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Diplomingenieur der Lebensmittel- und Biotechnologie

SNEV and hTERT: two different ways to extend the life span of human cells

durchgeführt am Institut für Angewandte Mikrobiologie
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betreut von
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„Der Langsamste, der sein Ziel nicht aus den Augen verliert, geht noch immer geschwinder, als jener, der ohne Ziel umherirrt“

Gotthold Ephraim Lessing

1729 – 1781

DANKE!

Nun hab ich es wirklich geschafft, nach Jahren diese Arbeit fertig zu schreiben, kaum zu glauben!
Unzählige Leute haben dabei mitgewirkt und haben mir auf dem Weg dahin geholfen, eine wissenschaftliche Arbeit als Teil meines Studiums endlich in den Händen halten zu dürfen.
All das hat begonnen im Februar 2005, als ich eben meine Diplomarbeit am IAM in der Arbeitsgruppe von Regina Grillari (dazumals noch Voglauer) begonnen habe. Die Arbeitsgruppe war einfach wirklich ein Traum und ich würde mir für meine zukünftige Arbeit genau so eine Gruppe wünschen, da war ich schon sehr verwöhnt!
Jeder ist für Fragen offen, am meisten waren für mich Regina und Matthias da. Und es hat sich durch meine Arbeitsschwerpunkte auch so ergeben, dass ich im Labor sehr viel mit Matthias zusammengearbeitet hab, einen „perfekteren Lehrer“ hätte ich mir da ja fast nicht wünschen können, da war einfach immer präzisestes Arbeiten angesagt und alles wurde super erklärt!
Dann war da noch Thomas, DER Alleswisser was Zellkultur betrifft, der immer wieder auch ein Auge auf meine retroviral infizierten Zellen geworfen hat.
Klaus und Giovanni waren die Ansprechpartner für meine Klonierungsgschichten im 4. Stock, da war ich ja eher der „Aussenseiter“, aber war auch mal ganz interessant, um hineinzuschnuppern.
Insgesamt war´s einfach eine tolle Erfahrung, dort gearbeitet haben zu dürfen, und ich danke wirklich allen, die mir im Laufe der acht Monate IAM mit Tipps, Ratschlägen und Erklärungen zur Seite gestanden sind!
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ABSTRACT

Normally growing cells in culture have a finite number of population doublings before entering a state of irreversible growth arrest also termed replicative senescence.

One mechanism which plays a major role in this irreversible growth arrest is the progressive erosion of telomeres, which are specialized structures at the ends of linear chromosomes. This mechanism occurs because telomerase is not active. Introduction of hTERT, the catalytic subunit of human telomerase, avoids erosion of telomeres and allows the creation of a large variety of different continuous cell lines.

Besides telomerase, other proteins have also been shown to influence the replicative life span of cells, such as SNEV (senescence evasion factor).

In this study we have for the first time established a continuously growing kidney epithelial cell line using hTERT alone. The cell line shows an extended life span, enhanced telomerase activity as well as elongated telomeres compared to normally growing RPTECs, but the morphology resembles that of the corresponding normal counterpart.

Thus, we established an important tool for research in aging and tumor pathogenesis of the human kidney.

Furthermore, we wanted to gain more insight into the role of SNEV in life span extension. Therefore, we measured the amount of DNA damage in SNEV overexpressing HUVECs (human umbilical vein endothelial cells) in comparison to normally growing HUVECs.

Measuring the amount of DNA damage via Comet assay brought the result, that SNEV overexpressing HUVECs show significantly lower DNA damage in comparison to normally growing HUVECs. These data indicate that a high SNEV level correlates with a decrease of accumulated DNA damage during *in vitro* cultivation and that the SNEV level might be involved in the life span extension.

1 INTRODUCTION

1.1 The cell cycle

Each eukaryotic cell which wants to proliferate has to pass through the cell cycle. The cell cycle can be separated into 4 phases (Figure 1.1), which are controlled by specific cell cycle regulators (reviewed in: Prescott, 1968).

The G_1 phase takes place between the last mitosis and the next beginning of DNA synthesis. The duration of this phase is very variable and it serves as a control checkpoint for the cell size and the cell environment. Depending on this information, the cell is going to continue with the next cycle or not. Cells, which do not proliferate anymore, rest in a quiescent state - the recovery phase G_0. Cells can stay in this phase for a few days or for years and under certain conditions they can re-enter the cell cycle.

The G_1 phase is followed by the S phase, where the DNA of the cell is replicated. The S phase takes about 7 to 8 hours.

After S phase the next gap phase follows, the G_2 phase. The two morphological characteristics of this phase are the big nucleus and the polarisation of the cell. At this point the cells check if the DNA replication was done correctly, the cell is big enough and the environment is suitable for cell division.

The division of the cell takes place in the subsequent M phase, where the cytoplasm is split up and the result of this phase are two identical daughter cells.

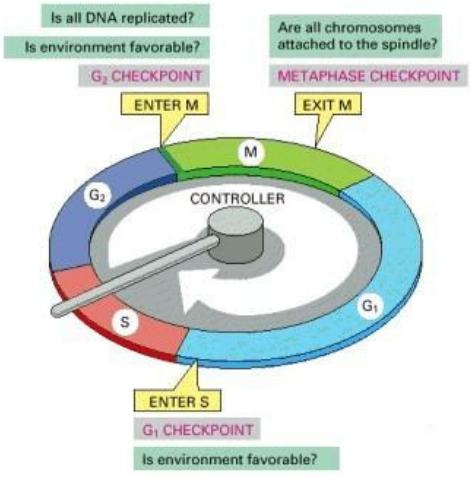

Figure 1.1: The four phases of the cell cycle: G_1 phase (gap phase), S phase (DNA replication), G_2 phase (gap phase) and M phase (division of the cell). [The figure was taken from Alberts, 4th edition]

But how is the cell cycle controlled?

There is a family of protein kinases, which is known as cyclin-dependent kinases (Cdks). The activity of these Cdks changes constantly with the progression of the cell cycle and is regulated by cyclins. Therefore, the level of cyclins oscillates during the cell cycle in contrast to the level of Cdks, which remains constant over the whole time.

Inhibitors of Cdks are called Cdk inhibitory proteins (CKIs).

P16 for example is a CKI which suppresses G_1-Cdks activity in G_1 phase. This protein is frequently mutated or deleted in a multitude of tumors, and is known to be an important tumor suppressor protein.

P21, which is transcriptionally activated by p53, is also a known CKI which suppresses G_1/S-Cdk and S-Cdk in G_1.

A gene regulatory protein, also known as tumor suppressor protein, which is also active in the cell cycle control system, is p53 (reviewed in: Sancar et al., 2004). It promotes transcription of genes that induce cell cycle arrest or apoptosis in response to DNA damage or other stress factors.

1.2 Normal human cells and replicative senescence

In contrast to most tumor cells and immortalized cells, the life span of normal human somatic cells is limited (Hayflick et al., 1961; reviewed in: Campisi, 1997). After a finite number of population doublings *in vitro* cells cease to grow and enter a state of irreversible growth arrest termed replicative senescence. Cells which have reached this state can remain in this viable, non-dividing state for months. The senescent phenotype is characterized by a flat and large morphology, dramatic changes in gene expression and a positive stain for β-galactosidase activity at pH 6.0 (reviewed in: Mathon et al., 2001; Dimri et al., 1995).

But what is behind this irreversible growth arrest? In literature, many different reasons for this phenomenon are described, which are probably all linked together.

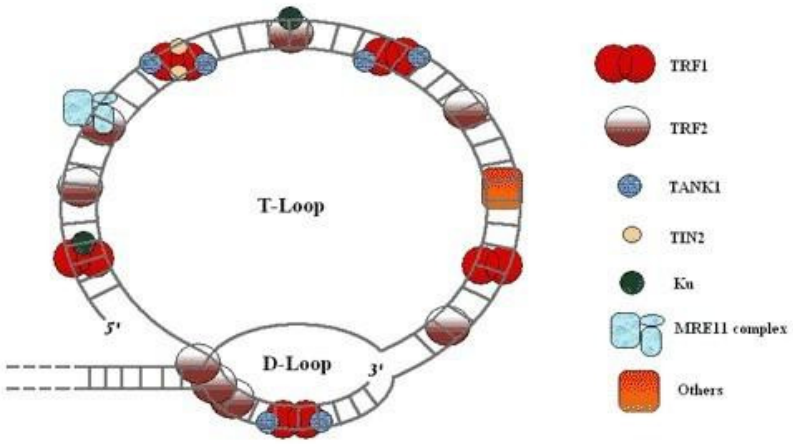

Figure 1.2: Duplex structure of telomeres consisting of a telomere (T)-loop and a displacement (D)-loop, associated with telomere binding proteins [The figure was taken from Lin Kah Wai, Medscape General Medicine 6(3), 2004]

It is long known that telomeres play an essential role in the aging of cells (Bodnar et al., 1998; Harley et al., 1990). Telomeres of human cells consist of TTAGGG DNA repeats (reviewed in: Blackburn et al., 1991) composed of double stranded DNA which ends with a short single-stranded 3´ overhang. The ends of telomeres are protected and regulated by telomere-binding proteins and form a special lariat-like structure called the t-loop (see Figure 1.2). This t-loop structure protects telomeres from being detected as a double-stranded break and avoids fusion and degradation. Two essential proteins which are associated with telomeres are the telomeric-repeat-binding factor 1 and 2 (TRF1 and TRF2). Whereas TRF1 is mainly involved in the control of telomeric length, TRF2 is mainly implicated in chromosome end protection by preventing end-to-end fusions (van Steensel et al., 1997; van Steensel et al., 1998; reviewed in: De Lange, 2002). In cultured, normal human cells, telomeres progressively shorten with each cell division and once they have reached a critical length, cells enter replicative senescence (reviewed in: Campisi, 2001).

The mechanism of telomere shortening can be easily explained (see Figure 1.3): DNA polymerases replicate only in a 5´ → 3´ direction, so the replication differs for the leading and the lagging DNA strand. The leading strand is replicated continuously in contrast to the lagging strand, where DNA polymerization starts from several RNA primers, which are elongated to create DNA fragments (Okazaki fragments). These RNA primers are finally degraded and replaced by DNA sequences. Removal of the terminal RNA primer on the lagging strand leaves a gap which is filled in by extension of the next Okazaki fragment. Because there is no

template for the “last” Okazaki fragment beyond the 5´end of the chromosome, one strand cannot be synthesized to its very end.

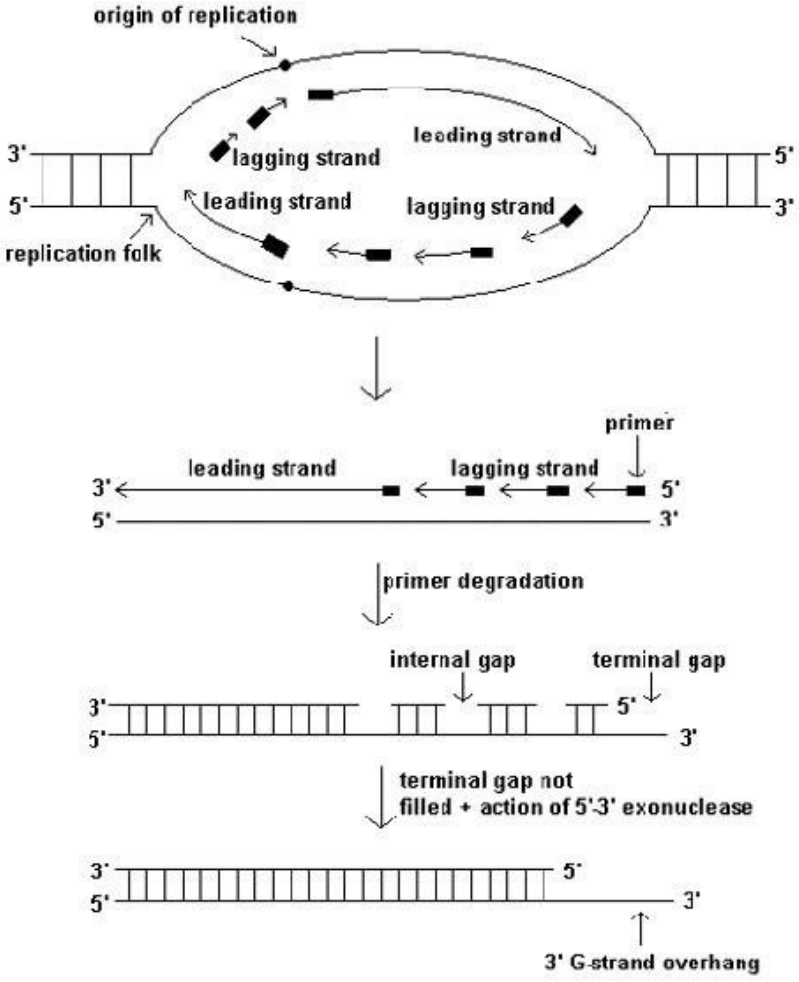

Figure 1.3: The mechanism of telomere shortening during DNA replication. The degradation of the primer on the lagging strand and the action of a putative 5´ to 3´ exonuclease lead to shortening of the 5´end of the telomere and the formation of a 3´-end overhang structure. [The figure was taken from Lin Kah Wai, Medscape General Medicine 6(3), 2004]

This so-called “end replication problem” illustrates the progressive reduction of chromosomal DNA at the 3´ ends during multiple cell cycles (reviewed in: Dahse et al., 1997; Watson et al., 1970).

As soon as telomeres become critically short, they are detected by the cellular DNA-damage machinery (reviewed in: De Lange, 2002). P53 and pRB (retinoblastoma protein) are the most important proteins of this machinery. The function of p53 and the pRB pathways in response to uncapped telomeres is very complex but when p53/pRB are over-activated senescence is induced either by p16 or p21.

Although telomere shortening seems to be the primary cause of cellular senescence, there are also other events like DNA damage (Di Leonardo et al., 1994), oncogene activation (Serrano et al., 1997) or tumor suppressor genes (Sugrue et al., 1997)

(see Figure 1.4), which can induce senescence independent of telomere erosion (reviewed in: Dimri 2005).

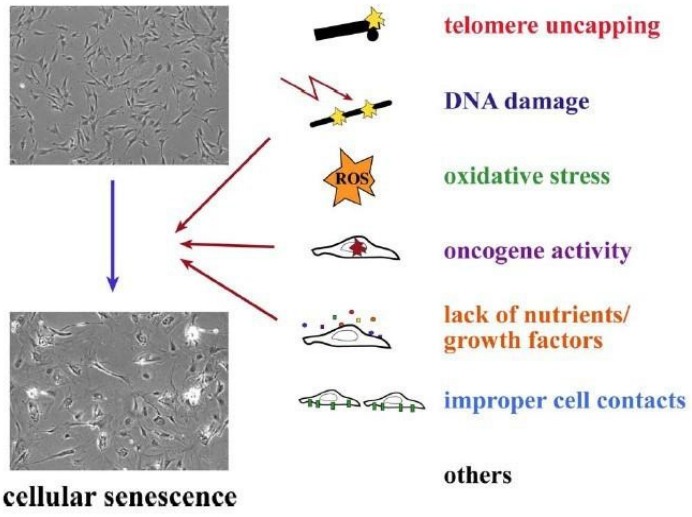

Figure 1.4: Different signals which are able to activate senescence independent of telomere erosion. The combined levels of stress determine how rapidly the entry into senescence will occur. [The figure was taken from Ben-Porath, 2005]

Additionally, activation of growth inhibitor genes, due to a variety of environmental stresses (sub-lethal doses of oxidants), can induce premature senescence (SIPS) or STASIS (stressor aberrant signaling-induced senescence).
Moreover, Zglinicki et al. describe that cross-talk between telomere dependent and independent pathways seems to exist (Zglinicki et al., 2000 and 2005).

1.3 Strategies for circumventing cellular senescence

Cellular senescence, a protective mechanism of cells, can be circumvented in different ways. *In vivo*, the circumvention of senescence plays a major role in carcinogenesis. Telomerase activity has been detected in approximately 90 % of all tumor cells (reviewed in: Shay and Bacchetti, 1997) confirming that telomerase plays an important role in carcinogenesis. Also activation of proto-oncogenes and inactivation of tumor-suppressor genes are involved in this multi-step process and due to such dysfunctions cells can progress to cancer.
In vitro, immortalization occurs spontaneously (10^{-12}). But cells can be immortalised by exogenous factors like chemical agents, DNA or RNA viruses, by ectopic

expression of telomerases (hTERT). New genes, such as SNEV due to life span extension.

1.3.1 HTERT

Telomerase, first discovered in *Tetrahymena thermophila* in 1985 (Greider and Blackburn, 1985), is an RNA-dependent DNA polymerase that synthesizes telomeric DNA sequences and is responsible for the unlimited proliferative potential. The enzyme consists of two essential components: the functional RNA component hTR (human telomerase RNA), which serves as a template for telomeric DNA synthesis (Feng et al., 1995), and a catalytic subunit called hTERT (human telomerase reverse transcriptase) with reverse transcriptase activity (see Figure 1.5) (Harrington et al., 1997b; Kilian et al., 1997; Lingner et al., 1997; Meyerson et al., 1997).

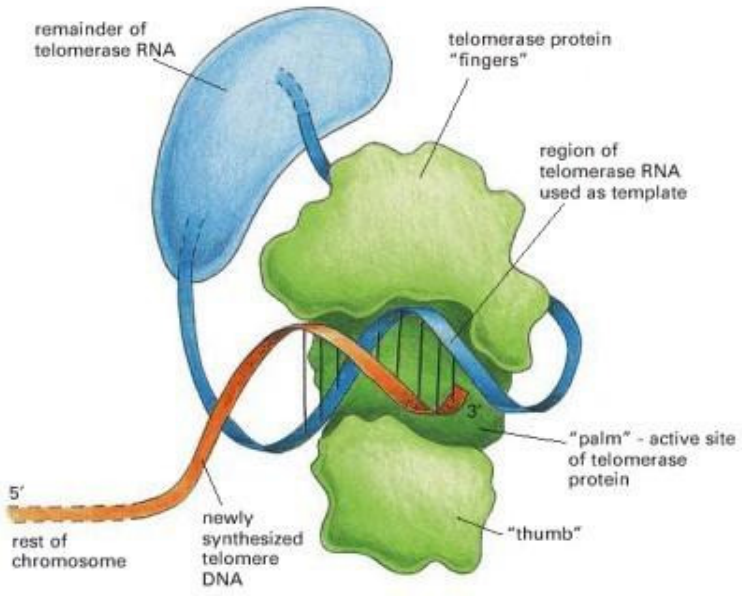

Figure 1.5: Human telomeraset consists of two essential components: The functional RNA component hTR (human telomerase RNA) and the catalytic subunit called hTERT (human telomerase reverse transcriptase). [The figure was taken from Alberts, 4th edition]

HTR is expressed in all tissues, independent of telomerase activity. In contrast, hTERT is generally repressed in normal human somatic cells and up-regulated in immortal cells, suggesting that hTERT is the regulatory determinant for telomerase activity (Cong et al., 1999).

Exceptional cases, where telomerase is also active in normal human somatic cells are male germ cells, activated lymphocytes and certain types of stem cells (Wright et al., 1996), but there are also other cell types with activated telomerase.

Telomerase avoids telomere shortening by recognizing the tip of a G-rich strand of an existing telomere DNA repeat sequence and elongating it in the 5´-to-3´ direction. Therefore, telomerase uses its RNA template. After extension of the parental DNA strand, replication of the lagging strand template can be completed by using these extensions as a template for synthesis of the complementary strand by a DNA polymerase (see Figure 1.6).

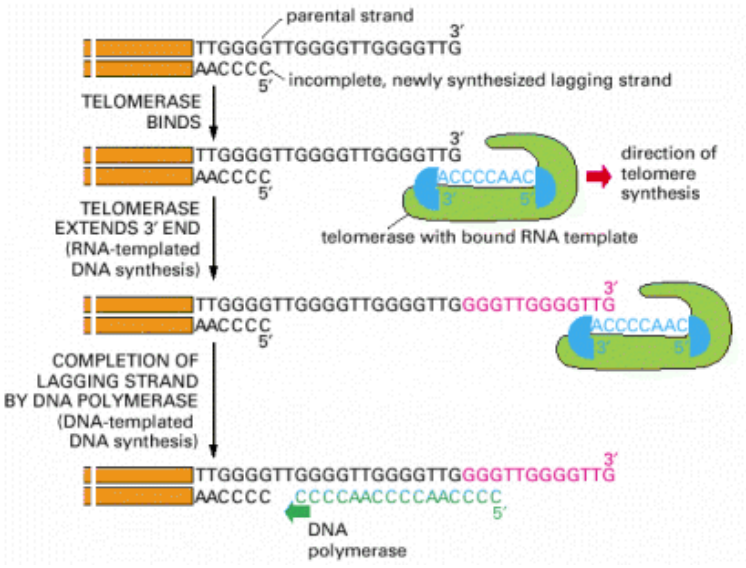

Figure 1.6: Telomere replication: Reactions involved in synthezising the repeating G-rich sequences that form the ends of the chromosomes of diverse eukaryotic organisms. [The figure was taken from Alberts, 4th edition]

Several different studies *in vitro* demonstrate that ectopic expression of hTERT alone is sufficient for the immortalization of many cell types (Bodnar et al., 1998; Ouellette et al., 2000; Yang et al., 1999; Chang et al., 2005; Wieser et al., 2008).

1.3.2 SNEV

Regulation of cellular processes which are caused and accompanied by variations of gene expression patterns are of great interest in cellular aging.

By screening for genes differently expressed in early passage versus senescent HUVECs, the protein SNEV (also known as PRP 19 (pre-mRNA processing 19), hPso4 (human psoralen sensitivity 4) and hNMP 200 (human nuclear matrix protein) has originally been identified as mRNA which is downregulated in HUVECs after entry into replicative senescence (Grillari et al., 2000) and as a component of the nuclear matrix (Gotzmann et al., 2000).

In comparison to the downregulated mRNA level of replicatively senescent cells, SNEV is higher expressed in early passage cells and several tumor cells (Voglauer et al,. 2006). As shown in Figure 1.7 stable overexpression of SNEV in HUVECs causes a significantly extension of their replicative life span without changing endothelial characteristics (Voglauer et al., 2006).

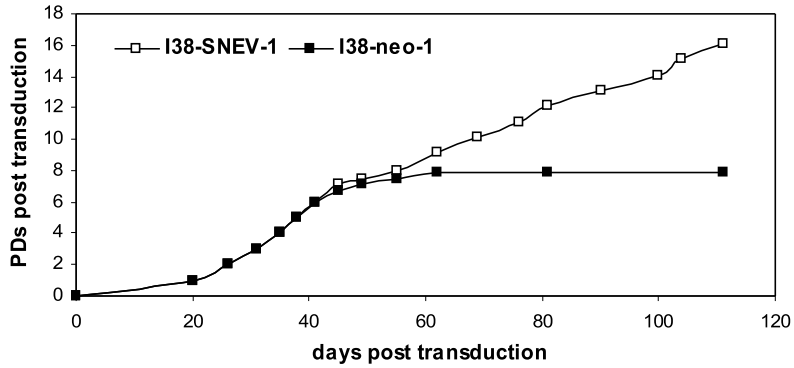

Figure 1.7: The growth curve clearly shows the life span extension of SNEV transduced HUVECs (I38-SNEV) in comparison to the non-transduced vector control cells I38-neo.

Sequence analysis of SNEV showed similarity to the *Saccharomyces cerevisiae* protein PRP 19, which is involved in pre-mRNA splicing and DNA repair. Because of sequence similarity, SNEV was also tested if it is involved in the human pre-mRNA splicing process. Indeed, SNEV is contained in a spliceosome associated complex and forms homo-oligomers, which can be inhibited by peptides consisting of the SNEV self-interaction domain. Addition of these peptides blocks pre-mRNA splicing *in vitro* (Grillari et al., 2005).

Figure 1.8 shows the domain architecture of SNEV which is highly similar to the one found for the yeast Prp19p (Ohi et al., 2003).

Figure 1.8: SNEV´s putative functional domains: U-box, a modified RING finger domain; SID, self interaction domain; LCR, low complexity region; CC, coiled coiled region; GL, globular domain; WD40, form a ß-propeller structure.

The SNEV domain consists of a 68 amino acid long U box domain (modified RING finger) at the N-terminus, a self interaction domain (SID), a low complexity region (LCR) and a globular domain (GL). 7 WD40 repeats (form a β-propeller structure) are localized at the C-terminus, which are proposed to mediate protein-protein

interactions. The putative SNEV coiled coiled region represents another segment with protein-protein interaction ability.

U box domains are found in a large variety of proteins which are conserved from yeast to humans and play an essential role in the process of ubiquitination (Aravind and Koonin, 2000). Indeed, it has been shown that SNEV is involved in ubiquitination and shows *in vitro* ubiquitin E3 ligase activity, the ubiquitin transfer therefore is dependent on the presence of the U box (Hatakeyama et al., 2001). SNEV escorts its substrate to the proteasome, a large multi-subunit protease, which plays a crucial role in intracellular protein degradation (Xie and Varshavsky, 2002). Because different E3 ligases have been reported to physically interact with the proteasome, Löscher and co-workers tested for an interaction of the E3 ligase SNEV with the proteasome. Indeed, the U box domain of SNEV physically and directly interacts with the β7 subunit of the proteasome (Löscher et al., 2005).

Furthermore, it has been demonstrated that SNEV has a direct role in DNA damage processing (Mahajan and Mitchell, 2003). SNEV is ubiquitinated upon DNA damage and its oligomerization is changed by its ubiquitination (Lu and Legerski, 2007). It interacts with terminal deoxynucleotide transferase (TdT) and binds sequence independently to double- but not to single-stranded DNA (Mahajan and Mitchell, 2003).

Moreover, SNEV and three other proteins (CDC5L, PLRG1 and SPF27) of the CDC5L-associated complex together with WRN, are necessary for early steps of DNA interstrand cross-link repair (Zhang et al., 2005). WRN, a DNA helicase with exonuclease activity which is involved in DNA repair and telomere maintenance, is mutated in patients suffering from Werner´s syndrome, a premature aging syndrome (Gray et al,. 1997; Suzuki et al., 1999; Yu et al., 1996). Stable overexpression of SNEV *in vitro* show an increased replicative life span - as mentioned above – while cells derived from Werner patients show reduced life spans.

Depletion of SNEV or CDC5L in HeLa cells resulted in the failure of these cells to form colonies (Zhang et al., 2005). Lu and Legerski showed that depletion of SNEV destabilizes CDC5L and PLRG1, and finally induces apoptosis (Lu and Legerski, 2007).

Because SNEV overexpression also correlates with increased resistance to the glutathione-depleting reagent BSO and the chemotherapeutic drug bleomycin (Voglauer et al., 2006), it is intriguing to think that the DNA interstrand cross link

repair function might be responsible for conferring longer life to cells (Grillari et al., 2007)

Considering all of the essential functions of SNEV, it is not surprising that SNEV is indispensable for early mouse development and that knock-out of SNEV in mice is early embryonic lethal (Fortschegger et al., 2007). The replicative life span of mouse embryonic fibroblasts (MEFs) from heterozygous $SNEV^{+/-}$ is reduced (Fortschegger et al., 2007) and hematopoetic progenitor show defects in proliferation and self-renewal (Schraml et al., 2008), which give us a further hints that SNEV plays a crucial role in influencing replicative life span.

1.4 Defense mechanisms against ROS

As cellular GSH (glutathione) is one of the most essential non-proteinous cell antioxidants, it is very important for cells to maintain the intracellular GSH level. Therefore, the availability of cysteine, necessary for the biosynthesis of GSH, is of high importance.

The metabolism of GSH is closely connected to Meister´s gamma-glutamyl-cycle (shown in Figure 1.9) (Meister, 1983) in which a critical role is played by membrane gamma-glutamyltransferase (GGT; EC 2.3.2.2), a cell surface protein.

GGT activity is present in the plasma membrane of almost all cells, whereas the highest enzyme levels are found in kidney cells (Hinchman and Ballatori, 1990).

GGT catalyzes the first step in the degradation of extracellular glutathione. The gamma glutamyl bond is hydrolyzed in glutamate and cysteine. At the same time cysteinyl-glycine will be released, and further cleaved to cysteine and glycine by plasma membrane dipeptidase activities. These two amino acids can then be taken up by the cell in an active process.

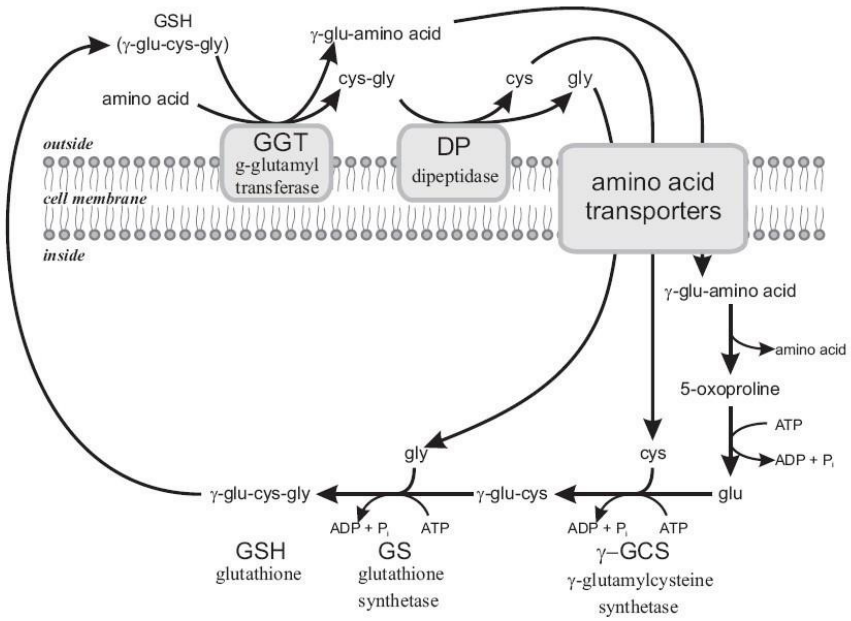

Figure 1.9: The glutathione cycle: GSH plays a central role in the protection of cells against oxidative stress. GGT therefore catalyzes the first step in the degradation of extracellular glutathione. [The figure was taken from Meister, 1983]

Besides GSH, aerobic organisms evolved both non-enzymatic and enzymatic defenses to minimize the damaging effects of ROS. Non-enzymatic defenses include compounds such as vitamin C and E, and ß-carotene. Enzymatic defenses include superoxide dismutase (SOD), catalases and peroxidases (reviewed in: Scandalios, 2002).

2 AIM OF THE STUDY

Project I

Numerous experiments showed that overexpression of the protein SNEV in HUVECs extends the cellular life span.

To get more informations about SNEV and its functions, we wanted to generate different deletion mutants. The aim was, to compare the individual deletion mutants with the wild type protein, having regard to the amount of DNA damage, the ROS level and the life span extension.

Project II

RPTECs, cultivated under serum-free conditions, approach senescence after approximately 22 population doublings. This short life time *in vitro* hampers the possibilities of working with RPTECs in research.

For this reason we wanted to immortalize human RPTECs with hTERT alone.

After positive immortalization, we wanted to characterize the newly established cell line.

3 MATERIAL AND METHODS

For recipes of buffer and solutions see Appendix

3.1 Cell culture

3.1.1 Description of cell lines

HUVECs, human umbilical vein endothelial cells

Endothelial cells (ECs) line the interior surface of blood vessels, forming an interface between circulating blood and the underlying tissues. They are involved in many activities of vascular functions, like the control of blood pressure, blood clotting, inflammation and the formation of new blood vessels. Furthermore, ECs function as gatekeepers, controlling the passage of particles and cells into and out of the bloodstream.

ECs are widely used as in vitro model for studying the vascular system and vascular disorders.

HUVECs, cultured according to the protocol described previously (Chang et al., 2005), proliferate for roughly 130 days, corresponding to 47 population doublings before they reach replicative senescence (Unterluggauer et al., 2007).

A hallmark for endothelial cells as well as for HUVECs, is the expression of vWF (van Willebrand Factor) (Hoyer et al., 1973) which is stored in the Weibel-Palade bodies (Weibel et al., 1964) and the expression of the 130-kDa transmembrane glycoprotein platelet endothelial cell adhesion molecule-1 (also known as PECAM-1 or CD31) (Albelda et al., 1990). In contrast to CD31, the level of vWF has been shown to decrease during ageing of ECs and can therefore be used as a marker for ageing.

The cells were isolated from human umbilical veins at the IAM according to Gimbrone et al. (Gimbrone et al., 1978). Cells were grown in gelatine (1% in PBS) pre-coated roux flasks in M199 medium supplemented with 4 mM L-Glutamine, 15 % fetal calf serum (FCS), 200 µg/ml endothelial cell growth supplement and 170 U/ml heparin in a humified atmosphere containing 7 % CO_2 at 37°C.

Transduced HUVECs (I38-SNEV and I38-neo), which were established by transduction of I38 cells with recombinant retrovirus encoding SNEV or the neomycin resistance gene only (Voglauer et al., 2005a), were grown in the above described

medium supplemented with 20 µg of the selection marker geneticin sulfate (G418)/ml.
The cells were passaged twice a week with a split ratio of 1:2 to 1:4, depending on the age of the cells.

RPTECs, Renal proximal tubular epithelial cells

The epithelium is a membranous tissue composed of one or more layers of cells separated by very little intracellular substance and forms the covering of most internal and external surfaces of the body and its organs.
Also the kidney is coated with such epithelial cells, called renal proximal tubular epithelial cells or RPTECs. These cells are responsible for re-absorption of water, glucose, solutes, proteins and advanced glycation end products. Additionally they express cytokeratins and aminopeptidase N (reviewed in: Riemann et al., 1999) and also the activity of gamma glutamyl transferase (GGT) is a marker protein for RPTECs (reviewed in: Tate and Meister, 1981).
RPTECs were isolated from the renal cortex as described by Ebert et al. (Ebert et al., 1990).The RPTECs were cultivated in serum-free SFM-N4 medium, consisting of DMEM/Ham´s F12, 4 mM L-Glutamine, 10 mM HEPES buffer, 10 ng/ml epidermal growth factor (EGF), 5 pM triiodothyronin (T3), 3.5 µg/ml ascorbic acid, 5 µg/ml transferrin, 25 ng/ml prostaglandine (PGE), 25 ng/ml hydrocortison, 5 µg/ml insuline, 8.65 ng/ml sodium-selenit also in a humified atmosphere containing 7 % CO_2 at 37°C.
These cells were split in a ratio of 1:2 to 1:4 twice a week, depending on the age of the cells.

RPTEC/TERT1, Renal proximal tubular epithelial cells transduced with hTERT

These RPTECs were transduced with recombinant retrovirus expressing hTERT at a population doubling level (PD) of 4.5. The cells were selected and cultivated in roux flasks in SFM-N4 supplemented with 100 µg G418/ml.
Cells were passaged at day 5 to 7 with a split ratio of 1:2.

HeLa cells, human epithelial cervix carcinoma cells

HeLa cells were established from a cervical epithelial carcinoma by George O. Gey in 1951 (Gey et al., 1951).

Cells were grown in roux flasks in Roswell Park Memorial Institute (RPMI 1640) medium supplemented with 4 mM L-Glutamine and 10 % fetal calf cerum (FCS) in humified atmosphere containing 7 % CO_2 at 37°C.
Cells were propagated twice a week with a split ratio of 1:10 to 1:20.

NIH 3T3 clone 4 cells, mouse fibroblast cells
Cells were cultivated in roux flasks in DMEM supplemented with 6 mM L-Glutamine and 10 % FCS.
The cells were propagated twice a week with a split ratio of 1:4 to 1:6.

BD RetroPackTM PT67 Cell line (CLONTECH)
The BD RetroPack PT67 cell line (Cat. No. 631510) is derived from a mouse fibroblast cell line (NIH 3T3) designed for stably producing high-titer retrovirus.
Cells were grown in DMEM supplemented with 6 mM L-Glutamine, 10 % FCS and 1 mM sodium pyruvat.
The cells were passaged twice a week with a split ratio of 1:20.

3.1.2 Detaching and subculturing of the cells

Passaging of HUVEC, HeLa, NIH 3T3 clone 4, BD RetroPackTM PT67 Cell line
The supernatant was discarded. The cells were washed with PBS to get rid of the serum and cells were detached from the culture flask by addition of 0.04 ml/cm^2 trypsin (0.1%) / EDTA (0.02%). To accelerate detaching, the culture vessel was incubated by 37°C for about 2 minutes. Cell detachment was monitored under the microscope.
Thereafter, the cells were suspended in fresh medium and a defined volume was transferred in the new culture flask with fresh medium to get the desired split ratio.
All needed solutions should be temperated to 37°C before use.

Passaging of RPTEC and RPTEC/TERT1 cells
The supernatant was discarded and the cells were washed twice with PBS to get rid of all media components. After the last washing step, PBS was removed with a pipette. Cells were detached by addition of 20 µl/cm^2 of trypsin (0.25 %) / EDTA (0.05 %) and incubated at 37°C for about 5 minutes. Trypsin action was stopped by addition of 8 µl/cm^2 of trypsin inhibitor. Thereafter, cells were diluted in fresh medium, transferred to a 10 ml centrifuge tube and centrifuged at 170 g for 10 minutes. The

supernatant was discarded, the pellet was re-suspended by tapping and the cells were resuspended in a defined volume to get the right split ratio. The diluted cells were transferred in new culture vessels with fresh medium and incubated at 37°C.

Detaching of cells using EDTA

After discarding the supernatant, the cells were washed twice with PBS. Cells were detached by adding EDTA (50 mM) and incubating at 37°C. Cells were further detached by tapping the culture vessel.

3.1.3 Cryo conservation

Freezing

After reaching confluence, the cells were trypsinized, re-suspended in fresh medium and centrifuged by 170 g for 10 minutes.

Cryo tubes were pre-chilled at –80°C. After centrifuging the supernatant was discarded and the pellet was re-suspended by tapping carefully. The cells were re-suspended in 1 ml, pre-cooled (4°C) freezing medium / 25 cm^2 cell suspension and transferred in the prepared cryo tube. The cells were frozen at –80°C for 24h, followed by a transfer to a liquid nitrogen tank for long time storage.

Thawing

7 ml culture medium in a 10 ml tube was pre-cooled at 4°C. The cryo tube from the liquid nitrogen tank was transferred in a vial filled with 70 % EtOH. Then the cells were rapidly thawed, transferred in the pre-cooled fresh medium and centrifuged at 170 g for 10 minutes. Thereafter, the supernatant was discarded and the pellet was re-suspended in fresh medium and incubated in humified atmosphere containing 7 % CO_2 at 37°C.

3.1.4 Calculation of the cell number and viability

A Bürker-Türk counting chamber (Hecht – Assistant) was used for determination of the cell number. 2 counting nets are engraved in the chamber base, each of them subdivided into 9 large squares. The large squares have an area of 1 mm^2 and are 0.1 mm in depth. With an optically plane cover glass a volume of 0.1 mm^3 (= 0.1 µl) suspension is defined. If a cover glass is placed on the external base, a capillary gap is produced between the underside of this cover glass and the chamber base.

For determination of the cell number, 100 µl of 0.5 % Trypan Blue was added to 500 µl cell suspension and mixed by pipetting (dilution factor of 1.2). Both sides of the chamber base were filled with cell suspension, and the number of viable cells was counted under a light microscope. At most about 200 cells per net can be counted, otherwise a dilution is necessary. The sum of cells was divided through the number of counted squares and multiplicated with 12 000 (chamber volume and dilution factor). The total cell number was determined according to the following equations:

$$viableCells/ml = \frac{viablecells}{counted\ major\ squares} \times 12000$$

$$Viability\ \ in\,\% = \frac{viable\ cells/ml}{total\ cell\ number/ml} \times 100$$

3.1.5 Calculation of population doubling level

The population doubling level (PDL) is defined as the total number of population doublings of a cell line. For the calculation of the population doublings in a single passage the following equation is used.

$$PDL = \frac{log(N/N_0)}{log2}$$

N = number of cells in the culture vessel at the end of a growth period
N_0 = number of cells plated in the culture vessel

3.1.6 Mycoplasma test with DAPI

Mycoplasma are the smallest and simplest self-replicating prokaryotes with a size of about 150 to 200 nm. In cell culture labs, mycoplasma tests are periodically necessary, because they are very infectious, difficult to eliminate and often have an effect on the observed result. DNA staining is a rapid and suitable method for detection of mycoplasma contaminations. In uninfected cells the fluorescently labelled DNA in the cell nuclei is visible against a dark background, while samples infected with mycoplasma show extra-nuclear fluorescence of mycoplasma DNA (fuzzy fluorescence of small filaments).

1 to 3 days before testing, cells were transferred into NUNC chamber slides, so that cells reached about 50-70 % confluence.

The cell culture medium was discarded and the cells were rinsed once with PBS containing Ca^{2+} and Mg^{2+} and once with staining solution (0.1 µg DAPI/ml methanol). For labelling 350 µl of staining solution per cm^2 was added and incubated at 37°C for

15 minutes. After this step, the staining solution was discarded, the removable polystyrene chamber was removed and the slide was air dried. For observation under the fluorescence microscope Olympus BH2 (40x Objective), one drop 50 % glycerine was placed onto the slide, covered with a cover slip.

3.2 Cloning of delta U–box in pLXSN

For transfecting BD RetroPack PT67 cell Line with a plasmid, the DNA must be in a useable vector such as pLXSN. So the Δ U-box was cut from the vector pGBKT7 and cloned into pLXSN.

Therefore, the Δ U-box and the vector was cut, cleaned and ligated.

	restriction digest of Δ U-box in pGBKT7	volume [µl]	restriction digest of Δ BD in pLXSN	volume [µl]
DNA		2.5		5.8
Restriction enzyme I	EcoRI	1.5	EcoRI	1.5
Restriction enzyme II	SalI	1.5	XhoI	1.5
BSA		0.5		0.5
buffer	10x buffer	5.0	EcoRI buffer	5.5
H_2O nuclease free		39.0		40.7

Table 3.1: mix for restriction digest of Δ U-box in pGBKT7 and Δ BD in pLXSN

Reaction mix for restriction digest (see Table 3.1) was pipetted into an Eppendorf tube, mixed on a vortex and incubated for 3 h at 37°C on the heat block. During this time a preparative gel were prepared. Therefore 2.5 g agarose and 5 ml 50x TAE was filled up with AD to 250 g. The gel was melted in the microwave oven till the solution was clear. Then it was cooled to about 50°C and 8 µl EtBr from a 10 mg/ml stock solution were added before casting the gels. After polymerisation the gel was transferred in 1x TAE buffer containing 3 mg/ml EtBr. Afterwards 6 µl λ 3 standard (Fermentas) and the digested samples with 6 µl 6x sample buffer (6x Loading Dye solution, Fermentas) were loaded, the gel was run at 80 V for about 1,5 h. The DNA was visualised under UV light using an UV transluminator. The band of the correct size was cut out from the gel and transferred in an Eppendorf tube.

Purification of the DNA (Wizard® SV Gel and PCR clean-up system)

To get rid of the EtBr, the DNA must be purified. Therefore the correct volume of membrane binding solution (10 µl of solution per 10 mg of agarose gel slice) was added to the gel and melted at 37°C at the heat block. The solution was mixed on a vortex to get a homogenous solution and transferred to a SV Minicolumn, which was placed in a collection tube and incubated 1 minute at RT. After centrifuging at 16 000

rcf for 1 minute the supernatant was discarded and 700 µl membrane wash solution, previously diluted with 95% ethanol, was added. The sample was centrifuged twice at the same conditions and the supernatant was discarded. This step was repeated with 400 µl membrane wash solution, after discarding the supernatant the column was centrifuged for 1 minute without solution, also at 16 000 rcf and the column was put on an Eppendorf tube, 50 µl AD was added and incubated for 1 minute at RT and centrifuged at 16 000 rcf for 1 minute. The dissolved DNA was stored at -20°C.

Ligation of delta U-box with pLXSN

Before ligation, the samples were loaded on a 1% agarose gel to get information about the concentration, which is necessary for ligation.

Ligation mixture:

2 µl vector pLXSN

20 µl insert Δ U-box

3 µl 10x ligase buffer

3 µl T4 ligase

2 µl H_20 nuclease free

The ingredients were pipetted in an Eppendorf tube an incubated 3 h at 16°C.

Precipitation

1 µl glycogen, 3 µl sodium acetate and 60 µl 96% EtOH (-20°C) was added to the ligation stock and incubated at -20°C for 0.5 h.

After precipitation the sample was centrifuged 15 minutes at maximum speed and the supernatant was discarded. 100 µl 70% ethanol was added and directly removed after. The pellet was centrifuged again 1 minute at maximum speed. The supernatant was discarded and the pellet was dried at RT. Afterwards the pellet was diluted in 10 µl nuclease free water.

Electroporation

900 µl SOC medium was prepared in an Eppendorf tube and 100 µl electro-competent bacteria were thawed on ice, in a pre-chilled electroporation cuvette. Then 5 µl of purified ligation product was mixed with the bacteria. The electroporation was performed immediately with an electroporation unit GenePulser (BioRad), used alignments see below (see Table 3.2).

Voltage	2,5 kV
Capacity	25 µF
Resistance	1 000 Ω
Time constant	15 – 20 opt

Table 3.2: Electroporation conditions

Immediately after electroporation, the bacteria suspension was transferred into the SOC medium and incubated at 37°C on the heat block for 30 minutes with shaking easily. After the incubation time the bacteria suspension was plated on LB-agar for plasmid selection through ampicillin resistance. The plates were incubated over night at 37°C.

Plasmid amplification and testing of positive clones

The colonies, which were grown over night, should contain the plasmid because of the ampicillin resistance.

Individual clones were picked with a pipette tip and dipped into a numbered PCR tube, filled with 25 µl of PCR reaction mix (see Table 3.3), which were mixed on ice:

Reagent	Concentration	Volume [µl]
Buffer (green) 10x	2x	5.0
Sense primer (pLXSN5´)	10 pmol/µl in H_2O	0.5
Anti sense primer (ΔWD40 as SalI)	10 pmol/µl in H_2O	0.5
dNTP´s	0.8 mmol/l	0.5
Go Taq polymerase	0.02 U/ml	0.25
Water	-	18.25

Table 3.3: PCR reaction mix

Afterwards the tip was given in a sterile test tube, filled with 4 ml LB medium supplemented with ampicillin. These tubes also were numbered like the PCR tubes. The test tubes were incubated over night at 37°C under shaking. The PCR was performed on a Biometra T-Gradient or T3-Thermocycler using time and temperature listed in Table 3.4.

	Temperature	Time [sec]
Denature	94	180
Cycling (30x)		
Denature	94	30
Annealing	58	30
Extension	72	90

Table 3.4: PCR temperature profile

After completion of the PCR run, the samples were loaded onto a 1% agarose gel and were run at 120V for about 50 minutes, to know which clones are positive. The correct size of the product should be 380 bp.

MiniPrep (Wizard plus mini prep DNA purification system, Promega)

Therefore 1.5 ml of each sample of the over night incubated bacteria were taken and transferred into an Eppendorf tube. After centrifuging at 10 000 g for 10 minutes, the supernatant was discarded and 250 µl of cell re-suspension solution were added to the pellet and mixed. Then, 250 µl cell lysis solution was added and the tube was inverted 4 times. At the next step, 10 µl alkaline protease solution was added and again inverted 4 times. After incubating the mix for 5 minutes at RT, 350 µl neutralising solution was added and centrifuged 10 minutes at maximum speed (RT). To bind the DNA, the cleared lysate, containing the plasmid DNA, was transferred into a minicolumn. The column was centrifuged 1 minute at maximum speed, the flow through discarded and the DNA washed by pipetting first 700 µl and after centrifuging 1 minute at maximum speed 200 µl wash solution onto the column. After the second centrifugation step (2 minutes, maximum speed), the column was put in an Eppendorf tube and 50 µl nuclease free water was added to elute the DNA. At least the column was centrifuged 1 minute at maximum speed and the diluted DNA was stored, correctly labelled, at -20°C.

Restriction digest

To proof, if there is the correct insert in the vector pLXSN, there was made a restriction digest (see Table 3.5) of the 6 samples with suitable restriction enzymes. These enzymes were chosen because of the vector map (see appendix) and the amino acid sequence of the Δ U-box:

Reagent	Amount	Volume [µl]
Plasmid DNA	1 µg	3.0
EcoRI	20 000 U/ml	0.8
BamHI	20 000 U/ml	0.8
EcoRI buffer	1x	1.0
BSA [10 mg/ml]	100 µg/ml	0.1
Water	-	4.4

Table 3.5: mix for restricion digest after performance of MiniPrep

Digest was performed by incubating 1 h at 37°C on the heat block. After incubation, the samples were loaded onto a 1% agarose gel and run about 50 minutes. Because of the result of the gel, one sample was singled out for doing Maxi Prep.

Maxi Prep using endo-free plasmid maxi kit (QIAGEN)

100 ml of LB medium was filled in a sterile culture vessel and 100 µl of ampicillin was added. A pipette tip of bacteria suspension, which was stored at -80°C was taken, put in the vessel and incubated over night at 37°C.

At the next day, the bacteria suspension was centrifuged at 6000 g for 15 minutes at 4°C, using a AVANTI J-20XP Beckman Coulter centrifuge.

The supernatant was discarded, the pellet was re-suspended in 10 ml buffer P1. After adding 10 ml of buffer P2 and inverting 4 times the solution was incubated for 5 minutes. The lysate then was supplemented with 10 ml of chilled buffer P3, again inverted 4 times and poured into the barrel of a capped QIAfilter cartridge. After incubation time of 10 minutes at RT, the cartridge was decapped, and the lysate was filtered with an inserted plunger into a clean 50 ml tube. After adding 2.5 ml of buffer ER and inverting 10 times, the lysate was incubated 30 minutes on ice. During incubation the QIAgen tip was equilibrated with 10 ml buffer QBT and afterwards the lysate was poured onto the tip. The tip was twice washed with 30 ml buffer QC and the elution followed with 15 ml of buffer QN in a clean tube. The precipitation was performed with 10.5 ml Isopropanol at RT, the solution was mixed and centrifuged at 3550 rpm for 30 minutes at RT. The obtained pellet was re-suspended in 5 ml 70% EtOH and again centrifuged at 2000 rpm for 25 minutes at RT. The supernatant was discarded in a laminar hood and after drying the pellet by air, it was re-dissolved in 50 µl sterile AD and stored at -20°C.

The concentration was identified spectrophotometrically with an Eppendorf BioPhotometer, with a 1:50 dilution of the plasmid DNA in water.

3.3 Virus generation of SNEV and its deletion mutants in RetroPack PT67 Packaging Cell Line

RetroPack PT67 Packaging Cell Line (Clontech), a NIH/3T3-based line, is used to produce infectious, replication-incompetent retrovirus and with this technique, a gene of interest can be introduced into a wide variety of mammalian cell types *in vitro* or *in vivo*. Besides, transfection by using retroviral gene transfer is one of the most powerful techniques for introducing genetic material into mammalian cells and also the transfection efficiency is very high.

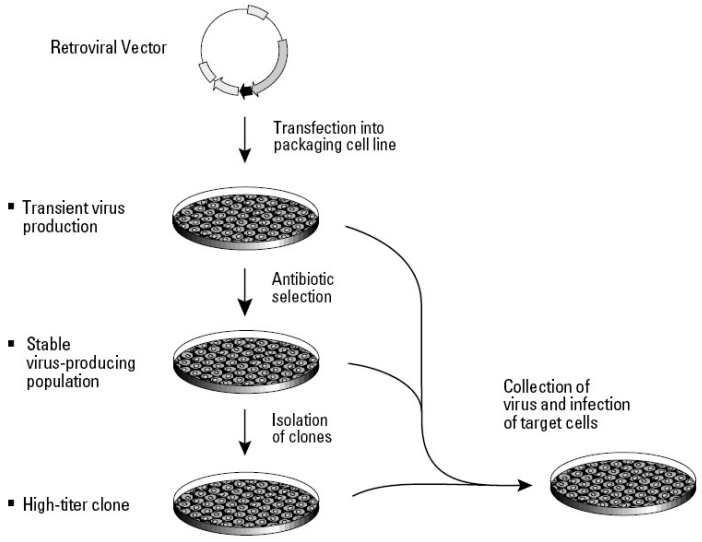

Figure 3.10: Procedure of transduction of PT67 cells, collection of virus and infection of target cells

The basic strategy for gene expression is shown in Figure 3.10. A retroviral vector containing the gene of interest is first transfected into PT67. By antibiotic selection, a population of cells that stably expresses the integrated vector can be isolated and virus produced by transfected packaging cells can then be used to infect target cells.

Pre-testing of cloning efficiency

Before the transfection was started, a preliminary test was performed. Therefore PT67 cells were seeded in a 96 well plate by making a dilution series of first 16 cells down to 0.5 cells per well to determine the cloning efficiency.

Transduction

One day before transduction was performed, PT67 cells were propagated 1:3 in 25 cm^2 roux flasks.

For transfection 18 sterile Eppendorf tubes (1 negative control) were filled with 0.5 ml Opti-MEM each. The plasmid DNA (Table 3.6) was pipetted in one tube with Opti-MEM (Invitrogen).

name	concentration [µg/µl]	volume [µl]
ΔGL2	1.2	5.4
WD40	1.0	6.5
ΔWD40	0.7	9.3
ΔBD	0.86	7.6
ΔU/BD	0.85	7.6
ΔU-Box	1.0	6.5
SNEV (pLXSN sense)	2.12	3.1
G418	1.3	5.0

Table 3.6: Concentrations of SNEV and its deletion mutants and the eliminated volumes of plasmid DNA, which were used for transduction of target HUVECs

The rest of tubes were filled with 20 µl of Lipofectamine 2000 (Invitrogen) each and incubated 5 minutes at RT. The plasmid DNA and Lipofectamine 2000 then were mixed and incubated for 20 minutes at RT. PT67 in 25 cm^2 roux flasks were rinsed once with Opti-MEM and then filled with 5 ml of the same medium. The PT67 cells then were incubated with the DNA / liquid mixture and incubated for 6 hours at 37°C in a humified atmosphere. After this incubation time, medium was changed to PT67 complete medium (DMEM, 10 % FCS, 6 mM L-Glutamine, 1 mM sodium pyruvat). One day after, the transduced cells were passaged into in two 25 cm^2 roux flasks, in a split ratio of 1:5 and 1:10 and 0.5 mg/ml G418 was added to the medium for selection of positive clones. Three days later, the medium was changed. Once the cells reached confluence, they were harvested, counted and diluted with medium, to get a statistically concentration of 3 cells per 200 µl. Each 200 µl were seeded into a 96 well by using a multichannel pipette. Wells, were only one clone was grown were marked and $1x10^5$ cells of 5 different clones of each transfectant were propagated in a 12 well plate as soon as they were confluent. After transfected PT67 reached confluency in the 12 well plates, they were propagated in 25 cm^2 roux flasks in a split ratio of 1:5.

Virus titer test

One day before testing the virus titer, NIH/3T3 was seeded in 24 well plates in a split ratio of 1:6. For performance of the virus titer test, 4 µg/ml polybrene (Hexadimethrine Bromide, Sigma) was added to NIH/3T3 complete medium, medium from the NIH/3T3 was discarded and each well was filled with 900 µl medium containing polybrene. The virus containing supernatant from PT67 transfectants was transferred to vials and filtered through a 0.45 µm filter and diluted 1:10 with medium. Six 10-fold serial dilutions were prepared by using a multichannel pipette. The PT67

transfectants were propagated 1:10. One day after the virus titer test was prepared, the medium was changed and 0.5 mg/ml G418 was added.
Because a bacteria contamination of PT67 transfectants was determined, double concentration of a penicillin/streptomycin solution was added to the medium, the cells were propagated twice a week at a split ratio of 1:10.
Every three days, the medium of the virus titer cells was changed and after 11 days the titer test was evaluated. The clones of PT67 transfectants which produced the highest titer were selected and expanded to 3 175 cm^2 roux flasks. At this last passaging step, the cells were seeded without any antibiotic. The virus containing medium from these cells was filtered through a 0.45 µm filter, aliquots of 5 ml were filled in vials (volumes of 5 ml) and frozen at -80°C.

Calculation of the virus titer

The titer of virus corresponds to the number of colonies, present at the highest dilution which contains colonies, multiplied by the dilution factor.

3.4 Retroviral transduction of HUVECs with SNEV and SNEV deletion mutants

Recombined viruses were used to transduce target cells, in our case HUVECs.
One day before transduction, HUVECs (101.4 PD 9.5) were seeded into 25 cm^2 roux flasks. The culture medium was removed, new medium, virus contained medium (concentrations are listed in Table 3.6) and 2 µl/ml polybrene was added to the cells. The total volume was 7 ml. For the negative control, only 7 ml medium was added to the cells. Cells were incubated over night and thereafter, they were passaged in a split ratio of 1:2 and 20 µg/ml G418 was added for selection of positive clones. Because SNEV deletion mutants didn´t grow very well, HUVECs were transduced again and after 24 h post-transduction, cells were lysed (protocol of cell lysation as described in 3.8.1) for performance of western blot.

3.5 Retroviral transduction of RPTECs with hTERT

The cDNA coding for hTERT in pLXSN was transduced in PT67 RetroPack Cell Line as described in 3.4 and retroviral particles were generated for transducing target cells.

RPTEC cells (PDL 5), grown in 25 cm^2 culture vessels, were separated from medium, 5 ml pre-aerated SFM-N4 medium, 4 µg/ml polybrene and 5 ml virus containing medium were added and incubated at 37°C in a humified atmosphere. Another 25 cm^2 culture vessel with RPTECs at PDL 5 was transduced with a vector, containing neomycin resistance only, as negative control. 24 hours after infection, cells were selected by addition of 100 µg/ml geneticin sulphate (G418).

3.6 Determination of DNA damage via Comet assay

The Comet assay is a common method for detection of DNA damage, both single and double strand DNA breaks. First described by Singh et al. in 1988, the principle is based on damaged DNA of an individual cell, which expands out of the cavity (Singh et al., 1988). The dimension of DNA damage can then be elicited by staining of DNA and counting out the individual cells.
Single and double stranded DNA damage in I38-SNEV and I38-neo cells was measured by single-cell gel elecrophoresis (comet assay) under alkaline conditions.

Preparation of the slides

A 1 % solution of agarose in PBS was prepared, melted in a water bath and cooled to 37°C. The day before, slides were pre-coated with a thin layer of 1 % agarose in PBS by dipping the slides into the agarose, wiping the back clean with a dry paper and let them dry at RT over night.
The slides were labelled and 2 drops of 100 µl 1 % agarose in PBS were pipetted quickly onto the slides followed by being covered with a cover slip (20x20 mm) and the slides were stored for about 5 minutes at 4°C.

Preparation of the cells

The cells (about 400 000 cells) were diluted in 140 µl low melting agarose (37°C). After removing the cover slips the cells were transferred as two roughly drops on each slide. Covered with a cover slip, the slides were stored at 4°C for about 5 minutes.

Cell lysis

The cover slips were removed from the slides and covered with cold lysis buffer for one hour at 4°C.

Alkaline treatment

The alkaline treatment was done in the electrophoresis tank at 4°C in electrophoresis buffer for 40 minutes in the dark.

Electrophoresis

The electrophoresis was carried out under stirring at a voltage of 25 constant for 30 minutes at 4°C, also protected from light.

Neutralisation

The slides were neutralized with neutralisation buffer for 5 minutes at 4°C 3 times.

Staining with SYBR Green I (Molecular probes)

The DNA was stained with SYBR Green I (diluted with TE buffer (10 mM TrisHCl, 1 mM EDTA, pH 7.5) 1:10 000). Therefore 20 µl was put on each slide and covered with a cover slip. The incubation followed light protected for 30 minutes at 4°C in a humified area.

Analysis

At least one hundred randomly selected cells were examined for the presence or absence of comets using fluorescence microscopy. The cells were scored as containing either no detectable DNA damage (no tail, Cat. I), only low (small tail, Cat. II) or high DNA damage (large tail, Cat. III).

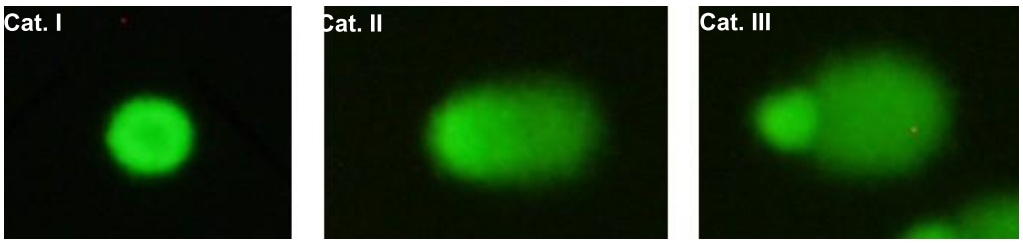

Figure 3.11: Comet assay: Cells were classified into 3 groups. Cat. I shows no damaged DNA (no tail), Cat. II displays a low (small tail), and Cat. III has accumulated high levels of DNA damage (long tail)

3.7 Measuring of ROS level after treatment with either BSO or Bleomycin using flow cytometry

Direct detection of ROS in biological samples has proven to be extremely difficult. ROS are very reactive molecules and are therefore extremely unstable, making it impossible to detect them directly. Thus detection of end products is one of the most

widely used methods for detection of ROS levels – either by chemiluminescence or by fluorescence – that are formed when specific compounds react with ROS (reviewed in: Freeman and Crapo, 1982; reviewed in: Pryor and Godber, 1991).

3.7.1 Application of BSO

Glutathione (GSH) is important for many cellular biochemical functions, including the regulation of gene transcription. When cells were treated with BSO (L-Butionine-[S, R]-sulfoximine, Sigma), which depletes GSH from cellular compartments by inhibition of cytosolic γ-glutamyl cysteine synthase (γ-GCS), a higher level of ROS was observed.

HUVECs were grown in 12 well plates in 7 % CO_2 at 37°C in a humified atmosphere to about 60 % confluence. Thereafter, the cells were treated with different concentrations of BSO (for preparation see appendix) (250 µM, 500 µM, 750 µM, 1 000 µM) in complete cell culture medium (composition of medium, 3.1.1) and incubated at 37°C for 48 h.

3.7.2 Application of Bleomycin

Bleomycin, produced from streptomyces verticullus, is used as chemotherapeuticum in cancer therapy, which has been reported to cause single- and double stranded DNA breaks (Hecht, 2000) as well as to activate oxidative stress pathways by producing ROS (Sikic, 1986).

After incubation with BSO, supernatant was discarded and the cells were treated with different dilutions of bleomycin (for preparation see appendix) (50 µg/ml, 75 µg/ml, 100 µg/ml, 150 µg/ml) in complete cell culture medium for further 24 h at 37°C in a humified atmosphere.

3.7.3 ROS staining

Staining with CM-H_2DCFDA (5-(and-6)-chloromethyl-2´,7´-dichlrorodihydrofluorescein diacetate, acetyl ester)

CM-H_2DCFDA is a cell-permanent indicator for ROS that is non-fluorescent until the acetate groups are removed by intracellular esterases. The oxidation occurs inside the cell. Oxidants like, hydrogen peroxide (H_2O_2), hydroxyl radical (HO), peroxyl radical (ROO•) and peroxynitrite anion ($ONOO^-$), can be detected by monitoring the increase in fluorescence with a flow cytometer.

First a solution of 1 µl DMSO in 10 ml PBS was made. CM-H_2DCFDA (for preparation see appendix) was diluted 1:10 in PBS and was stored light protected. The cell culture supernatant was discarded from the cells, which were then rinsed once with 1 ml of PBS per well. Each well was filled with 1 ml of the 1 µM staining solution, the negative control were filled with DMSO in PBS (1 µl/10 ml) and the cells were incubated for 30 minutes at RT, protected from light. Thereafter, the staining solution was removed and the cells were detached using 0.1% trypsin / 0.02% EDTA. Then the cells were re-suspended in 1 ml 10 % FCS in PBS, transferred to a tube and centrifuged at 170 g for 10 minutes. The cell pellet was re-suspended in 200 µl PBS and transferred into FACS tubes. Analysis was performed on a FACS Calibur (FL1 vs counts) using excitation sources and filters appropriate for FITC (488 nm/530 nm).

Staining with DHE (dihydroethidium)

The intracellular superoxide anion ($\bullet O_2^-$) concentration was detected by measuring the fluorescence intensity resulting from oxidation of DHE. The reagent has the ability to enter the cell, where it is oxidized to ethidium by $\bullet O_2^-$. Ethidium binds to DNA, which produces strong red fluorescence.

DHE (5mM/ml) was diluted in basal medium to a concentration of 20 µM and wrapped with aluminium foil to protect from light. Cell culture medium was removed, 0.5 ml staining solution was put in each well and the cells were incubated at 37°C for 30 minutes. The supernatant was transferred to an Eppendorf tube and the cells were harvested with 300 µl 50 mM EDTA at 37°C. Then, 700 µl PBS (Ca^{2+}/Mg^{2+}) was added and the solution was transferred to the tubes with the corresponding supernatant. The cells were centrifuged at 170 g for 10 minutes, the supernatant was removed and the cell pellet was re-suspended in 200 µl PBS and transferred into FACS tubes. Analysis was performed on a FACS Calibur (FL2 vs counts, 355 nm/420 nm).

3.8 Western blot

3.8.1 Cell lysis and preparation of samples

For western blot analysis, cells (grown to confluence) were lysed using DIGE lysis buffer. The cells were washed three times with PBS to get rid of the medium, afterwards cells were detached using EDTA. The cells were incubated at 37°C till they were detached. After tapping the flask, the cells were dissolved in 3 ml PBS

+Ca^{2+}/Mg^{2+} re-suspended and transferred in a 10 ml vial. The cell suspension was centrifuged at 170 g for 10 minutes and the supernatant was discarded. The pellet was re-suspended by tapping and dissolved in 1 ml PBS, transferred in an Eppendorf tube and centrifuged 30 second at maximum speed. The PBS was removed and the pellet was suspended in 50 µl DIGE lysis buffer by pipetting up and down and frozen at -80°C.

Before using the lysates, they were thaw on ice and centrifuged at maximum speed for 30 minutes. The supernatant was transferred in a new tube and the protein concentration was determined.

3.8.2 Bradford protein assay

To load equal amounts of protein onto the SDS page, the protein concentration of each sample was determined. The Bradford Assay is a rapid and accurate method commonly used to determine the total protein concentration of a sample.

Therefore the samples were diluted 1:50 in Bradford dilution buffer after the last centrifugation step of 3.8.1. A 96 well micro titer plate was taken and H1 was filled with 100 µl Bradford dilution buffer, H2 and H3 was filled with 100 µl 200 µg/ml BSA per well. 98 µl Bradford dilution buffer was pipetted in H4 to H11 for 1:50 sample dilution. Then 2 µl of each sample were added from H4 to H11 (8 samples per plate can be determined). The order of the samples should be noted carefully to avoid confusion. The rest of the wells from A to G were filled with 50 µl Bradford dilution buffer by using a multichannel pipette and pipetting reverse. Afterwards a dilution series was made by transferring 50 µl from H to A with a micro-titer pipette, the last 50 µl was discarded. At least Coomassie Brilliant Blue G was mixed with AD 1:5 and filtrated through a 0.45 µM filter. 150 µl of this Biorad staining solution was added from A to H by pipetting reverse with a multichannel pipette. The samples were incubated for about 5 minutes on a shaker and the absorbance was measured spectrophotometrically with a BioAssay Reader (Sunrise).

$$c(protein)[\mu g/\mu l] = \frac{c(protein)[\mu g/ml] * 50}{1000}$$

3.8.3 SDS PAGE

SDS-PAGE, the short form for sodium dodecyl sulfate polyacrylamide gel electrophoresis, is a very common form for analysing proteins according to their

molecular weight. Sodium dodecylsulfate is an anionic detergent which denatures proteins and binds to them quite specifically in a mass ratio of 1.4:1 and confers a negative charge to the polypeptide in proportion of its length. It is usually necessary to reduce disulfide bridges in proteins with either 2-mercaptoethanol (2-ME) or dithiothreitol (DTT) to avoid adopting of the random-coil configuration. The principle of polyacrylamide gel electrophoresis (PAGE) is similar to that of an agarose gel, separating proteins molecules according to their size. The so negatively charged protein molecules will move across a polyacrylamide gel to the positive electrode, when electric current is used. The polyacrylamide gel is a polymerized matrix, composed of acrylamid (AA) and a crosslinker bisacrylamide (BAA), the polymerisation of the two monomers is induced by ammonium persulfate (APS) and accelerated by TEMED, a stabiliser of free radicals. The pore size of the gel is determined by the ratio of AA/BAA and the concentration of acrylamide. The polyacrylamide mesh is accountable for the reason that smaller molecules can move on faster than the large ones through the gel.

For SDS-PAGE samples were thawed on ice and an aliquot of 10 µg was diluted in AD, pipetted in an Eppendorf tube and were mixed with the same volume of 2x SDS sample buffer, which was freshly supplemented with 10% 2-mercaphtoethanol. Following the samples were heated 10 minutes and immediately cooled on ice afterward .A 12% 1.0 mm NuPage Bis-Tris Gel (Novex, Invitrogen) was inserted in an XCell*SureLock*™ Mini-Cell (Novex, Invitrogen), which was then filled with MOPS buffer. This buffer was prepared with a 20 fold stock of NuPAGE MOPS SDS-Running Buffer (Invitrogen) according to the manufacturer´s protocol. The chambers were flushed before the centrifuged samples and the SeeBlue Plus2 pre-stained standard (Invitrogen) were loaded onto the gel. With a constant voltage of 200 V the proteins were separated for about 50 minutes.

3.8.4 Silver stain

Switzer et al., introduced silver staining in 1979. This technique provides a very sensitive tool for protein visualisation with a detection level down to the 0.3 – 10 ng level (Switzer et al., 1979). The detection of proteins depends on the binding of silver ions to the amino acid side chains, primary the sulfhydril and carboxyl groups of proteins, followed by reduction to free metallic silver.

After SDS page and blotting onto a PVDF membrane, the gel was fixed in a fixation solution (50 % EtOH, 10 % pure acetic acid) for 30 minutes. Next the gel was

incubated in a 0.26 % solution of glutaraldehyde for 15 minutes and afterwards washed 3 x 5 minutes with AD. After the washing step, the gel was incubated in a 0.02 % solution of formaldehyde for 10 minutes and flushed with AD. The gel then was given in a 0.02 % solution of formaldehyde for developing until the desired intensity of the bands was reached, immediately after, the reaction was stopped by flushing with AD and adding stop solution for at least 10 minutes.

3.8.5 Blotting

Proteins, separated in an SDS-PAGE can be transferred to a solid membrane for western blot analysis. Therefore an electric current is applied to the gel so that the separated proteins transfer through the gel onto the membrane. To detect the antigen blotted on the membrane, specific primary and secondary antibodies, which are conjugated with an enzyme that catalyzes chemiluminescence reactions, are used. Antibodies used are shown in Table 3.7.

After separating the proteins with SDS-PAGE, they were blotted onto a polyvinylidenefluoride (PVDF) membrane (Millipore) using the XCell II™ Blot module (Novex, Invitrogen). The membrane was tailored to a size of 6.5 x 8 cm and also two filter papers were cut to this size. The membrane was shortly immersed in methanol, rinsed with water and then soaked in 1x NuPAGE transfer buffer. The blotting pads should be soaked well with transfer buffer, so that they are free of air bubbles. Two soaked blotting pads and one shortly soaked filter paper was placed into the blot module. Afterwards the gel was put on a lantern slide and the membrane was placed onto the gel. Further a shortly soaked filter paper was put on the membrane and then this pack was put also in the blot module. Two more soaked blotting pads were added. The anode (+) core was placed on the top of the pads. By holding the blot module together, it was clamped in the blotting chamber and the blot module was filled with 1x Transfer buffer till the gel-membrane-assembly was covered. The outer chamber was filled with pre-cooled AD. The transfer was performed using 30 V constant for one hour. After this procedure, the membrane was shortly soaked in methanol, air dried and stored dry at RT.

The membrane was next blocked for one hour with 3% skim milk powder in 0.1% Triton X-100 (Sigma) in PBS (TPBS) and after removing blocking solution the membrane was incubated for 1 hour with the primary antibody, diluted in 1% TPBS. The blot was then 3 times for 5 minutes washed with TPBS and incubated with the secondary antibody, conjugated with either peroxidase or alkaline phosphatase,

diluted in 1% TPBS for one hour. After removing the secondary antibody, 3 washing steps were performed with 1% TBPS, each for 5 minutes. The detection was performed with either Chemiluminescent Peroxidase Substrate 1 (Sigma) or CDP Star reagent (New England BioLabs).

Primary antibody	Dilution	Company
mouse-anti-p16	1:500	Santa Cruz Biotech.
mouse-anti-p21	1:2000	US Biologicals
rabbit-anti-p27	1:500	US Biologicals
mouse-anti-p53Ser15	1:5000	Santa Cruz Biotech.
mouse-anti-SNEV	1:5000	
rabbit-anti-Prp19 867	1:5000	
mouse-anti-alpha 4 His	1:2000	
rabbit-anti-phospho-H2A.X(Ser139)	1:10000	Upstate
mouse-anti-beta actin	1:10000	Sigma
Secondary antibody	**Dilution**	**Company**
Anti mouse peroxidase	1:5000	Sigma
Anti rabbit alkaline phosphatase	1:5000	Sigma
Anti rabbit peroxidase	1:5000	Sigma
Anti mouse alkaline phosphatase	1:5000	Sigma

Table 3.7: Antibodies used for Western blotting

Detection with Chemiluminescent Peroxidase Substrate (Sigma)

The blot was placed on a lantern slide and the working solution was prepared by mixing one part of Chemiluminescent Reagent with 2 parts of the Chemiluminescent Reaction Buffer. The solution was mixed well and put onto the blot, the reagent was incubated for 5 minutes under protection of light. The blot was put in a plastic film and the detection was performed using the Roche LumiImager.

Detection with CDP Star reagent (New England BioLabs)

After washing the membrane 3 times with TPBS and once with CDP Star washing buffer for 5 minutes, 24 ml CDP Star washing buffer was mixed with 1 ml of CDP Star Assay Buffer and the membrane was applied to this solution for about 10 minutes. 1 ml of this solution was mixed with 10 µl CDP Star detection reagent, the membrane was placed on a lantern slide and incubated there for 5 minutes under protection of light.

3.9 Indirect immunofluorescence

To detect DNA damage, HeLa cells were grown on cover slips in 12 well plates, treated with 0.5 µM staurosporin in RPMI with 10 % FCS for 24 hours and incubated with anti-phospo-histone H2A.X (serine139) clone JBW301 (Upstate, catalog # 05-636). The characterisation of epithelial cells was done by using anti-pan-cytokeratin.

Used antibodies and their dilutions are listed in Table 3.8.

The supernatant was removed and the cells were washed twice gently with warm PBS for 5 minutes. The cells were fixed with methanol/acetone (1:1) for 2 minutes at RT. Afterwards the cells were washed once with PBS for 5 minutes and blocked with PBG (2 % fish gelatine, 5 % BSA in 1x PBS) for 30 minutes also at RT. Then the cells were treated with the anti-phospo-histone H2A.X (serine139) antibody in a wet chamber for 2 hours at RT. Therefore, 50 µl of the antibody, diluted 1:500 in PBG, were put on a para film, which were given in the wet chamber and the slides were positioned with the cells downside directly on the drop of antibody. For the negative control, the slides were incubated only in PBG.

After incubation time the slides were put back in the 12 well plate and washed three times with PBG. The slides were incubated with the second antibody, anti-rabbit-FITC, diluted 1:100 in PBG, for 1 hour at RT also in a wet chamber. Back in the 12 well plates, the slides were washed twice with PBG for 5 minutes and twice with PBS for 5 minutes. Finally the slides were air-dried, a drop of Fluoroprep was given on a slide and the cover slip was put on this drop. The analysis was done by using a Leica confocal microscope.

Primary antibody	**Dilution**	**Company**
mouse-anti-pan-cytokeratin	1:100	Sigma
rabbit-anti-phospho-H2A.X(Ser139)	1:500	Upstate
Secondary antibody	**Dilution**	**Company**
anti-rabbit-FITC	1:100	Sigma
goat-anti-mouse-FITC	1:100	Sigma

Table 3.8: Antibodies used for indirect immunofluorescence

3.10 Determination of GGT activity

Acivicin and 4-borono-2-amino-butanoic acid (L-ABBA) are specific inhibitors of GGT. For testing the GGT activity, RPTECs were grown in 12 well dishes.

A standard solution of 4 ml 10 % acetic acid, 1.6 ml 0.1 M TrisHCl pH 8.0 and 0.4 ml 0.1 M glycyl-glycine was prepared. The calibration was done by measuring several dilutions of para-nitroanilide (pNA) (1 mM, 100 µM, 50 µM, 25 µM, 12.5 µM and 6.25 µM) mixed with standard solution.

Acivicin (150 µM) and L-ABBA (100 nm, 10 µM and 1 µM) were diluted with RPTECs complete medium to get the correct concentration. Cells were treated for 2 h at 37°C in a humified atmosphere. After incubation, the cells were rinsed once with PBS and once with TrisHCl pH 8.0.

Cells then were incubated for 20 minutes at RT with the prior prepared substrate solution, containing 1 mM gamma-glutamyl-para-nitroanilide (GPNA), 60 mM TrisHCl pH 8.0 and 20 mM glycyl-glycine. After this last incubation step, 1 volume of substrate solution was mixed with 2 volumes 10 % acetic acid and the release of para-nitroanilide was determined by spectrophotometry at 405 nm on a Hitachi U 2001 photometer. Protein concentration was determined by using the Bradford assay as described in 3.8.2. GGT activity is expressed as nmoles pNA per minute per mg protein.

3.11 Real-time PCR TRAP assay

The PCR-based Telomeric Repeat Amplification Protocol (TRAP) permits telomerase detection with very low telomerase activity levels and enables the exponential amplification of the telomeric repeats generated in the telomerase reaction.
The telomerase adds specific DNA sequence repeats (TTAGGG) to the 3´ ends of DNA strands, which then were labelled with fluorescently nucleotides

Preparation of cell lysates

Cell lysis was performed using a modified standard protocol (Kim et al., 1994).
The cultivated cells were harvested and after determining the cell number, the required volume (100 000 cells) was transferred in a 1.5 ml Eppendorf tube. Now the cells were centrifuged at 170 g for 5 minutes and the supernatant was discarded. The cells were re-suspended in 0.5 ml PBS and again centrifuged at 1000 g for 10 minutes. After removing the liquid, 200 µl washing buffer was added to the pellet and the cells were then centrifuged at 10 000 g for 1 minute. After that, the supernatant was completely removed and the cells were re-suspended in 20 µl CHAPS lysis buffer. After 30 minutes incubation time on ice another centrifugation step (16 000 g, 30 minutes) followed. At least the supernatant was transferred in a Sarstedt tube and frozen at -80°C.

Real-time PCR TRAP assay

The lysates were thawed on ice and diluted with CHAPS lysis buffer. Because the samples were analysed in triplicates and also a negative control, digested with DNAse-free ribonuclease A (1 µg/µl) was analysed, 7 µl cell extract was diluted in 28 µl CHAPS lysis buffer, mixed and spinned down. 4 PCR tubes were filled with each 5 µl (5000 cells) of the diluted sample. Additional, 2 PCR tubes was filled with 5 µl

CHAPS lysis buffer and 2 other tubes with 5 µl H_2O_{dd} as no template control. Negative controls were treated with DNAse-free ribonuclease A to destroy the RNA-template, which the telomerase needs to form telomeric repeats, and incubated for 15 minutes at RT.

In the meantime, the PCR reaction mix was prepared (Table 3.9), mixed and spinned down, whereas the primer, SYBR Green I and the polymerase was added at least

As a positive control, a standard curve was established by decadic dilutions (1000, 100, 10 and 1 cell(s) in 5 µl) of HEK293 cell-lysates, analysed in duplicates.

From the PCR reaction mix a definite volume was removed, so that for each sample, which is digested with DNAse-free ribonuclease A, 13 µl can be added. The rest of the mix was diluted with AD, 2 µl AD for 13 µl PCR reaction mix. 13 µl PCR reaction mix was added to the DNAse-free ribonuclease A digested samples, the rest of the samples were mixed with each 15 µl of the diluted PCR reaction mix.

Before the analysis with a Rotor Gene 2000 Real-time Cycler (Corbett Research), with a 36 well carousel high speed rotor was started, all samples were incubated at RT for 30 minutes.

The samples and standards were denatured at 94°C for 2 minutes and 45 cycles were performed according to the temperature profile as seen below (Table 3.10).

Reagent	Final concentration	Volume/sample [µl]
TrisHCl pH 8.3	20.0 mM	0.40
$MgCl_2$	1.5 mM	0.30
KCl	63.0 mM	1.26
EGTA	1.0 mM	0.20
dNTPs	2.5 mM	0.50
TsmodIV primer	100 ng/µl	1.00
CXa primer	100 ng/µl	1.00
SYBR-green I	1:20 000	1.00
BSA [10 mg/ml]	0.30 µg/µl	0.60
Tween 20	0.005 %	2.00
Taq-polymerase	0.04 U	0.40
Sum		8.76
Sample volume		7.00
AD final volume		4.24
Total volume		20.0

Table 3.9: TRAP reaction mix

The diagram “fluorescence versus cycle” was acquired by measurement of the signal at 72°C and 65°C of each cycle. Practically semi-log amplification plots measured at 65°C (log increase in fluorescence versus cycle number) were used.

Action	Temperature [°C]	Time [sec]
Denature	94	120
Cycling		
Denature	94	10
Annealing	50	20
Elongation	72	60
Measuring	65	5

Table 3.10: PCR temperature profile of TRAP assay

The telomerase activity was expressed relative to HEK293 cells as % activity of HEK293.

3.12 Nuclear Flow FISH

The measurement of telomere length is possible with the fluorescence in situ hybridyzation technique (Flow FISH). This method was modified to the nuclear flow fish by Wieser et al. (Wieser et al, 2006), where only the nuclei were measured.
The principle of the nuclear flow fish technique is, that a fluorescein isothiocyanate (FITC)-labelled telomere specific peptide nucleic acid (PNA) probe is hybridized in a quantitative way to telomere repeats, followed by telomere fluorescence measurement by flow cytometry.

Sample preparation and hybridization

The cells, which were stored at –80°C in cryo tubes, were rapidly thawed at RT and transferred to 8 ml of cell culture medium. The cryo tube was rinsed with medium to loose as less cells as possible. The cells were centrifuged by 170 g for 10 minutes, the supernatant was removed and the pellet was re-suspended by tapping well. Then the cells were re-suspended in 5 ml PBS and again centrifuged under the same conditions. After that the supernatant was removed, the pellet was re-suspended by tapping and the cells were diluted in 3 ml 2 % Triton X-100/0.1 M citric acid coulter counter buffer, mixed on a vortex and incubated for 10 minutes at RT.
The formamide, stored at 4°C, was put to RT. The volume of hybridization buffer (75 % formamide, 1 % BSA (for preparation see appendix), 20 mM TrisHCl pH 7.1, 20 mM NaCl and 50 nM FITC conjugated $(C_3TA_2)_3$ PNA probe), needed, depends on the number of analysed samples. Handling with the hybridization buffer should be done under a flue, because of the formamide. 1800 µl hybridization buffer was necessary for each sample, because each sample was analysed in triplicates, and also 3 times without PNA for measuring of auto fluorescence. The loss of hybridization buffer by

pipetting should be considered by production. Hybridization buffer, which was needed for adding PNA was transferred in a 50 ml falcon tube.

The nuclei count was measured on a Multisizer™ 3 COULTER COUNTER (Beckman Coulter), therefore the cell solution was mixed on a vortex well and 100 µl of this solution was pipetted in a coulter counter tube. 9 ml of coulter counter buffer were added, each sample was measured twice and the mean was calculated. Only particles bigger than 2.5 µM were counted therefore. For telomere length analyses nuclei count of $5x10^5$ were used.

The nuclei now were centrifuged at 500 g, for 10 minutes, the supernatant was removed carefully. The pellet was re-suspended by tapping and diluted in the defined volume of PBS. Six Eppendorf tubes were filled with 500 µl from each sample and centrifuged at 800 g for 10 minutes at RT. The heat block was tempered to 80°C and the supernatant of the nuclei was removed carefully by holding the Eppendorf tube against the light. Soaking up of the pellet should be avoided. After that, the pellet was tapped easily and always three samples were filled with hybridization buffer without PNA and the other three containing PNA. The samples were put on the heat block at 80°C for 10 minutes under protection of light and incubated over night at RT, also under light protection.

The day after, PBS was warmed in a water bath at 40°C.

All samples were supplemented with 1 ml warm PBS under the flue and the Eppendorf tubes were inverted 4 times for mixing. Followed by incubation at 40°C for 10 minutes on the heat block under light protection and centrifuged at 500 g for 10 minutes. The supernatant was removed under the flue.

The pellet was washed with 1 ml of PBS, also with inverting after, heating on the heat block and centrifuging. This step was carried out three times. After the last step, the pellet was re-suspended in 200 µl DNA staining solution (for preparation see appendix) and the samples were analysed using flow cytometry tubes.

Flow cytometry

Analysis was performed on a FACS Calibur (Becton Dickinson). At first, the Quantum 24 FITC Low Level standard beads (Bang Laboratories) were analysed, and the voltage for FL1 was raised till a geomean of 55 of the standard beads with the highest fluorescence intensity. Then, samples stained with PI only were analysed at constant FL1 settings followed by the analysis of samples containing PI and PNA. In order to determine the relative telomere length, markers were set for calculating the

mean fluorescence intensities (MFI) of each beat population as shown in Figure 3.12 A. The values of relative fluorescence were then correlated to the defined MESF to derive a regression curve and the equation for MESF calculation of cell samples (Figure 3.12 B).

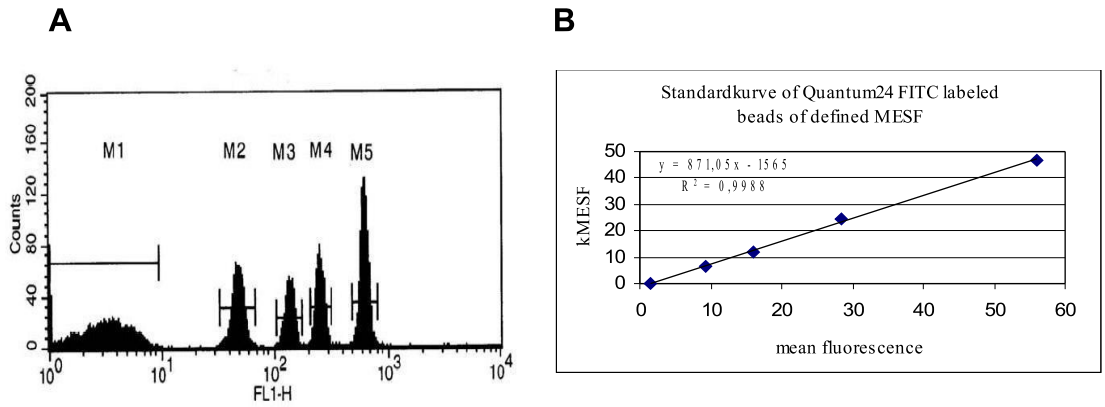

Figure 3.12: A: The mixed bead population was gated in a dot blot (FSC/SSC) and plotted in a FL-1 Histogram. Region M1 corresponds to the blank beads; regions M2 to M5 correspond to the FITC labelled beads with varying MESF values. B: A standard curve was established and linear regression was calculated to convert mean fluorescence intensities of samples into MESF.

Thereafter, G0/G1 cells were gated using a dot blot FCS-W vs. FL3, both with linear scaling (Figure 3.13 A). They are gated because they have the normal amount of DNA in contrast to the G2 population that has just finished DNA synthesis.

The fluorescence intensity of the G0/G1 cell population was then analysed using FL1 (PNA) and overlayed with the corresponding negative control (w/o PNA) (Figure 3.13 B). The MFIs of both, PNA probes and control samples were recorded. The MFI values of the control cells (M1) were subtracted from those of the PNA-probe labelled cells (M2), and converted into relative telomere length by the regression equation of the standard curve.

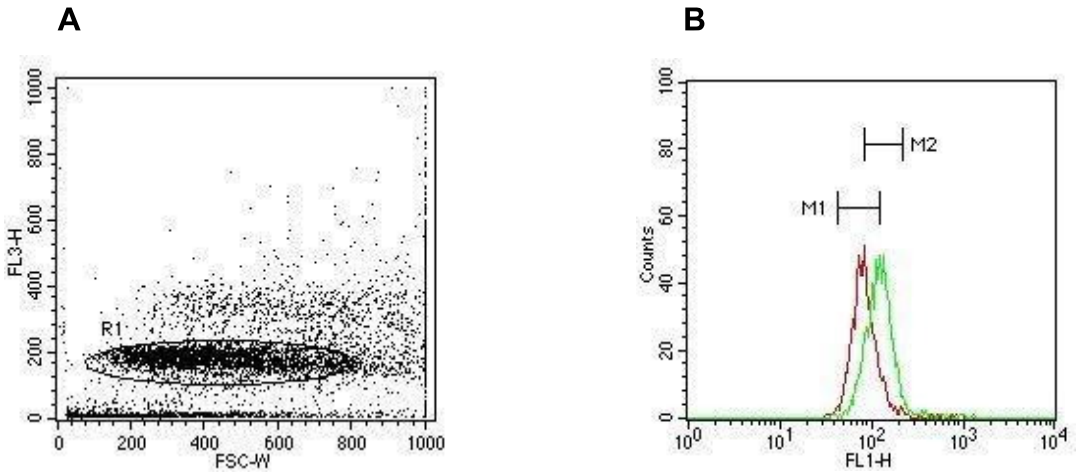

Figure 3.13: A: Dot plot of forward scatter (FSC-W) vs. PI fluorescence (FL3-H). Gated cells are in the G0/G1 phase cell cycle phase. B: FL-1 histogram of of G1/G0 phase cells showing the mean fluorescence intensity of cells labeled with PI only (red / M1) and the corresponding cells labeled with PNA+PI (green / M2)

4 RESULTS

4.1 Morphological characterization of cells

4.1.1 HUVECs

Early passage HUVECs show the typical cobblestone pattern (Figure 4.14 A). When HUVECs are freshly transferred into a new culture vessel, clusters of a few connected cells are built and from these clusters cells proliferate and form a homogenous monolayer of cells with close cell-cell contact (Gimbrone et al., 1974). Late passage HUVECs get more and more enlarged and flatten and the cells become inhomogenous in their appearance and finally, like all normal human somatic cells, they cease to grow and undergo replicative senescence (Figure 4.14 B).

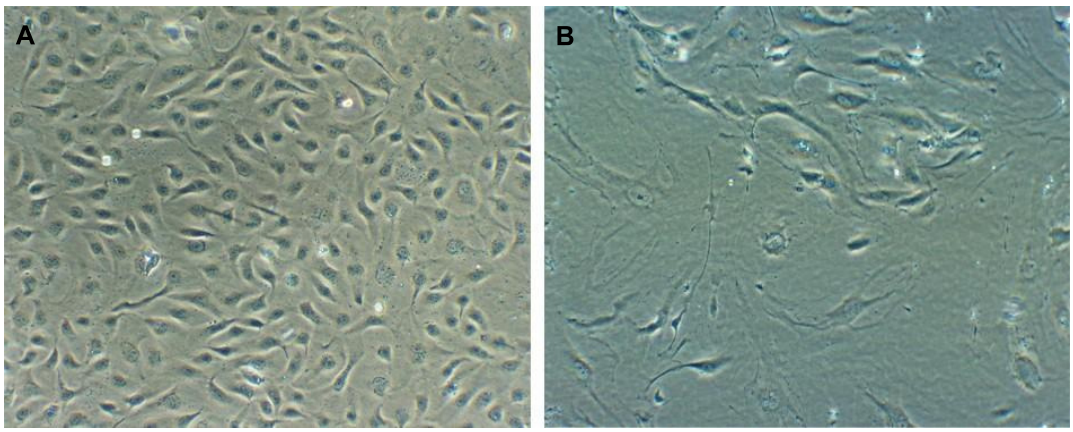

Figure 4.14: Picture A shows early passage HUVECs, in contrast to senescent HUVECs (picture B), which exhibit a large and flat morphology.

4.1.2 RPTECs

As shown in Figure 4.15 A, freshly transferred RPTECs form clusters of a few connected cells and from these clusters cells proliferate. RPTECs grow in small clusters (Figure 4.15 B), allowing them to maintain tight cell-cell contacts and when RPTECs are grown to high cell density, they form so called domes, as shown in picture C of Figure 4.15 (Lever, 1979).

RPTECs reach replicative senescence after approximately 24 population doublings in vitro and the growth arrest is accompanied by the typical enlarged morphology (Figure 4.15 D).

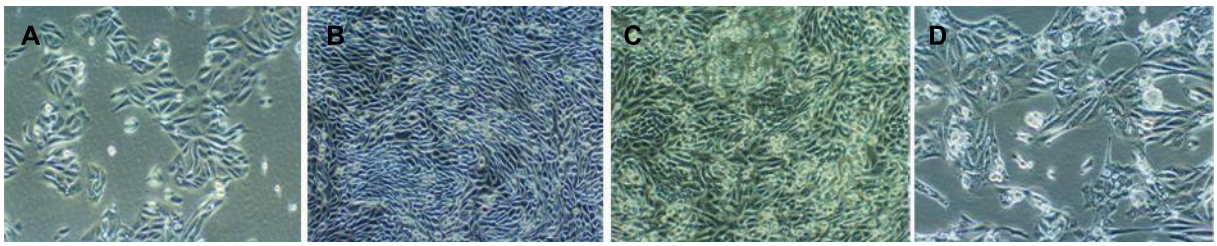

Figure 4.15: RPTECs in different passaging stages: (A)early passage cells, (B) confluent cells , (C) cells which form domes and (D)senescent RPTECs.

4.2 Cloning of delta U-box in pLXSN

After isolation of Δ U-box (about 1500 bp) from the vector pGBKT7 (7.3 kb) with the restriction enzymes EcoRI and SalI and vector pLXSN (5.9 kb) with the enzymes EcoRI and XhoI, DNA was loaded onto a preparative gel (shown in picture A and B of Figure 4.16) and the correct band of DNA was cut. Lambda DNA/EcoRI+HindIII Marker 3 (Fermentas) was used as standard.

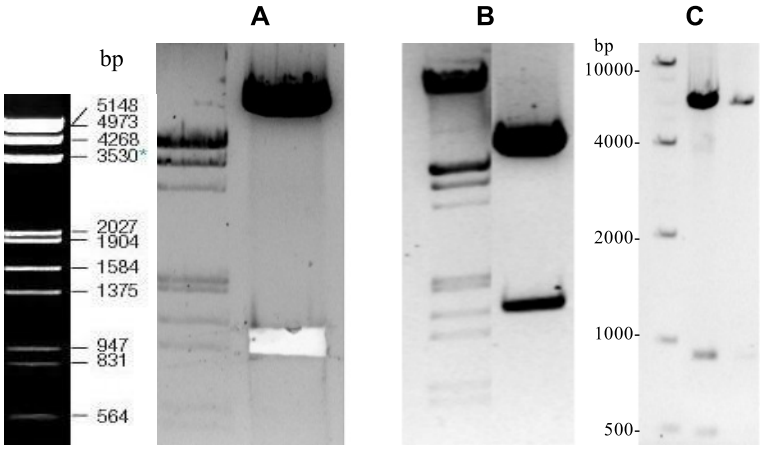

Figure 4.16: A: cut Δ U-box, (about 1500 bp) B: the vector pLXSN (5.9 kb) C: preparative gel of Δ U-box, also fast ruler, DNA Ladder, High Range was loaded for estimating the concentration.

The DNA containing cut gels were purified, Δ U-box and the vector pLXSN were ligated, precipitated and electroporation was performed. After plasmid selection with the antibiotic ampicillin, plasmid amplification of six colonies was performed by PCR. The picked clones were tested by loading them onto an agarose gel. Because there was a band at 380 bp at all six lanes, all samples were positv and MiniPrep was performed for purifying the DNA. Restriction enzymes EcoRI and BamHI, chosen because of the vector map, were used for performing restriction digest. Again, the DNA was loaded onto a gel, and one positive clone was selected for preparation of MaxiPrep. Finally, restriction digest was done and two different dilutions of the DNA

were loaded onto an agarose gel, as shown in Figure 4.16 C. For determination of the concentration, fast ruler DNA Ladder, High Range (Fermentas) was also loaded
The elicited concentration, according to the gel, is 0.4 µg/µl. The concentration, determined by an Eppendorf BioPhotometer, was 1.56 µg/µl. The mean of both elicited results was taken and defined as concentration of Δ U-box.

4.3 Transfection of PT67 RetroPack Packaging Cell Line and the amount of virus titer

Retroviral particles, containing plasmid DNA of SNEV and its deletion mutants, were produced by using RetroPack PT67 Packaging Cell Line.
Therefore a preliminary test was performed, as described in 3.3, for determining the right number of cells, which statistically have to be seeded in 96-well plates, to get one clone per well. Statistically 3 cells per well have to be seeded to get one clone.
PT67 cells were transduced with SNEV and its deletion mutants and a virus titer test was performed by infecting NIH/3T3 cells with retroviral particles as described in 3.3.
The individual results of the virus titer test are listed in Figure 4.17.

	Virus titer
SNEV	$2.0*10^3$
neo	$1.5*10^7$
delta GL 2	$4.0*10^6$
delta U-box + delta BD	$1.0*10^6$
delta U-box	$2.0*10^5$
delta WD 40	$1.1*10^2$
WD 40	$6.0*10^6$
delta BD	$1.0*10^4$

Figure 4.17: The amount of virus titer of SNEV and its deletion mutants.

4.4 Transduction of HUVECs with SNEV and its deletion mutants

Retroviral particles, produced with PT67 RetroPack Packaging Cell Line, were used to transduce HUVEC cells (101.4, PDL 9.5).
Cells were transduced and antibiotics were added after 24 hours. Two days after transduction, control cells, SNEV and neo-transduced cells looked well, but cells, transduced with deletion mutants, didn´t grow and looked very bad, as shown in Figure 4.18.

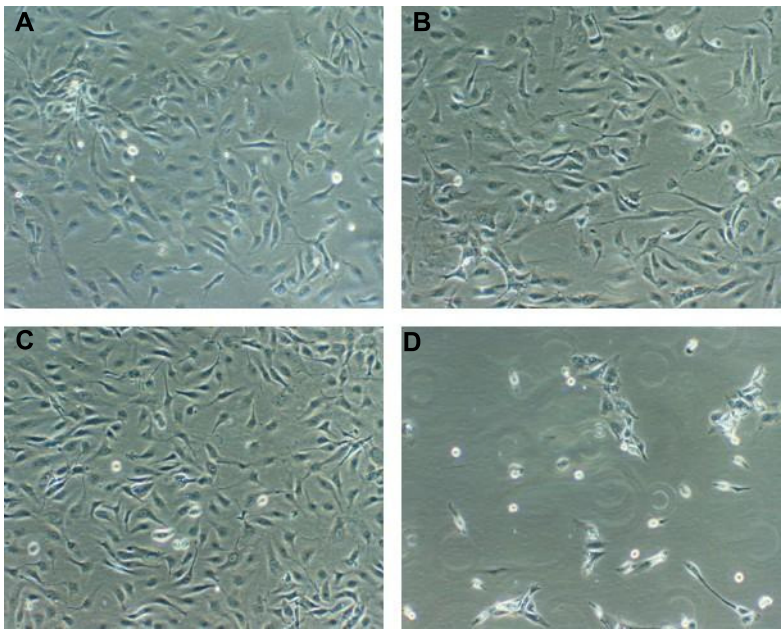

Figure 4.18: HUVECs, transduced with SNEV and its deletion mutants. A: control cells, B: SNEV transduced cells, C: neo-transduced cells, D: cells, transduced with Δ U-box/BD.

The transduction was abandoned. 101.4 cells (PD 15) were transduced with SNEV, neo and SNEV-deletion mutants again. After 24 h the cells were lysed. To determine the expression of SNEV a western blot was performed, showing no expression. No bands were detected after staining with Anti Prp19 867 rabbit antibody. Because His-tags were packaged in the plasmids, the blot also was treated with Anti α 4 His antibody, but there were also no bands (data not shown).

4.5 Determination of DNA damage

To look, if SNEV is involved in DNA damage repair processes, a Comet Assay was performed. Therefore, we analyzed I38-SNEV-1 cells and I-38-neo-1 cells approaching replicative senescence, both at PD 7 post transduction.

At least 100 cells were examined and assigned to Cat. I, II or III according to the forms of the DNA comets.

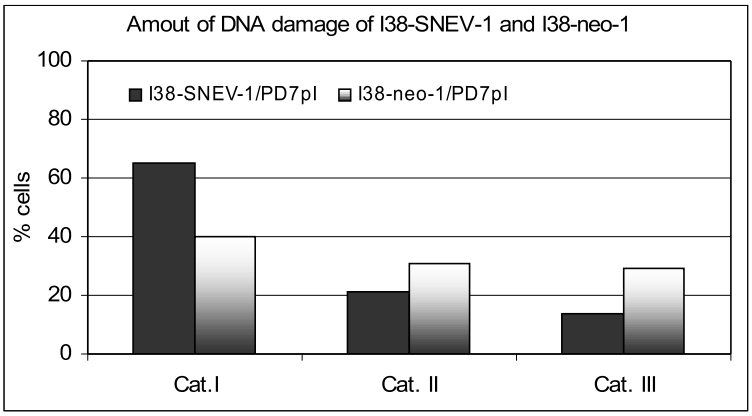

Figure 4.19: Analysis of at least 100 individual cells. The result clearly demonstrated higher percentages of undamaged I38-SNEV-1 cells at PD7pT, when compared to I38-neo-1 at same PD (Cat. I), while less cells were classified as Cat. II and Cat. III.

The result of the Comet assay is shown in Figure 4.19. 65 % of I38-SNEV-1 showed low level of DNA damage and were therefore assigned to Cat. I, only 40 % of I38-neo-1 showed no tail and were classified in the same category. Low DNA damaged cells were assigned to Cat. II. Whereas 30 % of I38-SNEV-1 and 21 % of I38-neo-1 were found within this group. Cells, which were classified Cat. III showed a long tail and their amount of DNA damage was very high. The percentage of I38-neo-1 in Cat. III (29 %) was two fold higher as I38-SNEV-1 (14 %). These data show that high level of SNEV correlates with a decrease of accumulated DNA damage during in vitro cultivation. Furthermore, we obtained also similar results in a second, independent experiment.

4.6 Determination of DNA double strand breaks using anti-phospho-H2AX-antibody

DNA double strand breaks (DSB) can be detected by analysis of gamma-H2AX foci in the nuclei of cells damaged with DSB-inducing agents. The H2A histone variant, H2AX, gets phosphorylated by the phosphatidylinositol 3-kinase-like protein kinases, ATM and ATR. The phosphorylated form, called gamma-H2AX, can be visualised as discrete foci with the use of specific antibodies.

For detection of gamma-H2AX foci with indirect immunofluorescence, we used HeLa cells, treated with either staurosporine or bleomycin of different concentrations. These chemicals are known to generate double strand breaks in mammalian cells. But unfortunately, it wasn´t possible for us, to visualise the typical, often in literature described, nuclear foci.

Therefore, we tried to detect gamma-H2AX by western blotting. HeLa cells were incubated with staurosporine or bleomycin of different concentrations and analyzed after diffterent time points. Lane 1 to 4 (Figure 4.20) show HeLa cells, which were treated with 0.5 µM staurosporine for either 1, 2, 3, or 4 hours. The time response of incubation is clearly reflected in the amount of gamma-H2AX. Lane 5 shows untreated HeLa cells, the strong signal of lane 6 shows cells, which were treated with 150 µg/ml bleomycin for 24 h. Cells from lane 7 to 10 were treated with either 0.1, 0.2, 0.4 or 0.5 µM staurosporin for 4 h. A concentration-dependent increase in gamma-H2AX was detected.

Although, it was not possible to detect DNA DSBs by indirect immunofluorescence, the western blot clearly shows, that gamma-H2AX detects DNA DSBs.

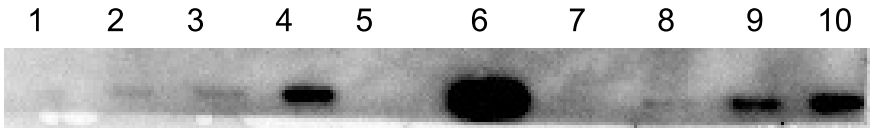

Figure 4.20: Lane 1 to 4 shows HeLa cells treated with 0.5 µM staurosporin for eiter 1, 2, 3 or 4 h. Cells at lane 5 were untreated. Lane 6 shows cells, treated with 150 µg/ml bleomycin for 24 h. Cells at lane 7 to 10 were incubated with either 0.1, 0.2, 0.4 or 0.5 µM staurosporin for 4 h.

4.7 ROS level

Cells were grown in 12 well plates and treated with either CM-H_2DCFDA or DHE before the ROS level was measured using flow cytometry as described in 3.7.3.

Figure 4.21 shows the ROS level of HUVECs at PDL 21 compared to the same cells at PDL 60. The level of peroxyl radicals (PO) and also the amount of superoxide anion (SO) are higher in late passage cells compared to the same cells at PDL 21. These results indicate that the ROS level of normally growing cells increases with the level of population doublings.

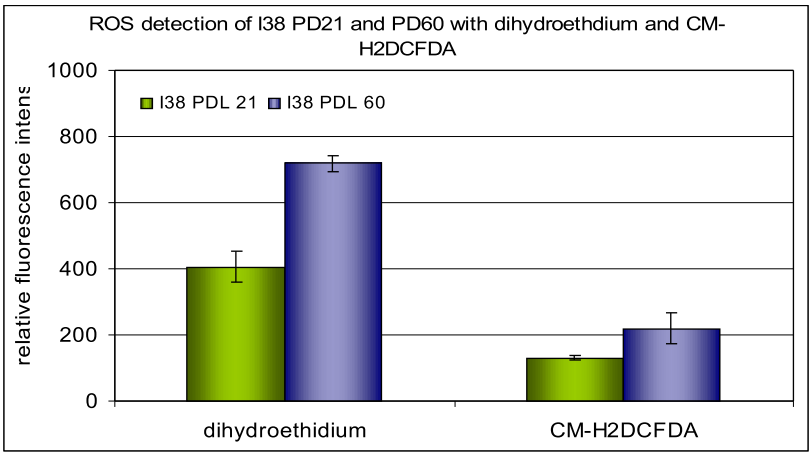

Figure 4.21: PO (CM-H_2DCFDA) and SO (DHE) level of early passage in comparison to late passage HUVECs. Both, PO and SO level are higher in late passage cells.

The ROS level of SNEV-transduced HUVECs was measured in comparison to neo-transduced HUVECs in order to evaluate if SNEV overexpressing cells are more resistant against stress. PO and also the SO level are significantly higher in neo-transduced cells at the same PDL as shown in Figure 4.22, whereas the difference of SO level between I38-SNEV-3 and I38-neo-3 is significantly higher compared to the PO level.

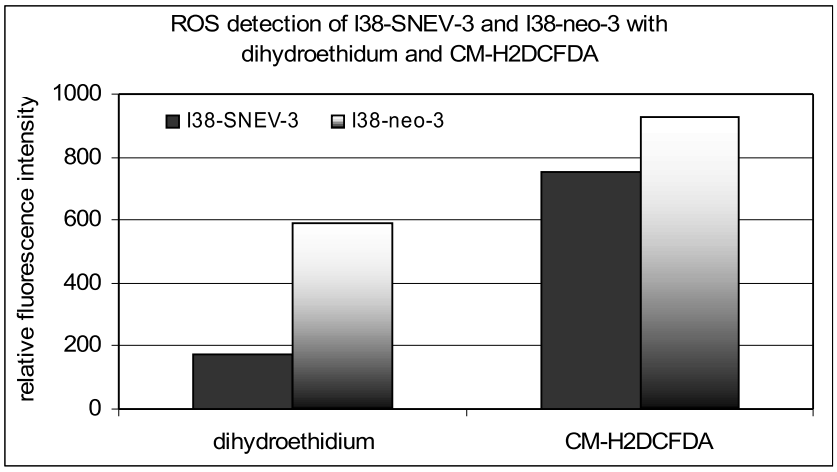

Figure 4.22: PO and SO level of SNEV-transduced HUVECs and vector control cells. PO level as well as SO level is lower in I38-SNEV cells compared to I38-neo-3.

The effect of treatment with bleomycin and BSO on the level of PO was also determined. Therefore, I38 cells at PDL 16 were treated with different concentrations of bleomycin (see Figure 4.23). The treatment with bleomycin alone didn´t show an effect on the PO level since treated cells have nearly the same level as non-treated cells.

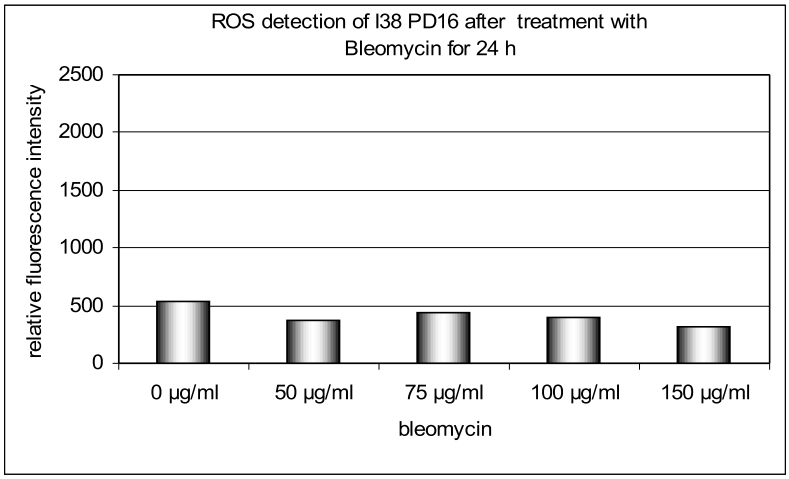

Figure 4.23: I38 cells (PDL 16) treated with different concentrations of bleomycin. The figure shows that treatment with bleomycin has no effect on the level of PO.

BSO is a selective inhibitor of glutathione (GSH) synthesis which depletes GSH. Figure 4.24 shows the PO level of BSO treated cells. It is clearly visible, that BSO treated I38 cells show a significantly higher level of PO. However, the PO level didn´t correlate with the concentration of BSO.

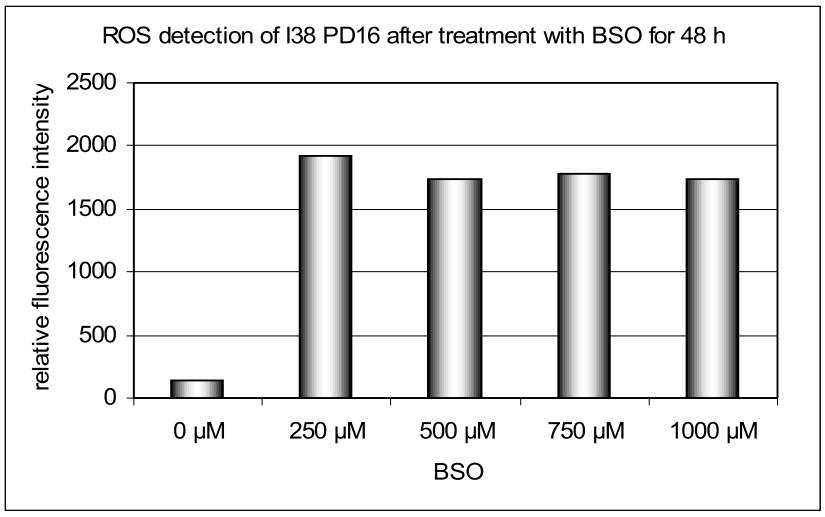

Figure 4.24: The PO level of I38 cells after treatment with different concentrations of BSO is significantly higher compared to non-treated cells.

Since it has been reported, that bleomycin regulates cellular glutathione (GSH), that acts against oxidative stress (Day et al., 2002), we further wanted to evaluate wether PO level changes, when cells were pre-treated with BSO (1 mM, 48 h) before incubation with bleomycin at different concentrations for 24 hours. The experiment observed that pre-treatment with BSO actually has an effect on PO level. In comparison to Figure 4.23, where cells were only treated with bleomycin, the PO

level is significantly higher when Glutathione was depleted by pre-treatment with BSO, as shown in Figure 4.25.

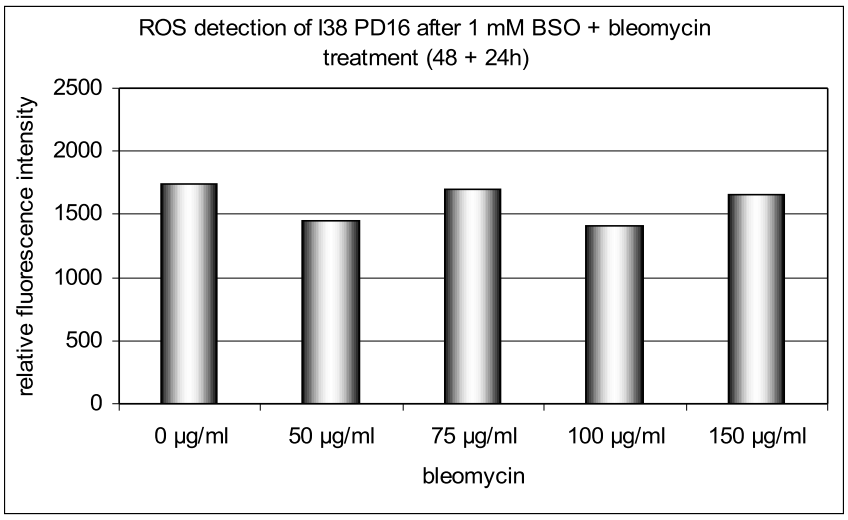

Figure 4.25: Cells were pre-treated with 1 mM BSO (48 h) for depletion of GSH, before they were treated with different concentrations of bleomycin (24 h). The figure shows, that the cytotoxic effect of bleomycin is enhanced, when GSH is depleted with BSO, because PO level is significantly higher in comparison to the PO level of bleomycin treated cells only (see Figure 4.23).

4.8 Expression of cell cycle regulator proteins

Expression levels of p16, p21, p53 and SNEV were analyzed to look how these cell cycle regulators are regulated through several passages of HUVECs. Therefore, 10 µg of total protein were separated by SDS-PAGE and Western blot analysis was performed.

The results, which are shown in Figure 4.26, indicate that p16 and p21 are not regulated throughout serial passages.

In contrast, the tumor suppressor protein p53 increases with advanced age of HUVECs. Additionally, the expression of SNEV shows a direct correlation to the PDL of the cells. It is clearly visible, that SNEV is higher expressed in early passage cells and decreases continuously with several passages.

Beta-actin was used as loading control.

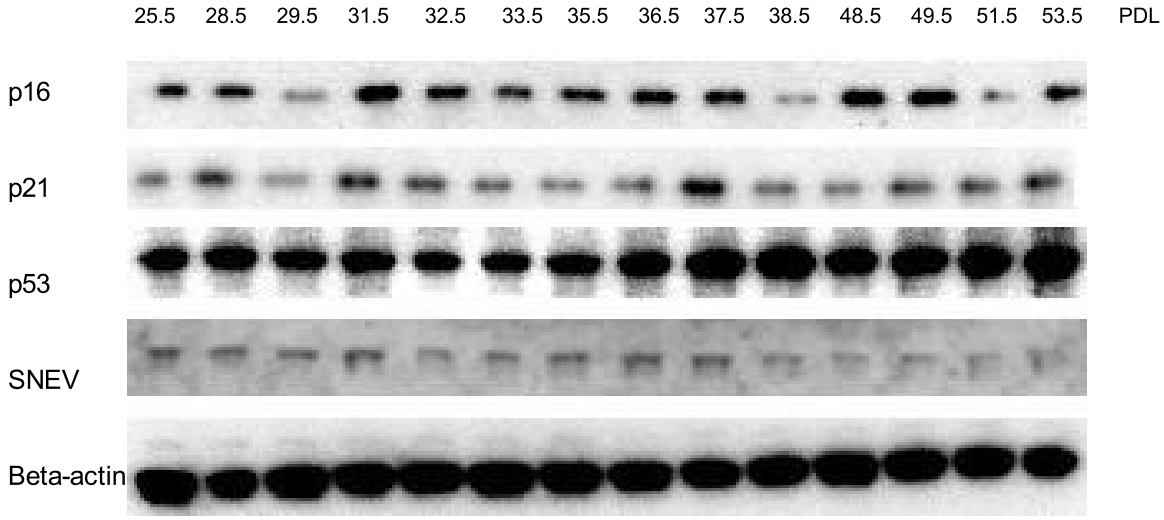

Figure 4.26: Expression of cell cycle regulators p16, p21, p53 and SNEV through serial passaging of HUVECs from PDL 25.5 to 53.5. P16 and p21 show no correlation with several passages. In contrast, p53 is higher expressed in late passage cells. Expression levels of SNEV decreases with increasing PDL of HUVECs.

4.9 Ecotpic expression of hTERT in RPTECs

RPTECs at PDL 5 were transduced with recombined retrovirus carrying hTERT. As negative control, cells were transduced with a vector carrying the neomycin resistance only. 24 h after infection, cells were selected by addition of geneticin sulphate.

Figure 4.27 shows the characteristic growth curve of RPTECs which enter growth arrest at PDL of 24 in comparison to RPTEC/TERT1, which grow continuously and have already reached more than 75 PDs without any signs of growth retardation.

Figure 4.28 shows the morphology of young and senescent RPTECs and RPTEC/TERT1 at two different time points after transduction.

Figure 4.28 A shows subconfluent normally growing RPTECs at PDL 5. The cells grow in clusters enabling cell-cell contact. RPTECs at PD 25 (Figure 4.28 B) which have already reached senescence, showed the typically enlarged morphology.

Picture C and D of Figure 4.28 show RPTECs after transduction with hTERT (8 PpI and 12 PpI). The cells are characterised by the typical cobblestone epithelial morphology, grow in clusters, which allow cell-cell contact.

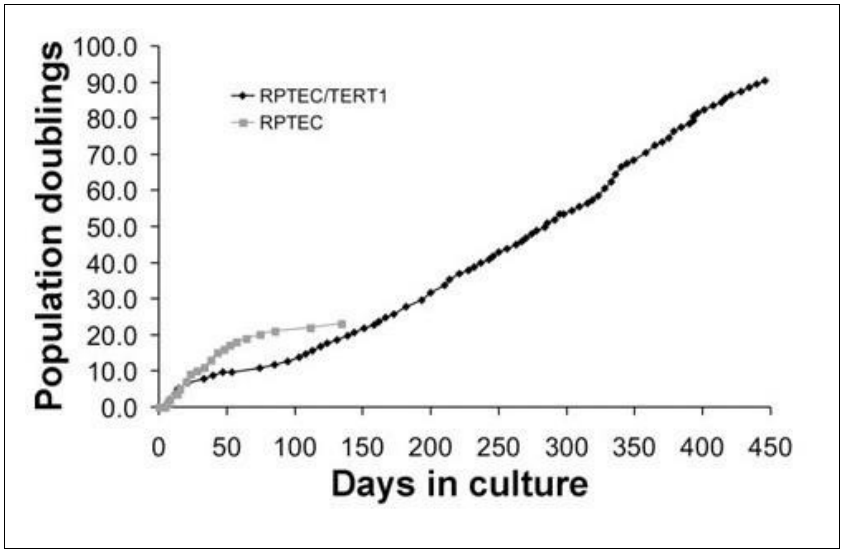

Figure 4.27: Growth curve of RPTECs which enter growth arrest at PDL 24. Compared to hTERT-immortalized cells, RPTEC/TERT1.

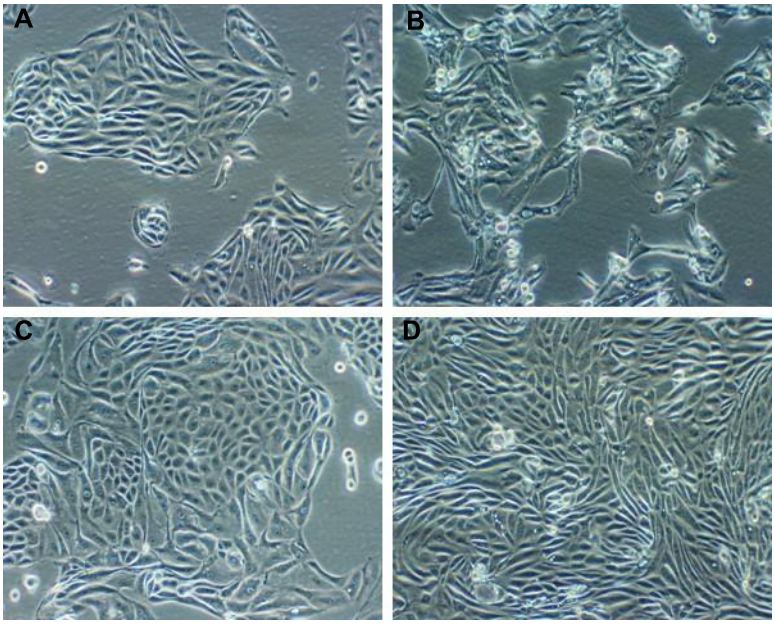

Figure 4.28: A: subconfluent RPTECs PDL 5 before transduction, they grow in clusters, B: senescent RPTECs, which have the typical enlarged morphology, C+D: hTERT transduced cells at two different PDpT (8 and 12).

4.10 Detection of cytokeratin

Expression of cytokeratins is a marker for cells of epithelial origin. These keratins form a complex network in the cytoplasma which extends from the surface of the nucleus to the cell membrane.

Cells were grown on slides and indirect immunofluorescence was done with a primary mouse anti-pan-cytokeratin antibody and a secondary FITC conjugated goat anti-mouse antibody.

Figure 4.29 shows that both, RPTECs as well as hTERT overexpressed cells form the typical cytokeratins.

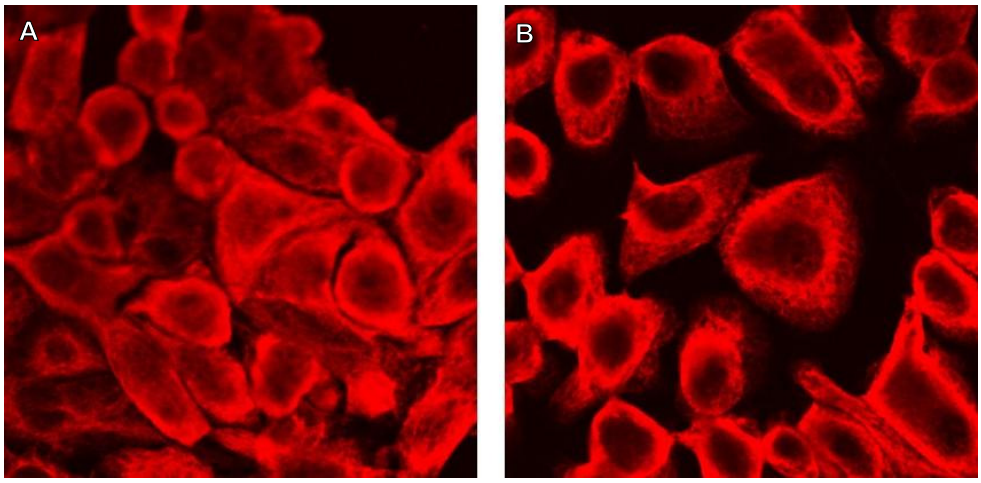

Figure 4.29: Both, RPTECs (picture A) and hTERT transduced cells (RPTEC/TERT1) (picture B) homogenously express cytokeratins.

4.11 GGT activity of RPTECs and RPTEC/TERT1

The activity of GGT was measured spectrophotometrically as described in 3.10. For inhibition of GGT, cells were treated with either acivicin or L-ABBA.

As shown in Figure 4.30, activity of GGT in RPTEC/TERT1 was comparable to the activity of late passage cells. However, early passage RPTECs (PD 11) exhibit a significantly lower level of GGT activity compared to late passage (PD 22) and hTERT transduced RPTECs (PpT 11).

The results, shown in Figure 4.31, demonstrate, that treatment with either 150 µM acivicin or 10 µM L-ABBA for 2 h significantly influences the GGT activity. Both, early passage (PD 10) and also hTERT transduced RPTECs had a significantly lower GGT activity after treatment.

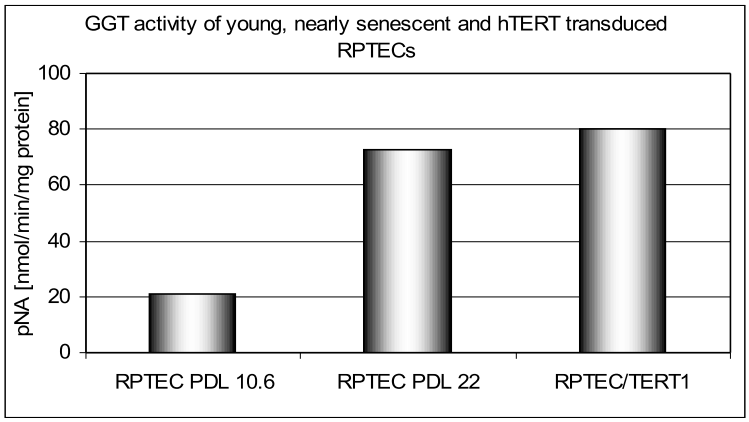

Figure 4.30: GGT activity in early passage RPTECs is significantly lower in comparison to nearly senescent RPTECs and RPTEC/TERT1.

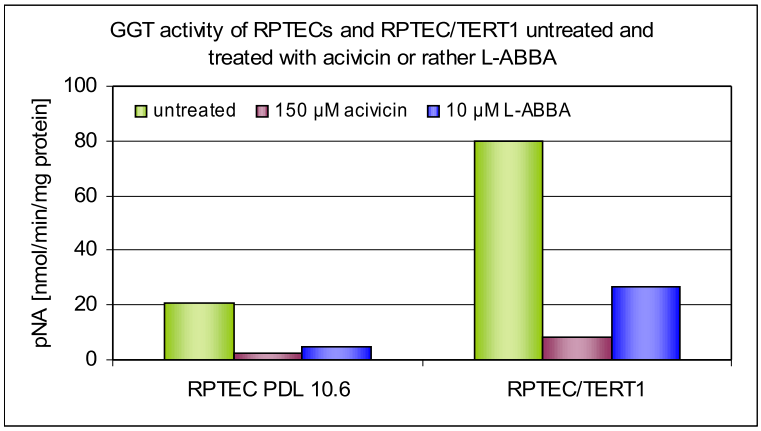

Figure 4.31: GGT activity of early passage RPTECs and RPTEC/TERT1 which were treated with either 150 µM acivicin or 10 µM L-ABBA for 2 h before measuring. Both cell lines show a significantly lower GGT activity after inhibition.

4.12 ROS detection in RPTECs and RPTEC/TERT1

As shown in Figure 4.32, PO are present at a high level in early passage RPTECs (PD 10.6), whereas senescent cells as well as RPTEC/TERT1 show reduced PO level. However, the level of SO increases with age and also hTERT transduced cells show a similar level as late passage cells (see Figure 4.33).

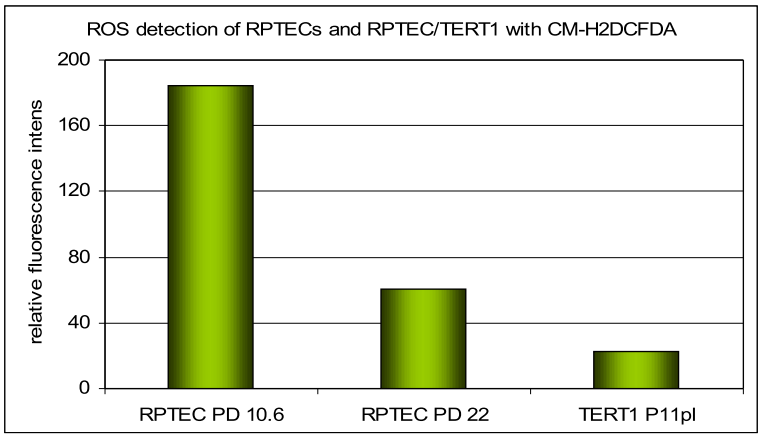

Figure 4.32: PO level of cells, treated with CM-H_2DCFDA. Early passage cells show a significantly higher PO level as senescent RPTECs and RPTEC/TERT1.

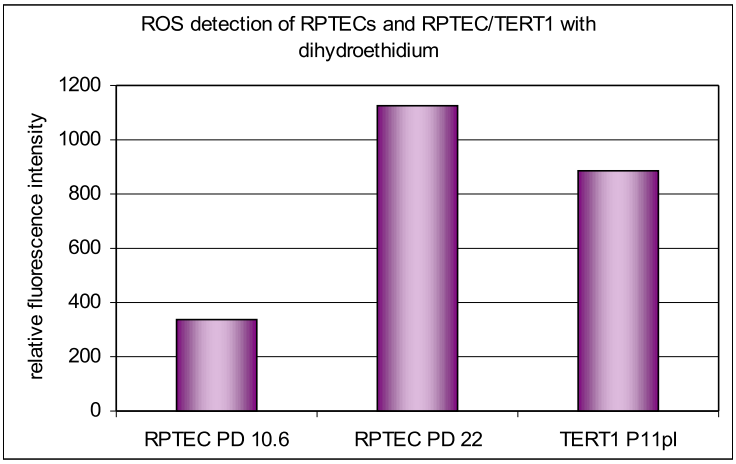

Figure 4.33: SO level: RPTEC/TERT1 shows a similar high level of SO as late passage RPTECs. In comparison, the SO level in early passage RPTECs was significantly lower.

4.13 Telomerase activity of RPTECs in comparison to RPTEC/TERT1

In order to test if hTERT overexpression leads to an induction of telomerase activity, real time TRAP assay was performed as described in 3.11. HEK293 cell lysates served as positive control, while RNAse treated lysates of all samples were used as negative controls.

As shown in Figure 4.34, RPTECs and vector control cells didn´t show any telomerase activity. In contrast, high telomerase activity could be detected in RPTEC/TERT1.

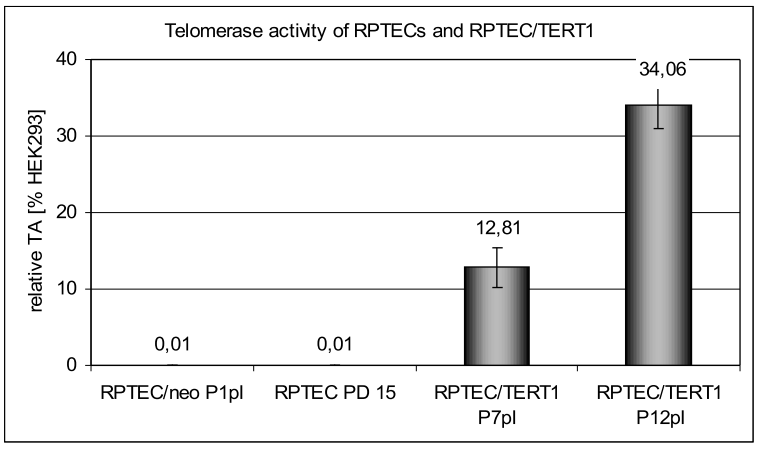

Figure 4.34: No telomerase activity could be detected in vector control cells and normally growing RPTECs. HTERT transduced RPTECs at two different PDpT showed high telomerase activity.

4.14 Telomere length of RPTECs compared with RPTEC/TERT1

Nuclear flow FISH was performed for determination of telomere length in RPTECs and hTERT transduced cells, RPTEC/TERT1.

Therefore, cells were harvested and stored at -80°C till analysis was performed.

As shown in Figure 4.35, telomere length declines during *in vitro* aging from 18.83 MESF at PD 14 to 12.83 MESF at PD 25.

In comparison to RPTECs, the telomere length of RPTEC/TERT1, was stable through several passages. RPTEC/TERT1 were analysed at PpT (population doublings post transduction) 4, 8, 12 and 15. The telomere length was stable there at a value of about 21 %.

These data verify the positive overexpression of hTERT alone in renal proximal tubular epithelial cells.

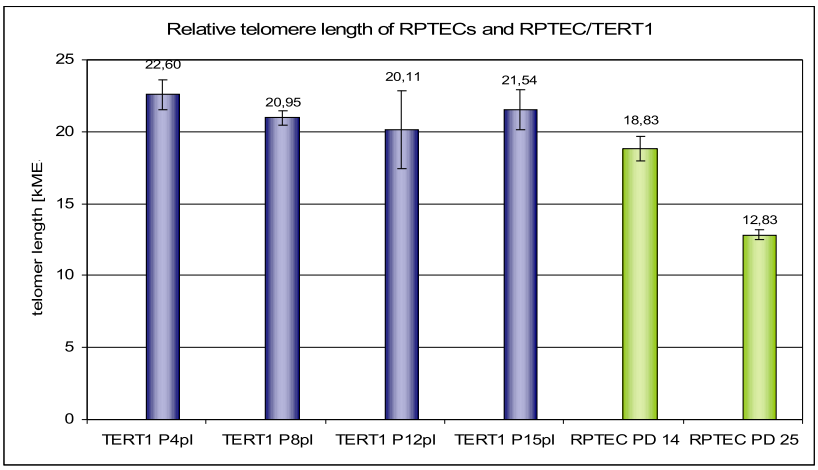

Figure 4.35: (blue bars) show the relative telomere length of RPTEC/TERT1, which is stable through several passages (about 21 MESF) in comparison to normally growing RPTECs (green bars). Telomere length of RPTECs decreases from 18.83 MESF (PD14) to 12.83 MESF (PD25).

5 CONCLUSION/DISCUSSION

5.1 The effect of SNEV transduced HUVECs

Replicative senescence and mechanisms that help to bypass this growth arrest are two main aspects which are intensively studied because such processes are suspected to play critical roles in both aging and tumor suppression (Campisi et al., 2005a; Campisi et al., 2005b)

By screening for genes downregulated in replicatively senescent HUVECs, Grillari and co-workers have isolated the protein SNEV (Grillari et al., 2000).

To get insight into the role of SNEV in replicative senescence, SNEV was stably overexpressed in HUVECs and the cellular phenotype was characterized.

Although the endothelial cell type-specific characteristics didn´t change, SNEV overexpression led to a significant increase of the life span when compared to the vector control cells (Voglauer et al,. 2006). Because neither telomerase activity nor elongated telomeres seemed to be the reason for the life span extension of SNEV overexpressed cells, we had a closer look on SNEVs role in DNA damage and its repair mechanism. Indeed, vector control cells, at the end of their replicative life span, had accumulated higher levels of single- and double-stranded DNA damage when compared to SNEV overexpressing cells of the same age. These results confirmed that SNEV overexpression correlates with higher stress resistance, based on the involvement of SNEV in DNA repair processes (Mahajan and Mitchell, 2003). This might explain the extension of the cellular life span upon SNEV overexpression.

As D´Adda di Fagagna analyzed DNA double strand breaks (DSBs) as little foci in indirect immunofluorescence analysis (D´Adda di Fagagna et al., 2003), we also tried analysis of DNA DSBs using anti-phospho-H2AX-antibody. But the analysis failed because numerous experiments of indirect immunofluorescence didn´t work. DSBs there should be visible as little foci in the nuclei of damaged cells, but no foci formation could be detected after treating cells with DSB-inducing agents. Although the antibody worked perfectly by using western blot analysis (Voglauer et al., 2005). A time - and concentration - dependent signal there could be detected by using DSB-inducing agents like staurosporine and bleomycin. The result of western blotting

indicates, that the antibody generally works. So, perhaps the used antibody is not convenient for indirect immunofluorescence or the used method is wrong.

Numerous studies have linked excess generation of ROS with cellular damage (Martinet et al., 2002). Therefore, we decided to determine the ROS level of treated and untreated cells. We wanted to know if there is a significant difference between SNEV transduced and un-transduced HUVECs. Furthermore, we wanted to look at the consequence of the ROS level by treating HUVECs with bleomycin and BSO.
The ROS level of SNEV tranduced HUVECs is significantly lower compared to the vector control cells at the same PDL, also indicating higher stress resistance of SNEV transduced cells. The measured ROS level of early passage HUVECs was lower in comparison to late passage HUVECs, which indicates more vitality of early passage compared to late passage cells, based on the better function of the detoxification machinery of early passage cells.
Bleomycin, used as an antitumor agent, exerts an influence on the glutathione system by increasing the expression level of γ-glutamylcystein synthase followed by higher GSH level (Day et al., 2002). Treating of I38 cells with different concentrations of bleomycin didn´t show an effect on the PO level, in contrast cells which were treated with BSO prior to bleomycin treatment. BSO therefore depletes the GSH level and makes cells more susceptible to the applied stress. Consequently, the cytotoxic effect of bleomycin is enhanced, when GSH is depleted with BSO, which could be reflected in the higher PO level.

The expression level of different cell cycle regulator proteins were analysed to look how they are regulated through several passages of HUVECs.
The expression level of p16 and p21 didn´t correlate with the progression of passaging, in contrast to p53 and SNEV, although p21 is a known target gene of p53. P53 is a tumor suppressor gene, which is involved in the regulation of the cell cycle and in the synthesis of ROS (Polyak et al., 1997) and it is also known as “the guardian of the genome” (Lane, 1992). The level of p53 increases through several passages of HUVECs. The result of increased p53 level in senescent cells was also found in literature (Atadja et al., 1995; Kulju and Lehmann, 1995). This result fits to the function of p53, which is involved in response to DNA damage, activation of DNA repair proteins, in initiating apoptotic cell death or inducing growth arrest (Hansen and Oren, 1997). P53 becomes activated in response to different types of stress,

which include but are not limited to DNA damage or oxidative stress. Late passage cells are more susceptible to DNA damage and cell stress, which causes “more work” for p53, which could be the reason for higher levels of p53 with several passages of the cells.

In contrast to p53, the level of SNEV decreases with several passages of HUVECs, as also identified by substractive hybridization by Grillari and co-workers, where the mRNA level of the “unknown gene 4” (SNEV) was increased in early passage versus senescent HUVECs (Grillari et al., 2000), and also Voglauer and co-workers observed a comparable result (Voglauer et al., 2005). These results indicate that SNEV down-regulation is connected to the entry into replicative senescence. Besides, downregulation of SNEV mRNA in replicative senescence was also found in epithelial kidney cells as well as human diploid skin fibroblasts (Voglauer et al., 2005), indicating that SNEV regulation during senescence is not restricted to a specific cell type.

Regarding to the different functional domains of the protein SNEV, we wanted to generate SNEV deletion mutants.
The transduction of HUVECs with SNEV deletion mutants didn´t work and also a western blot of transient transduced HUVECs didn´t show a positive result of SNEV-transduction.
The different characteristics of the individual deletion mutants would probably help, to get more informations into the mechanistic role of SNEV and its influence on cellular aging.

SNEV, an ubiquitously expressed protein, although not inducing immortalization, is sufficient to extend cellular life span without re-activating telomerase and influencing telomere dynamics (Voglauer et al., 2005). The extension of the cellular life span seems to be associated with increased resistance to stress and an improved DNA repair capacity.

5.2 Immortalization of RPTECs by introduction of hTERT and characterization of the newly established cell line

Human RPTECs represent a valuable in vitro model for toxicology, drug screening and development, as well as tissue engineering. But the short life time of RPTECs *in vitro* of about 24 population doublings presented a barrier for research.

This is the reason why several approaches have been performed to establish a continuously growing RPTEC cell line. Up to now, immortalization was only successful by introduction of viral oncogenes or combinations of SV40 and hTERT (Kowolik et al., 2004; Orosz et al., 2004; Racusen et al., 1997; Ryan et al., 1994).

In our experiment we could circumvent introduction of viral oncogenes. We achieved immortalization of human RPTECs by introduction of hTERT alone, resulting in the continuously growing cell line RPTEC/TERT1 (Wieser et al., 2008).

After immortalization we characterized this newly established cell line regarding to cell morphology and cell function.

RPTEC/TERT1 cells, which were cultivated in a hormonally defined medium like normally growing RPTECs, already reached 75 population doublings by looking like early passage cells with their typical cobblestone pattern without indicating any growth retardation (Qi et al., 2007; Sens et al., 1999; Taub and Sato, 1979; Taub and Sato, 1979; Taub and Sato, 1980). Furthermore, both parental and TERT-immortalized cells formed characteristic domes at high cell densities.

Visualising the cytokeratin network of RPTEC/TERT1 in comparison to normally growing RPTECs was the next step for characterization of the morphology. The result presented us, that the continuously cell line retained the same cytokeratin structure as before immortalization.

Several morphological tests demonstrated, that immortalization of RPTECs with hTERT alone didn´t change their morphological characteristics.

Measuring of the key enzyme in regulation of gluthathione synthesis, the gamma glutamyl transferase (GGT), which functions as intracellular antioxidant, demonstrates a cultivation time-dependent progression of GGT, whereas GGT activity in late-passage RPTECs, as also in RPTEC/TERT1 cells was increased compared to early-passage cells. A plausible explanation therefore would be, that induction of GGT activity only goes in hand with reduction of intracellular PO, whereas SO levels steadily increase. Increasing SO levels may therefore be responsible for the accelerated telomere erosion observed in late-passage cells.

Looking for ROS, especially peroxyl radicals (PO) and superoxide anion (SO), showed us, that early passage RPTECs had a significant higher level of PO compared to late passage RPTECs and RPTEC/TERT1 cells. In contrast, the SO level is decreased in early-passage RPTECs in comparison to late-passage RPTECs and RPTEC/TERT1 cells.

The results of testing GGT level, PO as well as SO level show, that RPTEC/TERT1 cells had rather the tendency to resemble late-passage RPTECs.

The activity of telomerase in early passage RPTECs, late passage RPTECs as well as in RPTEC/TERT1 cells was measured by real time TRAP assay normalized to HEK293 and the result confirmed that hTERT indeed resulted in activation of telomerase, the responsible enzyme for the successful immortalization.

By analysing telomere length, the telomere-dependent senescence of RPTECs was proofed. RPTEC/TERT1 cells were able to stabilize their telomere length, which fits to the result of the activated telomerase. Furthermore, the outcome indicates that RPTECs are suitable as additional novel cell model for aging research (Wieser et al., 2008). A really interesting aspect, because senescent kidney cells have been found to increase with age in vivo (Chkhotua et al,. 2003; Melk et al,. 2004) or rather with time after kidney transplantation, which inversely correlates with the functionality of the graft (Chkhotua et al., 2003).

In conclusion, RPTEC/TERT1 cells can be proposed as a general tool for studying basic cell biology, but also for toxicology, drug screening, aging and development as well as a potential source for tissue engineering and cell-based therapy approaches (Wieser et al., 2008).

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7 APPENDIX

7.1 Abbreviations

γ-GCS	gamma-glutamyl cysteine synthase
2-ME	2-mercaptoethanol
AA	Acrylamide
AD	Aqua destilata
APS	Ammonium persulfate
ATM	Ataxia-telangiectasia-mutated
ATR	Ataxia-telangiectasia-mutated and Rad3-related
BAA	bisacrylamide
bp	Base pair
BSA	Bovine serum albumin
BSO	L-Butionine-[S, R]-sulfoximine
CM-H_2DCFDA	5-(and-6)-chloromethyl-2´,7´-dichlrorodihydrofluorescein diacetate, acetyl ester
DAPI	4´, 6-diamino-2-phenylindol
DHE	dihydroethidium
DIGE	Differential gel electrophoresis
DMEM	Dulbecco´s Modified Eagle Medium
DMSO	dimethylsufoxide
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide
DTT	dithiothreitol
ECs	Endothelial cells
EDTA	Ethylendiaminetetraacetic acid
EGF	Epidermal growth factor
EtOH	Ethanol
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FISH	Fluorescence in situ hybridization
FITC	Flurescein isothiocyanate
G418	Geneticin sulfate
GGT	Gamma glutamyl transferase
GSH	glutathione
H_2O_2	hydrogen peroxide
HEK	Human embryonic kidney cells
HeLa	Henrietta Lacks
HEPES	[4-(2-hydroxyethyl)-piperazino]-ethanosulfonic acid
HO	Hydroxyl radical
hTERT	Human telomerase reverse transcriptase
HUVECs	Human umbilical vein endothelial cells
L-ABBA	4-borono-2-amino-butanoic acid
LB	Luria Bertani
MOPS	3-[N-morpholino] propanesulfonic acid
NaOH	Sodium hydroxide
neo	Neomycin phosphotransferase
$ONOO^-$	peroxynitrite anion

O_2^-	Superoxide anion
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Population doubling
PDL	Population doubling level
PDpT	Population doublings post transduction
PGE	prostaglandine
PECAM-1	Platelet endothelial cell adhesion molecule-1
PI	Propidium iodide
PNA	Peptide nucleic acid
PO	Peroxyl radicals
PVDF	Polyvinylidene difluoride
RB	Retinoblastoma protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Rounds per minute
RPMI	Roswell Park Memorial Institute
RPTECs	Renal proximal tubular epithelial cells
RT	Room temperature
SDS	Sodium dodecyl sulfate
SIPS	Stress induced premature senescence
SNEV	Senescence evasion factor
SO	Superoxide anion
T3	Triiodothyronin
TE	
TEMED	N,N,N´,N´-di-(dimethylamino)ethane
vWF	Van Willebrand Factor

7.2 Materials

7.2.1 Cell culture

Cell media

M199 (Biochrom AG, Berlin, Germany)
w 2.2 g/l $NaHCO_3$
w/o L-glutamine
DMEM/Ham´s F-12 (1:1) (Biochrom AG, Berlin, Germany)
w 2.4 g/l $NaHCO_3$
w/o L-glutamine
DMEM (Biochrom AG, Berlin, Germany)
w 3.7 g/l $NaHCO_3$
w/o L-glutamine
RPMI 1640 (Biochrom AG, Berlin, Germany)
w 2.0 g/l $NaHCO_3$
w/o L-glutamine

Supplements
L-glutamine (stored at -20°C)
FCS (sterile, pyrogen-free)
G418 solution 100 mg/ml
ECGS (stored at -80°C)
Sodium pyruvat
Polybrene
Hepes buffer (10 mM)
Epidermal growth factor (10 ng/ml)
Triiodothyronin (5pM)
Ascorbic acid (3.5 µg/ml)
Transferrin (5 µg/ml)
Prostaglandine (25ng/ml)
Hydrocortison (25 ng/ml)
Insuline (5 µg/ml)
Sodium-selenit (8.65 ng/ml)

Subculture

Reagents
0.1% Trypsin/EDTA solution
9.55 g PBS
1 g Trypsin
0.2 g EDTA-Na_4*$2H_2O$
ad 1 l with high quality AD (Biochrom AG, Berlin, Germany), sterile, pyrogen free
0.25% Trypsin/0.05% EDTA solution

PBS def. pH 7.2 (Dulbecco, Biochrom AG, Berlin, Germany)

Gelatine 1% in PBS

Devices
Laminar Flow, Holten Weighmaster 5 (Thermo Electron Corporation, Erlangen, Germany)
Incubator, Heracell CO_2 Incubator (Kendro Laboratory Products GmbH)

Cryopreservation and thawing

Cryo medium
10% FCS
10% DMSO (Sigma)
80% Cell culture medium

Devices
Centrifuge 4-15 (SIGMA Laboratory Centrifuges, Osterode, Germany)

Mycoplasma Test

Reagents
PBS def. pH 7.2 (Dulbecco, Biochrom AG, Berlin, Germany)
w Ca^{2+}/Mg^{2+}
50% Glycerol in PBS
FluoroPrep Mounting medium (Biomérieux, Marcy l´Etoile, France)

Staining solution
200 µl DAPI (5 µg/ml stock)
in 1 ml methanol

Devices
Confocal Microscope BH-2 RFCA (OLYMPUS, Hamburg, Germany)
Lab-Tek™ Chamber Slides™ (NUNC, Wiesbaden, Germany)

Induction of ROS

Bleomycin sulphate (Sigma):
10 mg (15 Units) of bleomycin sulphate were dissolved in 5 ml sterile physiological saline solution (0.9 % NaCl) and aliquots of 200 µl were filled in sterile glass tubes, which were then closed with sterile caps. These aliquots were stored at -20°C.

DL-Buthionine-[S,R]-sulfoximine (Sigma)
To get a solution of 225 mM (50 mg/ml) BSO, 50 mg BSO was weight in a 1.5 ml Eppendorf tube and 1 ml HQ water was added in the laminar hood. The powder was dissolved by holding the tube in an ultrasonic bath. Thereafter, the solution was sterile filtered through a 0.2 µm membrane and stored at 4°C.

0.9% NaCl, sterile filtered
high quality AD (Biochrom AG)

Devices
PBS sterile filter, 0.2 µm pore size (Nalgene)
12 well plate (NUNC, Wiesbaden, Germany)

ROS staining

Reagents
5x50 µg CM-H_2DCFDA (Molecular Probes):
2 x 50 µg CM-H_2DCFDA were diluted in 8.7 µl DMSO each in order to obtain a concentration of 10 mM. Aliquots of 3 µl were transferred to Eppendorf tubes containing inert N_2. Tubes were wrapped with aluminium foil and stored at -20°C.

5 mM dihydroethidium stabilized in DMSO (Molecular Probes)
1 ml of DHE, stabilized in DMSO was separated in aliquots of 30 µl in glass tubes, containing inert N_2. The tubes were stored at -20°C

PBS def.
DMSO (Sigma)
0.1% Trypsin/EDTA solution

Devices
FACS-Calibur (Becton Dickinson, Franklin Lakes, NJ, USA)
Cell Quest Software package (Becton Dickinson, Franklin Lakes, NJ, USA)
Centrifuge 4-15 (SIGMA Laboratory Centrifuges, Osterode, Germany)

Virus generation

Opti-MEM I reduced-Serum Medium (Gibco, Invitrogen)
Lipofectamine™ 2000 (Invitrogen)
G418 solution 100 mg/ml
Polybrene (Sigma)

Retroviral infection

Opti-MEM I reduced-Serum Medium (Gibco, Invitrogen)
Lipofectamine™ 2000 (Invitrogen)
G418 solution 100 mg/ml
Polybrene (Sigma)

7.2.2 Molecular Biology

Bradford protein Assay

Bradford dilution buffer
6.8 g/l KH_2PO_4
8.8 g/l NaCl
pH 7.2 (KOH)

200 µg/ml BSA stock solution

Protein assay dye reagent concentrate (BioRad)
diluted 1:5 with AD

Devices
96-well microtiter plates (NUNC)
Sunrice absorbance reader (Tecan)

SDS PAGE

DIGE Lysis buffer
3 ml Tris HCl (1 M)
15.22 g Thiourea
40.0 g Urea
4 g CHAPS
ad 100 ml with AD
adjust to pH 8.5 with HCl (1 M)

SDS sample buffer (2x)
100 g/l SDS
20% glycerine, 12.5% TRIS (1 M)
20% bromophenolblue (0.1%)
10% 2-ME was added immediately before use

Devices
Centrifuge 5451 (Eppendorf, Hamburg, Germany)
Xcell*SureLock*™ Mini-Cell (Novex, Invitrogen)

NuPAGE® 1 mm 12% Bis-Tris Gel (Novex, Invitrogen)

Sample buffer 2x SDS Tris/Gycerine
2.5 ml 0.5 M Tris pH 6.8
2 ml Glycerol
4 ml SDS (10%)
0.1 mg bromphenole blue
traces DTT
ad 10 ml with AD

Running buffer
50 ml 20x NuPAGE® MOPS SDS Running buffer (Novex, Invitrogen)
ad 1000 ml with AD

SeeBlue® Plus2 pre-stained Standard (Invitrogen)

Western Blot

TPBS washing buffer
8.0 g NaCl
0.2 g KCl
1.15 g Na_2HPO_4
0.2 g KH_2PO_4
ad 1000 ml with AD
1 ml Triton X-100 (Sigma)
adjust to pH 7.4 with HCl (1 M)

Blocking buffer
3% milk powder in TPBS buffer

Transfer buffer
50 ml 20x NuPAGE® Transfer buffer (Novex, Invitrogen)
100 ml Metanol
ad 1000 ml with AD

Antibodies
mouse-anti-p16 (Santa Cruz Biotech)
mouse-anti-p21 (US Biologicals)
rabbit-anti-p27 (US Biologicals)
mouse-anti-p53Ser15 (Santa Cruz Biotech)
mouse-anti-SNEV
rabbit-anti-Prp19 867
mouse-anti-alpha 4 His
rabbit-anti-phospho-H2A.X(Ser139) (Upstate)
mouse-anti-beta actin (Sigma)

Anti mouse peroxidase (Sigma)
Anti rabbit alkaline phosphatase (Sigma)
Anti rabbit peroxidase (Sigma)
Anti mouse alkaline phosphatase (Sigma)

CDP star washing buffer (New England BioLabs)
10 mM TrisHCl
10 mM NaCl
1 mM $MgCl_2$

Detection Kit
Chemiluminescent Peroxidase Substrate 1 (Sigma)

Devices
Xcell*SureLock*™ Mini-Cell (Novex, Invitrogen)

PVDF membrane (BioRad)
XCell II™ Blot module (Novex, Invitrogen)

Silver stain

Fixation solution
500 ml Ethanol (96%)
100 ml acetic acid
ad 1000 ml with AD

Incubation solution
150 ml Ethanol
34 g Na-acetate
1 g $Na_2S_2O_3$*H_2O
Add 500 ml with AD
Add 62.5 µl Glutaraldehyde to 25 ml Incubation solution immediately before use

$AgNO_3$ solution
0.05 g $AgNO_3$
10 µl Formaldehyde (35%)
ad 50 ml with AD

Developer solution
12.5 g Na_2CO_3
Add 500 ml with AD
Add 2.5 µl Formaldehyde (35%) to 25 ml Developer solution immediately before use

Stop solution
7.3 g EDTA
ad 500 ml with AD

Indirect Immunofluorescence

Reagents
PBS def.
Fixation solution (methanol/acetone 1:1)
PBG (2% fish gelatine, 5% BSA in 1x PBS)
FluoroPrep Mounting medium (Biomérieux, Marcy l´Etoile, France)

Antibodies
mouse-anti-pan-cytokeratin (Sigma)
rabbit-anti-phospho-H2A.X(Ser139) (Upstate)

anti-rabbit-FITC (Sigma)
goat-anti-mouse-FITC (Sigma)

Devices
Leica confocal microscope

Cloning

Preparative gel
2.5 g agarose
5 ml 50x TAE
ad with to 250 g with AD
add 8 µl EtBr (10 mg/ml) after cooling

6x Loading Dye solution (Fermentas)
Lamda DNA/EcoRI+HindIII Marker, 3 (Fermentas)

Purification of DNA with Wizard® SV Gel and PCR clean-up system (Promega)
According to the manufacturer´s protocol

Ligation
10x ligase buffer
T4 ligase
H_2O nuclease free

Reagents
Glycogen
Na-acetate
EtOH (96%)
EtOH (70%)

SOC medium
20 g/l Bacto Trypton
5 g/l yeast extract
10 mM NaCl, 3 mM KCl
10 mM $MgCl_2$, 20 mM glucose
10 mM $MgSO_4$

LB agar
1.5% Agar-Agar

LB medium
10 g/l Bacto Trypton
5 g/l yeast extract
10 g/l NaCl
pH 7.0 (NaOH)

MiniPrep with Wizard plus mini prep DNA purification system (Promega)
According to the manufacturer´s protocol

Restriction digest
Restriction enzymes
EcoRI
BamHI
EcoRI buffer
BSA [10 mg/ml]

MaxiPrep with Endo-free plasmid maxi kit (QIAGEN)
According to the manufacturer´s protocol

Devices
Electroporation unit GenePulser (BioRad)
Biometra T-Gradient or T3-Thermocycler
AVANTI J-20XP Beckman Coulter centrifuge
Eppendorf BioPhotometer

GGT activity

Standard solution
4 ml 10% acetic acid
1.6 ml 0.1 M TrisHCl pH 8.0
0.4 ml glycyl-glycine (0.1 M)

Reagents
para-nitroanilide
Acivicin (150 µM)
L-ABBA (1 µM)
PBS def.
TrisHCl pH 8.0

Incubation solution
Standard solution
1 mM gamma-glutamyl-para-nitroanilide
60 mM TrisHCl pH 8.0
20 mM glycyl-glycine

Devices
Hitachi U 2001 photometer

Real-time PCR TRAP Assay

CHAPS lysis buffer
10 mM TrisHCl, pH 7.5
1 mM $MgCl_2$
1mM EGTA
0.1 mM phenylmethylsulfonyl fluoride
5 mM β-mercaptoethanol
0.5% CHAPS
10% glycerol

Reagents
TrisHCl pH 8.3, $MgCl_2$, KCl, EGTA, dNTP´s
SYBR Green I 1:1000 dilution stock (Roche Diagnostics, Basel, Switzerland)
BSA (10 mg/ml stock)
Tween 20
Spice Taq Polymerase 2 U/µl (IAM)
DNAse free ribonuclease 10 mg/ml stock (Molecular Cloning, Sambrook)
Primers

CXa
5´-GTGTAACCCTAACCCTAACC-3´
TSmodIV
5´-AATCCGTCGAGAACAGTT-3´

Devices
Rotor Gene 2000 Real-time Cycler (Corbett Research)
Centrifuge 5451 (Eppendorf, Hamburg, Germany)

Nuclear Flow FISH

PBS def.
FITC labelled standard beads (Quantum™ 24, Bangs, Fishers, IN)

Dilution buffer
2% Triton X-100/0.1 M citric acid coulter counter buffer

Hybridization buffer
75% Formamide
1% BSA:
10 mg BSA/ml H_2O was weight out in an aluminium foil, transferred in a 10 ml vial and dissolved by centrifuging to avoid foaming of the BSA.
20 mM TrisHCl pH 7.1
20 mM NaCl
50 nM FITC conjugated $(C_3TA_2)_3$ PNA probe:
The PNA, which was stored at 4°C, was put out to let it come to RT. Thereafter, it was incubated on a 50°C heat block for 10 minutes under light protection.

DNA staining solution
10 µg/ml RNAse-A
0.1% BSA
in PBS
50 ng/ml Propidium iodide

10 mg/ml RNAse-A (DNAse free) (stored at –20°C) was diluted in 0.1 % BSA in PBS 1:1000, 1 ml of this solution was taken for preparing the beads. Finally, PI (25 µg/ml) was diluted 1:500 in PBS and added to the DNA staining solution.

Devices
Multisizer™ 3 COULTER COUNTER (Beckman Coulter)
FACS Calibur (Becton Dickinson)

Comet Assay

Reagents
1% agarose in PBS
PBS def.
Low melting agarose

Lysis solution
2.5 M NaCl
0.1 M EDTA
10 mM Tris
adjust to pH 10 (NaOH)
add 1ml Triton X-100 to 100 ml solution immediately before use

Electrophoresis buffer
0.3 M NaOH
1 mM EDTA

Neutralisation buffer

0.4 M Tris
pH 7.5 (HCl conc.)

DNA staining solution
SYBR Green I diluted 1: 10 000
in TE buffer (10 mM TrisHCl, 1 mM EDTA)
adjust to pH 7.5

Devices
Electrophoresis chamber
Microscope

7.2.3 Vector maps

pGBKT7 (Clontech)

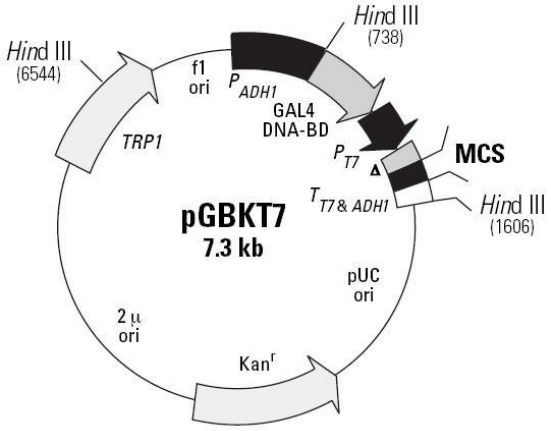


Figure 7.36: Vector map of pGBKT7. The figure was taken from http://www.clontech.com/images/pt/PT3248-5.pdf

pLXSN (Clontech)

The pLXSN vector, derived from Moloney murine leukemia virus, is designed for retroviral gene delivery and expression. The SV40 early promoter controls expression of the neomycin resistance gene which allows antibiotic selection in eukaryotic cells.

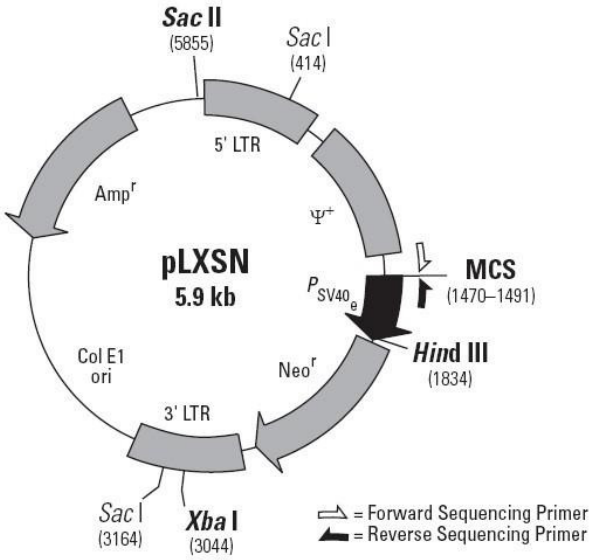
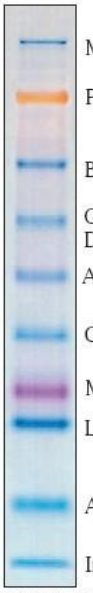


Figure 7.37: Vector map of pLXSN. The figure was taken from http://www.clontech.com/images/pt/PT3134-5.pdf

7.2.4 Protein and DNA markers

SeeBlue Plus 2 pre-stained (Invitrogen)



Protein	Approximate Molecular Weights (kDa)				
	Tris-Glycine	Tricine	NuPAGE® MES	NuPAGE® MOPS	NuPAGE® Tris-Acetate
Myosin	250	210	188	191	210
Phosphorylase	148	105	98	97	111
BSA	98	78	62	64	71
Glutamic Dehydrogenase	64	55	49	51	55
Alcohol Dehydrogenase	50	45	38	39	41
Carbonic Anhydrase	36	34	28	28	n/a
Myoglobin Red	22	17	17	19	n/a
Lysozyme	16	16	14	14	n/a
Aprotinin	6	7	6	n/a	n/a
Insulin, B Chain	4	4	3	n/a	n/a

NuPAGE® Novex
Bis-Tris 4-12% Gel

Figure 7.38: SeeBlue Plus 2 pre-stained. The figure was taken from http://products.invitrogen.com/ivgn/product/LC5925?ICID=search-product

Fast ruler, DNA Ladder, High Range (Fermentas)

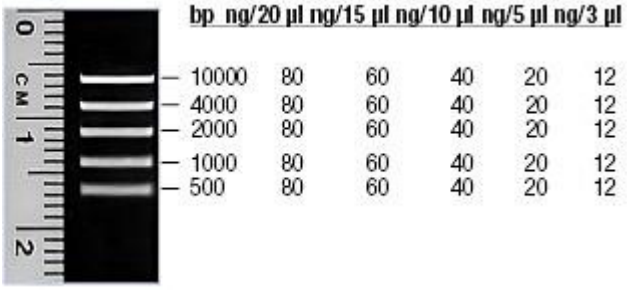


Figure 7.39: Fast ruler, DNA Ladder, High Range. The figure was taken from http://www.fermentas.com/en/products/all/dna-electrophoresis/fastruler-dna-ladders/sm112-fastruler-high

Lambda DNA/EcoRI+HindIII Marker, 3 (Fermentas)

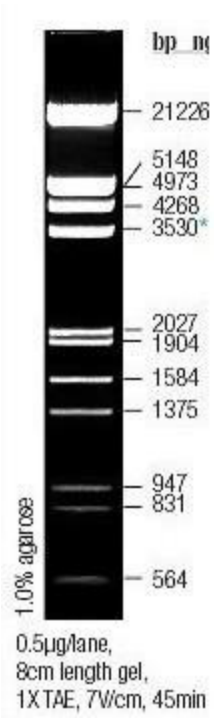


Figure 7.40: Lambda DNA/EcoRI+HindIII Marker 3. The figure was taken from http://www.fermentas.com/pdf/catalog2008/9_DNA_Electrophoresis.pdf

Magic Mark XP (Invitrogen)

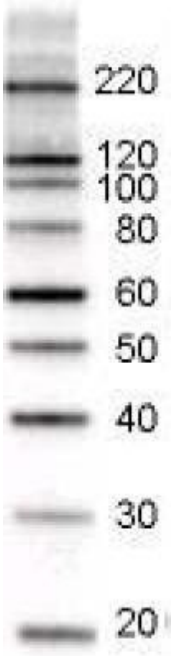


Figure 7.41: Magic Mark XP.The figure was taken from http://tools.invitrogen.com/content/sfs/manuals/magicmark_man.pdf

Immortalization of normal human kidney epithelial cells using the catalytic subunit of human telomerase

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Abstract

Normal human cells can be propagated in vitro only for a limited number of population doublings before entering a phase of irreversible growth arrest also termed replicative senescence. The primary cause for this irreversible growth arrest is the progressive erosion of telomeres, specialized structures at the ends of linear chromosomes, which, at a critical short length, trigger a DNA damage signal that induces senescence. One step by which cells can acquire unlimited proliferative potential is the activation of telomerase, the enzyme that adds lost telomere sequences, a process that is associated with 85% of tumors. The role of telomerase in cellular immortalization has further been demonstrated by introduction of the catalytic subunit of human telomerase (hTERT) in vitro, whereby a large variety of different continuously cell lines have been established. However, especially epithelial cells have proven difficult to immortalize since hTERT alone is insufficient to extend the in vitro life-span.

In this study we have used kidney epithelial cells (NHK) as a model to study aging and immortalization. NHK cells cultivated under serum-free conditions approach senescence after having reached about 22 population doublings, a process that is not triggered by the “classical” cell cycle regulators p16 and p21. Whereas, transfection of NHK cells using non-viral transfection methods, did not result in cellular immortalization, transduction with a retrovirus encoding hTERT resulted in a cell line with extended life-span, enhanced telomerase activity and elongated telomeres. Although the established cell line shows lower growth rates, the cellular morphology resembles that of the corresponding normal counterpart. Therefore, the new, continuously growing epithelial cell line is an important tool for studying aging and tumor pathogenesis of the human kidney.

Cell morphology and growth characteristics

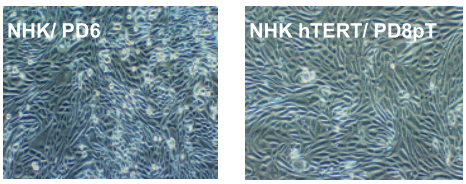

Figure 1 The cellular morphology of normal human kidney epithelial cells (NHK / PD6, left) is comparable to NHK cells transduced with retroviruses carrying hTERT (NHK hTERT / PD8pT, right).

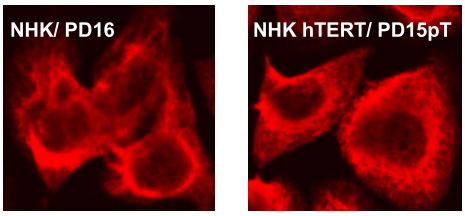

Figure 2 Normal NHK cells (PD16, left) as well as NHK hTERT cells (PD15pT, right) show positive staining for cytokeratin, a marker for cells of epithelial origin, as indicated by indirect immunofluorescence using an anti-cytokeratin antibody and a secondary-TRITC conjugated antibody.

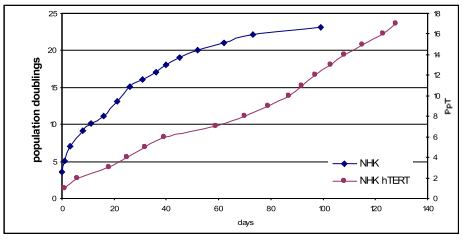

Figure 3 Growth rate analysis demonstrates that the non transfected NHK cells enter senescence after having reached about 23 population doublings, while NHK hTERT cells show no growth retardation so far.

Literature:
[1] Wieser et al. 2005: submitted.
[2] Wege et al. 2003: Nucleic Acids Res., 31 (3): E3-3

Acknowledgements:
This work was funded by Polymun Scientific.

Biochemical characteristics GGT and ROS

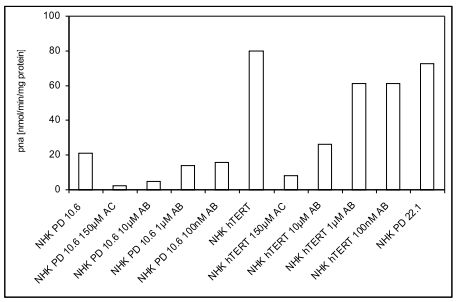

Figure 4 Determination of gamma glutamyl transferase (GGT) activity GGT inhibition with acivicin (AC) and l-abba (AB) shows that NHK hTERT cells have a similar level of GGT activity as senescent cells (PD22).

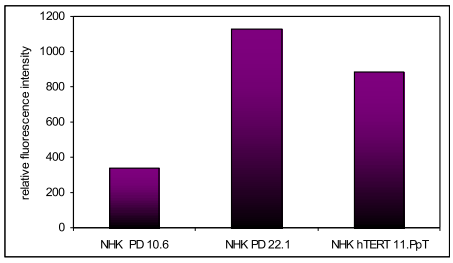

Figure 5 Detection of reactive oxygen species with dihydroethidium (DHE), which specifically detects O_3^-, shows that the O_3^--level increases with age as analyzed usinf flow cytometry. NHK hTERT cells show a similar high level of O_3^- when compared to cells at high PD (22).

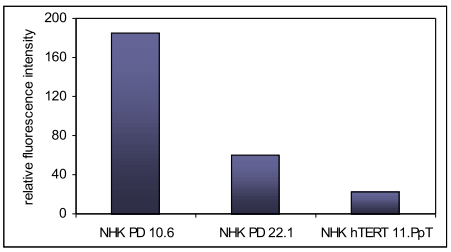

Figure 6 The level of H_2O_2, which was determined by incubation of the cells with CM-H_2DCFDA and flow cytometric analysis shows that H_2O_2 is present at a high level in early passage cells (PD10), whereas senescent cells as well as NHK hTERT cells show reduced H_2O_2 levels.

Telomere length and telomerase activity

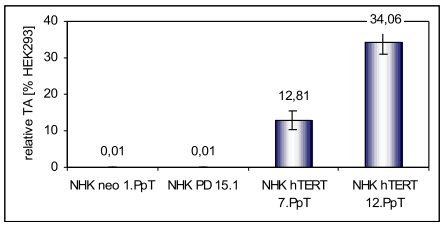

Figure 7 Whereas NHK cells and vector control cells (NHK neo) show very low telomerase activity (TA), introduction of hTERT significantly increases the TA. Hek293 cells served as positive control and TA activity was expressed relative to Hek293 cells [1].

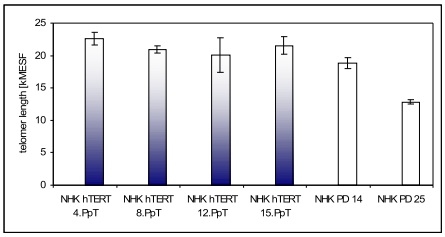

Figure 8 Whereas telomere length shorten upon in vitro cultivation of NHK cells (right), telomeres are stabilized after introduction of hTERT (right). Telomere length were measured by Nuclear Flow-FISH as described [2].

Conclusion

Introduction of hTERT….

- …is sufficient to bypass the phase of replicative senescence of normal human kidney epithelial cells
- …does not change the cellular morphology or growth characteristics
- …changes biochemical characteristics of the cells towards senescent cells
- … might allow the establishment of continuously growing cell lines of epithelial origin that are valuable for scientific as well as biotechnological purposes.