A study of telomere and telomerase biology in the dog and cat

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ABSTRACT

The primary aims of this project were to carry out a comprehensive investigation of telomere and telomerase biology in the dog and cat, and more specifically to investigate the potential of telomeres and telomerase as targets for novel cancer chemotherapeutics.

Telomeres are nucleoprotein structures that cap the ends of all eukaryotic chromosomes analysed to date. The mammalian telomere is composed of duplex, non-coding hexanucleotide DNA repeats of the sequence (TTAGGG)n, terminating in a 3′ single-stranded DNA overhang of varying length. The protein components of the telomere are involved in the maintenance of normal structure of the telomere, and the proper functioning of that structure within the cell.

Not all of the functions of the telomere are fully understood, however of primary interest is the regulation and restriction of cellular replication via a proposed telomere based 'mitotic clock.' This counting mechanism is based on the generally irretrievable telomere loss that accompanies cell division, referred to as the 'end replication problem.' The telomere attrition caused by end replication problem eventually triggers a replicative arrest and a characteristic cellular phenotype, referred to as senescence. Senescence has been linked to organ dysfunction and organismal ageing, and has therefore brought telomeres to the attention of a wide spectrum of research disciplines.

Whilst the majority of cell types suffer the effects of end replication problem, there are marked exceptions. Cells with high replicative burdens, germ line cells, and cancer cells do not show the replicative restrictions of other cell types. These cell types overcome end replication problem, in the majority of cases, by activation of a ribonucleoprotein complex called telomerase. This specialised reverse transcriptase enzyme replaces lost telomere sequence and is responsible, in approximately 80% of human tumours, for the unlimited replicative potential that is one of the hallmarks of cancer cells. Furthermore, inhibition of this complex has resulted in senescence and apoptosis of malignant cells, and overall telomerase has proven to be an excellent tumour marker, with potential diagnostic and prognostic applications, and is considered one of the most promising targets for anti-cancer therapy currently under investigation.

The majority of telomere and telomerase research carried out to date has been directly or indirectly for the benefit of human medicine. Research in the veterinary field lags far behind, and very little information was available on basic parameters such as telomere length and distribution of telomerase activity in dogs and cats at the beginning of the project. The first experiments carried out were therefore concerned with investigating mean telomere lengths in a wide age and tissue range of both the dog and cat. The protocol used for telomere length assessment was based on a DNA probe, Southern Blot and chemiluminescent technique referred to as Terminal Restriction Fragment analysis. Telomere lengths in peripheral blood samples taken from 112 dogs and 30 cats were found to range from 4.7 to 20.6 kb, and 9.6 to 23.5 kb respectively. These are similar to telomere lengths typically found in human samples (5-15 kb). The telomere lengths in a panel of normal canine and feline organ

samples, tumour samples, and primary fibroblast cultures also did not differ significantly from these values. Telomere lengths decreased significantly with increased age in both species, and whilst gender did not have a significant effect in either species, an intriguing finding was that breed of pedigree dog had a significant effect on telomere length. Primary canine and feline fibroblasts were found to cease replicating and assume a senescent phenotype *in vitro* after an average of 10 and 16 population doublings respectively. Over the course of these population doublings, telomere attrition was shown to occur in both canine and feline cells, and averaged 175 and 130 bp/cell division respectively. In summary, telomeres in the dog and cat are of a similar size to that found in humans, and telomeric attrition has been shown to occur in both species *in vivo* and *in vitro*. Furthermore, loss of telomeric sequence is associated with the triggering of a senescent phenotype in both canine and feline fibroblasts *in vitro*.

Telomerase activity studies used a commercially available assay, referred to as the Telomeric Repeat Amplification Protocol (TRAP). Telomerase activity was strongly down regulated in a wide range of somatic tissues of the dog and cat. Conversely, telomerase activity was detected in all canine and feline tumours assayed (19/19), and was also present in a panel of immortalised canine cell lines. These data linked telomerase with immortalisation and malignancy in the dog and cat, and have identified telomerase as a potential target for novel cancer chemotherapeutics in companion animals. A pilot study to assess the efficacy of a reverse transcriptase inhibitor (azidothymidine triphosphate) as a telomerase inhibitor in two canine telomerase dependant cell lines produced inconsistent inhibition of telomerase and no discernable effect on telomere lengths. However, future use of this drug in combination with agents that utilise different modalities for targeting telomerase may produce more favourable results. Such combinational therapies are currently producing the most promising results in the human field.

Expression of the catalytic component of human telomerase (hTERT) is sufficient to reconstitute telomerase activity and prevent senescence in a number of human tissues. This is definitive proof of a link between telomere attrition and senescence in humans. The canine and feline homologues of hTERT were not available to carry out this investigation in the dog and cat; however, heterologous expression of hTERT using a mammalian expression vector was used in canine fibroblasts to investigate the control of telomerase activity and the links with senescence in that species. Expression of hTERT mRNA was confirmed in the primary canine fibroblasts by reverse transcriptase PCR and sequence analysis; however the cells remained telomerase negative, and entered senescence normally. This may be due to sequence differences in the catalytic components of canine and human telomerase rendering hTERT ineffective in canine cells. Interestingly, telomerase activity was briefly reconstituted in an equine primary fibroblast culture that was subject to the same procedure. Telomerase was also successfully reconstituted in a human telomerase independent control cell line.

The studies described above went some way to addressing the biology of telomeres and telomerase in canine and feline cells, however, the wider implications of telomere attrition and telomerase

reactivation within cells remained unknown. The importance of an understanding of the possible knock-on effects of telomere length reduction on the transcriptome of the cell is important as potential telomerase inhibitors are likely to operate through telomeric attrition. Furthermore, telomerase reactivation has been suggested as a possible therapy for conditions when loss of replicative potential is part of the pathology, such as liver cirrhosis. These strategies will rely on the absolute safety of telomerase reactivation *in vivo*, and will also require knowledge of the wider effects of telomerase reactivation within the cell. These issues were addressed by examining the changes in the transcriptome of canine primary fibroblasts as they switched from actively replicating to senescent. The effect of telomerase reactivation in canine and feline primary cells also needs to be addressed, however, as described above, it was not possible to reconstitute telomerase activity in those cells. Instead, the effect of telomerase reactivation was investigated in a human telomerase independent cell line, as it is likely that many of the down-stream effects will be conserved between species. The experiments utilised DNA microarray technology (Affymetrix, Santa Clara, CA), as this allowed changes in mRNA expression levels of many thousands of genes to be monitored simultaneously. The experiments identified a number of genes of interest that warrant further investigation. Chief among these was the finding that mRNA levels of the gene product epiregulin were up regulated greater than 8-fold in telomerase positive, compared with telomerase negative cells. This is important, as epiregulin activity has been associated with cancer progression, and is therefore associated with a malignant phenotype. This immediately casts doubt over the safety of telomerase reactivation for therapeutic purposes. Genes of interest in senescent fibroblasts were thrombospondin-1, phosphatidic acid phosphatase type 2A, and ATPase, Na+/K+ transporting, beta 1 polypeptide. These gene products may be associated with inhibiting tumour angiogenesis, tumour inhibition, and regulation of senescent cell volume respectively. The upregulation of thrombospondin-1 and phosphatidic acid phosphatase type 2A in senescent canine fibroblasts may provide evidence of additional anti-tumour mechanisms in senescent cells, and the down regulation of Na+/K+ transporting, beta 1 polypeptide, which encodes a component of the Na+/K+, ATPase osmotic 'pump' may have a part to play in the increased volume of senescent cells.

Overall, the findings of this project indicate that telomeres and telomerase are directly involved with cancer development and progression in the dog and cat, and identify telomerase as a promising target for the development of future cancer chemotherapeutics in companion animals.

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DECLARATION

I declare that the work described in this thesis was carried out by me and is not that of any other person, and further has not been submitted in full or in part for consideration for any other degree or qualification.

Tom P. McKevitt

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PUBLICATIONS

Referred journals

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McKevitt, T. P., Nasir, L., Devlin, P., & Argyle, D. J. 2002, "Telomere Lengths in Dogs Decrease with Increasing Donor Age," *Journal of Nutrition*, vol. 132, no. 6, pp. 1604S-1606.

McKevitt, T. P., Nasir, L., Wallis, C. V., Argyle, D. J., 2003, "A cohort study of telomere and telomerase biology in cats," *American Journal of Veterinary Research*, vol. 64, no. 12, pp. 1496-1499.

Abstracts

"A study of canine telomere lengths: insights into the mechanisms of ageing and cancer," *British Small Animal Veterinary Association (BSAVA) congress*. Birmingham, UK, 2002

"Effects of a reverse transcriptase inhibitor on canine cell lines," *BSAVA congress*. Birmingham, UK, 2002.

"Canine telomeric attrition with age and breed specific differences in mean TRF," *Genomics and genetics of senescence and cancer*. Keystone, Colorado, USA, 2002.

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Chapter I

General introduction and review of the literature

1.1 Abstract

Telomeres are nucleoprotein structures that cap the ends of eukaryotic chromosomes. Under normal circumstances, cell division results in an irretrievable reduction in telomere length that eventually triggers loss of cellular proliferative potential. However, telomeres are occasionally maintained or even lengthened; in the vast majority of cases this is facilitated by a specialised ribonucleoprotein, telomerase. Telomeres and telomerase have been associated with ageing and malignant transformation respectively, and this Chapter reviews current evidence for and against these associations. The bulk of research in the field of telomere biology to date has been human based, and the review reflects this bias, however where possible, special reference is given to research of direct relevance to the dog and cat.

1.2 Telomere structure

Telomeres are specialised nucleoprotein structures that cap the ends of all eukaryotic chromosomes analysed to date. Telomeric DNA sequences and structure are highly conserved across otherwise wide species boundaries, and are based on a simple tandemly repeating unit. (Blackburn 1991) The sequence TTAGGG is the unit found in humans, other vertebrates, slime moulds and trypanosomes, whilst a wide range of organisms adhere to a telomeric repeat based on the simple formula $d(T/A)_{1-4}dG_{1-8}$, examples include the GGGGTT and GGGGTTTT sequences found in the ciliate protozoans *Tetrahymena* and *Euplotes* respectively (Blackburn & Szostak 1984) (Moyzis, Buckingham, *et al* 1988) (Blackburn 1990).

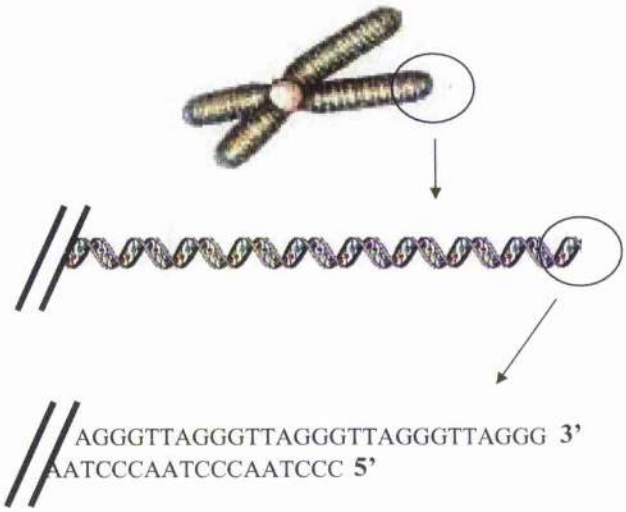
The G rich strand of eukaryotic telomeres analysed to date are all orientated in the 5′ to 3′ direction towards the terminus, and work carried out initially in *Oxytricha* showed that this strand produces an overhang beyond the complimentary C rich strand. The length of this overhang is species specific, from the exact 16 base pairs (bp) in *Oxytricha* to a variable 50-100 bp in humans and the mouse (Klobutcher, Swanton, *et al* 1981) (Greider 1999). The basic structure of the mammalian telomere is shown diagrammatically in Figure 1-1.

The overall length of telomeric sequence found in vertebrates typically runs to tens of thousands of base pairs, though there is considerable variation between species. Canine telomeres have been found in the range of 11 to 23 kilobases (kb) (Yazawa, Okuda, *et al* 2001) (Nasir, Devlin, *et al* 2001), and feline telomeres in the region of 12-23 kb (McKevitt, Nasir, *et al* 2003). Human germ line telomeres are 15-20 kb, whilst the laboratory mouse (*Mus musculus*) has unusually long telomeres ranging from 30 to >50 kb (Allshire, Dempster, *et al* 1989) (Campisi, Kim, *et al* 2001). The closely related interfertile species *Mus spretus* has telomeres that are slightly shorter than in humans, usually reaching 5-15 kb (Smogorzewska, van Steensel, *et al* 2000), illustrating the wide variation found between even closely related species. Aside from interspecies variation, diversity in telomere lengths is found at an organismal, tissue, cellular and even

chromosomal level, albeit with less marked variety, and telomere length is affected by cellular replicative history. Takubo *et al* demonstrated that telomere lengths are not significantly linked to tissue renewal times in humans. For example there is no typical telomere length for human myocardial tissue at a given age, despite this tissue being relatively static with respect to cellular turnover (Takubo, Izumiyama-Shimomura, *et al* 2002) (Cameron 1970). Furthermore, an individual with longer than average telomeres in one tissue is likely to have longer than average telomeres in a number of different tissues, illustrating telomere length variation at an individual organism basis around a species average (Takubo, Izumiyama-Shimomura, *et al* 2002).

Germ line cells have the ability to maintain or increase their telomere lengths, however the mean telomere lengths found in germ line cells remains constant for any given species (Kipling & Cook 1990) (Wright, Piatyszek, *et al* 1996 160 /id).

Figure 1-1. Structure of the mammalian telomere. Telomeres are specialised nucleoprotein structures found at chromosome ends, as shown diagrammatically below. The duplex telomeric DNA sequence is based on a repeating hexamer, and a single stranded G-rich 3′ overhang of variable length is found at the terminus.



1.2.1 G-quartets

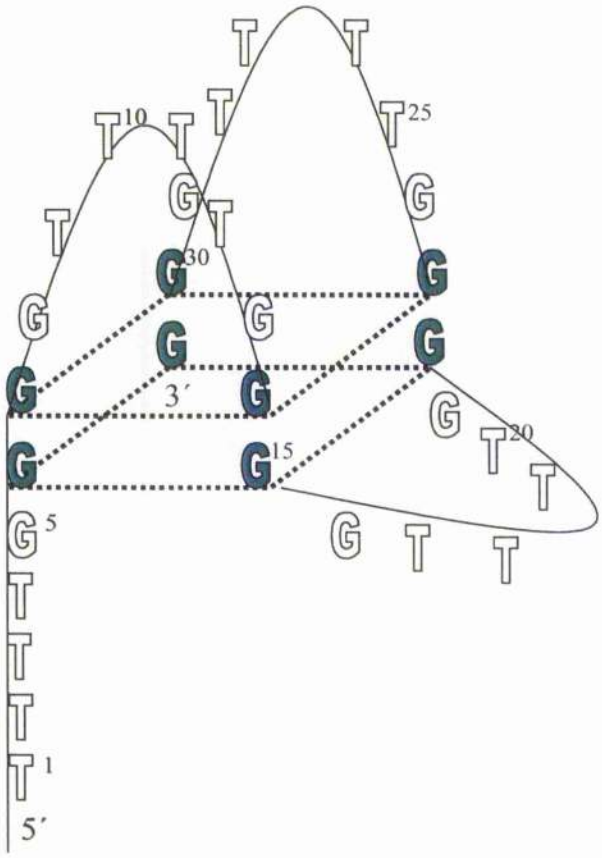
The G-quartet is the defensive structure hypothesised to be formed from the single stranded 3′ overhang at the end of the telomere, and it is believed to shield chromosome ends from the attentions of DNA repair complexes (Williamson, Raghuraman, *et al* 1989).

The suspicion that a specific telomeric sequence structure may be present at the telomeric terminus was initially aroused by the observation that oligonucleotides formed from the G-rich strand of telomeric sequence had greater electrophoretic mobility than would be expected for their size. Furthermore, the oligonucleotide Tet-4 formed from four Tetrahymena telomeric repeats, $d(TTGGGG)_4$ has been shown to contain G-G base pairs. (Henderson, Hardin, *et al* 1987). Experimental work based on these data has implied that *in vitro*, such G-G base pairs lead to the formation of the structure that has been termed the G-Quartet, under the influence of certain cations at physiological concentrations; the implication being that such structures may exist *in vivo* (Williamson, Raghuraman, *et al* 1989). The proposed structure is shown diagrammatically in Figure 1-2, and in this arrangement the common telomeric sequence elements, the G-strings (for example GGG in the human telomere) are the basis of the G-Quartet, whilst the variable elements, the T/A bases form loop regions around the Quartets. This structure has the potential to accommodate the telomeric sequences of a number of different species, as G-strings are a common feature in telomeric DNA (Blackburn & Szostak 1984).

1.2.2 Telomere associated proteins

In addition to a DNA repeat sequence, a number of proteins are also associated with the telomere. These telomeric proteins are believed to play important structural roles, and are integral to the proper functioning of the telomere (Greider 1999).

Figure 1-2. Proposed structure of the G-quartet. The diagram shows two G-quartets (linked by dashed lines) utilising the telomeric sequence of *Oxytricha sp.* d(TTTTGGGG). Variations in the telomeric sequence may be accommodated by variation in the number of stacked quartets, and by changes in the length of the connecting DNA loops. Every fifth base is numbered, and the deoxyguanosine residues involved in the quartets are shaded to clarify the arrangement of bases in the structure.



1.2.2.1 TRF1

(TTAGGG repeat binding factor 1) is a double stranded telomeric DNA binding factor that binds along the length of duplex telomeric DNA and was first identified and cloned in 1995. The discovery of TRF1 was the first evidence that telomeres form nucleoprotein complexes in vertebrates (Chong, van Steensel, *et al* 1995). The protein is described as being related to the proto-oncogene Myb due to its Myb-like DNA binding domain at its carboxy terminus, and acidic amino terminal domain. It is a ubiquitous protein, and has been found associated with telomeres at all stages of the cell cycle (Broccoli, Smogorzewska, *et al* 1997). Telomeric DNA has been shown to recruit TRF1 in the form of a tetramer, and at high protein concentrations *in vitro* will coat the entire length of available telomeric sequence with a 10 nm thick array of bound proteins. In addition, the presence of TRF1 attached to the telomere has the *in vitro* effect of promoting telomeric tracts to form pairs, with a strong bias for the parallel arrangement. (Griffith, Bianchi, *et al* 1998).

TRF1 has been shown to act as a negative regulator of telomere length, and it is proposed to carry out this function by inhibiting the ribonucleoprotein telomerase (vanSteensel & de Lange 1997). When new telomeres are transfected into cultured cells, these stretches of DNA are elongated, presumably by telomerase until they reach the average length for that type of cell. At this time, TRF1 is recruited to the new telomeres, and growth is halted. This provides an *in vitro* insight into the function and mode of action of TRF1 (Smogorzewska, van Steensel, *et al* 2000).

1.2.2.2 TRF2

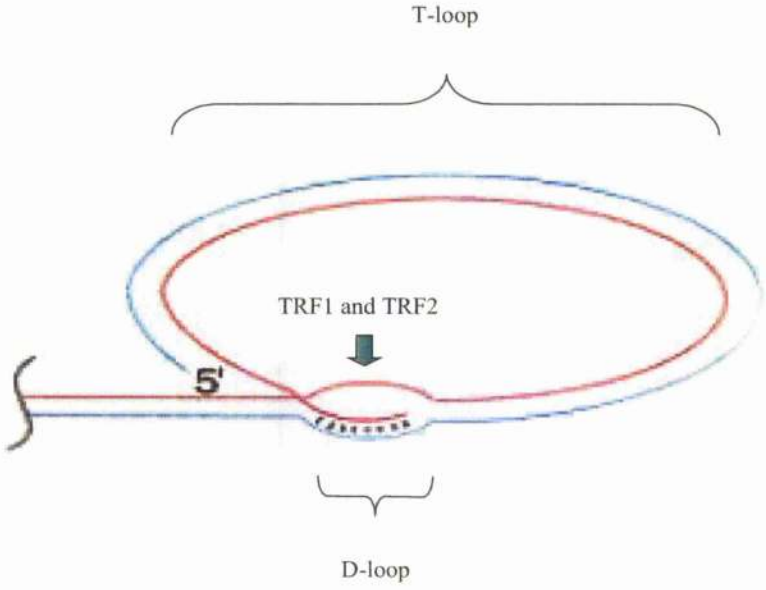
TRF2 was initially classified as a duplex DNA binding protein, and was first identified by Broccoli *et al* in 1997 (Broccoli, Smogorzewska, *et al* 1997). It is a distant homologue of TRF1 and has a similar Myb like DNA binding motif at its carboxy terminus. Unlike TRF1, however, it has a basic amino terminus. Inhibition of TRF2 has serious consequences for the cell, resulting in an

immediate threat of damage to the chromosomal ends by cellular activation of the ataxia telangectasia mutated (ATM)/p53-dependant DNA damage checkpoint pathway (Karlseder, Broccoli, *et al* 1999). Chromosomal end to end fusions follow, and the 3′ G rich single stranded DNA overhang at the telomere end is lost (Griffith, Comeau, *et al* 1999). This indicates that TRF2 interacts with both single and double stranded DNA, and supports the idea that TRF2 is crucial for the proper functioning of the telomere. Griffith *et al* used electron microscopy to demonstrate that TRF2 is capable of remodelling linear telomeric DNA into large duplex loops *in vitro* (Griffith, Comeau, *et al* 1999). As the presence of telomeric DNA *per se* is not enough to protect chromosomal ends from degradation, these telomeric DNA or 'T' loops are proposed to be the additional structure necessary for protection of the chromosomal ends (van Steensel, Smogorzewska, *et al* 1998).

Currently, the proposed view of telomeric structure *in vivo* is that the 3′, single stranded DNA overhang loops back to invade the duplex telomeric DNA and form a displacement loop in the order of a few hundred nucleotides. In many of the experimental cases the T-loops were very large and encompassed the entire length of the telomere. Whether this is always the case is unknown (Griffith, Comeau, *et al* 1999). TRF1 may also have a role to play in T-loop formation, as it has been shown to possess the ability to promote parallel pairing of telomeric tracts *in vitro*, which may help to stabilise the T-loop arrangement (Griffith, Bianchi, *et al* 1998). The structure of the T-loop is shown in Figure 1-3.

The fact that inhibition of TRF2 has such an immediately deleterious effect on the cell; (cells expressing a dominant negative allele of TRF2 rapidly undergo apoptosis (Karlseder, Broccoli, *et al* 1999)) led to difficulty assessing any potential role of this protein in regulation of telomere length. Smogorzewska *et al* circumvented this problem by examining the effect of over expression of TRF2, using a tetracycline inducible expression system in the human fibrosarcoma line HTC75. This study indicated that TRF2 is also a negative regulator of telomeric length, though in a transient way which is eventually corrected (Smogorzewska, van Steensel, *et al* 2000).

Figure 1-3. Structure of the T-loop. TRF1 stabilises a loop structure formed by the telomere and allows TRF2 to mediate invasion of the duplex DNA at the base of the telomere by the 3′ ssDNA overhang. This T-loop is thought to be important in allowing the cell to differentiate between a natural chromosomal end and a double stranded DNA break.



1.2.2.3 TANK1

TANK1, or tankyrase is a protein with homology to the catalytic domain of poly-adenosine phosphate ribose polymerase (PARP). This type of enzyme activity is usually associated with DNA repair (de Murcia & de Murcia 1994), and recombinant TANK1 has demonstrated PARP activity *in vitro*. TANK1 has been found to interact with TRF1 *in vitro*, and binding of TANK1 to TRF1 diminishes the ability of TRF1 to bind to telomeres. The long term over-expression of tankyrase in telomerase positive cell lines results in a gradual and progressive lengthening of telomeres, whilst PARP deficient forms of TANK1 do not demonstrate this effect. This suggests TANK1 regulates the negative effect of TRF1 on telomere length via ribosylation of TRF1, though it is possible TANK1 may act on other, as yet unidentified telomere associated factors as well (Smith, Giriat, *et al* 1998) (Smith and de Lange 2000). Tankyrase is likely to act by causing a structural change in the telomere end, which allows the enzyme telomerase access to the telomere sequence.

1.2.2.4 TANK2

This protein is a recently identified second tankyrase with telomere associations. This protein has a very similar amino acid identity to TANK1, but has a unique N-terminal domain. TANK2 also interacts with TRF1, however over expression of TANK2 leads to rapid cell death by necrosis, with loss of mitochondrial membrane potential. The PARP inhibitor 3-aminobenzamide blocks this effect *in vitro*, linking this activity with the cell death. If this is the case, it may be that the PARP activity of TANK1 is more efficiently regulated than that of TANK2, or that TANK2 acts on substrates that are not a target for TANK1 (Kaminker, Kim, *et al* 2001). This idea is supported by the fact that both TANK2 and TANK1 are not restricted to the telomere alone; both these proteins are abundant in the nuclear periphery and in the Golgi. It is therefore possible that they carry out non-telomere related activities in the cell, and so act on other substrates (Campisi, Kim, *et al* 2001).

1.2.2.5 Ku70 and Ku86

These proteins are components of DNA-dependant protein kinase (DNA-PK). DNA-PK is a trimeric complex that is essential for the repair of double stranded DNA breaks in the cell. Ku binds and stabilises the broken ends of DNA, and then recruits the catalytic component of the DNA-PK to carry out the necessary repair. Ku70 has been shown to specifically bind TRF1, and deficiency of Ku86 in mice causes genetic instability due to frequent telomere-telomere fusions. This is similar to the effect of TRF2 deficiency, however Ku86 deficiency is not associated with loss of the 3′ overhang, or loss of telomere sequence overall. This indicates that as well as its documented role in DNA repair, Ku is also important for protection of the terminal telomeric structure and that fusions associated with Ku deficiency are not mediated by loss of TRF2 function (Samper, Goytisolo, *et al* 2000).

1.2.2.6 TIN2 and HRAP1

TIN2 and HRAP1 also appear to be exclusively sited at the telomere, but in association with other proteins rather than the telomere directly. TIN2 interacts with TRF1, and HRAP1 with TRF2, and whilst their precise functions are unknown, they both play important roles in regulating telomere length (Campisi, Kim, *et al* 2001).

1.2.2.7 POT1

POT1 is a recently identified single stranded DNA binding protein first isolated in yeast. The human homologue, hPOT1 has been shown to bind specifically to the G-rich telomere strand, and acts as a positive regulator of telomere length in a telomerase dependant manner (Baumann, Podell, *et al* 2002), (Colgin, Baran, *et al* 2003).

1.3 Telomere function

A primary role of the telomere is to protect the integrity of chromosome ends. This idea was first proposed by McClintock (McClintock 1941) and later works have demonstrated the importance of this function in a wide variety of species (Cervantes & Lundblad 2002). It is likely this faculty evolved in response to the development of natural chromosomal ends that may be mistaken within the cell as double stranded DNA breaks (Zakian 1989) (Bouffler, Morgan, *et al* 1996).

Telomeres prevent loss of genetic information during replication of chromosomes by providing a non-coding DNA buffer that is expendable in the face of 'end replication problem,' (Blackburn & Szostak 1984) and they promote correct mitotic separation of sister chromatids during cell division (Kirk, Harmon, *et al* 1997).

1.3.1 End replication problem and telomere loss

End replication problem describes the difficulty encountered by a cell containing linear chromosomes in dealing with the established mechanics of cell division, specifically DNA replication. The semi-conservative mechanism of DNA replication describes how the strands of the DNA double helix separate and each acts as a template to direct the synthesis of a complementary daughter strand (Meselson & Stahl 1958). The point at which the DNA strands separate and synthesis of new DNA begins is termed the replication fork, two of which proceed bi-directionally from each other from an origin of replication. These single units of replicating DNA are referred to as replicons, and the typical mammalian cell may contain 50,000 to 100,000 of them with a size range of 40-200 kb. The DNA polymerases responsible for carrying out the replication are unidirectional in action in the 5′ to 3′ direction, and they require a short, labile tract of RNA to act as a primer. Whilst leading strand replication may proceed in the 5′ to 3′ direction to the end of the chromosome, lagging strand replication may only occur in the 5′ to 3′ direction in short fragments, termed Okazaki fragments. The Okazaki fragment includes the newly formed segment of

daughter strand DNA along with its RNA primer arranged 5′-RNA-DNA-3′ (Sugino & Okazaki 1973). The removal of these individual RNA primers leaves multiple internal gaps in the DNA sequence. These internal gaps are filled in by extension of the DNA and ligation, however a 5′ gap is left in the newly synthesised DNA strand as there is no terminal primer allowing it to be filled in (Olovnikov 1973) (Blackburn 1991). Despite the difficulty caused by end replication problem linear chromosome development may have facilitated the advent of meiosis, and the genetic diversity this enabled may well explain the evolutionary advantage provided by this otherwise unstable system (Griffith, Comeau, *et al* 1999) (Naito, Matsuura, *et al* 1998).

The model of telomere attrition by incomplete lagging strand synthesis predicts that 50% of chromosomes will have a G-rich overhang, and that chromosomal shortening will proceed at a rate of approximately 8-12 bases per cell division, a figure which is based upon the size of the RNA primer (Tseng, Erickson, *et al* 1979). However, experimental work using a variety of cell types has reported long overhangs of between 130-210 bases in >80% of the telomeres tested (Makarov, Hirose, *et al* 1997). In addition it is known that the telomeres of human fibroblasts shorten by 31-85 bp per cell doubling. (Harley, Futcher, *et al* 1990). This suggests that incomplete lagging strand replication is not alone in producing G-rich overhangs. Leading strand replication requires only one RNA primer, allowing DNA replication to proceed directly to the end of the sequence and resulting in no DNA sequence loss. To account for this, a revised model of telomere shortening has been proposed in which DNA is lost from both ends of the chromosome due to degradation of both 5′ strands of the DNA at the time of, or shortly after replication. This is termed the strand degradation hypothesis. A 5′-3′ exonuclease has been isolated from calf thymus tissue and advanced as the likely cause of the missing bases. This exonuclease may be active by itself or as a component of DNA polymerase, and it has been shown to act specifically on single strands of duplex DNA (Siegal, Turchi, *et al* 1992). The G-rich overhang may have developed at both ends of the chromosome to allow protection of the chromosome ends by T-loop formation (Campisi, Kim, *et al* 2001) (Klapper, Parwaresch, *et al* 2001).

1.3.1.1 Other causes of terminal sequence loss

Factors not considered in the strand degradation hypothesis are also likely to contribute to terminal sequence loss. Recent work has shown that oxidative damage is repaired less efficiently in telomeric DNA than elsewhere in the cell, and that oxidative stress accelerates telomeric attrition (von Zglinicki 2002). The converse has also been shown to be true, namely antioxidant (such as ascorbic acid 2-*O*-phosphate) treatment of cell lines attenuates telomere sequence loss (Furumoto, Inoue, *et al* 1998). These data have led some observers to the opinion that oxidative stress is an important modulator of telomeric attrition, and that telomere driven replicative senescence itself is primarily a stress response (von Zglinicki 2002). Evidence in support of this is found in data showing that some fibroblast cell lines lose as few as 10-20 bp per cell division, indicating that strand degradation and end replication problem may not always be the major determinant of telomeric sequence loss. It is possible that in those cell lines that are affected by greater sequence loss (and have higher peroxide levels) the major factor at work is oxidative stress (Lorenz, Saretzki, *et al* 2001). This additional factor does help explain the huge heterogeneity in replicative potential of clonally derived cell lines identified by Smith and Whitney (Smith & Whitney 1980). Evidence of such a stress response has been identified *in vivo* in the shortened replicative capacity of fibroblasts from subjects with Fanconi anaemia, a condition that is known to result in chronic oxidative stress. (von Zglinicki, Serra, *et al* 2000). It is likely that the effect of oxidative damage on the rate of telomeric attrition allows the individual cell to 'fine tune' entry into senescence to account for the greater risk of mutation, and therefore malignancy associated with higher levels of oxidative stress. This view appeals from an evolutionary standpoint, as it is a tumour suppressor mechanism by which those cells most at risk from malignant transformation may be removed from the replicating population more swiftly than would result from end replication problem alone.

1.3.2 Role of telomeres in chromosome separation

Kirk *et al* have demonstrated that telomeres are essential for chromosomal stability during mitosis in an experiment focused on the ciliated protozoan *Tetrahymena*. Telomeric DNA mutants were produced by expression of a telomerase RNA with an altered template sequence, and this prevented division of the germline nucleus in anaphase, consistent with a physical block to separation of the telomeres (Kirk, Harmon, *et al* 1997).

1.3.3 Role of telomeres in gene silencing

A transcriptional silencing mechanism has been proposed that is caused by proximity to the telomere. This has been termed the telomere position effect (TPE), and was first identified in *Saccharomyces cerevisae* where TPE can cause reversible gene silencing by a mechanism that is dependant both on telomere length and the proximity of the gene to the telomere (Tham & Zakian 2002). This has prompted investigations to identify if this effect can be demonstrated in mammalian cells. Promising results have been obtained using HeLa clones in which a luciferase reporter adjacent to a newly formed telomere expressed 10 times less luciferase than controls that have been randomly incorporated. Furthermore, lengthening of the telomere produced further reduction in reporter activity in the telomere adjacent reporters but not in the controls (Baur, Zou, *et al* 2001). Attempts to reproduce this effect in natural telomeric genes have not met with the same success; telomere length alone is not sufficient to determine the expression status of the genes investigated (Ning, Xu, *et al* 2003). However, this study has also identified a discontinuous pattern of gene expression during telomere shortening that may be related to senescence. Further work is necessary to clarify to what extent telomeres affect the expression level of genes located near to them, though the present evidence suggests that such proximity is a factor governing expression, and suggests another important role for the telomere in the mammalian cell.

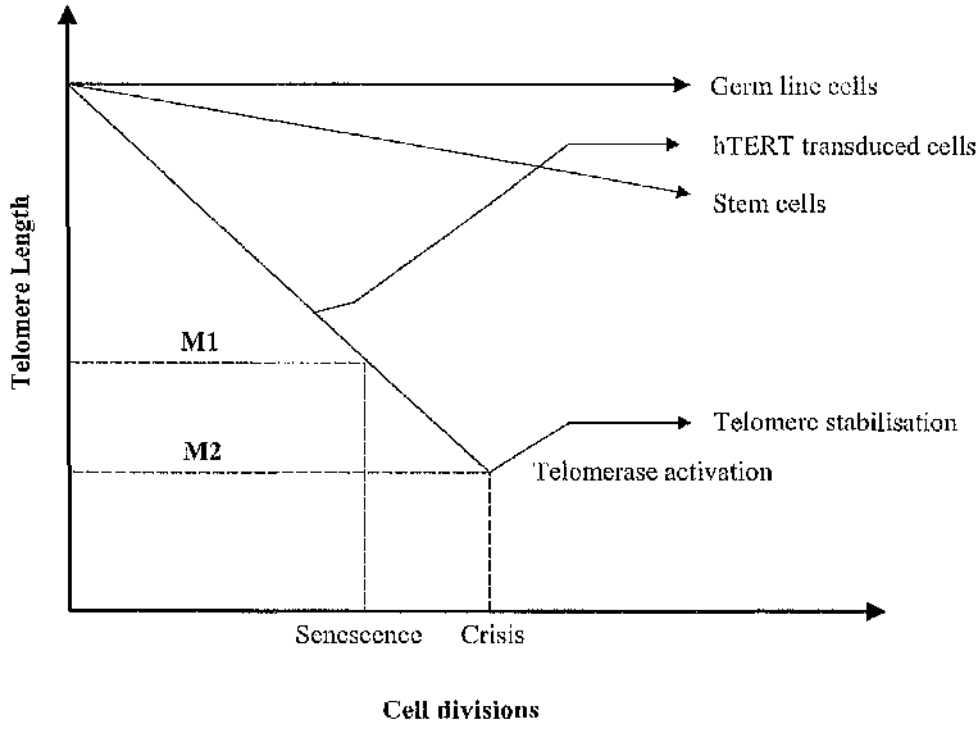
1.4 Replicative senescence

Senescence is the final phenotypic state adopted by a cell in response to several distinct physiological processes including proliferation, oncogene activation and oxygen free radical toxicity. In the context of this study, the qualified term replicative senescence is used to avoid confusion in cases where cells might for example be described as 'senescent' because they were derived from an aged individual, or because they are derived from post-mitotic tissue demonstrating age related decline in function.

The behaviour of individual cells in culture was initially thought to display immortality. The first flawed experiments in this field were carried out by Carrell in 1912, using cells dissociated from chicks and sub cultured in a medium derived from chick embryo extract. Carrell claimed these cells could be sub cultured indefinitely, and that organismal ageing and therefore mortality was a function of multicellularity. The cells cultured were in fact mortal, being sensitive to a wide variety of poor growth conditions and noxious substances. They were also being replenished regularly with a medium that was contaminated with fresh cells, and the cultures correspondingly underwent phases of growth when new, presenescent cells were added with the growth medium (Cristofalo & Pignolo 1993) (Hayflick 1965). Since then and despite advances in the field, Carrel's results have never been duplicated despite several attempts (Hayflick 1965) (Witkowski 1980). However, the view that the individual cell is inherently immortal persisted until the landmark experiments of Hayflick and Moorehead in 1961 and 1965, which proved conclusively that normal human fibroblasts have only a limited capacity for division. The process that eventually limits cell division has been termed replicative senescence, and with the exception of cells in the germ line and some stem cell types it affects all mammalian cells (Hayflick 1965) (Hayflick & Moorhead 1961) (Hayflick 1997). Olovnikov first proposed that loss of chromosomal end genes, or 'telogenes' was the trigger for Hayflick's limit, and linked telomeres to replicative senescence (Olovnikov 1973).

Replicative senescence occurs in two distinct phases in human fibroblasts, mortality stages 1 and 2 (M1 and M2, shown in Figure 1-4), and this model is the accepted sequence of events that occur in normal somatic cells approaching replicative exhaustion (Wright & Shay 1992). M1 probably occurs when telomeres are sufficiently short that they can no longer be effectively 'masked' from cellular DNA repair machinery and are treated as double stranded DNA breaks (Harley 1991). The replicative block that occurs at M1 is mediated by cell cycle checkpoint proteins that are associated with response to DNA damage, such as the p53 and p16/pRb pathways (Kohn 1999). As such, mutations or transforming oncogenes that inactivate these checks allow further cell division with concomitant telomeric attrition until M2 is reached (Lundberg, Hahn, *et al* 2000).

Figure 1-4. Two step hypothesis of cellular immortality. Germ line cells and pluripotent stem cells are telomerase positive, although pluripotent stem cells do not maintain their telomere lengths. Normal somatic cells are telomerase negative and undergo telomeric attrition. At a critical length this erosion triggers a replicative block termed Hayflick's limit, or mortality stage 1 (M1). Abrogation of cell cycle checkpoints such as p53 or p16/pRb allows further cell division and concomitant telomeric loss until a genetic crisis, or mortality stage 2 (M2) is triggered characterised by massive cell death. Almost all the rare cells that survive beyond this stage contain mutations that have led to telomerase reactivation.



M2 (crisis) represents the end result of critically short telomeres, and is characterised by end-to-end fusions, and chromosome breakage fusion cycles resulting in apoptosis (Wright & Shay 2000). The stringency with which replicative senescence is adhered to varies between species, for example whilst there are no confirmed reports of either human or chick fibroblasts from normal donors spontaneously immortalising, this occurs relatively frequently in cells derived from mice (Ponten 1971).

The onset of replicative senescence is determined by the number of times the cells divide, and not by calendar time. It is characterised by irreversible growth arrest in the G1 phase of the cell cycle and the ability to survive and remain metabolically active in this condition for a period of time that may extend to several years, despite an inability to synthesise DNA (Matsumura, Zerrudo, *et al* 1979). Senescent cells also appear enlarged and flattened, and express a β-galactosidase activity at pH6 (Dimri, Lee, *et al* 1995). These criteria are few, partly as other changes that occur in senescence are also seen in the reversible state of quiescence into which some cultured cells fall in the absence of growth factors (Berube, Smith, *et al* 1998). Even very young cultures are found to contain a small proportion of cells that are senescent, and this proportion slowly increases throughout the life span of the cell line until all the cells are affected.

It has been found that human fibroblasts, when treated with high concentrations of hydrogen peroxide stop dividing and become enlarged. This has been interpreted as a means by which replicative senescence may be triggered for experimental purposes. However as other cell states (such as terminal differentiation) may mimic the senescent phenotype, this means of inducing senescence should be treated with caution; at least until the underlying control mechanisms are more fully understood (Chen & Ames 1994) (Smith & Pereira-Smith 1996). In contrast, a recognised means for delaying the onset of replicative senescence is via cellular expression of DNA tumour viral genes, such as simian virus 40 (SV40) T antigen. SV40 acts as a dual inactivator of the genes p53 and RB1. The same effect is seen by treatment with antisense oligonucleotides to the tumour suppression genes p53 and RB1, or by expression

of a dominant negative p53 mutant (Smith & Pereira-Smith 1996). These treatments allow cells an extra 10-20 doublings compared to controls, though they are only effective if carried out on pre-senescent cells, as senescent cells cannot be stimulated to divide (Sager, Tanaka, *et al* 1983). After the extra population doublings treated cells will enter senescence normally, with the exception of SV40 treated cells, which will instead undergo a period of genetic crisis, usually leading to cell death.

1.4.1 How is senescence triggered?

When considering this subject, it should first be noted that whilst the finite replicative potential of normal somatic cells is now as much a central dogma as Carrel's view of cellular immortality was before 1961, there are still dissenting voices. Notably Harry Rubin states that the concept of a genetically predetermined number of human fibroblast replications, and its implied extension to other cells, is based on an artefact resulting from the damage accumulated by the explanted cells during their replication in the radically foreign environment of cell culture (Rubin 1998). Rubin is not alone in this view, and it must be accepted that the cell culture environment, improved though it is from the days of Carrel's work does represent a radically alien and crude environment for the cells grown within it; as such the true nature of cellular replicative potential may not lend itself entirely to one view or the other but lie somewhere between the two camps. That aside, replicative senescence as a phenomenon is widely accepted (Kipling & Wyndford-Thomas 1999), and explanations of how it is triggered usually fall into two broad camps. First, the cell is considered to play a passive role, having senescence thrust upon it as the result of accumulation of errors associated with, for example, imperfect repair of DNA damage and inadequate scavenging of harmful free radicals. Second, senescence is considered an active, programmed cellular process, and implies cellular ageing is part of an intrinsic genetic programme.

1.4.1.1 Mechanisms under consideration

Several hypotheses have been advanced to explain the link between telomeric attrition and replicative senescence. First, telomeres might bind transcription factors necessary for triggering senescence that are released upon loss of enough repeats. Whilst there is as yet no firm evidence for this in mammalian cells, this idea is based on experimental work carried out in yeast, where the telomere associated Rap1 protein sequesters silencing factors that normally act at non-telomeric sites (Marcand, Buck, *et al* 1996).

Second, the heterchromatic structure of DNA near the telomere may act to silence genes necessary for triggering the senescent phenotype. These genes would then become derepressed as telomeric sequence is lost, and heterochromatin diminishes. Again, the evidence put forward in support of this hypothesis is yeast-based research where there is strong evidence of desilencing of loci near telomeres (Shore 1995).

Finally, short telomeres may cause a DNA damage response that leads to cell cycle arrest, and replicative senescence. The key to this mechanism is believed to be the T-loop structure identified by Griffith *et al* and described previously (Griffith, Comeau, *et al* 1999). T-loops allow the telomere end to be sequestered from DNA repair complexes, and it is possible that loss of this loop forming ability due to telomeric attrition is the mechanism through which the senescence response is initiated. This is likely to occur through a p53 mediated DNA damage response.

1.4.1.2 Evidence in favour of a genetic basis to replicative senescence

Smith *et al* exploited *in vitro* hybrids between young and old cells to investigate how replicative senescence is controlled. Such hybrids were found to have no greater replicative potential than the old set of parental cells from which they were partly derived. This suggests that the senescent phenotype is dominant.

Further evidence of this is the observation that hybrids of two old cell lines never show greater replicative potential than their parental cells. In addition, fusion of immortal and mortal human fibroblasts produce hybrids that are invariably mortal, indicating that cellular immortality arises due to recessive mutations that allow escape from senescence (Pereira-Smith, Robetorye, *et al* 1990).

1.4.2 Replicative senescence and ageing

The link between replicative senescence and ageing was first postulated by Hayflick (Hayflick 1965), who linked the aged appearance of senescent cells to organismal ageing, and suggested replicative senescence may therefore play a part in the many phenotypic changes that occur during this process. This proved a popular idea, as it provided a mechanism by which cell culture studies could contribute to the investigation of the ageing process. The study of ageing, and more specifically age related disease is extremely important in the human field as we live in a world with an ageing population (HMSO 1995). Many of the improvements in human healthcare that have produced this effect have filtered into the veterinary field resulting in an increased demand for treatments for age related disease such as cancer (Bronson 1982). Hayflick's original hypothesis of ageing through the mechanism of replicative senescence indicates a cellular, genetic programme for how we age, but does not address why this might have evolved. The question of why we age is engaged more recently in the concept of antagonistic pleiotropy, which has the central tenet that evolutionary pressure does not have any effect on the post-reproductive age organism. This fits conveniently with Hayflick's idea, as replicative senescence would be strongly selected for as a potent anti-cancer mechanism and if any deleterious effects were co-selected they would be unchecked by the forces of evolution if they occurred in the older post-reproductive organism. The supposition is therefore that ageing may be the price paid for a cancer free reproductive lifespan (Krtolica, Parrinello, *et al* 2001).

A second idea is the disposable soma theory of ageing, in which evolution is viewed as the force behind the most efficient use of resources between

reproduction and somatic tissue repair (Kirkwood 1996). The theory is well illustrated by the mouse, a creature with a short lifespan, high fecundity and high predation rate. According to the theory, evolution has allocated the lion's share of resources to reproduction rather than a long-lived body, as a mouse with a body designed to live to for fifty years will in its natural environment most likely be eaten after two. Therefore the life spans of species may be a reflection of a logical use of resources in the face of predation and disease.

Aside from the hypotheses about why we age, Hayflick's idea about how this might happen has yet to be tested critically. Even if proved to play a part in the ageing process, replicative senescence cannot be the sole mechanism involved, as it does not provide an explanation for the age related changes that are clearly documented in post-mitotic tissues such as neurones (Smith & Whitney 1980). Yet cell culture has provided indirect evidence of a link between the two processes. First, a number of *in vitro* studies have shown an inverse relationship between donor age and the number of population doublings (PDs) until replicative senescence (Allsopp, Vaziri, *et al* 1992). Second, interspecies studies have shown a correlation between species life span and the replicative potential of fibroblast cell cultures derived from them. For example, whilst murine fibroblasts senescence after only 10-15 PDs, fibroblasts cultured from the long-lived Galapagos tortoise routinely undergo more than 100 PDs before exhausting their replicative potential (Soldstein 1974). This points to an overlap in the genes governing replicative senescence and organismal ageing (Rohme 1981). Allied to this data is the observation that cell lines derived from sufferers of the human progeric syndromes Werner's syndrome and Hutchinson-Gilford exhibit decreased proliferative potential compared to age matched controls. Such decreased proliferative potential is also found in Down's syndrome patients (Oshima, Campisi, *et al* 1995) (Goldstein & Harley 1979). Furthermore, recent research has demonstrated an association between loss of replicative potential and premature cardiac ageing in humans (Chimenti, Kajstura, *et al* 2003).

At a molecular level correlations have been uncovered between replicative senescence and ageing. The ability to respond to stressors by induction of heat shock protein 70 is attenuated in both senescent human fibroblasts and aged

rodent tissue (Choi, Lin, *et al* 1990) (Fawcett, Sylvester, *et al* 1994). Such observations are circumstantial only, but add to the overall weight of evidence.

Finally, a senescence associated marker enzyme (β-galactosidase activity at pH6, Dimri, Lee *et al* 1995) has been found to be more prevalent in cells from physiologically aged tissue of the Rhesus monkey (*Macaca mulatta*) than young equivalents, providing the first *in situ* evidence that replicative senescent cells accumulate in aging tissue. Crucially, this enzyme activity is not displayed in reversibly arrested cells, such as young, growing cells that have been affected by removal of growth factors. This allows clear differentiation between quiescence and true replicative senescence (Pendergrass, Lane, *et al* 1999).

The above evidence is not without contradiction; for example Cristofalo *et al* could not demonstrate an age effect on fibroblast replicative potential using a health screened donor population, and pointed out that weaknesses in candidate selection may give rise to false correlations (Cristofalo, Allen, *et al* 1998). One example of this is a study that could demonstrate an inverse relationship between the two parameters only when diabetics and pre-diabetics were included in the study group, even though diabetes is a disease that is known to increase cell turnover (Goldstein, Moerman, *et al* 1978).

Furthermore, whilst the decreased growth potential of cultures from patients with progeric syndromes would appear to provide strong circumstantial evidence of a link between replicative senescence and ageing, closer examination of early senescent cells from donors with perhaps the most documented condition, the Werner syndrome are found not to repress c-fos, which is a hallmark of normal senescence (Oshima, Campisi, *et al* 1995).

Perhaps most crucially of all, whilst the above evidence supports the idea of replicative senescence and ageing being co-dependent variables this does not imply causality; thus whilst telomeric attrition is associated with ageing it has not been shown conclusively that it is the major cause of ageing.

1.4.3 Senescence as a tumour suppressor mechanism

The ability of cells to replicate is essential for repairing and renewing the tissues of multi cellular organisms throughout life. However, this capacity to divide is also an opportunity for mutations to develop, and such mutations can lead to malignant transformation. The risk of malignant transformation increases with oxidative stress, environmental insults and errors in DNA replication. This is because all these factors cause damage to the genome, which increases the risk of mutation. It is mutation that may cause a genome to become unstable (hypermutable), and confer a growth or other survival advantage over normal cells that allows the development of cancer (Campisi 2001a).

The dividing cell has two strategies for dealing with the risk of malignant transformation. First, apoptosis or programmed cell death eliminates at risk cells from the population. Second cellular senescence eliminates risk by irreversibly arresting growth. The term 'cellular senescence' is used specifically in this case to denote a senescent phenotype that may be induced by a range of factors other than telomeric attrition, such as DNA damage, chromatin remodelling and strong mitogenic signals (Campisi 2000). Evidence linking cellular senescence and tumour suppression has been uncovered both *in vitro* and *in vivo*. DNA damage in the form of double strand breaks or oxidative damage will induce a senescent phenotype, and oxidative stress is known to shorten telomeres (von Zglinicki 2002). Agents that open or unravel chromatin structure will induce a senescent phenotype (Young & Smith 2001), and such agents have the capacity to cause loss of the gene silencing capacity of chromatin. Over expression of both the growth stimulatory transcription factor E2F1 and activated forms of the growth factor signal transducing protein Ras will induce a senescent phenotype (Dimri, Itahana, *et al* 2000) (Serrano, Lin, *et al* 1997). Malfunctions of these factors may contribute to the development of cancer; and this points to senescence being a general tumour suppressor mechanism with the capacity to respond to a wide range of signals from different sources within the cell.

The genes controlling cellular senescence give further insight into the importance of this process as a tumour suppressor mechanism. Genes governing cellular senescence encode two proteins that are at the heart of the two most important tumour suppressor pathways in the cell. Together, functional loss of the tumour suppressors p53 and pRb are the most disabled in mammalian cancers (Hickman, Moroni, *et al* 2002), and such functional loss has been shown to occur in canine cancers, for example canine melanoma (Koenig, Bianco, *et al* 2002). p53 controls the expression of genes that respond to genomic damage through cell cycle arrest or apoptosis, and p53 levels are increased in senescent cells (Bringold & Serrano 2000) (Lundberg, Hahn, *et al* 2000). pRb does a similar job of policing the genes controlling cell cycle progression and cellular differentiation, though by the indirect route of interacting with transcription factors such as E2F (Berube, Smith, *et al* 1998). Other oncogenes, such as c-myc, and the viral oncogenes E6 and E7, also act by overcoming senescence, underlining the importance of this process for tumour suppression in the cell (reviewed in Campisi, Dimri, *et al* 1996).

In vivo evidence of the link between senescence and tumour suppression has been demonstrated in the highly cancer prone state of mice that have been engineered to produce inactive p53 or $p16^{INK4a}$ proteins (Ghebranious & Donehower 1998). Conversely, premature senescence of mammary epithelial tissue suppresses the development of mammary cancer in young mice exposed to the mouse mammary tumour virus (Boulanger & Smith 2001).

Finally, a clear piece of evidence for the importance of replicative senescence as a tumour suppressor mechanism is the fact that cells with a finite replicative life span are orders of magnitude less likely to form tumours than immortal cells (Campisi 1997).

1.4.4 Cellular senescence as a facilitator of tumorigenesis

In light of the above evidence, it may appear strange to describe senescent cells as a cancer risk factor, however recent evidence shows this to be the case; not by

any direct action of the senescent cells themselves, but via adjacent cells that still have replicative potential (Krtolica, Parrinello, *et al* 2001).

Cellular senescence has been described as an example of antagonistic pleiotropy (Campisi 2001a). Therefore whilst replicative senescence may have evolved to provide the advantage of tumour suppression to organisms of breeding age, it may also have selected for undesirable characteristics, one of which may be a tendency to develop cancer in the older, post-reproductive organism. Krtolica *et al* using *in vitro* human fibroblast cultures provided the main experimental evidence of this effect. This work showed that both premalignant and malignant epithelial cells are stimulated to proliferate by senescent fibroblasts via factors that are at least in part secreted by the senescent cells (Krtolica, Parrinello, *et al* 2001). This effect was observed when the senescent cells comprised only 10% of the cell population as a whole, and was unaffected by the method by which senescence was induced (replicative exhaustion, oncogenic RAS, $p14^{ARF}$, and hydrogen peroxide).

1.5 Telomerase structure

Not all mammalian cells undergo telomeric attrition; for example germ-line cells and cancer cells do not. However these cells possess linear chromosomes, and are therefore prey to the end replication problem. The solution is the ribonucleoprotein telomerase, a complex composed of an RNA sequence that is complimentary to the telomere sequence, a catalytic component, and several other associated structural proteins with an estimated total molecular mass of 1000 kilodaltons (kDa) (Dhaene, Van Marck, *et al* 2000). Telomerase was identified in 1985, and the earliest purification of the active components occurred in *Euplotes aediculatus* (Greider & Blackburn 1985) (Lingner & Cech 1996). Telomerase activity allows maintenance of telomere length despite cell division, and accordingly activity is strongly associated with immortalised and cancerous tissues (Kim, Piatyszek, *et al* 1994) (Shay & Bacchetti 1997).

Telomerase is frequently described as a 'specialised' reverse transcriptase, and there are three main reasons for granting it specialised status. First, and unusually for a reverse transcriptase, telomerase utilises only a part of its RNA subunit functionally. Second, during the processive synthesis of telomeric repeats, the substrate translocates from one end of the template to the other by an unknown mechanism, and finally, the RNA subunit of telomerase is stably associated with the protein complex as a whole.

Telomerase acts by using the RNA sequence as an internal template, guiding the addition of nucleotides to the 3′ end of the telomere by the catalytic component, telomerase reverse transcriptase. The complex is then repositioned at the new telomere terminus and the process is repeated until the effects of end replication problem have been negated (Buys 2000). Accordingly, telomerase activity should be absent from those cell types that undergo telomeric attrition, and this is indeed the case (Reviewed in Dhaene, Van Marck, *et al* 2000).

1.5.1 Telomerase RNA

This component was first cloned in *Tetrahymena thermophila,* and the human analogue is referred to as hTR (human telomerase RNA component). In humans, the hTR transcript is 451 nucleotides in length and lacks polyadenylation. It contains a 'template' sequence that is complimentary to the telomere repeat, and in humans this sequence is 11 nucleotides in length (5'-CUAACCCUAAC-3') (Feng, Funk, *et al* 1995). Mammalian telomerase RNAs resemble small nucleolar RNAs (snoRNAs) due to the presence of a H/ACA box in their 3′ domain (Mitchell, Cheng, *et al* 1999). The vital nature of the RNA component to telomerase function was revealed first in *Tetrahymena*, where disruption of telomerase RNA was shown to lead to progressive telomere shortening (Ahmed, Sheng, *et al* 1998).

1.5.2 Telomerase reverse transcriptase

The telomerase reverse transcriptase gene (the human analogue is referred to as hTERT) consists of 16 exons and 15 introns and extends over 40 kb (Wick & Hagen 1999). Of the three major subunits that comprise the telomerase ribonucleoprotein, the hTERT gene product is considered to be the most common rate-limiting determinant of activity. This is due primarily to studies connecting telomerase activity directly with transcriptional activity of hTERT at all stages during the process of malignant transformation, in contrast with the hTR subunit that is expressed in many tissues irrespective of telomerase activity (Avilion, Piatyszek, *et al* 1996) (Wisman, De Jong, *et al* 2000). The reverse transcriptase domain has been shown to be essential for telomerase activity (Weinrich, Bodnar, *et al* 1997), however in humans both the amino and carboxyl terminus also have important functional roles to play, and even minor deletions in these regions may lead to loss of telomerase activity (Beattie, Zhou, *et al* 2000) (Banik, Guo, *et al* 2002).

The importance of the TERT component is further supported by studies demonstrating that forced expression of hTERT in telomerase negative cells is enough to reconstitute telomerase activity and extend replicative life span (Bodnar, Ouellette, *et al* 1998). This work was subsequently extended by Takakura *et al*, who showed conclusively that hTERT is significantly activated in cancer cells, but repressed in normal primary cells (Takakura, Kyo, *et al* 1999).

1.5.3 Human telomerase associated protein 1

TEP1, or hTEP1 in humans (human telomerase associated protein 1), is a 240 kDa protein which is associated with telomerase activity, but in common with hTERT, expression of this protein alone in a cell does not imply telomerase is active (Harrington, McPhail, *et al* 1997). hTEP1 is the human homologue of p80 in *Tetrahymena*, p80 being one of the first telomerase associated proteins identified. This protein was initially thought to be the catalytic component of

telomerase before TERT was identified (Dhaene, Van Marck, *et al* 2000). Some light has been shed on the possible function of this protein by recent work that has shown TEP1 to be identical to the 240 kDa vault protein. Vault proteins are large cytoplasmic ribonucleoprotein complexes, and whilst their function is as yet unknown the sharing of TEP1 between vault proteins and telomerase suggests TEP1 may play a structural role in ribonucleoproteins, or aid generally with their function or assembly (Kickhoefer, Stephen, *et al* 1999). In the same study, Kickhoefer *et al* demonstrated that vault proteins themselves display no detectable telomerase activity.

1.6 Telomerase activity

Many human tissues display telomerase activity during early embryonic development, however this period is short lived, and telomerase activity is repressed in most normal human somatic tissues after birth (Kim, Piatyszek, *et al* 1994) (Shay & Bacchetti 1997). Telomerase activity may still be found in specific tissues throughout adult life, and this pattern correlates with tissues that require a large replicative potential due to functional demand. For example, Tahara *et al* detected telomerase activity in normal colonic glandular epithelial crypt cells (Tahara, Yasui, *et al* 1999). Telomerase activity is induced by antigen activation of mature resting lymphocytes, (Weng, Palmer, *et al* 1997) and germ cells are telomerase positive, along with normal endometrial tissue (Dhaene, Van Marck, *et al* 2000).

1.7 Regulation of telomerase activity

1.7.1 Transcriptional regulation of the hTERT gene

As TERT is the primary determinant for telomerase activity the regulation of TERT expression has been the focus of many studies. The isolation and characterisation of the hTERT promoter (Wick & Hagen 1999) (Cong, Wen, *et al* 1999) led to transient transfection experiments that used hTERT promoter-luciferase constructs to show promoter inactivity in mortal and transformed

preimmortal cells, but activity in immortal cells, highlighting the importance of transcriptional control (Takakura, Kyo, *et al* 1999).

Regulation of hTERT promoter activity is likely to be a key point that is controlled at a number of different levels. The large numbers of transcription factor binding sites within the hTERT promoter provide evidence for this. Of these, several have been implicated in the control of hTERT expression and may be defined as transcriptional activators or repressors (Cong, Wen, *et al* 1999).

1.7.1.1 TERT transcriptional activators

1.7.1.1.1 C-Myc

C-myc is a well-characterised oncogene that promotes growth, proliferation and apoptosis (Grandori, Cowley, *et al* 2000). Alterations in the structure or expression of this gene have been linked to a wide variety of human cancers. The link between c-myc and hTERT transcription are the two E-box recognition sequences in the hTERT promoter sequence. This sequence (5′-CACGTG-3′) is recognised and bound by heterodimers formed by c-myc and the Max protein (Grandori, Cowley, *et al* 2000). C-myc has been shown to induce hTERT expression and telomerase activity in primary fibroblasts (Wang, Xie, *et al* 1998). These data provide clear evidence of a direct effect of c-myc on hTERT activation.

1.7.1.1.2 Sp1

Sp1 is a general transcription factor that binds to specific sequence areas of promoters termed GC boxes. It helps to initiate transcription of a large number of genes, particularly by aiding transcription of promoter sequences that are without the TATA-box binding protein, which is part of the general transcription machinery of the mammalian cell. The hTERT promoter is TATA-less, and Sp1 has been shown to cooperate with c-myc to activate transcription of hTERT in a cell-type dependant manner (Kyo, Takakura, *et al* 2000).

1.7.1.1.3 Human papillomavirus 16 E6 protein

Telomerase activity can be induced in a number of human cell types by the human papillomavirus 16 E6 protein, via up regulation of hTERT transcription. The mechanism by which E6 causes hTERT up regulation is as yet unknown, however it occurs independent of any interactions with either p53 or c-Myc (Klingelhutz, Foster, *et al* 1996).

1.7.1.1.4 Steroid hormones

Initial studies focusing on telomerase activity in normal human ovarian and endometrial tissues identified telomerase activity at those stages of the menstrual cycle that are under the influence of oestrogen. It has been recognised for some time that certain types of cancers are oestrogen dependant (Henderson, Ross, *et al* 1993), and so research has been directed at identifying whether a causal link between oestrogen influence and telomerase activity can be identified. Kyo *et al* were the first to demonstrate that oestrogen activates telomerase, and that this phenomenon is due to direct transcriptional regulation of hTERT expression in hormone sensitive tissues (Kyo, Takakura, *et al* 1999).

1.7.1.2 TERT transcriptional repressors

The crucial importance of TERT expression for reconstituting telomerase activity has been outlined above, and in accordance with the finding that telomerase activity is absent from most normal post-embryonic human somatic tissues it is considered likely that repression of hTERT transcription in these cell types is a key control point. Consistent with this hypothesis, fusion between normal somatic cells and some immortal telomerase positive cells results in repression of telomerase activity (Ishii, Tsuyama, *et al* 1999). Furthermore, down regulation of telomerase activity in cancer cells has been achieved via repression of hTERT expression by the transfer of specific chromosomes from normal cells, implying that normal cells express negative regulators of hTERT expression (Oshimura & Barrett 1997). To date several hTERT repressors have

been identified including Mad1, p53, pRb, E2F1, WT1, and anti-proliferation agents, as detailed below.

1.7.1.2.1 Mad1

The proteins c-Myc, Max and Mad1 are crucial to the proper cellular control of growth and differentiation. As has been described in Section 1.7.1.1.1 the heterodimers formed by c-myc and max have a positive effect on hTERT transcription. Mad/Max dimers act as the counterbalance to this relationship and produce downregulation of hTERT transciption through competitive inhibition of the c-myc/Max dimers (Xu, Popov, *et al* 2001).

1.7.1.2.2 p53

p53 is a tumour suppressor protein that acts by inducing cell cycle arrest or apoptosis and can respond to a variety of types of cellular damage. The importance of this protein is reflected in the fact that it is functionally disabled in 50% of human tumours (Asker, Wiman, *et al* 1999). Recent evidence has shown that p53 down regulates hTERT expression directly, and therefore its anti telomerase effects are not dependant on its cell cycle arrest or apoptosis functions (Xu, Wang, *et al* 2000) (Kusumoto, Ogawa, *et al* 1999).

1.7.1.2.3 pRb and E2F1

Over expression of both pRb and E2F1 resulted in repression of telomerase activity in a number of human cancer cell lines. The exact mode of action of this effect has yet to be elucidated, and whether pRb and E2F1 act independently or cooperate (Ying C.Henderson, , *et al* 2000) (Nguyen & Crowe 1999).

1.7.1.2.4 Wilms' tumour 1 tumour suppressor (WT1)

WT1 is a tissue specific (kidney, gonad and spleen) repressor of the hTERT promoter, inactivation of which may contribute to activation of telomerase during tumorigenesis in its target tissues (Oh, Song, *et al* 1999).

1.7.1.2.5 Antiproliferation and differentiation agents

Telomerase activity is reduced by cellular differentiation and cell cycle exit, therefore focus has been given to identifying possible inhibitory effects by factors governing these cellular changes on hTERT activity. Both Interferon-α and autocrine transforming growth factor β have been shown to exert a direct inhibitory effect on TERT expression in cell lines derived from human malignant cancers. Both these factors are involved in control of cellular proliferation, and it is possible many more such factors exert similar influence (Yang, Kyo, *et al* 2001).

1.7.2 Epigenetic regulation of TERT gene

DNA methylation is a common, programmed alteration of genomic sequences that has been shown to be essential for normal development (Reik & Dean 2001). The possibility that abnormal methylation may lead directly to up regulation of hTERT activity exists though whether this occurs remains to be resolved. It is clear that abnormal methylation down regulates tumour suppressors such as p16 and pRb and is associated with human cancers and so such a relationship may be implied (Laird & Jaenisch 1994). In addition, a reverse correlation has been shown between the degree of methylation of the hTERT promoter and telomerase activity in B-cell lymphocytic leukaemia (Bechter, Eisterer, *et al* 2002); it is possible such relationships are not rarities, but are as yet undiscovered.

1.7.3 Other TERT controls

Transcriptional regulation of hTERT is undeniably important for the regulation of telomerase activity in cells. However, the large volume of work that has been dedicated to categorising the telomerase activity status of normal human tissues has revealed other points of control. For example full-length hTR and hTERT mRNA are readily detectable in human lymphocytes, tonsils, peripheral B and T

cells and ovarian tissue without the presence of detectable telomerase activity (Ulaner, Hu, *et al* 2000) (Liu, Schoonmaker, *et al* 1999). This posttranscriptional control is likely to relate to phosphorylation of hTERT by serine/protein kinases, which is a modification necessary for activity that has been demonstrated by a number of different workers (Akiyama, Hideshima, *et al* 2002) (Minamino & Kourembanas 2001) (Haendeler, Hoffmann et al 2003). Interestingly, phosphorylation regulation of hTERT may exert control over telomerase activity in part by influencing the location of hTERT within the cell. hTERT tyrosine phosphorylation at position 707 has been linked with nuclear export of hTERT, and increased antiapoptotic activity (Haendeler, Hoffmann et al 2003). This also indicates novel effects of TERT within the cytosol that are unrelated to telomerase actvity, and demonstrate clearly that regulation of telomerase and its components is more complex than initially thought. Furthermore, the finding of hTERT transcripts in foetal tissues and tumour cell lines that would result in a truncated or inactive protein indicate that alternative splicing also has a role to play in regulating telomerase activity. These include an α transcript which lacks 36 nucleotides from the 5′ end of exon 6, the β transcript, which lacks exons 7 and 8 entirely, both α and β spliced transcripts and a number of insertional alternative transcripts (Ulaner, Hu, *et al* 1998) (Ulaner, Hu, *et al* 2001) (Ulaner, Hu, *et al* 2000) (Yokoyama, Wan, *et al* 2001).

The exact repressor gene or genes responsible for suppressing hTERT transcription have yet to be identified, however a gene/number of genes on the short arm of chromosome 3 suppress telomerase activity, (Tanaka, Shimizu, *et al* 1998) and produce permanent growth arrest in breast cancer cells (Cuthbert, Bond, *et al* 1999). Antagonism of growth hormone releasing hormone also produces down regulation of the hTERT gene in human glioblastomas, but the mechanism of suppression is not known (Kiaris & Schally 1999).

Initial investigations into the location of the telomerase gene placed it at a sub-telomeric region of the short arm of chromosome 5p (Bryce, Morrison, *et al* 2000). This data led workers to speculate that telomere position effect (TPE) might be the key to explaining transcriptional regulation. TPE has been characterised in both yeast and human cells, and refers to the reversible silencing

of a gene near the telomere (Tham & Zakian 2002) (Baur, Zou, *et al* 2001). More recent data indicates that the hTERT gene is positioned more than 2 Mega bases from the telomere, and so it is likely other control mechanisms are at work (Leem, Londono-Vallejo, *et al* 2002).

1.8 Telomeres, telomerase and cancer

The unchecked cellular division that is a feature of cancer cells indicates these cells have evolved a way to overcome the end replication problem. Telomerase reactivation is the most common way in which cancer cells overcome end replication problem, and telomerase activity in a growing mass is a strong indicator of malignancy (Shay and Bacchetti 1997). Bodnar *et al* described the first direct evidence of the ability of telomerase to extend the replicative life span of a cell line. This showed conclusively that forced expression of the catalytic component of human telomerase, hTERT was sufficient to reconstitute telomerase activity in the test cells, and that the newly telomerase positive cells were capable of dividing well beyond their accepted replicative limits. In addition to this the telomerase positive clones had elongated telomeres, and reduced β-galactosidase activity compared with their telomerase negative counter parts (Bodnar *et al* 1998).

A number of telomerase positive cell lines have shown continued telomeric attrition after immortalisation, which has lead to the opinion that another property of telomerase beyond telomere maintenance may contribute to the process of immortalisation (Zhu, Wang, *et al* 1999). Stewart *et al* examined the capacity of oncogenic H-Ras to transform previously immortalised cells and provided experimental evidence for this. Expression of H-Ras in a cell line immortalised by an alternative pathway did not cause transformation, however when these cells where forced to express hTERT and the experiment repeated successful transformation occurred and the cells developed a tumorigenic

phenotype. Crucially, this was also possible using a mutant form of hTERT that retains catalytic activity, but cannot maintain telomeres; providing clear evidence for a non-telomere based contribution of telomerase to tumorigenesis (Stewart, Hahn, *et al* 2002).

Whilst these data indicate telomerase activity acts as a facilitator of malignancy, telomerase activity *per se* is not sufficient to cause cancer, i.e. telomerase is not an oncogene. This is borne out by the observation that certain germ line cells and stem cells are telomerase positive, but are not cancerous (Kim, Piatyszek, *et al* 1994) (Broccoli, Smogorzewska, *et al* 1997). Telomerase activity does however confer unlimited replicative potential on a cell, which allows for the accumulation of mutations that may eventually lead to malignancy.

1.8.1 The Alternative Lengthening of Telomeres (ALT) pathway

Not all tumours, or tumour derived cell lines are telomerase positive. As many as 10% of human tumour derived cell lines are telomerase negative and rely on an alternative mechanism of telomere maintenance termed 'Alternative Lengthening of Telomeres,' (ALT) (Bryan & Reddel 1997). The mode of action of the ALT pathway is poorly understood, however cell fusion experiments have shown that ALT is triggered by recessive mutation(s) (Perrem, Bryan, *et al* 1999). The initial insight into the mechanism of ALT was gained by Murane *et al*, whose experimental work demonstrated fluctuations in telomere lengths of ALT positive cells consistent with a recombination mechanism (Murnane, Sabatier, *et al* 1994).

Human cells with an active ALT pathway characteristically have widely varying telomere lengths within the same cell, including telomeres so short as to be undetectable, and those so long as to be considered abnormal for that particular cell type (Grobelny, Godwin, *et al* 2000). This is in contrast to the situation in telomerase positive human cell lines, where mean telomere length is relatively homogenous around 5 kb (Chu, Piatyszek, *et al* 2000). The two modes of

telomere length maintenance are not mutually exclusive, and both telomerase activity and the ALT mechanism have been demonstrated in the same human cell line (Cerone, Londono-Vallejo, *et al* 2001).

Many ALT cell lines have also been found to contain nuclear structures referred to as ALT associated PML bodies (APBs) (Yeager, Neumann, *et al* 1999). PML (promyelocytic leukaemia) nuclear bodies (PNBs) have been implicated in oncogenesis and viral infection. They are usually 0.3-1 μm in diameter, and a typical mammalian nucleus may contain 20-30 such structures. (Ruggero, Wang, *et al* 2000) (Boisvert, Hendzel, *et al* 2000). APBs are disc or ring shaped PMLs with ALT specific contents, such as telomeric DNA and TRF1 and TRF2. APBs are the first PBLs found to contain DNA (Yeager, Neumann, *et al* 1999). In addition they have been found to contain a range of proteins involved in DNA replication and recombination (reviewed in Henson *et al* 2002). It is possible APBs may gather or modify the proteins required for functioning of the ALT mechanism. Recent work has shown that APBs may localise at nuclear foci in response to DNA damage, in association with factors that are associated with DNA repair such as RAD51 (Bischof, Kim, *et al* 2001).

Presently there are four proposed mechanisms by which homologous recombination may result in telomere replication. The first proposes inter-telomeric recombination events as the mechanism by which ALT maintains telomeres (Dunham, Neumann, *et al* 2000). As this mechanism involves the formation of a displacement loop, it raises the second possibility that the displacement loops created by the formation of the T-loops identified by Griffith *et al* (Griffith, Comeau, *et al* 1999), and described previously may be the basis of another mechanism of recombination dependant replication; in essence a self-replicating telomere (Henson, Neumann, *et al* 2002). The third and fourth hypotheses are based on an extra-chromosomal step involved in the copying of telomeric sequence. This mechanism involves extrachromosomal telomeric repeats (ECTR) in either a linear or circular format. ECTR have been identified in various types of cells, however they are generally restricted to ALT positive cells (Ogino, Nakabayashi, *et al* 1998). Circular ECTR has been utilised by *K.*

lactis to extend its telomeres, and linear ECTR has been identified in all ALT cell lines that have been tested (McEachern 2001) (Yeager, Neumann, *et al* 1999).

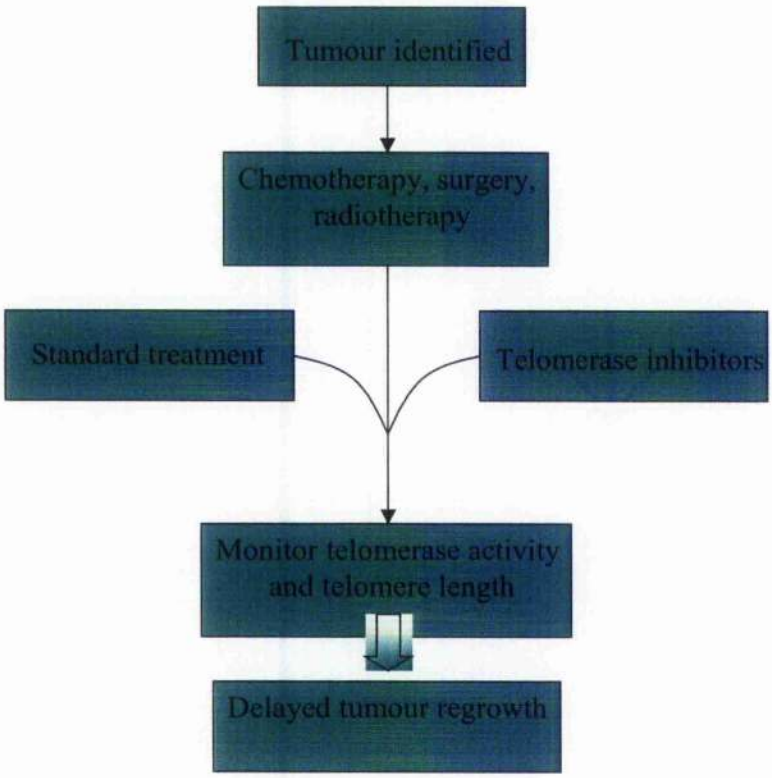
An important recent development in ALT research is the finding that ALT positive cell lines failed to produce macroscopic lung tumours even seven weeks after tail vein injection in mice using five different cell lines. By contrast once telomerase activity had been reactivated, the same five cell lines produced tumours large enough to kill the injected mice after 5 weeks. The conclusion reached is that ALT positive tumours, whilst capable of producing locally aggressive lesions appear to lack the ability to metastasise. This could be of great importance for the targeting of future cancer therapies based on anti telomerase therapy (Chang, Khoo, *et al* 2003).

1.8.2 Telomerase as a therapeutic target

The clear evidence linking telomerase activity to cellular immortalisation and cancer development makes telomerase an attractive target for anti-cancer therapeutic research (Yan, Coindre, *et al* 1999). The aim of this therapy would be to restore mortality to telomerase positive cancers via telomerase inhibition, allowing telomeric attrition to lead to cell senescence, or preferably a genetic crisis resulting in cell death. The therapeutic strategy is outlined in Figure 1-5. One of the main attractions of this type of therapy and a potential advantage over traditional methods is the very low levels of telomerase activity in normal tissues, allowing selective targeting of cancer cells with a minimum of toxic side effects (Bearss, Laurence, *et al* 2000). As previously mentioned, a range of normal tissues do retain telomerase activity, however selective tumour cell killing is still feasible as the majority of tumours investigated have shorter telomeres and higher proliferation rates than normal proliferating cell populations (Hastie, Dempster, *et al* 1990), (Bacchetti 1996). Thus, targeted anti-telomerase therapy aimed at a tumour cell population known to possess short telomeres would only require a short number of cell divisions before a therapeutic effect is seen, and this period of therapy should not effect the

telomere functions of the subject's population of germ and stem cells. The corollary of this is that anti-telomerase therapy may be ineffective in tumours with long telomeres, as the tumour may have progressed to a fatal outcome before senescence is triggered. It is possible that such tumours, whilst having substantial mean telomere lengths may have telomeres on specific chromosomes that are short enough to trigger a swift growth check. Despite this, the reality is that it is very unlikely all tumours will lend themselves to this type of therapy. These drawbacks, particularly the lag before therapeutic effect mean that antitelomerase therapy is unlikely to be a first line treatment for cancer, rather at present it is envisioned that these drugs will be deployed after primary therapy (such as surgery, radiotherapy, traditional chemotherapy) in a 'mopping up' role against surviving cancerous cells. Such therapy would be aimed at increasing remission times in clinical cases, or perhaps to affect a cure if the cancer was particularly sensitive to the agent (i.e. had short telomeres). In this regard, tandem research using existing therapies will focus on identifying which of these agents increase cancer cell vulnerability to telomerase inhibitors (Corey 2002).

Figure 1-5. Strategy for anti-telomerase cancer therapy. The aim of anti-telomerase therapy is to cause telomeric attrition in treated cancer cells, leading to senescence, or more favourably cell death. Such therapy is likely to be used as an adjunct to traditional treatment strategies, and the specificity of such agents will likely mean this therapy is particularly useful against metastatic disease and after primary debulking of a tumour.



1.8.3 Telomerase inhibitors

1.8.3.1 Reverse transcriptase inhibitors

The catalytic component of telomerase functions as a reverse transcriptase, and some of the earliest telomerase inhibition studies investigated the effects of drugs that are known to inhibit retroviral reverse transcriptase. The drugs used included Azidothymidine triphosphate, dideoxyguanosine, arabinofuranyl-guanosine, dideoxyinosine, dideoxyadenosine, didehydrothymidine and phosphonoformic acid. Whilst all these drugs are known inhibitors of retroviral reverse transcriptase, the emerging results with hTERT are not encouraging. Prolonged exposure of telomerase positive immortalised B- and T-cell lines to the panel of drugs resulted in telomerase inhibition in only two cases; with azidothymidine and dideoxyguanosine. Of these two, only dideoxyguanosine translated this effect into telomere shortening in every test culture, but crucially it had no effect on population doubling or cell survivability, even when drug exposure was extended to almost a year. The results indicated that the drug effect is caused by dideoxyguanosine binding to and competing for the nucleoside triphosphate binding site, and the results were not made more favourable by using combinations of the test drugs (Strahl and Blackburn 1996).

1.8.3.2 Telomerase RNA targeting

The majority of antitelomerase therapy tested to date has been directed against hTR. These agents are stably transfected antisense complementary DNA (cDNA), synthetic modified oligonucleotides with antisense polarity, targeted ribozymes and RNA interference (Feng, Funk, *et al* 1995), (Mata, Joshi, *et al* 1997), (Yokoyama, Takahashi, *et al* 1998).

1.8.3.2.1 Oligonucleotides

The RNA template region is considered a good target for antisense oligonucleotides for several reasons. First, the necessity of telomerase RNA to base pair with the telomere sequence for telomerase function indicates that this

region of the ribonucleoprotein must be accessible externally, and therefore it is a relatively available target within the cell. This is particularly the case when using oligonucleotides, as previous experience has given rise to compounds with favourable pharmacokinetics for cellular uptake, and furthermore chemically similar oligonucleotides have similar pharmacokinetics regardless of their target sequence (Corey 2002). Second, as the 11 base pair sequence of the RNA template region of hTR is known, design of the oligonucleotide itself is simple. Third, there are commercially available sources of oligonucleotides with widely varying chemical properties that may assist in achieving therapeutic effect, and as this is not a nascent technology, previous experience in this field will assist in development of clinical trials. Finally, use of oligonucleotides containing mismatched bases provides a convenient control of target specificity. An example of oligonucleotide technology already in development as an anti-cancer therapy is 'Gentasense,' being developed by Genta Corp. to target Bcl-2. This drug has shown promising results in trials against a number of different cancers (Dias & Stein 2003).

A hexameric phosphorothioate oligonucleotide has inhibited telomerase activity, lengthened cell-doubling time and induced apoptosis in a Burkitt's Lymphoma derived cell line *in vitro*. Using a xenograft human-nude mouse model to assess *in vivo* efficacy, this oligonucleotide caused a significant reduction in tumour size and splenic metastasis compared with mismatched sequence and saline only negative controls (Mata, Joshi, *et al* 1997). These results show that despite the drawbacks associated with telomerase inhibition therapy it is an area with potential for considerable success in the future.

1.8.3.2.2 Ribozymes

Preliminary results using hammerhead ribozyme technology have also gained promising results. Hammerhead ribozymes are small RNA motifs that have the ability to catalyse cleavage of the RNA phosphodiester chain at defined sites (Blount & Uhlenbeck 2002). Ludwig *et al* have reported the use of a hammerhead ribozyme that cleaved telomerase (hTERT) mRNA *in vitro* (Ludwig, Saretzki, *et al* 2001). This compound was stably expressed in a breast

cancer tumour cell line (MCF-7) using a mammalian expression vector delivery system, and in addition to resulting in reduced telomerase activity caused telomere reduction, growth inhibition and apoptosis.

1.8.3.2.3 RNA interference

RNA interference (RNAi) is a recent innovation that utilises cellular machinery that has evolved to target parasitic RNAs, including RNA viruses (Hutvagner & Zamore 2002). The mechanism is triggered by dsRNA, which becomes processed by double strand specific RNase to shorter RNA fragments. The shorter RNA fragments become incorporated into, and confer sequence specificity upon an RNase protein complex termed RISC that then targets the homologous RNA sequence for degradation (Hammond, Boettcher, *et al* 2001). The system may be triggered by artificially generated short dsRNAs, and these short interfering RNAs (siRNAs) have been tried against hTERT and the telomerase template RNA. Both agents reduced telomerase activity in a variety of human cancer cell lines, and although the effect was minor and short-lived, the result is interesting as it represents a success against a target thought to be restricted to the nucleus by a modality thought only to be effective in the cytoplasm (Kosciolek, Kalantidis, *et al* 2003).

1.8.3.3 Telomerase specific gene therapy

Recent studies have focussed on the potential to exploit the significant correlation between TERT promoter activity and telomerase activity to target telomerase specific suicide gene therapy (Gu, Kagawa, *et al* 2000). Considerable success has been achieved using a bacterial nitroreductase system under the control of the telomerase gene promoter for hTERT. This strategy has been shown to result in cell death *in vitro* in cell lines with high promoter activity, and result in significant tumour reduction *in vivo* using a xenograft model (Plumb, Bilsland, *et al* 2001).

1.8.3.4 Other compounds

Non-nucleoside low molecular weight molecules that interact with the proposed G-quadruplex structures have also been tested. One such molecule, a pentacyclic acridine (RHPS4) is a potent telomerase inhibitor. Such therapies target the four-stranded G-quadruplex structure formed by single stranded telomeric DNA. These drugs have been shown to be efficacious, and are hypothesised to act by stabilising a quadruplex structure formed by the first few hexanucleotide repeats synthesised by telomerase. This is inhibitory because telomerase requires a single stranded telomeric primer for positioning, and thus folding of the target sequence in a four-stranded structure prevents this (Gowan, Heald, *et al* 2001).

1.9 Mice as models for human telomerase studies

As described previously, the frequency of spontaneous immortalisation of human fibroblasts is vanishingly small (Wright & Shay 2000), and this is linked to the tight cellular controls on telomerase activity. However, telomerase activity is widespread in murine tissue, and yet murine cell lines that are telomerase negative actually immortalise with approximately ten million fold greater frequency than normal human cells. Furthermore, although the telomeres of the laboratory mouse, *Mus musculus* are several times the size of human telomeres (40-60 kb versus 5-15 kb), cell lines derived from these mice develop, after 10-15 population doublings, the characteristic phenotype and biochemical markers of senescent cells. This occurs in both wild type (telomerase positive) and murine telomerase RNA knockout ($mTR^{-/-}$) mice, and would seem to provide direct evidence to refute the idea of a telomere driven replicative senescence that may be overcome by telomerase activity (Artandi and DePinho 2000) (Blasco, Lee, *et al* 1997 36 /id).

Closer examination of this data reveals that mice may not represent a paradox after all. The postulated role of telomerase in cancer development is that telomerase activity allows the additional divisions necessary for genetic mutation to lead to full-blown malignant transformation. Such a mechanism is

only necessary if telomeric attrition represents an effective block to these extra replications. As pointed out by Wright and Shay (Wright and Shay 2000), this is clearly not the case in an animal with the large telomeric reserve of the mouse, and so telomerase down regulation has not evolved in normal murine tissue, as it would be ineffectual as an anti-cancer mechanism in this species.

In addition, it would appear murine tissue does not follow the established M1, M2 growth blocks that occur in human tissue, as M2 in the mouse appears to contribute to, rather than help to prevent tumour formation. Fifth and sixth generation $mTR^{-/-}$ mice with normal p53 activity provide the evidence for this. By the fifth generation, these mice have sufficiently short telomeres to produce a growth check, however the frequency of escape from crisis in sixth generation mice approaches 100%. This contrasts sharply with the figure of 10^{-7} for human cells that are telomerase negative at the equivalent crisis/M2 stage (Chin, Artandi, *et al* 1999) (Shay & Wright 1989).

The evolutionary advantage long telomeres confer on an organism is thought to be the facility to efficiently align chromosomes during meiosis, and is therefore completely independent of any cellular division counting mechanism (Wright & Shay 2000). It is postulated that telomerase repression evolved in tandem to maintain telomeres at the appropriate size for this function. Further to this line of argument, it is postulated that shorter telomeres, and the co-existent telomere driven growth arrest would be of little use to the mouse, first because the restriction on tissue repair would outweigh any potential anti-cancer benefit in an animal with a short life-span, and second, in an organism the size of a mouse a telomere based growth arrest with a lag period encompassing many rounds of cell division is unlikely to be of benefit when even a small mass in any part of the body is life-threatening (Wright & Shay 2000) (Takahashi, Kuro, *et al* 2000).

This intriguing explanation for the unusual telomere length and telomerase activity found in the mouse is based on work carried out in highly inbred laboratory strains, and has recently been countered by new evidence that inbreeding causes a net increase in telomere length with strain specific significant variation, and therefore the traditional mean telomere length of the

mouse (40-60 kb) may be an artefact (Hemann & Greider 2000) (Manning, Crossland, *et al* 2002). If this is proven to be the case, then the view of Wright *et al* (Wright & Shay 2000) that long telomeres are the evolutionary result of small size and short life span will need to be reviewed.

1.10 Aims of the project

There is much evidence to suggest that telomeres and telomerase are key components in the process of immortalisation and cancer progression, and possibly the ageing process. However, the majority of research carried out to date has been in the human field, and relatively little is known of the biology of telomeres and telomerase in the dog and cat. The aims of this project were to carry out a comprehensive investigation of telomere and telomerase biology as it relates to companion animals along three broad themes.

1. Telomere studies

The aims were to assess telomere lengths in a wide range of normal canine and feline tissues, peripheral blood samples, tumours and immortalised cell lines, and examine the effect of age, breed, and gender on telomere length in the dog and cat *in vivo*, whilst telomere attrition was also investigated *in vitro* using primary fibroblast cultures.

2. Telomerase studies

The aims were to assess the distribution of telomerase activity in a wide range of normal canine and feline somatic tissues, tumour samples, immortalised cell lines and primary cultures. The effects of a potential telomerase inhibitor were assessed in telomerase dependant canine cell lines, and the link between telomere attrition and senescence in the dog and cat was investigated using heterologous expression of the catalytic component of human telomerase in canine and feline primary cultures.

3. Gene expression studies

The aims were to carry out an investigation of changes in the transcriptome of ageing canine fibroblasts using a DNA microarray platform. The same technology was used to assess changes in gene expression levels concurrent with reactivation of telomerase in a human ALT cell line.

Materials and Methods

2.1 Materials

Materials in regular use such as equipment and general solutions are detailed in this section.

2.1.1 Cell culture materials

2.1.1.1 Sources of cell lines

CMT3, CMT7 and CMT8 cells were donated by G. Ruttemann, Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, University of Utrecht, PO Box 80.154, 3508 TD Utrecht, The Netherlands.
GM847 cells were donated by W. N. Keith, Department of Medical Oncology, Beatson Laboratories, University of Glasgow.
S22, SFA and EQ1 cultures were all generated from post mortem skin biopsy samples taken with informed owner consent at Glasgow University Veterinary School. The 3132T and GHK cell lines were generated from post-mortem biopsy samples taken with informed owner consent at GUVS.
CCL-176, CML10, MDCK, 293T, MCF7, D-17 and A-72 cells were all supplied by the American Type Culture Collection (ATCC), Manassas, VA.
AG08075, AG07906, AG07648 and AG08157 primary cultures were all supplied by Coriell Cell Repositories (CCR), 403 Haddon Ave., Camden, NJ 08103.

2.1.1.2 Plasticware

Tissue culture flasks, 6, 12, 24 well plates, Falcon conical centrifuge tubes (15 and 50 ml), and pipettes (5, 10, 25, and 50 ml) were supplied by Greiner (Gloucestershire, UK). Ninety-six well plates were supplied by Packard (Berkshire, UK).

2.1.1.3 Solutions, media and supplements

Gibco BRL Life Technologies and Sigma Genosys supplied all solutions and media, unless otherwise stated.

2.1.1.3.1 Media

All the following media were delivered as sterile solutions and stored at 4°C-
Dulbecco's Minimum Essential Medium (MEM) with Glutamax-L and 4500 mg/L glucose and pyridoxine
MEM-Alpha, with ribonucleosides and deoxyribonucleosides
MEM with Earle's salts
MEM with Earle's salts and $NaHCO_3$ without L-Glutamine
RPMI 1640 medium without L-glutamine.

2.1.1.3.2 Supplements

Foetal Calf Serum (FCS): virus and mycoplasma screened. FCS was stored in 50 ml aliquots at -20°C, and was used both with and without heat inactivation. When heat inactivation was required this was carried out at 56°C for 30 minutes before storage in aliquots.
L-glutamine: supplied as a 200 mM (100x) stock, and stored at -20°C in 5 ml aliquots.
Penicillin/streptomycin (P/S): supplied as a 100x stock of 10,000 units penicillin and 10,000 units streptomycin per ml and stored in 5 ml aliquots at -20°C.
G418 antibiotic: supplied as a 50 mg/ml solution and stored in 800 µl aliquots at -20°C. G-418 is an aminoglycoside antibiotic produced by *Streptomyces* that induces cytotoxicity by blocking translation.
Fungizone (Amphotericin B): supplied as a 100x stock solution and stored in 5 ml aliquots at -20°C, at a working concentration of 1.25 µg/ml.

Non-essential amino acids: supplied as a 100x stock solution and stored at 4°C. Concentrations and constituents of the 1x working solution are available from the supplier (GibcoBRL, Catalogue number 11140)

Trypsin-EDTA: supplied as a 100x stock solution and stored in 5 ml aliquots at -20°C.

Essential amino acids: supplied as a 50x stock solution and stored at 4°C. Concentrations and constituents of the 1x working solution are available from the supplier (GibcoBRL, Catalogue number 11130)

Sodium pyruvate: supplied as a 100 mM stock solution and stored at 4°C.

Vitamins for MEM media: supplied as a 100x stock solution and stored at 4°C.

2.1.2 General chemicals

Chemicals used were of analytic, ultrapure or molecular grade quality and were supplied by a range of companies.

2.1.3 Complete kits

QIAquick® Gel Extraction kit (QIAGEN, UK)

QIAamp® DNA blood midi and maxi kits (QIAGEN, UK)

EndoFree® Plasmid Maxi Kit (QIAGEN, UK)

TOPO TA Cloning® (Invitrogen, UK)

DNA Sequencing Kit Big Dye™ Terminator Version 3.0 Cycle Sequencing Ready Reaction (ABI, UK)

*Telo*TTAGGG PCR ELISA^{PLUS} Kit (Roche, UK)

RNeasy Total RNA Isolation Kit (QIAGEN, UK)

ENZO® Bioarray HighYield RNA transcript labelling kit (Affymetrix, CA)

2.1.4 Bacterial strains

2.1.4.1 *E.coli* One Shot® TOP10

Chemically competent *E.coli* cells (>1 x 10^9 colony forming units/μg) (Invitrogen). Genotype: F *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC)Φ80 *lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

2.1.5 DNA

Plasmid, molecular weight markers and oligonucleotide DNAs were stored at -20°C.

2.1.5.1 Plasmid vectors-PCI-neo Mammalian Expression Vector

PCI-neo Mammalian Expression Vector is supplied by Promega UK, and is derived from the pGEM®-3Zf(+) vector and contains a cytomegalovirus (CMV) immediate-early enhancer/promoter region with a downstream chimeric intron, T7 and T3 RNA polymerase promoters, an SV40 late polyadenylation signal and a neomycin phosphotransferase selectable marker which confers resistance to the antibiotic G-418, causing antibiotic inactivation through phosphorylation.

2.1.5.2 Molecular size standards

Molecular size standards used include a 1 kb DNA ladder (size range: 75-12,216 bp), 1 kb Plus DNA ladder™ (size range: 100-12,000 bp), 100 bp DNA Ladder (size range: 100-2072 bp), and a Low DNA Mass™ Ladder (size range: 100-2000 bp and 5-400 ng) all supplied by GIBCOBRL Life Technologies, and a CHEF DNA Ladder supplied by BIO-RAD Ltd (size range: 8-48.5 bp).

2.1.5.3 Oligonucleotide primers

Oligonucleotides for polymerase chain reaction (PCR) and cycle sequencing were supplied by both MWG Biotech and Sigma-Genosysis, and were delivered as lyophilised DNA. Primers were reconstituted in distilled water (dH_20) and stored at -20°C in 20 µl aliquots at 10 µM.

2.1.6 Enzymes

All enzymes were stored at -20°C and were removed from storage immediately before use.

2.1.6.1 Restriction enzymes

All enzymes and their associated buffers were supplied by GIBCO BRL (UK) and Invitrogen (UK). Details of restriction enzymes, their restriction sites and reaction conditions are shown in Table 2-1.

2.1.6.2 T4 DNA Ligase

T4 DNA ligase was provided by Promega UK, with a ligation buffer (used at a final concentration of 30 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM DTT and 1 mM ATP) to catalyse the joining of two strands of DNA between the 5′-phosphate and the 3′-hydroxyl groups of adjacent nucleotides in either a cohesive-ended or blunt-ended configuration.

2.1.6.3 Ready-To-Go™ PCR beads

Ready-To-Go™ PCR beads (Amersham, Pharmacia) are premixed and pre-dispensed reactions for PCR applications. When brought to a final volume of 25 µl, each reaction contains *Taq* DNA Polymerase (1.5 units), 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP and Bovine Serum Albumin (BSA).

Table 2-1. Restriction enzymes.

Restriction enzyme	Restriction site	Buffer	Incubation (°C)	Temp
Hinf I	[5′-G↓ANT C-3′]	REACT® 2	37	
Rsa I	[5′-GT↓AC-3′]	REACT® 1	37	
*Eco*R I	[5′-G↓AATT-3′]	Buffer 6	37	
Xho I	[5′-C↓TCGAG-3′]	Buffer 6	37	
Sal I	[5′-G↓TCGA-3′]	REACT® 10	37	

2.1.6.4 Murine Moloney Virus Reverse Transcriptase

Murine Moloney Virus Reverse Transcriptase (MMLV-RT) enzyme (GIBCOBRL) uses single stranded RNA in the presence of a primer to synthesise a complementary DNA strand. This enzyme is isolated from *E.coli* expressing a portion of the *pol* gene of the MMLV on a plasmid.

2.1.6.5 DNase I: DNA-*free*™

DNase I (Ambion) is a deoxyribonuclease I enzyme that cleaves both double stranded and single stranded DNA. Cleavage occurs preferentially adjacent to pyrimidine (C or T) residues, and the enzyme is therefore an endonuclease. Major products are 5′-phosphorylated di, tri and tetranucleotides. In the presence of Magnesium ions, DNase I hydrolyses each strand of duplex DNA independently, generating random cleavages. In the presence of Manganese ions, the enzyme cleaves both DNA strands at approximately the same site, producing blunt ends or fragments with 1-2 base overhangs. DNase I does not cleave RNA.

2.1.7 Equipment

2.1.7.1 Major Equipment

PCR Machines: GeneAmp PCR System 2400, 2700, and DNA Thermal Cycler 480 (Perkin Elmer, Boston MA, USA)
Stirrer: Magnetic Stirrer Hotplate (Stuart Scientific, Surrey, UK)
Balance: Precisa 100A-300M (Precisa Balances Ltd, Buckinghamshire, UK)
Power packs: PAB 35-0.2 (Kikusui electronics corporation, Yokohama, Japan)
Gel systems: Hoefer HE 33 Mini Horizontal Submarine Unit, CHEF system (Biorad, Hertfordshire, UK)
Incubator: B5042 (Heraeus, Surrey, UK)
Horizontal orbital shaker: 4628-ICE Labline Instruments Inc. (IL, USA)
Gel documentation system: Uvi tec (Thistle Scientific, Glasgow, UK)
Ultraviolet trans-illuminator: T2201 (Sigma Chemical Company, Surrey, UK)

Pipettes: Finnpipette Techpette (0.5-10, 5-40, 40-200, 200-1000 µl, UK)

Automatic Sequencing Apparatus: ABI 3100 (Amersham, UK)

Spectrophotometer: GeneQuant *pro* RNA/DNA calculator, Agilent 2100 Bioanalyzer, Nanodrop ND-1000 (Cheshire, UK)

Water baths: Sub36, and W6 (Grant, Cambridge, UK)

Microcentrifuges: Centrifuge 5402, 5415R and Minispin (Eppendorf, Cambridge UK)

Benchtop centrifuge: CPR Centrifuge (Beckman, Buckinghamshire, UK)

Biological safety cabinet (Microflow, Andover, UK)

Affymetrix: GeneChip® station and HGU133-A GeneChip® (Affymetrix UK)

2.1.7.2 Consumables

Eppendorf tubes: Flip-top and screw-top in both 1.5 ml and 0.5 ml sizes (Thermo Life Sciences, Basingstoke, UK)

Pipette tips: (Greiner, Gloucestershire, UK)

Filter tip pipette tips: A range of capacities (10, 100, 200, 1000 µl) supplied by Finntip (Thermo Lab Systems, Basingstoke, UK)

Petri dishes: (Sterilin, Staffordshire, UK)

Bijoux: (Greiner, Gloucestershire, UK)

Universals: (Greiner Gloucestershire, UK)

Scalpel blades: Schwann-Morton (Sheffield, UK)

Parafilm: (Sigma, Surrey, UK)

Phase lock gel: Eppendorf, Basingstoke, UK

2.1.8 Buffers, solutions and growth media

2.1.8.1 Water

Water for the preparation of general solutions and media was provided by a Vivendi Water systems (USF ELGA) filter system. Sterile water for more sensitive procedures such as PCR was supplied by Baxter Ltd.

2.1.8.2 Buffers and solutions

50x TAE buffer solution: 2M Tris base, 50mM Na_2EDTA, 1M glacial acetic acid. pH adjusted to 8.15 using glacial acetic acid and made up to a 2 litre volume.

TBE buffer solution: 0.09 M Tris Borate, 0.002 M EDTA

1 M Tris HCl: 121 g Tris base, 800 ml dH_2O. Adjusted to the desired pH with concentrated HCl and made up to 1 litre.

TE buffer: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA

1x Phosphate buffered saline (PBS): 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO4, 1.8 mM KH_2PO_4 (pH 7.3)

20x SSC: 3 M NaCl, 0.3 M Sodium citrate, pH 7.0

10x DNA Gel loading buffer: 20% w/v Ficoll 400, 0.1 M Na_2EDTA (pH 8.0), 1% w/v sodium dodecyl sulphate, 0.25% bromophenol blue. Stored at room temperature and used at a 1:10 dilution.

Ethidium bromide: made to a working dilution of 10 mg/ml with dH_20 in a fume cupboard. Stored away from light.

Transfast™ transfection reagent: Supplied as a dried lipid film to be reconstituted with 400 µl of water per vial to form multi-lamellar vesicles. Storage of dried and reconstituted reagent is at -20°C. (Promega)

Lipofectamine™ transfection reagent: Supplied in liquid form at a concentration of 2 mg/ml and stored at 4°C. (Invitrogen, UK)

DEPC water: 0.5 ml of Diethylpyrocarbonate was added to 500 ml water, left overnight in a fume cupboard and then autoclaved before storage at 4°C.

Lysis mix: 0.32 M sucrose, 10 mM Tris, 5 mM MgCl, 1% Triton X, pH of solution adjusted to 7.5 and stored at 4°C.

Nuclei lysis mix: 10 mM Tris, 0.4 M NaCl, 2 mM EDTA (pH 8.0), stored at room temperature.

2.1.8.3 Bacteriological media

Media were sterilised by autoclaving at 121°C for 15 minutes.

LB medium: 20 g tryptone, 20 g NaCl, 10 g yeast extract made up to 2 L with dH_20, pH then adjusted to 7.0 with NaOH

SOC medium: 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose. (Invitrogen, UK)

2.2 Methods

Methods used commonly throughout the thesis are detailed in this section whilst more specific methods are dealt with in the relevant chapter. Many of the methods described here are based on standard protocols that are detailed in Current Protocols in Molecular Biology Volumes 1 & 2 (Ausubel, Brent, *et al* 1994).

2.2.1 Growth of mammalian cells

2.2.1.1 Basic technique

All procedures involving mammalian cells were carried out in a laminar flow hood using standard aseptic procedures.

2.2.1.1.1 Cell counting

Cells were counted in a haemocytometer as follows. Cell pellets were suspended in an appropriate volume of media (usually 5 ml) to allow ease of counting in the haemocytometer chamber. A 25 µl volume of the cell suspension was then diluted 1:1 in 0.4% trypan blue (Sigma) and incubated at room temperature for 1 minute. The suspension was then introduced to the haemocytometer chamber and cell counts made using an inverted microscope with a 4 x 10 objective. Cells lying on the top and right side of the marked squares were not included in the count, those lying on the bottom and left perimeters were. Cell concentrations (cells/ml) were calculated by multiplying the mean numbers of cells per large marked square by 10^4 to account for the volume of the haemocytometer chamber and 2 to correct for the dilution factor. Dead cells were differentiated by uptake of the trypan blue stain and appear blue.

2.2.1.1.2 Passage and cryopreservation of cells

Stocks of cells for long-term storage were preserved over liquid nitrogen. Cells to be preserved were harvested using 0.05% Trypsin- 0.53 mM EDTA at mid-log phase growth and removed to a sterile 15 ml centrifuge tube. Cells were centrifuged at 1200 g for 2 minutes and the supernatant discarded. The cells were then re-suspended in freeze medium at a concentration of approximately 2 x 10^6 cells/ml. Freeze medium consisted of 45% culture medium, 45% foetal bovine serum, and 10% DMSO as a cryoprotectant. Cells were transferred in 1 ml aliquots to labelled cryovials (NALGENE™) and brought to -70°C at a controlled rate of -1°C per minute using a NALGENE™ Cryo 1°C Freezing container (NALGENE, USA). The vials were then transferred to a liquid nitrogen freezer. Cell stocks were revived by rapid thawing in a 37°C water bath and used following standard techniques as described. All cell lines were reseeded at a concentration of 1 x10^5 cells/ml after passaging.

2.2.1.2 Cell lines

All cell lines form an adherent monolayer in culture, and were cultured in 75 cm^2 tissue culture flasks kept at 37°C and in 5% CO_2 unless otherwise stated.

2.2.1.2.1 MDCK

S.H. Madin and N.B. Darby established the MDCK cell line from the kidney of an apparently normal female Cocker Spaniel in September 1958 (Gaush, Hard *et al* 1966). The cells were maintained in 20 ml Dulbecco's MEM with glutamax-1 medium supplemented with 10% FCS, 100 international units (IU)/ml penicillin and 100 IU/ml streptomycin and 1.25 µg/ml fungizone. Cultures were split, typically 1:9 every 3-4 days when sub-confluent. Cells were trypsinised and seeded into new flasks as described above.

2.2.1.2.2 AG07648

The primary canine fibroblast culture AG07648 was established from a post-mortem skin biopsy taken from the thorax area of a 6 year old female Beagle in May 1984. The species of origin was confirmed by chromosomal analysis; the karyotype was normal diploid female, 78XX. The cells were maintained in MEM Eagle medium with Earle's salts, 2 times normal concentration of essential and non-essential amino-acids (Section 2.1.1.3.2), MEM Vitamins, 2 mM L-glutamine, 100 IU/ml penicillin and 100 IU/ml streptomycin and 1.25 μg/ml fungizone and 20% FBS. Cultures were split, typically 1:5 every 5-7 days when sub-confluent. Cells were trypsinised and seeded into new flasks as described.

2.2.1.2.3 AG07906

The primary equine fibroblast culture AG07906 was established from a post-mortem sample taken from a skin biopsy sited at the right inner thigh of a 3 year old female thoroughbred horse. The sample was taken in June 1984. The species of origin has been confirmed by chromosome analysis, and the karyotype is confirmed as normal diploid female, 64XX. The cells were maintained in MEM Eagle medium with Earle's salts, 2 times normal concentration of essential and non-essential amino-acids (Section 2.1.1.3.2), MEM Vitamins, 2 mM L-glutamine, 100 IU/ml penicillin and 100 IU/ml streptomycin and 1.25 μg/ml fungizone and 20% FBS. Cells were trypsinised as standard, and reseeded at a ratio of approximately 1:5.

2.2.1.2.4 AG08075

The AG08075 primary canine fibroblast culture was established from a post-mortem skin biopsy from the abdomen of a 14-year-old female Beagle taken in March 1985. The species of origin and karyotype were established by chromosome analysis; the karyotype was normal diploid female (78XX). The cells were maintained in MEM Eagle medium with Earle's salts, 2 times normal concentration of essential and non-essential amino-acids (Section 2.1.1.3.2),

MEM Vitamins, 2 mM L-glutamine, 100 IU/ml penicillin and 100 IU/ml streptomycin, 1.25 µg/ml fungizone and 20% FBS. Cultures were split, typically 1:5 every 5-7 days when sub-confluent. Cells were trypsinised and seeded into new flasks as described.

2.2.1.2.5 GM847

The GM847 cell line is an SV40 transformed human fibroblast cell line. The cells were grown in DMEM medium supplemented with 10% FCS, 100 IU/ml penicillin and 100 IU/ml streptomycin and 1.25 µg/ml fungizone. Cells were trypsinised as standard every 3-4 days, and reseeded at a ratio of approximately 1:5.

2.2.1.2.6 CCL-176

The primary feline fibroblast culture CCL-176 was established from a post-mortem biopsy taken from the tongue of an embryonic female domestic cat. The cells were propagated in MEM Eagle medium with Earle's salts supplemented with a normal concentration of non-essential amino-acids (Section 2.1.1.3.2), and 10% FBS. Cultures were split, typically 1:5 every 3-4 days when sub-confluent.

2.2.1.2.7 CMT7

CMT7 is an immortal cell line established from a canine mammary tumour. CMT7 cells were maintained in RPMI medium supplemented with 10% FCS, 100 IU/ml penicillin and 100 IU/ml streptomycin and 1.25 µg/ml fungizone. Cells were trypsinised as standard every 3-4 days, and reseeded at a ratio of approximately 1:5.

2.2.1.2.8 SFA

The canine primary fibroblast culture SFA was derived from a post-mortem flank biopsy from a 13 year old male Staffordshire Bull Terrier at GUVS. Cells

were maintained in MEM-alpha medium supplemented with 10% FCS, 100 IU/ml penicillin and 100 IU/ml streptomycin, 1.25 µg/ml fungizone and 2 mM L-glutamine. Cells were passaged routinely after 5-7 days. The culture was confirmed to be canine in origin by RT-PCR and sequence analysis for canine cyclophilin.

2.2.1.2.9 S22

The feline primary fibroblast culture S22 was derived from an eight week old female DSH cat from a flank skin biopsy. Cells were maintained in MEM-Eagle medium supplemented with 10% FCS, 100 IU/ml penicillin and 100 IU/ml streptomycin, 1.25 µg/ml fungizone and 2 mM L-glutamine. Cells were passaged routinely after 5-7 days growth in culture.

2.2.1.2.10 293T

A human derived, SV40 transformed cell line sourced from renal epithelial cells. Cells were maintained in DMEM medium supplemented with 10% FCS, 100 IU/ml penicillin and 100 IU/ml streptomycin, 1.25 µg/ml fungizone and 2 mM L-glutamine. Cells were passaged routinely after 3-4 days growth in culture.

2.2.1.2.11 MCF7

The MCF7 cell line comprises epithelial-like cells derived from a human Caucasian breast adenocarcinoma patient. The cells were maintained in DMEM medium supplemented with 10% FCS, 100 IU/ml penicillin and 100 IU/ml streptomycin and 1.25 µg/ml fungizone. Cells were trypsinised as standard every 3-4 days, and reseeded at a ratio of approximately 1:5.

2.2.1.2.12 AG08157

This canine primary fibroblast culture was established from a post-mortem skin biopsy taken from a 42-day-old male Beagle dog in 1985. The cell line was maintained in MEM Eagle medium with Earle's salts, 2 times normal

concentration of essential and non-essential amino-acids (Section 2.1.1.3.2), MEM Vitamins, 2 mM L-glutamine, 100 IU/ml penicillin and 100 IU/ml streptomycin, 1.25 µg/ml fungizone and 20% FBS. Cultures were split, typically 1:5 every 5-7 days when sub-confluent. Cells were trypsinised and seeded into new flasks as described.

2.2.1.2.13 CMT8

CMT8 is an immortal cell line established from a canine mammary tumour. CMT7 cells were maintained in RPMI medium supplemented with 10% FCS, 100 IU/ml penicillin and 100 IU/ml streptomycin and 1.25 µg/ml fungizone. Cells were trypsinised as standard every 3-4 days, and reseeded at a ratio of approximately 1:5.

2.2.1.2.14 CMT3

CMT3 is also an immortal cell line established from a canine mammary tumour. Conditions for culture were as for CMT8.

2.2.1.2.15 CML10

This canine cell line contained epithelial-like cells and was derived from a melanoma biopsy taken from a 10-year-old female dog. The cells were maintained in DMEM medium supplemented with 10% FCS, 100 IU/ml penicillin and 100 IU/ml streptomycin and 1.25 µg/ml fungizone. Cells were trypsinised as standard every 3-4 days, and reseeded at a ratio of approximately 1:5.

2.2.1.2.16 D17

A canine osteosarcoma cell line derived from an eleven year old female poodle. Cells were maintained in DMEM medium supplemented with 10% FCS, 100 IU/ml penicillin and 100 IU/ml streptomycin and 1.25 µg/ml fungizone. Cells

were trypsinised as standard every 3-4 days, and reseeded at a ratio of approximately 1:5.

2.2.1.2.17 A72

An immortal canine cell line comprising cells of fibroblast morphology derived from an 8 year old female dog. Cells were maintained in DMEM medium supplemented with 10% FCS, 100 IU/ml penicillin and 100 IU/ml streptomycin and 1.25 μg/ml fungizone. Cells were trypsinised as standard every 3-4 days, and reseeded at a ratio of approximately 1:5.

2.2.1.2.18 GHK

A cell line comprising renal epithelial cells derived from a greyhound at GUVS, age and gender unknown. Cells were maintained in DMEM medium supplemented with 10% FCS, 100 IU/ml penicillin and 100 IU/ml streptomycin and 1.25 μg/ml fungizone. The cells were trypsinised as standard every 3-4 days, and reseeded at a ratio of approximately 1:5.

2.2.1.2.19 3132T

This immortal canine cell line comprised cells of an epithelial morphology and was derived from a lymphoma sample taken from a 10-year-old female dog. The cells were maintained in RPMI medium supplemented with 10% FCS, 100 IU/ml penicillin and 100 IU/ml streptomycin and 1.25 μg/ml fungizone. Cells were trypsinised as standard every 3-4 days, and reseeded at a ratio of approximately 1:5.

2.2.1.2.20 EQ1

This primary equine fibroblast culture was derived from a skin biopsy taken from a 7 year old male neutered horse at GUVS. Cells were maintained in MEM-alpha medium supplemented with 10% FCS, 100 IU/ml penicillin and 100 IU/ml streptomycin, 1.25 μg/ml fungizone and 2 mM L-glutamine. Cells were

passaged routinely after 5-7 days growth in culture. Species of origin was confirmed by RT-PCR and sequence analysis for equine major histocompatability complex class II, DQB locus (Szalai, Bailey, *et al* 1993).

2.2.2 Preparation of DNA from blood samples

2.2.2.1 Phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation

To each 3 ml blood sample 10 ml of lysis mix (Section 2.1.8.2) was added followed by gentle mixing and incubation on ice for 10 minutes. Samples were then centrifuged at 2800 g for 10 minutes at 4°C, the supernatant decanted and pellets resuspended in 500 µl of nuclei lysis mix (Section 2.1.8.2) and thoroughly mixed. A 100 µl volume of 10% SDS and 50 µl of proteinase K were then added and the samples were incubated at 37°C overnight. Samples were then deproteinised with an equal volume of a phenol/chloroform/isoamyl alcohol extraction mixture (25:24:1), followed by centrifugation at 2800 g for 10 minutes to separate the samples into aqueous and organic phases. The upper aqueous phase of each sample was then carefully transferred to a sterile 1.5 ml eppendorf tube and the DNA recovered by precipitation in an equal volume of molecular biology grade 100% ethanol. DNA was pelleted by centrifugation at 2800 g for 10 minutes at 4°C followed by a wash step in 70% ethanol. DNA was again pelleted and allowed to air dry, before resuspension in an appropriate volume of TE buffer (usually 50 µl) and storage at -20°C

2.2.2.2 DNA extraction using the QIAamp® DNA kit (QIAGEN, UK)

Both the Midi and Maxi kits were used for DNA extraction as per manufacturer's instructions. These kits are used for total DNA purification from 1-2 ml and 5-10 ml of whole blood respectively. Briefly, the protocol involved a lysis step, followed by adsorption of nucleic acid to a membrane contained within a specially designed QIAamp spin column through which lysate was

forced by centrifugal force. DNA bound to the membrane was then washed in two further centrifugation steps before elution in a concentrated form in elution buffer. All centrifugation steps were carried out at room temperature, and at 3000 rpm.

2.2.3 Recombinant DNA techniques

2.2.3.1 Storage and growth of bacteria

Plasmid DNA was maintained and stored in the *E.coli* strain One Shot®. Glycerol stocks were prepared from these bacteria and their transformants for long-term storage as outlined below.

The desired bacterial culture was streaked onto a 1.5% agar plate (15g agar in 1 litre LB medium); as the plasmid conferred ampicillin resistance the medium was supplemented with 50-100 μg/ml ampicillin. The plate was then incubated overnight at 37°C and the following day single colonies were picked using a sterile pipette tip into a sterile universal containing 3-5 ml LB medium supplemented with 50-100 μg/ml ampicillin. These cultures were then incubated at 37°C overnight in a horizontal orbital incubator at 225 rpm. Confirmation that this overnight culture was derived from bacteria containing the correct plasmid was confirmed by DNA isolation and restriction digestion. Glycerol stocks were prepared by addition of 90 μl of 50% glycerol to 210 μl of culture broth to produce a 15% glycerol mixture. These glycerol stocks were then stored at –20°C and –70°C. Bacterial stocks were subsequently revived by taking a surface sample of the glycerol stock and streaking this onto an agar plate.

2.2.3.2 Extraction and purification of plasmid DNA

2.2.3.2.1 Large-scale plasmid preparation

Large quantities of highly pure, endotoxin free plasmid necessary for applications such as transfections and sequencing were prepared using the EndoFree® Plasmid Maxi Kit (Qiagen,UK). A starter culture in 3-5 ml of LB broth was used to seed an overnight multiplier culture in 250 ml of LB broth

incubated at 37°C with constant agitation. These exponentially growing bacteria were harvested by centrifugation of the culture broth in 50 ml sterile centrifuge tubes at 2800 rpm. The remainder of the protocol was performed according to the manufacturers instructions. DNA was stored at -20°C.

2.2.3.2.2 Small-scale plasmid preparation

Requirements for small amounts of plasmid DNA, such as sequencing, were met using the QIAprep® PCR Spin Miniprep Kit (QIAGEN, UK). This kit isolated plasmid DNA from 4 ml LB broth cultures of exponentially growing bacteria. The bacteria were harvested by centrifugation (2800 rpm for 10 minutes), and the manufacturers protocol was then followed. The method involved lysis of the bacterial cells to release the DNA constructs, which were then harvested after centrifugation, filtration and wash steps into 50 µl of sterile water. DNA storage was at -20°C.

2.2.3.3 Determination of nucleic acid concentration and quality

2.2.3.3.1 Determination by spectrophotometry

Nucleic acid samples were diluted 1:20 by addition of 5 µl of resuspended nucleic acid in 95 µl of dH_2O. Optical density readings were taken at 260 nm and 280 nm, using blank dH_2O as a comparison. An optical density reading of 1.0 at 260 nm corresponds to an approximate nucleic acid concentration of 50 µg/ml for double stranded DNA, 40 µg/ml for RNA and 33 µg/ml for single stranded oligonucleotides. The ratio of the readings taken at 260 nm and 280 nm (OD_{260}/OD_{280}) was used to give an estimate of the purity of the nucleic acid. Pure preparations of DNA and RNA have an OD_{260}/OD_{280} of 1.8 and 2.0 respectively; a lower value suggests contamination, typically with protein or phenol.

2.2.3.3.2 Estimation of double stranded DNA concentration and quality by agarose gel electrophoresis.

This method was used when there were insufficient amounts of dsDNA for spectrophotometry, or when purity of a particular DNA fragment needed to be investigated. Agarose gel electrophoresis was carried out by standard methods as detailed elsewhere (Section 2.2.3.5), and the intensity of the fluorescence of the unknown DNA was compared to that of a known size marker (Low DNA Mass™ Ladder) following staining with ethidium bromide and visualisation by UV transillumination. Smearing of a DNA band indicated degradation of the sample and resulted in exclusion of that DNA from further analysis.

2.2.3.4 Restriction endonuclease digestion

The required amount of DNA was digested in a volume of 20 µl containing appropriate buffer for the restriction endonuclease(s), 4 units of each enzyme per microgram of DNA and dH_20 to make up the volume. The reactions were incubated at the appropriate temperature for a minimum of 1 hour.

2.2.3.5 Electrophoresis of DNA

DNA fragments of 0.1-22 kb were separated and identified by agarose gel electrophoresis using a Hoefer HE 33 Mini Submarine Electrophoresis Unit (Amersham Pharmacia Biotech, San Franciso, CA.). Typically 0.5 g of agarose was added to 50 ml of 0.5% TBE buffer and heated in a microwave oven for 1-2 minutes to produce a 1% gel. This was allowed to cool to 55°C before addition of 1.5 µl of 100 mg/ml ethidium bromide. The gel was then mixed and poured into a casting tray containing a comb with a suitable number of wells (10-20). After solidification the gel was transferred to the electrophoresis unit containing chilled 0.5% TBE buffer and the comb was carefully removed. DNA samples and molecular size standards were prepared by addition of a suitable amount of 10x DNA loading buffer (Section 2.1.8.2) and introduced to the wells using a micropipette. Gels were run at 100 V for approximately 20 minutes, then

removed from the electrophoresis unit and the DNA visualised using a UV transilluminator (Uvi tec, Thistle Scientific) and photographed using a Mitsubishi P91 photographic unit.

2.2.3.6 Purification of restriction enzyme fragments

When purification of DNA fragments was required for construction of recombinant plasmids, the DNA was purified from agarose gels using the QIAquick® Gel extraction kit (QIAGEN). DNA fragments of interest were cut from an agarose gel using a clean scalpel blade and the protocol was then performed according to the manufacturers instructions.

2.2.3.7 Ligation of vector and insert DNA

Fragments of DNA generated by restriction digestion were ligated with approximately 50-100 ng of vector DNA using T4 DNA ligase (Promega) according to the manufacturers instructions. The quantities of vector and insert DNA to be used were calculated to produce a molar ratio of between 1:1 and 1:5 using the equation:

$$\frac{X \text{ ng of vector} \times Y \text{ kb of insert}}{Z \text{ kb of vector}} \times \text{insert : vector ratio} = \text{ng of insert required}$$

Vector and insert were mixed with ligation buffer and 1 unit of DNA ligase in a total volume of 10 µl. Ligation reactions proceeded at 16°C overnight.

2.2.3.8 Transformation of bacteria with plasmid DNA

The TOP10 cells used in transformation experiments were stored in volumes of 25-50 µl in microcentrifuge tubes at -80°C. Aliquots were thawed on ice, and 0.5-5 µl of ligation reaction was added to the cells representing 1-20 ng of DNA. Mixing was carried out very gently to avoid damage to the bacterial cells. The cells were then left on ice for 30 minutes, followed by a 30 second heat shock at

42°C. The cells were then quenched on ice for 2 minutes, 200 µl of SOC medium was added and the tubes were incubated at 37°C for 1 hour with shaking at 225 rpm. The cells were streaked onto LB agar plates containing ampicillin at 100 mg/ml. The addition of ampicillin enabled selection of transformants as the plasmid used in this project conferred ampicillin resistance on the host bacteria.

2.2.4 Preparation of total RNA

The unstable nature of isolated RNA and the necessity for very pure isolates for downstream applications required that all materials used in RNA preparation be totally free from ribonuclease (RNAse) activity. RNAse is a very stable, ubiquitous enzyme that degrades RNA requiring no cofactors for function. Inhibition of this enzyme was carried out using RNAse ERASE (ICN Biomedicals Inc., Ohio), followed by rinsing with DEPC treated water. All plastic-ware used for RNA storage was pre-treated by an overnight soak in DEPC treated water followed by autoclaving and drying. Solutions were also prepared using DEPC treated water only, and gloves were used in all procedures involving RNA.

2.2.4.1 RNA extraction using RNAwiz™

Various methods have been described for the isolation of undegraded RNA, and progress in the field have led to the development of single step methods for the procedure (Chirgwin, Przybyla, *et al* 1979) (Chomcyznski & Saachi 1987). RNAwiz™ is a combination of denaturants and RNAse inhibitors for the isolation of total RNA, and it is effective for animal, plant and microorganism based RNA. The samples used for RNA isolation in this project were cell pellets harvested from tissue culture experiments, stored in 1.5 ml DEPC treated eppendorf tubes, as described above. These were homogenised directly in RNAwiz™ by simply pipetting vigorously several times. One ml of RNAwiz™ was used per 10^7 cells. The homogenate was then incubated at room temperature for 5 minutes to allow dissociation of nucleoproteins from the nucleic acids. Chloroform was then added (0.2x starting volume), and thorough mixing was

carried out by shaking the tube vigorously for approximately 20 seconds. The chloroform used was free from isoamyl alcohol and any other additives. The mixture was then centrifuged at 13000 rpm at 4°C for 15 minutes to allow separation of the mixture into three distinct phases, an upper aqueous phase containing the RNA, a semi-solid interphase containing most of the DNA, and a lower organic phase. The upper aqueous phase was removed carefully by pipetting and transferred to a clean DEPC treated 1.5 ml tube. RNase-free water was then added (0.5x starting volume), and thorough mixing carried out. This was followed by addition of 1x starting volume of isopropanol, mixing, and incubation of the sample at room temperature for 10 minutes. The RNA was then pelleted by centrifugation at 13000 rpm for 15 minutes at 4°C. The supernatant was decanted, and a wash step using 75% ethanol was carried out, followed by centrifugation as noted above.

The RNA samples were stored at -20°C in 70% ethanol until required. When required, the RNA samples were pelleted by centrifugation as above and the supernatant decanted. The pellets were then allowed to air dry for no longer than 10 minutes (complete drying of the pellet makes resuspension very difficult) and resuspended in 40 µl of DEPC water.

2.2.4.2 Assessment of RNA using agarose gel electrophoresis

An aliquot of each RNA sample was run on a 1% agarose TAE gel (10g agarose in 1 litre TAE buffer). Assessment of RNA quality was carried out by checking the integrity of the 18S and 28S ribosomal subunit bands, and examining their rate of migration in comparison to a 100 bp molecular weight standard (GIBCO BRL).

2.2.4.3 DNase treatment of RNA

RNA samples were freed from contaminating DNA by use of the DNA-free™ kit (Ambion). 0.1x volume of 10x DNase buffer and 2 units of DNase 1 were added directly to RNA samples, mixed and incubated at 37°C for 30 minutes.

The enzyme reaction was then halted by addition of 5 µl of well-mixed slurry of DNase Inactivation Reagent followed by a 2-minute incubation at room temperature. The inactivation reagent was then pelleted by centrifugation at 10,000 g for one minute and the RNA sample transferred to a clean DEPC treated eppendorf for storage.

2.2.5 Amplification of DNA by polymerase chain reaction

The polymerase chain reaction (PCR) is a technique that allows the amplification of a specific sequence of DNA and is effective even when the target DNA is very scarce. The technique was developed by Mullis *et al*, and allows large amounts of a single copy gene to be generated from genomic or viral DNA (Mullis, Faloona, *et al* 1986) (Mullis & Faloona 1987) (Saiki, Gelfland, *et al* 1988) (Kwok, Mack, *et al* 1987). The initial procedure used the Klenow fragment of DNA polymerase I. This enzyme needed to be replaced at each cycle as it was denatured by the reaction conditions. Efficiency was greatly improved by introduction of a thermostable *Taq* polymerase isolated from *Thermus aquaticus*, as this allowed automation of the procedure (Saiki, Gelfland, *et al* 1988).

PCR allows the amplification of any unknown DNA sequence by the simultaneous extension of primer pairs flanking the unknown sequence, each complementary to opposite strands of the DNA. An overview of PCR and its applications is available (Innis & Gelfland 1990), and although the basic technique has been developed to include other applications such as cloning (Scharf 1990), basic PCR remains widely used. An outline of the procedure is provided below.

2.2.5.1 Primer design

Primers were designed following the guidance of a standard text (Innis & Gelfland 1990). The goal of primer design is to produce an oligonucleotide that

will hybridise efficiently with the DNA sequence of interest, and not hybridise with any other sequence. The amount of sequence permissible between primers is flexible, and up to 10 kb is possible, however beyond 3 kb efficiency is decreased (Jeffreys, Wilson, *et al* 1988). The disadvantages of minimising the distance between the primers include reducing the ability to re-amplify the sequence using nested internal oligonucleotides, and short distances between primers result in less sequence information being gained from the PCR. For any given pair of primers, the annealing temperatures (Tm) and GC content were balanced.

Whilst in many PCR applications the primer design will be exactly complementary to the template DNA, in some cases, such as efforts to clone or detect homologues when sequence information is lacking, mismatches will be unavoidable. Independent of the reason for mismatches between template and primer DNA, it is best if these are located close to the 5′ end of the primer. The closer a mismatch is to the 3′ end of the primer the more likely it will prevent extension, as DNA polymerase will be acting in a 5′ to 3′ direction.

The annealing portion of primers should generally be between 18 and 30 nucleotides in length; any increase in size beyond this is unlikely to improve primer specificity significantly and this size of primer is sufficient for sequence as complex as the human genome. Other potential features of the primer sequence, such as restriction enzyme sites should be engineered onto the 5′ end of the primer. Such additions can have a detrimental effect on primer specificity at low temperatures, and so are best used when amplifying from a single template vector. Primer GC content should match that of the template as closely as possible. Unusual stretches of sequence such as internal complementarities can lead to secondary structures and a loss of annealing efficiency, and so should be avoided.

A common artefact in PCR is the formation of primer-dimers. A primer-dimer is the product of primer extension annealing on itself or on the other primer in the PCR. Since the primer-dimer product contains one or both primer sequences and their complementary sequences it provides an excellent template for further

amplification. Given the small size of the product, and that small products are copied more efficiently, primer-dimers can swamp the PCR and sequester primer from the real target on the template DNA. These can be avoided by not using primers with complementary sequences in the 3´ region. Primer dimers can be minimised by optimising the $MgCl_2$ concentration.

2.2.5.2 Preparation of PCR reactions

The sensitive nature of PCR means that very stringent steps must be taken if contamination is to be avoided. First, physical separation of the PCR area from bench space used for other work is recommended. In the case of PCRs carried out in this project, a dedicated PCR suite isolated from the main laboratory was used. In addition, a set of instruments used to aliquot reagents (micropipettes and their tips) were kept within the PCR suite and restricted to PCR use. Filter tip pipette tips were used to reduce the chance of carryover from one step to the next, and master mixes of reagents were used whenever possible to reduce the number of pipetting steps required per reaction. Reaction components including primers were aliquoted prior to use and stored at -20°C.

2.2.5.3 Reaction conditions

A number of PCR amplification kits containing all the necessary reagents were used according to the manufacturers' instructions. The reaction mixes were made up to either 50 or 25 µl and contained variable concentrations of primers, dATPs, dCTPs, dTTPs, dGTPs, $MgCl_2$, DNA polymerase, and 1x PCR buffer containing Tris-HCl, KCl, and gelatin. To this was added an appropriate volume of DNA or cDNA template. Two thermal cyclers were used; a Perkin Elmer 480 which requires a layer of mineral oil to be placed over the reaction mixes, and a Perkin Elmer 2400 with a heated lid that does not require any mineral oil. Reactions were placed in these machines in 0.5 ml and 0.2 ml thin walled eppendorf tubes respectively. The exact parameters of the reactions varied, but generally consisted of an initial denaturation at 94°C for five minutes followed by 25-45 cycles of the following; denaturation at 94°C for one minute, annealing

at 55-72°C for one minute, extension at 72°C for one minute thirty seconds followed by a final extension step at 72°C for 4-30 minutes. Reaction products were visualised using agarose gel electrophoresis using 5-10 µl of the reaction mix.

2.2.5.4 Purification and assessment of PCR products

Single PCR products were purified following the QIAquick® PCR purification kit protocol (QIAGEN). Briefly, the DNA band of interest was removed from the agarose gel with a sharp scalpel. Visualisation was by UV transilluminating. The DNA binds to the filter within the column, facilitating separation from all other components of the mixture by a series of washing steps. The PCR products are finally eluted in 30-50 µl of sterile water, 4 µl of which was assessed by 1% TAE agarose gel electrophoresis against a 100 bp molecular weight standard (GIBCO BRL).

2.2.5.5 First strand DNA synthesis for reverse transcriptase (RT)-PCR

Analysis of gene expression requires accurate determination of mRNA levels, but as PCR is based on amplification of DNA, the process of amplifying RNA sequence requires an initial step of conversion of the RNA to cDNA by reverse transcription. The MMLV reverse transcriptase enzyme was used to mediate the formation cDNA for RT-PCR in the experiments detailed in this thesis. Reverse transcriptase synthesises a DNA strand complementary to an RNA template when provided with a primer that is base paired to the RNA and has a free 3′-OH group. Three types of primers are used, namely random primers, gene specific primers (GSP) and oligo-dT primers. Oligo-dT primers pair with the poly A sequence found at the 3′-end of most eukaryotic mRNA molecules. All of the above primers can be used to initiate the synthesis of cDNA strands in the presence of the four dNTPs. The RNA-DNA hybrid is subsequently hydrolysed by either raising the pH (as DNA, unlike RNA is resistant to alkaline hydrolysis), or by using a ribonuclease. The 3′ end of the newly synthesised

DNA strand forms a hairpin loop that primes the synthesis of the complementary DNA strand. The hairpin loop is then removed by digestion with S1 nuclease, which recognises unpaired nucleotides.

The specific conditions used involved first a denaturation step of the RNA (in 9 µl of DEPC water) at 65°C for 5 minutes to ensure no secondary RNA structure remained that might interfere with the process. The sample was then quenched on ice, and the first strand synthesis was carried out in a total reaction volume of 25 µl containing the heat treated RNA, the MMLV reverse transcriptase enzyme and its buffer, DL-Dithiothreitol (DTT), dNTPs, an RNAse inhibitor and primers. The reaction conditions vary between protocols. A Perkin-Elmer thermal cycler 480 was used for the reaction, and the reaction mix was kept on ice immediately before use or at -20°C for more long-term storage.

2.2.6 DNA sequence analysis

2.2.6.1 Automated sequencing

Sanger dideoxy DNA sequencing is the most commonly used method for DNA sequencing, particularly for large-scale genomic sequencing (Sanger, Nicklen, *et al* 1977). A variation of automated DNA sequencing using dye-labelled terminators, in which the dyes are attached to the terminating dideoxynucleoside triphosphates has been used in this project (Prober, Trainor, *et al* 1987).

2.2.6.1.1 Sample preparation

During sample preparation, DNA fragments in a sample are chemically labelled with fluorescent dyes. The dyes facilitate the detection and identification of the DNA. Typically each DNA molecule is labelled with one dye molecule, but up to five dyes can be used to label the DNA sample. More specifically, PCR reactions were performed using DNA samples (200-500 ng) in a total volume of 20 µl containing 0.5 µM of primers, 40 mM Tris-HCl, 1 mmol/l $MgCl_2$ and 4 µl of Big Dye™ Terminator Cycle sequencing Ready Reaction (ABI Prism). Samples were prepared in the PE 2400 thermal cycler incorporating 25 cycles of

amplification, each cycle consisting of a denaturing step at 96°C for 10 seconds followed by an annealing temperature of 50°C for 5 seconds, and an elongation step of 60°C for 4 minutes. DNA was then purified by precipitation using ethanol (95%). Pelleted DNA (14000 rpm for 20 minutes) was washed in ethanol (70%) and repelleted before all ethanol was removed and the pellet dried at 90°C for 1 minute. Template Suppression Reagent (25 µl) was then added and the mixture heated to 95°C for 5 minutes and chilled before transfer to genetic analyser sample tubes.

2.2.6.1.2 Sample sequencing

Samples were loaded and run on the ABI PRISM® 3100 Genetic Analyzer (PE Applied Biosystems, UK) under standard sequencing conditions for generation of automated sequence data. The ABI PRISM® 3100 Genetic Analyzer is a multi-colour fluorescence-based DNA analysis system using the proven technology of capillary electrophoresis with 16 capillaries operating in parallel. The 3100 Genetic Analyzer is fully automated from sample loading to data analysis.

2.2.6.1.3 Sequence evaluation

The length of read is 750 bases at the 98.5% base calling accuracy with less than 2% ambiguity. The output is in the form of a chromas file. A series of different computational software were utilised for sequence analysis including the 'Blast' search engine within the NCBI database, Genetics Computer Group (GCG) and ClustalW (http://www.ch.embnet.org/software/ClustalW.html).

2.2.7 Estimation of protein concentration

Protein concentrations were estimated using a bicinchoninic acid (BCA, Sigma, UK) method (Smith, Krohn, *et al* 1985). The protocol combines the reduction of Cu^{2+} to Cu^{1+} caused by protein in an alkaline environment with the capability of BCA to form an intense purple complex with Cu^{1+}. The complex produced from

this reaction is stable and increases in a proportional fashion over a broad range of increasing protein concentration. It is also water-soluble and exhibits a strong absorbance at 562 nm, thus allowing the spectrophotometric quantification of protein in aqueous solution. A standard panel of 6 bovine serum albumin (BSA) dilutions from a 2 mg/ml stock solution (Sigma, UK) were used as a standard against which protein samples of unknown concentration could be compared. Standard concentrations were 80, 100, 200, 400, 1000, and 2000 µg/ml. A 10 µl volume of each standard solution was added in duplicate to individual wells of a 96 well plate. An additional 3 wells were used as blanks (ie. contained 10 µl of dH_2O). A 1:10 dilution of each protein sample to be tested was then made in dH2O and 10 µl of each diluted sample added to the test wells in duplicate according to a predetermined pipetting scheme.

The developing solution was a mixture of 5 ml of BCA and 100 µl of $CuSO_4$ (Copper (II) Sulphate pentahydrate, 4% w/v solution, Sigma, UK). A 200 µl volume of this solution was added to each of the standard, blank and test wells and the test plate then incubated at 37°C for 45 minutes to allow for colour development. Absorbance of wells was then measured using a microtitre plate reader and concentrations of test samples determined by comparison with the standard dilutions.

2.2.8 Electroblotting of DNA fragments to a positively charged nylon membrane

Electroblotting was necessary for the transfer of DNA fragments after polyacrylamide gel electrophoresis (Section 4.3.2.5) to a nylon membrane, as the small pore size of polyacrylamide gels does not allow the effective transverse diffusion of DNA required for successful Southern blotting.

After electrophoresis, the polyacrylamide gel was removed from its glass backing by laying a piece of wet Whatmann 3MM filter paper slightly larger than the gel onto the gel surface, being careful to avoid air bubbles. This allowed

the gel to be lifted safely from its backing without risk of breakage. Two Scotch-Brite pads and a further 7 pieces of 3MM Whatmann filter paper of the same size as the gel were presoaked for 15-30 minutes in 0.5% TBE, carefully removing air pockets from the Scotch-Brite pads by repeated squeezing and agitation. These pieces were necessary for building up a sandwich around the gel for electroblotting.

Electroblotting experiments carried out in this thesis used the Mini-PROTEAN® II cell (Bio-Rad) and non-denaturing polyacrylamide gels. The electroblot apparatus contains a gel holder consisting of hinged grey and black panels on which the components of the sandwich are built up on the grey panel as follows; Scotch Brite pad, 3x filter papers, filter paper with gel (gel surface uppermost), prewetted positively charged nylon membrane (Amersham, UK), followed by the remaining 4 pieces of filter paper and the second Scotch Brite pad. At each stage any air bubbles are carefully removed from between the layers, and the filter paper carrying the gel and the gel surface itself are flooded with 0.5% TBE before they are put into place. The gel holder is then closed and placed in the transfer cell with the grey panel facing the cathode. An ice block is included to prevent overheating of the transfer buffer. The cell is then filled with 0.5% TBE and electroblotted for 4 hours at 30 V. Following this the membrane is removed and denatured for 10 minutes, DNA side up in 0.4 M NaOH. The membrane is then rinsed in 2x SSC and is ready for downstream applications.

Chapter III

In vivo and *in vitro* canine and feline telomere studies

3.1 Abstract

The majority of telomere research carried out to date has focussed on humans, and has established links between telomere attrition and senescence. Senescence has been associated with organismal ageing, and failure or bypass of senescence has been associated with immortalisation and malignancy. Telomeres are therefore of great interest to both oncologists and gerontologists, however, despite a small number of studies demonstrating similarities between companion animal and human telomeres, the amount of information available to veterinary researchers is relatively very small. The experiments detailed in this Chapter describe investigations to establish the typical range of telomere lengths found in the dog and cat in both healthy tissues and tumour samples. Possible associations between age, gender, breed, life expectancy and telomere length were investigated. A separate set of experiments aimed at demonstrating and quantifying telomeric attrition *in vitro* in the dog and cat using primary fibroblast cultures were carried out, and senescent cells in both species were characterised using a number of established biomarkers. The major aim of this research was to investigate possible links between telomere attrition, senescence and ageing in the dog and cat and thereby establish the importance of telomere biology to current veterinary research.

3.2 Introduction

The study of canine and feline telomeres is still a relatively new area. Interest in this subject has been spurred by advances in human telomere biology that have identified telomeres and their cellular functions as being important to the process of malignant transformation, and perhaps ageing itself (Artandi & DePinho 2000) (Bearss, Laurence, *et al* 2000) (Corey 2002), and reviewed in (Campisi, Kim, *et al* 2001). In contrast to the large volume of work already dedicated to human telomere biology relatively little work has been carried out in other species, with the exception of the laboratory mouse. The experiments detailed in this chapter aim to improve understanding of canine and feline telomere biology as it relates to organismal ageing and cancer.

Yazawa *et al* in 2001 were the first to provide telomere length data for canine tumours and somatic tissues (Yazawa, Okuda, *et al* 2001). This study investigated telomere lengths by restriction endonuclease digestion and Southern blot and in a panel of 27 mammary gland tumours found telomere length to vary from 11.0 to 21.6 kb. A panel of 12 normal mammary gland sections were found to have slightly longer telomeres but the groups could not be compared, as a significant age difference existed between them. Increasing age was negatively correlated with telomere length in the control dogs, whilst such a relationship was not found in the tumour tissue sections. These data imply that the canine tumour samples investigated contained an active telomere length maintenance mechanism that is not found in the corresponding non-cancerous tissue (Yazawa, Okuda, *et al* 2001). Brummendorf *et al* examined the telomere lengths in peripheral blood leukocytes of cats ranging in age from newborn kittens to 10 year old animals using fluorescence *in situ* hybridisation and flow cytometry and again could demonstrate that the older animals had shorter telomeres in the cell types examined (Brummendorf, Mak, *et al* 2002).

A negative correlation between age and telomere length has been a major finding in human telomere research (Lindsey, McGill, *et al* 1991) (Hastie, Dempster, *et al* 1990), and these preliminary investigations of telomere biology in the dog and

cat have revealed similarities with human telomere biology, and represent an encouraging start. These data have also raised the possibility of using telomere length measurement as a novel way of determining age in wildlife, and a pilot study has produced promising results in birds (Haussmann & Vleck 2002). However studies to date have assumed a uniform rate of telomeric attrition with age for a given species, and as recent work has detailed that other, variable factors such as oxidative stress affect telomeric decline these initial data must be treated with caution (von Zglinicki, Pilger, *et al* 2000) (von Zglinicki 2002).

The study of ageing and age related disease is of major importance in human medicine, and the centrepiece of most theories of the ageing process revolves around DNA damage. Telomeric loss, such as that noted to occur in dogs and cats can lead to DNA damage and this fact has lead some researchers in the human field to conclude that the ageing process begins in the embryo when telomerase activity is repressed in the majority of human somatic tissues (Skulachev 1997) (Ahmed & Tollefsbol 2001). Evidence of an actual link between telomere length dynamics and the characteristic phenotypic changes associated with ageing is circumstantial only, so for example associating decreasing telomere length in human fibroblasts and B- and T- lymphocytes with the clearly age related changes of decreased wound healing and impaired immune function may be a case of confusing sequence with consequence (Allsop, Chang, *et al* 1995) (Weng, Levine, *et al* 1995).

There is also evidence of telomere association with age related disease. However the short telomeres found in artherosclerotic blood vessels and endothelium under haemodynamic stress, compared with healthy tissue counterparts, may be an effect of the disease process, rather than the cause of it. Such associations may be useful in future diagnostic protocols; for example telomere length has been found to be inversely proportional to arthersclerotic grade (Okuda, Khan, *et al* 2000). Recent work has also shown short telomeres in human peripheral blood leukocytes (PBLs) to be associated with an increased risk for the development of carcinomas in a number of organs (Wu, Amos, *et al* 2003).

Despite the lack of firm evidence of a link between telomeric attrition and ageing, it is interesting to hypothesise how such a relationship might work. A recent idea advances the prospect that the telomeric contribution to phenotypic ageing may revolve around a mosaic effect (Toussaint, Remacle, *et al* 2002). Accumulation of functionally impaired senescent cells may cause a reduction in the 'vitality' of the tissue as a whole that is sufficient to produce a phenotypic effect and yet does not have a large impact on mean telomere length of the cell population.

The ability to accurately identify senescent cells *in vitro* and *in vivo* is therefore central to telomere-based studies of ageing. The most commonly used biomarker is senescence associated β-galactosidase activity at pH 6.0, useful both *in vitro* and *in vivo* (Dimri, Lee, *et al* 1995). The expression of the cyclin dependant kinase inhibitor p21 increases as cells near senescence, and $p16^{INK4a}$ has been shown to accumulate in senescent cells. $p16^{INK4a}$ is also a cyclin dependant kinase inhibitor, and acts as an inhibitor of cell replication through its interaction with the retinoblastoma gene product pRb (Iluschtscha & Reddel 1999) This interaction results in the maintenance of pRb in a hypophosphorylated form in senescent cells, and this has been advanced as additional means by which senescent cells may be identified *in vitro* (Alcorta, Xiong, *et al* 1996).

More than one method has been described to determine telomere length. The bulk of telomere length research carried out to date, and all the experiments detailed in this chapter use a Southern Blot and hybridisation approach, though recent studies reflect a trend towards variations on the original technique or use of completely new methodologies. A discussion of the other methods currently in common usage is detailed in Section 3.2.1.

3.2.1 Alternative methods for determining telomere length in mammalian cells

3.2.1.1 Hybridisation protection assay

The hybridisation protection assay (HPA) is a rapid (45 minutes) and sensitive way to determine telomere lengths directly by a chemiluminescent method. Cell lysate or DNA solution is mixed with a hybridisation solution containing a light emitting (acridium ester labelled) telomere specific probe. A selection buffer then differentially hydrolyses unhybridised probe, allowing chemiluminescence of the sample to correlate directly with the amount of telomere repeats it contains (Nakamura, Hirose, *et al* 1999). As only telomeric DNA is included in the analysis, the concern over inclusion of sub-telomeric DNA that has been raised with reference to the Southern Blot method is avoided (Saldanha, Andrews, *et al* 2003). Shearing of the sample DNA has no effect on the result, and the DNA used does not require purification either from cells or tissue lysates. The entire procedure can be performed in a reaction tube. Results obtained using the HPA method compare favourably with those obtained by Southern Blot; however in common with Southern Blot this method does not allow measurement of telomeres in individual cells. The equipment required for the HPA method is not specialised beyond that available in most laboratories so it is not an expensive method to adopt (Nakamura, Hirose, *et al* 1999).

3.2.1.2 Fluorescent *in situ* hybridisation (FISH)

The original theme of the FISH method consisted of hybridising fixed cells to a fluorescent peptide nucleic acid probe that is complementary to the telomere sequence. This method has the advantage over the HPA method that the telomere lengths of individual cells may be assessed; the usefulness of it is clearly demonstrated by the number of variations that have been created. The variants in current use include quantitative FISH (Q-FISH), quantitative flow cytometry (Q-FISH^{FCM}) and flow cytometry and FISH (flow FISH) (Hultdin, Gronlund, *et al* 1998). The direct labelling of the telomere sequence with the fluorescent probe and its visualisation allows for greater accuracy in

measurement of telomere length than other methods, however the method requires the use of metaphase spreads thus introducing an inherent bias to the method as senescent cells are excluded from the sample population. The addition of flow cytometry allows small subsets of cells to be included in the analysis on an individual basis, however another level of complexity is added to an already technically demanding technique. The most complex variant includes the use of digital fluorescence microscopy in a Q-FISH^{FCM} technique. This method utilises both a telomere specific fluorescent PNA probe and the 4′, 6-diamidino-2-phenylindole (DAPI) dye specific for chromosomes allowing accurate telomere length measurements of individual chromosomes within the same cell to be performed.

3.2.1.3 Telomeric-oligonucleotide ligation assay (T-OLA)

This method is comparatively less complex than FISH derived methods, however telomeric DNA must be isolated from sub-telomeric sequence, a large amount of DNA (approximately 30 μg) is required, and the method requires the use of an electron microscope. The assay involves hybridisation of a highly specific ^{32}αP-labelled oligonucleotide to non-denatured DNA. The oligonucleotide binds in the presence of ligase to single stranded DNA with high base pairing specificity and the products are resolved on a denaturing polyacrylamide gel. The probe specificity for single stranded portions of non-denatured DNA allow this method to be used to estimate the length of the 3′ single stranded overhang found at the telomere terminus, and has an effective range from 24-650 nucleotides. As this method involves the use of radioactive nucleotides, safety is more of a concern than with the other procedures (Huffman, Levene, *et al* 2000) (Cimino-Reale, Pascale, *et al* 2001).

A summary of the methods detailed above is provided in Table 3-1.

Table 3-1. Methods currently used to measure telomere lengths and G-rich overhang. Southern Blot is the least complex technique and requires the least specialist equipment, however the higher throughput capabilities of the newer methods reduce time constraints and improve accuracy.

Technique	Summary
Southern Blot	Time consuming, labour intensive
HPA	Simple rapid and sensitive
Q-FISH	Labour intensive and more complex than Southern Blot and HPA
Q-FISH^{FCM}	Similar complexity to Q-FISH, however process takes approximately 30 hours
Q-FISH^{FCM} and digital fluorescence microscopy	Use of digital fluorescence microscopy reduces time required, however very complex technique
T-OLA	Less complex than FISH based methods, used primarily to measure G-rich overhangs

3.2.2 Chapter aims

The aims of the experiments detailed in this chapter were to evaluate telomere lengths in a number of different breeds of dogs and the domestic shorthaired cat (DSH) across a wide age range of individuals. This study allowed the effect of age, breed and gender on telomere length in the dog and cat to be investigated. *In vitro* studies aimed at exploring the replicative potential of a number of primary fibroblast cell lines and the interrelationship between replicative potential and the age of the donor animals concerned. In addition, the analysis of telomere lengths of necropsy specimens from healthy tissues, and a panel of canine and feline tumour specimens were carried out.

3.3 Materials and Methods

3.3.1 Sample details

3.3.1.1 Blood samples

Five ml jugular blood samples were taken from dogs and cats kept in environmentally enriched housing at the Waltham Centre for Pet Nutrition (WCPN). Samples also included excess blood from 2 ml jugular samples taken from clinical cases for routine biochemical analysis at GUVS. A total of 112 dogs and 30 cats were sampled. Sampling carried out at WCPN was in accordance with WALTHAM® research ethics guidelines. Cats ranged in age from 1 to 17 years and were all Domestic Shorthaired (DSH). Dogs sampled were aged from <1 to 15 years from five recognised breeds, the Labrador Retriever (n = 24), Miniature Schnauzer (n = 16), Beagle (n = 10), Golden Retriever (n = 22), and Great Dane (n = 4), and 37 dogs described as cross breed. All animals were clinically healthy at the time of sampling following a routine health inspection by a veterinary surgeon.

3.3.1.2 Necropsy specimens

Tissue samples were collected from canine and feline necropsy cases at GUVS. In all cases animals had either been euthanased for unrelated medical reasons at GUVS prior to necropsy or had been delivered for necropsy to the pathology department. All necropsies performed at the school are carried out with informed owner consent.

Approximately 1 cm^3 tissue samples were taken under aseptic conditions from as many organs as the particular necropsy would allow and immediately flash frozen in liquid nitrogen (LNO_2) before storage at -70°C. Details of individual animals are shown in Table 3-2.

Table 3-2. Details of necropsy sample animals. Canine (a) and feline (b) necropsy sampling was carried out with informed owner consent on each of the animals detailed. Tissue samples of approximately 1 cm^3 were harvested from as many organs as the particular necropsy would allow. Any evidence of post-mortem DNA degradation (OD_{260}/OD_{280} <1.7, or evidence of smearing after agarose gel electrophoresis) resulted in exclusion of the particular sample from the study.

(a)

	Breed	Age (years)	Gender	Cause of death
CN1	English Bulldog	0.25	Female	Aspirational pneumonia
CN2	Yorkshire terrier	0.75	Male	Tracheal collapse
CN3	Dachshund	6	Male	Vertebral disc collapse
CN4	Cross breed	11	Female neuter	Neoplasia
CN5	Staffordshire Bull terrier	13	Male	Trauma

(b)

FELINE	Breed	Age (years)	Gender	Cause of death
FN1	DSH	1	Male neuter	Trauma
FN2	DSH	1.5	Male neuter	Trauma
FN3	DSH	3	Male neuter	Protein losing nephropathy
FN4	DSH	13	Female neuter	Intestinal carcinoma
FN5	DSH	15	Male	Trauma

3.3.1.3 Tumour samples

Tumour samples were collected from two sources; necropsies carried out at GUVS and samples from excisional biopsies harvested at the time of surgery at the Peoples' Dispensary for Sick Animals (PDSA) hospital, Shamrock Street, Glasgow. All necropsies were carried out with informed owner consent, and decisions to perform excisional biopsies were made solely on clinical grounds and with informed owner consent, the samples used in this project representing surplus tissue beyond that required for histological diagnosis of tumour type as part of routine diagnostic work-up. Samples were flash frozen in LNO_2 at time of harvesting before storage at -70°C. A total of 21 tumour samples were used in this study, 17 canine and 4 feline. All tumours were identified by histopathology carried out at the pathology department of GUVS, and samples included examples of anaplastic sarcoma, chondrosarcoma, fibrosarcoma, haemangiosarcoma, leiomyosarcoma, liposarcoma, lymphoma, nephroblastoma, neurofibrosarcoma, rhabdomyosarcoma, spindle cell tumour, synovial cell sarcoma, mammary carcinoma and squamous cell carcinoma.

3.3.1.4 Cell lines

Cells used in this chapter include the SFA, AG08157, AG07648, AG08075, CCL-176 and S22, all of which are primary fibroblast cell cultures as detailed in Section 2.2.1.2. Cells were grown in T75 flasks and passaged routinely upon reaching approximately 80% confluence. The timing of passage is particularly important for telomere length studies, as evidence exists that prolonged confluence may affect telomeric attrition rate (Sitte, Saretzki, *et al* 1998). Cell numbers were counted (Section 2.2.1.1.1) upon harvesting, and all cell lines were seeded at 1 $x10^5$ cells/ml. 2 $x10^6$ cells were retained at each passage, transferred to 1.5 ml eppendorf tubes and pelleted at 500 g for 5 minutes. The growth media was discarded and the pellets washed in sterile PBS. A further 5-minute centrifugation step was performed; PBS was carefully aspirated from the cell pellet, which was then flash frozen in LNO_2 before storage at -70°C. DNA extracted from these pellets was used for telomere length estimation.

Cell counts at harvesting were used to generate growth curves for the cell lines according to the formula:

$$T_c = \frac{0.3T}{\text{Log}\,(A/A_0)}$$

Where T_c represents the doubling time for the cell population, T is the time elapsed, A is the number of cells at the time of harvesting and A_0 is the number of cells at an initial point (Wieder 1999).

3.3.2 DNA extraction

3.3.2.1 Isolation of DNA from peripheral blood samples

Canine DNA was isolated from 5 ml blood samples using either the QIAamp DNA blood maxi kit, or phenol/chloroform extraction as detailed in Section 2.2.2. DNA was resuspended in 50 µl TE buffer, and DNA quality and quantity were estimated using spectrophotometry (GeneQuant), and quality of all DNA isolates was confirmed by 1% agarose TBE gel electrophoresis (Section 2.2.3.3). DNA samples were then stored at -20°C in screw-top 1.5 ml Eppendorf tubes until required for analysis.

3.3.2.2 Isolation of DNA from necropsy specimens and cell pellets

Cell lysis and digestion was carried out in 1.5 ml eppendorf tubes using either 2 $x10^6$ cells or approximately 100-200 mg of tissue. Tissue samples were crushed in the eppendorf tubes using a sterile pestle. Digestion buffer consisted of 100 mM NaCl, 10 mM Tris HCl (pH 8), 25 mM EDTA (pH 8), 0.5% sodium dodecyl sulphate (SDS) and 0.1 mg/ml proteinase K (Roche). Approximately 1 ml of digestion buffer was used per tissue sample, whilst cell pellets required only 0.3 ml per sample. All samples were incubated with shaking at 50°C for between 12 and 16 hours in tightly capped tubes. Deproteination was achieved by equal volume phenol/chloroform/isoamyl alcohol (25:24:1) extraction. Separation of the phases was achieved by a 10 minute centrifugation at 1700 g;

separation was further aided by carrying out this step in 15 ml tubes containing Phase Lock gel (eppendorf) which forms a stable barrier between the upper, DNA containing aqueous phase and the lower organic phase. This allowed the aqueous phase to be easily transferred to a fresh tube for precipitation of the DNA in 2x(original) volumes of 100% ethanol and ½ volume of 7.5 M ammonium acetate. This precipitation step in the presence of high salt concentration helps reduce the amount of RNA in the DNA sample. The DNA was then pelleted by centrifugation at 1700 g for 2 minutes. A further wash step in 70% ethanol was then carried out before again pelleting the DNA, decanting the alcohol and allowing the DNA to air dry. DNA samples were then resuspended in 50-100 µl of TE buffer and quality and quantity checked as described in Section 2.2.3.3.

3.3.3 Telomere length analysis

3.3.3.1 Digestion of DNA with *Hin*f1/*Rsa*1

The selection of the restriction endonucleases *Hin*f1 and *Rsa*1 was based on the fact that these enzymes do not contain recognition sequences that cut within mammalian telomeric sequence. These enzymes are not alone in this respect, however, they are the most commonly used in the literature (Nakamura, Hirose, *et al* 1999) (Lauzon, Dardon, *et al* 2000). The use of these enzymes allows complete digestion of non-telomeric DNA, up to the first recognition site for the enzymes. The remaining intact telomeric DNA is referred to as a Telomere Restriction Fragment (TRF). Three µg of DNA was digested per sample at 4U of enzyme mix/µg of DNA for 12-16 hours at 37°C.

3.3.3.2 DNA fragment separation

3.3.3.2.1 Agarose gel electrophoresis and Southern blot

Separation of digested DNA was achieved by 0.8% agarose gel electrophoresis in 1x TAE buffer using highly pure agarose (SIGMA) following a standard protocol (The DIG system user's guide for filter hybridisation 2003) (Southern 1979). A DIG molecular weight marker was used (23.1 kb- 0.12 kb, Roche UK).

Equal amounts of sample DNA were run in each lane, and each sample was run in duplicate. Individual samples were made up to 20 µl using dH_20 and 2 µl of 10x DNA loading buffer (Section 2.1.8.2). Samples were run at 5 V/cm for between 4 and 5 hours until the loading dye was approximately 2 cm from the base of the gel.

Southern transfer (Southern 1975) of the digested DNA was carried out by capillary action using 20x SSC as a transfer buffer following a standard protocol (Current protocols in molecular biology) to a high quality positively charged nylon membrane (Amersham, UK). Gels were pre-treated first by submersion in 0.25 M HCl for 5-10 minutes; completion of this step was indicated by a change in colour of the bromophenol blue loading dye from blue to yellow. Following a rinse in dH_20 the gels were submerged in denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 30 minutes, with a change of solution after 15 minutes. This step was not complete until the loading dye colour had changed from yellow to blue. After a further rinse in H_20 the gels were submerged in a neutralisation solution (0.5 M Tris-HCl, 3 M NaCl at pH 7.5) for 30 minutes with a change to fresh solution after 15 minutes. All pre-treatment steps were carried out at room temperature and with gentle agitation using a rocking table (Luckham, UK).

Gels were flipped before Southern transfer as this reduced the likelihood of irregularities in the upper gel surface resulting in uneven DNA transfer. Transfer was carried out overnight, and the DNA was then UV cross linked with 120 mJoules of energy using a trans-illuminator (Sigma) to the nylon membrane. After twice washing the membrane with 2x SSC the blot was ready for probe hybridisation and chemiluminescent detection (Section 3.3.3.3).

3.3.3.2.2 Pulsed field agarose gel electrophoresis

Pulsed field electrophoresis was carried out using a CHEF-DR® II system (Biorad). This system allows resolution of large DNA fragments (approximately 10-2000 kb) beyond the capacity of simple gel electrophoresis and is necessary for resolution of DNA fragments beyond approximately 25 kb in size (Carle,

Frank, *et al* 1986). The pulsed field electrophoresis used in this Chapter is described as 'contour clamped homogenous electric field' (CHEF). In this system the gel is surrounded by a set of electrodes that allows alternation of the electric field to be coupled with a uniform direction of DNA migration (Chu, Vollrath, *et al* 1986). One percent agarose gels in 0.5% TBE as buffer were used in all procedures, and the apparatus includes a pump and cooling system to keep the buffer temperature steady at 14°C for the duration of the electrophoresis. The gels were run at 6 V/cm with a 1 to 6 second ramped switch time and a total run time of 15 hours. These parameters are within that recommended by the manufacturer to resolve DNA of 1-100 kb in length. An 8-48 kb CHEF DNA size standard (Bio-Rad) was used consisting of 13 bands: 8.3, 8.6, 10.1, 12.2, 15, 17.1, 19.4, 22.6, 24.8, 29.9, 33.5, 38.4 and 48.5 kb.

Following electrophoresis, gels were stained for 30 minutes in 1.0 µg/ml ethidium bromide to visualise the DNA size standard that was then photographed with the aid of UV transillumination (Uvi, Thistle Scientific, UK). These photographs allowed the position of the DNA marker bands to be accurately recorded on the final autoradiographs. Gels were UV treated in a UV cross-linker with 60 mJoules of energy to cleave the separated DNA fragments; this is necessary to allow efficient Southern transfer of fragments greater than approximately 20 kb. The gels were pre-treated in 0.4 M NaOH, 1.5 M NaCl for 15 minutes to reduce background and increase transfer efficiency then Southern blotted using the same solution as a transfer solvent onto a positively charged nylon membrane overnight. Following this the nylon membranes were neutralised in 0.5 M Tris, pH 7.0 for 5 minutes, then briefly rinsed in 2x SSC (Section 2.1.8.2) before proceeding with hybridisation. The hybridisation and chemiluminescent method are common to the standard and CHEF electrophoresis protocols.

3.3.3.3 Hybridisation and chemiluminescent detection

All hybridisation steps were carried out using a Hybaid Maxi hybridisation oven (Hybaid) and standard hybridisation flasks on a rotary mount. Pre-hybridisation

was carried out at 42°C for 45 minutes using 25 ml of DIG Easy Hyb solution (Roche, UK). This solution was then discarded and replaced with 10 ml of DIG Easy Hyb containing 2 µl of digoxigenin (DIG) labelled telomere probe ($TTAGGG_7$). Hybridisation was carried out at 42°C for 3 hours.

Two stringency washes were carried out; the first consisted of two washes in 2x SSC, 0.1% SDS for 5 minutes each at room temperature. This was followed by 2 washes in 0.2x SSC, 0.1% SDS at 50°C for 20 minutes each. Gentle agitation of the membrane was carried out during both stringency washes and throughout the detection procedure.

Membranes were rinsed in a washing solution (0.3% w/v Tween® 20, 0.1 M maleic acid, 0.15 M NaCl, pH 7.5) for 5 minutes at room temperature followed by a 30-minute incubation at room temperature in freshly prepared blocking buffer (Roche, UK) dissolved in maleic acid buffer solution (0.1 M maleic acid, 0.15 M NaCl, pH 7.5). Blots were then incubated with a DIG specific antibody (750 units/ml Anti-Digoxigenin, Fab fragments) covalently coupled to alkaline phosphatase (Anti-DIG-AP) (Roche, UK). The Anti-DIG-AP working solution was prepared to a final concentration of 75 mU/ml (1:10,000) in blocking buffer (Roche, UK). The antibody solution was centrifuged for 5 minutes at 13000 rpm before careful pipetting from the surface of the liquid to avoid background signal being generated by aggregated antibody.

Incubation in the antibody solution was followed by two further 15-minute washes at room temperature in wash buffer (Roche, UK), followed by a 5-minute incubation in detection buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl). Excess detection buffer was briefly blotted from the membrane by placing it DNA side up on a piece of 3MM Whatman filter paper. The blot was then placed on an acetate sheet and approximately 3 ml of substrate solution placed drop wise onto the DNA side before overlaying with another acetate. The system used the chemiluminescent alkaline phosphate substrate CSPD® (25 mM Disodium 4-chloro-3-(4-methoxyspiro(1,2-dioxetane-3,2'-(5'-chloro)tricyclo[$3.3.1.1^{3,7}$]decan)-4-yl) phenyl phosphate) (Roche, UK) diluted

1:100 in detection buffer. Any bubbles were carefully removed from between the sheets and the composite was incubated at room temperature for 10 minutes. Excess substrate solution was then squeezed out and the two plastic sheets sealed with sellotape. The sealed membrane was incubated at 37°C for a further 10 minutes to aid the chemiluminescent signal. The membranes were then used to generate autoradiographs. Exposure time varied between 5 and 20 minutes to achieve optimum image quality for analysis.

3.3.3.4 Analysis of autoradiographs

Autoradiographs were analysed by densitometry using TotalLab v2.01 software. Mean TRF (kb) values were determined from the densitometric readings according to the formula:

$$\text{TRF (kb)} = \frac{\Sigma(Od_i L_i)}{\Sigma(Od_i)}$$

Where Od_i is the chemiluminescent signal and Li is the length of the TRF fragment at position i. The calculation takes into account the higher signal intensity from larger TRF fragments because of multiple hybridization of the telomere specific probe. Duplicate analyses were carried out on each sample.

3.3.4 Confirmation of senescence

Replicative senescence in primary cultures was marked by cessation of cellular replication and morphological changes of the senescent cells, involving an increased cell size and a rounded, flattened appearance. In addition to these visual indicators, a number of biomarkers were employed to confirm the presence of senescent cells in cultures, and these are detailed below.

3.3.4.1 Senescence associated β-galactosidase (SA-β–GAL) staining at pH 6

Replicative senescence was detected in cell lines by staining for the presence of a senescence associated β-galactosidase activity at pH 6.0. This method was developed from that originally outlined by Dimri *et al* (Dimri, Lee, *et al* 1995) and detects SA-β–GAL using the compound X-Gal, which forms a perinuclear blue precipitate upon cleavage (Miller 1972). Cells were stained *in situ* in tissue culture flasks or grown on cover slips contained in 6 well plates, and were first washed three times in PBS, then fixed for 10 minutes at room temperature in 2% formaldehyde/0.2% glutaraldehyde in PBS. Cells were then washed a further three times in PBS and incubated at 37°C (without CO_2) in fresh SA- β –GAL staining solution consisting of 40 mM citric acid/sodium phosphate buffer (pH6.0), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM sodium chloride, 2 mM magnesium chloride and 2 mg/ml X-gal dissolved in dimethylformamide all made up in distilled water.

Staining was evident after 2-4 hours and maximal after 12-16 hours. Five fields of at least 100 cells were counted, and cells positive for SA- β –GAL activity expressed as a percentage of the total number counted. Staining was recorded by digital photography.

3.3.4.2 Immunocytochemistry

Immunocytochemistry (ICC) is a technique that allows identification of cellular or tissue antigens by means of a specific antigen-antibody interaction. The method has been greatly refined since the initial direct labelling experiments that employed a primary antibody conjugated directly to a fluorochrome (Coons, Creech, *et al* 1941). A common variation that uses an indirect immunoperoxidase labelling approach is used in these experiments (Sternberger, Hardy, *et al* 1970). This method involves a dual antibody system in which an unlabelled primary antibody is bound to a secondary biotinylated 'bridging' antibody. A complex formed from strept-avidin and horseradish peroxidase

(HRPO) is then conjugated to this secondary antibody to provide signal amplification at the site of the primary antibody. The final substrate for the HRPO is 3,3 diaminobenzidine (DAB) (Sigma, USA) which produces a brown end product at the antigen site that is highly insoluble in alcohol and other inorganic solvents. This method was used to identify the presence of $p16^{INK4a}$ and p21 in cells grown on cover slips in the primary canine culture SFA and the primary feline culture S22 (both detailed in Section 2.2.1.2). The primary $p16^{INK4a}$ antibody used was the H-156 purified rabbit polyclonal antibody (Santa Cruz Biotechnology) raised against a recombinant protein corresponding to amino acids 1-156 representing full length $p16^{INK4a}$ of human origin. The p21 antibody, SX118 (murine IgG_1) was sourced from BD PharMingen and is raised against the final 20 amino acids of human p21.

Accumulation of both $p16^{INK4a}$ and p21 has been associated with replicative senescence in human cultures (Alcorta, Xiong, *et al* 1996) (Huschtscha & Reddel 1999). Alcorta *et al* found that p21 accumulated as human fibroblasts approached senescence (in the last few passages), and then reduced in the senescent cells, concomitant with an increase in $p16^{INK4a}$ levels (Alcorta, Xiong, *et al* 1996). The staining carried out on the SFA and S22 cultures detailed in this chapter were on the final passages (p12 and p10 respectively) and the cells had been senescent, as determined by SA-β-GAL activity, for two weeks before staining was carried out.

3.3.4.2.1 Staining optimisation

This procedure involves identifying a concentration of primary antibody that provides the maximum amount of specific staining with the least amount of background. A range of dilutions (1/50, 1/100, 1/200, 1/400) is used to stain a positive control tissue for each antigen to be investigated. Repeating this procedure and omitting the primary antibody provides negative controls. Evaluation of control slides by light microscopy identified the most favourable dilution for each antibody, which was then used on all subsequent test cells. The H-156 (p16) antibody has previously been shown to cross react with canine $p16^{INK4a}$ in fibroblasts (Koenig, Bianco, *et al* 2002), and the MCF7 cell line

(Section 2.2.1.2) was used as a positive control for the p21 antibody as recommended in the product datasheet.

3.3.4.2.2 Immunocytochemical staining procedure

The antibody diluent used in this study consisted of 0.1 g BSA (BDH, UK) and 0.01 g sodium azide (BDH, UK) in 100 ml of 0.01 M TBS (pH 7.5). Test cells were incubated with primary antibodies for two hours at room temperature followed by three five minute wash steps in the wash buffer (Tween20, 0.01 M TBS at pH 7.5). A standard 1/200 dilution of the appropriate secondary antibody (polyclonal swine anti-goat/mouse/rabbit, biotinylated, Dako, UK) was then applied for a 45-minute incubation at room temperature. This was followed by another three five minute washes in wash buffer before signal amplification was achieved by a 45-minute incubation with the HRPO conjugated strept-avidin complex (Dako, UK). The chromagen DAB was reconstituted in 5 ml tap water as per manufacturers instructions as ions contained in the tap water enhance the reaction and improve the intensity of the final staining. The chromagen was then applied for 5 minutes to produce the stable brown insoluble product that may be viewed by light microscopy. Counterstaining was performed using Gills' haematoxylin followed by washing of the stained cells in water, dehydration via a series of graded alcohols and clearing in Histo-Clear (AGTC Bioproducts). Cover slips were then permanently mounted using DPX mountant (BDH, UK) onto microscope slides before examination by light microscopy.

3.4 Results

3.4.1 DNA quantity and quality

Genomic DNA was isolated from PBLs using both phenol/chloroform extraction with ethanol precipitation, and also using the QIAamp DNA blood maxi kit (QIAGEN, UK). Good quality DNA samples were obtained and checked by spectrophotometry to have an A_{260}/A_{280} value of >1.7. Agarose gel assessment was also carried out revealing intact, high molecular weight DNA with no evidence of smearing associated with DNA degradation, as shown in Figure 3-1a. Complete digestion of the DNA was confirmed by agarose gel electrophoresis to reveal a smear of DNA with no evidence of residual high molecular weight DNA. An example of a post digestion gel is shown in Figure 3-1b.

3.4.2 Comparison of CHEF electrophoresis and standard agarose gel electrophoresis

A number of samples were analysed using both standard agarose gel electrophoresis and CHEF electrophoresis (Figure 3-2). TRF results were found to be comparable in size using the two methodologies (average difference 0.6 kb, standard deviation 0.44). All future TRF investigations were performed using standard agarose gel electrophoresis.

3.4.2.1 Analysis of autoradiographs

A TRF value was determined for each sample as detailed in Section 3.3.3.4. Duplicate analysis was carried out for each sample, and the average difference between samples was 0.42 kb with a standard deviation of 0.42. A representative autoradiograph with telomere length analysis is shown in Figure 3-3.

Figure 3-1 (a) 1% agarose gel electrophoresis of genomic DNA isolated from canine PBLs, and (b) canine DNA after a 2-hour digestion with 4 iu/μg of *Hinf* I and *Rsa* I. Both gels include a 100 bp marker (100-2072 bp, Invitrogen)

(a) (b)

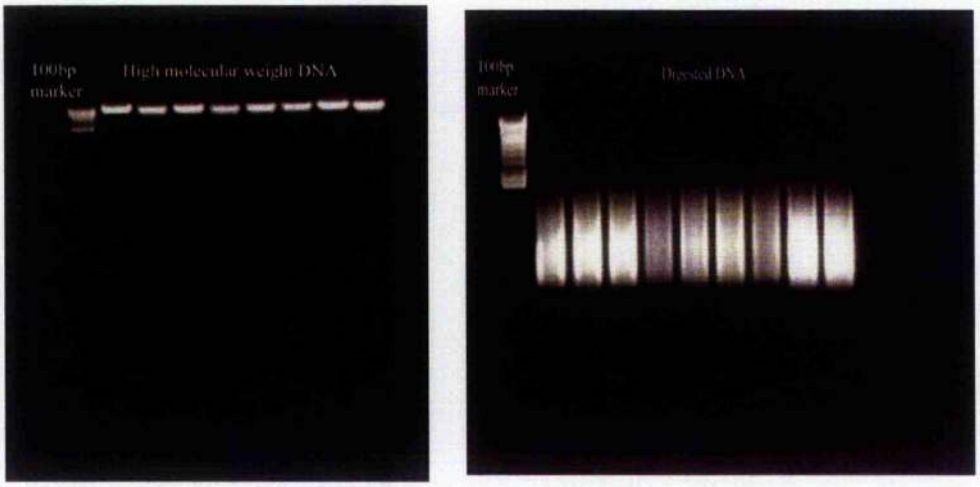


Figure 3-2. Comparison of CHEF and standard agarose gel electrophoresis
The autoradiographs below demonstrate comparable telomere smears generated using both CHEF and standard agarose gel electrophoresis. Both autoradiographs show necropsy samples from an 11-year-old female crossbred bitch. The autoradiograph shown on the left contains smears generated from specimens using CHEF electrophoresis as described in Section 3.3.3.2.2. The samples shown are duplicates of skeletal muscle (lanes 1 and 2), liver (3 and 4) and kidney (5 and 6). The same samples were re-analysed to produce the autoradiograph shown on the right using the same protocol except that these smears were separated by standard agarose gel electrophoresis as outlined in section 3.3.3.2.1. Skeletal muscle is shown in lanes 1 and 2, liver in lanes 3 and 4 and kidney in lanes 7 and 8. Lanes 5 and 6 contain a lung sample. Averages of the duplicates were taken to demonstrate that for each of the three samples the telomere lengths were comparable. Skeletal muscle was estimated at 22.7 kb using CHEF and 22.4 kb using standard gel electrophoresis, liver at 21.5 kb by CHEF and 20.4 kb by standard gel electrophoresis and kidney at 17.4 kb by CHEF and 17.8 kb by standard agarose electrophoresis.

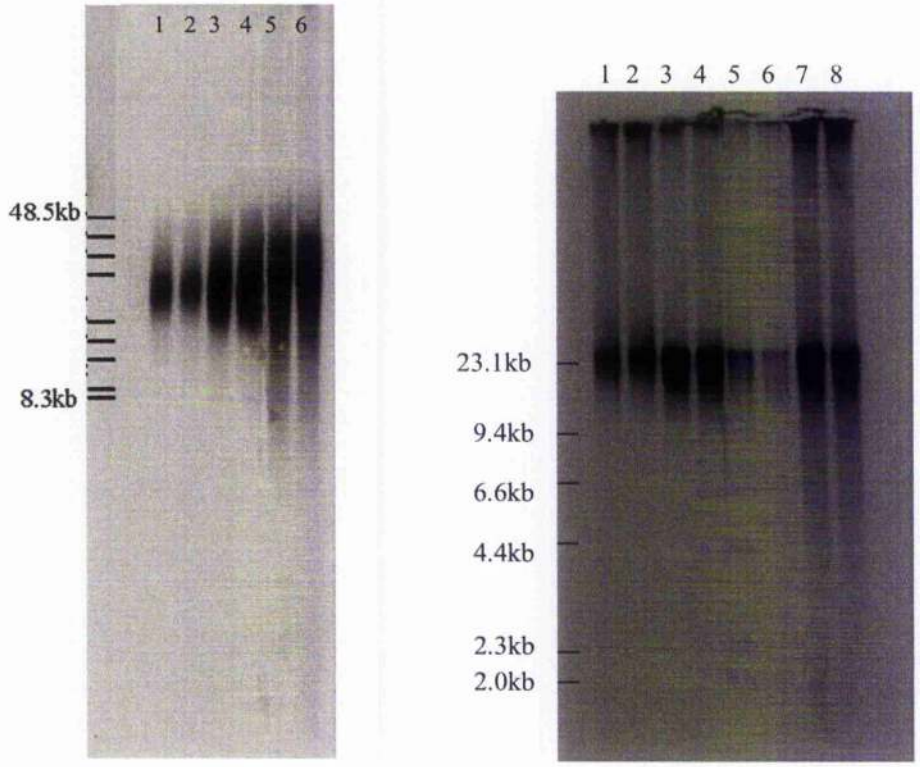
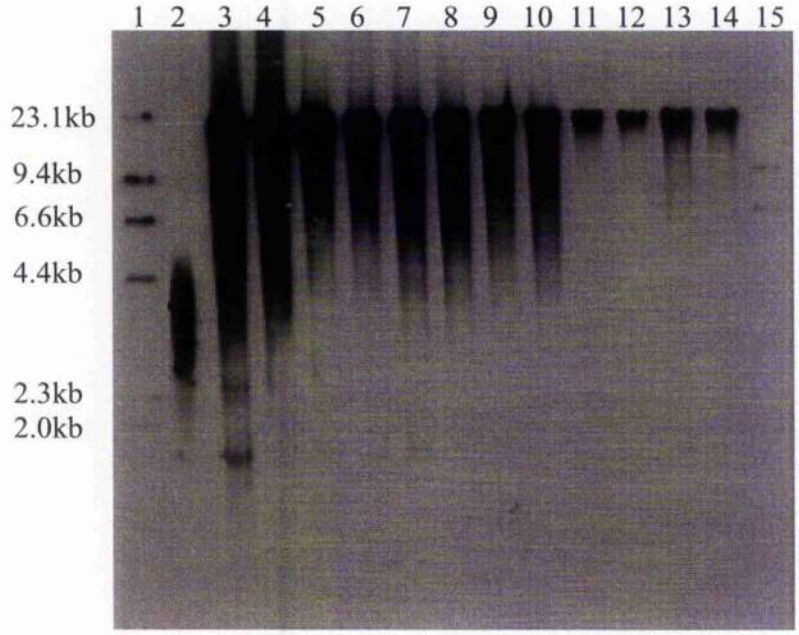


Figure 3-3. Autoradiograph of feline TRFs highlighted by chemiluminescence. Lanes order is as follows- Lane 1, DNA molecular weight marker II (Roche); lane 2, control DNA (3.9 kb, Roche, UK); lanes 3 and 4, 10 year old mean TRF 5.0 kb; lanes 5 and 6; 1 year old mean TRF 7.3 kb; lanes 7 and 8, 9 years old, mean TRF 6.4 kb; lanes 9 and 10, 9 years old, TRF 7.2 kb; lanes 11 and 12, 4 years old, TRF 16.3 kb; lanes 13 and 14, 1 year old, TRF 13.3 kb; lane 15, second marker lane.



3.4.3 *In vivo* telomere length studies

3.4.3.1 TRF analysis of PBL samples in the dog

Duplicate TRF analysis was carried out on a total of 112 canine PBL DNA samples. The final mean TRF value was then determined by averaging each pair of analyses. Canine samples were derived from five distinct breed groups and 36 cross breed dogs revealing a heterogeneous population of telomeres ranging from 9.6 kb to 23.5 kb. Of these individuals, 24 animals were Labrador Retrievers, comprising 15 females and 9 males with an age range of <1 to 13 years, and mean TRF values of 14.7 to 19.7 kb. Sixteen dogs belonged to the Miniature Schnauzer breed comprising 7 females and 9 males with an age range of <1 to 9 years and a mean TRF range of 9.7 to 19.9 kb. Two females and 8 males represented the Beagle breed, with an age range of 4 to 13 years and a mean TRF range of 14.9 to 22.3 kb. Four Great Dane dogs were also included, 1 female and 3 males, age range 2 to 3 years and TRF values of 10.7 to 18.5 kb. Gender data was unavailable for the Golden Retriever and crossbreed group. Golden Retrievers numbered 22, aged from 1 to 13 years and with mean TRFs ranging from 9.6 to 22.1 kb. Thirty-six crossbreed dogs made up the remainder of the canine PBL samples, ranging in age from 1 to 15 years and with a mean TRF range of 12 to 23.5 kb. TRF results for individual dogs are shown in Tables 3-3, 3-4, 3-5, 3-6, 3-7 and 3-8, corresponding to Labrador Retrievers, Miniature Schnauzers, Golden Retrievers, Beagles, Great Danes, and Crossbreeds respectively.

These data were used to examine the effect of age, breed and gender on telomere length in the dog. Regression plots of age against TRF in the canine PBL samples are shown in Figures 3-4 and 3-5. An analysis of covariance for all 112 dogs revealed no trend of changing telomere length with age (Figure 3-6), however a significant association between decreasing telomere length and increasing donor age was identified in all pedigree dogs ($p = 0.001$) when these were analysed in isolation. In contrast, the crossbreed group showed a trend of increasing telomere length with increasing age. An analysis of covariance including the five pedigree breed groups (Beagle, Great Dane, Golden Retriever,

Labrador Retriever and Miniature Schnauzer) revealed breed to have a highly significant effect on telomere length (p = <0.0001). This is the first report of breed specific differences in telomere length in the dog. There was no significant interaction between age and breed in the analysis, so the age distributions of the individuals within each breed group did not contribute to the breed effect on telomere length. A Least Squares means analysis was carried out on the pedigree dog PBL samples as shown in Table 3-9. This revealed that when mean TRF values are corrected for age the Great Dane group had the shortest telomeres, and that the telomeres of the Great Danes and the Miniature Schnauzers were significantly shorter than the Golden Retriever and Beagle group (p <0.05). The Miniature Schnauzer breed also displayed significantly shorter telomeres than the Labrador Retrievers (p <0.05).

Gender had no effect on telomere length within the canine PBL sample population.

Table 3-3. TRF analysis of PBL DNA isolated from Labrador Retrievers; 15 females (F), 9 males (M) ranging in age form <1 to 13 years old. TRFs in this group ranged from 14.7 to 19.7 kb.

Sample number	Age (years)	Gender	Mean TRF (kb)
1	<1	F	14.8
2	<1	F	18.5
3	<1	F	19.5
4	1	M	15.8
5	1	M	18.2
6	1	F	16.9
7	3	M	17.4
8	3	M	14.7
9	3	M	17.5
10	3	M	18.2
11	3	F	18.2
12	3	F	17.1
13	4	F	16.3
14	4	M	18.5
15	5	F	17.7
16	5	F	17.1
17	5	M	14.8
18	5	F	14.8
19	6	M	16.3
20	6	F	19.7
21	7	F	17.7
22	8	F	17.9
23	13	F	15.2
24	13	F	15.8

Table 3-4. TRF analysis of PBL DNA isolated from Miniature Schnauzers; 7 females (F), 9 males (M) ranging in age from <1 to 9 years. TRFs in this group ranged from 9.7 to 19.9 kb.

Sample number	Age (years)	Gender	Mean TRF (kb)
1	< 1	F	15
2	< 1	M	14
3	< 1	M	14.6
4	< 1	F	15.2
5	2	F	19.2
6	2	F	19.9
7	5	M	15.4
8	6	F	15.8
9	6	M	16.9
10	6	F	14.5
11	6	M	15.1
12	6	M	10.6
13	7	M	12
14	9	M	12.6
15	9	M	10.3
16	9	F	9.7

Table 3-5. TRF analysis of PBL DNA isolated from Golden Retrievers; ranging in age from 1 to 13 years, gender information for this group is not available. TRFs in this group ranged from 9.6 to 22.1 kb.

Number	Age (years)	Mean TRF (kb)
1	1	18
2	2	17.8
3	2	17.7
4	2	17.4
5	4	17.9
6	4	17.8
7	5	20.6
8	5	19.7
9	5	18.4
10	6	18.5
11	7	21.3
12	8	19.7
13	9	20.4
14	9	16.7
15	9	22.1
16	10	19.1
17	10	16.4
18	11	16.7
19	12	15.1
20	12	10.2
21	13	9.6
22	13	18

Table 3-6. TRF analysis of PBL DNA isolated from Beagles; 2 females (F) and 8 males (M), with an age range of 4 to 13 years, represent the breed. TRFs in this group ranged from 14.9 to 22.3 kb.

Number	Age (years)	Gender	Mean TRF (kb)
1	4	M	21.7
2	4	M	16.3
3	5	M	21.6
4	6	F	22.3
5	6	M	18.7
6	7	F	14.9
7	9	M	15.1
8	12	M	18.7
9	12	M	16.7
10	13	M	18.1

Table 3-7. TRF analysis of PBL DNA isolated from Great Danes, 3 males (M) and one female (F). TRFs ranged from 10.7 to 18.5 kb.

Number	Age (years)	Gender	Mean TRF (kb)
1	2	M	18.5
2	2	M	13.5
3	2	F	10.7
4	3	M	16.5

Table 3-8. TRF analysis of PBL DNA isolated from crossbreeds; gender information for this group is not available, ranging in age from 1 to 15 years. TRFs in this group ranged from 12 to 23.5 kb.

Number	Age (years)	Mean TRF (kb)
1	1	16
2	1	20
3	1	19.8
4	1	12
5	1	17.9
6	1	14
7	1	19.2
8	2	19.4
9	2	16.9
10	2	12.3
11	3	16.3
12	4	19.1
13	4	21.7
14	5	19.2
15	6	19.2
16	7	21.2
17	7	20.8
18	7	20.4
19	7	14.8
20	8	21.8
21	8	20.9
22	8	23.5
23	8	20.9
24	9	21.9
25	9	23
26	9	21.5
27	9	21.6
28	10	19.9
29	10	21.9
30	10	21.7
31	10	22
32	11	23.5
33	12	19.4
34	12	18.7
35	12	16
36	15	17.3

Figure 3-4. Regression plot of TRF against age for the Golden Retriever, Beagle and Crossbreed dogs. A regression plot of TRF against age for the Golden Retriever and Beagle PBL DNA samples both show a trend of decreasing TRF with increased age. However the group of 36 cross breeds show a trend of increasing TRF with increasing age. It is believed this is an artefact caused by a breed effect on TRF, therefore the apparent increase in TRF with increasing age is considered to reflect the breed backgrounds within the sample population and is not a true reflection of the effect of age on telomere length.

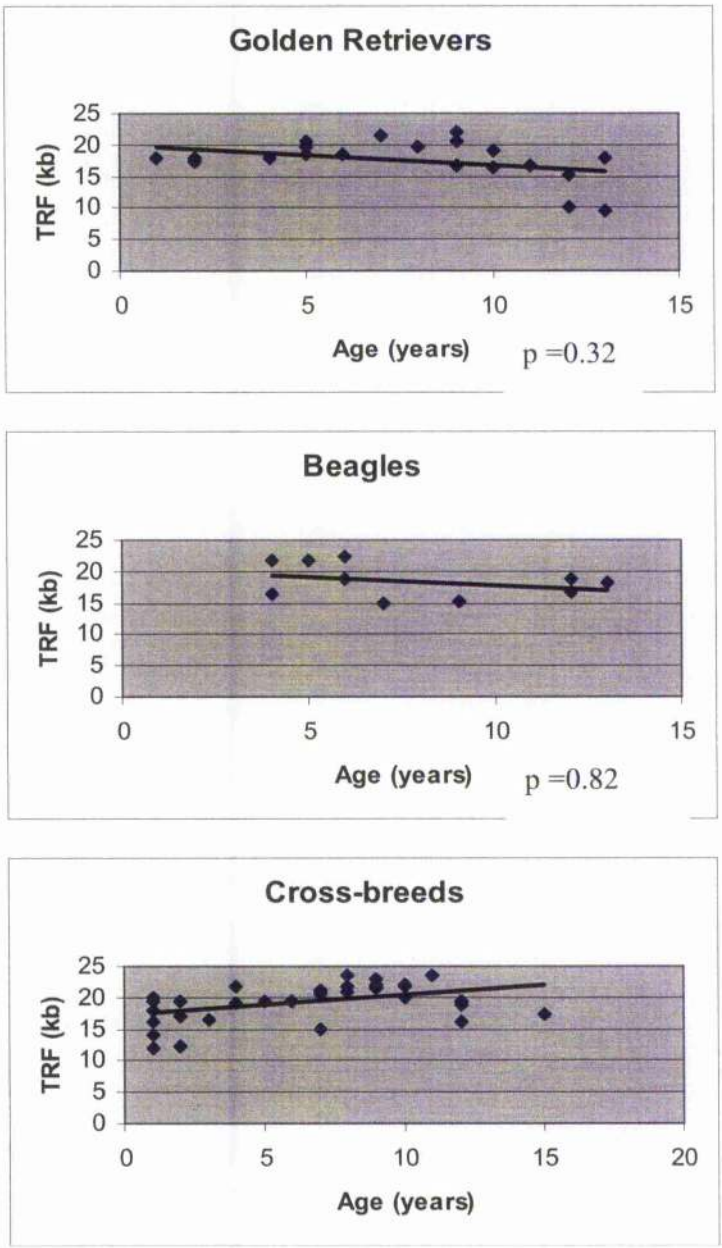


Figure 3-5. Regression plot of TRF length against age for Miniature Schnauzer and Labrador Retriever PBL DNA samples. In this case both breeds show a trend of decreasing TRF with increased age.

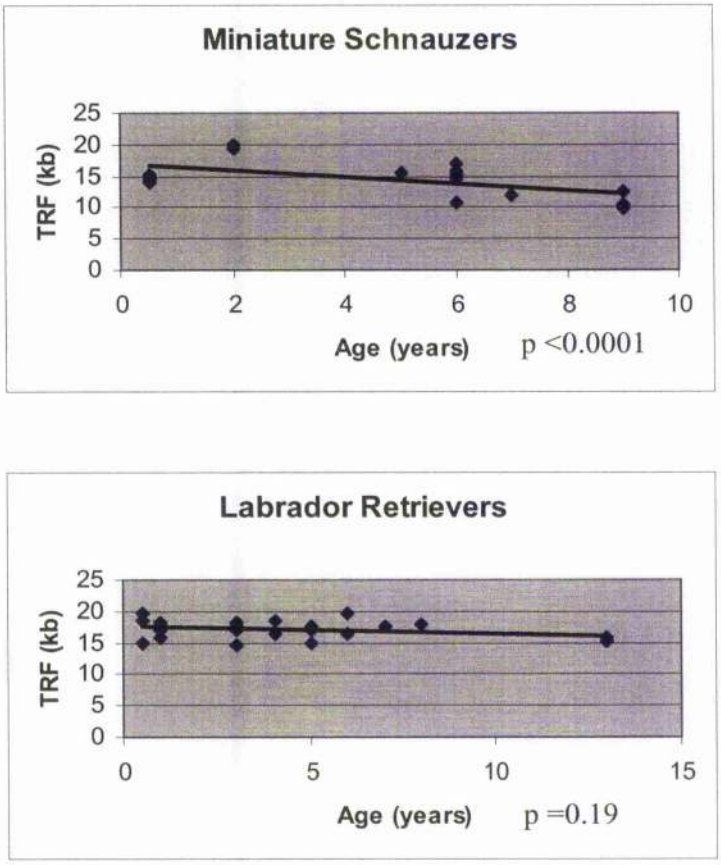
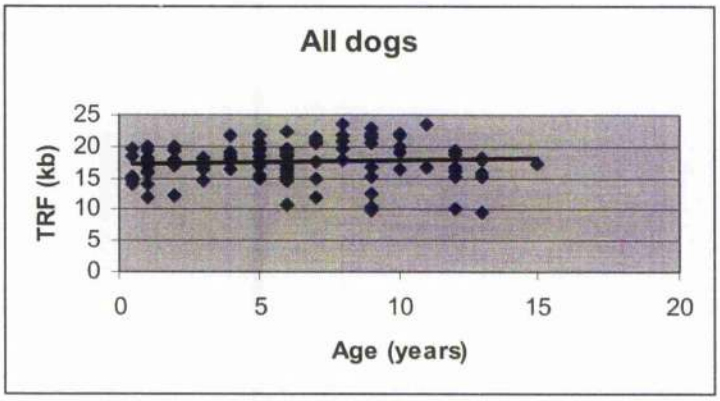


Figure 3-6. Regression plot of TRF length against age for all canine PBL DNA samples combined, including and excluding crossbreed animals. Analysis of all samples together does not reveal any trend of decreasing TRF with increasing age. However, removal of the crossbreed dogs from the analysis reveals an association between decreasing telomere length and increasing age (p = 0.001) in pedigree dogs. These data are likely a reflection of large genetic diversity in the crossbreed group, in effect some individuals inheriting longer than average telomeres, others shorter than average telomeres. Although it is not possible to examine whether there is any interaction between breed inheritance and age within the crossbreed dogs such an interaction could cause an overshadowing of the age effect on telomere length. These data underscore the importance of removing breed as a variable when examining the effect of age on telomere length in the dog.



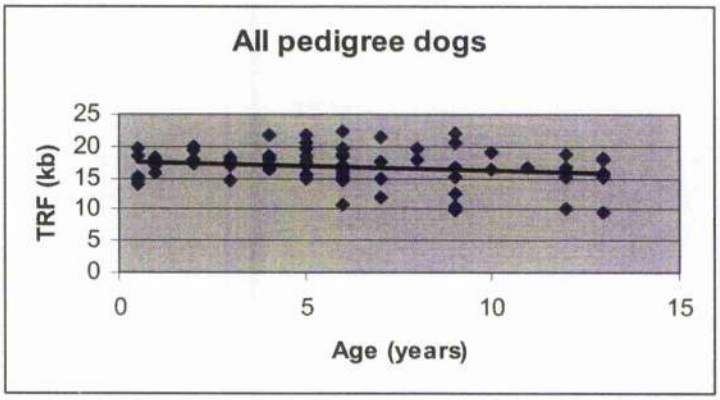


Table 3-9. Least Squares Means analysis of canine PBL TRF length from the five pedigree breed groups. Great Dane and Miniature Schnauzers had significantly shorter TRFs than the Golden Retriever and Beagle groups, and the Miniature Schnauzer group had significantly shorter TRFs than the Labrador Retrievers. Groups with overlapping confidence limits are denoted with the same superscript letter.

Breed	Mean TRF	95% confidence limits
Great Dane[ab]	13.8	11.4-16.3
Miniature Schnauzer[b]	14.2	13.0-15.4
Labrador Retriever[ac]	16.7	15.7-17.7
Golden Retriever[c]	18.2	17.1-19.2
Beagle[c]	19.0	17.5-20.6

3.4.3.2 TRF analysis of PBL samples in the cat

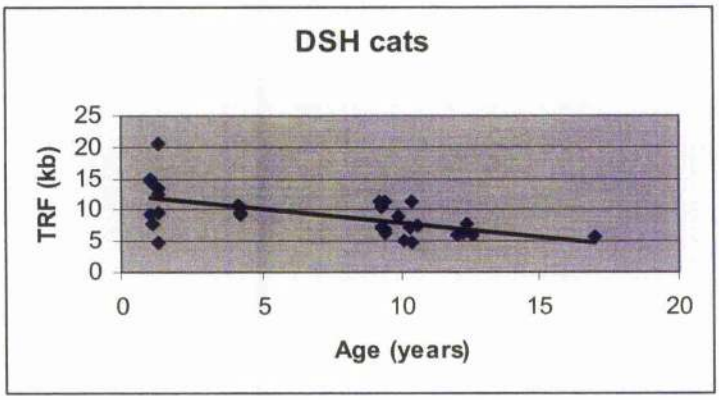
All thirty feline PBL DNA samples were derived from cats belonging to the DSH breed group. Of these, 12 were female and 18 male with ages ranging from 1 to 17 years and mean TRF values ranging from 4.7 to 20.6 kb. TRF results for individual cats are shown in Table 3-10. A regression plot of TRF against age (Figure 3-7) showed a significant association between decreasing TRF length and increased donor age (p =0.001).

An analysis of covariance was also carried out on the 30 feline samples to investigate the effect of age and gender on telomere length in the cat. Breed was not included in the analysis as all the cats were DSHs. Again age was shown to have a significant effect on telomere length (p = 0.001), whilst gender did not affect telomere length at a significant level. In addition the cats had significantly different PBL TRF values to the dogs sampled (p = <0.01).

Table 3-10. TRF analysis of PBL DNA samples taken from 30 DSH cats, 12 female (F) and 18 male (M). TRF values range from 4.7 to 20.6 kb.

Number	Age (years)	Gender	TRF (kb)
1	1	F	14.5
2	1	M	14.9
3	1	M	9.2
4	1	M	7.6
5	1	M	13.3
6	1	M	20.6
7	1	M	4.7
8	1	M	9.5
9	1	M	12.6
10	4	F	10.8
11	4	M	9.4
12	4	F	9.2
13	4	M	9.2
14	9	FN	11.4
15	9	M	10.4
16	9	FN	7.2
17	9	M	6.9
18	9	MN	11.3
19	9	FN	6.4
20	9	M	8.9
21	10	FN	5
22	10	M	7.1
23	10	FN	4.8
24	10	FN	11.3
25	10	F	7.4
26	12	M	6.1
27	12	M	6.1
28	12	M	7.6
29	12	F	6.1
30	17	FN	5.6

Figure 3-7. Regression plot of TRF length against age for the feline PBL DNA samples. The cats sampled all belong to the DSH breed type, with an age range of 1 to 17 years, TRFs ranged from 4.7 to 20.6 kb, and show an association of decreasing size with increasing age ($p = 0.001$).



3.4.3.3 TRF analysis of necropsy samples in the dog and cat

3.4.3.3.1 Normal tissue

Tissue samples were harvested from 5 dogs and 5 cats necropsied at GUVS for unrelated medical reasons. Details of donor animals are shown in Table 3-2. After DNA extraction and quality assessment as detailed in Section 2.2.2, 48 DNA samples were considered fit for duplicate TRF analysis comprising 26 canine and 22 feline samples. The canine samples included cardiac muscle (n = 2), liver (n = 4), skeletal muscle (n = 4), lung (n = 2), kidney (n = 4), small intestine (n = 3), spleen (n = 2), stomach (n = 3), testis (n = 1) and ovary (n = 1). The feline samples included brain (n = 2), cardiac muscle (n = 2), liver (n = 5), skeletal muscle (n = 3), lung (n = 4), kidney (n = 3), small intestine (n = 2), and testis (n = 1). TRF values for each sample are shown in Table 3-11. Regression plots of TRF against age of donor animal are detailed in Figures 3-8, 3-9, 3-10 and 3-11. These are included in cases where more than 2 samples per organ are available for comparison.

When all canine organ samples were analysed together (TRFs ranged from 11.6-22.8 kb) no trend in telomere length between the different ages of animal is visible, however when tissue samples were analysed individually the liver, kidney and small intestine samples showed a trend of decreasing TRF with increased age of donor animal. A repeat of this analysis using only the feline samples (TRFs ranged from 8.5-26.3 kb) revealed a trend of decreasing TRF with increased donor age even when the different tissue types were included in the same analysis. The canine and feline necropsy sample TRFs were not significantly different from the range of TRFs found in canine (9.6-23.5 kb) , and feline (4.7-20.6 kb) PBLs.

3.4.3.3.2 Tumour tissue

A total of 21 tumour samples, 17 canine and 4 feline were subjected to TRF analysis. TRFs ranged from 7.2 to 22.2 kb for the canine samples and 11.6 to

18.3 kb for the feline samples. Details of individual results are shown in Table 3-12.

Table 3-11 TRF analysis of necropsy samples. TRF analysis of necropsy samples collected from dogs (a) and cats (b) euthanased at GUVS for unrelated medical reasons. 26 canine and 22 feline samples were considered fit for TRF analysis, and the results shown below indicate an average of two TRF analyses per sample.

(a)

CANINE	Cardiac muscle	Liver	Skeletal muscle	Lung	Kidney	Small intestine	Testis	Spleen	Stomach	Ovary
CN1		22.7	15.2		22.8	14.6			13.7	12.3
CN2	14.9			14.7	22.8	20.3		22.6	14.7	
CN3	19	17.4	19.3	19.3	14.8	11.6				
CN4		20.5	22.6		17.3					
CN5		13.5	21.1				14.4	18.2	18.6	

(b)

FELINE	Brain	Cardiac muscle	Liver	Skeletal muscle	Lung	Kidney	Small intestine	Testis
FN1	18.7	12.6	10.1	19	26.3			
FN2	19.4	16.5	14.8		15			
FN3			22.7			22.8		
FN4			14.5	18	16.7	17.9	17	
FN5			9.4	11.3	11.5	13.7	8.5	15.6

Figure 3-8. Regression plot of TRF length against age for canine necropsy specimens (1). A trend of decreased TRF with increased donor age is present in both the canine liver (range 13.5 to 22.7 kb) and kidney samples analysed (range 14.8 to 22.8 kb). Both liver and kidney trends may reflect cell turnover in these organs. No such trend is seen with canine skeletal muscle samples, reflecting the post-mitotic nature of this tissue. The increased TRF in the samples from older animals may reflect individual variation in donor animals.

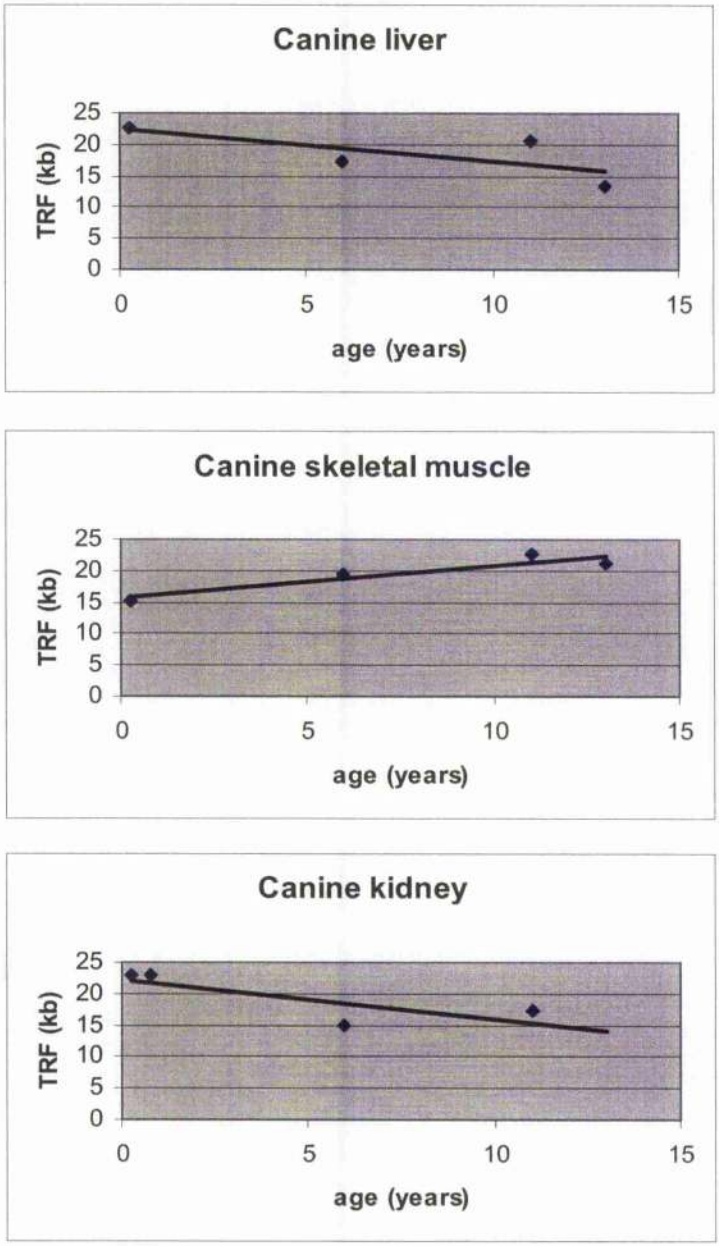


Figure 3-9. Regression plot of TRF against age for canine necropsy specimens (2). A trend of decreased TRF with increased donor age is present in the canine small intestine samples (range 11.6 to 20.3 kb), but not in the stomach samples (range 13.7 to 18.6 kb). A regression plot of TRF length against age for the entire canine necropsy samples combined displays no trend of changing TRF with age. This may reflect breed variation in the sample population.

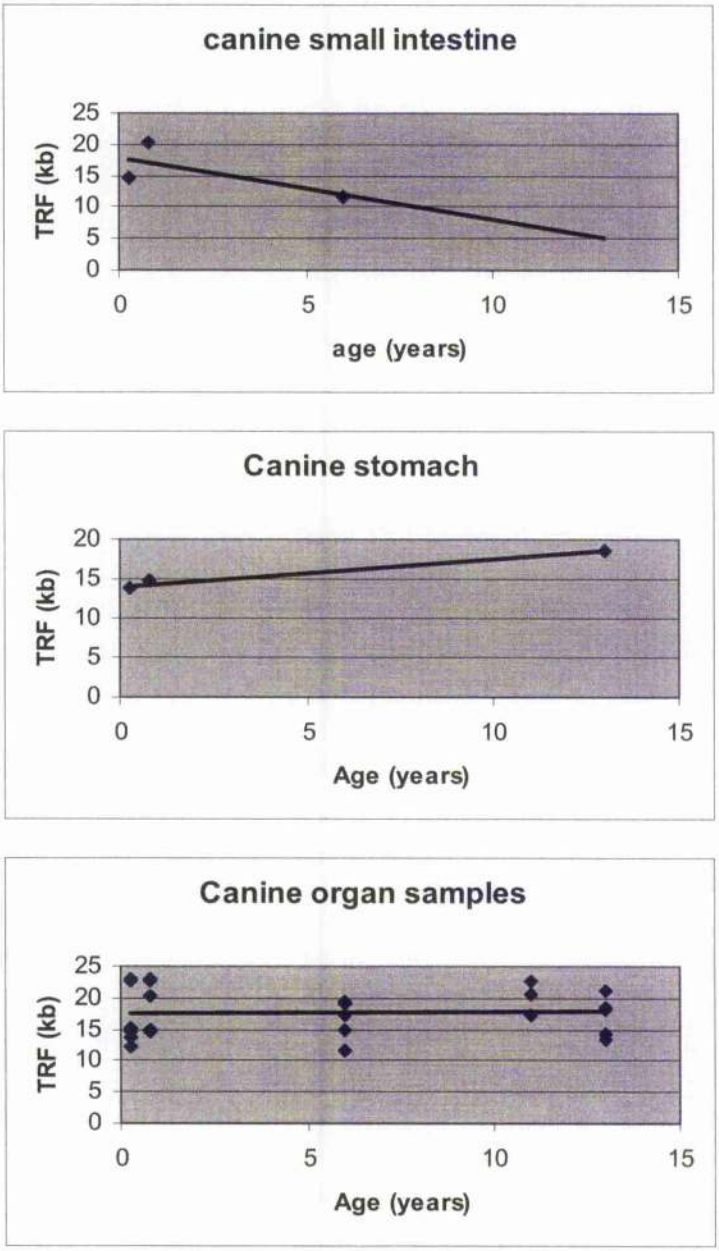
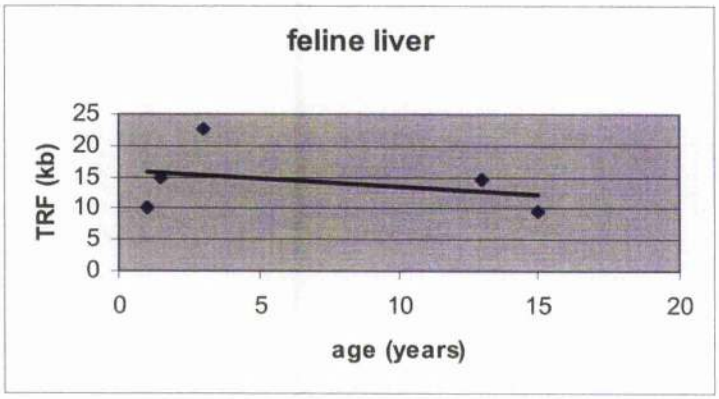
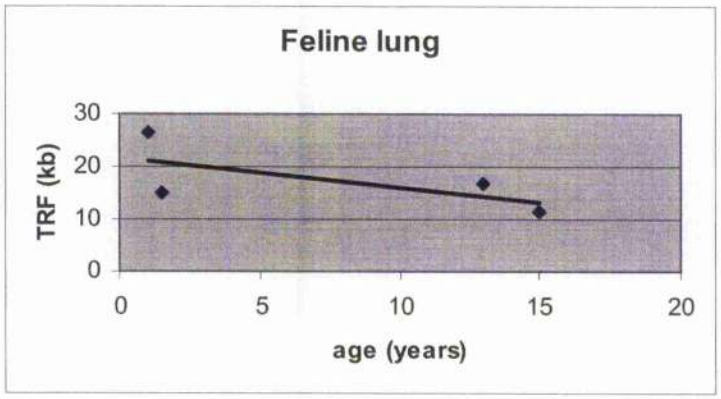


Figure 3-10. Regression plot of TRF length against age for feline necropsy specimens (1). Feline liver (range 9.2 to 22.7 kb), lung (range 11.5 to 26.3 kb) and skeletal muscle (range 11.3 to 19 kb) all show a trend of decreased TRF length with increased age of donor animal.





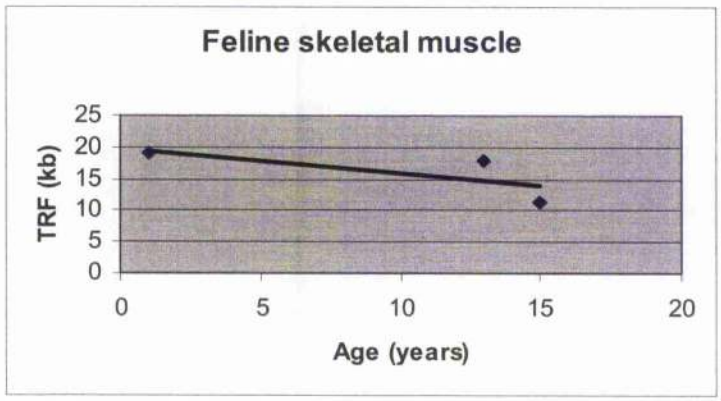
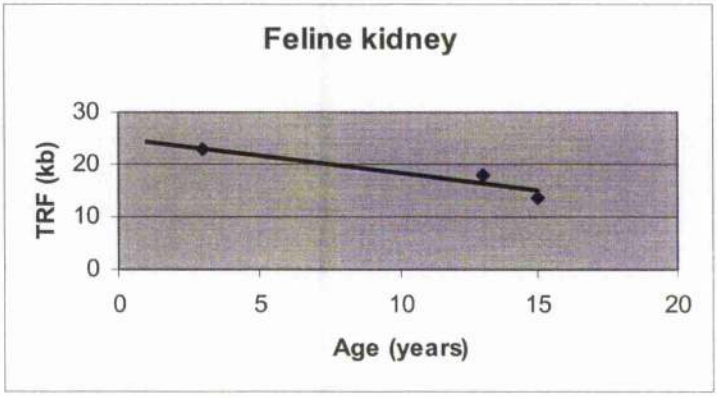


Figure 3-11. Regression plot of TRF against age for feline necropsy specimens (2). Feline kidney (range 13.7 to 22.8 kb) samples show a trend of decreasing TRF length with increased age of donor. Combining all feline organ samples (range 9.4 to 26.3 kb) reveals the same trend.



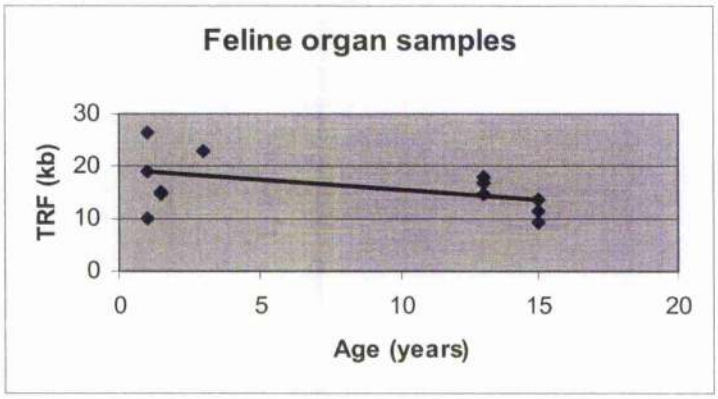


Table 3-12. TRF analysis of canine (a) and feline (b) tumours sampled during biopsy and at post-mortem examination. Matched normal tissue samples from each individual case were unavailable.

(a)

Number	Tumour type	TRF (kb)
1	Anaplastic sarcoma	18.3
2	Chondrosarcoma	17.6
3	Fibrosarcoma	20.9
4	Haemangiosarcoma	20
5	Leiomyosarcoma	14.7
6	Leiomyosarcoma	17.5
7	Leiomyosarcoma	19.4
8	Leiomyosarcoma	16.7
9	Liposarcoma	21.4
10	Lyphoma	19.1
11	Nephroblastoma	14
12	Neurofibrosarcoma	7.2
13	Rhabdomyosarcoma	15.5
14	Spindle cell tumour	22.2
15	Spindle cell tumour	19.5
16	Spindle cell tumour	12.8
17	Synovial cell sarcoma	22.2

(b)

Number	Tumour type	TRF (kb)
1	Mammary carcinoma	14.9
2	Squamous cell carcinoma	11.6
3	Squamous cell carcinoma	14.9
4	Squamous cell carcinoma	18.3

3.4.4 *In vitro* telomere studies

3.4.4.1 Growth of primary fibroblast cultures

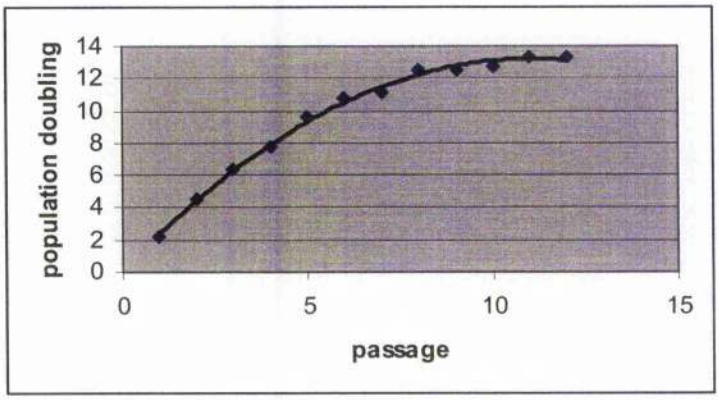
The canine primary fibroblast cultures AG08075, AG08157, AG07648, SFA and the feline primary fibroblast culture S22 (all detailed in Section 2.2.1.2) were maintained in culture until cell replication ceased. Growth curves were generated for each cell line and used to determine how many population doublings occurred in the cell population before replication arrest. This ranged from 4.7 to 16.6 population doublings, as shown in Figures 3-12 and 3-13. The AG08075, AG08157, AG07648 and SFA cultures were then maintained in the growth medium of the S22 culture, as it displayed the greatest replicative potential (16.6 PDs). The change of growth medium did not increase the replicative potential of the other cultures beyond that which had already been achieved.

3.4.4.2 TRF analysis of primary fibroblast cultures

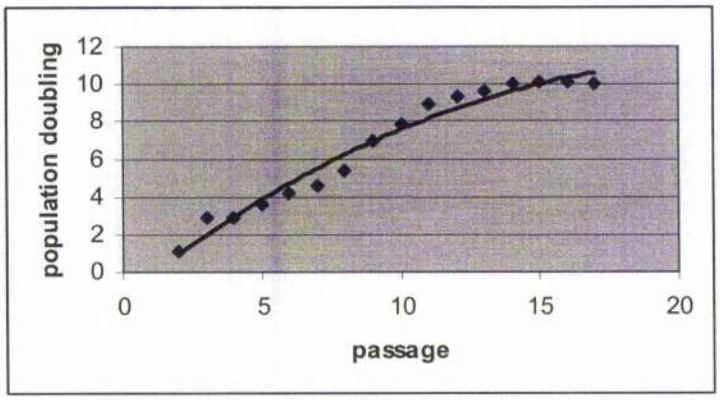
Telomere length analysis was carried out on selected passages of the SFA, S22 and AG07648 cell lines, as shown in Figures 3-14 and 3-15. The SFA canine primary fibroblast culture underwent a reduction in mean TRF from 20.5 kb at passage 2 to 17.5 kb at passage 13 during which the culture underwent 13.3 population doublings. This equates to a telomere loss of approximately 220 bp per cell division. The S22 feline fibroblast culture demonstrated telomere loss from 10.6 kb at passage 3 to 9.3 kb at passage 10, during which the cell line underwent 16.6 population doublings, in this case equating to approximately 80 bp per cell division. The AG07648 cell line also underwent telomere loss, from 11.0 kb at passage 2 to 9.7 kb at passage 15. This occurred over 10.1 population doublings, and thus equates to a telomere loss of approximately 130 bp per cell division. No correlation between age of donor animal and replicative potential of the *in vitro* culture derived from it could be identified.

Figure 3-12 Growth curves for canine primary fibroblast cultures SFA, AG07648, and AG08157. These cell lines underwent 13.3, 10.1 and 8.9 population doublings respectively before cell replication ceased.

SFA



AG07648



AG08157

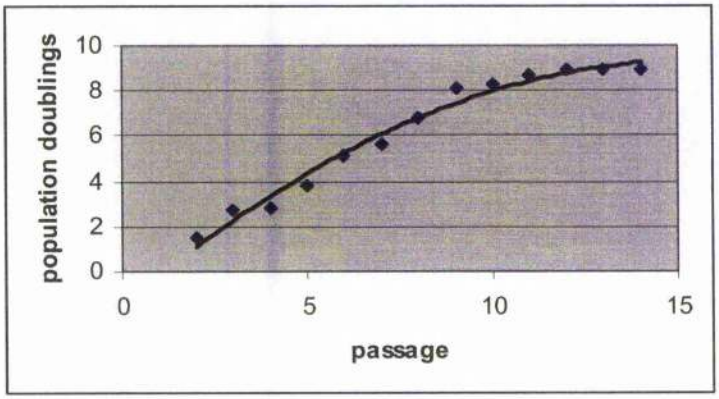
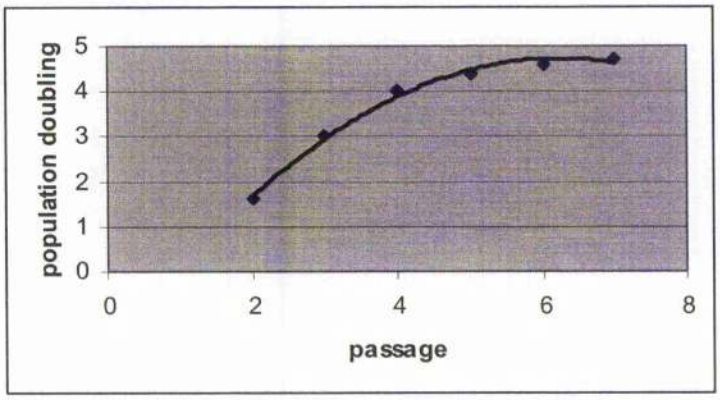


Figure 3-13. Growth curves for the canine primary fibroblast culture AG08075 and the feline primary fibroblast culture S22. These cell lines underwent 4.7 and 16.6 population doublings respectively before replication ceased.

AGO8075



S22

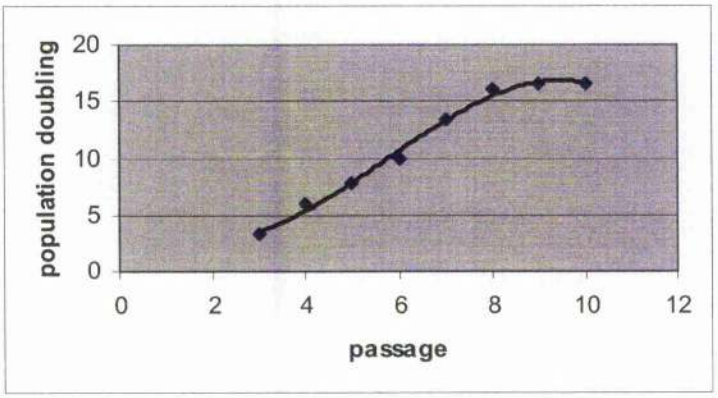


Figure 3-14. Autoradiographs demonstrating telomeric attrition in the SFA and AG07648 cell cultures. Lanes 1, 2, 3, 4, and 5 in the SFA autoradiograph represent passages 2, 4, 7, 10 and 12 respectively, and equate to telomere lengths of 20.5, 20.1, 19.7, 18.9, and 17.5 kb. The SFA cell culture ceased replicating at passage 13 after a total of 38 days in culture. Lanes 1 and 2 both represent passage 2 and lanes 3 and 4 passage 14 of the AG07648 cell culture and equate to telomere lengths of 11.0 and 9.7 kb respectively. The AG07648 culture was in culture a total of 98 days. Lane 5 is a standard TRF marker lane (DNA molecular weight marker II, Roche, UK).

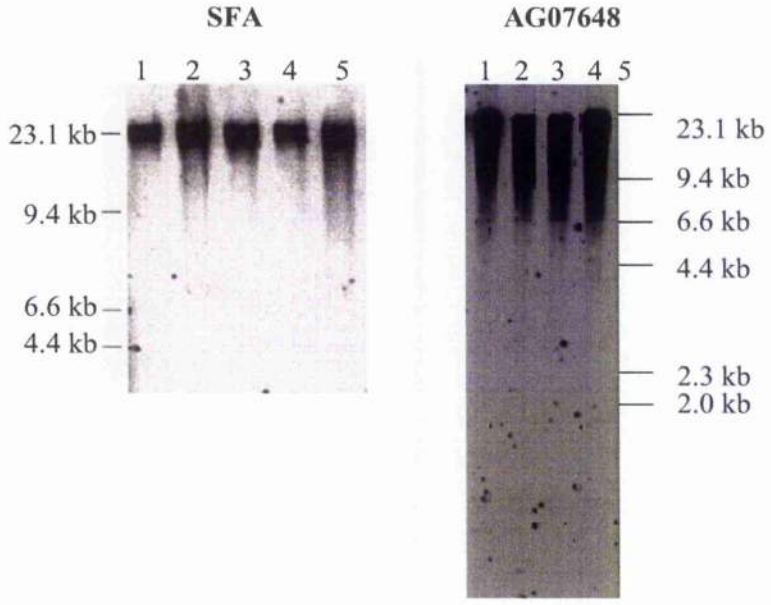
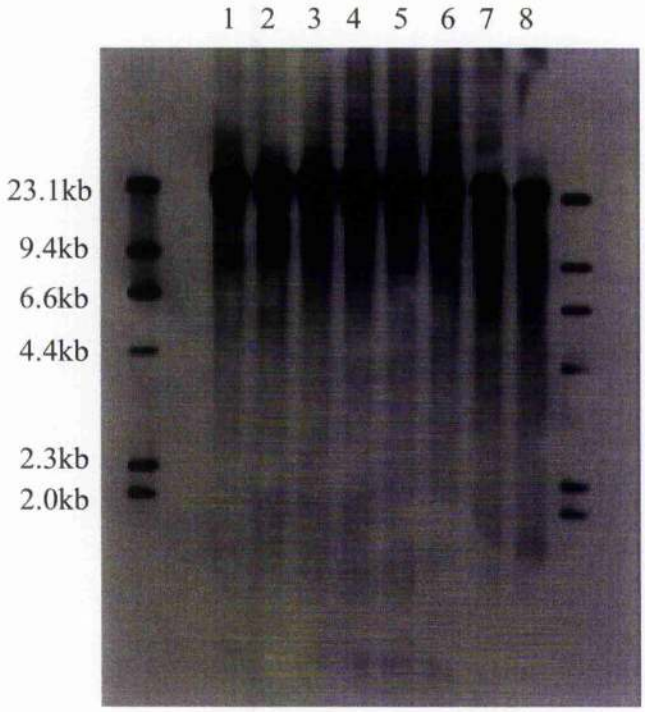


Figure 3-15 Autoradiograph demonstrating telomeric attrition in the S22 feline fibroblast culture. Cells were maintained in MEM Eagle medium (Sigma) supplemented with 10% heat inactivated FCS, penicillin-streptomycin and fungizone and passaged routinely upon reaching approximately 80% confluence. The cell line was initially seeded with 2 $x10^6$ cells, and reseeded after passage at a concentration of $1x10^5$ cells/ml. After 66 days in culture the cell line ceased replication and the majority of cells within it were shown to be senescent by SA-β-GAL staining. Autoradiograph lanes 1 and 2 show telomeric smears generated from passage 3 cells, 3 and 4 correspond to passage 5, 5 and 6 to passage 7 and lanes 7 and 8 to passage 9. These telomeric smears represent mean telomere lengths of 10.6, 10.7, 9.9 and 9.3 kb respectively.



3.4.4.3 Detection of senescence *in vitro*

SA-β-GAL staining was the main method by which the accumulation of senescent cells *in vitro* was recorded. Presence of a perinuclear blue dye indicates that a cell is senescent, and staining was found to be clear and easily interpreted in the cell lines used, as illustrated by Figure 3-16. Staining of the SFA primary culture was carried out at passage 2, 4, 10 and 12. For comparison purposes all other cell cultures were stained initially, again at passage 10 and when the culture had effectively stopped replicating. The S22 and AG08075 cell cultures did not grow beyond passage 10 and were therefore stained only twice. In addition, the replicatively immortal human cell line GM847 was stained as a negative control.

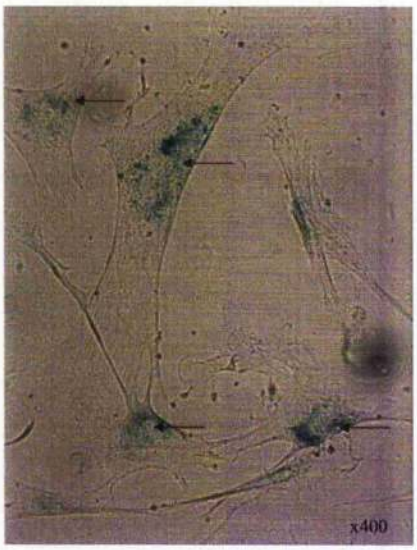
Initial staining, carried out as soon as possible after the cell lines were introduced to culture conditions revealed varying percentages of senescent cells. Both the SFA and S22 cultures contained less than 5% senescent cells, increasing to 20% at passage 4 in the SFA culture. The other cell cultures contained 14, 16 and 21% SA-β-GAL positive cells at passage 2 in the AG08157, AG08075 and AG07648 cultures respectively. The S22 and AG08075 lines were the first to cease replicating, and at passages 10 and 7 respectively both these cell lines contained >95% SA-β-GAL positive cells. At passage 10 AG08157, SFA and AG07648 were 43%, 80% and 65% SA-β-GAL positive, and these cell lines stopped replicating and reached >95% SA-β-GAL positive status at passages 14, 12 and 17 respectively. The GM847 cell line, as expected did not contain any SA-β-GAL positive cells.

Further confirmation of senescence in the primary fibroblast cultures was determined by ICC for $p16^{INK4a}$ and p21, as detailed in Section 3.3.4.2. Working dilutions of 1/50 were used for both the H-156 (p16) and the SX118 (p21) antibodies. ICC was carried out on the final passage of each culture tested, (p12 and p10 for SFA and S22 respectively). The test cells had been in a state of replicative senescence for 2 weeks prior to ICC, and had previously been shown to be SA-β-GAL positive, as described above. In both cell cultures, ICC for

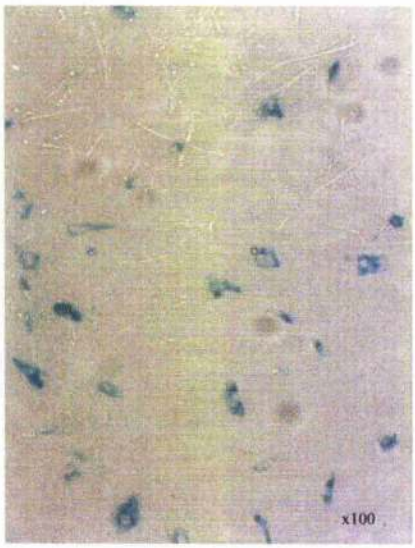
$p16^{INK4a}$ resulted in strong nuclear, and moderate cytoplasmic staining, indicating the accumulation of $p16^{INK4a}$ in the senescent cells. A representative image of this staining for the S22 culture is shown in Figure 3-17. In both cases the cultures were found to be negative for p21, as shown in Figure 3-18. These results follow the pattern noted by Alcorta *et al* in senescent human fibroblasts (Alcorta, Xiong, *et al* 1996).

Figure 3-16. Senescence associated β-galactosidase activity at pH 6. A stable perinuclear blue dye allows visual identification of cells that have entered replicative senescence, as indicated in image (a) by arrows. Five fields of at least 100 cells were counted, and cells positive for SA- β –GAL activity expressed as a percentage of the total number counted. Percentage positive varied greatly depending on the replicative age of the cell line, as shown in image (b), indicating the canine primary fibroblast cell line SFA at p4 with 20% of cells estimated to be SA-β-GAL positive and image (c), generated from the same cell line at p12, where 100% of the cell population appear to be SA- β –GAL positive.

(a)



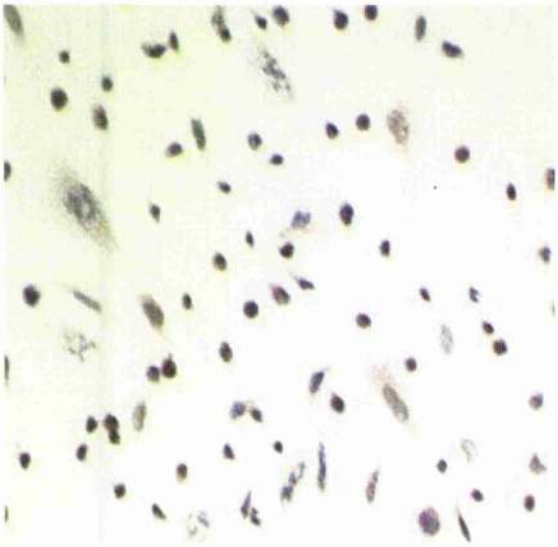
(b)



(c)

Figure 3-17. ICC for p16^{INK4a} in late passage S22 primary feline fibroblasts. Image (a) shows the presence of the brown insoluble DAB end product in passage 10 S22 feline fibroblasts using the H-156 anti-p16 primary antibody (Santa Cruz Biotech). This culture entered replicative arrest 2 weeks before staining was carried out, and staining was strongest in the nuclei of the senescent cells. Image (b) is taken from the same passage cells with omission of the primary (H-156) antibody. No uptake of stain is apparent, implying that the stain is binding specifically to the primary antibody. Counterstaining of nuclei used Gills haematoxylin. Both images are at 200x magnification.

(a)



(b)

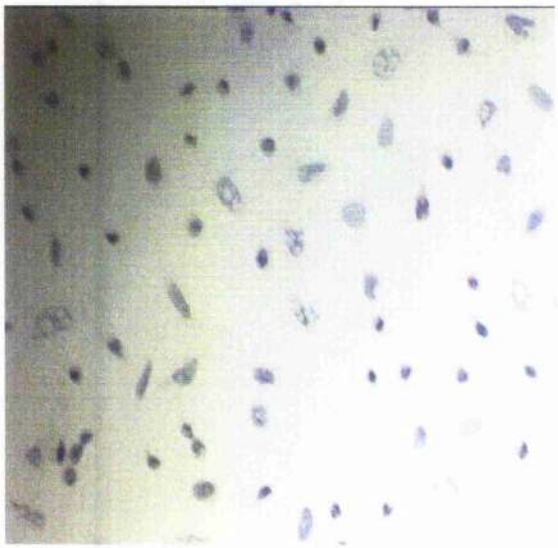
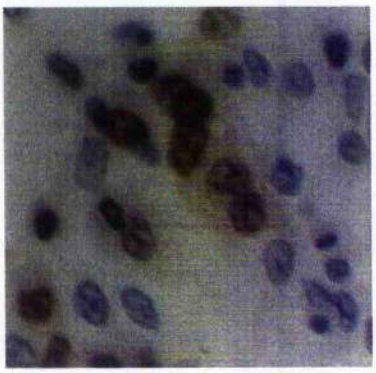


Figure 3-18. ICC detection of p21 in late passage canine and feline fibroblasts. Both the passage 12 canine fibroblasts from the SFA culture (a), and the passage 10 feline fibroblasts from the S22 culture (b) entered replicative senescence 2 weeks before staining. No DAB stain is visible in either culture, implying an absence of p21 in both cell cultures. Image (c) shows staining of the positive control cell line (MCF7), illustrating that the primary antibody (SX118, BD PharMingen) was working effectively. All images are at 400x magnification.

(a)

(b)

(c)



3.5 Discussion

The primary aims of this chapter were to investigate telomere lengths in the dog and cat and to determine what if any effect the variables of age, breed and gender have on telomeres in these two species. An analysis of 112 canine PBL DNA samples demonstrated that PBL telomeres range from 9.6 to 23.5 kb in the dog. Analysis of the same cell types from 30 DSH cats revealed a similar range, from 4.7 to 20.6 kb. Furthermore, this study found telomere lengths in a panel of 26 canine and 22 feline somatic tissues to range from 11.6 kb to 22.8 kb, and 8.5 kb to 26.3 kb respectively. These ranges are very similar to those typical of human telomeres (Blackburn 1990) (Harley 1995), and are in contrast with the much longer telomeres found in the mouse, which is a species commonly used to model human telomere biology (Kipling & Cook 1990).

3.5.1 Age effect on PBL telomere length in the dog and cat

This study has shown age to have a significant effect on telomere length in five pedigree dog breeds and the DSH cat, (p = 0.001 for both species). This age effect supports previous reports documenting the effect of age on telomere length in human PBLs (Rufer, Brummendorf, *et al* 1999), in canine mammary tissue (Yazawa, Okuda, *et al* 2001) and feline blood cells (Brummendorf, Mak, *et al* 2002).The rate of telomere loss in human PBLs is not linear throughout life, instead telomere loss appears accelerated in infancy. In granulocytes this corresponds to 3052 bp/year for the first 6 months of life, compared with an average of 39 bp/year over a human lifespan (Rufer, Brummendorf, *et al* 1999). Such a pattern is not apparent for the dog and cat PBLs investigated in this study; however the priority in this investigation was to encompass a broad age range of animals, and particularly in the cat this has resulted in the majority of the samples being from either old or young individuals. The use of animals from the tails of the age distribution is a powerful tool to investigate the relationship between age and telomere length as this experimental design emphasizes the

contribution of age to the variation in telomere length. The results indicate that old dogs and cats have shorter telomeres than young individuals. This implies that telomere length reduces with increasing age; however further work will be required to reveal whether this relationship is linear or more complex.

3.5.2 Breed effect on telomere length in the dog

In addition, this study has considered the effect of breed on canine telomere length. We have shown breed to have a significant effect in the pedigree canine PBL sample population ($p = <0.0001$). This is interesting as it correlates with findings in human research that have determined telomere length to be a heritable trait (Slagboom, Droog, *et al* 1994) (Jeanclos, Schork, *et al* 2000), and also because breed has an effect on lifespan in the dog. An age corrected analysis of the breeds examined revealed the Great Dane population to have the shortest mean TRFs, and the Beagle breed to have the longest. Of the five pedigree breeds examined the Great Dane also has the shortest life expectancy (Maynagh 1983) (Michell 1999). The same sources of breed specific data also credit the Beagle breed with the longest life expectancy (Maynagh 1983) (Michell 1999).

These correlations between breed specific telomere length and life expectancy provide evidence of a link between telomere length and ageing in the dog, albeit circumstantial evidence from a relatively small population, but nevertheless adding to the growing list of such evidence linking telomere dynamics with the ageing process (Rohme 1981) (Oshima, Campisi, *et al* 1995) (Benetos, Okuda, *et al* 2001). In addition these data provide an area for further investigation in canine telomere studies. It would be interesting to investigate if selective breeding such pedigrees on the basis of longer than average telomeres in the parental stock would result in an increased life expectancy in the progeny. It is realised such a study would require careful selection of candidates to avoid interference with existing breeding programmes aimed at reducing the prevalence of inherited disease.

3.5.3 Gender effect on telomere length in the dog and cat

Gender did not have a significant effect on telomere length in the sample population for which gender data was available. This is interesting as a gender effect on telomere length has been identified in humans (Benetos, Okuda, *et al* 2001), and more recently rats (Cherif, Tarry, *et al* 2003). In both these studies, males were found to have shorter telomeres than females in an age corrected analysis. This provides a novel mechanism for the gender related differences in lifespan that are known to exist in both humans and rats; in both species females have a greater life expectancy (Cherif, Tarry, *et al* 2003). The results of studies on the effect of gender on canine lifespan are complicated by factors such as entire versus neutered, and natural death versus euthanasia within the sample population. Michell found entire female dogs to have the greatest natural life expectancy in a panel of over 3000 individuals (Michell 1999), and whilst Moore *et al* found castrated male dogs to have the greatest life expectancy in a different study, this sample population of 927 military animals did not include any entire females (Moore, Burkman, *et al* 2001). Thus the effect of gender on canine lifespan is still unclear, and therefore the lack of gender effect on telomere length in the canine samples in this study does not add or detract from a possible link between telomere length and life expectancy in the dog.

The author is unaware of any research on the effect of gender on life expectancy in the DSH cat, and so the lack of relationship between telomere length and gender in the DSH population cannot be correlated with life expectancy at present.

3.5.4 Telomere length analysis of normal canine and feline organ samples

Necropsy sample analysis showed a trend of TRF length reduction with increased donor age in canine liver, kidney and small intestine, and all the feline samples for which a comparison was possible, including liver, lung, skeletal muscle and kidney. Tissue renewal times are not available for canine and feline

tissues, however these results correlate with findings in the adult, non-growing mouse where only neurones and myocardium were found to be static with respect to cell turnover (Cameron 1970). Other tissues would be expected to undergo a decline in telomere length proportional to their renewal rates. Human liver and renal cortex also show a trend of decreased telomere length with increased donor age (Takubo, Izumiyama-Shimomura, *et al* 2002), as does gastric mucosa, small and large intestinal mucosa and the spleen (Furugori, Hirayama, *et al* 2000) (Hiyama, Hiyama, *et al* 1996) (Takubo, Izumiyama-Shimomura, *et al* 2002). However, given the small sample size the experiment primarily provides an insight into the average size of telomeres in these organs in the dog and cat, as the trends noted may simply be a reflection of natural variation in the telomere lengths of the individuals concerned. This is clearly illustrated in the canine gastric mucosal samples. If tissue renewal times are similar in the dog and the mouse a greater telomeric loss would be expected in gastric mucosa than any of the other tissues examined, yet no such trend is displayed. This may simply be a reflection of individual variation and the small sample size ($n = 3$). However, it is also possible that the unusually high turnover of gastric mucosa may be key to explaining the lack of appreciable telomere loss with age. Telomerase activity has been detected in gastrointestinal mucosa in humans and in dogs (Section 4.4.1) (Bachor, Bachor, *et al* 1999), and so it is possible that transient telomerase activity in canine gastric mucosa counteracts the effect of end replication problem brought on by high cell turnover. This is also likely to be the case in human gastric mucosal samples, as despite what must be a higher cell turnover in this tissue, telomere loss is not significantly greater in gastric mucosa than any other human tissues that have been examined (Takubo, Izumiyama-Shimomura, *et al* 2002).

3.5.5 Telomere length analysis of canine and feline tumours

Telomere lengths in a panel of canine and feline tumours were also examined to determine if a quantifiable reduction in telomere length of the cancerous tissue had occurred before transformation. Unfortunately matched normal tissue

samples from the same individual were not available for comparison, and so it is not possible to gauge how many cell divisions were undergone in each case before transformation occurred. It is only possible to state that the tumours examined did not show any significant difference in telomere length to normal canine and feline organ or PBL samples.

Recent work has identified short PBL telomeres as a risk factor for the development of a number of diseases in humans, including cancer and myocardial infarction (Wu, Amos, *et al* 2003) (Reviewed in Wong & dePinho, 2003). This raises the possibility that assessment of telomere length in PBLs may provide a non-invasive aid to diagnosis of a range of diseases (Reviewed in Wong & dePinho, 2003). Furthermore, this research in the human field raises the possibility that a genetic predisposition to short telomeres may predispose a canine breed to, for example, cancer development. Whilst the situation *in vivo* will be more complex than this simple relationship it would be interesting to discover whether this single factor is important by determining whether a breed with an increased cancer risk also tends to display shorter than average PBL telomeres. If such an association were discovered, this would have great potential for use in evaluating disease risk in individuals, or aid non-invasive diagnosis.

3.5.6 *In vitro* telomere studies

The *in vitro* study carried out using canine and feline primary fibroblast cultures revealed these cultures to have much less replicative potential than their human counterparts. Normal human primary fibroblast cultures will enter replicative senescence after approximately 50 population doublings (Harley 1995), however the greatest replicative potential of any of the canine or feline primary cultures used was only 16.6 population doublings. Replicative potential of canine and feline primary fibroblast cell cultures has not been investigated before and it is possible the reduced proliferative potential of the cell lines studied in this project represent a genuine species difference between companion animals and humans. Rohme *et al* (Rohme 1981) demonstrated a correlation between species lifespan

and replicative potential of fibroblasts in a study that included mammalian species with a wide range of life expectancy; those findings indicate that the shorter the lifespan of the species the poorer the replicative potential of the fibroblast cell line derived from it. These data fit well with our findings, and provide an explanation for the comparatively poor replication of our canine and feline primary cultures.

Furthermore, no clear correlation between age of donor and replicative potential of cell line could be demonstrated in this study. Although the cell line with the greatest replicative potential was derived from an eight-week-old feline, a cell line derived from a six-week-old canine had less replicative potential than one derived from a thirteen year old dog. Whilst studies have presented evidence that a negative correlation between age and replicative potential does exist (Bruce, Deamond, *et al* 1986) (Cristofalo & Pignolo 1993), a more recent evaluation has not been able to repeat the findings (Cristofalo, Allen, *et al* 1998). It must also be borne in mind that the *in vitro* environment is a crude simulacrum of the situation encountered by the cell *in vivo*; even the best regulation of culture conditions and the constituents of growth media lag far behind physiological regulation in the living organism. It is therefore possible that the variation in replicative potential of the primary fibroblast cultures may be in part the product of sub-optimal conditions, or 'culture shock' (Sherr & dePinho 2000).

In addition, it is possible that the initial telomere length of the specific cell from which a culture is derived is the more important factor in determining its proliferative potential (Allsopp, Vaziri, *et al* 1992), however applying these criteria did not reveal any insight into the varying potentials of the cell lines studied. The S22 cell line initially contained mean TRFs of 10.6 kb, and although these were eclipsed by the mean TRF of the SFA cell line at the first passage (20.5 kb), the SFA cell line underwent 3.3 fewer population doublings before senescence. It is possible that inclusion of a larger number of cell lines in the experiment might reveal a relationship between initial mean telomere length of a culture and its replicative potential, however far fewer canine and feline primary fibroblast cell lines are available than human equivalents, and make such an analysis beyond the scope of this project.

All primary cell cultures demonstrated SA-β-GAL staining that increased with passage number and approached 100% once replicative exhaustion had been reached. Furthermore, >95% positive SA-β-GAL staining was shown to correlate with an accumulation of $p16^{INK4a}$ in primary canine and feline fibroblast cultures, thus confirming that senescence was the cause of the replicative arrest rather than quiescence (Dimri, Lee, *et al* 1995) (Alcorta, Xiong, *et al* 1996) (Huschtscha & Reddel 1999).

Telomere attrition was detected *in vitro* in the SFA, S22 and AG07648 cell lines despite the small number of population doublings before senescence. At 220, 80 and 130 bp per cell division respectively, only the SFA cell line demonstrated telomere loss greater than that usually detected in human cells *in vitro* (estimated at 30-200), and only by a small margin (Harley 1995). It is also likely that these values overestimate the rate at which telomeric attrition would occur under normal circumstances *in vivo*, as this has been shown to be the case in human tissues where a realistic estimate is closer to 10-50 bp per cell division (Harley 1995).

The choice of TRF analysis for these telomere length studies was governed by the fact that this is still widely regarded as the method of choice (Saldanha, Andrews, *et al* 2003), and also by the practical consideration of available equipment and expertise. However, the TRF method is not without its critics, and the chief query regarding the accuracy of the technique lies in the inclusion of sub-telomeric repeats in the analysis, up to the first restriction enzyme cutting site in the target DNA. Typically comments on the subject refer to a study carried out by Hultdin *et al* that estimated this sub-telomeric DNA component to average 3.2 kb in a number of human cell lines and cell suspensions by comparing a Southern Blot analysis with a fluorescence *in situ* hybridisation and flow cytometry ($Q\text{-}FISH^{FCM}$) protocol developed for the purpose (Hultdin, Gronlund, *et al* 1998). Given these data it must be conceded that the telomere length values presented in these experiments will encompass a small proportion of sub-telomeric DNA. However, the same study found that results gained by Q-

FISH^{FCM} correlated significantly with those gained by Southern blotting. This is evidence that the sub telomeric DNA content was consistent given the same experimental conditions across the whole sample population, and therefore does not exclude the use of TRF analysis for the accurate investigation of telomere length changes.

Without comparison of TRF analysis with telomere length estimation methods beyond the scope of this project it is impossible to state the actual amount of sub-telomeric DNA included in the canine and feline telomere length results documented, however human sub telomeric DNA has been found to vary between 2.5 and 4 kb in a review carried out by de Lange (de Lange 2003).

The TRF analysis method has been found to give reproducible results that meet the demands of the project, however it is clear that the labour intensive nature of the technique make it less suitable for larger scale applications. From receiving a tissue, blood or cell pellet sample to getting a TRF value takes three to four days, and the technique is prone to complications such as uneven transfer of DNA to the membrane, uneven probe and chemiluminescent substrate distribution, and high, uneven or mottled background all of which can interfere with analysis of the final blot and result in exclusion of a blot from the study. Even with experience of the technique and due attention paid to optimisation, the multi-step nature of the Southern Blot and chemiluminescent detection will inevitably result in loss of a number of blots to these complications. Whilst an investigation of the newer techniques for telomere length analysis has not been carried out in our laboratory it is envisioned that if techniques such as the hybridisation protection assay (Section 3.2.1.1) compare favourably with Southern Blot in terms of sensitivity and repeatability then the TRF method will not remain in common use for large scale telomere length analysis.

The decision to examine mean telomere length rather than investigate telomere length on individual chromosomes was again based on what was practically achievable within the scope of the project, however it is noted that although mean telomere length decreases as the canine and feline cells investigated divide it is likely, given research carried out in the mouse that the trigger for entering

senescence is the shortest telomere within a particular cell, rather than the average length of all the telomeres (Hemann, Strong, *et al* 2001).

3.6 Summary

The experiments described in this Chapter have determined the range of telomere lengths that are found in both canine and feline PBLs and normal, healthy organ samples taken from a wide spectrum of ages using a standard TRF method. A TRF analysis of PBL samples taken from 112 dogs of various breeds, and 30 DSH cats found TRFs to range from 4.7 to 20.6 kb, and 9.6 to 23.5 kb respectively. PBL telomere lengths in both the pedigree dog and DSH cat have been shown to decrease significantly with increased age, and whilst gender did not have a significant effect in either species, an intriguing finding was that breed of pedigree dog had a significant effect on telomere length. An age corrected analysis revealed that the shortest mean PBL TRFs in the sample population were sourced from the breed with the shortest life expectancy. Telomere lengths in a panel of canine and feline tumours were not found to be significantly different from telomere lengths of PBL or healthy organ origin. *In vitro* telomere length studies determined that the replicative potential of four canine primary fibroblast cultures was an average of 10 population doublings, whilst a feline primary fibroblast culture had a replicative potential of 16 population doublings. Once replicative potential had been exhausted, the cells adopted an enlarged and rounded appearance typical of senescence, were positive for SA-β-GAL activity and $p16^{INK4a}$. Telomere attrition was demonstrated *in vitro* in primary fibroblast cultures of canine and feline origin, and averaged 175 and 130 bp/cell division respectively. The *in vivo* and *in vitro* datasets have demonstrated that telomeric attrition occurs in both the dog and cat; that the telomere lengths of companion animals are similar to humans, and the data supports the idea that the telomere may be involved in companion animal ageing.

Chapter IV

In vivo and *in vitro* canine and feline telomerase studies

4.1 Abstract

Activity of the ribonucleoprotein telomerase is absent from the majority of normal human somatic tissues, however telomerase activity has been detected in approximately 80% of human malignancies. These findings have established a clear link between telomerase activity and cancer in humans. However, very little research has been carried out on the biology of telomerase in companion animal species, and the experiments described in this Chapter aim to address this lack of information. First, an investigation was carried out to determine if telomerase is active in normal canine and feline somatic tissues. Second, an investigation of telomerase activity in a panel of canine and feline tumour samples, and immortalised canine cell lines was undertaken to determine if telomerase activity in dogs and cats is associated with immortalisation and malignancy. Further to this work, a study on the potential use of a reverse transcriptase inhibitor to inhibit telomerase activity in two immortalised canine cell lines is described. Finally, an attempt was made to transfect the human telomerase reverse transcriptase sequence into primary canine and feline fibroblasts to reconstitute telomerase activity in those cells. This was undertaken in a bid to extend the replicative lifespan of the fibroblasts, and thereby provide direct evidence of a link between telomeric attrition and replicative senescence in the dog and cat.

4.2 Introduction

The ribonucleoprotein telomerase has been a major focus for research since its telomere specific DNA polymerase activity, initially identified in *Tetrahymena* was confirmed to have a specific association with immortal cells and cancer (Greider & Blackburn) (Kim, Piatyszek, *et al* 1994). This initial study led to a large volume of research aimed at determining exactly where this polymerase activity could be found in normal, pre-malignant and malignant tissues (for review see Dhaene, Van Marck, *et al* 2000). The conclusion of this large volume of work was, that with the exception of germ cells and the stem cells of renewable tissues, telomerase is not readily detectable in normal human tissue. In contrast telomerase activity is found in the vast majority of human cancers (Shay & Bacchetti 1997) and this has led to the identification of telomerase as a promising molecular target for therapeutic intervention in the field of oncology. At present this is one of three main applications that have been identified with respect to telomerase, the other two being cancer diagnosis and prognosis, and tissue engineering (Kelland 2001).

4.2.1 Telomerase and cancer

The six hallmarks of cancer are described as being a self-sufficiency in growth signals, insensitivity to antigrowth signals, avoidance of programmed cell death, sustained angiogenesis, invasion and metastasis and finally, limitless replicative potential (Hanahan & Weinberg 2000). The trait of limitless replicative potential, acquired by all cancer cells, is dependant in the majority of cases on telomerase and for this reason it has become a key player in cancer research today. In fact, less than 20 years after its discovery, the ectopic expression of its catalytic subunit is one of only three conditions required to successfully produce direct tumorigenic conversion of normal human epithelial and fibroblast cells (Hahn, Counter, *et al* 1999).

The strategies for targeting telomerase have been outlined elsewhere (Section 1.8.2), and the significant advances that have been made in the field appear to at

least partly justify the initial optimism with which researchers approached the subject. A number of companies involved in drug discovery have made significant investments into targeting telomerase for new cancer drugs. Geron Corporation has produced an oligonucleotide telomerase inhibitor (GRN163) that targets the template region of hTERT, and has been shown to inhibit telomerase activity and has passed initial safety tests. Geron is also in the process of developing a telomerase vaccine with Phase I clinical trials already underway (Goldman 2003). Isis pharmaceuticals, a drug discovery company that focuses entirely on RNA targeting have a number of oligonucleotide based anti-telomerase therapies in various stages of development, several of which have reached Phase II clinical trials (Corey 2002). In addition, telomerase positive cancer cells have been targeted using an adenovirus vector containing bacterial nitroreductase under the control of the hTR and hTERT promoters. Bacterial nitroreductase bioactivates the prodrug CB1954 into an active cytotoxic alkylating agent, and this approach has produced favourable results in both telomerase positive cell lines and *in vivo* (Bilsland, Anderson, *et al* 2003).

One of the major criticisms levelled at anti-telomerase therapies is termed the lag effect, meaning the time required after telomerase inhibition for natural telomere attrition to lead to senescence or apoptosis. For instance, one of the earliest strategies employed an antisense vector directed against hTR in the cervical cancer cell line HeLa; this did result in telomeric attrition and crisis (M2) however the strategy required up to 26 population doublings in the transfected cells to be effective. *In vivo* such a degree of tumour growth would almost certainly result in death of the patient before destruction of the transformed cells (Feng, Funk, *et al* 1995).

These data imply that it will be necessary to assess telomere length in individual cancers before the decision to use antitelomerase therapy can be made. Whilst this is certainly a drawback, a survey of telomeres across 60 human tumour cell lines in the US has revealed mean telomere length to be only 5 kb (Chu, Piatyszek, *et al* 2000), implying that a large proportion of human telomerase positive tumours would lend themselves to such therapy. In addition it has been found that functional p53 is not required for telomere driven apoptosis to occur,

thus allowing telomerase therapy to be effective in a much broader range of cancers, given that p53 function is known to be abrogated in a wide range of human malignancies (Zhang, Mar, *et al* 1999).

A second major concern over telomerase as a target for cancer therapy is the potential that the selection pressures such therapy would cause could result in the emergence of an ALT competent phenotype that would be resistant to the treatment. This may well be a problem in individual cases, however a number of studies already carried out have found no evidence that such a switch from telomerase to ALT under the selection pressure of antitelomerase therapy occurs (Herbert, Pitts, *et al* 1999) (Zhang, Mar, *et al* 1999). Furthermore, telomerase inhibitors that target the G-rich single strand overhang at the end of the telomere (G-quadruplex interacting compounds) may be less susceptible to ALT as a resistance mechanism as they target the substrate of telomerase, and do not interfere with the enzyme itself. An example of such a compound is telomestatin, which has shown favourable results *in vitro* using a number of human leukaemia cell lines (Tauchi, Kazuo, *et al* 2003). An important feature of this compound is that it appears to act at least partly by inducing telomere dysfunction rather than shortening, and therefore does not suffer a 'lag effect' in its mode of action (Tauchi, Kazuo, *et al* 2003). This may make it ideal choice to combine with other types of telomerase inhibitor, which may be potent but not immediate in their effect, for an overall improved efficacy.

4.2.2 Telomerase as a tumour marker and prognostic indicator

It is not surprising that the greatest part of research that has been carried out in this area has focussed on humans and the traditional model species, the mouse. However, a number of investigators have begun to examine the extent of telomerase activity in the tissues of the dog and cat, and this research has already uncovered interesting parallels between human, canine and feline telomerase biology (Nasir, Devlin, *et al* 2001) (Argyle & Nasir 2003) (Biller, Kitchell, *et al* 1998) (McKevitt, Nasir, *et al* 2002). Yazawa *et al* detected telomerase activity in

26/27 examples of mammary carcinoma in the dog, compared to telomerase activity in 4/12 examples of normal canine mammary tissue (Yazawa, Okuda, *et al* 2001). Funakoshi *et al* also investigated the telomerase activity of canine mammary tumours, and found telomerase activity in all the adenomas, benign mixed tumours and adenocarcinomas examined. In contrast to the findings of Yazawa, Funakoshi also found telomerase activity to be entirely absent from normal and hyperplastic canine mammary tissue, though interestingly telomerase activity was also absent from two malignant mixed tumours (Funakoshi, Nakayama, *et al* 2000). This is evidence that an ALT pathway may be present in a subset of canine cancers. In addition there was a relatively greater level of telomerase activity in the adenocarcinomas than any of the other positive tissues, raising the possibility that relative telomerase activity (RTA) could be used as a prognostic indicator. These findings are also reflected in the human literature, were telomerase is considered a useful marker for the detection of cancer cells, and in cancers were telomerase becomes up regulated during tumour progression telomerase is considered a useful prognostic indicator (reviewed in Hiyama & Hiyama 2003).

Telomerase activity is considered to be absent from normal human somatic tissues (Forsyth, Wright *et al* 2002), and this has been linked to the fact that the majority of mammalian somatic tissues do not require great replicative potential, and so telomerase activity has been tightly down regulated as an anti-cancer mechanism. However Leri *et al* have detected telomerase activity in a large proportion of canine cardiac myocytes (up to 20%) (Leri, Barlucchi, *et al* 2001). The animals included in the study were all suffering from a form of dilated cardiomyopathy, and these data alone raise an interesting question mark over the simple association between telomerase activity in somatic tissue and malignancy (Kim, Piatyszek, *et al* 1994). These data also imply that canine myocytes from non-growing adults are capable of division, as even in cells that are telomerase competent telomerase activity appears to be tightly correlated with entry into the cell cycle and replication (Chadeneau, Siegal, *et al* 1995).

In this context the intriguing findings of Leri *et al* (Leri, Barlucchi, *et al* 2001) underscore the importance of considering health screening of samples for

telomerase activity assays and make it clear that a simple association between telomerase activity, immortalisation and malignancy is by no means all encompassing. Indeed, the discovery of telomerase activity in the heart has been a substantial addition to the evidence calling into question the dogma that the heart is one of the two major organs in the body that are unable to undergo physiological repair (the other being the brain), due to the inability of myocytes and neurones to divide (Kajstura, Leri, *et al* 1998) (Horner & Gage 2000).

A number of studies have also investigated telomerase activity in the tissues of the cat. Cadile *et al* (Cadile, Kitchell, *et al* 2001) examined the potential of using telomerase activity as a marker for malignancy in feline tissues. The results are encouraging; 1/22 benign samples and 29/31 malignant samples were positive for telomerase activity, translating to a sensitivity and specificity for cancer detection of 94 and 95% respectively. In addition telomerase activity has been detected in normal feline lenses, and a number of feline immortalised cell lines (Colitz, Davidson, *et al* 1999) (Muleya, Nakaichi, *et al* 1998).

4.2.3 Telomerase therapy

Not all potential therapeutic interventions involving telomerase are concerned with switching off the enzyme. The ability of telomerase to extend the proliferative life of a cell population has lead to the possibility of using telomerase to intervene in disease states where the pathogenesis includes failure of cellular proliferation. A potential application for this approach is in the treatment of liver cirrhosis; a common disorder, which, left unchecked, results in end-stage liver failure and ultimately death (Williams & Iredale 1998). A major component of liver cirrhosis is the failure of hepatocyte proliferation, and it is likely that telomere attrition has a large part to play in this because the sustained hepatocyte turnover associated with liver disease results in accelerated telomere loss compared with a healthy organ (Miura, Horikawa, *et al* 1997). Rudolph *et al* have shown that adenoviral delivery of mTR inhibited the development of experimental liver cirrhosis in $mTR^{-/-}$ mice by enabling hepatocytes to overcome the proliferative block imposed by short telomeres (Rudolph, Chang, *et al* 2000).

The telomeres of human cirrhotic patients have been shown to be short enough to produce a proliferation check, and so it is feasible that a variation of this technique could be used to treat liver cirrhosis in humans, and that such an approach could be extended to the veterinary field (Rudolph, Chang, *et al* 2000).

Another area where therapeutic use of telomerase activity has already shown great promise is in bone regeneration and repair. A new and very effective treatment for large bone defects beyond the capacity of normal healing is the use of bone marrow stromal stem cells (BMSSCs). These are defined as pluripotent progenitor cells with the ability to differentiate into osteoblasts, chondrocytes, adipocytes, muscle and neural cells (Caplan & Bruder 2001). These cells must be expanded *ex vivo* to produce the large numbers necessary to aid bone repair upon cell transplantation to the site of a defect. The major restriction to the application of the technique is the limited replicative potential of BMSSCs in cell culture, coupled with a gradual loss of osteogenic potential (Bianco, Riminucci, *et al* 2001). Shi *et al* have demonstrated that ectopic expression of hTERT in these cells extended their lifespan and maintained their osteogenic potential (Shi, Gronthos, *et al* 2002). Again, clear potential exists to exploit this application of telomerase in both the medical and veterinary medical fields.

A major concern over the use of telomerase for therapeutic intervention is the potentially increased risk of malignant transformation in the target tissue (Kim, Piatyszek, *et al* 1994). In the examples described here, it is envisioned that the risk of developing cancer secondary to telomerase therapy will be slight. For example, when considering telomerase for the treatment of human liver cirrhosis it is likely that any telomerase therapy will be used only to prolong the life of a patient until such time as a liver transplant becomes possible, therefore minimising the long-term risk of exposure to the initial therapy. Furthermore, Shi *et al* have been able to show that the bone generated by their telomerase expressing stem cells is of a normal architecture (Shi, Gronthos, *et al* 2002). This is an important finding as lack of normal architecture and failure to differentiate would be indicative of genetic instability and therefore risk of malignant transformation. However, not all findings in this area have been so encouraging. Recent work carried out by Mondello *et al* found that ectopic expression of

hTERT resulted in karyotype instability and anchorage independent growth in human fibroblast cell lines (Mondello, Chiesa, *et al* 2003). These results indicate that a case-by-case approach will be necessary when assessing the safety of telomerase therapy in the future, and that such therapy might not be entirely without risk.

All these studies based on the analysis of telomerase activity have utilised a protocol originally designed by Kim *et al* termed the Telomeric Repeat Amplification Protocol (TRAP) (Kim, Piatyszek, *et al* 1994). Initially this technique was qualitative only, utilising an oligonucleotide primer as a substrate for telomerase. If the telomerase enzyme is active in a sample then telomeric repeats are synthesised and added to the 3′ end of the primer. These extension fragments are subsequently amplified by PCR and may be detected by polyacrylamide gel electrophoresis. The assay is sensitive enough to detect one telomerase positive cell in 10,000 negatives. The original technique has been modified and updated to improve sensitivity and quantification (Kim & Wu 1997), and Roche molecular biochemicals have produced a variant that includes an enzyme linked immunosorbent assay (ELISA) to allow semi-quantification of telomerase activity and more accurate comparison between samples.

Whether the TRAP assay can be used as the basis for clinical applications of telomerase testing is still debatable. Tseng *et al* determined the TRAP assay to be 100% sensitive and 90% specific in relation to the detection of malignant ascites secondary to ovarian cancer in a series of 97 cases. These results compared favourably to the 96% sensitivity and 100% specificity of cytology, and led the authors to recommend telomerase activity testing as an adjuvant to cytopathological methods (Tseng, Jain, *et al* 2001). Braunschweig *et al* carried out a similar test of the assay as a marker of malignancy in cytological effusions and in a total of 291 cases determined the TRAP assay to perform relatively poorly; only 76% sensitive and 82-91% specific (Braunschweig, Guilleret, *et al* 2001) when compared to routine histopathological diagnosis. The TRAP assay is vulnerable to false negatives caused by inadequate handling and storage of material for the assay. To ensure integrity of the telomerase RNA subunit it has

been proposed that samples should be snap frozen in liquid nitrogen and stored at -80°C within 20 minutes of collection, as the assay relies on the presence of functionally intact RNA (Cadile, Kitchell, *et al* 2001).

Telomerase is not an oncogene (Harley 2002), and therefore coupling a telomerase activity assay with other markers of abnormal behaviour in a tissue may be necessary to reduce the potential for false positives when associating telomerase activity with malignancy. Work carried out by Chu *et al* (Chu, Lin, *et al* 2001) on canine transmissible venereal tumour (CTVT) is a good example of why such a strategy may be necessary. CTVT occurs in two phases, a progressive growth phase (P-phase) followed by spontaneous regression (R-Phase). Telomerase activity is present in both phases of the disease, but is higher in P-phase tissue samples. These samples also have a greater mitotic index. However, despite the fact that P-phase tissue has high telomerase activity and an increased mitotic index, it inevitably regresses to the R-phase. Therefore whilst telomerase activity is broadly acceptable as a marker of malignancy it will be necessary to validate results in a case-by-case approach before the results of telomerase testing are used as a basis for making clinical decisions (Chu, Lin, *et al* 2001).

4.2.4 Chapter aims

4.2.4.1 To investigate telomerase activity in normal canine and feline somatic tissue and tumour samples

As described, to date only limited information is available on telomerase activity in the tissues of the dog and cat. In light of this, an investigation of telomerase activity was conducted encompassing a wide variety of tissue samples taken from healthy organs of the dog and cat. In addition telomerase activity was assessed in a panel of canine and feline tumour samples to ascertain if the widespread dependence on telomerase reactivation found in human tumours was mirrored in the dog and cat, such that telomerase status may be of diagnostic/prognostic significance in companion animals.

4.2.4.2 To assess if telomerase inhibition could be achieved using a potential telomerase inhibitor *in vitro*

Some of the earliest anti-telomerase strategies tested in human immortalised cell lines involved the use of reverse transcriptase inhibitors (RTIs) (Strahl & Blackburn 1996). A number of these drugs were found to be capable of inhibiting telomerase activity and reducing telomere length *in vitro*. In this chapter, the effect of the reverse transcriptase inhibitor 3′-Azido-3′-deoxythymidine triphosphate (AZT-TP) on telomerase activity was assessed in two telomerase positive canine cell lines.

4.2.4.3 Telomerase reactivation study

It is known that ectopic expression of the catalytic component of human telomerase, hTERT, greatly extends the lifespan of primary human cell cultures *in vitro* by causing reconstitution of telomerase activity in the transformed cells (Bodnar, Ouellette, *et al* 1998). Whilst at the time of this study the canine and feline homologous sequences to hTERT were not known, an investigation was conducted to determine if ectopic expression of hTERT in primary canine and feline cell lines is capable of reconstituting telomerase activity using heterologous gene expression, and whether this is sufficient to expand replicative potential *in vitro*.

4.3 Materials and Methods

4.3.1 Sample details

4.3.1.1 Normal somatic tissues

Normal somatic tissue samples were collected from the same animals that were described in Chapter III for telomere length assessment. These were 5 dogs (CN1-CN5) and 5 cats (FN1-FN5). Details including age, sex, breed and cause of death of individual animals from which samples were taken are presented in Table 3-2. The 26 canine samples included kidney (n = 4), liver (n = 5), ovary (n = 1), skeletal muscle (n = 4), small intestine (n = 4), stomach (n = 2), cardiac muscle (n = 3), lung (n = 2) and testis (n = 1). The 25 feline samples included brain (n = 4), cardiac muscle (n = 3), liver (n = 5), skeletal muscle (n = 4), lung (n = 4), small intestine (n = 3), testis (n = 1) and kidney (n = 1).

4.3.1.2 Tumour tissues

A total of 19 tumour tissue samples were collected from 15 dogs and 4 cats during necropsy examination at GUVS and surgical biopsy at the PDSA hospital, Shamrock Street, Glasgow. The canine samples included 10 examples of mammary carcinoma, 3 fibrosarcomas and 2 squamous cell carcinomas. The four feline samples included 1 mammary carcinoma and 3 squamous cell carcinomas. The tumour types were confirmed by routine histopathological examination carried out at GUVS, and all samples were harvested with informed owner consent. Each of the tissue samples was snap frozen in LNO_2 at the time of collection to ensure integrity of the telomerase RNA until required.

4.3.1.3 Primary cultures and cell lines

Primary fibroblast cultures of canine (SFA and AG08157), feline (S22 and CCL-176) and equine (EQ1) origin, and a human immortalised cell line (GM847) were used in the telomerase reactivation study. Additionally, a number of

immortal cell lines of both canine and human origin (results shown in Table 4-14) were used for telomerase activity testing. Two cell lines from this panel (MDCK and CMT7) were subsequently used to test the efficacy of a potential telomerase inhibitor *in vitro*. The sources of each of these cells are provided in Section 2.1.1.1, and individual details including culture requirements are provided in Section 2.2.1.2. Additional details of the investigation of telomerase activity in cell lines and the telomerase reactivation study are provided below.

4.3.1.3.1 Telomerase reactivation study

Primary fibroblast cell cultures were utilised for this study. These included the canine cultures SFA and AG08157 and the feline cell cultures S22 and CCL-176. All the primary fibroblast cultures were at passage 2 at the beginning of the experiment to preserve the greatest possible replicative potential for the experimental procedure. The human ALT competent cell line GM847 and the primary equine fibroblast culture EQ1 were included, GM847 as a positive experimental control and EQ1 as a further species control for the specificity of the hTERT sequence. A full-length hTERT clone was kindly donated by Robert Weinberg (Whithead Institute for Biomedical research, Cambridge, MA), and the mammalian expression vector PCIneo (Promega, UK), detailed in Section 2.1.5.1 was used as the vehicle for entry of hTERT into the test cells.

4.3.1.3.2 Telomerase inhibition study

The ability of a RTI to reduce telomerase activity was assessed in two immortalised canine cell line lines that were found to be telomerase positive in the telomerase activity study. The RTI AZT-TP was used in this study. The drug was supplied as a 10 mM solution of the tetralithium salt in water (Calbiochem, La Jolla CA), and was stored in a tightly closed container in aliquots of 60 µl at -70°C, and protected from light when not in use. The drug is considered toxic and was used only with appropriate safety precautions (within a fume cupboard and wearing protective clothing and gloves).

4.3.2 The TRAP assay

TRAP assay testing of all samples in this project used the TeloTAGGG Telomerase PCR ELISA^{PLUS} kit available commercially from Roche, UK.

4.3.2.1 Sample preparation for the TRAP assay

Samples used for telomerase activity analysis included cell pellets derived from cultured cells and protein extracts derived from tissue samples. Cells were harvested and counted as described elsewhere (Section 2.2.1). A total of 2 x 10^5 cells were harvested for each assay. Cells were pelleted at 3000 g for 5 minutes at 4°C, the supernatant decanted, and following a brief resuspension in 100 µl of PBS the cells were again pelleted and the supernatant carefully removed. Cell pellets were then resuspended in 200 µl ice-cold lysis reagent (Roche, UK) and incubated on ice for 30 minutes. The lysate was then centrifuged at 16000 g for 20 minutes at 4°C and the supernatant carefully removed with a micropipette, taking care not to disturb the pellet of cellular debris. To facilitate this, only 175 µl was removed per sample. Cell extracts were then either used immediately for the TRAP assay or snap frozen in LNO_2 and stored at -80°C for later use.

Normal tissue samples and tumour samples were placed in tumour pots and snap frozen in LNO_2 immediately after harvesting. Samples were then stored at -80°C. Such precautions in tissue handling were necessary to safeguard the integrity of telomerase RNA and catalytic subunit and thus reduce the likelihood of false negative results (Cadile, Kitchell, *et al* 2001). Thin slices of frozen samples were then removed (approximately 10 slices being sufficient) using sterile scalpel blades on disposable petri dishes and immediately transferred to 1.5 ml eppendorf homogenisation tubes containing 200 µl of ice-cold lysis reagent (Roche, UK). Samples were then thoroughly homogenised using a sterile pestle and stored on ice for 30 minutes to achieve thorough lysis.

Following lysis, samples were centrifuged at 16000 g for 20 minutes at 4°C and the protein rich supernatant carefully removed with a micropipette, as described for cell pellets. Protein concentration was then determined using a standard protocol, as described in Section 2.2.7. The tissue extracts were either immediately used for the TRAP assay or snap frozen in LNO_2 and stored at -80°C for later use.

4.3.2.2 Primer elongation and amplification

All preparation steps for the TRAP PCR procedure were carried out on ice, including the thawing of frozen samples prior to analysis. Unless otherwise indicated sample numbers were limited to six per individual assay to minimise the potential cost in reagents of failure of any assay. Master mixes were made up for all samples and controls consisting of 25 µl of a 2x reaction mixture (Roche, UK) and 5 µl of an internal standard (Roche, UK). A 30 µl volume of the mix was placed into a PCR tube for each sample and to this was added 1 µl of the cell extract or a volume equivalent to 5 µg of total protein. A 1 µl volume of two separate positive controls, one of high and one of low telomerase activity were used, and 1 µl of lysis buffer alone was used as a negative control. Analysis of a heat-inactivated version of each test sample provided an additional level of control. Heating these control samples to 85°C for 10 minutes inactivates telomerase protein within the sample and ensures any positive result is specific to telomerase activity. All reactions were then made up to a total volume of 50 µl using nuclease-free water (Roche, UK) and transferred to a DNA Thermal Cycler (Perkin Elmer) for the combined primer elongation/amplification reaction. Reaction conditions are shown in Table 4-1.

4.3.2.3 Hybridisation and ELISA

Hybridisation steps were carried out in 0.5 ml eppendorf tubes placed in colour coded racks to differentiate between test, internal standard and negative (including heat inactivated) controls. A 2.5 µl volume of amplification product from the previous step was added to 10 µl of a denaturation reagent (Roche, UK)

in the corresponding eppendorf tube. These mixtures were then incubated at room temperature for 10 minutes before addition of 100 µl of either a test hybridisation buffer (to test and control samples) or an internal standard buffer (to internal control samples). These buffers contained DIG-labelled detection probes specific for telomeric repeats and the internal standard respectively. Thorough mixing of the contents of all tubes was ensured by vortexing before 100 µl of the contents of each was removed and placed in a corresponding well of a streptavidin precoated microtitre plate (Roche, UK) according to a predetermined pipetting scheme. A plate contained 12 modules of 8 wells each, and modules could be removed to facilitate separate analysis such that more than one assay could be performed per kit.

The microtitre plate wells were then clearly labelled, covered with an adhesive strip to prevent evaporation and incubated at 37°C on a heated plate shaker (Dynatech, UK) rotating at 300 rpm for 2 hours. This allowed immobilisation of the telomerase and internal standard products with their corresponding detection probes to the streptavidin-coated walls of the plate wells, via the biotin label of the kit primer. Following hybridisation the solutions were completely removed from the wells, following which the wells were washed three times for a minimum of 30 seconds per wash with 250 µl of wash buffer, (Roche, UK) ensuring complete removal of the buffer from the wells between steps.

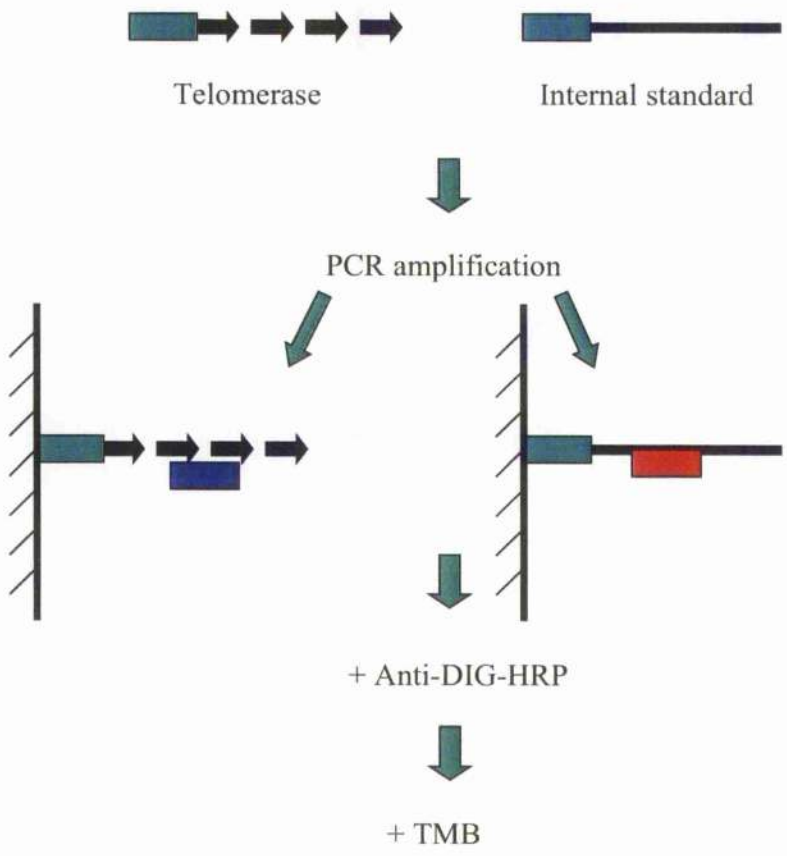
The anti-DIG working solution was a polyclonal antibody (Roche, UK) raised in sheep conjugated to HRP that was diluted to a concentration of 10 mU/ml in a conjugate dilution buffer (Roche, UK). A 100 µl volume of this anti-DIG-HRP working solution was then added to each of the wells that were again covered with adhesive film and incubated for 30 minutes at room temperature on a plate shaker rotating at 300 rpm. The working solution was then removed completely from the wells and five, 30-second wash steps carried out as described previously. Following this, 100 µl of a substrate solution, equilibrated to room temperature and containing the HRP sensitive compound 3,3′, 5,5′-tetramethylbenzidine (TMB) was added to each well and the plate covered and incubated for colour development at room temperature for 10 minutes with

rotation at 300 rpm. Without removal of the substrate solution from the well, 100 µl of a stop reagent (< 5% sulphuric acid, Roche, UK) was then added; this stopped the reaction and in addition caused a colour change of the reacted HRP substrate from blue to yellow, which increased the sensitivity of the reaction. The absorbance of the samples was then immediately measured at 450 nm, with a reference wavelength of 690 nm using a microtitre plate reader (Dynex Technologies, UK). An overview of the TRAP assay procedure is provided in Figure 4-1.

Table 4-1. Telomerase primer elongation/amplification reaction protocol

Step	Time	Temerature	Cycle
Primer elongation	20 minutes	25°C	
Telomerase inactivation	5 minutes	94°C	
Amplification			
Denaturing	30 seconds	94°C	30 cycles
Annealing	30 seconds	50°C	30 cycles
Polymerisation	30 seconds	72°C	30 cylces
	10 minutes	72°C	
Hold		4°C	

Figure 4-1. Overview of the TRAP assay. In the first step, telomerase adds telomeric repeats (TTAGGG) to the 3′ end of the biotin labelled synthetic primer (green rectangle). These elongation products, as well as the internal standard included in the same reaction vessel are amplified by PCR. The PCR products are split into two aliquots, denatured and hybridised separately to DIG labelled detection probes, specific for telomeric repeats (blue) and internal standard (red), respectively. The resulting products are immobilised to the streptavidin-coated microtitre plate wall via the biotin label. The immobilised amplicons are then detected with an antibody against DIG that is conjugated to HRP and the sensitive peroxidase substrate TMB.



4.3.2.4 Quantification of telomerase activity

The level of telomerase activity in positive samples was determined from the absorbance of each test sample and the absorbance obtained from 1 µl of the control templates. The control templates (TS8) were identical to a telomerase elongation product of 8 telomeric repeats and were at a concentration of 0.001 amol/ml (low activity) and 0.1 amol/ml (high activity). As amplification of the telomerase specific products and internal standards were competitive, both low and high controls were used in each experiment to cover for a potentially broad range of telomerase activity in the test samples. The most appropriate control was then used depending on the level of telomerase activity in the test samples. In practice this meant use of the high activity control in the majority of cases. RTA in a sample was then determined using the formula:

$$RTA = \frac{(A_S - A_{S0})/A_{S,IS}}{(A_{TS8} - A_{TS8,0})/A_{TS8,IS}} \times 100$$

where,

A_S = absorbance of sample

A_{S0} = absorbance of heat-treated sample

$A_{S,IS}$ = absorbance of the internal standard of the sample

A_{TS8} = absorbance of the control template (TS8)

$A_{TS8,0}$ = absorbance of lysis buffer

$A_{TS8,IS}$ = absorbance of the internal standard of the control template

The values $(A_{TS8} - A_{TS8,0})/A_{TS8,IS}$ obtained using 1 µl of the high and low control templates were considered acceptable if within the range of 2.0-4.0 and 0.2-0.5 respectively after 10 minutes of substrate reaction. Test samples with absorbance readings greater than three times the background reading (mean absorbance of the heat treated samples) were considered to be telomerase positive.

4.3.2.5 Detection of telomerase mediated DNA-ladder

The biotin labelled primer used in the TRAP assay allowed identification of the amplification products by polyacrylamide gel electrophoresis (PAGE). The Mini PROTEAN 3 vertical gel electrophoresis system (Bio-rad) was used for this procedure. First, 20 µl of the PCR product was mixed with a loading dye containing bromophenol blue and xylene cyanol. Separation of amplification products was achieved using a precast 12% non-denaturing polyacrylamide gel (Bio-Rad, Hertfordshire, UK) run for 30 minutes at 100 V. After electrophoresis, products were transferred by electroblotting (Section 2.2.8) to a positively charged nylon membrane (Amersham, UK), which was then blocked with a 2% blocking reagent (Roche, UK) for 30 minutes at room temperature to prevent binding at non-specific sites. Blocking solution was then discarded and the membrane incubated with 20 ml of a streptavidin-alkaline phosphate conjugate which bound to the biotin labels on the DNA fragments. Following this the membrane received two 15-minute rinses in 100 ml of wash buffer (0.1 M Maleic acid, 0.15 M NaCl pH 7.5; 0.3 v/v Tween 20) at room temperature before equilibration with 20 ml of detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5) for 2-5 minutes. The membrane was then placed DNA side up on an acetate sheet and 1 ml of CSPD ready-to-use (Roche, UK) applied drop wise. (CSPD ready-to-use is a chemiluminescent substrate for alkaline phosphatase at a concentration of 0.25 mM). A second acetate sheet was placed over the membrane, ensuring no air bubbles were trapped in the process, to help distribute the substrate solution evenly over the surface after which there was a 5 minute incubation at room temperature before excess CSPD solution was squeezed out and the acetate sheets sealed with sellotape. At this point an incubation of the damp membrane at 37°C for 10 minutes helped to enhance the chemiluminescent reaction. The membrane was then exposed to X-ray film for 15-20 minutes to produce an autoradiograph.

4.3.3 Telomerase inhibition study

The effect of the RTI AZT-TP on telomerase activity of canine cells was investigated *in vitro* using the immortalised canine cell lines MDCK and CMT7 (detailed in Section 2.2.1.2). An initial investigation assessed the toxicity of the drug to the test cells before any telomerase inhibitory effects could be examined.

4.3.3.1 Investigation of acute cytotoxic effect

Both test cell lines were maintained in two-fold dilutions of AZT-TP in a 96 well plate format to assess cytotoxicity of the drug. Each cell line was seeded at 1 $x10^5$ cells/ml in triplicate and allowed to settle overnight before exposure to concentrations of 480, 240, 120, 60, 30, 15, 7.5 and 3.75 μM AZT-TP in complete culture medium for a period of 7 days. Treated medium was replaced with fresh, drug containing solution after 3 days, following normal practice for growth medium replacement. Parallel cultures of the test cell lines, seeded at the same concentration were maintained in complete culture medium without the addition of the test drug. These cultures also received fresh medium after 3 days. This experiment allowed the identification of AZT-TP concentrations that did not kill the test cells outright, and the experiment was then repeated in duplicate using the identified concentrations of AZT-TP in a T25 flask format, as this allowed easier harvesting and counting of cells, given the relatively greater numbers. This second stage was used to identify the highest concentration of AZT-TP that did not effect cell morphology or growth rate, and this concentration was duly selected for the main study.

4.3.3.2 Investigation of effect of AZT-TP on telomerase activity

MDCK and CMT7 cells were maintained in T75 flasks under normal culture conditions. Each cell line was initially seeded at 1 x 10^5 cells/ml, and two batches of each cell line were maintained. One batch of each cell line was exposed to AZT-TP at a concentration previously determined not to exert any acute cytotoxic effects on the cells. A second batch was maintained in parallel in AZT-TP free medium. Cells were passaged normally as described elsewhere (Section 2.2.1) upon reaching 80% confluence, and at each passage cells were

counted (Section 2.2.1.1.1) and 2 x10^5 and 2 x10^6 cells from each treated and control cell line harvested and retained for analysis. These cells were pelleted at 3000 g for 5 minutes to facilitate removal of the culture medium. The pellets were then briefly resuspended in cold PBS, repelleted as described and the supernatant decanted to remove the last traces of culture medium. These precautions were necessary due to the theoretical possibility of the presence of Taq DNA polymerase inhibitors in the media used to culture the cells. Pellets were then snap frozen in LNO_2 and transferred to -80°C storage. The 2 x10^5 cell pellet was subsequently used to assess telomerase activity in the sample by the TRAP assay, and the 2 x10^6 pellet used for telomere length analysis. In addition, growth curves were generated for each cell line and any difference in growth rate between test and control cells noted. The techniques used for telomere length analysis and the production of growth curves have been detailed elsewhere (Sections 3.3.3 and 3.3.1.4).

4.3.4 Telomerase reactivation study

4.3.4.1 Cell lines

In addition to the canine and feline primary cultures used in this study (Section 4.3.1.3) a number of other cell lines were included as controls. Telomerase activity has been successfully reconstituted in the human ALT positive cell line GM847 by ectopic expression of hTERT (Perrem, Colgin, *et al* 2001), and this cell line was therefore included to ensure the experimental protocol was working effectively. In addition, the ability of hTERT to reconstitute telomerase activity was investigated in an equine primary fibroblast culture, EQ1. The aim here was to use equine cells as an additional control to determine whether heterologous TERT expression is sufficient to activate telomerase in a range of mammalian cells. Currently, there are no published data on the feline, equine or canine TERT sequences which limits the investigation of homologous TERT reconstitution analyses. However, during the preparation of this thesis, our group have isolated and cloned the cDNA encoding the canine TERT sequence (submitted for review, Nasir, Gault, *et al* 2004).

4.3.4.2 Cloning of hTERT cDNA into a mammalian expression vector

The PCIneo mammalian expression vector (Section 2.1.5.1) was selected to act as the plasmid for introducing hTERT into the test cells as it is designed specifically for mammalian gene expression studies and includes a strong, constitutively active CMV promoter, and has the capacity for selection of transfected cells via its neomycin phosphotransferase sequence. Furthermore, PCIneo has been successfully used to transfect several canine cell lines within the laboratory. PCIneo required linearization to facilitate insertion of the hTERT cDNA, and this was achieved by a 2-hour digestion of 5 µg of PCIneo with 4 IU/µg each of *Eco*R 1 and *Sal* I at 37°C. As these restriction enzymes have one recognition site each within the PCIneo sequence (at nucleotide positions 1096 and 1120 respectively), they were ideal for linearising the vector. Following digestion, linearised vector was subjected to 1% agarose gel electrophoresis (Section 2.2.3.5) with a 1 kb DNA ladder to confirm integrity of the DNA and size of fragment. The band containing the vector was visualised using a UV transilluminator (Sigma, UK), removed from the gel using a sterile scalpel blade and purified using the QIAquick® Gel extraction kit (QIAgen, UK) following the manufacturers recommended protocol. Concentration of plasmid DNA was then determined by a standard protocol (Section 2.2.3.3.2).

A full-length hTERT clone (Table 4-2) was kindly donated by Robert Weinberg (Whithead Institute for Biomedical research, Cambridge, MA), supplied cloned into the pBABE-puro retroviral vector. The hTERT cDNA was isolated from this vector using the same enzymes as described for the PCIneo, and vector and insert DNA were then ligated using T4 DNA ligase (Promega, UK) following the protocol detailed in Section 2.2.3.7. A molar ratio of vector: insert DNA of 1:3 was used. A 1 µl volume of the ligation products from each reaction were then used to transform *E. coli* TOP10 bacterial cells as described in Section 2.2.3.8. After overnight growth on agar containing ampicillin at 100 mg/ml individual colonies were picked and grown as described in section 2.2.3.1.

Table 4-2. hTERT clone sequence

ORIGIN

```
   1 GCAGCGCTGC GTCCTGCTGC GCACGTGGGA AGCCCTGGCC CGGGCCACCC CCGCGATGCC
  61 GCGCGCTCCC CGCTGCCGAG CCGTGCGCTC CCTGCTGCGC AGCCACTACC GCCAGGTGCT
 121 GCCGCTGGCC ACGTTCGTGC GGCGCCTGGG GCCCCAGGGC TGGCGCCTGC TGCAGCGCGG
 181 GGACCCGGCG GCTTTCCGCG CGCTGGTGGC CCAGTGCCTG GTGTGCGTGC CCTGGGACGC
 241 ACGGCCGCCC CCCGCCGCCC CCTCCTTCCG CCAGGTGTCC TGCCTGAAGG AGCTGGTGGC
 301 CCGAGTGCTG CAGAGGCTGT GCGAGCGCGG CGCGAAGAAC GTGCTGGCCT TCGGCTTCGC
 361 GCTGCTGGAC GGGGCCCGCG GGGGCCCCCC CGAGGCCTTC ACCACCAGCG TGCGCAGCTA
 421 CCTGCCCAAC ACGGTGACCG ACGCACTGCG GGGGAGCGGG GCCTGGGGGC TGCTGCTGCG
 481 CCGCGTGGGC GACGACGTGC TGGTTCACCT GCTGGCACGC TGCGCGCTCT TTGTGCTGGT
 541 GGCTCCCAGC TGCGCCTACC AGGTGTGCGG GCCGCCGCTG TACCAGCTCG GCGCTGCCAC
 601 TCAGGCCCGG CCCCCGCCAC ACGCTAGTGG ACCCCGAAGG CGTCTGGGAT GCGAACGGGC
 661 CTGGAACCAT AGCGTCAGGG AGGCCGGGGT CCCCCTGGGC CTGCCAGCCC CGGGTGCGAG
 721 GAGGCGCGGG GGCAGTGCCA GCCGAAGTCT GCCGTTGCCC AAGAGGCCCA GGCGTGGCGC
 781 TGCCCCTGAG CCGGAGCGGA CGCCCGTTCG GCAGGGGTCC TGGGCCCACC CGGGCAGGAC
 841 GCGTGGACCG AGTGACCGTG GTTTCTGTGT GGTGTCACCT GCCAGACCCG CCGAAGAAGC
 901 CACCTCTTTG GAGGGTGCGC TCTCTGGCAC GCGCCACTCC CACCCATCCG TGGGCCGCCA
 961 GCACCACGCG GCCCCCCCAT CCACATCGCG GCCACCACGT CCCTGGGACA CGCCTTGTCC
1021 CCGGGTGTAC GCCGAGACCA AGCACTTCCT CTACTCCTCA GGCGACAAGG AGCAGCTGCG
1081 GCCCTCCTTC CTACTCAGCT CTCTGAGGCC CAGCCTGACT GGCGCTCGGA GGCTCGTGGA
1141 GACCATCTTT CTGGGTTCCA GGCCCTGGAT GCCAGGGACT CCCCGCAGGT TGCCCCGCCT
1201 GCCCCAGCGC TACTGGCAAA TGCGGCCCCT GTTTCTGGAG CTGCTTGGGA ACCACGCCCA
1261 GTGCCCCTAC GGGGTGCTCC TCAAGACGCA CTGCCCGCTG CGAGCTGCGG TCACCCCAGC
1321 AGCCGGTGTC TGTGCCCGGG AGAAGCCCCA GGGCTCTGTG GCGGCCCCCG AGGAGGAGGA
1381 CACAGACCCC CGTCGCCTGG TGCAGCTGCT CCGCCAGCAC AGCAGCCCCT GGCAGGTGTA
1441 CGGCTTCGTG CGGGCCTGCC TGCGCCGGCT GGTGCCCCCA GGCCTCTGGG GCTCCAGGCA
1501 CAACGAACGC CGCTTCCTCA GGAACACCAA GAAGTTCATC TCCCTGGGGA AGCATGCCAA
1561 GCTCTCGCTG CAGGAGCTGA CGTGGAAGAT GAGCGTGCGG GACTGCGCTT GGCTGCGCAG
1621 GAGCCCAGGG GTTGGCTGTG TTCCGGCCGC AGAGCACCGT CTGCGTGAGG AGATCCTGGC
1681 CAAGTTCCTG CACTGGCTGA TGAGTGTGTA CGTCCTCGAG CTGCTCAGGT CTTTCTTTTA
1741 TGTCACGGAG ACCACGTTTC AAAAGAACAG GCTCTTTTTC TACCGGAAGA GTGTCTGGAG
1801 CAAGTTGCAA AGCATTGGAA TCAGACAGCA CTTGAAGAGG GTGCAGCTGC GGGAGCTGTC
1861 GGAAGCAGAG GTCAGCCAGC ATCGGGAAGC CAGGCCCGCC CTGCTGACGT CCAGACTCCG
1921 CTTCATCCCC AAGCCTGACG GGCTGCGGCC GATTGTGAAC ATGGACTACG TCGTGGGAGC
1981 CAGAACGTTC CGCAGAGAAA AGAGGCCGA GCGTCTCACC TCGAGGGTGA AGGCACTGTT
2041 CAGCGTGCTC AACTACGAGC GGGCGCGGCG CCCCGGCCTC CTGGGCGCCT CTGTGCTGGG
2101 CCTGGACGAT ATCCACAGGG CCTGGCGCAC CTTCGTGCTG CGTGTGCGGG CCCAGGACCC
2161 GCCGCCTGAG CTGTACTTTG TCAAGGACAG GCTCACGGAG GTCATCGCCA GCATCATCAA
2221 ACCCCAGAAC ACGTACTGCG TGCGTCGGTA TGCCGTGGTC CAGAAGGCCG CCCATGGGCA
2281 CGTCCGCAAG GCCTTCAAGA GCCACGTCTC ACGTCCAGTG CCAGGGGATC CCGCAGGGCT
2341 CCATCCTCTC CACGCTGCTC TGCAGCCTGT GCTACGGCGA CATGGAGAAC AAGCTGTTTG
2401 CGGGGATTCG GCGGGACGGG CTGCTCCTGC GTTTGGTGGA TGATTTCTTG TTGGTGACAC
2461 CTCACCTCAC CCACGCGAAA ACCTTCCTCA GCTATGCCCG GACCTCCATC AGAGCCAGTC
2521 TCACCTTCAA CCGCGGCTTC AAGGCTGGGA GGAACATGCG TCGCAAACTC TTTGGGGTCT
2581 TGCGGCTGAA GTGTCACAGC CTGTTTCTGG ATTTGCAGGT GAACAGCCTC CAGACGGTGT
2641 GCACCAACAT CTACAAGATC CTCCTGCTGC AGGCGTACAG GTTTCACGCA TGTGTGCTGC
2701 AGCTCCCATT TCATCAGCAA GTTTGGAAGA ACCCCACATT TTTCCTGCGC GTCATCTCTG
2761 ACACGGCCTC CCTCTGCTAC TCCATCCTGA AAGCCAAGAA CGCAGGGATG TCGCTGGGGG
2821 CCAAGGGCGC CGCCGGCCCT CTGCCCTCCG AGGCCGTGCA GTGGCTGTGC CACCAAGCAT
2881 TCCTGCTCAA GCTGACTCGA CACCGTGTCA CCTACGTGCC ACTCCTGGGG TCACTCAGGA
2941 CAGCCCAGAC GCAGCTGAGT CGGAAGCTCC CGGGCACGAC GCTGACTGCC CTGGAGGCCG
3001 CAGCCAACCC GGCACTGCCC TCAGACTTCA AGACCATCCT GGACTGATGG CCACCCGCCC
3061 ACAGCCAGGC CGAGAGCAGA CACCAGCAGC CCTGTCACGC CGGGCTCTAC GTCCCAGGGA
3121 GGGAGGGGCG GCCCACACCC AGGCCCGCAC CGCTGGGAGT CTGAGGCCTG AGTGAGTGTT
3181 TGGCCGAGGC CTGCATGTCC GGCTGAAGGC TGAGTGTCCG GCTGAGGCCT GAGCGAGTGT
3241 CCAGCCAAGG GCTGAGTGTC CAGCACACCT GCCGTCTTCA CTTCCCCACA GGCTGGCGCT
3301 CGGCTCCACC CCAGGGCCAG CTTTTCCTCA CCAGGAGCCC GGCTTCCACT CCCCACATAG
3361 GAATAGTCCA TCCCCAGATT CGCCATTGTT CACCCCTCGC CCTGCCCTCC TTTGCCTTCC
3421 ACCCCCACCA TCCAGGTGGA GACCCTGAGA AGGACCCTGG GAGCTCTGGG AATTTGGAGT
3481 GACCAAAGGT GTGCCCTGTA CACAGGCGAG GACCCTGCAC CTGGATGGGG GTCCCTGTGG
3541 GTCAAATTGG GGGGAGGTGC TGTGGGAGTA AAATACTGAA TATATGAGTT GTTCAGTTTT
3601 G
```

Plasmid DNA was then purified from each individual clone using the QIAprep® PCR Spin Miniprep Kit (QIAGEN, UK, detailed in Section 2.2.3.2.2). This kit allowed isolation of plasmid DNA from 4 ml LB broth cultures of exponentially growing bacteria. The bacteria were harvested by centrifugation (2800 rpm for 10 minutes), and the manufacturers protocol was then followed. The method involved lysis of the bacterial cells to release the DNA constructs, which were then harvested by centrifugation, filtration and wash steps into 50 µl of sterile water. DNA was stored at -20°C.

A 5 µl volume of each plasmid was then digested with 4 IU/µg of *Eco*R 1 and *Sal* I as described previously, and the digests run out on a 1% agarose gel to confirm the correct size of plasmid and insert. Plasmids found to contain the hTERT insert were then used to produce bulk stocks of purified PCIneo/hTERT (Section 2.2.3.2.1) and glycerol stocks of bacteria containing the construct as described in Section 2.2.3.1.

4.3.4.3 Stable transfection of cell cultures

Transfections were carried out using TransFast™ (Promega, UK) and Lipofectamine™/Plus™ transfection reagents (Invitrogen life technologies, UK). Test cell cultures were transfected with PCIneo/hTERT and PCIneo empty vector as a control.

4.3.4.3.1 TransFast™ reagent

The TransFast transfection reagent relies on interaction between liposomes and DNA to facilitate entry of the nucleic acids into mammalian cells. Liposomes are lipid bilayers that form colloidal particles in an aqueous medium. The lipids contained in TransFast include the synthetic cationic lipid, (+)-N,N [bis (2-hydroxyethyl)-N-methyl-N-[2,3-di(tetradecanoyloxy)propyl] ammonium iodide, and the neutral lipid L-dioleoyl phosphatidyethanolamine (DOPE).

The incubation of cationic lipid containing liposomes and nucleic acids results in rapid association and compaction of the nucleic acid; this is thought to be caused by electrostatic interactions between the negatively charged nucleic acid and the positively charged head group of the synthetic lipid. Thus the negative charge of the nucleic acid is shielded, allowing closer association with the target cell membrane. Entry of the liposome into the cell may then occur by either endocytosis or fusion with the cell membrane (Gao & Huang 1995). The neutral lipid DOPE, which allows the complexes to escape from endosomes and lysosomes into the cytoplasm, enhances this process (Farhood, Serbina, *et al* 1995). It is not known how the transfected DNA of DNA/liposome complex gains entry to the nucleus.

4.3.4.3.2 Transfection protocol using TransFast™ reagent

Cells were plated at normal concentration (1 x 10^5/ml) the day before transfection and allowed to settle overnight. The TransFast reagent was reconstituted with 400 µl of water on the same day to result in a 1 mM concentration of lipid. The reagent was then vortexed thoroughly to resuspend the lipid film and stored at -20°C overnight. The first stage of the transfection process involved optimisation of the conditions. This involved a trial of different amounts of DNA in the transfection reaction, and different charge ratios of TransFast reagent to DNA. This was done in a 24 well plate format, and for each cell line to be transfected, 0.25, 0.5, 0.75 and 1.0 µg of DNA was used with a 1:1, 2:1 and a 3:1 charge ratio. Each combination was also tested in complete medium, and medium without the addition of FCS. The conditions resulting in the most effective transfection were then adopted for the main study.

Master mixes adequate for 6 replicates of each DNA concentration and charge ratio were made up. First the required amount of medium (with or without FCS, and prewarmed to 37°C) was combined with the requisite amount of DNA and vortexed. To this was added the amount of TransFast reagent indicated by the charge ratio. For a 24 well plate format, the total volume of these 3 constituents was 200 µl per well. The mixture was allowed to incubate for 15 minutes at

room temperature, then growth medium was carefully aspirated from the test cells and after a final vortex the TransFast/DNA mixture was added to the cells, which were then incubated under normal culture conditions for 1 hour.

At the end of the incubation period the cells were overlaid with prewarmed complete medium (1 ml per well in the 24 well plate format). Cells were then returned to the incubator and left undisturbed for 48 hours. At the end of this period, selection for transfected cells was achieved by the addition of fresh medium containing the antibiotic G418 at a concentration previously determined to select strongly for transfected cells. In general, mammalian cells require a concentration of 400-600 μg/ml of G418 to achieve the selection criteria of >90% cell death in control cells within 5-7 days (Manufacturer's transfection guidelines). As such, control (untransfected) cells from each cell line were exposed to concentrations of 300, 350, 400, 500 and 600 μg/ml of G418 initially to determine the concentration of drug required for adequate selection.

Test cells were maintained in selective medium for 3-4 weeks with twice-weekly changes of medium to eliminate dead cells and debris until, if stable transfectants were achieved, distinct colonies of surviving cells appeared. At this point surviving cells were harvested and pooled to produce a cell line containing stable transfectants only. These were maintained in selective medium and otherwise cultured normally. In addition a parallel transfection with empty plasmid vector was carried out, to provide a negative control.

4.3.4.3.3 Lipofectamine™ and Plus™ reagents

Lipofectamine is a different liposome formulation consisting of the polycationic lipid 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA) and DOPE. It has the same mode of action as TransFast™, and it is recommended for use with the proprietary Plus™ reagent (Invitrogen, UK). The Plus reagent is used for precomplexing with DNA and has been found to enhance cationic lipid-mediated transfection. Optimisation

of conditions using Lipofectamine was determined in the same manner as detailed for the TransFast reagent.

4.3.4.3.4 Transfection using the Lipofectamine/Plus reagents

Cells were plated at normal concentration (1 x10^5/ml) the night before the procedure such that they would be 60-80% confluent at the time of transfection. Plasmid DNA was precomplexed with the Plus reagent; for each well of a 24 well plate format this involved dilution of 0.4 µg of DNA in 25 µl of medium without serum before the addition of 4 µl of the Plus reagent to the mixture, a thorough mixing of the components and a 15 minute incubation at room temperature.

A 1 µl volume of Lipofectamine reagent was then diluted in 25 µl of serum free medium in a second tube and mixed before this was added to the tube containing the precomplexed DNA. After a thorough mixing there then followed another 15-minute incubation at room temperature. During this incubation, the growth medium covering the test cells was replaced with 0.2 ml of transfection medium (normal or without FCS) per well. The DNA-Plus/Lipofectamine complexes were then added to the test wells, mixed gently and incubated under normal culture conditions for 3 hours. After the incubation, transfection medium was completely removed and replaced with normal growth medium for each of the cell types. Cells were then incubated under normal growth conditions for 72 hours, after which growth medium was replaced with selective medium containing G418 at the previously determined concentration.

Cell lines were maintained under normal growth conditions in the selective medium and passaged upon reaching 80% confluence. At each passage, cell pellets were retained for TRF and TRAP analysis, as described previously for the telomerase inhibition study (Section 4.3.3.2) Cell counts at passage were monitored and used to construct growth curves for each surviving test cell line.

4.3.4.4 Confirmation of transfection

Successful transfection of the hTERT gene sequence into test cell lines was confirmed by RT-PCR (Section 2.2.5). Any possible DNA contamination in the isolated RNA samples was removed by inclusion of an RNAse-free DNAse 1 digestion step for each sample before RT-PCR (Section 2.2.4.3). First strand cDNA synthesis was carried out using both random primers and the gene specific primers detailed below. The use of oligo-dT primers was not appropriate in this case as the mature hTERT transcript is not polyadenylated. The gene specific primer sequences were 5′-ACTGTTCAGCGTGCTCAACTA-3′ (DNHT001) and 5′-TCATTCAGGGAGGAGCTCTGCT-3′ (DNHT001R), corresponding to nucleotide numbers 1980-2000 and 2378-2399 respectively on the *Homo sapiens* telomerase reverse transcriptase mRNA sequence, accession number NM_003219. The PCR was then carried out at 95°C for 5 minutes followed by 25 amplification cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. The final polymerisation incubation at 72°C was carried out for 7 minutes, after which the PCR products were held at 4°C until further analysis. The PCR products were visualised by agarose gel electrophoresis to confirm they were of the correct size, and then gel purified before sequencing using the ABI PRISM® 3100 Genetic Analyzer (Section 2.2.6) and confirming the origin of the sequence using the 'Blast' search engine (Section 2.2.6.1.3).

4.4 Results

4.4.1 Telomerase activity profile of normal canine and feline tissues

Telomerase activity was assessed in a panel of tissues (26 canine and 25 feline) sampled from 5 dogs (CN1-5) and 5 cats (FN1-5) as detailed in Table 3-2. Not all the tissue types sampled were available from each of the 10 animals, due to the individual nature of each necropsy examination. RTA values were generated from absorbance readings of test, control and internal standard wells as detailed in the Materials and Methods (Section 4.3). A sample was described as telomerase positive if the value determined by subtraction of background absorbance (average of the absorbance of the heat inactivated samples, $\overline{A}_{S0}$) from the absorbance of the sample, A_S resulted in a value greater than twice the background; i.e. $A_S - \overline{A}_{S0} / \overline{A}_{S0} \geq 2.0$. To reduce the likelihood of false negatives due to this highly stringent standard, samples with $A_S - \overline{A}_{S0} / \overline{A}_{S0} \geq 1$-2 were said to contain low, or borderline telomerase activity. These samples contained telomerase activity that was between two and three times greater than the background. Background readings of less than 0.1 were acceptable, any higher reading indicated inadequate heat inactivation and resulted in repetition of the experiment. All experiments utilised the high activity positive telomerase control, and $(A_{TS8}-A_{TS8,0})/A_{TS8,IS}$ values after 10 minutes of colour development were between 2.0 and 4.0.

RTA values determined for the canine tissue samples from subjects CN1, CN2, CN3, CN4 and CN5 are shown in Tables 4-2 to 4-6. In all the subjects where kidney tissue samples were available for analysis, these were negative for telomerase activity. Similarly, no activity could be detected in liver, cardiac muscle, lung or skeletal muscle samples isolated from several dogs. Small intestinal tissue samples were available for analysis in 4 subjects and low levels of telomerase activity could be detected in 2 cases (CN2 and CN3). Similarly, low levels of activity were identified in a stomach sample from CN1, however a

stomach sample from CN5 was negative for telomerase activity. One ovarian sample was available for analysis (CN1), however this sample was negative. A single testis sample was also analysed (CN5), and this sample showed the presence of low levels of activity.

RTA values determined for the feline tissue samples from subjects FN1, FN2, FN3, FN4 and FN5 are shown in Tables 4-7 to 4-11. Of the feline samples examined, telomerase activity was not detected in the following tissue samples from several subjects; brain, cardiac muscle, liver, skeletal muscle, lung and small intestine. Telomerase activity was also absent from the single feline kidney specimen (FN5). The one sample that was telomerase positive out of the 25 examined was a testis sample taken from subject FN5.

The telomerase positive and low telomerase activity results for both dogs and cats were all confirmed by repeat TRAP analysis.

4.4.2 Telomerase activity profile of canine and feline tumour samples

Telomerase activity was assessed in a panel of 15 canine and 4 feline tumours utilising the same criteria as described above for normal tissues, including the use of the high activity positive control for all experiments. Canine tumours sampled included fibrosarcoma (n = 3), mammary carcinoma (n = 10), and squamous cell carcinoma (n = 2). Feline tumour samples included mammary carcinoma (n = 1), and squamous cell carcinoma (n = 3). All tumour samples were identified by histopathology carried out at the Pathology department at GUVS. All tumour samples were either harvested during surgical biopsy and immediately flash frozen in LNO_2, or in the case of tumours harvested post-mortem, the necropsy was carried out the same day as euthanasia.

High levels of telomerase activity were detected in 4 of 4 (100%) feline tumour samples, and in 15 of 17 (88%) canine tumour samples. The remaining two canine tumour samples contained low levels of telomerase activity, which was

confirmed by repeat TRAP analysis. The TRAP results for the canine and feline tumour samples are shown in Tables 4-12 and 4-13 respectively.

Table 4-2. TRAP analysis of necropsy specimens from canine CN1. A_S is the absorbance of the sample, A_{S0} the absorbance of the heat inactivated version of the sample, ΔA_S was determined by subtraction of the mean of the heat inactivated samples ($\bar{A}_{S0}$ or background) from A_S, ΔA_S / $\bar{A}_{S0}$ is the ratio of sample to background activity, and RTA the relative telomerase activity. Samples were considered telomerase positive if the difference in absorbance (ΔA_S) was greater than twice the background activity (ΔA_S / $\bar{A}_{S0} \geq 2.0$). Samples contained low telomerase activity if ΔA_S / $\bar{A}_{S0}$ was between 1 and 2. Using these criteria samples were designated positive (POS), low activity (LOW) or negative (NEG) for telomerase activity. The CN1 assay gave a background absorbance of 0.044, and a $(A_{TS8}-A_{TS8,0})/A_{TS8,IS}$ value of 2.52 after 10 minutes of colour development using the high activity positive control.

CN1	A_S	A_{S0}	ΔA_S	$\frac{\Delta A_S}{\bar{A}_{S0}}$	RTA	Result
Kidney	0.042	0.05	0	0	0	NEG
Liver	0.054	0.046	0.01	0.23	4	NEG
Ovary	0.076	0.043	0.032	0.73	3	NEG
Skeletal muscle	0.041	0.042	0	0	0	NEG
Small intestine	0.041	0.036	0	0	6	NEG
Stomach	0.090	0.046	0.044	1.05	3	LOW

Table 4-3. TRAP analysis of necropsy specimens from canine CN2. A_S is the absorbance of the sample, A_{S0} the absorbance of the heat inactivated version of each sample, ΔA_S was determined by subtraction of the mean of the heat inactivated samples ($\overline{A}_{S0}$ or background) from A_S, ΔA_S / A_{S0} is the ratio of sample to background activity, and RTA the relative telomerase activity. Samples were considered telomerase positive if the difference in absorbance (ΔA_S) was greater than twice the background activity (ΔA_S / $\overline{A}_{S0} \geq 2.0$). Samples contained low telomerase activity if ΔA_S / $\overline{A}_{S0}$ was between 1 and 2. Using these criteria samples were designated positive (POS), low activity (LOW) or negative (NEG) for telomerase activity. The CN2 assay gave a background absorbance of 0.046, and a $(A_{TS8}-A_{TS8,0})/A_{TS8.IS}$ value of 2.14 after 10 minutes of colour development using the high activity positive control.

CN2	A_S	A_{S0}	ΔA_S	$\frac{\Delta A_S}{\overline{A}_{S0}}$	RTA	Result
Cardiac muscle	0.042	0.045	0	0	0	NEG
Kidney	0.041	0.046	0	0	0	NEG
Liver	0.041	0.045	0	0	0	NEG
Small intestine	0.101	0.048	0.055	1.20	3	LOW

Table 4-4. TRAP analysis of necropsy specimens from canine CN3. A_S is the absorbance of the sample, A_{S0} the absorbance of the heat inactivated version of each sample, ΔA_S was determined by subtraction of the mean of the heat inactivated samples ($\overline{A}_{S0}$ or background) from A_S, ΔA_S / $\overline{A}_{S0}$ is the ratio of sample to background activity, and RTA the relative telomerase activity. Samples were considered telomerase positive if the difference in absorbance (ΔA_S) was greater than twice the background activity (ΔA_S / $\overline{A}_{S0} \geq 2.0$). Samples contained low telomerase activity if ΔA_S / $\overline{A}_{S0}$ was between 1 and 2. Using these criteria samples were designated positive (POS), low activity (LOW) or negative (NEG) for telomerase activity. The CN3 assay gave a background absorbance of 0.052, and a $(A_{TS8}-A_{TS8,0})/A_{TS8,IS}$ value of 3.58 after 10 minutes of colour development using the high activity positive control.

CN3	A_S	A_{S0}	ΔA_S	$\frac{\Delta A_S}{\overline{A}_{S0}}$	RTA	Result
Cardiac muscle	0.032	0.037	0	0	0	NEG
Kidney	0.063	0.073	0.011	0.2	0	NEG
Liver	0.034	0.037	0	0	0	NEG
Lung	0.091	0.042	0.039	0.75	5	NEG
Skeletal muscle	0.033	0.037	0	0	0	NEG
Small intestine	0.388	0.086	0.336	6.5	172	POS

Table 4-5. TRAP analysis of necropsy specimens from canine CN4. A_S is the absorbance of the sample, A_{S0} the absorbance of the heat inactivated version of each sample, ΔA_S was determined by subtraction of the mean of the heat inactivated samples ($\overline{A}_{S0}$ or background) from A_S, ΔA_S / $\overline{A}_{S0}$ is the ratio of sample to background activity, and RTA the relative telomerase activity. Samples were considered telomerase positive if the difference in absorbance (ΔA_S) was greater than twice the background activity (ΔA_S / $\overline{A}_{S0} \geq 2.0$). Samples contained low telomerase activity if ΔA_S / $\overline{A}_{S0}$ was between 1 and 2. Using these criteria samples were designated positive (POS), low activity (LOW) or negative (NEG) for telomerase activity. The CN3 assay gave a background absorbance of 0.055, and a $(A_{TS8}-A_{TS8,0})/A_{TS8,IS}$ value of 2.35 after 10 minutes of colour development using the high activity positive control.

CN4	A_S	A_{S0}	ΔA_S	$\frac{\Delta A_S}{\overline{A}_{S0}}$	RTA	Result
Cardiac muscle	0.071	0.067	0.016	0.3	<1	NEG
Kidney	0.061	0.070	0.006	0.1	0	NEG
Liver	0.058	0.038	0.003	0.1	3	NEG
Lung	0.086	0.063	0.031	0.6	3	NEG
Skeletal muscle	0.072	0.060	0.017	0.3	7	NEG
Small intestine	0.072	0.031	0.017	0.3	3	NEG

Table 4-6. TRAP analysis of necropsy specimens from canine CN5. A_S is the absorbance of the sample, A_{S0} the absorbance of the heat inactivated version of each sample, ΔA_S was determined by subtraction of the mean of the heat inactivated samples ($\overline{A}_{S0}$ or background) from A_S, ΔA_S / $\overline{A}_{S0}$ is the ratio of sample to background activity, and RTA the relative telomerase activity. Samples were considered telomerase positive if the difference in absorbance (ΔA_S) was greater than twice the background activity (ΔA_S / $\overline{A}_{S0} \geq 2.0$). Samples contained low telomerase activity if ΔA_S / $\overline{A}_{S0}$ was between 1 and 2. Using these criteria samples were designated positive (POS), low activity (LOW) or negative (NEG) for telomerase activity. The CN5 assay gave a background absorbance of 0.062, and a $(A_{TS8}-A_{TS8,0})/A_{TS8,IS}$ value of 3.00 after 10 minutes of colour development using the high activity positive control.

CN5	A_S	A_{S0}	ΔA_S	$\frac{\Delta A_S}{\overline{A}_{S0}}$	RTA	Result
Liver	0.070	0.069	0.008	0.1	<1	NEG
Skeletal muscle	0.059	0.068	0	0	0	NEG
Stomach	0.062	0.052	0	0	0	NEG
Testis	0.152	0.059	0.09	1.5	<1	LOW

Table 4-7. TRAP analysis of necropsy specimens from feline FN1. A_S is the absorbance of the sample, A_{S0} the absorbance of the heat inactivated version of each sample, ΔA_S was determined by subtraction of the mean of the heat inactivated samples ($\bar{A}_{S0}$ or background) from A_S, ΔA_S / $\bar{A}_{S0}$ is the ratio of sample to background activity, and RTA the relative telomerase activity. Samples were considered telomerase positive if the difference in absorbance (ΔA_S) was greater than twice the background activity (ΔA_S / $\bar{A}_{S0} \geq 2.0$). Samples contained low telomerase activity if ΔA_S / $\bar{A}_{S0}$ was between 1 and 2. Using these criteria samples were designated positive (POS), low activity (LOW) or negative (NEG) for telomerase activity. The FN1 assay gave a background absorbance of 0.063, and a $(A_{TS8}-A_{TS8,0})/A_{TS8,IS}$ value of 3.00 after 10 minutes of colour development using the high activity positive control.

FN1	A_S	A_{S0}	ΔA_S	$\frac{\Delta A_S}{\bar{A}_{S0}}$	RTA	Result
Brain	0.069	0.070	0	0	0	NEG
Cardiac muscle	0.063	0.053	0.01	0.2	2	NEG
Liver	0.072	0.067	0.005	0.1	<1	NEG
Skeletal muscle	0.103	0.059	0.04	0.6	8	NEG
Lung	0.119	0.063	0.056	0.9	22	NEG

Table 4-8. TRAP analysis of necropsy specimens from feline FN2. A_S is the absorbance of the sample, A_{S0} the absorbance of the heat inactivated version of each sample, ΔA_S was determined by subtraction of the mean of the heat inactivated samples ($\overline{A}_{S0}$ or background) from A_S, ΔA_S / $\overline{A}_{S0}$ is the ratio of sample to background activity, and RTA the relative telomerase activity. Samples were considered telomerase positive if the difference in absorbance (ΔA_S) was greater than twice the background activity (ΔA_S / $\overline{A}_{S0} \geq 2.0$). Samples contained low telomerase activity if ΔA_S / $\overline{A}_{S0}$ was between 1 and 2. Using these criteria samples were designated positive (POS), low activity (LOW) or negative (NEG) for telomerase activity. The FN2 assay gave a background absorbance of 0.063, and a $(A_{TS8}-A_{TS8,0})/A_{TS8,IS}$ value of 3.00 after 10 minutes of colour development using the high activity positive control.

FN2	A_S	A_{S0}	ΔA_S	$\frac{\Delta A_S}{\overline{A}_{S0}}$	RTA	Result
Brain	0.071	0.067	0.004	0.1	<1	NEG
Cardiac muscle	0.063	0.072	0	0	0	NEG
Liver	0.056	0.041	0.015	0.24	3	NEG
Lung	0.066	0.061	0.005	0.1	1	NEG

Table 4-9. TRAP analysis of necropsy specimens from feline FN3. A_S is the absorbance of the sample, A_{S0} the absorbance of the heat inactivated version of each sample, ΔA_S was determined by subtraction of the mean of the heat inactivated samples ($\overline{A}_{S0}$ or background) from A_S, ΔA_S / $\overline{A}_{S0}$ is the ratio of sample to background activity, and RTA the relative telomerase activity. Samples were considered telomerase positive if the difference in absorbance (ΔA_S) was greater than twice the background activity (ΔA_S / $\overline{A}_{S0} \geq 2.0$). Samples contained low telomerase activity if ΔA_S / $\overline{A}_{S0}$ was between 1 and 2. Using these criteria samples were designated positive (POS), low activity (LOW) or negative (NEG) for telomerase activity. The FN3 assay gave a background absorbance of 0.038, and a $(A_{TS8}-A_{TS8,0})/A_{TS8,IS}$ value of 2.27 after 10 minutes of colour development using the high activity positive control.

FN3	A_S	A_{S0}	ΔA_S	$\frac{\Delta A_S}{\overline{A}_{S0}}$	RTA	Result
Lung	0.037	0.046	0	0	0	NEG
Cardiac muscle	0.033	0.032	0	0	1	NEG
Brain	0.043	0.032	0.005	0.13	<1	NEG
Liver	0.039	0.035	0.001	0.03	5	NEG
Skeletal muscle	0.037	0.033	0	0	5	NEG
Small intestine	0.052	0.052	0.014	0.37	0	NEG

Table 4-10. TRAP analysis of necropsy specimens from feline FN4. A_S is the absorbance of the sample, A_{S0} the absorbance of the heat inactivated version of each sample, ΔA_S was determined by subtraction of the mean of the heat inactivated samples ($\overline{A}_{S0}$ or background) from A_S, ΔA_S / $\overline{A}_{S0}$ is the ratio of sample to background activity, and RTA the relative telomerase activity. Samples were considered telomerase positive if the difference in absorbance (ΔA_S) was greater than twice the background activity (ΔA_S / $\overline{A}_{S0} \geq 2.0$). Samples contained low telomerase activity if ΔA_S / $\overline{A}_{S0}$ was between 1 and 2. Using these criteria samples were designated positive (POS), low activity (LOW) or negative (NEG) for telomerase activity. The FN4 assay gave a background absorbance of 0.030, and a $(A_{TS8}-A_{TS8,0})/A_{TS8,IS}$ value of 3.97 after 10 minutes of colour development using the high activity positive control.

FN4	A_S	A_{S0}	ΔA_S	$\frac{\Delta A_S}{\overline{A}_{S0}}$	RTA	Result
Liver	0.025	0.035	0	0	0	NEG
Skeletal muscle	0.040	0.035	0.005	0.17	<1	NEG
Small intestine	0.027	0.021	0.006	0.2	<1	NEG

Table 4-11. TRAP analysis of necropsy specimens from feline FN5. A_S is the absorbance of the sample, A_{S0} the absorbance of the heat inactivated version of each sample, ΔA_S was determined by subtraction of the mean of the heat inactivated samples ($\overline{A}_{S0}$ or background) from A_S, ΔA_S / $\overline{A}_{S0}$ is the ratio of sample to background activity, and RTA the relative telomerase activity. Samples were considered telomerase positive if the difference in absorbance (ΔA_S) was greater than twice the background activity (ΔA_S / $\overline{A}_{S0} \geq 2.0$). Samples contained low telomerase activity if ΔA_S / $\overline{A}_{S0}$ was between 1 and 2. Using these criteria samples were designated positive (POS), low activity (LOW) or negative (NEG) for telomerase activity. The FN5 assay gave a background absorbance of 0.043, and a $(A_{TS8}\text{-}A_{TS8,0})/A_{TS8,IS}$ value of 2.69 after 10 minutes of colour development using the high activity positive control.

FN5	A_S	A_{S0}	ΔA_S	$\frac{\Delta A_S}{\overline{A}_{S0}}$	RTA	Result
Brain	0.048	0.043	0.005	0.12	<1	NEG
Kidney	0.041	0.042	0	0	0	NEG
Liver	0.041	0.040	0	0	0	NEG
Lung	0.044	0.044	0.001	0.02	0	NEG
Small intestine	0.047	0.041	0.004	0.09	<1	NEG
Skeletal muscle	0.037	0.047	0	0	0	NEG
Testis	0.171	0.047	0.128	2.98	7	POS

Table 4-12. TRAP analysis of canine tumour samples. A_S is the absorbance of the sample, A_{S0} the absorbance of the heat inactivated version of each sample, ΔA_S was determined by subtraction of the mean of the heat inactivated samples ($\overline{A}_{S0}$ or background) from A_S, ΔA_S / $\overline{A}_{S0}$ is the ratio of sample to background activity, and RTA the relative telomerase activity. Samples were considered telomerase positive if the difference in absorbance (ΔA_S) is greater than twice the background activity (ΔA_S / $\overline{A}_{S0} \geq 2.0$). Samples contained low telomerase activity if ΔA_S / $\overline{A}_{S0}$ was between 1 and 2. Using these criteria samples were designated positive (POS), low activity (LOW) or negative (NEG) for telomerase activity. The canine tumour assay gave a background absorbance of 0.071, and a $(A_{TS8}-A_{TS8,0})/A_{TS8,IS}$ value of 2.10 after 10 minutes of colour development using the high activity positive control. Low telomerase activity results were confirmed by repeat analysis.

Tumour	A_S	A_{S0}	ΔA_S	$\frac{\Delta A_S}{\overline{A}_{S0}}$	RTA	Result
Mammary carcinoma	0.335	0.07	0.264	3.72	11	POS
Mammary carcinoma	0.155	0.07	0.084	1.18	6	LOW
Mammary carcinoma	0.277	0.065	0.206	2.9	8	POS
Mammary carcinoma	0.301	0.07	0.23	3.24	14	POS
Mammary carcinoma	0.272	0.07	0.201	2.83	10	POS
Mammary carcinoma	0.256	0.075	0.185	2.61	7	POS
Mammary carcinoma	0.284	0.073	0.213	3.0	9	POS
Mammary carcinoma	0.15	0.064	0.079	1.11	3	LOW
Mammary carcinoma	0.709	0.074	0.638	8.99	29	POS
Mammary carcinoma	0.363	0.072	0.292	4.11	13	POS
Fibrosarcoma	0.835	0.067	0.764	10.8	54	POS
Fibrosarcoma	0.322	0.074	0.251	3.54	14	POS
Fibrosarcoma	0.646	0.073	0.575	8.10	36	POS
Squamous cell carcinoma	0.24	0.074	0.169	2.38	8	POS
Squamous cell carcinoma	1.428	0.072	1.357	19.1	81	POS

Table 4-13. TRAP analysis of feline tumour samples. A_S is the absorbance of the sample, A_{S0} the absorbance of the heat inactivated version of each sample, ΔA_S was determined by subtraction of the mean of the heat inactivated samples ($\overline{A}_{S0}$ or background) from A_S, ΔA_S / $\overline{A}_{S0}$ is the ratio of sample to background activity, and RTA the relative telomerase activity. Samples were considered telomerase positive if the difference in absorbance (ΔA_S) is greater than twice the background activity (ΔA_S / $\overline{A}_{S0} \geq 2.0$). Samples contained low telomerase activity if ΔA_S / $\overline{A}_{S0}$ was between 1 and 2. Using these criteria samples were designated positive (POS), low activity (LOW) or negative (NEG) for telomerase activity. The feline tumour assay gave a background absorbance of 0.04, and a $(A_{TS8}-A_{TS8,0})/A_{TS8,IS}$ value of 3.90 after 10 minutes of colour development using the high activity positive control.

Tumour	A_S	A_{S0}	ΔA_S	$\frac{\Delta A_S}{\overline{A}_{S0}}$	RTA	Result
Mammary carcinoma	0.510	0.037	0.47	11.8	13	POS
Squamous cell carcinoma	0.232	0.040	0.192	4.8	4	POS
Squamous cell carcinoma	0.481	0.043	0.441	11.0	10	POS
Squamous cell carcinoma	0.477	0.039	0.437	10.9	11	POS

4.4.3 Telomerase activity in immortalised cell lines

Telomerase activity was assessed in the canine immortalised cell lines CML10, MDCK, D17, A72, GHK, CMT3, CMT7 and CMT8, and in a number of immortalised cell lines of human origin (GM847, 293T and 3132T), which were included for comparison of telomerase activity levels. The canine primary fibroblast culture SFA was included as a negative control. Details of the entire panel of cell lines used are provided in Section 2.2.1.2, and the results of the TRAP assay are shown in Table 4-14. Telomerase activity was confirmed in all of the canine immortalised cell lines tested, with RTA values ranging from 3 in the A72 cells to 38 in the CMT3 cells. The human cell lines 293T and 3132T were also positive for activity, and with RTA values of 12 and 47 respectively, the human cell lines displayed a similar level of telomerase activity to their canine counterparts. No activity was detected in the human ALT competent cell line GM847 or the primary fibroblast culture, which were confirmed to be telomerase negative by repeat analysis. A representative autoradiograph showing TRAP PCR products form three immortalised canine cell lines is shown in Figure 4-2.

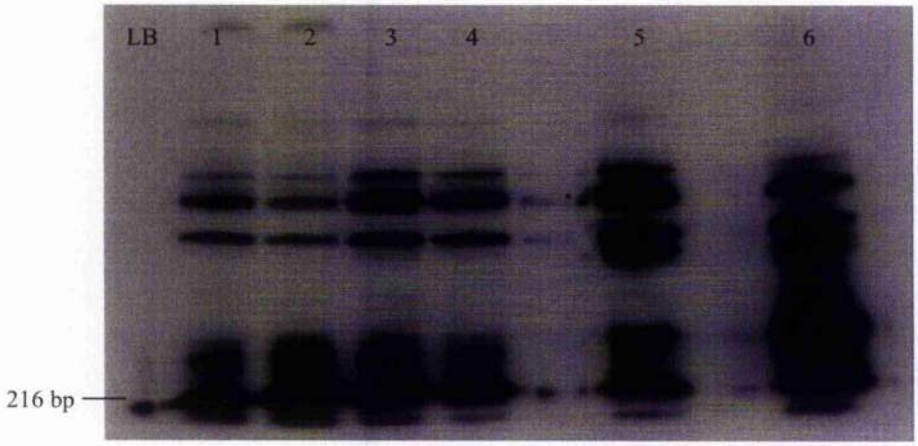
4.4.4 Effect of the RTI AZT-TP on telomerase activity in canine telomerase positive cells

Canine MDCK and CMT7 cells identified as positive for telomerase activity (Section 4.4.3) were used to test the telomerase inhibitory effects of AZT-TP. The initial step in this investigation was to assess the highest concentration of AZT-TP to which the test cell lines could be exposed without inducing an acute cytotoxic effect. To this end, cells were exposed to two-fold dilutions of AZT-TP in a 96 well plate format, whilst control cell cultures were maintained in drug free medium. The initial screening ran for 7 days. Concentrations of 120 μM and above resulted in death of both the cell lines. An AZT-TP concentration of 60 μM and below allowed survival of test cells, and the experiment was then repeated in a T25 flask format with cells seeded at 1 $x10^5$ cells/ml.

Table 4-14. TRAP analysis of immortalised cell lines. A_S is the absorbance of the sample, A_{S0} the absorbance of the heat inactivated version of each sample, ΔA_S was determined by subtraction of the mean of the heat inactivated samples ($\overline{A}_{S0}$ or background) from A_S, ΔA_S / $\overline{A}_{S0}$ is the ratio of sample to background activity, and RTA the relative telomerase activity. Samples were considered telomerase positive if the difference in absorbance (ΔA_S) was greater than twice the background activity (ΔA_S / $\overline{A}_{S0} \geq 2.0$) On these criteria samples were designated positive (POS) or negative (NEG) for telomerase activity. The cell line assay gave a background absorbance of 0.060, and a $(A_{TS8}\text{-}A_{TS8,0})/A_{TS8,IS}$ value of 2.78 after 10 minutes of colour development using the high activity positive control.

Cell line	A_S	A_{S0}	ΔA_S	$\frac{\Delta A_S}{\overline{A}_{S0}}$	RTA	Result
A72	0.287	0.062	0.225	3.75	3	POS
CML10	0.544	0.055	0.489	8.15	10	POS
CMT3	1.194	0.060	1.134	18.9	38	POS
CMT7	0.492	0.072	0.42	7	9	POS
CMT8	0.862	0.071	0.791	13.2	11	POS
D17	0.382	0.058	0.324	5.4	7	POS
GHK	0.605	0.057	0.548	9.1	11	POS
GM847	0.072	0.062	0.01	0.2	<1	NEG
MDCK	0.182	0.052	0.13	2.2	4	POS
SFA	0.064	0.066	0	0	0	NEG
293T	3.45	0.061	3.389	56.5	47	POS
3132T	1.190	0.060	1.13	18.8	12	POS

Figure 4-3. Autoradiograph image of a representative PAGE of TRAP assay PCR products. LB indicates the lysis buffer only negative control, lanes 1 and 2 are the CML10 cell line, lanes 3 and 4 are from the CMT7 cell line, and lanes 5 and 6 from the CMT3 cell line. The 216 bp internal standard is indicated. The banding pattern is caused by the presence of TTAGGG repeat sequences of varying length, produced by active telomerase in the samples.



This repetition was carried out to facilitate harvesting and counting of cells. Cells were passaged upon reaching approximately 80% confluence, and the doubling time for each test and control flask noted. A concentration of 60 μM of AZT-TP caused an increase in doubling time and in the number of dead cells in the culture, as shown in Table 4-15, compared with the control cell lines for both MDCK and CMT7 cells. A 30 μM concentration of AZT-TP did not result in any acute cytotoxic effect to either cell line. This concentration was subsequently used for the main study with both cell lines.

4.4.4.1 Effects of AZT-TP on cell growth parameters

In the main study, test cells were grown in T75 flasks and exposed to 30 μM of AZT-TP throughout the course of the experiment. Control cells were also maintained in T75 flasks in parallel with the test cultures and passage of each culture was carried out as normal. Cell counts and population doubling times for each of the cell lines were recorded at passage and are shown in Tables 4-16 and 4-17. These data were used to generate growth curves for comparison between control and test cell lines, as shown in Figures 4-3 and 4-4. Control CMT7 cells reached 36.2 population doublings over the time course of the experiment (70 days), whilst at 32.5 population doublings the AZT-TP treated CMT7 cells achieved only 3.7 fewer divisions. The more rapidly replicating MDCK control and test cells achieved 52.0 and 45.3 population doublings respectively, a difference of 6.7 population doublings over the 70 days of the experiment. No significant difference in the growth rates between control cells and cells cultured in the presence of AZT were discernible in either the MDCK cells or the CMT7 cells.

4.4.4.2 Effects of AZT-TP on telomerase activity

Telomerase activity was monitored in the control and AZT-TP treated versions of each cell line at a number of time points during the course of the experiment. These data were used to generate an RTA value for each cell type to allow a comparison between the telomerase activity of the control and treated cells, as

shown in Tables 4-18 and 4-19. Figure 4-5 illustrates that AZT-TP exposure did not result in a sustained, demonstrable reduction in telomerase activity in the CMT7 cell line. The equivalent analysis for the MDCK cell line is also shown in Figure 4-5 and established that whilst the AZT-TP treated cells had a generally lower RTA value than the control cells suggestive of a telomerase inhibitory effect, they retained demonstrable telomerase activity. Telomerase activity was found to be lower in the control MDCK cell line than the control CMT7 cell line, and whilst all the AZT-TP treated CMT7 cell pellets assayed for telomerase activity remain telomerase positive ($\Delta A_S / \overline{A}_{S0} \geq 2.0$), passage 32, 44 and 49 of the treated MDCK cell line had $\Delta A_S / \overline{A}_{S0}$ values of between 1 and 2, and are therefore classified as containing low telomerase activity.

4.4.4.3 Effects of AZT on telomere lengths

Telomere length was also monitored in the cell lines using 2 $x10^6$ cell pellets retained at passage. The analysis was carried out as described previously (Section 3.3.3) using standard agarose gel electrophoresis, and was undertaken to determine if exposure to the RTI translated into any reduction in telomere length in the test cells. Mean TRF length was found to be 5.2 kb and 5.1 kb in the control MDCK cells at the first and last passages used in the experiment (passage 30 and passage 48). The AZT-TP treated cells had a mean TRF value of 5.0 kb at passage 30, and 4.9 kb at passage 48. A similar result was obtained for the CMT7 cells. In these cells, both the control and AZT-TP treated cells were found to contain mean TRFs of 4.2 and 4.3 kb at the beginning and end of the experiment respectively (passage 49 to 67). Hence, no discernible change in telomere lengths could be detected in cells cultured in the AZT-TP. The TRF smears generated from the AZT-TP treated cells are shown in Figure 4-6.

Table 4-15. Investigation of acute cytotoxic effect of AZT-TP on CMT7 and MDCK cells. T_c is the average doubling time in hours for cell lines maintained in 0, 30, and 60 μM AZT-TP counted at day 3 and 7 of the experiment; these values are followed by the average percentage of cells found to be dead during cell counting as defined by uptake of trypan blue stain (Section 2.2.1.1.1).

Cell line	T_c 0	% dead	T_c 30	% dead	T_c 60	% dead
CMT7	40.9	<5	35.8	<5	59.4	14
MDCK	19.4	<5	19.3	<5	29.3	9

Table 4-16. Proliferation of CMT7 control and AZT-TP treated CMT7 cells. All cell counts are $x10^6$, T_c indicates the doubling time of the cell populations in hours since the previous time point, and PD indicates the cumulative population doublings undergone by the control and AZT-TP treated cells during the course of the experiment.

Time point	Cell count (control)	Cell count (treated)	T_c (control)	T_c (treated)	PD (control)	PD (treated)
1	3.6	3.0	25.7	30.2	1.9	1.6
2	2.6	3.6	52.1	38.8	3.3	3.5
3	3.2	2.0	42.8	71.8	5	4.5
4	3.0	2.8	60.4	64.4	6.6	6.0
5	3.6	2.0	38.8	71.8	8.5	7.0
6	3.2	3.4	57.0	54.2	10.2	8.8
7	2.7	2.4	50.1	56.8	11.6	10.1
8	6.2	4.6	45.4	54.3	14.2	12.3
9	3.2	3.6	28.5	25.9	15.9	14.2
10	3.4	2.6	81.3	104.1	17.7	15.6
11	2.0	2.0	95.7	95.7	18.7	16.6
12	5.6	5.4	19.2	19.7	21.2	19.0
13	4.2	3.4	34.7	40.6	23.3	20.8
14	7.0	6.6	42.6	43.9	26.1	23.5
15	6.0	5.4	64.8	68.8	28.7	25.9
16	1.6	1.6	105.8	105.8	29.4	26.6
17	6.6	6.6	35.1	35.1	32.1	29.3
18	3.4	3.2	40.6	42.8	33.9	31.0
19	4.8	2.8	31.7	48.3	36.2	32.5

Table 4-17. Proliferation of MDCK control and AZT-TP treated MDCK cells. All cell counts are $x10^6$, T_c indicates the doubling time of the cell populations in hours since the previous time point, and PD indicates the cumulative population doublings undergone by the control and AZT-TP treated cells during the course of the experiment.

Time point	Cell count (control)	Cell count (treated)	T_c (control)	T_c (treated)	PD (control)	PD (treated)
1	6.3	4.0	18.0	23.9	2.7	2.0
2	5.2	2.9	30.2	46.7	5.1	3.5
3	5.3	3.3	29.8	41.7	7.5	5.2
4	6.3	4.3	36.0	45.5	10.2	7.3
5	4.6	2.9	32.6	46.7	12.4	8.8
6	7.7	4.4	32.5	31.3	15.4	11.9
7	6.5	4.3	26.6	34.1	18.1	14.0
8	7.9	8.3	40.1	39.2	21.1	17.1
9	2.8	3.8	32.2	24.8	22.6	19.0
10	7.6	6.7	49.0	52.3	25.5	21.8
11	7.2	5.2	33.6	40.2	28.4	24.2
12	8.2	6.2	15.8	18.2	31.4	26.8
13	6.5	5.6	26.6	28.9	34.1	29.3
14	10.2	8.1	35.7	39.6	37.5	32.3
15	11.3	8.1	47.9	55.5	41.0	35.3
16	4.6	2.8	32.6	48.3	43.2	36.8
17	7.7	7.5	32.5	32.9	46.1	39.7
18	5.3	5.4	29.8	29.5	48.5	42.1
19	11.0	9.0	20.7	22.6	52.0	45.3

Figure 4-4. Growth rates of control CMT7 cells and CMT7 cells exposed to 30 μM AZT-TP. Population doubling of the control cells are represented by blue squares, whilst green squares represent test cells. No significant difference existed between the growth rates of the two cultures.

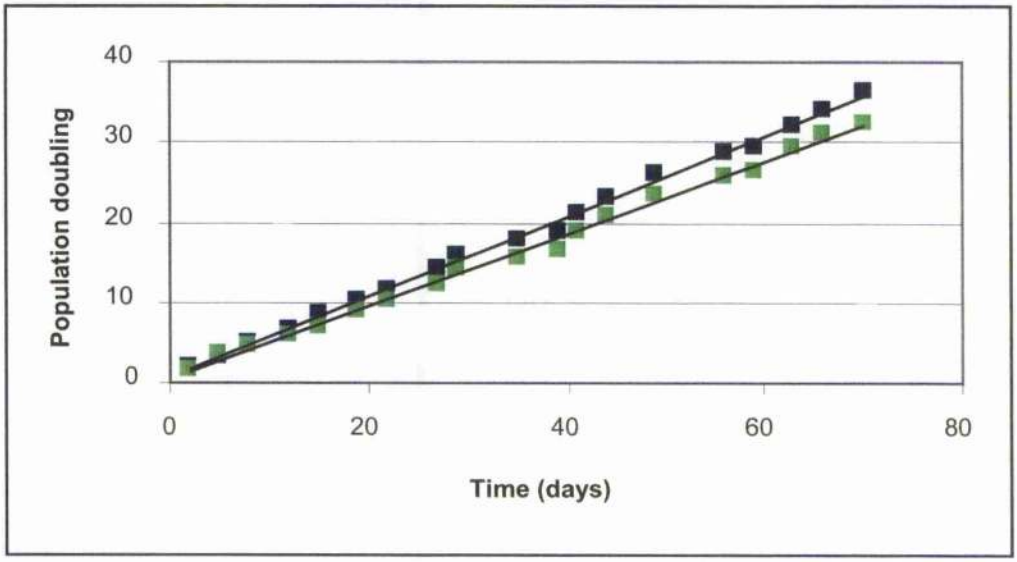


Figure 4-5. Growth rates of control MDCK cells and MDCK cells exposed to 30 μM AZT-TP. Population doubling of the control cells are represented by blue squares, whilst green squares represent the AZT-TP treated test cells. No significant difference in growth rate existed between the two cultures.

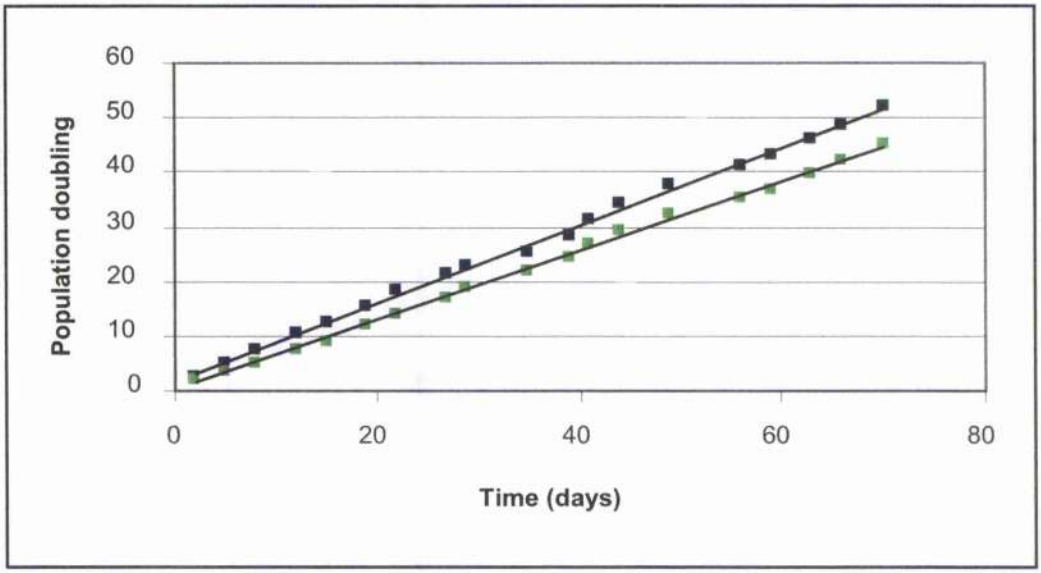


Table 4-18. Relative telomerase activity of AZT-TP treated and control CMT7 cells. A_S represents the absorbance of the sample, A_{S0} the absorbance of the heat inactivated control of the sample, $A_{S,IS}$ the absorbance of the internal standard of the sample and RTA the relative telomerase activity. (Tx) and (Cx) denote AZT-TP treated and control cells, respectively. The assay gave a background absorbance of 0.069, and a $(A_{TS8}-A_{TS8,0})/A_{TS8,IS}$ value of 2.11 after 10 minutes of colour development using the high activity positive control.

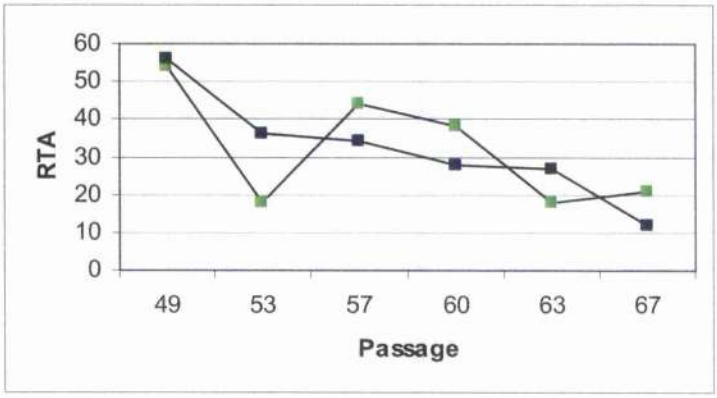
Passage	A_S (Tx)	A_S (Cx)	A_{S0} (Tx)	A_{SO} (Cx)	$A_{S,IS}$ (Tx)	$A_{S,IS}$ (Cx)	RTA (Tx)	RTA (Cx)
49	0.706	1.563	0.064	0.056	0.568	1.279	54	56
53	0.635	1.137	0.066	0.060	1.487	1.394	18	36
57	1.272	1.050	0.072	0.056	1.296	1.384	44	34
60	1.206	0.660	0.075	0.095	1.396	0.955	38	28
63	0.667	0.638	0.062	0.096	1.569	0.940	18	27
67	0.633	0.457	0.096	0.088	1.195	1.492	21	12

Table 4-19. Relative telomerase activity of AZT-TP treated and control MDCK cells. A_S represents the absorbance of the sample, A_{S0} the absorbance of the heat inactivated control of the sample, $A_{S,IS}$ the absorbance of the internal standard of the sample and RTA the relative telomerase activity. (Tx) and (Cx) denote AZT-TP treated and control cells, respectively. The assay gave a background absorbance of 0.075, and a $(A_{TS8}-A_{TS8,0})/A_{TS8,IS}$ value of 2.05 after 10 minutes of colour development using the high activity positive control.

Passage	A_S (Tx)	A_S (Cx)	A_{S0} (Tx)	A_{SO} (Cx)	$A_{S,IS}$ (Tx)	$A_{S,IS}$ (Cx)	RTA (Tx)	RTA (Cx)
30	0.342	0.154	0.074	0.066	2.278	2.728	6	2
32	0.163	0.699	0.074	0.080	1.476	2.134	3	14
37	0.279	0.346	0.068	0.077	2.658	2.166	4	5
39	0.305	0.834	0.076	0.070	1.577	1.401	7	27
41	0.304	0.330	0.073	0.079	1.973	1.786	6	7
44	0.158	0.659	0.078	0.079	2.503	1.534	2	11
49	0.192	0.244	0.075	0.079	2.229	1.624	3	5

Figure 4-6. Effect of AZT-TP on the RTA of CMT7 and MDCK cells. Control cells are represented by blue squares, whilst green squares denote AZT-TP treated cells. Comparison of RTA values established that AZT-TP is ineffective at reducing telomerase activity *in vitro* in the CMT7 cell line. Whilst RTA values of AZT-TP treated MDCK cells are generally lower than the matched control samples the test cells retain active telomerase.

CMT7



MDCK

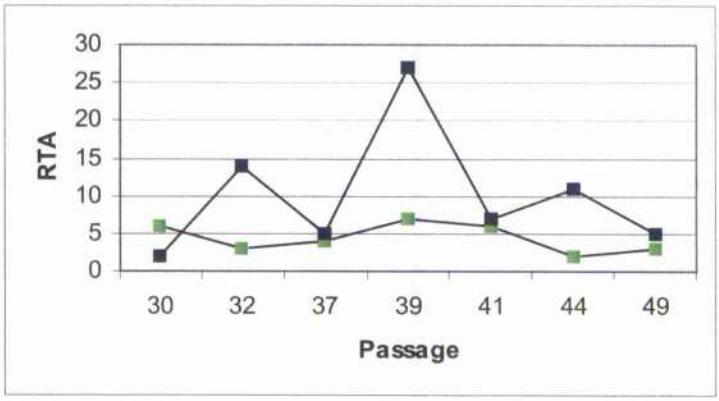
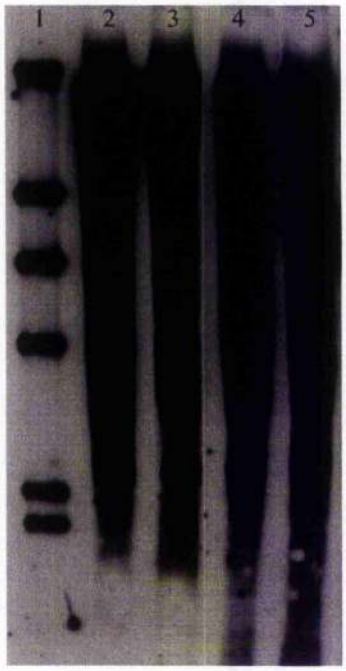


Figure 4-7. TRF analysis of first and last passage AZT-TP treated CMT7 and MDCK cells. Lanes 2 and 3 show passage 30 and passage 48 respectively of the MDCK cell line, corresponding to mean TRFs of 5.0 and 4.9 kb. Lanes 4 and 5 show passages 49 and 67 of the CMT7 cell line, corresponding to TRFs of 4.2 and 4.3 kb respectively. Lane 1 contains the DNA molecular weight marker II (Roche).



4.4.5 Ectopic expression of hTERT in primary canine, feline and equine fibroblasts

4.4.5.1 Generation of a PCIneo/hTERT expression vector

Prior to embarking on the experiments aimed at reconstituting telomerase activity in canine, feline and equine primary fibroblasts, the hTERT DNA was cloned into the PCIneo mammalian expression vector. PCIneo vector DNA was linearised by *Eco*R 1 and *Sal*I restriction digestion, as described in Section 4.3.4.2, and visualised following agarose gel electrophoresis. This generated a fragment of the expected size of approximately 5.6 kb that was then quantified by agarose gel electrophoresis using the Low DNA Mass Ladder (GibcoBRL) following gel purification, as shown in Figure 4-7. Purified hTERT clone was isolated from the pBABE-puro vector and quantified in the same manner and the expected size product of approximately 3.5 kb identified as shown in Figure 4-7. Vector and hTERT DNA were found to be at a concentration of 25 and 10 ng/µl respectively.

Following ligation and transformation, plasmid DNA was isolated from 12 overnight bacterial cultures and the presence of hTERT insert confirmed by *Eco*R 1 and *Sal*I digestion. Eleven of the 12 plasmids were confirmed to contain the hTERT insert as shown in Figure 4-8. The use of two different restriction enzymes ensured the correct orientation of the insert.

Following large sale purification of endotoxin free plasmid DNA (Section 2.2.3.2.1), the DNA quantity and quality was confirmed by UV spectrophotometry. This process was also carried out using PCIneo empty vector to provide sufficient empty vector to act as an experimental control. Plasmid DNA concentration was determined to be 0.12 and 0.14 µg/µl for the empty vector and PCIneo/hTERT construct respectively. A_{260}/A_{280} values for the suspensions were 1.71 and 1.79 respectively, indicating the plasmid DNA was of a sufficiently high quality to be used for transfection.

Figure 4-8. Quantification of PCIneo vector and hTERT insert. A 2 µl volume of the vector and insert were quantified by comparison with DNA fragments contained in the low DNA Mass™ Ladder (GibcoBRL). The ladder contained an equimolar mix of 6 blunt ended DNA fragments of 2000, 1200, 800, 400, 200 and 100 bp. Electrophoresis of a 2 µl volume of this ladder resulted in bands containing 100, 60, 40, 20, 10 and 5 ng of DNA respectively. Samples of unknown concentration were then subjectively quantified by comparing the intensity of the sample bands with the intensities of the bands in the ladder. By this method, the hTERT and vector samples shown were estimated to contain 10 and 25 ng/µl of DNA respectively. The 1 kb plus ladder illustrated that each fragment was of the correct size; 3.5 kb for hTERT and 5.6 kb for PCIneo vector.

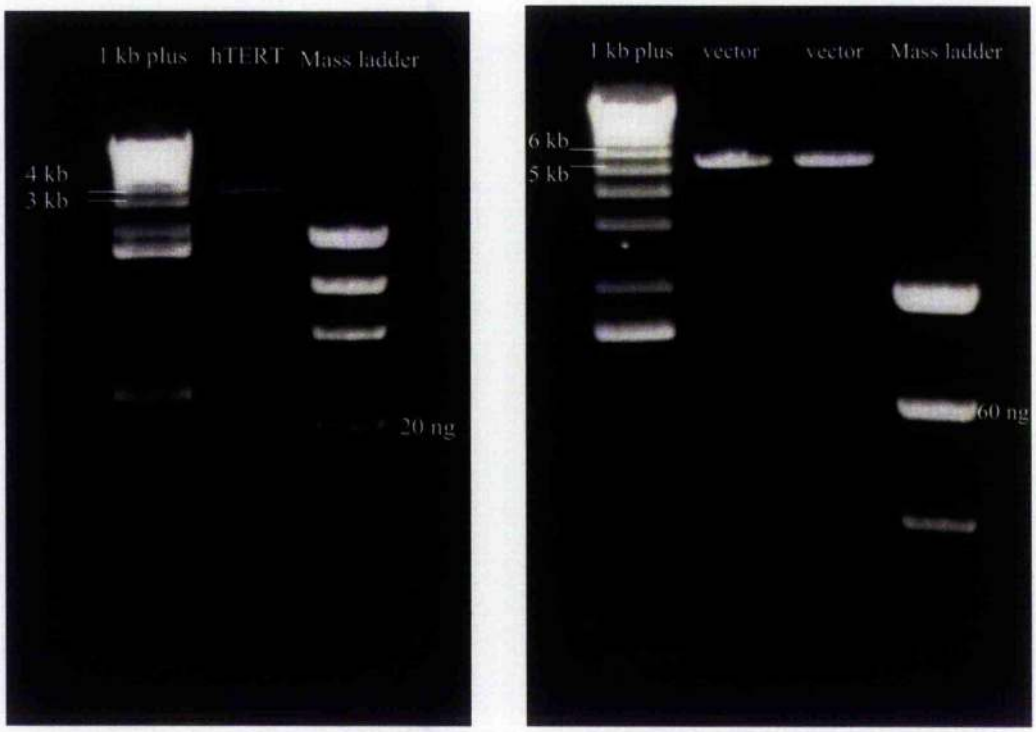
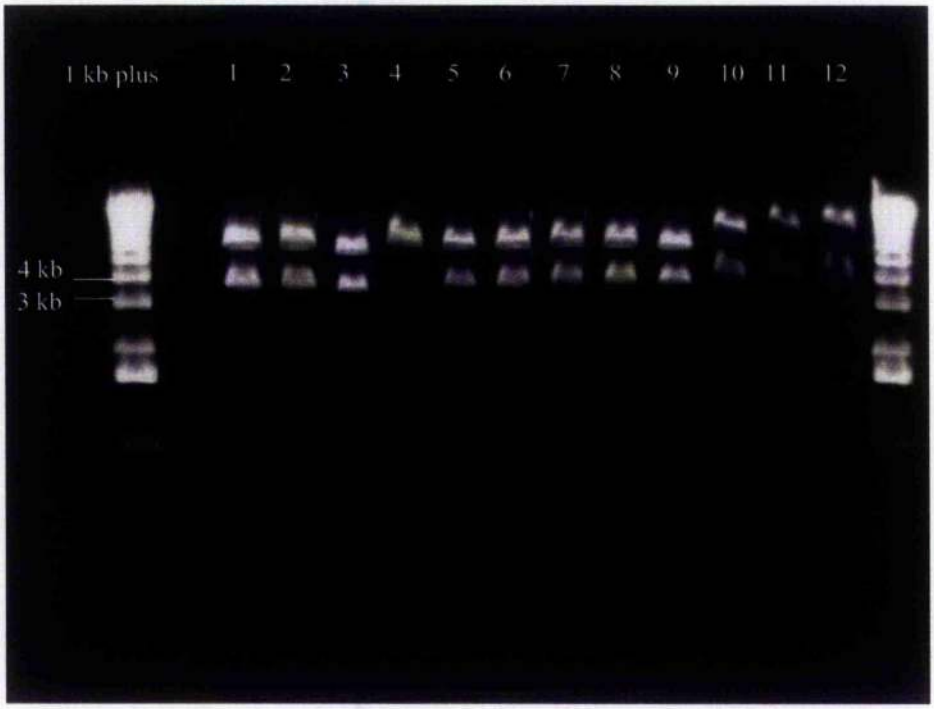


Figure 4-9. Confirmation of ligation of hTERT with PCIneo vector. Plasmid DNA isolated from 12 transformed bacterial colonies was digested with *EcoR* 1 and *Sal* I for 2 hours at 37 °C to cut insert DNA from the PCIneo plasmid backbone. Digestion products were then separated by gel electrophoresis to confirm that a correctly sized insert (3.5 kb) was present in the plasmid samples. This process confirmed the presence of hTERT insert in all samples except number 4.



4.4.5.2 Transfection of PCIneo-hTERT into cells using the Transfast® reagent

Several cell cultures were used to generate stable hTERT expressing cells. These included the canine primary fibroblasts (SFA, AGO8157), feline primary fibroblasts (S22, CCL-176), equine primary fibroblasts (EQ1) and the human ALT cell line (GM847). Further information on these cultures is provided in Section 2.2.1.2. The cells were initially exposed to varying concentrations of G418 antibiotic to determine the concentration required to produce selection for transfected cells. This was carried out in a 24 well plate format. Untransfected SFA, AG08157, S22, CCL-176, GM847 and EQ1 cells were seeded at 1 $x10^5$ cells/ml and after settling overnight were exposed to concentrations of 300, 350, 400, 500 and 600 µg/ml of G418. Control cells were maintained in parallel in drug free medium. In all cases, by day 7, a concentration of 400 µg/ml or above of G418 had resulted in the death of >90% of the cells, and this concentration was adopted for all subsequent experiments.

Each cell line was transfected using a wide variety of conditions to offer the greatest chance of success. In a 24 well plate format, each cell line was transfected with 0.25, 0.5, 0.75, and 1.0 µg of plasmid DNA using a 1:1, a 2:1 and a 3:1 charge ratio in serum free and complete growth medium as described in Section 4.3.4.3.2. After the application of selective medium containing 400 µg/ml of G418, three weeks were allowed for the development of distinct islands of surviving cells to form, representing clonal expansion of successfully stably transfected cells. Selective medium was replaced every 3 days during this period to facilitate removal of dead cells and debris.

The GM847 (human ALT), SFA (canine) and EQ1 (equine) cell lines all developed islands of surviving cells within this timeframe, providing evidence for the generation of stable transfectants. The cells surviving in selective media were trypsinised and pooled to form new cell lines. Each of these three cell lines produced successful transfectants using 1.0 µg of plasmid DNA and a 2:1 charge ratio (TransFast reagent to DNA) in medium containing serum, and the

transfection was also successful in the GM847 cell line using 0.75 μg of plasmid DNA in a 2:1 charge ratio in serum containing medium, and 1.0 μg DNA, with a 2:1 charge ratio in serum free medium.

Stable transfectants could not be generated in any of the other cell cultures (AG08157, S22 or CCL-176) using any of the varying conditions described above. Transfection of these cells was repeated in a 6 well plate format using the most successful conditions described above. This was carried out as the greater cell numbers provided a relatively improved opportunity for successful transfection. The repeat involved scaling the experiment up fivefold to account for the greater area of the wells in 6 well plates. Transfection time was reduced to 30 minutes in an attempt to reduce possible cell damage during the transfection process, but again after 3 weeks in selective medium, no successfully transfected cells were apparent in any of the cell lines, and all of the test cells had been killed by the selective medium. In summary, modifying various transfection protocol parameters including the quantity of DNA, the quantity of transfection reagent, transfection time, presence or absence of serum within the media and increasing the number of cells had no effect on transfection efficiency in the AG08157, S22 or CCL-176 cell cultures.

4.4.5.3 Transfection using the Lipofectamine and Plus reagents

For the cell cultures in which Transfast failed to generate stable transfectants, the transfection process was repeated using a second reagent to investigate if a different liposome formulation would improve efficiency. The AG08157, S22 and CCL-176 cell cultures were transfected as described in Section 4.3.4.3.4. After introducing selective medium to the test plates three weeks was again allowed for removal of dead cells and the emergence of colonies of surviving cells. Using this methodology, cells of the AG08157 fibroblast culture were successfully transfected, however neither of the two feline cultures (S22 and CCL-176) survived the process. These cells exhibited cell death following transfection with Lipofectamine/Plus as observed following transfection using Transfast reagent.

4.4.5.4 Evaluation of hTERT expression in stably transfected cells

To further confirm the generation of stable transfectants, the presence of hTERT mRNA was confirmed in the cells surviving in G418 supplemented medium by RT-PCR as detailed in Section 4.3.4.4, and shown in Figure 4-9. In all cells RT-PCR amplified a product of approximately 400 bp as expected, confirming expression of hTERT. This demonstrated that the transfection process had been successful in the SFA, GM847 and EQ1 cell lines. Too few cells remained after negative TRAP analysis of the hTERT-transfected AG08157 cells to carry out RT-PCR analysis for that cell line.

Further positive confirmation for the generation of hTERT expressing cells was provided by sequence analysis of the PCR products using the ABI PRISM® 3100 Genetic Analyzer as detailed in Section 2.2.6. A BLAST homology search demonstrated that the sequences isolated from the cells showed 100% similarity to the hTERT mRNA sequence. The sequence data is shown in Figure 4-10.

4.4.5.5 Propagation of transfected cell lines and investigation of telomerase activity

Stably transfected cell lines were maintained in normal growth medium supplemented with 400 µg/ml of G418 and passaged normally. However, after pooling of the initial islands of transfected cells, the SFA/hTERT and AG08157/hTERT cell lines did not require further passage as in both cases the cells developed a senescent appearance and ceased replicating before reaching confluence. TRAP analysis confirmed that both hTERT transfected canine cell lines remained telomerase negative.

The GM847/hTERT cell line and the EQ1/hTERT cell line were passaged normally upon reaching approximately 80% confluence, and at each passage cell pellets were retained for TRAP analysis. The results of this analysis for GM847/hTERT are shown in Table 4-20. The GM847/hTERT cell line was

passaged for 80 population doublings post transfection, and was shown to maintain a high level of telomerase activity throughout this time. The replication rate of the cell line remained steady with a mean population doubling time of 58 hours (standard deviation of 17.1 hours). In contrast, the EQ1/hTERT cell exhibited increasing population doubling time with increased passage, and after 23.2 population doublings replication ceased (Figure 4-11). An untransfected EQ1 culture maintained in parallel underwent 23.0 population doublings before entering senescence, as also shown in Figure 4-11. SA-β-GAL staining was carried out in the final passages of both these cell lines, which at 80 and 85% positive were found to contain a majority of senescent cells, as shown in Figure 4-12. Whilst TRAP analysis of the EQ1/hTERT cell line proved telomerase negative at three separate time points corresponding to passages 5, 9 and 15, duplicate TRAP analysis of first passage transfected EQ1/hTERT cells was telomerase positive, as shown in Table 4-21. RT-PCR carried out on RNA isolated from the three telomerase negative passages confirmed that hTERT mRNA could not be detected in any of the three, as shown in Figure 4-13.

In summary, generation of stable transfectants was successful in the SFA, AG08157, EQ1 and GM847 cell lines, as confirmed by RT-PCR and sequence analysis. Whilst hTERT expression was sufficient to reconstitute telomerase activity in a human ALT cell line, no telomerase activity was detected in the hTERT transfected canine fibroblasts. hTERT expression in the equine fibroblasts did reconstitute telomerase activity briefly, but this was not maintained, and the cells subsequently returned to a telomerase negative status.

Figure 4-10. RT-PCR confirmation of hTERT mRNA in SFA/hTERT, GM847/hTERT and EQ1/hTERT cell lines. Duplicate analysis for each cell line was carried out, using both random primers and gene specific primers for the generation of cDNA. Lanes 1 and 2 represent SFA/hTERT, lanes 3 and 4 GM847/hTERT, lanes 5 and 7 EQ1/hTERT, lane 6 is a negative control and lane 8 contains a 1 kb plus DNA ladder. The DNHT001 forward and reverse primers used for the amplification of the cDNA produce a 380 bp product in the presence of hTERT mRNA, therefore the results indicated successful transfection of the SFA, GM847 and EQ1 cell lines.

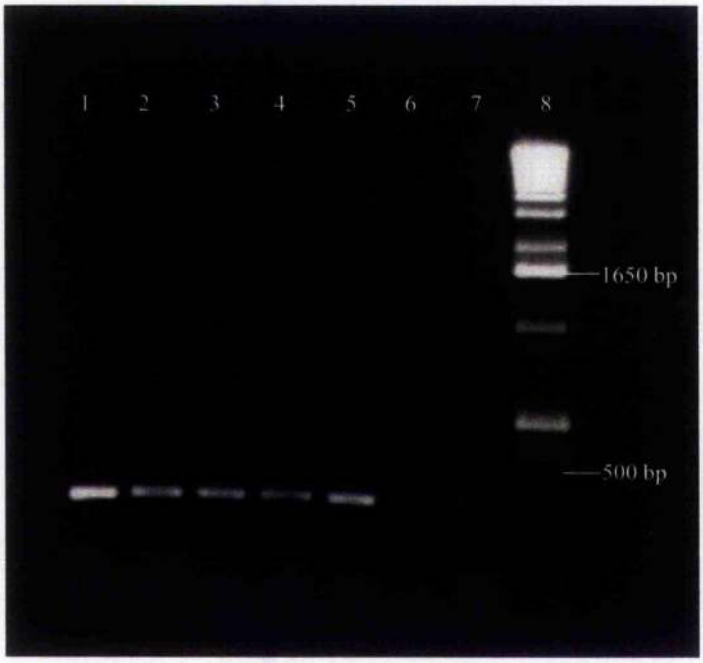


Figure 4-11. Sequence analysis of PCR products generated using the DNHT001 forward and reverse primers for hTERT mRNA. In all three cases the sequence data shared 100% similarity with the hTERT mRNA sequence, confirming the expression of hTERT mRNA in the EQ1/hTERT, GM847/hTERT and SFA/hTERT cell cultures.

EQ1/hTERT (nucleotide positions 2094-2454)

NGGNGGCGGGCGCCCGGCCTCCTGGGCGCCTCTGTGCTGGGCCTGGAACGATAT
CCACANTTNCCTGGCGCACCTTCGTGCTGCGTGTGCGGGCCCAGGAACCCGCCG
CCTGAGCTGTACTTTGTCAAGGTGGATGTGACGGGCGCGTACGACACCATCCCC
CAGGACAGGCTCACGGAGGTCATCGCCAGCATCATCAAACCCCAGAACACGTAC
TGCGTGCGTCGGTATGCCGTGGTCCAGAAGGCCGCCCATGGGCACGTCCGCAAG
GCCTTCAAGAGCCACGTCTCTACCTTGACAGACCTCCAGCCGTACATGCGACAG
TTCGTGGCTCACCTGCAGGAGACCAGCCCGCTGAGG

GM847/hTERT (nucleotide positions 2035-2422)

TACTGTTCAGCGTGCTCAACTACGAGCGGGCGCGGCGCCCCGGCCTCCTGGGCG
CCTCTGTGCTGGGCCTGGACGATATNCACAGGGCCTGGCGCACCTTCGTGCTGC
GTGTGCGGGCCCAGGACCCGCCGCCTGAGCTGTACTTTGTCAAGGTGGATNTNA
CGGGCGCGTACGACACCATCCCCCAGGACAGGCTCACGGAGGTCATCGCCAGCA
TCATCAAACCCCAGAACACGTACTGCGTGCGTCGGTATGCCGTGGTCCAGAAGG
CCGCCCATGGGCACGTCCGCAAGNCCTTCAAGAGCCACGTCTCTACCTTGACAG
ACCTCCAGCCGNACATGCGACAGTTTCGTGGCTCACCTGCAGGAGACCAGCCCG
TTGAGGGATG

SFA/hTERT (nucleotide positions 2084-2410)

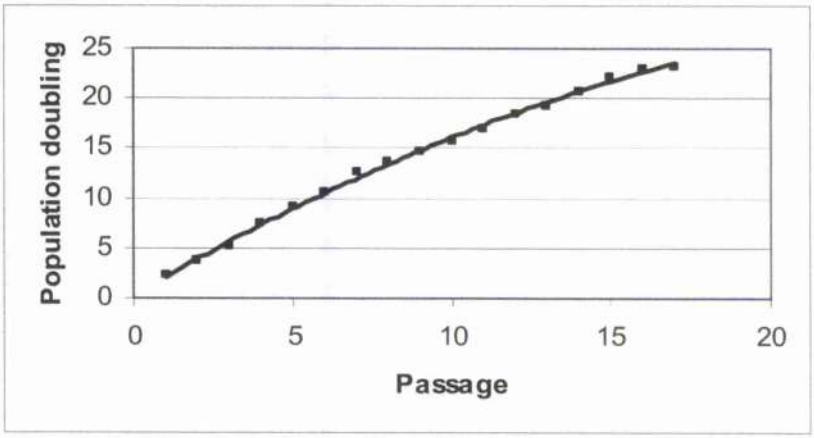
AGCGTGNTNAAACTACGANGCGGGCGCGGCGGCCCCGGCCTTCCTGGGCGCCTC
TGTNCTGGGCCTGGACGATATNCACAGGNCCTGGCGCACCTTCGTGCTGCGTGT
GCGGGCCCAGGACCCGCCGCCTGAGCTGTACTTTGTCAAGGTGGATGTGACGGG
CGCGTACGACACCATCCCCCAGGACAGGCTCACGGAGGTCATCGCCAGCATCAT
CAAACCCCAGAACACGTACTGCGTGCGTCGGTATGCCGTGGTCCAGAAGGCCGC
CCATGGGCACGTCCGCAAGGCCTTCAAGAGCCACGTCTCTACCTTGNCANNCCT
CCC

Table 4-20. Telomerase activity of GM847/hTERT cell line. TRAP analysis was carried out as described previously before transfection and at the passages indicated after transfection. RTA values are not included as the assays were carried out at different times. Telomerase activity was shown to be absent before transfection, and maintained at a high level after transfection. This human cell line control validated the suitability of the TransFast reagent for the transfection of PCIneo/hTERT. A_S represents the absorbance of the sample, and A_{S0} the absorbance of the heat inactivated control of the sample.

Passage	A_S	A_{S0}	ΔA_S	$\frac{\Delta A_S}{\bar{A}_{S0}}$	Result
Pretransfection	0.072	0.051	0.021	0.4	NEG
3	2.039	0.050	1.989	39.8	POS
20	1.627	0.077	1.550	20.1	POS
25	2.197	0.084	2.113	25.2	POS
29	1.430	0.072	1.358	18.9	POS
48	1.557	0.053	1.504	28.4	POS

Figure 4-12. Growth of untransfected and hTERT transfected EQ1 cells. Both cell lines had equivalent replicative potentials of 23 population doublings. The hTERT transfected cell line averaged 1.37 population doublings per passage (s.d. 0.51), whilst the untransfected cell line averaged 2.04 population doublings per passsage (s.d. 0.85).

EQ1/hTERT



EQ1

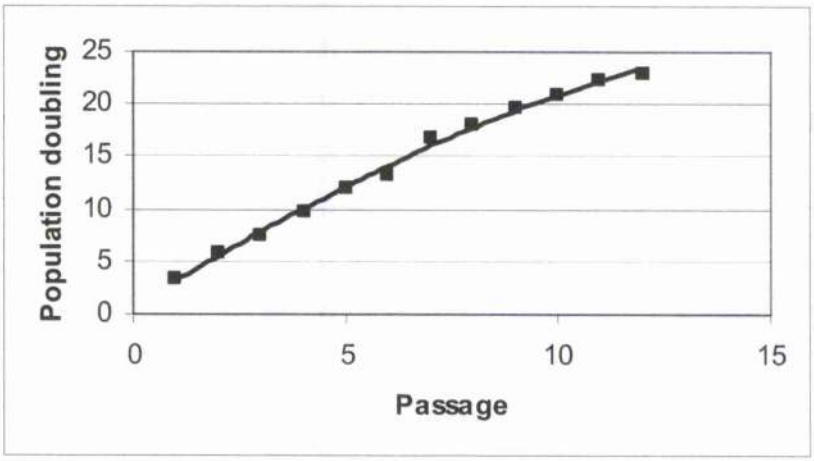


Figure 4-13. SA-β-GAL staining of EQ1 and EQ1/hTERT cell lines. Staining was carried out on the final passage (p12) of the EQ1 cell line, shown in (a) below, and the final passage (p17) of the EQ1/hTERT cell line shown in (b) below. These passages were found to contain 80 and 85% SA-β-GAL positive cells, confirming that both cultures were entering senescence.

(a)

(b)

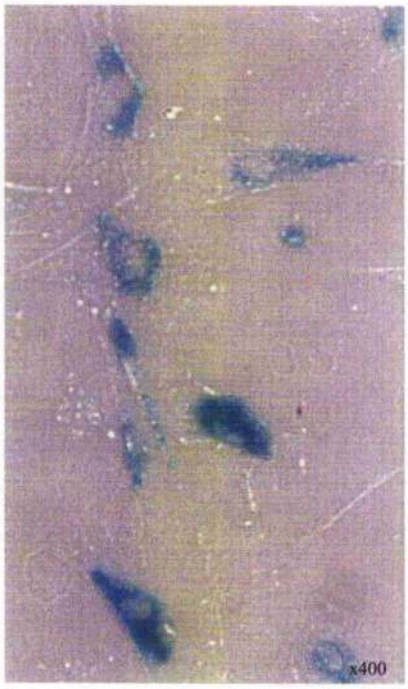
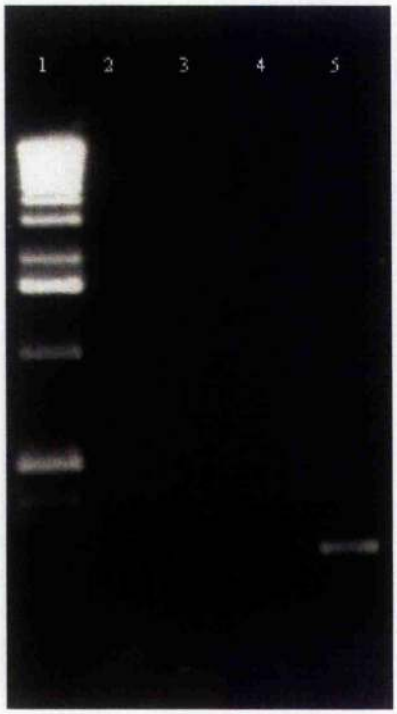


Table 4-21. TRAP analysis of EQ1/hTERT cell line. A_S is the absorbance of the sample, A_{S0} the absorbance of the heat inactivated version of the sample, ΔA_S was determined by subtraction of the mean of the heat-inactivated samples ($\overline{A}_{S0}$ or background) from A_S, and ΔA_S / $\overline{A}_{S0}$ is the ratio of sample to background activity. Samples were considered telomerase positive if the difference in absorbance (ΔA_S) was greater than twice the background activity (ΔA_S / $\overline{A}_{S0} \geq 2.0$). Using these criteria samples were designated positive (POS) or negative (NEG) for telomerase activity. The assays were carried out at different times, however all assays used the high activity positive control, and (A_{TS8}-$A_{TS8,0}$)/$A_{TS8,IS}$ values were between 2 and 4 for each assay. RTA values are not included as results of different assays are shown together.

Passage	A_S	A_{S0}	ΔA_S / $\overline{A}_{S0}$	Result
1	0.184	0.032	4.75	POS
1	0.113	0.030	2.77	POS
5	0.065	0.049	<1	NEG
9	0.049	0.047	<1	NEG
15	0.046	0.046	<1	NEG

Figure 4-14. RT-PCR investigation of hTERT mRNA expression in the EQ1/hTERT cell line. RT-PCR investigation of hTERT mRNA expression in passages 5, 9 and 14 of the cell line EQ1/hTERT, corresponding to lanes 2, 3, and 4 respectively. Random primers were used for the generation of cDNA, the production of which was confirmed by PCR for the equine major histocompatability complex. The DNHT001 forward and reverse primers were then used for the amplification of the cDNA to produce a 380 bp product in the presence of hTERT mRNA. Lane 5 is a positive control (GM847/hTERT), and lane 1 shows the 1 kb plus DNA ladder. These results demonstrate that hTERT mRNA is not expressed in any of the three passages.



4.5 Discussion

4.5.1 Assessment of telomerase activity in canine and feline somatic and tumour tissues

A primary aim of this chapter was to investigate the distribution of telomerase activity in canine and feline somatic and tumour tissues. To identify tissue specific telomerase activity in these species, TRAP analysis of post-mortem healthy organ samples from 5 dogs and 5 cats was carried out. This investigation found telomerase activity to be absent from a wide range of normal canine tissues including canine liver, cardiac muscle, lung, skeletal muscle and kidney, and feline brain, cardiac muscle, liver, skeletal muscle, lung, small intestine and kidney. The only canine sample in which telomerase activity was detected, resulting in an $\Delta A_S / \overline{A}_{S0}$ value of >2.0, was taken from the small intestine of a 6-year-old male dachshund. The positive feline sample was testicular tissue harvested from a 15-year-old DSH. In both these cases, the tissues in question have previously been found to harbour telomerase activity in human samples. Wright *et al* found the tissue of the human testis to be telomerase positive, whilst mature human spermatozoa were telomerase negative (Wright, Mieczyslaw , *et al* 1996). Similarly, stem cells of the lower intestinal crypt have been found to be telomerase positive using an immunohistochemical method in human sections (Forsyth, Wright, *et al* 2002). Overall, these data reveal that telomerase activity is absent from the majority of canine and feline somatic tissues, and highlight a marked similarity between the distribution of telomerase activity in human somatic tissues (Shay & Bacchetti 1997), and the tissues of the dog and cat.

Telomerase activity in the small intestine is likely a reflection of the replicative demand placed upon basal intestinal cells, and as this high turn over is common to the intestinal tracts of both species it is unsurprising that a canine sample was also shown to be telomerase positive. Telomerase activity in the intestine is likely to vary with the rate of cellular turnover, which in turn may vary depending on factors such as the constituents of the diet and the feeding patterns

of the individual animals. It is possible that these variables are the cause of the lower absorbencies of the six other small intestinal samples (four canine and two feline).

Given the function of the testis and ovary and considering previous findings of telomerase activity in human gonadal tissue, the telomerase positive result for feline testis was not surprising, however a canine testicular sample, and a canine ovarian sample were found to have $\Delta A_S / \bar{A}_{S0}$ values of < 2.0. Aside from the issue of telomerase RNA integrity, it is important to note that for a sample to have met the standard for classification as telomerase positive the requirement was for telomerase activity that was at least three times the level of background absorbance. This was a highly stringent system, and it would therefore be misleading to categorically declare all samples that did not meet this standard as telomerase negative. For example, the testicular sample mentioned had an $\Delta A_S / \bar{A}_{S0}$ value of 1.5, meaning that this sample contained telomerase activity that was 250 % greater than the background. It is for this reason that the low telomerase activity ($\Delta A_S / \bar{A}_{S0}$ values of between 1 and 2) class was included. Included in this class were a stomach and small intestinal sample from dogs CN1 and CN2 respectively, in addition to the second testicular sample.

Even with a two-tier classification scheme the ovarian sample from CN1 was still telomerase negative, however rather than this being the permanent state for that particular tissue it is likely that the activity of telomerase in canine and feline ovarian tissues fluctuates with time. This idea is based on recent findings in human ovarian epithelium where it has been shown that it is incorrect to regard this tissue as simply telomerase negative or positive; instead its status is dependant upon the level of the sex hormone oestrogen. This interaction is facilitated by oestrogen receptors that interact directly with a specific set of DNA sequences, the oestrogen response elements (EREs). The EREs are located in the 5′ flanking regions of hormone-regulated genes, and can cause transcriptional silencing or activation of the target. An ERE has been identified within the hTERT promoter, and it has recently been shown that addition of oestrogen to human ovary epithelium cell cultures results in the induction of

hTERT expression and telomerase activity (Misiti, Nanni, *et al* 2000). An equivalent hormonal control mechanism operating in the dog is likely to result in mostly telomerase negative results for canine ovarian tissue, given the long periods of anoestrus and associated periods of low oestrogen levels in the bitch (Johnson 2000). This applies directly to the ovarian sample from CN1, as the animal concerned was 3 months old at time of sampling and therefore still prepubescent with basal oestrogen levels.

Telomerase activity was also assessed in a panel of 15 canine and 4 feline tumour sections harvested during both necropsy examinations and at the time of excisional surgery. The specific association between malignancy and telomerase activity has long been established in the human literature (Kim, Piatyszek, *et al* 1994), and a large scale survey has found telomerase to be active in the majority of human cancers (approximately 80%, Shay & Bacchetti 1997). Given this evidence, the results of the survey carried out in this chapter point to a very similar association between telomerase activity and malignancy in the dog and cat. High telomerase activity (ΔA_S / $\overline{A}_{S0}$ >2.0) was found in 13/15 canine tumours, and 4/4 feline tumours, equating to 89% of the tumour sample population. These results indicate that telomerase is active in the majority of canine and feline tumours. The low telomerase activity (ΔA_S / $\overline{A}_{S0}$ 1-2) found in duplicate analyses of the remaining two canine tumours is very likely to be sufficient for continued cell division, however as work carried out in this chapter and elsewhere (Perrem, Colgin, *et al* 2001) has shown that ALT and telomerase activity can coexist *in vitro* it is possible that the lower telomerase activity in the two samples may be supported by other telomere maintenance mechanisms.

It is also possible that partial degradation of telomerase RNA might have lead to the two lower telomerase results, however considering that all the tumour samples were handled in the same manner this is thought to be an unlikely explanation. In both cases the internal standard of the PCR reactions worked normally indicating there were no inhibitors of the PCR reaction active in the samples. A third possibility is that the particular sample contained too few malignant cells to be detected by the assay. Whilst there will be inherent

variability in the cellular composition of different tumour samples this is not felt to be a major issue for two reasons; first all tumour sections were sampled by harvesting thin full transverse sections of the tissue thus ensuring that tumour cells in the core of the section, and not surrounding normal tissues were analysed. Second, the PCR based TRAP assay is known to be highly sensitive and capable of detecting telomerase activity in as few as 10 positive cells (Shay & Bacchetti 1997). The most plausible explanation is that the 'low' telomerase activity was simply sufficient for the replicative demands of the two tumour samples in question.

In addition, telomerase activity was assessed in a number of immortalised canine and human cell lines, and all of these were found to be telomerase positive with the exception of the human cell line GM847, which is known to use an ALT mechanism for continued proliferation (Bryan, Englezou, *et al* 1995). In this case the RTA values were useful in that they provided a semi-quantitative comparison of telomerase activities between the cell lines. This analysis showed that the CMT3 cell line had relatively the greatest telomerase activity of all the canine cell lines tested. The fact that a greater than 10-fold difference in RTA value between the canine cell lines with the highest and lowest activities (CMT3 and A72 respectively) existed clearly demonstrated that relatively low telomerase activity can allow cellular replication to proceed. That such a broad range exists reinforced the idea that the two borderline tumour samples discussed above might well overcome end replication problem by telomerase activity alone.

Overall, the results of the TRAP analysis have demonstrated that telomerase activity is absent from the majority of canine and feline somatic tissues. Tissues that did contain telomerase activity either contained germ line cells, or were from tissues that are subject to a high replicative burden. In contrast, telomerase activity was detected in all the samples from a panel of canine and feline tumours, and all canine immortalised cell lines that were tested, linking telomerase reactivation in the dog and cat with immortalisation and malignancy.

4.5.2 The *in vitro* inhibition of telomerase activity using the RTI AZT-TP

The use of RTIs to target telomerase in malignant cells is an approach that has recently shown some success in human leukaemia cell lines (Brown, Sigurdson, *et al* 2003). The experiment described in this chapter investigated the effect of a 30 μM concentration of the RTI AZT-TP on two telomerase positive immortal canine cell lines, MDCK and CMT7 (cell lines detailed in Section 2.2.1.2). The ultimate goal of such a treatment is to reduce telomerase activity in the target cells to such an extent that end replication problem will eventually result in growth arrest or apoptosis via critically short telomeres. The concentration of drug used was determined in an initial calibration study, and corresponded to the highest concentration of drug to which the cells could be exposed without inducing an acute cytotoxic effect. This concentration was significantly less than the 100 μM of AZT-TP to which human immortalised cell lines were exposed in a similar study, (Strahl & Blackburn 1996). This is likely a reflection of species differences between the human and canine cell lines.

The experiment ran over a course of 32.5 population doublings for the CMT7 and 45.3 population doublings for MDCK AZT-TP treated cell lines. The experiment was discontinued at these points due to the economic consideration of the cost of AZT-TP, in addition to which data gained from further population doublings would be of little relevance to the possible usefulness of the drug in the *in vivo* setting, as beyond these limits the majority of canine malignancies would have already proven fatal to the affected animal. The relatively poor effect of the drug on the replication rates of the treated cell lines, (i.e. 3.7 and 6.7 fewer population doublings over the entire experiment for the CMT7 and MDCK cell lines respectively), was reflected in the negligible effect on the telomere lengths of the treated cells. This is interesting as in the MDCK cell line, monitoring of RTA values indicated that at several time points in the experiment telomerase activity was reduced in the AZT-TP treated cells compared with the controls. This indicated that residual telomerase activity in the treated cell lines was sufficient to maintain telomere length in the face of partial inhibition. This

again indicated that a wide range of telomerase activity is capable of supporting replication in canine cell lines, and raised the potential that a successful single agent telomerase inhibitor may need to almost completely inhibit telomerase activity in target cells before this translates to a reduction in telomere length sufficient to result in growth arrest or cell death. Whilst these findings are disappointing, and similar to the results of an experiment carried out in human immortalised cell lines (Strahl & Blackburn 1996) it was important to determine that species differences did not effect the susceptibility of canine cells to the RTI inhibitor. Furthermore, the efficacy of RTI inhibitors such as AZT-TP may be greatly enhanced by the concurrent use of other agents that target the telomere itself (e.g. Telomestatin, Tauchi, Kazuo, *et al* 2003). The specific example of Telomestatin is given as this agent, which inhibits telomerase by the formation of stable G-quadruplexes at the telomere terminus, also appears to act at least partly by causing telomere dysfunction (Tauchi, Kazuo, *et al* 2003). This effect will complement the use of RTIs by helping to alleviate any 'lag effect' caused by the strategy of targeting telomerase alone.

4.5.3 Ectopic expression of hTERT in primary fibroblasts

The generation of stable transfectants was successful in the SFA, AG08157, EQ1 and GM847 cell lines, as confirmed by RT-PCR and sequence analysis. Whilst hTERT expression was sufficient to reconstitute telomerase activity in a human ALT cell line, no telomerase activity was detected in the hTERT transfected canine fibroblasts. hTERT expression in the equine fibroblasts did reconstitute telomerase activity briefly, but this was not maintained, and the cells subsequently returned to a telomerase negative status.

The successful entry of DNA into primary fibroblast cultures is difficult due to their limited replicative potential and relative resistance to DNA transfection (Quilliet, Chevallier-Lagente, *et al* 1996), however a number of different strategies have evolved to facilitate this process. These methods include lipid mediated transfection, calcium phosphate transfection, diethylaminoethyl

(DEAE)-dextran transfection, electroporation, biolistics, polybrene, and virus mediated transduction, (reviewed in Sambrook & Russell 2001) and (Ausubel, Brent, *et al* 1994). All these methods rely on different strategies to negate the repulsion between the negatively charged DNA and the negatively charged cell membrane to allow successful DNA entry to the target cells, or they facilitate this entry by a physical (electroporation and biolistics) rather than a biochemical means. Lipid mediated transfer strategies were used in this chapter as these techniques are recommended for use in difficult situations (i.e. primary cultures) where other methods have proved unreliable (Thompson, Frazier-Jessen, *et al* 1999).

Both of the liposome formulations used (TransFast and Lipofectamine) are commercially available, and both have been successful with a wide variety of cell types, including for the Lipofectamine reagent primary fibroblasts (Baker & Cotten 1997). The charge ratios and other conditions that were found to be successful for canine, equine and human cells were typical for those described in the manufacturer's recommended protocols, and validate the choice of reagents. However the manufacturers also note that individual variation in cell lines can result in failure of tested methodologies in specific cases, and this was true for the primary feline cell cultures. This particular type of culture has previously been shown to be resistant to transfection using a liposome formulation (Koksoy, Phipps, *et al* 2001). Furthermore, lipid mediated transfection is known to result in variable toxicity in different cell lines (Sambrook & Russell 2001), and the fact that the feline culture used in this project did not survive the procedure despite repeated attempts and the use of transfection medium that included serum would suggest that the feline fibroblast is at the upper end of this variable toxicity range.

As the main aim of the experiment was to investigate the species specificity of the hTERT gene product, further work concentrated on the successfully transfected canine cell lines (SFA and AG08157). The fact that neither of the transfected cell lines required passage immediately suggested that telomerase activity had not been reconstituted and that the cells had entered senescence. TRAP analysis confirmed that both cell lines remained telomerase negative after

transfection, and the phenotypic appearance of the cells indicated they were senescent. This could not be confirmed by SA-β-GAL staining as the number of cells available never exceeded one sub confluent T25 flask for each culture, and the overriding priorities were the confirmation of transfection and analysis for telomerase activity.

It is interesting that neither the SFA/hTERT or the AGO8157/hTERT cell line required passage despite how each entered the experiment at passage 2, with the majority of replicative potential intact. This may be explained by the very low transfection efficiency of the PCIneo/hTERT construct. In each case, it was noted that only three or four distinct 'islands' of transfected cells repopulated the test flasks after transfection and the introduction of selective medium. Considering that each of these islands derived possibly from only one cell, it is not surprising that the cultures reached replicative exhaustion before confluence. However, this did not occur with the equine cell line, EQ1/hTERT. This cell line underwent 17 passages before finally entering senescence, as confirmed by SA-β-GAL staining. This discrepancy is probably due to one or possibly two factors. First, as confirmed by the control EQ1 cell line (see Figure 4-11), the equine cells had almost twice the replicative potential of the two canine cell lines (see Figure 3-12). Second, transfection of the equine cell line was more efficient than the canine cell lines, resulting in a greater pool of cells to begin the new culture.

Despite the fact that telomerase activity was briefly detected in one early passage of the EQ1/hTERT cell line, this did not increase the replicative potential, as shown in Figure 4-11. Why this cell line should subsequently revert to a telomerase negative status was investigated with further RT-PCR analysis for hTERT mRNA. The negative results from three later passages of post transfection EQ1/hTERT cells implied that hTERT was no longer expressed in the cells, explaining the telomerase negative status. The reason for this loss of hTERT mRNA is likely due to the selection process that occurred after the introduction of selective medium containing G418. Despite the efforts made to confirm that the transfected vector contained the required insert (Figure 4-8) it is possible that some empty vector was also transfected into the primary equine cells, providing G418 resistance without expressing hTERT. These empty vector

cells might have a survival advantage and overgrown hTERT-expressing cells in the culture. It is also possible that the PCIneo/hTERT construct removed the hTERT insert post-transfection, thus producing cells containing empty PCIneo vector. Alternatively, mutation in the primary culture could have enabled untransfected cells to survive in selective medium, and again overgrow hTERT-expressing cells.

The finding that hTERT had the capacity, albeit briefly to reconstitute telomerase activity in a primary equine culture raises the question of why this was not possible in the primary canine cell lines in which hTERT mRNA was detected. The main issue surrounding this question is the fact that the experiment was carried out with the premise that hTERT regulation alone determined the telomerase status of the cell lines investigated. This was not an unreasonable presumption, given that ectopic expression of hTERT is sufficient to reconstitute telomerase activity in a number of human tissues (Bodnar, Ouellette, *et al* 1998), and given the experimental success detailed in this project with the GM847/hTERT cell line. However, evidence exists that hTERT status is not alone in determining whether telomerase is active (Liu, Schoonmaker, *et al* 1999). Successful activation of telomerase in the canine cell lines would require that no post-transcriptional inhibition of hTERT mRNA was active, and that the hTERT gene product associated successfully with mature canine telomerase RNA in a process that would require the participation of numerous cellular factors for both maturation of the RNA and association of this factor with the catalytic component (reviewed in Collins & Mitchell 2002). It is not unreasonable to suggest that one of these many factors, including cleavage of the primary telomerase RNA transcript; primary and secondary structural rearrangements to facilitate association with the catalytic component (Mitchell & Collins 2000), and association with other protein elements necessary for maturation (Martin-Rivera & Blasco 2001) may have been inactive, inhibited or otherwise unsuited to the production of functionally active telomerase. That the experiment was transiently successful in an equine cell line underscores the point that differing status of these co-factors, rather than species variation might be the cause of the continued telomerase inactivity in the hTERT transfected canine cell lines. An avenue for further investigation is to test this hypothesis by

repeating the experiment using the recently cloned cTERT sequence (submitted for review Nasir, Gault, *et al*, 2004).

4.6 Summary

The experiments detailed in this chapter have demonstrated that telomerase activity is strongly down regulated in a wide range of somatic tissues of the dog and cat. The only normal tissues found to contain telomerase activity at a low level were canine and feline testis, and canine small intestine and stomach. Telomerase activity in canine and feline tissues is however associated with immortalisation and malignancy; telomerase activity was detected in 19/19 tumour samples analysed. Telomerase activity was also detected in a panel of canine immortalised cell lines, and the relative telomerase activity in these cell lines was similar to that detected in immortalised cell lines of human origin. The attempt to reconstitute telomerase activity in canine and feline primary fibroblasts was ultimately unsuccessful. Whilst the hTERT sequence was successfully transfected into primary canine fibroblast cultures, the cells remained telomerase negative and entered replicative senescence normally. This may be a product of significant differences between the human and canine TERT sequences. Overall, the biology of telomerase in the dog and cat is very similar to that found in humans, and the research described in this Chapter has identified telomerase as a promising target for the development of future cancer treatments for companion animals.

Chapter V

Gene expression profiling in association with telomerase reactivation and the onset of replicative senescence

5.1 Abstract

Telomeres and telomerase show considerable promise as targets for new anti-cancer treatments; furthermore they may provide insights into the mechanisms of phenotypic ageing, and therapeutic reactivation of telomerase may have applications for the treatment of conditions where loss of replicative potential is a major part of the pathology, such as liver cirrhosis. This wide range of potential applications is reflected in the availability of a wealth of information on the biology of telomeres and telomerase in the human literature; however there is a paucity of information regarding the wider effects of replicative senescence and telomerase reactivation on the transcriptome of the mammalian cell. A wider view of the effects of telomeric attrition and telomerase reactivation than is currently available will be necessary to assess the safety of any potential therapeutic intervention involving telomeres and telomerase. Recent technological advances in the development of cDNA microarrays, typified by Affymetrix GeneChips® have allowed researchers to take a global view of changes in gene expression in human cells, and this technology can be used to investigate the cellular consequences of telomeric attrition and telomerase

reactivation at the level of the transcriptome. The experiments detailed in this Chapter describe the use Affymetrix technology to carry out an investigation of the changes in mRNA expression levels as canine primary fibroblasts change from actively replicating to senescent, and following the reconstitution of telomerase activity in a human cell line.

5.2 Introduction

Genome projects worldwide have vastly increased our knowledge of the genomic sequences of humans and other organisms, as well as the genes that they encode. However, until relatively recently the study of the regulation and function of these genes has been restricted to labour intensive step-by-step analysis of individual genes. The last decade has seen a major advance in the field with the advent of DNA microarrays, consisting of thousands of DNA probes immobilised on a solid surface and hybridised against fluorophore labelled cDNA or cRNA targets from template RNA sources. Although the technology is heralded as breakthrough it is in essence a variation of a standard Southern Blot (Southern 1979) with the traditional nitrocellulose membrane replaced by a glass slide. DNA microarrays, along with Serial Analysis of Gene Expression (SAGE) (Patino, Mian, *et al* 2003) have become the core technology used in gene expression profiling, and are also of major importance in the fields of comparative genomics and genotyping (Harrington, Rosenow, *et al* 2000). The main reason for the wide application of this technology is the growing awareness of the limitations imposed upon what may be extrapolated from research based around a 'gene by gene' approach, as typified by the Northern blot (Ausubel, Brent, *et al* 1994). If the aim of genomics is to advance understanding of the organisation and evolution of genomes (McKusick & Ruddle 1987) then attempting to place the relevance of each single gene individually is a hopelessly ineffective approach to a biological system with the complexity of a mammalian genome containing 30,000-40,000 genes (Venter, Adams, *et al* 2001) and with complexities such as polymorphic variation, time and place of expression of RNAs and intermolecular interaction of gene products.

DNA microarrays take a 'global' approach to gene expression studies, allowing a simultaneous readout of all the relevant components at a given time, thus allowing for rapid assessment of gene expression profiles in disease states and thereby representing a technological breakthrough in the analysis of biological specimens. The technology also allows researchers to take a broader view than

was possible before the advent of the technology, and this global approach allows the identification of complex patterns of interaction that are invisible at the individual component level. In effect, the more genes and biological conditions studied simultaneously the more obvious the underlying organisation becomes.

5.2.1 Applications of microarray technology

Microarray technology, despite being relatively new, has already proved very useful to researchers and has lead to significant advances in the molecular classification of tumours and the discovery of subsets previously not known to exist. Notably, gene expression profiling using a DNA microarray has led to the discovery of two distinct forms of diffuse large B-cell lymphoma likely arising from two distinct non-transformed cellular progenitors. These two new groups are associated with significantly different prognoses, and thus the use of the technology has directly lead to more reliable predictions of the clinical course of the disease and the expected treatment response (Alizedeh, Eisen, *et al* 2000).

Furthermore, use of a DNA microarray has allowed Sorlie *et al* to classify breast carcinomas by gene expression profile. This system is sufficiently robust to be used as a basis for judgements on prognosis in a subset of patients receiving uniform therapy and has lead to the identification of a subset of tumours based on an oestradiol receptor positive group not previously known to exist. This example is particularly interesting as it represents an advance in understanding directly attributable to the microarray approach where previous research based on individual genes in isolation could not correlate established findings with clinical outcome (Sorlie, Perou, *et al* 2001). Microarray technology also has potential for use in drug discovery applications by facilitating identification of novel drug targets. The technology has already proved useful in antibiotic development (Ivanov, Schaab, *et al* 2000).

A clear application of this technology to the field of telomere biology is the use of microarrays to help assess the safety of telomerase reactivation as a therapeutic approach to conditions such as liver cirrhosis (Rudolph, Chang, *et al*

2000) and large bone defects (Shi, Gronthos, *et al* 2002). Of paramount importance to such a therapeutic strategy will be the safety of reactivating telomerase in an already diseased tissue given the clear association between telomerase and cancer (Kim, Piatyszek *et al* 1994). Initial studies have identified an encouraging lack of cancer associated changes in the phenotype of telomerase immortalised cells (Jiang, Jimenez, *et al* 1999) (Morales, Holt *et al*, 1999), however an association between immortalisation by telomerase and activation of the oncogene c-myc has been found in human mammary epithelial cells (Wang,J, Hannon,G *et al* 2000). This study alone suggests that the use of telomerase reactivation for therapeutic purposes must be approached with caution. To this end, the global view of the transcriptome provided by microarray technology provides an excellent tool with which to identify previously overlooked consequences of telomerase reactivation and telomeric attrition.

5.2.2 Microarray platforms

The two main forms of arrays are cDNA, 'spotted' arrays and oligonucleotide arrays. The technology is essentially the same in both types, and consists of well characterised and annotated hybridisation probes arranged on glass microscope slides or nylon membranes, against which sample fluorophore labelled cDNA or cRNA is applied and allowed to hybridise. The advantage of the spotted arrays is that they may be manufactured in the laboratory, and therefore tailored to suit specific experimental needs. Such arrays are usually robotically spotted onto glass slides by a commercially purchased 'arrayer' robot. The main advantages of the oligonucleotide arrays are that they are much more comprehensive; the manufacturing process allows significantly smaller individual spots to be produced (50-150 um in diameter, McGall, Barone, *et al* 1997) allowing approximately 15,000-20,000 genes to be accommodated on each array, rather than the several hundred genes usually represented on spotted arrays. This allows interrogation of the entire transcriptome, allowing the detection of relevant changes that would be missed by a tailored array. Second, as oligonucleotide arrays are available commercially, this technology facilitates comparison of results between laboratories. Oligonucleotide array technology is

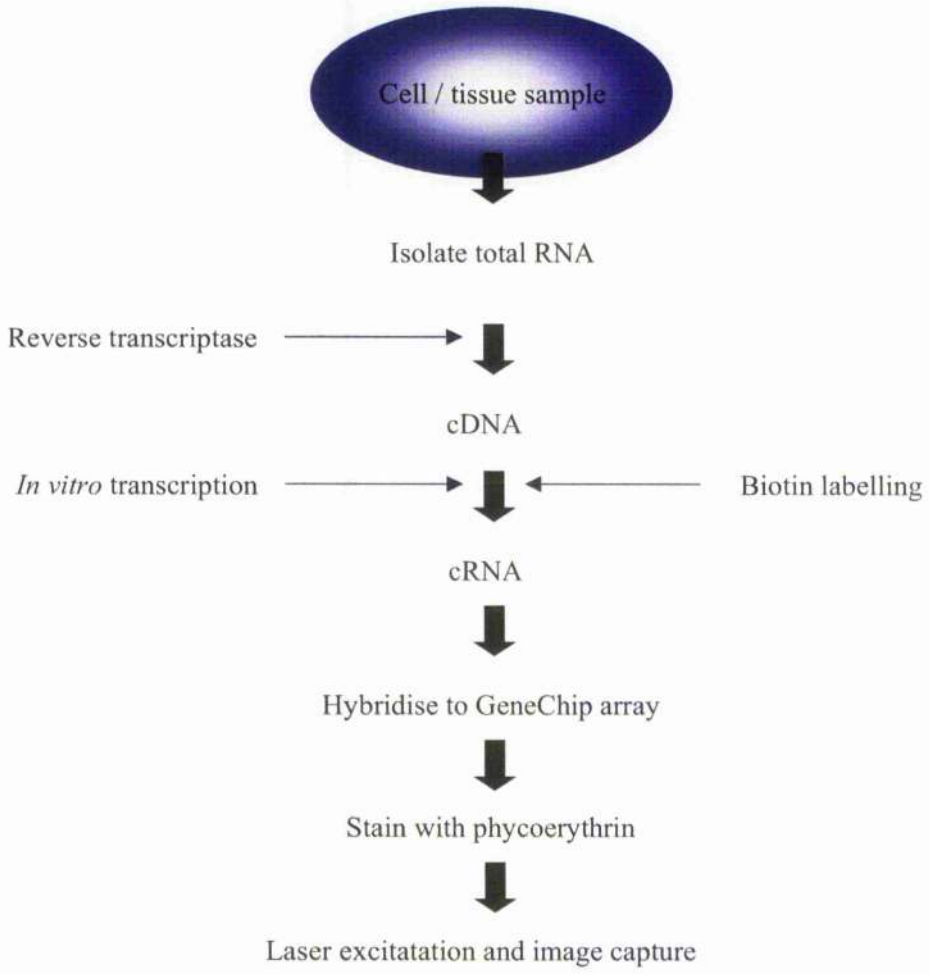
typified by Affymetrix GeneChip® arrays, an overview of which is provided in Figure 5-1. As these arrays were used in all the experiments detailed in the chapter this technology will be the focus of discussion.

5.2.3 Affymetrix GeneChip® arrays

The GeneChip® (Affymetrix, Santa Clara, CA) oligonucleotide arrays consist of high-density synthetic oligonucleotide probe sets on a 1.28 x 1.28 cm glass surface, with a capacity of up to one million probe sets per array in experimental versions. The system has the advantage that sequence data alone is sufficient for array manufacture without intermediate steps such as cloning or PCR. In addition as little as 200 to 300 bases of sequence is required to generate independent, usually non-overlapping 25 base oligonucleotide probe sets unique to the target. The sequence chosen for oligonucleotide formation is usually selected from the 3′ end of the gene sequence to reduce problems that might arise from the use of partially degraded mRNA.

A number of controls are built into the system to reduce the likelihood of false positives and interference from background signal. Crucial to the process is the use of probe redundancy, meaning that a number of oligonucleotides of varying sequence are designed to hybridise to different regions of the same mRNA. The use of multiple detectors for the same molecule augments results by improving signal to noise ratios (due to averaging over the intensities of multiple array features) and improves the accuracy of RNA quantitation (by averaging and outlier rejection). This greatly reduces the effects of cross-hybridisation and miscalls (Lipshutz, Fodor, *et al* 1999), and allows the system to provide an accurate representation of the relative abundance of each targeted transcript in the sample, referred to as the expression value.

Figure 5-1. Overview of a GeneChip® microarray experiment. The aim of any microarray experiment is to detect a given mRNA species (transcribed genes) in a tissue or cell type. In a GeneChip experiment this is achieved by reverse transcribing the RNA isolated from the sample to the more stable cDNA state. *In vitro* transcription is used to produce biotin labelled cRNA that is hybridised to the array surface, and stained with a fluorophore for detection.



A second control is the use of mismatch (MM) oligonucleotides for every perfect match (PM) contained on the array. MM oligonucleotide probes differ by only one base in a central position from their PM counterparts, and therefore allow a direct subtraction of background signal to be made for each probe set, in addition to providing another specificity safe-guard. The use of PM/MM probe sets are the best way to ensure that fluorescence signals are being generated by the intended target mRNA, and allow accurate quantification of the RNA molecule in the target solution (Lipshutz, Fodor, *et al* 1999).

5.2.3.1 Affymetrix data collection and normalisation

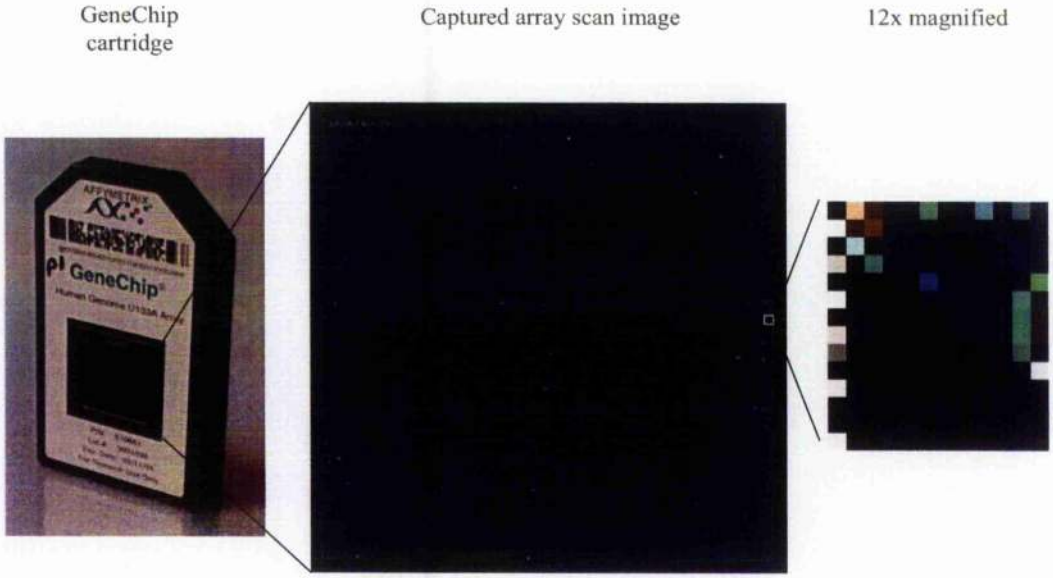
The fluorophore signal from the hybridised target cRNA (Figure 5-1) is stimulated by means of laser excitation directed through the back of the glass array support focussed at the interface of the array surface and the target solution. Fluorescence emission is collected by a lens coupled to a sensitive detector through a series of optical fibres allowing a two-dimensional image of hybridisation intensity to be obtained. These images must then be analysed to identify arrayed spots and measure the relative fluorescence intensities. A typical GeneChip® DNA array is the Human genome (HG)U133-A and B chipset, which contains over 1 million oligonucleotides targeting over 33,000 human genes in a two chip set. A representative scan image from a HGU133-A GeneChip is shown in Figure 5-2.

Each GeneChip® experiment presents researchers with a very large volume of information, so much so that initial problems with the technology tended to revolve around data analysis rather than experimental procedure. Data analysis software, designed to reliably extract biological information and aid meaningful interpretation has been developed in tandem with the technological hardware. The GeneChip® array images utilised in this chapter used Affymetrix® Microarray Suite software (MAS) for acquisition and processing of the GeneChip generated data. This software package also provides indicators of sample integrity, assay execution, and hybridisation performance through the assessment of control hybridisations. The MAS software evaluates the

abundance of each transcript represented on the array and labels it as either present, absent or marginal. An algorithm identifies and removes contributions from stray hybridisation signals, and combines the results from probes that interrogated different fragments of the same transcript. The statistical significance of each detection call is indicated by an associated p-value.

The software allows for adjustments to be made to sensitivity and specificity as appropriate for a particular experimental design, for example if the main thrust of the experiment is high sensitivity the parameters may be changed, requiring a less stringent p-value for a positive call. This has the effect of increasing the sensitivity, however the number of miscalls is increased.

Figure 5-2. Representative Affymetrix GeneChip array scan for HGU133-A chip. The chip contains a total of 22,283 oligonucleotide targets with which fluorescently labelled complimentary nucleic acid targets may bind. Subsequent laser excitation through the glass back of the array cartridge produces fluorescent emission that is recorded as the final image. The complexity of the resultant scan is only appreciable when magnified, as shown in the 12x-magnified image below on the right. Automated image capture and processing is carried out using microarray suite software (Affymetrix), and analysis of the processed results uses GD Expressionist Analyst and Refiner software (Genedata).



Interrogation of the processed results is then performed using GD Expressionist™ Refiner and GD Expressionist™ Analyst software (Genedata, Basel, Switzerland). The Expressionist software is a bundle of client server based programs that are specifically for use with Affymetrix technology (for tutorial see **http://www.msi.umn.edu/software/expressionist/**). The Refiner is a tool for assessing, standardising and tracking microarray data. It allows quality control when comparing data from different GeneChip microarrays, identifying and highlighting errors in individual microarrays and standardising the data quality from all arrays in the experiment. The Analyst component allows user defined investigation of the biological context of the standardised data. For example, this program allows a direct comparison of gene expression levels at different time points in an experiment using data obtained from separate microarrays.

5.2.4 Chapter aims

The aims and objectives of this chapter were

1. To investigate changes in gene expression with increasing cell division in a primary canine fibroblast culture (SFA) as the cells switched from actively replicating to senescent, and to identify potential genes that may be associated with cellular ageing and limiting cell proliferation.

2. To investigate changes in gene expression profiles in human ALT immortalised cells (GM847) following reactivation of telomerase activity, to identify genes that may be associated with an immortalised phenotype.

5.3 Materials and Methods

5.3.1 Sample details

5.3.1.1 SFA

Cell pellets containing 2 x 10^6 cells were harvested at passage from passages 2, 4 and 10 of the primary canine fibroblast cell culture SFA as described elsewhere (Section 3.3.1.4). Cells were washed in ice-cold PBS, repelleted and after removal of the supernatant were flash frozen in LNO_2 and stored at -80°C until required. These passages were chosen to reflect a change in the culture from actively replicating, (at passage 2 < 5% SA-β-GAL positive) to a majority of senescent cells (at passage 10 80% SA-β-GAL positive), as detailed in Section 3.4.4.3. Too few cells were available from the two later passages of the SFA culture for analysis.

5.3.1.2 GM847 and GM847/hTERT

Pellets of 2 x10^6 cells were harvested at passage and stored as described above. Four time points were analysed in this experiment, the first corresponding to the passage of the GM847 cell line that was used for the hTERT transfection experiment (p13). The remaining three time points utilised different passages of the hTERT expressing, telomerase positive cell line GM847/hTERT, including p1, p19 and p26.

5.3.2 Isolation of total RNA from canine fibroblasts

Total RNA was isolated from primary canine fibroblast cultures using the RNeasy Total RNA Isolation Kit (QIAGEN, UK) following the manufacturer's recommended protocol. Isolated RNA was eluted in 35 µl of RNAse free water and then precipitated by addition of 1/10 volume of 5 M NaOAc at pH 5.2 (Sigma, UK), and 2.5 volumes of 100% ethanol. Samples were thoroughly mixed and incubated at -20°C for one hour before pelleting at 14000 g for 20

minutes at 4°C. Glycogen (Ambion, UK) was added (1 µl of a 5 mg/mL solution) as a co-precipitant to aid visualization of the RNA pellet. RNA quality and quantity were confirmed using the Nanodrop and Agilent 2100 Bioanalyzer (Agilent Technologies). A_{260}/A_{280} ratios between 1.9 and 2.1 were considered acceptable.

5.3.3 Synthesis of double-stranded cDNA from total RNA

5.3.3.1 First strand cDNA synthesis

Synthesis reactions were carried out in 1.5 ml RNAse free tubes. A 5 µg amount of total RNA was used for each sample. This protocol uses only high performance liquid chromatography (HPLC)-purified T7 Oligo(dT)$_{24}$ primer (100 pmol/µl, Affymetrix) as this is essential for efficient downstream applications. RNA and primer were made up to a total volume of 20 µl with DEPC treated H_2O. Primer hybridisation proceeded for 10 minutes at 70°C followed by a brief centrifugation step and quenching on ice. To each sample 4 µl of 5x First strand cDNA buffer (Invitrogen, UK), 2 µl of 0.1 M DTT and 1 µl of 10 mM dNTP mix (Invitrogen) were added, samples were mixed well and then incubated at 42°C for 2 minutes. A volume containing 200 U of SuperScript II reverse transcriptase enzyme (Invitrogen) was then added to each sample, mixed thoroughly and incubated at 42°C for 1 hour.

5.3.3.2 Second strand cDNA synthesis

First strand reactions were placed on ice following a brief centrifugation step to bring down condensation on tube sides. To each sample was then added the components of the second strand reaction; 30 µl of 5x second strand reaction buffer (Invitrogen), 3 µl of a 10 mM dNTP mix, 10, 40 and 20 U each of the enzymes *E.coli* DNA Ligase, *E.coli* DNA polymerase I and *E.coli* RNAse H respectively (all sourced at Invitrogen), and 91 µl of DEPC treated water for a final total volume of 170 µl. Second strand reaction mixtures were gently mixed and then incubated at 16°C for 2 hours, after which 10 U of T4 DNA polymerase

was added to each sample, samples were returned to 16°C for a 5 minute incubation before addition of 10 µl of 0.5 M EDTA to each sample. Samples were then ready for a cDNA clean-up procedure.

5.3.4 Clean-up of double-stranded cDNA

The cDNA clean-up procedure involves a standard phenol/chloroform/isoamyl alcohol extraction and the use of phase lock gel (PLG) (Eppendorf, UK) in 1.5 ml eppendorf tubes. PLG forms an inert seal between the aqueous and organic phases and so allows complete recovery of the aqueous phase without contamination from the interphase. An equal volume of (25:24:1) phenol:chloroform:isoamyl alcohol saturated with 10 mM Tris-HCl pH 8.0, 1 mM EDTA was added to the final cDNA synthesis preparation and vortexed before transfer to the 1.5 ml eppendorf tube containing pelleted PLG. Samples were then centrifuged at 14000 g for 2 minutes, and the resulting upper aqueous phase transferred to a fresh 1.5 ml tube.

5.3.5 Ethanol precipitation

Precipitation of purified cDNA was achieved by addition of 0.5 volumes of 7.5 M NH_4OAc and 2.5 volumes of absolute ethanol (stored at -20°C) followed by vortexing. cDNA was immediately pelleted by centrifugation at room temperature for 20 minutes, followed by two wash steps in 80% ethanol (stored at -20°C). cDNA pellets were then air dried thoroughly before resuspension in 12 µl of RNAse-free water.

5.3.6 Synthesis of biotin-labelled cRNA

In vitro transcription (IVT) and biotin labelling were carried out using the ENZO® Bioarray HighYield RNA transcript labelling kit (Affymetrix). Briefly, 5 µl of each of the 12 µl cDNA samples were used per reaction, 7 µl being held in reserve. To each 5 µl sample was added 4 µl of 10x HY reaction buffer, 4 µl of biotin labelled ribonucleotides, 4 µl of 10x DTT, 4 µl of 10x RNAse inhibitor

mix, 2 µl of 20x T7 RNA polymerase and 17 µl of DEPC treated water for a total volume per reaction of 40 µl. Reactions were mixed by gentle pipetting, briefly spun to bring down mix from the sides of the reaction vessels and incubated at 37°C for 4 hours. This is the optimal time for producing good quality biotin labelled cRNA; overnight incubation has been found to produce relatively shorter products.

5.3.7 Clean-up and quantification of IVT products

5.3.7.1 Clean-up procedure

Clean up of IVT products are essential for 260 nm absorbency readings to truly reflect the quality of the cRNA product. Clean up facilitates this by removing all unincorporated NTPs from the reactions. Phenol/choloform extraction may not be used in this instance as the biotin label will cause some of the cRNA to partition into the organic phase and result in a low yield. Instead clean up was carried out using RNeasy spin columns (Qiagen) following the manufacturers' standard protocol. A 20 µl volume of each 40 µl cRNA sample was used in the clean-up procedure, the other 20 µl being held in reserve to minimise the chance of the sample being lost completely.

5.3.7.2 Quantifying the cRNA

Spectrophotometric analysis was carried out on 1 µl of each sample using the Nanodrop® ND-1000 system (Nanodrop technologies, Portland, DE). Yields of 0.6 µg/ul or above and A_{260}/A_{280} ratios between 1.9 and 2.1 were considered acceptable. Adjusted cRNA yields were calculated for each sample to reflect the carryover of unlabelled total RNA in each sample. Using an estimate of 100% carryover the true yield is determined by the formula:

adjusted cRNA yield = RNA_m-(total RNA_i)(y)
where,
RNA_m = amount of cRNA measured after IVT (µg)
total RNA_i = starting amount of total RNA (µg)

y = fraction of cDNA used in IVT reaction

5.3.8 Fragmentation of cRNA for target preparation

A 20 µg amount of cRNA was used per sample for fragmentation. A minimum concentration of 0.6 µg/µl of cRNA allows each reaction to be carried out in a 40 µl volume consisting of 8 µl of 5x fragmentation buffer, cRNA and RNAse-free water. Fragmentation was carried out at 94°C for 35 minutes after which samples were quenched on ice. A 1 µl aliquot from each sample was used for confirmation of fragmentation using the Agilent 2100 Bioanalyzer. Fragmented RNA was then stored at -20°C until hybridisation.

5.3.9 Target hybridisation

Array chips used in these experiments were standard size human genome HGU133A, requiring 15 µg of fragmented cRNA per hybridisation reaction. In addition the hybridisation cocktail contained 3 µl of 10 mg/ml Herring sperm DNA, 3 µl of 50 mg/ml acetylated BSA, 150 µl of 2x hybridisation buffer and 20 µl of hybridisation controls. These comprised 15 µl of a 20x Eukaryotic hybridisation control mix containing transcripts for the prokaryotic genes *BioB, bioC, bioD* and *cre. BioB, bioC* and *bioD* are genes from the biotin synthesis pathway in *E.coli*, and *cre* is the recombinase gene from P1 bacteriophage. The 20x hybridisation control mix was heated to 65°C for 5 minutes to completely resuspend the cRNA before aliquotting. A 5 µl volume of B2 control oligo (Affymetrix) completed the control sets. The final volume of the hybridisation cocktail was made up to 300 µl with RNase-free water.

Once complete the hybridisation cocktails for each sample were mixed by gentle pipetting and heated to 99°C for 5 minutes in a heat block. HU133A probe arrays were equilibrated to room temperature immediately before use and then filled with approximately 250 µl of 1x hybridisation buffer using a micropipette and appropriate tips. The probe array cartridge is filled using two pipette tips inserted through rubber sealed septa located on the back of the array cartridge. Solutions

enter through one septum and the second allows venting of air from the array to ensure the cartridge remains at atmospheric pressure. Filled array chips were incubated at 45°C for 10 minutes with rotation in a GeneChip® Hybridisation oven (Affymetrix).

The hybridisation cocktail was transferred from 99°C to a heat block at 45°C for 5 minutes, before centrifugation at 14000 g for 5 minutes to facilitate removal of any insoluble material from the hybridisation mixture. Buffer solution was then removed completely from the array cartridge and replaced with 200 µl of the clarified hybridisation cocktail. Array cartridges were then placed in the hybridisation oven at 45°C and rotated at 60 rpm for 16 hours.

5.3.10 Washing and staining of arrays

Washing and staining of the array cartridges was carried out using the GeneChip® Fluidics Station 400 (Affymetrix) following the manufacturer's recommended protocol. The fluidics station was operated using MAS software set to the specific array cartridges used in all experiments (HGU133-A). The fluidics station was primed with appropriate non-stringent (6x standard saline phosphate with EDTA, 0.01% Tween 20, and 0.005% antifoam) and stringent wash buffer (100 mM *N*-morpholinoethanesulfonic acid, 0.1 M NaCl, and 0.01% Tween 20), placed in reservoirs A and B respectively.

Following hybridisation, the hybridisation cocktail was removed completely from the array cartridges and filled completely with non-stringent wash buffer (approximately 250 µl required). Array cartridges were then ready for washing and staining procedures once they had equilibrated to room temperature.

5.3.11 Antibody amplification procedure for eukaryotic targets

This procedure is one of two protocols available for washing and staining the array sets. This procedure was chosen in preference to the non-amplification

protocol as this is recommended for HU133-A array cartridges due to the small size of the probe cells.

The staining solution comprised 600 µl of 2x 2-(N-Morpholino)ethanesulphonic acid (MES) stain buffer, 48 µl of 50 mg/ml acetylated BSA, 12 µl of 1 mg/ml streptavidin-phycoerythrin (SAPE) and 540 µl of distilled H_20 for a total volume of 1200 µl. Staining solution was mixed thoroughly and divided into two 600 µl aliquots per array cartridge. Antibody solution mix consisted of 300 µl of 2x MES stain buffer, 24 µl of 50 mg/ml acetylated BSA, 6 µl of 0.1 mg/ml normal goat IgG antibody, 3.6 µl of 0.5 mg/ml goat anti-streptavidin antibody, and 266.4 µl of distilled water for a total volume of 600 µl per array cartridge. The protocol used for washing and antibody amplification is outlined in Table 5-1.

The washing and staining protocol is semi-automated requiring only placement of required stains and antibody solutions when prompted by the LCD display on the fluidics station. When washing and staining is complete the probe arrays are ejected from the fluidics station and are stored at 4°C in the dark until scanning.

5.3.12 Probe array scan

Probe array cartridges were scanned using the GeneArray® Scanner. The scanner uses an argon-ion laser and is also controlled by the Affymetrix® MAS software. The laser performs a duplicate scan at a wavelength of 570 nm for each array cartridge, forming a final image that is an average of both scans. Cartridge windows may be carefully cleaned with a lint free tissue before scanning if this is required.

5.3.13. Data analysis

Data analysis was carried out using the standard data processing and analysis software detailed in Section 5.2.3.1. All user definable parameters remained at the Affymetrix default settings, and the search strategy used for the processed data is detailed in Section 5.4. The use of replicates as controls in microarray

experiments have been proposed at three different stages in experiment design, namely biological, or sample replicates, technical, or array replicates, and duplicate spots within individual arrays (Xiang, Yang *et al* 2003). These three levels of control are designed to reduce error attributable to biological variation in the sample tissue or cells, technical error during extraction, labelling and hybridisation of samples, and measurement error due to imperfections or blemishes such as dust on the array surface, respectively (Churchill 2002). It has been accepted that the cost of microarray experiments are such that the use of replicates are usually kept to the minimum required for meaningful interpretation of the data (Xiang, Yang *et al* 2003), and the design of the Affymetrix GeneChip array has reflected this concern with a number of inbuilt controls. First, the use of multiple probes interrogating non-overlapping areas of sequence of each RNA target at physically different areas on the array reduces error when measuring the fluorescent signal. Second, the control hybridisation cocktail, detailed in Section 5.3.9 allows quantification of technical error at the hybridisation stage of the experiment, and exclusion of GeneChips that do not meet an automatically defined standard required for inter-Chip comparison. Finally, the numbers of time points interrogated in both the SFA and GM847 experiments were increased beyond the 2 that were technically required in each experiment to investigate replicative senescence and telomerase reactivation respectively. In the SFA experiment, an additional time point at passage 4 was included, and in the GM847 experiment, three post-transfection time points corresponding to passages 1, 19 and 26 post-transfection were used. The Expressionist Analyst software was then used to isolate only those genes that displayed consistent patterns of expression, as detailed in Section 5.4.1. For the SFA experiment, this translated to those genes that displayed an increasing or decreasing level of expression in the cell population as the number of senescent cells increased. In the GM847 experiment, only those genes that were consistently up or down-regulated in all three of the post transfection time points were considered for further analysis.

Table 5-1. Antibody amplification protocol for eukaryotic targets. Wash buffer A refers to non-stringent wash buffer (6x standard saline phosphate with EDTA, 0.01% Tween 20, and 0.005% antifoam), and wash buffer B refers to stringent wash buffer (100 mM *N*-morpholinoethanesulfonic acid, 0.1 M NaCl, and 0.01% Tween 20).

Stage-	Comprises-
Post hybridisation wash 1	10 cycles of 2 mixes/cycle with wash buffer A at 25°C
Post hybridisation wash 2	4 cycles of 15 mixes/cycle with wash buffer B at 50°C
Stain	Stain the probe array for 10 minutes in SAPE solution at 25°C
Post stain wash	10 cycles of 4 mixes/cycle with wash buffer A at 25°C
Second stain	Stain the probe array for 10 minutes in antibody solution at 25°C
Third stain	Stain the probe array for 10 minutes in SAPE solution at 25°C
Final wash	15 cycles of 4 mixes/cycle with wash buffer A at 30°C, followed by the holding temperature of 25°C

5.4 Results

Each HGU133-A probe array contained targets for 22,283 gene sequences. Of these, approximately 15% are called present for the typical cross species array using canine samples, and approximately 45% are present using human derived samples (Chismar, Mondala, *et al* 2002). The results obtained with the 7 GeneChips used in this chapter followed this established pattern. For the canine primary fibroblasts, array experiments 1 (passage 2), 2 (passage 4), and 3 (passage 10) called 14.7, 13.6, and 13.2% of the target mRNA sequences present respectively. For the human GM847 cell line, array experiments 1 (pretransfection), 2 (passage 1), 3 (passage 19), and 4 (passage 26) called 46.4, 48.1, 49.7, and 47.7% present. In all cases, the arrays met the required standard for signal quality using the control sets detailed in Section 5.3.9, as automatically defined by the MAS software.

5.4.1 Initial data interrogation

Initial analysis of the microarray data was performed using a profile distance search. This method of viewing the data allowed identification of gene profiles that resembled a complex reference profile that could not be defined by any method other than drawing. Using this method, genes that were up regulated and down regulated as the SFA primary culture approached senescence were identified and isolated into appropriately labelled subgroups, as illustrated in Figure 5-3. The same process was applied to identify genes in the GM847 cell line that showed a consistent change in expression level after transfection. The reference profiles generated for genes identified as up regulated and down regulated in the SFA cell culture are shown respectively in Figure 5-4 and Figure 5-5. The corresponding reference profiles for the GM847 cell line are shown in Figure 5-6 and Figure 5-7.

5.4.2 Analysis of SFA subgroups

The SFA subgroups were defined by profile distance search as described above and could be divided into two categories; genes that were up regulated and genes that were down regulated as the cell line approached senescence. These groups included 627 and 689 genes respectively. The groups were then further filtered by an expression ratio analysis. This analysis selected genes from the two groups based upon highest ratio values. This value was calculated by dividing the highest expression value for an item by the lowest value for the same item, allowing genes with expression levels that had undergone the greatest change to be identified. The expression value is a measure of the relative abundance of each transcript. This tool was also used for identifying genes within the subgroups that had undergone small changes in expression levels (e.g. 2 fold change or less) allowing these possibly background variations to be removed from the analysis. Using this method, genes that underwent 4 fold and 8 fold or greater increase or decrease in mRNA expression over the time course of the experiment were identified as shown for up regulation in Figures 5-8 and 5-9, and for down regulation in Figure 5-10 (no SFA genes underwent an 8 fold or greater decrease in expression level over the course of the experiment). In total, mRNAs from 12 genes underwent a four fold or greater increase as the SFA culture approached senescence. A group of 5 mRNAs underwent the same ratio change of down regulation as the culture approached senescence. Ontologies of these genes were then compiled by an accession number search using the Gene Finder search engine provided by the Cancer Genome Anatomy Project (CGAP) (http://cgap.nci.nih.gov/). CGAP is a subsidiary of the National Cancer Institute, which is the U.S. federal government's principal agency concerned with cancer research. Details of the results of this search are provided in Table 5-2 and Table 5-3.

5.4.3 Analysis of GM847 subgroups

The same analytic strategy was applied to the GM847→GM847/hTERT experiment. The initial profile distance search identified 887 genes that had a

low expression value before transfection, switching to a consistently high expression value in the three post transfection time points. The converse profile; high expression before transfection switching to consistently low expression after transfection produced a group of 707 genes for analysis. Further selection was carried out by expression ratio analysis as detailed for the SFA experiment. The results of this analysis for 4-fold and 8-fold increase in mRNA expression are shown in Figures 5-11 and 5-12 respectively. Four-fold and 8-fold decrease in expression post transfection is shown in Figures 5-13 and 5-14 respectively. Descriptions of those genes undergoing 8 fold and greater decrease or increase in mRNA expression value post transfection (numbering 8 and 4 respectively) were accessed using the same search facility detailed for the SFA experiment, and are provided in Tables 5-4 and 5-5.

Figure 5-3. Overview of the sampling strategy for the SFA culture. The three time points sampled correspond to passages 2, 4 and 10 of the SFA primary culture, during which the culture went from containing less than 5% senescent cells (at passage 2) to 80% senescent cells (at passage 10), as defined by SA-β-GAL staining. These time points were chosen to allow comparison between an actively replicating cell population with most of its replicative potential intact and a cell population made up of a majority of senescent cells. The search strategy employed using Expressionist™ Analyst software was then to identify those genes with mRNA expression levels that had increased or decreased at least four fold over this time, thus highlighting those genes that experience the greatest change in expression level in association with the onset of replicative senescence in canine fibroblasts.

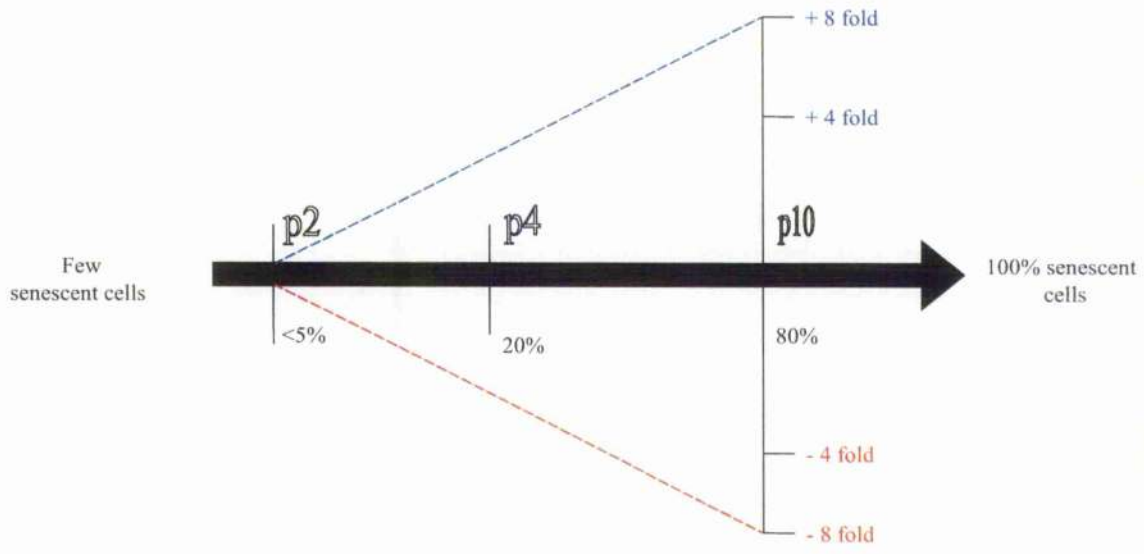


Figure 5-4. Profile distance search for the SFA cell culture highlighting up regulation. The linear display profile charts the expression level of mRNAs of each gene over the three time points corresponding to passages 2, 4 and 10 respectively. The black line may be set by the user and indicates the preferred expression profile, in this case up regulation. The software then isolates each gene in the entire group that fits the specified profile (highlighted in red), allowing this group of genes (numbering 689) to be isolated in a subgroup for further analysis.

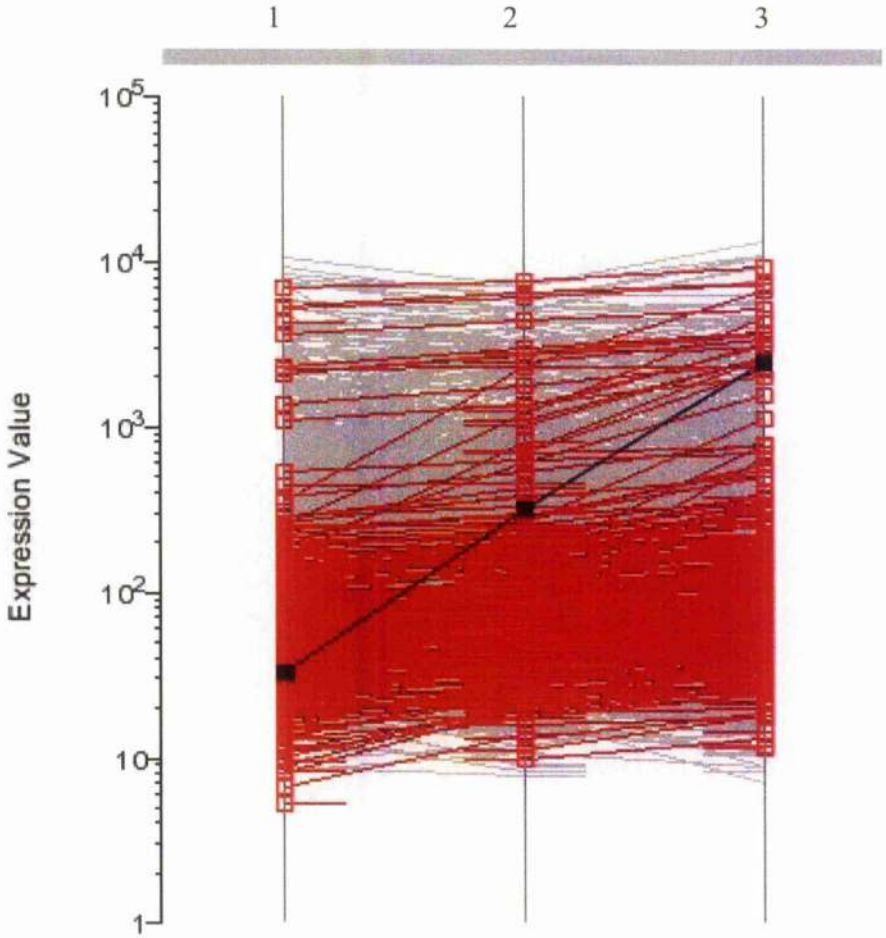


Figure 5-5. Profile distance search for the SFA cell culture highlighting down regulation. The linear display profile charts the expression level of each gene over the three time points corresponding to passages 2, 4 and 10 respectively. The black line may be set by the user and indicates the preferred expression profile, in this case down regulation. The software then isolates each gene in the entire group that fits the specified profile (highlighted in red), allowing this group of genes (numbering 627) to be isolated in a subgroup for further analysis.

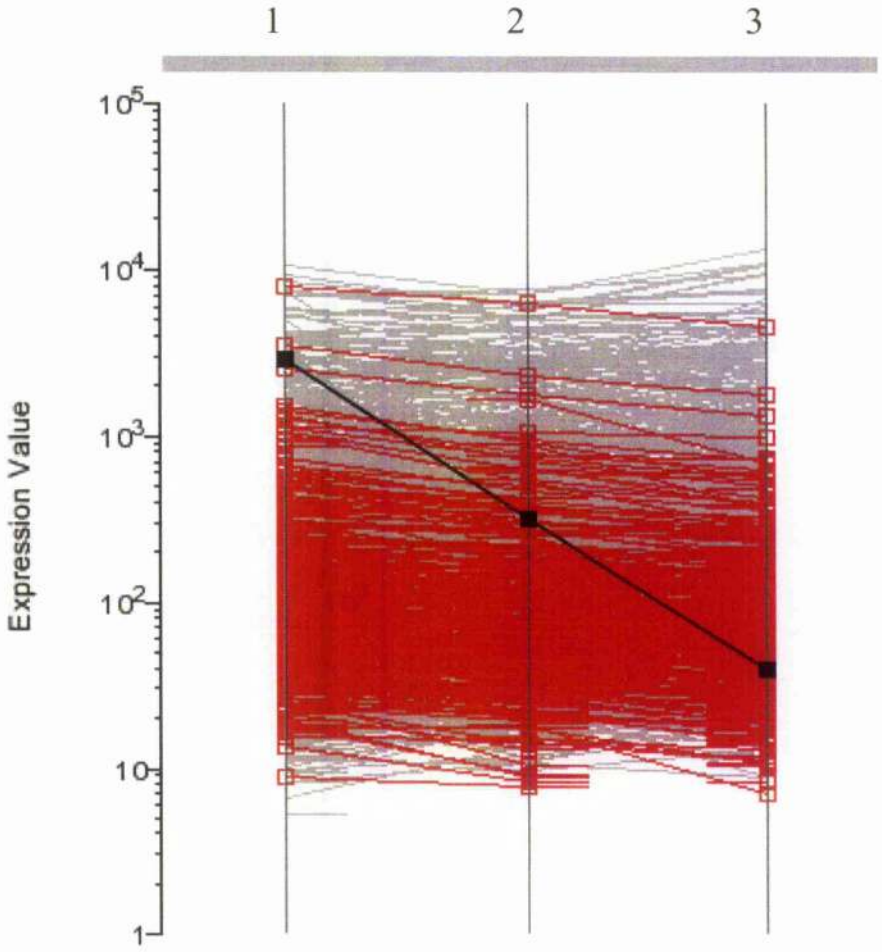


Figure 5-6. Profile distance search for the GM847 cell line highlighting up regulation. The linear display profile has been set to select for mRNAs that demonstrate sustained up regulation in post transfection cells compared with the original parent cell line. This pattern is reflected in the black line that represents the reference profile against which genes were selected. The group contained a total of 887 genes that are highlighted in red. This subgroup was then selected for further analysis by highest ratio profiling. Time points 2, 3 and 4 are all taken from passages of the successfully transfected GM847 cell line (p1, p19, and p26 respectively), whilst time point 1 is the passage of the parent GM847 cell line from which the transfected cell line was created.

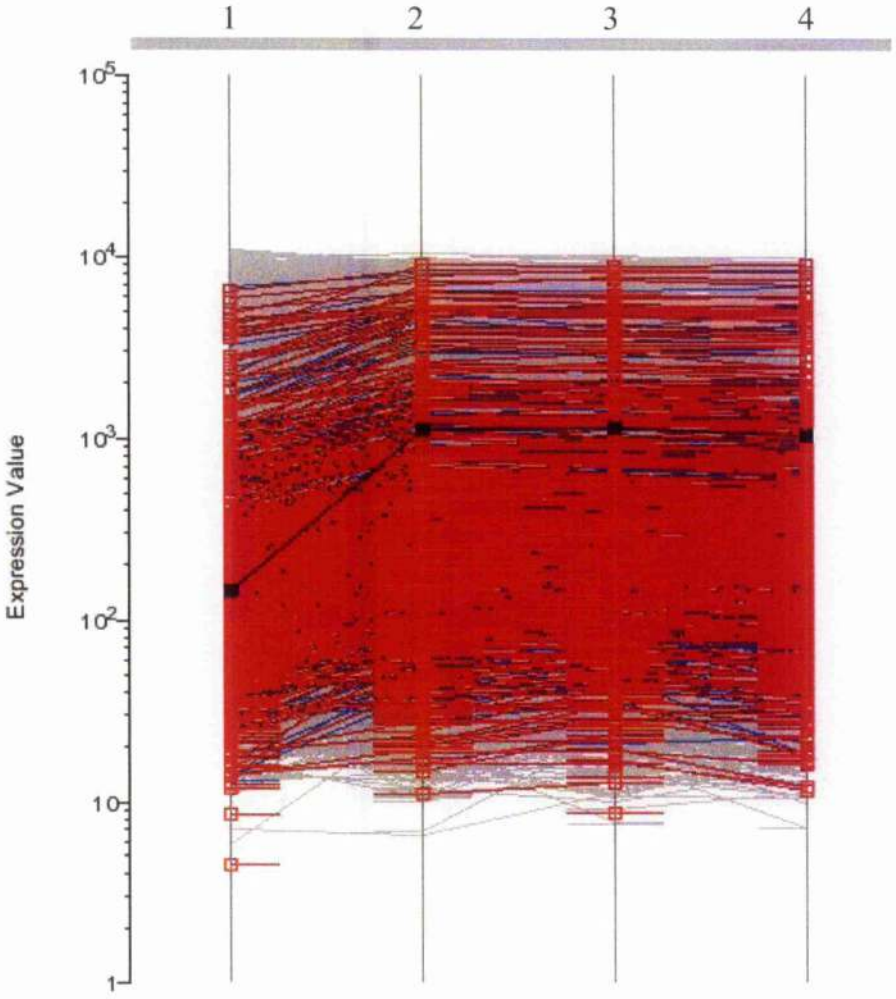


Figure 5-7. Profile distance search for the GM847 cell line highlighting down regulation. The linear display profile has been set to select for genes that demonstrate sustained down regulation in post transfection cells (time points 2, 3 and 4) compared with the original parent cell line (time point 1). This pattern is reflected in the black line that represents the reference profile against which genes were selected. The selected group contained a total of 707 genes that are highlighted in red. This subgroup was then selected for further analysis by highest ratio profiling.

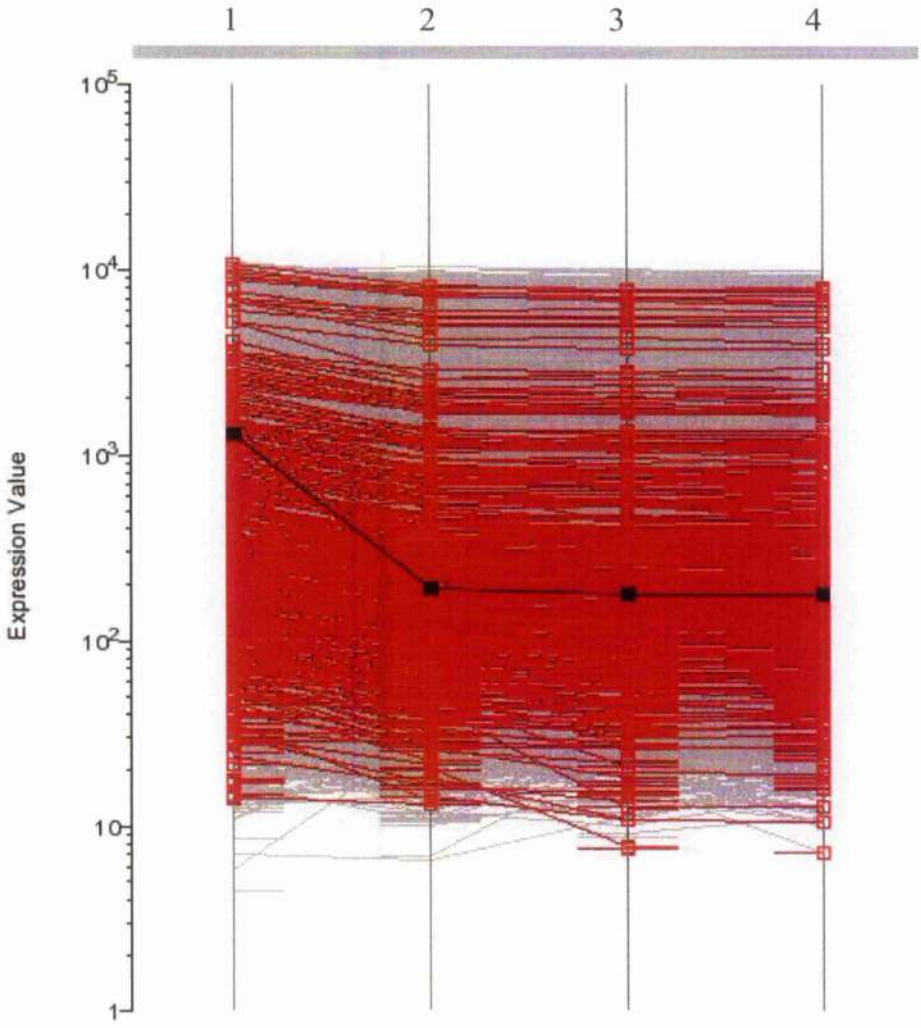


Figure 5-8. Gene group demonstrating four fold increase in expression value and above for mRNAs of the SFA cell culture. This figure illustrates a highest ratio analysis for increasing gene expression level in the SFA cell culture. The filter is set to select only those genes with mRNAs that have undergone a four-fold or greater increase in expression value over the course of the experiment. This group is highlighted in blue and totals 12 genes. Horizontal blue bars indicate genes not detected at all three time points; for example a horizontal blue bar to the left of time point 2 indicates expression of that gene was not detected at time point 1.

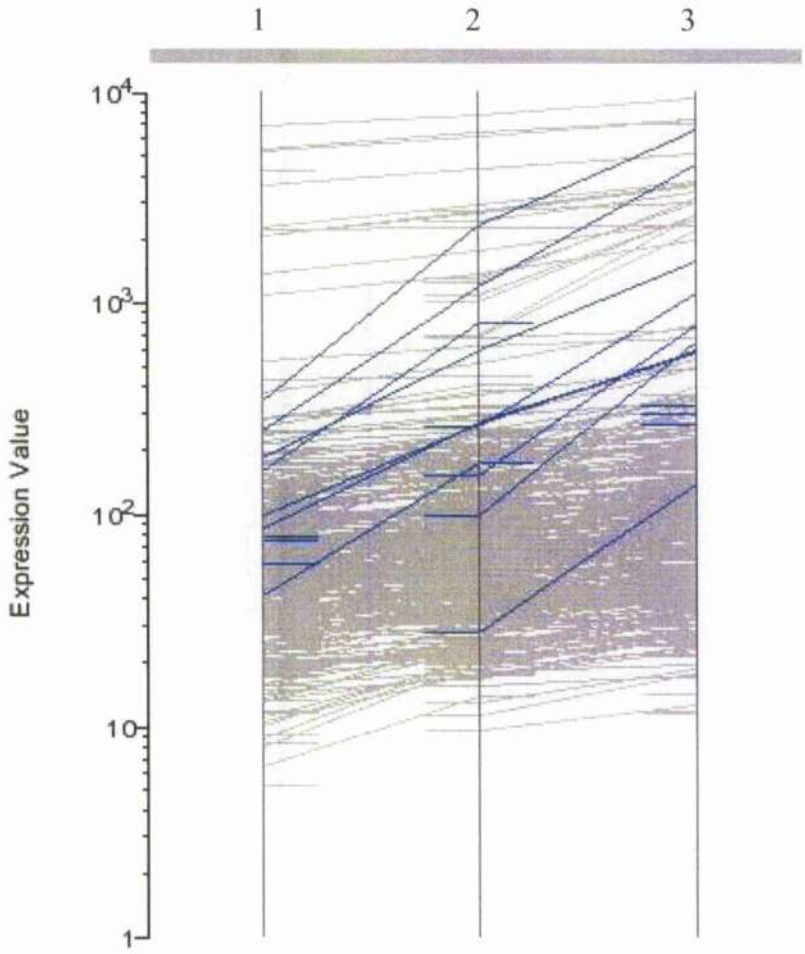


Figure 5-9. Gene group demonstrating eight-fold increase in mRNA expression and above for the SFA cell culture. The three genes in this group are highlighted in blue, whilst the grey background indicates genes below the highest ratio threshold of eight.

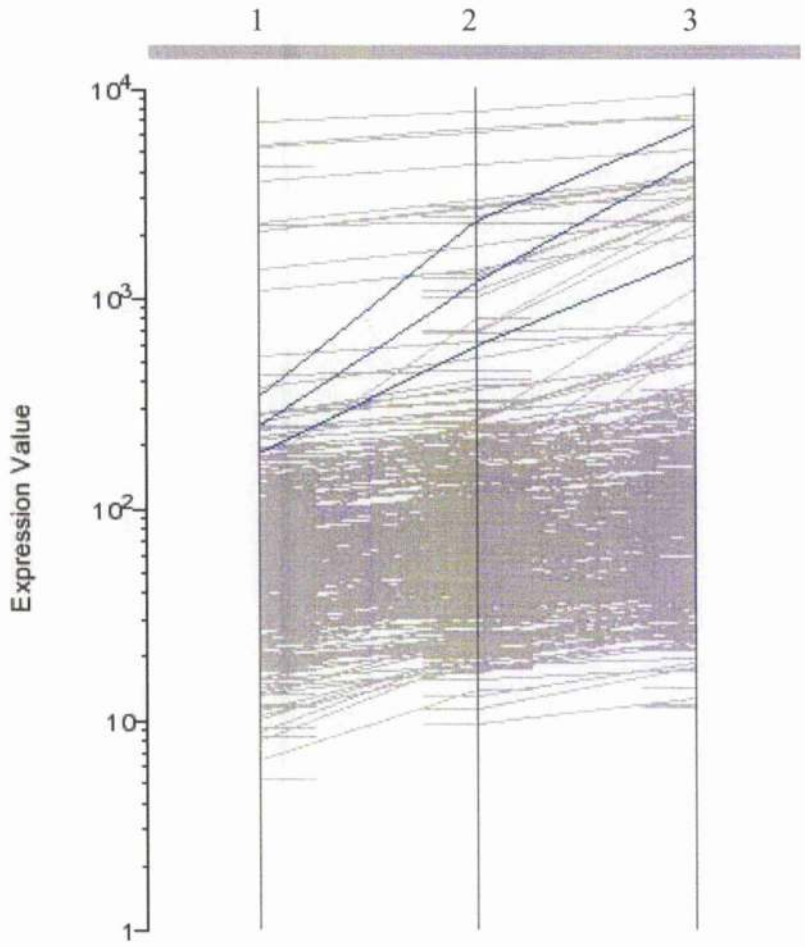


Figure 5-10. Gene group demonstrating between four and eight-fold decrease in mRNA expression for the SFA cell culture. Selected genes are highlighted in red, whilst unselected genes are shown in grey in the background.

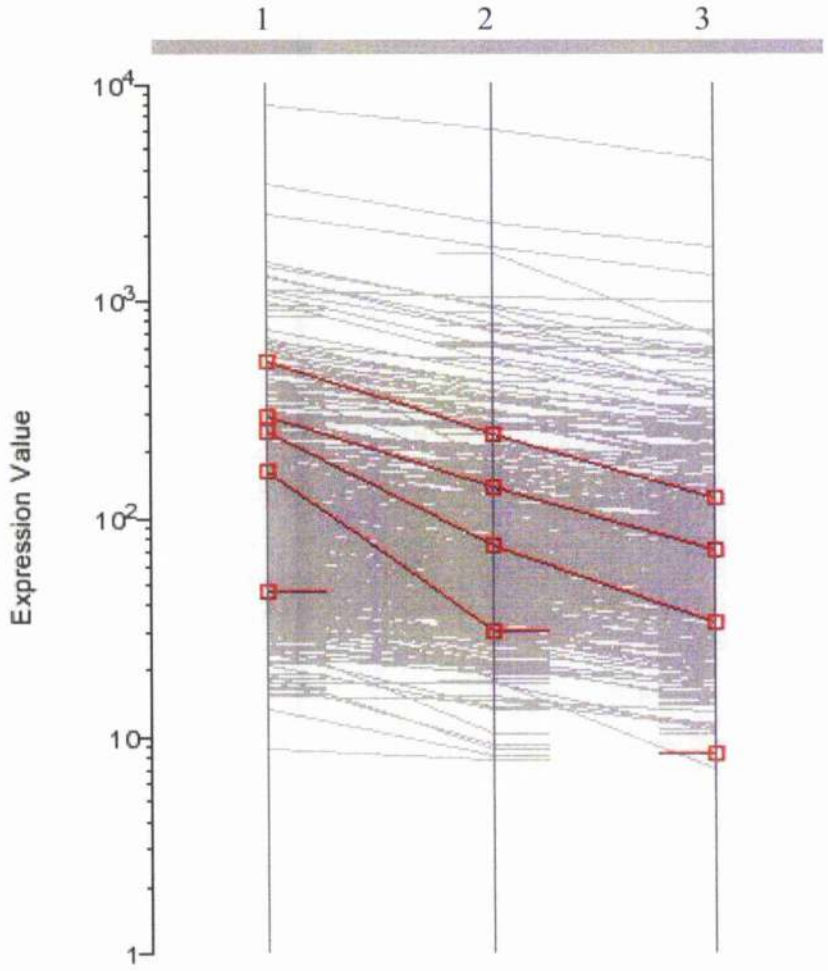


Figure 5-11. Gene group demonstrating four-fold or greater increase in mRNA expression value by highest ratio analysis in the GM847 cell line experiment. This group comprised 72 different genes.

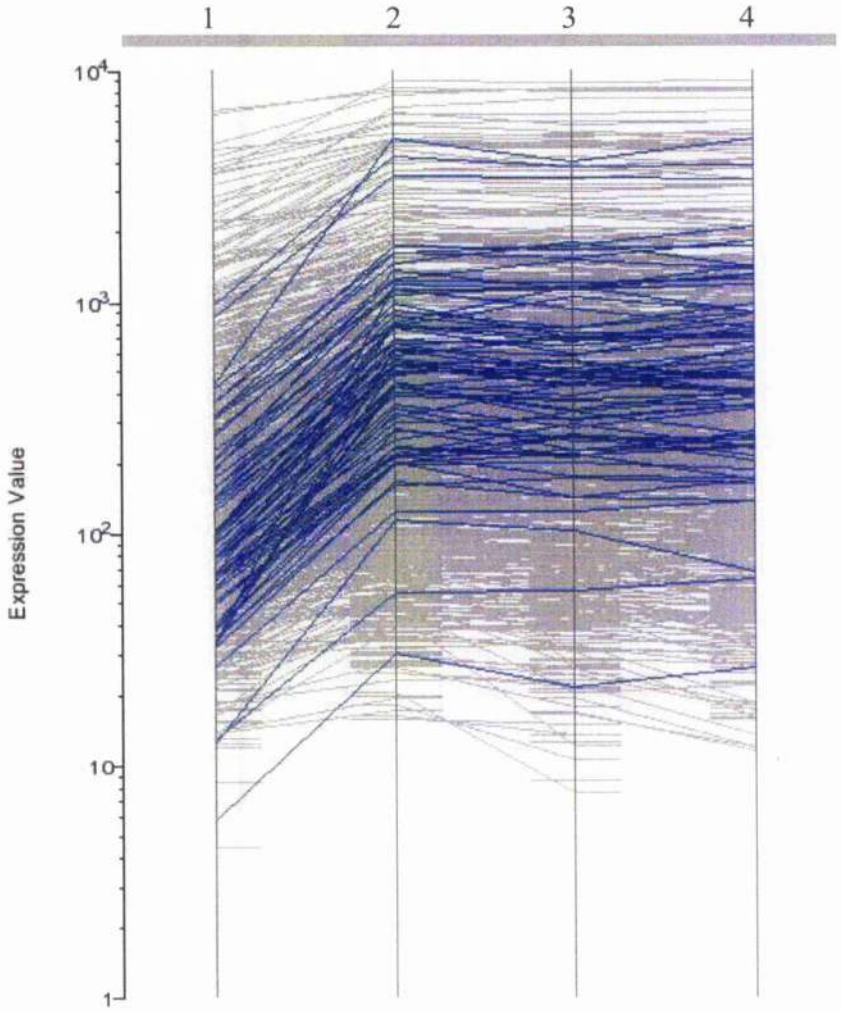


Figure 5-12. Gene group demonstrating eight-fold or greater increase in mRNA expression value by highest ratio analysis in the GM847 cell line experiment. This group comprised 8 different genes, highlighted in blue. Unselected genes in this analysis group are shown in grey in the background.

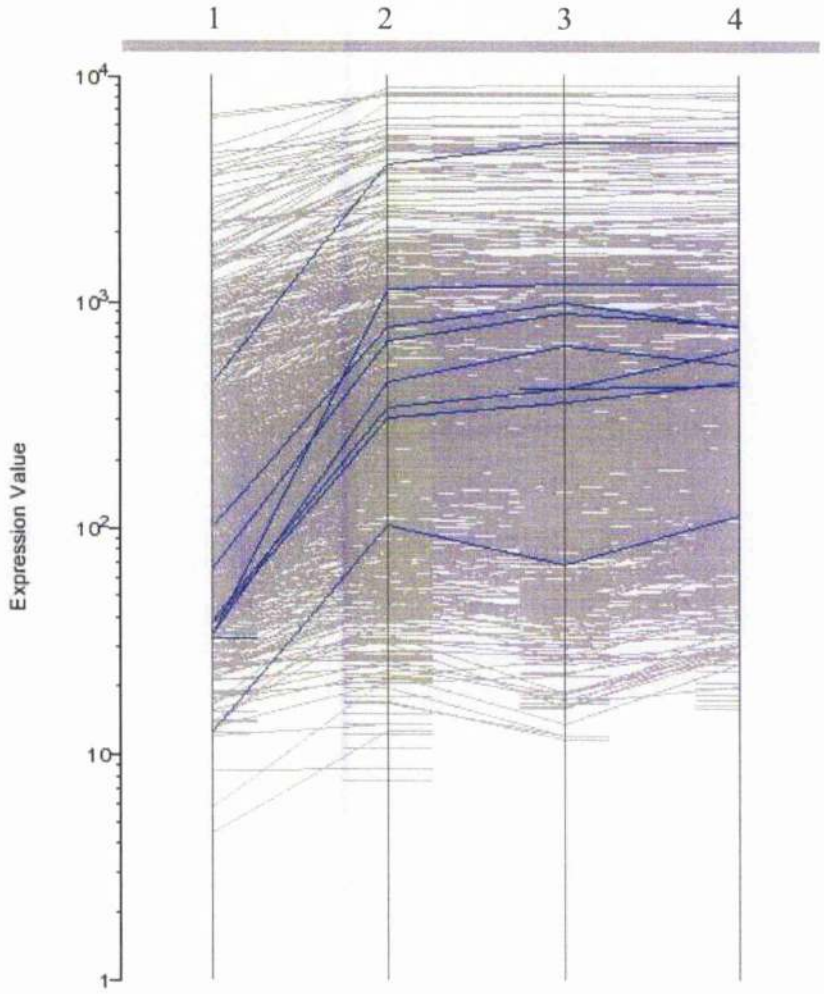


Figure 5-13. Gene group demonstrating four-fold or greater decrease in mRNA expression value for the GM847 cell line experiment. This group comprised 15 genes highlighted in red. Unselected genes are shown in grey in the background.

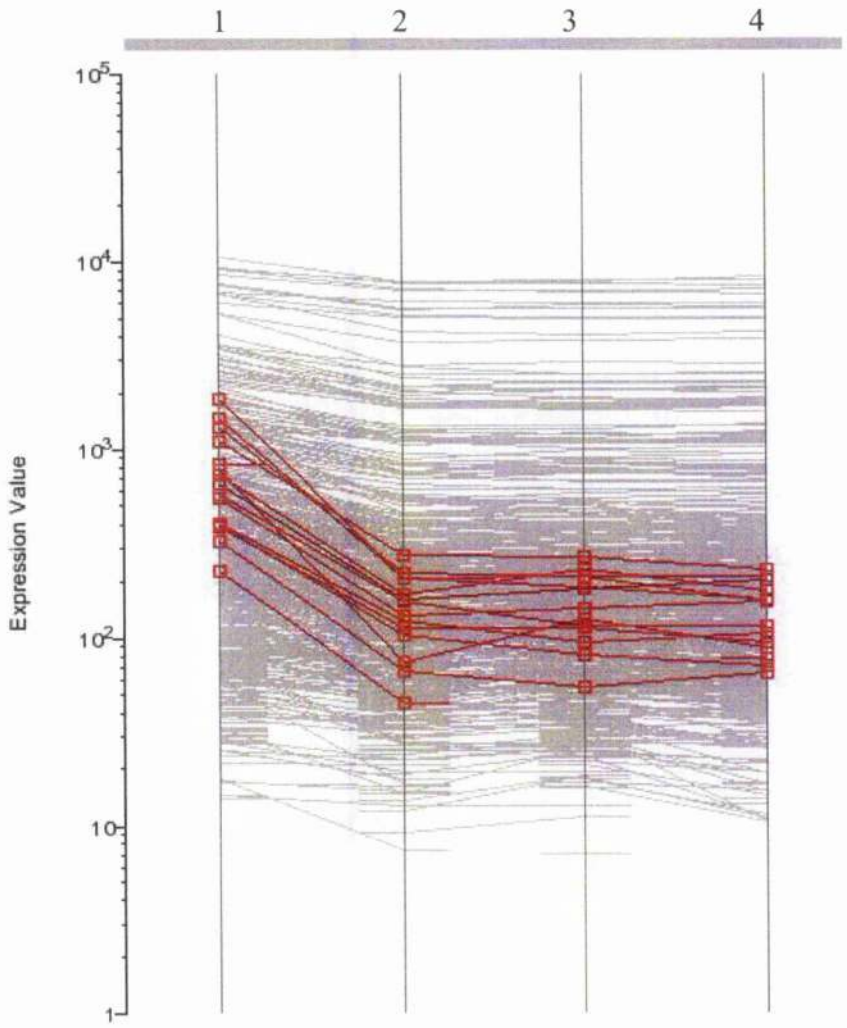


Figure 5-14. Gene group demonstrating eight-fold or greater decrease in expression value for the GM847 cell line. This group comprised 3 different genes, highlighted in red. Unselected genes are shown in grey in the background.

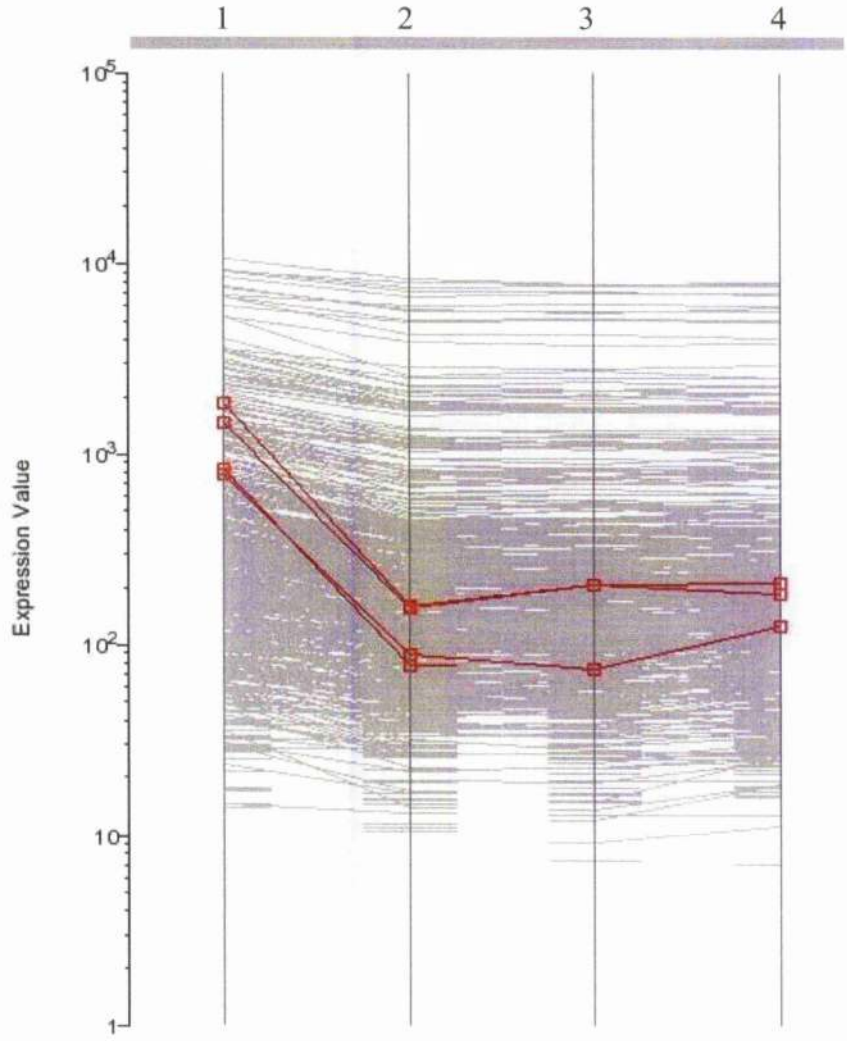


Table 5-2. Genes demonstrating four fold or greater increase in mRNA expression value between passages 2 and 10 of the SFA cell culture. Genes designated '+' demonstrated 4-8 fold expression increase, those designated '+ +' demonstrated eight fold or above increased expression.

Gene name (Accession number)	Gene description	Expression
Human 28S ribosomal RNA gene (M27830)	Encodes ribosomal RNA transcript	+ +
Mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isoenzyme B (BC051835)	Acetylglucosaminyltransferase activity	+ +
Thrombospondin 1 (NM_003246)	Inhibitor of angiogenesis	+ +
Synaptosomal-associated protein, 91kD homolog (mouse) (BC052454)	Synapse function	+
Insulin-like growth factor binding protein 2 (BC12769)	Negative regulator of proliferation in primary cells	+
Breast cancer anti-oestrogen resistance 3 (U92715)	Intracellular signalling, regulator of cell cycle, signal transduction	–
Serine (or cysteine) proteinase inhibitor (NM_030666)	Immune response, endopeptidase activity	+
Phosphatidic acid phosphatase type 2A (BC008787)	Lipid metabolism, cell growth and maintenance, tumour suppression	+
Double homeobox, 4 (NM_033178)	Regulation of transcription, transcription factor activity	+
Chromosome 11 open reading frame 10 (BC015968)		+
Amyotrophic lateral sclerosis 2 chromosome region, candidate 2 (NM_018571)	DNA repair failure and amyotrophic lateral sclerosis	+
Consensus sequence with transforming growth factor beta 1, mRNA (NM_003238)	Growth factor activity associated with inflammatory cells	+

Table 5-3. Genes demonstrating four fold or greater decrease in mRNA expression value between passages 2 and 10 of the SFA cell culture. No genes in this group underwent greater than a 8-fold decrease in mRNA expression values.

Gene name	Gene description	Expression
B-cell translocation gene 1, anti-proliferative (BC016759)	Cell growth and proliferation, up regulated by interferon	-
ATPase, Na+/K+ transporting, beta 1 polypeptide (NM_001677)	Component of Na/K pump for maintaining membrane potential and regulating cell volume	-
Methyl-CpG binding domain protein 2 (NM_003927)	Negative regulation of transcription	-
Discs, large (Drosophila) homolog 1 (NM_004087)	Intracellular signalling cascade, cytoskeletal protein binding	-
Adaptor-related protein complex 4, epsilon 1 subunit (NM_007347)	Endocytosis, non-selective vesicle transport, ubiquitously expressed	-

Table 5-4. Genes demonstrating 8 fold and above increase in mRNA expression in the GM847 cell line post hTERT transfection

Gene name	Gene description	Expression
Solute carrier family 16, member 3 (NM_004207)	Monocarboxylate transport, integral membrane protein	+ +
Melanoma antigen, family A, 6 (BC041599)	Molecular function unknown	+ +
HIRA interacting protein 5 (NM_015700)	Linked with Fe/S containing proteins	+ +
Coated vesicle membrane protein RNP24 (NM_006815)	Vesicular transport, integral to cell membrane	+ +
G protein-coupled receptor 61 (NM_031936)	Protein signalling pathway, integral to cell membrane, biological function unknown	+ +
Epiregulin (NM_001432)	Growth factor activity, angiogenesis, cell proliferation	+ +
Enolase 1, (alpha) (BC015641)	Transcription factor activity	+ +
Ceroid-lipofuscinosis, neuronal 2, late infantile (Jansky-Bielschowsky disease) (BC014863)	Lipid metabolism, proteolysis and peptidolysis	+ +

Table 5-5. Gene demonstrating 8 fold and above decrease in mRNA expression in the GM847 cell line post hTERT transfection

Gene name	Gene description	Expression
Mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isoenzyme B (BC051835)	Transferase activity, transferring hexosyl groups, carbohydrate metabolism	- -
H4 histone family, member G NM_003547)	DNA packaging and binding	- -
Eukaryotic translation initiation factor 5A (BC001832)	Protein biosynthesis	- -

5.5 Discussion

The primary aims of this chapter were to carry out investigations of the changes in mRNA expression in cell cultures in experiments concerned with two different areas of telomere related biology; namely the changes during *in vitro* cellular ageing and the onset of replicative senescence in canine fibroblasts, and the changes following reactivation of telomerase in a human telomerase independent ALT cell line. The Affymetrix technology utilised for this purpose is currently the market leader in terms of commercially available DNA arrays and allows a global picture of gene expression in human cells to be formulated. The HGU133-A and B Chip sets contain over 1 million oligonucleotides, representing more than 33,000 human genes (for product details refer to http://www.affymetrix.com/products/arrays/index.affx). The 'A' chip was used in these experiments as it contained the greatest number of oligonucleotides (22,283) targeted against well-characterised human genes. The lack of detailed information available on many of the genes, and the smaller number of genes represented on the remaining 'B' chip precluded its use in the experiments in terms of cost/benefit. Even at the subsidised prices available for academic research, each 'A' GeneChip alone cost approximately £500 at the time of the experiments, and this despite the cost of purchase being reduced by at least half since the creation of the HGU133 Chip set in 2001 (Hasseman 2002).

5.5.1 Use of the Affymetrix HGU133-A GeneChip for cross-species hybridisation

Currently, no canine specific DNA microarrays with the complexity of the GeneChip platform are commercially available; at present canine specific arrays are much less complex and are designed with specific applications, such as toxicology in mind, as exemplified by the canine arrays of Phase 1 Molecular Toxicology Inc., Santa Fe, NM (http://www.phase1tox.com/). Hence, investigation of large scale gene expression changes in canine samples is currently limited to using human specific arrays, and relies on cross species

hybridisations. There are few reports of the use of GeneChips for cross-species hybridisation experiments, and those that do exist tend to focus on non-human primates (Bigger, Brasky, *et al* 2001) (Kayo, Allison, *et al* 2001). The author is not aware of any published reports of the use of human genome array Chips for cross-species hybridisation with canine RNA samples and the present study therefore provides the first report of such an investigation, and demonstrates that approximately 15% of the human mRNAs probed by the HGU133-A GeneChip can consistently be identified in canine samples.

The design of Affymetrix technology is such that a gene is called present only if multiple oligonucleotide targets, in the vast majority of cases interrogating unique, non-overlapping units of sequence make a present call. In addition, negative calls must be made by the corresponding MM probe sets, which differ in only one base from their PM counterparts (Section 5.2.3). With the use of such positive and negative controls it is therefore expected that cross-species genomic differences will be reflected in a lower percentage of genes being called 'present' (as was identified in the SFA samples), rather than any degradation in the reliability of a positive call. This is corroborated by a study on the variability of results in cross-species GeneChip arrays using a non-human primate (*Rhesus macaque*) as the test species (Chismar, Mondala, *et al* 2002). In that study the percentage of genes called present in the non-human primate samples were 29.4% +/- 3.5, (average +/- standard deviation) compared with 46.4% +/- 3.3 for the human derived samples. Whilst a comprehensive comparison between the complete canine and human genomic sequences has yet to be undertaken (Kirkness, Bafna, *et al* 2003), it is likely that the smaller percentages present in the SFA chips (13.8% +/- 0.78) compared with the human samples analysed in these experiments (48.0 +/- 1.4) reflect the greater diversity between the human and canine genomes than between the human and *Rhesus macaque* genomes.

A second possibility is that the smaller percentages present in the canine samples reflect a genuine difference in canine and human biology in terms of numbers of genes expressed in the cell at a given time. Whilst variations will occur, they are unlikely to be great enough to account for the 34.2% difference in the number of genes found present in the two species. Instead, it is likely that a proportion of

this subset of genes that were typically called present in human samples yet typically called absent in canine samples are in fact active, but sequence differences between the two species results in a failure of Affymetrix to detect them. Some of the genes within this group will undoubtedly be of experimental and biological significance, therefore the use of cross species arrays do not allow examination of global gene expression changes in the canine test cells. Despite this, Affymetrix is undoubtedly the most effective tool available at present for these types of investigation.

5.5.2 Genes with significant changes in mRNA levels

In total, 28 genes that had undergone at least a four-fold change in mRNA levels during the course of both experiments were flagged by expression ratio analysis for further investigation. The biological function of each of these genes, to the extent of present knowledge, was then determined as described previously (Section 5.4.1). It was not possible, nor was it expected that each of these 28 genes could be in some way directly related to the biological phenomena (senescence and telomerase activation) that were under investigation. However, a number of this group have interesting functions, and their possible relation to telomere biology is discussed below.

5.5.2.1 Genes up regulated between passages 2 and 10 in the SFA culture

A total of 12 genes were identified from GeneChip analysis of the SFA culture with mRNA levels that increased at least 4 fold from passage 2 to passage 10. Included in this group was the gene that encodes phosphatidic acid phosphatase type 2A (PAP2), which is an integral membrane glycoprotein that hydrolyses a number of structurally related lipid phosphate substrates and appears partly responsible for the uptake of lipids from the extra cellular space (Roberts & Morris 2000). Interestingly, this gene product has also been implicated in a tumour suppressor role, and is down regulated in both colonic tumours and prostatic cancer cell lines (Leung, Tompkins, *et al* 1998) (Porkka & Visakorpi

2001). Considering these previous studies, the finding that PAP2 was strongly up regulated in senescent canine primary fibroblasts may implicate it in a tumour suppressive role in this species, and it may provide a further molecular marker for senescence in canine cells, in the same manner that $p16^{INK4a}$ has been found to accumulate in the senescent human and canine cells (Alcorta, Xiong, *et al* 1996) (Koenig, Bianco *et al* 2002).

A second gene in this group, with mRNA expression levels that increased over 8 fold between passages 2 and 10 is an inhibitor of angiogenesis, Thrombospondin 1 (TSP-1). This antiangiogenic factor appears to act by binding with, and subsequently reducing the bioavailability of angiogenic factors such as fibroblast growth factor (Margosio, Marchetti, *et al* 2003). Oncogenes act as inducers of tumour neovascularization in part by suppression of factors such as TSP-1 (Kalas, Gilpin, *et al* 2003), and so it is possible that a marked increase in TSP-1 mRNA levels as the canine fibroblasts approached senescence reflects an attempt by the cells to counteract any such action. It has even been suggested that restoration of TSP1 levels might be a means with which to inhibit tumour angiogenesis (Kalas, Gilpin, *et al* 2003).

Levels of mRNA encoding insulin-like growth factor binding protein 2 (IGFBP-2) were also increased over 4 fold in the passage 10 SFA cells compared with passage 2. Elevated IGFBP-2 levels are associated with malignancy in many different types of human tissue, particularly in the prostate (Richardsen, Ukkonen, *et al* 2003), and this molecule has even been proposed as a marker for malignant transformation in prostate epithelium (Richardsen, Ukkonen, *et al* 2003). The increased expression of its mRNA in the SFA culture was initially unexpected, however studies have shown that IGFBP-2 appears to play a different role in cultures of primary cells, were it has been shown to act as a growth inhibitor in more than one type of tissue (Hoflich, Lahm, *et al* 1998) (Moore, Wetterau, *et al* 2003). The results would indicate that IGFBP-2 might have a similar growth inhibitory effect in primary canine fibroblasts.

Whilst breast cancer anti-oestrogen resistance gene 3 is described in the CGAP ontology as involved with regulation of the cell cycle, as the name suggests this

has been primarily in association with breast cancer cells, rather than normal primary cultures (van Agthoven, van Agthoven, *et al* 1998), and so any significance of the increase in mRNA levels noted in this experiment is unclear.

Double homeobox 4 is linked with transcription factor activity (CGAP), however this particular gene transcript is not widespread, having been found in association with a type of muscular dystrophy (Gabriels, Beckers, *et al* 1999), so this gene cannot be readily linked to telomere biology. The remaining members of this group, as detailed in Table 5-2 include genes such as synaptosomal associated protein, 91 kD homolog (mouse), Mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isoenzyme B, and amyotrophic lateral sclerosis 2 chromosome region, candidate 2. These genes highlight the fact that technology as sensitive as Affymetrix will often highlight changes in the transcriptome that bear, with the extent of current knowledge, no relation to the area under investigation.

Another interesting finding in this group was the absence of the mRNAs of genes with products that have previously been associated with the onset of replicative senescence. Examples of this class have been described previously, such as $p16^{INK4a}$ and SA-β-GAL (Alcorta, Xiong, *et al* 1996) (Dimri, Lee, *et al* 1995); as these two are in common use as biomarkers of senescence. However, over expression of a number of other genes have also been associated with the onset of replicative senescence in human cells. Examples include interferon beta, which was found up regulated in a primary human fibroblast culture at the end of its replicative lifespan, and was therefore implicated in replication suppression (Tahara, Kamada, *et al*, 1995). Satoh *et al* identified preferential expression of the gene encoding vimentin in senescent human fibroblasts (Satoh, Kashimura, *et al* 1994). Vimentin is a cytoskeletal protein, and its overproduction in senescent fibroblasts has been suggested as a possible explanation for the enlarged and flattened phenotype that is characteristic of senescence (Nishio, Inoue, *et al* 2001). Senescent human endothelial cells were found to contain high levels of the transcript for interleukin-1 alpha, a known inhibitor of endothelial cell proliferation *in vitro*, providing a further marker for senescence in that cell type (Maier, Voulalas, *et al* 1990). Up regulation of mRNA and protein levels

for the glycoprotein Clusterin/apolipoprotein J have been detected in senescent human fibroblasts, and have been hypothesised to represent a secondary consequence of the senescent phenotype rather than a causal agent (Petropoulou, Trougakos, *et al* 2001). Gonos *et al* identified a number of genes that were up regulated in both senescent rat embryo fibroblasts and senescent human osteoblasts that included fibronectin, osteonectin, α1-procollagen, apolipoprotein J, SM22, and GTP-α (Gonos, Derventzi, *et al* 1998). Fibronectin, osteonectin and α1-procollagen are all extra cellular matrix genes, and the changes in expression levels of these are likely to be related to the cytoskeletal changes associated with the senescent phenotype. SM22 and GTP-α are both involved with negative regulation of calcium dependant signal transduction, and it has been suggested that the over expression of these genes may be responsible for the poor homeostasis of senescent cells (Gonos, Derventzi, *et al* 1998). In addition, Chang *et al* have described up regulation of a number of genes in association with growth arrested, SA-β-GAL positive cells produced by doxorubicin chemotherapy in the human colon carcinoma cell line HCT116. A number of these expression changes were found to correlate with changes in protein levels, and included maspin, keratin18, and cyclin D1 (Chang, Swift, *et al* 2002). Maspin is a secreted growth inhibitor, whilst keratin 18 is known to have antiapoptotic activity, and cyclin D1 has been associated with ageing colon tissue (Chang, Swift, *et al* 2002).

Whilst activity of SA- β-GAL and the presence of $p16^{INK4a}$ have been identified in late passage SFA cells, as detailed in Section 3.4.4.3, none of the other genes outlined above were up regulated. This is not considered to be an unusual finding; for example it is important to note that only one of the additional nine gene products listed above (Apolipoprotein J), was detected in more than one of the referenced studies. This raises the point that all senescent cultures will not display up regulation of exactly the same genes. Even within the same cell type, minute variations in the culture environment may alter the expression profile of the study culture. Instead, it will be more useful to generate a list of genes that are associated with senescence, and use this information to aid understanding of the phenotypic changes that are occurring in a culture as it ceases to be

replicatively active. Furthermore, it is possible that the inability of Affymetrix to detect the mRNAs of some of these gene products is an example of species differences in RNA sequence resulting in a failure of detection due to the stringent Affymetrix mismatch controls. The significantly lower percentage of genes called present in the SFA GeneChips compared with a typical human sample (approximately 15% compared with approximately 45%) implies that a large number of genes might fall into this category, and underlines the point that whilst the HGU133 Chipset has proved useful for cross species hybridisations, the results of these investigations cannot be said to encompass changes in global gene expression in canine cells.

5.5.2.2 Genes down regulated between passages two and ten in the SFA culture

A total of 5 genes were identified from GeneChip analysis of the SFA culture with mRNA levels that decreased at least 4 fold from passage 2 to passage 10. Changes in the mRNA levels of the ATPase, Na+/K+ transporting, beta 1 polypeptide included it in this group. The significance of this is unclear, however the Na+/K+ ATPase is involved with the regulation of cell volume via maintenance of ionic gradients (Hernandez & Cristina 1998), and changes in Na+/K+ ATPase activity have been associated with ageing of skeletal muscle in rats (Sun, Nagarajan, *et al* 1999). Whether a change in activity of the Na+/K+ ATPase system is in any way related to the increased volume of senescent cells *in vitro* (Mitsui & Schneider 1976), or whether this is purely a reflection of the lack of cell division is speculative. The control of cell volume is a complex field beyond the scope of this discussion, however it is recognised that the *in situ* mechanisms for governing cell volume will almost certainly extend beyond transcriptional regulation of a single gene product.

The finding that mRNA for the anti-proliferative B-cell translocation gene 1 was down regulated with the approach of senescence was surprising, as this gene is associated with negative regulation of proliferative activity (Maekawa, Nishida,

et al 2002). However, the full range of functions of this gene in all tissues is not understood, and the gene has also been associated with cell proliferation, as listed in the CGAP ontology. The remaining three genes in this group, namely Methyl-CpG binding domain protein 2, discs, large (Drosophila) homolog 1, and adaptor-related protein complex 4, epsilon 1 subunit, are all widely expressed, and as detailed in Table 5-3, involved with general molecular processes within the cell that do not link readily with telomere biology.

Down regulation of a number of genes not included in the above group have previously been associated with senescence, including the c-fos component of the AP1 transcription factor, the Id1 and Id2 negative regulators of helix-loop-helix transcription factors, the E2F-1 component of the E2F transcription factor and MAD-2, which is involved in progression of mitosis (Dimri, Testori, *et al* 1996) (Chang, Swift, *et al* 2002). As described previously for gene up regulation (Section 5.5.2.1), genes with expression changes associated with senescence will not follow a characteristic pattern in all senescent cell populations that are investigated (for example, the additional genes noted above do not overlap between the two studies from which they were sourced). However, as none of the genes down regulated in the SFA study could be linked directly to telomere biology, it is possible that all 5 of these genes are secondary to the senescent phenotype, rather than acting directly to trigger it.

5.5.2.3 Genes up regulated in association with reconstitution of telomerase activity

Microarray analysis of the human telomerase independent cell line GM847 identified a greater than 8 fold up regulation of eight genes post telomerase reactivation. The most interesting result in this group was the finding that epiregulin mRNA levels were greatly increased (> 8 fold) in GM847/hTERT cells assayed at three different time points compared with the parent GM847 cell line. Epiregulin is a potent growth factor belonging to the epidermal growth factor family and has been associated with the progression of certain carcinomas (Toyoda, Komurasaki, *et al* 1997). This result concurs with a previous cDNA

study of hTERT immortalised human fibroblasts were epiregulin was found to be highly expressed in hTERT immortalised fibroblasts, but not in normal primary fibroblasts. This discovery was listed as the principal finding of the research and lead the authors to conclude that therapeutic use of telomerase should be approached with caution, due to the association between epiregulin and tumorigenesis (Lindvall, Hou, *et al* 2003). In addition it was concluded that epiregulin up-regulation is required for sustained cell proliferation. The fact that up-regulation of this gene has now been identified in association with the process of immortalisation in two separate studies adds credence to the idea that epiregulin has an important role to play in telomerase mediated cellular immortalisation. Whether the same applies to ALT immortalised cells is open to question, as the parent ALT dependent cell line had the ability to replicate with a very much lower epiregulin mRNA level than was detected post transfection. However, the detection of epiregulin mRNA at any level contrasts with the complete absence of epiregulin activity detected in a previous study using human primary fibroblasts (Lindvall, Hou, *et al* 2003), and in this study using canine primary fibroblasts. It must be borne in mind that the hTERT mRNA copy number per cell of the hTERT transfected cells in this study will be much higher than that encountered *in vivo*, due to their expression being driven by a strong constitutively active CMV promoter. This high hTERT mRNA expression translated to high telomerase activity and was reflected in the TRAP results of the GM847/hTERT cells, which were by far the highest of all the telomerase positive samples assayed in this project (Table 4-20). It may be that the lower epiregulin levels detected in the original GM847 cells were enough to permit continued proliferation, and that epiregulin mRNA activity is associated with both telomerase, and ALT activity.

The increase in mRNA levels of enolase 1 (alpha) were also of interest, as this gene product has been associated with transcription factor activity; however this ubiquitously expressed gene has also been associated with glycolysis (CGAP), and so the relevance of the mRNA changes noted in this experiment were unclear, as they may simply reflect changes in cellular metabolism. A recent report found differential abundance of enolase 1 protein between human infiltrating ductal carcinoma of the breast and normal tissue (Somiari, Sullivan *et*

al 2003) however the pattern of protein abundance was not consistent in all of the test samples, and so any possible association between enolase 1 and tumorigenesis remains unclear.

The remaining 6 members of this group contained two genes of unknown biological function (Melanoma antigen family A, 6 and G protein-coupled receptor 61), whilst the remainder, as detailed in Table 5-4 are associated with very disparate functions, as defined by CGAP. These include intracellular transport (Solute carrier family 16, member 3 and coated vesicle protein RNP24), protein manufacture (HIRA interacting protein 5), and lipid and protein metabolism (Ceroid-lipofuscinosis, neuronal 2, late infantile (Jansky-Bielschowsky disease)). All these biological functions are concerned with general cellular metabolism, and are therefore likely to vary with time in a typical cell population.

5.5.2.4 Genes down regulated in association with reconstitution of telomerase activity

This group contained a total of three different genes with mRNA levels that decreased at least 8 fold in association with the reconstitution of telomerase activity. Two of the three genes in this group, Mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase isoenzyme B, and eukaryotic translation initiation factor 5A, are involved with metabolic processes such as carbohydrate metabolism and protein biosynthesis, processes that are associated with active, replicating cells, and so the down-regulation of these mRNAs post transfection is intriguing. However, these results were not reflected in any change in the population doubling time of the cell line, and likely represent background changes in cellular metabolism that are of little direct relevance to the transfection process. Similarly, the change in expression of the third member of the group, member G of the H4 histone family could not be linked directly to the experimental process.

5.5.3 Biological significance of Affymetrix generated data

DNA microarray analyses have identified genes of interest related to both the onset of replicative senescence and the reconstitution of telomerase activity that warrant further investigations. The primary concern of these investigations must be to ensure that the changes in the transcriptomes of the test cells that were identified in these studies were also reflected by changes in the proteomes. This is important as much modification and regulation occurs with mammalian gene products post transcription, and this raises the question of how related mRNA levels are to the biological activity of the proteins they encode. It may be misleading to interpret great changes in mRNA levels as equalling changes of great biological significance. It is known that mRNA abundance in a cell often correlates poorly with the amount of protein synthesised, and furthermore proteins rather than mRNA transcripts are the major effector molecules in the cell (Gygi, Rochon, *et al* 1999). Whilst a protein equivalent of the Affymetrix DNA microarray is not technically feasible at present due to the structural diversity and complexity of proteins compared with nucleic acids (Talapatra, Rouse, *et al* 2002) (Huber 2003), two-dimensional gel electrophoresis techniques have greatly improved identification and analysis of changes at the protein level in cells (Ong & Pandey 2001). Such techniques could be adopted to aid confirmation of the significance of the results gained by the use of Affymetrix technology.

Potentially the most significant finding of the experiments was the consistent up regulation of epiregulin mRNA in association with telomerase reactivation. The association between epiregulin and cancer progression that has been identified previously in carcinomas (Toyoda, Komurasaki *et al*, 1997), and the finding that epiregulin up-regulation was necessary for sustained proliferation of hTERT immortalised fibroblasts (Lindvall, Hou *et al* 2003), and the failure to detect epiregulin mRNA in either normal human fibroblasts (Lindvall, Hou *et al* 2003) or canine fibroblasts in these experiments all suggest that the hTERT immortalised cells may in fact show changes associated with a transformed

phenotype. Although validation of the results using the methods detailed above are an area for further investigation, the conclusion of the hTERT reactivation experiment detailed in this Chapter must be that any future therapeutic use of telomerase must be approached with caution.

Finally, the very large amount of data produced by these Affymetrix studies enforced a filtering of the results to facilitate interpretation. This was achieved by an expression ratio analysis, and excluded genes with mRNAs that had undergone a less than 4 fold change in expression. This is highly likely to exclude genes of biological significance, and again demonstrates that whilst the method detailed in this Chapter was useful for identifying genes of interest in the two studies, it cannot be said to have identified all the genes that are associated with either senescence or telomerase reactivation that were active in the two cell cultures. An improved version of the primary canine fibroblast study, using a canine specific array of the complexity of the HGU133-A GeneChip would undoubtedly uncover more genes of interest. At the time the experiments were carried out such technology was not available, however, the canine genome project and the interest it has generated within both the medical and veterinary medical research fields (Ostrander & Kruglyak 2000) will undoubtedly result in canine specific microarrays of the complexity of the GeneChip in the near future.

5.6 Summary

Affymetrix® GeneChip technology has proved very useful for the analysis of changes in mRNA expression in canine fibroblasts. The experiments detailed in this chapter have identified changes in mRNA expression of a number of genes in association with replicative senescence that warrant further investigation. These include thrombospondin-1, phosphatidic acid phosphatase type 2A, and ATPase, Na+/K+ transporting, beta 1 polypeptide. Additionally, changes in mRNA levels in association with the reconstitution of telomerase activity in a human cell line have been identified that are of relevance to the therapeutic use of telomerase reconstitution. Specifically, mRNA levels of the epiregulin gene were up regulated greater than 8 fold in the cell line in which telomerase had been reconstituted compared with the telomerase negative parent cell line. Epiregulin has been associated with the progression of human carcinomas, and is not detectable in normal human fibroblasts.

Chapter VI

General Discussion

6.1 Work described in this thesis

The aims of this thesis were to carry out a study of the biology of telomeres and telomerase in the dog and cat. The advances that have been made towards manipulating the biological functions of the telomere to provide novel treatments for cancer and age related disease in humans have spurred interest in this particular subject. In the veterinary field it is hoped that such a strategy could be used in a similar manner to provide new treatment regimes for the benefit of companion animals.

Given the relatively small amount of work in the literature concerning the telomeres of companion animals, the thesis was concerned with the gathering of background data rather than focussing on research with a directly clinical bias; however this does not imply that clinical considerations were not taken into account. The key factor of interest in human telomere biology is evidence that the telomere is the 'mitotic clock' at the centre of a replication control mechanism in cells (Vaziri *et al* 1994), and that the growth arrest associated with this effect is a potent anti-cancer mechanism (Ishikawa 2000). Phenotypic ageing may even be a side-effect of the process (Campisi 2001). The main mechanism at work is telomere attrition; this cellular countdown is believed to be the trigger for cellular senescence. It was therefore fundamental in this thesis to establish that the phenomenon of telomere attrition occurs in both the dog and cat, both *in vitro* and *in vivo*, and a large proportion of the work undertaken focussed on this. The TRF methodology used for these experiments

first identified a range of telomere lengths in the dog and cat that is comparable to that found in humans, and then comparison of samples taken from a wide age range of dogs and cats established that a significant decline in the telomere lengths of PBLs does occur as both dogs and cats get older. Blood samples were selected for this *in vivo* investigation primarily due to the twin considerations of ease of gathering the samples (within WCPN ethical policies) and a minimum of distress to the donor animals, allowing a much greater number of samples to be gathered than would otherwise have been possible. The only other route considered for sampling was cheek cell scraping, however this was abandoned due to the relatively small amount, and the generally poor quality of DNA harvested by this technique. Furthermore, it was subjectively felt that the animals sampled by cheek cell scrapings found this procedure more distressful than blood sampling.

In vitro investigations used primary fibroblast cell cultures. These are often used as the 'typical' cell for *in vitro* work, as is reflected in the human telomere literature (Allsopp *et al* 1992) (Hayflick 1965). Primary fibroblasts were also available within GUVS, which was a consideration given the relatively small number of commercially available primary cultures of companion animal origin, in comparison with cultures of human origin.

Using these *in vivo* and *in vitro* approaches it was established that telomeric attrition occurs in the dog and cat, and it was therefore plausible that an important link between telomeres, telomerase and cellular immortalisation exists in the companion animals, as has been found in humans (Artandi and DePinho 2000). The next logical step was to investigate the distribution of telomerase activity in the somatic tissues of the two species. Telomeric attrition will only occur to an effective degree in the absence of telomerase activity, and so for a telomere based replicative arrest to be active in canine and feline somatic tissues the majority must be telomerase negative. To this end, the highly sensitive TRAP assay was employed to categorise a wide range of normal somatic tissue in the dog and cat as either telomerase positive or negative. As is the established pattern in human tissues (Burger *et al* 1997), telomerase activity was found to be restricted to a handful of sites, all of which contained tissues with high replicative burdens (Section 4.4.1). Furthermore, high telomerase activity was detected in the vast majority of canine and feline malignant

tumours in a survey carried out on post-mortem and biopsy specimens, and in immortalised canine cell lines using the same protocol. Taken together these two findings demonstrate clearly that telomerase reactivation is central to continuing cellular proliferation in the dog and cat, and that telomerase activity is strongly down regulated in the vast majority of normal canine and feline somatic tissues.

All of these findings link the telomere with the control of cellular proliferation in the dog and cat, and verified that telomerase may be a target for novel anti-cancer strategies in the companion animal species. Therefore a study was undertaken to investigate if a RTI could affect cell-doubling time in two immortalised canine cell lines shown previously to be telomerase positive. This particular category of drug was chosen given previous findings that RTI inhibitors could demonstrably effect cell doubling times in human telomerase positive cell lines (Strahl and Blackburn 1996). Whilst the results of the study carried out in this project were disappointing in that exposure to AZT-TP did not have any discernable effect on the growth rate of the cell lines, this was in effect a pilot study, and lays the groundwork for possible future investigations into combinational therapy with a number of agents. A strategy that has received some recent attention is the use of agents to target both telomerase (e.g. AZT-TP) and its substrate, the telomere (e.g. G-quadruplex interacting compounds such as telomestatin, Tauchi *et al* 2003). The data recorded in the AZT-TP study may be combined with a future larger study using a number of potentially therapeutic agents.

A major finding in human telomere research has been the discovery that ectopic expression of the catalytic subunit of telomerase alone is sufficient to reconstitute activity in a number of different cell types (Bodnar *et al* 1998). Whilst the canine TERT gene and its promoter has now been cloned by this group, at the time of the experiments neither the canine or feline homologous sequences to hTERT were available. Instead, telomerase reactivation experiments in the dog and cat were carried out using a clone of the hTERT gene, and using a human ALT cell line and an equine fibroblast culture as controls. Whilst there were technical difficulties associated with transfecting feline primary fibroblasts, canine primary fibroblasts transfected with hTERT remained telomerase negative and entered senescence normally. The human cell line was successfully transfected using the same protocol employed for the canine

and feline cells, and subsequently was shown to be telomerase positive, thus demonstrating that the experimental protocol was adequate. It is possible that the canine fibroblasts remained telomerase negative due to sequence differences between the human and canine TERTs, as the cTERT and hTERT sequences show only 77% similarity; (Nasir, L 2003, unpublished report). Given that the cTERT sequence is now known, an interesting area for further investigation would be to repeat the experiment with the same protocol and substitute the cTERT sequence for the hTERT sequence in the canine fibroblasts. If species-specific sequence differences are to blame for the failure to reconstitute telomerase activity in the canine fibroblasts then the use of the homologous sequence should overcome this difficulty.

Targeting telomeres and telomerase for therapeutic intervention in a number of disease states may involve selection of co-factors and associated gene products in conjunction with targeting the telomere and the telomerase ribonucleoprotein directly. A good example of this is the recent finding of the association between epiregulin upregulation and continued cell proliferation in a cell population immortalised by ectopic expression of hTERT (Lindvall *et al* 2003). The upregulation of this gene product was identified by the use of a cDNA microarray, and this approach was also adopted in this project to investigate the changes associated with the approach of senescence in canine fibroblasts, and the reactivation of telomerase in an ALT cell line. Ideally, a canine fibroblast culture would have been used rather than the GM847 ALT cell line for the telomerase reactivation experiment, however given the failure of ectopic expression of hTERT to reactivate telomerase activity in the canine primary fibroblast culture SFA, this option was not available. As described previously, a study of the changes in gene expression in association with telomerase reactivation in a human cell culture has been carried out previously, however that study used a human primary fibroblast culture. The use of the immortal cell line GM847 in this thesis provided additional insight into the changes in mRNA levels found in association with telomerase reactivation beyond that already in place due to activation of the ALT mechanism.

6.2 Future studies

The work carried out in this project examined a number of different aspects of telomere biology, and within each area topics were identified that would benefit from further research. These are detailed below.

6.2.1 Telomere studies

It is felt that the most interesting finding of this work was the significant breed specific differences in telomere length in the dog. Although the sample numbers available were relatively small (n = 112), a trend was identified that relatively low life expectancy in a canine breed is associated with shorter mean TRF in age matched individuals (Section 3.5.2). Whilst the differences in life expectancies of breeds such as the Miniature Schnauzer and Beagle are small, and with a possibly large degree of variation, it is noted that certain breeds, generally grouped as the 'Giant breeds' (e.g. the Great Dane) do have a significantly shorter life expectancy than other breeds and crossbreeds (Michell 1999). The small sample number of Great Danes examined in this thesis did prove to have the shortest telomeres of all the breeds examined, and it is felt that it would be interesting to extend this work to a much larger study comparing the TRFs of Great Danes to those of a physically smaller breed. The main hurdles to the gathering of this data will be harvesting an adequate number of blood samples from healthy individuals, due to the relative scarcity of Giant breeds in Britain, and the natural reluctance of owners to submit healthy animals to a procedure such as blood sampling. Involving breed associations in such a study would be productive; as owners affiliated with such societies may be more receptive to the idea of research with potential health benefits for the breed as a whole. Specifically, if an association between telomere length and life expectancy could be conclusively proven in dog breeds, then not only would this benefit human research into the potential association between the telomere and the ageing process, but it also might allow the development of a selective canine breeding program, based on telomere lengths in the hope of producing progeny with greater life expectancy.

6.2.2 Telomerase studies

The specific association between telomerase reactivation and malignancy that has been identified in this thesis has verified that telomerase is a target for novel anti-cancer drug development in the dog and cat. The pilot study carried out using the RTI AZT-TP may be used as the basis for a study on the efficacy of combining a number of agents for a greater effect on telomerase activity, which should translate to a discernable effect on the growth rates of immortal canine and feline cells. Although other agents rather than AZT-TP could be selected to act as one of these potential therapeutic agents, the work described in this thesis has shown that AZT-TP was capable of affecting telomerase activity in the MDCK cell line, and so it may be worth persisting with this drug and combining it with other agents for a greater inhibitory effect. In addition, AZT-TP alone has been shown recently to have a significant effect on the growth rates of a human cancer cell line (Brown *et al* 2003), so this drug is not without promise. The nature of this expanded study would be concerned with one or possibly two strategies combined. First, the addition of a second RTI inhibitor to effect an overall greater telomerase inhibition in the test cell lines, and / or combining this approach with one of the newer drugs that target the availability of the telomere to the telomerase enzyme (e.g. Telomestatin, Tauchi *et al* 2003).

6.2.3 Gene expression studies

The Affymetrix work detailed in this thesis has identified a number of potential areas for further investigation. The most promising of these is the finding that epiregulin mRNA is upregulated in association with telomerase reactivation in the GM847 cell line. This corroborates with the findings of a previous study, and inhibition of epiregulin activity has been shown to have an inhibitory effect on the growth rate of human immortalised cell lines (Lindvall *et al* 2003). This gene product shows considerable promise for targeting in human immortalised cells, and it would be interesting to determine, using canine and feline immortalised cell lines whether such an effect can be demonstrated in companion animal cells. This approach of targeting potential telomerase co-factors could be combined with some of the therapies outlined above for the overall targeting of telomerase in canine and feline cancers.

GLOSSARY

293T	Human renal cell line
3132T	Canine lymphoma cell line
A_{260}/A_{280}	Measure of DNA/RNA quality
A72	Canine fibroblast cell line
AG07648	Primary canine fibroblasts
AG07906	Primary equine fibroblasts
AG08075	Primary canine fibroblasts
AGO8157	Primary canine fibroblasts
ALT	Alternative lengthening of telomeres
APB	ALT associated PMLs
ATCC	American Type Culture Collection
AZT-TP	3′-Azido-3′-deoxythymidine triphosphate
BCA	Bicinchoninic acid
BMSSCs	Bone marrow stromal stem cells
bp	base pairs
BSA	Bovine serum albumin
CCL-176	Primary feline fibroblasts
CCR	Coriell Cell Repositories
cDNA	Complementary DNA
CGAP	Cancer Genome Anatomy Project
CHEF	Contour clamped homogenous electric field
CML10	Canine melanoma cell line
CMT3	Canine osteosarcoma cell line
CMT7	Canine mammary tumour cell line
CMT8	Canine osteosarcoma cell line
CMV	Cytomegalovirus
D17	Canine osteosarcoma cell line
DAB	3,3 diaminobenzidine
DEPC	Diethylpolycarbonate

dH_2O	Distilled water
DIG	Digoxigenin
(D)MEM	(Dulbecco's) Minimum Essential Medium
DNA-PK	DNA dependant protein kinase
DOPE	L-dioleoyl phosphatidycthanolamine
DSH	Domestic Shorthaired (cat)
DTT	DL-Dithiothreitol
ECTR	Extra chromosomal telomere repeats
EQ1	Primary equine cells
EREs	Oestrogen response elements
FCS	Foetal calf serum
FISH	Fluorescent *in situ* hybridisation
GHK	Canine renal cell line
GM847	Human fibroblast cell line
GSP	Gene specific primers
GUVS	Glasgow University Veterinary school
HGU133	Human genome Affymetrix GeneChip
HPA	Hybridisation protection assay
HRPO	Horseradish peroxidase
ICC	Immunocytochemistry
IGFBP-2	Insulin-like growth factor binding protein 2
IU	International units
IVT	*In vitro* transcription
Kb	kilobase pairs
kDa	Kilodaltons
LNO_2	Liquid nitrogen
M1,M2	Mortality stages 1 and 2
MAS	Affymetrix microarray suite software
MCF7	Human mammary tumour cell line
MDCK	Madin Darby canine kidney cell line
MES	2-(N-Morpholino)ethanesulphonic acid
MM	Mismatch
MMLV-RT	Murine Moloney Virus reverse transcriptase

$mTR^{-/-}$	Murine telomerase RNA knockout
PAGE	Polyacrylamide gel electrophoresis
PAP2	phosphatidic acid phosphatase type 2A
PARP	Polyadenosine phosphate ribose polymerase
PBLs	Peripheral blood leukocytes
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDs	Population doublings
PLG	Phase lock gel
PM	Perfect match
PML	Promyelocytic leukaemia nuclear bodies
RNAi	RNA interference
RNAse	Ribonuclease
RTA	Relative telomerase activity
RTI	Reverse transcriptase inhibitor
RT-PCR	Reverse transcriptase PCR
S22	Primary feline fibroblasts
SA- β –GAL	Senescence associated β-galactosidase
SAGE	Serial analysis of gene expression
SAPE	Streptavidin-phycoerythrin
SDS	Sodium dodecyl sulphate
SFA	Primary canine fibroblasts
siRNAs	Short interfering RNAs
SV40	Simian virus 40
TEP1	Telomerase associated protein 1
TERT	Telomerase reverse transcriptase
Tm	Annealing temperature
TMB	3,3′, 5,5′-tetramethylbenzidine
TPE	Telomere position effect
TR	Telomerase RNA
TRAP	Telomeric repeat amplification protocol
TRF	Telomere restriction fragment
TSP-1	Thrombospondin 1

WCPN	Waltham Centre for Pet Nutrition
WT1	Wilms' tumour 1 tumour supressor

REFERENCES

Ahmed, A. & Tollefsbol, T. 2001, "Telomeres and Telomerase: Basic Science Implications for Aging", *Journal of the American Geriatrics Society*, vol. 49, no. 8, pp. 1105-1109.

Ahmed, S., Sheng, H., Niu, L., & Henderson, E. 1998, "Tetrahymena mutants with short telomeres", *Genetics*, vol. 150, no. 2, pp. 643-650.

Akiyama, M., Hideshima, T., Hayashi, T., Tai, Y. T., Mitsiades, C. S., Mitsiades, N., Chauhan, D., Richardson, P., Munshi, N. C., & Anderson, K. C. 2002, "Cytokines Modulate Telomerase Activity in a Human Multiple Myeloma Cell Line", *Cancer Research*, vol. 62, no. 13, pp. 3876-3882.

Alcorta, D., Xiong, Y., Phelps, D., Hannon, G., Beach, D., & Barrett, J. á. 1996, "Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts", *Proceedings of the National Academy of Sciences*, vol. 93, no. 24, pp. 13742-13747.

Alizedeh, A., Eisen, M., Davis, R., Chi, M., Lossos, I., Rosenwald, A., Boldrick, J., Sabet, H., Tran, T., Yu, X., Powell, J., Yang, L., Marti, G., Moore, T., Hudson, J., Lu, L., Lewis, D., Tibshirani, R., Sherlock, G., Chang, W., Greiner, T., Weisenburger, D., Armitage, J., Warnke, R., Levy, R., Wilson, W., Grever, M., Byrd, J., Botstein, D., Brown, P., & Staudt, L. 2000, "Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling", *Nature*, vol. 403, pp. 503-511.

Allshire, R. C., Dempster, M., & Hastie, N. D. 1989, "Human telomeres contain at least three types of G-rich repeat distributed non-randomly", *Nucleic Acids Research*, vol. 17, no. 12, pp. 4611-4627.

Allsop, R., Chang, E., Kashefi-Aazam, M., & Rogaev, E. 1995, "Telomere shortening is associated with cell division in vitro and in vivo", *Experimental Cell Research*, vol. 220, no. 1, pp. 194-200.

Allsopp, R. C., Vaziri, H., Patterson, C., Goldstein, S., Younglai, E. V., Futcher, A. B., Greider, C. W., & Harley, C. B. 1992, "Telomere length predicts replicative capacity of human fibroblasts", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, no. 21, pp. 10114-10118.

Argyle, D. & Nasir, L. 2003, "Telomerase: a potential diagnostic and therapeutic tool in canine oncology", *Veterinary Pathology*, vol. 40, no. 1, pp. 1-7.

Artandi, S. E. & DePinho, R. A. 2000, "Mice without telomerase: what can they teach us about human cancer?", *Nature Medicine*, vol. 6, no. 8, pp. 852-855.

Artandi, S. E. & DePinho, R. A. 2000, "A critical role for telomeres in suppressing and facilitating carcinogenesis", *Current Opinion in Genetics & Development*, vol. 10, no. 1, pp. 39-46.

Asker, C., Wiman, K. G., & Selivanova, G. 1999, "p53-Induced Apoptosis as a Safeguard against Cancer", *Biochemical and biophysical research communications*, vol. 265, no. 1, pp. 1-6.

Ausubel, F., Brent, R., Kingston, R., Moore, D., Seidman, J., Smith, J., & Struhl, K. 1994, in *Current protocols in molecular biology*, New York: Greene publishing associates Inc., and John Wiley and Sons Inc.: (Book, two volumes)

Avilion, A. A., Piatyszek, M. A., Gupta, J., Shay, J. W., Bacchetti, S., & Greider, C. W. 1996, "Human telomerase RNA and telomerase activity in immortal cell lines and tumor tissues", *Cancer Research*, vol. 56, no. 3, pp. 645-650.

Bacchetti, S. 1996, "Telomere maintenance in tumour cells", *Cancer Surveys*, vol. 28, pp. 197-216.

Bachor, C., Bachor, O., & Boukamp, P. 1999, "Telomerase is active in normal gastrintestinal mucosa and not up-regulated in precancerous lesions", *Journal of Cancer Research and Clinical Oncology*, vol. 125, pp. 453-460.

Baker, A. & Cotten, M. 1997, "Delivery of bacterial artificial chromosomes into mammalian cells with psoralen-inactivated adenovirus carrier", *Nucleic Acids Research*, vol. 25, no. 10, pp. 1950-1956.

Banik, S. S. R., Guo, C., Smith, A. C., Margolis, S. S., Richardson, D. A., Tirado, C. A., & Counter, C. M. 2002, "C-Terminal Regions of the Human Telomerase Catalytic Subunit Essential for In Vivo Enzyme Activity", *Molecular and Cellular Biology*, vol. 22, no. 17, pp. 6234-6246.

Baumann, P., Podell, E., & Cech, T. R. 2002, "Human Pot1 (Protection of Telomeres) Protein: Cytolocalization, Gene Structure, and Alternative Splicing", *Molecular and Cellular Biology*, vol. 22, no. 22, pp. 8079-8087.

Baur, J., Zou, Y., Shay, J., & Wright, W. 2001, "Telomere position effect in human cells", *Science*, vol. 292, pp. 2075-2077.

Bearss, D., Laurence H Hurley1, & Daniel D Von Hoff1 2000, "Telomere maintenance mechanisms as a target for drug development", *Oncogene*, vol. 19, no. 56, pp. 6632-6641.

Beattie, T. L., Zhou, W., Robinson, M. O., & Harrington, L. 2000, "Polymerization Defects within Human Telomerase Are Distinct from Telomerase RNA and TEP1 Binding", *Molecular Biology of the Cell*, vol. 11, no. 10, pp. 3329-3340.

Bechter, O., Eisterer, W., Dlaska, M., Kuhr, T., & Thaler, J. 2002, "CpG island methylation of the hTERT promoter is associated with lower telomerase activity in B-cell lymphocytic leukaemia", *Experimental Haematology*, vol. 30, pp. 26-33.

Benetos, A., Okuda, K., Lajemi, M., Kimura, M., Thomas, F., Skurnick, J., Labat, C., Bean, K., & Aviv, A. 2001, "Telomere Length as an Indicator of Biological Aging : The Gender Effect and Relation With Pulse Pressure and Pulse Wave Velocity", *Hypertension*, vol. 37, no. 2, pp. 381-385.

Berube, N. G., Smith, J. R., & Pereira-Smith, O. M. 1998, "The genetics of cellular senescence. [Review] [30 refs]", *American Journal of Human Genetics*, vol. 62, no. 5, pp. 1015-1019.

Bianco, P., Riminucci, M., Gronthos, S., & Robey, P. G. 2001, "Bone Marrow Stromal Stem Cells: Nature, Biology, and Potential Applications", *Stem Cells*, vol. 19, no. 3, pp. 180-192.

Bigger, C. B., Brasky, K. M., & Lanford, R. E. 2001, "DNA Microarray Analysis of Chimpanzee Liver during Acute Resolving Hepatitis C Virus Infection", *The Journal of Virology*, vol. 75, no. 15, pp. 7059-7066.

Biller, B., Kitchell, B., & Cadile, C. 1998, "Evaluation of an assay for detecting telomerase activity in neoplastic tissues of dogs", *American Journal of Veterinary Research*, vol. 59, pp. 1526-1529.

Bilsland, A., Anderson, C., Fletcher-Monaghan, A., Jeffry Evans, Ganly, I., Knox, R., Plumb, J., & Keith, W. 2003, "Selective ablation of human cancer cells by telomerase-specific adenoviral suicide gene therapy vectors expressing bacterial nitroreductase", *Oncogene*, vol. 22, no. 3, pp. 370-380. 1994.

Bischof, O., Kim, S., Irving, J., Beresten, S., Ellis, N. A., & Campisi, J. 2001, "Regulation and Localization of the Bloom Syndrome Protein in Response to DNA Damage", *The Journal of Cell Biology*, vol. 153, no. 2, p. 367.

Blackburn, E. H. & Szostak, J. 1984, "The molecular structure of centromeres and telomeres", *Annual Review of Biochemistry*, vol. 53, pp. 163-194.

Blackburn, E. H. 1990, "Telomeres and their synthesis", *Science*, vol. 249, pp. 489-490.

Blackburn, E. H. 1991, "Structure and function of telomeres", *Nature*, vol. 350, no. 6319, pp. 569-573.

Blasco, M., Lee, H., Hande, M., Samper, E., Lansdorp, P., dePinho, R., & Greider, C. 1997, "Telomere shortening and tumor formation by mouse cells lacking telomerase RNA", *Cell*, vol. 91, no. 1, pp. 25-34.

Blount, K. & Uhlenbeck, O. 2002, "The hammerhead ribozyme", *Biochemical Society Transactions*, vol. 30, no. 6, pp. 1119-1122.

Bodnar, A. G., Ouellette, M., Frolkis, M., Holt, S. E., Chiu, C. P., Morin, G. B., Harley, C. B., Shay, J. W., Lichtsteiner, S., & Wright, W. E. 1998, "Extension of Life-Span by Introduction of Telomerase into Normal Human Cells", *Science*, vol. 279, no. 5349, pp. 349-352.

Boisvert, F. M., Hendzel, M. J., & Bazett-Jones, D. P. 2000, "Promyelocytic Leukemia (PML) Nuclear Bodies are protein structures that do not Accumulate RNA", *The Journal of Cell Biology*, vol. 148, no. 2, p. 283.

Bouffler, S., Morgan, W., Pandita, T., & Slijepcevic, P. 1996, "The involvement of telomeric sequences in chromosomal aberrations", *Mutation research*, vol. 366, pp. 129-135.

Boulanger, C. A. & Smith, G. H. 2001, "Reducing mammary cancer risk through premature stem cell senescence", *Oncogene*, vol. 20, pp. 2264-2272.

Braunschweig, R., Guilleret, I., Delacretaz, F., Bosman, F., Mihaescu, A., & Benhattar, J. 2001, "Pitfalls in TRAP assay in routine detection of malignancy in effusions", *Diagnostic cytopathology*, vol. 25, no. 4, pp. 225-230.

Bringold, F. & Serrano, M. 2000, "Tumor suppressors and oncogenes in cellular senescence", *Experimental Gerontology*, vol. 35, no. 3, pp. 317-329.

Broccoli, D., Smogorzewska, A., Chong, L., & de Lange, T. 1997, "Human telomeres contain two distinct Myb-related proteins, TRF1 and TRF2", *Nature Genetics*, vol. 17, no. 2, pp. 231-235.

Bronson, R. 1982, "Variation in age at death of dogs of different sexes and breeds", *American Journal of Veterinary Research*, vol. 43, pp. 2057-2059.

Brown, T., Sigurdson, E., Rogatko, A., & Broccoli, D. 2003, "Telomerase Inhibition Using Azidothymidine in the HT-29 Colon Cancer Cell Line", *Annals of Surgical Oncology*, vol. 10, no. 8, pp. 910-915.

Bruce, S. A., Deamond, S. F., & Ts'o, P. O. 1986, "In vitro senescence of Syrian hamster mesenchymal cells of fetal to aged adult origin. Inverse relationship between in vivo donor age and in vitro proliferative capacity", *Mechanisms of Ageing & Development*, vol. 34, no. 2, pp. 151-173.

Brummendorf, T., Mak, J., Sabo, K., Baerlocher, G., Dietz, K., Abkowitz, J., & Lansdorp, P. M. 2002, "Longitudinal studies of telomere length in feline blood cells: Implications for haematopoeitic stem cell turnover in vivo", *Experimental Haematology*, vol. 30, no. 10, pp. 1147-1152.

Bryan, T. M., Englezou, A., Gupta, J., Bacchetti, S., & Reddel, R. R. 1995, "Telomere elongation in immortal human cells without detectable telomerase activity", *The EMBO Journal*, vol. 14, no. 17, pp. 4240-4248.

Bryan, T. M. & Reddel, R. R. 1997, "Telomere Dynamics and Telomerase Activity In In Vitro Immortalised Human Cells", *European Journal of Cancer*, vol. 33, no. 5, pp. 767-773.

Bryce, L., Morrison, N., Hoare, S., Muir, S., & Keith, W. 2000, "Mapping of the Gene for the Human Telomerase Reverse Transcriptase, *hTERT*, to Chromosome 5p15.33 by Fluorescence *in Situ* Hybridization", *Neoplasia*, vol. 2, no. 3, pp. 197-201.

Burger, A. M., Bibby, M. C., & Double, J. A. 1997, "Telomerase activity in normal and malignant mammalian tissues: feasibility of telomerase as a target for cancer chemotherapy", *British Journal of Cancer*, vol. 75, no. 4, pp. 516-522.

Buys, C. H. 2000, "Telomeres, telomerase, and cancer", *New England Journal of Medicine*, vol. 342, no. 17, pp. 1282-1283.

Cadile, C. D., Kitchell, B. E., Biller, B. J., Hetler, E. R., & Balkin, R. G. 2001, "Telomerase activity as a marker for malignancy in feline tissues", *American Journal of Veterinary Research*, vol. 62, no. 10, pp. 1578-1581.

Cameron, I. 1970, "Cell renewal in the organs and tissues of the nongrowing adult mouse", *Texas Reports on Biology and Medicine*, vol. 28, pp. 203-248.

Campisi, J., Dimri, G., & Hara, E. 1996, "Control of replicative senescence", in *Handbook of the biology of ageing*, E. Schneider & J. Rowe, eds., Academic Press, New York, pp. 121-149 (Book)

Campisi, J. 2000, "Cancer, ageing and cellular senescence", *In Vivo*, vol. 14, pp. 183-188.

Campisi, J. 1997, "The biology of replicative senescence", *European Journal of Cancer*, vol. 33, no. 5, pp. 703-709.

Campisi, J. 2001a, "Cellular senescence as a tumor-suppressor mechanism", *Trends in Cell Biology*, vol. 11, no. 11, pp. S27-S31.

Campisi, J. 2001, "From cells to organisms: can we learn about aging from cells in culture?", *Experimental Gerontology*, vol. 36, no. 4-6, pp. 607-618.

Campisi, J., Kim, S. h., Lim, C. S., & Rubio, M. 2001, "Cellular senescence, cancer and aging: the telomere connection", *Experimental Gerontology*, vol. 36, no. 10, pp. 1619-1637.

Caplan, A. I. & Bruder, S. P. 2001, "Mesenchymal stem cells: building blocks for molecular medicine in the 21st century", *Trends in Molecular Medicine*, vol. 7, no. 6, pp. 259-264.

Carle, G., Frank, M., & Olson, M. 1986, "Electrophoretic separations of large DNA molecules by periodic inversion of the electric field.", *Science*, vol. 232, no. 4746, pp. 65-68.

Cerone, M. A., Londono-Vallejo, J. A., & Bacchetti, S. 2001, "Telomere maintenance by telomerase and by recombination can coexist in human cells", *Human Molecular Genetics*, vol. 10, no. 18, p. 1945.

Cervantes, R. B. & Lundblad, V. 2002, "Mechanisms of chromosome-end protection", *Current Opinion in Cell Biology*, vol. 14, no. 3, pp. 351-356.

Chadeneau, C., Siegal, P., Harley, C., Muller, W., & Bacchetti, S. 1995, "Telomerase activity in normal and malignant murine tissues", *Oncogene*, vol. 11, pp. 893-898.

Chang, B-D., Swift, M. E., Shen, M., Fang, J., Broude, E. V. & Roninson, I. B. 2002, "Molecular determinants of terminal growth arrest induced in tumour cells by a chemotherapeutic agent", *Proceedings of the National Academy of Sciences*, vol. 99, no. 1, pp. 389-394.

Chang, S., Khoo, C., Naylor, M., Maser, R., & DePinho, R. A. 2003, "Telomere based crisis: functional differences between telomerase activation and ALT in tumor progression", *Genes and Development*, vol. 17, pp. 88-100.

Chimenti, C., Kajstura, J., Torella, D., Urbanek, K., Heleniak, H., Colussi, C., Di Meglio, F., Nadal-Ginard, B., Frustaci, A., Leri, A., Maseri, A., & Anversa, P. 2003, "Senescence and death of primitive cells and myocytes lead to premature cardiac aging and heart failure", *Circulation Research*, vol. 93, no. 7, pp. 604-613.

Chen, Q. & Ames, B. 1994, "Senescence-like growth arrest induced by hydrogen peroxide in human diploid fibroblast F65 cells", *Proceedings of the National Academy of Sciences*, vol. 91, pp. 4130-4134.

Cherif, H., Tarry, J. L., Ozanne, S. E., & Hales, C. N. 2003, "Ageing and telomeres: a study into organ- and gender-specific telomere shortening", *Nucleic Acids Research*, vol. 31, no. 5, pp. 1576-1583.

Chin, L., Artandi, S., Shen, Q., Tam, A., Lee, S., Gottlieb, E., Greider, C., & dePinho, R. 1999, "p53 deficiency rescues the adverse effects of telomere loss and cooperates with telomere dysfunction to accelerate carcinogenesis", *Cell*, vol. 97, no. 4, pp. 527-538.

Chirgwin, J., Przybyla, A., MacDonald, R., & Rutter, W. 1979, "Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease", *Biochemistry*, vol. 18, no. 24, pp. 5294-5299.

Chismar, D., Mondala, T., Fox, H., Roberts, E., Langford, D., Masliah, E., Salomon, D., & Head, S. 2002, "Analysis of result variability from high-density oligonucleotide arrays comparing same-species and cross-species hybridisations", *Biotechniques*, vol. 33, pp. 516-524.

Choi, H., Lin, Z., Li, B., & Liu, A. 1990, "Age-dependent decrease in the heat-inducible DNA sequence-specific binding activity in human diploid fibroblasts", *Journal of Biological Chemistry*, vol. 265, pp. 18005-18011.

Chomcyznski, P. & Saachi, N. 1987, "Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction", *Analytical Biochemistry*, vol. 162, no. 1, pp. 156-159.

Chong, L., van Steensel, B., Broccoli, D., Erdjument-Bromage, H., Hanish, J., Tempst, P., & de Lange, T. 1995, "A Human Telomeric Protein", *Science*, vol. 270, no. 5242, pp. 1663-1667.

Chu, C., Piatyszek, M. A., & Wong, S. 2000, "Telomerase activity and telomere lengths in the NCI cell panel of human cancer cell lines", *Proceedings of the American Association for Cancer Research*, vol. 41, p. 3405.

Chu, G., Vollrath, D., & Davis, R. 1986, "Separation of large DNA molecules by contour-clamped homogeneous electric fields", *Science*, vol. 234, no. 4783, pp. 1582-1585.

Chu, R., Lin, C., Liu, C., Yang, S., Hsiao, Y., Hung, S., Pao, H., & Liao, K. 2001, "Proliferation characteristics of canine transmissible venereal tumor", *Anticancer research*, vol. 21, no. 6(A), pp. 4017-4024.

Churchill, G. 2002, "Fundamentals of experimental design for cDNA microarrays", *Nature Genetics (Supplement)*, vol. 32, pp. 490-495.

Cimino-Reale, G., Pascale, E., Battiloro, E., Starace, G., Verna, R., & D'Ambrosio, E. 2001, "The length of telomeric G-rich strand 3'-overhang measured by oligonucleotide ligation assay", *Nucleic Acids Research*, vol. 29, pp. 1-6.

Colgin, L. M., Baran, K., Baumann, P., Cech, T. R., & Reddel, R. R. 2003, "Human POT1 Facilitates Telomere Elongation by Telomerase", *Current Biology*, vol. 13, no. 11, pp. 942-946.

Colitz, C., Davidson, M., McGahan, M., & Christine, M. 1999, "Telomerase Activity in Lens Epithelial Cells of Normal and Cataractous Lenses", *Experimental Eye Research*, vol. 69, no. 6, pp. 641-649.

Collins, K. & Mitchell, J. 2002, "Telomerase in the human organism", *Oncogene*, vol. 21, pp. 564-579.

Cong, Y. S., Wen, J., & Bacchetti, S. 1999, "The human telomerase catalytic subunit hTERT: organization of the gene and characterization of the promoter", *Human Molecular Genetics*, vol. 8, no. 1, p. 137.

Coons, A., Creech, H., & Jones, R. 1941, "Immunological properties of an antibody containing a fluorescent group", *Proceedings of the society for experimental biology and medicine*, vol. 47, pp. 200-202.

Corey, D. 2002, "Telomerase inhibition, oligonucleotides, and clinical trials", *Oncogene*, vol. 21, no. 4, pp. 631-634.

Cristofalo, V. J. & Pignolo, R. J. 1993, "Replicative senescence of human fibroblast-like cells in culture", *Physiological Reviews*, vol. 73, no. 3, pp. 617-638.

Cristofalo, V. J., Allen, R. G., Pignolo, R. J., Martin, B. G., & Beck, J. C. 1998, "Relationship between donor age and the replicative lifespan of human cells in culture: a reevaluation", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 18, pp. 10614-10619.

Cuthbert, A. P., Bond, J., Trott, D. A., Gill, S., Broni, J., Marriott, A., Khoudoli, G., Parkinson, E. K., Cooper, C. S., & Newbold, R. F. 1999, "Telomerase Repressor Sequences on Chromosome 3 and Induction of Permanent Growth Arrest in Human Breast Cancer Cells", *JNCI Cancer Spectrum*, vol. 91, no. 1, pp. 37-45.

de Lange, T. 1995, in *Telomeres*, E. H. Blackburn & C. Greider, eds., Cold Spring Harbor Press, New York, pp. 265-293.

de Murcia, G. & de Murcia, J. 1994, "Poly(ADP-ribose)polymerase: a molecular nick-sensor", *Trends in Biochemical Sciences*, vol. 19, pp. 172-176.

Dhaene, K., Van Marck, E., & Parwaresch, R. 2000, "Telomeres, telomerase and cancer: an up-date", *Virchows Archiv*, vol. 437, no. 1, pp. 1-16.

Dias, N. & Stein, C. 2003, "Potential roles of antisense oligonucleotides in cancer therapy. The example of Bcl-2 antisense oligonucleotides", *European Journal of Pharmaceutics & Biopharmaceutics*, vol. 54, no. 3, pp. 263-269.

Dimri, G. P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E. E., Linskens, M., Rubelj, I., Pereira-Smith, O., Peacocke, M., & Campisi, J. 1995, "A Biomarker that Identifies Senescent Human Cells in Culture and in Aging Skin in vivo", *Proceedings of the National Academy of Sciences*, vol. 92, no. 20, pp. 9363-9367.

Dimri, G. P., Testori, A., Acosta, M. and Campisi, J. 1996, "Replicative senescence, ageing and growth-regulatory transcription factors", *Biological Signals*, vol. 5, no. 3, pp. 154-162.

Dimri, G. P., Itahana, K., Acosta, M., & Campisi, J. 2000, "Regulation of a Senescence Checkpoint Response by the E2F1 Transcription Factor and p14ARF Tumor Suppressor", *Molecular and Cellular Biology*, vol. 20, no. 1, p. 273.

Dunham, M., Neumann, A., Fasching, C., & Reddel, R. 2000, "Telomere maintenance by recombination in human cells", *Nature Genetics*, vol. 26, no. 4, pp. 447-450.

Farhood, H., Serbina, N., & Huang, L. 1995, "The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer", *Biophysical Acta*, vol. 1235, p. 289.

Fawcett, T., Sylvester, S., Sarge, K., Morimoto, R., & Holbrook, N. 1994, *Journal of Biological Chemistry*, vol. 269, pp. 32272-32278.

Feng, J., Funk, W., Wang, S., Weinrich, S., Avilion, A., Chiu, C., Adams, R., Chang, E., Allsopp, R., Yu, J., Le, S., West, M., Harley, C., Andrews, W., Greider, C., & Villeponteau, B. 1995, "The RNA component of human telomerase", *Science*, vol. 269, pp. 1236-1241.

Forsyth, N., Wright, W., & Shay, J. 2002, "Telomerase and differentiation in multicellular organisms: turn it off, turn it on, and turn it off again", *Differentiation*, vol. 69, pp. 188-197.

Funakoshi, Y., Nakayama, H., Uetsuka, K., Nishimura, R., Sasaki, N., & Doi, K. 2000, "Cellular proliferative and telomerase activity in canine mammary gland tumors", *Veterinary Pathology*, vol. 37, no. 2, pp. 177-183.

Furugori, E., Hirayama, R., Nakamura, K., Kammori, M., Esaki, Y., & Takubo, K. 2000, "Telomere shortening in gastric carcinoma with ageing despite telomerase activation", *Journal of Cancer Research and Clinical Oncology*, vol. 126, pp. 481-485.

Furumoto, K., Inoue, E., Nagao, N., Hiyama, E., & Miwa, N. 1998, "Age-dependent telomere shortening is slowed down by enrichment of intracellular vitamin C via suppression of oxidative stress", *Life Sciences*, vol. 63, no. 11, pp. 935-948.

Gabriels, J., Beckers, M.-C., Ding, H., De Vriese, A., Plaisance, S., van der Maarel, S. M., Padberg, G. W., Frants, R. R., Hewitt, J. E., Collen, D., & Belayew, A. 1999, "Nucleotide sequence of the partially deleted D4Z4 locus in a patient with FSHD identifies a putative gene within each 3.3 kb element", *Gene*, vol. 236, no. 1, pp. 25-32.

Gao, X. & Huang, L. 1995, "Cationic liposome-mediated gene transfer", *Gene Therapy*, vol. 2, no. 10, pp. 710-722.

Gaush, C.R., Hard, W.L. & Smith, T.L. 1966, "Characterisation of an established cell line of canine kidney cells", *Proceedings of the Society for Experimental Biology and Medicine*, vol. 122, pp. 931-935.

Ghebranious, N. & Donehower, L. A. 1998, "Mouse models in tumor suppression", *Oncogene*, vol. 17, pp. 3385-3400.

Goldman, M. 2003, "The role of telomeres and telomerase in cancer", *Drug discovery today*, vol. 8, no. 7, pp. 294-296.

Goldstein, S. & Harley, C. 1979, "In-vitro studies of age related diseases", *Federation Proceedings*, vol. 38, no. 5, pp. 1862-1867.

Goldstein, S., Moerman, E. J., Soeldner, J. S., Gleason, R. E., & Barnett, D. M. 1978, "Chronologic and physiologic age affect replicative life-span of fibroblasts from diabetic, prediabetic, and normal donors", *Science*, vol. 199, no. 4330, pp. 781-782.

Gowan, S. M., Heald, R., Stevens, M. F. G., & Kelland, L. R. 2001, "Potent Inhibition of Telomerase by Small-Molecule Pentacyclic Acridines Capable of Interacting with G-Quadruplexes", *Molecular Pharmacology*, vol. 60, no. 5, pp. 981-988.

Grandori, C., Cowley, S. M., James, L. P., & Eisenman, R. N. 2000, "The Myc/Max/Mad network and the transcriptional control of cell behavior", *Annual Review of Cell and Developmental Biology*, vol. 16, no. 1, p. 653.

Greider, C. W. 1999, "Telomeres do D-loop-T-loop", *Cell*, vol. 97, no. 4, pp. 419-422.

Greider, C. & Blackburn, E. 1985, "Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts", *Cell*, vol. 44, pp. 405-413.

Griffith, J. D., Comeau, L., Rosenfield, S., Stansel, R. M., Bianchi, A, Moss, H., & de Lange, T. 1999, "Mammalian telomeres end in a large duplex loop", *Cell*, vol. 97, no. 4, pp. 503-514.

Griffith, J., Bianchi, A., & de Lange, T. 1998, "TRF1 Promotes Parallel Pairing of Telomeric Tracts in Vitro", *Journal of Molecular Biology*, vol. 278, no. 1, pp. 79-88.

Grobelny, J. V., Godwin, A. K., & Broccoli, D. 2000, "ALT-associated PML bodies are present in viable cells and are enriched in cells in the G(2)/M phase of the cell cycle", *Journal of Cell Science*, vol. 113, no. 24, pp. 4577-4585.

Gu, J., Kagawa, S., Takakura, M., Kyo, S., Inoue, M., Roth, J. A., & Fang, B. 2000, "Tumor-specific Transgene Expression from the Human Telomerase Reverse Transcriptase Promoter Enables Targeting of the Therapeutic Effects of the Bax Gene to Cancers", *Cancer Research*, vol. 60, no. 19, pp. 5359-5364.

Gygi, S. P., Rochon, Y., Franza, B. R., & Aebersold, R. 1999, "Correlation between Protein and mRNA Abundance in Yeast", *Molecular and Cellular Biology*, vol. 19, no. 3, pp. 1720-1730.

Hahn, W., Counter, C., Lundberg, A. S., Beijersbergen, R., Brooks, M., & Weinberg, R. 1999, "Creation of human tumour cells with defined genetic elements", *Nature*, vol. 400, pp. 464-468.

Hammond, S. M., Boettcher, S., Caudy, A. A., Kobayashi, R., & Hannon, G. J. 2001, "Argonaute2, a Link Between Genetic and Biochemical Analyses of RNAi", *Science*, vol. 293, no. 5532, p. 1146.

Hanahan, D. & Weinberg, R. 2000, "The hallmarks of cancer", *Cell*, vol. 100, pp. 57-70.

Harley, C. B., Futcher, A. B., & Greider, C. W. 1990, "Telomeres shorten during ageing of human fibroblasts", *Nature*, vol. 345, no. 6274, pp. 458-460.

Harley, C. B. 1991, "Telomere loss: mitotic clock or genetic time bomb?", *Mutation research*, vol. 256, no. 2-6, pp. 271-282.

Harley, C. 1995, "Telomeres and ageing", in *Telomeres*, E. Blackburn & C. Greider, eds., Cold Spring Harbor Press, New York, pp. 247-263.

Harley, C. 2002, "Telomerase is not an oncogene", *Oncogene*, vol. 21, pp. 494-502.

Harrington, C., Rosenow, C., & Retief, J. 2000, "Monitoring gene expression using DNA microarrays", *Current Opinion in Microbiology*, vol. 3, pp. 285-291.

Harrington, L., McPhail, T., Mar, V., Amgen EST Program, Bass, M., Arruda, I., & Robinson, M. 1997, "A mammalian telomerase associated protein", *Science*, vol. 275, pp. 973-977.

Hasseman, J. 2002, "Microarray technology: an array of opportunities", *Nature*, vol. 416, pp. 885-895.

Hastie, N., Dempster, M., Dunlop, M., Thompson, A., Green, D., & Allshire, R. C. 1990, "Telomere reduction in human colorectal carcinoma and with ageing", *Nature*, vol. 346, no. 6287, pp. 866-868.

Haussmann, M. & Vleck, C. 2002, "Telomere length provides a new technique for ageing animals", *Oecologia*, vol. 130, pp. 325-328.

Hayflick, L. & Moorhead, P. 1961, "The serial cultivation of human diploid cell strains", *Experimental Cell Research*, vol. 25, pp. 585-621.

Hayflick, L. 1965, "The limited *in vitro* lifetime of human diploid cell strains", *Experimental Cell Research*, vol. 37, pp. 614-636.

Hayflick, L. 1997, "Mortality and immortality at the cellular level: a review", *Biochemistry*, vol. 62, pp. 1180-1190.

Haendeler, J., Hoffmann, J., Brandes, R. P., Zeiher, A. M., & Dimmeler, S. 2003, "Hydrogen peroxide triggers nuclear export of telomerase reverse transcriptase via Src kinase family-dependent phosphorylation of tyrosine 707", *Molecular & Cellular Biology*, vol. 23, no. 13, pp. 4598-4610.

Hemann, M. T. & Greider, C. W. 2000, "Wild-derived inbred mouse strains have short telomeres", *Nucleic Acids Research*, vol. 28, no. 22, pp. 4474-4478

Hemann, M., Strong, M., Hao, L., & Greider, C. W. 2001, "The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability", *Cell*, vol. 107, pp. 67-77.

Henderson, B., Ross, R., & Pike, M. 1993, "Hormonal chemoprevention of cancer in women", *Science*, vol. 259, pp. 633-638.

Henderson, E., Hardin, C., Wolk, S., Tinoco, I., & Blackburn, E. 1987, "Telomeric DNA oligonucleotides form novel intramolecular structures containing guanine-guanine base pairs", *Cell*, vol. 51, pp. 899-908.

Henderson, Y.C., Ta-Jen Liu, R. L. B., & Clayman G.L. 2000, "Telomerase activity in head and neck tumors after introduction of wild-type p53, p21, p16, and E2F-1 genes by means of recombinant adenovirus", *Head and Neck*, vol. 22, no. 4, pp. 347-354.

Henson, J., Neumann, A., Yeager, T., & Reddel, R. 2002, "Alternative lengthening of telomeres in mammalian cells", *Oncogene*, vol. 21, no. 4, pp. 598-610.

Herbert, B. S., Pitts, A. E., Baker, S. I., Hamilton, S. E., Wright, W. E., Shay, J. W., & Corey, D. R. 1999, "Inhibition of human telomerase in immortal human cells leads to progressive telomere shortening and cell death", *Proceedings of the National Academy of Sciences*, vol. 96, no. 25, pp. 14276-14281.

Hernandez, J. A. & Cristina, E. 1998, "Modeling cell volume regulation in nonexcitable cells: the roles of the Na+ pump and of cotransport systems", *AJP - Cell Physiology*, vol. 275, no. 4, p. C1067-C1080.

Hickman, E. S., Moroni, M. C., & Helin, K. 2002, "The role of p53 and pRB in apoptosis and cancer", *Current Opinion in Genetics & Development*, vol. 12, no. 1, pp. 60-66.

Hiyama, E., Hiyama, K., Tatusumoto, N., Kodama, T., Shay, J., & Yokoyama, T. 1996, "Telomerase activity in human intestine", *International Journal of Oncology*, vol. 9, pp. 453-458.

Hiyama, E. & Hiyama, K. 2003, "Telomerase as tumor marker", *Cancer Letters*, vol. 194, no. 2, pp. 221-233.

HMSO 1995, in Technology foresight panel on health and life sciences. Progress through partnership. Technology foresight 4. London.

Hoflich, A., Lahm, H., Blum, W., Kolb, H., & Wolf, E. 1998, "Insulin-like growth factor-binding protein-2 inhibits proliferation of human embryonic kidney fibroblasts and of IGF-responsive colon carcinoma cell lines", *FEBS Letters*, vol. 434, no. 3, pp. 329-334.

Horner, P. & Gage, F. 2000, "Regenerating the damaged nervous system", *Nature*, vol. 407, pp. 963-970.

Huber, L. 2003, "Is proteomics heading in the wrong direction?", *Nature Reviews Molecular cell biology*, vol. 4, pp. 74-80.

Huffman, K., Levene, S., Tesmer, V., Shay, J., & Wright, W. 2000, "Telomeric shortening is proportional to the size of the G-rich telomeric 3' overhang", *The Journal of Biological Chemistry*, vol. 275, pp. 19719-19722.

Hultdin, M., Gronlund, E., Norrback, K., Eriksson-Lindstrom, E., Just, T., & Roos, G. 1998, "Telomere analysis by fluorescence in situ hybridization and flow cytometry", *Nucleic Acids Research*, vol. 26, no. 16, pp. 3651-3656.

Huschtscha, L. & Reddel, R. 1999, "p16 INK4a and the control of cellular proliferative life span", *Carcinogenesis*, vol. 20, no. 6, pp. 921-926.

Hutvagner, G. & Zamore, P. D. 2002, "RNAi: nature abhors a double-strand", *Current Opinion in Genetics & Development*, vol. 12, no. 2, pp. 225-232.

Innis, M. & Gelfland, D. 1990, *PCR protocols. A guide to methods and applications.* Academic Press, Inc, San Diego,CA (Book)

Ishii, Y., Tsuyama, N., Maeda, S., Tahara, H., & Ide, T. 1999, "Telomerase activity in hybrids between telomerase-negative and telomerase-positive immortal human cells is repressed in the different complementation groups but not in the same complementation group of immortality", *Mechanisms of Ageing and Development*, vol. 110, no. 3, pp. 175-193.

Ishikawa, F. 2000, "Aging clock: the watchmaker's masterpiece", *Cellular & Molecular Life Sciences*, vol. 57, no. 5, pp. 698-704.

Ivanov, I., Schaab, C., Planitzer, U., Teichmann, A., Machl, S., Theml, S., Meier-Ewert, B., Seizinger, H., & Loferer, H. 2000, "DNA microarray technology and antimicrobial drug discovery", *Pharmacogenomics*, vol. 1, no. 2, pp. 169-178.

Jeanclos, E., Schork, N., Kyvik, K., Kimura, M., Skurnick, J., & Aviv, A. 2000, "Telomere length inversly correlates with pulse pressure and is highly familial", *Hypertension*, vol. 36, pp. 195-200.

Jeffreys, A., Wilson, V., Neumann, R., & Keyte, J. 1988, "Amplification of human minisatellites by the polymerase chain reaction: towards DNA fingerprinting of single cells", *Nucleic Acids Research*, vol. 16, no. 23, pp. 10953-10971.

Jiang, X., Jimenez, G., Chang, E., Frolkis, M., Kusler, B., Sage, M., Beeche, M., Bodnar, A., Wahl, G., Tlsty, T., & Chiu, C. 1999, "Telomerase expression in human somatic cells does not induce changes associated with a transformed phenotype", *Nature Genetics*, vol. 21, pp. 111-114.

Johnson, C. 2000, "Disorders of the oestrus cycle," in *Essentials of small animal internal medicine*, R. Nelson & C. Couto, eds., pp. 633-636.

Kajstura, J., Leri, A., Finato, N., Di Loreto, C., Beltrami, C. A., & Anversa, P. 1998, "Myocyte proliferation in end-stage cardiac failure in humans", *Proceedings of the National Academy of Sciences*, vol. 95, no. 15, pp. 8801-8805.

Kalas, W., Gilpin, S., Yu, J. L., May, L., Krchnakova, H., Bornstein, P., & Rak, J. 2003, "Restoration of thrombospondin 1 expression in tumor cells harbouring mutant ras oncogene by treatment with low doses of doxycycline", *Biochemical and biophysical research communications*, vol. 310, no. 1, pp. 109-114.

Kaminker, P. G., Kim, S. h., Taylor, R. D., Zebarjadian, Y., Funk, W. D., Morin, G. B., Yaswen, P., & Campisi, J. 2001, "TANK2, a New TRF1-associated Poly(ADP-ribose) Polymerase, Causes Rapid Induction of Cell Death upon Overexpression", *Journal of Biological Chemistry*, vol. 276, no. 38, pp. 35891-35899.

Karlseder, J., Broccoli, D., Dai, Y., Hardy, S., & de Lange, T. 1999, "p53- and ATM-Dependent Apoptosis Induced by Telomeres Lacking TRF2", *Science*, vol. 283, no. 5406, pp. 1321-1325.

Kayo, T., Allison, D. B., Weindruch, R., & Prolla, T. A. 2001, "Influences of aging and caloric restriction on the transcriptional profile of skeletal muscle from rhesus monkeys", *Proceedings of the National Academy of Sciences*, vol. 98, no. 9, pp. 5093-5098.

Kelland, L. 2001, "Telomerase: biology and phase I trials", *The Lancet (Oncology)*, vol. 2, pp. 95-102.

Kiaris, H. & Schally, A. V. 1999, "Decrease in telomerase activity in U-87MG human glioblastomas after treatment with an antagonist of growth hormone-releasing hormone", *Proceedings of the National Academy of Sciences*, vol. 96, no. 1, p. 226.

Kickhoefer, V. A., Stephen, A. G., Harrington, L., Robinson, M. O., & Rome, L. H. 1999, "Vaults and Telomerase Share a Common Subunit, TEP1", *Journal of Biological Chemistry*, vol. 274, no. 46, pp. 32712-32717.

Kim, N. W. & Wu, F. 1997, "Advances in quantification and characterization of telomerase activity by the telomeric repeat amplification protocol (TRAP)", *Nucleic Acids Research*, vol. 25, no. 13, pp. 2595-2597.

Kim, N., Piatyszek, M., Prowse, K., Harley, C. B., West, M., Ho, P., Coviello, G., Wright, W., Weinrich, S., & Shay, J. W. 1994, "Specific association of human telomerase activity with immortal cells and cancer", *Science*, vol. 266, pp. 2011-2015.

Kipling, D. & Cook, H. 1990, "Hypervariable ultra-long telomeres in mice", *Nature*, vol. 347, pp. 400-402.

Kipling, D. & Wyndford-Thomas, D. 1999, "Telomere dependant senescence", *Nature Biotechnology*, vol. 17, no. 4, pp. 313-314.

Kirk, K. E., Harmon, B. P., Reichardt, I. K., Sedat, J. W., & Blackburn, E. H. 1997, "Block in Anaphase Chromosome Separation Caused by a Telomerase Template Mutation", *Science*, vol. 275, no. 5305, pp. 1478-1481

Kirkness, E. F., Bafna, V., Halpern, A. L., Levy, S., Remington, K., Rusch, D. B., Delcher, A. L., Pop, M., Wang, W., Fraser, C. M., & Venter, J. C. 2003, "The Dog Genome: Survey Sequencing and Comparative Analysis", *Science*, vol. 301, no. 5641, pp. 1898-1903.

Kirkwood, T. 1996, "Human senescence", *Bioessays*, vol. 18, pp. 1009-1016.

Klapper, W., Parwaresch, R., & Krupp, G. 2001, "Telomere biology in human aging and aging syndromes", *Mechanisms of Ageing and Development*, vol. 122, no. 7, pp. 695-712.

Klingelhutz, A., Foster, S., & Mc, J. 1996, "Telomerase activation by the E6 gene product of human papillomavirus type 16", *Nature*, vol. 380, pp. 79-82.

Klobutcher, L., Swanton, M., Donini, P., & Prescott, D. 1981, "All gene sized DNA molecules in four species of hypotrichs have the same terminal sequence and an unusual 3' terminus", *Proceedings of the National Academy of Sciences*, vol. 78, no. 5, pp. 3015-3019.

Koenig, A., Bianco, S., Fosmire, S., Wojcieszyn, J., & Modiano, J. 2002, "Expression and significance of p53, Rb, p21/*waf-1*, p16/*ink-4a*, and PTEN tumor suppressors in canine melanoma", *Veterinary Pathology*, vol. 39, pp. 458-472.

Kohn, K. 1999, "Molecular interaction map of the mammalian cell cycle control and DNA repair systems", *Molecular Biology of the Cell,* vol. 10, pp. 2703-2734.

Koksoy, S., Phipps, A. J., Hayes, K. A., & Mathes, L. E. 2001, "SV40 Immortalization of feline fibroblasts as targets for MHC-restricted cytotoxic T-cell assays", *Veterinary Immunology and Immunopathology* , vol. 79, no. 3-4, pp. 285-295.

Kosciolek, B. A., Kalantidis, K., Tabler, M., & Rowley, P. T. 2003, "Inhibition of Telomerase Activity in Human Cancer Cells by RNA Interference", *Molecular Cancer Therapeutics*, vol. 2, no. 3, pp. 209-216

Krtolica, A., Parrinello, S., Lockett, S., Desprez, P. Y., & Campisi, J. 2001, "Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 21, pp. 12072-12077.

Kusumoto, M., Ogawa, T., Mizumoto, K., Ueno, H., Niiyama, H., Sato, N., Nakamura, M., & Tanaka, M. 1999, "Adenovirus mediated p53 gene transduction inhibits telomerase activity independant of effects on cell cycle arrest and apoptosis in human pancreatic cancer cells", *Clinical Cancer Research*, vol. 5, pp. 2140-2147.

Kwok, S., Mack, D., Mullis, K., Poiesz, B., Ehrlich, G., Blair, D., Friedman-Kien, A., & Sninsky, J. 1987, "Identification of human immunodeficiency virus sequences by using in vitro enzymatic amplification and oligomer cleavage detection", *Journal of Virology*, vol. 61, no. 5, pp. 1690-1694.

Kyo, S., Takakura, M., Kanaya, T., Zhuo, W., Fujimoto, K., Nishio, Y., Orimo, A., & Inoue, M. 1999, "Estrogen activates telomerase", *Cancer Research*, vol. 59, pp. 5917-5921.

Kyo, S., Takakura, M., Taira, T., Kanaya, T., Itoh, H., Yutsodo, M., Ariga, H., & Inoue, M. 2000, "Sp1 cooperates with c-Myc to activate transcription of the human telomerase reverse transcriptase gene (hTERT)", *Nucleic Acids Research*, vol. 28, pp. 669-677.

Laird, P. & Jaenisch, R. 1994, "DNA methylation and cancer", *Human Molecular Genetics*, vol. 3, pp. 1487-1495.

Lauzon, W., Dardon, J., Cameron, D., & Badley, A. 2000, "Flow cytometric measurement of telomere length", *Cytometry*, vol. 42, pp. 159-164.

Leem, S., Londono-Vallejo, J., Kim, J., Bui, H., Tubacher, E., Solomon, G., Park, J., Horikawa, I., Kouprina, N., Barrett, J., & Larionov, V. 2002, "The human telomerase gene: complete genomic sequence and analysis of tandem repeat polymorphisms in intronic regions", *Oncogene*, vol. 21, pp. 769-777.

Leri, A., Barlucchi, L., Limana, F., Deptala, A., Darzynkiewicz, Z., Hintze, T. H., Kajstura, J., Nadal-Ginard, B., & Anversa, P. 2001, "Telomerase expression and activity are coupled with myocyte proliferation and preservation of telomeric length in the failing heart", *Proceedings of the National Academy of Sciences*, vol. 98, no. 15, pp. 8626-8631.

Leung, D., Tompkins, C., & White, T. 1998, "Molecular cloning of two alternative spliced forms of human phosphatidic acid phosphatase cDNAs that are differentially expressed in normal and tumour cells", *DNA and cell biology*, vol. 17, pp. 377-385.

Lindsey, J., McGill, N., Lindsey, L., Green, D., & Cooke, H. 1991, "In vivo loss of telomeric repeats with age in humans", *Mutation research*, vol. 256, pp. 45-48.

Lindvall, C., Hou, M., Komurasaki, T., Zheng, C., Henriksson, M., Sedivy, J. M., Bjorkholm, M., Teh, B. T., Nordenskjold, M., & Xu, D. 2003, "Molecular Characterization of Human Telomerase Reverse Transcriptase-immortalized Human Fibroblasts by Gene Expression Profiling: Activation of the Epiregulin Gene", *Cancer Research*, vol. 63, no. 8, pp. 1743-1747.

Lingner, J. & Cech, T. R. 1996, "Purification of telomerase from Euplotes aediculatus: Requirement of a primer 3' overhang", *Proceedings of the National Academy of Sciences*, vol. 93, no. 20, pp. 10712-10717.

Lipshutz, R., Fodor, S., Gingeras, T., & Lockhart, D. 1999, "High density synthetic oligonucleotide arrays", *Nature Genetics*, vol. 21 (supplement), pp. 20-24.

Liu, K., Schoonmaker, M., Levine, B., June, C., Hodes, R., & Weng, N. 1999, "Constitutive and regulated expression of telomerase reverse transcriptase (hTERT) in human lymphocytes", *Proceedings of the National Academy of Sciences*, vol. 96, pp. 5147-5152.

Lorenz, M., Saretzki, G., Metzkow, S., & von Zglinicki, T. 2001, "BJ fibroblasts display high antioxidant capacity and slow telomere shortening independent of hTERT transfection", *Free radical biology and medicine*, vol. 31, no. 5, pp. 824-831.

Ludwig, A., Saretzki, G., Holm, P. S., Tiemann, F., Lorenz, M., Emrich, T., Harley, C. B., & von Zglinicki, T. 2001, "Ribozyme Cleavage of Telomerase mRNA Sensitizes Breast Epithelial Cells to Inhibitors of Topoisomerase", *Cancer Research*, vol. 61, no. 7, pp. 3053-3061.

Lundberg, A. S., Hahn, W. C., Gupta, P., & Weinberg, R. A. 2000, "Genes involved in senescence and immortalization", *Current Opinion in Cell Biology*, vol. 12, no. 6, pp. 705-709.

Maekawa, M., Nishida, E., & Tanoue, T. 2002, "Identification of the Anti-proliferative Protein Tob as a MAPK Substrate", *Journal of Biological Chemistry*, vol. 277, no. 40, pp. 37783-37787.

Maier, J. A., Voulalas, P., Roeder, D., & Maciag, T. 1990, "Extension of the life-span of human endothelial cells by an interleukin-1 alpha antisense oligomer", Science, vol. 249, no. 4976, pp. 1570-1574.

Makarov, V. L., Hirose, Y., & Langmore, J. P. 1997, "Long G tails at both ends of human chromosomes suggest a C strand degradation mechanism for telomere shortening", *Cell*, vol. 88, no. 5, pp. 657-666.

Manning, E., Crossland, J., Dewey, M., & Van Zant, G. 2002, "Influences of inbreeding and genetics on telomere length in mice", *Mammalian Genome*, vol. 13, pp. 234-238.

Marcand, S., Buck, S. W., Moretti, P., Gilson, E., & Shore, D. 1996, "Silencing of genes at nontelomeric sites in yeast is controlled by sequestration of silencing factors at telomeres by Rap 1 protein", *Genes and Development*, vol. 10, no. 11, pp. 1297-1309

Margosio, B., Marchetti, D., Vergani, V., Giavazzi, R., Rusnati, M., Presta, M., & Taraboletti, G. 2003, "Thrombospondin-1 as a scavenger for matrix-associated fibroblast growth factor-2", *Blood* published online August 28.

Martin-Rivera, L. & Blasco, M. A. 2001, "Identification of Functional Domains and Dominant Negative Mutations in Vertebrate Telomerase RNA Using an in Vivo Reconstitution System", *Journal of Biological Chemistry*, vol. 276, no. 8, pp. 5856-5865.

Mata, J. E., Joshi, S. S., Palen, B., Pirruccello, S. J., Jackson, J. D., Elias, N., Page, T. J., Medlin, K. L., & Iversen, P. L. 1997, "A Hexameric Phosphorothioate Oligonucleotide Telomerase Inhibitor Arrests Growth of Burkitt's Lymphoma Cellsin Vitroandin Vivo", *Toxicology and Applied Pharmacology*, vol. 144, no. 1, pp. 189-197.

Matsumura, T., Zerrudo, Z., & Hayflick, L. 1979, "Senescent human diploid cells in culture: survival, DNA synthesis and morphology", *Journal of Gerontology*, vol. 34, no. 3, pp. 328-334.

Maynagh, E. 1983, "Great Danes," in *Medical and genetic aspects of purebred dogs*, First edition, R. Clark & J. Stainer, eds., Veterinary medicine publishing company, pp. 233-239.

McClintock, B. 1941, "The stability of broken ends of chromosomes in Zea mays", *Genetics*, vol. 26, pp. 234-282.

McEachern, M. 2001, "Recombinational telomere elongation in the yeast K. lactis," in *Telomeres and telomerases: cancer and biology*, First edition, G. Krupp, ed., Georgetown.

McGall, G., Barone, A., Digglemann, M., Fodor, S., Gentalen, E., & Ngo, N. 1997, "The Efficiency of Light-Directed Synthesis of DNA Arrays on Glass Substrates ", *Journal of the American Chemical Society*, vol. 119, no. 22, pp. 5081-5090.

McKevitt, T. P., Nasir, L., Devlin, P., & Argyle, D. J. 2002, "Telomere Lengths in Dogs Decrease with Increasing Donor Age", *Journal of Nutrition*, vol. 132, no. 6, pp. 1604S-1606.

McKevitt, T. P., Nasir, L., Wallis, C. V., Argyle, D. J., 2003, "A cohort study of telomere and telomerase biology in cats", *American Journal of Veterinary Research*, vol. 64, no. 12, pp. 1496-1499.

McKusick, V. & Ruddle, F. 1987, "Toward a complete map of the human genome", *Genomics*, vol. 1, no. 2 (Editorial), pp. 103-106.

Meselson, M. & Stahl, F. 1958, "The replication of DNA in Escherichia coli", *Proceedings of the National Academy of Sciences*, vol. 44, pp. 671-682.

Michell, A. 1999, "Longevity of British breeds of dog and its relationship with sex, size, cardiovascular variables and disease", *Veterinary Record*, vol. 145, pp. 625-629.

Miller, J. 1972, *Experiments in Molecular genetics* Cold Spring Harbor Laboratory Press, New York.

Minamino, T. & Kourembanas, S. 2001, "Mechanisms of telomerase induction during vascular smooth muscle cell proliferation", *Circulation Research*, vol. 89, no. 3 pp. 237-243.

Misiti, S., Nanni, S., Fontemaggi, G., Cong, Y. S., Wen, J., Hirte, H. W., Piaggio, G., Sacchi, A., Pontecorvi, A., Bacchetti, S., & Farsetti, A. 2000, "Induction of hTERT Expression and Telomerase Activity by Estrogens in Human Ovary Epithelium Cells", *Molecular and Cellular Biology*, vol. 20, no. 11, pp. 3764-3771.

Mitchell, J., Cheng, J., & Collins, K. 1999, "A box H/ACA small nucleolar RNA-like domain at the human telomerase RNA 3' end", *Molecular and Cellular Biology*, vol. 19, pp. 567-576.

Mitchell, J. & Collins, K. 2000, "Human telomerase activation requires two independent interactions between telomerase RNA and telomerase reverse transcriptase", *Molecular cell*, vol. 6, pp. 361-371.

Mitsui, Y. & Schneider, E. L. 1976, "Relationship between cell replication and volume in senescent human diploid fibroblasts", *Mechanisms of Ageing and Development*, vol. 5, pp. 45-56.

Miura, N., Horikawa, I., Nishimoto, A., Ohmura, H., Ito, H., Hirohashi, S., Shay, J. W., & Oshimura, M. 1997, "Progressive Telomere Shortening and Telomerase Reactivation During Hepatocellular Carcinogenesis", *Cancer Genetics and Cytogenetics*, vol. 93, no. 1, pp. 56-62.

Mondello, C., Chiesa, M., Rebuzzini, P., Zongaro, S., Verri, A., Colombo, T., Giulotto, E., D'Incalci, M., Franceschi, C., & Nuzzo, F. 2003, "Karyotype instability and anchorage-independent growth in telomerase-immortalized fibroblasts from two centenarian individuals", *Biochemical and biophysical research communications*, vol. 308, no. 4, pp. 914-921.

Moore, G., Burkman, K., Carter, M., & Peterson, M. 2001, "Causes of death or reasons for euthanasia in military working dogs: 927 cases (1993-1996)", *Journal of the American Veterinary Medical Association*, vol. 219, no. 2, pp. 209-214.

Moore, M., Wetterau, L., Francis, M., Peehl, D., & Cohen, P. 2003, "Novel stimulatory role for insulin-like growth factor binding protein-2 in prostate cancer cells", *International Journal of Cancer*, vol. 105, pp. 14-19.

Morales, C., Holt, S., Ouellette, M., Kiran, J., Yan, Y., Wilson, K., White, M., Wright WE, & Shay JW 1999, "Absence of cancer–associated changes in human fibroblasts immortalized with telomerase", *Nature Genetics*, vol. 21, no. 1, pp. 115-118.

Moyzis, R. K., Buckingham, J. M., Cram, L. S., Dani, M., Deaven, L. L., Jones, M. D., Meyne, J., Ratliff, R. L., & Wu, J. R. 1988, "A highly conserved repetitive DNA sequence, (TTAGGG)n, present at the telomeres of human chromosomes", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 85, no. 18, pp. 6622-6626.

Muleya, J., Nakaichi, M., Sugahara, J., Taura, Y., Murata, T., & Nakama, S. 1998, "Establishment and characterization of a new cell line derived from feline mammary tumor", *Journal of Veterinary Medical Science*, vol. 60, no. 8, pp. 931-935.

Mullis, K., Faloona, F., Sharf, S., Saiki, R., Horn, G., & Erlich, H. 1986, "Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction", *Cold Spring Harbor Symposium on Quantitative Biology*, vol. 51, pp. 263-273.

Mullis, K. & Faloona, F. 1987, "Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction", *Methods in Enzymology*, vol. 155, pp. 335-350.

Murnane, J. P., Sabatier, L., Marder, B. A., & Morgan, W. F. 1994, "Telomere dynamics in an immortal human cell line", *The EMBO Journal*, vol. 13, no. 20, pp. 4953-4962.

Naito, T., Matsuura, A., & Ishikawa, F. 1998, "Circular chromosome formation in a fission yeast mutant defective in two ATM homologues", *Nature Genetics*, vol. 20, pp. 203-206.

Nakamura, Y., Hirose, M., Matsuo, H., Tsuyama, N., Kamisango, K., & Ide, T. 1999, "Simple, rapid, quantitative, and sensitive detection of telomeric repeats in cell lysate by a hybridisation protection assay", *Clinical Chemistry*, vol. 45, pp. 1718-1724.

Nasir, L., Devlin, P., Mckevitt, T., Rutteman, G., & Argyle, D. J. 2001, "Telomere lengths and telomerase activity in dog tissues: a potential model system to study human telomere and telomerase biology", *Neoplasia (New York)*, vol. 3, no. 4, pp. 351-359.

Nasir, L., Gault, E., Campbell, S., Veeramalai, M., Gilbert, D., McFarlane, R., Munroe, A., & Argyle, D. 2004, "Isolation and expression of the reverse transcriptase component of the *Canis familiaris* telomerase ribonucleoprotein (canTERT).", *Gene-submitted for review.*

Nguyen, D. & Crowe, D. 1999, "Intact functional domains of the retinoblastoma gene product (pRb) are required for down regulation of telomerase activity", *Biochimica et Biophysica Acta*, vol. 1445, pp. 207-215.

Ning, Y., Xu, J. f., Li, Y., Chavez, L., Riethman, H. C., Lansdorp, P. M., & Weng, N. p. 2003, "Telomere length and the expression of natural telomeric genes in human fibroblasts", *Human Molecular Genetics*, vol. 12, no. 11, pp. 1329-1336.

Nishio, K., Inoue, A., Qiao, S., Kondo, H., & Mimura, A., 2001, "Senescence and cytoskeleton: overproduction of vimentin induces senescent-like morphology in human fibroblasts", *Histochemistry & Cell Biology*, vol. 116, no. 4, pp. 321-327.

Ogino, H., Nakabayashi, K., Suzuki, M., Takahashi, E. i., Fujii, M., Suzuki, T., & Ayusawa, D. 1998, "Release of Telomeric DNA from Chromosomes in Immortal Human Cells Lacking Telomerase Activity", *Biochemical and biophysical research communications*, vol. 248, no. 2, pp. 223-227.

Oh, S., Song, Y., Yim, J., & Kim, T. K. 1999, "The Wilms' Tumor 1 Tumor Suppressor Gene Represses Transcription of the Human Telomerase Reverse Transcriptase Gene", *Journal of Biological Chemistry*, vol. 274, no. 52, pp. 37473-37478.

Okuda, K., Khan, M. Y., Skurnick, J., Kimura, M., Aviv, H., & Aviv, A. 2000, "Telomere attrition of the human abdominal aorta: relationships with age and atherosclerosis", *Atherosclerosis*, vol. 152, no. 2, pp. 391-398.

Olovnikov, A. M. 1973, "A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon", *Journal of Theoretical Biology*, vol. 41, no. 1, pp. 181-190.

Ong, S. E. & Pandey, A. 2001, "An evaluation of the use of two-dimensional gel electrophoresis in proteomics", *Biomolecular Engineering*, vol. 18, no. 5, pp. 195-205.

Oshima, J., Campisi, J., Tannock, T., & Martin, G. 1995, "Regulation of c-fos expression in senescing Werner syndrome fibroblasts differs from that

observed in senescing fibroblasts from normal donors", *Journal of Cellular Physiology*, vol. 162, no. 2, pp. 277-283.

Oshimura, M. & Barrett, J. 1997, "Multiple pathways to cellular senescence: role of telomerase repressors", *European Journal of Cancer*, vol. 33, pp. 710-715.

Ostrander, E. A., Kruglyak, L. 2000, "Unleashing the canine genome," *Genome Research*, vol. 10, no. 9, pp. 1271-1274.

Patino, W. D., Mian, O. Y., Shizukuda, Y., & Hwang, P. M. 2003, "Current and Future Applications of SAGE to Cardiovascular Medicine", *Trends in Cardiovascular Medicine*, vol. 13, no. 4, pp. 163-168.

Pendergrass, W., Lane, M., Bodkin, N., Hansen, B., Ingram, D., Roth, G., Yi, L., Bin, H., & Wolf, N. 1999, "Cellular proliferation potential during ageing and caloric restriction in Rhesus monkeys (*Macaca mulatta*)", *Journal of Cellular Physiology*, vol. 180, pp. 123-130.

Pereira-Smith, O. M., Robetorye, S., Ning, Y., & Orson, F. M. 1990, "Hybrids from fusion of normal human T lymphocytes with immortal human cells exhibit limited life span", *Journal of Cellular Physiology*, vol. 144, no. 3, pp. 546-549.

Perrem, K., Bryan, T., Englezou, A., Hackl, T., Moy, E., & Reddel, R. 1999, "Repression of an alternative mechanism for lengthening of telomeres in somatic cell hybrids", *Oncogene*, vol. 18, no. 22, pp. 3383-3390.

Perrem, K., Colgin, L. M., Neumann, A. A., Yeager, T. R., & Reddel, R. R. 2001, "Coexistence of Alternative Lengthening of Telomeres and Telomerase in hTERT-Transfected GM847 Cells", *Molecular and Cellular Biology*, vol. 21, no. 12, pp. 3862-3875.

Petropoulou, C., Trougakos, I. P., Kolettas, E., & Gonos, E.S. 2001, "Clusterin/apolipoprotein J is a novel biomarker of cellular senescence that does not affect the proliferative capacity of human diploid fibroblasts", *FEBS Letters*, vol. 509, no. 2, pp. 287-297.

Plumb, J., Bilsland, A., Kakani, R., Zhao, J., Glasspool, R., Knox, R., Evans, T., & Keith, W. 2001, "Telomerase-specific suicide gene therapy vectors expressing bacterial nitroreductase sensitize human cancer cells to the pro-drug CB1954", *Oncogene*, vol. 20, no. 53, pp. 7797-7803.

Ponten, J. 1971, "Spontaneous and virus induced transformation in cell culture", *Virology Monographs*, vol. 8, pp. 1-253.

Porkka, K. & Visakorpi, T. 2001, "Detection of differentially expressed genes in prostate cancer by combining supression subtractive hybridisation and cDNA library array", *Journal of Pathology*, vol. 193, pp. 73-79.

Prober, J., Trainor, G., Dam, R., Hobbs, F., Robertson, C., Zagursky, R., Cocuzza, A., Jensen, M., & Baumeister, K. 1987, "A system for rapid DNA sequencing with fluorescent chain-terminating dideoxynucleotides", *Science*, vol. 238, no. 4825, pp. 336-341.

Quilliet, X., Chevallier-Lagente, O., Eveno, E., Stojkovic, T., Destee, A., Sarasin, A., & Mezzina, M. 1996, "Long-term complementation of DNA repair deficient human primary fibroblasts by retroviral transduction of the XPD gene", *Mutation research*, vol. 364, no. 3, pp. 161-169.

Reik, K. & Dean, W. 2001, "DNA methylation and mammalian epigenetics", *Electrophoresis*, vol. 22, pp. 2838-2843.

Richardsen, E., Ukkonen, T., Bjornsen, T., Mortenses, E., Egevad, L., & Busch, C. 2003, "Overexpression of IGFBP2 is a marker for malignant transformation in prostate epithelium", *Virchows Archive*, vol. 442, pp. 329-335.

Roberts, R. Z. & Morris, A. J. 2000, "Role of phosphatidic acid phosphatase 2a in uptake of extracellular lipid phosphate mediators", *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*, vol. 1487, no. 1, pp. 33-49.

Rohme, D. 1981, "Evidence for a relationship between longevity of mammalian species and life spans of normal fibroblasts in vitro and erythrocytes in vivo", *Proceedings of the National Academy of Sciences*, vol. 78, pp. 5009-5013.

Rubin, H. 1998, "Telomerase and cellular lifespan: Ending the debate?", *Nature Biotechnology*, vol. **16**, no. 5, pp. 396-397.

Rudolph, K. L., Chang, S., Millard, M., Schreiber-Agus, N., & DePinho, R. A. 2000, "Inhibition of experimental liver cirrhosis in mice by telomerase gene delivery", *Science*, vol. 287, no. 5456, pp. 1253-1258.

Rufer, N., Brummendorf, T., Kolvraa, S., Bischoff, C., Christensen, K., Wadsworth, L., Schulzer, M., & Lansdorp, P. 1999, "Telomere fluorescence measurements in granulocytes and T lymphocyte subsets point to a high

turnover of hematopoietic stem cells and memory T cells in early childhood", *Journal of Experimental Medicine*, vol. 190, no. 2, pp. 157-167.

Ruggero, D., Wang, Z-G., & Pandolfi, P. 2000, "The puzzling multiple lives of PML and its role in the genesis of cancer", *Bioessays*, vol. 22, no. 9, pp. 827-835.

Sager, R., Tanaka, K., Lau, C., Labina, Y., & Anisowicz, A. 1983, "Resistance of human cells to tumorigenesis induced by cloned transfroming genes", *Proceedings of the National Academy of Sciences*, vol. 80, no. 24, pp. 7601-7605.

Saiki, R., Gelfland, D., Stoffel, S., Scharf, S., Higuchi, R., Horn, G., Mullis, K., & Erlich, H. 1988, "Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase", *Science*, vol. 239, no. 4839, pp. 487-491.

Saldanha, S., Andrews, L., & Tollefsbol, T. 2003, "Assessment of telomere length and factors that contribute to its stability", *European Journal of Biochemistry*, vol. 270, pp. 389-403.

Sambrook, J. & Russell, D. 2001, "Introducing cloned genes into cultured mammalian cells," in *Molecular cloning: a laboratory manual*, Third edition, Cold Spring Harbor Laboratory Press, New York.

Samper, E., Goytisolo, F. A., Slijepcevic, P., van Buul, P. P. W., & Blasco, M. A. 2000, "Mammalian Ku86 protein prevents telomeric fusions independently of the length of TTAGGG repeats and the G-strand overhang", *EMBO Reports*, vol. 1, no. 3, pp. 244-252.

Sanger, F., Nicklen, S., & Coulson, A. 1977, "DNA sequencing with chain-terminating inhibitors", *Proceedings of the National Academy of Sciences*, vol. 74, no. 12, pp. 5463-5467.

Satoh, Y., Kashimura, M., Kaneko, S., Karasaki, Y., Higashi, K., & Gotoh, S., 1994, "Cloning of cDNAs with possible association with senescence and immortalisation of human cells", *Mutation Research*, vol. 316, no. 1, pp. 25-36.

Scharf, S. 1990, "Cloning with PCR," in *PCR protocols. A guide to methods and applications*, Academic Press Inc, San Diego, CA, pp. 84-91.

Serrano, M., Lin, A., McCurrach, M., Beach, D., & Lowe, S. 1997, "Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a", *Cell*, vol. 88, no. 5, pp. 593-602.

Shay, J. W. & Wright, W. E. 1989, "Quantitation of the frequency of immortalization of normal human diploid fibroblasts by SV40 large T-antigen", *Experimental Cell Research*, vol. 184, no. 1, pp. 109-118.

Shay, J. W. & Bacchetti, S. 1997, "A Survey of Telomerase Activity in Human Cancer", *European Journal of Cancer*, vol. 33, no. 5, pp. 787-791.

Sherr, C. & dePinho, R. 2000, "Cellular senescence: mitotic clock or culture shock", *Cell*, vol. 102, no. 4, pp. 407-410.

Shi, S., Gronthos, S., Chen, S., Reddi, A., Counter, C., Robey, P., & Wang, C.-Y. 2002, "Bone formation by human postnatal bone marrow stromal stem cells is enhanced by telomerase expression", *Nature Biotechnology*, vol. 20, pp. 587-591.

Shore, D. 1995, "Telomere position effect and transcriptional silencing in the yeast S.cerevisae", *Cold Spring Harbor Press* pp. 247-263.

Siegal, G., Turchi, J. J., Myers, T. W., & Bambara, R. A. 1992, "A 5' to 3' Exonuclease Functionally Interacts with Calf DNA Polymerase {varepsilon}", *Proceedings of the National Academy of Sciences*, vol. 89, no. 20, pp. 9377-9381.

Singh, D., Febbo, P., Ross, K., Jackson, D., Manola, J., Ladd, C., Tamayo, P., Renshaw, A., D'Amico, A., Richie, J., Lander, E., Loda, M., Kantoff, P., Golub, T., & Seller, W. 2002, "Gene expression correlates of clinical prostate cancer behavior", *Cancer Cell*, vol. 1, pp. 203-209.

Sitte, N., Saretzki, G., & von Zglinicki, T. 1998, "Accelerated telomere shortening in fibroblasts after extended periods of confluency", *Free Radical Biology & Medicine*, vol. 24, no. 6, pp. 885-893.

Skulachev, V. 1997, "Ageing is a specific biological function rather than the result of a disorder in complex living systems: Biochemical evidence in support of Weismann's hypothesis", *Biochemistry (Moscow) online*.

Slagboom, P., Droog, S., & Boomsma, D. 1994, "Genetic determination of telomere size in humans: a twin study of three age groups", *American Journal of Human Genetics*, vol. 55, pp. 876-882.

Smith, J. R. & Pereira-Smith, O. M. 1996, "Replicative senescence: implications for in vivo aging and tumor suppression", *Science*, vol. 273, no. 5271, pp. 63-67.

Smith, J. & Whitney, R. 1980, "Intraclonal variation in proliferative potential of human diploid fibroblasts: stoachistic mechanisms for cellular ageing", *Science*, vol. 207, pp. 82-84.

Smith, P., Krohn, R., Hermanson, G., Mallia, A., Gartner, F., Provenzano, M., Fujimoto, E., Goeke, N., Olson, B., & Klenk, D. 1985, "Measurement of protein using bicinchoninic acid", *Analytical Biochemistry*, vol. 150, no. 1, pp. 76-85.

Smith, S., Giriat, I., Schmitt, A., & de Lange, T. 1998, "Tankyrase, a Poly(ADP-Ribose) Polymerase at Human Telomeres", *Science*, vol. 282, no. 5393, pp. 1484-1487.

Smith, S. & de Lange, T. 2000, "Tankyrase promotes telomere elongation in human cells", *Current Biology*, vol. 10, no. 20, pp. 1299-1302.

Smogorzewska, A., van Steensel, B., Bianchi, A., Oelmann, S., Schaefer, M. R., Schnapp, G., & de Lange, T. 2000, "Control of Human Telomere Length by TRF1 and TRF2", *Molecular and Cellular Biology*, vol. 20, no. 5, pp. 1659-1668.

Soldstein, S. 1974, "Aging in vitro. Growth of cultured cells from the Galapagos tortoise", *Experimental Cell Research*, vol. 83, no. 2, pp. 297-302.

Somiari, R., Sullivan, A., Russell, S., Somiari, S., Hu, H., Jordan, R., George, A., Katenhusen, R., Buchowiecka, A., Arciero, C., Brzeski, H., Hooke, J., & Shriver, C. 2003, "High-throughput proteomic analysis of human infiltrating ductal carcinoma of the breast", *Proteomics*, vol. 3, no. 10, pp. 1863-1873.

Sorlie, T., Perou, C. M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Thorsen, T., Quist, H., Matese, J. C., Brown, P. O., Botstein, D., Lonning, P. E., & Borresen-Dale, A. L. 2001, "Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications", *Proceedings of the National Academy of Sciences*, vol. 98, no. 19, pp. 10869-10874.

Southern, E. 1979, "Gel electrophoresis of restriction fragments", *Methods in Enzymology*, vol. 68, pp. 152-176.

Southern, E. 1975, "Long range periodicities in mouse satellite DNA", *Journal of Molecular Biology*, vol. 94, pp. 51-69.

Sternberger, L., Hardy, P. J., Cuculis, J., & Meyer, H. 1970, "The unlabeled antibody enzyme method of immunohistochemistry: preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-antihorseradish peroxidase) and its use in identification of spirochetes", *Journal of histochemistry and cytochemistry*, vol. 18, no. 5, pp. 315-333.

Stewart, S. A., Hahn, W. C., O'Connor, B. F., Banner, E. N., Lundberg, A. S., Modha, P., Mizuno, H., Brooks, M. W., Fleming, M., Zimonjic, D. B., Popescu, N. C., & Weinberg, R. A. 2002, "Telomerase contributes to tumorigenesis by a telomere length-independent mechanism", *Proceedings of the National Academy of Sciences*, vol. 99, no. 20, pp. 12606-12611.

Strahl, C. & Blackburn, E. H. 1996, "Effects of reverse transcriptase inhibitors on telomere length and telomerase activity in two immortalized human cell lines", *Molecular and Cellular Biology*, vol. 16, no. 1, pp. 53-65.

Sugino, A. & Okazaki, R. 1973, "RNA-linked DNA fragments *in vitro*", *Proceedings of the National Academy of Sciences*, vol. 70, no. 1, pp. 88-92.

Sun, X., Nagarajan, M., Beesley, P. W., & Ng, Y. C. 1999, "Age-associated differential expression of Na(+)-K(+)-ATPase subunit isoforms in skeletal muscles of F-344/BN rats", *Journal of Applied Physiology* , vol. 87, no. 3, pp. 1132-1140.

Szalai, G., Bailey, E., Gerber, H., & Lazary, S. 1993, "DNA sequence analysis of serologically detected ELA class II haplotypes at the equine DQB locus", *Animal Genetics*, vol. 24, pp. 187-190.

Tahara, H., Kamada, K., Sato, E., Tsuyama, N., Kim, J. K., Hara, E., Oda, K., & Ide, K. 1995, "Increase in expression levels of interferon-inducible genes in senescent human diploid fibroblasts and in SV40-transformed human fibroblasts with extended lifespan", *Oncogene*, vol. 11, no. 6, pp. 1125-1132.

Tahara, H., Yasui, W., & Tahara, E., 1999, "Immuno-histochemical detection of human telomerase catalytic component, hTERT, in human colorectal tumor and non-tumor tissue sections", *Oncogene*, vol. 18, no. 8, pp. 1561-1567.

Takahashi, Y., Kuro, O., & Ishikawa, F. 2000, "Aging mechanisms", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 23, pp. 12407-12408.

Takakura, M., Kyo, S., Kanaya, T., Hirano, H., Takeda, J., Yutsudo, M., & Inoue, M. 1999, "Cloning of Human Telomerase Catalytic Subunit (hTERT) Gene Promoter and Identification of Proximal Core Promoter Sequences Essential for Transcriptional Activation in Immortalized and Cancer Cells", *Cancer Research*, vol. 59, no. 3, pp. 551-557.

Takubo, K., Izumiyama-Shimomura, N., Honma, N., Sawabe, M., Arai, T., Kato, M., Oshimura, M., & Nakamura, K. I. 2002, "Telomere lengths are characteristic in each human individual", *Experimental Gerontology*, vol. 37, no. 4, pp. 523-531.

Talapatra, A., Rouse, R., & Hardiman, G. 2002, "Protein microarrays: challenges and promises", *Pharmacogenomics*, vol. 3, no. 4, pp. 527-536.

Tanaka, H., Shimizu, M., Horikawa, I., Hiroyuki, K., Yokota, J., Barrett, J., & Oshimura, M. 1998, "Evidence for a putative telomerase repressor gene in the 3p14.2-p21.1 region", *Genes, Chromosomes and Cancer*, vol. 23, no. 2, pp. 123-133.

Tauchi, T., Kazuo, S., Sashida, G., Sumi, M., Nakajima, A., Shimamoto, T., Ohyashiki, J., & Ohyashiki, K. 2003, "Activity of a novel G-quadruplex-interactive telomerase inhibitor, telomestatin (SOT-095), against human leukemia cells: involvement of ATM-dependent DNA damage response pathways", *Oncogene*, vol. 22, pp. 5338-5347.

Tham, W. & Zakian, V. 2002, "Transcriptional silencing at Saccharomyces telomeres: implications for other organisms", *Oncogene*, vol. 21, pp. 512-521.

Thompson, C., Frazier-Jessen, M., Rawat, R., Nordan, R., & Brown, R. 1999, "Evaluation of Methods for Transient Transfection of a Murine Macrophage Cell Line, RAW 264.7", *Biotechniques*, vol. 27, pp. 824-832.

Toussaint, O., Remacle, J., Dierick, J.-F., Pascal, T., Frippiant, C., Zdanov, S., Magalhaes, J., Royer, V., & Chainiaux, F. 2002, "From the Hayflick mosaic to the mosaics of ageing. Role of stress-induced premature senescence in human ageing", *The International Journal of Biochemistry and Cell Biology*, vol. 34, pp. 1415-1429.

Toyoda, H., Komurasaki, T., Uchida, D., & Morimoto, S. 1997, "Distribution of mRNA for human epiregulin, a differentially expressed member of the epidermal growth factor family", *Biochemical journal*, vol. 326, pp. 69-75.

Tseng, B., Erickson, J., & Gouilan, M. 1979, "Initiator RNA of nascent DNA from animal cells", *Journal of Molecular Biology*, vol. 129, no. 4, pp. 531-545.

Tseng, C. J., Jain, S., Hou, H. C., Liu, W. w., Pao, C. C., Lin, C. T., Horng, S. G., Soong, Y. K., & Hsueh, S. 2001, "Applications of the Telomerase Assay in Peritoneal Washing Fluids", *Gynecologic Oncology*, vol. 81, no. 3, pp. 420-423.

Ulaner, G., Hu, J., Vu, T., Giudice, L., & Hoffmann, A. 1998, "Telomerase activity in human development is regulated by human telomerase reverse transcriptase (hTERT) transcription and by alternate splicing of hTERT transcripts", *Cancer Research*, vol. 58, pp. 4168-4172.

Ulaner, G., Hu, J., Vu, T., Oruganti, H., Giudice, L., & Hoffmann, A. 2000, "Regulation of telomerase activity by alternate splicing of human telomerase reverse transcriptase (hTERT) in normal and neoplastic ovary, endometrium and myometrium", *International Journal of Cancer*, vol. 85, pp. 330-335.

Ulaner, G., Hu, J., Vu, T., Giudice, L., & Hoffmann, A. 2001, "Tissue specific alternate splicing of human telomerase reverse transcriptase (hTERT) influences telomere length during human development", *International Journal of Cancer*, vol. 91, pp. 644-649.

van Agthoven, T., van Agthoven, T. L. A., Dekker, A., van der Spek, P. J., Vreede, L., & Dorssers, L. C. J. 1998, "Identification of BCAR3 by a random search for genes involved in antiestrogen resistance of human breast cancer cells", *The EMBO Journal*, vol. 17, no. 10, pp. 2799-2808.

van Steensel, B., Smogorzewska, A., & de Lange, T. 1998, "TRF2 protects human telomeres from end to end fusions", *Cell*, vol. 92, pp. 401-413.

vanSteensel, B. & de Lange, T. 1997, "Control of telomere length by the human telomeric protein TRF1", *Nature*, vol. 385, pp. 740-743.

Vaziri, H., Dragowska, W., Allsopp, R. C., Thomas, T. E., Harley, C. B., & Lansdorp, P. M. 1994, "Evidence for a mitotic clock in human hematopoietic stem cells: loss of telomeric DNA with age", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 21, pp. 9857-9860.

Venter, J. C., Adams, M. D., Myers, E. W., Li, P. W., Mural, R. J., Sutton, G. G., Smith, H. O., Yandell, M., Evans, C. A., Holt, R. A., Gocayne, J. D., Amanatides, P., Ballew, R. M., Huson, D. H., Wortman, J. R., Zhang, Q., Kodira, C. D., Zheng, X. H., Chen, L., Skupski, M., Subramanian, G.,

Thomas, P. D., Zhang, J., Gabor Miklos, G. L., Nelson, C., Broder, S., Clark, A. G., Nadeau, J., McKusick, V. A., Zinder, N., Levine, A. J., Roberts, R. J., Simon, M., Slayman, C., Hunkapiller, M., Bolanos, R., Delcher, A., Dew, I., Fasulo, D., Flanigan, M., Florea, L., Halpern, A., Hannenhalli, S., Kravitz, S., Levy, S., Mobarry, C., Reinert, K., Remington, K., Abu-Threideh, J., Beasley, E., Biddick, K., Bonazzi, V., Brandon, R., Cargill, M., Chandramouliswaran, I., Charlab, R., Chaturvedi, K., Deng, Z., Francesco, V. D., Dunn, P., Eilbeck, K., Evangelista, C., Gabrielian, A. E., Gan, W., Ge, W., Gong, F., Gu, Z., Guan, P., Heiman, T. J., Higgins, M. E., Ji, R. R., Ke, Z., Ketchum, K. A., Lai, Z., Lei, Y., Li, Z., Li, J., Liang, Y., Lin, X., Lu, F., Merkulov, G. V., Milshina, N., Moore, H. M., Naik, A. K., Narayan, V. A., Neelam, B., Nusskern, D., Rusch, D. B., Salzberg, S., Shao, W., Shue, B., Sun, J., Wang, Z. Y., Wang, A., Wang, X., Wang, J., Wei, M. H., Wides, R., Xiao, C., Yan, C., Yao, A., Ye, J., Zhan, M., Zhang, W., Zhang, H., Zhao, Q., Zheng, L., Zhong, F., Zhong, W., Zhu, S. C., Zhao, S., Gilbert, D., Baumhueter, S., Spier, G., Carter, C., Cravchik, A., Woodage, T., Ali, F., An, H., Awe, A., Baldwin, D., Baden, H., Barnstead, M., Barrow, I., Beeson, K., Busam, D., Carver, A., Center, A., Cheng, M. L., Curry, L., Danaher, S., Davenport, L., Desilets, R., Dietz, S., Dodson, K., Doup, L., Ferriera, S., Garg, N., Gluecksmann, A., Hart, B., Haynes, J., Haynes, C., Heiner, C., Hladun, S., Hostin, D., Houck, J., Howland, T., Ibegwam, C., Johnson, J., Kalush, F., Kline, L., Koduru, S., Love, A., Mann, F., May, D., McCawley, S., McIntosh, T., McMullen, I., Moy, M., Moy, L., Murphy, B., Nelson, K., Pfannkoch, C., Pratts, E., Puri, V., Qureshi, H., Reardon, M., Rodriguez, R., Rogers, Y. H., Romblad, D., Ruhfel, B., Scott, R., Sitter, C., Smallwood, M., Stewart, E., Strong, R., Suh, E., Thomas, R., Tint, N. N., Tse, S., Vech, C., Wang, G., Wetter, J., Williams, S., Williams, M., Windsor, S., Winn-Deen, E., Wolfe, K., Zaveri, J., Zaveri, K., Abril, J. F., Guigo, R., Campbell, M. J., Sjolander, K. V., Karlak, B., Kejariwal, A., Mi, H., Lazareva, B., Hatton, T., Narechania, A., Diemer, K., Muruganujan, A., Guo, N., Sato, S., Bafna, V., Istrail, S., Lippert, R., Schwartz, R., Walenz, B., Yooseph, S., Allen, D., Basu, A., Baxendale, J., Blick, L., Caminha, M., Carnes-Stine, J., Caulk, P., Chiang, Y. H., Coyne, M., Dahlke, C., Mays, A. D., Dombroski, M., Donnelly, M., Ely, D., Esparham, S., Fosler, C., Gire, H., Glanowski, S., Glasser, K., Glodek, A., Gorokhov, M., Graham, K., Gropman, B., Harris, M., Heil, J., Henderson, S., Hoover, J., Jennings, D., Jordan, C., Jordan, J., Kasha, J., Kagan, L., Kraft, C., Levitsky, A., Lewis, M., Liu, X., Lopez, J., Ma, D., Majoros, W., McDaniel, J., Murphy, S., Newman, M., Nguyen, T., Nguyen, N., & Nodell, M. 2001, "The Sequence of the Human Genome", *Science*, vol. 291, no. 5507, pp. 1304-1351.

von Zglinicki, T., Serra, V., Lorenz, M., Saretzki, G., Lenzen-Grobimlighaus, R., Gebner, R., Risch, A., & Steinhagen-Thiessen, E. 2000, "Short telomeres in patients with Vascular dementia: an indicator of low antioxidant capacity and a possible risk factor?", *Laboratory Investigation*, vol. 80, no. 11, pp. 1739-1747.

von Zglinicki, T., Pilger, R., & Sitte, N. 2000, "Accumulation of single-strand breaks is the major cause of telomere shortening in human fibroblasts", *Free radical biology and medicine*, vol. 28, no. 1, pp. 64-74.

von Zglinicki, T. 2002, "Oxidative stress shortens telomeres", *Trends in Biochemical Sciences*, vol. 27, no. 7, pp. 339-344.

Wang, J., Xie, L. Y., Allan, S., Beach, D., & Hannon, G. J. 1998, "Myc activates telomerase", *Genes and Development*, vol. 12, no. 12, p. 1769.

Wang, J., Hannon, G., & Beach, D. 2000, "Risky immortalisation by telomerase", *Nature*, vol. 405, pp. 755-756.

Weinrich, S., Bodnar, A., Lichtsteiner, S., Kim, N., Trager, J., Taylor, R., Carlos, R., Andrews, W., Wright, W., Shay, J., Harley, C., & Morin, C. 1997, "Reconstitution of human telomerase with the template RNA component hTR and the catalytic protein subunit hTRT ", *Nature Genetics*, vol. 17, no. 4, pp. 498-502.

Weng, N-P., Palmer, L., Levine, B., Lane, H., June, C., & Hodes, R. 1997, "Tales of tails: regulation of telemere length and telomerase activity during lymphocyte development, differentiation, activation, and aging", *Immunological Reviews*, vol. 160, pp. 43-54.

Weng, N., Levine, B. L., June, C. H., & Hodes, R. J. 1995, "Human Naive and Memory T Lymphocytes Differ in Telomeric Length and Replicative Potential", *Proceedings of the National Academy of Sciences*, vol. 92, no. 24, pp. 11091-11094.

Wick, M. & Hagen, D. 1999, "Genomic organization and promoter characterization of the gene encoding the human telomerase reverse transcriptase (hTERT)", *Gene*, vol. 232, pp. 97-106.

Wieder, R. 1999, "Selection of methods for measuring proliferation," in *Cell growth, differentiation and senescence*, First edition, G. Studzinski, ed., Oxford, pp. 1-32.

Williams, E. J. & Iredale, J. P. 1998, "Liver cirrhosis", *Postgraduate Medical Journal*, vol. 74, no. 870, pp. 193-202.

Williamson, J., Raghuraman, M., & Cech, T. R. 1989, "Monovalent cation-induced structure of telomeric DNA: The G-Quartet model", *Cell*, vol. 59, pp. 871-880.

Wisman, G., De Jong, S., Meersma, G., Helder, M., Hollema, H., de Vries, E., Keith, W., & van der Zee, A. 2000, "Telomerase in (pre)neoplastic cervical disease", *Hum Pathology*, vol. 31, no. 10, pp. 1304-1312.

Witkowski, J. 1980, "Dr.Carrel's immortal cells", *Medical History*, vol. 24, no. 2, pp. 129-142.

Wright, W. E. & Shay, J. W. 2000, "Telomere dynamics in cancer progression and prevention: fundamental differences in human and mouse telomere biology.", *Nature Medicine*, vol. 6, no. 8, pp. 849-851.

Wright, W., Piatyszek, M., Rainey, W., Byrd, W., & Shay, J. 1996, "Telomerase activity in human germline and embryonic tissues and cells", *Developments in genetics*, vol. 18, pp. 173-179.

Wright, W. E. & Shay, J. W. 1992, "The two-stage mechanism controlling cellular senescence and immortalization", *Experimental Gerontology*, vol. 27, no. 4, pp. 383-389.

Wu, X., Amos, C. I., Zhu, Y., Zhao, H., Grossman, B. H., Shay, J. W., Luo, S., Hong, W. K., & Spitz, M. R. 2003, "Telomere Dysfunction: A Potential Cancer Predisposition Factor", *JNCI Cancer Spectrum*, vol. 95, no. 16, pp. 1211-1218.

Xiang, Z., Yang, Y., Ma, X., & Ding, W. 2003, "Microarray expression profiling: Analysis and applications", *Current Opinion in Drug Discovery & Development*, vol. 6, no. 3, pp. 384-395.

Xu, D., Wang, Q., Gruber, A., Bjorkholm, M., Chen, Z., Zaid, A., Selivanova, G., Peterson, C., Wiman, G., & Pisa, P. 2000, "Downregulation of telomerase reverse transcriptase mRNA expression by wild type p53 in human tumour cells", *Oncogene*, vol. 19, pp. 5123-5133.

Xu, D., Popov, N., Hou, M., Wang, Q., Bjorkholm, M., Gruber, A., Menkel, A., & Henriksson, M. 2001, "Switch from Myc/Max to Mad1/Max binding and decrease in histone acetylation at the telomerase reverse transcriptase promoter during differentiation of HL60 cells", *Proceedings of the National Academy of Sciences*, vol. 98, pp. 3826-3831.

Yan, P., Coindre, J. M., Benhattar, J., Bosman, F. T., & Guillou, L. 1999, "Telomerase Activity and Human Telomerase Reverse Transcriptase mRNA Expression in Soft Tissue Tumors: Correlation with Grade, Histology, and Proliferative Activity", *Cancer Research*, vol. 59, no. 13, pp. 3166-3170.

Yang, H., Kyo, S., Takatura, M., & Sun, L. 2001, "Autocrine Transforming Growth Factor {beta} Suppresses Telomerase Activity and Transcription of Human Telomerase Reverse Transcriptase in Human Cancer Cells", *Cell Growth and Differentiation*, vol. 12, no. 2, p. 119-127.

Yazawa, M., Okuda, M., Setoguchi, A., Iwabuchi, S., Nishimura, R., Sasaki, N., Masuda, K., Ohno, K., & Tsujimoto, H. 2001, "Telomere length and telomerase activity in canine mammary gland tumours", *American Journal of Veterinary Research*, vol. 62, no. 10, pp. 1539-1543.

Yeager, T. R., Neumann, A. A., Englezou, A., Huschtscha, L. I., Noble, J. R., & Reddel, R. R. 1999, "Telomerase-negative Immortalized Human Cells Contain a Novel Type of Promyelocytic Leukemia (PML) Body", *Cancer Research*, vol. 59, no. 17, p. 4175-4179.

Yokoyama, Y., Takahashi, Y., Shinohara, A., Lian, Z., Wan, X., Niwa, K., & Tamaya, T. 1998, "Attenuation of telomerase activity by a hammerhead ribozyme targeting the template region of telomerase RNA in endometrial carcinoma cells", *Cancer Research*, vol. 58, no. 23, pp. 5406-5410.

Yokoyama, Y., Wan, X., Takahashi, Y., Shinohara, A., & Tamaya, T. 2001, "Alternatively spliced variant deleting exons 7 and 8 of the human telomerase reverse transcriptase gene is dominantly expressed in the uterus", *Molecular Human Reproduction*, vol. 7, pp. 853-857.

Young, J. I. & Smith, J. R. 2001, "DNA Methyltransferase Inhibition in Normal Human Fibroblasts Induces a p21-dependent Cell Cycle Withdrawal", *Journal of Biological Chemistry*, vol. 276, no. 22, pp. 19610-19616.

Zakian, V. 1989, "Structure and function of telomeres", *Annual Review of Genetics*, vol. 23, pp. 579-604.

Zhang, X., Mar, V., Zhou, W., Harrington, L., & Robinson, M. O. 1999, "Telomere shortening and apoptosis in telomerase-inhibited human tumor cells", *Genes and Development*, vol. 13, no. 18, pp. 2388-2399.

Zhu, J., Wang, H., Bishop, J. M., & Blackburn, E. H. 1999, "Telomerase extends the lifespan of virus-transformed human cells without net telomere lengthening", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 7, pp. 3723-3728.