Activity and Regulation of Telomerase in Malignant Cells

ANNA LINDKVIST
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Abstract

An important step in tumorigenesis is the acquisition of cellular immortality. Tumor cells accomplish this by activating the enzyme telomerase, and thereby avoiding replicative senescence. The aim of this thesis was to study the activity and regulation of telomerase in a panel of malignant cell types.

We found that TGF-β1 (transforming growth factor-β1) mediated differential effects on telomerase activity in five ATC (anaplastic thyroid carcinoma) cell lines. Cells that harbored a p53 mutation responded by up-regulation of telomerase activity after TGF-β1 treatment, whereas cell lines displaying wt p53 responded by down-regulation of telomerase activity. Thus, these results indicate a possible connection between p53 genotype and telomerase response to TGF-β1 treatment. Furthermore, the decreased telomerase activity appeared to be due to transcriptional repression of the hTERT promoter and the increased activity possibly involved hTERT activation via phosphorylation.

We have previously shown that IFNs (interferons) sensitize MM (multiple myeloma) cells to Fas-mediated apoptosis. In the present investigation both IFN-α and IFN-γ down regulated telomerase activity in the MM cell line U-266-1970. The mechanism underlying the reduction of telomerase activity by IFN was shown to be transcriptional repression of the hTERT gene. We suggest that one potential mechanism whereby IFN sensitize MM cells to Fas-mediated apoptosis is by repressing hTERT activity at the transcriptional level.

In the next study we demonstrated that basal telomerase activity is not a key determinant of sensitivity to cytotoxic drugs in ESCC (esophageal squamous cell carcinoma) cell lines. Furthermore, we observed no correlation between c-Myc amplification, p53 mutations and high telomerase activity levels in these cell lines.

Finally, neuroblastoma cell lines were shown to up-regulate telomerase activity in response to hypoxic exposure and the main regulatory mechanism was not mediated by increased hTERT mRNA expression. This finding might constitute an adaptive stress response of tumor cells exposed to hypoxia.

Keywords: apoptosis, ATC, c-Myc, drug sensitivity, ESCC, hTERT, hypoxia, IFN, MM, neuroblastoma, p53, telomerase, TGF-β

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List of papers

This thesis is based on the following papers, referred to in the text by their roman numerals:


* These authors contributed equally to this work

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## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT</td>
<td>Alternative lengthening of telomeres</td>
</tr>
<tr>
<td>ATC</td>
<td>Anaplastic thyroid carcinoma</td>
</tr>
<tr>
<td>ATM</td>
<td>ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>DN</td>
<td>dominant negative</td>
</tr>
<tr>
<td>ds</td>
<td>double stranded</td>
</tr>
<tr>
<td>ESCC</td>
<td>esophageal squamos cell carcinoma</td>
</tr>
<tr>
<td>FMCA</td>
<td>Fluorometric Microculture Cytotoxicity Assay</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone acetyltransferase</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase complex</td>
</tr>
<tr>
<td>HIF-1</td>
<td>Hypoxia-inducible factor 1</td>
</tr>
<tr>
<td>HSP</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>hTERT</td>
<td>Human telomerase reverse transcriptase</td>
</tr>
<tr>
<td>hTR</td>
<td>Human telomerase RNA</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferons</td>
</tr>
<tr>
<td>IGF-2</td>
<td>Insulin-like growth factor 2</td>
</tr>
<tr>
<td>MM</td>
<td>Multiple myeloma</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>POT1</td>
<td>protection of telomeres</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td>ss</td>
<td>single-stranded</td>
</tr>
<tr>
<td>TEP</td>
<td>telomerase-associated protein</td>
</tr>
<tr>
<td>t-loop</td>
<td>Telomeric loop</td>
</tr>
<tr>
<td>TRAP</td>
<td>Telomeric repeat amplification protocol</td>
</tr>
<tr>
<td>TRF</td>
<td>telomeric repeat binding factor</td>
</tr>
<tr>
<td>TPG</td>
<td>total product generated</td>
</tr>
</tbody>
</table>
Cancer includes a heterogeneous group of disorders with different biological properties. Cancer has widely been thought to arise from a single cell, with the transformation of normal human cells into malignant tumors perceived as a multistep process. With each step reflecting genetic changes that confer different types of growth advantages, ultimately leading to the conversion of normal cells into tumor cells.

During recent years an alternative model has evolved. It suggests that tumors arise from small populations of cancer stem cells. Genetic or epigenetic changes of a stem cell could give rise to cancer stem cells with the ability to generate phenotypically diverse cancer cells. This would explain the heterogeneous nature of some human tumors. Stem cells make up an attractive precursor of cancer since stem cells already have at least one characteristics of tumor cells \emph{i.e.} the ability of self renewal \cite{1, 2}.

Tumor cells have been suggested to manifest six characteristics: unlimited replicative potential, self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of programmed cell death, sustained angiogenesis and tissue invasion and metastasis (reviewed in \cite{3}). 90% of all cancer cells acquire unlimited replicative potential by activating the enzyme telomerase, and the present thesis will deal with the activity and regulation of telomerase in malignant cells \cite{4}. 

**Introduction**
Background

Chromosomal endings

Telomeres and their associated proteins

The ends of linear chromosomes are composed of repetitive G-rich sequences (TTAGGG in humans [5]) and their associated proteins (Table 1), together known as telomeres. Telomeric DNA is composed of several thousand repeats of the TTAGGG sequences and estimated to vary in length from 5 to 15 kilobases depending on factors such as tissue type and age. The telomere is double stranded except for the extreme 3’end which consist of a G-rich overhang of 130-210 bases in length [5-9]. This single-stranded 3’overhang invades the double stranded telomeric DNA and forms a large duplex loop-back structure called the t-loop (telomeric loop) [10] (Fig. 1). This chromatin structure is stabilized through the binding of specific proteins (reviewed in [11, 12]).

Figure 1. The t-loop at the end of the chromosome. The 3’-end of the human telomere folds back and invades the ds telomeric DNA, generating a protective and stabilizing structure referred to as the telomeric-loop (t-loop).
Formation of t-loops is stimulated by the telomeric repeat binding factor (TRF) 1 and 2, which both bind as dimers to double stranded (ds) telomeric DNA. TRF1 has the ability to bend telomeric DNA \textit{in vitro} and it has been suggested that binding of several TRF1 units to the telomeres could result in the folding back of the telomere on itself. TRF2 can form t-loops \textit{in vitro} and has been proposed to promote the invasion of the 3’overhang [10, 13-18]. TRF1 and TRF2 both associates with other proteins when bound to the telomeric DNA (Table 1) [19-25].

The single-stranded (ss) TTAGGG binding protein; protection of telomeres (POT1) interacts with the G-rich strand and positively effects chromosome stability. POT1 can propose both bind the 3’ telomeric overhang and the displaced TTAGGG repeats at the base of the t-loop [26, 27]. POT1 has also been suggested to be recruited to the telomeres by the TRF1 complex [28, 29].

The t-loop functions as a protective cap at the chromosome ends, allowing cells to distinguish between random DNA breaks and natural chromosome ends and thereby protecting the telomeres from cellular activities such as DNA damage checkpoints and DNA repair enzymes. The t-loop structure also explains the observations that the telomeres protect chromosomes from degradation and end-to-end fusions [10].
# Table 1. Telomere associated proteins in humans.

<table>
<thead>
<tr>
<th>Telomere-binding factor</th>
<th>Postulated function(s)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRF1</td>
<td>Binds ds TTAGGG</td>
<td>Griffith et al., 1999 [10]</td>
</tr>
<tr>
<td></td>
<td>Facilitates t-loop formation</td>
<td>Chong et al., 1995 [14]</td>
</tr>
<tr>
<td></td>
<td>Negative regulator of telomere length</td>
<td>König et al., 1998 [15]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bilaud et al., 1996 [16]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bianchi et al., 1997 [17]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Smogorzewska et al., 2000 [30]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>van Steensel et al., 1997 [31]</td>
</tr>
<tr>
<td>TRF2</td>
<td>Binds ds TTAGGG</td>
<td>Griffith et al., 1999 [10]</td>
</tr>
<tr>
<td></td>
<td>Facilitates t-loop formation</td>
<td>Smogorzewska et al., 2000 [30]</td>
</tr>
<tr>
<td></td>
<td>Negative regulator of telomere length</td>
<td>Broccoli et al., 1997 [18]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bilaud et al., 1997 [13]</td>
</tr>
<tr>
<td>Tankyrase 1 and 2</td>
<td>Associates with TRF1</td>
<td>Smith et al., 1998 [20]</td>
</tr>
<tr>
<td></td>
<td>Positive regulator of telomere length</td>
<td>Kaminker et al., [21]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cook et al., [19]</td>
</tr>
<tr>
<td>TIN2</td>
<td>Associates with TRF1/TRF2</td>
<td>Kim et al., 1999 [22]</td>
</tr>
<tr>
<td></td>
<td>Negative regulator of telomere length</td>
<td>Ye et al., [25]</td>
</tr>
<tr>
<td>PinX1</td>
<td>Associates with TRF1</td>
<td>Zhou et al., 2001 [23]</td>
</tr>
<tr>
<td></td>
<td>Inhibits telomerase activity</td>
<td></td>
</tr>
<tr>
<td>RAD50/MRE11/NBS1</td>
<td>Associates with TRF2</td>
<td>Zhu et al., 2000 [24]</td>
</tr>
<tr>
<td></td>
<td>Facilitates t-loop formation</td>
<td></td>
</tr>
<tr>
<td>RAP1</td>
<td>Associates with TRF2</td>
<td>Li et al., 2000 [32]</td>
</tr>
<tr>
<td></td>
<td>Negative regulator of telomere length</td>
<td></td>
</tr>
<tr>
<td>POT1</td>
<td>Binds ss TTAGGG</td>
<td>Baumann et al., 2001 [26]</td>
</tr>
<tr>
<td></td>
<td>Chromosome stability</td>
<td>Loayza et al., 20004 [27]</td>
</tr>
<tr>
<td></td>
<td>Associates with TRF1</td>
<td>Ye et al., 2004 [28]</td>
</tr>
<tr>
<td></td>
<td>Negative/Positive regulator of telomere length</td>
<td>Liu et al., 2004 [29]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Colgin et al., 2003 [33]</td>
</tr>
</tbody>
</table>

Since the list of known proteins that specifically bind the telomere is rapidly increasing, only a selection is shown. The catalytic subunit of telomerase is excluded in this table, see table 2.
The telomere hypothesis

Each cell division shortens the telomeric sequence with 30 to several hundred base pairs [7, 34-36]. This shortening has been shown to be proportional to the size of the G-rich telomeric 3′-overhang, i.e. the telomeres shortens faster in cells with long overhangs [36].

Telomeric shortening has been suggested to be caused by the “end replication problem” and is due to the inability of DNA polymerases to fully replicate the extreme ends of linear chromosomes. DNA polymerase replicates only in the 5’ to 3’ direction and utilizes RNA primers to start synthesis of the new daughter strands. The leading strand is synthesized continuously and proceeds all the way to the end generating blunt ends. However, synthesis of lagging strand happens discontinuously with series of Okazaki fragments. Removal of the extreme 5’ primer from the lagging strand leaves a gap that cannot be filled in by DNA polymerase and this makes the 5’end of the lagging strand shorter than the parental strand. This problem with conventional DNA replication was first described by Olovnikov (1971) and Watson (1972) [37] (reviewed in [34, 38, 39]) (Fig. 2).

Figure 2. Telomeric loss during DNA replication. During DNA replication removal of the extreme 5’ primer from the lagging strand leaves a gap that makes the 5’end of the lagging strand shorter than the parental strand. Degradation of the 5’ strand by exonucleases results in even shorter telomeres and a reestablished 3′ overhang on the leading strand.
Recent data suggests that loss of telomeric repeats during replication not only is a problem at the lagging strand but also at the leading strand. As mentioned above the leading strand is blunt ended after synthesis, i.e. it has lost its 3’ overhang upon replication and is therefore shorter than the parental strand. Moreover, the t-loop can’t be formed unless this 3’ overhang is reestablished. The observation that both ends of the human chromosome contain long G-tails indicates the occurrence of processing events on the blunt ended leading strand in order to reestablish the 3’ overhang. Evidence from the yeast model system suggests the involvement of exonucleases activity on the C-rich strand generating a G-rich overhang [9, 40-42] (reviewed in [38]) (Fig. 2). Taken together this indicates that both lagging and leading strand shortens after DNA replication, causing shrinking of the chromosomes with each cell division.

The telomere hypothesis suggests that telomeric shortening function as a mitotic clock, which limits the lifespan of normal cells. The restricted proliferative capacity of normal human somatic cells was first described by Hayflick and Moorhead in 1961 [43]. They discovered that cells, after approximately 40-70 population doublings, terminate DNA-replication and enter cellular senescence; a viable, irreversible growth arrested state [43, 44]. But how is senescence triggered by telomeric shortening?

Initially it was thought that senescence was induced by the telomeric length per se, but recent data suggest that it is the altered telomere state that is responsible. Expression of TRF2 molecules that lack the ability to bind to the telomere has been shown to induce senescence [45]. Telomeric shortening reduces the amount of TRF2 bound at the telomere; less TRF2 eventually leads to the opening of the t-loop an event that could initiate the senescence. Without the protective feature of the t-loop the end of the chromosome would induce a DNA damage signal similar to that induced by double-strand DNA breaks. This signals is mediated by p53 and the ATM (ataxia telangiectasia mutated) pathway eventually leading to G1 arrest, apoptosis or senescence [45-47] (reviewed in [48, 49].

It has also been shown that normal human fibroblasts maintain their G-rich overhang at senescence, implying that a reduction in overhang length is not the molecular signal that triggers replicative senescence [50]. Furthermore, over-expression of TRF2 increased the rate of telomeric shortening without inducing senescence. Increasing the number of TRF2 molecules on the telomere could lead to a stabilization of the t-loop, hence there is a protective effect of TRF2 on short telomeres [47].

**Telomerase**

A consequence of the telomere hypothesis is that immortal cells (tumors, germ line and stem cells) must have the capability to overcome telomeric
loss; this can be achieved by activating the enzyme telomerase. Telomerase is an RNA-dependent DNA polymerase that adds new telomeric repeats to the ends of chromosomes [51]. Immortal cells thereby maintain their telomere length and avoid replicative senescence [52].

Telomerase reverse transcriptase and the telomerase RNA part

The telomerase enzyme consists of two major components, the telomerase reverse transcriptase (TERT) [53-55] and an RNA part (TR) which acts as a template [56]. The length of human TR (hTR also referred to as hTERC and hTER) is approximately 450 base pairs and has a template region consisting of 11 nucleotides (5´-CUAACCCUAAC-3´) complementary to the human telomere sequence [56].

Using the template region; hTR recognizes and binds to the telomeres 3’end. Part of the template region is then used by hTERT to catalyze the polymerization of dNTPs onto the end of the chromosome. hTERT extends the telomeric DNA until the end of the RNA template is reached. This is followed by translocation of the template, after which hTERT extends the DNA by another six bases. The three steps during telomerase catalysis: recognition, elongation and translocation are repeated until the telomerase complex dissociates. Telomere elongation of the 3’end enables the DNA replication machinery to further elongate the 5’end of the chromosome (reviewed in [57]) (Fig. 3).

Figure 3. The telomerase complex at the end of the telomere. hTR recognizes and binds to the 3’-overhang and hTERT to catalyze the polymerization of new telomeric repeats onto the end of the chromosome, with hTR as a template.

It is believed that DNA replication and telomere extension cooperates during replication at the telomere. Several reports have shown that the telomeres replicate throughout S-phase and that the overall pattern follows that of total DNA synthesis [40, 58, 59]. Furthermore, it has been shown that hTR and
hTERT accumulate at intranuclear sites separate from telomeres throughout most of the cell cycle. Data indicates that during this period hTR and hTERT are sequestered away from one another, residing in different compartments. During S-phase both hTR and hTERT are recruited to the telomeres, suggesting a cell cycle regulated trafficking of telomerase to the telomeres [60]. A hypothesis in favor of a replicative cooperation, states the necessity of DNA replication in order to avoid overextension of G-tails by telomerase. In order for telomerase to be able to elongate the G-rich strand the telomeres needs to be in an open accessible state and as cells exit S-phase the t-loop is reestablished. An interesting question is whether the t-loop in itself send an active signal for cell cycle progression or if progression is inhibited before t-looping is completed (reviewed in [38])?

**Telomerase-associated proteins**

Several proteins have been described to associate with the telomerase core, many of which the physiological role still is unclear (Table 2) (Fig. 3). The heat shock proteins (HSP) 90, HSP 70 and p23 functions as chaperones and specifically binds to hTERT and influence its proper assembly with hTR. HSP 70 dissociates from hTERT upon assemble with hTR. However, HSP 90 and p23 remain associated with the functional telomerase complexes [61]. Dyskerin has been proposed to play a role in ribosomal processing and have been shown to bind to hTR. Cells that express a mutated dyskerin gene have lower telomerase activity and shorter telomeres than the corresponding controls. Furthermore, dyskerin has been suggested to be involved in processing or stability of the telomerase RNA [62]. hStau and L22 are additional RNA-binding proteins that have been discovered, implicated in nucleocytoplasmic transport and hTR processing [63]. TEP1 (telomerase-associated protein) is a telomerase RNA-binding protein with a possibly role in RNA stability [64, 65].
Table 2. *The telomerase core and associated proteins in humans.*

<table>
<thead>
<tr>
<th>Component</th>
<th>Postulated function(s)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>TR</td>
<td>Template RNA Binds to the telomeres 3’ end</td>
<td>Feng <em>et al.</em>, 1995 [56]</td>
</tr>
<tr>
<td>Dyskerin</td>
<td>RNA binding protein Involved in ribosomal processing</td>
<td>Mitchell <em>et al.</em>, 1999 [62]</td>
</tr>
<tr>
<td>hStau/L22</td>
<td>RNA binding protein RNA stability, processing and telomerase assembly</td>
<td>Le <em>et al.</em>, 2000 [63]</td>
</tr>
<tr>
<td>HSP90/70 and p23</td>
<td>Chaperones, binds to hTERT and influence its proper assembly with hTR</td>
<td>Forsythe <em>et al.</em>, 2001 [61]</td>
</tr>
</tbody>
</table>

Since the list of known proteins that specifically associates with telomerase is rapidly increasing, only a selection is shown.

### Regulation of telomerase

**Regulation of the hTERT transcript**

hTERT it is the only part of the telomerase complex shown to correlate with telomerase activity, and expression is in general restricted to immortal cells. Whereas both hTR and TEP1 are commonly expressed in both malignant and normal cells [53, 54, 66-68]. Furthermore, ectopic expression of hTERT is sufficient for restoring telomerase activity in certain telomerase negative cells [52]. It has therefore been suggested that regulation of telomerase activity mainly occurs through hTERT.

The *hTERT* gene consists of 16 exons and 15 introns spanning approximately 35 kb, it contains seven reverse transcriptase (RT) motifs conserved among RTs, and one telomerase specific T motif [53-55, 69, 70]. The *hTERT* promoter region is GC-rich, lacks traditional TATA and CAAT boxes but is rich in transcription factor binding sites [69, 70] (reviewed in [71]).

One of the transcription factors important in transcriptional regulation of *hTERT* is the oncogene c-Myc, which activate *hTERT* transcription. c-Myc forms a complex with the Max protein and bind as heterodimers to the two E-boxes located in the proximal *hTERT* promoter, and thereby activate gene expression
transcription. Sp1 has been implicated to cooperate with c-Myc in the activation of hTERT. This is supported by the Sp1 binding sites located between the two proximal E-boxes [71]. The Myc/Max complex has been shown to physically interact with the hTERT promoter in electrophoretic mobility shift assays [72-76]. An additional E-box binding protein Mad1, also forms heterodimers with Max and Mad1 have been shown to negatively regulate hTERT expression. The Mad1/Max complex replaces the c-Myc/Max complex at the hTERT promoter E-boxes and represses transcription [75-77]. hTERT repression by Mad is also thought to be mediated by chromatin condensation through the recruitment of histone deacetylases (HDAC). This would result in diminished acetylation of histones and repression of gene transcription. In the same manner c-Myc might recruit histone acetyltransferases (HAT), leading to an increased acetylation of histones and thereby stimulating transcription [75, 78]. Mad1 has been reported to function as an inhibitor of hTERT under physiological conditions, since inhibition of Mad1 with siRNA in hTERT negative cells activated the expression of hTERT mRNA [79] (Fig. 4).

![Diagram of transcriptional regulation of hTERT](image)

*Figure 4. Transcriptional regulation of hTERT.* Some various mechanisms that can act on the hTERT promoter are schematically shown. The c-Myc/Max complex binds to E-boxes and activates transcription, whereas Mad1/Max binding represses transcription. Sp1 has been implicated to cooperate with c-Myc in the activation of hTERT. p53 inhibits Sp1 binding to the hTERT promoter and represses transcription. Mad1 might recruit histone deacetylases (HDAC) as another way of inhibiting hTERT transcription. c-Myc may stimulate transcription by the recruitment of histone acetyltransferases (HAT).

Another repressor of hTERT transcription is the anti-oncogenic protein p53. Overexpression of wild type (wt) p53 has been shown to repress hTERT
promoter activity as well as hTERT mRNA expression and telomerase activity [80-84]. Interestingly, repression of hTERT by p53 is not dependent on the presence of the two potential p53 binding sites located at position -1945 and -1317. However, elimination of all of the Sp1 binding sites in the proximal promoter of hTERT resulted in a marked loss of p53 repression. Suggesting that Sp1 plays a role in the p53-mediated transcriptional repression of hTERT [81]. The involvement of Sp1 has been confirmed in a recent report by Xu et al. that showed that p53 inhibits Sp1 binding to the hTERT promoter by forming a p53-Sp1 complex. It is possible that this interaction affects the myc-regulatory pathway of hTERT expression and thereby represses transcriptional activity of hTERT [84]. The physiological relevance of p53 has been questioned by Lin et al. since depletion of p53 with siRNA in hTERT negative cells failed to activate hTERT expression [79] (Fig. 4).

Alternative splicing of the hTERT mRNA has been implicated as a regulatory mechanism of telomerase activity. Alternative splicing of hTERT produces mRNAs lacking critical RT motifs. The α-splice variant contains a 36 bp deletion within one RT motif; the β-splice message causes a truncated protein lacking several RT motifs, and none of these transcripts codes for an active transcript. Only cells expressing full-length hTERT transcripts containing complete RT motifs exert telomerase activity. It has been demonstrated that cells can shift from expressing the active full-length transcript to the inactive β-splice variant [68, 85-88].

Phosphorylation of the hTERT protein
Akt kinase is a downstream effector in the phosphoinositide 3-kinase (PI3K) pathway, involved in signal transduction (reviewed in [89]). Akt protein kinases have been shown to increase telomerase activity through direct phosphorylation of the hTERT protein. This phosphorylation is stabilized by HSP90. Akt, HSP90 and hTERT forms a complex in the nucleus and HSP90 maintains Akt in its phosphorylated and active state, enabling Akt to phosphorylate and activate hTERT. Disruption of the HSP90 binding to Akt induces Akt dephosphorylation and reduced telomerase activity [90-93].

Regulation of telomerase by telomeric proteins
An additional step of regulation involves the recruitment of telomerase to the telomere and the accessibility of the telomere to telomerase (Table 1) (reviewed in [12]). Both TRF1 and TRF2 negatively regulate telomere length. Overexpression of either of these proteins results in a progressive shortening of telomere length. Furthermore, telomere elongation is induced by expression of dominant-negative TRF1. Neither TRF1 nor TRF2 inhibited the enzymatic activity of telomerase indicating that telomerase-dependent regulation is blocked by sequestration of the 3’ end in the t-loop [30, 31, 47].
Tankyrase 1 and 2 are poly (ADP-ribose) polymerases that ADP-ribosylates TRF1 and thereby inhibits its binding to the telomere. Overexpression of Tankyrase 1 induced loss of TRF1 and lengthening of telomeres. Suggesting that tankyrases function as positive regulators of telomere length, by releasing TRF1 from the telomere and opening up the telomeric complex allowing telomerase access to the telomere [19, 20]. TIN2 has been described as a negative regulator of telomere length by protecting TRF1 from being modified by tankyrases [12, 22]. PinX1 binds TRF1 and negatively regulates telomere elongation. PinX1 forms stable complexes with hTERT/hTR and inhibits telomerase activity, depletion of PinX1 increases telomerase activity and telomere length [23, 94]. Of the TRF1 complex PinX1 is so far the only component shown to directly affect telomerase activity.

POT1 has been described as both a negative and positive regulator of telomere length [26, 27, 33]. By binding to the 3’ overhang POT1 might block access of telomerase to the telomere and thereby inhibiting elongation. POT1 has also been implicated in stabilizing the t-loop [26, 27]. As a positive regulator POT1 has been proposed to recruit telomerase to the telomere, alternatively by stabilizing the “open” structure of the chromosome [33]. Yeast Rap1p acts as a negative regulator of telomere length, and hRap1 seems to share both structural and functional similarities with yeast Rap1p. Suggesting that hRap1 also might function as a negative regulator [32].

**Telomerase and cancer**

Although immortalization is a fundamental step during transformation, forced expression of hTERT alone immortalize but fails to transform human cells. Telomerase expressing cell clones retain normal growth control and have a normal karyotype [52, 95-97]. Transformation of normal human epithelial and fibroblast cells is accomplished by ectopic expression of hTERT in combination with the simian virus 40 large-T oncoprotein and an oncogenic allele of H-ras. Cells transfected without hTERT were not transformed, suggesting that telomerase is essential for the formation of human tumors [98].

Approximately 90% of all tumors analyzed so far exhibit telomerase activity[4]. Furthermore, telomerase activity is normally not detected in normal somatic cells, with the exception for highly proliferative cells such as germ line cells and stem cells. Telomerase is repressed during early human embryonic development and cells induced to differentiate show decreased telomerase activity levels [85, 99] (reviewed in [100]). It has also been shown that T cells express telomerase upon activation, however these activity levels are insufficient to confer immortality and telomeres from these cells shorten continuously [101, 102]. The same observation has been made for hemato-
poietic stem cells which have telomerase activity and still display telomeric shortening [7, 103]. Taken together this indicates that those levels of telomerase are not sufficient to prevent telomeric shortening.

The developmental switching of telomerase in humans has been postulated to be an intrinsic step towards initiating the telomeric clock, where telomere attrition to critically short lengths serves to limit tumor progression (reviewed in [100]). Interestingly, recent data suggest that telomerase could contribute to tumorigenesis by a telomere length-independent mechanism. Immortal human fibroblasts that maintain their telomeres by an alternative mechanism fail to be transformed upon expression of the simian virus 40 large-T oncoprotein and an oncogenic allele of H-ras, suggesting that telomere elongation is not sufficient for tumorigenesis. Furthermore, expression of a mutant telomerase enzyme in these cells unable to elongate telomeres forms tumors at the same efficiency as a functional enzyme [104]. Cao et al. reported that expression of a mutant hTERT significantly decreased telomerase activity but rescued cells from apoptosis [105]. In a recent report by Del Bufalo et al. both expression of wt hTERT and expression of catalytically and biologically inactive hTERT mutants in human breast carcinoma cells protected cells from apoptosis mediated by inhibition of Bcl-2 [106]. This indicates that hTERT inhibits apoptosis regardless of telomerase activity and its ability to lengthening telomeres [105, 106].

Immortalization of human cells is a prerequisite for the eventual progression to a tumorigenic state but what is the origin of telomerase activation in cancer? Could the telomere length in various tumor cells give an answer to that? It has been reported that tumor cells have extremely short but stable telomeres; e.g. the telomere length in colorectal carcinoma (7.12 kb) is shorter than normal control (9.25 kb) (reviewed in [107]). This would suggest that telomerase is activated after a considerable period of telomeric shortening in the originating telomerase negative cell. Such a cell would in this case supposedly evade hTERT repression supposable arising from mutational or epigenetic disruption of hTERT repressor genes. Another possibility is that human cancers arise from stem cells, which already have active telomerase. Many researchers now favor the possibility that genetic or epigenetic changes of stem cells give rise to cancer stem cells with the ability to generate phenotypically diverse cancer cells. In support of this is the information that some tumors have longer telomeres than normal tissue for example intracranial tumors and basal cell carcinomas of the skin. This indicates that the tumor cells have not undergone enough divisions to induce significant telomere shortening [1, 2, 107, 108]. It is however, possible that both of these mechanisms exist in parallel.
Telomerase as a prognostic marker

Several studies have proposed detection of telomerase activity as a predictor of clinical outcome and indicator of malignancy (reviewed in [109]). Most cases of malignant gliomas have active telomerase, this is in contrast to grade I gliomas where telomerase is not detected [109]. Furthermore, telomerase activity can also be used to distinguish benign from malignant thyroid tumors and telomerase activity may also correlate with tumor invasiveness [109-113]. There is also a strong correlation between high telomerase activity and poor clinical prognosis in neuroblastomas, gastric cancer and non-small cell lung cancer [109]. In neuroblastomas telomerase activity has been shown to be capable of distinguishing between good and poor outcome with regard to all tumor stages [114-117]. Furthermore, full length hTERT mRNA is a strong independent prognostic factor in neuroblastoma [118]. High telomerase activity have been associated with shorter survival in multiple myeloma (MM) patients and MM cells with high telomerase activity levels and short telomeres defines a subgroup of patient with poor prognosis [119]. Short telomeres has also been associated with an unfavorable outcome in chronic lymphocytic leukemia [120]. Furthermore, increased telomerase activity have been associated with the progression of squamous esophageal carcinoma [121, 122]. However, a recent report by Hsu et al. conclude that telomerase expression does not correlate to tumor stage and prognosis in squamous esophageal carcinoma [123]. Interestingly, it has been shown that high telomerase activity in the urine correlates with the presence of bladder tumors in men [124].

Inhibition of telomerase

A number of different approaches have been developed to inhibit telomerase activity, i.e. targeting of core telomerase components or the telomere. Many methods still rely on telomere shortening which is a relative slow process, resulting in a lag phase before growth arrest or apoptosis of the cells (reviewed in [125, 126]).

Targeting of hTERT

Over-expression of hTERT has been reported to confer resistance towards apoptosis induced by different stimuli i.e. oxidative stress, serum deprivation and DNA break inducing agents [127-133]. This makes inhibition of hTERT an attractive target in therapy and different approaches have been tested. Expression of a dominant negative (DN) hTERT in human cancer cell lines results in complete inhibition of telomerase activity, reduction in te-
lomere length and tumor cell death. The onset of cellular arrest was related to the cell lines initial telomere length, i.e. cells with long telomeres demanded several population doublings before growth arrest was initiated [134, 135]. Human acute leukemia cells expressing DN hTERT underwent apoptosis after 50 days of cultivation. Furthermore, early passages of DN-hTERT expressing cells showed higher induction of daunorubicin induced apoptosis compared to control cells, indicating that inhibition of telomerase activity sensitize cells to daunorubicin [136].

Inhibition of telomerase activity using hTERT antisense in a human thyroid cancer cell line resulted in inhibition of cell growth and an increased rate of apoptosis after approximately 20 population doublings. At 30 population doublings soft agar colony formation and tumor growth in nude mice were reduced compared to control cells [137]. Interestingly, reduction of endogenous hTERT protein by RNA interference has been shown to increase the apoptotic fraction already after 24h of treatment with a Bel-2 inhibitor [106]. Support for the notion that inhibition of hTERT can induce short-term apoptosis without affecting the telomere length have been presented by Kraemer et al. [138] and Saretzki et al. [139].

The use of reverse transcriptase inhibitors to decrease telomerase activity has been widely tested and the results vary from exhibiting only weak inhibitory activity and mild proliferative impairment to increasing the number of apoptotic cells [125, 126].

Targeting hTR
Six-base oligonucleotides TTAGGG have been shown to inhibit telomerase activity, presumably by competing with the telomere in binding to hTR. Cells pretreated with TTAGGG oligonucleotides showed enhanced apoptotic response induced by staurosporine, Fe^{2+} and amyloid β-peptide [140]. Hammerhead ribozymes cleaves specific RNA sequences. Ribozymes targeting hTR efficiently decreases telomerase activity and induces telomeric shortening. Furthermore, the cells showed reduced replicative capacity and cellular changes typical of apoptosis. These effects were however telomere length dependent [141]. An attractive approach is the simultaneously targeting of telomeres and telomerase. Shortening of telomeres with paclitaxel combined with inhibition of telomerase by hTR antisense resulted in reduced cell growth rate and enhanced the cytotoxicity of paclitaxel [142]. This might be a possible solution in avoiding the lag-phase observed in other telomerase inhibition studies were apoptosis is dependent on telomere length reduction.

GRN163 is an oligonucleotide that forms stable duplexes with hTR and thereby inhibits telomerase activity [143]. The effects of GRN163 on cell survival have been shown to be dependent on initial telomere length in the treated cells [143-145]. Wang et al. showed that GRN163 treatment for 7-14 days resulted in growth inhibition and apoptosis in cells with short telomeres.
(1.7-5.4kb) but not in cells with long telomeres (9-11kb) [144]. A lipid modification of GRN163 (GRN163L) resulted in a more rapid loss of telomere length and cell growth than GRN163 [146, 147]. Furthermore, pretreatment of hepatoma cells with GRN163L 5-14 days before administration of doxorubicin lead to sensitization to doxorubicin-induced apoptosis [147]. GRN163L has also been shown to inhibit telomerase activity, tumor growth and prevent lung metastases in nude mice [147, 148].

G-quadruplex stabilization

A strategy to inhibit telomerase activity is the use of agents that stabilize G-quadruplexes. Telomestatin inhibits telomerase activity by facilitating the formation or stabilization of intermolecular G-quadruplex, including those produced between telomeric sequences [149]. MM cells treated with telomestatin exhibit a lag phase of 7-14 days followed by apoptotic cell death. This suggests that telomestatin induces gradual telomeric shortening leading to a critical telomere length [150]. Furthermore, in human leukemia cells telomestatin efficiently decreased telomerase activity and telomere length followed by apoptosis. Early-passage telomestatin treated cells exhibited enhanced chemosensitivity toward daunorubicin and cytosine-arabinoside [149]. Inhibition of telomerase apparently sensitize tumor cells to a variety of agents such as daunorubicin, staurosporine, Fe^{2+}, amyloid β-peptide, paclitaxel and doxorubicin [147] [142] [140] [136].

Therapeutic potential

Taken together the results from the different approaches to inhibit telomerase activity and function indicate that apoptosis can be induced by both telomere length dependent and independent mechanisms. The telomere length independent mechanism could possibly be due to uncapping of the telomere or by altered telomerase mediated regulation of gene expression [126]. Telomerase inhibition has a promising cancer therapeutic potential although there are some problems.

Approximately 10% of all human tumors are able to maintain their telomeres by the ALT mechanism (see next section for details). The use of telomerase inhibitors for treatment of telomerase-positive tumors may provide a selective advantage to cells, which can activate the ALT mechanism and thereby evading apoptosis. This suggests that the combination of telomerase and ALT inhibitors might be the most successful therapy in cancer treatment [126, 151]. In addition, there might also be unwanted effects on normal telomerase-positive cell such as stem cells. It is however thought that anti-telomerase treatments eliminate the proliferative potential of cancer cells before the telomere lengths of stem cells is critically shorten. Since stem
cells only rarely divide their telomeres should shorten at a slower rate than telomerase inhibited proliferating cancer cells [152]. Moreover, in a recent report by Preto et al. [153], it was shown that thyroid cancer cells with wt p53 exhibited growth arrest but not apoptosis upon telomere erosion caused by the use of a DN hTERT. This raises the possibility that cancer cells with a functional wt p53 will have a different response to telomerase inhibition therapy [153].

**Alternative lengthening of telomeres**

Approximately 10% of all human cancers and 30% of all immortal cell lines lack active telomerase, but show no telomere shortening. They maintain their telomeres by one or more mechanisms referred to as alternative lengthening of telomeres (ALT). The telomere length phenotype of ALT cells is extremely heterogeneous with telomeres ranging from very short to extremely long [154, 155]. ALT cells have been reported to contain promyelocytic leukemia bodies. These contain telomeric DNA, TRF1, 2, replication factor A and proteins involved in recombination [156], indicating the involvement of recombination in ALT. This is supported by data presented by Dunham et al. [157], they showed that telomeric DNA is copied from telomere to telomere in ALT cells.

Telomeres in ALT cells could also be elongated using other telomeres as a copy template. One strand of a telomere may invade another telomere and anneal with the complementary strand. The invaded telomere is then used as a template for DNA synthesis, thereby extending the invading strand. Another alternative is rolling-circle replication in which the telomere is extended on an extra chromosomal circular template (reviewed in [151, 158]).

Interestingly, some telomerase positive tumors have also been reported to have a telomere phenotype similar to those seen in ALT cells. Indicating the coexistence of these mechanism either within a cell or within different regions of a heterogeneous tumor[155]

**Cytokines**

Cytokines are signaling proteins secreted by a cell, which affect their own activity or that of other cells. The following presentation briefly describes the cytokines used in the present investigation.
Interferons

Interferons (IFNs) are pleiotropic cytokines mediating a range of functions including antiviral, antiproliferative, antitumor and immunomodulatory activities. They are divided into type I (α and β) and type II (γ) IFNs. IFN signaling involves activation of the Janus kinases family of tyrosine kinases that phosphorylate substrate proteins called STATs (signal transducers and activators of transcription), which in turn activate IFN-stimulated genes [159, 160].

IFN-α has been widely used in the treatment of multiple myeloma (MM), and has been demonstrated to have antitumor activity. The overall effect on survival is modest and the benefits of IFN-α treatment remain controversial [161]. Furthermore, depending on different cellular contexts IFNs can either promote or inhibit apoptosis [159]. However, it has been shown that both IFN-α and -γ sensitize MM cell lines to Fas induced apoptosis, the mechanism behind this is not known [162]. It is possible that IFNs could be used to increase the efficiency of future cancer therapies.

Transforming growth factor-β

Transforming growth factor-β (TGF-β) is a cytokine expressed in three different isoforms, β1, β2 and β3. Binding of TGF-β to specific serine-threonine kinase receptors results in activation of downstream signaling pathways mediated by Smads. The Smad proteins enter the nucleus where they regulate transcription, either through direct DNA binding or by interacting with other transcriptional activators/repressors [163, 164].

TGF-β is involved in the regulation of cell growth, differentiation, matrix production and apoptosis in a variety of cell types. It is also important during embryonic development [99, 164]. TGF-βs role in carcinogenesis is complex, having both tumor suppressor and oncogenic activities. It causes growth arrest in normal cells and at early stages of tumorgenesis. Often during malignant progression, cells acquire insensitivity to growth inhibition by TGF-β, then acting as a tumor promoter by stimulating angiogenesis and immunosuppression [164].

Tumorhypoxia

Solid tumors contain hypoxic regions, i.e. regions with lower pO2 levels than the normal tissue of the corresponding organ. This is mainly due to the fact that tumor cells grow faster than the developing tumor capillary network. The result is an excess of cells in relation to the limitations for diffusion of oxygen from the capillary network. The hypoxic regions are heterogeneously distributed within the tumor mass and are also characterized by low glucose
levels, acidic pH and an increased interstitial tissue pressure. In these regions, pathways are activated resulting in cellular changes which adapt the tumor cells to the hypoxic micro environment. It has been suggested that this evolutionary process promotes tumor progression [165-167]. The extent of tumor hypoxia has also been shown to correlate with tumor malignancy and increased frequency of metastasis [168, 169]. Furthermore, hypoxic regions of tumors show increased resistance to radiation and chemotherapy [165, 166, 170].

Ample processes involved in the hypoxic response are mediated by hypoxia-inducible factor 1 (HIF-1) a key regulator of O₂ homeostasis and the related transcription factors HIF-2 and HIF-3. During normoxia the α subunits of HIF are hydroxylated which leads to HIF interaction with a ligase-ubiquitination complex followed by degradation of the ubiquinated HIFs via the proteosomal pathway. At least three dozen HIF-1 regulated genes have been identified so far including insulin-like growth factor 2 (IGF-2) and vascular endothelial growth factor (VEGF). The HIF-1 regulated genes are involved in angiogenesis, vascular reactivity, energy metabolism, erythropoiesis, cell proliferation and survival [165, 170]. Recent studies suggest that hTERT could be a new target for HIF-1 [171-173].
The present investigation

Aim:
Telomerase has been implicated in several cellular processes, such as immortalization, apoptosis resistance and malignancy grade all important attributes in tumor progression. The general aim of this thesis was to study the activity and regulation of telomerase in a panel of malignant cell types. More specifically we wanted to;

- Investigate the effect of TGF-β1 on telomerase activity and the mechanisms involved.
- Explore hTERT as a target for IFN-induced sensitization to apoptosis.
- Investigate if basal telomerase activity levels correlate with drug sensitivity.
- Investigate the effect of hypoxia on telomerase activity

Experimental methodology
Measurement of telomerase activity
A sensitive method to detect telomerase activity designated the TRAP assay (telomeric repeat amplification protocol), was developed in 1994 by Kim and coworkers [4]. This assay is a PCR (polymerase chain reaction) based method utilizing the telomerase enzymes ability to elongate 3´ ends with TTAGGG repeats. The reaction is a one buffer, two enzyme system performed in a single reaction tube.

In the first step of the assay telomerase adds telomeric repeats onto a non-telomeric oligonucleotide (TS) primer. In the second step these extension products serves as templates for PCR amplification using a reverse primer and the TS primer. The PCR reaction generates a ladder of products with 6 base increments starting at 50 bp (Fig. 5). With the TRAP assay telomerase activity can be detected from small quantities [4]. There is now a commercial available TRAP-assay the TRAPeze Telomerase Detection Kit, which
makes it possible to quantitate telomerase activity, due to the co-amplification of an internal control in the PCR, generating a product of 36 bp.

Relative telomerase activity of each extract can be determined by taking the ratio of the entire telomerase ladder to that of the internal control. An additional method to calculate telomerase activity is by including one sample of the TSR8 control template. TSR8 is an oligonucleotide comprised of the TS primer extended with 8 telomeric repeats. This oligonucleotide can be amplified using reverse and TS primer, and serves as a standard for estimating the amount of TS primers with telomeric repeats extended by telomerase in a given extract. By correlating the relative telomerase activity to the TSR8 control, telomerase activity will be expressed in TPG (total product generated) units. Each unit of TPG corresponds to the number of TS primers extended with at least 4 telomeric repeats by telomerase.

In order to accurately quantitate telomerase activity using the TRAP assay it is important to be in the linear range of the PCR reaction. To validate this, dilution series for each cell line were performed in order to determine the correct amount of protein to be used in each assay.

**Step 1**

<table>
<thead>
<tr>
<th>TS-Telomerase product</th>
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<tbody>
<tr>
<td>TS-primer</td>
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<tr>
<td>5’-AATCCGTCGAGCAGAGTT</td>
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</tbody>
</table>

**Step 2**

| 5’-AATCCGTCGAGCAGAGTT | ag ggtag ggtag ggtag ggtag -3’ |
|------------------------|
| Reverse-primer |
| TS-primer |

*Figure 5. Principles of the TRAP-assay, a method for detection of telomerase activity. Step 1, telomerase synthesizes telomeric repeats onto the nontelomeric oligonucleotide TS. Step 2, amplification of the TS-telomerase product by PCR using reverse and TS-primer.*
Results and discussion

Paper I: Differential effects of TGF-β1 on telomerase activity in thyroid carcinoma cell lines

Anaplastic undifferentiated thyroid carcinoma (ATC) is a rare but highly aggressive solid tumor. The disease has a deadly clinical course because of the rapid, invasive growth of the ATC primary tumors and its failure to respond to treatment. Nearly all patients die within 6 months of diagnosis [174]. Telomerase activity has been detected in 57% of ATC tumors (8/14) [111-113, 175-178]. The objective of the present study was to investigate the effect of TGF-β1 on telomerase activity in a panel of human ATC cell lines. Four of the cell lines i.e. KAT 4, C 643, HTh 74 and KTC-1, respond with growth inhibition upon TGF-β1 treatment [179, 180]. One of the cell lines, HTh 7, has been shown to lack responsiveness to TGF-β1 induced growth inhibition [179].

TGF-β1 treatment of HTh 74 and KTC-1 resulted in decreased telomerase activity levels. This is in agreement with previous studies where TGF-β1 has been shown to repress telomerase activity in a wide variety of cell types [88, 181, 182]. Furthermore, TGF-β1 has also been shown to down regulate telomerase activity during human placental differentiation [99]. In contrast to HTh 74 and KTC-1 an increase of telomerase activity was detected in C 643 and HTh 7 cells after TGF-β1 treatment. A stimulatory effect on telomerase activity by TGF-β1 has to our knowledge not been reported earlier, and may reflect deregulation in a more malignant genotype. In addition, we observed that TGF-β1 had no effect on telomerase activity in the KAT 4 cell line. Thus, our results indicate that the investigated ATC cell lines differ in telomerase response upon TGF-β1 treatment. Furthermore, the growth inhibitory response induced by TGF-β1 did not correlate with the effect on telomerase activity, indicating that TGF-β1 is able to regulate proliferation and telomerase activity separately, at least in ATC cells.

To elucidate possible mechanisms explaining the opposite effects of TGF-β1 on telomerase activity in the ATC cell lines, C 643 and HTh 74 were selected for further studies. HTh 74 showed a decrease both in hTERT mRNA and protein levels after TGF-β1 treatment, suggesting that repression of telomerase activity was due to inhibition of hTERT expression. This is in line with previously performed studies that demonstrated a decrease of hTERT expression after TGF-β1 treatment in skin keratinocytes and during trophoblast differentiation [88, 99]. In contrast, TGF-β1 had no effect on hTERT protein or mRNA levels in C 643. Taken together, our results indicated that TGF-β1 inhibits telomerase activity in HTh 74 through transcriptional repression of the hTERT promoter.

To test this further, the two ATC cell lines were transfected with luciferase reporter constructs containing full length or deletion parts of the
Addition of TGF-β1 decreased promoter activity in the hTERT full-length construct in HTh 74. Thus, we conclude that TGF-β1 inhibits telomerase activity through transcriptional repression of the hTERT promoter. Using the five different deletion constructs we were able to find two regions in the promoter responsible for the inhibitory effect. These regions harbor two Myc/Max sites (E-boxes) at position -235 and at -27 [69].

Earlier reports have shown that c-Myc activates hTERT transcription and that TGF-β1 is able to down regulate c-Myc expression in certain cell lines [74, 75, 88, 183-187]. Furthermore, it has been suggested that a switch from Myc/Max to Mad/Max binding represses hTERT expression [75]. RT-PCR analysis of c-Myc expression revealed decreased mRNA levels after TGF-β1 treatment, supporting our theory of the involvement of Myc/Mad switching in TGF-β1 regulation of telomerase in HTh 74. Recent data presented by Hu et al. [187] suggest the existence of two mechanisms by which TGF-β could effect repression of the rat TERT gene expression. In the first mechanism, TGF-β1-induced Smad3 inhibits c-Myc expression and thus TERT activity. The second effect is suggested to be direct inhibition of TERT by Smad3 binding to the TERT promoter [187]. Thus, the potential effects of Smad3 on the hTERT promoter in ATC need to be evaluated in future studies.

In the transfection studies of C 643 we observed no differences in hTERT promoter activity in full length or deletion constructs between TGF-β1 treated and control cells. Interestingly, transfection of C 643 cells with the shortest hTERT promoter deletion fragment (only containing intron 1 and 37bp of exon 2) resulted in elevated levels of the basal luciferase activity compared to the other constructs. Thus, suggesting the presence of inhibitory site(s) in the upstream part of the promoter affecting activity. In line with the basal telomerase activity in C 643, the hTERT transcriptional activity was higher in C 643 cells compared to HTh 74. In C 643 cells c-Myc levels initially increased after TGF-β1 treatment followed by a decrease after 72 h of treatment, indicating an insensitivity regarding hTERT promoter regulation in this particular cell line. In summary; this study demonstrates that TGF-β1-mediated up-regulation of telomerase activity is not due to transcriptional regulation of hTERT in C 643.

It has been shown that telomerase activity is enhanced by direct phosphorylation of the hTERT subunit by Akt protein kinase, a downstream effector in the PI3-kinase pathway [89, 92]. Furthermore, hypoxia induced up-regulation of telomerase activity is abrogated by MEK-inhibition in certain cell types [188]. To evaluate the possible involvement of these mechanisms in the observed up-regulation of telomerase activity after TGF-β1 treatment in C 643, cells were treated with either the PI3-kinase inhibitor LY294002 or the MEK-inhibitor U0126. Both inhibitors efficiently abrogated TGF-β1-mediated up-regulation of telomerase activity. Interestingly, TGF-β1 has been suggested to activate both the MAPK and the PI3-kinase pathway [189]. Thus, the present study suggests a possible involvement of hTERT
phosphorylation as well as the MAPK pathway in regulating the increase of telomerase activity observed in C 643.

Next we aimed to search for genetically differences between the five ATC cell lines, which might explain the observed opposite effects of TGF-β1 on telomerase activity. Several studies have reported that wt p53 down-regulates telomerase activity [79, 80, 83, 84, 190, 191] and in this context it is interesting to note that p53 mutations have been found in ATC cell lines. A high incidence of p53 mutations has been reported in ATC compared to differentiated carcinomas and mutations of this tumor-suppressor gene might be important in triggering progression from differentiated to anaplastic carcinomas [174].

We therefore performed sequence analyses of the p53 gene in the ATC cell lines. The data revealed that HTh 74 displays wt p53 and it has previously been shown by Kurebayashi et al. [192] that KTC-1 also has a wt p53 genotype. Furthermore, we found that HTh 7, KAT 4 and C 643 all harbored mutations in the p53 gene. Thus, three of the five ATC cell lines harbored p53 mutations. Interestingly, we observed that the cell lines responding by up-regulation of telomerase activity after TGF-β1 treatment harbored p53 mutations whereas cell lines displaying wt p53 responded by down-regulation of telomerase activity. Our present data might indicate a possible connection between p53 mutations and the inability to respond by down-regulation of telomerase activity upon TGF-β1 treatment. Future studies inhibiting wt p53 in HTh 74 will elucidate if deregulation of the TGF-β1 inhibitory effect occurs.

In conclusion, the present study shows that TGF-β1 mediates differential effects on telomerase activity in ATC cell lines possibly reflecting deregulation of TGF-β1 signaling in a more malignant genotype. Inhibition of telomerase activity mediated by TGF-β1 is caused by transcriptional repression of the hTERT gene, most likely due to decreased c-Myc levels. Furthermore, our data suggest the involvement of the PI3-kinase and MAPK-pathways in regulating TGF-β1 mediated increase of telomerase activity.

Paper II: Interferon-Induced Sensitization to Apoptosis is Associated with Repressed Transcriptional Activity of the hTERT promoter in Multiple Myeloma

Multiple myeloma (MM) is a B cell disease, characterized by the accumulation of malignant plasma cells in the bone marrow. Complications of the disease include bone pain, skeletal lesions and anemia. Conventional therapy can extend patient survival to 3-4 years but few patients are cured. Although MM cells initially are sensitive to therapies, prolonged exposure leads to drug resistance and hence a poor clinical outcome [193, 194]. Elevated levels of telomerase activity has been associated with shorter survival in MM...
patients and higher telomerase levels are generally observed in MM cells from patients with relapsed disease [119].

We have previously demonstrated that IFNs sensitize a panel of MM cell lines and primary tumors to Fas induced apoptosis [162, 195]. Decreased levels of telomerase activity have been suggested to increase susceptibility to apoptosis [106, 128, 130, 136, 141, 149]. The aim of the present investigation was to explore hTERT as a target for IFN-induced sensitization to apoptosis in MM.

To test this hypothesis the highly Fas-resistant interleukin 6 (IL 6) dependent MM cell line U-266-1970 was selected. In the present study we show that IFN-α as well as IFN-γ down-regulated telomerase activity in U-266-1970 cells, although with different kinetics. IFN-α has previously been shown to decrease telomerase activity in malignant hematopoietic cell lines [196]. During the years contradictory results concerning the cell cycle dependent regulation of telomerase expression have been reported [60, 196-201]. Both IFN-α and -γ exert growth inhibitory effects on U-266-1970, raising the possibility that the IFN induced repression of telomerase activity might be a consequence of cell cycle arrest. However, we did not observe a straight correlation between accumulation of cells in G1 and down-regulation of telomerase activity after IFN-γ treatment. Furthermore, mimose-induced G1 arrest was not sufficient for down-regulation of telomerase activity. We conclude that in the MM cell line U-266-1970, G1 arrest alone is not sufficient for down-regulation of telomerase activity.

After IFN-α or IFN-γ treatment hTERT mRNA levels were rapidly decreased, but no differences in splicing pattern were observed. Thus, suggesting that the IFNs may inhibit telomerase activity through transcriptional regulation at the hTERT promoter. To test this hypothesis U-266-1970 cells were transiently transfected with full length and different deletion constructs of the hTERT promoter. Using the full length construct both IFN-α and γ repressed promoter activity, thus confirming our hypothesis. This is in concordance with previous results were IFN-α was suggested to induce repression of hTERT mRNA independent of de novo protein synthesis [196]. Furthermore, IFN-γ was previously reported to repress the full length hTERT promoter in HPV E6 and E7 positive cervical carcinomas [202]. We conclude, by the use of hTERT promoter analysis, that both IFNs repress hTERT at the transcriptional level in U-266-1970.

The hTERT promoter contains transcription factor binding sites for Myc, Mad, AP1, NF-κB and STAT proteins [71] which are all known to be implicated in the regulation by IFNs [159, 203-206]. Further analysis using a panel of hTERT promoter constructs suggested a minor inhibitory site located between fragment -145 and +228, and one of the Myc/Max sites is located in this region. However, we observed only a small effect of IFNs on L-Myc mRNA and Mad mRNA levels were decreased at late time points, therefore these data do not support the Myc/Mad switch to be a predominant
underlying mechanism of IFNs induced hTERT repression. It has been demonstrated that v-Myc activity and DNA-binding properties can be regulated through dephosphorylation of the protein whereas the v-Myc protein and mRNA levels were unaffected [207]. This indicates that we cannot rule out the involvement of Myc after IFN treatment.

Since the IFNs continued to inhibit promoter activity with all of the analyzed deletion constructs we suggested that important regulatory site(s) is located within the proximal region of the hTERT gene. Inhibition of NF-κB by the use of a dominant negative IkBα expression vector efficiently repressed hTERT promoter activity in the full length and the P+288 construct. Thus, confirming the presence of functional NF-κB binding sites within this region. However, we could not demonstrate a direct effect of either IFN-α or IFN-γ on NF-κB activity. Therefore, we suggest that the observed inhibition of telomerase activity by IFNs is not due to repression of NF-κB binding to the hTERT promoter.

Previous studies have suggested that IFN-γ may down-regulate hTERT expression in HPV E6 and E7 positive cervical carcinomas via interferon regulatory factor 1 (IRF-1) [202, 208]. This was not confirmed in our study, although Western blot analysis of U-266-1970 cells showed an increase of IRF-1 protein levels after both IFN-α and IFN-γ treatments, no effect on transcriptional activity of the putative IRF-1-binding sites within the hTERT promoter was observed in the present study (data not shown).

In the present study exploring the impact of apoptosis sensitization on telomerase activity we demonstrated that pretreatment with IFN-α prior to the administration of the Fas agonistic antibody CH-11 further reduced the telomerase activity as compared to IFN-α treatment alone. The addition of CH-11 alone did not, however, alter the telomerase activity in U-266-1970 cells. These data show that telomerase activity is repressed in MM cells sensitized to Fas-mediated apoptosis. Furthermore, the dominant negative IkBα expression vector demonstrated to efficiently down-regulate hTERT via transcriptional targeting resulted in augmentation of Fas-mediated apoptosis in MM cells. However, we cannot exclude that reduced NF-κB activity induces additional effects besides transcriptional repression of hTERT.

We conclude that one potential mechanism whereby IFNs sensitize MM cells to Fas-mediated apoptosis is by repressing hTERT activity at the transcriptional level, making hTERT an attractive target for MM therapy aiming at lowering the threshold for apoptosis.
Cancer of the esophagus is the seventh most common cause of cancer related death in the western world. The prognosis is poor i.e. most individuals die within three years of diagnosis. Two major types of esophageal cancer exist: squamous carcinoma and adenocarcinomas [121, 209]. Chemotherapeutic treatment in patients with esophageal carcinoma often results in diverging responses, ranging from complete response to progression of the disease [121, 209, 210]. Chemosensitivity of tumor cells involves multiple factors that influence the response to different drugs. Theses factors includes genes involved in drug uptake, drug metabolism, DNA repair and apoptosis [211]. High telomerase activity levels have been suggested to be associated with resistance to drug-induced apoptotic cell death [126]. The objective of this study was to asses the relationship between telomerase activity and sensitivity to standard cytotoxic drugs in 10 human esophageal squamous cell carcinoma (ESCC) cell lines. Telomerase activity was measured using the TRAP-assay and drug sensitivity was measured by the Fluorometric Microculture Cytotoxicity Assay (FMCA). FMCA is based on measurement of fluorescein which is generated by hydrolysis of fluorescein diacetate by cells with intact plasma membranes [212].

All of the ten cell lines expressed telomerase activity to varying degrees, with overall high activity levels. Telomerase activity has been detected in 79-100% of esophageal tumors [123, 210, 213, 214]. The activity levels of telomerase in the ESCC cell lines in the present study were in general high compared to reference cell lines. It has previously been described that ESCC cell lines have extremely high telomerase activity levels [214] and that increased telomerase activity was associated with the progression of squamous esophageal carcinoma [121, 122]. Furthermore, Li et al. reported that telomerase activity correlated with nodal metastasis [122]. We determined the cell proliferation rates of the ten ESCC cell lines as a value of tumor aggressiveness. In the present study no correlation between telomerase activity and doubling time was found, this is in concordance with the results presented by Asai et al. [215]. Neither did the cell lines expressing the lowest telomerase activity correspond to cell lines derived from well differentiated tumors. Our data should however be interpreted with caution since the in vitro cell line system not might be representative of the clinical situation. Discrepancy between in vitro and in vivo systems might be the result of selection under cell culture conditions.

The transcription factor c-Myc is a known activator of hTERT transcription and hence telomerase activity [74, 75, 77]. Four of the cell lines used in the present work have previously been described to harbour amplification of the c-Myc oncogene [216]. Interestingly, we did not find a correlation be-
tween high telomerase activity and amplification of c-Myc. Furthermore, eight of the ESCC cell lines have been found to harbour p53 mutations [217]. Several studies have shown that wt p53 down regulates telomerase activity in a variety of cell lines and cells with mutations in the p53 gene have increased telomerase activity levels [80-82, 84, 190, 218]. Furthermore, telomerase activity has been shown to be significantly associated with p53 mutations in colorectal carcinomas and in non-small cell lung carcinoma [191, 219]. We did however not find a correlation between p53 mutations and telomerase activity levels in the investigated ESCC cell lines. It is possibly that the telomerase activity levels in the cell lines not correspond to the telomerase activity levels in the primary tumors, hence the lack of correlation with p53 status. However, Sood et al. observed that there were no differences in telomerase positivity between tumors with wt p53 and tumors with mutations within critical sites of p53 [220]. Furthermore, in a study by Roos et al. high telomerase activity was significantly associated with p53 protein accumulation, but not with p53 gene mutation [120]. Furthermore, Lin et al. were unable to confirm that p53 is a repressor of hTERT under physiological conditions [79]. Thus at present diverging results concerning the correlation with telomerase and p53 status exist.

The 20 cytotoxic drugs investigated in the present study included the key drugs used in the clinic for treatment of squamous cell oesophageal carcinoma i.e. cisplatin and 5-fluorouracil [209]. No correlation could be found between the basal level of telomerase activity and sensitivity to cytotoxic drugs, including cisplatin (p=0.9) and 5-fluorouracil (p=0.8). Interestingly, Asai et al. reported that cells with high telomerase activity was more sensitive to cisplatin [215]. But, in concordance with our data they observed no correlation with sensitivity towards 5-fluorouracil and telomerase activity levels [215]. One of the drugs, mitoxantrone, analysed in our study had a p-value towards a statistical correlation with telomerase activity (p=0.054). However, with the significance level set at 0.05 one would expect 5% i.e. 1 out of 20 to falsely give a positive correlation [221]. If there is a true correlation between telomerase activity levels and sensitivity to mitoxantrone, needs to be evaluated in future studies.

To conclude, basal telomerase activity is not a key determinant of sensitivity to cytotoxic drugs in ESCC cell lines. However, our data does not exclude a role for telomerase activity in sensitivity to cytotoxic drugs since analyzes were performed on cell lines and this might not correspond to the clinical situation. Future studies should preferable include tumor samples and normal biopsies from patients.
Paper IV: Hypoxia mediated up-regulation of telomerase activity in neuroblastoma cell lines

Neuroblastoma is one of the most common solid tumors of childhood; it affects approximately 1 out of 8000 children with the highest incidence in the first five years of life. The tumors are derived from neural crest cells in the sympathetic nervous system. The malignant potential varies, some tumors spontaneously regress and others show resistance to aggressive chemotherapy [222]. It has been shown that telomerase activity correlates with poor prognosis in neuroblastoma [114-117]. Furthermore, the presence of full length hTERT mRNA is a strong independent prognostic factor in neuroblastoma [118]. Tumor hypoxia has been suggested to dedifferentiate neuroblastoma cells toward an immature and neural crest-like phenotype. Dedifferentiation of neuroblastoma cells caused by hypoxia could be one mechanism behind the progression of a tumor from being responsive to treatment to become refractive [223, 224]. Furthermore, hypoxia has been suggested to promote tumor progression by activating signaling pathways enabling cells to overcome the hostile microenvironment and to favor unrestricted growth [165-167]. The aim of the present investigation was to study the effect of hypoxia on telomerase activity in two neuroblastoma cell lines, SK-N-AS and SK-N-DZ, and possible mechanisms involved in the regulation.

In this study we demonstrate that both SK-N-AS and SK-N-DZ up-regulate telomerase activity in response to hypoxia (0.1% O2). Since telomerase heals the telomeres, confers genome stabilization and is implicated in conferring cellular resistance towards apoptosis [71, 128-130, 136, 149], up-regulation of telomerase activity levels might be one mechanism whereby cells adapt to a stressful environment such as hypoxia. Hypoxia has previously been demonstrated to increase telomerase activity levels for a variety of cells e.g. vascular smooth muscle cells, the placenta and cervical cancer [171-173, 188, 225].

To elucidate the mechanisms involved in the observed hypoxia mediated up-regulation of telomerase activity, hTERT mRNA expression in cells cultured under normoxic or hypoxic conditions were investigated. We observed an increased level of total hTERT mRNA in one of the neuroblastoma cell lines (SK-N-DZ) whereas the mRNA level was unaffected in SK-N-AS cells. The hTERT mRNA status have been tightly connected to telomerase activity in cancer cells [69, 71, 86, 115, 171, 172]. However, in SK-N-DZ cells telomerase activity was not significantly increased at 18h and 32h when the elevated levels of hTERT mRNA was detected. Interestingly, the hTERT mRNA increase, at least in partly, could be confirmed to be mediated by alternative splicing i.e. switching in splicing pattern in favor of the active full length variant. Anderson et al. recently reported a similar regulatory splicing mechanism in hypoxia exposed ovarian carcinoma cell lines al-
though they did not observe any changes in the level of total hTERT mRNA [173]. Seimiya et al. did not detect any changes of total hTERT mRNA levels in colon adenocarcinomas or ovarian carcinoma cell lines, although telomerase activity was up-regulated after hypoxia exposure. They suggested in their study that hypoxia up-regulated telomerase activity via MAPK signaling [188]. This result demonstrates that regulation of hTERT and telomerase activity upon hypoxia is multifactorially regulated and might be cell specific.

Insulin-like growth factor 2 (IGF-2) is a target gene of hypoxia-inducible factor 1 (HIF-1) and signals through the MAPK and PI3 kinase pathway [170, 226]. Both of these pathways have been shown to mediate increased telomerase activity levels through phosphorylation of hTERT [90, 92, 188]. In the present work hypoxia efficiently increases IGF-2 expression in both SK-N-AS and SK-N-DZ. Increased expression was observed in both cell lines during the time when telomerase activity was up-regulated, making the involvement of IGF-2 regulating pathways possible in the hypoxia-mediated regulation of telomerase. Interestingly, IGF-1 that activate the same pathway as IGF-2, was shown to stimulate telomerase activity through a dual mode of action in prostate cancer cells, including early rapid effects probably involving phosphorylation of hTERT and later effects involving up-regulation of hTERT expression [227]. To further test this IGF-2 was exogenously added to SK-N-AS cells under normoxic conditions followed by assessment of telomerase activity. We observed a significant increase in telomerase activity upon IGF-2 treatment, demonstrating that IGF-2 indeed has the capacity to regulate telomerase activity in neuroblastoma cells and to our knowledge this mechanism has not been reported earlier. Thus, indicating a possibility of the involvement of an IGF-2 autocrine loop in telomerase regulation under hypoxia. Future experiments using inhibitors of the PI3K, MAPK pathways or specific inhibitors of the receptor for IGF-2 will clarify the involvement of these factors in the hypoxia-mediated up-regulation of telomerase activity.

To conclude we demonstrate that telomerase activity is up-regulated in two neuroblastoma cell lines in response to exposure to hypoxia. The main regulatory mechanism of telomerase activity was not mediated by increased hTERT mRNA expression in either of the two cell lines.
General conclusions

With this thesis I provide new insights about the complexity in the regulation of telomerase activity in tumor cells regarding cytokine treatment and exposure to hypoxia. Furthermore, the relationship between telomerase activity levels and drug sensitivity was investigated.

TGF-β1 was demonstrated to mediate differential effects on telomerase activity in five ATC cell lines. We hypothesize that this could reflect a deregulation of TGF-β1 signaling in a more malignant genotype. Cells that harbored a \textit{p53} mutation responded by up-regulation of telomerase activity after TGF-β1 treatment, whereas cell lines displaying wt \textit{p53} responded by down-regulation of telomerase activity. Our data might indicate a possible connection between the \textit{p53} genotype of a cell and its response to cytokine treatment. Future studies depleting wt \textit{p53} will elucidate if deregulation of the TGF-β1 inhibitory effect occurs.

We suggest that one potential mechanism whereby IFNs sensitize MM cells to Fas-mediated apoptosis is by repressing \textit{hTERT} activity at the transcriptional level. This makes \textit{hTERT} an attractive target in aiming at lowering the threshold for apoptosis. Combination therapy with \textit{hTERT} inhibition and conventional therapy would therefore be interesting tumor treatments in the future.

Interestingly, basal telomerase activity was not found to be a key determinant of sensitivity to cytotoxic drugs in ESCC cell lines. Furthermore, we observed no correlation between \textit{c-Myc} amplification, \textit{p53} mutations and high telomerase activity levels in these cell lines. Our data does not exclude a role for telomerase activity in sensitivity to cytotoxic drugs since analyzes were performed on cell lines and this might not correspond to the clinical situation.

Finally, neuroblastoma cell lines were shown to up-regulate telomerase activity in response to hypoxic exposure. This finding might constitute an adaptive stress response of tumor cells exposed to hypoxia.
Sammanfattning på svenska

Telomeras, cellens biljett till evigt liv

Förvandlingen av en normal mänsklig cell till en cancercell är en process som sker i flera steg. Under den här processen får cancercellen egenskaper som leder till olika fördelar jämfört med en normal cell. En av dessa egenskaper är förmågan att dela sig oändligt många gånger, detta till skillnad mot normala celler som har en begränsad delningsförmåga. 90% av alla cancerceller blir odödliga med hjälp av enzymet telomeras. Enzymer är protein som behövs för all kemiska reaktioner i kroppen. Enzymer kan bygga upp eller bryta ner kroppens struktur. Den här avhandlingen handlar om enzymet telomeras och dess reglering.

I kroppens celler finns DNA, vår arvsmassa, som bestämmer vilka egenskaper vi får. DNAt har formen av en dubbel spiral och ändarna på spiralen består av långa bitar oviktigt (icke kodande) DNA. Dessa bitar som kallas telomerer skyddar det viktiga kodande DNAt. Varje gång en cell delar sig, försvinner en bit av telomererna. Efter 50 celldelningar har så mycket av telomererna försvunnit att ytterligare celldelningar hindras. Normala celler har således en begränsad förmåga att dela sig. Cancerceller undviker detta genom att ta hjälp av telomeras. Telomeras är ett enzym som finns inuti cellen och som kan bygga på nytt DNA på ändarna av telomererna.

Vi kan jämföra en celldelning med att åka ett varv i en karusell. Vid varje varv måste cellen ”betala” med en ”biljett” i form av en bit DNA och efter 50 varv har vanliga celler inga ”biljetter” kvar utan måste hoppa av karusellen. Cancerceller behöver också betala med en bit DNA, men de får även en ny bit DNA från telomeras efter varje varv. Detta leder till att cancercellen kan åka hur många varv på karusellen som helst och får därmed oändlig celldelningsförmåga. Det är viktigt att förstå vad det är som reglerar telomeras och dess aktivitet. Denna kunskap kan i slutändan användas för att behandla cancer, genom att stänga av telomeras och därmed hindra cancerceller från att dela sig obeegränsat.

TGF-β1 är ett ämne som spelar en viktig roll i regleringen av tillväxt i olika typer av celler. Dess roll i tumörutveckling är komplex då TGF-β1 både kan hämma och främja tumörtillväxt. I arbete I har vi studerat hur telomerasaktiviteten påverkas av TGF-β1 i fem cellinjer från sköldkörtelcancer. Våra resultat visar att TGF-β1 kan ger olika effekter på telomeras, då

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I arbete II har vi undersökt vilken effekt två interferoner (IFN), har på telomerasaktiviteten. IFN ärämnen som påverkar tillväxten negativt. Våra resultat visar att IFN minskar telomerasaktiviteten i den studerade cellinjen. Tidigare resultat har visat att celler som behandlas med IFN lättare svarar på olika dödssignaler, signaler som påverkar cellen att begå självmord. Vi tror att en av orsakerna till detta är att IFN hämmer telomeras, som i vanliga fall skyddar mot dessa dödssignaler. Vi visar i arbete II att även hämning av telomerasgenen ger ett starkare svar på dödssignaler. Detta gör att hämning av telomeras i kombination med vanlig kemoterapi kan leda till att cellerna lättare dör och skulle i framtiden kunna utnyttjas som cancerterapi.


Många tumörer har områden där syrenivåerna är mycket lägre än i de omkringliggande regionerna. Dessa områden i tumören är elakartade och svåra att behandla. I delarbete IV har vi undersökt hur låga syrenivåer påverkar telomeras i två cellinjer från neuroblastom (nervvävnadstumörer). Bågge cellinjerna svarar med en ökad aktivitet av telomeras efter att cellerna har utsatts för låga syrenivåer. De exakta mekanismerna bakom denna effekt är inte klar, men data tyder på att ämne som heter IGF2 kan vara inblandat. Vi visar att IGF2 ökar vid låga syrenivåer samt att IGF2 kan öka telomerasaktiviteten. Vi föreslår att celler ökar aktiviteten av telomeras vid låga syrenivåer för att skydda sig mot de skadliga effekterna av syrebrist.
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