containing spot, and all other applicable data or comments. To ensure that identical records are made for each printing, we record all data for our microarrays onto a standardized form that we have developed over the past several years. We are also developing a two-dimensional bar-coding system to keep detailed track of our inventory of microarrays.

Image Acquisition and Data Processing

The work just described supports the process of data generation; the following sections describe the mining of the resulting data.

Array Normalization and Background Subtraction

As our arrays are based on a colorimetric detection method, a high-resolution scanner is used to scan them into digital images. Before a normal office scanner is used, it is important to ensure that it digitizes accurately without transforming the image (26). If the image is transformed by the scanner, mathematical correction transformation should be applied to the result. Following acquisition, the digitized images can be normalized and subtracted as desired. We have developed a software program, GeneAnalyzer, which accomplishes background subtraction, array normalization, and quantification. When colorimetric microarrays are analyzed, several types of background must be considered; for instance, regional background subtraction is useful when the array shows differential intra-array background expression, whereas global background subtraction is suitable when the background value is constant within arrays but variable between arrays, as a result of experimental conditions. For interarray comparison to be supported, arrays may be normalized by several methods, including reference to housekeeping gene levels and median chip values. However, investigators should think carefully about the effects of performing such background standardization or normalization before they start analyzing their results; they should especially consider the effect of background subtraction on diminishing the signal of low-expression genes.

Software for Microarray Data Acquisition

In general, image acquisition and data analysis include the following processes: (a) image grabbing and digitizing; (b) image processing; and (c) data mining, including a qualitative and quantitative analysis of all digitized images, and a statistical analysis of data. We developed our software with a user-friendly interface and a limited number of preset functions, to enable researchers to analyze their own data. The main features of our program are as follows.

First, we provide users a PIN number, which allows optimal security of their data and access to the interactive functions of our web server facility. Second, users can upload their electronic images from remote sites over the Web. Third, our system processes the users' initial data to enhance the image profile, through standard computer software such as MatLab. Fourth, our system supports the users' data archiving and database organization for the next stage of data analysis.

Statistical Analysis and Data Mining

Although it is necessary to use statistical methodologies to analyze voluminous microarray data, it is equally important to generate adequate microarray-derived data to support statistical testing. Thus it is recommended for most studies to use at least three mice (or individuals) chosen at random from each test group (e.g., young vs old mice). RNA extracted from each donor tissue source (e.g., lung) is then subjected to three separate RT reactions, and each of these nine independently obtained cDNAs is used as targets for three different microarrays. Because on our microarray each amplicon is spotted in triplicate, we have a total of 81 (3^4) spots, or data points, for each gene being analyzed (RNA from three mice multiplied by three RT reactions multiplied by three microarrays multiplied by three spots on each slide). The use of at least triplicates for each step of microarray fabrication enables us to obtain statistically meaningful data. Without this replication, simple variations in the efficiency of the RT reaction, interanimal variation, or misprinting of a spot could all result in falsely perceived changes in gene expression.

Because the statistical analysis of microarrays presents a challenge to many biologists, it is recommended that a statistician be consulted as necessary. Statistical consultants can be extremely helpful, not only at the final stage of data analysis, but also at the initial experimental design step; for example, they may provide answers to fundamental questions, such as how many animals are needed to establish a statistically significant data analysis, or whether or not one may pool RNA samples.

Once microarray data are processed through statistical analysis, data entry points deemed of true "significant" value, that is, gene expression changes as effects of an experimental physiological change, should be subjected to the next level of data analysis, now popularly termed the data mining process (30). Many established methods have been popularized among microarray users, including principal component analysis (31–33), hierarchical clustering (34–

37), multidimensional scaling (38–40), and self-organizing maps (41). In general, the selection of any of these methods is dependent on the individual investigator's preference and expertise. For example GeneSpring software, sold by Silicon Genetics, Inc. (San Carlos, CA), and Significance Analysis for Microarrays from Stanford University (42), are preferred for many gene screening data mining tasks because they can analyze data generated by several different microarray platforms. These data mining software packages enable researchers to display their data in forms suitable for publication, easily conveying the essence of the results.

Following data mining, microarray data that seem to be significant should be validated by using one of two popular methods: Northern blotting or quantitative RT-PCR. In general, it is advisable that microarray data be validated by the selection of four or five randomly designated genes from each of three categories: those showing high, intermediate, and low levels of significant difference. Because we use amplicons to generate our probes, we can easily validate our results, using the same primers used to generate our amplicons by quantitative or semi-quantitative RT-PCR.

During data analysis, special consideration must be given to low-expression genes, which generally exhibit the greatest variance in expression levels. On any given microarray, these genes show very weak intensities, and in some cases they are barely visible above the background value. Here, standard global normalization and thresholding are not practical, because the signals are so weak. Often we find that global thresholding is too crude, allowing in one case the gene expression to be quantified as a gain, and in another case allowing the same gene expression to be quantified as a loss. One possible solution for this problem is to use "segmental thresholding," localized thresholding for each individual weak spot. Then the local background level is calculated against the global background level to obtain confidence level indices. The actual gene expression level for these low-abundance genes is then the "minithresholding level" divided by the confidence level. We realize that this is not a perfect solution; often we have to disregard these data points altogether.

Conclusions

The notion that the bioinformatics of microarray studies is still in its infancy pertains not only to studies in the aging area, but also to many other biological systems as well. The entire field of microarrays is experiencing volatile changes in methodological approaches, technological design, and bioinformatics development for data interpretation. Part of the growing pains in the use of microarray technology is the constant need for new cutting-edge technology and the re-evaluation of methodology. Therefore, for any designer microarray projects, it is necessary to be vigilant for any new methodology and technology developments, to improve the design and fabrication process as well as the bioinformatic aspects of gene screening tasks.

As with all technologic advances, the microarray approach is not an end in itself; it is just a beginning. Obviously, one wants to know whether the genes identified as significantly changed at the RNA level are truly manifested at the protein level. For this purpose, the recent explosion of proteomic technology is certainly a testimony to the need for follow-up to microarray data. Ultimately, gene expression microarray studies have to be followed with experiments to examine protein changes, thus permitting a comprehensive examination of gene expression changes from RNA to protein levels.

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Address correspondence to Dr. Eugenia Wang, Department of Biochemistry and Molecular Biology, University of Louisville School of Medicine, 570 South Preston Street, Baxter Building Room 304, Louisville, KY 40292. E-mail: Eugenia.Wang@Louisville.edu

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