Replicative Lifespan in
Rodent Cells

Nicole Frances Mathon

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Medical Research Council Laboratory of
Molecular and Cellular Biology,
University College London
Abstract:

Replicative limits have been thought to constrain the extended proliferation of primary cells. After dividing a set number of times, cells irreversibly withdraw from the cell cycle and adopt a characteristic phenotype in a process defined as cellular senescence. The certainty that all cells will ultimately senescence has resulted in a finite replicative capacity being used as one of the defining features of primary cells. In this thesis I show that primary rat Schwann cells can proliferate indefinitely in culture without acquiring immortalizing mutations. These results demonstrate that senescence can no longer be considered an unavoidable barrier to primary cell culture.

The only known mechanism of limiting replicative lifespan is telomere shortening, which occurs upon division of telomerase negative cells. Other uncharacterised cell-intrinsic mechanisms were thought to regulate lifespan in cells that normally express telomerase yet senesce in culture. I show that senescence can be induced in primary Schwann cells, which express telomerase, by altering the conditions in which they are cultured. These results provide the first demonstration that extrinsic as well as intrinsic factors can regulate replicative lifespan and has fuelled speculation that senescence in telomerase positive cells may also be induced by external conditions rather than an intrinsic cell division timer.

The cyclin-dependent kinase inhibitor p16^{INK4A} is progressively upregulated in most primary cell types as they divide in culture. This induction was thought to be a response to an intrinsic timer that operated to limit replicative capacity. I demonstrate that p16^{INK4A} induction in rat Schwann cells can be uncoupled from a senescent arrest. Moreover, the rate and level of p16^{INK4A} induction is dependent on the conditions in which the cells are cultured. These results demonstrate that p16^{INK4A} induction in primary Schwann cells is a response to extrinsic signals rather than an intrinsic cell division timer.
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Abbreviations

NS Normal Schwann
HDF Human Diploid Fibroblast
MEF Mouse Embro Fibroblast
RF Rat Fibroblast
OPC Oligodendrocyte Precursor Cell
Imm Immortalized rat fibroblast line
HMF Human Mammary Fibroblast
PD Population doubling
P Passage
GGF Glial Growth Factor
FCS Foetal Calf Serum
SA-β-Gal Senescence Associated β-Galactosidase activity at pH6
dnp53 Dominant negative p53
TERT Telomerase catalytic subunit
hTR Telomerase RNA template
CDK Cyclin Dependent Kinase
CDKI Cyclin Dependent Kinase Inhibitor
ROS Reactive Oxygen Species
L-NGFR Low affinity nerve growth factor receptor
Chapter 1: General Introduction

At the time I began the work described in this thesis, it was generally accepted that normal primary somatic cells had a limit to their replicative capacity. Indeed, a defining feature of primary cells was that, after a set number of divisions, they would permanently exit the cell cycle and enter a characteristic state termed cellular senescence. Senescence was thought to reflect aging at the cellular level because, similar to all animals that age and eventually die, the senescence barrier determined the lifespan of populations of all primary cells. An understanding of the mechanisms that determine such a replicative limit has provoked much interest, primarily for two reasons:

1. A replicative limit could protect against the clonal expansion required for tumour formation. Tumorigenesis is a multistep process, thought to require acquisition of four to seven oncogenic lesions (Hanahan and Weinberg, 2000). Calculations based on mutation rates and probabilities of acquiring cooperating mutations suggest that senescence should act as an effective barrier to cellular division before a tumour could reach full malignancy (Wright and Shay, 2002).

2. Cellular "ageing" could contribute to organismal ageing: the accumulation of senescent cells within the body, coupled with a reduction in viable regenerative cells could contribute to the gradual deterioration in organismal function associated with ageing.

Since cellular senescence was first defined, advances in the field have increased our understanding of cellular mechanisms that can operate to limit replicative capacity. Therefore, in this introduction I will first present a history of senescence, and then provide a summary of the state of the senescence field at the time of commencing this thesis.
1: The History of Senescence:

1.1a: Hayflick's definition of cellular senescence:

40 years ago when Hayflick began his studies on cultured cells, it was assumed that all cells had the capability to proliferate indefinitely *in vitro* with failure to culture cells for extended periods being ascribed to technical problems (Carrel and Ebeling, 1921), (Shay and Wright, 2000). However, Hayflick observed that the majority of the established cell lines of the day had karyotypic abnormalities. When he restricted his studies to freshly isolated cultures of human foetal diploid fibroblasts, after a finite number of divisions in culture the cells ceased to divide. This replicative limit was dependent on division number rather than time in culture and appeared to be inherent in each set of fibroblasts that Hayflick isolated as proliferation always ceased after the same number of divisions, irrespective of whether the cells had been frozen during their replicative lifespan (Hayflick, 1964). Furthermore, this limit was not influenced either by changes in media or surrounding cells. Mixed cultures of "old" male cells and "young" female cells were always overgrown by female cells. These observations led Hayflick to conclude that primary diploid cells had an innate limit to their replicative capacity and that the ability of established cell lines to divide indefinitely resulted from acquisition of genetic abnormalities, equivalent to those occurring in tumorigenesis. Possession of a finite replicative limit thus became an integral part of the definition of a primary somatic cell (Hayflick, 1964). Hayflick's observation that foetal fibroblasts had greater replicative potential than cells derived from adults led to his proposal that this phenomenon of primary cells was likely to represent aging at the cellular level (Hayflick, 1964). The term cellular senescence was thus coined.
1.1b: The senescent phenotype:

Hayflick described a characteristic phenotype of the arrested cells in his senescent cultures. This senescent phenotype has since been widely used to identify cells at the end of their replicative lifespan. Although the senescent cells were irreversibly arrested, they remained metabolically active. In fact, these arrested cells could be stably maintained in culture over many years (Hayflick and Moorhead, 1961). Phenotypically, the senescent cells were enlarged, lost their polarization, appearing flattened, and often contained prominent stress fibres (Hayflick and Moorhead, 1961). The frequency of nuclear abnormalities also increased - a high proportion of senescent cells were multinucleated or had enlarged nuclei (Hayflick and Moorhead, 1961).

More recently, a marker of senescence has been described. Senescent cells, but not differentiated or quiescent cells, were found to express β-galactosidase activity at pH 6 (termed Senescence Associated -β galactosidase, SA-β-gal) (Dimri et al., 1995). This marker is readily detectable as a perinuclear stain using an X-gal based staining protocol, correlates tightly with senescence in a number of cell types and has been used to identify senescent cells in vivo (Dimri et al., 1995). Despite disputes over the specificity of this marker, and a lack of understanding of its significance in senescence (Severino et al., 2000), (Krishna et al., 1999), (Kurz et al., 2000), (Yegorov et al., 1998), SA-β-gal is now used almost universally as a definitive marker of senescent cells.

In addition to changes in expression of regulators that maintain the senescent arrest, alterations in genes apparently unrelated to induction of the arrest have also been observed in senescent cells (Shelton et al., 1999). For example, senescent fibroblasts are found to secrete elevated levels of the enzyme collagenase (West et al., 1989). Analysis of the altered gene expression profiles in different cell types revealed that these senescent profiles were cell type specific (Shelton et al., 1999).
1.2: Telomere shortening is a cell intrinsic mechanism for regulating replicative lifespan:

As most primary somatic cells that were cultured in vitro were observed to senesce, the proposal that primary somatic cells possess a finite "Hayflick limit" soon became readily accepted. However, a mechanism by which cell division could be counted, and thus lifespan determined, was only elucidated relatively recently:

1.2a: The end-replication problem:

During DNA replication, new bases are added to both the leading and lagging strand DNA templates in the 5'-3' direction (Fig 1.1). Replication of the leading strand occurs by production of one continuous molecule that is extended to the end of the DNA template. The lagging strand is synthesized by ligation of a series of short DNA fragments (Okazaki fragments), produced by extension of RNA primers bound to the template. The primers are subsequently excised and the resulting gaps filled in by DNA polymerase. As a result of this process the DNA under the terminal primer at the 3' end of the chromosome remains unreplicated. Therefore, in the absence of any corrective mechanism, linear eukaryotic chromosomes will reduce in length each time their DNA is duplicated. The relevance of the self-limiting nature of this progressive loss of terminal DNA was already realized soon after Hayflick proposed his limit (Olovnikov, 1973). However, a more comprehensive understanding of how the end-replication problem may limit proliferative lifespan was not obtained until the advent of telomere biology.

1.2b: Telomeres:

Telomeres are the DNA-protein complexes found at the end of linear eukaryotic chromosomes. They serve two important functions: They protect the chromosome ends from being recognized as double strand breaks which elicit cellular DNA-damage responses, and they provide a means of circumventing the end-replication problem (McEachern et al., 2000). The DNA-protein complex is dynamic and can exist in either
Template DNA = Newly synthesized leading strand.

Terminal DNA remains unreplicated. As the lagging strand is synthesized by extension of primers, the end of the template which hybridizes to the terminal primer and DNA 3’ to this remains unreplicated.

Figure 1.1: The End Replication Problem
DNA can only be synthesized in the 5’-3’ direction. During DNA replication the leading strand (red) is synthesized as one continuous molecule (5’ to 3’ direction) whereas the lagging strand is formed from a discontinuous set of Okazaki fragments (blue), each requiring a primer for synthesis. After excision of the primers the gaps between Okazaki fragments are filled and the fragments are ligated to make one continuous strand. The DNA at the 3’ end of the chromosome and under the most 3’ primer cannot be replicated due to the absence of any more 3’ template DNA to provide further priming events. Therefore, in the absence of any corrective mechanism, DNA will progressively shorten with each replication event.
a functional, protective state defined as being "capped", or in the inverse "uncapped" state (Blackburn, 2001; Blackburn, 2000). The factors that confer the capped state are still incompletely understood, but it is clear that the conformation of the higher order DNA-protein structure is important. Although most work to address this issue has been carried out in yeast, I will only discuss what is known about mammalian cells, as the capping strategy in lower eukaryotes appears to differ significantly (Shore, 2001).

The DNA component of mammalian telomeres consists of tandem repeats of TTAGGG (Moyzis et al., 1988). The number of these repeats is maintained in the germline, but varies from species to species. In humans the telomere tract comprises approximately 10kb whereas telomeres of laboratory mice can be at least ten times longer. As a result of the end-replication problem, the 3' end of the telomeric DNA exists as a G-rich single-stranded overhang (Henderson and Blackburn, 1989). This single stranded DNA is susceptible to degradation by exonucleases and can also trigger DNA damage responses. It is therefore important that it is concealed in a protective protein cap.

TRF1 and TRF2 are homodimeric proteins that bind to double stranded telomeric DNA via myb domains (Chong et al., 1995), (Broccoli et al., 1997), (Smith and de Lange, 1997). These proteins play an important role in maintaining the telomere: over expression of the wild type proteins results in telomeric shortening whereas a TRF1 DNA binding mutant induces elongation (Karlseder et al., 2002), (Smogorzewska et al., 2000), (van Steensel et al., 1998). The telomeric-binding function of TRF2 is essential for cellular viability. Cells expressing a dominant negative form cell cycle arrest or undergo apoptosis (van Steensel et al., 1998), (Karlseder et al., 1999).

Although proteins that bind the single strand telomeric overhang had been identified in yeast, mammalian homologues could not be detected. It was thus unclear how this vulnerable stretch of DNA was protected within the telomere. However, the mechanism became evident upon microscopic examination of the structure of purified human DNA terminal regions. These studies revealed that the telomere loops back upon itself (forming a "t-loop") that allows the single-stranded region to invade the duplex of more centromeric telomere repeats. The 3'-overhang is thus stabilized in a "D-loop" (Fig 1.2) (Griffith et al., 1999), (Greider, 1999). The juncture at the D-loop is stabilized by TRF2 (Stansel et al., 2001), a finding that could explain the essential requirement for this protein in telomere capping (Karlseder et al., 1999). Pot1 and hnRNPA1, two
Figure 1.2: Mammalian telomeric structure
The DNA-protein complex at the ends of linear chromosomes forms the telomere cap. The terminal DNA loops back on itself (“t-loop”) to allow the vulnerable single-stranded 3’ end to invade more centromeric duplex telomere repeats, forming a “D-loop”. The D-loop juncture is stabilized by the TRF2 homodimer. TRF1 also binds double-stranded DNA at the telomere and Pot1 and hnrNPA1 bind single-stranded DNA. TRF1 and TRF2 recruit other proteins (examples are depicted above) to the telomere, many of which are implicated in regulating telomere length and stability. In addition, telomerase and its associated protein complex (including Tep1, dyskerin, ribosomal L22 protein) may be periodically bound at the telomere.
Adapted from Blackburn, 2001.
mammalian single-strand DNA binding proteins, have since been identified but their role in capping is still unclear (Baumann and Cech, 2001), (LaBranche et al., 1998).

Besides the proteins bound directly to DNA, there are numerous proteins recruited to the telomeric complex via protein-protein interactions (Fig 1.2). These include TIN2 (Kim et al., 1999) and tankyrase (Smith et al., 1998), both of which bind to and regulate TRF1 function. Tankyrase is an ADP-ribose polymerase which ribosylates TRF1 and thus affects its ability to bind telomeric DNA (Smith and de Lange, 2000). hRap1, a protein that is recruited to the telomeric complex by TRF2 has also been identified. This protein is homologous to a yeast negative regulator of telomere length, scRap1, which binds directly to duplex telomeric repeats (Li et al., 2000). Interestingly, components of the cellular DNA repair machinery have also been located within the telomeric complex. These include the RAD50/MRE11/NBS1 complex, involved in double-strand break repair (Zhu et al., 2000) and Ku protein complexes that are involved in non-homologous end joining (Hsu et al., 2000). In addition, the components of the ribonuclease complex that comprise telomerase (see section 1.2c) also bind at telomere ends.

It remains to be determined precisely how these telomeric components contribute to the capped state. It appears that a functional telomere is dependent on many factors: The binding of proteins to single-stranded and duplex DNA both at the tip and throughout the telomere; the length of the telomeric tract; the formation of higher order structure and the presence of active telomerase and DNA damage/repair components (Blackburn, 2001).

The finding that telomeres of various cultured human cells progressively shortened in culture (Harley et al., 1990), (Hastie et al., 1990), (de Lange et al., 1990) strengthened the hypothesis that the end-replication problem may confer the limit on cellular division.

1.2c: Telomerase:

If the end-replication problem were to operate in the absence of any corrective mechanism, not only would telomeres shorten during the lifetime of an animal but this loss of terminal DNA would also accumulate throughout successive generations of animals, ultimately leading to loss of essential coding DNA. However, as animals can
reproduce indefinitely passing on intact DNA throughout the propagation of the species, mechanisms have clearly evolved to prevent progressive telomere attrition. The mechanism that has developed to counteract this progressive attrition involves expression of telomerase enzymatic activity.

Telomerase was first identified as an enzyme that catalyzes the addition of hexameric repeats to the 3' end of telomeres, and thus was immediately recognized as providing a possible solution to the end-replication problem (Greider and Blackburn, 1985). This enzyme was found to be a ribonucleoprotein complex consisting of an RNA component, hTR, and a catalytic protein subunit, hTERT (Greider and Blackburn, 1987), which may function as a homodimer (Wenz et al., 2001), (Beattie et al., 2001). The RNA component exists in a conserved, complex secondary structure (Chen et al., 2000) and provides a template on which new telomeric repeats are assembled (Yu and Blackburn, 1991). hTERT is a reverse transcriptase which catalyzes the addition of nucleotides onto the RNA template (Nakamura et al., 1997) (Fig 1.3).

Although hTERT and hTR alone are sufficient to confer activity in vitro (Weinrich et al., 1997), there are numerous proteins that have been found complexed with the telomerase core. Most of these proteins, which include TEP1, Dyskerin, C1/C2, Staufen and L22 (Liu et al., 2000), (Mitchell et al., 1999), (Ford et al., 2000), (Le et al., 2000), bind to the RNA component. The role of these proteins remains unclear.

1.2d: Telomerase expression and regulation:

The telomere hypothesis postulated that telomere shortening is responsible for limiting cellular proliferative lifespan. For this hypothesis to hold, it was important that telomerase activity was not expressed in those cells that undergo senescence. Conversely, cells known to divide indefinitely (immortalized cell lines, germ cells and some stem cells) should maintain telomere length. Consistent with the telomere hypothesis early studies showed that various human somatic cell types, including fibroblasts, did not express active telomerase (Allsopp et al., 1992), (Counter et al.,) but cells immortalized in culture and cells derived from tumours had detectable activity (Kim et al., 1994), (Shay and Bacchetti, 1997). Furthermore, telomerase activity was detected in the human germ line and developing embryonic tissues, but was found to be downregulated in the same adult cell types (Wright et al., 1996). From these
Figure 1.3: Addition of telomere repeats by telomerase

The telomerase core enzyme consists of the catalytic component, TERT, and the RNA template, TR. Telomerase binds at the end of telomeres and the TERT reverse transcriptase catalyses the assembly of telomere repeats onto the 3' end of single stranded DNA. The enzyme complex then translocates along the telomere, allowing proccessive addition of new telomere repeats. Other proteins e.g. TEP1 (telomerase-associated protein) and Dyskerin are found associated with telomerase but are not essential to reconstitute telomerase activity in vitro.

Adapted from Mathon and Lloyd, 2001.
findings, it became generally accepted that human somatic cells are telomerase negative.

More recently it has become clear that characterizing cellular telomerase status is a complex issue for a number of reasons (Forsyth et al., 2002), (Collins et al., 2002), (Greider, 1998). Firstly, the finding that telomerase activity is downregulated when tumour cells are induced to enter the quiescent or differentiated state (Holt et al., 1996), (Sharma et al., 1995) suggested the need to differentiate between "telomerase silent" and "telomerase competent" cells. Telomerase silent cells lack telomerase activity regardless of their cell cycle state. Telomerase competent cells have active telomerase when cycling, but may be mistaken for being telomerase negative if analysed in vivo when most somatic cells are quiescent or differentiated. Secondly, the telomerase status of a cell in culture does not necessarily reflect telomerase activity of the same cell type in vivo. Telomerase activity may be increased or decreased upon transfer into culture (Chadeneau et al., 1995), (Wright et al., 1996). Finally, the original finding that hTERT expression was limiting for telomerase activity (Weinrich et al., 1997) has led to the misleading use of hTERT levels to indicate enzyme activity. It is now clear that telomerase is regulated at many levels (see below) and that this assumption in many cases has proved erroneous (Liu et al., 2001), (Colgin et al., 2000), (Yi et al., 2000).

With these factors taken into consideration, the current view is that the majority of human somatic cells are telomerase silent. However, numerous telomerase competent somatic cell types have also been identified. These include haematopoietic cells and epithelial cells in the skin, the gastrointestinal tract, the endometrium, the prostate, kidneys, trachea and bronchi (Kolquist et al., 1998). These are generally cell types that are required to undergo proliferation throughout life and thus require a greater telomere reserve. For example telomerase is present in the regenerative basal layer of epidermis in skin and in the mitotic regions of hair follicles but is absent in the differentiated dermis (Harle-Bachor and Boukamp, 1996), (Ramirez et al., 1997). In many cases telomerase activity clearly correlates with proliferative requirement, suggesting tight regulation of this activity. For example telomerase is tightly regulated throughout the menstrual cycle in the endometrium and is silenced in menopausal women (Brien et al., 1997), (Tanaka et al., 1998). Another example is T lymphocytes that have basal levels of telomerase activity that can be rapidly elevated 500-1000-fold in response to cytokine exposure (Bodnar et al., 1996), (Buchkovich and Greider, 1996). Interestingly
this transient activation in T-lymphocytes is insufficient to maintain telomere length throughout a lifetime as telomeres have been observed to shorten in these cells (Vaziri et al., 1994; Vaziri et al., 1993). Although there are differences in telomerase status in normal somatic cells, it is clear that in 90% of tumours derived from various cell types, telomerase activity is high (Shay and Bacchetti, 1997). In tumours that do not express telomerase, telomeres are stabilized by alternative means (Alternative Lengthening of Telomeres, ALT) that are probably based on recombination mechanisms (Bryan et al., 1997). Therefore telomere maintenance appears to be an essential requirement in all tumour cells.

In laboratory mice, the pattern of telomerase expression is different. Telomerase activity is detectable in the majority of tissues in vivo and in cells cultured in vitro (Greenberg et al., 1998), (Chadeneau et al., 1995), (Prowse and Greider, 1995). Although there does appear to be some level of tissue-specific telomerase regulation (lower levels of activity are present in the kidney and spleen and are undetectable in the brain (Prowse and Greider, 1995), this may reflect the quiescent state of these cell-types in vivo, as mTERT is detectable in all cases (Martin-Rivera et al., 1998), (Klapper et al., 2001). Interestingly, although levels of telomerase in murine cells are sufficient to maintain telomere length and replicative lifespan of these cells is not controlled by telomere shortening (see section 1.4a), telomerase is also upregulated in murine tumours (Blasco et al., 1996), (Chadeneau et al., 1995).

The mechanisms involved in regulating telomerase activity are very complex and still incompletely understood (Poole et al., 2001), (Aisner et al., 2002). The primary mechanism of regulation is at the level of TERT expression (Weinrich et al., 1997). The TERT promoter has been cloned (Horikawa et al., 1999) and various transcription factors that positively regulate expression identified. These include c-Myc (Wu et al., 1999), (Wang et al., 1998), Sp1 (Misiti et al., 2000), oestrogen (Kyo et al., 1999) and progesterone (Wang et al., 2000). The involvement of the Myc oncogene (Wu et al., 1999), (Wang et al., 1998) in telomerase regulation may contribute to the elevated levels observed in tumour cells and the involvement of oestrogen could be important for normal regulation of telomerase levels in the endometrium (Tanaka et al., 1998). The identification of negative regulators of TERT is also of interest in understanding how TERT is downregulated in most somatic tissues. Transcription factors that can repress TERT expression include WT1 (Oh et al., 1999), p53 (correlating with its tumour

* Greenberg et al., 1999
suppressive function) (Kanaya et al., 2000) and Mad 1, which antagonizes the positive TERT regulation by Myc (Oh et al., 2000), (Xu et al., 2001).

It is becoming clear that telomerase activity is not only regulated at the transcriptional level. Various inactive splice variants of TERT have been identified, one of which has been shown to act as a dominant negative (Colgin et al., 2000), (Yi et al., 2000). This finding raised the possibility that the balance of TERT splice variants can modulate overall telomerase activity. The association of various proteins with the telomerase ribonucleoprotein complex (see section 1.2c) presumably plays a role in regulating activity or accessibility to the telomere substrate but details remain obscure. There have also been numerous reports suggesting regulation by post-translational modifications (e.g. phosphorylation) (Kang et al., 1999). In one study the tyrosine kinase c-Abl was shown to specifically phosphorylate and thus inhibit TERT in response to ionizing radiation (Kharbanda et al., 2000). A final level of regulation is implemented by TERT subcellular localization. Recent studies of GFP-tagged telomerase demonstrated that telomerase is sequestered in nucleolar sites but is released into the nucleoplasm when telomere replication is required during the cell cycle. This form of telomerase regulation appears to be absent in transformed cells. DNA damage was observed to enhance the nucleolar localization presumably to prevent telomerase acting on non-telomeric substrates thus ensuring correct DNA repair (Wong et al., 2002). In addition, activation of telomerase in stimulated lymphocytes is reported to occur by phosphorylation and subsequent translocation of TERT into the nucleus (Liu et al., 2001). 14-3-3 proteins have been implicated in maintaining TERT nuclear localization (Seimiya et al., 2000).

1.2e: Proof of the telomere hypothesis:

Although there was significant correlative evidence in favour of telomere length determining proliferative lifespan, the telomere hypothesis was not definitively proven until human TERT was cloned (Nakamura et al., 1997) and retrovirally expressed in telomerase negative human cells. Introduction of the hTERT subunit into human fibroblasts, endothelial cells and retinal pigment epithelial cells was sufficient to confer telomerase activity, reverse telomeric shortening and allow unlimited proliferation in these cells (Bodnar et al., 1998), (Yang et al., 1999), (Vaziri et al., 1998). Importantly,
exogenous telomerase activity did not induce any of the changes associated with transformation (karyotypic abnormalities or loss of cell cycle checkpoints, mitogen dependency, contact inhibition, or anchorage dependency), or alter the differentiation status of the immortalized cells (Jiang et al., 1999), (Morales et al., 1999), (Yang et al., 1999). A more recent finding that TERT immortalized fibroblasts cultured over very long time periods ultimately express elevated levels of the Myc oncogene probably reflects progressive selection in culture for cells with a proliferative advantage rather than a direct oncogenic response to telomerase activity (Wang et al., 2000).

1.3: Telomere shortening in vivo:

The identification of telomere shortening as an intrinsic mechanism of limiting replicative lifespan fuelled investigations into its role in vivo. The objective of these studies was to investigate the hypothesis that replicative limits had evolved to protect against cancer. In addition, investigations into telomere-dependent senescence in vivo could address the question of whether such a replicative limit could contribute to organismal aging.

1.3a: Does telomere shortening occur in vivo?

The original findings that cells derived from old donors had reduced replicative potential compared with cells from young donors initiated the hypothesis that senescence may play a role in aging and cancer (Hayflick, 1964). However, these experiments have proved difficult to repeat. Although the telomere length of donor cells correlates tightly with proliferative lifespan in culture, little correlation between telomere length and donor age has been found (Hayflick, 1964); (Allsopp et al., 1992), (Cristofalo et al., 1998). Furthermore, as it is now clear that many proliferative human cell types are telomerase competent (e.g. many epithelial cell types)(see section 1.2d) it is possible that telomerase is regulated to ensure significant telomere shortening does not occur in vivo.
1.3b: The role of telomere shortening in cancer:

There is evidence to suggest that telomere maintenance is essential for the sustained proliferation of tumour cells: 90% of tumour cells are found to have stabilized telomere length by expression of high telomerase levels (Kim et al., 1994), (Shay and Bacchetti, 1997) whilst the remainder of tumour samples maintain telomere length by alternative mechanisms (Bryan et al., 1997). Treatment with telomerase inhibitors or expression of dominant negative forms of TERT in telomerase positive tumour cells triggers apoptosis, demonstrating that telomerase activity is essential for their viability (Hahn et al., 1999), (Herbert et al., 1999), (Shammas et al., 1999). Furthermore, although TERT expression alone does not confer a transformed phenotype, telomerase activity is an essential requirement (in addition to Ras, SV40-large T and SV40-small t) for generation of transformed cells in vitro (Jiang et al., 1999), (Yang et al., 1999), (Morales et al., 1999), (Hahn et al., 1999), (Elenbaas et al., 2001).

Although telomere maintenance appears to be important for sustained tumour viability, it is still unclear whether a telomere-imposed replicative limit acts as a barrier to tumorigenesis. One indication that telomere shortening may have occurred during tumorigenesis is the observation that in some cases tumour cells have reduced telomere lengths compared to their surrounding non-malignant cells (Hastie et al., 1990), (Sommerfeld et al., 1996).

In an attempt to address the role of telomere shortening in vivo, and to perhaps resolve debate about the putative tumour suppressive function, telomerase knockout mice were generated by deletion of mTR or mTERT (reviewed by (Artandi and DePinho, 2000)), (Blasco et al., 1997), (Liu et al., 2000), (Nikaido et al., 1999). The knockouts were bred for at least four generations before a phenotype became evident, demonstrating the large telomere reserve of murine cells (Lee et al., 1998), (Rudolph et al., 1999). Rather than resolving the function of telomere shortening, the findings from the telomerase knockouts complicated matters: surprisingly, late generation TERT-/- mice were found to have an increased incidence of cancer, suggesting that telomere shortening promoted rather than protected against cancer (Rudolph et al., 1999). It is thought that the critically short telomeres in these mice generate genetic instability (similar to that observed in human fibroblasts approaching crisis -see section 2.3a of this chapter (Counter et al., 1992) that accelerates accumulation of oncogenic mutations.
Consistent with this suggestion, analysis of MEFs from late generation mice revealed increased incidences of chromosomal abnormalities (Blasco et al., 1997), (Hande et al., 1999), (Niida et al., 1998). Moreover, in a p53-/- genetic background where the survival of genetically unstable cells is promoted, the cancer incidence of late generation telomerase negative mice is further increased (Chin et al., 1999), (Artandi and DePinho, 2000). However, the generation of further mouse models for telomere shortening (summarized in Table 1.1) demonstrated that telomere shortening can have opposite effects depending on genetic background. Therefore, it seems that a telomere limit can protect against the expansion of premalignant cells carrying certain mutations (e.g. loss of the INK4A locus) but telomere shortening can also promote tumorigenesis by generating genetic instability. It is unclear which effect predominates in humans. Dyskeratosis Congenita (DKC) is a human genetic disorder that is associated with reduced telomerase activity (Mitchell et al., 1999), (Vulliamy et al., 2001), (Shay and Wright, 1999), (Marciniak et al., 2000), (Collins and Mitchell, 2002). Interestingly, much like the telomerase knockout mouse, DKC patients show an increased incidence of cancer, suggesting that telomere shortening in humans can also promote cancer (Marciniak et al., 2000).

The role of telomere shortening in cancer has been further complicated by recent studies suggesting that telomerase may have additional proliferative or survival functions that are independent of its role in telomere maintenance (Gonzalez-Suarez et al., 2001), (Oh et al., 2001), (Artandi et al., 2002). These additional functions could explain why telomerase is progressively upregulated in mouse and human tumour progression, (Kolquist et al., 1998), (Blasco et al., 1996), (Chadeneau et al., 1995) and why humans tightly regulate telomerase so that it is only active when cells are proliferating and require maintenance of telomere reserve (see section 1.2d).

Although, the role of telomere shortening in human tumorigenesis is extremely complex it is clear that telomere maintenance is essential for tumour survival and that in the majority of tumour cells this is achieved by telomerase expression. There are currently many ongoing investigations into exploiting this finding for cancer diagnosis (by detection of activity) and treatment (by inhibiting activity)(reviewed in (Mathon and Lloyd, 2001), (Bearss et al., 2000), (Shay et al., 2001)).
Table 1.1: The cancer phenotypes of telomerase knockout mouse models:

<table>
<thead>
<tr>
<th>Mouse model for telomere shortening</th>
<th>Cancer phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TERT-/-</td>
<td>Increased frequency of spontaneous tumours compared to wild type.</td>
<td>(Rudolph et al., 1999)</td>
</tr>
<tr>
<td>TERT -/-; p53 -/-</td>
<td>Increased frequency of spontaneous tumours compared to p53/- mice.</td>
<td>(Chin et al., 1999), (Artandi and DePinho, 2000)</td>
</tr>
<tr>
<td>TERT -/-</td>
<td>Decreased frequency of epithelial tumours in carcinogen treated mice compared to carcinogen treated wild type mice.</td>
<td>(Gonzalez-Suarez et al., 2000)</td>
</tr>
<tr>
<td>TERT -/-; INK4A/-</td>
<td>Decreased frequency of carcinogen induced tumours compared to carcinogen treated INK4A/- mice.</td>
<td>(Greenberg et al., 1999)</td>
</tr>
<tr>
<td>TERT -/-; ApcMin</td>
<td>Increased frequency of benign microscopic adenomas but a decreased frequency of these progress to malignancy compared with the Min mouse (Su et al., 1992).</td>
<td>(Rudolph et al., 2001)</td>
</tr>
</tbody>
</table>

1.3c: The role of replicative senescence in ageing:

Hayflick's original suggestion that ageing at the cellular level could have wider implications for organismal ageing seemed an attractive hypothesis: whilst a finite replicative capacity could limit the regenerative potential of tissues, the accumulation of senescent cells in the body might also hinder function of cells that had not yet reached their replicative limit. The finding that senescent cells have altered expression profiles also raised the possibility that these cells could actively contribute to the degeneration associated with ageing (Shelton et al., 1999). For example, senescent fibroblasts secrete elevated levels of collagenase and may thus play a role in the decreased elasticity of aging skin (West et al., 1989). However, it has not yet been convincingly demonstrated that senescent cells with critically short telomeres accumulate with age: measurements of telomere length have been inconclusive and although there have been reports that
senescent β-gal positive cells accumulate in aged skin, these have been difficult to reproduce (Schneider and Mitsui, 1976), (Allsopp et al., 1992), (Cristofalo et al., 1998), (Dimri et al., 1995), (Severino et al., 2000).

The most commonly quoted evidence in favour of a role for replicative senescence in ageing has been provided by the symptoms of DKC patients and the phenotypes of late generation telomerase knockout mice. Although DKC patients tend to die of bone marrow defects many of the other associated symptoms such as hair graying and osteoporosis are features of premature ageing. These are closely mirrored by the phenotypes of TERT-/- mice - see Table 1.2. However, many of these defects (e.g. reduced subcutaneous fat and hair loss) could be a secondary response to malnutrition due to gut defects that are not particularly associated with normal human ageing.

Furthermore, replicative ageing clearly cannot account for the degeneration of human post-mitotic tissues (e.g. the brain and heart) or indeed for normal ageing of mice (due to their excessive telomere reserve) or post-mitotic organisms such as drosophila. Therefore, if telomere shortening does play a role in human ageing it can only be a contributory factor.
**Table 1.2**: Phenotypes of DKC patients and late generation TERT-/- mice compared with normal human ageing (adapted from (Marciniak et al., 2000), see also (Rudolph et al., 1999), (Lee et al., 1998), (Herrera et al., 2000)).

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Human ageing</th>
<th>DKC</th>
<th>Late generation TERT-/- mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shortened lifespan</td>
<td>NA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Defective wound healing</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hair loss and greying</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Decreased subcutaneous fat</td>
<td>+</td>
<td>?</td>
<td>+</td>
</tr>
<tr>
<td>Increased cancer</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Osteoporosis</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Gut defects</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Type II diabetes</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Atherosclerosis</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cataracts</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bone marrow failure</td>
<td>-</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>Testicular atrophy</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**1.4: Telomere shortening does not regulate replicative lifespan of all cell types**

If telomere shortening were the only mechanism regulating proliferative lifespan, we would expect all cells expressing telomerase, and maintaining telomere length to be immortal. However, the findings discussed below demonstrate that in certain cultured cell types this is not the case:

**1.4a: Senescence of murine cells is telomere-independent:**

Murine cells have a significantly reduced proliferative lifespan in culture compared to human cells. For example, mouse embryo fibroblasts (MEFs) undergo classical senescence after only 8-10 population doublings (compare to 60 PDs in human
cells) (Blasco et al., 1997). This arrest is clearly independent of any telomere shortening because murine cells have active telomerase and maintain extremely long telomeres in culture (Zijlmans et al., 1997). Furthermore, MEFs derived from telomerase knockout mice senesce and then spontaneously immortalize at exactly the same rate as wild type MEFs. Telomerase negative MEF cell lines can be maintained in culture for at least 200 population doublings, indicating that the telomere reserve in these cells is more than adequate to sustain long-term proliferation and cannot be responsible for the rapid senescent arrest observed in primary cells (Blasco et al., 1997). Finally, unlike human fibroblasts in which overexpression of telomerase is sufficient to confer immortality, MEFs overexpressing TERT senesce at the same rate as wild type cells in culture (Artandi et al., 2002). Therefore, although telomere shortening is clearly responsible for limiting the lifespan of some human cells, it cannot explain the senescence that occurs in cultured murine cells.

1.4b: Senescence of some human cells is telomerase-independent:

Although human fibroblasts can undergo about 60 divisions before arresting, some cultured epithelial cells senesce much more rapidly - for example 20PDs in human mammary epithelial and only 3PDs in thyroid epithelial cells (Kiyono et al., 1998), (Jones et al., 2000). Unlike fibroblasts, epithelial cells tend to spontaneously emerge from this senescent arrest at a high frequency and immortalized lines are found to have inactivated the Rb pathway by downregulation of p16INK4A, usually by methylation of the p16INK4A promoter (Kiyono et al., 1998), (Jarrard et al., 1999), (Farwell et al., 2000), (Dickson et al., 2000), (Foster et al., 1998). Cells that have emerged from senescence in this way are however not immortal. They continue to divide and demonstrate telomere shortening until reaching a second proliferative barrier (Romanov et al., 2001). Studies in which hTERT was expressed in a variety of epithelial cell types (including mammary epithelial cells, keratinocytes, thyroid epithelial cells and adenoid epithelial cells) confirmed that the mechanism of limiting lifespan in these cell types differed from that in fibroblasts. Activation of telomerase and telomere elongation was insufficient to bypass the first p16INK4A-dependent proliferative barrier. Abrogation of the Rb pathway (by p16INK4A inactivation) was required in addition to telomere maintenance to confer immortality (Kiyono et al., 1998), (Farwell et al., 2000), (Dickson et al., 2000). These
findings led to the proposal that in certain human cell types an additional cell-intrinsic mechanism to telomere shortening operates to limit replicative capacity. As this additional arrest appeared to be dependent on p16^{INK4A} and p16^{INK4A} levels were observed to gradually increase in these cells as they neared senescence, it was suggested that p16^{INK4A} accumulation formed the basis for this alternative cell division counting mechanism, (Jones et al., 2000), (Dickson et al., 2000).

1.5: Extrinsic factors can rapidly induce a senescent phenotype in cultured cells, independent of their replicative history.

Since Hayflick first defined senescence as the arrest occurring at the end of a proliferative lifespan, subsequent investigations have revealed that induction of a senescence phenotype is more complex, as it is not exclusively induced in response to cellular division. There are now numerous reported examples where exposure of cultured cells to external stresses at any point in their replicative history leads to induction of a senescence phenotype that is indistinguishable from that induced by division. DNA damage induced by γ-irradiation or reagents that cause DNA-double strand breaks can rapidly induce senescence in early passage HDFs (Di Leonardo et al., 1994), (Robles and Adami, 1998). Oxidative stresses (e.g. induced by exposure to hydrogen peroxide) also induce senescence, possibly reflecting the resultant DNA damage (Chen and Ames, 1994), (Severino et al., 2000), (Toussaint et al., 2000). Furthermore, in experiments designed to investigate resistance of primary cells to a single oncogene, it was discovered that the arrest induced by oncogenic Ras in keratinocytes and fibroblasts was accompanied by a classical senescence phenotype (Serrano et al., 1997), (Lin and Lowe, 2001), (Farwell et al., 2000). Other external senescence-inducing insults include ceramide and TGFβ (Venable et al., 1995), (Katakura et al., 1999), (Mouton and Venable, 2000). All these factors mediate their cellular response by induction of cell-cycle inhibitors and overexpression of these CDKIs alone is sufficient to cause a senescent arrest (McConnell et al., 1998) (see section 2.2b in this chapter).
1.6: Redefining senescence:

Describing the arrest triggered by cell division, DNA damage, oxidative stress, and oncogenic stimulation indiscriminately as senescence is uninformative and in some circumstances confusing. However, establishment of a refined definition in itself has been a matter of debate.

Because the senescent phenotype is indistinguishable, whatever the stimulus, it was suggested that the term senescence should be used to describe the arrest *per se*. However, to distinguish between arrests induced by a cell-intrinsic mechanism at the end of a proliferative lifespan and arrests that can be triggered at any point in a cell's replicative history, it is suggested that the term senescence should be prefaced with "replicative" or "premature" as appropriate (Fig 1.4) (Mathon and Lloyd, 2001), (Lloyd, 2002), (Serrano and Blasco, 2001).

On the other hand, as the word senescence implies an ageing process, it has been suggested that this term should be reserved to describe the arrest occurring at the end of a replicative lifespan in response to a cell-intrinsic mechanism. The term "stasis" (*Stimulation And Stress Induced Senescence*) has been supplied as an alternative to describe the arrest triggered by external factors (e.g. response to γ-irradiation) (Wright and Shay, 2002), (Drayton and Peters, 2002). The limitation of this definition is that it does not supply a term for cells demonstrating a senescent phenotype, but for which the stimulus for this arrest is unknown. For example, this definition cannot be used to describe the SA-β-galactosidase positive cells detected in aged skin, because it is unclear whether these are the result of stasis or senescence. In contrast, by the first definition these cells can simply be described as senescent. The alternative prefixes (replicative/premature) would allow refinement of this description once the stimulus for the arrest had been established. Therefore, for the purposes of this thesis, we will use the term senescence to describe the characteristic phenotype originally described by Hayflick. In cases where the stimulus that induced this phenotype is known, we will include the terms replicative or premature, as appropriate.
Redefining senescence

The senescent phenotype describes an enlarged, flattened G1-arrested cell, that is frequently multinucleated and stains positively for SA-β-gal. An identical senescent phenotype can be induced by intrinsic factors such as telomere shortening, defined as "Replicative Senescence" or by extrinsic factors such as oxidative stress, expression of oncogenic Ras or agents that induce DNA damage, defined as "Premature Senescence".

**Figure 1.4:** Redefining senescence

REPLICATIVE SENESCENCE

- Induced by: Intrinsic factors
  - e.g. telomere shortening

PREMATURE SENESCENCE

- Induced by: Extrinsic factors
  - e.g. Oxidative stress
  - Oncogenic stress
  - DNA damage
2: Cell Cycle Regulators Involved in Senescence:

2.1: Introduction to the cell cycle:

Eukaryotic cellular proliferation is a tightly regulated process, involving the coordination of extracellular and intracellular cues to ensure that division only occurs in appropriate circumstances. In higher organisms the adverse consequences of failure of this exquisite regulation are demonstrated by proliferative disorders such as cancer. This level of control is achieved by regulating passage through the cell cycle. The cell cycle can be divided into four phases: DNA is replicated during S-phase and is correctly distributed between the two daughter cells in M-phase. The two intermittent phases, G1 and G2, allow the cell to grow, and to sense, by a series of feedback mechanisms, whether it is appropriate to proceed through to the next phase of the cell cycle (Malumbres and Barbacid, 2001). These mechanisms, termed checkpoint controls, converge on cyclin-dependent kinases (CDKs). CDKs are serine/threonine kinases that regulate transition through the cell cycle by phosphorylation of specific substrates that mediate the essential features of cellular division (DNA replication, mitosis and cytokinesis). Checkpoint activation can result in arrest at different phases until conditions again become suitable for the cell cycle to continue e.g. once DNA damage has been repaired. Alternatively, programmed cell death may be initiated to prevent improper division or a cell may completely exit the cell cycle (e.g. upon mitogen withdrawal) and enter a quiescent state termed Go. Checkpoints can be activated throughout the cell cycle in response to a variety of stimuli including lack of mitogen, incomplete DNA synthesis, DNA damage, expression of oncogenic Ras and misalignment of chromosomes on the spindle during mitosis. As senescence results in G1 arrest, we will mainly introduce regulation of the G1-S transition.
2.1a: CDK regulation:

Transition through the cell cycle is controlled by the sequential activation of different CDKs. CDKs 4 and 6 are involved in passage through early G1 (Matsushime et al., 1992), (Meyerson and Harlow, 1994) whilst CDK2 is required to complete G1, initiate entry into S-phase and allow progression through S-phase (Dulic et al., 1994), (Pagano et al., 1992). CDK1 regulates progression through G2 and M phase. To ensure tight cell-cycle control, CDKs are regulated at many levels both by positive and negative regulators (Malumbres and Barbacid, 2001), (Ekholm and Reed, 2000):

CDK activity is entirely dependent on heterodimerization with cyclin subunits that also direct their substrate specificity. The levels of these unstable proteins are regulated throughout the cell cycle. CDK4 and CDK6 associate with cyclins D1, D2 and D3 to form active complexes. The levels of cyclin D proteins are regulated by signaling through both the MAPK and PI3K pathways: Signaling through the MAPK pathway leads to cyclin D synthesis (Cheng et al., 1998) whereas signaling through the PI3K pathway blocks their degradation (by inhibiting GSK-3β, the kinase which stimulates cyclin D export from the nucleus for degradation) (Diehl et al., 1998), (Alt et al., 2000). The association of CDK 4 and 6 with cyclin Ds thus allows integration of cell cycle control with levels of external mitogens and growth factors. CDK2 is activated by dimerization with cyclins E1 and E2 at the G1/S transition and with cyclins A1 and A2 during S phase (Dulic et al., 1992), (Zariwala et al., 1998), (Pagano et al., 1992), (Sweeney et al., 1996).

CDKs are regulated both positively and negatively by phosphorylation: activation requires phosphorylation on a specific threonine near the active site, catalyzed by the CDK7-cyclin H complex, also known as CDK-Activating Kinase (CAK) (Nigg, 1996) and dephosphorylation by CDC25 phosphatases on adjacent threonine and tyrosine residues. The inhibitory dual specific kinase responsible for this phosphorylation step is Weel (Malumbres and Barbacid, 2001).

CDK activity is further regulated by interaction with two families of CDK inhibitors (CDKIs) (Sherr et al., 1999). The INK4 family includes p16INK4A (Serrano et al., 1993), p15INK4b (Hannon and Beach, 1994), p18INK4c (Guan et al., 1994) and p19INK4d (Chan et al., 1995). These small proteins specifically bind to CDK4 and 6 and inhibit their binding to cyclin D to form active complexes. The Cip/Kip family includes p21Cip1
(Harper et al., 1993), p27Kip1 (Polyak et al., 1994), (Polyak et al., 1994) and p57Kip2 (Lee et al., 1995). Whilst this family can bind to and inhibit most CDK-cyclin complexes, they are also essential assembly factors for Cyclin D-CDK4/6 complexes (Cheng et al., 1999) and in vivo seem to be most important as inhibitors of Cyclin E-CDK2 complexes. The INK4 family thus plays a secondary inhibitory role on CDK2: binding of these inhibitors to CDK4/6 leads to displacement of the Cip/Kip inhibitors from active cyclin D complexes onto CDK2-cyclin complexes (McConnell et al., 1999).

2.1b: The G1-S transition:

The regulation of the G1-S transition (Fig 1.5) is particularly stringent. It is important that conditions for division are favourable before entry into S-phase when DNA replication commits a cell to divide. The point at which this assessment occurs is in late G1 and is known as the "restriction point". Beyond the restriction point the cell is no longer dependent on external signals (e.g. presence of growth factors) to complete the cell cycle.

The critical mediator of transition from G1 to S phase is the tumour suppressor protein Rb, which is the primary substrate of CDK4, 6 and 2 during G1 progression (Dyson, 1998). p107 and p130, the other members of the Rb family, show some functional redundancy with Rb but also appear to have distinct roles in cell cycle progression. For example p130 appears to be important in maintaining the quiescent state. In its hypophosphorylated state Rb can bind to the E2F transcription factor to form a complex that actively represses genes required for cell cycle progression (Dyson, 1998). Mitogenic stimulation leading to elevated cyclin D levels triggers the phosphorylation of Rb on specific residues by CDK4/6-cyclin D complexes. This Rb phosphorylation impairs active gene repression by the Rb-DP-E2F complex leading to transcription of some genes required for cell cycle progression, including cyclin E (Zhang et al., 2000), (Harbour et al., 1999). CDK2 associates with the newly transcribed cyclin E. Inhibition of these complexes by the Cip/Kip family of CDKIs is limited as these inhibitors are sequestered within the active cyclin D-CDK4/6 complexes. Active CDK2 positively regulates its own activity by phosphorylating p27Kip1 and targeting it for destruction (Sheaff et al., 1997). The generation of active cyclin E-CDK2 complexes allows complete phosphorylation of Rb that leads to its dissociation from DP-E2F.
Figure 1.5: Regulation of the G1-S transition:
In quiescent cells the E2F and DP transcription factors are maintained in an inactive state because they are bound by the Rb family member, p130. In G1 they are predominantly bound by Rb. Mitogenic stimulation leads to Cyclin D synthesis and thus formation of active Cyclin D/CDK4/6 complexes which phosphorylate Rb. Partial Rb phosphorylation leads to derepression by Rb-DP-E2F complexes, resulting in transcription of certain genes, including Cyclin E. The resulting active Cyclin E/CDK2 complexes complete Rb phosphorylation, allowing the release of DP-E2F and thus induction of the E2F responsive genes required for the G1-S transition. The Cip/Kip family of CDKIs inhibit CDK2 complexes. The INK4A family bind to and inhibit CDK4/6, but also lead to the displacement of Cip/Kip proteins from Cyclin D/CDK4/6 complexes (where they act as assembly factors) onto Cyclin E/CDK2 complexes.
R = Restriction Point - where the cell enters the mitogen-independent phase of the cell cycle.
(Adapted from Malumbres and Barbacid, 2001).
Lundberg and Weinberg, 1998), (Harbour et al., 1999). Release of E2F from the Rb complex allows induction of E2F responsive cell cycle genes. Transcription of these genes is essential for transition into S-phase.

2.2: G1 Checkpoints:

Activation of checkpoints in the G1 phase of the cycle may induce apoptosis (e.g. in response to DNA damage), withdrawal from the cycle into Go (e.g. after serum deprivation) or a cell cycle arrest frequently accompanied by a senescent phenotype. Cells in this senescent state are resistant to apoptosis (Chen et al., 2000) and differ from quiescent cells, as the cell cycle arrest is irreversible (Seshadri and Campisi, 1990). The key cell cycle inhibitors involved in mediating these G1 checkpoints are frequently found to be mutated in immortalized cultured cells and thus established cell lines are deficient in normal G1 checkpoint responses.

2.2a: The key players involved in mediating G1 checkpoints:

p53:

The p53 transcription factor is a critical mediator of stress-induced G1 checkpoints and consistent with this function it is a tumour suppressor (Malkin et al., 1990), (Malkin et al., 1990) and is inactivated in the majority of established cell lines. Unlike most other tumour suppressors, p53 tends to be inactivated in tumours by point mutation. These mutant forms can act as dominant negative inhibitors of wild type p53 which functions as a tetramer. (Vousden and Lu, 2002). Whilst other members of the p53 family (p73 and p63) play roles in development (Melino et al., 2002) the primary function of p53 appears to be to prevent inappropriate proliferation under stressful conditions (Vousden and Lu, 2002). Various stresses including DNA damage, inappropriate oncogenic stimulation, ribonucleotide depletion, loss of anchorage/survival signals and hypoxia result in p53-mediated inhibition of cell cycle progression via induction of cell cycle arrest, apoptosis, differentiation or senescence (Vousden and Lu, 2002), (Giaccia and Kastan, 1998). Although in most cases p53
mediates withdrawal of damaged cells from cycle, it also contributes to DNA repair mechanisms allowing rehabilitation of cells back into the proliferative pool.

The predominant mechanism of p53 activation is by stabilization and thus elevation of p53 levels (Maltzman and Czyzyk, 1984). In dividing cells p53 is maintained at low levels by ubiquitination that targets it for destruction by the proteasome. In particular the Mdm2 ubiquitin ligase is instrumental in maintaining p53 inactivity, both by targeting it for destruction and by directly blocking its transcriptional activity (Momand et al., 1992), (Kubbutat et al., 1997). Mdm2 is itself induced by p53 and thus p53 activity is controlled by an autoregulatory loop (Barak et al., 1993). p53 activity is further regulated by subcellular localization (Pearson et al., 2000), (Fogal et al., 2000) and posttranslational modification including phosphorylation (Oda et al., 2000), acetylation (Prives and Manley, 2001) and sumoylation (Gostissa et al., 1999), (Rodriguez et al., 1999). The cellular outcome of p53 activation varies depending on context and cell type. It is not yet understood how the complexities of p53 regulation determine outcome but recent studies are beginning to provide some clues. For example phosphorylation of p53 on serine 46 is required for induction of its apoptotic effector genes but not for the genes involved in cell cycle arrest (e.g. the CDKI, p21CIpl) (Oda et al., 2000). In addition, interaction of p53 with various regulatory proteins (e.g. the ASPP family and other p53 family members) may target its specificity to different gene promoters (Oda et al., 2000), (Flores et al., 2002).

Cyclin Dependent Kinase Inhibitors:
CDKI induction is essential to mediate most G1 checkpoints. However, the particular CDKIs involved vary depending on cell type and stimulus. The lack of a developmental phenotype of the p21CIpl knockout mice would suggest that the role in development of this CDKI is secondary to its function mediating checkpoint responses (Deng et al., 1995), (Brugarolas et al., 1995). In contrast the other members of the Cip/Kip family p27Kip1 (Chen and Segil, 1999), (Durand et al., 1998) and p57Kip2 (Zhang et al., 1997), (Yan et al., 1997) appear to have more important roles in development. In the majority of checkpoint responses that p21CIpl regulates, it acts as the downstream effector of p53 to induce cell cycle arrest. The G1-arrest response to DNA damage is almost entirely dependent on p53-dependent p21CIpl induction (Dulic et al., 1994), (Deng et al., 1995), (Brugarolas et al., 1995), (el-Deiry et al., 1993). p21CIpl also operates downstream of p53 in the arrest induced in primary fibroblasts, Schwann cells
and keratinocytes by expression of oncogenic Ras (Serrano et al., 1997), (Lloyd et al., 1997), (Lin and Lowe, 2001). Interestingly, in certain contexts induction of p21Cip1 in this checkpoint response can be p53 independent (Sewing et al., 1997), (Woods et al., 1997), (Olson et al., 1998). Although p53 is an important tumour suppressor (Donehower et al., 1992), p21Cip1 is not (Deng et al., 1995).

The signals involved in mediating induction of the INK4 family of inhibitors remain largely obscure, with the exception of p15INK4B, which is upregulated in response to the cytokine TGFβ in epithelial cells (Li et al., 1995), (Massague and Wotton, 2000). Most attention has focused on the cell cycle role of p16INK4A as it is known to act as a tumour suppressor. In particular loss of p16INK4A is associated with melanoma (Kamb et al., 1994), (Sharpless et al., 2001). p18INK4C and p19INK4D are only rarely mutated in cancer and although p15INK4B mutations are observed in tumours, these are often large deletions that also abrogate p16INK4A function (Ruas and Peters, 1998). However, p15INK4B knockout mice do show a slightly increased susceptibility to cancer (Latern et al., 2000). p16INK4A induction can occur secondary to p53 activation by an unknown mechanism in DNA damage responses. DNA damage triggers transient p53 and p21Cip1 activity that is followed by sustained elevation of p16INK4A (Robles and Adami, 1998). In addition, p16INK4A may also be induced in response to UV irradiation (Pavey et al., 1999). p16INK4A induction also plays a role in the oncogenic Ras induced arrest in primary cells but the relative importance of this CDKI in mediating the arrest varies depending on cell type (Brookes et al., 2002), (Sharpless et al., 2001), (Krimpenfort et al., 2001). In HDFs, oncogenic Ras stimulates rapid p53-independent induction of p16INK4A (Lin et al., 1998), (Zhu et al., 1998), (Wei et al., 2001).

ARF:

Unusually, the IN4Ka locus that encodes the p16INK4A CDKI also encodes another tumour suppressor, p19ARF (p14ARF in humans) from an alternate reading frame (Sherr, 1998), (Kamijo et al., 1997). As these distinct proteins are encoded by different reading frames, they show no homology to each other. p16INK4A is encoded by exon 1α spliced to exon 2 whereas an alternative upstream exon 1β spliced to exon 2 encodes p19ARF (Quelle et al., 1995). Recent work seems to suggest that most of p19ARF function is conferred by exon 1β (Lohrum et al., 2000), (Weber et al., 2000). Point mutations that inactivate p16INK4A whilst maintaining p14ARF function are observed in human cancers but p14ARF specific mutations have not yet been identified (Kamb et al., 1994), (Ruas...
and Peters, 1998). However, as oncogenic mutation usually involves loss of the whole INK4a locus it has been difficult to dissect the precise roles of the two cell cycle inhibitors encoded from this locus, but it is clear that both are important tumour suppressors (Kamijo et al., 1997), (Sharpless et al., 2001). In the majority of cases ARF function appears to be mediated by its induction of p53. ARF physically interacts with mdm2 and blocks both mdm2-mediated p53 degradation and transactivational silencing (Pomerantz et al., 1998), (Stott et al., 1998), (Zhang et al., 1998). It was originally suggested that p19ARF stabilizes p53 levels by sequestering mdm2 in the nucleolus and thus inhibiting mdm2-induced p53 degradation in the cytoplasm (Weber et al., 1999), (Roth et al., 1998), (Lohrum et al., 2000). However, recent work has demonstrated that ARF-dependent p53 stabilization does not require relocalization of mdm2 to the nucleolus (Llanos et al., 2001). The ability of ARF to inhibit the mdm2 ubiquitin ligase activity appears to be sufficient to mediate p53 stabilization (Honda and Yasuda, 1999). It has also recently been demonstrated that p53 stabilization may not be essential for ARF to mediate p53 transcriptional activity (Korgaonkar et al., 2002).

ARF is induced in response to various oncogenes including Myc, E1A, activated Ras and v-Abl (Zindy et al., 1998), (de Stanchina et al., 1998), (Palmero et al., 1998), (Radfar et al., 1998) and is essential in mediating the resultant p53-dependent arrest or apoptosis. However, in one study in murine keratinocytes, p53 was induced independently of ARF by oncogenic Raf activation (Roper et al., 2001). That p53 can be activated independently of ARF by oncogenic stimulation has been corroborated in a recent study using an in vivo epithelial model: p53-dependent apoptosis (triggered by selective inactivation of the Rb family by an SV40T mutant) was equivalent in mice expressing or lacking ARF (Tolbert et al., 2002). As ARF is regulated by E2F it acts at the intersection between the Rb and p53 signaling pathways (DeGregori et al., 1997), (Bates et al., 1998). Furthermore, recent work has demonstrated that ARF can act upstream of both the Rb and p53 pathways, inducing an Rb-dependent cell cycle arrest, independently of p53. These findings suggest that ARF may have other targets than mdm2 and p53 (Weber et al., 2000), (Carnero et al., 2000), (Korgaonkar et al., 2002). The primary function of ARF appears to be in mediating p53-dependent oncogenic checkpoints, as its involvement in other p53-dependent checkpoints such as the response to UV or IR is debatable (Kamijo et al., 1997), (Khan et al., 2000).
2.2b: Pathways involved in inducing senescence during stress-induced checkpoints:

**DNA damage:**

DNA damage may be caused by both intrinsic factors (e.g. inaccurate DNA replication and reactive cellular metabolites) and external insults (e.g. ultraviolet, UV and ionizing radiation, IR). The cellular components involved in directly sensing DNA damage are still obscure but details are becoming clear about the downstream pathways (Bartek and Lukas, 2001). Originally it was thought that all G1- DNA damage checkpoints were solely mediated by p53. However, more recent findings have led to the proposal of a "two-wave" model in which the initial rapid DNA response is p53-independent, but then the resultant arrest is subsequently sustained by p53-dependent pathways (Bartek and Lukas, 2001). DNA damage leads to activation of the ATM and ATR kinases that phosphorylate Chk2 and Chkl respectively. Phosphorylation of the Cdc25a by Chkl and 2 targets it for destruction and thus very rapidly inhibits G1 CDK activation (Mailand et al., 2000), (Falck et al., 2001). DNA damage may also stimulate APC mediated cyclin D1 destruction by unknown mechanisms and thus also contribute to initiate arrest by this alternative pathway in some cell types (Agami and Bernards, 2000). Chk1 and 2 can also phosphorylate p53 on serine 20, uncoupling it from Mdm2 and thus resulting in its activation (Chehab et al., 2000), (Hirao et al., 2000), (Shieh et al., 2000). ATM and probably ATR also directly phosphorylate p53 and Mdm2 to activate p53 (Maya et al., 2001), (Zhang et al., 2002). Active p53 instigates transcription of genes involved in mediating cell cycle arrest, primarily p21C^kip1 and thus maintains the arrest initiated by Cdc25a and cyclin D1 degradation. The finding that E2F1 and ARF can also be induced in response to ATM activation could indicate that this pathway may have a role in amplifying the p53 response (Lin et al., 2001), (Khan et al., 2000). A delayed induction of p16INK4A, occurring after the p53 damage response, has also been observed (Pavey et al., 1999), (Robles and Adami, 1998). As yet the significance and mechanism of the induction is undetermined, although in one report it was suggested that p16INK4A may be required to mediate the arrest (Shapiro et al., 1998). The pathways involved in the DNA damage response are summarized in Fig 1.6.
Figure 1.6: The DNA damage checkpoint

A rapid arrest (left-hand side) is induced in response to DNA damage by activation of ATM and ATR kinases which phosphorylate Chk2 and Chk1. These in turn phosphorylate cdc25A (ser123) and Cyclin D1, targeting them for ubiquitination and destruction by the proteosome. The resulting constitutive inhibitory phosphorylation of CDK2 on tyrosine 15, in combination with a redistribution of p21\textsuperscript{Cip1} from CyclinD/CDK4/6 complexes to Cyclin E/CDK2, blocks the G1-S transition. The G1 arrest is sustained by a p53-dependent pathway. Activation of ATM and ATR leads to Chk2/Chk1 dependent phosphorylation of p53 on serine 20 (uncouples from Mdm2). In addition, ATM (and probably also ATR) directly phosphorylates Mdm2 (serine 395) (inhibits) and p53 (serine 15) (activates). The result is transcription of p53 downstream effectors, such as p21\textsuperscript{Cip1}, which maintain the G1 arrest. Maintenance of the arrest may also involve a delayed induction of p16\textsuperscript{INK4A}, which inhibits CyclinD1/CDK4/6 activity, and p19\textsuperscript{ARF}.

Adapted from Bartek and Lucas, 2001.
Figure 1.6: The DNA damage checkpoint
Oxidative stress:

Oxidative stress is induced in response to high levels of ROS (reactive oxygen species) that may be generated intracellularly (e.g. as by-products of aerobic respiration or to function as second messengers) or derived from external sources. Intermediate levels of ROS result in cell cycle arrest whereas higher levels trigger apoptosis or necrosis (Martindale and Holbrook, 2002), (Chen et al., 2000). ROS can directly or indirectly modulate functions of numerous enzymes and transcription factors including ATM, ERK, JNK, p38, PLCY1, PI3K, JAK, AKT, HSF1, NFκB and p53 (Martindale and Holbrook, 2002). The magnitude and duration of oxidative stress in addition to the cell type determine the cellular outcome to this stress. The responses to oxidative stress are thus complex and largely remain obscure. Whether the induction of oxidative stress induced senescence is solely a result of ROS induced DNA damage or involves other ROS responsive signaling pathways remains unclear.

Moderate doses of H₂O₂ induce p53 and p21Cip1 in HDFs and trigger senescence one week after treatment. Higher doses result in greater elevation of p53 levels and trigger apoptosis in the absence of p21Cip1 induction (Chen et al., 2000), (Chen et al., 1998), (Chen and Ames, 1994). Although both Rb and p53 are required for the G1 arrest (abrogation of either allows entry into S phase), inhibition of both pathways cannot restore replicative capacity (Chen et al., 1998). The initial p53 induction is transient but results in a sustained elevation of p21Cip1 and Rb hypophosphorylation (Chen et al., 1998). Interestingly, whilst p53 is not required for establishment of the enlarged senescent phenotype, Rb family members and continued protein synthesis appear to be important (Chen et al., 2000).

Oncogenic stress:

Consistent with the multiple mutations required during tumorigenesis, primary cells are resistant to transformation by a single oncogene (Lloyd, 1998), and, depending on the oncogenic stimulus will either apoptose (e.g. in response to Myc (Evan et al., 1992)) or arrest. This oncogenic checkpoint is believed to be a vital tumour suppressive mechanism. Expression of oncogenic Ras and thus overstimulation of the MAPK pathway in primary cells results in cell cycle arrest that can be accompanied by differentiation (Bar-Sagi, 1995), de-differentiation (M Harrisingh, personal communication) or senescence, depending on cell type. Human and murine fibroblasts
and keratinocytes undergo senescence upon stimulation by oncogenic Ras (Serrano et al., 1997), (Lin and Lowe, 2001), (Farwell et al., 2000).

The Ras-induced arrest has been best characterized in MEFs due to the availability of cells from mouse knockouts. In MEFs, expression of oncogenic Ras is accompanied by elevated levels of p19ARF, p53, p21Cip1, p16INK4A, p15INK4B and the tumour suppressor PML (Palmero et al., 1998), (Serrano et al., 1997), (Malumbres et al., 2000), (Pearson et al., 2000). The senescent arrest is completely dependent on the p53 pathway as fibroblasts lacking p53 or p19ARF, which is required to mediate p53 activation, are resistant to Ras induced arrest (Serrano et al., 1997), (Kamijo et al., 1997), (Palmero et al., 1998). The mechanism by which p19ARF is induced in response to Ras is still unclear but Myc, E2F and DMP1 have all been implicated (Zindy et al., 1998), (Dimri et al., 2000), (Inoue et al., 1999), (Inoue et al., 2000). Recently it has been demonstrated that the PML tumour suppressor can also contribute to Ras-dependent p53 activation. Induction of PML in response to oncogenic Ras results in recruitment of p53 to ternary complexes containing PML and the CBP acetyltransferase in specific nuclear structures termed PML nuclear bodies. This relocalization may promote the acetylation of p53 on lysine 382, a p53-activating event (Pearson et al., 2000). Loss of PML alone is sufficient to render MEFs insensitive to Ras-arrest. Interestingly, in this scenario elevated p53 levels are still observed, but the finding that these levels are insufficient to mediate the arrest would suggest that PML-mediated p53 activation as well as p53 stabilization is required (Pearson et al., 2000). Although, activated p53 transcriptional activity is required for the Ras arrest, its transcriptional target p21Cip1 is not and therefore other p53 inducible genes must be involved in mediating the Ras arrest (Pantoja and Serrano, 1999). The role of the regulators of the Rb pathway in mediating oncogenic checkpoints is less clear. Although loss of Rb alone is not sufficient to overcome arrest, (as demonstrated in MEFS from the Rb knockout mouse), this may be due to functional compensation by other Rb family members, as cells lacking Rb and p107 are resistant to Ras arrest (Peeper et al., 2001). Of the INK4 family of CDKIs p15INK4B is required for the arrest (Malumbres et al., 2000) whereas p16INK4A is not (Sharpless et al., 2001), (Krimpenfort et al., 2001). The mechanism by which these INK4 inhibitors are induced is unknown. Interestingly, in the context of a high intensity Raf signal in MEFs the resultant arrest is dependent on p21Cip1 which is upregulated in a p53 independent manner. The mechanism of p21Cip1 induction in this
case is unclear, but may be mediated by the E2F1 or Sp1 and Sp3 transcription factors (Woods et al., 1997), (Kivinen et al., 1999), (Gartel et al., 2000).

The mechanism of the Ras-induced arrest in HDFs seems to differ significantly from that in MEFs. In MEFs, the p53 pathway plays a primary role in mediating the arrest whereas in HDFs this pathway appears to be less important. Although in many reported cases p53 levels increase upon oncogenic Ras expression in HDFs, loss of p53 function is not sufficient to overcome the arrest (Serrano et al., 1997), (Lin et al., 1998), (Wei et al., 2001) (it should be noted that upregulation of p53 is controversial - see Brookes et al 2002). In addition, elevation of ARF is not observed, suggesting that p53 activation is not mediated by the ARF-Mdm2 pathway (Wei et al., 2001), (Brookes et al., 2002). It is unclear how p53 is activated, but PML may also play a role in Ras-dependent activation of p53 in human cells. However, in HDFs, Ras-induction of PML seems to promote phosphorylation of p53 on serine 15 rather than acetylation (Ferbeyre et al., 2000). The elevated levels of p21\textsuperscript{Cip1} are dependent on p53 and, consistent with the susceptibility of p53-/− cells to Ras-induced arrest, loss of p21\textsuperscript{Cip1} is also not sufficient to prevent Ras-induced senescence (Wei et al., 2001), (Zhu et al., 1998). The role of p16\textsuperscript{INK4A} in Ras arrest in HDFs is controversial. Initial results in primary HDFs suggested that, as for p53, abrogating the function of this tumour suppressor was not sufficient to overcome the Ras-arrest (Serrano et al., 1997). However a very recent study in human fibroblasts derived from a melanoma patient lacking p16\textsuperscript{INK4A} expression demonstrated that these cells are resistant to Ras-induced arrest (Brookes et al., 2002). It is unclear why there is a discrepancy in these results but, although the cells from the melanoma patient appeared to have functional p53 and ARF, other cooperating mutations cannot be excluded. Whatever the result of this debate, it is clear that abrogation of both the Rb and p53 pathways prevents oncogenic Ras induced arrest (Serrano et al., 1997). Recent work has contributed to a greater understanding of the signals connecting Ras with p16\textsuperscript{INK4A} induction. The Ets transcription factors and their negative regulators the Id proteins have been implicated in p16\textsuperscript{INK4A} regulation. In primary HDFs p16\textsuperscript{INK4A} transcription is maintained at low levels because Id1 inactivates the Ets1/2 transcription factors. An oncogenic Ras signal leads to constitutive phosphorylation and thus activation of Ets2 that overrides the inhibitory effect of Id 1 and leads to elevated p16\textsuperscript{INK4A} levels (Ohtani et al., 2001) (Fig 1.8). In addition, the observation that PML overexpression leads to elevated p16\textsuperscript{INK4A} levels and senescence
could suggest that this tumour suppressor may also contribute to Ras induced p16^{INK4A} upregulation (Ferbeyre et al., 2000).

Oncogenic Ras expression in murine keratinocytes induces a senescent phenotype (Lin and Lowe, 2001), (Paramio et al., 2001). The findings of Lin and Lowe would suggest that the pathways involved resemble those employed in Ras arrest in MEFs: the arrest is accompanied by induction of p19^{ARF}, p53, p21^{Cip1} and p16^{INK4A} and abrogation of the p53 pathway by loss of p19^{ARF} or p53 is sufficient to overcome arrest. These findings were contradicted in a study by Paramio and colleagues in which they demonstrated that loss of the INK4 locus (i.e p16^{INK4A} and p19^{ARF}) was not sufficient to overcome the Ras-arrest. Additional loss of p21^{Cip1} was also required. Therefore in this study the checkpoint mechanism appears to differ from that in MEFs. Characterizing the pathways involved has been further complicated by the finding that oncogenic stimulation of Raf, the downstream effector of Ras in the MAPK pathway, results in a p53-dependent arrest which in this case appears to be p19^{ARF} independent (Roper et al., 2001).

In summary, activation of checkpoints can initiate arrest or apoptosis by employing common cell cycle regulators (p53, ARF, CDKIs). The cellular outcome and the cell cycle components involved vary depending on stimulus, cell type and context. In situations where a G1 arrest is elicited and maintained, it is usually accompanied by a senescent phenotype. In fact, recently it has been shown that directly triggering a G1 arrest by overexpression of CDKIs in HDFs is sufficient to induce an irreversible senescent phenotype (McConnell et al., 1998), (Dai and Enders, 2000).

2.3: Pathways involved in senescence induced by division of human cells in culture:

2.3a: Senescence triggered by telomere shortening:

The finding that HDFs can be immortalized by telomerase expression alone indicated that signals induced by short telomeres are responsible for triggering replicative senescence. Examination of the cell cycle regulators commonly involved in mediating checkpoints has contributed to our understanding how the telomere-
dependent arrest is induced but the signals connecting short telomeres with these cell cycle regulators are less well characterized.

Analysis of cell cycle regulators in HDFs approaching replicative senescence revealed a transient elevation of p21\(^{Cip1}\) in these cells that was followed by elevated p16\(^{INK4A}\) levels (Alcorta et al., 1996), (Hara et al., 1996). Although p53 levels do not appear to change as HDFs near the end of their replicative lifespan, specific activating modifications of the p53 protein become evident; phosphorylation of ser-15, thr-18 and ser-376 increases whereas ser-392 becomes dephosphorylated and modification by poly (ADP-ribose) polymerase becomes evident (Vaziri et al., 1997), (Webley et al., 2000). Activation of p53 rather then stabilization is consistent with the finding that ARF levels do not change as cells near senescence (Wei et al., 2001). This mode of p53 stabilization is reminiscent of the mechanism employed during Ras-induced premature senescence of HDFs (see section 2.2b). Also reminiscent of Ras induced premature senescence, abrogation of both the Rb and p53 pathways, but not either one alone, allows significant extension of the proliferative lifespan of HDFs (Shay et al., 1991), (Hara et al., 1991). However, these cells are not completely immortal and, after continued telomere shortening, ultimately enter a state termed "crisis" where continued division is balanced by high levels of cell death and thus there is no net increase in cell number. Crisis is accompanied by high levels of genetic instability, presumably caused by critical telomere shortening (Fig 1.7). Cells that emerge from crisis and are able to continue proliferating are all found to have stabilized their telomeres (Counter et al., 1992), (Wright et al., 1989). It has been reported that loss of p21\(^{Cip1}\) alone is sufficient to extend lifespan of HDFs until they reach crisis (Brown et al., 1997). However, as generation of the p21\(^{Cip1}\) /- HDFs used in this study was a lengthy process during which selection for further mutation may have occurred, it is difficult to interpret these results. Although it is clear that Rb is important in mediating replicative senescence (Hara, 1991), the role of p16\(^{INK4A}\), a regulator of the Rb pathway is less clear. Human fibroblasts naturally lacking p16\(^{INK4A}\) expression appear to show some extension of replicative lifespan (Brookes et al., 2002). It has been generally assumed that in HDFs lacking p53, the senescent arrest is mediated by the elevated levels of p16\(^{INK4A}\). However, in p21\(^{Cip1}\) /- HDFs p16\(^{INK4A}\) upregulation is maintained, but these levels are insufficient to trigger a senescent arrest (Wei et al., 2001). A recent study using CDK4/6 mutants incapable of binding p16\(^{INK4A}\) has suggested that p16\(^{INK4A}\) is not responsible for activating the Rb.
Figure 1.7: Telomere shortening determines proliferative capacity in HDFs.

HDFs do not express telomerase and so their telomeres progressively shorten. Short telomeres trigger entry into senescence. Senescence can be overcome by inactivation of both the Rb and p53 pathways, allowing continued proliferation and telomere shortening. Eventually, critically short telomeres induce a period of genetic instability and increased cell death termed crisis. Exogenous expression of telomerase at any stage in this progression is sufficient to confer immortality.

pathway during senescent arrest (Morris et al., 2002). If this is indeed the case, it remains to be established how the Rb pathway is activated in the absence of p53 to trigger senescence. One possibility that has been suggested is that a Cdc25 dependent pathway, similar to that observed in the first wave of the DNA-damage response (see section 2.2b) could be important (Morris et al., 2002).

One commonly favoured hypothesis is that, as telomeres shorten, and the probability that they will become uncapped increases (Blackburn, 2000), the uncapped telomeres are perceived as damaged DNA, triggering a DNA-damage response that results in the senescent arrest. This is consistent with the recent finding that it is telomere uncapping and thus exposure of chromosome ends, rather than telomere shortening per se which seems to induce replicative senescence (Karlseder et al., 2002). This hypothesis has been further strengthened by recent investigations into the effects of artificially-induced-telomere uncapping: the finding that telomere capping is dependent on maintenance of an intact t-loop structure mediated by TRF2 (see section 1.2b) has allowed investigations of the cellular response to uncapping induced by a TRF2 dominant negative (de Lange, 2002). Much like DNA damage, introduction of dnTRF2 results in different outcomes depending on cell type. In primary lymphocytes, ATM-p53 dependent apoptosis occurs (Karlseder et al., 1999). This dependency on ATM is again consistent with uncapped telomeres triggering a DNA damage response. In human fibroblasts dnTRF2 induces a senescence response that is phenotypically indistinguishable from replicative senescence (Karlseder et al., 1999). Closer analysis revealed that the mechanism of dnTRF2-induced arrest paralleled that of replicative senescence as p53, p21^{Cip1} and p16^{INK4A} are upregulated and the arrest is mediated either by the Rb pathway or by p53: abrogation of both pathways is required to overcome the arrest (Smogorzewska and de Lange, 2002). Interestingly, unlike the apoptotic response in lymphocytes, senescence and p53 activation was not entirely dependent on ATM (Smogorzewska and de Lange, 2002). It is possible that in this context other known damage sensors such as ATR may be required to cooperate to transduce the signal from telomeres to p53.

The finding that p16^{INK4A} is induced in this system might suggest that elevated p16^{INK4A} in senescent HDFs is likely to be a direct response to a telomere signal. Although experiments with dnTRF2 have not provided any clues to the signals connecting p16^{INK4A} upregulation with short telomeres, recent studies into regulation of
the p16INK4A promoter have suggested that these signals are likely to differ from those activated in response to oncogenic Ras. Oncogenic Ras appears to upregulate p16INK4A via constitutive activation of the Ets2 transcription factor. However, during telomere shortening and replicative senescence, a progressive upregulation of Ets1 and a concomitant downregulation of its negative regulator Id1 is more likely to be responsible for the elevated p16INK4A levels. The signals that determine the balance between Ets and Id levels and how they vary with replicative age, remain to be established (Ohtani et al., 2001), (Zebedee and Hara, 2001) (Fig 1.8).

The expression of dnTRF2 in wild type MEFs resulted in senescence. However, in murine cells derived from telomerase knockout mice (see section 1.3b) that are forced to undergo critical telomere shortening by extensive proliferation (after overcoming telomere-independent senescence), apoptosis is induced (Chin et al., 1999). This cellular response to critical telomere shortening is likely to be analogous to crisis in HDFs. The senescent response to dnTRF2 expression was found to be solely dependent on the p53 pathway (Smogorzewska and de Lange, 2002). Although critical telomere shortening is extremely unlikely to occur naturally in a murine lifespan, it is interesting to note that murine cells appear to be intrinsically more dependent on p53 pathways to mediate checkpoint responses than human cells in which p53 is required but is not sufficient to mediate the arrest.

The use of dnTRF2 may provide a valuable tool to further investigate the pathways involved in replicative senescence. However, it is still unclear whether this system can be considered the equivalent to replicative senescence. The finding that the p53 modifications involved in replicative senescence are similar but not identical to those occurring during a DNA damage response (Webley et al., 2000) suggests that these two checkpoints are not equivalent. An investigation of the p53 modifications occurring during the dnTRF2 response has not yet been published.

In summary, telomere-induced replicative senescence in HDFs seems to resemble aspects of both the checkpoint arrests caused by DNA-damage and oncogenic Ras. The similarities and differences in regulation of the cell cycle during Ras induced or DNA-damage induced premature senescence and telomere-induced senescence are illustrated in Table 1.3.
Young HDFs Premature senescence Induced by activated Ras

Figure 1.8: Regulation of $p16^{INK4A}$ by Ets and Ids during senescence

A: In young HDFs $p16^{INK4A}$ is maintained at low levels because its positive regulator, Ets2 is inhibited by Id1

B: Expression of oncogenic Ras leads to phosphorylation of Ets2, resulting in increased levels and activity, that overrides the negative effect of Id1. Therefore, $p16^{INK4A}$ is induced.

C: As HDFs approach their replicative limit levels of Ets1 are progressively upregulated by an unknown signal, and levels of Id1 decline. The result is $p16^{INK4A}$ induction.

Adapted from Zebedee and Hara, 2001.
2.4: Pathways involved in senescence induced by division of cultured murine cells:

Unlike human fibroblasts, where the trigger for senescence is clear (i.e telomere shortening), the stimulus in murine cells is still unknown. However, the availability of MEFs derived from mouse knockouts and characterization of their replicative capacities, has enabled extensive investigation into the cell cycle regulators involved in mediating the senescent arrest in murine cells.

Entry into senescence of cultured MEFs is accompanied by increases in p53 (Mendrysa and Perry, 2000), p19ARF (Zindy et al., 1998) and p16INK4A (Zindy et al., 1997), (Kamijo et al., 1999). However, p21cip1 levels do not increase (Pantoja and Serrano, 1999). The proliferative arrest is strongly dependent on the p19ARF-p53 pathway, as p53/- and p19ARF/- MEFs (Kamijo et al., 1997) do not senesce in culture and the majority of spontaneously immortalized MEF lines mutate p53, whereas the remainder lose expression of the INK4a locus and thus p19ARF function (Kamijo et al., 1997). However, the senescent arrest must involve other downstream p53 targets than p21cip1 as p21cip1/- MEFs display wild type characteristics (Pantoja and Serrano, 1999). Although p15INK4B plays a significant role in Ras induced premature senescence in MEFs, it does not seem to be important for limiting lifespan in culture (Latres et al., 2000). Similarly, p16INK4A does not play a primary role, as senescence is not averted in MEFs from the p16INK4A knockout (Krimpenfort et al., 2001), (Sharpless et al., 2001). However, consistent with findings from p16INK4A-antisense studies, these cells appear to spontaneously emerge from senescence at a higher frequency than wild type cells and, in a minority of cases p19ARF-p53 pathways remain intact (Sharpless et al., 2001), (Carnero et al., 2000). These results raise the possibility that an unidentified factor can cooperate with p16INK4A to mediate senescence via the Rb pathway, independently of the p53 pathway. That the Rb pathway is important in mediating the senescence arrest has been demonstrated by the lack of senescence in MEFs deficient in all three Rb family members (Sage et al., 2000). The maintenance of a senescent arrest in MEFs from the Rb single knockout suggests that the other Rb family members can functionally compensate for Rb to mediate senescence in culture (Sage et al., 2000). Intriguingly p19ARF may operate upstream of both the Rb and p53 pathways during senescence as arrest can be induced by reexpression of p19ARF in p19ARF/-p53/-, but not in p16INK4A/-
Rb-/− MEFs (Carnero et al., 2000). The most commonly held view is that DNA damage response checkpoints in MEFs do not require p19ARF function and thus differ from culture-induced senescence in this respect (Kamijo et al., 1997) but see also (Khan et al., 2000). However, other findings suggest similarities between DNA damage responses and senescence of cultured MEFs: loss of proteins required for DNA repair (e.g. ATM) accelerate senescence presumably due to increased levels of DNA damage. Consistent with this hypothesis, p53-/−; ATM-/− cells in which the checkpoint response to DNA damage has been abrogated are immortal, despite impaired DNA repair mechanisms (Xu and Baltimore, 1996), (Westphal et al., 1997). A comparison of checkpoint responses involve in DNA damage, expression of oncogenic Ras and senescence in cultured MEFs is given in Table 1.4.

Although most studies on murine replicative lifespan have been carried out in cultured MEFs, it is important to appreciate that there are likely to be differences in other cell types. For example, senescence in cultured mouse bone marrow derived macrophages appears to depend on p16INK4A (Randle et al., 2001).
Table 1.3: Comparison of G1 checkpoints in HDFs: All references are provided in the text unless otherwise specified within the table.

<table>
<thead>
<tr>
<th>Associated with induction of:</th>
<th>TELOMERE INDUCED SENESCENCE</th>
<th>DNA-DAMAGE INDUCED SENESCENCE</th>
<th>ONCOGENIC RAS-INDUCED SENESCENCE:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Role of p53</td>
<td>- p53 activation but not stabilization (acetylation and phosphorylation)</td>
<td>- p53</td>
<td>- p53 (Serrano et al., 1997)</td>
</tr>
<tr>
<td>Hara et al, 1991;</td>
<td>- No increase in ARF</td>
<td></td>
<td>- No increase in ARF</td>
</tr>
<tr>
<td>Serrano et al, 1997;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DiLeonardo et al, 1994</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Role of Rb</td>
<td>- E1b or E6: Arrest</td>
<td>- p53-/-: No arrest</td>
<td>- dn p53: Arrest</td>
</tr>
<tr>
<td>Shay et al, 1991;</td>
<td>- p53 antisense: Arrest</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hara et al, 1991;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serrano et al, 1997</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Role of Rb +p53</td>
<td>- E1a or E7: Arrest</td>
<td>?</td>
<td>- Inhibition Rb pathway (cyclin D1 and constitutively active CDK4 overexpression); Arrest</td>
</tr>
<tr>
<td>Shay et al, 1991;</td>
<td>- Rb antisense; Arrest</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hara et al, 1991;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serrano et al, 1997</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Role of ARF</td>
<td>- p53-/-; Rb -/-: No arrest</td>
<td>- p53-/-; Rb -/-: No arrest</td>
<td>- p53-/-; Rb -/- (E1a): No arrest</td>
</tr>
<tr>
<td>Wei et al, 2001;</td>
<td>(antisense Rb and p53/SV40LT/ E1A+E1B/E6+E7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brookes et al, 2002</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Role of p21</td>
<td>NOT INVOLVED</td>
<td>?</td>
<td>NOT INVOLVED</td>
</tr>
<tr>
<td>Brown et al, 1997;</td>
<td>- p21-/-: No arrest (see text for caveats)</td>
<td>- p21-/-: No arrest</td>
<td>- p21-/-: Arrest</td>
</tr>
<tr>
<td>Wei et al, 2001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Role of p16</td>
<td>- p16 -/-: Arrest but extended lifespan</td>
<td>?</td>
<td>- p16 -/-: No Arrest</td>
</tr>
</tbody>
</table>

E1a binds Rb family and disrupts p53-dependent p21 transcription; E1b binds p53 family; E7 inhibits Rb; E6 binds p53 family; SV40LT binds Rb and p53 family.
Table 1.4: Comparison of G1 checkpoints in MEFs: All references are provided in the text unless otherwise specified within the table.

<table>
<thead>
<tr>
<th>SENESCENCE INDUCED BY DIVISION IN CULTURE</th>
<th>DNA DAMAGE-INDUCED SENESCENCE:</th>
<th>ONCOGENIC RAS-INDUCED SENESCENCE:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Associated with induction of:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Role of p53</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kamijo et al, 1997; Serrano et al, 1997</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- p53-/-: No arrest</td>
<td>- p53-/-: No arrest</td>
<td>- p53-/-: No arrest</td>
</tr>
<tr>
<td>Role of Rb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sage et al, 2000; Peeper et al, 2001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Rb-/-: Reduced arrest</td>
<td>- Rb-/-: Reduced arrest</td>
<td>- Rb-/-: Reduced arrest</td>
</tr>
<tr>
<td>- Rb-/-; p107-/-: p130-/-: No arrest</td>
<td>- Rb-/-; p107-/-; p130-/-: No arrest</td>
<td>- Rb-/-; p107-/-: No arrest</td>
</tr>
<tr>
<td>Role of Rb +p53</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- p53-/-; Rb -/-: No arrest</td>
<td>- p53-/-; Rb -/-: No arrest</td>
<td>- p53-/-; Rb -/-: No arrest</td>
</tr>
<tr>
<td>Role of ARF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kamijo et al, 1997</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- p19-/-: No arrest</td>
<td>- p19-/-: Arrest (Kamijo et al)</td>
<td>- p19-/-: No arrest</td>
</tr>
<tr>
<td>- p19-/-: No arrest (Khan et al; 2000)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Role of p21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pantoja et al, 1999; Brugarolas et al, 1995</td>
<td>- p21-/-: Arrest</td>
<td>- p21-/-: No arrest</td>
</tr>
<tr>
<td>Role of p16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Krimpenfort et al, 2001; Sharpless et al, 2001</td>
<td>- p16 -/-: Arrest</td>
<td>- p16 -/-: Arrest</td>
</tr>
</tbody>
</table>
3: Introduction to this thesis:

It has been generally accepted that primary somatic cells possess a finite replicative limit in culture. In keeping with Hayflick's original hypothesis primary somatic cells were found to senesce in culture and required acquisition of immortalizing mutations in order to divide indefinitely. It was well established that in telomerase negative human fibroblasts, the mechanism responsible for limiting replicative lifespan was telomere shortening. Exogenous expression of telomerase was sufficient to immortalize these cells in the absence of any other changes. However, it was unknown what mechanism was responsible for inducing senescence in rodent cells that naturally express telomerase and in certain human cell types that senesced despite artificial TERT expression. A popular hypothesis was that in these cells an uncharacterized intrinsic mitotic counter was linked to progressive accumulation of CDKIs, particularly p16\textsuperscript{INK4A}, which ultimately causes the senescence arrest. It was commonly assumed that senescence operated as an important intrinsic self-limiting mechanism in all primary somatic cells and therefore that it was likely to play an important role in vivo.

In this thesis we investigated the mechanisms regulating proliferative lifespan in telomerase expressing rodent cells. We found that primary rat Schwann cells, naturally expressing telomerase can divide indefinitely in culture in the absence of any genetic changes and thus demonstrated that not all primary somatic cells have intrinsic replicative limits. Furthermore, we found that, although no intrinsic mechanisms limit proliferation in this cell type, rat Schwann cells senesce when cultured in altered conditions. This finding provides evidence that extrinsic factors are as likely as intrinsic mechanisms to regulate proliferative capacity in telomerase expressing cells. The progressive upregulation of CDKIs that occurs in both telomerase positive and negative cultured cells has been proposed to be linked to an intrinsic mitotic counter which ultimately determines proliferative capacity. We found that CDKIs are also upregulated in rat Schwann cells despite their unlimited replicative potential. However, our finding that p16\textsuperscript{INK4A} induction can be uncoupled from population doublings demonstrated that p16\textsuperscript{INK4A} induction in rat Schwann cells is not linked to an intrinsic timer. The findings presented in this thesis therefore demonstrate the importance of extrinsic factors in regulating the replicative capacity of cultured cells and provide a basis for further
studies to investigate what these factors are and how they signal to induce cellular senescence.
Chapter 2: Materials and Methods

All cell culture reagents were from Gibco BRL and all other reagents from Sigma unless otherwise stated.

2.1: Isolation of pure cell cultures:

Dissection:
Schwann cells and perineural fibroblasts were isolated from postnatal day 7 Sprague Dawley rats (Cheng et al., 1995): sciatic nerves were dissected from 20 rats under sterile conditions. Nerves were minced and then incubated in trypsin (0.025%)/DNase (0.004%)/collagenase (0.1%) in EBSS (37°C). After 35 mins the enzymatic reaction was inhibited by addition of 10%FCS (Autogen Bioclear). Cells were pelleted, resuspended in L-15, 0.1%BSA and filtered through sterile 20μm nylon gauze (UV sterilized). The filtered cell suspension was sequentially applied to immunopanning dishes.

Purification:
Immunopanning dishes were prepared as described in (Cheng et al., 1995). Single cell suspensions were incubated in panning dishes coated with OX42 (1μg/ml; Sera-Lab Ltd) (30 mins, room temperature) to remove contaminating macrophages. The unbound population was then sequentially incubated on 3 dishes coated with OX7 mAb (0.9μg/ml) (Cheng et al., 1995) (30mins, room temperature each). Perineural fibroblasts remaining bound to the OX7 dishes were incubated overnight (37°C, 10% CO₂) in normal fibroblast medium (see below). The Schwann cell suspension resulting from the immunopanning procedure was pelleted, resuspended in the appropriate Schwann cell medium and plated on Poly-D-Lysine (PDL, 2.4μg/ml) coated dishes (see below) and incubated overnight (37°C, 10% CO₂).
2.2: Cell culture:

Coating dishes:

Poly-D-Lysine (PDL)
Plastic tissue culture dishes (Nunc) or baked glass coverslips were incubated with PDL solution (2.4μg/ml in sterile water) for 15 minutes at room temperature. Plates were then washed three times in sterile water before leaving to air dry.

Laminin
Dishes or coverslips that had previously been PDL coated were incubated with laminin solution (50μg/ml in medium) for 2 hours. Plates were washed once with medium before the addition of cells.

Schwann cells in 3% serum:
Schwann cells were cultured on PDL (2.4μg/ml) coated dishes in DMEM with 1000mg/ml glucose, supplemented with 3% charcoal stripped foetal calf serum (FCS, Autogen Bioclear), 4mM glutamine (ICN), kanomycin (100μg/ml), gentamycin (2μg/ml), forskolin (1μM, Calbiochem) and GGF (20ng/ml, R&D systems). Cells were maintained in a humidified incubator at 37°C, 10% CO₂, 20% O₂. Cells were passaged every three days (before reaching confluence) by trypsinizing and replating at 4.8x10⁵ cells per 9cm dish. Cells were counted using a Coulter counter. Cells plated after the first trypsinisation after isolation were defined as Passage 1.

Schwann cells in 20% serum:
Cells were cultured essentially as described above, but in medium containing 20% FCS. Cells were also passaged every three days, as described above.

Schwann cells in SATO:
Schwann cells were isolated in the complete absence of serum, using trypsin inhibitor. Cells were cultured on plates coated with PDL (2.4μg/ml) and laminin (50μg/ml, Sigma). Cells were grown in SATO (DMEM with glucose supplemented with 100μg/ml BSA, Gibco BRL, 60ng/ml progesterone, 16μg/ml putrescine, 50ng/ml thyroxine, 50ng/ml triiodothyronine, 40ng/ml selenium, 10μg/ml insulin (Gibco BRL) and 100μg/ml transferrin (Bottenstein et al., 1979). Transferrin and insulin were freshly added to medium just before use. SATO medium was supplemented with forskolin (1μM, Calbiochem) and GGF (20ng/ml, R&D systems). The GGF was added the day after the cells had been trypsinized and re-plated. Cells were trypsinized once a week, counted with a Coulter Counter and replated at 1x10⁶ cells per 9cm dish. Trypsinization
was carried out using trypsin inhibitor (0.25mg/ml, Boehringer Mannheim), in DMEM, 1% BSA (Gibco BRL) to avoid exposure to serum. Cells treated with trypsin inhibitor were centrifuged before resuspending in SATO medium and replating.

**Perineural fibroblasts:**
Perineural fibroblasts were cultured on plastic in DMEM supplemented with 10% FCS (Autogen Bioclear), 4mM glutamine (ICN), kanomycin (100μg/ml), gentamycin (2μg/ml). Cells trypsinized off the panning antibody were defined as Passage 1. Cells were trypsinized every three days and were replated at 5.0x10^5 cells per 9cm dish.

**GP+E producers and Human Mammary Fibroblasts**
These cells were cultured under the same conditions as the rat perineural fibroblasts. HMFs were passaged every five days, GP+Es were passaged at confluence.

**T24 Hybridoma:**
T24 hybridoma (for production of α-mouse Thy1.1) was cultured in suspension in flasks in RPMI medium supplemented with 10% foetal calf serum. Supernatant was collected, filter sterilized and stored at 4°C.

**Producing Schwann cell clones:**
5x10^3 primary Schwann cells at different passages, or Schwann cells expressing dominant negative p53 (from Alison Lloyd) (Lloyd et al., 1997), were seeded onto PDL and laminin-coated 9cm dishes in 3% serum Schwann cell medium (see above), supplemented with 20% filtered conditioned medium obtained from confluent Schwann cell cultures. Numbers (mean of counts from triplicate plates) of colonies at the two-cell stage were counted to determine plating efficiency. Colonies were isolated by trypsinization using cloning rings and transferred to 1cm dishes. These clones were then expanded as described above.

### 2.3: Proliferation assays:

**Adapted 3T3 protocol**
Schwann cells and fibroblasts were seeded on triplicate plates at 4.8x10^5 and 5.0x10^5 cells per 9cm dish respectively. Every three days, triplicate plates were trypsinized and the cell number for each plate counted using a Coulter counter. The fold increase in cell number at each passage was calculated.
BrdU incorporation

Cells seeded on PDL and laminin coated coverslips were incubated with bromodeoxyuridine (BrdU) (10μM) for differing time periods (cumulative label) or for 16 hours (Schwann cells) and 9 hours (fibroblasts) for a pulse label. Cells were then fixed (3.7% formaldehyde, 15mins) and permeabilized (2M HCL, 0.5% Triton X-100, 30mins). BrdU incorporation was visualized by direct immunofluorescence (see below), staining with anti-BrdU antibody (RPN202, Amersham, 1/20 dilution, 1 hour, room temperature) followed by Cy3-conjugated anti-mouse secondary (Jackson Immunoresearch, 1/200 dilution, 1 hour, room temperature) and Hoescht label (see below). The percentage of nuclei that had incorporated BrdU was calculated by counting at least 500 cells on triplicate coverslips.

Tritiated thymidine incorporation:

Triplicate samples of cells were pulse labeled with ^3H-thymidine (0.5μCi/ml, Amersham) for four hours. Cells were then lysed with 1%SDS. DNA was precipitated by addition of 15% ice cold TCA and then incubation for at least 5 minutes on ice. Lysates were transferred to filter paper using a vacuum pump. Filters were repeatedly washed with ice-cold 5% TCA and finally with absolute ethanol. Dried filters were transferred to scintillation vials for counting on a liquid scintillation counter (Packard).

2.4: Immunofluorescence and cell staining

Microscopy:

Phase contrast pictures of cells were taken using an inverted microscope (Leica) and Openlab software. Immunofluorescence was visualized using a Zeiss Axioscope microscope.

Immunofluorescence for Schwann cell and fibroblast markers.

Schwann cells were grown on glass coverslips coated with PDL and laminin. Fibroblasts were grown directly on glass coverslips. Cells were fixed in 4% paraformaldehyde (10 minutes, room temperature). Cells were incubated with primary antibody diluted in 4% FCS, 0.1% azide in PBS for 1 hour at room temperature. Antibodies used were: anti-S-100 (diluted 1/400, Charles River PharmserVICES); anti-NGF (diluted 1/4, gift from Anne Mudge (Cheng et al., 1995); anti-Thy1.1 (Ox7 supernatant)(diluted 1/2, gift from Anne Mudge (Cheng et al., 1995)). Coverslips were
washed in PBS (x9) and incubated with appropriate fluorescence-conjugated secondary antibodies (see table 2.1)(1 hour, room temperature, diluted 1/200). Cells were simultaneously stained with Hoescht to visualize nuclei (see below). All secondary antibodies were from Jackson Immunological Research labs. Coverslips were washed in PBS (x9) before a final fixation stage (5% acetic acid in ethanol, 10 minutes, -20°C). Coverslips were washed a further 9 times and then mounted in Citifluor (Citifluor). The staining protocol for S-100 required an additional permeabilization step (10 minutes, ice cold methanol, -20°C) before incubation with primary antibody. In all cases, controls in which the primary antibody had been omitted were included. For double labeling, staining for cell-surface antigens was performed first and then followed by the protocol for the intracellular antigen (S-100). To identify percentages of cells positively labeled for antigenic markers, more than 200 cells were counted on each of triplicate coverslips.

**Immunofluorescence for p53**

Cells grown on PDL and laminin coated glass coverslips were fixed in ice cold methanol (10 minutes, -20°C) and otherwise were stained essentially as described above. The primary antibody was anti-p53, 421, Calbiochem, diluted 1/20.

**Staining cells for senescence associated β-Galactosidase activity**

Cells were washed in PBS (x2) before fixation (2% formaldehyde/0.2% glutaraldehyde in PBS, 5 minutes, room temperature). Cells were washed (3x with PBS) and incubated with β-galactosidase staining solution (40mM sodium citrate, 40mM sodium phosphate, 5mM potassium ferrocyanide, 5mM potassium ferricyanide, 150mM sodium chloride, 2mM Magnesium chloride, 1mg/ml X-gal, pH adjusted to 6.0 with HCl) at 37°C in the dark for 8-10 hours. Incubation times were optimized to prevent background staining.

**Visualizing nuclei using Hoescht dye.**

Cells were fixed (in 4% paraformaldehyde for 10 minutes) and permeabilized (in 0.5% Triton in PBS for 5 minutes) before incubating with Hoescht dye (0.3μg/ml in PBS, 1 hour). Cells were washed and mounted as described above.

**Metaphase spreads**

Cells were seeded on 6cm dishes. At the exponential growth phase demecolcine was added (0.05μg/ml). After a 2 hour incubation cells were trypsinized and gently centrifuged in nuncatron (Nunc) coated centrifuge tubes (500rpm, 20 minutes). Cells were carefully resuspended in 0.075M (0.56%) warm KCl and incubated at room temperature for 12 minutes to allow cell swelling. Cells were then gently centrifuged for
5 minutes (500rpm) and then carefully resuspended in freshly made, ice cold, Carnoy's fixative (3:1 dried methanol: glacial acetic acid) and incubated for 30 minutes at room temperature. Cells were carefully washed twice in fixative before being resuspended in a small volume of fixative (300μl). 20μl cell suspension was dropped onto ultra clean superfrost slides (BDH) held at an angle, from a height of >40cm. Slides were dried and then washed in 70% acetic acid. Spreads were mounted in Vectashield (contains DAPI) and analysed by fluorescence microscopy. More than 40 spreads were counted for each of triplicate slides.

2.5: Western Blotting

**Protein extraction:**
Cells were washed twice in ice cold PBS before scraping into 1ml PBS. Cells were pelleted in a chilled microcentrifuge (Hereaus, 1 minute, 13 000rpm) and resuspended in 1N lysis buffer (Table 2.2). After 15 minutes on ice (with vortexing) lysates were cleared by centrifugation and protein concentration determined using a Bradford assay (BioRad) at OD 595nm. Lysates were equalized for protein concentration, combined with sample buffer (see Table 2.2) and boiled for 5 minutes.

**Protein separation:**
30μg of each protein sample was resolved by SDS-PAGE on 15% gels. Proteins were transferred to PVDF membranes (Immobilon P. Millipore), which were blocked overnight at 4°C. See Table 2.2 for Running buffer, Transfer buffer and Block.

**Protein detection:**
Membranes were incubated with primary antibody, diluted to the required concentration (see Table 2.1) in block for 1 hour at room temperature. Membranes were washed (3x 5 minutes) in PBST or TBST, depending on the stringency required (see Table 2.1), before incubation with HRP-conjugated secondary antibody (Amersham)(diluted 1/200 in block, 1 hour room temperature). Washes were repeated (including an additional final wash in PBS/TBS only) before developing the blot (5 minute incubation in ECL, Amersham (normal or plus depending on antibody (see Table 2.1)), room temperature). Blots were exposed to film (Biomax ML, Kodak), which was developed in an Agfa automatic processor.
Table 2.2: Solutions used for protein extraction and western blotting:

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>1N lysis buffer</td>
<td>1% NP-40, 150mM NaCl, 50mM Tris pH 8, 20mM NaF, 100μg/ml PMSF, 15μg/ml aprotinin and 1mM Na₃VO₄.</td>
</tr>
<tr>
<td>4x sample buffer</td>
<td>200mM, Tris pH 6.8, 8% SDS (BioRad), 40% glycerol, 400mM DTT, 0.25% Bromophenol blue.</td>
</tr>
<tr>
<td>10x Running Buffer</td>
<td>2.5M Glycine (BDH), 250mM Tris 1% SDS</td>
</tr>
<tr>
<td>10x Transfer buffer</td>
<td>200mM Tris 1.5M glycine, 20% methanol (BDH)</td>
</tr>
<tr>
<td>Milk Block</td>
<td>5% milk, 0.05% Tween-20 (BioRad) in PBS</td>
</tr>
<tr>
<td>Stripping buffer</td>
<td>200mM glycine pH 2.5, 0.4% SDS</td>
</tr>
<tr>
<td>1x PBS Tween wash</td>
<td>0.05% Tween-20 in PBS</td>
</tr>
<tr>
<td>20x TBS Tween</td>
<td>200mM Tris pH 8, 3M NaCl, 1% Tween-20</td>
</tr>
<tr>
<td>PBS</td>
<td>137mM NaCl, 2.7mM KCl, 1.47mM KH₂PO₄, 8.1mM Na₂HPO₄</td>
</tr>
<tr>
<td>20x TBS</td>
<td>200mM Tris pH 8, 3M NaCl</td>
</tr>
</tbody>
</table>

2.6: RT-PCR

RNA extraction:
Total RNA was isolated using RNAserts kit (Promega). The protocol provided in the kit was followed: cells (or freshly dissected sciatic nerves) were lysed in denaturing solution and lysates subjected to phenol:chloroform extraction (phenol:chloroform:isoamyl alcohol 125:24:1, buffered with 42mM sodium citrate). The aqueous phase containing the RNA was removed and the RNA precipitated with isopropanol. Precipitated RNA was washed in 70% ethanol and RNA concentration was obtained by spectrophotometry (Beckman) at OD 260nm (A₂₆₀ = 40μg/ml RNA).
Synthesis of cDNA:
cDNA was generated from 2μg total RNA using Superscript Preamplification System (Gibco BRL). The protocol provided in the kit was followed: RNA was incubated with and without (RT- negative control) reverse transcriptase in the presence of random primers and dNTPs (42°C, 50 mins) to allow production of cDNA. Successful generation of DNA (and absence in RT- control) was confirmed by PCR for GAPDH (see below).

PCR amplification of cDNA:
2μl cDNA was amplified using the following reaction mix (all reagents from Promega):
- 5μl 10x PCR buffer
- 3μl 25mM MgCl<sub>2</sub>
- 1μl dNTP mix
- 1μl sense and 1μl antisense primers (10μM)
- 0.3μl α<sup>32</sup>P-dCTP
- 0.5μl Taq DNA polymerase (5u/μl) (added before or after a hot start as required)
The reaction mix was made up to 50μl with water. PCR was carried out in a Techne PCR machine, based on the following program (annealing temperature was optimized for each set of primers - see Table 2.4):
- Denaturation: 94°C, 1 minute
- Annealing: 52°C, 1 minute
- Elongation: 72°C, 1 minute

Where required a hot start of 3 minutes at 94°C was included (see Table 2.4)

One reaction lacking cDNA was included as a negative control. The appropriate number of cycles which would allow sufficient amplification without saturation was determined for each PCR reaction by comparing DNA levels in aliquots taken from PCR reactions after a range of different cycles. Each PCR reaction was run using two different numbers of cycles to verify that saturating levels had not been reached.

Amplified DNA was resolved by running 5μl PCR product combined with loading dye (20% w/v Ficoll 400, 1% w/v SDS, 0.25% w/v Bromophenol blue and 0.25% Xylene cyanol) on a 4% acrylamide gel (0.5x TBE gel, polymerized with 0.12% APS and 25μl TEMED (BioRad)). Gels were run for about 5 hours at 60V before soaking in destain solution (7% v/v acetic acid, 5% v/v methanol, 88% v/v water; 20 minutes) and drying (80°C, 2 hours). DNA was visualized by exposing dried gels to film or a phosphoimager detection screen. The signal detected on the phosphoimager screen was quantified using an FX phosphoimager and Quantity-One quantification software.
Equalizing levels of cDNA:
PCR was carried out, as described above, to quantify GAPDH levels in all samples. Levels were determined for both cycle numbers used during the PCR reaction and results were only considered acceptable if the fold differential between samples was maintained at both cycle numbers. PCR for GAPDH was repeated on cDNA samples that had been equalized for GAPDH levels to confirm that equivalent DNA levels were used in each reaction.

Expression analysis by RT-PCR:
Equivalent levels of cDNA (as determined above) were amplified in PCR reactions using primers and conditions specific for each reaction (see Table 2.3). Each reaction was carried out at two different cycle numbers to ensure saturation was not occurring. Each experiment was repeated at least two times, using freshly prepared cDNA for each experiment.

Table 2.3: Conditions and primers used for RT-PCR:

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Primer sequence</th>
<th>Annealing temperature</th>
<th>Cycles</th>
<th>Hot start</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>5': AAAAGGGTCATCATCTCCGC 3': GGATGCAGGGATGATGTTCT</td>
<td>58°C</td>
<td>20 and 22</td>
<td>No</td>
<td>350bp</td>
</tr>
<tr>
<td>p16INK4A</td>
<td>5': CTCCGAGAGGAAGGCGAACTCG 3': GGTGCAGTACTACCAGAGTG</td>
<td>50°C</td>
<td>24 and 26</td>
<td>Yes</td>
<td>350bp</td>
</tr>
<tr>
<td>p19ARF</td>
<td>5': GCCACTGCTGGGAGAGGTCG 3': GGTGCAGTACTACCAGAGTG</td>
<td>50°C</td>
<td>24 and 26</td>
<td>Yes</td>
<td>380bp</td>
</tr>
</tbody>
</table>
2.7: Retroviral infection:

Cells were infected by co-cultivation at approximately a 1:2 ratio with the producer cell line (GP+E-pBabePuro, GP+E-pBabePuro-H-Ras\textsuperscript{V12}, GP+E-LXSN, GP+E-LXSN-H-Ras\textsuperscript{V12} (from Alison Lloyd) (Lloyd et al., 1997), (Morgenstem and Land, 1990), (Markowitz et al., 1990) which had been pretreated with 20\mu g/ml of mitomycin C (2 hour treatment). After three days, producer cells were removed by positive immunopanning (see cell isolation above) with plates coated with anti-mouse Thy1 (supernatant from T24 hybridoma, (Le Gros et al., 1984). The purified, infected cells were replated and after a further 24 hours cells were transferred into selective medium. Appropriate drug concentrations were determined for each cell type: Schwann cells: G418: 0.4mg/ml. Fibroblasts: puromycin: 2.5\mu g/ml. When selection was judged to be complete (i.e. when no further death was observed in infected cells and all cells in an uninfected control had died), cells were treated with BrdU for a proliferation assay (see above) or were stained for senescence-associated \(\beta\)-galactosidase activity (see above). Three independent infections were carried out for each experiment.

2.8: \(\gamma\)-irradiation of cells:

Cells were seeded into flasks 24 hours before irradiation. Flasks were exposed to 12Gy X-rays (using facility at ICRF, London) for 2 minutes (appropriate doses to elicit a response were previously determined in dose response experiments in both fibroblasts and Schwann cells), or were mock irradiated. After a 20 hour recovery period cells were pulsed with \(^3\)H-thymidine for four hours and analysed in a proliferation assay (see above). Irradiation experiments were repeated at least twice.

2.9: Telomerase assay:

A standard TRAP assay was performed as described previously (Kim et al., 1994). Solutions used are described in Table 2.4. The following primers were used:
The TS primer acts as the template for telomere repeat addition and as the 5' primer in subsequent PCR amplification:

5'-AATCCGTCGAGCAGAGGT-3'
The ACT primer binds the extended TS template and primes the reverse reaction during PCR amplification:

5'-GCGCGG[CTAACC]3-3'

All reagents for PCR were from Promega.

**Preparing cell extracts:**

Cells were trypsinized, counted, resuspended in CHAPS lysis buffer and incubated on ice for 30 minutes. Cell suspensions were centrifuged in a chilled microcentrifuge (13,000rpm, 20 minutes) and supernatants were stored at -80°C.

**End-labeling TS primer:**

For each telomerase reaction, 1μl TS primer, 0.05μl T4 polynucleotide kinase (10μ/ml), and 0.25μl γ-32P-ATP (3000Ci/mmol) were combined in kinase buffer (total volume 2μl) and incubated at 37°C for 20 minutes followed by 85°C, 5 minutes.

**Extension of template by addition of telomere repeats:**

For each telomerase reaction the labeled TS primer (2μl) was incubated with 2μl cell extract, 1μl dNTPs mix (2.5mM of dCTP, dATP, dGTP, dTTP), 2μl ACT primer in 50μl TRAP buffer (room temperature, 30 minutes). For each cell extract a duplicate heat-treated (80°C, 20 minutes) negative control was included. In addition a CHAPs only negative control was included.

**PCR amplification of extended template:**

The reaction mix described above was amplified using the following PCR conditions:

- **Hot start** 94°C, 4 minutes
- **Denaturation** 94°C, 15s
- **Annealing** 60°C, 30s
- **Elongation** 72°C, 30s
- 35 cycles.

**Visualization of reaction products:**

25μl reaction products (with loading dye) were resolved by PAGE (10% non denaturing gel). Gels were dried and exposed to film.
Table 2.4: Solutions for telomerase assay

<table>
<thead>
<tr>
<th>Solution</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHAPs lysis buffer</td>
<td>10mM Tris-HCl, pH7.5; 1mM MgCl₂; 1mM EGTA; 0.1mM Benzamidine; 5mM β-mercaptoethanol; 0.5% CHAPs; 10% Glycerol.</td>
</tr>
<tr>
<td>10X TRAP reaction buffer</td>
<td>200mM Tris-HCl, pH8.3; 15mM MgCl₂; 630mM KCl; 0.5% Tween 20; 10mM EGTA.</td>
</tr>
<tr>
<td>10X Loading dye</td>
<td>50% Glycerol; 0.25% bromophenol blue; 0.25% xylene cyanol; 50mM EDTA, pH8.</td>
</tr>
</tbody>
</table>

2.10: Telomere length:

Hybridization of telomere probe:

FITC-conjugated PNA probes were from Applied Biosystems:

Telomere probe: Flu-OO-CCCTAACCTAACCTAA
Alphoid probe: Flu-OO-CCCATAAACCAC

Cells were trypsinized, counted, washed (2x PBS) and 10⁵ cells were pelleted in a benchtop microfuge. Cells were resuspended in 300μl hybridization mix, containing either the telomere, or alphoid (background) probe at 0.3μg/ml. DNA was denatured by incubating the hybridization mixture in a shaking heat block (80°C, 10 minutes) and reactions were then incubated at room temperature in the dark for at least 2 hours. Cells were washed twice in Wash Buffer 1 (pellet in bench top microfuge, 7 minutes, 6500 rpm, 16°C) and once in Wash Buffer 2 (5 minutes, 6500 rpm, 16°C). Cells were resuspended in 250μl PI solution, incubated in the dark at room temperature for 2-4 hours and then stored at 4°C before analysis on the flow cytometer.
Table 2.5: Solutions for hybridization of telomere probe:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hybridization mix</td>
<td>70% Formamide; 1% BSA; 20mM Tris-HCl, pH 7 in PBS</td>
</tr>
<tr>
<td>Wash Buffer 1</td>
<td>70% Formamide; 0.1% BSA; 10mM Tris HCl, pH 7; 0.1% Tween in PBS</td>
</tr>
<tr>
<td>Wash Buffer 2</td>
<td>0.1% BSA; 0.1% Tween in PBS</td>
</tr>
<tr>
<td>PI solution</td>
<td>6µg/ml Propidium Iodide; 0.1% BSA; RNAase A (0.2u/µl)</td>
</tr>
</tbody>
</table>

Flow cytometry:
Analysis was performed in a FACS Calibur flow cytometer, using the FL3 channel for detection of propidium iodide and the FL1 channel for detection of the fluorescein signal from the FITC conjugated probe. No compensation was set. Nuclei were gated on the FL3 channel. 5000 events were collected and analysed on the FL1 channel using Cell-Quest software (Becton Dickinson). The telomere signal was defined as the mean signal detected on the FL1 channel after the background signal from the same sample hybridized to the alphoid probe was subtracted. All signals were then expressed relative to the signal obtained for HMF-TERT cells within the same experiment to control for variations in the flow cytometer between independent experiments. Experiments to determine telomere length were repeated at least three times (using freshly prepared cell samples each time) and the mean of these experiments used to determine relative telomere length.

Hybridization of telomere probe to metaphase spreads:
Metaphase spreads were prepared as described above. Slides were fixed in 4% paraformaldehyde (2 minutes, room temperature), washed (2x PBS) and then treated with pepsin (1mg/ml, pH2, 10 minutes, 37°C). The formaldehyde fixation and wash steps were repeated. Slides were dehydrated in 100% ethanol and left to dry before treating with hybridization mix (Table 2.5), transferred to 80°C, 10 minutes and then incubated in the dark (room temperature, 2 hours). Slides were washed on a shaker, using 3x 15 minute washes in 1ml Wash Buffer 1 (Table 2.5) followed by 3x 5 minute washes in 1ml Wash Buffer 2 (Table 2.5). Finally slides were rinsed in deionized water and then 100% ethanol before mounting in Vectashield.
Table 2.1: Antibodies used during immunofluorescence and western blotting.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Dilution</th>
<th>Development of signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>p16^{NK4A}</td>
<td>Santa Cruz (sc1207)</td>
<td>1/200</td>
<td>Anti-rabbit HRP, TBST, ECL plus</td>
</tr>
<tr>
<td>p21^{Cip1}</td>
<td>Santa Cruz (sc937)</td>
<td>1/1000</td>
<td>Anti-goat HRP, PBST, ECL plus</td>
</tr>
<tr>
<td>p19^{ARF}</td>
<td>AbCAM: custom produced</td>
<td>1/1000</td>
<td>Anti-rabbit HRP, PBST, ECL plus</td>
</tr>
<tr>
<td>p27^{Kip1}</td>
<td>Santa Cruz (sc1641)</td>
<td>1/1000</td>
<td>Anti-mouse HRP, PBST, ECL plus</td>
</tr>
<tr>
<td>p53 (421)</td>
<td>Calbiochem (0P03)</td>
<td>1/20</td>
<td>Anti-mouse HRP, TBST, ECL plus</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>Santa Cruz (sc2821)</td>
<td>1/1000</td>
<td>Anti-mouse HRP, PBST, ECL plus</td>
</tr>
<tr>
<td>Cyclin D2</td>
<td>Santa Cruz (sc593)</td>
<td>1/300</td>
<td>Anti-rabbit HRP, PBST, ECL normal</td>
</tr>
<tr>
<td>Cyclin D3</td>
<td>Santa Cruz (sc182)</td>
<td>1/300</td>
<td>Anti-rabbit HRP, PBST, ECL normal</td>
</tr>
<tr>
<td>Cyclin E</td>
<td>Santa Cruz (sc481)</td>
<td>1/200</td>
<td>Anti-mouse HRP, PBST, ECL normal</td>
</tr>
<tr>
<td>Cyclin A</td>
<td>Santa Cruz (sc596)</td>
<td>1/200</td>
<td>Anti-rabbit HRP, PBST, ECL plus</td>
</tr>
<tr>
<td>CDK2</td>
<td>Santa Cruz (sc163)</td>
<td>1/1000</td>
<td>Anti-rabbit HRP, PBST, ECL normal</td>
</tr>
<tr>
<td>CDK4</td>
<td>Santa Cruz (sc260)</td>
<td>1/1000</td>
<td>Anti-rabbit HRP, PBST, ECL normal</td>
</tr>
<tr>
<td>CDK6</td>
<td>Santa Cruz (sc177)</td>
<td>1/1000</td>
<td>Anti-rabbit HRP, PBST, ECL plus</td>
</tr>
<tr>
<td>S-100</td>
<td>Charles River Pharm services</td>
<td>1/400</td>
<td>FITC-conjugated anti rabbit</td>
</tr>
<tr>
<td></td>
<td>(see (Cheng et al., 1995))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NGF</td>
<td>from Anne Mudge (see(Cheng et al., 1995))</td>
<td>1/5</td>
<td>FITC-conjugated anti mouse</td>
</tr>
<tr>
<td>Thy1.1 (Ox7)</td>
<td>from Anne Mudge (see (Cheng et al., 1995))</td>
<td>1/2</td>
<td>Cy3 conjugated anti mouse</td>
</tr>
</tbody>
</table>
Chapter 3: Primary rat Schwann cells divide indefinitely in culture:

Senescence was generally thought to present an inevitable intrinsic barrier to the proliferation of cultured primary cells (Campisi, 1997), (Wynford-Thomas, 1997). After a set number of divisions, that appeared to be defined for each cell type, cells stopped dividing and became senescent (Hayflick and Moorhead, 1961), (Hayflick, 1964). A senescent culture was easily distinguished from quiescent cells (for example cells arrested by serum deprivation) because the arrest was irreversible: senescent cells could not be induced to reenter the cell cycle by mitogenic stimulation (Hayflick and Moorhead, 1961). Senescent cells could also be distinguished by their characteristic phenotype. Typically they had an enlarged, flattened morphology; showed nuclear abnormalities; expressed elevated CDKI levels and stained positively for the senescence marker β - galactosidase activity at pH 6 (Hayflick and Moorhead, 1961), (Dimri et al., 1995), (Alcorta et al., 1996).

Although senescence was observed in all the somatic cell types characterized, (with the exception of certain stem cells), proliferating cells could eventually be expanded from many of these senescent cultures. These immortal cell lines were found to have by-passed senescence as a result of "immortalizing" mutations (Hayflick and Moorhead, 1961), (Kamijo et al., 1997). Although some cell types, particularly rodent cells (Wright and Shay, 2000), immortalized at relatively high frequency, a static or slow growth period indicative of senescence was still readily detectable in all cultures tested.

The only primary cell types that could be expanded indefinitely in culture were germ cells and embryonic stem cells (Campisi, 1997), (Thomson et al., 1998), (Brustle et al., 1999). The lack of a proliferative limit in these cell types was understood to directly relate to the proliferative requirements of these cells in vivo. Consistent with the finding that immortalizing mutations were mostly in tumour suppressor genes, it was assumed that senescence acted as an important barrier to tumorigenesis in somatic cells (Stamps et al., 1992). It was thus commonly thought that the proliferative capacity of a cell type in vitro should provide some indication of the replicative limits operating on the same cell type in vivo.
The number of population doublings that occurs before a culture senesces varies depending on cell type. For example mouse embryo fibroblasts senesce very rapidly (after only a few PDs) (Blasco et al., 1997) whilst human fibroblasts can undergo at least 50PDs before arresting (Hayflick, 1964). The certainty that all somatic cells will ultimately senesce has restricted cell culture studies to cell lines that have undergone immortalizing mutations or, to the period of proliferation before primary cultures enter senescence. However, we observed that rat Schwann cells cultured in our laboratory could proliferate in culture over long periods without obviously entering senescence (Lloyd et al., 1997). Therefore this cell type appeared to behave differently from other rodent cell types that generally have very short replicative lifespans. This observation prompted us to investigate whether these cells could present the first known example of a primary differentiated cell type with an unlimited replicative capacity.

**Investigating the replicative capacity of rat Schwann cell cultures:**

**Pure cultures of Schwann cells and fibroblasts can be isolated from rat sciatic nerves.**

Schwann cells are the glial cells of the peripheral nervous system. They can be readily purified in large numbers from rat sciatic nerves (Brockes et al., 1979), (Cheng et al., 1998). Upon dissociation from the nerve and transfer into culture Schwann cells partially de-differentiate and re-enter the cell cycle. They can be maintained in a proliferative state in culture in the presence of low levels of serum and specific Schwann cell mitogens: glial growth factor (GGF) (Maurel and Salzer, 2000) and forskolin which leads to elevated cAMP levels (Howe and McCarthy, 2000).

Pure primary cultures of Schwann cells were obtained by dissecting the sciatic nerves from p7 rats (Fig 3.1). The dissociated nerves contain a mixture of Schwann cells and fibroblasts of the nerve connective tissue (Brockes et al., 1977). We chose to culture the perineural fibroblasts in parallel with the Schwann cells, as we predicted that, like other rodent fibroblasts, they would rapidly senesce and thus provide a positive control for detection of senescence. Any contaminating macrophages were removed by immunopanning with α - Ox42 coated dishes (Cheng et al., 1995). The fibroblasts were subsequently separated from the Schwann cells by immunopanning.
Figure 3.1: Schwann cells are isolated from rat sciatic nerves:
Sciatic nerves were dissected from p7 rats. The dissociated cells were purified by sequential immunopanning: contaminating macrophages were removed by binding α-Ox42 coated dishes. The remaining mixture of cells was sequentially passed over three α-Thy1.1 coated dishes to bind all perineural fibroblasts. The remaining unbound population consisted of Schwann cells. The day following isolation, the Schwann cells and fibroblasts were trypsinized and plated at densities of $4.8 \times 10^5$ and $5.0 \times 10^5$ cells per 9cm dish respectively.
using an antibody recognising Thy1.1, which is expressed by the fibroblasts but not Schwann cells (Brockes et al., 1977), (Cheng et al., 1995). The unbound population of Schwann cells was plated on laminin - coated dishes. The day following isolation, both cell types were trypsinised and replated at set densities (see below). The resulting cell cultures were homogeneous and the two cell types had clearly distinguishable morphologies: The fibroblasts were flattened compared to the spindle-shaped Schwann cells (Fig 3.1).

To assess the purity and verify the identity of the cell cultures obtained by this technique immunostaining for cell - specific markers was carried out (Fig 3.2). Schwann cells express the low affinity NGF receptor (L-NGFR) and the intracellular S100 antigen. Fibroblasts express Thy 1.1 (Brockes et al., 1977) but not S100 (Brockes et al., 1977) or L-NGFR (Yan and Johnson, 1988). Schwann cell cultures were found to be 99.9% pure by staining with α-S100, α-NGFR and α-Thy 1.1 and the low percentage of contaminating cells were Thy 1.1 expressing fibroblasts. In fibroblast cultures (99.6% pure), the contaminating cells were L-NGFR-expressing Schwann cells. As Schwann cells are highly dependent on autocrine survival factors (Cheng et al., 1998) it would be unlikely that the few Schwann cells present in fibroblast cultures would survive. The extremely low numbers of fibroblasts present in Schwann cell cultures were not detectable at later stages in culture probably because they could not proliferate at low density in Schwann cell growth conditions. Furthermore, we might expect any fibroblast expansion to be rapidly halted by entry into senescence (see below).

**Schwann cells maintain a constant proliferative rate throughout long - term culture:**

To investigate proliferative rate and capacity a modified 3T3 protocol was carried out. Cells were seeded at a fixed number per 9cm dish (4.8 x 10^5 for Schwann cells and 5.0 x 10^5 for fibroblasts). This number was carefully chosen to minimize selective pressures in culture by ensuring that Schwann cells were replated before reaching confluency (every three days) and reseeded at a sufficient density to optimize autocrine survival effects. Fibroblasts were grown in 10% foetal calf serum whilst Schwann cells were cultured in 3% foetal calf serum supplemented with forskolin and
A Fibroblast culture isolated by positive Thy1.1 immunopan:

Stain with: α-Thy1.1
0.38% cells stain for NGF

B Schwann cells isolated as unbound population after Thy1.1 immunopan:

Stain with: α-NGF
Stain with: α-S100
0.09% cells stain for Thy1.1

Figure 3.2: Primary cultures of Schwann cells are 99.9% pure:
A: Perineural fibroblasts at passage 1 were immunostained with both α-Thy1.1 (red) and α-NGF (green). 0.38% of cells expressed NGF. The remainder expressed Thy1.1.
B: Schwann cells at passage 1 were immunostained with α-Thy1.1 (red) and α-NGF (green) or α-Thy1.1 (red) and α-S100 (green) 0.09% cells expressed Thy1.1. The remainder expressed NGF and S100.
Nuclei were visualized with Hoescht (blue).
Percentages are means of triplicate counts of at least 200 cells.
Glial Growth Factor (GGF). The cells plated the day after isolation from the animal were defined as Passage 1. Every three days the cells were passaged by trypsinising, counting and re-seeding at the set density. Minimal cell death was observed after trypsinisation of both cell types. The replicative capacity of more than four independently isolated batches of Schwann cells and five batches of fibroblasts have been analysed in this way.

The perineural fibroblasts showed proliferation curves characteristic of most cultured rodent cells (Fig 3.3B): Initially, they proliferated at a high rate but after only about 3 passages (= 8 PDs) the rate decreased until cell numbers ceased to increase and the cultures appeared to have senesced. This proliferative arrest was observed in all of seven batches investigated. In all cases, after continual passaging (re-plating) of the arrested cultures, proliferative rates began to increase and eventually reached levels equivalent or higher to those of freshly isolated cells. These apparently "immortalized" cell lines have been maintained in culture for at least 100PDs.

In contrast, the Schwann cells divided at a constant rate, showing no indication of proliferative arrest at any stage (Fig 3.3A). At least four batches of Schwann cells have been maintained in culture for more than 50 passages (75 PDs) - equivalent to the original population expanding $10^{20}$ times.

To confirm the constant proliferative rate suggested by the Schwann cell proliferation curve, BrdU uptake of Schwann cells at various stages in culture was measured. BrdU labeling provides an indication of the rate of DNA synthesis and thus gives another measure of proliferative rates in addition to cell counts. Consistent with the growth curves, the percentage of cells that incorporated BrdU into their DNA after a fixed BrdU pulse remained constant, irrespective of passage number (Fig 3.3C).

It remains a possibility that a replicative limit would eventually be reached beyond the stage at which the Schwann cells were cultured. However, the Schwann cells have been maintained in culture for more than the maximum number of population doublings reported for all other primary rodent cells which typically senesce after only a few population doublings. Furthermore, as the number of progeny generated by 75PDs is more than twice the number of cells estimated to make up an average rat, it seems very unlikely that any replicative limit beyond this point could be of any in vivo relevance.
Figure 3.3: Schwann cells proliferate at a constant rate in culture.
Figure 3.3: Schwann cells proliferate at constant rate in culture

A modified 3T3 assay was carried out on Schwann cells (A) and fibroblasts (B). Cells were cultured in DMEM supplemented with 3% FCS, GGF and forskolin (Schwann cells) or 10% FCS (fibroblasts). Triplicate plates of cells were trypsinized every three days and cells were counted and reseeded at 4.8x10^5 (Schwann cells) or 5.0x10^5 (fibroblasts) cells per 9cm dish. Each point on the growth curves represent the mean of triplicate counts.

C: Schwann cell BrdU uptake was measured at different passage numbers. Cells were plated at a set density on coverslips in either 3% or 1% serum and incubated with BrdU for 8 hours, 24 hours after plating. The percentage of cells that incorporated BrdU was counted after immunostaining with α-BrdU and Hoescht.

Numbers represent means for three coverslips. At least 500 cells were counted per coverslip.
Schwann cells can be cloned at high efficiency:

The rapid entry into senescence of most rodent cells should inhibit expansion of clones derived from single cells past a certain colony size. Cloning efficiencies have thus often been used to investigate replicative capacity (Wright and Shay, 2002). In order to verify that Schwann cells did not senesce in culture we generated and expanded Schwann cell clones (Fig 3.4). Schwann cells at various stages in culture were seeded at low density. Mutations in p53 are sufficient to immortalize most rodent cells (Kamijo et al., 1997). Therefore a Schwann cell line expressing a dominant negative form of p53 (dn-p53) (generated by A. Lloyd: (Lloyd et al., 1997)) was used as a control. Although the plating efficiencies were low (=7% for Schwann cells at all passages), this was to be expected as sensitivity to stress is increased in cells at low density and results in low cloning efficiencies, even for cells with large replicative capacities (e.g. human diploid fibroblasts) (Wright and Shay, 2002). In addition, Schwann cells secrete an autocrine survival factor and thus at clonal density are particularly sensitive to the harsh trypsinisation and replating procedure (Cheng et al., 1998). The dn-p53 Schwann cells showed slightly higher survival rates (=12%), consistent with their impaired p53-dependent damage response pathways. Of the normal Schwann cells that survived plating, the percentage that proliferated to produce large colonies was high (>50%) and approximately equivalent to dn-p53 cells (Fig 3.4). Six of these clones were expanded in culture (at least 50PDs) and had morphologies and proliferation rates that were indistinguishable from those of pooled Schwann cell cultures. The finding that the cloning efficiency remained high and similar at all passages indicates that senescence is not acting as a barrier to prevent clonal expansion and that the proliferative capacity of Schwann cells remains the same at various stages in culture.
Figure 3.4: Schwann cells can be cloned at high efficiency.

At the passage numbers indicated Schwann cells were seeded at low density in triplicate on laminin-coated dishes in normal Schwann cell medium supplemented with 20% conditioned medium from confluent Schwann cells. Plating efficiencies were equivalent for all passages of normal Schwann cells (~7%) and slightly higher for cells expressing dominant negative p53 (NS dn p53) (~12%). Cloning efficiency was calculated as indicated. Numbers are means of three experiments.
Examining Schwann cell cultures for the presence of senescent cells:

The constant proliferative rates observed for pools of cultured cells suggested that Schwann cells do not undergo a senescence-associated proliferative arrest. To confirm that the arrest observed in the fibroblasts was associated with senescence and to further investigate Schwann cell cultures for any evidence of senescence it was necessary to examine cultures at the single cell level. We therefore inspected cultures at various passages for cells displaying the characteristic senescent phenotype.

Schwann cells maintain an identical morphology throughout long-term culture:

Senescent cells are usually easy to detect in cultures as they are very large, flat cells and often contain visible stress fibres. The majority of fibroblasts observed between passages 3-6 (when cells had stopped dividing - Fig 3.3B) had adopted this characteristic senescent morphology (Fig 3.5B). As the proliferation rate of the cells started to increase (passage 7-10, Fig 3.3B) smaller, more uniform cells began to appear until at late passages the cultures appeared homogeneous. Each of the immortalized batches had a slightly different morphology, suggestive of a clonal origin (Fig 3.5B). In contrast the Schwann cells in our cultures always maintained their characteristic spindle-shaped morphology (Fig 3.5A). Appearance of enlarged cells was very rare, (<1 in $10^4$ cells), and the frequency did not vary with passage number.

Schwann cells do not express the senescence marker β-galactosidase activity at pH 6:

A commonly used marker for senescence is expression of a form of β-galactosidase that is active at pH 6, commonly termed Senescence Associated β-galactosidase (SA-β-gal) (Dimri et al., 1995) This marker has been used to detect senescent cells both in vivo and in vitro (Dimri et al., 1995).

We stained Schwann cells and fibroblasts at different passages in culture for SA-β-gal activity (Fig 3.6). A significant number of SA-β-gal positive cells (≈15%) first became evident in perineural fibroblast cultures at around passage 3, correlating
Figure 3.5: Schwann cells maintain identical morphology throughout long term culture.
A: Representative Schwann cell morphology at different stages in culture
B: Perineural fibroblast morphology at early stages of culture (p1), senescence (p4) and of the three immortalized cell lines Imm1, Imm2 and Imm3 (>p60)
Size bar: 50μm
with the onset of proliferative arrest in these cells (Fig 3.6C). The majority of cells with
the characteristic senescent morphology stained positively for this senescence marker
whereas the smaller more refractile cells did not. The percentage of SA-β-gal positive
cells increased until around passage 7 (≈43%) but at later passages this number was
reduced to background levels in the homogeneous immortalized cultures (Fig 3.6C).
Although the number of SA-β-gal positive cells correlates tightly with the number
showing a senescent morphology, there is a time lag between maximal staining and
onset of proliferative arrest. These results are consistent with findings that some of the
characteristics of a senescent phenotype (cell enlargement and SA-β-gal activity)
develop after the initial senescent cell cycle arrest (Dai and Enders, 2000), (McConnell
et al., 1998).

Schwann cell cultures were stained for SA-β-gal activity at passages 2, 3, 4, 7,
10, 17, 21, 23, 25 and 29 (Fig 3.6A). The numbers of SA-β-gal stained Schwann cells
were consistently low (<1%) at all passages investigated (Fig 3.6C). Staining was
detected in a minimal fraction of cells, demonstrating that the protocol worked in rat
Schwann cells. This is an important internal control, as there are reports that certain cell
types, for example senescent mouse fibroblasts, cannot be stained with standard
protocols (Dimri et al., 1995).

Schwann cells do not show nuclear abnormalities at any stage in culture.

An increased frequency of nuclear abnormalities (multiple nuclei, giant nuclei
and irregularly shaped nuclei) is observed in senescent cell cultures (Hayflick and
Moorhead, 1961). Hoescht staining was used to visualize the nuclei of fibroblasts and
Schwann cells at various stages in culture (Fig 3.7A). Significant numbers of
multinucleate cells (10-24%) and giant nuclei (10-14%) were detected in fibroblast
cultures between passages 6-10, consistent with these cells being senescent. At later
stages in culture when fibroblast cultures had regained proliferative capacity through
immortalization, the numbers of abnormal nuclei were consistently low (3.7B).
Schwann cell nuclei were examined at passages p5, p10, p20, p23 and p29. In all cases
the nuclei were uniformly small, with less than 1% of cells showing any irregularities.
Figure 3.6: Schwann cells do not stain positively for Senescence Associated β-Galactosidase.

X-gal staining at pH6 for 10 hours was used to detect a senescent phenotype. Fibroblasts (B) stained positively between passages 3-10 but not at early passage or after immortalization. Schwann cells at any stage in culture, represented by p10 (A), did not stain. Schwann cells treated with aphidicolin (1μg/ml), for 5 days arrested and stained positively for SA-β-Gal (A-right panel).

C: Quantitation of SA-β-Gal positive cells. Numbers represent the mean of triplicate counts. More than 200 cells were counted. The percentages of fibroblasts, but not Schwann cells, labeling at different passages was significantly different (2-tailed t-test, P<0.01).
Figure 3.7: Schwann cells do not show nuclear abnormalities during culture.
A: Schwann cell and fibroblast nuclei were stained with Hoescht at different stages in culture. Schwann cells maintained uniform nuclei at all passages (represented by p10 and p20). Fibroblasts showed increased frequencies of giant nuclei and multinucleate cells at senescence (represented by p10).
B: Quantitation of nuclear abnormalities. Numbers are means of triplicate counts of at least 200 cells. The percentages of fibroblasts, but not Schwann cells, showing abnormalities at different passages was significantly different (2-tailed t-test, P<0.01).
These results are consistent with the lack of any other aspect of the senescent phenotype at any stage of Schwann cell culture.

**Schwann cells maintain the ability to induce a senescence phenotype:**

It was important to establish whether Schwann cells in our cultures expressed the signalling pathways required to induce a senescence response. A senescent phenotype can be induced in cultured primary cells that have undergone a prolonged cell cycle arrest in response to stress, for example by oxidizing agents or chemicals that induce DNA damage (Di Leonardo et al., 1994), (Robles and Adami, 1998), (Chen and Ames, 1994) (see also section 1.4a). Schwann cells were treated with aphidicolin, a DNA polymerase inhibitor that blocks cells in S-phase (Ikegami et al., 1978) to investigate whether a prolonged cell cycle arrest would induce senescence in these cells. The majority of aphidicolin treated Schwann cells adopted a senescent morphology and stained positively for SA-β-gal (Fig 3.6A). These results demonstrate that, although Schwann cells possess the signalling pathways required to induce a senescent phenotype, this program is not activated by proliferation *in vitro*.

In summary, we have demonstrated that primary rat Schwann cells have unlimited replicative capacity *in vitro*. Cultures of these cells maintain constant proliferative rates for at least 75 PDs with no significant numbers of senescent Schwann cells detectable at any stage in culture. These cells maintain the ability to induce a senescent response, but these pathways are not activated during normal Schwann cell culture. Therefore, these findings suggest that intrinsic replicative barriers may not operate in all normal primary somatic cells.

Our findings have been supported by work carried out in collaboration with Tang et al in rat oligodendrocyte precursors (OPCs). Rat OPCs provide another example of a rat glial cell type that can be expanded indefinitely in culture. Similar analysis to that described here failed to detect senescent cells in the OPC cultures (Tang et al., 2001). This work lends weight to our finding that replicative limits can no longer be assumed to limit the proliferation of primary somatic cells.

A previous study claiming that mouse embryo fibroblasts (MEFs) could be expanded indefinitely in defined culture conditions is frequently cited as an example of
a cell type that appeared to be immortal (Loo et al., 1987), (Wright and Shay, 2002). In these experiments cell cultures were produced from whole embryos and were expanded in defined medium. However, the identity of the proliferating cell types was not verified and it was assumed that these immortal cultures consisted of MEFs. In later studies it became clear that the culture conditions used in this study had selected for outgrowth of an embryonic stem cell population expressing nestin, a neuroepithelial stem cell marker (Loo et al., 1994). The findings in this study were thus consistent with other reports that embryonic stem cells lack replicative limits. Although it is accepted that embryonic stem cells can divide indefinitely it is unclear whether adult stem cells possess replicative limits (Campisi, 1997), (Thomson et al., 1998), (Brustle et al., 1999).

In conclusion, the work described here in rat Schwann cells and that carried out in parallel with rat oligodendrocyte precursors provide the only known examples of mature somatic cells that do not senesce in extended culture. These findings suggest that it may no longer be appropriate to include a finite replicative limit in the definition of a primary cell.
Chapter 4: Investigating the basis for the unlimited replicative capacity of cultured rat Schwann cells.

A: Do Schwann cells acquire immortalizing mutations in culture?

In the previous chapter we demonstrated that a senescent arrest could not be detected in cultured rat Schwann cells, whereas perineural fibroblasts rapidly stopped proliferating and underwent classical senescence. Early and late passage Schwann cells were indistinguishable in terms of morphology (Fig 3.5A), mitogen dependence (Fig 3.3C), cloning efficiencies (Fig 3.4) and ability to differentiate (M Harrisingh, personal communication), suggesting that they had not acquired genetic changes affecting these responses during the observed culture period. However, it was possible that the Schwann cells may have acquired other mutations conferring unlimited replicative capacity. Our finding that immortalized lines could be produced from every batch of perineural fibroblasts isolated is consistent with the high frequency of spontaneous immortalization observed in rodent cells (Wright and Shay, 2000). The slow emergence of proliferating cells from the senescent fibroblast cultures and the differing characteristic morphologies of each of the resultant lines (Fig 3.5B) might suggest that they had arisen from a single mutant cell. However, if the spontaneous mutation rate in Schwann cells was significantly higher than in fibroblasts such an immortalization process might not be so readily detectable. Therefore, it was important to determine whether the cultured Schwann cells had acquired immortalizing mutations.

When Hayflick first defined the process of senescence, he distinguished primary cells that senesced from immortal cell lines that had undergone genetic lesions. The established cell lines that Hayflick examined demonstrated karyotypic abnormalities and thus Hayflick incorporated heteroploidy into his definition of a cell line (Hayflick and Moorhead, 1961). However, it has become clear that although acquisition of immortalizing mutations frequently correlates with karyotypic abnormalities (especially in cases where p53 or other components of DNA damage pathways are mutated (Fukasawa et al., 1996), (Levine, 1997)), this is not always the case.
The mutations present in immortalized cell lines have been best characterized in murine cells and most commonly involve inactivation of p53 or loss of the INK4A locus. In one study of immortalized MEF lines 75% were found to have inactivated p53 whilst the remaining 25% had lost expression of both products of the INK4A locus (Kamijo et al., 1997). It appears that murine cells only require one genetic change to overcome senescence, as abrogation of the p53 pathway is sufficient to confer immortality (Kamijo et al., 1997). The precise nature of the immortalizing mutation may not always be obvious in established cell lines (Franza et al., 1986). However, because immortalizing mutations occur in genes that mediate normal cell cycle checkpoint responses, immortalized cells can usually be distinguished from primary cells by defects in these checkpoints. For example, immortalized cell lines may fail to respond to DNA damage (Kamijo et al., 1997) or may have lost resistance to transformation by a single oncogene (Serrano et al., 1997).

To investigate whether Schwann cell cultures had undergone immortalizing mutations, late passage cells were examined for maintenance of diploid status and intact cell cycle checkpoints. The Schwann cell clones (see Figure 3.4) were similarly analysed. All cells within the same clone would be predicted to carry the same genetic change if immortalizing mutation had been acquired during generation of these clones. Therefore, analysis of cell cycle controls within these clones would allow detection of mutations that might be undetectable in subpopulations of pooled cultures. Three batches of fibroblasts that had emerged from senescence (Imm 1, Imm 2 and Imm 3) provided positive controls of cells predicted to be defective in cell cycle regulation. These cells would allow identification of the genetic changes commonly occurring during rat cell immortalization.

Schwann cells maintain diploid status in culture:

Metaphase spreads were generated to determine cell ploidy. Overlapping or very dispersed spreads were not included in the analysis (examples of fields used for chromosome counting are illustrated in Fig 4.1A). Late passage Schwann cells maintained the normal rat diploid number of 42 chromosomes (Fig 4.1B). In contrast, one of the fibroblast lines, Imm 2, had abnormal, elevated chromosome numbers. Slight variation in chromosome counts results from errors in resolving individual
Figure 4.1: Schwann cells maintain diploid status during long-term culture.
Metaphase spreads were generated for both Schwann cells and fibroblasts at different stages in culture. Representative examples used to count chromosome number are illustrated in A. Excessively dispersed or overlapping spreads were discarded. Rat diploid number = 42.
B: Representation of chromosome counts. Chromosomes in 40 metaphase spreads were counted for each sample.
chromosomes within spreads but is clearly distinguishable from the disperse distribution observed in the karyotypically abnormal Imm 2 cells.

Schwann cells maintain expression at the INK4A locus:

The INK4A locus is frequently deleted in cell lines (Ruas and Peters, 1998). This locus encodes two distinct tumour suppressors from alternate reading frames: the CDKI, p16\textsuperscript{INK4A}, and p19\textsuperscript{ARF}, which acts to increase levels of active p53. Both these genes play roles in senescence: MEFs lacking p19\textsuperscript{ARF} (in the presence of functional p16\textsuperscript{INK4A}) are immortal (Kamijo et al., 1997). MEFs lacking p16\textsuperscript{INK4A} alone still senesce, but immortalization is accelerated suggesting that p16\textsuperscript{INK4A} may be important in maintaining the senescent state (Krimpenfort et al., 2001), (Sharpless et al., 2001), (Sherr, 2001). Although loss of p19\textsuperscript{ARF} appears to be sufficient to overcome senescence, it is extremely rare to find inactivation by point mutation in established lines: more frequently expression of both transcripts from this locus is lost (Ruas and Peters, 1998), (Kamijo et al., 1997), (Tanaka et al., 1997), (Gemma et al., 1996).

To investigate whether loss of the INK4A locus may be a selected event during Schwann cell expansion, we investigated expression of both the tumour suppressors encoded by this locus in Schwann cell clones and late passage pooled populations. It was not possible to examine p19\textsuperscript{ARF} protein expression due to lack of a suitable antibody. Therefore we used semi-quantitative RT-PCR as p19\textsuperscript{ARF} protein expression has been shown to correlate with mRNA levels (Palmero et al., 1998). PCR conditions were carefully optimized to ensure that amplified cDNA levels were representative of mRNA levels in the cell: PCR was carried out at two different numbers of cycles to guard against saturation and cDNA extracts were equalized for GAPDH levels prior to amplification (see methods for more detail).

All six Schwann cell clones and late passage cultures maintained equivalent levels of p16\textsuperscript{INK4A} protein (revealed by western blotting) and p19\textsuperscript{ARF} mRNA (Fig 4.2). Analysis of the three fibroblast lines revealed that Imm 1 had lost expression at the INK4A locus as both p16\textsuperscript{INK4A} protein and p19\textsuperscript{ARF} mRNA were completely undetectable, even upon overexposure. Imm 2 fibroblasts expressed elevated levels of both p16\textsuperscript{INK4A} and p19\textsuperscript{ARF} compared to senescent cultures and Imm3 cells. Overexpression of p19\textsuperscript{ARF} in
Figure 4.2: Schwann cells maintain expression at the INK4A locus.
Schwann cell clones (1-6) and pools (p3 and p15) (A) and immortalized fibroblast lines (Imm 1-3) and passage 7 pools (p7) (B) were analysed for p16\textsuperscript{INK4a} protein expression by western blotting and p19\textsuperscript{ARF} mRNA expression by semi-quantitative RT-PCR. 30µg protein extract was loaded in each lane for western blotting. RNA extracts were equalized for GAPDH levels prior to amplification. Results are representative of at least two separate experiments.
other cell lines has previously been correlated with defects in p53 function (Kamijo et al., 1997), (Quelle et al., 1995).

**Schwann cells maintain p53 activity:**

The p53 tumour suppressor is a critical mediator of cellular damage responses and loss of p53 activity is the genetic event that occurs most frequently in establishment of rodent cell lines (Kamijo et al., 1997), (Giaccia and Kastan, 1998). The predominant mechanism of p53 activation upon checkpoint activation involves its stabilization, but p53 is also regulated by posttranslational modification and subcellular localization (see chapter 1, section 2.2a). p53 inactivation during establishment of cell lines almost always occurs by point mutations that generate dominant negatives, rather than loss of the whole locus (Vousden and Lu, 2002).

p53 levels in various passages of Schwann cells were barely detectable by western blotting and were only visible after treatment with ALLN, a proteasome inhibitor which blocks p53 degradation (Ravid et al., 2000) (Fig 4.3A). Similarly, nuclear p53 staining was only visible after ALLN treatment (Fig 4.3B). p53 is normally maintained at very low background levels by its rapid turnover whereas mutant forms of p53 are often more stable than the wild type (Kubbutat and Vousden, 1998), (Jones et al., 1994). The observation that late passage cells have low p53 background levels might suggest they express the less stable, active form. This argument was strengthened by the finding that elevation of p53 levels by ALLN treatment led to increases in the p53-transcriptional target p21^CIP1 (Fig 4.3A).

We used a functional assay to confirm whether p53 was active in these cells. The advantage of this strategy is that it can also verify whether upstream p53 regulators and downstream p53 effectors remain intact. The p53-dependent DNA-damage response checkpoint was thus investigated in Schwann cell pools and clones by exposing them to X-rays, which is known to result in activation of a p53-dependent cell cycle arrest in primary cells (Kuerbitz et al., 1992). Proliferation was measured by ^3H-thymidine uptake 24 hours after X-ray irradiation. Cell division in all six Schwann cell clones and late passage Schwann cells pools was inhibited by X-ray treatment (more than 8-fold reduction in ^3H-thymidine uptake compared to mock irradiated cells)
Figure 4.3: Schwann cells maintain expression of active p53
A: Schwann cells at different stages in culture were treated with ALLN (100μM) or carrier for 6 hours before protein was extracted for western blotting. 30μg protein was loaded in each lane and probed with α-p53 and α-p21Cip1. Results are representative of 3 separate experiments.
B: p53 was detected in ALLN treated (as in A) late passage Schwann cells by immunofluorescence. Staining in untreated cells was undetectable.
Figure 4.4: Schwann cells maintain p53-dependent DNA damage response checkpoints.
A: Schwann cell clones, late and early passage pools and dnp53-expressing cells were treated with x-rays (12Gy) or were mock-irradiated. 20 hours after irradiation cells were pulsed with $^3$H-thymidine, 0.5$\mu$Ci/ml, for 4 hours and then $^3$H-thymidine uptake measured. Results are means of triplicate counts.
B: The three immortalized fibroblast lines were analysed similarly to the Schwann cells, as described above.
This response was entirely dependent on p53 activity as a Schwann cell line expressing dn-p53 (Lloyd et al., 1997) was completely insensitive to X-ray exposure.

Similar treatment of the fibroblast lines revealed that Imm 2 had a much reduced p53 response (only ~2 fold reduction in ^H-thymidine uptake compared to mock irradiated cells) (Fig 4.4), suggesting that these cells were likely to be mutated in an effector of this damage pathway. This finding is consistent with the abnormal chromosome numbers (Fig 4.1) and the elevated expression from the INK4A locus (Fig 4.2) observed in this line, as these abnormalities have previously been observed to correlate with defects in p53 pathways (Levine, 1997), (Fukasawa et al., 1996), (Quelle et al., 1995).

**Rat Schwann cells arrest in response to oncogenic Ras:**

Unlike immortalized cell lines, primary cells are resistant to transformation by a single oncogene (Lloyd, 1998). Expression of oncogenic Ras in primary cells results in a p53 and CDKI dependent cell cycle arrest (Serrano et al., 1997), (Lloyd et al., 1997), (Ridley et al., 1988), (Lin and Lowe, 2001), (Paramio et al., 2001). The mechanism by which this arrest is implemented differs between cell type and species (Lloyd, 1998). Although the mechanism of Ras-induced arrest in primary rat Schwann cells is still incompletely understood, it is known to involve p21^Cip1 induction and is dependent on functional p53 (Lloyd et al., 1997). The response of primary rat fibroblasts to oncogenic Ras is likely to resemble that of primary MEFs (Serrano et al., 1997). The Ras induced arrest in primary MEFs is accompanied by a classic senescence phenotype (staining positively for SA-β-gal); requires elevation of active p53, p19^ARF and p15^INK4B and is also associated with elevated levels of p16^INK4A (Palmero et al., 1998), (Serrano et al., 1997), (Malumbres et al., 2000), (Kamijo et al., 1997).

To determine whether rat Schwann cells maintained a negative response to Ras activation, H-Ras V12 was retrovirally expressed in all the Schwann cell clones and early and late passage cells. The proliferative rate was measured by BrdU uptake 5 days after infection. In all cases Schwann cells demonstrated a clear cell cycle arrest in response to oncogenic Ras compared to cells infected with vector alone (Fig 4.5). The arrested cells adopted the characteristic refractile morphology associated with Ras expression (Fig 4.5) (Lloyd et al., 1997) and could not be expanded in culture. A
Figure 4.5: Schwann cells maintain arrest in response to oncogenic Ras.
Early and late passage Schwann cell cultures and Schwann cells expressing dominant negative p53 were retrovirally infected with vectors containing activated Ras or empty vector (LXSN/RasLXSN). Cells expressing activated Ras developed a characteristic morphology (B). After drug selection proliferation rate was measured by BrdU incorporation after a 16 hour BrdU pulse (A). Results are means of triplicate experiments and more than 600 cells were counted per experiment.
Schwann cell line expressing dominant negative p53 (Lloyd et al., 1997) was completely refractory to Ras expression and could be efficiently expanded in culture, confirming the p53-dependence of this response.

Similar analysis of the immortalized fibroblast lines demonstrated that Imm1 and Imm 3 had lost the ability to respond negatively to oncogenic Ras: These Ras-infected cells assumed a classical transformed morphology (Fig 4.6), exhibited loss of contact inhibition and proliferated at a greater rate than the vector control infected cells (Fig 4.6). As Imm1 has lost expression of both the tumour suppressors encoded by INK4a, it seems that rat fibroblasts, similar to MEFs, are also likely to depend on expression at this locus to mediate the Ras-induced arrest (Serrano et al., 1996). Attempts to express H-Ras V12 in primary perineural fibroblasts as a control proved unhelpful as senescent cells began to appear in the cultures before the infection protocol (co-cultivation, purification from producers and drug selection) had been completed. However, surprisingly one of the immortalized lines (Imm 2) maintained a negative response to H-Ras V12. These cells demonstrated a more than 13-fold reduction in proliferation (Fig 4.6). Similar to Ras-infected primary human and mouse fibroblasts (Serrano et al., 1997) the arrested cells adopted a senescent morphology and stained positively for SA-β-Gal. Although it is surprising that these cells maintained this checkpoint, especially as we had demonstrated defects in their DNA-damage-response pathways (Fig 4.4), there is another documented example of an immortalized rat cell line, REF 52 (Franza et al., 1986), (Serrano et al., 1997), which also maintains a negative H-Ras V12 response. It is unclear which immortalizing mutation in Imm 2 cells leaves the checkpoint response to oncogenic Ras intact but results in a defective DNA damage response.
Figure 4.6: Immortalized fibroblast lines are transformed by oncogenic Ras.
The three immortalized fibroblast lines were retrovirally infected with vectors containing activated Ras or empty vector: Ras-Babe-puro (Ras-BP)/Babe-puro (BP).
A: After drug selection proliferation rate was measured by BrdU incorporation after a 16 hour BrdU pulse. Results are means of triplicate experiments and more than 600 cells were counted per experiment.
B: Cells were stained for SA-β-Gal activity after drug selection.
In summary, we have demonstrated that late passage Schwann cells and all the Schwann cell clones maintain diploid status and intact cell cycle checkpoints. These findings indicate that these cells are extremely unlikely to have acquired immortalizing mutations. In contrast all three fibroblast lines examined were found to be defective in their cell cycle regulation:

1) Imm 1 cells have lost expression at the INK4A locus.
2) Imm 2 cells demonstrate defective p53 damage response pathways and have karyotypic abnormalities.
3) Imm 3 cells have lost the ability to arrest in response to activated Ras. These cells have thus undergone mutation in an unidentified oncogene or tumour suppressor that can cooperate with oncogenic Ras to allow transformation.

**B: Do primary rat Schwann cells lack intrinsic replicative barriers?**

The experiments described in part A of this chapter indicate that the unlimited replicative capacity of rat Schwann cells is extremely unlikely to be the result of immortalizing mutations and therefore suggest that there is no intrinsic mechanism limiting the lifespan of this cell type. To address the question of why this cell type differs from other rodent cells that senesce, we compared intrinsic mechanisms that have previously been implicated in regulating replicative lifespan in the Schwann cells and fibroblasts.

The only known cell-intrinsic mechanism for determining entry into senescence is telomere shortening. In cells that lack telomerase expression, telomere shortening occurs with every cell division (Harley et al., 1990), (Hastie et al., 1990), (de Lange et al., 1990) until critically short telomeres initiate entry into senescence. Retroviral expression of telomerase in HDFs was sufficient to overcome telomere-induced senescence and confer immortality in the absence of any other genetic changes (Bodnar et al., 1998), (Yang et al., 1999), (Vaziri et al., 1998).

In all murine cells characterized, telomerase is active and telomeres are very long (Greenberg et al., 1998), (Chadeneau et al., 1995), (Prowse and Greider, 1995). Telomere-based mechanisms have been excluded from limiting replicative capacity of
these cells, because they senesce rapidly despite their excessive telomere reserve and because they are not immortalized by telomerase overexpression (Blasco et al., 1997), (Artandi et al., 2002).

The telomerase status of rat cells has not been well characterized. Furthermore, telomerase activity varies significantly depending on cell type and proliferative status (see introduction) (Collins et al., 2002). Therefore, to determine whether telomere-dependent mechanisms regulate the proliferative capacity of rat Schwann cells and fibroblasts it was important to characterize their telomerase status.

**Rat Schwann cells and fibroblasts maintain active telomerase throughout culture:**

Standard TRAP (Telomere Repeat Amplification Protocol) assays were used to determine whether cultured rat Schwann cells express active telomerase. In these assays cell extracts are incubated with an artificial telomere oligonucleotide template, which, in the presence of telomerase activity is extended by the addition of telomere repeats at the 3' end. The reaction products are amplified by PCR and resolved by running on a gel. The presence of a "ladder" identifies cell extracts containing telomerase activity. The "ladder" represents the various extended forms of the template, each differing by the number of added telomere repeats (i.e multiples of 6bp) (Kim et al., 1994).

Both early passage Schwann cells and perineural fibroblasts were found to express telomerase activity. The level of activity was found to be roughly equivalent in the two cell types by titration of cell number used for extract preparation (Fig 4.7A). It was important to determine whether telomerase activity is maintained at later passages as some cell types downregulate telomerase activity in response to transfer into culture (Wright et al., 1996). Analysis of cells at various stages in culture demonstrated that telomerase activity was maintained in late passage Schwann cells, senescent fibroblasts and the three immortalized fibroblast lines (Fig 4.7B). The telomerase activity remained at approximately constant levels throughout culture and was at least equivalent to that observed in a human tumour cell line used as a positive control (Fig 4.7B).
Figure 4.7: Schwann cells and fibroblasts maintain equivalent levels of telomerase activity throughout culture.
A: Standard TRAP assays were carried out on extracts from early passage Schwann cells (p3) and fibroblasts (p1). Activity was detectable in extracts from 5000 and 500 cells. H = heat inactivated control.
B: TRAP assays were carried out on extracts prepared from 5000 cells of the indicated cell types and passages. Alternate unlabelled lanes are heat inactivated controls.
C = Human tumour cell line positive control.
Rat Schwann cells and fibroblasts maintain long telomeres throughout culture:

Despite the finding that both Schwann cells and fibroblasts maintain high levels of telomerase activity throughout culture, it was important to verify that these levels were adequate to maintain telomere length. Levels of telomerase in haematopoietic cells, which were detectable by standard protocols, were insufficient to maintain telomere length over long time periods (Vaziri et al., 1994; Vaziri et al., 1993), (Chiu et al., 1996).

Accurate methods for measuring telomere length have only been developed relatively recently. Techniques based on southern blotting and telomere-specific oligonucleotide probes require large numbers of cells and are often difficult to interpret due to the smeary results and the contribution of sub-telomeric DNA sequences. This technique is also biased for detection of short telomeres due to the low transfer efficiency of large fragments of DNA and thus is less suited to rodent studies where telomeres are often very long (Zijlmans et al., 1997). As a result, this technique for measuring telomere length has largely been superseded by two methods based on hybridizing fluorescently labeled telomere probes. Q-FISH involves in situ hybridization of fluorescent probes to metaphase spreads followed by calculation of length from the intensities of fluorescent signals and specifically designed algorithms (Lansdorp et al., 1996), (Poon et al., 1999), (Zijlmans et al., 1997). Alternatively, telomere length can be determined in populations of cells by hybridization of fluorescent probes followed by flow cytometry to measure the fluorescence signal. We used an adaptation of a flow cytometry-based protocol (Rufer et al., 1998), (Hultdin et al., 1998).

Cells were hybridized with a telomere specific FITC-labelled PNA (Peptide Nucleic Acid) probe. An FITC-conjugated PNA probe specific for alphoid repeat sequences on the X-chromosome was used as a control for background fluorescence levels (Rufer et al., 1998). Signals obtained using this probe were undetectable by fluorescence microscopy whereas the telomere-specific probe bound specifically to the telomeres at the end of chromosomes and was easily visible (Fig 4.8). The intensity of the fluorescent signal obtained from telomere-bound-PNA can be measured by flow cytometry and correlates tightly with telomere lengths (Rufer et al., 1998), (Hultdin et al., 1998). As this technique does not give an absolute measure of telomere length a
**Figure 4.8:** The PNA telomere probe binds specifically at the end of telomeres. Metaphase spreads were prepared from Imm1 fibroblasts and hybridized with either the FITC-PNA-telomere probe or FITC-PNA-alphoid probe (green). Nuclei were visualized with Hoescht (blue).
standard was required against which telomere lengths could be compared. We used human mammary fibroblasts, HMFs (gift from Mike O'Hare), which do not express telomerase in culture (Fig 4.9A) and undergo senescence after a defined number of passages. This senescent arrest is dependent on telomere shortening, as an HMF line artificially expressing telomerase (HMF-TERT) (gift from Mike O'Hare) has unlimited replicative capacity (personal communication from Mike O'Hare). We established that telomerase negative HMFs underwent senescence (as demonstrated by 100% cells staining for SA-β-Gal - Fig 4.9B) after we had passaged them 15 times. HMF cells at passage 9 were thus used as an example of cells with telomeres approaching critically short lengths. HMF-TERT cells were also included in every telomere length assay as an example of a cell type with sufficiently long telomeres to prevent entry into senescence. All fluorescence signals were expressed relative to those obtained for HMF-TERT samples. As expected, the telomere fluorescence signal from HMF-TERT cells was consistently stronger than the signal from HMF p9 cells (Fig 4.10).

Having established this assay, we used it to determine whether changes in telomere length could be important in regulating replicative lifespan of rat Schwann cells and fibroblasts. Typical profiles obtained in the flow cytometry assay are shown in Fig 4.10A and a graphical summary of at least three separate experiments is shown in Fig 4.10B. Both Schwann cells and fibroblasts maintained approximately equivalent telomere lengths throughout culture. The rat cells not only had much longer telomeres than those of human cells approaching telomere-induced senescence, but also maintained lengths greater than human fibroblasts immortalized by TERT expression. The telomere lengths of both rat cell types are thus more than sufficient to sustain long-term proliferation in culture. Therefore, differences in telomere length are unlikely to explain the different replicative capacities of these cells.

In summary, we have shown that rat Schwann cells and fibroblasts maintain high levels of telomerase activity during culture. This telomerase activity maintains telomeres at constant lengths that are sufficient to sustain long-term proliferation. We cannot exclude the possibility that one chromosome within the cell has significantly shorter telomeres that would be undetectable by our methods. As the shortest telomere rather than average telomere length appears to determine cellular viability, this could be an important consideration (Hemann et al., 2001). However, as telomerase levels were sufficient to maintain average telomere length and, as telomerase preferentially extends
Figure 4.9: Human mammary fibroblasts approaching senescence are an example of cells with critically short telomeres.

A: HMFs do not express telomerase activity in culture. Standard TRAP assays were carried out on extracts prepared from 5000 HMFs or RFs. H = Heat inactivated control.

B: HMFs that do not express telomerase senesce in culture. HMFs and HMFs stably expressing exogenous hTERT were stained for SA-β-Gal at pH 6 (8 hours). HMFs passaged 15 times in our laboratory stained positively.
Figure 4.10: Rat Schwann cells and fibroblasts maintain long telomeres in culture.
Figure 4.10: Rat Schwann cells and fibroblasts maintain long telomeres in culture.

A: Cells were fixed and hybridized with either alphoid-specific or telomere-specific FITC-conjugated PNA probes. Propidium iodide staining was used to gate nuclei during flow cytometry analysis. Intensity of 5000 gated nuclei was measured by flow cytometry and representative profiles are illustrated in A.

x-axis = fluorescence intensity; y-axis = number of counts.

B: Quantification of results from at least three independent experiments. Mean FITC intensity was determined for each sample by subtracting the mean intensity of the alphoid signal for that sample. Intensities were expressed relative to that of HMF-TERT expressing cells (defined as having an intensity = 1)
short telomeres (Hemann et al., 2001), it seems unlikely that such an unusually short telomere would reach critical levels.

Our findings would suggest that replicative lifespan in cultured rat Schwann cells and fibroblasts, like in all other rodent cells characterized, is not dependent on telomere-based mechanisms. This intrinsic mechanism therefore cannot explain the difference in replicative potentials between these cell types.

**Schwann cells induce p16^INK4A in culture:**

The progressive upregulation of CDKIs is a common feature observed in cultured primary somatic cells as they approach the end of their proliferative lifespan. In most cell types accumulation of p16^INK4A (inhibitor for cyclin D/CDK4/6 complexes) and p21^Cip1 (inhibitor of CDK2 complexes) is observed. Levels of p16^INK4A (Zindy et al., 1997), (Kamijo et al., 1997) and p19^ARF (Zindy et al., 1998) increase upon transfer of MEFs into culture. The accumulation of p19^ARF as MEFS approach senescence is required to induce the senescent arrest (Kamijo et al., 1997), whereas, despite being upregulated, p16^INK4A is not required for the arrest: MEFs from p16^INK4A knockouts senesce at the same rate as wild type cells (Sharpless et al., 2001), (Krimpenfort et al., 2001). Because CDKI upregulation occurs in all cells approaching senescence and because these CDKIs appear to play an essential role in limiting replicative capacity, we were interested to investigate whether these cell cycle inhibitors were also upregulated in cultured rat Schwann cells. Expression of both the cell cycle inhibitors encoded by the INK4A locus was analysed in Schwann cells and perineural fibroblasts. Neither of these genes was expressed in either cell type in the intact nerves of p7 rats (Fig 4.11 B and C) (negative result was confirmed by overexposure of autoradiographs). Unsurprisingly, similar to the situation in MEFs, p16^INK4A was induced in the perineural fibroblasts as they neared senescence: p16^INK4A protein first became evident at about passage 3, concurrent with entry of the fibroblasts into senescence and then increased until passage 7 when levels stabilized (Fig 4.11 A and B). Similarly, p19^ARF was also induced in cultured fibroblasts: mRNA was detectable almost immediately upon transfer into culture (Fig 4.11 C). Surprisingly, both cell cycle inhibitors were also induced in Schwann cells, despite the maintenance of constant proliferative rates in these cells (Fig 4.11 A,B,C). As in the fibroblasts, p19^ARF mRNA was evident almost
Figure 4.11: p16^{INK4A} and p19^{ARF} are induced in cultured Schwann cells and fibroblasts:
A: Analysis of p16^{INK4A} protein expression by western blotting.
B: Analysis of p16^{INK4A} mRNA expression by semi-quantitative RT-PCR.
C: Analysis of p19^{ARF} mRNA expression by semi-quantitative RT-PCR.
D: Analysis of p21^{CIP1} protein expression by western blotting.
All results are representative of at least three independent experiments. 30μg protein was analysed in western blotting. mRNA levels were equalized for GAPDH.
N = extract from sciatic nerve.
immediately, but upregulation of p16\textsuperscript{INK4A} protein was not detectable until p8. After the initial induction the levels of p16\textsuperscript{INK4A} protein and p19\textsuperscript{ARF} mRNA remained stable. The maximal levels of p16\textsuperscript{INK4A} and p19\textsuperscript{ARF} induced in Schwann cells were approximately equivalent to those observed in fibroblasts. p21\textsuperscript{Cip1} is not induced in cultured MEFs (Pantoja and Serrano, 1999). Similarly, levels of p21\textsuperscript{Cip1} were consistently equally low in both the Schwann cells and fibroblasts (Fig 4.11 D). Slight variations in p21\textsuperscript{Cip1} levels were observed in both cell types but there was no consistent trend that correlated with passage number.

Schwann cells express elevated levels of Cyclin D1, CDK4 and CDK6 compared to perineural fibroblasts:

The finding that Schwann cells and fibroblasts upregulated CDKIs to equivalent levels demonstrated that differential induction of cell cycle inhibitors cannot explain the different replicative capacity of these cells. To investigate why Schwann cells and fibroblasts respond differently to elevated CDKI expression, the levels of the positive cell cycle regulators that are inhibited by p16\textsuperscript{INK4A} were determined. p16\textsuperscript{INK4A} specifically binds to CDK4 and CDK6, preventing their association with cyclin D complexes and thus inhibiting kinase activity (Ruas and Peters, 1998). This disruption of cyclin D/CDK4/6 complexes also results in displacement of Cip/Kip CDKIs onto CDK2 complexes and thus has a secondary inhibitory effect on CDK2 activity (McConnell et al., 1999).

A comparison of fibroblasts and Schwann cells demonstrated that levels of cyclin D1, CDK4 and CDK2 were several fold higher in Schwann cells (Fig 4.12). This finding presents the possibility that Schwann cells are "resistant" to elevated p16\textsuperscript{INK4A} levels because the targets of this inhibitor in this cell type are expressed in excess to those in other cells which senesce in culture. To confirm this hypothesis it would be necessary to analyse the proportion of CDKs bound in active cyclin complexes and the inactive proportion bound by p16\textsuperscript{INK4A} in both Schwann cells and fibroblasts. In addition kinase activities of immunoprecipitated complexes from these cells should be compared.
Figure 4.12: Schwann cells express higher levels of Cyclin D1, CDK4 and CDK2 than fibroblasts:
Lysates prepared from Schwann cells and fibroblasts at passage 10 were analysed for expression of the proteins indicated by western blotting.
Results are representative of three experiments.
30μg protein was loaded in each lane.
S = Schwann cell  F = Fibroblast.
In summary, we have demonstrated that the unlimited replicative capacity of cultured rat Schwann cells is extremely unlikely to be conferred by immortalizing mutations. Moreover, as telomere shortening does not seem to regulate proliferation in rodent cells and as no differences in telomere length or telomerase activity were found between Schwann cells and fibroblasts, the basis of Schwann cell immortality cannot be linked to telomere control.

Interestingly, we observed that rat Schwann cells, like all other cultured primary somatic cells, upregulated CDKIs with time in culture. This finding would suggest that Schwann cells are responsive to the same signals that induce senescence in other cell types. However, in rat Schwann cells these signals are not translated into a senescence response. A possible explanation for this difference is suggested by our findings that Schwann cells naturally express elevated levels of positive cell cycle regulators compared to the fibroblasts. Similarly, rat OPCs, which do not senesce in culture, also induce CDKIs (p21^{CIP1}, p27^{KIP1}, p57^{KIP2}, p18^{RbK4C} and p19^{RbK4D}). These cells also express high levels of cyclins and CDKs (Cyclins D1, D2, D3, E and A and CDK2 and CDK4) compared to perineural fibroblasts (Tang et al., 2001)). Further investigations based on this finding may increase our understanding of why different rat cell types have different replicative capacities.
Chapter 5: Proliferative capacity and $p16^{INK4A}$ induction in Schwann cells depend on external culture conditions and not an intrinsic mitotic timer.

Culture conditions determine Schwann cell proliferative capacity:

To date, telomere shortening is the only known cell-intrinsic mechanism of limiting replicative capacity. However, the finding that many cell types, artificially or naturally expressing telomerase, (e.g. human epithelial cells and most rodent cell types) undergo telomere-independent senescence has led to the assumption that other unidentified intrinsic mechanisms are responsible for limiting the replicative lifespan of these cells (Kiyono et al., 1998), (Farwell et al., 2000), (Dickson et al., 2000), (Blasco et al., 1997), (Artandi et al., 2002).

Recently, it has become clear that senescence is not exclusively triggered in response to cellular division: an identical senescent phenotype can be induced at any point in a cells' replicative history by external insults such as oxidative stress, DNA damaging agents, and oncogenic stress (Chen and Ames, 1994), (Severino et al., 2000), (Robles and Adami, 1998), (Serrano et al., 1997). Many similar cellular stresses may be encountered in the unphysiological tissue culture environment. It is generally acknowledged that the conditions in which cells are grown in vitro differ greatly from the environment they encounter in vivo (Rubin, 1997): Pure cultures of cells are grown in two dimensions on plastic dishes, whereas the same cell types in the body interact in three dimensions with a variety of different cell types. Cells grown in vitro are maintained in 20% oxygen whereas physiological oxygen concentrations are much lower (2-5%). The majority of cell types are cultured in high levels of serum that contains a rich mixture of mitogens not normally encountered in the body, except in cases of wounding. Finally the regular process of trypsinizing and replating that is required to propagate cells in culture is a stress never encountered in vivo. It is thus likely that this artificial environment induces cellular stress responses (Rubin, 1997), (Rubin, 2002), many of which resemble those shown to rapidly induce a senescent phenotype. In support of this hypothesis, transfer of most cells into culture results in
altered gene expression profiles to those observed *in vivo* and many of these changes in gene regulation can be correlated with cellular stress responses. For example, it has been suggested that the upregulation of p53, the central mediator of cellular damage responses, in cultured cells may be a response to the hostile tissue culture environment (Mendrysa and Perry, 2000), (Wright and Shay, 2002). Furthermore, that cells accumulate stress in culture has been directly demonstrated by the finding that levels of cellular oxidative damage increase with time spent in culture (Chen et al., 1995).

Although it has been generally assumed that a cell intrinsic division counting mechanism similar to telomere shortening limited the lifespan of rodent cells, there is very little evidence in favour of such a mechanism. In fact, in many cases such an intrinsic limit (that only allows 8PDs in cultured MEFs) would be inconsistent with the proliferative requirements of cells *in vivo*. We speculated that, as external stresses can induce senescence, perhaps the senescence occurring in cultured rodent cells could be a response to the external stresses encountered in the tissue culture environment and may have been misinterpreted as an intrinsic replicative barrier. The lack of an intrinsic replicative limit that we had demonstrated in rat Schwann cells could thus be a more widespread feature in other rodent cells. Although others have also proposed that senescence can result from "Culture Shock" rather than an intrinsic "mitotic clock", direct proof in its favour remained to be established (Sherr and DePinho, 2000). As we speculated that the differences in replicative capacities between rat Schwann cells and fibroblasts are unlikely to reflect different intrinsic mechanisms of regulating lifespan, but are more likely to reflect responses to the external culture environment, we cultured these cells in a variety of different conditions to test this hypothesis.

**Schwann cells demonstrate a senescent phenotype when cultured in high serum concentrations:**

In chapter 3 we showed that Schwann cells have unlimited replicative capacity when cultured in low serum concentrations in the presence of specific Schwann cell mitogens. One similarity between the culture conditions used to expand the rat Schwann cells, and rat OPCs and mouse neuroepithelial stem cells that also divide indefinitely, is the absence or low concentration of serum (Tang et al., 2001), (Loo et al., 1987). In contrast, most other cell types that senesce, are cultured in higher serum
concentrations (routinely 10%). On the basis of this observation we were interested to
determine whether culturing Schwann cells in elevated serum concentrations would
affect their replicative capacity. Therefore, we isolated and cultured primary rat
Schwann cells in medium containing 20% serum.

Schwann cells grown in 20% serum proliferated at a greater rate (as measured
by BrdU incorporation) than when cultured in 3% serum (Fig 5.1). However, after
about two weeks in these conditions large, flattened multinucleate cells with a
characteristic senescent morphology began to appear (Fig 5.2A). These cells stained
positively for the senescence marker SA-β-Gal activity (Fig 5.2B). Therefore,
senescence in Schwann cells can be induced by long-term proliferation in high serum.
These findings have been corroborated in rat oligodendrocyte precursors that are
normally grown in defined conditions in the absence of serum. Transferring these cells
into serum-containing medium also induced senescence (Tang et al., 2001). These
results provide the first indications that extrinsic factors can determine cellular
replicative capacity.

**Appropriate culture conditions to extend fibroblast proliferative lifespan have not yet been discovered:**

The finding that senescence in rat Schwann cells can be induced by proliferation
in high serum suggests that the senescence observed in perineural fibroblasts could also
be triggered by extrinsic factors encountered in the culture environment. To test this
hypothesis we attempted to optimize fibroblast culture conditions to extend replicative
lifespan. Rat glial cell types have unlimited replicative capacity when cultured in low
serum or serum free conditions but senesce in the presence of high serum concentrations
(Tang et al., 2001). On the basis of these findings it seemed reasonable to culture
fibroblasts in low serum or in completely defined serum-free conditions. Despite
investigating a variety of commonly used serum-free conditions (Celis, 1998), (Loo et
al., 1987) (Table 5.1) none were found that would sustain survival and proliferation of
perineural fibroblasts. A more extensive search for defined fibroblast conditions is
beyond the scope of this thesis although it is of interest that numerous other attempts to
culture fibroblasts in the absence of serum have also not succeeded (C Sherr personal
communication).
Figure 5.1: Schwann cells proliferate at a greater rate in high serum concentrations:
A: Schwann cells at passage 15 that had been continuously cultured in 3% serum or 20% serum were incubated with BrdU for different time periods before being fixed and stained with α-BrdU. The percentage of cells that had incorporated BrdU at each time point was calculated and is represented graphically. Each point represents the mean of counts for cells on three coverslips. 400 cells were counted on each coverslip. The results are representative of two independent experiments.
Figure 5.2: Schwann cells grown in 20% serum develop a senescence phenotype.
A: Morphology of Schwann cells continuously grown in 20% serum (after 2 weeks) compared to cells in 3% serum.
Scale bar: 60μm.
B: Schwann cells grown in 20% serum (2 weeks) and cells in 3% serum stained for SA-β-Gal activity (8 hour stain).
Scale bar: 30μm.
Table 5.1: Perineural fibroblasts do not proliferate when cultured in different serum free conditions. Freshly isolated perineural fibroblasts were transferred into the culture conditions indicated above. Attempts to maintain survival for more than one passage in these conditions were unsuccessful. SATO is defined medium containing transferrin (100 µg/ml), insulin (10 µg/ml), selenium (40 ng/ml), BSA (100 µg/ml), putrescine (16 µg/ml), thyroxine (50 ng/ml), triiodothyronine (50 ng/ml) and progesterone (60 ng/ml) (Bottenstein and Sato, 1979).
These findings provide the first direct evidence that varying culture conditions can alter cellular proliferative capacity: rat Schwann cells divide indefinitely in low serum, but senesce in high serum concentrations. These results raise the possibility that senescence in all cell types expressing telomerase could be a response to external stimuli rather than a response to an intrinsic mitotic counter. To definitively prove this hypothesis it would be necessary to identify optimal culture conditions to allow unlimited proliferation of these cell types. Although our attempts to identify optimal conditions for the fibroblasts were unsuccessful, it is possible that, by testing a greater variety of conditions, an appropriate culture environment for extended proliferation may be identified.

These findings in rat glial cells have since been corroborated by studies in certain human cell types. Recently, it has been demonstrated that telomere-independent senescence observed in certain cultured human cell types is also a response to the culture environment: artificial overexpression of TERT in human foetal lung fibroblasts, mammary epithelial cells and keratinocytes was insufficient to allow immortalization of these cells (Ramirez et al., 2001), (Wright and Shay, 2002). However, when these cells expressing telomerase were grown in altered culture conditions they could be expanded indefinitely. The improved culture conditions involved growing the epithelial cells on feeder layers and maintaining the fibroblasts in lower oxygen concentrations. These human cells were capable of undergoing numerous population doublings (50PDs in the fibroblasts) before entering culture-induced senescence. Growth in sub-optimal conditions may therefore lead to a gradual accumulation of cellular damage that ultimately induces senescence.
p16\textsuperscript{INK4A} induction in rat Schwann cells is dependent on culture conditions:

The progressive induction of CDKIs as cells approach senescence is observed in all cell-types, irrespective of whether senescence is telomere dependent or independent (Alcorta et al., 1996), (Hara et al., 1996), (Zindy et al., 1997), (Zindy et al., 1998). In Chapter 4 we demonstrated that rat Schwann cells cultured under standard conditions progressively upregulate p16\textsuperscript{INK4A} with time in culture, despite lacking any intrinsic replicative limit. This interesting finding might suggest that, although the culture conditions are optimised to allow indefinite Schwann cell proliferation, these cells are still exposed to the same types of stimuli that can induce CDKIs and ultimately senescence in other cultured cells.

Unlike many other CDKIs that seem to play important roles in development (for example p27\textsuperscript{Kip1} (Durand et al., 1998), the physiological function of p16\textsuperscript{INK4A} has remained obscure, even though it is clear that it is an important tumour suppressor (Ruas and Peters, 1998), (Sharpless et al., 2001), (Krimpenfort et al., 2001). Studies in adult mice revealed that p16\textsuperscript{INK4A} expression is increased and becomes more widespread with age (Zindy et al., 1997), suggesting that p16\textsuperscript{INK4A} induction both \textit{in vivo} and \textit{in vitro} may occur as a function of cellular ageing.

Whereas signals responsible for regulating other CDKIs (for example p53-dependent p21\textsuperscript{Cip1} induction (el-Deiry et al., 1993) and TGF\textbeta-dependent p15\textsuperscript{INK4B} elevation (Li et al., 1995), (Massague and Wotton, 2000) have been characterized, very little is known about what intra/extracellular stimuli may be responsible for p16\textsuperscript{INK4A} induction. In fact, upregulation of p16\textsuperscript{INK4A} upon division in culture appeared to be the stimulus associated most tightly with induction of this CDKI (Ruas and Peters, 1998) and it has thus been suggested that the primary role of this CDKI is to regulate proliferative lifespan. This role was thought to be particularly important in human epithelial cells that undergo telomere-independent senescence. In these cells senescence is accompanied by considerable increases in p16\textsuperscript{INK4A} expression and the majority of cells that emerge from this arrest have specifically downregulated p16\textsuperscript{INK4A} by promoter methylation, suggesting that this telomere-independent arrest is dependent on p16\textsuperscript{INK4A} (Kiyono et al., 1998), (Jarrard et al., 1999), (Farwell et al., 2000), (Dickson et al., 2000), (Foster et al., 1998). In rodent cells, which also undergo telomere-independent senescence, the role of p16\textsuperscript{INK4A} in regulating cellular lifespan has been determined by
examining MEFs derived from the p16^{INK4A} specific knockout mouse. These cells senesce at the same rate as wild type cells, demonstrating that any role that p16^{INK4A} play in senescence of MEFs can only be contributory (Sharpless et al., 2001), (Krimpenfort et al., 2001).

On the basis of the commonly held belief that all cells possess intrinsic replicative barriers and on the evidence supporting a contributory role for p16^{INK4A} in inducing senescence, it was generally thought that the progressive induction of p16^{INK4A} in culture formed part of an intrinsic mitotic counting mechanism: the level of p16^{INK4A} expression was thought to directly correspond to the number of cellular divisions that had occurred (Jones et al., 2000), (Dickson et al., 2000).

If p16^{INK4A} induction is a response to a mitotic timer, it would appear that Schwann cells maintain this intrinsic mechanism although they do not respond with a senescent arrest. Alternatively, we speculated that, as Schwann cell replicative capacity is not determined by intrinsic mechanisms but instead is dependent on external stimuli, the induction of p16^{INK4A} in these cells could also be a response to extrinsic signals. Interestingly, rat OPCs, which also lack intrinsic replicative limits, do not induce p16^{INK4A} when cultured in the absence of serum (Tang et al., 2001). As serum exposure determines the replicative capacity of both Schwann cells and OPCs, we were interested whether the differential induction of p16^{INK4A} in these cells might reflect differences in their serum exposure: Schwann cells were maintained in serum whereas the OPCs were never exposed to serum. Therefore, to investigate whether p16^{INK4A} induction might be a response to the culture environment, the levels of this CDKI were investigated in Schwann cells grown both in the complete absence of serum, or in the high serum concentrations that induce senescence in these cells.

Schwann cells can be cultured in the complete absence of serum:

To analyse the effect of culture in complete absence of serum, defined serum-free conditions were established in which Schwann cells could be expanded in vitro. Schwann cells were isolated from sciatic nerves in the absence of serum. These cells could be cultured on laminin in defined medium (SATO) with added Schwann cell mitogens (GGF and forskolin (Bottenstein et al., 1979), (Maurel and Salzer, 2000), (Howe and McCarthy, 2000). Trypsinization was carried out using trypsin inhibitor to
prevent any exposure to serum. Cells were passaged once a week and replated at high density. Under these conditions Schwann cells appeared to proliferate indefinitely at a constant rate (maintained in culture for at least 26 passages, 126 days).

**Determination of proliferative rates of Schwann cells cultured in different serum concentrations:**

To investigate p16<sup>INK4A</sup> induction in different serum concentrations, Schwann cells were isolated from rats and cultured in the absence of serum in the conditions described above (SATO), in standard culture conditions containing 3% serum and in 20% serum. As the concentration of serum is likely to influence the rate of cell division, it was important to determine the proliferative rate in these three conditions to allow comparisons of p16<sup>INK4A</sup> levels at equivalent population doublings. However, accurate determination of proliferative rate by counting cell number at trypsinization proved difficult in serum-free conditions because cells tended to aggregate and form clumps during trypsinization, which reduced replating efficiencies. The proliferation rates determined from these cell counts were thus under-representative of real division rates. Therefore rates of BrdU incorporation were measured to obtain accurate comparisons of proliferative rate. BrdU was added to cells that had been plated at the appropriate density on the previous day.

Schwann cells were found to proliferate at almost identical rates in 3% serum and in the defined serum-free conditions: Maximal BrdU incorporation occurred after approximately the same time in both conditions (Fig 5.3A). Schwann cells grown in 20% serum divided much more rapidly, with maximal labelling occurring at least 20 hours in advance of the 3% and serum-free cells. Approximations of cell cycle times in the different serum concentrations (calculated as described in (van Heyningen et al., 2001)) were 43 hours, 48 hours and 19 hours for cells in SATO, 3% and 20% respectively (Fig 5.3B).
Figure 5.3: Schwann cell proliferation rate depends on serum concentration:
A: Schwann cells at passage 15 that had been continuously cultured in serum free conditions (SATO), 3% serum or 20% serum were incubated with BrdU for different time periods before being fixed and stained with α-BrdU. The percentage of cells that had incorporated BrdU at each time point was calculated and is represented graphically. Each point represents the mean of counts for cells on three coverslips. 400 cells were counted on each coverslip. The results are representative of two independent experiments.
B: Enlargement of first four time points in A to allow estimation of cell cycle time from best-fit lines. Cell cycle time = 1/gradient (Van Heynigen et al, 2001)
p16INK4A induction is significantly delayed in Schwann cells grown in the absence of serum:

A comparison of levels of p16INK4A and p19ARF protein in Schwann cells grown in serum-free conditions and in 3% serum revealed that, while both of these cell cycle inhibitors were readily detectable in 3% cells, they were completely undetectable (p16INK4A) or negligible (p19ARF) in SATO cells that had undergone significantly more divisions (compare Day 56 in SATO with Day 27 in 3%) (Fig. 5.4A). After extensive passaging of cells in SATO, low levels of p16INK4A mRNA eventually became detectable. However, these levels were considerably lower than in cells cultured in 3% serum, although the SATO cells had undergone more than double the PDs of 3% cells (compare Day 102 in SATO with Day 42 in 3%) (Fig. 5.4B). Analysis of serum-free cells at later stages in culture would be required to determine whether p16INK4A RNA is eventually induced to the levels observed in late passage 3% cells.

p16INK4A is induced more rapidly and to higher levels in Schwann cells cultured in high serum concentrations:

A comparison of the levels of p16INK4A mRNA in Schwann cells grown in 3% and 20% serum revealed that p16INK4A was induced more rapidly in cells grown in the higher serum concentration: p16INK4A is first detectable at PD8 in 20% cells but does not become visible until PD12 in 3% cells (Fig 5.5). Furthermore, p16INK4A is induced to higher levels in the 20% cells: in 3% cells, after the initial induction, levels of p16INK4A stabilize after about 16 - 18PDs (~passage 11-12/day 33-36) (Fig 5.4B, Fig 3.8). However, in 20% serum the levels of p16INK4A continue to increase until at least PD28 and reach considerably higher levels than those expressed in 3% cells (compare PD28 in 20% with PD21 in 3%). It is possible that with further passaging in 20% serum p16INK4A levels may increase even further. Alternatively, levels may ultimately stabilize as they do in the 3% cells.

Due to lack of a suitable antibody it was not possible to analyse p16INK4A induction at the cellular level in different serum concentrations. It would be interesting to determine whether the observed induction corresponds to elevated levels in all cells.
**Figure 5.4:** p16\(^{INK4A}\) and p19\(^{ARF}\) induction in Schwann cells is delayed in the absence of serum.

A: p16\(^{INK4A}\) and p19\(^{ARF}\) protein expression analysed by western blotting. 30μg protein were loaded in each lane.

All results are representative of at least two experiments.
d = day in culture.

B: p16\(^{INK4A}\) mRNA expression in Schwann cells cultured in 3% serum or in absence of serum analysed by semi-quantitative RT-PCR. Levels of RNA were equalized for GAPDH.

RT- = Reverse transcriptase negative control.
Figure 5.5: p16\textsuperscript{INK4A} is induced to higher levels in Schwann cells cultured in high serum concentrations: p16\textsuperscript{INK4A} expression in Schwann cells cultured in 3% serum and 20% serum, analysed by semi-quantitative RT-PCR. RNA extracts were equalized for GAPDH levels. RT- = Reverse transcriptase negative control. Results are representative of three independent experiments repeated for different batches of cells.
or an increased proportion of those expressing p16\(^{INK4A}\), and, in the 20% cells how this correlates with entry into senescence. It is possible that the senescent Schwann cells observed in 20% cultures express an elevated threshold level of p16\(^{INK4A}\) sufficient to trigger senescence.

In summary, these results demonstrate that the rate at which p16\(^{INK4A}\) is induced and the level at which it is ultimately expressed are dependent on the serum concentration in which Schwann cells are cultured but is independent of the rate of division. Therefore, at least in cultured Schwann cells, a cell-intrinsic mitotic counter is not involved in p16\(^{INK4A}\) regulation. Induction of this CDKI is more likely to reflect a response to accumulated stress after prolonged time in unfavourable environments.

Our findings have been corroborated by recent work demonstrating that p16\(^{INK4A}\) induction can also be uncoupled from cell division in certain human epithelial cells: human mammary epithelial cells and keratinocytes cultured on plastic induce p16\(^{INK4A}\) and undergo a senescent arrest that is p16\(^{INK4A}\)-dependent but telomere-independent (Ramirez et al., 2001), (Kiyono et al., 1998), (Dickson et al., 2000), (Foster et al., 1998). Culturing these cells on feeder layers delays p16\(^{INK4A}\) induction and allows proliferation until the telomere limit (Ramirez et al., 2001).

The work described here provides a basis to further our understanding of the signals induced by the extrinsic factors that regulate proliferative lifespan in rodent cells. Our findings that p16\(^{INK4A}\) induction is a response to extracellular conditions suggest that levels of this CDKI could be used as a marker for activation of culture-induced checkpoints. Schwann cells cultured in SATO and 3% serum provide a valuable comparative system that could be used to investigate these checkpoints. The advantage of this system is that these cells divide at similar rates and do not senesce, yet they differentially activate checkpoints in response to their culture conditions. Therefore, using these culture conditions, it will be possible to distinguish the cell cycle regulators involved in activating culture-induced checkpoints from those involved in controlling normal proliferation rates or those altered secondary to a senescent arrest. This system will thus provide an invaluable tool with which to further investigate the antiproliferative signals induced by division in culture.
Chapter 6: Discussion

6.1: Updating views on senescence:

Since Hayflick first defined cellular senescence forty years ago, it has become generally accepted that somatic cultured cells have intrinsic replicative limits. Indeed, this finite replicative capacity was used as one of the defining features of primary somatic cells and was commonly used to distinguish primary cells from cell lines that had acquired immortalizing mutations. Replicative limits were thought to be important in protecting against tumorigenesis and had also been suggested to play a role in organismal ageing. To date, the only known intrinsic mechanism of limiting cellular proliferative capacity is telomere shortening. The telomere independent senescence seen in some human cell types and in rodent cells was thought to be induced by an undefined cell intrinsic mitotic counter, linked to the progressive upregulation of CDKIs.

In this thesis we have challenged many of these assumptions by demonstrating that:

1) primary rat Schwann cells divide indefinitely in culture whilst maintaining intact cell cycle checkpoints. These findings challenge the dogma that all primary somatic cells have an intrinsic replicative limit, demonstrating that a finite replicative capacity can no longer be used as a defining feature of primary somatic cells.

2) primary rat Schwann cell replicative capacity can be determined by culture conditions. Our finding that Schwann cells senesce in elevated serum concentrations demonstrates that the only replicative limits imposed on this cell type are those induced by the external environment. We have presented the first experimental evidence in favour of the hypothesis that "culture shock" determines proliferative limits in some cells.

3) the rate of p16INK4A induction in Schwann cells can be uncoupled from proliferative rates by altering culture conditions. Therefore p16INK4A induction in primary rat Schwann cells is dependent on external conditions and not an intrinsic timer.
The work presented in this thesis in combination with a similar study, carried out in collaboration with Tang et al, in rat oligodendrocyte precursor cells and with later studies in certain human cell types has led to an updated model of senescence (illustrated in figure 6.1). Senescence can be induced by intrinsic factors, termed Replicative Senescence, or extrinsic factors, termed Premature Senescence (because it can curtail any intrinsic replicative capacity). Replicative senescence induced by telomere shortening has been characterized in human diploid fibroblasts, which are immortalized by telomerase expression alone (Bodnar et al., 1998), (Yang et al., 1999), (Vaziri and Benchimol, 1998). Premature senescence was originally identified in response to acute insults such as treatment with agents that induce DNA damage or oxidative stress, or by expression of oncogenic Ras (Robles and Adami, 1998), (Severino et al., 2000), (Toussaint et al., 2000), (Serrano et al., 1997). We find that in rat Schwann cells and OPCs (which naturally express telomerase) there are no intrinsic replicative limits (Tang et al., 2001). However, the environment in which these cells are cultured can determine their proliferative capacity. Culture in high serum levels resulted in the induction of premature senescence in these cells.

Similar findings have been demonstrated in certain human cell types. Human keratinocytes, mammary epithelial cells and foetal lung fibroblasts in their standard culture conditions undergo telomere independent senescence as was demonstrated by the inability to immortalize these cells by telomerase expression. However if the epithelial cells are grown on feeder layers and the fibroblasts are cultured in low oxygen tensions they divide until they reach their telomere limit. Telomerase expression under these conditions is sufficient to allow immortalization (Ramirez et al., 2001), (Wright and Shay, 2002). Therefore in five different cell types culture conditions have been shown to be responsible for inducing premature senescence. This culture induced premature senescence has been termed "culture shock" by some researchers in the field (Sherr and DePinho, 2000).

In telomerase positive rodent cell types where the stimulus for senescence is unknown (e.g. rodent fibroblasts), proliferative lifespan may be determined by an as yet uncharacterized intrinsic mitotic timer. Alternatively senescence in these cells may be a similar form of premature senescence to that observed in rat OPCs, rat Schwann cells, human epithelial cells and human foetal lung fibroblasts under certain culture
Figure 6.1: Pathways to senescence:
Cellular senescence may be induced in response to cell intrinsic mechanisms (Replicative Senescence), or in response to extrinsic factors (Premature Senescence). Agents that induce DNA damage, oxidative stress, or expression of oncogenic Ras can induce premature senescence. Telomere shortening induces replicative senescence. Human dermal fibroblasts (-TERT) undergo telomere-dependent replicative senescence and can be immortalized by telomerase expression (+TERT). Human foetal lung fibroblasts and epithelial cells and rat OPCs and Schwann cells (which naturally express telomerase) cultured in certain conditions undergo telomere-independent senescence. Altering the culture conditions for these cell types allows the rat cells to divide indefinitely and the human cells to divide until they reach their telomere limit. Telomerase expression (+TERT) in the human cells in these optimal conditions allows immortalization. Therefore senescence in these cells can be a response to the culture environment, termed "Culture Shock". It is unclear whether senescence in telomerase expressing rodent cells (e.g., fibroblasts) is due to culture shock (premature senescence) or due to an intrinsic timer (replicative senescence). If extrinsic factors are involved, it is possible that these cells could divide indefinitely in optimized culture conditions.
conditions. If this is the case it is possible that optimal culture conditions could be found for these cell types that would allow their unlimited proliferation.

Before culture-induced senescence was identified as limiting the proliferation of some cell types, intrinsic mitotic timers were thought to determine the proliferative capacity of all primary somatic cell types. In cells that lacked telomerase activity telomere shortening provided this intrinsic mechanism. However in cells that maintained telomere length the mitotic timer remained unidentified. It was observed that p16\(^{INK4A}\) was progressively upregulated in these cells as they proliferated in culture (Zindy et al., 1997). Therefore, although the signals responsible for p16\(^{INK4A}\) induction in telomerase expressing cells were poorly understood, it was thought that this progressive upregulation formed part of the unidentified mitotic timer that limited the proliferation of these cells.

We found that p16\(^{INK4A}\) was also induced in cultured rat Schwann cells. However, this induction was not dependent on a mitotic timer, but instead was determined by culture conditions. From these findings, we would propose the model illustrated in Figure 6.2 in which p16\(^{INK4A}\) induction directly correlates with activation of the pathways responsible for activating the "culture shock" checkpoint. When Schwann cells are cultured in 3% serum they are exposed to factors in the culture environment that lead to limited activation of cell cycle checkpoints as demonstrated by a moderate induction of p16\(^{INK4A}\). However, this level of signalling is insufficient to induce withdrawal from the cell cycle and thus senescence. When Schwann cells are cultured in 20% serum the level of antiproliferative signalling is increased as demonstrated by the rapid induction of p16\(^{INK4A}\) to high levels. In a proportion of cells activation of checkpoints to this extent is sufficient to induce senescence. In contrast, when Schwann cells are cultured in the absence of serum the exposure to checkpoint activating signals is significantly reduced although not completely absent, as these cells ultimately induce a very low level of p16\(^{INK4A}\).

Our findings relating p16\(^{INK4A}\) induction to culture conditions have been corroborated by recent work in human epithelial cells. In human keratinocytes and mammary epithelial cells grown on plastic, p16\(^{INK4A}\) is induced rapidly and to high levels, peaking when these cells enter culture-induced senescence.
Figure 6.2: \( p_{16}^{INK4A} \) is induced in response to culture conditions and not an Intrinsic timer in some primary cells

The rate and level to which \( p_{16}^{INK4A} \) is induced in rat Schwann cells and human epithelial cells is dependent on their culture conditions. In rat Schwann cells the concentration of serum in the culture medium regulates \( p_{16}^{INK4A} \) induction whereas in human epithelial cells the substratum these cells are cultured on determines the \( p_{16}^{INK4A} \) induction rate. The same extrinsic factors also regulate the replicative capacities of these cell types. Therefore \( p_{16}^{INK4A} \) induction directly correlates with activation of the premature senescence checkpoint in rat Schwann cells and human epithelial cells.
Figure 6.2: p16\textsuperscript{INK4A} is induced in response to culture conditions and not an intrinsic timer in some primary cells.
(Ramirez et al., 2001). If culture-induced senescence is avoided by culturing these cells on feeder layers the induction of p16\textsuperscript{INK4A} is delayed until these cells approach their telomere limit (Fig 6.2). Similar to rat Schwann cells, the replicative capacity of rat OPCs is dependent on exposure to serum in the culture environment. The finding that p16\textsuperscript{INK4A} is not induced in these cells when they are cultured in the absence of serum could indicate that induction of this CDKI in OPCs is also dependent on culture conditions (Tang et al., 2001).

These findings demonstrate that a dependency for p16\textsuperscript{INK4A} induction on culture conditions in other cell types may be more widespread. Therefore, consistent with proposals that culture conditions may limit the lifespan of most telomerase expressing cells, it is possible that the induction of p16\textsuperscript{INK4A} in these cells may also be a response to the extracellular environment. The model we have described (Fig 6.2) thus suggests that p16\textsuperscript{INK4A} induction could be used as a general indicator of the level of checkpoint activation triggered by the culture environment.

6.2: Relevance of lifespan studies in rat Schwann cells to other cell types:

The senescent phenotype induced in response to premature (including culture-induced) senescence and replicative senescence is indistinguishable. Therefore it is extremely difficult to identify whether senescence has been induced in cultures in response to an intrinsic limit or by external conditions. If we had initially cultured Schwann cells in the higher serum concentrations routinely used to grow other cell types we might easily have misinterpreted the senescence observed in these cells as an intrinsic replicative barrier. That the stimulus for senescence in Schwann cells could have been mistaken in this way, highlights the possibility that senescence in other telomerase positive cell types might similarly be misunderstood. Indeed, human foetal lung fibroblasts provide an example of a cell type in which senescence was incorrectly attributed to an intrinsic mitotic timer (Wright and Shay, 2002). This cell type (which ironically was used by Hayflick to define cellular senescence) does not express telomerase and thus it was assumed that the replicative limit reached after about 50 PDs was a response to critically short telomeres. However, it was very recently demonstrated that these cells could not be immortalized by telomerase expression. It was found that the senescence response in these cells was in fact a response to growth in 20% oxygen.
Culturing these cells in lower oxygen tensions extended their proliferative lifespan until they reached a telomere-imposed limit (Wright and Shay, 2002). This example demonstrates how culture-induced premature senescence may occur only after extended periods of proliferation, which in this case led to the mistaken assumption that a telomere-based mechanism was responsible for the senescence response. These examples lend weight to our hypothesis that culture-induced senescence may be more widespread in other cell types as well as in the five cell types in which it has now been demonstrated.

Although it is tempting to postulate that telomere shortening is the only intrinsic mechanism of limiting replicative lifespan, it is worth considering whether unidentified intrinsic mechanisms could also be responsible for the differences between the replicative lifespan of rat Schwann cells and other rodent cell types. In view of this possibility, it is interesting that the only rodent cell types demonstrated to have unlimited replicative capacity both have a similar function in vivo: Schwann cells are the myelinating cells of the peripheral nervous system whereas oligodendrocytes perform the same function in the central nervous system. This correlation between unlimited replicative lifespan in vitro and cellular role in vivo could suggest a particular requirement for an unlimited replicative capacity associated with glial cell function. Thus it is possible that, whilst most rodent cell types evolved intrinsic replicative limits, it was important that glial cell types did not.

However, it is difficult to predict whether, consistent with the above hypothesis, glial cells are required to undergo significantly more divisions than other cell types during a normal rodent lifespan. Schwann cells are a regenerative cell type that is required to proliferate in vivo upon nerve damage (Love et al., 1998). However, it is unclear whether they would undergo sufficiently more divisions than other cell types that are also stimulated to divide in response to wounding but have limited replicative capacities in culture (e.g. fibroblasts).

The strongest argument in favour of intrinsic mechanisms controlling the proliferative capacity of most rodent cells, is that no cultures conditions have yet been identified to overcome senescence in these cells (without inducing mutation). Despite attempts in this thesis and by others, appropriate conditions for extending the proliferative lifespan of rodent fibroblasts remain to be discovered. If intrinsic
mechanisms of limiting lifespan operate in these cells, it would not be surprising that alterations in culture conditions would have no effect on their proliferative potential.

However, it is also possible to explain the difficulties in optimizing fibroblast culture conditions without invoking intrinsic replicative barriers in these cells. Schwann cells and fibroblasts may not differ with respect to intrinsic mitotic timers but they may differ in their susceptibility to signals from the external culture environment. We found that although Schwann cells and fibroblasts induce equivalent levels of p16\(^{INK4A}\) they display different replicative capacities. If p16\(^{INK4A}\) induction in fibroblasts is a "read-out" for checkpoint activation induced by proliferation in culture (as we have demonstrated in Schwann cells) our findings would suggest that Schwann cells and fibroblasts are exposed to equivalent levels of culture-induced antiproliferative signals. However, the differential senescence responses of these two cell types would suggest that there are cell type differences in the pathways that mediate the senescent arrest. This is not necessarily surprising considering that cell type differences have been demonstrated for the cell cycle regulators that are involved in mediating senescence in culture. For example senescence in murine macrophages is dependent on p16\(^{INK4A}\) whereas in MEFs it is not (Randle et al., 2001).

We demonstrated that one possible explanation for the differential response of Schwann cells and fibroblasts to equivalent inductions is that Schwann cells express much higher levels of the positive cell cycle regulators that p16\(^{INK4A}\) inhibits. Therefore, if the balance of cell cycle regulators in fibroblasts is set differently from that in Schwann cells, these cells may be intrinsically more sensitive to activation of checkpoints induced by proliferation in culture. Finding culture conditions that reduce activation of these checkpoints sufficiently to bypass a senescent arrest may therefore be very difficult.

A similar hypothesis based on species differences in DNA repair efficiencies has been proposed to explain the different replicative capacities of MEFs and HDFs (Shay et al., 2001). Although these cell types are routinely cultured in the same conditions, most HDF strains do not undergo telomere-independent senescence whereas MEFs do. There is evidence to suggest that murine cells have less efficient DNA repair mechanisms than human cells and are more sensitive to oxidative stress (Hart and Setlow, 1974), (Kapahi et al., 1999), (Shay et al., 2001). Therefore, it was proposed that levels of stress induced by proliferation in culture may be sufficient to cause premature
senescence in MEFs, but can be tolerated by HDFs that divide until reaching their telomere limit.

In summary, intrinsic mechanisms of limiting the proliferative capacity of telomerase expressing cells have not yet been identified. However, we have presented evidence that external conditions can regulate proliferative capacity in some cell types. Therefore, as there is little evidence in favour of alternative, telomere-independent mitotic timers, we suggest that senescence in telomerase expressing cells is a response to the culture environment. However, our work suggests that intrinsic differences between cell types may determine susceptibility to culture-induced senescence.

6.3: Implications of culture-induced senescence for proliferation of cells in vitro and in vivo

6.3a: Cellular proliferation in vitro:

Research in cellular biology has been hampered by what appeared to be the unavoidable replicative limits present in cultured cells. Studies in primary cells have been restricted to the period of proliferation before entry into senescence, which, in the case of rodent cells, may be only a few passages. For this reason many studies have been conducted on immortalized cell lines. However, the immortalizing mutations may have unknown effects on the cellular processes being investigated and thus the results of these studies must be treated with caution. Our findings that senescence in Schwann cells, and potentially other cell types (see discussion above) is a response to the external cellular environment, raises the possibility that culture conditions could be identified that would allow the unlimited expansion of many primary cell types. This would be of obvious benefit to all cell-based research.

In addition, the ability to expand primary cells indefinitely could be of therapeutic advantage: much attention has focused on embryonic stem cell research with the aim of providing unlimited resources of various normal cell types which could be used to treat disease. For example neuronal precursors could be expanded to generate different types of neurons to treat Alzheimer's disease, spinal cord injuries or Parkinson's disease (Fuchs and Segre, 2000). Studies into generating glial precursors for
treatment of myelination diseases (e.g. Pelizaeus-Merzbacher disease) are already in progress (Brustle et al., 1999). However, if primary somatic cells could be expanded indefinitely for this purpose instead, many of the ethical and technical problems associated with embryonic stem cell research could be circumvented.

6.3b: Cellular proliferation in vivo:

We demonstrated that extracellular factors can induce premature senescence in cultured primary cells. It is possible that cells proliferating in vivo could also be exposed to similar factors. For example, culture-induced senescence in human foetal lung fibroblasts was demonstrated to be a response to high oxygen tensions (Wright and Shay, 2002). It is possible that certain cell types would be exposed to similarly high oxygen concentrations within the lung. Therefore these cells may be equally susceptible to oxidative-stress induced senescence in vivo as they are in vitro. When Hayflick first defined senescence, it was suggested that cell-intrinsic mechanisms of limiting replicative capacity had evolved to protect against the clonal expansion required for tumorigenesis, but also, as an unselected side effect, could promote ageing. As a result, many studies have focused on the role of telomere shortening in vivo (see introduction). However, as premature senescence could also be an important mechanism limiting proliferation of cells within the body, the role of premature senescence in cancer and ageing should also be considered.

Premature senescence in ageing:

Although the role of telomere shortening in human ageing is controversial, it is generally accepted that many stress-inducing factors are likely to be responsible for accelerating ageing (Johnson et al., 1999). The same stresses that induce premature senescence in culture such as DNA damaging agents and oxidative stress also have an important role in organismal ageing (Johnson et al., 1999), (Finkel and Holbrook, 2000), (Ames et al., 1993). Cumulated oxidative damage is believed to be the main source of age-related DNA damage. In line with this hypothesis, mice lacking p66<sup>shc</sup>, a protein that is important in maintenance of cellular oxidant levels, have extended lifespans (Migliaccio et al., 1999). Rodent lifespans can also be extended by caloric restriction and this dietary restriction has been shown to reduce the accumulation of genetic lesions
that occurs with age (Sohal and Weindruch, 1996), (Hamilton et al., 2001). It is thus thought that in these mice induction of lower metabolic rates leads to reductions in free radical metabolic by-products and thus less DNA damage. That DNA damage could be a major contributor to ageing has also been suggested by the premature ageing of mutant mice defective in DNA repair mechanisms (de Boer et al., 2002). These mice show many of the characteristic ageing phenotypes e.g. premature greying, osteoporosis, cachexia. In addition, the accelerated ageing phenotype of mice expressing an activated form of p53 could be a reflection of the increased susceptibility of these mice to various p53-mediated cellular stress responses, for example senescence or apoptosis (Tyner et al., 2002).

On the basis of this evidence, it is tempting to speculate that senescence in vivo occurring in response to cumulated oxidative damage/DNA damage, may contribute to organismal ageing by reducing regenerative potential and because accumulated senescent cells could hinder the actions of surrounding functional cells. Because the senescent phenotype induced by stress or telomere shortening is indistinguishable, it is possible that senescent cells that have been detected in ageing human skin (Dimri et al., 1995) could in fact be cells that had undergone a damage response rather than, as was suggested, cells that had reached telomere limits. Furthermore, unlike replicative senescence, senescence induced by extrinsic factors could explain the ageing of tissues with low mitotic rates. In some tissues, for example endothelia, cells may be exposed to more damage than others and these levels of stress could be sufficient to induce senescence in the absence of significant division. However, although the possibility that stress-induced senescence could contribute to ageing is an attractive hypothesis, there is very little direct proof in support of this suggestion.

**Premature senescence in cancer:**

Any mechanism that results in the removal of damaged cells from the proliferative pool could be predicted to act as a tumour suppressor. Therefore, if senescence occurs in response to extracellular factors in vivo it should act to protect against tumorigenesis. Consistent with such a tumour suppressive role for premature senescence, this G1 cell cycle arrest is induced in response to oncogenic activation, upon expression of oncogenic Ras and Raf (Serrano et al., 1997), (Zhu et al., 1998), to confer resistance to transformation in many primary cell types. That many of the cell
cycle regulators that mediate the senescent arrest are tumour suppressors is in keeping
with this protective role for premature senescence. Perhaps the best example is p16\textsuperscript{INK4A}
because the induction of this CDKI has been particularly associated with premature
senescence (although it is also induced during telomere-dependent senescence). p16\textsuperscript{INK4A}
is a potent tumour suppressor but the means by which it mediates this protective
function has been unclear (Ruas and Peters, 1998), (Sharpless et al., 2001),
(Krimpenfort et al., 2001). The most favoured hypothesis is that it is its role in
regulating proliferative lifespan that confers this tumour suppressive function (although
this is likely to be cell-type dependent (Randle et al., 2001)). In favour of this
hypothesis, culture-induced premature senescence of breast epithelial cells seems to be
dependent on p16\textsuperscript{INK4A}, and, levels of this CDKI are specifically down regulated
following an elevation in levels during the early stages of breast cancer development
(Kiyono et al., 1998), (Foster et al., 1998), (Ramirez et al., 2001), (Hui et al., 2000).
These observations would argue that overcoming p16\textsuperscript{INK4A}-dependent premature
senescence might be an important step during tumorigenesis. It is unclear what the
extracellular signals leading to p16\textsuperscript{INK4A} induction during breast carcinogenesis might
be. In cultured breast epithelial cells p16\textsuperscript{INK4A} was induced in response to proliferation
on an inappropriate substratum (Ramirez et al., 2001). Perhaps the induction of p16\textsuperscript{INK4A}
observed in breast carcinogenesis is also a response of these cells to proliferation in the
abnormal tumour microenvironment.

Despite correlative evidence arguing in favour of a tumour suppressive role for
premature senescence, it has been extremely difficult to demonstrate that this arrest does
protect against the expansion of pre-malignant cells \textit{in vivo}. Perhaps the most
convincing evidence that premature senescence can operate \textit{in vivo} is provided by
recent experiments in mouse models of B-cell lymphoma (Schmitt et al., 2002). Treating
these mice with chemotherapeutic drugs induces p53-dependent apoptosis in
the tumour cells, which temporarily halts tumour progression. However, if the apoptotic
response downstream of p53 is disabled by overexpression of bcl-2, drug treatment
induces cell cycle arrest. This arrest is mediated by p53 and p16\textsuperscript{INK4A} and is
accompanied by a classical senescent phenotype. Interestingly, mice with tumours that
responded to drug treatment with a senescent arrest had better prognosis after
chemotherapy than those in which senescence mechanisms were disabled (e.g in the
case of mutated p53 or INK4A). These findings indicate that premature senescence can
operate in vivo and is likely to act as an important fail-safe barrier to tumour progression in the case of defective apoptotic responses. It is possible that in other cell types that are less prone to apoptosis, senescence could predominate as the primary tumour suppressive mechanism (Schmitt et al., 2002).

**A link between senescence ageing and cancer:**

Although at present little is known about the roles of senescence in ageing and cancer, it is likely that they are closely linked. Cancer incidence increases rapidly during old age. An accumulation of senescent cells could play a part in this trend (DePinho, 2000). There have been reports that senescent cells can provide a more permissive environment for tumour progression: senescent fibroblasts in culture (both premature and replicative) were found to stimulate proliferation of surrounding malignant and premalignant but not normal epithelial cells. Senescent fibroblasts also promoted the ability of these epithelial cells to form tumours in mice (Krtolica et al., 2001).

**6.4: Further work:**

A better understanding of the mechanisms involved in culture-induced senescence will be of great value if, as we predict, this phenomenon is more widespread in other cell types. By elucidating the nature of the stresses induced during in vitro culture it may be possible to bias the selection of culture conditions to efficiently determine optimal conditions for other cell types, thus allowing the long term culture of many different primary cell types for research and therapeutics. In addition, it seems likely that senescence induced by the extracellular environment may also determine cellular proliferative capacity in vivo. A greater understanding of the pathways connecting extracellular signals with the senescence arrest may increase our understanding of its role in vivo. Ultimately we may be able to manipulate this mechanism to exploit it as a tumour suppressor or alternatively to avert senescence and delay the ageing process.
6.4a: Investigating the factors responsible for senescence of Schwann cells in high serum:

In the human epithelial cells and foetal lung fibroblasts shown to undergo culture-induced senescence, this arrest could be overcome by growing the cells on feeder layers or at lower oxygen concentrations (Ramirez et al., 2001), (Wright and Shay, 2002). Therefore these studies suggested that the lack of signals from surrounding cells and oxidative damage may be some of the stresses responsible for culture-induced senescence. Our finding that the proliferative lifespan and the rate and level of induction of p16\(^{INK4A}\) in Schwann cells is dependent on the levels of serum in which they are cultured would suggest that it is the exposure to serum that is responsible for activating senescence checkpoints in these cells. Our knowledge of the factors that can induce premature senescence (DNA-damaging agents, oxidative stress, inappropriate mitogenic stimulation) may contribute to our understanding of what aspect of culture in serum is responsible for the senescence observed in Schwann cells (Robles and Adami, 1998), (Severino et al., 2000), (Toussaint et al., 2000), (Serrano et al., 1997).

Culture in high serum concentrations could lead to inappropriate mitogenic stimulation.

Signaling through the MAPK pathway can induce different cellular responses depending on the level and duration of MAPK activation (Sewing et al., 1997), (Woods et al., 1997), (Zhu et al., 1998). Whilst activation of this pathway is required for normal proliferation, expression of oncogenic Ras and thus overstimulation of the MAPK pathway can result in senescence in primary cells (Serrano et al., 1997), (Lin and Lowe, 2001), (Farwell et al., 2000). Serum contains a rich mixture of non-specific mitogens that are probably supplied at saturating levels in culture. It is thus possible that these mitogens lead to inappropriate activation of both the MAPK and PI3K downstream pathways in cultured cells, that differs to that occurring in more physiological contexts. On the other hand, in Schwann cells grown in low serum or in the absence of serum, in the presence of specific mitogens, the level of mitogenic stimulation may provide the correct balance of signals for sustained proliferation. In support of this hypothesis, we found that Schwann cells in 20% serum and fibroblasts in 10% serum proliferated at much greater rates than Schwann cells in low serum before they entered senescence,
perhaps indicative that the levels of mitogenic stimulation in high serum differ from those required for long-term proliferation. It would be interesting to compare the relative stimulation of the MAPK and PI3K pathways in fibroblasts in 10% serum; in Schwann cells in SATO, 3% and 20% serum and in both these cell types after expression of oncogenic Ras. It may also be informative to investigate the effect on replicative capacity of reducing either MAPK or PI3K signaling with specific chemical inhibitors.

**Culture in high serum concentrations could lead to production of oxidative stress or DNA damage:**

Culture in high serum concentrations could either directly result in exposure to high levels of ROS present within the serum or, the increased metabolic rates that might accompany growth in these conditions could lead to increased production of ROS metabolic by-products which may accumulate to ultimately induce senescence. It would be interesting to compare the proliferative capacities of the rat cells cultured in hypoxic conditions or in the presence of free-radical scavengers (e.g. N-acetyl-cysteine). In addition it may be possible to directly measure the level of intracellular ROS using fluorescent dyes sensitive to free radicals (e.g. DCF) (Macip et al., 2002).

Rates of accumulation of DNA-damage might also be increased by exposure to high serum, either as a secondary response to increased metabolic rates or because of specific mutagenic agents present within the serum.

It is possible that one specific factor present in serum is responsible for causing the senescence-inducing cellular signals, in which case culturing cells in fractionated serum could provide a route to identification of this factor. However, it is more likely that the gradual activation of checkpoints in cultured cells reflects a general response to the unphysiological conditions provided by the serum-containing environment. In this respect, it would be interesting to compare the expression patterns of Schwann cells cultured in SATO and 3% serum. As these cells proliferate indefinitely at the same rate, but are exposed to different levels of antimitogenic signals (as indicated by $p16^{INK4A}$ induction) this provides an ideal system to investigate culture-induced senescence. It has been shown, using gene array technology that different cellular responses lead to induction of different patterns of genes (Shelton et al., 1999), (Iyer et al., 1999), (Lee et
Comparing these patterns in the presence and absence of serum should prove informative about the type of response induced in these cultured cells. For example, increased expression of genes involved in DNA-damage responses in the 3% cells compared to SATO cells would implicate mutagenic factors as the major culprits in causing culture-induced senescence. Examining these gene profiles could also help identify upstream components in the pathways regulating the cell cycle proteins that mediate the senescent arrest (e.g. the factors responsible for p16^{INK4A} induction).

Using p16^{INK4A} induction as a marker of culture-induced checkpoint activation:

We demonstrated that induction of p16^{INK4A} in Schwann cells is a response to the culture environment, and this induction is accelerated and amplified in the presence of high serum concentrations. This provides a useful tool with which to investigate the intracellular pathways activated in response to proliferation in culture. Investigating the stimuli for culture induced senescence by examining proliferative capacity is not only time consuming, but is also dependent on investigating strong signals that induce threshold signaling levels sufficient to trigger an arrest. In addition, analysis of the pathways involved would be complicated by the cell cycle arrest itself. Our system is ideally suited to use p16^{INK4A} induction as a marker for culture-induced checkpoint activation because upregulation of this CDKI can be altered by extrinsic factors and, in addition, can be investigated in the absence of a senescent arrest. Therefore, unlike in human epithelial cells where p16^{INK4A} induction has been uncoupled from cellular division but not cell cycle arrest (Ramirez et al., 2001), it is clear that p16^{INK4A} induction in Schwann cells operates upstream to contribute to culture-induced senescence rather than occurring as a downstream response once senescence pathways have already been initiated. By using p16^{INK4A} induction in cultured Schwann cells as a marker for culture-induced checkpoint activation, it would be possible to investigate the extrinsic factors and pathways involved in culture-induced senescence. Furthermore, analysis of the signals directly upstream of p16^{INK4A} induction should enable a more defined strategy to elucidate the signaling pathways involved in this particular checkpoint response.

Generally, very little is known about the mechanisms by which p16^{INK4A} is induced. Upon DNA damage in HDFs, a delayed but sustained induction of p16^{INK4A} follows a transient p53/p21^{G1} elevation (Robles and Adami, 1998). In contrast,
expression of oncogenic Ras in HDFs results in a rapid p53-independent induction of p16\(^{INK4A}\) (Lin et al., 1998), (Zhu et al., 1998), (Wei et al., 2001). Whether the progressive telomere-independent induction of p16\(^{INK4A}\) observed in cultured cells resembles either of these responses is unclear. Recently it has been demonstrated that, in HDFs, the pathways involved in replicative (telomere-induced) and premature (oncogenic-Ras-induced) senescence can be differentiated at the level of p16\(^{INK4A}\) induction. In this study telomere shortening was found to trigger p16\(^{INK4A}\) via upregulation of its positive regulator Ets1 and concomitant downregulation of its negative regulator Id1. In contrast the induction of p16\(^{INK4A}\) observed after expression of oncogenic Ras seemed to result from increased levels and activity of Ets2 (Ohtani et al., 2001). A similar analysis of the regulators responsible for upregulation of p16\(^{INK4A}\) in cultured Schwann cells could increase our understanding of the factors responsible for culture-induced senescence.

In addition, it would be interesting to investigate the contribution of other candidates that have been implicated in p16\(^{INK4A}\) regulation: Bmi1, a member of the polycomb group of transcriptional repressors, appears to be important in downregulation of the INK4A locus and it is this function that allows it to cooperate with Myc in tumorigenesis (Jacobs et al., 1999). JunB can upregulate p16\(^{INK4A}\) expression by binding AP-1 sites in its promoter and MEFs expressing elevated levels of JunB induce higher levels of p16\(^{INK4A}\) in culture and senesce more rapidly (Passsegue and Wagner, 2000). The finding that overexpression of PML can induce p16\(^{INK4A}\) in HDFs suggests that this tumour suppressor can also be involved in p16 regulatory pathways. (Ferbeyre et al., 2000). Finally, the downregulation of 14-3-3-\(\sigma\), a member of the 14-3-3 family that is specifically expressed in epithelia, leads to reductions in p16\(^{INK4A}\) expression and by-pass of telomere independent senescence in human keratinocytes (Dellambra et al., 2000). In addition to investigating the roles of all these candidates in p16\(^{INK4A}\) regulation in cultured Schwann cells, it would also be interesting to determine whether the induction of p16\(^{INK4A}\) in response to proliferation in culture in this system occurs secondary to p53 activation, as seems to be the case in DNA damage responses (Robles and Adami, 1998).
In conclusion, we have demonstrated that primary rat Schwann cells can divide indefinitely in culture. Therefore, a finite replicative capacity can no longer be used as one of the defining features of primary cells. Although Schwann cells possess no intrinsic replicative limits, we have demonstrated that external factors can regulate their proliferative capacity. These findings raise the possibility that the senescent arrest seen in other telomerase expressing cell types could also be a response to external conditions rather than an intrinsic replicative barrier.

In addition, we demonstrated that induction of p16\(^{INK4A}\) in Schwann cells is a response to the culture environment. It is thus possible that induction of this CDKI in many other cell types could also be determined by extracellular factors rather than, as was commonly thought, an intrinsic timer.

We demonstrated that although cultured rat Schwann cells and fibroblasts have different replicative capacities, they induce equivalent levels of p16\(^{INK4A}\). Schwann cells may thus be more resistant than fibroblasts to the antiproliferative signals induced by proliferation in culture. Our finding that Schwann cells express higher levels of positive cell cycle regulators than the fibroblasts might provide an explanation for this intrinsic difference.

Finally, we have identified a valuable system to further investigate how factors in the extracellular environment can activate the senescence checkpoint. Using p16\(^{INK4A}\) induction as a marker for activation of these signals, we can directly compare two different culture conditions in which Schwann cells proliferate at the same rate yet differentially activate these checkpoints. Using this system to investigate antiproliferative signals from the extracellular environment may aid identification of conditions that allow the unlimited proliferation of many cultured primary cell types. In addition, it is likely that premature senescence checkpoints also operate in vivo. A greater understanding of the pathways involved in this checkpoint could determine the role of premature senescence in tumour suppression and organismal ageing.
References:


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