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Development of Adenoviral Vectors for Monitoring Telomerase Activity in Living Cells

Anna Helena Edqvist

2007

Department of Cell and Organism Biology
Lund University
Sweden

A doctoral thesis at a university in Sweden is produced either as a monograph or as a collection of papers. In the latter case, the introductory part constitutes the formal thesis, which summarises the accompanying papers. These have already been published or are manuscripts at various stages (in press, submitted, or in preparation).

Cover: Glioblastoma multiforme cells transduced with
Ad5F35-EGFP virus and stained with DRAQ5.
Picture taken by Bo Holmqvist (Pathology, Lund)

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LIST OF PAPERS

This thesis is based on the following papers, referred to by their Roman numerals:

I: **Anna Edqvist**, Bo Holmqvist, Ineke Sloots, Dongping Tian, Bengt Widegren, Leif G. Salford and Xiaolong Fan. **Tropism dependent gene transfer and penetration efficacy of adenoviral vectors in primary glioblastoma multiforme cells.** Submitted.

II: **Anna Edqvist**, Johan Rebetz, Marcus Järås, Anna Rydelius, Gunnar Skagerberg, Leif G. Salford, Bengt Widegren and Xiaolong Fan. **Detection of cell cycle- and differentiation stage-dependent human telomerase reverse transcriptase expression in single living cancer cells.** Molecular Therapy 2006; Vol. 14: 139-148.

III: Marcus Järås, **Anna Edqvist**, Johan Rebetz, Leif G. Salford, Bengt Widegren, Xiaolong Fan. **Human short-term repopulating cells have enhanced telomerase reverse transcriptase expression.** Blood 2006; Vol. 108: 1084-1091.

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ABBREVIATIONS

ALT	alternative lengthening of telomeres
bp	base pairs
CAR	coxsackie- and adenovirus receptor
d2EGFP	destabilised enhanced green fluorescence protein
DKC	dyskeratosis congenita
GBM	glioblastoma multiforme
HSC	haematopoietic stem cell
hTERC	human telomerase RNA component
hTERT	human telomerase reverse transcriptase
hTERT	human telomerase reverse transcriptase gene
ITR	inverted terminal repeat
kb	kilo base pairs
LT-HSC	long-term haematopoietic stem cells
M1	mortality phase 1
M2	mortality phase 2, 'crisis'
MOI	multiplicity of infection
POT1	protection of telomerase 1
RGD	Arginine-Glycine-Asparagine
SRC	NOD/SCID mice bone marrow re-populating cells
ST-HSC	short-term haematopoietic stem cells
TP	terminal protein
TRAP	telomeric repeat amplification protocol
$TRF2^{\Delta B \Delta M}$	dominant-negative form of telomeric-repeat factors 2

GENERAL INTRODUCTION

Hallmarks of cancer

In development of cancer, normal cells need to acquire accumulation of genetic traits necessary for altering normal cell physiology into a malignant phenotype. Most cancer cells acquire six essential alterations of functional capabilities; they provide themselves with growth signals, develop insensitivity to growth-inhibitory signals, escape programmed cell death (apoptosis), achieve immortality, ensure a constant formation and development of new blood vessels, and last but not least, attain powers of tissue invasion and metastasis (Hanahan and Weinberg, 2000). And then, there are two types of genetic events important for initiation of the carcinogenic process; **i**) activation of oncogenes as a consequence of point mutation, amplification or chromosomal translocation and **ii**) inactivation of tumour suppressor genes by deletion, mutation or epigenetic silencing (Vogelstein and Kinzler, 2004). Activation of oncogenes provides the cell with enhanced capacity for proliferation and drives cancer progression by "stepping on the gas". Inactivation of tumour suppressor genes on the other hand, sets aside intrinsic protection mechanisms for inappropriate growth, such as apoptosis and cell cycle-arrest (quiescent state), and drives cancer progression by removing the "brakes". Genetic instability is an attribute in human cancer cells that contributes to accumulation of genetic alterations and drives progression of carcinogenesis.

Involvement of tissue restricted stem and progenitor cells in cancer

The existence of a distinct tumourigenic cell population was first observed in acute myeloid leukaemia (ALM), a malignancy of the haematopoietic system, and later in breast and brain tumours (Al-Hajj *et al.*, 2003; Lapidot *et al.*, 1994; Singh *et al.*, 2003). These tumourigenic cells have capacity for self-renewal, constantly replenishing the heterogeneous cell populations within the tumour mass by differentiation, and driving a continued expansion of tumour cell population. These tumourigenic cells resemble normal stem cells in self-renewal, capability to develop into multiple lineages, and

potential to proliferate extensively, therefore, they are termed cancer stem cells (Pardal *et al.*, 2003). The identification of cancer stem cells supports the notion that cancers are derived from a single transformed stem cell or from a differentiated progenitor cell that has regained the self-renewal capacity. In a self-renewal cellular division, at least one of the daughter cells maintains the same proliferative and differentiation capacity as the mother cell. Long-living stem cells undergo hierarchical differentiation process to maintain adult tissue with mature cells, which have a lifespan of days or weeks. Likewise, if a tumour is viewed as an organ, the few long-living cells that constantly replenish the tumour mass are then the cancer stem cells. The self-renewal division is strictly regulated in normal stem cells, whereas it is most likely deregulated in cancer stem cells, which results in continuous self-renewal of these and expansion of relatively differentiated non-tumourigenic cancer cells essential for the tumour-growth (Al-Hajj and Clarke, 2004). This non-tumourigenic subset of the tumour lacks the ability to self-renew and is blocked at certain differentiation steps towards mature cells.

Unquestionably, anticancer therapies need to effectively identify, target and eliminate the cancer stem cells to prevent regeneration of malignancy. Further, self-renewal and extensive proliferation capacities are distinct characteristics that provide possibilities for detection of cancer stem cells and normal stem cells and also to distinguish them from normal mature somatic cells.

Cell proliferation and lifespan

In the 1960s, Hayflick reported that the proliferative capacity of fibroblasts *in vitro* was pre-determined and this led to the hypothesis that cells possess an internal biological clock (Hayflick, 1965). This “clocking” mechanism registers the number of cellular divisions an individual cell has passed and halts further divisions when the so-called Hayflick limit is reached, thus limiting the proliferating life span of the cell. A culture of normal human cells, for example fibroblasts, stops dividing after about 50-70 generation doubling times.

The end replication problem

A few years later, both Olovnikov and Watson, independently, hypothesised that every round of replication of a linear chromosome would result in a loss of a length of base pairs (bp) at the ends of chromosome (Olovnikov, 1973; Watson, 1972). This is due to the 5´to 3´directed DNA synthesis and the need of a short RNA primer for DNA polymerase to start replication from (i.e. replication cannot start *de novo*) (**Figure 1**). This theoretical integrity problem of linear chromosomes was termed "the end replication problem". Each round of replication results in an inevitable loss between 30-150 bp at both ends of the chromosome (Harley *et al.*, 1990; Huffman *et al.*, 2000).

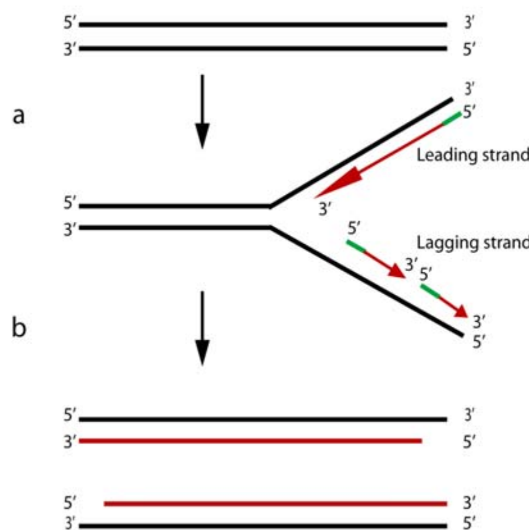

Figure 1. *The end replication problem.* The DNA double helix is replicated semi-conservatively by DNA polymerase. Each of the DNA strands serves as a template, called parental strands (shown in black), and each of the two new daughter chromosomes consists of one old (shown in black) and one newly synthesised strand (shown in red).

a) There is continuous leading strand synthesis starting from one short RNA primer (shown in green). The discontinuous DNA synthesis of the lagging strand is made as a series of short DNA sequences, called *Okazaki fragments;* all starting from a short RNA primer. To produce a continuous DNA chain, the RNA primers in front of a newly synthesised Okazaki fragment are erased and replaced by DNA.
b) Consequently, the gaps appearing after removal of the most distal RNA primers at the very 5´end of newly synthesised strands cannot be filled since DNA polymerase has no primers to start from. This results in an inevitable loss of base pairs at the end of chromosomes.

Telomeres

Already in the late 1930s, the work by Müller and McClintock independently had surmised that natural chromosome termini displayed special capping characteristics since chromosomes with broken ends were unstable and could form dicentric- and ring chromosomes (McClintock, 1938; McClintock, 1941; Muller, 1938). Müller termed the chromosome ends "telomeres" from the Greek "telos" for end and "meros" for part. The first telomeric DNA to be sequenced was from the ciliated protozoa *Tetrahymena thermophila* and it was revealed that telomeres consist of a non-coding sequence that protects the coding sequences on the chromosomes (Blackburn, 1991; Blackburn and Gall, 1978).

Telomere shortening and the biological clock

The progressive telomere shortening due to the "end replication problem" directly supports the hypothesis of an internal biological "clocking" mechanism. When the telomeres reach a critically short length they induce replicative senescence in a cell, which is characterised by an arrest in proliferation with maintained metabolic activity (Allsopp *et al.*, 1992; Harley *et al.*, 1990; Hayflick, 1965; Wright and Shay, 1992).

Telomere structure

The telomeric sequence of a great majority of eukaryotes, including unicellular organisms, plants and mammals, is characterised by short tandem repeats at both ends of the chromosome. In vertebrates, this tandem repetitive array consists of a GT-rich sequence, TTAGGG (Moyzis *et al.*, 1988). The overall telomere length varies greatly between species and it may measure between 5-15 kilo bp (kb) in humans and between 20- 150 kb in mice (Blackburn, 1991; de Lange *et al.*, 1990; Starling *et al.*, 1990). The natural ends of the double-stranded telomere consist of a 3´single-stranded telomeric overhang, which in humans are approximately 150 bp (Makarov *et al.*, 1997). This telomeric overhang can fold back and invade the adjacent double-stranded telomeric repeats forming a protective capped T-loop structure (Griffith *et al.*, 1999). This T-loop

structure protects the chromosome ends but it is not yet clarified if all telomeres are T-looped, and if it is a dominant or an optional structure.

Telomere associating proteins

Hitherto, six major telomere-associating proteins: telomeric-repeat factors 1 and 2 (TRF1 and TRF2), repressor activator protein 1 (RAP1), TRF1-interacting protein 2 (TIN2), protection of telomeres 1 (POT1) and tripeptidyl peptidase 1 (TPP1) have been identified. Together, they form a complex called shelterin, which participates in telomere length regulation and affects the structure of telomeric DNA (Liu *et al.*, 2004; Ye *et al.*, 2004). Both TRF1 and TRF2 bind to double-stranded telomeric repeats and POT1 binds to single-stranded telomeric overhang (Smogorzewska and de Lange, 2004). TRF2 protein was seen to be involved in the formation of T-loop structure *in vitro*, but with low efficiency (Stansel *et al.*, 2001). This suggests a probable requirement of additional factors such as other proteins or even the whole shelterin complex. On the other hand, inhibition of TRF2, by expression of a dominant-negative form of the protein ($TRF2^{\Delta B \Delta M}$) resulted in chromosomal fusions, aberrant mitosis, and aneuploidy leading to replicative senescence, and apoptosis mediated by p53-dependent DNA-damage response pathway, both *in vitro* and *in vivo* (Karlseder *et al.*, 1999; Lechel *et al.*, 2005; Smogorzewska and de Lange, 2002; van Steensel *et al.*, 1998). This suggests that TRF2 does have an important role in structure determination. Furthermore, checkpoints factors taking part in DNA damage response pathway have been observed at telomeres; both after inhibition of TRF2 and also when telomeres become critically short (d'Adda di Fagagna *et al.*, 2003). Thus, not only the length but also the structure of telomeres is important for replicative lifespan of cells.

Taken together, the T-loop structure with associated proteins creates a capped telomere structure that masks telomeres and prevents them from being considered as broken or damaged DNA. Thus, they shield chromosomes from recombination, end-to-end fusion, and degradation by exonuclease and are prerequisite for continued cell proliferation (Blackburn, 2001; Karlseder *et al.*, 1999).

Telomere lengthening

In 1985, a novel telomere terminal transferase, later called telomerase, was found to be involved in the *de novo* elongation of telomeres, thus compensating telomeric loss, in *Tetrahymena hermophila* (Greider and Blackburn, 1985). Since then, telomerase has now been identified and characterised in several species, including humans (Morin, 1989). It is a ribonucleoprotein enzyme and consists of two major components: a RNA template for telomeric repeat synthesis (the telomerase RNA component, TERC) (Feng *et al.*, 1995) and a catalytic component with reverse transcriptase activity (the telomerase reverse transcriptase, TERT) (Meyerson *et al.*, 1997). In humans, the genes coding for TERC (*hTERC*) and TERT (*hTERT*) are located on chromosomes 3 and 5, respectively (Meyerson *et al.*, 1997; Soder *et al.*, 1997). Besides these components telomerase is associated with a set of accessory proteins too; dyskerin which is involved in the biogenesis and stability of telomerase, and human ever shorter telomeres 1A (hEST1A), which is most likely involved in remodelling the telomere cap structure and makes the telomere sequence accessible for telomerase (Reichenbach *et al.*, 2003; Smogorzewska and de Lange, 2004).

Telomere lengthening mechanisms

Telomeres are extended during DNA synthesis (S-) phase of cell cycle through repetitive reverse transcription (by TERT) of a short segment of the RNA template (TERC) and thereby adding multiple copies of telomeric repeats to the 3'-end of DNA. This is followed by synthesis of the complementary strand by DNA polymerases to create double-stranded telomeric DNA (**Figure 2**). Telomere length is controlled by a negative feedback loop involving the shelterin complexes, where the single-stranded DNA binds POT1 components and acts as an inhibitor of telomerase activity (Smogorzewska and de Lange, 2004). Long telomeres contain extended numbers of shelterin complexes with increased probability for the POT1 components to bind to the telomeric overhang and block its access to telomerase. In addition, the shelterin complexes may inhibit telomerase activity by promotion of T-loop formation in which

the 3' overhang is unavailable since it is tucked into the double-stranded DNA part of the telomere.

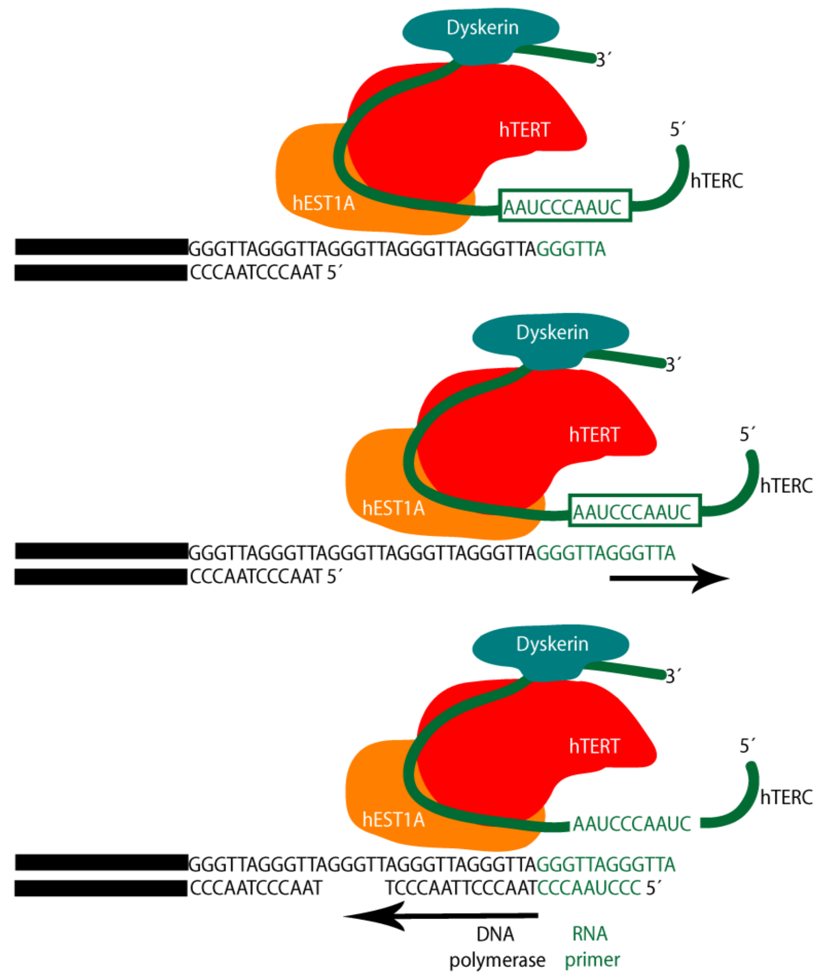

Figure 2. *Telomere lengthening by telomerase.* Telomerase consists of a RNA component (hTERC), a catalytic component with reverse transcriptase activity (hTERT), and accessory proteins such as dyskerin and hEST1A. The absolute 3' ends of double-stranded DNA molecule are single-stranded and extended by repetitive reverse transcription of the RNA template. This is followed by traditional DNA synthesis of a complementary strand by DNA polymerase to achieve double-stranded telomeric sequence. Telomerase prolongs the telomeres at both ends of a linear chromosome, with tandem repetitive array of TTAGGG sequences, and is thereby an evolutionary solution to "the end replication problem" (Telomerase structure adapted from Smogorzewska and de Lange, 2004).

Telomerase independent mechanisms of telomere maintenance have also been found in several eukaryotes. *Drosophila melanogaster,* the fruit fly, uses retrotransposons and the mosquito, *Anopheles gambiae,* uses homologous recombination to elongate their

telomeres (Mason and Biessmann, 1995; Roth *et al.*, 1997). Human cells have a telomerase independent mechanism as well, the alternative lengthening of telomere (ALT), which can compensate for the telomeric loss. ALT mechanism is based on homologous recombination where DNA sequence can be copied from one telomere to another (Dunham *et al.*, 2000). This mechanism is found in tumours and immortalised cell lines (Bryan *et al.*, 1997; Dunham *et al.*, 2000) and these cells usually have extensively long telomeres.

Telomerase activity

In adults, telomerase remains active in immature germline cells, certain stem- and progenitor cells in renewal tissues and in the highly proliferative cells, such as lymphocytes, while normal somatic cells often possess undetectable or very low levels of telomerase activity (Bekaert *et al.*, 2004; Hiyama and Hiyama, 2007; Kim *et al.*, 1994). A study of normal, early passage human fibroblasts showed a transient upregulation of *hTERT* and telomerase activity during their transition through S-phase of the cell cycle (Masutomi *et al.*, 2003). But this cell-cycle dependent transient expression of hTERT protein was not involved in stabilising the overall telomere length; instead it had a stabilising effect on the telomere cap structure (Masutomi *et al.*, 2003). Thus hTERT protein possesses cell survival functions that are unrelated to the maintenance of telomere length and are rather associated with an increased genomic stability and an enhanced DNA repair (Li *et al.*, 2005; Sharma *et al.*, 2003).

Telomerase activity in adult stem and progenitor cells

Relatively low levels of telomerase activity have been detected in various cell populations containing adult stem cells, including haematopoietic (HSC), neuronal, skin, hair follicle, intestinal crypt, mammary epithelial, pancreas, adrenal cortex and kidney. Its function and regulation during tissue stem cell self-renewal and differentiation is not well characterised, but there is indication of a telomerase necessity for maintenance of stem cell functions and self-renewal, especially in the

haematopoietic system. HSCs are multipotential cells, which give rise to the myeloid and lymphoid cell lineages during haematopoiesis. HSCs can be divided into two different types of populations depending on their capacity to reconstitute the bone marrow of sub-lethally irradiated recipients. These are termed long-term repopulating HSCs (LT-HSCs) and short-term repopulating HSCs (ST-HSCs), both with self-renewal capacity. The first step in haematopoiesis involves a gradual loss of self-renewal capacity from LT-HSCs to ST-HSCs (Christensen and Weissman, 2001; Morrison and Weissman, 1994) and the next step is differentiation into multi-potent progenitor cells (Morrison *et al.*, 1997). HSCs are thought to be critically dependent on telomerase activity (Chiu *et al.*, 1996; Engelhardt *et al.*, 1997; Hiyama *et al.*, 1995b), however the activity seems to be insufficient for a complete prevention of telomere loss because age-related telomere shortening has been found in HSCs (Engelhardt *et al.*, 1997; Hiyama *et al.*, 1995b; Yui *et al.*, 1998). Therefore, cancer/leukaemic stem cells may be compromised by telomere shortening and genomic instability and need to achieve elevated telomerase activity for further progression.

Regulation of telomerase activity

The hTERC component of telomerase is ubiquitously expressed in nearly all cell types and its expression does not correlate with telomerase activity; therefore it is a poor predictor of telomerase activity status in cells (Avilion *et al.*, 1996). In contrast, there is a strong correlation between the presence of detectable hTERT mRNA and telomerase activity (Ducrest *et al.*, 2002; Meyerson *et al.*, 1997; Ulaner *et al.*, 1998). Thereupon, it is suggested that regulation of telomerase activity is mainly dependent on transcription levels of *hTERT* (Cong *et al.*, 2002; Ducrest *et al.*, 2002; Horikawa *et al.*, 1999; Meyerson *et al.*, 1997).

The promoter region of *hTERT* gene contains a 181 bp long core promoter that is sufficient for transcriptional activation in cancer cells (Horikawa *et al.*, 1999; Takakura *et al.*, 1999). This region lacks the traditional TATA or CAAT box but possesses relatively high density of CpG dinucleotides and two E-boxes, the consensus binding sites of the transcription factors Sp1 and c-Myc, respectively (Takakura *et al.*,

1999). There is a direct activation of the *hTERT* promoter at these E-boxes by c-Myc/Max-factor heterodimer in normal proliferating cells (Bouchard *et al.*, 1998; Horikawa *et al.*, 1999; Wu *et al.*, 1999). Sp1 expression is upregulated during cellular transformation and has a co-operative role with c-Myc (Horikawa *et al.*, 1999; Kyo *et al.*, 2000). In addition to activation of *hTERT* transcription, c-Myc possesses an important role in activation of growth checkpoint phase 1 (G1) cyclin-dependent kinases (CDKs) during cell cycle, which are also important for cell proliferation (Wu *et al.*, 1999).

In a genetic screen of HeLa cells, three genes, all known to be involved in tumour suppressing or oncogene activation pathways, were found to limit hTERT expression (Lin and Elledge, 2003). The first, Mad1 protein represses *hTERT* promoter through competing for the ubiquitous binding partner Max-factor and hindering its binding to c-Myc in the Mad1/c-Myc pathway (Gunes *et al.*, 2000; Oh *et al.*, 2000; Poole *et al.*, 2001). The second, *SIP1,* a downstream transcriptional target of the TGF-β pathway, plays a partial role in TGF-β-mediated repression of *hTERT* (Lin and Elledge, 2003). The third, *MEN1* a tumour suppressor gene, with its gene product Menin acting as a direct repressor of *hTERT*.

The presence of a CpG island in the *hTERT* regulatory region suggests a possible role for epigenetic regulation of *hTERT* gene expression. It is proposed that the *hTERT* gene is first transiently silenced in the differentiating cells through histone deacetylation, and then by a stable inactivation by DNA methylation (Lopatina *et al.*, 2003). Another factor that also could have an impact on telomerase activity is post-transcriptional regulation of hTERT; it is only the full-length transcript that acts as a functional telomerase protein (Ulaner *et al.*, 1998). Likewise, the assembly of active telomerase ribonucleoprotein complex and its accessibility to the telomere ends via association of shelterin complexes with the telomeric DNA was seen to have an impact on the overall telomerase activity in cells (Smogorzewska and de Lange, 2004).

Telomere shortening during ageing and chronic diseases

The observations that telomere shortening limits the replicative lifespan of primitive human cells in cultures have fuelled the hypothesis of a biological "clocking" mechanism that implicates ageing. Telomere shortening occurs in most human organs and tissues, which are mitotically active and undergoing cell division during ageing. The *in vivo* situation is not fully characterised but a study on humans by Cawthon *et al.* (Cawthon *et al.*, 2003) suggests that telomere shortening contributes to a higher mortality in many age-related diseases, such as heart and infectious diseases.

Telomere shortening is detected in the different cell types of human organs affected by chronic diseases, such as peripheral blood cells in different bone marrow failure syndromes, in hepatocytes of patients with cirrhosis, and in colon epithelial cells of patients with colitis ulcerosa (Djojosubroto *et al.*, 2003). This telomere shortening in chronic disease might be due to elevated rate of cellular turnover and thereby limit the regenerative capacity of affected organ over time. In addition, intracellular oxidative stress could also contribute to an active telomere shortening (Passos *et al.*, 2006). Furthermore, different mutations in telomerase components decrease telomerase activity and cause acceleration of telomere shortening. Dyskeratosis congenita (DKC) is a severe form of inherited aplastic anaemia and can be either X-linked with mutations in the *DKC1* gene or autosomal dominant with mutations in the *hTERC* gene (Heiss *et al.*, 1998; Vulliamy *et al.*, 2001). The *DKC1* gene encodes dyskerin that is involved in ribosomal RNA processing and associates closely with the hTERC component in the telomerase complex (Yamaguchi, 2007). Patients with DKC suffer from abnormal skin pigmentation, chromosomal aberrations, and increased incidence of cancer and bone marrow failure (Cawthon *et al.*, 2003; Marrone *et al.*, 2004). Both types of mutations give rise to decreased telomerase activity because one normal allele is insufficient for the production of adequate amounts of telomerase for telomere maintenance with the consequence of telomere shortening, stem cell exhaustion and premature ageing (Marrone *et al.*, 2004).

Together, even if a disturbed telomere maintenance gives rise to a phenotype that resembles the phenotype in human ageing, this observation is not a proof that telomere shortening has a causal role in normal human ageing. Nevertheless, there are

indications that compromised telomere maintenance may play a critical role in the regenerative capacity of organs and tissues during ageing and chronic diseases.

Immortalisation and tumourigenesis

The prevalence of human cancer increases with advanced age and major hallmarks of most cancers are chromosomal instability with numerous genetic alterations. These often affect growth-controlling genes in early stage of tumour development (Hanahan and Weinberg, 2000). Chromosomal instability is linked to the functional status of the telomere structures, which plays a dual role in tumourigenesis. Short telomeres lose their protective telomeric cap structure and consequently, undergo end-to-end fusions, forming dicentric chromosomes, and anaphase bridges (Gisselsson *et al.*, 2001; McClintock, 1938). Continued cellular division allows cascades of repeated breakage-fusion bridge cycles of abnormal chromosomes followed by DNA fragmentation and further random gene gains and/or losses. Consequently, dysfunctional chromosomes accumulate and via chromosomal instability, promote tumourigenesis**.** Simultaneous to this promotion, DNA damage response pathways are also stimulated. These pathways involve pRb and p53 signalling and are upregulated early in tumourigenesis both in humans and mTERC$^{-/-}$ mice (Artandi *et al.*, 2000; Bartkova *et al.*, 2005; Rudolph *et al.*, 2001) inducing replicative senescence or apoptosis (the Hayflick limit also called mortality phase one, M1) (**Figure 3**) (Blackburn, 2001; Bodnar *et al.*, 1998; d'Adda di Fagagna *et al.*, 2003; Gisselsson *et al.*, 2001; Karlseder *et al.*, 1999; Rudolph *et al.*, 2001; Shay *et al.*, 1991).

Replicative senescence is thought to be an essential anti-tumour barrier that limits the inappropriate growth of normal cells and is one of the two proliferative checkpoints in the telomere hypothesis (**Figure 3**) (Shay *et al.*, 1991; Stewart and Weinberg, 2006; Wright and Shay, 2001). Despite these limitations, cell proliferation beyond senescence would be possible in cells which have activated oncogene and/or inactivated tumour suppressor genes. It has been shown that inactivation of DNA damage checkpoint kinases, such as CHK1 and CHK2 can restore cell-cycle

progression into S-phase allowing for continued cell proliferation (d'Adda di Fagagna *et al.*, 2003).

Eventually, second proliferative checkpoint called 'crisis' (or M2) is activated; characterised by uncontrolled chromosomal instability and massive cell death (**Figure 3**). Cells that survive checkpoint crisis/M2 are capable of stabilising their telomeres, most frequently by re-activation of telomerase, and thus are able to continue and support the progression of tumours (Artandi *et al.*, 2000). Approximately 90 % of all human tumours have high telomerase activity and expression of hTERT, which maintain telomere structures stable, encouraging an abnormal, increased number of cell divisions (Kim *et al.*, 1994; Lin and Elledge, 2003; Shay and Bacchetti, 1997). Another way to prolong and stabilise the telomeres and achieve immortality is through the ALT mechanism, but it was observed in about only 7-10 % of all tumours in human (Bryan *et al.*, 1997; Dunham *et al.*, 2000; Shay and Bacchetti, 1997).

Furthermore, introduction of a dominant-negative version of hTERT (DN-hTERT) protein was found to inhibit telomerase activity in various human tumour cell lines, such as LoVo (colon) and SW613 (breast), leading to telomere shortening and cell death by apoptosis (Hahn *et al.*, 1999b). But the immortalised human cell line, GM847, that used ALT mechanism to prolong its telomeres was not affected by the expression of DN-hTERT, confirming that DN-hTERT exerts its effects on telomerase with telomere shortening as the result (Hahn *et al.*, 1999b).

Successful immortalisation of normal human cells *in vitro* was achieved with ectopic hTERT expression that upregulated telomerase activity and prolonged the telomere length, but the cells did not achieve a tumourigenic phenotype; they had a normal karyotype (Bodnar *et al.*, 1998; Morales *et al.*, 1999; Vaziri and Benchimol, 1998). To achieve transformation of normal primary human cells *in vitro*, introduction of an oncogenic allele of H-ras and the SV40 large-T oncoprotein, which inactivates both the pRB- and p53- pathways, was necessary in combination with the ectopic expression of hTERT (Hahn *et al.*, 1999a). The classical telomere hypothesis with two proliferative checkpoints or mortality stages (M1 and M2) in response to moderate or severe telomere dysfunction is further supported by findings where low levels of TRF2$^{\Delta B \Delta M}$ expression evoke p53-dependent senescence, whereas high levels of

$TRF2^{\Delta B \Delta M}$ expression induced p53-independent apoptosis *in vivo* (Lechel *et al.*, 2005; Shay *et al.*, 1991).

Taken together, these results suggest that the expression of hTERT is necessary but not sufficient for malignant transformations and that telomere length can influence the replicative lifespan of cells. Moreover, considering that activation of hTERT expression or ALT mechanism is required for transformation of telomerase negative cells *in vitro*, it implies that maintenance of telomeres is essential for the unlimited proliferative potential of tumour cells. The fact that nearly all tumours express hTERT might be due to:

i) Tumour cells with re-activated telomerase activity survive crisis (M2)

ii) Tumours arise from telomerase-positive cells.

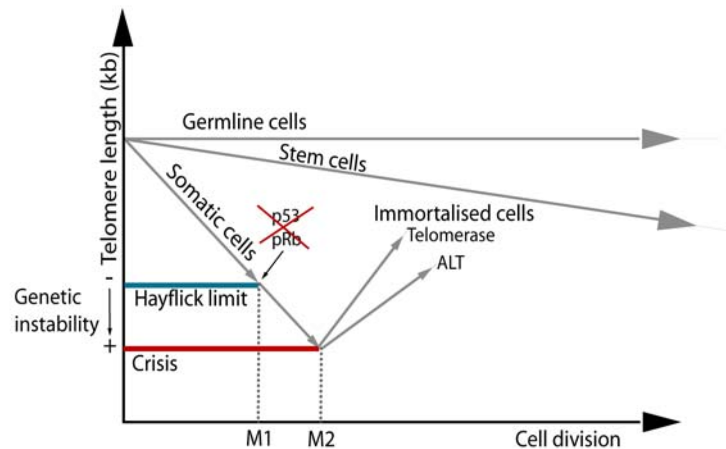

Figure 3. *The telomere hypothesis.* Cellular division (*abscissa*) in normal cells with no or low telomerase activity have a progressive shortening of the telomere length (*ordinate*). The first proliferative checkpoint, senescence or M1, is a permanent growth arrest and is induced by short or dysfunctional telomeres. Oncogene activation or tumour suppressor gene inactivation (p53, pRb) facilitates further cellular division beyond senescence, leading to shorter telomeres and increased genetic instability. Eventually, the second proliferative checkpoint, crisis or M2, is reached and p53-independent massive cell death occurs. Cells surviving crisis have a re-established telomere maintenance either via telomerase or ALT mechanism (Adopted from Stewart and Weinberg, 2006).

Clinical implications of telomerase

Telomerase activity has been investigated as exclusive bio-marker for cancer as almost all human tumour tissues have elevated expression levels compared to normal tissues. Their value as a diagnostic tool needs to be evaluated carefully since normal stem cells as well as progenitor cells in renewal tissues and proliferating lymphocytes also express telomerase activity to some extent. The most commonly used method for detection of telomerase activity in cells from various types of tissues is the sensitive telomeric repeat amplification protocol (TRAP) assay (Kim *et al.*, 1994; Wright *et al.*, 1995). The TRAP assay has been used to detect telomerase activity in urine for diagnosis of bladder cancer with some success (Sanchini *et al.*, 2005). Since elevated telomerase activity or hTERT expression has been clarified as a later event of malignancy progression and correlates well with severity of the disease, it might be a useful prognostic marker (Engelhardt *et al.*, 2000; Hiyama *et al.*, 1995a; Ohyashiki *et al.*, 1997).

The elevated telomerase activity in cancer is an attractive target for development of anti-cancer therapies, maybe in combination with other traditional methods such as chemo- and radiotherapy. Different strategies for telomerase inhibition have been elaborated including interfering with assembly of the ribonucleoprotein enzyme, localisation and concentration of telomerase to its target site in the nucleus, association of telomerase with telomeres, and targeting the major components hTERC and hTERT. The hTERT component is a preferred target because of its good correlation with telomerase activity in several types of malignancies. Safety is a particularly important issue since normal stem and progenitor cells would also be targeted giving adverse side effects through damage to healthy tissues. Inhibition of telomerase activity eventually induces apoptosis or senescence, but such strategies suffer from long lag periods before sufficient telomere shortening in treated tumour cells are reached. This lag period is dependent on the initial telomere length in tumour cells and the cell population doubling time, which can be several days or even weeks (Glanz *et al.*, 2007).

Alternatively, strategies aiming to decrease high expression levels of telomerase in cancer cells evoke rapid cell response. Both, hairpin-short-interfering RNA (siRNA) targeting the hTERC RNA and peptide nucleic acids (PNAs) targeting the hTERT

RNA diminish the levels of functional telomerase ribonucleoprotein complex, which leads to a rapid cell growth inhibition and induction of apoptosis. These treatments do not cause any telomere shortening, telomere uncapping, or p53 dependent DNA damage response; instead there is a rapid down-regulation in expression of genes involved in cell cycle. Together, these findings suggest that elevated telomerase activity is involved in some other cancer-proliferative functions, which are unrelated to the telomeres; e.g. through regulation of genes involved in tumour growth (Li *et al.*, 2005; Sharma *et al.*, 2003).

Another therapeutic approach that has been used in gene therapy trials is to use the *hTERT* core promoter to localise and restrict expression of a suicide or a pro-apoptotic gene to cancer cells only (Komata *et al.*, 2001). Furthermore, conditionally replicating adenoviral oncolytic vectors with an intact viral *E1* gene, essential for viral replication, have been constructed to replicate specifically in tumour cells. Applications of these vectors in conjunction with the *hTERT* promoter that controls transcription of the viral *E1* gene, promoting cell lysis and then augmenting viral spread to the neighbouring tumour cells, have led to significant inhibition of tumour growth in tumour xenografted mice (Lanson *et al.*, 2003; Wirth *et al.*, 2003).

Gene transfer into cancer and tissue restricted stem cells using viral vectors

The development of different strategies for targeting and for detection of telomerase activity in clinical use or in basic research requires a gene transfer system for genetic manipulation of cells. Today, the vast majority of gene transfer system is based on viral vectors where genes essential for viral replication are deleted to avoid unwanted viral spread. The deletion also vacates space on the viral genome so there is capacity to accommodate transgenes. The most important characteristic of a gene transfer vector is high gene transfer efficiency. Other requirements and criteria, such as long term or transient gene expression, vary according to the purpose of gene transfer and are reflected in the choice of vector.

Currently, there are several vector systems available, which are based on different genus of retroviruses such as the mammalian and avian C-type retroviruses

(also referred to as oncoretroviruses), lentiviruses and spuma viruses. All these viruses are enveloped particles with genomes consisting of a linear single-stranded RNA molecule of 7-11 kb (Kay *et al.*, 2001). The simplest retroviral genome consists of three genes; *gag*, *pol* and *env* genes, which encode the structural proteins, nucleic-acid polymerases/integrases and surface glycoproteins, respectively. In retroviral vectors, all three genes are deleted to minimize the risk of replication-competent vector creation and also to provide space for the transfer gene, such that up to 8 kb of exogenous DNA can be inserted. The production of retroviral vectors take place in a packaging cell line where the deleted genes are expressed *in trans*. The retroviral single-stranded RNA genome is first reverse transcribed into a double-stranded DNA pre-integration complex, which is then integrated into the host genome catalysed by viral integrase. The major characteristic of retroviral-based vectors is this integration of their genome into the host genome. Hence, transduced cells maintain the transgene expression for a long period of time and the transgene is not lost at cellular division as it is passed on to daughter cells. To gain access to the host genome, the integration complex fully relies on the dissociation of the nuclear membrane during mitosis, and consequently retroviral vectors cannot transduce quiescent cells. Lentivirus, however, rely on active transport into the nucleus, which enable them to transduce quiescent cells. Additionally, retroviral gene transfer is prone to insertional mutagenesis, which can lead to uncontrolled cell proliferation due to activation of a proto-oncogene (Hacein-Bey-Abina *et al.*, 2002; Hacein-Bey-Abina *et al.*, 2003).

Other viral vector systems that are widely used for gene transfer are based on adenovirus.

Adenovirus

Adenoviruses were originally described in the early 1950s and belong to the family *Adenoviridae*. Hitherto, there are 51 different human adenoviral serotypes known and they are classified into six species, A through F, according to their biological and genetic properties. Primary adenoviral infections occur in early childhood and give a lifelong immunity to the infecting serotype. Adenoviruses cause infections in the respiratory- (species C, E and some from B), gastrointestinal- (species A and F), urinary tracts (species B), and in the eyes (species D) (Kojaoghlanian *et al.*, 2003). Due to previous respiratory and gastrointestinal infections and fever related illnesses in childhood and later as adults, more than 90 % of the human population carries a pre-existent humoral immunity against adenoviruses and almost 50 % of humans have antibodies against Ad5 and Ad2 serotypes (Chirmule *et al.*, 1999; Russell, 2000). The tonsils, lymphoid tissues on either sides of the throat are the most probable sites for persistent adenoviral infection. Most adenoviral vectors are based on either serotype 5 or serotype 2; both belonging to species C.

Structure

Adenovirus is a non-enveloped virus with an icosahedral capsid and external knobbed fibres, which project out from the capsid (**Figure 4**). The capsid consists of two types of major proteins: hexon (protein II) and penton base (protein III). The fibre (protein IV) in its turn consists of three domains: tail, shaft and the knob. Besides these three major proteins, there are several types of minor capsid proteins, which have a role in capsid structure and function. Proteins IIIa, VI, VIII and IX stabilise the capsid structure and take part in nuclear import of hexon proteins. They may also play a role in mechanisms behind viral escape from endosomes into the cytoplasm (Vellinga *et al.*, 2005). Proteins V, VII, μ, the terminal protein (TP) and a non-covalently associated cysteine protease are packed together with the condensed viral DNA (Mangel *et al.*, 1993).

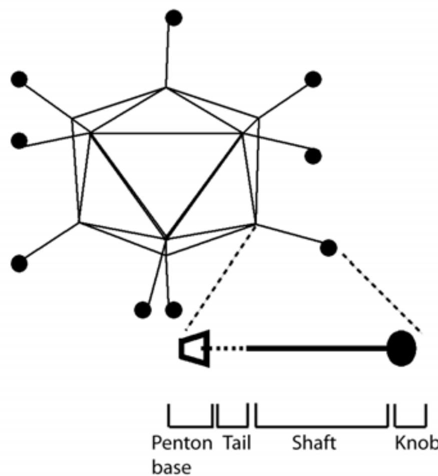

Figure 4. *Adenoviral structure.* The structure of the adenoviral particle is a symmetrical icosahedral with three major types proteins: hexon, penton base and fibre. The penton base and its Arg-Gly-Asp (RGD) motif interact with integrins receptors on host cells mediating viral uptake by endocytosis, dissociation of fibre proteins and viral escape from endosomes. The fibre protein consists of three domains; tail, shaft and knob, where the knob domain facilitates the initial attachment to host cell and thereby predominantly determines the viral tropism.

Genome

The adenovirus genome consists of a 36 kb double-stranded linear DNA molecule. Both ends of the genome contain long inverted terminal repeat (ITR) sequences which include the viral origin of replication and one copy of the TP covalently bound to each 5´end, which function as primers for adenoviral genome DNA replication. The viral genome can be divided into early transcription regions (*E1A, E1B*, *E2A*, *E2B*, *E3* and *E4*), delayed early transcription regions (*IX* and *IVa2*) and late transcription regions (*L1, L2, L3, L4* and *L5*) (**Figure 5**). These regions overlap each other and are located on both DNA strands. Every early region codes for multiple mRNA transcripts achieved by alternative splicing and by using substitute start codons. *E1A* is activated shortly after infection and E1A proteins are required for transcriptional activation of all other viral genes. The *E2* region encodes the viral DNA polymerase, required for viral replication. The *E3* transcription unit encodes several immunomodulatory proteins that repress the host immune response against the virus-infected cells. These

proteins interfere with antigen presentation and T-lymphocyte recognition and protect the cells from apoptosis induced by TNF-α (Burgert and Blusch, 2000). The proteins encoded by the *E4* region interacts with viral and cellular regulatory components that affect a series of events including cell cycle control, DNA repair, apoptosis, transcription and posttranslational modifications (Tauber and Dobner, 2001).

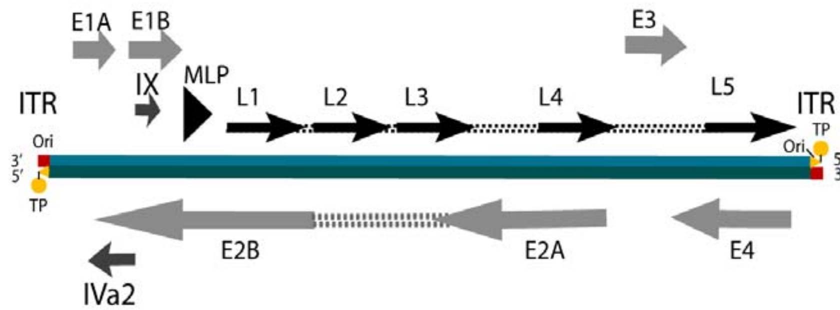

Figure 5. *Adenoviral genome.* Organisation of Ad5 genome with inverted terminal repeats (ITR) including origins of replication (Ori) and terminal protein (TP), localised at each end of the double-stranded DNA molecule. Arrows indicate the direction of transcriptional regions and dotted lines linking the arrows indicate intervening sequences that are spliced out during mRNA maturation. Early transcription regions (E1-E4) and delayed early transcription region (IX and IVa2) are transcribed in both directions and transcription is initiated by E1A; thus the E1A protein is necessary for all other viral transcriptional activation. Transcription of late transcriptional region (L1-L5) starts from a common major late promoter (MLP) producing one long mRNA encoding all but one (pIX) of the virion structural proteins.

Life cycle

Infection is initiated predominantly by the binding of the viral knob domain of the fibre to a primary attachment receptor on the host cell. For most human serotypes this attachment receptor is the Coxsackie- and Adenovirus receptor (CAR) (Bergelson *et al.*, 1997; Roelvink *et al.*, 1998). CAR is a component of the tight junctions predominantly expressed in cells of epithelial origin, and may take part in regulation of epithelial permeability and tissue homeostasis (Cohen *et al.*, 2001; Raschperger *et al.*, 2006). Serotypes from species B use CD46 as primary attachment receptor (Gaggar *et al.*, 2003; Segerman *et al.*, 2003). In humans, CD46 is a membrane cofactor protein with complement regulatory functions and expressed, although at low levels, on all cells except on erythrocytes (Liszewski *et al.*, 2005).

Following attachment, cellular uptake of viral particles is mediated through clathrin-mediated endocytosis. The uptake of viral particles is triggered by interaction between a conserved RGD motif on the penton base and a secondary receptor on the cell that belongs to the α_v integrin family (Wickham *et al.*, 1993). This interaction leads to a disassociation of the fibre proteins and conformational changes in the penton base, which enable viral escape from the endosomes into the cytoplasm (Medina-Kauwe, 2003; Shayakhmetov *et al.*, 2005). The viral trafficking towards the nucleus varies in time and routes depending on adenoviral species; this is most likely due to the use of different attachment and secondary receptors. Adenovirus from species B resides and accumulates for a longer period of time in late endosomes/lysosomes. It uses these compartments to achieve localisation near the nucleus, whereas adenovirus from species C escape from the endosomes at an earlier stage and make their way along the microtubule tracks to the nucleus and achieve nuclear localisation more quickly (Miyazawa *et al.*, 1999; Shayakhmetov *et al.*, 2003). Retrograded transport to the host cell surface has also been observed for adenoviruses belonging to species B (Shayakhmetov *et al.*, 2005). After the virus capsid has entered the cell, where the microenvironment is more reducing, the viral cysteine protease located in the capsid is reactivated and conducts the final dissociation of the capsid by degradation of protein VI. This leads to release of viral DNA from the capsid (Greber *et al.*, 1996). The uncoated viral DNA is finally imported alone into the nucleus through the nuclear pore complex (Greber *et al.*, 1997).

Upon viral infection, the host cell starts to produce antiviral cytokines such as interferons (IFN α and β). High concentration of interferons is found at the site of infection and cells that have responded to IFN are said to be in an antiviral state. IFN can induce transcription of several genes involved in inhibition of cell proliferation and induction of apoptosis. Adenovirus can counteract these antiviral responses by the action of E1A protein, which inhibits transcriptional regulators of IFN response genes and establish an optimal milieu for efficient production of progeny virus and also stop premature lysis of infected cells (Muruve, 2004). The E1A proteins are involved also in forcing the host cell to enter the S-phase of cell cycle, inhibited synthesis of host cellular proteins, thus optimise the environment for viral protein synthesis and

replication (Flint and Shenk, 1989). Viral replication starts about 5-6 hours (h) post-infection with the onset of transcription of the late transcription region by a common major promoter. A long transcript is produced, which is spliced into the different structural parts of the viral particle. Assembly of virion particles in the nucleus starts about 8 h after infection. The life cycle of the virus is completed about 30-40 h post-infection with the release of progeny particles through cell lysis.

Adenoviral vectors as a tool for gene transfer

For decades, recombinant adenoviral vectors have been gradually developed, improved and simplified for optimum gene transfer. The first-generation adenoviral vectors have their *E1* transcription region deleted in order to make space for the transgene expression cassette and are therefore replication deficient. An additional deletion in the *E3* transcript region, which is dispensable for the viral life cycle, can give a transgene capacity of 8 kb (Volpers and Kochanek, 2004). The production of vectors takes place in a packaging cell line with the *E1A* gene provided *in trans*. Moreover, the transgene expression cassette is integrated into the viral vector by homologous recombination. A major disadvantage of these vectors is the expression of their remaining viral genes, which induces cellular immune response leading to elimination of adenoviral-transduced cells by the host (Yang *et al.*, 1994).

To reduce the host cellular immune response and achieve a stable transgene expression, the second-generation adenoviral vectors with additional deletions of the *E2* (Engelhardt *et al.*, 1994) and/or *E4* (Wang *et al.*, 1997) genes were produced. The usage of these vectors gave an improved persistence of transgene expression and decreased inflammatory responses in animal studies.

To further lower the inflammatory responses and toxicity in the host, high-capacity adenoviral vectors with only the ITRs and the packaging signal left as viral elements were developed. These high-capacity adenoviral vectors, also called gutless vectors, can accommodate up to 36 kb of non-viral DNA. Production of gutless adenoviral vectors is more complicated since all viral gene except E1A (provided by the packaging cell line) need to be provided *in trans*. This is achieved by the use of a

helper-virus in the protocol. After production, the helper-virus must be removed using ultracentrifugation and this inevitably results in low levels of contamination with helper-virus giving inferior and lower titers of viral particles. Likewise, homologous recombination between vector and helper-virus DNA can occur during DNA replication resulting in compromised vector production and higher helper-virus contamination.

First line of host defence, the innate immune response, is triggered by interaction between viral capsid proteins and host cellular receptors and is independent of viral gene transcription (Liu and Muruve, 2003). Therefore, all different generations of adenoviral vectors, viral genes deleted or not, induce the innate immune response of the host. This induction is dose dependent, where high titers of administered adenoviral vector lead to extensive inflammation in transduced tissue with rapid loss of vector and transgene. To overcome inflammatory effects as much as possible, low virus titers are recommended, and therefore it is important to use a vector with high gene transfer efficiency.

Taken together, adenoviral vectors allow high gene transfer due to their efficient nuclear entry mechanism and their capability to transduce quiescent cells (Volpers and Kochanek, 2004). They are also feasibly manipulated and can be produced at high titers. The viral genome stays as an episomal element in the nucleus and therefore the risk for insertional mutagenesis is low. Limitations of this vector system are an uncontrolled gene transfer because the vectors have a broad tropism and are difficult to target specifically. The gene expression, as it is of transient nature, decreases with time due to the loss of the viral genome during cell division. Also, the induction of host innate and adoptive immune responses restricts readministration.

AIM OF THE THESIS

General aim

The aim of this thesis was to develop adenoviral vectors for transduction of living cancer cells and for subsequent detection of *hTERT* transcriptional activity.

Specific aims

Selection of an adenoviral vector tropism for efficient transient transduction into tumour cells grown as monolayer or spheres.

Development of adenoviral vectors for monitoring *hTERT* transcriptional activity in single living cells.

Utilisation of the developed adenovirus to investigate expression pattern of *hTERT* in human cancer and HSCs cells.

GENERAL DISCUSSION

The contents of this thesis focus on the development of adenoviral vectors with capability of detecting living cells with *hTERT* transcriptional activity. First, a traditional Ad5 vector is compared to an Ad5 vector genetically modified with Ad35 tropism and the most efficient is selected for transduction into tumour cells (paper **I**). Second, the selected Ad5 vector with Ad35 tropism is developed further for monitoring transcriptional activity of *hTERT* promoter in single living cells (paper **II**). And finally, the developed *hTERT*-reporter vector is used to monitor hTERT protein expression pattern in cancer cells, and stem cells of haematopoietic hierarchy as an example (papers **II** and **III**).

Selection of vector tropism

Adenoviral vectors have several attractive features for gene transfer compared to retroviral vectors. They are conveniently generated and can be produced at high titers. Additionally, adenoviral vectors transduce quiescent as well as dividing cells, and they rarely integrate into the host genome. Retroviral vectors only transduce dividing cells and the reverse transcribed retroviral genome integrates into the host genome. This normally gives a long-lasting gene expression; although retroviral vector mediated gene transfer is associated with risk of insertional mutagenesis. In contrast, adenoviral vectors are not associated with such risk and are a better choice when transient expression is desired.

The most commonly used adenoviral vectors are based on Ad5 or Ad2 serotypes, both of which belong to species C. The transfer efficiency of adenoviral vector is predominantly determined by its primary attachment receptor on the host cells. This is the major limiting factor for Ad5 and Ad2 vectors since the expression of their primary attachment receptor, CAR, is highly variable in target tissues and cells, such as solid tumours and haematopoietic cells. Therefore, several strategies to attain CAR-independent and higher gene transfer efficiency have been developed. **(1)** The vector tropism can be re-directed to a specific receptor on the host cell by

incorporation of high-affinity peptide motifs into the C-terminal end or the HI loop of the fibre protein (Dmitriev *et al.*, 1998; Wickham *et al.*, 1997). **(2)** Bi-specific molecules that target both adenoviral fibre protein and a specific cell surface receptor have been used (Miller *et al.*, 1998; Watkins *et al.*, 1997; Wickham *et al.*, 1996). **(3)** Structural proteins of the virion particle, most commonly the fibre protein, can be genetically exchanged with structural counterparts from other adenoviral serotypes, which alters attachment receptor usage of resulting adenoviral vectors. Strategies **1** and **2** for Ad5 and Ad2 have one major drawback; true re-direction of vector tropism cannot be obtained since native tropism is still maintained. To overcome this problem these vectors need to be mutated in both the fibre knob domain and in the penton base RGD motif to eliminate native interaction with CAR and α_v integrins, respectively (van Beusechem *et al.*, 2002). The incorporation of foreign peptide motifs into the fibre protein, as in strategy **1**, was found to prevent fibre trimerisation and virus maturation, and thus is not an optimal site for addition of foreign peptides (Hong and Engler, 1996). Strategy **2** is very versatile because with the continued identification of new high-affinity peptide ligands and antibodies, the number of targets available is constantly increasing. Its limitations are the additional need for production of bi-specific molecules since they are not an integral part of the virion and relatively high multiplicity of infections (MOIs) are used for efficient transduction of cells. This latter causes complexity with respect to toxicity and inflammatory responses. Strategy **3** has so far been the most successfully used approach for re-directing vector tropism and is superior to strategy **1** due to feasible generation of high-titer vector preparations and to strategy **2** due to one-step production and a lower MOIs usage for efficient transduction.

Fibre swapped Ad5 vectors with counterparts from adenovirus belonging to species B efficiently transduce human cell types that are relatively refractory to infection with fibre non-modified Ad5 vectors, including haematopoietic cells and tumour cells (Brouwer *et al.*, 2007; Miyazawa *et al.*, 1999; Nilsson *et al.*, 2004b; Shayakhmetov *et al.*, 2000; Ulasov *et al.*, 2006). Fibre swapped Ad5 vector with tropism from Ad35, termed Ad5F35, is dependent on CD46 receptor, widely expressed on host cells, for attachment. The Ad5F35 vector was previously shown to be superior to the

fibre non-modified Ad5 vector in transducing haematopoietic cells (Nilsson *et al.*, 2004a) and we wanted to compare these vectors in glioblastoma multiforme (GBM) cells in the context of viral tropism. We demonstrated that the Ad5F35 vector had substantially better transduction and penetration capacity in both, monolayer and sphere cultures of GBM cells (paper **I**).

Thus the usage of the Ad5F35 vector with CD46 as attachment receptor for gene transfer into tumour cells, instead of the Ad5 vector with CAR as attachment receptor is preferential for various reasons. **First**, Ad5F35 vectors are more efficient in transduction of quiescent cells compared to Ad5 vectors that predominantly transduce dividing cells (Nilsson *et al.*, 2004b). This is especially advantageous concerning gene transfer into GBM cells, since most GBM cells are relatively slow growing and have a population doubling time of about 4 days. **Second**, the expression of CD46 is elevated cells of certain tumour types compared to their normal counterparts, whereas expression of CAR is low or variable in tumour cells (Fishelson *et al.*, 2003; Kinugasa *et al.*, 1999; Ni *et al.*, 2006). **Third**, Ad5F35 vector infects cells with high CD46 expression more efficiently compared to cells which have a low CD46 expression (Anderson *et al.*, 2004).

GBM cells grown as three-dimensional spheres become more closely associated to each other and so the viral vectors administrated into the growth medium have a limited access to cells internal to the sphere. Furthermore, spheres might contain a more heterogeneous population of tumour cells, where primitive and differentiated tumour cells are in close association with each other. Also a spherical geometry is more representative of a solid tumour mass than cells grown as monolayer in a culture. Therefore, spheres are an alternative *in vitro* model and more relevant for assessing gene transfer efficiency and penetration capacity of viral vectors into tumour cells than monolayer cultures. Alternatively, *in vivo* models with human xenografts grown in rodents can also be used. Here, the gene transfer efficiency can be additionally evaluated in a more complex microenvironment, such as connective tissue cells and extracellular components.

Development of Ad5F35 vectors for monitoring *hTERT* transcriptional activity

Telomerase activity has long been thought as an exclusive bio-marker for cancer as almost all tumour tissues have elevated expression levels compared to normal tissues. The most commonly used method for detection of telomerase activity is the TRAP assay (Kim *et al.*, 1994). The method is a PCR-based assay with capability to detect telomerase activity in protein extracts from as few as 10 cells (Wright *et al.*, 1995). In addition, RT-PCR analysis of hTERT mRNA expression has also been used (Ulaner *et al.*, 1998). Both of these methods are quantitative and represent telomerase activity or hTERT mRNA expression respectively in a population of cells. They do not distinguish either heterogeneous telomerase activity or hTERT mRNA expression patterns between individual cells. Alternatively, immunohistochemical methods using antibodies directed against hTERT protein are applicable for detection of *hTERT* gene expression at cellular level. Unfortunately, several commercially available antibodies against hTERT protein suffer from poor specificity, which can be due to cross-reactivity with other antigens (Yan *et al.*, 2004). Wu *et al* presented evidence that one of the available antibodies, which has been extensively used, binds to the nucleolin protein, a nucleolar organiser involved in cellular growth and proliferation, and not at all to the hTERT protein as assumed (Wu *et al.*, 2006).

We were interested in developing a method where telomerase activity could be detected without killing the cells. Such a protocol would facilitate characterisation of cells in the context of their telomerase status. We chose to work with the first generation of adenoviral vectors because they are relatively easy to manipulate and also give a high, yet a transient gene transduction in living cells. We continued to utilise the Ad5F35 vector for high gene transfer efficiency into a various types of cells. To assess telomerase activity we used the *hTERT* promoter, including the core promoter region and the up-stream negative regulatory sequence (Fujimoto *et al.*, 2000; Horikawa *et al.*, 1999; Komata *et al.*, 2001; Takakura *et al.*, 1999). In normal cells the *hTERT* promoter is active during S-phase of cell cycle and we aimed to assess this dynamical regulation through the *hTERT* promoter controlling transcription activity of a reporter gene. We chose the destabilised enhanced green fluorescence protein with a very short half-life

of 2 h (d2EGFP). Dynamic regulation of the *hTERT* promoter could be monitored quantitatively through d2EGFP levels in cells due to its rapid turnover, because a PEST-domain is fused at the c-terminal end of the EGFP to generate d2EGFP (Li *et al.*, 1998). The vector was termed Ad5F35-hTERT-d2EGFP.

In order to shield the *hTERT* promoter from potential effects of viral enhancer elements, which overlap with ITRs and packaging signal of the adenoviral genome, bovine growth hormone transcription stop signal or chicken β-globin HS4 insulator were used (Chung *et al.*, 1997; Steinwaerder and Lieber, 2000; Vassaux *et al.*, 1999). An insulator located between a promoter and an enhancer establishes separate domains and blocks their interaction with each other (Gaszner and Felsenfeld, 2006). Therefore, the two different types of insulator sequences were inserted in front of the *hTERT* promoter in Ad5F35 vector construct. Thus, the created new vectors were termed Ad5F35-bovins-hTERT-d2EGFP and Ad5F35-chins-hTERT-d2EGFP. The comparison between insulator shielded promoters and the unshielded promoter when tested in transduced fibroblasts cell lines, which have undetectable hTERT mRNA transcript and low telomerase activity, showed no significant difference in d2EGFP expression. An explanation might be that the adenoviral vector genome stays as an episomal element in the nucleus and therefore an insulator element would not have the same impact compared to in a chromosomal integration situation. The fact that the expression level of d2EGFP is very low in all 3 (Ad5F35-hTERT-d2EGFP, Ad5F35-bovins-hTERT-d2EGFP and Ad5F35-chins-hTERT-d2EGFP) vector type transduced fibroblasts, implicate that the viral enhancers might not have any detectable effects on the *hTERT* promoter; at least not in fibroblasts.

It can be argued whether the assessment of *hTERT* transcription activity with our hTERT reporter vector represent the actual telomerase activity in cells, since post-transcriptional events such as alternative splicing of the hTERT transcript, incorrect assembly of telomerase components, and POT1 inhibition of telomerase activity at the telomeres might have an impact on the activity. Nevertheless, our reporter gene expression correlates well with both telomerase activity and hTERT mRNA transcript expression in bulk cells. Furthermore, extensive copies of reporter vector genome might cause squelching of transcription factors required for endogenous *hTERT*

expression. We did not observe such difference between reporter vector transduced cells and non-transduced cells both assessed using TRAP assay or hTERT mRNA expression levels. This is important if hTERT-reporter vector transduced cells are used for further analysis.

Monitoring *hTERT* promoter expression in cancer and stem cells.

The vast majority of cancers express high levels of telomerase, which we confirmed at single living cell level in various cancer cell lines transduced with the hTERT-reporter vector (paper **II**). In addition, our hTERT-reporter vector was able to monitor dynamic cell cycle-regulation of the *hTERT* transcriptional activity in promyelocytic leukaemic HL-60 cells with elevated d2EGFP levels in S/G2/M phase (paper **II**). Furthermore, hTERT-reporter vector could monitor *hTERT* transcriptional repression during retinoic acid enforced granulocytic differentiation of HL-60 cells, which had significant correlation with decreasing telomerase activity and hTERT mRNA expression (paper **II**). As a proof of principle, we transduced two freshly isolated xenograft GBM cell lines with our hTERT-reporter vector. To keep the tumour forming capacity of such cells, consecutive *in vivo* passaging in SCID mice is needed. The frequencies of d2EGFP$^+$ cells differed considerably between the two GBM lines tested, which might be due to different cell doubling times or even different levels of more “stem-progenitor-cell-like” cells with tumour forming capacity within the GBM cell population (paper **II**). Interestingly, upon repeated hTERT-reporter vector transduction of later passages of the two xenograft GBM lines maintained as *ex vivo* cultures, the frequencies of d2EGFP$^+$ cells were remarkably diminished (unpublished data). This is coincided with a gradual loss of tumour forming capacity of such GBM cells under *in vitro* culture conditions, and could be interesting to test further.

Telomerase activity is found in cells undergoing rapid expansion, such as lymphocytes, germline cells and in transient amplifying cells from different compartments of stem cells. Nevertheless, age-related telomere shortening has been found in human HSCs, which may limit haematopoietic re-generation (Engelhardt *et al.*, 1997; Yui *et al.*, 1998). Different mutations in telomerase components in DKC

patients cause decreased telomerase activity and accelerated telomere shortening, which compromises the maintenance of HSC compartments with bone marrow failure as a consequence (Marrone *et al.*, 2004; Vulliamy *et al.*, 2001).

The haematopoietic system has an outstanding re-generative capacity, which depends on a quite small population of HSCs consistently replenishing all the mature cells in myeloid and lymphoid cell lineages. Most HSCs are quiescent *in vivo* but have a capacity for self-renewal and extensive proliferation. HSC transplantation can be applied to restore a normal haematopoietic system in leukaemia and autoimmune diseases. Traditionally, isolation of HSCs and their immediate progenitor cells is based on expression of cell-surface markers and the early stages of the hierarchical structure of haematopoietic system can be assessed in transplantation studies. Human self-renewing HSCs are assessed as surrogate NOD/SCID mice bone marrow Repopulation Cells (SRCs), due to their capacity to re-populate the bone marrow of sub-lethally irradiated NOD/SCID mice. Hence, human HSCs are divided into LT-HSCs or ST-HSCs based on their re-populating capacity. The assumed role of telomerase activity in human progenitor haematopoietic cells was previously founded on assessment of phenotypically defined population of primitive haematopoietic cells, using TRAP assay (Morrison *et al.*, 1996). It was demonstrated that isolated $CD34^{+}CD38^{-}$ cells had low telomerase activity, whereas $CD34^{+}CD38^{+}$ cells had higher levels of telomerase activity, which increased upon differentiation of such cells (Engelhardt *et al.*, 1997). Besides, the $CD34^{+}CD38^{-}$ cell population contains only of a minor fraction of re-populating HSCs with distinct engraftment and differentiation potentials assessed as SRCs in irradiated NOD/SCID mice. Whether these SRCs possess telomerase activity and how it is regulated within the SRC compartment has not yet been clarified. Likewise, whether telomerase activity is directly required by re-populating HSCs or down-stream progenitors has not been possible to assess.

Following transduction with the hTERT-reporter vector, we were able to separate a distinct population of core blood $CD34^{+}$ cells with elevated *hTERT* transcription activity as monitored by d2EGFP expression. This cell population contained cells with the ability to re-populate the bone marrow of primary but not of secondary NOD/SCID mice recipients, and therefore they most likely represent the

ST-HSCs and down-stream progenitors (paper **III**). Such cells have upregulated telomerase activity due to higher proliferation rate and represent the first step in haematopoiesis, from LT-HSCs to ST-HSCs characterised by the gradual loss of self-renewal capacity. Nevertheless, this population of cells might contain a very small population of LT-HSCs, because even if most LT-HSCs are quiescent (Glimm *et al.*, 2001) they occasionally undergo self-renewal divisions and telomerase is most likely expressed upon such occasions, however, their frequency in the sorted population was below the detection limit of the method. Since previous studies assessing telomerase activity have been based on TRAP assay on cell populations identified by cell surface marker expression, it has not been possible to distinguish individual re-populating HSCs with telomerase activity. Our studies contribute with an intrinsic gene expression based strategy to distinguish between LT-HSCs and ST-HSCs. Moreover, our studies show that the LT-HSCs express low or undetectable levels of telomerase and that the telomerase activity is only upregulated in primitive haematopoietic cells upon active cell proliferation.

CONCLUSIONS

Adenoviral vector with Ad35 tropism was superior to Ad5 tropism for transient transduction into tumour cells grown as monocultures or spheres (paper **I**).

The developed hTERT-reporter vector with Ad35 tropism had ability to transduce a broad variety of somatic cells and monitor *hTERT* transcriptional activity in single living cells (paper **II**).

The expression of reporter gene could monitor a cell cycle- and differentiation stage-dependent *hTERT* expression in successfully transduced cancer cells. The hTERT transcription activity was upregulated in S/G2/M phase of cell cycle and repressed during enforced granulocytic differentiation of HL-60 cells (paper **II**).

The hTERT-reporter vector could monitor upregulated *hTERT* expression in cycling $CD34^+$ haematopoietic cells (paper **III**).

Upregulation of *hTERT* expression within the human HSC compartment was associated with reduced self-renewal capacity (paper **III**).

The hTERT-reporter vector could be utilised to separate long-term from short-term repopulating HSCs (paper **III**).

POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Vår arvsmassa finns lagrad som linjära dubbelsträngade DNA-molekyler, kromosomer, i våra celler. Celler i vävnader och organ förnyas ständigt genom celldelning. Kromosomerna i en cell måste replikeras för att kunna ge upphov till två nya celler. Replikeringsmaskineriet har bara förmågan att arbeta i en riktning längs med DNA-strängarna och kräver en kort sekvens utav RNA att starta ifrån. Konsekvensen blir att i var ände av kromosomerna är det en av DNA-strängarna som inte kan replikeras till fullo och vid varje celldelning går därför en bit DNA förlorad. Den här ständiga DNA förlusten kallas för "*änd-replikeringsproblemet*" och skulle snabbt få förödande konsekvenser för cellen och vår arvsmassa. Lösningen på problemet är att varje kromosomände består av många kopior av en kort DNA-sekvens som inte utgör någon kodande information och denna sekvens kallas för telomer. För att vår arvsmassa inte skall ta skada även om telomererna blir farligt korta avstannar celldelningsprocessen, vilket medverkar till åldrandet av vävnader och organ. Vissa celler i kroppen har förmågan till ständig celldelning, bland annat blodstamcellerna som förser kroppen med nya blodceller livet ut. För att bibehålla längden på telomererna i dessa celler uttrycks ett enzym som kallas för telomeras, vars uppgift är att förlänga telomererna genom att addera fler kopior av den korta DNA-sekvensen till varje kromosomände. Även cancerceller som anses ha en oändlig och okontrollerad celldelningskaraktär måste behålla telomerernas längd och uttrycker därför oftast telomeras i höga koncentrationer. Således är telomeras en mycket lovande markör för att upptäcka cancerceller och är även ett eventuellt mål för cancerterapi.

Huvudsyftet med avhandlingsarbetet var att utveckla adenovirala vektorer med förmågan att upptäcka uttrycket av telomeras i cancerceller. Fördelen med att använda ett vektorsytem baserat på adenovirus är många, bland annat är det ett dubbelsträngat DNA-virus som väldigt sällan integreras i värdcellens arvsmassa och kan infektera delande så väl som icke-delande celler. Vi har använt oss av en kontrollregion, en promotor, som vanligtvis styr genuttrycket utav en huvudkomponent av

telomerasenzymet. I vektorerna styr denna promotorn istället uttrycket av en reportergen vars produkt är ett grönt fluorescerande protein. Dessutom är ett ytprotein i vektorn genetiskt modifierat för att vektorn ska infektera cancerceller och blodceller effektivare via en alternativ receptor på cellytan. Detta innebär att om de celler som vanligtvis uttrycker telomeras infekteras med vektorn kommer det gröna fluorescerande proteinet att produceras och vi kan hitta dessa celler och sortera ut dem. Med hjälp av denna vektorn har vi på cellnivå undersökt telomeras aktiviteten i cancerceller och konstaterat att uttrycket av telomeras är cellcykel- och differentierings beroende. Den här vektorn använde vi även för att undersöka uttrycket av telomeras hos blodstamceller. Vi kunde visa att de humana blodstamceller som har ett högt uttryck av telomeras även delvis har förlorat sin självförnyande förmåga och blivit mer mogna. Denna vektorn ger oss potentiella möjligheter att kunna separera de mest primitiva blodstamcellerna från de mer mogna för att kunna genomföra vidare studier. Sammanfattningsvis har vi i detta avhandlingsarbete utvecklat adenovirala vektorer som ger oss möjlighet att studera reglering av telomerasuttrycket i normala celler, cancerceller och blodstamceller.

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REFERENCES

Al-Hajj, M., and Clarke, M. F. (2004). Self-renewal and solid tumor stem cells. Oncogene *23*, 7274-7282.

Al-Hajj, M., Wicha, M. S., Benito-Hernandez, A., Morrison, S. J., and Clarke, M. F. (2003). Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A *100*, 3983-3988.

Allsopp, R. C., Vaziri, H., Patterson, C., Goldstein, S., Younglai, E. V., Futcher, A. B., Greider, C. W., and Harley, C. B. (1992). Telomere length predicts replicative capacity of human fibroblasts. Proc Natl Acad Sci U S A *89*, 10114-10118.

Anderson, B. D., Nakamura, T., Russell, S. J., and Peng, K. W. (2004). High CD46 receptor density determines preferential killing of tumor cells by oncolytic measles virus. Cancer Res *64*, 4919-4926.

Artandi, S. E., Chang, S., Lee, S. L., Alson, S., Gottlieb, G. J., Chin, L., and DePinho, R. A. (2000). Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice. Nature *406*, 641-645.

Avilion, A. A., Piatyszek, M. A., Gupta, J., Shay, J. W., Bacchetti, S., and Greider, C. W. (1996). Human telomerase RNA and telomerase activity in immortal cell lines and tumor tissues. Cancer Res *56*, 645-650.

Bartkova, J., Horejsi, Z., Koed, K., Kramer, A., Tort, F., Zieger, K., Guldberg, P., Sehested, M., Nesland, J. M., Lukas, C*., et al.* (2005). DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. Nature *434*, 864-870.

Bekaert, S., Derradji, H., and Baatout, S. (2004). Telomere biology in mammalian germ cells and during development. Dev Biol *274*, 15-30.

Bergelson, J. M., Cunningham, J. A., Droguett, G., Kurt-Jones, E. A., Krithivas, A., Hong, J. S., Horwitz, M. S., Crowell, R. L., and Finberg, R. W. (1997). Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. Science *275*, 1320-1323.

Blackburn, E. H. (1991). Structure and function of telomeres. Nature *350*, 569-573.

Blackburn, E. H. (2001). Switching and signaling at the telomere. Cell *106*, 661-673.

Blackburn, E. H., and Gall, J. G. (1978). A tandemly repeated sequence at the termini of the extrachromosomal ribosomal RNA genes in Tetrahymena. J Mol Biol *120*, 33-53.

Bodnar, A. G., Ouellette, M., Frolkis, M., Holt, S. E., Chiu, C. P., Morin, G. B., Harley, C. B., Shay, J. W., Lichtsteiner, S., and Wright, W. E. (1998). Extension of life-span by introduction of telomerase into normal human cells. Science *279*, 349-352.

Bouchard, C., Staller, P., and Eilers, M. (1998). Control of cell proliferation by Myc. Trends Cell Biol *8*, 202-206.

Brouwer, E., Havenga, M. J., Ophorst, O., de Leeuw, B., Gijsbers, L., Gillissen, G., Hoeben, R. C., ter Horst, M., Nanda, D., Dirven, C., *et al.* (2007). Human adenovirus type 35 vector for gene therapy of brain cancer: improved transduction and bypass of pre-existing anti-vector immunity in cancer patients. Cancer Gene Ther *14*, 211-219.

Bryan, T. M., Englezou, A., Dalla-Pozza, L., Dunham, M. A., and Reddel, R. R. (1997). Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. Nat Med *3*, 1271-1274.

Burgert, H. G., and Blusch, J. H. (2000). Immunomodulatory functions encoded by the E3 transcription unit of adenoviruses. Virus Genes *21*, 13-25.

Cawthon, R. M., Smith, K. R., O'Brien, E., Sivatchenko, A., and Kerber, R. A. (2003). Association between telomere length in blood and mortality in people aged 60 years or older. Lancet *361*, 393-395.

Chirmule, N., Propert, K., Magosin, S., Qian, Y., Qian, R., and Wilson, J. (1999). Immune responses to adenovirus and adeno-associated virus in humans. Gene Ther *6*, 1574-1583.

Chiu, C. P., Dragowska, W., Kim, N. W., Vaziri, H., Yui, J., Thomas, T. E., Harley, C. B., and Lansdorp, P. M. (1996). Differential expression of telomerase activity in hematopoietic progenitors from adult human bone marrow. Stem Cells *14*, 239-248.

Christensen, J. L., and Weissman, I. L. (2001). Flk-2 is a marker in hematopoietic stem cell differentiation: a simple method to isolate long-term stem cells. Proc Natl Acad Sci U S A *98*, 14541-14546.

Chung, J. H., Bell, A. C., and Felsenfeld, G. (1997). Characterization of the chicken beta-globin insulator. Proc Natl Acad Sci U S A *94*, 575-580.

Cohen, C. J., Shieh, J. T., Pickles, R. J., Okegawa, T., Hsieh, J. T., and Bergelson, J. M. (2001). The coxsackievirus and adenovirus receptor is a transmembrane component of the tight junction. Proc Natl Acad Sci U S A *98*, 15191-15196.

Cong, Y. S., Wright, W. E., and Shay, J. W. (2002). Human telomerase and its regulation. Microbiol Mol Biol Rev *66*, 407-425, table of contents.

d'Adda di Fagagna, F., Reaper, P. M., Clay-Farrace, L., Fiegler, H., Carr, P., Von Zglinicki, T., Saretzki, G., Carter, N. P., and Jackson, S. P. (2003). A DNA damage checkpoint response in telomere-initiated senescence. Nature *426*, 194-198.

de Lange, T., Shiue, L., Myers, R. M., Cox, D. R., Naylor, S. L., Killery, A. M., and Varmus, H. E. (1990). Structure and variability of human chromosome ends. Mol Cell Biol *10*, 518-527.

Djojosubroto, M. W., Choi, Y. S., Lee, H. W., and Rudolph, K. L. (2003). Telomeres and telomerase in aging, regeneration and cancer. Mol Cells *15*, 164-175.

Dmitriev, I., Krasnykh, V., Miller, C. R., Wang, M., Kashentseva, E., Mikheeva, G., Belousova, N., and Curiel, D. T. (1998). An adenovirus vector with genetically modified fibers demonstrates expanded tropism via utilization of a coxsackievirus and adenovirus receptor-independent cell entry mechanism. J Virol *72*, 9706-9713.

Ducrest, A. L., Szutorisz, H., Lingner, J., and Nabholz, M. (2002). Regulation of the human telomerase reverse transcriptase gene. Oncogene *21*, 541-552.

Dunham, M. A., Neumann, A. A., Fasching, C. L., and Reddel, R. R. (2000). Telomere maintenance by recombination in human cells. Nat Genet *26*, 447-450.

Engelhardt, J. F., Ye, X., Doranz, B., and Wilson, J. M. (1994). Ablation of E2A in recombinant adenoviruses improves transgene persistence and decreases inflammatory response in mouse liver. Proc Natl Acad Sci U S A *91*, 6196-6200.

Engelhardt, M., Kumar, R., Albanell, J., Pettengell, R., Han, W., and Moore, M. A. (1997). Telomerase regulation, cell cycle, and telomere stability in primitive hematopoietic cells. Blood *90*, 182-193.

Engelhardt, M., Mackenzie, K., Drullinsky, P., Silver, R. T., and Moore, M. A. (2000). Telomerase activity and telomere length in acute and chronic leukemia, pre- and post-ex vivo culture. Cancer Res *60*, 610-617.

Feng, J., Funk, W. D., Wang, S. S., Weinrich, S. L., Avilion, A. A., Chiu, C. P., Adams, R. R., Chang, E., Allsopp, R. C., Yu, J., and *et al.* (1995). The RNA component of human telomerase. Science *269*, 1236-1241.

Fishelson, Z., Donin, N., Zell, S., Schultz, S., and Kirschfink, M. (2003). Obstacles to cancer immunotherapy: expression of membrane complement regulatory proteins (mCRPs) in tumors. Mol Immunol *40*, 109-123.

Flint, J., and Shenk, T. (1989). Adenovirus E1A protein paradigm viral transactivator. Annu Rev Genet *23*, 141-161.

Fujimoto, K., Kyo, S., Takakura, M., Kanaya, T., Kitagawa, Y., Itoh, H., Takahashi, M., and Inoue, M. (2000). Identification and characterization of negative regulatory elements of the human telomerase catalytic subunit (hTERT) gene promoter: possible role of MZF-2 in transcriptional repression of hTERT. Nucleic Acids Res *28*, 2557-2562.

Gaggar, A., Shayakhmetov, D. M., and Lieber, A. (2003). CD46 is a cellular receptor for group B adenoviruses. Nat Med *9*, 1408-1412.

Gaszner, M., and Felsenfeld, G. (2006). Insulators: exploiting transcriptional and epigenetic mechanisms. Nat Rev Genet *7*, 703-713.

Gisselsson, D., Jonson, T., Petersen, A., Strombeck, B., Dal Cin, P., Hoglund, M., Mitelman, F., Mertens, F., and Mandahl, N. (2001). Telomere dysfunction triggers extensive DNA fragmentation and evolution of complex chromosome abnormalities in human malignant tumors. Proc Natl Acad Sci U S A *98*, 12683-12688.

Glanz, C., Rebetz, J., Stewenius, Y., Persson, A., Englund, E., Mandahl, N., Mertens, F., Salford, L. G., Widegren, B., Fan, X., and Gisselsson, D. (2007). Genetic intratumour heterogeneity in high-grade brain tumours is associated with telomere-dependent mitotic instability. Neuropathol Appl Neurobiol *33*, 440-454.

Glimm, H., Eisterer, W., Lee, K., Cashman, J., Holyoake, T. L., Nicolini, F., Shultz, L. D., von Kalle, C., and Eaves, C. J. (2001). Previously undetected human hematopoietic cell populations with short-term repopulating activity selectively engraft NOD/SCID-beta2 microglobulin-null mice. J Clin Invest *107*, 199-206.

Greber, U. F., Suomalainen, M., Stidwill, R. P., Boucke, K., Ebersold, M. W., and Helenius, A. (1997). The role of the nuclear pore complex in adenovirus DNA entry. Embo J *16*, 5998-6007.

Greber, U. F., Webster, P., Weber, J., and Helenius, A. (1996). The role of the adenovirus protease on virus entry into cells. Embo J *15*, 1766-1777.

Greider, C. W., and Blackburn, E. H. (1985). Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. Cell *43*, 405-413.

Griffith, J. D., Comeau, L., Rosenfield, S., Stansel, R. M., Bianchi, A., Moss, H., and de Lange, T. (1999). Mammalian telomeres end in a large duplex loop. Cell *97*, 503-514.

Gunes, C., Lichtsteiner, S., Vasserot, A. P., and Englert, C. (2000). Expression of the hTERT gene is regulated at the level of transcriptional initiation and repressed by Mad1. Cancer Res *60*, 2116-2121.

Hacein-Bey-Abina, S., Le Deist, F., Carlier, F., Bouneaud, C., Hue, C., De Villartay, J. P., Thrasher, A. J., Wulffraat, N., Sorensen, R., Dupuis-Girod, S.*, et al.* (2002). Sustained correction of X-linked severe combined immunodeficiency by ex vivo gene therapy. N Engl J Med *346*, 1185-1193.

Hacein-Bey-Abina, S., Von Kalle, C., Schmidt, M., McCormack, M. P., Wulffraat, N., Leboulch, P., Lim, A., Osborne, C. S., Pawliuk, R., Morillon, E.*, et al.* (2003). LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Science *302*, 415-419.

Hahn, W. C., Counter, C. M., Lundberg, A. S., Beijersbergen, R. L., Brooks, M. W., and Weinberg, R. A. (1999a). Creation of human tumour cells with defined genetic elements. Nature *400*, 464-468.

Hahn, W. C., Stewart, S. A., Brooks, M. W., York, S. G., Eaton, E., Kurachi, A., Beijersbergen, R. L., Knoll, J. H., Meyerson, M., and Weinberg, R. A. (1999b).

Inhibition of telomerase limits the growth of human cancer cells. Nat Med *5*, 1164-1170.

Hanahan, D., and Weinberg, R. A. (2000). The hallmarks of cancer. Cell *100*, 57-70.

Harley, C. B., Futcher, A. B., and Greider, C. W. (1990). Telomeres shorten during ageing of human fibroblasts. Nature *345*, 458-460.

Hayflick, L. (1965). The Limited in Vitro Lifetime of Human Diploid Cell Strains. Exp Cell Res *37*, 614-636.

Heiss, N. S., Knight, S. W., Vulliamy, T. J., Klauck, S. M., Wiemann, S., Mason, P. J., Poustka, A., and Dokal, I. (1998). X-linked dyskeratosis congenita is caused by mutations in a highly conserved gene with putative nucleolar functions. Nat Genet *19*, 32-38.

Hiyama, E., and Hiyama, K. (2007). Telomere and telomerase in stem cells. Br J Cancer *96*, 1020-1024.

Hiyama, E., Yokoyama, T., Tatsumoto, N., Hiyama, K., Imamura, Y., Murakami, Y., Kodama, T., Piatyszek, M. A., Shay, J. W., and Matsuura, Y. (1995a). Telomerase activity in gastric cancer. Cancer Res *55*, 3258-3262.

Hiyama, K., Hirai, Y., Kyoizumi, S., Akiyama, M., Hiyama, E., Piatyszek, M. A., Shay, J. W., Ishioka, S., and Yamakido, M. (1995b). Activation of telomerase in human lymphocytes and hematopoietic progenitor cells. J Immunol *155*, 3711-3715.

Hong, J. S., and Engler, J. A. (1996). Domains required for assembly of adenovirus type 2 fiber trimers. J Virol *70*, 7071-7078.

Horikawa, I., Cable, P. L., Afshari, C., and Barrett, J. C. (1999). Cloning and characterization of the promoter region of human telomerase reverse transcriptase gene. Cancer Res *59*, 826-830.

Huffman, K. E., Levene, S. D., Tesmer, V. M., Shay, J. W., and Wright, W. E. (2000). Telomere shortening is proportional to the size of the G-rich telomeric 3'-overhang. J Biol Chem *275*, 19719-19722.

Karlseder, J., Broccoli, D., Dai, Y., Hardy, S., and de Lange, T. (1999). p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2. Science *283*, 1321-1325.

Kay, M. A., Glorioso, J. C., and Naldini, L. (2001). Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. Nat Med *7*, 33-40.

Kim, N. W., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L., Coviello, G. M., Wright, W. E., Weinrich, S. L., and Shay, J. W. (1994). Specific association of human telomerase activity with immortal cells and cancer. Science *266*, 2011-2015.

Kinugasa, N., Higashi, T., Nouso, K., Nakatsukasa, H., Kobayashi, Y., Ishizaki, M., Toshikuni, N., Yoshida, K., Uematsu, S., and Tsuji, T. (1999). Expression of membrane cofactor protein (MCP, CD46) in human liver diseases. Br J Cancer *80*, 1820-1825.

Kojaoghlanian, T., Flomenberg, P., and Horwitz, M. S. (2003). The impact of adenovirus infection on the immunocompromised host. Rev Med Virol *13*, 155-171.

Komata, T., Kondo, Y., Kanzawa, T., Hirohata, S., Koga, S., Sumiyoshi, H., Srinivasula, S. M., Barna, B. P., Germano, I. M., Takakura, M.*, et al.* (2001). Treatment of malignant glioma cells with the transfer of constitutively active caspase-6 using the human telomerase catalytic subunit (human telomerase reverse transcriptase) gene promoter. Cancer Res *61*, 5796-5802.

Kyo, S., Takakura, M., Taira, T., Kanaya, T., Itoh, H., Yutsudo, M., Ariga, H., and Inoue, M. (2000). Sp1 cooperates with c-Myc to activate transcription of the human telomerase reverse transcriptase gene (hTERT). Nucleic Acids Res *28*, 669-677.

Lanson, N. A., Jr., Friedlander, P. L., Schwarzenberger, P., Kolls, J. K., and Wang, G. (2003). Replication of an adenoviral vector controlled by the human telomerase reverse transcriptase promoter causes tumor-selective tumor lysis. Cancer Res *63*, 7936-7941.

Lapidot, T., Sirard, C., Vormoor, J., Murdoch, B., Hoang, T., Caceres-Cortes, J., Minden, M., Paterson, B., Caligiuri, M. A., and Dick, J. E. (1994). A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. Nature *367*, 645-648.

Lechel, A., Satyanarayana, A., Ju, Z., Plentz, R. R., Schaetzlein, S., Rudolph, C., Wilkens, L., Wiemann, S. U., Saretzki, G., Malek, N. P.*, et al.* (2005). The cellular level of telomere dysfunction determines induction of senescence or apoptosis in vivo. EMBO Rep *6*, 275-281.

Li, S., Crothers, J., Haqq, C. M., and Blackburn, E. H. (2005). Cellular and gene expression responses involved in the rapid growth inhibition of human cancer cells by RNA interference-mediated depletion of telomerase RNA. J Biol Chem *280*, 23709-23717.

Li, X., Zhao, X., Fang, Y., Jiang, X., Duong, T., Fan, C., Huang, C. C., and Kain, S. R. (1998). Generation of destabilized green fluorescent protein as a transcription reporter. J Biol Chem *273*, 34970-34975.

Lin, S. Y., and Elledge, S. J. (2003). Multiple tumor suppressor pathways negatively regulate telomerase. Cell *113*, 881-889.

Liszewski, M. K., Kemper, C., Price, J. D., and Atkinson, J. P. (2005). Emerging roles and new functions of CD46. Springer Semin Immunopathol *27*, 345-358.

Liu, D., O'Connor, M. S., Qin, J., and Songyang, Z. (2004). Telosome, a mammalian telomere-associated complex formed by multiple telomeric proteins. J Biol Chem *279*, 51338-51342.

Liu, Q., and Muruve, D. A. (2003). Molecular basis of the inflammatory response to adenovirus vectors. Gene Ther *10*, 935-940.

Lopatina, N. G., Poole, J. C., Saldanha, S. N., Hansen, N. J., Key, J. S., Pita, M. A., Andrews, L. G., and Tollefsbol, T. O. (2003). Control mechanisms in the regulation of telomerase reverse transcriptase expression in differentiating human teratocarcinoma cells. Biochem Biophys Res Commun *306*, 650-659.

Makarov, V. L., Hirose, Y., and Langmore, J. P. (1997). Long G tails at both ends of human chromosomes suggest a C strand degradation mechanism for telomere shortening. Cell *88*, 657-666.

Mangel, W. F., McGrath, W. J., Toledo, D. L., and Anderson, C. W. (1993). Viral DNA and a viral peptide can act as cofactors of adenovirus virion proteinase activity. Nature *361*, 274-275.

Marrone, A., Stevens, D., Vulliamy, T., Dokal, I., and Mason, P. J. (2004). Heterozygous telomerase RNA mutations found in dyskeratosis congenita and aplastic anemia reduce telomerase activity via haploinsufficiency. Blood *104*, 3936-3942.

Mason, J. M., and Biessmann, H. (1995). The unusual telomeres of Drosophila. Trends Genet *11*, 58-62.

Masutomi, K., Yu, E. Y., Khurts, S., Ben-Porath, I., Currier, J. L., Metz, G. B., Brooks, M. W., Kaneko, S., Murakami, S., DeCaprio, J. A.*, et al.* (2003). Telomerase maintains telomere structure in normal human cells. Cell *114*, 241-253.

McClintock, B. (1938). The Production of Homozygous Deficient Tissues with Mutant Characteristics by Means of the Aberrant Mitotic Behavior of Ring-Shaped Chromosomes. Genetics *23*, 315-376.

McClintock, B. (1941). The Stability of Broken Ends of Chromosomes in Zea Mays. Genetics *26*, 234-282.

Medina-Kauwe, L. K. (2003). Endocytosis of adenovirus and adenovirus capsid proteins. Adv Drug Deliv Rev *55*, 1485-1496.

Meyerson, M., Counter, C. M., Eaton, E. N., Ellisen, L. W., Steiner, P., Caddle, S. D., Ziaugra, L., Beijersbergen, R. L., Davidoff, M. J., Liu, Q.*, et al.* (1997). hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. Cell *90*, 785-795.

Miller, C. R., Buchsbaum, D. J., Reynolds, P. N., Douglas, J. T., Gillespie, G. Y., Mayo, M. S., Raben, D., and Curiel, D. T. (1998). Differential susceptibility of primary and

established human glioma cells to adenovirus infection: targeting via the epidermal growth factor receptor achieves fiber receptor-independent gene transfer. Cancer Res *58*, 5738-5748.

Miyazawa, N., Leopold, P. L., Hackett, N. R., Ferris, B., Worgall, S., Falck-Pedersen, E., and Crystal, R. G. (1999). Fiber swap between adenovirus subgroups B and C alters intracellular trafficking of adenovirus gene transfer vectors. J Virol *73*, 6056-6065.

Morales, C. P., Holt, S. E., Ouellette, M., Kaur, K. J., Yan, Y., Wilson, K. S., White, M. A., Wright, W. E., and Shay, J. W. (1999). Absence of cancer-associated changes in human fibroblasts immortalized with telomerase. Nat Genet *21*, 115-118.

Morin, G. B. (1989). The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. Cell *59*, 521-529.

Morrison, S. J., Prowse, K. R., Ho, P., and Weissman, I. L. (1996). Telomerase activity in hematopoietic cells is associated with self-renewal potential. Immunity *5*, 207-216.

Morrison, S. J., Wandycz, A. M., Hemmati, H. D., Wright, D. E., and Weissman, I. L. (1997). Identification of a lineage of multipotent hematopoietic progenitors. Development *124*, 1929-1939.

Morrison, S. J., and Weissman, I. L. (1994). The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. Immunity *1*, 661-673.

Moyzis, R. K., Buckingham, J. M., Cram, L. S., Dani, M., Deaven, L. L., Jones, M. D., Meyne, J., Ratliff, R. L., and Wu, J. R. (1988). A highly conserved repetitive DNA sequence, (TTAGGG)n, present at the telomeres of human chromosomes. Proc Natl Acad Sci U S A *85*, 6622-6626.

Muller, H. J. (1938). The remaking of chromosomes. . The Collecting Net *13*, 181, 183-195, 198

Muruve, D. A. (2004). The innate immune response to adenovirus vectors. Hum Gene Ther *15*, 1157-1166.

Ni, S., Gaggar, A., Di Paolo, N., Li, Z. Y., Liu, Y., Strauss, R., Sova, P., Morihara, J., Feng, Q., Kiviat, N.*, et al.* (2006). Evaluation of adenovirus vectors containing serotype 35 fibers for tumor targeting. Cancer Gene Ther *13*, 1072-1081.

Nilsson, M., Karlsson, S., and Fan, X. (2004a). Functionally distinct subpopulations of cord blood CD34+ cells are transduced by adenoviral vectors with serotype 5 or 35 tropism. Mol Ther *9*, 377-388.

Nilsson, M., Ljungberg, J., Richter, J., Kiefer, T., Magnusson, M., Lieber, A., Widegren, B., Karlsson, S., and Fan, X. (2004b). Development of an adenoviral vector system

with adenovirus serotype 35 tropism; efficient transient gene transfer into primary malignant hematopoietic cells. J Gene Med *6*, 631-641.

Oh, S., Song, Y. H., Yim, J., and Kim, T. K. (2000). Identification of Mad as a repressor of the human telomerase (hTERT) gene. Oncogene *19*, 1485-1490.

Ohyashiki, J. H., Ohyashiki, K., Iwama, H., Hayashi, S., Toyama, K., and Shay, J. W. (1997). Clinical implications of telomerase activity levels in acute leukemia. Clin Cancer Res *3*, 619-625.

Olovnikov, A. M. (1973). A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon. J Theor Biol *41*, 181-190.

Pardal, R., Clarke, M. F., and Morrison, S. J. (2003). Applying the principles of stem-cell biology to cancer. Nat Rev Cancer *3*, 895-902.

Passos, J. F., von Zglinicki, T., and Saretzki, G. (2006). Mitochondrial dysfunction and cell senescence: cause or consequence? Rejuvenation Res *9*, 64-68.

Poole, J. C., Andrews, L. G., and Tollefsbol, T. O. (2001). Activity, function, and gene regulation of the catalytic subunit of telomerase (hTERT). Gene *269*, 1-12.

Raschperger, E., Thyberg, J., Pettersson, S., Philipson, L., Fuxe, J., and Pettersson, R. F. (2006). The coxsackie- and adenovirus receptor (CAR) is an in vivo marker for epithelial tight junctions, with a potential role in regulating permeability and tissue homeostasis. Exp Cell Res *312*, 1566-1580.

Reichenbach, P., Hoss, M., Azzalin, C. M., Nabholz, M., Bucher, P., and Lingner, J. (2003). A human homolog of yeast Est1 associates with telomerase and uncaps chromosome ends when overexpressed. Curr Biol *13*, 568-574.

Roelvink, P. W., Lizonova, A., Lee, J. G., Li, Y., Bergelson, J. M., Finberg, R. W., Brough, D. E., Kovesdi, I., and Wickham, T. J. (1998). The coxsackievirus-adenovirus receptor protein can function as a cellular attachment protein for adenovirus serotypes from subgroups A, C, D, E, and F. J Virol *72*, 7909-7915.

Roth, C. W., Kobeski, F., Walter, M. F., and Biessmann, H. (1997). Chromosome end elongation by recombination in the mosquito Anopheles gambiae. Mol Cell Biol *17*, 5176-5183.

Rudolph, K. L., Millard, M., Bosenberg, M. W., and DePinho, R. A. (2001). Telomere dysfunction and evolution of intestinal carcinoma in mice and humans. Nat Genet *28*, 155-159.

Russell, W. C. (2000). Update on adenovirus and its vectors. J Gen Virol *81*, 2573-2604.

Sanchini, M. A., Gunelli, R., Nanni, O., Bravaccini, S., Fabbri, C., Sermasi, A., Bercovich, E., Ravaioli, A., Amadori, D., and Calistri, D. (2005). Relevance of urine telomerase in the diagnosis of bladder cancer. Jama *294*, 2052-2056.

Segerman, A., Atkinson, J. P., Marttila, M., Dennerquist, V., Wadell, G., and Arnberg, N. (2003). Adenovirus type 11 uses CD46 as a cellular receptor. J Virol *77*, 9183-9191.

Sharma, G. G., Gupta, A., Wang, H., Scherthan, H., Dhar, S., Gandhi, V., Iliakis, G., Shay, J. W., Young, C. S., and Pandita, T. K. (2003). hTERT associates with human telomeres and enhances genomic stability and DNA repair. Oncogene *22*, 131-146.

Shay, J. W., and Bacchetti, S. (1997). A survey of telomerase activity in human cancer. Eur J Cancer *33*, 787-791.

Shay, J. W., Pereira-Smith, O. M., and Wright, W. E. (1991). A role for both RB and p53 in the regulation of human cellular senescence. Exp Cell Res *196*, 33-39.

Shayakhmetov, D. M., Eberly, A. M., Li, Z. Y., and Lieber, A. (2005). Deletion of penton RGD motifs affects the efficiency of both the internalization and the endosome escape of viral particles containing adenovirus serotype 5 or 35 fiber knobs. J Virol *79*, 1053-1061.

Shayakhmetov, D. M., Li, Z. Y., Ternovoi, V., Gaggar, A., Gharwan, H., and Lieber, A. (2003). The interaction between the fiber knob domain and the cellular attachment receptor determines the intracellular trafficking route of adenoviruses. J Virol *77*, 3712-3723.

Shayakhmetov, D. M., Papayannopoulou, T., Stamatoyannopoulos, G., and Lieber, A. (2000). Efficient gene transfer into human CD34(+) cells by a retargeted adenovirus vector. J Virol *74*, 2567-2583.

Singh, S. K., Clarke, I. D., Terasaki, M., Bonn, V. E., Hawkins, C., Squire, J., and Dirks, P. B. (2003). Identification of a cancer stem cell in human brain tumors. Cancer Res *63*, 5821-5828.

Smogorzewska, A., and de Lange, T. (2002). Different telomere damage signaling pathways in human and mouse cells. Embo J *21*, 4338-4348.

Smogorzewska, A., and de Lange, T. (2004). Regulation of telomerase by telomeric proteins. Annu Rev Biochem *73*, 177-208.

Soder, A. I., Hoare, S. F., Muire, S., Balmain, A., Parkinson, E. K., and Keith, W. N. (1997). Mapping of the gene for the mouse telomerase RNA component, Terc, to chromosome 3 by fluorescence in situ hybridization and mouse chromosome painting. Genomics *41*, 293-294.

Stansel, R. M., de Lange, T., and Griffith, J. D. (2001). T-loop assembly in vitro involves binding of TRF2 near the 3' telomeric overhang. Embo J *20*, 5532-5540.

Starling, J. A., Maule, J., Hastie, N. D., and Allshire, R. C. (1990). Extensive telomere repeat arrays in mouse are hypervariable. Nucleic Acids Res *18*, 6881-6888.

Steinwaerder, D. S., and Lieber, A. (2000). Insulation from viral transcriptional regulatory elements improves inducible transgene expression from adenovirus vectors in vitro and in vivo. Gene Ther *7*, 556-567.

Stewart, S. A., and Weinberg, R. A. (2006). Telomeres: cancer to human aging. Annu Rev Cell Dev Biol *22*, 531-557.

Takakura, M., Kyo, S., Kanaya, T., Hirano, H., Takeda, J., Yutsudo, M., and Inoue, M. (1999). Cloning of human telomerase catalytic subunit (hTERT) gene promoter and identification of proximal core promoter sequences essential for transcriptional activation in immortalized and cancer cells. Cancer Res *59*, 551-557.

Tauber, B., and Dobner, T. (2001). Molecular regulation and biological function of adenovirus early genes: the E4 ORFs. Gene *278*, 1-23.

Ulaner, G. A., Hu, J. F., Vu, T. H., Giudice, L. C., and Hoffman, A. R. (1998). Telomerase activity in human development is regulated by human telomerase reverse transcriptase (hTERT) transcription and by alternate splicing of hTERT transcripts. Cancer Res *58*, 4168-4172.

Ulasov, I. V., Tyler, M. A., Zheng, S., Han, Y., and Lesniak, M. S. (2006). CD46 represents a target for adenoviral gene therapy of malignant glioma. Hum Gene Ther *17*, 556-564.

van Beusechem, V. W., Grill, J., Mastenbroek, D. C., Wickham, T. J., Roelvink, P. W., Haisma, H. J., Lamfers, M. L., Dirven, C. M., Pinedo, H. M., and Gerritsen, W. R. (2002). Efficient and selective gene transfer into primary human brain tumors by using single-chain antibody-targeted adenoviral vectors with native tropism abolished. J Virol *76*, 2753-2762.

van Steensel, B., Smogorzewska, A., and de Lange, T. (1998). TRF2 protects human telomeres from end-to-end fusions. Cell *92*, 401-413.

Wang, Q., Greenburg, G., Bunch, D., Farson, D., and Finer, M. H. (1997). Persistent transgene expression in mouse liver following in vivo gene transfer with a delta E1/delta E4 adenovirus vector. Gene Ther *4*, 393-400.

Vassaux, G., Hurst, H. C., and Lemoine, N. R. (1999). Insulation of a conditionally expressed transgene in an adenoviral vector. Gene Ther *6*, 1192-1197.

Watkins, S. J., Mesyanzhinov, V. V., Kurochkina, L. P., and Hawkins, R. E. (1997). The 'adenobody' approach to viral targeting: specific and enhanced adenoviral gene delivery. Gene Ther *4*, 1004-1012.

Watson, J. D. (1972). Origin of concatemeric T7 DNA. Nat New Biol *239*, 197-201.

Vaziri, H., and Benchimol, S. (1998). Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. Curr Biol *8*, 279-282.

Vellinga, J., Van der Heijdt, S., and Hoeben, R. C. (2005). The adenovirus capsid: major progress in minor proteins. J Gen Virol *86*, 1581-1588.

Wickham, T. J., Mathias, P., Cheresh, D. A., and Nemerow, G. R. (1993). Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. Cell *73*, 309-319.

Wickham, T. J., Segal, D. M., Roelvink, P. W., Carrion, M. E., Lizonova, A., Lee, G. M., and Kovesdi, I. (1996). Targeted adenovirus gene transfer to endothelial and smooth muscle cells by using bispecific antibodies. J Virol *70*, 6831-6838.

Wickham, T. J., Tzeng, E., Shears, L. L., 2nd, Roelvink, P. W., Li, Y., Lee, G. M., Brough, D. E., Lizonova, A., and Kovesdi, I. (1997). Increased in vitro and in vivo gene transfer by adenovirus vectors containing chimeric fiber proteins. J Virol *71*, 8221-8229.

Wirth, T., Zender, L., Schulte, B., Mundt, B., Plentz, R., Rudolph, K. L., Manns, M., Kubicka, S., and Kuhnel, F. (2003). A telomerase-dependent conditionally replicating adenovirus for selective treatment of cancer. Cancer Res *63*, 3181-3188.

Vogelstein, B., and Kinzler, K. W. (2004). Cancer genes and the pathways they control. Nat Med *10*, 789-799.

Volpers, C., and Kochanek, S. (2004). Adenoviral vectors for gene transfer and therapy. J Gene Med *6 Suppl 1*, S164-171.

Wright, W. E., and Shay, J. W. (1992). Telomere positional effects and the regulation of cellular senescence. Trends Genet *8*, 193-197.

Wright, W. E., and Shay, J. W. (2001). Cellular senescence as a tumor-protection mechanism: the essential role of counting. Curr Opin Genet Dev *11*, 98-103.

Wright, W. E., Shay, J. W., and Piatyszek, M. A. (1995). Modifications of a telomeric repeat amplification protocol (TRAP) result in increased reliability, linearity and sensitivity. Nucleic Acids Res *23*, 3794-3795.

Wu, K. J., Grandori, C., Amacker, M., Simon-Vermot, N., Polack, A., Lingner, J., and Dalla-Favera, R. (1999). Direct activation of TERT transcription by c-MYC. Nat Genet *21*, 220-224.

Wu, Y. L., Dudognon, C., Nguyen, E., Hillion, J., Pendino, F., Tarkanyi, I., Aradi, J., Lanotte, M., Tong, J. H., Chen, G. Q., and Segal-Bendirdjian, E. (2006). Immunodetection of human telomerase reverse-transcriptase (hTERT) re-appraised: nucleolin and telomerase cross paths. J Cell Sci *119*, 2797-2806.

Vulliamy, T., Marrone, A., Goldman, F., Dearlove, A., Bessler, M., Mason, P. J., and Dokal, I. (2001). The RNA component of telomerase is mutated in autosomal dominant dyskeratosis congenita. Nature *413*, 432-435.

Yamaguchi, H. (2007). Mutations of telomerase complex genes linked to bone marrow failures. J Nippon Med Sch *74*, 202-209.

Yan, P., Benhattar, J., Seelentag, W., Stehle, J. C., and Bosman, F. T. (2004). Immunohistochemical localization of hTERT protein in human tissues. Histochem Cell Biol *121*, 391-397.

Yang, Y., Nunes, F. A., Berencsi, K., Furth, E. E., Gonczol, E., and Wilson, J. M. (1994). Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. Proc Natl Acad Sci U S A *91*, 4407-4411.

Ye, J. Z., Donigian, J. R., van Overbeek, M., Loayza, D., Luo, Y., Krutchinsky, A. N., Chait, B. T., and de Lange, T. (2004). TIN2 binds TRF1 and TRF2 simultaneously and stabilizes the TRF2 complex on telomeres. J Biol Chem *279*, 47264-47271.

Yui, J., Chiu, C. P., and Lansdorp, P. M. (1998). Telomerase activity in candidate stem cells from fetal liver and adult bone marrow. Blood *91*, 3255-3262.