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Changes in Telomere Length in Rabbit Osteoblasts Following Distraction Osteogenesis

With a review of literature relating to Telomeres, Aging and Cancer

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Contents

Declaration

I confirm that this thesis :

a) is composed in its entirety by myself
b) is my own work and, where noted, utilises resources provided by my research supervisors
c) has no part which has been submitted for any other degree or professional qualification

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ABSTRACT OF THESIS

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The current and preceding two centuries of medicine can be thought of as three separate ages; The Age of Amputation, The Age of Replacement and The Age of Regeneration. The field of medicine is only just starting to explore the possibilities of regrowth, whether it be missing limbs or degrading neurones. Stem cell research is still controversial but has some potentially exciting applications for future treatment.

As it becomes possible to regrow missing body parts and replace failing ones, the life expectancy of people in developed countries may increase to previously unknown ages. However, simply replacing organs or body parts is unlikely to be the solution to fully unlocking the so-called ‘secrets of immortality’. To achieve this goal it is necessary to understand how the body ages and why it does so.

One of the foundations of research into aging is the study of telomeres, DNA repair mechanisms and their role in disease and cancer. In this thesis, recent research regarding telomeres will be reviewed. The effects of distraction osteogenesis procedures on the length of telomeres, and hence the cells' biological age, will also be investigated.

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Introduction

In the year 1860, a man crosses the street and his leg is crushed beneath the wheels of a wooden cart. He presents to his local physician with an open fracture of his tibia and is given a grave diagnosis; the leg must be amputated to prevent it becoming septic and risking the ill humour spreading to the rest of his body. He will undergo surgery without anaesthetic or sterile conditions and, even if the amputation goes as smoothly as can be expected, he has a poor prognosis for the coming weeks.

One hundred years later, in 1960, an elderly gentleman falls down some stairs and fractures his proximal femur. He is admitted to hospital and is kept comfortable with a femoral nerve block and opiate analgesics. In the morning, he is taken to a sterile operating theatre and rendered unconscious with a general anaesthetic before his fractured femoral neck and head are excised and replaced with a synthetic, metal and plastic prosthesis. His recovery is swift, although he is likely to need further surgery in future as the implant wears and begins to fail.

A further fifty years later, in 2010, a man loses the tip of his small finger due to an accident with a sharp kitchen knife. Unable to reattach the tip, physicians try an experimental procedure to re-grow the missing part of finger. They apply a powder, partially composed of pig bladder cells, and in several weeks time, report that he has re-grown the missing finger tip, complete with nail bed. He suffers no ill effects of the procedure and returns to his daily activities as usual, with a bit more care when using a sharp knife.

These three stories give examples of generalised names that can be given to the current and preceding two centuries of medicine; The Age of Amputation, The Age of Replacement and The

Age of Regeneration. The field of medicine is only just starting to explore the possibilities of regrowth, whether it be missing limbs or degrading neurones. Stem cell research is still controversial but has some potentially exciting applications for future treatment.

As it becomes possible to regrow missing body parts and replace failing ones, the life expectancy of people in developed countries may increase to previously unknown ages. However, simply replacing organs or body parts is unlikely to be the solution to fully unlocking the so-called 'secrets of immortality'. To achieve this goal it is necessary to understand how the body ages and why it does so.

The question of why our bodies decline when we age can be answered by thinking about our natural life span - the life expectancy of someone who lived in the Stone Age would be around 35 to 40 years. The Theory of Evolution states that organisms evolve with the most energy efficient method of continued existence and function. Therefore, it would not be of benefit for an organism to waste the vast amounts of energy needed to continue mechanisms for maintenance and repair of a body past its expected life-span. Thus, humans have evolved with regenerative processes which keep our bodies healthy during our time maturing, mating and raising our offspring. When this job is done, the human body begins the slow decline that is associated with age, if other limiting factors have not played their part, such as disease, predation and injury.

It is only by the intervention of modern science and medicine that we have extended this expected life-span and enter the time period where the body has not evolved protective mechanisms.

In the last century, the human life expectancy in developed countries has doubled, mainly due to improvements in sanitation and medical advances in neonatal care, infection control and treatment of cardiovascular and respiratory disease. The population of the world that is over 60 years of age is expected to reach 1.2 billion by 2025. As medical professionals have to manage an increasing number of age-related diseases, it will become more critical to understand the science behind aging and how it relates to disease.

One of the foundations of research into aging is the study of telomeres, DNA repair mechanisms and their role in disease and cancer. In this thesis, recent research regarding telomeres will be reviewed.

As we enter the age of regeneration, new medical problems may present themselves, arising from the previously unknown effects of replacing diseased organs. It is still not known whether any detrimental effects will occur by taking a differentiated adult cell, such as an osteoblast, and forcing it to multiply many times to fill the required area of a new organ construct or area of tissue loss. The laboratory study carried out in this thesis aims to determine whether there is an effect on the telomere length of osteoblasts following a distraction procedure.

Part 1

Telomeres, Aging and Cancer

Review of Literature Relating to Telomeres, Aging and Cancer.

A review of current and recent research into the above topics was carried out with an emphasis on telomeres. Therefore the primary search term that was input into the boolean search was 'Telomeres'. This returned 56,047 results, which shows the intense interest that is currently invested in telomere research. For the purposes of this review, a more defined search was therefore required. Entering 'Telomeres' AND 'Senescence' returned 7,954 results, which was still a substantial number with a large percentage of results being of little relevance to this review. The addition of 'Aging' to the above terms lessened the results to 4,194, while adding 'Cancer' lessened them to 1,307. Adding the two search terms together, making a search string of 'Telomeres' AND 'Senescence' AND 'Aging' AND 'Cancer', returned 642 papers that were highly relevant to the literature review.

Further identification of important papers was possible by filtering for 'most cited', and recent developments identified by filtering by 'date published'.

Part 1.1

Senescence

The word senescence derives from the latin 'senescere', 'to grow old'. When applied to a single cell, the term senescence refers to the process by which a cell ceases to be capable of reproduction or division. When applied on the level of the organism, the term refers to a long term, progressive reduction in the functional abilities of the organism to maintain tissue homeostasis and mount the necessary physiological response to stress.

In humans, this decline in function correlates with many of the characteristic physiological changes associated with age, such as impaired wound healing, compromised immune response and muscle atrophy.

The human body has a very high potential for replication and repair in response to stresses. This is achieved primarily by the undifferentiated stems cells in somatic tissues. This capacity varies with the tissue type and is highest in tissues with a high level of cell turnover, such as the gut or liver, as stem cells produce large numbers of specialised precursor cells. In lower turn-over tissues, such as the heart and brain, there is a smaller reservoir of specialist stem cell progeny.

The aforementioned age-related decline is partly a result of a decrease in the proliferative capacity and partly due to inappropriate differentiation of stem cells. In the musculoskeletal system, age-related decline includes osteopenia and sarcopenia, resulting in impaired mobility. There is also a reduction in the regenerative ability of muscle after injury, as myogenic fibres are replaced with fibrous tissue, rather than muscle tissue. The haemopoietic system has

decreased/altered cell lineages, resulting in a normocytic anaemia and impaired immune response, leading to decreased capacity to combat infection. The liver and kidneys are unable to process drugs and toxins as effectively. Lower turn-over organs, such as the brain, show a decrease in neurogenesis, meaning fewer new neurones are generated and there is an associated decline in function.

Senescence begins in an organism after it is fully developed and consists of a decreasing ability to respond to stresses, diseases and physical demands, as well as an increase in age-related disease such as cancer. The chronological age of a mammal is not directly related to the degree of senescence displayed in their tissues, as there are a great many external and internal factors which can determine this. These include external factors, such as dietary intake and internal factors, such as reactive oxygen species and the diverse genetic phenotypes governing DNA repair[1].

Hayflick et al noted in experiments in the 1960s that cells appeared to have a limit to the number of times they were able to replicate in vitro[2]. After a certain number of replications, differing depending on the type of tissue and the organism it came from, the cells entered a state of irreversible growth arrest and could no longer be stimulated to divide. The cells continued their regular metabolic functions as normal but when their usual lifespan was complete they spontaneously died without division. Hayflick found this number to be around fifty divisions for human fibroblast cells. This maximum number of divisions, which seemed to restrict the replicative potential for certain lines of cells, was therefore named the Hayflick Limit.

Hayflick and Moorehead therefore hypothesised that there was an intracellular factor or messenger that was progressively lost with every cell division and was responsible for limiting the proliferation of the cell.

The Hayflick Limit does not, however, apply to all cell lines. In certain tissues and types of cells, there exists no limit to the number of cells that may be produced. Cell lines with a high turnover due to their function, such as gut epithelial cells, have a much higher or non-existent limit. It has also been identified that in many tissues this limit does not exist until the organism reaches its maximum expected maturity and size. This is due to the necessity of organisms, such as humans, to grow from the two cells of sperm and egg into the many billions of cells present in an adult body.

An important aspect of senescence is that although the cell is unable to divide, it still continues its normal cell cycle. This means that in vitro cells may remain viable indefinitely, in the absence of external influence. The situation in vivo is more complex as many factors can affect whether a cell continues to function or undergoes apoptosis. Examples of the two ends of the spectrum include senescent melanocytes of naevi, which can exist for years, and senescent tumour cells of liver carcinomas, which are rapidly removed by phagocytes[3].

This variation in replicative capacity also occurs between organisms of differing species. Small rodents such as mice have a much higher replicative capacity than humans while prokaryote organisms have an infinite capacity.

The reasons for the Hayflick limit and its variations between tissues and organisms were not understood until the discovery of telomeres. Although telomere induced senescence appears to

be a very important factor in cellular senescence, there are many other causes for a cell to be driven into the permanently non-dividing state. These include many types of cellular stress and in the cancer suppression response in cells with activated oncogenes[4].

Non telomere-related senescence may be responsible for protection against melanoma. Pollock et al[5] showed that most skin naevi have both a mutation of the BRAF oncogene and markers of cellular senescence[6]. However, they were not found to have shortened telomeres[7]. This indicates that there must be another mechanism responsible for initiating senescence, other than telomere dysfunction.

Part 1.2

What are telomeres?

The process by which a chromosome is replicated during cell division is an imperfect one. As the single, long strand of DNA that comprises the chromosome is replicated, small numbers of base-pairs at its termini are unable to be transcribed, due to a failing in the process of replication known as the 'end replication problem'.

To understand this problem, it is necessary to look at the way in which chromosomes are replicated. Since a chromosome is comprised of two strands of DNA in a helical structure, the separate strands are identified by labelling their ends as either three prime (3′) or five prime (5′). The strands are arranged opposite one another so the 3′ end of one strand corresponds with the 5′ end of the other. With this labelling, it is possible to identify a strand by describing it as the 3′-to-5′ strand or the 5′-to-3′ strand.

Synthesis of DNA uses enzymes called DNA polymerases which 'move' along the original piece of DNA, known as the template strand, in the direction of 3′ to 5′. The synthesis begins at many sites along the DNA, known as primer sites. At these sites, the template DNA separates its two strands to allow the polymerase to bind and initiate replication on both strands. Synthesis therefore occurs simultaneously at many sites and in both 3′-to-5′ and 5′-to-3′ directions, until the individual sections of replication meet.

Synthesis is straightforward on the 3′ to 5′ template strand, as it is the direction the polymerase acts in, producing new DNA called the leading strand in a continuous, unbroken segment. However, problems arise with the 5′ to 3′ strand, as synthesis cannot be initiated at its termini.

This means that the process has to 'wait' until the template chromosome is 'opened' enough to allow synthesis to occur opposite to the direction of replication. Small sections of RNA attach to points on the template strand and act as primers to allow polymerase to start synthesis. The production of this DNA, known as the lagging strand, is therefore discontinuous, producing many small fragments of DNA, called Okazaki fragments. These are later 'stitched' together by DNA ligase and it is at this stage that the loss of base pairs occurs. DNA must be present in front of an RNA primer for DNA ligase to stitch the RNA in place. This occurs at the ends of all the Okazaki fragments but not at the end of the lagging strand, where an RNA primer sits on its own. The RNA is later degraded and therefore leaves a deficit of around 200 bases at the end of the lagging strand. This is often called the End Replication Problem.

The end replication problem could be harmful to an organism if the DNA that was lost in replication was of importance to its survival. The ends of chromosomes are therefore protected by long segments of simple tandem DNA repeats that do not code for any gene product[9], known as telomeres. This portion of chromosome is essentially 'junk DNA' as loss of any part of it does not affect the function or survival of the cell.

Telomeres therefore act as protective 'caps' on the ends of the vital DNA, the centromere, and are sacrificed a few base pairs at a time to allow a perfect copy of the important chromosomal DNA to be produced. They can be thought of as similar to the plastic bands on the ends of a shoelace which are themselves worn by use, but protect the inner portion from fraying.

The presence of telomeres on the termini of a chromosome also distinguishes its ends from unwanted breaks in the DNA. If DNA breaks are identified in a normal cell, cell cycle arrest

occurs and DNA repair mechanisms are activated or cell apoptosis occurs if damage is too great. Telomeres protect the chromosomes from incorrect activation of these mechanisms[8]. A minimum length of telomere is required for this function.

Another vital function of telomeres is to limit the inevitable, inbuilt errors that occur during DNA replication. Transcription errors are more likely to occur at the ends of a sequence of DNA and so a non-vital buffer of repeating DNA at the chromosome termini allows these errors to occur harmlessly.

Telomeres are also vital to avoid homologous recombination, where the ends of chromosomes attach to their other end and form a circle, and non-homologous end joining, where the ends attach to another chromosome entirely. This is a problem in eukaryotes as they have linear chromosomes with two ends, unlike the majority of prokaryotes whose chromosomes are circular, with no telomeres at all.

Most eukaryotic telomeres consist of six to eight base-pair long repeats, containing mainly guanine. In vertebrates, the telomeres are made up of a sequence of 6 bases, TTAGGG, repeating up to 2000 times.

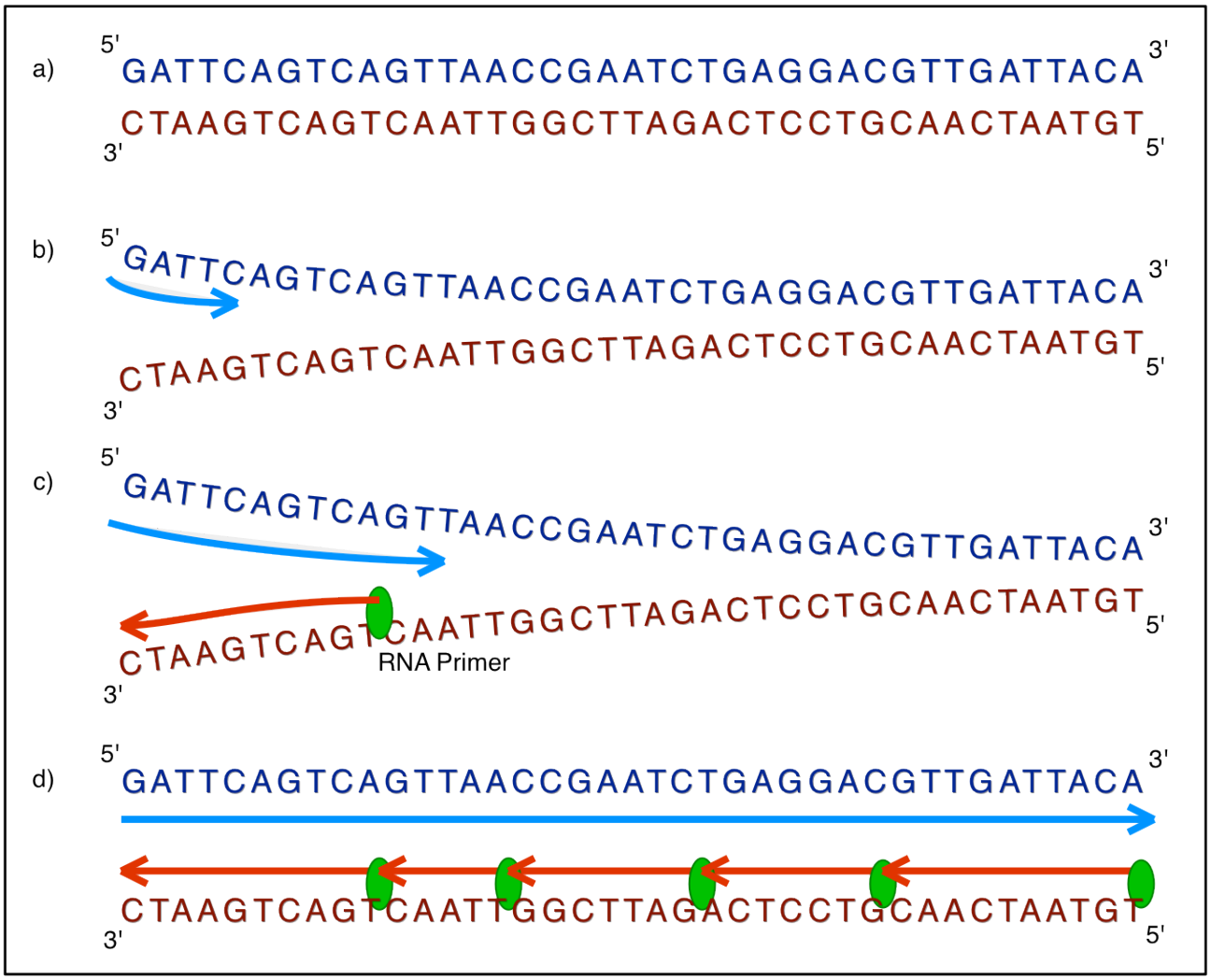
To counter this loss of telomere DNA, an enzyme called telomerase exists which can add repeat sequences to the 5' end of a telomere. It consists of an RNA primer (AAUCCC), which produces the TTAGGG repeat, and a protein called telomere reverse transcriptase, which catalyses the process[9,10]. This enzyme is normally only present with significant activity during embryogenesis and in certain adult cells, such as germ line cells[11], but is thought to be highly active in a large proportion of cancer cells, which will be discussed later. Telomerase is active in

all adult tissues but its level of activity is so low as to have little or no effect on the progressive telomere attrition that occurs from replication.

The importance of telomerase in maintaining telomere length for replicative capacity was highlighted in experiments using human fibroblasts[12]. In normal circumstances in vitro, the division of fibroblasts results in progressive telomere shortening and subsequent cellular senescence, once the Hayflick limit is reached. When a mutation that induces enforced expression of TERT (the catalytic subunit of telomerase) is introduced, the telomere length is stabilised and the cells have an unlimited replicative capacity, thus bypassing the Hayflick limit.

The Hayflick limit therefore prevents the unchecked proliferation of normal non-cancerous cells but also, crucially, prevents the proliferation of pre-cancerous cells that are undergoing neoplastic change. This provides a major obstacle to tumours developing their characteristic malignant and infiltrative properties and almost all human cancers have developed some way of bypassing the limit by stabilising telomere lengths, usually by expressing high levels of telomerase[13].

Figure 1.2a



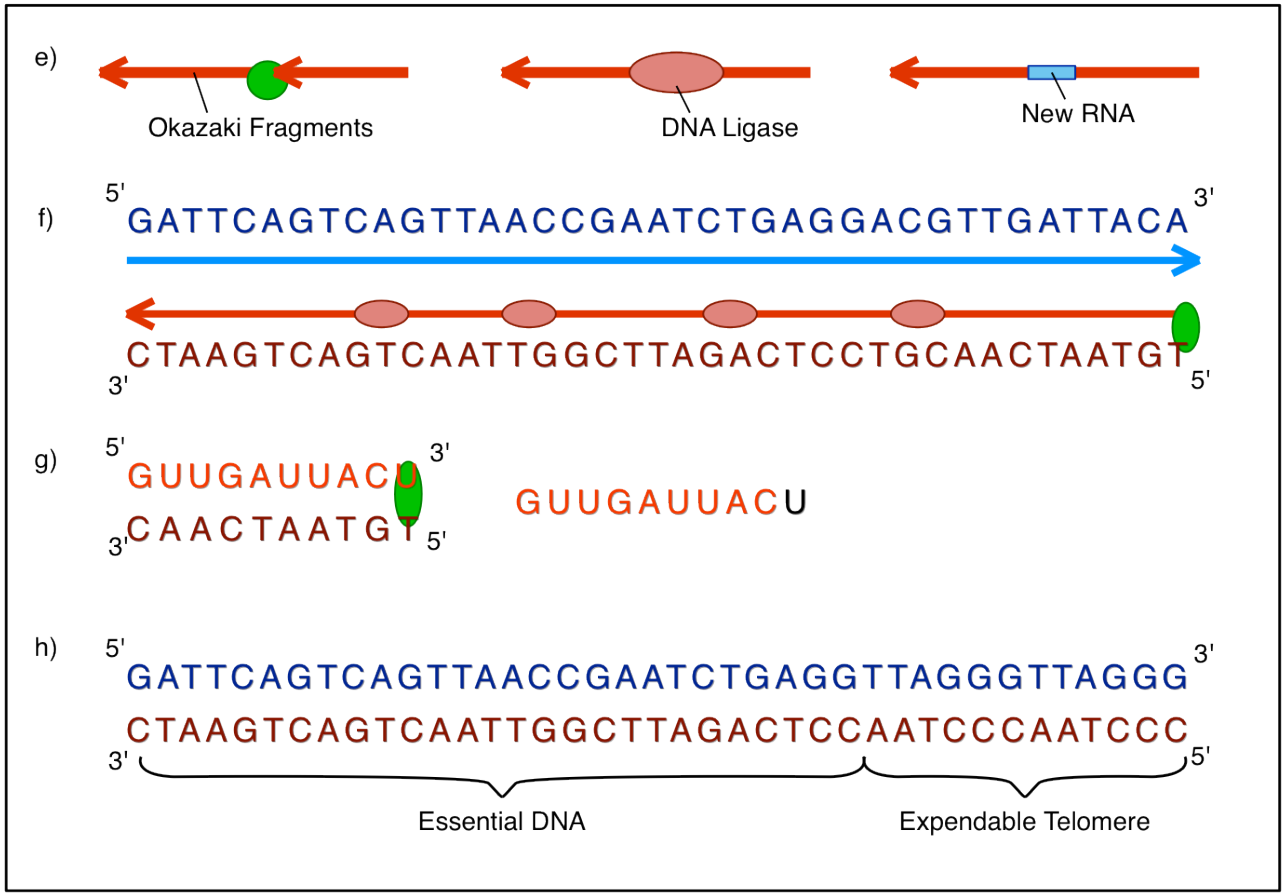
a) DNA is arranged in a double strand, opposite to one-another, with ends of each strand labelled 5-prime and 3-prime.

b) When the ribosome binds to the DNA it separates the strands and, since it works from 5′ to 3′, immediately begins synthesising RNA from the leading (blue) strand.

c) When the ribosome 'opens' the DNA further, an RNA primer is encountered on the trailing strand and RNA synthesis can begin in the opposite direction to the leading strand.

d) The ribosome encounters RNA primers intermittently along the length of the trailing strand so many small sections of RNA are synthesised, known as Okazaki fragments.

Figure 1.2b



e) An enzyme called DNA ligase binds to gaps between the Okazaki fragments in order to 'stitch' them together into one long strand.

f) This process requires DNA/RNA on both sides of the gap so no DNA ligase binds at the 5' end of the trailing strand.

g) As the DNA ligase does not bind at the end of the strand, the final base is not transcribed and hence is lost in the synthesis

h) To combat this, chromosomes have telomere end-caps. This means that the DNA on the peripheries is expendable 'junk' and no important information is lost.

Part 1.3

The Role of Telomeres in Aging

Experiments in the 1960s demonstrated a phenomenon of cells spontaneously entering a state of irreversible growth arrest[2]. As stated in the previous section, this state occurs after a finite number of cell divisions, known as replicative senescence or the Hayflick Limit, and is accelerated under certain conditions such as oxidative stress[14,15]. It was not until the discovery of telomeres in the 1990s that a reason for this senescence was understood[12,16].

Also, as discussed previously, small portions of telomere are lost with every cell replication, resulting in a reduction in the length of telomeres over time[11]. In humans, this appears to be around 40-100 base pairs of the terminal telomeric repeat DNA with every division. When the telomeres reach a critical length, they lose their protective capacity and senescence occurs. This is likely to be due to increased activity of the oncogene p53, identifying short telomeres as DNA damage and triggering senescence[17]. Senescence itself is when the cells enter a state of permanent growth arrest, meaning they can no longer divide, but are capable of continuing to be metabolic activity[18].

The idea that telomere length was responsible for replicative senescence was given further support when it was found that when telomerase was expressed in foreskin fibroblasts, replicative senescence was avoided entirely[12].

As humans become older, the number of times their cells have replicated increases. This means that the telomeres of cells that lack telomerase activity become shorter and closer to the critical length mentioned above. Human fibroblasts have a Hayflick limit of around 50-70 divisions[11]

but this varies for different cell types. Some tissues display significant telomere shortening over time, such as peripheral blood cells and hepatocytes, but some show very little shortening at all, such as human brain tissue[19]

Although telomeres are responsible for cellular senescence, it is still a matter of debate as to their role in organismal senescence[17]. However, it is widely viewed that telomeres and telomerase have a significant role in the formation and proliferation of cancerous cells.

A possible role of cell senescence in organismal aging could be in accumulation of senescent cells and a decrease in the regenerative potential of the stem cell reservoir[3]. As a person gets older, the number of senescent cells in their tissues increases, possibly reaching a level which compromises the tissue's overall ability to respond to stresses. Aging could be produced by either this accumulation of senescent cells or by the exhaustion of the regenerative potential of stem cells.

Compared to humans, mice have a significantly shorter lifespan but, despite this, have a high incidence of cancer. They also have much longer telomeres and a high activity of telomerase[17]. When telomerase activity is suppressed in knock-out studies in mice, an increase in cancer resistance is found. Conversely, when over-expression of telomerase is achieved, a relative increase in cancer incidence is found[20]. A similar relationship seems to exist with expression of the p53 oncogene. As discussed previously, the p53 gene is likely to be responsible for inducing senescence in the presence of DNA damage. Mice that lack p53 have an increased cancer incidence and those with an over-expression of the gene are short-lived but cancer resistant[21]. Senescence therefore seems to be a tumour suppressor mechanism controlled largely by

telomere length, telomerase activity and the p53 gene. Large animals have a higher number of cells and hence a greater number of targets for cancerous change. This means that they benefit from telomerase suppression with a greater cancer resistance but at a price of age-related changes. Smaller animals, such as rodents, benefit from high telomerase activity but have high predation rates in the wild so are not adversely affected by the associated increase in cancer incidence[21].

Studies on cancer resistant mice have shown that telomerase activity and telomere length have a direct effect on lifespan and physiological age. Mice with an over-expression of TERT, the catalytic subunit of telomerase, had a higher median lifespan and a physiological appearance characteristic of a younger mouse, with increased epidermal thickness, higher epidermal stem cell proliferative function and decreased associated gastric and dermatological disease[22]. Comparisons between mice with naturally occurring long and short telomeres have shown differences in functional decline in the presence of TERT over-expression. Over-expression in mice with long telomeres results in a prevention of age-related functional decline, while in mice with short telomeres, a decline occurs in highly regenerative organs such as the intestine, testes and haematopoietic system[23].

Human studies into telomere length and aging have been carried out by identifying progeroid syndromes which induce premature aging. These include Werner syndrome, ataxia telangiectasia and dyskeratosis congenita.

Werner syndrome is rare autosomal recessive disorder, caused by a mutation in the WRN gene, on chromosome 8, which is involved in DNA repair and telomere maintenance. This mutation

also results in a failure to control DNA recombination and chromosome aberrations, causing genome instability. The reduced capacity for telomere maintenance results in rapid telomere attrition and premature senescence, as demonstrated in fibroblasts of Werner's sufferers[24]. The role of telomere shortening in the causation of senescence is shown by the fact that premature senescence can be avoided in vitro by enforced TERT expression.

Patients with Werner's have no physiological differences until puberty, when they cease normal growth and begin to exhibit a series of multi-organ, progressive pathologies that are normally associated with age. These include senile cataracts, osteoporosis, skin atrophy, heart disease and cancer, leading to a significantly decreased median life expectancy of 47-48 years[23].

Ataxia telangiectasia is also an autosomal recessive disorder, characterised by cerebellar degeneration, skin abnormalities and multiple malignant neoplasms. It is caused by mutations in the ATM (Ataxia Telangiectasia Mutated) gene, resulting in abnormalities of DNA damage-signaling/repair and telomere maintenance. As with Werner's cells, premature senescence can be avoided in vitro by enforced TERT expression[24].

The relevance of telomerase in the process of aging has been investigated extensively with the use of telomerase knock-out mice. These mice had TERC (Telomerase RNA Component) gene deletions which prevent the expression of telomerase. First generation TERC knock-out mice showed very little phenotypical difference to control mice, only showing only modest stem cell dysfunction in highly proliferative organs and having a near-normal lifespan. This was thought to be due to the naturally long length of telomeres, thus allowing a telomere reserve that retained end-capping. However, in the subsequent generations of knock-out mice,

progressively worsening telomere dysfunction was noted, with the associated shortened lifespan and widespread, premature age-characteristic tissue degeneration[25]. The degree and severity of these premature degenerative changes could be directly correlated with the degree of telomere dysfunction, as measured by anaphase bridging.

Part 1.4

Telomeres and Cancer

In experiments in vivo, the process of telomere dysfunction-based senescence, that leads to cellular senescence and cessation of growth, requires several cell cycle checkpoints[6]. Most importantly, these require the correct functioning of the previously mentioned p53 pathway and the pRB pathway. When these pathways are intact, telomere shortening leads to permanent growth arrest and eventually apoptosis.

If the above processes are interrupted, telomeres continue to shorten and become progressively dysfunctional. Instead of the normal process of telomere dysfunction-based senescence, the unprotected, dysfunctional telomeres cause the cell to enter a state called telomere-based crisis[26]. Although crisis and senescence both result from shortening of telomeres, crisis differs in that the cell does not enter the characteristic cessation of growth and DNA synthesis. Instead, the short telomeres cause end-to-end fusions of chromosomes, resulting in mitotic catastrophe, multipolar cell division and gross aberrations in chromosome number[6]. The oncogenic mechanisms which interrupt the cell cycle checkpoints allow the cell to bypass normal senescence and avoid apoptosis.

Although a cell in crisis does not undergo normal cycle arrest and apoptosis, evidence suggests that it can still be a cancer-preventative mechanism. To convert normal human cells to a tumourigenic state, experiments have used SV40 T (a DNA virus which binds to, and inactivates, p53 and pRB) and Ras (a GTPase signal transduction protein which causes cells to

develop malignant properties such as invasiveness and proliferation)[6,27]. In these experiments, the resultant tumours grew in size but the cells subsequently entered crisis. Further proliferation when transferred to a different host did not occur, even when rescue from crisis was attempted by activation of telomerase[28].

Formation of anaphase bridges has been used as a marker of telomere dysfunction[29]. Most human cancers have cells containing anaphase bridges but some normal, non-neoplastic cells also contain low numbers, with an inverse relationship between frequency of anaphase bridges and telomere length[30,31]. The fact that active normal cells show signs of telomere dysfunction without senescence suggest that telomere-based crisis may be a secondary protective measure which limits cancerous growth when telomere dysfunction-based senescence fails.

It is only when cancerous cells mutate to the point where telomerase expression is high enough to prevent telomere dysfunction, that the cell can bypass crisis and replicate without limit. This can happen by mutations or by activation of oncogenes such as c-Myc[32].

When compared to other organisms, such as rodents, humans have relatively short telomeres. Telomerase activity is also minimal in the majority of human cells, resulting in a progressive shortening of length with each cell division. The only cells which show a higher telomerase activity are stem cells, and even these do not have a high enough level to continue activity in the cells that they differentiate into.

This lack of telomerase activity and the relatively short length of telomeres is an important cancer-suppression mechanism. Shortened, dysfunctional telomeres cause inhibition of

tumourigenesis by either telomere dysfunction-related senescence or telomere-based crisis, meaning the growth of a potentially lethal cancer is suppressed as unchecked cell division results in senescence or crisis. This occurs in cells which are pre-cancerous unless there is an oncogenic mutation.

The reason why a replicative limit is tumour protective lies in the fact that a cell must undergo multiple mutations for it to become malignant[33]. When a pre-malignant mutation occurs, a cell must divide at least 20 times before the population of mutant cells reaches a level where a second mutation is likely to occur. As most mutations occur as a single allele, they are recessive and hence require a further 20 or so division for elimination of the native allele and homogeneity to occur. The homgenous mutant cells would then need a further 20 divisions for the population to expand again.

This process requires a great number of divisions for malignancy to occur. The result of this is that most pre-malignant cells reach the Hayflick limit with only 1 or 2 mutations, undergo senescence and hence do not progress to cancer[34]. A method to bypass this limit is therefore present in more than 90% of human cancers, namely telomerase up-regulation, as previously discussed. By extending its proliferative life-span, a mutant cell line is given the time for oncogenic mutations to accumulate.

If prevention of cancer by replicative limit is so effective, then why is the limit as high as 50 divisions? The reason is that, although a very low replicative limit would prevent almost all cancerous changes, it would impair the function of the organism in general. The process has therefore evolved as a balance between cancer suppression and allowing cells sufficient

proliferative capacity for growth, maintenance and repair, over the expected life-time of the organism.

What then, are the advantages of having long telomeres and active telomerase in animals with short life-spans? Small animals that have high predation rates are, firstly, more likely to suffer an incapacitating injury. If this injury is not rapidly repaired then the animal will quickly be picked out by a predator and eaten. Therefore the extensive proliferative abilities associated with telomerase activity are beneficial in early healing and return to function.

The triple-G sequences in telomeres are also a preferred site for oxidative damage[35]. If an organism has very long telomeres, the repetition of the triple-G sequence every six base pairs means that oxidation is much more likely to occur harmlessly here, rather than at an important site in the genomic DNA.

Many of the premature aging diseases mentioned previously have a defect in the DNA repair mechanism which results in a loss of genome integrity. The role of the tumour suppressor gene p53 can be highlighted by the fact that, in mice bred with similar DNA repair mechanism defects, the p53 response to DNA damage and subsequent degenerative aging is almost completely reversed when p53 is deleted[23].

The p53 tumour suppressor has been called the guardian of the genome as it is the main cellular mechanism to initiate apoptosis and growth arrest in response to genotoxic stresses. These stresses include not only DNA damage and telomere dysfunction, but also UV radiation,

reactive oxygen species and hypoxia. It is also not limited to initiating cell apoptosis but is responsible for signalling DNA repair pathways, depending on the severity of the stress.

Deletion of p53 in mice with critically short telomeres, who would otherwise show signs of premature aging, results in a decrease in apoptosis and an increase in proliferation, evidenced by decreased organ dysfunction in highly proliferative tissues. Further, late generation telomerase knock-out mice, such as those discussed previously, have similar phenotypes to wild mice when the p53 gene is also knocked-out as the cellular senescence and apoptosis pathways are no longer initiated in response to telomere dysfunction.

The increase in stem cell function when p53 is deleted in mice with short telomeres does not result in an increase in mean life-span. This is because the cells lose their tumour suppression and the mice have a much higher incidence of cancer[36]. The types of cancer that are caused in telomere dysfunctional mice with knock-out p53 and TERC are similar to the spectrum that affects elderly humans, mainly epithelial cancers of the skin, GI tract and breast[36].

The role of p53 in both cancer and aging has also been shown at the opposite end of the spectrum; in mice which have been engineered with hyperactive p53. These mice develop phenotypes of premature aging but are cancer resistant, showing increased numbers of senescent cells in all tissues[37].

Following on from the research carried out on knock-out mice, human cell studies have confirmed the role of telomere dysfunction and telomerase in cancer; ie. that programmed telomere shortening, resulting in senescence, is an important system for tumour suppression. Support for this can be evident as most human cancers negate the shortening of telomeres

through increased expression of telomerase. When telomerase is inhibited in already cancerous cells, they regress to pre-cancerous rates of telomere shortening and senescence.

As has been mentioned previously, humans and mice have differing requirements for tumour suppression, due to their life-spans and opposite ends of the food-chain. The lifetime risk of cancer can be thought of as proportional to the size of an organism (ie. the number of cells it contains and hence the number of sites for mutation to occur) and its average lifespan (the length of time a cell line has for mutations to occur)[34]. A 70kg human with a life expectancy of 70 years should therefore require 125,000 times the cancer resistance of a 20g mouse that lives for 2 years[38].

This means that mice cells do not need to regulate their tumour burden through telomere attrition, instead gaining the benefits of unlimited proliferation and long telomeres. Humans, however, benefit from the the tumour suppressive advantages of senescence. Therefore human cells employ multiple pathways to activate senescence in response to telomere dysfunction. This can be shown in experiments with human cell cultures which have either the p53 or Rb pathways inhibited[39]. If p53 activity is suppressed, the cell cultures gain a small number of extra cell divisions but eventually the dysfunctional telomeres trigger senescence by inhibiting phosphorylation of Rb. Conversely, if the Rb pathway is interrupted, the p53 pathway can enforce senescence.

Multiple mechanisms for triggering senescence, even in the absence of one or more pathways, provides a redundancy in the active regulation of telomere dysfunction.

Part 2

Distraction Osteogenesis

Limb lengthening is a procedure used to lengthen or correct deformities of long bones. It was first described in 1905 by Codivilla, an Italian surgeon, and later refined by Ilizarov, who described distraction osteogenesis. The process involves division (osteotomy) of the target bone and fixation with a distraction device which can separate the ends of the osteotomy gradually over several weeks. As the bone ends are slowly pulled apart, new bone tissue is formed between them. Once the desired length is reached, the new bone is allowed to heal in a consolidation phase before the fixator is removed. This consolidation phase can take three to four times the length of time of the distraction phase, which itself can take a month per centimetre of new bone.

The procedure has traditionally used the Ilizarov apparatus, an external fixator device. This consists of a series of rings which surround the bone, above and below the osteotomy site. The rings are fixed to the bone with percutaneous pins and tensioned wires and to one another with rigid struts. The struts which span the osteotomy site have a screw system incorporated so as to allow a gradual extension of their length, whilst providing a stable construct. Once in place, the screws are turned to distract the bone.

The optimum rate of distraction has been found to be around 1mm per day. Studies by Ilizarov in the 1980s[40] experimented with various factors affecting the quality and success of distraction. Canine tibia were subjected to a limb lengthening procedure with both open osteotomy and closed osteoclasis. The speed at which the distraction was carried out, measured in millimetres per day, and the incrementes of distractions per day were varied.

Ilizarov found that premature consolidation occurred when the distraction rate was 0.5mm per day. In these dogs, there was dense bone regeneration at the osteotomy sites which consolidated by only 23 days into distraction, preventing further lengthening and resulting in traction fractures at the interface between original and new bone. In those dogs that had a distraction rate of 2mm per day, the space between the bone ends was filled with dense fibrous tissue but no evidence of osteogenic activity.

The balance between early consolidation and fibrous infiltration was found to be at 1mm per day. However, this rate still had variations in quality, as measured by observing osteogenic activity with histological staining, depending on the number of steps of distraction in a day. Similar results to the 2mm per day group were found when the distraction was done in a single step every 24 hours. The best outcome was found by carrying out the distraction of 1mm in 60 steps through a 24 hour period, with a formation of cortex that was 'almost identical to the original bone thickness and density'.

This difference in quality of bone formation depending on distraction frequency is an important limiting factor in the use of Ilizarov frames. Difficulties arise when trying to increase the distraction frequency, as the process of tightening screws around the frame can be complex. However, many patients are able to cope with four-times daily distractions. The advent of self-lengthening intra-medullary devices, as mentioned below, combat this difficulty to some extent and allow for a higher frequency of distraction. This occurs in very small increments, either automatically or by utilising the normal movement of a joint to activate the lengthening.

Additionally, in the above experiment, all of the open osteotomy sites showed some evidence of fibrous infiltration at all stages of the distraction, whereas only a limited number of the closed osteoclasis sites exhibited this.

Although external fixators have proven effective in distraction procedures, they have significant co-morbidities and impact on a patient's quality of life. Pain, pin site infections and hardware failure are common and the fixator itself is large and bulky. The frames are usually required for long periods of time, from many months to over a year. They cause problems with clothing, mobility and have psychological implications as patients are very conscious of their presence.

In recent years, internal fixation devices, called intra-medullary skeletal kinetic distractors, have been developed in attempts to address some of the complications of external fixators. These devices are intra-medullary nails that telescope to the desired length either mechanically by a specific movement at the ankle (such as orthofix) or by a radio signal activating an electric motor (such as fitbone).

Studies by Cole et al[41] in the late 80s investigated the clinical outcomes of ISKDs, with favourable results. They carried out lengthening procedures on femurs and tibias, in patients who had suffered trauma, infection, polio and burns, all with a subsequent limb length-discrepancy. All patients in the study eventually went on to union, even with lengthenings of up to 110mm. The only complications they found in this study was implant failure, which was corrected in subsequent cases by a design change. They concluded that the ISKD represented a safe and cost-effective option that 'reduces lifestyle disruption and complications'.

Later studies have not been so favourable[42,43], with new complications occurring when compared to external fixators. They found that, although fixator-induced morbidity can be reduced with ISKDs, there are complications associated with nail guidance and operative technique. A maximum distraction of perhaps 4cm for the tibia and 8cm for the femur has been suggested when using an ISKD[42] in order to limit weak consolidation and contractures. This means that if greater distraction distances are needed then multiple ISKDs can be used in sequential nail exchange operations. Binding of bone regenerate at the osteotomy site was also noted as a complication[43], leading to failure of lengthening and a need for re-osteotomisation.

Cosmetically, patients often have a better outcome with ISKDs compared to external fixators, as they have no visible external sign of the device during the distraction process and there is less scarring when the process is finished.

Distraction osteogenesis has mainly been used after trauma, such as when a fracture occurs at the epiphyseal plate or after bone loss, but can also be used to correct congenital and developmental deformities, such as congenital short femur and rickets. New bone can also be grown following resection of neoplastic disease, such as osteosarcoma, or bone destruction in osteomyelitis.

Distraction osteogenesis has been shown to be effective in treating non-unions with bone loss[44]. Historically, tibial non-unions were difficult to treat due to their associated complications of deformity, chronic osteomyelitis and length-discrepancies. Often it was difficult to treat these, even with bone grafting and rigid internal fixation, resulting in a need for amputation. As

Ilizarov frames and distraction procedures were developed, these problems could be effectively addressed.

Recently, correction of deformity has been facilitated with the use of hexipod devices, such as the Taylor-Spacial Frame. This is a ring-structure external fixator with six calibrated telescoping struts 'zig-zagging' between the rings either side of the deformity. Prior to these frames, it was difficult to correct deformities accurately as they often occur in multiple planes, making simple hinged distraction devices insufficient to correct anatomically.

The process of distraction creates an area of new bone to form and fill the gap created between the original bone ends. This tissue is formed by cells at the ends of the osteotomy dividing and proliferating at a greatly increased rate than would normally be required. This could have profound effects on the telomere length and increase the risk of malignant transformation or senescence in the cells. The aim of this study is to identify if this increased rate of division results in a decreased average length of telomere in the cells of the newly formed tissue. If this were the case, the reduction in telomere length would have implications for neoplastic change in the new tissue. This would be especially important since, in some patients, the procedure is used to fill the bone defect created following excision of bone tumours.

Part 3

Changes in Telomere Length in Rabbit Osteoblasts Following Distraction Osteogenesis

Part 3.1

Hypothesis

In rabbit tibias subject to distraction osteogenesis, the increased cell turnover at the site of new bone formation causes an artificial reduction in telomere length in osteoblasts due to replicative attrition of telomeres.

Part 3.2

Reasons for Research

Following injury, the normal response of bone healing follows a well established pattern of reaction, repair and remodelling. Initially, blood collects around a fracture site and forms a haematoma which organises with the proliferation of fibroblasts. These fibroblasts are responsible for the formation of granulation tissue. After a few days, cells from the periosteum develop into chondroblasts and osteoblasts. These cells make the fracture callus (composed of hyaline cartilage and woven bone) which is a stable but mechanically weak construct.

Following this, the newly formed cartilage and woven bone are gradually replaced by lamellar bone and later remodelled by osteoclasts/osteoblasts over a course of years into compact bone.

The healing process outlined above normally occurs over relatively short distances, requiring bone ends in a fracture to be within a few milimetres of one another. If greater distances occur, then the bone will fail to unite and instead will either form atrophic bone ends or progress to fibrous interposition/non-union.

When a bone is subjected to a distraction procedure, the two ends of the fracture or osteotomy are first placed in direct contact so as to allow the normal healing process to begin. However, before the consolidation phase is reached, the fracture callus is pulled apart, increasing the distance between the original bone ends. This induces the cells of the callus in between the bone ends to proliferate to fill the gap that forms.

The osteoblasts themselves may be forced to replicate many times, potentially reaching their replicative limit early. It may also be the case that the pool of stem cells in the tissue that osteoblasts arise from may be prematurely exhausted or brought close to depletion.

This study is therefore aimed at identifying whether there is a measureable difference in the physiological/replicative age of osteoblasts in bone subjected to a distraction. This is measured by comparing the length of telomeres from osteoblast at various points in the distraction, at either end of the distraction site and from control sites of bone on the contralateral side.

The bone first needs to be demineralised to allow processing in a DNA isolation pathway, before carrying out a telomere detection assay. The overall physiological age of the tissue was to be measured by staining for numbers of senescent cells in each sample.

Part 3.3

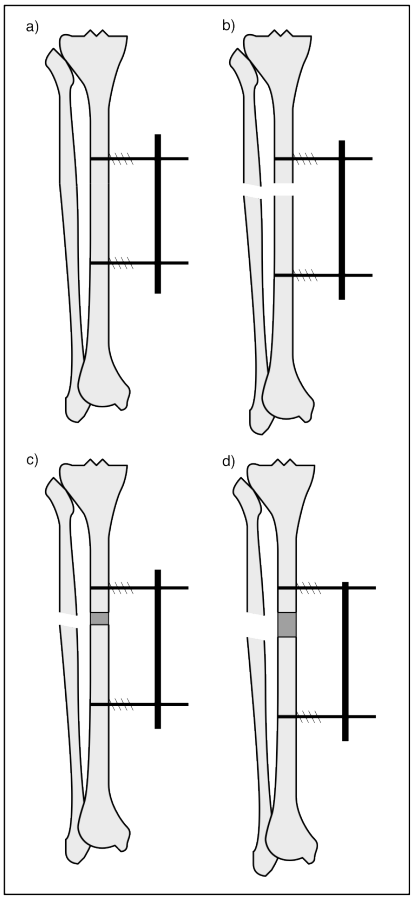
Methods

1) Distraction Procedure
2) Identification of Suitable Bone Growth
3) Harvesting of Bone Samples
4) Decalcification of Compact Bone
5) Isolation of Genomic DNA
6) Telomere Length Assay
7) Chemiluminescence Detection

Distraction Procedure

This was carried out on a total of 6 rabbits by Professor Simpson. Under anaesthetic, each rabbit had an osteotomy performed on a single tibia and two screw-pins inserted at either side of the osteotomy. A distraction frame was then applied to the pins. After the period of callus formation (approximately 1 week) the distraction process was started. This required increasing the gap between the pins by 0.7mm per day for a period of 10 days, resulting in a total distraction of 30mm.

Figure 3.3a - Distraction



a) Ex-fix applied
b) Osteotomy made
c) Distraction at 1mm/day
d) Consolidation phase once desired length reached

Identification of Suitable Bone Growth

A period of 6 weeks was then left to allow for consolidation and following this, the quality of the bone between the osteotomy sites was assessed using radiographs. At this stage there was only one animal with sufficient bone growth to allow for measurement. This animal was then humanely killed in accordance with animal handling protocols. One other animal had poor to moderate bone growth and had samples harvested for analysis to check that the procedure was viable.

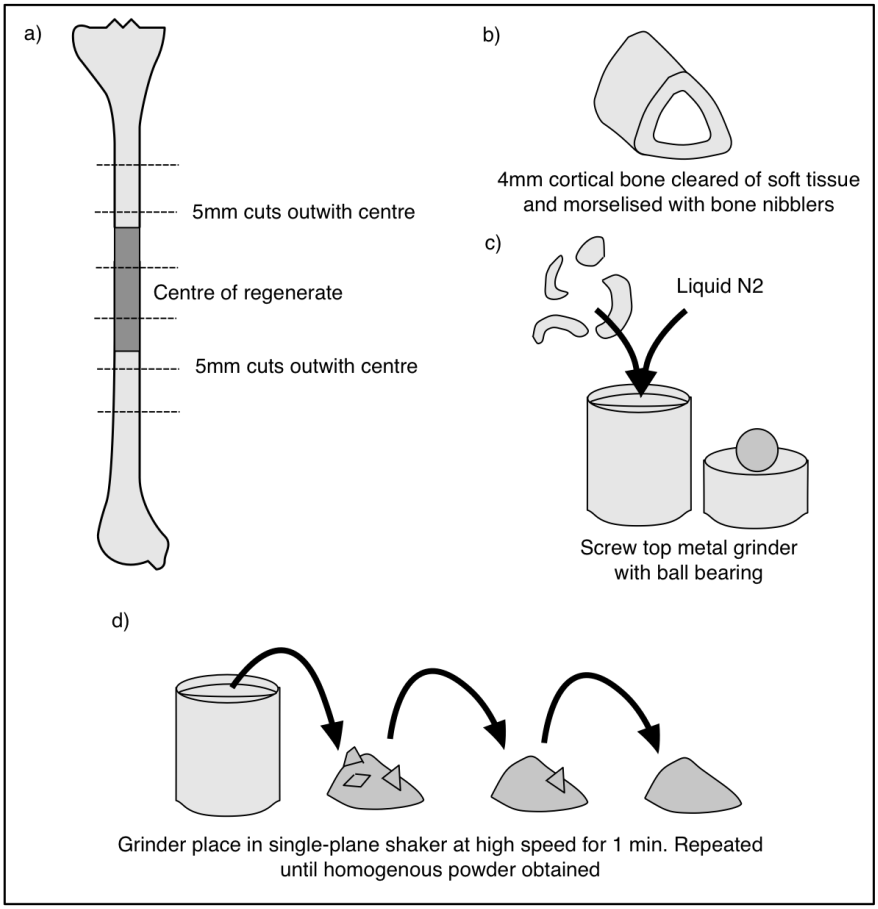
A further three rabbits had distraction procedures carried out at a later date to provide more samples for analysis.

Harvesting of Bone Samples

Both lower limbs of the rabbits were harvested and fresh frozen directly following death. These were kept at -40°C in the university tissue storage unit. At the time of harvesting, the samples were removed from the freezer and prepared once thawed to room temperature.

All soft tissues were stripped from the tibia and the bone marrow was removed. This was carried out with scalpels, bone 'nibblers' and curettage. At this stage the importance of good quality bone consolidation was identified as the poor quality samples proved difficult to prepare. In these samples, there was a lot of fibrous tissue ingrowth into the callus and between the bone ends which made differentiation between suitable sample tissue and unwanted soft-tissue

Figure 3.3b – Sample Preparation



a) Centre of regenerate identified and 4mm sample taken, with adjacent samples
b) Bone cleared of soft tissue and morselised
c) Fragments placed in grinder with liquid nitrogen
d) Grinding repeated until powder homogenous

problematic, although this immature distraction osteogenesis material should have theoretically undergone the same number of cell divisions.

Sandpaper was used as a final step in removing all soft tissues from the bone sample.

The contralateral tibia to the distracted one was also stripped and prepared to provide a control sample for comparison and standardisation in each animal.

Once stripped of soft tissue, the new bone between the osteotomy ends was identified. This area of bone was cut into 3 samples of 3-4mm each, using a fine-bladed bone-saw. A further 2 samples were taken from either side of this and a 1cm sample of bone from the same level on the contralateral tibia was taken as a control.

Decalcification of Compact Bone

The protocol used to isolate genomic DNA does not allow for samples from bone in its standard form. A technique for decalcifying the bone was therefore used which allowed the processed bone to be treated as a tissue sample, for the purposes of lysis and isolation.

Following soft tissue stripping, each sample of bone was morsellised into as small pieces as possible, using bone nibblers.

At this stage, the bone was still in pieces of around 2-3mm in size and the decalcification required the bone to be in as fine a powder as possible. The samples were therefore ground to powder using a metal blender.

The morsellised bone samples were placed into the chamber of a 1cm wide metal blender and covered in liquid nitrogen, up to half the volume of the blender. The metal ball bearing was

then placed in the blender and the top securely screwed in place. Next, the blender was fixed onto a single-plane vibration 'shaker' and run at high speed for 1 minute. This caused the ball in the blender to oscillate at high speed and pulverise the frozen bone pieces.

Following each step in the shaker, the bone was emptied from the blender and examined for consistency/particle size. On average the bone samples needed five repetitions of the blending process to obtain a homogenous powder that would be suitable for the decalcification process.

The decalcification protocol that was used was designed for 5g of bone but, due to the small size of the samples, a maximum of 1.5g of bone powder was obtained. This meant that all measurements in the procedure were reduced to one quarter of their given value.

Between 1g and 1.5g of each sample of bone from each animal was placed in individually labelled, 50ml sterile polypropylene tubes along with 10ml of 0.5 M EDTA at pH 7.5. This was the agent used for decalcification. Following this, the samples were agitated on a rotator at 40°C for 24 hours.

After agitation, the samples were centrifuged at 2000 x g for 15 minutes and the resulting supernatant was discarded. A further 10ml of 0.5 M EDTA was then added and the samples returned to the rotator for 24 hours. This was repeated to allow for full decalcification which was monitored by adding a saturated solution of ammonium oxalate, at a pH of 3.0, to the supernatant and assessing the resulting colour. If the solution remained clear then the process was complete.

This was the most time consuming part of this step as a total of four to five repetitions were needed for complete decalcification, meaning a period of three to four days.

Once decalcification was complete, each sample was centrifuged as above but no further EDTA was added. Instead, the pellets in the polypropylene tubes were washed with 10ml of sterile de-ionised water to remove ions that had accumulated during decalcification. The samples were then centrifuged again at 2000 x g for 15 minutes and the supernatant discarded. This washing procedure was repeated a further three times. The pellets were then stored in the -60°C freezer prior to DNA isolation. The final step before isolation was to add 80μl of buffer ATL from the Qiagen QIAamp DNA Mini Kit to the still frozen samples.

Isolation of Genomic DNA

The QIAamp Tissue DNA Mini Kit was chosen as the method for DNA isolation as it uses gravity-flow, which allowed the processing of multiple samples in parallel is possible. The process for each sample took approximately 3 hours.

The genomic tips used in the kit required a specific weight of sample as it was possible to overload the tips and clog the filters. This was not much of a concern during the procedure as only small volumes of sample were obtained and were suitable for the smallest genomic tip size.

The isolation procedure consisted of five stages;

i) Lysis and digestion with protease

ii) DNA binding

iii) Washing

iv) Elution

v) Precipitation.

For digestion, proteinase K was chosen due to its stability and suitability for short digestion times. It is a protease isolated from the fungus Tritirachium album. At binding, the lysate was added to the genomic tips and the DNA bound to the filter, with degraded RNA, cellular proteins and metabolites passing through to the supernatant. The genomic tip was then washed with several different buffers to remove residual contaminants. The final stages involve elution of the DNA and precipitation in isopropanol. It was then suitable for Southern blot analysis.

Lysis and Digestion with Protease.

This step caused lysis of the cell nuclei and denatured proteins to strip the DNA of all bound proteins.

A combination of Buffer G2 and the RNase A stock solution (provided with the kit) was made by adding 4μl of RNase to 2ml Buffer G2.

Each numbered sample of powdered bone was placed into a new 10ml screw-cap tube along with 2ml of Buffer G2 (plus RNase) and 0.1ml Proteinase K.

The tubes were then incubated in a 50°C water-bath for 3 hours.

Following incubation, the samples were centrifuged at 5000g for 10 minutes (at 4°C) to remove particulate matter.

DNA Binding

For each sample, a genomic-tip (Quiagen genomic-tip 20/G) was equilibrated by adding 1ml of Buffer QBT to it and allowing it to pass through by gravity, into a waste tray.

Each sample of lysate was then centrifuged on full power for 10 seconds to reduce further the amount of particulate material. The clear precipitate was added to the equilibrated genomic-tips promptly.

The samples were allowed to pass through by gravity.

Washing and Elution

The genomic-tips were then placed over a clean 10ml collection tube and washed using 1ml of Buffer QC under gravity flow. This process was repeated 3 times to ensure all contaminants were removed.

The genomic-tips were placed over a clean 10ml collection tube and 1ml of Buffer QF was added and passed by gravity flow. This step was repeated twice.

Precipitation

The tips were then placed over the final clean 10ml collection tube, which collected the processed DNA. The protocol that was used was designed for obtaining around 1ml of DNA but it was predicted that only 500μl would be possible from this process. All other steps prior to this used the volumes of buffer for 1ml of DNA but this step halved the volume of isopropranol for precipitation, to allow for greater concentration of DNA at the end. Thus 750μl of isopropranol was used, rather than 1.4ml.

The 750μl of isopropranol was added to the genomic-tip and it was immediately placed, with the collection tube in a centrifuge for 15 minutes at 8000G at 4°C.

The supernatant was then carefully removed, leaving a pellet at the bottom of the collection tube.

This pellet of DNA was then washed further by adding 1ml of 70% Ethanol at 4°C, to remove precipitated salt. This was vortexed for 3 seconds to mix and then centrifuged for 10 minutes at 5000G at 4°C.

The supernatant was again removed carefully with a pipette and the pellet was allowed to air dry for 5 minutes.

The pellet was resuspended in 50μl of Buffer TE at pH 8 as it dissolves best in slightly alkaline conditions. This was placed on a shaker table for 12 hrs at 55°C.

Measurement of DNA Yield

At the end of the DNA Isolation process the yield of DNA was determined for each sample from the concentration of DNA measured by absorption at 260nm on a spectrophotometer. This was expected to be between 0.1 to 1.0 for the next step to be accurate so each sample was diluted to the same concentration. The purity of the DNA was also assessed by a ratio of absorbance at 260 to 280 nm (A260/A280). Pure DNA has a ratio of between 1.7-1.9. The purified DNA was then available for telomere length assay and detection by chemoluminescence.

Telomere Length Assay

A number of methods have been described to detect and measure telomere length. The method used here is the current standard. The TeloTAGGG Telomere Length Assay from Roche was used in this instance. This process utilises three main steps;

1) Digestion of genomic DNA
2) Separation of DNA fragments and Southern blotting
3) Hybridisation, incubation and chemiluminescence detection

Handling of all collection tubes during this process was carried out on ice.

Digestion of Genomic DNA

Following the steps described previously, the purified genomic DNA was digested by a mixture of frequently cutting restriction enzymes which do not cut telomeric DNA. The non-telomeric-DNA was digested to low molecular-weight fragments.

The genomic DNA was centrifuged at maximum speed for 5 minutes and the supernatant was placed in a fresh collection tube.

A digesting enzyme mixture was prepared by mixing the enzymes Hinf I and Rsa I together in a 1:1 ratio, with a resulting concentration of 20 U/μl for each enzyme. 1μl of this mixture was required for each individual sample.

The next step required that each sample and the control DNA (provide with the assay kit) be diluted to a volume of 17μl. Therefore, 15μl of control DNA (1.5μg) was added to 2μl of nuclease-free water. 1.5μg of each sample DNA was added to nuclease free water to achieve a final volume of 17μl.

2μl of digestion buffer 10x and 1μl of the enzyme mixture was added to each sample and mixed with pulse vortex for 5 seconds.

The reaction mixture was then incubated for 2hrs at 37°C.

The digestion reaction was then halted by the addition of 5μl of gel electrophoresis loading buffer 5x and mixed by pulsed vortex.

Separation of DNA Fragments

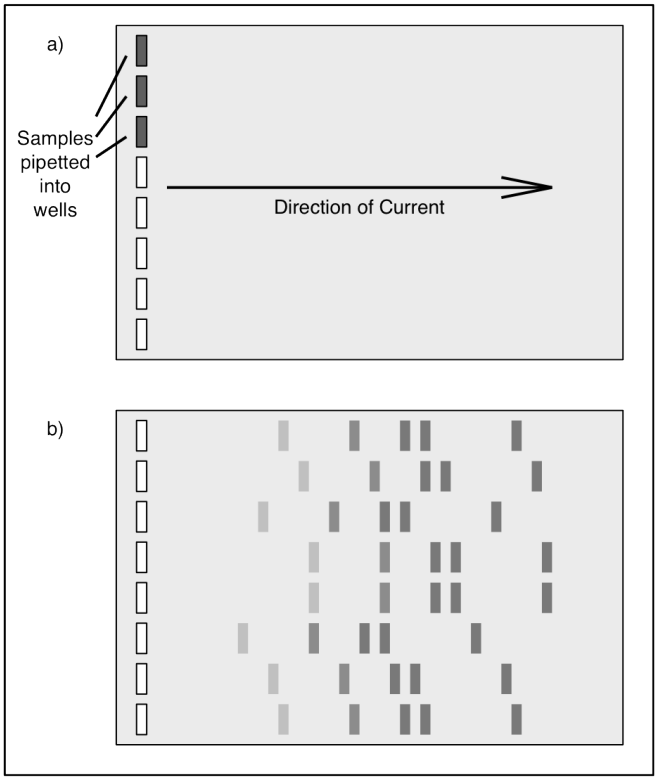
The separation of DNA fragments was achieved using standard gel electrophoresis.

A 0.8% highly pure, nucleic acid grade agarose gel 15cm in length was prepared and placed in TAE buffer. 4μl DIG molecular weight marker, 12μl nuclease-free-water and 4μl 5x loading buffer was mixed in a reaction vial and vortexed.

2μl of each DNA sample, at equalised concentrations, was then placed in each lane. 10μl of the diluted DIG molecular marker was then placed in each of the lanes at the far sides of the gel, to allow for accurate length measurement.

The gel was then run at 5V/cm in 1x TAE buffer for around 3 hours, until the blue marker has migrated around 10cm down the gel.

Figure 3.3c - Gel Electrophoreses



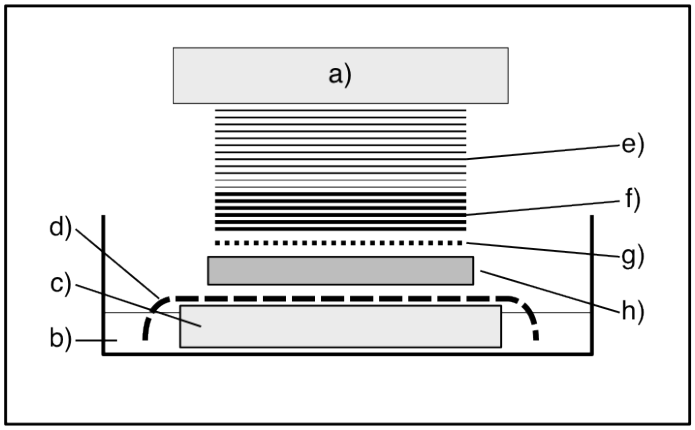
a) Samples individually pipetted into wells and current passed through gel
b) Larger fragments of DNA pass slowly through the gel while smaller fragments pass faster, reaching further from the wells.

Once gel electrophoresis was complete, the Southern transfer of the DNA was performed in standard fashion, with a positively charged nylon membrane. The gel was first submerged in HCl solution and agitated for 10 minutes at room temperature. This caused the blue tracking

dye to turn yellow. Next, the gel was washed with de-ionised water and submerged in denaturation solution for 15 minutes. This step was repeated and then the gel was submerged in the neutralisation solution for 2 periods of 15 minutes.

Once the gel had been washed, it was placed in the Southern blotting apparatus and left to transfer for approximately 6 hours, or overnight. Following blotting, the DNA was fixed to the membrane by UV cross-linking and washed twice with 2x SSC solution.

Figure 3.3d - Southern Blot



a) Weight
b) Buffer solution
c) Plastic block
d) Filter paper
e) Absorptive paper towel
f) Filter paper
g) Nylon Membrane
h) Agarose gel

Hybridisation

35ml of DIG Easy Hyb solution was pre-heated to 42°C and the blotting membrane was agitated in 18ml of this solution for 40 minutes, whilst the temperature was maintained at 42°C.

The hybridisation solution was prepared by adding 1μl of telomere probe to 5ml of the DIG Easy Hyb solution. The membrane was then transferred straight into the hybridisation solution and incubated/agitated for 3 hours at 42°C

Following hybridisation, the membrane was washed with the stringent washing buffer I by agitation for 5 minutes. This was repeated with fresh buffer and then washed twice with stringent washing buffer II for 5 minutes, agitated at 50°C.

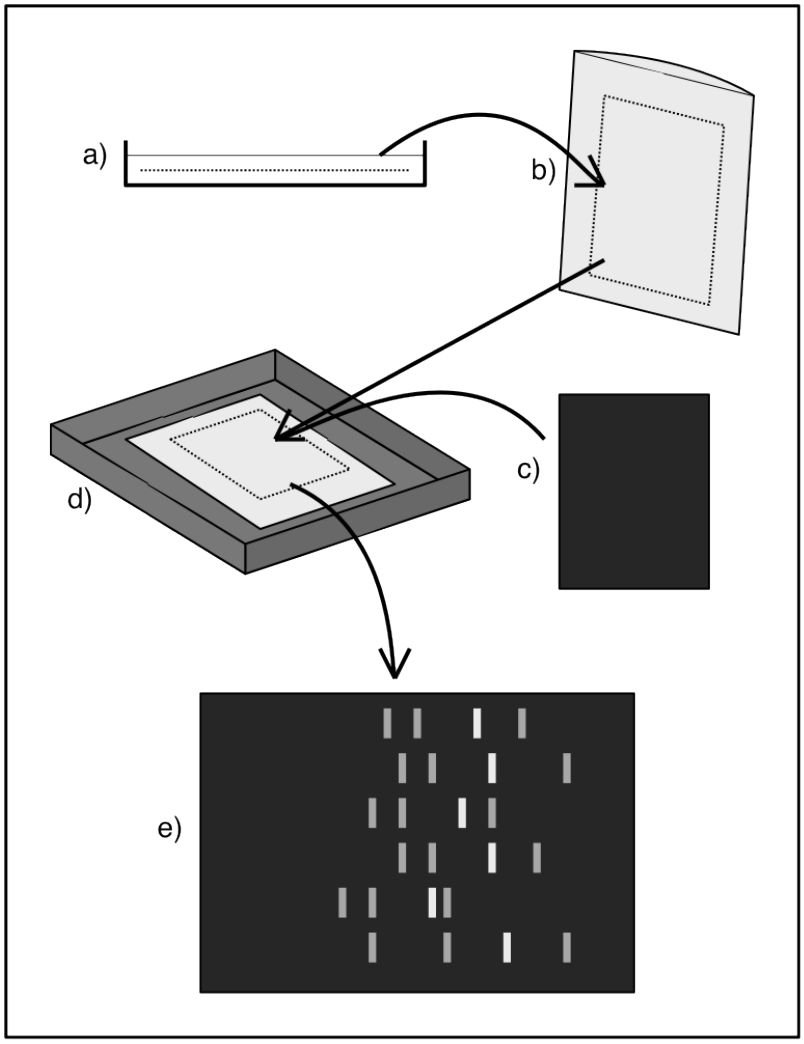
Chemoluminescence Detection

The membrane was once again washed with 200ml of 1x washing buffer and then placed in 100ml blocking buffer, to be incubated / agitated for 30 minutes at room temperature.

Next the membrane was incubated in Anti-DIG-AP working solution for 30 minutes as above. It was then washed twice with 100ml 1x washing buffer.

Finally, the membrane was incubated with 100ml of 1x detection buffer for 5 minutes at room temperature. The membrane was then removed from the buffer and placed onto an absorbant sheet of paper, but not allowed to dry. Instead it was quickly placed into a hybridisation bag, DNA side up. 40 drops of substrate solution were then

Figure 3.3e - Chemiluminescence



a) After washing steps, membrane placed in hybridisation fluid
b) Membrane placed in hybridisation bag
c) Bag placed in radiograph cassette and X-ray sensitive films placed on top. This was left for a minimum of 6 hours
d) Film developed and highest intensity bar used to calculate telomere length.

added to the DNA side and the bag closed, making sure to avoid trapping bubbles of air. Excess solution was squeezed out and the edge of the bag was sealed.

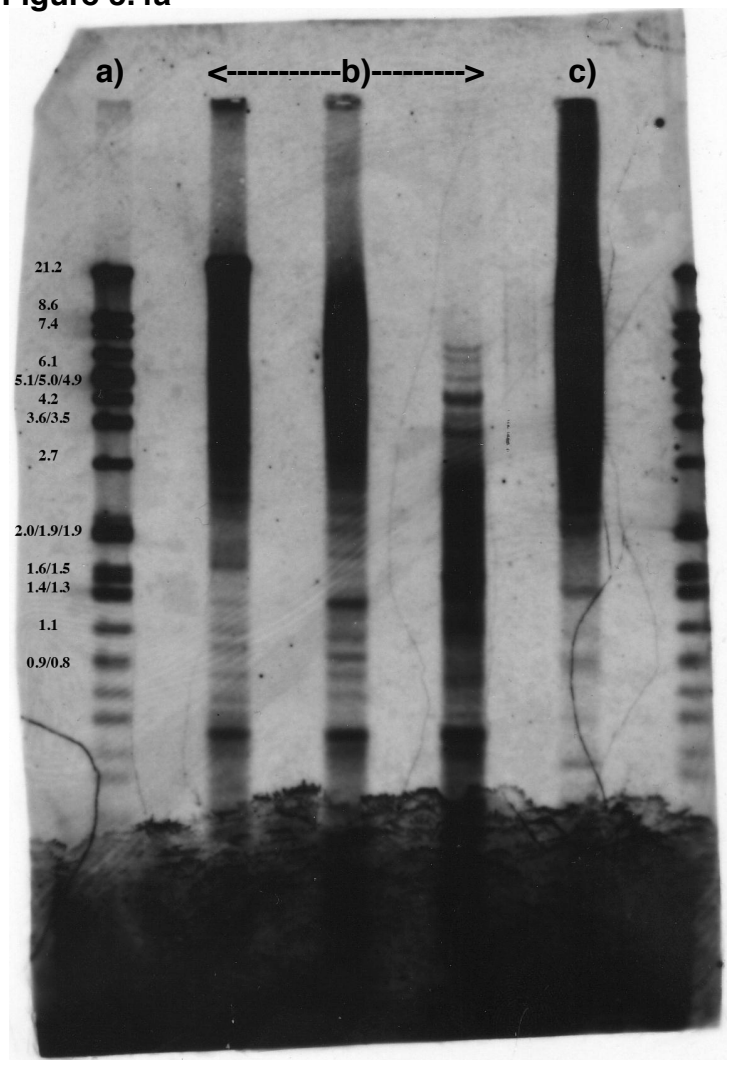
Detection of labelled telomeres was achieved by exposing the membrane to X-ray sensitive film. In a dark-room, under a red light, the hybridisation bag was placed flat in a radiograph cassette and x-ray sensitive film placed on top. The luminescence peaks over the first 6 hours so the membrane was left in the sealed dark box with the film for 6 hours, carefully avoiding shaking the box. The film was then developed and the highest intensity bar of each sample was compared to the DIG molecular weight marker to calculate mean telomere length.

Part 3.4

Results

The first two sets of rabbit samples that were processed had poor results. Identification of new bone growth was difficult as the new regenerate was a mix of fibrous tissue and immature bone. When the samples were processed, a large amount of fibrous material was included, which clogged the metal grinder and made it difficult to pulverise the bone. This led to decreased efficiency in decalcification, meaning that it had to be repeated further times than would otherwise have been necessary.

Figure 3.4a



a) Molecular weight marker
b) Control markers (D17, SB, 3132)
c) Control sample

The small quantity of actual bone harvested from the samples with low mineralisation led to very low yields of purified DNA. This meant that the subsequent steps of DNA digestion and telomere length assay were rendered inefficient and produced highly variable results, with at least 2 of the samples failing to show any result at all at chemo-luminescence detection.

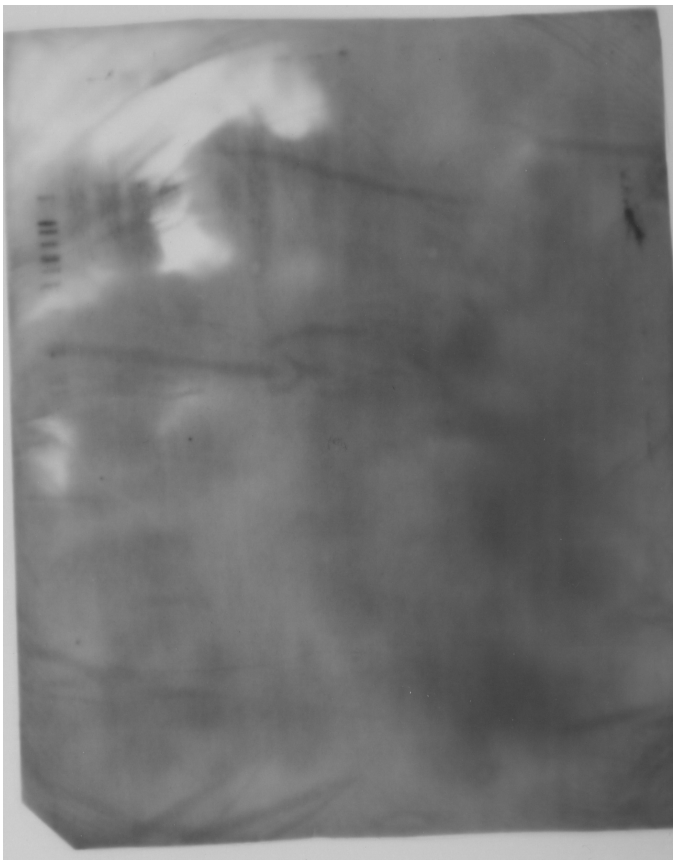
The samples taken from the contralateral tibias of these rabbits were processed with adequate

results, however. The expected yields of purified DNA were obtained and telomere length assay succeeding in producing detectable results.

Each rabbit tibia yielded a total of 5 samples – a sample from the centre of regeneration and 2 from either side. Each sample was further split into 3 samples in an attempt to reduce loss from error. This meant that for a single rabbit tibia, 15 samples were obtained and processed.

A total of 6 rabbit tibias (3 distraction and 3 control) were processed during the research period. This number required the cleaning/preparation of bone to be performed on 18 samples (5 from each distraction, 1 per control tibia), DNA elution/purification process to be carried out on 48 samples (15 from each distraction tibia plus 1 per control tibia) and the electrophoresis/southern blot/chemoluminesence on 48 samples in 3 batches (16 lanes per batch).

Figure 3.4b



Example radiograph following chemoluminesence demonstrating molecular weight marker in leftmost lane and diffuse spread of sample DNA fragments in lanes 2-17

A radiograph of a control sample with labelled markers is shown in figure 3.4a, demonstrating adequate DNA yield was obtained and that the process was successful. This control sample was a large piece of bone, which yielded a much higher volume of DNA than the later samples.

The samples that were processed showed a diffuse spread of DNA fragments on detection, compared with the control, as shown in figure 3.4b. This may be due to a number of problems. Firstly, the low yield of DNA would not have provided enough DNA fragments for visible bands to develop. Secondly, the difficulties in preparing the bone may have limited the efficacy of the digestion process and hybridisation.

As it was only possible to carry out the procedures on 2 rabbits at a time, there were large time intervals between obtaining sets of samples. After noting the immature state of the regenerate in the first pair of samples, radiographs were taken sequentially of each tibia to ensure the distraction was progressing well and adequate bone was consolidating. On examination of a further 2 samples, the radiographs showed immature regenerate and hence further time was allowed for consolidation following the end of the distraction period.

Unfortunately, the increased time required for identification and preparation of samples meant that only the initial samples were processed by the end of the period of degree time, and hence no significant or useful results were obtained.

Part 4

Plans for Future Research

Part 4.1 - Method for Measuring Telomere Lengths in Cortical and Cancellous Bone

The method outlined above was adapted from 3 pathways to decalcify bone, purify the DNA from its cells and measure the length of the telomeres involved. In carrying out the literature review, no similar method was found to be described previously. If this method is reliable and repeatable, it may be useful in future research.

To identify this, we plan to carry out the process on a number of samples and ascertain whether it is viable. If this is successful we hope to publish the technique as a manuscript.

Part 4.2 - Continuation of Current Research

Due to the difficulties encountered in completing the laboratory work, only 3 of the rabbit samples have been fully processed so far. The other samples are still held in the -60°C storage freezer in the Chancellor's Building at the Edinburgh Royal Infirmary, awaiting processing. As the process of decalcification, DNA isolation and telomere length assay has been shown to work, I would plan to finish processing these samples when time and laboratory availability allows.

Part 4.3 - Smoking, Osteoporosis and Telomere length

I have also been looking at the relationship between smoking and telomere length and plan to investigate if there is a link between smoking, reduced telomere length and osteoporosis.

Smoking has been long recognised as a major risk factor in many chronic diseases, including cancer, heart disease and respiratory disease, leading to 5 million deaths per annum, globally. Cigarette smoke is a complex mix of over 5 thousand different chemicals, including oxidants and free radicals. These, in combination with the increase in generation of reactive oxygen species, cause damage to lipids, proteins and DNA in cells throughout the smoker's body. Clinical and epidemiological studies have shown that shorter mean telomere lengths in leukocytes are associated with cardiovascular disease, obesity, insulin resistance and dementia[45]. Oxidative stress and inflammation are known to accelerate telomere shortening and both are direct effects of cigarette smoking. Recent studies have shown a direct relationship between smoking and peripheral leukocyte telomere length, with significantly shorter telomeres in healthy individuals who smoked, compared with those who did not[46]. Telomere length was found to be approximately 5 base-pairs shorter per 'pack-year' when adjusted for age and other risk factors[47].

Previous research has established links between smoking and reduced telomere length in many tissues and diseases. There has been little study into whether or not smoking causes a decrease in telomere length in cells of the axial skeleton, or if there is a link between reduced telomere length and risk of osteoporosis.

Information on smoking history is routinely gathered from patients when they enter hospital, either at the pre-assessment stage for elective operations or on clerk-in questioning, if emergent.

Discard material from femoral heads can be obtained following elective or post-traumatic arthroplasty. The mean telomere length in the discard material tissue can be measured and plotted against the smoking history to identify any trend.

The severity of osteoporosis could also be ascertained if the patient has had, or is due to have, a DEXA scan. It may also be possible to obtain an assessment of bone density by taking radiographs of the bone section and comparing their x-ray penetration to a calibrated measure. This could be done by placing a metal wedge on the x-ray film with increasing steps of known density. This method is being used in ongoing research.

For the research into smoking and osteoporosis, 2-3 cm3 samples of femoral neck cancellous bone will be obtained from trauma and elective arthroplasty patients. The samples taken will be part of discard material so will be covered under the 'discard material' ethics application, already in place in the unit.

Once collected, the samples will be kept in a -60C freezer until the decalcification procedure can be carried out. Each sample will be split into 3 separate samples to be processed individually, to try to eliminate error and avoid loss from failed assay.

A smoking history will be obtained from each patient as outlined above and follow them up to assess for osteoporosis, either using the radiographic method or by bone scan.

Part 5

References and Appendices

Part 5.1

References

1	**Kenyon, C.** The Genetics of Aging. Nature 467 622-622 (2010)
2	**Hayflick, L, Moorhead, PS.** The serial cultivation of human diploid cell strains. Exp. Cell Res. 1961;25:585–621
3	**Collado M, Blasco MA,** Serrano M. Cellular Senescence in Cancer and Aging. Cell 2007;130:223-233
4	**Muller M.** Cellular senescence: molecular mechanisms, in vivo significance, and redox considerations. Antioxid Redox Signal 2009;11:59–98
5	**Pollock PM, Harper UL, Hansen KS, et al.** High frequency of BRAF mutations in nevi. Nat Genet 2003;33:19–20.
6	**Hornsby PJ.** Cellular Aging and Cancer. Critical Reviews in Oncology/Hematology 2011;79:189–195
7	**Michaloglou C, Vredeveld LC, Soengas MS, et al.** BRAFE600- associated senescence-like cell cycle arrest of human naevi. Nature 2005;436:720–4.
8	**Jiang H, Ju Z, Rudolph KL.** Telomere shortening and ageing. Zeitschrift Fur Gerontologie Und Geriatrie 2007;40-5:314-24
9	**Feng JL et al.** The RNA component of human telomerase. *Science* 1995;269-5228:1236-41
10	**Nakamura TM et al.** Telomerase catalytic subunit homologs from fission yeast and human. *Science* 1997;277-5328:955-9.
11	**Allsopp RC et al.** Telomere length predicts replicative capacity of human fibroblasts. *Proceedings of the National Academy of Sciences of the USA* 1992;89-21:10114-8
12	**Bodnar, A. G. et al.** Extension of life-span by introduction of telomerase into normal human cells. Science 1998;279:349–352
13	**Stewart, SA, Weinberg, RA.** Telomeres: cancer to human aging. Annu. Rev. Cell Dev. Biol. 2006;22:531–557.
14	**Vonzglinicki T, Saretzki G, Docke W, Lotze C.** Mild hyperoxia shortens telomeres and inhibits proliferation of fibroblasts - a model for senescence. *Experimental Cell Research* 1995;220-1:186-93.

15	**Finkel T, Holbrook NJ.** Oxidants, oxidative stress and the biology of ageing. *Nature* 2000*;*408-6809:239-47*.*
16	**Meyerson M.** Telomerase enzyme activation and human cell immortalization. *Toxicology Letters (Shannon)* 1998*;*102-103-0:41-5*.*
17	**de Magalhaes JP.** From cells to ageing: a review of models and mechanisms of cellular senescence and their impact on human ageing. *Experimental Cell Research* 2004*;*300-1:1-10*.*
18	**Cristofalo VJ, Pignolo RJ.** Replicative senescence of human fibroblast like cells in culture. *Physiological Reviews* 1993*;*73-3:617-38*.*
19	**Djojosubroto MW, Choi YS, Lee HW, Rudolph KL.** Telomeres and telomerase in aging, regeneration and cancer. *Molecules and Cells* 2003*;*15-2:164-75
20	**Gonzalez-Suarez E et al.** Increased epidermal tumors and increased skin wound healing in transgenic mice overexpressing the catalytic subunit of telomerase, mTERT, in basal keratinocytes. *Embo Journal* 2001*;*20-11:2619-30*.*
21	**Pawelec G, Solana R.** Are cancer and ageing different sides of the same coin? Conference on Cancer and Ageing. *Embo Reports* 2008*;*9-3:234-8*.*
22	**Tomas-Loba, A. et al.** Telomerase reverse transcriptase delays aging in cancer-resistant mice. Cell 2008;135:609–622
23	**Ergün Sahin & Ronald A. DePinho.** Linking functional decline of telomeres,mitochondria and stem cells during ageing. Nature 2010;464:520-528
24	**Martin, G. M.** Genetic modulation of senescent phenotypes in Homo sapiens. Cell 2005;120:523–532
25	**Blasco, M. A. et al.** Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. Cell 1997;91:25–34
26	**Shay JW, Wright WE.** Senescence and immortalization: role of telomeres and telomerase. Carcinogenesis 2005;26:867–74
27	**Sun B, Chen M, Hawks CL, Pereira-Smith OM, Hornsby PJ.** The mini- mal set of genetic alterations required for conversion of primary human fibroblasts to cancer cells in the subrenal capsule assay. Neoplasia 2005;7:585–93
28	**Sun B, Huang Q, Liu S, et al.** Progressive loss of malignant behavior in telomerase-negative tumorigenic adrenocortical cells and restoration of tumorigenicity by human telomerase reverse transcriptase. Cancer Res 2004;64:6144–51.

29	**Gisselsson D,Jonson T, Petersen A,et al.**Telomeredysfunctiontriggers extensive DNA fragmentation and evolution of complex chromosome abnormalities in human malignant tumors. Proc Natl Acad Sci USA 2001;98:12683–8.
30	**Kammori M, Poon SS, Nakamura K, et al.** Squamous cell carcinomas of the esophagus arise from a telomere-shortened epithelial field. Int J Mol Med 2007;20:793–9.
31	**Aida J, Izumo T, Shimomura N, et al.** Telomere lengths in the oral epithelia with and without carcinoma. Eur J Cancer 2009;46:430–8.
32	**Kyo S, Takakura M, Fujiwara T, Inoue M**. Understanding and exploit- ing hTERT promoter regulation for diagnosis and treatment of human cancers. Cancer Sci 2008;99:1528–38
33	**Vogelstein, B. & Kinzler, K.W**. The multistep nature of cancer. Trends Genet. 1993;9:138–141
34	**Wright WE, Shay JW.** Historical claims and current interpretations of replicative aging. Nature Biotechnology 2002;20:682-688
35	**Oikawa, S, Kawanishi, S.** Site-specific DNA damage at GGG sequence by oxidative stress may accelerate telomere shortening. FEBS Lett. 1999;453:365–368
36	**Artandi, S. E. et al.** Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice. Nature 2009;406:641–645
37	**Tyner, S. D. et al.** p53 mutant mice that display early ageing-associated phenotypes. Nature 2002;415:45–53
38	**Shay, JW, Wright, WE.** Aging. When do telomeres matter? Science 2001;291:839–840
39	**Shay, JW, Pereira-Smith,O.M. and Wright,WE**. A role for both RB and p53 in the regulation of human cellular senescence. Exp. Cell Res. 1991;196:33-39
40	**Ilizarov GA**. The tension stress effect on the genesis and growth of tissues 2. The influence of the rate and frequency of distraction. Clln Ortho Rel Res 1989;239;263-285
41	**Cole JD. et al.** The intramedullary skeletal kinetic distractor (ISKD): first clinical results of a new intramedullary nail for lengthening of the femur and tibia. Injury 2001;32:129-139
42	**Leidinger B.** Limb lengthening with a fully implantable mechanical distraction intramedullary nail. Zeitschrift Fur Orthopadie und Ihre Grenzagebiete 2006;144:419-426
43	**Kubiak EN. et al.** Early complications encountered using a self-lengthening intramedullary nail for the correction of limb length inequality. Diseases and Related Surgery. 2007;18:52-57
44	**Paley D et al.** Ilizarov Treatment of Tibial Nonunions With Bone Loss. Cln Ortho Rel Res 1989;241;146-165

45	**Babizhayev, MA, Yegorov, YE.** Smoking and health: association between telomere length and factors impacting on human disease, quality of life and life span in a large population-based cohort under the effect of smoking duration. Fundamental and Clinical Pharmacology 2011;25:425-442
46	**McGrath M., Wong J.Y., Michaud D., Hunter D.J., De Vivo I.** Telomere length, cigarette smoking, and bladder cancer risk in men and women. Cancer Epidemiol. Biomarkers Prev. 2007;16:815–819
47	**Valdes A.M., Andrew T., Gardner J.P. et al.** Obesity, cigarette smoking, and telomere length in women. Lancet 2005;366:662–664
48	**Effros RB, Pawelec G.** Replicative senescence of t cells: does the Hayflick Limit lead to immune exhaustion? Immunol Today 2009 18: 450–454
49	**Pawelec G, Koch S, et al.** Immunosenescence, suppression and tumour progression. Cancer Immunol Immunother 2006 55: 981–986
50	**Pinkston-Gosse J, Kenyon** c DaF-16/FOXO targets genes that regulate tumor growth in Caenorhabditis elegans. Nat Genet 2007 39: 1403–1409
51	**Solana R, Pawelec G, Tarazona R** Aging and innate immunity. Immunity 2006;24: 491–494
52	**Tyner SD et al** p53 mutant mice that display early ageing-associated phenotypes. Nature 2002;415: 45–53
53	**Von Zglinicki T, Saretzki G, Docke W, Lotze C** Mild hyperoxia shortens telomeres and inhibits proliferation of fibroblasts: a model for senescence? Exp Cell Res 1995;220: 186–193
54	**Alcorta, D. A. et al.** Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts. Proc. Natl Acad. Sci. USA 1996;93, 13742–13747.
55	**Hara, E. et al.** Regulation of p16CDKN2 expression and its implications for cell immortalization and senescence. Mol. Cell. Biol. 1996;16, 859–867.
56	**Mooi, W. J. & Peeper, D. S.** Oncogene-induced cell senescence—halting on the road to cancer. N. Engl. J. Med. 2006;355, 1037–1046.
57	**Collado, M. & Serrano, M.** The power and the promise of oncogene-induced senescence markers. Nature Rev. Cancer 2006;6, 472–476.

58	**Bartkova, J. et al.** Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. Nature 2006;444, 633–637.
59	**Di Micco, R. et al.** Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. Nature 2006;444, 638–642.
60	**Xue, W. et al.** Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. Nature 2007;445, 656–660.
61	**Ventura, A. et al.** Restoration of p53 function leads to tumour regression in vivo. Nature 2007;445, 661–665.
62	**Roninson, I. B.** Tumor cell senescence in cancer treatment. Cancer Res. 2003;63, 2705–2715.
63	**Dimri, G. P. et al.** A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc. Natl Acad. Sci. USA 1995;92, 9363–9367.
64	**Herbig, U, Ferreira, M.et al.** Cellular senescence in aging primates. Science 2006;311, 1257.
65	**McMurray, M.A. Gottschling,D.E.** An age-induced switch to a hyperrecombinational state. Science 2003;301, 1908–1911.
66	**Curtis, H.J.** Biological mechanisms underlying the aging process. Science 1963;141, 686–694.
67	**Tyner, S.D.et al.** p53 mutant mice that display early ageing-associated phenotypes. Nature 2002;415, 45–53.
68	**Maier, B. et al.** Modulation of mammalian lifespan by the short isoform of p53. Genes Dev. 2004;18, 306–319.
69	**Garcia-Cao, I. et al.** ‘‘Super p53’’ mice exhibit enhanced DNA damage response, are tumor resistant and age normally. EMBO J. 2002;21, 6225–6235.
70	**Matheu, A. et al.** Delayed ageing through damage protection by the Arf/p53 pathway. Nature 2007;448, 375–379.
71	**Cao, L., Li, W., Kim, S. et al** Senescence,aging,andmalignant transformation mediated by p53 in mice lacking the Brca1 full-length isoform. Genes Dev. 2003;17, 201–213

72	**de Boer, J. et al.** Premature aging in mice deficient in DNA repair and transcription. Science 2002;296, 1276–1279.
73	**Dunham, M. A., Neumann, A. A., Fasching, C. L. & Reddel, R. R.** Telomere maintenance by recombination in human cells. Nature Genet. 2000;26, 447–450.
74	**Harley, C. B., Futcher, A. B. & Greider, C. W.** Telomeres shorten during ageing of human fibroblasts. Nature 1990;345, 458–460.
75	**Allsopp, R. C. et al.** Telomere length predicts replicative capacity of human fibroblasts. Proc. Natl Acad. Sci. USA 1992;89, 10114–10118.
76	**Cawthon, R. M. et al.** Association between telomere length in blood and mortality in people aged 60 years or older. Lancet 2003;361, 393–395.
77	**Bodnar, A. G. et al.** Extension of life-span by introduction of telomerase into normal human cells. Science 1998;279, 349–352.
78	**Blasco, M. A. et al.** Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. Cell 1997;91, 25–34.
79	**Lee, H.-W., Blasco, M. A. et al.** Essential role of mouse telomerase in highly proliferative organs. Nature 1998;392, 569–574.
80	**Herrera, E. et al.** Disease states associated to telomerase deficiency appear earlier in mice with short telomeres. EMBO J. 1999;18, 2950–2960.
81	**Flores, I., Cayuela, M. L. & Blasco, M. A.** Effects of telomerase and telomere length on epidermal stem cell behavior. Science 2005;309, 1253–1256.

Part 5.2

Appendices

Solution Constituents for DNA/Telomere Assays

Isolation of Genomic DNA

Before the isolation procedure was started, the necessary buffers were prepared as follows and stored at room temperature:

Name	Constituents
Buffer 'G2'	800 nM guanidine HCL, 30nM Tris-Cl at pH 8.0, 30 nM EDTA at pH 8.0, 5% Tween-20, 0.5% Triton X-100
Buffer 'QBT'	750 nM NaCl, 50 nM MOPS at pH 7.0, 15% isopropanol, 0.15% Triton X-100
Buffer 'QC'	1.0 M NaCl, 50 nM MOPS at pH 7.0, 15% isopropanol
Buffer 'GF'	1.25 M NaCl, 50 nM Tris-Cl at pH 8.5, 15% isopropanol

Telomere Length Assay (Taken from Roche TeloTAGGG Telomere Length Assay)

Name	Constituents
TAE Buffer	0.04M Tris-acetate, 0.001 M EDTA, pH8
HCl Solution	0.25M HCl
Denaturation Solution	0.5M NaOH, 1.5M NaCl
Neutralisation Solution	0.5M Tris-HCl, 3M NaCl, pH7.5
20x SSC	3M NaCl, 0.3M Sodium Citrate, pH7.0
2x SSC	Dilute 20x SSC 1:10 with autoclaved, redistilled water

Name	Constituents
DIG Easy Hyb	Granules provided with kit - reconstituted with 64ml autoclaved, redistilled water and incubated at 37°C. Pre-warmed to 42°C the night before use.
Stringent Wash Buffer I	2x SSC, 0.1% SDS
Stringent Wash Buffer II	0.2x SSC, 0.1% SDS
Washing Buffer 1x	Dilute 10x washing buffer (as provided with kit) 1:10 with autoclaved, distilled water
Blocking Solution 1x	Dilute 10x blocking buffer (as provided with kit) 1:10 with maleic acid buffer 1x (as prepared below)
Maleic Acid Buffer 1x	Dilute 10x maleic buffer (as provided with kit) 1:10 with autoclaved, distilled water
Anti-DIG-AP working solution	Anti-DIG-AP vial (as provided with kit) spun at 13000rpm for 5 minutes then diluted with blocking solution 1x (as prepared above) at 1:10,000 (10μl in 10ml)
Detection Buffer 1x	Detection buffer 10x (as provided with kit) 1:10 with autoclaved, distilled water

Animal License Details
License number 60/3396
Departmental license for animal handling
Individual license held by Dr David Cottrell, who performed the procedures on the rabbits
I did not carry out any procedures on live animal subjects