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Expansion of the replicative lifespan of human muscle cells by retroviral transduction of the catalytic telomerase subunit gene.

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A thesis submitted to the University of Glasgow in part fulfilment for the degree of Doctor of Philosophy

September, 2004

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Beatson Institute for Cancer Research

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And

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Declaration

I am the sole author of this thesis. All the references have been consulted by myself in the preparation of this manuscript. Unless otherwise acknowledged, all the work presented in this thesis was performed personally.

Acknowledgements

Firstly I would like to thank my two supervisors, Ken Parkinson and Mina Behan, for all there patience and kindness. Thanks to Ken for all of his invaluable advice, for his encouragement when things weren't going to plan and for the support he has shown me over the past years. I would like to thank Mina for all guidance she has given from my undergraduate degree until now. You have always believed in my capabilities even when I didn't.

There are numerous people who without their help I would never had finished. Thanks to all the members of R9 past and present, Hazel Ireland, Vivienne Morrison, Sara Fitzsimons, Nighean Barr, Jim McCaul , Fay Minty ,Nick Forsyth, Steve Bryce, Kirsty Wing and Nicola Craig. Thanks to for all the advice, chats, technical help and brilliant secret Santa gifts. The pink fluffy bag is still my favourite. In particular June Munro, I would have been at a loss without her organisation skills and technical expertise. I have to express my gratitude to Katrina Gordon, without your constant support and critical review of this thesis I would have never have finished. Your patience, advice and knowledge has been priceless. I cannot thank you enough.

I would like to thank Ann O'Dowd, Fiona Curtis, Debbie Lee for all the great advice. Thanks to Ann Macleod and Olive Scullion, you always knew how to cheer me up. Thanks to Professor Barry Gusterson for his kindness. Although I never had the opportunity to work with her, I would like to thank Karen Steeghs for the production of the initial hTERT HMC cell lines. Thanks are due to Gerry Graham, thank you for the career advice and your kindness. Thank you for taking a chance and let me work for you, not only gaining the knowledge and technical skills, but also many friends.

I would also like to express my gratitude to Norwich Union, in particular Dr W Jack for great kindness in providing financial support.

Finally I would like to thank my family and friends, your thoughts and prayers have been tremendous. Thanks to Annmarie, Jim, Phil, Marion, Gary and Kathleen. Thank you all for your love and support over the past years. Fiona thanks for giving me access to your computer, and listening to all my moans. A special thanks to Brain. Philip, Kevin and Clare, you make me realise what is important in life. Thanks to Sarah, Ruth, Christine, Andrea, Gayle and Jackie. You have all been a massive support to me.

A special thanks to Paul. Thank you for listening to my complaints, sitting through my presentations and telling me to get back to work when needed.

This thesis is dedicated to my Mum and Dad. I bet you never thought you'd be pensioners and still have a kid at 'school'. Your advice that 'it's better to try and fail, than not try' has been invaluable. Without your love, support and honesty I would never have completed this task. Thank you both, and who would have thought that all those hours spent at the dinning room table would have paid off.

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Abbreviations

ALT	Alternative lengthening of telomeres
ATP	Adenosine triphosphate
ATM	Atxia Telangiectasia Mutant
BFB	Breakage fusion breakage
BICR	Beatson Insitute for Cancer Research
BM	Beckers muscular dystrophy
cdk	cycklin dependent kinase
CHK	Checkpoint kinase
C-terminal	Carboxy-terminal
DMD	Duchenne muscular dystrophy
DNA	Deoxyribonucleic acid
DSB	Double strand breaks
E.Coli	*Escherishia Coli*
HFF	Human fetal fibroblasts
HEK	Human embryonic kidney
HMC	Human muscle cell
HPV	Human papillomavirus
hTERT	Human telomerase reverse transcriptase
IgG	Immunoglobulin G
LOH	Loss of herterozygosity
M0	Mortality stage zero
M1	Mortality stage 1
M2	Mortality stage 2
pRB	Retinoblastoma
RNA	Ribonucleic acid
SABG	Senescence Assoicated β-galactosidase

SV40	Simian virus 40
TERT	Telomerase reverse transcriptase
UV	Ultraviolet radiation

Reagents

BrdU	5-Bromo-2'-deoxy-uridine
BSA	Bovine serum albumin
CO_2	Carbon dioxide
DAPI	4',6-Diamidino-2-phenylindole
DH2O	De-ionised water
DMEM	Dulbeccos modified Eagle's medium
DMSO	Dimethylsulphoxide
EDTA	Ethylenediaminetetra-acetic acid
FITC	Flurescein Isothiocyanate
FBS	Foetal bovine serum
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulphonic acid)
HCl	Hydrogen Chloride
LB	Luria Bertani
MES	2-(N-Morpholino) ethane sulfonic acid
MOM	Mouse on mouse kit
NaOH	Sodium hydroxide
PBS	Phosphate buffered saline
PCR	Polmerising chain reaction
SDS	Sodium dodecyl sulphate
SSC	Sodium chloride-sodium citrate
TBE	Tris, boric acid, ethylendiaminetetra-acetic acid
TE	Tris, ethylenediaminetetra-acetic acid

TEMED	Tetramethylenediamine
Tris	2-amion-2-(hydroxymethyl)propane-1,3-diol
Tris-HCl	2-amion-2-(hydroxymethyl)propane-1,3-diol in Hydrogen choride
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

Units

A	absorbancy
bp	base pair
Bq	Becquerel
c	centi
Da	dalton
g	gram
g	gravity
Gy	Grey
hrs	hours
k	kilo
kb	kilo base
l	litre
M	molar
M	mega
μ	micro
m	milli
m	metre
μl	micro liter
ml	milli liter
M	molar

mM	milli molar
min	minute
n	nano
°C	degree Celsius
MPD	mean population doublings
OD	optic density
rpm	revolutions per minute
RT	room temperature
s	seconds
U	unit
UV	ultra violet
V	volts
v/v	volume for volume
W	watts
w/v	weight for volume

Abstract

Duchenne Muscular Dystrophy (DMD) is a genetic disorder which is caused by mutations in the dystrophin gene. One of the functions of this protein is to anchor the cell membrane to the sacrolemma. In DMD, the absence of this protein leads to muscle damage during contractions, resulting in continuous regeneration and repair of the muscle until the replicative ability of the muscle is exhausted. The consequence of this loss is loss of muscle mass and death of the patients in the third decade of life due to respiratory failure.

There have been many attempts to cure this disorder including the strategy of gene and cell therapy. Although these approaches have had some success in animal models there has been no improvement in human trials. At present the treatment of DMD consists of steroid therapy and supportive treatment.

The aim of my work was to test the hypothesis that the transduction of telomerase in to muscle cells could produce a non transformed muscle cells with unlimited growth potential that could in principal, could serve as a target cell population for human dystrophin gene therapy.

The expression of the catalytic subunit of telomerase (hTERT) has been successfully used to extend the lifespan of a number of different cell types without malignant transformation. However previous attempts to immortalise muscle cells in this manner had failed. Here I report the successful immortalisation of normal human skeletal muscle cells by retroviral infection of hTERT. The telomerase positive cells display an extended lifespan, with 4/5 clones exceeding 120 MPD. The clones show no feature of transformation *in vitro*, retain a stable diploid karyotype, have wild type unmethylated CDKN2A genes and do not express muscle specific markers desmin and spectrin. *In vivo*, they can repair and reconstitute muscle in immunosuppressed RAG-1 mice. These results suggest that telomerase expression can extend lifespan of human muscle cells and could aid attempts at gene therapy for muscle diseases.

Introduction

A major advance in medical research has been the ability to remove cells from human and animal tissues and grow them in an artificial environment. The development of various media and the use of specialized incubators have allowed cell types to be examined in terms of their DNA, their growth properties and the changes which occur in them in diseased states. It was thought that under optimal growth conditions all cells had an unlimited ability to replicate until the work of Hayflick and Moorhead (1, 2) showed that this was not so: on the contrary, with certain exceptions cells have a strictly limited growth potential. The exceptions are germline cells, immortalized tissue culture cell lines and cancer cells which will indeed proliferate continuously. *In vitro*, normal somatic cells eventually age and stop proliferating, i.e. become senescent.

The replicative capacity of cells limits the lifespan of key systems in the body and the genetic programme that directs this process was elucidated by the work of Hayflick and others (1, 2). They were the first to show that, as predicted by the German biologist August Weismann (3), somatic cells of higher animals have a limited doubling potential (as cited in (4)). Weismann had no experimental evidence for his hypothesis but he stated that "death takes place because a worn-out tissue cannot forever renew itself and because a capacity for increase by means of cell division is not everlasting but finite"

This important experimental work of Hayflick *et al* confirmed that cells in culture replicate only for a finite number of divisions. In their seminal studies they took fibroblasts in culture and divided them into two parts, in one continual growth was allowed, until it ceased. The other sample was preserved cryogenically. When the latter cells were brought back to culture conditions and grown, they underwent the same number of doublings as the other set and then stopped growing. It thus appeared that the cells had been programmed to grow for a set number of divisions and then stop (1).

These workers suggested that their findings represented aging at the cellular level. Their aim was to identify the putative cell division counting mechanism which they postulated must exist (5) but it is only in the last few years that this has been achieved. It has now been confirmed that the shortening of telomeres at each round of DNA replication that occurs in normal cells in *vivo* and *in vitro* represents the functioning "mitotic clock". Rounds of DNA replication are measured until a limit is reached: "the Hayflick limit"(2).

The arrest of cell division which eventually occurs represents the finite replicative lifespan of the cell and leads to the process called replicative or cell senescence.

Hayflick deduced several important points from his work: first, that there is an inverse relationship between donor age and population doublings (of great importance in considering cell transfer therapy, as will be discussed later). Secondly, the same changes in biological activity which are seen *in vitro* can be recognised as characteristic of aging in the human. Thirdly, there is a direct relationship between species lifespan and the population doublings of their cultured normal cells, suggesting that aging does not only represent terminal differentiation but is related to other events occurring in these cells which may limit their usual abilities (2).

It has become apparent that although the fields of aging and cancer research differ greatly they are linked by studies into cellular aging, proliferative capacity and the development of malignancy (6). A common hypothesis has been suggested based on the view that telomere shortening is the molecular measure (mitotic clock) of the proliferative capacity remaining in cells (6). Telomeres can be maintained without any shortening by the enzyme telomerase. This enzyme is not expressed in the majority of normal human somatic cells but it is found in germ line cells, immortal cells in culture and malignant cells. Most malignant tumours acquire immortality and then proliferate continuously and this ability depends on allowing the re-expression of telomerase. Without this, telomeric DNA is lost from the ends of chromosomes every time cells divide due to the end replication problem. The cells become senescent in this state, they may still remain metabolically active and resist apoptotic death for long periods (7). Research into the mechanism and regulation of telomerase has been extensive, in the hope that it will provide an insight into the processes of aging, immortalisation and cancer (6).

1.1. Mitotic clock

Hayflick's concept of the mitotic clock (1, 2, 5), was seminal in the study of cell replication: much experimental work now supports his view that cellular aging is dependent on the number of cell divisions while total cellular life span is measured by the number of population doublings or cell generations, not by chronological time. The mitotic clock functions by counting the number of such cell divisions. In eukaryotic cells, the process involves evaluating the progressive loss of telomeric repeats at the ends of the chromosomes (see figure 1.1), which occurs during the aging process *in vitro* and *in vivo*.

Evidence indicates that this loss eventually induces antiproliferative signals, resulting in cellular senescence (6).

The mitotic clock is therefore a timing mechanism. It regulates cellular senescence based on the normal, progressive loss of telomeres. In support of this hypothesis, the growth of most continuously replicating cells depends on activation of telomerase, a ribonucleoprotein enzyme which maintains telomere length and stability.

Figure 1.1 Schematic representation of the telomere structure

Demonstrates the position of the telomere, the subtelomeric region, the coding DNA and the centromere.

Adapted from Checkpoint Controls and Cancer, Volume 2, Activation and regulation protocols, Edited by Axel H. Schonthal, Page 340

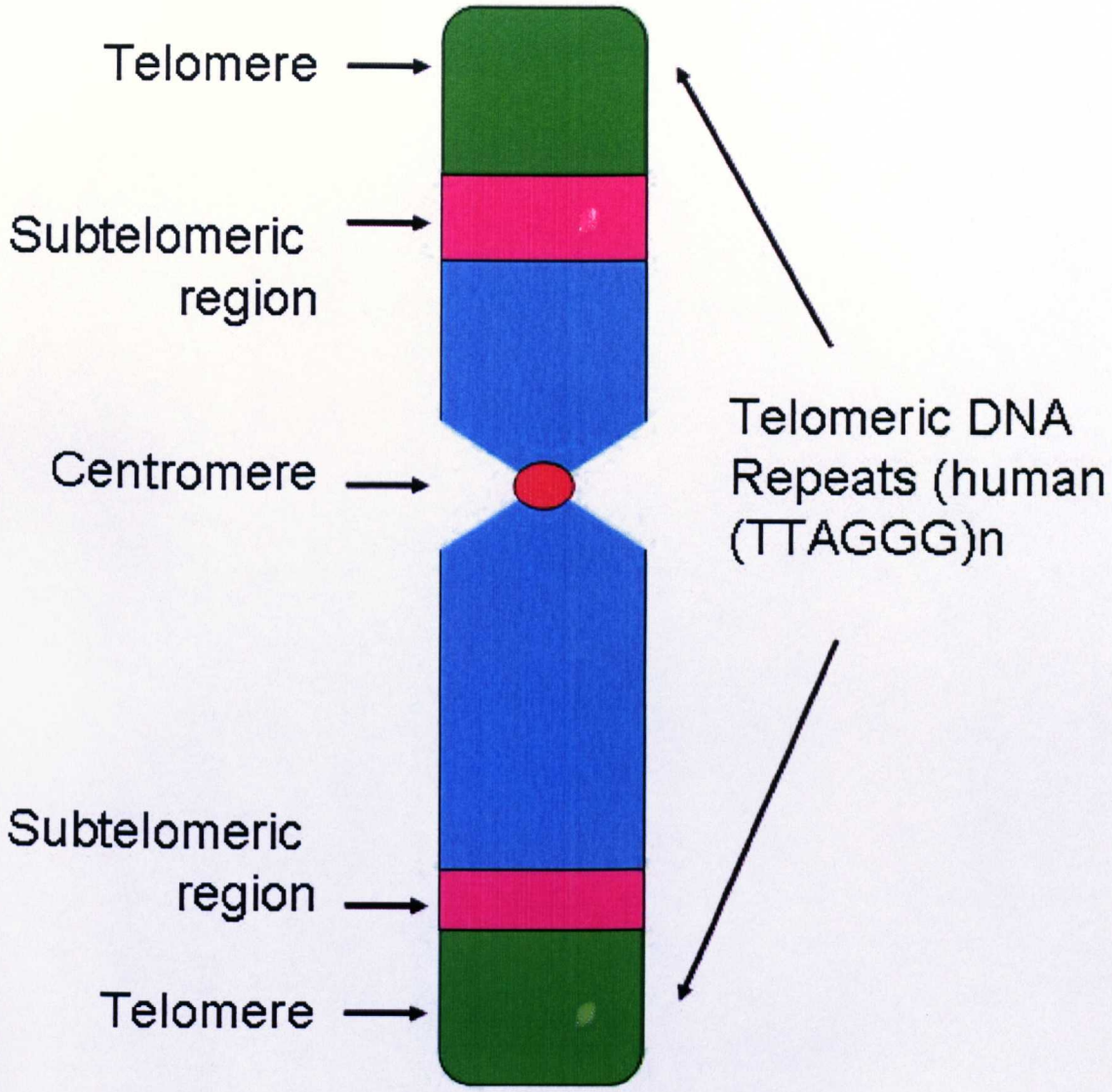

Figure 1.1 Schematic representation of the telomere structure

1.2 Cellular lifespan and senescence

Replicative or cellular senescence is the irreversible growth arrest which occurs when human diploid cells reach the end of their proliferative potential when serially cultured (5). It is thought that this mechanism has evolved to protect the cell against tumorogenesis (8). A senescent cell not only stops replicating but displays a different phenotype (9). The cells become enlarged, multi-nucleated and flat and they also show increased staining for β-galactosidase at pH 6 (7). As the cell approaches senescence, there is also an increase in the cyclin-dependent kinase inhibitors $p16^{INK4a}$ and $p21^{Cip1}$ which leads to cell cycle arrest (see below) (10).

1.2.1 Cell cycle arrest

The cell cycle is a highly ordered and structured process. DNA must be accurately replicated and identical chromosomes distributed into two daughter cells. The cycle is divided into four distinct phases i.e. G0, G1, Mitosis and S phase (DNA synthesis). G1 is the gap where the cell can exit the cycle and stay quiescent (G0) (see figure 1.2). During G1 there is a restriction point where gene transcription is tightly regulated (see figure 1.3) (11-13). INK4a/ARF encodes two distinct proteins, $p16^{INK4a\ and}$ $p19^{ARF}$ (mouse) or $p14^{ARF}$ (human), which when expressed result in cell cycle arrest (figure 1.3 and 1.4). p16 binds directly to the cyclin dependent kinases (Cdks) 4 and 6 and blocks the phosphorylation of the retinoblastoma gene product (pRB) and the release of the transcription factor E2F. This results in the cells arresting in the G1 phase of the cell cycle. $p14^{ARF}$ / $p19^{ARF}$ interact directly with MDM2 and lead to the stabilisation of p53 and cause the cell to arrest in G1 or G2. The accumulation of p53 also leads to the increased expression of the p53-regulated gene $p21^{Cip1}$.

Figure 1.2 The cell cycle

Schematic diagram of the cell cycle, showing the position of the G1 restriction point in relation to the other phases.

Adapted from Stem Cells 2001;19: 1, Pages 88 -91, E.D. Israels & L.G. Israels, The Cell Cycle

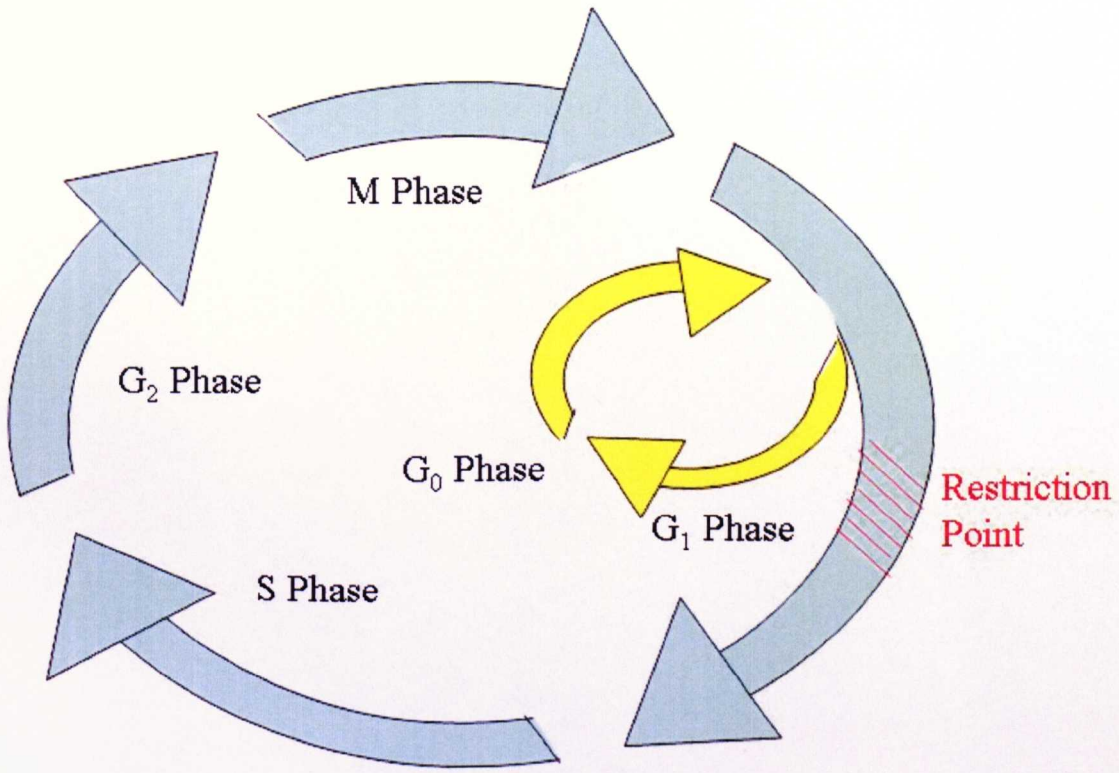

Figure 1.2 The cell cycle

Figure 1.3 The G1 restriction checkpoint

Schematic diagram showing the relationship between the cell cycle regulators and the phosphoyrlation of RB. The yellow arrow indicates a positive response on the following activator, while the pink arrow represents an inhibitory response.

Adapted from Stem Cells 2001;19: 1, Pages 88 -91, E.D. Israels & L.G. Israels, The Cell Cycle

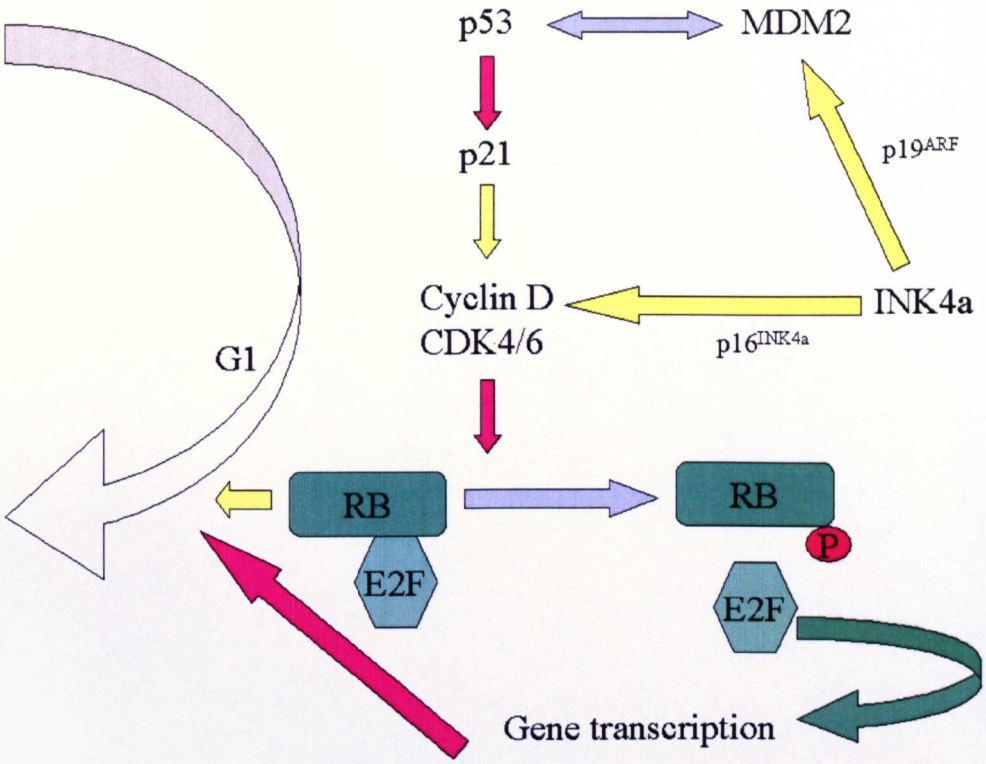

Figure 1.3 The G1 restriction checkpoint

Figure 1.4 The structure of the INK4a locus

The splicing pattern to generate the 2 proteins is shown below the schematic. Note that $p14^{ARF}$ and $p16^{INK4a}$ are translated from exon 2 in different reading frames and terminate in different exons. $p14^{ARF}$ is terminated in exon 2 while p16 stops in exon 3.

Adapted from Voorhoeve, P. M (2003) The tumour-suppressor functions of the human INK4A locus, Cancer Cell, 4; 4: 311 – 319

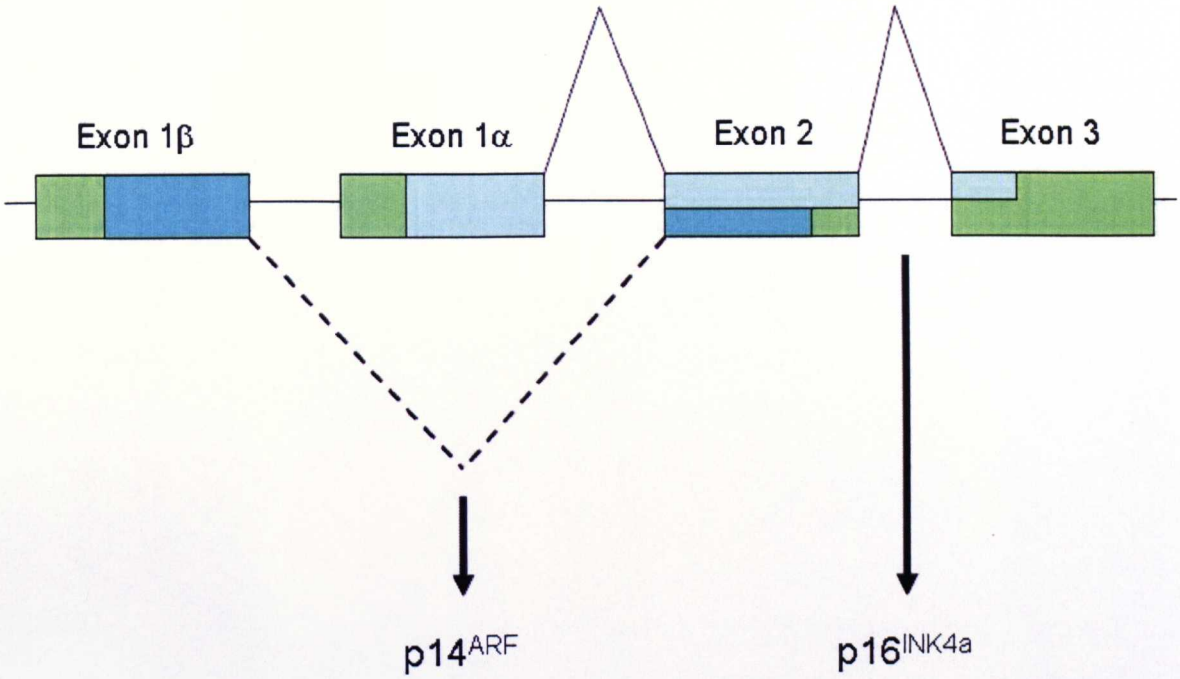

Figure 1.7 The structure INK4a locus

1.2.2 Mortality checkpoints

Cellular senescence can be divided into 3 components: mortality stage 0 (M0), mortality stage 1 (M1) and mortality stage 2 (M2) (see figure 1.5) (10). In culture normal diploid cells will undergo a number of population doublings before they arrive at a barrier to proliferation. As a cultured epithelial cell approaches senescence it accumulates p16 and reaches M0. This checkpoint is dependent on p16. If a cell has dysfunctional p16 it can bypass this barrier and continue growing until it reaches M1 or senescence (14). At this stage the checkpoint is dependent on p53 and pRB. Disruption of these proteins allows the cell to proliferate until M2 or crisis (13). Unlike senescence, there is cellular proliferation during crisis (15), however, there is also a high rate of cell death resulting in no net growth in the cultures. M2 can be overcome if telomerase is reactivated or if the telomeres are extended due to ALT (Alternative Lengthening of Telomerase) (16). If a cell can reach this point, it is immortalised. It has been noted that the effects of the cell cycle regulators involved in the senescence checkpoint can be overcome by the effect of oncogenic agents, such as SV40 T antigen, or papilloma virus E6 and E7 proteins. In addition, mutations in the key regulators of the checkpoint, p53, pRB and p16 are common in many different tumour cell types.

Figure 1.5 Models of senescence and immortalisation.

Graph showing the growth arrest checkpoints M0, M1 and M2 in relation to MPD, the alterations that must take place for the cells to escape from these checkpoints and the outcome for those cells which do escape.

Adapted from European Journal of Cancer, 1997, 33; 5: pages 703 – 709, The Biology of Replicative Senescence.

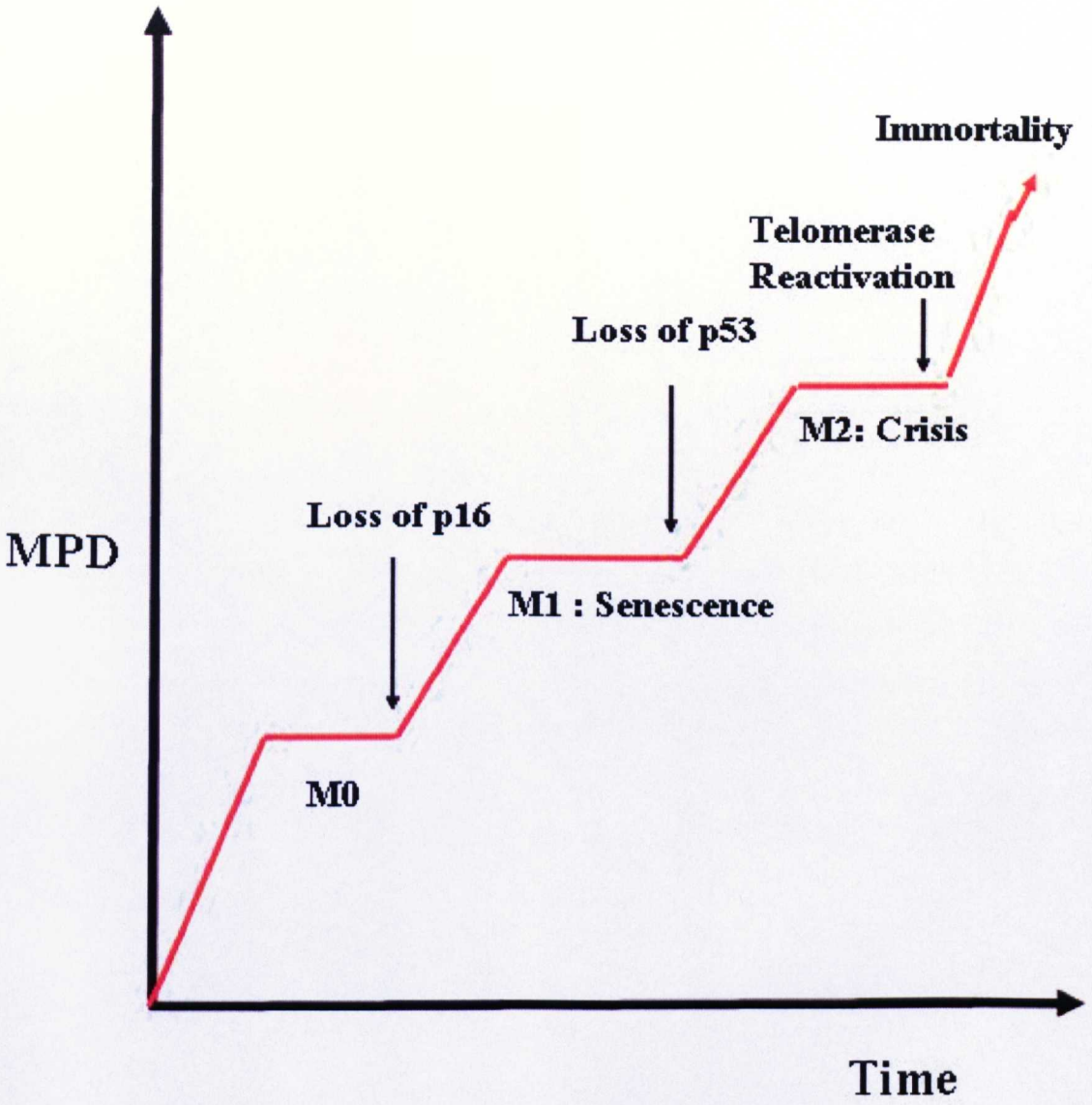

Figure 1.5 Models of senescence and immortalisation

1.2.2.1 M0 checkpoint and p16/p19ARF regulation

As indicated above, p16 is coded on the INK4A locus along with $p19^{ARF}$ (12). These are a family of CDK inhibitors with p16 inhibiting CDK4/6 and $p19^{ARF}$ by binding to MDM2 preventing the destruction of p53. This results in an increase in p53 which then leads to an increase in p21 due to the inhibition of CDK6 and CDK4. The inhibition of CDK4/6 by p16 prevents phosphorylation of RB and the cell remains in G1 and cannot replicate (17). If p16 is inactivated by methylation or is mutated (18, 19) the cells will continue proliferating and bypass M0 to go on to M1.

1.2.2.2 M1 and p53/RB regulation

RB is known as the "gatekeeper" protein in cell cycle regulation (12, 17). RB is a pocket protein which when unphosphorylated binds to E2F. While E2F is bound to RB it cannot bind to DNA and activate E2F responsive genes. RB can be phosphorylated by CDK4/6 and the cyclin D complex. This results in the release of E2F and the cell can continue through the cell cycle. Transcription factor p53 is another important regulator and is known as "guardian of the genome" (13). In the presence of DNA damage, p53 interrupts the cycle and allows time for the DNA repair mechanisms. This is achieved by p53 inhibiting RB phosphorylation and preventing transcription. In M1, if RB and p53 normal function is blocked, for example by introducing SV40 T-antigen into the cells, then the cells will continue to proliferate.

1.2.2.3 M2 and telomerase

By the time a cell has gone through M0 and M1 there has been a great amount of genetic alterations and many cell cycle regulators are lost (12). Throughout this proliferative period the telomeric repeats at the ends of the chromosomes will become increasingly shorter (discussed in detail later). At M2 the telomeres reach a critically short length and the majority of cells have entered crisis. Crisis is different from senescence as there is continued growth (15). However, this is accompanied by apoptosis so that there is no net increase in cell number. Most cells will stay in a state of crisis but a rare cell may bypass this by reactivating telomerase and increasing the length of its telomeres. These cells will now become immortal.

1.3 Telomeres

1.3.1 Telomeric DNA and replication

Telomeres are structures at the ends of eukaryotic linear chromosomes (see figure 1.1). They derive their name from the Greek word *telos* meaning end and *meres*, a component. They consist of the simple, repetitive sequence of TTAGGG (20), varying in size from 6-26 kilobase pairs in humans and other vertebrates, together with specialised proteins, and are highly conserved throughout evolution (21). The presence of such structures was predicted before they were identified by Olovnikov (22) and Watson (23) since it had been already shown that, before a cell divides, it must replicate its DNA. However the telomeres at the ends of the chromosomes are not completely replicated due to the 'end replication problem' (see figure 1.6). DNA polymerases replicate DNA in a 5' to 3' direction only and require an 8-12 base pair RNA primer to initiate DNA synthesis. The replication of the leading strand is continuous but the lagging strand is synthesized by the ligation of short DNA fragments (Okazaki fragments). The removal of the RNA primers at the 5' ends of the newly synthesized strand results in a short single stranded 3' overhang in the other strand. The progressive loss of telomeric sequences continues each time a normal somatic cell divides until a critical length is reached and the cell will no longer divide i.e. has reached senescence (24-26). In addition, telomeric sequences may also be lost by exonuclease digestion (27) and oxidative damage (28). The constant shortening of the telomere results in the telomere t-loop being unable to form. The telomere can no longer protect the ends of the chromosomes and these results in chromosomes fusing and degrading. The result of this is that the cells enter growth arrest (29).

The end replication problem is overcome by germ cells and the majority of cancer cells by the activation of the enzyme complex telomerase, which maintains telomere lengths above a threshold and allows the cells to proliferate indefinitely.

Figure 1.6 The end replication problem

A. DNA polymerase replicates DNA in a 5' to 3' direction utilising an RNA primer. The leading strand is replicated continuously whereas the lagging strand is synthesised from short DNA fragments (Okazaki fragments) which are subsequently joined together by DNA ligase.

B-C. Removal of the RNA primer at the 5' end of the newly synthesised strand results in a 'gap' creating a 3' overhang in the complimentary strand.

D. During each cycle of DNA replication there is progressive shortening of the 5' ends (red and green) which contributes to the shortening of telomeric sequences in the absence of telomerase.

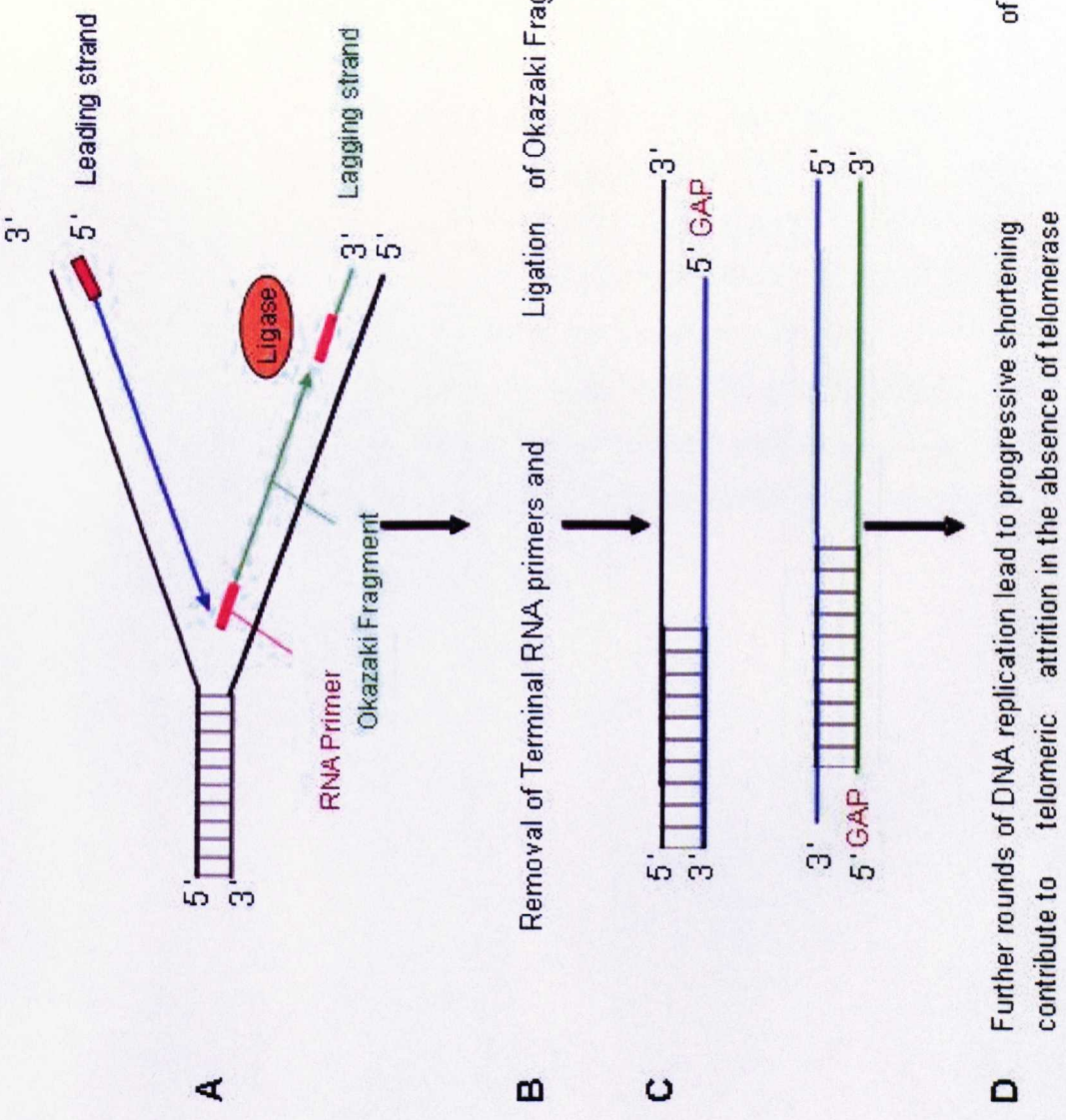

Figure 1.6 The end replication problem

1.3.2 Telomere function

Telomeres are capping structures which protect the ends of the chromosome against degradation and prevent them from being recognised as double strand breaks (DSB) by the DNA repair machinery (21, 30). Their other functions are in preventing the loss of heterozygosity (31), chromosomal translocations (32), deletions and loss of information, all of which could lead to cell death (33). Without telomeres, the ends of chromosomes are subject to the same degradation or random fusion which leads to activation of the DNA repair system and disruption of cell cycle checkpoints. Short dysfunctional telomeres are interpreted by the cell as a DSB and fuse resulting in dicentric chromosomes. The dicentric chromosomes are replicated during S-phase and result in anaphase bridging during mitosis. The dicentric chromosome then breaks during telophase and can enter into a breakage fusion breakage (BFB) cycle in further rounds of replication (see figure 1.7). In normal somatic cells these DSBs would be recognised by the DNA repair machinery and repaired or the cell would undergo apoptosis. However in cancer cells which may have lost cell cycle checkpoints (e.g. p53 and pRB) the increase in DSB and BFB cycle contributes to genomic instability and the gains and losses associated with cancer. It can be seen that telomeres provide the major role in protecting the coding DNA in chromosomes.

Figure 1.7 Telomeric erosion and the generation of dicentric chromosomes and anaphase bridges

As cells divide in the absence of telomerase, the telomere shortens as depicted in red (A). This exposes the ends of the chromosomes and these are seen as double strand breaks (DSB) by the DNA repair machinery. They fuse to form a dicentric chromosome e.g. fusion of two sister chromatids (green and purple) (B). The dicentric chromosome is replicated during S-phase (C) and these results in anaphase bridging during segregation in mitosis (D and E). The dicentric chromosome breaks when pulled to opposite spindle poles resulting in a double strand break [DSB (F)] it causes in changes in gene dosage [amplifications –amps and deletions –del (G)]. The broken chromosome must be repaired again and can enter a breakage fusion breakage (BRB) cycle and facilitate the genomic instability associated with cancer.

Taken from Checkpoint Controls in Cancer 2004 p344

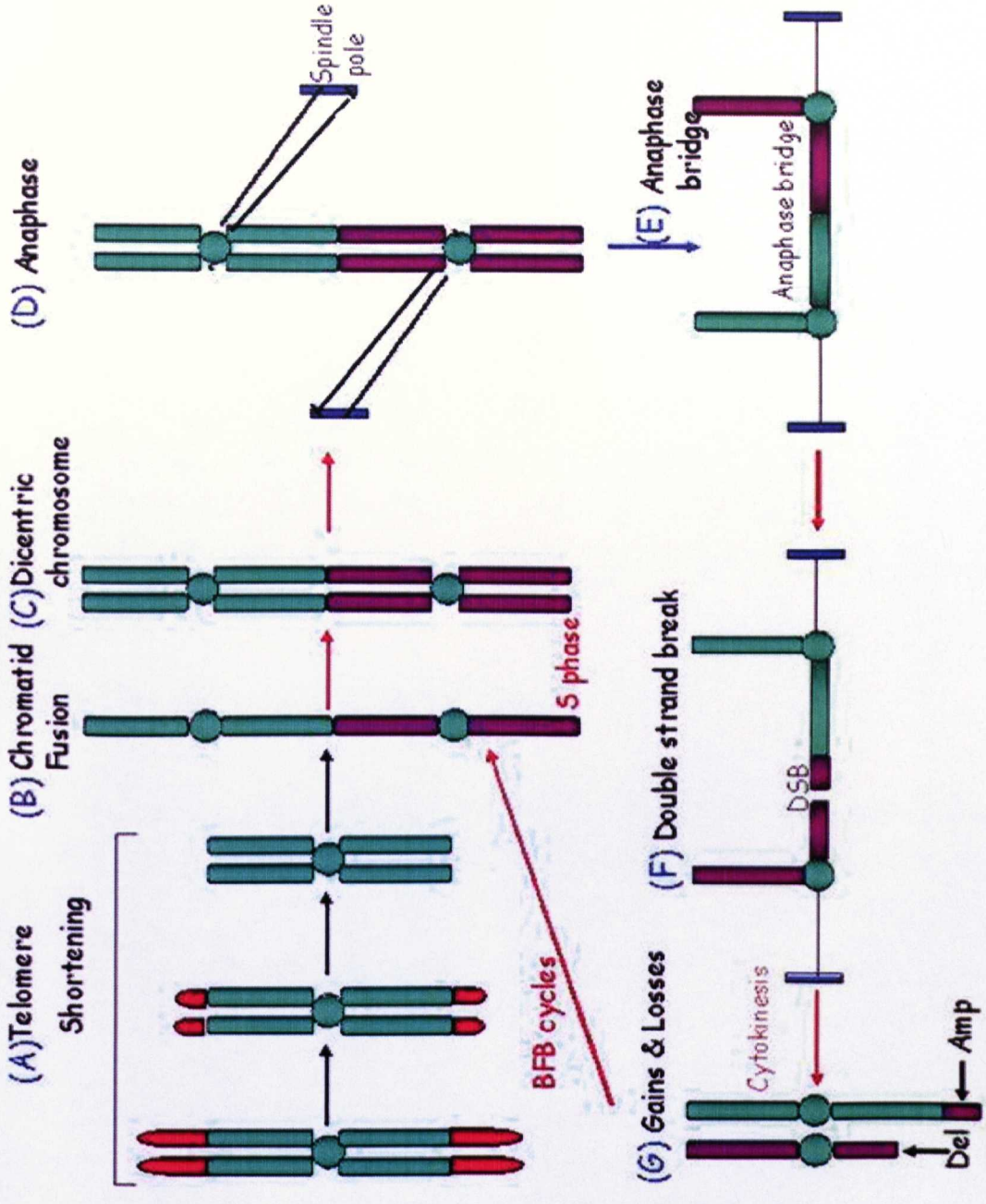

Figure 1.7 Telomeric erosion and the generation of dicentric chromosomes and anaphase bridges

1.3.3 Telomeric structure

It can be seen from the above that the structure of telomeres is very important in maintaining genomic stability. Their ends are arranged in a large loop called the telomeric t loop (see figure 1.8). Electron microscopy has shown that the loop can be several kb large while indirect evidence has suggested that the 3' overhang is buried deep within the junction between the t loop and the tail (34). It is thought that this structure prevents the 3' overhang from being seen as a DNA break and therefore degraded. The single stranded 3' overhang invades and hybridises with a region of double strand telomeric DNA which displaces genomic DNA to form a structure called a D-loop. The formation of t loops depends critically on TRF2 and disruption of its function can cause tumour cells to die. The disruption of the t loop structure results in critically short telomeres, leading to cell death (35) or senescence (36).

1.3.4. Telomere-associated proteins

Many proteins associate with telomeres to differing degrees. The two proteins which are involved exclusively in mammals are TRF1 and TRF2 (37, 38). These bind the double stranded telomeric DNA. TIN2 and hRAP1 localise to telomeres but this is through binding to TRF1 or TRF2 (39, 40). It is thought that these four proteins have a role in maintaining in regulating the length and structure of the telomere. Some proteins which are associated with telomeres also localize to other sites. An example is TANK1 (also known as tankyrase). This is a poly-ADP ribosylase which is associated with DNA repair. Recently it has been discovered that this also interacts with TRF1 under certain circumstances (41). Other proteins which are involved with double strand breaks have also been localised to TRF1 including Ku (42). Ku is essential for repair of double strand breaks by non-homologous end binding. It is thought that this protects the terminal telomeric structure. As yet only a few mammalian telomeric-associated proteins have been identified, however, the extensive list of yeast telomere-associated proteins suggests that this list is still to be expanded (43).

Figure 1.8 Telomeric t-loop and associated binding proteins

Schematic diagram of the telomere structure and associated proteins. Most of the telomeric DNA is packed into nucleosomes, however the end is attached to associated proteins. The basic t-loop and d-loop structure is seen in A and the placement of associated proteins in B.

Adapted from EMBO Reports, 2002, 3:12; pages 1139-1145, Rhodes et al, Telomere architecture

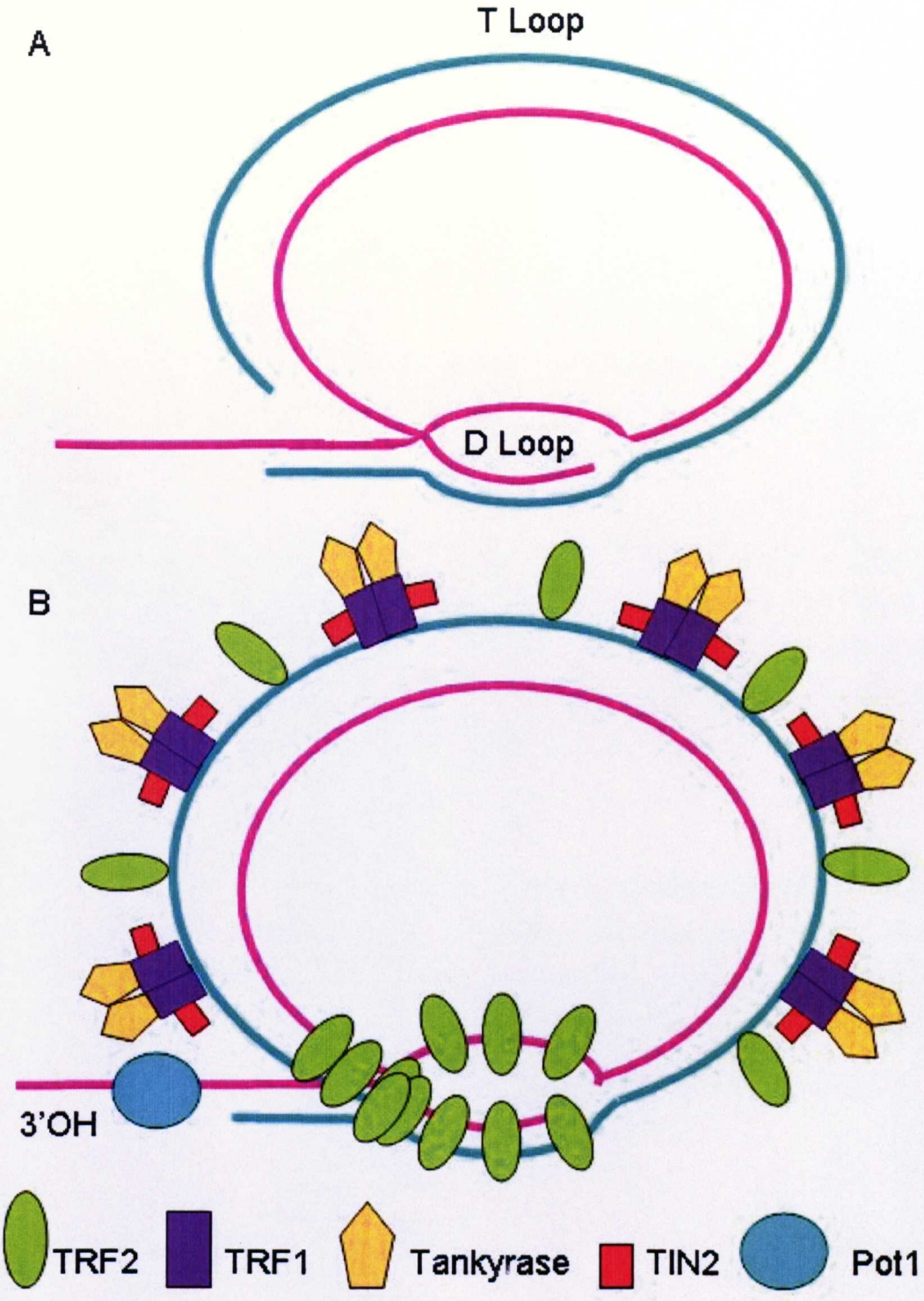

Figure 1.8 Telomeric t-loop and associated binding proteins

1.3.5 Telomere length and cellular lifespan

There are hundreds of thousands of telomeric repeats in mammalian cells and these correlate with the life span of the cell (30, 44, 45) . Cells with longer telomeres undergo more divisions than those with shorter telomeres. Telomere shortening is also seen *in vivo* - in a variety of tissues, telomeres are shorter in older individuals than in younger ones (46-48).

Regulation of telomere length in mammalian cells is complex. Human sperm telomeres are longer than those in somatic cells. Length also differs between normal somatic and malignant cells. The average telomere length in colorectal carcinoma and ovarian carcinoma is shorter than that in the corresponding normal tissue from the same individual (6).

There is also variation between species: In mice telomere restriction fragment lengths up to several hundred kilo base pairs are found (45, 49). The length is similar in different tissues in the newborn mouse, but differs in the tissues of the adult. It is interesting that immortalisation of primary mouse cells in culture appears to be relatively easy compared to its achievement in human cells and this may reflect the presence of telomerase in the tissues (see later).

Regulation of proliferation is thought to play a role in protection against carcinogenesis: Cancer incidence increases with age in keeping with a requirement for "multiple hits" in its development. Long-lived species may have more complex control mechanisms to limit cellular proliferation than short-lived ones (50, 51). Inhibition of telomerase activity may be one mechanism which exists in humans, to limit replicative potential (52).

1.3.6 Telomere attrition

There are thousands of the repeats of the sequence TTAGGG in human cells but the mean telomere length decreases by 50 - 150 base pairs at each division (attrition). As previously described, a cell will replicate until the telomeres reach a critical short length when a damage signal is triggered which will induce senescence. Not all telomeres in the cell will be shortened to the same length but it is the shortest telomere, rather than the average telomere length, which determines whether the cell continues replication or enters senescence (45, 53, 54). The mechanism for this is not completely understood but it is thought to involve the uncapping of the telomere since an uncapped telomere can trigger cell cycle arrest or apoptosis. Short telomeres are seen as uncapped. Uncapping of telomeres can be induced by disruption of the telomere structure and this can be achieved by expression of a dominant negative form of TRF2 REF. In contrast, synthesis and capping of the telomeres can be induced by telomerase expression and the dynamic state of telomeres can then be maintained.

Another proposed mechanism by which shortening of the telomere may signal growth arrest, is that the short telomere is recognised as a DSB and this induces a p53 dependent growth arrest (24, 55, 56)

1.3.7 Telomere hypothesis

There has been great interest in the telomere hypothesis of cellular aging. This hypothesis assumes that telomerase, the enzyme responsible for maintaining telomere length, is active during gametogenesis (allowing for long-term viability of the germ cell life) but is repressed during somatic cell differentiation, accounting for the loss of telomeric DNA associated with the finite replicative capacity of these cells (25, 57). Thus telomere length and telomerase activity provide valuable biomarkers of the replicative capacity of cells and may be causally involved in cellular aging, immortalisation and neoplasia (58).

Two key assumptions underlie the hypothesis:- (1) a small amount of DNA from telomeres is lost with each round of DNA replication and (2) deletions accumulate, eventually leading to cellular senescence. With regard to germ line and tumour cells, which are immortal, the hypothesis proposes that special mechanisms exist in them to prevent deletions and senescence (10).

1.4 Telomerase

1.4.1 Definition

Telomerase is a reverse transcriptase ribonucleoprotein which synthesises telomeric DNA onto the ends of chromosomes. It copies an RNA template into DNA but is unusual in that it contains its own RNA template as an integral part of the enzyme. The enzyme was first discovered in Tetrahymena (59) but was later found to be present in all eukaryotes, and although varying between species, it showed conservation of structure and function (60).

Telomerase is comprised of 2 subunits: hTER which is the RNA component acting as a template for the polymerase activity of the enzyme, and hTERT which is the catalytic component with reverse transcriptase activity (61). Both components are required *in vitro* to reconstitute telomerase activity. However it is thought that hTERT is the rate-limiting step in telomerase activity and for this reason hTERT gene expression and regulation is now at the forefront of telomerase research. Telomerase is expressed in germline cells and in developing tissue: During some point in development, thought to be at differentiation, the cell will switch off the expression of telomerase (62).

1.4.2 Isolation, identification and measurement of telomerase Activity

Telomerase was first identified by Greider and Blackburn (59) who reported a novel activity which added TTGGGGG repeats onto synthetic telomere primers in Tetrahymena cell-free extracts. They proposed that terminal transferase was involved in the addition of the telomeric repeats necessary for the replication of chromosomal ends in the eukaryotes.

Over the next few years these two workers and other colleagues set out to search for telomerase in various species, including humans (57). They identified telomerase in many single-cell organisms but, to their surprise; found that many human cells lacked the enzyme. Greider, Harley and co-workers examined normal human somatic cells, however, and then realised that they lost segments of their telomeres as they divided in culture, in keeping with absence or inactivity of telomerase. The exception was germ line cells, in which the telomeres remained intact and telomerase activity was detectable (25, 48).

Although reduction of telomere length and proliferative capacity suggests a major involvement in human aging, it may not be the main factor. A different suspicion as to its primary function, has been raised i.e. could the loss of proliferative capacity observed in human cells lacking telomerase have endured not to age us but to help us to avoid cancer? Indeed work has revealed that telomerase can be identified in most human cancer cell lines and that, in contrast to most normal human cells, cancer cells can express telomerase, retain their telomeres and have the potential to survive indefinitely (6). This is discussed below (1.3.4.1).

The increasing interest in telomerase activity demanded the development of a specific, sensitive and reliable method for its extraction and detection in tissues and cells. Such an assay was first described by Kim and colleagues (63) and then refined by Piatyszek and co-workers (64). In brief, the method was PCR-based and used a single tube reaction in which telomerase first synthesised extension products, followed by the use of these products as templates for PCR amplification. The refined method was designated the TRAP (telomeric repeat amplification protocol) assay and shown to give high sensitivity and a faster result.

Although it is a one tube PCR-based protocol, it is performed in two steps: (1) telomerase-mediated extension of an oligonucleotide primer (TS) which serves as a substrate for telomerase and (2) hot start PCR amplification of the resultant product (an incremental 6-nucleotide single-stranded DNA ladder) with the oligonucleotide primer pair, TS (forward) and CX (reverse). The oligonucleotides were designed to minimise primer-dimer artefacts. Extraction is by a gentle detergent-based lysis. The TRAP assay has been of inestimable help in measuring telomerase activity in various tissues, acting as a significant "marker". In future this may be of diagnostic and prognostic value, including in therapy where the presence of telomerase could be established before therapeutic inhibition by anti-telomerase is used (see figure 1.9)

Figure 1.9 Diagram of the TRAP assay

In the first step tissue or cell extracts to be tested are incubated with an end-labelled oligonucleotide (TS Primer) for 30 minutes at 30°C. If telomerase activity is present in the extracts then TTAGGG repeats will be added to the labelled primer. The second step is PCR amplification of these extended products. These products can then be separated by eletrophoresis on a polyacrylamide gel and the characteristic 6bp ladder is observed in telomerase positive extracts.

Taken from Checkpoint controls and Cancer Vol 2 2004 Page 335

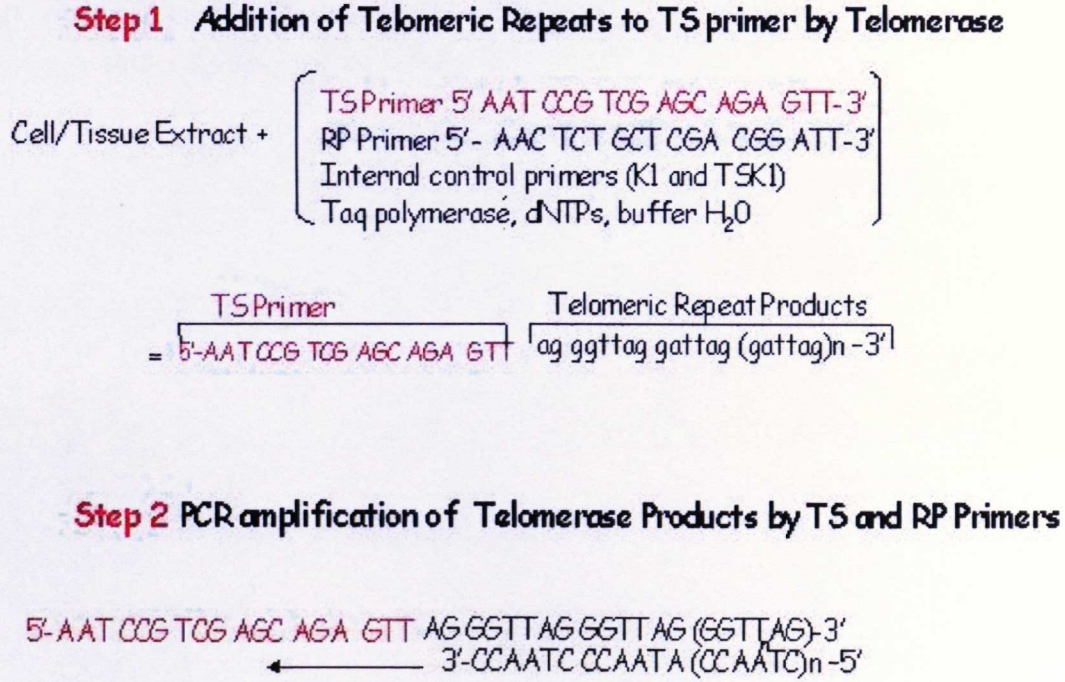

Figure 1.9 Diagram of the TRAP Assay

1.4.3 Structure

The active telomerase complex contains several protein components as well as an RNA subunit, the catalytic component TERT (telomerase reverse transcriptase). The catalytic subunit has been cloned from a variety of different species and is a specialised reverse transcriptase (RT) that uses an associated RNA to provide a template for DNA telomeric repeats (21). In humans it is now known as hTERT but it has also been called HTCS1, hTRT, hTCS1, TP2 and hEST2. TERT proteins vary in size ranging from 103-134 kDa and all share sequence motifs in common with conventional RTs, as well as unique sequence features in keeping with their telomerase activity (45). Their polymerisation activity is restricted to copying a short sequence of RNA template repeatedly, a function made use of in the TRAP assay (64).

The intrinsic RNA component (TR, telomerase RNA) is associated with several protein components as well as TERT (65). All TRs contain a characteristic sequence complementary to one unit of the telomeric repeat. Over 30 TRs have been cloned varying in size from 159 nucleotides in Tetrahymena to 1300 nucleotides in yeast. Human and mouse TR are about 400 nucleotides in length. TRs show low sequence homology but share a conserved secondary structure consisting of a stem, a pseudo-knot and a set of stem-loop structures.

In addition to the TERT and TR which form the catalytic core of telomerase, several other proteins have been identified in different species (45). These lack structural and primary sequence conservation. For instance, TP1 in mouse and human are dissimilar to TLP1 in rat which is a large protein (230-240 k Da) binding TR. TP1/TLP1 is also a component of vaults, ubiquitous cytoplasmic ribonucleoprotein particles of unknown function. Indeed, the function of the telomerase-associated proteins is not known but they probably act to regulate its activity. Chaperones p23 and hsp90 also associate with the human telomerase complex by binding to TERT: and *in vitro* and *in vivo* studies reveal that their role is essential in assembling active telomerase in humans.

1.4.4 Function

It appears that telomerase is expressed routinely by cells of the germ line in the developing embryo. Once cell differentiation occurs in the body, however, telomerase is repressed in many somatic cells with resultant telomere shortening. When telomeres reach a critical length, the uncapped telomere is thought to provide a signal which stops further replication and then the cell becomes senescent. It has been demonstrated that if telomerase is switched on in senescent cells then the cell can elongate its telomeres and re-enter the cell cycle and proliferate (66, 67).

If, however, cancer-promoting genetic mutations have occurred which block the danger signal, cells will bypass senescence and continue to divide (6). These cells will also presumably continue to lose telomeric sequences and undergo chromosomal changes which may allow further carcinogenic mutations to arise. However as the telomeres reach critically short lengths the cells will reach a point at which crisis occurs and they die. If, however, in the precrisis period cells can activate telomerase, the cells will maintain their telomeres: they will be kept, albeit shortened, and although genetically disturbed will continue to replicate showing the immortality characteristic of cancer (other features are discussed below).

Apart from germ cells and most malignant tumours, low telomerase activity is found in some normal somatic cells including stem cells in renewable tissues, e.g. intestinal crypts and the basal layer of the skin, haematopoietic tissues and activated lymphocytes (68). The telomerase activity is low and, although it provides for an increase in proliferative capacity, it still does not prevent telomere shortening. In contrast to human tissues, significant telomerase activity is present in most normal mouse tissues suggesting different regulatory mechanisms (45).

1.4.5 Telomerase knockout mouse

Different species, like different cell types, have varying lengths of telomeres. Mice have telomeres which are three times longer than human telomeres. For this reason the generation of a telomerase knockout mouse was a great achievement (69-72). First the knockout mouse lacking the RNA component of telomerase was created and then it was allowed to reproduce for six generations until short telomeres were achieved. This animal model supports the telomere hypothesis: It has an aged phenotype, there is an increase in grey hairs, skin lesions and alopecia with decreased body mass, impaired wound healing, infertility, abnormalities in the gastrointestinal tract and defects in the haematopoietic system.

1.4.6 Role of telomerase in certain diseases

Telomerase activity has been studied in various human diseases i.e. in several types of malignancy, especially colorectal carcinoma (73); in cirrhosis of the liver, where there is severe tissue damage accompanied by continuous regeneration (74); in AIDS (75) and in rare diseases like dyskeratosis congenita where the telomeres are abnormally short (76, 77)

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1.4.6.1 Neoplasia

Telomerase activity is found in 95% of all malignant tumours (57) but it is very important to realise that this is not what makes normal cells malignant. Telomerase expression only allows the cells to grow indefinitely: the transformation to malignancy has already occurred. Expression of telomerase may, however, contribute to the severity and outcome of the disease since it allows mutated cells to continue dividing and accumulate more genetic abnormalities including those leading to metastatic growth and angiogenesis, in other words, adding to the malignant features of the original tumour (50, 73, 78). The high incidence of telomerase expression in cancer has promoted the idea of using anti-telomerase therapy. Since most normal somatic tissues do not express telomerase, inhibiting telomerase may provide a strategy for the treatment of cancer (79-81).

1.4.6.2 Cirrhosis of the liver

Cirrhosis of the liver affects several hundred million people every year. The main causes of the disease are chronic viral hepatitis, followed by alcoholism. Damaged liver will

regenerate to a degree but fibrous scarring and architectural distortion set in. Once the normal anatomy is badly disrupted liver failure ensues. At this point the only hope for a patient is a liver transplant. As would be expected, the length of telomeres in the cirrhotic liver is much shorter than those in a healthy individual (82). To test whether or not this feature was contributing to the disease state, an experiment was carried out using telomerase knockout mice (82, 83). Cirrhosis was induced in animals by one of three mechanisms: chemicals, surgical damage or due to a genetic disorder. This was achieved by subjecting mice to repeated exposure to hepatoxins such as CCl_4, or removing two thirds of the liver or using a genetically engineered mouse, an albumin-directed urokinase plasminogen activator (Alb-uPA) mutant. Alb-uP expression has been shown to cause widespread death of hepatocytes and liver failure in new-born mice. It was found that, compared to controls, the mTR-/- mice had a more severe phenotype of the disease and could not reconstitute the liver effectively. To verify that cirrhosis in each case was in fact due to the lack of telomerase and short telomeres, the mice were infected with an adenovirus containing mTR. The mice with short dysfunctional telomeres now had telomerase activity and telomere function: the damage to the liver was reversed, liver function improved and disease progression slowed.

1.4.6.3 Dyskeratosis congenita

Dyskeratosis congenita (DKC) is a very rare genetic disorder characterised by abnormalities in skin pigmentation, nail growth, learning difficulties, pulmonary disease, greying and hair loss. There are also abnormalities of the gastrointestinal tract, hypogonadism, osteoporosis and an increase in epithelial and haematological cancers. Death usually occurs by the age of 16 years, although there have been cases of patients living until 50 years old (76). DKC is caused by a genetic defect which results in the absence of a protein called dyskerin. Dyskerin is expressed ubiquitously and thought to function in the formation of ribosomes in the nucleolus, as well as leading to reduction in levels of hTERT, the RNA template of telomerase. As a result of the latter, sufferers from DKC have abnormally short telomeres explaining why the disease shows characteristics of advanced aging. Death is usually due to bone marrow failure or malignancy. This disorder highlights the importance of telomere length in the replicative lifespan of cells in normal development (77).

1.4.7 The telomerase catalytic subunit (TERT) and reconstitution of telomerase activity

Cloning of the mouse telomerase component was reported in 1997(84, 85). A mouse genomic TERT was identified by hybridisation to a 450-nucleotide (nt) probe from the transcribed region of the human telomerase RNA gene. (This region was 65% homologous with the human RNA gene) (61, 84, 85).

Isolation and characterisation of the catalytic subunit of mouse telomerase or TERT (mouse telomerase reverse transcriptase) an essential component of the telomerase complex, was reported in 1997 (85, 86).

Studies soon showed that reconstitution of telomerase activity could be achieved in normal human cells (66, 67, 87). Retroviral-mediated expression of hTERT resulted in functional telomerase activity in normal aging human cells. In addition, when reconstruction was achieved *in vivo*, elongation in telomeric DNA was identified and cellular lifespan was shown to be increased. Of the greatest importance, transfection of hTERT into normal cells was shown not to lead to malignant transformation.

Bodnar et al (67) transfected skin fibroblasts and retinal pigment epithelial cells also, with vectors encoding the human telomerase catalytic subunit. In contrast to telomerase-negative control clones which exhibited telomere shortening and cell senescence, telomerase-expressing clones had elongated telomeres, continued to divide and showed reduced staining for β-galactosidase (a marker for senescence). Their karyotype was normal and they exceeded their expected lifespan by at least 20 doublings. Again the workers categorically stated that normal human cells were maintained in a "phenotypically youthful state", and they outlined the important applications this could have for research.

Expression of the human telomerase catalytic component hTERT in normal human somatic cells can reconstitute telomerase activity and extend their replicative potential. Jiang and colleagues (88) showed that, at twice the normal life span, telomerase-expressing human skin fibroblasts (BJ-hTERT) and retinal pigment epithelial cells (RPE-hTERT) retained normal growth control in response to serum deprivation, high cell density, G1 or G2 phase blockers and spindle inhibitors. No cell growth was observed in soft agar and no tumour formation *in vivo*. Thus it was concluded that telomerase expression in normal cells does not appear to induce changes associated with a malignant phenotype. However, it has recently been shown that if cells are allowed to grow in vitro for 150 MPDS, it can lead to

the production of a selective neoplastic variant (89). A cell in culture for this length of time results in expanding a cell population by 10^{16}, this is far greater than would ever be required.

1.5 Skeletal muscle

Skeletal muscle is the major tissue in the human body. It consists of long, striated (striped) fibres attached to the skeleton by tendons, usually under voluntary control. The main muscle groups form the pelvic and shoulder girdles, the limb muscles and those of the axial skeleton.

1.5.1 Anatomy

Muscle fibres are composed of multinucleate cells up to 10 cm or more in length with diameters ranging from 10 to 100 μm. They run from one end of the muscle to the other and are polygonal in shape. The main structural features are shown in figure 1.10.

The multinucleated individual muscle fibres are surrounded by a cell membrane, the *sarcolemma* or sarcolemmal membrane, which actually consists of two layers: a plasma membrane attached to the muscle cytoplasm or sarcoplasm (the plasma-lemma) and an outer basement membrane (basal lamina). Between the two layers lie the nuclei of the satellite cells, the cells with proliferative potential (see figure 1.10b and 1.11).

Up to 90% of the volume of the muscle fibre is composed of the contractile elements, the myofibrils. These have a mean diameter of 1 μm and are composed of myofilaments (see figure 1.10c) and thin actin filaments. Together they form the contractile elements of the fibre and, in series, in regular sequence are known as the *sarcomere* (33, 90, 91).

Figure 1.10 The structure of skeletal muscle, illustrating the appearance and organisation.

A. Gross anatomy of skeletal muscle. The muscle is attached to the bone via a tendon and deep fascia. The skeletal muscle consists of 3 layers of connective tissue, an outer epimysium, a central perimysium and an inner endomysium. The perimysium divides the skeletal muscle into a series of compartments which contain the fascicles, the bundles of muscle fibres.

B Diagram of an individual fascicle. Within the fascicle the endomysium surrounds each fibre and attaches fibres to one another. Stem cells are scattered among the fibres to repair any damage.

C. Diagram of the skeletal muscle myofibrils showing a detailed electron micrograph through a skeletal muscle cell. The regular cross striations can be seen. Below is a schematic representation of the single sarcomere showing the interaction between the light and dark bands, and the Z disc and M line.

Adapted from Molecular Biology of the Cell, Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson, J.D, Third Edition p853

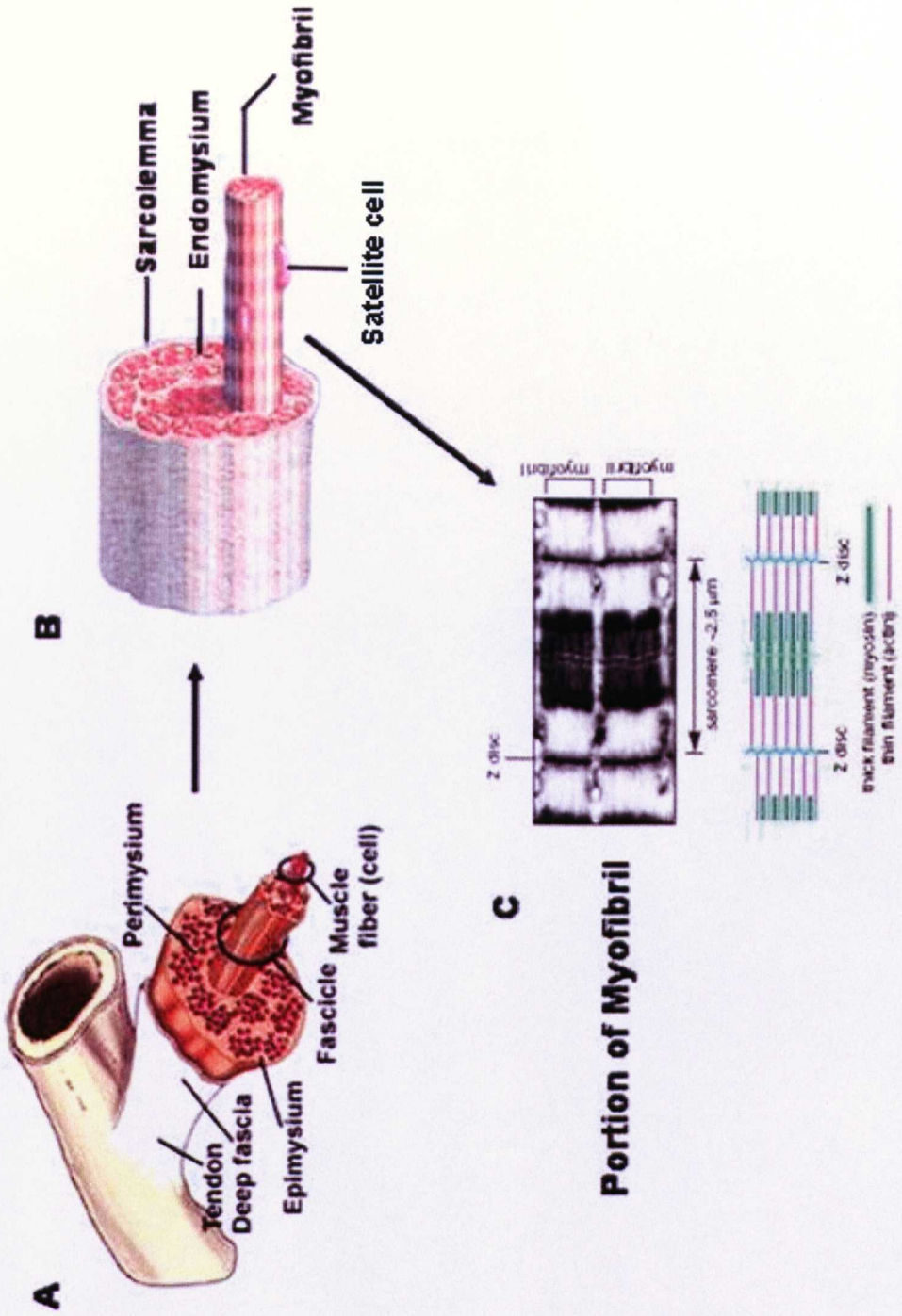


Figure 1.10 The structure of skeletal muscle, illustrating the appearance and organisation.

1.5.2 Function

Apart from the usual organelles (mitochondria, Golgi apparatus etc.) the sarcoplasm contains two specialised internal membrane systems: The transverse (T) tubular system, consisting of invaginations of the surface membrane into the fibre and the sarcoplasmic reticulum which is in close contact with it and forms lateral sacs or *terminal cisternae* at the surface of the myofibril (see figure 1.11). These two interconnected systems are concerned with the excitation of muscle during contraction and relaxation. The "sliding filament" theory describes muscle action (92): an electrical impulse travels down the nerve and reaches the terminal cisternae of the sarcoplasmic reticulum, via the T system. Calcium is released and bindings to troponin on the thin filaments which changes its shape to uncover cross bridging sites on actin. These bind to myosin which has been energised by splitting ATP into ADP + P + energy, by myosin ATPase. The crossbridge bends producing a power stroke which pulls the thin filament inward, shortening the muscle sarcomere i.e. contraction occurs (see figure 1.12).

Figure 1.11 T tubules and the sarcoplasmic reticulum

A. Schematic of the two systems of membranes which relay the signal to contract from the muscle plasma membrane to all of the myofibrils in the cell.

B.Electron micrograph showing two T tubules.

Adapted from Molecular Biology of the Cell, Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson, J.D, Third Edition p853

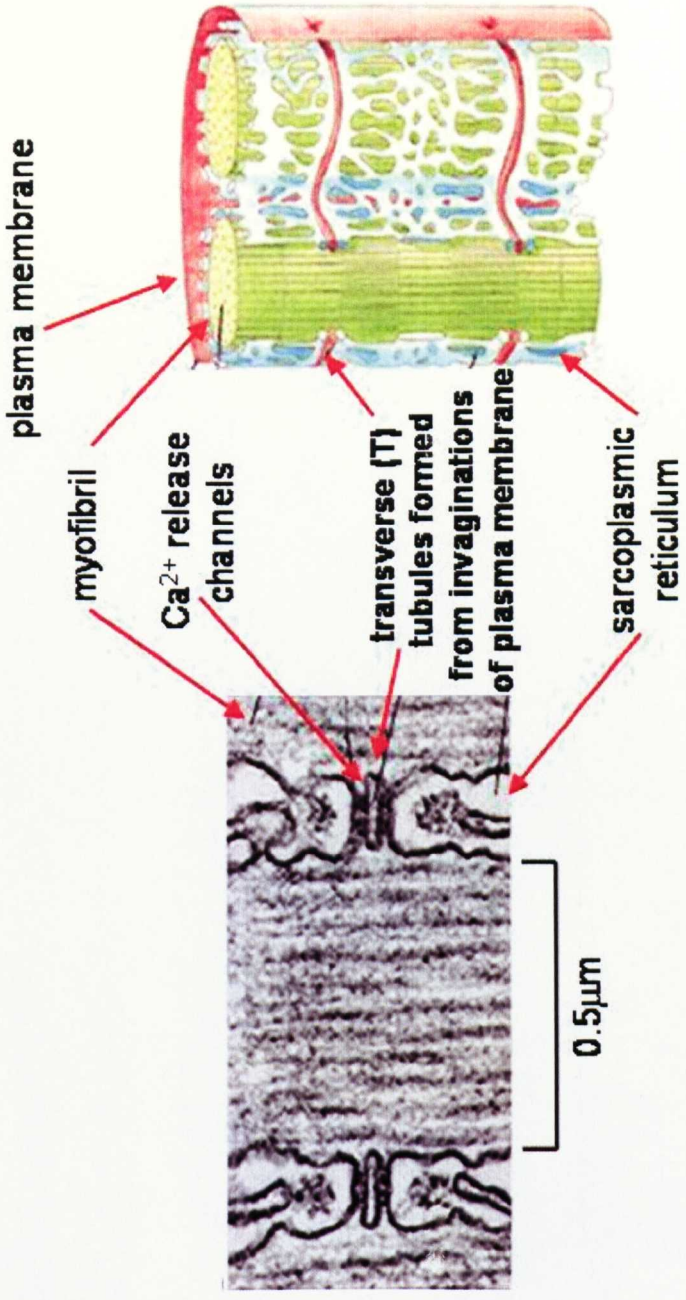


Figure 1.11 T tubules and the sarcoplasmic reticulum

Figure 1.12 The cycle of crossbridging during muscle contraction

A Attached. At the start of the cycle the myosin head is bound tightly to the actin filament. This is terminated by the binding of ATP to the myosin head

B Released. The ATP molecule binds to the back of the head of the myosin and this result in a change in conformation of the head, allowing it to move along the filament.

C Cocked. The cleft closes around the ATP molecule resulting in the head being displaced approximately 5nm along the filament. Hydrolysis of ATP occurs but the ADP and inorganic phosphate (Pi) remain attached to the head.

D Force-generating. The weak binding of the actin to the myosin head causes the release of the Pi, resulting in the bond strengthening. The myosin head is now attached to a new actin site. The release of the Pi results in the power stroke and during this ADP is lost and the myosin head regains its initial conformation.

E Attached. At the end of the cycle the myosin head is again tightly locked to the actin filament in its new position.

Adapted from Rayment I. Structure of the actin-myosin complex and its implications for muscle contraction. Science. (1993) 261; 5117: p 58-65.

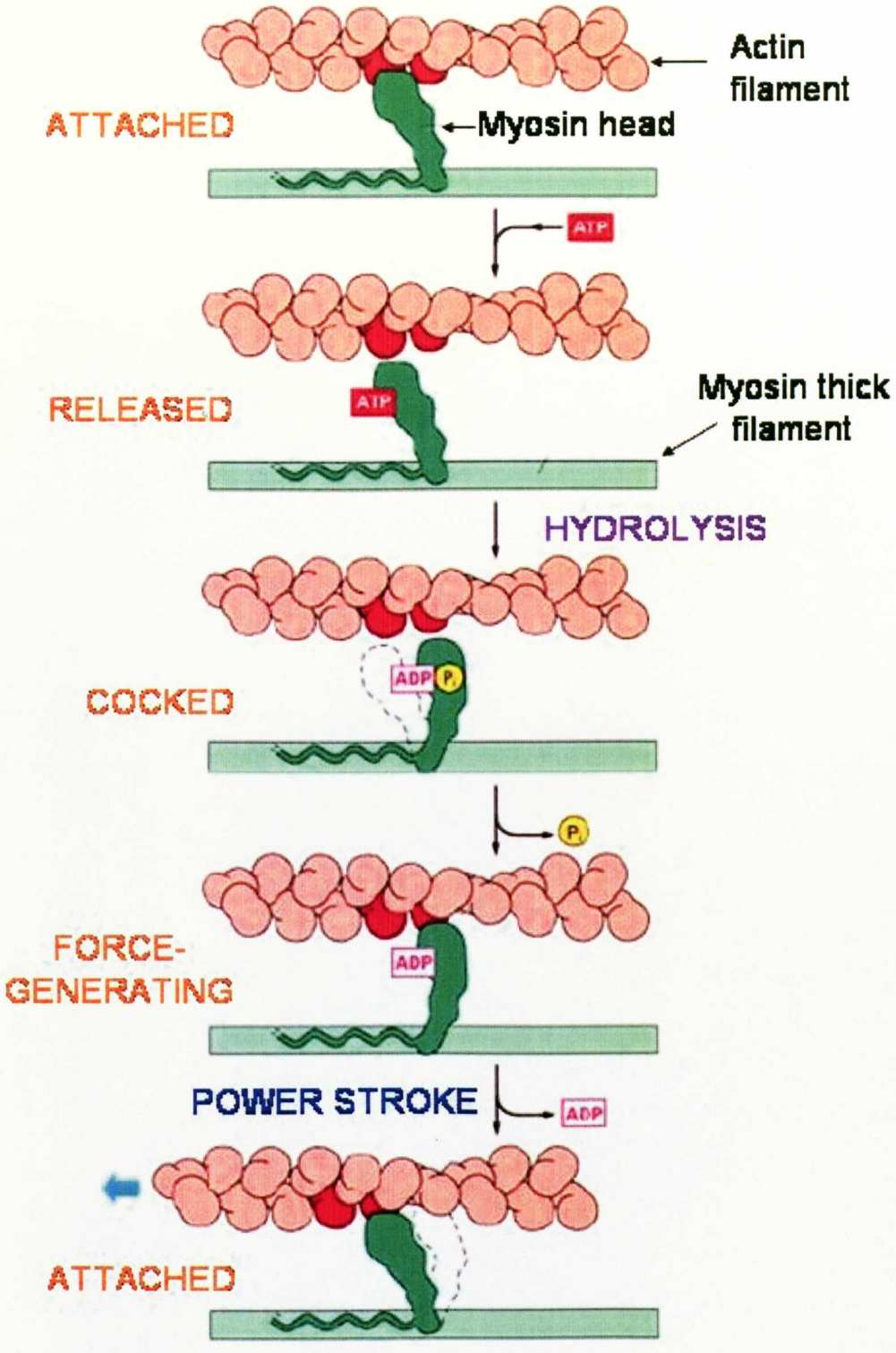


Figure 1.12 The cycle of crossbridging during muscle contraction

1.5.3 Satellite cells

Muscle has a limited proliferative ability dependent on the function of the "satellite" cells (93) These, as stated above, lie between the plasma and basement membranes, usually adjacent to peripheral myonuclei. They have nuclei with dense peripheral heterochromatin and a small amount of cytoplasm with a few organelles but no contractile myofilaments.

They are usually in the G0 phase of the cell cycle but can leave it to enter the mitotic cycle and differentiate to express muscle-specific proteins. Their activation and differentiation is tightly regulated by myogenic regulatory factors of the MyoD family and the cyclin-dependent kinases and their inhibitors (94). These are in turn controlled by growth factors. Satellite cells are obviously key players in muscle fibre growth, regeneration and hypertrophy. There is evidence that they are a heterogeneous cell population in terms of mitotic cycle duration and ability to differentiate.

The numbers of muscle fibres do not increase postnatally and growth occurs by fibre enlargement so that the contribution of satellite cells is critical. There is evidence of age-related alterations with the average number of progeny produced inversely proportional to donor age. Satellite cells from old rats initiate DNA replication more slowly *in vitro* than cells from young or foetal rats and divide less frequently. *In vivo* the number of satellite cells in mouse soleus muscle drops from 4.6% at 8 months of age to 2.4% at 30 months.

Various growth factors have been described as having an effect in culture i.e. basic fibroblast growth factor, insulin-like growth factor, platelet-derived growth factor and transforming growth factor (95).

The process of satellite cell differentiation includes withdrawal from the cell cycle, expression of muscle-specific markers and fusion with one another to form myotubes or with existing myotubes or myofibres. Differentiation is accompanied by decreased apoptosis.

The muscle specific proteins are expressed at specific stages of muscle development (see figure 1.13) (94). The first ones appear before withdrawal from the cell cycle: i.e. desmin, the muscle-specific intermediate filament protein, is seen first. The myogenic transcription factors of the MyoD family, including MyoD, MRF, myf-5 and myogenin are then expressed in order (96). MyoD is the first to appear and marks the onset of terminal myoblast differentiation. Myogenin is the last to appear. Expression of these factors is

followed by cell cycle arrest, then phenotypic differentiation and then cell fusion. As will be seen, satellite cells play a crucial role in DMD.

1.6 Duchenne Muscular Dystrophy

Duchenne muscular dystrophy (DMD) is the most common and most severe of a group of inherited disorders characterised by chronic, progressive muscle wasting and degeneration (97, 98). It is the second most common genetic disorder, affecting 1 in every 3,500 live male births. It leads to a wheelchair existence at the end of the first decade of life and death from cardiac and respiratory failure by the end of the third.

Anatomists in the early 19th century first studied selective involvement of various muscle groups in disease (99). The clinical and pathological features of DMD were described in the mid 19th century, due to the work of the English physician Edward Meryon (1807-1880) and the French physician, Duchenne (1806-1875). Meryon (1851) first described the systemic clinical and pathological findings in the disease, reporting four families with a large number of affected males. He reported the progressive muscle weakness and wasting, beginning in early childhood and leading to premature death. He also stressed the fact that it had a predilection for males and was frequently familial. He identified the disease as a primary muscle disorder and showed on microscopy that, unlike other cases of what was termed "muscular paralysis", the spinal cord and nerves were completely normal while the muscle was replaced with adipose and fibrous tissue.

Concepts of genetics were poorly understood at the time. Although Mendel had published his work in 1866 it was not known to the scientific community until 1900 (100). The similarity between the inheritance of DMD and haemophilia apparently did not strike Meryon although the latter was very well known, and he described cases of DMD simply as familial.

Twelve years after publishing his initial report, Meryon expanded his observations on DMD to form a chapter in his book (101) but it was the French physician, Duchenne de Boulogne who made personal and extensive observations on DMD. He produced excellent photographs of his cases, reported electrophysiological results derived from the apparatus he had constructed and biopsy findings using the muscle biopsy needle which he made. It is his name which is now attached to the disease

Duchenne believed that Meryon, with whose work he was familiar, was describing a different disease (progressive muscular atrophy) although Meryon refuted this in his 1864 book. Indeed, Meryon must be regarded as having more insight into the pathogenesis of DMD because he described it as a primary muscle disorder characterised by what he described as "granular degeneration" of fibres whereas Duchenne thought that the abnormality was in the nervous system and the muscle showed simply hyperplasia of the fibrous and fatty tissue: an effect which is secondary (102).

DMD, and its allelic, less severe variant, Becker's muscular dystrophy (BMD) has attracted intense interest since it was first described and the landmarks in its study are summarised in the Table 1.1 (98).

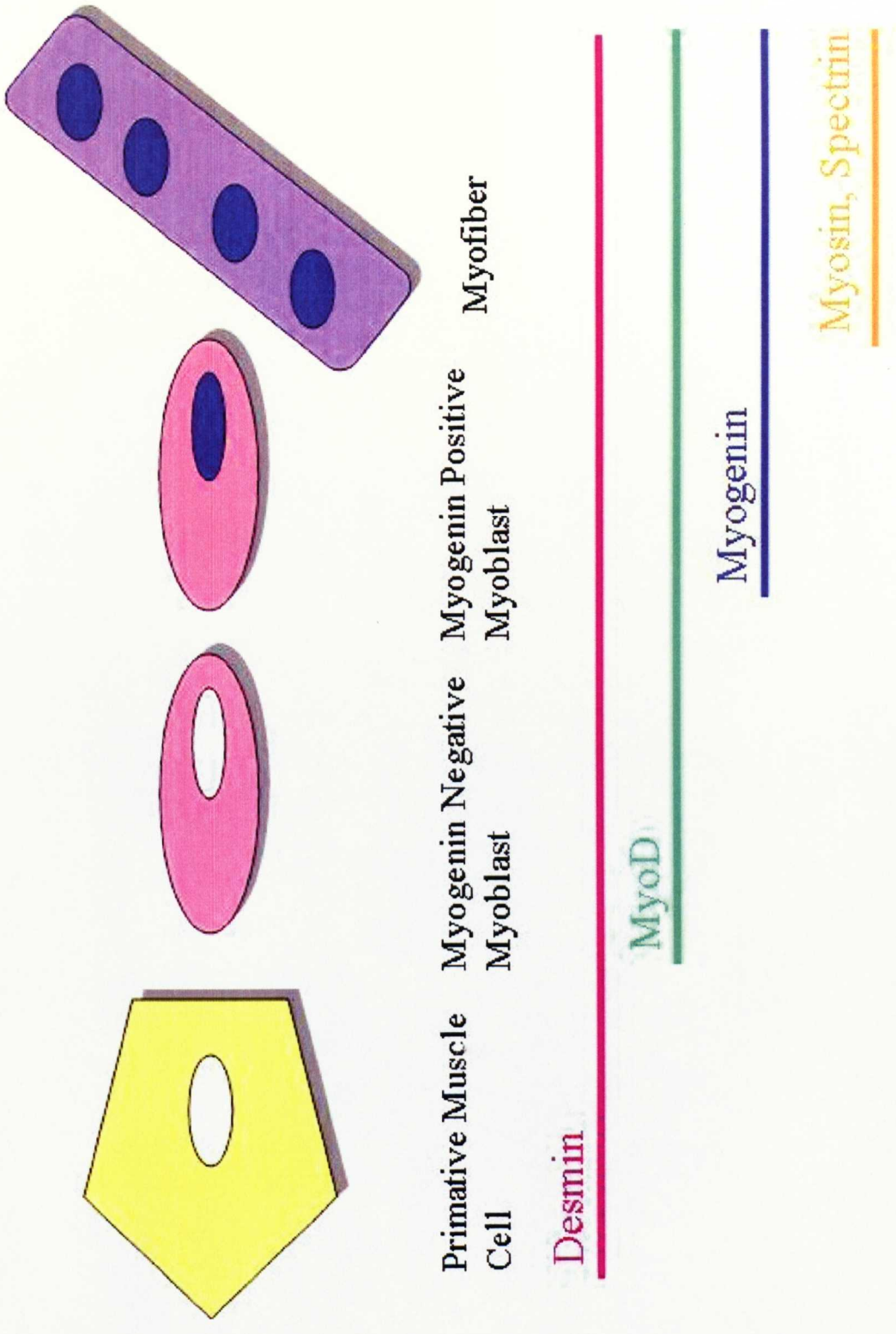


Figure 1.13 Sequence at which muscle specific proteins are expressed

Demonstrates when specific muscle proteins are expressed

Table 1.1 Landmarks in the history of Duchenne and Becker muscular dystrophies taken from The History of a Genetic Disease, Emery & Emery.

1830-	Muscular dystrophy recognised as a disorder and DMD as a specific type of dystrophy
1860-1900	Microscopic myopathology defined
1930-1960	Resolution of heterogeneity by clinical and discriminant analysis BMD defined (1955) X-linked inheritance confirmed by segregation analysis (1959
1959-1960	Serum creatine kinase for diagnosis and carrier detection
1966-1968	Introduction of Bayesian statistics for risk estimation
1975-1980	Defect localised to muscle and other membranes by electron microscopy and biochemistry
1979-1982	Gene localised to Xp21 Linked RFLP identified (1982)
1983-1984	DMD and BMD shown to be allelic
1983-1985	Linked RFLPs first used for carrier detection (1983), and prenatal diagnosis (1985)
1985	Responsible gene isolated (gene-specific probes)
1987	Gene product (dystrophin) identified
1988	Dystrophin localised to sarcolemma, absent in DMD and abnormal in BMD
1989	Myoblast transfer in *mdx* mouse
1990	Gene therapy in *mdx* mouse

1.6.1 Pathogenesis

If dystrophin is absent, muscle tears when it contracts. Before the child with DMD shows clinical symptoms, muscle biopsy reveals large, hypercontracted, eosinophilic and prenecrotic fibres which have arisen because calcium enters the fibre through the sarcolemmal defect. This leads to mitochondrial overload and fibre death and then more muscle damage due to activation of proteases. Engel et al (103) provided the seminal work showing that the sarcolemmal membrane was indeed defective in the prenecrotic fibres and that this was the primary lesion in DMD. Their ultrastructural studies showed focal disruption or absence of the plasma membrane while the basement membrane was preserved.

Muscle biopsy from a case of DMD will reveal complete absence of dystrophin. The disease was later discovered to be caused by a mutation in the dystrophin gene (104). In BMD, the protein expressed seems to be partially functional, explaining the differences in severity of the two diseases (105). In both diseases, fibre necrosis is followed by regeneration. Regeneration ceases at a certain time, however, and the muscle tissue is replaced with fibrous and fatty tissue.

1.6.2 Clinical features

At birth, the baby appears normal but the parents note that he is slow to walk. The child then gradually develops the characteristic calf enlargement (hypertrophy) and has difficulty in climbing stairs. He starts to show Gower's manoeuvre (see Fig1.14) i.e. in a sitting position he raises himself by climbing up along his own legs. By the early teens, muscle wasting is such that the child is in a wheelchair permanently. The disease is fatal by the late twenties or early thirties due to respiratory or heart failure. In very few cases DMD is associated with mental retardation (106).

The muscles most severely affected are those of the pelvic and shoulder girdles, the proximal upper and lower limbs and the trunk muscles. The latter leads to exaggerated lumbar lordosis and scoliosis. It is the pelvic and lower limb muscle involvement which is responsible for the child demonstrating Gower's manoeuvre. Respiratory muscle involvement is relatively late, beginning at the end of the second decade.

Figure 1.14 Schematic diagram of Gowers Manoeuvre

Demonstrates the ability of children with DMD to move from a seated position on the floor, to standing position. The child first has to roll over on to all fours and then push himself up with his hands. The child then walks inward and uses upper body strength to regain an upright position.

Taken from W.R.Gowers' Pseudo-hypertrophic Muscular Parlaysis, 1879, Clinical sign for the development of DMD Figure 1.14 Schematic diagram of Gowers Manoeuvre Figure

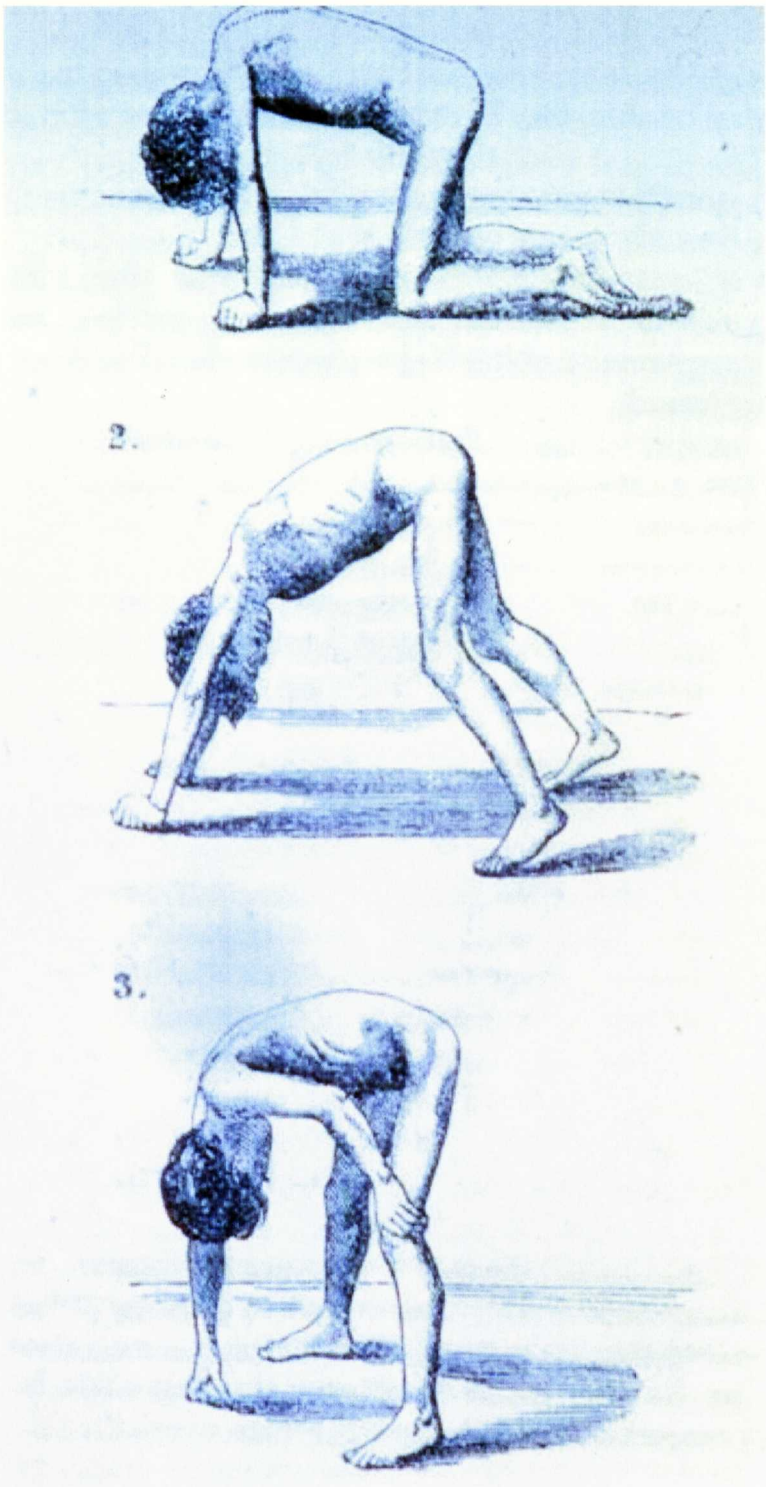


Figure 1.14 Schematic diagram of Gowers Manoeuvre

1.6.3 Pathology

The pathological features of DMD depend on continuous cycles of degeneration and regeneration. This is due to the lack of dystrophin in the cell membrane which results in it being weakened, therefore when the muscle contracts it tears (107). Segmental necrosis of muscle fibres occurs in the late stage, resulting in only a few clusters of tiny and large fibres remaining, all surrounded by adipose and fibrous tissue. Prenecrotic fibres can be identified in the early stages when there is conspicuous evidence of regeneration (108). As the disease progresses, the regenerating (satellite) cells with large nuclei, basophilic cytoplasm and immunohistochemistry positively of N-CAM, HLA class 1 and MyoD, become less and less common until they are completely absent (94). At this stage there is no satellite cell multiplication and no muscle regeneration. Adipose and fibrous tissues with scant clusters of fibres are all that remain.

The pathological picture, when seen with the clinical features, is almost diagnostic and becomes complete when immunohistochemistry reveals the characteristic complete lack of dystrophin. In BMD, the allelic variant of DMD, dystrophin is present but fragmented and in variable amount.

1.6.4 Inheritance

The disease is passed from mother to son (figure 1.15). The mother herself is unaffected because she has one normal dystrophin gene (90). If the mutation is passed to her daughter, she is unaffected also but, like her mother, is a "carrier". Very rarely a carrier has muscle problems in which case she is known as "manifesting carrier". This occurs when the normally random inactivation of the X chromosome is biased. Extremely rare cases of female patients with typical DMD have been reported. This occurs when an X autosomal translocation has occurred.

In two-thirds of cases there is a family history of an affected uncle or brother. In one-third, however, a new mutation has occurred in the massive dystrophin gene (109). Thus genetic counselling cannot eliminate this disorder.

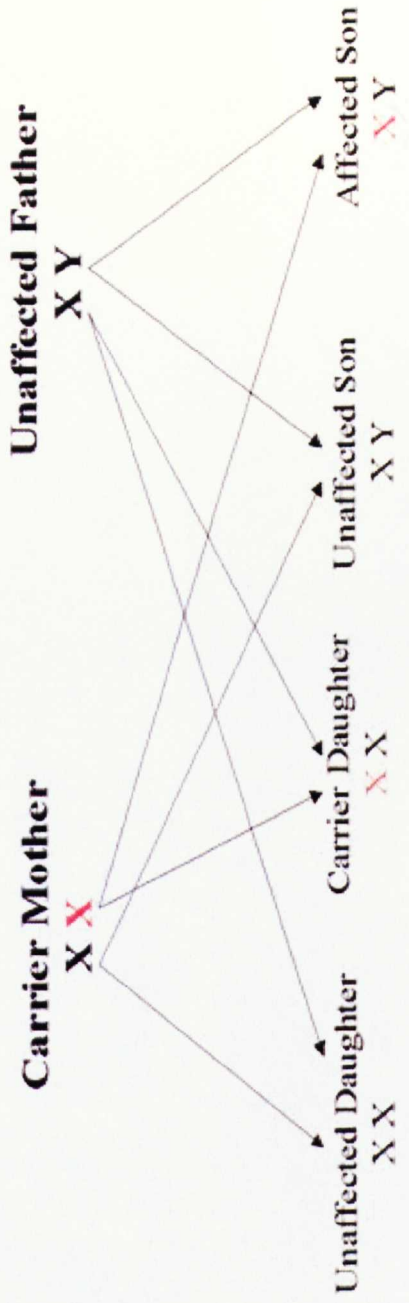


Figure 1.15 Pattern of inheritance

Figure demonstrates how the defective gene is passed from mother to son resulting in the disease, and from mother to daughter resulting in the daughter becoming a carrier.

1.6.5 Dystrophin gene

The dystrophin gene was discovered in 1986, located at Xp21.1 (110, 111). It is the largest gene known at 2.4mB with 79 exons, of mean size 0.2 kB, and introns of mean size 35 kB (see figure 1.16). The actual coding sequence represents less than 1% of its nucleotide composition which is transcribed into a 14kB DNA. It is probably the large size of the gene which makes it liable to the effect of mutagenic agents and accounts for the relatively high mutation rate of around 1 in 10,000 male births. The site and type of mutation are important. Deletions affecting the central rod domain are more likely to lead to a milder BMD phenotype whereas those affecting the C-terminal result in DMD. The gene takes up to 24 hours to be transcribed and forms about 0.1% of the total human genome. It shows strong homology to utrophin and spectrin, contains multiple promoters and has six alternative sliced exons in the C-terminal domain. In 65% of the cases of DMD, the mutation is due to a deletion or duplication, while 5% of cases arise from duplication; the remaining 30% have point mutations. In all cases of DMD the reading frame is lost and this leads to premature termination of translation. In BMD, the less severe variant, the reading frame is not lost and a truncated form of the protein is produced (112).

Figure 1.16 Dystrophin gene

A: Genomic organisation of the dystrophin gene, located in Xp21. The black vertical lines represent the 79 exons of the dystrophin gene distributed over about 2·5 million bases. The arrows indicate the various promoters: in particular brain (B), muscle (M), and purkinje (P) promoters; R, B_3, S, and G represent the Dp260 (retinal), Dp140 ($brain_3$), Dp116 (schwann cells), and Dp71 (general) promoters. B: The domain composition of the various dystrophin proteins is indicated. The amino-terminal domain is followed by the spectrin-like domain, the cysteine-rich, and the carboxy-terminal, domains.

Taken from Muntoni F., Dystrophin and mutations: one gene, several proteins, multiple phenotypes, Lancet Neurology, 2003, 2; 12: p 731 – 740

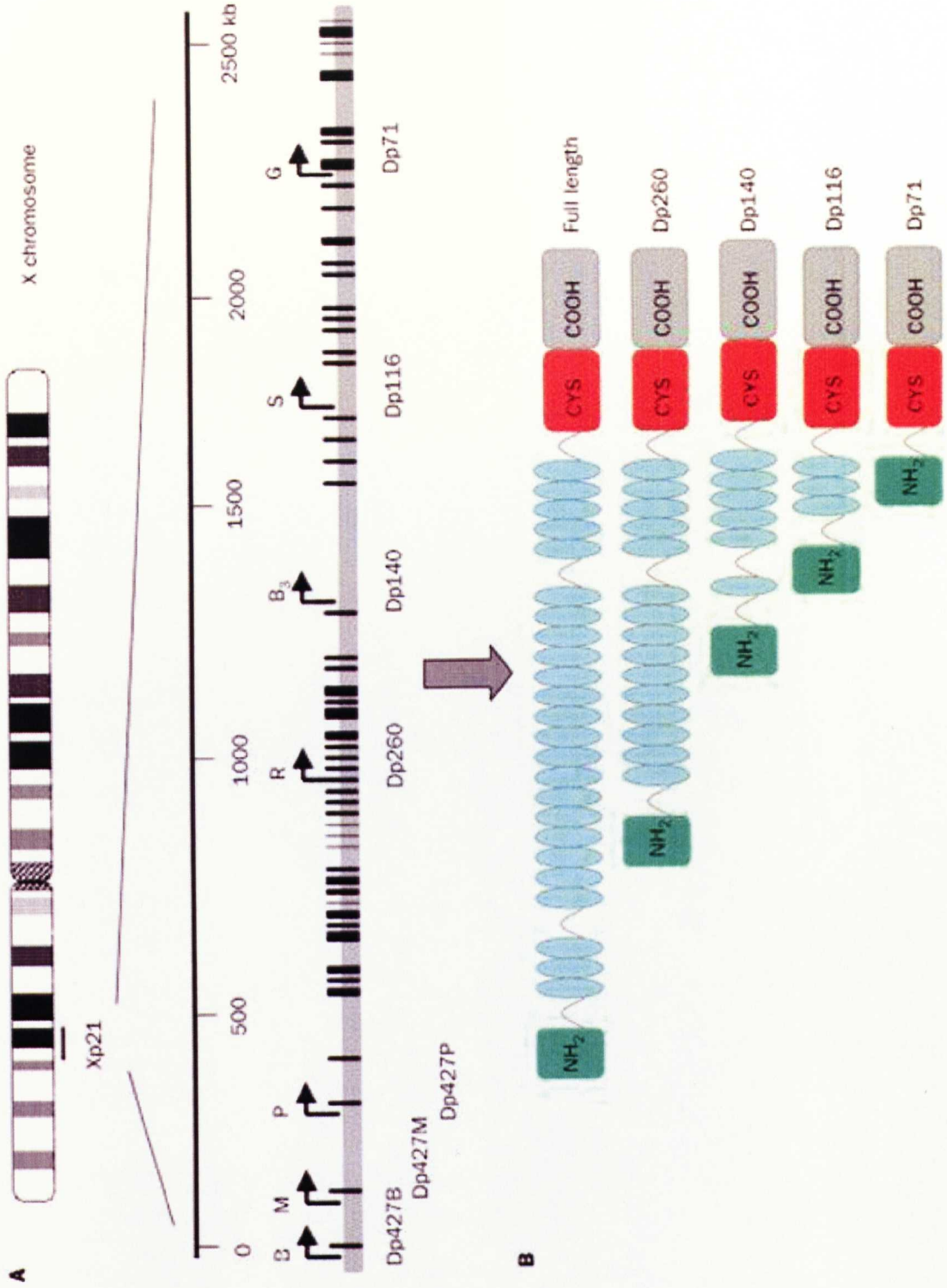


Figure 1.16 Dystrophin gene

1.6.6 Dystrophin protein

The major site of expression of dystrophin is at the inner surface of the sarcolemma of skeletal muscle. Full length transcripts can also be found in the brain and purkinje cells but in much lower abundance. The dystrophin gene contains multiple promoters and shorter transcripts are found in the retina, brain, kidney, schwann cells and in most non-muscle tissue (see figure 1.16). Dystrophin is a 427 kDa protein which can be divided into four domains: an N-terminal actin domain (240 amino acids) a rod-shaped triple-helical spectrin-like domain (3000 amino acids), an α-actin-like cysteine rich domain (280 amino acids) and a unique C-terminal domain (420 amino acids) (113, 114). In muscle, its role is as a link between the actin-associated cytoskeleton and the dystrophin glycoprotein complex (DGC) (see figure1.17) (115). It forms 0.002% of muscle protein.

1.6.7 Dystrophin glycoprotein complex

Dystrophin is not directly attached to the sarcolemmal membrane but interacts through a complex of glycoproteins, the DGC (figure 1.17) (116, 117). The DGC can be divided into 3 domains, extracellular (lamin and α-dystroglan), transmembrane (β-dystroglyan and sarcoglycan proteins) and submembrane (dystrophin, utrophin, dystrobrevins and the syntrophins). β dystroglycan spans the sarcolemmal membrane binding at its extracellular end to the α-dystroglycan and at the intracellular, to the C-terminal of dystrophin. α-dystroglycan then links to the extracellular matrix component, laminin. Thus, there is a network of interactive proteins providing a link from the intracellular contracting muscle fibre to the extracellular matrix (see figure1.17).

The submembrane domain of DGC, the dystrobrevins and syntrophin, act to bring assorted components of the DGC together by interaction amongst themselves and dystrophin. In DMD, there is an absence or reduction in all the components of DGC while in BMD, this is seen to a lesser degree. Individual components have now been shown to be absent in certain other inherited muscle disorder diseases.

Figure 1.17 The dystrophin glycoprotein complex

Shows the relationship between the dystrophin protein and the structure of the muscle complex. When the muscle contracts, dystrophin anchors the actin fibre to the sarcolemma via the complex.

Adapted from Roush.W, Muscular Dystrophy: Backup gene may help muscles help themselves. Science (1997); 276: 5309 p35

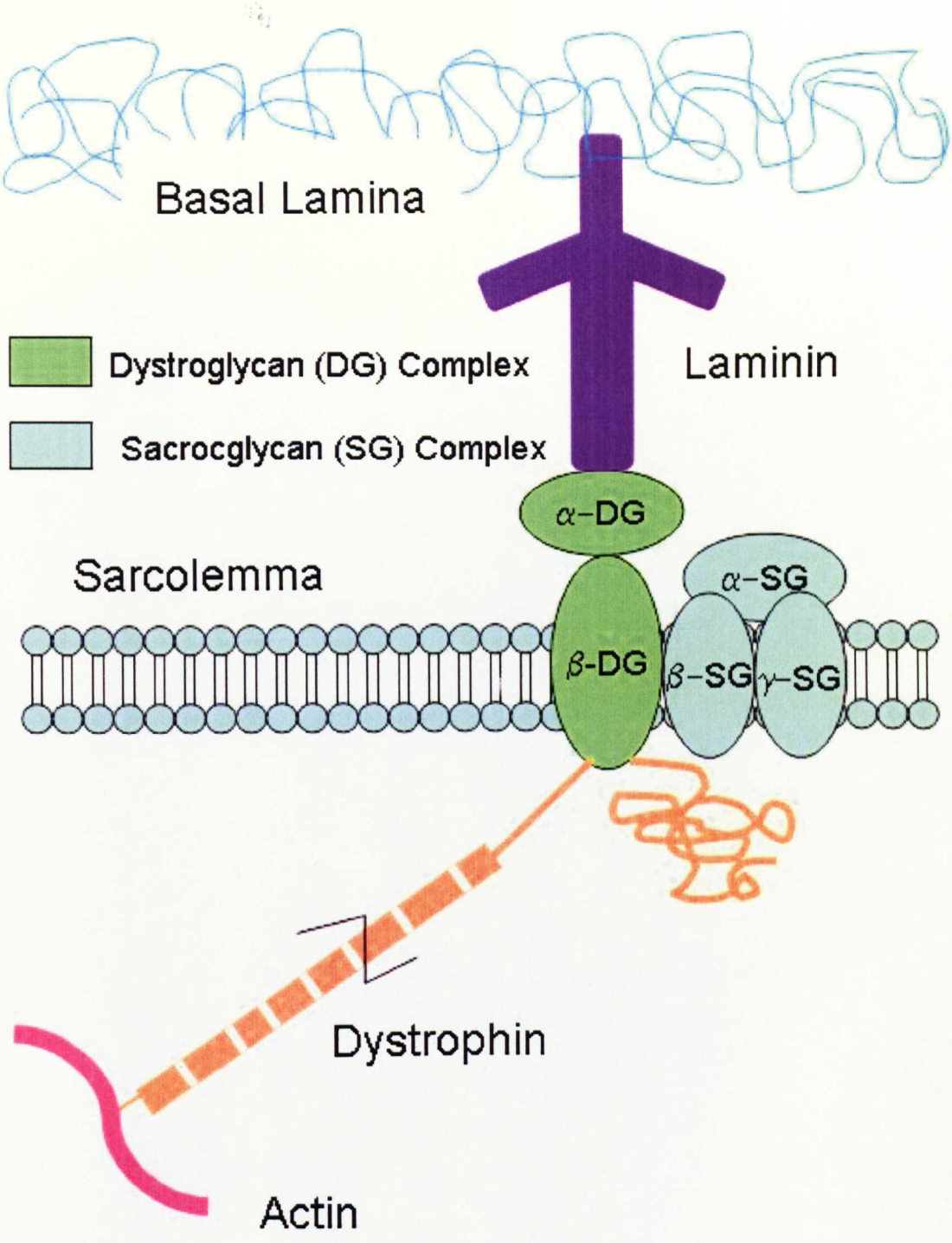


Figure 1.17 The dystrophin glycoprotein complex

1.6.8 Satellite cells in DMD

Almost all the muscles in the affected boy consist finally of a few muscle fibres of varying size, surrounded by fat and fibrous tissue. Before this occurs, several workers have reported increased numbers of satellite cells (SC) compared to controls (94). In the first two to three years of life, regeneration keeps pace with fibre destruction but as the disease progresses there is more and more evidence of inadequate replacement.

Satellite cells are the only muscle cells which replicate; they form approximately 5-6% of the total muscle nuclei in the adult (95). Satellite cell replicative capacity is diminished in DMD (94). The normal age-related decline has been shown to be accelerated in DMD myoblasts: (118). The average myoblast from a 5 year old healthy boy is capable of 56 doublings or a potential yield of 10^{17} cells but in patients with DMD as young as 2 years (the typical age of clinical onset) only 6% of myoblasts were found to be capable of 50 doublings and by the age of seven, only rare myoblasts could achieve even 10 divisions.

Study of telomere length provides an indication of how many cell divisions a cell has undergone, and, once the normal number of mean population doublings (MPDs) is known, what regenerative capacity remains. The rate of telomere shortening in myonuclei from skeletal muscle satellite cells at ages from 5 days to 86 years has been established: replicative capacity and telomere length decrease in parallel with age for the first two years of life but then remain remarkably stable with a regular loss of telomere sequences of 75-155 bp per cell division. In boys with DMD there is a marked decrease in satellite cell telomere length by the age of 2 years: as early as this, telomere loss is 14 times greater than in satellite cells from healthy two year olds (119) (187 bp/year versus 13 bp/year). However there is some controversy surrounding this as other groups have suggested that replicative senescence has no role in DMD, and the telomeres remain at a normal length (120) This group has suggested that it is the lack of differentiation in DMD which results in onset of the phenotype, not failure to replicate. It is difficult to determine which group is correct. However discrepancy may be due to the insensitive nature of southern blotting.

1.6.9 Animal models

Naturally occurring and experimental laboratory models of DMD are available, with the latter being much more frequently used.

1.6.9.1 Xmd golden retriever and cats

A naturally occurring animal model for DMD is seen in the golden retriever dog (121). It shows the phenotype of the disease before the age of one year with stiff hind legs and progressive muscle wasting. This model has the closest clinical resemblance to the human disease. The pathological picture is also similar and immunohistochemistry confirms the absence of dystrophin. Ethical approval for working with canines can however prove difficult. There are also reported natural cases of felines with DMD which, like the canine model resemble the human disease more closely than that in the *mdx* mouse. These are rarely used in research.

1.6.9.2 The mdx mouse

Currently the most popular animal model for DMD is the *mdx* mouse. The mouse model was first introduced by Bulfield et al, in 1984 (122) as a murine glycolytic mutation with histological evidence of myopathy. The mdx mouse has a premature stop codon in exon 23 of the dystrophin gene. It has no detectable levels of dystrophin, although it has been reported that dystrophin can be seen in revertant fibres. No dystrophin is present during immunoblotting, but some fibres are positive for dystrophin under fluorescent microscopy. On further analysis, it was shown that they were expressing dystrophin despite the mutation in the gene (123). The mdx mouse does not show the phenotypic wasting seen in the human disorder, however it has been reported that the limb muscles of the mdx mouse are susceptible to injury and this increases with age (124).

This model has been used extensively in the development of new therapies for DMD.

1.6.10 Current therapies for DMD

At present the treatment of DMD and BMD is purely supportive directed at maintaining a better standard of life for the patient but providing no cure for the disease. The main medication used is steroid therapy which may help to protect the necrotic muscle. The other options are mainly orthopaedic procedures. Trunk deformities like scoliosis appear,

especially when a wheelchair is required. This causes respiratory problems and to counteract this, a steel pole may be fused with the spine to straighten it. The patient can then sit in an upright position so that the onset of respiratory failure may be postponed for a number of years. Another therapy is the use of oxygen in the home. In the latter stages of the disease the children often suffer from sleep apnoea. By allowing the child to breath pure oxygen, he can obtain a full night's sleep. It has been shown that this can greatly improve in the child's standard of life. Also very important in the treatment of this disorder is the role of physical therapy. This helps to prevent the onset of contractures. Because this is a hereditary disease genetic screening for families at risk and genetic counselling is an important aspect of the treatment (125).

Due to the nature of this disorder, there is at present no therapeutic cure, and it is unlikely there will be. However there is extensive research being performed in the development of gene therapy or cell therapy to aid this disorder but, as of yet, these have been unsuccessful in clinical trials. These will be discussed in more detail in the discussion.

1.7 Aims

This disease would appear to be an ideal candidate for gene therapy, but this has not yet been achieved. The constant necrosis and regeneration of the muscle cells in these children result in the lack of replicative capacity. We feel this may play a major role in the problem. Therefore the aim of this project was to assess if it was possible to immortalise normal human muscle cells by transfection with hTERT alone. The muscle cell lines produced would then be tested to assure that they had not adapted a malignant phenotype using cell culture techniques and karyotyping. This is important as the immortalisation had to be achieved without inducing malignant transformation. Previous attempts using hTERT alone had been unsuccessful. If the experiments were successful, we would then investigate how our technique had differed from others. The cell lines would be further characterised to asses how the introduction of telomerase had changed there cell cycle properties by examining the cell cycle regulators. Finally the differentiation capacity of these cells line would be explored in culture and in an animal model. If successful this suggests a way of producing long-lived cell lines for research and hopefully for cell therapy in the future.

Chapter 2 Materials and Methods

2.1 Materials

General cell culture media and supplies

Supplier	Reagent
A/S Nunc Botolph Claydon, UK	1ml Cryotubes
Sigma Chemical Company Fancy Road, Poole, Dorset, UK	Penicillin G (benzylpenicillin) sodium salt, Cat No. P3032 Streptomycin Sulphate BP, Cat No. S9137
Becton Dickinson Labware Plymouth, UK	Falcon tissue culture dishes, full range
Fisher Scientific UK Bishop Meadow Road, Leicestershire, Loughborough, UK LE11 5RG	Dimethylsulfoxide ($CH_3.SO.CH_3$) Cat No. BPE231-1
Gibco BRL Life Techologies Paisley, UK	DMEM, Cat No. 21969-035 HEPES Buffer, Cat No. 15630-056 L-Glutamine, Cat No. 25030-032 Geneticin (G418 Sulphate) Cat No. 11811-098
Worthington Biochemical Corporation, Reading, UK Autogen Bioclear	Trypsin, Cat No 39J3128 Fetal Bovine Serum

Holly Ditch Farm, Mile Elm, Calne, Wiltshire, UK	Cat No S011S Horse serum Cat No 7.10

Specialized Cell Culture Techniques

Supplier	Reagents
Costar Bucks, UK	5mm filter membrane
Nalge Nunc International Corp 2000 North Aurora Road, Naperville. IL, 60563-1796 USA	8 Well Permanox ® Slide, Cat No. 177445
Sigma Chemical Company Fancy Road, Poole, Dorset, UK	Ploybrene (Hexadimethrine), Cat No H9268 Hoeschst 33258 Cat No. B1155 Puromycin dihydrochloride Cat No.P8833

Molecular Biology Kits

Supplier	Reagent
QIAGEN Ltd Boundary Court, Gatwick Road, Crawley, West Sussex, RH10 2AX UK	Plasmid Midi Kit Cat No 12143

Antibodies

Supplier	Reagent
Vector Laboratories Inc 16 Wulfic Square, Bretton, Peterborough, PE3 8RF UK	DAB Substrate kit for peroxidase, 3,3'-diaminobenzidine Cat No SK-4100 Vectashield with DAPI, Cat. No. H-1200 **Vectastain ABC Kit (Mouse IgG)** **Cat. No. PK-4002** Vector MOM basic kit Cat. No. BMK-2202
Novo Castra Balliol Park West, Benton Lane, Newcastle upon Tyne, NE12 8EW UK	Spectrin Ab (Broad Spectrin). Mouse Monoclocal, Cat No. NCL-SPEC2 Spectrin Ab, Mouse Monoclonal, Cat No. NCL-SPEC1
DAKO 6392 Via Real, Carpiteria, CA, USA	Desmin Ab, Mouse Monoclonal, Cat No. D33

Boehringer Mannhiem UK (Diagnostics & Biochemicals) Ltd Bell Lane, Lewes, East Sussex, BN7 1LG UK	5-Bromo-2-deoxy-uridine Labelling and Detection Kit 1, Cat No 1296 736
Santa Cruz Autogen Bioclear Holly Ditch Farm, Mile Elm, Calne, Wiltshire,UK	p16 antibody Mouse monoclonal, Cat No. sc-9968

Western Blot

Supplier	***Reagent***
Sigma Chemical Company Fancy Road, Poole, Dorset, UK	Aprotin, ***Cat No. A6279*** Leupeptin, Cat No. L2023 Pepstatin A, Cat No. P4265 PMSF, Cat No. P7626 Sodium Fluoride, Cat No. S1504 Okadaic Acid, Cat No. O7760 DTT, Cat No. D0632 Benzamidine, Cat No. B6506 EGTA, Cat No. E4378

Chemicals

Supplier	Reagent
Agar Sceintific Ltd 66a Cambridge Road, Stanstead, Essex, CM24 BDA	Paraformaldehyde ($(CH_2O)nH$-OH) Cat No. R1018
Amersham International Little Chalfont, UK	Redivue [α^{32}P]dCTP~ 3000Ci/mmol Cat No. AA0005
BioFine Ltd London, UK	X-Gal
James Burrough (F.A.D) Ltd 70 Eastways Industrial Park, Witham, Essex, CM8 3YE UK	Ethanol (C_2H_5OH) Cat No. SIN 1170
Fisher Scientific UK Bishop Meadow Road, Loughborough, Leicestershire, LE11 5RG UK	Magnesium Chloride ($MgCl_2.6H_2O$) Cat No. M/0600/53 Methanol (CH_3OH) Cat No. BPE1105-1 Sodium Chloride (NaCl) Cat No. BPE358-1
Sigma Chemical Company Fancy Road, Poole, Dorset, UK	Ethidium Bromide Cat No. E1510 Giemsa's Stain Cat No. GS500 Glacial Acetic Acid ($C_2H_4O_2$) Cat No. A6283 Mes (2-[N-Morpholino] ethanesulfonic acide) Cat No. M8250

Sigma Chemical Company Fancy Road, Poole, Dorset, UK	TEMED (N,N,N,N- Tetramethyl-ethylenediamine) Cat No. T8133 EDTA (Ethylenediaminetetra-acetic acid, $C_{10}H_{14}N_2O_8Na_2.2H_20$) Cat No. ED2SS Potassium Ferrocyanide ($K_4Fe(CN)_6.H_20$) Cat No. P9387 Potassium Ferricyanide ($K_3Fe(CN_6)$) Cat. No. P3667

PCR Reagents

Supplier	Enzyme
PE Allied Biosystems Warrington, Cheshire, UK	AmpliTaq® DNA Polymerase Cat No. N801-0060 10X PCR Buffer & $MgCl_2$ Solution Cat No. N808-0010

Laboratory Plasticware

Supplier	Material
Becton Dickinson Labware Plymouth, UK	Falcon Tubes
Bibby-Sterilin Stone, UK	5ml Bijous 5ml & 20ml Universals
Elkay Galway, Eire	Microcentrifuge tubes Pipette tips
Greiner Labortechnik Stonehouse, UK	Filter pipette tips

Electrophoresis Gels

Supplier	Reagent
Sigma Chemical Company Fancy Road, Poole, Dorset, UK	Kodak X-OMAT Film Cat No. F5263
Whatman International Maidstone, UK	3mm Chromatography paper Cat No. 3030 917

Microbial Host, Media and Supplies

Supplier	Material
Difoc Central Avenue, West Molesey, Surrey, KT8 2SE UK	Tryptone, Bacto Cat No. 0123-15 Yeast Extract, Bacto Cat No. 0127-15
Bibby-Sterilin Stone,UK	Petri dishes
Gibco BRL Life Techologies Paisley,UK	Library Efficiency DH5α™ Competent Cells Cat No. 18263-012 S.O.C. Medium Cat No. 15544-018
Sigma Chemical Company Fancy Road, Poole, Dorset, UK	Ampicillin Cat No. A9393

Cell Lines

Source	Cell line	Origin
ATCC 12301 Paklawn Drive Rockville, Maryland, 20852 USA	NIH 3T3 cells NRK	Swiss Mouse Embryos Cat. No. CCL92 Mycoplasma Indicator
Dr Oliva Periera-Smith, Baylor College of Medicine, Houston, Texas, USA	HeLa	Cervical Carcinoma

Dr G.P. Nolan, Stanford University Medical Center, San Francisco, California, USA	Phoenix A	Retroviral Packaging Cell Line
Dr EK Parkinson CRC Beatson Insitute, Bearsden, Glasgow, UK	BICR 31	Alveolus Carcinoma

Internet Links

National Centre for Biotechnology Information (NCBI)
http://www.ncbi.nlm.nih.gov/

Plasmids

pBabest2 and pBabe Neo retroviral plasmids were obtained from H. Vaziri and S.Benchimol, Standford University School of Medicine, Department of Molecular Pharmacology, Edwards Building, 300 Pasteur Drive Standford, California, CA 94305-5332, USA.

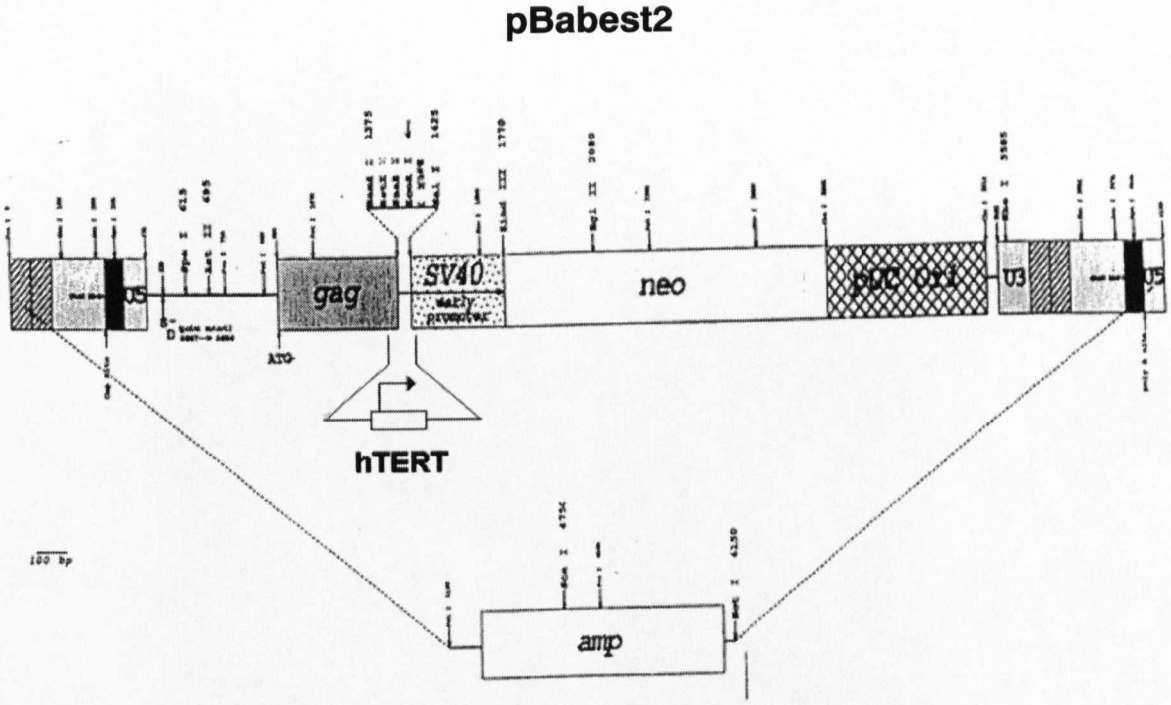


Figure 2.1 Schematic diagram of pBabest2 retroviral plasmid

pBabe Neo retroviral plasmid is the same structure as pBabest2 with the hTERT insert missing.

2.2 Cell Culture Techniques

All cell culture work was carried out using strict aseptic technique in Class II laminar flow hoods (Medical Air Technology Ltd, Manchester, UK). Reteroviral work with human muscle cultures was carried out in category II containment facilities.

2.2.1 Human muscle biopsy explant cultures

Human muscle biopsies were obtained from a healthy 23 year old male (line 1) and 28 year old female (line 2) from the vastus lateralis muscle of the thigh. The tissue was placed in a vial of Dulbecco's modified Eagle's medium (DMEM) with 10% foetal bovine serum (FBS), 20mM HEPES buffer, 2mM L-glutamine, 50U/ml penicillin and 20ng/ml streptomycin for transportation. The muscle biopsies were cut into cubes of approximately 5mm by 5mm. The muscle samples were spread out among petri dishes and covered in 2ml FBS, or enough to just coat the plates and the samples. The plates were then put in a 5% CO_2 humidified incubator for 2 hours. After this time the human muscle biopsies were removed and the plate was covered in 5ml of DMEM with 10% FBS 20mM HEPES buffer, 2mM L-glutamine, 50U/ml penicillin and 20ng/ml streptomycin and left in the incubator overnight. The next day the medium was replaced with 10ml fresh media.

2.2.2 Optimisation of growth conditions for human muscle cells

The initial culture conditions for the growth of human muscle cultures (HMC) were established by Karen Steeghs as reported in Wootton et al 2003. Muscle cell cultures were grown in 10% DMEM supplemented with 10% (v/v) FBS, 20mM HEPES buffer, 2mM L-glutamine, 50U/ml penicillin and 20ng/ml streptomycin. The optimal growth conditions for the muscle cell cultures were determined by growing hTERT-infected and uninfected HMCs in different media and serum concentrations for 20 days (see Table 1). The MPD of the cells were recorded to determine the optimum medium conditions (See fig 1a). All medium used had 20mM HEPES buffer, 2mM L-glutamine, 50U/ml penicillin and 20ng/ml streptomycin added.

Table 2.1 Comparison of different culture conditions for growth of HMC

Media	Serum
Dulbecco's modified Eagle's medium	10% FBS 20% FBS
Ham's F10	10% FBS 20% FBS
Ham's F12	10% FBS 20% FBS

The MPD that cells achieved was recorded to determine the optimum medium conditions for cell growth. The formula was according to Paul et al 1975

$$MPD = (\log Nb - \log Na) \times 3.32$$

where Na = initial cell number

Nb = final cell number

It was determined that 10% (v/v) foetal bovine serum in DMEM was optimum for growth (see figure 3.4)

2.2.3 Passaging of human muscle cells

The HMCs were routinely cultured in DMEM supplemented with 10% (v/v) FBS, 20mM HEPES buffer, 2mM L-glutamine, 50U/ml penicillin and 20ng/ml streptomycin at 37°C in a 5% CO_2 humidified incubator. Medium changes were carried out every 3-4 days and cells were passaged when they reached 70-80% confluence. To passage the cells, medium was removed and the cells rinsed with 1X PBS to remove any residual serum. Trypsin/EDTA was added and the cells incubated for 3-5 mins or until the cells were rounded up and became detached from the plate. 10% DMEM was added to inactivate the trypsin, cells counted and pelleted by centrifugation at 1000rpm for 5 mins. The pellet was resuspended in 10% DMEM and seeded at an appropriate density for experiments.

2.2.4 Cryopreservation and thawing of muscle cells

Stocks of cells were cryopreserved at different MPDs. Cell were maintained in culture and when ready to be cryopreserved they were trypsinised, neutralised with medium and pelleted. Cells were preserved at 10^6 cells in 1ml of freezing medium (10% (v/v) DMSO in growth medium with 20% FBS) in cryotubes, 1ml per vial. The cryotubes were wrapped in

cotton wool and placed in a -70°C freezer for 24 hrs to freeze slowly. The following day the cells were stored in liquid nitrogen.

To thaw the cells the cryotubes were removed from liquid nitrogen and immersed in a large volume of 37°C water. The cells were resuspended in 10ml of normal growth medium (10% DMEM) and then pelleted by gentle centrifugation at 1000rpm for 5 mins, to remove the DMSO. Finally the cells were resuspended in growth medium and plated.

2.2.4 Growth of cells in low oxygen

Cells were removed from normal culture conditions and placed in low oxygen conditions. The cells used where split in the normal fashion and then placed in DMEM supplemented with 10% (v/v) FBS, 20mM HEPES buffer, 2mM L-glutamine, 50U/ml penicillin and 20ng/ml streptomycin and media antioxidant N-(tert)-butyl hydroxylamine at a concentration of 100μM. This removes the oxygen from the media. The cells were then placed in an incubator at 37°C at 5% oxygen.

2.3 Mycoplasma testing of muscle cell lines

Antibiotic-free medium which had been in contact with the cells to be tested, for at least 48hrs, was removed. This medium was incubated with 10^4 normal rat kidney (NRK) indicator cells. The NRK cells were incubated in at 37°C tissue culture incubator for 2-3 days. The medium was then removed and the cells were washed in PBS twice. The cells were fixed in methanol/acetic acid (3:1), added slowly then poured off, and replaced with another 5ml of fixative. This step was repeated and finally the fixative was left on for 10mins. After the fixative was removed 0.05μg/ml Hoechst 33258 in PBS was added and left for 10 mins. Cells were then washed twice in distilled H_2O and mounted in McIlvanies's buffer (0.2M Na_2HPO_4, 0.1M citric acid). Cells were examined using a fluorescence microscope.

2.4 Production of retroviruses

Phoenix A packaging cells were plated $2X10^6$ cells in a 6cm plate. 24 hrs later 8μg of retroviral plasmid DNA was added to 100μl of OPTI-MEM in one falcon tube (Bectin Dickinson) while in another 25μl of lipofectamine in 75ul of OPTI-MEM was added. The 2 solutions were mixed and incubated at room temperature (RT) for 45 mins to form complexes The cells had their medium removed and were washed twice with 1 X PBS. 0.8ml of OPTI-MEM was added to the complexes and the mixture added to the Phoenix A cells. The cells were then incubated for 5hrs in a CO_2 incubator at 37°C. The transfection mixture was removed and the cells were washed twice with growth medium (10% DMEM) and incubated overnight in 10% DMEM. The medium was replaced 24 hrs later with 10% FBS in DMEM. 24 hrs later the medium was removed and filtered through a 4.5μ filter (Gelman Science). The virus-containing medium was flash frozen on dry ice in 1ml aliquots and stored at -70°C until required.

2.4.1 Titration of Viruses

The day before titration, 1 X 10^5 NIH 3T3 fibroblasts were plated on 6 cm dishes. Next day serial dilutions of the virus were mixed with 10% FBS/DMEM with 4μg/ml polybrene (Sigman Aldrich, Poole, UK). Polybrene is a cationic reagent which aids in the 'sticking' of the virus to the cell membrane and therefore improves infection efficiencies. 2ml of each of the dilutions were placed on the NIH 3T3 cells and incubated in a CO_2 incubator for 3hrs. To minimise the toxic effects of the polybrene, it was diluted by adding 4ml of growth medium to the dishes. All of the plates were re-fed the next day with growth medium. 24 hrs later the appropriate concentration of antibiotic was added and the cells selected for 2 weeks or until uninfected control NIH 3T3 plates were dead (see table 2). The plates were fixed in 3% (v/v) paraformaldhyde/PBS and stained in 10% (v/v) giemsa for 30 mins. The plates were washed in double distilled H_2O and allowed to dry. The number of colonies on each plate were counted and expressed as colony-forming units/ml of supernatant. The muscle cells were infected at a 1:1 ratio of virus:cell where possible.

2.4.2 Determination of antibiotic selection concentration

Kill curves for each of the different cell types/cultures were established to determine the appropriate concentration of antibiotic to use during selection and generation of stable pools and clones. Cells were plated at a density of 10^5 cells in 60mm plates and a range of antibiotic concentrations which resulted in complete cell death after 2 weeks was taken as the appropriate antibiotic concentration to use during selection. These concentrations are listed in table 2.2

Cell line	Antibiotic	Concentration
NIH 3T3	G418 (Invitrogen, Paisley, UK)	1 mg/ml
NIH 3T3	Puromycin (Sigam, Dorset)	1μg/ml

Table 2.2 Concentration of antibiotic used in transfections

2.4.3 Retroviral infection of human muscle cells

The cells to be infected were seeded at a density of 10^5cell/6cm dish 72hrs prior to infection. Polybrene (8mg/ml) was added to the viral supernatant which contained between 10^5 and 10^4 viral particles and the total volume made up to 5mls with 10% DMEM. The cells and the viral supernatant were incubated at 37°C for 4 hrs and the medium replaced with fresh 10% DMEM and the cells left overnight. Next day the cells were harvested and plated at a density of 3 X 10^5/9cm dish, allowed to recover for a further 72 hrs and then placed under appropriate selection until resistant colonies had appeared. Pools of resistant colonies were frozen down or individual clones were picked and expanded prior to cryopreservation in 1ml cryotubes (Nunc, Wiesbaden, Denmark). The ampoules were stored in the liquid nitrogen tank until required.

2.5 Giemsa staining of cultured cells

Cells were washed twice in PBS and fixed in 10% (v/v) formaldehyde/PBS for 30 mins. The cells were then washed in milli-Q H_2O (Millipore Milli-Q Systems, UK) and stained with 10% (v/v) giemsa/milli-Q H_2O for 30 mins. After 2 further washes in milli-Q H_2O the plated were air-dried and observed under a low power (X 10 magnification) light microscope (Zeiss) and colonies counted.

2.6 Soft agar colony forming assay

Agar was prepared at 0.8%w/v and 1.6%w/v with Bactoagar (DIFCO, Detroit, MI) and distilled water. This was autoclaved and allowed to cool to 40°C. Double strength DMEM was made up with 40% FBS. The 1.6%w/v agar was mixed with the 2X DMEM/40% FBS in a ratio of 1:1 giving a final concentration of 0.8% agar. This was used to coat the bottom of the plates (10mls per each 9cm plate), which were put in the incubator at 37°C and 5% CO_2 to set. The 0.8%w/v agar was mixed with the 2X DMEM/40% FBS in a ratio of 1:1 giving a final concentration of 0.4%w/v agar. Cells were mixed with this top layer of agar and poured onto the coated plates. The cells were plated at 1 X 10^4 for HeLa cells and 1 X 10^5 for HeLa cells, HFF cells, HMC T2 and HMC T15. The plates were placed in and incubator with 5% CO_2 at 37°C. They were fed weekly with 0.8% agar mixed equally with 2X DMEM/40% FBS. The colonies were counted and photographed with a digital camera after 5 weeks.

2.7 Low serum assay

Cells were plated at 5 X 10^4 in 3cm dishes in DMEM with 10% (v/v) FBS or 0.2% (v/v) FBS. Each week the cells were trypsinised and passaged as indicated in section 2.3.3. The cells were counted and their MPDs were recorded each week for 10 weeks.

2.8 Senescence associated β-galactosidase(SABG) assay

Cells were maintained in culture until growth rates ceased as indicated by MPD or in the case of hTER- infected cells at 44MPD. The cells were washed twice in PBS and fixed in 3% formaldehyde (v/v) in PBS for 5 mins at RT. Cells were then rinsed again in PBS and stained for SABG as described by (7). Cells were incubated with 2ml of X-gal staining solution (50mM MES, 150mM NaCl, 2mMMgCL_2, 5mM K_4Fe $(CN)_6.H_2O$ (potassium ferrocyanide), 5mM $K_3Fe(CN_6)$ (potassium ferricyanide) and 1mg/ml X-gal) at pH 5.8 in a 6cm dish. The plates were incubated at 37°C in a humidity box for 16 hrs. The cells were rinsed in milli-QH_2O and visualised with a light microscope, scored for blue β-galactosidase positive cells and photographed.

2.9 5-Bromo-2'-deoxy-uridine incorporation analysis

5-Bromo-2'-deoxy-uridine (BrdU) incorporation analysis was carried out using the BrdU labelling and detection kit (Roche, Germany). Reagents were supplied as kit components. Cells were plated in 8 chamber slides (Nalge Nunc, USA) at a density of 3 X 10^3 cells per well. The cells were maintained in growth medium for 48 hrs. BrdU labelling reagent was added to sterile medium at a concentration of 10μmol per ml. This was incubated on the cells for 48 hrs at 37°C in a 5% CO_2 incubator. The cells were then washed three times in PBS and fixed in 70% (v/v) ethanol (in 50mM Glycine Buffer, pH 2.0) for 20 mins at -20°C. The slides were washed again in PBS and incubated overnight at 4°C with anti-BrdU mouse monoclonal antibody diluted (1:10) in incubation buffer (66mM Tris-buffer, 0.66mM MgCl2 and 1mM 2-mercaptoethanol). Following a further 2 washes in PBS wash buffer, the cells were incubated with an FITC-conjugated anti-mouse IgG antibody (diluted 1:10 with PBS) for 10 mins at RT in the dark, in a humidifier box. The cells were washed again with PBS wash buffer and air-dried. The gasket was removed and the slides were mounted in Vecashield/DAPI (Vector Laboratories, Peterborough, UK). Slides were sealed using clear nail polish and cover slips, then stored at 4°C until ready to view. Visualisation of slides was carried out using a Zeiss Axioskop fluorescent microscope. Images were captured using a CCD Camera System (Princeton Instruments).

2.10 Immunohistochemistry

2.10.1 Preparation of frozen sections

8μm frozen sections of muscle from implanted mice, untreated RAG-1 mice and from a normal human donor were cut on the cryostat.

2.10.2 Preparation of paraffin tissue sections

Muscle biopsies were fixed in formalin (10%(v/v) formaldehyde in PBS pH 7.6) for 24 hrs before embedding in paraffin using standard procedures. 6μm sections were cut using a Leica microtome. The sections were floated in a 50°C water bath and collected on glass slides. The sections were dewaxed and rehydrated in coplin jars using standard procedures. Briefly, sections were dewaxed in two changes of Histoclear and rehydrated through graded alcohols, 100%, 70%, 50% ethanol/milli-Q H_20 (v/v)

2.10.3 Histological Staining of Muscle Sections

Mayer's haematoxylin-eosin staining of muscle tissue was routinely performed to examine the morphology of the muscle tissue. The sections were placed in haematoxylin solution for 5mins and washed for 3mins. The sections were counterstained with eosin for 30-60secs, rinsed in running tap water for 30 secs, then dehydrated through graded alcohols and cleared in Histoclear and placed in mounting medium.

2.10.4 Desmin staining of muscle cells

Cells were grown on chamber slides at a density of 1 X 10^3 and washed 3 times in PBS. The slides were then fixed in acetone at 4°C for 20 mins and allowed to air dry. The sections were blocked with 2 % horse serum (Autegen Bioclear, UK) in PBS for 30 mins. The slides were incubated for 60 mins at RT with a mouse monoclonal antibody directed against desmin (clone D33, at a dilution of 1:100, Dako, USA). After washing with PBS the cells were incubated with secondary and tertiary antibodies from the avidin biotin complex (Vectastain Peroxidase Mouse IgG ABC kit, Vector Laboratories Inc,USA) as per the manufacturer's instructions and counterstained with 3, 3'diaminobenzidine (DAB, Vector Laboratories, UK).

2.10.5 Spectrin staining of muscle tissue

8μm frozen sections of muscle from the implanted mice, untreated RAG-1 mice and from a normal human donor were blocked with 2 % (v/v) horse serum in PBS for 30mins. All tissues were incubated with the primary antibody spectrin (NCL – SPEC2, at a dilution of 1:100, Novocastra Labs, UK) overnight at 4°C. The slides were washed and stained using the Vector MOM Immunodetection Kit following the manufacturer's instructions (Vector Laboratories Inc,UK). Tissue was visualised using DAB. The tissue was examined using a light microscope and digitally photographed.

2.10.6 p16 staining of muscle cells

Cells were grown on chamber slides at a density of 1 X 10^3 and washed 3 times in PBS. The slides were then fixed in acetone at 4°C for 20 mins and allowed to air dry. The sections were blocked with 2 % horse serum (Autogen Bioclear, UK) in PBS for 30 mins. The slides were incubated for 60 mins at RT with a mouse monoclonal antibody directed against p16 (clone 50.1, at a dilution of 1:50, SantaCruz). After washing with PBS the cells

were incubated with secondary and tertiary antibodies from the avidin biotin complex (Vectastain Peroxidase Mouse IgG ABC kit, Vector Laboratories Inc,USA) as per the manufacturer's instructions and counterstained with 3, 3'diaminobenzidine (DAB, Vector Laboratories, UK).

2.11 Microbiological Techniques

2.11.1 Transformation of bacteria with plasmid DNA

E coli DH5α competent cells were thawed on ice and 20μl transferred into a prechilled 1.5ml microcentrifuge tube. Plasmid DNA diluted to a final concentration of 10ng/μl-20ng/μl was added to the DH5α cells, mixed and incubated on ice for 30 mins. The cells were then heat-shocked for 40 s in a 42°C water bath and then placed on ice for 2 min. 80μl of S.O.C. Medium (GibcoBRL, Paisley, UK) was added and the mixture agitated in a shaker at 225rpm for 1hr at 37°C to allow the induction of the antibiotic resistance gene ampicillin. The cells were then spread on LB* medium plates contining 1.5% (w/v) agar supplemented antibiotic. Plates were incubated in an inverted position at 37°C and clonies were transferred into LB medium the next day.

* LB Medium is also known as Luria-Bertani which consists of 10g bacto-tryptone (Difco, Surrey, UK), 5g bacto-yeast extract (Difco, Surrey, UK), 10g NaCl in 1ltr of deionised H_2O at pH 7.0. LB Medium is prepared in-house at the BICR.

2.12 DNA extraction and analysis

2.12.1 Preparation of plasmid DNA

Plasmid DNA was extracted using the QIAGEN Midi Kit(alkali method described by (126). 500ml of LB medium containing 100μg/ml ampicillin (Sigma, Poole, UK) in 15mls sterilin tubes were inoculated with a single bacterial colony from the agar plate (previously transformed, see above section 2.12). The cultures were then placed into a 37°C shaker for 16 hrs. The bacterial cells were then harvested from the broth by centrifugation at 6000 X g for 15 mins. The supernatant was removed and the pellets were resuspended in 10ml of buffer P1 (50mM Glucose 25mM Tris-HCl pH8, 10mM EDTA pH8). An equal vol of buffer P2 (0.2M NaOH/1%(w/v) SDS) was added to lyse the cells and denature the DNA. The tubes were gently mixed by inversion and then incubated for 5mins at room

temperature. 10ml of cold buffer P3 (potassium acetate/glacial acetic acid, 3M and 2M respectively) was then added and then incubated on ice for 20 mins. The tubes were centrifugated at 20,000 X g for 30 min at 4°C to separate the plasmid DNA from the cell debris. The supernatant was removed and the pellet was re-spun at 20,000 X g for 15 mins at 4°C. 10ml of buffer QBT was added to the QIAGEN-tip 500 and allowed to empty by gravity. On completion of the spin the supernatant was removed and added to the QIAGEN-tip and allowed to enter the column by gravity. The column was then washed twice with 30ml of buffer QC and the DNA was eluted from the column by the addition of 15ml of buffer QF. The DNA was collected in a 30ml glass tube and precipitated by the adding of 0.7 volumes of isopropanol. The mixture was centrifuged at 15,000 X g for 10mins at 4°C and the supernatant removed. This left a DNA pellet which was washed with 5ml of 70% (v/v) ethanol and then centrifuged again for 10 mins at 15,000 X g, 4°C. The supernatant was removed and the pellet was then dissolved in TE buffer (Tris/EDTA), pH 8.0 and plasmid concentration was determined (see section 2.13.4)

2.12.2 Preparation of genomic DNA from tissue culture cells

$1X10^6$ cells were harvested and pelleted by centrifugation (see section 2.2.3). The cell pellets were resuspended in 1ml of 1 X PBS and transferred to 1ml eppendorf tubes and spun at 13,000g in a microcentrifuge for 10 mins at 4°C to re-pellet the cells. The PBS was removed, the cell pellets resuspended in 0.5ml of digestion buffer (10mM TRIS-HCl pH7.9, 1mM EDTA, 0.3M sodium acetate, 1% 9w/v) SDS and 200μg/ml proteinase K) and digested overnight at 37 °C in a shaking incubator. The following day undigested cell debris was pelleted by centrifugation at 13,000g for 10 mins at 4 °C. The supernatant was removed and 1ml of 100% ethanol added, inverted several times until threads of DNA were visible. The DNA was either spooled out using a glass pasteur pipette or pelleted by centrifugation at the same speed and temperature as above for 15 mins. The DNA was then washed with 70% (v/v) ethanol and allowed to air dry for 10 mins. The DNA was resuspended in 500μl of TE buffer, incubated at 65 °C for 10 mins and the tubes flicked to disperse it. The DNA was then stored at -20 °C and used for PCR and southern blotting.

2.12.3 Synthesis of Oligonucleotides

Oligonucleotides were synthesised at the BICR as a core service on a 394 RNA/DNA synthesizer (Applied Biosystems,UK) using Cruachem reagents. Oligonucleotides were deprotected and lyophilised using Hybaid Proligo system and supplied in this form. The oligonucleotide was resuspended in 500µl of milli-Q H2O and the concentration determined as described below (section 2.13.4).

2.12.4 Quantitation of DNA Concentration

DNA was quantified by spectrophotomeric determination of its UV light absorbency. 5µl of sample was added to 495µl of de-ionised water and the absorbency of the solution was measured at 260 nm and 280 nm in a quartz cuvette, using de-ionised water as a blank. The concentration of the solution was calculated using Beer's law which states that an optical density of 1.0 at 260 nm corresponds to a concentration of 50mg/ml for double-stranded DNA and 30mg/ml for single-stranded oligonucleotides. Pure preparations of DNA have an absorbance ratio between 1.5 and 1.9 at A260/A280.

2.13 Electrophoretic Techniques

2.13.1 Agarose gel electrophoresis of non radioactive PCR products

Non-radioactive PCR products were separated on the agarose gels and visualized by staining with ethidium bromide and UV transillumination. Gels were prepared by melting the appropriate amount of agarose in 1 X TAE (40 mM tris-acetate and 2mM EDTA pH8) by microwaving on a medium heat for 2-3 min. A final concentration of 0.5µg/ml of ethidium bromide (Sigma,Poole, UK) was added to the agarose and the gel was poured into the appropriate casting tray. The gel was then allowed to set and once solid, placed in the electrophoresis tank containing 1 X TAE running buffer. 1/5 vol of loading buffer (50% glycerol, 100mM EDTA, 1 X TAE, 0.1% bromophenol blue) was added to each sample. The samples were loaded into the appropriate wells and electrophoresis performed for 1hr at 100V to ensure separation of bands. Samples were run against an appropriate mass ladder to estimate the size of the fragments.

2.13.2 Denaturing Polyacrylamide Gel Electrophoresis

The radio-labelled PCR products of polymorphic markers (see section 2.15 below) were resolved on a range of different percentage polyacrylamide gels under denaturing conditions. This range was dependent on the length of the PCR product (See table 3). A gel solution was prepared from the Flowgel (Flowgen Instruments, Staffordshire, UK) stock solutions using 7.5M Urea (Flowgel Diluent), 25% w/v acrylamnide solution 19:1 acryl:bisarcryl and 7.5M urea (Flowgel Concentrate), and Flowgel Sequencing Buffer comprising 7.5M urea, 1M tris-borate and 20mM EDTA pH8.3. The solution was polymerised by the addition of 650µl of 10% w/v ammonium persulphate and 100µl of TEMED per 100ml of gel solution. This solution was then poured between glass plates separated by 0.4mm spacers and allowed to set at RT for approximately 1 hr. Before loading the gels were pre-run at 60W for 45 mins in 1 X TBE (90mM tris-borate and 2mM EDTA pH 8.0) to warm the gels. Denaturing gel-loading buffer (98% v/v formamide, 10mM EDTA pH 8.0, 0.1% w/v xylene cyanol and 0.1% w/v bromophenol blue) was added to the completed PCR reactions (5ul added to 10ul reaction) and this mixture denatured by heating at 95°C for 5 mins and immediately quenched on ice. 20µl of this mixture was subjected to electrophoresis at 50W for approximately 4 hrs depending upon the size of the PCR product. The gel was then transferred to a sheet of whatman 3M paper and dried under vacuum at 80°C for 45 mins. The PCR products were detected by autoradiography using Kodak X-omat film (Kodak, UK).

2.14 PCR and analysis of amplification products

2.14.1 Microsatellite Analysis

Total reaction volumes were 10µl containing 40ηg genomic DNA, 10mM tris-HCl, pH 8.3, 50mM KCl, 1.5mM MgCl2, 10% (v/v) dimethylsulphoxide, 1µM of each oligonucleotide primer, 200µM of each deoxynucleotide triphosphate, 0.4µl of [α^{32}P]dCTP (24.7kBq/µl Amersham, UK) and 0.5 units of Taq polymerase (PE Allied Biosystems, UK). Reactions were subjected to an initial denaturation step of 5 min at 94°C, followed by 30 cycles of 30 sec at 94°C; 30 sec at 55-65°C; and 30 sec at 72°C, and a final extension step of 7 min at 72°C. Radiolabelled samples were electrophoresed on 4-10% polyacrylamide gels under denaturing conditions. Gels were dried then exposed to X-ray film to visualise the resolved reaction products. The following primers were used to

examine specific cell cycle regulators: D17S1353B (p53), D13S153 (pRB), D11S922 (p57), D6S1051(p21) and D9S1870 (INK4A)(see table 2.3)

Table 2.3 Summary of Different percentages of polyacrylamide gels used to separate microsatellite products

Microsatelite marker	% of polyacrylamide in gel	Size of product
D9S1870 INK4A	6%	179 – 221bp
D11S922 p57	8%	88 – 138bp
D6S1051 p21	5%	223 – 239bp
D13S153 pRB	7%	155bp
D175S1353 p53	6%	184 – 122bp

2.15 Protein analysis

2.15.1 Western blotting and antibodies

This was performed essentially as described by Munro et al (127, 128). 100 μg of protein was subjected to electrophoresis on 10% (c-myc) or 17% (CDKIs) non-denaturing polyacrylamide gels. Primary antibodies were: $p16^{INK4A}$ (C-20; Santa Cruz Biotechnology, California, U.S.A.), ERK2 (Transduction Laboratories) and c-myc (N-262; Santa Cruz Biotechnology). The SV40-immortalised human keratinocytes line SV6-1 HFK was used as a positive control for $p16^{INK4A}$ and the human squamous cell carcinoma line BICR31 as a negative control. BICR31 harbours a homozygous deletion of the INK4A locus. The HaCaT line is an immortal human skin keratinocyte line that expresses high levels of $p15^{INK4B}$. The human squamous cell carcinoma line BICR78, which does not express $p15^{INK4B}$ mRNA, served as a negative control for $p15^{INK4B}$. HFF UV are human foetal skin fibroblasts irradiated with 8 milli-joules of ultraviolet light from a stratalinker 2400 (Stratagene, USA) and harvested 16h later.

2.15.2 Telomerase Activity

Cellular extracts were prepared and assayed for telomerase activity using the TRAPeze kit (Intergen, Company, UK) following the manufacturer's instructions. The PCR products were resolved on 10% (v/v) non-denaturing polyacrylamide gel. To semi-quantitate telomerase activity, the exposure of the 36bp internal standard to the telomerase ladder was compared. The intensity of the bands were measured using the Bio-Rad molecular imager and quantified by Quant One imaging software.

2.16 Regeneration of striated muscle in vivo

The main part of this experiment was carried out by Dr D Watt at Imperial College. I provided the cells for injection, cut the sections, stained, examined and photographed them. Dr Watt provided the RAG-1 mice, injected the hTERT-transfected and control cells, sacrificed the animals and dissected the area affected.

RAG-1 mice were selected because they would not reject the implanted human muscle cell and could with stand the procedure. The RAG-1 mice were irradiated with a single dose of X-radiation, to inhibit regeneration of the mouse own cells, before the procedure. The grafted cells are then more likely to regenerate damaged muscle (Watt et al 1982). Barium chloride, a myotoxic agent, was administered by injection. Barium chloride was used as it stimulates necrosis of the muscle without damaging the basement membrane (Caldwell et al 1990). Forty eight hrs after injection, the right tibialis anterior (TA) was implanted with cells. The cells were first trypinsied and counted. $3x10^5$ cells were pelleted by centrifuge. The skin over the tibialis anterior muscle was surgical excised and the pellet of cells was delivered. The cells were placed using a glass PCR pipette and injected through the epimysium into the muscle. The tip of the pipette was stained with red dye to allow identification of the injection site latter (Gibson et al 1995). Six TA muscles received T 15 cells, two, T2 cells and three, control human muscle cells. 25 days after implantation, the recipients were sacrificed and frozen sections prepared. These were stained with H+E or, for spectrin by immunochemical methods. The number of spectrin-positive fibres, indicative of myofibre fusion, was observed by counting at least 5 fields adjacent to the injection site. Only fibres positive for spectrin on all sides were considered positive. At least 1000 cells were scored per sample.

3 Results

3.1 Extension of replicative lifespan of HMCs by hTERT expression

HMCs was explanted from a needle biopsy specimen, from healthy volunteers. Ethical permission was obtained from the North Glasgow Hospital University Trust. Cells were allowed to grow out of the biopsy and transferred into petri dishes. After 25 mean population doublings (MPD) the HMCs were infected with either a retrovirus containing hTERT or with a neomycin (NEO) selection marker alone. Previously a line of human muscle cells had been infected in the same manner from a different donor after 5 MDPs by Dr K Steeghs who carried out preliminary work before the start of my PhD. Mass culture and selected culture were grown under G418 selection. From the first line, five hTERT expressing clones (T1, T2, T3, T4 and T15) and 3 empty vector controls expressing the neomycin resistance gene (NEO 1, NEO 2 and NEO 3) were studied and the mean population doublings (MPD) was determined.

Before and after transfection with the hTERT the muscles cells retained the same appearance. The cells were mono-nucleated and grew in a single layer. When plated at low density the muscle cells had a large oval shape; however as the cells approached confluence they became more spindle shaped. The cells always remained mono-nucleated even at this point. The cells were never allowed to reach full confluence. When a plate was 70-80% confluent the cultures were split. In the case of T15, the plate occasionally required splitting twice a week. Each time the cultures were split the cells MPD was calculated and recorded. The growth rates of the clones can be seen in Figure 1a. As seen from the graph the NEO clones all senesced at approximately 37 MPDs. All of the hTERT expressing cells had bypassed senescence although there were variations in cell proliferation rate. Clone T1 showed an initial slow growth rate but it did not reach senescence. In fact over time the growth rate of T1 increased. This alteration in growth rate suggests that a secondary event had taken place which allowed it to overcome senescence. T2, T3 and T4 all showed a consistent linear growth rate, which did not change considerably over the course of time. The most striking result was seen in T15. It displayed a consistently fast growth rate and has bypassed 120 MPDs. All of the hTERT expressing clones display extension of proliferative lifespan of at least 60 MPDs.

Figure 3.1 Growth curve of hTERT clones and NEO clones

Telomerase expressing HMCs clones show variable growth rate. Each clone is indicated on the graph. In the first set of transfections (A) all of the Neo-infected clones senesced after 21-28 MPDs, whereas the hTERT-infected clones have completed between 45 (T1) and 125 (T15) MPDs. In the second transfection (B) the neo pool (HMCN) senesced at 36 MPDs while and the neo clone (NA), senesced at 40 MPDs. The hTERT transfected pool (HMCT) continued to proliferate and at last calculation had reached 57 MPDs and the hTERT clone (TC) had by passed 48 MPDs.

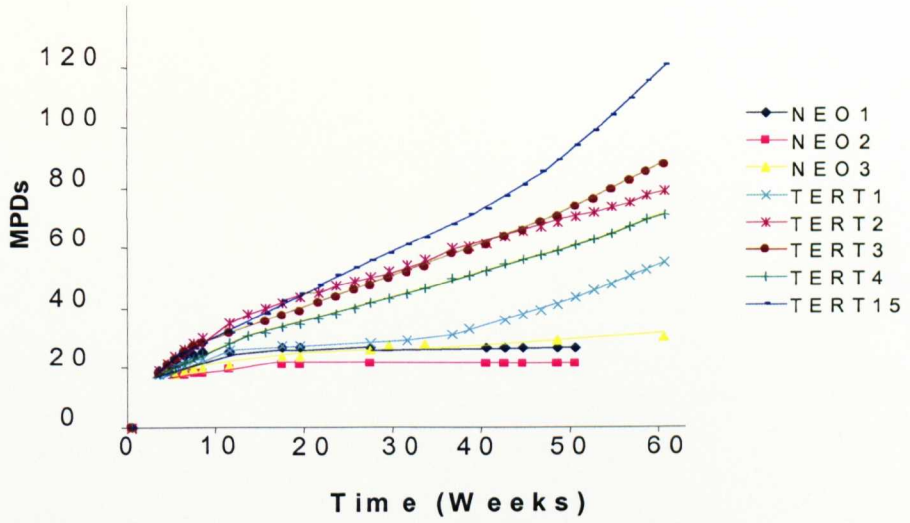


Figure 3.1A The growth curve of the first infected cell strain

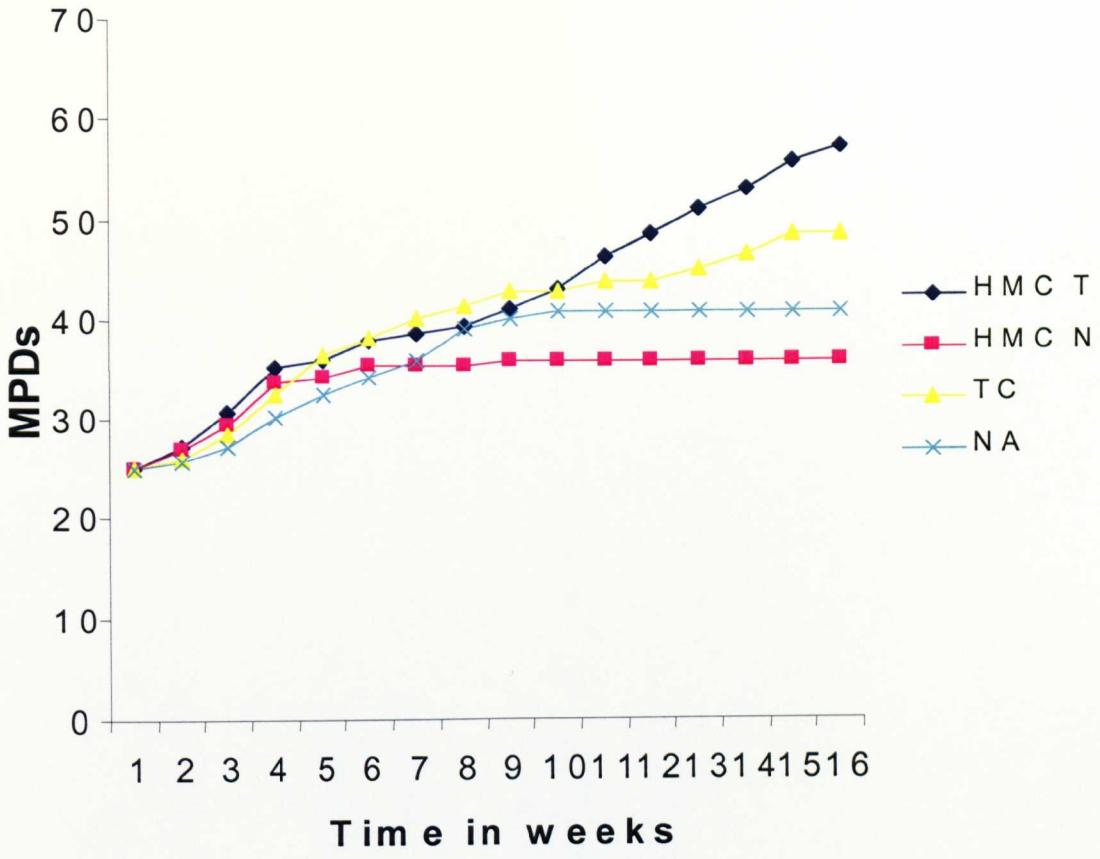


Figure 3.1B Growth curve of the second infected cell strain

To determine that the cells were in a cycling state, the levels at which they incorporated bromodeoxyuridine (BrdU) was measured. This can be seen in Figure 3.2. Using HeLa cells as a control it was seen that the incorporation of BrdU was low in Neo 3 after 21-28 MPDs, with considerable higher incorporations in all of the hTERT expressing clones. The level of BrDU incorporation was directly comparable to the clones growth rate. With T15 showing incorporation at the same level as the HeLa control cells.

Transfected cells were tested for the expression of β-galactosidase, which is a marker for senescence. The results for this experiment can be seen in Figure 3.3. HMC Neo cells stained positive while HMC hTERT cells remained negative. On observation of the cells, all of those which had been infected with hTERT had a spindle shaped appearance which is characteristic of proliferating cells. While those which were transfected with NEO or control HMCs as time passed, showed the characteristic flattening and widened shape of senescent cells.

Figure 3.2 BrdU incorporation in cycling cells

The incorporation of bromodeoxyuridine (BrdU) into the different HMC clones and cultures demonstrates the difference in the growth rates. With T15 the fastest growing line, showing the highest level of incorporation and T1, the slowest growing line, demonstrating the lowest level of incorporation.

BrdU incorporation was analysed at 25 weeks after initial transfection. The experiment was performed 4 times with approximately 5 % variation between experiments.

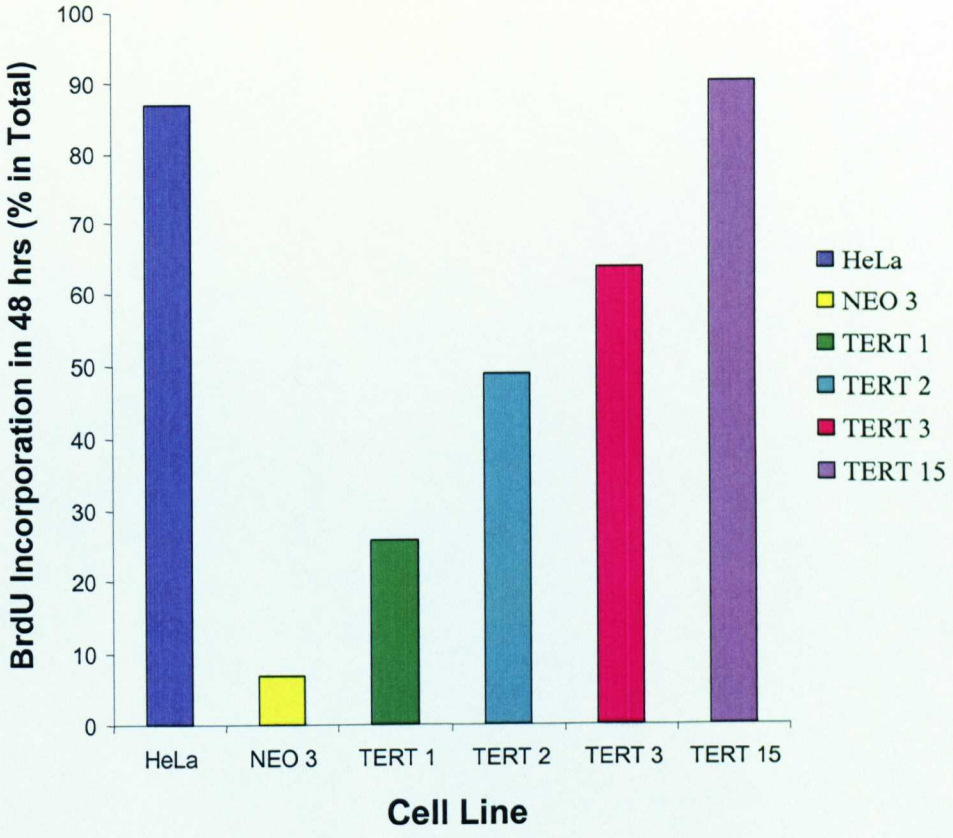


Figure 3.2 BrdU incorporation in cycling cells

Figure 3.3 β-galactosidase incorporation in transfected and non transfected cells

Staining of the Neo-control HMC line 2 after 37 MPDs for senescence-associated β-galactosidase shows numerous flat cells that stain positively and appear blue on the photograph (A) In contrast the hTERT infected HMC cells showed few positive cells (B). Arrows denote positive staining.

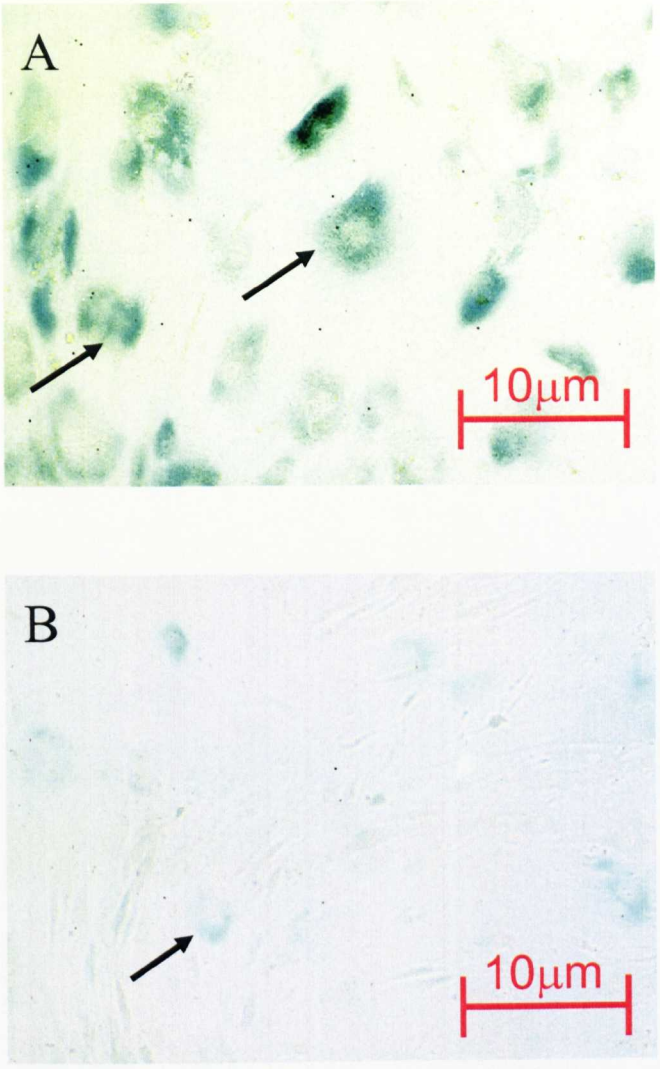


Figure 3.3 β-galactosidase incorporation in transfected and non transfected cells

3.1.1 Discussion

It would have been expected that the rate of growth would remain constant and this was the case for T2, T3 and T4, however, this was not true of all the clones. The most interesting aspect of the cell growth rates was the difference between the different TERT clones. In most studies showing immortalisation, this difference in growth rate has not been discussed because full populations of cells are examined and not clones derived from an individual cell (129). When examining Werner syndrome fibroblast (a disorder characterised with early ageing) it was discovered that these cells had limited replicative potential, thought to be due to chromosome instability. To assess the role of telomere length in the senescence of these cell single telomeres of individual clones were examined, rather than examining a pool of cells. There were great differences between the rates of erosion of telomeres from clone to clone (between normal ranges and four times normal range). It was suggested that in the pools of fibroblasts, cells with faster eroding telomeres were being overpopulated by the still replicated 'normal' cells. Therefore the results were not giving a clear picture. In the hTERT cells, if pools of cells were examined rather than clones, it is easy to assume that T15 may have taken over the population and altered the results.

The change in some of the growth rates may be due to a secondary alteration. This seemed the most plausible reason behind the growth of T1 which suddenly accelerated after 20 MPDs. This cell line appeared to be approaching senescence but escaped and continued to proliferate for another 30 MPDs.

T15 was the most interesting clone due to its high proliferation rate. This was much faster than the other clones, reaching 120 MPDs. In fact, T15 has not stopped proliferating to date. The success of this clone is unknown but it could be due to the fact that the cell was immortalised at a primitive stage in the myogenic lineage. This would mean that the cell would already have long telomeres and therefore be able to maintain these with the help of hTERT. Also using retroviral vectors means that you cannot be sure where the gene has inserted, i.e. it may have inserted into multiple sites. This may result in the expression of other genes due to insertion mutagenesis. This may be giving T15 an advantage over the other clones. Another possibility is that T15 is expressing more telomerase than the other clones, and this could occur if the retroviral vector had entered at a site near to a strong promoter which is highly transcribed.

3.2 Growth conditions for transfected cell lines

The cells lines were established in DMEM with 10% foetal calf serum, with added penicillin and streptomycin. When examining the literature for the culture of human muscle cells it was discovered that F10 was commonly used as the chosen medium. Previous attempts at immortalisation with the same retroviral vectors, in Ham's F10 had proven unsuccessful. Therefore, to test the importance of medium in these experiments normal HMC cells was transferred into Ham's F10 after 25 MPDs, T2 was transferred after 70 MPDs and HMC T15 after 115 MPDs. The cells were grown for more than 100 days and their MPDs were calculated, as seen in figure 3.4. All the cell lines grew more quickly in DMEM compared to F10. HMC cells grew for 4 more MPDs in F10 but grew for greater than 10 MPDs in DMEM. T15 and T2 both entered senescence after being grown in F10. This experiment was repeated using 15% and 20% foetal bovine serum (FBS) instead of 10% foetal bovine serum. In the case of HMC this increased the MPD by 2.5 for 20% FBS but this did not alter the result from the TERT clones. Both of the TERT clones examined underwent senescence after 6 MPDs in the case of T2, and 1 MPD in T15. This result was not altered when the concentration of serum was increased to 20%. Therefore for the rest of the experiments cells were grown in DMEM as described.

To further establish the importance of growth conditions, cells were also grown in low concentrations of oxygen to see if this favoured the growth rate of the cells. Preliminary results are shown (see table 3.1), and it appears to have no effect on cell growth rates.

Cell Line	MPDs in normal oxygen conditions over 3 weeks	MPDs in low oxygen conditions over 3 weeks
HMC	4.5	4.2
T15	7.8	8.2

Table 3.1 Effects of low oxygen on growth rates of HMC and T15 cells.

Figure 3.4 Growth rate of cells in different medium

A) HMCs show a reduced replicative lifespan in Ham's F10 medium compared with DMEM. HMC line 2 (a) was cultured in DMEM with 10% foetal bovine serum and after 25 MPDs was either kept in DMEM (pink squares) or transferred into Ham's F10 (blue diamonds). Clone T2(b) was compared in the two media as in (a) but it was transferred at 70 MPDs and clone T15 (c) was transferred into F10 at 115 MPDs.

B) Comparison of T15 and HMC in different culture conditions and their MPDs resulting from these changes.

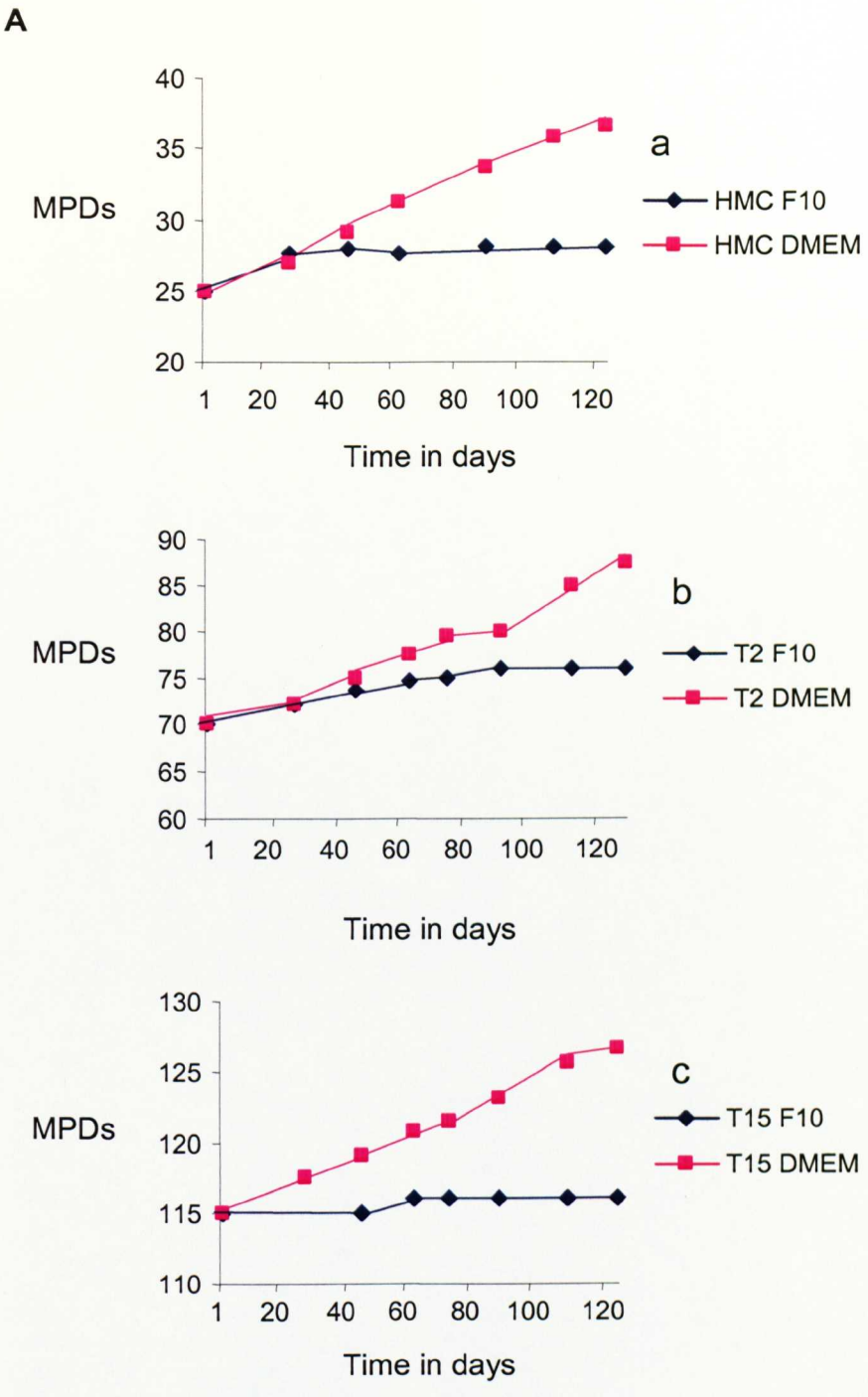


Figure 3.4 Growth rate of cells in different medium

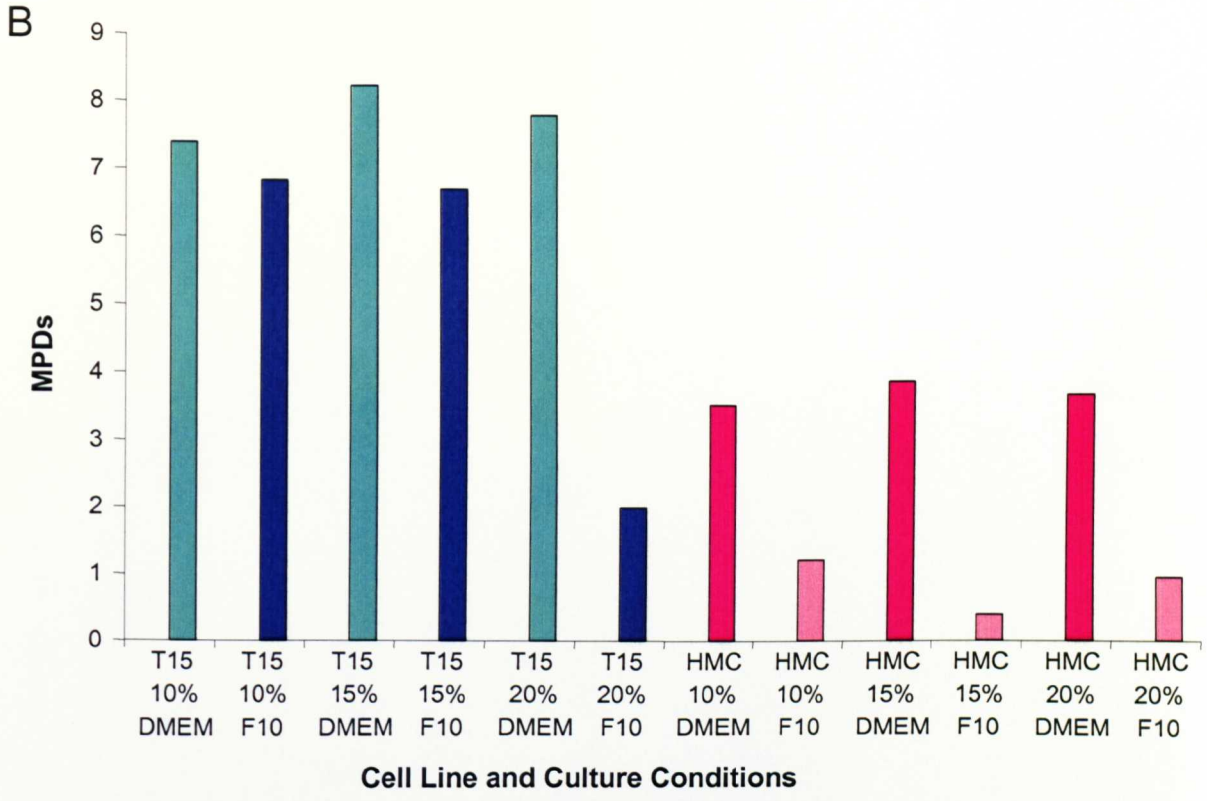


Figure 3.4 Growth rate of cells in different medium

3.2.1 Discussion

There have been previous attempts to immortalise HMCs with hTERT, which were unsuccessful (130). Why our protocol was successful and others have failed, is not completely clear. However, what was evident is how the use of different media made a difference in the ability of these cells to grow. It was merely by serendipity that the HMCs were grown in DMEM since, this was the standard medium used in our laboratory for growing fibroblasts. It was not until I searched the literature, that I was aware that F10 and F12 are the media more commonly used. It was at this point that I decided to see if different media would alter the growth rates. I initially anticipated that the use of F10 or F12 would allow some of the slower growing clones to grow faster, but the opposite was true. The use of F10 results in senescence of the hTERT clones. Therefore it is reasonable to suggest that the choice of medium used may have led partly to the failure of other groups to immortalise these cells. There are many differences in the formulations of Ham's F10 and DMEM including the inclusion of biotin, Thiamine, and vitamin B12 in Ham's F10 and six fold higher concentration of calcium in DMEM. It is likely however that not only the medium but also the incubators, the reagents and other in-house factors may play a role.

Another explanation for the success of this experiment was the early removal of the selecting agent G418. G418 was removed as soon as the mock infected cultures died. It has been shown that in normal human keratinocytes, if G418 is kept on the cells the replicative lifespan of the cells decreases (131). Therefore this may have played a role in the viability of the cells.

It was recently suggested that low oxygen concentrations may allow cells to grow for longer (132, 133). To test this, a pilot experiment was set up. This initial experiment did not seem to have any effect on the growth rate, however, and was hard to maintain as the cells were susceptible to fungal infections. However I would not rule this out as I feel that I may have not perfected the technique and would have liked to repeat this experiment on a bigger scale and include cell line controls that are known to grow better in low oxygen.

3.3 hTERT-expressing muscle cells lack transformed properties

It has previously been reported that fibroblasts can have extended replicative lifespan by transfection with hTERT without the malignant transformation (134). However since there is such a strong relationship between telomerase activity and malignancy, the transfected cell lines were tested for transformation.

3.3.1 Effect of low serum on MPD

Cells were grown in 0.2% FBS in DMEM over 10 weeks. Every 2 weeks cells were split and MPD recorded. Figure 3.5 shows the total MPD over the 10 weeks using normal HMC and HeLa cells as a control. As can be seen HMC control cells grew for 3 MPDS while all hTERT infected cell lines could only manage less than 2 MPDs in 10 weeks. In contrast T15, the fastest growing cell line, proliferates for 18 MPDs in the 10 weeks in 10% serum.

Figure 3.5 Observation of the growth in low serum

Proliferation of HMC TERT clones in 0.2% FBS in DMEM is minimal over 10 weeks, and is less than early passage HMC. HeLa cells were able to undergo considerable proliferation under the same culture conditions. HeLa cells were used as a positive control and normal HMCs at early passage used as a negative control. For comparison of the ability of cells to grow in 0.2% FBS, T15 in 10% FBS in DMEM is shown. These results are from one experiment with four replicates.

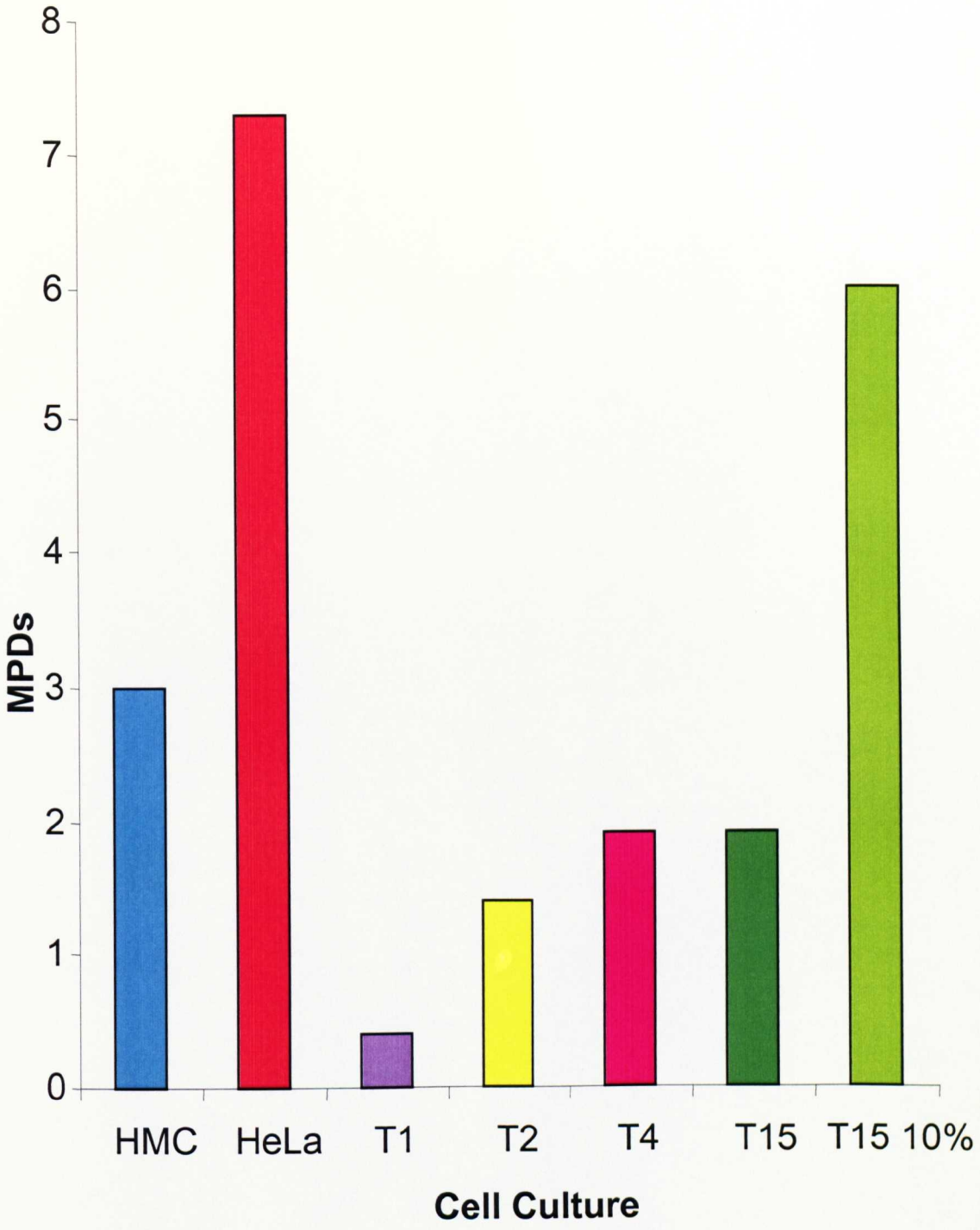
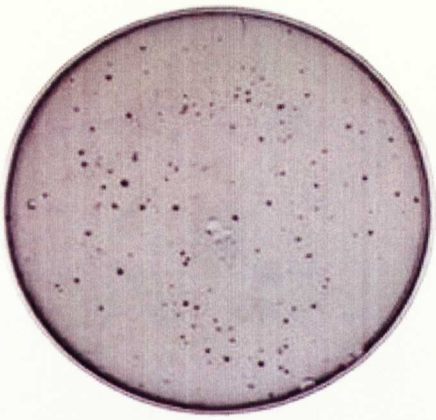


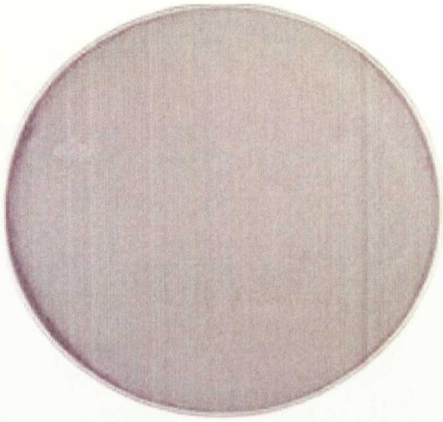
Figure 3.5 Observation of the growth in low serum

3.3.2 Cell growth in agar

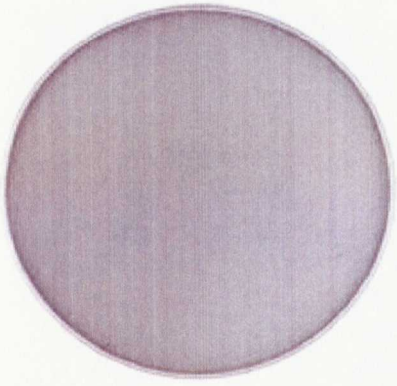
The cell lines were then grown in agar to test for anchorage dependency. Human foetal fibroblasts (HFF) and HeLa cells were used as negative and positive controls respectfully. The HeLa cells grew in numerous colonies (as can be seen in Figure 3.6), however the transfected cells failed to grow in agar. Colony efficiency of the HeLa cells was determined (see Figure 3.7).



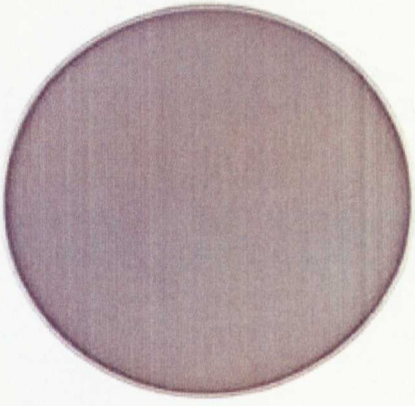
HeLa



HFF



HMC T2



HMC T15

Figure 3.6 Results of Agar assay.

Cells were grown on plates coated with agar. After 4 weeks the visible colonies were counted.

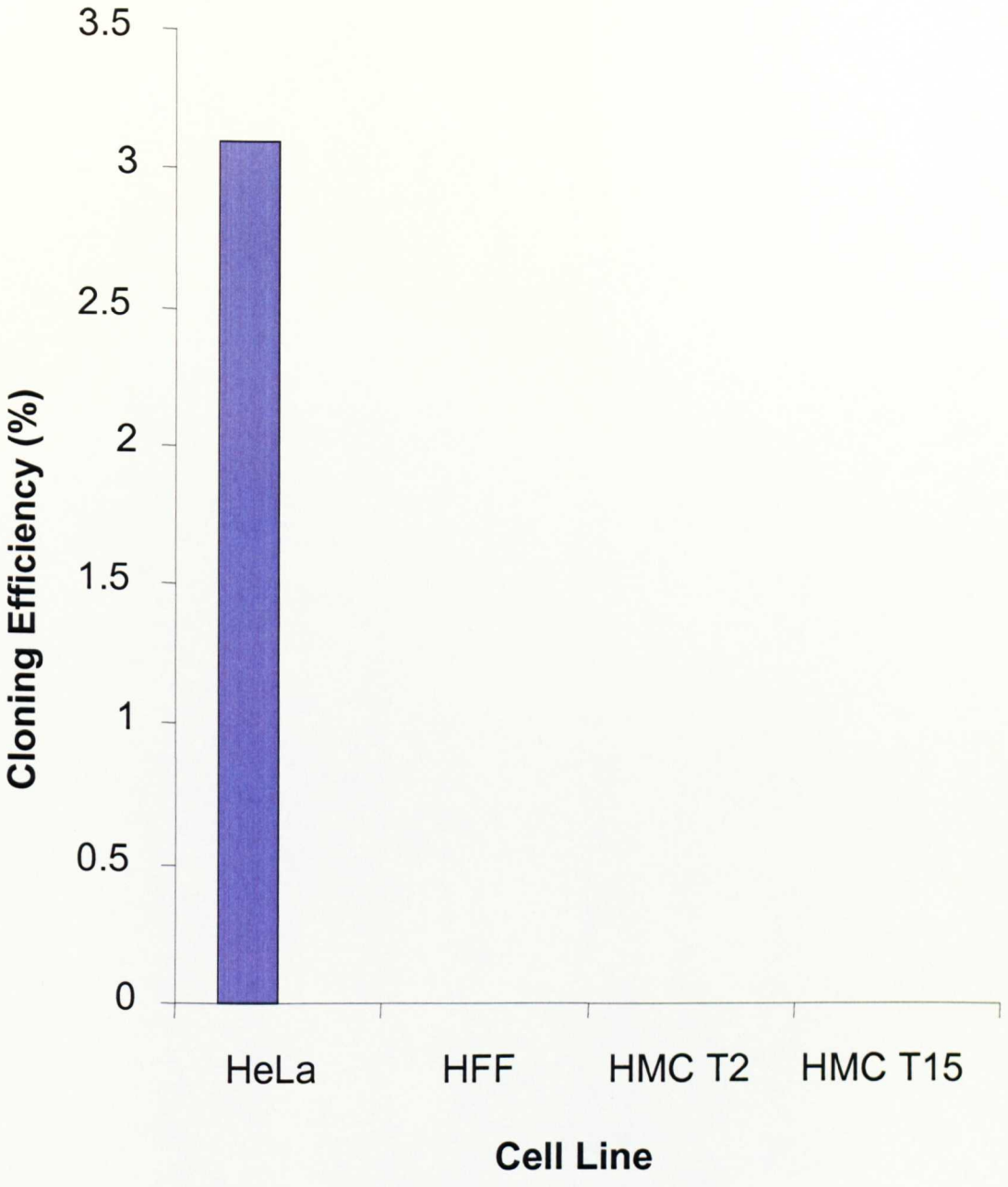


Figure 3.7 Graph summarising the cloning efficiency in soft agar

Count of all visible colonies was performed and the cloning efficiency determined

3.3.1 Discussion

It was very important to examine the cells for signs of malignant transformation. None was found as identified by lack of growth in agar and low concentrations of serum. This is in keeping with reports of other cell types transfected with hTERT, including fibroblasts and retinal epithelium. Immortalisation of cells is not a straight forward exercise. Different cell types require different techniques. For example, some cell lines are actually developed from malignant cells, eg. HeLa cells and rhabdomyosarcoma cells. Some normal cells have been transfected with an oncogene, e.g. myocardial fibroblasts, mammary epithelial cells and keratinocytes with HPV and lymphocytes, with EBV. In contrast, there are those cells which are transfected with hTERT i.e. fibroblasts and endothelial cells. These cells are immortalised with hTERT alone. They tend to have fewer abnormalities than those immortalised by other means. Therefore it was not surprising but promising that no sign of malignancy was found in the hTERT-transfected HMCs. Even when the fastest growing clone was selected, no signs of malignancy were found. My results are in keeping with those of other workers.

3.4 Determination of Cell Type

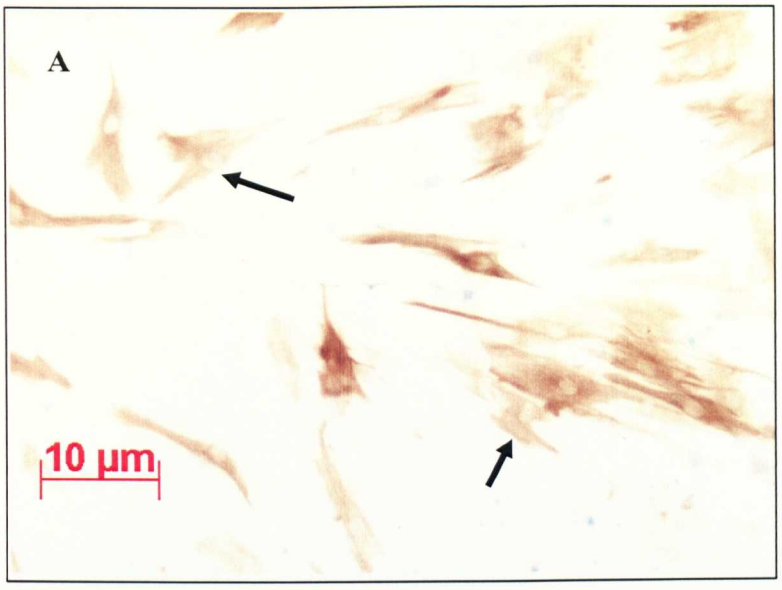
The TERT transfected cells lines were grown on glass chamber slides, fixed and stained for Desmin. Desmin was used as it is one of the first proteins expressed when muscle cells enter the myogenic lineage (see figure 1.13). All cells were desmin positive with characteristic cytoplasmic staining (figure 3.8). Normal human muscle cells were used as a positive control and human foetal fibroblasts (HFF) as negative control.

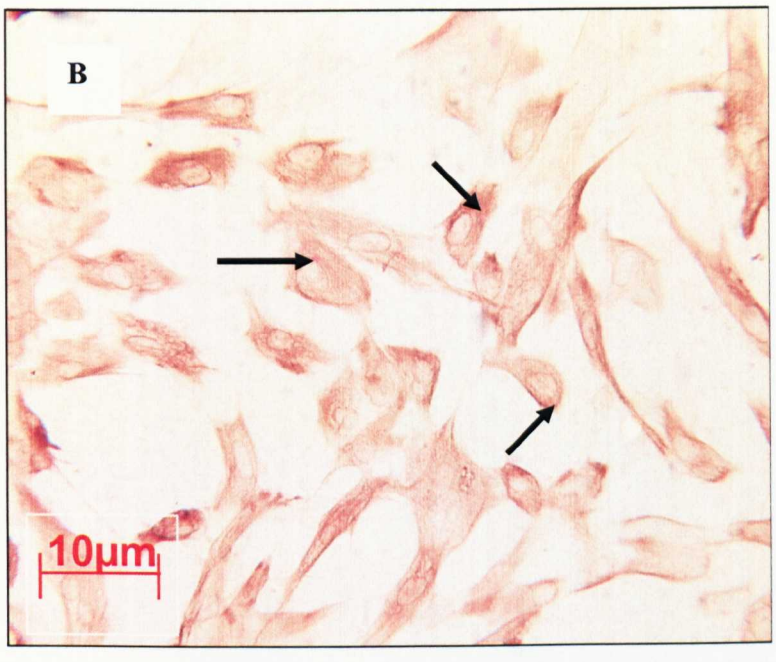
3.4.1 Karotyping of Cell Lines

The chromosomes of all the cells lines were stained by Hazel Ireland and then I counted to determine if there were any chromosomal abnormalities. Over 50 chromosomes spreads where counted for four of the cell lines at 50 – 70 MPD and the number of chromosomes in each spread noted. Figure 3.9 shows the hTERT expressing HMC cells had a modal chromosome number of 46 and figure 3.10 a typical normal karyotype from T15.

Figure 3.8 Desmin staining

Positive desmin staining on control of normal HMC (A), positive staining on clone T15 cells (B) and a negative control of HFF (C). The arrows show the positive staining cells.


A
10 µm


B
10µm

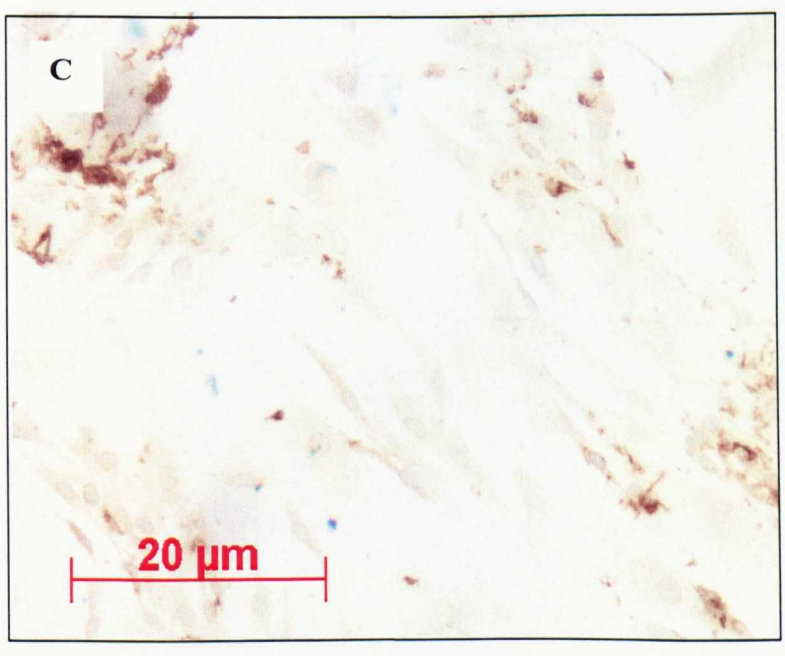

C
20 µm

Figure 3.9 Showing the normal model number of chromosomes

Each graph denotes a clone of hTERT transfected HMCs: A – T1, B- T2, C – T3, D-T4 and E-T15. As can be seen from the graphs all have a modal chromosome number of 46. These results are from one experiment.

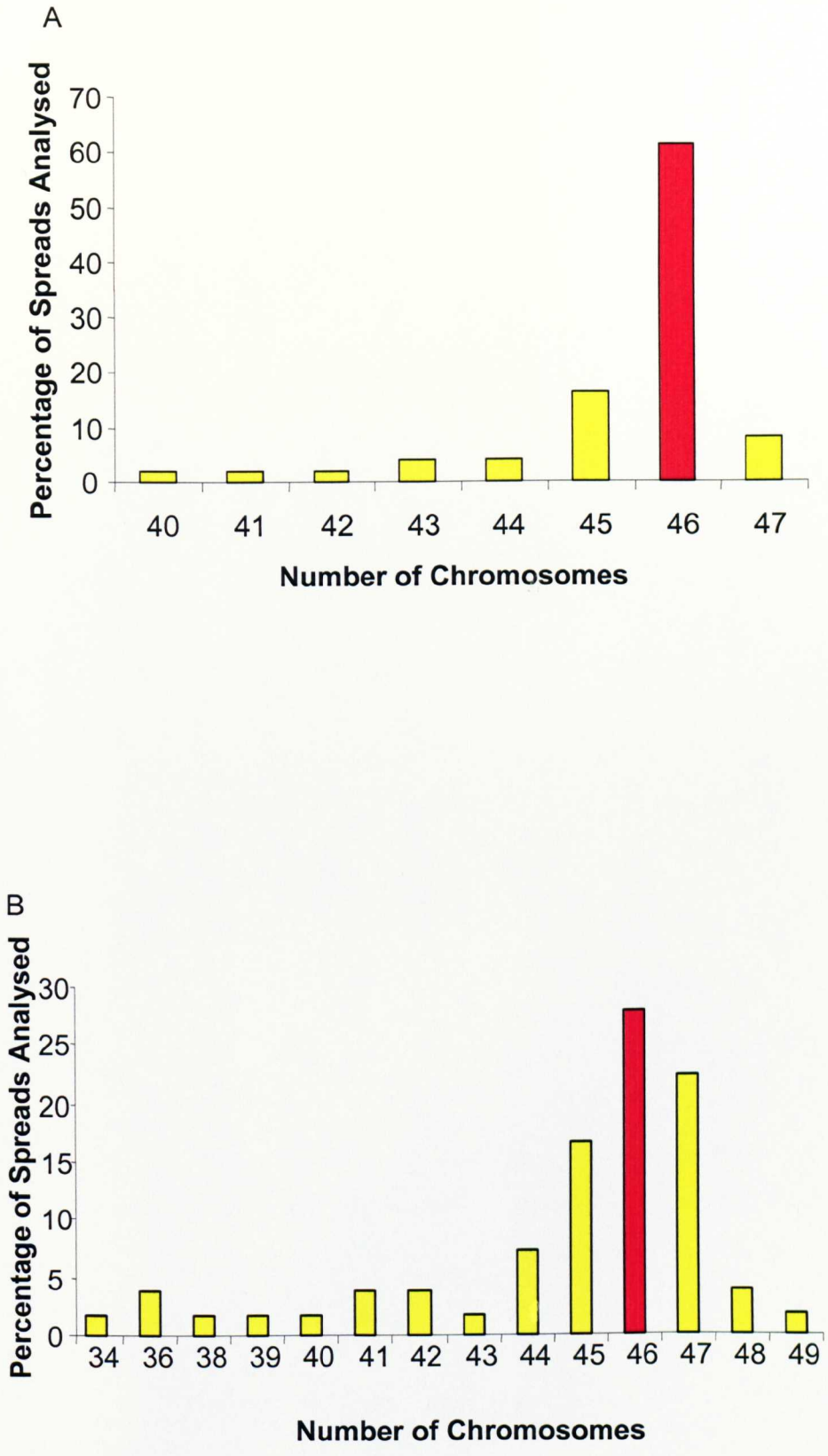
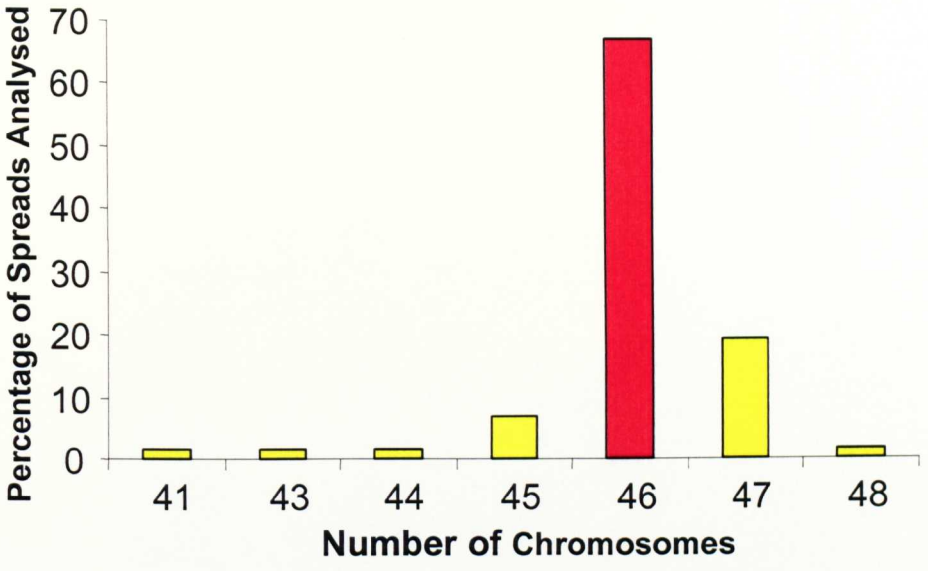


Figure 3.9 Showing the normal modal number of chromosomes for each of the clones studied (A-B).

C



D

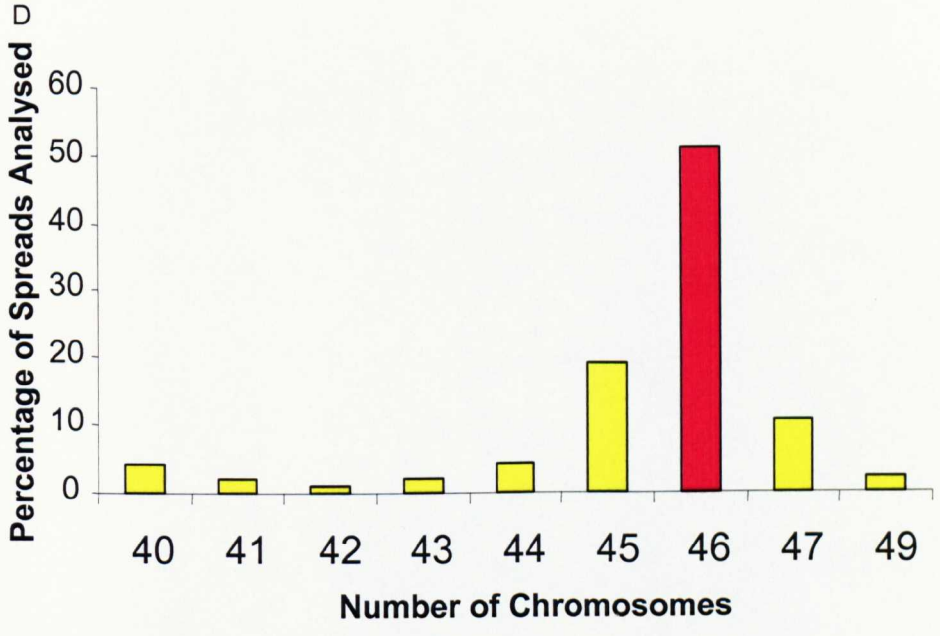


Figure 3.9 Showing the normal modal number of chromosomes for each of the clones studied (C-D).

E

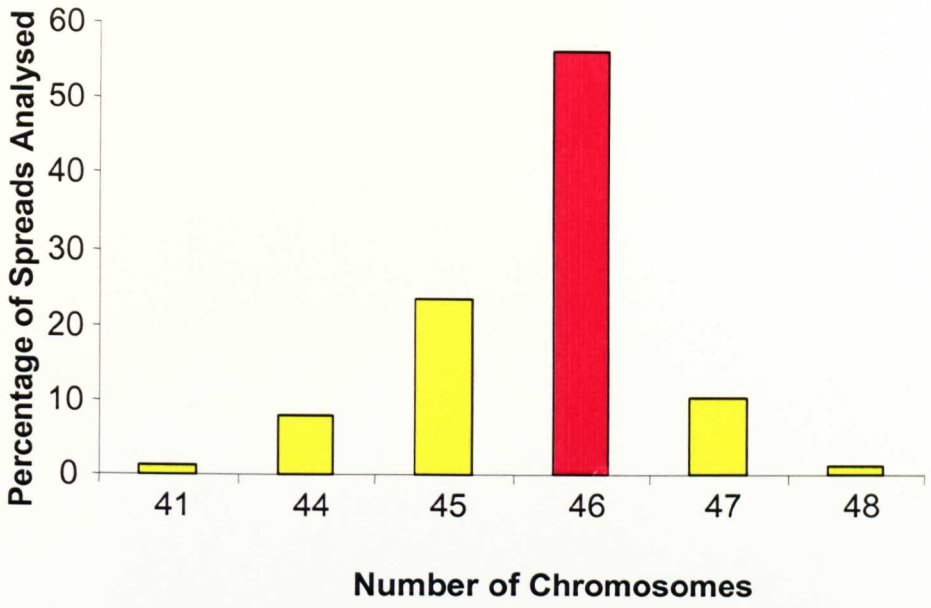


Figure 3.9 Showing the normal modal number of chromosomes for each of the clones studied (E).

Figure 3.10 Typical karyotype for T15

The G-banded karyotype of HMCT15 was typical of eight metaphases examined in detail and revealed no obvious abnormalities. The analysis of the G-banding was carried out by Hazel Ireland.

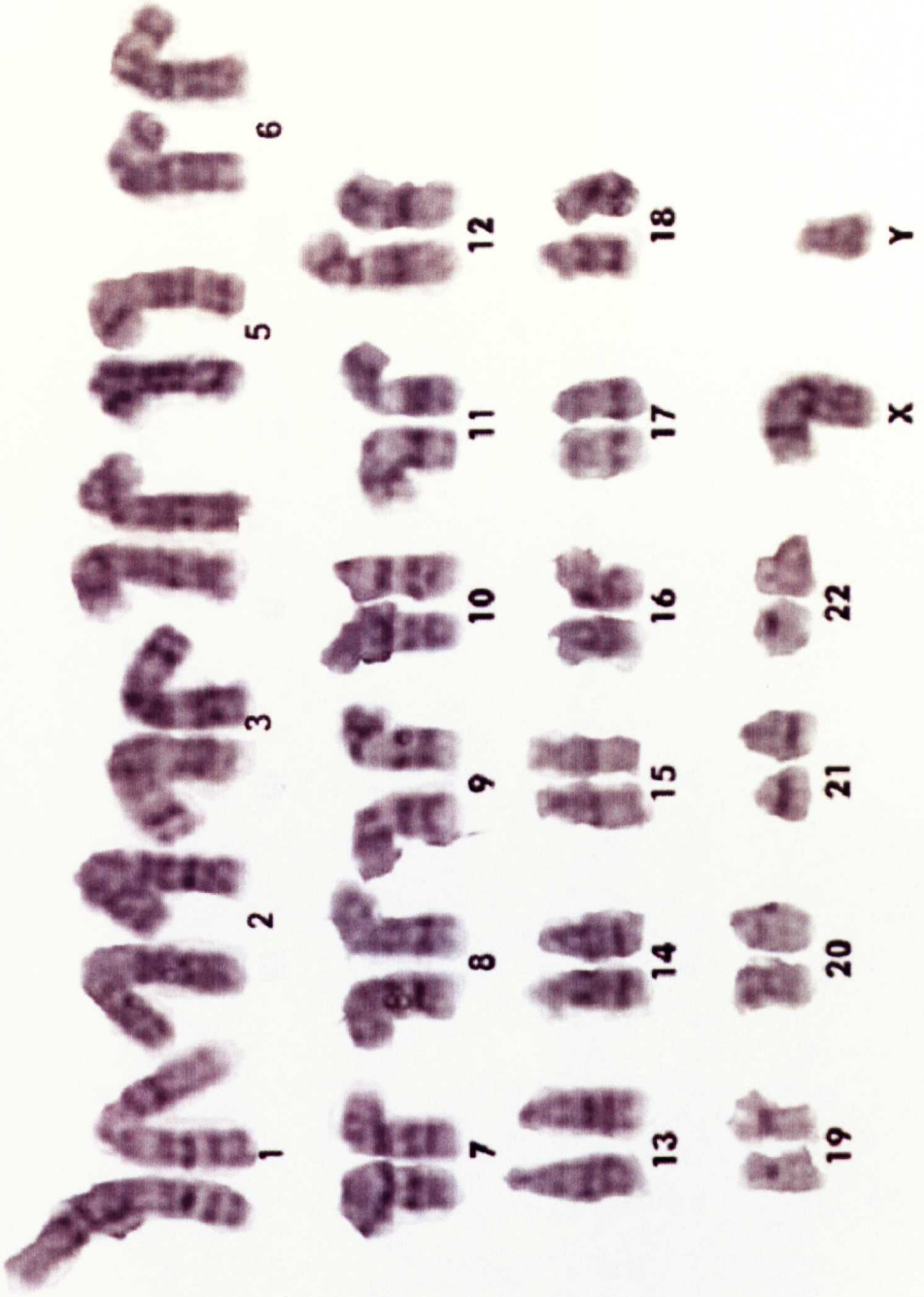


Figure 3.10 Typical karyotype for T15

3.4.2 Discussion

Desmin was used as the muscle specific marker to determine if the cells were of the myogenic lineage. The reason for this is that it is expressed early on in the differentiation of cells committed to this lineage. It was expected that the cells from the healthy adult volunteer would be at this stage *in vitro*. Desmin is also useful because it is expressed in myogenic cells throughout their lifespan, unlike other markers e.g. myogenin and myf-5. It is not only expressed in myocytes but also myotubes. As myocytes differentiate, they join to become multinucleated and form myotubes.

By analysis of the model chromosome number and by specific examination of the cell karyotype, no abnormalities could be seen. All chromosomes were present and there was no genetic abnormalities were evident.

3.5 Expression of telomerase and telomere length

To assess how the introduction of telomerase had affected the telomeres, telomere length was calculated and telomerase expression was measured. The initial experiment was carried out by Dr Katrina Gordon and repeated by me. It was important to assess if the introduction of telomerase could increase telomere length, and also if the transfection had resulted in successful expression of telomerase. As can be seen from Figure 3.11 all of the HMCs infected with hTERT are expressing telomerase. The cells which were infected with the Neo control vector had no telomerase activity as indicated by the lack of TRAP ladder.

On examination of the telomere lengths it could be seen that the extension in telomere length did not collate to the growth rate. T15 had telomere lengths which were similar to that of T4, but T4 grew much slower than T15. Also surprisingly T3, which had undergone 88 MPDs, had telomeres shorter than senescent HMCs.

Figure 3.11 TRAP assay and TRF blot for hTERT clones

A: Shows the TRAP assay performed on HMC Neo and HMC hTERT pools and clones. CHAPS is a buffer alone control and TSR8 is a positive control supplied by the manufactures of the assay. HI is the heat-treated control for each sample. 36bp band at the bottom of the gel is an internal positive control to assess if the PCR has worked.

B: TRF Southern blot showing the average telomere length. The follow cell lines were used as controls for TRF lengths: GM847 cells, long TRF lengths (TRF>10Kb), HeLa intermediate TRF length (4-6Kb) and A1898 short TRF length (4-6Kb) and A1898 short TRF length (2-3Kb).

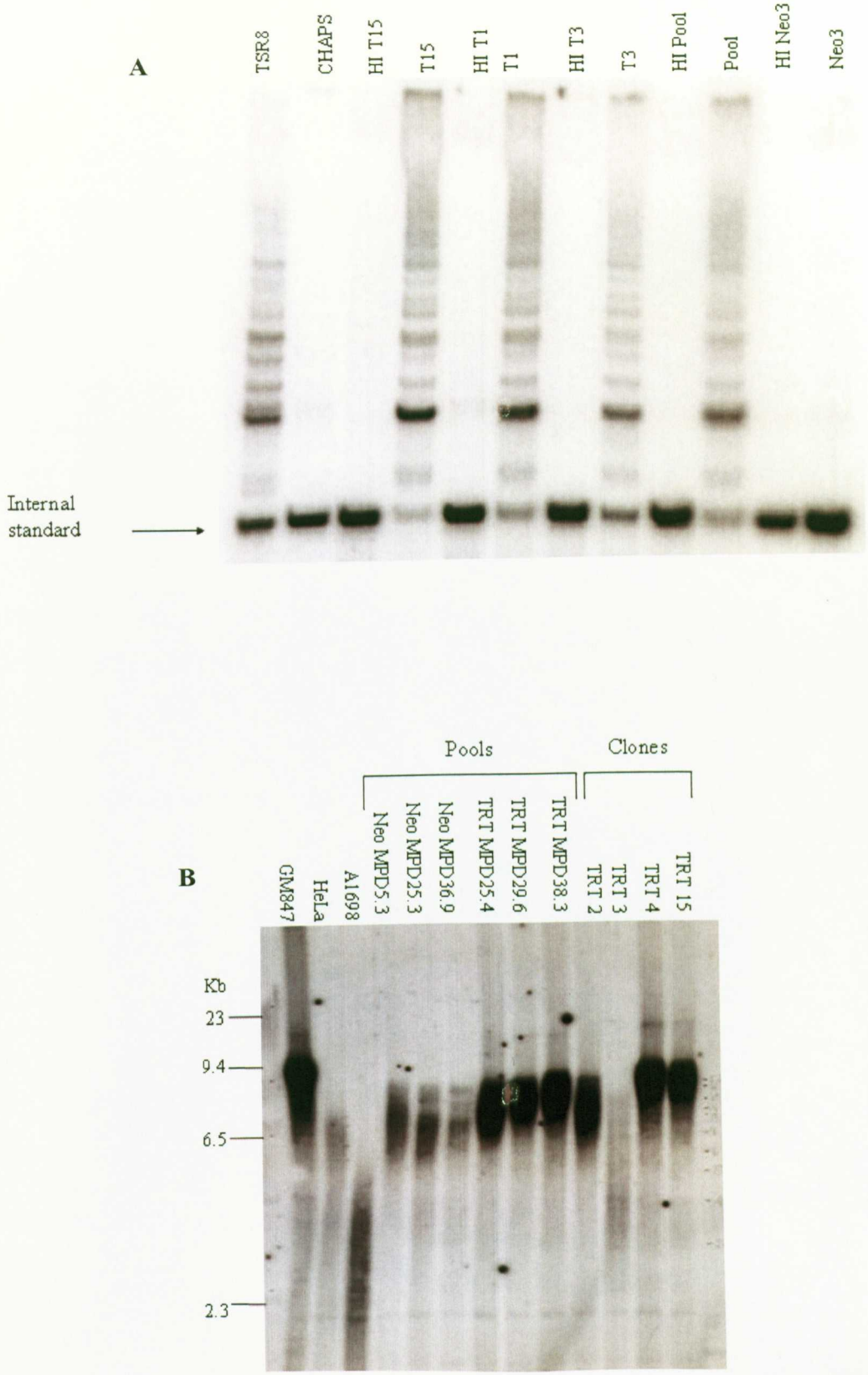


Figure 3.11 TRAP assay and TRF blot for hTERT clones

3.5.1Discussion

The results from the TRF blot show progressive shortening of the telomeres as the Neo pool they approach senescence, while the serial cultivation of the hTERT pools show a slight increase in telomere length. However there does not seem to be direct relationship between telomere length and growth rate. Also T15 does not appear to express higher amounts of telomerase than the other clone. The telomere length for T3 suggests that this clone has the ability to replicate with short telomeres and recruit telomerase.

3.5.2 The changes in growth rate for T1

As can be seen from Figure 3.1 the growth rate of T1 has changed considerably over time. At 30 weeks it was thought that the cell would senescence at 30 MPDs, however at this point the growth rate changed and the clone has continued to proliferate. It is likely to assume that this clone has undergone a secondary alteration. To try and address this TRAP assays were performed on T1 at 20 MPDs and T1 at 44 MPDs, to find out if after this change the cell was now expressing more telomerase. Using T2 as a control, the concentration of protein to be used was assessed by assaying serial dilutions of the protein extracts. If too much protein is used the PCR reaction becomes saturated thus making any quantification of TRAP products invalid. The titration TRAP assay can be seen in Figure 3.12A and the TRAP assay for early and late T1 in figure 3.12B

Figure 3.12 TRAP assay to asses for changes in T1 telomerase expression

A: Titration of T2 Protein Extract to assess appropriate concentration of protein to be uses.

B: Trap for early passaged T1 cells and late passaged T1 cells. T2 and T15 were used as positive controls. In both cases CHAPS is a buffer alone control and TSR8 is a positive control supplied by the manufactures of the assay. HI is the heat-treated control for each sample. Band of positively at the bottom of the gel is an internal positive control to assess if the PCR has worked.

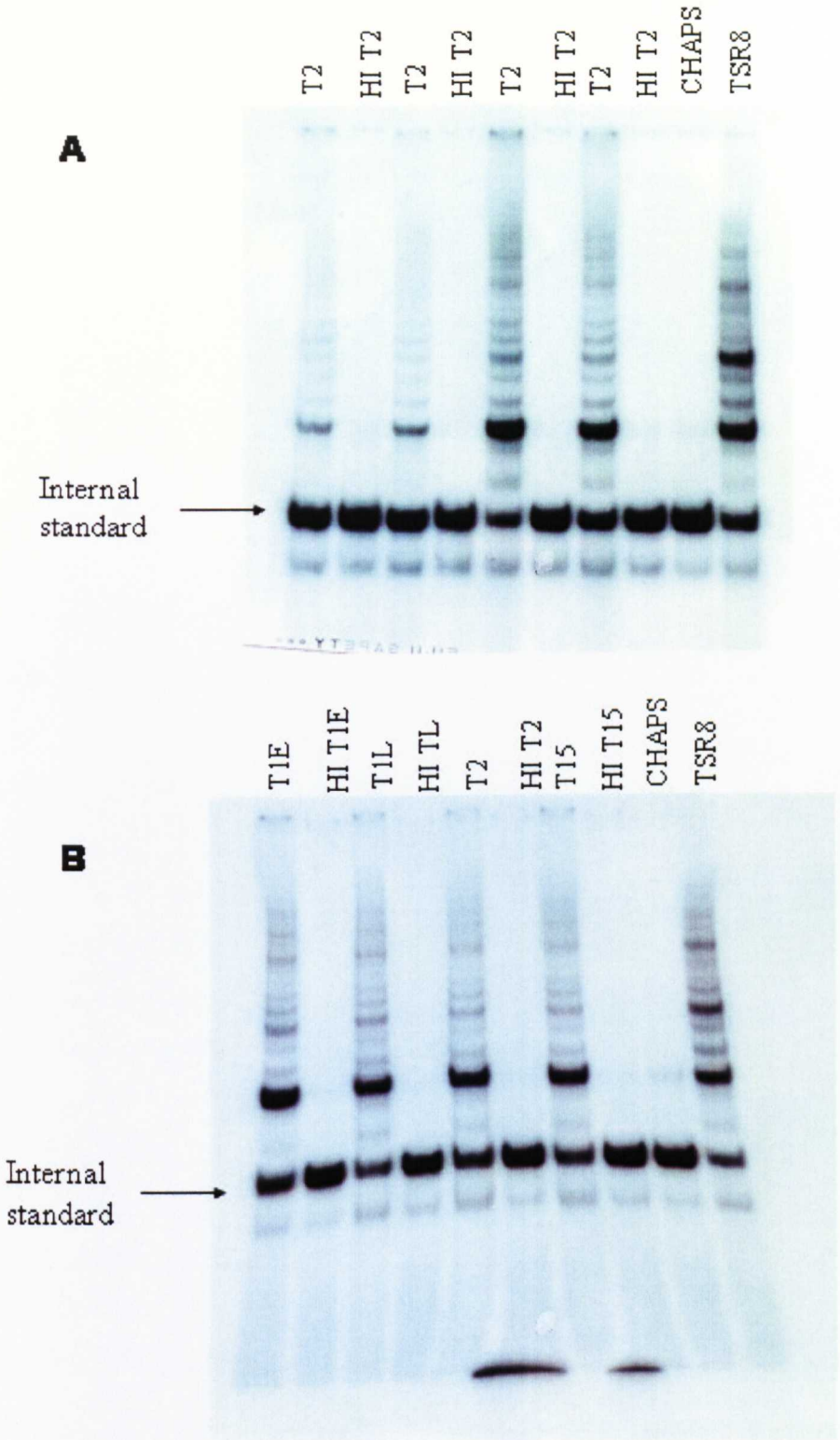


Figure 3.12 TRAP assay to determine telomerase activity in T1 cell line.

3.5.2.1 Discussion

The concentration of protein to use was determined to be 0.3μg/μl, which was within the linear range for the assay. The TRAP assays of the early and late passage T1 cells showed that there was no significant increase in the level of telomerase activity between early and late cultures. Therefore this can be ruled out as a mechanism for the increase in proliferations rate. If more time had permitted I would have liked to have examined other aspects of late and early T1 cells, in particular if the telomeres had extended further in the late passage cells.

3.6 No general loss of heterozygosity at genetic loci encoding the cell cycle proteins involved in senescence

The cell lines were all assessed to find out if there was loss of heterozygosity at specific loci. The loci chosen were those associated with the cell cycle proteins $p21^{WAF}$, $p57^{KIP2}$, p53, pRB-1 and INK4. There was no evidence of loss of heterozygosity at the $p21^{WAF}$, $p57^{KIP2}$, p53 or pRB-1 loci (Figure 3.13). However, it was noted that the fastest growing clone T15 did show allelic loss at the INK4 locus ($p15^{INK4B}$, $p14^{ARF}$ and $p16^{INK4A}$), despite possessing a normal unmethylated $p16^{INK4A}$ gene (135). The improved proliferation rate of HMCT15 relative to the other clones might therefore have been be due to reduced expression of the $p16^{INK4A}$ or $p15^{INK4B}$ proteins brought about by haplo-insufficiency. However, surprisingly the levels of $p14^{ARF}$ $p15^{INK4B}$ and $p16^{INK4A}$ in HMC T15 are similar to early passage HMC cultures. Taken together, the data show that the HMC TERT clones are genetically very stable and do not need to lose $p16^{INK4A}$ to develop and extend replicative lifespan.

Figure 3.13 Loss of heterozygosity

Allelic analysis of genetic loci involved in cell cycle regulation and senescence. This figure shows the retention of p53, pRB-1. p57KIP2 and p21WAF loci. However at the INK4A locus there is loss of heterozygosity in T15. The arrows indicate the position of each allele and the informative microsatellite illustrated is given above each panel next to the locus name. Each of the markers was repeated three times and gave the same results as shown here.

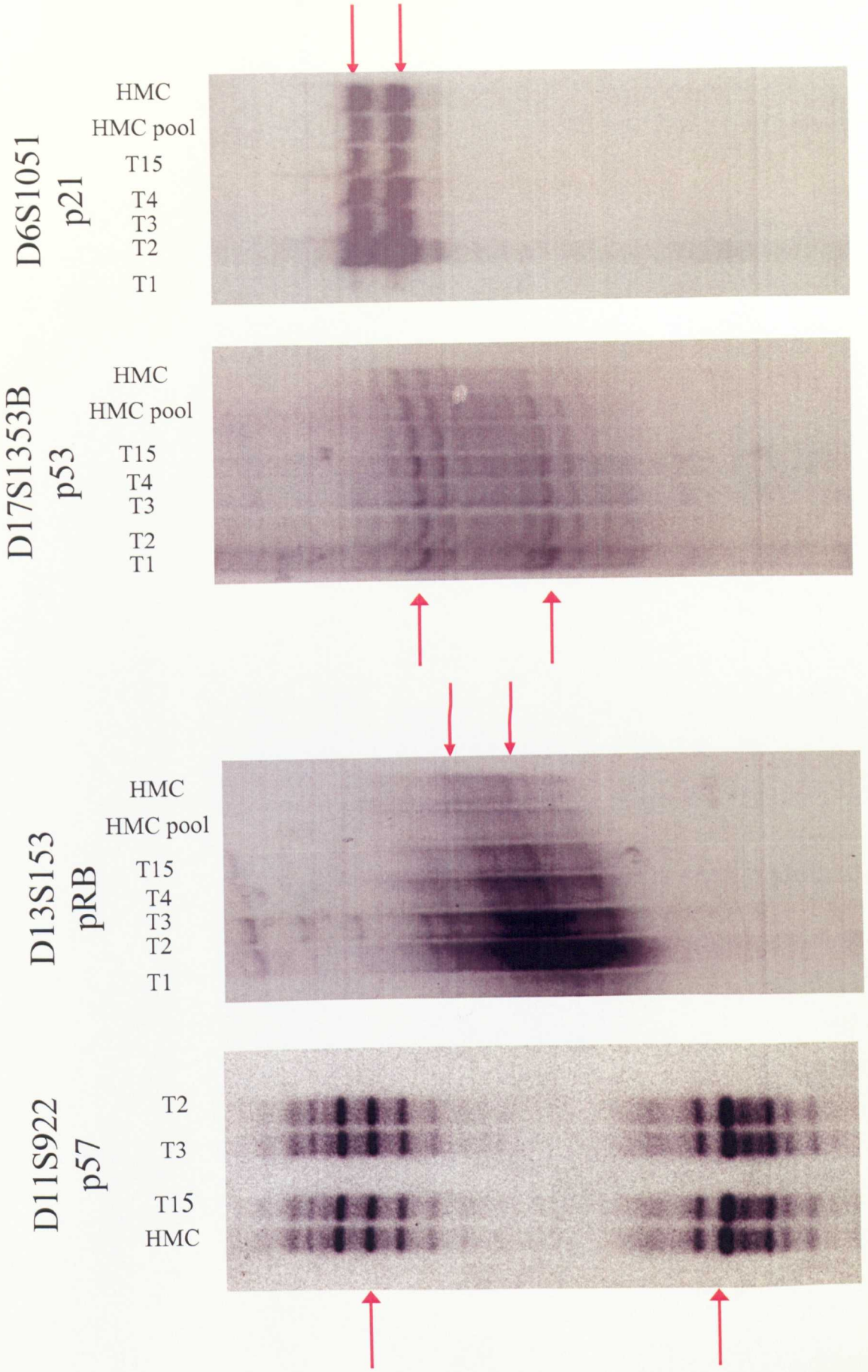


Figure 3.13 No loss of heterozygosity

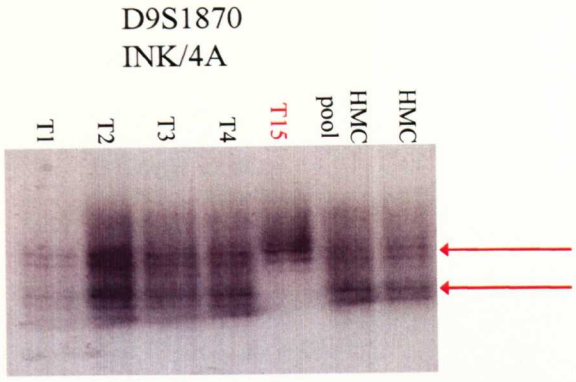


Figure 3.13 Loss of heterozygosity

3.6.1 Loss of INK4 in TERT 15

To further examine the importance of this loss of heterozygosity of the INK4 locus in T15 three cell line were transfected with E6 and E7 to see if this had an effect on the proliferation rate of the cell line. The clones examined were T2, T4 and T15. The cell lines were infected using a Puromycin resistant only or with E6 or E7 expression vectors. They were infected with E6, or E7 or vector alone. The MPD for each cell line can be seen in Figure 3. As can be seen in the graph the introduction of E6 and E7 has made only a slight difference in the growth rate of T2 and T4 and very little to T15.

3.6.2 Discussion

E6 and E7 can improve the growth rate of slow growing clones slightly, but not T15 suggesting that the cell cycle controls may have been subtly altered in T15. However, the growth rates of E6/E7 T2 ; E6/E7 T4 cultures growth rate did not match that of T15, suggesting that if the pRb/p53/p21 pathways are slightly more compromised in T15 than the other clones this does not account for the faster growth rate of T15.

Figure 3.14 Growth rates from transfection with E6/E7

The three graphs below show the growth rates from T2 (A), T4 (B) and T15 (C) cells transfected with E6, E7 or puromycin vector alone. These results are from one experiment only.

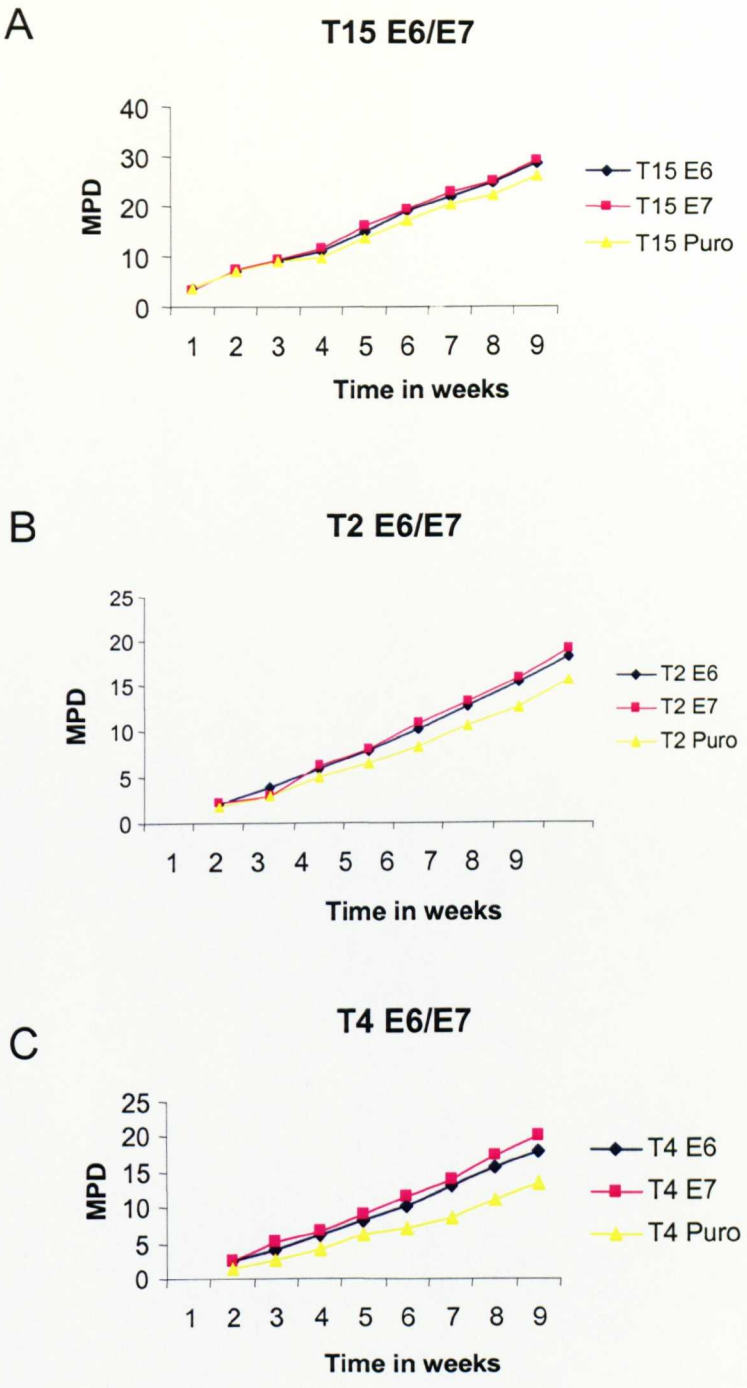


Figure 3.14 Growth rates from transfection with E6/E7

3.7 Staining for P16

As discussed in Wootton *et al* in the immortalisation of keratinocytes there is also a slow proliferation rate to start which then takes off. In keratinocytes this is due to the increase and then decrease of p16. Western blot of p16INK4A were performed at different MPDs, after the hTERT clones had bypassed senescence. The levels of p16 were measured by June Munro and there was no direct correlation between MPD, growth rate and p16 expression. To ensure that the levels of p16 does not increase as the cells approach senescence immunohistochemistry staining of human muscle cells was done at different time points. As can be seen from figure 3.15 there is no marked increase or decrease in the staining levels of HMCs compared to controls at different MPDs.

Figure 3.15 Analysis of $p16^{INK4A}$

The western blot (A) was performed by Dr J Munro showing there is no increase in the accumulation of p16, which is often seen in cells in culture. BICR31 was used as a positive control

Immunohistochemistry staining also demonstrated that there was no increase in p16 in HMC as they approach senescence. B is cells at early passage while C is cells approaching senescence. BICR6 (D) cells were used as a negative control since they do not express p16 and HeLa cells were used as a positive control.

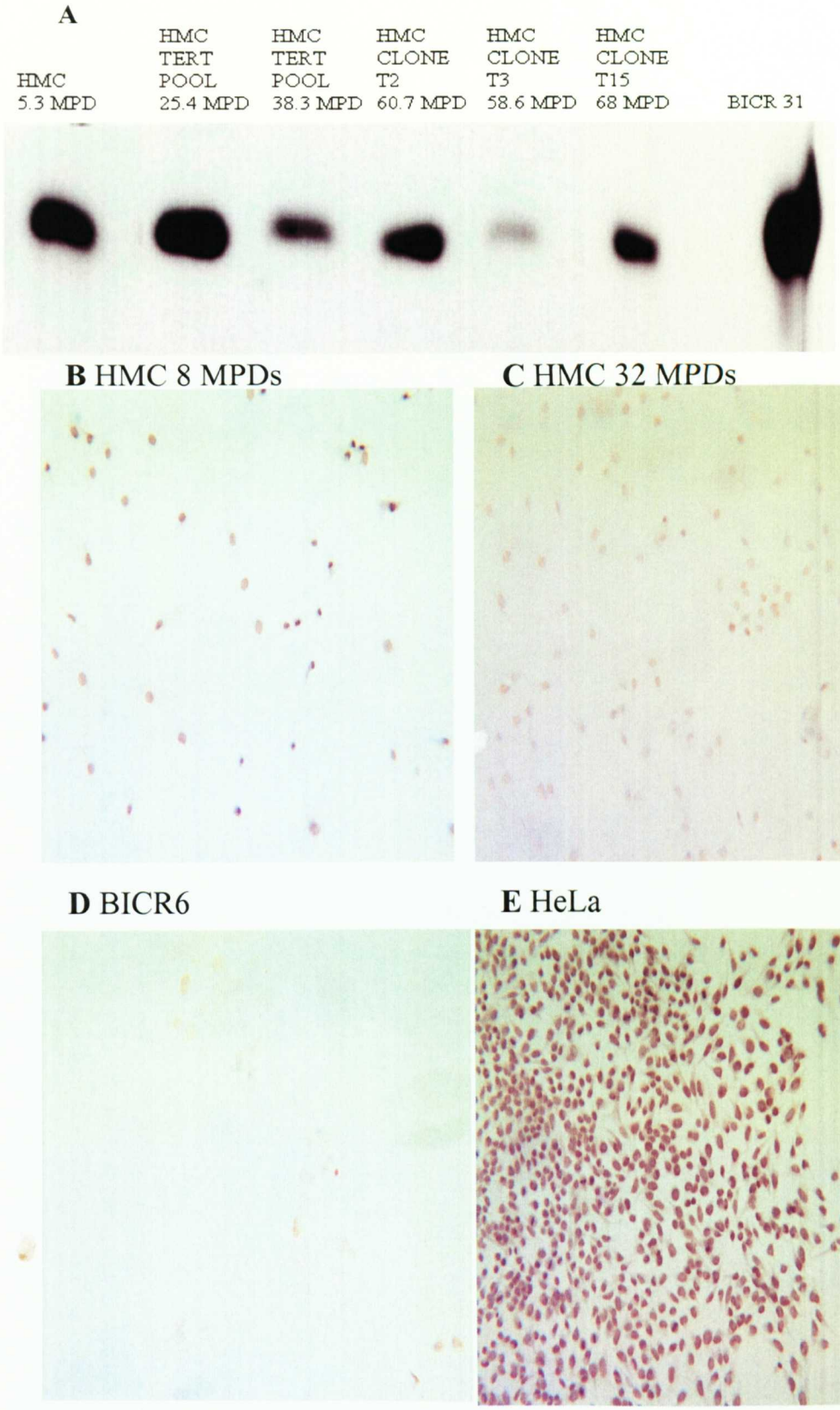


Figure 3.15 Analysis of $p16^{INK4A}$

3.7.1 Discussion

From these experiments it was determined that the proliferation rate in the hTERT clones is unrelated to $p16^{INK4A}$. The difference in the clones growth rates was initially thought to be due to the accumulation of $p16^{INK4A}$, as seen in keratinocytes(136). It has also been shown in TERT-expressing epithelial cells that the cells will not bypass senescence unless $p16^{INK4A}$ has been inactivated (137). Therefore it was encouraging to see that one of the hTERT-expressing clones (T3) showed levels of $p16^{INK4A}$ lower than that of earlier passaged cells. However there was no clear correlation between the levels of $p16^{INK4A}$ and the proliferation rate. Also, although T15 has an allele missing in the INK4A locus, it did not reduce the level of expression of $p16^{INK4A}$ compared to the other clones. Dr J Munro then sequenced three exons of the $p16^{IN4A}$ locus and it was shown to be wild type and unmethylated ($p16^{INK4A}$ is often silenced by methylation). Although these results suggest that there are no alterations in the $p16^{INK4A}$/RB pathway, we cannot rule out other secondary changes. However there is no straight forward relationship between the growth rates of each clone and the $p16^{INK4A}$ status. Other cell cycle regulators were later examined by Dr J Munro (Wootton et al, 2003), these included $p15^{INK4B}$, c-myc and $p21^{WAF}$. Again these were not abnormal in their levels of expression.

3.8 TERT-expressing HMCs can regenerate striated muscle in vivo.

The expression of hTERT has been reported not interfere with the differentiation programme in HMCs without lifespan extension (138). The cells were first tested for their ability to differentiate in vitro. The cells were grown on glass chamber slides and once the cells had settled their medium was changed to 2% horse serum. The cells were then fixed and stained with desmin using the standard protocol. As can be seen in Figure 3.16 myotubes with numerous nuclei can be seen. The numbers of positive fibres were not as successful as hoped. When reviewing the literature it was suggested that telomerase is switched off before myocyte differentiation can take place. This may have been the reason for the poor success rate.

We then went on to test whether our cultures with extended lifespan could still differentiate and re-constitute human striated muscle *in vivo*. For this part of the experiment I provided the TERT expressing HMCs in culture medium and these were transported to Dr D Watt at The Imperial College. Here they were transplanted into RAG-1 mice (139) in which a

localised area of trauma had been produced. A pervious attempt using SCID mice was proven unsuccessful as they could not withstand the procedure.

T15 engrafted more efficiently, in keeping with its high proliferation rate *in vitro*. Figure 3.17 shows the presence of healthy mature muscle fibres in grafts from both T2 (Figure 3.16 and T15 (figure 3.16). In contrast, grafts of late passage control HMCs and ungrafted controls (Fig 3.16 show no evidence of regeneration. The human grafts also stained positively with a human specific spectrin antibody confirming their human origin (Figure 3.17) in contrast to the non-grafted controls and normal mouse muscle. The submembranous staining pattern in the T2 engrafted muscle was observed in 38% of the muscle fibres present in the graft close to the injection site and resembled that of normal human muscle. Thus the HMC clones that display a considerable extension of proliferative lifespan can still undergo fusion and re-constitute mature striated muscle, *in vivo* and these observations are consistent with previous reports that telomerase expression does not disrupt the differentiation programme of HMC cells (*130, 138*). *Table 3.2 shows the* percentage of spectrin positive fibres in the grafts in normal muscle cell grafts and hTERT muscle cell grafts.

Muscle Graft	Number of spectrin-positive fibres/total number of fibres	Percentage of spectrin-positive fibres
Normal cultured human muscle cells	26/105	24.8
HMC T2	312/1099	28.4

Table 3.2 Comparison of the number of fused muscle cells in xenografts of normal HMCs and the telomerase-expressed HMC clone T2

Figure 3.16 In vitro differentiation of T15 stained with desmin

The figure shows the development of myotubes with multi nuclei within the cell. The characteristic branching of the differentiated cells can also be seen. Arrows indicate the multiple nuclei. Figure 3.16 B is a high power photograph of A.

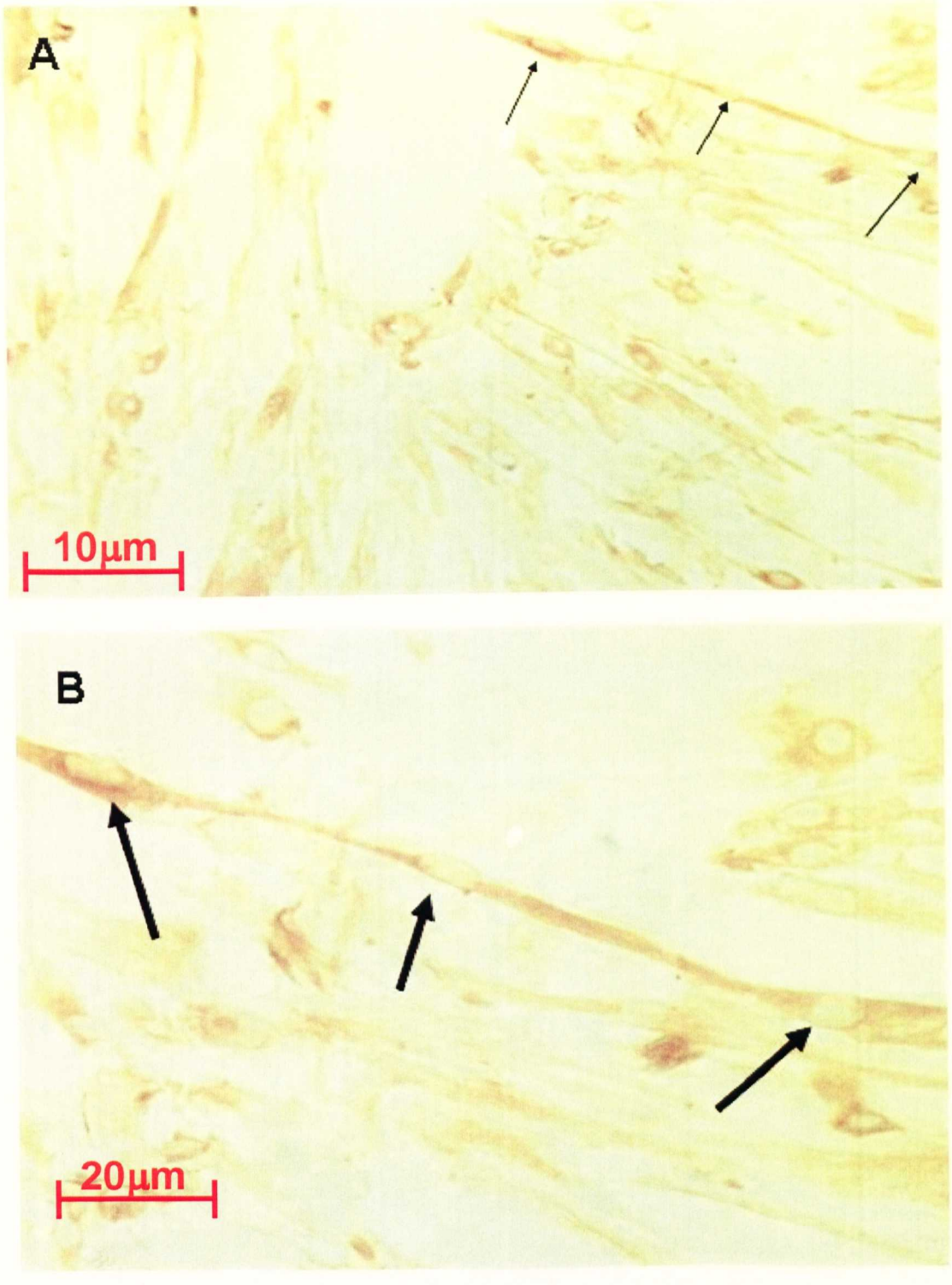


Figure 3.16 In vitro differentiation of T15 stained with desmin

Figure 3.17 H+E staining of mouse sections implanted with human muscle cells

The figure show the results of experiments in which HMC clones T2 and T15 were grafted into the damaged muscle of irradiated RAG-1 mice. Figure A-C shows hematoxylin and eosin stained muscle grafts and control. A: T2 showing evidence of healthy muscle fibres, B: T15 showing healthy muscle fibres and C: un-injected control showing no mature or healthy regenerating muscle fibres.

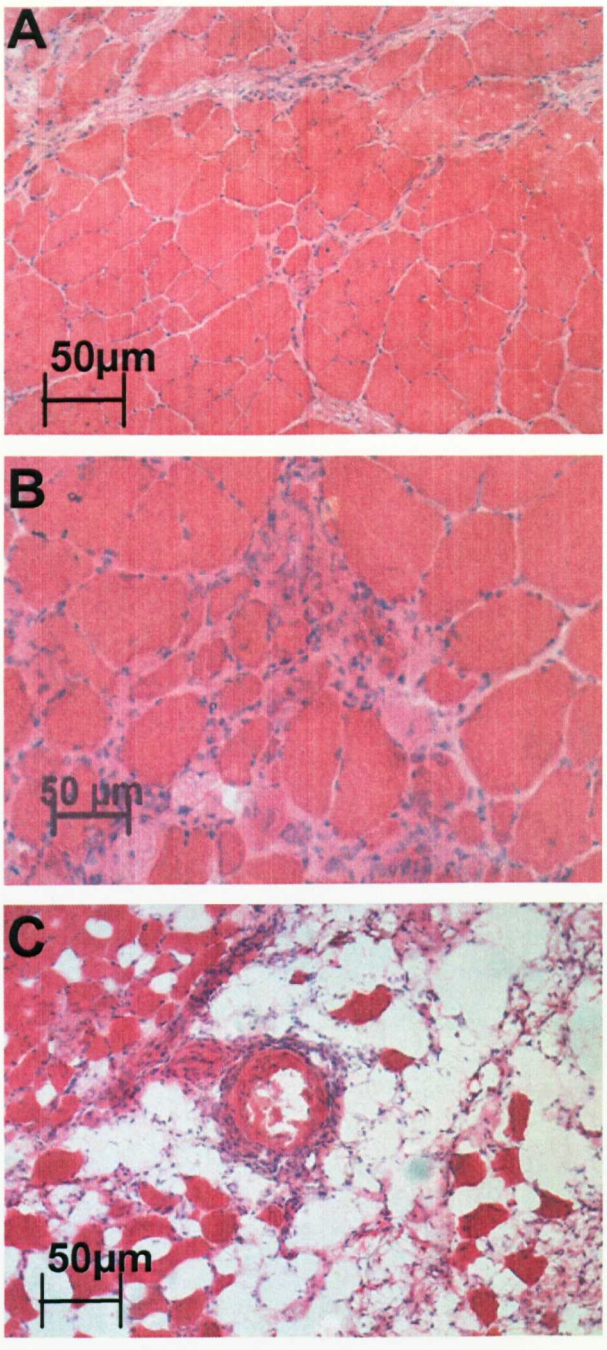


Figure 3.17 H+E staining of mouse sections implanted with human muscle cells

Figure 3.18 Staining of Spec 1 on mice injected with human muscle cells

A: Graft of T2, stained with a human specific anti-spectrin antibody. Showing evidence of submembranous accumulation of the protein

B: Normal human muscle control stained with the same antibody

C: A control which was not injected with human cells showing no specific staining

D: Normal mouse muscle showing no specific staining

Arrows indicated spectrin positive fibres which have fused.

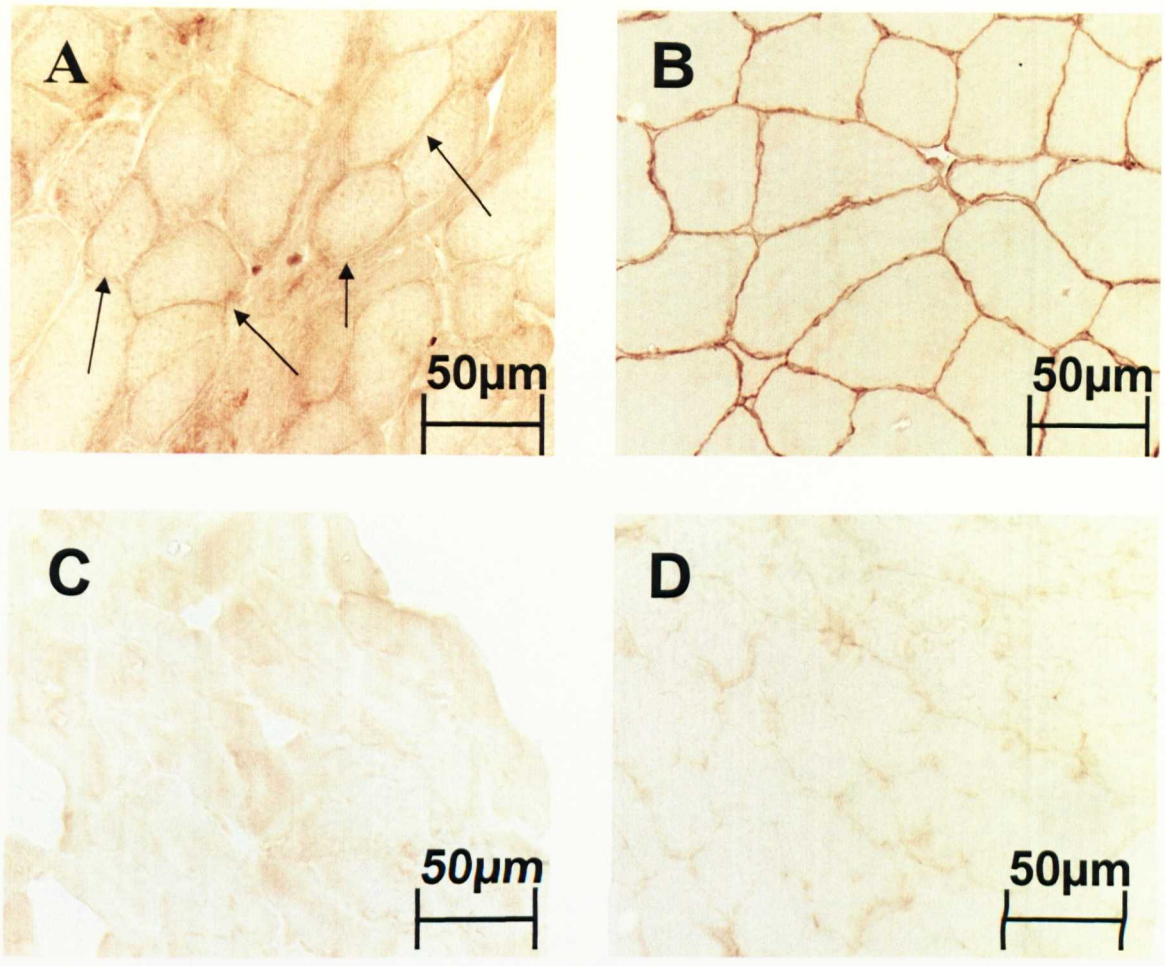


Figure 3.18 Staining of Spec 1 on mice injected with human muscle cells

3.8.1 Discussion

The TERT clones and HMCs were placed in a medium that encouraged differentiation (2% horse serum in DMEM). The TERT clones (T2 and T15) did not differentiate as efficiently as the control cells (C2C12). Examination of the literature suggests that when muscle cells differentiate, telomerase is switched off. This may explain why the results for the *in vitro* differentiation are not as successful as one would have hoped. Nonetheless they did confirm that the cells were of the myogenic lineage. For this reason we still went ahead with *in vivo* differentiation.

We collaborated with Dr D Watt who had developed the technique. It had been previously established that muscle cells will unite with damaged myotubes *in vivo*, even between species. This is a delicate experiment that requires immuno-compromised mice and a defined area of trauma to undergo regeneration.

The result of the *in vivo* work was successful. The difference from the *in vitro* results may be due to the fact that the artificial signalling is not adequate to overcome the continued telomerase expression, while in the animal model, the cells can overcome this due a more appropriate environment. The regeneration of the human muscle in the murine model was localised to the site of injection. This localisation appears to be a common problem in the use of cell therapy.

4 Discussion

Research into DMD and the other muscle dystrophies has provided some insight into the disease itself and novel therapeutic approaches to try and provide some form of treatment for the disease. I have summarised the most promising therapeutic approaches which are under investigation at present and discuss the merits and pitfalls of these approaches. I also discuss the potential importance of my research in the context of existing therapeutic approaches and provide an insight into the way forward.

4.1 Therapeutic attempts in DMD

The potential uses of gene and cell therapy were starting to be considered as treatments for DMD by the late 1980's (140). In both cases the aim was the same i.e. replace the missing gene product.

4.1.1 Gene Therapy

4.1.1.1 Dystrophin gene replacement

At present the hope for a cure in a hereditary disease like DMD is based on gene therapy. Indeed, this disease would appear to be an ideal candidate but there are major obstacles. The dystrophin gene is the largest known, therefore obtaining a vehicle to deliver the full size gene is a major problem (141). It has been achieved in the *mdx* mouse but only where the expression was at a level 50 times greater than normal. Apparently this did reverse the phenotype although this is controversial. Delivery of full length gene in a human at such high levels would be difficult. To overcome this, a number of minigenes have been developed as well as a naked DNA approach (142, 143). An unexpected difficulty identified is that, although DMD sufferers do not express functional dystrophin, they do express isoforms of the protein. This causes an immune response in the naked DNA method (144).

The development of minigenes has focused on the production of small functional genes which can correct the main problems in DMD. By analysis of mutations seen in Becker's muscular dystrophy (BMD), a milder form of the disease, the important domains could be recognised. These truncated forms of dystrophin were generated on an mdx mouse background and the motor function of the animal assessed. This included the maintenance

of strength and force and the presence of centrally located nuclei on histology. It was concluded that the removal of the C-terminal domain (CT) or repeats 4-23 was compatible with normal function (145).

However, although these minigenes are functional, transgenic animal studies have shown that they cannot maintain muscle strength (146). The focus is again on larger constructs and better delivery systems are now being investigated. Another major difficulty has been the large target area and the multiple sites affected.

But the immunological problem still remains. The use of adenoviral vectors results in a $CD8^+$ T-cell mediated response and the DNA is often not incorporated into the host's cells genome. The result of this is that the DNA is lost during the muscle turnover, which is characteristic of DMD. Also retroviral vectors have been shown to invoke an immune response and in some cases result in tumours derived from retroviral-producer cells. In an effort to overcome these problems, a hybrid system of adenoviral and retroviral vectors has been developed. It acts to stimulate the muscle cells to produce the retrovirus *in vitro*. To overcome the immunological problem, a study has been performed on young mice (147).

There has been great development in the use of other strategies for gene therapy, including the use of oligonucleotides to repair genetic mutations (148), and muscle-specific targeting with adenoviral vectors (149). However all of these have their own limitations and none as yet has been totally successful.

Another problem for gene therapy in DMD is the protocol which has been successful in mice. The cells are first removed from the mouse and allowed to expand *in vitro*. Then the gene is introduced and the cells are placed into the host (150). It has been documented that in children with DMD the muscle cells have limited replicative capacity. Therefore the transfer of this technique to clinical trials may prove difficult.

4.1.1.2 Utrophin overexpression

Utrophin has genetic similarities to dystrophin in that they both share the same C-terminal domain binding proteins, namely the dystrophin associated protein complex (DAPC) (151). It has been suggested that upregulation of utrophin might compensate for the deficiency of dystrophin since this is normal in DMD. Due to the constant regeneration which occurs in DMD, utrophin is increased in the muscle overall where it binds with components of the DGC at the sarcolemmal membrane.

Could utrophin act as a replacement for dystrophin? Utrophin has been shown to work in a complementary fashion with dystrophin, i.e. as the levels of utrophin decrease as foetal muscle develops the levels of dystrophin increases. Also in dystrophic muscle, it has been noted that the levels of utrophin increase, compensating for the lack of dystrophin. However as disease progresses, it cannot compensate for the normal levels of dystrophin. Therefore the aim of this therapy is to induce overexpression of the protein (152) in the hope that this could prevent the damage to muscle by playing the role of dystrophin. Evidence to support this ideal came with the development of a transgenic mouse which overexpressed utrophin but was breed on the mdx mouse. This animal had a less severe phenotype than the mdx mouse, and showed less evidence of fibrosis and necrosis in the diaphragm (153). The numbers of fibres positive for central nuclei (sign for regenerating fibres) was also lower and the levels of serum kinase were close to normal. The increased levels of utrophin in the mdx mouse would only have to be 2 to 3 times higher than in wild type to illicit an effect (152). Also it was reported that the high ubiquitous expression of utrophin has no toxic effect on the other organs. Drugs are now currently being developed which will activate the expression of utrophin (154). Attempts have included using glucocorticoids to upregulate levels of utrophin by post- translation mechanisms. This can increase the level of utrophin by 40% in normal and dystrophic human muscle cells (155).

There has been many attempts at gene therapy focused on the expression of utrophin, and adenoviral delivery of utrophin in muscle has been more successful in the mdx mouse than with dystrophin (152). However as with dystrophin-directed gene therapies there are many hurdles to overcome before this therapy could be used in humans.

4.1.2 Cell therapy

Somatic cell therapeutic approaches have consisted of injecting normal myoblasts into the muscle of affected boys, so-called myoblast transfer (156, 157). It was already known, that in *mdx* mice, the dystrophic fibres would fuse with normal muscle fibres and it was thought that the injection might therefore result in the expression of dystrophin in the child. Partridge et al (158) had shown that in the mouse fusion did occur but he pointed out the formidable problems involved in treatment of DMD, such as immune rejection and the fact that all muscles were involved (159). Law (1988) had carried out similar experiments using a different mouse mutant but he claimed that not only fusion occurred, but also that it produced clinical improvement (160). This report led to enormous worldwide interest in applying the technique to boys affected with DMD. Several trials were set up, using normal myoblasts from fathers, brothers or histocompatible donors which were cultured and then injected (157).

The definitive trial was carried out in 1995 by Mendell et al (157). Once a month for six months, 1g of muscle was taken from the fathers or brothers of 12 boys with DMD. The myoblasts were cultured and allowed to divide to yield 1 $x10^6$ cells. These were injected in a grid formation at 55 sites, each 5mm apart, into one arm. In the other arm control injections were carried out. Voluntary muscle strength was tested in each arm once a month. The results revealed that myoblast transfer had no effect on muscle strength. Immunosuppressive therapy produced no difference in strength between the control and myoblast-injected arms. Investigation of dystrophin expression revealed no significant differences between the myoblast-injected and control injected arms. Occasional dystrophin positive fibres were identified, less than 1% of the total. One boy showed 10% dystrophin-positive fibres but even he had no increase in muscle strength.

It was concluded that myoblast transfer failed to improve strength in patients with DMD. The fact that several specific variables had to be considered was pointed out i.e. immunosuppression and the fact that the localisation site was massive. These workers did not address the importance of another variable addressed in this thesis, i.e. whether or nor the transplanted myoblasts were capable of replicating in the child (157, 161). The results of this trial put an end to the prospect that simple myoblast transfer techniques could be beneficial to DMD patients.

4.1.3 Tissue regeneration by embryonic stem cells and bone marrow.

The importance of stem cells was first reported in the work of Till and McCulloch in 1961 (162). Their work on the haematopoetic system demonstrated that the bone marrow contained clones of cells which could reconstitute the blood in patients exposed to irradiation. Through the years this has been developed and bone marrow transplants are common practice in the treatment of leukaemia and other haematopoeitc disorders. Now there is a greater interest on developing cells of a more primitive origin, namely the totipotent stem cells.

It has long been known that stem cells have three functions: to regenerate damaged cells, to differentiate into new cell lineages and to self renew the population. It has been postulated that by introducing stem cells into a diseased tissue, they may be able to repair the damage. This is very promising, particularly in disorders such as neurological diseases where there is no cell regeneration. To this end embryonic stem cells have been developed from the inner cell mass of the blastocyst. (163). This provides stem cells which can act as totipotent when introduced into the host. However at present not enough is known about the process involved in the development of these cells to organs, and therefore manipulation of this is not possible. Recent studies have suggested that the use of these cells may not be as straightforward as once thought. Although stem cells are thought of as being immunologically naïve it has been demonstrated that once the cells start to differentiate they switch on the MHC genes of the donor and therefore can be rejected by the recipient. Also, *in vitro* these cells can develop neoplastic properties (164, 165).

Therefore although there is much hope for these cells as possible tools in curing many disorders, this work is at a very early stage and much caution must be exercised in DMD. As was first discovered by the use of bone marrow cells, there can be host rejection if the donor and recipient are not matched. Therefore the use of embryonic stem cells may only be completely risk free if, at birth the patient has the stem cells harvested from the umbilical cord and stored. This is now being offered at some hospitals to new parents (166).

4.1.3.1 Stem Cell Therapy

There are two types of stem cell: those derived from the embryonic blastocyst (embryonic stem cells, ES) and those originating at various sites in the adult, like bone marrow (163).

Both possess two abilities, i.e. maintenance of precursor cells with the ability to divide and provision of such cells, which will then be able to differentiate. ES cells have not been used in DMD.

Bone marrow, however, can give rise to myogenic progenitors which migrate into degenerating muscle and regenerate it (167). Labelling of bone marrow mesenchymal cell has shown that there is a subset of cells which can participate in myogenic differentiation. These cells have been identified in the skeletal muscle of *scid/bg* mice two weeks after muscle regeneration has been stimulated. There is also evidence of bone marrow-derived cells in the skeletal and cardiac muscle of *mdx* mice after bone marrow transplantation. This supports the idea that the haematopoietic cells can become myogenic and migrate to the site of injury (168). Thus the hope of a bone marrow transplant as a potential therapy seems very promising. However when a bone marrow transplant from a healthy mouse was given to *mdx* mice there was no significant increase in the number of dystrophin-positive fibres (169). Also, the quantification of dystrophin-positive fibres arising from the bone marrow was difficult to determine, due to the presence of revertant fibres in the mdx mouse. When the experiment was changed to use *mdx*4cv mice which have no positive dystrophin fibres the outcome was not as successful. The percentage of positive-dystrophin fibres averaged at 0.25%. PCR analysis of the muscle showed that the repair by BM-derived muscle cells did not exceed 1% (170, 171).

However recent developments have suggested that cells derived from bone marrow do not in fact reconstitute new cell populations but fuse to existing cells. An example of this was shown in bone marrow-derived hepatocytes. The bone marrow cells could repopulate the liver of mice suffering from furnarylacetoacetate hydrolase deficiency and could correct the disorder. However, this was due to the fusion of bone marrow cells rather than the development of new hepatocytes (172). This may be able to explain why the bone marrow transplant was unsuccessful in the *mdx* mouse.

The way forward for stem cell therapy may lie in the purification of muscle precursor cells which could then be implanted into patients (159). Early muscle cells have been purified by preplating cells and selecting those which are desmin-positive (an early myogenic marker). These cells have been shown to regenerate muscle after injection into a mouse host. They seem to home to the site of injury more rapidly than bone marrow-derived cells. The number of cells required to replace the damaged muscle still has to be determined, however this approach remains promising.

4.2 Lessons from animal models

4.2.1 The mdx Mouse

The most common animal model used in the study of DMD is the *mdx* mouse. This animal was found to have a spontaneous, non-sense mutation in exon 23 (109, 121). Dystrophin is absent as in the human, but the model does not show the phenotype of DMD. Muscle is regenerated continuously and there is no influx of fibrous or fatty tissue. Also there is a high incidence of revertant fibres, which express dystrophin. The affected skeletal muscle appears to be in a constant state of successful regeneration.

To try and mimic DMD, there have been a number of new models developed, all based on the *mdx* mouse. *mdx*2-5cv and *mdx*4cv have been generated by chemical mutagenesis as has the *mdx*/utr-/- which has no utrophin. The *mdx*2-5cv and *mdx*4cv are characterised a stop codon in exon 53 of the dystrophin gene. These models have no revertant fibres (173). The *mdx*/utr-/- mice (also known as dko), have a more severe phenotype with marked kyphosis. It is thought that these animals may die from respiratory failure at 20 weeks, however at this point the muscles of the tongue are not strong enough to allow for feeding, and there may be cardiac defects also (174). These models are closer to DMD but still do not show the phenotype observed in the human DMD.

Mouse cells have telomeres up to three times as long as human cells and they have greatly increased replicative potential. The fact that the *mdx*/utr-/- is closer in phenotype to DMD, may be due to the observation that, the normal phenotype in the *mdx* mouse is due to long telomeres and the mice may have the ability to use utrophin to compensate for the lack of dystrophin better than humans. The failure of the *mdx* mouse to display phenotype of the disease has been recognised by scientists working in other fields (109). It has been proposed that the poor results seen with the transplantation of BM-derived cells, may be due to the mdx mouse having hypertrophic muscle and continuous regeneration (170).

This discrepancy between an animal model and a human disease has been observed in other disorders. Heterozygote mouse models for many tumour suppressor genes show no abnormality but, if the mutation is homozygous, the defects are lethal. These include BRCA1, BRCA2, WT1, VHL and E-CAD (175). In other models, other factors can compensate for the defect. This is seen in the mouse model for retinoblastoma. In the human, heterozygosity for RB results in a predisposition for retinoblastoma, a hereditary tumour of the eye. However in the mouse model, retinoblastoma does not develop because

in mice, the p107 gene suppresses the development of the tumour (176). Mouse models do not always replicate human disease even when the same germline mutation is present. In the case of DMD, however, studies on the mdx mouse suggest that continual regeneration may have the beneficial effect: this adds to the value of our work in extending the replicative lifespan of skeletal muscle cells.

It recently has been suggested that there is another population of myoblasts, similar to the satellite cells but with stem cell-like properties. When injected into mdx mice, these cells show enhanced proliferation and it has been proposed that selecting for this population will make the uptake of cells into the host graft more successful (159). These muscle progenitor cells are now being used in gene therapy as it has been suggested that by using these cells there is a better uptake of the gene into the muscle (177).

4.2.2 Immunosuppression and gene delivery

Recent advances with animal models have shown that the choice of immunosuppression can be optimised and is of great importance in how well the graft takes (178, 179). The importance of the immune response was highlighted during clinical trails for myoblast transfer. It was shown that even when the donor and recipient were matched at the major histocompatiblity locus, there was still the development of antibodies against dystrophin and signs of immune rejection (180). Use of the patient's own cells in genetic manipulation, however may overcome this problem. This underlines the importance of our results.

The angle of injection or the use of enzymes which degrade the extracellular matrix might improve migration (181). Delivery to multiple sites however still proves to be a great problem. The replicative ability of the myoblasts, which was not considered, is likely to have also been a major factor.

4.3 Importance of cell culture conditions

There has been a great interest in whether the culture conditions for the growth of the muscle cells are optimal and how much a role this could play in successful cell therapy approaches. This has also been debated in culturing of skin keratinocytes and breast epithelium. It had been suggested that these cells could only be immortalised via telomerase activation and removal of $p16^{INK4A}$/pRB pathway. However it has been shown that these cells can be immortalised by telomerase alone, if the correct culture conditions

are used (136). As can be seen from our results, the use of different media greatly improved the lifespan of the hTERT-infected cells. It has also been suggested that the levels of oxygen in incubators are too high and this can result in oxidative stress and lead to damage to the chromosomes resulting in DSBs and senescence. To assess the importance of this, other groups have changed the conditions in which they grow the cells. It has been documented by Minamina et al (133) that hypoxic conditions can extend the lifespan of vascular smooth muscle cells by activating telomerase and reconstituting telomeres. It has also been suggested that the levels of oxygen *in vitro* can hinder the replicative potential of lung fibroblasts. This leads to the suggestion that it is not only the end replication problem that is regulating the length of telomeres but also oxidative damage (132). To test this, we performed preliminary experiments of our hTERT-infected muscle cells in low oxygen conditions. However our results were not conclusive and given more time this would have been an avenue that I would have liked to explore.

4.4 Previous attempts at extension of lifespan of human skeletal muscle

Decary et al 2000 has already shown that human myoblasts have a limited proliferative capacity and low level of nuclear turnover. They went on to measure telomere length in dystrophic patients of various ages. They showed that at the age of 2 yrs the myoblasts of a patient with DMD were already 14 times shorter than a normal child of the same age (119). However there have been suggestions that it is not in fact replicative senescence which has caused the onset of the disorder but rather the inability of the dystrophic satellite cells to differentiate (120, 182). This explains the failure of myoblast transfer but fails to answer why the absence of dystrophin in the *mdx* mouse did not prevent the constant regeneration and differentiation seen in this model. A way to resolve this conflicting view and potentially create a better mouse representation of DMD would be to cross the mdx mouse with the short telomere mouse. If this new cross showed the progressive muscle wasting, with necrosis, fatty tissue influx and premature death, then replicative senescence would be the contributing factor. If this cross did not show the phenotype of DMD then the role of failure to differentiate could be further investigated.

Despite these conflicts of ideas, other groups have previously attempted to immortalise human skeletal muscle. In 1999 Louchmuller et al reported the extension of lifespan of human myoblasts due to the expression of E6 and E7 genes of the human papillomavirus. The use of these DNA tumour viruses did extend the lifespan, but they failed to report if

the cells' karyotype had changed, if the infection had resulted in any malignant transformation or if *in vivo* differentiation was possible (183). A year later Seigneurin et al reported that it was not possible to immortalise DMD muscle with SV40 T-antigen (Tag), but the lifespan could be extended somewhat. It was only when the Tag-transformed cells were also transfected with hTERT that the cells could bypass senescence. Again absence of malignant transformation was not reported (130). Further work suggested that the lifespan of muscle cells could be extended slightly by transfection with hTERT, but the group did not see the same extension as in our study. However, interestingly, they did have some extension with DMD muscle cells, and these cells were able to constitute muscle fibres in SCID mice (138).

We have shown here that the expression of telomerase can result in the extension of lifespan of HMCs from two separate donors. These cells have bypassed senescence without any malignancy and can reconstitute human muscle fibres in mice. The introduction of telomerase has resulted in the extension of telomere (Dr K Gordon, Wootton et al, 2003) without reduction in p16 levels, or methylation of p16 or an increase in c-myc (Dr J Munro, Wootten el al, 2003). We have demonstrated that it is possible to extend the lifespan of HMCs with hTERT alone, providing the correct culture conditions are used.

4.5 Final summary and future work

The nature of the disease has been analysed by the study of microarrays of DMD muscle compared to normal skeletal muscle. Interestingly of the 110 genes examined more were overexpressed than underexpressed. Naturally dystrophin was underexpressed, however, gene coding for immune response signals, and those associated with regeneration were overexpressed (184). Therefore the use of gene therapy may have to be readdressed to accommodate the newly detailed progression of the disease and the numerous factors involved.

The fast growth rate of T15, compared to the other clones, was of great interest, and I would have liked to examine this further. During the course of the project it was discovered that the only difference between T15 and the other cell lines, was it had an allele missing at the INK4A locus. Although this did not affect the levels of p16, it may have allowed selective advantage over the other clones. The question as to why this clone has achieved such a growth rate will not be answered until microarray analysis of this clone compared to other clones and normal HMCs is carried out. If more time had

permitted and there was limitless resources I would have liked to examine the hTERT clones via DNA array, micro-array and eventually proteomics. There may be subtle differences between each clone, and this would fully answer the question, how has the introduction of telomerase into the cells altered them.

I believe that this technique for immortalisation could be perfected further and I wished I had more time to explore the use of low oxygen culture conditions. It is now becoming evident that cellular senescence is more complicated than first thought. The end replication problem is not the only mechanism controlling the fate of a cell lifespan, and there is increasing evidence that oxidative damage initiating the DNA repair mechanism also play a role. I think that by adapting culture techniques, we may see better results.

This work has provided a mechanism by which long-lived cell lines can be produced for use in all aspects of muscle research. Obtaining a biopsy is invasive and normal muscle has a limited replicative lifespan, so that this will hopefully provide a resource for many researchers.

The development of immortal muscle cells may not only provide valuable tools for research but may also in the future provide strategies for therapy. Although our cells were permanently transfected with hTERT, this could be refined by using a Cre recombinase and hTERT flanked by loxP sites (89). This would allow the hTERT to be introduced and then removed once the telomeres had been extended. This would be beneficial as it is understandable that for clinical use there would be concerns about transplanting a telomerase-positive cell line into a child. Using this system, the cells could be taken by biopsy, transfected with hTERT, their telomeres extended, and then the hTERT removed. At this point the cells could be reintroduced into the patient and allow for more successful gene therapy. Therefore if time and resources had allowed I would have liked to try this technique to immortalise muscle from a BMD patient and from a DMD patient.

Wither you believe that the onset of DMD is due to an inability for cells to differentiate, or if it is due to lack of replication by the muscle cells, it cannot be argued that this is a complex disease which will not be cured by one line of therapy alone. It will take collaboration from gene, cell and stem cell therapy to find a cure.

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Publications

HUMAN GENE THERAPY 14:1473–1487 (October 10, 2003)
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Telomerase Alone Extends the Replicative Life Span of Human Skeletal Muscle Cells Without Compromising Genomic Stability

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ABSTRACT

Continuous cycles of muscle fiber necrosis and regeneration are characteristic of the muscular dystrophies, and in some cases this leads to premature replicative senescence of myoblasts *in vitro*. The molecular mechanism of senescence in human myoblasts is poorly understood but there is evidence to suggest that telomeric attrition may be one of the ways by which this is achieved. We report here, for the first time, the extension of normal human skeletal muscle cell replicative life span by the reconstitution of telomerase activity. The telomerase-expressing cells show no features of transformation *in vitro* and have stable genomes with diploid karyotypes, do not express exceptionally high levels of c-*myc* and have wild-type, unmethylated CDKN2A genes. *In vivo*, they regenerate to repair muscle injury in immunosuppressed RAG-1 mice. This work suggests that telomerase expression to repair short telomeres may aid the expansion of diploid human muscle cells and consequently attempts at gene therapy for muscle diseases.

SUMMARY OVERVIEW

In the muscular dystrophies, continuous cycles of muscle damage and regeneration cause the replicative capacity of the myoblasts to decline and this may be linked to telomeric attrition. Because premature myoblast senescence may frustrate attempts at gene therapy, we tested the ability of telomerase to elongate muscle cell telomeres and extend their replicative life span. We report here, for the first time that telomerase extends the life span of normal human skeletal muscle cells without transforming the cells or compromising genomic stability. The cells can regenerate striated muscle when injected into immunosuppressed mice. These results suggest that the expression of telomerase may be a useful aid to gene therapies for muscle diseases, especially the muscular dystrophies.

INTRODUCTION

HUMAN TELOMERES are composed of tandem repeat $(TTAGGG)_n$ sequences of DNA. In cells that are "immortal," such as the human germline, a stable telomere length is maintained through the action of telomerase (Counter, 1996). Early in human development, telomerase is downregulated in most somatic cells, and this, at least in part, results in the progressive shortening of telomeres that culminates in replicative senescence (Harley *et al.*, 1990; Wright *et al.*, 1996; Bodnar *et al.*, 1998). In the absence of extensive cell division *in vivo*, telomeres do not shorten, as exemplified by the fact that telomere length remains constant in telomerase-negative tissues, such as the brain and striated muscle (Decary *et al.*, 1997). When human muscle cells (HMC) are grown *in vitro*, the cells replicate for a limited number of population doublings, during

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which time telomeres shorten until, although the cells remain viable, they will no longer divide (Decary *et al.*, 1997). Proliferative capacity and telomere length have been correlated in human satellite cells and an age-related decline in each has been shown (Decary *et al.*, 1997).

In patients with Duchenne's muscular dystrophy (DMD), the fatal loss of muscle is caused by the decline in muscle regenerative capability; this may be a consequence of premature replicative senescence. The role of telomeric attrition in the premature senescence of DMD myoblasts is controversial (Decary *et al.*, 2000; Oexle and Kohlschutter, 2001) but telomere shortening could explain the premature senescence of the HMC cultures from these patients (Webster and Blau, 1990).

There is considerable interest in developing an adequate gene therapy strategy for DMD and it was recently shown that wild-type dystrophin could correct the muscle phenotype of the DMD laboratory model (*mdx* mouse) when introduced using an adeno-associated virus carrying dystrophin minigenes (Wang *et al.*, 2000a). However, murine cells have long telomeres (Kipling and Cooke, 1990), and therefore, the *mdx* mouse muscle cells are unlikely to experience critical telomere loss during a normal life span. As a consequence, the premature replicative senescence characteristic of human DMD muscle cells may not occur. This may explain why *mdx* mice only exhibit the degenerative phase of the human disease in the early stages of life and from then on seem capable of continuous regeneration.

Attempts have already been made to extend the replicative capacity of myogenic cells using DNA tumor viruses (Morgan *et al.*, 1994; Lochmuller *et al.*, 1999; Seigneurin-Venin *et al.*, 2000b) but such approaches cannot be countenanced for human transplantation. Inactivation of p53 by the viral genes, even if expressed transiently, would likely create genetic instability (Kastan *et al.*, 1991; Yin *et al.*, 1992). It might be predicted that as myoblasts are a similar cell type to fibroblasts, they should be readily immortalized by the expression of the catalytic component of telomerase, hTERT, without sustaining chromosomal alterations or transformed properties (Bodnar *et al.*, 1998; Vaziri and Benchimol, 1998). However, not all groups report the successful immortalization of human fibroblasts using this approach (O'Hare *et al.*, 2001). Furthermore, attempts to reconstitute telomerase by ectopic expression of hTERT did not result in extension of replicative life span in either normal or DMD myoblasts (Seigneurin-Venin *et al.*, 2000a).

Here, using a different culture medium and protocol to that used above, we describe the successful extension of the life span of human skeletal muscle cells for more than 120 mean population doublings (MPDs) by means of telomerase reconstitution and show that these cells are genetically stable, non-transformed, and have the ability to regenerate muscle in the RAG-1 mouse.

MATERIALS AND METHODS

HMC culture and retroviral infection

The human muscle satellite cell cultures were established from a biopsies taken from a 23-year-old male (line 1) and a 28-year-old female (line 2) by explant culture using special techniques to prevent the outgrowth of fibroblasts (Edington *et al.*, 1995). The culture medium was Dulbecco's modified Eagle's medium (DMEM) containing 20 m*M* HEPES, glutamine, antibiotics, and 10% vol/vol fetal bovine serum. The cells were then released from the culture vessel using 0.1% trypsin and 0.01% ethylenediaminetetraacetic acid (EDTA) and designated MPD 0 cells. The MPDs were calculated as previously described (Munro *et al.*, 2001). The cells were confirmed as HMCs by their immunoreactivity with desmin antibodies (see below) and were routinely passaged at 5×10^4 cells per 5 cm. diameter dish (Nunc or Falcon). The HMCs were infected with the pBabeNeo and pBabeEst2 retroviruses (Vaziri and Benchimol, 1998) as described previously (Munro *et al.*, 2001) and the infected cells were selected with 200 μg/ml G418. The G418 was removed once the mock-infected plates were dead. In the pBabeEst2 retroviral vector hTERT is transcribed from the retroviral LTR (Vaziri and Benchimol, 1998). All experiments were performed with the pBabeEst2 (hTERT)-infected myoblast clones when they had completed between 51 and 68 MPDs after infection with the retroviruses. Senescent HMC cultures were defined as those that incorporate bromodeoxyuridine (BrdU) into 5% of the cells, or less in 48 hr (Stein, 1985) and failed to undergo 1 MPD in 4 weeks. Some cultures were also stained for the presence of the senescence associated β-galactosidase as described by Dimri and coworkers (Dimri *et al.*, 1995).

BrdU labeling and detection

BrdU labeling was carried out exactly as described previously (Munro *et al.*, 2001).

Senescence-associated β-galactosidase assay

Senescence-associated β-galactosidase assay was carried out by the method of Dimri and coworkers (Dimri *et al.*, 1995) as modified by Munro and coworkers (Munro *et al.*, 2001), except that the assay was carried out at pH 5.8 instead of 6.0. pH 5.8 gave superior staining in the senescent HMCs.

Agar colony assay

The ability of the cells to form colonies in suspension was tested by suspending the cells in 0.4% agar (DIFCO Bactoagar, Detroit, MI) in DMEM containing 20% vol/vol fetal bovine serum at a density of 10^5 cells per 5-cm diameter dish.

Low serum assay

The cells were plated in DMEM containing 0.2% vol/vol fetal bovine serum at 5×10^4 cells per 3-cm diameter dish for 3 weeks prior to the commencement of the experiment because human cells can take 10–14 days to become fully quiescent (Seshadri and Campisi, 1990). The cells were disaggregated and counted weekly and the MPDs calculated as detailed above. HeLa cells were used as a positive control line and normal HMC cells at early passage were used as negative controls.

Immunohistochemistry

Cells were grown on chamber slides and washed three times in phosphate-buffered saline (PBS). The slides were then fixed in acetone at 4°C for 20 min and air-dried. The cells were

blocked with 2% horse serum in PBS for 30 min. The slides were incubated for 60 min at room temperature with a mouse monoclonal antibody directed against desmin (clone D33, at a dilution of 1:100, Dako Corp., Carpinteria, CA). After washing with PBS the cells were incubated with secondary and tertiary antibodies from the avidin-biotin complex (Vectastain Peroxidase Mouse IgG ABC kit, Vector Laboratories Inc., Burlingame, CA) per the manufacturer's instructions and stained with 3,3′diaminobenzidine (DAB).

Muscle sections were blocked with 2% horse serum in PBS for 30 min and were incubated with the primary antibody spectrin (NCL–SPEC2, at a dilution of 1:100; Novocastra Labs, Newcastle upon Tyne, UK) overnight at 4°C. The slides were washed and stained using the Vector MOM Immunodection Kit as described (BMK-2202, Vector Laboratories Inc.) and stained with DAB.

Western blotting and antibodies

This was performed essentially as described by Munro and associates (Munro *et al.*, 1999, 2001). One hundred micrograms of protein was subjected to electrophoresis on 10% (c-*myc*) or 17% (CDKIs) gels. Primary antibodies were: $p16^{INK4A}$ (C-20; Santa Cruz Biotechnology, Santa Cruz, CA), ERK2 (BD Biosciences, Heidelberg, Germany) and c-*myc* (N-262; Santa Cruz Biotechnology). The SV40-immortalized human keratinocyte line SV6-1 HFK was used as a positive control for $p16^{INK4A}$ and the human squamous cell carcinoma line BICR31 as a negative control. BICR31 harbors a homozygous deletion of the *INK4A* locus. The HaCaT line is an immortal human skin keratinocyte line that expresses high levels of $p15^{INK4B}$. The human squamous cell carcinoma line BICR78, which does not express $p15^{INK4B}$ mRNA, served as a negative control for $p15^{INK4B}$. HFF UV are human fetal skin fibroblasts irradiated with 8 mJ of ultraviolet light from a Stratalinker 2400 (Stratagene, La Jolla, CA) and harvested 16 hr later.

$p16^{INK4A}$ PCR and DNA sequencing

The sequencing of exons 1α, 2, and 3 of the *INK4A* locus was performed as described previously (Munro *et al.*, 1999).

Bisulfite modification of $p16^{INK4A}$ and PCR

Genomic DNA was modified using the CpG™ DNA Modification kit (Intergen Company, Oxford, UK) following the manufacturer's instructions with the following alterations. DNA modification reagent I included urea (Paulin *et al.*, 1998) and the modified DNA was eluted in 40 μl of 10 m*M* Tris pH 7.5, 0.1 m*M* EDTA.

PCR was carried out on 1 μl of modified DNA using the primers of Bauer and associates (Baur *et al.*, 1999). PCR conditions were according to manufacturer's instructions using Immolase DNA Polymerase (Bioline Ltd., London, UK) and either 1.75 m*M* $MgCl_2$ (methylated modified form primers) or 2 m*M* $MgCl_2$ (unmethylated modified form primers) depending on primer sets used. The PCR cycles were 95°C for 7 min followed by 35 cycles comprising 94°C for 30 sec, 60°C (methylated modified form primers) or 58°C (unmethylated modified form primers) for 30 sec and 72°C for 30 sec. There was a 72°C extension time for 7 min. Products were sequenced using sequencing conditions as described (Munro *et al.*, 1999). The human squamous cell carcinoma line SCC-15 has a completely methylated $p16^{INK4A}$ gene promoter and the DNA from this line served as a control for the methylated PCR primers and as a negative control for the unmethylated ones. DOK is a human keratinocyte line derived from a squamous dysplasia of the oral cavity and expresses a mutant $p16^{INK4A}$ protein. DOK DNA was therefore used as a positive control for the unmethylated PCR primers and as a negative control for the methylated ones.

Telomere length measurement

This was performed as described previously (Munro *et al.*, 2001), except that the blot was hybridized with a radiolabeled oligonucleotide probe $(TTAGGG)_3$ at 42°C and subsequently washed to a stringency of 6xSSC/1% sodium dodecyl sulfate (SDS) at 42°C. The telomere lengths were measured as described (Munro *et al.*, 2001).

Telomerase activity

Cellular extracts were prepared and assayed for telomerase activity using the TRAPeze kit (Intergen, Company, UK) following the manufacturer's instructions.

Microsatellite analysis

Total reaction volumes were 10 μl containing 40 ng genomic DNA, 10 m*M* Tris-HCl, pH 8.3, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 10% dimethyl sulfoxide, 1 μ*M* of each oligonucleotide primer, 200 μ*M* of each deoxynucleotide triphosphate, 0.4 μl of [α32P]dCTP (24.7 kBq/μl) and 0.5 units of *Taq* polymerase (Advanced Biotechnologies, Epsom, Surrey, UK). Reactions were subjected to an initial denaturation step of 5 min at 94°C, followed by 30 cycles of 30 sec at 94°C; 30 sec at 55–65°C; and 30 sec at 72°C, and a final extension step of 7 min at 72°C. Samples were electrophoresed on 4–10% polyacrylamide gels under denaturing conditions. Gels were dried then exposed to x-ray film to visualize the resolved reaction products. The following markers were used. D17S1353B (p53), D13S153 (pRB), D11S922 (p57), D6S1051 (p21), and D9S1870 (*INK4A*).

Cytogenetics

Medium was changed 24 hr prior to harvesting to enhance cell division. Demicolchicine (0.01 μg/ml) was added for 4 hr to arrest the cells in metaphase. Metaphases were harvested and G-banded using conventional techniques.

Induction of regeneration in host muscle and implantation of cells

Irradiated RAG-1 mice were chosen as hosts to receive the cellular implants because they can accept xenografted cells without immune rejection of the implanted cells. Under these conditions the grafted cells are more likely to regenerate muscle after myotoxicity (Watt *et al.*, 1982; Wakeford *et al.*, 1991). Barium chloride, an agent that is myotoxic and causes the muscle fibers to degenerate was administered as described (Caldwell *et al.*, 1990). Forty-eight hours after barium chloride administration the right tibialis anterior (TA) muscle of host mice was implanted with 3×10^5 cells as described (Gibson *et al.*,

1995). Six TA muscles received HMCT15 cells, two HMCT2 cells, and 3 were implanted with control human muscle cells. Twenty-five days after implantation of the cells, recipients were sacrificed, the TA muscle removed and frozen sections prepared. Sections were stained with hemotoxylin and eosin or by the immunocytochemical detection of spectrin (see above). The number of spectrin positive fibers, indicative of muscle cell fusion, were quantitated by examining at least five fields adjacent to the injection site. Only muscle fibers that were positive for spectrin on all sides were considered positive. At least 1000 cells were scored per sample.

RESULTS

Extension of replicative life span of skeletal satellite cells by hTERT expression

Skeletal HMCs were obtained from needle biopsies of patients with no neuromuscular disease. After 5 MPDs HMC line 1 was infected with either a retrovirus containing hTERT or with the neomycin-selectable marker alone and HMC line 2 was similarly infected after 25 MPDs. The mass cultures or clones were then selected on G418. Both the HMCNeo mass cultures senesced after approximately 37 MPDs but the corresponding hTERT-infected cultures have currently extended their life span to more than 60 MPDs (HMC line 1, Fig. 1a) and more than 57 MPDs (HMC line 2, Fig. 1b). The senescent cultures of both HMC lines contained predominantly flat cells that expressed the senescence-associated β-galactosidase (Fig. 1c), whereas the corresponding TERT-expressing HMC cells were predominantly composed of small, spindle-shaped cells that did not express senescence-associated β-galactosidase (Fig. 1d). Three neo- (NEO1, NEO2, and NEO3) expressing and five hTERT- (TERT1, TERT2, TERT3, TERT4, and TERT15) expressing clones were studied further (Fig. 2). After 21–28 MPDs, all the Neo clones had a low BrdU incorporation rate over 48 hr (Fig. 2a) and stopped proliferating (Fig. 2a). In contrast, all five of the HMCTERT clones had a higher BrdU incorporation rate and one clone, HMCT15, was comparable to the immortal HeLa cell line control (Fig. 2b). Four of the five HMCTERT clones bypassed senescence immediately (Fig. 2a) and have now achieved more than 120 MPDs. The TERT clone HMCT1, displayed a slowed proliferation at the same time as the Neo control clones and incorporated BrdU into only 25% of the population in 48 hr but after several weeks it also escaped senescence (Fig. 2a), consistent with a requirement for a secondary alteration in this clone. All of the TERT-infected HMC clones, including HMCT1 possessed robust telomerase activity, as assessed by the TRAP assay in contrast to the Neo-infected clones (Fig. 3a).

Telomerase extension of proliferative life span is not always associated with TRF length maintenance

It was important to establish how telomerase extends proliferative life span, because the ability to repair short telomeres will be important for gene therapy. After 5 MPDs *in vitro*, the HMCs had average TRF lengths of 8 kb, which had shortened to 6 kb in those approaching senescence. This is similar to the length at senescence previously reported (Decary *et al.*, 1997) and somewhat longer than that reported for senescent fibroblasts (Harley *et al.*, 1990). The signal intensity of the TRF smear also decreased upon senescence.

It can be seen from Figure 3b that three of the four hTERT-expressing clones that bypassed senescence had TRF lengths equivalent to, or longer, than in the young HMC cultures and had a much stronger TRF signal. Clone T3, however, reached at least 88 MPDs and had an average TRF length less than that in the near-senescent mass cultures of the neo controls (Fig. 3b). There are precedents for this finding (Zhu *et al.*, 1999; Ouellette *et al.*, 2000), which have been attributed first to the ability of cells to retain capped and functional telomeres, even when TRF are short (Zhu *et al.*, 1999) and second, to the recruitment of telomerase to the shortest telomeres (Ouellette *et al.*, 2000). The latter is thought to occur when TERT transgenes become methylated and the telomerase activity decreases (Ouellette *et al.*, 2000). In any event, our results show that the proliferative life span of HMCs can sometimes be extended without the complete maintenance of average TRF length. Furthermore, there was no correlation between the average TRF length of each clone and its growth rate (Figs. 2a and 3b). In particular, clones HMCT15 and HMCT4 had the longest average TRF lengths but had very different growth rates.

Telomerase extension of proliferative life span is dependent on the culture conditions

Previous studies of telomerase-expressing HMC cells cultured in the Ham's F10-based MCDB 120 medium failed to detect an extension of replicative life span (Seigneurin-Venin *et al.*, 2000a). The retroviral vectors used by Seigneurin-Venin and ourselves were essentially identical and led to telomere elongation in some of the experiments of Seigneurin-Venin and associates (Seigneurin-Venin *et al.*, 2000b), so the telomerase transgene was functioning normally in their experiments. However, one recent paper has highlighted the importance of culture conditions in the successful immortalization of several human cell types with telomerase alone (Ramirez *et al.*, 2001). To test whether the difference between our results and those of Seigneurin-Venin and coworkers (Seigneurin-Venin *et al.*, 2000a) could be because of the choice of culture medium, we transferred normal HMC cells into Ham's F10 after 25 MPDs, HMC T2 after 70 MPDs, and HMC T15 after 115 MPDs. The normal HMCs grew through a further 4 MPDs in F10 medium but showed a considerably truncated replicative life span when compared to the same cells cultured in DMEM (Fig. 4a). Increasing the fetal bovine serum concentration from 10–20% increased the replicative life span by 2.5 MPDs in both types of media (data not shown). Both the telomerase-expressing clones underwent senescence after 6 (HMCT2; Fig. 4b) and 1 MPDs (HMCT15; Fig. 4c), respectively, after transfer into Ham's F10 and this result was not influenced by the fetal bovine serum concentration. These results may explain the discrepancy between our results and previous reports by others.

The proliferation rate in TERT-expressing HMC clones which bypass senescence is unrelated to c-myc, $p16^{INK4A}$, $p15^{INK4B}$, or $p21^{WAF}$ levels

The HMCTERT clones differed in their proliferation rates but all were less than that of early-passage HMC cultures (Fig. 2a). A similar phenomenon has been noted in TERT-express-

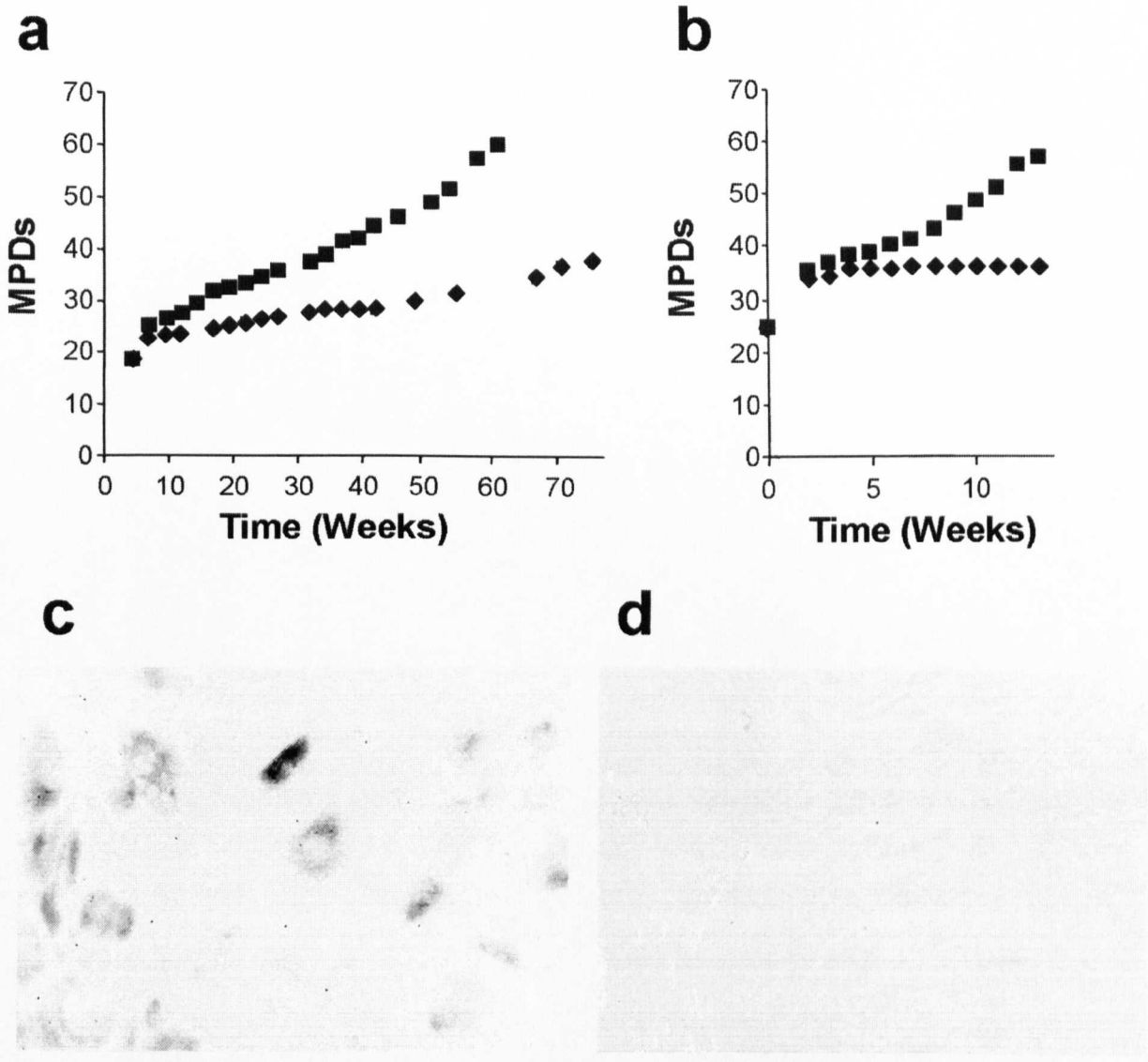

FIG. 1. Human skeletal muscle cell (HMC) cultures expressing high levels of telomerase show an extended replicative life span. Growth curves of HMC mass cultures derived from two individuals and infected with either pBabeNeo alone or pBabeEst2 after 5 (line 1) (**a**) or 25 (line 2) (**b**) mean population doublings (MPDs). In both cases the replicative life span of the TERT-infected mass cultures (red squares) has been extended well beyond the normal life span of the controls (blue diamonds) and the cultures are still proliferating. Staining of the Neo-control HMC line 2 cultures after 37 MPDs for senescence-associated β-galactosidase shows numerous large flattened cells that stain positively and appear blue on the photograph (**c**) but in contrast the TERT-infected HMC cells showed few positive cells (**d**).

ing human keratinocytes that have bypassed senescence (Dickson *et al.*, 2000; Ramirez *et al.*, 2001), where the slow proliferation rate was attributed to an accumulation of the cyclin-dependent kinase inhibitor (CDKI), $p16^{INK4A}$. When the TERT-expressing keratinocytes increased their proliferation rate, $p16^{INK4A}$ was lost or downregulated (Dickson *et al.*, 2000). In other culture systems also, TERT-expressing epithelial cells senesce, unless $p16^{INK4A}$ is inactivated (Foster *et al.*, 1998; Kiyono *et al.*, 1998).

We measured the levels of $p16^{INK4A}$ in HMC mass cultures and clones (Fig. 5a). The hTERT-expressing HMC mass cultures increased $p16^{INK4A}$ expression until they proliferated beyond the normal senescence checkpoint of 30 MPD, whereupon $p16^{INK4A}$ was downregulated to early-passage levels in the mass cultures. Two of five HMC clones (HMCT3 and HMCT4) showed less $p16^{INK4A}$ expression than the early-passage hTERT-expressing cells but unlike the situation with keratinocytes (Dickson *et al.*, 2000), there was no clear relationship between the levels of $p16^{INK4A}$ and the clonal proliferation rate. To test the integrity of the $p16^{INK4A}$ gene directly, we sequenced the three exons of $p16^{INK4A}$ in all four of the HMC clones and found it to be wild-type (data not shown). As $p16^{INK4A}$ is often silenced by methylation (Merlo *et al.*, 1995), especially *in vitro* (Foster *et al.*, 1998), we also performed

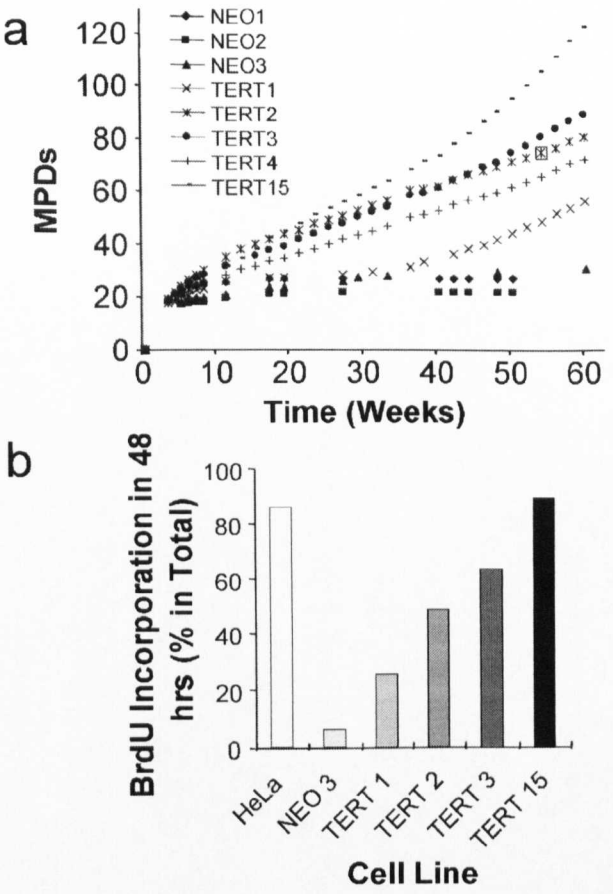

FIG. 2. Telomerase-expressing human muscle cell (HMC) clones show variable growth rates. **a**: Clones of pBabe Neo- and pBabeEst2-infected HMC cultures from the experiment shown in Figure 1. The number of each clone is indicated on the graph. All the Neo-infected clones senesced after 21–28 mean population doublings (MPDs), whereas the TERT-infected clones have so far completed between 45 (HMC T1) and 125 (HMC T15) MPDs. **b**: The incorporation rate of bromodeoxyuridine (BrdU) into the different HMC clones and cultures shows that the differences in culture growth rates in (a) can largely be explained by differences in the size of the proliferating fraction and hence the extent to which senescence is blocked.

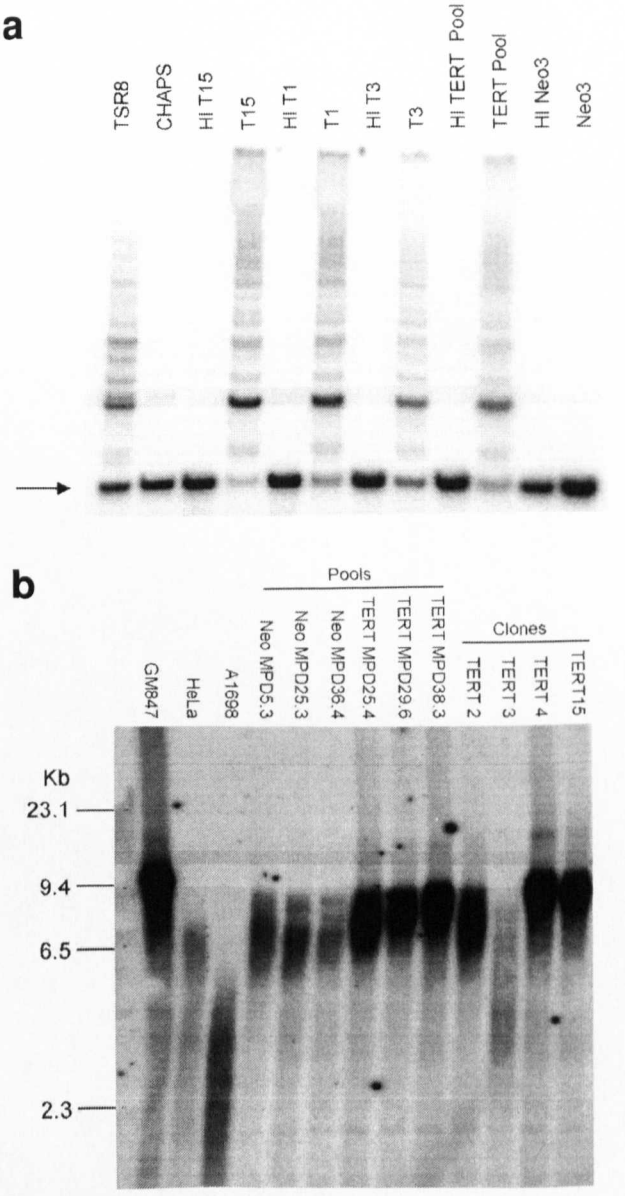

FIG. 3. Human muscle cells (HMCs) infected with pBabeEst2 express telomerase and generally maintain or extend average TRF lengths. **a**: TRAP assays performed on the HMCNeo and HMCTERT mass cultures and clones showing robust telomerase activity in all the cultures that bypassed senescence. The Neo 3 clone illustrated was representative of six such clones and was negative for telomerase. The lower arrow represents the internal standard product, which is used to check for adequate polymerase chain reaction (PCR) conditions. CHAPS is the buffer alone negative control; TSR8 is the kit positive control; HI indicates the heat-treated control for each sample. **b**: TRF Southern blot showing that the average TRF length of the HMCNeo mass culture declines with serial cultivation. This trend was progressively reversed in the mass cultures infected with pBabeEst2 (HMCTERT). The HMCTERT mass culture and all of the clones (except HMCT3) at least maintained average TRF lengths at presenescent levels. Clones HMCT4 and HMCT15 extended their TRF lengths to greater than presenescent levels.

bisulfite modification/sequencing of the genomic DNA and four of the HMC clones contained unmethylated $p16^{INK4A}$ genes (Fig. 5b). We cannot rule out other secondary alterations that may have affected the $p16^{INK4A}$/pRb pathway in the HMCTERT clones but there appears to be no straightforward relationship between the proliferation rate of each clone and $p16^{INK4A}$ status. The $p15^{INK4B}$ levels were also sustained in the HMCTERT mass cultures that had bypassed senescence and in most of the HMC clones (Fig. 5a). HMCT4 again showed the lowest levels of this CDKI and was one of the slower growing clones (Fig. 2a). Therefore, the levels of $p15^{INK4B}$ in the HMCTERT clones do not explain the variation in growth rate. There was also no relationship between clonal growth rate and the levels of $p21^{WAF}$ or p53, as the

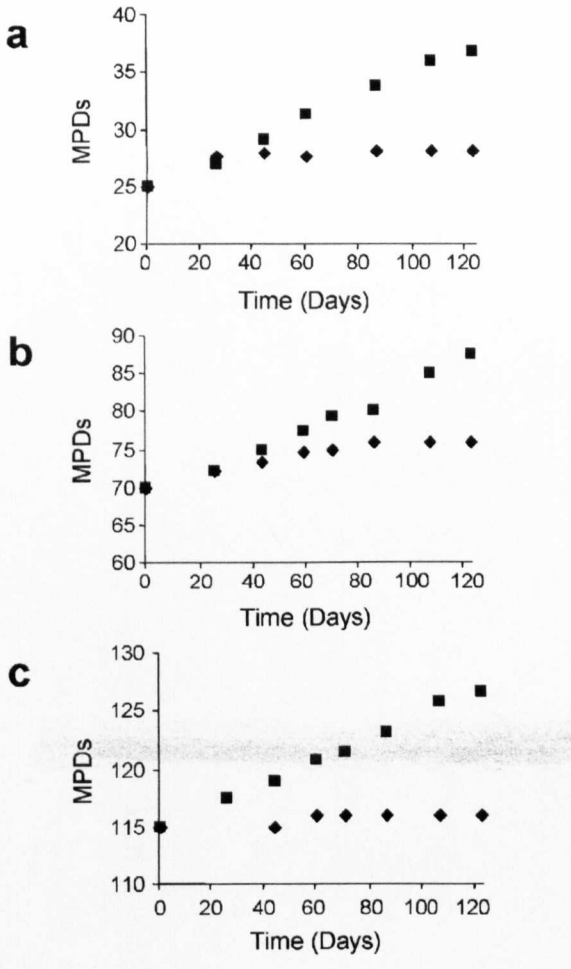

FIG. 4. Human muscle cells (HMCs) show a reduced replicative life span in Ham's F10 medium and cannot be immortalized with telomerase alone. **a**: HMC line 2 was cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and after 25 mean population doublings (MPDs), either kept in Dulbecco's (squares) or transferred to Ham's F10 (diamonds). **b**: Clone HMCT2 growth was compared in the two media as in (a) except that the cells were transferred into F10 at 70 MPDs. Symbols are as in (a). **c**: Clone HMCT15 was compared in the two media as in (a) except that the cells were transferred into F10 at 115 MPDs. Symbols are as in (a).

fastest growing clone, HMCT15, had the most $p21^{WAF}$. The levels of p53 were also examined and there was no evidence of altered expression (data not shown). Although not entirely conclusive, together with the $p21^{WAF}$ data, this result suggests an absence of p53 mutations.

Another issue of importance when considering the use of hTERT-expressing HMC cells in the treatment of human muscle disease, is the status of the proto-oncogene c-*myc*, which when upregulated can bypass the $p16^{INK4A}$ cell cycle checkpoint (Alevizopoulos *et al.*, 1997) and upregulate endogenous telomerase levels (Wang *et al.*, 2000b). Moreover, it is thought that the upregulation of c-*myc* may be responsible for the inability to extinguish telomerase activity in TERT-immortalized mammary cells after removal of the TERT transgene (Wang *et al.*, 2000b). We therefore examined the levels of c-*myc* protein in our clones. We found that the levels of c-*myc* were approximately threefold higher in the HMCTERT mass cultures once they had bypassed senescence and the HMCTERT clones expressed 1.7- to 6.0-fold more c-*myc* than the HMC controls. However, the levels of c-myc in HMCT15 were still 2- to 15-fold lower than the levels found in the immortal cancer lines HT1080, Jurkat and HeLa (Fig. 5c). Furthermore, there was no relationship between c-*myc* levels and the proliferation rate of each clone (Fig. 2a). Therefore, the proliferation characteristics of the various HMC clones expressing telomerase are not explained by their c-*myc* status alone.

TERT-expressing HMC clones bypassing senescence are cytogenetically normal

Human fibroblasts immortalized by the ectopic expression of hTERT are karyotypically normal and retain cell cycle checkpoint function (Jiang *et al.*, 1999; Morales *et al.*, 1999) and HMCs expressing hTERT must also display these characteristics, if they are to be a useful adjunct to gene therapy.

After 50–70 MPDs all five of the hTERT expressing HMC clones had a modal chromosome number of 46. The G-banded karyotype of HMCT15 shown in Figure 6a was typical of eight metaphases examined in detail and revealed no obvious abnormalities. Similarly, there was no evidence of loss of heterozygosity at the $p21^{WAF}$, $p57^{KIP2}$, p53, or pRB-1 loci (Fig. 6b). However, it was noted that the fastest growing clone HMCT15 did show allelic loss at the *INK4* locus ($p15^{INK4B}$, $p14^{ARF}$, and $p16^{INK4A}$), despite possessing a normal unmethylated $p16^{INK4A}$ gene. The improved proliferation rate of HMCT15 relative to the other clones might therefore have been caused by reduced expression of the $p16^{INK4A}$ or $p15^{INK4B}$ proteins brought about by haplo-insufficiency. However, the levels of both $p15^{INK4B}$ and $p16^{INK4A}$ in HMCT15 are similar to early passage HMC cultures (Fig. 6a). Taken together, the data show that the HMCTERT clones are genetically stable.

HMC cells expressing telomerase show no evidence of neoplastic transformation

In contrast to HeLa cells, the HMCTERT clones all failed to proliferate in 0.2% serum-containing medium (Fig. 7a). They resembled normal early-passage HMCs and if anything proliferated slightly less than the HMC controls under these conditions. The same clones also failed to proliferate in agar, unlike HeLa cells (Figs. 7b and 7c). Therefore the HMCTERT clones have not undergone neoplastic change.

TERT-expressing HMCs can regenerate striated muscle in vivo

The expression of hTERT has been reported not to interfere with the differentiation program in HMCs without life span extension (Seigneurin-Venin *et al.*, 2000a). To test whether our cultures with extended life span could still differentiate and reconstitute human striated muscle *in vivo*, we transplanted them

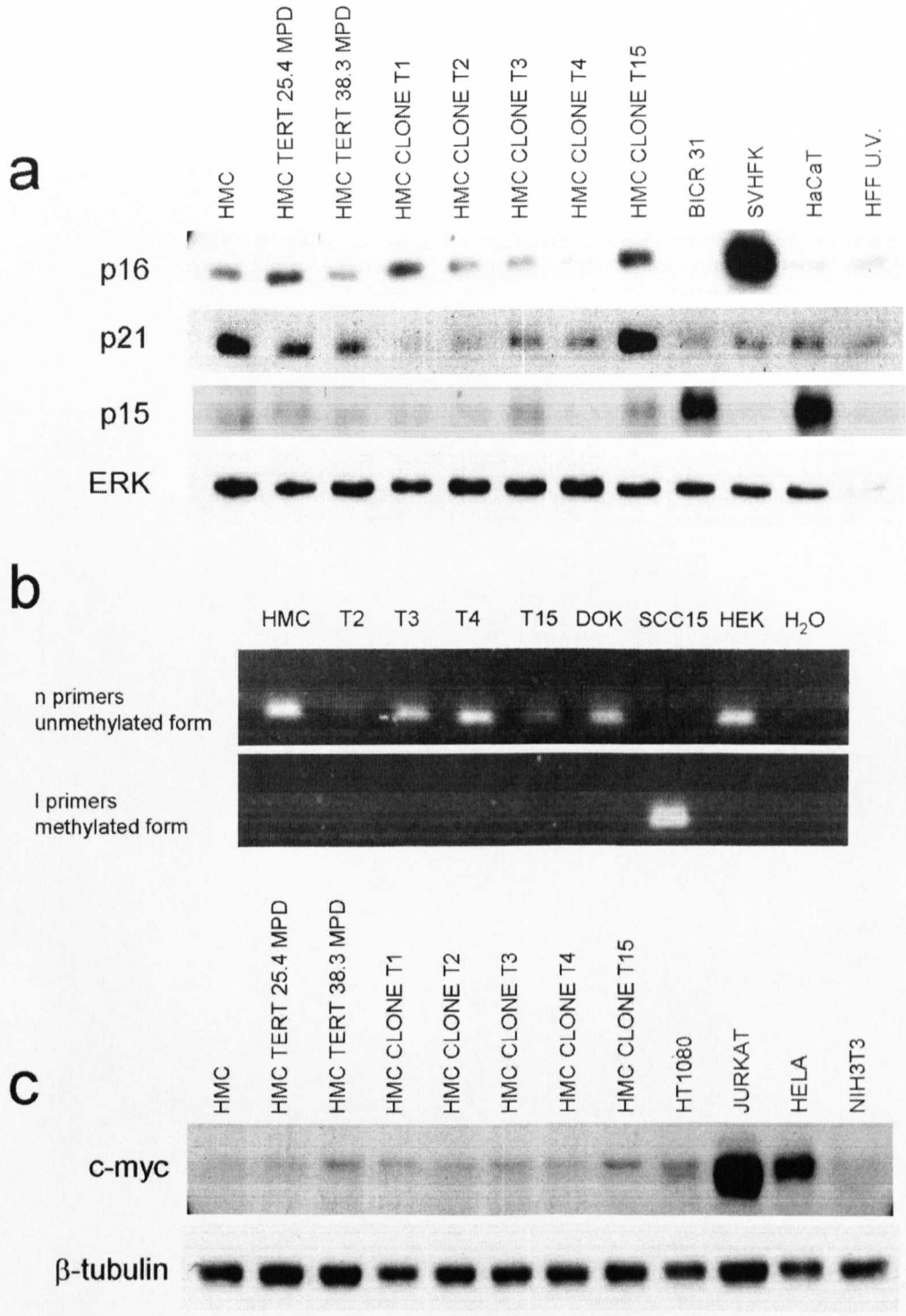

FIG. 5. The cyclin-dependent kinase inhibitor and c-*myc* status of the HMCTERT cultures. **a**: Western blots of $p16^{INK4A}$, $p15^{INK4B}$, and $p21^{WAF}$ maintained for up to 70 mean population doublings (MPDs) in culture (30 MPDs beyond the senescence checkpoint for HMCNeo cultures). The negative control for $p16^{INK4A}$ was BICR31, a cell line that is deleted for the *INK4A* locus (Munro *et al.*, 1999, 2001). The positive control was SV6-1HFK. ERK2 was used as a loading control. **b**: The figure shows the $p16^{INK4A}$ products obtained using n (unmethylated form) and l (methylated form) sets of primers (Baur *et al.*, 1999). Similar results were seen with m (unmethylated form) and k (methylated form) sets of primers (data not shown). Line SCC-15 served as a positive control for methylated $p16^{INK4A}$ and cell line DOK and normal human epidermal keratinocytes (HEK) as controls for the unmethylated $p16^{INK4A}$. **c**: Western blot of c-*myc* levels in the HMCTERT cultures that shows no large increase in the c-*myc* levels for up to 70 MPDs in culture. The loading control was β-tubulin.

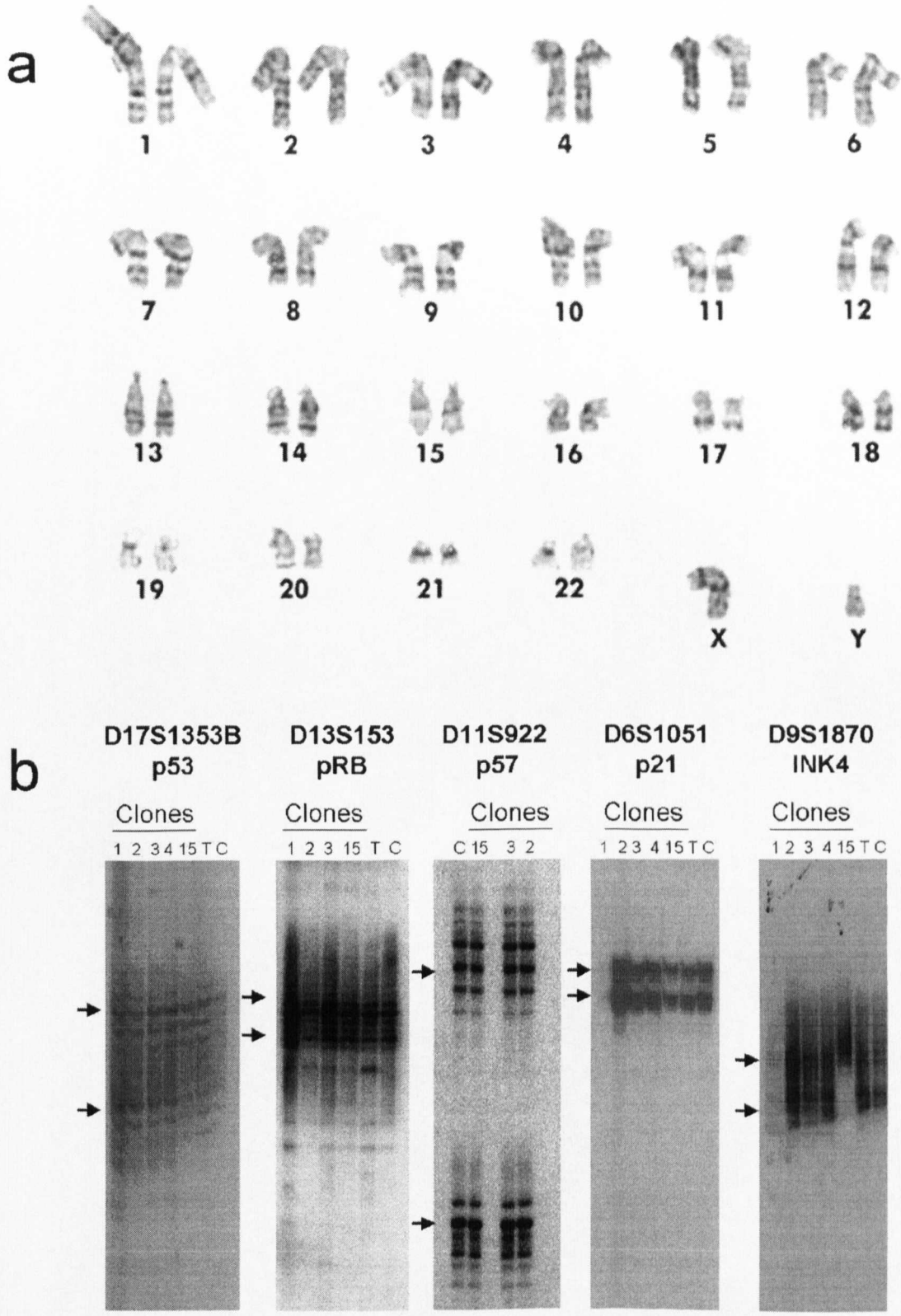

FIG. 6. The genotype of HMCTERT cultures is stable. **a**: G-banded karyotype of clone HMCT15 showing no obvious abnormalities. **b**: Allelic analysis of genetic loci involved in cell cycle regulation and senescence. This figure shows almost exclusive retention of heterozygosity at the p53, pRB-1, $p57^{KIP2}$, and $p21^{WAF}$ loci, but clone HMCT15 did show loss of heterozygosity at the *INK4* locus. The arrows indicate the position of each allele and the informative microsatellite illustrated is given above each panel next to the locus name. The clone numbers are each HMCTERT clone; T = HMCTERT pool; C = normal HMC cells.

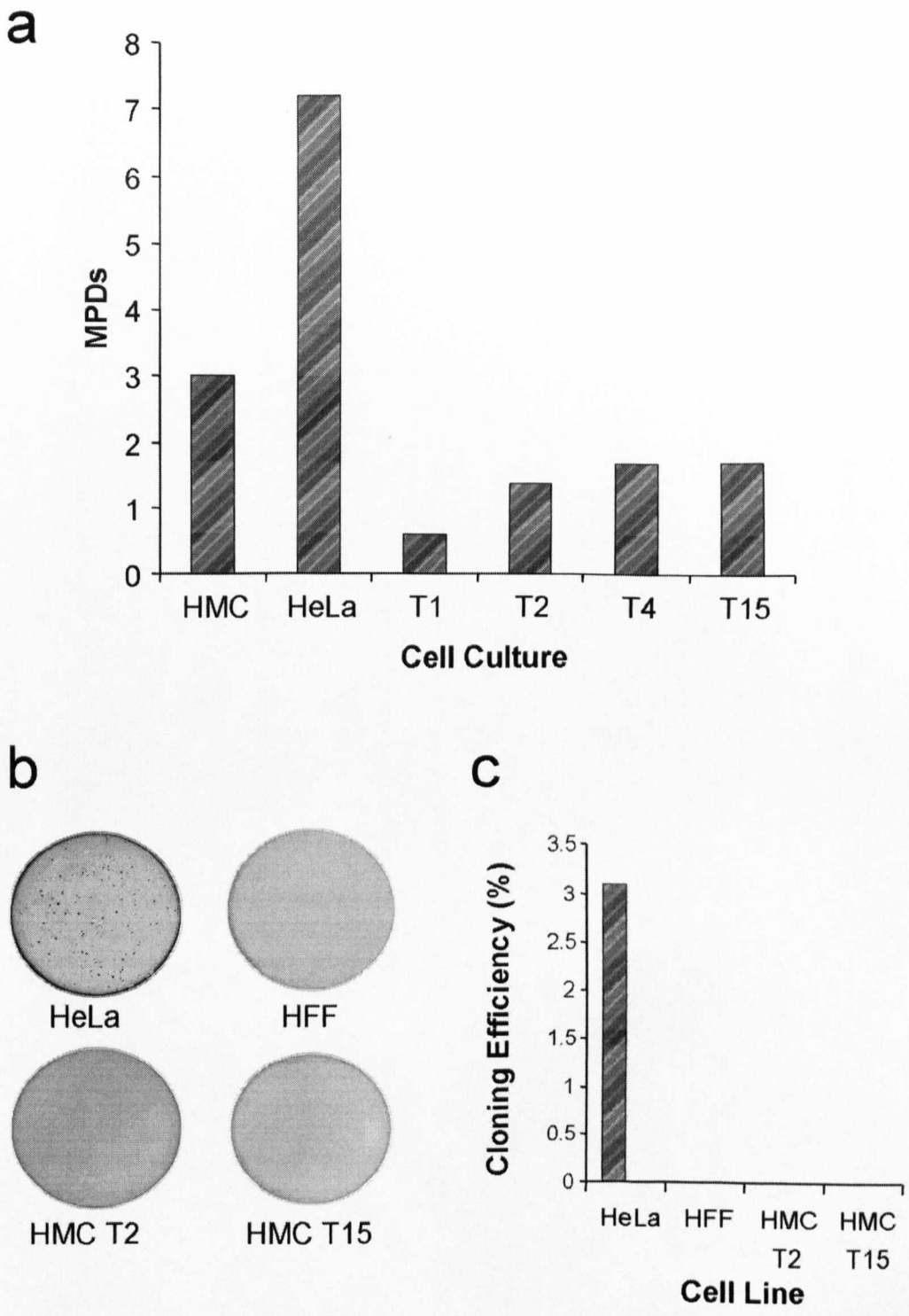

FIG. 7. Expression of hTERT in human muscle cells (HMC) does not result in neoplastic transformation. **a**: Proliferation of HMCTERT clones in low levels of fetal bovine serum is minimal and is less than early passage HMC cells. HeLa cells were able to undergo considerable proliferation under the same culture conditions. HeLa cells were used as a positive control line and normal HMC cells at early passage were used as negative controls. **b**: The HMCTERT clones fail to proliferate in suspension culture in contrast to the HeLa cell line controls that showed a cloning efficiency in agar of 3%, consistent with previous reports. The colonies were counted and photographed after 5 weeks. **c**: Quantification of the experiment shown in Figure 5b.

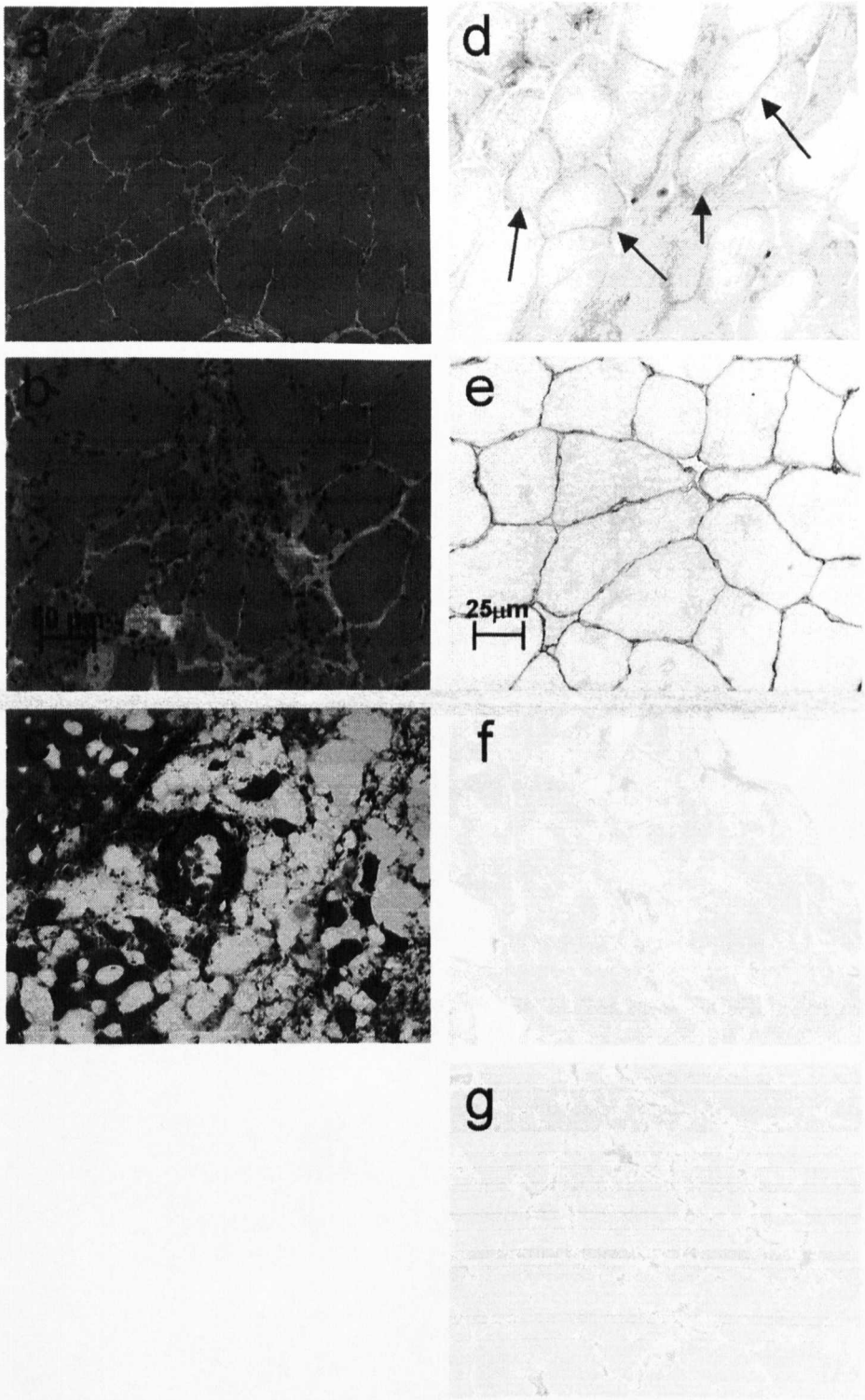

FIG. 8. The HMCTERT clones can reconstitute fully differentiated muscle fibers *in vivo*. This figure shows the results of experiments in which HMC clones T2 and T15 were grafted into the damaged muscle of irradiated RAG-1 mice. **a–c**: Hematoxylin and eosin stained muscle grafts and control. a: HMCT2 showing evidence of healthy muscle fibers. b: HMCT15, as in (a). c: Ungrafted muscle showing no mature or healthy regenerating muscle fibers. **d**: Graft of HMCT2, stained with a human specific antispectrin antibody and showing evidence of a submembranous accumulation of the protein. **e**: Normal human muscle stained with the same antibody. **f**: A control graft with no added human muscle cells shows no specific staining. **g**: Normal mouse muscle showing no specific staining. Scale bar = 50 μm (a–c) and 25 μm (d–g). Arrows in (d) indicate human spectrin positive muscle fibres that were considered to have fused (see Table 1).

into RAG-1 mice (Watt *et al.*, 1982) in which a localized area of trauma had been produced. HMCT15 engrafted more efficiently, in keeping with its high proliferation rate *in vitro*. Figure 8 shows the presence of healthy mature muscle fibers in grafts from both HMCT2 (Fig. 8a) and HMCT15 (Fig. 8b). In contrast, grafts of late-passage control HMCs and ungrafted controls (Fig. 8c) show no evidence of regeneration. The human grafts also stained positively with a human specific spectrin antibody confirming their human origin (Fig. 8d), in contrast to the nongrafted controls (Fig. 8f) and normal mouse muscle (Fig. 8g). The submembranous staining pattern in the HMCT2-engrafted muscle was observed in 28% of the muscle fibers present in the graft close to the injection site and resembled that of normal human muscle (Fig. 8e). Quantitation of the number of spectrin positive fibers present in the grafts (Table 1) showed that the number of fused muscle fibers in the telomerase expressing clone HMCT2 was at least as high as in the early passage HMC controls (28.4% vs. 24.8%). Thus the HMC clones that display a considerable extension of proliferative life span can still undergo fusion and reconstitute mature striated muscle *in vivo*, and these observations are consistent with previous reports that telomerase expression does not disrupt the differentiation program of HMC cells (Seigneurin-Venin *et al.*, 2000a,b).

DISCUSSION

There are encouraging signs that gene therapy for DMD and perhaps other diseases may be possible (Wang *et al.*, 2000a) but there is also evidence to suggest that muscle gene therapy in humans may fail, unless the telomeres of the recipient cells are repaired, to allow sufficient replicative capacity of the genetically corrected cells (Webster and Blau, 1990; Decary *et al.*, 2000). Therefore, we decided to investigate whether the replicative life span of human muscle cells could be extended by the ectopic expression of hTERT, without compromising genomic stability, analogous to the previous observations made with human fibroblasts (Bodnar *et al.*, 1998; Vaziri and Benchimol, 1998; Jiang *et al.*, 1999; Morales *et al.*, 1999).

We have shown here that the expression of hTERT can extend the telomeres of HMCs and extend the proliferative life span of both mass cultures derived from two different donors and four of five of the clones tested. Although it is well established that telomerase expression itself does not cause genomic instability (Jiang *et al.*, 1999; Morales *et al.*, 1999) and that it protects cells from chromosomal fusions (Mitchell *et al.*, 1999; Artandi *et al.*, 2000), cells immortalized by telomerase under certain culture conditions do show karyotypic changes (Farwell *et al.*, 2000). Furthermore, all previous successful attempts to extend the replicative life span of human myoblasts used DNA tumor viruses and presented no data on the karyology of the cells (Lochmuller *et al.*, 1999; Seigneurin-Venin *et al.*, 2000b). Significantly, therefore, we show here that hTERT can extend the life span of HMCs for up to 40 MPDs, without causing gross karyotypic alterations. The cells were not transformed and were still able to reconstitute human striated muscle when grafted into RAG-1 mice, confirming earlier studies, which showed that the ectopic expression of hTERT does not disrupt the ability of HMCs to reconstitute normal striated muscle *in vivo* (Seigneurin-Venin *et al.*, 2000a,b).

Epithelial cells cannot be fully rejuvenated to early-passage growth rates with hTERT alone (Kiyono *et al.*, 1998; Dickson *et al.*, 2000; Ramirez *et al.*, 2001), and generally, rapid growth rates in TERT-expressing epithelial cells is accompanied by the loss of $p16^{INK4A}$. Interestingly, the fastest growing clone, HMCT15, did show loss of heterozygosity (LOH) at the *INK4* locus but no reduction in the levels of $p15^{INK4B}$ or $p16^{INK4A}$, at least prior to 70 MPDs. Furthermore, HMCT15 showed a normal karyotype so the LOH of *INK4* was not the result of an obvious karyotypic change. The levels of CDKI expression did not correlate with HMCTERT clonal growth rate. Furthermore, there was no evidence of $p16^{INK4A}$ mutation, deletion, or methylation in any of the HMC clones studied. The levels of c-*myc* protein, which is sometimes upregulated in hTERT-expressing cells (Wang *et al.*, 2000b), did not correlate with the growth rate of the HMCTERT cultures and was much lower than the levels expressed by cancer cell lines. The growth rate of the TERT-expressing mass HMC cultures and four of the five clones studied was stable, arguing against secondary events in the extension of life span in these clones. However, in one of the clones, HMCT1, unknown secondary events did appear to contribute to the bypass of senescence, as the clone assumed a much faster growth rate at later passage.

Our experiments led to the bypass of senescence in HMCs after the expression of hTERT alone, where previous attempts did not, despite an increase in the average TRF length (Seigneurin-Venin *et al.*, 2000a,b). One possible explanation is that we used DMEM, while other groups have used Ham's F10, or derivatives thereof and it has recently been reported that the choice of culture conditions can influence the impact of telomere-independent senescence mechanisms (Ramirez *et al.*,

TABLE 1. COMPARISON OF THE NUMBER OF FUSED MUSCLE CELLS IN XENOGRAFTS OF NORMAL HMCS AND THE TELOMERASE-EXPRESSING HMC CLONE, HMCT2

Muscle graft	*Number of spectrin-positive fibers/total number of fibers*	*Percentage of spectrin-positive fibers*
Normal cultured human muscle cells	26/105	24.8
HMC T2	312/1099	28.4

HMC, human muscle cells.

2001). We showed that when our HMC cells were cultured in Ham's F10 medium rather than DMEM they showed a truncated replicative life span and that two of the telomerase-expressing HMC clones (HMCT2 and HMCT15) rapidly underwent senescence when transferred from DMEM to Ham's F10 medium. These observations underline the importance of developing improved culture media for the growth of HMC cells in the future. There are many differences in the formulations of Ham's F10 and DMEM, including the inclusion of biotin, thiamine, and vitamin B_{12} in the former and a sixfold higher calcium concentration in the latter. Testing the impact of these variables may aid the quest for improved HMC culture conditions in the future. Another difference between our work and that of previous investigators is that we removed G418 selection as soon as the mock-infected cultures had died and it has been documented that maintaining human keratinocytes under continuous G418 selection reduces their replicative life span (Morgan *et al.*, 1987).

The great variability in the proliferation rate of the HMC-TERT clones that bypassed the senescence checkpoint could not be completely explained by c-*myc*, CDKI, or telomerase levels, or by average TRF length. However, it is possible that hTERT is able to bypass senescence more efficiently in HMC, which are at a particular stage of differentiation. With respect to this, it will be interesting to transduce hTERT into bone marrow HMC stem cells (Ferrari *et al.*, 2001).

Our results could have implications for the expansion of human muscle cell populations in general but in particular as an aid to the gene therapy of the muscle dystrophies, where the proliferative capacity of the target HMC is low because of premature replicative senescence (Webster and Blau, 1990; Decary *et al.*, 1997). However, it should be stressed that although the culture conditions we have reported here are superior to those used previously, they are still not fully capable of rejuvenating normal diploid myoblasts and it is possible that the generation of reactive oxygen species in cell culture may be responsible for the slow growth rate of most of the hTERT-expressing myoblast clones (Ramirez *et al.*, 2001). This problem may eventually be solved by the use of antioxidants or culturing cells under low oxygen conditions (Wei *et al.*, 2001).

A further problem is that although telomerase does not transform cells (Jiang *et al.*, 1999; Morales *et al.*, 1999), the expression of telomerase may facilitate the development of tumors by contributing to the immortalization process (Counter, 1996; Counter *et al.*, 1998) or by other, as yet unidentified, mechanisms (Gonzalez-Suarez *et al.*, 2001; Stewart *et al.*, 2002). The transient expression of hTERT using the Cre recombinase and hTERT flanked by *loxP* sites (Wang *et al.*, 2000b) could be used to extend the length of short telomeres in myoblasts and thus increase their replicative potential. These rejuvenated HMCs would then make better targets for various forms of gene therapy. However, the use of hTERT in such therapies is dependent on the establishment of the improved myoblast culture conditions described above and our data represents an important step toward this objective.

Our work also opens up the opportunity to obtain long-lived, genetically stable HMC lines from biopsies of patients with muscular diseases to serve as valuable human *in vitro* models of these diseases.

ACKNOWLEDGMENTS

The authors would like to thank Professors John Wyke and Paul Harrison for critical review of the manuscript. We thank H. Vaziri for the gift of the pBabeEst2 retroviral construct. We are deeply indebted to Cancer Research UK, EMBO, Mr. William Jack and the Norwich Union Insurance Company for financial support of the work.

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Received for publication May 20, 2002; accepted after revision August 21, 2003.

Published online: September 12, 2003.

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