The role of the cell attachment in the regulation of telomerase during keratinocyte differentiation

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THE ROLE OF CELL ATTACHMENT IN THE REGULATION OF TELOMERASE DURING KERATINOCYTE DIFFERENTIATION

Thesis submitted with regulations
for the degree of doctor of philosophy

by

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May 2010

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To my cousin Steve,
And to his favourite protein

Abstract

The catalytic subunit of telomerase TERT has non-canonical functions, which are independent of telomere elongation and sometimes telomerase activity. In skin keratinocytes, both *TERT* expression and anoikis are regulated by extracellular matrix molecules and their integrin receptors, but the effect of *TERT* deregulation on anoikis has not been previously investigated.

HaCaT cells expressing wild-type *TERT*, *TERT*-HA (non-canonical function only) Dn*TERT* (catalytically inactive TERT) and the empty vector (PURO) were created by retroviral transduction. HaCaT anoikis was monitored after disengagement of integrins following anchorage deprivation by nuclear staining and FACS. The expression of both *TERT* and *TERT*-HA, and to a lesser extent Dn*TERT*, significantly decreased the appearance of apoptotic HaCaT cells in suspension. This showed that *TERT* could mute anoikis even in the absence of telomere lengthening but that it may require telomerase activity for optimum effect. *TERT/TERT*-HA did not mute cisplatin-induced HaCaT apoptosis, suggesting that the effects are specific to anoikis.

Telomerase activity in the anchorage-deprived samples significantly decreased compared to the controls at time zero in all the samples. h*TERT* mRNA expression dropped in all groups in suspension compared to the zero time empty vector controls but the expression was still higher in those cells over expressing *TERT/TERT*-HA than the controls at zero time, suggesting a relationship between *TERT/TERT*-HA expression and resistance to anoikis. However, *TERC* expression increased in suspension similarly in all experimental groups. Therefore, these results suggest that the downregulation of *TERT* and not *TERC* contributes to the downregulation of telomerase in suspension, although the downregulation of ectopically expressed *TERT* also suggests that post-transcriptional mechanisms are involved. *TERT* splice variants were rare and not strikingly regulated in suspended keratinocytes. Western blot and FACS

analyses indicated that TERT does not act by affecting integrin expression or their density on the keratinocyte surface, suggesting a point of action downstream of integrins.

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Acknowledgements

I would first like to thank my supervisor Ken for his constant support, optimism and passion for this project that helped me pass from difficult times to exciting results and really love this subject.
I would also like to thank Prof. Fortune for giving me the opportunity to follow this research in such an inspriring environment, and the Support for Oral Science from the Institute of Dentistry, from whom this study has been founded.
Thanks to all the people who collaborated or helped a lot in this study, without whom I would have never gone so far: Prof. Petra Boukamp and her group for the validation of TERT-HA model and the opportunity of discussion and improvement of this project during the European Telomerase meeting; Dr. John Marshall, Dr. Linda Hammond and Dr. Gary Warnes for the support during FACS analysis; Dr. Teck Teh for the infinite patience he had while teaching me real-time PCR.

My "telomerase partners", old and new, in particular Caroline, Fay, Hara and Ann-Marie for all the help of course, but especially for all the fun times and numerous chats and alternative-organic-raw food breaks which I am really going to miss.
Thanks to all but especially to some really amazing CDOS people: Eleni for all the help, advice and simply for her beyond the limit kindness she showed me during these years; Simon and Hong, because not only did they incredibly survived to my loud laughs, but they were always there to help me out and share their lab experience with me; Alan and Steve, because they always dealt with my countless help requests and complaints with an ironic comment about the stupidity of the request (Alan) or by telling me off (Steve), but eventually they always surrendered!
Thanks to all my PhD friends for sharing with me the pains and the joys of this experience, in particular Cecilia para compartir muchas barras de chocolate conmigo, Emilios for being my favourite colleague of all times (exclusively when I need a buffer, right?), and Bilal, because his variety of stories during lunch time will be stuck in my mind forever!

During these years, I was also extremely lucky to meet or know better some gorgeous people, like Alessia, Francesca, Emanuela and Ryan. I simply cannot thank them enough for letting me be part of their life, sharing memorable experiences, and for being there every time I needed them. What would I have done without you?!
Also, I would like to thank all my friends from Italy for the emails, the unexpected phone calls, the daily chats, the holidays together and for everything else that never let me feel alone up here.

Lastly, I would love to thank my parents, my sister and all my family for the love they show me every day and for always supporting my passion for science that sent me far from home and my Nobel Prize ambitions during all these years of study. I'm not sure about getting the Prize soon, but I hope I'll make you proud anyway.

Author's declaration

I declare that I am the sole author of this thesis and that all the work presented herein was performed by myself, unless otherwise stated. This thesis has not been submitted for consideration for any other degree in this, or any other university.

Table of abbreviations

AKT	Thymoma viral proto-oncogene 1
ALT	Alternative lengthening of telomeres
amole	Attomole
ANOVA test	Analysis of variance test
AP-1	Transcription activator protein-1
AP-2	Activating enhancer-binding protein-2 alpha
APAF1	Apoptotic peptidase activating factor 1
Apo2L	Apoptosis-inducing ligand 2
ASV	Alternative splice variants
Bag-1	Bcl-2-associated athanogene
Bax	Bcl-2–associated X protein
Bcl-2	B-cell leukemia/lymphoma 2
Bcl-Xl	Bcl-2-like 1
BID	BH3 interacting domain death agonist
BLAST	Basic Local Alignment Search Tool
Bp	Base pairs
BSA	Bovine serum albumin
c-Abl	c-abl oncogene 1, receptor tyrosine kinase
cDNA	Complementary DNA
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CtBP	C-terminal binding protein
Cy5	Cyanine 5
DC	Dyskeratosis congenita
dH_2O	Distilled water
DISC	Death-inducing signaling complex
DKC1	Dyskeratosis congenita 1, dyskerin
DMBA	7,12-dimethylbenz[a]anthracene
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DnTERT	Dominant-negative TERT
dNTP	Deoxyribonucleotide triphosphate
E2F1	E2F transcription factor 1
E3	Ubiquitin ligase
EBV	Epstein-Barr virus
ECL	Enhanced Chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol tetraacetic acid
ERE	Estrogen-responsible element
ESD	Homo sapiens esterase D/formylglutathione hydrolase
EST (family member)	Mitogen-activated protein kinase kinase kinase 8-family member

FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
fos	FBJ osteosarcoma oncogene
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GRB2-SOS	Growth factor receptor-bound protein 2
GRHL2	Grainyhead-like 2
GSK3	Glycogen synthase kinase 3 beta
H_2O_2	Hydrogen peroxide
HA	Influenza virus haemagglutinin
Her2/*neu*	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog
HI	Heat inactivated
HIF-1	Hypoxia-inducible factor-1
hnRNP	heterogeneous nuclear ribonucleoprotein
HPRT1	Homo sapiens hypoxanthine phosphoribosyltransferase 1
HPV	Human papillomavirus
Hr/min/sec	Hour/minute/second
Hsp90	heat shock protein 90 kDa
IAP	Inhibitor of apoptosis
IC	Internal control
IFN-α	Interferon-α
JNK	c-Jun-NH2-kinase / mitogen-activated protein kinase 8
Kb	Kilobase
KBM	Keratinocyte basal medium
KGM	Keratinocyte growth medium
KIP1	cCyclin-dependent kinase inhibitor 1B
Mad1	MAX dimerization protein 1
MAP	Mitogen-activated protein
MAPK/ERK	Mitogen-activated protein kinase
max	Myc associated factor X
MDCK	Madin-Darby Canine Kidney Epithelial Cells
MEK	Mitogen-activated protein kinase kinase
MES	2-(N-morpholino)ethanesulfonic acid
MKRN1	Makorin RING finger protein 1
mRNA	Messenger RNA
MSH2	MutS homolog 2
mtDNA	Mitochondrial DNA
mTERT/hTERT	Murine TERT/ human TERT
Myc	v-myc myelocytomatosis viral oncogene homolog
NCBI	National Center for Biotechnology Information
NCI/NCII	Non canonical function of telomerase (type I and II)
NFX1	Nuclear transcription factor, X-box binding 1
NHEK	Normal human epidermal keratinocytes
nm	Nanometer
$p14^{ARF}$	INK4A alternative reading frame protein
$p15^{INK4b}$	Cyclin-dependent kinase inhibitor 2B
$P16^{INK4a}$	Cyclin-dependent kinase inhibitor 2A
$p21^{waf}$	Cyclin-dependent kinase inhibitor 1A

p23	Prostaglandin E synthase 3
P53	Tumor protein p53
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide
PI3K	Phosphotidyl Inositol-3Kinase
Pin2/TRF1	Telomeric repeat binding factor 1
PinX1	PIN2/TERF1 interacting, telomerase inhibitor 1
PKC	Protein kinase C
POLR2A	Homo sapiens polymerase (RNA) II (DNA directed) polypeptide A
polyHEMA	Polyhydroxyethylmethacrylate
POT1	Protection of telomeres 1
PP2A	Protein phosphatase 2A
qPCR	Quantitative PCR
Raf	v-raf-leukemia viral oncogene
Ras and HRAS	Harvey rat sarcoma virus oncogene
Rb	Retinoblastoma protein
RGD	Arginylglycylaspartic acid
RMRP	RNA component of mitochondrial RNA processing endoribonuclease
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RT	Retrotranscriptase
RTA	Relative telomerase activity
S100A8 and S100A9	S100 calcium binding protein A (8 and 9)
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SFK	Src-family kinases
SH2 domain	Src Homology 2 domain
shRNA	Small hairpin RNA
siRNA	Small interfering RNA
SCC	Squamous cell carcinomas
SV40	Simian vacuolating virus 40
SYBR green	N',N'-dimethyl-N-[4-[(E)-(3-methyl-1,3-benzothiazol-2-ylidene)methyl]-1-phenylquinolin-1-ium-2-yl]-N-propylpropane-1,3-diamine
TBE	Tris/Borate/EDTA buffer
TBS/TBS-T	Tris buffered saline (TBS-Tween 20
TEMED	Tetramethylethylenediamine
TERC	Telomerase RNA Component
TERT	Telomerase Reverse Transcriptase
TGF	Transforming growth factor
TPA	12-O-Tetradecanoylphorbol-13-acetate
TPP1	Tripeptidyl peptidase 1

TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
TRAP	Telomere Repeat Amplification Protocol
TRF	Telomere Restriction Fragment
TRF2	Telomere repeat binding factor 2
Tris	Tris(hydroxymethyl)aminomethane
UV	Ultraviolet
WaB	Wash buffer
WB	Western blot
WT1	Protein Wilms' tumor 1 tumor suppressor
YAP1	Homo sapiens Yes-associated protein 1

Chapter 1:

Introduction

Chapter 1:

Introduction

1.1 The telomere and telomerase: functions and regulation

1.1.1 The telomere

The word "telomere", derived from the two Greek nouns "telos", end, and "meros", part, was coined for the first time by Hermann Müller in 1938 to describe the extreme part of the chromosome which showed a certain level of protection from chromosomal damage induced by X-rays in *Drosophila* (Chuarie 2006).

Later on, the "end of replication problem" aroused new interest about the extremity of chromosomes, where DNA polymerase seemed unable to complete the replication of the linear DNA molecules because of the lack of a DNA template, according to the independent thoughts of Alexei Olovnikov in 1971 and James D. Watson the following year (Olovnikov 1996).

In the majority of eukaryotic organisms, such the protozoan *Tetrahymena thermophila* where telomere sequence was first indentified by the Nobel Prize winner Elizabeth Blackburn in 1978, and fungi, insects, plants and vertebrates, telomere structure is well conserved in the similar composition of short DNA repeats with a sequence of $(d[T/A]_{1-4}dG_{1-8})_n$ and a G-rich strand overhanging at the 3' end. The length of the telomere

repeat sequence can vary from few base pairs in yeast to the very long murine telomeres (Louis and Vershinin 2005).

In humans, telomeres typically consist of a tandem repeating base sequence (TTAGGG) between 3 to 20 kb in length, varying with cell type, development and individuals, and decreasing during cell divisions both *in vivo* and *in vitro*. In skin keratinocytes, for example, mean telomere length varies from approximately 10 kb of samples from infant foreskin, to approximately 6-7 kb after 30 cell divisions, when the cells started to senesce. Keratinocytes from adult foreskin or trunk instead show a much more heterogeneous length, from 5 to 10 kb (Krunic, Moshir et al. 2009).

The heterogeneity of telomere fragment length, the continuous erosion of this length during cell division, and the different response to telomere shortening in different types of cells can be explained by understanding the role of the telomere in cells, and what the difference in its length means in term of cell survival and behaviour.

A first insight into the role of the telomere was provided in the early 1970s by Olovnikov, who first proposed a correlation between the end replication problem and the Hayflick limit, suggesting a progressive shortening at the end of the chromosome at each DNA replication which eventually leads to cell senescence. The region of DNA where this shortening was supposed to happen was indeed the telomere, as later on proved by Harley in 1990 with studies of the telomere length in cultured human fibroblasts (Harley, Futcher et al. 1990).

The main (canonical) function of telomeres has been widely studied since then. Telomeres ensure that the end of the chromosome is not interpreted by the cell as a DNA double-strand break leading to chromosomal instability and cell cycle arrest. The key role of the extremity of the chromosome must be therefore accurately regulated, so that only cells which are supposed to be in a continuous proliferative state, such as stem cells or cells in the germ line, will avoid telomere shortening and cell death. In these cells telomeres are kept at a certain

length by two different mechanisms discussed below. On the contrary, somatic cells need final senescence to keep the correct homeostasis of the tissue they belong to, and the whole organism (Blackburn 2001). Senescence can be also seen as a form of tumour suppression, since different studies *in vivo* and *in vitro* have detected the presence of senescence cells in the premalignant lesions, but not in the cancer cells. If also the functions of p16/Rb or p53 is impaired, then the premalignant cells will progress to malignancy (Campisi 2005).

Protection of the telomere is achieved by the involvement of a complex of six proteins, named shelterin, and a variety of supplementary proteins which directly or indirectly binds the telomeric DNA sequence and, together with a higher level structure called "t loop", they provide a reversible capping of the telomere. Capping is generally temporally lost when the structure of the telomere needs to be accessible for the replicating machinery to proceed (Blackburn 2001).

1.1.2 Telomere lengthening: ALT and telomerase

Lengthening of the telomere does not occur just in the germ line and stem cells, but also in cancer cells, which manage to escape the proliferative block due to telomere shortening in two main ways.
The majority of cancer cells maintain a certain telomere length by reactivation of the enzyme telomerase, which is able to extend the telomeric repeats and overcome the loss of those during cell divisions (Blackburn 2001). The action of this enzyme will be analyzed in detail in the following chapters.

However, a smaller subset of tumours does not show detectable amount of telomerase and therefore utilizes an alternative lengthening of telomeres (ALT) system to overcome Hayflick limit (Bryan, Englezou et al. 1995; Reddel 2003).

This process can proceed in two different ways, which explains the typical presence of the highly heterogeneous telomere lengths in cells performing ALT (Bryan and Reddel 1997). In the first case there is still a small portion of telomeric DNA in the chromosome. This chromosome with short telomeres recombines with a complementary telomeric sequence in another chromosome where telomeres are still long (Dunham, Neumann et al. 2000). This long telomeric sequence can act as a primer for the further synthesis of the leading strand of the shorter telomere. Finally, the lagging strand will be duplicated as well leading lengthening of the previously short telomere.

In case the chromosome has already lost all the telomeric and some subtelomeric DNA during cell divisions, a TTAGGG repeat present more distantly will recombine to a chromosome with longer telomeres, and telomeric elongation will proceed from that point. However, the subtelomeric part previously lost will not be regained, leading to a loss of genomic material (Dunham, Neumann et al. 2000).

The ALT mechanism has been shown in a small subset of cells derived from tumours, such as glioblastoma, osteosarcoma, breast carcinoma, and adrenocortical carcinoma. ALT also acts in approximately 28% of immortalized cell lines *in vitro* (Bryan, Englezou et al. 1997).

1.1.3 Telomerase

In the majority of cases, telomere lengthening is carried out by a large ribonucleoprotein complex called telomerase. The enzyme was first discovered in the ciliated protozoan T*etrahymena thermophila* (Greider and Blackburn 1985), where it was hypothesized to act with a terminal transferase action in its short minichromosomes.

The human telomerase complex (figure 1) includes a 451-nucleotide RNA molecule, *TERC* (Telomerase RNA Component, also known as *hTR*, *TRC3*, *SCARNA19*), which serves as a template for addition of TTAGGG repeats, a catalytic component, TERT (Telomerase Reverse Transcriptase, also known as EST2, TCS1, TRT), and numerous other proteins (Collins and Mitchell 2002; Keith, Bilsland et al. 2002).

Human *TERC* is localized on human chromosome 3q26.3 (Soder, Hoare et al. 1997). In mouse and human, *TERC* is a transcribed by RNA polymerase II, as demonstrated by the presence of both TATA and CAAT boxes in the upstream flanking regions (Hinkley, Blasco et al. 1998), whereas in ciliates it is a product of RNA polymerase III (Greider and Blackburn 1989). The 5'-end of the template region, longer in the human than the murine *TERC*, is hypothesized to affect the processivity of the enzyme, allowing a correct interaction of the DNA substrate with the telomerase complex (Hinkley, Blasco et al. 1998).

The group of proteins interacting with the telomere and the two main components of telomerase *TERC* and TERT is quite composite, formed by the six-protein "shelterin" complex and probably several unidentified proteins. This highly multifunctional complex of proteins which bind double strand or single strand telomeric DNA, or establish protein-protein interactions, intervenes in maintaining telomere length, telomere capping by formation of the t-loop, and in promoting DNA repair (De Boeck, Forsyth et al. 2009) .

TERT is the catalytic core of telomerase. The structure of the macromolecule has been investigated with comparative studies, which show a general pattern where binding with *TERC* is possible by the RT (retrotranscriptase) domain and the N-terminal domain, and DNA binding is promoted by the both C and N-terminal domains. Interaction with other proteins of the telomerase complex is instead carried out in other regions of the TERT macromolecule (Podlevsky, Bley et al. 2008; De Boeck, Forsyth et al. 2009).

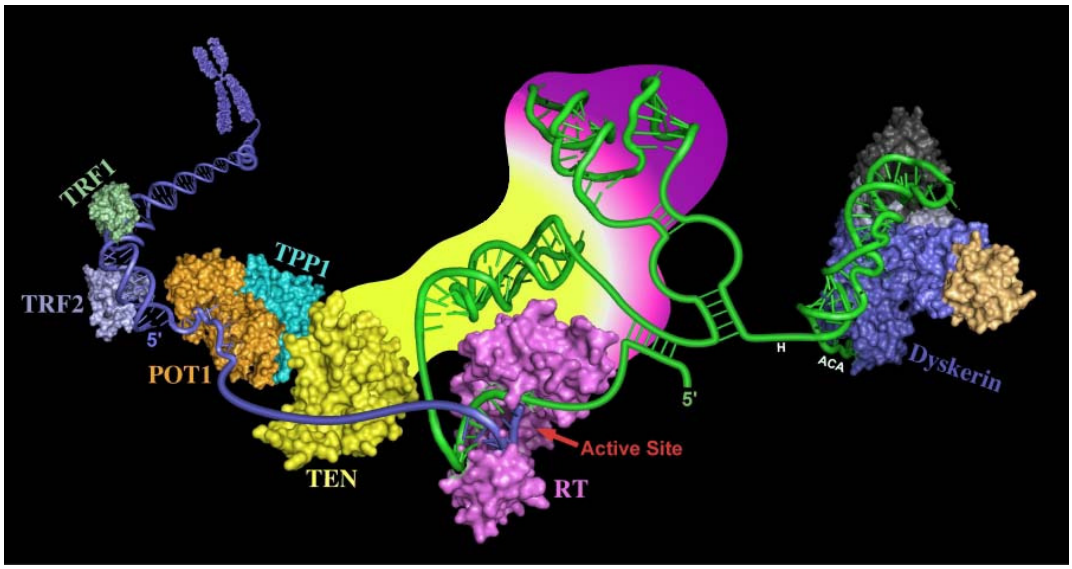

Figure 1 : Structure of human telomerase

Human telomerase is composed of the reverse transcriptase hTERT (yellow and pink, at the centre), and the RNA component *TERC* (or hTR, green). Proteins forming shelterin bind at the N-terminal domain of TERT (TEN, in yellow). Some of these proteins directly bind the telomeric DNA, such as POT1, which binds single-strand telomeric DNA, and TRF1 and TRF2, which bind double-strand telomeric DNA. The other three proteins involved in the shelterin complex, among which TPP1 shown here, bind to the first set of proteins. In addition, the complex formed by the protein dyskerin, which is involved in the binding to *TERC* by the binding to the H/ACA consensus sequence, is shown on the right (figure modified from (Podlevsky, Bley et al. 2008)).

1.1.4 Functions of telomerase

1.1.4.1 Canonical functions of telomerase

Telomerase can be detected at high levels in germ line cells, where the enzyme is always active and serves its canonical function of lengthening chromosome ends, while in most somatic cells telomerase activity is almost undetectable. In somatic cells, the progressive shortening of telomeres leads to instability of the shelterin complex and DNA damage signalling (de Lange 2005). Forced expression of *hTERT* results in reconstitution of functional telomerase activity in normal aging human cells (Weinrich, Pruzan et al. 1997) and *in vivo* this activity leads to elongation of telomeric DNA and to extension of cellular replicative life span (Bodnar, Ouellette et al. 1998). Other evidence for a possible role in cellular homeostasis of telomerase *in vivo* comes from the study of *TERC* deficient mice (*TERC*-/- mice). *TERC*-/- mice show progressive telomere shortening with each successive generation. This is associated with age-related genetic instability, reduced wound healing, deficiency of the reproductive and haematopoietic systems, and increased incidence of spontaneous malignancies (Blasco, Lee et al. 1997; Lee, Blasco et al. 1998; Rudolph, Chang et al. 1999). These results prove the need for both the RNA and catalytic subunit of telomerase in the execution of its canonical function of telomere elongation, but the presence of *TERC* does not seem to be required for the neoplastic transformation of mouse embryo fibroblasts *in vitro* (Blasco, Lee et al. 1997) or cancer formation *in vivo* (Greenberg, Chin et al. 1999) but the tumour yield is reduced (Gonzalez-Suarez, Samper et al. 2001) and the tumours show less propensity to metastasise (Chang, Khoo et al. 2003). Short telomeres have also been shown to suppress cancer by the induction of senescence as mediated by p53 (Feldser and Greider 2007).

Finally, it has also been shown that *TERC* associates with the nuclear protein dyskerin through the H/ACA consensus sequence present in both

TERC and in the class of small nucleolar RNAs (snoRNAs) processed by dyskerin (Mitchell, Wood et al. 1999) in Dyskeratosis congenita (DC). DC is a rare inherited bone marrow failure syndrome exhibiting clinical and genetic heterogeneity, such as abnormalities of the skin, fingernails, and tongue. X-linked recessive, autosomal dominant and autosomal recessive forms are recognized and studied (Dokal and Vulliamy 2003; Kirwan and Dokal 2008). X-linked DC involves a mutation in *DKC1*, the gene encoding dyskerin, reduced levels of *TERC* and decrease in telomere lengths (Mitchell, Wood et al. 1999), presenting effects which are very similar to pathogenic *TERC*-/- mice (Blasco, Lee et al. 1997; Kipling and Faragher 1999). It has been argued that telomerase effects are secondary to the DC phenotype rather than causal (Shay and Wright 1999). However, definitive evidence that telomere dysfunction was causal came from studies of autosomal dominant DC. This form of the disease mapped to chromosome 3q (Vulliamy, Marrone et al. 2001), the same chromosome arm as *TERC* (Soder, Hoare et al. 1997), and mutations in the *TERC* gene were also found (Vulliamy, Marrone et al. 2001).

1.1.4.2 Non canonical functions of telomerase

Telomere lengthening may therefore be defined as the canonical function of the telomerase. The catalytic subunit TERT, however, has also been investigated for "non-canonical" functions (NC) which are independent from telomere lengthening in several studies (figure 2). These functions can be divided in two groups, functions requiring the presence of the RNA component *TERC*, and therefore telomerase activity (NC I), and functions which do not have this requirement (NC II) (Parkinson, Fitchett et al. 2008).

- **Non-canonical functions of telomerase that still require enzyme activity (NC I)**

Non-canonical functions of telomerase, that require the presence of *TERC* but are independent of telomere lengthening (NC I), have been investigated firstly by studies in mice, and subsequently *in vitro*.

The very long mouse telomeres, reaching up to 150 kb (Kipling and Cooke 1990) and therefore the absence of critical telomere shortening offered a good system to study the modulation of the two telomerase components in a lengthening-independent way.

Subsequent treatment of mice that are highly responsive to the initiating and promoting effects of 7,12-dimethylbenz[a]anthracene (DMBA) and 12-O-Tetradecanoylphorbol-13-acetate (TPA) respectively (referred as two-stage chemical tumourigenesis mouse model) leads to formation of papillomas, which are hyperplastic skin lesions showing activation of the proto-oncogene *ras* (Balmain, Ramsden et al. 1984). The majority of these papillomas and the carcinomas deriving from them induced by DMBA initiation possess a mutation in codon 61 of *ras* (Quintanilla, Brown et al. 1986).

Mice treated with the tumour promoting chemical TPA show a high level of telomerase activity in the skin papillomas. In particular, the enzymatic activity of telomerase was correlated with the progression and the genomic instability of the papillomas and couldn't be attributed to the increased proliferation of the cells only (Bednarek, Budunova et al. 1995). Similarly, mouse mammary carcinomas promoted by a *wnt-1* transgene show increased expression of *TERC* and a 10-fold higher telomerase activity than non-malignant tissues (Broccoli, Godley et al. 1996).

Furthermore, the necessity of *TERC* in the progression of carcinogenesis is shown by *TERC*-deficient first generation mice, which are more resistant to DMBA and TPA-induced papilloma formation. However, the

tumour suppressor phenotype is more evident in the later generations mice, where shorter telomeres are correlated with a near disappearance of papillomas and p53 activation (Gonzalez-Suarez, Samper et al. 2000), suggesting the importance of canonical and non-canonical functions of telomerase in the promotion and progression of skin neoplasias.

Moreover, Tert seems to play an important role for the formation of these papillomas independently from its canonical function of telomere lengthening, since mice treated with DMBA and TPA and overexpressing *mTERT*, show not only an increase in telomerase activity, but also in skin tumourigenesis and wound healing (Gonzalez-Suarez, Samper et al. 2001). These effects are not seen when the same mice are deprived of *TERC*, demonstrating the requirement for both the telomerase subunits in this mouse cancer model (Cayuela, Flores et al. 2005).

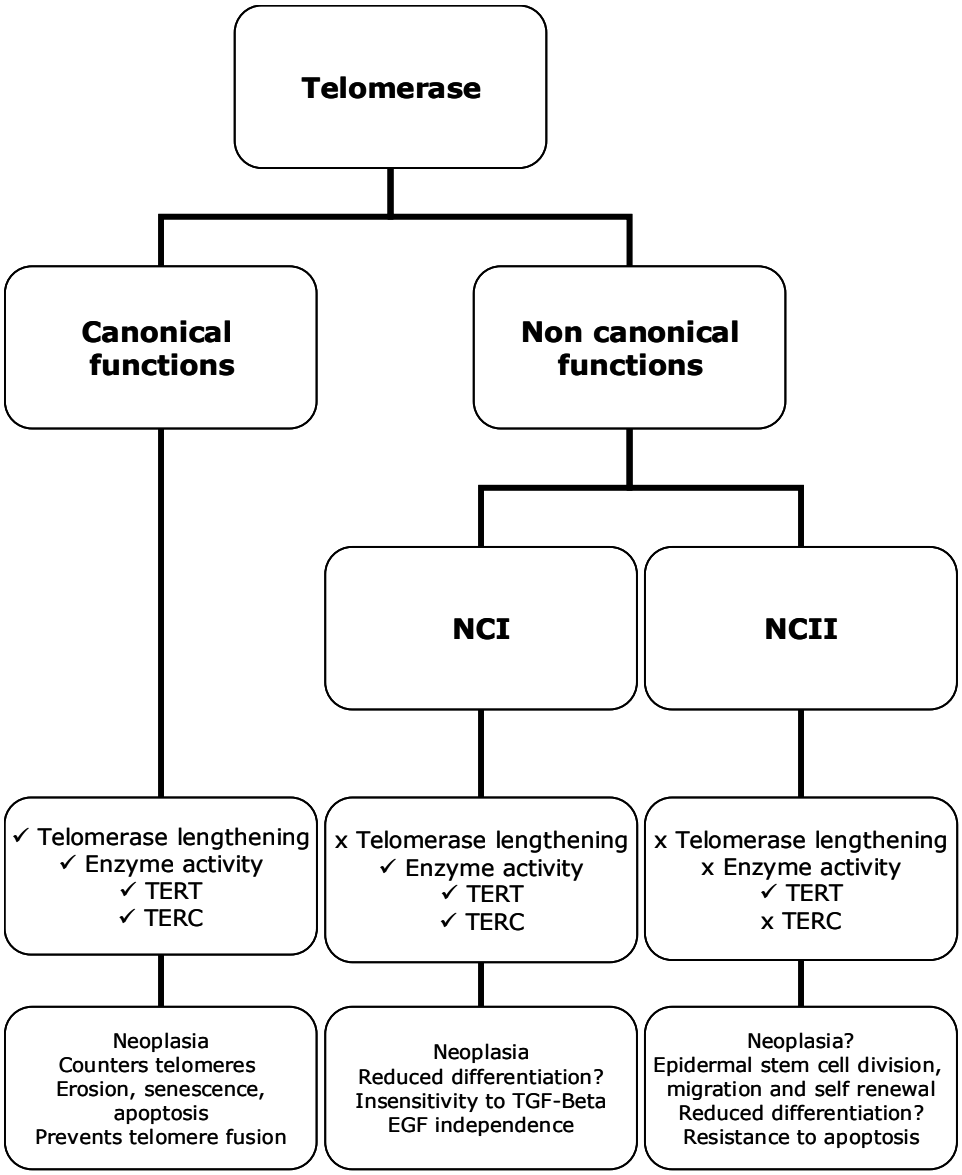

Figure 2: Canonical and non-canonical functions of telomerase

In the scheme, the functions of the enzyme are listed together with the components required for those functions. Also, the phenotypes which were shown in the literature to be correlated with each function are shown. As shown, both non-canonical functions are independent from telomerase elongation, but can be subdivided in functions requiring the presence of *TERC* (NC I) or functions independent from *TERC* (NC II).

Similarly, the ectopic expression of m*TERT* in DMBA-treated mice is associated with spontaneous development of mammary carcinogenesis, especially in late generation females (Artandi, Alson et al. 2002).

In vitro, ectopic expression of *mTERT* has been associated with *TERC*-dependent increased colony formation and immortalization of mouse embryo fibroblasts and with the insensitivity to TGF-β (Geserick, Tejera et al. 2006), confirming previous studies showing a reduced requirement for TGF-β in the growth of mammary epithelial cells (Stampfer, Garbe et al. 2001).

Ectopic *hTERT* expression has also been correlated with enhanced DNA repair capacity in normal human oral fibroblasts (Shin, Kang et al. 2004) and with immortalization of normal human keratinocytes, (Dickson, Hahn et al. 2000) and mammary epithelial cells (Smith, Coller et al. 2003) after loss of $p16^{INK4a}$. Furthemore, overexpression of *hTERT* into the latter cell lines promotes the expression of fibroblast and epidermal growth factor to stimulate cell proliferation (Smith, Coller et al. 2003).

Down regulation of *hTERT* with shRNA in different cancer lines has shown an association with downregulation at the transcription level of cyclin D1 (Jagadeesh and Banerjee 2006). The transcription of this cyclin is regulated by the pathway regulated by Ras, Src, MEK, AKT, and ERK, suggesting a possible regulation of TERT at some point in this oncogenic signalling, as well as EGF (Fu, Wang et al. 2004). Therefore, expression of cyclin D1 appears to be dependent on the presence of *TERT* and telomerase activity. On the other hand, dependence on the presence of *TERC* for the expression of the cyclin has been recently shown by the use of a telomerase inhibitor, which prevents the binding between *TERC* and the telomere, and it is associated with downregulation of the expression of cyclin D1 (Tokcaer-Keskin, Dikmen et al. 2010).

Another line of evidence supporting a possible role of telomerase independent from telomere-lengthening derives from the genetic reconstruction of tumorigenic fibroblasts from their normal counterparts.

In this experiment mutant *Ras*, SV40 T antigen and *TERT* are required for tumorigenesis (Hahn, Counter et al. 1999), but when *Ras* and SV40 T antigen are introduced into fibroblasts maintaining their telomeres by ALT (Alternative Lengthening of Telomeres) few tumours result (Stewart, Hahn et al. 2002). The addition of TERT, however, restores tumorigenity (Stewart, Hahn et al. 2002).

Additional proof that the effect of TERT does not require telomerase lengthening comes from the use of TERT-HA *in vitro* (Counter, Hahn et al. 1998). TERT-HA is a C-terminal hemaglutinin (HA) tagged hTERT which is catalytically active but fails to elongate telomeres and to extend cellular life span, while the specificity and processivity for an oligonucleotide substrate is very similar to wild-type hTERT (Ouellette, Aisner et al. 1999). The ectopic expression of *TERT*-HA can convey a tumorigenic phenotype in normal human fibroblasts and in cells that maintain telomeres by a telomerase-independent mechanism (ALT, Alternative Lengthening of Telomeres)(Stewart, Hahn et al. 2002).

Also, recent findings show that TERT-HA is able to immortalize primary human foreskin keratinocytes, when telomerase is interacting with the HPV E7 oncoprotein (Liu, Dakic et al. 2009).

These results show that the presence of telomerase activity does not necessarily require telomere maintenance and supports a novel telomere elongation-independent role for telomerase.

Furthermore, the first discovery of the presence of TERT in normal human fibroblasts associated the presence of the enzyme with a delay in the entrance in senescence (Bodnar, Ouellette et al. 1998). This effect is not due to telomere elongation alone but also to protection of the telomere structure (Masutomi, Yu et al. 2003).

Since *TERT* expression is detected exclusively during the S phase of the fibroblast cell cycle, it has been proposed that the increase in DNA damage response may be due to modulation of chromatin by TERT due to

modification of the histones (Masutomi, Possemato et al. 2005). Normal human fibroblasts where TERT is downregulated are less able to repair the DNA, and show an increase in radiosensitivity and chromosomal disruptions (Masutomi, Possemato et al. 2005). However this proposed mechanism does not explain the insensitivity to TGF-β growth inhibition cited above, suggesting that there may be other non-canonical mechanisms of telomerase.

- **Non-canonical TERT functions that are independent of catalytic activity or *TERC* (NC II)**

In other experiments, evidence for a role of TERT in the absence of the RNA component *TERC* and in the absence of changes in telomere length have been observed in mice lacking for *TERC* (*TERC*-/-). In these mice, induction of *TERT* has been associated with a developmental transition in the hair follicle cycle. With ectopic induced *mTERT* overexpression, stem cells of the hair follicle bulb undergo proliferation leading to the anagen phase of hair (Sarin, Cheung et al. 2005); these were also associated with promotion of hair growth when mice were constitutively overexpressing *mTERT* in a *TERC-/-* background (Sarin, Cheung et al. 2005). These effects, therefore, support a function of TERT independent from the RNA component (Flores, Cayuela et al. 2005; Sarin, Cheung et al. 2005) and from the catalytic activity (reverse transcriptase function) of the enzyme (Choi, Southworth et al. 2008) in the promotion of stem cell self-renewal and functional maintenance without inducing neoplasia. It has also been suggested that regulation of *mTERT* in the anagen phase of the mouse hair cycle is mediated through regulation of Wnt signalling (Choi, Southworth et al. 2008). More recently, it has been shown that Tert directly associates with the BRG protein to enhance Wnt signalling and that the *Tert*-/- mice have developmental defects that are consistent with a physiological role for Tert in this pathway (Park et al Nature 2009).

TERT has also been associated with promotion of cell survival of cells independent of the presence of the RNA template and therefore telomere lengthening. A previous study using the *TERT*-HA model together with wild-type *TERT,* showed that *TERT* overexpression has in fact been correlated in mouse embryonic fibroblasts and motor neurons with an increased resistance to apoptosis initiated by staurosporine and NDMA (N-methyl-D-aspartic acid); neither the canonical function of the enzyme nor the catalytic activity of TERT was not required (Lee, Sung et al. 2008). Similar results were also seen in cortical neurons, where *TERT* overexpression has been associated with resistance to ischaemic brain injury and NMDA-Induced neurotoxicity (Kang, Choi et al. 2004). In both the cases, it was hypothesised that TERT may protect apoptosis by acting at mitochondrial level, but the exact mechanism has not been identified yet.

- **Telomerase and the mitochondria**

The interplay between telomerase, the mitochondria, apoptosis and cellular senescence has been investigated by numerous groups, but it is still one of the most discussed topics in the telomerase field. This is due to the variety of hypotheses and results that are continuously produced and not agreed upon by the whole scientific community.

One of the first studies that demonstrated a possible correlation between telomerase and the mitochondria showed that apoptosis induced by reactive oxygen species (ROS) was correlated with reduction of the mitochondrial transmembrane potential and telomerase shortening in HeLa cells; however, no decrease in telomerase activity was observed, suggesting a more direct interaction between the ROS and the telomere in the specific telomere shortening. On the other hand, if telomerase activity was inhibited, the cells became more sensitive to ROS-induced apoptosis, which was also shown to be independent from caspase-3 activation (Ren, Xia et al. 2001).

According to another study, also carried out with in HeLa cells, overexpression of *TERT* protects cells from apoptosis due to the DNA damaging agent etoposide. Etoposide is a DNA strand breakage agent that also involves release of cytochrome c from the mitochondria and most importantly the activation of caspase-3; this study has also hypothesized a protection by TERT at an early step of the apoptotic cascade, before release of the cytochrome c and its dependence on a functional RT domain in TERT protein (Zhang, Chan et al. 2003).

A further insight into the mechanism of ROS-induced apoptosis in *TERT*-transfected fibroblasts showed increased damage in the mitochondrial DNA (mtDNA) compared to the undamaged nuclear DNA in the affected cells (Santos, Hunakova et al. 2003), suggesting a more direct involvement of telomerase and the organelle.

A possible confirmation of the role of telomerase in the mitochondria biology was shown with the colocalization of TERT in the mitochondria, due to its N-terminal mitochondrial targeting sequence (Santos, Meyer et al. 2006). Recent evidence shows that TERT is indeed localized on the membrane of the organelle (Haendeler, Drose et al. 2009). The export of TERT from the nuclei to the cytoplasm through the nuclear pores is dependent on the presence of ROS. It involves phosphorylation of the protein by the Src kinase, and it has been correlated with an increase of apoptosis in transformed human embryonic kidney cells (Haendeler, Hoffmann et al. 2003). Recently, the same group showed that levels of mitochondrial TERT are downregulated by exogenous H_2O_2 in endothelial cells, and that Src kinase is also present in the mitochondria, therefore a similar regulation of TERT could be carried out in the nucleus and in the organelle (Buchner, Zschauer et al. 2010).

The presence of TERT in the mitochondria was therefore linked with increased levels of ROS-induced mtDNA damage, leading to the hypothesis that TERT could sensitize the cells to ROS-induced apoptosis (Santos, Meyer et al. 2004). For this function, which also extends to

etoposide-induced apoptosis, telomerase must be catalytically active (Santos, Meyer et al. 2006).

However, the role of telomerase in inducing apoptosis through the mitochondrial pathway is not unanimously accepted yet. Different studies had observed the opposite situation, since inhibition of *hTERT* by siRNA in HeLa cells induces the mitochondrial apoptotic pathway through the activation of Bax, after cisplatin treatment (Massard, Zermati et al. 2006), and similarly in a bladder cell line, together with induction of cytochrome c release into the cytosol (Park, Kim et al. 2008).

To confirm this, a more recent study showed that ablation of *TERT* by shRNA in endothelial cells also increased the rate of ROS produced by the mitochondria, therefore emphasizing the protective effect of the protein on this pathway of apoptosis. *In vivo,* heart mitochondria derived from second generation TERT-/- mice show an impaired respiration and lung fibroblasts from the same mice are less resistant to UVB radiations. Furthermore, the study shows that the protection by TERT against damage to the mtDNA is dependent on the RT activity of the enzyme (Haendeler, Drose et al. 2009). In these studies therefore, the role of telomerase is thought to be protecting cells from mitochondrial cell death.
Other studies support this theory: von Zglinicki's group (Ahmed et al JCS 2008) showed a reduction in the damage to the mitochondrial DNA after *TERT* overexpression, correlated with a decrease in the production of ROS and an increase of the transmembrane potential (Passos, Saretzki et al. 2007). Furthermore, expression of a mutant of TERT which cannot be exported from the nuclei leads to an increased level of ROS released from the mitochondria and, independently from this phenomenon, to an early cell senescence in normal fibroblast, and to nuclear DNA damage in primary fibroblast, In addition, even if the enzyme is catalytically active *in vitro*, telomerase fails in immortalize the fibroblasts (Kovalenko, Caron et al. 2010).

However, stress induced on the cell by ROS is still thought to be an essential cause of telomere shortening (Passos and von Zglinicki 2005) and to replicative senescence**,** which in turn is strictly linked with the nuclear export of TERT from the nuclei, since antioxidants can inhibit it (Haendeler, Drose et al. 2009).

Intracellular levels of ROS could also have a further importance in a particular form of cell death due to deprivation of the cells from the extracellular matrix, termed anoikis. For further details on this topic, see section 1.4.3.

1.1.5 Regulation of telomerase activity

As explained above, telomerase activity can be detected at very different levels amongst different tissues in the body, and therefore the regulation of the expression of all the components of the big holoenzyme must be coordinated and carried out within multiple stages. Up to date, the regulation of telomerase, especially of the catalytic component, has been found at transcriptional, post-transcriptional, and post-translational levels.

1.1.5.1 Regulation at transcriptional level

Gene amplification of *TERT* is the most evident regulation of the level of gene expression and therefore telomerase activity. *TERT* amplification has been detected in at least 30% of tumour cell lines and primary tumours (Zhang, Zheng et al. 2000). This amplification can be due to amplification of the chromosome 5 carrying *TERT* gene, subregions of the chromosome, or amplification of the single gene locus (Cong, Wright et al. 2002).

- **Activators of transcription**

Activation of the *TERT* promoter is one of the most common types of gene regulation, thanks to the many binding sites present in the DNA sequence (Cong, Wright et al. 2002). c-Myc is one of the most studied activators of transcription. This factor has been shown to activate *TERT* transcription and telomerase activity in human mammary epithelial cells and normal human diploid fibroblasts by dimerization with Max. (Wang, Xie et al. 1998). The regulation of *TERT* by c-Myc is complex and not fully understood yet, but *TERT* is a direct target of Myc since the activation is independent from the synthesis of the TERT protein (Cerni 2000; Kyo, Takakura et al. 2008).

Other binding sites in *TERT* promoter suggests regulation from different cellular transcription factors, such as Enhancer-binding Protein-2 (AP-2), Hypoxia-inducible factor-1 (HIF-1), and transcription activator protein (AP-1), which can also act as a transcription repressor (Kyo, Takakura et al. 2008).

The presence of an estrogen-responsive element (ERE) in the *TERT* promoter is associated with regulation of transcription by hormones, in particular estrogen, which promotes the nuclear accumulation of TERT in an AKT-dependent way (Kimura, Ohmichi et al. 2004). Progesterone instead activates *TERT* transcription through the MAP kinase pathway in the malignant cells containing receptor for this hormone (Wang, Kyo et al. 2000).

Also oncogenes can have a role in promoting *TERT* expression. The protein E6 of the human papilloma virus (HPV) can induce telomerase activity independently from Myc as shown by different studies (Kyo, Takakura et al. 2008), as well as Her2/*neu*, Raf and Ras when expressed

together with a protein of the EST family, in a MAP kinase-dependent way (Goueli and Janknecht 2004).

Recently, new regulators of telomerase activity have been found by mass spectrometry studies (Kang, Chen et al. 2009). This proteins are MutS homolog 2 (MSH2), heterogeneous nuclear ribonucleoprotein (hnRNP) D, hnRNP K and grainyhead-like 2 (GRHL2), and their expression is upregulated in parallel with *hTERT* in head and neck squamous cell carcinoma. MSH2 is a protein involved in the DNA repair machinery, and it is necessary for genetic stability, cell proliferation, and maintenance of the capping of the telomere. GRHL2 regulates the formation of the stratified epithelia, and the two ribonucleoproteins are transcriptional regulators that binds to the promoter of the target gene (Kang, Chen et al. 2009).

- **Repressors of transcription**

Genes involved in the repression of telomerase activity are mostly studied with a chromosomal transfer approach, where regions of a chromosome are transferred in a telomerase-positive cell lines, and the activity of the enzyme is detected afterwards, indicating if that transferred region contains a repressor of telomerase (Cong, Wright et al. 2002).

A quite predictable repressor of *TERT* transcription is Mad1, a protein that dimers with Max, and competes with the dimer and transcriptional activator Myc/Max for its binding with the DNA. The repression induced by Mad1 has also been correlated with a decreased acetylation of the histones at the *TERT* promoter (Xu, Popov et al. 2001).

The protein Wilms' tumour 1 tumour suppressor (WT1), which is essential in regulation of differentiation in the kidney and gonad cells, also acts as a repressor of *TERT* transcription (Oh, Song et al. 1999).

The tumour suppressor p53 also inhibits *TERT* transcription, but the mechanisms involved are not clarified yet. The repression could act either directly or indirectly on *TERT* promoter, and it may require association with Sp1, which is interestingly considered also a weak activator of *TERT* transcription (Cong, Wright et al. 2002).

Moreover, repression on *TERT* promoter can be carried out by the presence of the cytokine Transforming Growth Factor-β (TGF-β), even if different studies still debate on the nature (direct or indirect) of its interaction with *TERT* promoter (Kyo, Takakura et al. 2008).

Furthermore, in haemapoietic cells, interferon-α (IFN-α), an inhibitor of cellular proliferation, directly acts on *TERT* promoter to repress gene transcription (Xu, Erickson et al. 2000).

In particular, in head and neck squamous cell carcinoma, overexpression of p53, E2F1, and $p21^{waf}$ are associated with inhibition of telomerase activity (Ying, Randall et al. 2000). In this malignancy, E2F1 acts together with retinoblastoma protein (pRB) (Crowe and Nguyen 2001), which represses the transcription by modification of the chromatin and histone deacetylation (Magnaghi-Jaulin, Groisman et al. 1998).

- **Epigenetic regulation**

Apart from specific proteins acting like activators or repressors of transcription, modification at transcriptional level of a gene can be carried out also by epigenetic regulation, which is modification on the chromatin state, DNA methylation and histone de/acetylation.

Many studies have shown that *TERT* transcription, at least in some cell types, can be silenced by methylation of the DNA, consistent with the presence of clusters CpG sites in the promoter (Kyo, Takakura et al. 2008). Also, the recruitment of histone deacetylases by transcription

repressors such as Mad1 and Sp1 and use of an inhibitor of histone deacetylase showed that modification of the status of the histones around *TERT* promoter is a mechanism to regulate the transcription of the gene (Cong, Wright et al. 2002; Kyo, Takakura et al. 2008).

Recently, a new repressor of transcription has also been identified in the protein NFX1. One of the two isoforms of this protein, NFX1-123, coactivates *TERT* promoter if cotransfected with *c-Myc*, while the isoform NFX-91 is a target of ubiquitination in the presence of the HPV protein E6 (Gewin, Myers et al. 2004). The degradation through deubiquitination is carried out by the complex E6-AP, an E3 ubiquitin ligase which is the product of the interaction between the viral protein E6 and the cellular protein AP. Therefore, activation of *TERT* promoter by E6 is carried out by degradation of NFX1 (Gewin, Myers et al. 2004). A new study by the same group have shown that the repression of *TERT* transcription by NFX1 is due to its interaction with the histone deacetylase mSin3A at the level of the *TERT* promotorer (Xu, Luo et al. 2008).

The discovery of this repressor is in agreement with the recent observation that *TERT* activation is dependent on the *in vivo* tissue environment in keratinocytes. *In situ*-grown epidermis or in organotypic cultures activation of transcription of *hTERT* is active, while in the less favourable growth conditions in conventional surface cultures, *hTERT* is silenced through deacetylation of its promoter (Krunic, Moshir et al. 2009).

Furthermore, other evidence supports the presence of a still unknown repressor of telomerase present on chromosome 3p (Cuthbert, Bond et al. 1999) and this repressor may act on *hTERT* by regulating the status of chromatin and therefore inhibiting its transcription (Szutorisz, Lingner et al. 2003). The mechanisms involved in this repression are still under investigation, however it has been shown that the regulation does not involve modification of c-Myc activity (Ducrest, Amacker et al. 2001).

- **Regulation of *TERC***

Normal human undifferentiated cells express high amount of the RNA component of the telomerase, and the expression decreases to almost undetectable level in differentiated normal diploid cells (Yashima, Maitra et al. 1998); however, cancer cells show a reactivation of *TERC* expression (Avilion, Piatyszek et al. 1996), implying a regulation of the transcription of this component as well as of *TERT*. The half-life of *TERC* is the highest amongst all RNAs studied up to date, reaching up to 4 weeks in certain cancer cell lines; this half-life is thought to increase through the expression of TERT, without the transcription rate being affected (Yi, Tesmer et al. 1999).

Repression of *TERC* is mainly carried out by methylation of the promoter; however, this is not sufficient to completely block transcription in the cell lines maintaining telomeres by the ALT mechanism. In this case, *TERC* transcription is effectively blocked by hypermethylation of the promoter in more than 90% of its CpG sites (Hoare, Bryce et al. 2001).

In addition, various transcription factors are involved in the regulation of *TERC* promoter: NF-Y interacts with the conserved CCAAT sequence in *TERC*, promoting the transcription of the RNA; also pRb activates the promoter, and this effect is inhibited by mutating the CCAAT sequence, but the protein does not interact directly with the promoter (Zhao, Glasspool et al. 2000).

In addition, Sp1 and Sp3 also interact with different regions in the promoter and induce a dose-dependent activation and repression of the promoter, respectively (Zhao, Glasspool et al. 2000). Sp1 and Sp3 bind to the sites Sp1 in the *TERC* promoter, and their binding has been shown recently to be regulated by JNK (c-Jun-NH2-kinase) (Bilsland, Stevenson et al. 2006).

More recently, another activator of transcription has been identified in the adenoviral protein E1A, which does not bind directly to the promoter, but

to other transcriptional regulators, such as pRb. The mechanisms through which E1A activates *TERC* transcription are not clear yet, however E1A is thought to promote transcription by sequestering the corepressor CtBP, a protein that binds to the specific Sp1 sites in *TERC* promoter (Glasspool, Burns et al. 2005).

1.1.5.2 Regulation at post-transcriptional level: mRNA alternative splice variants

The human *TERT* gene is localized on the antisense strand of the short arm of chromosome 5 (5p15.33), as localized using a probe for fluorescence *in situ* hybridization (Bryce, Morrison et al. 2000). The gene is composed of 48881 bp (NCBI Reference Sequence: NG_009265.1), formed by 16 exons (from 62 to 1354 bp length) and 15 introns (from 104 to 8616 bp length) (Wick, Zubov et al. 1999).

The first exon contains the translation start codon, while the end codon is localized on exon 16, together with the 3'-untranslated region. The telomerase specific motif, or T motif, is localized on the exon 3, while the reverse transcriptase (RT) motifs are located on exon 4 (motifs 1 and 2), on exon 9 (motif B', also known as 4), exon 10 (motif C, or 5), exon 11 (motifs D, or 6, and E, or 7). Motif A, also known as motif 3, is the only motif located in two exons, 5 and 6 (Lingner, Hughes et al. 1997; Wick, Zubov et al. 1999).

In addition to the full-length mRNA transcript, multiple splice variants have been identified, including deletions of part of or whole exons, and insertions in the intronic regions (Saeboe-Larssen, Fossberg et al. 2006). The result is mostly premature termination of translation due to frame shifts. Two different alternative splice variants (ASV), designed α and β deletions, have been studied in further detail and cover a role of bigger

importance in the possible regulation of telomerase activity by alternative splicing (figure 3).

The α splice variant consists in an in-frame deletion of 36 bp, corresponding to 12 amino acids, in the reverse transcriptase motif A. This is due to the presence of an alternative splice acceptor site in exon 6. Due to the lack of this essential RT motif, the protein is inactive; it is also been shown to act as dominant negative because overexpression of this splice variant in cells negative for telomerase activity (where *TERC* is present but TERT is not detectable) cannot reconstitute telomeric activity, whereas overexpression of the product from the full length *hTERT* can (Colgin, Wilkinson et al. 2000). Also, exogenous overexpression of the α splice variant in cancer cells (where *hTERT* is already overexpressed) reduced the amount of telomerase activity and it was correlated with progressive shortening of the telomere and apoptosis (Colgin, Wilkinson et al. 2000; Yi, White et al. 2000)

The product of the α splice variant could also inhibit expression of telomerase activity in both telomerase-positive fibrosarcoma cells and in SV40-immortalized fibroblasts, addressing the cells to senescence or cell death, respectively (Colgin, Wilkinson et al. 2000). This observation suggests that this splice variant may be generated to cover more complex regulatory roles of cell function other than the inhibition of telomerase activity.

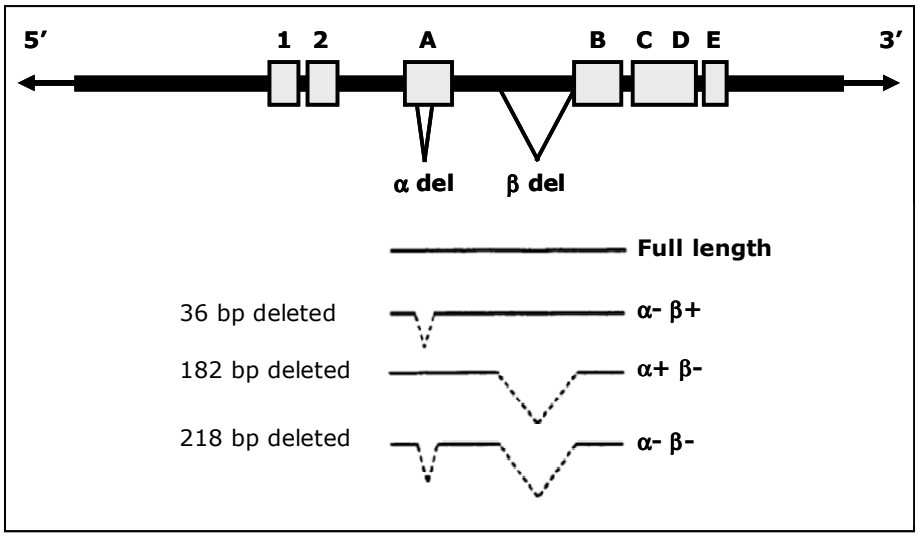

Figure 3: Diagram of the hTERT mRNA alternative splice variants

In the top of the diagram, the schematized structure of *hTERT* mRNA with the conserved domains is shown. The two major splice variants due to deleted regions in the mRNA are shown below. A splice variant (α-β+) is due to the deletion of 36 bases from the RT motif A. Therefore the product will be a shorter protein lacking of catalytical activity. The β splice variant (α+β-) is due to the deletion of 182 bases located before the motif B, therefore leading to a nonsense mutated product. Another nonsense product without RT activity is formed by the combination of the two deletion processes during the splicing (α-β-).

The β splice variant consists in a 182 bp deletion before the B motif which results in a nonsense mutation and the creation of a truncated, potentially unstable, protein. A possible role for this splice variant has not been identified yet, but its expression seems to vary between cell lines and may provide an important role in telomerase regulation.

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- **The problem of detecting *hTERT* ASV**

To try to investigate the role of the splice variants in telomerase regulation, different PCR methods have been utilized to estimate firstly the amount of ASVs in different cell types and then to correlate telomerase activity with the presence of the splice variants; however,

differences between the techniques utilized do not make the comparison between studies easy.

Various assays utilized a primer set flanking the regions α and β, therefore amplifying all the possible splicing combinations in the same polymerase reaction and detecting the different amount of products by further electrophoresis on agarose or polyacrylamide gels. This protocol can interfere with the nature of the PCR reaction (all the products will be amplified after enough number of cycles without reflecting the original mRNA amount), therefore it does not seem accurate enough for the measurement of five different products (full-length, α, β, and the combinations of these). One of these studies (Yi, Shay et al. 2001) showed little variation in the ratio of splice variant expression between the panel of normal, *in vitro* immortalized and cancer cell lines, but the amount of β deleted variant interestingly represented the majority of the transcript in all telomerase-positive cell lines (approximately 80-90% of the total amount of hTERT mRNA), compared to the full-length transcript (approximately 5%). Correlation with telomerase activity was found only with the full-length variant only (in the presence of the RNA component *TERC* which is necessary for the enzymatic activity). On the other hand, a more recent study showed an equal presence of all splice variants in telomerase-positive lung carcinoids, with a higher presence of full-length transcript, but higher expression of the β deleted splice variant was detected in telomerase-negative lung carcinoids. Therefore the correlation between telomeric activity and the presence of a splice variant was not excluded, where the β deleted splice variant could cover a role of telomerase negative regulation (Zaffaroni, Villa et al. 2005).

Other assays utilize real-time methods based on a dual-labelled probe system. In this method, primers are designed for each specific splice variant and the resulting products given by the amplification of the cDNA by the possible primers combinations are detected by a probe which is identical for all the splice variants. In the study by Ohyashiki *et al*., *hTERT* inactive splice variants are expressed in lower amounts in leukaemia patients with high telomerase activity, hypothesizing a

possible loss of telomerase regulation in the progression of the malignancy and confirming a possible role of splice variants as regulator of telomerase activity (Ohyashiki, Hisatomi et al. 2005). To confirm this observation, a more recent study using the same primers/probe approach showed that in non-neoplastic cell lines all *hTERT* splice variants were expressed in a lower amount than in adjacent lung carcinoma samples (Mavrogiannou, Strati et al. 2007), confirming the observations made previously by Zaffaroni et al. (Zaffaroni, Della Porta et al. 2002) in samples taken from a non-neoplastic breast tissue adjacent to the neoplastic area.

The difference in the outcome of the splice variant detection after the two different techniques is even more relevant if I consider the study by Mavrogiannou *et al*., where it was possible to detect a higher amount of full-length transcript (approximately 50% of the total amount of *hTERT* mRNA) compared to the β deleted splice variant (approximately 4%), in contrast to the aforementioned study by Yi et al. (Yi, Shay et al. 2001). This could be due the higher specificity of primers/probe assay, which permits the unique amplification of each splice variant, in contrast to the first method described where all the ASV are detected simultaneously.

Similarly to the primers/probe approach, a new technique using specific primer combinations and SYBR green real-time PCR has been developed, avoiding the use of probes and allowing the possibility of a melting curve analysis to check the specificity of the template. Also, the quantitation of the products is more specific because a reference gene analysis is included in the assay. Studies on melanoma cell lines using this technique showed a similar expression of all the full-length and splice variants in all the samples, where the full-length transcript and β deleted splice variant were relatively more expressed then the other variants compared to the total amount of *hTERT* mRNA (approximately 48% and 44% respectively) (Lincz, Mudge et al. 2008). Also, telomerase activity was directly correlated with the presence of the full-length variants and inversely correlated with the β deleted splice variant.

An interesting study by Cerezo *et al*. (Cerezo, Kalthoff et al. 2002) hypothesized a regulation of *hTERT* splice variant by TGF-β1 signalling specifically in skin keratinocytes. The expression of the ASV transcripts was measured in this study according to the first protocol mentioned in this chapter, which is the detection of all the different ASV after amplification with a general primer pair. HaCaT keratinocytes overexpressing *Myc* and treated with TGF-β1 showed a reversible decrease of full-length *hTERT* transcript and an increase of the β splice variant. However, the contribution of Myc in this modulation is not fully understood. In fact, entire HaCaT sheets (not overexpressing Myc) detached from the surface of a culture dishes, and resembling a normal tissue disruption and cell differentiation, also show a shift of expression from full-length *hTERT* to the inactive β splice variant. Furthermore, the use of organotypic cultures that resemble the opposite phenomenon of epidermal re-formation, similar to what happens *in vivo* showed that regeneration of the different layers of epidermis is accompanied with a shift from the β splice variant to the full-length variant of *hTERT*. The correlation with TGF-β1 was therefore suggested, since this factor is involved in numerous processes involved in wound healing, especially during proliferation and mobilization of keratinocytes during tissue re-epithelialisation (Barrientos, Stojadinovic et al. 2008).

In conclusion, the aforementioned study by Cerezo *et al.* gives a further insight of a possible role of the β splice variant in regulation of telomerase activity, and the utilization of an organotypic model seems appropriate for a partial correlation with the complex phenomenon of re-epithelialisation *in vivo*. However, a more standardized and accurate method of detecting the expression of *hTERT* splice variants is necessary before a possible elucidation of the role of the spliced transcript can be made.

The utilization of unique primer pairs for each splice variants, the analysis of each ASV separately with a real-time PCR protocol and the utilization of one or more housekeeping genes as a standard references should therefore be considered the method of choice for a reliable quantification.

1.1.5.3 Regulation at post-translational level

Although *TERT* regulation is greatly carried out at the transcriptional level, some studies have also demonstrated a further level of regulation after the synthesis of the protein. Post-translational regulators of telomerase activity have revealed different proteins interacting with TERT in a direct or indirect way (interaction mediated by the accessories protein in the holoenzyme), as shown by several studies both *in vivo* and *in vitro*.

The overexpression of the protein PinX1, which interacts with Pin2/TRF1, a member of the telomeric complex, has been associated with inhibition of telomerase activity and telomere shortening, and it has been observed that the regulation is due to the direct binding of this PinX1 to TERT (Zhou and Lu 2001).

In a similar way, the protein KIP, also known as KIP1, a protein mediating the interaction between DNA and DNA-dependent protein kinases, directly or indirectly interacts with TERT but the interaction on this case stimulates telomerase activity and telomeres lengthening both *in vivo* and *in vitro*, therefore counteracting somehow the role of PinX1 (Lee, Yu et al. 2004).

Also the correct assembly of TERT protein is necessary for the function of the enzyme. The molecular chaperones p23 and Hsp90 associate with the presence of telomerase activity and their inhibition also inhibits the telomerase activity (Holt, Aisner et al. 1999).

Furthermore, Ku, an heterodimer involved in DNA repair, contributes to telomerase activity also in the absence of *TERC*, therefore associating directly with the catalytic component (Chai, Ford et al. 2002).

Telomerase activity *in vitro* is also enhanced after phosphorylation of the serine/threonine sites present in TERT by the activated Akt kinase (Kang,

Kwon et al. 1999). On the other hand, telomerase activity is inhibited by the interaction with the tyrosine kinase c-Abl, which is activated by DNA double strand breaks (Kharbanda, Kumar et al. 2000) and, reversibly, by protein phosphatase 2A (PP2A) (Li, Zhao et al. 1997).

Furthermore, different protein kinase C (PKC) isoforms are involved in the cell specific activation of telomerase activity, as demonstrated by the use of specific inhibitors of these isoforms (Ku, Cheng et al. 1997).

In addition, phosphorylation may act as a regulatory mechanism in the translocation of hTERT from the nucleus to the cytoplasm, which is initiated after oxidative stress and it has been correlated with an apoptosis. Components of the Src kinase family, activated by ROS, can in fact phosphorylate hTERT and trigger the nuclear export of the macromolecule (Haendeler, Hoffmann et al. 2003).

Moreover, the identification of a Makorin RING finger protein 1 (MKRN1) showed that TERT can undergo ubiquitination, which is degradation of the protein by the proteasome complex. MKRN1 mediates the translocation of TERT to the proteasome (Kim, Park et al. 2005), suggesting that further ways of regulating telomerase can provide an even wider involvement of other cellular processes and pathways.

In particular, in skin keratinocytes, a further way of regulating telomerase expression due to differentiation has been hypothesized. During differentiation, calcium levels increase in these cells. A parallel increase in the two calcium binding proteins S100A8 and S100A9 is also shown during this process; therefore it has been hypothesized that when level of calcium increase during differentiation, the ion is detected by the complex of the two S100 proteins, leading to the temporary release of S100A8. This protein in turn will directly interact with telomerase and reduce the enzymatic activity; on the other hand, when calcium levels are low, S100A9 interacts with S100A8 and prevent its bound with telomerase. Therefore, S100A8 and S100A9 have an antagonistic role in the regulation of telomerase activity (Rosenberger, Thorey et al. 2007).

1.2 The epidermis

The epidermis is a stratified epithelium mainly composed of keratinocytes organized in multilayers (figure 4). The layer located closer to the basement membrane and to the derma is the basal layer (or stratum germinativum), composed of nucleated proliferating undifferentiated cells which continually migrate to the upper layers. Following an increasing gradient of differentiation, the following layer is the prickle cell layer (or stratum spinosum), also composed of nucleated and proliferating keratinocytes with characteristic spines preventing attacks from bacteria. Next is the granular layer (or stratum granulosum), where the cells lose the spines and the nuclei, and produce keratin. On the most external surface of the epidermis, the horny (or stratum corneum) layer is composed of anucleated dead cells, which will eventually shed and desquamate (Fuchs 1995).

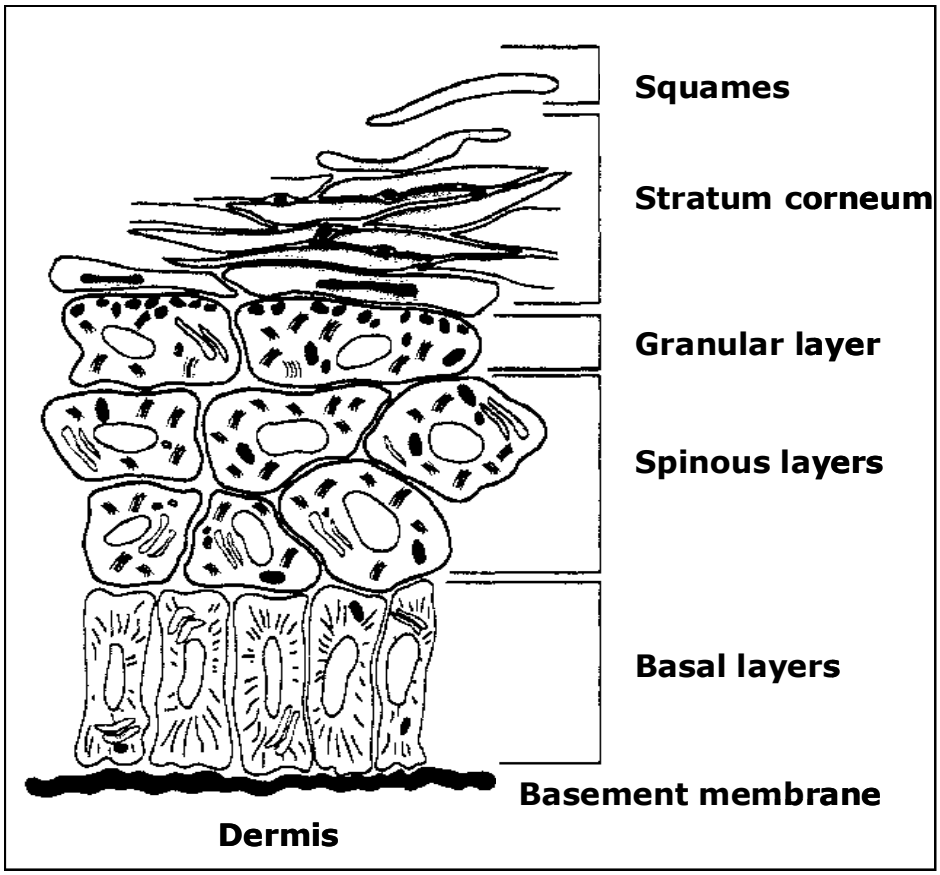

Figure 4: Structure of the epidermis

The epidermis is the outer surface of the skin, located above the derma, and it is composed by several layers of mostly keratinocytes migrating upwards during differentiation. The lower layer of the epidermis in contact with the dermis is the basal cell layers, where cells are continuously proliferating and moving to the next layer, the spinous layer. In this layer cells are shaped more irregularly and keep proliferating and migrating during skin regeneration. The next layer, granular layer, is characterized by an increase flattening of the keratinocytes, which also start accumulating keratin. Keratin production is maximised in the cells in the outer layer, the stratum corneum, where cells are dead, flattened and form overlapping layers to carry on the waterproof and barrier function of the skin. Regularly, dead cells are shed from the skin surface in the form or squames (figure from (Fuchs 1995)).

1.3. Human squamous cell carcinoma and its mouse model

1.3.1 Human squamous cell carcinoma

Head and neck cancer is the sixth most common cancer worldwide, and the majority of these cancers are squamous cell carcinomas (SCCs). Head and neck cancer can start in a different set of oral tissues, such as oral cavity, pharynx, larynx, salivary gland, tongue, thyroid, affecting the treatment and the severity of the disease (Hennessey, Westra et al. 2009). In the rest of the body, SCC can affect sun-exposed areas like part of the trunk or extremities, and genitals (Johnson, Rowe et al. 1992).

SCC affects mostly older men, and it has been associated directly or indirectly with many risk factors, such as UV exposure, immunosuppression, tobacco, alcohol use, albinism, genetic skin diseases, and chronic inflammation. Also infectious aetiologies, such as Epstein-Barr virus (EBV) and human papillomavirus (HPV), represent a high risk factor for SCC (Dubina and Goldenberg 2009; Hennessey, Westra et al. 2009; Chute and Stelow 2010).

In the skin, SCC can arise in the absence of precursor lesions or after the occurrence of actinic keratoses patches, which are premalignant lesions due to sun-exposure. In the most common *in situ* variant of SCC, the affected epidermis presents increased thickness, hyperkeratosis and pink patches of the epidermis but no invasion into the dermis (Johnson, Rowe et al. 1992) .

In the oral cavity, the most common premalignant lesions are leukoplakia (adherent white patches on the mucous membranes) and erythroplakia (red patch or lesion). The latter, even if less common, is almost always correlated with a future transformation into malignancy. These lesions histologically are formed of hyperplastic (excessive cell proliferation) or

dysplastic (abnormality in the maturation of the cells) cells (Jon and Albrecht 2003).

Histological evaluation has allowed investigators to distinguish different variants of SCC, such as verrucous carcinoma, papillary SCC, basaloid SCC, spindle cell carcinoma, adenosquamous carcinoma, acantholytic (adenoid/pseudovascular) SCC, and undifferentiated carcinoma, highlighting the difficulty in diagnosing each specific class and the need for finding a specific treatment for each one (Chute and Stelow 2010).

1.3.1.1 Molecular markers in human SCC

A wide variety of molecular markers are used to diagnose SCC in head and neck, among which are: epidermal growth factor receptor (EGFR), p53 status, cell cycle proteins such as cyclin D and $p16^{INK4a}$, transcription factors and oncogenes such as c-*Myc* and *Ras*, apoptotic proteins such as Bax and Bcl-2, presence of HPV, and telomerase activity (Nadal and Cardesa 2003; Gold and Kim 2009).

- **EGFR**

Overexpression of EGFR is found in more than 90% of head and neck SCC (Gold and Kim 2009) and it is associated to a poor prognosis, a positive nodal stage of the cancer and a lower survival in the patients. This receptor, when dimerizing with another subunit or with a receptor of the ErbB family like ErbB2, autophosphorylates and activates ERK1 and ERK2, part of the MAPK pathway, regulating in turn the growth, progression and malignancy of the tumour (Mahmoud, Mehmet et al. 2008).

- **p53**

Mutations in the tumour suppressor protein p53 are frequent in various human carcinomas. In head and neck SCC, mutations are found in 40% to 87% of tumours, depending on the sensitivity of the technique (Boyle, Hakim et al. 1993; Waridel, Estreicher et al. 1997). P53 mutations increase with the progression of the tumour, and can be found not only at the invasive stage of the malignancy, but also at non-invasive stages, and in normal tissues adjacent to malignant tissue, indicating that mutations in the tumour suppressor are early events in the development of SCC (Nees, Homann et al. 1993). However, not all studies agree that there is a correlation between p53 mutation and the tumour stage or clinical outcome (Gold and Kim 2009).

- **Cyclin D and $p16^{INK4a}$**

During tumour progression, the entry into the S phase of the cell cycle is essential for the progression and growth of the tumour. Cyclin D1 is one of the essential regulators of this transition, and the amplification of the gene for the cyclin is detected in 35-64% of laryngeal SCC (Papadimitrakopoulou, Izzo et al. 2001). The gene amplification is correlated with protein overexpression and with the progression of the disease. Cyclin D overexpression is found on mild and moderate dysplasias (Papadimitrakopoulou, Izzo et al. 2001), and in carcinomas at invasive stages (Jares, Fernandez et al. 1994; Papadimitrakopoulou, Izzo et al. 2001), associating with a lower survival of the patient. This suggests that cyclin D is already overexpressed at an early stage of the neoplastic transformation, and it increases during the progression of the malignancy. Cyclin D is inhibited by $p16^{INK4a}$ via inhibition of the phosphorylation of the protein pRb and blockage in the release of E2F. Different kinds of alterations on $p16^{INK4a}$ are found in nearly 70% of head and neck SCC, with some differences according to the kind of modification (such as loss of heterozygosity, point mutations, and deletions). Even if no correlation with the outcome of the malignancy has been observed, mutation on this key protein may confer an advantage to the tumour cells, such as increased growth rate (Hardisson 2003).

However, other studies showed a correlation between loss of the p16 and tumour progression. A retrospective study found alterations in the two tumour suppressor *p53* and $p16^{INK4a}$ in premalignant laryngeal lesions that later became neoplastic (Gallo, Santucci et al. 1997), and mutation, loss of heterozygosity or hypermethylation of $p16^{INK4a}$ and $p14^{ARF}$ (encoded by the same locus in the chromosome 9) were also found in samples of severe dysplasia (Kresty, Mallery et al. 2002) that subsequently progressed to SCC.

- **c-Myc and *RAS***

The role of the transcription factor c-Myc has often been studied in parallel with Ras. Mutations in *RAS* are seen in 35% of oral SCC in the Asiatic population (Saranath, Chang et al. 1991), but even if this is correlated with different causes, such as the effect of chewing betel quid or increased tobacco assumption compared to the Western population where *RAS* mutations are almost undetectable (Hardisson 2003), a reason for is still to be defined. However, since overexpression of *Ras* is found in the dysplastic mucosa as well as in the more advanced stages of the tumour (Wendell, Carol et al. 1994; Hardisson 2003) indicating that this event, similarly to the expression pattern of cyclin D, is also an early event in tumourigenity.

Also *c-MYC* amplification and the overexpression of the transcription factor is found at different levels in head and neck cancer, and, even if generally amplification of *c-MYC* leads to a worse outcome for the patient (Wang, Xue et al. 2010), the reason for the variance in the results is not fully comprehended yet. One possibility is, that *c-MYC* is part of an amplicon that includes the gene for focal adhesion kinase, FAK, and the *FAK* gene is amplified more often than *c-MYC* (Agochiya, Brunton et al. 1999) and so *FAK* and not *c-MYC* may account for the poor prognosis of the tumours. In support of this a recent analysis of several microarray studies of head and neck SCC revealed a link between FAK and its associated focal adhesion pathways and poor prognosis (Thurlow, Peña Murillo et al. 2010 (in press)).

- **Bcl-2 and Bax**

The protein family Bcl covers a mayor role in the apoptotic fate of the cells. B-cell leukemia/lymphoma 2 (Bcl-2), of the most important member of this family, covers a role as inhibitor of apoptosis which can be modulated by its heterodimerization with Bcl-2–associated X protein (Bax), a proapoptotic protein (Hardisson 2003). If the apoptotic signalling eventually starts, Bcl-2 will start the caspase cascade.

Overexpression of Bcl-2, but also Bax, and of proteins of the same family with analogue functions, is found in different amounts in samples from head and neck SCC, but even if this suggests that this protein family covers an important role in the apoptotic rate of the malignant cells, correlation with survival in the patients or with the stage of the cancer has not been unequivocally demonstrated (Hotz, Bosq et al. 1999).

- **HPV**

As mentioned before, HPV is viewed more and more as an etiological factor for a defined population of head and neck SCC, also shown by recent epidemiologic data (D'Souza, Kreimer et al. 2007), and it associates with a better prognosis compared to non-HPV SCC (Psyrri, Gouveris et al. 2009; Ha Linh, Andrew et al. 2010). The virus triggers the continuous expression of the two viral protein E6 and E7, which in turn repress the transcription of both p53 and pRb (Psyrri, Gouveris et al. 2009).

In particular, in high-risk HPV, E6 accelerates the rate of degradation of p53 by promoting the ubiquitin-dependent proteolysis in a ATP-dependent mechanism (Scheffner, Werness et al. 1990; Munger and Howley 2002). Similarly, the viral protein E7 from high-risk HP promotes the ubiquitin-dependent proteolysis of the tumour suppressor pRB (Munger, Werness et al. 1989; Boyer, Wazer et al. 1996; Munger and

Howley 2002) and inhibit $p16^{INK4a}$-induced cell cycle arrest by G1 block (Giarre, Caldeira et al. 2001).

- **Apoptosis**

In addition, the apoptotic index in SCC is interestingly elevated, and correlates with the progression of the disease from mild dysplasia to carcinoma. Also proliferation and angiogenesis progressively increase, but at a different rate, promoting the progression and invasion of the tumour even in presence of high apoptosis (Macluskey, Chandrachud et al. 2000). This is being shown also at a more molecular level by studies on proteins regulating apoptosis.

The anti-apoptotic protein Bag1 is downregulated in its nuclear localization in SCC compared with normal oral epithelium, while the amount of protein present in the cytoplasma does not vary in the malignancy. This may reflect a different localization of some of Bag-1 isoforms between nucleus and cytoplasm, and this may be correlated with the differentiation of the tumour (Hague, Packham et al. 2002).

Caspase-3 expression, is rare and basilar in normal epithelia, but nuclear DNA degradation is found in all the upper layers of the epidermis and probably necessary for the frequent turnover of the cells. However, true apoptosis, as diagnosed by caspase 3 activation, increases in oral squamous cell carcinoma and it is correlated with the differentiation stage of the tumour (Hague, Eveson et al. 2004).

- **Telomerase and its regulators**

Expression of *hTERT* mRNA and telomerase activity are both elevated in a variety of human cancers, as previously stated (Blackburn 2001). Telomerase activity is found on approximately 90% of head and neck SCC (Hohaus, Cavallo et al. 1996), and it is associated with the stage of the malignancy (Kim, Piatyszek et al. 1994; Kang, Chen et al. 2009; Strazisar, Mlakar et al. 2009), although increased in many dysplasias

(Mao, El-Naggar et al. 1996). However, telomerase activity can be found also on the mucosa adjacent to the neoplastic cells, showing a gradient of activity that is independent from the presence of dysplasia (Hohaus, Cavallo et al. 1996).

Interestingly, *hTERT* overexpression is associated with the overexpression of the regulators of telomerase activity MSH2, hnRNP D, hnRNP K and GRHL2, demonstrating the critical role of hTERT in SCC and the necessity for a fine regulation (Kang, Chen et al. 2009).

In addition, the expression of the gene coding for the calcium-binding protein S100A2 is up-regulated in approximately 80% SCC and parallels the up-regulation of *hTERT* (Strazisar, Mlakar et al. 2009), further emphasising the importance of the S100 protein family in the regulation of telomerase in squamous tissue (Rosenberger, Thorey et al. 2007), as mentioned above in section 1.1.5.3.

1.3.1.2 The mouse two-stage tumourigenesis system

As discussed in the previous sections, the formation and progression of squamous cell carcinoma covers a wide variety of proteins and cellular pathways, which are complex to identify. For this aim, different groups have studied a simpler mouse system, defined as mouse two-stage tumourigenesis system, where the tumour formation and progression can be analyzed more in detail and under controlled conditions, after administering the initiating carcinogen DMBA and the tumour promoter TPA. As already explained briefly in section 1.1.4.2, the first stage is the formation from normal skin keratinocytes of a papilloma, and the second stage is the progression of the papilloma into squamous cell carcinoma (Yuspa 1998).

The initiation stage triggered by DMBA consists of the formation of stable adducts of the drug with the DNA and it is therefore irreversible (Devanesan, RamaKrishna et al. 1993), while the promotion stage triggered by TPA, which activates protein kinase C (Kikkawa, Kishimoto et al. 1989), is reversible and it is not sufficient to initiate the papillomas. The irreversible nature of initiation for at least a year suggests that the initiation event takes place in the permanent stem cells of the epidermis and recent evidence has shown that these reside in the hair follicle (Argyris 1980; Brown, Strathdee et al. 1998; Jaks, Barker et al. 2008).

The initial formation of papillomas in mice after repeated TPA administration reflects a change in the keratinocytes, that stop differentiating and become hyperplastic (Yuspa 1998).

The most important feature, detected in more than 90% of tumours, is however the presence of the mutation in the *ras* oncogene (Quintanilla, Brown et al. 1986), which is followed by the overexpression of Cyclin D (Robles and Conti 1995).

Cultured mouse keratinocytes overexpressing an activated *ras* secrete TGFα, which is a ligand for EGFR (Lee, Punnonen et al. 1992). The overexpression of TGFα preceeds the overexpression of EGFR in the early stages of the tumour (Rubin Grandis, Tweardy et al. 1998), suggesting that both these events are necessary for the initial stages of the tumour promotion. EGFR, even if not required for the mitogenic response in *in vitro v-ras* transformed keratinocytes, is necessary for the growth of the papillomas in the two-stage tumourigenesis system (Dlugosz, Hansen et al. 1997).

The progression of the papillomas towards squamous cell carcinoma can happen spontaneously from a small subpopulation of high-risk papillomas, and it is associated with genetic alterations, such as trisomy of the chromosome 6 and 7 (carrying *ras*), and aneuplody, followed by progressive absence of differentiation markers (Aldaz, Conti et al. 1988; Yuspa 1998).

An important oncogene which is essential for the progression into carcinoma is *fos*, which works together with *ras* in skin carcinogenesis. The product of *fos* is crucial in the transcriptional regulation of many genes, since it can dimerize with jun to form the transcriptional factor AP-1 (Greenhalgh, Welty et al. 1990), which in turn targets several genes involved in cell proliferation, differentiation, and tumourigenesis (Saez, Rutberg et al. 1995).

High-risk papillomas also show loss of both TGF-β1 and TGF-β2, which correlates with hyperproliferation of the basal and suprabasal strata, respectively (Glick, Kulkarni et al. 1993).

Increased expression of the integrin α6β4 in the suprabasal strata can also be detected (Tennenbaum, Weiner et al. 1993), suggesting the importance covered by the interactions between the transforming cells and the extracellular matrix in malignant progression.

Furthermore, overexpression of a mouse homolog to the anti-apoptotic protein Bcl-2 was detected in the basal layer of papillomas and in most of the cells of the carcinomas (Rutberg, Lee et al. 1997).

Correlation between the speed of progression of papillomas to malignancy has also been correlated with loss of p53. However, the loss of this tumour suppressor does not correlate with the initial promotion stage (Kemp, Donehower et al. 1993). Also mutations or deregulation of the tumour suppressors *p16*^{INK4a} and *p15*^{INK4b} are associated with the progression of the malignancy, since these alterations are found only in the latest stage of mouse SCC, the spindle carcinoma (Linardopoulos, Street et al. 1995).

Interestingly, telomerase does not appear to affect the progression rate of papillomas to carcinomas but augments the formation of papillomas giving clues as to its mode of action (Gonzalez-Suarez, Samper et al. 2001) There is abundant evidence that papilloma development is

impeded by TGF-β (Cui, Fowlis et al. 1996) and that non-canonical functions of telomerase can render cells resistant to the anti-proliferative effects of TGF-β. However, I have not been able to show that *TERT* expression affects TGF-β sensitivity in either normal or HaCaT human keratinocytes (E.K. Parkinson, unpublished data).

1.4 Apoptosis and anoikis

1.4.1 Apoptosis

Apoptosis, also known as "programmed cell death", is an innate response of the cell essential for normal development, homeostasis and protection of the rest of the organism from potentially damaging agents (Ashkenazi 2002; Gewies 2004; Jin and El-Deiry 2005). A cell undergoing an apoptotic process presents an initial chromatin condensation and segregation, cytoplasm condensation and convolution of the cell membrane. Later on the surface of the cell will develop buds, originating apoptotic bodies which contain pieces of cytoplasm, and fragmented chromatin, and later on these cell fragments are phagocytosed.

Apoptosis is activated through two major signalling pathways, which create a complex network (figure 5) exhibiting some cross-talk. The extrinsic pathway depends on the stimulation of death receptors of the TNFR superfamily by a pro-apoptotic ligand, such as endogenous Apo2L/TRAIL (apoptosis-inducing ligand 2 or tumour necrosis factor-related apoptosis-inducing ligand), following the interaction receptor-ligand, a molecular complex known as DISC is being formed, and caspases 8 and 10 are activated. Caspases are proteolytic enzymes which degrade endonuclear, stromal and enzymatic proteins important for cell viability. Thus activated initiator caspases 8 and 10 activate downstream effector caspases 3, 6, and 7, leading to apoptosis (Ashkenazi 2002; Gewies 2004; Jin and El-Deiry 2005).

The intrinsic pathway involves mitochondria and the endoplasmic reticulum. In this pathway, the tumour-suppressor gene *p53* activates members of the pro-apoptotic Bcl-2 family, eventually leading to the release of cytochrome c and SMAC/DIABLO from the mitochondria. Cytochrome c binds APAF-1, forming an “apoptosome” that activates the initiator caspase 9. Caspase 9 activates downstream effector caspases,

including the same caspases 3, 6, and 7 present in the extrinsic pathway. SMAC/DIABLO, interacts with inhibitor of apoptosis (IAP) proteins, permitting the apoptotic process (Ashkenazi 2002; Gewies 2004; Jin and El-Deiry 2005).

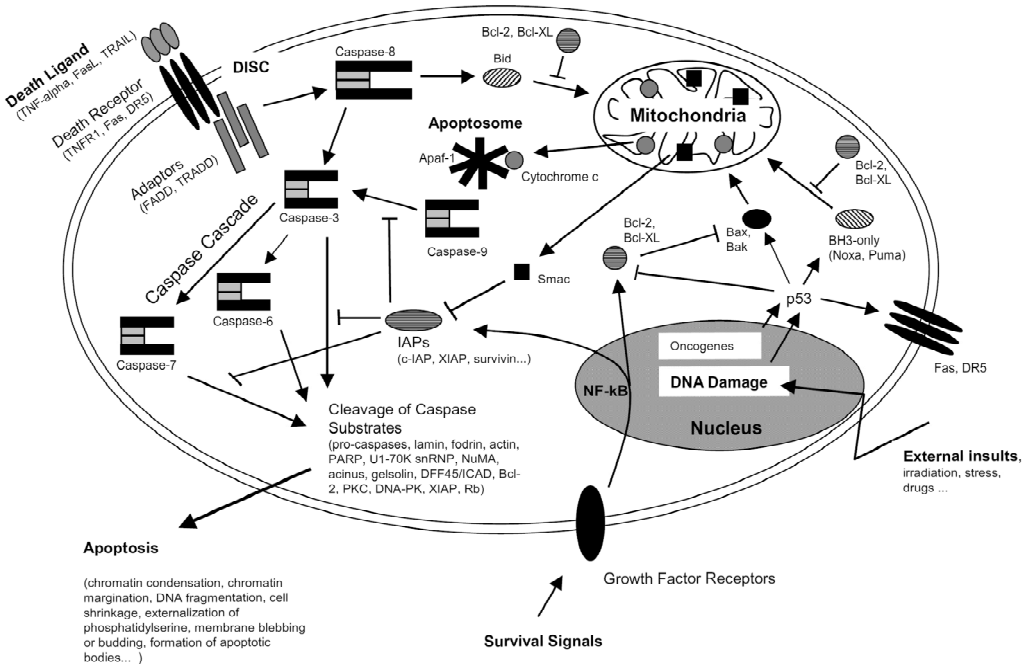

Figure 5: Schematic representation of some major apoptotic signalling pathways

The two major apoptosis signalling pathways, extrinsic (involving the death receptors) and intrinsic (involving the mitochondria) converge at the level of the effector caspases 3, 6, and 7. The extrinsic and intrinsic pathways can also cross-talk when caspase 8 cleaves the pro-apoptotic Bcl-2 family member BID, which leads to activation of caspase 9 via mitochondria and further activate caspases 3, 6, and 7. The effector caspases can also activate caspase 8 and 10, forming a positive amplification loop (Stennicke and Salvesen 2000). Figure from (Gewies 2004).

1.4.1 Anoikis

The lack of cell attachment to the extracellular matrix (ECM) can induce apoptosis in different systems *in vitro*, and this process has been designated as anoikis (from the Greek term "homelessness") (Frisch and Francis 1994). Different cell types *in vitro* present different sensitiveness to anoikis, and in particular it has been shown that epithelial cells become more resistant to anoikis after oncogenic transformation (Frisch and Screaton 2001). Anoikis *in vivo* therefore may prevent dysplastic growth of epithelial cells, by deleting them before they reattach to new matrix, but its meaning may be more complex when other cell types are considered (Frisch and Ruoslahti 1997).

Different mechanisms, most of them cell type-specific, have been hypothesised for anoikis (Frisch and Ruoslahti 1997; Frisch and Screaton 2001; Stupack and Cheresh 2002). Signalling starting from integrins can lead to a cascade of events through the activation of the FAK pathway, activating PI3K and Akt, or Ras, Raf and ERK1/ERK2. In both cases, the final results will be the expression of the anti-apoptotic proteins Bcl2, Bcl-xL, or cIAPs (Stupack and Cheresh 2002).

Inhibition of EGF signalling, a modification in the structure of cytoskeleton, and a role of death-receptors to understand the initial point of activation of caspases have also been investigated (Frisch and Ruoslahti 1997; Frisch and Screaton 2001). Some of the pathways hypothesized for anoikis are shown in figure 6.

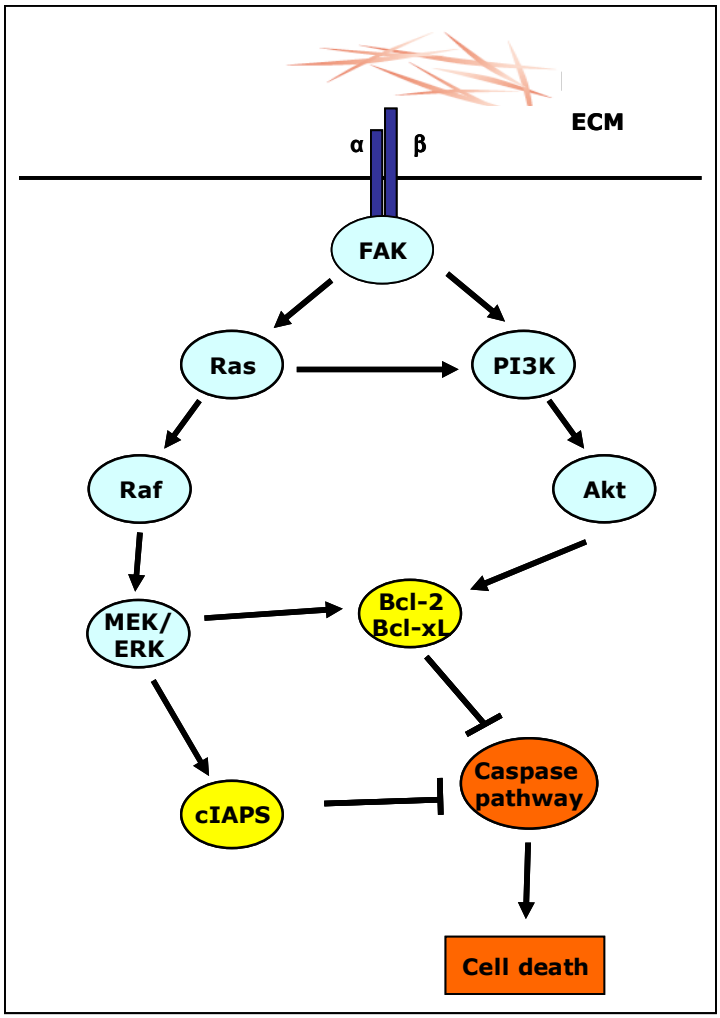

Figure 6: Schematic representation of some hypothesized anoikis signalling pathways

Autophosphorylation of FAK triggered by activation of integrins leads to the Ras-dependent activation of PI3K and further activation of Akt. Akt, together with NF-kB (not shown) can target the expression of the anti-apoptotic proteins of the Bcl family. Alternatively, FAK can activate the Raf-MEK-ERK pathway. Erk phosphorylates Bcl-2 inhibiting its degradation, and also promote the expression of the anti-apoptotic proteins cIAPs.

1.4.3 Role of ROS production in anoikis

Few studies have shown a role of the production of ROS in anoikis. An increase in the production of ROS has been correlated with an augmented sensitivity in cell death due to anchorage deprivation in endothelial cells. Therefore, antioxidants could be promoting the survival and proliferation of these cells in a matrix-deprived environment (Schafer, Grassian et al. 2009). This would confirm an earlier study showing a rise in ROS production from the mitochondria after detachment of endothelial cells from the ECM. ROS were hypothesized to intervene at an point upstream to caspase activation (Li, Ito et al. 1999). However, another study showed that an induced increase the concentration of ROS is followed by an increase in the number of cells resistant to anoikis. ROS, in fact, activates the Src survival pathway, and ERK and Akt signalling, similarly to the pathways triggered by the integrin signalling during cell attachment to the ECM (Giannoni, Buricchi et al. 2008).

1.5. The extracellular matrix and the role of integrins

The extracellular matrix (ECM) is defined as the part of a tissue which is external to the cells. ECM is formed by numerous proteins and polysaccharides which can vary in amount and type according to the adjacent tissue or singular cell, to reflect the individual need of a specific tissue or cell shape, development, proliferation or function (Alberts 2002). Each one of these functions is carried out by a combination of different proteins of the ECM, revealing the complex interaction happening between the cell and the ECM and how influential a disruption of this signalling network can be on the cell behaviour.

As explained above, a lack of interactions between the cell and the ECM triggers an apoptotic phenotype known as anoikis. The components of the ECM that have been studied more extensively in anchorage deprivation-induced cell death are integrins because of the different variety of effects initiated by their expression and binding to specific ligands.

Integrins are composed by a large number of transmembrane proteins, which bind to the cellular cytoskeleton on the cytoplasmic tail, and to specific ligands on the ECM on the other end. The affinity with the ligand is quite weak, but the concentration of integrins on the cell surface is so elevated that it can warrant a highly regulated reversible binding between the cell and the ECM (Alberts 2002). Integrins are formed of many variants of two distinct subunits, α and β, which then dimerize on the cell surface to form a complete receptor. Some of the subunits, such as the widely studied $\beta 1$, are transported to the Golgi complex and is glycosylated at the N-terminus, to produce the mature subunit that can move to the cell surface and eventually form a receptor (Gu and Taniguchi 2004).

Once the ligand is recognized, the activated integrins cluster to form defined structures on the cell membrane called focal adhesions, which

also encompass a number of accessory proteins, like kinases (such as focal adhesion kinase, FAK, or the Src kinases) or scaffolding proteins. On the inside of the cell, other proteins are recruited from the active integrins, especially with scaffolding and signalling functions (Desgrosellier and Cheresh 2010). As detailed in figure 7, the signal triggered by integrins is complex and ramified, and involves several important transcription factors, such as Jun or NF-kB, different kinases, such as ERK, AKT and RAF, and oncogenes such as RAS. The combinations of a specific ligand, and the accessory proteins recruited, will trigger a specific signal which can result in cell migration, survival, proliferation, all of these happening during the cell development, differentiation, and transformation.

The role of integrins in cancer has therefore been widely studied by numerous groups. Expression of integrins is modified in malignant cells, but also in the surrounding tissues and host cells, such as fibroblasts and the cells in the vascular system (Desgrosellier and Cheresh 2010), not only to confer anti-apoptotic and proliferative advantages to the cancer cells, but also to create a favourable environment for these cells to proliferate and invade other tissues.

In the neoplastic cells, integrins can cooperate with a variety of oncogenes, amplifying the signal triggered by the activation of these. For example, the integrin α6β4 works together with EGFR through the Src family kinase Fyn to promote migration of SCC cells under EGF stimulation (Mariotti, Kedeshian et al. 2001; Mercurio and Rabinovitz 2001); with ErbB2 in mammary and ovarian human carcinoma, possibly to promote invasion (Falcioni, Antonini et al. 1997); and with Met, the receptor for the hepatocyte growth factor, even if studies are not concordant on this (Trusolino, Bertotti et al. 2001; Chung, Yoon et al. 2004).

In addition, deregulation of integrin signalling is not always carried out by overexpression of the receptor. For example, the expression of integrin α2β1 is reduced during the progression of breast adenocarcinoma, and its

reintroduction can restore the non-malignant phenotype in breast epithelial cells (Zutter, Santoro et al. 1995).

Integrin overexpression is, however, the most frequent case of regulation of the signalling during cancer formation and progression. Even if tumour cells usually present mutations in oncogenes or loss of heterozygosity of other genes, and therefore gaining an increase proliferation, migration, or survival, and becoming rather independent from the binding with the ECM, it has been proposed that an increased integrin signalling could still be an advantage for the malignant cells, conferring extra proliferation and other malignant characteristics (Guo and Giancotti 2004).

For example, overexpression or maintenance of the expression level of integrin α6β4, important for the formation of hemidesmosomes, can be found in several malignancies, among which SCC, and in some of those it is correlated with the survival of the patient and the severity of the tumour (Mercurio and Rabinovitz 2001). Also, in SCC, downregulation of the integrin αvβ5 is associated with upregulation of the integrin αvβ6 in the regulation of anoikis. The two integrins provide antagonistic roles since αvβ5 would promote anoikis, while αvβ6 would inhibit it (Janes and Watt 2004).

On the other hand, gain of function mutations in the domains of integrins in the neoplastic cells are rare events (Wellcome Trust Sanger Institute Cancer Genome Project). In a particular cell line derived from SCC from the tongue, SCC-4, mutations in the integrin β1 confers an enhanced binding affinity for the ligand and eventually a inhibition of differentiation, which is associated with a worse prognosis (Evans, Perkins et al. 2003).

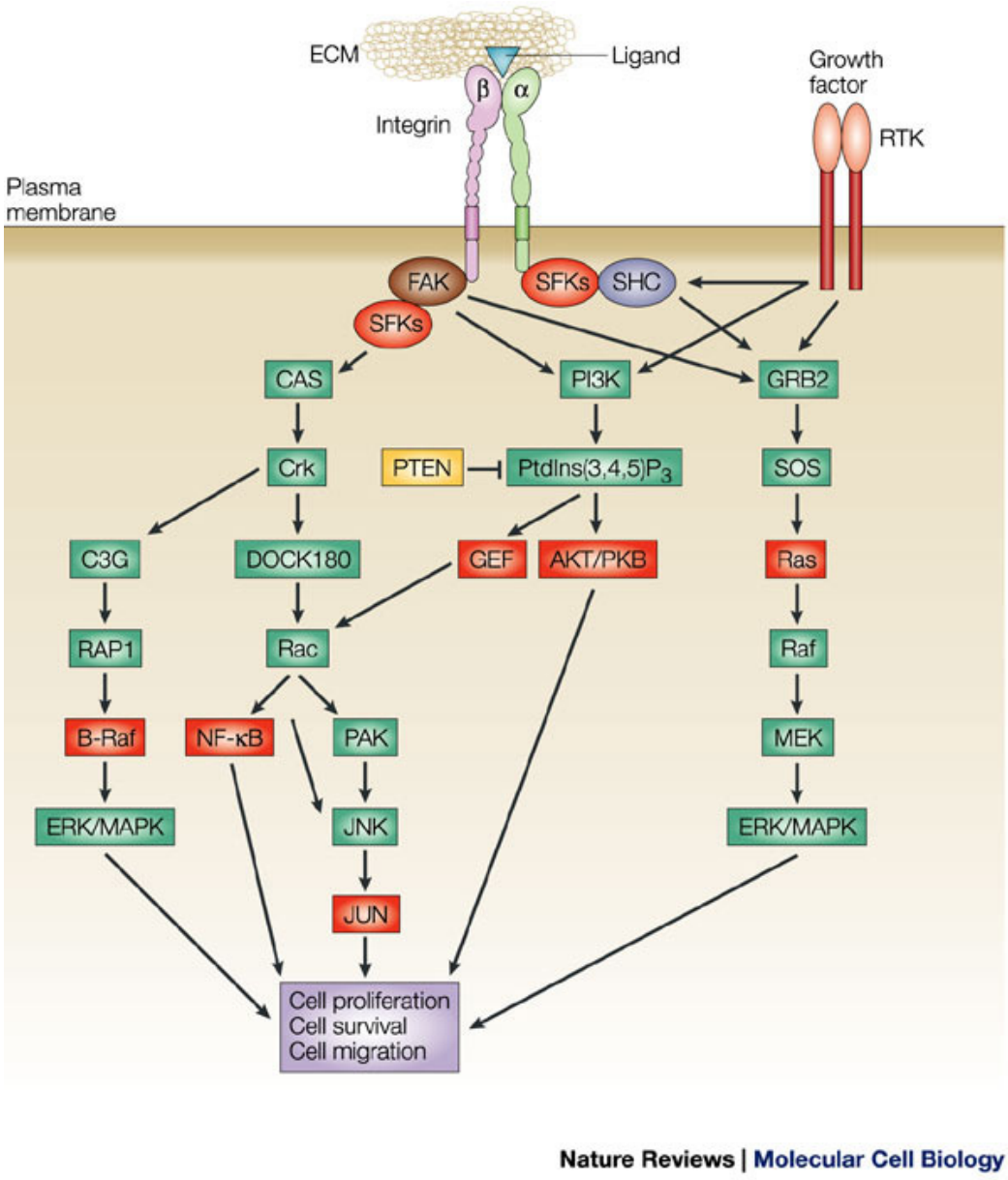

Figure 7: The integrin pathway

The different pathways initiated by integrins can lead to three different cell processes: cell proliferation, cell survival, and cell migration (together with cytoskeletal organization). After dimerization of two α and β subunits and binding with the specific ligand, clusters of integrins are formed on the surface as focal adhesion, and binding proteins in the cytoplasm interact with the cytoplasmatic tail of the integrin receptor and the cytoskeleton. This interaction activates the oligomerization and autophosphorylation of FAK, and thus the following binding to Src-family kinases (SFKs), thanks to the presence of the specific SH2 domain in this protein family, and the activation by phosphorylation of a variety of FAK-associated proteins. Through a cascade signalling, this pathway leads to

the activation of Raf, ERK and the regulators of transcription NF-κB and Jun to regulate cell growth and differentiation.
FAK, independently from the interaction with Src, can also activate other protein with the SH2 domain, such as Phosphotidyl Inositol-3Kinase (PI3K), which eventually leads to the activation of AKT, a regulator of cell survival.
In addition, Src can activate the G-protein Ras through phosphorylation of FAK, which bind to the growth-factor-receptor-bound protein complex,the GRB2-SOS. Activation of Ras triggers the MAPK pathway, eventually promoting cell migration. Figure from (Guo and Giancotti 2004).

1.6 Aim of the thesis: interplay between telomerase and extracellular matrix in the modulation of apoptosis and anoikis in skin

A possible involvement of telomerase in the mechanism of apoptosis and the regulation of the enzyme by loss of attachment with the extracellular matrix has firstly been suggested by observations in anchorage-deprived normal human epidermal keratinocytes and squamous cell carcinoma lines. In these cell lines, anchorage deprivation inhibits telomerase activity, while increasing ERK1 activity and leading the cells to cell cycle arrest. Re-adhesion to type IV collagen via specific integrin subunits α2β1 inhibits ERK1 activity and telomerase repression (Crowe, Nguyen et al. 2005).

Decreased ß1 integrin expression has also been associated with loss of basement membrane attachment and terminal differentiation of these same cell types (Jones and Watt 1993). A study from Tiberio *et al.* (Tiberio, Marconi et al. 2002) indicates that keratinocytes enriched for stem cells are protected from anoikis via β1 integrin, in a Bcl-2 dependent manner, and that disruption of β1 integrin activates the extrinsic apoptotic pathway.

Secondly, the importance of the pathway involving the focal-adhesion kinase FAK has also been correlated with cell survival, being able to suppress suspension-induced cell death (anoikis) and transform kidney epithelial (MDCK) cells (Frisch and Francis 1994). Also, FAK is activated by autophosphorylation upon integrin-mediated cell attachment and the signalling which follows FAK activation leads to the activation of the MAP kinases ERK1 and ERK in human kidney epithelial cells and mouse fibroblasts (Schlaepfer and Hunter 1996).

Thirdly, in the same two-stage chemical carcinogenesis mice model cited above, a reduction in FAK protein expression in the epidermis has been directly associated with a reduction in the acquisition of malignancy and

an increase keratinocyte cell death (McLean, Brown et al. 2001; McLean, Komiyama et al. 2004), leading to investigate the role of FAK in the progression of these tumours. FAK is known to be involved in signalling from growth-factor receptors and to influence the adhesion mechanics regulated by integrins, working together with the Src-family kinases (McLean, Carragher et al. 2005).

Collectively from these data and from what I already know about anoikis, I can say that there is considerable experimental support that telomerase is regulated by ECM; a possible survival mechanism involving integrins and FAK/Src kinases, *Ras* and ECM; a confirmation of this pathway in the survival of skin cancer cells, since FAK (Frisch and Ruoslahti 1997), *Src* (Frisch and Francis 1994) and *Ras* all mute anoikis (Frisch and Francis 1994).

The first aim of the study was to establish that my chosen model of immortal HaCaT keratinocytes did show a down regulation of telomerase activity in anchorage-deprived conditions and if so how this down regulation occurred.

The second aim of this study is to investigate whether telomerase could augment neoplasia in human squamous epithelia and in mouse models of this tumour such as the two stage epidermal tumourigenesis by protecting keratinocytes from anoikis.

The final aim of the project was to begin to investigate how telomerase was able to mute anoikis and in particular whether telomere lengthening or catalytic functions of the enzyme were required.

Chapter 2:

Materials and methods

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Materials and methods

2.1 Cell culture

HaCaT cells expressing wild-type *TERT* (possessing both canonical and non-canonical function), *TERT*-HA (non canonical function only), Dn*TERT* (double negative *TERT*) and the empty vector (pBabePuro) were created by retroviral transduction in our group by Dr. Fitchett and Dr. Bergin. HaCaT are immortalized skin keratinocytes and they were maintained at 37ºC in 5% CO_2 in RPMI 1640 medium (Invitrogen, Paisley, UK) containing 10% v/v Fetal Clone II, 2 mM L-glutamine, 25 mM HEPES.
SVFN, a clone of SvpgC2a, was kindly donated by Dr. Muy-Teck Teh and used as a positive control for *hTERT* mRNA expression level. SvpgC2a are pre-malignant oral keratinocytes infected with SV40 virus (Kulkarnl, Sundqvist et al. 1995) and maintained at 37ºC in 5% CO_2 in DMEM medium (Lonza Biologics, Slough, UK) containing 10% v/v bovine serum, 2 mM L-glutamine.

VA13 was used as a negative control for *hTERC* expression level and are lung fibroblasts lacking detectable amounts of the RNA component of telomerase and elongate telomeres via the ALT mechanism (Bryan, Marusic et al. 1997; Atkinson, Hoare et al. 2005). Cells were maintained at 37ºC in 5% CO_2 in DMEM medium (Lonza Biologics, Slough, UK) containing 10% v/v bovine serum, 2 mM L-glutamine.

GM847 was used as a negative control for telomerase activity and are SV40-immortalized skin fibroblast line, which maintains telomere length via ALT (Bryan, Marusic et al. 1997). It presents almost undetectable amount of *TERC* (Atkinson, Hoare et al. 2005) Cells were maintained at

37ºC in 5% CO_2 in DMEM medium (Lonza Biologics, Slough, UK) containing 10% v/v bovine serum, 2 mM L-glutamine.

SCC-25 and NHEK were used as a positive control for integrin expression. SCC-25 (squamous cell carcinoma line) cells were maintained at 37ºC in 5% CO_2 in DMEM medium containing 10% v/v bovine serum, 2mM L-glutamine. NHEK (normal human epidermal keratinocytes) were maintained at 37ºC in 5% CO_2 in Keratinocyte basal medium (KBM) (Cambrex) supplemented with bulletkit (hydrocortisone, insulin, bovine pituitary extract, EGF, antibiotics, Cambrex) to make Keratinocyte growth medium (KGM).

All cells were cryopreserved in liquid nitrogen in a freezing medium (10% DMSO, 20% serum, 70% complete culture medium), after a gradual dropping in temperature at -80°C overnight. This was done by placing the cells in ampoules at 1-2x10^6 cells/ml and wrapping them in cotton wool in a plastic box. All cells were routinely subcultured by rinsing briefly with 0.02% EDTA and then incubating in 0.05% trypsin/0.01% EDTA at 37^{o}C until the cells completely detached from the culture vessel. To obtain single HaCaT cell suspensions for experiments the HaCaT cells were incubated for 10 mins in 0.1% EDTA at 37^{o}C and then disaggregated by incubation in 0.05% trypsin and 0.05% EDTA for 30-40 mins.

2.2 Quantitative real-time PCR

2.2.1 RNA extraction and cDNA synthesis

Total RNA from HaCaT-PURO, HaCaT-*TERT*, HaCaT-*TERT*-HA*,* VA13, SFVN at time 0, and after 20 hr in suspension for HaCaTs, was extracted using an RNA RNeasy Mini Kit (Qiagen, Crawley, UK) and the concentration evaluated with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Loughborough, UK). First strand cDNA was synthesized DyNAmo™ cDNA Synthesis Kit (Finnzymes, via New England Biolabs, Hitchin, UK) according to the manufacturer's instructions.

2.2.2 Creation of standard curve

The expression of *hTERT* and *hTERT* splice variants (alpha deletion or α-β+, beta deletion or α+β-, alpha/beta deletion or α-β-) and *TERC* was compared to the expression of a set of the two housekeeping genes *HPRT1* and *ESD.* For the creation of housekeeping gene standards, the PCR reaction was additionally performed on the cDNA samples derived from the HaCaT-*TERT* cell line, using TITANIUM Taq DNA Polymerase (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France) and gene specific primer pairs (table 1), which were previously analysed with a BLAST search of GenBank to ensure specificity, and the presence of a single specific PCR band was verified by electrophoresis on a 2% agarose gel. Real-time PCR conditions were as follows: denaturation at 95°C for 1 min; amplification of 40 cycles at 95°C for 30 sec, 60°C for 1 min, annealing at 65°C for 3 min.

PCR products were purified using a Qiagen spin column (Qiagen, Crawley, UK) and eluted in dH_2O. The DNA concentration was measured with a

NanoDrop spectrophotometer (Thermo Fisher Scientific, Loughborough, UK) and the total number of gene copies was measured according to the equation:

$$\left[\frac{\frac{6.02\times10^{4}}{(basepair)(660)}\times DNA\,conc.\times 2\times vol.}{10^{11}}\right] - vol. =$$

volume required to dilute original stock DNA to give 10^{11} per 2µl concentration

The initial stock of 10^{11} copies/2 µl was subsequently diluted using 25 µg/ml tRNA (Sigma-Aldrich, Poole, UK) as carrier nucleic acid to give a dilution series of 10^{9}, 10^{7}, 10^{6}, 10^{5}, 10^{4}, 10^{3}, 10^{2} copies/2 µl.

2.2.3 mRNA expression analysis with real-time qPCR

For real-time qPCR analysis, 2 µl of cDNA derived from the samples or from each standard dilution was used in a 10 µl reaction in a real-time PCR Roche LightCycler system (Roche Diagnostics, Burgess Hill, UK). dH_20 was used as a negative control. PCR reactions were carried out with the SYBR Green I Master Mix (Roche Diagnostics, Burgess Hill, UK) and the specific primer sets (table 1), and melting curve analysis of products was additionally performed to ensure specificity. All primers were used at a concentration of 5 µM.

For *HPRT1, ESD* and *TERC* mRNA quantification, real-time PCR conditions were as follows: denaturation at 95°C for 5 min; amplification of 45 cycles at 95°C for 10 sec, 60°C for 10 sec, 72°C for 10 sec; melting curve at 95°C for 5 sec, 65°C for 1 min; cooling down at 40°C for 30 sec. Acquisition time for the detection of the fluorescence corresponded to the last step of the amplification stage.

For *hTERT* mRNA quantification, the real-time PCR conditions were as follows: denaturation at 95°C for 5 min; an amplification of 45 cycles at

95°C for 10 sec, 60°C for 10 sec, 72°C for 10 sec; a melting curve at 95°C for 5 sec, 65°C for 1 min; cooling down at 40°C for 30 sec. Acquisition time for the detection of the fluorescence was performed at 88°C for 1 sec. The higher acquisition temperature for *hTERT* mRNA expression was chosen to minimize the acquisition of primer dimers which are easy to form due to the length of the template (182 bp).

For *hTERT* splice variant mRNA quantification, real-time PCR conditions were as follows: denaturation at 95°C for 5 min; amplification of 55 cycles at 95°C for 10 sec, 67°C for 10 sec, 88°C for 10 sec; melting curve at 95°C for 5 sec, 65°C for 1 min; cooling down at 40°C for 30 sec. Combinations of four primers are required for the detection and analysis of all the splice variants (see section 1.1.5.2). Blast analysis was performed to identify the correct position of the primers in the total *hTERT* mRNA and to predict the length of the amplicons (see table 2).

2.2.4 Data analysis

The samples were analysed in triplicate, and absolute quantification of the gene of interest and the single housekeeping genes was possible with comparison with a standard curve using standard dilution of the same gene. Relative amount of the copy number of the gene of interest of the unknown sample was calculated by normalizing the absolute quantification of the gene of interest with the normalization factor based on the geometric mean of multiple housekeeping genes. The geNorm VBA applet for Microsoft Excel (http://medgen.ugent.be/~jvdesomp/genorm/) was used to determine the stability of expression of the selected housekeeping genes and to calculate the normalization factor used to estimate the mRNA expression of the genes of interest. The result was proven to be statistically significant by one way ANOVA test and Tukey-Kramer multiple comparisons test, with different levels of significativity (***: highly significant, $p<0.001$; **: very significant, $p<0.01$; * significant, $p<0.05$; ns: non significant).

Genes	Sequence (5'-3')	Position on gene (BLAST alignment)	Product size (bp)
HPRT1-F	TGACACTGGCAAAACAATGCA	496-516	93
HPRT1-R	GGTCCTTTTCACCAGCAAGCT	589-569	
ESD-F	TCAGTCTGCTTCAGAACATGG	387-407	71
ESD-R	CCTTTAATATTGCAGCCACGA	458-438	
hTERT-F	AGAGTGTCTGGAGCAAGTTGC	1791-1811	183
hTERT-R	CGTAGTCCATGTTCACAATCG	1973-1953	
α+	TGTACTTTGTCAAGGTGGATGTG	2175-2197	
α-	CTGAGCTGTACTTTGTCAAGGAC	2169-2188, 2226-2227	
β+	GTACGGCTGGAGGTCTGTCAA	2373-2354	
β-	GGCACTGGACGTAGGACGTGG	2346-2341 2543-2529,	
hTR-F1	ACCCTAACTGAGAAGGGCGTAG	49-70	121
hTR-R1	GTTTGCTCTAGAATGAACGGTG	170-149	

Table 1: Primer sets for real-time PCR

For amplicon length of splice variants, see table 2.
HPRT1: Homo sapiens hypoxanthine phosphoribosyltransferase 1 (Lesch-Nyhan syndrome), NM_000194.1
ESD: Homo sapiens esterase D/formylglutathione hydrolase, NM_001984.1
hTERT: Homo sapiens telomerase reverse transcriptase (*TERT*), transcript variant 1, NM_198253.2
hTR: Homo sapiens telomerase RNA component (*TERC*), telomerase RNA NR_001566.1

NCBI Reference Sequence: NM_198253.2
Homo sapiens telomerase reverse transcriptase (*TERT*), transcript variant 1, mRNA, 4018 bp

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[…]
1501 gcacaacgaa cgccgcttcc tcaggaacac caagaagttc atctccctgg ggaagcatgc
1561 caagctctcg ctgcaggagc tgacgtggaa gatgagcgtg cgggactgcg cttggctgcg
1621 caggagccca ggggttggct gtgttccggc cgcagagcac cgtctgcgtg aggagatcct
1681 ggccaagttc ctgcactggc tgatgagtgt gtacgtcgtc gagctgctca ggtctttctt
1741 ttatgtcacg gagaccacgt ttcaaaagaa caggctcttt ttctaccgga agagtgtctg
1801 gagcaagttg caaagcattg gaatcagaca gcacttgaag agggtgcagc tgcgggagct
1861 gtcggaagca gaggtcaggc agcatcggga agccaggccc gccctgctga cgtccagact
1921 ccgcttcatc cccaagcctg acgggctgcg gccgattgtg aacatggact acgtcgtggg
1981 agccagaacg ttccgcagag aaaagagggc cgagcgtctc acctcgaggg tgaaggcact
2041 gttcagcgtg ctcaactacg agcgggcgcg gcgccccggc ctcctgggcg cctctgtgct
2101 gggcctggac gatatccaca gggcctggcg caccttcgtg ctgcgtgtgc gggcccagga
2161 cccgccgcCT GAGCTGTACT TTGTCAAGgt ggatgtgacg ggcgcgtacg acaccatccc
2221 ccaggACagg ctcacggagg tcatcgccag catcatcaaa ccccagaaca cgtactgcgt
2281 gcgtcggtat gccgtggtcc agaaggccgc ccatgggcac gtccgcaagg ccttcaagag
2341 CCACGTctct accttgacag acctccagcc gtacatgcga cagttcgtgg ctcacctgca
2401 ggagaccagc ccgctgaggg atgccgtcgt catcgagcag agctcctccc tgaatgaggc
2461 cagcagtggc ctcttcgacg tcttcctacg cttcatgtgc caccacgccg tgcgcatcag
2521 gggcaagtCC TACGTCCAGT GCCaggggat cccgcagggc tccatcctct ccacgctgct
2581 ctgcagcctg tgctacggcg acatggagaa caagctgttt gcggggattc ggcgggacgg
2641 gctgctcctg cgtttggtgg atgatttctt gttggtgaca cctcacctca cccacgcgaa
[…]
```

α deletion

β deletion

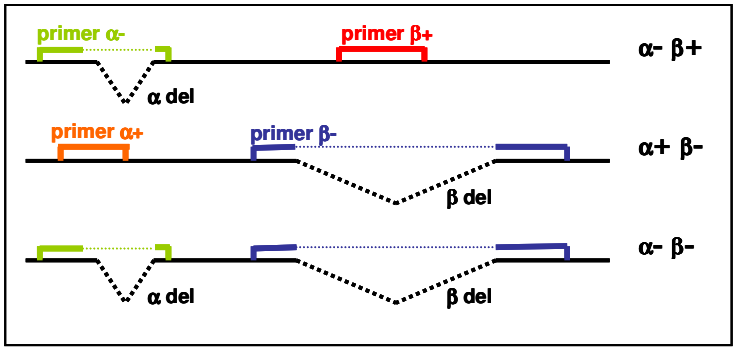

Table 2: Blast analysis of primers used for the detection of *hTERT* and *hTERT* splice variant mRNA expression

Expression of *hTERT* mRNA was performed on real-time qPCR using the reverse and forward primers for h*TERT* (highlighted in yellow in the table).
Expression of *hTERT* splice variants was performed using a combination of primers α+, α-, β+, β-. In the table, the 36 bp deletion called the "alpha deletion" is highlighted in green; the 182 bp deletion called the "beta deletion" is highlighted in light blue. The same deletion sites are marked as a dotted line in the lower graph. To analyze all the possible scenarios, one primer is located entirely, or partly, on the deletion site (underlined primers, primers α+ and β+), and one primer is flanking the deletion site (primers in bold, uppercase font, α- and β-).
Primer combinations and amplicons obtained are as follows:
α+/β-, beta deleted mRNA variant: 187 bp
α-/β+, alpha deleted mRNA variant: 169 bp
α-/β-, alpha and beta deleted mRNA variant: 156 bp

2.3 Telomere Repeat Amplification Protocol (TRAP)

2.3.1 Conventional TRAP assay

The Telomeric Repeat Amplification Protocol, or TRAP, is a two-step PCR-based methodology to determine the telomerase activity *in vitro,* originally developed by Kim *et al.* (Kim, Piatyszek et al. 1994). In the first step of the assay, telomerase, if present in the sample lysate, adds telomeric repeats (GGTTAG) to a template substrate. In the second step, amplification of these adducts is performed with specific forward and reverse primers, together with amplification of an internal standard PCR control (with separate forward and reverse primers). The amount of the internal control product detected is inversely correlated with the amount of telomeric product, and can be utilized to check the equal loading of the lysates, hence normalize the results, and identify false-negative samples due to Taq polymerase inhibitors.

In a conventional TRAP assay, telomeric repeats, representing the enzymatic activity, are subsequently shown on a polyacrylamide gel as a ladder pattern of PCR products with a spanned size of 6 bp, starting from a size of 50 bp. The internal control band is shown at 36 bp. The higher the telomerase activity, the stronger the signal of the ladder will be.
A non-radioactive TRAP assay was utilized in our study, adapted from the protocol of Herbert *et al.* (Herbert, Hochreiter et al. 2006).

- **Preparation of samples**

Half a million cells were pelleted, washed twice with PBS, and stored at -80°C in RNAse-free tubes until further usage. After thawing, cell pellets were immediately resuspended in 200 µl of CHAPS Lysis Buffer (from TRAPeze Kit, Chemicon Europe Ltd, Chandlers Ford, UK) and incubated

on ice for 30 min. The suspension was then spun at 12,000 g for 20 min at 4°C to remove cell debris, and 160 µl of the supernatant were used to determine the protein concentration. Protein determination was performed with DC protein assay (BIO-RAD, Hemel Hempstead, UK), according to the manufacturer's instructions. A standard curve calculated with a known BSA concentration, using the CHAPS Lysis Buffer was first established. Following this, the protein concentration in the samples was estimated according to the standard. Optimization of the amount of protein required was first established by serial titrations of the lysates, so that the assay could be sensitive over the broad activity of our samples. Aliquots of the lysates were stored at -80°C in RNAse-free tubes until subsequent use and from which appropriate dilutions were made..The optimal protein concentration was estimated to be 100 ng/µL.

- **Positive and negative controls**

The positive control cell pellet (10^6 cells) provided in the Chemicom TRAPeze kit (Chemicon Europe Ltd, Chandlers Ford, UK) was suspended in 200 µL of CHAPS Lysis Buffer and the same procedure of sample preparation was followed. The lysate was diluted 1:1 with RNase-free water before loading in the assay to avoid overloading of the system.

A specific negative control for the absence of telomerase activity was obtained by cell lysate of GM847 cells, which possess ALT phenotype and lacks detectable telomerase activity.

A negative control was performed to check the presence of primer-dimer PCR artefacts or the presence of PCR contamination by substitution of the cell extract with the same amount of CHAPS Lysis Buffer.

Heat treatment of each sample was also used as a third negative control, as telomerase is heat sensitive. Every sample extract was incubated at 85°C for 10 minutes prior to the assay to inactivate telomerase.

- **Cy5 fluorescent-based PCR reaction**

Two µl of sample lysates (100 ng/µl) were used in each 50 µl PCR reaction, according to table 3. The fluorescent dye Cy5, fluorescent in the red region (~650 nm excitation/670 nm emission), was attached to the 5'-end of the primer TS to permit final quantification of the PCR products on the gel. Primers details are shown in table 3.

- **Visualization of PCR products**

After PCR, 5 µl of loading dye (0.25% bromophenol blue, 50% glycerol, 50 mM EDTA) were added to the samples, and 25 µl of this mix were loaded to a 12% non denaturing PAGE gel.

12% polyacrylamide gel was cast in a disposable plastic cassette (Invitrogen, Paisley, UK) by mixing 12% of PolyPAGE-40 Acrylamide/Bis (19:1) (Polysciences, Eppelheim, Germany), 6% of 1X TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA), 0.01% of 10% ammonium persulfate, 0.001% of TEMED and dH_2O.

The gel was run on a XCell SureLock™ Mini-Cell vertical electrophoresis system (Invitrogen, Paisley, UK), in 0.5X TBE for approx. 1 hour at 140-160 V, protected from the light. Subsequent fixation of the gel to avoid DNA diffusion was performed by incubation of the gel in 0.5 M NaCl, 50% (vol/vol) ethanol and 40 mM sodium acetate (pH 4.2) for 15 min at room temperature (RT) in the dark.

Visualization of the TRAP ladder on the gel was performed using a Phosphorimager (Typhoon 9400 scanner, GE Healthcare, Chalfont, UK) reading Cy5 fluorescence at 633 nm. The intensity of the 6 bp ladder representing telomerase products and the 36 bp IC band was estimated with ImageQuant software (Molecular Dynamics Inc., Sunny Vale, CA) on the 4^{th} smallest band in the ladder. The relative telomerase activity (RTA)

was calculated as the ratio of the intensity of the TRAP ladder to the intensity of the internal control.

The extension products were created and amplified by PCR in a five-stage reaction: incubation at 30°C for 1 hour, where telomerase is allowed to add repeats to the substrate; telomerase inactivation at 95°C for 5 min; 24 cycles at 95°C for 30 sec (denaturation), 52°C for 30 sec (annealing) and 72°C for 30 sec (extension); final elongation at 72°C for 2 min; hold at 4°C.

2.3.2 Real-time TRAP assay

A modification of the conventional TRAP assay using a real-time qPCR technique allows a faster and more accurate quantification of the telomerase activity in the samples. Despite the similarity between the real-time and non real-time methods, the real-time protocol is adjusted to permit the relative quantification of the activity compared to a standard curve of known concentrations of adducts, and the fluorometric detection of the products labelled with fluorescein (485 nm excitation/535 nm emission). In our study, I have used a commercial kit called TRAPEZE® RT (Chemicon Europe Ltd, Chandlers Ford, UK, catalogue No. S7710). The increase of fluorescence in the PCR reaction is due to the presence of Amplifluor® primers (included in the kit) in the reaction and the concept of energy transfer. In this technique, the fluorophore and quencher molecules are spatially close due to the hairpin structure of the primer itself; therefore the fluorescence emission is constantly quenched. However, during PCR amplification, the hairpin is unfolded by the polymerase causing fluorescence emission. This fluorescence is therefore correlated with the amount of accumulation of telomeric products in the reaction.

Component	Supplier	Concentration	Volume (µl) per reaction Total 50 µl
RNase-free H_2O	Qiagen, Crawley, UK	-	37.7
TRAP buffer *	-	10X	5
dNTP mix	New England Biolabs, Hitchin, UK	10 mM each primer	0.5
Cy5-TS	Eurofins MWG Operon, London, UK	100 ng/µl	1
NT primer	Eurofins MWG Operon, London, UK	100 ng/µl	1
ACX primer	Eurofins MWG Operon, London, UK	100 ng/µl	1
TSNT template	Sigma-Aldrich, Poole, UK	10^{-20} mol/µl	1
BSA	Ambion, Huntingdon, UK	50 mg/ml	0.4
Taq polymerase	New England Biolabs, Hitchin, UK	5 U/µl	0.4
Sample	-	100 ng/µl	2

Table 3: Components of PCR reaction for TRAP assay

The main components of the PCR reaction of the TRAP assay are the 10X TRAP buffer (200 mM Tris-HCl pH 8.3, 15 mM $MgCl_2$, 630 mM KCl, 10 mM EGTA, 0.5% Tween-20) to lyse the cells of the sample and collect TERT; the different primers, the function of which is shown in table 4; the substrate TS to which telomerase adds the telomeric repeats; and the Taq polymerase to amplify the product added by telomerase in the first step of the reaction.

Primer name	Sequence	Function
Cy5-TS	5'-Cy5-AATCCGTCGAGCAGAGTT-3'	Substrate oligonucleotide/ forward primer
ACX	5'-GCGCGGCTTACCCTTACCCTTACCCTAACC-3'	Reverse primer
TSNT	5'-AATCCGTCGAGCAGAGTTAAAAGGCCGAGAAGCGAT-3'	template for internal control
NT	5'-ATCGCTTCTCGGCCTTTT-3'	Reverse primer for internal control

Table 4: Role of the primers used in the PCR reaction for TRAP assay

The four primers utilized in the PCR reaction have got specific function that allows the addition of the telomeric repeat and the following amplification for further detection of their amount and correlation with telomerase activity. Active telomerase binds to the TS substrate and a number of telomeric repeats (GGGTTA) are added to TS according to the degree of activity of the enzyme. In the PCR step of the assay, the products generated, formed of TS and AG$(GGGTTA)_n$, are amplified with the TS primer, that is attached to a fluorophore for the following detection, and with the reverse primer ACX. In addition, the sequence TNST serves as a template for an internal control which is amplified with the reverse primer NT and the forward primer TS. Therefore, using the same TS primer, this internal control competes with the telomeric repeats in the amplification process. Its expression will be inversely correlated with the amount of telomeric products, thus with the activity of the telomerase.

- **Preparation of samples and controls**

The lysates, sample preparation and controls are identical to those of the conventional TRAP assay (see section 2.3.1).

- **Generation of TSR8 Standard Curve**

TSR8 is an internal control included in the kit that allows the creation of a standard curve to calculate the amount of adducts extended by telomerase in the different samples. The TSR8 sequence is composed of the TS primer sequence extended with 8 telomeric repeats $AG(GGTTAG)_7$. The stock TSR8 concentration (20 amoles/µL) was serially diluted 1:10 with CHAPS Lysis Buffer to obtain TSR8 concentrations of 2 amoles/µL, 0.2 amoles/µL and 0.02 amoles/µL. Two µL of each of the four TSR8 dilutions were used per assay.

- **Real-time qPCR reaction**

Two µl of sample lysates (100 ng/µl) were used in each 25 µl PCR reaction, according to table 5.

The extension products were created and amplified using a real-time PCR Roche LightCycler system (Roche Diagnostics, Burgess Hill, UK) in a four-stage reaction: initial denaturation at 37°C for 30 min; telomerase inactivation at 95°C for 2 min; 45 cycles at 94°C for 15 sec (denaturation), 59°C for 1 min (annealing) and 45°C for 10 sec (extension); cooling down at 37°C for 30 sec. Acquisition time for the detection of the fluorescence corresponded to the last step of the amplification stage.

- **Data analysis**

The logarithmic plot of the standard curve was created by calculating the $\log_{10}$ of the amoles per well for each reaction (see table 6). The logarithmic value was plotted against the Cp measurement (second derivative cycle-threshold) at the corresponding standard as detected by the qPCR machine using Microsoft Excel®. A linear regression plot was then calculated and utilized to extrapolate the arbitrary telomerase units of the unknown samples from the known Cp measurement. The result was proven to be statistically significant by one way ANOVA test and Tukey-Kramer multiple comparisons test, with different levels of significativity (***: highly significant, $p<0.001$; **: very significant, $p<0.01$; * significant, $p<0.05$; ns: non significant).

Component	**Supplier**	**Concentration**	**Volume (µl) per reaction Total 25 µl**
TRAPEZE® RT Reaction Mix*	Chemicon Europe Ltd, Chandlers Ford, UK	5X	5
Titanium Taq Polymerase	Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France	5 U/µl	0.4
Nuclease Free Water	Chemicon Europe Ltd, Chandlers Ford, UK	-	17.6
Sample**	-	100 ng/µl	2

Table 5: Components of real-time qPCR reaction for real-time based TRAP assay

*5X TRAPEZE® RT Reaction Mix is composed of TS primer, RP tailed primer, Amplifluor® primer dA, dC, dG and dTTP, diluted in 10X Reaction Buffer S Plus, 10 mM MgCl2 with 0.5 mg/ml BSA
**Samples included were: TSR8 dilutions, positive and negative controls, and experimental samples

Like in a conventional TRAP assay, the two major primers are TS, acting also like a substrate for the addiction of telomeric repeats, and RP (corresponding to the primer ACX in the conventional TRAP assay). In this real-time qPCR-based TRAP, the internal control is missing, because of the impossibility to quantify two distinct products, but the control is provided by independent amplification of a serial dilution of the sequence TSR8, composed of 8 telomeric repeats, which creates a standard curve for the quantification of the number of repeats added by telomerase.

TSR8 dilution	**Correspondent copies of TSR8**	**Log_{10}**	**Experimental average Cp**
40 amoles	24088000	7.381800743	25.75
4 amoles	2408800	6.381800743	28.13
0.4 amoles	240880	5.381800743	29.97
0.04 amoles	24080	4.381656483	31.9

Table 6: Data for generation of a TSR8 standard curve

One complete standard curve was taken as reference for samples repeats. Accuracy of the efficiency was confirmed by correspondence of the Cp from the second and third dilution in the subsequent run.
Telomerase activity extrapolated in this way was then subtracted from the water sample.

2.4 Anoikis assay

This assay permits the analysis of the cell behaviour in a environment where the cell-matrix engagement is disrupted. For this aim, I have used bacteriological dishes coated with PolyHEMA (poly(2-hydroxyethylmethacrylate), Sigma-Aldrich, Poole, UK), an non-ionic hydrogel that, at a concentration of 10 mg/ml (1 mg/cm^2) precludes matrix deposition and therefore cell attachment (Bretland, Lawry et al. 2001).

2.4.1 Coating of the dishes and cell seeding

PolyHEMA (Sigma-Aldrich, Poole, UK) was firstly dissolved 10% (w/v) in 95% acetone to obtain the stock solution. One ml of working solution (0.4% (v/v) in acetone:90% alcohol) was then homogenously applied to a petri dish and let it dry in the tissue culture hood before adding the cell suspension.

Adapting from Frisch's protocol (Frisch and Francis 1994), cell lines were grown to different confluences: 20%, 70% and 100% confluence. Single cells were obtained by treatment with 0.1% EDTA for approx. 3 mins and trypsinization with 0.05% trypsin/ 0.05% EDTA. Two x 10^6 cells were counted and plated onto 100-mm diameter petri dishes coated with polyHEMA in duplicate, and in parallel the same number of cells were used as a control at time 0.

To analyze the hallmarks of apoptosis, DNA content was measured by Hoechst staining and morphological characterization of the nuclei or independently by propidium iodide (PI, Sigma-Aldrich, Poole, UK) staining and fluorescence activate cell sorting (FACS). After 20 hrs, cells can be seen floating in the medium or loosely attached to the surface of the dishes, still as single cells. Both these type of cells were combined

together in a single tube by gently scraping the dish and pipetting the floating cells before further fixation.

2.4.2 Morphological characterization of the apoptotic nuclei

For morphological characterization of the nuclei at the microscope, cells at 0 hr and 20 hrs were fixed with 3.7% paraformaldehyde for 15 mins at 4°C, washed in sterile PBS and dropped on microscope slides to let air-dry at 60°C for 2 hr. Cells were then stained with or 0.1 µg/ml Hoechst 33258 (Sigma-Aldrich, Poole, UK), and washed twice in PBS 1X before the coverslip was applied. Stained cells were then blindly counted and photographed on a Nikon Eclipse TE200-S. Blindness of the experiment was achieved by covering the sample names with removable tape and randomizing the slides before the count.

2.4.3 Analysis of the sub-G1 peak by fluorescence-activated cell sorting for fluorescence-activated cell sorting (FACS)

Cells at 0 hr and 20 hrs were fixed with ice cold absolute ethanol while gently vortexing to avoid clustering, and washed twice with 100 mM sodium citrate (Sigma-Aldrich, Poole, UK). The cells were suspended in PI RNAse mix, (500 µg/ml propidium iodide (Sigma-Aldrich, Poole, UK), 5 mg/ml RNase A (Sigma-Aldrich, Poole, UK), 100 mM sodium citrate, 1X PBS) and filtered through a 100 µm cell strainer. Analysis of the PI staining in the sub-G1 peak, corresponding to the percentage of dead cells in the sample, was performed by the Flow Cytometry Core Facility, at the Institute of Cell and Molecular Sciences, Bart's and the London School of Medicine and Dentistry, by Dr. Gary Warnes. A minimum of 25000 cells were analyzed by LSRII system (BD Biosciences, Oxford, UK) at a wavelength of 585 nm.

For detection of cisplatin-induced apoptosis, cells were treated with 0 μM, 10 μM, 20 μM and 35 μM cisplatin (Sigma-Aldrich, Poole, UK) before plating in regular tissue culture dishes. After 0 h and 20 hrs, cells were collected from the dishes by gently scraping and pipetting. Adherent cells were combined with floating cells before fixation and analysis of the sub-G1 peak was performed as cited above.

2.4.4 Data analysis

For morphological characterization of apoptotic nuclei, blind scoring of >300 nuclei per sample was used to detect membrane blebbing, cellular shrinkage, chromatin condensation and nuclei fragmentation, hallmarks of anchorage deprivation induced cell death, as reported (Frisch and Francis 1994). The statistical significance of the differences were analyzed using one way ANOVA test and Tukey-Kramer multiple comparisons test, with different levels of significativity (***: highly significant, $p<0.001$; **: very significant, $p<0.01$; * significant, $p<0.05$; ns: non significant).

For FACS analysis, a minimum of 25000 events was measured for each sample and percentage of apoptotic cells were calculated after analysis of Sub-G1 peak. The statistical significance of the differences were analyzed using Anova test as above mentioned.

2.5 Western blotting

Western blotting, or protein immunoblot, is a technique used to detect the expression and localization of specific proteins in a sample of tissue homogenate or cell extract. Proteins are first separated by electrophoresis in denaturing or native conditions before being immobilized on a membrane and detected using specific antibodies against the target protein.

2.5.1 Sample preparation

Whole cell lysates were obtained by washing the cells growing in the flask/dish from medium with sterile PBS 1X and incubation with RIPA buffer (1% NP-40, 0.1% SDS, 50 mM Tris pH 7.3, 150 mM NaCl, protease inhibitors (Complete Mini EDTA-free tablets, Roche Diagnostics, Burgess Hill, UK) for 20 min at 4°C.

The lysates were then spun at 12,000 g for 20 min at 4°C to remove cell debris, and 160 µl of the supernatant were used to determine the protein concentration. Protein determination was performed with the DC protein assay (BIO-RAD, Hemel Hempstead, UK), according to the manufacturer's instructions. A standard curve was calculated with a known BSA (New England Biolabs, Hitchin, UK) concentration using the sample lysis buffer. Following this, the protein concentration in the samples was estimated according to the standard.

2.5.2 Preparation of controls

NHEK and SCC-25 were chosen as positive control for integrin expression Positive controls were performed by culturing the cells to 70% confluence

in their specific growth medium, and cell lysates were obtained as above mentioned.

2.5.2 Preparation of SDS-PAGE gels

Proteins were separated on either precast 4-12% resolving gels under denaturing and reducing conditions (NuPAGE Novex Bis-Tris Pre-Cast Gels, Invitrogen, Paisley, UK) in MES SDS Running Buffer (NuPAGE, Invitrogen, Paisley, UK), or on a hand-made 8% gel.

The hand-made 8% separating gel (0.25 M Tris pH 8.8, 26% of (30% acrylamide/0.8% bis-acrylamide) 0.4% SDS, 1% ammomium persulfate, 0.06% TEMED) was allowed to polymerized before topping with stacking gel (0.1 M Tris pH 6.8, 17% of (30% acrylamide/0.8% bis-acrylamide) 0.4% SDS, 1,2% ammomium persulfate, 0.3% TEMED). The running buffer for this gel was composed of 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3.

2.5.3 Protein separation and detection

Total cellular proteins (10 µg) from the samples were boiled for 5 min in SDS sample buffer (2X, 0.5 M Tris HCl, pH 6.8, 10% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.1% bromophenol blue) and separated on the precast or hand-made gel in the appropriate running buffer at 100-130 V. Six µl of ladder (Dual colour, BIO-RAD, Hemel Hempstead, UK) were also separated as reference for the protein molecular weight.

Separated proteins were electroblotted to 0.45 µm PVDF membranes (Immobilon, Millipore, Watford, UK) at 30 V at 4°C for 90 min using a chilled transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol).

The membrane was then blocked in 5% low fat (<1.5%) milk in TBS-T (1 M TRIS pH 8.0, 5 M NaCl, 0.05% Tween-20) at RT for 1 hr.

Primary antibody was added at the appropriate dilution in milk/TBS-T. The membrane was incubated at 4°C overnight and then washed extensively with TBS-T.

The corresponding horseradish peroxidase-conjugated secondary antibody was added at appropriate dilution in milk/TBS-T. The membrane was incubated at room RT for 1 h and then washed extensively with TBS-T. Antigen-antibody complexes were detected using ECL system for medium-strong signals, or SuperSignal West Femto Maximum Sensitivity Substrate for weak signals (Pierce, Thermo Fisher Scientific, Loughborough, UK), according to the manufacturer's instructions. The antibodies used are shown in table 7.

2.5.4 Data analysis

Estimation of the difference the level of protein expression in each sample was evaluated by Labworks 4.6 software (UVP Bioimaging System) or with ImageJ software and amount of proteins were normalized with the estimated amount of expressed GAPDH in the loading control.

2.6 Integrin analysis by FACS

Cells from an approximately 70% confluent plate were trypsinized using 0.05%/0.25% trypsin/EDTA. After neutralization of trypsin with DMEM/0.1% BSA/0.1% sodium azide (Wash Buffer, WaB), the cells were spun and washed with WaB at 1200rpm for 3 mins. Cells were then resuspended to get a concentration of $4x10^6$ cells/ml in WaB and kept on ice.

In 15 ml tubes, 50 µl of cells ($2x10^5$) were aliquoted and 50 µl of the primary antibody of the appropriate dilution were added to the sample. The cells are mixed by gently flicking the tube and kept on ice to incubate for 45-60 min. Specification of the antibodies and the optimal dilutions are shown in table 7.

After incubation, the cells were washed twice with 2 ml of WaB and spun at 300 g for 3'. The supernatant was discarded both times in one flick of the tube, leaving approximately 50 µl on the cell pellet. 50 µl of second antibody at the appropriate dilution were added to the sample. The cells were mixed by gently flicking the tube and kept on ice to incubate for 30 min. Specification of the antibodies and the optimal dilutions are shown in table 7. Cells were washed twice in WaB as above. After the final wash, cells were transferred in a total suspension of 500 µl in WaB in appropriate FACS tubes (BD Biosciences, Oxford, UK).

2.6.1 Data analysis

Flow cytometry was performed in the Core Cell Sorting Facility laboratory, at the John Vane Science Centre, Bart's and the London School of Medicine and Dentistry, by Dr. Linda Hammond (Tumor Biology, Institute of Cancer) on a BD FACSCalibur platform (BD Biosciences, Oxford, UK). Data analysis was performed using Cell Quest software.

Primary antibodies for FACS, Western blot and anoikis assay				
Antibody name	**Supplier and cat no.**	**Type**	**Host**	**Dilution and application**
Integrin α1 (clone FB12)	Chemicon Europe Ltd, Chandlers Ford, UK # MAB1973Z	Primary monoclonal	mouse	1:1000 FACS
Integrin α2 (clone P1E6)	Chemicon Europe Ltd, Chandlers Ford, UK # MAB1950Z	Primary monoclonal	mouse	1:1000 FACS
Integrin α3 (clone P1B5)	Chemicon Europe Ltd, Chandlers Ford, UK # MAB1952Z	Primary monoclonal	mouse	1:1000 FACS
Integrin α5 (clone P1D6)	Chemicon Europe Ltd, Chandlers Ford, UK # MAB1956Z	Primary monoclonal	mouse	1:1000 FACS
Integrin α6 antibody (clone GoH3)	Abcam, Cambridge, UK # Ab19765	Primary monoclonal	rat	1:1000 FACS
Integrin α9β1 (clone Y9A2)	Chemicon Europe Ltd, Chandlers Ford, UK # MAB2078Z	Primary monoclonal	mouse	1:1000 FACS
Integrin αVβ5 (clone 15F11)	Chemicon Europe Ltd, Chandlers Ford, UK # MAB2019Z	Primary monoclonal	mouse	1:1000 FACS
Integrin αVβ6 (clone 10D5)	Chemicon Europe Ltd, Chandlers Ford, UK	Primary monoclonal	mouse	1:1000 FACS

	# MAB2077Z			
integrin β4	Abcam, Cambridge, UK # ab29042	Primary monoclonal	mouse	1:1000 WB FACS
integrin β1 (CD29)	Abcam, Cambridge, UK # ab30394	Primary monoclonal	mouse	1:1000 WB FACS
integrin αV (CD51)	Abcam, Cambridge, UK # ab55991	Primary polyclonal	rabbit	1:500 WB
GAPDH	Abcam, Cambridge, UK # ab9485	Primary polyclonal	rabbit	1:2000 WB
integrin β1 (clone 6S6)	Millipore, Watford, UK #MAB2253Z	Primary monoclonal	Mouse	1:250 anoikis assay

Table 7: Primary antibodies utilized in western blot and FACS analysis and anoikis assay

In the table, appropriate dilutions for the different techniques are shown. The only blocking antibody was against β1 integrin used on the anoikis assay.
WB : western blot
FACS: fluorescence-activated cell sorting

Isotype controls and secondary antibodies for FACS and Western blot				
Antibody name	**Supplier and cat no.**	**Type**	**Host**	**Dilution and application**
Mouse IgG1 Negative Control (clone Ci4)	Chemicon Europe Ltd, Chandlers Ford, UK # MABC002	Primary monoclonal	mouse	1:1000 FACS
Rat IgG2b (kappa [RTK4530])	Abcam, Cambridge, UK # Ab18536	Primary monoclonal	rat	1:1000 FACS
Alexa Fluor® 488 anti-mouse IgG	Invitrogen, Paisley, UK # A-21121	Secondary	goat	1:250 FACS
Alexa Fluor® 488 anti-rat IgG	Invitrogen, Paisley, UK # A-21208	Secondary	donkey	1:250 FACS
HRP-conjugated anti-mouse IgG	Pierce, Thermo Fisher Scientific, Loughborough, UK #31430	Secondary	goat	1:2000 WB
HRP-conjugated anti-rabbit IgG	Pierce, Thermo Fisher Scientific, Loughborough, UK #31460	Secondary	goat	1:2000 WB

Table 8: Isotype controls and secondary antibodies utilized in western blot and FACS analysis and anoikis assay

In the table, appropriate dilutions for the different techniques are shown.
WB : western blot
FACS: fluorescence-activated cell sorting

Chapter 3:

Results 1

Chapter 3:

Results 1

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3. Characterization of telomerase components in the model cell lines

The aim of this project is to determine a possible new role of telomerase in the protection from anoikis in HaCaT skin keratinocytes. To do this, I decided to use as a model immortalized human skin keratinocytes (HaCaT) overexpressing the following constructs: wild type *TERT*, the flagged *TERT*-HA, which has telomerase activity *in vitro* but cannot lengthen telomeres *in vivo*, the catalytically dead dominant-negative TERT mutant, Dn*TERT*, which has neither catalytic activity nor the ability to lengthen telomeres and as a control HaCaT carrying an empty vector.

To adopt this model as a good system to identify exclusively non-canonical functions of telomerase, I investigated the ability to elongate telomeres *in vitro,* the activity of the enzyme *in vitro*, the presence of the two main components TERT and *TERC*. In addition, as the splice variants of *TERT* have been reported to regulate telomerase activity (Ulaner, Hu et al. 1998; Colgin, Wilkinson et al. 2000; Cerezo, Kalthoff et al. 2002), I investigated the three most common forms, the α variant, the β variant and the $\alpha\beta$ variant.

Additionally, to determine the relationship between telomerase regulation in my model system and in intact epidermis *in vivo* I also tested the above in anchorage deprived conditions that is designed to simulate the earliest events in squamous differentiation.

3.1 Characterising telomere length in HaCaT

The HA-flagged *TERT* construct, *TERT*-HA, has previously been reported to be incapable of extending the length of telomeres and immortalising human cells, despite possessing telomerase activity *in vitro* (Counter, Hahn et al. 1998). To evaluate the adequacy of the system used to distinguish between canonical (dependent from telomere lengthening) and non-canonical functions (independent from telomere lengthening) of telomerase, the inability in lengthening telomeres by the flagged protein TERT-HA was tested in HaCaT cells overexpressing the corresponding gene, and compared with HaCaT overexpressing wild-type *hTERT*, HaCaT carrying the empty vector, and with untransduced HaCaT. This experiment was carried out by the group of Professor Petra Boukamp on cells that were prepared by our group and sent to her (unpublished data).

Telomere Restriction Fragment Length (TRF) analysis permits the evaluation of telomere length in the samples carrying different *hTERT* constructs, according to a previous study (Gordon, Ireland et al. 2003). Briefly, genomic DNA is digested using the restriction enzymes HinF1 and Rsa1. Telomeric and subtelomeric regions lack of the restriction sites from these enzymes, therefore those regions of DNA will not be affected by the enzymatic digestion and will produce a long smear when the digested DNA is resolved by gel electrophoresis and detected by Southern blotting.

The average telomere repeat fragment length increases in HaCaT-*TERT* cells from an average of 4 kb to 6.5 kb in 5 weeks *in vitro*, whereas HaCaT, HaCaT-Puro (empty vector) and HaCaT-*TERT*-HA showed no telomere extension (figure 8) showing that the fusion protein hTERT-HA is incapable of lengthening telomeres in intact cells as previously shown for fibroblasts (Counter, Hahn et al. 1998).

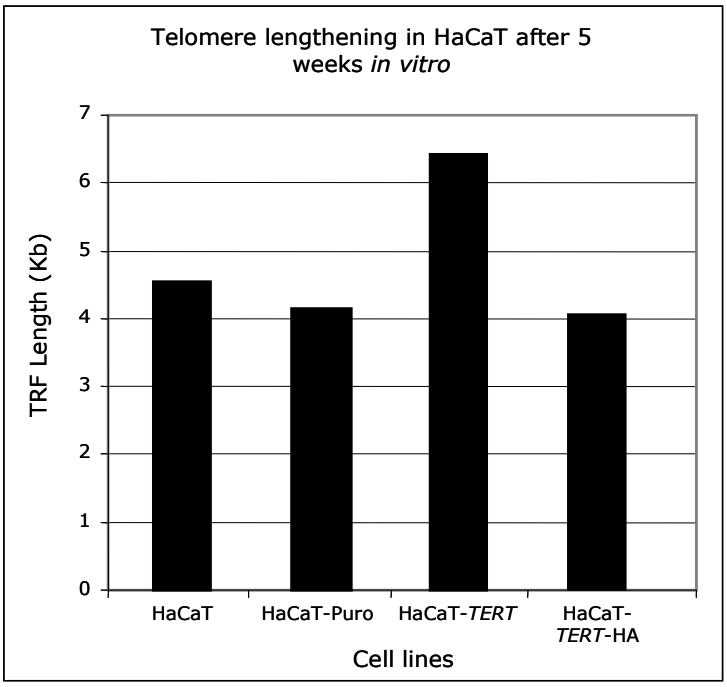


Figure 8: Variation in Repeat Fragment Lengths (TRF) between different *hTERT* constructs in HaCaT grown for 5 weeks *in vitro*

This experiment was carried out by the group of Professor Petra Boukamp (unpublished data). Telomere repeat fragment length increases in HaCaT-*TERT* cells from an average of 4 kb to 6.5 kb after 5 weeks *in vitro*, whereas HaCaT-*TERT*-HA shows no telomere extension and the telomere length in these cells is comparable to the untransduced HaCaT and the empty vector control HaCaT-Puro.

3.2 Characterising telomerase activity in HaCaT following anchorage deprivation

To confirm and extend previous observations that telomerase activity in keratinocytes is dependent on anchorage and attachment to the extracellular matrix (Crowe, Nguyen et al. 2005), I measured the activity of TERT in HaCaT-Puro, HaCaT-*TERT*, HaCaT-*TERT*-HA, HaCaT-Dn*TERT* at time 0 (referred in the graph as 0 hr), and after the cells were cultured in suspension for 20 hrs (referred in the graph as 20 hrs).

The activity of the enzyme can be measured *in vitro* by introducing a primer that acts as a substrate for telomerase-mediated addition of TTAGGG repeats in a PCR reaction. Two methods, both defined as the Telomeric Repeat Amplification Protocol (TRAP), may be used for this aim: a conventional PCR followed by resolving the telomerase products on a polyacrylamide gel (figure 9), and a real-time qPCR (figure 10).

Even if the outcome of telomerase activity among the samples is similar between the two techniques, there is a difference in detection and sensitivity between them. Real-time qPCR TRAP assay is not as routinely used as conventional TRAP (in our case a Cy5-based TRAP), however the sensitivity of the assay is much higher and the quantitation is more accurate than the analysis of the band intensity derived from a conventional TRAP.

However, since conventional TRAP is still the method of choice in the detection of telomerase activity, I decided to perform both the techniques to get a reliable and accurate measure of the enzymatic activity.

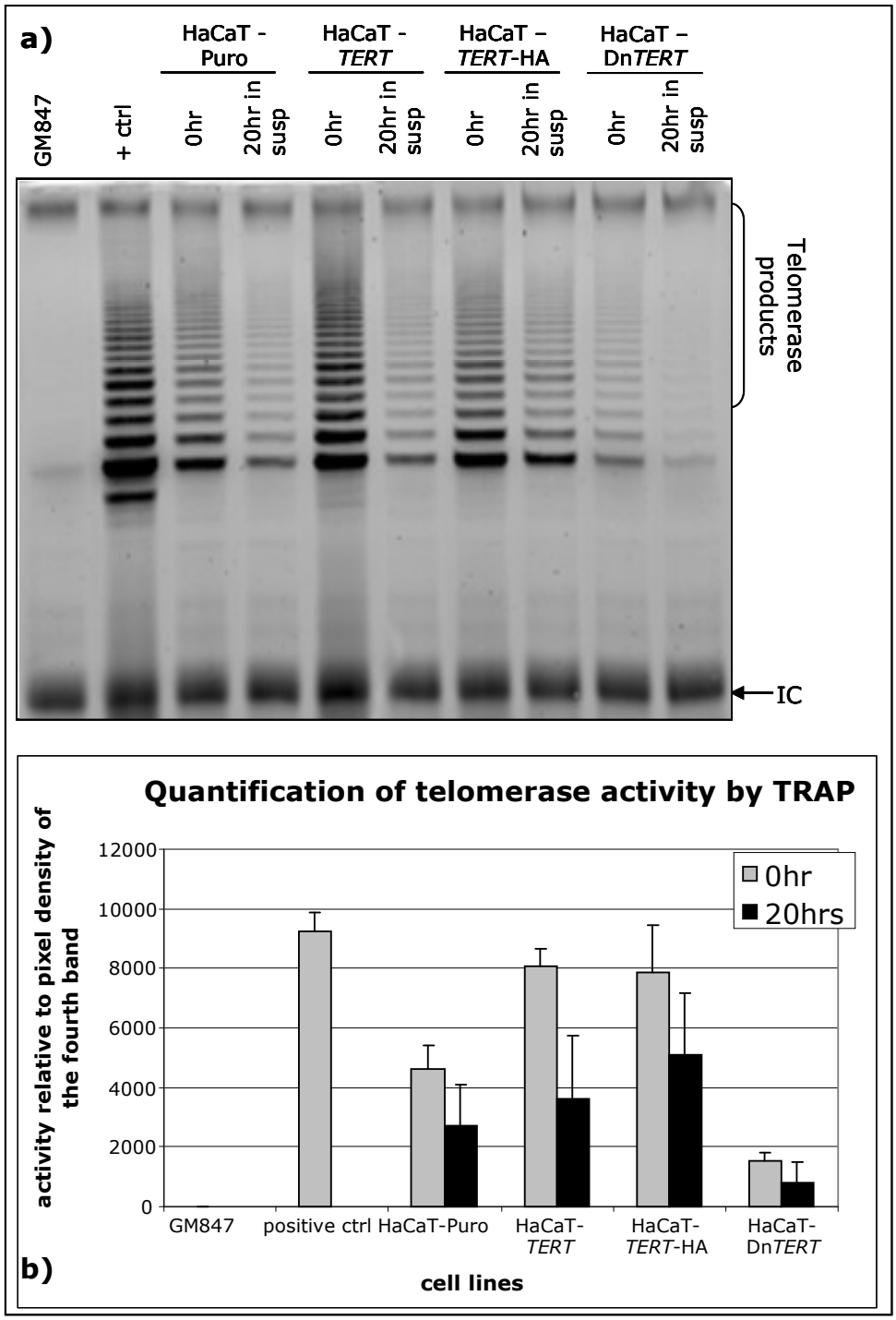
Conventional PCR-based TRAP did not show any detectable telomerase activity in GM847, as expected from previous studies (Bryan, Marusic et al. 1997); however, TRAP performed with real-time qPCR method show a minor amount of activity in this cell line. This is in line with recent

findings of a low amount of *TERT* expression in this cell line (Atkinson, Hoare et al. 2005).

On a Cy5-TRAP plot (figure 9) at the control time (0 hr, as shown in the graphs) HaCaT-Puro showed nearly half the amount of telomerase activity compared to the telomerase-positive cells used as a control in the commercial TRAPeze Kit (Chemicon Europe Ltd, Chandlers Ford, UK), and to the activity of HaCaT cells overexpressing *TERT* and *TERT*-HA. HaCaT cells expressing a dominant-negative version of *TERT,* Dn*TERT*, showed a three fold reduction of telomerase activity compared to HaCaT-Puro, as expected from previously published work on other cell lines (Hahn, Counter et al. 1999).

When the cells are placed for 20 hrs in suspension (20 hrs, as shown in the graphs) and undergoing anoikis, telomerase activity decreases in a similar fashion in all the cell lines, with an average of a 1.8 fold reduction compared to the corresponding cell lines at time 0. However, telomerase activity in the HaCaT-*TERT* and HaCaT-*TERT*-HA in the cells at 20 hrs remains higher than or comparable to the HaCaT-Puro cells at zero time, where the level of anoikis is low.

A similar pattern of enzymatic activity but more striking differences between positive and negative values are also shown by real-time qPCR-based TRAP (figure 10). Also in this case, telomerase activity drops dramatically when the cells are placed in suspension and undergo anoikis, but in the HaCaT-*TERT* and HaCaT-*TERT*-HA the activity is still more elevated than in HaCaT-Puro under the same experimental conditions and comparable to HaCaT-Puro at zero time.

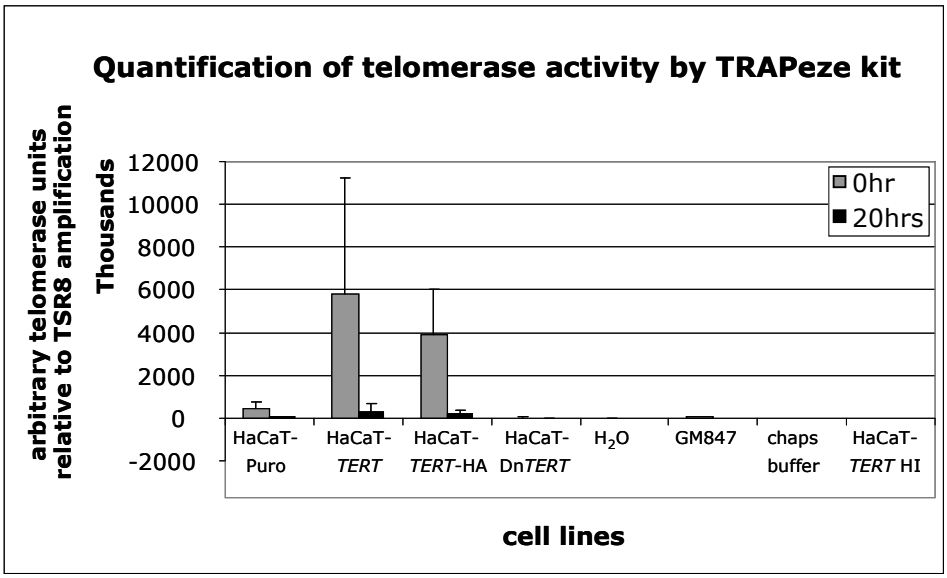

a)
GM847
+ ctrl
HaCaT - Puro
0hr
20hr in susp
HaCaT - TERT
0hr
20hr in susp
HaCaT – TERT-HA
0hr
20hr in susp
HaCaT – DnTERT
0hr
20hr in susp
Telomerase products
IC
Quantification of telomerase activity by TRAP
activity relative to pixel density of the fourth band
12000
10000
8000
6000
4000
2000
0
0hr
20hrs
GM847
positive ctrl
HaCaT-Puro
HaCaT-TERT
HaCaT-TERT-HA
HaCaT-DnTERT
cell lines
b)

c) Cell lines	Average of telomerase activity (%)		Standard deviation	
	0 hr	20 hrs	0 hr	20 hrs
GM847	0	-	0	-
positive control	9255.80	-	620.06	-
HaCaT-Puro	4620.48	2707.78	783.63	1354.41
HaCaT-*TERT*	8064.62	3598.54	592.39	2138.03
HaCaT-*TERT*-HA	7853.23	5076.38	1582.11	2081.82
HaCaT-Dn*TERT*	1562.98	806.03	220.48	697.40

d) HaCaT cells: significance	P value
TERT 0 hr - *TERT* 20 hrs	P<0.05 *
Puro 20 hr – *TERT-HA* 0hr	P<0.01 **
Puro 20 hr – *TERT* 0hr	P<0.01 **
TERT 0 hr - Dn*TERT* 0 hr	P<0.001 ***
TERT 0 hr - Dn*TERT* 20 hrs	P<0.001 ***
TERT 20 hrs - *TERT*-HA 0 hr	P<0.05 *
TERT-HA 0 hr - Dn*TERT* 0 hr	P<0.001 ***
TERT-HA 0 hr - Dn*TERT* 20 hrs	P<0.001 ***
TERT-HA 20 hrs - Dn*TERT* 20 hrs	P<0.05 *

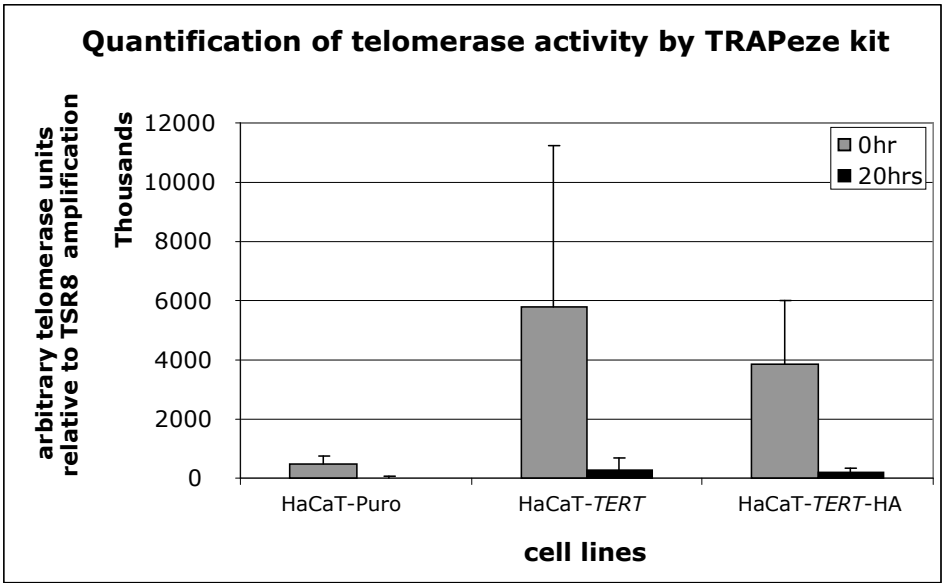
Figure 9: Analysis of telomerase activity using Cy5-TRAP

a) Example of polyacrylamide gel after Cy5-TRAP for detection of telomerase activity in HaCaT cells at control times (0 hr) and after 20 hrs in suspension during anoikis assay (20 hrs). Positivity for telomerase activity is shown by the 6-bp incremental TRAP ladder. A positive control available with TRAPeze kit (Chemicon Europe Ltd, Chandlers Ford, UK) were used as a negative and positive control, respectively. The 36-bp internal standard control (IC) is indicated by an arrow at the bottom of the gel to confirm equal loading of the samples. 200 ng of cell lysates were used in the assay, after previous optimization by serial dilutions (not shown).
b) and table c) Quantification of telomerase activity using Cy5-TRAP. The absolute intensity of the fourth band of the ladder in each sample, obtained by graphic software, was divided by the intensity of the IC band. Average of the values of three independent repeats and standard deviation (SD) are shown. ***: highly significant; **: very significant; * significant; ns: non significant.



a)

b) cell lines	Average activity of telomerase (arbitrary units relative to TSR8 amplification)		standard deviation	
	0 hr	20 hrs	0 hr	20 hrs
HaCaT-Puro	450310.98	34395.65	335386.71	31702.63
HaCaT-*TERT*	5777341.78	307299.82	5434091.96	373521.71
HaCaT-*TERT*-HA	3865639.63	194612.53	2129273.10	148708.96
HaCaT- Dn*TERT*	19149.47	-1128.86	17253.62	6739.04
H_2O	0.00	-	0.00	-
GM847	57440.71	-	-	-
chaps buffer	9006.62	-	-	-
HaCaT-*TERT* HI	-5071.81	-	-	-



c)

d)

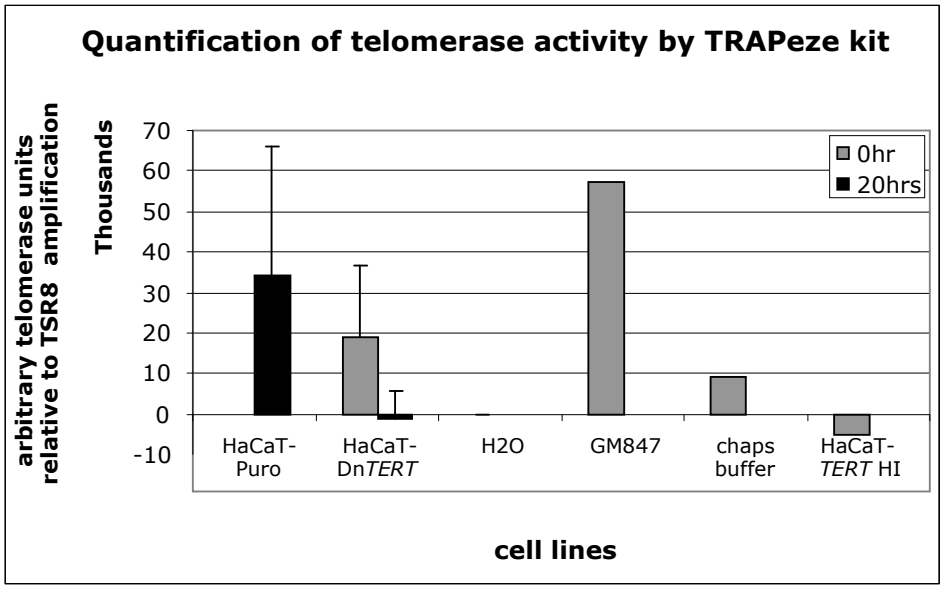


Figure 10: Analysis of telomerase activity using TRAPeze-RT detection kit

a) and more in detail in c) and d) Detection of telomerase activity in HaCaT cells at control times (0 hr) and after 20 hrs in suspension during anoikis assay (20 hrs). The mean ratio of telomerase activity relative to the empty vector HaCaT-Puro at time 0 is shown. The assay was performed in triplicate samples with 200 ng of total cell lysate, and four negative controls were performed (water, GM847, lysis buffer (chaps buffer), and a heat-inactivated lysates (HaCaT-*TERT* HI). The data are presented as means of three independent repeats and SD, and the values are reported in table b). Between each comparison group (comparison at different time points and between different cell lines), the p value is < 0.0001, considered extremely significant.

3.3 Detection of h*TERT* mRNA expression

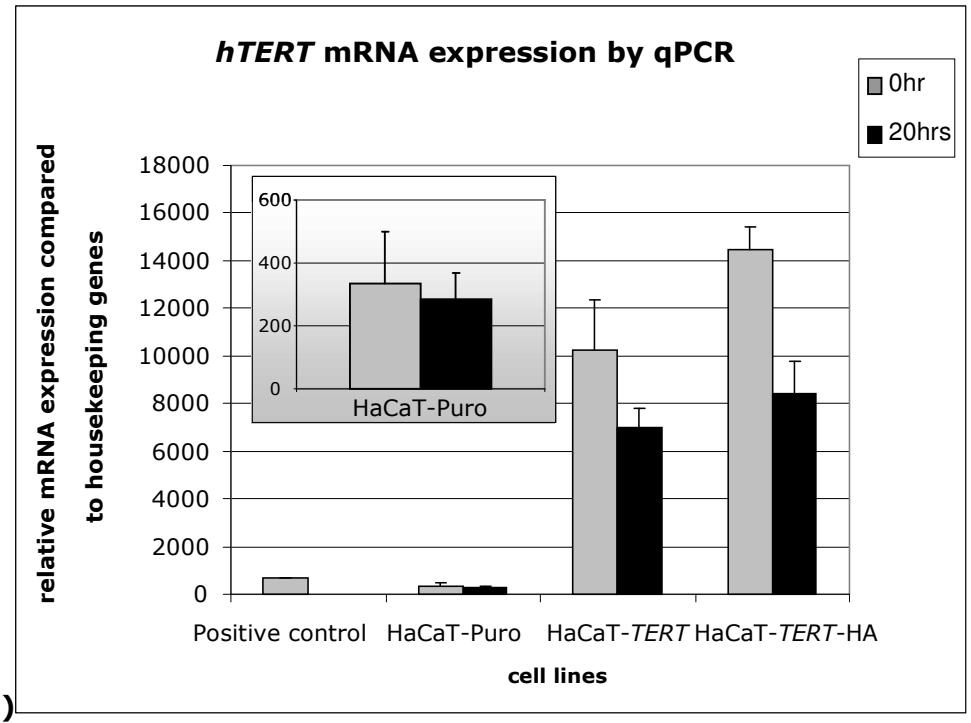
Normally, somatic cells express almost undetectable amounts of TERT, however a basal level of mRNA expression should always be detectable as shown in normal fibroblasts during their passage through the S phase of the cell cycle (Masutomi, Yu et al. 2003). On the other hand, cells expressing a construct with *hTERT* or *hTERT*-HA should highly express the corresponding mRNA. Therefore, I investigated *hTERT* and *hTERT*-HA mRNA expression in HaCaT carrying the empty vector and carrying *hTERT* and *hTERT*-HA by real-time PCR.

Quantitative real-time qPCR requires normalization of cDNA to reflect the same amount of initial RNA in each reaction, which may easily vary due to the high sensitivity of the method and the difficulty in quantifying with the same sensitivity, the amount of the starting material (Vandesompele, De Preter et al. 2002). Also, the difference in the expression in different cell lines illustrates the need for a standard for mRNA expression to use as a normalization tool. To increase the accuracy of the normalization, at least two control genes should be used, and their stability in the studied sample should be evaluated before proceeding with the gene of interest. mRNA levels of 4 housekeeping genes (*YAP1, POLR2A, HPRT1, ESD*, see Table 1, in the Materials and Methods section) were chosen from previous studies (Saviozzi, Cordero et al. 2006; Allen, Winters et al. 2008) and analysed using real-time PCR. Evaluation of the stability (M) of the gene expression profiles for each of the housekeeping genes was carried out using geNORM (Vandesompele, De Preter et al. 2002). M is a measure of how stable each housekeeping gene is with respect to the other housekeeping genes in the data set. A lower M value corresponds to a bigger stability; therefore that housekeeping gene is more suitable for normalization. All the 9 possible combinations of normalization using 4, 3 or 2 housekeeping genes (*YAP1-POLR2A-HPRT1-ESD; YAP1-POLR2A-HPRT1; POLR2A-HPRT1-ESD; POLR2A-ESD* etc.) were performed and the groups presenting the lowest average of M value were considered. However, *YAP1* was eventually removed form data analysis, since a

recent study has shown the involvement of this protein in apoptosis (Danovi, Rossi et al. 2007), which may interfere with our results. Therefore, the pair of housekeeping genes *ESD-HPRT1* (average M=0.668) was chosen for further normalization of *hTERT* mRNA expression in HaCaT cell lines and in the positive control (SVFN4 cells) (figure 11). As shown in figure 4, table b), at time 0, *hTERT* gene expression is approximately 30-fold and 43-fold increased in *hTERT* or *hTERT*-HA overexpressing HaCaT keratinocytes compared to control (HaCaT-Puro) cells, respectively.

After 20 hrs in suspension, anchorage-deprived cells show a lower amount of exogenous *hTERT* (or *hTERT*-HA) mRNA, corresponding to an average 1.6-fold decrease in *hTERT* or *hTERT*-HA. However, there was a non significant difference in the endogenous *hTERT* mRNA expressed by HaCaT-Puro at zero time and after 20 hrs of anchorage deprivation. Significantly, in anchorage-deprived cells, *hTERT* and *hTERT*-HA gene expression is still more abundant than in the empty vector control cells at zero time. The mRNA relative expression is approximately 21-fold and 25-fold increased in *hTERT* or *hTERT*-HA overexpressing HaCaT keratinocytes compared to control (HaCaT-Puro) cells, respectively. Results portrayed represent the means and standard deviations from 3 independent experiments.

a)



b) cell lines	relative h*TERT* mRNA expression compared to housekeeping genes		standard deviation	
	0 hr	20 hrs	0 hr	20 hrs
positive control	704.58	-	0.00	-
HaCaT-Puro	334.51	284.73	165.07	82.50
HaCaT-*TERT*	10224.22	6997.85	2128.07	793.12
HaCaT-*TERT*-HA	14460.60	8398.19	964.51	1358.98

g) HaCaT cells: groups for comparison	P value
Puro 0 hr - Puro 20 hrs	p>0.05 ns
Puro 0 hr - *TERT* 0 hr	P<0.001 ***
Puro 0 hr - *TERT* 20 hrs	P<0.001 ***
Puro 0 hr - *TERT*-HA 0 hr	P<0.001 ***
Puro 0 hr - *TERT*-HA 20 hrs	P<0.001 ***
Puro 20 hrs - *TERT* 0 hr	P<0.001 ***
Puro 20 hrs - *TERT* 20 hrs	P<0.001 ***
Puro 20 hrs - *TERT*-HA 0 hr	P<0.001 ***
Puro 20 hrs - *TERT*-HA 20 hrs	P<0.001 ***
TERT 0 hr - *TERT* 20 hrs	P<0.05 *
TERT 0 hr - *TERT*-HA 20 hrs	P<0.01 **

TERT 20 hrs - *TERT*-HA 0 hr	P<0.001 ***
TERT 20 hrs - *TERT*-HA 20 hrs	p>0.05 ns
TERT-HA 0 hr - *TERT*-HA 20 hrs	P<0.001 ***

Figure 11: Quantification of *hTERT* mRNA expression by real-time qPCR in HaCaT cells at control times (0 hr) and after 20 hrs in suspension during anoikis assay (20 hrs)

In graph a) and table b), relative amount of *hTERT* mRNA expression compared to HaCaT-Puro at time 0. Absolute values of mRNA copy numbers were normalized with the two set of housekeeping genes *ESD* and *HPRT1*. In the inner graph, inset of the relative amount of *hTERT* mRNA expression in HaCaT-Puro. SVFN (SV40-infected premalignant oral keratinocytes) were used as a positive control for *hTERT* expression.
The data are presented as means of three independent repeats and SD, and the values are reported in table b). ***: highly significant; **: very significant; * significant; ns: non significant.

3.4 Detection of h*TERT* splice variants mRNA expression

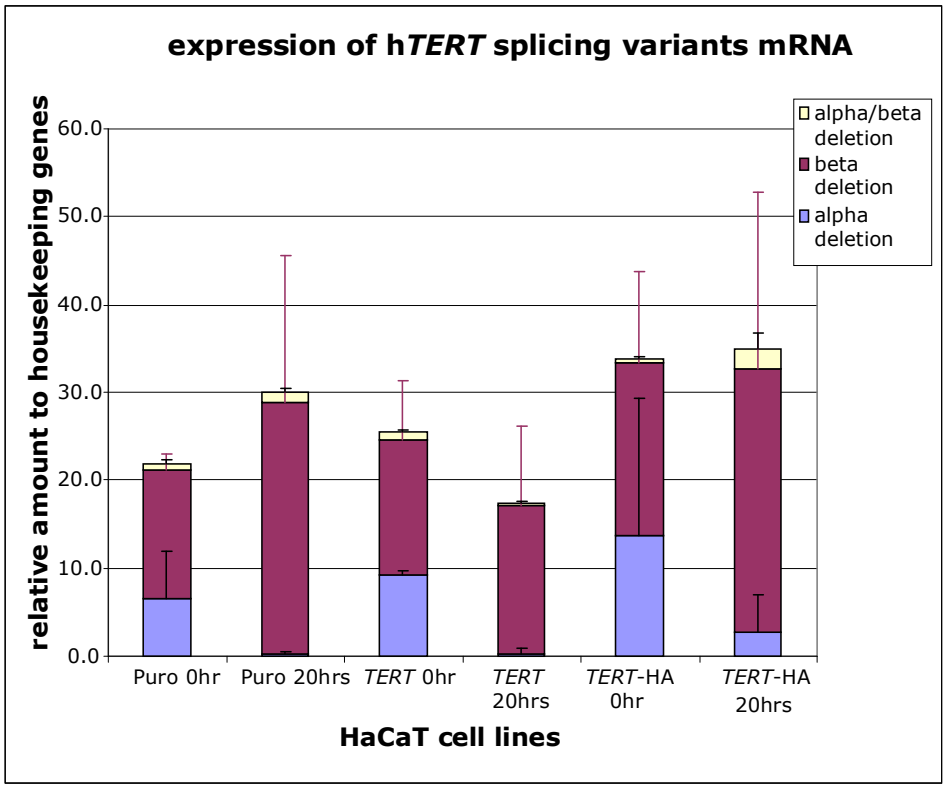
As explained in the introduction, section 1.1.5.2, alternative splice variants (ASV) of h*TERT* have been identified in numerous cell lines. A possible role in regulation of telomerase due to these ASV was therefore hypothesized by a number of studies. In particular, a study by Cerezo *et al.* (Cerezo, Kalthoff et al. 2002) suggests a possible implication of the beta (β) splice variant in regulation of telomerase activity in HaCaT cell lines.

Therefore, to investigate a possible role of alternative splice variants in our cell lines undergoing anoikis in an accurate way, I developed a real-time qPCR protocol that utilizes unique primer pairs for each splice variant. The expression of each variant is detected independently and quantified against the pair of housekeeping genes *ESD* and *HPRT1*. It is however necessary to consider that a precise quantification of h*TERT* splice variants is difficult considering the very low amount of these transcripts. Also, HaCaT-*TERT* and HaCaT-*TERT*-HA do not show any exogenous ASV, due to the presence of a constitutive promoter in the retrovirally-transduced vector.

As shown in figure 12, all HaCaT cells show a reduction in the alpha (α) splice variants (dominant negative variant) when deprived of anchorage. However, the decrease is not significant, and since this splice variant represents less than 2% of the endogenous h*TERT* transcript in HaCaT-Puro at time 0, it is possible to exclude a regulatory role of this ASV in h*TERT* expression.

The beta (β) splice variant represents 4.4% of the total endogenous h*TERT* transcript in HaCaT-Puro at time 0, but its expression does not significantly change at 20 hrs, or in the *TERT*/*TERT*-HA overexpressing cells.

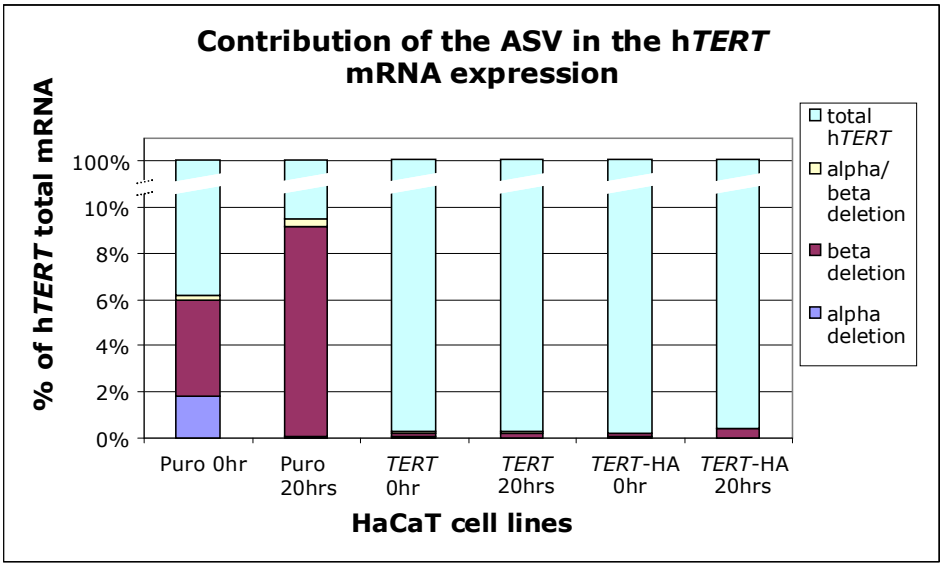
The alpha/beta (αβ) deletion contributes minimally to h*TERT* gene expression (less than 0.5% in HaCaT-Puro at time 0) and it is not significantly altered in any cell lines.



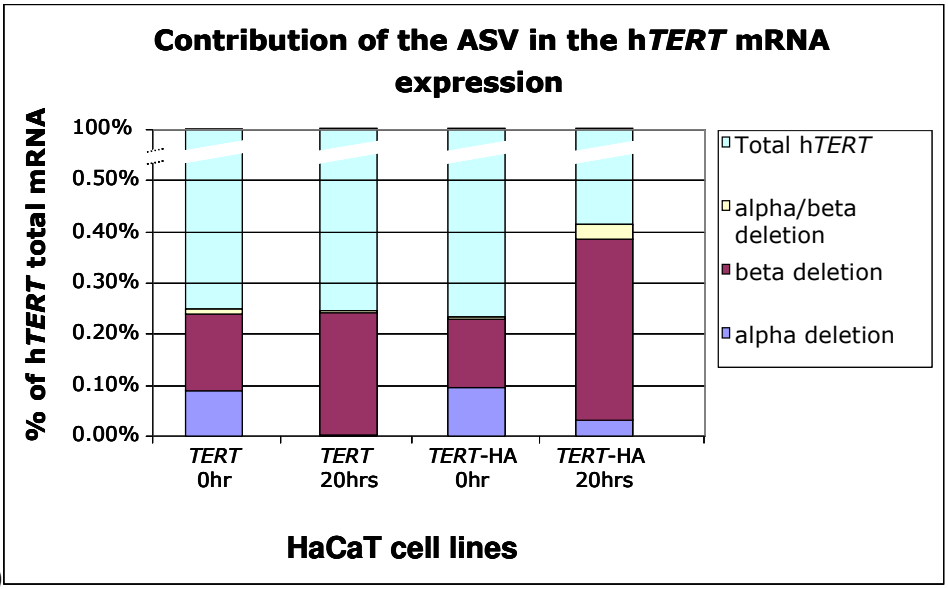
a)

b) Each splice variant expression is calculated as concentration relative to the pair of housekeeping genes				
cell lines	**average of alpha deletion expression**		**standard deviation**	
	0 hr	20 hrs	0 hr	20 hrs
HaCaT-Puro	6.46	0.21	5.56	0.18
HaCaT-*TERT*	9.23	0.29	0.46	0.50
HaCaT-*TERT*-HA	13.73	2.66	15.61	4.39
cell lines	**average of beta deletion expression**		**standard deviation**	
	0 hr	20 hrs	0 hr	20 hrs
HaCaT-Puro	14.82	28.71	1.70	16.57
HaCaT-*TERT*	15.31	16.77	6.89	9.02
HaCaT-*TERT*-HA	19.70	29.95	10.25	20.06
cell lines	**average of alpha/beta deletion expression**		**standard deviation**	
	0 hr	20 hrs	0 hr	20 hrs
HaCaT-Puro	0.68	0.99	0.39	0.47
HaCaT-*TERT*	0.99	0.26	0.29	0.23
HaCaT-*TERT*-HA	0.37	2.26	0.25	1.79

c)



d)



e) cell lines	% total h*TERT* mRNA	% alpha del ASV	% beta del ASV	% alpha/beta del ASV
HaCaT-Puro 0 hr	93.43	1.93	4.43	0.20
HaCaT-Puro 20 hr	89.50	0.07	10.08	0.35
HaCaT-*TERT* 0 hr	99.75	0.09	0.15	0.01
HaCaT-*TERT* 20 hrs	99.75	0.00	0.24	0.00
HaCaT-*TERT*-HA 0 hr	99.77	0.09	0.14	0.00
HaCaT-*TERT*-HA 20 hrs	99.58	0.03	0.36	0.03

Figure 12: Quantification of *hTERT* splice variants mRNA expression by real-time qPCR in HaCaT cells at control times (0 hr) and after 20 hrs in suspension during anoikis assay (20 hrs)

In graph a) and table b), relative concentration of *hTERT* splice variants normalized to the pair of housekeeping genes *ESD* and *HPRT1*. The data are presented as means and SD and average of three independent experiments.
In graphs c), d) and table e), relative contribution in percentage of each splice variant to the correspondent total (endogenous and exogenous) amount of h*TERT* mRNA is represented. In graph d), inset of *TERT* and *TERT*-HA expressing cells is shown. Variations in the mRNA amount between the gropus is not statistically significant ($p>0.05$). Contribution of ASV in the total h*TERT* mRNA is so low that a possible regulative role may be excluded in our cell lines.

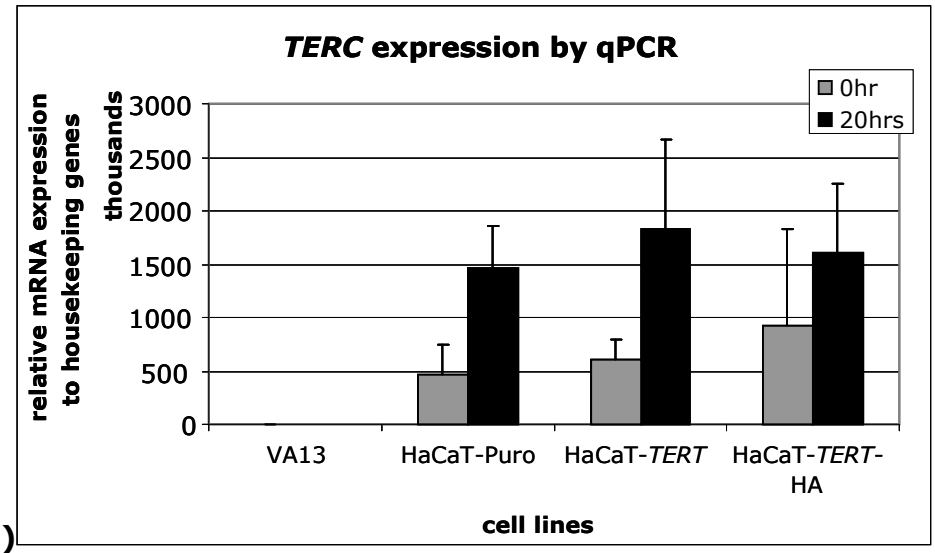
3.5 Detection of *TERC* RNA expression

Non-canonical functions of telomerase can be divided into two subgroups: the one requiring the presence of the RNA component *TERC* or the one which is independent from it (Parkinson, Fitchett et al. 2008).

As I showed above, exogenously-expressed telomerase is highly active even when the cells are deprived of anchorage, but to further categorize any observed phenotype in *TERT/TERT*-HA overexpressing HaCaT and relate it to a defined subgroup in the range of non-canonical functions, it is necessary to measure *TERC* expression in the cell at control time (0 hr, as shown in figure 13) and undergoing anoikis (20 hrs).

The expression of *TERC* was normalized against the pair of housekeeping genes *ESD* and *HPRT1*. HaCaT cells expressing the different constructs do not show a significant difference in *TERC* expression at control time, but show an increase when deprived of anchorage. The increase could be due to the stability of the *TERC* mRNA, which is known to have one of the longest half-lifes of all human mRNAs (Yi, Tesmer et al. 1999), or possibly to an unidentified stabilizing mechanism operating under conditions of anchorage deprivation.

a)



b) cell lines	average of *TERC* expression		standard deviation	
	0 hr	20 hrs	0 hr	20 hrs
VA13	48.21	-	0.00	-
HaCaT-Puro	452813.18	1459167.49	288817.24	398974.75
HaCaT-TERT	610826.61	1824166.86	177486.55	838392.54
HaCaT-TERT-HA	916887.20	1602932.45	910882.48	647018.22

Figure 13: Quantification of *TERC* expression by Real-time qPCR in HaCaT cells at control times (0 hr) and after 20 hrs in suspension during anoikis assay (20 hrs)

In graph a) and table b), relative amount of *TERC* expression compared to HaCaT-Puro at time 0. Absolute values of RNA copy numbers were normalized with the two set of housekeeping genes *ESD* and *HPRT1*. VA13 was used as a negative control for *TERC* expression, as VA13 is known to use the ALT system and lacks the RNA component of telomerase (Atkinson, Hoare et al. 2005).
The data are presented as mean of three independent repeats and SD, and the values are reported in table b). Variation among the samples is not statistically significantly ($p>0.05$).

3.6 Summary of results part 1

As explained in the section 1.1.4 of the introduction, telomerase has been involved in a variety of functions in germs, stem, cancer and even somatic cells. The main (canonical) function of telomerase is the ability to elongate the telomeres, however the enzyme has got other non-canonical functions, which are independent from telomere lengthening and can be either dependent on, or independent from, the RNA component *TERC*.

A study on the two-stage chemical epidermal tumourigenesis mouse model, showed the mice were more prone to form papillomas and show increased wound healing when *TERT* was overexpressed in the basal keratinocytes (Gonzalez-Suarez, Samper et al. 2001). This could be explained by a new function of TERT, independent of telomere lengthening (mice already having long telomeres), in the promotion of tumour formation. However, *TERT* overexpression did not affect the malignant conversion of the papillomas (Gonzalez-Suarez, Samper et al. 2001). In particular, TERT could act by giving the malignant cells an increased resistance towards a form of cell death specific to anchorage deprivation, termed anoikis. This is supported by a possible regulation of *ras*, which has been shown to be involved both in the two-stage chemical tumourigenesis model, where a mutation on the gene is necessary for the initiation of carcinogenesis and papilloma formation (Brown, Quintanilla et al. 1986), and in anoikis, where it inhibits the cascade leading to cell death (Frisch and Francis 1994).

To identify the role of telomerase in the protection against anoikis in skin keratinocytes, and define this function as novel and independent from telomere lengthening, different aspects of telomerase activity and the main components of this enzyme were measured.

The system I used permits the investigation of non-canonical functions of telomerase only thanks to the expression of a modified form of h*TERT*, *TERT*-HA. The influenza virus haemagglutinin (HA) epitope tag added at the carboxy-terminus does not affect the telomerase activity *in vitro* but

annuls the ability to elongate telomeres, as also shown by Counter's group (Counter, Hahn et al. 1998).

The drop in telomerase activity occurred in all HaCaT lines when cultured for 20 hrs in suspension, but the higher activity retained in *TERT* and *TERT*-HA overexpressing cells at this time point compared to the empty vector at time 0 suggests the necessity of an active TERT in protection against anoikis that I later observed (see result chapter 2, section 4.1). However, the ability of telomerase to elongate the telomere is not necessary in the protective function, as the same function also is observed in HaCaT-*TERT*-HA, whose telomerase cannot carry out its canonical function of telomere elongation.

The expression of the RNA component *TERC* has also been investigated to determine if TERT regulates *TERC* expression during anoikis. *TERC* was interestingly elevated in the cells that undergo anoikis after 20 hrs in suspension, demonstrating that the lack of telomere lengthening is not due to the lack of the RNA component but of a different function of the telomerase when cells are deprived of anchorage. It also confirm a previous study showing a possible stabilization of *TERC* due to the overexpression of the catalytic component (Yi, Tesmer et al. 1999).

Therefore, the catalytic component TERT seems to be the only direct candidate in the protection against anoikis observed in HaCaT keratinocytes overexpressing *TERT*/*TERT*-HA. In fact, although h*TERT* expression decreases after 20 hrs in suspension, it is still higher than the control.

The hypothesis that h*TERT* expression was regulated at transcriptional level was rejected because TERT was not downregulated in the HaCaT Puro controls in suspension. In addition the alternative splice variants in our cells were shown not to play a major role as had been suggested by previous semi-quantitative studies (Cerezo, Kalthoff et al. 2002). This would seem to be in contrast with what was shown in a previous study, which showed an increase in the inactive β splice variant when HaCaT cell

sheets are detached from the basement membrane (Cerezo, Kalthoff et al. 2002), and such a manipulation would trigger anoikis (Marconi, Atzei et al. 2004). However, I believe that the methods I have used to determine the expression of the splice variants, using independent primers for each splice variant, is more accurate than the one used by Cerezo *et al.*, where all splicing patterns were identified by a single pair of primers and the products quantified by densitometry following gel electrophoresis. Furthermore, the β splice variant produces an out-of-frame protein that may be recognised as foreign and not expressed at high enough levels to be a functional dominant-negative as suggested (Cerezo, Kalthoff et al. 2002). There is fact no evidence that any of the splice variants are naturally expressed as proteins at significant levels.

From my results, it is suggested that some post-transcriptional and post-translational mechanisms may regulate *TERT* mRNA and telomerase expression respectively during HaCaT anoikis.

Indeed there is evidence that a balance between the calcium-binding proteins S100A8 and S100A9 controls telomerase activity at the post-translational level (Rosenberger, Thorey et al. 2007) in differentiating keratinocytes. Furthermore, it has been shown that telomerase activity is not regulated by *TERT* mRNA levels in cervical cancer *in vivo* (Matthews, Shera et al. 2000), and *TERT* and *TERC* mRNA levels do not decrease immediately upon detachment from the basement membrane in epidermis whereas telomerase activity does (Harle-Bachor and Boukamp 1996).

My results from the enzymatic assay show telomerase activity in HaCaT cells at control times, confirming earlier findings detecting telomerase activity in the epidermis (Taylor, Ramirez et al. 1996). In addition, the drop of telomerase activity that I could detect when HaCaT are anchorage-deprived for 20 hrs is comparable with the results *in vivo* by Boukamp's group, where telomerase activity is detected in the regenerative basal layer of the skin but it is absent in the upper layers,

where keratinocytes differentiate and lose contact with the extracellular matrix (Harle-Bachor and Boukamp 1996).

The presence of telomerase activity in the epidermis, however, is not fully correlated with the expression of the mRNA for the two telomerase components *TERT* and *TERC*. In fact, both *TERT* and *TERC* can be detected not only in the basal layer of the epidermis, where telomerase activity is present, but also in the suprabasal of the epidermis, and the expression of TERT, in particular, follows a gradient of expression as detected by *in situ* hybridization (Nakano, Watney et al. 1998). These studies confirm my results, showing an invariant expression of *TERT* mRNA when control HaCaT are anchorage-deprived for 20 hrs compared to the time zero, and a decrease, but not annulment, of TERT expression in HaCaT overexpressing exogenous TERT or TERT-HA. I also detected the expression of *TERC* both when HaCaT are growing anchored to the ECM and when they grow detached from it. The increase of *TERC* in HaCaT after 20 hrs in suspension has been previously discussed in this section.

My results partly contradict the previous study by Crowe (Crowe, Nguyen et al. 2005), where telomerase activity is undetectable at time 0, then is suddenly detected at 8 hrs in suspension, and again is undetected at 24 hrs in suspension in NHEK. The levels of TERT mRNA measured by reverse transcription-PCR and not real-time qPCR, vary between undetectable at time 0, to high levels at 2 hrs in suspension, to a complete drop at 4 hrs, and a peak in expression at 8 hrs, followed by a lack of activity in the expression at 24 hrs. These results are not accompanied by an analysis of the expression of *TERC*, so it is difficult to determine a correlation between the variation in TERT mRNA levels and the telomerase activity found by this group. In addition, the detection of TERT protein by western blot cannot be evaluated, since a later study highlighted the inefficiency and non-specificity of the antibody use by Crowe (Wu, Dudognon et al. 2006).

Chapter 4:

Results 2

Chapter 4:

Results 2

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4. The role of *TERT* expression in the protection against anoikis (apoptosis in anchorage-deprived cells)

As more extensively explained in the introduction, a possible involvement of telomerase in the mechanism of apoptosis and the regulation of the enzyme by loss of attachment has been shown in normal human epidermal keratinocytes and squamous cell carcinoma lines, where anchorage deprivation inhibits telomerase activity, while increasing ERK1 activity in this setting contributes to cell cycle arrest. Re-adhesion to type IV collagen via specific integrin subunits α2β1 inhibits ERK1 activity and telomerase repression (Crowe, Nguyen et al. 2005).

Decreased ß1 integrin receptor density also been associated with loss of basement membrane attachment and terminal differentiation of these same cell types (Jones and Watt 1993). Also, keratinocytes enriched for stem cells are protected from anoikis via β1 integrin, in a Bcl-2 dependent manner, and disruption of β1 integrin activates the extrinsic apoptotic pathway (Tiberio, Marconi et al. 2002).

The importance of the pathway involving the focal-adhesion kinase FAK has also been correlated with cell survival, being able to suppress anoikis and transform kidney epithelial cells (Frisch and Francis 1994). Also, FAK activation is dependent on integrin-mediated cell attachment and the following signalling cascade leads to the activation of the MAP kinases ERK1 and ERK in human kidney epithelial and mouse fibroblast (Schlaepfer and Hunter 1996). Additionally, there is evidence that

epidermal growth factor (EGF) and its downstream effectors mitogen activated protein kinase (MAPK) and ERK can mute anoikis in HaCaT keratinocytes (Jost, Huggett et al. 2001).

FAK is known to be involved in signalling from growth-factor receptors and to influence the adhesion mechanics regulated by integrins, working together with the SRC-family kinases (McLean, Carragher et al. 2005).

Moreover, reduction in FAK protein expression in the epidermis has been directly associated with a reduction in the acquisition of neoplasia and malignancy and an increase keratinocyte cell death in the same two-stage chemical carcinogenesis mouse model (McLean, Brown et al. 2001; McLean, Komiyama et al. 2004) where a *Ras* mutation is known to be the initiating event (Brown, Quintanilla et al. 1986; Quintanilla, Brown et al. 1986). FAK is overexpressed in a number of human cancers, including squamous cell carcinoma (Agochiya, Brunton et al. 1999), as is the upstream effector of Ras, the EGF receptor (Gusterson, Cowley et al. 1985).

Telomerase is also over-expressed in papillomas and carcinomas generated by the mouse epidermal two-stage tumourigenesis protocol (Parkinson, Fitchett et al. 2008) and forced expression of *TERT* in the epidermis leads to improved wound repair and an increase in the number of papillomas but not carcinomas (Gonzalez-Suarez, Samper et al. 2001). Thus *TERT* promotes neoplasia rather than malignancy in this model. Telomerase is also expressed in human squamous dysplasias and carcinomas (Mao, El-Naggar et al. 1996).

Collectively current data support hypotheses encompassing telomerase regulation by ECM and a possible survival mechanism involving integrins and FAK/SRC kinases, *HRAS* and ECM. The involvement of these pathways in the survival of skin cancer cells is strongly indicated, since FAK (Frisch and Ruoslahti 1997), *Src* (Frisch and Francis 1994), EGFR (Jost, Huggett et al. 2001), and *HRAS* all mute anoikis (Frisch and Francis 1994).

Therefore the aim of this part of my study was to test the hypothesis that TERT mutes anoikis to enhance two-stage epidermal tumourigenesis. Moreover, protection against anoikis, if shown in the cell line model of HaCaT cells over expressing *TERT* and *TERT*-HA characterized above, would be a novel non-canonical function of telomerase.

To this hypothesis, I set up an experimental assay where I analyzed the behaviour of the HaCaT-Puro, HaCaT-*TERT*, HaCaT-*TERT*-HA, and HaCaT-Dn*TERT* when they are deprived from anchorage for 20 hours. I determined the specificity of cell death (anoikis rather than general apoptosis) after treatment of HaCaT cells in surface cultures with cisplatin, and two independent methods were utilized to confirm the results, studying two different sets of cells derived from two independently-transduced HaCaT populations.

4.1 The effect of disruption of cell-matrix interactions on phenotypic features of apoptosis

According to Frisch's protocol (Frisch and Francis 1994), HaCaT-Puro, HaCaT-*TERT*, HaCaT-*TERT*-HA cells were plated on petri dishes that had been coated with polyhydroxyethylmethacrylate (polyHEMA). PolyHEMA is in fact known to prevent cell attachment because its uniformly nonionic nature prevents matrix deposition (Folkman and Moscona 1978). Live and apoptotic cells were harvested for further analysis at time 0 (right after trypsinization) and at 20 hr. At this final time, some cells were loosely attached to the bottom of the coated dish, however these cells remained as single round cells and did not spread on the dish. Other cells were floating in the medium but all the cells were collected for the analysis.

The percentage of apoptotic cells was quantified by two independent methods, by morphological analysis of the nuclei, and by flow cytometry, as described in Materials and Methods. The results have been statistically analyzed independently with one way analysis of variance (ANOVA) test.

- **Morphological evaluation of the apoptotic nuclei**

Firstly, classical hallmarks of apoptosis, such as membrane blebbing, cellular shrinkage, chromatin condensation and nuclei fragmentation, have been detected by Hoechst staining of the nuclei in at least 300 nuclei per sample (figure 14). Blind analysis of 19 independent experiments was performed, and the results were subjected to the statistical test. All HaCaT samples harvested at time 0 present a minimal percentage of apoptotic cells (less than 1%), which is normally expected in recently trypsinized cells. On the other hand, HaCaT cells that underwent anchorage deprivation and overexpress *hTERT* (canonical function of telomerase) or *hTERT*-HA (non-canonical function of telomerase) show an average percentage of apoptotic cells of

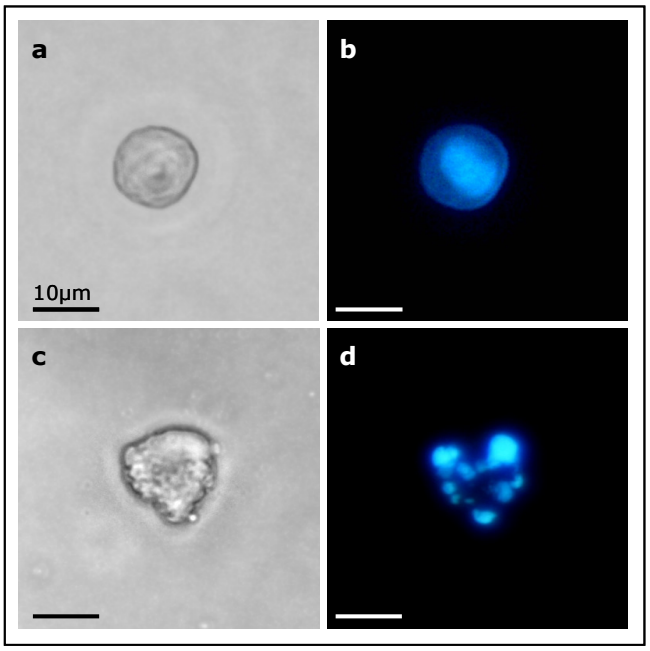
approximately 19 and 16-fold higher than the control at 0 hr, respectively, compared to an approximately 34-fold higher rate of apoptotic cells seen in the same suspended HaCaT-Puro compared to the same control at 0 hr. Therefore, *hTERT* overexpression was shown to increase resistance to apoptosis in HaCaT cells by an average of approximately 1.9 fold compare to HaCaT-Puro, and the result was proven to be statistically significant by one way ANOVA test and Tukey-Kramer multiple comparisons test (figure 14, table g).

- **Detection of the apoptotic nuclei by FACS**

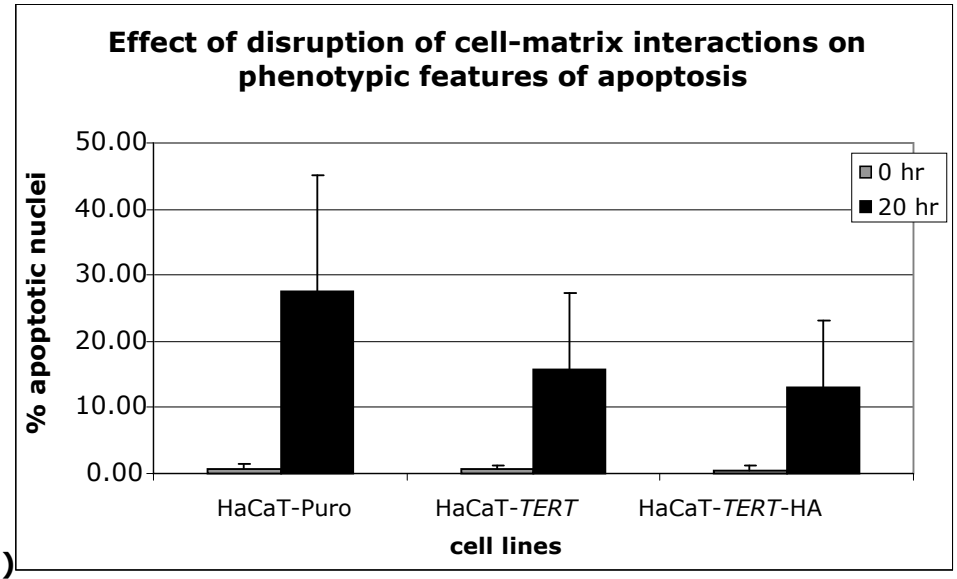
Secondly, flow-cytometric analysis of propidium iodide-stained cells was performed to detect the percentage of apoptotic cells present in sub-G1 peak of cell cycle analysis (figure 15) in 10 independent experiments, sorting at least 25000 cells. The bigger number of events analysed with FACS permitted a more accurate detection of apoptotic cells, and therefore there was no need for a bigger number of experiments to be performed to acquire a significant result. All HaCaT samples harvested at time 0 showed a minimal percentage of apoptotic cells (maximum 2%). On the other hand, HaCaT cells that underwent anchorage deprivation and overexpressed *hTERT* or *hTERT*-HA showed an average percentage of apoptotic cells that was approximately 8 and 6-fold that of the control at 0 hr respectively, compared to approximately 11-fold in the HaCaT-Puro controls. Therefore, *hTERT* overexpression was shown to increase resistance to anoikis in HaCaT cells by an average of approximately 1.6 fold compared to HaCaT-Puro, and the result was proven to be statistically significant compared to HaCaT-*TERT*-HA ($p < 0.05$) by one way ANOVA test and Tukey-Kramer multiple comparisons test (figure 15, table d).

It should be noted that different percentages of apoptotic cells were detected by the two methods used above. The bigger percentage of apoptosis estimated by morphological evaluation of the cells could be due to the inferior accuracy of the method and to the smaller number of cells

analyzed compared to FACS analysis. However, it is also possible that the visual observation method can detect cells in an earlier stage of apoptosis and can detect nuclei which are highly reduced in size, which the FACS method could mistakenly detect as debris. Furthermore, the level of significance in the rate of apoptotic cells between HaCaT-Puro and HaCaT-*TERT* after 20 hrs after detection of apoptosis by FACS is not as high as after morphological evaluation. This may due to the smaller sample size, the bigger variability of the assay and, as said before, the different amount of apoptotic cells detected by this method. Nevertheless, the significance of the results is shown at least with one sample, HaCaT-*TERT*-HA, making the pattern of the results obtained by both methods consistent, and strengthening the conclusions drawn. Therefore, I believe that a reduction in the variability of the assay (see section 4.4) and a bigger sample size would prove that the same pattern of resistance towards anoikis could be shown with FACS as well as with morphological evaluation of the nuclei.



e)



f) Cell lines	Average of cell death (%)		Standard deviation	
	0 hr	20 hrs	0 hr	20 hrs
HaCaT-Puro	0.82	27.49	0.82	27.49
HaCaT-*TERT*	0.63	15.87	0.63	15.87
HaCaT-*TERT*-HA	0.59	13.10	0.59	13.10

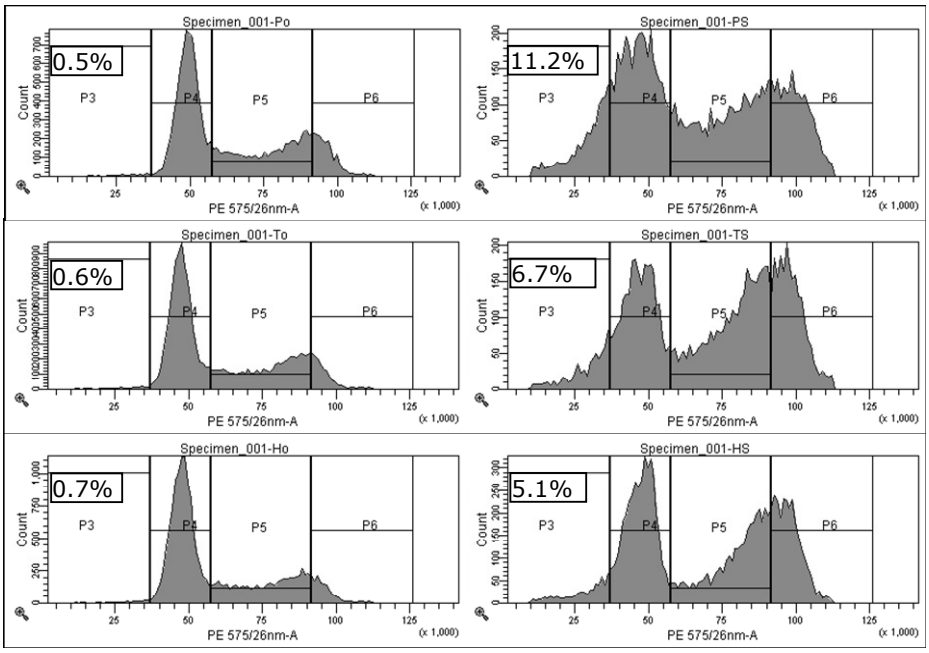
g) HaCaT cells: groups for comparison	P value
Puro 0 hr - Puro 20 hrs	P<0.001 ***
TERT 0 hr - *TERT* 20 hrs	P<0.001 ***

TERT-HA 0 hr - *TERT*-HA 20 hrs	P<0.01 **
TERT 20 hrs - Puro 20 hrs	P<0.01 **
TERT-HA 20 hrs - Puro 20 hrs	P<0.001 ***
TERT 20 hrs - *TERT*-HA 20 hrs	p>0.05 ns

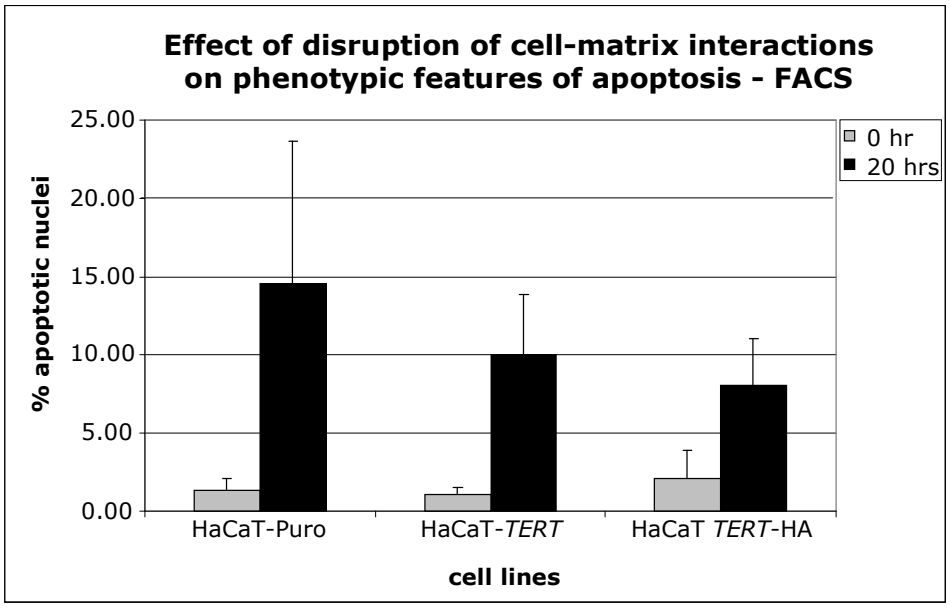
Figure 14: Effect of disruption of cell-matrix interactions on phenotypic features of apoptosis by Hoechst staining of nuclei

HaCaT cells (approximately 2 million cells) were plated onto petri dishes which had been coated with polyHEMA and after 20 hrs hallmarks of apoptosis in the nucleus were detected in the fixed cells and compared with cells fixed at time 0, as described in Materials and Methods.
Top picture: Comparison between a normal nuclei of an unaffected cell (a, b) with an apoptotic nucleus, showing membrane blebbing, cellular shrinkage, chromatin condensation and nuclei fragmentation (c, d).
e), table f) and table g). Anchorage-deprived HaCaT cells expressing exogenous *TERT* or *TERT*-HA are approx 1.9 fold more resistant to apoptosis than HaCaT expressing the empty vector. Results portrayed represent mean and standard deviation (SD) for 19 independent experiments and significance is shown as follows; p value showing no significant difference between the wild-type *TERT* construct and HA-flagged *TERT* in the response to anoikis after 20 hours of growth in suspension. ***: highly significant; **: very significant; * significant; ns: non significant.

a)



b)



c) Cell lines	Average of cell death (%)		Standard deviation	
	0 hr	20 hrs	0 hr	20 hrs
HaCaT-Puro	1.32	14.52	1.32	14.52
HaCaT-*TERT*	1.08	10.06	1.08	10.06
HaCaT-*TERT*-HA	2.13	8.05	2.13	8.05

d) HaCaT cells: groups for comparison	P value
Puro 0 hr - Puro 20 hrs	P<0.001 ***
TERT 0 hr - *TERT* 20 hrs	P<0.001 ***
TERT-HA 0 hr - *TERT*-HA 20 hrs	P<0.05 *
TERT 20 hrs - Puro 20 hrs	P>0.05 ns
TERT-HA 20 hrs - Puro 20 hrs	P<0.05 *
TERT 20 hrs - *TERT*-HA 20 hrs	p>0.05 ns

Figure 15: Effect of disruption of cell-matrix interactions on phenotypic features of apoptosis analysed by FACS

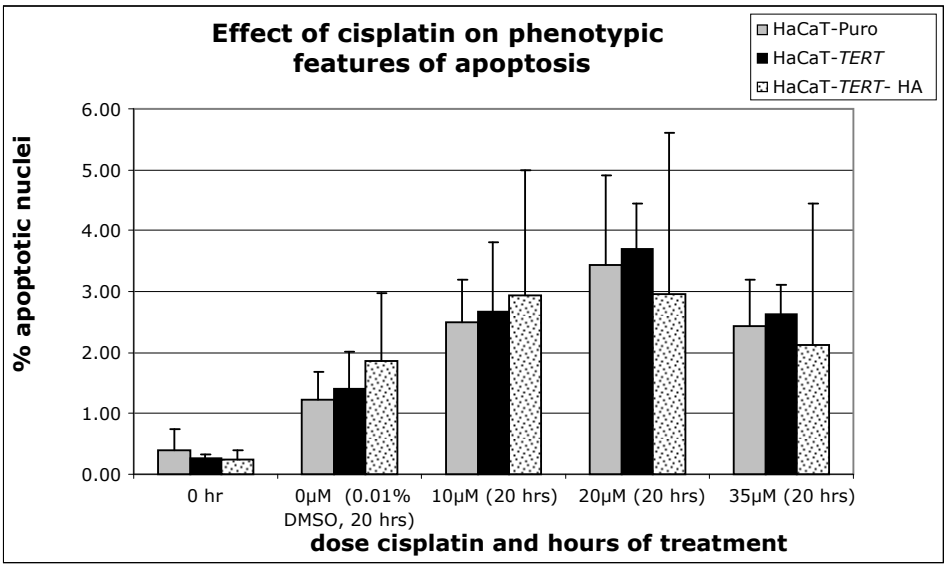
a) HaCaT cells (approximately 2 million cells) were plated onto petri dishes which had been coated with polyHEMA and after 20 hrs PI staining analysis to determine the percentage of apoptotic cells (Sub-G1 phase) was performed and compared with cells fixed at time 0, as described in Materials and Methods. In the left panel; representative FACS analysis of HaCaT-Puro (empty vector, P0), HaCaT-*TERT* (T0) and HaCaT-*TERT*-HA (H0) at time 0. Apoptotic cells are in P3 sector, corresponding to SubG1 peak; G1 cells in sector P4; S-phase cells in sector P5; M phase cells in sector P5. Apoptotic cells are less than 3% of the total events sorted. In the right panel, representative FACS analysis of HaCaT-Puro (empty vector, PS), HaCaT-*TERT* (TS), and HaCaT-*TERT*-HA (HS) harvested after 20 hr in suspension in polyHEMA coated dishes.
b) and table c). Anchorage-deprived HaCaT cells expressing exogenous *TERT* or *TERT*-HA are approximately 1.9 fold more resistant to apoptosis than HaCaT cells expressing the empty vector. Results portrayed represent mean and SD for 10 independent experiments and significance is as follows; p value showing no significant difference between the wild-type *TERT* construct and HA-flagged *TERT* in the response to anoikis after 20 hours of growth in suspension. ***: highly significant; **: very significant; * significant; ns: non significant.

4.2 Determination of nature of cell-death: general apoptosis or anoikis?

To test whether the resistance to cell death due to *hTERT* or *hTERT*-HA overexpression was directly correlated with anchorage-deprivation and was not a general form of apoptosis, treatment with non toxic doses (0 µM, 10 µM, 20 µM and 35 µM) of cisplatin was performed on HaCaT cells growing in adherence to tissue culture dishes (figure 16). Most forms of apoptosis are induced by drugs such as cisplatin, which cross-links DNA in several different ways, interfering with cell division in mitosis, although the mechanism has not yet been fully elucidated (Gonzalez, Fuertes et al. 2001)

In surface cultures cisplatin-treated *hTERT* or *hTERT*-HA overexpressing cells do not show any protection in drug-induced cell death compared to the corresponding control carrying the empty vector; therefore I can deduce that *hTERT* or *hTERT*-HA overexpression did not protect HaCaT cells against apoptosis induced by cisplatin. This suggests that the anoikis resistance phenotype induced by TERT is independent of apoptotic mechanisms associated with DNA damage.

a)



b)

Dose cisplatin	**Average of cell death (%)**		
	HaCaT-Puro	**HaCaT-*TERT***	**HaCaT-*TERT*-HA**
0 μM (0 hr)	0.40	0.40	0.40
0 μM (0.01% DMSO, 20 hrs)	1.23	1.23	1.23
10 μM (20 hrs)	2.50	2.50	2.50
20 μM (20 hrs)	3.43	3.43	3.43
35 μM (20 hrs)	2.43	2.43	2.43
Dose cisplatin	**Standard deviation**		
	HaCaT-Puro	**HaCaT-*TERT***	**HaCaT-*TERT*-HA**
0 μM (0 hr)	0.35	0.35	0.35
0 μM (0.01% DMSO, 20 hrs)	0.45	0.45	0.45
10 μM (20 hrs)	0.69	0.69	0.69
20 μM (20 hrs)	1.47	1.47	1.47
35 μM (20 hrs)	0.76	0.76	0.76

Figure 16: Effect of *TERT* on apoptosis analysed by Hoechst staining of nuclei in cisplatin-treated cells

HaCaT cells (approx 2 million cells) were plated onto tissue culture control dishes. Cells were treated with 0 μM, 10 μM, 20 μM and 35 μM of cisplatin and after 20 hrs hallmarks of apoptosis in the nucleus were detected in the fixed cells and compared with cells fixed at time 0, as described in Materials and Methods. Exogenous *hTERT* or *hTERT*-HA expression do not protect cells from cisplatin-induced cell death in HaCaT cells. Results portrayed represent mean and SD for 2 independent experiments.

4.3 The effect of the dominant negative (catalytically dead) *TERT* overexpressing HaCaT on anoikis

As some non-canonical functions of telomerase require the catalytic activity of telomerase (Gonzalez-Suarez, Samper et al. 2001), and some do not (Sarin, Cheung et al. 2005; Choi, Southworth et al. 2008; Park, Venteicher et al. 2009), I additionally tested whether telomerase activity was required to mute HaCaT anoikis. The catalytically-dead, dominant-negative TERT mutant, DnTERT, which has neither catalytic activity nor the ability to lengthen telomeres, was included in a second set of anoikis assays, together with a second population of transduced HaCaT-Puro, HaCaT-*TERT*, HaCaT-*TERT*-HA cells.

As shown in figure 17 (black bars), due to the modest difference in the percentage of apoptotic cells between the samples, a bigger sample size would be required to repeat exactly the result as before, where the protection due to *TERT* and *TERT*-HA expression was estimated approximately 1.9 fold compared to HaCaT-Puro. However, the same trend can be seen also in this experiment, where HaCaT-*TERT* and HaCaT-*TERT*-HA are on average 1.7 fold more resistant to anoikis than the control HaCaT-Puro at time 0.

The presence of the dominant negative version of TERT did not show the same level of protection, since anoikis resistance can be quantified as approximately 1.4 fold higher than HaCaT-Puro. This may suggest that the lack of a catalytic activity of TERT reduces the protection against anoikis and that catalytic activity is required for the observed phenotype even in the absence of telomere lengthening. However, the results may be confounded by the inhibition of the canonical functions of telomerase in telomerase-positive HaCaT cells and the Dn*TERT*-HaCaT cells do show evidence of increased anaphase bridge formation, which is consistent with telomere shortening and dysfunction (E. K. Parkinson – unpublished data). The last hypothesis is further supported by the observation that the Dn*TERT*-expressing HaCaT cells were not sensitised to anoikis, which

would be predicted if the DnTERT had no protective functions and that the protective function of TERT against anoikis required telomerase activity.

4.4 The Role of Cell Density on the Effect of *TERT* on Anoikis

Although the data showing TERT-induced protection is significant, the anoikis assay on HaCaT cells show great variability, and the reasons for this were not initially clear. Maintaining the same experimental conditions is essential as in any other study, but identifying the reason for a major variability in this assay could be useful for future work.

The possible role of cell culture confluence in modulating the rate of apoptotic cells during the assay was hypothesized during routine cell culture. HaCaT cells with the different *TERT* constructs behave differently during routine culture, as it has been observed by my colleagues in our group: HaCaT-*TERT* and HaCaT-*TERT*-HA seem to undergo a faster proliferation when the cells are at a higher confluence, while HaCaT-Puro and HaCaT-Dn*TERT* seem to proliferate quicker at a lower confluence. Also, other experiments undertaken by my colleagues have shown a possible dependence of cell confluence. These hints prompted me to perform the same anoikis assay with cells growing under different levels of confluence. The experiment was repeated three times and significance was evaluated by two-tailed ANOVA test.

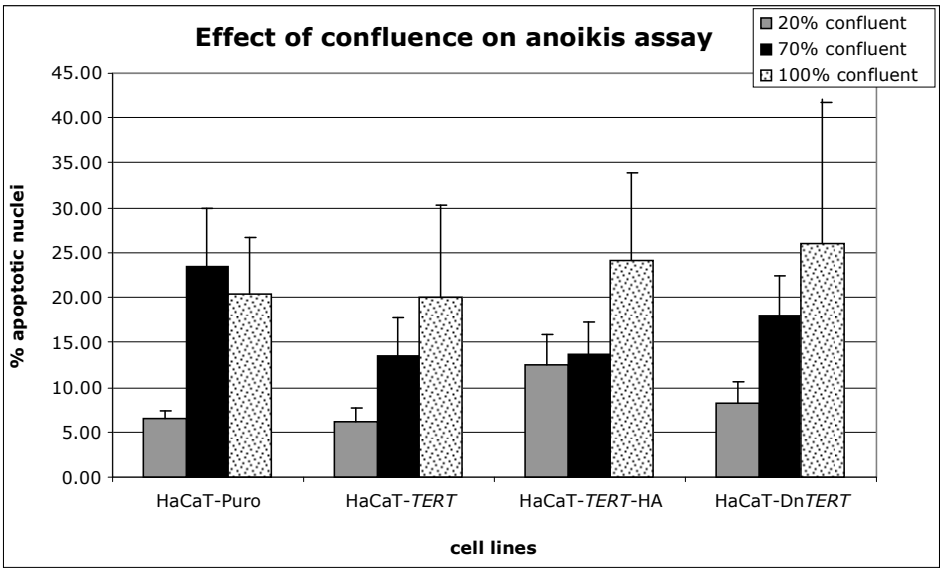
When all our experimental groups are harvested for the anoikis assay at low density (20% confluent), so that in the flask the interaction between the still small colonies is minimized, the resistance to anoikis is very high (figure 17). More than 90% of HaCaT are still alive after 20 hrs in suspension, and the presence of any *TERT* transgene in the cells does not significantly influence the apoptotic rate.

On the other hand, when cells were harvested at full confluence (100%), prior to being placed in suspension, so that in the culture flask HaCaT form a multilayer of cells and the interaction between the cells is maximal, the resistance to anoikis significantly decreases compared to the lowest confluence. Approximately 77% of HaCaT are still alive after 20 hrs in suspension, and also in this case the presence of any *TERT* transgene expressed in the cells does not significantly influence the apoptotic rate. Also, at this confluence variability between the experiment in the same cell line is greater than at the lowest confluence. To confirm that cell death of HaCaT at this confluence wasn't related to the depletion of the nutrients in the culture media, fresh medium was added the day before the experiment.

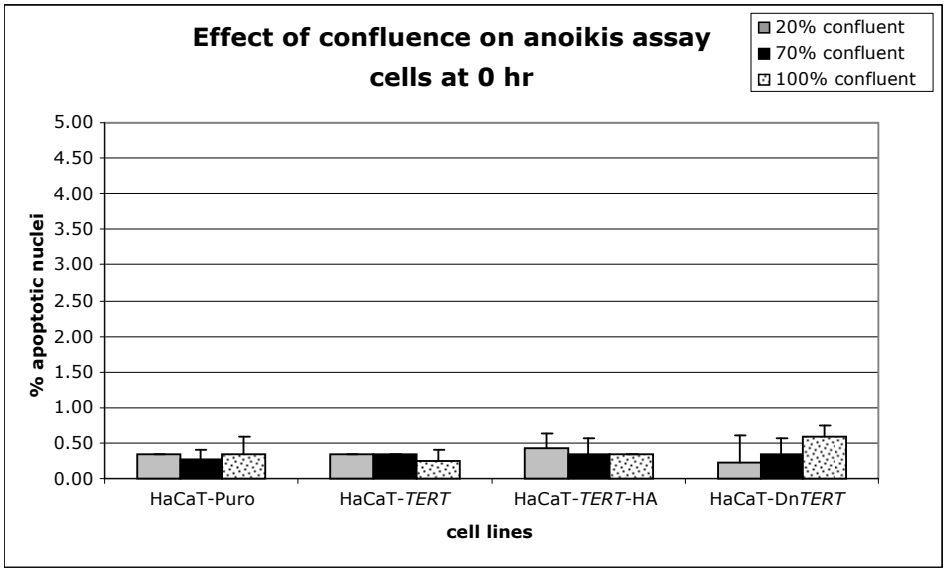
However, when the density of the cell culture (approximately 70% confluence) permits the cells to be in a proliferating state but also to gain inter-cellular interactions, an effect of TERT overexpression on cell apoptosis can be more reproducibly seen. As shown by section 4.1 and 4.3 of this result chapter, *TERT* expression confers significant resistance to anoikis after 20 hrs in suspension to HaCaT-*TERT* and HaCaT-*TERT*-HA, while HaCaT-Dn*TERT* is less effective.

From this data I can conclude that one aspect to consider when performing this kind of anoikis assay is the cell density prior to the experiment. Variation depending on density could be due to the increasing inter-cellular interactions within the culture, which promotes anoikis in an overconfluent culture (Frisch and Francis JCB 1994), or to the different cell-cycle and density-dependent expression of the proteins targeted by TERT to confer protection against anoikis.

a)



b)



c) Cell lines	**Average (%) of apoptotic cell death after 20 hrs in suspension**		
	Cell confluence before assay		
	20%	**70%**	**100%**
HaCaT-Puro	6.49	23.36	20.33
HaCaT-*TERT*	6.15	13.49	20.09
HaCaT-*TERT*-HA	12.52	13.73	24.16
HaCaT-Dn*TERT*	8.16	18.02	26.03
Cell lines	**Standard deviation**		
	Cell confluence before assay		
	20%	**70%**	**100%**
HaCaT-Puro	0.86	6.66	6.34
HaCaT-*TERT*	1.62	4.27	10.19
HaCaT-*TERT*-HA	3.44	3.59	9.73

HaCaT-Dn*TERT*	2.46	4.48	16.12

d) HaCaT cells: groups for comparison	**P value**
20% confluence	
Puro 20 hrs – *TERT* 20 hrs	P>0.05 ns
TERT 20 hrs – *TERT*-HA 20 hrs	P<0.05 *
Puro 20 hrs - *TERT*-HA 20 hrs	P<0.05 *
Puro 20 hrs - Dn*TERT* 20 hrs	P>0.05 ns
TERT 20 hrs - Dn*TERT* 20 hrs	P>0.05 ns
TERT-HA 20 hrs - Dn*TERT* 20 hrs	P>0.05 ns
70% confluence	
Puro 20 hrs – *TERT* 20 hrs	P<0.05 *
TERT 20 hrs – *TERT*-HA 20 hrs	P<0.05 ns
Puro 20 hrs - *TERT*-HA 20 hrs	P<0.05 *
Puro 20 hrs - Dn*TERT* 20 hrs	P>0.05 ns
TERT 20 hrs - Dn*TERT* 20 hrs	P>0.05 ns
TERT-HA 20 hrs - Dn*TERT* 20 hrs	P>0.05 ns
100% confluence	P=0.89 ns

e) Cell lines	**Average (%) of apoptotic cell death at 0 hr**		
	Cell confluence before assay		
	20%	**70%**	**100%**
HaCaT-Puro	0.33	0.26	0.33
HaCaT-*TERT*	0.33	0.33	0.25
HaCaT-*TERT*-HA	0.44	0.33	0.33
HaCaT-Dn*TERT*	0.22	0.33	0.58
Cell lines	**Standard deviation**		
	Cell confluence before assay		
	20%	**70%**	**100%**
HaCaT-Puro	0.00	0.15	0.27
HaCaT-*TERT*	0.00	0.00	0.17
HaCaT-*TERT*-HA	0.19	0.23	0.00
HaCaT-Dn*TERT*	0.38	0.23	0.17

Figure 17: Effect of confluence of the cell culture in the rate of apoptotic assay performed with the anoikis assay

As in fig. 14, HaCaT cells (approximately 2 million cells) were plated onto petri dishes which had been coated with polyHEMA and after 20 hrs hallmarks of apoptosis in the nucleus were detected in the fixed cells and compared with cells fixed at time 0, as described in Materials and Methods.
Differently from fig. 14, these are a new population of cells transduced with the different transcripts. Also the new transcript Dn*TERT* has been included in the cell line HaCaT-Dn*TERT*, characterized by the expression of a catalytically inactive TERT which is unable to elongate telomeres.
Before plating the cells in the polyHEMA dishes, HaCaT were cultured at different confluences: low density (20% confluent), medium density (70% confluent) and high density (100% confluent).
a), b), table b), c) and d) Anchorage-deprived HaCaT cells expressing exogenous *TERT* or *TERT*-HA are respectively approximately 1.7 fold more resistant to apoptosis than HaCaT expressing the empty vector and compared to HaCaT-Dn*TERT*, but this effect is seen just when the cells derive from 70% confluence. At the lowest confluence, the percentage of apoptotic cells is approximately 6%, and it is not significantly affected by the presence of *TERT*. At full confluence, the percentage of apoptotic cells approximately 23%, and it is not affected by the presence of *TERT* either. Results portrayed represent mean and SD for 3 independent experiments for 20% and 100% confluence, and 5 repeats for 70% confluence. In the table c), the significance values are shown for each relevant groups of comparison. Significance is as follows; p value showing no significant difference between the wild-type *TERT* construct and HA-flagged *TERT* in the response to anoikis after 20 hours of growth in suspension. ***: highly significant; **: very significant; * significant; ns: non significant. The significance of the cells growing at 70% confluence prior to the anoikis assay is concordant to the results shown in figure 14, confirming once more the phenotype observed. It was not possible to perform Anova test on the last set of experimental group (100% confluence prior to the anoikis assay) because of the big variation and the sample size.

4.5 Summary of results part 2

Normal human epidermal keratinocytes and squamous cell carcinoma (SCC) lines have been shown to be sensitive to programmed cell death when deprived from anchorage to the ECM (Tiberio, Marconi et al. 2002), although SCC cells are more resistant than normal keratinocytes (Rheinwald and Beckett 1980). This particular form of apoptosis has been termed anoikis and it can be distinguished from other forms of apoptosis by treating cells with cisplatin, a DNA damaging agent that induces apoptosis initially through the release of cytochrome c from the mitochondria and only later through the exogenous pathways mediated by caspase 8.

The regulation of anoikis by extracellular molecules has been already proved by several studies and pathways deriving from FAK/SRC kinases and therefore *HRAS* have been involved as well in the phenomenon.

Since *TERT* expression has been shown to be regulated by integrin expression in keratinocytes, it could be hypothesised that telomerase covers a role in the apoptotic phenotype by regulation of integrin expression of the downstream derived pathway. In particular, considering the involvement of the various abovementioned kinases not only in anoikis but also in the two-stage chemical tumourigenesis mouse model, TERT may act in promoting neoplasia and malignancy in this model by protecting the cells towards anoikis by regulating HRAS and FAK/SRC kinases pathway.

HaCaT cells are spontaneously immortalized but non-transformed human keratinocytes that have been widely used as a model for studies on keratinocytes differentiation. As explained in results part 1, wild-type h*TERT*, *TERT*-HA (active *in vitro* but unable to elongate telomeres of cultured HaCaT cells) and Dn*TERT* (dominant negative *TERT*) were transduced into the cells, and the rate of cells undergoing anoikis, after

disruption with the matrix, was evaluated in the three cell lines and compared with HaCaT-Puro (empty vector, control).

After 20 hrs in suspension, when integrins are disengaged from the ECM, cells die at a different rate; *hTERT* and *TERT*-HA overexpression was shown to significantly increase resistance to anoikis in HaCaT cells by an average of approx 1.9 fold compared to HaCaT-Puro, however this protection was not seen in HaCaT-Dn*TERT*. This particular form of cell death was exclusive to anchorage deprivation and TERT overexpression did not protect adherent HaCaT cells treated with subtoxic doses of cisplatin, confirming that the observed phenomenon is anoikis and not general apoptosis.

The data suggest that TERT must be catalytically active to optimally promote resistance to anoikis in HaCaT, and the resistance effect is independent of telomere lengthening but dependent on density in cell culture.

The protection against anoikis due to *TERT* overexpression could be argued to be subtle, but it was observed over a big number of repeats (19 repeats), with two different methods and in some cases with two different batches of retrovirally-transduced HaCaT cells.

First, the variability of the anoikis assay needs to be considered: difference in the cell density before the experiment was shown to influence greatly the amount of apoptotic cells, and the protection to anoikis was particularly observed when the cells before the experiment were growing in the exponential phase, up to 70% confluence. Also, *TERT* is not completely repressed in HaCaT-Puro, as shown in results part 1, so its expression could attenuate the difference between the cell lines. Finally, my anoikis assays showed a lower rate of apoptotic cells compared to a different anoikis assay in polypropylene tubes performed in a previous study (Marconi, Atzei et al. 2004), which showed up to 60% of HaCaT undergoing anoikis against 30% in my experiment after microscopic evaluation of the nuclei.

Therefore it can be concluded that TERT may provide a new role in the promotion of neoplasia and malignancy, protecting the cells that overexpress it against anoikis, therefore making them more resistant and potentially able to migrate and invade other tissues. This phenomenon is independent from telomere lengthening, therefore this role may be part of the reason by which mice treated with DMBA and TPA and overexpressing TERT in the basal keratinocytes are more prone to skin carcinogenesis in spite of the murine long telomeres, as shown by a previous study (Gonzalez-Suarez, Samper et al. 2001).

The effects of TERT on anoikis are similar to those reported by Lee *et al.* (Lee, Sung et al. 2008) for motor neurones in that the effects of telomerase in muting anoikis do not absolutely require telomerase catalytic activity. As TERT requires *TERC* (and hence telomerase activity) to enhance wound healing and the production of papillomas in the two-stage carcinogenesis system, this suggests that the muting of anoikis may not be sufficient for these phenotypes although it is still possible that it is essential.

Furthermore, the observation that anoikis resistance induced by TERT can be affected by the state of confluence in the cells before the experiment could be related to recent studies on a relation between TERT and Wnt pathway.

Recently, an involvement of TERT in the Wnt/β-catenin signalling has been shown and correlated with transition from telogen to anagen in the hair follicle of mice conditionally expressing *TERT*; in addition, this function does not require RT activity of the enzyme (Sarin, Cheung et al. 2005; Choi, Southworth et al. 2008). In this animal model, *TERT* expression regulates the expression of a series of genes involved in development, signal transduction and adhesion, which are closely related to the genes similarly regulated by Myc and Wnt pathways (Choi, Southworth et al. 2008). Further studies have shown that TERT occupies position in the promoters of genes targeted by Wnt, and can therefore

coact with β-catenin to regulate transcription in these genes (Park, Venteicher et al. 2009).

β-catenin is a multifunctional protein that changes localization dependently on the presence of Wnt signal. In the absence of Wnt, β-catenin binds to cadherin and it becomes part of the complex of adherens junctions, necessary for adhesion and growth of epithelial cells. The free β-catenin that is not bound to cadherin is subject to degradation after the binding with the protein GSK3. In the presence of a Wnt signal, a cascade of events leads to the inhibition of GSK3, and therefore β-catenin, not degraded, can enter the nucleus and bind to specific factors to activate the transcription of Wnt target genes, among which Myc to promote proliferation (Alberts 2002). In addition, Wnt has been shown to protect cells against chemotherapy-induced apoptosis by recruiting β-catenin for the activation of transcription of its target genes in human colorectal cancer cells (Chen, Guttridge et al. 2001).

Therefore, for my observation on the state of confluence of HaCaT cells, it is possible to speculate the presence of a fine balance between the rate of β-catenin involved in adherence junctions (in bigger number when the cells are confluent) and free β-catenin involved in the Wnt pathway. If TERT is able to coact with β-catenin in the Wnt signalling, to produce an anti-apoptotic signal, then cells at a lower confluence which have a higher amount of free cytoplasmic β-catenin, would also possess an increased resistance towards apoptosis, consistent with my results. This hypothesis is testable in a number of ways, such as western blot, immunoprecipitation, or reporter assays.

However, the pathways through which TERT regulates the apoptotic fate of the cells are under investigation. Since anoikis involves a complex cascade of protein starting from interaction with the ECM, integrins are the first choice for targeting the pathway and to indentify exactly at which point TERT acts, so this was investigated in the next result chapter.

Chapter 5:

Results 3

Chapter 5:

Results 3

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5. Testing the regulation of integrin expression by *TERT* overexpression in the protection against anoikis

Human keratinocytes located in the basal layer of the epidermis are still undifferentiated and express different type of integrins (see Table 9). All the receptors studied up to date are expressed in the basal layer of the epidermis, with the only exception being the receptor for vitronectin αvβ8, that is expressed in the suprabasal layer (Watt 2002).

The correct balance between cell proliferation and terminal differentiation (homeostasis) is maintained by the regulated expression of these receptors for extracellular matrix molecules, as confirmed by a variety of malignancies showing a deregulation of integrin receptors, such as squamous cell carcinoma, where α6β4 is expressed in the suprabasal level of the epidermis and promotes the progression of the malignancy from the initial benign papilloma (Tennenbaum, Weiner et al. 1993).

In keratinocytes, integrin expression has been correlated with adhesion to the substrate, migration, motility, wound healing, and with progression to malignancy (Watt 2002).

However, the main function of integrin expression in neoplasia and malignancy is in the process known as terminal differentiation, where the keratinocytes migrate from the basal layer to the outer surface and acquire a cornified envelope (Adams and Watt 1990). During this process, the maturation and transport of integrin β1 is inhibited (Hotchin,

Gandarillas et al. 1995) and therefore the presence of the receptors associated with this integrin in the cell membrane is reduced. The importance of the specific integrin β1 has been confirmed after showing that terminal differentiation can be inhibited with the addition of fibronectin (Adams and Watt 1989) or with an antibody against the β1 subunit (Watt, Kubler et al. 1993).

Table 9: Expression of integrins in skin keratinocytes	
Receptor specificity	**Integrin dimer**
collagen and laminin	α2β1
epiligrin and laminin	α3β1
fibronectin	α5β1
fibronectin	αvβ1
tenascin	α9β1
tenascin, fibronectin and vitronectin	α8β1
vitronectin	αvβ5
fibronectin and tenascin	αvβ6
vitronectin	αvβ8
associated with hemidesmosomes and intermediate filament system	α6β4

Table 9: Integrins detected in skin keratinocytes

Integrins are receptors formed by the interaction of two subunits, α and β. The specificity of the ligand recognized by integrins depends on the subunits forming the receptor. Integrins are cell and tissue specific; in this table, the receptors expressed in skin keratinocytes are shown.

Different studies have compared anoikis and terminal differentiation in keratinocytes (Gandarillas 2000) and have found parallels in the level of importance of integrins and other pathways in the two processes. However, there are also many differences between anoikis and terminal differentiation, such as the lifetime of the cell undergoing the process (which is as long as weeks in differentiation compared to hours in

anoikis) and the cell morphology before anucleation (Gandarillas, Goldsmith et al. 1999).

As explained in the introduction of this chapter, β1 integrin in particular is crucial in the apoptotic destiny of different types of cells, not only keratinocytes, but in a variety of different cell types. Reduced expression of this integrin in subclones of pancreatic cancer cells confers a resistance in anoikis. This resistance is specific to β1 integrin, and independent from the reduction of the α5 and α6 integrins (Walsh, Clynes et al. 2009). Anchorage deprivation-induced apoptosis is also inhibited in islets of Langerhans after treatment with an anti-β1 integrin antibody or RGD peptides (that mimic the ECM molecules) by preventing the activation of caspase-3 (Pinkse, Bouwman et al. 2006). Also in endothelial cells growing in suspension, anoikis (in this paper from 1993 anoikis was still defined as programmed cell death) is inhibited when cells are plated on dishes coated with a β1 integrin antibody (Meredith, Fazeli et al. 1993). In fibroblasts β1 integrin has been implicated in the survival pathway when the cells are growing in collagen matrix, by regulating the (PI3K)/Akt pathway and triggering apoptosis (Tian, Lessan et al. 2002; Xia, Nho et al. 2004).

Specifically, α3β1 integrin is shown to be necessary and sufficient in protecting from apoptosis in MK cells, a keratinocyte cell line derived from wild-type and α3-knockout mice, via FAK and ERK activation and through inhibition of caspase-3 activation (Manohar, Shome et al. 2004). In addition, skin keratinocytes enriched for stem cells are protected from anoikis via β1 integrin in a Bcl-2 dependent manner, and disruption of β1 integrin activates the extrinsic apoptotic pathway (Tiberio, Marconi et al. 2002).

In a different study Janes and colleagues showed that in squamous cell carcinoma, a change in the expression of integrin from avβ5 to avβ6 is related to an increased protection against anoikis. In particular, anoikis is promoted by avβ5 by inhibition of Akt; Akt activation is increased when caspase-8 is inhibited, but the apoptotic pathway does not involve signals

from the death receptors, that is the most common feature of the caspase-8 –related intrinsic apoptotic pathway (Janes and Watt 2004).

From these observations, it is possible to hypothesise a possible involvement of integrins in the resistance to anoikis shown in my experimental HaCaT cell lines overexpressing *TERT* and *TERT*-HA.
I therefore analyzed the expression of a panel of surface integrins in HaCaT-Puro, HaCaT-*TERT*, HaCaT-*TERT*-HA and HaCaT-Dn*TERT* by FACS analysis, and the expression of the total amount of integrins from whole cell lysates in the same cell lines, at control time (0 hr) and after the cells were cultured in suspension for 20 hrs.

5.1 Analysis of integrin expression on the cell surface of HaCaT cells after *TERT* and *TERT*-HA overexpression

To investigate the involvement of integrins in the resistance of HaCaT cell lines overexpressing *TERT* and *TERT*-HA to anoikis, I first tested whether there was a correlation between *TERT* overexpression and integrin expression in normal surface cell culture conditions.

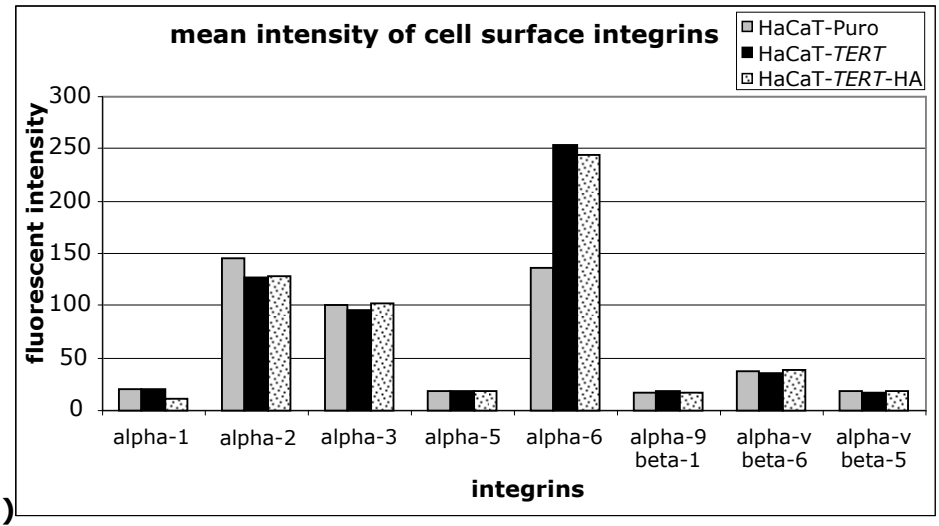
A panel of integrins was first analyzed by FACS on a first generation batch of HaCaT-Puro, HaCaT-*TERT* and HaCaT-*TERT*-HA cells and compared with the appropriate isotype controls. The choice of selection for this panel was partly the availability in our lab and in Dr. John Marshall's laboratory. Also, the wide range of alpha subunits embraces the majority of the receptors formed with the integrin β1 in human keratinocytes.

In this preliminary experiment (figure 18), HaCaT showed positivity for the integrin subunits α2, α3, α6 and for integrin αvβ6, the receptor for fibronectin and tenascin. Particularly interesting was the case of the integrin subunit α6, which expression was almost double in HaCaT-*TERT* and HaCaT-*TERT*-HA compared to HaCaT-Puro. The expression on the cell

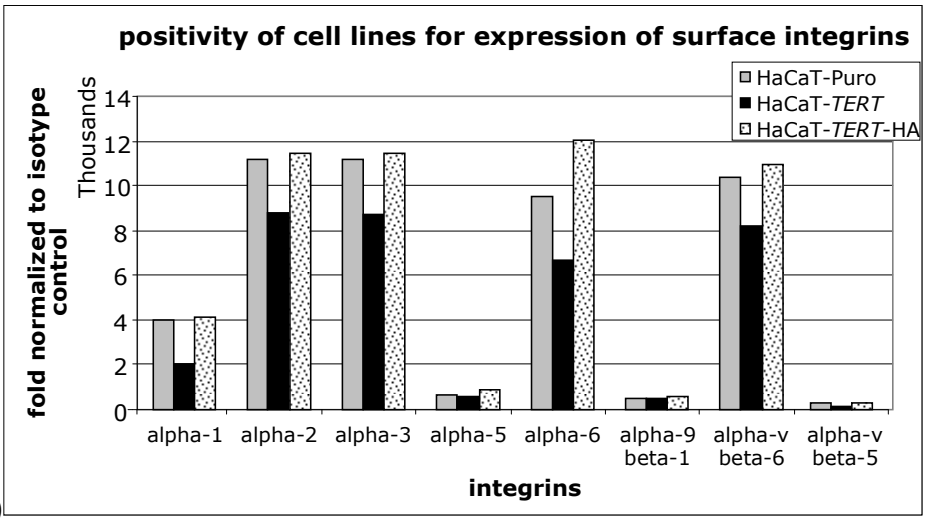
surface in the other subunits was not affected by the overexpression of *TERT* and *TERT*-HA in HaCaT.

The expression analysis on the cell surface of the subunit α6 was therefore repeated together with the subunits α4, β1 and β4 in a second batch of HaCaT-Puro, HaCaT-*TERT*, HaCaT-*TERT*-HA, and HaCaT-Dn*TERT.* The second batch of HaCaT lines were transduced with the retroviral constructs independently from the first batch but showed a similar level of protection against anoikis by the *TERT* constructs (see section 4.4). The experiment was repeated three times to increase the statistical significance (figure 19). Even allowing for the big variability in the percentage of cells positive for integrin α6 in the three experiments, the *TERT*-dependent increase in expression of this subunit was not confirmed with the new population of cells. The other two subunits which were positively expressed on the cell surface of HaCaT were β1 and β4. However, no significant difference in the expression of these subunits on the cell surface of HaCaT due to *TERT* overexpression could be detected.

a)



b)



c) Integrins	Fold mean intensity of cell surface integrins expression		
	HaCaT-Puro	HaCaT-*TERT*	HaCaT-*TERT*-HA
α1	20.37	19.89	11.17
α2	145.54	126.98	128.82
α3	99.90	95.57	102.82
α5	18.57	18.47	18.49
α6	136.69	254.16	244.33
α9β1	17.14	18.00	17.73
αvβ6	36.79	35.51	38.70
αvβ5	17.85	17.21	18.77

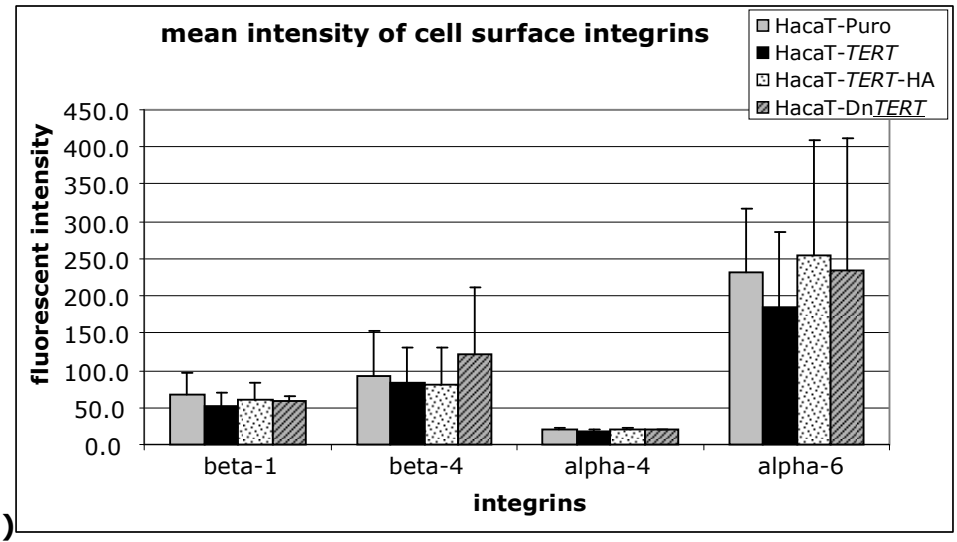
d) Integrins	**Fold mean positivity of cell surface integrins expression normalized to isotype controls**		
	HaCaT-Puro	HaCaT-*TERT*	HaCaT-*TERT*-HA
α1	3998.88	2066.67	4139.08
α2	11197.75	8763.16	11471.26
α3	11151.69	8718.42	11440.23
α5	686.52	581.58	870.11
α6	9493.33	6706.04	12043.37
α9β1	511.24	516.67	571.26
αvβ6	10364.04	8221.05	10968.97

e) controls	**Fold mean intensity of isotype controls expression normalized to unstained cells**		
	HaCaT-Puro	HaCaT-*TERT*	HaCaT-*TERT*-HA
unstained	100.00	100.00	100.00
IgG1	111.38	107.78	107.77
IgG2	103.09	101.76	106.21

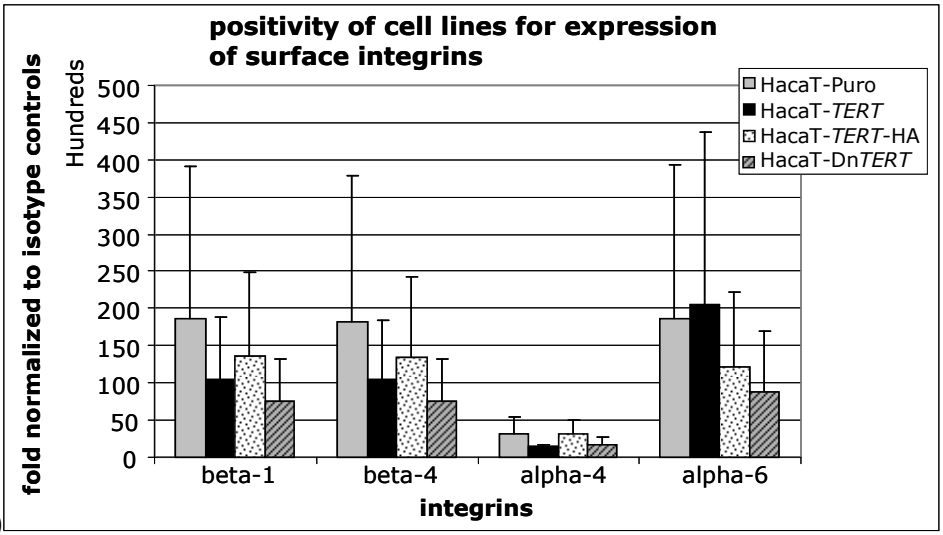
Figure 18: Preliminary analysis of the effect of *TERT* overexpression in regulating the expression of integrins on the cell surface of HaCaT

Graph a) and table c): analysis of the intensity of integrins expression on cell surface. Expression in HaCaT overexpressing *TERT* and *TERT*-HA were compared with expression of the same subunits in HaCaT-Puro. The downregulation of integrin α1 in HaCaT-*TERT*-HA was not further investigated because the positivity for this integrin is not particularly elevated in any of the three cell lines. The only striking increase is shown in the case of integrin α6, which is overexpressed in HaCaT-*TERT* and HaCaT-*TERT*-HA.
Graph b) and table d): analysis of the positivity of integrins expression on cell surface. Positivity was compared between HaCaT-Puro, HaCaT-*TERT* and HaCaT-*TERT*-HA after independent normalization with the appropriate isotype controls. HaCaT showed positivity for the integrin subunits α2, α3, α6 and for integrin αvβ6, even with great variability amongst the cell lines.
Table e): control analysis for the negativity of the appropriate isotype controls compared with unstained HaCaT.

a)



b)



c) Integrins	Fold mean intensity of cell surface integrins expression			
	HaCaT-Puro	HaCaT-*TERT*	HaCaT-*TERT*-HA	HaCaT-Dn*TERT*
β1	67.76	52.64	61.80	57.97
β4	92.92	83.00	81.18	120.85
α4	20.18	18.78	20.26	20.26
α6	231.30	185.09	254.56	234.87
Integrins	**Standard deviation**			
	HaCaT-Puro	HaCaT-*TERT*	HaCaT-*TERT*-HA	HaCaT-Dn*TERT*
β1	29.64	17.39	21.06	6.85
β4	61.01	47.12	48.23	89.74
α4	2.32	1.50	1.27	1.05
α6	85.45	99.98	155.69	176.80

d) Integrins	**Fold mean positivity of cell surface integrins expression normalized to isotype controls**			
	HaCaT-Puro	HaCaT-*TERT*	HaCaT-*TERT*-HA	HaCaT-Dn*TERT*
β1	18574.95	10561.26	13589.66	7484.29
β4	18106.38	10488.55	13456.78	7546.90
α4	3145.59	1421.59	3096.50	1726.89
α6	18605.49	20496.84	12169.56	8863.30
Integrins	**Standard deviation**			
	HaCaT-Puro	HaCaT-*TERT*	HaCaT-*TERT*-HA	HaCaT-Dn*TERT*
β1	20579.23	8243.32	11257.63	5682.44
β4	19752.26	7924.20	10913.67	5698.28
α4	2301.98	340.41	1944.88	977.18
α6	20800.02	23291.85	9923.51	8119.16

e) controls	**Fold mean intensity of isotype controls expression normalized to unstained cells**			
	HaCaT-Puro	HaCaT-*TERT*	HaCaT-*TERT*-HA	HaCaT-Dn*TERT*
unstained	100.00	100.00	100.00	100.00
IgG1	100.75	94.48	100.96	100.78
IgG2	100.98	129.00	105.17	101.98
controls	**Standard deviation**			
	HaCaT-Puro	HaCaT-*TERT*	HaCaT-*TERT*-HA	HaCaT-Dn*TERT*
unstained	0.00	0.00	0.00	0.00
IgG1	15.85	17.74	16.23	16.28
IgG2	14.57	17.16	18.79	16.62

Figure 19: Analysis of the effect of *TERT* overexpression in regulating the expression of integrins on the cell surface of HaCaT

Graph a) and table c): analysis of the intensity of integrins expression on cell surface. Expression in HaCaT overexpressing *TERT* and *TERT*-HA were compared with expression of the same subunits in HaCaT-Puro. The downregulation of integrin α1 in HaCaT-*TERT*-HA was not further investigated because the positivity for this integrin is not particularly elevated in any of the three cell lines. The only striking increase is shown in the case of integrin α6, which is overexpressed in HaCaT-*TERT* and HaCaT-*TERT*-HA.

Graph b) and table d): analysis of the positivity of integrins expression on cell surface. Positivity was compared between HaCaT-Puro, HaCaT-*TERT* and HaCaT-*TERT*-HA after independent normalization with the appropriate isotype controls. HaCaT showed positivity for the integrin

subunits α2, α3, α6 and for integrin αvβ6, even with great variability amongst the cell lines.

Table e): control analysis for the negativity of the appropriate isotype controls compared with unstained HaCaT.

5.2 Analysis of integrin expression in whole cell extracts after *TERT* and *TERT*-HA overexpression in cell culture and during anoikis

To analyze the total amount of integrin expressed in the entire cell, both immature in the cells and when the integrins cluster together to form a mature receptor on the surface of the cells, western blot analysis of various integrin subunits was performed. Western blot with integrins is a problematic procedure, due to the different forms of these proteins which are glycosylated in the Golgi apparatus before moving to the cell surface. Therefore, a wider panel of integrin antibodies was studied at first, but the inefficiency of the antibodies and their low signal to background ratio restricted the panel to the subunits αv, β1, β4, and β6.

The lysates were taken from HaCaT-Puro, HaCaT-*TERT* and HaCaT-*TERT*-HA at 0 hr, after 20 hrs culture in suspension (anoikis assay) and after 20 hrs in a cell culture control dish (growing adherent) for integrin αv and integrin β6, and at 0 hr and 20 hrs in suspension for integrins β1 and β4. Controls were represented by cell lysates form NHEK and SCC-25, where possible, and expression was normalized to GAPDH expression. Expression of β-actin was first contemplated as a good housekeeping control, but the variability of its expression during cells in apoptosis excludes the possibility of using the expression of this protein as an internal control (figure 20, a).

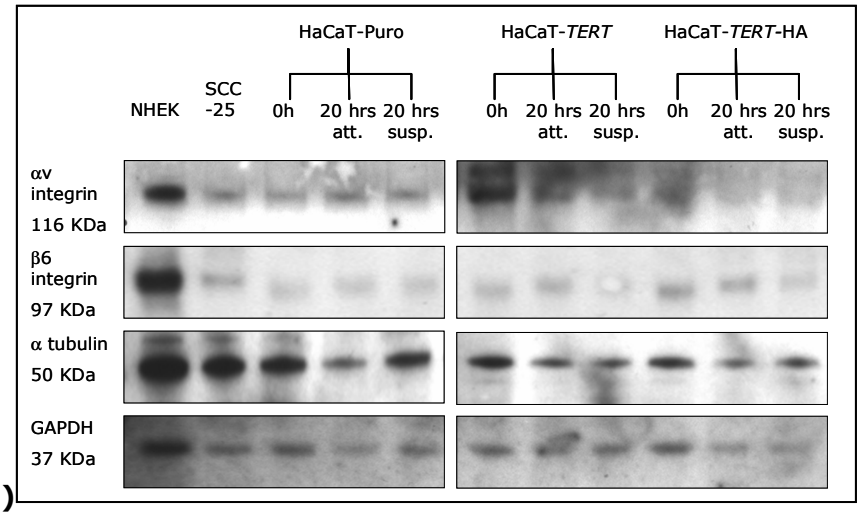
The expression of integrin αv (figure 20, a) is regulated by the presence of TERT at the control times: HaCaT cells containing the empty vector express lower amount of this integrin compared to HaCaT-*TERT*-HA and even less if compared to HaCaT-*TERT*. Furthermore, when HaCaT-Puro undergo anoikis, no difference in the αv integrin expression is seen; however, in HaCaT overexpressing *TERT* and *TERT*-HA, anoikis is correlated with a more than two fold decrease in αv integrin expression.

On the other hand, integrin β6 expression is not regulated by *TERT* overexpression at control times (figure 20, a) and does not vary when

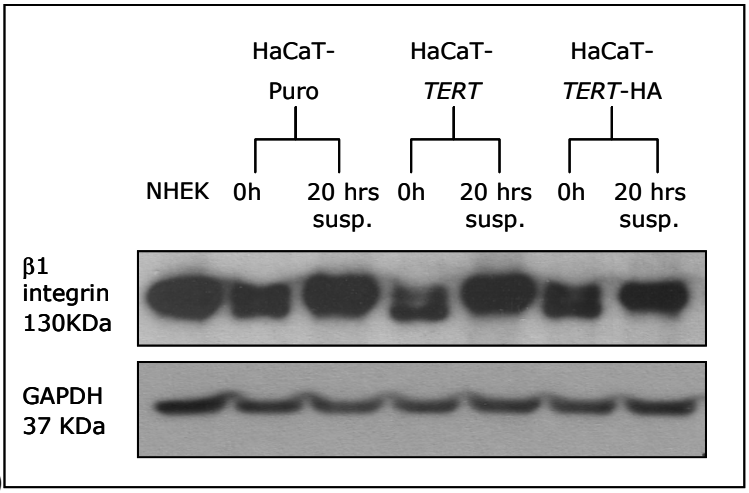
the cells undergo anoikis compared to normal culture conditions at the same time point or to the control time point. However, a shift from the non glycosylated (non mature) form to the glycosylated (mature) form of the integrin is shown when all the three cell lines undergo anoikis.

Integrin β1 expression does not appear to significantly vary at controls time between the three cell lines. A slight decrease of expression may be seen in HaCaT-*TERT* and *TERT*-HA, but the experiment should be repeated to confirm the significance of the result; however, there is an evident regulation in the expression of the glycosylated form of this subunit when the cell lines undergo anoikis among the three cell lines. Also in this case, it is not possible to estimate if the small decrease in expression of this subunit in *TERT* and *TERT*-HA overexpressing cells is significant.

Interestingly, while expression of integrin β4 is not significantly affected by the presence of *TERT* or *TERT*-HA at control times, it decreases when the cells are cultured in suspension for 20 hrs and undergo anoikis (figure 20, c). The expression of the integrin β4 appears to be strongly affected during anoikis by the overexpression of *TERT* and *TERT*-HA.


HaCaT-Puro
HaCaT-TERT
HaCaT-TERT-HA
NHEK
SCC -25
0h
20 hrs att.
20 hrs susp.
0h
20 hrs att.
20 hrs susp.
0h
20 hrs att.
20 hrs susp.
αv integrin
116 KDa
β6 integrin
97 KDa
α tubulin
50 KDa
GAPDH
37 KDa

a)


HaCaT-Puro
HaCaT-TERT
HaCaT-TERT-HA
NHEK
0h
20 hrs susp.
0h
20 hrs susp.
0h
20 hrs susp.
β1 integrin
130KDa
GAPDH
37 KDa

b)

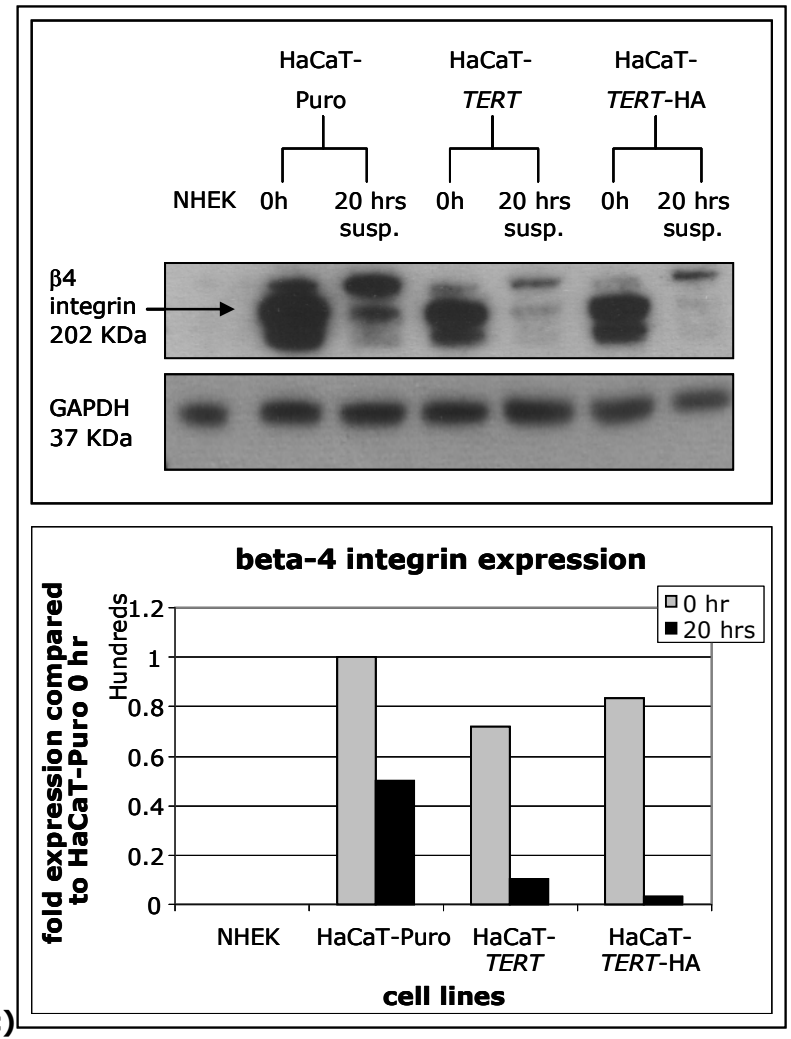


Figure 20: Western blot analysis of the regulation of integrin expression in whole cell after *TERT* and *TERT*-HA overexpression in cell culture and during anoikis

a): panel of integrin and their expression in HaCaT-Puro, HaCaT-*TERT* and HaCaT-*TERT*-HA at time 0 hr, after 20 hrs of conventional cell culture in adherence on tissue culture dishes, and after 20 hrs in suspension (anoikis assay). The expression was normalized to the expression of the housekeeping protein GAPDH, since β-actin expression is not stable when cells undergo anoikis. At control times, expression of integrin αv is increased in TERT and TERT-HA overexpressing HaCaT compared to the control. When cells undergo anoikis, the expression of the integrin does not vary in HaCaT-Puro, but it is reduced about two fold in HaCaT-TERT and HaCaT-TERT-HA. Expression of integrin β6 does not vary with TERT presence, but a shift to the glycosylated (higher molecular weight) form can be seen when all the three experimental group undergo anoikis.
b): Integrin β1 is expressed in two forms, glycosylated (upper band) and unglycosylated. At 0 hr, *TERT* and *TERT*-HA expression is correlated with a slight decrease in the expression of the glycosylated form, but the

difference may not be significant and would need further confirmation. When the cells, independently from *TERT* overexpression, undergo anoikis, there is a shift in the expression of the glycosylated form in all the three cell lines. In this case, a moderate downregulation is shown in HaCaT-*TERT* and HaCaT-*TERT*-HA compared to HaCaT-Puro at 20 hrs in suspension.
c): the expression of integrin β4 is stable in the three cell lines at time 0 hr, suggesting there is no regulation due to *TERT* or *TERT*-HA overexpression during normal cell culture. However, when the cells undergo anoikis, the expression of this protein significantly decreases in the three cell lines, and the decrease appears to be greater in cells expressing *TERT* or *TERT*-HA.

5.3 The ability of TERT to block anoikis is independent of the β1-integrin

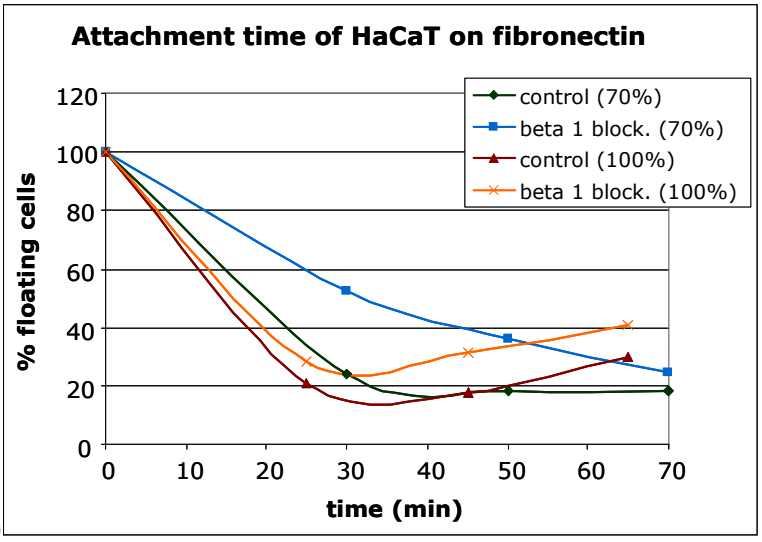
As discussed before, the role of β1 integrin has been proven to be essential in the regulation of cell death due to anchorage deprivation. The specificity of the function of this integrin has been shown with different methods, such as by coating dishes with an β1-integrin antibody (Meredith, Fazeli et al. 1993) or by adding a neutralizing antibody against this integrin to the cultures (Adams and Watt 1990).

To determine a possible involvement of integrin β1 in the anoikis model, I therefore utilized a neutralizing antibody against β1 integrin. Firstly, analysis of the cell attachment time was investigated by treating HaCaT-Puro cells growing on cell culture dishes coated with fibronectin with the antibody diluted 1:250 in serum free medium, and comparing with untreated cells growing on the same type of coated dishes. Cells were grown at confluency and subconfluency before the experiment, as I have previously demonstrated that these variables strongly influence the ability of *TERT* to mute anoikis (see section 4.4).
As shown in figure 21, the antibody was more effective when cell were kept in subconfluent culture compared to complete confluence prior to trypsinization and plating on the coated dishes. In particular, at the 70% confluence that was chosen for the anoikis assay, the β1 neutralizing antibody could increase the time required for maximal attachment of HaCaT Puro by more than two fold and increased the number of unattached cells at 30 minutes by two fold. This showed that the dose of neutralizing antibody used was effective against HaCaT cells.

The second step was to determine the involvement of integrin β1 in the resistance to anoikis was to repeat the assay in the presence of the antibody in the four cell lines HaCaT-Puro, HaCaT-*TERT*, HaCaT-*TERT*-HA and HaCaT-Dn*TERT*, and to compare resistance to anoikis with the untreated cells. In all our cell lines, blocking the function of the β1 integrin increased the percentage of apoptotic cells, especially in our

control cells (figure 22). However, there was no difference in the pattern when compared to the untreated cells undergoing anoikis.

a)

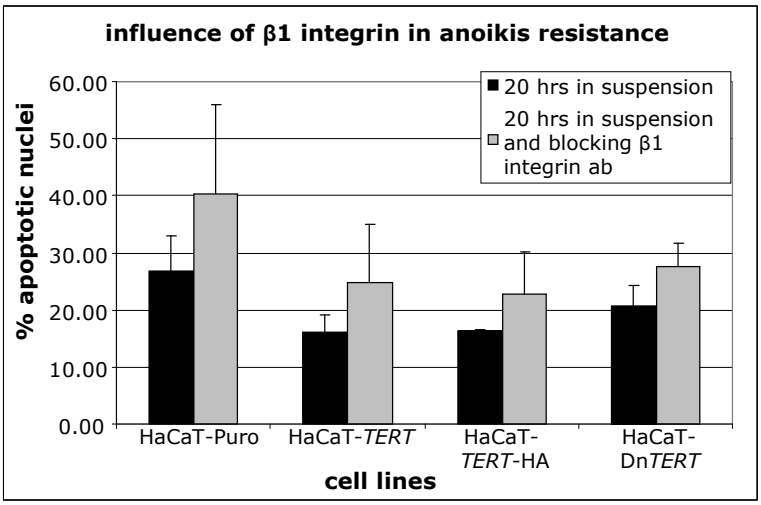


b) Attachment time of HaCaT-Puro on fibronectin coated dishes				
time (min)	% floating cells in the fibronectin dish			
	70% confluence		**100% confluence**	
	control	β1 blocking ab	control	β1 blocking ab
0	100.00	100.00	100.00	100.00
25	-	-	20.90	28.35
30	24.19	52.51	-	-
45	-	-	18.00	31.20
50	18.20	36.19	-	-
65	-	-	30.00	40.82
70	18.09	24.79	-	-

Figure 21: Attachment time is reduced when cells are treated with an antibody blocking β1-integrin

Figure a) and table b): HaCaT-Puro were grown to two different levels of confluence (70% and 100%) before trypsinisation and plating into fibronectin coated dishes. Time of adhesion to the cell surface was analyzed by measuring the percentage of floating cells after defined time points after treatment with a β1-integrin neutralizing antibody. The antibody resulted in more efficient blocking of adhesion of the cell to the coated dish surface when cells were previously grown to subconfluence (squared blue line) compared to when they were grown to complete confluence (crossed orange line).

a)



b) Cell lines	Average (%) of apoptotic cell death after 20 hrs in suspension	
	Without β1 blocking antibody	With β1 blocking antibody
HaCaT-Puro	26.86	40.43
HaCaT-*TERT*	16.20	24.74
HaCaT-*TERT*-HA	16.32	22.81
HaCaT-Dn*TERT*	20.71	27.68
Cell lines	**Standard deviation**	
	Without β1 blocking antibody	With β1 blocking antibody
HaCaT-Puro	5.97	15.45
HaCaT-*TERT*	2.90	10.29
HaCaT-*TERT*-HA	0.36	7.22
HaCaT-Dn*TERT*	3.46	4.02

Figure 22: Effect of disruption of cell-matrix interactions on phenotypic features of apoptosis by Hoechst staining of nuclei after treatment with a neutralizing antibody against integrin β1

Figure a) and table b): HaCaT cells (approx 2 million cells) were plated onto petri dishes which had been coated with polyHEMA and after 20hrs hallmarks of apoptosis in the nucleus were detected in the fixed cells and compared the same cells treated with a blocking antibody raised against integrin β1 (dilution 1:250).

In all the cell lines, treatment with the antibody increased the rate of apoptotic cells but did not change the pattern of the results, as HaCaT cells overexpressing the different *TERT* transcripts still showed a reduced level of anoikis. This suggests that some ECM ligands for integrin β1, such as fibronectin and collagen are produced by HaCaT cells in suspension to mute anoikis but also that the *TERT* transgenes do not

work by increasing the expression of these ligands or integrin receptors containing the β1 subunit. This is consistent with the FACS and western blot data presented in sections 5.1 and 5.2. The result also suggests that TERT mutes anoikis independently of the β1 subunit at point downstream of integrins.

5.4 Summary of results part 3

HaCaT cells overexpressing *TERT* and *TERT*-HA show resistance to anoikis when compared to their empty vector controls. However, the mechanisms through which *TERT* operates to promote this resistance are not yet understood.

The ability of TERT to mute anoikis may partially explain how it augments papilloma formation in mice treated with TPA and DMBA, and it is essential to understand the pathway involved in this resistance. As explained in the introduction, section 1.4.1, and in the chapter 4, the importance in anoikis held by integrins, HRAS, Src, FAK and all the other kinases involved in the cascade, together with the final effector caspases, has been shown by numerous papers. I therefore first investigated the role of integrins in the anoikis resistance due to *TERT* overexpression to determine the specific point of regulation of TERT in the anoikis death pathway.

As discussed in the introduction of this result chapter, for many cell types, including keratinocytes, cell survival depends on intracellular signals triggered by integrins. Attachment to the ECM via integrins promotes proliferation and cell survival; on the other hand, when cell lose their contact with the ECM, the apoptotic form of anoikis will be triggered.

Several papers emphasize the role of the integrin subunit β1, therefore I focused my study especially on this particular protein. FACS analysis of cells cultured under normal culture conditions not only showed that there is no contribution of *TERT* in the expression of integrin β1 in the surface of HaCaT cells, but also didn't show any difference in the expression of any of the other considered integrins.

Western blot analysis of HaCaT cells under normal culture conditions and when induced to undergo anoikis showed no difference in the density of

integrins αvβ6 and αvβ5 in surface culture after ectopic *TERT*/*TERT*-HA expression and there was no difference in the number of HaCaT cells over-expressing the integrins in the different groups. However, ectopic *TERT*/*TERT*-HA expression did result in an apparent increase in the level of αv protein as detected by western blotting compared to the empty vector controls at zero time but the levels still fell after the induction of anoikis in a similar manner to the empty vector controls. This result is significant when one considers that avβ5 and avβ6 have been reported to have antagonistic roles in regulating anoikis, with avβ5 inducing anoikis and avβ6 inhibiting in a malignant oral SCC line (Janes and Watt 2004) . However, the data I have assembled so far does not support the idea that ectopic *TERT*/*TERT*-HA expression mutes anoikis by either downregulating avβ5 or upregulating avβ6. β1 integrin showed both glycosylated and non glycosylated forms in the control times, and an increase in the higher molecular weight glycosylated form when the cells undergo anoikis. However, the possible regulation of this integrin expression by *TERT* is not certain, and western blot analysis needs to be repeated to have more confident results.

Interestingly, however, I could see a strong downregulation of β4 integrin expression in whole lysates when HaCaT-*TERT* and HaCaT-*TERT*-HA undergo anoikis. This downregulation seems to be correlated with the overexpression of *TERT* and *TERT*-HA in the cell lines model, and therefore it could be hypothesised that expression of β4 integrin is regulated during anoikis by *TERT*, independently from the canonical function of this enzyme. α6β4 integrin is been shown to have both pro and anti-apoptotic functions, depending on the cell type and the status of p53 (Bachelder, Ribick et al. 1999). In particular, a study showed that α6β4 carries an AKT/PKB–dependent anti-apoptotic function, in p53-deficient carcinoma cells, while in cells expressing wild-type p53, apoptosis by α6β4 is promoted by activation of the caspase-3 pathway (Bachelder, Ribick et al. 1999).

Treating the cells with an antibody blocking the functions of β1 integrin could allow an insight into the mechanisms by which *TERT* acts to

promote the anoikis. When HaCaT cells were treated with this antibody before inducing them to undergo anoikis, the same resistance pattern in HaCaT overexpressing *TERT*, *TERT*-HA and Dn*TERT* was shown, indicating that *TERT* acts downstream of β1 integrin in the resistant phenotype.

A further study would include the analysis by FACS of the expression on the cell surface of the same integrins, or at least of the one positive for the expression in HaCaT, and including integrin β4, in the four experimental cells at time 0 hr and after 20 hrs in suspension. Therefore it would be possible to analyze if a modulation in the integrin expression can be seen dependently from *TERT* expression just when the cells are undergoing the apoptotic phenotype. However, it wasn't possible at the time of the writing to perform such an experiment, because of the numerous variables of the experiment (cell confluence, elevated number of cells to be plated, amount of reagent needed) which need to be considered and will constitute a long-term experiment.

Chapter 6:

Discussion

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Discussion

The main function of telomerase (also known as the canonical function) can be been defined as the maintenance of telomere length throughout multiple cell cycles. However, other phenotypes, which are independent from telomere lengthening, have been investigated and correlated with the catalytic component of telomerase TERT, leading to new non-canonical functions (NC) of this protein. Type I NC functions are dependent on the presence of the RNA component *TERC* and have been correlated with some forms of neoplasia, while type II NC functions are independent from *TERC* and have not been associated with carcinogenesis yet (Parkinson, Fitchett et al. 2008). It has been hypothesized that TERT can promote epithelial stem cell self-renewal and functional maintenance without inducing neoplasia. Inhibition of anoikis by telomerase may contribute to this phenotype allowing the self-renewal of the stem cells that are migrating out of the niche (Flores, Cayuela et al. 2005; Sarin, Cheung et al. 2005). To support this hypothesis, the inhibition of apoptosis by telomerase has been shown (Fu, Begley et al. 1999) and telomerase downregulation has been correlated with keratinocyte differentiation *in vivo* or ECM detachment *in vitro* (Crowe, Nguyen et al. 2005).

The aim of my project was the determination of the mechanism through which telomerase promotes papilloma formation in the two stage mouse model of epidermal tumourigenesis of squamous cell carcinoma and by inference its human counterpart. I hypothesized that the catalytic component of telomerase TERT could regulate cell survival in skin keratinocytes, in particular by promoting resistance to a particular form of apoptosis induced by anchorage-deprivation, defined as anoikis, to the

cells. Having established this I went on to test whether the ability of TERT to antagonise anoikis was independent from the canonical function of telomerase (telomere elongation) and the catalytic activity of the enzyme.

Squamous cell carcinoma is one of the most frequent human malignancies, affecting the epidermis and therefore the cells composing it, the keratinocytes. Generally, the formation of this tumour starts from hyperplastic lesions, which progress in the human to dysplasias of increasing grade and finally malignant SCCs (Califano, Westra et al. 2000). In the mouse, hyperplastic lesions progress into papillomas, a fraction of which eventually can evolve into the malignant SCCs (Yuspa 1998).

In vitro, the study of SCC is more complex due to the limitations in the culture of normal epidermal keratinocytes, which undergo senescence after few population doublings and are constantly changing with passage (Rheinwald and Green 1975). Normal keratinocytes are also hard to transfect making many experiments difficult, so for the preliminary steps of the investigation I used one of the more characterized keratinocytes cell lines, HaCaT, an immortalized keratinocyte line derived from normal human skin (Boukamp, Petrussevska et al. 1988). The advantage of using this cell line compared to normal keratinocytes is the lack of senescence and the similar differentiation pattern to skin keratinocytes that can be seen during cell culture (Boukamp, Petrussevska et al. 1988).

On the other hand, the pathways involved in the formation and progression of SCC are now well characterized and studied *in vivo* after the development of a two-stages carcinogenesis mouse model. In this system, the mutation of the oncogene *ras* is the most distinctive feature of the initial stage on the tumour formation (Quintanilla, Brown et al. 1986), but other mechanisms can participate to this event and to the subsequent growth and progression of the cancer, such as the overexpression of cyclin D (Robles and Conti 1995), the activation of the

oncogene *fos* (Greenhalgh, Welty et al. 1990) and the increased expression of the integrin α6β4 (Tennenbaum, Weiner et al. 1993).

The involvement of the enzyme telomerase in the tumour formation was also found by several groups. High telomerase activity is detected in skin carcinoma derived from TPA-treated mice, and the enzymatic activity correlates with progression and the genomic instability of the papillomas (Bednarek, Budunova et al. 1995). The activity of the telomerase requires both the components of the enzyme TERT (catalytic component) and *TERC* (RNA component), as demonstrated by several studies performed on mouse models (Gonzalez-Suarez, Samper et al. 2000; Gonzalez-Suarez, Samper et al. 2001; Cayuela, Flores et al. 2005).

This particular function of telomerase in promoting neoplasia is in line with evidence showing that telomerase does not just elongate the telomere (defined as canonical function of telomerase) but covers many other non-canonical functions (Parkinson, Fitchett et al. 2008). Some of these require the presence of both TERT and *TERC* (defined as NC I), while some others don't require the presence of the RNA component (defined as NC II).

To create a good system that allows the identification of non canonical functions of telomerase, I decided to use as a model HaCaTs overexpressing the following constructs: wild type *TERT*, the flagged *TERT*-HA, which has telomerase activity *in vitro* but cannot lengthen telomeres *in vivo*, the catalytically dead dominant-negative TERT mutant, *DnTERT*, which has neither catalytic activity nor the ability to lengthen telomeres, and as a control HaCaT carrying an empty vector.

The ability of telomerase to elongate telomeres in HaCaT was firstly investigated by Professor Petra Boukamp's group, and I carried out the characterization of HaCaT with the different constructs by the investigation of the activity of the enzyme *in vitro* and the level of expression of the two main components *TERT* and *TERC*. In addition, as the splice variants of TERT have been reported to regulate telomerase

activity (Ulaner, Hu et al. 1998; Colgin, Wilkinson et al. 2000; Cerezo, Kalthoff et al. 2002), I investigated the three most common forms, the α variant, the β variant and the αβ variant. I characterized in this way all the experimental groups at control times (0 hr) and after 20 hrs of growth in suspension after anchorage deprivation.

Firstly, the work from Professor Petra Boukamp's group proved that HaCaT-*TERT*-HA, where *TERT*-HA is a modification of wild-type TERT by attachment of a haemagglutinin tag at the C-terminal of the protein, is an effective system to study non-canonical functions of telomerase. The cells carrying this construct, in fact, cannot elongate telomeres in cell cultured for 5 weeks *in vitro*, even if the telomerase complex is active *in vitro*, as I later demonstrated. However, the reasons for TERT-HA inability to elongate the telomere are not known yet (Counter, Hahn et al. 1998).

From my first set of results, I could confirm previous studies showing a particular pattern of expression of the components of telomerase in the skin. In those studies, *TERT* is expressed both in the basal and in the suprabasal layers of epidermis (Nakano, Watney et al. 1998), where cells start the differentiation process and lack contact from the extracellular matrix and the dermis. *TERT* expression follows a gradient which progressively decreases to the upper layers of the epidermis, where cells lose the nuclei and start keratinisation (Fuchs 1995). This was confirmed by my detection of *TERT* expression by real-time qPCR in HaCaT, both at control times, when cells grow attached to a surface, and after 20 hrs in suspension, when they are deprived from the anchorage. In the control HaCaT, no drop in *TERT* mRNA expression was detected after 20 hrs, while in *TERT* and *TERT*-HA overexpressing HaCaTs, the amount of *TERT* mRNA expression at 20 hrs decreased but was not less than the control time and that HaCaT-Puro at time 0. These data not only confirm the presence of *TERT* at two different stages of keratinocyte differentiation, but also support the arguement that this system is a good model to investigate the effect of *TERT* overexpression in the two different time points and when the cells are subjected to anchorage deprivation.

Similarly, the expression of *TERC* detected by the abovementioned study was detected both at basal and suprabasal layers of the epidermis, with a strong *in situ* hybridization signal that did not show any gradient in the expression (Nakano, Watney et al. 1998). From my results, *TERC* is expressed both at the control time and after 20 hrs of growth in suspension. Differently from *TERT* mRNA expression, there is no drop in the expression of *TERC* when the cells are deprived from the anchorage; instead, there is a massive increase of the expression. This could be explained by a previous study showing that the half-life of *TERC* is very long, and can be further stabilized by the presence of TERT (Yi, Tesmer et al. 1999).

The presence of both TERT and *TERC* in the basal and suprabasal layers of the epidermis would lead us to believe that the telomerase holoenzyme is active in the same region of skin. However, a previous study showed that the suprabasal layers lack telomerase activity (Harle-Bachor and Boukamp 1996). Telomerase is active just in the basal layer of the epidermis, where keratinocytes show high proliferation rate and contribute to the continuous regeneration and proliferation of the skin and it is not needed when the cells start differentiating (Flores, Cayuela et al. 2005). I could confirm these observations by the detection of telomerase activity in HaCaTs at control times and after 20 hrs growth in suspension. In fact, the modest telomerase activity of HaCaT dramatically drops when the cells are deprived from the anchorage, and at 20 hrs the activity is almost undetectable. This pattern of expression in reproducible also in the presence of exogenous *TERT* and *TERT*-HA, even if the activity is not annulled at 20 hrs because of the very high activity already detected at time 0. These results are in partial disagreement with a previous study from Crowe, who detected a reduction in TERT mRNA in anchorage-deprived keratinocytes (Crowe, Nguyen et al. 2005). However, my results are in line with the *in situ* hybridization studies from Nakano (Nakano, Watney et al. 1998) and more coherent with the differentiation pattern of the skin keratinocytes, that wouldn't justify the

need of telomerase activity described by Crowe in the upper layers of the epidermis.

Since the localization of telomerase activity does not correspond to the expression pattern of *TERT* and *TERC*, there should be a mechanism for the regulation of the activity of the enzyme. *TERC* but mostly *TERT* are finely regulated by numerous factors, leading to transcriptional regulation and, in the case of TERT, to post-translational regulation (see section 1.1.5 of the introduction for further details).

I considered one of the possible regulatory mechanisms of telomerase activity, which is the preferred expression of one of the numerous inactive or dominant-negative splice variants of *TERT* rather than the wild-type. After careful review of the previous studies for the detection of *TERT* splice variants using different techniques, I adopted the work done by Lincz *et al.*, who use independent primer sets for each of the three most characterized splice variants, alpha, beta, and alpha/beta (Lincz, Mudge et al. 2008). I quantified the expression of the three splice variants both at 0 hrs and in suspension, considering a previous study that correlated the presence of the beta variant with regulation of telomerase activity in HaCaT (Cerezo, Kalthoff et al. 2002). From my results, the two main splice variants alpha and beta are expressed at low levels in all the HaCaT cells, and, particularly in HaCaT-Puro, they represent less than 6% of the total *TERT* mRNA. The increase of the amount of beta splice variant when the cells are deprived from anchorage from the ECM is not significant, in my opinion, since the level of expression is still very low compared to the amount of full-length *TERT* and unlikely to be sufficient to produce any effect on telomerase regulation.

From the characterization of the components of telomerase in the HaCaT for my model, I could ensure that any phenotype that is shown when cells get deprived from anchorage is associated not only with the presence of an active form of TERT, but also permit the classification of

any new function of TERT associated with the phenotype in canonical or non-canonical function, thanks to the availability of HaCaT-*TERT*-HA.

Since *TERT* expression decreases with cell differentiation (Cerezo, Stark et al. 2003), and differentiation of skin keratinocytes is regulated by the attachment with the cell matrix (Crowe, Nguyen et al. 2005), it is possible to hypothesise an association between *TERT* expression and the ECM. In addition, it is well known that epithelial cells undergo a specific form of apoptosis called anoikis when they are deprived from the ECM (Frisch and Francis 1994). Therefore, in neoplastic cells, I can hypothesise that TERT confers an advantage to neoplastic keratinocytes by promoting resistance to anoikis itself, making the cells able to grow independently from the attachment with the ECM.

To test this hypothesis, I studied the behaviour of HaCaT in a situation of anchorage deprivation in the presence or absence of exogenous TERT.

While control HaCaT at time zero show less than 1% of apoptosis, possibly due to the response to the trypsin treatment, I could detect up to 45% of apoptotic nuclei in control HaCaT after 20 hrs in suspension, by morphological staining of the nuclei; with FACS analysis, the percentage of apoptotic nuclei in the same experimental group was lower, corresponding to 14% of the total cell nuclei. Those two percentages are not as elevated as the one reported by the first study on anoikis (Frisch and Francis 1994), where 60% of apoptotic nuclei where detected in HaCaT by analysis of DNA laddering, but this may be due to the difference in the detection techniques.

When *TERT* is overexpressed in HaCaT, I could detect an almost two-fold significant protection towards apoptosis in the cell lines at 20 hrs after anchorage-deprivation. The same level of protection was detected when HaCaTs overexpress *TERT*-HA, therefore this indicates that the phenotype that I detected is directly associated with the presence of TERT but it is independent from its canonical function of telomere elongation.

Later, I confirmed that the apoptotic phenotype observed in the cells when deprived of their anchorage with ECM, and characterized by several changes in the cell structure and in the nucleus morphology, is not a general form of apoptosis, but can be defined as anoikis. In fact, the pattern of resistance of HaCaT carrying *TERT* and *TERT*-HA is not repeated when cells are treated with cisplatin, a general inducer of apoptosis (Gonzalez, Fuertes et al. 2001), rather than being placed in suspension.

In addition, when HaCaTs carry a construct with a dominant-negative form of *TERT*, *DnTERT*, the cells, which show very low levels of telomerase activity, as explained above, carry a mixed phenotype, showing a level of resistance to apoptosis that is bigger than the control HaCaTs, but smaller than *TERT/TERT*-HA overexpressing HaCaT. This may suggest that catalytic activity is still required for the observed phenotype even TERT does not elongate telomeres. In a previous study, regulation of anoikis by TERT in motor neurons was shown not to require the catalytic activity of the enzyme (Lee, Sung et al. 2008), but it is possible that changes in the response towards anoikis is not sufficient to promote the formation of papillomas in the two-stage carcinogenesis system, and to augment wound healing; and this is consistent with the requirement for *TERC* to produce these two phenotypes (Rudolph, Chang et al. 1999; Gonzalez-Suarez, Samper et al. 2001).

In conclusion, from my data I could confirm the hypothesis that a catalytically active TERT confers an advantage to the immortalized HaCaT cells by promoting resistance towards anoikis, and this phenomenon is due to a new non-canonical function of telomerase. This may explain why pre-malignant dysplasias (Edington, Loughran et al. 1995) and malignant SCC (Rheinwald and Beckett 1980; Tiberio, Marconi et al. 2002), and therefore have an advantage in the formation and progression of the tumour.

Several proteins are involved in the mechanism of anoikis, therefore characterizing a mechanism through which TERT confers resistance to the

cells towards this form of cell death can be difficult. Anoikis for definition involves the receptors of the ECM components, integrins. When the cells are attached to the matrix, different signalling pathways are triggered from the activated integrins, and, after activation of the Src/FAK, involve the Ras-Raf-Erk pathway or the PI3K-Akt pathway. Both these pathways can lead to the transcription of anti-apoptotic factors and block the caspase-induced death signal (Frisch and Ruoslahti 1997; Frisch and Screaton 2001; Stupack and Cheresh 2002).

To investigate the point of intervention of TERT in the apoptotic machinery, I analyzed changes in expression of the integrin subunits in whole cell lysates and on the cell surfaces.

On the integrin subunits that I took into consideration, FACS analysis of HaCaTs cultured under normal culture conditions did not show any contribution of *TERT* in the expression of integrins on the cell surface. In particular, I couldn't detect any difference in the expression of the integrin β1, which is been previously involved in the keratinocytes terminal differentiation by several studies (Adams and Watt 1989; Watt, Kubler et al. 1993; Hotchin, Gandarillas et al. 1995)

The expression of integrin αv has been shown to be involved in the metastatic stage of different malignancies (Desgrosellier and Cheresh 2010). From my results on total cell lysates, however, overexpression of *TERT* and *TERT*-HA in HaCaT is correlated with a with a more than two fold decrease in αv integrin expression when the cells are growing in suspension, but even at time 0 the overexpression of *TERT/TERT*-HA is associated with an upregulation of this integrin subunit compared to the control HaCaTs. Therefore protection against anoikis by TERT may is unlikely to be mediated by the upregulation of this integrin.

The analysis if integrin β6 expression showed a shift from the non glycosylated (immature) form to the glycosylated (mature) form of the integrin when the cells lines grow in suspension for 20 hrs, but this change is not muted by the expression of *TERT* or *TERT*-HA. A similar

patter of glycosylation is shown by the integrin β1, which is expressed in both the mature and immature forms at time 0, but only the mature form is expressed after 20 hrs in suspension.

It is therefore likely that in our system TERT protects against anoikis by intervening downstream of integrin signalling. I could also confirm the independence from this particular integrin subunit by analysing the response to anoikis after the blockage of the integrin with a specific anti-β1 antibody. The pattern of resistance among the cell lines didn't vary in presence of this antibody, therefore excluding the possible regulation of this receptor or any of is ligands in the apoptotic phenotype.

The only relevant difference in the expression of integrins when HaCaT are grown detached from the matrix for 20 hrs concerns the subunit β4, which is donwregulated in *TERT/TERT*-HA overexpressing HaCaTs after 20 hrs compared to HaCaT-Puro and with all the groups at control time. This result could be consistent with previous studies that showed a p-53 status dependent pro- and anti-apoptotic functions of α6β4 integrin (Bachelder, Ribick et al. 1999).

In addition, I've observed that the resistant phenotype is modulated by the status of confluence of the cell culture before they are deprived from the anchorage and undergo anoikis. This has introduced the possibility of the involvement of β–catenin and the Wnt pathway in the TERT-induced resistance to anoikis, confirming recent studies that showed an interplay between TERT and this pathway (Sarin, Cheung et al. 2005; Choi, Southworth et al. 2008; Park, Venteicher et al. 2009).

As β–catenin nuclear signalling is down regulated when epithelial cells form stable junctions (Kuphal and Behrens 2006) my results may be explained by TERT delaying the the induction of the anoikis programme, which is known to be dependent on cell-cell contacts (Frisch and Francis 1994). TERT might achieve this by its recently described ability to increase Wnt and β–catenin nuclear signalling through its interaction with BRG (Park, Venteicher et al. 2009) (see figure 23). BRG is a chromatin-

remodeling factor that is recruited by β–catenin in the activation of the transcription factor TCF/LEF and the transcription of Wnt target genes (Barker, Hurlstone et al. 2001). The interaction with TERT would also be consistent with the ability of DnTERT to partially mimic the wild-type. The model of interaction and interplay between TERT and β–catenin is explained in detail in figure 24.

TERT has been shown to be essential for an adequate DNA damage response and its absence sensitises cells to DNA damage and also provokes premature senescence (Masutomi, Possemato et al. 2005). It has also been shown that TERT enters the mitochondria and protects mitochondrial DNA from damage and the organelle itself from oxidative stress (Zhang, Chan et al. 2003). However, these phenotypes require the catalytic activity of TERT and hence the presence of *TERC*, whereas my results indicated that the catalytic activity of TERT was largely indispensable for its ability to mute anoikis.

More recently, it has been shown that TERT can generate micro RNAs (miRNAs) by interacting with the RNA component of mitochondrial RNA processing endoribonuclease (RMRP) (Maida, Yasukawa et al. 2009) but the relevance of this to TERT's ability to mute anoikis is not yet known.

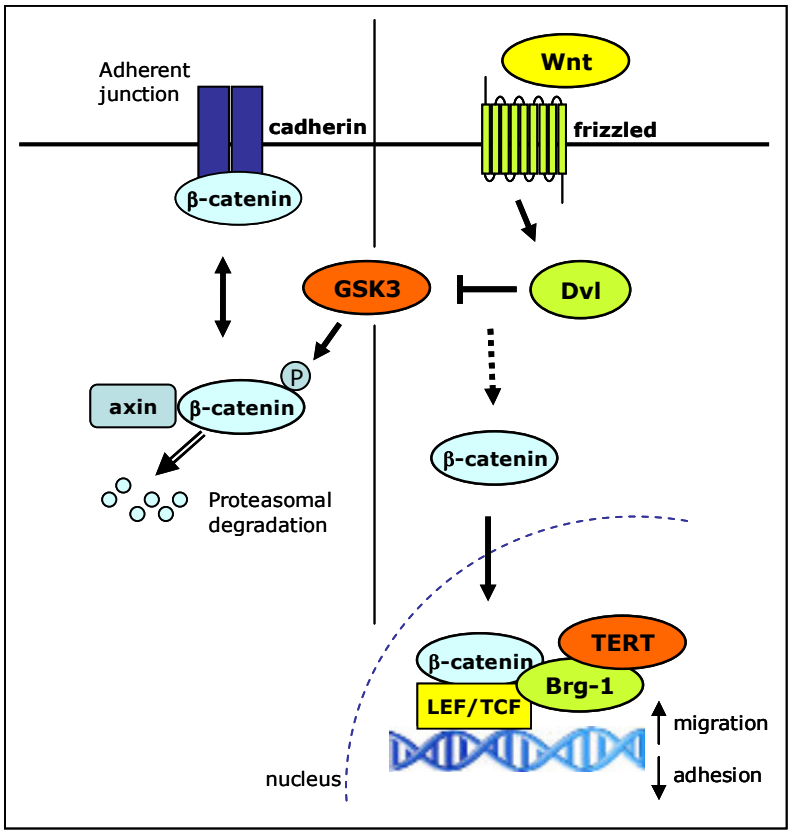

Figure 23: The possible role of TERT in the regulation of Wnt and β–catenin nuclear signalling

In absence of Wnt signalling, β–catenin can bind to cadherin to form adherent junctions, fundamental for intercellular adhesion or, if bound to the protein axin, it is subjected to phosphorylation by GSK3 and subsequent proteasomal degradation. In presence of Wnt ligand, the active receptor for Wnt, frizzled, activate dishevelled (Dvl). Dvl prevents the phosphorylation of β–catenin by inhibition of GSK3, letting β–catenin enter the nucleus. In the nucleus, β–catenin recruits the chromatin-remodeling factor Brg-1 and interacts with the transcription factors LEF/TCF to permit the transcription of Wnt target genes. Since TERT has been shown to interact with Brg-1, it could act as a coactivator or transcription of Wnt target genes under the presence of Wnt signal and when β–catenin is not totally occupied in the formation of adherent junctions.

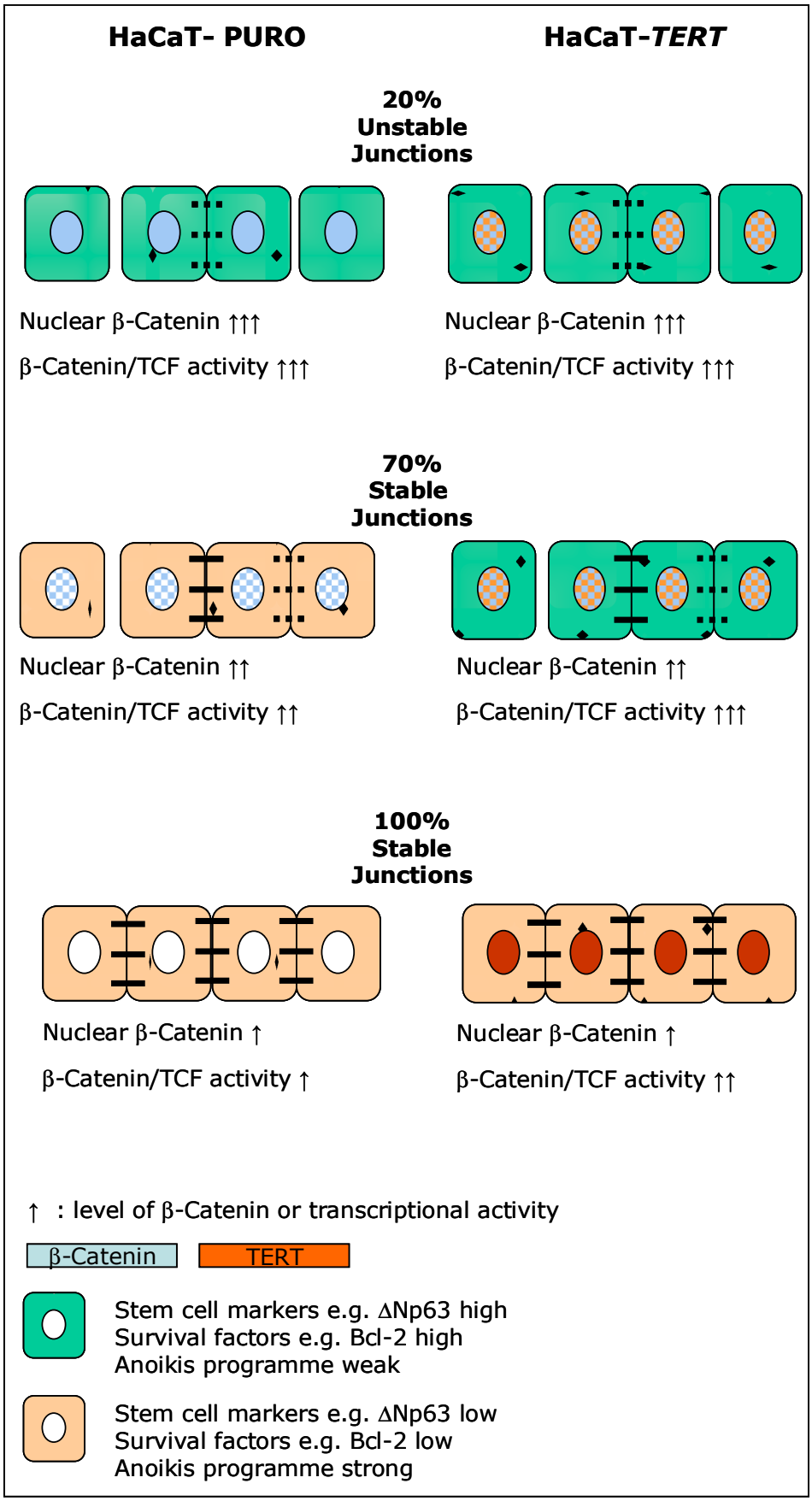
HaCaT- PURO
HaCaT-TERT
20%
Unstable
Junctions
Nuclear β-Catenin ↑↑↑
β-Catenin/TCF activity ↑↑↑
Nuclear β-Catenin ↑↑↑
β-Catenin/TCF activity ↑↑↑
70%
Stable
Junctions
Nuclear β-Catenin ↑↑
β-Catenin/TCF activity ↑↑
Nuclear β-Catenin ↑↑
β-Catenin/TCF activity ↑↑↑
100%
Stable
Junctions
Nuclear β-Catenin ↑
β-Catenin/TCF activity ↑
Nuclear β-Catenin ↑
β-Catenin/TCF activity ↑↑
↑ : level of β-Catenin or transcriptional activity
β-Catenin
TERT
Stem cell markers e.g. ΔNp63 high
Survival factors e.g. Bcl-2 high
Anoikis programme weak
Stem cell markers e.g. ΔNp63 low
Survival factors e.g. Bcl-2 low
Anoikis programme strong

Figure 24: Possible regulation of the anoikis programming of HaCaT by *TERT* overexpression

As seen in Figure 23, the amount of β–catenin free to enter the nucleus and initiate transcription is dependent of the total amount of β–catenin, which can also be engaged in the formation of adherent junctions. In HaCaT-Puro, the amount of nuclear β–catenin decreases with the cell confluence because of the cell-cell junctions, which increase in number and stability in parallel with the cell confluence. Therefore, in the situation of higher confluence, the transcriptional activity regulated by nuclear β–catenin gradually decreases. If the the cells are influenced in their apoptotic programme by the transcription of specific Wnt/β–catenin target genes, then elevated transcriptional activity at lower confluence would inhibit anoikis. In our model, the elevated levels of TERT in the nucleus in HaCaT-*TERT*, by its proposed interaction with BRG would keep levels of β–catenin transcriptional activity high even when levels of β–catenin are not as elevated, such in a situation of sublconfluence. Therefore, at this state of 70% cell confluence, the anoikis programme would still be weak compared to the corresponding control cells. In summary it is proposed that TERT may affect anoikis programming rather than the process of anoikis itself.

6.1 Conclusion

The catalytic subunit of telomerase, TERT, can cover canonical and non-canonical functions. Non-canonical functions encompass every role that in independent from telomere elongation; however, some of these non-canonical functions still require the presence of the RNA component *TERC* and therefore the catalytic activity of the enzyme.

I have characterized in this study a new non-canonical function of telomerase, requiring the catalytic activity of the enzyme, which confers protection against cell death to immortal skin keratinocytes during anchorage-deprivation. This particular from of cell death is defined anoikis, and the resistance towards it would represent a major advantage in the growth and proliferation of malignant cells in a tissue, since it would render the cells less dependent from the contact with the extracellular matrix.

Furthermore, this resistance to anoikis could be a mechanism through which TERT, overexpressed in both dysplasia and SCC cell lines and tissues, as well as mouse papillomas and SCCs, could promote the formation of these tumours. The exact point in the apoptotic pathway that is regulated by TERT has not been identified yet. In adherent cells, several pathways are triggered from activated integrins that binds to the ECM and converge to the blockage of caspases through the transcription of anti-apoptotic factors. From my study, I could not detect a significant change in the expression of integrins, thus I hypothesize that TERT acts downstream of these receptors to regulate anoikis in HaCaT cell lines, or alternatively influences the programming of anoikis potential when the cells are attached.

It would be interesting to test if the TERT-induced resistance to anoikis happens also in normal human keratinocytes, because this would have relevance to normal stem cell function and wound repair, as well as neoplasia. In addition, the use of organotypic cultures with HaCaT cells

could give a further inside of the role of TERT overexpression in the keratinocytes differentiation, in a system that is more directly comparable with the *in vivo* situation.

To underpin the mechanisms involved in the regulation of anoikis, a systematic study of the most important proteins involved in apoptosis, in the integrin signalling pathways, and in the Wnt/β-catenin pathway should be carried out, so that any change of expression could give a further insight of the exact point of action of TERT. It would also be interesting to test the role of miRNAs in the TERT-induced resistance to anoikis by knocking down DICER, the enzyme responsible for much miRNA generation.

These and other experiments will be required to establish the mechanism of TERT-induced resistance to anoikis. Understanding the manner by which TERT operates to influence an array of biological processes, independently of telomere length, will be critical in finding appropriate ways to manipulate telomere function both positively and negatively, for the purposes of tissue regeneration and cancer therapy, respectively.

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