GENE REGULATION BY POST-TRANSCRIPTIONAL PROCESSING DURING CELLULAR SENESCENCE IN HUMAN DIPLOID FIBROBLASTS

By

SHANAZ ADI GHANDHI

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy,

The City University of New York.

2006

UMI Number: 3204983

UMI Microform 3204983

Copyright 2006 by ProQuest Information and Learning Company.
All rights reserved. This microform edition is protected against unauthorized copying under Title 17, United States Code.

ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

© 2006

Shanaz Adi Ghandhi

All rights reserved

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

Date	**Chair of Examining Committee** **Dr. Karen Hubbard** **The City College of New York**
Date	**Executive Officer** **Dr. Richard L. Chappell**
	Dr. Harvey Ozer, **The UMDNJ-New Jersey Medical School**
	Dr. Jerry Guyden, **The City College of New York**
	Dr. David Calhoun, **The City College of New York**
	Dr. Jill Bargonetti-Chavarria, **Hunter College**

THE CITY UNIVERSITY OF NEW YORK

Abstract

Gene regulation by post-transcriptional processing during cellular senescence in human diploid fibroblasts

By

SHANAZ ADI GHANDHI

Advisor: Dr. Karen Hubbard

Primary human fibroblasts undergo an extended period of senescence at the end of their replicative life span which is characterized by the complete loss of proliferative potential accompanied by specific morphological and molecular changes. The expression of a gene from the time it is transcribed to the moment a functional protein is made can be modulated at various steps and alternative splicing of pre-mRNA, mRNA transport and regulation of mRNA half-life are important steps that ultimately regulate the levels of functional protein that a cell expresses. We are interested in a family of proteins called Heterogeneous Nuclear Ribonucleoproteins, specifically type A1 and A2 that appear to have roles in many steps of mRNA biogenesis mentioned above. We have identified that levels of these proteins are significantly changed in senescent fibroblasts. However there is very little information regarding their function in senescent cells. My proposed hypothesis was that the observed changes in levels of hnRNP A1 and A2 could have significant effects on the gene expression patterns of senescent cells where their levels are significantly lower and that identifying these changes could help elucidate the mechanism by which cells age.

In order to study altered gene expression patterns of cell cycle regulatory proteins, we performed quantitative RT-PCR and immunoblotting to study specific target genes and products. To identify gene expression changes on a more global scale we performed cDNA array experiments and compared expression profiles among senescent cell populations and fibroblasts that over-express hnRNP A1. We have identified cyclin D1 as a candidate mRNA that may be regulated by age and post-transcriptional processing. In order to identify target mRNA that are bound by hnRNPA1 and maybe age-regulated, we performed RNA co-immunoprecipitation experiments using hnRNP A1 as the bait protein. With this approach we identified human mdm2 mRNA as a binding target for hnRNP A1 in young and old fibroblasts. This is a novel and important finding because a large number of mRNA isoforms are made from the human mdm2 pre-mRNA. Despite its importance in regulation of p53, little is known about the role of the alternatively spliced isoforms of human mdm2.

ACKNOWLEDGEMENTS

I owe an enormous debt to the many people who have helped, assisted, guided and mentored me in my course of study. The people who have been most instrumental in this endeavor are Dr. Karen Hubbard, my thesis advisor and members of my committee, Dr. Jerry Guyden and Dr. Harvey Ozer, MD. I would also like to thank Dr. Jill Bargonetti-Chavarria and Dr. David Calhoun for their guidance and involvement in this important stage of my career. This work would not have been possible without the kind help and unstinting support of Dr. Michael Desmond Samms, Dr. Powrnima Joshi and future doctors Vimaris Dejesus, Ivelise Rijo, Charles Utti and Dmitry Oulianov. This work was a personal journey, yet I have had the privilege of crossing paths with a very special group of graduate and under-graduate students who have helped me to get to this point.

My family has been most supportive and words would be inadequate to express my gratitude for their contribution to my career and personal goals. Air Marshal Adi Ghandhi, PVSM, AVSM, VrC (Ret'd), Mrs. Matilda Ghandhi and Meher Adi Ghandhi are the reason that I have been able to undertake and fulfill this very important step in my life and career.

TABLE OF CONTENTS

List of Tables……viii

List of Figures……ix

Chapter 1……54

Chapter 2……85

Chapter 3……145

Summary and Conclusions……...……..181

References……..184

Detailed table of contents

List of Tables

Materials and Methods

Table1. PCR primers and conditions…….……. 47

Chapter 2

Table 2.1 Expression of Cell cycle and growth

regulatory genes is altered in cellular senescence…….…….125-126

Table 2.2. Summary of gene expression changes in

RNA metabolism genes in senescent IMR90 fibroblasts…….128

Chapter 3

Table 3.1 Cloning of PCR products and clone analysis……161

Table 3.2. Sequence analysis and results of similarity search..169-170

List of Figures

Introduction……..1

Figure 1. Lifespan of normal human diploid fibroblasts…….3

Figure 2. Pre-mRNA splicing model with RNA binding proteins……..12-13

Figure 3. Model of vertebrate mRNA transport…….15

Figure 4. Schematic representation of hnRNPA1 and hnRNPA2 proteins and gene structure……18-19

Figure 5. Cellular localization pattern of hnRNPA1 and hnRNPA2 are altered in senescent fibroblasts……23-24

Figure 6. Cell cycle control by tumor suppressors and oncogenes and the *Ink4a/Arf* locus……..…...30

Chapter 1……..54

Figure 1.1 Alternative splicing at the caspase-2 locus…… 57

Figure 1.2 Exon structure of fibronectin gene…….59

Figure 1.3 mRNA levels and protein levels of hnRNPA1 are altered in senescent fibroblasts…….63-64

Figure 1.4. mRNA analysis of hnRNPA1 and hnRNPA2 in other fibroblasts cell strains such as WI38 normal human diploid fibroblasts……..65-66

Figure 1.5. hnRNP C protein is expressed at the same levels in young, G0 arrested and senescent IMR90 fibroblasts……67-68

Figure 1.6. hnRNP A1 protein is over-expressed as
a fusion product in IMR90 fibroblasts……70-71
Figure 1.7. Schematic representation of caspase-2 locus
and mRNA analysis of alternatively splice products……..74-75
Figure 1.8. RNase protection assay on IMR90 fibroblasts……...76-77
Figure 1.9 Analysis of the fibronectin locus and
alternative splicing decisions at the EDA and EDB exons……..80-81
Chapter 2…….85
Figure 2.1. Verification of RNA quality(a)……98-99
Figure 2.2. Verification of RNA quality(b)……100-101
Figure 2.3. Representation of data obtained from
comparison of 192 genes from cDNA arrays……104-105
Figure 2.4. Heirarchical cluster analysis of genes (A)……..….106-107
Figure 2.4. Heirarchical cluster analysis of genes (B)…….109
Figure 2.5. Validation of CCND1 gene expression
changes by real-time PCR……112-113
Figure 2.6. Analysis of INK4A locus……116-118
Figure 2.7. Analysis of caspase-7 mRNA and
protein profiles in IMR90……..122-123
Figure 2.8. Affymetrix array analysis and real-time
PCR validation of gene expression patterns on
up-regulated genes after hnRNPA1 over-expression
in IMR90 fibroblasts……..130-132

Figure 2.9 Affymetrix array analysis of gene expression patterns on down-regulated genes after hnRNPA1 over-expression in IMR90 fibroblasts……135-136

Chapter 3……..145

Figure 3.1. A. Protein levels of hnRNPA1 and actin in young and old IMR90 cell lysates……..158-160

Figure 3.2. RT-PCR analysis on isolated RNA and RE digest sample result……..163-165

Figure 3.3. PESX analysis……..171-172

Figure 3.4. Model for hnRNPA1 effects on alternative splicing of human mdm2 mRNA and cell growth……178-179

Introduction

Human Diploid Fibroblast model of replicative senescence

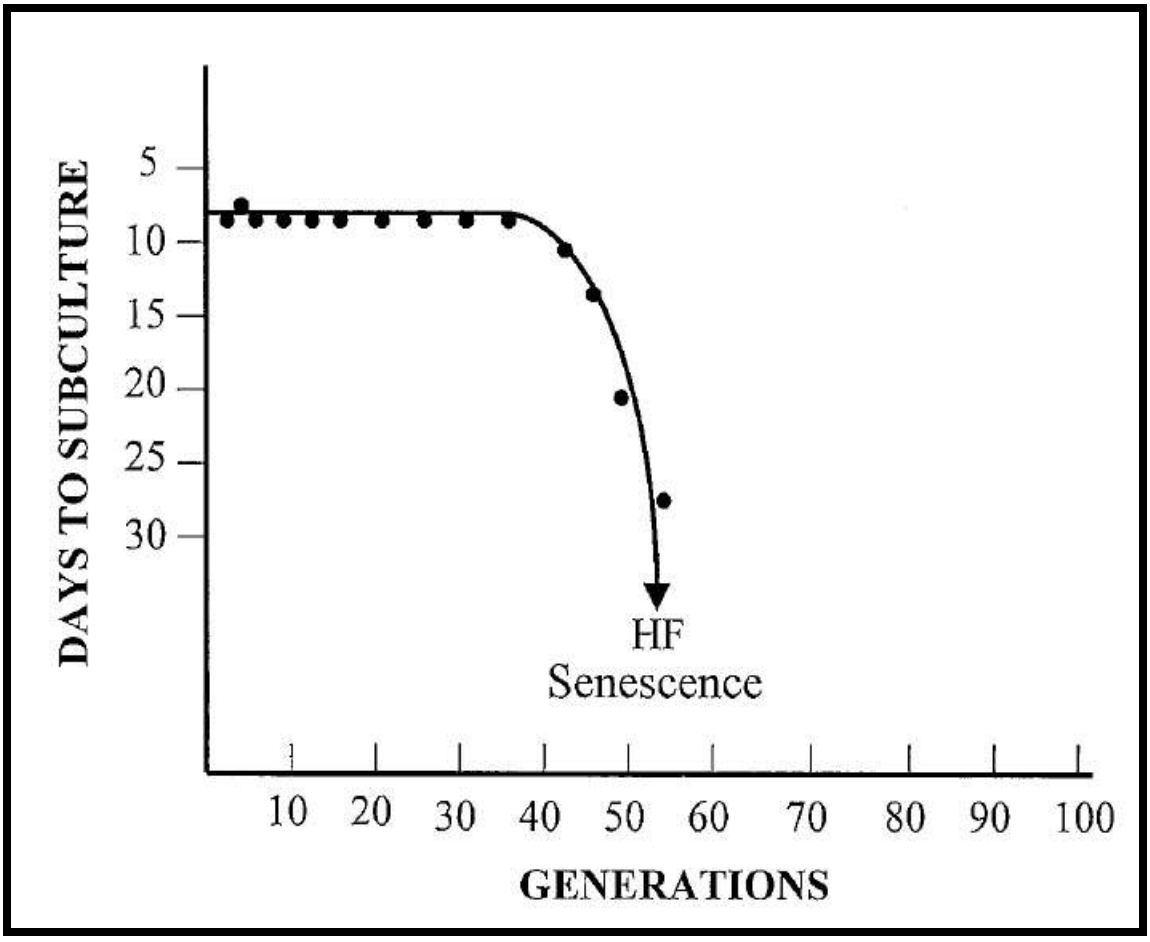
The seminal observation made by Leonard Hayflick, was that human fibroblast cells in culture have a finite replicative capacity in defined environments (Hayflick 1961). The stage at which cells stop dividing after continuous sub-cultivation was called the Hayflick limit (Hubbard 1997), later renamed replicative or cellular senescence and also M1 (Mortality checkpoint 1) (Hubbard 1997; Cristofalo 1988). Onset of replicative senescence is characteristic of all normal human cells in culture and varies among individual cell types. Cellular or replicative senescence can be defined as a state of permanent or irreversible growth arrest in G0/G1 of the cell cycle, in which cells assume an enlarged, flattened morphology, exhibit an altered pattern of gene expression and yet remain metabolically active. The hallmark of cellular senescence is the inability of senescent cells to proliferate in response to mitogenic stimulation.

The question of whether cellular aging parallels organismal aging is still an open one, but there are some suggestive correlations in the literature (Cristofalo 1998). Organismal aging is an important issue in this era of increased life expectancy and resulting age-related diseases. There are two types of theories to explain how and why cells in general age: stochastic aging theories that are based on the gradual and inevitable accumulation of random events that ultimately lead to metabolic dys-regulation and breakdown and non-stochastic aging theories or programmed aging (e.g. a genetic program). There are many ways to cause cells to undergo premature senescence such as oxidative stress,

cross-linked DNA, ceramide, PI-3K inhibitors, HDAC (histone deacetylase) inhibitors and DNA de-methylating agents (Zhou 2003). Depending on the causative agent, the phenotype of treated cells can then be defined by a variety of senescence associated changes. Therefore, the senescent state is sometimes difficult to characterize. This is because the phenotype has not yet been defined clearly for various cell types and investigations into a subset of biomarkers of cellular aging (e.g. senescence associated β-galactosidase activity at low pH and reduced levels of *c-fos* mRNA) in organisms have led to conflicting conclusions (Cristofalo 1998).

Figure 1. Lifespan of normal human diploid fibroblasts.

Normal fibroblasts (**HF**) undergo repeated population doublings that can be measured as a function of the passage number. As the culture becomes older the number of days the cells will need to come to confluence stays constant, these are considered young and mid-passage cells. The cells then enter late-passage when the number of days to confluence in each sub-cultivation steadily increases until they are senescent and they are unable reach confluence at all. (from Jha 1998).



Gene expression patterns in senescent cells.

The senescent phenotype is dominant over young cells as evidenced by cell fusion experiments. All combinations of cells at different replicative ages were fused and growth characteristics recorded. The young/old hybrid behaved like the old/old hybrid control and was able to undergo 4-5 further replicative cycles. In contrast, the young/young hybrid control continued dividing 40-50 times more. From this, it is believed that old cells were able to suppress the program of young cells (Hubbard 1997). Old cells were also able to suppress the immortalization program of malignant cells in a similar experiment. These fusion experiments were expanded into a complementation experiment in which different immortal cell lines were fused and senescence onset was expected to be restored in the hybrid (Vojta 1996). The assumption was that different genetic alterations exist in different immortal cell lines and that these defects could be complemented in synkaryons created when immortal cells presumably carrying different alterations were fused (Vojta 1995). Thirty one different immortal cell lines have been assigned to four complementation groups A, B, C and D, for indefinite division potential (Vojta 1995). The hypothesis proposed was that if similar changes are assumed for members of the same complementation group then it may be possible to map the altered locus to an individual chromosome (Vojta 1995). The complementation groups so defined were further studied for presence of senescence associated genes and these and other studies established that there are regions of the human genome that are required for a cell to undergo senescence in a timely manner (Vojta 1995). Chromosomes carrying tumor

suppressor genes may mimic this effect by controlling cell division, which was may contribute to senescence onset and maintenance.

It is now believed that differential regulation of many pathways is required to ultimately cause cells to undergo senescence. Onset of senescence can be modulated by multiple events involving gene regulation, chromosome dynamics (telomere hypothesis and DNA repair), protein activity and degradation, mitochondrial mechanisms and other metabolic pathways. It is possible to induce a senescence–like phenotype in cultured cells (Cristofalo 1998, Vojta 1995, Michishita 1999), for example in the case of keratinocytes life span is shorter in culture than fibroblasts and senescence can be induced by over-expression of specific genes. In the case of keratinocytes , senescence may be controlled differently because keratinocytes are already in a differentiation program controlled by of a different set of genes. It has been suggested that since $p21^{WAF1}$ (a negative cell cycle and growth regulator) has as role in differentiation as well as growth arrest that the observed up-regulation of p21 prior to onset of senescence may be an inherent differentiation program cells undergo in culture concomitant with senescent specific events. It has also been shown by microarray display of gene expression in senescent fibroblasts and skeletal muscle cells that there is minimal overlap of gene expression changes between these two cell types suggesting that they probably undergo aging through different mechanisms (Marx 2000). H-ras-V12 (an activated oncogene) activates the senescent checkpoint through $p16^{INK4a}$/$p19^{ARF}$ in mouse primary cells but in human fibroblasts can induce senescence through mitogen kinase signaling pathways. In mouse

embryonic fibroblasts, the mouse equivalent of human primary fibroblasts in culture, the p53-p19^{ARF} pathway appears to be more important in onset of senescence. This differs in human cells where telomere dynamics is proposed to have a more significant contribution. Human cells in contrast to rodent cells have very low frequency of immortalization. Hence, we believe that senescence may be a tumor suppressive mechanism. This also implies that senescence in mammalian cells is manifested and controlled by different pathways.

There are multiple defects in signaling pathways in senescent cells as seen in WI-38 HDF (lung) in which *c-fos* (an early-G1 response gene) was not induced by certain signals such as mitogens but was induced by UV irradiation (Cristofalo 1998). Also mitogenic stimulation can initiate an abortive attempt at G1 but fails to induce DNA replication. Introduction of poly(A)+ RNA from senescent fibroblasts into early passage fibroblasts inhibits DNA synthesis. This activity led to the identification of p21^{WAF1} as a senescence mediating protein. Therefore, there are multiple levels of checkpoints to cell cycle progression and growth that are turned on in senescent cells. I will discuss this in further detail later in the description of the INK4a locus and its significance in cellular senescence.

In all, the replicative senescent phenotype is characterized by changes in morphology to large, refractive cells and general dys-regulation of coordinated genetic processes. This is extensively catalogued in Cristofalo 1998, however, creating a genome-based profile of gene expression in senescent cells using

microarrays may help to elucidate the information catalog by investigating many genes that may work together in a coordinated cellular pathway .

Telomere hypothesis of replicative senescence.

The hypothesis proposes that progressive telomere shortening during division of human fibroblasts in vitro leads to activation of a senescence program defined by the Hayflick limit (~60-80 population doublings for HDF) (Hayflick 1961). Ectopic expression of telomerase halts this telomere shortening, stabilizes telomere lengths and ultimately immortalizes normal human fibroblasts (Hubbard 1997, Harley 1997). It is believed that a telomere length sensing mechanism involves a cellular monitor capable of recognizing diminished telomeres as double strand breaks in DNA. Critical genes proximal to telomeres could also be structurally altered as chromosomes progressively erode. In yeast, this is called the telomere silencing effect and is characteristic of yeast genome dynamics, but there is no known analog in mammalian genomes. The DNA damage signal at eroded telomeres is recognized and transduced to tumor suppressor controlled pathways of p53 and pRb (Retinoblastoma protein) which are capable of triggering cell cycle arrest. These events are proposed to precede onset of senescence and are observed to be impaired when cells bypass senescence (Chin 1998).

Telomere binding proteins and telomere maintenance mechanisms

Telomeres are located at both ends of linear chromosomes and serve to protect chromosome ends. In mammals, telomeres are G-rich regions of double and single stranded DNA. In human somatic cells the size range is between 3-18

kilobasepairs of duplex DNA with a 130-210 base long 3' G-rich overhang. The telomere is exclusively made up of $(TTAGGG)_n$ repeats. In mouse cells telomeres are longer at ~30 kilobasepairs in length (O'Reilly 1999, Price 1999, Colgin 1999, Prescott 1999, Perrem 2004, Artandi 2000 and Harley 1997).

In vertebrates TRF1, TRF2 and tankyrase (Price 1999) are known to bind and regulate telomeres. It is now known that hnRNPA1 (heterogeneous nuclear ribonucleoproteins A1) and its proteolytic by-product UP1 may be components of telomeric structure. hnRNPA1 is one of several proteins that were identified as putative telomere proteins because of high affinity for single-stranded TTAGGG sequences (Hubbard 1995, Price 1999). That hnRNPs A, D and E (identified telomeric sequence binding hnRNPs) bind RNA better than DNA and show affinity for telomeric sequences. In the case of hnRNPA1, there is also a proteolytic by-product called UP1 that is known to bind telomerase and in certain conditions promote maintenance of telomere length. The crystal structure of UP1 bound to a 12-nucleotide single-stranded telomeric DNA has been determined (Ding 1999; LaBranche 1998). Whether the telomere binding capability of hnRNPs is merely fortuitous or functionally significant remains to be seen.

Post-transcriptional regulatory mechanisms of gene expression.

Different protein products can be generated from a single gene encoding pre-mRNA. Cells use alternative splicing, RNA editing, alternative poly(A) site selection and alternative translation initiation to achieve diversity in gene products from a relatively few number of genes (Siomi 1997).

Structure and function of heterogeneous nuclear Ribonucleoproteins.

The hnRNPs were first described as a major group of abundant, nuclear, chromatin-associated RNA-binding proteins (Dreyfuss 1993; Krecic 1999). There are six core hnRNPs { A1 (34 kDa), A2 (36 kDa), B1 (37 kDa), B2 (38 kDa), C1 (41kDa) and C2 (43 kDa)} that package and transport nascent RNA polymerase II transcripts called hnRNA into hnRNP particles. These proteins fall into more than twenty groups according to structure and function. The most prominent structural feature of hnRNPs is that they contain RNA-binding motifs as well as auxiliary domains that may mediate protein-protein interactions. The non-RNA binding glycine rich domain of hnRNPA1 is a case in point. The hnRNP genes and their products are alternatively spliced, phosphorylated and methylated resulting in great diversity of binding-properties and other functions. hnRNP particles form nucleosome-like fibrils with nascent transcripts. They package ~700 nucleotides in each particle. hnRNPs have multiple functions in cellular metabolism including:

1. Packaging of RNA into hnRNP fibrils.
2. Transcription. hnRNP K is a TATA -BP binding protein.
3. Telomere maintenance. In a transformed mouse erythroleukemia cell line, loss of hnRNPA1 due to retroviral insertion at the *HNRPA1* locus correlates with short telomeres; reconstitution of hnRNPA1 restores telomere length (LaBranche 1998).
4. Alternative pre-mRNA splicing. hnRNPA1 is a concentration dependent antagonist of the serine-arginine (SR) protein ASF/SF2 which is an important

splicing regulator. hnRNPA1 can act as an exonic splicing repressor and can affect alternative splicing of its own pre-mRNA. Both 3' and 5' splice site usage, exon inclusion and exclusion can be altered by hnRNPs.

5. Coordinate transcription and alternative splicing. In interphase nuclei there are believed to be spatially and functionally distinct domains called speckles that are enriched in splicing factors. It is proposed that hnRNPs may also be concentrated in these regions to create mRNA "factories" inside the nucleus when cells are transcribing genes actively (DeJong 1996; Lewis 2000).
6. Pre-mRNA 3' end processing. hnRNP I can bind to the polyadenylation efficiency element, positioned upstream or downstream of the poly(A) site.
7. mRNA translation and turnover. hnRNPs E1 and E2 can mediate translational silencing of specific genes. hnRNP D and hnRNP A1 can bind AU-rich elements that mediate RNA stability (Hamilton 1997).

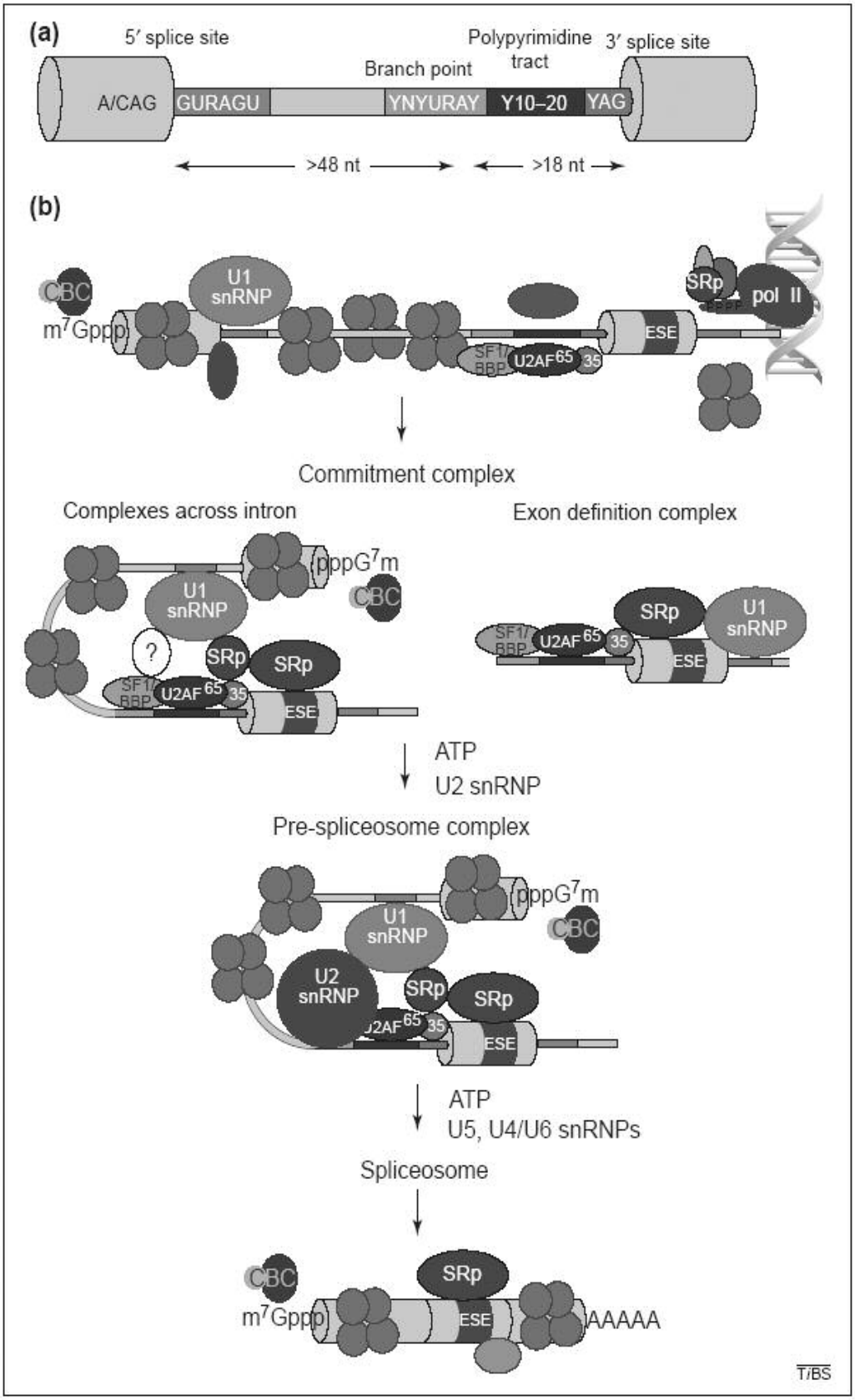
Alternative splicing.

Alternative splicing can give rise to functionally distinct proteins from a single gene. It is believed to occur in 35-50% of all human genes (Misteli 2000). The regulation can be dependent on sex, developmental stage, tissue type, in response to growth stimuli, hormones, cytokines and extra cellular pH (Konig 1998). It can involve simple or extensive sequence elements for example, in the case of a K^+ channel regulator in birds, there can be 576 alternative isoforms of the protein produced from a single gene by alternative splicing (Smith 2000). Nascent mRNA transcribed by RNA polymerase II is immediately bound by various hnRNPs, cap-binding complex and splicing factors. U1 snRNP (small

nuclear ribonucleoprotein) binds to the 5' splice site (GU), SFI/BBP (splicing factor/branchpoint binding protein) binds the branchpoint, $U2AF^{65}$ (U2 snRNP auxiliary factor) binds the polypyrimidine tract and $U2AF^{35}$ binds the 3' splice site (AG). These proteins assemble on pre-mRNA to form the splice commitment complex. SR (serine-arginine rich) proteins promote the formation of commitment complexes, by assisting U1 snRNP and U2AF to form a bridge between the 5' and 3' splice sites. ATP hydrolysis and binding of U2 snRNP to the branchpoint forms the pre-spliceosomal complex. Subsequent binding of U5, U4/U6 snRNPs accompanied by ATP hydrolysis allows the assembly of a mature spliceosome across the intron. The spliced mRNA is released and changes in hnRNP particle composition can occur to facilitate mRNA transport.

Figure 2. Pre mRNA splicing model with RNA binding proteins

This figure shows splice-site elements and splicing complex assembly. (a) consensus splice site elements for a typical metazoan intron. (b) nascent mRNA is immediately bound by various hnRNP , cap binding complex and splicing factors. SR proteins are found initially with polyadenylation factors , later they promote assembly of commitment complexes and might form a bridge between 5' and 3' splice sites. After splicing, changes in hnRNP complexes can occur which might facilitate nucleocytoplasmic export and even cytoplasmic events. (From Smith 2000)

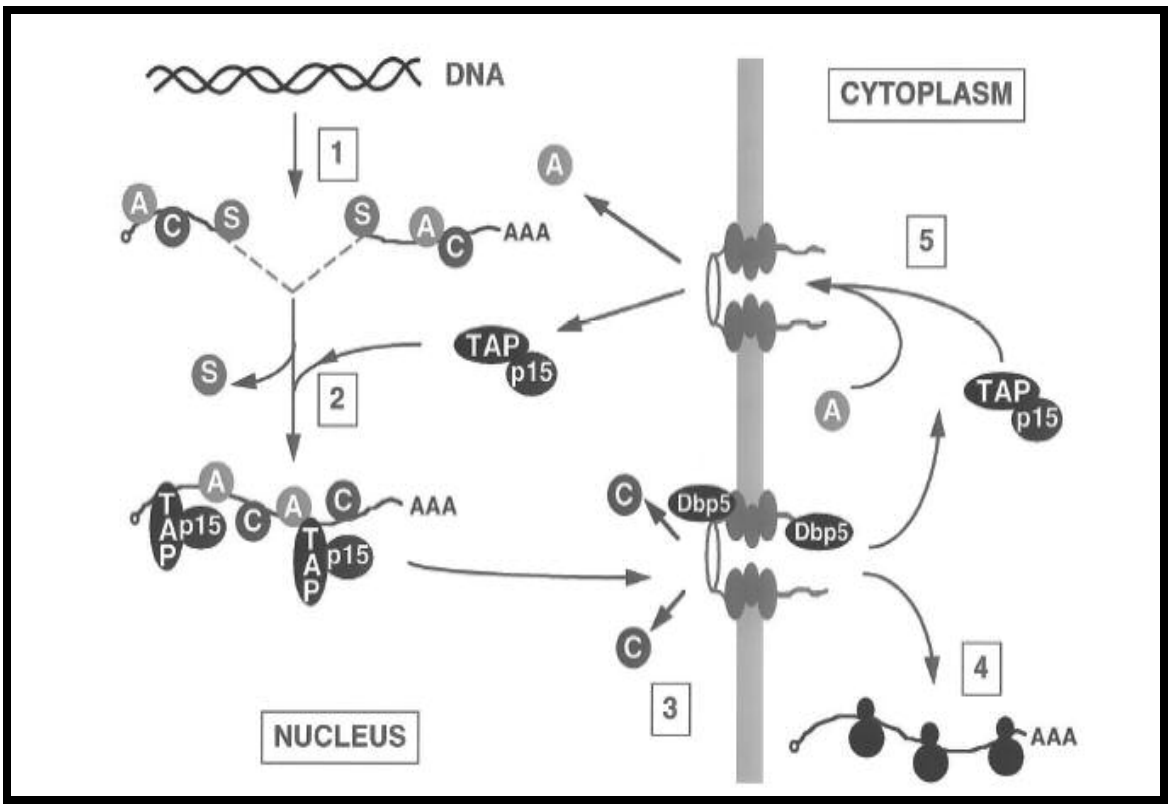

(a)
5′ splice site
Polypyrimidine tract
3′ splice site
Branch point
A/CAG
GURAGU
YNYURAY
Y10–20
YAG
>48 nt
>18 nt
(b)
CBC
m7Gppp
U1 snRNP
SRp
pol II
ESE
SF1/BBP
U2AF65
35
Commitment complex
Complexes across intron
Exon definition complex
pppG7m
?
ATP
U2 snRNP
Pre-spliceosome complex
U2 snRNP
Spliceosome
AAAAA
TiBS

mRNA transport.

hnRNP proteins were observed to shuttle between the nucleus and cytoplasm suggesting that they may have a role in mRNA export. Export of mRNA has been shown to be independent of RNA synthesis suggesting that the active process is hnRNP or protein mediated transport and RNA transcripts are passive cargo. The M9 sequence in hnRNPA1 signals both import and export. hnRNP A1 is imported through interaction with the import receptor tap (transportin) (Stutz 1998). The export receptor for hnRNPA1 is not known. In contrast to hnRNPA1, hnRNP C is completely nuclear and does not shuttle. The current model of vertebrate mRNA transport proposes that once pre-mRNA is committed to splicing, accessibility of RNA to export factor is excluded. Once splicing is completed commitment factors are released and export factors Tap (transportin) and p15 are recruited to the mature mRNA (Cullen 2000). hnRNPs A1 and C are still bound to the mRNA. Before leaving the nucleus hnRNP C is released and hnRNPA1 accompanies the mRNA into the cytoplasm. Cytoplasmic RNA binding proteins replace hnRNPA1 and Tap, to target the mRNA to translation. hnRNPA1 and Tap are then recycled back into the nucleus.

Figure 3. Model of vertebrate mRNA transport: relevant RNA binding proteins and transportins.

mRNA transport is mediated by hnRNP A1 and transportins. hnRNPA1 is bound to both pre-mRNA and mRNA and facilitates export of mRNA to the cytoplasm through the NPC (Nuclear Pore Complex). A and C are hnRNPs (from Cullen 2000).

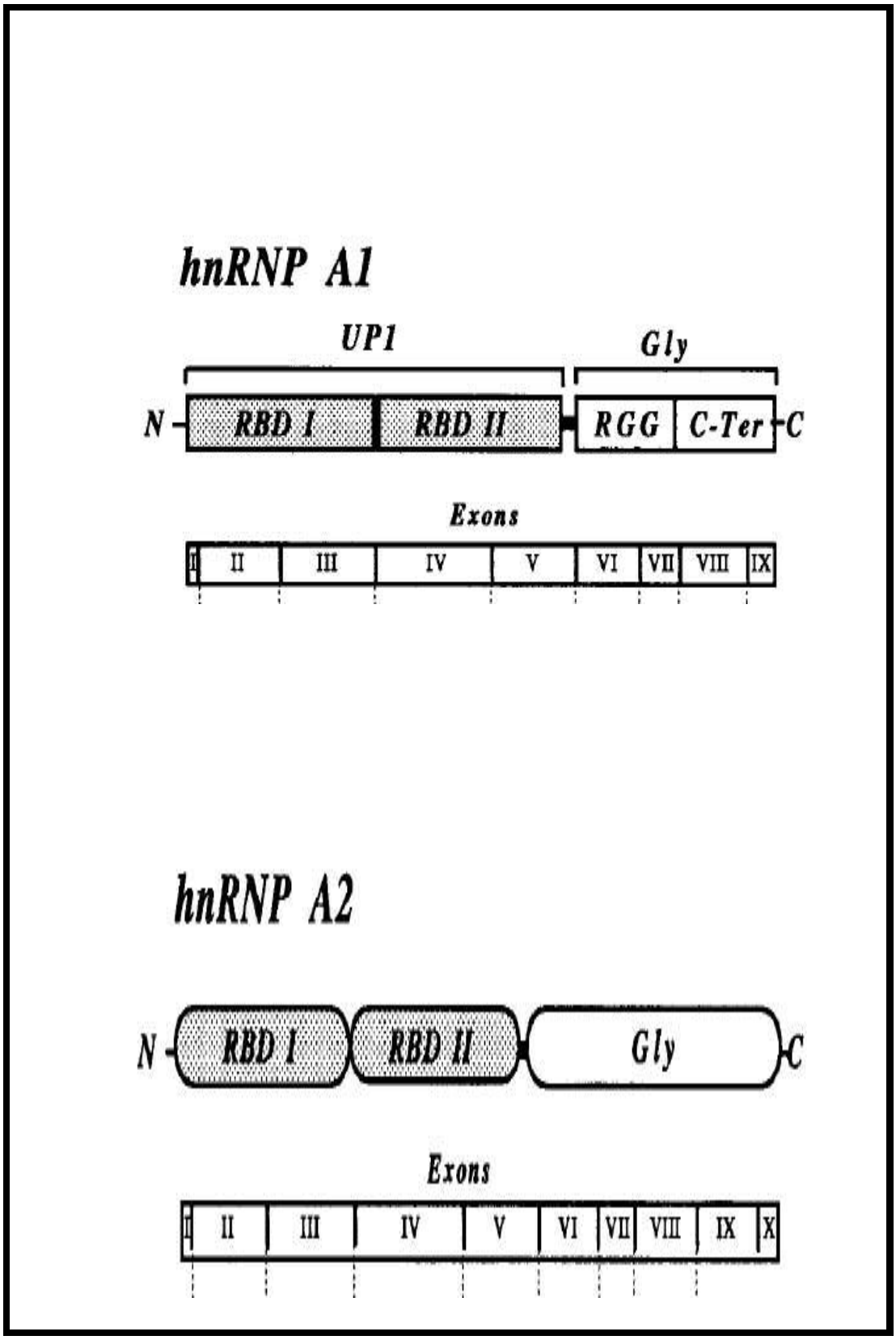


Heterogeneous nuclear ribonucleoproteins A1 and A2.

hnRNPs A1 and A2 are similar proteins that have a modular structure consisting of two tandemly arranged RNA binding domains (RBD) linked to a glycine-rich region at the carboxyl terminal. The RBD are highly conserved sequences while the auxiliary gly-domain can be variable. This suggests a role for the glycine-domain in specialization of function. Both genes have multiple isoforms through alternative splicing. The *HRNPA1* gene is found at chromosome 12q13.1 and encodes hnRNP A1 and hnRNP $A1^B$ proteins. The hnRNP A1 protein isoform excludes exon 7B and hnRNP $A1^B$ isoform includes the same exon, this splicing event is influenced by conserved elements in intron CE1 and CE6 (Blanchette 1999). Recent evidence for the presence of a single nucleotide polymorphism (SNP) in the 5' upstream promoter element of the gene suggests that the levels of hnRNPA1 expression may be higher in tissue from sporadic human colorectal cancers. 18 out of 30 cases expressed hnRNPA1 at greater than 2 fold levels compared with normal colon tissues, however the role of hnRNPA1 in this cancer is unknown (Ushigome 2005). The *HRNPA2/B1* gene is found at chromosome 7p15 and encodes hnRNP A2, hnRNP B1 and hnRNP B2 proteins. In addition, hnRNPA1 can be proteolytically cleaved to give UP1 (made of the two RNA binding domains only) (Biamonti 1994). hnRNP A1 also carries a 38 amino acid nuclear shuttling signal called M9, near the C-terminal that facilitates its movement to and from the nucleus.

hnRNP A1 modulates alternative splicing by favoring distal 5' splice site selection and through exon skipping/inclusion (Yang 1994). hnRNPA1 may also have a role in 3' splice site usage (Bai 1999).

Figure 4. Schematic representation of hnRNPA1 and hnRNPA2 proteins and genes structure. There is much similarity between these two proteins and their genes. Both encode more than one mRNA isoform and protein (from Krecic 1999). The figure depicts the major isoforms of each protein hnRNPA1 and hnRNPA2, both have two RNA binding domains (RBD) and within each domain there are two RNA recognition motifs (RRM). The C-termini of both proteins are Glycine rich domains. The M9 nuclear shuttling sequence is present within this domain in hnRNP A1.


hnRNP A1
UP1
Gly
N
RBD I
RBD II
RGG
C-Ter
C
Exons
I
II
III
IV
V
VI
VII
VIII
IX
hnRNP A2
N
RBD I
RBD II
Gly
C
Exons
I
II
III
IV
V
VI
VII
VIII
IX
X

hnRNPA1 function.

The ratio of splicing factors SF2/ASF relative to hnRNPA1 controls 5' splice site selection. It appears that hnRNP A1 may be responsive to stress in human fibroblasts after exposure to osmotic shock or UV irradiation. hnRNP A1 rapidly exits the nucleus and accumulates in the cytoplasm (VanOordt 2000). hnRNP B1 also relocalizes to the cytoplasm whereas other hnRNPs remain nuclear. This redistribution is mediated by phosphorylation of hnRNPA1 through the classic stress response pathway involving MKK $_{3/6}$ and p38 MAPK (Misteli 2000 and VanOordt 2000). The effect is a specific stress-driven response as mitogenic activators do not elicit the same re-localization response. The redistribution of hnRNPA1 into the cytoplasm was able to affect 5' splice site usage of the adenoviral E1A pre-mRNA reporter construct as expected when hnRNPA1 is titrated away from the nucleus.

Another function of hnRNP A1 is binding to DNA and RNA, especially telomeric sequences as described above (Eversole 2000). Protein modification by methylation of hnRNPA1 at specific arginine residues can affect the ability of hnRNPA1 to bind RNA (Kim 1998). Interestingly, hnRNPA1 appears to have a role in cytoplasmic transcription and replication of a cytoplasmic RNA virus, mouse hepatitis virus (MHV) (Shi 2000). In a study on viral mRNA transport, a dominant-negative hnRNPA1 mutant was used that is it lacked the nuclear transport domain, and was able to cause global inhibition of viral transcription and replication. hnRNPA1 may also be able to influence 3'splice site decisions. In a study using the calcitonin gene construct (Bai 1999), it was found that the over-

expression of the SR protein ASF/SF2 caused the increased usage of a proximal 3' splice site and this was antagonized by the co-transfection of an hnRNPA1 plasmid. hnRNPA1 protein can inhibit both 5' and 3' splice site decisions and may achieve this by different mechanisms depending on the mRNA species and availability of other splicing factors.

hnRNP A1 can participate in the splicing of CD44, a transmembrane glycoprotein whose splice variants have been implicated in tumor progression (Blencowe 2000 and Matter 2000). hnRNPA1 is capable of interacting with regulatory splice elements in CD44 exon v5. hnRNPA1 over expression resulted in exon skipping which could be reversed by co-expression of oncogenic forms of the small G proteins p21Ras and cdc42. In mouse cells, cdc42 is required for ras signaling. If hnRNPA1 does function in a coordinate complex with these proteins, then this would be the first evidence of a link between cell growth signaling pathways and hnRNPA1. In my study I hope to investigate if hnRNPA1 and/or A2 have a direct role in mediating cell cycle and growth control through the INK4a locus. Zhu 2002, published that the ratios of $p16^{INK4a}/p14^{ARF}$ mRNA using over expression of hnRNPA1 and A2 in HDF show differential regulation. It was also shown that the localization of both hnRNPA1 and hnRNPA2 are altered in senescent fibroblasts, see Figure 5. Since specific splicing factors like SF2 and SC35 promote proximal 5' splice site usage antagonistically with hnRNPA1 levels, all these studies necessitate inclusion of comparisons of these antagonists (Inoue 1994 and Blencowe 2000).

Splicing factor and hnRNP A1 and A2 expression patterns.

It is believed that gene regulation mediated by altered levels of splicing regulatory proteins may be responsible for changes in gene expression. It is now known that over expression of hnRNP A2/B1 is an early biomarker of lung cancer (Sato H 2000). In another study, relative levels of hnRNPA1/$A1^B$ to ASF/SF2 were investigated in myometrium of the pregnant human uterus. It appears that there are substantial differences in expression patterns in the upper and lower uterine segments and this may be related to specific myometrial contractile protein expression as in the case of adenylyl stimulatory G protein $G_s\alpha$ (Pollard 2000).

Changes in ratios of SR protein ASF/SF2 to hnRNPA1 are seen in different cell types in mouse tissues (Elliott 2000). In the testes of mouse, hnRNPA1 is expressed only in nuclei of spermatogonia and Sertoli cells, not in later stages of germ cell development (Venables 1999).

Figure 5. Cellular localization pattern of hnRNPA1 and hnRNPA2 are altered in senescent fibroblasts. In situ localization of hnRNP A1 and A2 proteins in HS74 human diploid fibroblasts. Cellular localization studies of hnRNPA1 and A2 were done using 4B10 and 10D1 monoclonal antibodies, respectively. All fields are at a magnification of 400X. The localization pattern of hnRNPA1 appears to be exclusively nuclear in young cells, as compared with senescence in which the protein is spread in the cytoplasm. (From Zhu 2002)

Figure 5. Localization of hnRNPA1 and A2 are altered in senescent human diploid fibroblasts.

Silencing of hnRNP A1 and A2: effects on growth of cells.

There has been much interest in the role of splicing in gene regulation of cell cycle and cancer-related pathways. The levels of various protein components of alternative splicing complexes are critical to effects on alternative splicing and it has been the goal of many researchers to perturb levels of these components in ways that will be informative to disease-specific patterns of post-transcriptional gene regulation (Kashima 2003). RNA silencing has offered a powerful tool for performing studies where the effects of lack of specific mRNA leads to dramatic down-regulation in protein levels without altering the parent genomic DNA sequences of the gene. Additionally, silencing of RNA (using small interfering RNA called siRNA) is capable of maintaining its effect for generations and this is especially useful when studying the effects of down-regulation of genes on cell growth characteristics (Meister 2004, Elbashir 2002, 2001). In the case of hnRNP A1 and hnRNP A2, silencing by siRNA in human transformed cells and normal human fibroblast strains, it was observed that the combined knockdown of hnRNP A1 and hnRNP A2 in human transformed cells promotes apoptosis in a time-dependent manner. In contrast, a similar reduction in hnRNP A1 and hnRNP A2 expression in normal human cell strains did not induce cell death (Patry 2003, 2004). Furthermore, a variety of mouse cell lines displayed the same effect and were resistant to cell death in the absence of mouse hnRNP A1 and hnRNP A2. hnRNP A1 and hnRNP A2 are believed to have a role in regulating cell growth characteristics when silenced at the same time, but when individually silenced do not have the same degree of effect on cell death. This is indicative of independent

roles of hnRNP A1 and hnRNPA2 in normal cell metabolism, which is interesting to our study as we focus on the role of hnRNP A1 protein effects on gene expression and it would be interesting to enquire whether the same genes are targeted using hnRNP A2. Studies on Colo16 carcinoma cells and HaCat immortalized keratinocytes, identified a role for hnRNP A2 specifically in negatively regulating cell growth without causing cells to die. Therefore, hnRNP A2 may be more important for cell proliferation in immortalized cells than hnRNPA1, in a cell-type dependent manner (Yaowu 2005). Another example of a splice site decision affected by levels of hnRNPA1 is the *SMN1* gene that encodes a survival motor neuron 1 protein, that causes the neurodegenerative disease spinal muscular atrophy (SMA) in humans. The homozygous loss of this gene is the genetic cause of this disease but there is another nearly identical gene *SMN2* that produces a functional protein but at levels insufficient to compensate for loss of SMN1. The SMN2 gene has a single nucleotide transition C/T in exon 7 and this change is sufficient to prevent efficient exon 7 splicing. This change appears to create an exon splicing silencer sequence in exon 7 that can be recognized by hnRNPA1 that represses its splicing and leads to exclusion of the exon. This was shown to be regulated by manipulating levels of splicing factors, by the use of siRNA against hnRNPA1 and hnRNPA2 which showed that hnRNPA1 was more effective at causing the exon to be excluded than hnRNPA2 (Yaowu 2005). In all, these studies lead us to believe that there is an essential and varied role for hnRNPA1 and hnRNPA2 in cells across species and cell-type. In fact, the simplicity with which an exon splicing event can be performed or

prevented as in the case of *SMN2* exon 7 (Yaowu 2005) to change gene expression patterns in significant way, highlights the importance of competition between negative regulators of splicing such as hnRNPA1 and positive regulators of splicing such as ASF/SF2 (Zhu 2001).

Senescence and cell cycle control.

The cell cycle is regulated by checkpoints at every stage to ensure integrity and continuity of cell growth. At the molecular level there is a sensitive relationship between genes, their products and transduction of outside signals. These internal monitors are the result of genetic regulation of specific pathways that exist in cells throughout their life. Breakdown in these pathways leads to abnormal cell growth. Senescence is believed to be a natural stage in the life of all somatic cells *in vivo* and primary cells in culture. It is an inevitable consequence of repeated cell division resulting in cells that do not divide or die, not unlike differentiated cells in vivo. It is believed that onset of this non-replicative stage in normal cells is preceded by an event of growth arrest in G1 or G2 called G0 arrest (Crompton 1997 and Sherr 2000). In human diploid fibroblasts there has been extensive study of cell cycle related molecular mechanisms. Primarily the role of cyclins, CDK (cyclin dependent kinases), CDKI (cyclin dependent kinase inhibitors), TS (tumor suppressors), oncogenes, DNA and RNA metabolism regulatory proteins have been studied and in the more recent literature, signal transduction mediators. In the case of HDF, a two-stage senescence mechanism is recognized based on CDKIs of CIP (CDK Inhibitor protein) and INK4a (inhibitor of CDK4a) families. p21 $^{WAF1/SDI1/CIP1}$ is a CIP member and is induced

in epithelial cells as they approach senescence causing the cells to arrest in G0 (Stein 1998). Subsequently, cellular $p16^{INK4a}$ expression is induced and $p21^{WAF1/SDI1/CIP1}$ levels decline. $p16^{NK4a}$ (an INK4a family member) is believed to be necessary for senescence maintenance in HDF. The up regulation of $p16^{INK4a}$ is believed to be a key event in HDF senescence onset and maintenance (Serrano 2000). Expression of $p21^{WAF1/SDI1/CIP1}$ can be induced by p53 and in the levels of p53 are also high prior to senescence. Although $p21^{WAF1/SDI1/CIP1}$ levels fall during senescence, cells that overcome this checkpoint continue to express $p21^{WAF1/SDI1/CIP1}$ throughout their extended life span. The level of $p21^{WAF1/SDI1/CIP1}$ is at a maximum in cells at culture crisis, when cells start dying, and is lowered only in event of immortalization. It is interesting and relevant to this work that a recent study on p53 alternative splicing into an amino-terminus deleted isoform called p47 (Ghosh 2004) that can inhibit p53 mediated cell growth arrest via p53 ubiquitination and cell localization patterns. Figure 4 summarizes the pRb and p53 pathways to cause growth arrest in response to hyperproliferative signals and DNA damage.

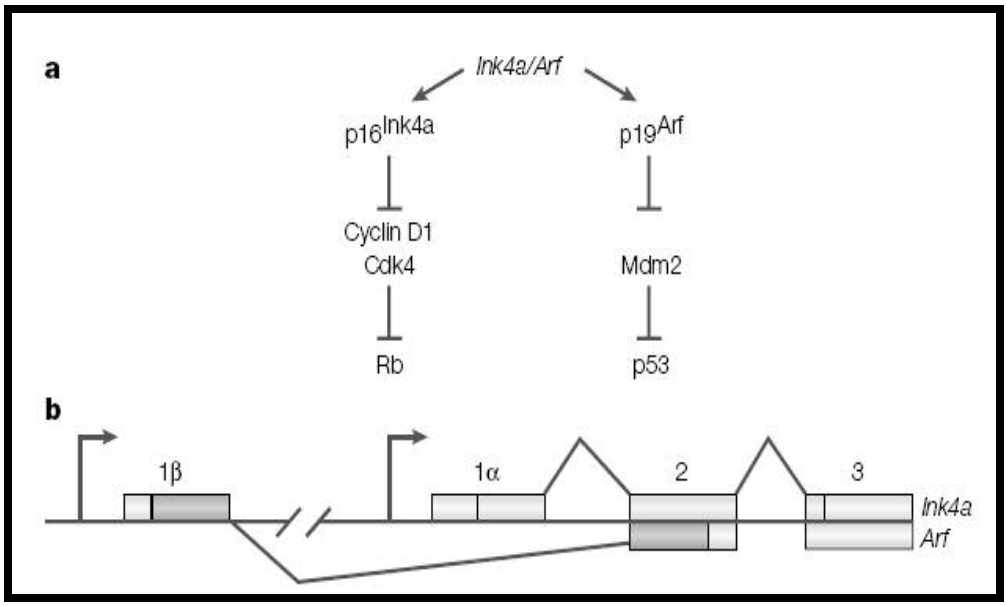
The *Ink4a/Arf* locus.

This locus was identified by genetic analyses in familial melanoma kindreds that established a link to the tumor suppressor at 9p21, a chromosomal hotspot (Chin 1998). The unique gene structure at this locus generates two distinct ORF (open reading frames) that initiate in different first exons and continue in alternative reading frames in a common second exon. Use of common RNA

sequences to encode completely different proteins is unusual regulation of a mammalian gene (Sharpless 1999).

The two products of the mouse *Ink4a/Arf* locus, p16Ink4a and $p19^{ARF}$ ($p14^{ARF}$ in human) indirectly regulate the retinoblastoma protein pRb and p53, respectively. Alternative first exons (1α and 1β) that are transcribed from different promoters specify the 5' ends of the *Ink4a* and *Arf* transcripts, respectively. These are spliced to the same acceptor site in exon 2, which is translated in alternative frames. *Ink4a* coding sequences in exons 1α, 2 and 3, and *Arf* coding sequences in exons 1β and 2 (Sherr 2001).

Figure 6. Cell cycle control by tumor suppressors and oncogenes and the *Ink4a/Arf* locus. a. The two products of the mouse *Ink4a/Arf* locus, $p16^{Ink4a}$ and $p19^{ARF}$ ($p14^{ARF}$ in human) indirectly regulate the retinoblastoma protein (Rb) and p53,respectively. b. Alternative first exons (1α and 1β) that are transcribed from different promoters(arrows) specify the 5' ends of the *Ink4a* and *Arf* transcripts, respectively. These are spliced to the same acceptor site in exon 2, which is translated in alternative frames. *Ink4a* coding sequences in exons 1α, 2 and 3 are denoted by light shading, and *Arf* coding sequences in exons 1β and 2 are indicated by dark blue shading. The schematic is not drawn to scale, and in both the human and mouse genomes, exons 1α and 1β are separated by >15 kb (Sherr 2001).



It appears that through protein-protein interactions and resultant gene expression these cell cycle regulators should be able to shut down cell cycle progress completely when signaled to do so. How these mechanisms are initiated and maintained is the next question. Stimuli that cause growth arrest of cells can potentially be triggers for cell senescence also. In senescence, the event of growth arrest is believed to be due to differential regulation of a number of cell cycle genes but the signals that trigger these changes have not yet been defined. The irreversible nature of this growth arrest lends credit to the hypothesis that senescence onset and maintenance are preceded by multiple events involving cell cycle changes and dramatic dysregulation of other pathways.

Studies of cells in which cell cycle checkpoints are abrogated show that they are capable of overcoming or bypassing senescence (Brummelkamp 2002). Human tumor viruses can inactivate p53 and pRb to prevent senescence and lead cells to crisis/ genetic catastrophe/M2 (mortality checkpoint 2). The cells that overcome senescence temporarily continue to lose telomere length. Thus, telomere maintenance is not necessary for escape from senescence in the case of viral transformation. Deletion of $p21^{WAF1/CIP1/SDI1}$ in human fibroblasts prevents onset of senescence. The INK4A locus produces $p16^{INK4a}$ and $p14^{ARF}$, growth inhibitor proteins that serve to maintain active p53 and pRb. Both $p16^{INK4a}$ and $p14^{ARF}$ expression increase during senescence (Crompton 1997). Constitutively active *ras* has been shown to induce senescence in primary human fibroblasts and this correlates with the production of $p16^{INK4a}$ and p53 (Sharpless 1999).

In a study to identify negative regulators of the INK4a proteins , a gene determining chromatin structure in mammals called *bmi-1* encodes a protein that has been proposed as a potential inhibitor of $p16^{INK4a}$ (Jacobs 1999). Mice that are *bmi-1*$^{-/-}$ have developmental defects of the lymphoid and nervous systems. Cells from these animals senesce prematurely in vitro. Animals carrying *bmi-1* mutations were crossed with animals having disruptions at their INK4a/ARF locus. Cells from these animals did not undergo senescence and it is believed that this gene needs INK4a gene products for its activity. There are some implications for Bmi-1 as a chromatin repressor which could be a mechanism for regulation at the INK4a /ARF locus (Jacobs 2000). Another study has used this ability of *bmi-1* lacking cells to senesce in culture in a cDNA library screen for senescence bypass (Jacobs 2000). A cDNA was selected, cloned and identified as TBX2. In these cells levels of $p16^{INK4a}$ and $p19^{ARF}$ are lowered. This is attributed to lack of function of the protein TBX2. This gene is a preferential inhibitor of $p19^{ARF}$ as TBX2-immortalized mouse embryonic fibroblasts have levels of $p16^{INK4a}$ that are comparable to those in senescent cells.

Not much is known about transcriptional induction of this locus and intermediate regulatory processes in formation of the alternative protein products. It is known that the gene is methylated and this event can be transcriptionally repressive (Iida 2000). Also E2F1 transcription factor activates $p14^{ARF}$ and can induce growth arrest in the presence of p53 (Dimri 2000). From the nature of splice site decision it appears as if hnRNPA1 might have a role in splicing regulation. This is under investigation in our laboratory. In general, study of

regulation events prior to the formation of active protein is required to expand our knowledge of these two gene products as tumor suppressors and as growth arrest genes.

Senescence and cell death.

Cells can die by necrosis or apoptosis. Apoptosis requires an extensive set up of genetically co-ordinated pathways and cells must be committed to apoptosis to activate the process. Cells recognize external and /or internal signals to trigger these events in a step-wise and highly regulated manner. Senescent cells are believed to be resistant to apoptotic signals. This has been observed in keratinocytes and human diploid fibroblast model systems. It is believed that senescence, in addition to being an anti-proliferative mechanism and a potential tumor suppressive mechanism may also make cells resistant to apoptosis. The relationship between senescence and apoptosis, however, is very difficult to investigate as senescent cells are already in a state of dys-regulation. Also, depending on the cell type, different signals can be recognized as apoptotic which makes the issue of senescent cell response to these signals difficult to characterize. A case in point are the studies on keratinocytes compared for apoptosis response at different ages in their life- span, which showed that senescent cells and those that underwent growth arrest were resistant to apoptotic signals by UV irradiation (Chaturvedi 1999). Other studies on human diploid fibroblasts show that senescent cells may undergo apoptosis when death inducing signals are used to trigger cell death (Dejesus 2002, Tepper 2000). Another study using lymphoid cells from Werner syndrome patients showed that these

cells in culture are not susceptible to FasL induced apoptosis (Wu 1998). Werner syndrome is due to a defect in a helicase and is manifested as a premature aging syndrome.

Mechanism of apoptosis and major players.

Apoptosis requires initiation, commitment and execution. The cellular participants of apoptosis can be classified accordingly. One of the most important molecular components of apoptosis are the caspases, a group of enzymes that have cysteine protease activity and form a multi gene family in mammals. They cleave specific targets, some of which are downstream caspases themselves. They are zymogens that undergo a protein cleavage event to form the active enzyme. The role of caspases in cell death can be initiation or execution of proteolytic events. Expression of caspase-1 induces apoptosis in rat fibroblasts and this can be inhibited by bcl-2 that has anti-apoptotic function and crmA, a serpin that specifically inhibits some members of the caspase family (Miura 1997). Caspase–2/ NEDD 2/ Ich-1 (Interleukin–1β converting enzyme homology protein 1) can induce apoptosis but its gene also encodes an anti-apoptotic mRNA isoform called caspase-2L. This is because the caspase-2 pre-mRNA is alternatively spliced into two forms. One mRNA species (short form) encodes a longer polypeptide of 435 amino acid residues called caspase-2L. This form is homologous to caspase-1 and CED-3 (the death executioner of *Caenorhabditis elegans*) . An exon inclusion event, or alternative 5' splice site usage results in a polypeptide of 312 amino acids residues called caspase-2S from the other mRNA. This form terminates 21 amino acid residues after the pentapeptide QACRG that

is the signal for cleavage by caspases. Caspase-2 appears to have autolytic capability, that is it can cleave other procaspase-2 molecules (Miura 1997).

Another gene that undergoes alternative splicing to give multiple protein products is the *bcl-x* gene (Reed 1999). This is a member of the bcl-2 family of genes that are important regulators of apoptosis. They normally control various aspects of apoptosis, however, alterations in their expression are associated with diseases. Bcl-x is over expressed in several common types of cancer. The gene has a three-exon structure . An alternative 5' splice site in exon 2 can be used to generate the alternative isoform Bcl-xS. The two products are Bcl-xL of 241 amino acids (an anti-apoptotic protein) and Bcl-xS of 178 amino acids (a pro-apoptotic protein). It is not clear how the pro-apoptotic Bcl-xS functions but it has been proposed that it hinders the ability of Bcl-xL to interact with other Bcl-2 family proteins. Fas (CD95), the membrane bound receptor for FasL, can be alternatively spliced to produce Fas(ΔTM), that lacks the transmembrane domain, causing it to be secreted rather than inserted into the plasma membrane. Fas(ΔTM) acts as a soluble decoy for FasL and reduces the cells ability to respond to the death signal. Increased Fas (ΔTM) mRNA have been reported in the autoimmune disease lupus, in which T-cells become resistant to apoptotic signals (Reed 1999).

Using anti-sense RNA against various mRNA has shown that post-transcriptional processing is a powerful method of controlling alternate formation of proteins that ultimately have a role in deciding cell fate. A study of caspase-2 gene regulation at the level of alternative splicing showed that hnRNPA1 levels

relative to SC35 (a general splicing regulator and functional antagonist of hnRNPA1) could affect splicing of caspase-2 pre-mRNA in HeLa cells. As expected from the structure of the gene higher hnRNPA1 levels through over expression promoted exon inclusion and increased the ratio of caspase-2S to 2L (preference for anti-apoptotic form) (Kumar 1999). Higher levels of SC35 promoted exon exclusion and decreased the ratio of caspase-2S to 2L (preference for the pro-apoptotic form). It could be argued that higher hnRNPA1 levels may affect mRNA transport efficiency and mRNA stability that also contribute to the differences in caspase-2 mRNA levels. The effect of over expression of hnRNPA1 in HeLa cells should not significantly change RNA metabolism in these cells as hnRNPA1 is already highly abundant in these cells (Kumar 1999). hnRNPs represent an entirely new class of proteins that can regulate apoptosis, in addition to those directly involved in the cell death machinery.

Extra-cellular Matrix (ECM) and aging.

The ECM is a macromolecular network which surrounds cells in tissues and is known to be organized by surrounding cells in a genetically determined manner that is environmentally dependent and is specific to the cells that secrete the material. The secretion and composition of extra-cellular matrix varies with age and developmental stage of the surrounding cells. The role of ECM in the remodeling of tissues can be disease-specific and age-related. One of the most important macromolecular components of the ECM is fibronectin which has multiple roles in the arrangement and interactions at the cell surface. In particular, fibronectin is believed to be altered in expression and behavior as tissues age and

the expression of fibronectin protein is believed to be under regulation at the post-transcriptional level in an age-related manner. There is some evidence for the role of gene expression of fibronectin in aging skin fibroblast cell cultures where there was an observed increase in the fibronectin transcript produced as cells age. The observed increase in gene expression of fibronectin in dermal connective tissue may also be induced by environmental conditions such as UV exposure and sunlight, suggesting a protective role for the protein in such tissues (reviewed in Labat-Robert 2004). In addition, there is evidence of increased fibronectin expression at both the protein and mRNA level in senescent cultured fibroblasts and Werner syndrome fibroblasts. It has also been suggested that the role of proteins such as fibronectins in aged tissues is different and more detrimental to tissue integrity through the secretion of fibronectin into the ECM accompanied by the up-regulation of enzymes such as matrix metalloproteinases that may act in a pro-inflammatory manner. This has led to the investigation of differential gene expression of such enzymes and fibronectin in mouse models to define the role if any of these ECM components in tissues as they age (Muro 2003).

Fibronectin (FN) and alternative splicing

Fibronectin is a large extra-cellular protein that is proposed to have a role in cellular signaling. It is a ubiquitous and abundant extra-cellular matrix protein (ECM) that is secreted by cells as a soluble dimer that is subsequently assembled into an insoluble matrix at the cell surface. The fibrils that form are a result of arrangements and interactions between fibronectin and integrins at the cell surface. Fibronectin fibril are made up of several protein forms that differ in both

their primary structure and post-translational modifications. The amino acid sequence variations are the consequence of alternative splicing of fibronectin pre-mRNA at three sites. These are extra domain B (EDB) or extra type III homology B (EIIIB or EDII), extra domain A (EDA or EIIIA or EDI) and type III homologies connecting segment (IIICS or the V region in rodents). The EDA and EDB exons are single exons that code for other type III repeats in the fibronectin protein, reviewed in Muro 2003, see Figure 1.2 for structure of the fibronectin gene and alternatively spliced exons. The IIICS region undergoes more complicated splicing that is species dependent and in humans there are more than 20 different isoforms that are generated after splicing. There are two forms of fibronectin that are produced from the fibronectin gene, a plasma form called pFN that is soluble and dimeric, but lacks both EDA and EDB domains. This is a secreted form of fibronectin that is found in the bloodstream and is synthesized by hepatocytes. Cellular fibronectin called cFN is also dimeric but can be cross-linked in a multimeric form and the proportion of EDA and EDB containing molecules differ in various tissues (Muro 2003). The amount of fibronectin produced by cells and secreted varies with species, age, developmental stage and disease. In fact, the EDA- and EDB- forms of fibronectin are more abundant as cells age and in a study to compare the exclusion of these two exons it was found that the EDB exon appears to be excluded more often (Caputi 1995). Using gene targeting, Muro and colleagues have generated a mouse strain that constitutively includes EDA exon, which caused a major decrease in the amount of FN in all tissues. However, mice without the EDA exon showed abnormal wound healing.

Both mutant types had significantly shorter life spans than wild-type fibronectin strains and this indicated that the normal splicing regulation of fibronectin may have a role in the development and maintenance of biological functions in mice (Muro 2003, Chauhan 2003). In human fibroblast cells there are age-related changes in ability of fibronectin to promote cell adhesion (Magnuson 1992). In addition, there are changes in alternative splicing that are associated with age and growth arrest due to serum deprivation. It was found that the fibronectin produced by senescent cells is slightly smaller than fibronectin produced from young cells, indicating that there is a decrease in exon inclusion in these forms (Magnuson 1992).

The goal of this study was to investigate the role, if any, of heterogeneous nuclear ribonucleoprotein A1 as a representative mRNA processing regulatory protein and establish that gene expression in aging cells is powerfully modulated by such regulatory mechanisms. We investigated specific gene expression patterns of caspase-2 and fibronectin as they are proteins that express multiple isoforms, are involved in cell growth and aging mechanisms and are potential substrates for hnRNPA1 regulation. We then proceeded to broaden this study using microarrays to establish gene expression profiles of senescent fibroblasts and also cells that over-express hnRNPA1. Eventually it is our goal, to compare between these profiles age-specific genes that indicate mRNA processing is a significant mechanism to regulate gene expression in aging. We also anticipated that there may be differences in specific target RNA for hnRNP A1 binding in young and old fibroblasts which if critical to cell growth regulation, would

indicate direct substrates for hnRNPA1 activity. Changes in the RNA sub-populations that are available for hnRNPA1 recognition and that are capable of binding hnRNP A1 could elucidate the role of this protein as a significant player in aging mechanisms and more importantly set the stage for individual mRNA processing factors in onset and maintenance of senescence.

Materials and Methods

1. Cell culture, transfection, harvesting of cells for RNA and protein.

HS74 (Human fetal bone marrow fibroblasts), IMR-90 (Human Lung fibroblasts) and WI-38 (Human fetal lung fibroblasts) are human diploid fibroblasts that conform to the Hayflick model of cell growth in culture and reach senescence between 60-65 population doublings (Hayflick 1961). Cell cultures were maintained in DMEM-HAM F10 (Invitrogen) in 1:1 proportion, supplemented with $NaHCO_3$, 1% penicillin- streptomycin (Invitrogen) and 10 % Fetal Bovine Serum (Sigma, JRH) at 5% CO_2 at 37°C in a humid incubator. Media was replaced twice weekly for young cells subcultivated at 1 : 5 per passage and more often for late passage and senescent cells (Hubbard 1997). Cells were also cryogenically preserved to achieve continuity of raw materials for all experiments. Cells were seeded at low density of $2.5x10^5$ cells per 35mm dish or multi-well plate when required for in situ assays e.g. TUNEL (FragEL™ DNA Fragmentation Detection Kit, Colorimetric - TdT Enzyme, EMD Biosciences, USA). G0 arrested cells were kept in 0 -1 % FBS for 72 hrs prior to harvesting. All comparisons included G0 arrested cells in order to assess changes that were observed through these experiments as senescence-specific. Some investigators choose to use serum starved HDF as senescent, others even use extended life-span HDF on the premise that the changes that mediate senescence in culture are extended and maintained beyond that stage (Shelton 1999). In our protocol, senescent fibroblasts were harvested after maintaining them on plates for >6 weeks without acheiving confluence. Transfections were performed on young log

phase fibroblasts (Zhu 2002) using Lipofectamine™ (Invitrogen) protocol. In this method cells were washed in DMEM- HAM F10 medium without antibiotics and fetal bovine serum. Plasmids for over-expression of hnRNPA1 and A2 were pEGFP-A1 (fusion protein of GFP and hnRNPA1 under the CMV promoter) and pEGFP-A2 (fusion protein of GFP and hnRNPA2 under the CMV promoter) and pEGFP alone as control vector (Zhu 2002). 10 μg of plasmid vector was mixed with Lipofectamine reagent in serum-free, antibiotic-free media for 45 minutes then poured over the cells in a total volume of 8 mL media. The cells were incubated at 37°C for 6 hours for transfection to occur and then the media was replaced with 1% antibiotic, 10% FBS containing medium. RNA was isolated 48 hours post-transfection and cells were harvested for protein at 72 hours post-transfection by standard protocols (Sambrook 1989). RNA was isolated using Fenozol (Active Motif, CA) a one-step isolation procedure. RNA was quantified by spectrophotometry and OD 260nm/280nm ratios were checked to ensure RNA quality. RNA was then stored in water at -80°C.

2. RNA isolation and RNA-PCR to check for genomic contamination.

After RNA was isolated as detailed above, in order to ensure that there was no genomic contamination, an RNA – PCR procedure was performed. 2μL of template RNA was added to a PCR mixture using β-actin primers which would detect genomic sequence if present. The total PCR reaction composed of 2 μl of template RNA, 5 μl of 10 X RT-PCR Buffer (100 mM Tris-HCl, pH 8.3, 500mM KCl,15 mM $MgCl_2$), 2.5 μl of dNTP mix (2.5 mM each dNTP), 0.25 μM each PCR primer, 0.5 unit of Thermostable DNA Polymerase (Novagen) was run at

50°C annealing for 20 cycles of PCR extension. The complete PCR volume was loaded onto a 1% agarose gel with 0.5 μg/mL ethidium bromide (Sambrook 1989). The reactions were electrophoresed until the molecular marker was separated and the gel checked for absence of any PCR products. If the reaction did not show any products, the RNA sample was considered free of genomic DNA contamination.

3. TUNEL assay protocol.

Cells were plated at a seed density of 2 X 10^5 cells per mL and then treated with okadaic acid (50 mM) and Tumor necrosis factor α (10 mg/mL), for 24 hours prior to fixation. Cells were washed three times with 1X PBS at room temperature and then fixed using 4% para-formaldehyde. Cells were permeablized using 0.1% Triton X-100 in 0.1% sodium citrate for 2 minutes. The following steps were according to the instructions from EMD Biosciences (FragEL™ DNA Fragmentation Detection Kit, Colorimetric-TdT Enzyme (EMD Biosciences, USA). Endogenous peroxidases were inactivated using a 30% solution of hydrogen peroxide in methanol. Terminal deoxynucleotidyl transferase (TdT) binds to exposed ends of DNA fragments generated in response to apoptotic signals and catalyzes the template-dependent addition of biotin-labeled and unlabeled deoxynucleotides. The cells were incubated with a reaction mix containing TdT enzyme. Biotinylated nucleotides are detected using streptavidin-horseradish peroxidase (HRP) conjugate present in the labeling mix. The reaction was stopped using 0.5 M EDTA at pH 8.0. The coverslip was blocked using 4% BSA in PBS and then labeled with the conjugate containing peroxidase

streptavidin for 30 minutes at room temperature. DAB was added to give the color reaction as diaminobenzidine reacts with the labeled sample to generate an insoluble colored product at the site of DNA fragmentation. Incubation with DAB-hydrogen peroxide solution was carried out for 15 minutes and then the coverslips were counterstained for visualization. Counterstaining with methyl green aids in the morphological evaluation and characterization of normal and apoptotic cells (EMD Biosciences).

3. RT-PCR protocols.

RNA isolation was done according to the one-step protocol of Chomczynski and Sacchi (1987) with modifications. Cells from different cells lines and at specified population doubling levels were lysed in 4.0 M guanidinium thiocyanate, 0.5 % Sarkosyl, 0.7 % β-mercaptoethanol (guanidinium thiocyanate prevents RNase activity). RNA was separated from DNA and protein by phenol : chloroform extraction. RNA is precipitated with isopropyl alcohol and stored at –80°C.

Reverse-transcription reaction. Each RT-driven cDNA reaction (Sambrook 1987) was set up as follows: 5 μg template RNA, 4 μl dNTP mix (2.5 mM each dATP, dCTP, dGTP and dTTP), 2 μl of 50 μM first strand primers (either random decamers or oligo $d(T)_{18}$), 3 μl of Reverse transcriptase buffer /Alternate 10X first-strand buffer (500 mM Tris-HCl, pH 8.3/ 750 mM KCl/ 30 mM $MgCl_2$/ 50 mM DTT), 1 μl of 10 units /μl Placental RNase Inhibitor, 1 μl of 100 units/μl Moloney-Murine Leukemia Virus reverse transcriptase. Reaction mix was pre-incubated for 3 minutes at 80°C to denature before addition of RNase Inhibitor

and Reverse Transcriptase. The reverse transcription reactions was carried out for 1 hour at 42°C and then heated to 95°C for 10 minutes to terminate the reaction. Samples was cooled to 4°C for regular PCR amplification or stored at -20°C until needed.

Polymerase Chain Reaction. In order to compare the levels of β-actin, c-fos, c-myc, hnRNP A1, hnRNPA2, $p14^{ARF}$, $p16^{ARF}$, $p21^{WAF1/CIP1/SDI1}$, Ich-1 and SF2/ASF in young, G0 arrested, senescence, immortal, pEGFP vector only control, hnRNP A1 and hnRNP A2 over expressing cells, the cDNAs obtained by reverse transcription were used as templates in PCR (Sambrook 1987). In order to quantify abundance of mRNA in the amount of final PCR product, the amount of starting templates and PCR cycle number are mentioned in Table1 (see below). In order to normalize mRNA abundance a standard PCR for Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β–actin was included in each experimental set. Briefly, PCR reaction was performed in 50μl reaction volumes. Each reaction contained 2 μl of template cDNA, 5 μl of 10 X RT-PCR Buffer (100 mM Tris-HCl, pH 8.3, 500mM KCl, 15 mM $MgCl_2$), 2.5 μl of dNTP mix (2.5 mM each dNTP), 0.25 μM each PCR primer , 0.5 unit of Thermostable DNA Polymerase (Novagen). 10 μCi of deoxyadenosine 5'-triphosphate [α-^{32}P dATP] (NEN Products) was used when quantification was performed using radio-isotopes. Each PCR cycle consisted of a heat-denaturation step at 94°C for 30 seconds, a primer-annealing step at 50°C to 60°C for 30 seconds, and an extension at 72°C for 2 minutes. Primer sequences (forward and reverse), the PCR product sizes, amount of template cDNA (RT-product), annealing

temperature, and number of extension cycles are also listed in the Table 1 (see below):

Table1. PCR primers and conditions

Gene	Primer sequences (5' to 3')	Product size (Base pair)	cDNA	Annealing Temperature	#of PCR
ACTB	CGCCGCCCTAGGCACCA TTGGCCTTAGGGTTCAGGGGGG	243	2 μL	50°C	20
CFOS	GAATAAGATGGCTGCAGCCAAGTGC AAGGAAGACGTGTAAGCAGTGCAGC	698	3 μL	60°C	30
GAPDH	CGGAGTCAACGGATTTGGTCGTAT AGCCTTCTCCATGGTGGTGAAGAC	306	2 μL	50°C	20
HRNPA1	CTAAAGAGCCCGAACAGCTGAG TCAGTGTCTTCTTTAATGCCACCA	329	2 μL	55°C	20
HRNPA2	GGAACAGTTCCGTAAGCTCTTTAT GCCAAAGCCTCTTTTCTTTCCAG	415	2 μL	55°C	20
$p14^{ARF}$	CCGGCGACCGGCTGAGTGG CTGCGTCATGGACTGGACTGG	222	3 μL	55°C	20
$p16^{INK4A}$	CAACGCACCGAATAGTTACG CGTGTCCAGGAAGCCC	166	3 μL	50°C	30
$p21^{WAF1/CIP1}$	GCTAGCCATGGCAGAACCGGCTGGGGATGT CTCGAGGATCCTTAGGGCTTCCTCTTGGAG	500	2 μL	60°C	30
SF2/ASF	ACCAAGGACATTGAGGACGTG AACCACTCTGTTTTCAGACCG	288	2 μL	50°C	30
CASP2	ATGCTAACTGTCCAAGTCTA GTCCATCTTCATCAACTCC	200	2 μL	42°C	30
FN-HA-1 **FN-HA2**	AGAGCATAGACACTCACTTCA AAACAGAAATGACTATTGAAGGCTTG	604/334	2 μL	54°C	19
FN-HB1 **FN-HB2**	TAATATCAGAAAAGTCAATGCCAGTTG ATTACTGGTTATAGAATTACCACAACC	775/502	2 μL	54°C	19
FN-CS1	TTTAAAGCCTGATTCAGACATTCGTT	318-796	2 μL	54°C	19
FN-CS2 **FN-CS4**	GGGAACCGAATATACAATTTATGTCA TAGTCTACATCTTCCCTGGG	318-796	2 μL	54°C	19

Gel analysis of PCR products. The entire reaction volume was electrophoresed using agarose gels and visualized with ethidium bromide at 0.5 μg/mL. Alternatively, samples were electrophoresed on 5% polyacrylamide gels and visualized and quantified with the SYBR™ green stain. SYBR™ green can be visualized by Blue fluorescence (Molecular Dynamics, Amersham) and quantified with ImageQuant software (Amersham). The abundance of each mRNA was determined by comparing the ratio of signals between the amplified target cDNA and internal control (GAPDH or β-actin).

4. RNase Protection Assay.

The ribonuclease protection assay allows us to detect and quantify specific mRNA (Sambrook 1989). It is a highly sensitive and specific method and this method is more sensitive to levels of mRNA for apoptosis genes as they are usually expressed at very low steady state levels in cells. The template sets were purchased from BD Pharmingen and they included selected apoptosis gene sequence sets. The first template set hAPO-1b containing the specific sequences for caspases1, 2 (Ich-1S and L), 3,5,6,7,8,9, and Granzyme B and template set hAPO-2b containing specific sequences for bcl-W, bcl-x (S), bcl-X (L), bfl1, BID, bik, bak, bax, bcl-2 and mcl1 . In addition all template sets include internal control templates for the L32 (large ribosomal subunit RNA) and GAPDH. The templates were used to generate a T7- RNA polymerase-driven, $\alpha^{32}P$ – labeled anti-sense RNA probe set. The reaction was set up for each template set as follows: 1 μl of 40 units/ml RnaseIn, activated in 2μl of 100 mM DTT, 1 μl of GACU pool (GAC: 2.75 mM each and 61 mM U), 4 μl 5X transcription buffer, 1 μl template set, 10 μl

of 40 μM α-^{32}P-dATP and 1 μl of 20 units /ml T7 RNA polymerase. This was incubated at 37°C for 1 hr. The reaction was terminated using DNase (1 unit/μl). Probe RNA was extracted using tRNA as helper and phenol chloroform: isoamyl alcohol to clear away protein and DNA contamination. The probe set was hybridized in excess to target RNA pools (isolated as in method 6) for 12 –16 hours at 56°C. Free probe and other single stranded RNA was digested with RNase A and T1 as follows: 0.3 μl RNase A + T1 (80 ng/μl RNase A and 250 units /μl RNase T1), 125 ml of RNase buffer and hybridization reaction. The remaining RNase protected probes were treated to Proteinase K digestion to get rid of protein contamination that may contribute to gel shift and extracted and separated on a denaturing 5 % polyacrylamide gel containing 7M Urea, vacuum dried at 80°C, exposed to PhosphoImaging (Molecular Dynamics) or autoradiography.

5. In vitro splicing assay.

Preparation of nuclear and cytoplasmic extracts. This protocol is adapted from Dignam JD, 1983. At least fifty 100 mm plates of cells were grown, harvested and re-suspended in buffer A (10 mM 1-Piperazineethane sulfonic acid, 4-(2-hydroxyethyl)- monosodium salt called HEPES (pH 7.9),1.5 mM Magnesium chloride, 10.0 mM Potassium chloride, 0.5 mM Dithiothreitol and 0.5 mM Phenylmethylsulphonyl Fluoride). Cells were then lysed using a homogenizer dounce and centrifuged. The pellet was resuspended in a controlled volume of buffer C (20 mM HEPES at pH 7.9, 25 % glycerol, 0.42 M Sodium chloride, 1.5 mM Magnesium Chloride, 0.2 mM Ethylenediaminetetracetic acid, 0.5 mm Dithiothreitol and 0.5 mM Phenylmethylsulphonyl Fluoride). The lysate was kept

at 4°C for 30 minutes , centrifuged to remove nuclear debris and dialyzed against buffer D (20 mM HEPES at pH 7.9, 20 % v/v glycerol, 0.1 M Potassium Chloride, 0.2 mM Ethylenediaminetetracetic acid, 0.5 mM Dithiothreitol and 0.5 mM Phenylmethylsulphonyl Fluoride) for 45 minutes on ice. The nuclear extract thus obtained was frozen at –80°C. The cytoplasmic extract can also be prepared by dialyzing the supernatant of the first centrifugation step against buffer D, this is called the S100 fraction.

In vitro transcription. The protocol for making template is mMessage mMachine^{TM} (Ambion). The reaction was set up as follows: 2 µl of 10 X transcription buffer, 10 µl of 2X ribonucleotide mix (containing 8 mM cap analog,10 mm each of ATP, CTP, UTP and 2 mM GTP), 4.5 µl of α^{32}P- UTP (at 40 mM at any specific activity), 1 µg of linearized template (plasmid vector carrying partial/full length cloned templates for formation of pre-mRNA), 2 µl of 10X enzyme mix containing SP6/T7/T3 RNA polymerase. This reaction was incubated at 37°C for 2 hr. The reaction was stopped using DNase I. Full length pre-mRNA was extracted with phenol: chloroform : isopropyl alcohol and re-suspended in water for the splicing reaction.

Splicing reaction. Splicing reactions were set up as recommended by Mayeda 1998 as follows: 5.0 µl of 13% polyvinyl alcohol, 1 µl of 80 mM $MgCl_2$, 1 µl of 25X ATP/CP mix, 1 µl of (10 units /µl) Creatine Kinase, 1.25 µl of 0.4 M HEPES-KOH at pH 7.3 , 20 femtomol of labeled mRNA and 7.25 µl of nuclear/cytoplasmic extract from cells. These were incubated at 30°C for 30 to 60 minutes. Resulting RNA was extracted again and centrifuged. The RNA were separated on a 5%

polyacrylamide / 7M urea gel at 15 watt. The gel was exposed to a PhosphorScreen or autoradiography.

6. Immunoblotting and protein analysis.

Standard western blotting protocols (Harlow 1999) were followed to isolate, estimate, separate and analyze proteins in cells. To detect levels of the following proteins hnRNPA1, hnRNPA2, SF2, SC35, p21, pRb, $p14^{ARF}$, $p16^{INK4a}$,caspase 2/Ich-1 L ,caspase 2/Ich-1 S, fibronectin and p53 prepared extracts from fibroblasts at different culture age were used. For making protein lysates, cells were washed 3 times with 1X cold PBS and then placed on ice. Cold RIPA (radioimmunoassay buffer containing NP-40 at 1%, sodium deoxycholate at 1%, sodium dodecyl sulphate at 0.1%, NaCl at 150 mM and Tris-HCl at 10 mM with protease inhibitors leupeptin at 0.1 μg/ml, pepstatin at 0.1 μg/ml and phenylmethylsulphonyl fluoride at 1mM) was poured over the cells and cells were scraped into cold microfuge tubes. The lysate was passed through a 21 gauge syringe needle to ensure that all cells had lysed. Lysates were centrifuged at 10,000Xg for 10 minutes at 4°C. The cleared lysate was collected and aliquots prepared to estimate the amount of protein by the Bradford protein assay (BioRad). Lysates were run on 8-12 % acrylamide gels and then transferred in an electroblot apparatus. Membranes (PVDF, Osmonics) were blocked with 5% non-fat milk in PBS. The following antibodies were used for primary detection: monoclonal antibody 4B10 (1:10,000) for hnRNPA1/A1B; apoptosis sampler kit 2 (BD Biosciences) containing monoclonal antibodies against caspase-2 (1:1000), caspase-3 (1:1000), caspase-7 (1:1000), DFF45 (1:500), FADD (1:250), FasL (1:5000), PARP (1:500), RIP (1:1000) and

TRADD (1:250); actin (1:5000) from Chemicon. Secondary antibody, goat IgG or mouse IgG conjugated with HRP was used for visualization of bands using the ECL kit (Amersham).

7. Immuno-precipitation protocol. Radioimmunoassay precipitation buffer (RIPA) was prepared fresh for each use (Harlow 1999). RIPA components are 1% NP-40, 1% Deoxycholic acid, sodium salt, 0.1% sodium dodecyl sulphate, 150 mM NaCl and 10 mM Tris-HCl (pH 7.4). Protease inhibitors were added fresh before use, PMSF at a final concentration of 1mM, pepstatin at a final concentration of 0.1 μg/ml and leupeptin at a final concentration of 50 μM. The cells were washed three times with 1X PBS twice and then RIPA buffer added to the cells. The plates were rocked at 4°C for 30 minutes. Cells were scraped and collected into microcentrifuge tubes. Lysates were spun at 15,000 Xg for 10 minutes at 4°C. Protein samples were mixed with CL-4B beads (Amersham) that were pre-incubated with antibody. The cell lysates were incubated for 1 hour at 4°C and then washed thoroughly. Beads were washed three times with 1 ml of RIPA with protease inhibitors and protein was eluted using sample loading buffer. Protein samples were analyzed by polyacrylamide gel electrophoresis and processed for western blotting as above.

8. Labeling index of HDF. This protocol (Calbiochem, USA) measures the percentage of actively dividing cells and is a colorimetric assay that allows us to determine the percentage of mitotic cells in a population. Cells were plated in 6 well plates at 2×10^5 cells per well. A BrdU solution at 1:2000 dilution in fresh tissue culture media was prepared and layered over the cells. After overnight

incubation, cells were fixed at room temperature for 30 minute. Anti-BrdU antibody was added and incubated for 1 hour, cells were washed in wash buffer. The horse radish peroxidase conjugate was prepared and layered over the cells for 1 hour. Cells were washed thoroughly and then substrate solution containing diaminobenzidine was added and incubated for 1 hour. Cells were observed in the microscope and stained nuclei were counted as a proportion of the total number of cells in the field. Cells were used as senescent if the percentage of labeled cells in the population were less than 5%.

Chapter 1

Gene expression patterns and profiles of potential target genes regulated by Heterogeneous nuclear ribonucleoprotein A1

Introduction

In order to establish age related gene expression patterns in known genes such as caspase-2 and fibronectin we studied alternative splicing patterns of mRNA from these loci.

Caspase-2 locus and alternative splicing.

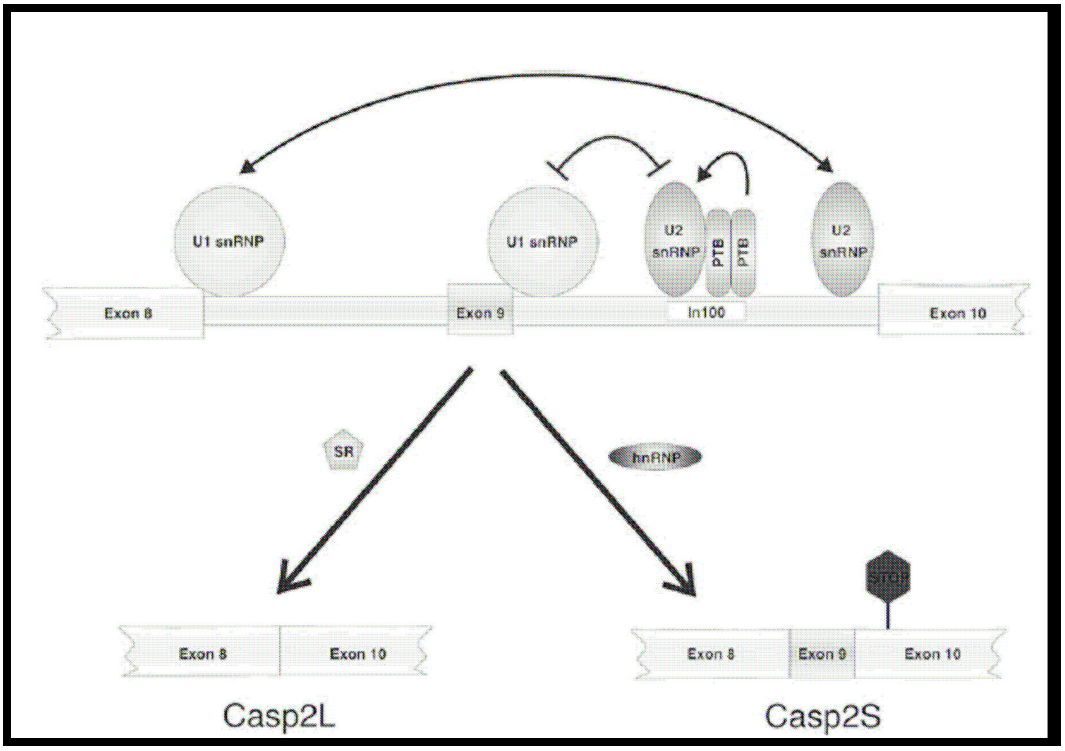
Caspase–2/ NEDD 2/ Ich-1 (Interleukin–1β converting enzyme homology protein 1) can induce apoptosis but its gene also encodes an anti-apoptotic mRNA isoform called caspase-2L. This is because the caspase-2 pre-mRNA is alternatively spliced into two forms. One mRNA species (the short form) encodes a longer polypeptide of 435 amino acid residues called caspase-2L. This form is homologous to caspase-1 and CED-3 (the death executioner of *Caenorhabditis elegans*). An exon inclusion event, or alternative 5' splice site usage results in a polypeptide of 312 amino acids residues called caspase-2S from the other mRNA. This form terminates 21 amino acid residues after the pentapeptide QACRG that is the signal for cleavage by caspases. Caspase-2 appears to have autolytic capability that is it can cleave other procaspase-2 molecules (Miura 1997) and therefore is believed to be anti-apoptotic. The inclusion of the exon number 9 results in a pre-mature stop codon. By employing a caspase-2 mini-gene strategy it was shown that the SR proteins SC35 and ASF/SF2 promote exon skipping of exon 9 and an increase of the anti-apoptotic casp-2L, whereas hnRNP A1 facilitated exon 9 inclusion increasing casp-2S (reviewed in Schwerk 2005). Experiments performed to elucidate the mechanism of this alternative splicing event has lead to the discovery of an important sequence within the intron downstream of exon 9 called

In100 (Schwerk 2005). This 100 nucleotide sequence has a decoy 3' splice site upstream to a polypyrimidine tract binding protein binding site and both part function to repress the inclusion of exon 9 as seen when the levels of SR protein are higher in cells (Cote 2001). It is still unknown whether there is a competitive interaction between the antagonistic splicing factors hnRNP A1 and SC35 or ASF/SF2 at this exon inclusion/exclusion event.

In old cells, we anticipate that the levels of hnRNP A1 are sufficiently low as compared to other SR splicing factors that we might see the shift in old cells towards the pro-apoptotic form. This would be mediated by the presence of other splicing factors and mRNA processing factors that could have a significant role to play in this decision.

Figure 1.1 Alternative splicing at the caspase-2 locus.

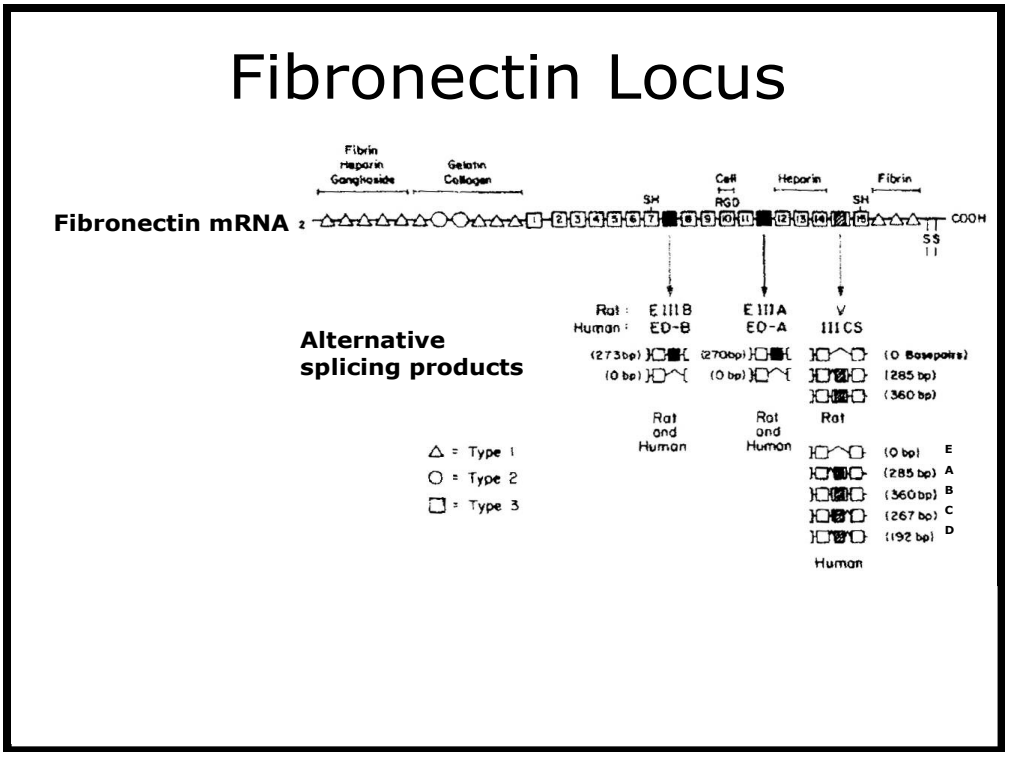
In this schematic model of exon inclusion mediated by hnRNPA1 the exon 9 splicing decision is affected by splicing factors such as SC35 and hnRNPA1. The U2 snRNP binds to In100 and forms a non-productive splicing complex which is promoted by binding of PTB to this sequence, promoted by SR protein and the formation of the anti-apoptotic casp-2S. In the other case hnRNP A1 competes with SC35 and promotes inclusion of exon 9, forming the pro-apoptotic casp-2L (Schwerk 2005).



Fibronectin locus and alternative splicing.

The fibronectin locus is known to undergo alternative splicing in an age-related, developmental stage specific and species specific manner. It is believed that the production and secretion of fibronectin as a major macromolecular component of extra-cellular matrix may have a role in many human physiological processes such as wound healing, cell matrix assembly, secretion of ECM, cell adhesion, cell differentiation, tissue injury and inflammation, cell cycle progression and mitogenic signal transduction (reviewed in Muro 2003). In addition it is has been shown that there may be a role for this protein as a determinant of life-span in mice where it regulation by alternative splicing was perturbed and the mice showed a reduction in life-span and ability to perform maintenance functions such as wound healing. Most of the alternative splicing mechanisms that are found in human at the fibronectin gene involve the alternative splicing of either EDA or EDB exons and the studies mentioned above on life span were based on changes at the EDA exon. However the EDB exon is known to be alternatively spliced and excluded in human diploid fibroblasts and it has been proposed that there may be a role for a splicing regulatory protein such as hnRNP A1 in this mechanism (Magnuson 1991). It has also been shown that there are alternative splice regulatory elements called PRE1 and PRE2 that are required for proper 5' splice site usage at the EDB exon (Kuo 1999).

Figure 1.2 Exon structure of fibronectin gene. There are 3 different types of repeat homologies. Exons EIIIB, EIIIA and IIICS are alternatively and differentially spliced depending on tissue and genus type (Magnuson 1991).



In this study it was observed that the regions of exon EDB may have a role to play in the splice site decision and that this event may be mediated by serine-arginine rich (SR) splicing factors. It is known that there is an antagonistic relationship between general splicing factors such as SR proteins and hnRNPA1 in choice of splice site and exon inclusion/exclusion decisions (Zhu 2001). We propose that there is a potential role of hnRNPA1 in the splicing regulation of fibronectin in an age-specific manner.

Results

The goal of this study is to identify and study the effects if any of post-transcriptional processing via hnRNP A1 on gene expression of candidate genes as a function of the age of cells.

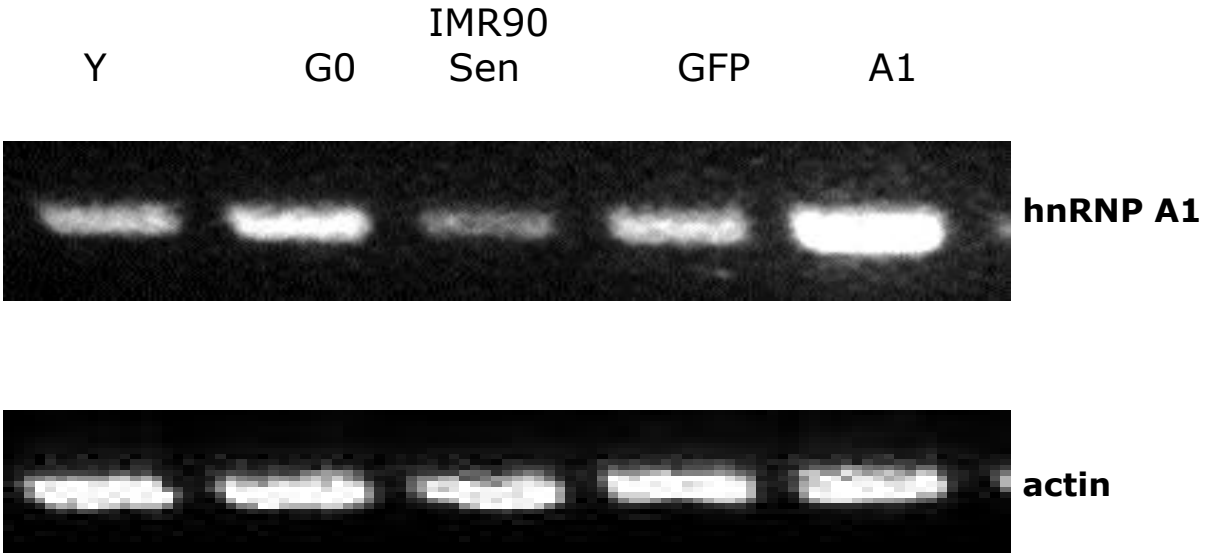
mRNA and protein levels of hnRNPA1 are altered in senescent IMR90 fibroblasts.

The levels of steady state mRNA and protein of hnRNPA1 were confirmed to be altered in senescent fibroblasts by basic molecular biology techniques. We used G0 arrested cells to distinguish whether the changes observed were indeed senescence-specific. IMR90 and WI38 fibroblasts were also analyzed for p21WAF1 and $p16^{INK4a}$ mRNA levels that are up-regulated in cells as they age (Stein 1999). The levels of $p16^{INK4a}$ mRNA and protein are very low in fibroblasts and it is more accurate to study the protein levels and assess up-regulation of the protein in senescent IMR90 (see Figure 2.6, panels A and B). Senescence of fibroblasts was also confirmed by testing the labeling index of human diploid fibroblasts and confirming that there are less than 5% dividing cells in senescent cell populations (Hubbard 1997). Figure 1.3 depicts the results of reverse-transcriptase PCR, panel A and western results, panel B and shows that there is a decrease in mRNA of hnRNPA1 in cell as they age. This is not specific to IMR90 cells and has been observed in HS74 cells studied (Zhu 2002, Hubbard 1995), and also in WI38 fibroblasts (see Figure 1.4). The decrease in hnRNPA1 levels is believed to be the result of down-regulation of gene expression at a post-transcriptional stage which then results in down-regulation of protein products,

hnRNPA1 and hnRNPA1^{B}. There may also be translational control of the mRNA due to alternatively spliced products and lack of availability of other splicing factors in the cell (Meshorer 2000). We also confirmed that the levels of hnRNP C, another member of the core hnRNPs that is constitutively nuclear is also subject to age-related down-regulation in expression (see Figure 1.5). Also, we tested WI38 cells for levels of general splicing factors ASF/SF2 at the mRNA level and we found that the pattern of down-regulation of hnRNP A1 mRNA appears to be unique to the hnRNP A/B sub-family of proteins (see Figure 1.4).

Figure 1.3 mRNA levels and protein levels of hnRNPA1 are altered in senescent fibroblasts. A. RNA was isolated from cells under different condition and analyzed by semi quantitative reverse transcription polymerase chain reaction for gene expression of hnRNPA1 and actin. **B.** Total cell lysates were analyzed for hnRNPA1 levels, amount of protein is 20μg per lane, and membranes were stripped and re-probed for actin protein. Lanes are M, molecular weight marker, Y, young IMR90, G0, G0 arrested IMR90 fibroblasts and Sen, senescent IMR90 fibroblasts.

Figure 1.3 A. mRNA analysis in IMR90 fibroblasts.



B. Western analysis on IMR90 fibroblasts

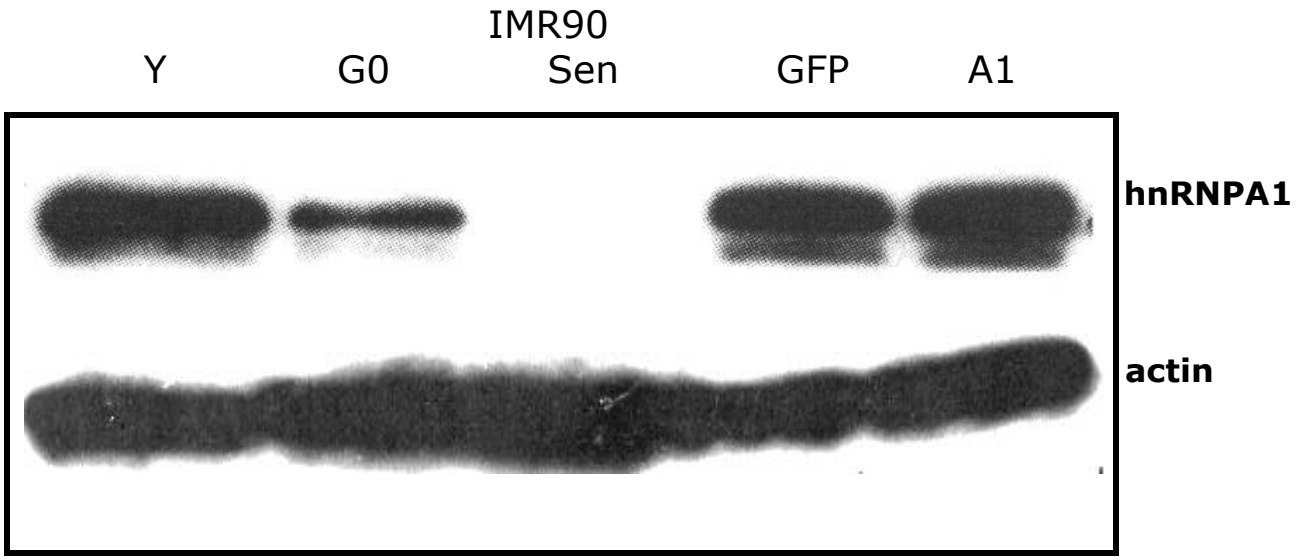


Figure 1.4. mRNA analysis of hnRNPA1 and hnRNPA2 in other fibroblasts cell strains such as WI38 normal human diploid fibroblasts. mRNA analysis of hnRNPA1 and other splicing factors was performed. RNA was isolated and then analyzed by semi-quantitative reverse transcription polymerase chain reaction (Materials and Methods Table 1) for gene expression of hnRNPA1, hnRNPA2, ASF/SF2 and actin. Lanes are M, molecular weight marker, Yo, young WI38, G0, G0 arrested WI38 fibroblasts and Sen, senescent WI38 fibroblasts.

Figure 1.4 mRNA analysis in WI38 fibroblasts

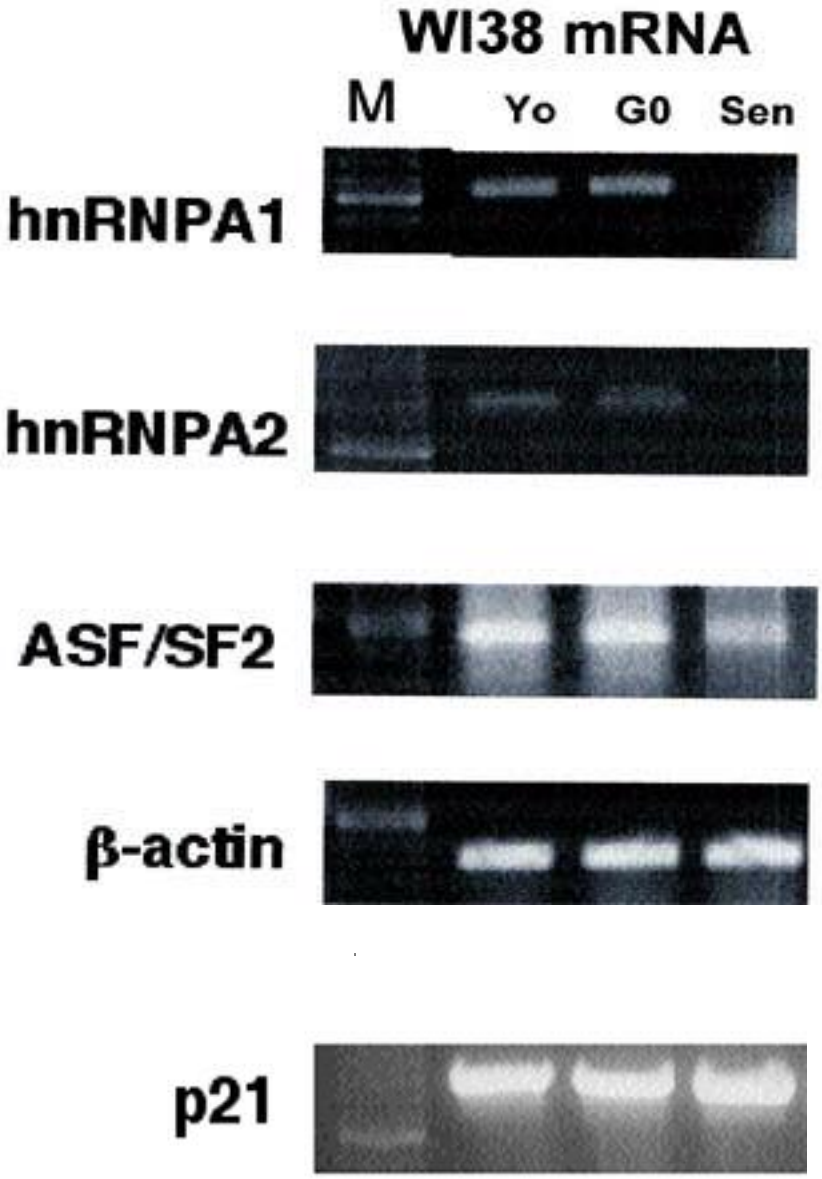
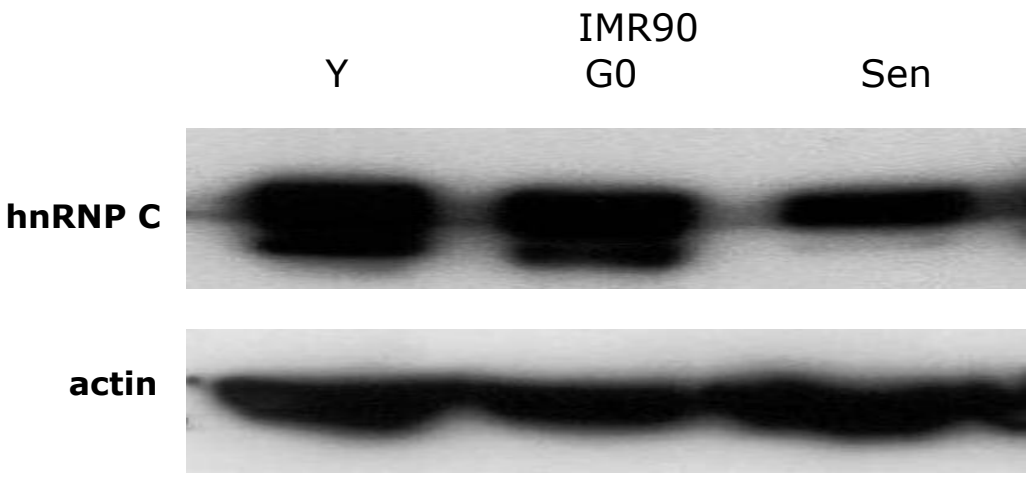


Figure 1.5. hnRNP C protein is expressed at the same levels in young, G0 arrested and senescent IMR90 fibroblasts. Total cell lysates from Y, young, GO, G0 arrested and Sen, senescent IMR90 were analyzed for hnRNP C levels using mouse monoclonal antibody, 4F4, at concentration of 1:5000, amount of protein is 30 μg per lane; membranes were stripped and re-probed for actin protein.

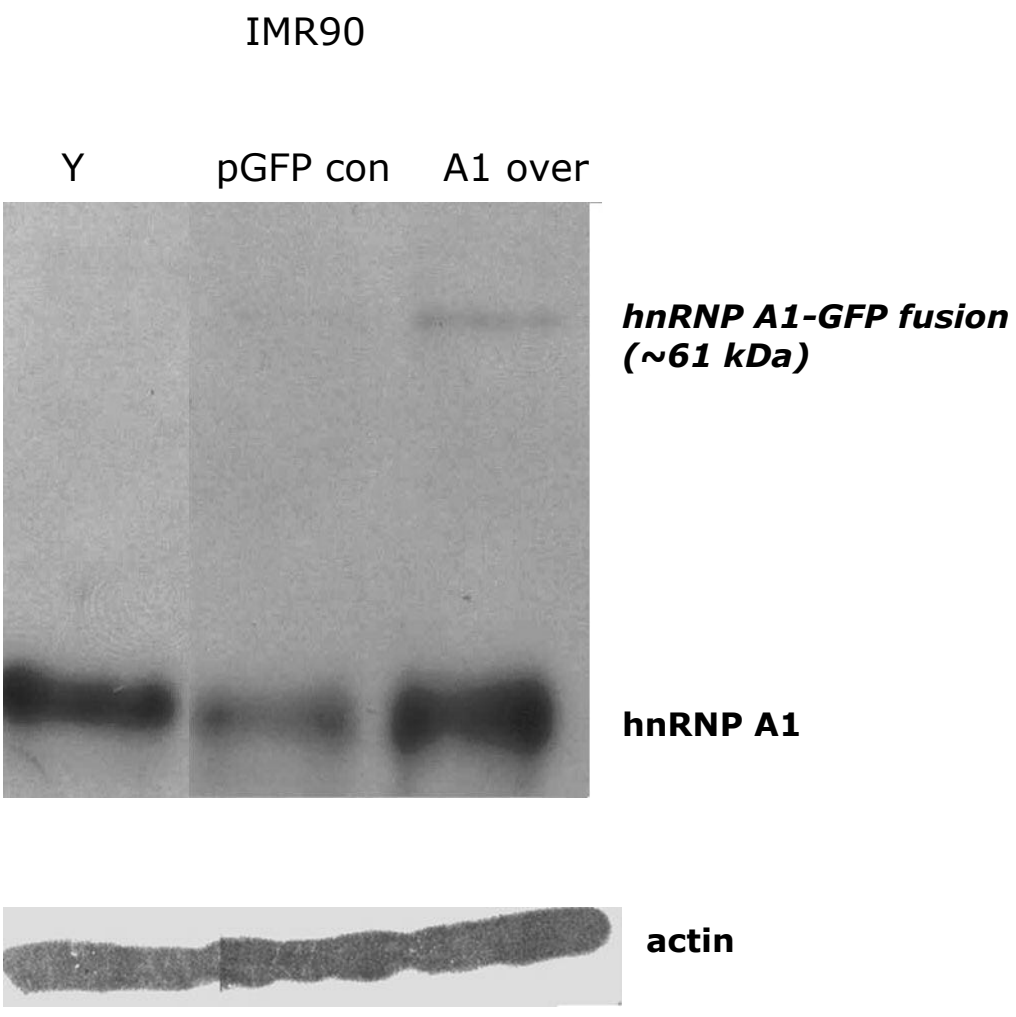
Figure 1.5 Western analysis in IMR90 fibroblasts



We also confirmed that the levels of hnRNPA1 were increased in IMR90 cells that over-expressed hnRNPA1 using the pEGFP-hnRNPA1 constructed plasmid. GFP protein is a 27 kDa protein that is used in this instance as part of a fusion product that is made from the over-expression plasmid for hnRNPA1. This result can be seen in Figure 1.6, western analysis of cells that over-express hnRNPA1 show that there is an additional band not observed in young IMR90 at ~60 KDa which could be the band of GFP-hnRNPA1 fusion product that is made specifically from the constructed vector. There appears to be lower levels of hnRNPA1 protein in the GFP control lane, the reason for which can be that the levels of endogenous hnRNPA1 vary by culture in young fibroblasts. However, the levels of hnRNPA1 protein are much higher in over-expressing cells but appears to co-migrate with endogenous hnRNPA1. We have been able to assess the higher levels of hnRNPA1 expression in over-expressing cells by mRNA and by western analysis, we believe that the fusion protein may be cleaved which would explain the higher levels of what appears as endogenous hnRNPA1 in over-expressing cells.

Figure 1.6. hnRNP A1 protein is over-expressed as a fusion product in IMR90 fibroblasts. Cells were transfected with pGFP empty control vector and pGFP-A1 fusion vector. Protein was isolated from cells 72 hours post-transfection and analyzed by western analysis using mouse monoclonal antibody 4B10 at 1:10,000. 20 μg of protein was loaded per lane. Blots were stripped and re-probed for actin. Lane Y indicates young IMR90 fibroblast samples at equivalent population doubling levels for comparison.

Figure 1.6. Western analysis on hnRNPA1 over-expressing IMR90 fibroblasts.



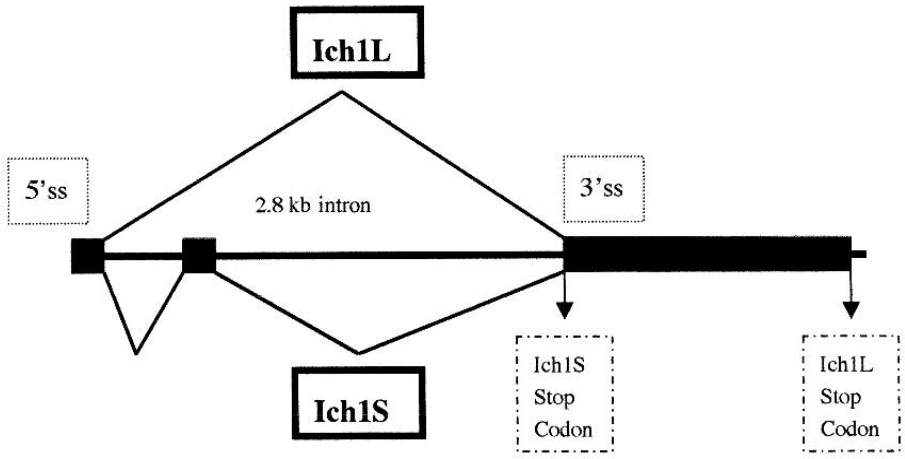
Alternatively spliced mRNA isoforms of the caspase-2 locus are unchanged by age in IMR90 fibroblasts.

The products of the caspase-2 locus are casp-2L and casp-2S, the long and short form respectively. In order to assess the relative levels of these mRNA in senescent IMR90 we performed RNase protection assays using human Apo-1a probe set from BD Pharmingen to compare the relative levels of caspase-2 mRNA in young and senescent IMR90. The signal was very low in all cells, but when compared to the house-keeping gene (L32 and GAPDH) we could assess the relative levels of the isoforms of mRNA. Figure 1.8 depicts a result of RNase protection assay. In this figure, there are two bands that show a downshift after treatment with RNase T1. These are caspase 2S and caspase 2L marked in bold. They were identified by molecular weight and migration in the image. Caspase-2S mRNA includes alternate exon 9 and therefore makes a longer mRNA than caspase-2L. The inclusion of the exon causes the in-frame addition of a premature stop codon and the protein product of this mRNA is shorter than caspase-2L that reads through to exon 10 and makes a longer protein. Previous studies have shown that caspase-2 pre-mRNA is subject to post-transcriptional regulation by hnRNP A1 and SC35 (Jiang 1998). We proposed that the levels of hnRNPA1 being low in senescent fibroblasts would drive the alternative splicing decision to favor exclusion of the exon in the mRNA leading to formation of the longer pro-apoptotic protein isoform caspase-2L. Our observations did not show an age-related increase in the levels of caspase-2S relative to caspase-2L which suggests that

hnRNPA1 may not have a role to play at the level of post-transcriptional processing of the locus.

Figure 1.7. Schematic representation of caspase-2 locus and mRNA analysis of alternatively splice products. This locus is also called Ich-1 has an alternatively spliced exon 9 in the centre. **A.** The inclusion of this exon causes the recognition of the Ich1S stop codon as shown below. The splicing inclusion of exon 9 was promoted by hnRNPA1 levels (Jiang 1998) which would lead to a lowered caspase -2L to -2S ratio in young fibroblasts. In contrast, lower levels of hnRNPA1 would promote exon exclusion and preference for the caspase-2L form. **B.** Results of RNase protection assay are summarized here, the ratio of caspase-2L to -2S isoforms of RNA were measured, three independent replicates were used for each condition. Error bars denote standard error.

Figure 1.7 A.


Ich1L
5'ss
2.8 kb intron
3'ss
Ich1S
Stop
Codon
Ich1L
Stop
Codon
Ich1S

B.

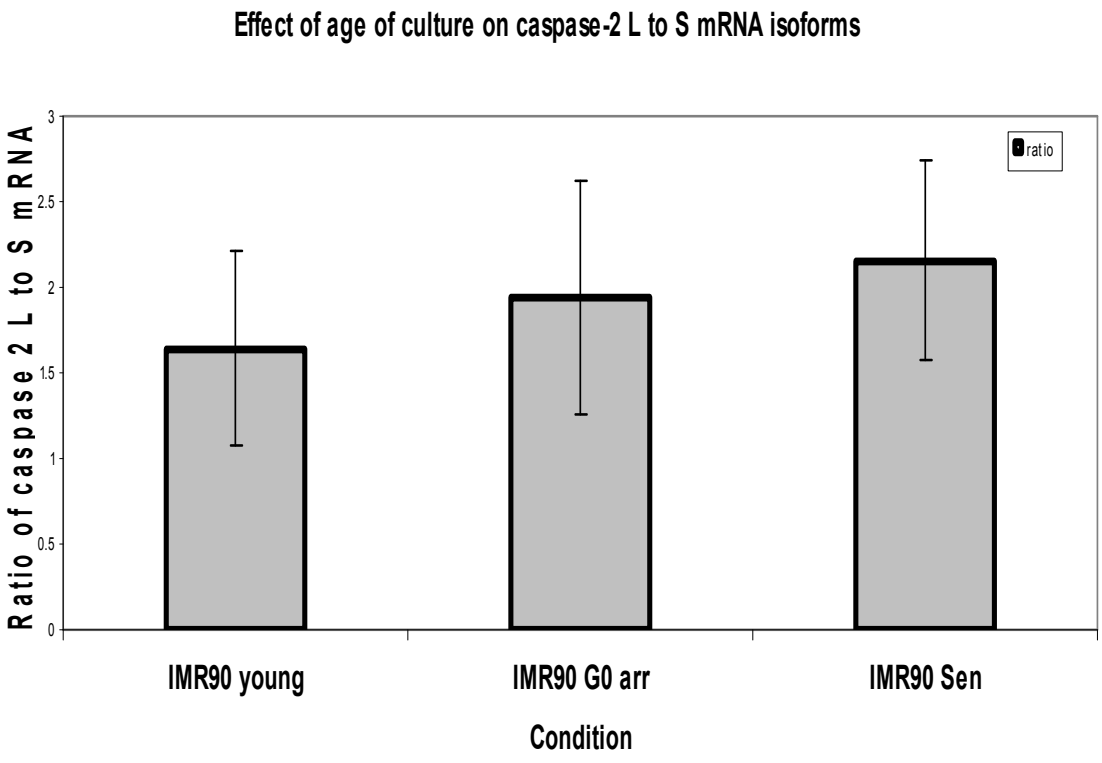
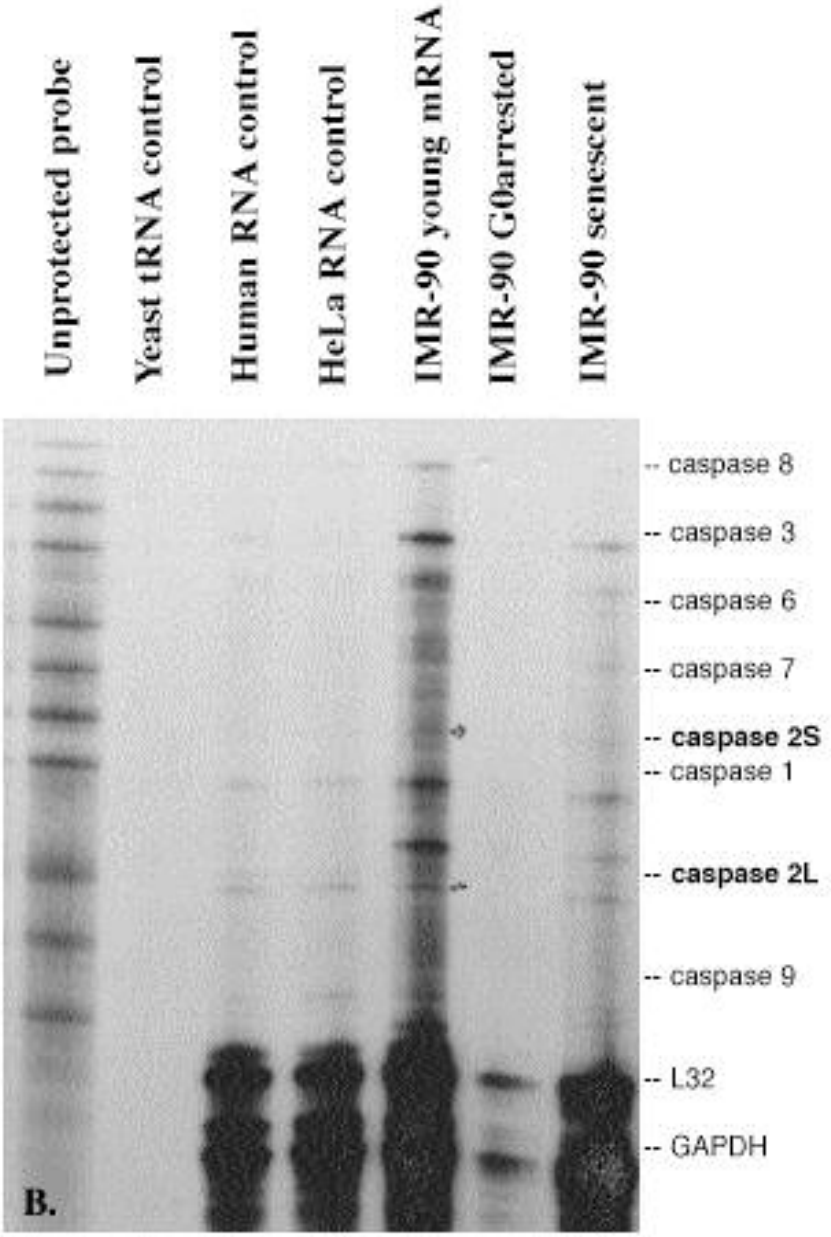

Effect of age of culture on caspase-2 L to S mRNA isoforms
Ratio of caspase 2 L to S mRNA
3
2.5
2
1.5
1
0.5
0
ratio
IMR90 young
IMR90 G0 arr
IMR90 Sen
Condition

Figure 1.8. RNase protection assay on IMR90 fibroblasts. RNA was isolated from cells under various conditions of age and the controls used were yeast tRNA and human and HeLa cell RNA. RNA was hybridized to the probe set, RNAse treated and then denatured. RNA was electrophoresed and probe signal imaged using a PhosphorImaging screen. Relative mobility in the gel was graphed to the molecular weights of the probe set and bands in sample lanes identified using this as a standard. Bands for caspase-2S and -2L are depicted and were measured for intensity of signal using ImageQuant software. Relative levels of mRNA were calculated using GAPDH as a calibrator and ratios of caspase-2L to -2S isoforms in different conditions were averaged.

Figure 1.8.


Unprotected probe
Yeast tRNA control
Human RNA control
HeLa RNA control
IMR-90 young mRNA
IMR-90 G0arrested
IMR-90 senescent
-- caspase 8
-- caspase 3
-- caspase 6
-- caspase 7
-- caspase 2S
-- caspase 1
-- caspase 2L
-- caspase 9
-- L32
-- GAPDH
B.

Analysis of Fibronectin locus.

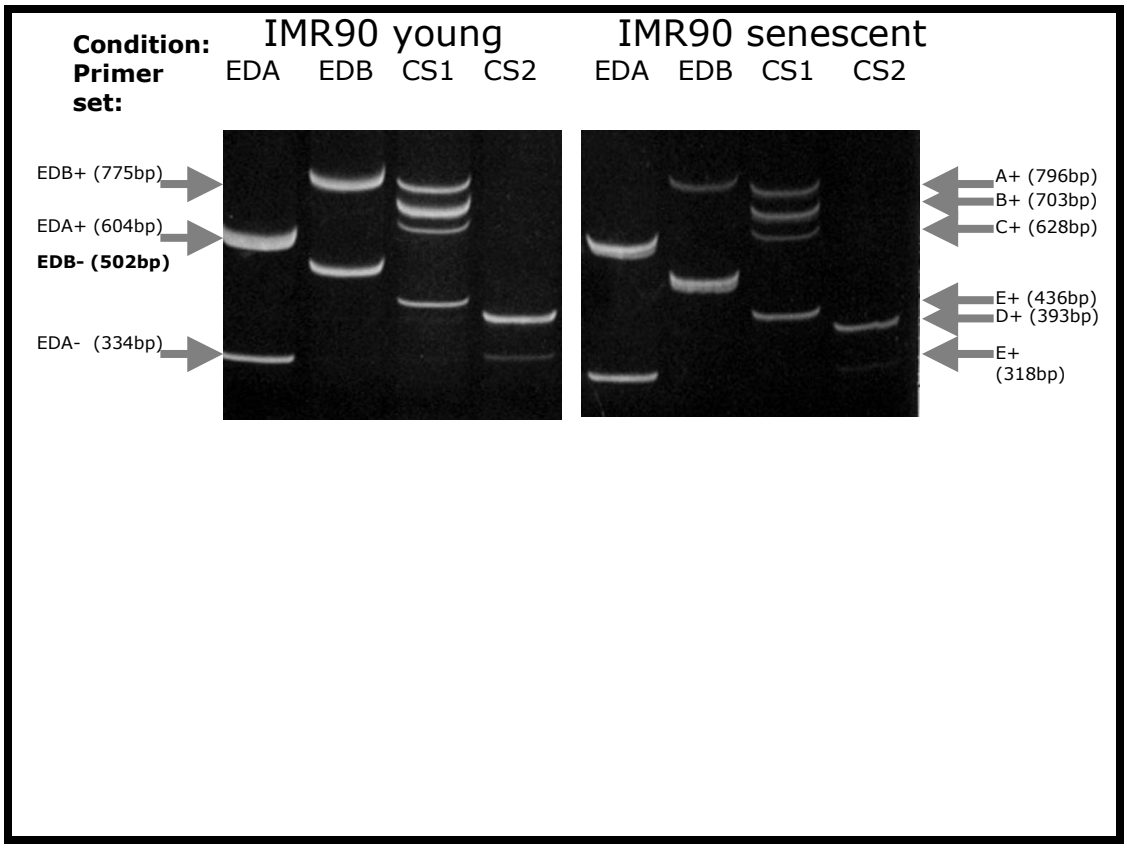
The fibronectin locus was analyzed for alternatively spliced mRNA and our experimental objective was to identify a possible role for hnRNPA1 in this alternative splicing pattern. The locus produces many different types of RNA (see Figure 1.2) and these can represent the various types of fibronectin that are produced by cells under differing conditions of growth. This gene encodes a glycoprotein present in a soluble dimeric form in plasma, and in a dimeric or multimeric form at the cell surface and in extra-cellular matrix. Fibronectin is involved in cell adhesion and migration processes including embryogenesis, wound healing, blood coagulation, host defense, and metastasis. The gene has three regions subject to alternative splicing, with the potential to produce 20 different transcript variants. We performed quantitative reverse transcription-polymerase chain reaction analysis to compare the relative levels of alternatively spliced products using EDA, EDB and IIICS exons for splice site decisions. We found that IMR90 fibroblasts express all variants of exon inclusion/ exclusion choices at all three exons known to undergo alternative splice decisions, a result that confirmed previous observations (Magnuson 1991). In our study we anticipated that the role of hnRNPA1 at the post-transcriptional level could potentially influence alternative splicing by exon inclusion/exclusion events. We proposed that the results of hnRNPA1 over-expression in young fibroblasts would shift the transcription profile of fibronectin towards the production of EDB+ form, which was not observed. Figure 1.9, B, lane 5, A1, which after quantitation did not show a significant shift to produce relatively more EDB+ transcript. Therefore we conclude that fibronectin

splicing is under age-dependent gene expression regulation in IMR90 fibroblasts but not affected by higher levels of hnRNPA1 protein.

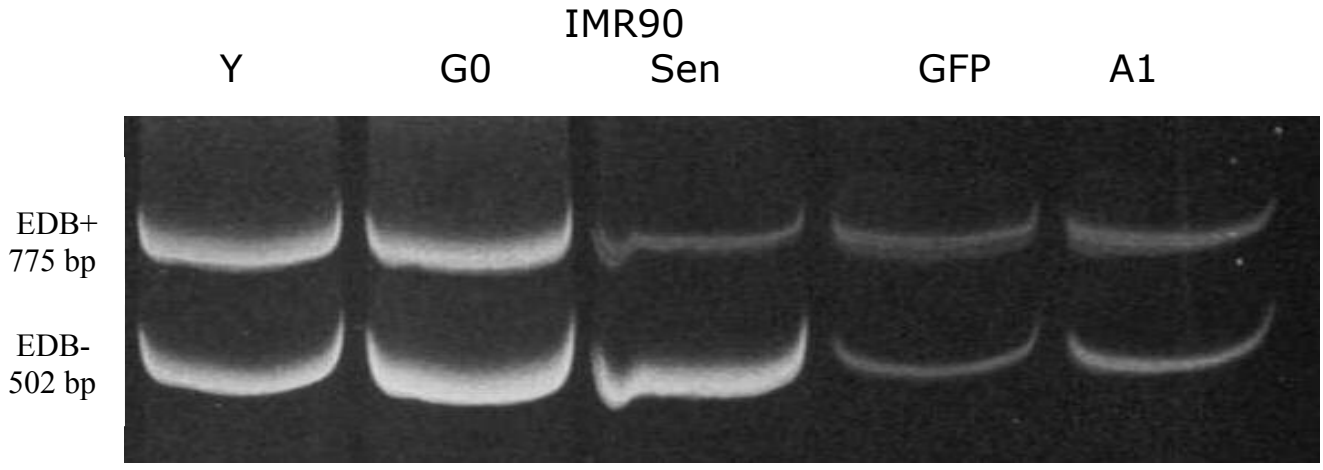
Figure 1.9. Analysis of the fibronectin locus and alternative splicing decisions at the EDA and EDB exons. A. IMR90 young and senescent fibroblasts were harvested and RNA isolated. The RNA was analyzed for fibronectin transcripts by RT-PCR, the products of which were electrophoresed and analyzed. The left panel depicts alternative splicing events that occur in young fibroblasts, the relative levels of PCR products were quantified and the ratio of EDA+ to EDA- determined. The panel on the right depicts alternative splicing event that occur in senescent fibroblasts and once again splicing products were compared. **B**. The results of RT-PCR were once again confirmed and compared between five conditions, IMR90 young, IMR90 G0 arrested, IMR90 senescent, IMR90–pGFP control vector, IMR90 transfected with pGFP-hnRNPA1 fusion construct for over-expression of hnRNPA1. Experimental conditions were the same in all reactions.

Figure 1.9

A. RT-PCR analysis of fibronectin EDA and EDB exon splicing in IMR90 cells.



B. RT-PCR analysis of FN EDB exon splicing products.



Discussion

We based these studies on the hypothesis that hnRNPA1 as a representative post-transcriptional regulator may be responsible for splicing patterns observed in human diploid fibroblasts. We anticipated that perturbations in the level of this protein as seen in senescent fibroblasts and experimentally induced by over-expressing hnRNPA1 in fibroblasts could in fact change the alternative splicing patterns of the loci we chose to study. At the commencement of these studies, little was known about in vivo substrates for alternative splicing regulation by hnRNPA1, and we postulated that any known targets of hnRNPA1 that could have effects on cell cycle and growth could be critical to the senescent mechanism via post-transcriptional processing. Our studies have confirmed the splicing patterns and effects of hnRNPA1 over-expression seen previously in fibroblast cells (Jiang 1998 and Magnuson 1992). In the case of caspase-2, over-expression of hnRNPA1 was shown to have an effect on the splicing of a caspase-2 minigene construct, and this effect could be competed out by the antagonistic splicing factor 35 (Jiang 1998). This was an important observation because it identified caspase-2 as a substrate for hnRNPA1 binding and alternative splicing regulation, the only other mammalian gene at the time known to be subject to hnRNPA1 levels affecting splicing patterns was hnRNPA1 itself (Blanchette 1999). Since that time, there has been an increased interest in the role of alternative splicing as a mechanism to regulate gene expression in many diverse biological conditions such as apoptosis and cell cycle regulation that are also critical to aging mechanisms (Meshorer 2000). Though there may be a role for hnRNPA1 as a splicing regulator of caspase-

2 in a mini-gene construct, there does not appear to be any effect on caspase-2 splicing as cells age and when hnRNPA1 is over-expressed in IMR90 fibroblasts. There is no defined role for the potentially anti-apoptotic form of caspase-2 and it would be interesting to investigate this protein in the aging context. This is because aging cells are capable of responding to specific cell death signals (Dejesus 2002) and the balance between the alternative products of a dual modulator such as caspase-2 may be critical to the response of senescent cells in vivo to forces that ultimately determine tissue integrity and ability to respond to adverse environmental cues.

We also proposed fibronectin (FN1) as a locus that undergoes age-dependent gene expression changes to be regulated by post-transcriptional processing that could be perturbed by the over-expression of hnRNPA1. There were many reasons to believe that FN1 alternative splicing is central to its role in localization and secretion. An in vivo model for alterations in the ability of fibronectin EDA exon to be spliced in an accurate manner during development of mice caused developmental difficulties and affected life-span in this model system (Muro 2003). Also, it was observed that the antigenic determinants of fibronectin in tissue can be altered by age and this is highly specific to the age of tissues that were studied (Muro 2003). In cultured cells, the secreted form of fibronectin appears to be higher than the cytoplasmic form and older cells produce a shorter fibronectin monomer that is believed to be the result of exon exclusion events (Magnuson 1992). Therefore, we proposed that fibronectin alternative splicing provides a choice model pre-mRNA for hnRNPA1 ability to influence splicing. We found that

although there was a definite age-related shift to producing a smaller mRNA that excluded EDB exon, we were not able to define a role for hnRNP A1 in this splicing event. It is possible that the effects of hnRNPA1 may be capable of influencing alterations of fibronectin levels and forms in old cells but our results indicate that this does not happen at the level of alternative splicing of the EDB exon.

Our studies indicated that although little is known about specific genes that are altered at the post-transcriptional level in cells as they age, it is important to investigate the role of alternative splicing in the production of altered protein isoforms that can affect the unique gene expression pattern that is associated with aging. Also, most alternative splicing studies are performed using gene constructs that include the most obvious sequence elements for splicing such as exon-intron boundaries and binding sites for general splicing factors. Though these approaches have been instrumental in elucidating the mechanisms of splicing complex components both RNA and protein, they have ignored the contribution of secondary structure and in vivo context. The environment in which a gene is altered in expression is highly critical to the nature of the alteration and our studies attempted to address these changes in the cellular context. Our result are an important step towards understanding gene regulation of caspase-2 and fibronectin at the cellular level and it would be very interesting to further these studies at the tissue and organism level.

Chapter 2

Modulation of gene expression by heterogeneous nuclear ribonucleoprotein A1 and age in IMR90 human fibroblasts

Introduction

Genetic control of Cellular Senescence in Human Diploid fibroblasts

Cellular senescence is a permanent cell growth arrest that occurs after a limited number of population doublings in culture. It is accompanied by typical changes at the morphological level such as an enlarged and flattened morphology, increased β-galactosidase activity and changes at the chromatin level that are typical to senescent cells. Senescent cells also exhibit gene expression changes in the p53 and pRb-$p16^{INK4a}$ pathways that lead to increased levels of $p21^{WAF1}$ and growth arrest. In addition to the gene expression changes that may be causative to the senescent phenotype, the shortening and dysfunction of telomeres in cells as they age may contribute to the onset of senescence. There is also some evidence that in some cell types, the role of telomere length in triggering senescence may be less significant and that senescence may be initiated due to changes in the environment, also called Stress Induced Pre-mature Senescence or SIPS. As mentioned earlier, pre-mature senescence can be induced by activated oncogenes, DNA damage and ROS (reactive oxygen species) (Cristofalo 1998). Recent studies have shown that oncogenic-ras is capable of arresting fibroblasts in a Raf-1/MEK/p38MAPK dependent manner (Mason 2004). In all, there are many genetic changes that occur in the onset and maintenance of senescence and it is important to establish how these genetic changes are common to other biological processes that a cell may undergo, but also to identify and characterize a broad range of genetic changes that reflect that senescence is a multi-faceted dynamic mechanism.

Analysis of gene expression pattern in cellular senescence

Gene expression studies using microarrays have been used in all field of biology as part of a large-scale genomics approach to establish molecular signatures of cells under various conditions. A systematic analysis of the RNA variations from these microarray studies have given valuable insight into gene function and expression within and across metabolic pathways (Nair 2003). One of the first studies on human fibroblasts (Iyer 1999) was to analyze the effects of fetal bovine serum on normal human diploid fibroblast cells derived from foreskin, ATCC CRL 2091, which were serum deprived for 48 hours then grown in 10% FBS. In this way cells were semi-synchronized and response to serum over the following 24 hours were analyzed at the gene expression level. The transcriptional response to serum suggested that the fibroblast is an active participant in the network of cells and processes that occur in inflammation, angiogenesis and wound healing responses. Following these initial studies on genomics of aging there have been a number of articles published on genomics of various aging model systems and the findings indicate that aging at the molecular level is species-specific, tissue-specific and subject to chemical and biological influences that can alter the age phenotype of cells through various pathways such as cell cycle, signal transduction and other cell metabolism processes (Nair 2003). Also gene expression studies on whole animal tissues do not address the variation in gene expression profiles when tissues are used indicating that the inherent heterogeneity due to presence of different cell types can be responsible for the broad range of expression levels that is often observed. This has shifted the focus of studying gene expression profiles from the

organismal level to the cellular level, to avoid the source of misinterpretation due to changes in tissue composition. In a study on response to DNA damage in normal aging tissue cells (Kyng 2005) and Werner syndrome cells in culture, it was found that the genetic response to agents of DNA damage was significantly altered in old cells. The overall result of this study was that aging can be genetically defined by alterations in gene expression patterns that compromise the ability of old cells to respond to insults such as DNA damage that can further compromise basic protective mechanisms such as stress response and tumor suppression. In two other studies that were done using human diploid fibroblasts as the model system for aging it was found that changes in gene expression were consistent with the results previously obtained using traditional non-genomic methods and in the process they also were able to discover new target genes as having a role in the process of aging in those cells studies. The study on senescent WI38 fibroblasts, by Semov in 2002, used a specially designed microarray for those genes that contained an E-box (CACGTG) in their regulatory region as a binding site for c-Myc, a transcription factor. It was found that using a subset of genes that carried a specific regulatory sequence allowed for the establishment of a role for Myc transcription factor in senescent cells via activation of various genes but also a defining role for Myc regulated gene expression between quiescent and senescent fibroblasts. The other study, done using IMR90 fibroblasts by Mason, 2004 established a molecular signature for cells in which oncogenic-ras was induced and a time-course for changes in gene expression investigated to identify genes that were involved in senescence induced specifically by oncogenic-ras. It was established through this

study that various genes that underwent changes in expression level were senescence specific and also that there are genes whose expression changes but whose role in senescence is not immediately apparent. These were topoisomerase IIα and HDAC9, both genes whose primary function is in regulation of chromatin topology and composition, via which they can regulate gene expression. It is possible that the functions of gene products in senescent cells are altered and distinct from those in young fibroblasts and it would be interesting to progress these studies in the context of senescence associated gene expression. The objectives of these studies were to characterize molecular and cellular changes in senescent fibroblasts in order to gain insight into the mechanisms of senescence and to identify candidate senescence biomarkers. The studies mentioned here are only two among many that have been performed on aging but they are the most relevant to my work for comparison and contrast.

Genomics of alternative splicing and alternatively spliced genes.

There is much interest in the levels of gene expression that give rise to multiple diverse gene products that are able to perform multiple and diverse functions. That gene expression is developmentally regulated, tissue specific and physiology-specific is not new, however, the realization that the human genome contains only ~30,000 genes has led scientists to enquire about the various levels of gene regulation that can give rise to the diversity of gene expression seen under various biological conditions. Alternative splicing in particular, leads to formation of different gene products from a single gene by the production of more than one mRNA that then encode more than one polypeptide. Genomics has allowed

researchers to identify changes in gene expression, but the overall effort has not extended into addressing the various spliced forms of each gene under study. This has led to some innovative approaches to addressing the role of alternative splicing, using expressed sequence tags (EST), that are derived from fully processed mRNA in high-throughput studies. These studies have led to conclusion that there is a high rate of alternative splicing of genes in all cells, approximately 50% of all human genes can undergo alternative splicing and ~90% of those genes result in the production of alternate proteins (Modrek 2002).

Another approach to identify possible alternative spliced isoforms of gene on a genomic scale has been the development of ribonomics (Tenenbaum 2002). In this method mRNPs (mRNA containing ribonucleoprotein particles) are isolated, components characterized and RNA sequenced and analyzed for functional relationships. As the composition of RNP complexes is dynamic, they are difficult to study and characterize. In these studies a combination of protein targeting followed by genomics to study the RNA targets has only confirmed the dynamic nature of such complexes in the cell and it is important to understand the nature of these complexes in order to understand the co-ordinated steps of gene-regulation at the level of alternative splicing. As there appears to be infrequent correlation between mRNA levels called the transcriptome of a cell and the protein output termed the proteome, it is believed that studies that can elucidate the many steps in between mRNA maturation and translation of a protein will play an important role in understanding gene expression variations under differing biological conditions.

It is the objective of this work to identify and characterize human diploid fibroblasts as they age in culture and to identify targets genes that may be subject to alternative splicing in the senescent context. In our studies we have increased the levels of heterogeneous nuclear ribonucleoprotein A1, a potential regulator protein in alternative splicing, and it is our hypothesis that observed changes in alternatively spliced genes may be the result of regulation by hnRNP A1. We also proposed that there may be genes whose expression is affected by the levels of hnRNPA1 in cells may also have an age-specific pattern of expression, helping us to understand a genetic mechanism of senescence in fibroblasts.

Materials and Methods:

1. Clontech array procedure

Probe synthesis from total RNA. The Clontech Atlas array cDNA protocol was followed and 5 μg of total RNA was routinely used as the starting amount of template for all samples. A reaction mix of cDNA reaction buffer, 2.5 mM each dTTP, dCTP and dGTP, [α-^{32}P] dATP at 10 mCi/mL, DTT at 5mM, was added to RNA with 1μL of commercially made primer mix. RNA and primers were first pre-heated at 70°C and the temperature was slowly ramped down to 48°C in a water bath. During this time, 5 units MMLV-reverse transcriptase was added to the reaction mix tube and then mixed with the denatured RNA. The complete reaction was further incubated at 48°C for 25 minutes after which time the reaction was stopped using a reaction termination buffer containing EDTA.

Probe chromatography. The prepared probe was diluted to a volume of 0.2 mL with an equilibrating buffer NT2. NucleoSpin extraction spin columns were set up with collection tubes and the probe pipetted onto the spin column. After three repeats of washing the column the probe was collected into an elution buffer NE and radioactivity of the probe was checked by scintillation counting. The probes always had total counts of 2-10 X10^6 cpm. to be used in the hybridization step.

Hybridization of cDNA probes to the array. A pre-hybridization step was performed using the hybridization solution provided by the manufacturer and sheared and denatured salmon testes DNA. The DNA was denatured at 95°C for 5 minutes and then quickly chilled on ice, it was then added to the hybridization buffer and kept at 68°C till further use. The Atlas array membrane was wet with

water and then placed in a hybridization bag. 5 mL of pre-hybridization buffer containing blocking DNA was added to the membrane and incubated at 68°C for 30 minutes. The probe was incubated at 95°C briefly then placed on ice. A denaturing solution of 1M NaOH, 10mM EDTA was added incubated at 68°C for 20 minutes. 5 mL of C_ot-1 DNA was added to the probe, followed by neutralizing solution containing 1M NaH_2PO_4 at pH 7.0. The probe was further incubated at 68°C for 10 minutes before adding to the hybridization bag containing the array membrane. Hybridization was performed for 16 hours at 68°C. Membranes were washed at high stringency 2X SSC, 1%SDS at 68°C repeatedly to remove background noise. Without drying the membrane, it was wrapped carefully in saran-wrap and then exposed to the PhosphorImaging screen for 24 hours. The image was read using a Molecular Dynamic Storm 840 (Amersham).

2. Image analysis and statistical analysis of data

The image was generated as a file in ImageQuant and converted to a .tiff type file. The image was then aligned and a data report was generated using the AtlasImage software (Clontech, BD Biosciences). The data report was opened by a spreadsheet reading program such as Microsoft excel and ratios and normalized against house-keeping gene expression levels. Further statistical analysis was performed in the R package.

3. Using Affymetrix arrays for hnRNPA1 over-expression studies.

Cell pellets were collected from IMR90 cells that were transfected as above and washed with 1X PBS. The cell pellets were transferred to the Genomics microarray core facility at Memorial Sloan Kettering Cancer Center for RNA

isolation using Trizol (Invitrogen) and further analysis using the human genome U133 plus 2.0 cDNA array that contains spots for more than 47000 transcripts. Three independent trials of each pGFP control vector and pGFP-A1 over-expression vector were performed. The analysis of absolute expression levels for the genes represented on the array was performed using Affymetrix MicroArray Suite 2.0 software. Scaling and normalization procedures were performed in order to compare results from different experiments. Gene expression levels were analyzed by GCOS (Genomics Core Operating System) and fold change of genes were obtained. For each experiment the results obtained from three independent replicate and identically treated samples were filtered. Genes whose expression was not modulated by a factor of two (either as an increase or as a decrease) were filtered out of the data set. In addition there were genes that were filtered as absent as they exhibited higher P-values and these were not considered for further analysis.

4. Confirmation of gene expression using Real-time PCR

Real time PCR experiments on selected genes were performed using an Applied Biosystems 7500 real-time PCR system using Taqman gene expression assays for the following genes : CCND1 (Hs00277039_m1); CHI3L1 (Hs00609691_m1); CXCL5 (Hs00171085_m1); EDG7 (Hs00173857_m1); PTGS2 (Hs00153133_m1); RAP1A (Hs00234123_m1); RSAD2 (Hs00369813_m1); IL1B (Hs00174097_m1); HNRPA2 (Hs00242600_m1); Human ACTB (beta-actin) Endogenous Control (FAM / MGB (4333762F); Human GAPD (GAPDH) Endogenous Control FAM / MGB (4333764F). The reactions were set up according to the protocol using the universal 10X PCR Taqman mix, at a final reaction volume of 25 μL. The reactions

are standardized to run at the same conditions, 40 cycles of 94°C for 20 seconds, 60°C for 20 seconds and 72°C for 60 seconds. mRNA was quantified using the formula for comparative C_T method, the amount of target normalized to an endogenous reference and relative to a calibrator is given by $\mathbf{2^{-\Delta\Delta C_T}}$.

Results

Gene profiling of IMR90 young and senescent fibroblasts.

The goal of these experiments was to establish a molecular signature of cellular senescence and identify novel targets of post-transcriptional regulation using cDNA arrays.

The quality of the starting material, RNA, is a critical factor in microarray analysis and contaminants such as genomic DNA and in some cases lipids can give rise to non-specific binding (Nair 2003). Several protocols suggest that RNA must be DNAse treated prior to reverse-transcription and in our studies we found that this step depleted the mRNA pool so that we could not get consistent signals even on normally abundant mRNA such as housekeeping genes. Instead we chose to perform a gene-specific PCR on the RNA sample in order to assay whether there was presence of contaminating genomic sequences. Figure 2.1, panel A shows the results of the RNA-PCR in which β-actin primers were used to look for genomic sequences. Taq polymerase enzyme is known to have minimal ability to use RNA as a template and in our studies we saw no PCR products after gel electrophoresis which indicates a complete absence of genomic contamination. In Figure 2.1, panel B, the same RNA samples from IMR90 fibroblasts at young, G0 arrested and senescent stages were reverse-transcribed to cDNA and then analyzed by PCR for β-actin. We were able to observe comparative levels of the transcript in all three conditions as expected for a house-keeping gene. In our studies all RNA samples used were subjected to the same rigorous analysis in order to assure RNA quality.

Furthermore, RNA isolated from young, growth arrested and old cells were analyzed for biomarkers of senescence, see Figure 2.1, and we observed the up-regulation of senescence associated RNAs such as p21. Also since our studies assume that the levels of hnRNPA1 mRNA in cells is low, we established that the levels of HRNPA1 transcript are indeed lowered in old cells and up-regulated in hnRNPA1 over-expressing cells, see Figure 2.2, and panel B.

Figure 2.1 (a) Verification of RNA quality. RNA was checked for genomic DNA contamination by PCR. Panels A and B are 1.5% agarose gels stained with 0.5 μg/mL ethidium bromide. In Panel A, RNA were extracted from young IMR90 (lane 1), G0 arrested IMR90 (lane 2) and senescent IMR90 (lane 3) IMR90 HDF and were tested for DNA contamination by PCR using β-actin primers. In Panel B, the same RNA samples were first reverse transcribed then used as template in a PCR with β-actin primers. The arrow (246 bp) indicates amplified β-actin from cDNA.

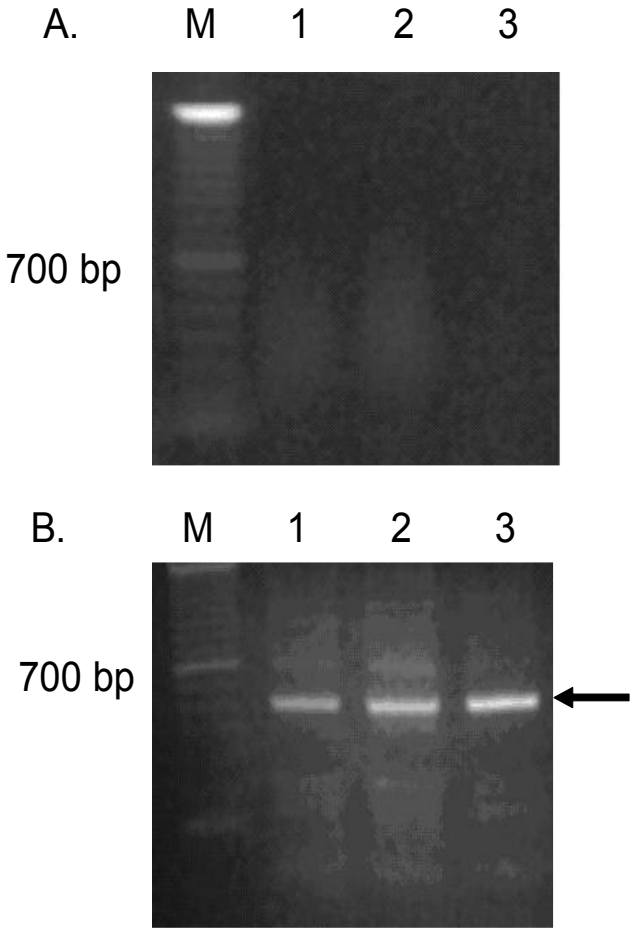
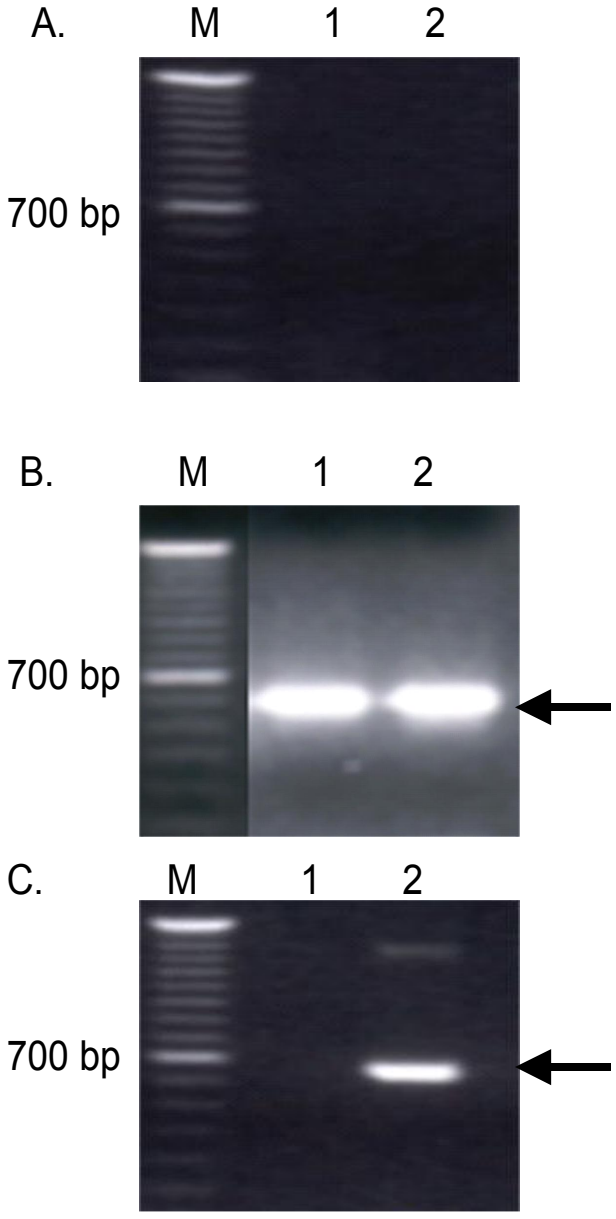

A.
M
1
2
3
700 bp
B.
M
1
2
3
700 bp

Figure 2.2(b) Verification of RNA quality. RNA was analyzed for genomic DNA contamination by PCR. Panels A, B and C are 1.5% agarose gels stained with 0.5μg/mL. In Panel A, RNA extracted from IMR90 transfected with pEGFP (lane 1) and IMR90 transfected with pEGFP-A1 (lane 2) were tested for DNA contamination by PCR using GAPDH primers. In Panel B, the same RNA samples were first reverse transcribed then used as template in a PCR with GAPDH primers. In Panel C, the expression of hnRNP A1 was detected by PCR. The levels of hnRNP A1 mRNA are shown to be much higher in cells transfected with A1 than in the pEGFP control.


A.
M
1
2
700 bp
B.
M
1
2
700 bp
C.
M
1
2
700 bp

Comparison of data sets from young and old IMR90 fibroblasts revealed expected and unexpected genes whose expression was altered by age.

The resulting images of array analysis were collected and analyzed using standard recommended software, AtlasImage from Clontech. In this analysis, the images from ImageQuant were converted from .gel type files to .tiff files and saved. These images could then be opened in AtlasImage software and compared and raw signals obtained. Upon further analysis, it appeared as if there was no consistent housekeeping gene in the included spots on the arrays and it was decided that normalization would be performed to the median of all data points in a gene set. This method is more reliable because house-keeping genes show inherent variation depending on experimental conditions and more consistent house-keeping genes would have been required for comparisons. Other methods for normalization include normalizing to the sum total of signals on the array. This method relies on the fact that the total mRNA made by cells under various conditions does not really change and can be a reliable constant when the relative signal ratio of gene expression is compared under different experimental conditions. This method was not applicable to our studies because of increasing heterogeneity in cell populations as cell cultures grow older and that our array studies focused on a subset of genes and did not represent all genes being expressed in cells at the time. Hence, it was decided that normalization methods would be applied according to the median of each data set and further analysis performed based on correlation values in each data set. Figure 2.3, depicts the gene expression patterns of 192 genes in young IMR90, G0 arrested IMR90 and old IMR90 fibroblasts. This displays the amount of

information obtained from all experiments and emphasizes the need for cluster analysis of data to sort patterns of gene expression that are similar.

Figure 2.3. Representation of data obtained from comparison of 192 genes from cDNA arrays. Conditions on the x-axis are 1, IMR90 young, 2, IMR90 G0 arrested and 3, IMR90 senescent. This figure was generated after data transformation and normalization the median of each gene data set, through GeneSpring (Silicon Genetics).

Figure 2.3

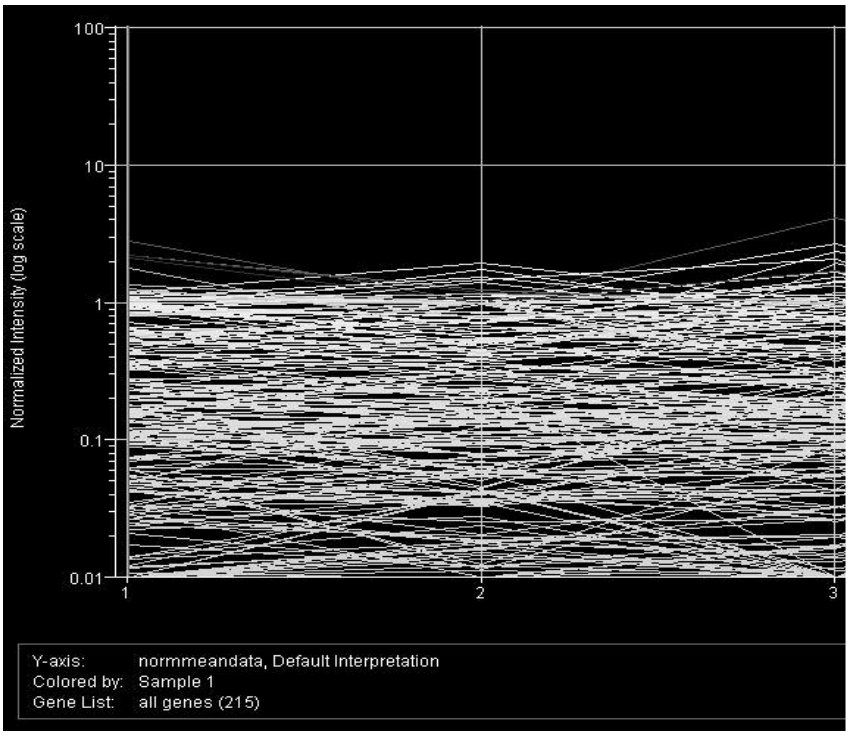
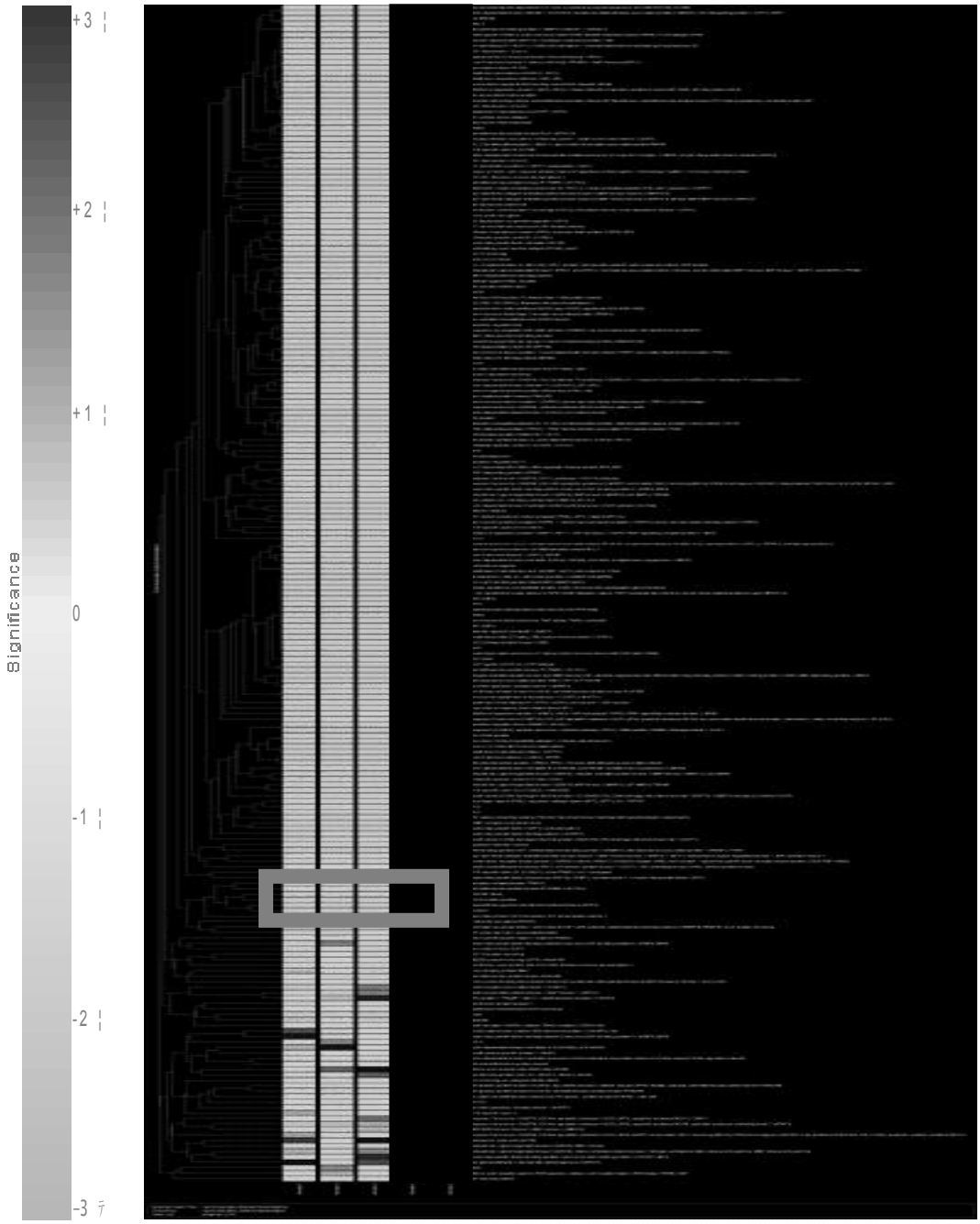

100
10
1
0.1
0.01
Normalized Intensity (log scale)
1
2
3
Y-axis: normmeandata, Default Interpretation
Colored by: Sample 1
Gene List: all genes (215)

Figure 2.4. A and B Heirarchical cluster analysis of genes, genes with similar patterns of expression were grouped together and a similarity tree can be seen (in blue) that shows the distance of similarity and dissimilarity between genes. The first column are 192 genes analyzed in young IMR90, second column are the same set of genes analyzed in G0 arrested cells and third column are genes from IMR90 senescent fibroblast samples. Cyclin D1, boxed in red, is up-regulated in senescent fibroblasts, and this is depicted by the green colors of the boxes in the first two columns and red color box in the last column. Figure 2.4 B depicts the same figure at higher resolution, to see individual gene expression patterns.

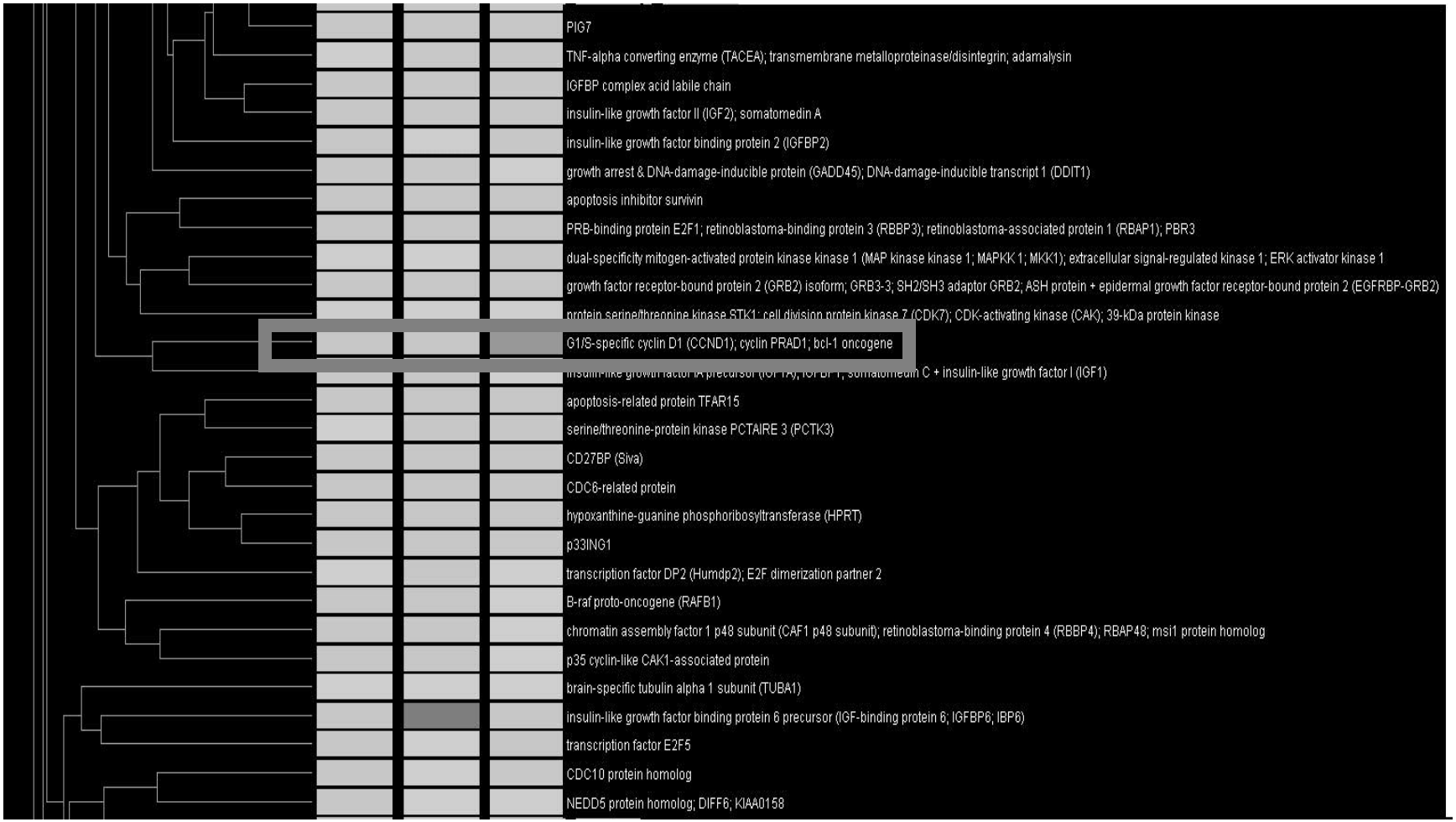
Note on Scale: The scale to the left of the figure below is a key to Figure 2.4, it shows that the colors indicate a significance increase of +3 in red and decrease of -3 in green over the median of the data set.


Significance
+3
+2
+1
0
-1
-2
-3

In Figure 2.4, all data sets are shown after hierarchical cluster analysis and as seen marked in yellow, most gene expression are unchanged in age. However, when observed more closely there are some genes that did show age-related changes. Cyclin D1 (also called bcl-1 oncogene) is a G1/S phase cyclin that is tightly regulated in cell cycle and is a known oncogene. It is up-regulated in IMR90 senescent fibroblasts and not in G0 arrested cells. Figure 2.4, B, shows the heirarchical cluster analysis in the region around this gene. The blue similarity tree to the left of the figure indicates that there are other genes with similar pattern of gene expression but not with comparable significance of expression. This is an important point when analyzing gene expression patterns and in the attempt to assemble functional pathways and relationships, new genes can be identified in a pathway that is already well studied.

In our studies most genes were found to have low expression levels and from the heirarchical cluster analysis this can be seen in Figure 2.4. However, many genes are normally expressed at very low levels in cells and even though they may not express transcripts at comparable levels to other genes, small changes in the number of transcripts available in cells can have significant effects on the amount of protein produced. This can alter gene expression patterns of other genes which then alter the overall phenotype of a cell or its ability to respond to biological and environmental influences. As a result we were careful not to ignore genes that were expressed at low levels, instead we analyzed all genes that showed changes at the transcriptional level.

Figure 2.4 B.


PIG7
TNF-alpha converting enzyme (TACEA); transmembrane metalloproteinase/disintegrin; adamalysin
IGFBP complex acid labile chain
insulin-like growth factor II (IGF2); somatomedin A
insulin-like growth factor binding protein 2 (IGFBP2)
growth arrest & DNA-damage-inducible protein (GADD45); DNA-damage-inducible transcript 1 (DDIT1)
apoptosis inhibitor survivin
PRB-binding protein E2F1; retinoblastoma-binding protein 3 (RBBP3); retinoblastoma-associated protein 1 (RBAP1); PBR3
dual-specificity mitogen-activated protein kinase kinase 1 (MAP kinase kinase 1; MAPKK 1; MKK1); extracellular signal-regulated kinase 1; ERK activator kinase 1
growth factor receptor-bound protein 2 (GRB2) isoform; GRB3-3; SH2/SH3 adaptor GRB2; ASH protein + epidermal growth factor receptor-bound protein 2 (EGFRBP-GRB2)
protein serine/threonine kinase STK1; cell division protein kinase 7 (CDK7); CDK-activating kinase (CAK); 39-kDa protein kinase
G1/S-specific cyclin D1 (CCND1); cyclin PRAD1; bcl-1 oncogene
C + insulin-like growth factor I (IGF1)
apoptosis-related protein TFAR15
serine/threonine-protein kinase PCTAIRE 3 (PCTK3)
CD27BP (Siva)
CDC6-related protein
hypoxanthine-guanine phosphoribosyltransferase (HPRT)
p33ING1
transcription factor DP2 (Humdp2); E2F dimerization partner 2
B-raf proto-oncogene (RAFB1)
chromatin assembly factor 1 p48 subunit (CAF1 p48 subunit); retinoblastoma-binding protein 4 (RBBP4); RBAP48; msi1 protein homolog
p35 cyclin-like CAK1-associated protein
brain-specific tubulin alpha 1 subunit (TUBA1)
insulin-like growth factor binding protein 6 precursor (IGF-binding protein 6; IGFBP6; IBP6)
transcription factor E2F5
CDC10 protein homolog
NEDD5 protein homolog; DIFF6; KIAA0158

Other methods of analysis of gene expression patterns are K-means clustering and self-organizing maps. We performed both types of analysis to group the patterns of gene expression observed and found that similar results can be obtained by other methods of cluster analysis (data not shown). As mentioned, CCND1, cyclin D1 expression levels were observed to increase in senescent IMR90, other genes also regulated by age were analyzed further, the INK4a locus being of particular interest because the products of this gene are alternatively spliced and involved in cell cycle arrest mechanism that are important to the senescent phenotype.

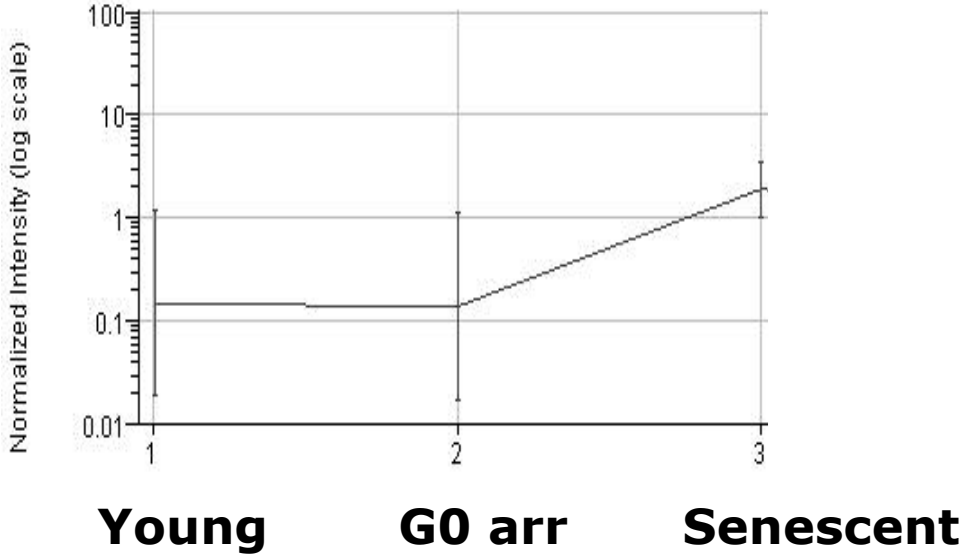
Analysis of individual genes from the age-genomic study and validation of results.

There are many statistical issues related to microarray studies and it is important to validate the findings using independent methods. It is not feasible to validate all observations, however, using alternate methods such as semi-quantitative PCR and real-time PCR on a selection of genes can help confirm previously observed changes and validate microarray data. In order to do this, we performed further analyses on specific genes such as cyclin D1 and the products of INK4A locus. In Figure 2.5 A, the levels of CCND1 are shown to change in old cells, as observed by gene array studies. In Figure 2.5 B, real-time PCR was performed on CCND1 to validate the observed changes. In other studies (Iyer 1999) using such validation methods it has been observed that the fold changes in comparisons can be changed depending on the method used. Therefore, the changes seen in panel A and B show that the pattern of gene expression is affected by age

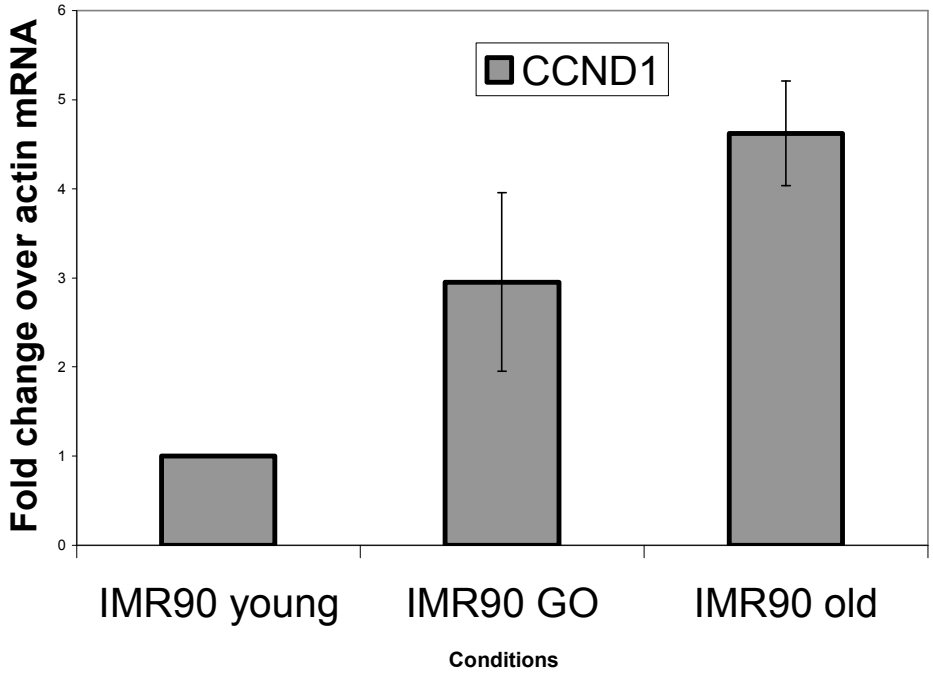
but as neither method addresses the absolute levels of CCND1 transcript in cells there are method based differences in fold changes.

Figure 2.5. Validation of CCND1 gene expression changes by real-time PCR. Panel A is a representative graph for the gene expression levels from array studies. In the graph the levels of log signal intensity are shown, IMR90 young, G0 arrested and senescent fibroblasts were used in these studies. RNA was isolated and parallel samples were processed for both cDNA array (panel A) analyses and real-time PCR (panel B). Array experiments were repeated 5 times for each condition and real-time PCR was performed on 3 of the replicates. Error bars indicate standard error.

Figure 2.5A. Array results



B. Real-time PCR results



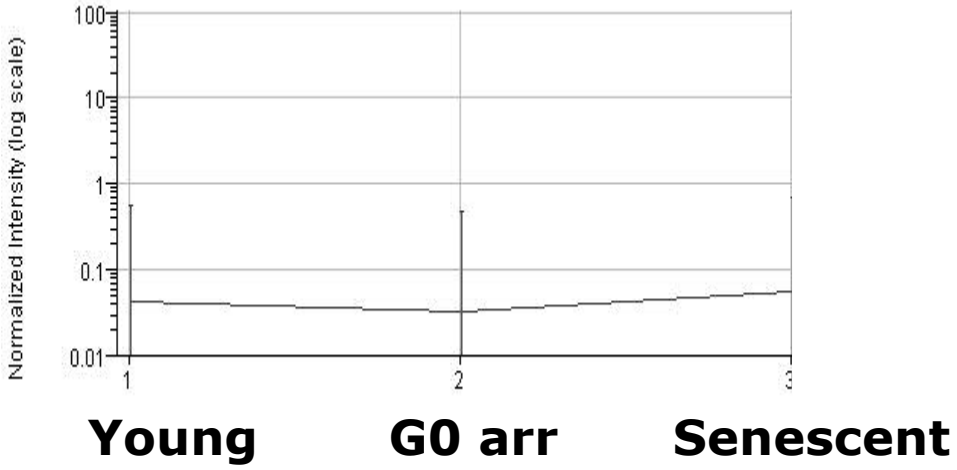
In the above figure it can be seen that there is a fold change in old cells at the level of transcription of CCND1. From array data there would appear to be >100 higher fold change in signal in old cells, whereas the levels of CCND1 transcript in old cells from real-time data do not indicate the same fold change at ~4 times the amount of transcript that is present in young IMR90. There is also higher variability in signal intensity in both young and growth arrested IMR90 in the case of array results, suggesting sensitivity of the method to variations in hybridization signals and membrane arrays. Also in the case of growth arrested cells there appears to be some variation in signal that is consistent between both methods of validation, which indicates that the variation has a biological reason and may be a result of cell population heterogeneity. However, the overall pattern of gene expression change appears to be age-dependent and not due to cell growth characteristics because the change in old cells is dramatic and consistent. This was a surprising result because CCND1 is induced to pass cells through G1 and to S phase of the cell cycle. Old cells are terminally growth arrested with the majority of cells having G1 levels of genomic content. It was interesting that the levels of CCND1 transcript were higher in old cells which may have implications for post-transcriptional processing events and translational control of the expression of cyclin D1.

The other locus that we investigated further, at the level of translation of mRNA was the INK4A locus. As mentioned in the introduction, this gene produces more than one transcript and the proteins made are dissimilar in structure but similar in function. Both products $p16^{INK4a}$ and $p14^{ARF}$ regulate the cell cycle through other tumor suppressors, pRb and p53. It was interesting to note that the

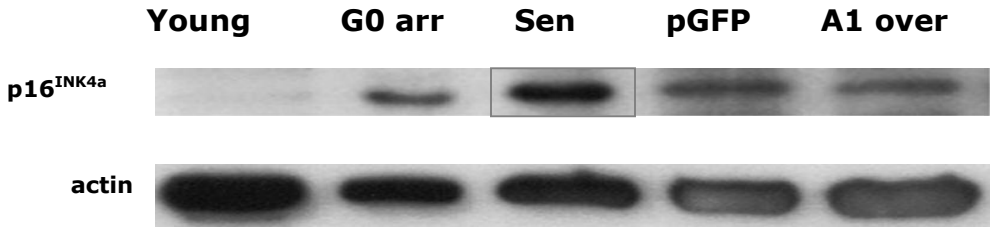
levels of transcript of these two genes were not changed in IMR90 as they age. However, immunoblot analysis on the protein levels produced by these isoforms revealed that both transcripts are further processed post-transcriptionally, at the level of mRNA transport and stability, a result that was indicated in earlier studies by Zhu 2002, and at translation to produce higher levels of $p16^{INK4A}$ protein in old IMR90cells, and lowered levels of $p14^{ARF}$ in old IMR90 cells.

Figure 2.6. Analysis of INK4A locus. Panel A is a representative graph for the gene expression levels from array studies. In the graph the levels of log signal intensity are shown, IMR90 young, G0 arrested and senescent fibroblasts were used in these studies. RNA and protein was isolated and parallel samples were processed for both cDNA array (Panels A and C) and western blot analysis (Panels B and D). Array experiments were repeated 5 times for each condition. Error bars indicate standard error. Western analysis was performed using standard protocols.

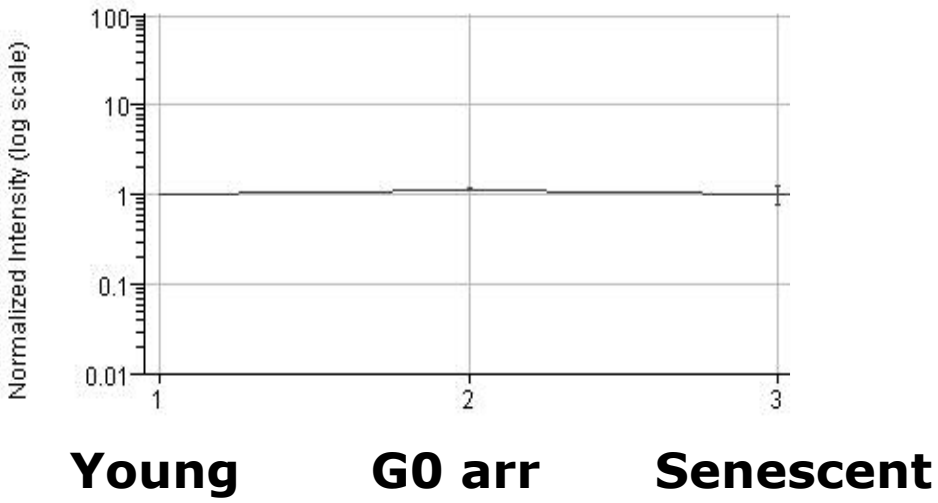
Figure 2.6A. Array results on $p16^{INK4a}$ mRNA.



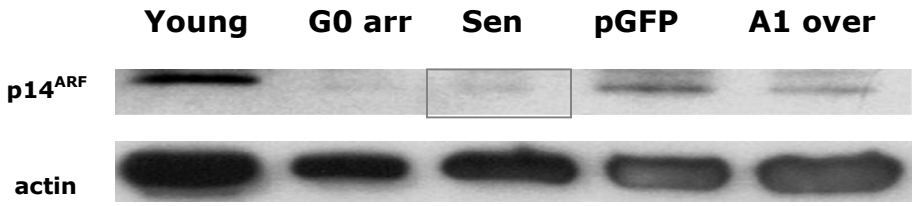
B. Protein levels of $p16^{INK4a}$ are higher in old IMR90.



C. Figure 2.6 (cont'd) Array results on $p14^{ARF}$ mRNA.



D. Protein levels of $p14^{ARF}$ are lower in old IMR90.



As seen in Figure 2.6. There are age related changes that occur at the protein level in senescent fibroblasts that do not reflect the changes if any at mRNA levels. In fact, the results of array analysis indicate the levels of both transcripts at steady state are comparative in young, G0 arrested and senescent fibroblasts. If the signal intensities between the two isoforms are compared, then the level of mRNA of $p14^{ARF}$ may be >100 fold higher than the overall transcription level of $p16^{INK4a}$ mRNA. This would indicate a level of gene regulation that precedes the formation of mRNA, that is at the level of heterochromatin and start of transcription. The data also show that the G0 arrest state of senescent cells can be significant if the change in levels of protein in G0 arrested cells reflect functional changes. Therefore when cells are growth arrested, there does not appear to be a change at the transcription, but it is followed by a dramatic change in the levels of the protein. In G0 arrested cells, $p16^{INK4a}$ protein immediately starts to be synthesized even though the levels of transcript are low, indicating that the mRNA may acquire stability or some other modification that results in an increase of translation. The same effect to a more dramatic degree could account for the higher levels of $p16^{INK4a}$ in senescent IMR90, but that $p16^{INK4a}$ protein may have additional stability in old cells is indicated. So there is a mechanism to keep the levels of $p16^{INK4a}$ mRNA low in young cells and also inactive to translate. As the cells respond to growth signals such as deprivation of serum, the mRNA is triggered and translation occurs to give a stable and functional protein that checks cell growth. In the case of $p14^{ARF}$, the levels of

transcript are relatively high in all cells, which could be tissue specific or cell type specific. In young cells the levels of mRNA are high as is protein indicating that $p14^{ARF}$ may have other functions in young cells that are independent of growth regulation. As cells are growth arrested by serum deprivation some change occurs to destabilize the mRNA and prevent its translation. Also there could be events that destabilize any available $p14^{ARF}$ protein, further suggesting that $p14^{ARF}$ may have more than one function in young cells. As cells age, the levels of $p14A^{ARF}$ protein are still maintained at a low level which would indicate that $p14^{ARF}$ is not as important as $p16^{INK4a}$ in IMR90 in growth control. It is important to always consider the effects of different promoters on the levels of transcription of these isoforms because even though this locus provides an elegant and clear substrate for regulation at the level of alternative splicing, regulation of INK4a gene products may not always be subject to alternative splicing as a mechanism to switch between INK4a and ARF proteins.

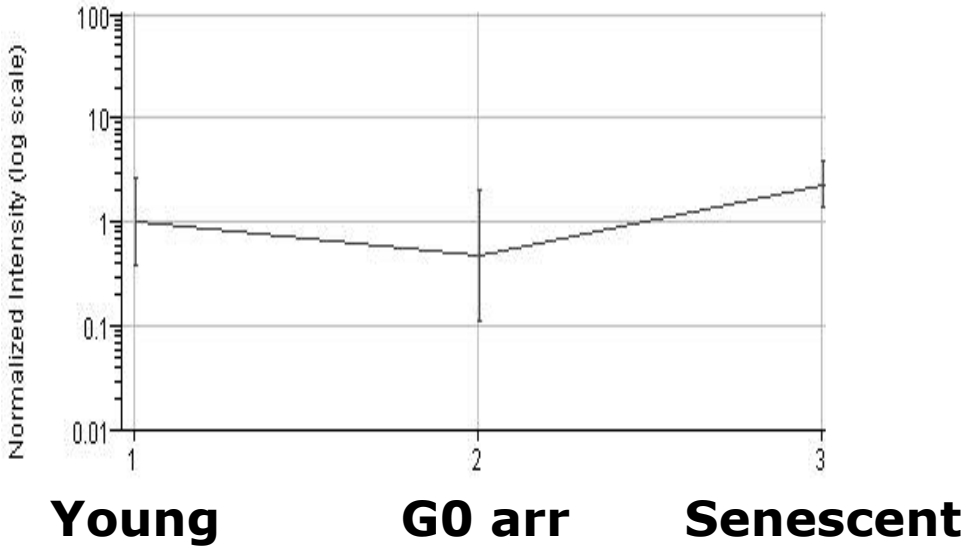
Validation of apoptosis related genes at the protein level.

As described for a selected set of genes above, we also studied the protein levels of another gene that may be altered in cells as they age. The caspase-7 gene showed the most significant change in gene expression level from our genomics studies and we analyzed the translational profile of this gene. Caspases are proteins that are activated and activate processes in the cells after they have been committed to die. Caspase-7 mRNA was found to be up-regulated in old IMR90 (see Figure 2.7 panel A) and down-regulated at the protein level in old IMR90 (see Figure 2.7, panel B). Most studies on the levels of caspases in aging have focused on protein

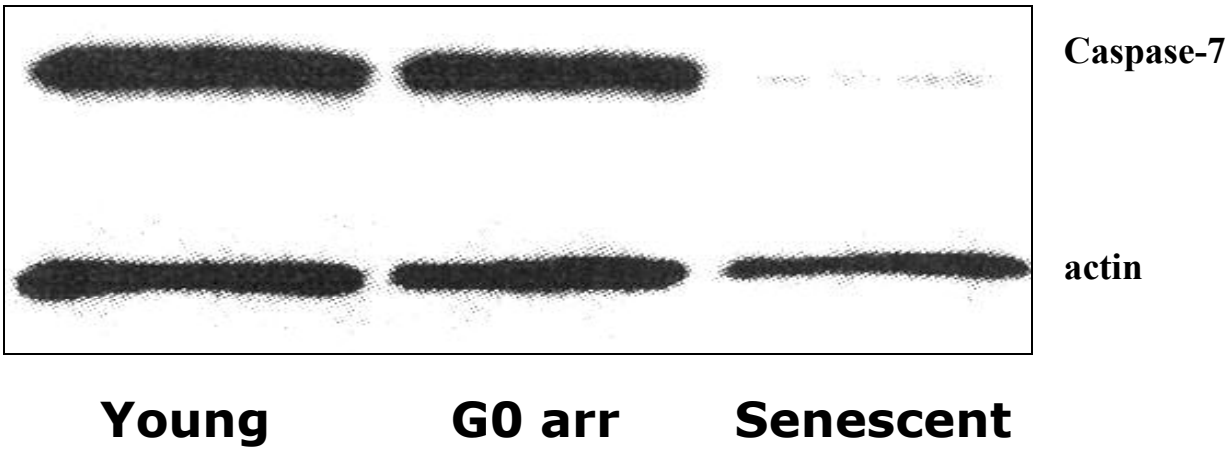
levels and activation of caspase, that trigger downstream effects that will result in death of cells. In our studies we were interested in changes of mRNA through alternative splicing and other mRNA processing mechanisms that would provide insight into the role of such proteins in cells as they age and how they are regulated.

Figure 2.7. Analysis of caspase-7 mRNA and protein profiles in IMR90. Panel A is a representative graph for the gene expression levels from array studies. In the graph the levels of log signal intensity are shown, IMR90 young, G0 arrested and senescent fibroblasts were used in these studies. RNA and protein was isolated and parallel samples were processed for both cDNA array (Panel A) and western blot analysis (Panel B). Array experiments were repeated 5 times for each condition. Error bars indicate standard error. Western analysis was performed using standard protocols.

Figure 2.7A. Array results on caspase-7 mRNA.



B. Protein levels of caspase-7 are lower in old IMR90



In our studies, we find that the caspase-7 transcription is slightly up-regulated in old IMR90 and down-regulated at the protein level, suggesting a regulatory mechanism that suppresses translation of the protein via post-transcriptional processing or mRNA transport. Also this gene showed a sharp down-regulation of protein levels in aged IMR90 that was distinct and opposite to what was observed at the mRNA level. In comparison the levels of mRNA in G0 arrested cells appears to be variable but lowered the level of protein is maintained in G0 cells, indicating that the protein must be stable in young cells even when they are insulted by serum deprivation. This stability of the protein may be lost as cells age and this locus provides us with an interesting case study for an aging biomarker that is also a definitive marker for the ability of a cell to undergo death in response to environmental stimuli.

Summary of age-genomics study by functional grouping of genes.

The importance of such a study using a genomics approach to studying cell growth regulation during aging is through the establishment of a signature of genes that are up-regulated and down-regulated exclusively by age. The following Table 2.1, lists the genes that were found to be changed by age and some that were not. The goal of this study is to order genes by functional groups and patterns of gene expression and to provide insight into possible genes that have not yet been studied in the age context.

Table 2.1 Expression of Cell cycle and growth regulatory genes is altered in cellular senescence. The following data was generated by standard statistical analysis of correlation between 6 independent replicates of senescent and young IMR90.Fold change was calculated using GeneSpring software and statistical methods, genes that did not show more than two fold change and less than 0.5 fold change were filtered as unchanged, denoted by - - in the table.

Genes up-regulated in senescence	**Fold change**
G1/S-specific cyclin D1 (CCND1); cyclin PRAD1; bcl-1 oncogene	20.3
Cell division G1/S-specific cyclin E (CCNE)	16.1
G1/S-specific cyclin D2 (CCND2)	4.1
Ion protein kinase 3	2.5
Genes down-regulated in senescence	**Fold change**
G2/mitotic-specific cyclin G1 (CCNG1; CYCG1)	0.6
p35 cyclin-like CAK1-associated protein	0.4
Genes unchanged in senescence	**Fold change**
cell division control protein 2 homolog (CDC2) cyclin-dependent kinase 1	--
cyclin-dependent protein kinase 2 (CDK2); p33 protein kinase	--
cell division protein kinase 5 (CDK5)	--
cell division protein kinase 7 (CDK7); CAK	--
cyclin-dependent kinase 5 activator isoform	--
cell division cycle protein 25A (CDC25A)	--
CDC25B; CDC25HU2; M-phase inducer phosphatase 2	--
CDC25C; M-phase inducer phosphatase 3	--
CDC-like kinase 1 (CLK1)	--
CDC-like kinase 2 (CLK2)	--
CDC-like kinase 3 (CLK3)	--
cell division protein kinase 9 (CDK9)	--
G2/mitotic-specific cyclin A (CCNA; CCN1)	--
G2/mitotic-specific cyclin B1 (CCNB1)	--
G1/S-specific cyclin C	--
cyclin G2 (CCNG2)	--
cyclin H (CCNH)	--
cyclin-dependent kinase inhibitor 1 (CDKN1A) p21	--
cyclin-dependent kinase 4 inhibitor (CDK4I; CDKN2); p16-INK4a	--
cyclin-dependent kinase 4 inhibitor B (CDKN2B); p15-INK4B	--
cyclin-dependent kinase 4 inhibitor D (CDKN2D); p14-INK4D	--

Table2.1 (continued) Apoptosis genes

Genes up-regulated in senescence	Fold change
caspase-7 precursor (CASP7)	3.7
BAD protein	2.0
Genes down-regulated in senescence	**Fold change**
caspase-4 precursor (CASP4); ICH-2 protease	0.4
caspase & rip adaptator with death domain (CRADD), (RAIDD)	0.4
Genes unchanged in senescence	**Fold change**
BCL-2 binding athanogene-1 (BAG-1)	--
activator of apoptosis harakiri (HRK)	--
caspase-2 precursor (CASP2); ICH-1L protease + ICH-1S protease	--
caspase-3 (CASP3); apopainCPP32; YAMA protein	--
caspase-9 precursor (CASP9)	--
caspase-10 precursor (CASP10) (FLICE2)	--
tumor necrosis factor receptor 1-associated death domain protein TRADD)	--
receptor interacting protein cell death protein RIP	--
tumor necrosis factor precursor (TNF-alpha; cachectin)	--
tumor necrosis factor-beta (TNF-beta)	--
fas antigen ligand (FASL)	--
TNF-related apoptosis inducing ligand (TRAIL)	--

Table 2.1, indicates known genes and some new genes that have age-related changes in gene expression but also those genes that are known to be changed in an age-related context that do not show changes at mRNA level. This result confirms the results of previous studies using genomics to study aging that the proteome of a cell does not correlate with its transcriptome.

Genomic study on RNA binding proteins using Atlas array HA 1.2 III.

We also investigated, with the help of cDNA arrays, levels of transcription of RNA binding proteins that were altered in senescent IMR90 fibroblasts. We found that using an array with ~1200 spots, to compare the gene expression signatures between IMR90 young and old, showed that there are some RNA processing genes that are themselves regulated by age, hnRNPA1 as we know is lowered as cells age. We were able to compare ratio fold change using IMR90 young as the calibrator and the following table is a summarization of the results found.

Table 2.2. Summary of gene expression changes in RNA metabolism genes in senescent IMR90 fibroblasts. Three independent trials of IMR90 young and old cells were averaged and compared and the fold changes calculated by standard statistical methods. In this data unchanged genes were at a fold change of 1.0 with respect to IMR90 young fibroblasts.

Genes up-regulated in senescence	Fold change
Heterogeneous nuclear ribonucleoprotein M (hnRNP M)	3.0
Heterogeneous nuclear ribonucleoprotein F (hnRNP F)	2.0
Genes down-regulated in senescence	**Fold change**
Heterogeneous nuclear ribonucleoprotein A1 (hnRNP core protein A1)	0.3
Dead box protein 3, y-chromosomal	0.3
Heterogeneous nuclear ribonucleoprotein L (hnRNP L)	0.4
Heterogeneous ribonucleoprotein C (hnRNP core protein C)	0.4
Heterogeneous nuclear ribonucleoprotein A0 (hnRNP A0)	0.5
Heterogeneous nuclear ribonucleoproteins A2/B1 (hnRNP A2 and B1)	0.5
Heterogeneous nuclear ribonucleoprotein H (hnRNP H)	0.5
Splicing factor U2AF 35 kDa subunit (U2 auxiliary factor 35 kDa subunit)	0.6

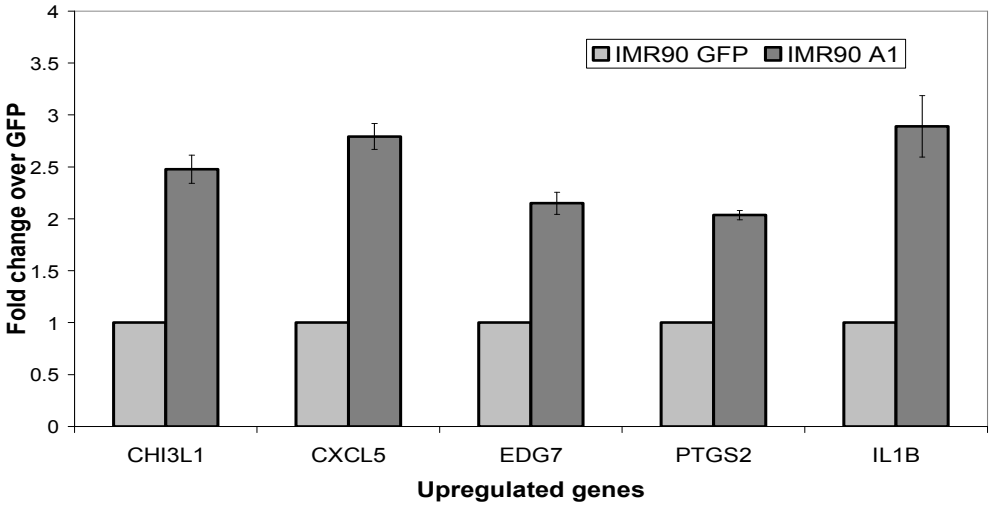
Genes unchanged in senescence	Fold change
Eukaryotic initiation factor 4a-II (eIF-4a-II)	1.0
Splicing factor, arginine/serine-rich 3 (splicing factor SRP20)	1.0
Splicing factor, arginine/serine-rich 2 (splicing factor SC35) (SC-35)	1.0
Heterogeneous nuclear ribonucleoprotein U (SAF-A)	1.0
Cleavage stimulation factor, 64 kDa subunit (CSTF 64 kDa subunit)	1.0
Heterogeneous nuclear ribonucleoprotein G (HNRNP G)	1.0
Hu-antigen C	1.0

Genomics of hnRNPA1 over-expressing cells identify new targets for RNA processing and aging.

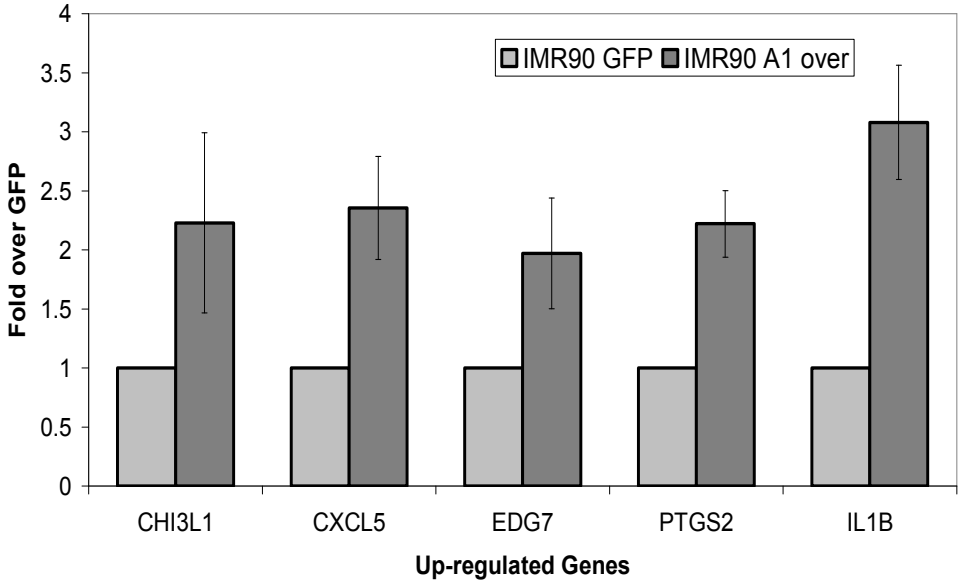
The goal of this study was to identify changes in gene expression that were affected both by age and post-transcriptional mechanisms. In order to accomplish this objective we used hnRNP A1 over-expressing cells as a changing factor in cell, whose specific role is to affect mRNA levels of genes. The hypothesis was that the changes observed after over-expressing hnRNPA1 in IMR90 cells would potentially be opposite to the effects of lowered hnRNPA1 in cells as seen in senescence. In addition senescent cells have alterations of localization and post-translational modification that can lead to altered RNA binding activity and therefore altered gene expression. Affymetrix, whole genome array, U133 plus 2.0 arrays that provide analysis of ~47000 transcripts were used to compare samples that contained the vector pGFP plasmid and pGFP-hnRNPA1 plasmid. RNA samples were analyzed for genomic contamination, see Figure 2.2, housekeeping gene glyceraldehyde-3 phosphate dehydrogenase (GAPDH) and hnRNPA1. Gene expression levels were analyzed using Affymetrix and GCOS (Genomics Core Operating System) and fold changes in three trials obtained. The following Figure 2.8 shows 5 genes that were consistently up-regulated in hnRNPA1 over-expressing cells.

Figure 2.8. Affymetrix array analysis and real-time PCR validation of gene expression patterns on up-regulated genes after hnRNPA1 over-expression in IMR90 fibroblasts. IMR90 fibroblasts were transfected with pGFP control vector and pGFP-hnRNPA1 expression construct. RNA was isolated and reverse-transcribed to cDNA. Hybridization and image analysis followed and signals were obtained. The results of the analysis are shown here. In panel A and B, the calibrator is IMR90 young cells that carry the pGFP vector control, fold changes are indicated on the y-axis and error bars indicate standard error. In panel C, a comparison using real-time PCR of the same set of genes between young and old IMR90 was performed. In this graph the y-axis indicates fold change over young IMR90 and error bars indicate standard error.

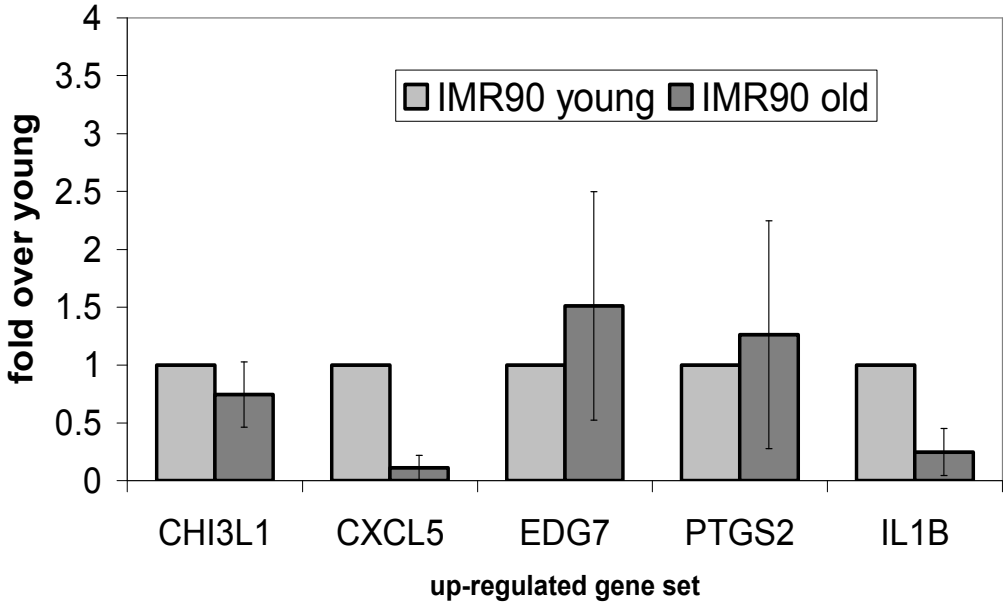
Figure 2.8 A. Affymetrix array result showing up-regulated genes



B. Real time PCR results on up-regulated genes



C. Real-time PCR on young and old IMR90, on genes that were up-regulated by hnRNPA1 over-expression



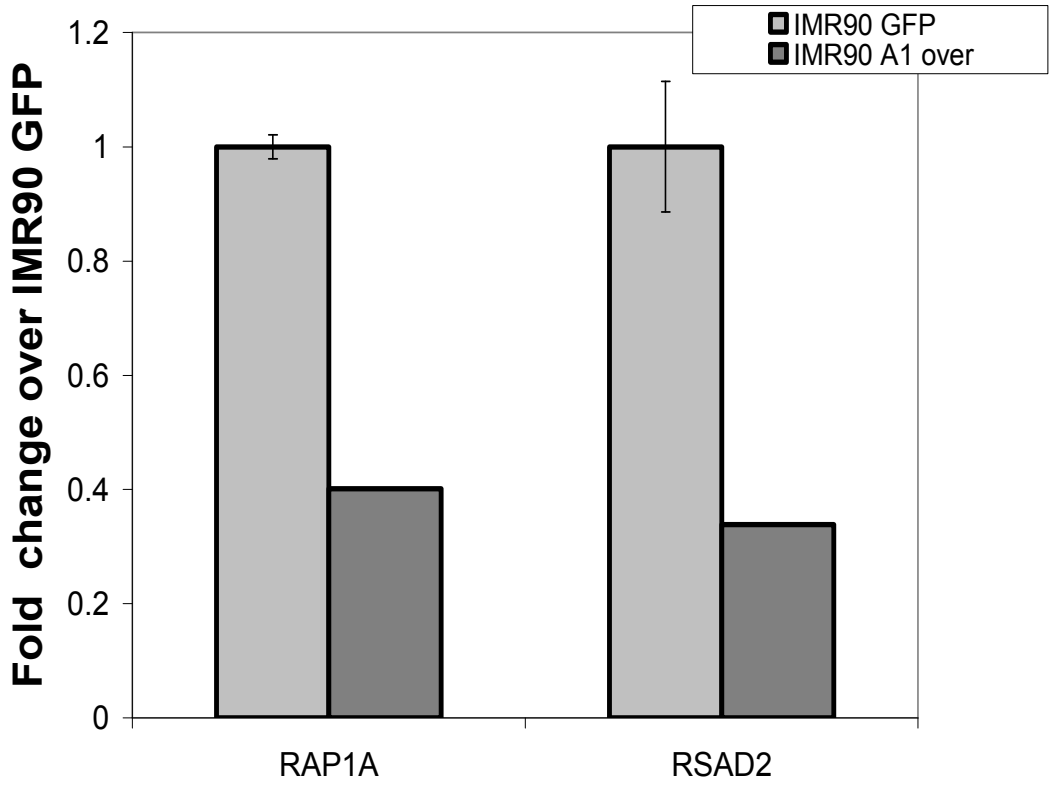
We found that 5 genes were consistently up-regulated at the mRNA level in IMR90 that over-express hnRNPA1. The overall results show that the fold change in mRNA levels using a hybridization based technique such as array analysis and an amplification based technique such as real-time PCR can give similar output, increasing the level of confidence in the results. The following genes were found to be up-regulated in IMR90 hnRNPA1 over-expressing cells: CHI3L1, CXCL5, EDG7, PTGS2 and IL1B. The following genes were found to be consistently down-regulated in IMR90 hnRNPA1 over-expressing cells: RAP1A and RSAD2. Our goal was to identify changes in gene expression affected by hnRNP A1 and in order to do so we analyzed mRNA levels of RAP1A and RSAD2 with the help of real-time PCR in young and old IMR90 fibroblasts. In old cells the fold change of RAP1A as compared to young cells was 12.6 ± 8 and that of RSAD2 was >100 fold, a result that would have to be re-investigated but could reflect biological changes in mRNA and the high level of variation that could be due to a mixed cell population.

The observations on the effects of age on the same set of genes that are up-regulated by hnRNPA1 over-expression showed that there is variation in gene expression associated with age and that this variation may be the result of many diverse mechanisms of gene regulation not merely those that involve RNA processing and this is consistent with previous literature (Kumar 1992). The specific genes that showed a down-regulation with age were CXCL5 and IL1B, both of which showed a fold change of less than 0.5. This provides us with potential genes for further study which may follow the pattern of our hypothesis, that is

genes are regulated by a variety of processes in aging cells and that RNA processing has a definitive genetic regulatory role in the senescence mechanism.

Figure 2.9 Affymetrix array analysis and real-time PCR validation of gene expression patterns on down-regulated genes after hnRNPA1 over-expression in IMR90 fibroblasts. IMR90 fibroblasts were transfected with pGFP control vector and pGFP-hnRNPA1 expression construct. RNA was isolated and reverse-transcribed to cDNA. Hybridization and image analysis followed and signals were obtained. The results of the analysis are shown here. The calibrator is IMR90 young cells that carry the pGFP vector control, fold changes are indicated on the y-axis and error bars indicate standard error.

Figure 2.9 Affymetrix array results for genes that were down-regulated by hnRNPA1 over-expression



Discussion

Aging is a complex and multi-faceted phenotype that is an area of intense investigation and there is already a substantial body of evidence that points to gene expression as a powerful program to cause senescence and maintain them as terminally growth arrested. It was our endeavor to identify gene expression patterns in senescent IMR90 as an effort to establish the molecular signature of cells as they undergo senescence in a time-dependent manner after being in culture for more than 60 population doubling. This is important because many other studies on the gene expression of replicative senescent cells use induced senescence or stress induced premature senescence (SIPS) as an aging phenotype and assume that these cells will represent aging cells in tissue and organisms. We believe that our system of culturing cells to their replicative limit will help define the genetic programs that are inherent to a "molecular clock" mechanism, this being the closest model system to aging cells in vivo. In these studies we identified up-regulated genes such as CCND1 that is known to have a role in cellular senescence as suggested by previous studies (Afshari 1993, Morisaki 1999). That cyclin D1 is a known oncogene (isolated as PRAD1 oncogene by mapping sites of amplification in parathyroid adenomas) and is regulated by cell cycle is well documented, however there is little information regarding its up-regulation in senescent fibroblasts at the mRNA level (Solomon 2003). Cyclin D1 is also alternatively spliced to produce cyclin D1b which is completely homologous to the more abundant form cyclin D1a but differs in the C-terminal sequence (Solomon 2003). The locus is capable of producing more than one isoform and there is also little information regarding the

decision that causes this event of gene regulation in cells. In fact, cyclin D1b is constitutively nuclear and is present in S-phase. The role of cyclin D1 protein is to regulate pRb phosphorylation and inactivation. There are two studies that report conflicting results on the regulatory activity of pRb by cyclin D1b (Lu 2003, Solomon 2003) and therefore it would be interesting to investigate the role if any of this alternatively spliced form in senescent fibroblasts as a potential regulatory player in the senescent mechanism.

Another target gene for study is caspase -7. This gene encodes a protein which is a member of the cysteine-aspartic acid protease (caspase) family. Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis. Caspases exist as inactive pro-enzymes which undergo proteolytic processing at conserved aspartic residues to produce two subunits, large and small, that dimerize to form the active enzyme. The precursor of this caspase is cleaved by caspase 3 and 10. It is activated upon cell death stimuli and induces apoptosis. Alternative splicing results in four transcript variants, encoding three distinct isoforms (Del Bello 2003). The role of this protein in senescence is not known and could provide an interesting target for RNA binding proteins and their effect at the post-transcriptional processing level. As mentioned earlier, the protein is much lower in senescent fibroblasts indicating that there may be a level of regulation at post-transcriptional processing that suppresses the production of precursor caspase-7.

Another study on genomics of aging identified RNA binding proteins as potential targets for a role in aging fibroblasts and these are: hnRNP M and hnRNP

F (see table 2.2). hnRNP F is a protein that has three repeats of quasi-RRM domains that bind to RNAs which have guanosine-rich sequences. This protein is very similar to the family member hnRNP H. In a study on splicing of bcl-x (a member of the bcl-2 family of apoptosis genes), into isoforms Bcl-X_S and Bcl-X_L, when hnRNP F was over-expressed there was a preference for formation of the pro-apoptotic form Bcl-X_S (Garneau 2005). hnRNP M is a gene that encodes a protein that has three repeats of quasi-RRM domains that bind to RNAs. Multiple alternatively spliced transcript variants are known for this gene but only two transcripts have been isolated (Datar 1993). It is not known whether this gene can affect mRNA processing of other genes. It is interesting that hnRNP H another gene that is known to bind sequences that regulate splicing of Bcl mRNA is down-regulated in senescent IMR90 (Garneau 2005) but this may reflect a difference in regulatory mechanisms for the expression of these very similar proteins. Also there may be some redundancy in their roles in cell metabolism. Overall the results of this study emphasized that genomics studies can identify new and unknown targets of gene expression regulation and also that the protein levels and activities of each gene must be further investigated to get a complete idea of their role in cellular senescence.

The identification of new genes as putative targets for hnRNPA1 regulation by the simple method of over-expressing hnRNPA1 in IMR90 cells is a novel finding in the field of alternative splicing regulation of gene expression. In this study we were able to analyse mRNA levels of 5 genes that were up-regulated in hnRNPA1 over-expressing IMR90 fibroblasts. These were: CXCL5, CHI3L1,

IL1B, PTGS2 and EDG7. CXCL5 and IL1B are genes that encode proteins involved in inflammation response, an interesting observation as fibroblasts are also known to have a role in inflammation of tissues. CXCL-5 was first identified as ENA78 and the gene is very similar to the gene for IL-8 (Chang 1994). The protein encoded by the CXCL5 gene is an inflammatory chemokine that belongs to the CXC chemokine family. This chemokine is produced concomitantly with interleukin-8 (IL8) in response to stimulation with either interleukin-1 (IL1) or tumor necrosis factor-alpha (TNFα) and binds to IL-8 type B receptor in activating neutrophils where it is a potent chemotaxin (Imaizumi 2004). An interesting observation about CXCL5 and IL1B mRNA expression is that in IMR90 cells that undergo pre-mature senescence due to induction of oncogenic-ras, the levels of these mRNA are highly induced (Mason 2004, supplemental data). The protein encoded by the IL1B gene is a member of the interleukin 1 cytokine family. This cytokine is produced by activated macrophages as a proprotein, which is proteolytically processed to its active form by caspase 1 (CASP1/ICE). This cytokine is an important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis. The induction of cyclooxygenase-2 (PTGS2/COX2) by this cytokine in the central nervous system (CNS) is found to contribute to inflammatory pain hypersensitivity. This gene and eight other interleukin 1 family genes form a cytokine gene cluster on chromosome 2 (Allan 2000). IL1B mRNA is highly expressed in IMR90 fibroblasts as they age (Kumar 1992), in fact mRNA encoding IL1β may be stabilized by post-transcriptional regulatory mechanisms. In mouse

embryonic fibroblasts the same result was observed in conjunction with up-regulation of p38 MAPK a known senescence inducing gene in mice fibroblasts (Uekawa 2004). Our result is unexpected if hnRNP A1 has a role to play in IL1B induction which should result in lowered levels of IL1B mRNA in hnRNPA1 over-expressing cells in contrast to senescent IMR90 that have lower levels of hnRNPA1 protein and higher levels of IL1β . It is possible that other mechanisms of IL1B induction are functional in the context of hnRNPA1 over-expression because the mechanism is independent of direct hnRNPA1 effects on mRNA of IL1B. Another level of regulation is through the expression of telomerase enzymes that results in an up-regulation of IL-1β mRNA in normal human fibroblasts (Kanzaki 2003). In a study on centenarians and inflammatory gene clusters, it was found that there was no correlation between allelic variation seen in the IL1 gene cluster and longevity (Francheschi 2005). The expression of this gene requires further study to understand the exact role in senescence and why it is induced by high levels of hnRNPA1 protein.

Another gene induced by hnRNPA1 over-expression is CHI3L1, a secretory glycoprotein and member of the chitinase protein family. It is associated with late stages of monocyte and macrophage maturation (Rehli 1997). There are few studies that address the role of CHI3L1 in growth of normal cells but there is some evidence for stimulating fibroblast proliferation (Junker 2005). PTGS2 is a gene that encodes Prostaglandin-endoperoxide synthase (PTGS), also known as cyclooxygenase, is the key enzyme in prostaglandin biosynthesis, and acts both as a dioxygenase and as a peroxidase. There are two isozymes of PTGS: a constitutive

PTGS1 and an inducible PTGS2, which differ in their regulation of expression and tissue distribution. Human PTGS2 is expressed in a limited number of cell types and regulated by specific stimulatory events, suggesting that it is involved in inflammation and mitogenesis. The expression of this gene is deregulated in epithelial tumors (Samoha 2005). Interestingly, this gene, also called COX-2 is up-regulated in aging human kidney tissues (Melk 2004), therefore if hnRNPA1 is able to induce expression of this gene in IMR90 fibroblasts this may be cell-type specific phenomenon, also the variation due to presence of interactions of cell types in whole tissues can be entirely different than that observed in cell culture. The EDG7 (endothelial differentiation G-protein 7) gene encodes a member of the G protein-coupled receptor family, as well as the EDG family of proteins. This protein functions as a cellular receptor for lysophosphatidic acid and mediates lysophosphatidic acid-evoked calcium mobilization. This receptor couples predominantly to G (q/11) alpha proteins. There is evidence to suggest that endothelial differentiation gene EDG-7 regulates Lysophosphatidic acid (LPA)-mediated mesangial cell proliferation and suggest that EDG-7 and EDG-2 receptors play a diverse role in mesangial cell proliferation (Xing 2004).

There were two genes that were down-regulated in cells that over-express hnRNPA1. These were RAP1A and RSAD2. The product of the RAP1A gene belongs to the family of RAS-related proteins. These proteins share approximately 50% amino acid identity with the classical RAS proteins and have numerous structural features in common. The most striking difference between RAP proteins and RAS proteins resides in their 61st amino acid: glutamine in RAS is replaced by

threonine in RAP proteins. The product of this gene counteracts the mitogenic function of RAS because it can interact with RAS GAPs and RAF in a competitive manner. Two transcript variants encoding the same protein have been identified for this gene, rap1A and rap1B are the mRNA isoforms that are products of two different genes located on chromosome 1 and 12 respectively (Mitra 2003). Rap1 protein is up-regulated in proliferative arrest of human keratinocytes and this observation appears to be unique to inhibition of growth and independent of differentiation in these cells. When Rap1 was inactivated by the addition of rapGAP, enhanced proliferation was observed, which led to the conclusion that Rap1 is important to growth regulation of specific cell-types (D'Silva 2003). RSAD2 also called viperin (virus inhibitory protein, endoplasmic reticulum associated and interferon inducible) is an ISG (interferon stimulated gene) that has anti-viral properties against human cytomegalovirus (HCMV). It appears to undergo relocalisation in cells following infection by HCMV and is proposed to be anti-viral due to its localization within the cytoplasm before infection.

The mRNA levels of a sub-set of human genes appears to be responsive to both age and hnRNPA1 over-expression in IMR90 fibroblasts. There are many unique and typical gene expression changes that occur as cells age and it was our endeavor to identify these genes and establish mRNA expression patterns of aging IMR90 fibroblasts and those that are putative targets for post-transcriptional processing. It is important to stress the need for further studies; now that these genes have been identified there may be underlying mechanisms that are in common between the genes that are subject to relative levels of a post-

transcriptional regulator such as hnRNPA1. Understanding these mechanisms is important to enhance our knowledge of the genetic basis of aging and also highlights the importance of potential gene regulation mechanisms that can depend on competition between negative and positive regulators of mRNA processing.

Chapter 3

Identification of RNA species that bind to the heterogeneous nuclear ribonucleoprotein A1 in normal human fibroblasts: an age -related study.

Introduction:

Heterogeneous nuclear ribonucleoprotein A1 effects gene expression by recognition of RNA consensus sequences

Several studies have proven that hnRNP A1 recognizes consensus RNA sequences and is able to influence post-transcriptional processes such as splicing and transport to alter gene expression levels in very specific ways. In addition there are other proteins such as SR proteins, so called because they have serine-arginine rich domains that are typical of RNA binding motifs which act in concert with hnRNPs to affect gene expression through splicing and RNA transport. The SR family of proteins are involved in splice site (SS) selection and in particular two members of this family, SC35 and ASF/SF2 are known to promote proximal exon splicing by recruiting general splicing factors such as U2AF65 to an upstream 3'SS or U1 snRNP to a downstream 5'SS (Guil 2003). As nascent mRNAs are made in nuclei during transcription they are bound by heterogeneous nuclear ribonucleoproteins (hnRNPs). hnRNPs act as packaging proteins and are components of pre-mRNP complexes within the nucleus. Many studies show that hnRNPs have RNA binding properties and in some chimeric and synthetic reporter systems, hnRNP A1 has been shown to bind to specific sequences in mRNA. The best studied model for binding, post-transcriptional processing and alternative splicing regulation by hnRNPA1 is the hnRNPA1 transcript itself (Chabot 1997). Recently, there have been several studies on the HIV tat mRNA and alternative splicing and RNA transport as affected by cytoplasmic hnRNPA1, but the first mammalian target for hnRNP A1 binding and splicing effects is cellular ras (Guil S,

2003). In this study components of the ras intron splicing sequence complex were identified and hnRNPA1 was shown to be an effective regulator of ras splicing.

Sequence elements in m RNA are binding targets for RNA binding proteins that co-operate to post-transcriptionally regulate gene expression.

There are many mechanisms whereby expression of mature mRNA is affected by RNA binding proteins. In particular, the role of heterogeneous nuclear ribonucleoproteins in splicing seems to be to inhibit the splicing reaction through direct competition for binding RNA sequences that enhance splicing. The competitive factors are splicing promoting factors such as SC35 and ASF/SF2 (Blanchette 1999). hnRNP A1 can also bind and process its own mRNA by binding regions around a normally silent exon, repressing the splicing of that exon. In another study, hnRNP A1 was shown to promote co-operative assembly of hnRNP containing complexes which interfered with the action of enhancer sequences and their recognition by splicing promoting complexes (Zhu 2001). These studies and others have established that there are sequence specific binding sites for hnRNPA1 activity and that these binding sites have a role in the regulation of the gene expression by enhancing or silencing the inclusion of exons. Such sequences have been identified for some genes, in particular CUAGACUAGA in the ESS of HIV tat exon 2, UACCUUUAGAGUAGG in the ISS of hnRNP A1 (for 5' SS selection), UUAGAUUAGA in the mouse hepatitis virus RNA transcription regulatory region and UAGGGCAGGC in an exon of human FGF receptor

(reviewed in Guil 2003). These sequences all contain the hnRNP A1 putative binding sequence UAG(G/A) which appears to be repeated within most known

hnRNPA1 complex binding regions on mRNA. In our studies, we looked for these regions within the sequences that we had identified as bound to hnRNP A1 containing complexes. That these sequences may function as binding sites for hnRNP A1 and whether they may actually influence splicing decisions in these genes remains to be determined.

hnRNP A1 complexes bind pre-mRNA and help in nuclear mRNA export.

Newly made mRNA in the nucleus of cells is immediately coated with RNA binding proteins to form a complex called the heterogeneous ribonucleoparticle. This is a protective and processing step in the maturation of nascent transcripts within the nucleus. It is believed that the composition of the packaging complex is altered as the complex with its bound RNA is processed and transported out of the nucleus. In a study to identify the components of the packaging particle and the re-arrangements of the players, these particles were isolated and analyzed (Mili 2001). In this study, RNP complexes were shown to contain the transcripts for actin and TAFII30 among others, at different stages of maturation by studying the inclusion or exclusion of exons as the mRNA mature and are transported out of the nucleus. The conclusion of this work was that hnRNP A1 protein along with other components of the ribonucleoprotein complex undergo compositional alterations as the ribonucleoparticle with its mRNA undergoes biogenesis within the nucleus of cells and as the mature mRNA is transported out of the nucleus into the cytoplasm. This is the first study where an hnRNP A1 complex was purified from human cells and the bound RNA identified. The RNA binding ability of the proteins that shuttle along with the RNA, such as hnRNP A1 (a shuttling hnRNP) also undergo change

implying that the RNA components of these particles is also altered as they undergo maturation. This is important to the any physiologically related gene expression study as these changes may contribute to alterations at the level of protein products of specific genes. In our study particularly, the genetic contribution to the aging phenotype is our focus and therefore such changes can be significant to the expression of genes under study. In my proposal I extended the same reasoning to address the question of whether the RNA targets bound by hnRNP A1 containing complexes are altered as cells age. In previous studies and in Chapter 1 of this thesis, hnRNP A1 undergoes age-related changes in gene expression and in cellular localization pattern. It is also known to have an age-related alteration in phosphorylation status (unpublished data) that may affect the ability of hnRNP A1 to form complexes with other proteins that normally would bind in young cells. In these ways, hnRNP A1 may have altered function in cells as they age and this may affect its ability to directly bind RNA or indirectly through other protein in the complex.

Therefore, this study has implications on the RNA processing capabilities of old cells and the changes that occur may manifest themselves at the gene expression level and perhaps even greater implications for the genetic basis of the senescent phenotype.

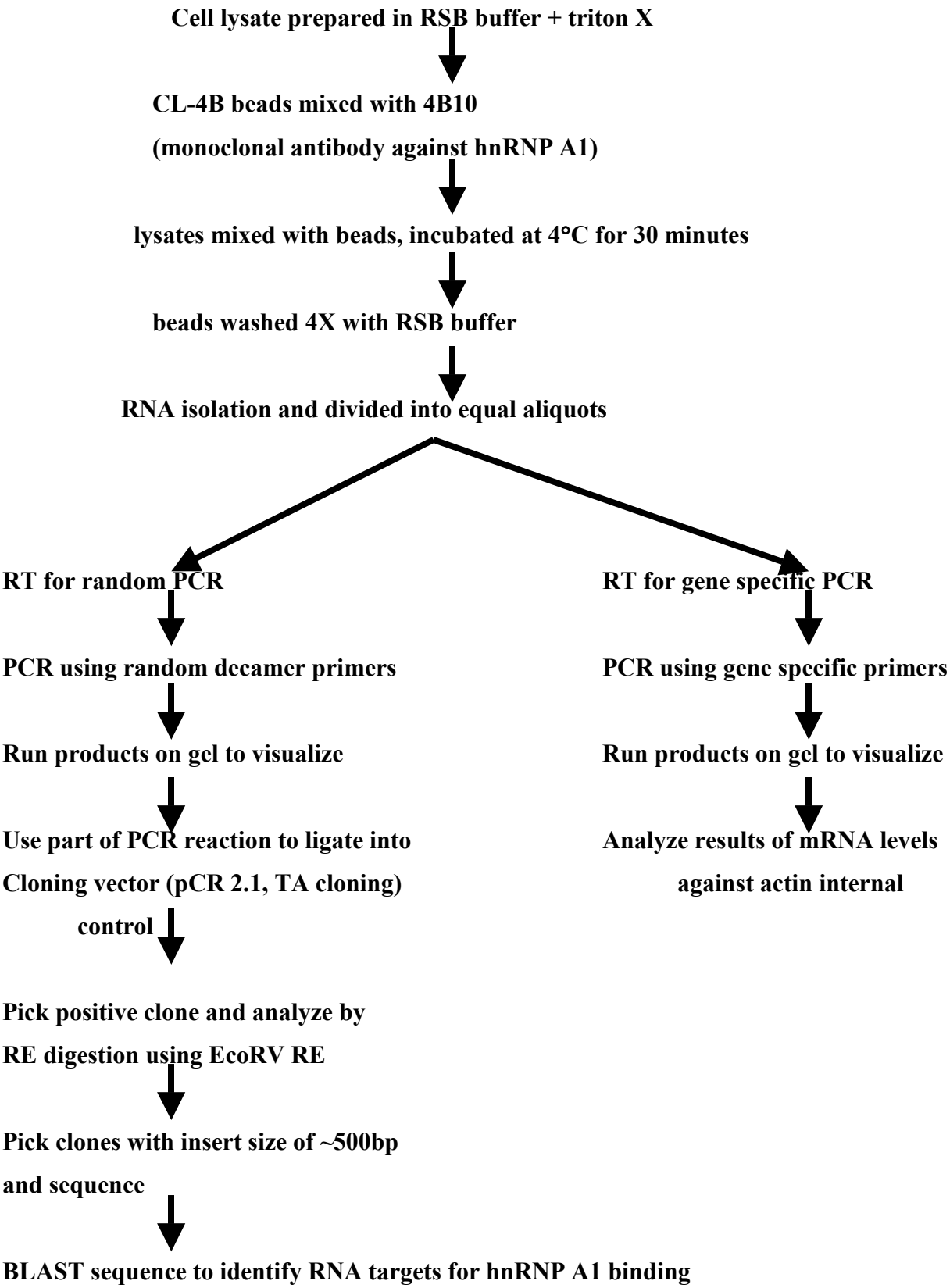
Materials and Methods:

1. RNA co-immunoprecipitation protocol.

The following flow chart outlines the steps of the methodology modified from the original protocol to identify RNA binding targets of heterogeneous nuclear ribonucleoprotein A1. This protocol is a kind gift from Dr. Serafin Pinol-Roma (refer Mili 2001). It includes a short immunoprecipitation step that minimizes degradation of protein-associated RNA and so speed and care are paramount in this protocol. All steps were performed on ice. Briefly fresh agarose beads were washed thoroughly to minimize RNase in RSB+ 2.0% Triton X-100 buffer (10 mM Tris, pH 7.4; 100 mM NaCl; 2.5 mM $MgCl_2$) and bound to an hnRNPA1 specific antibody . Cell lysate were prepared using the buffer RSB without proteinase inhibitors. Cells were lysed by passing the cell suspension through a narrow gauge syringe needle. The total cell lysates were incubated with the antibody coated beads for a minimal period of 10 to 15 minutes to ensure sufficient yield and maintain the integrity of bound RNA. The complexes were eluted from the beads and immediately processed for RNA isolation using phenol: chloroform and ethanol to precipitate the RNA. For reverse transcription and polymerase chain reaction, the resulting RNA were treated with DNase and then processed for the first-strand reaction. The resulting cDNA was cloned and then sequenced.

*(Abbreviations used in the flow chart below: **CL-4B**: are sepharose beads conjugated with protein G; **RSB**: minimum salt buffer, see below in detailed protocol 3; **pCR**: cloning vector from the TA cloning kit (Invitrogen); **TA**: dT overhangs on the ends of PCR products are used for cloning into the vector; **RE**: restriction enzyme; **BLAST**: Basic Local Alignment Sequence Tool)*

Flow sheet of RNA co-immunoprecipitation protocol.



Detailed method of co-immunoprecipitation of hnRNP and RNA.

2. RNA isolation.

After the last wash the beads were drained and 75 μL of Tris-EDTA buffer and 1% sodium dodecyl sulphate were added. The sample was heated at 65°C for 3 minutes. 225μL of TE (10mM Tris, 1mM EDTA) was added. The sample was extracted using a phenol/chloroform solution twice and then RNA precipitated with the help of 3M sodium acetate at pH 5.2, two volumes of ethanol and 20 μg of glycogen.RNA was precipitated overnight at -20°C, centrifuged and then resuspended in 20 μl of Tris-EDTA buffer, followed by heating at 65°C and processed further in a reverse transcriptase reaction for identifying RNA precipitated with protein complexes.

3. Reverse-transcription reaction.

Each RT-driven cDNA reaction was set up as follows: 5 μg template RNA, 4 μl dNTP mix (2.5 mM each dATP, dCTP, dGTP and dTTP), 2 μl of 50 μM first strand primers (either random decamers or oligo $d(T)_{18}$), 3 μl of Reverse transcriptase buffer /Alternate 10X first-strand buffer (500 mM Tris-HCl, pH 8.3/ 750 mM KCl/ 30 mM $MgCl_2$/ 50 mM DTT), 1 μl of 10 units /μl Placental RNase Inhibitor and 1 μl of 100 units/μl Moloney-Murine Leukemia Virus reverse transcriptase. The reaction mix was pre-incubated for 3 minutes at 80°C for denaturation before addition of RNase Inhibitor and Reverse Transcriptase. The reverse transcription reactions was carried out for 1 hour at 42°C and then heated to 95°C for 10 minutes to terminate the reaction. Samples was cooled to 4°C for regular PCR amplification or stored at -20°C until needed.

4. Polymerase Chain Reaction. The cDNA that was isolated was divided into two equal aliquots.

a. Random PCR. In order to amplify random sequences that were bound by hnRNP A1 random decamers were used as the primers in a PCR. The reaction was optimized at 40 repeat cycles denaturation at 94°C for 30 second, annealing at 42°C for 90 second and extension at 72°C for 120 second. The PCR products were run on 1% agarose gel. The PCR products were cloned as explained in the cloning procedure below.

b. Gene specific PCR. In order to compare the levels of β-actin, and hnRNP A1 in young, senescence, pEGFP vector only control, hnRNP A1, the cDNAs obtained by reverse transcription were used as templates in PCR. In order to quantify abundance of mRNA in the amount of final PCR product, the amount of starting templates and PCR cycle number are mentioned in Table1 (Materials and Methods). In order to normalize mRNA abundance a standard PCR for Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or beta-actin was included in each experimental set. Briefly, PCR was performed in 50μl reaction volumes. Each reaction contained 2 μl of template cDNA, 5 μl of 10 X RT-PCR Buffer (100 mM Tris-HCl, pH 8.3, 500mM KCl,15 mM $MgCl_2$), 2.5 μl of dNTP mix (2.5 mM each dNTP), 0.25 μM each PCR primer , 0.5 unit of Thermostable DNA Polymerase (Novagen). Each PCR cycle consisted of a heat-denaturation step at 94°C for 30 seconds, a primer-annealing step at 50°C to 60°C for 30 seconds, and an extension at 72°C for 2 minutes. Primer sequences (forward and reverse); the PCR product sizes, amount of

template cDNA (RT-product), annealing temperature, and number of extension cycles are also listed in the Materials and Methods table 1.

c. Confirmation of gene expression using real time

Real time PCR experiments on selected genes were performed using an Applied Biosystems 7500 real-time PCR system that utilizes Taqman gene expression assays for the following genes : mdm2; Human GAPD (GAPDH) Endogenous Control FAM / MGB (4333764F). The reactions were performed according to standard methods using the universal 10X PCR Taqman mix, at a final reaction volume of 25 μL. Reactions were standardized to run at the same conditions, 40 cycles of 94°C for 20 seconds, 60°C for 20 seconds and 72°C for 60 seconds. mRNA was quantified using the formula for comparative C_T method, in which the amount of target is normalized to an endogenous reference and relative to a calibrator is given by $\mathbf{2^{-\Delta\Delta C_T}}$ (Applied Biosystems).

5. Cloning protocol and sequencing.

PCR products were ligated into the pCR 2.1 (Invitrogen, TA cloning kit) cloning vector that utilizes the single dT overhangs that are a by-product of PCR reactions catalysed by Taq polymerase. The ligation reaction was performed at 14°C overnight using T4 DNA ligase and 3 μL of fresh PCR product (Invitrogen protocols). SURE cells were transformed by heat shock at 42°C for 90 seconds. The cells were recovered and plated onto Luria broth + 50 μg/mL kanamycin plates + 40 μL of 40 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) + 40 μL of 100 mM IPTG (isopropyl-beta-D-thiogalactopyranoside). White colonies were selected and grown in selective media. Plasmids were isolated using a

mini-prep spin column from Qiagen (Qiagen Quick-spin plasmid miniprep manual) and sequenced using an Applied Biosystems sequencer and the M13 universal primer to start the sequencing reaction (Invitrogen).

6. Sequence analysis using BLAST.

Vector sequences were subtracted from the sequences obtained. The rest of the sequence was compared against known sequences using the BLAST tool (www.ncbi.nlm.nih.gov). Sequences were chosen on the basis of being previously identified as human genes and either in the coding regions or the flanking regions of the mRNA sequences of known genes.

Results

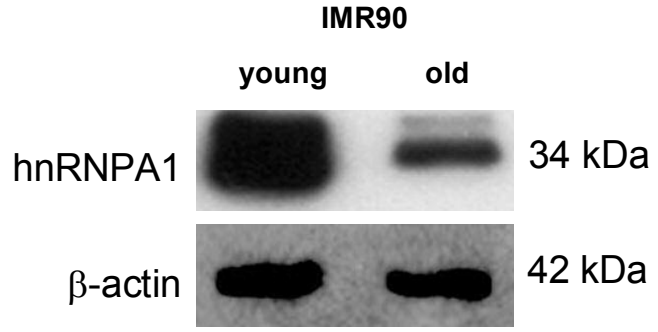
Co-immunoprecipitation of RNA bound complexes using heterogeneous nuclear ribonucleoprotein as bait protein.

We sought to identify putative mRNA substrate for hnRNPA1 by identifying sequences which directly bind to hnRNPA1 protein. In order to accomplish this co-immunoprecipitation experiments were performed whereby hnRNPA1-mRNP complexes were immuno-precipitated by antibodies specific to hnRNPA1. The co-immunoprecipitation method used the monoclonal antibody 4B10 against hnRNPA1 protein as the bait which had been conjugated to sepharose beads. This procedure is a modification of the protocol previously used for characterization of these complexes, Mili 2001. In their experiments more than one protein was believed to be part of complexes precipitated in the nuclear compartment that associated with different nuclear structures. The composition of the complex was further altered as the particle with its bound RNA targets was transported out of the nucleus into the cytoplasm. This was shown by immuno-precipitation of hnRNPA1 sub-cellular fractions and the identification of protein components of the complexes and bound RNA species. In our experiments we isolated hnRNP A1 containing protein complexes with their bound RNA targets from the pool of all such complexes present in total fibroblast cell lysates. From earlier studies, it was established that hnRNP A1 and actin mRNA are known binding targets for hnRNP A1 protein complexes. We tested RNA pools by RT-PCR for these transcripts. The results are shown in Figure 3.1c, where the relative

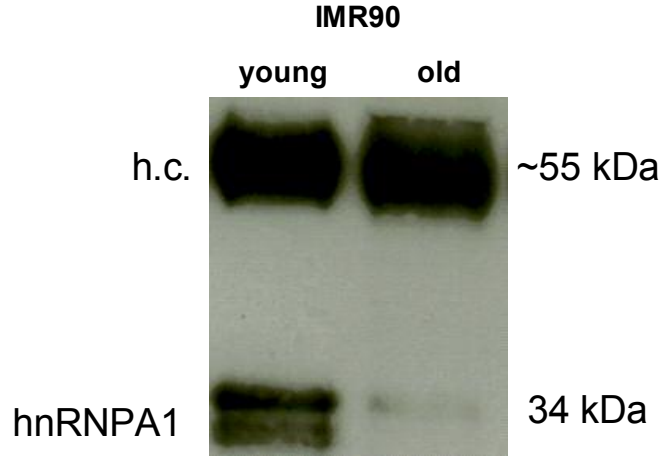
levels of actin and hnRNP A1 mRNA are comparatively expressed between young and senescent IMR90 fibroblasts. Figure 3.1 a and b show that the levels of hnRNPA1 are significantly lower in old IMR90 and after immunoprecipitation using the same amount of monoclonal antibody the levels of isolated hnRNPA1 is low but not completely absent, panel b. The observed difference in signal intensities of hnRNPA1 in panel a compared with panel b, where hnRNPA1 was immuno-precipitated can explained by the differences in efficiency of immunoprecipitation. We anticipate then that there should be some hnRNPA1 containing complexes in the cell lysates from old IMR90 and that these will be associated with RNA species. Figure 3.1, panel c, shows the results of PCR amplification of gene specific sequences from the isolated complexes and it appears that there no change in the ability of hnRNPA1 containing complexes to bind actin and hnRNPA1 mRNA. The lower protein levels in senescent cells may be due to faulty post-transcriptional processing or translational processes in old cells, since we were able to observe the same level of steady state binding to mRNA species.

Figure 3.1. A. Protein levels of hnRNPA1 and actin in young and old IMR90 cell lysates before immunoprecipitation show that the level of hnRNP A1 protein is significantly lower in old IMR90 fibroblasts (major band at ~34 kDa is hnRNPA1). The relative levels of actin, a cytoskeletal protein does not change in old cells and is used as a loading control for Western analysis. **B**. Post-immunoprecipitation levels of hnRNPA1 are also low in old cells, see band at ~34 kDa, and h.c., heavy chain of immunoglobulin is at the same intensity in both samples, implying that the amount of antibody in each reaction was the same .**C**. RT-PCR on cDNA transcribed from RNA isolated post immunoprecipitation, using hnRNPA1 and b-actin primer sets. In both young and old lysates there appears to be comparable amounts of hnRNPA1 mRNA species in the isolatedhnRNPA1 containing complexes, suggesting that the mechanism by which hnRNPA1 itself is regulated in old cells may be post-transcriptional and even post-translational. Equivalent amounts of protein were loaded on each SDS-PAGE, at 20 μg in panel A and equivalent volumes loaded in panel B, which was post-IP. The starting amounts for the RT-PCR were also used at 2 μL of the RT reaction as template. **D**. Results of RNA-PCR, RNA was used in PCR reaction with actin primers and no products were seen, as would have been expected if there was genomic DNA contamination

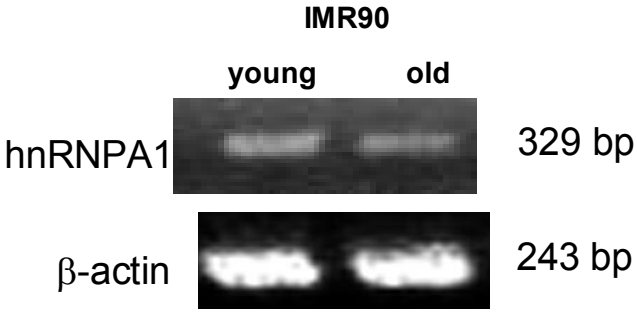
A.


IMR90
young
old
hnRNPA1
34 kDa
β-actin
42 kDa

B.

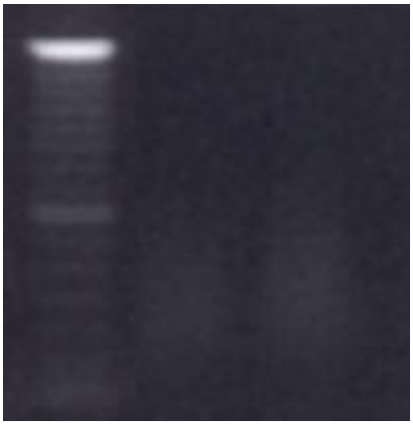

IMR90
young
old
h.c.
~55 kDa
hnRNPA1
34 kDa

C.


IMR90
young
old
hnRNPA1
329 bp
β-actin
243 bp

D. RNA-PCR analysis

M 1 2



Identification and analysis of hnRNPA1 complex bound RNA

RNA that was isolated from the complexes immediately following the co-immunoprecipitation step was reverse transcribed and then amplified by PCR using random decamers in order to amplify all cDNA sequences. An aliquot of the reaction was electrophoresed on a 1% agarose gel to visualize the products. The amount of cDNA template used for PCR was increased because it was not always possible to visualize PCR products in the old samples. There was a correlative increase in PCR products as seen in Figure 3.2, lanes 4 and 5 with the increase in cDNA template used in the PCR reaction. PCR products were immediately ligated into pCR2.1 cloning vector as fresh PCR products maintain the 3'A overhangs that are added by Taq polymerase enzyme in a non-template driven manner.

The following table summarizes the results of the cloning experiments performed and the number of clones identified as positive for containing inserts by restriction enzyme digest of the site of cloning.

Table 3.1 Cloning of PCR products and clone analysis

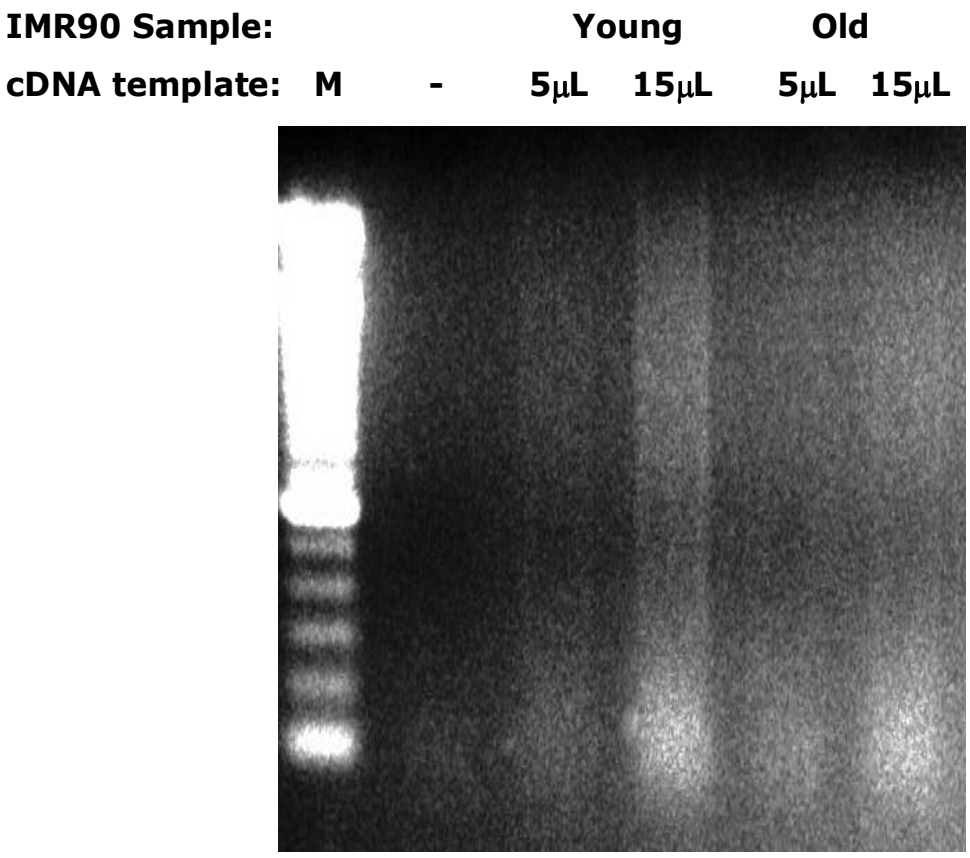
Sample	**Insert containing clones (total number of clones)**					
Trials	**1**	**2**	**3**	**4**	**5**	**% of total clones**
IMR90 young	2(15)	2(11)	0(6)	10(10)	6(6)	41.6%
IMR90 old	4(15)	0(11)	0(10)	3(10)	2(10)	16.0%

Plasmids were analyzed by restriction enzyme digestion to show that they contained inserts (see Figure 3.2, B). The number of positive clones was calculated to assess whether there was a difference between the numbers of positive clones isolated from young as compared to old cells. There appears to be a 3-fold difference in the

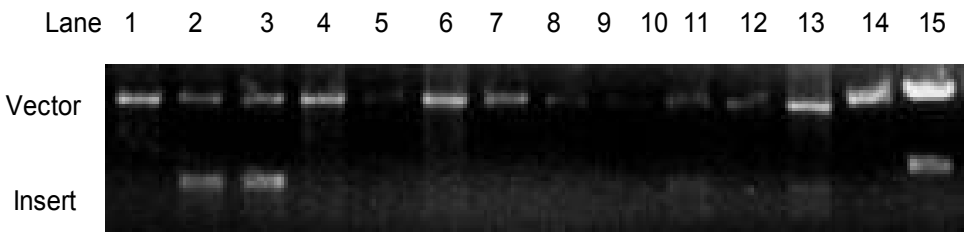
percentage of positives isolated from young cells which may reflect the change in mRNA species that bind to hnRNPA1 in young compared with old cells. However, we cannot ignore that the differences observed are subject to cloning efficiencies, which could be a factor in the differences observed.

Figure 3.2. RT-PCR analysis on isolated RNA and RE digest sample result. A. The RNA isolated from hnRNPA1 complexes were reverse transcribed to cDNA and amplified by PCR using random decamers as the primers for the reaction. Sample aliquots were electrophoresed on a 1% agarose gel containing 0.5 μg/mL ethidium bromide to visualise products. The figure shows that there is no amplification of transcripts in the lane (-) where there is no cDNA template. The young (5 μL and 15 μL cDNA was used as template) and old (5 μL and 15 μL cDNA was used as template) samples show that as the template in the reverse transcription is increased there is a correlative increase in the products formed as a result of PCR amplification. All other reaction conditions and components were maintained constant. **B**. Restriction enzyme analysis on clones isolated from IMR90 young cell lysates. This figure shows a selection of plasmid clones that were digested using a restriction enzyme to confirm the presence of an insert. In most cases the insert was not present.

A. Agarose gel analysis of amplified products from RNA isolated after co-immunoprecipitation with 4B10 monoclonal antibody



B. Restriction enzyme analysis on clones isolated from IMR90 young cell lysates



There may also be changes in availability of hnRNP A1 protein in old cells (refer Figure 3.1 A and B) and hnRNPA1 may also be modified. The change in level of PCR products could be explained by many technical reasons of the cloning procedure such as the ability or random primers to find complementary sequences and ligation efficiency. There is an additional possibility that rearrangements of the components of the hnRNPA1-mRNA ribonucleoparticles may occur as cells age which could alter specific and non-specific mRNA sequences bound to the particles.

Identification and analysis of unknown RNA binding targets for hnRNP A1 containing complexes.

We then sought to identify the cloned inserts by sequencing which was performed on the positive clones that contained inserts and Table 3.2 summarizes the results of sequencing and BLAST analysis. In this table, IMR90 fibroblast cells were harvested at different ages, young <35 PDL and old >64 PDL. The clone # indicates the clones selected for presence of an insert which following sequencing and BLAST homology analyses had sequences that were part of coding regions and flanking regions of known genes in humans. There were four human genes that were found in both young and old samples and these are listed below and in Table 3.2. The reason for displaying more than one gene sequence from the similarity search was that there appeared to be some overlap in the sequences that we were able to clone. The overlap was between the following four genes and the table summarizes the overlap region (grey highlighted area). Scores were marked as positive if the similarity score was more than 200 (Altschul 1990).

1. **mdm2 (A):** AF144029 Homo sapiens MDM2 gene, intron 9 and exon 10, partial sequence. Human mdm2 is a cellular oncogene which has the ability to inhibit tumor suppressor p53 by targeting it to degradation, among other functions to promote cell growth and division. This sequence contains the beginning of exon 10 of human mdm2, and a part of intron 9 preceding the exon (Tamborini 2001)

2. **Asthma (B):** AF095853 Homo sapiens asthmatic clone 1 mRNA, 3' UTR. This region was identified in a study performed to identify genes that were differentially expressed in a clinical study using eosinophil cell populations of asthmatic and non-asthmatic patients (Kilty 1999).

3. **UBE3 (C):** AF018071 Homo sapiens D15S1506 ca repeat region, complete sequence. This region may flank a region of chromosome 15 that was previously identified to contain the ubiquitin E3 ligase that may be mutated in Angelman syndrome and Prader-Willi syndrome, both imprinting diseases of humans (Sutcliffe 1997).

4. **Hairless (D):** AJ277249 Homo sapiens partial HR gene for hairless protein, exon 2 and joined cds. This region was identified in a study to clone and analyze genes that are involved in congenital alopecia. The mRNA for this gene undergoes alternative splicing and that the regulation of this gene may be tissue specific (Cichon 1998)

As these four human genes were the only human gene candidates, we chose to score the sequences by the number of times a positive hit occurred in the similarity search. The sequence mdm2 (A) occurred most often and we further analyzed the sequence for potential splicing enhancer/silencer sequences using the PESX utility (http://cubweb.biology.columbia.edu/pesx/) described in Zhang 2004, 2005. The sequence for 3'UTR was also analyzed in the same utility, because it would be interesting to know if there are sequences in flanking regions to a coding region that can potentially affect splicing of exons and thereby affect gene expression. The results are seen in Figure 3.3, a and b that show regions marked in red as putative silencers and green as putative enhancers. In addition, the sequences were scanned for potential hnRNPA1 binding sites using known genes, as explained in the introduction to Chapter 3 of this thesis. This sequence is UAG(G/A). There are several matches to these sequences within the isolated RNA from hnRNPA1 complexes.

Table 3.2. Sequence analysis and results of similarity search (see following pages).

Sample	Clone #	Sequence (highlighted region is the query match with overlap)	Homology score (see Note) mdm2(A)	Asthma(B)	UBE3A(C)	Hairless(D)
Young	1	CATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTC GGCTTAAGGCTCTCGAAAGCCGAATTCCAGCACACTGGCGGC				
Young	2	GNTGAATCACTACTATAGGGCGAATTGGGCCCTCTAGATGC ATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAAT TCGGCTTAGAGAAGCCGAATTCCAGCACACTGGCGGCCGTTAC	+ +	+		
Young	3	GATGNNACGACTCNTATAGGGCGAATTGGGCCCTCTAGAAT GCATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAA TTCGGCTTTGTAAGCCGAATTCCAGCACACTGGCGGCCGTTA	+ +		+	
Young	4	ATNTTGATCGATCATATAGGGCGAATGGGCCCTCTAGATGC AATTCGGATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCA GCTTCGCACCCCAAAGCCGAATTCCAGCACACTGGCGGCC	+ +		+	
Young	5	GNTGNTACGACTCCTATAGGGCGAATTGGGCCCTCTAGATGC ATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAAT TCGGCTTATATAAGCCGAATTCCAGCACACTGGCGGCCGTTA	++	+	+	
Young	6	ATACGACTCACTATAGGNGCGAATTGGGCCCTCTAGAATGC ATGCTCCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAAT TCGGCTTATNGNTGCTAANNCNAATTCCATNACACTGGCNGCN				+
Young	7	ATACGACTCACTATAGGGCGAANTGGGCCCTCTAGATGCAT GCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGG CTTAGCAAGCCGAATTCCAGCACACTGGCGGCCGTTACTAG	+ +	+		
Young	8	NTGATCCATCATATAGGGCGAATGGGCCCTCTAGATGCATGCT CGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGGCTT AAGCCGAATTCCAGCACACTGGCGGCCGTTACTAGTGGA	++			

Table 3.2 continued

Sample	Clone #	Sequence (highlighted region is the query match with overlap)	Homology score (see Note) mdm2 (A)	Asthma (B)	UBE3A(C)	Hairless(D)
Old	1	NTGATCACTACTATAGGGCGAATTGGGCCCTCTAGATGCATG CTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGGC TTACCACAGTGTCTAAGCCGAATTCCAGCACACTGGCGGC	++	+		
Old	2	TCACTACTTAGGGCGAATTGGGCCCTCTAGATGCATGCTCGA GCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGGCTTACCA CAGTGTCTAAGCCGAATTCCAGCACACTGGCGGCCGTTA	++		+	
Old	3	NTGATCACTATATAGGGCGAATTGGGCCCTCTAGATGCATG CTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGGC TTAGAGGGGCATACAAGCCGAATTCCAGCACACTGGCGGC			+	
Old	4	GATGATCGACTACTATAGGGCGAATTGGGCCCTCTAGATGC ATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCG GCTTCTGCGCGGTTTCAAGCCGAATTCCAGCACACTGGCG			+	
Old	5	ATGATCNCTATATAGGGCGAATTGGGCCCTCTAGATGCA TGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGG CTTTTCAGGCCTGTAGAAGCCGAATTCCAGCACACTGGCGGC			+	
Old	6	CTNCTATAGGGCGAATTGGGCCCTCTNGCATGCATGCTCGAG CGGCTCGCCAGTGTGATGGATATCTGCAGAATTCGGCTT AACAAGCCGAATTCCAGCACACTGGCGGCCGTTACTAGTGGAT	++	+		

[Note: All scores were assessed and bit scores were used as a threshold for similarity. ++ *indicates a score of >200 and* + *indicates a bit score of <200].*

Figure 3.3. PESX analysis. PESX (Putative exon splicing enhancer/silencers) is a utility that can predict regions of an mRNA that may be involved in exon splicing to enhance the inclusion or exclusion of an exon. In addition to identifying the putative splicing regulatory sequences, the sequences were also scanned for putative hnRNPA1 binding sites [UAG (G/A)] on the basis of known binding sites (see introduction) and these sequence are marked in enlarged letters below.

A. Homo sapiens MDM2 gene, intron 9 and exon 10, partial sequence

aacgc**cagctggcg**aaaggggga**tgtgctgcc**aaggcgattaagttgggtaacgccagggttttcc

cagtcacgacgttgtaaaacgacggccagtgaa**ttgtaat**acgactc**acta*tagg***gcgaattgg

gccctc*tag**a***tgcatgctcgagcggccgccag**t**gtgatggat**atc**tgcagaattcgg**ttatatatt**

tttcttgcagagcacagggatgagt*tagg***g**aaacaga**t**acagaggtcaagaggtg**a**tgtttatcaa

at**t**cttgttt*tag**a***tcttgatg**c**tggtgtaag (*1- 294)*

B. Homo sapiens asthmatic clone 1 mRNA, 3' UTR

cgagc**tc**ggatccactagtaacggccgccagtgtgctggaattcggcttattaaccctcactaaatgct
ggggatacaggcatccaccaccacgcccggttaatttcg**t**a**tt**tttaatagaggtggggtt**t**ctccatgt
tggtcaggctggtctcgaactccsgacyt**c**agctgatttgcccgccttggcctcccaaagtgctggaat
tacaggcgtgagccacccacacccagccccccaa**gtt**tt**ctt**ttctttg*taga***g**atgg**g**ggtctc
gctggtgttgcccagg*tagg*tctcaaactcctggtcctcaagtaatcctcckgccttggcctccca
aagtgttgagattacaggcatgagccaccatgcccagcctccctgttttcaattcccctgttttaccattat
gagctaaagaactctgactgcctgggtaa**a**acaaggacaatagtgtsgtastagcatrggatagtcat
gaaga*taga*atgcaytaaagctataaagcatgtcgcattacagtaggtgctaaagttaatgc**t**a**t**tt
tctctcacctcttccactaaaacccagagagttaatgacttagtgcttaactagtagaa**c**ttccggcaaca
gccaacttctctgctttg**c**acagacattacgaatgtctcagcatt*taga*cacttactggcttaaagaa
aaagcacc**t**cagaggaattgaaacccagctgccggccatccccagcatttagtgagggttaataagc
cgaat**t**ctgcagatatccatcacactggcggccgctcgagcatgcatc*taga*gggcccaattcg
ccgtatagtgagt *(1-849)*

[Note: Regions highlighted in blue are sequences that were found in the homology search after clones were selected from the co-immunoprecipitation method; Enlarged letters in *italics* represent the UAG(G/A) sequence that is known to be the consensus sequence for recognition by heterogeneous nuclear ribonucleoprotein A1; the boxed region in the mdm2 gene sequence is the exon 10 boundary; Red: Potential Exon Splicing Silencer (PESS); Green: Potential Exon Splicing Enhancer (PESE) ; Underlined and bold : First bases of PESE/PESS.]

Discussion

Previous studies have shown that hnRNPA1 may have altered binding ability depending on cell state, for example in senescent fibroblasts hnRNP A1 and A2 proteins less abundant than in young cells and their ability to bind RNA is lowered but they are able to bind single stranded DNA such as telomeric repeats with higher affinity as compared to young cells (from Hubbard 1995). It is also known that mRNA are packaged in a complex assembly as they are made in the nucleus of cells in complexes that are made up of RNA and RNA binding proteins such as hnRNPA1. As mRNA mature and are transported out of the nucleus the composition of these hnRNP particles change and this may affect the ability of the protein components of these particles to recognize and bind various mRNA. In our studies, we have used previously applied approaches to identify hnRNP complex bound mRNA species that may be altered in fibroblasts as they age. We then utilized sequence analysis to allow us to identify regions of bound RNA that could be potential targets for hnRNPA1 binding.

The results of our study indicated that there is some similarity in the RNA species bound by hnRNPA1 containing complexes in young and old fibroblasts. This result was validated by real-time PCR on human mdm2 mRNA sequences and we found that in two independent trials on both young and old IMR90 samples, the presence of mdm2 mRNA was confirmed, however, as the comparative amount of mdm2 mRNA appeared was highly variable, these results would need further validation and confirmation. Also this analysis did not rule out the possibility that the target sequences bound to the complex are not ssDNA, for which RNA binding

proteins such as hnRNPA1 may have some affinity. There may also be small fragments of DNA that were precipitated with hnRNPA1 containing complexes but we tested this by RNA-PCR and were able to verify that sequences that are common to both DNA and RNA such as actin were not observed in A PCR reaction that preferentially uses DNA as the template, see figure 3.1, panel d. However, it is unlikely that any DNA sequences would be isolated using the co-immunoprecipitation method, as it would be of large molecular weight and would not be detectable in the final eluate. We anticipated that only short fragments of nucleic acid such as mRNA would be bound by the complex and detectable in our system, also the stringency of our method is considerable as shown by the lack of detectable products after PCR amplification in immunoprecipitation without antibody (see Figure 3.2, A lane 2).Thus the presence of target sequences seems to be due to binding of mRNA species that are specific to hnRNPA1 containing complexes. There was some difference in the number of insert containing clones obtained after RT-PCR between young and old samples and that may be attributed to the difference in the state of hnRNPA1 in old cells, which is known to be altered in phosphorylation status (unpublished data) and localization pattern being largely cytoplasmic in cells as they get older (Zhu 2002).

It appears as if there was no change in the levels of hnRNPA1 mRNA and β-actin mRNA that were detectable by this method, indicating that if the protein levels are much lower in old cells that there are events that take place post-transcriptionally and perhaps during translation. In the case of actin a universally expressed cytoskeletal protein there has been some evidence for variation in

expression level due to cell type or physiological state but in our system both the mRNA and protein levels are maintained making it a good house-keeping gene for comparison. It is possible that hnRNPA1 mRNA is also regulated at the level of nuclear transport and that the shuttling ability of hnRNPA1 itself could be altered in cells as they age. The exact mechanism which regulates hnRNPA1 levels in old cells remains to be studied which would give an insight into the regulatory mechanisms of this multi-functional gene and its product.

The mRNA targets that were isolated and showed homology to various known transcripts were further analyzed and it is remarkable that the regions that were identified by similarity search seem to be regulatory or flanking regions to regulatory regions in the mRNA.

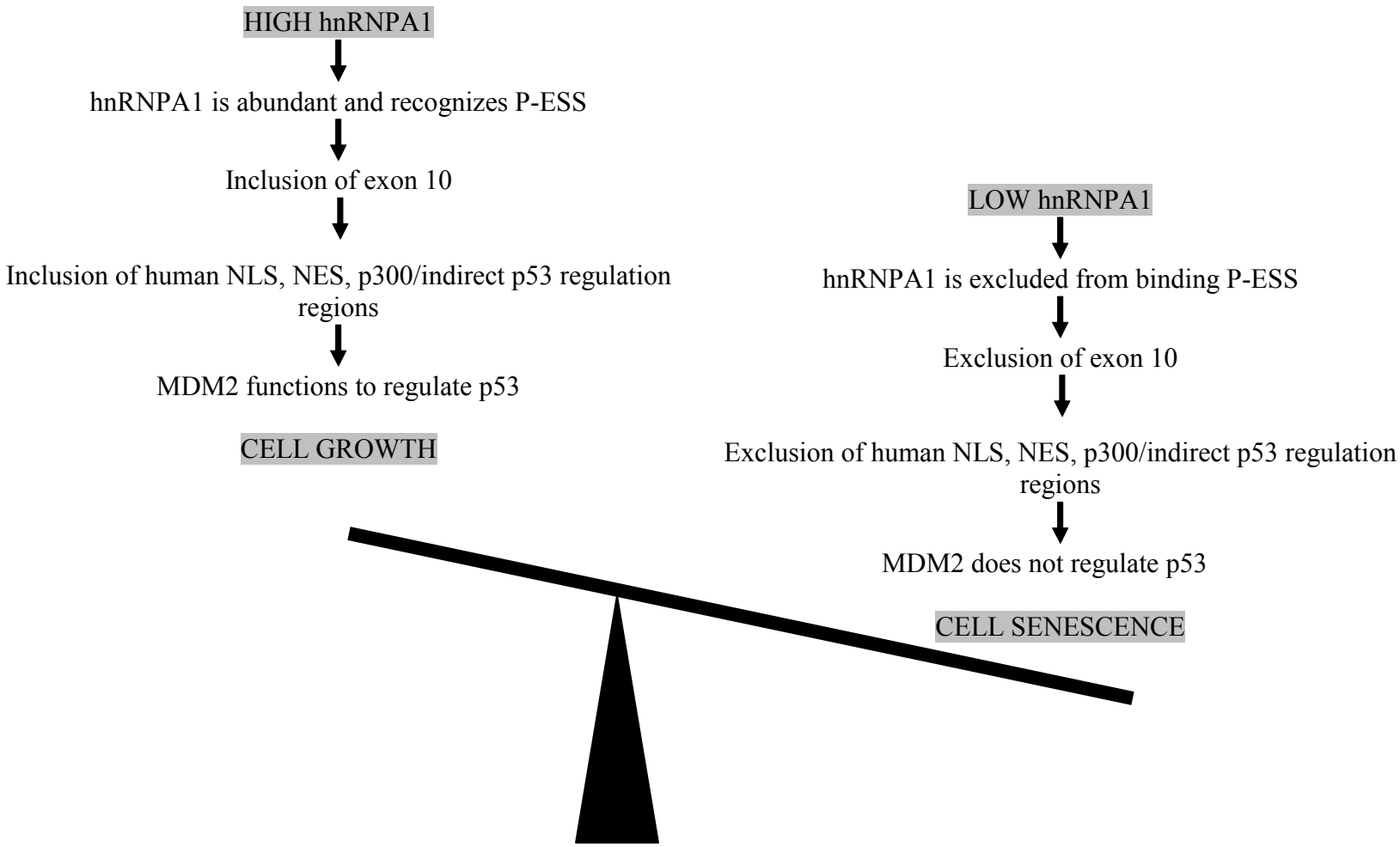
Human mdm2 (called *HDM2*) is the human homolog to *mouse double minute 2 (MDM2),* and is a known oncogene in humans. It is a protein of ~90 kDa size and is usually localized in the nucleus of cells. It was found to bind to the tumor suppressor p53 protein and inhibit the trans-activation of p53. Over-expression of mdm2 can inactivate p53 in cells causing the cells to grow uncontrolled. Once it binds to p53 it acts as a ubiquitin ligase and facilitates the proteasomal degradation of p53 (reviewed in Bartel 2002). In addition to p53, mdm2 also participates in a network of interactions whereby it can affect cell fate. The full length protein contains regions that can interact with other proteins such as p53, p73, E2F1, DP1 (partner to E2F1), $p14^{ARF}$, L5 ribosomal ribonucleoprotein particle and RNA (Bartel 2002). In addition there are functional regions of the protein that act as nuclear localization signals and nuclear export signals. The gene

that encodes human MDM2 is made up of two promoter regions that can initiate transcription. Transcripts that are initiated from promoter P1 lack exon 2, whereas transcripts that are initiated from the p53-sensitive promoter P2 generate mRNA that lack exon 1. These two promoters make mRNA that are different only in the 5' end of the transcript and do not affect the start site of translation which begins in exon 3, but there are some differences in the translation efficiencies of the transcripts initiated from different promoters. Human MDM2 also contains two growth inhibitory domains called ID1 and ID2, deletion of either of these domains stabilizes the expression of MDM2 and appears to promote regulation of growth (Bartel 2002). The gene for human MDM2 is made up of 12 exon and intervening intron sequences, exons 3 to 12 are coding exons that give rise to the full length protein. In our studies we identified a region that lies in intron 9 (see figure 3.3, mdm2 analysis) and ~100 nucleotides upstream of the intron 9-exon 10 junction. Intron 9 is spliced in most *MDM2* mRNA species detected (Bartel 2002) and in a study done on human ovarian cancers, revealed alternative as well as aberrant splicing of *MDM2* mRNA (Sigalas I 1996). It was observed that the products of these alternative transcripts of *MDM2* lacked the nuclear localization signal, nuclear export signal and this overlapped with ID1 (the growth inhibitory region) (Sigalas 1996). In addition the proteins produced were unable to bind p53 which may have contributed to the lack of cell growth regulation seen in these cells. We propose that the alternatively spliced forms seen in vivo may be the result of relative levels of proteins involved in splicing decisions such as RNA binding proteins and in particular hnRNPA1. If there is a role of hnRNPA1 in the onset and maintenance of

senescence through regulation of splicing of cell cycle regulatory and growth proteins, then the possible contribution of alternative MDM2 proteins with altered function, whose splicing was regulated by hnRNPA1 binding, could be critical to the senescent phenotype.

Transcripts for human *MDM2* (AF144029) and hairless gene (AJ277249) are both known to undergo alternative splicing suggesting a role for hnRNP A1 effects as a splicing regulator protein. We further undertook to analyze the region of similarity and found that there are potential exons splicing enhancer/silencer sequences that are present in the region of homology. Also in the case of human mdm2 there is a putative UAG(G/A) hnRNPA1 binding site at the intron 9-exon 10 boundary that may be a potential regulatory site for recruiting splicing factors and preventing the splicing of exon 10. This may also be the region that is bound by hnRNPA1 in RNP complexes that we identified and requires further study. We further propose a model whereby the levels of hnRNPA1 could have a role in this splicing decision and this is diagrammed as below.

Figure 3.4. Model for hnRNPA1 effects on alternative splicing of human mdm2 mRNA and cell growth. In the model, we propose that there maybe a stage in the regulation of MDM2 mRNA in cells where the splicing decision and inclusion of exon 10 and nearby coding regions is affected by the levels of hnRNPA1 protein. Therefore, in young fibroblasts, where the levels of hnRNPA1 are high relative to other splicing factors, the inclusion of exon 10 may be preferred leading to the formation of MDM2 protein that contains functional regions for its localization in the nucleus and its ability to bind and inactivate p53. In this case, the cells would show normal regulation of growth as is observed in young growing fibroblasts. In the case of senescent cells where the levels of hnRNPA1 are low, the splice site regulatory regions in the intron 9-exon 10 boundary hnRNPA1 binds to the splice site regulatory region but due to altered function of hnRNPA1 protein, altered post-translational modifications and abundance of other competing splicing factors, leads to the exclusion of exon 10. As a result, the regions of MDM2 protein required for its normal function in the nucleus may be missing and therefore the protein is unable to regulate p53 activity and the cell is growth arrested and enters senescence. *[Note: NLS: Nuclear localization signal; NES: Nuclear export signal; P-ESS: putative-exon splicing silencer sequence]*


HIGH hnRNPA1
hnRNPA1 is abundant and recognizes P-ESS
Inclusion of exon 10
Inclusion of human NLS, NES, p300/indirect p53 regulation regions
MDM2 functions to regulate p53
CELL GROWTH
LOW hnRNPA1
hnRNPA1 is excluded from binding P-ESS
Exclusion of exon 10
Exclusion of human NLS, NES, p300/indirect p53 regulation regions
MDM2 does not regulate p53
CELL SENESCENCE

It would be interesting and of importance to see whether the MDM2 protein localization patterns are changed in cells as they senesce. There is some evidence that normal MDM2 protein is sequestered in the nucleolus of cells by $p14^{ARF}$, and in this way, p53 remains present and active in the nucleus to regulate the cell cycle. It would be interesting to see if there is an altered pattern of localization of MDM2 relative to p53 and $p14^{ARF}$ in senescent cells, and if so what role it has to play in the senescent phenotype. In addition further investigation of the distribution of spliced isoforms of MDM2 in senescent cells could reveal the role if any, of hnRNPA1 as a splicing regulator of *MDM2* mRNA and this could be the first instance of a known mechanism for *MDM2* mRNA splicing and have implications in the area of p53 regulation via MDM2.

Summary and Conclusions.

Senescence of cells in culture is an important and relevant model system for study of age-related pathologies and basic aging mechanisms. It is also useful for modeling application strategies and analyzing effects of therapeutic trials on a cell-type basis. Much of what is now known about neurodegenerative diseases and cancers has been based on studies of cells in culture and then applied to in vivo systems such as mice and primates. There is no doubt that the mechanisms that cause an organism to age are multi-faceted and complex. By using cell culture models for aging such as normal human diploid fibroblasts we have gathered a significant body of information about the genetic regulatory mechanisms, oxidative stress response and environmental agents that can affect the course and timing of aging of an organism. We know that there is a genetic component to the aging mechanism that regulates aging onset in a species-specific, tissue-specific and age-specific manner. In any genetic study, it is important to set up the fundamental genetic background in which perturbations of minor or major players can be informative to the phenotype as a whole. This is the quest for genetic and cellular bio-markers that are uniquely age-related in a time-dependent replication-dependent context.

In our studies, we took the approach that post-transcriptional mechanisms are stages of gene expression regulation that can have widespread effects on the ability of cells to differentiate, commit apoptosis and immortalize which are all forces that occur in a time-environment continuum to cause organisms to age. In the post-genomic era it has been proposed that observed changes in gene expression are powerfully modulated by alternative splicing and mRNA processing. In fact the role of alternative splicing in producing the varied gene expression patterns and proteins that are observed in nature is

believed to be highly significant in the context of the much fewer genes available to make so many different types of proteins. Also the effect of alternative splicing can be to shift between forms of a protein with opposing effects creating a genetic switch mechanism that can lead to change in cell fates. The SMN2 gene is an example where a single nucleotide change in this gene can lead to the decision of altered splicing that can cause the neurodegenerative disease spinal muscular atrophy (Kashima 2003). Another example of a protein whose expression is affected by alternative splicing is the Hutchinson-Gilford progeria syndrome (HGPS) gene that undergoes an alternative splicing event in the lamin A encoding gene (Blencowe 2000) and this affords a effective model for the study of alternative splicing in the context of aging. There are many ways in which the profile of gene expression in cells can be altered under various circumstances and at the genomic levels it has started to become clearer that single nucleotide polymorphisms genes and their flanking regions can lead to subtle and strong alterations at the expression level.

Initially our studies showed that when specific genes that showed age-related alterations or mRNA-processing dependent alterations in expression were examined, that the patterns of gene expression are subject to a variety of influences, cell culture conditions, cell-type and also age. We believe that our results are indicative of gene regulation patterns at the caspase-2 and fibronectin loci in the cellular context but they may well be altered by tissue context and in vivo.

We expanded this logic to studying gene expression patterns on a more global scale and we found that there were expression patterns that correlated with previous reports in the case of the INK4a locus (Zhu 2002). Furthermore we were able to identify novel targets for gene expression such as CXCL5 and IL1B that were not only regulated

by age but also by the presence of an excess of hnRNPA1 protein that could be a post-transcriptional regulator of these genes through direct effects on mRNA. Alternately, the up-regulation of these genes could be a by-product of the effects of other factors that act as negative regulators of these loci. We believe that these genes are interesting target genes to study in the context of aging because their gene products are involved in cell-cell signaling and response to stimuli, both of these processes being age-dependent and specific.

Our investigations into the putative target RNA at which hnRNPA1 protein may have a role to play in the production of mature mRNA yielded a set of interesting mRNA, human MDM2 and a region of a gene for alopecia. Both these genes are subject to alternative splicing and provide obvious and intriguing systems for further investigations. We believe that the putative role of hnRNPA1 as a splicing regulator of human MDM2 mRNA could provide much insight on the regulation of a gene whose product is so important to the p53 mechanism of action. It is surprising that there is little known about the splicing of this gene and potential regulators of splicing events of this gene. We propose that further investigations of the pre-mRNA for human MDM2 and components of its splicing mechanism may have widespread implications on studies of aging and cancer.

References

Afshari CA, Vojta PJ, Annab LA, Futreal PA, Willard TB, Barrett JC. (1993) Investigation of the role of G1/S cell cycle mediators in cellular senescence. *Exp. Cell Res*. 20992): 231-237.

Allan SM. (2000) The role of pro- and anti- inflammatory cytokines in neurodegeneration. *Ann N Y Acad Sci.* 917:84-93.

Alcorta DA, Xiong Y, Phelps D, Hannon G, Beach D, Barrett JC (1996) Involvement of the cyclin-dependent kinase inhibitor p16(INK4a) in replicative senescence of normal human fibroblasts. *PNAS-USA*. 93: 13742-13747.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic Local Alignment search tool. *J. Mol Biol*. 215(3): 403-410.

Artandi SE, DePinho RA (2000) A critical role for telomeres in suppressing and facilitating carcinogenesis. *Curr Opin in Genet and Dev*. 10: 39-46.

Bagga PS, Wilusz J (1998) Northwestern screening of expression libraries. In *Methods in Molecular Biology*, vol. 118: RNA-protein interaction protocols (Haynes S. Ed.). Humana Press, pp. 245-256.

Bai Y, Lee D, Yu T, Chasin LA (1999) Control of 3' splice site choice in vivo by ASF/SF2 and hnRNPA1. *Nucleic Acids Res.* 27(4); 1126-1134.

Bartel F, Taubert H, Harris LC (2002) Alternative and aberrant splicing of MDM2 mRNA in human cancer. *Cancer Cell*. 2: 9-15.

Bates S, Phillips AC, Clark PA, Stott F, Peters G, Ludwig RL, Vousden KH (1998) $p14^{ARF}$ links the tumor suppressors pRb and p53. *Nature*. 395: 124-125.

Bentley D. (1999) Coupling RNA polymerase II transcription with pre-mRNA processing. *Curr Opin in Cell Biol*. 11: 347-351.

Bertucci F, vanHulst S , Bernard K, Loriod B, Granjeaud S, Tagett R, Starkey M, Nguyen C, Jordan B, Birnbaum D (1999) Expression scanning of an array of growth control genes in human tumor cell lines. *Oncogene*. 18: 3905-3912.

Biamonti G, Ruggiu M, Saccone S, Della Valle G, Riva S (1994) two homologous genes originated by duplication encodes the human hnRNP proteins A2 and A1. *Nucleic Acid Res*. 22:11:1996-2002.

Biggiogera M, Bottone MG, Pelliciari C (1998) Nuclear RNA is extruded from apoptotic cells. *J Histochem. Cytochem.* 46: 9: 999-1005.

Biggiogera M, Pelliciari C (2000) Heterogeneous ectopic-RNP-derived structures (HERDS) are markers of transcriptional arrest. *FASEB J*. 14; 828-834.

Blanchette M, Chabot B. (1999) Modulation of exon skipping by high-affinity hnRNPA1 binding sites and by intron elements that repress splice site utilization. *EMBO J.* 18: 1939-1952.

Blencowe BJ. (2000) Exonic splicing enhancers: mechanism of action, diversity and role in human genetic diseases. *TIBS* 25: 106-110.

Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindsten T, Turka LA, Mao X, Nunez G, Thompson CB (1992) *bcl-X*, a *bcl-2* related gene that functions as a dominant regulator of apoptotic cell death. *Cell*. 74: 597-608.

Brachmann RK, Boeke JD (1997) Tag games in yeast: the two hybrid system and beyond. *Curr. Opin. Biotech.* 8: 561-568.

Brenner C, Kroemer G (2000) Mitochondria and death signal integrators. *Science*. 289 : 1150-1151.

Brockstedt E, Rickers A, Krostka S, Laubersheimer A, Dosken B, Wittman-Leibold B, Boumert K, Otto A (1998) Identification of apoptosis-associated proteins in a human Burkitt lymphoma cell line. *J Biochem Chem*. 273: 43: 28057-28064.

Brooks SA, Rigby WFC (2000) Characterization of the mRNA ligands bound by the RNA binding protein hnRNP A2 utilizing a novel *in vivo* technique. *Nucleic Acids Res*. 28: 10: e49i-ix.

Brummelkamp TR, Kortlever RM, Lingbeek M, Trettel F, MacDonald ME, van Lohuizen M, Bernards R. (2002) TBX-3, the gene mutated in Ulnar-Mammary Syndrome, is a negative regulator of p19ARF and inhibits senescence. *J. Biol. Chem.* 277(8): 6567-6582.

Caputi M, Baralle FE, Melo CA (1995). Analysis of the linkage between fibronectin alternative spliced sites during ageing in rat tissues. *Biochim. Biophys. Acta*. 1263: 53-59.

Chalfie W, Kain S (1999) The glow spreads throughout biology. *TIBS*. 24: 251.
Chaturvedi V, Qin J-Z, Denning MF, Choubey D, Diaz MO, Nickoloff DJ (1999) Apoptosis in proliferating, senescent and immortalized keratinocytes. *J Biochem Chem*. 274: 33 : 23358-23367.

Chang M, McNinch J, Basu R, Simonet S (1994) Cloning and characterization of the human neutrophil activating peptide (ENA-78) gene. *J Biol. Chem*. 269(41) 25277-25282.

Chauhan AK, Iaconig A, Baralle FE, Muro AF (2003) Alternative splicing of fibronectin: a mouse model demonstrates the identity of in vitro and in vivo systems and the processing autonomy of regulated exons in adult mice. *Gene* 324: 55-63.

Chin L, Pomerantz J, DePinho RA (1998) The INK4A/ARF tumor suppressors: one-gene two-products two-pathways. *TIBS*. 23: 291-296.

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem*. 162(1): 152-9.

Cichon, S, Anker, M, Vogt, I.R, Rohleder, H, Putzstuck, M, Hillmer, A, Farooq, S.A. Al-Dhafri, K.S., Ahmad,M., Haque, S, Rietschel, M, Propping, P. Kruse, R. and Nothen,M.M. (1998) Cloning, genomic organization, alternative transcripts and mutational analysis of the gene responsible for autosomal recessive universal congenital alopecia. *Hum. Mol. Genet*. 7 (11), 1671-1679.

Colgin LM, Reddel RR (1999) Telomerase maintenance mechanisms and cellular immortalization. *Current Opin in Genet and Dev*. 9: 97-103.

Cote J, Dupuis S and Wu JY (2001) Polypyrimidine tract binding protein binding downstream of caspase-2 alternative exon 9 represses its inclusion. *J. Biol. Chem*. 276: 8535-8543.

Cristofalo VJ, Volker C, Francis MK, Tresini M (1998) Age–dependent modifications of gene expression in human fibroblasts. *Critical Reviews in Euk Gene Expr*. 8: 1, 43-80.

Cristofalo VJ, Allen RG, Pignolo RJ, Martin BG, Beck JC (1998) Relationship between donor age and the replicative life-span of human cells in culture: A reevaluation. *PNAS-USA*. 95: 10614-10619.

Crompton NEA (1997) Telomeres, senescence and cellular radiation response. *Cell Mol. Life. Sci*. 53: 568-575.

Cullen BR (2000) Nuclear RNA export pathways. Mol. Cell. Biol. 20:12: 4181-4187.

Datar KV, Dreyfuss G, Swanson MS. (1993) The human hnRNP M proteins: identification of a methionine/arginine-rich repeat motif in ribonucleoproteins. *Nucleic Acids Res*. 21(3): 439-46.

Dejesus V, Rios I, Davis C, Chen Y, Calhoun D, Zakeri Z, Hubbard K (2002) Induction of apoptosis in human replicative senescent fibroblasts. *Exp. Cell Res*. 274 (1): 92-99.

DeJong L, Grande MA, Mattern KA, Schul W, van Driel R (1996) Nuclear domains involved in RNA synthesis, RNA processing and replication. *Crit. Rev. Euk. Expr*. 6: 2 &3: 215-246.

Del Bello B, Valentini MA, Comporti M, Maellaro E. (2003) Cisplatin-induced apoptosis in melanoma cells: role of caspase-3 and caspase-7 in Apaf-1 proteolytic cleavage and in execution of the degradative phases. *Ann N Y Acad Sci*. 1010:200-4.

Dignam JD, Leibovitz RM, Roeder R (1983) Accurate transcription by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res*. 11-1475-1489.

Dimri GP, Hahana K, Acosta M, Campisi J (2000) Regulation of a senescence checkpoint response by the E2F1 transcription factor and $p14^{ARF}$ tumor suppressor. *Mol. Cell. Biol*. 20: 1: 273-285.

Ding J, Hayashi MK, Zhang Y, Manche L, Krainer AR, Xu R-M (1999) Crystal structure of the two-RRM domain of hnRNPA1 (UP1) complexed with single stranded telomeric DNA. *Genes and Development*. 13: 1102-1115.

Dreyfuss G, Matunis M, Pinol-Roma S, Burg CG (1993) hnRNP proteins and the biogenesis of mRNA. *Annu Rev. Biochem*. 62: 289-321.

Dreyfuss G, Struhl K (1999) Nucleus and gene expression. Multiprotein complexes mechanistic connections and nuclear organization. *Curr Opin in Cell Biol*. 11: 303-306.

D'Silva NJ, Mitra RS, Zhang Z, Kurnit DM, Babcock CR, Polverini PJ, Carey TE. (2003) Rap1, a small GTP-binding protein is upregulated during arrest of proliferation in human keratinocytes. *J Cell Physiol*. 196(3): 532-40.

Elbashir SM, Harborth J, Weber K, Tuschl T (2002) Analysis of gene function in somatic mammalian cells using small interfering RNAs. *Methods*. 26(2): 199-213.

Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature. 411 (6386): 494-498.

Elliott DJ (2000) Splicing and the single cell. *Histology and Histopathol*. 15: 239-249.

Eversole A, Maizels N (2000) *In vitro* properties of the conserved mammalian protein hnRNP D suggest a role in telomere maintenance. *Mol. Cell. Biol*. 20: 15: 5425-5432.

Franceschi C, Olivieri F, Marchegiani F, Cardelli M, Cavallone L, Capri M, Salvioli S, Valensin S, De Benedictis G, Di Iorio A, Caruso C, Paolisso G, Monti D.(2005) Genes involved in immune response/inflammation, IGF1/insulin pathway and response to oxidative stress play a major role in the genetics of human longevity: the lesson of centenarians. *Mech Ageing Dev.*126(2): 351-61.

Frederickson RM (1998) Macromolecular matchmaking: advances and related technologies. *Current Opinion in Biotech*. 9: 90-96.

Garneau D, Revil T, Fisette J, Chabot B(2005)Heterogeneous nuclear ribonucleoprotein F/H proteins modulate the alternative splicing of the apoptotic mediator Bcl-x. *J Biol Chem*. 280(24): 22641-50.

Garrett MD, Fattaey A (1999) CDK inhibition and cancer therapy. *Current Opinion in Genetics and Development*. 9: 104-111.

Gerhold D, Rushmore T, Caskey CT (1999) DNA chips: promising toys have become powerful tools. *TIBS*. 24: 168-173.

Ghosh A, Stewart D, Matlashewski G (2004) Regulation of human p53 activity and cell localization by alternative splicing. *Mol Cell Biol.* 24(18): 7987-7997.

Guil S, Gattoni R, Carrascal M, Abian J, Stevenin J, Bach-Elias M (2003) Roles of hnRNPA1, SR proteins, and p68 helicase in c-H-*ras* alternative splicing regulation. *Mol. Cell Biol*. 23 (8): 2927-2941.

Hamilton JB, Burns CM, Nichols RC, Rigby WC. (1997) Modulation of AUUUA response element binding by heterogeneous nuclear ribonucleoprotein A1 in human T lymphocytes. *J Biochem Chem*. 272: 45: 28732-28741.

Harley CB (1997) Human ageing and telomeres. *Ciba Foundation Symposium*. 21:1 129-139.

Harlow, E. and Lane, D. (1999). Using Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Hayflick L, Moorhead PS (1961) The serial cultivation of human diploid cell strains. *Experimental Cell Research*. 25: 565-621.

Horn TL, Bhattacharjee A, Schook LB, Rutherford MS (20000 Altered hepatic mRNA expression of apoptosis genes during dimethylnitrosamine exposure. *Toxicological Sciences*. 57: 240-249.

Hubbard K, Ozer HL (1997) Senescence and immortalization of human cells. In: Studzinski, GP, ed. Cell Growth and Apoptosis: A Practical Approach. Blackwell Synergy press.

Hubbard K, Dhanaraj SN, Sethi KA, Rhodes J, Wilusz J, Small MB, Ozer HL. (1995) Alteration of DNA and RNA binding activity of human telomere binding proteins occurs during cellular senescence. *Exp. Cell Res*. 218(1): 241-7.

Hubbard K, Ozer HL (1999) Mechanism of immortalization. *Age*. 22: 65-69.

Iida S, Akiyama Y, Nakajima T, Ichikawa W, Nihei Z, Sugihara K, Yuasa Y (2000). Alterations and hypermethylation of the p14ARF gene in gastric cancer. *Int. J. Cancer*. 87: 654-658.

Imaizumi T, Hatakeyama M, Taima K, Ishikawa A, Yamashita K, Yoshida H, Satoh K.(2004) Effect of double-stranded RNA on the expression of epithelial neutrophil activating peptide-78/CXCL-5 in human endothelial cells. *Inflammation.* 28(4): 215-9.

Inoue K, Ohno M, Shimura Y (1994) Aspects of splice site selection in constitutive and alternative pre-mRNA splicing. *Gene Expression*. 4: 177-182.

Ito Y, Ide T, Mitsui Y (1998) Expressional changes in alternative splicing affecting genes during cell passage of human diploid fibroblasts. *Mechanisms of Ageing and Development*. 105: 105-114.

Ito A, Uehara T, Nomura Y (2000) Isolation of Ich-1s (caspase-2S) binding protein that partially inhibits caspase activity. *FEBS letters*. 470: 360-4.

Iyer VR, Eisen MN, Ross DT, Schuler G, Moore T, Lee JCF, Trent JM, Staudt LM, Hudson Jr. J, Boguski MS, Lashkari D, Shalon D, Botstein D, Brown P (1999) The transcriptional program in the response of human fibroblasts to serum. *Science* 283: 83-87.

Jacobs JJL, Kieboom K, Marino S, DePinho RA, vanLohuizen m (1999) the oncogene and polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. *Nature*. 397: 164-168.

Jacobs JJL, Keblusek P, Robanus-Maandag E, Kristel P, Lingbeek M, Nederlof PM, van Welsem T, van de Vijver MJ, Koh EY, Daley GQ, van Lohuizen M (2000). Senescence bypass screen identifies TBX2, which represses Cdkn2a (p19 ARF) and is amplified in a subset of human breast cancers. *Nature Genetics*. 26: 291-299.

Jha KK, Banga S, Palejwala V, Ozer HL (1998) SV40 mediated immortalization. *Exp. Cell Res*. 245(1): 1-7.

Jiang Z-H, Zhang W-J, Rao Y, Wu JY (1998) Regulation of Ich-1 pre-mRNA alternative splicing and apoptosis by mammalian splicing factors. *PNAS-USA*. 95: 9155-9160.

Junker N, Johansen JS, Andersen CB, Kristjansen PE. (2005) Expression of YKL-40 by peritumoral macrophages in human small cell lung cancer. *Lung Cancer.* 48(2):223-31.

Kanzaki Y, Onoue F, Sakurai H, Ide T. (2003) Telomerase upregulates expression levels of interleukin (IL)-1alpha, IL-1beta, IL-6, IL-8, and granulocyte-macrophage colony-stimulating factor in normal human fibroblasts. *Biochem Biophys Res Commun*. 305(1):150-4.

Kashima T, Manley JL (2003) A negative element in SMN2 exon 7 inhibits splicing in spinal muscular atrophy. *Nature Genetics.* 34(4): 460-463.

Khan J, Bittner ML, Chen Y, Meltzer PS, Trent JM (1999) DNA microarray technology: the anticipated impact on the study of human disease. *Biochimica et Biophysica Acta*. 1423: M17-M28.

Konig H, Ponta H, Herrlich P (1998) Coupling of signal transduction to alternative pre-mRNA splicing by a composite splice regulator. *The EMBO journal*. 17: 10: 2904-2913.

Kilty IC, Vickers PJ (1999) Studies of differential gene expression in clinically derived eosinophil populations. *Clin. Exp. Allergy*. 29(12): 1671-1680.

Kim S, Park GH, Paik WK (1998) Recent advances in protein methylation: enzymatic methylation of nucleic acid binding proteins. *Amino Acids*. 15: 291-306.

Kish VM, Pederson T, Isolation and characterization of ribonucleoprotein particles containing heterogeneous nuclear RNA. (REF)

Kitamura Y, Shimohama S, Kamoshima W, Ota W, Matsuoka Y, Nomura Y, Smith MA, Perry G, Whitehouse PJ, Tamiguchi T. (1998) Alterations of proteins regulating apoptosis, Bcl-2, Bcl-X, Bax, Bax, Bad, Ich-1 and CPP32 in Alzheimer's disease. *Brain Research*. 780: 260-269.

Kiyono T, Foster SA, Koop JI, McDougall JK, Galloway DA, Klingelhultz AJ (1998) Both pRb/ $p16^{INK4a}$ inactivation and telomerase activity are required to immortalize human epithelial cells. *Nature*. 396: 84-88.

Korhonen L, Hammer S, Olsson P-A, Lindholm D (1997) Bcl-2 regulates the levels of the cysteine proteases Ich and CPP32/Yama in human neuronal precursor cells. *European Journal of Neuroscience*. 9 : 2489-2496.

Krecic AM, Swanson (1999) hnRNP complexes: composition, structure and function. *Current Opinion in Cell Biology*. 11: 363-371.

Kumazaki T, Mitsui Y, Hamada K, Sumida H, Nishiyama M (1999) Detection of alternative splicing of fibronectin mRNA in a single cell. *Journal of Cell Science*. 112: 1449-1453.

Kumar S (1999) Mechanisms mediating caspase activation in cell death. *Cell Death and Differentiation*. 6: 1060-1066.

Kumar S, Millis AJ, Baglioni C.(1992) Expression of interleukin 1-inducible genes and production of interleukin-1 by aging human fibroblasts. *Proc Natl. Acad. Sci U S A*. 89(10): 4683-7.

Kuo BA, Norton PA (1999) accurate selection of a 5' splice site required sequences within fibronectin alternative exon B. *Nucleic Acids Res.* 27: 3945-3952.

Kyng KJ, May A, Stevnsner T, Becker KG, Kolvra T, Bohr VA (2005) Gene expression responses to DNA damage are altered in human aging and in Werner syndrome. *Oncogene* 24: 5026-5042.

Labat-Robert J (2004) Cell-matrix interactions in aging: role of receptors and matricryptins. *Ageing Res. Reviews*. 3: 233-247.

LaBranche H, Dupuis S, Bed-David Y, Bani M-R, Wellinger RJ, Chabot B (1998) Telomere elongation by hnRNPA1 and a derivative that interacts with telomeric repeats and telomerase. *Nat. Genet*. 19: 199-202.

Ladomery M (1997) Multifunctional proteins suggest that connections between transcriptional and post-transcriptional processes. *Bioessays*. 19: 10: 903-909.

Lewis JD, Tollervey D (2000) like attracts like: getting RNA processing together in the nucleus. *Science.* 288 : 1385-1389.

Li H, Bergerson K, Cryns V, Pasternack MS, Zhu H, Shi L, Greenberg A, Yuan J (1997) Activation of caspase-2 in apoptosis. *Journal of Biological Chemistry*. 272: 34: 21010-21017.

Lindstrom MS, Klangby U, Inoue R, Pisa P, Wiman KG, Asker CE (2000) Immunolocalization of human $p14^{ARF}$ to the granular component of the interphase nucleolus. *Experimental Cell Research*. 256: 400-410.

Liu H-S, Jan M-S, Chou C-K, Chen P-H, Ki N-J (1999) Is green fluorescent protein toxic to the living cells. *Biochemical Biophysical Research Communications*. 260: 712- 717.

Lopez-hoyos M, Carrio R, Merine J, Merino R (1998) Regulation of B-cell apoptosis by Bcl-2 and bcl-xL and its role in the development of auto-immune diseases. *International Journal of Molecular Medicine*. 1: 475-483.

Lu F, Gladden AB, Diehl JA (2003) An alternatively spliced cyclin D1 isoform, cyclin D1b is a nuclear oncogene. *Cancer Res.* 63: 7056-7061.

Magnuson VL, Young M, Schattenberg DG, Mancini M, Chen D, Steffenson B, Klebe R (1991) The alternative splicing of fibronectin pre-mRNA is altered during aging in response to growth factors. *J. Biol. Chem.* 266: 14654-14662.

Marone M, Ferrandina G, Macchia G, Mozzetti S, de Pasgna A, Bennedetti- Panici P, Mancuso P, Scambia G (2000) Bcl-2, Bax, Bcl-xL and Bcl-xS expression in neoplastic and normal endometrium. *Oncology*. 58 : 161-168.

Marx J (2000) Chipping away at the causes of aging. *Science*. 287: 2390.

Mason DX, Jackson TJ, Lin AW. (2004) Molecular signature of oncogenic ras-induced senescence. *Oncogene*. 23: 9238-9246.

Matter N, Marx M , Weg-Remers S, Ponta H, Herrlich P, Konig H (2000) HnRNPA1 is part of an exon specific splice-silencing complex controlled by oncogenic-signaling pathways. *Journal of Biological Chemistry*. Aug 24.

McConnell BB, Starborg M, Brookes S, Peters G (1998) Inhibitors of cyclin-dependent kinase induce features of replicative senescence in early passage human diploid fibroblasts. *Current Biology*. 8: 351-354.

Meister G, Tuschl T (2004) Mechanisms of gene silencing by double stranded RNA. *Nature.* 431 (7006): 343-349.

Melk A, Schmidt BM, Takeuchi O, Sawitzki B, Rayner DC, Halloran PF. (2004) Expression of p16INK4a and other cell cycle regulator and senescence associated genes in aging human kidney. *Kidney Int.*65(2):510-20.

Mercatante D, Kole R (2000) Modification of alternative splicing pathways as a potential approach to chemotherapy. *Pharmacology and Therapeutics*. 85 : 237-243.

Meshorer E, Soreq H. (2000) Pre-mRNA splicing modulations in senescence. Aging Cell. 1: 10-6.

Michishita E, Nakabayashi K, Suzuki T, Kaul SC, Ogino H, Fujii M, Mitsui Y, Ayusawa D (1999) 5-Bromodeoxyuridine induces senescence- like phenomena in mammalian cells regardless of cell-type or species. *J. Biochemistry*. 126: 1052-1059.

Misteli T. (2000) Different site, different splice. *Nature Cell Biology*. 2: E98-E100.

Mitra RS, Zhang Z, Henson BS, Kurnit DM, Carey TE, D'Silva NJ. (2003)Rap1A and rap1B ras-family proteins are prominently expressed in the nucleus of squamous carcinomas: nuclear translocation of GTP-bound active form. *Oncogene*. 22(40): 6243-56.

Miura M, Hisahara S, Araki T, Okano H (1997) Execution mechanisms of programmed cell death by caspase (ICE-CED3) family proteases. *Heart Vessels, Suppl*. 12: 66-70.

Modrek B, Lee C (2002) A genomic view of alternative splicing. *Nature Genet*. 30: 13-19.

Molecular biology of Aging, Basic Life Sciences (2000) vol. 35 (Woodhead AD, Blackett AD, Hollaender A, Eds) Chapter 1: Aging research: the challenge of the twenty-first century. Plenum Press, NY and London

Morisaki H, Ando A, Nagata Y, Perriera- Smith O, Smith JR, Ikeda K, Nakanishi M (1999) Complex mechanisms underlying impaired activation of cdk4 and cdk2 in replicative senescence: roles of p16, p21 and cyclin D1. *Experimental Cell Research*. 253: 503-510.

Munro J, Stott FJ, Vousden KH, Peters G, Parkinson EK (1999) Role of the alternative INK4A proteins in human keratinocyte senescence : evidence for the specific inactivation of $p16^{INK4A}$ upon immortalization. *Cancer research*. 59: 2516-2521.

Munro TP, Magee RJ, Kidd GJ, Carson JH, Barbarese E, Smith LM, Smith R (1999) Mutational analysis of a heterogeneous nuclear ribonucleoprotein A2 response element for RNA trafficking. *Journal of biological chemistry*. 274: 18: 34389-34395.

Muro AF, Chauhan AK, Gajovic S, Iaconig A, Porro F, Stanta G, Baralle F (2003) Regulated splicing of the fibronectin EDA exon is essential for proper skin wound healing and normal lifespan. J Cell Biol. 162: 149-160.

Nair PN, Golden T, Melov S (2003) Microarray workshop on aging. *Mech. Aging Dev*. 124: 133-138.

Nicholson DW (1999) Caspase structure, proteolytic substrates and function during apoptotic cell death. *Cell Death and Differentiation*. 6: 1028-1042.

Patry C, Bouchard L, Labrecque P, Gendron D, Lemieux B, Toutant J, Lapointe E, Wellinger R, Chabot B. (2003) Small interfering RNA-mediated reduction in heterogeneous nuclear ribonucleoparticule A1/A2 proteins induces apoptosis in human cancer cells but not in normal mortal cell lines. *Cancer Res*. 63(22): 7679-7688.

Patry C, Lemieux B, Wellinger RJ, Chabot B (2004)Targeting heterogeneous nuclear ribonucleoparticule A1 and A2 proteins by RNA interference promotes cell death in transformed but not in normal mouse cell lines. *Mol Cancer Ther.* 3910): 1193-1199.

Pollard AJ, Sparey C, Robson SC Krainer AR, Europe-Finner GN (2000) Spatio-temporal expression of the trans-acting splicing factors ASF/SF2 and heterogeneous nuclear ribonucleoproteins A1/$A1^B$ in the myometrium of the pregnant human uterus: A molecular mechanism for regulating regional protein isoform expression in vivo. *The journal of Clinical Endocrinology and Metabolism*. 85:5: 1928-1936.

O'Connell J, Goode T, Shanahan F (2000) Quantitative measurement of mRNA expression by competitive RT-PCR. *Methods in Molecular Biology*, vol. 92 : PCR in Bioanalysis (Meltzer ST, Ed.). Humana press, pp. 183-193.

O'Reilly M, Teichmann SA, Rhodes D (1999) Telomerases. *Current opinion in structural biology*. 9: 56-65.

Perrem K, Reddel RR (2004) Telomeres and cell division potential. *Prog. Mol. Subcell. Biol*. 24: 173-189.

Pollack R, Perou CM, Alizadeh AA, Eisen MB, Pergamenschikov A, Williams CF, Jeffrey SS, Botstein D, Brolon PO (1999) Genome –wide analysis of DNA copy-number changes using cDNA microarays. *Nature Genetics*. 23: 41-46.

Prescott JC, Blackburn EC (1999) Telomerase: Dr. Jekyll or Mr. Hyde? *Current Opinion in Genetics and Development*. 9: 368-373.

Price CM (1999) Telomeres and telomerase: broad effects on cell growth. *Current Opinions in Genetics and Development*. 9: 218-224.

Reed JC (1999) Splicing and dicing apoptosis genes. *Nature Biotechnology*. 17: 1064-1065.

Reed R, Chiara MD (1999) Identification of RNA –protein contacts within functional ribonucleoprotein complexes by RNA site-specific labeling and UV cross linking. *Methods: A companion to Methods in Enzymology*. 18: 3-12.

Rehli M, Krause SW, Andreesen R (1997) Molecular characterization of the gene for human cartilage gp-39 (CHI3L1) a member of the chitinase protein family and marker for late stages of macrophage differentiation. *Genomics,* 43: 221-225.

Robertson KD, Jones PA (1999) Tissue specific alternative splicing in the human INK4A/ARF cell cycle regulatory locus. *Oncogene*. 18: 3810-3820.

Sambrook, J., Fritsch, E.F., and Maniatis, T., in Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, NY, Vol. 1, 2, 3 (1989).

Samoha S, Arber N. (2005) Cyclooxygenase-2 inhibition prevents colorectal cancer: from the bench to the bed side. *Oncology.* 2005; 69 Suppl 1:33-7.

Sato H, Kaman H, Ishikawa H, Horiguchi H, Fujiwara M, Yamashita YT, Ohtsuka M, Sekezawa K (2000) Expression of hnRNP A1/B1 proteins in cancer cell lines. *International Journal of Oncology*. 16: 763-767.

Schwerk C and Schulze-Ostoff K (2005) Regulation of apoptosis by alternative pre-mRNA splicing. *Molecular Cell*. 19: 1-13.

Sedivy JM (1998) Can the ends justify the means? Telomeres and the mechanisms of replicative senescence and immortalization in mammalian cells. *PNAS-USA*. 95: 9078-9081.

Semov A, Marcotte R, Semova N, Ye X, Wang E (2002) Microarray analysis of E-box binding-related gene expression in young and replicatively senescent human fibroblasts. *Anal. Biochem*. 302: 38-51.

Sengupta DJ, Zhang B, Kraemer B, Pochart P, Fields S, Wickens M (1996) A three-hybrid system to detect RNA-protein interactions *in vivo*. *PNAS-USA*. 93-8496-8501.

Sengupta DJ, Wickens M, Fields S (1999) Identification of RNAs that bind to a specific protein using the yeast three-hybrid system. *RNA*. 5: 596-601.

Serrano M, Massague J (2000) Networks of tumor suppressors. *EMBO Reports*. 1:2: 115-119.

Sharpless NE, dePinho RA (1999) The INK4a /ARF locus and its two gene products. *Current opinion in Genetics and Development*. 9: 22-30.

Shelton DW, Chang E, Whittier PS, Choi D, Funk WD (1999) Microarray analysis of replicative senescence. *Current Biology*. 9: 939-945.

Sherr CJ (2000) The Pezcoller Lecture: Cancer cell cycles revisited. *Cancer research*. 60: 3689-3695.

Sherr CJ (2001) The *INK4a*/*ARF* network in tumor suppression. *Nat. Rev. Mol. Cell Biol*. 2: 731-737.

Sherr CJ, Weber JD (2000) The ARF/p53 pathway. *Current Opinion in Genetics and Development*. 10: 94-99.

Shi ST, Huang P, Li H-P, Lai MMC (2000) Heterogeneous nuclear ribonucleoprotein A1 regulates the synthesis of a cytoplasmic virus. *The EMBO Journal* 19: 17: 4701-4711.

Sigalas, I., Calvert, A.H., Anderson, J.J., Neal, D.E., Lunec, J. (1996).Alternatively spliced mdm2 transcripts with loss of p53 binding domain sequences: transforming ability and frequent detection in human cancer. *Nat. Med*. 2, 912–917.

Siomi H, Dreyfuss G (1997) RNA-binding proteins as regulators of gene expression. *Current Opinion in Genetics and Development*. 7: 345-353.

Smith CWJ, Vacate J (2000) Alternative splicing the logic of combinatorial control. *Trends Biochem Sci*. 25(8): 381-8.

Solomon DA, Wang Y, Fox SR, Lambeck TC, Giesting S, Lan Z, Senderowicz AM, Knudsen ES (2003) Cyclin D1 splice variants. *J Biol. Chem.* 278(32): 30339-30347.

Stein GH, Drullinger LF, Soulard A, Dulic V (1999) Differential roles for cyclin-dependent kinase inhibitors p21 and p16 in the mechanisms of senescence and differentiation in human fibroblasts. *Mol. Cell. Biol*. 19: 3: 2109-2117.

Stutz F, Rosbash M (1998) Nuclear RNA export. *Genes and Development*. 12: 3303-3319.

Subramaniam A, Ranganathan P, Diamond SL (1999) Nuclear targeting peptide scaffolds for lipofection of non-dividing mammalian cells. *Nature Biotechnology*. 17: 873-877.

Sumantran VN, Lee DS, Woods Ignatoski KM, Ethier SP, Wicha MS (2000) A *bcl-Xs* adenovirus selectively induces apoptosis in transformed cells compared to normal mammary cells. *Neoplasia*. 2 : 3 : 251-260.

Sutcliffe,J.S. Jiang,Y.-H, Galjaard,R.-J, Matsuura,T, Fang,P, Kubota,T, Christian,S.L, Bressler J, Cattanach B, Ledbetter D.H and Beaudet A.L (1997) The E6-Ap ubiquitin-protein ligase (UBE3A) gene is localized within a narrowed Angelman syndrome critical region. *Genome Res*. 7(4): 368-377.

Swanson MS, Dreyfuss G (1999) Preparation of Heterogeneous nuclear ribonucleoprotein complexes. *Methods in Molecular Biology*, vol. 118: RNA-protein interaction protocols (Haynes S, Ed.). Humana Press, pp. 299-308.

Tamborini F, Della Torre G, Lavarino C, Azzarelli A, Carpinelli P, Pierotto MA, Pilotti S (2001) Analysis of the molecular species generated by mdm2 gene amplification in liposarcomas. *Int. J. Cancer*. 92(6): 790-796.

Tan J, Wooley JC, LeSturgeon WM (2000) Nuclear matrix-like filaments and fibrogranullar complexes form through the rearrangements of specific nuclear ribonucleoproteins. *Mol. Biol. Cell*. 11: 1547-54.

Taylor JK, Zhang QQ, Wyatt JR, Dean NM (1999) Induction of endogenous *bcl-xS* through the control of Bcl-X pre-mRNA splicing by antisense oligonucleotides. *Nature Biotechnology*. 17: 1097-1100.

Tenenbaum SA, Lager PJ, Carson CC, Keene JD (2002) Ribonomics: identifying mRNA subsets in mRNP complexes using antibodies to RNA-binding proteins and genomic arrays. *Methods*. 26: 191-198.

Tepper CG, Seldin M, Mudryj M (2000) Fas-mediated apoptosis of transiently growth–arrested and senescent normal human fibroblasts. *Experimental Cell Research*. 260: 9-19.

Thullberg M, Welcker M, Bartkov J, Kjerulff AA, Lukas J, Hogberg J, Bartek J (2000) Monoclonal antibody probes for $p21^{WAF1/CIP1}$ and the INK4A family of cyclin-dependent kinase inhibitors. *Hybridoma*. 19:1: 63-72.

Ting-Lee M-L, Kuo FC, Whitmore KC, Sklar J (2000) Importance of replication in microarray gene expression studies: statistical methods and evidence from repetitive cDNA hybridizations. *PNAS-USA* 97: 18: 9834-9839.

Tugores A, Belmonte JCI (1999) Differential display of eukaryotic mRNAs. In *Methods in Molecular Biology*, vol. 97: Molecular Embryology: Methods and protocols. (Sharpe PT, Mason I, Eds.). Humana press, pp. 575-590.

Uekawa N, Nishikimi A, Isobe K, Iwakura Y, Maruyama M.(2004) Involvement of IL-1 family proteins in p38 linked cellular senescence of mouse embryonic fibroblasts.
FEBS Lett. 575(1-3): 30-4.

Ushigome M, Ubagai T, Fukuda H, Tsuchiva N, Sugimura T, Takatsuka J, Nakagama H (2005) Up-regulation of hnRNPA1 gene in sporadic human colorectal cancer. *Int J. Oncol*. 26(3) : 635-640.

VanOordt W-H. Diaz-Meco MT, Lozano J, Krainer AR, Moscat J, Caceres JF (2000) The $MKK_{3/6}$-p38-signaling cascade alters the sub cellular distribution of hnRNP A1 and modulates alternative splicing regulation. *J. Cell Biol*. 149: 2: 307-316.

Vaziri H, Benchimol S (1999) Alternative pathways for the extension of cellular life span: inactivation of p53/pRb and expression of telomerase. *Oncogene*. 18: 7676-7680.

Venables JP, Eperon IC (1999) The roles of RNA - binding proteins in spermatogenesis and male infertility. *Current Opinion in Genetics and Development*. 9: 346-354

Vijg J (1999) Profiling aging by gene arrays. *Mech. Age Dev.*. 112: 1-4.

Vogt M, Haggblom C, Yeargin J, Christiansen-Weber T, Haas M (1998) Independent induction of senescence by $p16^{INK4a}$ and $p21^{CIP1/WAF1}$ in spontaneous immortalized human fibroblasts. *Cell Growth and Differentiation*. 9: 139-146.

Vojta P, Barrett JC (1995) Genetic analysis of cellular senescence. *Biochimica et Biophysica Acta*. 1242: 29-41.

Wang L, Miura M, Bergerson, Zhu H, Yuan J (1994) ICH-1 an ICE/CED3 related gene encodes both positive and negative regulators of programmed cell death. *Cell*. 78: 739-750.

Wang J, Manley JL (1997) Regulation of mRNA splicing in metazoa. *Current Opinion in Genetics and Development*. 7: 205-211.

Waterhouse N, Kumar S, Song Q, Strike P, Sparrow L. Dreyfuss G, Alneuri ES, Litwack G, Lavin M, Watters D (1996) Heterogeneous nuclear ribonucleoproteins C1 and C2 , components of the spliceosome are specific targets of Interleukin 1b-converting enzyme–like proteases in apoptosis. *Journal of Biological Chemistry*. 271: 46: 29335-29341.

Wu J, He J, Mountz JD (1998) Effect of age and apoptosis on the mouse homologue of huWRN gene. *Mechanisms of ageing and development*. 103: 27-44.

Wu J, He J, Mountz JD (1998) Effect of age and apoptosis on the mouse homologue of the hnWRN gene. *Mechanisms of ageing and development*. 103: 27-44.

Xing Y, Ganji SH, Noh JW, Kamanna VS. (2004) Cell density-dependent expression of EDG family receptors and mesangial cell proliferation: role in lysophosphatidic acid-mediated cell growth. *Am J Physiol Renal Physiol*. 287(6):F1250-7.

Yang L, Embree LJ, Hickstein DD (2000) TLS-ERG leukemia fusion protein inhibits RNA splicing mediated by serine-arginine proteins. *Mol. Cell. Biol*. 20:10: 3345-3354.

Yang X, Bani M-R, Lu S-J, Rowan S, Ben-David Y, Chabot B. (1994) The A1 and $A1^B$ proteins of heterogeneous nuclear ribonucleoproteins modulate 5' splice site selection *in vivo*. *PNAS-USA*. 91: 6924-6928.

Yaowu H, Brown MA, Rothnagel JA, Saunders NA, Smith R (2005) Roles of heterogeneous nuclear ribonucleoprotein A and B in cell proliferation. *J Cell Biol*. 118: 317303183.

Zhang XH, Kangsamaksin T, Chao MS, Banerjee JK, Chasin LA. (2005) Exon inclusion is dependent on predictable exonic splicing enhancers. *Mol Cell Biol*. 25(16):7323-32.

Zhang XH, Chasin LA. (2004) Computational definition of sequence motifs governing constitutive exon splicing. *Genes Dev*. 18(11):1241-50.

Zhou XZ, Perrem K and Lu KP (2003) Role of Pin2/TRF1 in telomere maintenance and cell cycle control. *J Cell Biochem*. 89 (1): 19-37.

Zhu J, Mayeda A, Krainer A (2001) Exon identity established through differential antagonism between exonic splicing silencer bound hnRNP A1 and enhancer bound SR proteins. *Mol. Cell* 8: 1351-1361.

Zhu D, Xu G, Ghandhi SA, Hubbard K (2002) Modulation of the expression of p16INK4a and p14ARF by hnRNP A1 and A2 RNA binding proteins: implications for cellular senescence. *J. Cell Physiol*. 193(1): 19-25.

Zong Q, Schummer M, Hood L, Morris DR (1999) Messenger RNA translation state: the second dimension of high-throughput screening. *PNAS-USA*. 96: 10632-10636.