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**Telomeres, DNA damage signaling molecules and cellular aging**

**By: Homayoun Vaziri**

**A Thesis submitted in conformity with the requirements for the Degree of Doctor of  
Philosophy in the University of Toronto**

**Department of Medical Biophysics  
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**To “Iconoclasts” of the past and future**

Gilgamesh, I will reveal unto thee a hidden thing,  
Namely, a secret of gods will I tell thee:  
There is a plant that grows under water, it has prickle  
like a thorn, like a rose its thorns will wound your hands.  
If thy hands will obtain that plant, then your hands will  
hold that which restores his lost youth to a man.  
.....Gilgamesh tied heavy stones to his feet; they pulled  
him down into the deep, and he saw the plant. He took  
the plant, though it pricked his hands. ....I will take it  
to Uruk of the strong walls; there I will give it to the old  
men to eat. Its name shall be "The Old Men Are Young Again".

*The Epic of Gilgamesh, 3<sup>rd</sup> millennium B.C.*

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## **Abstract**

### **Telomeres, DNA damage signaling molecules and cellular aging**

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Doctor of Philosophy, 1998

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Normal human cells have a finite life span and undergo senescence after a fixed number of divisions. This process appears to involve some form of genetic memory by which normal cells count the number of divisions they undergo before senescence is reached. The telomere hypothesis proposed that loss of telomeric DNA at the end of human chromosomes acts as a mitotic clock, counting each cell division. Once a critical length of telomeric DNA is reached, senescence is initiated. Questions addressed in this thesis are: 1) How does telomere shortening cause cell cycle exit? 2) Is telomere shortening one of the factors which causes senescence?

To address the first question, a model is proposed in chapter two in which telomere shortening is perceived by the cell as DNA damage and that this signal activates a DNA damage signaling pathway leading to senescence. In chapter three, we show that post-translational activation of p53 protein is one factor responsible for upregulation of p21<sup>WAF1</sup> in aging cells and PARP (poly (ADP-ribose) polymerase) is involved in the regulation of p53 protein. We found that, either inhibition of PARP or loss of p53 led to extension of life span in normal human fibroblasts. Loss of three genes in our model (PARP, p53 and p21) led to extension of cellular life span. In contrast, loss of ATM gene led to accelerated telomere shortening and premature senescence. We conclude that these DNA damage signaling molecules are involved in regulation of cellular senescence.

Answering the second question required reconstitution of telomerase activity in normal human cells. We show in chapter four of this thesis that telomerase activity can be reconstituted in normal cells by forced expression of hTERT, the catalytic subunit of human telomerase. This activity is sufficient to elongate telomeric DNA and extend the replicative life span of cells. These findings provide evidence consistent with the telomere hypothesis and indicate that telomere shortening is one factor which initiates cellular senescence by activation of a DNA damage signaling cascade. Furthermore they indicate that telomere elongation may be sufficient to prevent senescence and render normal human cells immortal.

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## **List of Abbreviations:**

Mean Terminal restriction fragment length	TRF
Normal human diploid fibroblasts	HDF
Telomere amplification protocol	TRAP
Population doublings	PDs
Fluorescent in situ hybridization	FISH
Spectral karyotyping	SKY
Telomerase induced extended life span fibroblasts	TIELF
SV40 large T antigen	SV40LT
Poly(ADP-ribose) polymerase	PARP
Cyclin dependent kinase	CDK
Ataxia Telangiectasia	AT
Ataxia Telangiectasia gene product	ATM
Base pairs	BP
Proliferating cell nuclear antigen	PCNA
American Tissue Culture Collection	ATCC
Human Papiloma Virus	HPV
Horseradish peroxidase	HRP
Single strand	SS
Double strand	DS
Wild Type	WT

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## **Chapter 1**

### **Introduction and historical overview**

## **1.1 Do normal somatic cells have a finite or infinite division potential?**

In 1881 August Weismann hypothesized that normal somatic cells of higher animals have a finite life span and aging occurs as a consequence of the failure of tissue to self renew indefinitely (Weismann, 1891). In 1912 Alexis Carrel, a Nobel Laureate and surgeon, established a chicken heart fibroblast culture and showed that these cells can undergo indefinite division (Carrel, 1913). He was able to keep these cells in culture for 34 years, after which the cultures were terminated on purpose. These findings generated public attention and steered the scientific community into believing that normal cells are intrinsically immortal and that aging does not arise as result of events occurring inside individual cells as Weismann suggested, and is due to unknown complex interaction between cells when organized as tissues.

As a result of Carrel's experiments, and for many years thereafter, many investigators believed in the dogma that somatic cells are inherently immortal and the reason they can not be propagated indefinitely in culture is because of imperfect culture conditions. This was despite the fact that many other research groups had confirmed that they were unable to maintain their primary cell cultures indefinitely (Parker, 1961).

In 1961, Leonard Hayflick published a paper which cast doubt on the validity of Carrel's experiments (Hayflick and Moorhead, 1961). He showed that normal human fibroblasts are capable of undergoing a finite number of divisions after which they become large and unable to divide further. He showed that this phenomenon (they termed phase III) was independent of culture conditions and that it represented aging at the cellular level. Hayflick also proposed that normal cells both in vitro and in vivo are mortal and immortal clones can arise from normal cells by further

genetic and adaptive changes. Therefore immortal cells in culture like cancer cells in vivo are capable of indefinite divisions. This proposal was fundamental to our understanding of aging and cancer. Hayflick's experiments also indicated that the number of doublings human diploid fibroblasts (HDFs) undergo is inversely proportional to the age of the donor from whom they are driven (Hayflick, 1965).

It is possible that Carrel's chicken cells may have been immortalized by contaminating oncogenic viruses present in chicken embryo extracts. Others have found avian leukosis virus in spontaneously transformed chicken cells. However it is intriguing that Carrel never reported any sign of "crisis" and did not attempt to re-establish another line of immortal chicken cells.

Today we know that immortalization of chicken or human fibroblasts is an extremely rare event even in the presence of oncogenic viruses or carcinogens. If Hayflick's theory was correct, then "senescence" acts as a growth suppressor mechanism to limit the life span of normal cells and to prevent genomic instability. But what initiates the senescence program and why do human cells always undergo a fixed number of doublings before senescence?

## **1.2 The end-replication problem**

In the cold winter of 1966, Aleksay Olovnikov, a Russian biologist at the Gamaleya Institute of Epidemiology and Microbiology, suggested that the ends of linear chromosomes can not be replicated fully during each round of DNA replication and that this may be the cause of cell senescence. Here is the story in his own words:

*Now the enigma of the phenomenon really struck me like thunderstorm. All the way back from the university on foot, I thought hard at feasible causes of the effect. As I remember, the first signs of forthcoming winter slowly pancake on earth that day as the big but rear snowflakes. Of course, the point was in DNA, But how? Suddenly, now I was in metro station, I realized that I caught the solution: polymerase cannot move along the very end of the template like the locomotive that cannot rolls all its wheels along the very end of the rail, not being lost the contact with it. So, the end helix was the clue point. Long ago after that, I cannot see at metrotrains without remembering of the same thought.(SIC)*

In a theoretical paper he proposed that in somatic cells the ends of the chromosomes are not fully replicated during DNA synthesis, resulting in the loss of the ends of linear DNA molecules with each cell division (Olovnikov, 1971). Based on this observation, he made several conclusions: 1) this loss may be the cause of the Hayflick limit; 2) the solution to the problem may be the evolution of very specialized buffering DNA structures at the chromosome ends; 3) tumor cells and some other immortal cell types have a specialized polymerase that is responsible for synthesis of the DNA ends. Olovnikov's theory went unnoticed until two decades later.

A year later, James Watson, independently arrived at a similar conclusion. He realized that the reason T7 bacteriophage DNA forms concatamers in bacteria may be to prevent incomplete replication of the ends of the linear T7 DNA. Watson recognized that since DNA polymerase can only synthesize DNA in the 5' to 3' direction, and because it relies on an RNA primer, this would cause a problem on the lagging strand of the DNA molecules. Once the last Okazaki fragment is removed, there would be no 3' end to prime synthesis (Watson, 1972). Therefore during each round of replication the terminal region of the DNA could not be fully replicated. This was named "the

end-replication problem". The end-end joining of T7 bacteriophage genomes enables them to reduce the loss of DNA caused by the end replication problem. However, unlike Olovnikov, Watson did not envision the link between end-replication and cell aging, nor did he propose the existence of a specialized polymerase which could replicate the chromosome ends.

### 1.3 Telomeres

Early work in the 1930s on *Drosophila melanogaster* by Mueller (Mueller, 1938) and McClintock (McClintock, 1941) on Maize indicated that chromosomes with no heterochromatic ends are recombinogenic, unstable and frequently lost. Mueller coined the term "telomere" derived from the Greek words, *telos* (the end) and *meros* (the region or part) meaning end-region for the ends of chromosomes. In 1978, Elizabeth Blackburn at Yale was interested in finding out how rDNA molecules in *Tetrahymena* are replicated as linear DNA molecules. She was able to demonstrate that the ends of these rDNA molecules consist of simple repetitive sequences  $(TTGGGG)_n$ , and that these are indeed the same sequence that cap the end of telomeres at the chromosome ends (Blackburn, 1978).

In 1988, Robert Moyzis' group at Los Alamos was able to clone the telomeres from human chromosomes and showed that they contain tandem repeats of  $(TTAGGG)_n$  (Moyzis et al., 1988) which are highly conserved in vertebrates during evolution (Meyne et al., 1989).

## 1.4 Telomerase

In 1985, Carol Greider in Blackburn's Laboratory identified an enzymatic activity in *Tetrahymena* that was able to synthesize telomeric repeats. The enzyme had its own RNA template and was able to synthesize telomeric repeats de novo. They called this enzyme "telomere terminal transferase" or "telomerase" for short. Telomerase activity was also later found in the human cervical carcinoma HeLa cell line (Morin, 1989).

Telomerase is a holoenzyme containing multiple components in both humans and ciliates (Nugent and Lundblad, 1998). Human telomerase consist of an RNA component called hTR (Feng et al., 1995) and a catalytic reverse transcriptase subunit designated hTERT (Harrington et al., 1997; Kilian et al., 1997; Meyerson et al., 1997; Nakamura et al., 1997). The complex contains a potein of unknown function (TEP1) which interacts with the hTR (Harrington et al, 1997). The hTR and hTERT by themselves are sufficient to restore telomerase activity in vitro and in vivo (Weinrich et al., 1997).

Not all eukaryotic cells use telomerase to extend their telomeres. Telomere maintenance in *Drosophila* is accomplished by retrotransposition by LINE like retroposons HeT-A and TART (Mason & Biessmann, 1992). Other insects like the mosquito *Anopheles gambiae* seem to maintain their telomeres by homologous recombination (Roth et al, 1997). Although telomere maintenance in *S. Cerevisiae* is performed by telomerase, interference with telomere elongation by inactivation of EST1 leads to telomere shortening and senescence. Cells surviving senescence have tandem amplifications of subtelomeric repeat sequences called Y elements through a process involving homologous recombination (Lundblad and Blackburn, 1993). Recently telomerase negative

immortal human cell lines have also been identified (Bryan, 1993).

### **1.5 The rise of the “telomere hypothesis of cell aging and immortalization”**

In 1989, Calvin Harley and colleagues showed that telomeric DNA is gradually lost during replicative senescence of HDFs in culture (Harley et al., 1990). This loss occurred in cells as a function of age in vivo as well (Allsopp et al., 1992; Vaziri et al., 1993). There was also a striking correlation between replicative life span of HDFs and the initial length of their telomeric DNA (Allsopp et al., 1992). Remarkably loss of telomeric DNA did not occur in germ line DNA as a function of age (Allsopp et al., 1992). In addition tumor DNA had shorter telomeres compared to their adjacent normal tissue (Hastie et al., 1990). If the loss of telomeric DNA in normal cells initiates senescence then immortal cells should presumably maintain their telomeres by some mechanism.

When normal human cells are transfected with SV40 Large T antigen, the cells bypass senescence, and acquire an extended life span and finally enter an irreversible phase of cell cycle arrest called “crisis”. Senescent cells differ from cells at crisis by several different criteria: 1) Senescent cells are G1 and G2/M arrested whereas cells at crisis due to aneuploidy do not show a distinct phase of cell cycle arrest. 2) Cells at senescence are viable for long periods of time without significant cell death whereas cells at crisis undergo slow cell growth and death. 3) Senescent cells have significantly longer telomeres than cells at crisis. 4) Senescent cells are nearly diploid whereas at crisis cells are highly aneuploid and have an unstable genome. At a rare frequency of  $< 3 \times 10^{-7}$  human cells can undergo further genetic change, overcome crisis and become immortal (Shay and Wright, 1989). Telomeres continue to shorten after expression of SV40 LT in normal human cells

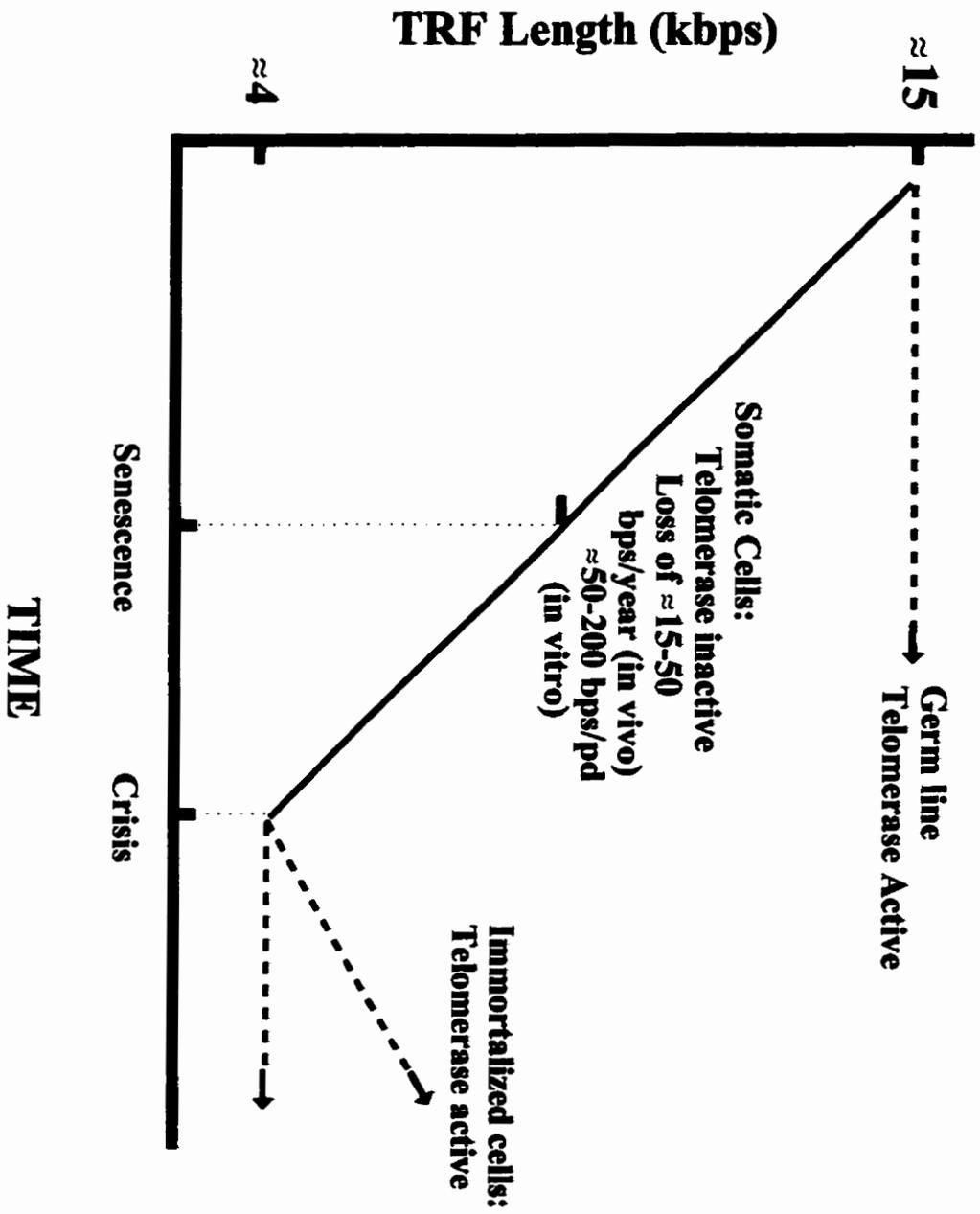
during the extended life span phase until crisis occurs. After crisis the telomeres remain short but are stably maintained. This telomere maintenance is correlated with expression of telomerase in post-crisis cells and not in the pre-crisis phase (Counter et al., 1992).

Recently, it has been shown that maintenance of telomeric DNA can also occur via an alternative pathway independent of telomerase (Bryan et al., 1995). Therefore, immortal cells in culture are able to maintain their telomeres via telomerase dependent and independent pathways.

Harley and co-workers formed the telomere hypothesis (Harley, 1991; Harley et al., 1992) which proposed the following:

1. Telomeres are maintained during gametogenesis by telomerase and this prevents shortening of telomeres between the generations of the organism.
2. During somatic differentiation of most tissues telomerase activity is repressed.
3. With each division normal human cells lose telomeric DNA due to the end-replication problem and lack of telomere maintenance by telomerase. Critical loss of telomeric repeats would lead to generation of a check point signal which initiates senescence and cells stop dividing.
4. Transforming events like mutations and viral oncogene expression can overcome senescence and cells continue to lose telomeric DNA until a critical loss of repeats triggers crisis and subsequent death.

5. In post-crisis immortal cells and cancer cells, this loss of telomeric DNA is counteracted by expression of telomerase activity. Hence, expression of telomerase may be required for cell immortality.



**Figure 1.** Schematic diagram of the telomere hypothesis of cell aging and immortalization. The vertical axis shows the mean terminal restriction fragment length (TRF) which is a measure of the length of telomeric DNA. The horizontal axis represents time in cell divisions. Telomeres of germline cells are maintained by telomerase (dotted line). Somatic cells however lack telomerase and undergo telomere shortening with each cell division. When one or more telomeres lose a critical amount of (TTAGGG)<sub>n</sub> repeats cells undergo a G1 arrest (senescence). This block can be bypassed by SV40LT antigen or mutations that allow telomere loss to continue until crisis, at which point most of (TTAGGG)<sub>n</sub> repeats are lost. This loss becomes lethal at crisis unless telomerase is activated to prevent telomere loss. Therefore, only the cells which have maintained telomere length are able to survive crisis and become immortal.

## 1.6 Testing the Telomere hypothesis

What is the mechanism by which telomere shortening activates the senescence G1 block? The fact that SV40 LT antigen can extend the life span of HDFs by overcoming senescence suggested that the p53 gene may be involved in the signaling pathway. SV40 LT antigen is known to bind to p53 and neutralize its function. Furthermore, HDFs which express SV40 LT continue to lose telomeric DNA until crisis (Counter et al., 1993). This suggests that the telomere shortening signal may pass through p53 to the cell cycle machinery. In 1985 it was shown that p53 gene is frequently inactivated by retroviral insertion of Friend erythroleukemia virus (Mowat et al., 1985) leading to the notion that p53 acts as a tumor suppressor and not an oncogene (Munroe et al., 1990). Subsequently in 1993 p53 was found to act as a “guardian of the genome” by sensing DNA strand breaks through an yet unknown mechanism and activating the appropriate cellular response to cope with the damage . DNA damage activates p53 and leads to cell cycle arrest ( Kuerbitz, 1992). Wild type (wt) p53 protein transactivates the expression of a gene called p21<sup>Waf1/Cip1</sup> (El-Deiry et al., 1993), which encodes an inhibitor of cyclin dependent kinases (Harper et al., 1993). Surprisingly Noda et al identified p21 as a gene which is upregulated during senescence (Noda et al., 1994). Thus one important question to answer was: “Does the induction of p21 observed at senescence depend on p53?”. I synthesized these separate observations into a model called the “Telomere loss/DNA damage model of cell aging”. In this model telomere loss is perceived as DNA damage by the cell and this loss activates a DNA damage signaling cascade which initiates senescence. It is worthy to mention that generation of only one unrepaired double strand break in a single HDF is sufficient to arrest the cells permanently in G1 phase of the cell cycle (Di Leonardo, 1994).

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## Overview of Chapter 2

The telomere hypothesis states that a short telomere at the end of at least one chromosome can signal the cell cycle arrest of senescence. The mechanism behind this signal however was unknown. We proposed that a short telomere is perceived by the cell as DNA damage, leading to activation of a DNA damage pathway. In this model, one of the consequences of telomere loss is activation of the p53 protein and subsequent transactivation of its downstream target p21<sup>Waf1/Sdi1/Cip1</sup>. We also proposed that other molecules which are involved in recognition of DNA strand breaks play a role in the initiation of a senescence cascade in response to telomere shortening. Based on this model, it was also suggested that ectopic expression of telomerase in normal cells overcomes senescence by preventing the senescence associated DNA damage signal. This work constitutes chapter 2 of this thesis and was published as (Vaziri H&Benchimol, S. (1996)“From telomere loss to p53 induction and activation of a DNA-damage pathway at senescence: The telomere loss/DNA damage model of cell aging. *Experimental Gerontology*, Vol 31, pp 295-301). Experimental evidence in support of various aspects of this model is presented in chapters 3-5 of this thesis.

## **Chapter 2**

### **The telomere loss/DNA damage model of cell aging**

## **2.1 Abstract**

The Telomere hypothesis proposes that critically short telomeres may act as a mitotic clock to signal the cell cycle arrest at senescence (Harley, 1991). Here we extend the telomere hypothesis and propose a model which unifies several areas of cell aging . We propose that telomere shortening is perceived by the cells as DNA damage and this in turn activates a p53 dependent or independent DNA-damage pathway that leads to the induction of a family of inhibitors of cyclin dependent kinases (including p21 and p16) and the eventual G1 block of senescence.

## **2.2 Telomere loss and cell cycle arrest**

The mechanism by which telomere shortening leads to cell cycle arrest in G1 is unknown. Two models have been proposed to link telomere loss with the G1 arrest at senescence. In one theoretical model, loss of telomeric DNA and continued shortening of at least one single chromosome leads to deletion of an unknown gene which signals the G1 cell cycle arrest (Allsopp & Harley 1995). In a second model (Wright & Shay 1992) heterochromatin shifting due to telomere loss alters the expression of certain subtelomeric genes which may cause cellular senescence. Although some evidence suggests that this may be the case in yeast (Gottschling et al., 1990), it is not clear if this is true for mammalian cells.

Based on the observations that dicentric and ring chromosomes are seen at a relatively high frequency (30-70%) at senescence and that this coincides with a critical telomere length (Allsopp, 1995), we wish to propose a third model "The Telomere loss/DNA-damage Hypothesis", in which

the loss of a critical amount of telomeric DNA from any subset of chromosomes generates dicentric and rings through an yet unknown mechanism and eventual DNA breakage during the next mitosis. These DNA breaks generate a signal which activates a "DNA damage" response pathway culminating in G1 arrest and senescence. We recognize that the DNA breaks induced by breakage and fusion of dicentric chromosomes may be different and repaired differently compared with DNA breaks induced by genotoxic agents.

The product of the p53 tumor suppressor gene has been shown to mediate a G1 arrest in response to DNA damaging agents including gamma irradiation in HDFs (Dulic et al., 1994; Di Leonardo et al., 1994). However, both p53-dependent and p53-independent pathways may contribute to the control of the G1-S transition (Kastan et al., 1992)

### **2.3 p53, p21 and the G1 checkpoint**

p53 has been implicated in the regulation of the G1-S transition. Multiple targets have been identified down stream of p53, including p21<sup>*sdi1/Waf1/cip1*</sup>, MDM2 and cyclin G. p21 was independently cloned by several groups using different systems (El-Deiry et al., 1994; Noda et al., 1994; Harper et al., 1994).

p21 is a potent inhibitor of CDK2,4-Cyclin A,E kinase activity (Xiong et al., 1994). Interestingly p21 was also cloned as a gene upregulated at senescence (Noda et al., 1994). Recently it was also shown that gamma irradiation of HDFs leads to induction of p53 and p21, which may cause a G1 block. Interestingly these cells also show morphological changes reminiscent of cellular senescence (Leonardo et al., 1994). Whether induction of p21 has a causal role in initiation of senescence or maintenance of the senescent phenotype remains to be elucidated.

## **2.4 P53 and Senescence**

It has been suggested that p53 is a senescence associated gene. These conclusions were based on experiments in which cells, transfected with SV40 large T antigen or HPV-E6/E7 escaped senescence and had an extension of life span (Shay et al., 1991). SV40 large T antigen binds p53 protein and interferes with its DNA binding and transcription function. The HPV E6 protein also binds to p53 protein and promotes the ubiquitin-dependent proteolytic degradation of p53. Hence, both proteins target p53 and disrupt its function. In addition, HDFs obtained from cancer prone individuals with Li-Fraumeni syndrome who have a germline p53 gene mutation and are heterozygous at the p53 locus, have an extended life span in culture (Rogan et al., 1993). In light of the role of p53 in regulating the G1-S transition (Lane, 1994) and the fact that in all the above experiments p53 was inactivated (either through mutations or through its interaction with SV40-LT or HPV-E6) in young, early passage cells, it is possible that escape from senescence could have occurred due to genomic instability which is known to be one consequence of p53 gene inactivation (Yin et al., 1992; Livingstone et al., 1992). Therefore the role of p53 as a senescence associated gene needs to be investigated more rigorously.

HDFs enter a G1 arrest in response to gamma irradiation and other DNA damaging agents (Di Leonardo et al., 1994; Dulic et al., 1994). G1 arrest in response to irradiation has been shown to be dependent on p53. Irradiation results in an induction of p53 protein and is associated with an increase in the expression of the p53-inducible gene p21. If critical telomere shortening is somehow perceived as a DNA damage signal by the cell, then it is possible that p53 protein is induced in

response to the signal (or is post-translationally modified) and is involved in the G1 arrest observed at senescence. Interestingly the p21<sup>Waf1/Sdi1/Cip1</sup> gene, a transcriptional target of p53 protein has been observed to be upregulated at the mRNA level (Noda et al., 1994). Therefore it is possible that the induction of p21 at senescence is mediated directly by p53 protein binding to the p21 promoter.

The level of p53 protein does not seem to change at senescence (Afshari et al., 1993; see chapter 3). There is even a slight down regulation of p53 mRNA observed during senescence (Irving et al., 1992). p21<sup>Waf1/Sdi1/Cip1</sup> can also be induced through a p53 independent pathway (Michieli et al., 1994). Hence, the upregulation of p21 during senescence may also occur through a p53 independent pathway. Loss of p53 function by E6 or SV40 large T antigen which have been shown to extend cellular lifespan may promote genetic instability and lead to activation of a p53-independent pathway controlling p21. SV40 immortalized cells which have bypassed senescence have been shown to upregulate p21 when they enter crisis (Rubelj & Pereira-Smith 1994). The presence of T antigen which complexes with p53 and inactivates it makes it likely that the induction of p21 during crisis is occurring via a p53-independent process. One must bear in mind that cells at crisis have also undergone further changes which may be responsible for activation of a p53 independent process. Upregulation of p21 both at senescence and crisis suggests that senescence and crisis may not be two completely distinct checkpoints as thought previously. The involvement of p21 at senescence and crisis could indicate the presence of common signaling pathways.

## 2.5 pRb, p16 and cellular senescence

Rb becomes hyperphosphorylated in the latter part of the G1 phase of the cell cycle in HDFs. Hypophosphorylated forms of pRb are the active forms of pRb which inhibit E2F dependent transcription leading to G1 arrest (Sherr, 1994). Cyclin E-CDK2 and Cyclin D-CDK4 are believed to be involved in phosphorylation of pRb.

One of the more firmly established hallmarks of senescence is the failure of senescent cells to phosphorylate pRb (Stein et al., 1990). The recent identification of p21 as an inhibitor of CDK2 and CDK4 and the knowledge that these proteins are likely responsible for the phosphorylation of pRb explains why pRb is found in a hypophosphorylated and active state in senescent cells. As stated earlier, p21<sup>Waf1/Sdi1/Cip1</sup> protein is induced in senescent cells and could lead to inhibition of CDK activity (see chapter 3). It is also pertinent to note that T antigen not only binds to p53 but also binds and inactivates pRb. HPV E7, which cooperates with HPV E6 to bypass senescence, also binds and neutralizes Rb.

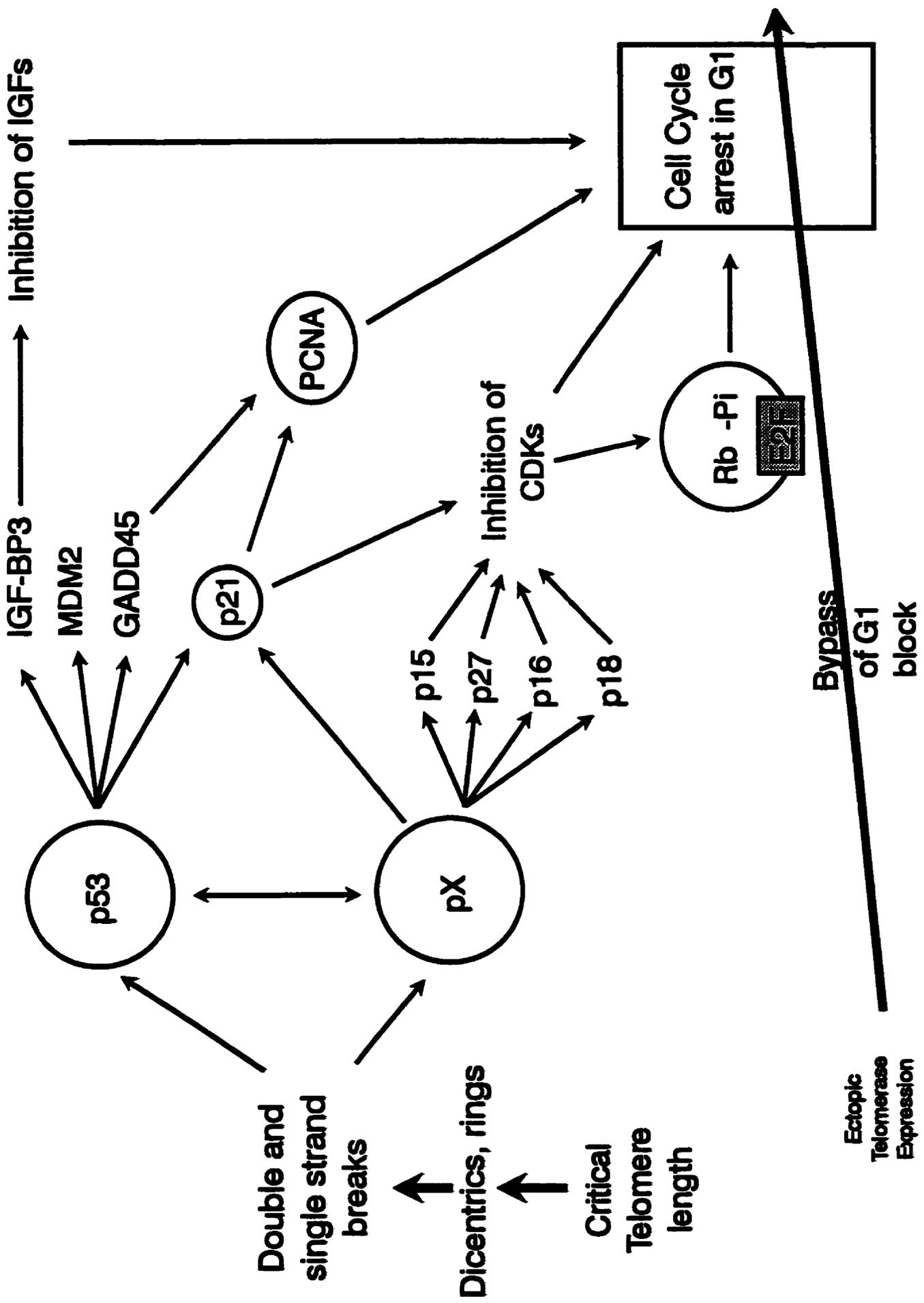
Expression of another CDK inhibitor p16<sup>INK4a</sup> (an inhibitor of CDK4/6 which are known to phosphorylate pRb) is also upregulated in senescent cells (Hara et al, 1996; Alcorta et al, 1996). This suggests that multiple independent pathways may be involved in promoting the hypophosphorylation and activation of pRb protein. Loss of p16 has been associated with extended life span (Noble, 1996) and is thought to be mediated through hypermethylation of the 5' CpG island in the p16 promoter (Foster et al, 1998).

## **2.6 The telomere loss/DNA damage model of cellular senescence**

We propose the following hypothesis:

1. Critically short telomeres in HDFs on a subset of any given set of chromosomes lead to the formation of true telomere-telomere dicentric and ring chromosomes (Benn, 1976; Sherwood et al., 1989).
2. These unstable structures are broken during the next mitosis leading to the generation of DS and SS breaks in the DNA which can be telomeric or non-telomeric in origin. Cycles of breakage and fusion lead to accumulation of more DNA breaks.
3. Generation of these ss or ds breaks in the DNA leads to activation of a DNA damage pathway either through a p53 dependent or p53 independent process.
4. Activation of a DNA damage pathway leads to upregulation of p21, and other inhibitors of cyclin dependent kinases and subsequent hypophosphorylation of pRb and the ultimate G1 arrest at senescence.

This model suggests that ectopic expression of telomerase in young HDFs could prevent telomere shortening and formation of dicentric chromosomes and subsequent DNA strand breaks. These cells will not activate the senescent program and therefore never reach senescence (Figure 2).



IGF-BP3 → Inhibition of IGFs

MDM2

GADD45

p53

p21

Double and single strand breaks

PCNA

pX

Dicentric, rings

p15

p27

p16

p18

Inhibition of CDKs

Critical Telomere length

Cell Cycle arrest in G1

Rb -Pi

E2F

Bypass of G1 block

Ectopic Telomerase Expression

**Figure 2.** A schematic model of molecules which may be involved at senescence. The telomere length signal can go through either p53 or some other protein independent of p53 (pX) and induce p21 . P21 may inhibit either the CDKs and/or directly bind to PCNA and inhibit DNA synthesis. Px may also be involved in the induction of other negative regulators like p15, p16, p18 and p27. These negative regulators may exert their effects through inhibition of multiple cyclin/cdks. P18 and p16 may be specifically involved in the inhibition of the CDKs involved in the phosphorylation of pRb. Hence their expression leads to hypophosphorylation of pRb and binding of E2F. All the above eventually leads to the G1 arrest. This model predicts that forced expression of telomerase in normal human cells prevents the initiation of the senescence cascade.

## **2.7 The negative regulators of CDKs and senescence.**

The recent discovery of new negative regulators of CDKs such as p27, p16, p15 (Hunter, 1994) raises the interesting possibility of their involvement in cellular senescence. Based on our model (Fig 1.) initiation of cellular senescence by a critical telomere length leads to activation of a DNA-damage pathway which leads to CDK inhibition and failure to phosphorylate pRb or Rb-like proteins. CDK inhibitors like p21 may play a critical role in the initiation and maintenance of senescence. It is likely that multiple pathways exist for senescence and that inactivation of one pathway (eg Rb) will not be sufficient for escape from senescence. **These findings suggest that senescence may have been evolved to act as an anti-tumor mechanism in vivo.**

## **2.8 Summary**

We propose a model which connects several areas of cell aging with tumor suppressor genes Rb and p53. A critical telomere length in senescent HDFs leads to generation of DNA breaks and subsequent activation of a DNA-damage pathway(s) which in turn cause the induction of multiple negative regulators of CDKs, hypophosphorylation of pRb and therefore the eventual G1 arrest at senescence.

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### Overview of Chapter 3

This chapter demonstrates that three proteins (p53, ATM and PARP) involved in recognizing and mediating the cellular response to DNA damage are also involved in regulation of cellular life span. I also show that p21<sup>Waf1/Sdi1/Cip1</sup>, a gene whose expression is increased with cell age, can be upregulated in a p53-dependent and p53-independent manner. These findings provide evidence for the model proposed in Chapter 2. This chapter has been published as:

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## **Chapter3**

**Regulation of p53 protein and telomere dynamics in aging cells: Telomere loss is perceived by the cell as DNA damage**

### 3.1 Abstract

The loss of telomeres that occurs during cell aging has been proposed as a mechanism for counting cell divisions in normal somatic cells. How such a mitotic clock initiates the intracellular signaling events that culminate in G1 cell cycle arrest and senescence to restrict the life span of normal human cells is not known. We investigated the possibility that a critically short telomere length activates a DNA damage response pathway involving p53 and p21<sup>WAF1</sup> in aging cells. We show that the DNA binding and transcriptional activity of p53 protein increases with cell age in the absence of any marked increase in the steady state level of p53 protein, and that p21<sup>WAF1</sup> promoter activity in senescent cells is dependent on p53 and the transcriptional co-activator p300. Moreover, we detected increased specific activity of p53 protein in AT fibroblasts, which exhibit accelerated telomere loss and undergo premature senescence, compared with normal fibroblasts obtained from age-matched siblings. We investigated the possibility that PARP ( Poly(ADP-ribose) polymerase), an enzyme that is activated in response to DNA damage, may be involved in senescence and in the post-translational activation of p53 protein in aging cells. We show that p53 protein can associate with PARP *in vitro* and *in vivo*, and that inhibition of PARP activity leads to abrogation of p21 and mdm2 expression in response to DNA damage. Moreover, treatment of HDFs with PARP inhibitors leads to a significant extension of cellular life span. In contrast, hyperoxia, a potent activator of PARP, is associated with accelerated telomere loss, activation of p53 and premature senescence. We propose a model in which p53 is post translationally activated not only in response to DNA damage but also in response to the critical shortening of telomeres that occurs during cellular aging.

### 3.2 Introduction

Normal human diploid fibroblasts (HDFs) are mortal and undergo a limited number of population doublings in culture (Hayflick and Moorhead, 1961) after which they enter a viable but non-proliferative phase known as senescence. Senescent cells arrest primarily in the G1 phase of the cell cycle (Sherwood *et al.*, 1988). This proliferative block can be overcome upon expression of the SV40 large T antigen or combination of both the HPV-16 E6 and E7 proteins resulting in the extension of cellular replicative potential (Shay *et al.*, 1993). SV40 large T antigen is known to bind the tumor suppressor p53 protein and to interfere with its DNA binding and transcriptional activation function. HPV-16 E6 protein also binds p53 protein and promotes its degradation by the ubiquitin-dependent proteolytic pathway. Hence, both proteins disrupt the function of p53 suggesting that p53 protein likely serves as a key regulator of cellular senescence. Consistent with this idea are the findings that spontaneous loss of p53 alleles in HDFs is associated with extension of cellular lifespan (Rogan *et al.*, 1995) and that expression of certain dominant negative p53 alleles can extend the cellular replicative lifespan of human (Bond *et al.*, 1994; Gollahon and Shay, 1996) and rat fibroblasts (Rovinski and Benchimol, 1988).

Aging in HDFs (Harley *et al.*, 1990; Lindsey *et al.*, 1991; Allsopp *et al.*, 1992) and in human hematopoietic cells (Hastie *et al.*, 1990; Vaziri *et al.*, 1993; Vaziri *et al.*, 1994; Metcalfe *et al.*, 1996) is accompanied by the progressive loss of telomeric DNA. Expression of the enzyme telomerase in immortal cells provides one mechanism for the prevention of telomere shortening (Morin, 1989; Counter *et al.*, 1992; Kim *et al.*, 1994). Persistent loss of telomeres, in the absence of telomerase, could lead to a critically short telomere length, cell cycle arrest and senescence (Allsopp and Harley, 1995). However, it is unclear how shortened telomeres give

rise to the anti-proliferative signals that result in senescence. One clue was provided by Benn (1976) and (Sherwood *et al.*, 1988) who reported an increase in the incidence of dicentric chromosomes in aging cell populations. Dicentric chromosomes are likely the result of recombination occurring at the shortened and exposed telomeric ends of chromosomes through yet unknown mechanisms. We suggested previously that dicentric chromosomes, upon breakage at mitosis, might activate a p53-dependent DNA damage pathway leading to cell cycle arrest (Vaziri and Benchimol, 1996). Several lines of evidence are consistent with this model. First, the p21<sup>WAF1</sup> gene, which encodes an inhibitor of cyclin-dependent kinases (CDK) (Harper *et al.*, 1993; Dulic *et al.*, 1994), is transcriptionally regulated by p53 (El-Deiry *et al.*, 1993) and is over-expressed in senescent cells (Noda *et al.*, 1994). Overexpression of a transfected p21<sup>WAF1</sup> gene in recipient cells results in G1 cell cycle arrest (Yang *et al.*, 1995). Second, exposure of HDFs to  $\gamma$ -irradiation leads to a p53-dependent, prolonged G1 arrest and induction of p21<sup>WAF1</sup> expression that is reminiscent of senescence (Di Leonardo *et al.*, 1994). Third, DNA binding experiments indicate that binding of p53 protein to the p53 responsive element present on the ribosomal gene cluster (RGC) DNA fragment increases in senescent cells (Atadja *et al.*, 1995). The significance of the presence of a p53 consensus sequence in the the RGC remains unclear. Together, these results suggest that p53 protein may be activated during cell aging to promote transcription of the p21<sup>WAF1</sup> gene. p21<sup>WAF1</sup> expression, however, is known to be regulated through p53-dependent and p53-independent pathways (Michieli *et al.*, 1994; Datto *et al.*, 1995). In this study, we have investigated further the role of p53 and p21<sup>WAF1</sup> in cell aging and demonstrate that the DNA binding activity of p53 protein on the p53 responsive element present in the human p21<sup>WAF1</sup> promoter increases during cell aging. In addition, we show that p21<sup>WAF1</sup> expression at senescence

is dependent on p53 as well as on the transcriptional co-activator p300.

A number of genes encode proteins that may be capable of sensing DNA damage. ATM and poly(ADP-ribose) polymerase (PARP) represent two such proteins and both have been implicated in the p53-dependent DNA damage response pathway (Kastan *et al.*, 1992; Lu and Lane, 1993; Wesierska *et al.*, 1996). HDFs from individuals with ataxia-telangiectasia (AT), an autosomal recessive disease characterized by sensitivity to ionizing radiation, sterility, immune dysfunction, degeneration of cerebellum, telangiectasia and premature aging, undergo premature senescence in culture (Shiloh *et al.*, 1982). The gene that is defective in AT patients called ATM is related to the *TEL1* gene of *S.cerevisiae* and encodes a protein with similarity to the phosphatidylinositol 3-kinase family of proteins. Inactivation of *TEL1* in *S.cerevisiae* leads to rapid telomere loss (Greenwell *et al.*, 1995). Furthermore fibroblasts from mice lacking ATM undergo premature senescence in culture (Xu and Baltimore, 1996). We demonstrate that telomeric DNA is lost rapidly in human AT fibroblasts and that this telomere shortening is associated with premature senescence and increased DNA binding activity of p53 protein.

PARP is strongly activated by binding to DNA strand breaks produced by various DNA damaging agents including hydrogen peroxide and undergoes rapid automodification by synthesizing long branches of poly(ADP-ribose) (Lindahl *et al.*, 1995). We report that PARP and p53 protein associate *in vitro* and *in vivo*. Hyperoxia, which is known to activate PARP (Zhang *et al.*, 1994), leads to accelerated loss of telomeres through an unknown mechanism, activation of p53 protein and premature senescence. Furthermore, inactivation of either p53 or PARP in HDFs leads to extension of replicative lifespan. Together, these data implicate ATM, PARP, p53 and p21<sup>WAF1</sup> as key components in a telomere loss/DNA damage signaling pathway

that is activated in human cells during cell aging.

### **3.3 DNA binding activity of p53 protein is altered during cellular aging**

Nuclear injection of linearized plasmid DNA or circular DNA with a large gap is sufficient to induce p53-mediated growth arrest, a process that is known to depend on the DNA binding activity of p53 (Huang *et al.*, 1996). Moreover, the generation of double-stranded DNA breaks in response to ionizing radiation has been shown to increase the DNA binding activity of p53 through an unknown mechanism (Reed *et al.*, 1995). If double-stranded breaks or large gaps in chromosomal DNA occur during the process of cellular aging in culture, one might expect to see increased DNA binding activity of p53 as a consequence of such DNA damage. Therefore, we examined the DNA binding activity of p53 protein using an electrophoretic mobility shift assay (EMSA) with a <sup>32</sup>P-labelled double stranded oligonucleotide containing the p53 consensus binding sequence (p53CON) (Funk *et al.*, 1992) and nuclear extracts prepared from the human fibroblast strain MRC-5 at different population doublings (PD). Inclusion of the p53-specific monoclonal antibody PAb421 in the binding reaction facilitated visualization of the p53-DNA complex. An increase in the DNA binding activity of p53 protein was observed as MRC-5 cells approached senescence (Figure 3A,B,F). This observation was confirmed in two other human fibroblast strains, WI-38 and BJ (Figure 3C). Competition with an excess of unlabelled p53CON oligonucleotide but not with an equivalent amount of unrelated oligonucleotide with similar nucleotide composition as p53CON confirmed that binding was specific (Figure 1A). As a further control for specificity, binding to p53 was observed when an extract from the p53-

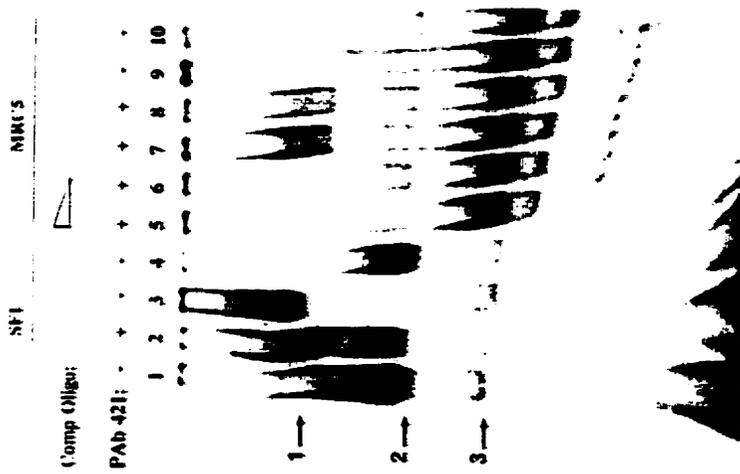
overexpressing cell line SF1 was used (Figure 3A) but not when an extract prepared from p53-null cells was used (data not shown).

When the DNA binding activity was normalized to the amount of p53 protein present in extracts from young and old cells by Western blotting (Figure 3D), we found a 1.9-fold increase in the DNA binding activity of p53 in old MRC-5 cells ( $n=2$ ), a 5.5-fold increase in old WI-38 and a 1.5-fold increase in old BJ cells relative to their young counterparts (Figure 3E). Hence, the increase in DNA binding cannot be ascribed to elevated levels of p53 protein and could reflect an age-related post-translational change in the p53 protein.

The number of dicentric chromosomes was shown previously to increase in aging MRC-5 cultures (Benn, 1976) possibly as the result of telomere shortening (Harley, 1991). In Figure 3F, we superimpose our data showing the age-related changes in the DNA binding activity of p53 protein with the frequency of dicentric chromosomes in aging MRC-5 cells. The DNA binding data was obtained from five independent experiments. The activity peaked in near senescent cultures as cellular proliferation declined and then decreased in cells that had reached senescence. The incidence of dicentric chromosomes near senescence increases at the same time as the DNA binding activity of p53 (Figure 3F). It is of interest to note that extracts prepared from aging WI-38 cells, which have a higher incidence of dicentric chromosomes than MRC-5 cells (Benn, 1976), also exhibit a higher level of DNA binding by p53 after normalization for p53 protein content (Figure 3E). Since dicentric chromosomes undergo breakage and fusion cycles during mitosis, it is possible that the resulting DNA strand breaks activate the latent DNA binding activity of p53 protein. We suggest that senescence-associated DNA damage (SAD) (Figure 3F), acting through p53, could initiate the events that lead to cellular senescence.

Since the increased DNA binding activity of p53 was measured in the presence of PAb421 antibodies, we considered the possibility that the DNA binding assay reflected greater accessibility of p53 protein to PAb421 antibodies perhaps due to a conformational change and/or post-translation modification of p53 in old cells. While Western blotting revealed similar levels of p53 protein in young and old cells, immunoprecipitation analysis revealed increased levels of PAb421-reactive p53 protein in old cells (Figure 3D, middle panel). We turned, therefore, to studies of p21<sup>WAF1</sup> expression and transcriptional reporter assays in which RNA expression, while dependent on the DNA binding activity of p53 *in vivo*, occurs independently of added PAb421 antibodies.

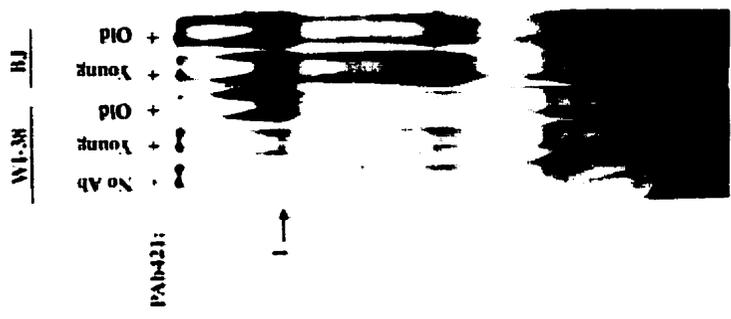
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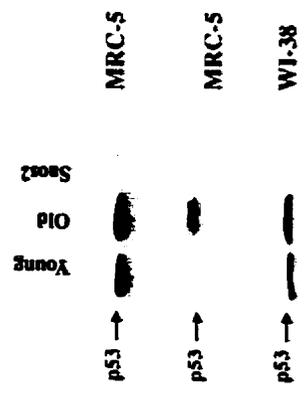
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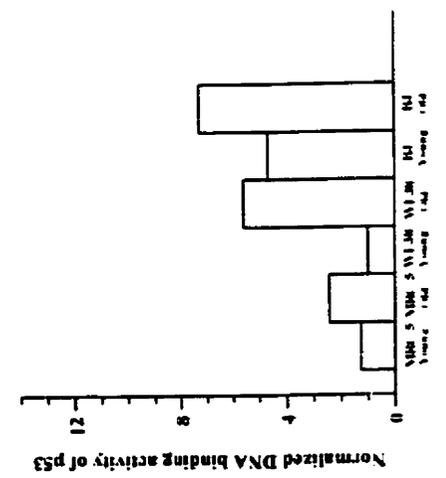
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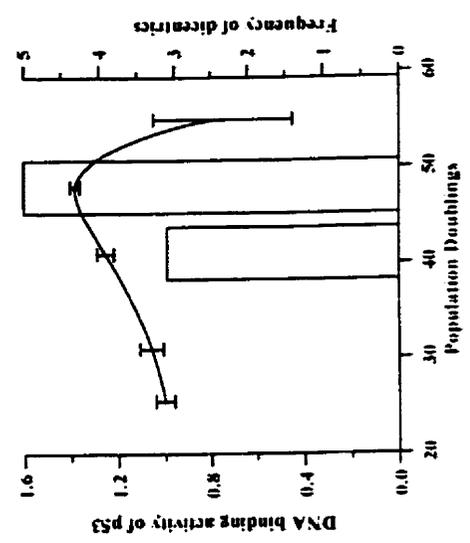
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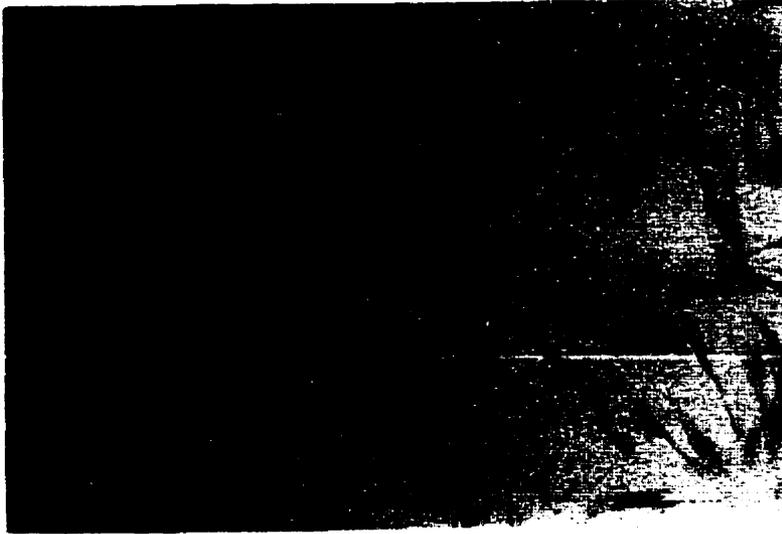


F



**Figure 3.** DNA binding activity of p53 protein increases with cell age. (A) Nuclear extracts (5  $\mu$ g) prepared from SF1 or MRC-5 cells were incubated with a  $^{32}$ P-labelled double stranded oligonucleotide containing the p53 consensus binding sequence (p53CON) with (+) or without (-) the p53-specific monoclonal antibody PAb421 and analyzed by EMSA. SF1 cells which overexpress p53 protein were used as controls in lanes 1-4. MRC-5 cells at PD32 were used in lanes 8 and 10, and at PD47 in lanes 5-7, 9. The binding reactions were supplemented with the human p53-specific monoclonal antibody PAb1801 (lane 3) or control IgG antibodies (lane 4). Lanes 5 and 6 contained cold competitor p53CON at 50-fold and 10-fold molar excess over the labelled p53CON substrate; lanes 7 and 8 contained a 50-fold excess of an unrelated, cold double-stranded mutated oligonucleotide with the same nucleotide composition as p53CON. Arrow 1 on the left indicates the position of the supershifted PAb421-p53 protein-DNA complex, arrow 2 is the p53 protein-DNA complex and arrow 3 is a non-responsive band. (B) Nuclear extracts prepared from MRC-5 cells at different population doublings (PD) were tested for p53 DNA binding activity; arrow 1 indicates the position of the PAb421-p53 protein-DNA complex. (C) DNA binding activity of p53 in nuclear extracts prepared from young and old WI-38 and BJ cells; arrow 1 indicates the position of the PAb421-p53 protein-DNA complex. (D) Western immuno-blot analysis of p53 protein in young and old MRC-5 cells (top panel) and in young and old WI-38 cells (bottom panel). The p53-negative cell line, Saos2, was included as a control. Total cell lysates (300  $\mu$ g protein) were resolved by SDS-polyacrylamide gel electrophoresis; p53 protein was detected by immuno-blotting with PAb1801 and visualized by enhanced chemiluminescence. In the middle panel, p53 was immunoprecipitated with PAb421 from young and old MRC-5 cells prior to Western blotting. (E) The p53 DNA binding activity in young and old cells was determined by EMSA and normalized to the amount of p53 protein present in the nuclear extracts by Western blotting. (F) Nuclear extracts were prepared from MRC-5 cells at different population doublings on five separate occasions. Each mean value shown on the curve is based on 5 independent samples (5  $\mu$ g protein) each measured for DNA binding activity at least once within the linear range of the DNA binding assay. Binding activity was determined by measuring the amount of radioactivity present in the PAb421 supershifted complex using a Molecular Dynamics PhosphorImager and Image Quant software. DNA binding activity is expressed relative to that seen at the earliest time point (PD 24-27). Error bars represent the standard error of the mean. Also shown is the frequency of dicentric chromosomes (bars) which increase during cell aging in culture. The data for the frequency of dicentric chromosomes (50 metaphase spreads/PD) in aging MRC-5 cells was obtained from Benn (1976).

**A**



**B**



**C**

1 2 3 4 5



**Figure 4** p21 protein expression during cell aging. (A) Young and (B) near senescent MRC-5 cells (which become larger) were fixed and p21 protein was detected immuno-histochemically using SC-187 monoclonal antibody and biotinylated HRP-conjugated secondary antibody. Same magnification was used in both cases. (C) p21 protein from SIC cell extracts (1 mg protein) was immunoprecipitated with SC-187 antibodies and detected by immuno-blotting using rabbit polyclonal antibodies against p21 (Pharmingen) and HRP-conjugated anti-rabbit antibodies. Lanes 1 and 2, near senescence (PD58) cultures of two SIC clones transfected with HPV-16 E6; lanes 3 and 4, two near senescence cultures of SIC cells; lane 5, young SIC cells (PD25).

### **3.4 Involvement of p53 and p300 in the regulation of p21<sup>WAF1</sup> promoter activity in aging human fibroblast strains**

p21<sup>WAF1</sup> mRNA levels were shown previously to increase as HDFs approached senescence (Noda *et al.*, 1994). Analysis of p21 protein levels in MRC-5 cells by immunohistochemistry (Figure 4A,B) and in SIC cells by Western immuno-blotting (Figure 4C) revealed an accumulation of p21 protein in near senescent cells. While p21 protein was localized primarily in the nucleus of young cells, both nuclear and cytoplasmic staining were seen in the older cells.

To examine the involvement of p53 in regulating p21 levels in aging fibroblasts, clones of the human fibroblast strain SIC expressing HPV-16 E6 were generated and p21 protein levels were measured. SIC/E6 clones produced less p21 protein compared with non-transfected SIC cells at the same population doubling (Figure 4C). p21 protein expression, however, was not completely abolished in these cells, possibly due to residual p53 activity or the existence of a p53-independent pathway for p21<sup>WAF1</sup> induction. It should be noted that SIC/E6 cells underwent senescence and did not exhibit a significant extension of their life-span.

In order to examine the mechanism underlying the transcriptional activation of the p21<sup>WAF1</sup> gene in aging cells, transient expression studies were performed using a vector (p21P-luc) in which the human p21<sup>WAF1</sup> promoter (2.4 kb fragment) containing a resident p53 responsive element was linked upstream of a luciferase reporter gene. A related reporter plasmid in which a 72-bp region encompassing the p53 consensus sequence was deleted from the p21<sup>WAF1</sup> promoter (p21 $\Delta$ P-luc) was used in parallel. All measurements of luciferase activity were normalized with respect to plasmid copy number in the transfected cells, using a modified Hirt assay, to control for variations in transfection efficiency. Measurement of luciferase activity 72 h

after transfection in young and old cells revealed approximately 3-fold higher levels of luciferase in cells transfected with the full length promoter compared with cells transfected with the deleted promoter (Figure 5A). Hence, the 72-bp fragment of the p21<sup>WAF1</sup> promoter that contains a p53 responsive element is required for maximal luciferase activity in both young and old cells. Luciferase activity was about 2-fold higher in old cells compared with young cells (Figure 5A,D). The elevated expression of luciferase in old cells was observed with both the full length and the truncated promoters suggesting that induction of luciferase activity in old cells might involve sequences that lie outside the 72-bp fragment of the luciferase promoter. El-Deiry *et al.* (1993) identified a second p53 recognition element in the human p21<sup>WAF1</sup> promoter that may be involved in this age-related induction. However, it remains possible that the age-related increase in p21 promoter activity involves a p53-independent pathway. To address this further, we examined the Box-A promoter of the IGF-BP3 gene (a minimal promoter fragment with the p53 consensus sequence for IGF-BP3) which is reported to be a target for p53 (Buckbinder *et al.*, 1995) and to be over-expressed in senescent HDFs (Goldstein *et al.*, 1991). Luciferase expression directed by the IGF-BP3 promoter was also higher in old MRC-5 cells compared with younger cells (Figure 5A). The SV40 promoter, which does not contain a p53 consensus binding site, provided a control for specificity in this series of transfection experiments and showed no age-related activation.

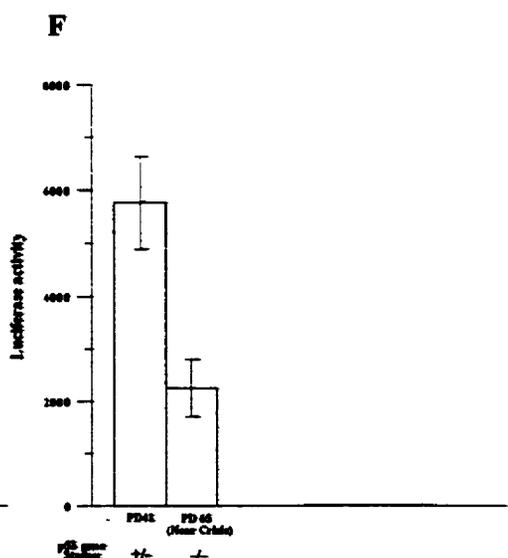
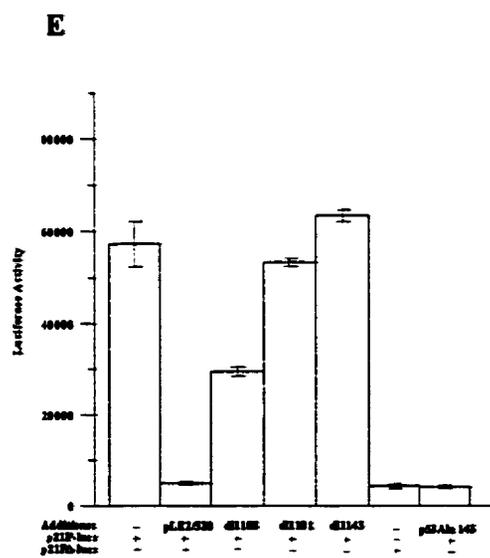
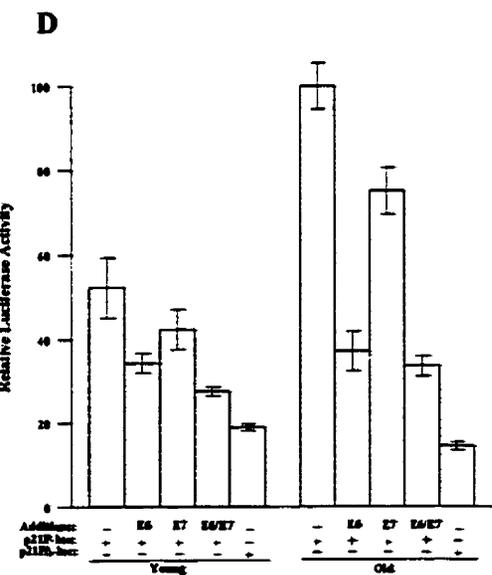
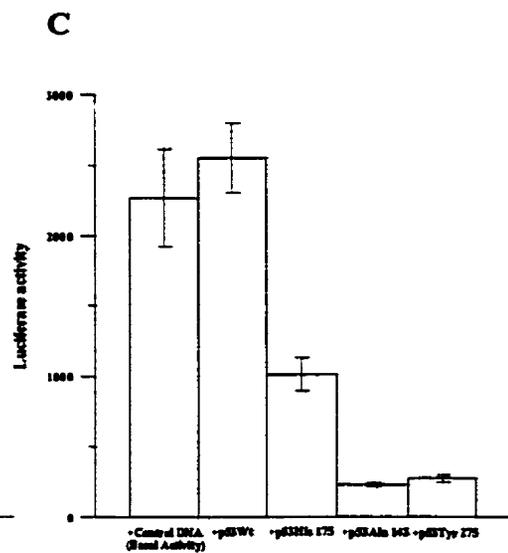
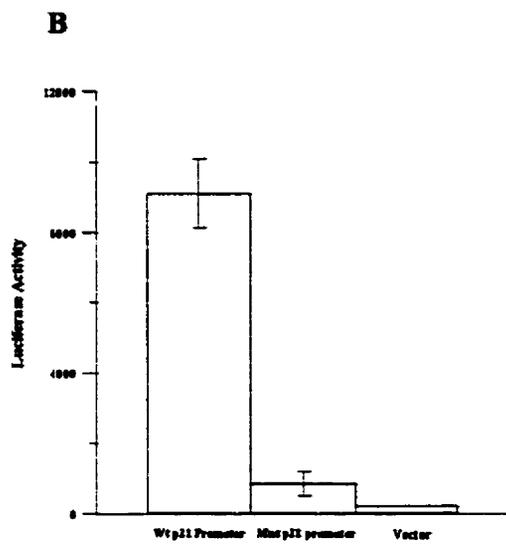
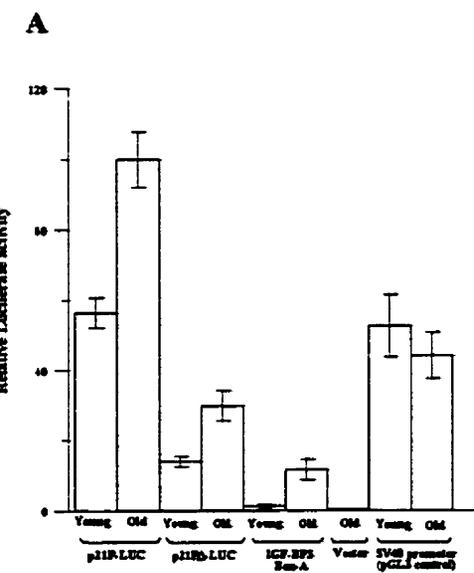
The ability of the 72-bp fragment containing the p53 consensus binding site to confer maximal activity on the p21<sup>WAF1</sup> promoter was confirmed through the use of MRC-5 clones stably transfected with pSV2neo and either p21P-luc or p21 $\Delta$ P-luc. Luciferase activity in pooled, stable, G418<sup>R</sup> clones was 9-fold higher in cells that contained the intact p21<sup>WAF1</sup> promoter

compared with cells that contained the deleted promoter (Figure 5B).

To test directly the involvement of p53 protein in regulating the activity of the p21<sup>WAF1</sup> promoter present in p21P-luc, we chose to disrupt endogenous p53 function in near senescent MRC-5 cells through transient over-expression of various dominant negative mutant p53 cDNAs. p21<sup>WAF1</sup> promoter activity was significantly repressed upon expression of p53Ala143, p53His175 and p53Tyr275 polypeptides but not by wild-type p53 expression (Figure 5C).

An alternative means of disrupting endogenous p53 function involves expression of HPV-16 E6 protein which is known to promote ubiquitin-dependent degradation of p53 protein. Accordingly, young and old cells were co-transfected with p21P-luc and one of either E6, E7 or a combination of E6/E7. E6 reduced p21<sup>WAF1</sup> promoter activity more profoundly in old cells than in young cells (Figure 5D). E7 had no significant effect on p21<sup>WAF1</sup> promoter activity in young cells but a reproducible repression of p21<sup>WAF1</sup> promoter activity was observed in old cells. The combination of E6 and E7 in old cells was not better than E6 alone in repressing luciferase expression. E7 protein is known to bind to a number of cellular proteins including pRB, pRB-related proteins and the transcriptional adapter protein p300. To determine if any of these proteins is involved in regulating p21<sup>WAF1</sup> promoter activity, we made use of several adenovirus E1A protein variants that have either lost the ability to bind pRB and pRB-related proteins yet retain the ability to bind p300 (dl1108) or lost the ability to bind p300 while retaining the ability to bind pRB and pRB-related proteins (dl1101, dl1143) (Howe *et al.* 1990). Of the three E1A variants tested, only the one encoded by dl1108 repressed the p21<sup>WAF1</sup> promoter in old cells (Figure 5E). Wild-type E1A (pLE2/520) repressed the activity of the p21<sup>WAF1</sup> promoter as effectively as p53Ala143. Wild-type E1A and the dl1108 variant also share the ability to induce

DNA synthesis in quiescent fibroblasts (Howe *et al.*, 1990). Together, these data implicate both p300 (or other p300 related molecules) and p53 proteins in the regulation of the p21<sup>WAF1</sup> promoter in human fibroblasts.



**Figure 5** Activation of the p21 promoter requires both p53 and p300. Values obtained can only be compared within the given graph and can not be compared with other graphs due to different genetic backgrounds and experimental conditions.

(A) Young and old MRC-5 cells were transfected with 5 µg of reporter plasmid in which the luciferase coding sequence was placed downstream of the wild-type p21 promoter (p21P-luc), a mutant p21 promoter (p21ΔP-luc), the IGF-BP3 promoter element bearing Box A, no promoter (vector), or the SV40/pGL3 control promoter. Luciferase activity was measured in triplicate, 72 h after transfection, using a luminometer and normalized for differences in plasmid copy number determined using a modified Hirt procedure as described in Materials and methods. The highest level of normalized luciferase activity was seen in old cells. This value was set at 100% and all other values are expressed proportionately. Error bars represent the SEM.

(B) Young MRC-5 cells were stably transfected with wild-type or mutant p21 promoter-luciferase plasmids. After selection in G418 the resulting 25 mutant promoter clones and 20 wild-type promoter clones were pooled and luciferase activity was measured.

(C) MRC-5 cells nearing senescence were co-transfected with the p21 promoter-luciferase reporter plasmid (p21P-luc) and one of various human p53 cDNA plasmids (2 µg) in which expression was controlled by the CMV promoter. Normalized values of luciferase activity were determined as described in (A) and are expressed in relative light units (RLU).

(D) Young and old cells were co-transfected with 5 µg of p21P-luc plasmid together with 10 µg of one of the following expression plasmids: pSV2-E6, pSV2-E7, pSV2-E6/E7, or control vector plasmid. 72 h after transfection, luciferase activity was measured as described in (A).

(E) Cells nearing senescence were transfected with the p21P-luc plasmid together with plasmids expressing wild-type or mutant Ad E1A protein. dl1108 can bind p300 but fails to bind pRB or p107; dl1101 and dl1143 both fail to bind to p300 but do bind to pRB and p107. pLE2/520 expresses wild-type E1A protein.

(F) The cell strain 2675T, was derived from a Li-Fraumeni patient and bears a heterozygous p53 gene mutation. Upon prolonged passage in culture, these cells lose the wild-type p53 allele. The p53 gene status of these cells at PD 41 and at PD 65 was examined and found to be heterozygous (+/-) at the earlier passage with loss of heterozygosity (-/-) occurring at the later passage. Cells at PD 41 or at PD 65 were transfected with p21P-luc and luciferase activity was measured as described in Materials and methods.

### **3.5 Loss of a wild-type p53 allele leads to reduced p21<sup>WAF1</sup> promoter activity and extension of cellular lifespan**

Expression of SV40 large T antigen or HPV-16 E6 and E7 combination of proteins in human fibroblasts leads to an extension of cellular lifespan. These cells bypass the senescence checkpoint but eventually enter a second phase of arrest termed crisis that, unlike senescence, is associated with cell death. Elevation in p21<sup>WAF1</sup> RNA levels has been observed in cells entering crisis as well as in cells entering senescence (Rubelj and Pereira-Smith, 1994). Having shown that p53 protein is involved in the regulation of p21<sup>WAF1</sup> gene transcription in aging fibroblasts, we next wished to determine if p53 played any role in the induction of p21<sup>WAF1</sup> RNA at crisis. We made use of fibroblasts obtained from an individual with the Li-Fraumeni syndrome. Fibroblasts from Li-Fraumeni patients commonly contain one wild-type p53 allele and one mutant, non-functional p53 allele. When placed in culture, these cells have been shown to bypass senescence (Bischoff *et al.*, 1990) upon loss of the remaining wild-type p53 allele (Rogan *et al.*, 1995). The 2675T strain carries a heterozygous mutation at codon 245 that converts glycine to aspartic acid (Srivastava *et al.*, 1990; Mirzayans *et al.*, 1995). The p53Asp245 protein is stable, displays an altered conformation, and fails to bind DNA (Friend, 1994). The 2800T strain was obtained from a normal individual. However, it too carries a heterozygous p53 mutation, at codon 234, that replaces tyrosine with cysteine (Mirzayans *et al.*, 1995). The p53Cys234 protein appears to have retained a normal conformation (Friend, 1994). We cultured the 2800T and 2675T strains and noticed that 2800T entered senescence after 56 PDs while 2675T continued to divide for a longer period of time prior to entering crisis after 68 PDs (as

judged by having no distinct G1 or G2/M phases, short telomeres and presence of cell death). Analysis of the p53 gene revealed that the senescent 2800T cells remained heterozygous at the p53 locus, while 2675T lost the normal p53 allele at some point prior to entering crisis between PD41 and PD65. The 2675T cells remained in crisis for 230 days and no immortal (ie post-crisis) clones arose from this strain. 2675T cells at PD41 and PD65 were transfected with p21P-luc and luciferase was measured 72 h later. Luciferase activity was lower at PD65 than at PD41 (Figure 5F). These data indicate that complete loss of wild-type p53 gene expression in human fibroblasts is associated with a reduction in p21<sup>WAF1</sup> promoter activity and with extension of cellular lifespan.

### **3.6 Short telomeres, premature senescence and increased DNA binding activity of p53 protein in AT strains**

The ATM gene product has been proposed to lie upstream of p53 protein in the DNA damage response pathway that leads to cell cycle arrest at the G1/S boundary (Kastan *et al.*, 1991; Lu and Lane, 1993). HDFs from individuals with ataxia-telangiectasia display increased sensitivity to  $\gamma$ -irradiation and certain strains undergo accelerated senescence in culture (Shiloh *et al.*, 1982). In addition, disruption of the yeast *TEL1* gene, which shows homology with the human ATM gene, results in accelerated loss of telomeric DNA (Greenwell *et al.*, 1995). As a result of these findings, we wished to investigate further the connection between p53 function, aging and telomere loss in AT cells.

Fibroblasts were obtained from five AT patients and compared with fibroblasts from either their normal siblings or unrelated age-matched controls. For each cell strain, we

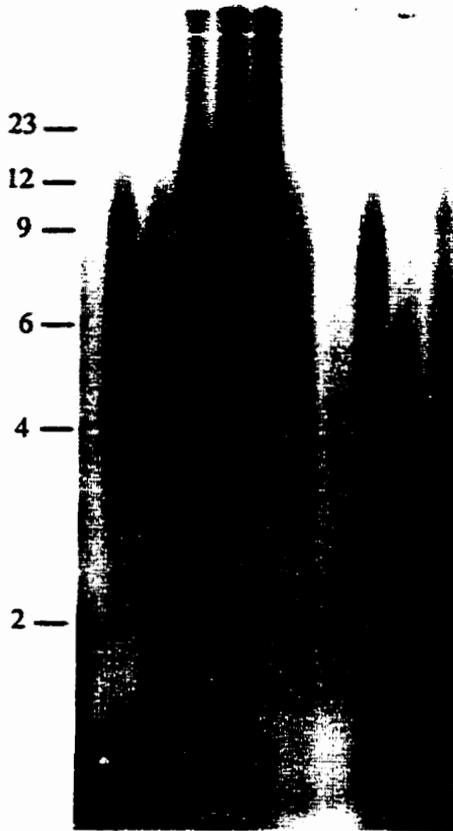
determined the number of population doublings that preceded senescence, mean terminal restriction fragment (TRF) length, the amount of p53 protein and the DNA binding activity of p53 protein. The results are presented in Figure 6. As expected all the AT fibroblast strains reached senescence earlier than the normal strains. The mean TRF length, which reflects the length of telomeres, was shorter at the same PD in the AT strains 3487C and 1937B than in their sibling controls 3492 and 3400 cells (Figure 6A,D). Of the remaining 3 AT strains, 3395B had a short TRF length and displayed premature senescence, while 2052B and 2530 had longer telomeres (longer than controls) at early passage and continued to divide until they reached PD 35 and 38, respectively, at which time shortened telomeres were present (Figure 6D). This finding prompted us to compare the rate of telomere loss in the AT strain 2530 with its age-matched normal control 3400. Accelerated telomere loss was observed in 2530 (267 bp/pd) compared with 3400 cells (60 bp/pd). While these data strengthen the association between AT and the senescent phenotype, they indicate that the involvement of the ATM gene in regulating telomere length and replicative senescence is likely to be complex.

In Figure 3, we showed that the DNA binding activity of p53 protein increased during cell aging in the absence of a marked increase in the steady state level of p53 protein. Since certain AT strains have short telomeres and display accelerated aging in culture, we compared the DNA binding activity and steady state level of p53 protein in the 3487C AT strain with its normal sibling strain 3492, at approximately the same population doubling. Enhanced DNA binding activity was evident in 3487C even though these AT cells contain less p53 protein than the normal sibling control 3492 (Figure 6B). These results indicate that the specific DNA binding activity of p53 protein is higher in the 3487C AT strain than in the normal sibling 3492

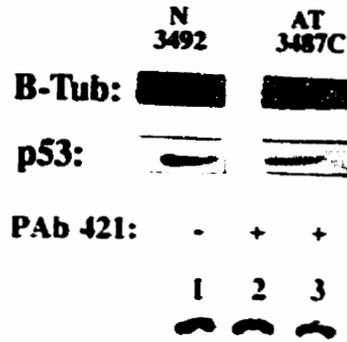
strain. Increased DNA binding activity was similarly observed in 1937B AT cells compared with the age-matched, sibling control 3400 cells (data not shown). These data support the view that ATM is involved in the regulation of telomere length and that the DNA binding activity of p53 protein is post-translationally activated in aging cells.

**A**

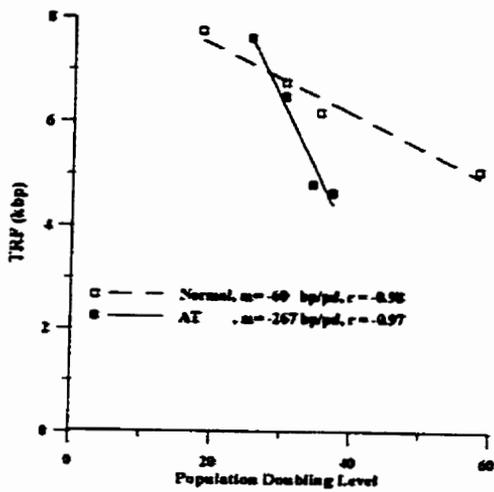
	Normal				AT					
	F1	3492	3400	P4	F28	1937	3487C	3395B	2052B	2530
Age(yr):	26	7	11	12	8	8	24	13	15	8
PD#:	26	21	18	24	20	22	20	20	35	38



**B**



**C**



**D**

Cell Strain	Lane#	Age (yrs)	Status	PD matched			Senescence	
				TRF (kbp)	SD (n=2)	PD#	TRF (kbp)	PD Max
F1	1	26	N	7.0	0.1	26	-	>52
3492	2	7	N	5.6	0.1	21	-	>36
3400	3	11	N	7.4	0.3	18	-	>40
P4	4	12	N	8.5	0.8	24	-	>62
F28	5	8	N	8.9	1.4	20	-	>56
3487C	6	24	AT	5.1	0.1	22	-	23
1937B	7	8	AT	5.0	0.4	20	-	22
3395	8	13	AT	5.5	0.2	20	-	29
2052B	9	15	AT	6.7	1.3	23	4.5	35
2530	10	8	AT	9.0	1.3	25	4.9	38

**Figure 6** Analysis of replicative lifespan, telomeric DNA length, and DNA binding activity of p53 protein in AT HDFs.

(A) Genomic DNA from 5 normal (lanes 1-5) and 5 AT strains (lanes 6-10) were digested with *HinfI/RsaI* and telomeric DNA was detected using a  $\gamma$ -<sup>32</sup>P-labelled (C<sub>3</sub>T<sub>2</sub>A<sub>2</sub>)<sub>3</sub> probe. The mean terminal restriction fragment (TRF) length was determined from these data as described in Materials and methods, and are indicated in (D).

(B) In the bottom panel, EMSAs were performed with 5  $\mu$ g of nuclear extract from an AT strain (3487C) and its normal age-matched sibling (3492). Nuclear extracts were prepared at PD 23 for both cell strains and were added to reactions containing end-labelled p53CON oligonucleotide and the p53-specific antibody PAb421. Lane 1, 3487C cell extract without antibody; lane 2, 3492 cell extract; lane 3, 3487C cell extract. The top panel represents a Western blot in which cell lysates (300  $\mu$ g protein) were subjected to electrophoresis on an SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and subsequently blotted with DO-1 antibody to detect p53 and a  $\beta$ -tubulin control antibody.

(C) Mean TRF length of AT strain 2530 was measured throughout its lifespan and compared with an age-matched normal control strain 3400.

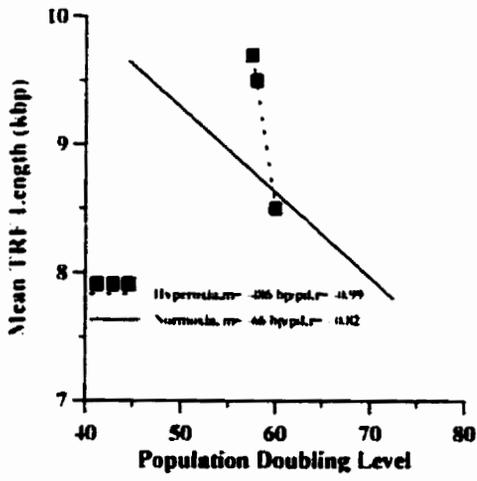
(D) Summary of data collected on normal and AT strains. 3 AT strains had shorter telomeres and were entering senescence while 2 AT strains with longer telomeres continued to proliferate further before entering premature senescence (indicated by PD max).

### **3.7 Hyperoxia leads to accelerated telomere loss, functional activation of p53 protein and premature senescence.**

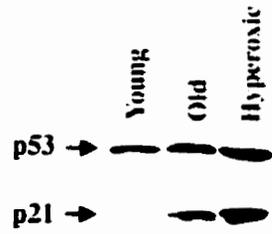
The cellular lifespan of human diploid fibroblasts can be affected by the oxygen concentration at which the cells are cultured. Cells have an extended life-span under low oxygen conditions and a shortened life-span under high oxygen (Chen *et al.*, 1995; Saito *et al.*, 1995). In addition, telomeres of HDFs grown under hyperoxia have been shown to undergo an accelerated rate of shortening (von Zglinicki *et al.*, 1995). We wished to determine if the association between shortened telomeres, p53 activation and senescence could also be demonstrated in cells grown under hyperoxic conditions. WI-38 and BJ cells were cultured under 20% oxygen or exposed to hyperoxia (40% oxygen). WI-38 and BJ cells were exposed to hyperoxia at PD 27 and 57, respectively, and cultured under hyperoxic conditions for the duration of their life-span. Both WI-38 and BJ cells lost telomeric DNA at an accelerated rate under hyperoxia and entered senescence prematurely (Figure 7A). BJ cells lost telomeric DNA at a rate of 66 bp/pd when grown at 20% O<sub>2</sub> or at a rate of 486 bp/pd in hyperoxia (Figure 7A); WI-38 cells lost telomeric DNA at a rate of 70 bp/pd under normal oxygen or at a rate of 240 bp/pd in hyperoxia (data not shown). We then measured the relative level of p53 protein by Western blot analysis and the DNA binding activity of p53 by EMSA in cells grown under normal or hyperoxic conditions (Figure 7B,C). Normalization of the DNA binding values to the level of p53 protein, revealed a 13-fold and a 2-fold increase in the specific DNA binding activity of p53 protein in WI-38 cells and BJ cells, respectively, upon exposure to hyperoxia. Consistent with this finding, the amount of p21 protein increased in hyperoxic cells to a level even higher than that seen in old cells

(Figure 7B). Together these results indicate that hyperoxia leads to an accelerated loss of telomeric DNA beyond the level which can be explained by the end-replication problem. The loss of telomeric DNA is associated with an increase in the specific activity of p53 protein, elevation of p21 protein expression and premature senescence.

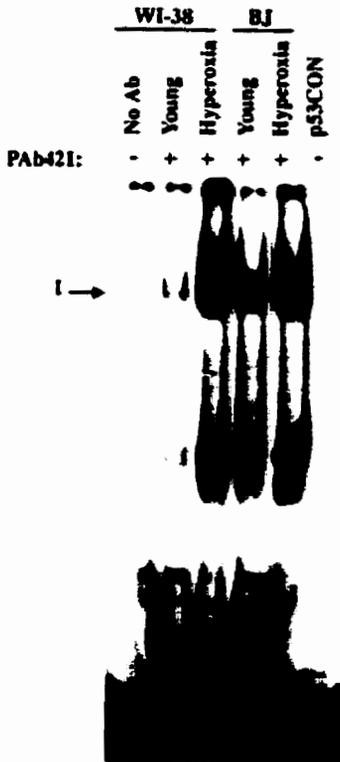
**A**



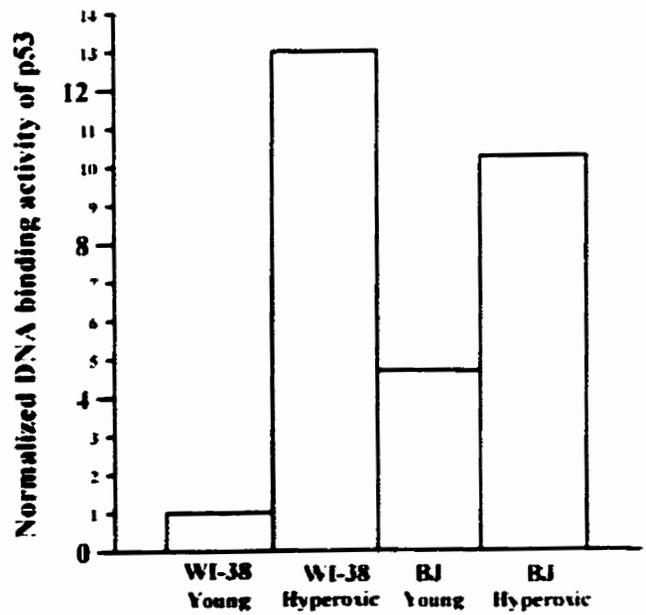
**B**



**C**



**D**



**Figure 7** Hyperoxia leads to accelerated telomere loss, activation of p53 protein and premature senescence. (A) 1  $\mu$ g of *HinfI/RsaI*-digested genomic DNA from WI-38 and BJ cells under normoxia and hyperoxia were resolved on a 0.5% agarose gel and subsequently probed with a  $\gamma$ -<sup>32</sup>P-labelled (C<sub>3</sub>TA<sub>2</sub>)<sub>3</sub> probe. Quantification of mean TRF length over the indicated PD range is shown for BJ cells under hyperoxia (dotted line and solid squares) and under normal oxygen (solid line and open circles). The rate of TRF loss observed under normal oxygen was 66 bp/pd,  $r = -0.82$  and under hyperoxia, 486 bp/pd,  $r = -0.99$ . (B). Immuno-blot analysis of p53 (DO-1) and p21 (SC-187) protein in young WI-38 cells under normal conditions and hyperoxic conditions, and in old cells and normal conditions. (C) EMSA showing the increase in DNA binding activity of p53 for p53CON (p53 binding consensus sequence) in young and hyperoxic cells as described in Figure 3. Arrow 1 indicates the position of the supershifted band. (D) Quantification of DNA binding activity of p53 under normal and hyperoxia. Values obtained by integration of supershifted band (band 1) in Figure 7C and normalized to the values obtained from quantification of p53 protein in Figure 7B.

### **3.8 Poly(ADP-ribose) polymerase and p53 protein interact in vitro and vivo**

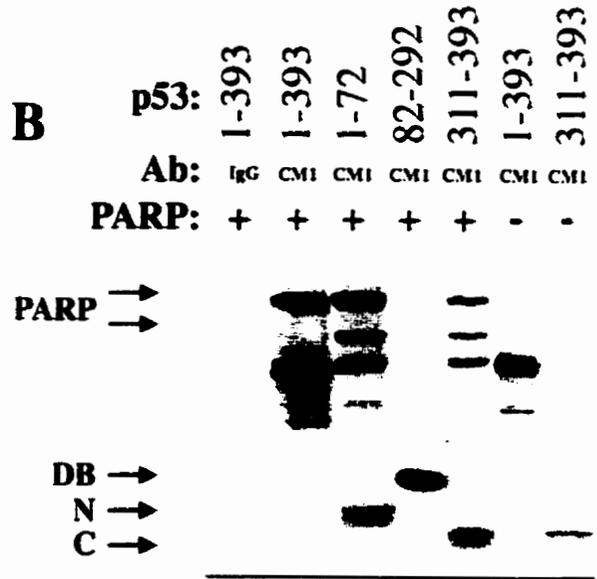
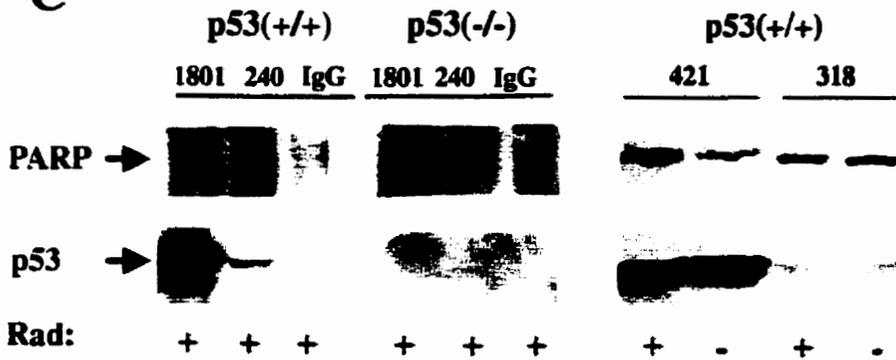
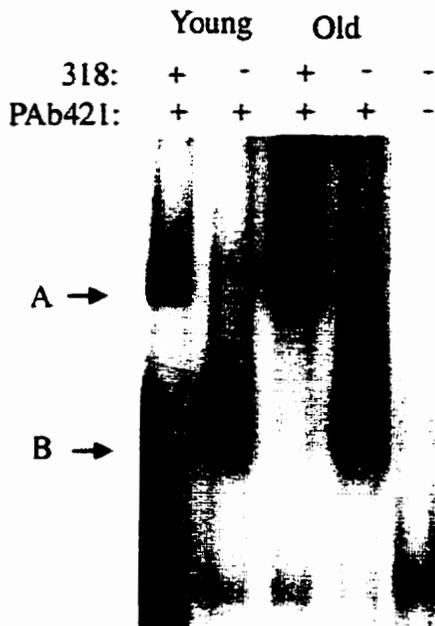
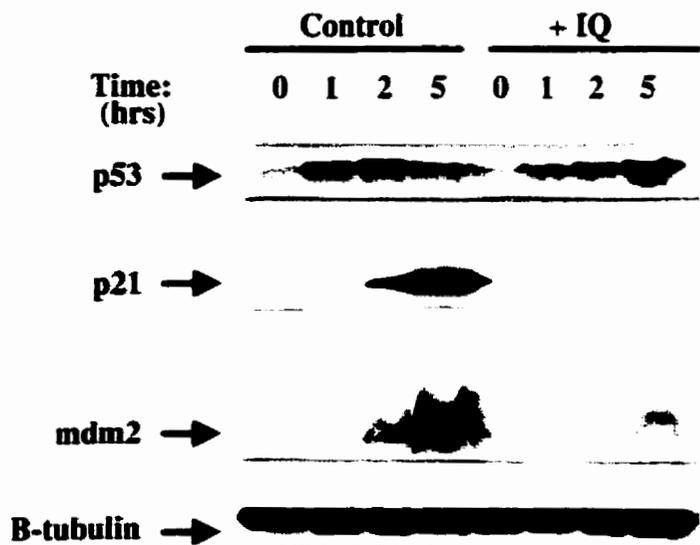
The senescence-associated activation of p53 protein function seen in aging fibroblasts, AT fibroblasts and hyperoxic fibroblasts, may be analogous to the post-translational activation of p53 protein that is observed in cells exposed to DNA damaging agents. Modifying enzymes that are activated in response to DNA damage such as DNA-PK, SAPK and PARP have been shown to modify p53 protein *in vitro* (Anderson, 1993; Wesierska *et al.*, 1996) and hence, represent potential physiological activators of p53 protein during aging or after DNA damage. To investigate the interaction of p53 and PARP, purified full length human p53 was mixed with purified full length PARP (Huletsky *et al.*, 1989) The mixture was subjected to immunoprecipitation with CM1 polyclonal antibody against p53, resolved by polyacrylamide gel electrophoresis and processed for Western blotting to detect p53 (with a mixture of PAb7 and PAb240 antibodies) and bound PARP (C2-10 antibody). The immunoblot presented in Figure 8A shows that PARP co-immunoprecipitated with p53. In order to map the region of p53 that interacts with PARP, several truncated forms of p53 protein were prepared and mixed with full length PARP for immunoprecipitation/Western blot analysis. As shown in Figure 8B, both an N-terminal p53 fragment (amino acids 1-72) and a C terminal p53 fragment (311-393), but not the DNA binding domain of p53 (82-292), were able to interact with PARP (although very weak binding was observed up on longer exposures). The binding with PARP was much stronger with the N-terminal fragment of p53.

To investigate this interaction *in vivo*, cell extracts were prepared from the wild-type p53-expressing human cell line OCI/AML-3 (Fu *et al.*, 1996) and subjected to immunoprecipitation

with antibodies against p53 (PAb1801, PAb240 or PAb421) or PARP (318). The immunoprecipitates were resolved by polyacrylamide gel electrophoresis, proteins were transferred to nitrocellulose membranes and probed with a mixture of antibodies against PARP (C2-10 and 318) or p53 (PAb7). The results of this co-immunoprecipitation/Western blot analysis indicate that p53 protein and PARP form a complex *in vivo*. Three antibodies directed towards different epitopes of p53 co-immunoprecipitated bound PARP (Figure 8C, top panel) in addition to p53 protein (Figure 8C, bottom panel). Moreover, in the reciprocal experiment, antibodies against PARP co-immunoprecipitated bound p53. An extract prepared from the p53 null cell line SKOV3 served as a control. Although certain extracts used for this experiment were prepared from  $\gamma$ -irradiated cells (2 Gy or 6 Gy) in order to increase the amount of p53 protein and facilitate detection of complexes with PARP, the interaction between p53 and PARP was also seen in non-irradiated cells. Of the 3 p53 antibodies used, PAb1801 was the least effective in co-immunoprecipitating the bound PARP. One explanation for this observation is afforded by the peptide mapping experiments which revealed that PARP bound tightly to the N-terminal fragment of p53. PAb1801 also binds to the N-terminus of p53 and could disrupt the interaction between PARP and p53. Alternatively the interaction between p53 and PARP may prevent access of PAb1801 to its binding site. Two other antibodies that recognize epitopes at the N-terminus of p53, DO-1 and DO-7 failed to co-immunoprecipitate PARP (data not shown). Immunoprecipitation using Pab240 antibody against the DNA binding domain of p53 revealed that more PARP was co-immunoprecipitated (Fig 8C). This indicates that the core DNA binding domain of p53 protein is most likely not masked by PARP-p53 interactions.

The PARP/p53 interaction was investigated further using gel mobility shift experiments

to assay for p53-DNA binding activity to the previously described p53 binding sequence p53CON. Nuclear extracts from young and old HDFs were incubated with p53CON. The binding reactions were supplemented with PAb421 or a mixture of PAb421 and 318 antibodies. The results presented in Figure 8D show that p53-DNA complexes can be recognized and supershifted with antibodies to p53 (band B) and that these supershifted complexes can be further shifted upon incubation with antibodies to PARP (band A). Inclusion of the PARP antibody alone in the binding reaction resulted in a supershifted band with a mobility very similar to that seen with the PAb421 antibody (data not shown). These results confirm the existence *in vivo* of a p53-PARP complex with DNA binding activity.

**A****B****C****D****E**

**Figure 8** Interaction of p53 and PARP in vitro and in vivo (A) Interaction of full length human p53 with PARP *in vitro*. 0.5 µg of p53 and PARP were mixed, incubated and subsequently immunoprecipitated with CM1 antibody against p53 as described in Materials and methods. DnaseI was added to ensure interaction did not depend on DNA. Immune complexes were subjected to immunoblotting with PAb7 and C2-10 to detect p53 and PARP, respectively. (B) Full length purified PARP was mixed with different purified fragments of p53 protein as indicated and subjected to immunoprecipitation and immunoblotting with PAb7 and PAb240. DB (DNA binding domain, residues 82-292), N (residues 1-72), C (residues 311-393). (C) Interaction between PARP and p53 protein *in vivo*. Cell extracts were prepared from untreated or γ-irradiated wild type p53 expressing cells (OCI/AML-3) or p53-null cells (SKOV3), 1 to 2.5 h after treatment with a dose of 2 or 6 Gy. Proteins were immunoprecipitated with antibodies against p53 (PAb1801, PAb240, PAb421), antibodies against PARP (318) or control IgG antibodies. Immunoprecipitated proteins were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes and blotted with a mix of C2-10 and 318 antibodies specific for PARP (top) or with PAb7 antibodies directed against p53 (bottom). (D) Nuclear extracts were prepared in low pH buffer from young and old cells and mixed with <sup>32</sup>P-labelled p53CON. The DNA binding reactions were supplemented with PAb421 antibodies or a mixture of PAb421 and 318 antibodies, and analyzed on a native polyacrylamide gel (pH 6.8). (E) OCI/AML-3 cells were incubated in the presence of 200 µM IQ or in DMSO (control) for 1 h prior to γ-irradiation (2 Gy). 20 minutes after irradiation, a second equivalent dose of IQ was added to the cells that had received IQ earlier. At different times after irradiation, cell extracts were prepared and samples containing 400 µg protein were subjected to sequential immunoblotting with the following antibodies: PAb1801 (p53), SC-187 (p21), 2A10 (mdm2), and anti-β tubulin.

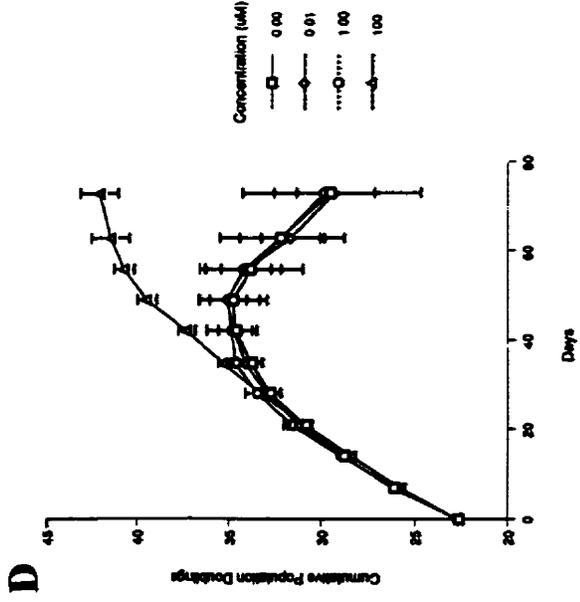
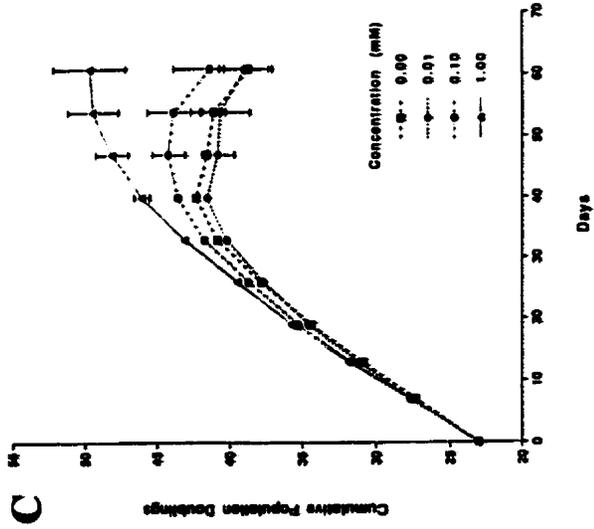
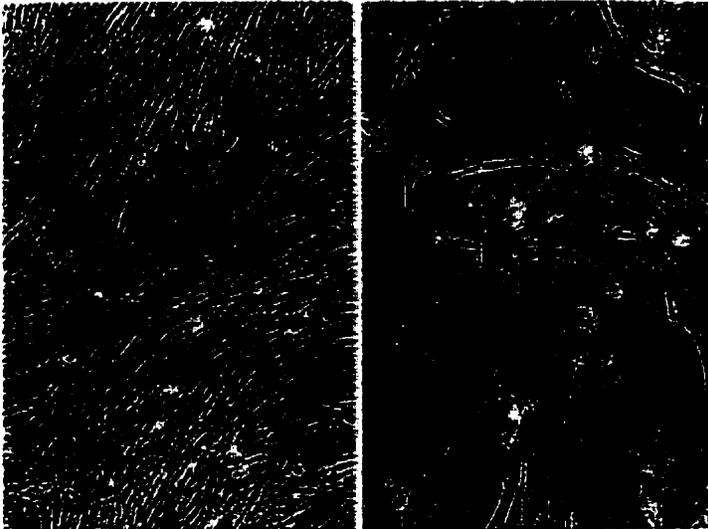
### **3.9 Inactivation of PARP leads to extinction of p21 and mdm2 expression in response to DNA damage**

If the interaction with PARP regulates the activity of p53 protein in response to DNA damage, then it is possible that inhibition of PARP leads to abrogation of p53-DNA binding activity and of the expression of down-stream targets of p53. Cells irradiated with 2 Gy showed an increase in the amount of p53 protein and an elevation in the level of p21 and mdm2 proteins (Figure 8E). However, prior treatment of the cells with the specific PARP inhibitor 1,5-dihydroxyisoquinoline (IQ), prevented the increase in expression of p21 and mdm2 after irradiation without affecting the accumulation of p53 protein (a significant increase in the stability of p53 protein was observed in the presence of IQ after careful quantitation, data not shown). Treatment with IQ also diminished and delayed the increased DNA binding activity that is normally seen after irradiation (data not shown). This indicates that mechanisms involved in the accumulation of p53 protein in response to DNA damage are uncoupled from those affecting its activation.

### **3.10 Inactivation of PARP and p53 leads to extension of lifespan in HDFs**

The demonstration that p53 is involved in cellular senescence coupled with the observation that PARP, an enzyme that is strongly activated by binding to DNA single- or double-strand breaks, can associate with p53 protein *in vivo*, raises the question of PARP involvement in modulating cellular lifespan. PARP may sense DNA damage directly and relay the signal to p53 protein resulting in functional activation of p53. One prediction of this model is that in the presence of a PARP inhibitor, p53 may not become activated, and cells would have an extended lifespan. To

test this model, HDFs were treated with inhibitors of PARP, namely 3-amino benzamide (3AB) having an IC50 of 23  $\mu$ M, and the more specific inhibitor 1,5- dihydroxyisoquinoline (IQ) having an IC50 of 0.39  $\mu$ M (Banasik *et al.*, 1992). We recognize the possibility that these two inhibitors may target NAD-dependent enzymes other than PARP. However, it has been shown previously that IQ has specificity for PARP *in vivo* and that it does not significantly change the NAD<sup>+</sup> pool *in vivo* (Shah *et al.*, 1996). 3AB-treated cells showed a significant extension of cellular lifespan as did the IQ-treated cells (Figure 9). It is evident in Figure 9D that there is significant variation in the cumulative number of doublings reached before senescence. We believe that this may be due to initial variation in the calculated PDs of the starting cell population. Pooling of the maximum PD achieved between different sources of WI38 could be responsible for the downward growth of the curves (Fig 9D). Removal of the PARP inhibitors led to restoration of normal lifespan and subsequent senescence (data not shown). Hence, functional inactivation of PARP or functional inactivation of p53 (section 3.5) leads to a common phenotype, namely, extension of cellular lifespan. This supports the idea that PARP and p53 are both components of a senescence determining pathway.



**Figure 9** Extension of cellular lifespan by PARP inhibitors. WI-38 cells were grown in the presence of 100  $\mu$ m IQ (A) or in the absence of IQ (B).

Growth curves of WI-38 cells incubated in presence of different concentrations of 3AB (C) or IQ (D).

### 3.11 Discussion

There is substantial evidence that cellular senescence is associated with elevated expression of the p21<sup>WAF1</sup> gene and with a decrease in the size of telomeres. The p53 nuclear phosphoprotein has also been implicated in senescence since it can activate expression of the p21<sup>WAF1</sup> gene by binding to the p53-responsive element within the p21<sup>WAF1</sup> promoter. Moreover, the DNA binding activity of p53 has been shown to increase in aging fibroblasts, and disruption of p53 protein function has been reported to extend the proliferative lifespan of human fibroblasts. The data we present here extend these observations and demonstrate that binding of p53 to a physiologically relevant binding site in the p21<sup>WAF1</sup> promoter increases in old cells. We show that expression of the p21<sup>WAF1</sup> gene in aging fibroblasts is regulated by p53 and by the transcriptional co-activator p300 which was previously shown to regulate the p21<sup>WAF1</sup> promoter independently of p53 (Missero *et al.*, 1995). The increased p53-dependent transcriptional activity of the IGF-BP3 promoter in old cells (Figure 5A) also provides an explanation for the upregulation of IGF-BP3 with cell age (Goldstein *et al.*, 1991). In agreement with previous studies we find that the steady state level of p53 protein does not change markedly as cells age in culture. These observations suggest that p53 protein is post-translationally activated in aging cells and that one of its functions is to control expression of p21<sup>WAF1</sup>.

We and others have detected higher levels of p53 protein in old cells compared with young cells when extracts were immunoprecipitated with PAb1801 or PAb421 antibodies prior to Western blotting (Kulju and Lehman, 1995; Vaziri and Benchimol, 1996). Immuno-blotting without prior immunoprecipitation revealed similar levels of p53 protein in young and old cells.

These observations suggest that p53 protein undergoes a change in conformation during cellular aging that exposes epitopes at the termini of the molecule. The altered conformation may have relevance with respect to the increased DNA binding (Figure 3) and transcriptional activity of p53 (Figure 5).

In AT cells that have short telomeres and consequently reach senescence after fewer population doublings, we found that p53 protein had increased DNA binding activity compared with age-matched normal controls at the same population doubling. These data are consistent with the results obtained with aging normal fibroblasts and demonstrate an association between the DNA binding activity of p53 and cellular lifespan that is independent of p53 protein level.

Amongst the 5 AT fibroblast strains examined, we found heterogeneity with respect to telomere length. 3 strains had short telomeres and senesced prematurely while two strains with longer telomeres at the time of analysis displayed a higher proliferative capacity in culture. Nevertheless, these strains had an accelerated rate of telomere shortening and underwent premature senescence. These data are consistent with the idea that the ATM gene plays a role in determining cellular lifespan. However, the involvement of ATM in regulating telomere length is likely to be complex.

A number of studies have concluded that the p53-dependent DNA damage response is defective or attenuated in  $\gamma$ -irradiated AT cells (Kastan *et al.*, 1991; Lu and Lane, 1993). It may not be appropriate, however, to compare the activity of p53 protein in response to  $\gamma$ -irradiation in AT and normal cells, or even the radiosensitivity of AT cells with normal cells. AT cells have a shorter lifespan than normal cells and, hence, at the time of analysis, these cells will have completed a far greater proportion of their total lifespan than normal cells. AT cells may be

approaching the end of their proliferative lifespan while normal cells at the same population doubling will still retain considerable proliferative potential. It has been shown, for example, that the radiation sensitivity of normal fibroblasts could change with age in culture (Holliday, 1991).

Treatment of cells with hyperoxia results in premature senescence and an accelerated rate of telomere shortening that cannot be accounted for by the end replication problem associated with the inability to fully replicate DNA at the ends of chromosomes during each round of DNA replication. Premature senescence resulting from hyperoxia, like the premature senescence seen in AT fibroblasts, is associated with shortened telomeres and with the post translational activation of p53 protein.

We provide several lines of evidence to show that p53 and PARP interact: (i) binding occurs *in vitro* using purified components, (ii) binding *in vivo* can be demonstrated by co-immunoprecipitation of PARP using 3 antibodies against distinct epitopes of p53, and by co-immunoprecipitation of p53 with an antibody against PARP, and (iii) PARP can be detected on p53-DNA complexes by antibody supershift experiments. An association between p53 and PARP *in vitro* was recently reported (Wesierska *et al.*, 1996). Chemical inhibition of PARP activity with 1,5-dihydroxyisoquinoline resulted in the abrogation of the p53-mediated induction of p21 and mdm2 expression that is normally seen in  $\gamma$ -irradiated cells. Our findings are consistent with the observation that the p53 response to DNA damage is defective in cells with PARP deficiency (Whitacre *et al.*, 1995). These data indicate that the interaction between PARP and p53 is critical for p53 function in response to DNA damage signals. While p53 activity was disrupted by the inhibition of PARP activity, the accumulation of p53 protein in response to

irradiation was not greatly affected. This provides compelling evidence that the accumulation and activation of p53 protein in response to irradiation represent separate events that can be completely uncoupled in one direction. PARP activity is required for p53 protein activation but is dispensable for p53 protein accumulation.

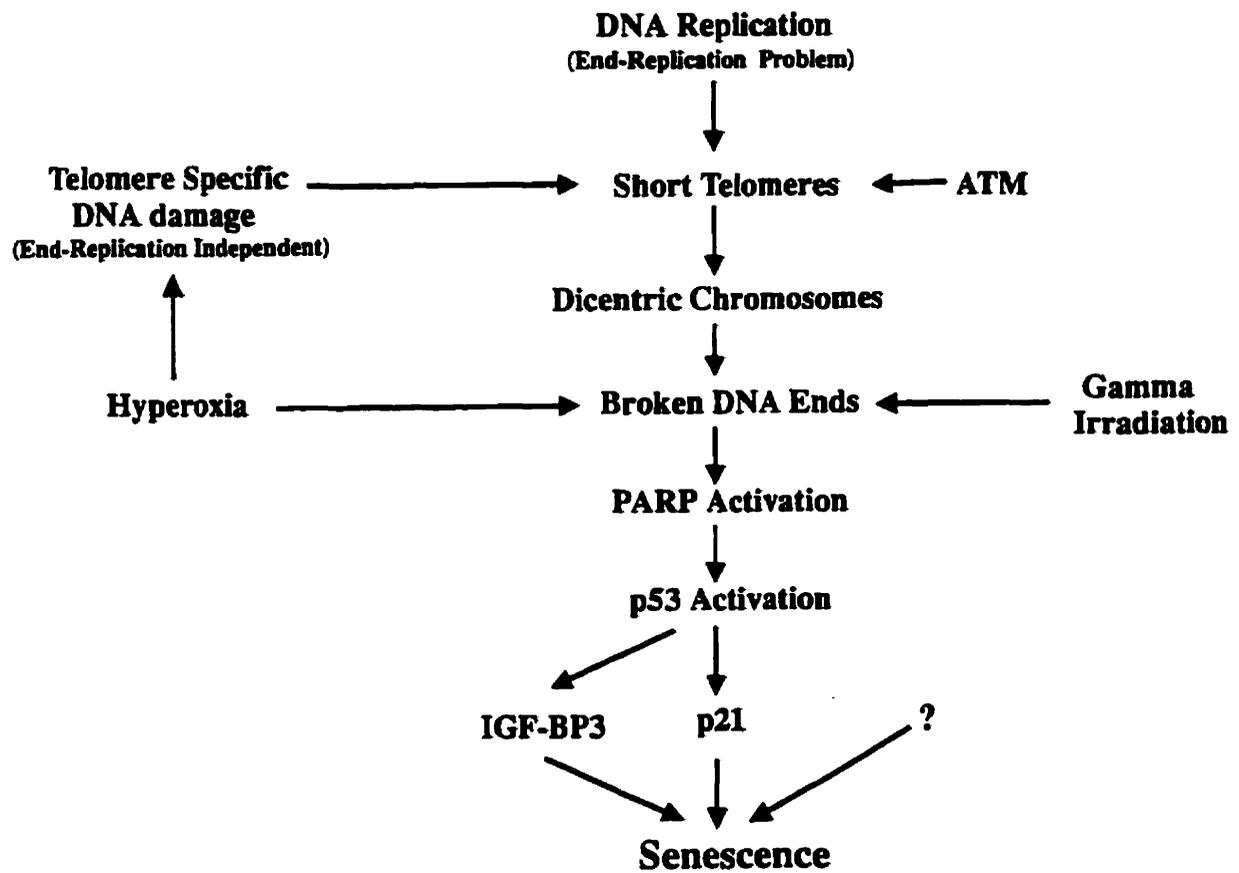
Since PARP is known to become activated in response to DNA damage, it is possible that PARP acts as a DNA damage sensor that relays the telomere loss signal to p53. To investigate the presumed involvement of PARP in senescence, we rendered cells deficient in PARP activity through the use of two chemical inhibitors, 3-aminobenzamide and 1,5-dihydroxyisoquinoline. Both inhibitors were effective in extending cellular lifespan. We conclude that PARP is involved in cellular aging. Hence, inactivation of at least two proteins, p53 and PARP leads to a similar phenotype, namely extension of cellular lifespan. These findings suggest a model in which PARP, in response to the DNA ends which accumulate in aging cells, possibly as a consequence of dicentric chromosome breakage, activates p53 protein (Figure 10).

The p53-PARP interaction may affect p53 protein function in at least two ways. First, it is possible that p53 is ADP-ribosylated by PARP in response to DNA damage or cellular aging. ADP-ribosylation may be the mechanism through which p53 protein is post-translationally activated in aging cells or in cells that have acquired DNA damage. Inhibition of poly (ADP-ribose) synthesis would prevent the activation of p53 and hence, no mdm2 or p21 expression would be triggered. In a second, alternative model, PARP may regulate p53 function in the absence of enzymatic modification. PARP is known to bind tightly to DNA ends and strand breaks, and requires automodification (ie ADP-ribosylation) for release from DNA. Binding of p53 to PARP at such sites will similarly sequester p53 and prevent it from acting as a

transcriptional factor. The addition of an inhibitor of poly (ADP-ribose) synthesis will prevent the release of PARP and associated proteins such as p53 from the DNA ends. Under these conditions, p53 would be incapable of promoting transcription. Further experiments are needed to determine which of these two models is correct. It will be important, for example, to determine if p53 protein is ADP-ribosylated *in vivo*.

Mice deficient in PARP activity have been generated (Wang *et al.*, 1995). These mice are born healthy and fertile. However, the proliferation of primary embryonic fibroblasts in culture was impaired and the proliferation of thymocytes *in vivo* following  $\gamma$ -irradiation was delayed. While the normal lifespan of these mice may seem to be at odds with the model presented in Figure 10, we believe that it is inappropriate to compare the lifespan of inbred mice having long telomeres in excess of 100-kb with that of human cells which have much shorter telomeres. It should be noted in this context that PARP activity is positively correlated with the life-span of various species (Grube and Burkle, 1992). PARP activity may be dispensable in animals with short life-span.

In summary our results provide a model for events which lead to activation of the genetic program of cellular aging and identify several key molecules involved in the p53 pathway for growth arrest. Knowledge of the molecular mechanisms involved in cell aging will be important for future drug design to extend the lifespan of normal cells for therapeutic intervention and treatment of aging-associated diseases. Furthermore these studies have relevance for the therapeutic eradication of immortal tumor cells through the re-initiation of the senescence pathway.



**Figure 10** Model connecting telomere loss signal and senescence-associated growth arrest. Telomere loss as a consequence of end-replication problem or direct damage to telomeres by free oxygen radicals may initiate series of events leading to formation of dicentric chromosomes which upon subsequent breakage activate PARP and p53 protein. p53-dependent/independent transcriptional activation of genes such as p21 and IGF-BP3 leads to cessation of proliferation and the cell cycle arrest associated with senescence.

### **3.12 Materials and methods**

#### *Cell culture and transfection*

Cells were grown at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. All cells were cultured in  $\alpha$ -minimal essential medium supplemented with 10% FBS, except for AT strains which were grown in 20% FBS. Subconfluent cultures were split 1:8 in early passage and 1:4 or 1:2 in mid to late passage, using 0.25% trypsin/EDTA. Phosphate buffered saline (PBS), contained no calcium or magnesium. The normal HDFs used in this study included: MRC-5 (ATCC), WI-38 (ATCC); S1C (age 45 y), F1 (age 26 y), P4 (age 12 y), F28 (age 8 y), and BJ (fetal foreskin). The 3400 (age 11 y) and 3492 (age 7 y) cells were obtained from the Coriell Institute. The following AT strains were obtained from the Coriell Institute: 1937B (age 24 y), 3395B (age 13 y), 3487C (age 8 y), 2530 (age 8 y), and 2052B (age 15 y). The 2675T and 2800T fibroblasts strains were derived from members of a Li-Fraumeni syndrome family and were kindly provided by Dr. M. C. Paterson (University of Alberta). OCI/AML-3 is a wild-type p53 expressing cell line established from the primary blasts of a patient with acute myelogenous leukemia (Fu *et al.*, 1996). SF1 is an SV40 immortalized HDF cell line.

Cells were defined as being young if they had completed <30-40% of their lifespan and as being old if they had completed >85-90% of their lifespan. Senescence was defined as the inability to divide over a three week period. FACS analysis by PI staining and BrdU pulse labelling was used to confirm that old cells were arrested at the G1/S and G2/M boundaries as described previously (Sherwood *et al.*, 1988).

Transfection experiments were carried out by electroporation (to generate stable clones) or by the DEAE dextran method (Kriegler, 1991) using triplicate 10cm<sup>2</sup> dishes per plasmid DNA for transient expression studies. For electroporation, HDFs, when 70% confluent, were trypsinized, washed in PBS, collected and mixed with the appropriate plasmids: p21P-luc, p21ΔP-luc, or pSV2-E6 (20 μg) in combination with pSV<sub>2</sub>neo (2 μg). Cells were electroporated using a Bio-Rad gene pulser at 150-300 V, 960 μF. Cells were selected in media containing 400 μg/ml of G418 and clones were isolated using cloning rings.

For inhibition of PARP, varying amounts of 3-aminobenzamide (Sigma) (0, 10 μM, 100 μM and 1 mM) or 1,5-dihydroxyisoquinoline (Aldrich) (0, 0.01μM, 1μM, 100μM) were added to the cells which were plated at a density of 10<sup>5</sup> cells per cm<sup>2</sup> in 6-well plates.

#### *Hyperoxic conditions*

A tri-gas water-jacketed incubator from Forma Scientific (Model 3327) was used for the culturing of cells under hyperoxia. BJ and WI-38 cells were exposed to hyperoxia (40% oxygen) at 50% confluency at PD 57 and 27, respectively. After several weeks in culture, the BJ and WI-38 cells stopped dividing at PD 60 and 32, respectively. Control cells were maintained under normal oxygen conditions. Cells were grown in DMEM-M199 medium supplemented with 10% FBS and Gentamycin (50 mg/l). Cells were re-fed weekly and split 1:2 upon achieving confluency.

#### *Plasmid constructs*

All plasmids were purified using Qiagen columns and quantified by a fluorometer (Turner Model

450) used within the linear range. A 2.4 kb DNA fragment derived from the endogenous human p21<sup>WAF1</sup> promoter present in the plasmid wwp-luc (El-Deiry *et al.*, 1993) was digested with *HindIII* and subcloned in the *HindIII* site of the pGL3-Basic vector (Promega) in the correct orientation to generate p21P-luc. p21ΔP-luc was constructed by digestion of p21P-luc with *SacI* which cuts once within the p21<sup>WAF1</sup> promoter at the extreme 5'-end and once within the polylinker of the vector followed by re-ligation of the plasmid. The plasmids were sequenced and the deletion of a 72 bp fragment in p21ΔP-luc containing the p53 consensus binding site was confirmed. The pGL3-control vector expresses luciferase under control of the SV40 promoter. CMV-wtp53, p53Ala143, p53Tyr275 are plasmids containing full length p53 cDNA under control of the CMV promoter. pSV2-E6, pSV2-E7, pSV2-E6/E7 contain genes derived from HPV-16 under control of the SV40 promoter (Watanabe *et al.*, 1989). pLE2/520 expresses wild type E1A; dl1101 and dl1143 express E1A deletion mutants competent for binding to pRB but unable to bind p300; dl1108 expresses a mutant E1A protein which binds to p300 but is unable to bind pRB (Barbeau *et al.*, 1994).

*Plasmid construction and purification of human p53 proteins* The nucleotide sequences encoding residues 1-72, 82-292, 311-393 of wild-type human p53 were subcloned into the pET19b vector using standard techniques and their correct incorporation confirmed by sequencing. The resulting plasmids express 10 histidine residues followed by a linker containing an enterokinase cleavage site and a His-Met dipeptide immediately N-terminal to the p53 sequence. *Escherichia coli* BL21(DE3)-(pLys-S) harbouring the desired plasmid were grown in 2-litre batches of Luria broth at 37°C (p53: 1-72 and p53: 311-393) or 25°C (p53: 82-292) and protein production was

induced with 1mM isopropyl-thiogalactose at an optical density (600 nm) of 0.6-0.7. Three hours post induction, cells were harvested by centrifugation at 6000g for 30 min. His-tagged proteins were purified from cell extracts by Nickel affinity chromatography and dialyzed for 24 h at 4°C against 4 litres of buffer (25 mM sodium phosphate, 100 mM NaCl, pH 7.0 or 7.4) using Spectra/por 3000 molecular weight cut off dialysis membrane. Protein concentrations were determined spectrophotometrically using the appropriate theoretical extinction coefficient.

#### *Electrophoretic mobility shift assays (EMSAs)*

Nuclear extracts were prepared from cells washed with PBS and immediately lysed on ice in buffer A (20 mM Tris pH 7.4, 20% glycerol, 10 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 5 mM EDTA, 0.1% NP-40, 2 mM Pefabloc (Boehringer Mannheim), 70 µg/ml aprotinin, 50 µg/ml leupeptin). The nuclear pellet was resuspended in buffer B (same as buffer A except for 100 mM NaCl, 1% NP-40). The mixture was kept on ice for 15 min, spun and the supernatant was used immediately. The binding reaction contained 0.1 µg of PAb421, 1 µg of poly(dI-dC), 5 µl of nuclear extract (4 µg of protein), <sup>32</sup>P-labelled double-stranded p53CON oligonucleotide (10<sup>5</sup> c.p.m.) containing the p53 consensus binding site (Funk *et al.*, 1992), shown underlined

(GGATCCAAGCTTGGACATGCCCGGGCATGTCCCTCGAGGGATCC) in a final concentration of 100 mM NaCl, 5 mM EDTA, 20 mM Tris pH 7.4. A fill-in reaction was used to label the oligonucleotide using Klenow DNA Polymerase, α-<sup>32</sup>P-dCTP and the antisense oligonucleotide (GGATCCCTCGAG). In some experiments a competitor oligonucleotide of the same base composition and length as p53CON was used. The reactions were incubated at room temperature for 20 min and samples were analyzed on a 4% non-denaturing polyacrylamide gel

run at 200 V for 3 h. Gels were dried and exposed to a PhosphorImager screen for 3-5 days.

Supershifted bands generated within the linear range of the DNA binding assay were quantified on a PhosphorImager (Molecular Dynamics) using Image Quant software.

#### *Immunoprecipitation, Western blot analysis and immunohistochemistry*

Cells washed on ice with PBS were lysed either in lysis buffer C (1% NP-40, 150 mM NaCl, 20 mM Tris pH 8.0, 2 mM Pefabloc (Boehringer Mannheim), 70 µg/ml aprotinin, 50 µg/ml leupeptin, pepstatin A 20 µg/ml, 500 mM EDTA) or SUG buffer (3% SDS, 125 mM Tris pH 6.8, 6% urea, 10% glycerol and all of the above protease inhibitors). For immunoprecipitations, lysates (1 mg) prepared using lysis buffer C were mixed with one of the following antibodies: PAb1801 (human p53-specific); PAb421 (panspecific p53); SC-187 (p21-specific antibody); 318 (PARP-specific polyclonal antibody). The immune complexes were collected with 100 µl of protein G-sepharose beads (Pharmacia). For analysis of PARP, the samples were subjected to sonication for 20 sec prior to immunoprecipitation. Protein quantification was performed using a modified Lowry assay (Sigma). An equal volume of 2X protein sample buffer was added to cell extracts adjusted to contain equivalent amount of protein, boiled and loaded on 10% polyacrylamide gels containing SDS. Proteins were transferred to nitrocellulose membranes. The p53 protein was detected using DO-1, PAb1801 or PAb7 antibodies (Oncogene Science), CMI is a polyclonal rabbit antibody raised against human p53 (Dimension Labs). PARP was detected using C2-10 monoclonal antibody. The protein-antibody complexes were detected using an HRP-conjugated secondary antibody using the super-signal enhanced chemiluminescence system (Pierce).

p21 protein was detected by immunohistochemistry using the Vectastain ABC kit (Dimension Labs) as suggested by the manufacturer. In brief, cells were fixed with 70% ethanol, blocked with 5% horse serum, washed with PBS containing 0.05% tween 20. After incubation in a 1:200 dilution of SC-187 primary antibody (Santa Cruz) for 30 min, cells were incubated with a biotinylated secondary antibody for 10 min and treated with 3% H<sub>2</sub>O<sub>2</sub> for 1 min.

### *Luciferase assay*

Cells were lysed 72 h after transfection with p21P-luc or p21ΔP-luc and the protein content of each extract was determined. Luciferase activity was measured using the luciferase assay reagent (Promega). Light emission over a 30 sec interval was measured in a Berthold LB 9507 luminometer. A modified Hirt procedure (Matsuoka *et al.*, 1990) was used to extract plasmid DNA from cells. Linearized plasmid DNA was run on a 0.8% agarose gel; the gel was dried and subsequently probed with a  $\gamma$ -<sup>32</sup>P-end labelled oligonucleotide (5'ATTACCAGGGATTTTCAGTCG) specific for the luciferase coding sequence. Band intensities were quantified on a PhosphorImager (Molecular Dynamics) to provide an estimate of relative plasmid copy number in the transfected cells and hence, a measure of transfection efficiency. This is preferred over co-transfection with a  $\beta$ -gal vector, for example, for several reasons: endogenous  $\beta$ -gal activity in HDFs increases with age (Dimri *et al.*, 1995); measurement of  $\beta$ -gal activity provides only an indirect measure of transfection efficiency; and p53 is known to modulate the activity of a large number of different promoters in co-transfection experiments. The RLUs (relative light units) obtained from the luciferase assay were normalized for differences in transfection efficiency in this way.

### *Isolation of DNA, SSCP analysis, and measurement of telomeric DNA length*

Cells were washed three times in PBS. Cell pellets were resuspended in proteinase K digestion buffer (100 mM NaCl, 10 mM Tris pH 8, 5 mM EDTA, and 0.5% SDS) containing proteinase K at a final concentration of 0.5 mg/ml. After incubation at 48°C overnight, the DNA was extracted once with phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform. DNA was precipitated with 95% ethanol and dissolved in TE (10 mM Tris pH 8, 1 mM EDTA pH 8).

DNA (10 µg) was digested with *Hinf*I and *Rsa*I (20 U each; BRL), extracted as described above, precipitated with 95% ethanol, washed in 70% ethanol, resuspended in TE and quantified by fluorometry using a Turner model 450 fluorometer. One µg of digested DNA was resolved by electrophoresis on a 0.5% agarose gel poured on a Gel bound membrane (FMC Bioproducts) and run for 700 V-h. Gels were dried, denatured, neutralized and probed with a  $\gamma$ -<sup>32</sup>P-5'-end labelled (CCCTAA)<sub>3</sub> oligonucleotide as described (Vaziri *et al.*, 1993). Gels were exposed to a PhosphorImager screen and the hybridization signals were digitized and subdivided into 1-kbp to 21-kbp, for calculation of the mean TRF length (L) by using the formula  $L = \sum (OD_i * L_i) / \sum OD_i$ , where  $OD_i$  = integrated photon signal in interval i and  $L_i$  = TRF length at the midpoint of interval i.

SSCP analysis of p53 genomic DNA on Li-Fraumeni cells was performed as previously described (Mitsudomi *et al.*, 1992) in young cells and during senescence and crisis.

### **3.13 References**

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## Overview of Chapter 4

The second part of the hypothesis proposed in chapter 2 predicted that ectopic expression of telomerase in normal human cells should lead to extension of cellular life span. We tested this hypothesis by forced expression of the catalytic domain of telomerase hTERT gene in normal human fibroblasts. Consistent with the telomere hypothesis and our own model, we were able to extend telomere length and increase the cellular life span significantly. The results of these experiments are presented in the next chapter. This chapter has appeared elsewhere as: **Vaziri, H & Benchimol, S. "Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span " (1998).**  
*Current Biology, Vol 8, 279-282.*

## **Chapter 4**

**Reconstitution of telomerase activity in normal human cells and life span extension:**

**“Evidence for the telomere hypothesis”**

## 4.1 Summary

Normal somatic cells have a finite life-span (Hayflick and Moorhead, 1961) and lose telomeric DNA present at the ends of chromosomes each time they divide as a function of donor age *in vivo* or in culture (Harley et al., 1990; Hastie et al., 1990; Vaziri et al., 1993). In contrast, many cancer cells and cell lines established from tumors maintain their telomere length by activation of an RNA-protein complex called telomerase, an enzyme originally discovered in *Tetrahymena* (Greider and Blackburn, 1985), that synthesizes telomeric repeats (Counter et al., 1992; Kim et al., 1994; Morin, 1989). These findings led to the formation of the “telomere hypothesis” which proposes that critical shortening of telomeric DNA due to the end-replication problem (Olovnikov, 1971) is the signal for the initiation of cellular senescence (Harley, 1991; Harley et al., 1992). In yeast the *Est2* gene, the catalytic subunit of telomerase, is essential for telomere maintenance *in vivo* (Counter et al., 1997; Lendvay et al., 1996; Lingner et al., 1997). The recent cloning of the cDNA encoding the catalytic subunit of human telomerase hTRT/hEST2 (Meyerson et al., 1997; Nakamura et al., 1997) makes it possible to test the telomere hypothesis. In this study we expressed the catalytic subunit of human telomerase hTRT/hEST2 in normal human diploid fibroblasts, which lack telomerase activity, to determine if telomerase activity could be reconstituted leading to extension of replicative life-span. Our results show that retroviral-mediated expression of hTRT/hEST2 resulted in functional telomerase activity in normal aging human cells. Moreover, reconstitution of telomerase activity *in vivo* led to an increase in the length of telomeric DNA and to extension of cellular life-span. These findings provide direct evidence in support of the telomere hypothesis, indicating that telomere length is

one factor that can determine the replicative life-span of human cells.

## **4.2 Reconstitution of telomerase activity, telomere elongation and extended life span**

hTERT/hEST2 cDNA was subcloned in both sense and anti-sense orientation in the retroviral vector pBabe under the control of the promoter present in the Moloney murine leukemia virus LTR (Morgenstern and Land, 1990). The plasmids, pBabe (viral backbone alone), pBabest2 (sense hTERT virus) and pBabest2-AS (antisense hTERT virus), were transfected into the packaging cell line Phoenix-E and viral supernatants were harvested. The normal human diploid fibroblast strain BJ, previously transfected with the ecotropic virus receptor gene (Albritton et al., 1992), was infected with viral supernatants (moi = 4) at approximately PD (population doubling) 75-79 . These cells have approximately 10-15 PDs remaining before reaching senescence. Colonies resistant to both G418 and hygromycin were selected, isolated with cloning cylinders and expanded. BJ cells like other fibroblasts do not normally have telomerase activity (Kim et al., 1994), but they do express the RNA subunit (hTR) of the telomerase complex (Nakamura et al., 1997). We reasoned, therefore, that BJ cells, which normally reach senescence after 87-90 PDs represented a suitable recipient cell strain in which to express hTERT/hEST2, reconstitute telomerase activity, and to test for elongated telomeres and extended life-span.

pBabe and pBabest2-AS virus-infected BJ cells formed sparse and smaller colonies (Figure 11 a,b). In contrast, pBabest2-infected cells gave rise to many larger colonies (Figure 11c).

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**Figure 11** Colony forming ability of BJ cells infected with retroviruses encoding the human telomerase catalytic subunit. BJ cells infected with pBabest2-AS (A), pBabe (B) or pBabest2 (C) were replated and selected in G418 and hygromycin. Cells were fixed and stained with methylene blue to detect colonies. The total number of colonies (containing  $\geq 400$  cells) in duplicate dishes was: pBabest2-AS,  $n=14.5$ ; pBabe,  $n=31$ ; pBabest2,  $n=244$ .

Ten drug-resistant colonies, each consisting of approximately 250-350 cells, were isolated from each of the infected cultures and expanded. In this secondary plating assay, none of the 20 colonies derived from the pBabe or pBabest2-AS infected cultures were capable of dividing sufficiently to reach confluence even after 14-20 days. These cells appeared to have reached senescence after an estimated 87-90 PDs on the basis of increased cell size, failure to divide and increased endogenous  $\beta$ -galactosidase activity (Figure 12b,c) (Dimri et al., 1995).

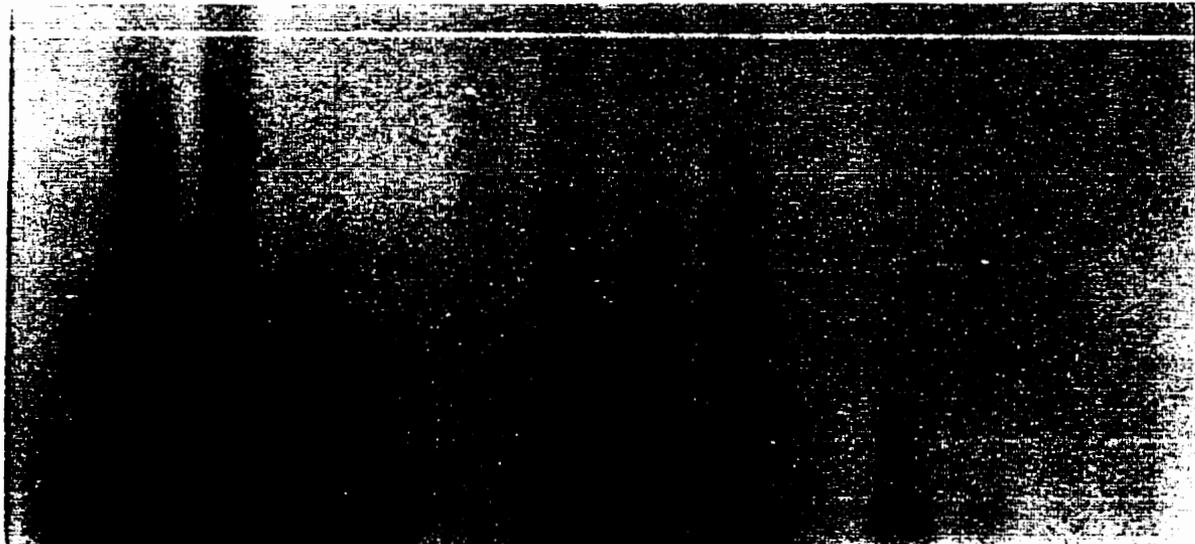
In contrast, 9 of 10 colonies obtained from the pBabest2 infected cultures reached confluence in the secondary plating assay. To test for extended proliferative potential, single cells were isolated by limiting dilution from the 9 surviving clones and grown in a tertiary plating assay. These cells have now been in continuous culture and have reached a minimum of 116 PDs (Table 1). The mean PD number for the 10 pBabest2 infected clones was 28 PDs higher than the 20 control clones ( $p < 10^{-5}$  by student's T test). No evidence of  $\beta$ -galactosidase activity has been detected in these dividing cultures (Figure 12a). To date these cultures show no sign of senescence and continue to divide. These cells so far show no sign of aneuploidy, and like normal fibroblasts they growth arrest in response to low serum and undergo contact inhibition at high density (data discussed in next chapter).



**Figure 12** Endogenous  $\beta$ -galactosidase activity in BJ cells infected with retroviruses encoding the human telomerase catalytic subunit and controls. (A) Cells were infected with the sense pBabest2 and assessed at PD 117, (B) infected with pBabest2-AS and assessed at  $\approx$  PD 87, (C) infected with pBabe and assessed at  $\approx$ PD 87.

BJ clones infected with pBabest2 virus had reconstituted telomerase activity detected by the TRAP assay (Telomere Repeat Amplification Protocol; telomeric repeats synthesized by telomerase are PCR amplified and resolved) (Figure 13), or by the conventional primer extension assay (data not shown), whereas BJ cells infected with pBabe or pBabest2-AS had no detectable activity (Figure 13). Reconstitution of telomerase activity has also been observed in other telomerase-negative cells (Weinrich et al., 1997). We refer to the pBabest2-infected clones as TIELF cells for telomerase induced extended life-span human fibroblasts. TIELF cells continuously expressed telomerase activity over time at PD 111, PD 117 and PD 123 (Figure 13). Extracts prepared from varying numbers of cells indicated that the level of telomerase activity in the TIELF cells was comparable to that of the established, adenovirus5-transformed human cell line 293 (Figure 13). Telomerase activity was sensitive to RNase and heat treatment (Figure 13).

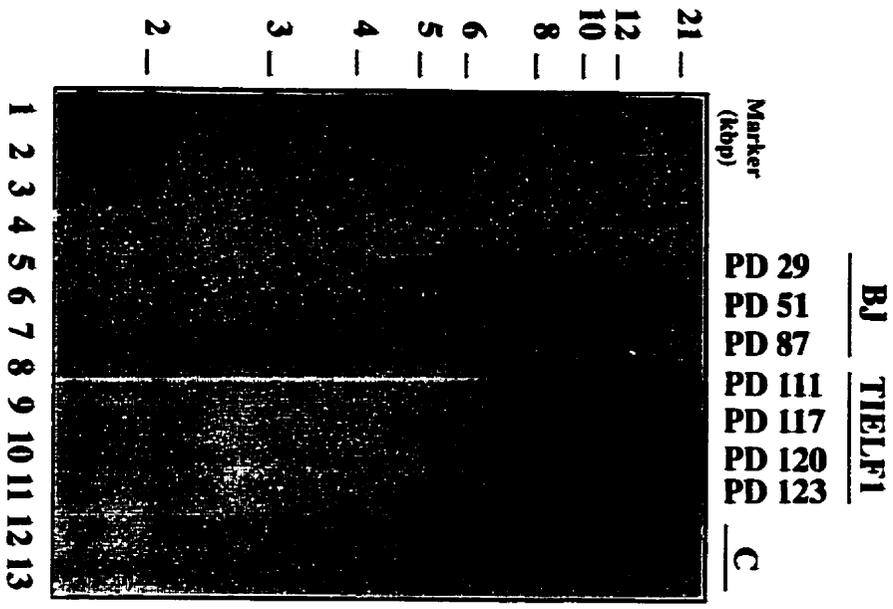
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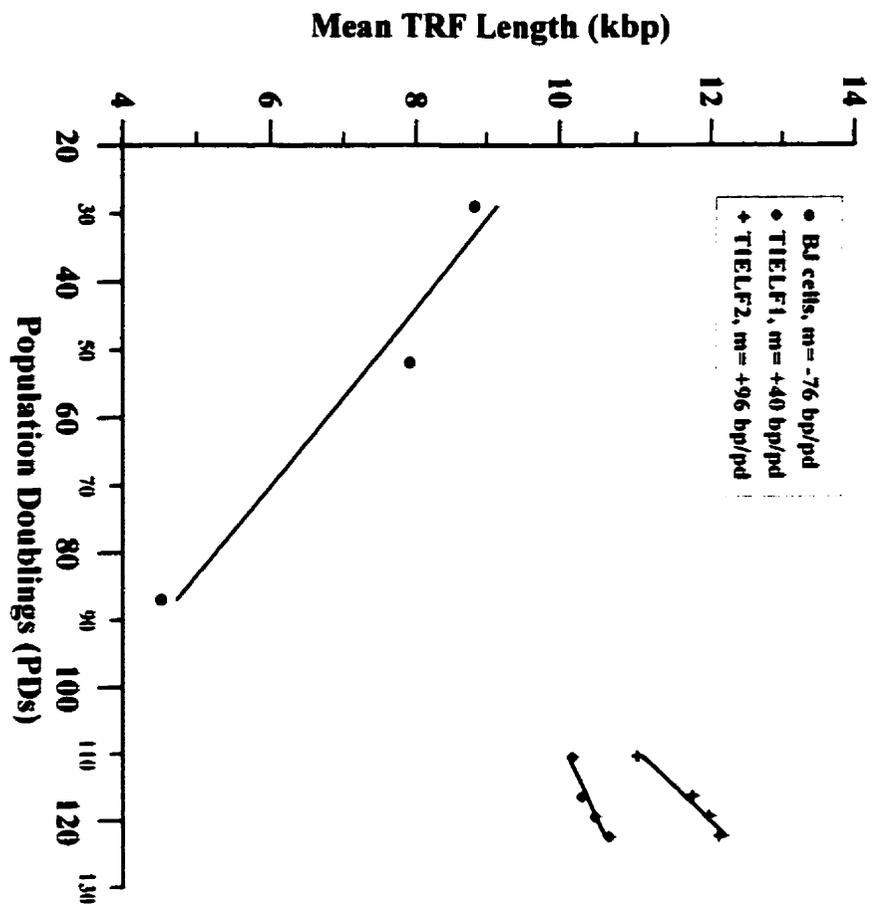
**Figure 13** Reconstitution of telomerase activity in BJ human diploid fibroblasts. TRAP assays were performed as described previously (Kim and Wu, 1997). Lane 1, RNase-treated 293 cell extract serving as a negative control; lanes 2-5, 293 cell extracts corresponding to  $10^5$ ,  $10^4$ ,  $10^3$ , and  $10^2$  cells, respectively; lane 6, RNase-treated TIELF cell extract; lanes 7-9, cell extracts derived from  $10^3$  TIELF cells at PD 111, PD 117 and PD 123, respectively; lane 10, TIELF cell extract heated at  $80^\circ\text{C}$  for 3 minutes; lanes 11-12, cell extract corresponding to 100 or 50 TIELF cells at PD 123; lanes 13-14, cell extracts corresponding to  $10^5$  BJ cells infected with pBabest2-AS and pBabe virus, respectively.

Telomere length was measured using the terminal restriction fragment (TRF) length assay as described previously (Vaziri et al., 1993). Genomic DNA was extracted from BJ cells and from two independent TIELF clones at several PDs and the mean TRF length was determined. As expected, control BJ cells lose telomeric DNA with each population doubling at a rate of -76 bp/pd (Figure 14 a,b), similar to what we reported previously in these cells (Vaziri, 1997). However, TIELF1 and TIELF2, two clones derived from pBabest2-infected BJ cells, acquired very long telomeres rapidly in the initial expansion phase and continued to elongate with increasing PD number; the approximate rates were +40 bp/pd and +94 bp/pd in clones TIELF1 and TIELF2, respectively (Figure 14 a,b). An increase in the size and intensity of the TRF signal from these two clones is evident on the Southern blot shown in Figure 14a.

**A**



**B**



**Figure 14** Analysis of telomeric DNA (TRFs) in BJ and BJ derived TIELF cells. Genomic DNA, purified by DNazol (BRL), was digested with *HinfI* and *RsaI*, quantitated in triplicate by fluorometry and 1  $\mu$ g of DNA/lane was resolved on a 0.5% agarose gel. A  $\gamma$ -<sup>32</sup>P-end labelled (C<sub>3</sub>TA<sub>2</sub>)<sub>3</sub> probe was used to detect and measure the length of telomeric DNA as previously described (Vaziri et al., 1993). **(a)** Lanes 1-2, DNA size markers; lanes 3-4, blank; lane 5, young BJ cells at PD 29; lane 6, BJ cells at PD 51; lane 7, old BJ cells at PD 87; lanes 8-11, TIELF cells with increasing PDs as indicated; lane 12, BJ cells infected with pBabest2-AS and pBabe at  $\approx$ PD 50-55. **(b)** Quantitative analysis of telomeric DNA in normal BJ cells and the TIELF cells. Normal BJ cells lost telomeric DNA at a rate of -76 bp/pd (solid circles),  $r = -0.98$ . Two TIELF cell clones increased their telomere length at a rate of +94 bp/pd (cross),  $r = 0.98$ , and +40 bp/pd (black diamonds),  $r = 0.96$ .

### **4.3 Discussion**

Our results provide direct evidence for the telomere hypothesis. They show that forced expression of hTERT/hEST2 cDNA in normal human cells results in telomerase activity, elongation of telomere length and an extended life-span. Thus, normal human cells can bypass the Hayflick limit and increase their replicative life-span upon expression of telomerase activity. It is notable that the length of telomeric DNA was not merely maintained but increased in TIELF cells. After submission of this work, similar results were reported (Bodnar et al., 1998). In contrast to this study in which young/mid-life cells were used to extend life-span, our study used older cells that had completed 80% of their lifespan and hence, demonstrates that senescence can be prevented even in old cells. Our results are reminiscent of studies with germ cells, which, unlike somatic cells, express telomerase activity, have long TRFs ( $\approx 15\text{kb}$ ) and show a net increase in the length of the telomeric DNA with age (Allsopp et al., 1992). It is possible that expression of telomerase in normal human cells might be associated with processes related to de-differentiation and could lead to generation of cells with the stem cell property of indefinite self renewal. TIELF cells could replace genetically unstable, established cell lines currently used in a wide variety of applications. Ectopic expression of telomerase in normal cells may be successfully used in gene therapy to increase the life-span of cells carrying the desired transgene. Similar approaches can also be used for treatment of aging-related diseases and cancer.

## 4.4 Materials and Methods

### *Cell culture*

The neonatal human fibroblast cell strain (BJ) attained a maximum life-span of approximately 87-90 PDs under our conditions. BJ cells with or without the ecotropic receptor (mCAT1) gene were grown in  $\alpha$ -minimal essential medium supplemented with 10% FBS. Older cells were grown with 15% FBS. Cells were split at a 1:4 or 1:8 ratio at early passage or at a 1:2 ratio in later passages. PBS contained no calcium or magnesium. Plating efficiency for BJ cells was > 90%. PD# was calculated by the count/split method or as  $PD = \text{Log}(N_f/N_0)/\text{Log}_2$ , where  $N_f$  is the final cell number and  $N_0$  is the initial number of seeding cells

### *Retroviral infection*

The retroviral constructs were packaged using the highly efficient and helper-free cell lines Phoenix-A and Phoenix-E (ATCC). Packaging cells were transfected when approximately 80% confluent. Phoenix cells were incubated in 25  $\mu\text{M}$  chloroquine 5 minutes prior to transfection with 10  $\mu\text{g}$  of retroviral plasmid DNA by the calcium phosphate technique. 72 hrs post-transfection, the virus-containing medium was collected and the virus titre determined using NIH3T3 cells. Titres of  $2-4 \times 10^6$  TU/ml (Transducing units) were obtained. BJ cells were infected in the presence of polybrene (4  $\mu\text{g}/\text{ml}$ ) using viral supernatants at a  $\text{moi} = 4$ . Throughout this work the transfection efficiencies were monitored by a CMV-EGFP construct. BJ cells were incubated for 20 hrs at 32°C in virus-containing medium. Fresh medium was added and the cells were incubated for a further 24 hours in virus-free medium prior to

trypsinization and plating in medium supplemented with G418 (400  $\mu\text{g/ml}$ ) and Hygromycin (20-50  $\mu\text{g/ml}$ ). Drug resistant colonies were isolated with cloning rings approximately 2-3 weeks later.

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## Overview of Chapter 5

In the previous chapter I showed that telomere elongation leads to extension of life span in normal cells. The model described in chapter 2 predicts that ectopic expression of telomerase in normal cells should overcome the requirement for alteration of check point control genes involved in the senescence arrest. A corollary of this model is that extension of life span by telomerase should not cause alteration of normal checkpoint controls. In this chapter, evidence is provided that TIELF cells are chromosomally stable and have an intact G1 checkpoint in response to DNA damage. Moreover, TIELF cells have a normal ability to join double strand DNA breaks in response to ionizing radiation and are not tumorigenic. These results are consistent with the model proposed in chapter 2.

### Acknowledgments:

Dr. Tej Pandita provided 6432 rabbit anti-hTERT antibody. Dr. Squire performed the cytogenetic analysis, Dr. Hill's lab performed the comet, radiation survival and tumorigenicity assays.

## **Chapter 5**

### **Characterization of telomerase induced extended life span fibroblasts**

## 5.1 Abstract

Life span determination in normal human cells may be regulated by nucleoprotein structures called telomeres (Harley, 1991), the physical ends of eukaryotic chromosomes (Blackburn and Greider, 1995). Telomeres have been shown to be essential for chromosome stability and function (Blackburn and Greider, 1995) and shorten with each cell division in normal human cells in culture (Harley et al., 1990) and with age *in vivo* (Allsopp et al., 1992; Harley et al., 1990; Hastie et al., 1990; Vaziri et al., 1993). Reversing telomere shortening by forced expression of telomerase in normal cells has been shown to extend replicative life span (Bodnar et al., 1998; Vaziri and Benchimol, 1998). This is associated with generation of unusually long telomeres (Bodnar et al., 1998; Vaziri and Benchimol, 1998). We present data which suggests that telomere elongation in normal human cells by forced expression of telomerase does not lead to chromosomal instability. These cells are not tumorigenic, they have normal radiosensitivity and DNA break rejoining activity, and they display an intact G1 checkpoint in response to ionizing radiation. Thus forced expression of telomerase in normal human cells does not lead to genomic instability.

## 5.2 Introduction

Telomeres, the nucleoprotein structures which protect the ends of eukaryotic chromosomes are composed of tandem repeats which are maintained by telomerase, an RNA-protein complex which synthesizes telomeric repeats *de novo*. Vertebrate chromosomes terminate in tandem repeats of (TTAGGG)<sub>n</sub> (Moyzis et al., 1988) which are bound to a unique family of telomere binding proteins (Chong et al., 1995). Due to incomplete replication of the DNA termini (Olovnikov, 1971; Watson, 1972), human somatic cells lose telomeric DNA each time they divide (Harley et al., 1990). The telomere hypothesis proposes that shortening of telomeres of one or more chromosomes will eventually lead to senescence (Harley, 1991). This hypothesis also proposes that expression of telomerase activity in cancer cells may be required for cell immortality (Counter et al., 1992; Harley, 1991).

In yeast the catalytic domain subunit of telomerase is required for telomere maintenance *in vivo* (Lendvay et al., 1996; Lingner et al., 1997). The human telomerase complex contains at least two components, the RNA template component hTR (Feng et al., 1995) and a conserved catalytic subunit hTERT (Harrington et al., 1997; Kilian et al., 1997; Meyerson et al., 1997; Nakamura et al., 1997).

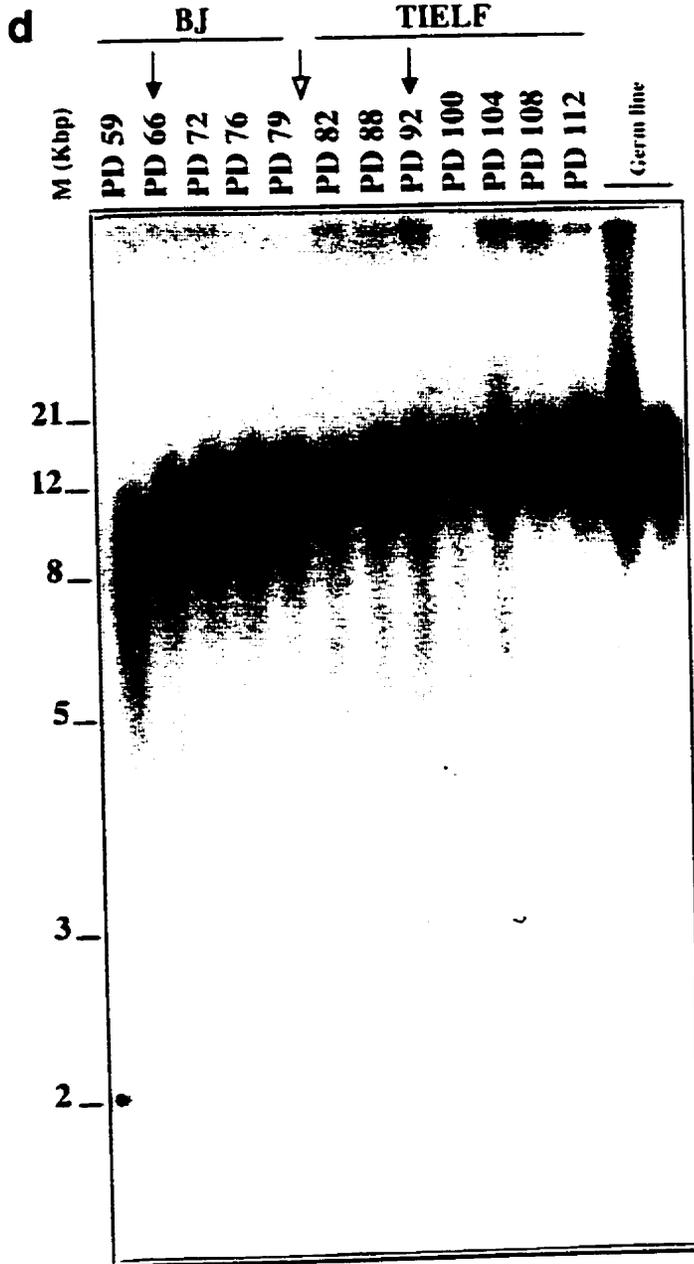
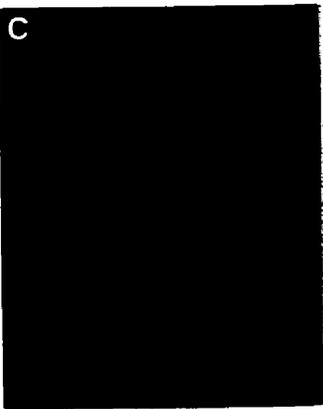
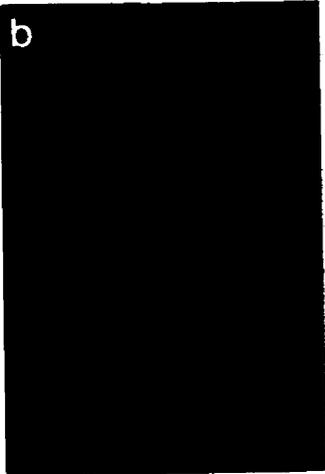
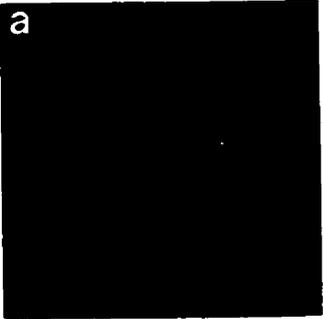
Recently it has been shown that hTR and hTERT are sufficient for reconstitution of telomerase activity (Weinrich et al., 1997) and forced expression of hTERT in normal human cells leads to reconstitution of telomerase activity, telomere elongation and extended replicative potential (Bodnar et al., 1998; Vaziri and Benchimol, 1998). By G-banding analysis of metaphase chromosomes, the cells with extended life span appear to have a normal banding

pattern (Bodnar et al., 1998). In this work we investigated the possibility that forced expression of telomerase, leading to generation of long telomeres and extension of cellular life span may result in genomic instability and checkpoint related defects in TIELF (Telomerase Induced Extended Life span Fibroblasts) cells.

### **5.3 Analysis of telomeric DNA by TRF analysis and telomere FISH**

TIELF cells were generated by infection of BJ cells with pBabest2 retrovirus expressing hTERT as described (Vaziri and Benchimol, 1998). In this experiment, BJ cells infected with the antisense hTERT virus (pBabest2-AS) senesced at approximately PD80. Hence hTERT expressing cells with PDs beyond this value are considered TIELF cells. Majority of TIELF cells expressed the hTERT protein assayed by immunohistochemistry with a rabbit polyclonal antibody (6432) (Figure 15a). No staining was observed in the parental strain expressing the antisense construct (Figure 15b). Analysis of telomeric DNA indicated that after hTERT expression the mean TRF length (terminal restriction fragment length) increased at a rate of approximately +116 bps/pd (average of two independent experiments) between PDs 59-112 (Figure 16d). TIELF cells at PD112 had a mean TRF length of  $16.8 \pm 1$  kb which was comparable to and slightly longer than germline (Figure 16d, Last 2 lanes). Analysis of telomeric DNA on individual telomeres by fluorescent in situ hybridization (Figure 16, left panel) revealed that: 1) young cells (Figure 16a,) have a substantially more intense fluorescent signal than old cells (Figure 16b) and 2) that the telomeric signal on most chromosomes in TIELF cells (Figure 16c) are more homogeneous and intense than either young or old cells.

**Fig 15.** hTERT staining of TIELF cells (a) and control BJ cells (b). Confluent BJ and TIELF cells were fixed and stained with an anti hTERT polyclonal rabbit antibody (6432) at 1:1000 dilution using the vectastain ABC system.

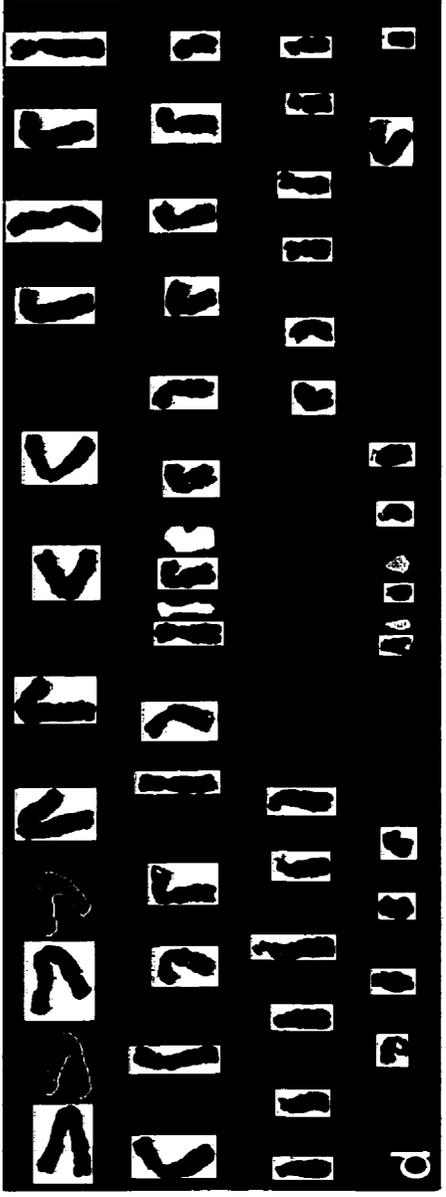
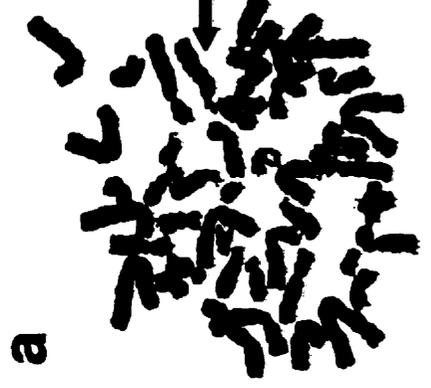
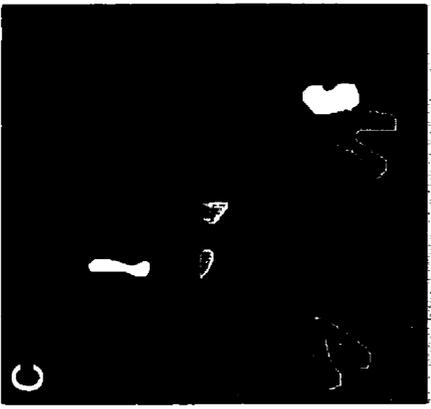
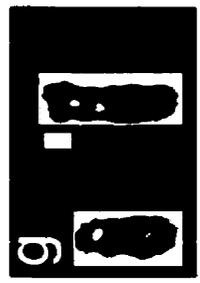
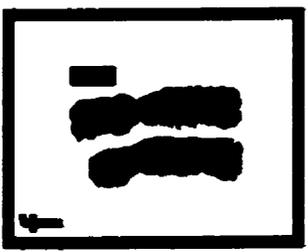
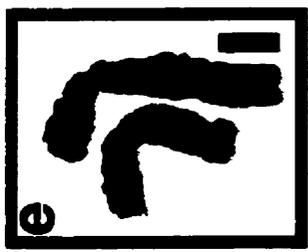


**Figure 16** Analysis of telomeric DNA during life span extension of TIELF cells. Left panel, detection of interphase telomeres by telomere FISH with superimposed DAPI staining. Telomere signal intensity diminished with increasing passage and was significantly restored in TIELF cells. Young BJ31(a), Old BJ 78(b) and TIELF(c) were grown on the same chamber slide. A telomere specific probe (Oncor) was used to detect (TTAGGG)<sub>n</sub> repeats as previously described (Henderson et al., 1996) and according to Oncor's protocols. d, TRF analysis of BJ cells expressing hTERT with increasing PD. Genomic DNA, purified by spooling using DNAzol, was digested with *HinfI* and *RsaI*. 1 μg of DNA quantitated by fluorometry was resolved on a 0.5% agarose gel. Telomeric DNA was detected using an end labeled γ(<sup>32</sup>P)-(C<sub>2</sub>TA<sub>2</sub>)<sub>3</sub> probe and quantitated as described (Vaziri et al., 1993) using TELORUN 1.4 software (written by Vaziri&Harley 1990-1993), solid arrows indicate the times at which cells were analyzed for chromosomal aberrations, open arrow is the approximate PD at which parental BJ cells normally senesce. Last two lanes: sperm DNA TRFs.

#### **5.4 Chromosomal stability in TIELF cells**

The question of chromosomal stability in TIELF cells was addressed by cytogenetic G-banding analysis of metaphase chromosomes in young control BJ31 cells (BJ cells at PD31, without telomerase activity), BJ66 cells (telomerase positive cells at PD 66; TRF=11.9±0.8 kbp) and TIELF cells at PD 92 (TRF=14.1±0.6 kbp). Analysis of metaphase spreads by G-banding revealed no significant differences between BJ31, BJ66 (n=1/105) and TIELF cells (n=4/94, 50 of these metaphases were obtained from TIELF cells at PD 140) (Fisher Exact Test, p=0.2, two tailed). The nonclonal chromosomal aberrations which were detected in TIELF cells included 5p+, 2q+ and balanced translocation t(12;14) (Figure 17). A more detailed analysis of possible chromosomal aberrations was performed by spectral karyotyping (SKY). This analysis uses colored fluorescent chromosome-specific paints that provide a complete analysis of the human chromosomal complement. Thus, chromosomal rearrangements can be identified by the juxtaposition of different colors along a single chromosome. The SKY analysis confirmed the results of the G-banding analysis (Figure 17) and revealed one additional structural change 12p- in TIELF cells (SKY analysis is more sensitive than G-banding). Even with the addition of this new aberration to our previous data set no significant statistical difference between BJ (n=1/105) and TIELF cells (n=5/94) were present (Fisher Exact Test, p=0.1, two tailed). None of the aberrations detected was identical and hence do not represent clonal changes. The frequency of aberrations detected was identical and hence do not represent clonal changes. The frequency of aberrations found in TIELF cells (5%) is comparable to that of other normal fibroblast strains (3-8%) (Wolman et al., 1964). We also analyzed numerical changes as a measure of chromosomal

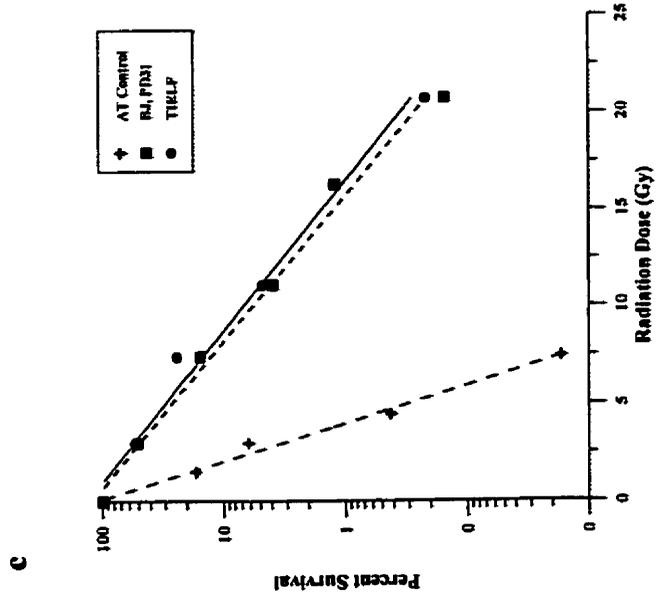
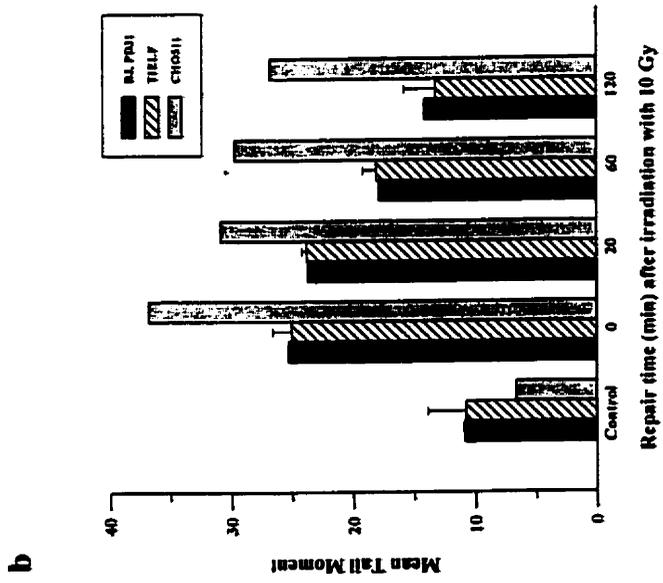
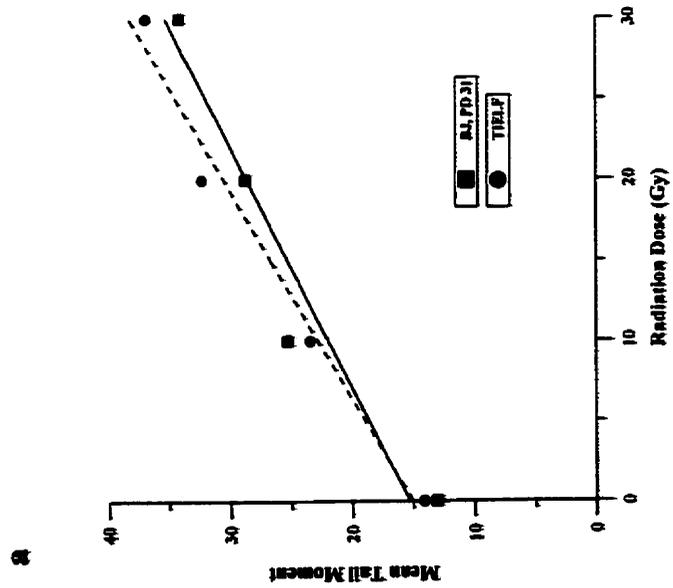
stability using a chr8 specific centromeric probe (D8Z2, Vysis) by interphase FISH analysis. Analysis of 400 cells from each strain did not reveal any evidence of aneuploidy (BJ31; 93%, BJ66;92% and TIELF;95%, measured by the number of normal cells with double signals).



**Figure 17.** G-banding and SKY analysis of metaphases from TIELF cells containing a t(12;14) translocation (arrow) and other aberrations. Structural chromosome aberrations were detected in metaphases from TIELF cultures. Cells were grown as described (Vaziri and Benchimol, 1998), split 1:16 in growth media without G418 for 24 hrs before any treatment. Well-spread G-banded metaphases (550 band level) were analyzed from BJ31, BJ66 and TIELF strains. These were coded prior to analysis and scored double blind. a, G-banding of the t(12;14). b,c, SKY analysis of the same translocation. d, SKY and G-banding of a. e, 2q+ , structural aberration shown by the solid bar. f, 5p+ aberration. g, 12p- deletion. A SKY™ hybridization and detection kit (SD-200 bio-system; Applied Spectral Imaging Inc) was used to visualize all human chromosomes in 23-24 colors. Preparation of chromosome slides, combination of fourier spectroscopy, CCD imaging and computerization to excite and measure the emission spectra simultaneously for all dyes in the spectral range and from all points in the metaphase spread was performed (Schrock et al., 1996). Images were analyzed using SKYVIEW software.

## **5.5 DNA strand break rejoining activity and radiation sensitivity is intact in TIELF cells**

To determine if TIELF cells have a deficiency in DNA repair, the following experiments were performed. TIELF and BJ31 control cells were exposed to increasing doses of ionizing radiation (0-30 Gy) and DNA strand breaks and rejoining were analyzed by the comet assay (Olive et al., 1990). There was a linear relationship between the tail moment (a measure of the DNA double strand breaks) and the radiation dose and this relationship was identical between the BJ31 and TIELF cells (Figure 18). Second, cells were tested for their ability to rejoin DNA double strand breaks following 10Gy of ionizing radiation. The normalized tail moments, measured at different times after irradiation were identical in BJ31 and TIELF cells (Figure 18). Similar results were also obtained for SS break rejoining (data not shown). Finally, we used continuous low dose ionizing radiation to measure long term clonogenic survival and relative radiosensitivities. There was no difference between the survival of BJ31 or TIELF cells at these biologically relevant doses of ionizing radiation (Figure 18).



**Figure 18** The response of BJ31 and T1ELF cells to ionizing radiation: a, generation of DNA double strand breaks; b, rejoining kinetics of double strand breaks; c, clonogenic survival after low dose rate irradiation. In all assays BJ31 and T1ELF cells behave identically.

a, Ionizing radiation dose vs tail moment of BJ31 and T1ELF cells as measured immediately after irradiation by the neutral comet assay. b, Mean tail moment as a function of time after a dose of 10 Gy in BJ31, T1ELF and positive control CHO511 cells (Ku-70 deficient) which have impaired DNA repair and show higher tail moments. Exponentially growing cells were trypsinized, resuspended in PBS at  $2 \times 10^4$  cells/ml and irradiated on ice. After irradiation 0.5 ml of the cell suspension was mixed with 1.5 ml of 1% low melting point agarose and pipetted onto a glass slide. For the neutral comet assay, cells were lysed, washed by submersion in TBE buffer, followed by electrophoresis and detection (Olive et al., 1990). The individual nuclei with broken DNA drawn out of the nucleus by the electrophoresis form a "comet-like" structure. The comets were digitized and the tail moment was calculated as described (Olive et al., 1990) by Northern Eclipse software (Empix). One hundred comets from each sample were analyzed for each dose or time point. The normalized tail moment was used as a parameter to indicate DNA damage (Olive et al., 1990). Three independent experiments were done and the mean  $\pm$ SD was expressed in the final plot.

c, clonogenic survival as a function of ionizing radiation dose. Cells were irradiated with  $\text{Co}^{60}$  gamma rays at a low dose rate of 0.025 Gy/min (LDR) at  $37^\circ\text{C}$ . This dose-rate was chosen to be low enough that cell survival could be influenced by repair of radiation damage occurring during the treatment.  $2 \times 10^6$  cells were grown to confluency. Following irradiation the cells were held at  $37^\circ\text{C}$  for 24 hrs before being trypsinized, counted, diluted and plated for colony formation. Survival was calculated using the cell count obtained just prior to plating. The cells were plated to assay for colony formation and then incubated for 10-14 days before being stained. Colonies containing greater than 50 cells were counted. Survival was expressed as the ratio of plating efficiency between irradiated and control cells.

## **5.6 Preserved radiation induced G1 checkpoint**

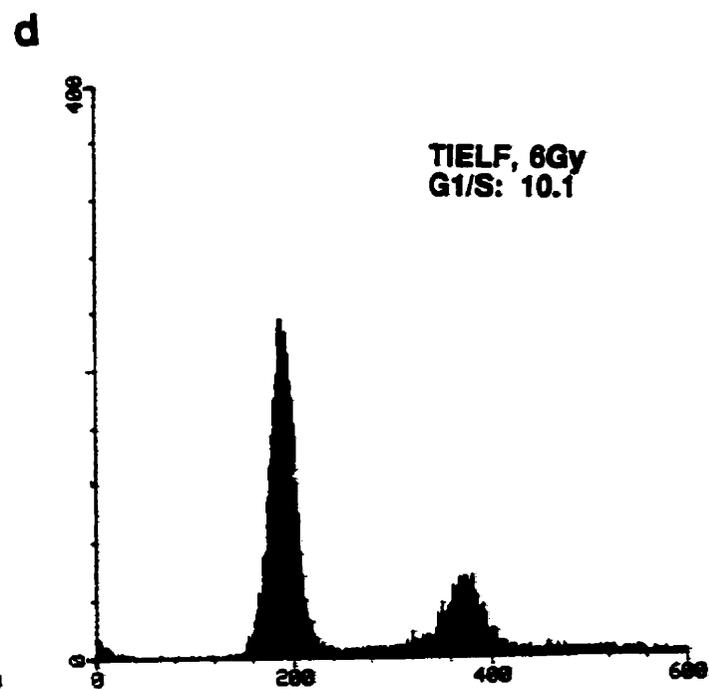
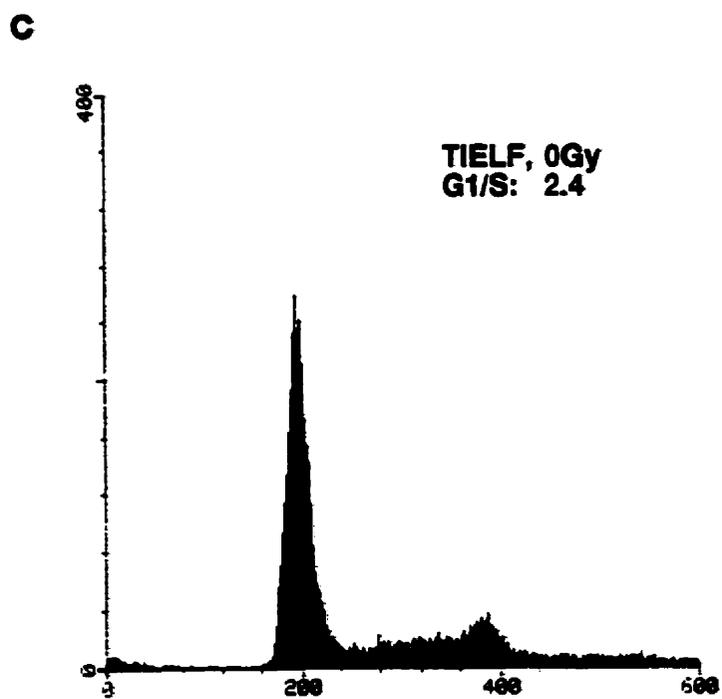
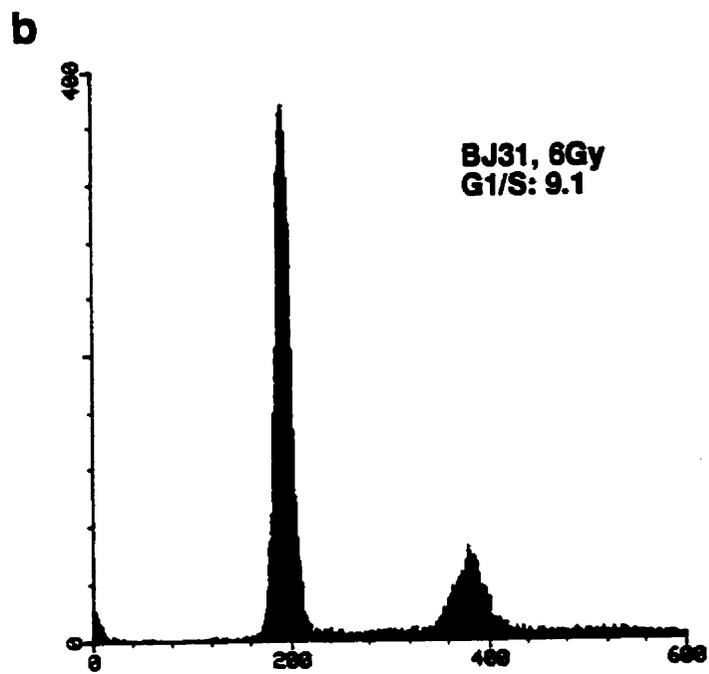
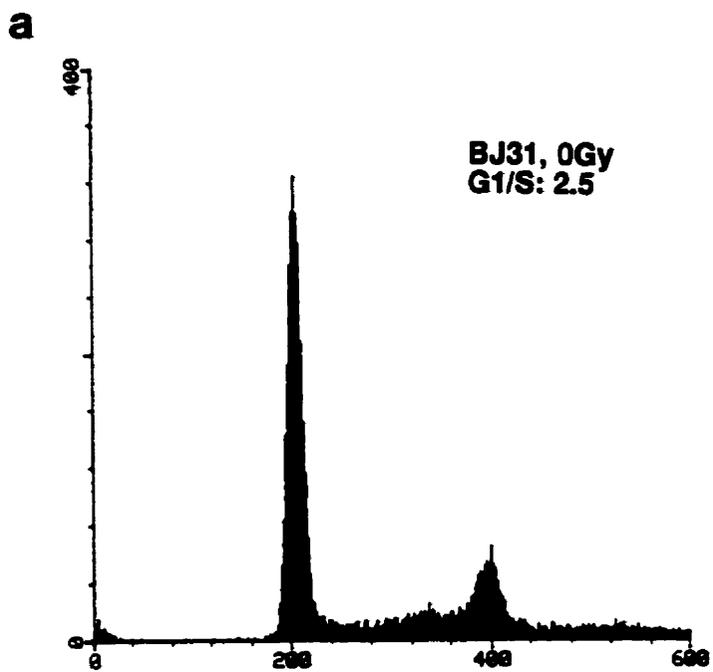
To determine if the DNA damage G1 checkpoint was retained in TIELF cells, cell cycle analysis was performed 24 hrs after exposure to 6Gy of ionizing radiation (Figure 19). Both the BJ31 and TIELF cells undergo a G1 arrest as measured by the increased G1/S ratio. There was no significant difference in the G1/S ratio between the two cell strains (BJ, G1/S=9.1 TIELF, G1/S=10.1). Therefore the radiation-induced G1 checkpoint appears to be intact in both strains.

## **5.7 Lack of tumorigenicity in TIELF cells**

We investigated as well the tumorigenic potential of TIELF cells by injection of  $2 \times 10^6$  BJ31 and TIELF cells into the leg muscle of 10 CB17 scid mice. No tumors were formed after 150 days.

We conclude that on the basis of five criteria: cytogenetic analysis, radiation sensitivity, DNA break rejoining activity, radiation induced G1 checkpoint and tumorigenicity, TIELF cells appear identical with their normal young counterparts. It is probable that further division, constitutive telomere elongation and clonal succession in TIELF cells would eventually lead to chromosome instability.

These possibilities can not be ruled out until TIELF cells at higher population doublings are analyzed for genomic instability.



**Figure 19** Cell cycle analysis of BJ31 and TIELF cells in response to ionizing radiation. Cells were fixed with 70% ethanol, washed in PBS and resuspended in PBS with 0.1% Triton X-100, 0.12mM EDTA containing 50 µg of Rnase. After PI addition (50 µg/ml), the DNA content was measured without gating. The cell cycle was quantitated using the fully automated MODFIT program and the G1/S ratio was calculated. a, BJ31 cells without treatment. b, BJ31 cells exposed to 6 Gy of ionizing radiation. c, TIELF cells without treatment. d, TIELF cells + 6 Gy.

## **5.8 Materials and Methods**

### *Comet Assay*

Briefly,  $2 \times 10^4$  exponentially growing cells were trypsinized, and suspended in PBS and irradiated on ice. After irradiation an aliquot of 1.5 ml of 1% low melting point agarose held at 50°C was added to the tube and the suspension was rapidly pipetted onto a glass microscope slide. For neutral comet assay, cells were lysed at 55°C for 2 hours in buffer containing 30 mM EDTA and 0.5% SDS, pH 8.5, washed by submersion in TBE buffer (90 mM Tris, 2 mM EDTA, 90 mM boric acid, pH 8.5) for 3 h with three changes of buffer. This was followed by electrophoresis in fresh TBE buffer at 0.6 V/cm for 25 min. Then the slides were rinsed with distilled water and stained for 30 min in 2.5 µg/ml PI.

Comets were digitized and the tail moment was calculated using Northern Eclipse software. One hundred comets from each sample were analyzed for each dose or time point. No attempt was made to select comets, other than to avoid obvious debris or comets spaced too closely or overlapped. The normalized tail moment was used as parameter to indicate DNA damage. It is the same definition as that of Olive et. al. 1990. Three independent experiments were done and the mean +/-SD was expressed in the final plot.

### *Radiation Survival Assay*

Cells were irradiated with Co60 gamma rays at a fractionated low dose rate of 0.025 Gy/Min (LDR) at 37°C. This dose was chosen to be low enough that repair of radiation damage would be possible.  $2 \times 10^6$  cells were grown in T75 flasks until they reached confluency. Following irradiation the cells were held at 37°C for 24hrs before being trypsinized, counted, diluted and plated for colony formation. Survival was calculated using the cell count obtained just prior to plating. The cells were

plated for colony formation assay at three consecutive ten-fold dilutions and then incubated for 10-14 days before being stained with methylene blue in 50% ethanol. Colonies containing greater than 50 cells were counted as survivors and percent survival calculated as the ratio of plating efficiency for the treated group divided by that for an untreated control.

#### *Cell cycle analysis*

Cells were fixed with 70% ethanol, washed in PBS and resuspended in PBS with 0.12% Triton X-100, 0.12mM EDTA containing 50µg of Rnase. After PI addition (50µg/ml), the DNA content was measured without gating. The cell cycle was quantified using the fully automated MODFIT program and the G1/S ratio was calculated.

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## **Chapter 6**

### **Conclusions and future direction**

## 6.1 Conclusions

This thesis addresses a fundamental question: What signals and initiates cellular aging *in vitro*? Previous studies on the role of telomere loss in cell aging were essentially correlative and lacked a direct approach. These studies were mostly performed by measurement of mean TRF length and correlating the values with either the donor age or the *in vitro* age of cells. Direct testing of the telomere hypothesis required reconstitution of telomerase activity in normal human cells and testing them for telomere elongation and extended life span. To many this appeared to be a daunting task, since telomerase appeared to consist of many subunits including an RNA template. Recent cloning of the catalytic subunit of *Euplotes* and *S. Cerevisiae* telomerase (Lingner et al., 1997) led to cloning of human catalytic subunit hTERT (Harrington et al., 1997; Kilian et al., 1997; Meyerson et al., 1997; Nakamura et al., 1997). This allowed the reconstitution of telomerase activity *in vitro* (Beattie et al., 1998; Weinrich et al., 1997) and *in vivo* (Vaziri and Benchimol, 1998; Weinrich et al., 1997). In order to test the telomere hypothesis, I made a simple and major assumption during the course of this work: that the expression of the catalytic subunit of telomerase (hTERT) is the limiting factor for telomere elongation in normal cells. This assumption proved to be valid. Expression of hTERT in normal human fibroblasts led to restoration of telomerase activity and telomere elongation. Even more strikingly BJ cells acquired a significant extend life span (Vaziri and Benchimol, 1998). These findings provide evidence consistent with the telomere hypothesis of cell aging and immortalization. One can therefore conclude that telomeres are one factor which are involved in cellular life span regulation.

However, details of cellular senescence and its prevention are still not very well understood. I attempted to dissect partially the mechanism by which telomere shortening can potentially lead to

senescence by proposing a model in which telomere loss is perceived by the cell as DNA damage.. If this model is correct, the same molecules involved in DNA damage response pathways should be involved in telomere regulation and hence regulation of life span. I found evidence that at least three molecules (p53, ATM and PARP) are involved in this process. Inactivation of any one of these molecules leads to significant extension (p53, PARP) or shortening of life span (ATM). I also found that upregulation of p21<sup>Waf1/Sdi1/Cip1</sup>, the down stream target for p53 protein is dependent on p53 and p300 during aging *in vitro*.

Taken all together, these results provide a coherent model for initiation and execution of a cellular senescence cascade in normal cells. Telomere shortening as a consequence of the end replication problem may trigger a signal which is relayed by DNA damage signaling molecules like p53 to p21<sup>Waf1/Sdi1/Cip1</sup>, subsequently leading to inhibition of CDKs and DNA synthesis and subsequent cell cycle exit.

## **6.2 TIELF cells as a new tool for research**

Immortalization of human cells in the past involved use of SV40 LT antigen or HPV-16 E6/E7. Both methods are known to cause genomic instability in normal cells and yield immortal clones at a very rare frequency. The resulting immortal cell lines derived by these methods or tumor derived lines also suffer from genomic instability, and this has always been a major problem in the use of such lines as model systems.

In the process of testing the telomere hypothesis, I generated TIELF cells by forced expression of hTERT in normal human fibroblasts. As shown in chapter 5, TIELF cells are genetically stable.

TIELF cells may therefore prove invaluable substitutes for the many genetically unstable cell lines currently used in research, and allow experiments to be carried out in a normal diploid background. Moreover, overexpression of hTERT in cell strains that are difficult to establish such as dendritic cells, may facilitate the establishment of cell lines. It should be noted that extension of life span and survival in certain lineages may not always be possible by overexpression of hTERT alone. Telomere elongation factors may have a cell type specific pattern of expression, and generation of TIELF like cells may require more than hTERT alone. Finally, generation of TIELF-like cells should make it possible to perform homologous targeted recombination to inactivate multiple genes in a diploid background without replicative exhaustion. These tools would be invaluable for many different areas of research.

### **6.3 TIELF cells in gene or cell-based therapy**

Replicative senescence may be a potential problem in gene therapy. Forced expression of hTERT in combination with the expression of the desired transgene may allow maintenance of the transgene, sustaining the desired phenotypic on the assumption that TIELF cells will maintain their genomic integrity *in vivo*. Similar approaches may also be used to increase the success of cell-based therapies.

### **6.4 Future directions: Have we proven the telomere hypothesis?**

In this thesis I have merely provided evidence consistent with the telomere hypothesis and have

identified several molecules which are involved in life span regulation in normal human fibroblasts. The “proof” of telomere hypothesis would certainly require further experimentation. We have not yet clearly shown that telomere shortening is the cause of senescence.

We need to focus on several issues in the future. First, it is possible that overexpression of hTERT in normal human cells may have consequences in addition to telomere elongation. These many include inactivation of certain pathways which are required for senescence (without causing immediate genomic instability). My preliminary experiments have shown this be unlikely (data not shown). Expression of hTERT with an HA tag epitope does not influence the telomerase activity but interferes with telomere elongation *in vivo* and does not extend cellular life span. This additional control indicates that most likely telomere elongation is required for life span extension. These possibilities can not be fully ruled out unless mutant versions of hTERT or the RNA template (hTR) are expressed in HDFs and the cells are tested for telomere elongation and extended life span.

Generation of an inducible system for hTERT expression will also aid in answering several questions. For instance, a further test of the telomere hypothesis is that extinction of hTERT expression in an inducible manner should lead to telomere loss and initiation of senescence.

Finally, the mechanism by which hTERT elongates telomeres *in vivo* and how this prevents senescence must be deciphered. Telomere elongation may be controlled by several negative and positive regulators. Identification of these factors and the mechanism of their cross-talk with the DNA damage signaling molecules may be the key to understanding how telomere shortening initiates senescence and how telomere elongation leads to its prevention.

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