DISSERTATION

TELOMERE LENGTH REGULATION BY TELOMERASE IN TRANSFORMED AND NON-TRANSFORMED RAT CELL LINES: IDENTIFICATION AND CHARACTERIZATION OF THE TERT-GENE AND PROMOTER FROM RAT

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In Gedenken an meine Betreuerin

Frau Prof. Christa Cerni

und

für meine liebe Familie

Maria, Adrian und Victoria

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1. ZUSAMMENFASSUNG

Die Enden der Chromosomen, die sogenannten Telomere, bestehen bei höheren Eukaryonten aus 3´-TTAGGG-5´ wiederholten Einheiten (Repeats). Durch die unvollständige Replikation der DNA gehen bei jeder Zellteilung 50-200 Nukleotide verloren. Diese progressive Telomerverkürzung führt *in vitro* zu Seneszenz und Zelltod. Die Telomerlängen geben damit den Rahmen für das replikative Potential einer Zelle vor. Telomerase, ein Holo-Enzym bestehend aus einer RNA- und einer katalytischen Untereinheit (TERT), synthetisiert durch reverse Transkription die Telomeren-Repeats und gleicht den Verlust der Nukleotide aus. Hohe Telomerase-Aktivität ist nicht nur in normalem Hodengewebe, sondern vor allem in humanen Tumoren bzw. Tumorzell-Linien zu finden. Die Aktivierung der Telomerase gilt als Voraussetzung für die maligne Transformation von humanen Zellen.

Um den Einfluss verschiedener immortalisierender und/oder transformierender Onkogene auf die Telomerase-Aktivität (TA) und die Telomeren-Länge zu studieren, habe ich Ratten-Embryonalzellen (RECs) mit verschiedenen immortalisierenden (c-Myc, HPVs und mutiertes p53 usw.) und/oder transformierenden (c-Ha-ras) Onkogenen transfektiert. Anschließend wurden die isolierten Zell-Linien auf Telomerase-Aktivität mittels eines in dieser Arbeit entwickelten modifizierten TRAP-ASSAYS (Telomeric Repeat Amplification Protocol) untersucht. Die entsprechenden TRFs(Telomeric Restriction Fragments) wurden nach Isolierung der genomischen DNA durch Pulse-Field-Elektrophorese bestimmt. c-Myc/c-Ha-ras transfektierte Zell-Linien zeigten eine höhere TA und längere TRFs im Vergleich zu anderen Zell-Linien.

Das Myc/Max/Mad Netzwerk spielt eine wichtige Rolle bei der Regulation der Genexpression über die Transkription und ist bei der Zellteilung, dem Zellwachstum,

dem Zelltod (Apoptose) und der Differenzierung von Zellen beteiligt. Während die Myc Proteine den Zellzyklus positiv beeinflussen, sind Mad Proteine negative Regulatoren der Zellpoliferation. Ich habe während meiner Dissertationsarbeit ein induzierbares Ratten Zellsystem (c-Myc^{ER}/ras, c-Myc/MMTV ras und c-Myc/c-Ha-ras/mad1^{ER}) entwickelt und konnte mit diesem System eine positive transkriptionale Regulation des TERT-Genes in Verbindung mit dem Myc/Max-Heterodimer nachweisen. Ich konnte Mad1 als Antagonist zu Myc identifizieren und nachweisen, dass Mad1 eine negative Regulation des Genes bewirkt.

Es wurden ebenfalls eine Reihe von stabil expremierenden hTERT Klonen aus einer Zelllinie hergestellt. Durch die ektopische Expression von hTERT zeigten diese eine höhere Wachstumsrate als die Ausgangszelllinie. Ausserdem wurde durch die ektopische Expression mittels Transfektion von hTERT in Telomerase- und ALT-negativen Lungenkrebszelllinie eine enorme Verlängerung der Telomerenlängen bewirkt. Dabei zeigten die Telomere aller Chromosomen innerhalb einer Zelle eine einheitliche Telomerenlänge (ca. 14 Kbp). Somit dürfte die ektopische Expression von hTERT einen neuen Mechanismus der Telomerasefunktion darstellen. Diese Eigenschaft könnte die erhöhte Aggresivität mancher Tumorzellen erklären, welche in Telomerase negativen Tumorzellen und in Tumorzellen, die nicht den ALT-Mechanismus verwenden, gefunden wird. Die exakte medizinische Bedeutung und der biologische Einfluß des erhöhten Wachstumspotentials unter dem Einfluss der hTERT Expression ist Gegenstand weiterer Untersuchungen.

Im weiteren Teil meiner Arbeit wurde die vollständige Ratten TERT-cDNA mittels einer *in silico* Analyse und reverser Transkriptase Polymerase Ketten Reaktion (RT-PCR) kloniert. Analog wurde der "Core"-Promotor des TERT Genes der Ratte in ein Reporter Plasmid kloniert. Mittels gezielter Mutagenese wurde ein Myc-bindendes Motif (E-Box) aus dem Promotor entfernt. Ich konnte mittels Luciferase Assays die

Aktivierung des Ratten TERT-Promotors in Abhängigkeit von c-Myc *in vitro* nachweisen. Zur Detektion und Quantifizierung der Transcripte des Ratten-TERT und TERC Genes mittels RT-PCR wurde im Rahmen dieser Arbeit neue Methoden entwickelt.

Zusammenfassend zeigen meine Daten dass, 1) Mad1 ist in der Lage, eine Myc/Ras vermittelte Transformation *in vivo* zu unterdrücken 2) ektopisch experimierte Telomerase fähig ist, die einzelne Telomer- Längenregelung zu überwinden.

2. ABSTRACT

The finite life span of organisms is regulated by telomere reduction due to the "end replication problem" of DNA. The reduction of telomeres can be counterbalanced by the activation of telomerase or by other less defined mechanisms, e.g. alternative telomere lengthening (ALT). Most somatic human cells lack telomerase activity because they do not express the catalytical subunit of the telomerase reverse transcriptase (hTERT) gene. Conversely, most cancer cells reexpress hTERT and are therefore telomerase positive. Telomerase is a specific multi-subunit ribonucleoprotein that synthesizes TTAGGG telomere DNA onto chromosomal ends by using a RNA component (TERC) as a template for telomere specific sequences and the catalytic subunit.

I was interested in the need for telomerase activity in self-developed immortalized and/or transformed cell lines derived from primary rat embryo cells (RECs). First, I determined telomerase activity by improving a modified TRAP assay. As a model for normal cells, untransfected RECs cultivated as mass cultures were analysed. The extend of telomerase activity reflected the growth potential of RECs: it was high at the first few population doublings, declined at senescence and was almost undetectable during cell crisis. The eventual resumption of growth capacity was paralleled by increased telomerase activity.

Furthermore, I analysed a series of individual cell lines derived from RECs upon transfer of various oncogenes. Interestingly, none of several $p53^{val135}$-immortalized cell lines which differed in their growth behaviour, amount and localization of mutated p53 protein, and which were derived from various transfection experiments expressed significant amounts of telomerase activity. The additional expression of a c-Ha-ras oncogene, leading to highly tumorigenic cell lines, further decreased

telomerase activity. In contrast, immortalisation of RECs by activated oncogenic c-Myc with or without a co-operating activated ras oncogene was clearly associated with very high telomerase activity. This high activity exceeded that of human HeLa cells, which served as positive control and for standardisation of the assay. Cell lines expressing E1A (early gene 1A of adenovirus) had high telomerase activity, coexpression of ras oncogene, however, yielded cell lines with low telomerase activity.

Furthermore, c-Myc as a transcriptional regulator and a member of the Myc/Max/Mad network plays an essential role in cell proliferation, growth, apoptosis, and differentiation. Whereas Myc proteins affect cell cycle progression positively, Mad proteins are negative regulators of cell proliferation. I established an oncogene inducible rat-cell-systems (c-Myc^{ER}/ras, c-Myc/MMTV ras und c-Myc/c-Ha-ras/mad1^{ER}) and detected a positive regulation of the TERT gene by Myc proteins, whereas Mad proteins as known antagonist to Myc caused a negative regulation of the gene.

I conclude that at least in primary rat embryonal cells 1. (re-)activation of telomerase activity clearly depends on the nature of the immortalising oncogenes and 2. that the concomitant expression of a mutated Ras-oncogene in general reduces telomerase activity and 3. in contrast c-Myc in co-operation with c-Ha-Ras is the most potent inducer of telomerase activity. My data further imply that there is at least one additional p53-dependent-mechanism in higher eukaryotic cells to maintain efficiently telomere lengths and that this non-telomerase mechanism appears to be rather regulated by special oncogenes than to depend on a specific cell type.

Further, *in silico* techniques and partial genomic sequence data from rat genom project were used to clone the cDNA encompassing the complete rat TERT transcription unit and the core region of the rat TERT promoter.

Sequence analysis revealed that the rat core TERT promoter is GC-rich, lacks TATA and CAAT boxes and contains one E-box immediately upstream of the TERT transcription start site (ATG). The Myc gene family members encode transcription factors which, upon dimerization with Max protein, bind to the DNA sequence 5'-CACGTG-3', termed E-box, or to close related sequences. The E-boxes in the TERT gene locus are preferred target sites for Myc/Max heterodimers und TERT becomes upregulated by overexpressed c-Myc, which could be counteracted by deletion or mutation of the E-box within the rat TERT core promoter.

We were also interested in the effects of exogenous hTERT on telomerase activity and telomere elongation in a telomerase-negative background and transfected a hTERT gene into suitable human lung cancer cell lines. Our data indicate that under certain circumstances i.e. when the regulation of telomere length individuality is relaxed or abolished, ectopic telomerase expression and activity extends all chromosomal ends to almost the same length. Interestingly, this seems to be a new mechanism for telomerase function, as this is found in non-telomerase and non-ALT tumor cells. The clinical or biological impact needs to be further investigated.

Summerizing the data indicate that 1). Mad1 is able to suppress Myc/Ras-mediated transformation under *in vivo* conditions 2. ectopic telomerase expression and activity is capable to overcome individual telomere length regulation .

3. INTRODUCTION

Multicellular organisms have a finite replicative capacity that is regulated at the single cell level. Hayflick and Moorhead were the first to report on the limit of *in vitro* growth potential of human embryonic cells explanted from various tissues [1]. The proliferative potential of normal cells in culture is limited to a finite number of population doublings, a phenomenon known as cellular senescence or Hayflick limit [2]. The number of successive divisions in culture depends on the species, age, tissue, cell type and genetic background of the donor. Thus, it might vary considerably within a few doublings of differentiated cells from an elderly donor. Human cells are estimated to have the potential to undergo on average 60-70 mitosis and at this point the cells enter growth arrest and senescence [2, 3]. This state of senescence is characterized by telomere shortening and a high frequency of nuclear abnormality [4, 5]. Telomeres are repetitative sequences at the end of chromosomes that are essential for the stability and integrity of linear chromosomes [6]. Telomere shortening has been proposed as the mitotic clock that marks the progress of a cell towards the end of its replicative life span. The enzyme telomerase is responsible for maintenance of the chromosome ends. It is a reverse transcriptase, in particular a RNA dependent DNA polymerase.

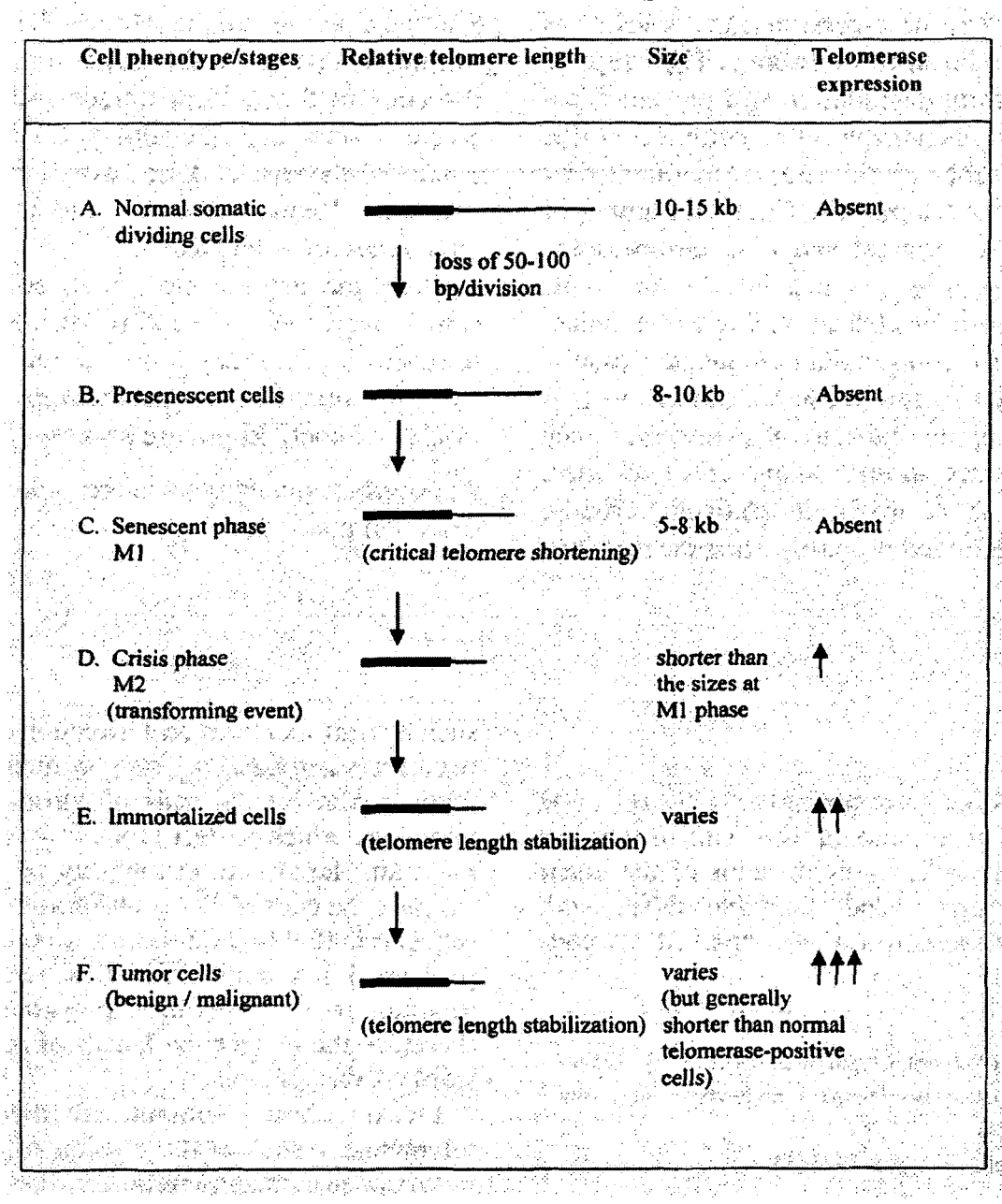

Fig.1. **Influence of telomerase activity and telomere length on the processes of cellular aging, senescence, immortalisation and tumorigenesis.**

The effects of telomerase expression on telomere length in various cell types are depicted (figure 1). The broad solid line represents the 3′ terminal portion of a chromosome and the narrow solid line, the telomere length basal or low levels of telomerase are indicated by single upward arrows, double arrows indicate an intermediate level of telomerase expression, and elevated levels of telomerase are represented by three upward arrows. **(A)** In the absence of telomerase in most normal somatic cells, cellular division is accompanied by the loss of telomeric

repeats due to the end replication problem. **(B)** Repeated cell division leads to the attrition of telomere length resulting in cells acquiring a pre-senescent phenotype approaching senescence. **(C)** With further telomeric attrition to a critical telomere length, cells approach the senescent stage M1. Some cells in this phase can escape senescence and become immortal. However, these cells eventually undergo apoptosis or cell death in the absence of telomerase. **(D)** Cells in the M1 phase that do not escape senescence enter the M2 crisis stage (towards cell death). **(E)** A few rare cells in this phase (M2) may escape crisis and become immortal with the reactivation of telomerase. **(F)** During transformation the telomere lengths are stabilized and vary depending on the cell type. The telomere of transformed cells are short and in most cases are nearly equal to or less than the length at the M2 threshold stage [6]. They are also much shorter than those of telomerase-positive normal cells [7]. It is the reactivation and up-regulation of telomerase that maintains the stability of the short telomere lengths. Finally, the transforming events (inactivation of tumor suppressor genes, up-regulation of certain oncogenes, such as ras) along with the up-regulation of telomerase impart an immortal and tumorigenic (benign/malignant) phenotype to the cells [8].

Among the multitude of acquired features of human cancer cells [9], the maintenance of telomere length is a crucial pre-requisite since it counteracts the otherwise physiological erosion of telomere [10].

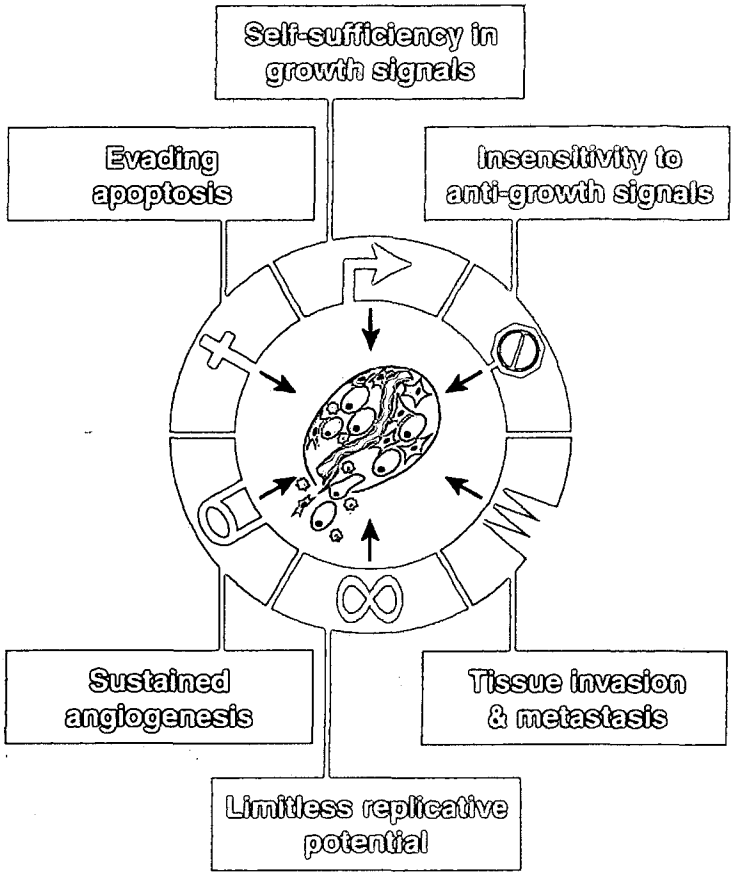

Fig.2. **Acquired capabilities of cancers**

Hanahan and Weinberg suggest that most if not all cancers acquire the same set of functional capabilities (figure 2) during their development, albeit through various mechanistic strategies [9].

3.1 TELOMERES

3.1.1 Composition and structure

The telomeres, the ends of linear eukaryotic chromosomes, have defined important roles of these structures in many cellular processes. Failure of any of them could lead to genetic instability, a possible prelude to cancerous cell transformation, or cell death. Telomeres are specialized nucleoprotein complexes that serve for protection,

replication and stabilization of the chromosome ends. They are also believed to protect the ends of chromosomes against exonucleases, prevent fusions and illegitimate recombination, and play an important role in the separation of chromosomes during mitosis [11-13].

The inability of DNA polymerase to replicate the termini of linear molecules [14, 15] predicts loss of telomeric DNA sequences and shortening of telomeres with each round of semi-conservative DNA synthesis - "the end replication problem" [4, 5, 16, 17].

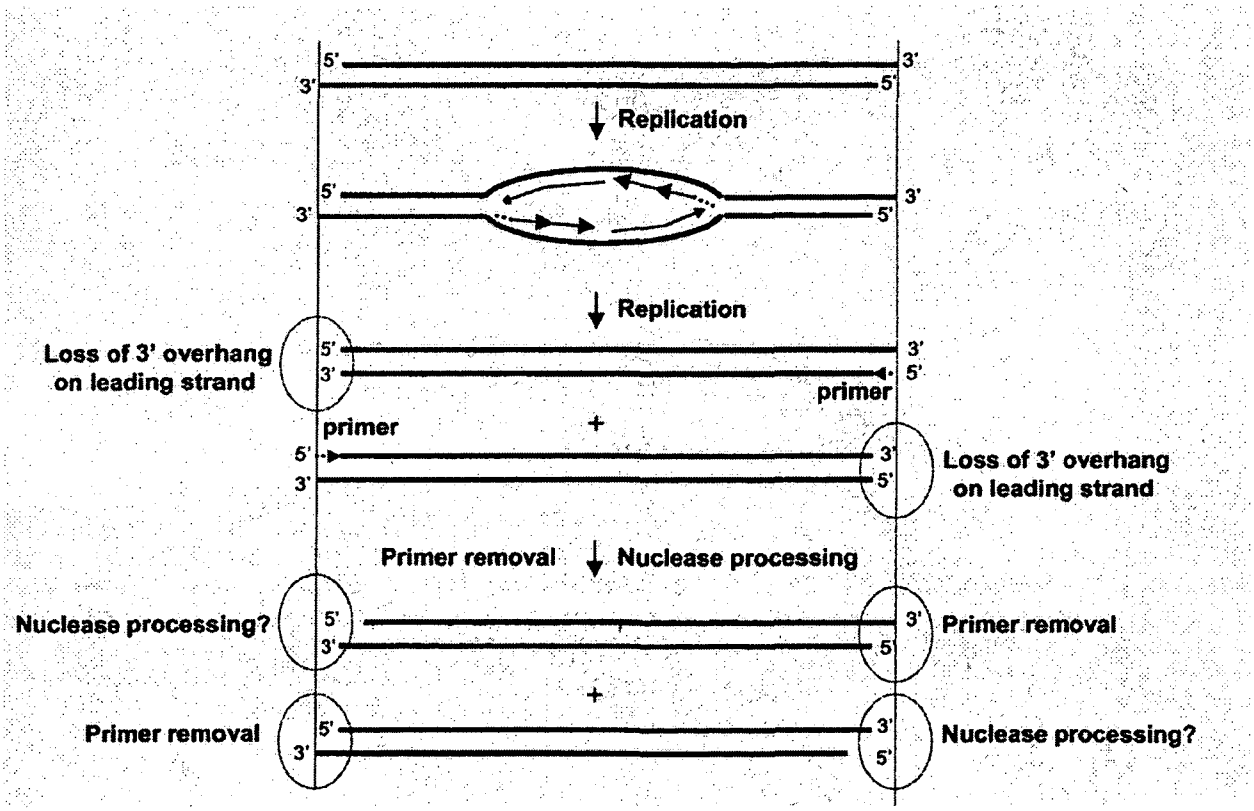

Fig.3. **End replication problem**

DNA replication by conventional polymerase proceeds in the 5'- to- 3' direction (figure 3). The newly synthesized leading strands would not generate overhangs, but the newly synthesized lagging strands would lose their extreme 3' end after RNA

primers are removed. In addition, both parental strands might also be subject to nuclease processing [18].

In most organisms studied, telomeres contain tandemly repeated simple DNA sequences composed of a G-rich-strand. This simple sequence repeat in telomeres $(TTAGGG)_n$ is highly conserved and appears to be the same in all vertebrates. The evolutionary conservation of telomeric DNA suggests that the repeat sequence is critical for telomere function. For most species they consist of 6-8 bp short monotonously repeated elements but there is a broad variability in the number of repeats ranging from a few as in ciliates up to several thousand in vertebrates [11, 19].

The telomeric DNA sequences have generally been subdivided into three distinct classes: 1.) telomere associated sequences (TAS) 2.) double stranded telomeric repeats and 3.) 3' overhangs. TAS are the repeated elements proximal to the terminus specific sequences and are usually described as the area between the first identifiable single copy gene and the telomeric repeats. The TAS family members are relatively homologous within the families of each species, but the may differ in various species. In addition, there is a big variability of the actual amounts of TAS between the chromosomes of the same species. It became evident that TAS can provide alternative mechanisms for telomere maintenance via recombination or transposition in case telomerase is absent [14, 20-22].

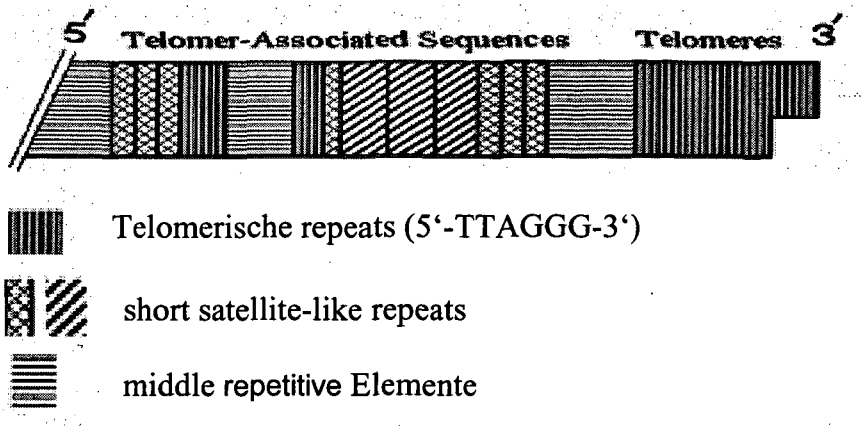

Fig.4. **Organization of eukaryotic telomeres**

The ends of telomeres consist of repetitive telomeric sequences with single-stranded 3′ overhangs (figure 4). Subtelomeric regions are composed of various short satellite-like repeats and middle-repetitive elements, interspersed with telomeric repeats [23].

Telomeric repeats are synthesized by telomerase, a ribonucleoprotein capable of synthesizing telomeres de novo. This is the main telomere replication mechanism [4].

3.1.2 Configuration and telomeres associated proteins

Telomeres have a unique t-looped configuration at the end where the telomere bends back on itself [24]. The overhanging guanine-rich single strand is tucked into the double stranded telomeres. This creates a second smaller d-loop by displacing one of the strands. This structure appears to protect the telomeres from end to end fusion with other chromosomes and from cell cycle checkpoints that would otherwise recognize the telomere as chromosome breaks requiring repair [25, 26].

Several proteins have been identified that associate with mammalian telomeres. Most of these regulate one or more aspect of telomere structure or telomere length. Some associate exclusively with telomeres, whereas others localize to additional subnuclear or subcellular sites.

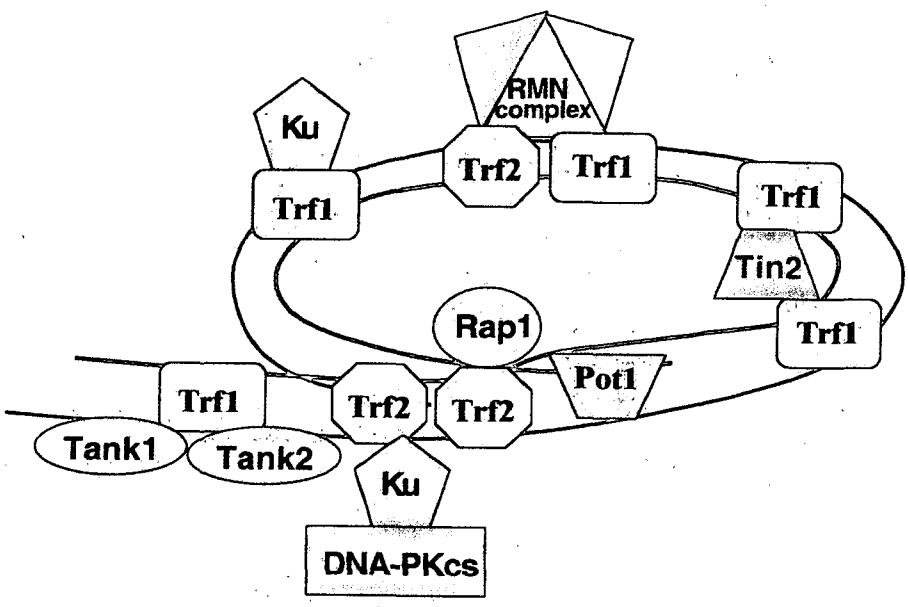

Fig.5. **Telomere-associated proteins**

Mammalian telomeres are thought to end in a large t-loop whose structure and stability may depend on a number of proteins, such as TRF2, TRF1, POT1, TIN2 and RAP1 (figure 5). Non-classical PARPs, such as TANK1 and TANK2 may also associate with the telomeres through their interaction with TRF1. In addition, TRF1 and TRF2 recruit to the telomeres a number of proteins known to participate in DNA repair, most notably the RAD50-MRE11-NBS1 (RMN) complex and the DNA-dependent protein kinase, which is composed of a catalytic subunit (DNA-PKCs) and a DNA end binding subunit (Ku) [27].

Two mammalian proteins, TRF1 and TRF2, bind directly to double stranded telomeric DNA [28, 29], TRF1 and TRF2 contain a similar C-terminal Myb domain

that mediates sequence-specific binding to telomeric DNA. TRF1 is involved in telomere length regulation via a negative feedback, over-expression results in shortened telomeres while loss of functional TRF1 causes elongated telomeres [30, 31]. Loss of TRF2 shortens telomeres and causes telomere-telomere associations. It is more conserved than TRF1 and its main function is to maintain the 3'-overhangs at the telomere ends [32].

A third protein, POT1, binds specifically to the single stranded 3'-overhang [33]. POT1 binding is regulated by the TRF1 complex in response to telomere length. A mutant form of POT1 lacking the DNA-binding domain abrogated TRF1-mediated control of telomere length, and induced rapid and extensive telomere elongation [34, 35]. TRF2 and POT1 appear to be particularly important for stabilizing the telomeric structure and protecting chromosome ends from degradation and fusion [32, 33, 36, 37]. Two additional mammalian telomere-associated proteins, TIN2 and hRAP1, also localize specifically to telomeres, TIN2 interacting with TRF1 and co-localized with TRF1 in nuclei and metaphase chromosomes. A mutant TIN2 that lacks amino-terminal sequences effects elongated human telomeres in a telomerase-dependent manner. TRF1 is insufficient for the control of telomere length in human cells, and TIN2 is an essential mediator of TRF1 function [38]. hRAP1 is located at telomeres, affects telomere length and is recruited to telomeres by TRF2 [39]. These proteins, together with TRF1, appear to be particularly important for regulating the telomere length. They do not appear to act on telomerase, but rather appear to regulate the telomeric structure and hence the ability of telomerase to access the 3'-overhang. TRF1, RAP1 and TIN2 are also very likely to help stabilizing the telomere structure.

Two non-classical poly-ADP ribose polymerases (PARPs), enzymes commonly associated with DNA repair and maintenance of chromosome stability [40, 41], also interact with TRF1 [42, 43]. However, these proteins, TANK1 and TANK2, are most

abundant at the nuclear periphery, in Golgi vesicles, and, like classical PARPs, localize to centrosomes during mitosis [43-45]. Thinking on largely extranuclear localization, TANKs may function in non-telomeric cellular processes. On the other hand, their location at the nuclear periphery and their PARP activity raises the possibility that TANKs participate in repairing, or signalling the occurrence of dysfunctional telomeres. Several proteins known to participate in DNA repair were recently found at telomeres. One example is Ku, the DNA end-binding component of a DNA-dependent protein kinase (DNA-PK), which is essential for DSBs (double strand breaks) repair by non-homologous end joining [46]. The 70 kD Ku subunit binds TRF1 and TRF2 in cells, and a significant fraction of Ku associates with mammalian telomeres [47-49]. Cells from mice deficient in either Ku subunit, or the DNA-PK catalytic subunit, are genomically unstable owing to frequent telomere fusions [50]. Thus, DNA-PK, in addition to its role in DNA repair, may also play a role in telomere maintenance. This idea is supported by recent data showing that DNA-PK, together with TRF2, is required for strand-specific processing of the telomeres after they are replicated in S phase [37]. Likewise, RAD50, MRE11 and NBS1 (RMN), another important DNA repair complex, may function at telomeres. This complex associates with mammalian telomeres, at least during S phase, very likely owing to an interaction between NBS1 and TRF1 [51] and TRF2 [52].

3.2 TELOMERASE

3.2.1 Function and composition

Telomerase is a ribonucleoprotein complex with a RNA component (Telomerase RNA; TR) and a catalytic subunit (Telomerase Reverse Transcriptase; TERT) as

core components, which synthesizes telomeric sequence repeats onto chromosomal ends. The basic function of telomerase is to extend the 3´-end of telomeres, the end of linear chromosomes, by de novo synthesis of the G-rich telomeric repeat DNA. Telomerase represents a highly specialised RNA dependent DNA polymerase as it synthesises only one sequence and does not use an exogenous RNA template. Assembly of functional telomerase and elongation of telomeres are probably multi-step processes that involve other telomerase components and associated proteins to form a functional, multi-subunit telomerase holo-enzyme.

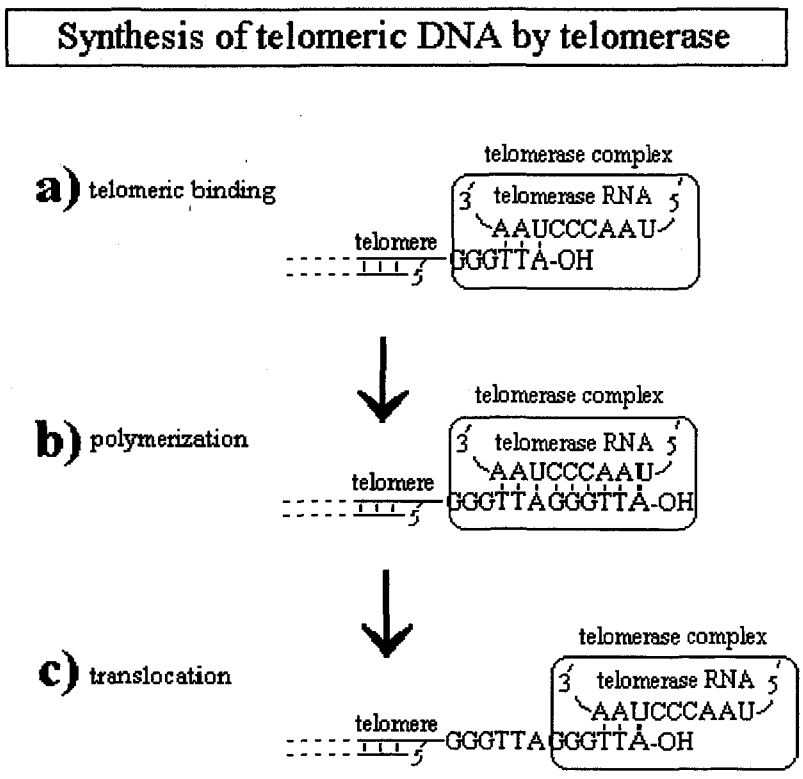

Fig.6. **Elongation of telomere by telomerase**

As a first step, three nucleotides of the template region of the RNA component of telomerase hybridize to the last three nucleotides of the telomeric 3′ overhang (figure 6). **(a)** The telomere is elongated by the action of reverse transcriptase of the catalytic component of telomerase. **(b)** Reitinerated rounds of hybridisation and

synthesis lead to successive elongation of the telomere. **(c)** translocation and newly synthesis of telomere.

3.2.2 The RNA subunit of telomerase (hTR or hTERC for human)

Telomerase activity depends on an essential RNA subunit. As template for synthesis of telomere DNA functions a CA-rich region, complementary to the GT-rich telomeric sequence, in the RNA strand of telomerase. The telomerase RNA was the first telomerase enzyme component to be cloned; in ciliates [53], followed by budding yeast [54] and mammals [55, 56].The mammalian telomerase RNAs are widely expressed in many tissues and throughout development, even in those tissues without telomerase activity [55, 57].

Phylogenetic comparison of the telomerase RNAs from various species reveals a surprising conservation of secondary structure, despite a large divergence in length and primary sequence [58-61] (Fig 7).

Despite, there is a wide variability in length, from 150 to 200 nucleotides in the ciliates to about 450 in mammals and approximately 1300 nucleotides in S. cerevisia for telomerase RNAs, they all contain a putative template specific for the repeat sequence of each species. In vitro, a two-site binding model for telomerase in which the 3′end of the primer is bound on the template region of the RNA while the 5′end is bound in a separate anchor site that has specifity for telomeric sequences has been proposed [62, 63]. It is supposed that the anchor site can bind duplex as well as single-stranded DNA, which may be critical for its function at the chromosome ends [61, 64].

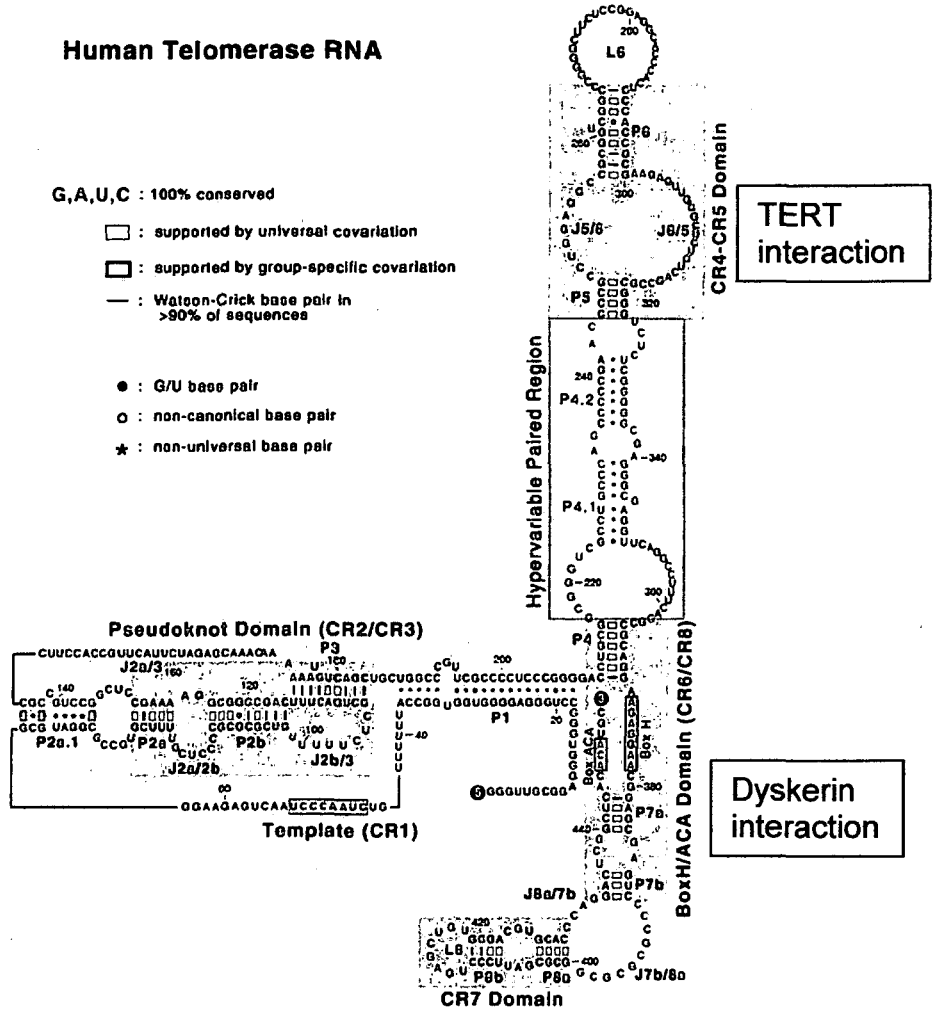

Fig.7. **Proposed Secondary Structure of human Telomerase RNA.**

The proposed secondary structures of human telomerase RNA war determined by phylogenetic comparative analysis (figure 7). Paired regions (P) are numbered from 59 to 39 as P1–P8. The junction regions (J) between two paired regions are named with reference to the flanking paired regions (figure 7). Invariant nucleotides are shown with red letters in bold. Base pairs supported by co-variation evidence are shown as green filled boxes. Base pairs supported by co-variation only within a given group of species are shown as blue open boxes. Dashes represent potential

Watson-Crick base pairs that exist in more than 90% of the sequences. Filled circles indicate G/U base pairs, while open circles represent non-canonical pairs. An asterisk between base pairs in a potential paired region represents the non-universal base-pairings. The non-universal paired regions exist only in an individual class of species and no homologous helical region could be confidently identified in other groups. The four universal structural domains described in the text are shaded in grey and labelled. The template region, Box H and Box ACA motifs are labelled and the conserved nucleotides are boxed. Every twentieth nucleotide of the human RNA is numbered [61].

3.2.3 The catalytic subunit of telomerase

The catalytic subunit of telomerase was initially purified biochemically from *Euplotes aediculatus* as p123 [65, 66]. Sequencing analysis revealed that p123 contains reverse transcriptase motifs and is homologous to the yeast protein EST2, which is one of four EST (ever shorter telomeres), a protein required for telomere maintenance that was initially identified by genetic screening of yeast mutants with reduced telomere length and the senescence phenotype [67]. Introduction of single-amino-acid substitutions within the reverse transcriptase motifs leads to telomere shortening and senescence in S. cerevisiae, indicating that these motifs are important for catalysis of telomere elongation *in vivo* [68]. The catalytic subunit of human telomerase hTERT (human telomerase reverse transcriptase) was identified first 1997 [69, 70]. The human protein is a reverse transcriptase similar to the catalytic subunits of unicellular eukaryotes *S. cerevisiae*, Tetrahymena and of mouse and reveals in comparison with other RTs (reverse transcriptases) seven conserved protein domains that are part of a protein fold and forms the active site of the

enzyme. There is one additional telomerase specific domain called the T-motif. TERT proteins represent a subgroup of RTs and are in general most similar in structure and function to retroviral and retrotransposon RTs [71-73]. Although the RNA component is present in most cells and tissues most normal cells have undetectable levels of telomerase activity and do not express TERT. Regarding studies of hTR in different tissues and tumors it is concluded that only small amounts of hTR are sufficient for telomerase activity and hTERT protein is the only limiting factor of telomerase activity. Competitive RT-PCR data indicate that most cancer cells contain 15.000-60.000 molecules of hTR and 1-30 molecules of hTERT mRNA [74, 75].

The telomerase catalytic subunits from different organisms are phylogenetically conserved in their reverse transcriptase motifs with other reverse transcriptases [69], but are more related to each other than to other reverse transcriptases and therefore form a distinct subgroup within the reverse transcriptase family [76, 77].

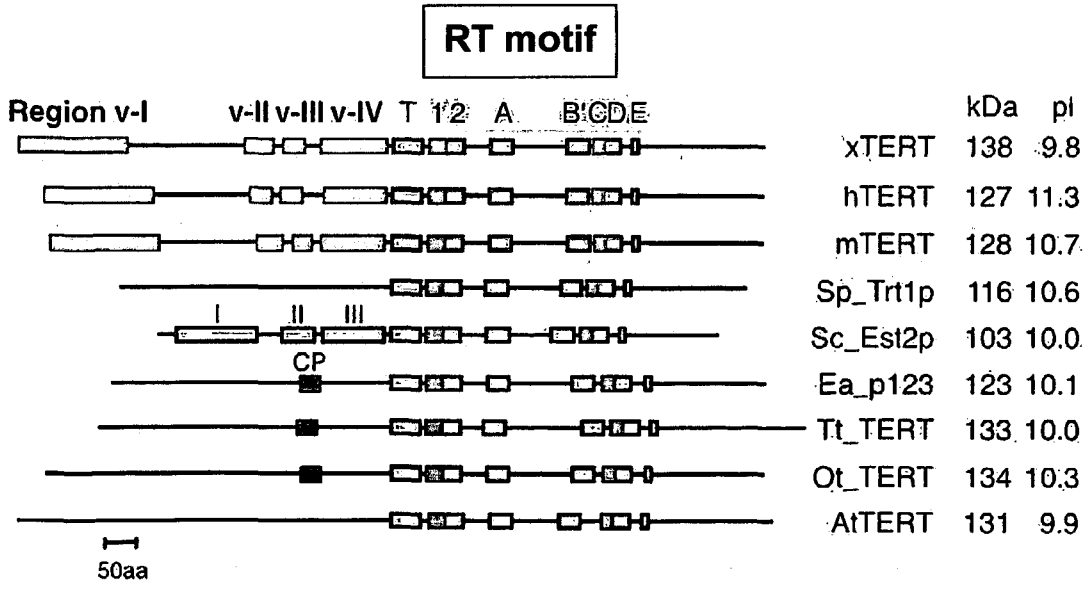

Fig.8. **Conserved regions found in vertebrate TERT amino-terminal halves**

Schematic structures of TERT proteins derived from diverse species (figure 8). Regions v-I, v-II, v-III and v-IV identified in this study to be conserved among vertebrate TERT proteins, as well as motif CP previously identified as ciliate TERT specific motif [78] are shown. The region I, II and III in Sc_Est2p were identified by the random mutation experiment [79, 80].

Several features distinguish the telomerase catalytic subunit: (i) all of the reverse transcriptase motifs are located in the C-terminal half of the proteins; (ii) a conserved telomerase-specific region, termed the T motif, is located just N-terminal to the reverse transcriptase motifs; and (iii) a large N-terminal region contains conserved, functionally important domains [69, 79, 81-83].

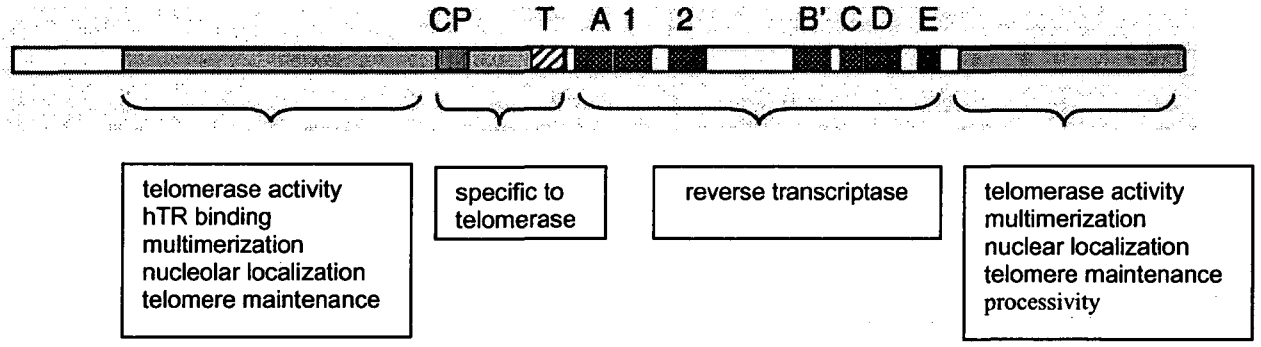

Fig.9. **Schematic architecture of the telomerase reverse transcriptase:** Schematic of some important residues within the telomerase reverse transcriptase [84].

3.2.4 Localization and organization of hTERT gene

The hTERT gene is present as a single copy on chromosome band 5p15.33 (Fig. 10), the most distal band on the short arm of chromosome 5p [85, 86]. The mapping of the hTERT gene to the subtelomeric region led to the speculation that telomere

positional effect may contribute to the repression of hTERT gene expression. Telomere position effect, which results in the reversible silencing of a gene near the telomere, has been well characterized in the yeast S. cerevisiae [87] and was recently observed in human cells [88]. However, the complete genomic sequence of hTERT indicates that the hTERT gene is more than 2 Mb away from the telomere on the short arm of chromosome 5 [89]. This is much farther away from a telomere than previously thought.

The hTERT gene consists of 16 exons and 15 introns and extends over 40 kb [90, 91]. A similar gene organization of telomerase catalytic genes has been reported for *Tetrahymena thermophila* and *Schizosaccharomyces pombe*, with 18 and 15 introns, respectively [78, 92], while the *Oxytricha trifallax*, *Euplotes aediculatus*, and *S. cerevisiae* TERT genes contain no introns [78].

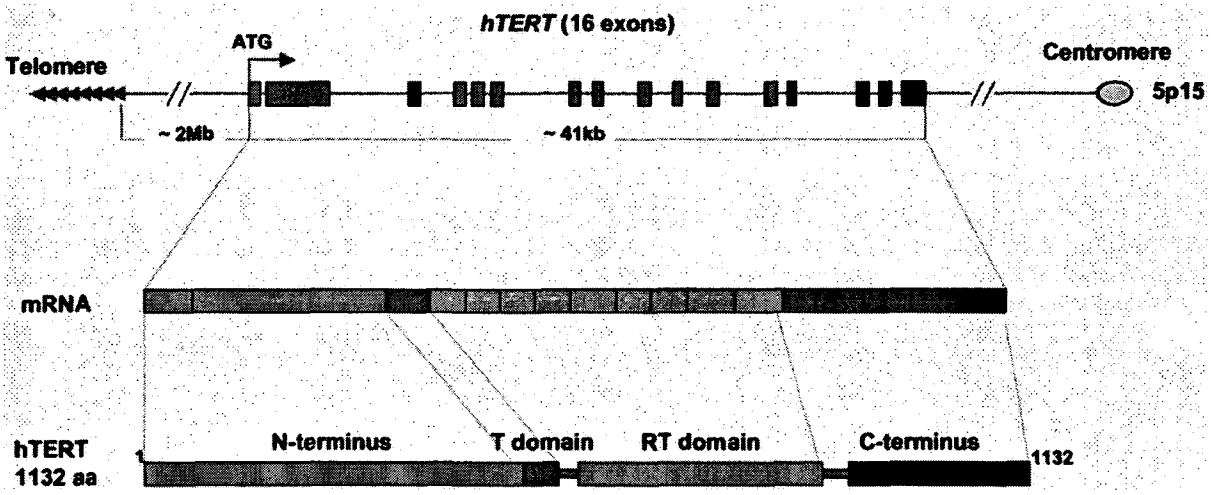

Fig.10 **Gene organization of the hTERT gene**

The human hTERT gene consists of 16 exons and 15 introns located on the short arm of chromosome 5 (5p15.33), approximately 2 Mb away from the telomere (figure 10). It is transcribed towards the centromere. The specific telomerase domain (T

domain), reverse transcriptase domain (RT domain), and the C-terminal region of the hTERT protein are indicated [18].

3.2.5 Telomerase-associated proteins

Telomerase-associated proteins have been cloned from several organisms, including ciliates, yeast and mammals, but as yet there is no conserved set of proteins that appear universally associated with the murine telomerase (TERT and TR) in all organisms [73]. Both biochemical and genetic studies suggest the existence of additional protein subunits of telomerase that may be involved in the biogenesis or assembly of active telomerase and may mediate or regulate the access of telomerase to its substrate, the telomeres (Table 1) [18, 84].

Protein	Interacting region	Function
hTERT associated		
TEP1	aa 1–350, 601–927	Unknown
P23/p90	aa 1–195	Assembly/conformation
14-3-3	aa 1004–1132	Nuclear localization
hTR associated		
TEP1	nt 1–871	Unknown
hGAR1	hTR H/ACA domain	Stability, maturation, localization
Dyskerin/NAP57	hTR H/ACA domain	Stability, maturation, localization
hNOP10	hTR H/ACA domain	Unknown
hNHP2	hTR H/ACA domain	Stability, maturation, localization
C1/C2	nt 33–147	Stability, maturation, localization
La	nt 1–205, 250–451	Accessibility to telomeres?
A1/UP1	nt 1–208	Unknown
hStau	nt 64–222	Accessibility to telomeres?
L22	nt 64–222	hTR processing, localization?

aa, amino acids; nt, nucleotides.

Table1. **Human telomerase-associated proteins[18]**

3.2.6 Telomerase activity in normal human cells and in cancers

About 85% of all human cancers are telomerase-positive, and of the residue some use alternative lengthening and some do not appear to have any mechanisms to maintain telomere length. Some tumors use both, alternative lengthening of telomeres and telomerase to maintain telomere length.

Normal somatic tissues are in general telomerase negative, but some types of normal cells express telomerase activity, including haematopoietic progenitor cells, intestinal crypt cells, endometrial cells and basal layer cells of skin and cervical keratinocytes. The common feature of these telomerase-positive normal cells is their highly regenerative capacity. These cells exhibit continuous or cyclic regeneration throughout human life. Telomerase activity in these cells is tightly associated with cellular proliferation.

For normal somatic cells in vitro, the telomeric restriction fragment (TRF) length progressively declines at a rate of 40 - 200 base pairs (bp) per cell division to 5-8 kb at senescence [93-95]. In normal human cells, telomerase activity appears to be strictly regulated during development [95]. Telomerase activity is extinguished during embryonic differentiation in most somatic cells but remains active in some tissues, such as male germ cells, activated lymphocytes, and certain types of stem cell populations [95-97]. Consistent with the telomere hypothesis, the high proliferative potential of these normal tissues would entail a special need for telomerase to maintain telomere length and genetic stability. Deregulation of telomerase expression has been directly linked to human diseases [98].

The physiological roles of this proliferation-dependent telomerase regulation in normal cells remain unclear. During expansion of haematopoietic cells, telomere length decreases despite the presence of telomerase activity, suggesting that

telomerase activity alone is insufficient to completely prevent telomere shortening in these cells [99]. However, the rate of base pair loss per population doubling decreases in the presence of telomerase activity during expansion, indicating that telomerase activation may slow down the rate of telomere erosion in these cells. Telomerase activation may thus be an adaptive response to protect excessive telomere loss and possibly may help to extend the proliferative life span of highly regenerative cells.

3.2.7 Regulation of telomerase activity

The regulation of telomerase activity occurs at various levels, including transcription, mRNA splicing, maturation and modifications of TR and TERT, transport and subcellular localization of each component, assembly of active telomerase ribonucleoprotein, and accessibility and function of the telomerase ribonucleoprotein on telomeres.

Importantly, among the core components of human telomerase, only the catalytic component hTERT seems to be the limiting determinant of telomerase activity, as other components are usually expressed ubiquitously. In most cases, hTERT expression is closely correlated with telomerase activity, with cancer initiation and progression. It is transcriptionally repressed in many normal cells and is re-activated or up-regulated during immortalization. Substantial experimental data demonstrate that the transcriptional regulation of hTERT expression represents the primary and rate-limiting step in the activation of telomerase activity in most cells [86, 90, 100, 101].

3.2.7.1 Transcriptional regulation of hTERT

Transcriptional regulation of hTERT is thought to be the major mechanism of telomerase regulation. The hTERT promoter is a highly GC-rich, TATA- or CAAT-less promoter. The GC-rich region forms a large CpG island around the ATG [90, 100, 101]. Deletion analyses in reporter assays show that the proximal region of the hTERT promoter is responsible for most of the transcriptional activity. Indeed, the 200-bp proximal region, designated as the hTERT core promoter, contains the basal transcriptional activity of hTERT [101]. In the core promoter, multiple E-boxes and Sp1 binding sites are located. c-Myc binds to these E-boxes through heterodimer formation with Max proteins and activates transcription of hTERT [102-105]. This is a direct effect of c-Myc that does not require de novo protein synthesis. Mad proteins are antagonists of c-Myc and switching from Myc/Max binding to Mad/Max binding decreases promoter activity of hTERT [106-109]. Sp1 is also a key molecule that binds to GC-rich sites on the core promoter and activates hTERT transcription [106]. Co-operative action of c-Myc and Sp1 is required for full activation of hTERT promoter. Over-expression of c-Myc is frequently observed in a wide variety of tumor types, and usually results from chromosome translocation involving the c-Myc genes in addition to gene amplification [110].

A growing number of findings have defined the hTERT gene as an important target for a variety of cellular and viral oncogenic mechanisms. Some of the candidate regulators may represent true "physiological" and "endogenous" regulators to repress or activate the hTERT transcription, but others may not (for review see ref. [111]).

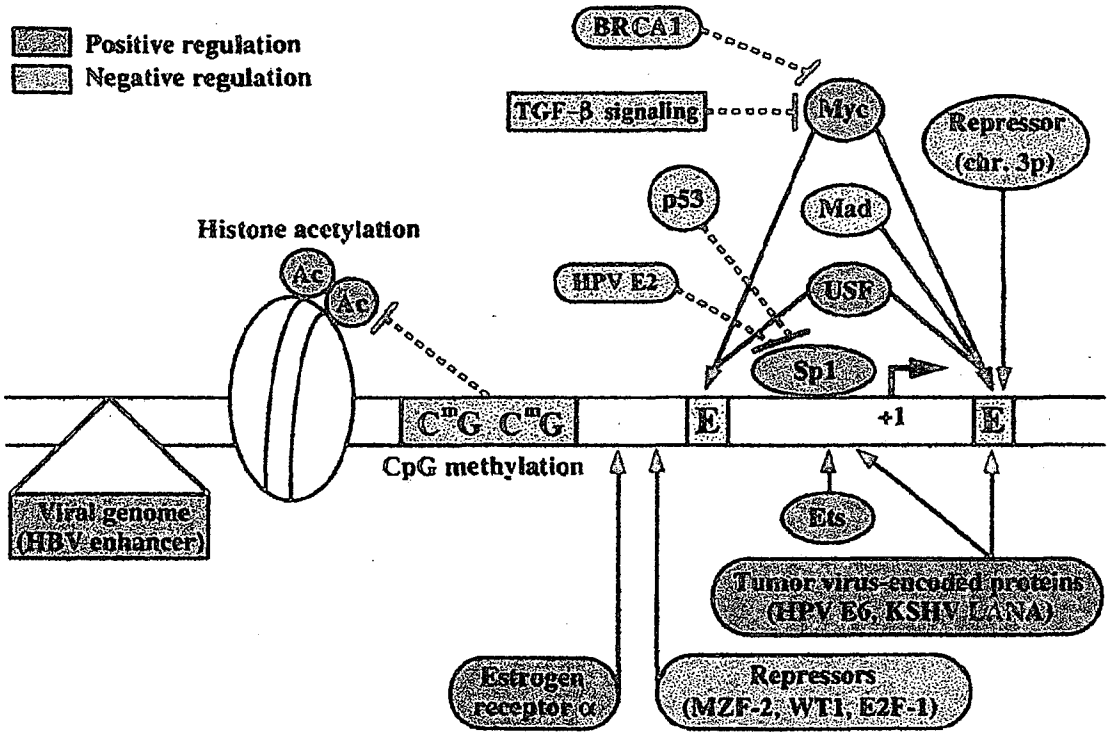

Fig. 11 **Multiple mechanisms of the transcriptional regulation of the hTERT gene**

Schematically shown are various mechanisms that can act on the hTERT promoter to regulate the hTERT transcription (figure 11). Some activators (e.g. Myc) and repressors (e.g. Mad) may function through recruitment of HAT and HDAC, respectively. In addition to its activator function in cancer cells, Sp1 may recruit HDAC to repress the hTERT transcription in normal cells. E, represents two canonical E-box (CACGTG) elements upstream and downstream of the transcription initiation site (+1) [100, 111, 112].

3.2.7.2 Alternate splicing of hTERT

Alternate splicing of hTERT transcripts appears to have at least some role in telomerase regulation. The hTERT transcript has at least six alternate splicing sites (four insertion sites and two deletion sites), and variants containing both or either of the deletion sites are present during development as well as in a panel of cancer cell lines [113]. One deletion (β-site) and all four insertions cause pre-mature translation terminations, whereas the other deletion (α-site) is 36 bp and lies within a reverse transcriptase (RT) motif. However, introduction of splicing variants that contain the α, β or both α and β deletion sites fail to reconstitute telomerase activity in telomerase-negative cells [114], suggesting that alternate splicing may be one mechanism of telomerase regulation. Interestingly, splicing variants that lack the α-site function as dominant-negative inhibitors of telomerase causing telomere shortening and eventually cell death [91, 114, 115].

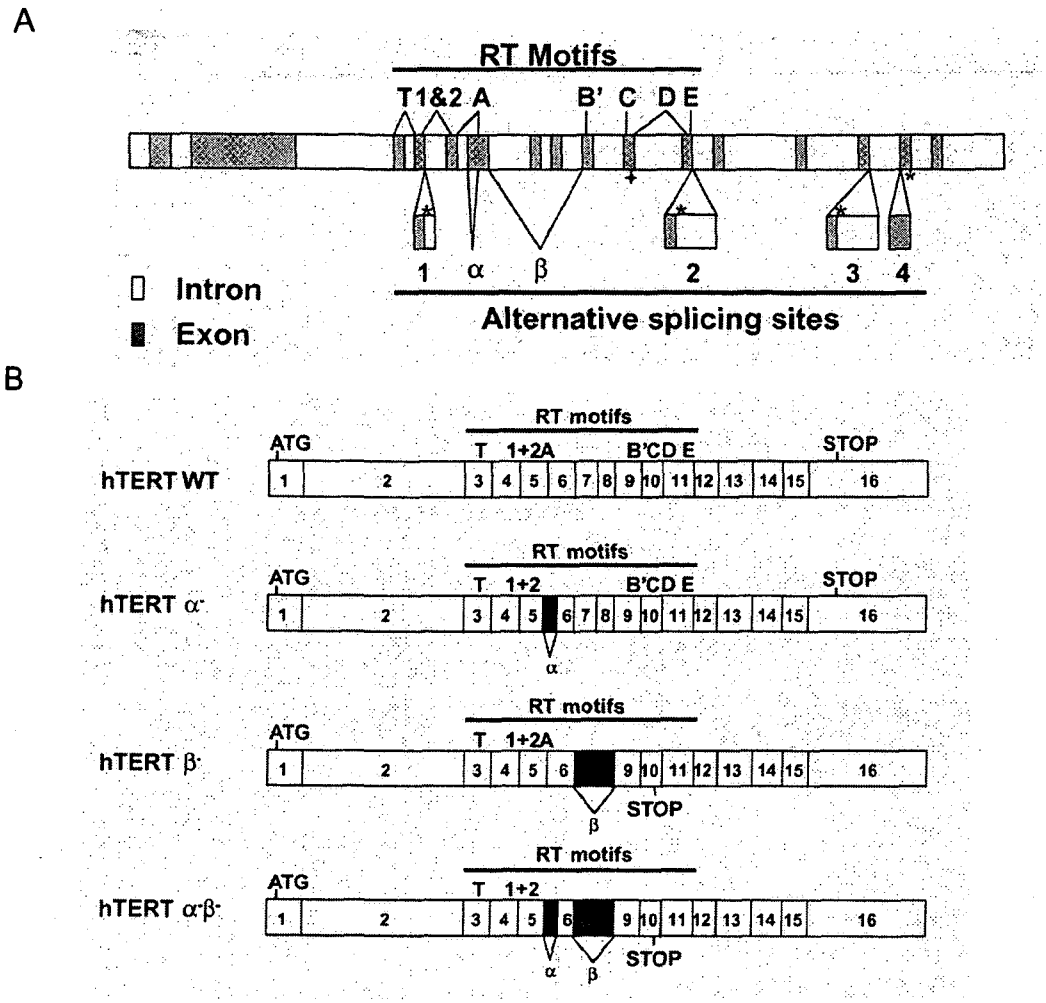

Fig.12. **Genomic organization of hTERT gene**

Alternative splicing sites of the human telomerase catalytic subunit (figure 12) **(A)** Solid boxes are exons; shaded boxes are 5′ UTR or 3′ UTR; the locations of the motifs (T, 1 and 2, A, B′, C, D and E) are indicated on top; and the locations of alternative splicing sites (insertions 1-4 and deletions α and β) are marked on the bottom. The locations of termination codons that correspond to the variants containing insertions are marked with *, and + marks the location of the termination codon for the variant containing deletion β; the deletion α does not cause a frameshift. **(B)** Four possible combinations of hTERT alternative splicing variants containing the deletions.

3.2.7.3 Epigenetic regulation of hTERT transcription

DNA methylation of promoters for certain genes, such as cell cycle regulators and tumor suppressor genes is responsible for the loss of expression of these genes in some cancers. The presence of a large CpG island with dense GC-rich content in the hTERT promoter suggests that DNA methylation and chromatin structure may play a role in the regulation of hTERT expression. However, a generalized pattern of site-specific or region-specific methylation correlating with expression of the hTERT gene has not been identified [116, 117], suggesting that this type of regulation is not a major mechanism involved in telomerase regulation.

Xu et al. and Devereux et al showed that the hTERT promoter was completely or partially methylated in a variety of human normal, immortal, and cancer cell lines. Moreover, treatment of hTERT-negative cells with DNA de-methylating agent 5-AZC (AZC) and histone deacetylase inhibitor TSA induced hTERT expression [109, 116]. These results indicate that hTERT promoter methylation is involved in the regulation of hTERT expression and telomerase activity, at least in some cells [111, 118].

3.2.7.4 Posttranslational modification of hTERT

Phosphorylation of hTERT protein may be one mechanism of hTERT activation. Telomerase activity in human breast cancer cells is markedly inhibited by the treatment with protein phosphatase 2A [119]. Some protein kinases, such as Akt kinase and protein kinase C (PKC) have been reported to mediate phosphorylation of hTERT protein, leading to telomerase activation [120, 121].

Akt protein kinase is involved in activation of human telomerase [120, 122]. Two putative Akt kinase phosphorylation sites within hTERT (protein sequences 220-

GARRRGGSAS-229 and 817-AVRIRGKSYV-826) have been identified. Activated Akt kinase is able to phosphorylate these hTERT peptides and activate telomerase activity in vitro, while the Akt kinase inhibitor Wortmannin inhibits telomerase activity in human melanoma cells in a dose-dependent manner [120].

3.2.7.5 Chaperone-mediated regulation

The Hsp90 chaperone complex, including Hsp90, Hsp70 and p23, is functionally associated with telomerase [123]. In an *in vivo* reconstitution system for telomerase activity, the addition of purified components of the Hsp90 chaperone complex to extracts from cells with weak telomerase activity significantly increased telomerase activity [124].

3.2.8 Telomere maintenance without telomerase – alternative lengthening of telomeres (ALT)

Telomerase activation is not the only mechanism by which cells can stabilize their telomeres. As many as 10% of human tumor-derived cell lines are telomerase-negative and rely on an alternative mechanism of telomere maintenance termed ALT [125-128]. ALT has been identified in about one third of cell lines immortalised *in vitro* using simian virus 40 (SV40), human papillomavirus (HPV) and chemical carcinogens or spontaneously immortalised cells [127].

Telomerase-independent mechanisms of telomere length maintenance also exist in other organisms. Drosophila and mosquitoes use transposable elements to maintain telomeres [129, 130]. In mutant yeast lacking telomerase, survivors use one of two independent pathways of telomere length maintenance that require either Rad50 or

Rad51 [131-134]. Type I survivors contain multiple tandem copies of the Y' element and very short terminal tracts, whereas Type II survivors (10% of total) require Rad50p and have long and heterogeneous telomeres with terminal tracts of 12 kb or longer [133, 134]. The Type II survivors are thought to maintain telomere length in a similar fashion as that found in human cells using the recombination-based pathway of telomere maintenance [135]. Embryonic stem cells derived from telomerase knockout mice can also activate a telomerase-independent mechanism to maintain telomere length [136].

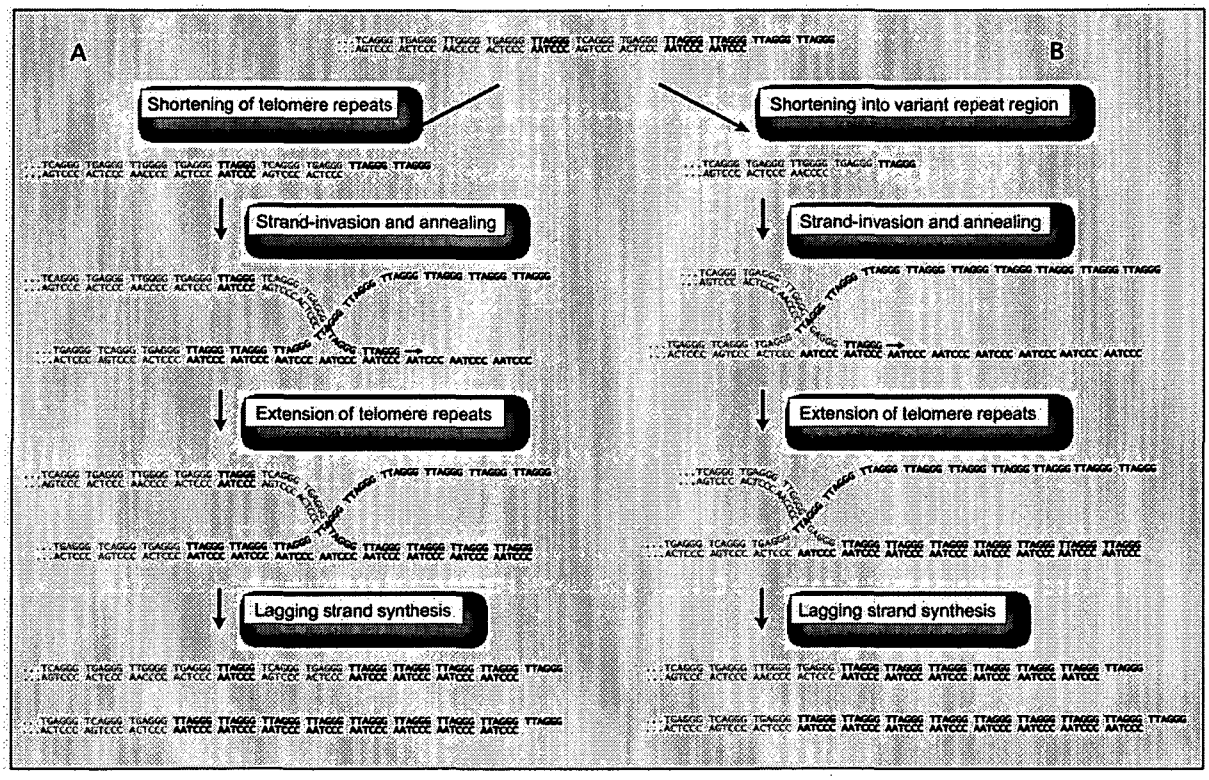

Fig.13. **Recombination-mediated lengthening of telomeres in ALT cells.**

Available data are consistent with a mechanism in which telomeres use other telomeres (or extrachromosomal telomeric DNA) as a copy template (figure 13). **(A)** One strand of a shortened telomere may invade another telomere and anneal with the complementary strand. This acts as a primer for extension of the invading strand using the invaded telomere as a copy template for DNA synthesis, after which the

other strand can be filled in. Evidence for this mechanism was provided by inserting a DNA tag into telomeres of ALT cells and showing that the tag was copied on to other telomeres [137]. **(B)** If telomere shortening extends into the subtelomeric region that contains variant repeat sequences, recombination-mediated lengthening would be predicted to result in replacement of distal variant repeats by TTAGGG repeats. This prediction was confirmed by sequencing subtelomeric regions of cells before and after activation of ALT and escape from crisis [138, 139].

Several characteristics can be used to identify human cells using the recombination-based pathway of telomere length maintenance. First, they have no detectable telomerase activity and lack expression of the catalytic protein component hTERT, or in some cases, they lack both hTERT and the integral RNA component, hTR [140]. Second, cells using the recombination-based pathway to maintain telomeres have very long and heterogeneous telomeres ranging in length from less than 2 kb to 50 kb[141]. Third, they contain extrachromosomal telomere repeats (ECTRs) that may be linear double-stranded fragments of telomeric DNA [142]. Fourth, cells using the recombination-based pathway to maintain telomeres have a novel type of pro-myelocytic leukaemia (PML) nuclear bodies called ALT-associated PML bodies (AAPBs) that contain PML protein, TRF1, TRF2, replication factor A, Rad51, and Rad52 [143]. It is not clear how characteristics associated with the recombination-based pathway of telomere maintenance are generated, and the molecular mechanisms involved also remain unclear.

Two studies in cancer models have suggested that tumors that use alternative lengthening of telomeres may not be fully malignant. When SV40-immortalised human cells that were positive for alternative lengthening were transduced with a mutant *RAS* oncogene, they were unable to form subcutaneous tumors in

immunocompromised animals unless telomerase activity was induced by the exogenous telomerase catalytic subunit (TERT). Consistent with other data suggesting that telomerase contributes more to the malignant phenotype than maintenance of telomere length alone, a mutant TERT that is unable to lengthen telomeres but presumably retains other functions was also able to render the cells tumorigenic. In addition, the TERT-transduced cells had a significantly increased growth rate under adverse cell-culture conditions [144, 145].

3.2.9 Diagnostic and therapeutic consideration

Telomerase may play an important clinical role in assessing the extent of tumor margins. Biopsy specimens from a tumor bed show that telomerase activity was detectable in 10% of tissue areas that were presumed disease-free based on morphologic review. This means that margins that were declared free of tumor may not in actuality be free of tumor. An assay for telomerase could theoretically provide a molecular way of determining margins, and thus identifying patients who are at increased risk for local recurrence [146].

The emerging understanding of the role of telomeres and telomerase in the pathophysiology of human cancer indicates that strategies directed against telomeres and telomerase hold much promise for both diagnostic and therapeutic uses. In particular, the restricted expression of telomerase and hTERT make this molecule an especially attractive target for intervention. However, exploiting telomere biology for these uses will require integrating the evolving understanding of the multiple roles of telomeres and telomerase in the physiology of normal and malignant cells.

Functional characterization of telomerase in rat and human cells

Implication	Findings	Implications
Diagnostic		
Lung cancer	The telomerase activity in bronchoalveolar lavage fluid has a higher sensitivity (78% vs 65% for cytology).[51]	Telomerase assays might play a potentially useful adjunct role in noninvasive screening and diagnosis of cancers.
Breast cancer	The telomerase activity in breast fine-needle aspirates has higher sensitivity (86% vs 70% for cytology) [53] and is detectable in stage 0-I cancer cells.[52]	
Colon cancer	Telomerase is detected in the intestinal lavage fluid of patients (58%) with colorectal carcinoma.[54]	
Other cancers	Telomerase is expressed in most cancers of urinary bladder (90%), prostate (80%), and kidney (69%);[55] thyroid (82%);[56] *pancreas (95%);[57] liver and biliary system;[59,61] and uterine* cervix.[63]	
Therapeutic		
Lung cancer	Treatment with ceramide can inhibit telomerase activity in human lung adenocarcinoma cell line.[94]	Telomerase inhibition might be useful in the treatment of various cancers with telomerase-positive cells.
Breast cancer	Immortalization of breast epithelial cells from women with Li-Fraumeni syndrome (which immortalize spontaneously) is reduced by treatment with antitelomerase agents.[78]	
Colon cancer	No data available.	
Other cancers	Treatment with sodium butyrate and antisense phosphorothioate oligonucleotides can reduce telomerase activity and proliferation of human liver and prostate cancer cells.[82,95]	

Table 2. **Diagnostic and Therapeutic Implications of Telomerase and Telomerase Inhibition on Major Human Cancers:** for review see [147].

3.2.10 References to introduction and Discussion

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4. THESIS OBJECTIVES

The observation that more than 85 % of tumor express telomerase has led to two hypotheses: (i) telomerase activity is necessary for the proliferation of cancer cells; and (ii) telomerase expression provides an excellent tool for the diagnosis, prognosis, and treatment of cancer with specific inhibitors and thus provides a powerful strategy for cancer chemotherapy. Telomerase detection in cells derived from breast fine needle aspirates, bronchial washes, and pancreatic juices show high sensitivity and specificity for cancer detection. In tissue samples, the level of telomerase activity is a useful prognostic indicator in certain adult cancers such as gastric and colon. Immunohistochemical detection of hTERT will facilitate exact diagnosis of the telomerase positive cells and expand the application of telomerase in cancer diagnosis. Several experimental studies reported are very promising and show that telomerase exactly fulfills the requirements for a good diagnostic marker (i.e. urinary bladder carcinoma). Telomerase is considered also as a potential target of cancer therapy. In particular, malignant gliomas are one of the best candidates for telomerase-targeted therapy. It is because malignant gliomas are predominantly telomerase-positive, while normal brain tissues do not express telomerase.

In view of the current interest in developing anti-cancer drugs directed against telomerase, it is of particular importance to analyse the individual tumors with regard to their respective telomere maintenance mechanism. For cancer cells the maintenance of telomeres is a pre-requisite for unlimited proliferation potential. In the majority of human malignancies this is achieved by re-activation of the catalytic component of telomerase, hTERT.

(1) As the field of telomerase research in rather young and controversial reports appear in the literature I first was interested to summerize all aspects about targeted inhition of human cancer in a review.

(2) I investigated telomerase activity and telomere lengths in a series of rat cell lines constitutive and/or inducible expressing various viral and cellular oncogenes. Transformation of embryonic rat cells (RECs) with c-myc and c-H-ras is accompanied by increased telomerase activity, which is clearly dependent on the level of c-Myc protein. Mad proteins play a role as antagonists to c-Myc in the Myc/Max/Mad network regarding cell proliferation, growth, apoptosis, and differentiation. Since the Myc and Mad proteins are expressed during different growth conditions and have opposite effects on cell proliferation, it has been suggested that they constitute a molecular switch for proliferation versus growth arrest and/or differentiation. To study the effects of Mad1 expression on tumor cell outgrowth and aggressivity of tumor cells transformed cell lines were generated by co-transfection of c-myc, c-Ha-ras, and a chimeric mad1ER construct into RECs (MRMad1ER cells). These cells were used to study the effect of Mad1 activation on proliferation of the tumorigenic cells *in vitro* and *in vivo*.

(3) We were interested in the effects of exogenous hTERT on telomerase activity, processivity and telomere elongation in a telomerase- and ALT-negative background and transfected a hTERT gene into a suitable human lung cancer cell line, MSPG. It was the aim of my study to investigate whether a hTERT expression vector could be of benefit for TA and/or telomere elongation in TA-negative cells and how far telomerase-negative cancer cells might benefit from the introduction of transgenes involved in telomerase regulation. Therefore, I determined the telomerase activity (TA) in a large series of different human

cancer cells lines and developed a telomerase detection assay based on a modified TRAP assay. In order to determine the underlying mechanism for the lack of TA in the investigated human lung cancer cell lines, I performed RT-PCR for the RNA component of telomerase, hTERC, and the various splice variants of the catalytic component of telomerase, hTERT.

(4) We further were interested in cloning the complete rat TERT cDNA and rat TERT promoter to perform expression studies. For this purpose we employed *in silico* analysis and established an RT-PCR assay to measure rat TERT and TERC expression levels.

5. RESULTS

REVIEW

5.1 TARGETED INHIBITION OF TELOMERASE IN HUMAN CANCER: WILL IT BE A DOUBLE-EDGED SWORD?

Short title:

Telomerase and cancer

Key words:

telomeres; telomeric repeats; telomerase; telomerase in cancer; hTERT; Myc; immortalization; ALT;

5.1.1 Zusammenfassung

Mehr als 80% der menschlichen Malignome exprimieren das Enzym Telomerase, während normale Zellen im allgemeinen Telomerase-negativ sind. Bei jeder normalen Zellteilung erfolgt ein Verlust von DNA-Sequenzen an den Enden der Chromosomen, eine Folge des "End-Replikation-Problems" von DNA-Polymerasen. Eine kritische Verkürzung der Telomere führt schließlich zum Stopp im Zellzyklus und zum Zelltod. Telomerase, ein Ribonukleoprotein-Komplex mit einer RNA (TERC) und einer katalytischen Untereinheit (TERT) als Hauptbestandteile, kann an die äußersten Enden der Chromosomen Telomer-Sequenzen ansynthetisieren. Die Aktivierung der Telomerase scheint daher ein massgeblicher Schritt in der Tumorentstehung zu sein. Tatsächlich führte die Transfektion von TERT-Konstrukten in verschiedenen humane Zellarten zu Telomerverlängerung bzw. Stabilisierung und, bemerkenswerterweise, zur einer Immortalisierung der Zellen. Umgekehrt bewirkte, zumindest in den ersten *in vitro* Versuchen, die experimentelle Hemmung der Telomerase einen Wachstumsstillstand von transformierten Zellen. Diese Erfolge lassen eine zukünftige medikamentöse Hemmung der Telomerase als eine aussichtsreiche, neuartige Krebstherapie erscheinen. Es gibt jedoch einige berechtigte Bedenken bezüglich ihrer generellen Anwendbarkeit. Sie beruhen auf der Tatsache, dass (i) in 20% der humanen Tumoren die Telomerlängen durch einen Telomerase-unabhängigen, alternativen Mechanismus konstant gehalten werden, (ii) in manchen Tumoren die Telomerlängen im Laufe der Zeit unvorhersehbar variieren, und (iii) ein überexprimiertes c-Myc Onkogen, wie es in einer Vielzahl von Malignomen gefunden wird, die Telomerase aktiviert. Dies könnte sehr leicht den tatsächlichen Telomer-Erhaltungsmechanismus in Tumorzellen verschleiern. Die unberechenbare Dynamik der Regulation von Telomerlängen

schränkt daher den generellen Einsatz einer zukünftigen Anti-Telomerase-Therapie einigermaßen ein. Durch entsprechend umsichtige Analysen von Tumorgewebe sollten überzogene Erwartungen aber auszuschließen sein.

5.1.2 Summary

More than 80% of human malignancies express telomerase activity, while normal somatic tissues in general lack it. During each normal cell division, there is a constant loss of DNA sequences at chromosomal ends, which is due to the "end-replication problem" of conventional DNA polymerase. Critical shortening of telomeres induces cell cycle arrest and eventually cell death. Telomerase, a ribonucleoprotein complex with a RNA (TR) and a catalytic subunit (TERT) as core components, is able to add reitineratedly telomeric repeat sequences to the very ends of chromosomes. It was suggested that activation of telomerase in tumor cells has a major impact on their continuous growth. Indeed, transfection of TERT-constructs into various normal human cell types led to telomere elongation or stabilization and, most importantly, cellular immortalization. Conversely, inhibition of telomerase in tumor cell lines induced growth arrest, at least in first experimental settings. Such initial success implies that drug-mediated abrogation of telomerase action might be an ideal adjuvant treatment for cancer patients. There are, however, legitimate concerns about the generalization of such an approach. They origin from the facts that (i) in 20% of human tumors telomere lengths are maintained constant by a telomerase-independent, alternative mechanism, (ii) in some tumor cell lines, telomere lengths fluctuate with time with puzzling dynamics, and (iii) over-expressed c-Myc oncogene, an obligatory finding in tumors, activates telomerase which could well mask the actual telomere lengthening strategy of a tumor cell population. This bewildering versatility of telomere length regulation limits the aptitude of a future telomerase-based treatment as a straightforward concept against cancer. However, appropriate analysis of the tumor cells by the clinician prior to treating the affected patient should help to avoid unreasonable expectations.

5.1.3 Introduction

In the last years several promising therapeutic approaches evolved from comparative molecular biological analysis of tumor cell deregulation *in vivo* and in vitro. Despite a diversified genetic history reflected by distinct biological features of a given cancer, there are six essential cellular circuits, which have to become deregulated in a normal cell in order to endow autonomous, aggressive and unlimited growth: (i) independence of replication toward exogenous growth factors, (ii) insensitivity toward growth arrest signals, (iii) resistance toward induction of apoptosis, (iv) unlimited lifespan, (v) neo-angiogenesis, and (vi) cellular mobility and growth in foreign tissue (1). Each of them might serve – at least theoretically – as target for more tailored cancer therapies and severe endeavours are currently made to find or create molecules that interfere with each one of them. Above all, the unlimited growth potential of tumor cells appears a most attractive target since its regulation is based on one component of the "hard-ware" of a cell, the telomeres, while the other five features involve cellular signalling pathways, components of the complex cell "soft-ware".

5.1.4 Mechanism of finite life span

Almost four decades ago Hayflick and Moorhead were the first to report on the limited *in vitro* growth potential of human embryonic cells explanted from various tissues (2). They found that after a period of logarithmic growth, mitotic rates of the cultures gradually declined and finally ceased. This phenomenon, termed replicative senescence, is the final fate of normal human cells in culture and has been confirmed by many laboratories and with many different cell types. No spontaneous

escape from senescence and eventual cell death has ever been observed with human cells. The mechanistic correlate for the postulated mitotic clock, which controls lifespan and can be retarded (f.i. by transfection with SV40) but not switched off in human cells, was found to reside in the ends of chromosomes, the telomeres.

5.1.5 Function and structure of telomeres

The study of telomeres, the ends of linear eukaryotic chromosomes, has revealed important roles of these structures in many cellular processes (for review see 3). Failure of any of them could lead at best to cell cycle arrest and cell death or at worst to genetic instability, a possible prelude to malignant cell transformation. Telomeres and their associated proteins are specialized nucleoprotein complexes that serve for protection, replication and stabilization of chromosomal ends. These complexes bundle and anchor telomeres in the nuclear matrix near nuclear pores and protect against exonucleases, prevent chromosomal fusion and illegitimate recombination and play an important role in chromosome separation during mitosis. Although they represent, at least formally, a DNA double strand break, telomeres are not recognized by the DNA damage recognition systems.

In most organisms studied, telomeres consist of 6-8 bp short, monotonously repeated DNA sequences, composed of a G-rich strand and the complementary C-rich strand. The simple repeat $(5'\text{-TTAGGG-}3')_n$ of telomeres is highly conserved from unicellular organisms to men and is identical in all vertebrates. There is, however, a broad variability in the number of repeats ranging from a few as in ciliates up to several thousands in vertebrates (for review see 3).

Telomeres are generally subdivided into three distinct areas: (i) telomere associated sequences (TAS), (ii) double stranded telomeric repeats, and (iii) 3´-overhangs. TAS are complex arrays of various repeated elements, including telomeric ones (4) and can represent a convenient source of supply for telomeric sequences in case when telomerase is absent, as telomeres can be maintained via recombination or transposition (5; and see below).

5.1.6 The "end-replication-problem" and ageing

The most obvious function of telomeres is to provide an abundant, yet limited reservoir of non-coding DNA sequences for the "end replication problem" of DNA synthesis. This is due to the need of known DNA polymerases for a transient RNA primer to start replication and their inability to completely replicate the ends of a linear template once the most terminal primer has been removed. This results in loss of at least 8 to 12 nucleotides – or even an entire Okazaki fragment of about 200 nucleotides - at the chromosomal ends during each round of DNA replication. Another possibility to create long 3'-overhangs is under discussion: a putative exonuclease might remove stretches of nucleotides (6). In any case, telomeres shorten continuously in the course of cell proliferation if no other compensatory mechanisms are available (7).

The telomere hypothesis proposes that telomere loss represents the mitotic clock, which counts the number of cell divisions. It was observed that with each cell division telomeres shorten by an average of 65 bp (7). Similar loss rates were found with a variety of other cell types and at many occasions. Thus, the amount of available telomeric sequences of the shortest telomere in a cell dictates the number of successful division. Attrition of one single telomere below a critical threshold

length is sufficient to signal cell cycle arrest and induces irreversible senescence and eventually cell death as observed with yeast cells. Due to the heterogeneous composition of chromosomal ends in each cell, size fractionation of telomeres in agarose gels yields a "smear" of telomeric DNA.

5.1.7 Function and composition of telomerase

Telomerase is a ribonucleoprotein complex with a RNA (TR) and a catalytic subunit (TERT) as core components, which synthesizes de novo telomeric, repeats onto the very ends of chromosomes. With its reverse transcriptase activity it represents a highly specialized RNA-dependent DNA polymerase as it synthesizes only one sequence. The RNA subunit has been determined for various ciliates, fungi, mouse and human (8-10). It is surprising that despite the highly conserved function of telomerase in eukaryotes there is – apart from the template region - little similarity among the various RNA sequences. This suggests that the secondary structure of RNA might be more conserved (11). Indeed, *in vivo* reconstitution of chimeric telomerase with TR and TERT from different species revealed functional enzyme complexes (12).

While the genes for the RNA component have been detected readily in various species on the basis of known template sequences, search for the genes coding for the catalytic components turned out to be difficult. It was only in 1997 when the catalytic subunit TERT (telomerase reverse transcriptase) of human telomerase was eventually identified (13,14). Comparison of the human and murine protein with the catalytic subunits of unicellular eukaryotes and other reverse transcriptases (RT) revealed seven conserved protein domains that are part of the active site of the enzyme and one additional telomerase specific domain called the T-motif (13, 15).

Telomerase activity is usually determined by a more or less modified TRAP assay (Telomeric Repeat Amplification Protocol), originally developed by Kim et al. (16). Interestingly, the RNA component is present in the majority of human cells and tissues. However, since normal somatic human cells do not express constitutively the catalytic component, they lack telomerase activity.

5.1.8 Telomerase-induced immortalization

Since hTERT protein is the only limiting factor of telomerase activity, the question arose whether exogenous, over-expressed hTERT could extend or abrogate the limited lifespan of otherwise mortal human cells. Bodnar et al. succeeded indeed in immortalising human retinal pigment epithelial cells as well as human foreskin fibroblasts, both initially telomerase-negative (17). Similar results were obtained with some other human cell types (18-20). It should be emphasized that the cell strains used were mostly of adult origin, whereas human embryonic cell strains in similar experimental settings appeared rather reluctant toward immortalization. In IMR90 embryonic lung fibroblasts, a hTERT construct readily induced telomerase activity, but failed to prevent progressive telomere shortening and senescence (18). Constitutive expression of hTERT appears therefore not a universal and straightforward strategy for immortalizing human cells in vitro.

Remarkably all the cell strains immortalized by exogenous hTERT retained normal growth control and did not show any characteristics of a transformed phenotype (19, 20). This finding is in accordance with the fact that transformation of human cells is a multistep process. Only very recently the group of Weinberg succeeded in transforming human cells using a combination of hTERT, SV40 large T antigen and

the oncogenic Ras (21). This was the first report on achieving controlled cellular transformation by a defined set of genes.

5.1.9 Protection of chromosomes by telomerase

In addition to its telomere-lengthening function, telomerase affects chromosomal ends in a way that differs from the well-understood synthesis of telomeric repeats. It was recently observed that co-expression of hTERT and SV40 in human fibroblasts induced telomerase activity and prompt immortalization without any net increase of telomeric sequences. Contrary to expectation, telomeres continued to shorten progressively for more than 40 doublings - well beyond the crisis point - despite presence of telomerase. Since the enzyme activity was necessary for efficient immortalization without counterbalancing the telomeric loss, a telomere capping function of telomerase was postulated that protects eroded chromosomes from fusion (22). Such a postulated protective function of telomerase during immortalization is reminiscent of puzzling telomerase activation in normal organs with highly proliferative cellular compartments such as skin, endometrium, intestinum and lymphoid system (23-25). If the observed up-regulation of telomerase in normal cells, which are enforced to divide, exerts a similar function, then, drug-mediated inhibition of telomerase will definitely cause harmful side effects, probably identical to those experienced with conventional chemotherapy.

5.1.10 Regulation of endogenous telomerase activity

It is obvious that an enzymatic activity, the de-regulation of which represents a definitive danger for the entire organism, must be tightly regulated at several levels.

Among the supposedly large number of factors involved in the regulation of telomerase activity, a few of them have already been characterized. Two human telomeric repeat factors, TRF1 and TRF2, have been isolated and shown to play a decisive role in telomere maintenance. TRF1 is involved in telomere length regulation by a negative feedback mechanism: over-expression results in shortened telomeres while loss of functional TRF1 causes telomere elongation (26). The action of TRF2, which is structurally similar to TRF1, appears quite opposite since loss of TRF2 shortens telomeres and causes telomere-telomere fusions, leading to cell cycle arrest and cell death (27). Very recently, electronmicroscopic studies of telomeric structures deciphered the mechanistic action of these two proteins. The 3'-overhang is folded back and invades upstream the duplex telomeric DNA, obviously by aid of TRF2. This, in turn, leads to an out-looping of most or all telomeric DNA, sized by TRF1 and by support of TIN2, the currently youngest member of the telomere-associated protein family (28). The sizes of telomeric loops correspond to the telomeric length of species, with large loops of murine telomeres and smaller one of human telomeres (29).

As seen with many other essential genes, the transcriptional regulation of the TERT gene is likely of similar complexity. Therefore, a major breakthrough was the recent finding that Myc proteins target the TERT promoter. The members of the Myc family play a prominent role in control of cell proliferation. Upon hetero-dimerization with Max, Myc/Max proteins bind to a recognition site, termed E-box (3'-CACGTG-5'), and are acting as potent transcription factors (for review see 30). Introduction of a Myc-construct into IMR90 fibroblasts and mammary epithelial cell yielded high telomerase activity (18). In a study with murine cells, c-Myc and m-TERT were found concordantly regulated during erythroleukemia differentiation (31). Conversely, antisense oligonucleotides of c-Myc were able to suppress telomerase activity in

three formerly telomerase-positive leukemic cell lines (32). Even though activation of hTERT through c-Myc contributes to the immortalizing effects of c-Myc, hTERT is not able to substitute c-Myc as an immortalizing agent (31). IMR90 fibroblasts were readily immortalized by an introduced Myc-construct, whereas constitutive expression of hTERT failed to do so.

Sequence analysis of the hTERT locus revealed the genetic basis for the link between Myc and TERT expression. hTERT carries altogether a series of 29 possible Myc/Max binding sites, a number exceeding by far that found in other Myc-regulated target genes. Two of the 18 canonical and 11 non-canonical (3'-CAC/TGTG-5') E-boxes are located upstream of the ATG-site of TERT and seem to be the predominant binding sites for Myc/Max transactivation complex (33).

Consistent with its central role in normal cell cycle progression is the fact that up-regulation of Myc genes by mutations, translocation or amplification is a obligatory finding in malignancies. Constitutive expression of c-Myc is driving cells into continuous proliferation. Moreover, in some tumors high expression level of Myc is associated with unfavourable prognosis. The long-known ability of c-Myc to immortalize primary cells indicated that Myc oncogenes might regulate expression of genes that enable cells to bypass replicative senescence. Therefore, the excitement about finding a direct link between Myc- and TERT-activities was reasonable. It obscured, however, the fact that for the control of normal dividing cells appropriate levels of Myc might induce endogenous TERT as well. Indeed at closer inspection, as mentioned above, a variety of proliferating cells and tissues with supposedly high Myc levels showed considerable telomerase activity. With regard to anticipating a targeted inhibition of telomerase in cancer cells, the question of overriding importance is whether in normal proliferating cells up-regulation of telomerase by Myc is an inadvertent side effect or an essential prerequisite for repeated cell cycle

progression. If the latter is the case, abrogation of telomerase function will definitely be harmful to treated patients.

5.1.11 Maintenance of telomere lengths in absence of telomerase

As observed in a variety of experimental systems and the majority of human malignancies, the main mechanisms to counterbalance the continuous loss of telomeric sequences is, of course, re-activation of telomerase. However, this is not the unique strategy for maintaining telomere lengths constant. It was shown that about 40% of *in vitro* immortalised human cell lines and a smaller fraction of human tumors showed no detectable telomerase activity. Nevertheless, these tumor cells had stable and sometimes even abnormally long telomeres (34). From these data it is clear that apart from telomerase re-activations other

mechanisms, which are telomerase-independent, must exist as well. Due to the lack of knowledge on their nature or regulation, they were named collectively and tentatively "alternative lengthening of telomeres"-mechanism (ALT) (35). The ability of cells to use ALT mechanism(s) instead of telomerase appears to reside in some tissue specific traits. Mesenchymal tumors were much more often found telomerase-negative than cancers originating from epithelial cells. Although the precise nature of the ALT mechanism(s) is currently unknown, it was assumed that recombination and retro-transposition might be involved and an attractive model for recombination-mediated lengthening of human telomeres was suggested recently by Reddel et al. (34).

5.1.12 Telomere lengths and telomerase in tumors

From 1994 on, when Kim et al. (16) have provided a first survey on telomerase activity in various human malignancies, the number of publications on this topic has been increasing exponentially. In the meantime almost all types of human malignancies were analysed for telomerase activity, often accompanied by determination of telomere lengths, and in general, about 80% of human tumors were found telomerase-positive (for review see 36). Due to space limit for this article, only a short summary of the most important findings is provided. What can be inferred from all these studies is that in many tumor types there is a clear correlation between progression of disease and telomerase activity both, quantitatively and qualitatively. Classical examples for a parallel increase in the number of telomerase-positive tumors and disease stage are colon and cervical carcinoma and melanoma, with a few telomerase-positive tumors in early stages and almost 100% in late disease. In some tumor types there is also a clear correlation between the extent of telomerase activity and tumor stage as examplified by neuroblastoma. Thus, determination of telomerase in a given tumor sample or small tumor specimen such as exfoliated tumor cells can materially contribute to improve diagnosis and prognosis. In contrast to successful correlation between enzyme activities and cancer stages, attempts to relate telomere lengths to telomerase activity yielded contradictory results. Moreover, some of the studies revealed unexpectedly high degrees of telomere length variation between parallel cultures and dynamic changes in telomere length during continuous growth (37). Several theoretical considerations have been raised to explain the disadvantageous versatility of telomeres, yet none of them has been proved vigorously. At the moment, these puzzling findings are

hampering to a certain extent the concept that drug-mediated inhibition of telomerase will cause telomere shortening in a straightforward manner.

5.1.13 Possible targets for inhibiting telomerase activity

At present, data on the effects of telomerase inhibition on the growth of human cancer cells are scarce. In principle, three potential targets can be envisaged: the RNA component RT, the catalytic subunit TERT and regulating proteins.

In one of the first studies, an antisense construct, directed against the RT component of telomerase, was transfected into HeLa cells and subclones were investigated with regard to their proliferation potential. Several, but not all of them, ceased growing after 23 to 26 generations. A number of other studies applying antisense constructs to tumor cell lines have been published but unmodified oligonucleotides turned out to be too leaky or unstable, since in none of the cases faithful abrogation of telomerase was achieved. To some surprise, a modified RT-antisense oligomer, 2'-O-methyl-RNA (2'-O-meRNA), was able to inhibit the RNA component of telomerase in a sequence-selective way and more efficiently than the corresponding DNA, complementary RNA and analogous peptide nucleic acids (38). Very recently further support for this promissing approach was provided by Herbert et al. (39). They reported on the growth potential of a series of 2'-O-meRNA-treated tumor cells lines, which have been selected on basis of their differently long telomeres. Upon treatment and in accordance with their initial telomere lengths, tumor cells ceased growing with time. Interestingly, the effect was reversible since ommitment of the inhibitors from culture medium restored both, telomerase activity and telomere lengths. Further modifications of such drugs, which f.i. might facilitate their cellular up-take, are awaited with great interest.

Another way to inhibit telomerase activity is to target the TERT component by reverse transcriptase inhibitors. Strahl & Blackburn (37) were able to induce progressive telomere shortening in malignant leukemic cells by synthetic peptides, yet the cells continued growing in presence of the drugs and despite eroding telomeres. Although limited data are available for drug-mediated TERT inhibition, it appears that affection of the TERT component by tailored chemicals is not that successful. Perhaps transfection of dominant-negative TERT DNA constructs into human tumor cell lines might reveal the expediency of the catalytic subunit of telomerase as target. Although extension of such an experimental approach to human cancer will be hampered by the well-known boundaries of gene therapy in men, it would definitely provide important information.

An ideal gene-based strategy for inactivating telomerase in human malignant cells will certainly be natural inhibitors. Transfer of whole chromosome-3 or subchromosomal fragments into telomerase-positive cells abolished very efficiently hTERT expression in the majority of hybrid clones, leading to senescence and cell death (40). Cloning and characterization of the gene(s) will only be a matter of time.

5.1.14 Prerequisites for targeted telomerase inhibition in cancer patients

Patients with minimal or residual disease will probably benefit most from a drug-targeted inhibition of telomerase, but it is quite conceivable that more advanced diseases might be successfully treated as well. There are, however, several prerequisites for its application to cancer patients. A major part concerns the tumor cells to be treated: (i) tumors must, of course, be telomerase-positive, (ii) the tumor cell telomeres must be short, at best 2-3 Kb. The tumor will anyway initially continue

to grow and the space until a critical attrition of telomeres will be achieved depends on the initial telomere lengths. This time must be sufficiently short or otherwise the patient will not benefit from therapy. (iii) c-Myc expression should be low. It is conceivable that high Myc expression might counterbalance the aspired inactivation of telomerase. (iv) p53 of tumor cells should be wild type. As observed with telomerase-negative mice and in several experimental cell systems transfected with DNA tumor viruses, abrogation of p53 function prolongs the lifespan of cells for several generations. (v) ideally, an alternative telomere lengthening mechanism should be excluded. Due to the current lack of insight into this/these mechanism(s) it is difficult to imaging how this could be preformed in a practical way. However, recent studies with telomerase-inhibitors in culture have revealed that the frequency with which ALT is activated upon inhibition of telomerase is neglectably low.

5.1.15 Conclusion

With regard to all the enigmas circling around telomerase regulation, it is obvious that actual application of telomerase-inhibiting drugs to patients will still take some time. The idea per se is, however, so attractive that major efforts towards realization of such a new therapeutic concept are undertaken by many universities and much better funded private companies. One of the main obstacles, I believe, is to find suitable *in vivo* models. The well-characterized rodents, which are supplied with ultra-long telomeres and less stringently regulated telomerase, appear of limited help in this respect. If appropriate multicellular organisms with convenient telomere lengths and human-like telomerase regulation were found and all, or at least most, of the current uncertainties removed by compelling evidence, than clinicians will be

challenged. The immediate goal is now to investigate some of the issues raised above so that drug-mediated telomerase inhibition may one day be realised.

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5.2 REPRESSION OF IN VIVO GROWTH OF Myc/Ras TRANSFORMED TUMOR CELLS BY Mad1

Running title: Repression of tumor growth by Mad1

Key words: Mad1ER; Myc; cell cycle; gene repression; telomerase activity; tumor growth

5.2.1 Abstract

The Myc/Max/Mad network of transcriptional regulatory proteins plays an essential role in cell proliferation, growth, apoptosis, and differentiation. Whereas Myc proteins affect cell cycle progression positively, Mad proteins are negative regulators of cell proliferation. It has been shown in several *in vitro* systems that Mad proteins antagonize c-Myc functions. In this report we describe the inhibition of tumor cell outgrowth *in vivo* by Mad1 expression. Transformed cell lines were generated by co-transfection of c-myc, c-H-ras, and a chimeric mad1ER construct into primary rat embryo cells (MRMad1ER cells). Activation of Mad1 by 4-Hydroxy-Tamoxifen (OHT) resulted in abrogation of telomerase activity, reduced cloning efficiency, and decreased proportion of cells in S-phase. Injection of MRMad1ER cells into syngenic rats induced aggressively growing tumors after a short latency period. This tumor growth was inhibited by OHT-treatment of animals, with the extent of inhibition correlating with the amount of OHT injected. No effect of OHT on tumor growth was observed with similarly transformed Myc/Ras cell lines which did not express Mad1ER. These data demonstrate that Mad1 is able to suppress Myc/Ras-mediated transformation under *in vivo* conditions.

5.2.2 Introduction

The development and homeostasis of a multicellular organism is guided by four cellular processes: proliferation, growth (i.e. increase in cell mass), differentiation, and apoptosis. Compelling evidence indicates that the Myc/Max/Mad network plays a key role in the regulation of each of these (for review see Henriksson and Lüscher, 1996; Facchini and Penn, 1998; Johnson et al., 1999; Eilers 1999; Grandori et al., 2000; Lüscher, 2001). The proteins of this network belong to the basic/helix-loop-helix/leucine-zipper (bHLHZip) class of transcription factors. Max plays a central role for network function because of its abundance and its versatility to form homodimers and to associate with Myc (c-, N-, and L-Myc), Mad (Mad 1, Mxi1, Mad 3 and Mad4), Mnt/Rox, and Mga (Blackwood and Eisenman, 1991; Ayer et al., 1993; Zervos et al., 1993; Hurlin et al., 1995; 1997; 1999; Meroni et al., 1997). The different dimers bind to E-box sequences (3'-CACGTG-5') in promoters and regulate transcription (for review see Lüscher and Larsson, 1999; Grandiori et al., 2000; Lüscher, 2001). Binding of Myc/Max contributes to gene activation through the N-terminal transactivation domain (TAD) of Myc whereas Mad/Max and Mnt/Max dimers repress transcription. Myc/Max heterodimers activate a number of genes that are directly or indirectly involved in cell cycle progression (for review see Cole and McMahon, 1999; Grandiori et al., 2000; Amati et al., 2001; Lüscher, 2001). Among the increasing number of Myc-regulated genes, the recently discovered E-box sequences in the promoter of the gene encoding the catalytic component of human telomerase, hTERT (Wu et al., 1999; Greenberg et al., 1999), are of special interest since re-activation of telomerase is an obligatory finding in many human malignancies (for review see Shay and Bacchetti, 1997). It is an attractive hypothesis that overexpressed Myc promotes cellular immortalization at least in part through re-

activation of hTERT (for review see Cerni, 2000). Indeed, myc genes are deregulated in a large number of human tumors and affect both the development and progression of hyperproliferations (see DePinho et al 1991).

Myc stimulates the G1-S-phase transition of the cell cycle by positively modulating cyclin D/CDK (cyclin dependent kinase) and cyclin E/CDK2 complexes and by negatively regulating the CDK inhibitors p21 and p27 (for review see Amati et al., 2001; Lüscher, 2001). Conversely, cell cycle exit and cellular differentiation are usually associated with downregulation of myc. While c-Myc normally is expressed in proliferating cells, Mad proteins are expressed in differentiating and resting cells, and terminal differentiation is closely linked to upregulation of mad genes (Ayer and Eisenman 1993; Larsson et al., 1994, Hurlin et al., 1995; Västrik et al., 1995; Cultraro et al., 1997; Quéva et al., 1998; Pulverer et al., 2000). Mad proteins are negative regulators of cell growth and inhibit the proliferation of a variety of human and rodent cell lines and the transformation of rat fibroblasts by c-Myc/c-H-Ras and other combinations of oncoproteins (Cerni et al., 1995; Chen et al., 1995; Hurlin et al., 1995; Koskinen et al., 1995; Lahoz et al., 1994; Roussel et al., 1996; Roy and Reisman, 1995; Västrik et al., 1995; Quéva et al., 1999; Bejarano et al., 2000; Gehring et al., 2000). It has also been shown that Mad1 expressed from an adenoviral vector is able to inhibit the growth of a human tumor cell line transplanted into immunocompromised mice (Chen et al., 1995). In addition, Mad1 inhibits apoptosis induced in different cell systems and under different experimental conditions (Bejarano et al., 2000; Gehring et al., 2000). Furthermore, granulocytic progenitor cells of mice with a homozygous deletion of mad1 display extra rounds of mitotic divisions prior to terminal differentiation. However the number of mature granulocytes is normal in these animals most likely due to enhanced apoptosis (Foley et al., 1998).

The repressive effect of Mad and Mnt is mediated by their N-terminal mSin3 interaction domains (SID). The mSin3 proteins in turn recruit corepressor molecules and histone deacetylases, and Mad and Mnt are therefore thought to exert their repressing activity at least in part by modification of chromatin structure (Alland et al., 1997; Hassig et al., 1997; Heinzel et al., 1997; Laherty et al., 1997; Sommer et al., 1997). The function of Myc has also been coupled to modulation of chromatin structure through its binding to the TRRAP protein which is part of a SAGA-like complex containing the histone acetyltransferase hGCN5 (McMahon et al., 1998; McMahon et al., 2000; Bouchard et al., 2001). Since the Myc and Mad proteins are expressed during different growth conditions and have opposite effect on cell proliferation, it has been suggested that they constitute a molecular switch for proliferation versus growth arrest and/or differentiation (Ayer and Eisenman, 1993). According to this hypothesis, the abundance of Myc- versus Mad-containing heterodimers determines at least in part whether cells enter a differentiation pathway or remain in a proliferative state.

To investigate the effect of Mad1 on growth of tumorigenic cells we generated c-Myc/c-H-Ras transformed rat cell lines that express an inducible Mad1 protein. These cells were used to study the effect of Mad1 activation on proliferation of the tumorigenic cells *in vitro* and *in vivo*.

5.2.3 Results

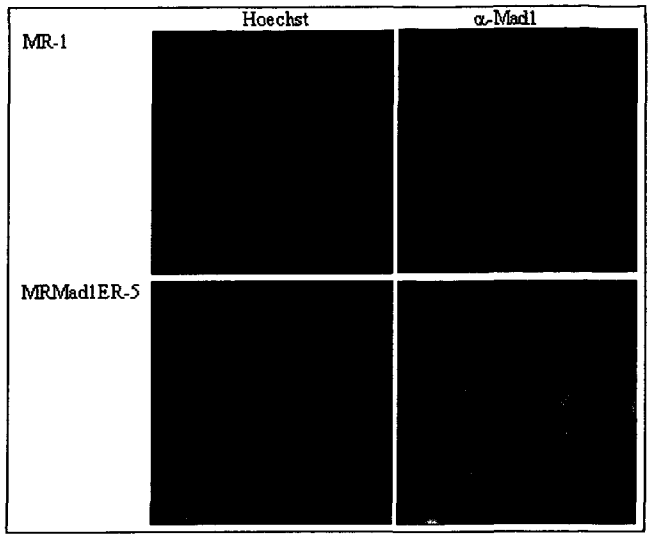
5.2.3.1 Generation and characterization of Mad1ER cell lines

We transfected a construct encoding a chimeric Mad1-estrogen receptor ligand binding domain (Mad1ER) into rat embryo cells (REC) together with vectors

encoding c-Myc (M) and c-H-Ras (R). The generation of transformed REC is strictly dependent on the coexpression of both c-Myc and an activated Ras (Land et al., 1983; Cerni et al., 1995). After G418 selection 50 colonies were picked and screened for Mad1 expression by immunofluorescence. Only two clones, MRMad1ER-5 and MRMad1ER-6, showed Mad1 fluorescence while the remaining 48 clones were negative (Figure 1a, lower panel and data not shown). In contrast, transformed control c-myc/c-H-ras cell lines (MR lines), that were not transfected with the mad1ER plasmid, did not stain with the Mad1-specific antibody (Figure 1a, upper panel and data not shown).

Northern blot analysis revealed that both MRMad1ER clones expressed the chimeric mad1ER mRNA to a similar extent (Figure 1b). For comparison four transformed control cell lines, MR-1, -2, -3, and -6 were concomitantly analyzed. In all four cell lines the expression levels of exogenous c-myc were similar while the levels of ras were significantly lower compared to the two Mad1ER lines (Figure 1b). Similarly, lower levels of ras were observed in 15 additional MR lines (data not shown), suggesting that the enhanced ras expression was specific for the MRMad1ER lines. As in MRMad1ER cells, addition of OHT to the transformed control cell lines altered neither myc nor ras expression (Figure 1b, and data not shown). Western blot analysis demonstrated that the size of the Mad1ER fusion protein, expressed in MRMad1ER cells, was identical to that expressed in transiently transfected COS-7 cells (Figure 1c).

a



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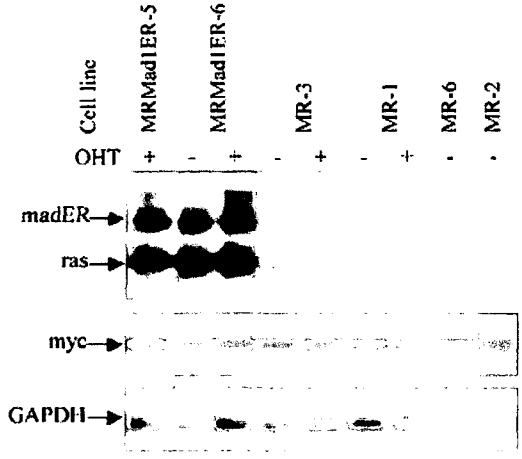

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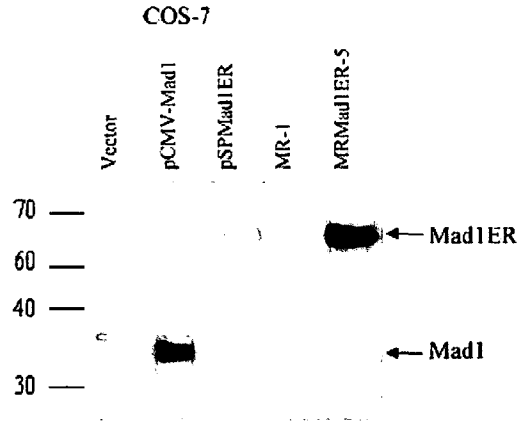


Figure 1 **Expression of Mad1ER and the transgenes in Myc/Ras transformed cells.** REC were transfected with plasmids encoding c-Myc, c-H-Ras, and Mad1ER together with a plasmid conferring neomycin resistance. (a) After G418 selection individual clones were screened for Mad1 expression by immunofluorescence in the presence of OHT. Nuclei were stained with Hoechst. A Myc/Ras transformed control cell line (MR-1) was negative for Mad1 expression (upper panel) whereas MRMad1ER-5 cells stained positive (lower panel). (b) MRMad1ER-5 and -6 cells and four MR cell lines were analysed by Northern blotting using probes for mad1 and c-H-ras (upper panel), c-myc, and GAPDH (middle and lower panel). Where indicated cells were grown in the absence or presence of OHT for 4 days. (c) MR-1 and MRMad1ER-5 cells were grown in absence of OHT and analysed by Western blotting using a polyclonal antibody against Mad1. Extracts from COS-7 cells transfected with CMV

vector, CMVmad1, or pSPmad1ER constructs were used as controls. Mad1ER and Mad1 are indicated.

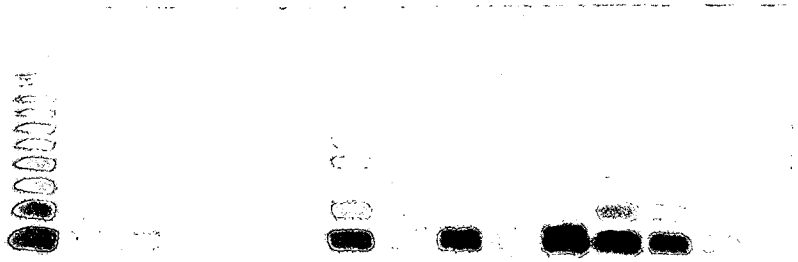
5.2.3.2 Activation of Mad1ER results in repression of TERT activity

In order to examine the functionality of the activated Mad1ER fusion protein we analyzed whether it would repress a known Myc target gene. It was recently found that the 5' region of the human gene encoding the catalytic domain of telomerase, hTERT, contains an array of E-box sequences and that some of these are targeted by Myc/Max and Mad1/Max complexes (Greenberg et al., 1999; Wu et al., 1999; Xu et al., 2001). The TERT protein is the rate limiting factor for telomerase activity (Avilion et al., 1996; Ulaner et al., 1998), and Myc expression has been shown to induce hTERT expression and activity (Wang et al., 1998; Falcetti et al., 1999). Conversely, overexpressed Mad1 represses enzyme activity (Oh et al., 2000). We therefore analyzed the activity of the rat TERT gene in the MRMad1ER-5 cells before and after OHT induction. Although the locus of the rat TERT is not characterized at present, conserved regulation of TERT genes by c-Myc can be deduced from the identical localization of relevant E-box sequences in the murine and human TERT promoters (Greenberg et al., 1999; Wu et al., 1999). Indeed, we have found that transfection of c-myc/c-H-ras into primary REC can induce high levels of telomerase activity (unpublished observation). The telomerase activity in MRMad1ER-5 cells was approximately 20% of that observed in HeLa cells (Figure 2a and c). Similar results were obtained with cells that were kept for several days in ethanol, the solvent for OHT (Figure 2a and c, lane 12-14). In contrast, OHT-treatment of the MRMad1ER-5 cells almost completely abolished telomerase activity (Figure 2a and c, lane 2-6). Since MR-1 cells, used throughout this study as

preferential control, had a priori only low levels of endogenous telomerase activity, we instead used MR-2 cells as control. As expected, presence of OHT had no effect on telomerase activity in MR-2 cells (Figure 2b, c). This finding corroborated our data that the OHT-induced Mad1ER inhibited TERT activity in the MRMad1ER-5 cells. Taken together, our results demonstrate that the transcriptional repressor function of Mad1ER is activated by OHT.

a

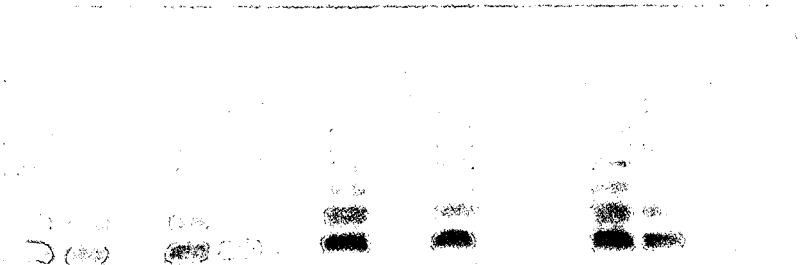
MRMad1ER-5



Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	HeLa	+OHT					-OHT					+EtOH			LB
Protein [µg]	0.5	5	5	2	0.5	0.2	5	5	2	0.5	0.2	5	2	0.5	
RNase			+					+							

b

MR-2



Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	HeLa	+OHT					-OHT					+EtOH			LB
Protein [µg]	0.5	5	5	2	0.5	0.2	5	5	2	0.5	0.2	5	2	0.5	
RNase			+					+							

c

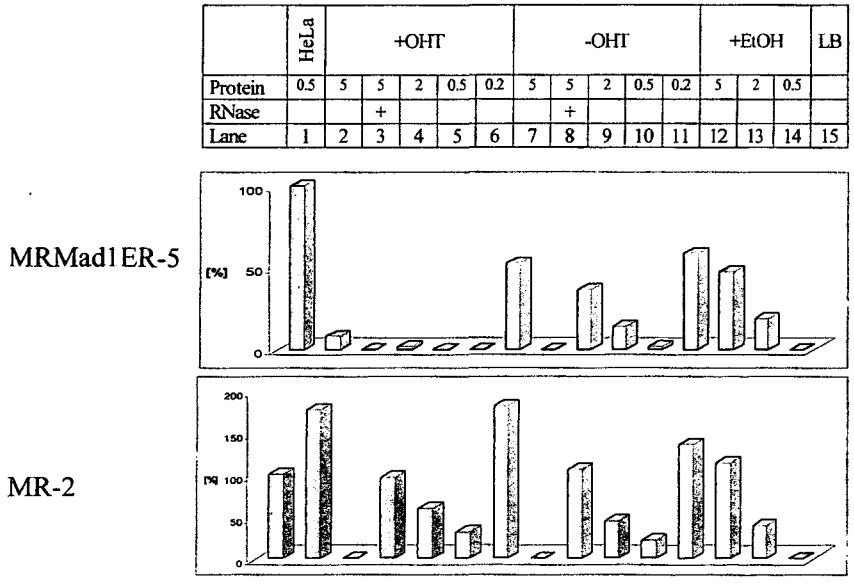


Figure 2 **Activation of Mad1ER represses telomerase activity in Mad1ER-5 cells.** Telomerase activity was determined in Mad1ER-5 (a) and MR-2 cells (b) and was semiquantitatively evaluated by ImageQuant 5.0 (c). Cells were cultured for 7 days in 2% FCS either in the absence or presence of OHT or supplemented with the appropriate

concentration of ethanol (EtOH), the solvent for OHT. Various amounts of cell lysates were subjected to PCR-based TRAP assay. The amplification products were separated on a 10% nondenaturing polyacrylamide gel and stained with Vistra Green. 0.5 mg of HeLa cell extract was used for comparison. Lysis buffer (LB) served as negative control. One out of three independent experiments is shown.

5.2.3.3 Inhibition of proliferation by Mad1ER in vitro

To examine the effect of Mad1 activation on cell proliferation, MRMad1ER cells were seeded at low cell numbers and incubated in the presence or absence of OHT for 10 days prior to staining with Giemsa. OHT-induction of Mad1 resulted in a significant reduction in both the number and the size of the colonies (Figure 3, lower panel). No influence of OHT on colony formation was observed with MR-1 cells (Figure 3, upper panel). The effect of Mad1 on cell growth was density-dependent since Mad1 did not significantly inhibit colony growth when cells were seeded at high densities (data not shown).

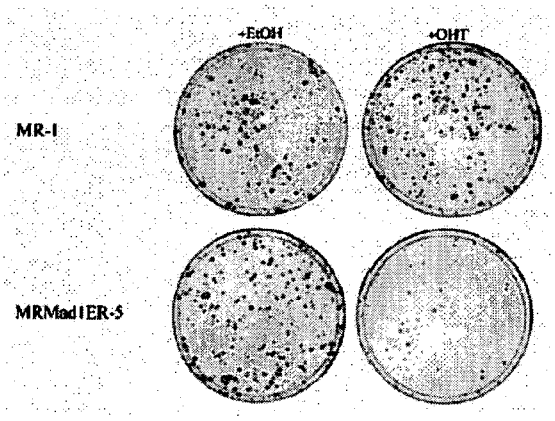


Figure 3 **Inhibition of clonal growth by Mad1ER activation**. MR-1 (upper panel) and MRMad1ER-5 cells (lower panel) were seeded in petri dishes and cultured in medium containing 10% FCS in the absence (left panel) or presence of OHT (right panel). After 10 days cell colonies were stained by Giemsa. One representative experiment out of six is shown.

We then performed FACS analysis in order to analyze the effect of Mad1ER on cell cycle distribution. MRMad1ER-5 cells have a diploid karyotype despite their highly transformed morphology (Figure 4a), while MRMad1ER-6 cells are mainly tetraploid (Figure 4c). In such heteroploid cells, the overlap of the G2/M fraction of diploid cells with the G1 fraction of tetraploid cells restricts the cell cycle analysis to cells in S-

phase. The fraction of cells in S-phase decreased from 51% to 25% and from 58% to 43% in clone MRMad1ER-5 and -6 cells, respectively, upon activation of Mad1ER (Figure 4a-d). A significant increase in the number of G0/G1 cells and a concomitant decrease in G2/M cells were seen in diploid MRMad1ER-5 cells (Figure 4a and b). No effect of OHT was observed in MR-1 cells or in normal diploid REC (Figure 4e-h). Thus activation of Mad1ER resulted in a reduced fraction of cells in S-phase in both cell lines but the effect was more pronounced in MRMad1ER-5 cells which also accumulated in G1 (Figure 4). The differences in S-phase reduction in presence of OHT was also reflected in growth curves performed during 5 days of exponential growth, with MRMad1ER-5 cells being more affected by OHT than MRMad1ER-6 or control cells (data not shown).

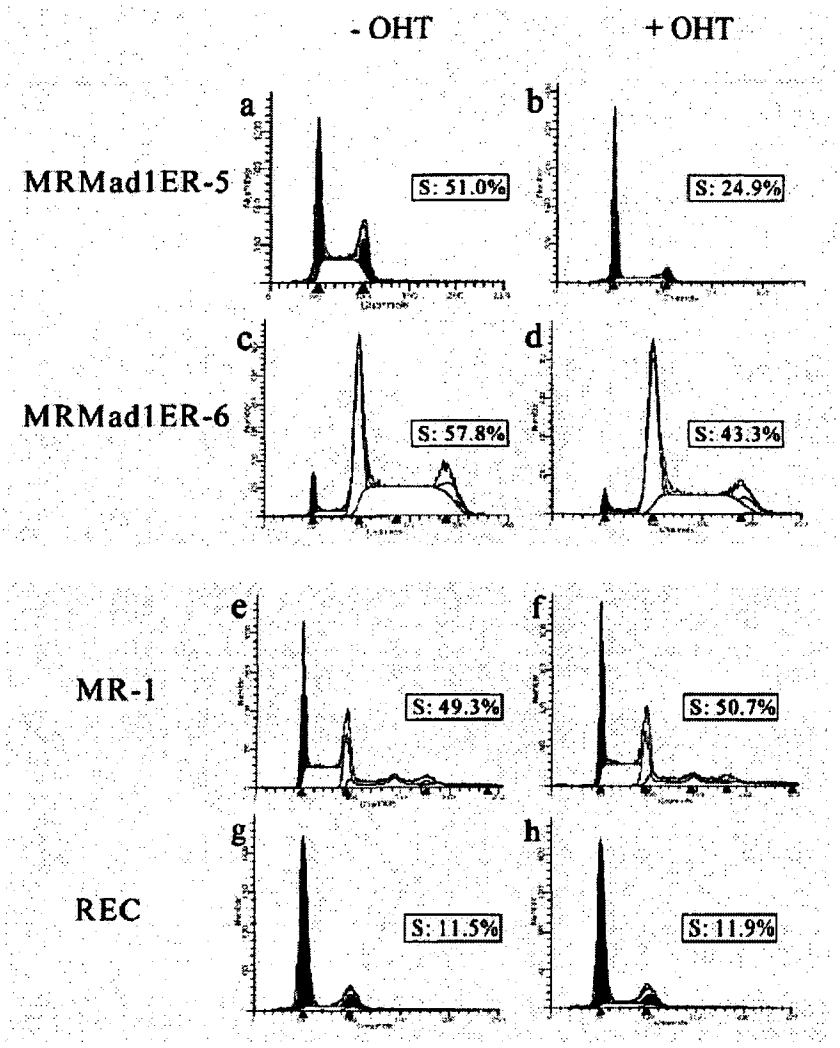


Figure 4 **Cell cycle analysis of Mad1ER cells.** MRMad1ER-5, MRMad1ER-6, MR-1, and REC were grown for 48 h in 10% FCS in the absence (a,c,e,g) or presence (b,d,f,h) of OHT. Cells were harvested, fixed, stained with propidium iodide and the DNA content was analysed by FACS. The percentage of cells in S phase is indicated. One experiment out of two is shown.

5.2.3.4 Activation of Mad1ER results in inhibition of Myc/Ras-dependent tumor Growth

The Myc-antagonizing effects of Mad1 in the MRMad1ER cells *in vitro* encouraged us to investigate whether activation of Mad1 could inhibit the *in vivo* growth of the cells. A small number ($5x10^4$) of MRMad1ER-5 cells was injected into syngenic 7 day

old male Fisher rats. Concomitantly animals received intraperitoneally (i.p.) OHT injections (0.3, 1.2, and 2.4 mg/kg) or PBS that were repeated every other day throughout the experiment.

On day 5 after tumor cell injection the first small nodules were detected in the control, group A, that received PBS and group B that received 0.3 mg/kg OHT (Figure 5a and b). By day 12 all animals of these two groups had palpable tumors. The tumors grew rapidly reaching diameters of 20 mm and more after a few days. Of the seven animals in group C, which were treated with 1.2 mg/kg OHT, four rats developed tumors with a similar latency period as observed in the control and group B (Figure 5c). In three rats the first palpable tumors arose after a longer latency period. However, the growth kinetic of all tumors in group C was rapid and similar to those of the tumors in groups A and B. The doubling time of tumor size in groups A, B, and C was two to three days. In the animals of group D, that were treated with 2.4 mg/kg OHT, the first two tumors become detectable on day 12 after injection and only by day 19 small tumor nodules were palpable in the remainders (Figure 5d). Thus, the average latency period of tumor appearance in this group was doubled. The initially retarded outgrowth of injected MRMad1ER cells was followed by slow tumor growth. In all but one animal the doubling time of tumor size increased significantly to up to two weeks. Comparison of tumor growth in the four groups showed a clear dose dependent effect of OHT on the proliferation rate of MRMad1ER cells *in vivo*. Three slowly growing tumors of group D (animal No. 2, 4 and 6, respectively) started to grow fast after approximately 40 days which might indicate the outgrowth of a subpopulation of more aggressive tumor cells. Figure 5e summarizes the successive increase of mean tumor diameters in the various treatment groups. While low concentrations of OHT appeared rather to stimulate tumor growth, the highest OHT-concentration significantly inhibited the proliferation

of MRMad1ER cells *in vivo*. The mean values of tumor diameters between groups A and D differed significantly ($P<0.05$) on day 19 after tumor cell injection. The fact that the body mass of OHT-treated rats increased in line with solvent-treated animals (Figure 5f) and untreated rats (data not shown) argues against biasing side-effects of repeated i.p. injections or OHT-applications. When MRMad1ER-6 cells were injected into animals in an analogous experiment using 2.4 mg/kg OHT, an increase in latency period and a reduction of tumor growth were observed, albeit less dramatic than with the MRMad1ER-5 tumors (data not shown). This is consistent with the observed differences in reduction of S-phase cells as determined by FACS analysis (Figure 4a- d).

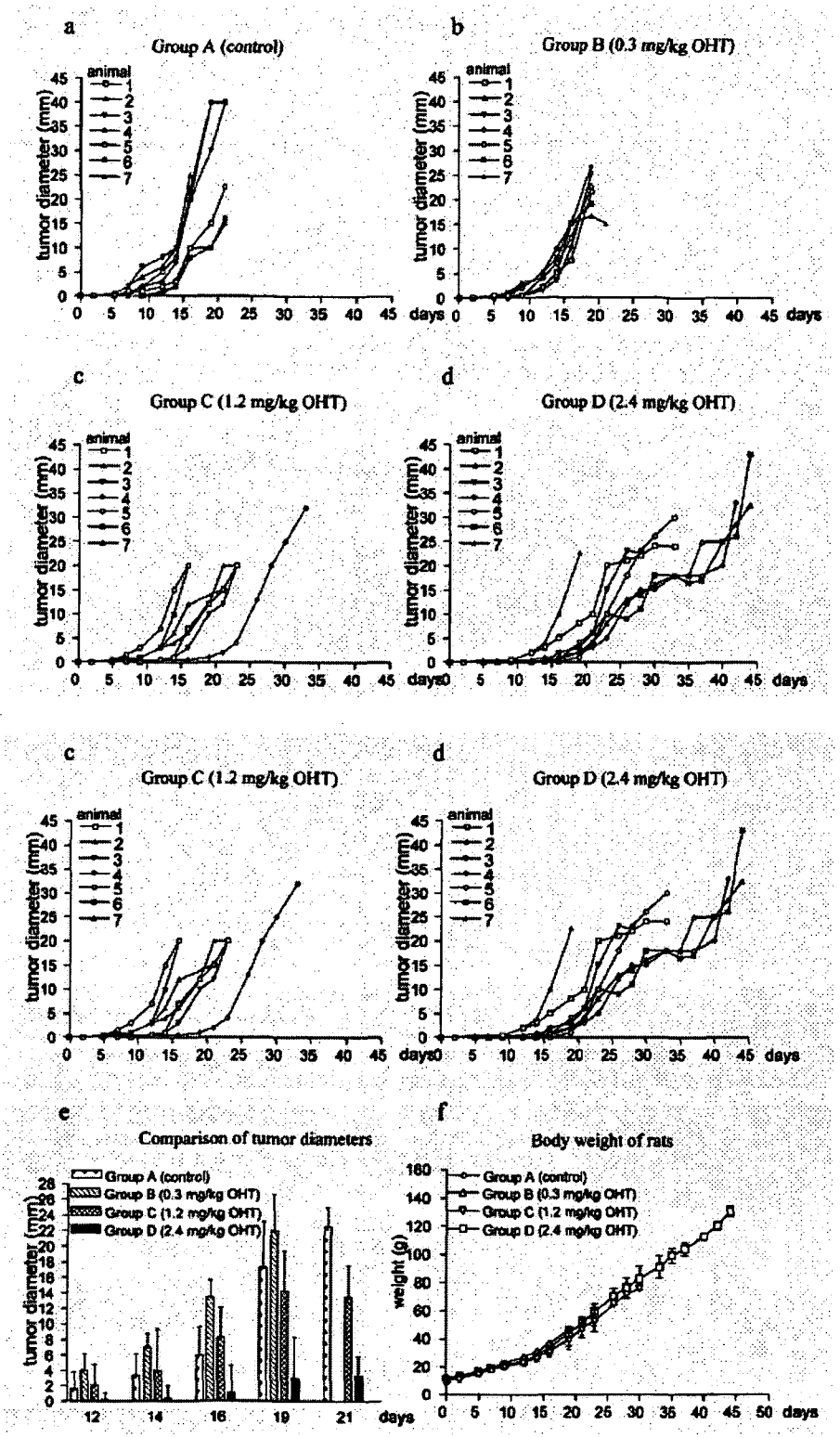


Figure 5 **Inhibition of tumor growth of MRMad1ER-5 cells upon induction of Mad1ER.** Seven days old, syngenic male rats were injected subcutaneously with $5x10^4$ MRMad1ER-5 cells. Control animals received PBS with 4% ethanol intraperitoneally every other day (**a**). Group B received 0.3 mg/kg OHT (**b**), group C 1.2 mg/kg OHT (**c**) and group D 2.4 mg/kg OHT (**d**). Tumor appearance, tumor growth and animal weight was recorded regularly. Comparison of the average tumor diameters in the four treatment groups (e). There was no difference among the groups with regard to animal weight (f).

It is known that tamoxifen, a clinically applied anti-estrogenic drug that is metabolized to OHT, can inhibit tumor cell growth both *in vivo* and *in vitro* in an unspecific manner, i.e. by mechanisms that are independent of the presence of estrogen receptors (Gunimeda et al., 1996; Treon et al., 1998; Lee et al., 2000). Although in previous *in vitro* experiments, a series of transformed c-myc/c-H-ras cell lines were found unresponsive to OHT application in culture as determined by growth curves and FACS analysis (Figure 4e, f and data not shown), we investigated the effect of the highest OHT-concentration on the *in vivo* growth of MR-1 cells (Figure 6). For comparison with the previous animal experiments, the number of injected cells was kept constant at $5x10^4$ cells per rat although the low ras expression of MR-1 cells (Figure 1b) was suggestive of a probably reduced tumorigenicity. Indeed, one animal in each group, i.e. No. 5 in group E and No. 2 in group F, respectively, remained tumor-free. However, among the arising tumors, there was no difference between solvent- and OHT-treated rats neither with regard to tumor onset nor growth kinetics (Figure 6). Note that the *in vivo* proliferation of untreated MR-1 and MRMad1ER-5 cells was similar despite their different ras mRNA expression (Figure 1b).

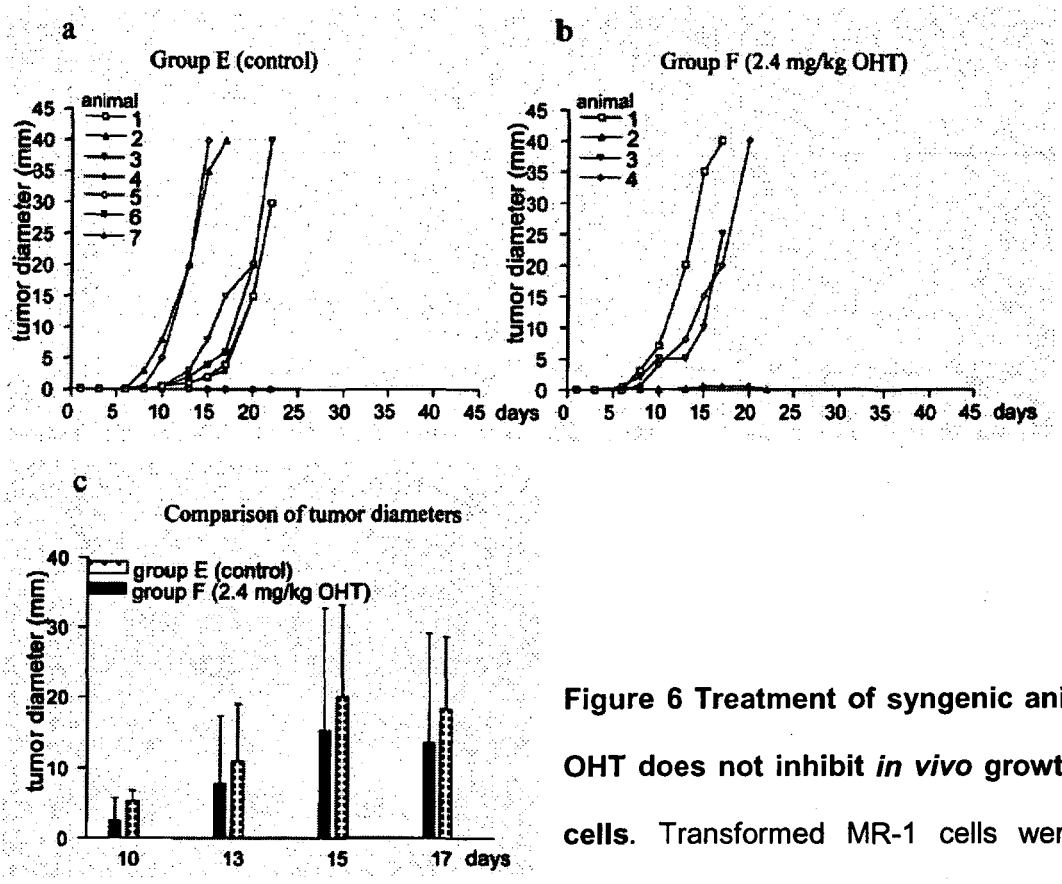
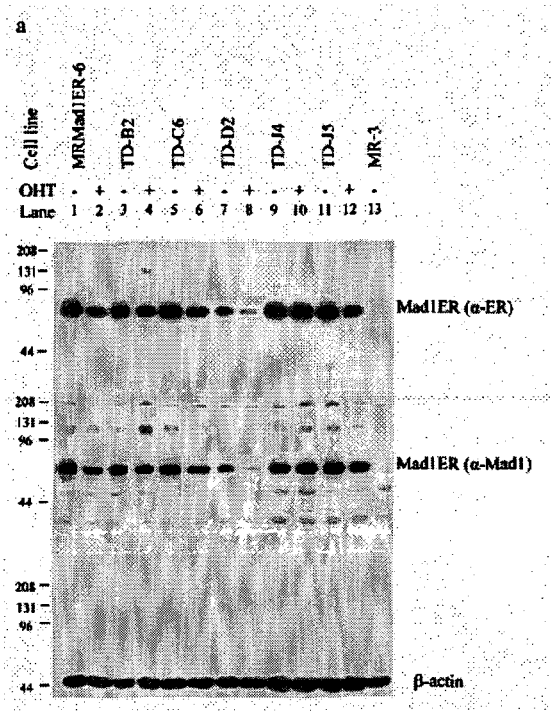


Figure 6 Treatment of syngenic animals with OHT does not inhibit *in vivo* growth of MR-1 cells. Transformed MR-1 cells were injected under the same conditions as MRMad1ER-5 cells. Animals received PBS with 4% ethanol (a) or 2.4 mg/kg OHT (b). There was neither an inhibitory effect of OHT administrations on tumor growth (c) nor on animal weight (data not shown).

5.2.3.5 Analysis of tumor-derived cell lines

We then examined whether the *in vivo* passage had altered the geno- and/or phenotype of MRMad1ER cells. Of nine isolated tumors from the different treatment groups, seven were successfully re-established in culture. All of them resembled the parental cells with regard to morphology (data not shown). Western blot analysis indicated that the tumor-derived (TD) lines still expressed the transfected c-myc and c-H-ras oncogenes to a similar extent as the parental cells and the chimeric mad1ER construct (Figure 7a and b). We observed that OHT reduced the expression of the Mad1ER protein in some of the cell clones. The reason for this is unclear at present. Since this was also found in the parental MRMad1ER-6 cells one may speculate that

this could be part of a compensatory autoregulatory mechanism to limit Mad1 activity. We also performed FACS analysis and Table 1 shows the percentage of cells in S-phase in the different TD lines. With exception of TD-A5, the overall percentage of cells in S-phase was lower than in the parental cells. Three TD lines (TD-A5, TD-B2, and TD-C6) still responded to OHT as determined by the reduction in the number of S-phase cells while the three others did not. We analyzed the expression of some cell cycle-relevant proteins that could be responsible for the reduction of S-phase cells in presence of OHT, with special interest on the still inducible TD-cell lines. However, Mad1 did not consistently affect the expression of p27, cyclin D1, or p21 (Figure 7b and data not shown). One cell line, TD-D2, deviated from the overall picture. These cells were derived from the tumor of animal No. 2 in group D, in which tumor growth was clearly accelerated after 37 days, indicating that some prominent genetic alterations had occurred. Indeed, addition of OHT resulted in a reduction of Mad1 expression (Figure 7a), induction of cyclin D1 (Figure 7b), and a concomitant increase of cells in S-phase (Table 1). These data indicate that selective pressure against Mad1-mediated growth inhibition was exerted *in vivo*, with TD-D2 as a clear example.



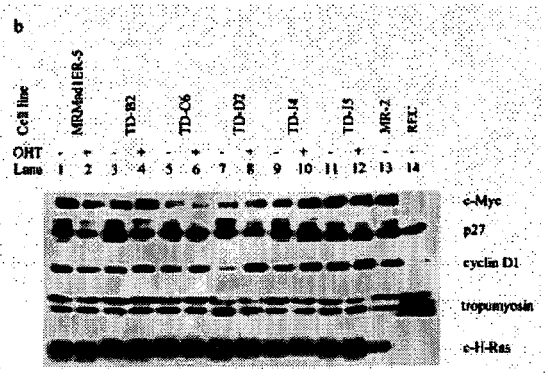


Figure 7 **Expression of Mad1ER and cell cycle regulatory proteins in tumor-derived cell lines.** Tumors from injected MRMad1ER-5 and -6 cells were excised and replated in culture in the absence or presence of OHT. Cell lysates

were prepared and analysed for protein expression. (**a**) Two identical blots were incubated in parallel with an ER antibody (upper panel) and a Mad1 antibody (lower panel). (**b**) Cell lysates were analysed for expression c-Myc, p27, cyclin D1, tropomyosin, and c-H-Ras. TD-J4 and TD-J5 cells were re-established from MRMad1ER-6 tumors of rats treated with 2.4 mg/kg OHT.

Table 1 FACS analysis of tumor-derived cell lines

TD cell line	parental cell line	animal group (animal No.)	*in vivo* treatment	% of cells in S-phase		Ratio
			OHT (mg/kg)	- OHT *in vitro*	+ OHT *in vitro*	
TD-A4	MRMad1ER-5	Group A (No. 4)	PBS	45.0	43.6	0.97
TD-A5	MRMad1ER-5	Group A (No. 5)	PBS	50.6	24.0	0.48
TD-B2	MRMad1ER-5	Group B (No. 2)	0.3	30.4	20.8	0.68
TD-C6	MRMad1ER-5	Group C (No. 6)	1.2	23.4	16.0	0.69
TD-D2	MRMad1ER-5	Group D (No. 2)	2.4	32.0	38.4	1.20
TD-J5	MRMad1ER-6	Group J (No. 5)	2.4	21.8	21.4	0.98

Tumors from the various treatments were excised, minced and re-plated in culture in DMEM with 10% FCS. Tumor-derived (TD) cell lines were incubated *in vitro* for at least 48 hours in the absence or presence of OHT before FACS analysis. The percentages of cells in S-phase and their ratios are indicated.

5.2.4 Discussion

We have generated transformed cell lines derived from primary rat embryo cells (REC) that express the human c-myc and c-H-ras genes together with a mad1-estrogen receptor fusion construct, mad1ER. Using these cells we have analyzed the effect of activated Mad1ER on cell proliferation *in vitro* and *in vivo*. Our findings demonstrate that functional Mad1 can be expressed in REC concurrently with high-level expression of c-Myc and c-H-Ras and that activated Mad1 inhibited telomerase activity and proliferation of the transformed cells in vitro. Injection of the cells into syngenic rats induced aggressively growing tumors after a short latency period. In contrast, continuous activation of Mad1 prolonged the latency period of tumor appearance and reduced the growth rate of the tumors in an OHT-dose dependent

manner. These effects were specific for Mad1 since no effect of OHT was observed with transformed MR-1 control cells. Taken together, our results provide further evidence for a growth inhibitory role of Mad1 and extend its function to the inhibition of myc-expressing tumors.

In our attempts to generate c-myc/c-H-ras transformed REC cell lines that express the chimeric mad1ER transgene, only two out of 50 clones analyzed clearly stained positive for the Mad1ER protein. One possibility for the low yield is that the primary REC used as target cells represent a mixture of different cell types and it is feasible that the proliferation of some (or most) might be sensitive to even low amounts of Mad1. Although the transfected REC were kept in phenol red-free medium supplemented with low-estrogen or charcoal-absorbed serum, remaining traces of estrogen in the sera might have been sufficient to activate the Mad1ER fusion protein and consequently inhibit the outgrowth of potential founder cells. We have previously observed such inherent sensitivity toward low Mad1 levels in established cell lines (Gehring et al., 2000). Another possibility is that basal Mad1 levels can be tolerated only in the presence of high c-Myc levels. Abundant Myc/Max complexes might efficiently antagonize Mad/Max dimers, thereby allowing cell proliferation. In support of this hypothesis, Cultraro et al. (1997) reported that expression of Mad1 could only be obtained in the presence of c-Myc in mouse erythroleukemia cells. However, this does not seem to be the explanation in our cells since we found that the amount of c-myc mRNA in the two established MRMad1ER clones was similar to that expressed in four MR cell lines. In contrast, the former cells expressed unusually high levels of ras mRNA and protein. In this context, a recent study on the cooperation of oncogenic ras with endogenous c-myc is of interest. Bazarov et al. (2001) reported that high levels of a c-H-Ras mutant (G12V) could compensate for low c-Myc levels in anchorage-independent growth of Rat1a cells as evidenced by

efficient cell growth in semi-solid medium. Thus, if low Mad1 levels in our cells compromised some of the c-Myc functions relevant for the transformed phenotype (which was the criterion for clonal isolation), then high c-H-Ras levels might have been the necessary prerequisite for clonal outgrowth. This assumption is not only supported by the high level of ras mRNA and protein (Figure 1 and 7), but also by the stable morphology of the MRMad1ER cells and their tumor-derived cell lines, irrespective of Mad1 activation (data not shown). Furthermore, tropomyosin levels, which are high in normal, flat REC (Figure 7b, lane 14) and generally low in their transformed derivatives remained stable in the MRMad1ER cells and their TD-lines upon OHT treatment (Figure 7b and unpublished observation). The expression of tropomyosin has also been reported previously to decrease upon transformation (Prasad et al., 1999). Taken together, our data indicate that Mad1 does not alter the transformed morphology of our cell lines which might be due to the high level of Ras expression.

Analysis of cell cycle distribution of the MRMad1ER cells revealed a reduction in the cell fraction in S-phase upon Mad1 activation. The cells were however not arrested since they continued to cycle, albeit at a slower rate. A decrease in the number of S-phase cells was also observed in response to Mad1 expression in astrocytoma and NIH3T3 cells (Chen et al., 1995; Roussel et al., 1996). In contrast to the modest effects during normal growth conditions, we found that Mad1 inhibited cell proliferation very efficiently when the MRMad1ER cells were plated at low cell density. When the number of plated cells was increased, this effect was no longer observed. We have made similar observations demonstrating more pronounced effects of Mad1 on proliferation during restrictive growth conditions in two other model systems (Bejarano et al., 2000; Gehring et al., 2000). These data suggest that Mad1 function is most clearly manifested under restrictive growth conditions.

To further characterize the consequences of Mad1ER activation we studied the effect on rat telomerase activity. As anticipated, the telomerase activity of MRMad1ER-5 cells was reduced to almost undetectable levels upon addition of OHT, but remained high in the transformed control MR line under the same conditions. Similarly, we and others have recently reported reductions in telomerase expression and activity in two human tumor cell lines, U937 and HL60, in the course of drug-induced differentiation concomitantly with Mad1 upregulation (Günes et al., 2000; Xu et al., 2001). In addition, hTERT expression in normal and malignant human cells was found to have an inverse correlation with Mad1 expression (Cong and Bacchetti, 2000; Oh et al., 2000; Günes et al., 2000). The Mad1-induced repression of hTERT transcription is mediated by the N-terminal SID of Mad1 that recruits histone deacetylases to chromatin (Cong and Bacchetti, 2000). This indicates that acetylation/deacetylation of histones contributes to the regulation of hTERT expression.

The inhibition of telomerase activity in response to Mad1 might contribute to the reduced *in vivo* growth of MRMad1ER-5 cells. REC are equipped with up to 150 kb ultralong telomeres that further increase by transfection of c-myc and c-H-ras (unpublished observation). Nevertheless, cell proliferation could well be affected by the lack of telomerase, probably even in case of long telomeres. Besides the effect of telomerase on telomere elongation, the enzyme exerts in addition a protective capping function on the telomeric ends that is independent from synthesis of telomeric repeats (Zhu et al., 1999). However, oncogene-transformed murine cells derived from animals without functional telomerase are as tumorigenic as their wild type counterparts, arguing against a contribution of telomerase activity for tumorigenic growth of rodent cells (Blasco et al., 1997). Thus at present it is unclear

whether telomerase inhibition upon Mad1 induction *in vivo* contributes to the reduced tumor growth.

Considering the complexity of cellular requirements for autonomous growth *in vivo*, several possibilities are conceivable for Mad1-mediated tumor inhibition. Transformed cells will not give rise to progressively growing tumors unless their survival in the host organism is guaranteed by sufficient supply of blood vessels. Since normal tissue generally produces more inhibitors than stimulators of neoangiogenesis, tumor cells must shift the balance in favor of angiogenic factors. Overexpression of c-Myc, a common finding in many different kinds of experimental and clinical malignant tumors, can suppress transcription of thrombospondin-1 (Tsp-1), a secreted inhibitor of angiogenesis (Good et al., 1990). It was recently shown that c-myc-transformed Rat-1a fibroblasts provoke neovascularisation in vivo when injected into immunodeficient mice or immunpriviledged sites (Ngo et al., 2000). It is likely that downregulation of angiogenic inhibitors such as Tsp-1 or similar factors by deregulated Myc contribute to the aggressive growth potential of myc-expressing tumor cells. Conversely, inhibition of c-Myc function by Mad1 could hamper the outgrowth of Myc expressing cells as a consequence of limited blood supply. Although determination of the nature and amount of angiogenic factors in the MRMad1ER cell lines is a future project, it is likely that the cells produce the potent angiogenic VEGF due to high c-H-ras expression (Rak et al., 1995; Larcher et al., 1996). Since neovascularization requires a coordinated array of a multitude of distinct proteins, upregulation of angiogenesis inhibitors such as for instance Tsp-1 by compromised c-Myc function could contribute to reduced tumor growth potential.

Mad1 might also oppose some c-Myc functions relevant for cell cycle progression. However, when we compared cell cycle distribution patterns of MRMad1ER cells and protein data from the founder and the tumor-derived cell lines, no consistent picture

emerged. One explanation for our results might be that the high amounts of c-H-Ras in MRMad1ER cell lines compensated some of the Mad1-compromised c-Myc functions, analogous to the reported attachment-independent growth of c-myc deficient, yet c-H-ras transformed Rat1a cells (Bazarov et al., 2001). Similar to the findings reported here, we could not detect any differences in expression of cell cycle regulatory molecules in Mad1-induced versus uninduced human tumor cells (Gehring et al., 2000). In contrast, Quéva et al. (1999) reported that mouse embryo fibroblasts derived from mad1 transgenic mice displayed increased levels of p21 and p27 and reduced CDK2 and CDK4 activities. We also observed an increase in p27 expression and a reduction in CDK2 activity in Mad1-expressing NIH3T3 cells (Bejarano et al., 2000). These data support the notion that Mad1 exerts at least some of its functions through negative regulation of CDK activity. It remains to be determined if Ras oncoproteins are able to abolish or compensate the effects of Mad1 on the activity of CDK complexes.

Recently, a number of studies have identified Myc target genes involved in cell cycle regulation, cell death, immortality, matrix adhesion, protein synthesis, and metabolism (see Grandiori et al., 2000; Coller et al., 2000; O´Hagan et al., 2000; Guo et al., 2001). The generation of Rat1-derived cell lines with homozygous deletion of c-myc allels revealed new insights into c-Myc functions. Comparison of gene expression between exponentially growing c-myc-null and wild type Rat1 cells identified 188 genes up-regulated and 95 genes down-regulated, directly or indirectly by endogenous c-Myc (Guo et al., 2001). However, when Rat1 cells over-expressing a c-myc transgene were analyzed and compared to the wild type Rat1 cells, a smaller set of genes was found activated, with only partial overlap with the latter (Guo et al., 2001). These data therefore suggest that endogenous c-Myc might regulate a different set of genes than ectopically overexpressed c-Myc. It is possible

that Mad1 interferes preferentially with the genes regulated by endogenous c-Myc rather than with those induced by overexpressed oncogenic Myc. A decrease, but not complete inhibition of cells in S-phase would then be expected *in vitro* and, in turn, result in slower tumor growth *in vivo*, which is what we observed.

An inverse correlation between Myc- and Mad expression was found in a series of specimen of differently advanced human breast cancers. This correlated with the clinical stage, with high Mad1 in benign or early diseases and high Myc levels in more advanced cases (Han et al., 2000). In invasive ductal carcinoma, Myc expression was prominent in cancerous tissue and proliferating cells, while Mad expression was sporadic and restricted to non-proliferating cells. In the more differentiated tumor cells, Mad expression was high and found in more cells, while it was reduced in less differentiated tumor cells. It was concluded that loss of Mad1 expression might contribute to malignant transformation of human mammary epithelial cells. These data, taken together with our results showing that activation of Mad1 in c-myc/c-H-ras transformed cells significantly retarded tumor appearance and reduced tumor growth, indicates that Mad1 might play a role in preventing tumorigenesis.

5.2.5 Materials and Methods

5.2.5.1 Plasmids and antibodies

CMVmad1, pSPmyc and pSPmax have been described previously (Cerni et al., 1995). pSPmyc and pVZ1mad1 were obtained from L. Kretzner and D. Ayer, respectively. The mad1 insert of pVZ1mad1 was cloned into ERBS6 (a kind gift from J. Lüscher-Firzlaff) generating mad1ER. The mad1ER insert was sequenced and

cloned into pSP generating pSPmad1ER. From the plasmid pVEJB which expresses an activated c-H-ras oncogene and a neo resistance gene (Cerni et al., 1990), the BamHI inserted neo gene was removed and the plasmid religated. pRSVneo was used as the neomycin resistance encoding plasmid (Gorman et al., 1983)

Polyclonal antibodies recognizing Mad1 (C-19), c-Myc (N-262), p27 (F-8), ER (F-10) were from Santa Cruz and the mouse monoclonal antibodies against Myc (Ab-2) and cyclin D1 (Ab-1) were obtained from Neomarkers. The 5C9 monoclonal antibody recognizing Mad1 has been described previously (Sommer et al., 1997). Anti-tropomyosin (TM311) was from Sigma and anti-human $p21^{ras}$ was obtained from Dako. DTAF- and Cy^3-labeled secondary antibodies were from Amersham Pharmacia Biotech., and anti-mouse and anti-rabbit IgG coupled to horse radish peroxidase were obtained from Calbiochem and Amersham Pharmacia Biotech., respectively.

5.2.5.2 Cell culture and transfection

Primary rat embryo cells (REC) were obtained from 15.5 gestation day old Fischer rat embryos by fractionated trypsinization as described (Cerni et al., 1990). REC were grown in phenol-red-free DMEM (Gibco) in 10% low-estrogen FCS (Bioconcept) and transfected with 2.5 µg pVEJB, 2.5 µg of pSPmyc, 5 µg of pSPmad1ER and 1 µg of pRSVneo per 60 mm petri dish using the calcium phosphate technique. In parallel REC were transfected with 2.5 µg pVEJB, 2.5 µg of pSPmyc and 1 µg of pRSVneo per 60 mm petri dish. 20 hrs after transfection the cells were trypsinized and replated at $3x10^5$ per 60 mm dish. Two days later 200 µg/ml G418 (Geneticin, Gibco) was added and the cells were fed with fresh medium

containing G418 every two to three days. After 14 days colonies were picked and checked for Mad1 expression using immunofluorescence. Two positive clones MRMad1ER-5 and -6 were identified, expanded and further characterized. Several Myc/Ras transformed clones (MR-1 etc) were also picked and expanded. The REC clones were maintained in phenol-red-free DMEM medium (Gibco) supplemented with 10% low estrogen FCS (Bioconcept) or charcoal-treated FCS (PAA, Austria) and penicillin/streptomycin (10 U/ml). 4-Hydroxy-Tamoxifen (OHT; Calbiochem) was added at a concentration of 1 µg/ml medium. HeLa cells were grown in DMEM supplemented with 10% FCS.

5.2.5.3 Immunofluorescence

Cells were grown on coverslips in the presence of OHT for 24 to 48 hrs, fixed in phosphate buffered saline (PBS) containing 4% paraformaldehyde, and permeabilized in PBS with 0.2% Triton X-100. Subsequently, cells were incubated in PBS containing 20% horse serum (blocking buffer). Antibody stainings were performed in blocking buffer. The DNA was stained with Hoechst 33258 (1 µg/ml in PBS) and the coverslips mounted with Moviol (Merck) in PBS containing 2.5% N-propylgallate (Sigma).

5.2.5.4 Northern blotting

Total RNA was isolated by Trizol (Gibco) according to the manufacturer's instructions. Ten µg of total RNA was separated on a glyoxal/dimethylsulfoxide agarose gel and blotted onto GeneScreen membranes (New England Nuclear), which were hybridized to random-primed cDNA probes of mad1, c-myc, c-H-ras and

glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Hybridizing mRNA was visualized by autoradiography.

5.2.5.5 Western blotting

Immunoblot analysis was preformed as described previously (Bejarano et al., 2000).

5.2.5.6 Telomeric Repeat Amplification Protocol (TRAP) Assay

Cell lysates were prepared using the CHAPS detergent lysis method (Kim et al., 1994). The protein concentration of lysates was measured using the BioRad (Bradford) protein assay. The PCR-based TRAP assay for detection of telomerase activity was performed according to Kim et al., (1994) with some modifications. 0.2 to 5 µg of lysates were subjected to 25 PCR cycles. The amplification products were separated on a 10% nondenaturing polyacrylamide gel, stained with Vistra Green (Amersham Life Science) and visualized in a FluorImager 595 (Molecular Dynamics) using ImageQuant 5.0. Semiquantitative densitometric evaluation was performed by ImageQuant 5.0.

5.2.5.7 Colony formation assays

Cells were seeded in petri dishes at low cell numbers (100 or 500 cells per 10 cm dish) and cultured in media containing 10% FCS in the absence or presence of OHT. After 10 days cell colonies were stained by Giemsa.

5.2.5.8 FACS analysis

Cells were seeded in 60 mm dishes in DMEM with 10% FCS and cultured for at least 24 hrs before induction. The cells were then incubated in FCS or charcoal-depleted FCS for at least 48 hrs in the absence or presence of OHT. Cells were harvested and fixed overnight in 70% ice-cold ethanol, washed in PBS and resuspended in 1 ml PBS containing 5 µg/ml propidium iodide (Sigma) and 100 µg/ml RNaseA (Sigma). Samples were incubated for 30 min at 37° C and placed at 4° C before flow cytometry analysis of the DNA profile. All samples were analyzed in a Becton Dickinson FACSCalibur system using CELLQuest software.

5.2.5.9 Animal experiments

Seven-day-old male Fisher rats received into the back subcutaneous injections of $5x10^4$ transformed cells in PBS. OHT was dissolved in ethanol and diluted with PBS to concentrations of 3 µg/ml, 6 µg/ml, 12 µg/ml and 24 µg/ml. Since the latter contained 4% ethanol, the ethanol concentration was adjusted to 4% in the other OHT-dilutions to avoid a probably biasing variable. On day 0, animals were injected with the tumor cells followed by i.p. applications of 0.3, 0.6, 1.2, and 2.4 mg/kg OHT or PBS with 4% ethanol. Treatment was repeated every other day with freshly prepared OHT solutions. Animal weight and tumor size were monitored regularly. Animals were killed by cervical dislocation when tumor diameters were approximately 20 mm for younger and 40 mm for older rats or before an unfavorable tumor localization would have restricted rats' comfort. Mean tumor diameters were statistically compared by the Kruskal-Wallis-test using Graph Pad Prism. The animal

experiment was performed according to the animal experiment approval GZ 66.009/98-Pr/4/00 given by the Austrian Ministry of Culture and Education.

5.2.5.10 Re-establishment of tumor-derived cell lines

Several tumors were excised, minced and replated in DMEM with 10% FCS without G418. A few days after explantation cells started to grow out from the tissue fragments. When small colonies had formed, cultures were carefully rinsed to remove abundant debris and floating tumor pieces. The morphology of attached, growing cells was indistinguishable from the initially injected cell lines. Since a first set of re-cultured tumors underwent apoptosis upon addition of G418 to the culture medium, we omitted the selection drug thereafter. Although the phenotype of these tumor-derived (TD) lines was clearly transformed, they had obviously lost the *neo* resistance gene in the course of the *in vivo* passage.

5.2.6 References

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5.3 UNIFORM AND SATURABLE TELOMERE ELONGATION BY EXOGENOUS hTERT IN TELOMERASE- AND ALT-NEGATIVE HUMAN LUNG CANCER CELLS

Running title: telomere elongation by exogenous hTERT

Key words: human lung cancer cell lines; hTERT; hTERT splice variants; telomere elongation; processivity of telomerase; FISH

5.3.1 Abstract

For cancer cells the maintenance of telomeres is a pre-requisite for unlimited proliferation potential. In the majority of human malignancies this is achieved by re-activation of the catalytic component of telomerase, hTERT. A small fraction of tumors uses an alternative telomere lengthening mechanism (ALT) that is characterised by highly heterogeneous telomere lengths resulting from intertelomeric recombinational events. However, some tumor cells obviously preserve their telomeres without telomerase activity or ALT. We were interested in the effects of exogenous hTERT on telomerase activity, processivity and telomere elongation in a telomerase-and ALT-negative background and transfected a hTERT gene into a suitable human lung cancer cell line, MSPG. Among a number of isolated MSPG-derived clones, two exerted high telomerase activity, which in turn elongated the telomeres considerably. Initially, the gain of telomeric sequences was more than 400bp per cell generation. This process slowed down by time until an upper limit of approximately 14kb long telomeres at all chromosomal ends was achieved. FISH analysis confirmed that the chromosomes had indeed acquired homogeneously long telomeres. These data indicate that ectopic telomerase is able to overcome individual telomere length regulation and extend all chromosomal ends to almost the same length.

5.3.2 Introduction

Among the multitude of acquired features of human cancer cells [1], the maintenance of telomere length is a crucial prerequisite since it counteracts the otherwise physiological erosion of telomeres [2]. Shortening of telomeres, due to the "end-replication problem" of DNA polymerases, leads eventually to irreversible cell cycle arrest and senescence [3,4]. In cancer prone cells, constant or sufficient long telomeres are usually achieved by re-activation of the catalytic component of telomerase, hTERT [5-8]. Approximately 80% of human malignant cells are telomerase positive [9,10]. Indeed, introduction of hTERT expression vectors into different normal cells endow them with an unlimited, yet normal proliferation potential [11-13]. This indicates that "immortalization" is an essential and unique requirement for tumor cells which is usually achieved by re-activation of telomerase [14].

Since inappropriate activation of telomerase in a normal cell represents an enormous danger for the organism, the control and regulation of the holo-enzyme and its associated proteins must be under stringent control. In a very recent report three tumor suppressor pathways were identified which negatively regulated hTERT expression at the transcriptional level. Efficient inhibition of these hTERT-suppressing proteins results in telomerase activity in originally telomerase-negative tumor cells and certain normal cell strains [15]. An additional control level of telomerase activity is alternate splicing of the hTERT transcript [16,17]. This mechanism appears important during development [16]. A functional TERT protein requires a full-length transcript. Within the genetic region for the reverse transcriptase domain, two potential splice sites were identified. The α-splice site causes a 36bp deletion, which affects the conserved

reverse transcriptase motif A. Its lack abolishes the reverse transcriptase activity of hTERT. The β-splice site results in a 182bp deletion and a frame shift, yielding a truncated, non-functional protein, which lacks the conserved reverse transcriptase motifs B, C, and D. The α+β splice variant combines both. Interestingly, expression of a full-length hTERT transcript is usually accompanied by the presence of one or all splice variants [17]. Especially the inactive β-splice variant is often detectable, even in telomerase-negative, normal tissue.

An alternative telomere lengthening mechanism (ALT) has been postulated for those malignant diseases, which lack telomerase activity [18,19]. Initially, ALT was defined as alternative(s) to telomerase-mediated telomere maintenance, however, in the last years ALT became confined to tumor cells which are characterised by an extreme heterogeneity of telomere lengths accompanied by special nuclear structures referred to as ALT-associated PML bodies (APBs) [reviewed in 20]. The mean terminal restriction fragments (TRFs) of classical ALT cells are around 20 kb with TRFs ranging from less than 1 kb to over 50kb [18-22]. Thus, telomeric DNA of ALT cells appears in gel electrophoresis as a bulk of high-molecular weight DNA with an extensive smear. The ultra-long TRFs obviously derive from some kind of inter-telomeric recombinational events [20,23].

Apart from re-activation of telomerase or activation of ALT, several other proteins participate in telomere length regulation, with the telomere repeat factors, TRF1 and TRF2, as central proteins for the stabilisation of telomere structure [24-26]. Both proteins bind in a complex with other proteins to telomeric DNA. TRF1 has been shown to interact with Tin2 [27], Tankyrase 1 and 2 [28,29] and PINX [30], whereas TRF2 acts in a complex with hRap1 [31]

and Mre11 [32]. Recently, POT1 was identified as an additional regulatory protein in the TRF1 complex, which determines the access of telomerase to the telomere terminus [33,34].

The physiological telomere erosion in a normal proliferating cell affects all telomeres to almost the same extend [35], however, the highly different individual telomere length of each chromosomal end is preserved during life time [36]. This indicates an additional level of telomere length control, which is obviously locally regulated. Also tumor cells with re-activated telomerase maintain their telomere length pattern during proliferation, even when the majority of telomeres is very short and in a range that would be critical for pre-senescent cells [37]. We were interested in the effects of the catalytic subunit of human telomerase, hTERT, transfected into telomerase-negative human lung cancer cell lines, on some of the telomere length control mechanisms. We report here that the efficient expression of hTERT in a stably telomerase- and ALT-negative cell line MSPG resulted in high telomerase activity which in turn leads to abolishment of local telomere length regulation. The extension of telomeres by exogenous telomerase was more than 400bp per cell generation after hTERT transfection, slowed down thereafter until the upper limit of telomere lengths was achieved. hTERT expression in the MSPG-derived sublines resulted in almost equally long telomeres of approximately 14kb on all chromosomal ends.

5.3.3 Results

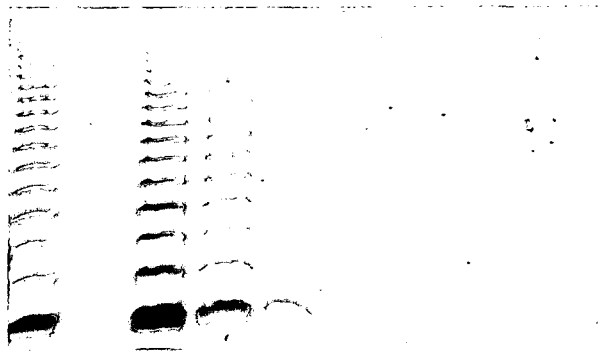
5.3.3.1 Telomerase activity and growth potential of human lung cancer cell lines

The aim of the study was to investigate whether and in how far telomerase-negative cancer cells might benefit from the introduction of transgenes involved in telomerase regulation. Therefore, we determined first the telomerase activity (TA) in a large series of different human cancer cells lines piled up in the last years in the institute (data not shown). Fig.1A shows the TAs of the 6 chosen lung cancer cell lines, which represent the spectrum of TA expected from cultured cancer cells [9]. Compared with HeLa cells as a standard, LPHE and MBSJ had lower values, whereas LSWW cells had even higher TA levels. Three cell lines were chosen, which were repeatedly TA-negative (HTB57, ZYGF and MSPG) as was the normal human embryonic lung fibroblast strain, WI38 (Fig. 1B).

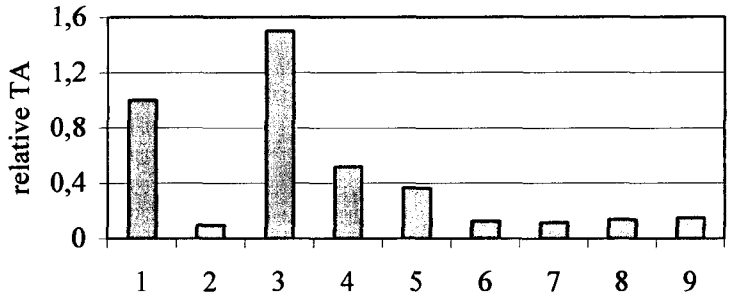
The growth potential of the cell lines *in vitro* corresponded to their respective TA status (Fig.1, C and D). The three TA-positive line proliferated more readily than their TA-negative counterparts, with ZYGF-cells having the lowest PDL value during exponential growth (Fig. 1D). This is in agreement with very recent reports which show that telomerase is able to regulate certain growth-promoting genes [38,39]. Similarly, the cell densities at confluence varied between the two groups, due to cell's plasticity. Whereas TA-negative lines retained their initial cell shape, TA-positive cells appeared to become quite small at the stationary phase of growth (data not shown). This suggests some additional differences between the two groups, perhaps in cytoskeleton dynamics or integrin composition.

A

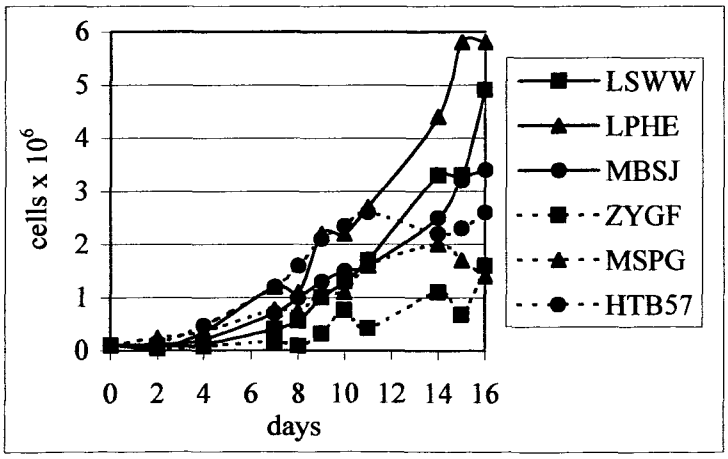
Cell lines	HeLa	HTB 57	LSWW	LPHE	MBSJ	ZYGF	MSPG	WI38	LB
Lane	1	2	3	4	5	6	7	8	9



B



C



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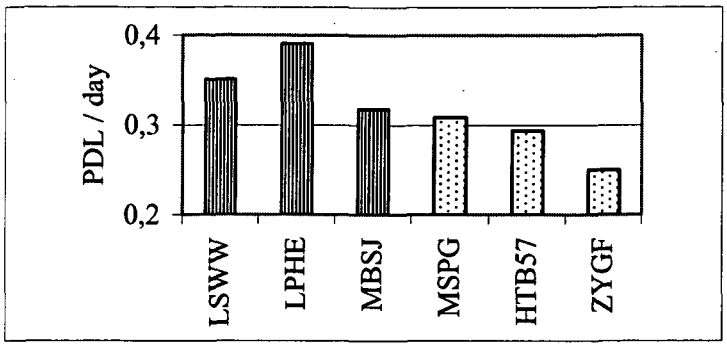


Fig.1. **Characterisation of selected lung cancer cell lines.** A. TRAP assay of six selected lung cancer cell lines (lane 2-7), telomerase-positive HeLa (lane 1) and telomerase-negative WI38-cells (lane 8). Lane 9 contains only lysis buffer. One of three independent experiments is shown. B. Densitometric evaluation of A. The amount of telomerase activity relative to that of HeLa cells was calculated. C. Growth curves of the cell lines. The solid lines correspond to telomerase-positive cell lines, the dotted lines to telomerase-negative cell lines. D. Population doubling levels (PDL) per day of the six lung cancer cell lines, calculated for their logarithmic growth phases. Lined bars: telomerase-positive cell lines; dotted bars: telomerase-negative cell lines.

5.3.3.2 Telomerase regulation in human lung cancer cell lines

In order to determine the underlying mechanism for the lack of TA, we performed RT-PCR for the RNA component of telomerase, hTERC, and the various splice variants of the catalytic component of telomerase, hTERT. In HTB57 as well as in the control WI38-cells, no hTERT signals could be detected. Two of the three TA-negative cell lines, however, expressed the inactive β-splice variant of 275 bp (Fig. 2, lane 11 and 13). This indicated that at least the hTERT promoter regions and a portion of the coding regions were

intact. As expected, the TA-positive lines showed a band at 457bp, which corresponds to the functional full-length transcript of hTERT. In addition, the α+β- and the inactive β-splice variants were also expressed (Fig. 2, lane 1, 5, 7, and 9). A soft band of the dominant negative α-variant was also detectable in LSWW- and LPHE-cells (Fig. 2, lane 5 and 7). With regard to expression of hTERC, no striking differences were found among the various lines. This indicates that the lack of telomerase activity could not be associated with repression of the hTERC promoter [40, 41].

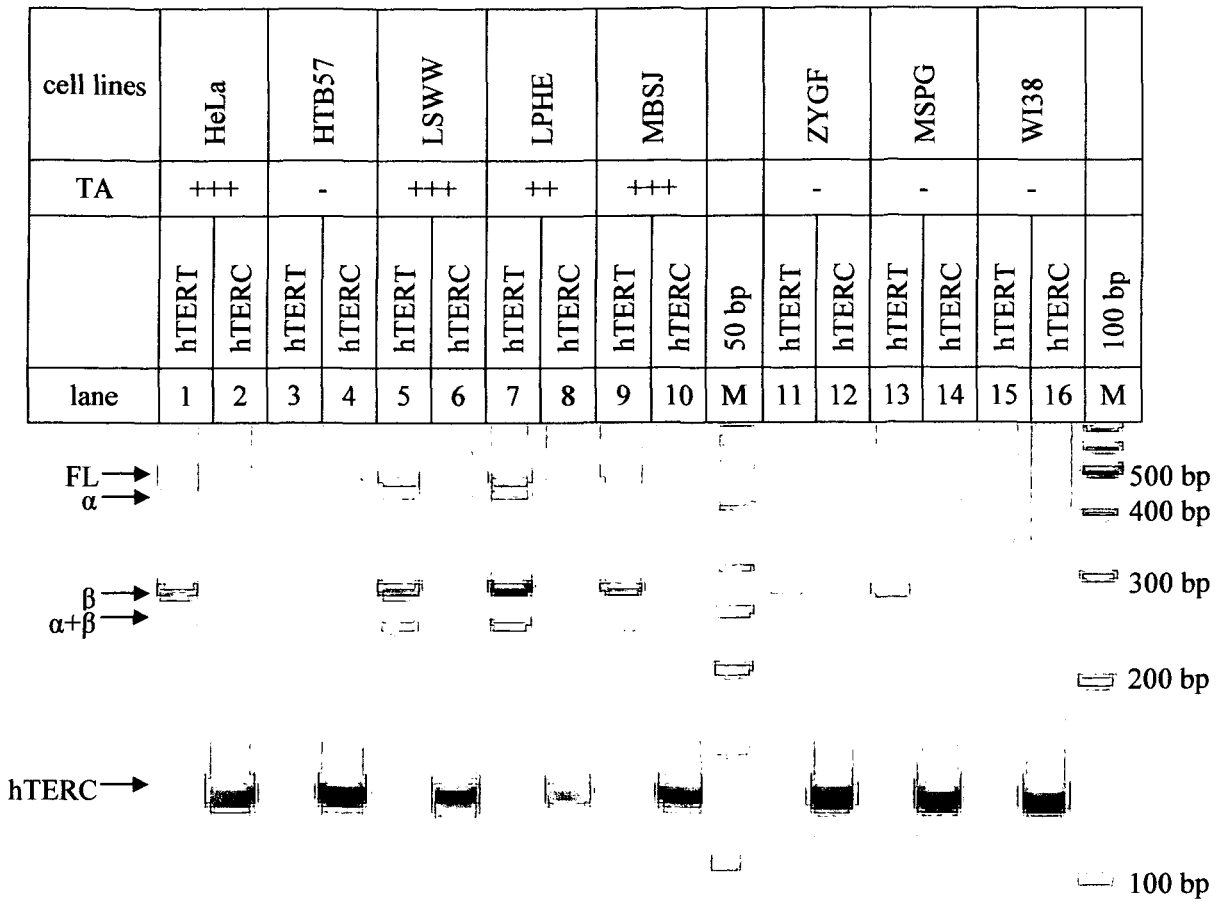


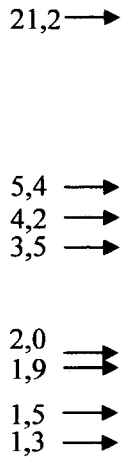
Fig.2. **Detection of hTERC and various splice variants by RT-PCR**. All telomerase-positive cell lines (lane 1 and 2; lane 5 to 10) have a band of 457bp, corresponding to a functional hTERT full length transcript (FL), and in addition the α+β- (239bp) and β-splice variant (275bp), respectively. The telomerase-negative ZYGF-and MSPG-cells express only the β-splice variant. HTB57 and the normal embryonic lung fibroblast strain WI38 express none of the hTERT transcripts. In the equal lanes, the signals of hTERC of 126 bp are shown. Two markers of 50bp and 100bp are indicated. TA indicates the telomerase status.

5.3.3.3 Telomere lengths of human lung cancer cell lines

We determined the telomere lengths of the cell lines and investigated them at least at two occasions during continuous passage in culture to confirm not only the results but also the stability of telomere lengths in the proliferating cancer cells (Fig. 3, A+B). All but one cell lines revealed the obligatory DNA smear covering a few kb. The average terminal restriction fragments (TRFs) of four lung cancer cell lines and those of HeLa cells were in the range of 4 to 6kb. Extremely short but stable telomeres were detected in the TA-positive MBSJ cell line. With the TA-negative HTB57 cells, a bulk of telomeric DNA of approximately 20kb was found. Both, EtBr-staining of the gel as well as digestion with the exonuclease Bal31 (data not shown) indicated correct digestion and the telomeric nature of the DNA. The lack of TA together with the presence of long telomeres are the classical characteristics for the ALT mechanism of telomere elongation, which is operating in this cell line. As expected the telomeres of all but one of the cancer cell lines remained fairly constant during several passages irrespective of their TA status. In contrast and to some surprise, this was not the case with the TA-negative ZYGF-cells. The telomeres become shorter during continuous culturing with an average loss of approximately 150bp per cell generation (Fig. 3 B).

A

cell lines	HeLa	HTB57		LSWW		LPHE		MBSJ		ZYGF		MSPG		WI38 / 41
PDL difference		+ 6		+ 21		+ 11		+ 8		+ 22		+ 18		
lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14



B

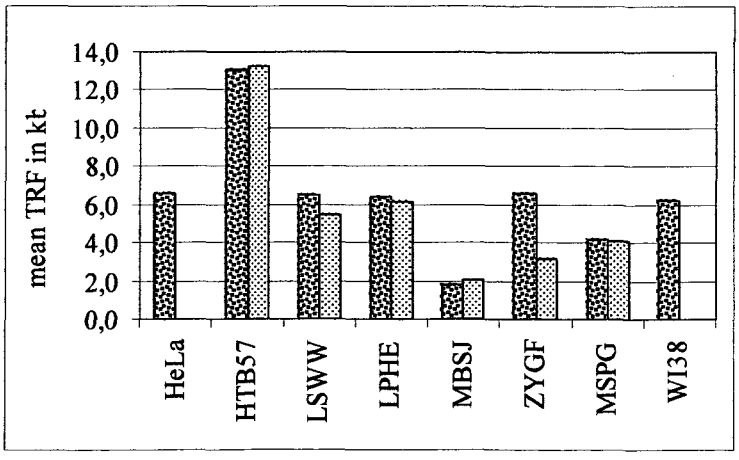


Fig.3. **Telomere lengths of lung cancer cell lines**. A. Determination of telomere lengths in lung cancer cell lines by Pulsed-field gel electrophoresis (PFGE). HinfI- and RsaI-digested DNA, corresponding to $2x10^6$ cells, was separated by PFGE and

telomeres visualised upon hybridisation with a telomeric probe. The lung cancer cell lines were analysed at two different occasions and the number of additional population doubling level (PDL) is indicated. WI38 were analysed in passage 41. B. Calculation of mean terminal restriction fragment (TRF) size in kb by densitometric evaluation of the Southern blot. The darker bars represent the mean TRFs at early cell passages, the dotted bars the later ones.

5.3.3.4 Effects of exogenous hTERT in TA-negative MSPG cells

It was of interest to study whether a hTERT expression vector could be of benefit for TA and/or telomere elongation in TA-negative cells. For these experiments we chose the MSPG cell line because the presence of the inactive β-splice variant appeared to exclude potentially TA-inhibiting factors in the cells. MSPG cells were transfected with various amounts of hTERT and a neo-coding gene for selection. Since mutations in the endogenous TERC gene could a priori not be excluded, we transfected also both genes in one experimental setting. In addition, c-Myc was shown to be able to induce endogenous TERT via E-box elements located close to the TERT promoter [reviewed in 42]. Thus, a c-MycER construct was also included in one of the DNA transfection mixtures. After the selection period in G418-containing medium, a number of colonies were isolated from each group, further propagated and analysed with regard to TA, gene expression and telomere lengths.

Among 12 analysed MSPG-sublines, subline 733 and 1012, which derived from cultures transfected with two different amounts of hTERT-DNA, showed high telomerase activity (Fig. 4, lane 5 and 11). The TA of subline 733 was similarly high as reference HeLa cells. Also subline 822 had a modest TA. The other subclones as well as the two isolated vector control sublines, 1111 and 1112, were TA negative. In order to prove the stability of telomerase expression in

sublines 733 and 1012 during cell culturing, we determined their TA at regular intervals. We found constant activities over more than 35 PDL as presented in Fig. 4, lane16-19. RT-PCR of hTERT expression of the 12 sublines (analogous to the experiments shown in Fig. 2) indicated a correlation between the acquired TA and the expression of hTERT full length transcripts in sublines 733, 822 and 1012 in addition to the parental inactive β-splice variant (data not shown). Growth curves of the TA-positive sublines showed that the efficient TA expression in subline 733 and 1012 provided a clear growth advantage compared to the TA-negative parental cells or sublines (data not shown).

5.3.3.5 *Transient hTERT expression in ZYGF cells*

We transfected the various transgenes into the TA-negative ZYGF cells, which also expressed the hTERT β-splice variant, and established a number of clones. Most of them were found to be TA-positive. However, the parental cells showed low TA at passage 28, a time point when their telomeres became quite short (Fig. 3, lane11). The spontaneous, late reactivation of telomerase in ZYGF parental cells, whose regulation is currently under investigation, hampered a valid analysis of the isolated sublines. In order to investigate whether ZYGF were nevertheless able to express a hTERT transgene, we transfected the cells transiently at low passage number and determined TA. As shown in Fig. 4, lane 20, transient expression of an exogenous hTERT gene was sufficient for high TA.

hTERT			X	X	X	X	X	X			X	X					X	X	X	X	hTERT	control
hTERC						X	X	X														
c-mycER									X	X												
vector											X	X	X	X					X	X		
MSPG sublines	HeLa	MSPG	711	721	733	811	812	822	912	921	1012	1021	1111	1112	LB		733 PDL 15	733 PDL 47	1012 PDL 15	1012 PDL 40	ZYGF	
lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		16	17	18	19	20	21

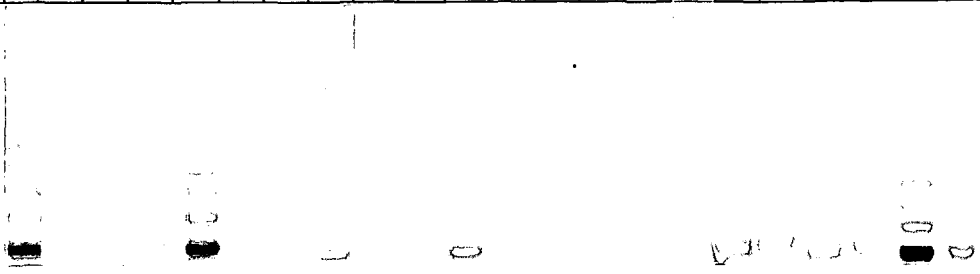


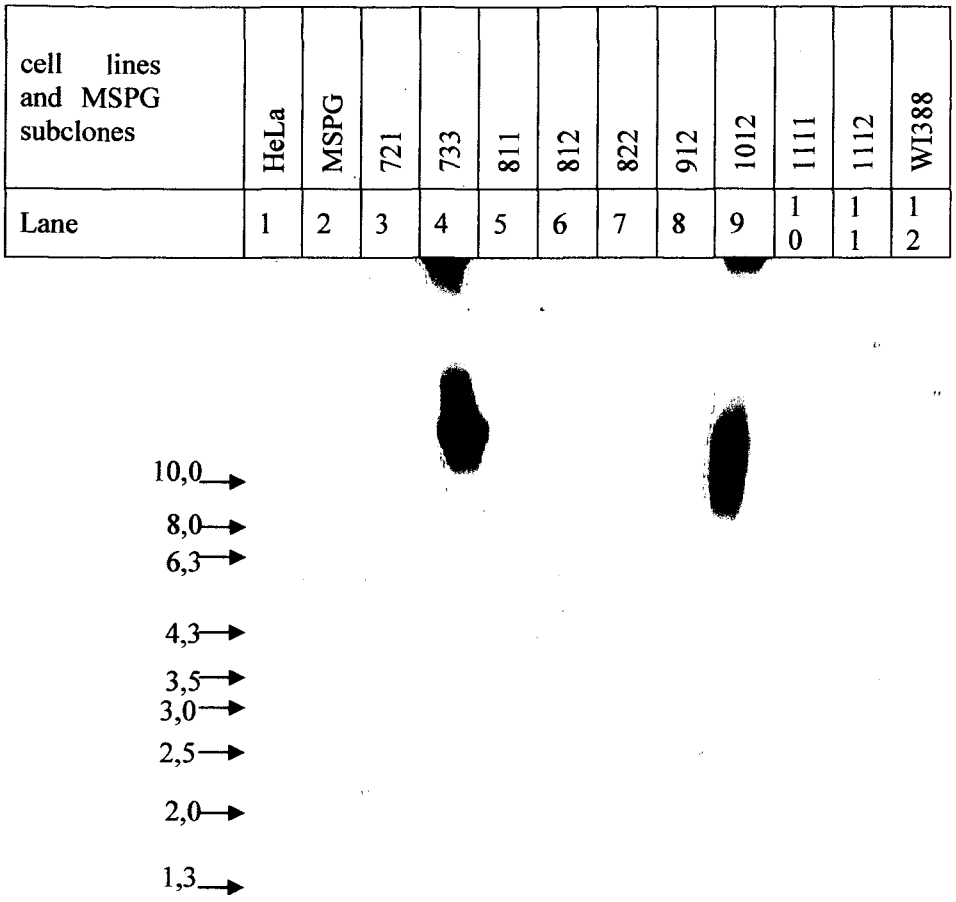
Fig.4. **TRAP assay of MSPG sublines:** Lane 2-14: telomerase activity in parental MSPG cells and 12 MSPG-derived sublines, transfected with hTERT with/without hTERC, compared with telomerase activity in HeLa cells (lane 1). Lane 15: lysis buffer (LB). Lane 16-19: Comparison of telomerase activity in subline 733 and 1012, respectively, during continuous cell growth. The population doubling level (PDL) after transfection with DNA is indicated. Lane 20 and 21: Telomerase activity in ZYGF-cells transiently transfected with hTERT DNA (lane 20) or vector (lane 21). The transfected genes are indicated by "X".

5.3.3.6 Enforced hTERT expression induces long, compact telomeres

We determined the telomere lengths in the various MSPG-sublines at the earliest convenience, i.e. when sufficient cells were available for DNA analysis (Fig. 5). This corresponded to about 20 cell generations between transfection with DNA, the selection period, clonal isolation and expansion. While the TA-negative sublines had mean telomere lengths of 4kb similar to the parental cells, the telomeric DNAs of the TA-positive sublines 733 and 1012 appeared as dense bulks of DNA, with mean TRFs of approximately 11 and 12kb, respectively (Fig. 5A, lane 4 and 9). Ethidium bromide staining of the gel as well as digestion of DNA with exonuclease Bal31 confirmed proper digestion and the

telomeric origin of the bulky DNAs. Since the 822 subline with its modest TA had telomere lengths similar to the parental cells (Fig. 5A, lane 7; and B), the efficient addition of telomeric sequences to the end of chromosomes obviously depended on a certain amount and/or activity of telomerase.

A



B

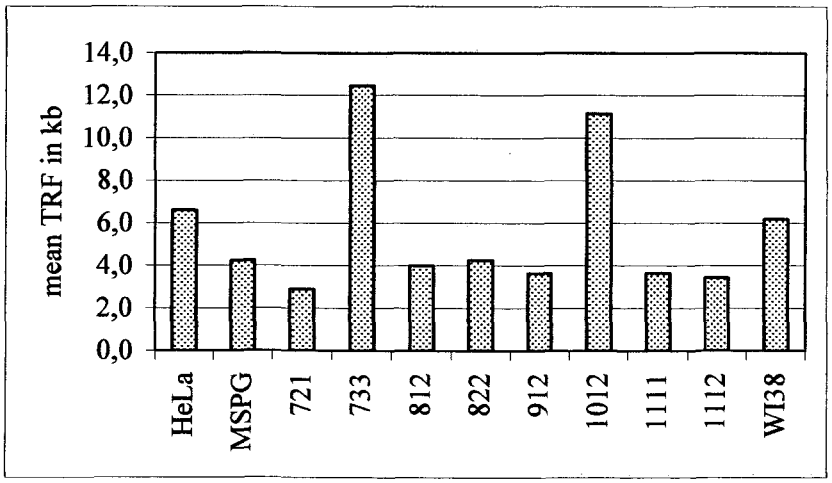


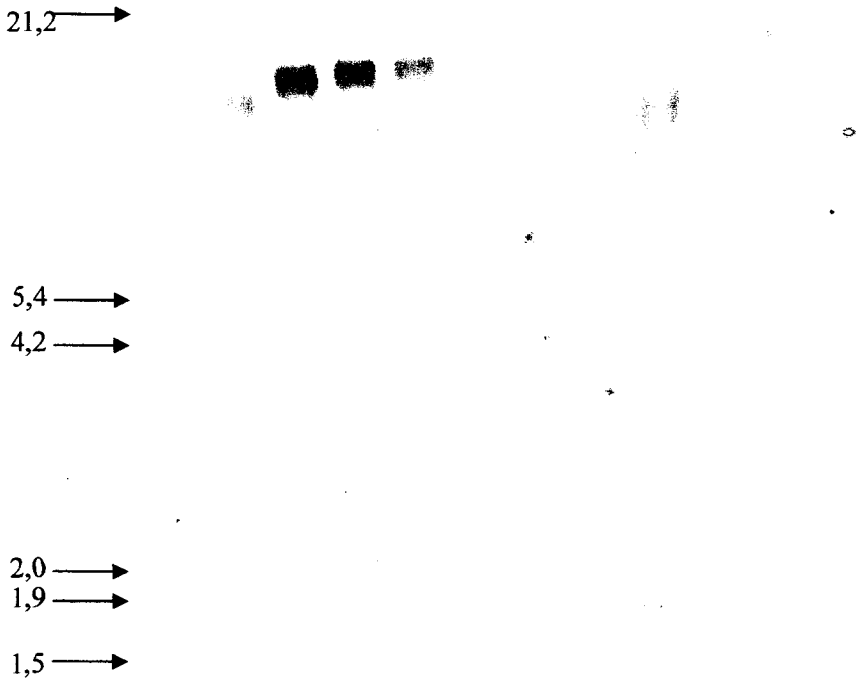
Fig.5. **Telomere lengths in MSPG sublines**. A. *Determination of telomere lengths in* MSPG sublines by PFGE as described in Fig. 3A. Transfected genes are the same as indicated in Fig. 4. B. Calculation of mean TRF in kb by densitometric evaluation of the Southern blot.

5.3.3.7 Processivity of exogenous hTERT

It was of interest to investigate whether the TA in the two sublines increased the telomeres constantly over time or whether there was an upper limit of telomere lengths. Fig. 6 shows that the latter is the case. The determination of telomere lengths in the course of continuous cell growth of both sublines indicated an upper limit of approximately 14kb. Moreover, assessment of the dynamics of telomere elongation revealed the addition of an average of 450bp during each of the first 20 cell generations (i.e. after DNA transfection), followed by a modest telomere elongation during the next 20 population doublings until the plateau level of 14-15kb was eventually reached. In comparison with ALT-HTB57 cells, both sublines differed also in another respect. Whereas HTB57 cells showed the obligatory DNA smear of telomeres, ranging from 2 to 20kb, the spectrum of telomere lengths in the two MSPG sublines was smaller. Specially in subline 733, the telomeres appeared uniformly long.

A

	MSPG	733				1012					HTB57	
PDL		19	30	39	47	16	24	31	36	40		+6
Lane	1	2	3	4	5	6	7	8	9	10	11	12



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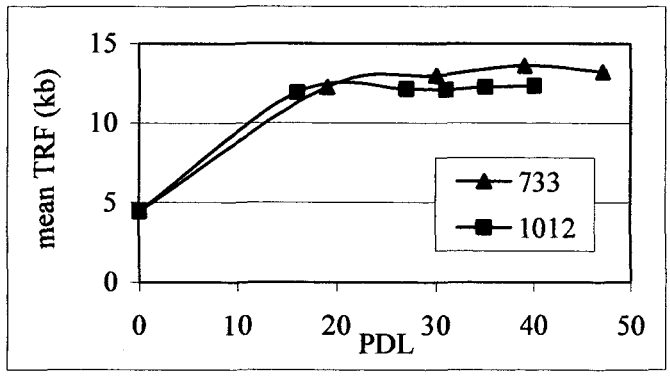
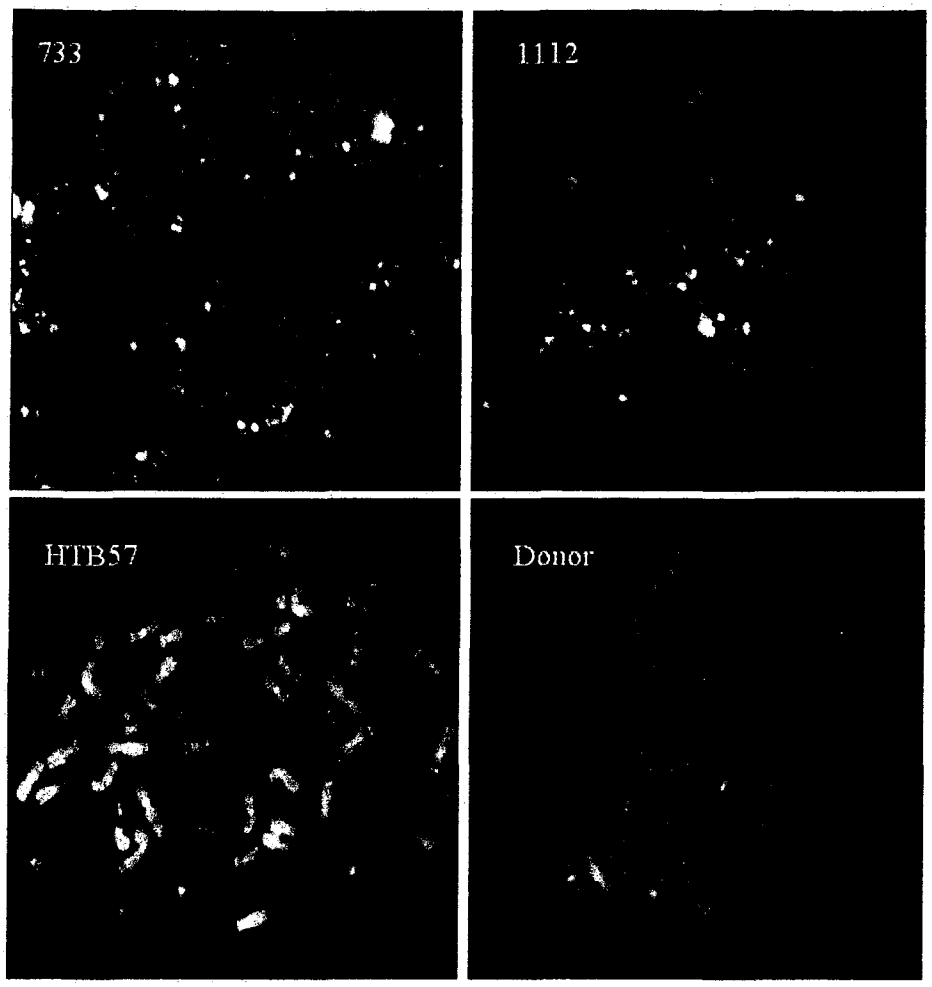


Fig.6. **Dynamics of telomere elongation by exogenous hTERT.** A. Subline 733 and 1012, respectively, were continuously grown in culture and genomic DNA prepared at regular intervals. The population doubling level (PDL) of the clonal founder cell is indicated. DNA was prepared as described in the legend to Fig. 3A and separated by PFGE. For comparison, HTB57 cells are shown in lane 11 and 12. B. Calculation of mean TRF from A for subline 733 (triangles) and 1012 (squares), respectively, in the course of increasing PDLs. 0: mean TRF of parental MSPG (lane 1 in A).

5.3.3.8 Q-FISH analysis of telomere lengths

We performed Q-FISH staining experiments of telomeres in order to visualise and confirm the results obtained by Southern blot analysis. As shown in Fig. 7A (right upper panel) and Fig. 7B (left panel) the MSPG-vector control subline 1112 had very short to almost undetectable telomeres which corresponded well to the Southern blot results (Fig. 5A). An identical staining pattern was also obtained with the parental MSPG cells (data not shown). In contrast, all chromosomes of subline 733 had almost equally long telomeres (Fig. 7A, left upper panel). A similar uniformity of telomere lengths was also observed with the other telomerase-positive MSPG subline 1012 (data not shown). The homogeneity of telomere length was especially obvious when comparing the stained telomeres of subline 733 with those of HTB57 cells (Fig. 7A, left lower panel, and Fig. 7B, right panel) which stabilise their telomeres by ALT. In HTB57 cells, corresponding to the bulky DNA and DNA smear revealed by Southern blot analysis (Fig. 3A and Fig. 6A), a broad variation of telomere lengths was evident in the prepared metaphases, ranging from intensively stained, long telomeres to almost undetectable ones. For comparison, lymphocytes from a normal donor were included in the analysis (Fig. 7A, right lower panel). Despite the differences in telomere length between the normal cells and the lung cancer cell lines, the variation in telomere lengths of the normal cells was similar to the MSPG vector control subline (Fig. 7B, right panel). In contrast, the variation of telomere lengths in the subline 733 was greatly reduced. In sum, these data suggest that telomerase is not only able to extend individual telomeres, but moreover to overcome local telomere length regulation, leading to homogeneously long telomeres.

A



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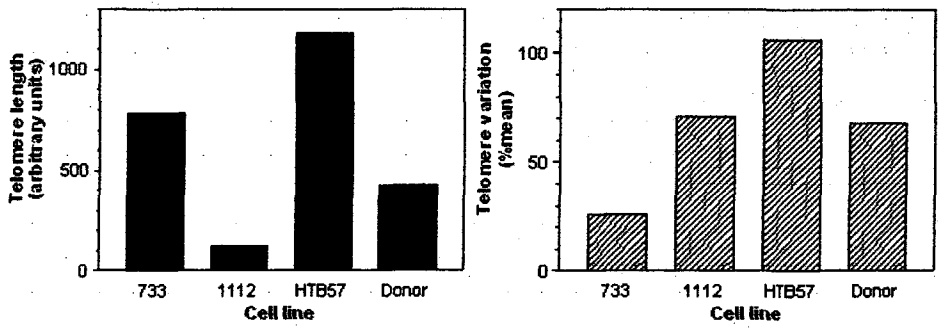

Fig.7. **Analysis of telomere lengths by Q-FISH.** Metaphase spreads were prepared and telomeric repeats detected by Q-FISH as described in Material and Methods. A. Images of representative metaphases derived from cell clones of the telomerase- and ALT-negative lung cancer cell line MSPG transfected stably with hTERT (733) or the control vector (1112), a lung adenocarcinoma cell line characterised by an ALT mechanism of telomere stabilisation (HTB57), and of lymphocytes from a healthy

person (donor) are shown. B. The lengths of at least 400 individual telomeres derived from at least three different metaphases were analysed by quantifying fluorescence intensity of single telomeres using the QWin quantification software. Telomere lengths are shown as mean arbitrary fluorescence units (left panel) and telomere variation was calculated as percent mean by the formula SD/mean x 100 (right panel).

5.3.4 Discussion

The current model on the maintenance of telomeres in cancer cells comprises two independent cellular strategies. One is the robust re-activation of telomerase, the other – usually in absence of telomerase – the induction of an alternative lengthening mechanism (ALT). The two mechanisms are not mutually exclusive since they can operate in the same cell [22,43,44]. For those tumor cells, which have neither of these mechanisms activated, it was suggested that immortalisation resulting from stabilisation of telomeres has not yet taken place [19]. Indeed, our results with the ZYGF cells support such a consideration. The originally telomerase-negative ZYGF cells lost continuously telomeric sequences and became spontaneously telomerase-positive after prolonged culturing. The fact, that the hTERT promoter in these cells was transcriptionally active as evidenced by the presence of the β-splice variant of hTERT, might have facilitated the shift to hTERT full length expression and, in turn, telomerase activity. We are currently analysing genetic differences between early- and late-passage-ZYGF cells. However, a third mechanism of telomere maintenance that is telomerase- and ALT-independent, is suggested by the presented data of MSPG cells and their sublines.

Although MSPG cells share the intact, activated hTERT promoter and the short telomeres with late-passage ZYGF cells, MSPG cells did not re-activate endogenous telomerase during long-term culture. This indicates absence of

telomere crisis and thus, other routes for telomere maintenance must exist. A previous analysis of 18 telomerase-negative tumors of different origin revealed abnormally long TRFs, characteristic for ALT, in only 4 samples whereas 14 tumors had TRFs in the normal range [19]. However, no data on telomere dynamics, TERT promoter activation or probably retarded telomerase re-activation are available for these tumors. In support of our data with MSPG cells, it was recently described that even in telomerase-positive cells telomere maintenance can be telomerase-independent. In one human ovarian cancer cell line, telomerase was efficiently inhibited during 15 weeks, however, the lack of telomerase did not affect the telomere lengths of the treated cells [45]. Thus, it is obvious that a subset of telomerase-negative and -positive tumor cells preserve their telomere lengths without activating either telomerase or ALT for that purpose.

The question arises how these tumor cells like MSPG-cells maintain their telomeres. It could be that the telomeric loss per cell generation is very modest and escapes measurement [46]. Alternatively discrete intra-telomeric recombinational events could counterbalance the loss. The latter possibility resembles telomerase-deficient SaccharoMyces cerevisiae Type I survivors. Deletion of the yeast RNA component results in cellular senescence. Rare survivors can be categorised into two groups with respect to their telomere structures. Type I survivors have short telomeres which are maintained by intratelomeric recombinational events, whereas type II survivors are characterised by long stretches of telomeric sequences resulting from intertelomeric recombination [47,48]. In addition, expression of ectopic telomerase in Type I survivors restores wild-type telomeres quickly, but only very slowly in Type II survivors [48].

The telomerase- and ALT-negative background of MSPG cells allowed investigation of the effect of enforced telomerase activity on certain parameters of telomere length regulation such as telomerase processivity and individual telomere length maintenance. In the telomerase-positive MSPG sublines, the telomeres were elongated very efficiently, with approximately 450bp per cell generation during the first 16 (in subline 1012) and 19 cell generations (in subline 733), respectively. When normal pre-senescent human cell strains were transfected with various hTERT constructs which resulted in telomerase expression and telomere elongation associated with extended life span, the processivity of telomerase was not more than maximal 200bp per cell generation corresponding to a range of a few to 30 telomeric repeats [11,49,50]. This value is lower than the initial 450bp that we had observed. However, the efficiency of telomerase depends on several other proteins, some of which might be present only in suboptimal concentrations in the normal human cell strains compared to established human cancer cell lines. The capacity of the ectopic telomerase in the MSPG sublines, which added about 75 telomeric repeats per cell division, is probably an underestimation due to the experimentally unavoidable gap in telomere length determination between the founder cells of the sublines and sufficient successors for appropriate DNA analysis. It can be speculated that the initial increase in telomere lengths is rather exponential than linear. It was repeatedly observed that telomerase prefers elongation of populations of short telomeres [22,43,44]. Also in ALT cells with ectopic expression of telomerase, the short telomeres appear to be preferential substrates for telomerase [43]. One protein relevant for the accessibility of telomerase to telomeric ends is the recently described POT1, which acts as a negative regulator of telomerase [33,34]. On short telomeres

the low amount of POT1 molecules, which are associated with TRF1, allows telomere elongation by telomerase. Conversely, on long telomeres with abundantly bound TRF1 complexes, POT1 inhibits telomerase activity. POT1 has a single- and double-stranded DNA binding region with which it contacts telomeric DNA. When many telomeric repeats are present, then POT1 is transferred to the 3'-single stranded DNA and there it might act as a negative regulator by directly inhibiting the catalytic activity of telomerase or by blocking access of telomerase to its substrate [33]. Thus, elongation of telomeres hinders more and more the access of telomerase to the 3'-ends of telomeres until a steady state between telomere loss and newly synthesised repeats is achieved. The upper limit of telomerase-regulated telomere lengths in human cells appears to be in the range of 12 to 19kb where this equilibrium is accomplished [11,14,15,34,49,50,51].

To some surprise, the efficient expression of telomerase in the MSPG sublines was able to overcome local telomere length control which is responsible for the individuality of telomere lengths. This regulation is independent of telomerase activity since in the majority of telomerase-positive tumors isolated telomeric DNA appears as smear in gel electrophoresis covering several kb [37,51]. First data on the regulation of individual telomere length have been reported only recently [35,36]. A detailed analysis on the telomere profiles of 20 individuals showed significant similarities among the individuals, irrespective of the cell type investigated [36]. This is suggestive for a continuous, local control of the individuality of telomere length that is genetically determined. How this is achieved and preserved over many cell generations is not understood. There are however some indications that the chromosome specific differences in telomere length are governed by factors located very distally on the

chromosome arms [36]. Whatever the regulatory mechanisms might be, they are relaxed or abolished in both telomerase-positive MSPG-sublines, especially in subline 733, which enables telomerase to act unrestrictedly on all chromosomal ends. Such a dominant telomerase activity was also observed with a hTERT-transfected ALT cell line [44]. FISH analysis of several hTERT-expressing subclones revealed that besides very intensively stained telomeres (corresponding to very long telomeres) almost all originally undetectable telomeres became detectable upon the action of ectopic telomerase.

The data presented here provide new insights into the regulation of telomere lengths in human tumor cells by telomerase and also suggest a telomerase- and ALT-independent telomere maintenance mechanism which might operate in a subset of tumor cells. In view of the current interest in developing anticancer drugs directed against telomerase, it will be of particular importance to analyse the individual tumors with regard to their respective telomere maintenance mechanism.

5.3.5 Material and Methods

5.3.5.1 Cell culture and electroporation

The normal human embryonic lung fibroblast strain, WI38, and human lung cancer cell line, HTB-57 (synonym for the SK-LU-1 cell line) [19] derived from an adenocarcinoma, were obtained from ATCC and grown in MEME. All other cell lines were cultivated in RPMI1640 at 37°C and 5% CO_2. Media were supplemented with 10% FCS and 10U/ml penicillin/streptoMycin. Four human

lung cancer cell lines, LSWW, MSPG, MBSJ, and ZYGF, were from squamous cell carcinoma, LPHE was derived from a large cell lung carcinoma. For electroporation (Equibio EasyjecT Plus), $1x10^6$ cells of ¾-confluent cell layers were mixed with a total amount of 25µg DNA in 1ml growth medium. Electroporation was performed at 280V and 1500µF. The DNA mixture contained 5µg pVV2, coding for a neo-resistance gene, and either 10 or 20µg pSP-hTERT, coding for the catalytic component of telomerase, and 10µg pSP-hTERC, coding for the RNA component of telomerase. One group received 10µg c-MycER-DNA, coding for c-Myc fused to a modified Tamoxifen-inducible oestrogen receptor (kindly provided by Dr.Downwards). Where necessary, the empty vector pSP was used to adjust to 25µg DNA. After electroporation, cells were seeded immediately into three 6cm-petri dishes and medium was replaced the next day to remove cell debris. 24h later, G418 (Calbiochem) was added at a concentration of 200µM and medium was changed twice a week. Monoclonal colonies were isolated by means of steel cylinders approximately four weeks after electroporation and further propagated in selection medium.

Growth curves were obtained by seeding $1x10^5$ cells in 6cm-petri dishes and cell numbers were determined every third day using a counting chamber. Medium was changed regularly.

5.3.5.2 Plasmids

To obtain pSP-hTERT and pSP-hTERC expression vectors, the plasmids pGRN145 and pGRN83 (both kindly provided by Geron Inc.) were cut with EcoRI and the purified inserts cloned into the EcoRI site of the pSP expression vector [52].

5.3.5.3 Telomeric Repeat Amplification Protocol (TRAP) Assay

Cell lysates were prepared using CHAPS detergent lysis method [9]. The protein concentration of lysates was measured using the BioRad (Bradford) protein assay. The PCR-based TRAP assay for detection of telomerase activity was performed with some modifications. 0.5µg of lysates were subjected to 25 PCR cycles. The amplification products were separated on a 10% nondenaturing polyacrylamide gel, stained with VistraGreen (Amersham Bioscience) and visualized in a FluorImager 595 (Molecular Dynamics) using ImageQuant 5.0. Semiquantitative densitometric evaluation was performed by ImageQuant 5.0.

5.3.5.4 Analysis of terminal restriction fragment (TRF) length

Preparation of genomic DNA. Cell lines were grown to confluence and washed once in buffer TD (137mM NaCl, 5mM KCl, 0.34mM $Na_2HPO_4.2H_2O$, 25mM Tris-HCl). Cell membranes and proteins were digested by incubation of the cell suspension with 250µg proteinase K and 0.7% SDS for 16h at 50°C. Genomic DNA was extracted by phenol-chloroform method and 10µg of DNA was

digested by 30U Hinf1 (Invitrogen) and 30U Rsa1 (Invitrogen) in REAct 1 buffer for 16h at 37°C.

Pulsed-field gel electrophoresis (PFGE). Digested DNA samples were run on a 1.0% pulsed-field-grade agarose (BioRad) gel in 0.5x Tris borate EDTA for 7h at constant 9V/cm, using a pulse time of 0.1sec. Electrophoresis buffer was kept at 14°C. A CHEF-DR III PFG apparatus (BioRad) was used.

Hybridisation of gels. Following electrophoresis and EtBr staining, gels were dried on filter papers for 1h at 60°C. Oligonucleotides $(TTAGGG)_4$ were end-labeled with [γ-^{32}P]ATP and then purified using NICK Columns Sephadex G-50 DNA grade (Amersham Bioscience). Gels were prehybridised for 4h at 37°C in10mM Na_2HPO_4, 0.84mM $Na_4P_2O_7$, 5x Denhardt's solution and 4x SSC, and hybridised with the probe for 24h at 37°C in 20ml prehybridisation solution. Then, gels were washed twice for 20min in 0.1x SSC at 37°C. Image analysis was performed using a PhosphorImager (Molecular Dynamics) and ImageQuant 5.0 for TRF quantification.

5.3.5.5 RT-PCR

cDNA was synthesised using 5µg total RNA isolated from growing cells by TRIzol reagent (Invitrogen) in a 20µl RT-reaction mixture (RevertAid First Strand cDNA synthesis kit, MBI). 1µl of the RT-reaction mixture was subjected to PCR. Primers for detecting hTERC, hTERT-total transcripts, hTERT-splice variants and hGAPDH and the corresponding expected PCR-products are listed below (Tab. 1). The following PCR conditions were used for all primers in a PCR-Express (Thermo Hybaid): Hot start by adding taq-polymerase (Roche Diagnostics) after an initial heating at 94°C for 2min, followed by 10 cycles

denaturation at 94°C for 30sec, annealing at 65°C for 30sec and extension at 72°C for 30sec. Then the PCR was continued with 15 cycles at the same conditions as described before except an annealing temperature of 60°C for 30sec. After cycling, a final extension step at 72°C for 5min was performed. PCR products were separated on a 5% non-denaturating polyacrylamid gel in a Protean-III electrophoresis chamber (BioRad), stained with VistraGreen (Amersham Biosciences), visualised on a FluorImager595 (Molecular Dynamics) and adapted for print with ImageQuant 5.0 (Molecular Dynamics) and Photoshop 7.0 (Adobe). Amplification of hTERT splice variants and hGAPDH was performed in a parallel reaction for internal control of the amount of applied cDNA.

	Primer	product
hTERT splice variants	GCCTGAGCTGTACTTTGTCAA CGCAAACAGCTTGTTCTCCATGTC	457bp (full-length) 421bp (α-splice variant) 275bp (β-splice variant) 239bp (α+β splice variant)
hTERC	TCTAACCCTAACTGAGAAGGGGCG GTTTGCTCTAGAATGAACGGTGGAAG	126bp
hGAPDH	CGGGAAGCTTGTGATCAATGG GGCAGTGATGGCATGGACTG	357bp

Tab.1 List of primers and expected products

5.3.5.6 Q-FISH analysis of metaphase chromosomes

Metaphase spreads from lymphocytes of a healthy donor were prepared by standard methods. Tumor cell lines were seeded at low density, cultured in growth medium with 10 % FCS for 24h. Colcemid (10ng/ml) was administered for 60 min and metaphase spreads prepared according to standard methods. Slides were aged at room temperature for 48h and at 90°C for 1h. Telomere length based on telomere signals of individual chromosomes was detected by quantitative fluorescence in situ hybridisation (Q-FISH) using the Telomere PNA FISH Kit/FITC according to the manufacturer's instructions (Dako). Metaphases were captured by QFISH software with a Leica DMRXA fluorescence microscope (Leica Mikroskopie und System, Wetzlar, Germany) equipped with appropriate epifluorescence filters and a COHU charged-coupled device (CCD) camera. Images were analysed by the Leica Q Win software employing the image analysis and particle detection functions. Means of fluorescence intensity of at least 400 individual telomeres are given in arbitrary units of telomere length. Variation of telomere length was calculated by the formula standard deviation (SD)/mean x 100.

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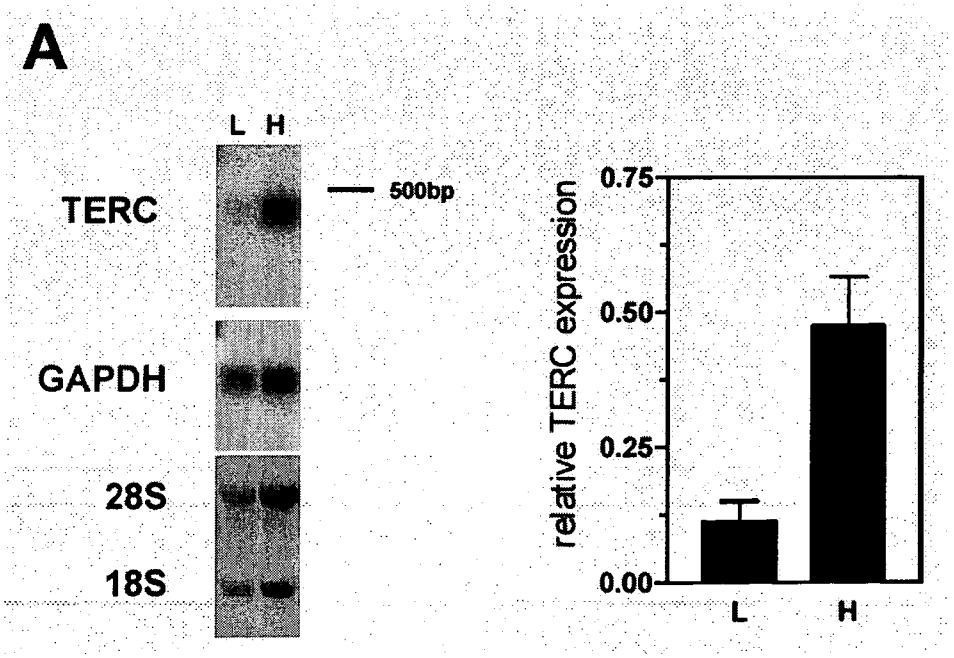
5.4 DETECTION AND QUANTIFICATION OF TRANSCRIPTS FOR THE CATALYTIC SUBUNIT TERT AND THE RNA COMPONENT OF TELOMERASE IN RAT TISSUE

Abbreviations: TERT, telomerase reverse transcriptase; TERC, telomerase RNA component; BLAST, Basic Local Alignment Search Tool; NCBI, National Center for Biotechnology Information

The enzyme telomerase is responsible for maintaining the ends of chromosomes i.e. telomeres, and has been found involved in cancer and immortalization (1-3). The telomerase complex comprises among other proteins two main subunits which provide the enzymatic core function, the telomerase reverse transcriptase (TERT)[2] (4) and an RNA component (TERC) (5). TERC is necessary for enzyme activity as demonstrated by appropriate anti-sense experiments and was shown to function as template for the telomeric oligo-repeat sequences (5). While TERC is ubiquitously expressed in normal somatic human cells, expression of TERT is limited to highly proliferative tissues such as germ cells and stem cells. Thus, the presence of TERT is rate limiting for telomerase activity. Importantly, up to 95% of cancer cells express TERT and telomerase activity (4). Expression of both, TERT and TERC, has been studied in human, mouse and unicellular organisms. There are, however, only scarce data on telomerase genes in the rat. The TERC and TERT genes from human (5-8) and mouse have been cloned (9-11) and sequence information is available. Recently the sequence of the rat TERC gene has been reported (12). Primers for the 3'-part of the rat TERT cDNA have been described (13, 14), but no sequence information about the rat TERT gene is yet available. Therefore we set out to develop tools which allowed investigations of both, the TERT and TERC mRNA expression, in the rat.

For that purpose, we searched with the published rat TERC sequence from GenBank accession number AF221916 (12) the EST database (NCBI, http://www.ncbi.nlm.nih.gov/) and found an EST cDNA clone encoding 95% of the rat TERC gene within a 408 bp fragment (GenBank accession numbers BF552926 and AI072290). The respective EST cDNA clone number IMAGE:1786369 was obtained from German Human Genome Project Resource Center (RZBD, Berlin, Germany) and used as hybridization probe for Northern analysis of rat total RNA samples. Fig.

1A shows a representative Northern blot experiment analyzing two oncogene-transformed rat cell lines: one clone with low telomerase activity (clone L) and one with high (clone H) as measured by a modified telomeric repeat amplification protocol (TRAP; 15) (Fig. 1B). The TERC probe hybridized to a 450 bp transcript which is in agreement with the size of the published rat TERC mRNA (12). For control purpose and for normalization of rat TERC expression, the RNA blots were hybridized with a probe for rat GAPDH (Fig. 1A *middle panel*). The integrity of the analyzed RNA was proven by the presence of intact 28S and 18S rRNA (Fig. 1A *lower panel*). Signals for GAPDH, 28S and 18S rRNA allowed normalization of rat TERC expression. Identical data were obtained by analyzing the TERC mRNA level relative to either the house keeping or the ribosomal genes (data not shown). The telomerase activity of the rat cell lines shown in Fig. 1B correlated with the TERC gene expression.



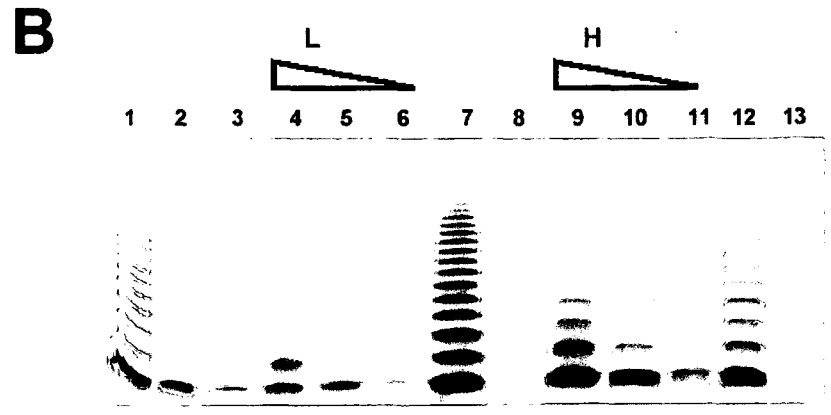


Fig.1 **Detection of rat TERC mRNA expression by Northern blot analysis in cell lines with low (L) and high (H) telomerase activity. A,** 5µg total RNA were separated on a 1% denaturing formaldehyde agarose gel, transferred with PressureBlot System (Stratagene, La Jolla, CA) onto nylon membranes (Amersham Pharmacia Biotech, UK), immobilized with Stratalinker UV crosslinker (Stratagene) and hybridized using ExpressHyb hybridization solution (Clontech) as recommended by the manufacturer. Autoradiograms generated by PhosphoImager (Molecular Dynamics, Sunnyvale, CA) using random labeled probes for rat TERC (*upper panel*) and rat GAPDH (*middle panel*). EtBr-stained 28S and 18S rRNAs was detected by FluorImager595 (Molecular Dynamics) (*lower panel*). Signals were quantified by densitometry and TERC mRNA levels were calculated relative to GAPDH expression. Means and SEM of two independent experiments are shown (*right panel*). **B,** various amounts of cell lysates were subjected to PCR-based TRAP assay. Amplification products were separated on a 10% non-denaturing polyacrylamide gel, stained with Vistra Green and detected by FluorImager595: Lane 1, lysate of HeLa cells; lanes 4-6 and 9-11 decreasing amounts of lysates (5.0, 2.0 and 0.5 µg protein) indicated by open triangles obtained from L and H cell lines, respectively; negative controls for telomerase activity (containing 5µg cell lysate and RNase): lane 2, HeLa cells; lane 3, clone L; lane 8, clone H. Lane 13 contains lysate buffer only. Positive controls contain a mixture of 0.5 µg HeLa lysate and 0.5 µg of clone L (lane 7) or H (lane 12).

In order to identify the rat TERT gene we searched public databases available at NCBI with sequences of human and mouse origin. A single GeneBank entry designated as rat TERT fragment was found which contained sequence information of 1.8 kb from the 3'-part of the TERT cDNA (accession AF247818). However, the fact that this sequence was completely identical to the published mouse TERT sequence, raised some doubt about its proper designation. Thus, we further

searched for rat genome sequences using the "rat genome BLAST" (NCBI, http://www.ncbi.nlm.nih.gov/). Indeed, for most parts of the mouse TERT cDNA we found corresponding rat genome sequences. The respective fragments were obtained from a database containing all rat raw whole genome shotgun (WGS) and bacterial artificial chromosome (BAC) sequence data. All these sequence traces represent single pass reads with a mean size of approximately 800 bp. The rat TERT fragments obtained by BLAST search were aligned to the mouse cDNA which allowed us to identify *in silico* most of the rat TERT cDNA as well as the exon/intron boundaries. The similarity between mouse and rat TERT coding region was found to be as high as 80%. The first intron within the TERT coding region was completely covered by several rat genome clones and was estimated to be a 101 bp sequence. Therefore we used exon 1 and 2 for designing PCR primers (rTERT-forward 5′-GGTCTTCCGCACGTTGGTTG-3′ and rTERT-reverse 5′-CAGCAGGTAGAGCGC ACAGT-3′) to amplify a specific cDNA fragment (349 bp), which distinguished it from both, the corresponding unspliced RNA and the genomic DNA fragment (450 bp). Total RNA and genomic DNA were isolated from rat cell lines and used to amplify the corresponding TERT fragments (Fig. 2A). PCR products were cloned into pCR2.1-TOPO vector (Invitrogen, Groningen, The Netherlands) for automated sequencing (VBC Genomics, Vienna, Austria) and data (DNA and cDNA) deposited in GenBank/EMBL/DDBJ (accession AJ440966 and AJ440965, respectively). In Fig. 2B the sequences of the PCR products (DNA and cDNA) for the rat TERT gene were aligned to those for mouse and human and the encoded rat TERT protein fragment is indicated. Consensus sites for splicing were identified at the exon/intron boundaries. Exon sequences for mouse, human and rat TERT were highly similar in contrast to the non-coding intron sequences. The sequenced fragment can be

estimated to represent approximately 10% of the complete coding region expected for the rat TERT gene.

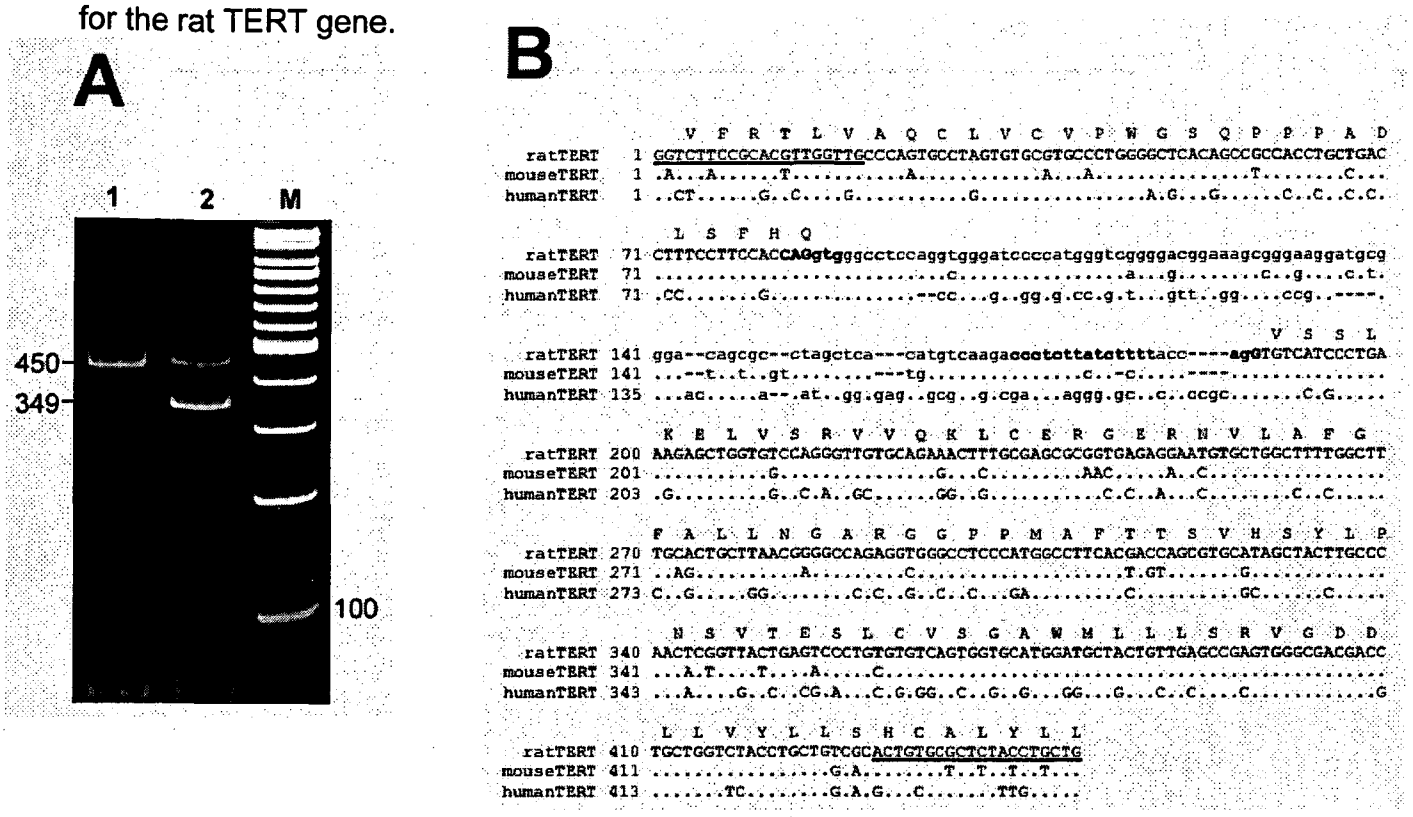


Fig.2 **Identification of the rat TERT gene. A**, TERT fragments from rat MR-2 cells (15) were amplified by PCR and RT-PCR using rTERT-forward and -reverse primer, separated on a 6% polyacrylamide gel and visualized by EtBr staining. Lane 1, PCR product from 0.1 ng genomic DNA; lane 2, RT-PCR product of total RNA reverse-transcribed with hexanucleotide primers and MMLV reverse transcriptase (Clontech); lane M, 100 bp DNA ladder marker. PCR was performed with PCR Master Mix (Promega, Madison, WI) as recommended by the manufacturer, T_A= 60°C and amplification time was 1.5 minutes per cycle. **B**, Rat TERT sequence from genomic and cDNA fragments (450 bp and 349 bp, respectively) aligned with sequences from the mouse and human TERT genes. Differences in the sequence of mouse and human compared to the sequence of rat are indicated. Dots represent identities whereas dashes are deletions. Exon and intron sequences are marked by capital and small letters, respectively. Consensus sequences important for splicing (acceptor, lariat and donor sites) are in bold. Positions of primers are underlined. Above the rat sequence the postulated reading frame of rat TERT is shown.

Expression levels for the TERT gene have been reported to be in the range of 0.2-30 molecules per cancer cell (16, 17). Because of this low abundance and the expected size of the transcript overlapping with 28S rRNA, we established a sensitive semi-

quantitative RT-PCR assay to quantify the TERT expression levels in relation to the GAPDH expression. In addition, for amplification of a specific 369 bp cDNA fragment of the rat TERC gene the primers rTERC-forward 5'-GTCTAACCCTATTGTTATAGC TG-3' and rTERC-reverse 5'-CAGGTGCACTTCCCACATCT-3' were employed. First strand cDNA synthesis was performed with MMLV reverse transcriptase and random hexanucleotide primers.

As shown in Fig. 3A aliquots of cDNA obtained from normal tissues and from transformed rat cell lines were amplified with primer pairs specific for TERT, TERC and GAPDH (Clontech, Palo Alto, CA), using 34, 32-34 and 27-28 cycles, respectively. Linear range of the RT-PCR assay was established for each primer set by performing 20-40 cycles in 2 cycle steps. After having established the appropriate cycle numbers for each primer set, linearity was confirmed by serial dilutions of cDNA. In most tissues analyzed the expected 349 bp fragment of the TERT cDNA was amplified. In liver and kidney tissue as well as *in vitro* cultured fibroblasts, an additional weak band of 450 bp was detectable. This does more likely originate from an unspliced TERT RNA as from traces of genomic DNA because cDNA had been synthesized from poly A+ RNA. Normalization to GAPDH allowed the comparison of the relative TERT and TERC expression levels among diverse tissues and cells (Fig. 3B). TERT and TERC were found to be coordinately expressed in rat tissues whereby higher expression levels were detected in liver, spleen, lung, testis and kidney. From the eight tissues analyzed the liver exhibited the highest amounts of the transcripts. This is in agreement with high telomerase activity observed in adult rat hepatic epithelial cells (18) and high TERT transcript levels found in adult mouse liver (19).

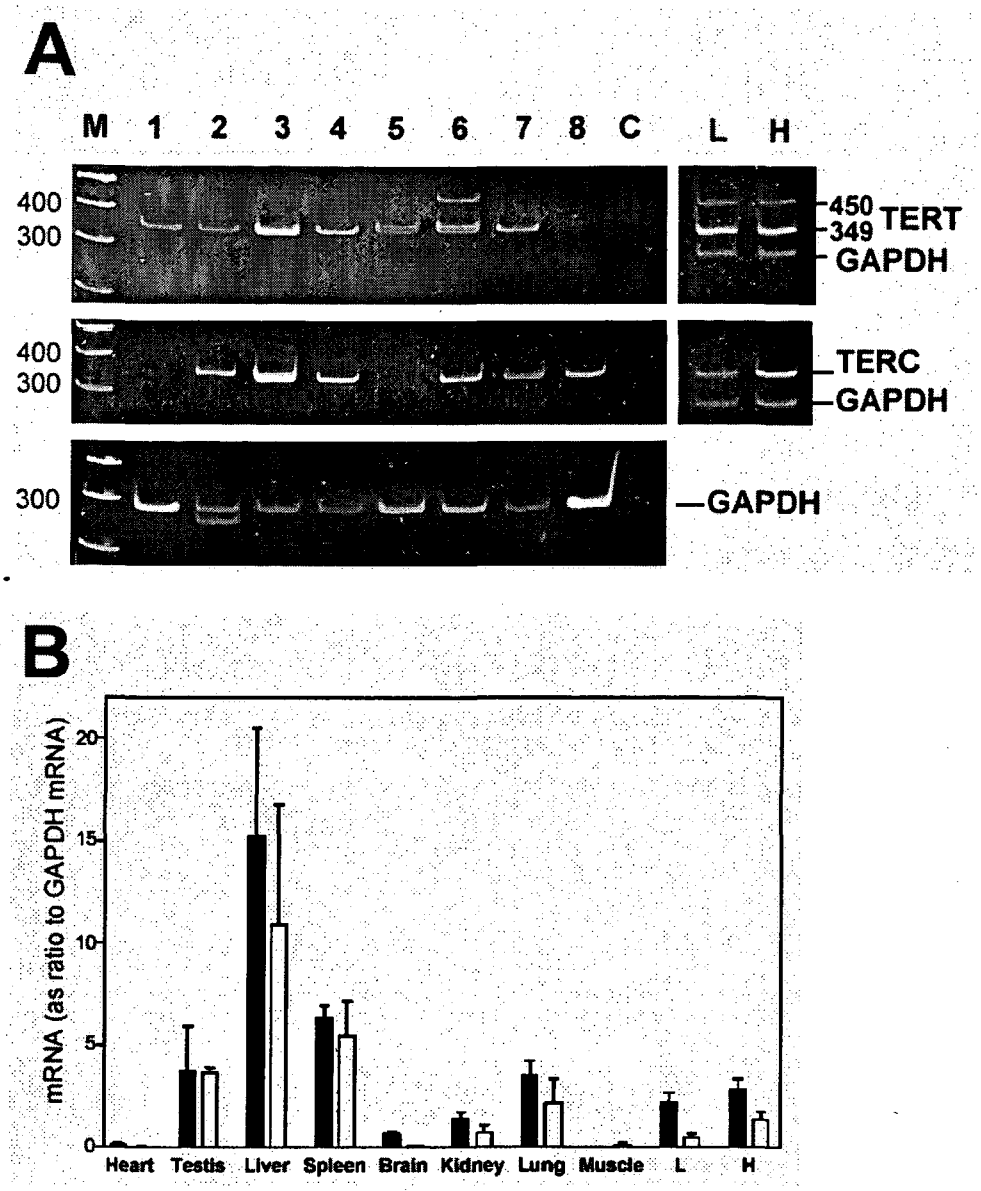


Fig.3 Detection **of rat TERT and TERC mRNA expression by RT-PCR. A,** Normalized cDNA panels derived from polyA+ RNA from heart (lane 1), testis (2), liver (3), spleen (4), brain (5), kidney (6), lung (7) and skeletal muscle (8) or no cDNA as control (C), as well as cDNA from transformed rat cell lines with low (L) and high (H) telomerase activity were amplified by RT-PCR. Product separation, visualization, and PCR conditions were as described in Fig. 2. At least three independent experiments were performed and representative results are shown. **B,** Expression levels of telomerase gene mRNAs were normalized to GAPDH mRNA: the 349 bp fragment of TERT (black boxes) and TERC (open boxes). Band quantitation was performed by densitometry within the linear range using a FluorImager595 (Molecular Dynamics). Means and SEM of three independent experiments are indicated.

By the use of *in silico* data, we have developed a sensitive, specific RT-PCR assay which allows measuring mRNA expression levels for the essential telomerase components, TERT and TERC, in rat cells and tissues.

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5.5 CLONING AND CHARACTERIZATION OF THE RAT TELOMERASE CATALYTIC SUBUNIT (RnTERT) GENE AND IDENTIFICATION OF THE PROXIMAL CORE PROMOTER REGION

By the use of in silico data analysis, we have amplified and cloned the full-length rat TERT cDNA (around 3400 bp) and the core region of the promoter (GenBank Accession No. AJ488949) by RT-PCR using specific primers.

Sequence analysis revealed that the rat TERT promoter is GC-rich, has no detectable TATA or CAAT boxes but contains binding sites for several transcription factors. Notable among this is one binding site (E-Box) for the c-myc oncogene, which is known to induce TERT expression. A sequence of approximately 750 bp upstream of the rat TERT start codon was cloned in a luciferase reporter vector (pGL3-basic). To investigate the role of c-Myc protein in the ratTERT gene transcription, a human c-myc cDNA expression plasmid was cotransfected with the rat TERT promoter-luciferase constructs into telomerase-negative FR3T3 cells. My preliminary data indicate that c-Myc has a positive effect of rat TERT expression. Deletion- and mutation analysis of the E-Box region confirmed these results. The *in vitro* translation analysis using [^{35}S] Methionine and standard rabbit reticulocyte lysate systems (RRL) indicate that the rat TERT protein is approximately 120 kDa. This result corresponds to the expected size and thus confirm the correct cloning procedure. Additionally, the function of rat TERT protein was examined *in vivo*.

6. DISCUSSION

A number of recent observations support the identification of the hTERT gene as a c-Myc target: (*i*) ectopic expression of c-Myc induces hTERT expression and activates telomerase activity in human cells [105]; (*ii*) the specific Myc binding motifs, E-boxes, are present in the hTERT promoter, and the activity of the promoter is significantly increased by transient expression of c-Myc [90, 101-103]; (*iii*) ectopic expression of the Mad1 protein results in repression of hTERT expression [107, 108]; and (*iv*) *in vivo* interaction between N-Myc and the hTERT promoter was recently observed in a neuroblastoma cell line transfected with N-myc [148].

To determine whether the endogenous rat TERT promoter is regulated by exogenous c-Myc and Mad1 proteins in cooperation with exogenous c-Ha-ras transformed cell lines derived from primary rat embryo cells (RECs) that express the human c-myc and c-H-ras genes together with a mad1-estrogen receptor fusion construct, mad1ER, were generated. The effect of activated mad1 on cell proliferation was investigated *in vitro* and *in vivo*. Our data demonstrate that activated Mad1 inhibits *in vitro* telomerase activity and proliferation of the transformed cells. *In vitro* assays indicate that RECs with high expression level of c-Myc and c-Ha-ras result in aggressively growing tumors after a short latency period. In contrast, continous activation of Mad1 prolonged the latency period of tumor appearance and reduces the growth rate of tumors in an OHT-dose (4-Hydroxy-Tamoxifen) dependent manner. Mad1 did not only inhibit tumor growth but also downregulates myc-expression in tumors. High telomerase activity leads in turn to elongation of telomeres, however in a disproportional manner. I conclude that c-myc

mediated telomerase exerts its effect mainly on long telomeres. Since a reduction in c-Myc levels causes rapid loss of telomeric sequences of the longest telomeres (manuscript in preparation), it is possible that c-Myc upregulates also other proteins involved in telomere length maintenance such as TRF1 or TRF2. Alternatively, c-Myc-mediated telomerase activity might counterbalance other cellular mechanisms which are aimed at telomere reduction

The current model on the maintenance of telomeres in cancer cells comprises two independent cellular strategies. One is the robust re-activation of telomerase, the other – usually in absence of telomerase – the induction of an alternative lengthening mechanism (ALT). The two mechanisms are not mutually exclusive since they can operate in the same cell [149-151]. For those tumor cells which have neither of these mechanisms activated, it was suggested that immortalisation resulting from the stabilisation of telomeres has not yet taken place [125]. The findings of my thesis indicate that the telomerase negative human lung cancer cell MSPG did not re-activate endogenous telomerase during long-term culture compared to ZYGF cells. The originally telomerase-negative ZYGF cells lost continuously telomeric sequences and became spontaneously telomerase-positive after prolonged culturing. The fact, that the hTERT promoter in these cells was transcriptionally active as demonstrated by the presence of the β-splice variant of hTERT, might have facilitated the shift to hTERT full length expression and, in turn, telomerase activity.

The question arises how these tumor cells like MSPG-cells maintain their telomeres. It could be that the telomeric loss per cell generation is very modest and escapes measurement [133]. Alternatively discrete intra-telomeric recombinational events could counterbalance the loss. The telomerase- and ALT-negative background of MSPG cells allowed investigations on the effect of enforced telomerase activity and on certain parameters of telomere length regulation such as telomerase processivity

and individual telomere length maintenance. In the telomerase-positive MSPG sublines, telomeres were elongated very efficiently with approximately 450bp per cell generation (in subline 1012 and 733), respectively. When normal pre-senescent human cell strains were transfected with various hTERT constructs which resulted in telomerase expression and telomere elongation associated with extended life span, the processivity of telomerase was not more than maximal 200bp per cell generation [105, 152, 153].

To some surprise, the efficient expression of telomerase in the MSPG sublines was able to overcome local telomere length control, which is responsible for the individuality of telomere lengths. This regulation is independent of telomerase activity since in the majority of telomerase-positive tumors isolated telomeric DNA appears as a smear in gel electrophoresis covering several kb [5, 154].

The data presented here provide new insights into the regulation of telomere lengths in human tumor cells by telomerase and also suggest a telomerase- and ALT-independent telomere maintenance mechanism, which might operate in a subset of tumor cells. In view of the current interest in developing anti-cancer drugs directed against telomerase, it will be of particular importance to analyse the individual tumors with regard to their respective telomere maintenance mechanism.

7. PUBLICATIONLIST

7.1 MANUSCRIPTS

7.1.1 Fuhrmann, G., C. Leisser, G. Rosenberger, M. Grusch, S. Huettenbrenner, T. Halama, I. Mosberger, **S. Sasgary**, C. Cerni, and G. Krupitza. "Cdc25a Phosphatase Suppresses Apoptosis Induced by Serum Deprivation." *Oncogene* 20, no. 33 (2001): 4542-53.

7.1.2 Leisser, C., G. Fuhrmann, G. Rosenberger, M. Grusch, T. Halama, **S. Sasgary**, C. Cerni, and G. Krupitza. "Cdc25a Mediates Survival by Activating Akt Kinase." *ScientificWorldJournal* 1, no. 1 Suppl 3 (2001): 94.

7.1.3 **Sasgary, S.**, G. Schmidt, B. Skrzypek, and C. Cerni. "Processivity of C-Myc-Induced Telomerase Activation in Rat Cell Lines." *ScientificWorldJournal* 1, no. 1 Suppl 3 (2001): 71.

7.1.4 **Sasgary, S.**, M. Wieser, and C. Cerni. "Targeted Inhibition of Telomerase in Human Cancer: Will It Be a Double-Edged Sword?" *Onkologie* 24, no. 1 (2001): 22-6.

7.1.5 Cerni, C., B. Skrzypek, N. Popov, **S. Sasgary**, G. Schmidt, L. G. Larsson, B. Luscher, and M. Henriksson. "Repression of in Vivo Growth of Myc/Ras Transformed Tumor Cells by Mad1." *Oncogene* 21, no. 3 (2002): 447-59.

7.1.6 Holzmann, K., W. Berger, D. Mejri, C. Cerni, and **S. Sasgary**. "Detection and Quantification of Transcripts for the Catalytic Subunit Tert and the Rna Component of Telomerase in Rat Tissue." *Anal Biochem* 317, no. 1 (2003): 120-3.

7.1.7 Sommer, K. W., C. J. Schamberger, G. E. Schmidt, **S. Sasgary**, and C. Cerni. "Inhibitor of Apoptosis Protein (Iap) Survivin Is Upregulated by Oncogenic C-H-Ras." *Oncogene* 22, no. 27 (2003): 4266-80.

7.1.8 Brachner, A., **S. Sasgary**, C. Pirker, M. Wieser, C. Cerni " Uniform and saturable telomere elongation by exogenous hTERT in telomerase- and ALT-negative human lung cancer cells." submitted to FASEB

7.2 ABSTRACTS

7.2.1 Cold Spring Harbor Meeting" Cancer Genetics and tumor suppressor genes", 19.- 23.08.1998, USA
„Presence or lack of telomerase activity in immortalized and transformed rat cell lines depend on the transfected oncogenes"
S.Sasgary, J.Gadek-Wasierski, C.Cerni

7.2.2 Fakultätavorlesung 16.10.1998, AKH Wien
„Lack of telomerase activity in transformed rat cell lines expressing human or murine p53 mutants"
S.Sasgary, J.Gadek-Wasierski, C.Cerni

7.2.3 Proc.Nature Biotech. Winter Symposium, Oxford Press (Abstract), 2000
„c-Myc-mediated telomerase activation is not essential for malignant transformation of rat cells"
S.Sasgary, M.Wieser, C.Cerni

7.2.4 AACR/Nature Genetics Joint Conference, Tucson, Arizona, 25.-27.01.2001, USA
„Kinetics of expression analysis during myc-mediated apoptosis"
K.Holzmann, C.Chamberger,G.Schmidt, B.Skrzypek, **S.Sasgary**, C.Cerni

7.2.5 Nature Biotechnology conference, Miami, 4.-8.2.2001, USA
"Processivity of c-Myc-induced telomerase activation in rat cell lines"
S.Sasgary, G.Schmidt, B.Skrzypek, C.Cerni

7.2.6 International Conference on Apoptosis, Kongress des IIAR, 25.-28.05.2001, Greece
„Apoptosis in transformed myc/ras rat cell lines upon inhibition of MEK1
G.Schmidt, C.Chamberger, , K.Holzmann,K.Sommer, B.Skrzypek, **S.Sasgary**, C.Cerni

7.2.7 Jahrestagung der österreichischen Biochemischen Geselschaft (ÖBG), Innsbruck 24.-27.9.2001
"Analysis of myc-mediated apoptosis in myc/ras transformed cells by cDNA arrays"
K.Holzmann, C.Chamberger, **S.Sasgary**, G.Schmidt, B.Skrzypek, C.Cerni

7.2.8 Symposium „Grundlagen der biologischen Alterungsmechanismen", Bad Hofgastein 26.-27.3.2002
„Age-dependent Telomeric loss in embryonic rat cells is counterbalanced by activated c-Myc"
C.Chamberger, **S.Sasgary**, M.Wieser, M.Hajek, G.Schmidt, C.Schreiner, C.Cerni

7.2.9 Joint Annual Meeting 2003 , Gemeinsame Jahrestagung 2003, 21-24.10.2003 Graz
„Overexpression of the catalytic subunit of human telomerase, hTERT, in human lung cancer cells results in homogeneously long telomeres"
A.Brachner, **S.Sasgary**, C.Pirker, W.Berger, M.Wieser, C.Cerni

7.2.9 Joint Annual Meeting 2003, Gemeinsame Jahrestagung 2003, 21-24.10.2003 Graz
„Inhibition of apoptosis protein (IAP) survivin is upregulated by oncogene c- H-ras"
K.Sommer, C.Schamberger, G.Schmidt, **S.Sasgary**, C.Cerni

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