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Molecular Changes Involved in Oral Cancer Progression and their Relevance to Keratinocyte Immortalisation

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A thesis submitted to the University of Glasgow in part fulfilment for the degree of Doctor of Philosophy

September 2002

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To Eleni

"When trouble is sensed well in advance, it can be easily remedied; if you wait for it to show itself, any medicine will be too late because the disease will have become incurable.

As the doctors say of a wasting disease, to start with it is easy to cure but difficult to diagnose; after a time, unless it has been diagnosed and treated at the outset, it becomes easy to diagnose but difficult to cure"

Nicolo' Machiavelli, 1513

Acknowledgements

I am intellectually and emotionally indebted to several people that have given me their support and help during these three years. My supervisor Paul Harrison deserves special mention not only for his constant guidance, unfailing patience and constructive criticism on my work, but also for giving me the enthusiasm, ego-boosts and career advise that keep leading me down this route. His critical reading of this manuscript has also been greatly appreciated. To my advisor Ken Parkinson, for enlightening me with his invaluable scientific knowledge and challenging me in brainstorming discussions. I have always wondered whether my progress was due to my personal efforts or rather to such admirable supervision, and still have not found an answer. To Jim O'Prey earlier and Janis Fleming later I owe all of my technical ability, which will never be more than a fraction of theirs. I truly appreciated their help, patience, suggestions and ability to answer my trivial questions without making me feel stupid. I am very grateful to Brad Ozanne for his encouragement and references, to Fiona McGregor and Katrina Gordon for constructive collaboration, to June Munro and Hazel Ireland for technical help. Thanks are also due to Ronnie McKail, Trish Wheeler, Lynn McGarry, Joe Winnie, Robert McFarlane, Liz Gordon, Anne McNealis, Laraine Hughes, Bill Simpson, Michael McTaggart, Iain White, Peter McHardy, Margaret O'Prey and all the other people that have helped me in many ways and that will remain unmentioned for reasons of brevity.

On a more personal sphere, I want to thank my family for always supporting and encouraging my career choices an for believing in my intellectual capacity ways in excess of its actual value.

Aurnab Ghose has played a relevant role in the progress of my PhD not only as a friend but also as a guide: our late evening discussions on scientific and non-scientific topics have been font of great enlightment, other than fun. Thanks are owed to my "mate" Maisie McGhee, for keeping me sane during this long boring writing up period with innumerable nights out clubbing and on a more serious note for sharing many introspective meditations. Also, all of my friends and well-wishers have kept my spirits up and will not be forgotten.

Finally, the White Lily Foundation, Cancer School, Glasgow, the Assessorato della Pubblica Istruzione, Regione Autonoma della Sardegna, Cagliari, and the Beatson Institute for Cancer Research, Glasgow are gratefully acknowledged for providing the necessary funding.

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Abbreviations

°C	degrees centigrades
Ab	antibody
ABI	anaphase bridge index
ARF	alternative reading frame
ATM	ataxia teleangectasia mutated
ATR	ataxia teleangectasia related
bp	base pair
BrdU	5-Bromo-2'-deoxy-uridine
cdk	cyclin dependent kinase
CMV	cytomegalovirus
CRABP	cellular retinoid acid binding proteins
CRBP	cellular retinoid binding proteins
C-terminal	carboxy terminal
DMSO	dimethylsulphoxide
DMEM	Dulbecco's modified Eagles medium
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ERK	extracellular signal regulated protein kinase
FAD	flavin adenine dinucleotide
FAM	6-carboxy-fluorescein
FBS	foetal bovine serum
FISH	fluorescence in-situ hybridisation
FITC	fluorescein isothiocyanate
G0	quiescence phase
G1	gap/growth phase 1
G2	gap/growth phase 2
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
Gy	Grays
HEK	human embrionic kidney
HF	human fibroblast
HK	human keratinocyte
HMEC	human mammary epithelial cell

HN	head and neck
HPV	human papilloma virus
hTERT	human telomerase reverse transcriptase
hTERC	human telomerase rna component
IgG	immunoglobulin G
IHC	immuno histochemistry
ISD	information and statistics division
JAK	Janus kinase
kb	kilobases
LOH	loss of heterozygosity
M	molar
μg	microgram
ng	nanogram
NHS	national health system
ml	millilitre
μl	microlitre
mm	millimeter
mM	millimolar
μM	micromolar
mRNA	messenger ribonucleic acid
mTR	mouse telomerase RNA
N-terminal	amino terminal
PCR	polymerase chain reaction
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PD	population doublings
PI	propidium iodide
PLK	polo-like kinase
RAR	retinoic acid receptor
Rb	retinoblastoma protein
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
rRNA	ribosomal ribonucleic acid
RXR	retinod receptor
S	synthesis phase
SABG	Senescence Associated β-Galactosidase

SCC	human squamous cell carcinoma cell
SDS	sodium dodecylsulphate
sfm	serum-free medium
SSC	standard saline citrate
Stat	signal transducers and activators of transcription
SV40	simian virus 40
TAMRA	6-carboxy-tetramethyl-rhodamine
TRF	telomere restriction fragments
TGF	transforming growth factor
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling
UV	ultraviolet radiation
UTP	uracil triphosphate
WT	Wilms' tumour

Abstract

Oral cancer has caused great concern in all of the western countries over the past two decades because of its progressively increasing incidence, mainly in young males, and its consistently low 5-year survival rate. Oral premalignant lesions (dysplasias) are thought to precede the development of cancer, but no clinical or histological criteria is at present available to predict their potential for malignant transformation. Therefore, it would be of diagnostic and therapeutic relevance to understand the molecular events involved at different stages of oral cancer.

Using a unique series of primary cultures from biopsies of normal oral mucosa, dysplasias and squamous cell carcinomas we have identified molecular changes characteristic of early oral cancer progression. Our group reported previously that acquisition of the immortal phenotype is an early event in oral cancer development (McGregor et al. 1997): data obtained during the course of this thesis project indicate that about half of oral dysplasia cultures are immortal and this is associated with loss of expression of retinoic acid receptor (RAR)-β and the cell cycle inhibitor, $p16^{INK4a}$, p53 mutations and increased levels of telomerase/hTERT mRNA. In contrast, increased expression of the EGF receptor (EGF-R), known to be a characteristic of oral cancer, does not occur until after the dysplasia stage in squamous cell carcinomas (SCCs). Interestingly, one atypical mortal dysplasia with a considerably extended lifespan has lost expression of RAR-β and $p16^{INK4a}$, but it still expresses wild-type p53 (albeit at higher level than normal) and has not activated telomerase. Further experiments demonstrate that retroviral transduction of hTERT immortalises D17 and this is associated with telomere lengthening but p53 remains wild-type. In contrast, transduction of hTERT does not extend the lifespan of two other typical mortal dyplasia cultures (that retain RAR-β and $p16^{INK4a}$ expression), even though telomeres are lengthened. Thus, telomerase activation/telomere maintenance is not sufficient per se to immortalise mortal dysplasias without concomitant loss of RAR-β and/or $p16^{INK4a}$, but p53 mutation is not required if telomerase is activated exogenously.

However, further work is required to clarify whether the molecular changes associated with the acquisition of an immortal phenotype in culture have any clinical or prognostic significance.

1 Oral cancer

1.1 Epidemiology and risk factors

Oral cancer is estimated as the 10th most frequent cancer worldwide, representing 2.6% of all cancers in 1990 (Parkin et al. 1999). Despite this low percentage, a high level of concern arose recently from the observation that the incidence of oral cancer, both worldwide and particularly in Scotland, was raising progressively over the years. The general trend was of a 1.5 fold increase in one decade, with the trends relating to Scotland closely mirroring those of Europe and of the whole world, but being in excess of both (fig. 1). Males are more susceptible to oral cancer than females, and, although an increase in incidence was seen for both genders, it was particularly dramatic in males belonging to a middle age group (data from ISD Scotland NHS statistics division). The reasons for such increases are not clear and the European School of Oncology Advisory report in 1995 strongly suggested that this matter needed further investigation. In keeping with this, a significant increase in the incidence of tongue cancer was observed in individuals younger than 40 years in the USA (Schantz and Yu 2002).

The most common risk factors for oral cancer have been assessed since 1988 in a series of reports, reviewed and summarized by Boyle (Boyle et al. 1992) and by McFarlane (Macfarlane et al. 1995). There is strong consensus identifying tobacco in all forms, both smoked and chewed, as the major risk factor for oral cancer. In India, the high incidence of this condition is associated with the widespread habit of chewing tobacco or betel nuts, whereas in other countries the strongest association was reported with length of time of exposure to cigarette smoking or to different types of cigarettes smoked. Almost all of these studies also report alcohol consumption as being an important risk factor, independently of the type of alcohol consumed, its effects being synergistic rather than additive to those of tobacco. For other risk factors, like mouthwash use and poor oral hygiene, the evidence accumulated is not enough to draw strong conclusions.

In the light of all this background information, it is particularly interesting to notice that the "western world" trend in the past decade has been a reduction in the percentage of tobacco smokers and alcohol drinkers. In particular, the Scottish Health Survey of 1998 reported a sharp decline in the prevalence of male smokers of all age groups, although a slight increase of alcohol drinkers was also reported.

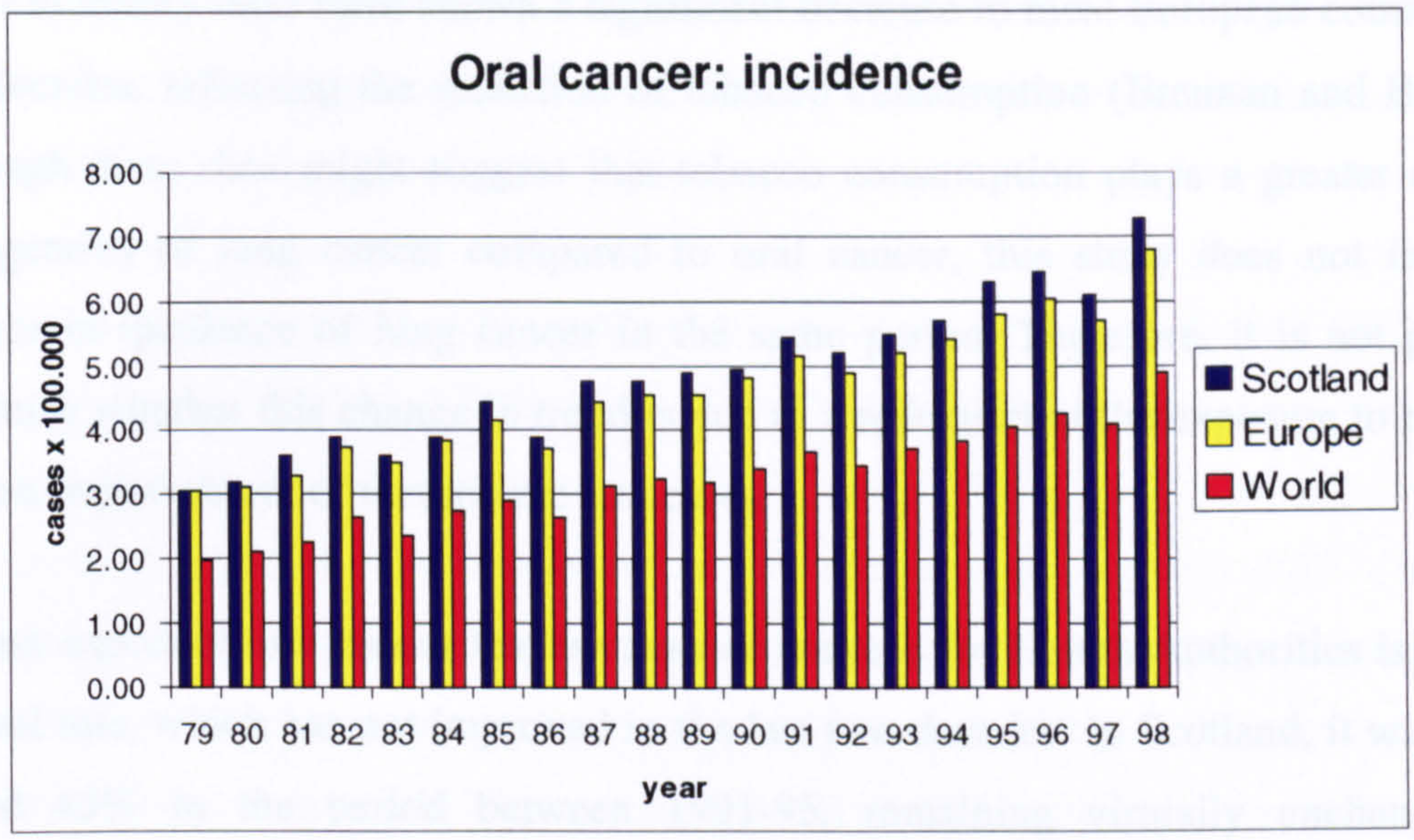


Fig. 1: Incidence of oral cancer in Scotland, Europe and the whole world in the last two decades.

Graphic illustration of the incidence of oral cancer in the last two decades. Data relative to Scotland, Europe and the world is compared.
A progressive increase in the number of cases diagnosed per year is observed for all three groups. In particular, oral cancer appears to be increasing in Europe at a higher rate than in the world. The trend observed in Scotland closely mirrors that of the european countries, but the number of cases reported in the former are higher.
From the ISD Scotland NHS Statistics Division (http://www.show.scot.nhs.uk/isd/cancer).

The reduction of alcohol and tobacco consumption are known to be important preventive measures for oral cancer, but the apparent discrepancy between reduction of risk factors and epidemiological trend observed in the last decade suggests other, as yet unidentified, risk factors being implicated in the pathogenesis of these condition. Interestingly, lung cancer mortality rates have shown a significant decrease in most European countries in the past decades, reflecting the reduction of tobacco consumption (Brennan and Bray 2002). Although these data might suggest that tobacco consumption plays a greater role in the pathogenesis of lung cancer compared to oral cancer, this study does not indicate the changes in incidence of lung cancer in the same period. Therefore, it is not possible to determine whether this change in trend is due to a reduction of the exposure to risk factors or to an improvement of therapeutic strategies.

Another aspect of oral cancer that is cause of concern for Health Authorities is the 5-year survival rate, which has not improved in the last two decades. In Scotland, it was assessed around 45% in the period between 1991-95, remaining virtually unchanged when compared to the 43% figures reported for 1976-80 and considerably lower than the 52.3% figure assessed in the USA in the period between 1993-1990. The reasons for this figures remaining stationary over the years are largely unknown. A report on the British Medical Journal (Zakrzewska 1999) attributed this phenomenon to the poor public awareness about this cancer, despite the easy accessibility of the oral cavity for medical inspection. This causes delays in diagnosis and reduced survival times. No major improvement in therapeutic strategies was reported in recent decades (Boyle et al. 1995), and the importance of examining new radio-therapeutic and chemo-therapeutic modalities was strongly suggested.

1.2 Clinical and histological aspects

The most common histotype of oral cancer is squamous cell carcinoma (SCC), which accounts for 91% of all oral cancers (Silverman and Gorsky 1990). In the majority of cases, SCCs are not preceded by premalignant lesions, but it is quite common to observe areas of hyperkeratosis, the so called "white patches", or red velvety lesions, named erythroplakias, in the oral mucosa surrounding the established carcinoma. In addition to this, premalignant lesions might precede the development of oral cancer by several months or years. Therefore, there is a strong need for a proper clinical/histologic classification of premalignant lesions and a long-term assessment of their risks of malignant transformation.

The characterisation of oral premalignant lesions as leukoplakias and erythroplakias is purely clinical. Leukoplakias are white plaques of the oral mucosa with histologic features of hyperkeratosis, which is an increased thickness of the epithelial layer producing keratin; erythroplakias are bright red, velvety plaques often showing marked epithelial atrophy. Both are defined as idiopathic, as they cannot be characterised clinically or histologically as any other disease. Erythroplakias may be associated with leukoplakias, in which case they are referred to as erythroleukoplakias.

Leukoplakias are by far the most common premalignant lesions of the oral cavity. A large number of studies assessing their rates of malignant transformation have reported huge variability (0.13%-6%) (Silverman et al. 1984). Interestingly, Silverman and collaborators have observed a 17.5% of malignant transformation rate over a group of 257 patients, but when this data was limited to homogeneous leukoplakias the percentage reduced to 6.5%, quite similar to the previously reported figures. On the other hand, the transformation rate for erythroplakias was 4 fold higher than that reported for leukoplakias. In keeping with this, three other studies have reported a 5-7 fold higher percentage of malignant transformation in erythroleukoplakias compared to leukoplakias (Kramer 1969) (Banoczy 1977) (Roed-Petersen 1971).

Histologically, oral premalignant lesions designated with the general term of dysplasia may present with a number of alterations in the epithelial architecture and in cellular morphology. The classification of oral dysplasias as mild, moderate, severe or carcinoma *in situ* is complex, and is based on several internationally adopted criteria: 1) loss of polarity of the basal cells, 2) increased nuclear/cytoplasmic ratio, 3) altered nuclear polarization, 4) irregular epithelial stratification, 5) increased number of mitotic figures, 6) presence of mitotic figures in the superficial half of the epithelium, 7) cellular pleiomorphism 8) nuclear hyperchromatism, 9) enlarged nucleoli, 10) abnormal keratinisation, 11) reduced cellular cohesion and 12) invasion of the lamina propria (Kramer et al. 1978). Not all of these alterations are seen in any one case, and indeed not all of the premalignant lesions show features of epithelial dysplasia at all. Erythroleukoplakias are more likely to present a wider spectrum of dysplastic features, and are associated with a higher risk of cancer development (36.4%) (Silverman et al. 1984). Therefore, a more cautious clinical approach is advised for those patients presenting with dysplasias of moderate to high grade, particularly if located in the floor of mouth and ventral tongue, since these two sites have been shown to be more prone to malignant transformation, even if the supporting histological evidence is often unclear (Silverman et al. 1984).

1.3 Treatment of oral dysplasias

The standard treatment for oral dysplasias entails a needle biopsy of the lesion for histological classification and a periodical follow up of the patient. Local treatments, like laser ablation or surgical resection, are not always appropriate, because a large number of patients present with multiple lesions. This phenomenon was initially named field cancerization, and was considered a result of widespread damage to the oral epithelium by carcinogens (Slaughter et al. 1953). However, it remains unclear whether these lesions arise from multiple altered clones or to the migration of an altered cell throughout the oral mucosa. In addition, local therapies do not act on the remaining oral mucosa, which, according to the field cancerization concept, has been exposed to the same risk factors as the site of the dysplasia and is therefore prone to develop multiple lesions synchronously. It is therefore necessary to develop chemopreventive strategies designed to reverse oral premalignant lesions or to prevent their malignant transformation.

Retinoids are the most prominent chemopreventive drugs analysed in clinical trials (Hong et al. 1986), (Stich et al. 1988), (Han et al. 1990), (Lippman et al. 1993), (Chiesa et al. 1992). Their efficacy in reversing oral premalignant lesions varied between 27% and 87% depending on the compounds and doses used in each study. The main drawback of treatment with retinoids is their toxicity. Serious side effects have been reported with high-dose treatments, often resulting in patient drop-out. In addition, high rates of relapse have been observed within a few months from suspension of treatment (Hong et al. 1986).

1.4 Conclusions

Oral premalignant lesions are easily diagnosed at clinical examination and readily accessible for biopsy: these two characteristics render them an ideal model to study the multistep process of oral carcinogenesis. Nevertheless, it is as yet impossible to assess precisely the risk of cancerization of these lesions, based only on histological, epidemiological or environmental parameters. The pharmacological treatments presently available, although of proven efficacy, do not invariably revert all of the premalignant lesions, they cause heavy side effects and they fail to sustain clinical response after suspension. Therefore, it would be clinically desirable to identify additional molecular markers to be used for cancer risk assessment in these patients.

2 Retinoids and their receptors in Oral Cancer

2.1 Structure and biochemical functions of epithelia

Epithelial tissues like that of the skin, oral cavity, trachea and lung are known as stratified squamous epithelia. They are organised in a multistratified architectural structure, with the stem cells residing in the basal layer. In response to as yet unidentified signals, basal cells undergo a series of morphological and biochemical changes and migrate to more superficial compartments of the epithelium, forming the spinous and granular layers. This differentiation process ultimately leads to the production of dead, flattened, impermeable squames organised in a layer called stratum corneum. This layer is absent in non-keratinising epithelia, like that of the oral cavity mucosa.

The pattern of keratinocyte protein expression varies in different epithelia and in different layers of the same epithelium. Basal keratinocytes invariably express keratins 5 and 14; in addition, they express keratin 19 in oral mucosa. Suprabasal spinous keratinocytes express keratin 1 and 10 in skin, keratin 4 and 13 in oral mucosa and all four of them in keratinising epithelia of the oral cavity like hard palate and gengiva. In addition, skin keratinocytes express transglutaminase type I activity and produce glutamine and lysine rich envelope proteins, like involucrin. Spinous cells stop producing keratins and envelope proteins and specialize in generating proteins involved in macrofibrillar aggregation.

An abnormal pattern of expression of squamous differentiation markers has been described in head and neck squamous cell carcinoma (HNSCC) *in vitro*. In particular, two squamous cell carcinoma (SCC) cell lines from non-keratinising epithelia of the buccal mucosa and the retromolar trigone expressed keratin 1, transglutaminase type I and involucrin, whereas the normal keratinocytes from the same areas do not express any of those markers (Poddar et al. 1991). In addition, 6/7 SCC cell lines from non keratinising epithelium were reported to express lower levels of keratin 19 than their respective normal keratinocyte counterparts (Hu et al. 1991).

2.2 Biological effects of Retinoids

Vitamin A and its analogues (retinoids) are known to have several biological effects in different organs. In stratified squamous epithelia they are mainly involved in the maintainance of appropriate cellular growth and differentiation, as originally suggested by

the aberrant squamous metaplasia observed in humans after prolonged vitamin A deprivation.

In vitro, retinoids inhibit squamous cell differentiation in both normal skin keratinocytes (Rubin and Rice 1986), (Choi and Fuchs 1990) and HNSCC keratinocytes, as demonstrated by their effect on reducing the expression of squamous differentiation markers like keratin 1, transglutaminase I and involucrin (Zou et al. 1994) (Poddar et al. 1991). As stated above, expression of these specific markers in HNSCC is aberrant and indicative of an abnormal pattern of differentiation. Therefore, the inhibitory effect of retinoids in this cellular context could suggest their involvement in restoring the normal pattern of differentiation. Considering that the onset of squamous differentiation normally requires cessation of cellular proliferation (Fuchs 1990), it was considered potentially interesting to also investigate the effects retinoids have on cellular growth. This showed that retinoids exert opposite effects on keratinocytes from normal oral mucosa and HNSCC, the former being stimulated (Sundqvist et al. 1991) and the latter being inhibited (Zou et al. 1994). A possible explanation for this is that in malignant cells the link between cellular growth and differentiation is not as tight as in normal cells.

The biological effects of retinoids are mediated by a complex group of receptors. Two families of cytoplasmic receptors, Cellular Retinoid Acid Binding Proteins I and II (CRABP I–II) and Cellular Retinol Binding Protein I and II (CRBP I-II) are responsible for binding these molecules on the cellular surface, metabolising and transporting them to the nuclear surface. CRBP II is expressed exclusively in the small intestine, and is therefore thought to participate in the absorption of vitamin A (Crow and Ong 1985). On the nuclear surface, vitamin A and retinoids interact with nuclear receptors, comprising dimers of the RAR and/or RXR families, which belong to the steroid thyroid hormone receptor superfamily. Both RARs and RXRs are composed by three isotypes, α-β-γ. Alternative splicing and usage of different promoters gives rise to further isoforms of each isotype. The ligand binding pattern of these two families of receptors is also different: RARs bind both retinoic acid (RA) and 9-*cis*-RA, whereas RXRs bind only the latter and other synthetic analogues. Retinoid nuclear receptors are formed by either RXRs homodimers or RXR-RAR heterodimers. The so formed receptors then act as ligand-dependent DNA binding transcription factors, whose target genes are largely unknown (summarised in (Leid et al. 1992)).

2.3 Differential expression of Nuclear Retinoid Receptors in oral cancer progression

Nuclear retinoid receptors seem to be the direct mediators of the action of retinoids on gene expression. Therefore, a great interest has been shown over the past decade in trying to identify patterns of differential expression in normal oral mucosa, premalignant tissues and SCCs. Such knowledge would be crucial not only in understanding the physiological activities of retinoids, but also in targeting treatment or prevention of oral malignancies with receptor specific drugs.

Prominent studies on retinoid receptors in oral cancer have been carried out by Reuben Lotan's group. Their first work on this topic was carried out in 1991 (Crowe et al. 1991; Hu et al. 1991). Normal keratinocytes were cultured from the non-keratinising epithelium of the oral mucosa and from the keratinising epithelium of the epidermis. Expression of RARα-γ mRNA could be detected in all the cultures, and no significant changes were observed in both receptors expression in relation to levels of retinoic acid present in the medium. The expression of RARβ was more variable: it was detected at very low levels in epidermal keratinocytes and at very high levels in oral keratinocytes. In the latter, RARβ levels dramatically dropped when cells were cultured in medium containing delipidized serum, but were restored upon external addition of RA to the medium. This data might suggest that while RARα-γ are likely to regulate functions common to all keratinocytes, RARβ might be involved in regulating different patterns of suprabasal differentiation in stratified non-keratinising squamous epithelia.

In parallel with this work, the expression of RAR subunits α-β-γ was investigated *in vitro* in oral leukoplakias and SCCs (Hu et al. 1991). This study demonstrated loss of RARβ expression in 5/7 SCC cultures derived from non keratinising epithelium of the oral cavity, while cultures from normal mucosa from the same sites expressed RARβ. Cultures of two oral leukoplakias from similar sites regularly expressed RARβ, but the number of samples was insufficient to be sure that this was a trend. On the other hand, no significant changes were reported in RARα–γ expression in SCC and leukoplakia cultures compared to their normal counterparts, other than a slight reduction in levels of RARγ. Therefore, this study established loss of RARβ as a frequent event in oral cancer.

These studies were then extended to the *in vivo* scenario (Xu et al. 1994). RAR α-β-γ and RXR α-β expression was assessed *in situ* in a large number of biopsy specimens from

normal oral mucosa, normal tissue adjacent to SCC lesions, hyperplasias, dysplasias and SCCs. All 5 receptors were detected in all of the biopsies taken from healthy volunteers with no significant changes found in the expression of RARγ and RXRα-β across the panel of samples analysed except for a slight decrease in the percentage of premalignant and malignant samples expressing RARα. The major variations were found in RARβ expression: in keeping with the *in vitro* findings, expression of this receptor was lost in 65% of carcinoma samples analysed, and interestingly also in 44% of oral dysplasias and in 27% of hyperplasias. This study was of crucial importance not only because it extended the *in vitro* findings to an *in vivo* situation, but also because it suggested a potential involvement of RARβ loss at early stages of oral cancer progression.

In the light of all this background knowledge, previous work from our group (McGregor et al. 1997) has analysed RAR/RXR α-β-γ expression in a series of primary cultures derived from normal oral mucosa, normal tissue adjacent to SCC lesions, dysplasias and SCCs (derived by Paul Harrison's and Ken Parkinson's groups at the Beatson Institute for Cancer Research). The novel information available in our study was the proliferative fate of all the samples analysed. All of our cultures were serially passaged until they reached growth arrest after 5-30 population doublings (and defined mortal) or until they proliferated beyond the 100 population doublings threshold (and defined immortal). RXRα-γ expression was not detectable in any of the cultures. The levels of RARα-γ and RXRβ did not show any consistent changes during cancer progression. RARβ showed a much more complex pattern of expression. In cultures from normal oral mucosa, RARβ was barely detectable *in vitro* unless the cells were treated with retinoic acid, but it was readily detectable in the corresponding biopsies *in vivo*. A 2-10 fold induction of RARβ expression upon retinoic acid treatment has been reported also for human normal mammary epithelial cells (Swisshelm et al. 1994). In mortal dysplasias, both erythroplakias and leukoplakias, and in SCC, RARβ expression was associated with the proliferative fate of the cultures. Mortal cultures expressed RARβ constitutively, i.e. independently of retinoic acid treatment, whereas all of the immortal cultures had lost RARβ expression. The only tumour expressing RARβ was also the only one that senesced in culture. Recently (McGregor et al. 2002), our group has extended these findings to a wider panel of premalignant and malignant lesions, confirming that RARβ loss of expression occurs at the dysplasia stage of oral cancer progression and is associated with the acquisition of the immortal phenotype. The only exception to this general trend is our culture D17, that proliferates for a considerably extended life-span (45 population doublings) but is still mortal. The features of D17 will be discussed in detail in the Results chapter of this thesis.

More recently, a very interesting interventional study carried out by Reuben Lotan's group (Wan et al. 1999) further refined the general knowledge on the role of RARβ in mediating the biological effects of retinoids. A human HNSCC cell line that naturally expresses RXRα-β-γ and RARα-γ but lacks RARβ expression was stably transfected with a retroviral vector transducing $RAR\beta_2$. In most of the infected clones the expression of squamous differentiation markers could be suppressed with doses of retinoids that were 1/10.000 of those required to exert the same effect on the parental line. No significant difference could be observed in the sensitivity to the growth inhibitory effects of retinoids after restoration of RARβ expression.

To corroborate the importance of RARβ in carcinogenesis, it has been reported that transgenic mice overexpressing an antisense $RAR\beta_2$ transgene are prone to developing lung tumours (Berard et al. 1996). In keeping with this, expression of RARβ in epidermoid lung cancer-derived cells reduced their tumourigenicity in nude mice (Houle et al. 1993).

2.4 RARβ and senescence

The role of RARβ in maintaining the mortal phenotype and the consequent need for RARβ silencing to allow the acquisition of the immortal phenotype is suggested by another finding reported by our group (McGregor et al. 2002). Keratinocytes from normal oral mucosa cultured from early stages to senescence showed a progressive increase of expression of RARβ, consistent with a role in the cellular senescence programme. Similarly, an up-regulation of RARβ expression has been reported in senescing normal human mammary epithelial cells (Swisshelm et al. 1994).

2.5 Conclusions

The evidence summarised suggests that RARβ is involved in several biological processes in human cells: squamous differentiation, cellular senescence and tumourigenicity. In oral cancer, loss of RARβ expression is a frequent event in premalignant lesions and SCCs *in vivo*, and is associated with the acquisition of an immortal phenotype *in vitro*.

3 The role of Epidermal Growth Factor Receptor in oral carcinogenesis

3.1 Receptor structure and signalling

The epidermal growth factor receptor (EGFR) family is composed of four members, EGFR, c-erbB2, 3 and 4. They are located on the cellular surface and their cytoplasmic domains have an intrinsic protein tyrosine kinase activity. Amongst these four receptors, EGFR is the only one able to bind epidermal growth factor (EGF) and transforming growth factor α (TGFα) and to mediate their biological activities. In response to ligand binding, EGFR forms homodimers or heterodimers with its preferential binding partner c-erbB2 and becomes activated through auto-phosphorylation at multiple tyrosine residues (summarised in (Klapper et al. 2000)). The precise mechanisms through which the ligand induced surface signals are transmitted to the nucleus and exert their effects are not fully understood, although the Stat (signal transducers and activators of transcription) signalling pathway seems to be involved. The Stat family is composed by seven members: Stat 1, 2, 3, 4, 5a, 5b and 6) (reviewed in (Aaronson and Horvath 2002)). They are activated in response to receptor-ligand coupling by phosphorylation, mainly through the Janus kinase (JAK) receptor associated cytoplasmic proteins. Upon activation, Stat proteins dimerize and translocate to the nucleus, where they bind to DNA response elements and therefore regulates gene expression. The EGFR has an intrinsic tyrosine kinase activity and it has been shown to directly activate Stat 1, 3 and 5 (David et al. 1996). Increased activation of Stat 3 has been reported in a variety of tumours, including breast cancer, lung cancer and haematologic neoplasias (summarised in (Bowman et al. 2000)). Evidence for interactions between EGFR/TGFα and Stat3 in squamous cell carcinogenesis has been brought by interventional studies (Grandis et al. 1998)(a): blocking the EGFR/TGFα signalling pathway by either TGFα neutralizing antibodies or EGFR-specific tyrosine kinase inhibitor resulted in a decrease in levels of activation of Stat3, suggesting that Stat3 activation is indeed linked to EGFR signalling.

3.2 Biological effects in keratinocytes

EGFR and its ligands are involved in positively regulating keratinocyte growth. This is primarily suggested by the absolute requirement for EGF when keratinocytes are maintained in culture. Further evidence comes from the fact that modulation of EGFR

using several strategies, such as the use of inhibitors of EGFR kinase activity (Bos et al. 1997), monoclonal antibodies or antisense oligonucleotides, results in inhibition of cellular proliferation in HNSCC cells but not in normal mucosal epithelial cells, as assessed by incorporation of (^{3}H) thymidine (Grandis et al. 1997). The same effect were observed using antisense oligonucleotides against TGFα (Grandis et al. 1998)(b). A possible explanation to this comes from the observation that EGFR is overexpressed in SCC cells, so their growth might be more dependent on the EGFR pathway than it is in normal cells.

In keeping with its role as a downstream effector in the EGFR signalling pathway, Stat 3 also seems to play a role in stimulating keratinocyte growth, since transfection of HNSCC cells with a dominant negative construct of Stat3 results in growth arrest (Grandis et al. 2000).

3.3 EGFR/TGFα differential expression in squamous cell carcinoma and premalignant lesions

Considering the importance of EGFR and its ligands in stimulating keratinocyte growth, it was a plausible hypothesis that a quantitatively or qualitatively abnormal expression of this receptor or production of its ligands might be a mechanism utilised by squamous cancer cells to acquire a proliferative advantage over their normal counterparts. Therefore, a number of studies have been carried out over the years to investigate EGFR and TGFα expression in HNSCCs.

EGFR gene amplification and protein overexpression was initially described in HNSCC samples by Ozanne and co-workers (Ozanne et al. 1986). Amplification of the EGFR was also reported in 10/12 HNSCC cell lines by Yamamoto and co-workers (Yamamoto et al. 1986), but only in 4/21 HNSCC cell lines in a later study (Ishitoya et al. 1989). In the same experiments, EGFR protein overexpression was found in approximately half of the biopsies from well differentiated carcinomas but in none of the moderately or poorly differentiated HNSCCs. Following these early studies, an increasing amount of evidence has since accumulated confirming the differential regulation and expression of both EGFR and TGFα in head and neck squamous cell carcinogenesis suggested earlier.

Grandis and co-workers (Grandis and Tweardy 1993) analysed EGFR and TGFα mRNA expression in cultures of normal oral mucosa and HNSCC. They found that both markers were barely detectable in normal mucosa, but they appeared upregulated in HNSCC. In

keeping with this, Stanton and collaborators in our Institute described EGFR upregulation in 8/10 cultures from oral SCCs, but normal levels of expression in 2/2 erythroplakia cultures (Stanton et al. 1994).

Changes in EGFR and TGFα expression were further clarified by a few ***in situ*** studies on fresh biopsy material. Using IHC analysis, Shin and co-workers (Shin et al. 1994) reported a 2-fold increase in EGFR expression in normal epithelium adjacent to tumours compared to normal epithelium from healthy individuals. In samples from normal individuals, the EGFR expressing cells were confined to the basal layer of the epithelium, while in apparently normal samples adjacent to SCCs EGFR expressing cells were also found in the suprabasal layers. The upregulation of EGFR in macroscopically normal mucosa adjacent to tumours could be an effect of the field cancerization, since all of these patients had a history of heavy tobacco use, while the healthy donors had never smoked. Similarly, EGFR was localised in basal and suprabasal epithelial layers in hyperplastic and dysplastic lesion. At the carcinoma stage, a further increase in EGFR expression (3-fold) was observed, and the distribution of EGFR positive cells was extended to all epithelial layers, including the most superficial ones. Similar data were reported by Grandis and collaborators for the expression of the ligand for EGFR, TGFα (Grandis et al. 1996) (Grandis et al. 1998)(c). In normal oral mucosa from non-smoking donors, EGFR was barely detectable and TGFα staining was confined to the suprabasal layers of the epithelium. In premalignant lesions, EGFR was slightly upregulated in mild dysplasias (1.5 fold) and moderate/severe dysplasias (2.5 fold); TGFα was slightly upregulated in mild dysplasias (1.5 fold) with no further upregulation in more severe dysplasia. In HNSCC biopsies, strong staining could be observed for both markers throughout all epithelial layers, particularly at the advancing margins of the tumours, although the absolute increase in both markers was only 2-fold compared to normal mucosa from healthy donors. The increased production of both EGFR and its ligand TGFα in all the mucosal layers in HNSCC is the most relevant finding of these works, suggesting a switch from a paracrine to an autocrine growth pathway. This would confer cancer cells a greater proliferative capacity compared to normal keratinocytes, and a relative independency from the levels of circulating growth factors.

Higher levels of EGFR and TGFα protein expression were also found to be negative predictors of patients survival (Grandis et al. 1998)(d).

3.4 c-erbB-2 receptor subunit in oral cancer

Following the observation that EGFR/c-erbB2 heterodimers are more stable and therefore dissociate from the ligand more slowly than any other combination of subunits, a few studies have assessed the involvement of c-erbB2 expression in SCC. Xia and co-workers (Xia et al. 1997) reported a strong association between c-erbB-2 expression and poor survival in patients with oral SCC. In a more recent study by the same group, protein expression of all the EGFR family members was assessed *in situ* in a large number of specimens from primary oral SCC (Xia et al. 1999). The authors reported that c-erbB2 expression was correlated with lymph node metastasis and poor disease outcome. Similarly, O-charoenrat and co-workers describe a strong association between overexpression of three members of the EGFR family (EGFR, c-erbB2 and c-erbB3) and advanced stages of HNSCC, particularly with nodal metastases (O-charoenrat et al. 2002). High levels of c-erbB2 have also been reported in three cell lines derived from oral SCC (Xia et al. 1999).

3.5 Conclusions

EGFR gene amplification and protein overexpression are frequent events in HNSCC *in vitro*. *In vivo*, EGFR is barely detectable in normal samples and confined to the basal layers, and its ligand TGFα is expressed at low levels only in the suprabasal epithelial layers. Slight increases of EGFR and TGFα expression are observed in dysplasias. In contrast, SCC express high levels of both EGFR and its ligand TGFα in all layers of the epithelium, consistent with the development of an autocrine pathway. Both markers are indicators of poor prognosis in HNSCC.

4 Cell cycle regulators

Mammalian cells are simultaneously exposed to a large number of stimuli from the extracellular microenvironment: growth factors, anti-mitogens and differentiation inducers. Cells are sensitive to such stimuli until a stage in late G1 phase of cell cycle, named restriction point, when they irreversibly commit to either entering the S phase and completing cell division, or to arrest in a quiescent state (G0). The cell cycle machinery governing cellular proliferation is activated and regulated by a complex interaction between cyclins, cyclin-dependent-kinases (cdks) and negative regulators, like cdk inhibitors, p53 and other players. These molecules exercise their functions at specific stages of the cell cycle: G1/S transition, S phase, G2/M transition, mitosis. The following sections summarise in a simplified way how the most relevant of these molecules interact and result in cells completing the mitotic division or pausing in a growth arrest status (fig. 2).

4.1 G1 cyclins and cdks

D-type cyclins (D1, D2 and D3) are key regulators of the G1-S phase of the cell cycle (reviewed in (Sherr 1994)). They are expressed in a lineage-specific manner, and the different subtypes show a greater intra subtype conservation in evolution than inter-subtype variation within a species, suggesting that they are not functionally redundant. Their synthesis is strictly dependent upon cellular exposure to mitogenic stimuli: it begins during the G0/G1 transition, reaches a peak level when cells approach the S phase and then remains constant during later phases of the cell cycle (Matsushime et al. 1994). Upon mitogen withdrawal, their levels drop dramatically. They assemble into functional complexes with two different cyclin-dependent-kinases: cdk4 and cdk6. Again, assembly of the two subunits into an active kinase complex requires the presence of mitogens. The association of D-type cyclins and their cdk partners appears to be transient, allowing cdks to associate with other partners during different phases of the cell cycle (Sherr 1993).

D-type cyclins are able to bind directly to retinoblastoma (Rb) protein and phosphorylate it through their catalytic component cdk4/6 (Kato et al. 1993). The phosphorylated form of Rb, pRb, is no longer able to bind to, and inhibit the functions of, transcription factors, like E2F, involved in G1/S transition (Matsushime et al. 1994).

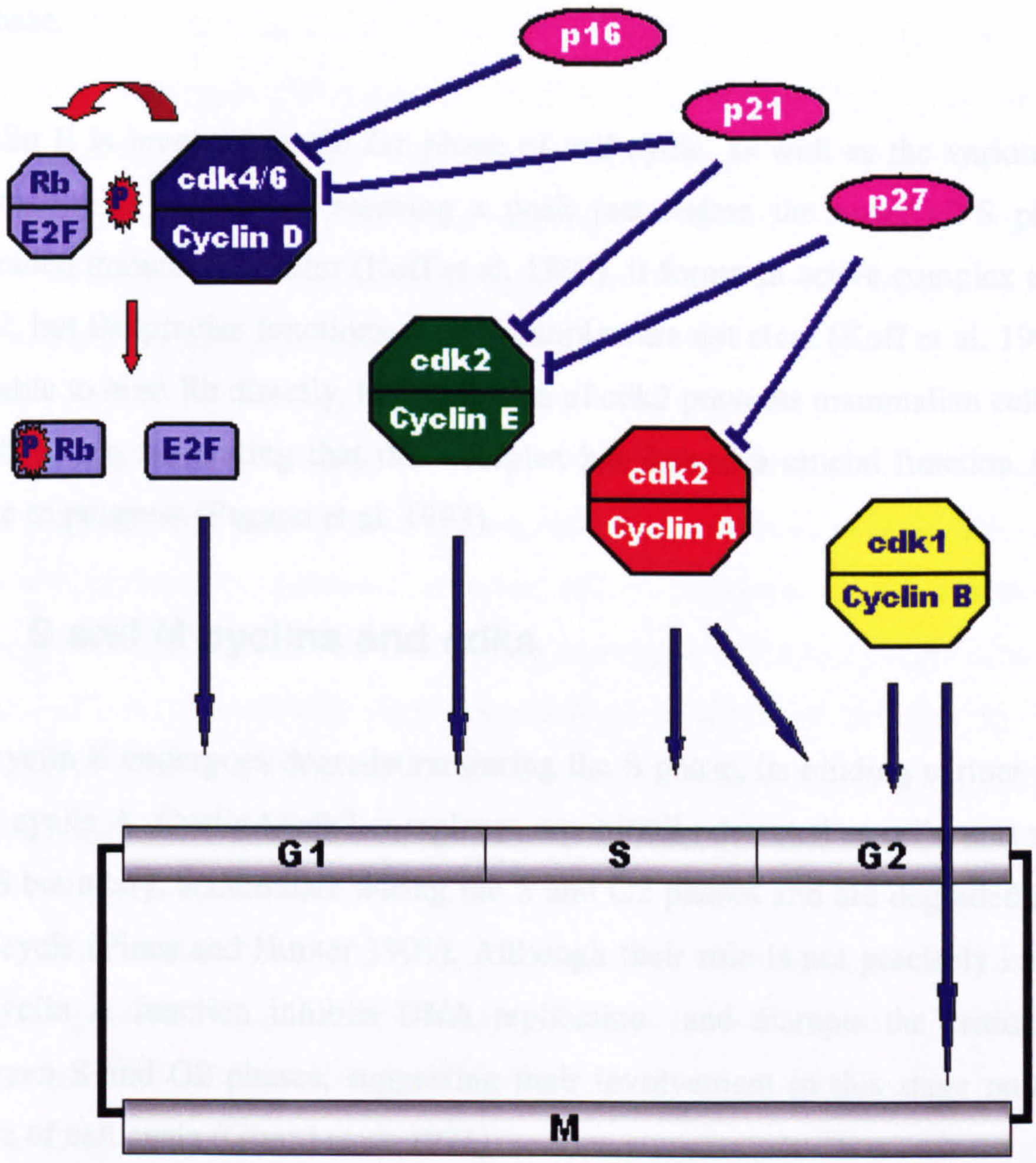


Fig. 2: Cell cycle regulators.

Graphic illustraton of the cell cycle and its regulators. The major cyclins, cyclin dependent kinases and cdk inhibitors are indicated (see text for discussion). G1: "Gap" phase 1; S: DNA synthesis phase; G2: "Gap" phase 2; M: mitosis.

As cells approach the G1/S boundary, the kinase activity of cyclinD/cdk4-6 complex gradually increases, Rb is phosphorylated, E2F is released from the inhibitory complex with Rb, target genes involved in S phase initiation are transcribed and cells proceed to the S phase.

Cyclin E is involved in the G1 phase of cell cycle, as well as the various cyclin Ds. Its expression is periodical, reaching a peak just before the onset of S phase and being degraded immediately after (Koff et al. 1992). It forms an active complex exclusively with cdk2, but the precise functions of this complex are not clear (Koff et al. 1992). Cyclin E is not able to bind Rb directly, but inhibition of cdk2 prevents mammalian cells from entering the S phase, suggesting that this complex has indeed a crucial function in allowing cell cycle to progress (Pagano et al. 1993).

4.2 S and M cyclins and cdks

As cyclin E undergoes degradation during the S phase, its binding partner cdk2 assembles with cyclin A. CyclinA/cdk2 complexes are initially detected as cells start approaching the G1/S boundary, accumulate during the S and G2 phases and are degraded at late stages of cell cycle (Pines and Hunter 1991). Although their role is not precisely known, inhibition of cyclin A function inhibits DNA replication and disrupts the critical temporal gap between S and G2 phases, suggesting their involvement in this stage rather than the G1 phase of cell cycle (Girard et al. 1991).

On the other hand, cyclin B is known to be involved both in entry to and in exit from mitosis. It is synthesized during S phase of cell cycle, forms active complexes with cdk1 (also named cdc2) and exerts its kinase activity during M phase (Pines and Hunter 1991). At anaphase, cyclin B is rapidly degraded and cdk1 released in its inactive form. This process is thought to regulate cellular exit from mitosis (Murray 1989).

4.3 Inhibitors of cyclins and cdks

An additional level of regulation on cell cycle progression is obtained through two families of cell cycle inhibitors, the INK4 family with its members $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, $p14^{ARF}$ (reviewed in (Roussel 1999)) and the CIP/KIP family with its members $p21^{CIP1/WAF1}$, $p27^{KIP1}$ and $p57^{KIP2}$ (reviewed in (Sherr and Roberts 1995)). The INK4 family inhibits specifically the cyclin D binding partners cdk4 and cdk6, whereas the CIP/KIP family is able to inhibit all cdks.

p21 is considered a universal cdk inhibitor, since it is capable of binding both cyclin D type dependent kinases cdk4/cdk6 and cyclin E and A dependent kinase cdk2 (Harper et al. 1995). In addition, p21 is able to inhibit DNA synthesis directly by binding PCNA (Xiong et al. 1992) which is a subunit of DNA polymerase δ and functions both in DNA replication and repair. p21 expression is positively regulated by p53 and its levels increase in response to p53 mediated DNA damage signals (el-Deiry et al. 1993). Considering its dual ability to inhibit DNA synthesis both directly (through PCNA) and indirectly (through cdks/Rb/E2F pathway) and its regulation by p53, p21 is considered as a mediator of p53 dependent cell cycle arrest in G1.

p27 is thought to be involved the cellular decision-making process between quiescing in G0 or entering G1, rather than in the regulation of cell division itself. The levels of p27 are known to increase in response to a variety of anti-mitogenic signals and to remain elevated during G0 (Firpo et al. 1994). When cells commit to cell cycle progression and enter G1/S phase, p27 is dramatically downregulated as a result of titration by cyclinD/cdk4-6 complexes. This drop in levels of p27 also causes activation of cyclinE/cdk2 complexes, allowing the cells to proceed into the following phases of cell cycle (Polyak et al. 1994).

The INK4a locus is unusually complex in its genomic organization, since it encodes two different proteins regulated by different promoters and translated with different reading frames: $p16^{INK4a}$ and $p14^{ARF}$ ($p19^{ARF}$ being its mouse homologue) (Quelle et al. 1995). p16 results from the transcription of exons 1α-2-3, whereas $p14^{ARF}$ transcription starts at exon 1β (located upstream of exon 1α), and proceeds with the same exons 2-3 as p16 (fig. 3).

Both p16 and $p14^{ARF}$ are considered tumour suppressor proteins, but their roles in cell cycle regulation are completely different. p16 specifically binds and inhibits cdk4 and cdk6. As previously discussed, these kinases are the major catalytic partners for D-type cyclins, and collaborate with cyclin E/cdk2 in controlling the G1/S transition in mammalian cells. p16 mainly binds cdk4, destabilizing cyclinD/cdk4 complexes and therefore preventing Rb phosphorylation and leading cells to G1 arrest (Serrano et al. 1993). A negative feedback loop seems to exist between Rb and p16: human cells lacking functional Rb through mutations or expression of viral oncoproteins have high levels of p16, sufficient for sequestering all the available cdk4/cdk6 and preventing them from binding with D-type cyclins (Li et al. 1994) (Parry et al. 1995). p16 is also implicated (and probably better known) for its role in the process of cellular senescence, which will be discussed in a later chapter.

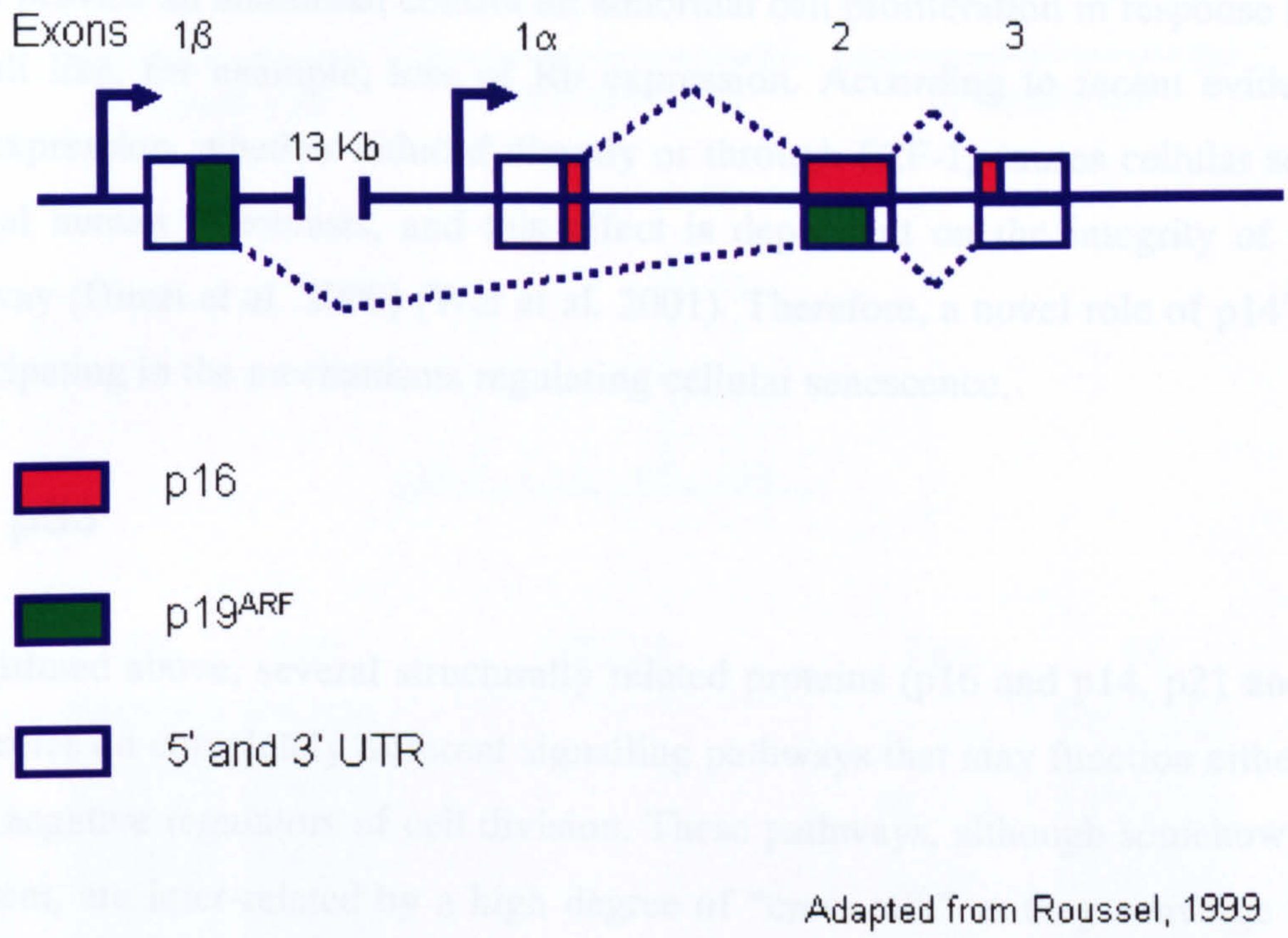


Fig. 3: The INK4a locus in the mouse.

Graphic illustration of the INK4a genomic organization. p16 is encoded by exons 1α, 2 and 3 (red boxes), $p19^{ARF}$ (the mouse homologue of human $p14^{ARF}$) is encoded by exons 1β, 2 and 3 (green boxes). The white boxes correspond to non-coding 5' and 3' regions. Splicing between the exons is indicated by the connecting lines. In the mouse, exons 1α and 1β are separated by an intronic sequence of approximately 13 Kb length.

$p14^{ARF}$ does not bind to cdks, but rather interacts with mdm2 and stabilizes p53 (discussed in more detail below), thus participating in the p53 pathway leading cells to G1 or G2 arrest or to apoptosis. $p14^{ARF}$ expression is activated by cellular oncogenes like Myc (Zindy et al. 1998), E1A (de Stanchina et al. 1998) and E2F-1 (Bates et al. 1998), and mediates p53-dependent cell cycle arrest or apoptosis in response to these stimuli. This could provide an additional control on abnormal cell proliferation in response to oncogenic stimuli like, for example, loss of Rb expression. According to recent evidence, $p14^{ARF}$ overexpression, whether induced directly or through E2F-1, causes cellular senescence in normal human fibroblasts, and this effect is dependent on the integrity of the p53-p21 pathway (Dimri et al. 2000) (Wei et al. 2001). Therefore, a novel role of $p14^{ARF}$ is that of participating in the mechanisms regulating cellular senescence.

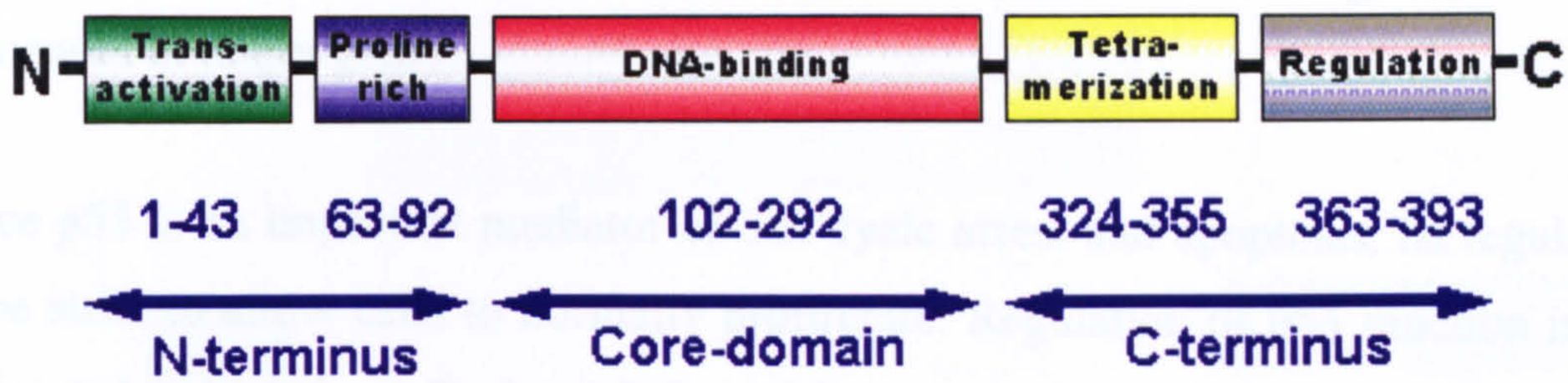
4.4 p53

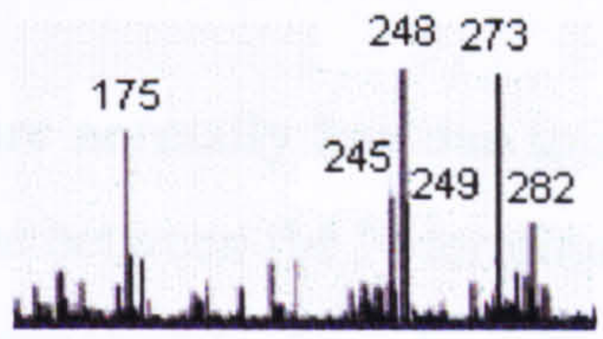
As outlined above, several structurally related proteins (p16 and p14, p21 and p27) exert their roles on completely different signalling pathways that may function either as positive or as negative regulators of cell division. These pathways, although somehow distinct and different, are inter-related by a high degree of "cross talk" as they converge to provide a complex mechanism that governs cellular proliferation.

p53 is a transcription factor that acts as a central relay in this intricated net of upstream and dowstream signals, leading to cell cycle arrest or apoptosis in response to a variety of stimuli. p53 also has a role in cellular senescence, as do some of the other molecular markers previously described, and this will be discussed in a subsequent chapter.

4.4.1 Structure and regulation

The full length p53 protein consists of 393 amino acids and can be divided into four functional domains (reviewed in (Levine 1997) and (Ryan et al. 2001)) (fig. 4). The 43 amino terminal residues are involved in transcriptional activation of several downstream target genes, and they also represent the binding site for negative regulators of p53 function, like MDM2. The DNA binding domain of p53 is located between amino acid residues 102 and 292, and their importance for the function of this protein is testified by the fact that over 90% of mutations described are located in this domain. p53 is a tetramer in solution, and aminoacid residues 324-355 are responsible for its tertiary structure.





T	63	140	15216	184	18
M	50.8%	40.7%	76.6%	34.2%	50%
N	3.1%	15.7%	6.4%	40.2%	0%
S	8%	3.6%	4.5%	2.2%	16.7%
O	27%	40%	12.5%	23.4%	33.3%

Adapted from Olivier and Hainaut, International Agency of Research on Cancer (http://www.iacr.fr) p53 mutation database, 2002

Fig. 4: p53 protein and localization of mutations.

Graphic illustraton of the p53 protein and the mutations reported in human cancer (IACR database, updated to year 2002).
p53 protein and the aminoacids codifying for the different functional domains are illustrated in the top section of the figure.
In the bottom section, the total number of mutations identified in each domain is reported (T), divided into the respective percentage of missense mutations (M), nonsense mutations (N), silent mutations (S) and other mutations (O). A comparative illustration of the specific codons mutated in the DNA binding domains is also presented.
Most of the p53 mutations are missense mutations, localized in the DNA-binding domain.

The carboxy terminal 26 aminoacid residues are involved in regulating the ability of p53 core domain to bind to specific DNA sequences: in a functionally inactive protein, it folds back and "covers" the central domain, preventing it from binding to DNA consensus sequences of target genes.

Since p53 is an important mediator of cell cycle arrest and apoptosis, its regulation needs to be strict to allow cells to normally proliferate. Regulation of p53 function is exerted at different levels and entails degradation of the protein by nuclear export and ubiquitination or stabilization mainly through phosphorylation and acetylation.

The levels of p53 in cells are normally low due to its short half-life, and its rapid turnover is mainly due to interactions between the N-terminus of p53 and MDM2 protein (Kubbutat et al. 1997). MDM2 is responsible for ubiquitin ligation of p53, nuclear export and subsequent degradation by the cytoplasmic proteolytic machinery (Roth et al. 1998). A feedback loop exists between MDM2 and p53, the latter being a positive regulator of MDM2 transcription (Wu et al. 1993).

Another level of p53 regulation is represented by its post-translational modifications. As mentioned in the structural description of p53, the amino terminus has a binding site for MDM2. Phosphorylation of this domain at serine 15 and 37 or at serine 20 does not affect the DNA binding properties of p53, but it decreases its affinity to MDM2, therefore preventing its degradation and increasing the protein levels (Shieh et al. 1997). The carboxy terminus of p53 is regulated by phosphorylation at serine 392 (Kapoor and Lozano 1998) and acetylation at lysine residues 320 and 382 (Sakaguchi et al. 1998). These modifications lead to activation of the DNA binding domain, possibly by interfering with its folded structure. Nevertheless, phosphorylation of p53 does not appear to be essential for its transcriptional activity: several C-terminal and/or N-terminal phosphorylation site p53 mutants lost the ability to be phosphorylated at the corresponding mutant site but retained the transcriptional activation function (Ashcroft et al. 1999).

4.4.2 Upstream regulators

p53 is activated by a variety of cellular stresses, including DNA damage, viral and cellular oncogenes (fig. 5). In response to these stimuli, p53 mediates cell cycle arrest to allow for DNA repair mechanisms to take place or, when the extent of the damage exceeds the possibility of repair, leads the cell to apoptosis.

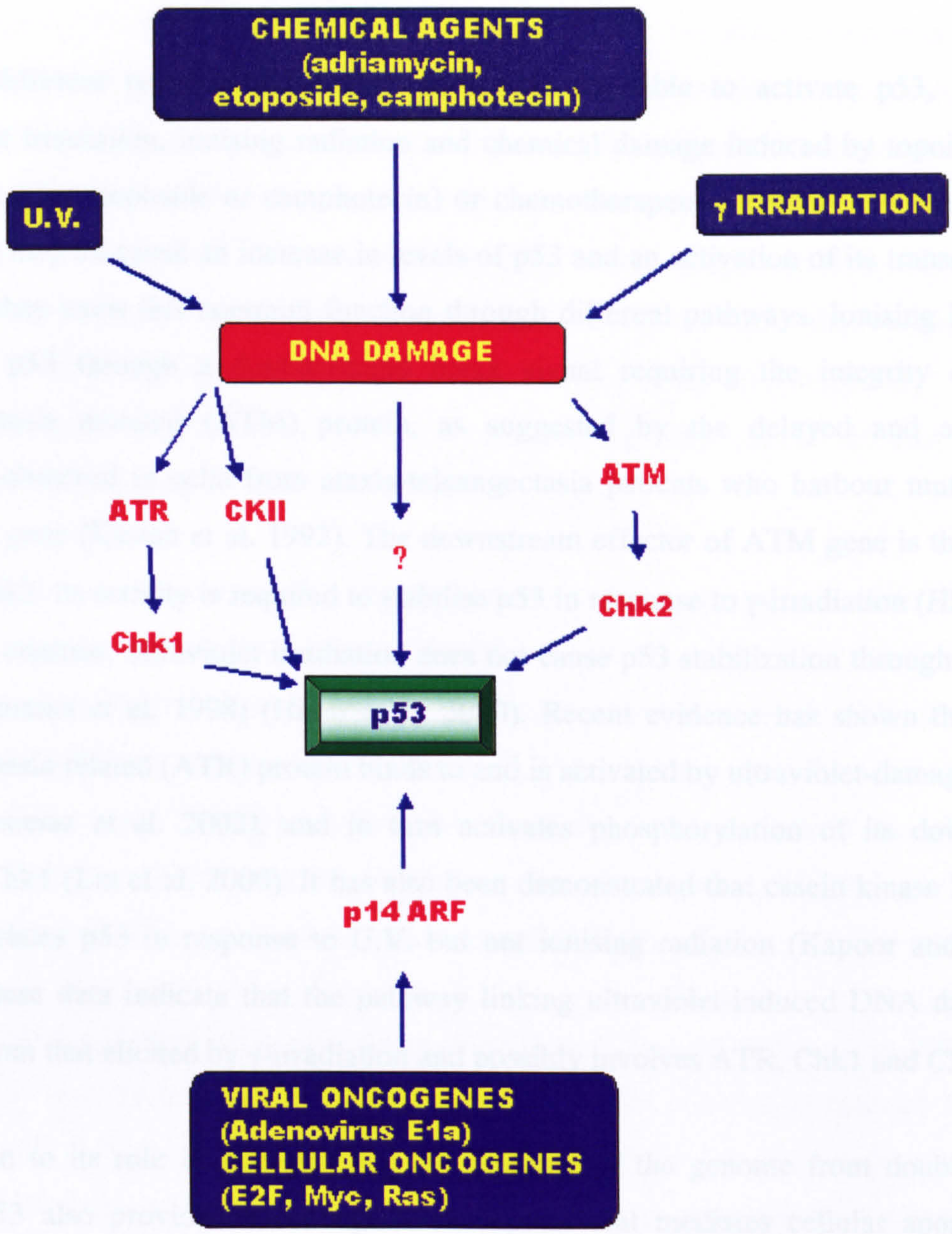


Fig. 5: Upstream regulators of p53

Graphic illustration of the main upstream activators of p53 (see text for discussion). Signals creating a DNA damage effect or an uncontrolled proliferation are known to activate p53 and mediate cell cycle arrest or apoptosis.

These functions of p53 provide an efficient mechanism to inhibit the proliferation of those cells that show genetic abnormalities (for a recent review, see (Woods and Vousden 2001)).

Several different types of DNA damage signals are able to activate p53, including ultraviolet irradiation, ionising radiation and chemical damage induced by topoisomerase inhibitors (like etoposide or camphotecin) or chemotherapeutic agents (like adriamycin). Although they all cause an increase in levels of p53 and an activation of its transcriptional activity, they exert this common function through different pathways. Ionising Radiation activates p53 through a double-strand break signal requiring the integrity of ataxia teleangectasia mutated (ATM) protein, as suggested by the delayed and attenuated response observed in cells from ataxia-teleangectasia patients who harbour mutations in the ATM gene (Kastan et al. 1992). The downstream effector of ATM gene is the protein kinase Chk2: its activity is required to stabilise p53 in response to γ-irradiation (Hirao et al. 2000). In contrast, ultraviolet irradiation does not cause p53 stabilization through ATM or Chk2 (Canman et al. 1998) (Hirao et al. 2000). Recent evidence has shown that ataxia teleangectasia related (ATR) protein binds to and is activated by ultraviolet-damaged DNA (Unsal-Kacmaz et al. 2002), and in turn activates phosphorylation of its downstream effector Chk1 (Liu et al. 2000). It has also been demonstrated that casein kinase II (CKII) phosphorylates p53 in response to U.V. but not ionising radiation (Kapoor and Lozano 1998). These data indicate that the pathway linking ultraviolet-induced DNA damage is distinct from that elicited by γ-irradiation and possibly involves ATR, Chk1 and CKII.

In addition to its role in safe-guarding the integrity of the genome from double strand breaks, p53 also provides an oncogene checkpoint that mediates cellular apoptosis in response to hyperproliferative stimuli. Viral oncogenes like the adenovirus protein E1A and cellular oncogenes like Myc, Ras and E2F are able to activate p53 through a $p14^{ARF}$ mediated signalling pathway, as reported by several studies in humans and mice (Bates et al. 1998) (de Stanchina et al. 1998) (Zindy et al. 1998). Once activated by oncogenes, ARF is able to stabilize p53 through direct interaction with the carboxy terminus of MDM2, which in turn binds p53 with its own amino terminal domain. This complex interaction closes the circuit between cell cycle promotion through pRb and apoptotic or growth-arrest responses to hyperproliferative stimuli occurring during oncogene expression.

4.4.3 Downstream targets

Once p53 is activated, a series of downstream events take place, most of which lead to cell cycle arrest in G1 or G2, DNA repair or apoptosis (fig. 6). The main downstream target of p53 in the induction of G1 cell cycle arrest is p21. Its role in inhibiting cyclinD/cdk4-6 and cyclinE/cdk2 complexes has been discussed above. p53-induced G2 arrest in response to DNA damage is mediated by both p21 and 14-3-3σ. The latter exerts its effect by binding to cyclinB/cdc2 complexes and sequestering them to the cytoplasm, thus inhibiting cell cycle progression (Chan et al. 1999). Recently it has also been shown that the roles of p21 and 14-3-3σ in mediating the G2-M block triggered by p53 are complementary: 14-3-3$\sigma^{-/-}$ p21$^{-/-}$ cells died significantly sooner than cells lacking only one of the two genes in response to DNA damage (Chan et al. 2000).

Another function of activated p53 is that of leading to programmed cell death. The ability of p53 to directly activate BAX protein, which is a member of the Bcl-2 family, has been known for years (Miyashita and Reed 1995). Another member of the Bcl-2 family, NOXA (Oda et al. 2000)(a) and two novel genes, PUMA (Nakano and Vousden 2001) and p53AIP1 (Oda et al. 2000)(b) have recently been described to mediate the p53-related apoptotic response. These proteins localise to the mitochondria, trigger cytochrome C release, activation of the caspases and subsequent cell death.

Once the DNA damaged cells have stopped in G1 or G2 phase of cell cycle, DNA repair mechanisms need to be triggered before the cells can be allowed to proceed to further stages of cell division. Although the pathways involved are not completely clear, it has been speculated that p53 may be also directly involved in these regulatory mechanisms.

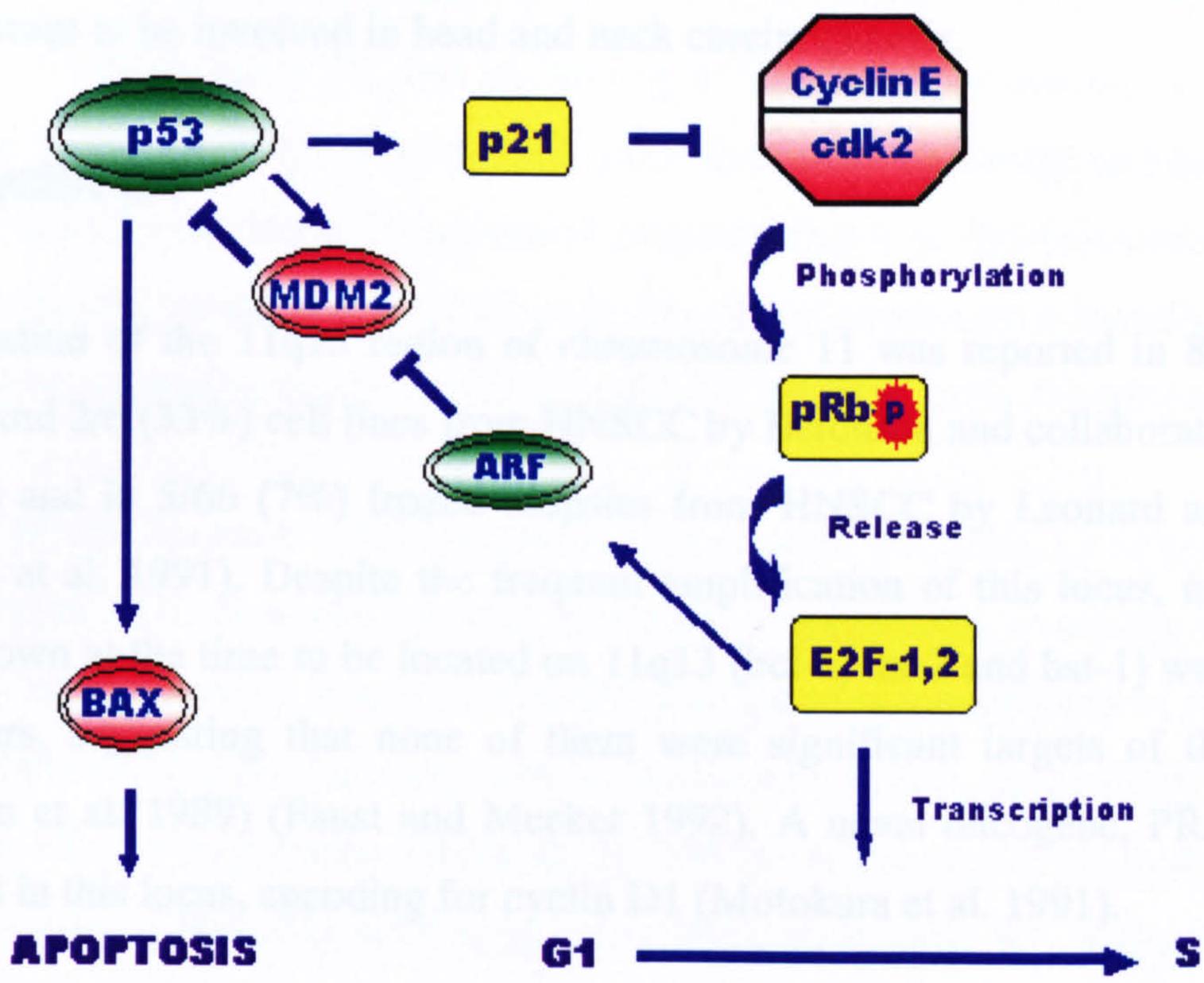


Fig. 6: Mediators of p53 dependent cell cycle arrest and apoptosis

Graphic illustration of the main mediators of the effects of p53 in cell cycle regulation and apoptosis (see text for discussion). p53 exerts its control on cell cycle progression mainly through p21 and its inhibitory effect on cyclinE/cdk2. Regulation of apoptosis implicates several p53 activated molecules, BAX being the best known. p53 also participates in regulation of its own stability through a regulatory feed-back loop involving MDM2 and $p14^{ARF}$.

5 Changes in cell cycle regulators in oral cancer

Out of the many cell cycle regulators described in the previous chapter, dysregulation of a number seem to be involved in head and neck carcinogenesis.

5.1 Cyclin D1

Amplification of the 11q13 region of chromosome 11 was reported in 8/23 (35%) fresh samples and 2/6 (33%) cell lines from HNSCC by Berenson and collaborators (Berenson et al. 1989) and in 5/66 (7%) frozen biopsies from HNSCC by Leonard and collaborators (Leonard et al. 1991). Despite the frequent amplification of this locus, none of the three genes known at the time to be located on 11q13 (bcl-1, int-2 and hst-1) was overexpressed in tumours, suggesting that none of them were significant targets of the amplification (Berenson et al. 1989) (Faust and Meeker 1992). A novel oncogene, PRAD-1, was later identified in this locus, encoding for cyclin D1 (Motokura et al. 1991).

Since then, amplification of PRAD-1 and overexpression of cyclin D1 gene have been reported by several studies of HNSCC ***in vivo***. Callender (Callender et al. 1994) has described a 2-10 fold amplification in 34% of fresh samples from HNSCC using southern blotting and Okami (Okami et al. 1999) reported a 2-6 fold amplification in 4/11 tumours using Fluorescence *In situ* Hybridization (FISH). A 2-12 fold amplification of PRAD-1 was also reported in 17/46 (37%) laryngeal tumours by Jares (Jares et al. 1994), and a significant association was found between gene amplification and mRNA over-expression. This report adds a functional significance to the occurrence of PRAD-1 locus amplification. In fact, similar percentages of HNSCCs have been reported as overexpressing cyclin D1 protein *in vivo*: 48% by Bartkova (Bartkova et al. 1995), 40% by Koontongkaew (Koontongkaew et al. 2000) and 26% by Schoelch (Schoelch et al. 1999). Recently, PRAD-1 amplification and cyclin D1 overexpression have been described in oral premalignant lesions and carcinomas *in vivo* (24-45%), but no correlation was found between percentage of samples with amplified cyclin D1 gene and increasing degrees of dysplasia or malignant transformation (Rousseau et al. 2001).

The evidence for cyclin D1 expression in oral cancer cell lines ***in vitro*** is not as definitive. Both Bartkova (Bartkova et al. 1995) and Okami (Okami et al. 1999), in two separate studies have reported cyclin D1 gene amplification in HNSCC cell lines, but the number of

samples analysed in each study is very small (3 and 4 respectively) and requires a cautious interpretation.

The study by Okami and collaborators also analysed cyclin D1 in the context of the p16/Rb pathway, of which cyclin D1 and its binding partner cdk4 are crucial components (Okami et al. 1999). These authors reported that amplification of cyclin D1 and inactivation of p16 are not mutually exclusive, being found in concordance in 2/4 cell lines and 4/4 tumour samples. On the contrary, loss of Rb and loss of p16 were never found together. Overall, 21/23 tumours contained at least one alteration in the p16/cyclin D1/Rb pathway.

In view of its frequent amplification/overexpression in HNSCC, several authors have analysed cyclin D1 expression *in vivo* in relation to **prognosis.** In a large cohort of patients with SCC of the anterior tongue, cyclin D1 was found overexpressed in 68% of cases, and this correlated with high tumour grade and reduced survival, particularly when associated with loss of p16 expression (Bova et al. 1999). A retrospective study carried out by Michalides in 1995 (Michalides et al. 1995) involving 47 patients with HNSCC demonstrated a clear correlation of overexpression of cyclin D1 with disease recurrence and shortened survival. Finally, an association between cyclin D1 overexpression and advanced tumour stage and regional lymph-node metastasis has recently been reported (Carlos de Vicente et al. 2002). Overall, these data indicate that overexpression of cyclin D1 is an indicator of poor prognosis in HNSCC.

5.2 Polo-Like-Kinase

Polo-like kinase (PLK) is a member of a family of serine/threonine kinases. It represents the mammalian homologue of Polo kinase in Drosophila Melanogaster and of CDC5 in Saccharomyces cerevisiae. Its role in regulating G2-M transition in mammals is suggested by its increased total levels and phosphorylation status when cells approach the M phase of cell cycle. It is also involved in bipolar spindle formation, as suggested by its transient association with the spindle apparatus and the centromere region of mitotic chromosomes. It has also been reported that levels of PLK expression are correlated with the proliferative activity of the cells, and a dramatic downregulation is seen under conditions of terminal differentiation (Yuan et al. 1997).

Following a report suggesting a prognostic significance of PLK mRNA expression in lung cancer (Wolf et al. 1997), this molecular marker was analysed in HNSCC (Knecht et al. 1999). In this study, PLK1 mRNA was found to be overexpressed in the majority of SCC

of the oropharynx and larynx, and to be strictly correlated with the metastasising potential of the tumour.

5.3 p16

As a tumour suppressor gene, p16 is often inactivated in carcinogenesis, through gene loss or mutation. The presence of a tumour suppressor gene on chromosome 9p had been suggested by the fact that deletions can frequently be found in several types of human tumours. By screening a large number of HNSCC with several markers localised on both arms chromosome 9, Van der Riet and co-workers were able to further refine the minimal area of loss to 9p21-22 (van der Riet et al. 1994). A few years later, the same group reported LOH involving this locus not only in the vast majority of the invasive carcinoma samples analysed (73%), but also in 20% of hyperplastic lesions, 57% of dysplasias and 80% of CIS (Califano et al. 1996). A candidate tumour suppressor gene for the INK4A locus on chromosome 9p21-22 was p16, although this locus encodes for both p16 and $p14^{ARF}$ which have distinct functions.

A smaller proportion of carcinomas from the HN also showed homozygous deletion of the INK4a locus (33%) suggesting that this is a frequent mechanism of p16 inactivation, even though this percentage is not as high as that found in other types of tumours (Cairns et al. 1995). Point mutations in one allele of the p16 gene have been described as a frequent event in oesophageal SCC (50% of cases) (Mori et al. 1994), but they seem to occur with lower frequency in HNSCC: Zhang and co-workers (Zhang et al. 1994) report point mutations harboured in the p16 gene in only 11/68 (16%) HNSCC tumour samples.

Another mechanism of p16 inactivation is transcriptional silencing through promoter methylation. DNA methylation at the 5' CpG island has been described in lung cancer and HNSCC biopsy samples and cell lines (Merlo et al. 1995). *In vitro*, methylation was associated with complete transcriptional silencing, and this was reversed by treatment with the demethylating agent 5-deoxyazacytidine.

A very comprehensive study investigating all of the genetic and epigenetic modifications of p16 gene and their effect on gene expression in HNSCC ***in vivo*** has been published by Reed (Reed et al. 1996). They describe loss of protein expression as assessed by IHC in 24/29 tumours sections. Of these, 16 showed homozygous deletion of p16 locus, 2 had LOH at the same locus and 5 showed promoter methylation, suggesting the first as the most important mechanism of p16 inactivation in this type of tumour. In contrast, a later

study by Miracca (Miracca et al. 1999) reported p16 inactivation in 20/32 HNSCC samples. Homozygous deletion occurred in only one case, whereas promoter hypermethylation and LOH were found in most of the remaining cases (13/20), followed by hypermethylation and mutation (4/20) and mutation and LOH (2/20).

p16 gene inactivation has also been described in oral premalignant lesions *in vivo*. Using IHC methods, Papadimitrakopoulou and co-workers (Papadimitrakopoulou et al. 1997) described loss of protein expression in 38% of oral leukoplakias, and a good correlation was found with LOH at 9p21 (80% of cases). This is consistent with Califano's data, reporting LOH at 9p21 as a frequent event in oral dysplasias, followed by a sharp increase in the invasive tumour stages. Analysing all these data, loss of p16 appears to be an early event in oral cancer progression.

Work carried out in our Institute has clarified the dysfunctional events involving p16 in HNSCC *in vitro* (Loughran et al. 1996) (McGregor et al. 1997) (McGregor et al. 2002). Loss of p16 expression was found in 19/19 immortal carcinomas and 6/7 immortal dysplasias, but only in 1/6 mortal carcinomas and 1/8 mortal dysplasias. In carcinomas, the mechanism responsible for inactivation of p16 was homozygous deletion in 8/18 cultures, whereas in the remaining 10 the absence of mRNA suggested a transcriptional silencing mechanism. In two of these cell lines, p16 transcripts were readily detectable after treatment with demethylating agents (Loughran et al. 1996). In addition to this, our group has demonstrated that p16 was re-expressed after demethylation treatment in 2/5 immortal dysplasia cultures but in none of the immortal carcinomas (McGregor et al. 2002). The combined results of these studies suggest a correlation between loss of p16 and acquisition of immortality rather than malignant phenotype, and confirm the importance of homozygous deletion and DNA methylation as mechanisms of p16 inactivation, in keeping with what described in literature.

However, the INK4a locus encodes for both p16 and $p14^{ARF}$, so further work was required to clarify which of the two proteins was the crucial target for inactivation in human cancer. In the 10 HNSCC cell lines described by Loughran (Loughran et al. 1996) as lacking large homozygous deletions of the INK4a locus, 4 had specifically inactivated p16 and another 4 had mutations or deletions in exons 2 and/or 3 that are shared by both proteins (Munro et al. 1999). Mutations in these exons are anticipated to alter the function of p16 but not $p14^{ARF}$ (Quelle et al. 1997). The remaining 2 cell lines had lost expression of $p14^{ARF}$, but no mutations of exon 1β (which is specific for $p14^{ARF}$) could be found. In keeping with this, a recent study by Shahnavaz (Shahnavaz et al. 2001) describes deletion of exon 1α

and/or exon 2 in 7/9 (72%) in oral carcinomas *in vivo*, but specific deletion of exon 1β was not found in any of the samples. These studies indicate that specific inactivation of p16 rather than $p14^{ARF}$ plays a role in human cancer. Nevertheless, the role of p14ARF inactivation in cancer development can not be excluded in mice: ARF-null mice that normally expressed p16 were prone to developing tumours at an early age (Kamijo et al. 1997), similarly to the phenotype described by Serrano and collaborators (Serrano et al. 1996) in INK4a-null mice.

A very interesting **interventional study** has further clarified that the loss of p16 expression in cancer development plays a direct role in allowing cellular proliferation. Several human carcinoma cell lines were transfected with a vector containing $p16^{INK4}$ cDNA. As a result, the colony forming efficiency was reduced, and the p16 expressing transfectants were selected against with continuous passage in culture (Okamoto et al. 1994).

The **clinical significance** of this molecular event in HNSCC has been assessed by a few studies, but it remains controversial. According to Bova (Bova et al. 1999), loss of expression of p16 is a marker of poor prognosis in carcinoma of the anterior tongue, particularly when associated with overexpression of Cyclin D1. In contrast with this, Yuen and collaborators (Yuen et al. 2002) found no clinical significance for loss of p16 expression in a group HNSCC lesions from different sites. This marker also seems unsuitable as a predictor of malignant transformation in premalignant laryngeal lesions, unless its loss occurs in concomitance with p53 overexpression (Gallo et al. 1997).

5.4 p53

The molecular mechanism most frequently involved in p53 inactivation is, similarly to other tumour suppressor genes, mutation of one allele followed by deletion of the other allele, leading to production of a non-functional protein. Not surprisingly, loss of heterozygosity at p53 locus (17p13) has been reported in 50-55% of HNSCC in two studies from the same group (Nawroz et al. 1994) (Califano et al. 1996), the latter also reporting an increasing percentage of LOH in premalignant lesions of increasing grade.

The occurrence of p53 mutations in human cancer is an extremely common event, and a large body of literature is available describing their nature, localization, frequency and worldwide distribution in all different types of cancer. A comprehensive and updated database of all the published literature on this topic is available through the Marie Curie Cancer Research Institute Paris website. In addition, a recent summary on mutations in p53

gene has been presented by Hainaut and Hollstein (Hainaut and Hollstein 2000). By and large, the most common type of human cancer mutation is a single base substitution (87%) but more complex mutations like small insertions or deletions are also found in 13% of cases. Point mutations are most frequently found at guanine nucleotides, consistent with the known action of benzopyrenes and nitrosamines present in cigarette smoke and with the importance of the smoking habit in the aetiology of HNSCC (Burns et al. 1993) (Boyle et al. 1993). In keeping with this, p53 mutations occur with a higher frequency in tobacco users than in patients that have never smoked (Lazarus et al. 1996) (Brennan et al. 1995), with a further increase when cigarette smoke is accompanied by alcohol intake (Brennan et al. 1995). The DNA binding domain, encoded by exons 5-8, is a mutational hotspot, harbouring 93% of the described mutations. This suggests that transcriptional activation of p53 is essential for its tumour suppressor activity. Although in most cases loss or mutation of both alleles is required to determine loss-of-function of p53, increasing evidence points out that some mutations might be sufficient in the heterozygous state, exerting a dominant negative effect on the wild type allele (Zambetti and Levine 1993) (Forrester et al. 1995).

Wild type p53 has a very short half life and as a consequence of this is almost undetectable with conventional Western blotting or immunohystochemical assays. Missense mutations often increase the half life and stability of p53 (Finlay et al. 1988), causing protein overexpression in tissues or protein samples. Therefore, p53 protein overexpression might be considered synonymous with gene mutation, and this assumption has been commonly made in literature. Nevertheless, it has to be taken into account that mutations which result in deletion or truncation of the protein do not cause its accumulation, leading to false negative results if the assessment of p53 mutations is based only on measurement of protein levels.

p53 mutations have often been described in HNSCC, but the reported frequencies might be underestimated considering that most studies limited their analysis to exons 5-8, the known mutational hotspots. A very early study (Sakai and Tsuchida 1992) analysed 15 oral SCC cultures and 5 of the biopsies from which the cultures were derived, finding homozygous mutations in almost all cases (14/15 cultures and 5/5 of the biopsies).

In our Institute, the p53 status of a large number of biopsies from HNSCC and their corresponding cultures has been analysed by K. Parkinson's group (Burns et al. 1993). Protein overexpression was reported in 8/24 (33%) of the samples analysed *in vivo* and in 9/18 (50%) of the corresponding cultures. Mutations in the p53 gene could be detected not only in all of the cultures overexpressing p53 but also in five cultures that did not express

high levels of p53. Upon sequencing, the mutations found in these five cultures either created premature stop codons or altered the reading frame, creating a truncated protein. The absence of the carboxy terminal region, which is responsible for protein oligomerization, might account for the absence of protein stabilization and consequent overexpression. The cultures overexpressing p53 had point mutations leading to a single base substitution. In most cases (11/13) no expression of the normal allele could be detected and the mutation was assumed to be homozygous. The same group has later reported that p53 overexpression is associated with the immortal but not the senescent phenotype in the same group of HNSCC cultures (Edington et al. 1995).

In a very interesting study carried out in Sidransky's laboratory, p53 gene has been sequenced in a large number of fresh biopsies and archival samples from all stages of head and neck cancer progression (Boyle et al. 1993). The authors report an increasing percentage of p53 mutations in the transition from dysplasias (15%) to carcinoma *in situ* (21%) to invasive SCC (43%), suggesting that the occurrence of p53 mutations starts in premalignant lesions and increases in frequency during the evolution to fully developed carcinoma. Another study reports the occurrence of p53 mutations in none of six hyperplasias analysed, but in 5/14 (36%) dysplasias and 26/33 (79%) SCCs (Kashiwazaki et al. 1997).

In another study, the percentage of samples staining positive for p53 progressively increased with increasing degrees of dysplasia (28-54%), but showed no further increase in carcinoma *in situ* and invasive SCCs (Sauter et al. 1994). A similar increase in the percentage of samples overexpressing p53 was reported in normal epithelium, hyperplasias and dysplasias all adjacent to SCC lesions (21-45%), with no further increase in the SCC samples (Shin et al. 1994). When assessed in oral dysplasias, p53 expression was detected in 5/14 (36%) samples by Kashiwazaki (Kashiwazaki et al. 1997), 9/20 (45%) samples by Shin (Shin et al. 1994) and 58/145 (40%) samples by Sauter (Sauter et al. 1994). In this last study, a clear increase in frequency of p53 overexpression was noted in the transition from mild (28%) to moderate (47%) to severe dysplasias (54%).

The **prognostic significance** of p53 overexpression in HNSCC seems controversial. In four different studies (Kaur et al. 1998) (Gluckman et al. 1994) (Sauter et al. 1995) (Field et al. 1993), p53 overexpression was correlated with a reduction of survival time after diagnosis with end stage disease, but p53 mutation was not correlated with clinical outcome (Sauter et al. 1995). In contrast, another study reported p53 mutations but not p53 overexpression to be correlated with patients survival in HNSCC (Mineta et al. 1998).

Three studies are focused on the association between p53 mutations and invasive potential in HNSCC. Burns et al. (Burns et al. 1994) have analysed three patients with SCC of the tongue, finding correspondence between the mutation in the primary tumour and in the lymph node metastasis. The high degree of congruence between mutations in primary and metastatic oropharingeal tumours has been confirmed by Zariwala et al. (Zariwala et al. 1994), indicating that p53 mutations precede metastatic spread. In contrast, Kropveld et al. (Kropveld et al. 1996) found no correspondence between p53 mutations in a group of 15 primary tumours and lymph node metastasis of HNSCC. Since these studies analysed quite small cohorts of patients, it is difficult to assess the actual significance of the data reported.

5.5 Conclusions

Several genetic events are involved in head and neck cancer development. Overexpression of cyclin D1 and PLK are frequently described at the carcinoma stage *in vivo*, both associated with poor prognosis, but no definitive data is available for either marker *in vitro*. p16 is often inactivated through deletion or promoter methylation both in premalignant and malignant lesions *in vivo*, and this event is associated with cellular immortality *in vitro*. Nevertheless, the clinical significance of p16 inactivation remains controversial. $p14^{ARF}$, which is encoded by the same genetic locus as p16, does not seem to be specifically targeted for inactivation in oral cancer development, both *in vivo* and *in vitro*. The frequency of p53 mutation and overexpression progressively increases in dysplasias of higher histological grade and carcinomas *in vivo*. In culture, mutation of p53 is often associated with the immortal phenotype. The prognostic significance of p53 in HNSCC remains unclear.

6 Telomeres and telomerase: an overview

The first indication that normal human somatic cells have a limited proliferative lifespan in culture goes as far back as four decades ago (Hayflick 1965) and is known as the Hayflick limit. The authors observed that embryonic cells explanted from various tissues proliferated for a finite number of population doublings before entering a non-dividing but viable state called replicative senescence. Proliferative potential *in vitro* was subsequently demonstrated to depend on species, tissue of origin and donor age (Martin et al. 1970) and was therefore thought to be genetically defined. Over the last decade, an impressive body of data has been obtained providing further insights into the molecular mechanisms governing the initial observation by Hayflick. It is now well accepted that the regulation of cellular lifespan involves specialized DNA structures at the end of chromosomes known as telomeres. The purpose of this chapter is to give an overview of the structure, functions and components of both telomeres and telomerase, the enzyme responsible for telomere maintainance (for a recent review on the general aspects of this topic, see (Kim et al. 2002)). The role of telomerase activity in cellular immortalisation will be discussed in more depth in chapter 7, in the context of all the other molecular markers known to participate in this process.

6.1 Structure and function of telomeres

6.1.1 Telomeric DNA

Telomeres are sequences of non coding DNA located at the end of chromosomes. They consist of repetitive 5'-TTAGGG-3' sequences, their length being extremely variable amongst species and, to a lesser extent, amongst different cells within a species. In human germ cells, telomeres measure 10-15 Kb, whereas in laboratory mice they exceed 30 Kb in length. Most of the telomere is composed of double stranded DNA, but a short tract of single stranded DNA overhangs at its 3' end. This phenomenon is explained by the well known "end replication problem" (fig. 7). DNA polymerases are able to replicate DNA only in the 5' to 3' direction and they initiate this process through a 8-12 base sequence of RNA hybridised to each DNA strand. While the replication of the leading strand is continuous and follows the replication fork, replication of the lagging strand is obtained through short fragments (Okazaki fragments) synthesised while the replication fork opens.

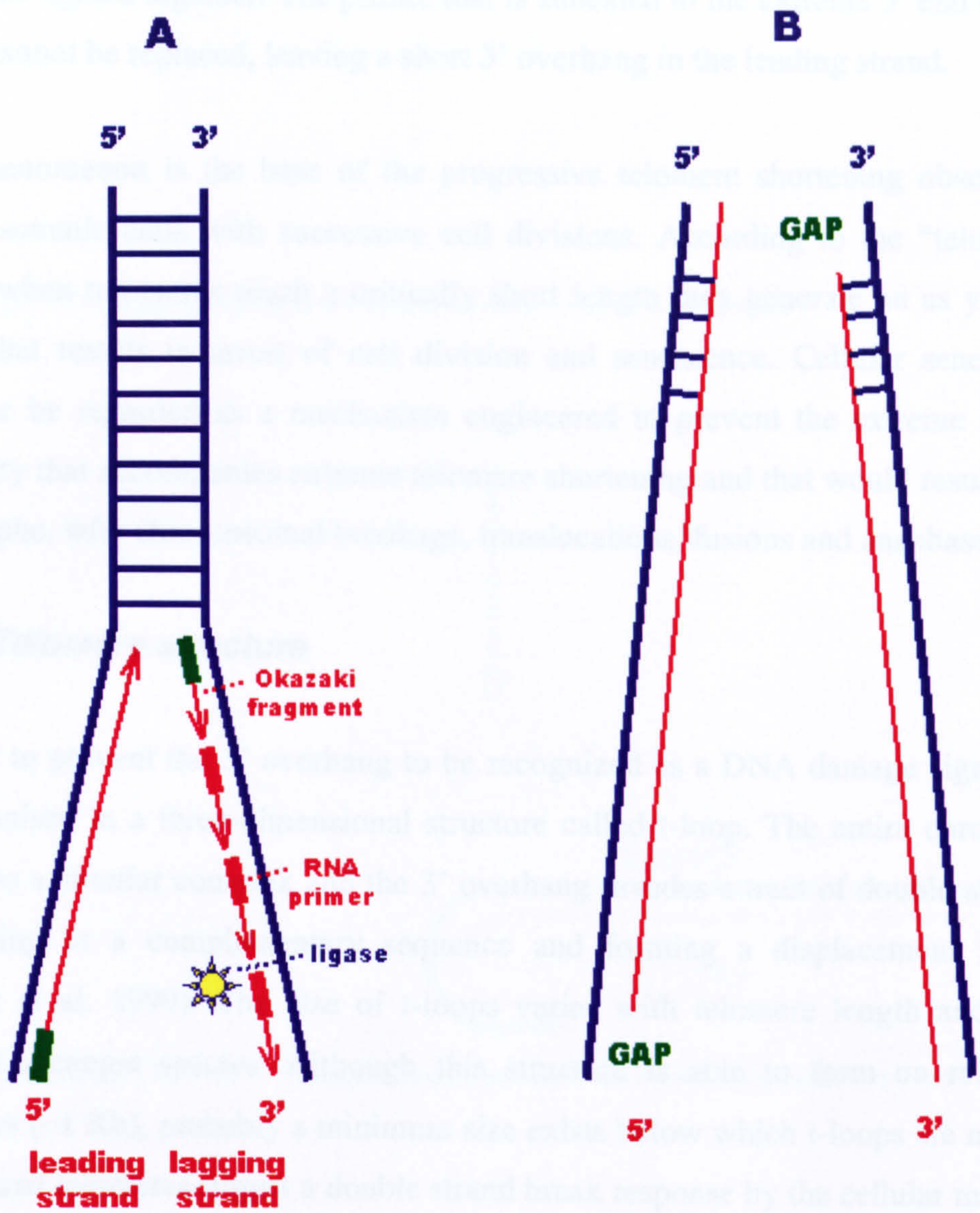


Fig. 7: The end replication problem

A) As the replication fork opens, lagging strand synthesis proceeds from 3' to 5' as the overall result of removal of RNA primers and ligation of the individual 5' to 3' synthesised Okazaki fragments. Leading strand synthesis is continuous, starting from a single RNA primer. **B)** After removal of terminal RNA primers, gaps remain at the 5' end of both the lagging and the leading strand.

The RNA primers utilised to start DNA polymerisation are later removed and the Okazaki fragments ligated together. The primer that is annealed to the extreme 3' end of the lagging strand cannot be replaced, leaving a short 3' overhang in the leading strand.

This phenomenon is the base of the progressive telomere shortening observed in most human somatic cells with successive cell divisions. According to the "telomeric clock" model, when telomeres reach a critically short length they generate an as yet unclarified signal that results in arrest of cell division and senescence. Cellular senescence could therefore be regarded as a mechanism engineered to prevent the extreme chromosomal instability that accompanies extreme telomere shortening and that would result in a genetic catastrophe, with chromosomal breakage, translocations, fusions and anaphase bridges.

6.1.2 Telomere structure

In order to prevent the 3' overhang to be recognized as a DNA damage signal, telomeres are organised in a three-dimensional structure called t-loop. The entire chromosome end folds into a circular complex and the 3' overhang invades a tract of double stranded DNA hybridising to a complementary sequence and forming a displacement loop (fig. 8) (Griffith et al. 1999). The size of t-loops varies with telomere length and is therefore different amongst species. Although this structure is able to form on relatively short telomeres (~1 Kb), probably a minimum size exists below which t-loops are no longer able to form and telomeres trigger a double strand break response by the cellular machinery.

6.1.3 Telomere associated proteins

Mammalian telomeres are associated with several proteins, regulating one or more of their functions (fig. 9) (reviewed in (Kim et al. 2002)). TRF2 binds directly to telomeric double stranded DNA and its function is thought to be crucial for the formation of the t-loop. RAP1 also localizes to the telomeres, but it does so by binding to TRF2. TRF1 is another protein known to bind directly to telomeric double stranded DNA. TIN2 forms complexes with TRF1 and thus indirectly associates to the telomeric structure. Finally, POT1 is known to bind directly and specifically to the single stranded 3' overhang. TRF1, TIN2, RAP1 and POT1 are thought to co-operate with TRF2 is stabilizing the t-loop, and their functions are still under investigation.

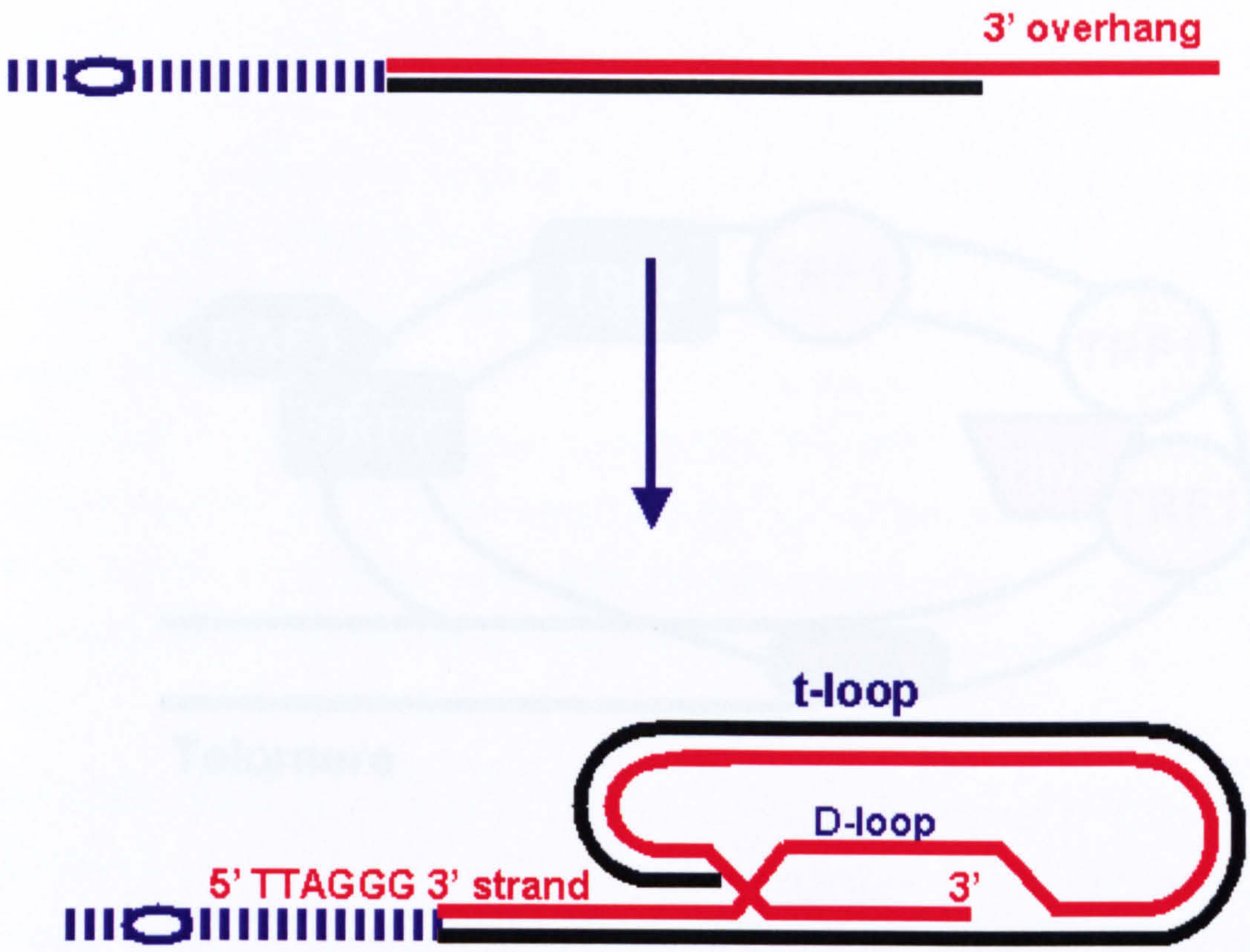


Fig 8: t-loop formation

In order to protect the 3' single stranded DNA from being recognised as a DNA damage signal, a 2-25 Kb of double stranded DNA folds back in a circular complex (t-loop). This allows the single stranded tail to displace a tract of double stranded DNA hybridizing to its complementary sequence (D-loop).

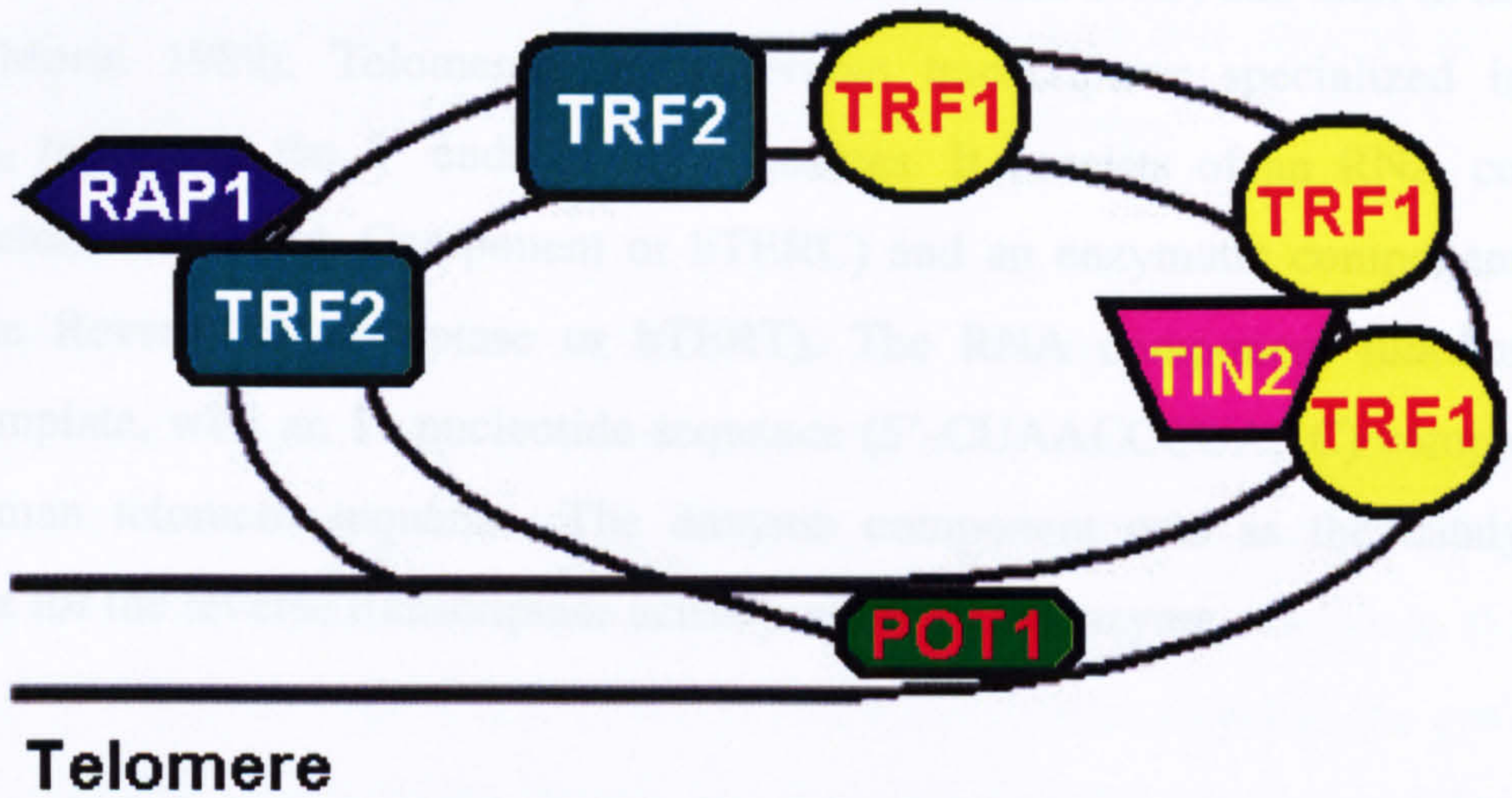


Fig. 9: Telomere associated proteins

Mammalian telomeres end in a t-loop structure which is stabilised by several proteins. TRF1 and TRF2 bind directly to the double stranded DNA, whereas TIN1 and RAP1 do so by forming complexes with TRF1 and TRF2 respectively. POT1 binds specifically to the 3' overhang of the t-loop.

6.2 Telomerase: its structure, functions and relevance to cancer

In order to prevent continuous loss of telomeric DNA that would cause chromosomal instability and cellular senescence, cells require the activity of an enzyme capable of synthesizing telomere ends, telomerase. This enzyme was initially identified in cell free extracts of *tetrahymena thermophila* (Greider and Blackburn 1985) and later in human cell extracts (Morin 1989). Telomerase is a reverse transcriptase specialized in adding $TTAGGG_n$ repeats at the 3' ends of chromosomes. It consists of an RNA component (human Telomerase RNA Component or hTERC) and an enzymatic component (human Telomerase Reverse Transcriptase or hTERT). The RNA component functions as an internal template, with an 11 nucleotide sequence (5'-CUAACCCUAAC) complementary to the human telomere sequence. The enzyme component acts as the catalytic unit, responsible for the reverse transcriptase activity of the hholoenzyme.

The ability of telomerase to add $TTAGGG_n$ repeats at the 3' ends of telomeres has been exploited to develop a PCR based assay to measure its activity using protein extracts, the Telomeric Repeat Amplification Protocol (TRAP) (Kim et al. 1994). Since the development of this assay, telomerase activity has been tested in a vast number of normal human tissues, tumour biopsies and cell cultures (summarized in (Bacchetti and Counter 1995) and in (Shay and Bacchetti 1997)). This was a crucial step in clarifying the pathways connecting telomerase activity, cellular proliferation, immortality and tumourigenesis. The aim of the following paragraphs is to summarize the scientific observations related to the role of telomerase activity in these biological processes. All the interventional studies aimed at immortalising human cells using hTERT and other genetic elements will be discussed in the following chapter.

6.2.1 Telomerase is activated in germline tissues and in the majority of cancers

Telomerase activity was tested in a broad range of tissues and cell lines as soon as a specific method for its measurement became available. Kim and collaborators (Kim et al. 1994) analysed 22 normal cultures derived from a variety of tissues, consistently failing to detect telomerase activity. In contrast, 94 naturally occurring immortal cell lines derived

from tumours of corresponding tissues, telomerase activity was invariably positive. Similarly, Yi and co-workers were able to detect telomerase activity in 16/16 immortal cell lines from different tumours (Yi et al. 2001). Telomerase activity was also detected in ovarian carcinoma cell lines (Counter et al. 1994) and in cells from myelomas and lymphomas (Nilsson et al. 1994). Data collected in our Institute from K. Parkinson's group and our group is in agreement with these findings. We have previously reported that our cultures from normal oral mucosa are invariably telomerase negative (McGregor et al. 2002). In our series of cultures from premalignant and malignant lesions of the HN, telomerase activity was positive in all of the immortal cultures and negative in all of the mortal ones, regardless of their premalignant/malignant phenotype.

When assayed *in vivo*, telomerase activity was not detected in most normal tissues, comprising normal skin, but high levels of enzyme activity were detected in testis, ovaries and haematopoietic stem cells (Kim et al. 1994). In contrast with this initial finding, Harle-Bachor and Boukamp (Harle-Bachor and Boukamp 1996) reported that when skin layers were dissected, relatively high levels of telomerase activity were found in the proliferative basal layer, whereas the quiescent dermis and the differentiated suprabasal layers lacked telomerase activity. These findings suggested that telomerase activity is present in those normal tissues or tissue compartments that are continuously proliferating, either to maintain tissue integrity or to produce gametes or blood cells.

Since those initial reports, telomerase activity has been analysed in a wide variety of tumours by several authors (summarized in (Bacchetti and Counter 1995) (Shay and Wright 1996) (Shay and Bacchetti 1997)). They report that telomerase activity is found in the vast majority (85-90%) of tumour tissues analysed. In oral cancer, there is overall agreement on the fact that most of the malignant lesions show detectable holoenzyme activity, with percentages of 95% (Miyoshi et al. 1999), 85% (Mutirangura et al. 1996) and 100% (Sumida et al. 1999). Only a few studies are available on telomerase activity in oral premalignant lesions. Telomerase activity was detected in 4/11 samples by Miyoshi (Miyoshi et al. 1999), 10/26 by Mutirangura (Mutirangura et al. 1996) and 5/7 by Sumida (Sumida et al. 1999).

These data clearly indicate that telomerase activity is a feature of the majority of cancer cells as well as highly proliferating tissue compartments. It could be speculated that stem cells might be the precursors of immortal or tumour cells *in vivo*, being *per se* endowed of

the ability to express telomerase and maintain their telomere length. On the other hand, telomerase positive malignancies might develop through reactivation of telomerase in cells that are normally telomerase negative. At the current stage of scientific knowledge, both hypotheses could hold true and are an interesting field for future investigation.

6.2.2 Telomerase activity is correlated with hTERT levels

The next question to be addressed was the relationship between telomerase activity and expression of its two components, hTERC and hTERT. hTERC was analysed through northern blotting in a variety of different tumour samples and normal tissues (Feng et al. 1995) (Avilion et al. 1996). Its expression, although slightly upregulated in tumours, was fairly ubiquitous and unrelated to the presence of telomerase activity. Work carried out in our Institute has demonstrated amplification of the hTERC locus in only 4/73 tumour samples analysed, of which 30 were HNSCCs (Soder et al. 1997). In these experiments, high levels of hTERC expression were demonstrated by *in situ* hybridisation in a lung tumour and its metastasis with hTERC gene amplification. The same group also analysed hTERC expression in a wider panel of tumour samples of epithelial origin, and reported that its patterns of expression were tumour specific (Soder et al. 1998). In lung cancer, hTERC overexpression was frequent in non-small cell lung cancers (41%) but not in adenocarcinomas (8%), consistent with the low hTERC expression reported for adenocarcinomas of the breast (13%) and ovary (18%). On the contrary, no significant difference was observed between SCCs (44%) and adenocarcinomas (32%) in cervical cancer. When hTERC was analysed by RT-PCR in a series of samples from normal oral mucosa, premalignant and malignant lesions, its expression was detected in all of the samples analysed, regardless of the presence or absence of telomerase activity (Sumida et al. 1999).

The catalytic subunit of telomerase was analysed in normal tissues and tumours from different sites through northern blotting, using polyA mRNA (Meyerson et al. 1997). hTERT mRNA was detectable only in those normal tissues that had previously been reported as having telomerase activity (Kim et al. 1994). It was also detected at high levels in a 6/6 breast and ovarian tumours analysed. Similarly, a good correlation between telomerase activity and hTERT mRNA expression, detected through RT-PCR, was reported by Sumida and co-workers (Sumida et al. 1999) in carcinomas, dysplasias and normal tissues from the oral cavity. Kolquist and co-workers (Kolquist et al. 1998) analysed the expression of hTERT through *in situ* hybridisation in a variety of normal tissues. hTERT expression was found to be high in haematopoietic stem cells but

undetectable in the majority of other adult tissues. In the skin, hTERT mRNA levels were high in the proliferative basal layer, but no expression was seen in the more superficial layers. These data is consistent with the previously described data for telomerase activity (Kim et al. 1994) (Harle-Bachor and Boukamp 1996).

Summarizing, hTERC expression is found ubiquitously *in vivo,* whereas hTERT expression widely varies amongst different tissues and different compartments of the same tissue. hTERT expression levels also correlate with levels of telomerase activity. Taken together, these observations suggest that although expression of both components is required for holoenzyme activity, the catalytic subunit is the actual limiting factor.

6.2.3 Lessons from knock-out mice

Recent efforts have lead to the generation of mTR (the mouse analogue of hTERC) knock-out mice. The phenotype of these mice initially appeared to challenge the involvement of telomerase in cellular immortalisation, oncogenesis and stem cell proliferation. Telomerase null transgenic mice were viable, able to develop normally, fertile and their cells could be efficiently immortalised and transformed *in vitro* (Blasco et al. 1997). These findings, however, were inherently misleading, since mouse telomeres are very much larger than human telomeres, thus potentially delaying the onset of any phenotype related to telomere erosion. Indeed, by the sixth generation these mice showed defective spermatogenesis and haematopoiesis (Lee et al. 1998) and a relative resistance to multi-stage skin carcinogenesis (Gonzalez-Suarez et al. 2000). Telomere length in fifth generation $mTR^{-/-}$ mice was only 41% of that observed in normal skin: it would therefore be reasonable to deduce that the phenotypic effects described develop once telomeres become sufficiently short. These findings underline the essential role of telomerase in long term viability of high-renewal tissues and carcinogenesis.

Late generation $mTR^{-/-}$ mice exhibit a high degree of chromosomal instability due to further telomere shortening, resulting in a slightly higher susceptibility to tumours in highly proliferating cell types (lymphomas, teratocarcinomas) than their normal counterparts (Rudolph et al. 1999). Human dyskeratosis congenita is consistent with this mouse model. This disease is characterized by low levels of hTERC, compromised telomerase function, telomere shortening and chromosomal instability. The affected patients show defects in highly regenerative tissues such as skin and bone marrow, and predisposition to tumours. These observations are in concordance with the role played by

telomerase activity in protecting the chromosomes from degradation and end to end fusion (Griffith et al. 1999).

6.2.4 Regulators of hTERT/Telomerase activity

As previously discussed, a wealth of literature is now available indicating a strong correlation between telomerase activity and expression of its catalytic subunit, suggesting that hTERT represents the limiting factor for holoenzyme activity. It is also accepted that telomerase activation occurs in cellular immortalisation and carcinogenesis. These two postulates have generated a considerable scientific interest in investigating the regulatory mechanisms of hTERT expression, both at a transcriptional and at a post-transcriptional level. Therefore, it appears appropriate to examine the current knowledge on the regulation of hTERT at a transcriptional level through activators (E6, c-Myc) and inhibitors (Mad, WT1, unidentified chromosomal repressors) and also at the post-transcriptional level by alternative splicing.

The expression of HPV-16 E6 protein has also been shown to stimulate telomerase activity in human mammary epithelial cells and keratinocytes, but not in fibroblasts (Klingelhutz et al. 1996).The authors also examined the effects of several E6 mutants, and were able to show that E6 is able to activate telomerase independently of its effect on p53 degradation.

A correlation between c-Myc oncogene expression and telomerase activity has been initially suggested by Wang and collaborators (Wang et al. 1998). The authors provided evidence that telomerase negative human mammary epithelial cells infected with a retroviral vector transducing c-Myc showed a 50-fold upregulation of hTERT transcription. This resulted in activation of telomerase activity to the levels measured in breast carcinoma cell lines. Subsequently, several c-Myc binding sites were identified in the hTERT promoter region (Wu et al. 1999), of which an evolutionary conserved proximal E-box (5'-CACGTG-3') seems required for c-Myc induced promoter activation (Greenberg et al. 1999).

Mad1 is another component of the Myc/Max/Mad protein network. Both c-Myc and Mad1 form stable heterodimers with Max protein, and the effects of Mad1/Max complexes are antagonistic to those of c-Myc/Max, ultimately resulting in suppression of gene transcription. Therefore, the identification of Mad1 as a potential repressor of hTERT transcription was not surprising. In telomerase negative cells WI38, activation of a conditional c-Myc/Estrogen receptor fusion protein increased the expression of hTERT

mRNA, but a sharp downregulation of hTERT was observed after infection of the same cells with a retrovirus transducing Mad. This was consistent with the fact that the immortal cell lines analysed by the same group endogenously expressed high levels of c-Myc and low levels of Mad, whereas the mortal cell lines had low levels of c-Myc and high levels of Mad1 (Oh et al. 2000).

Another potential repressor of hTERT is Wilms' Tumour 1 tumour suppressor protein (WT1). Ectopic expression of WT1 caused a negative regulation of hTERT transcription and telomerase activity, but this effect was not observed in HeLa cells that do not normally express WT1 protein (Oh et al. 1999). Therefore, WT1 might act as a transcriptional repressor of hTERT in a cell-specific manner.

RA treatment suppresses expression of hTERT and telomerase activity in human myeloblastic leukaemia (Mano et al. 2000) and acute promyelocytic leukaemia cell lines (Pendino et al. 2001). This effect is independent from tumour cell maturation, as it was also observed in a cell line that does not undergo terminal maturation in response to RA treatment; but it is dependent on RAR signalling pathway, because it cannot be evoked by RXR selective agonists (Pendino et al. 2001).

The idea that hTERT transcription might be under the control of an unidentified chromosomal repressor, lost in cancer cells, came from an early observation by Bryan and co-workers (Bryan et al. 1995). The authors reported that in telomerase positive tumour lines hTERT transcription could be extinguished by creating fusion hybrids with telomerase negative cells. Since then, chromosome transfer approaches have been used in a number of studies to try and identify putative telomerase repressors. The results are often contradictory and difficult to interpret because different studies testing the same chromosome have not always used the same donor cells (for a summary, see (Ducrest et al. 2002)); moreover, microscopic rearrangements of the chromosome used for transfer into the donor cell cannot be excluded. Chromosome 3 was reported to repress telomerase activity and induce permanent growth arrest in breast, renal and cervical carcinoma cell lines. Using allelotype analysis of microsatellite markers in rare immortal revertants allowed the identification of two candidate regions where the putative telomerase repressor might be located: 3p21.3-22 and 3p12-21.1 (Cuthbert et al. 1999). Both regions are reported as sites of LOH in human cancers, but the distal one is the most likely candidate, since 3p14 is known to be a fragile site. Chromosome 6 has recently been reported to suppress both telomerase activity and hTERT expression in an HPV16 immortalised keratinocyte cell line and in a cervical cancer cell line (Steenbergen et al. 2001). No other

reports are available on the effects of chromosome 6 transfer in other cancer cell lines or further refining the locus for the putative telomerase repressor.

hTERT expression is also regulated post-transcriptionally. Several variants of hTERT mRNA have been identified, arising from alternative splicing of the transcriptional product (Kilian et al. 1997) (fig. 10). The α splice site generates a 36bp deletion from the RT domain A of the hTERT gene and has been described as a dominant negative inhibitor of telomerase activity (Colgin et al. 2000). The β splice site generates a 182bp deletion resulting in a premature stop codon. Four insertion variants have also been described, also generating a premature stop codon (Kilian et al. 1997). All four insertion variants, the β and the α-β deletion variants encode for an inactive reverse transcriptase. Recently, it has been shown that telomerase activity corresponded with expression of full length hTERT transcript during the development of human heart, kidney and liver, but splicing variants of hTERT were still produced in the absence of telomerase activity in developing kidney (Ulaner et al. 1998). This observation suggests that different tissues express different profiles of hTERT transcripts, and that these splicing variants could play a role in tissue development. The role of hTERT splice variants in regulating telomerase activation during carcinogenesis remains an interesting field for future investigation.

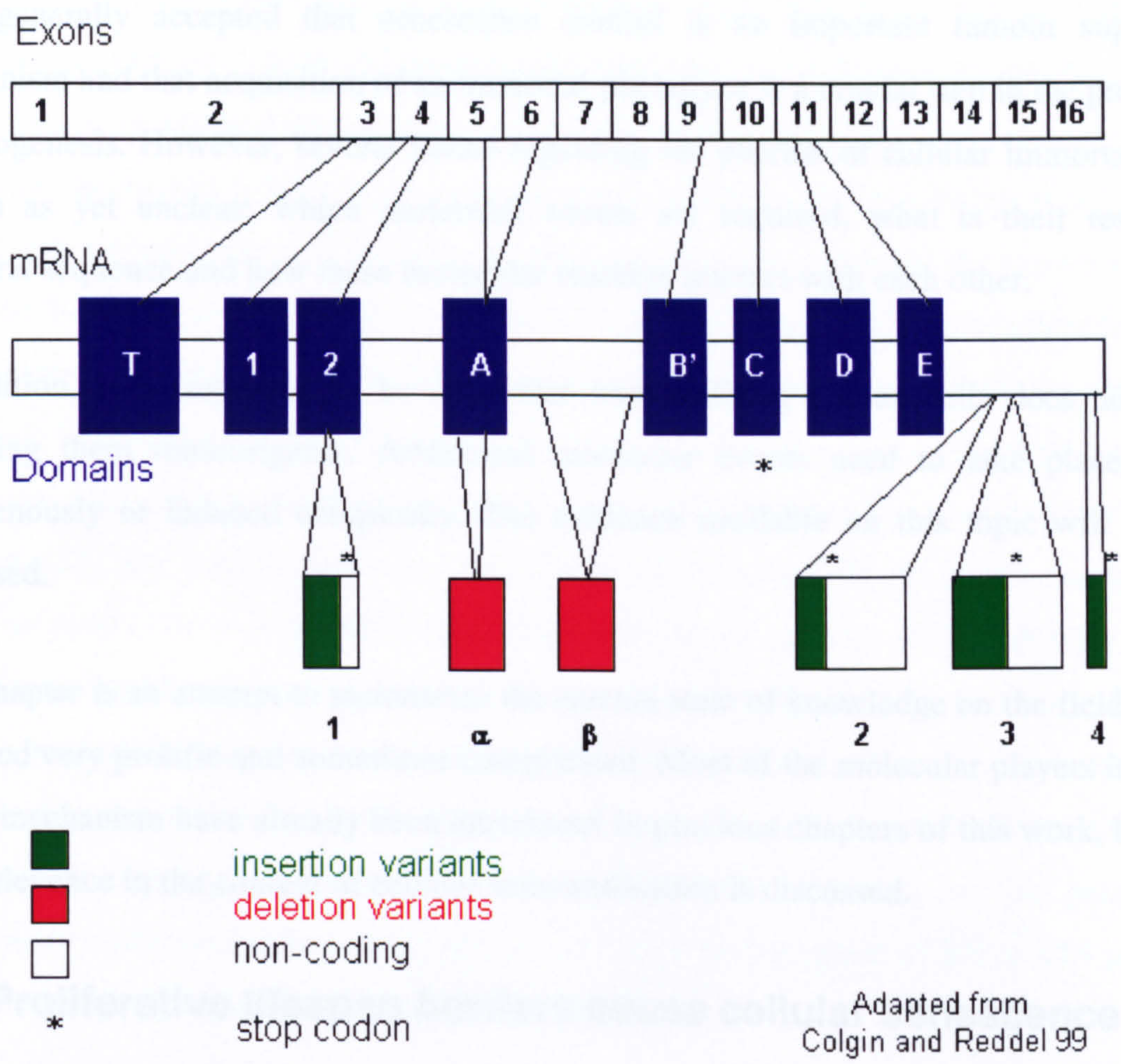


Fig. 10: Organization of the hTERT gene and post-transcriptional variants.

The hTERT gene comprises 16 exons and 8 domains. Domain T is telomerase specific, whereas domains 1, 2 and A through E are similar to those found in other reverse transcriptases. The assignment of these motifs to the exons encoding them is shown. The deletion variant α has been reported as having a dominant negative effect, whereas the β deletion variant generates a premature stop codon on domain C. All four insertion variants generate premature stop codons, as indicated in the figure.

7 The genetic basis of cellular immortalisation

It is generally accepted that senescence control is an important tumour suppressor mechanism and that acquisition of an immortal phenotype is a crucial step in the process of carcinogenesis. However, several issues regarding the process of cellular immortalisation remain as yet unclear: which molecular events are required, what is their respective temporal sequence and how these molecular markers interact with each other.

In addition, it is important to be clear that immortalising human cells does not mean rendering them tumourigenic. Additional molecular events need to take place, either endogenously or induced ectopically. The evidence available on this topic will also be discussed.

This chapter is an attempt to summarize the current state of knowledge on the field, which is indeed very prolific and sometimes complicated. Most of the molecular players involved in this mechanism have already been introduced in previous chapters of this work, but here their relevance in the context of cellular immortalisation is discussed.

7.1 Proliferative lifespan barriers cause cellular senescence

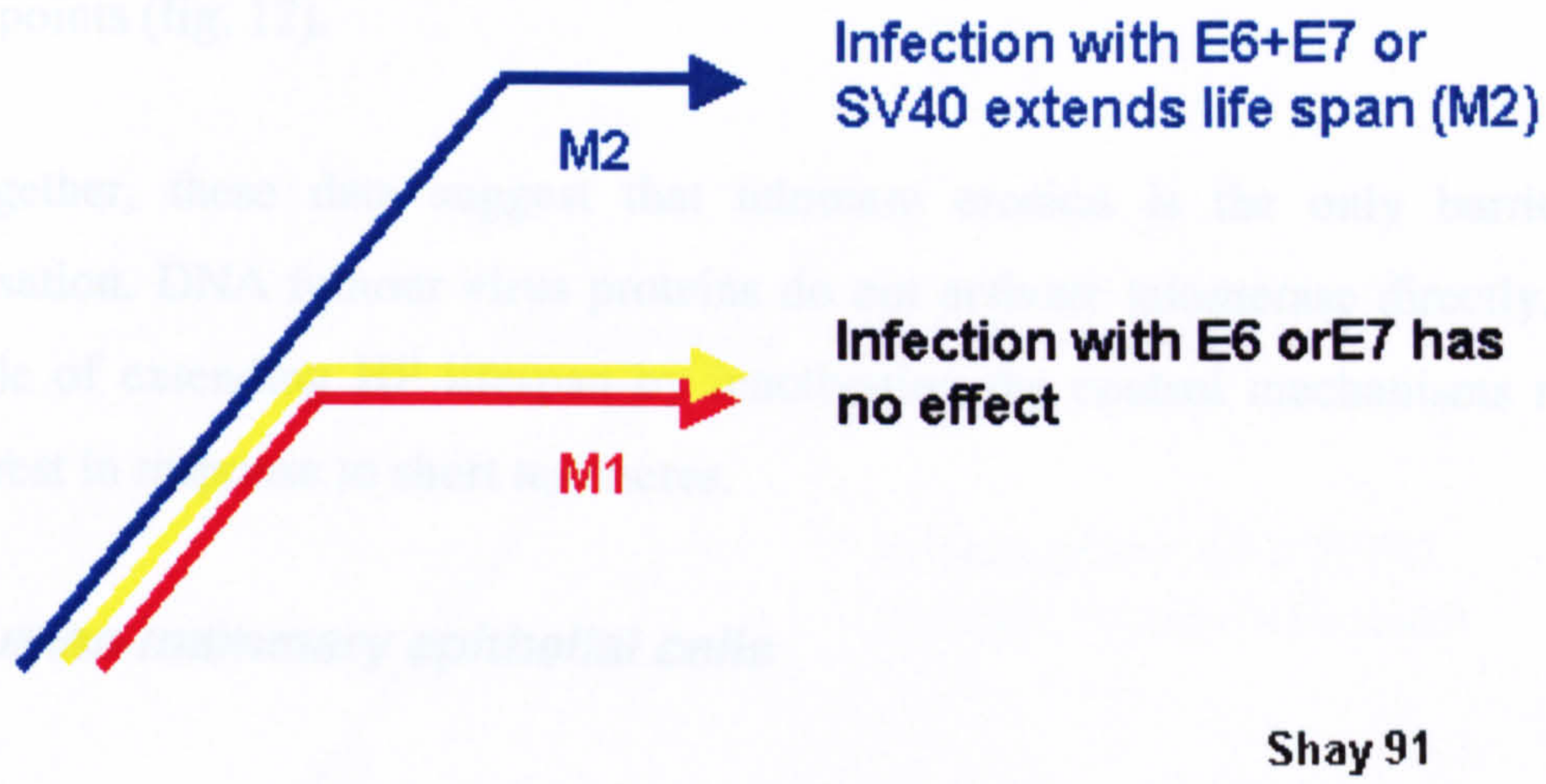
Almost 4 decades ago Hayflick provided evidence that cultured human diploid cells proliferate for a finite number of population doublings and then enter a proliferative lifespan barrier called senescence (Hayflick 1965). Since then, the molecular events regulating cellular replicative fate have been extensively analysed to understand how cellular senescence is triggered and therefore what molecular events are required to bypass it and reach immortality. Different cell types exhibit different regulatory mechanisms for cellular senescence (reviewed in (Wynford-Thomas 1999)). Therefore, the evidence accumulated in the past years is presented separately for different cellular settings.

7.1.1 Human primary fibroblasts

Human primary fibroblasts (HFs) are the most extensively used cell type in studies designed to clarify senescence/immortalisation mechanisms because of their easy propagation in cell culture. These cells are able to proliferate for 50-60 population doublings (PD) in culture and then enter senescence, remaining in a viable but non-dividing state for several months (Hayflick 1965).

The idea that multiple proliferative life span barriers existed in normal human fibroblasts has been suggested by Shay and collaborators (Shay et al. 1991). They demonstrated that HFs infected with SV40 tumour virus T-antigen were able to escape senescence (or mortality checkpoint 1-M1) and keep proliferating for an additional 30-40 PD, but they were invariably arrested by the onset of another mortality checkpoint, named crisis (or mortality checkpoint 2-M2). Very rare immortal clones spontaneously appeared amongst the growth arrested cells and could be cultured indefinitely. Amongst the known functions of SV40 T-antigen are its ability to bind both Rb and p53 and interfere with their functions. In the same experiments, cellular life span could not be extended by interfering with either of these two pathways individually (fig. 11). Transfection with plasmids expressing individual viral proteins binding Rb, like human papilloma virus E7 or adenovirus E1A, or binding p53, like human papilloma virus E6 or adenovirus E1B, still gave rise to clones arresting in M1. In contrast, co-transfections with E6/E7 or with E1A/E1B both allowed the cells to proliferate beyond the M1 checkpoint and arrest in M2. Similarly, SV40 T-antigen mutants having lost their ability to bind either Rb or p53 were able to overcome M1 when co-transfected into fibroblasts, but not when transfected individually. These experiments, although informative, have to be interpreted cautiously as viral proteins are notoriously multifunctional and the effects attributed to disruption of Rb or p53 pathways could actually be due to other unknown mechanisms.

The hypothesis that both p16/Rb and p53 pathways had to be disrupted in order to bypass M1 was somewhat challenged by later reports. Bond and collaborators infected human pre-senescent fibroblasts with a dominant negative p53 mutant viral construct (Bond et al. 1994) or HPV E6 protein (Bond et al. 1999) in two separate experiments (fig. 11). Such cells were able to escape senescence, but ceased proliferating after a significant lower number of PD than reported for cells in crisis (17 versus 30-40). In addition, their morphologic features upon reaching growth arrest resembled those of cells in senescence rather than in crisis, suggesting the existence of an intermediate arrest state termed $M^{int}E6$ by the same authors. In these cells p16 levels increased beyond the levels observed in their pre-infection counterparts, giving a plausible explanation for the restored growth arrest status after an incomplete M1 bypass and despite loss of p21 expression. In the same experiments, an incomplete rescue from senescence was also observed upon infection with HPV E7. These cells showed a limited extension in lifespan and eventually arrested in a crisis-like state named $M^{int}E7$. $M^{int}E7$ occurred much sooner than M2 and was accompanied by increased levels of p16, p53 and p21.



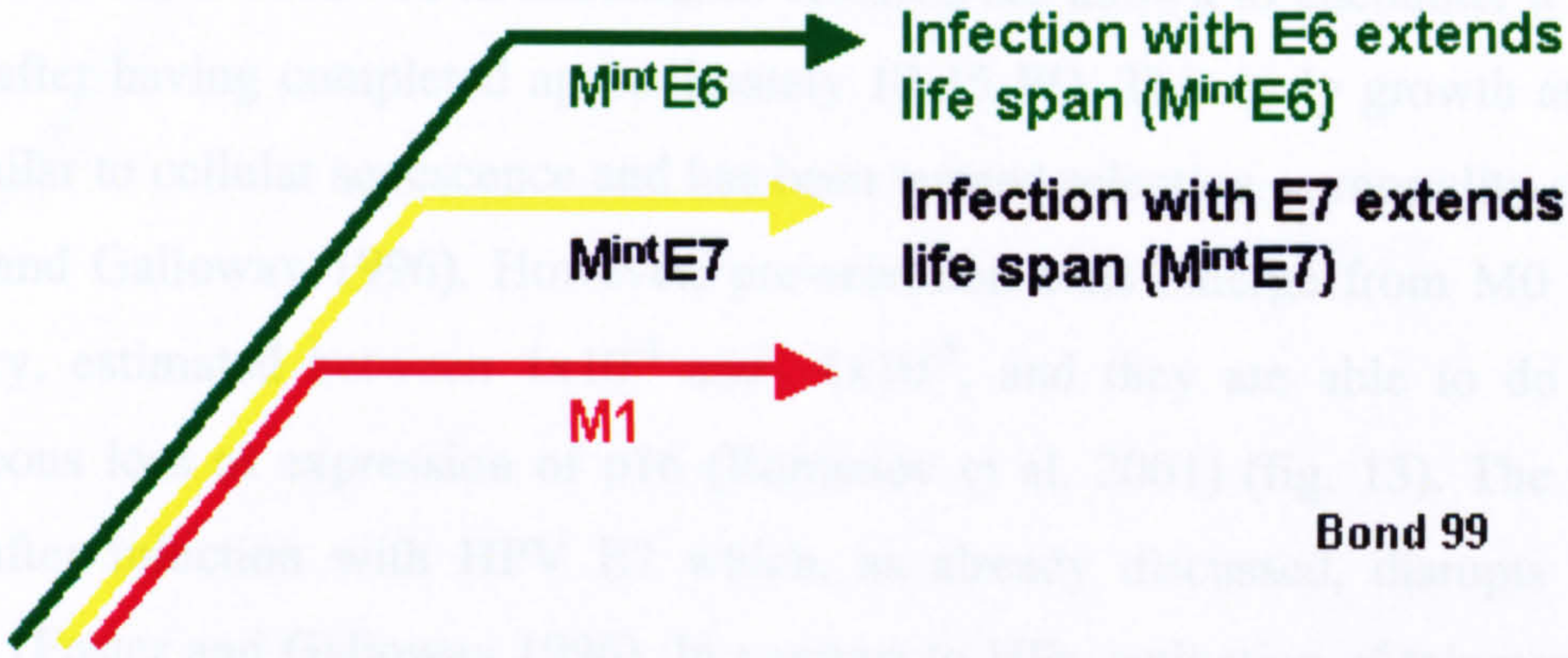


Fig. 11: Attempts to immortalise human fibroblasts

Graphic representation of the effect of different transduction experiments on cellular lifespan in fibroblasts. The red arrows indicate the proliferative fate of human fibroblasts in culture. The yellow, green and blue arrows show the effects of different interventional procedures (indicated next to the arrows) on fibroblast life span.
The corresponding reference is also indicated.

A less complex scenario was found when human fibroblasts were infected with retroviruses transducing the enzyme component of telomerase hTERT (Vaziri and Benchimol 1998) (Bodnar et al. 1998). hTERT expression resulted in activation of telomerase, elongation of telomeres and cellular immortalisation, bypassing both M1 and M2 checkpoints (fig. 12).

Taken together, these data suggest that telomere erosion is the only barrier to HF immortalisation. DNA tumour virus proteins do not activate telomerase directly, but they are capable of extending HF lifespan by inactivating the control mechanisms regulating growth arrest in response to short telomeres.

7.1.2 Human mammary epithelial cells

In human mammary epithelial cells (HMECs) senescence is regulated by somewhat different molecular mechanisms and the effects of viral oncoproteins are also different compared to those observed in fibroblasts. HMECs are known to encounter a first growth plateau after having completed approximately 10-15 PD. This early growth arrest state is very similar to cellular senescence and has been termed selection or mortality stage 0 (M0) (Foster and Galloway 1996). However, pre-selection cells emerge from M0 with a high frequency, estimated between $1x10^{-4}$ and $1x10^{-5}$, and they are able to do so through spontaneous loss of expression of p16 (Romanov et al. 2001) (fig. 13). The same effect occurs after infection with HPV E7 which, as already discussed, disrupts the p16/Rb pathway (Foster and Galloway 1996). In contrast to HFs, activation of telomerase through hTERT transduction at this stage of HMECs proliferative fate failed to extend their life span (Kiyono 98). In the same experiments, infection with E6 alone had no effect, but combined infection with E6/E7 generated immortal clones.

Post-selection HMECs show a progressive increase in levels of total p53 throughout the second phase of exponential proliferation, after which they reach a second growth arrest state with features of cellular crisis (Romanov et al. 2001). This mortality checkpoint is known as senescence or M1, but the same authors proposed to rename it agonescence after its similarities to crisis were appreciated. Infection of post M0 HMECs with HPV E6 or with a retroviral construct transducing hTERT causes immortalisation without any feature of crisis, hTERT with a higher efficiency than HPVE6 (Kiyono et al. 1998) (fig. 13). Kingelhultz and co-workers have demonstrated that HPV E6 is able to activate the transcription of hTERT in several epithelial cell types, but not in fibroblasts, and this occurs independently of its effect on p53 (Klingelhutz et al. 1996).

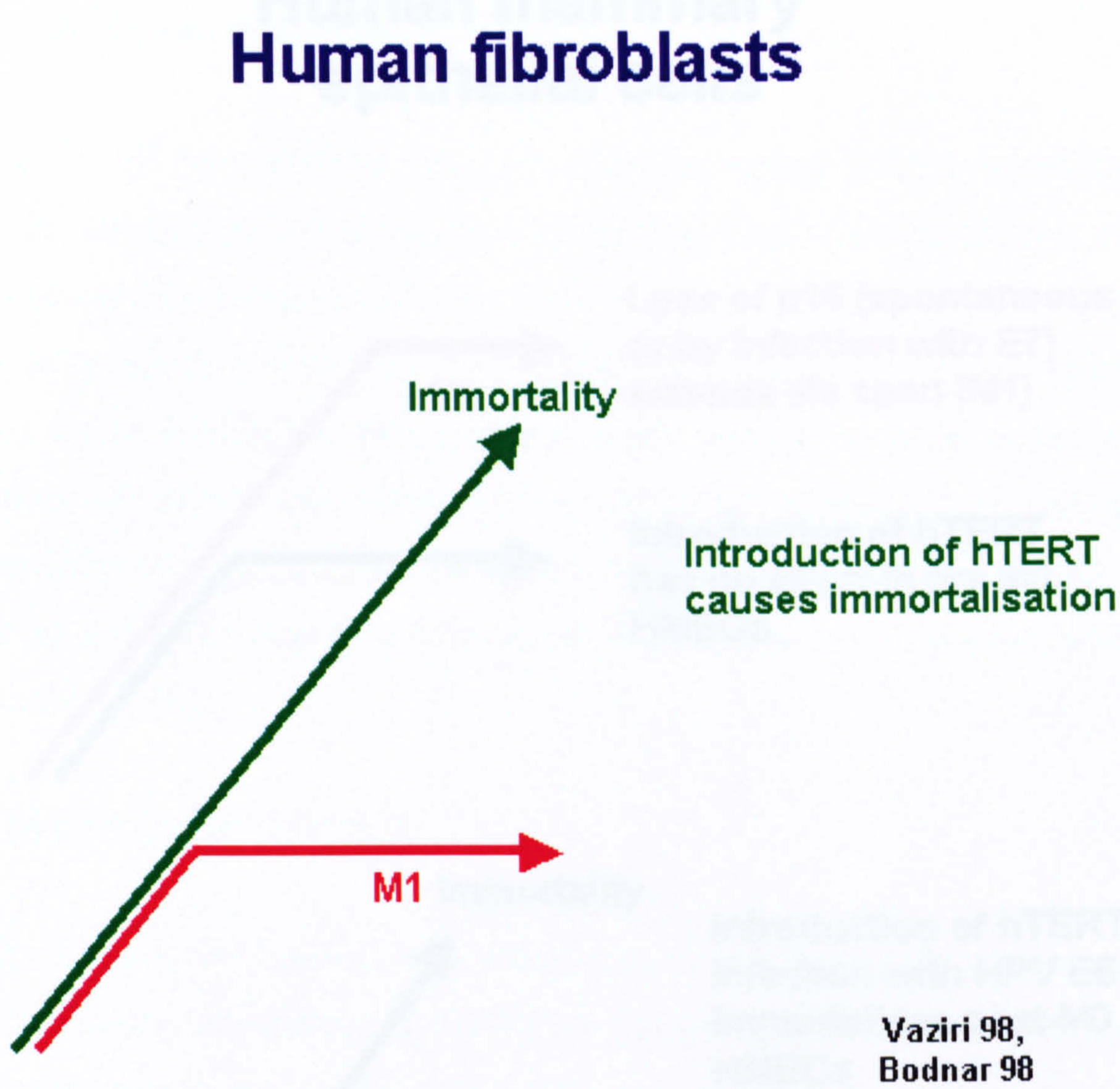


Fig. 12: Immortalisation of human fibroblasts

Human fibroblasts are efficiently immortalised by infection with an hTERT-transducing retroviral vector, as independently reported by the two indicated authors.

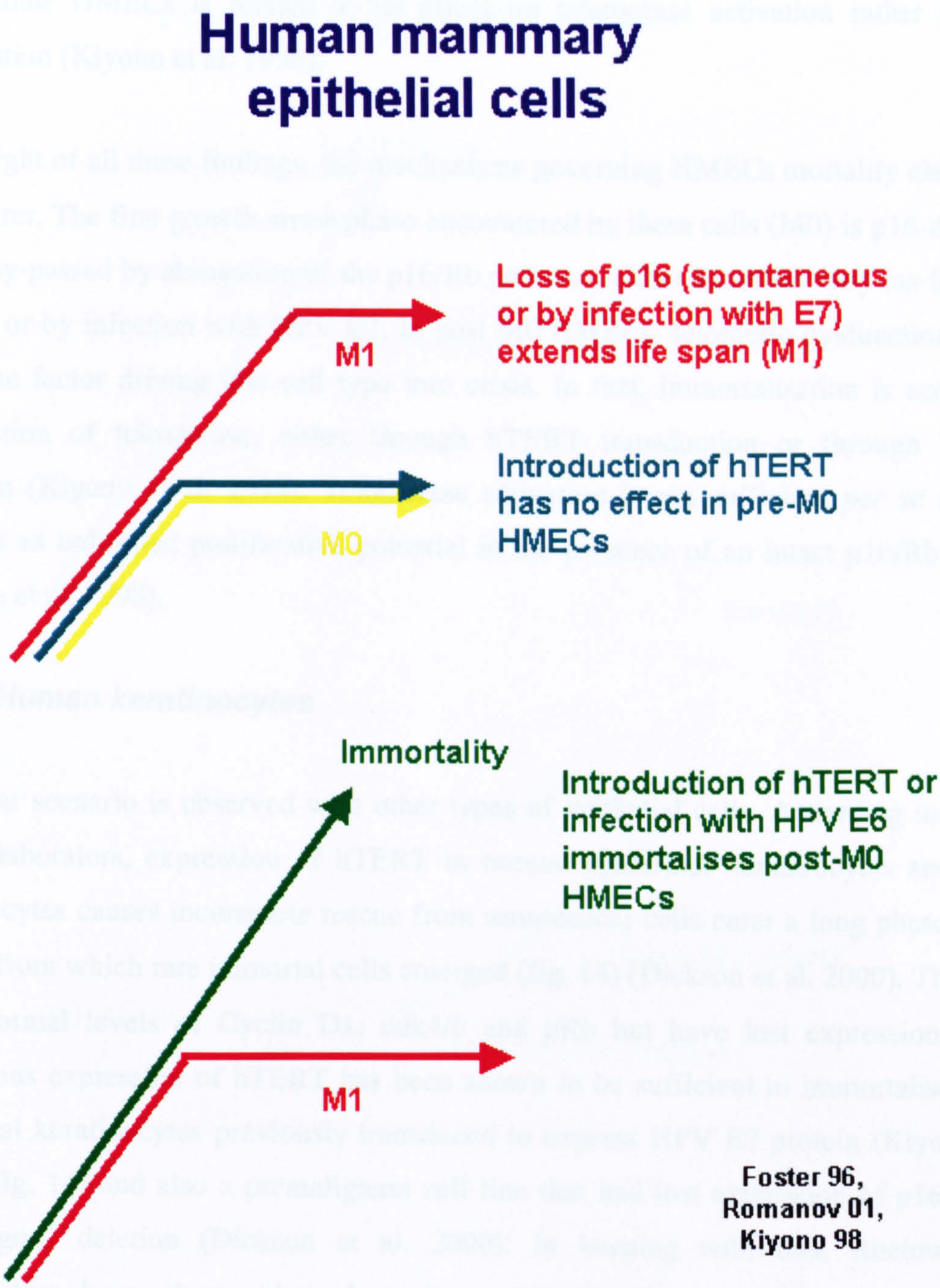


Fig. 13: Immortalisation of human mammary epithelial cells

Graphic representation of the effect of different transduction experiments on cellular lifespan in human mammary epithelial cells. M0 is by-passed by spontaneous or HPV E6-induced loss of p16. Immortalisation occurs when post M0 HMECs are transduced to express hTERT.
The corresponding references are cited.

Using several E6 mutants, it was possible to demonstrate that the ability of HPV E6 to immortalise HMECs is related to its effect on telomerase activation rather than p53 degradation (Kiyono et al. 1998).

In the light of all these findings, the mechanisms governing HMECs mortality checkpoints are clearer. The first growth arrest phase encountered by these cells (M0) is p16-dependent and is by-passed by abrogation of the p16/Rb pathway, either spontaneously (as frequently occurs) or by infection with HPV E7. In post M0 HMECs, telomeric dysfunction appears to be the factor driving this cell type into crisis. In fact, immortalisation is acquired by reactivation of telomerase, either through hTERT transduction or through HPV E6 infection (Kiyono et al. 1998). Telomerase activation is not sufficient *per se* to confer HMECs an unlimited proliferative potential in the presence of an intact p16/Rb pathway (Kiyono et al. 1998).

7.1.3 Human keratinocytes

A similar scenario is observed with other types of epithelial cells. According to Dickson and collaborators, expression of hTERT in normal epidermal keratinocytes and in oral keratinocytes causes incomplete rescue from senescence: cells enter a long phase of slow growth from which rare immortal cells emerged (fig. 14) (Dickson et al. 2000). These cells show normal levels of Cyclin D1, cdk4/6 and pRb but have lost expression of p16. Exogenous expression of hTERT has been shown to be sufficient to immortalise normal epidermal keratinocytes previously transduced to express HPV E7 protein (Kiyono et al. 1998) (fig. 14) and also a premalignant cell line that had lost expression of p16 through homozygous deletion (Dickson et al. 2000). In keeping with this, Rheinwald and collaborators have shown that abrogation of both p16 and p53 pathways extends keratinocyte life span, but immortalisation occurs only after hTERT transduction (Rheinwald et al. 2002).

Recent evidence by K. Parkinson's group (in press) better defines the features of crisis in HNSCC. *In vitro*, loss of p16 and mutation of p53 is associated with growth arrest in M2 crisis, with high grade of telomere dysfunction as assessed by the Anaphase Bridge Index (ABI). Restoration of telomerase activity at this stage causes cellular immortality, accompanied by lowering of the ABI index. These observations suggest that epidermal and oral keratinocytes need both reactivation of telomerase and loss of expression of p16/Rb pathway in order to acquire immortal phenotype.

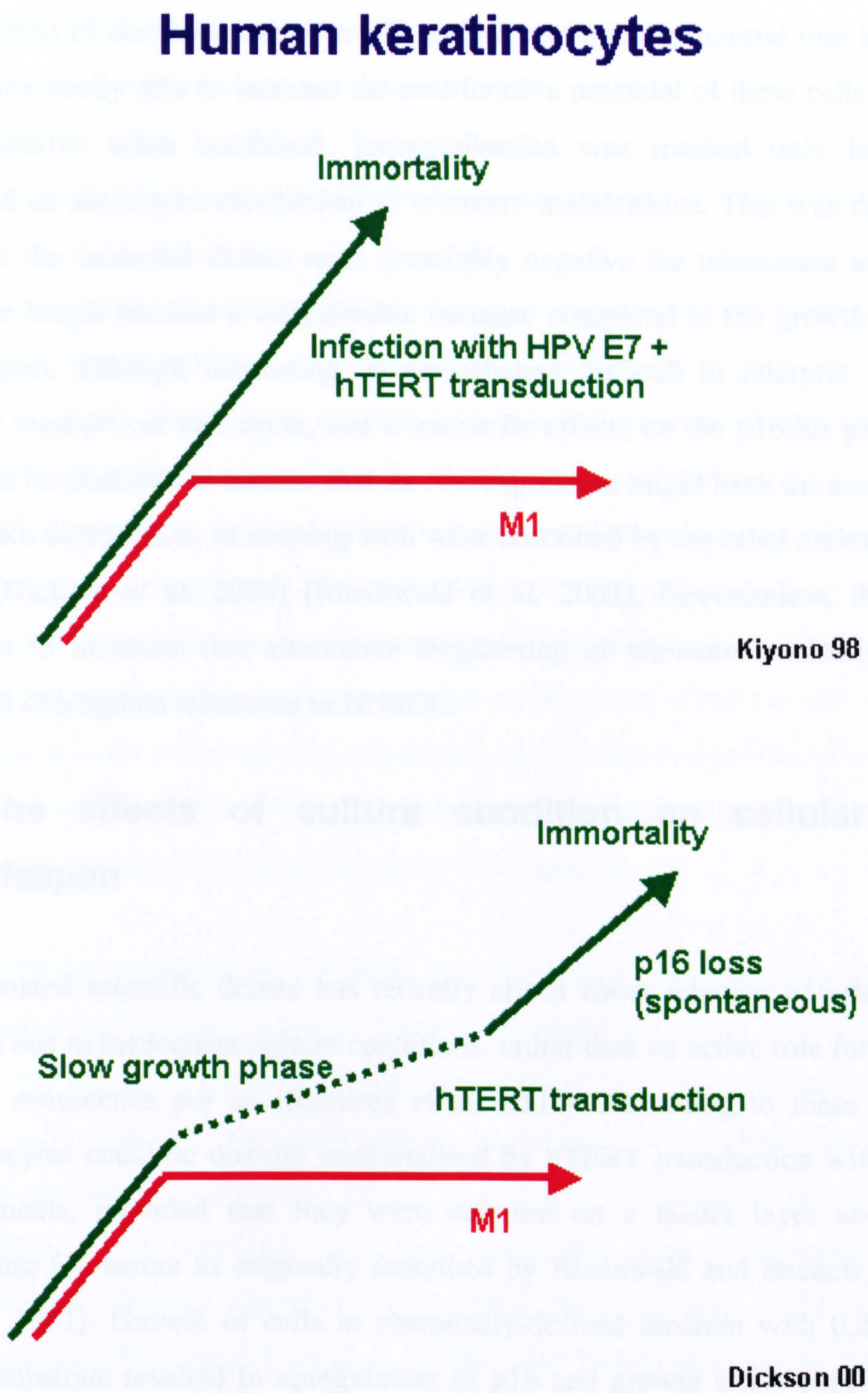


Fig. 14: Immortalisation of human keratinocytes

Graphic representation of the effect of the main transduction experiments on cellular lifespan in keratinocytes. Infection with HPV E7 combined with transduction of hTERT caused keratinocyte immortalisation. In contrast, hTERT transduction alone extended cellular life span through a long slow-growth phase. Immortal cells emerged only through spontaneous loss of p16.
The corresponding references are cited.

Recently, Opitz and collaborators (Opitz et al. 2001) have demonstrated that the transduction of dominant negative p53 and of cyclin D1 in normal oral keratinocytes are each individually able to increase the proliferative potential of these cells but their effects were additive when combined. Immortalisation was reached only later, when cells activated an alternative mechanism of telomere maintenance. This was deduced from the fact that the immortal clones were invariably negative for telomerase activity, but their telomere length showed a considerable increase compared to the growth arrested clones. This report, although interesting, is nevertheless difficult to interpret. Cyclin D1 is a positive regulator of cell cycle, and it exerts its effects on the p16/Rb pathway. It would therefore be plausible to assume that its overexpression might have the same effects of p16 loss or Rb degradation, in keeping with what described by the other authors (Kiyono et al. 1998) (Dickson et al. 2000) (Rheinwald et al. 2002). Nevertheless, there is no other evidence in literature that alternative lengthening of telomere mechanisms are used to maintain or lengthen telomeres in HNSCC.

7.2 The effects of culture condition on cellular replicative lifespan

An animated scientific debate has recently arisen about whether p16-dependent growth arrest is due to inadequate culture conditions, rather than an active role for p16 in inducing cellular senescence *per se* (Ramirez et al. 2001). According to these authors, human keratinocytes could be directly immortalised by hTERT transduction without any further requirements, provided that they were cultured on a feeder layer and with medium containing 5% serum as originally described by Rheinwald and Beckett (Rheinwald and Beckett 1981). Growth of cells in chemically-defined medium with 0.25% serum on a plastic substrate resulted in upregulation of p16 and growth arrest both before and after transduction with hTERT expression vector (Ramirez et al. 2001). In addition the authors reported that the p16 dependent M0 growth arrest stage observed in HMECs could be prevented by culturing these cells on a feeder layer in the presence of 10% serum. This suggests that p16 upregulation mediates a premature growth arrest state due to inadequate culture conditions, both in keratinocytes and in HMECs.

However, this conclusion is presently controversial. Many reports from different groups underline the importance of p16/Rb as a telomere independent growth arrest mechanism *in vitro* and the need to interfere with this pathway to immortalise different types of human epithelial cells (see paragraphs 1.2 and 1.3 of this chapter). In addition, p16 loss is

frequently found in HNSCCs and oral premalignant lesions *in vivo*, as discussed in chapter 5 of this introduction. It seems therefore unlikely that the involvement of p16 in cellular senescence is merely a culture artefact.

7.3 Cellular immortalisation and malignant transformation

The acquisition of an immortal phenotype is considered a crucial step in the process of carcinogenesis. Nevertheless, immortality and malignancy are not synonymous. In oral cancer, cellular immortality is observed in about 1/3 of premalignant lesions and in 9/10 carcinomas (combined data from K. Parkinson's and our group). Therefore, not all immortal cultures are cancers and cancers are not necessarily immortal. In keeping with this, Opitz and co-workers (Opitz et al. 2001) reported that exogenous overexpression of cyclin D1 and inactivation of p53, together with spontaneous activation of an alternative lengthening of telomeres mechanism, leads to immortalisation but not transformation of oral keratinocytes. Although the acquisition of an unlimited proliferative potential occurs more frequently in fully developed cancer than in premalignancies, additional or different genetic events must be involved in cancer development.

The molecular mechanisms governing human cellular transformation have recently been investigated by Weinberg's group. The authors tested the ability of SV40 large-T antigen, hTERT and H-ras to transform normal human fibroblasts (BJ cells) and human embryonic kidney cells (HEKs). The simultaneous expression of all three oncogenes permitted efficient anchorage-independent growth in soft agar and rapid tumour formation in nude mice (Hahn et al. 1999)(a). In a later study, the same investigators extended their observation to HMECs (Elenbaas et al. 2001). This cell type could be transformed by expression of SV40 large-T antigen, hTERT and H-ras, similarly to BJ and HEK cells; however, transformed HMECs showed a consistent amplification of c-myc locus, which was not observed in similarly treated BJ and HEK cells. This data suggest that in humans, multiple pathways involved in cellular proliferation and lifespan must be simultaneously disrupted in order to create a tumour cell. Nevertheless, it is unlikely that SV40 large-T antigen participates in this transformation process solely through its effects on p16/Rb and p53 pathways, since Morales and collaborators (Morales et al. 1999) have reported that combined expression of E6/E7, hTERT and H-ras was not sufficient to allow normal human fibroblasts to grow in soft agar. The importance of telomerase activity in maintaining the transformed phenotype has been underlined in another study in Weinberg's laboratory (Hahn et al. 1999)(b), in which inhibition of telomerase activity

through infection with a dominant negative hTERT expression vector resulted in inhibition of cellular growth and loss of their tumourigenic capacity in nude mice.

7.4 Conclusions

A number of interventional studies have been carried out in the past decade to clarify the requirements and timing of cellular immortalisation in different human cellular settings. Shay and collaborators (Shay et al. 1993) have proposed that two mortality checkpoints (M1 and M2) need to be by-passed to reach immortality. This two-stage mechanism applies to all cell-types, but the molecular mechanisms controlling M1 and M2 are cell type specific, although with clear similarities. In human fibroblasts, senescence control is mainly regulated by telomere erosion, as ectopic activation of telomerase efficiently immortalises these cells. In human mammary epithelial cells, inactivation of the p16/Rb pathway is an additional requirement for immortalisation, along with activation of telomerase, and it is frequently a spontaneous event. Similarly, human keratinocytes can be immortalised by disruption of p16/Rb pathway and activation of telomerase, but it has been recently suggested that p53 dysfunction is also required (Rheinwald et al. 2002). In addition, both human fibroblasts and human epithelial cells require additional and not completely clarified molecular events to develop a malignant phenotype.

8 Materials and Methods

8.1 Cell culture

All cell culture work was performed using strict aseptic techniques inside a laminar flow hood (Class II Microbiological Safety Cabinets, Medical Air technology Ltd., Manchester, UK). Cell culture work involving the use of retroviral constructs was performed in category II containment suites. Cells were incubated at 37°C in a dry atmosphere containing 5% (v/v) CO_2 (Heraeus, Essex, UK).

HeLa cells were maintained in modified Dulbecco's medium (Beatson formulation) (Gibco BRL Europe Life Technologies, Paisley, UK) supplemented with 10% foetal bovine serum (Harlan Sera-Lab, Crawley Down, Sussex, UK) and 1mM glutamine (Gibco BRL Europe Life Technologies, Paisley, UK). WI38 cells were maintained in modified Dulbecco's medium, supplemented with 5% foetal bovine serum and 1mM glutamine.

The derivation, characterisation and maintenance of the primary human keratinocyte cultures have been described previously (Edington et al. 1995) (McGregor et al. 1997). All cells were maintained on irradiated (60Gy from a Co^{60} radiation source) Swiss 3T3 feeders. The growth media used were flavine adenine dinucleotide (FAD) growth medium, composed by 1:3 Ham's F12/Dulbecco's modified Eagles medium (DMEM) (both from Gibco BRL Europe Life Technologies, Paisley, UK) supplemented with 10% foetal bovine serum (FBS), 0.4 μg/ml hydrocortisone (Sigma-Aldrich, Poole, Dorset, UK), 5 μg/ml insulin (Gibco BRL Europe Life Technologies, Paisley, UK), 5 μg/ml transferrin (Sigma-Aldrich, Poole, Dorset, UK), 10^{-10}M cholera toxin (Sigma-Aldrich, Poole, Dorset, UK), 1.8 x 10^{-4} adenine (Sigma-Aldrich, Poole, Dorset, UK) and 10 ng/ml epidermal growth factor (Gibco BRL Europe Life Technologies, Paisley, UK) and 10H growth medium, composed by DMEM supplemented with 10% FBS and 0.4μg/ml hydrocortisone. Each culture was tested and then maintained with whichever of these two media proved best for their growth. Immediately prior to experimentation all keratinocyte cultures were grown on FAD medium to avoid complications arising from growth in different media. The irradiated 3T3 feeders were carefully removed by treatment with phosphate buffered saline (PBS)/0.02% EDTA for 15-20 seconds and vigorous pipetting before analysis of the keratinocyte cultures. Swiss 3T3 cells were maintained in 10C medium (Dulbecco's MEM plus 10% donor calf serum (Sigma-Aldrich, Poole, Dorset, UK).

To freeze cell stocks for storage, semi-confluent cultures were trypsinized, pelleted and the pellet resuspended at a concentration of approximately 10^6 cells/ml in chilled medium containing 50% serum and 10%(v/v) dimethylsulfoxide (DMSO) (Fisher Scientific, USA). Suspensions were aliquoted into 1ml cryotubes (Nunc, Wiesbaden, Denmark) and placed in a polystyrene box and frozen, well insulated, at -70°C overnight to ensure a slow rate of cooling. The ampoules were then transferred to a liquid nitrogen tank until required.

Frozen stocks were recovered by removing the ampoules from liquid nitrogen and placed into a small, covered bucket of water at 37°C. Once thawed, the cells were added to 10 ml of the appropriate pre-warmed growth medium, centrifuged, resuspended in fresh growth medium and transferred to 90 mm Falcon culture dishes (Becton Dickinson, Franklin Lakes, USA).

8.2 Retrovirus production and ectopic expression of hTERT

The retroviruses were produced by infecting the amphotropic packaging line Phoenix A with pBabeNeo or pBabest2 (hTERT) retroviral constructs (Vaziri and Benchimol 1998) and selecting with the minimum concentration of G418 (Invitrogen, Paisley, UK) found to kill the uninfected cultures. The virus titers were determined using 3T3 cells: one infectious unit corresponded to the volume of retroviral supernatant capable of generating one G418-resistant colony. Dysplasia cultures were infected at about 80% confluency with 1.35×10^4 infectious units/ml in the presence of 4 μg/ml of polybrene (Sigma Chemicals, Poole, UK). The dysplasia cells were plated on G418-resistant 3T3 feeder cells, selected 48h later with 200 μg/ml of G418 and cultured under constant selection. Pooled G418-resistant colonies were used in all experiments to avoid variability due to clonal heterogeneity of the population.

8.3 Senescence Associated β-Galactosidase (SABG) assay.

Cells were maintained in culture for a minimum of three weeks to confirm proliferative growth had ceased. The 3T3 feeder layer was carefully removed and the cells fixed in 3% formaldehyde (Fisher Scientific, USA) in PBS for 2-5 minutes. Subsequently, cultures were stained essentially as described by Dimri and collaborators (Dimri et al. 1995): cells were incubated with SABG staining solution for approximately 16h at 37°C in a humid box, washed with milli-Q H_2O (Millipore MilliQ System, UK) and stored in milli-Q H_2O until visualised with a light microscope and photographed.

8.4 BrdU labelling index

The proliferative index of colonies was determined by Bromo deoxy Uridine (BrdU) labelling as previously described by Munro and collaborators (Munro et al. 2001). After incubating the cells for 48h with 10μM BrdU (Sigma Chemicals, Poole, UK), the irradiated 3T3 feeders were carefully removed and the cells fixed in 1:1 acetone/methanol (Fisher Scientific, USA) for 10 minutes. Subsequently, cells were washed in PBS for 10 minutes, permeabilised in 0.25% Tween 20 (Sigma Chemicals, Poole, UK) in PBS for 10 minutes in a humidity box and denatured in 1M HCl (Fisher Scientific, USA) at 60° for 5 minutes. After blocking with 10% FBS, cultures were incubated with an anti-BrdU antibody (Harlan Sera-Lab, Loughborough, UK) at 4°C overnight. The following day, cells were incubated with an FITC-conjugated anti-rat IgG (Sigma Chemicals, Poole, UK) at a dilution of 1/125 for 1 hour in the dark in a humidity box. After each antibody incubation, cells were washed with 10% FBS/1xKRH/Tween 80 as previously described (Munro et al. 2001). The nuclei were counterstained with propidium iodide (1ng/ml), finally the cells were mounted with Vectashield (Vector Laboratories, Peterborough, UK) and visualised with a fluorescence microscope.

8.5 TUNEL assay

Apoptosis was measured by the Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) assay using the ApoTag® kit (Intergen, Oxford, UK). After careful removal of the irradiated 3T3 feeders, cells were processed according to the manufacturer's protocol. Cells were fixed for 10 minutes in 1% paraformaldehyde (TAAB laboratories, UK) and washed 2 x 5 minutes in PBS. The cells were post-fixed in pre-cooled Ethanol/Acetic Acid (James Burrough Ltd., UK and Fisher Scientific, USA respectively) for 5 minutes at –20°C, drained and washed as above. The nuclei were counterstained with propidium iodide (1ng/ml), the cells were mounted in Vectashield and visualised on a fluorescence microscope.

8.6 RNA Analysis

RNA extraction and Northern blotting was carried out as previously described (McGregor et al. 1997). The hTERC probe was kindly provided by Dr. Fiona McGregor (Beatson Institute, UK). The RAR-β probe was made from a plasmid containing the full length RAR-β2 cDNA sequence provided by Prof. P. Chambon (Strasbourg, France).

8.7 Western Blot Analysis

Protein extraction and Western blotting was carried out as described previously (McGregor et al. 1997). Primary antibody incubations were carried out using the following antibodies: $p16^{ink4A}$ (C-20: Santa Cruz Biotechnology, Santa Cruz, CA, USA); EGF-R (Santa Cruz Biotechnology); c-erbB2 (Ab-17: Neomarkers, Fremont, CA, USA), PLK1 (Transduction Laboratories, Lexington, KY, USA); cyclin D1 (Ab3, Oncogene Research Products, Boston, MA, USA); p53 (D01, SantaCruz Biotechnology); ser 15 phospho-specific p53 (16G8, New England Biolabs, Hitchin, Hertfordshire, UK). Membranes were re-probed with a polyclonal antibody against total ERKs1&2 (ERK1(C-16)-G, Santa Cruz Biotechnology) or α-Tubulin (Sigma-Aldrich, Poole, Dorset, UK) to ensure even loading and transfer since they were found to be uniformly expressed in all keratinocyte cultures regardless of proliferative fate. Unless otherwise stated, protein was extracted from cultures during the proliferative phase of growth.

8.8 p53 sequencing.

This was performed using proof-reading DNA polymerase (Quiagen, Crawley, UK) and the following primers: exon 2, 2F/2R for amplification and sequencing; exon 3, 3F/4R for amplification and sequencing; exon 4, 3F/4R for amplification, 3F/4F/4R for sequencing; exons 5 & 6, 5F/5R for amplification and sequencing; exon 7, 7F/8R for amplification, 7F/7R for sequencing; exons 8 & 9, 7F/8R for amplification, 8F/8R for sequencing; exon 10, 10F/10R for amplification and sequencing; and exon 11, 11F/11R for amplification, 11F/11BR for sequencing. Primer sequences are as follows: 2F (5'-CTCAAAGAAGTGCATGGCTGG-3'); 2R (5'-CAGGAAGTCTGAAAGACAAGAGC-3'); 3F (5'-GAAGCGAAAATTCATGGGACTGAC-3'); 4R (5'-CATTGAAGTCTCATGGAAGCCA-3'); 4F (5'-ACCTGGTCCTCTGACTGCTCT-3'); 5F (5'- CGTGTTCCAGTTGCTTTATCTG-3'); 5R (5'-GGAGGGCCACTGACAACCA-3'); 7F (5'-AGGCGCACTGGCCTCATCTT-3'); 7R (5'-AGGGGTCAGCGGCAAGCAGA-3'); 8F (5'-TTGGGAGTAGATGGAGCCT-3'); 8R (5'-AACTTTCCACTTGATAAGAGGTC-3'); 10F (5'-GTACTGTGAATATACTTACTTCTCC-3'); 10R (5'-CAAAGAAGTATCCACACTCGTC-3'); 11F (5'-CACTCATGTGATGTCATCTCTCC-3'); 11R (5'-CAAGACTTGACAACTCCCTC-3'); 11BR (5'-ATCTAAGCTGGTATGTCCTACTC-3'). Sequencing of fragments in both directions was performed according to the manufacturer's protocols (ABI prism big dye terminator

reaction kit, Applied Biosystems, Foster City, CA, USA). Where a mutation was found, the result was confirmed using an independent PCR amplification product.

8.9 Telomerase assay.

Telomerase activity was measured in cell lysates containing 100-200ng of protein, using the TRAPeze telomerase detection kit (Intergen, Oxford, UK) according to the manufacturer's recommendations. The feeder layer of irradiated 3T3 fibroblasts was carefully removed before analysis to eliminate any residual contaminating activity from these cells. WI38 and HeLa cells were used as negative and positive controls, respectively. The PCR products were analysed by electrophoresis on a 12.5% polyacrylamide gel and visualised using ethidium bromide staining.

8.10 Semi-quantitative reverse transcription (RT)/polymerase chain reaction (PCR) assay for hTERT mRNA.

This assay was performed using the GeneAmp RNA PCR core reagent kit (Perkin Elmer, Branchberg, NJ, USA) according to the manufacturer's protocols. The hTERT primers used were MS113 (5'-AGAGTGTCTGGAGCAAGTTGC-3') and MS114 (5'-CGTAGTCCATGTTCACAATCG-3'), yielding a 183-bp fragment corresponding to nucleotides 1789-1971, as described by Dome and collaborators (Dome et al. 1999). The levels of GAPDH mRNA were also measured as an internal control, and the primers used were AAGGCTGAGAACGGGAAGCTTGTCATCAAT and AGCCCCAGCCTTCTCCATGGTGGTGAAGAC.

8.11 Quantitative reverse transcription (RT)/polymerase chain reaction (PCR) assays for hTERT mRNA.

This was performed using the Taqman reverse transcription reagents and Taqman PCR core reagent kit (Perkin Elmer, Branchberg, NJ, USA), according to the manufacturer's protocols. This method measures the number of PCR cycles required to obtain exponential amplification of specific PCR product and comparing this with a standard curve. For each experiment, an initial amplification reaction was carried out to measure the quantity of 7S rRNA present in each sample, as an internal control. To obtain a standard curve, four known quantities of 7S rRNA were reverse-transcribed and then PCR amplified: 10ng, 1ng, 100pg, 10pg. The 7S rRNA, the primers and the probe for cDNA PCR amplification

were supplied in the Taqman Ribosomal RNA control kit, Perkin Elmer, Branchberg, NJ, USA). Similarly, 5ng of RNA extracted from each sample and measured by optical density were reverse transcribed and PCR amplified. The values obtained from the quantification of the samples were always interpolated within those obtained for the corresponding standard curve. Subsequently, our gene of interest hTERT was quantified in the same set of samples. Initially, hTERT DNA was excised from an hTERT expression construct cloned in a pBluescript KS plasmid vector (kindly provided by Dr. Lisa Bryce, Beatson Institute for Cancer Research) and quantified by optical density. To obtain a standard curve, four known quantities of hTERT DNA were reverse trancribed: 1pg, 100fg, 10fg, 1fg. The cDNA was then PCR amplified using the primers ACAGCACTTGAAGAGGGTGCA and TTCCCGATGCTGCCTGAC and the fluorescent probe FAM-TGCGGGAGCTGTCGGAAGCA- TAMRA (VH Bio Ltd., Newcastle upon Tyne, UK). Similarly, 1μg of RNA extracted from each sample and measured by optical density was reverse transcribed and PCR. As described for rRNA, the values obtained from the hTERT quantification of the samples were always interpolated within those obtained for the corresponding standard curve. Finally, the relative levels of hTERT to ribosomal RNA and the standard deviation was calculated.

8.12 TRF measurement.

TRF measurement was carried out essentially as described by Munro and collaborators (Munro et al. 2001). 4 μg of genomic DNA was digested with *RsaI* and *HinfI* restriction enzymes (Roche diagnostics, Indianapolis, USA), separated on a 0.7% agarose gel and transferred to a Hybond N membrane (Amersham International, Amersham, UK) in 20xSSC. The blot was prehybridised for 15 minutes at 42°C in PerfectHyb Plus solution (Sigma Chemicals, Poole, UK), hybridised for 4 hours at 42°C with a radiolabelled oligonucleotide probe $(TTAGGG)_3$ using the same hybridisation solution and subsequently washed in 6xSSC/1%SDS at 42°C. The molecular weight markers used for determining the size of the TRFs were λ/hindIII and 1Kb ladders (Gibco BRL Europe Life Technologies, Paisley, UK).

9 Features of the primary human keratinocyte cultures used in this work: a descriptive summary

This project was designed and developed with the aim of investigating two main aspects of oral cancer development: 1) what molecular markers are involved in the transition from normal oral mucosa to dysplastic lesions and subsequently to squamous cell carcinoma, and 2) which molecular changes are required for keratinocyte immortalisation and the timing and hierarchy of their occurrence.

In order to answer these two questions, an *in vitro* model of oral cancer progression rendered available by previous work in our Institute was used (Edington et al. 1995) (McGregor et al. 1997). The purpose of this introductory chapter is to describe the derivation and characterization of the primary cultures from normal oral mucosa, mortal dysplasias, immortal dysplasias and squamous cell carcinomas used in this work, and their features are summarised in Table 1. The oral dysplasias were all derived and analysed by previous work in our group. In addition, several primary cultures derived from HNSCC biopsies were kindly made available by K. Parkinson's group at the Beatson Institute and extensively used throughout this project.

9.1 Features of the biopsy samples

All of the biopsy samples utilised for the derivation of corresponding cultures were excised at the Canniesburn Hospital or Glasgow Dental Hospital and School by our collaborator, Dr. E. Felix, and their histological features assessed by Prof. G. McDonald at the Glasgow Dental School. Biopsies from normal oral mucosa were obtained from three healthy volunteers (FNB, FNB3, FNB5, FNB6), one of whom donated two biopsy from different sites of the oral cavity (FNB and FNB3). Two additional biopsies were obtained from morphologically normal tissue adjacent to tumours and the corresponding cultures made (NB1 and NB5).

The fifteen biopsies of dysplasias were obtained from different sites of the oral cavity, mostly from those that are known to carry a high risk of malignant transformation like tongue and floor of mouth but in one case from buccal mucosa.

Type	Dysplasia #	Site	Dysplasia Pathology	Mortality phenotype (max. PDs)
Leukoplakias	D41	Posterolateral tongue	mild	3.5
	D6	Posterior tongue	Moderate/ severe	25
	D8	Ventral tongue	mild/ moderate	9
	D25	Floor of mouth	severe	28
	D30	Floor of mouth	mild	30
	D47	Floor of mouth	moderate	20
	D17	Buccal mucosa	mild/ moderate	45
	D48	Ventral tongue	moderate/ severe	25.5
	D20	Lateral tongue	moderate	immortal
	D34	Postero-lateral tongue	moderate	immortal
	D9	Ventral tongue	mild/ moderate	immortal
	D4	Ventral tongue/floor of mouth	early SCC	immortal
	D38	Lateral tongue	mild	immortal
Erythroleukoplakias	D19	Lateral tongue	severe/CIS	immortal
	D35	Floor of mouth/ ventral tongue	severe/CIS	immortal

Table 1: Features of the dysplasias used in this work.

Macroscopically, thirteen of the dysplasias appeared like leukoplakias, i.e. white hyperkeratotic patches on the oral mucosa, and two appeared like erythroleukoplakias, i.e. red velvety lesions with areas of leukoplakia admixed. Microscopically, they were classified as mild, moderate, severe dysplasias and carcinoma *in situ* (CIS) depending on several internationally adopted criteria: increased nuclear/cytoplasmic ratio, altered nuclear polarization, irregular epithelial stratification, increased number of mitotic figures, nuclear hyperchromatism, enlargement of nucleoli, abnormal keratinisation, reduced cellular cohesion and invasion of the lamina propria (see chapter 1).

Only limited amount of information was available regarding the clinical outcome of the excised dysplasias, due to strict regulations on patients right to privacy and confidentiality issues. Three of the dysplasias, D19, D35 and D48, evolved into fully developed squamous cell carcinomas within three years of onset. In particular, D19 had a very poor clinical outcome, with lymph-node invasion, requiring lateral neck dissection.

9.2 Establishment of primary cultures

Primary cultures were derived from biopsies using the irradiated 3T3 feeder layer system developed and optimised by Rheinwald and Beckett (Rheinwald and Beckett 1981). This system has been shown to facilitate growth of keratinocytes from normal oral mucosa as well as malignant lesions maintaining the characteristics of the original tumours. The growth medium utilised for the establishment of the cultures was either flavine adenine dinucleotide growth medium (FAD) supplemented with 10% foetal bovine serum (FBS), 0.4 μg/ml hydrocortisone, 5 μg/ml insulin, 5 μg/ml transferrin, 10^{-10}M cholera toxin, 1.8 x 10^{-4} adenine and 10 ng/ml epidermal growth factor, or DMEM supplemented with 10% FBS and hydrocortisone (10H). Each culture was tested and then maintained with whichever of these two media proved best for their growth. Other media formulations had been previously tested in our Institute (Edington et al. 1995), but the elimination of hydrocortisone or FBS had proved detrimental for growth of carcinoma cultures.

Another culture medium often utilised for growth of keratinocytes is the K-sfm serum-free chemically-defined medium, supplemented with 25 μg/ml bovine pituitary extract, 0.2 ng/ml EGF and 0.4 mM $CaCl_2$. This medium was found to be inadequate for the growth of carcinoma cultures because of the absence of serum, as explained above, and therefore not utilised.

The possibility to derive primary cultures from all stages of oral cancer progression and compare their features offers an invaluable advantage over other epithelial cancer progression models, like colon cancer, where cells from normal colonic mucosa can be grown only with extreme difficulty, and breast cancer, where the same difficulty is encountered in growing primary neoplastic cells. Another advantage offered by this culture system is that it prevents the premature growth arrest occurring when keratinocyte are grown in a chemically defined medium on plastic culture dishes, without the 3T3 feeder layer (Ramirez et al. 2001).

The 3T3 feeder layer was removed and replaced on a weekly basis or immediately before each assay. Keratinocytes were harvested when they had reached approximately 80% of confluence in culture dishes and during the exponential phase of their growth. Standardization of these two parameters was considered necessary to ensure that all of the molecular changes observed amongst the cultures were possibly related to different stages of cancer progression rather than different ages in culture or different percentages of confluence.

9.3 *In vitro* features of primary cultures

9.3.1 Proliferative potential

All of the primary cultures successfully derived from oral biopsies as described above were serially passaged in tissue culture plates and monitored for life-span. The four cultures obtained from healthy volunteers (FNB, FNB3, FNB5, FNB6) proliferated for 10, 3, 20 and 11.5 population doublings (PD) respectively, after which they showed a rapid deterioration of their growth rate. The two cultures derived from apparently normal tissue adjacent to tumours (NB1 and NB5) reached growth arrest after 20.5 and 13 PD respectively. Combining our data with Ken Parkinson's data, a total of 19 dysplasias were available, 13 classified as leukoplakias, 2 as erythroleukoplakias and 4 as erythroplakias. 5/13 of the leukoplakias and both the erythroleukoplakias showed an unlimited proliferative potential: they were still proliferating after having completed 100 PD and were therefore considered immortal. The remaining 8 leukoplakias and 4 erythroplakias reached a growth arrest status after between 4 and 30 PD, with the exception of D17 that had a considerably extended life span and proliferated for 45 PD (table 1). No clear correlation could be observed between proliferative potential and grade of dysplasia (mild, moderate, severe, CIS) or smoking/drinking habits. Both of the erythroleukoplakias cultures isolated in our group were immortal, but this does not appear to be a general trend

as four other erythroplakia cultures previously isolated in the Institute were mortal (Edington et al. 1995). Also, there was also no clear correlation between immortal phenotype and clinical outcome: only two of the seven dysplasias that evolved into SCC were immortal in culture.

9.3.2 Senescence or crisis?

As discussed in chapter 7, human epithelial cells proliferate for a finite number of population doublings and then enter a growth arrest state called M1 (mortality stage 1 or senescence). This phenomenon is characterized by a decline in the number of cells actually dividing, while the others exit from cell cycle and arrest in G0. They remain metabolically active, resistant to apoptosis and acquire some peculiar features like enlarged and flattened shape with occasional multinuclei, decrease in nuclear/cytoplasm ratio (Dimri et al. 1995). Abrogation of Rb/p16 and p53 tumour suppression pathways are capable of overcoming the M1 block and extending cellular life span. Cells then encounter a second proliferative checkpoint called M2 (mortality stage 2 or crisis), characterised by increased cell death and karyotypic abnormalities. Only a small number of cells are able to overcome M2 and acquire unlimited growth potential, and this event is thought to be dependent upon reactivation of telomerase and maintenance of telomere length. In order to understand the role of different molecular markers in the multistep process that leads to keratinocyte immortality, it was of crucial importance to assess whether the growth arrest observed in all the mortal cultures was due to senescence or crisis.

Three assays were used to answer this question. The first assay, Senescence Associated β-Galactosidase (SABG) staining, is positive in cells undergoing growth arrest irrespectively of their senescence or crisis status (Dimri et al. 1995) (Romanov et al. 2001). The second assay, Bromodeoxyuridine (BrdU) incorporation over a 48h period, is an index of DNA synthesis and therefore cellular proliferation. It better distinguishes between the two mortality checkpoints, since ~20% of normal senescent keratinocytes are positive in this assay compared to >80% of keratinocytes in crisis (K. Parkinson, personal communication). The third assay, TUNEL assay, is specific for DNA fragmentation that occurs during cellular apoptosis, which is a typical feature of cellular crisis but not senescence.

All of the mortal dysplasias were maintained in culture for a minimum of three weeks after their growth rate had decreased and then analysed by the assays described above. SABG staining was positive for all of the cultures. BRDU incorporation was found in <2% of

cells, with the exception of D17 that had a slightly higher percentage of BRDU incorporation (10-20%). Virtually no apoptosis could be detected by TUNEL assay. Figure 15 shows a representative example of these stainings in D17. These combined results suggest that all of our mortal dysplasia cultures enter growth arrest status because they reach senescence rather than crisis.

9.3.3 Invasive potential

In order to assess at what stage of oral cancer progression cells acquire invasive potential, an *in vitro* matrigel invasion assay was performed with or without the presence of EGF as a chemoattractant in the medium above the matrigel (McGregor et al. 2002). The percentage of cultures of normal oral mucosa, mortal and immortal dysplasias capable of invasion was negligible (<0.5%). However, the percentage of SCC and lymph-node metastasis showing invasion was significantly higher (2%), particularly with the metastatic culture (6%). Therefore, the invasive potential appears to be acquired at the carcinoma stage of oral cancer progression, and increases further in metastasis.

9.3.4 Chapter summary

- A description of the features of primary human keratinocyte cultures previously established and extensively used in this work is presented in this chapter.

- A series of oral biopsies from normal mucosa, macroscopically normal mucosa adjacent to tumours and premalignant lesions were collected from high-risk sites and corresponding cultures established.

- Half of the dysplasias were mortal in culture and the remaining half were immortal. The acquisition of immortality can therefore be an early event in oral cancer progression.

- The growth arrest status reached by the mortal dysplasias was due to senescence rather than crisis.

- The acquisition of invasive potential is a feature of SCC and metastatic lesions, thus a later event in oral carcinogenesis.

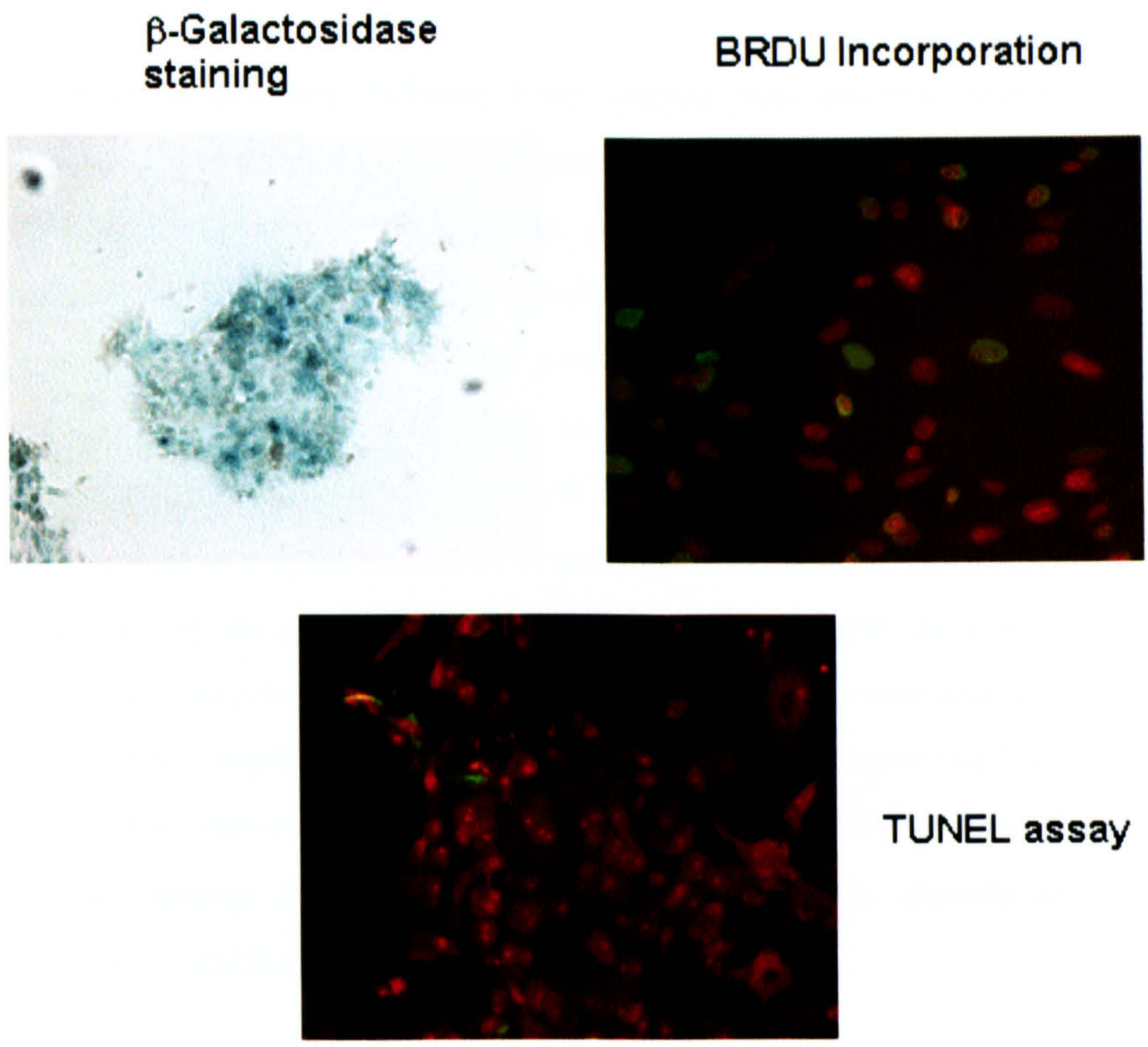


Fig. 15: SABG staining, BRDU incorporation, TUNEL assay in D17.

D17 was maintained in culture for a minimum of three weeks to confirm cessation of proliferative growth. In order to understand if the growth arrest status was due to senescence or crisis, SABG staining, BRDU incorporation and the TUNEL assay were performed, as illustrated above.
A) Cells that have reached growth arrest, both due to senescence or to crisis, stain positive for SA-βGal. staining, whereas actively proliferating cells stain negative.
B) Actively proliferating cells incorporate BRDU (green label), whereas cells that have reached growth arrest are only visualised by the nuclear DNA staining with propidium iodode (red label). The percentage of cells scoring positive for BRDU incorporation was assessed on a minimum of 500 cells.
C) Apoptotic cells are visualised by the DNA fragments label (green) and the nuclear DNA by the propidium iodide staining (red).Virtually no apoptotic cells are visible in any of the colonies.
The positive SABG staining, the low percentage of cells still proliferating and therefore incorporating the green BRDU label (21%) and the absence of apoptotic cells incorporating the green DNA-fragments label (TUNEL assay) are characteristics of senescence rather than crisis.
Similar evidence was obtained for all the other mortal dysplasias (Janis Fleming, personal communication).

10 Analysis of markers differentially expressed at different stages of oral cancer progression.

Our unique series of primary cultures from normal oral mucosa, mortal dysplasias, immortal dysplasias and squamous cell carcinomas represents an ideal system to carry out a detailed analysis on several features of oral carcinogenesis *in vitro*. The first and main advantage offered by this system is the possibility to analyse those molecular changes occurring in the transition from normal oral mucosa to premalignant lesions and squamous cell carcinomas. Another clear advantage is the availability of detailed information regarding the proliferative life span for all of the cultures. The acquisition of unlimited proliferative potential is a frequent, even if not universal, feature of cancer cells and, as previously reported by our group, it most commonly occurs at the dysplasia stage of oral cancer progression. Comparing molecular features of mortal and immortal dysplasias could therefore be of crucial importance in understanding those phenomena implicated in cellular immortality. Finally, information about the clinical outcome of some of the dysplasias from which our cultures were derived could prove useful in identifying markers of potential prognostic significance for early oral lesions.

A large number of literature reports describe several molecular events occurring during oral cancer progression. This study had several purposes: understanding the stage of oral cancer progression at which these molecular changes occur, investigating the potential correlations between these markers and the mortal/immortal proliferative potential and clarifying the molecular differences between erythroplakias and leukoplakias. For this reason, a representative panel of cultures from normal oral mucosa, mortal dysplasias, immortal dysplasias and SCC was selected and analysed for expression of the following markers:

- Retinoic Acid Receptor β (RARβ)
- Epidermal Growth Factor Receptor (EGFR) and c-erbB2
- Polo-like Kinase (PLK)
- Cyclin D1
- p16
- p53
- Telomerase activity, hTERC and hTERT.

For two of these markers (p16 and RARβ), an involvement in oral cancer had already been suggested through previous work carried out in our group and this was confirmed at the outset of my study.

10.1 RAR β is lost in immortal dysplasias and SCC

Retinoids (vitamin A analogues) and their nuclear receptors are known to play a central role in maintaining appropriate cellular growth and differentiation in non keratinising epithelia like the oral cavity mucosa. Several studies carried out *in vivo* have reported a progressive decreàse in RARβ expression in samples from normal, premalignant and malignant head and neck epithelia (Xu et al. 1994) (Lotan et al. 1995). *In vitro,* loss of RARβ seems to be a frequent finding in SCC (Hu et al. 1991). Interventional studies carried out by Wan and collaborators (Wan et al. 1999) were also able to demonstrate that transfection of an RARβ expression vector in HN cancer cells restores their sensitivity to suppression of squamous differentiation by retinoids.

Previous studies carried out in our group have thoroughly analysed the expression of RARβ in our series of normal samples, premalignant lesions and carcinomas (McGregor et al. 1997) (McGregor et al. 2002) and shown differential regulation of this marker at different stages of oral cancer progression. RARβ mRNA is detectable *in vivo* in samples from normal oral mucosa. In cultures derived from those biopsies RARβ mRNA was not expressed if the cells were cultured in a retinoid-free medium, but expression was restored upon retinoid treatment. Also, when these cultures were analysed at different stages of their life span, expression of the receptor increased as cells approached senescence (McGregor et al. 2002). Therefore, expression of RARβ in cultures from normal oral mucosa appears to be retinoic acid-dependent when cells are actively proliferating, whereas it becomes constitutive at later stages of their life span even in the absence of retinoids. In mortal dysplasia cultures, RARβ expression is constitutively active and its expression is detectable independently of retinoid treatment (McGregor et al. 1997). One interesting exception to this general principle is D17, a mortal culture that senesces after a considerably extended lifespan. The characteristics of this particular cell line will be discussed in greater detail in later chapters. In all of our immortal dysplasias and carcinomas, RARβ mRNA expression is undetectable *in vitro* and can not be restored with retinoid treatment.

Taking into account all these observations, the pattern of RARβ expression in normal oral mucosa and mortal dysplasias *in vitro* is not completely clear. Normal cultures might lack receptor expression as a result of absence of exposure to the ligand. This is suggested by its presence in normal biopsies which would be exposed to physiological levels of circulating retinoids and by its expression upon retinoid treatment. As cells approach senescence, higher levels of RARβ expression might be required to mediate the arrest of proliferative growth and terminal differentiation. Mortal dysplasias show a different pattern of regulation of RARβ expression, as they are able to express high levels of receptor in the complete absence of ligand. A possible explanation to this phenomenon is that mortal dysplasias behave like senescent normal cells, but have acquired some as yet unknown features that allow them to proliferate for longer. Conversely, the association between loss of expression of RARβ and immortal phenotype in culture appears quite strong, as it occurred with just one exception in our oral premalignant lesions.

The constitutive expression of RARβ mRNA in mortal dysplasias and its loss in immortal dysplasias and SCC was confirmed at the outset of my project (fig. 16). RARβ protein expression, although potentially more specific, could not be assessed because of the unavailability of a specific antibody.

10.2 Epidermal Growth Factor Receptor is upregulated in SCC

The EGF Receptor family is composed of 4 members EGF-R, c-erbB2, 3 and 4; of these EGF-R homodimers or EGF-R/c-erbB-2 heterodimers form receptors for EGF. The EGF Receptor complex and its ligands EGF and TGFα are involved in cellular growth and differentiation. As for many other growth factor receptors, over-expression of EGF receptor complex could be a potential mechanism enabling cells to acquire a proliferative advantage and respond to low amount of ligands. A vast body of literature (Grandis and Tweardy 1993) (Todd et al. 1989) (Ishitoya et al. 1989) including work done from our Institute (Stanton et al. 1994) has demonstrated that EGF-R is up-regulated in HNSCC, both *in vitro* and *in vivo*. EGF-R expression has also been analysed in oral premalignant lesions, both *in vitro* and *in vivo* (Grandis et al. 1998)(d), and its up-regulation in these lesions appears to be correlated with increasing degrees of dysplasia. It has also been suggested that c-erbB-2 may be a better prognostic index for oral cancer development than EGF-R subunit itself (Xia et al. 1999).

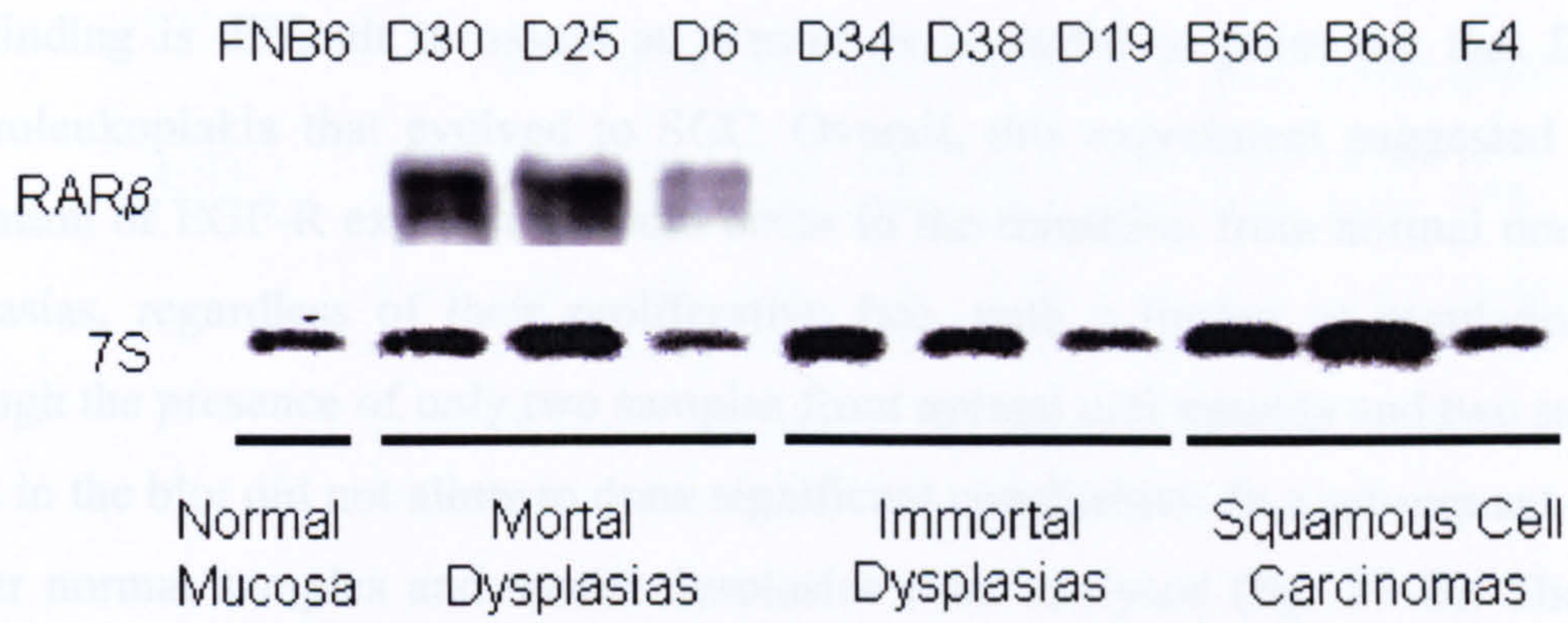


Fig. 16: RARβ expression in a panel of cultures from normal oral mucosa, mortal dysplasias, immortal dysplasias and squamous cell carcinomas.

Northern blot showing RARβ mRNA expression in a randomly selected group of cultures from normal oral mucosa, mortal dysplasias, immortal dysplasias and squamous cell carcinomas. The level of Ribosomal RNA subunit 7S was also determined to ensure equal loading of samples. Cells were cultured in a Retinoid-free (i.e. not supplemented) standard medium and samples were collected within the exponential growth phase for each culture. Names of individual cultures are shown on the upper part of the figure.

As shown in this figure, in the absence of RA treatment, RARβ is constitutively expressed in the mortal dysplasias and virtually undetectable in the normal culture, all of the immortal dysplasias and SCCs.

Data presented is representative of 2 independent experiments.

EGF-R expression was analysed by Western Blotting in selected cultures of normal oral mucosa, mortal dysplasias, immortal dysplasias and SCCs (fig. 17 A). After quantification of the EGFR bands and normalisation for the loading control, the average values were 0.5 for the normal samples, 2.2 for the mortal dysplasias, 3.3 for the immortal dysplasias and 7.9 for the SCCs. Amongst the immortal dysplasias, D19 showed particularly high levels of expression of EGFR, similar to those observed in SCCs. Although the significance of this finding is difficult to assess at present, it is useful to point out that D19 was an erythroleukoplakia that evolved to SCC. Overall, this experiment suggested that an up-regulation of EGF-R expression could occur in the transition from normal oral mucosa to dysplasias, regardless of their proliferative fate, with a further up-regulation in SCCs, although the presence of only two samples from normal oral mucosa and two samples from SCCs in the blot did not allow to draw significant conclusions. In a subsequent experiment, all our normal samples and mortal dysplasias were analysed (fig. 17 B). The difference between levels of EGFR expression in FNB5 and FNB6 observed in the previous experiment was not as marked in this second experiment. The levels of EGFR expression in mortal dysplasias were fairly consistent in the two experiments, but a slight degree of inter-experiment variation could still be observed, particularly for D6. The average values calculated were 0.8 for normal samples and 1.4 for mortal dysplasias. Therefore, the lower levels of EGFR expression in normal oral mucosa compared to mortal dysplasias suggested by the previous experiment was not a consistent finding.

A similar conclusion emerged from the analysis of levels of expression of c-erbB2, the favourite binding partner for EGF-R. No major differences were observed between normal samples (1.7 on average) and mortal dysplasias (2.4 on average) in the first experiment (fig. 18 A). Similarly, only slight differences could be observed amongst the different groups in the second experiment (fig. 18 B): the only normal sample analysed had a value of 1.0, the three mortal dysplasias had an average value of 1.7, the three immortal dysplasias an average value of 0.9 and the two SCCs an average value of 1.3. Again, strong conclusions could not been drawn due to the low number of normal samples and SCCs included in the blot. In addition, a moderate degree of variation could be observed in levels c-erbB2 expression for D30 and D25 in the two separate experiments, and the significance of this finding is unknown.

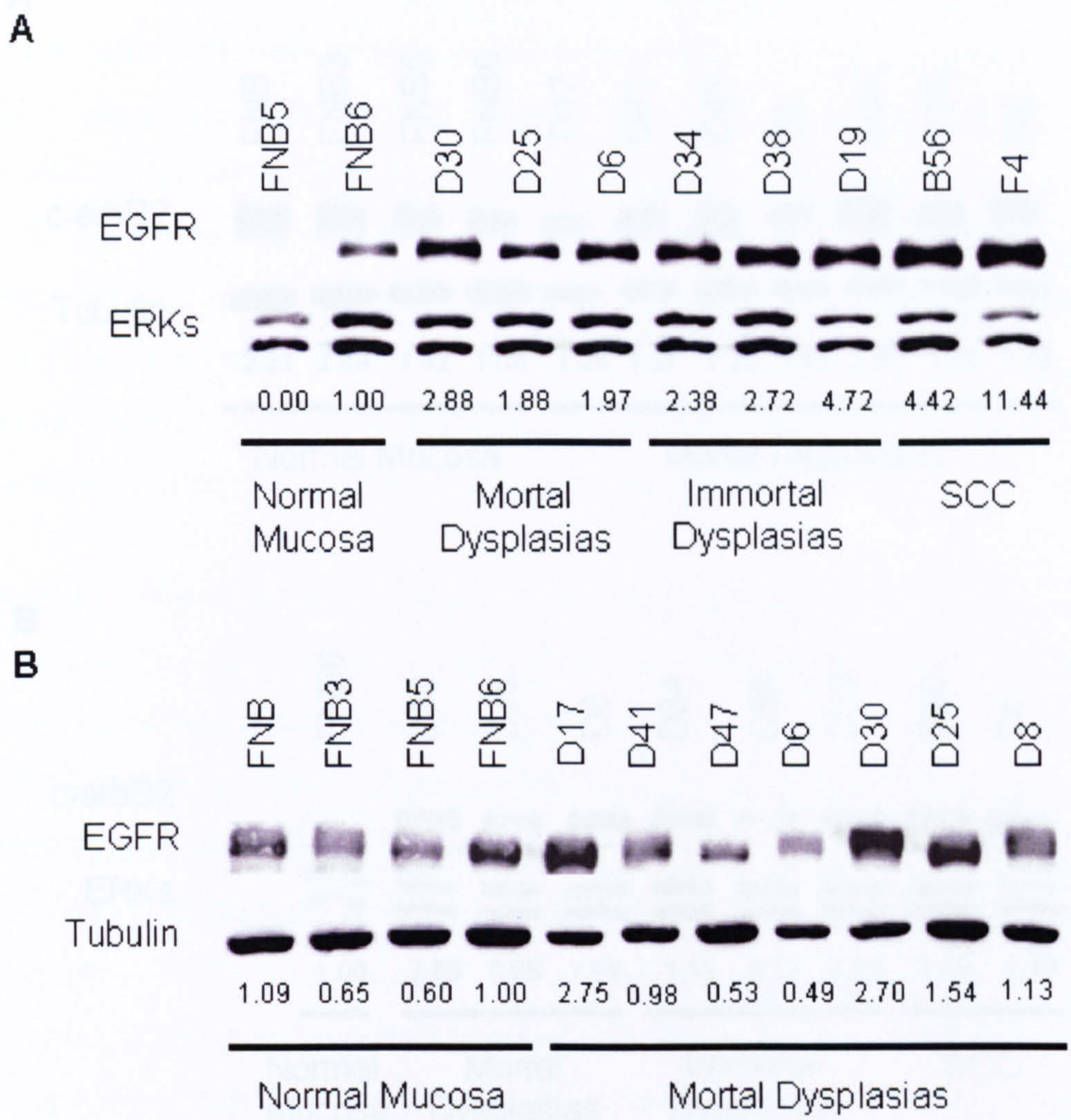


Fig. 17: EGFR expression in a panel of cultures from normal oral mucosa, mortal dysplasias, immortal dysplasias and SCCs.

A) The expression of EGFR was assessed through western blot in a selected panel of cultures from normal oral mucosa, mortal dysplasias, immortal dysplasias and squamous cell carcinomas. The names of individual cultures are shown on the upper part of each figure. ERK 1,2 levels were also measured to ensure equal sample loading. The quantification of the EGFR bands normalised for the loading control is shown in the bottom part of each figure. EGFR appeared to be overexpressed in dysplasias compared to normal samples, with further upregulation in carcinomas. To test the consistency of this finding, EGFR expression was assessed in a wider panel of normal samples and mortal dysplasias **(B)**. α–Tubulin was used as a loading control.
As shown above, EGFR protein expression is upregulated in SCCs but not in premalignant lesions.
Data presented is representative of 2 and 3 independent experiments (A and B respectively).

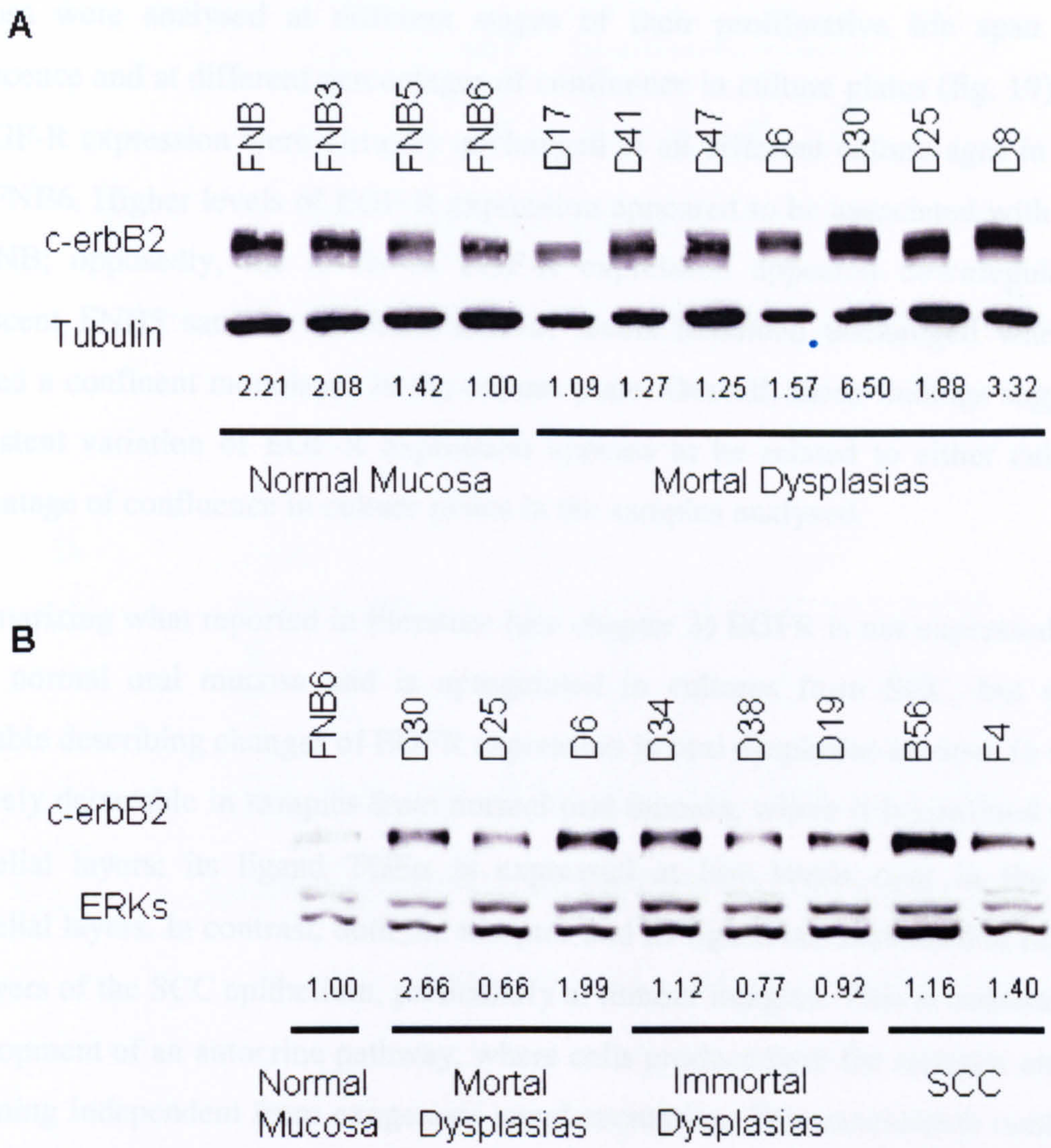


Fig. 18: c-erbB2 expression in a panel of cultures from normal oral mucosa, mortal dysplasias, immortal dysplasias and squamous cell carcinomas.

Western blots comparing c-erbB2 expression in all of our normal samples and mortal dysplasias (**A**) and in a randomly selected group of cultures from normal oral mucosa, mortal dysplasias, immortal dysplasias and squamous cell carcinomas (**B**). α–Tubulin and ERK 1,2 were used as a loading control (A and B respectively).
Names of individual cultures are shown on the upper part of each figure. The quantification of the c-erbB2 bands normalised for the loading control is shown in the bottom part of each figure.
No consistent changes in protein expression can be observed in the panel of samples analised, with the exception on FNB 6 in (B), which could be due to sample underloading.
Data presented is representative of 2 independent experiments.

To exclude that the differences in EGFR or c-erbB2 expression between normal cultures were not due to unintended differences in culture, four different samples of normal oral mucosa were analysed at different stages of their proliferative life span comprising senescence and at different percentages of confluence in culture plates (fig. 19). The levels of EGF-R expression were virtually unchanged at all different culture ages in both FNB3 and FNB6. Higher levels of EGF-R expression appeared to be associated with senescence in FNB; opposedly, the levels of EGF-R expression appeared downregulated in the senescent FNB5 sample. In FNB, EGF-R levels remained unchanged when the cells formed a confluent monolayer in the culture plate. Overall, these findings suggest that no consistent variation of EGF-R expression appears to be related to either culture age or percentage of confluence in culture plates in the samples analysed.

Summarizing what reported in literature (see chapter 3) EGFR is not expressed in cultures from normal oral mucosa and is upregulated in cultures from SCC, but no data are available describing changes of EGFR expression in oral dysplasias *in vitro*. *In vivo*, EGFR is barely detectable in samples from normal oral mucosa, where it is confined to the basal epithelial layers; its ligand TGFα is expressed at low levels only in the suprabasal epithelial layers. In contrast, both the receptor and its ligand are expressed at high levels in all layers of the SCC epithelium, particularly at tumour margins. This is consistent with the development of an autocrine pathway, where cells produce both the receptor and its ligand becoming independent from exogenous supplementation. This mechanism confers tumour cells a proliferative advantage over their normal counterparts and would explain the higher expression of both EGFR and TGFα at tumour margins. In dysplasias, only slight increases of EGFR have been described *in vivo*, not exceeding 2.5-fold.

On the basis of the new experimental findings described in this section, the EGFR upregulation in oral cancer progression appears to occur at the SCC stage: a 4.5 and 11.5 upregulation was found in two different carcinoma samples compared to a normal sample. In keeping with the development of an autocrine pathway, cultures from SCC grow well in medium not supplemented with EGF, whereas cultures from normal oral mucosa and dysplasias do not. No consistent difference was found in the expression of EGFR between samples from normal oral mucosa and dysplasias. The discrepancy between the data reported in literature *in vivo* and our findings *in vitro* might be due to the fact that cells from normal oral mucosa do not normally proliferate *in vivo*, but are forced to proliferate in culture and therefore to express EGFR. Alternatively, the cultures isolated from normal oral mucosa could be composed mainly by basal layer cells, that do express EGFR like cells from the basal layers of dysplasias and carcinomas.

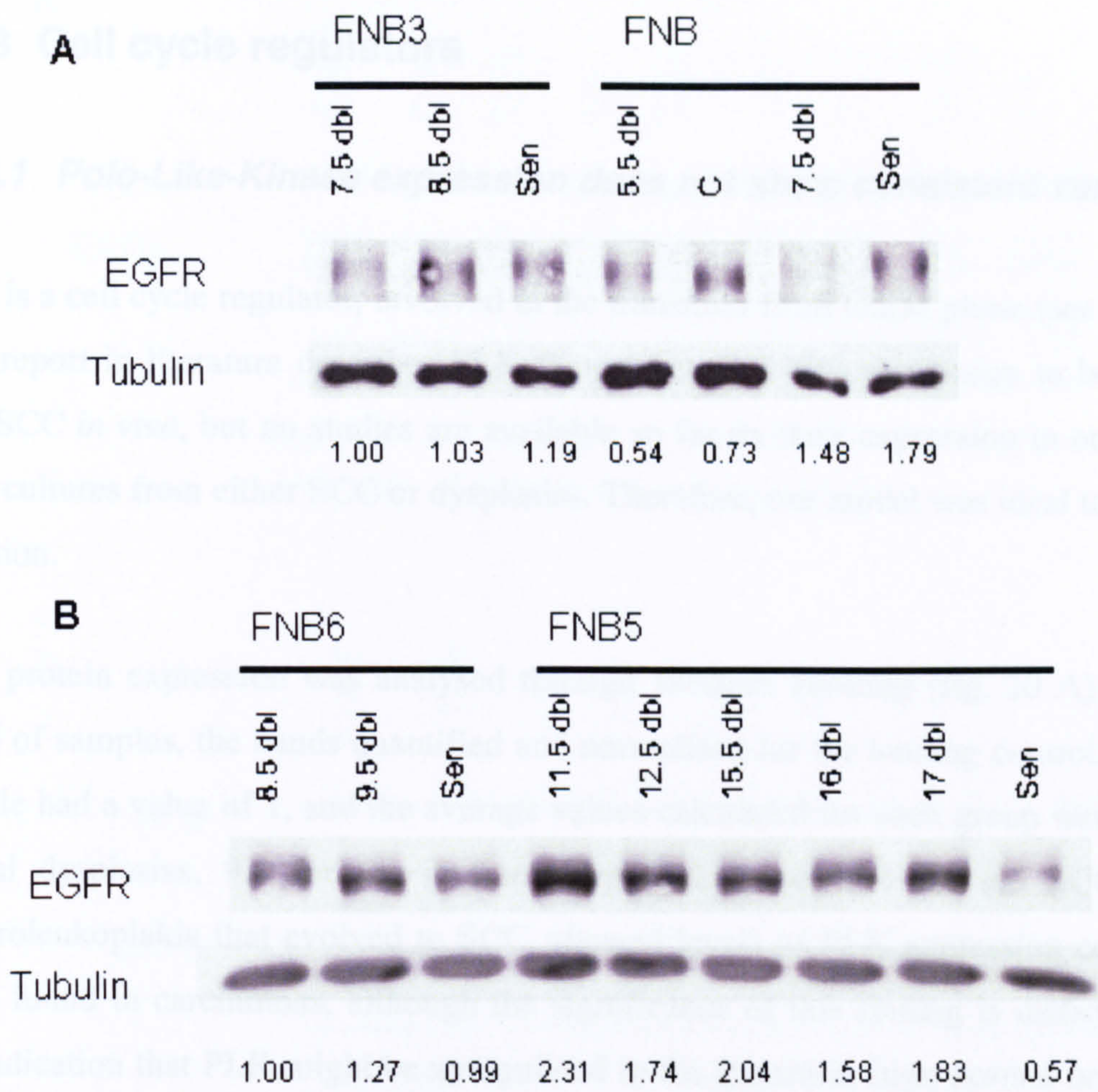


Fig. 19: EGFR expression in samples of normal oral mucosa at different ages and percentage of confluence in culture.

In order to determine whether EGFR expression varied depending on age in culture or percentage of confluence, all of our samples from normal oral mucosa were grown under standard culture condition and harvested at several different proliferative ages towards senescence. Sample collection was also standardised at 80% of confluence, with the exception of sample indicated as FNB C that was harvested after cells had formed a complete monolayer.
On the upper part of each figure are indicated names of individual cultures and number of population doublings completed in culture or senescence status.
The expression of EGFR was assessed by western blotting. α–tubulin was used as a loading control. The quantification of the EGFR bands normalised for the loading control is shown in the bottom part of each figure. No consistent variations in EGFR expression were observed in all of the cultures analysed depending on different stage of replicative life span or different percentage of confluence.

10.3 Cell cycle regulators

10.3.1 Polo-Like-Kinase expression does not show consistent variations

PLK is a cell cycle regulator, involved in the transition from G2/M phase (see chapter 5.2). One report in literature describes PLK (Knecht et al. 1999) expression to be elevated in oral SCC *in vivo*, but no studies are available so far on their expression in oral dysplasias or in cultures from either SCC or dysplasias. Therefore, our model was ideal to address this question.

PLK protein expression was analysed through Western Blotting (fig. 20 A) in the usual panel of samples, the bands quantified and normalised for the loading control. The normal sample had a value of 1, and the average values calculated for each group were 8.7 for the mortal dysplasias, 9.4 for the immortal dysplasias and 16.4 for the SCCs. D19, an erythroleukoplakia that evolved to SCC, showed levels of PLK expression comparable to those found in carcinomas, although the significance of this finding is difficult to assess. An indication that PLK might be upregulated in the transition from normal oral mucosa to early premalignant lesions, and further in SCCs emerged from this experiment, although only 1 normal sample and 2 SCCs were analysed. However, a more extensive analysis of this marker in normal samples and mortal dysplasias did not show major differences between the two groups (fig. 20 B): the two normal samples used in this second experiment showed a great variability in their levels of PLK expression (6.6 on average), and the mortal dysplasias were 13.17 on average. The apparent marked difference between levels of PLK expression in FNB5 and FNB6 were not confirmed in the following experiments, so the interpretation of this finding is unclear.

When PLK protein expression was analysed in samples from normal oral mucosa at different stages of their life span and percentages of confluence (fig. 21), no relevant age-specific changes were found, but a downregulation of this protein could be observed in a sample extracted from a confluent monolayer of cells. This finding requires confirmation, but if substantiated it suggests an involvement of PLK in regulatory mechanisms governing cellular proliferation rather than replicative senescence or premalignant-malignant transformation.

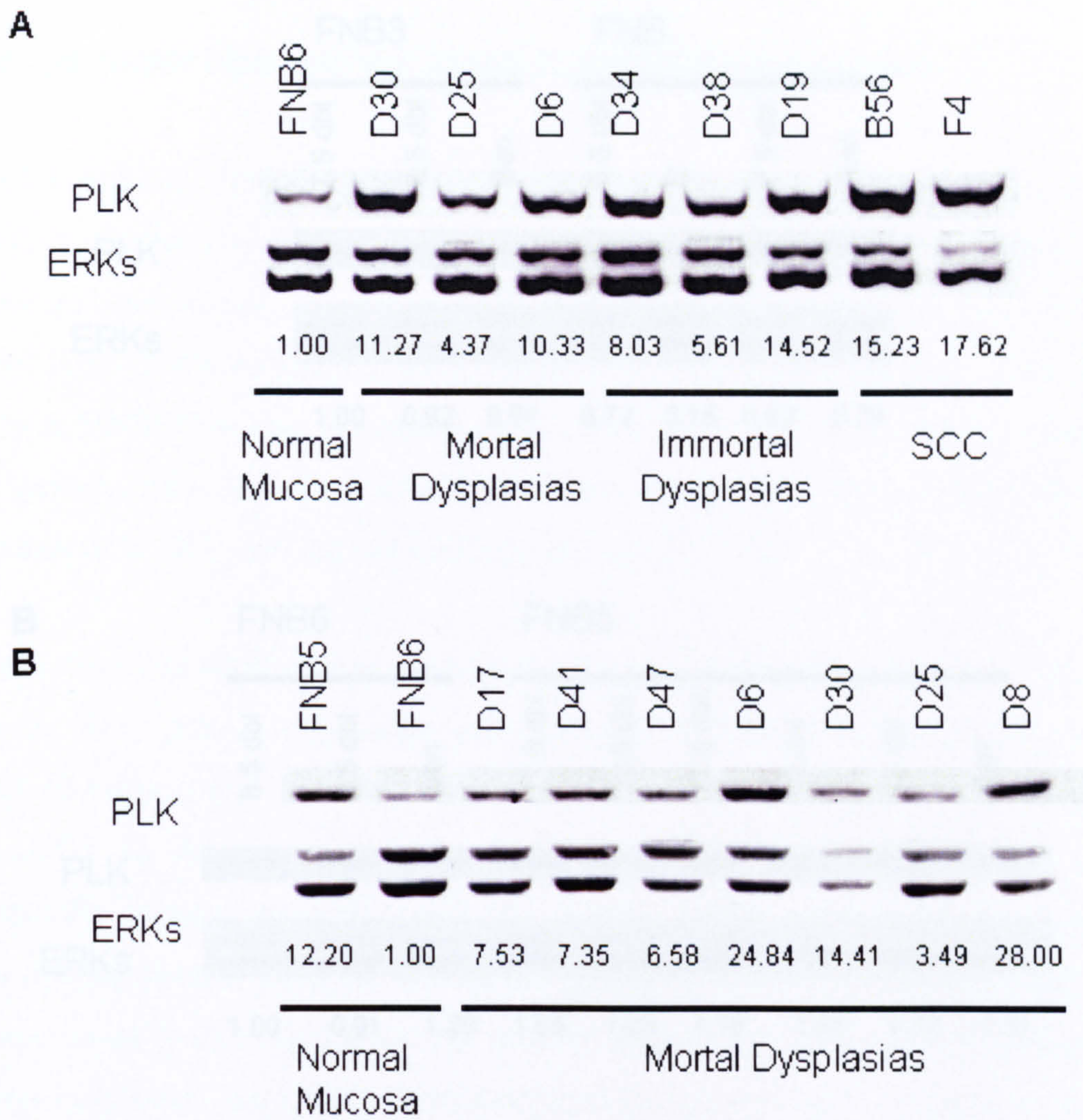


Fig. 20: Polo-Like Kinase expression in a panel of cultures from normal oral mucosa, mortal dysplasias, immortal dysplasias and squamous cell carcinomas.

A) The expression of PLK was assessed by western blot in a panel of cultures from normal oral mucosa, mortal dysplasias, immortal dysplasias and squamous cell carcinomas. The names of the cultures are shown on the upper part of each figure. ERK 1,2 were also measured to ensure equal sample loading. The quantification of the PLK bands normalised for the loading control is shown in the bottom part of each figure. Allowing for a slight underloading in samples at the extremities of the blot, PLK overexpression appeared to be a feature of dysplasias and carcinomas, compared to normal oral mucosa. To determine whether this was a consistent difference in expression levels, a wider variety of samples from normal oral mucosa and mortal dysplasias was analysed **(B).** As shown in the figure, PLK protein overexpression in neoplastic lesions was not confirmed in this second experiment.
Data presented is representative of 3 and 4 independent experiments (A and B respectively).

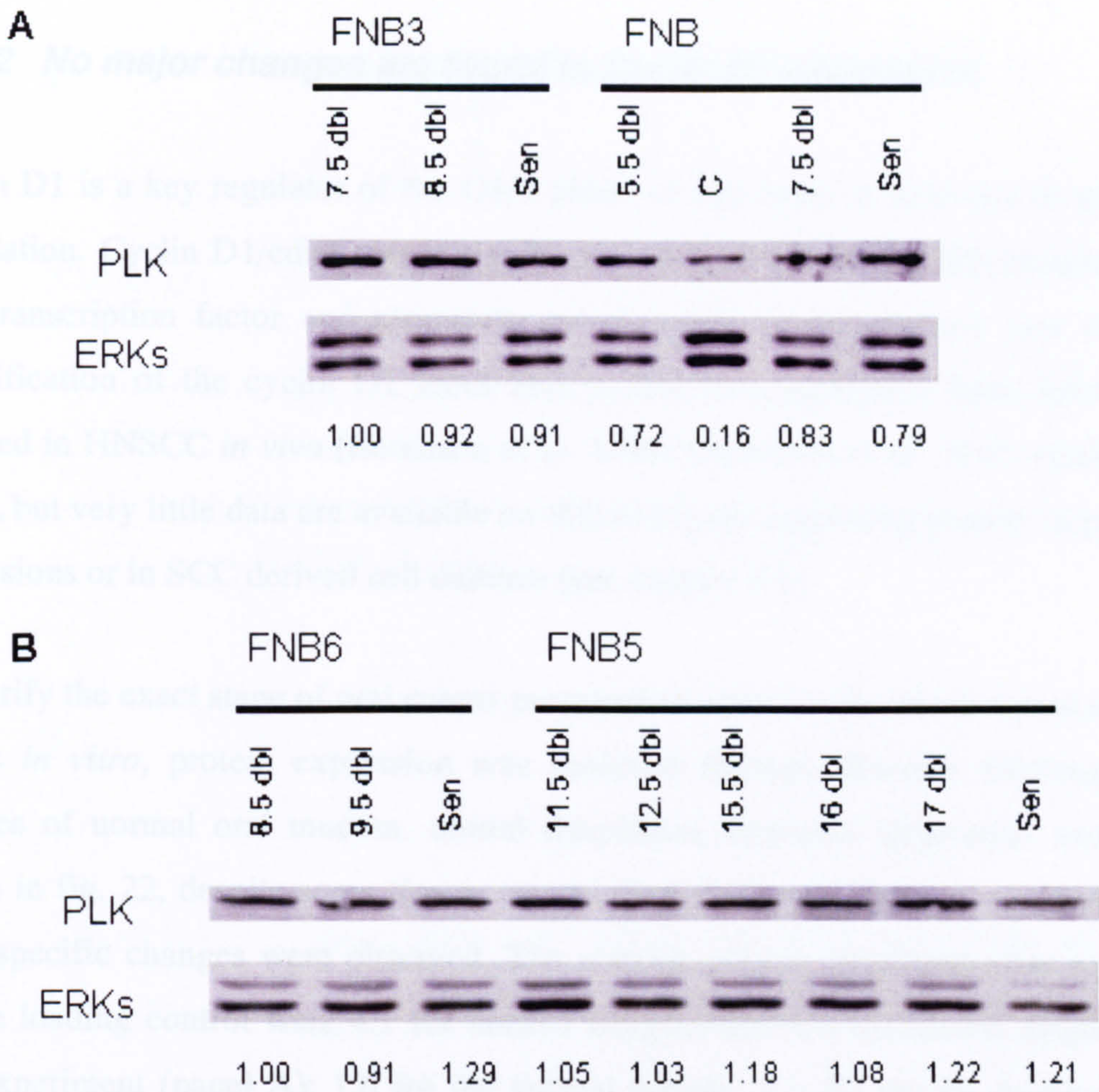


Fig. 21: Polo-Like-Kinase expression in samples of normal oral mucosa at different ages and percentage of confluence in culture.

PLK protein expression was monitored in four different cultures of normal oral mucosa at different proliferative ages in culture, comprising senescence. Protein extracts were obtained from cultures at 80% of confluence, with the exception of sample FNB C that was harvested after the cells had formed a complete monolayer. On the upper part of each figure are indicated name of individual cultures and number of population doublings completed in culture or senescence status. Levels of PLK protein were assessed through Western Blotting, using ERK 1,2 as a loading control. The quantification of the PLK bands normalised for the loading control is shown in the bottom part of each figure.
No consistent variation in PLK expression was observed in all of the cultures analysed regardless of the stage of replicative life span. A slight downregulation of PLK protein expression was observed in the sample obtained from the confluent monolayer (FNB C).

10.3.2 No major changes are found in Cyclin D1 expression

Cyclin D1 is a key regulator of the G1/S phase of cell cycle in response to growth factor stimulation. Cyclin D1/cdk4 complexes lead to phosphorylation of Rb protein, release of E2F transcription factor and ultimately enhancement of mitogenesis (see chapter 4.1). Amplification of the cyclin D1 locus and protein overexpression have been frequently reported in HNSCC *in vivo* (Berenson et al. 1989) (Bartkova et al. 1995) (Callender et al. 1994), but very little data are available on this cell cycle regulating protein in premalignant oral lesions or in SCC derived cell cultures (see chapter 5.1).

To clarify the exact stage of oral cancer progression at which the involvement of cyclin D1 occurs *in vitro,* protein expression was analysed through Western Blotting in several samples of normal oral mucosa, mortal dysplasias, immortal dysplasias and SCCs. As shown in fig. 22, despite some degree of variation observed amongst samples, overall no stage specific changes were observed. The average values calculated after normalisation for the loading control were 4.1 for normal samples and 4.9 for mortal dysplasias in the first experiment (panel A); 1.0 for the normal sample, 2.0 for mortal dysplasias, 0.9 for immortal dysplasias and 1.9 for SCCs in the second experiment (although only two SCC samples were analysed) (panel B).

It emerges from the literature reports (see chapter 5.1) that amplification of cyclin D1 locus and protein overexpression occur in 25-48% of premalignant lesions or SCC *in vivo,* but the data published on *in vitro* findings is inconclusive. When grown in culture, cells are all stimulated to grow, and this could mask the differences between protein expression in normal samples and neoplastic lesions observed *in vitro.*

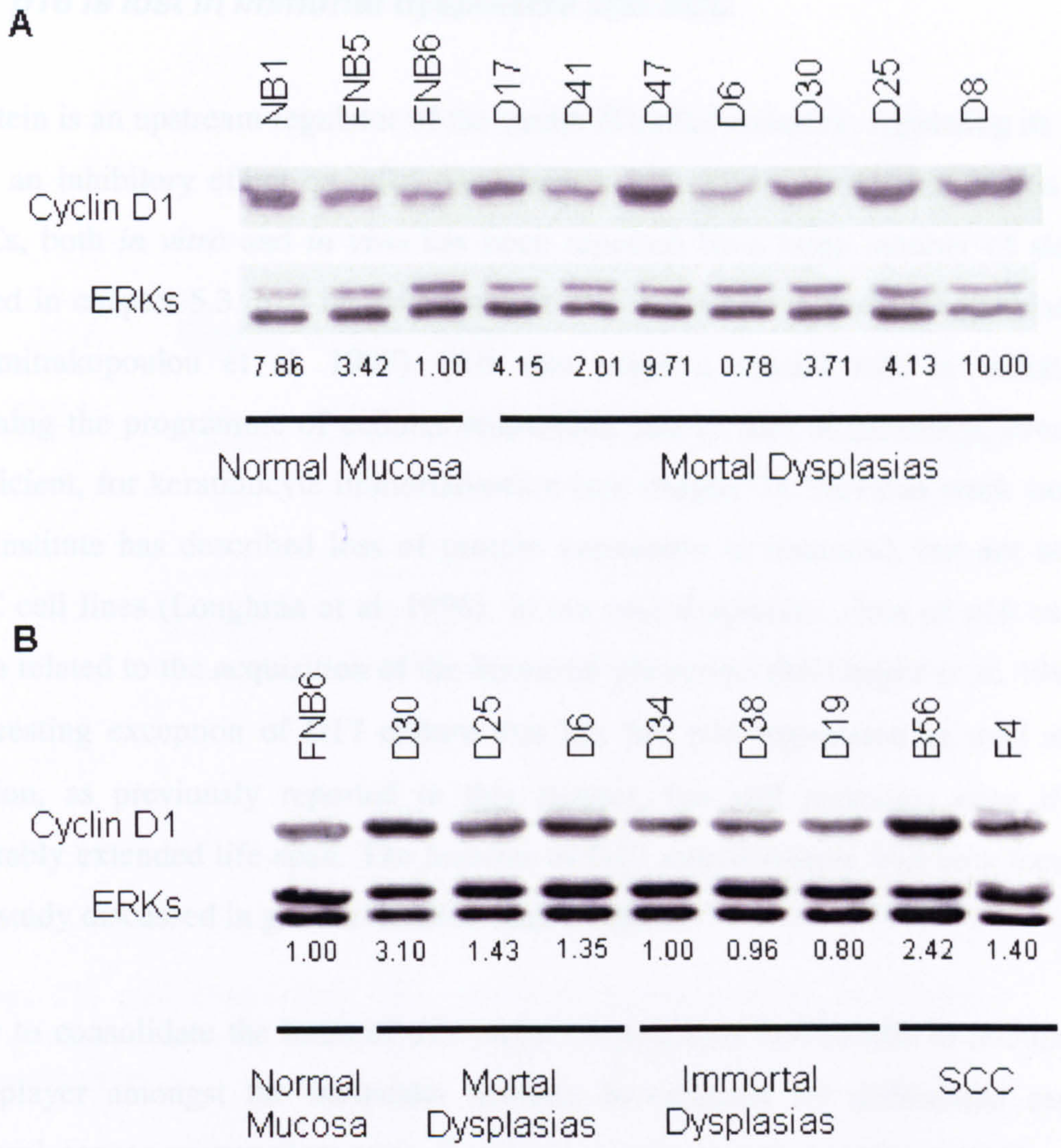


Fig. 22: Cyclin D1 expression in a panel of cultures from normal oral mucosa, mortal dysplasias, immortal dysplasias and squamous cell carcinomas.

Western blots comparing cyclin D1 expression in all of our normal samples and mortal dysplasias (**A**) and in a group of cultures from normal oral mucosa, normal mucosa adjacent to tumour (NB1), mortal dysplasias, immortal dysplasias and squamous cell carcinomas (**B**). Names of individual cultures are shown on the upper part of each figure. ERK 1,2 were used as a loading control. The quantification of the cyclin D1 bands normalised for the loading control is shown in the bottom part of each figure.

As shown in the figures, protein expression does not show major changes in the panel of samples analysed.

Data presented is representative of a single experiment (A) and 2 independent experiments (B).

10.3.3 p16 is lost in immortal dysplasias and SCC

p16 protein is an upstream regulator of the cyclin D1/cdk4 complex, regulating its function through an inhibitory effect on cdk4 (see chapter 4.3). Loss or mutation of this gene in HNSCCs, both *in vitro* and *in vivo* has been reported by a large number of studies, as described in chapter 5.3. p16 inactivation was also described in oral leukoplakias *in vivo* (Papadimitrakopoulou et al. 1997). p16 also plays a crucial role in initiating and maintaining the programme of cellular senescence and its loss is necessary, even though not sufficient, for keratinocyte immortalisation (see chapter 7). Previous work carried out in our Institute has described loss of protein expression in immortal, but not senescent, HNSCC cell lines (Loughran et al. 1996). In our oral dysplasias , loss of p16 expression was also related to the acquisition of the immortal phenotype (McGregor et al. 1997), with the interesting exception of D17 culture that has lost p16 expression as well as RARβ expression, as previously reported in this chapter, but still senesces, even if after a considerably extended life span. The features of D17 mortal culture will be a focus of the present study discussed in greater detail in later chapters.

In order to consolidate the basis of this project it appeared worthwhile to include such a crucial player amongst the molecular markers investigated for differential expression during oral cancer progression, even if only as a reiteration of previous findings in our group. p16 protein expression was analysed through Western Blotting in a small representative group of our samples. Cellular extracts were obtained during the exponential phase of growth of each culture, to avoid changes in protein expression due to cells approaching the senescence status.

In agreement with what previously described, p16 was present in normal samples and mortal dysplasias but undetectable in immortal dysplasias and SCCs (fig. 23). This corroborates the extensively described and important role played by p16 in cellular immortalisation.

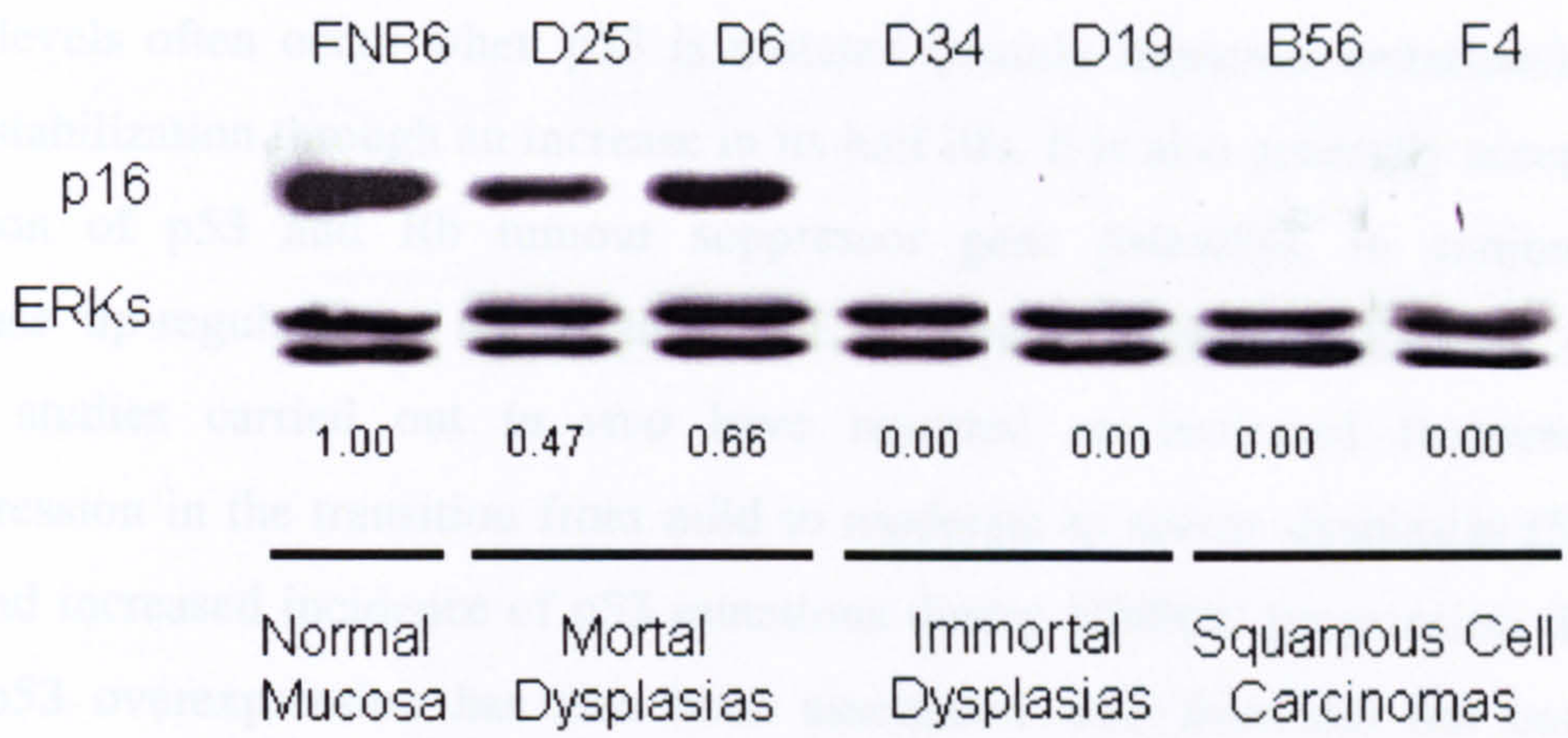


Fig. 23: p16 expression in a panel of cultures from normal oral mucosa, mortal dysplasias, immortal dysplasias and squamous cell carcinomas.

Western Blot showing p16 protein expression in a group of cultures from normal oral mucosa, mortal dysplasias, immortal dysplasias and squamous cell carcinomas. The samples were obtained from each culture during the exponential phase of growth. Names of individual cultures are shown on the upper part of the figure. ERK 1,2 was also measured to ensure equal loading of samples. The quantification of the p16 bands normalised for the loading control is shown in the bottom part of the figure.
The figure shows p16 protein expression in samples from normal mucosa and mortal dysplasias, but not in immortal dysplasias and carcinomas.
Data presented is from a single experiment, but confirms previous findings in the group.

10.3.4 p53 is overexpressed in immortal dysplasias and SCC

p53 mediates cell cycle arrest or apoptosis in response to a variety of cellular stresses (DNA damage, hypoxia, viral and cellular oncogenes) (see chapter 4.4). Elevated p53 protein levels often occur when p53 is mutated (mainly missense mutations) that cause protein stabilization through an increase in its half life. It is also generally accepted that an abrogation of p53 and Rb tumour suppressor gene pathways, in conjunction with telomerase up-regulation, are required for keratinocyte immortality (see chapter 7). Several studies carried out *in vivo* have reported an increased frequency of p53 overexpression in the transition from mild to moderate to severe dysplasias (Sauter et al. 1994) and increased incidence of p53 mutations during HNSCC progression (Boyle et al. 1993). p53 overexpression has also been associated with immortal but not senescent phenotype in HNSCC cell lines (Edington et al. 1995).

p53 protein expression was analysed through Western Blotting in normal samples, premalignant lesions and SCCs. Consistently low levels of protein were found in all of the normal samples (2.7 on average) and mortal dysplasias (3.3 on average), with the exception of D17 (9.45) (fig. 24 A). As reported earlier in this chapter, this mortal dysplasia senesces after a considerably extended life span and, also in contrast to all other mortal dysplasias, has lost expression of RARβ and p16. p53 protein expression was also considerably elevated in immortal dysplasias (15.6 on average) and SCCs (16.7 on average) (fig. 24 B).

Thus p53 protein analysis suggested a strict correlation between p53 over-expression and immortal phenotype as early as the premalignant stage of oral cancer progression. What remained to be tested was the actual correspondence between protein over-expression and gene mutation. Genomic DNA was obtained from all of our mortal and immortal dysplasias and the entire p53 coding region (exons 2-11) was sequenced (table 2). In all of the mortal dysplasias, comprising D17, the wild type status of p53 was confirmed. The immortal dysplasias harboured point mutations, affecting the DNA binding domain of the protein or, in one example, the tetramerization domain. These mutations were heterozygous in 3 of the cultures and homozygous in the remaining 3. Mutations were defined as homozygous when no expression of the normal allele could be detected upon sequencing.

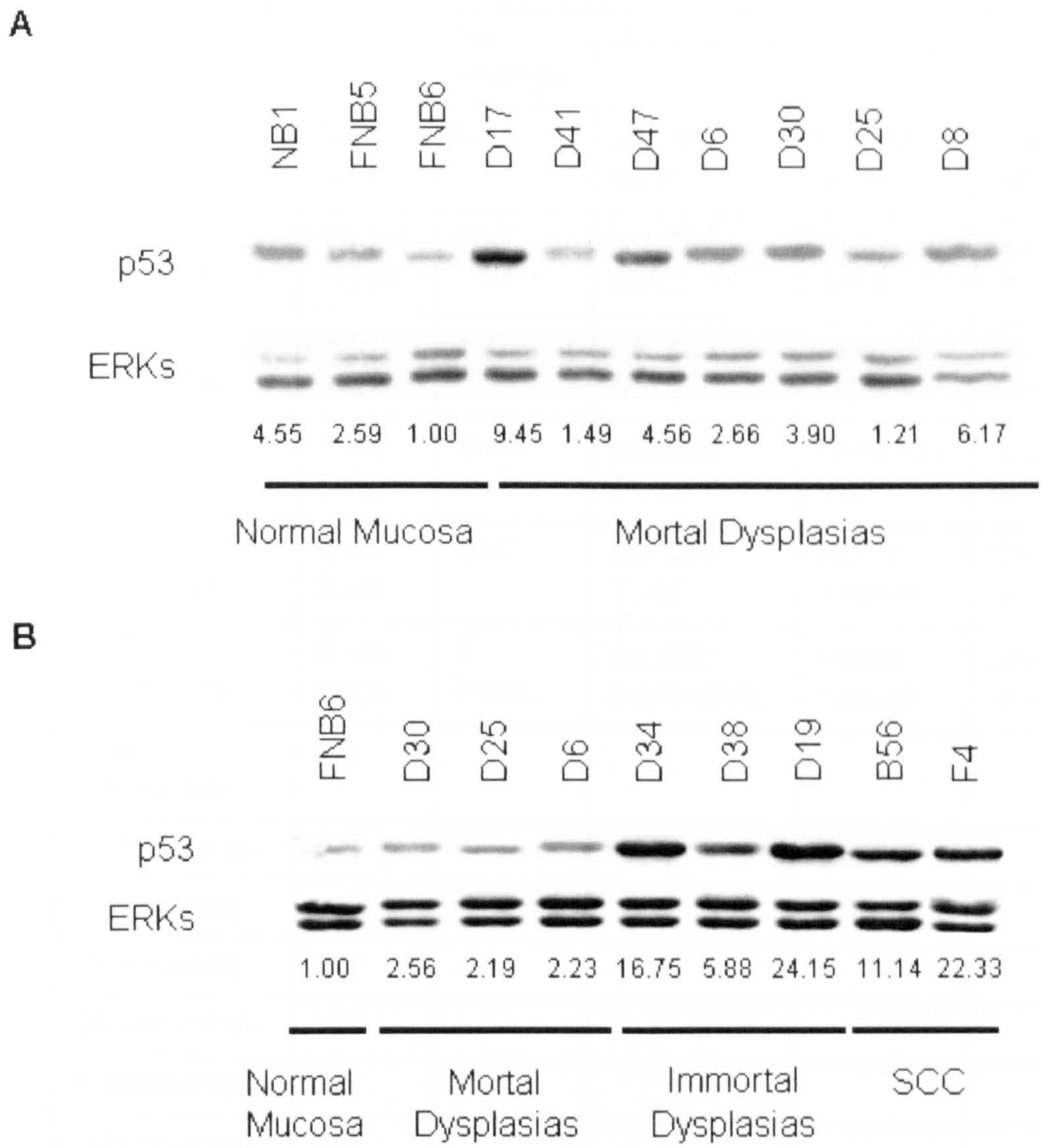


Fig. 24: p53 expression in a panel of cultures from normal oral mucosa, mortal dysplasias, immortal dysplasias and squamous cell carcinomas.

Western blots illustrating the expression of p53 protein in normal oral mucosa, mortal dysplasias, immortal dysplasias and squamous cell carcinomas. ERK 1,2 were used as a loading control. **A)** p53 expression levels are fairly homogeneous in all normal samples and mortal dysplasias analysed, with the exception of D17 which shows high levels of p53 expression. As described in the text, this culture, although still mortal, shows a considerably extended life span compared to all other mortal cultures. **B)** p53 overexpression is evident in all of the immortal dysplasias and carcinomas analysed, as a synonimous of protein stabilization due to gene mutation.
Names of individual cultures are shown on the upper part of each figure. Data presented is representative of 4 and 2 independent experiments (A and B respectively).

Dysplasia (prol. fate)	Exon	Nt change	AA change	Zigosity	Domain
D4 (immortal)	5 (nt 489)	C>A	aa 163 stop	Heterozy gous	DNA binding
D9 (immortal)	10 (nt 1009)	C>T	aa 337 R>C	Heterozy gous	tetramer ization
D19 (immortal)	5 (nt 488)	A>G	aa 163 Y>C	Homo zygous	DNA binding
D20 (immortal)	7 (nt 743)	G>A	aa 248 R>Q	Heterozy gous	DNA binding
D34 (immortal)	4 (nt 374)	C>T	aa 125 T>M	Homo zygous	DNA binding
D35 (immortal)	6 (nt 600)	T insert.	aa 200 frameshift	Homo zygous	DNA binding
D38 (immortal)	WT				
D17 (mortal)	WT				
D6 (mortal)	WT				
D8 (mortal)	WT				
D25 (mortal)	WT				
D30 (mortal)	WT				
D41 (mortal)	WT				
D47 (mortal)	WT				
D48 (mortal)	WT				

Table 2: p53 sequencing in cultures from mortal dysplasias and immortal dysplasias.

The entire coding region of p53 (exons 2-11) was sequenced in all our dysplasias, in collaboration with Janis Fleming. This table illustrates the exon and nucleotide number where the mutation was found, the consequent nucleotide and aminoacid change, the zigosity status and the protein domain affected by the mutation. WT indicates the wild type status of the p53 gene.

Nevertheless, this type of analysis does not allow to discriminate between a trace generated by two identical alleles (in which case the mutation would be truly homozygous) and a trace generated by a single allele, the other one being deleted (in which case it would be more appropriate to define the mutation as hemizygous). The only exception to the general trend of all immortal cultures harbouring mutations in p53 gene was the immortal culture D38, which retained wild type p53. The significance of this finding will be discussed later in the context of other data in the general discussion chapter of this work.

This findings are in concordance with the generally accepted crucial role played by the p53 tumour suppressor protein in acquisition of cellular immortality. Mutations occurring in our immortal SCC cell lines have already been reported and in the majority of cases they were homozygous (Edington et al. 1995). This work has extended the observation to a wide spectrum of oral premalignant lesions, where p53 overexpression and gene mutation are related to the transition from mortal to immortal phenotype. An apparent discordance seems to exist between levels of p53 protein expression and wild type status of p53 gene in D17 culture. One possible explanation for this finding is that the loss of p16 expression observed in D17 allows cells to bypass the initial growth arrest checkpoint regulated by p16. They undergo further telomere shortening, up-regulate p53 and indeed activate it through phosphorylation at serine residue 15 (Webley et al. 2000), this event finally leading to growth arrest. Alternatively, the over-expressed p53 might be transcriptionally inactive because of a concomitant increase in the levels of mdm-2, consistent with an auto-regulating feedback loop (Blaydes et al. 1997). Increased levels of p53 have also been reported in post-selection human mammary epithelial cells (HMECs), that have spontaneously lost expression of p16 (Romanov et al. 2001).

10.4 Telomerase

A vast body of literature has been produced over the past years on the role of telomerase in cellular immortalisation and cancer progression (see chapter 6.2). In the specific field of oral carcinogenesis, telomerase activity has been reported in a large percentage of SCCs and in a smaller percentage of oral leukoplakias *in vivo* (Miyoshi et al. 1999) (Mutirangura et al. 1996).

In view of this evidence in the literature, telomerase activity and levels of the two components of telomerase hTERT and hTERC were analysed in cultures from normal oral mucosa, oral premalignant and malignant lesions.

10.4.1 Telomerase activity is high in immortal dysplasias and SCC

The levels of telomerase activity were investigated using the semiquantitative method TRAPeze assay. This assay is a two step system utilising the polymerase chain reaction (PCR). In the first step of the reaction, telomerase adds a number of telomeric repeats (GGTTAG) onto the 3' end of a substrate oligonucleotide (TS) provided with the assay kit. In the second step, the extended products are amplified by PCR using the TS and RP (reverse) primers, generating a ladder of products with 6 base pair increments. The TRAPeze telomerase detection kit also includes an internal standard, a 36bp band (S-IC), in every lane. The internal control band appears weaker in those samples with high telomerase activity because amplification of the TRAP products and the S-IC band are semi-competitive. Being based on PCR amplification of substrates, this assay can be saturated by the amounts of telomerase in the samples, rendering a precise quantitation of the enzyme activity very difficult. For this reason, different amounts of protein extracts from our samples were tested in separate experiments. WI38 and Hela cells were included as negative and positive control, as described in literature (Kim et al. 1994). When 200ng of protein extract were used, telomerase activity was found to be undetectable or barely detectable in normal oral mucosa and mortal dysplasias and readily detectable in immortal dysplasias and SCCs (fig. 25). A second experiment using 100ng of whole cell extract from the same samples (fig. 26) revealed that the telomerase activities of immortal dysplasias and SCCs varied considerably, some of them showing higher levels than HeLa (D19, D20, D35, D38).

This finding suggests a close correlation between telomerase activity and cellular immortalisation at the dysplasia stage of oral cancer progression. The percentage of our oral dysplasia cultures that are telomerase positive (50%) is similar to the figure previously reported *in vivo* (Miyoshi et al. 1999) (Mutirangura et al. 1996) (Sumida et al. 1999).

To gain further insight into the mechanisms of telomerase activation, the two components of telomerase, hTERC (RNA template) and hTERT (reverse transcriptase) were subsequently analysed.

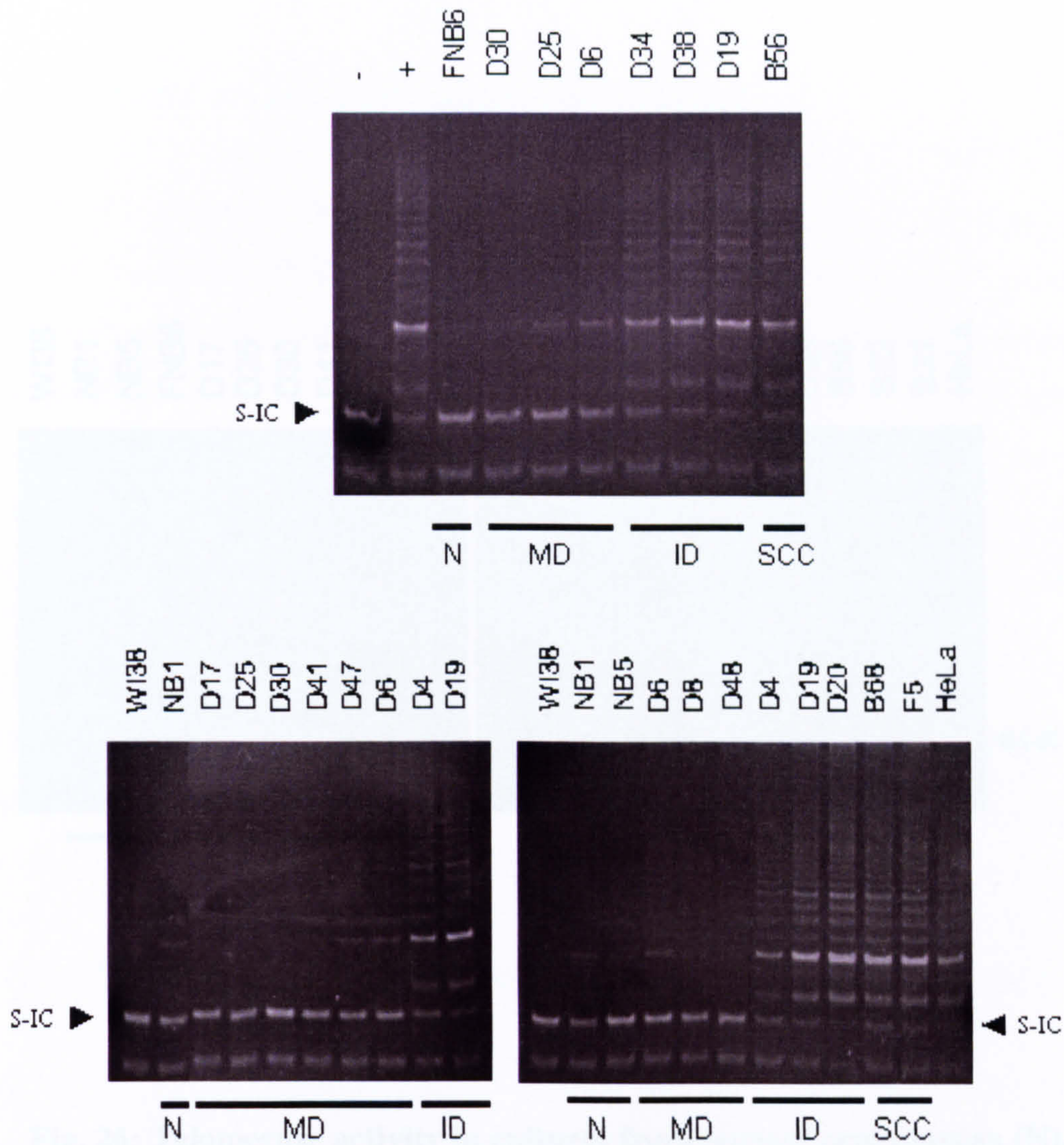


Fig. 25: Telomerase activity in cultures from normal oral mucosa (N), mortal dysplasias (MD), immortal dysplasias (ID) and squamous cell carcinomas (SCC).

TRAP assay illustrating telomerase activity in a large group of cultures from normal oral mucosa, mortal dysplasias, immortal dysplasias and squamous cell carcinomas. The gels show PCR products generated from 200ng of protein extracts after staining with ethidium bromide. The internal control (S-IC) is visible as a 36bp band and its intensity is inversely correlated to the telomerase activity in the sample. In the upper panel, protein extract from a positive control provided in the assay kit before and after heat inactivation were used respectively as positive and negative controls. In the lower panels, protein extracts from HeLa and WI38 were used as positive and negative controls respectively. Names of individual cultures are shown on the upper part of each figure.
Telomerase activity was consistently found barely detectable or undetectable in normal samples and mortal dysplasias and readily detectable in immortal dysplasias and carcinomas.

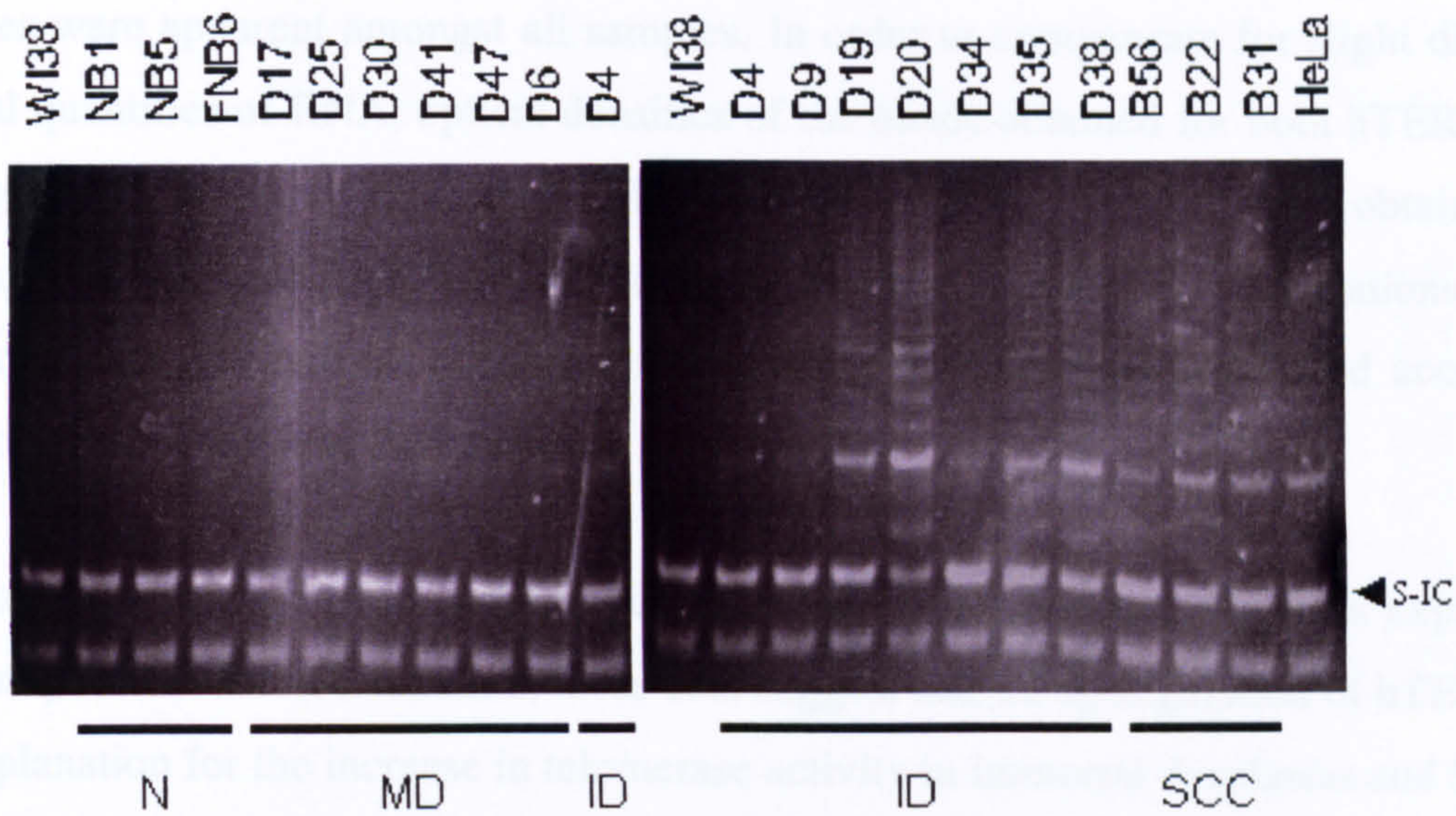


Fig. 26: Telomerase activity in cultures from normal oral mucosa (N), mortal dysplasias (MD), immortal dysplasias (ID) and squamous cell carcinomas (SCC) using 100 ng. of protein extract.

In order to avoid overlooking differences amongst samples that showed high levels of telomerase activity (see previous experiment), lower amounts of protein extracts were analysed for all of the cultures. The gels show PCR products generated from 100ng of protein extracts after staining with ethidium bromide. HeLa and WI38 were used as positive and negative controls respectively. Names of individual cultures are shown on the upper part of each figure.
Telomerase activity levels were found to be undetectable in normal samples and mortal dysplasias. In immortal dysplasias and SCCs telomerase activities were considerably variable, some of them being barely detectable with this amount of extract and others showing higher levels than HeLa.

10.4.2 No major changes are observed in hTERC

The levels of telomerase RNA component were analysed by Northern Blotting in cultures of biopsies from all different stages of oral cancer progression (fig. 27 A). No major changes were apparent amongst all samples. In order to compensate for slight differences in total quantities of RNA, optical densities of the bands obtained for both hTERC and 7S loading control were calculated. The relative hTERC RNA levels obtained after normalization against 7S RNA levels (fig. 27 B) revealed only minor variations amongst individual samples and no specific changes when samples where grouped according to stage of progression (fig. 27 C).

Although hTERC is a crucial component of telomerase holoenzyme and its expression is necessary for telomerase function, these data suggest that an up-regulation of hTERC is not the explanation for the increase in telomerase activity in immortal dysplasias and SCCs.

10.4.3 hTERT is highly upregulated in immortal dysplasias and SCC

hTERT mRNA expression was initially investigated by semiquantitative RT-PCR since our attempts to measure hTERT through northern blotting were repeatedly unsuccessful, due to its extremely low levels even in SCCs. At the point in the project when this work was performed, specific hTERT antibodies were still unavailable: although hTERT protein expression had been reported in one study *in situ* (Kolquist et al. 1998), there was not enough evidence on the reproducibility of this finding.

Several splicing or insertion variants of hTERT mRNA have been described (Kilian et al. 1997) some of which act as dominant negatives (Colgin et al. 2000). In the light of these reports, the primer set chosen for RT-PCR amplification of hTERT mRNA does not encompass any regions where splicing or insertion variants have been described. The levels of hTERT in normal oral mucosa and mortal dysplasias were undetectable or barely detectable (fig. 28). A low level of expression could be detected in the mortal dysplasia D6, which was also the mortal dysplasia that showed a barely detectable telomerase activity (fig. 28 and 25). In all of the immortal dysplasias and SCCs analysed, hTERT mRNA levels of expression were considerably higher than those found in mortal dysplasias and normal oral mucosa (fig. 28).

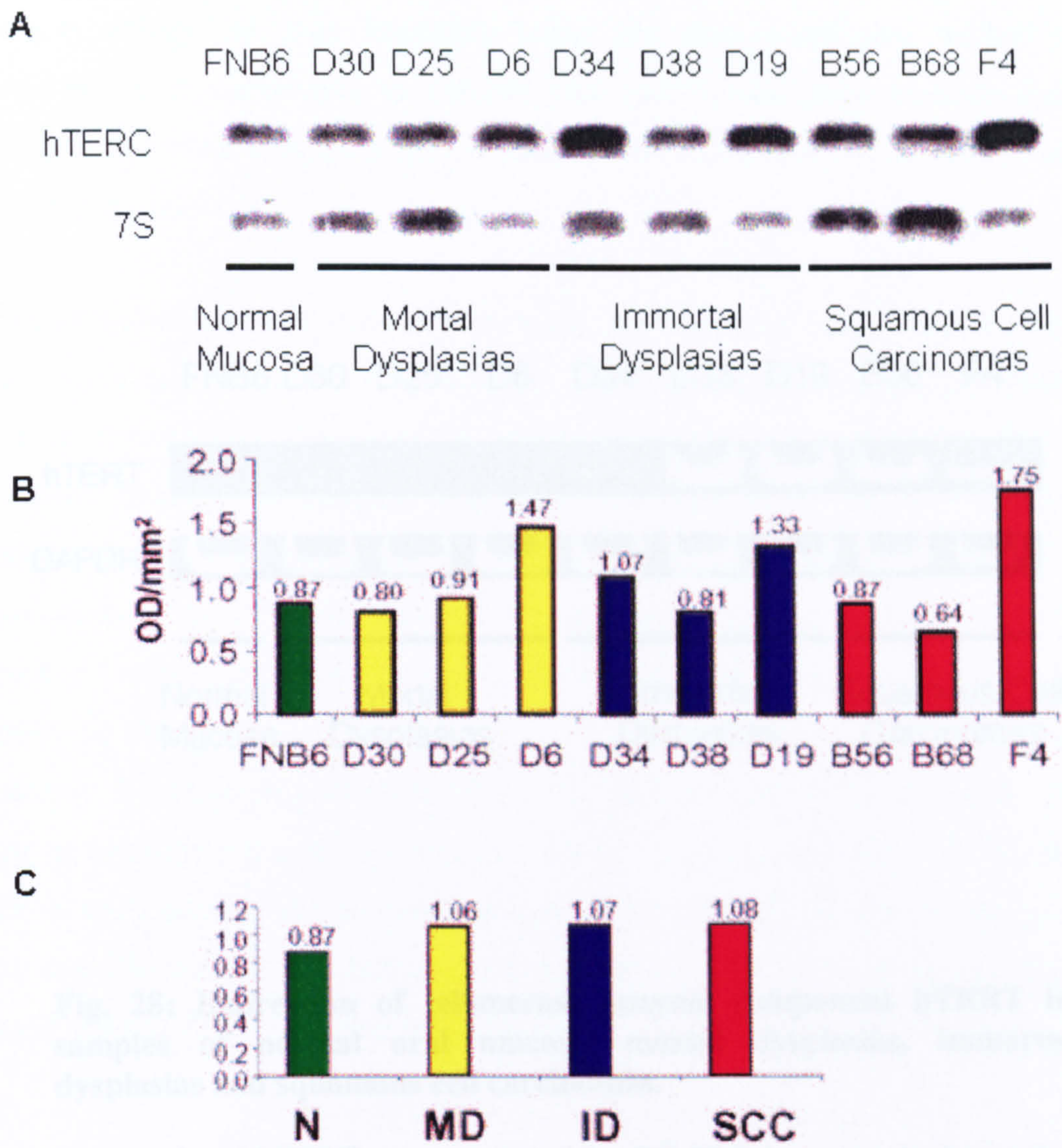


Fig. 27: Levels of telomerase RNA component hTERC in cultures of normal oral mucosa, mortal dysplasias, immortal dysplasias and squamous cell carcinomas.

A) Northern Blot analysing levels of hTERC in a panel of samples from normal oral mucosa, mortal dysplasias, immortal dysplasias and squamous cell carcinomas. Ribosomal subunit 7S RNA was also measured to ensure equal loading of samples. Names of individual cultures are shown on the upper part of each figure. Data presented is representative of 2 independent experiments.
B) and **C**) Graphical comparison of the levels of hTERC as assessed by Northern Blot after measurement of the optical densities of the bands and normalization against the 7S loading control. Data is presented for individual samples in B) and for groups in C).
No significant variation in hTERC levels was found amongst all samples even after normalization.

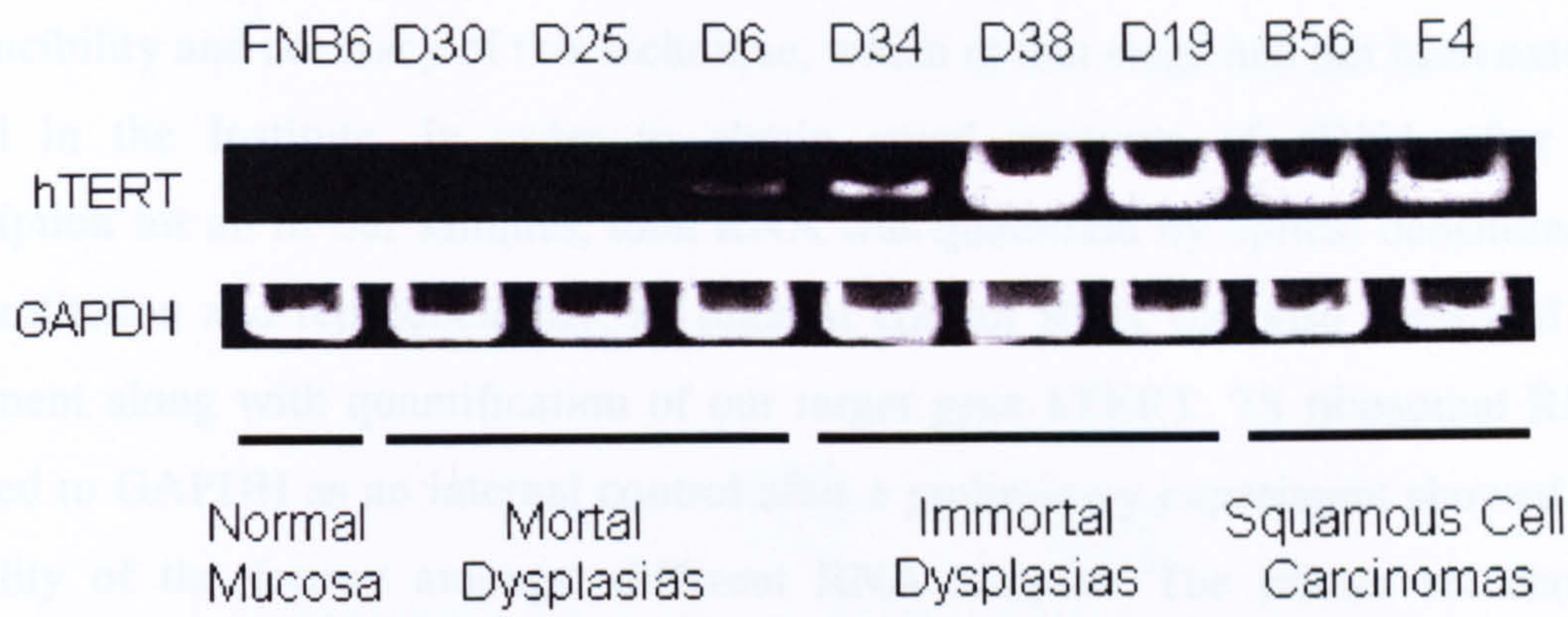


Fig. 28: Expression of telomerase enzyme component hTERT in samples of normal oral mucosa, mortal dysplasias, immortal dysplasias and squamous cell carcinomas.

The levels of hTERT were analysed by RT-PCR in a panel of samples from normal oral, mortal dysplasias, immortal dysplasias and squamous cell carcinomas. The gels show the PCR products generated after amplification with MS113 and MS114 primers (see Materials and Methods) and stained with ethidium bromide. GAPDH mRNA levels were also measured as an internal control.
Names of individual cultures are shown on the upper part of each figure. Data presented is representative of 5 independent experiments

This suggested a close correlation between levels of hTERT mRNA expression and telomerase activity, its only limitation being the semi-quantitative method used. It therefore appeared worthwhile to confirm this data by the more accurate Real Time Quantitative RT-PCR technique. An illustration of the principles of this technique is presented in fig. 29.

A series of preliminary experiments were performed to optimise the methods and ensure reproducibility and accuracy of this technique, which at that stage had not been extensively utilised in the Institute. In order to obtain equal amounts of cDNA after reverse transcription for all of our samples, total RNA was quantified by optical densitometry. To aid quantitation and reproducibility, an internal control RNA was also measured in each experiment along with quantification of our target gene hTERT. 7S ribosomal RNA was preferred to GAPDH as an internal control after a preliminary experiment showed a lower variability of the former amongst different RNA samples. The primer set chosen for Quantitative RT-PCR amplification of hTERT mRNA anneals to a region where no splicing or insertion variants have been described. After quantification of 7S rRNA and hTERT in each sample, the relative amounts of hTERT/rRNA were finally calculated. Typical amplification curves obtained for a standard curve, a mortal dysplasia, an immortal dysplasia and a SCC are shown in fig. 30 A and B.

The data presented in fig. 31 shows the average of the results obtained for the entire panel of dysplasias in three separate experiments performed as described above. In keeping with the findings reported for semi-quantitative RT-PCR, hTERT is highly upregulated in immortal dysplasias and SCCs compared to mortal dysplasias and normal oral mucosa. Despite a substantial heterogeneity amongst immortal dysplasias, levels of hTERT in the lowest expressing immortal dysplasia (D34) are still 10 fold higher than those of the mortal dysplasia expressing the highest levels of hTERT mRNA (D6). One important observation emerging from these results is that the cultures with the highest telomerase activity are the ones that express the highest levels of hTERT mRNA (see fig. 26 and 28). This suggests that hTERT overexpression and therefore telomerase activity upregulation occur at the dysplasia stage of oral cancer progression and is related to the acquisition of the immortal phenotype.

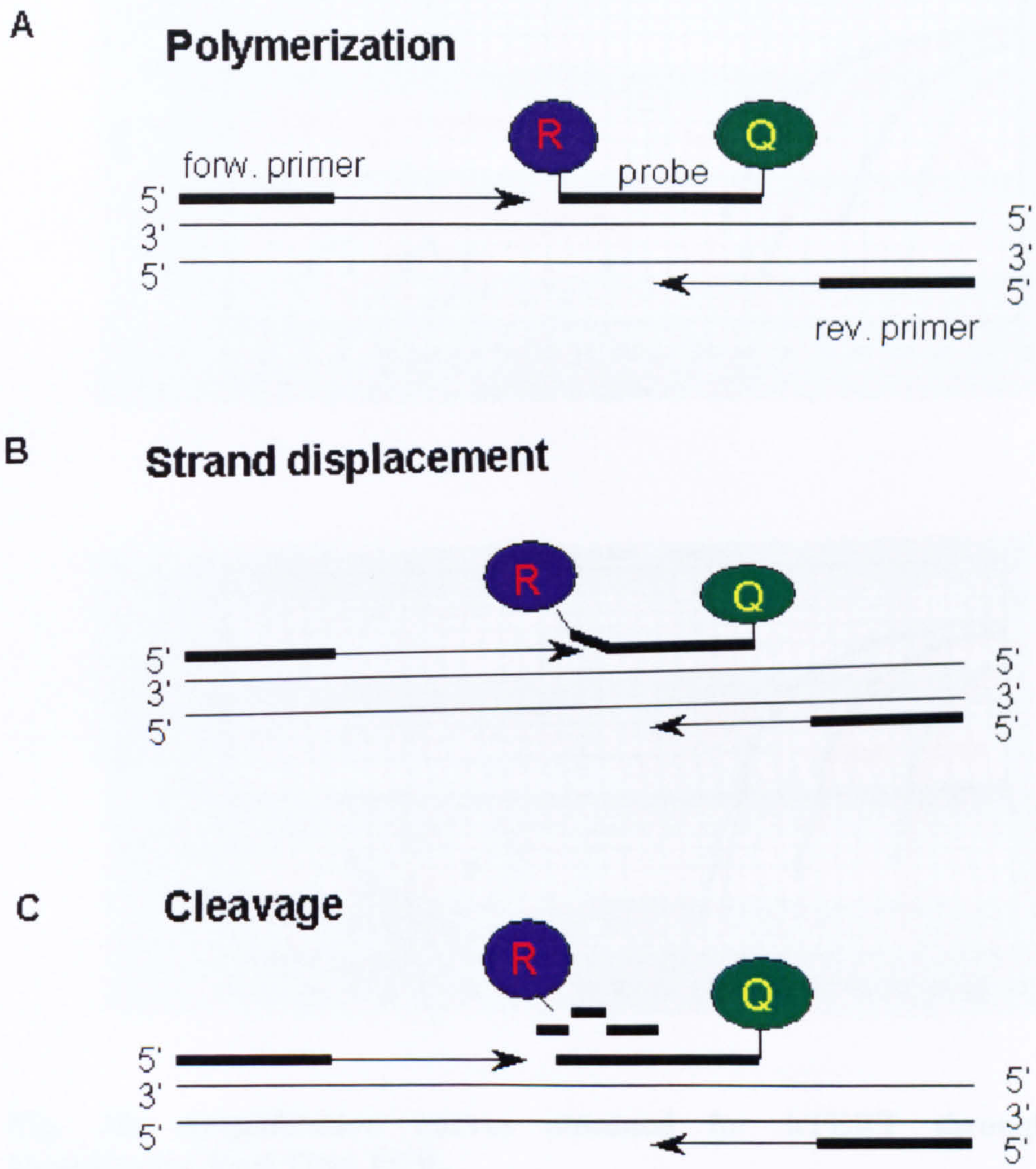
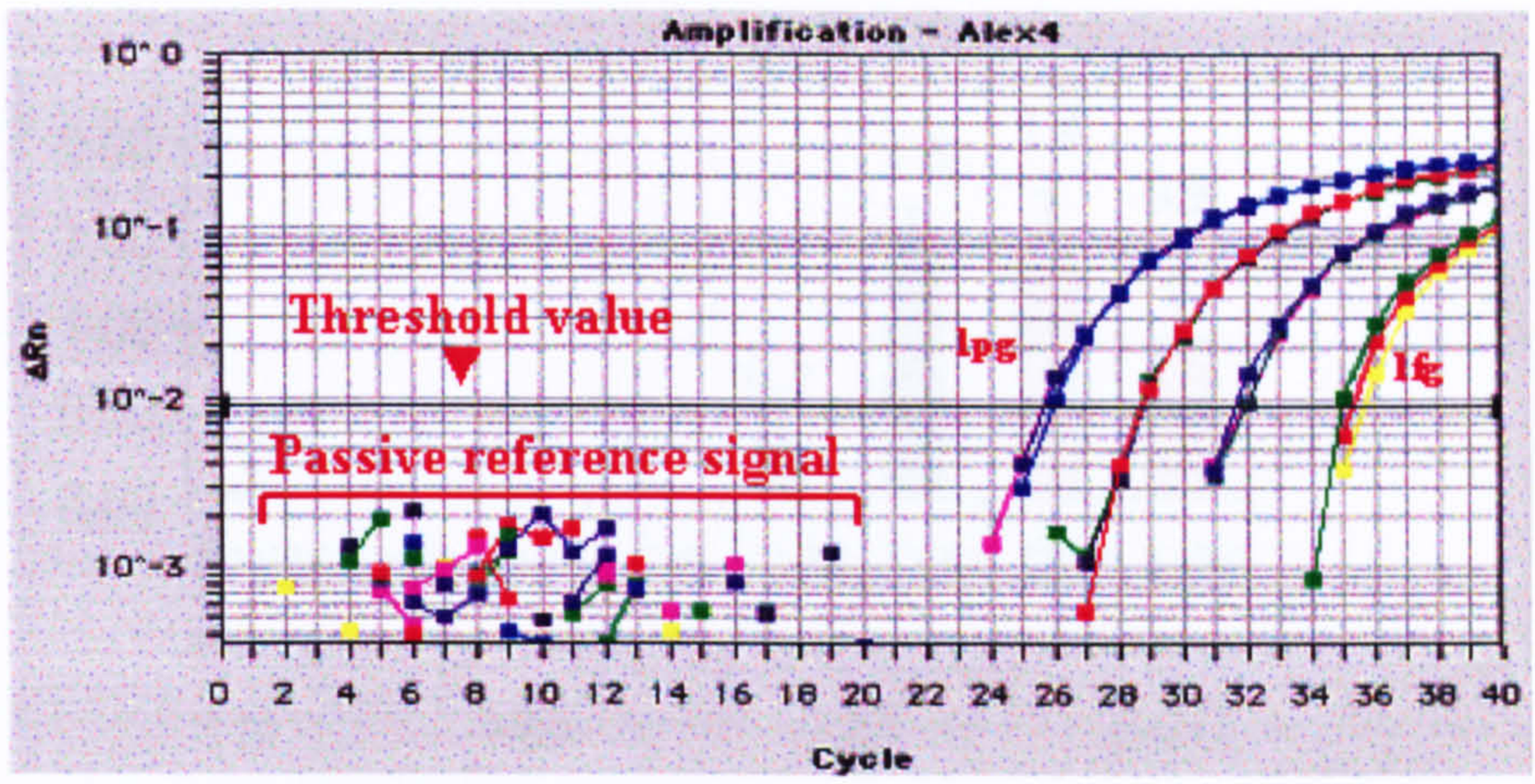


Fig. 29: Quantitative Real Time PCR.

In the Real time quantitative PCR a set of primers and an oligonucleotidic probe are designed to anneal with the target gene. The taqman probe is covalently linked with a fluorescent dye at its 5' end and with a quencher dye at its 3' end. The quencher dye suppresses the fluorescence of the reporter until cleaved by the amplitaq gold enzyme, which has a 5'-3' nuclease activity. Therefore, the fluorescence of the reporter dye progressively increases during the amplification reaction.
The instrument compares the intensity of the fluorescent signal at each cycle to a standard curve. The fewer cycles it takes to reach a detectable level of fluorescence, the greater the initial copy number of the target gene.

A



B

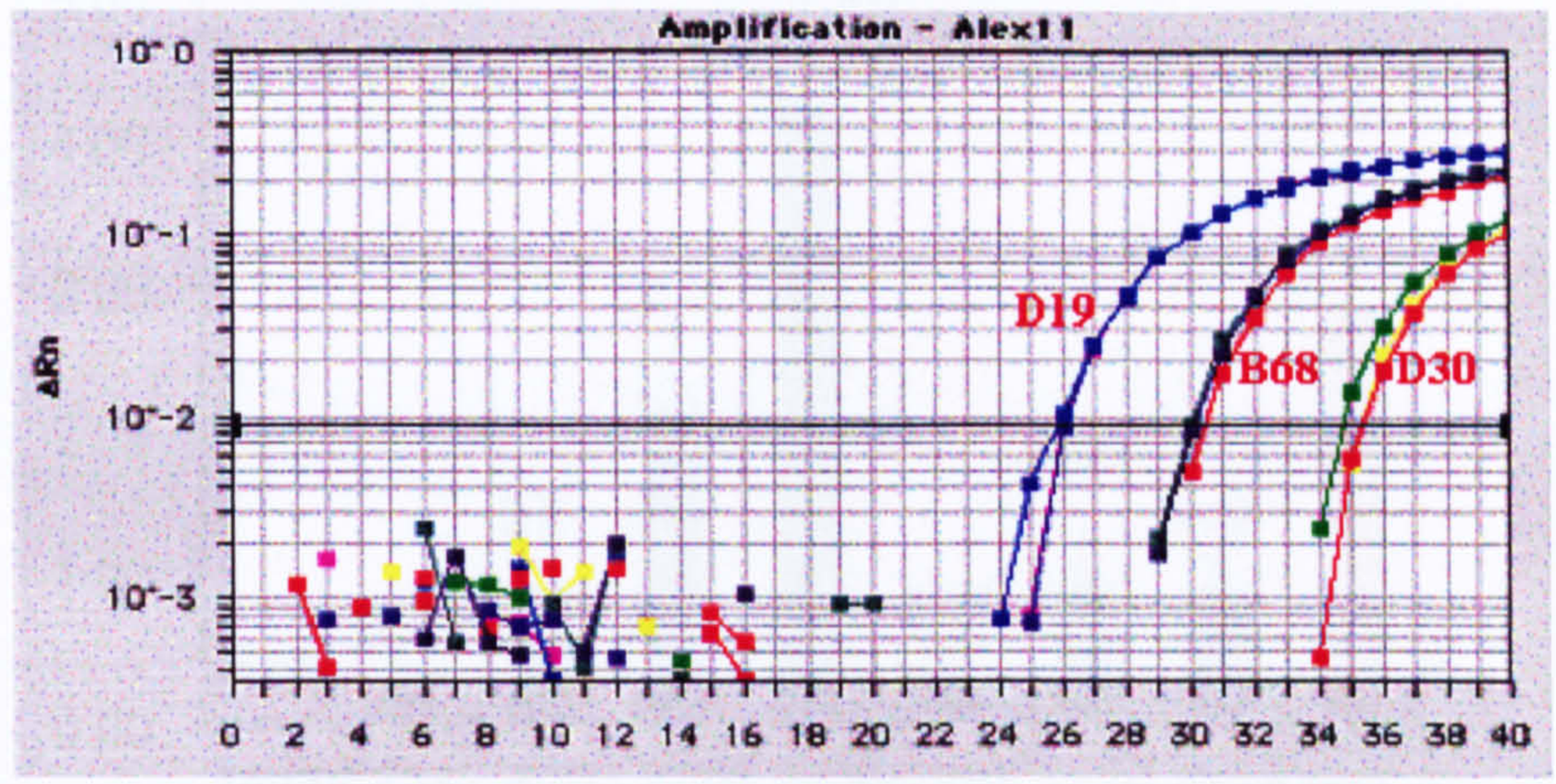


Fig. 30: Amplification curves obtained for hTERT through Quantitative Real Time PCR.

In most quantitative PCR detection systems the fluorescent signal obtained during the first 15 amplification cycles is considered as a passive reference signal. In successive amplification cycles, the instrument detects the increase in signal associated with exponential growth of PCR product and defines a threshold value. The fewer cycles it takes to reach the threshold value, the greater the initial copy number of the target gene.
A) Example of an amplification reaction carried out to obtain a standard curve. Four samples of known template amount (1pg, 100fg, 10fg, 1fg of hTERT insert) were amplified in triplicate, each generating overlapping curves. **B)** Example of an amplification reaction of three different samples containing an unknown quantity of hTERT, each analysed in triplicate. The three curves correspond to one immortal dysplasia (D19), one carcinoma (B68) and one mortal dysplasia (D30). The values obtained from this quantification are interpolated within those obtained for the corresponding standard curve.

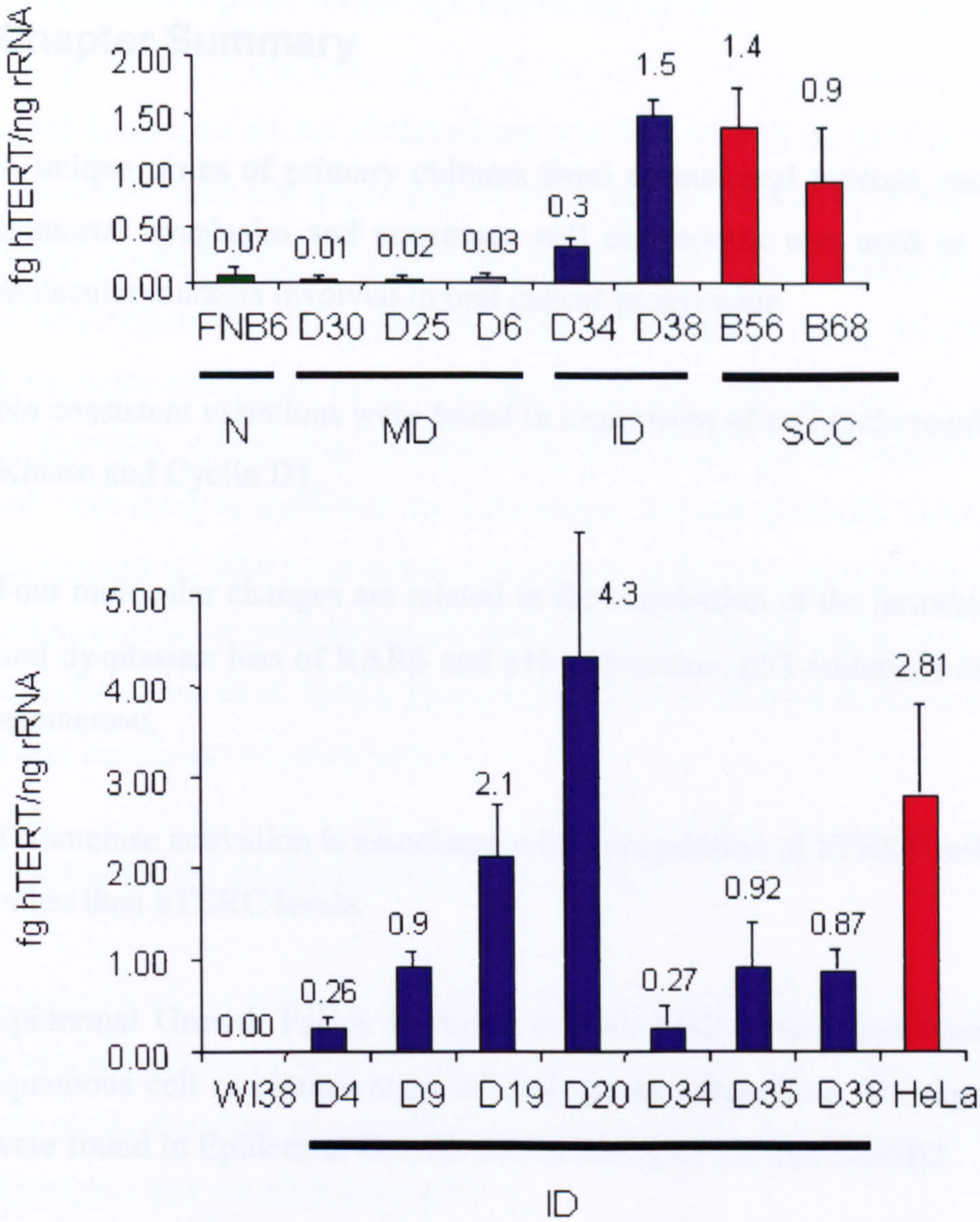


Fig. 31: Levels of hTERT in samples of normal oral mucosa (N), mortal dysplasias (MD), immortal dysplasias (ID) and squamous cell carcinomas (SCC) measured through Real Time quantitative PCR.

Summary illustrating the levels of hTERT mRNA in samples of normal oral mucosa, mortal dysplasias, immortal dysplasias and squamous cell carcinomas. The relative amounts of hTERT are expressed after normalization for 7S ribosomal RNA measured independently in each sample. Hela and WI38 were used as positive and negative controls respectively. Standard deviations are also shown.
Names of individual cultures are shown on the lower part of each figure. Data presented is the average of 3 independent experiments for each panel.

10.5 Chapter Summary

- A unique series of primary cultures from normal oral mucosa, mortal dysplasias, immortal dysplasias and squamous cell carcinomas was used to analyse several molecular markers involved in oral cancer progression.

- No consistent variations were found in expression of cell cycle regulators Polo Like Kinase and Cyclin D1.

- Four molecular changes are related to the acquisition of the immortal phenotype in oral dysplasias: loss of RARβ and p16 expression, p53 mutations and activation of telomerase.

- Telomerase activation is associated with upregulation of hTERT mRNA expression rather than hTERC levels.

- Epidermal Growth Factor Receptor subunit EGF-R overexpression occurs at the squamous cell carcinoma stage of oral cancer progression. No significant changes were found in Epidermal Growth Factor Receptor subunit c-erbB2.

11 Functional interventional studies of keratinocyte immortalisation

As described in chapter 10, our observational studies had thus far identified four molecular changes associated with the acquisition of the immortal phenotype in oral dysplasias: loss of RARβ and p16 expression, mutation of p53 and activation of telomerase. This scientific evidence was informative, but did not allow to determine what molecular events are required for immortalisation and what others are consequential to it. In addition, the literature provides contrasting evidence on this topic, some authors reporting that telomerase activation is sufficient to immortalise normal epithelial cells (Bodnar et al. 1998) (Ramirez et al. 2001) and others reporting that loss of p16 is required in addition to telomerase activation in order to immortalise epithelial cells (Kiyono et al. 1998) (Dickson et al. 2000) (Romanov et al. 2001). This section of our work was aimed at addressing these issues in oral neoplastic keratinocytes.

11.1 Features of our mortal dysplasias

The vast majority of our mortal dysplasias (7/8) proliferated for 5-30 population doublings and then reached a growth arrest status due to senescence (see chapter 9). They showed none of the molecular changes associated with immortality: they expressed RARβ and p16, had wild type p53 and no telomerase activity.

The remaining mortal culture, D17, showed cellular and molecular features that could be considered intermediate between the mortal and the immortal phenotype. It proliferated for a considerably extended life span (45 PD) but still eventually reached growth arrest due to senescence. In addition, it had lost expression of RARβ and p16, like immortal cultures, but had no telomerase activity and retained wild type p53, like mortal cultures. The loss of RARβ and p16 in D17 is not to be considered a cultural artefact, as loss of expression of these two molecular markers has been shown *in vivo* in the biopsy from which D17 was derived (K. Hunter, personal communication). D17 was therefore an example of a naturally occurring dysplasia that had acquired *per se* two of the four molecular features associated with the immortal phenotype but still remained mortal, suggesting that loss of p16/RARβ is not sufficient for keratinocyte immortalisation.

Our series of typical mortal cultures and our intermediate phenotype culture D17 provided a useful model to test whether telomerase activation was sufficient to confer cellular

immortality or it needed to occur in combination with other molecular changes, like p53 mutation, loss of expression of p16 and/or RARβ.

11.2 Ectopic expression of hTERT in two typical mortal cultures and in D17

To test this hypothesis, amphotrophic retroviral vectors transducing either the hTERT gene (pBabeEST) or the Neomycin resistance gene (pBabeNeo) were used to infect D17 along with the typical mortal dysplasia D30. In parallel, Janis Fleming in the group has performed the same experiments on another typical mortal dysplasia, D25.

The retroviral vectors were produced by infecting the packaging cell line Phoenix A with pBabeNeo (neomycin resistance gene) or pBabeEst (hTERT) constructs, selecting with G418 and titrating the virus production so that equal amounts of infectious units could be used to infect different cultures. A series of preliminary experiments were carried out to establish the minimum concentration of Neomycin (G418) that was able to kill the uninfected D30, D25 and D17 cultures. This concentration (200 μg/ml) was later used to select the infected cultures and to maintain retroviral expression. After infection, the G418-resistant colonies (typically 50-70) were pooled and used for all of the subsequent experiments to avoid variations due to clonal heterogeneity of the population. The number of G418-resistant colonies obtained after infection with the hTERT construct was similar to that obtained with the empty vector.

The typical mortal dysplasia culture D30 had an expected life span of 30 population doublings and was infected at 8.5, 9, 17 and 18 population doublings in four separate experiments. D25 had an expected life span of 25 population doublings and was infected at 7, 9 and 18 population doublings in three separate experiments. The atypical mortal dysplasia culture D17 had an expected life span of 45 population doublings and was infected after having reached 34.5 and 39 population doublings in two separate experiments. In each experiment, 8 plates of cells were seeded at a concentration of $5x10^5$ cells and treated as follows: two were infected with the hTERT vector, two were infected with the NEO vector, two were used as uninfected controls and two were left uninfected but treated with the same concentration of G418 used to select the infected plates, to confirm that no non-infected clones survived selection.

11.2.1 Assessing the functionality of hTERT vector

In order to assess whether infection with the hTERT vector resulted in activation of telomerase, protein extracts were obtained from each culture and checked for telomerase activity as soon as a sufficient number of cells were available. As shown in figures 32 and 33, no telomerase activity could be detected in D17 and D30 uninfected cultures and NEO infectants. Enzyme activity was readily detectable in the hTERT infectants, testifying for telomerase activation upon hTERT transduction.

A point that remained to be clarified was whether or not telomerase activation had any effect on telomere maintainance or elongation. To address this question, genomic DNA was extracted from the uninfected D17 and D30 cultures at different stages of their life span, comprising senescence, from the NEO infectants when they had reached growth arrest and from the EST infectants. TRF lengths were measured for all samples through Southern Blotting (fig. 34 and 35). In both cases, telomeres underwent progressive shortening with continuous passage in culture, and were significantly and progressively elongated in the hTERT infectants but not in the "mock" infectants.

It is evident from these results that infection with the hTERT vector but not with the "empty" vector is effective in maintaining/elongating the telomeres as well as activating telomerase.

11.2.2 Monitoring for proliferative fate of the cultures

The uninfected controls and infected cultures for D30, D25 and D17 were subsequently monitored for life span by successive passaging in culture plates.

With both D30 and D25 dysplasias, no difference in growth rate or proliferative fate was observed amongst the uninfected cultures, the cultures infected with the empty vector and those transduced with hTERT. The uninfected and infected cultures of both dysplasias showed a very slow proliferation rate from as early as discreet colonies were visible. They were maintained in culture for three weeks, during which time the colonies failed to reach confluence, suggesting that growth arrest status had been reached. Fig 36 shows a representative example of SABG positive staining in uninfected and hTERT transduced D30 colonies after cessation of proliferative growth. BRDU incorporation assay could not be carried out because of the limited amount of material available.

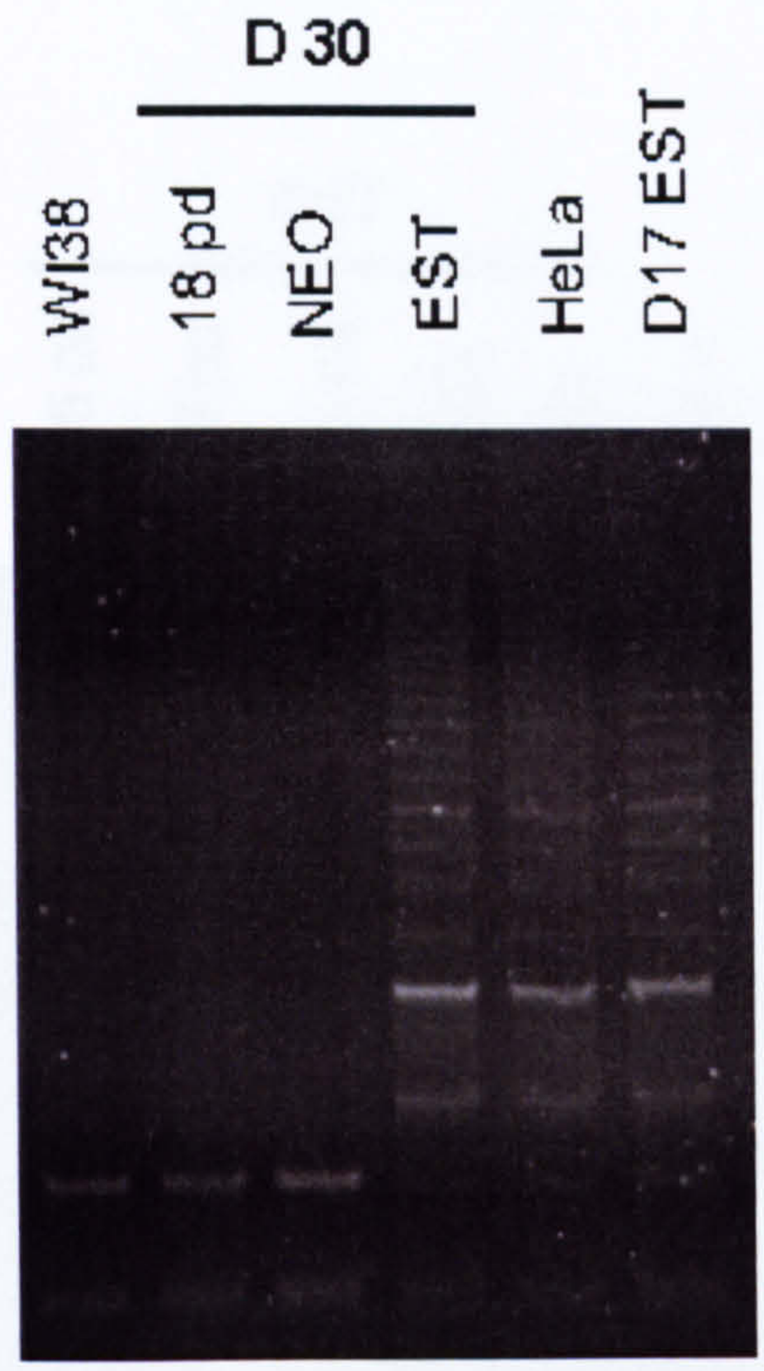


Fig. 32: Telomerase activity in D30 cultures before and after infection with hTERT expression vector.

TRAPeze assay was performed on 400ng of protein extract. The gels show the PCR products generated after staining with ethidium bromide. Protein extracts were obtained from the uninfected control at 18 PD, from the empty vector infectant, transduced with the neomicin resistance gene alone (NEO) at 24 PD and from the hTERT infectant (EST) at 24 PD. WI38 cells were included as negative control, HeLa cells and D17 EST infectants were included as positive controls.
Telomerase activity is readily detectable in the hTERT infectant and undetectable in the uninfected control and in the mock infectant.

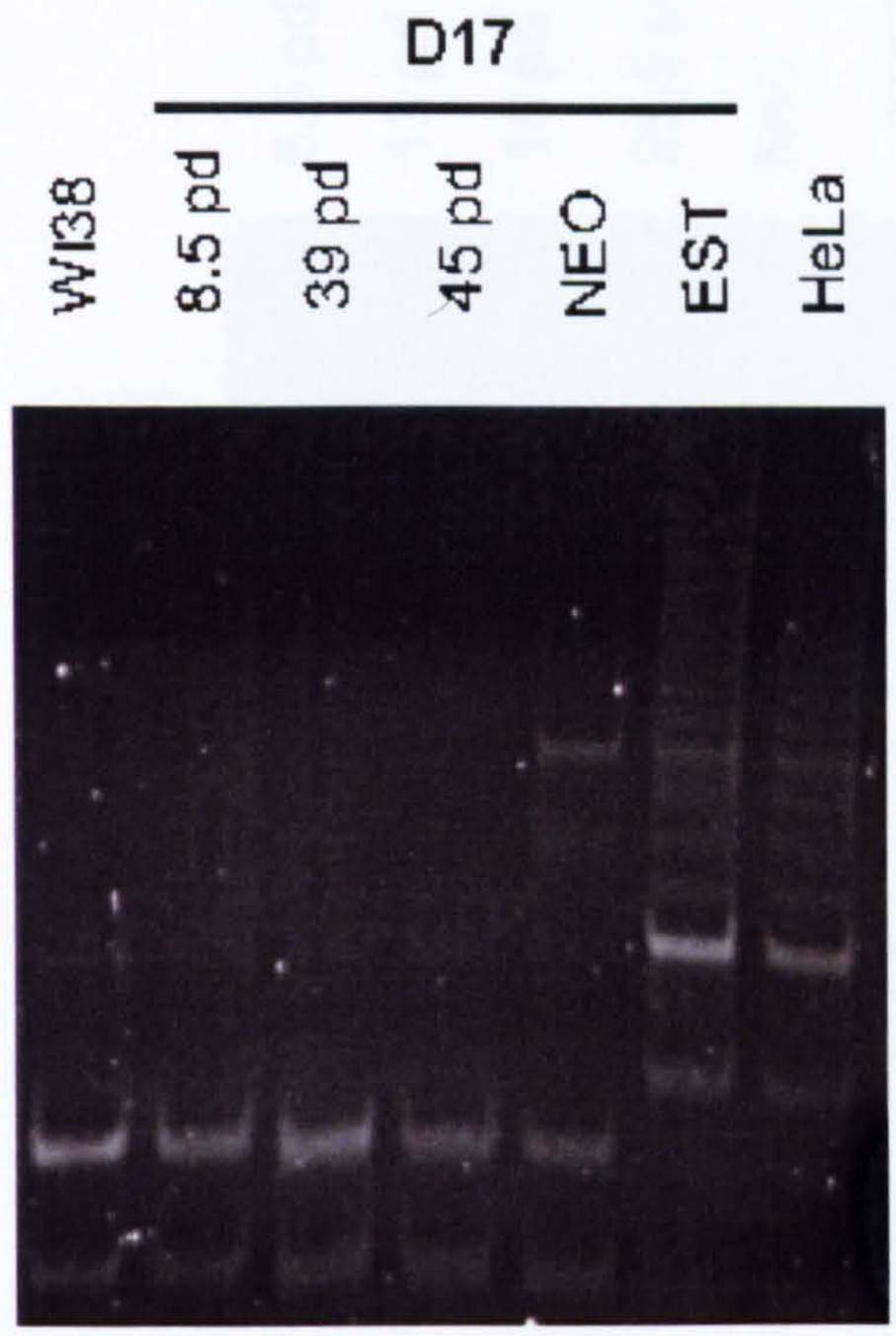


Fig. 33: Telomerase activity in D17 cultures before and after infection with hTERT expression vector.

TRAPeze assay was performed on 200ng of protein extract. The gels show the PCR products generated after staining with ethidium bromide. Protein extracts were obtained from the uninfected control at different stages of its life span (the number of population doublings is indicated for each sample), from the empty vector infectant transduced with the neomicin resistance gene alone (Neo) at 54 population doublings and from the hTERT infectant (EST) at 55 population doublings. WI38 and HeLa cells were included as telomerase negative and positive controls respectively.
Telomerase activity is readily detectable in the hTERT infectant and undetectable in all of the uninfected controls and in the mock infectant.

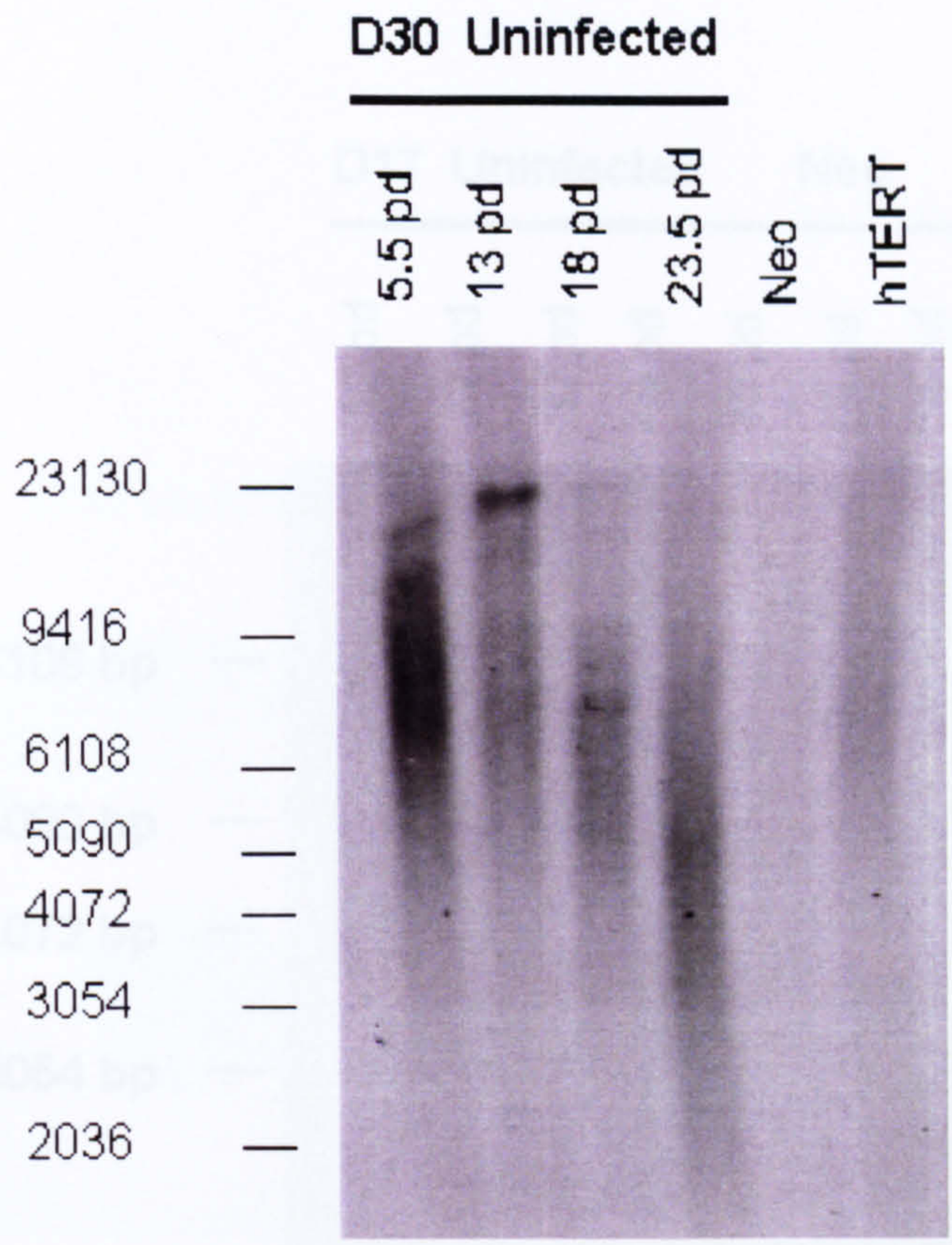


Fig. 34: Telomere length in D30 cultures before and after infection with hTERT expression vector.

Southern blot illustrating TRF length measurements in D30. Genomic DNA samples were obtained from D30 cultures at different stages of their life span (the number of population doublings is indicated for each sample), after infection with the empty vector (NEO) and with the hTERT transducing vector (EST). 1 Kb DNA fragments and λ/HindIII were used as reference molecular weight markers.
As shown in the figure, telomeres underwent a progressive shortening with increasing age in culture, but were considerably elongated after hTERT ectopic activation.
The ratio of telomeric TTAGGG repeats versus total nuclear DNA is lower in cells with short telomeres than in cells with long telomeres. Therefore, the measurement of telomere length in different samples using equal amounts of total nuclear DNA results in lower intensity of signals for cells with shorter telomeres. This, along with a limited sample availability, might explain the weaker signal observed in the senescing D30 NEO infectant.

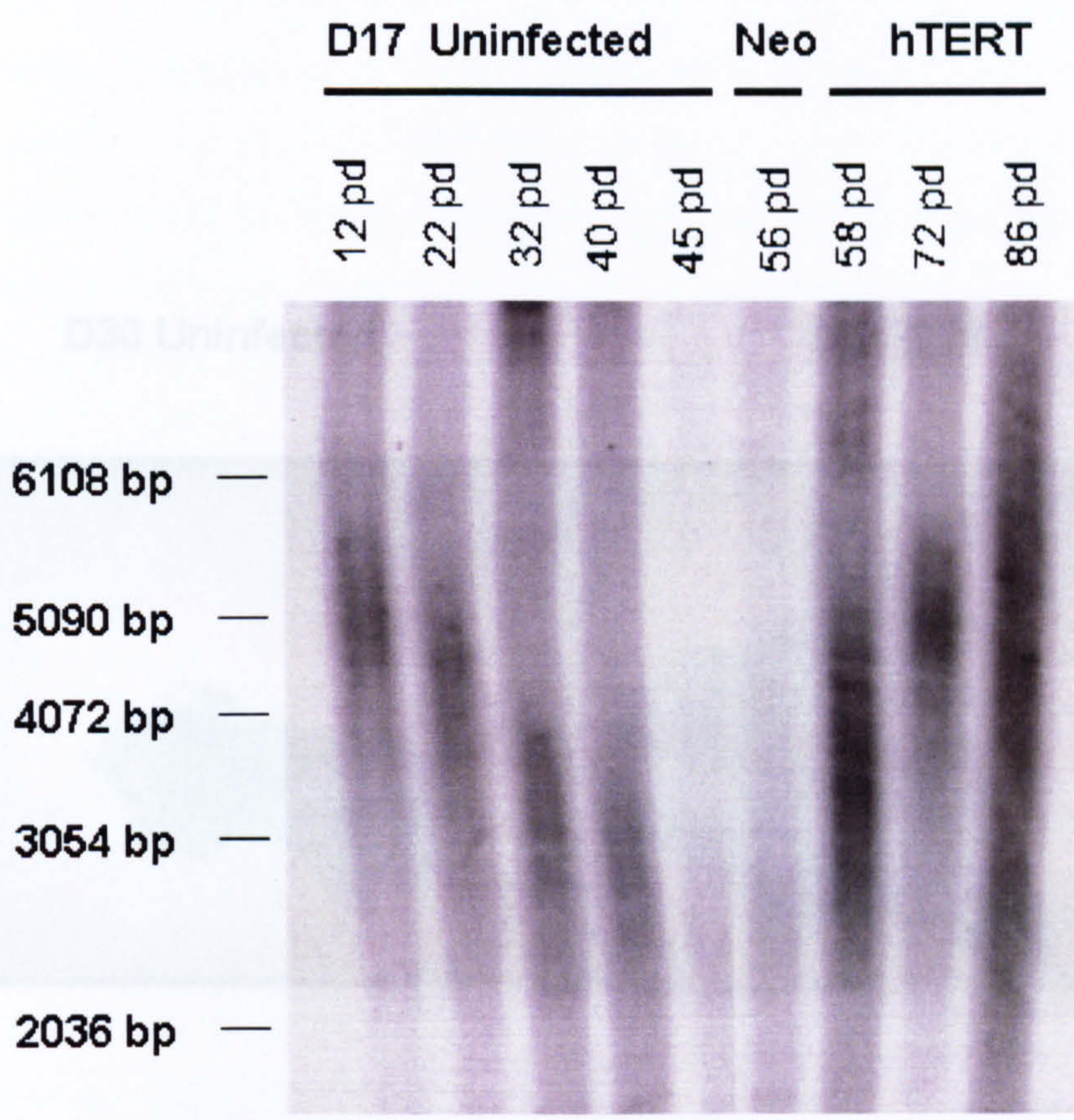


Fig. 35: Telomere length in D17 cultures before and after infection with hTERT expression vector.

Southern blot illustrating TRF length measurements in D17. Genomic DNA samples were obtained from D17 cultures at different stages of their life span towards senescence (the number of population doubling is indicated for each sample), after infection with the empty vector (NEO) and with the hTERT transducing vector (EST). 1 Kb and λ/Hind III DNA fragments were used as reference molecular weight markers.
As shown in the figure, telomeres underwent a progressive shortening as cultures approached senescence, but were considerably elongated after hTERT ectopic expression. The weaker signal observed in the D17 uninfected control and NEO infectant is due to the senescent status of the cultures.

D30 Uninfected D30 EST

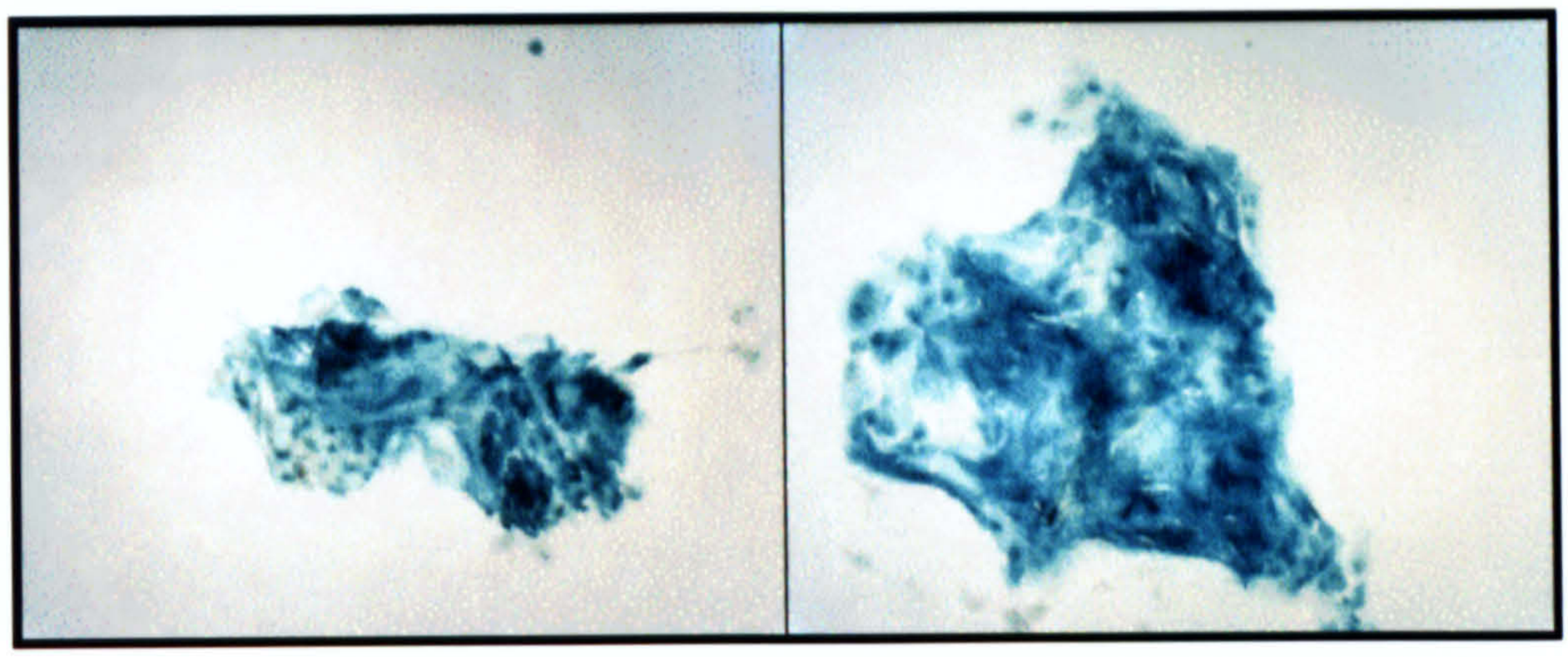


Fig. 36: SABG staining in D30 uninfected control and EST infectant.

Cells were maintained in culture for a minimum of three weeks after they appeared to be stationary to confirm cessation of proliferative growth. Subsequently, they were fixed, stained and photographed with a 10X magnifying lens.
The positive SABG staining confirms the growth arrest status reached by both the uninfected control and the EST infectant.

With D17, no substantial difference was observed between the growth rates of uninfected D17 and Neo infectants. However, the cells infected with the hTERT vector kept proliferating beyond 120 PD, at which point the cells were considered immortal. As expected, the uninfected D17 reached growth arrest after 45 PD. The NEO infectant stopped growing but having completed 56 PD in culture. One possible explanation for the difference between the number of population doublings completed at growth arrest by the D17 uninfected culture and the NEO infectant could come from the method used for counting the number of PD in infected cultures. G418 resistant colonies started emerging from selection and were counted as soon as visible under light microscopy. The assumption was made that each colony was derived from expansion of a single resistant cell. The number of resistant cells so obtained was then utilised for estimating the population doublings completed in culture when cells were subsequently harvested and counted. This method involves some approximations, not taking into account 1) those resistant cells that are not visible upon observation under the light microscope and 2) the possibility that one single colony could derive from more than one resistant cell. Both these factors could lead to the overestimation of cellular proliferation.

When the uninfected D17 and the NEO infectants entered a slow growth phase, they were maintained in culture for a minimum of three weeks to confirm proliferative growth had ceased. At that stage, hTERT infectants had reached 85 PD. Plates from all three cultures were assayed for SABG staining, BRDU incorporation and TUNEL assay to better define if the terminal growth arrest reached by the controls and the mock infectants was due to senescence or crisis, as discussed in chapter 9.3 (fig. 37). Both these cultures stained positive for SABG, had a low BRDU incorporation index (~ 20%) and showed virtually no apoptosis as assessed by TUNEL assay, suggesting they had arrested in senescence rather than in crisis. The hTERT infectants stained negative for SABG, had a high BRDU incorporation index (84%) and showed no apoptosis, as expected from rapidly proliferating cells, indicating that these cells had bypassed the replicative senescence checkpoint.

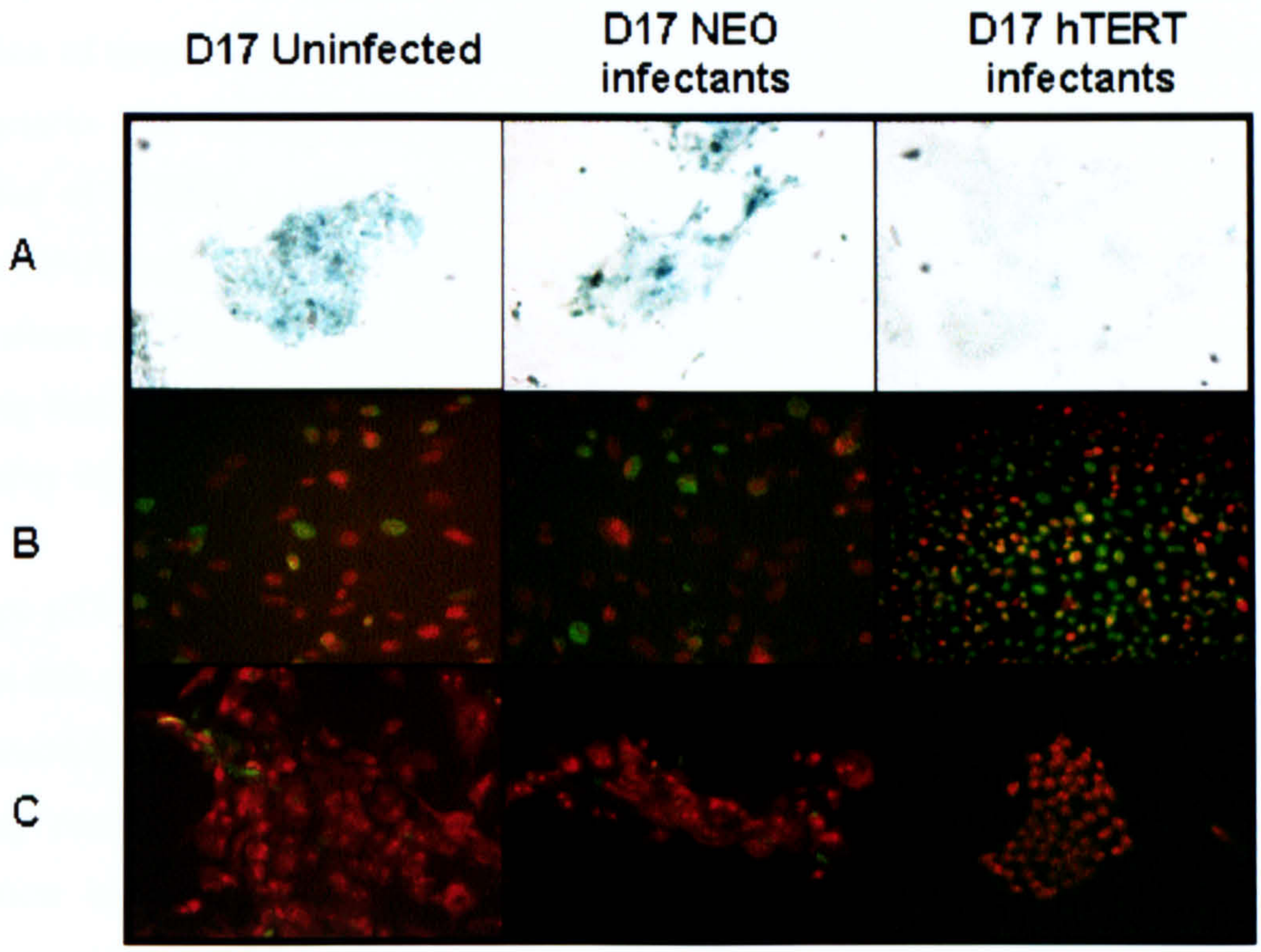


Fig. 37: SABG staining, BRDU incorporation and TUNEL assay in D17 before and after infection with the *hTERT expression* vector.

The aim of this experiment was to understand if proliferative senescence in D17 uninfected control and NEO infectants could be overcome by infection with hTERT.
SABG staining **(A),** BRDU incorporation **(B)** and TUNEL assay **(C)** were performed on D17 uninfected controls, NEO and hTERT infectants. When this experiment was carried out, D17 uninfected controls and NEO infectants had been stationary in culture for three weeks.
A) D17 uninfected controls and NEO infectants stain positive for SABG staining, whereas hTERT infectants stained negative.
B) The percentage of cells scoring positive for BRDU incorporation was assessed on a minimum of 500 cells: 21% of uninfected controls, 22% of Neo infectants and 84% of hTERT infectants.
C) Virtually no apoptotic cells are visible in either D17 uninfected controls, NEO infectants or hTERT infectants.
These combined results suggest that when the three assays were carried out D17 uninfected controls and Neo infectants had reached senescence, whereas hTERT infectants have bypassed this mortality checkpoint and were actively proliferating.

11.2.3 Investigating p53 status and phosphorylation at serine 15 in D17

In our previous work we have shown that p53 mutations are often associated with the acquisition of immortality in naturally occurring oral dysplasias. It still remained unclear if in a scenario like the one just described, where immortality is acquired after ectopic expression of hTERT, a selective pressure exists towards inactivation of p53. Therefore, genomic DNA samples were obtained from hTERT infectants after they had completed 86 PD in culture and the whole p53 coding region was sequenced. No mutations were found, indicating that mutation of p53 gene is not an obligatory requirement for the acquisition of immortality when telomerase is activated exogenously.

Wild type p53 has been reported to downregulate hTERT mRNA expression in carcinoma cells, but this could not be observed with mutant p53 (Xu et al. 2000). Therefore, mutation of p53 could be necessary to allow telomerase activation and immortalisation in a naturally occurring context, but not when hTERT is ectopically expressed. Another possible explanation is that the elongation of critically short telomeres as obtained through exogenous telomerase activation abolishes the stimulus leading to p53 mediated growth arrest and therefore the necessity to mutate p53 in order for cellular proliferation to continue.

It was recently shown that p53 response to the DNA damage-like signal generated by telomere erosion acts through phosphorylation of its serine-residue 15 in human fibroblasts (Webley et al. 2000). Therefore, it seemed worthwhile to investigate p53 expression and serine-residue 15 phosphorylation in D17 during its normal aging process and after immortalisation (and telomere elongation) with hTERT.

D17 was serially passaged in culture and analysed at different stages of its life-span comprising senescence. At each time point chosen for this experiments the cellular biology features of D17 were assessed through SABG staining and BRDU incorporation assay (fig. 38). As shown in the figure, the combined outcome of these two assays indicates that D17 is actively proliferating at 12.5, 22 and 32.5 PD and has reached senescence at 45 PD. Protein samples were extracted from plates grown in parallel with the ones utilised for the stainings and analysed through Western Blotting (fig. 39).

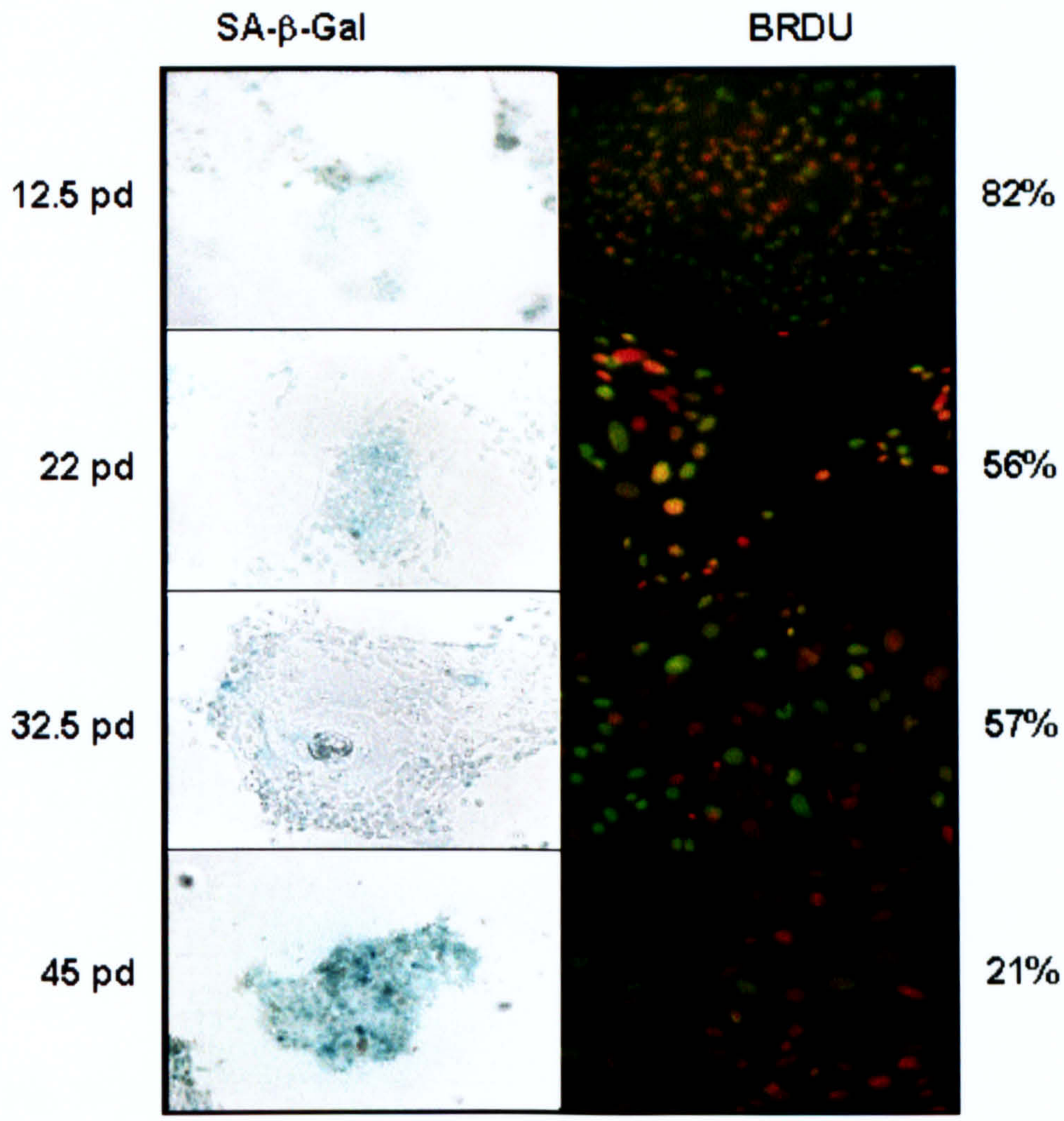


Fig. 38: SABG staining and BRDU incorporation in D17 at different culture ages.

D17 was grown in culture and harvested at different stages of its life span. SABG staining (left panels) and BRDU incorporation (right panels) were performed at the same time as the collection of samples to assess variations in p53 expression and p53-serine15 phosphorylation with increasing age in culture.
For each sample, the number of population doublings completed in culture and the percentage of cells scoring positive for BRDU incorporation are indicated on the left and right part of the figure respectively.
Taken together, these results confirm that D17 is actively proliferating at 12.5, 23 and 32.5 PD and has reached senescence at 45 PD.

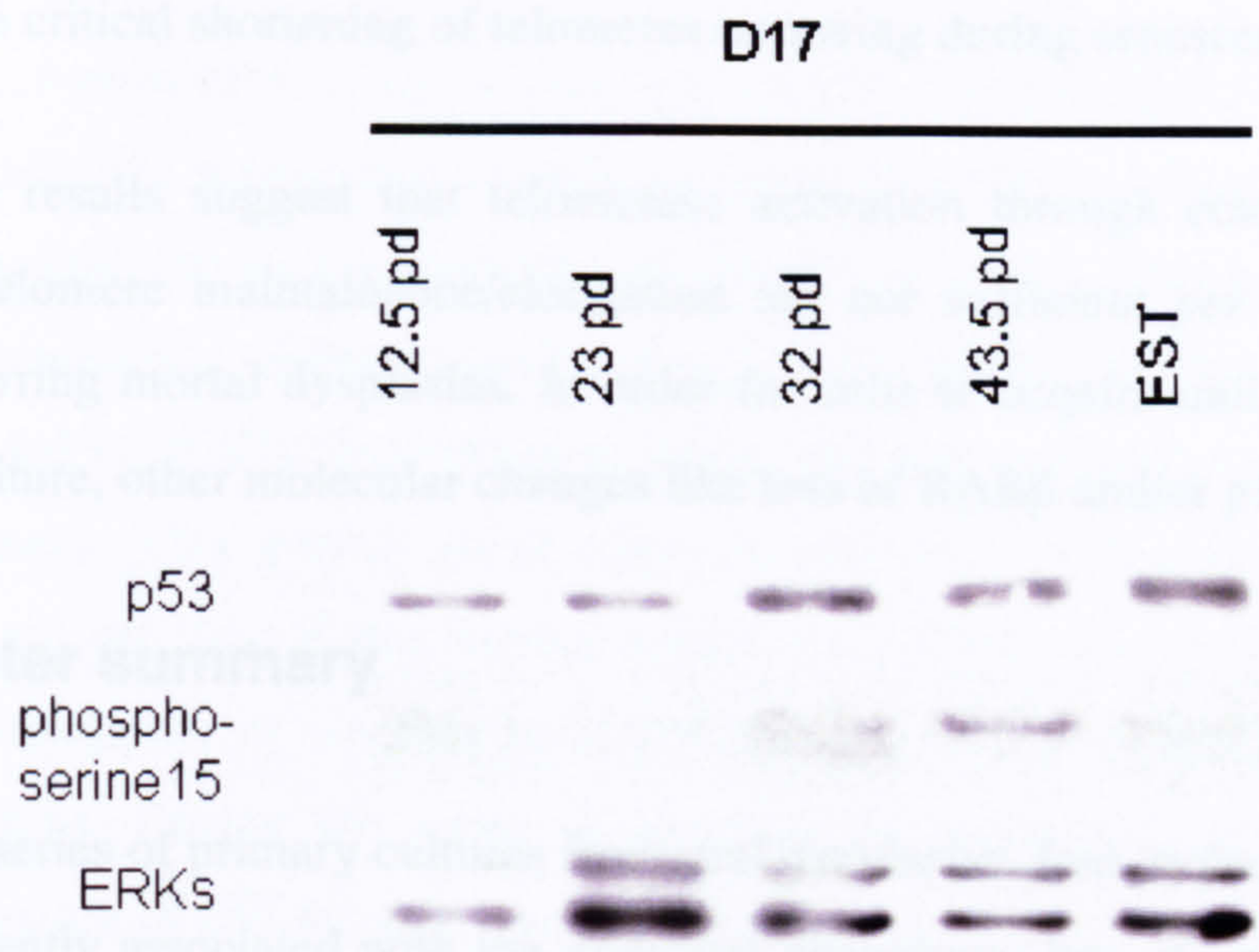


Fig. 39: p53 and p53 phosphorylation at serine15 in D17.

Western blots illustrating the expression of total p53 protein and p53 phosphorilation at serine residue 15 (detected through a phospho specific antibody) in D17 at different culture ages and after infection with hTERT expression vector. ERK 1,2 were also measured to ensure equal loading of samples.
As illustrated in the upper panel, the expression of total p53 protein is fairly uniform at all the different culture ages analysed.
In the lower panel, p53 phosphorylation at serine residue 15 increases as cultures approach senescence, and decreases after infection with hTERT expression vector. This trend closely mirrors that of telomere length, suggesting a telomere shortening dependent mechanism of p53 activation through phosphorilation at serine residue 15.

As shown in the figure, p53 protein expression is fairly homogeneous amongst all samples analysed. In contrast, p53 phosphorylation at serine residue 15 increases as cells approach senescence, but this stimulus seems to be abrogated upon telomere elongation following infection with hTERT. This result suggests that in human keratinocytes, as well as human fibroblasts, signalling through phosphorylation of serine 15 residue of p53 is triggered in response to the critical shortening of telomeres occurring during senescence.

Overall, these results suggest that telomerase activation through ectopic expression of hTERT and telomere maintainance/elongation are not sufficient *per se* to immortalise naturally occurring mortal dysplasias. In order for cells to acquire unlimited proliferative potential in culture, other molecular changes like loss of RARβ and/or p16 are necessary.

11.3 Chapter summary

- In our series of primary cultures from oral dysplasias, four molecular changes were consistently associated with the immortal phenotype: loss of expression of RARβ and p16, mutation of p53 and activation of telomerase.

- One atypical dysplasia (D17) senesced after a considerably extended life span (45 PD) and had molecular features that were intermediate between mortal and immortal phenotype: loss of expression of RARβ and p16, wild type p53 and no telomerase activity.

- D17 was immortalised by retroviral transduction of hTERT. This was associated with activation of telomerase and elongation of telomeres.

- Immortal hTERT transduced D17 clones retained wild-type p53, suggesting that p53 mutations are not an absolute requirement for cellular immortalisation when telomerase is activated exogenously.

- Immortalisation of D17 was associated with abrogation of the increase in levels of p53 phosphorylation at serine residue 15, that otherwise occurs during dysplasia senescence.

- Two typical mortal dysplasias (D30 and D25) that express RARβ and p16 were not immortalised by retroviral transduction of hTERT, despite activation of telomerase and elongation of telomeres.

12 General discussion

12.1 Features of the growth arrest status observed in our mortal dysplasia cultures

In recent years, considerable progress has been made in defining the cellular and molecular features of replicative senescence in different types of cells. As already described in the introduction section, human keratinocytes are known to reach a first mortality checkpoint, called M1 or senescence, characterised by positivity for SABG staining, a low proliferation index as assessed by BRDU incorporation index, and low apoptosis index as assessed by the TUNEL assay. This growth arrest status is associated with overexpression of p16, although it is not yet clear if this tumour suppressor protein plays a role in initiating the senescence programme, or whether its overexpression is a consequence of sub-optimal culture conditions. During the onset of keratinocyte senescence, telomere shortening causes an activation of p53 through phosphorylation of its serine residue 15. This causes a G1 growth arrest mediated by p21. A recent report suggests that p53 also participates in M1 through a telomere length-independent mechanism, associated with increased levels of $p14^{ARF}$ expression (Rheinwald et al. 2002). If senescence is bypassed, keratinocytes encounter a second mortality check-point (M2) called crisis, still characterised by positivity for SA-β-gal staining, but showing a high index of cellular proliferation and apoptosis by BRDU and TUNEL assays, respectively. Crisis is thought to be governed by critical telomere shortening causing a high degree of genetic instability, which can be overcome by activation of telomerase.

Our *in vitro* model of oral premalignant lesions has proved very useful in further defining the molecular changes associated with these two different mortality check-points. Summarising data from previous work in the group and that described in this thesis, all of our 8 mortal dysplasia cultures are invariably telomerase negative. The majority of these dysplasias (7/8) expressed RARβ, p16, and wild type (WT) p53 and reached senescence after 5-30 population doublings. The remaining mortal dysplasia, D17, had lost expression of p16 and RARβ but retained WT p53, and still senesced, although after an extended life span (45PD). In addition to this, Ken Parkinson's group in the Institute has described a mortal carcinoma culture (BICR 7) which is telomerase negative, has lost expression of p16 and has mutated p53 (no data is available on RARβ expression). Interestingly, this culture reached growth arrest in a crisis-like state. On purely observational grounds, these data suggest that loss of expression of RARβ and/or p16 in naturally occurring

premalignant/malignant lesions is still associated with a senescence like growth arrest status, whereas features of crisis are observed when p53 mutation occurs together with loss of p16. This is in keeping with the interventional studies recently published by Rheinwald and collaborators (Rheinwald et al. 2002), suggesting that both p16 and p53 functions have to be disrupted to evade M1 growth arrest in normal keratinocytes.

12.2 Molecular changes associated with immortality

Senescence control is widely regarded as a tumour suppressor mechanism. This process breaks down during tumourigenesis, yielding cells that have acquired an unlimited proliferative potential. Our analysis has revealed that this event occurs at early stages of oral cancer progression, since 7/15 of our dysplasias are immortal in culture. Four molecular changes are consistently found in association with the immortal phenotype: loss of expression of RARβ and p16, mutation of p53 and activation of telomerase. Only one immortal dysplasia (D38) represents an exception to this general rule, and will be discussed separately.

12.2.1 The role of RARβ in cellular immortality and cancer

The initial suggestion that retinoids might prevent the development of cancer came from the observation that prolonged vitamin A deprivation in humans predisposes to epithelial cancers. Direct evidence for the specific involvement of RARβ in prevention of malignant transformation was obtained through an interventional study. Transgenic mice expressing an antisense RARβ2 transgene were generated, and reported to be more prone to developing lung tumours than the non-transgenic mice (Berard et al. 1996). Nevertheless, the specific pathways linking retinoids and their receptors to cancer formation remain unclear.

Retinoids are known to inhibit aberrant squamous differentiation in HNSCC keratinocytes *in vitro*, and strong evidence points out RARβ as the mediator of this effect. In fact, transfection of RARβ2 in a human RARβ negative HNSCC cell line restored its sensitivity to the downregulation of squamous differentiation markers induced by RA treatment (Wan et al. 1999). Although abnormal differentiation is a common feature of malignant lesions arising in the oral cavity mucosa, the link between the effects of retinoids in suppressing squamous differentiation and preventing carcinogenesis has not yet been elucidated. It is possible to speculate that RARβ may mediate the effects of retinoids on the expression of

differentiation markers that are peculiar to oral epithelial cells, like keratin 19 in the basal layer or keratin 4 and 13 in the suprabasal layers. Although some reports suggest a correlation between RARβ and keratin 19 expression in normal oral keratinocytes, the same is not true for SCC from the oral cavity, where keratin 19 expression was detected even in cell lines that have lost expression of RARβ (Crowe et al. 1991) (Hu et al. 1991). *Keratin 4 and 13 expression is found in the suprabasal layer, which is composed of cells* that have already committed to differentiate (Fuchs 1990), so they could be reasonable candidates as downstream targets of RARβ. This potential avenue has not been investigated as yet.

The effects of retinoids on cellular growth are even more complicated. In HNSCC, they inhibit cellular proliferation *in vitro*, but this effect is not necessarily mediated by RARβ. In fact, ectopic expression of RARβ2 in HNSCC cells lacking endogenous expression does not significantly affect their growth rate in response to retinoid treatment (Wan et al. 1999). Our studies are novel in relating expression of RARβ to the proliferative potential of premalignant and malignant lesions from the oral cavity. In our *in vitro* model, cultures from normal oral mucosa express higher levels of RARβ when they are exhausting their proliferative potential, independently of retinoic acid treatment. Mortal dysplasias express RARβ even at early passages in culture, like senescent normal cells. We have speculated that RARβ expression could be required to mediate growth arrest in normal oral keratinocytes, but in the context of dysplastic lesions it might be no longer sufficient to exert this effect. This change in the regulation of RARβ expression is not due to expression of different RARβ isoforms, as both normal oral mucosa and mortal dysplasias predominantly expressed the RARβ2 isoform (Judith Brown and Paul Harrison, personal communication). In keeping with this hypothesis, we have demonstrated a striking association between loss of RARβ and cellular immortality: this molecular event had occurred in our immortal dysplasias with no exceptions. Loss of RARβ has been reported *in vivo* both in oral dysplasias (44% of cases) and in oral carcinomas (65% of cases). Therefore, our finding is unlikely to be a culture artefact. Our work provides evidence suggesting that RARβ might be involved in regulating the mortal to immortal transition. Functional experiments are required to determine if loss RARβ is causal or merely consequential to oral keratinocyte immortalisation.

12.2.2 Involvement of p16 and $p14^{ARF}$ in immortality and cancer

The INK4a locus codifies for two distinct proteins both involved in regulation of cellular replicative senescence and tumour suppression: p16 and $p14^{ARF}$. As a tumour suppressor protein, p16 is often lost or inactivated in several different types of tumours and in HNSCC (Cairns et al. 1995) (Califano et al. 1996) (Reed et al. 1996). Evidence for its direct involvement in carcinogenesis is provided by an animal model: mice engineered to bear a germline homozygous deletion of the INK4a locus are known to develop tumours with a higher frequency than their normal counterparts (Serrano et al. 1996). The functional relevance of p16 expression in regulating cellular growth has also been assessed *in vitro*. Transfection of a $p16^{INK4}$ cDNA into several carcinoma cell lines resulted in growth inhibition, and a strong selection pressure develops against the p16 expressing transfectants with continuous passage in culture (Okamoto et al. 1994).

In previous chapters of this thesis I have discussed the role of p16 in regulating growth arrest at M1 (replicative senescence) and the possibility that p16 overexpression might be due to inadequate culture conditions (see chapter 7.2). In our cultures from normal oral mucosa, p16 is expressed at high levels since the earliest available passages in culture and no further upregulation of protein expression occurs at later stages of their proliferative lifespan. This is in contrast with the accumulation of p16 reported in human mammary epithelial cells when approaching M0 and in keratinocytes when approaching senescence (Kiyono et al. 1998) (Munro et al. 1999) (Romanov et al. 2001). A possible explanation to this is the adult age of the donors from whom our cultures were derived. Alternatively, upregulation of p16 might be involved in the initiation of replicative senescence at an earlier stage than the dysplasias we were able to obtain.

With the exception of D17, our mortal dysplasias express high levels of p16 throughout their proliferative lifespan, like normal cultures. It is very unlikely that p16 overexpression in our cultures is due to inadequate culture conditions, as they were all established and maintained on a layer of irradiated feeder cells in the presence of serum in the growth medium. Therefore, p16 might be genuinely involved in regulating M1 growth arrest in our cultures from normal oral mucosa and mortal dysplasias.

Our dysplasias showed a strong association between acquisition of immortal proliferative potential and loss of p16 expression, the only exception being D38. This is in agreement with a large body of literature indicating that abrogation of the p16/Rb pathway is an indispensable requirement for immortalisation of mammary epithelial cells and oral

keratinocytes, along with telomerase activity (Kiyono et al. 1998) (Dickson et al. 2000) (Romanov et al. 2001). The evidence using our oral dysplasias is strengthened by work carried out by another group, describing loss of p16 expression in 38% of oral dysplasia biopsies *in vivo* (Papadimitrakopoulou et al. 1997). This percentage is very similar to the figure we have reported in oral dysplasia cultures, correlated with cellular immortality.

However, a recent report by Rheinwald and collaborators (Rheinwald et al. 2002) has provided evidence that p16 dysfunction is insufficient per se to significantly extend the proliferative life span of normal epidermal and oral keratinocytes. In order to interfere with the p16-related growth arrest mechanism, the authors have transduced keratinocytes with a p16-insensitive mutant cdk4 expression vector. Similarly, only a 10 PD life span extension was observed in normal human fibroblasts using p16-insensitive cdk4 and/or cdk6 mutants (Morris et al. 2002). One possible limitation of this approach is that it does not abolish all of the functions of p16, but only those mediated by cdk4 and/or cdk6. Rheinwald and collaborators (Rheinwald et al. 2002) also report that p16 dysfunction combined with telomerase activation fails to immortalise these cell types in the presence of functional p53. In these experiments, abrogation of the p16 pathway lead to accumulation of $p14^{ARF}$, ultimately resulting in stabilization of p53 and growth arrest. Therefore, p53 might participate to the replicative senescence control through two different mechanisms, one mediated by $p14^{ARF}$ and the other mediated by telomere shortening. Interference with both p16 and p53 functions may allow senescence to be bypassed, with cellular immortalisation being achieved through a concomitant activation of telomerase.

The $p14^{ARF}$ status of all our naturally occurring immortal dysplasias has not been investigated, although p16 and $p14^{ARF}$ are known to be frequently co-deleted or inactivated in immortal SCC cultures (45% and 20% of cases respectively) (Munro et al. 1999). The intermediate phenotype culture D17 lacked expression of p16 (and RARβ) and was successfully immortalised by ectopic activation of telomerase, despite retaining a functional p53. In fact, D17 has also lost expression of $p14^{ARF}$ (Janis Fleming and Paul Harrison, personal communication) and is therefore presumably unable to activate p53 through the proposed telomere length independent mechanism. Thus the requirements for D17 immortalisation are consistent with the recent conclusions of Rheinwald and collaborators (Rheinwald et al. 2002).

12.2.3 The atypical immortal culture D38

In previous chapters, D38 has often been referred to as an atypical immortal dysplasia. In fact, it is the only example amongst our immortal dysplasia cultures that does not conform to the molecular phenotype associated with the acquisition of an unlimited proliferative potential. D38 has lost RARβ expression and activated telomerase, like the other 6 immortal dysplasias, but it differs from them in having retained expression of p16 and wild type p53.

As discussed in previous sections, abrogation of the p16/Rb pathway is frequently reported in literature as a necessary requirement for immortalisation of human epithelial cells. Therefore, detecting high levels of p16 expression in an immortal dysplasia is unusual. The entire coding region of p16 was sequenced in previous work from our group, but no mutations could be found (Fiona McGregor and Paul Harrison, personal communication). p16 is a tumour suppressor protein that negatively regulates the activity of cdk4/cyclin D1 complexes, ultimately resulting in inhibition of the phosphorylation of Rb and the consequent release of E2F transcriptional activity. D38 shows normal levels of expression of cyclin D1, but the activity of the cyclin D1/cdk4 complex has not been tested. A naturally occurring cdk4 point mutant (cdk^{R24C}) that retains its kinase activity despite having lost the ability to bind p16 has recently been used to abolish p16 control on the cell cycle (Rheinwald et al. 2002). Thus it is not excluded that cyclin D1/cdk4 complex in D38 is constitutively active, or that cdk4 bears a mutation rendering it p16 insensitive but functionally active. Inactivation of p16 or pRb have been reported as mutually exclusive events in HNSCC (Okami et al. 1999). It is also not known whether Rb is lost in D38, which would render the upstream Rb control pathways ineffective. These are all hypotheses that need to be tested. Alternatively, the molecular phenotype of D38 could imply that inactivation of p16 is not a crucial step in cellular immortalisation, whereas loss of RARβ or other as yet unknown molecular changes might be required instead. However, at present, this is a radical interpretation.

The finding of WT p53 in D38 was not as surprising. As discussed above, the selective pressure to mutate p53 derives from its ability to sense critical telomere shortening and mediate a G1 cell cycle arrest before severe telomere dysfunction occurs. Activation of telomerase could *per se* reverse the telomere shortening mechanism associated with senescence and thus render inactivation of p53 unnecessary. The recent evidence that p53 is also involved in regulating proliferative senescence through a telomere-independent $p14^{ARF}$-mediated mechanism (Rheinwald et al. 2002) raises the possibility that D38 might

harbour a mutations in exon 1β of the INK4A locus, which uniquely encodes for $p14^{ARF}$ and is essential for its function. In this case inactivation of the $p14^{ARF}$/p53 pathway would be obtained through loss of $p14^{ARF}$ expression in alternative to mutation of p53.

12.3 Attempts to immortalise mortal dysplasias

Our *in vitro* model of oral cancer progression has identified several molecular changes that occur in association with cellular immortality. However, although informative, such analysis was not sufficient to distinguish the events that are merely consequential from those that have a causal importance in the process of immortalisation. In addition, the literature reports addressing this question are frequently in confliction, so that an univocal conclusion cannot be drawn. To briefly summarize the two main points of debate in this field, Shay's group reports that transduction of hTERT is sufficient to immortalise normal keratinocytes, p16 overexpression being a result of inadequate culture conditions (Ramirez et al. 2001) whereas Rheinwald's group provides evidence that p16 is upregulated independently of culture conditions in cells approaching senescence, and its loss is required in addition to telomerase activation to achieve keratinocyte immortalisation (Rheinwald et al. 2002).

As previously described, only 1/8 of our mortal dysplasias showed any of the changes that were found in immortal dysplasias. D17 was atypical, as it showed loss of RARβ and p16 and a considerably extended life span. This suggests that the acquisition of two out of four of the molecular changes associated with immortality is still not sufficient to confer an unlimited proliferative potential. D17 was therefore an ideal model to test whether ectopic activation of telomerase was sufficient to immortalise naturally occurring oral dysplasias, or whether other changes like p53 mutation and loss of RARβ and p16 are also required.

Initially, a retroviral hTERT expression vector was transduced in two typical mortal dysplasia cultures, that retained normal p16 and RARβ expression. Despite maintaining them under adequate culture conditions, activation of telomerase and the consequent telomere elongation did not allow these cultures to proliferate beyond their expected life span. In contrast, ectopic activation of telomerase in D17 under the same conditions caused immortalisation, suggesting that when loss of RARβ and/or p16 occur in concomitance with telomerase activation, oral dysplasias become immortal.

Another important point emerging from the analysis of D17 is that mutation of p53 is not an absolute requirement for immortalisation. In fact, the D17 hTERT infectants never

selected in favour of p53 mutation, and were shown to retain wild-type p53 after having completed 120 population doublings in culture. Certainly, activation of telomerase/ telomere elongation in D17 abrogated the increased phosphorylation of p53 at serine 15 that otherwise occurs at senescence. This could be an efficacious mechanism to bypass the selective pressure against wild type p53 in indefinitely proliferating cells. The most recent findings from our group have added $p14^{ARF}$ as an additional player in this scenario. It could be speculated that in D17 the concomitant dysfunction of p16 and $p14^{ARF}$ allows the cells to proliferate for longer, undergoing further telomeric attrition and ultimately reaching a further senescence checkpoint that involves telomere mediated phosphorylation of p53 at serine 15.

12.3.1 p53 mutation and telomerase activation in immortality and cancer

The involvement of both p53 and telomerase in human carcinogenesis is widely accepted and described in a copious body of literature. The human Li Fraumeni syndrome, due to an inherited heterozygous mutation of p53, is known to be characterized by predisposition of affected individuals to various types of cancers. Similarly, p53 knock out mice are more prone to developing tumours than their normal counterparts. Furthermore, an early intervention experiment presented evidence that functional p53 inhibits the growth of cancer cells: when WT p53 was transfected in a colon carcinoma cell line that had inactivated p53, a substantial inhibition of cell growth and colony forming efficiency was noticed, as assessed by H^3 thymidine incorporation, along with a selection pressure to mutate the exogenous p53 (Baker et al. 1990). More recent efforts have lead to the generation of transgenic mice that overexpress hTERT in their basal keratinocytes. These mice showed an increased susceptibility to chemically induced skin carcinogenesis (Gonzalez-Suarez et al. 2000; Gonzalez-Suarez et al. 2001). Not surprisingly, expression of a mutant form of hTERT in human cancer cell lines resulted in inhibition of their growth (Hahn et al. 1999)(b).

Our most recent data has identified a close correlation between mutation of p53, activation of telomerase activity (through hTERT upregulation) and cellular immortality. Telomerase activity was observed in immortal dysplasias with no exceptions, whereas the atypical culture D38 was the only one retaining wild type p53 along with an immortal phenotype.

The interactions between p53 and hTERT have not yet been fully elucidated. The hTERT promoter lacks p53 consensus binding sites (Cong et al. 1999), but p53 might regulate hTERT via interfering with the hTERT transcriptional activator Sp-1: recent evidence has

demonstrated that wild type p53 is able to form complexes with Sp-1 and thus could prevent it from binding to the hTERT promoter (Xu et al. 2000). In the same experiments, transfection of wild type p53 into Burkitt lymphoma cells lacking a functional p53 resulted in a rapid dowregulation of hTERT transcription. On the other hand, the critical shortening of telomeres observed as a result of cellular senescence activates p53 through phosphorylation of its serine residue 15, which mediates a p53-dependent G1 arrest in response to DNA damage (Webley et al. 2000). This can be circumvented by telomerase activation, as demonstrated with D17 in the present studies.

This background information suggests at least two possible routes to cellular immortality: through hTERT/telomerase activation and/or through mutation of p53. By spontaneously mutating p53, cells may acquire the ability to release the interference on Sp-1 mediated hTERT transcription by WT p53, and thus activate telomerase. Alternatively, telomerase activation might remove the selection pressure to inactivate p53 by correcting telomere dysfunction, and therefore abrogate the cascade of events that leads to p53-mediated cell cycle arrest. Although these two options are not mutually exclusive, the second is probably not of frequent occurrence in human keratinocytes since spontaneous activation of telomerase has never been reported in this cell type.

Activation of telomerase does not have to be considered solely in association with loss of function of p53. Several telomerase positive and negative regulators of hTERT expression/telomerase activity have been described in literature (discussed in chapter 6.2), and loss of expression of a telomerase inhibitor or gain of function of a telomerase activator could be responsible for telomerase activation in our immortal cultures. The expression of c-Myc and Mad1, reported to be respectively a positive and a negative regulator of hTERT, was previously analysed in all of our cultures from mortal and immortal dysplasias, but no changes were found (Judith Brown and Paul Harrison, personal communication).

12.3.2 Proposed future experiments

Using D17 to investigate the genetic basis of keratinocyte immortalisation offers several clear advantages, along with a few disadvantages. This culture has peculiar features *in vitro*, as it has spontaneously acquired two of the molecular changes associated with the immortal phenotype and retains an extended but still limited proliferative potential. Finding these molecular changes in a naturally occurring dysplasia is certainly more informative than artificially creating the same scenario through ectopically expressing

oncogenes, whose biological effects are not clearly and completely defined. The availability of the frozen biopsy sample from which this culture was derived enabled us to prove that loss of RARβ and p16 have occurred *in vivo* (Keith Hunter and Paul Harrison, personal communication), thus allowing us to rule out the possibility of a cultural artefact. Nevertheless, we can not exclude that other as yet unknown molecular events have taken place in D17 and might play a role in determining its cellular or molecular phenotype. In addition to our earlier findings, most recent experiments on D17 have identified loss of expression of $p14^{ARF}$, which might be involved in mediating the p53 regulatory effects on replicative senescence, as recently reported observations suggest (Rheinwald et al. 2002). It remains to be clarified whether all three molecular changes in D17 are required in addition to hTERT expression for immortalisation. In fact, we have not observed any mortal dysplasias that have lost just RARβ or p16; their $p14^{ARF}$ status needs to be investigated through further experiments.

Several interventional procedures could be carried out in the future to clarify these points.

RARβ expression could be exogenously abolished in a typical mortal dysplasia, creating a situation where the molecular status of our four markers of interest is RARβ-, p16+, telomerase- and wt p53. An RARβ2 mutant (R269Q) acting with dominant negative effects has been described in literature, but its inhibitory function is exerted on all RAR subtypes. If this mutant were to be used, it would be impossible to distinguish between the effects due to loss of expression of RARβ and those due to loss of expression of RARα/γ. A Similar problem would be encountered using expression vectors transducing RARβ4. This RARβ isoform lacks a DNA binding domain but retains its ability to interact with RXRs, rendering them unavailable for binding with other functional RARs. Alternatively, antisense mRNA or RNA interference approaches could be used. An antisense construct specific for the RARβ2 isoform has been designed and utilised by Berard and co-workers (Berard et al. 1996), and it proved successful in reducing the levels of expression of RARβ2 message in transgenic mice. A novel technique has been recently developed, using duplexes of 21-nucleotide RNAs, known as short interfering RNAs (si-RNAs): they have proved to efficiently inhibit endogenous gene expression when introduced in mammalian cells (Sui et al. 2002) (Yu et al. 2002). Considering what reported in literature, it is very unlikely that the sole abrogation of RARβ function would create an immortal phenotype, unless ectopic RARβ loss of expression resulted in activation of telomerase. Our observations seem to indicate that telomerase is not a direct target of RARβ since cultures

from normal oral mucosa and our mortal dysplasia D17 do not express RARβ but still have no detectable telomerase activity.

Alternatively, RARβ expression could be induced in an immortal dysplasia, to investigate if the artificial RARβ+, p16-, telomerase+ and mutated p53 combination restores the mortal phenotype. If this were the case, it would be then interesting to assess whether or not normal p16 expression and wt p53 are required in addition to RARβ for cells to be mortal. This information could be gathered by comparing the effects of RARβ ectopic expression in a typical mortal dysplasia and in our atypical mortal dysplasia D38, which retains p16 and wt p53. Such experiments have already been attempted in the group by transfection methods before the retroviral infection technique was developed, but proved inconclusive because of the very low transfection efficiencies. A retroviral vector expressing RARβ2 (LNSβ) has recently been generated and utilised in HNSCC cell lines by Lotan's research group, which had also encountered similar difficulties with transfection approaches. The only potential disadvantage of using this system would be the long period of time between the infection procedure and the manifestation of those biological effects imputable to RARβ expression. This would not enable us to distinguish between its direct effects and those mediated by other unidentified effectors. An inducible RARβ expression system could be used instead. Recently, an ecdysone-inducible mammalian expression system has been optimised by Van Craenenbroeck and co-workers (Van Craenenbroeck et al. 2001). This system entails transfection with an expression vector transducing a retinoid X receptor (RXR) and a modified ecdysone receptor, controlled by the constitutive RSV (Rous sarcoma virus) and CMV (cytomegalovirus) promoters respectively. After addition of ecdysone, the two receptors heterodimerize and bind to the ecdysone-responsive elements, resulting in transcription of the gene of interest. Although this system could potentially be useful, it has several drawbacks. The exogenous expression of RXR receptor might alter the physiological balance between the levels of expression of RXR/RAR subunits. In addition, RARβ expression would need to be maintained for a long period of time before senescence would be expected to occur, rendering difficult to discriminate between its direct and indirect effects. Also, if RARβ were to trigger senescence, a selection against those clones expressing RARβ could be expected. Nevertheless, this approach could still prove useful in identifying other possible target genes of RARβ. This could be accomplished by comparing the mRNA expression of a culture pre and post transfection with the inducible RARβ expression system through microarray analysis. This quite novel approach would be in keeping with our group's

ongoing work to identify genes implicated in mortality control through microarray analysis of our panel of mortal and immortal dysplasias.

As discussed earlier in this chapter, a certain degree of "cross talk" seems to occur between p53 and telomeres/telomerase activity. In keeping with this, we have observed that wild type p53 is found in association with low or undetectable telomerase activity and mortal phenotype, and mutated p53 is associated with high telomerase activity and immortal phenotype. To clarify whether the association between these markers is direct or only coincidental, we propose to interfere with p53 function in our atypical mortal dysplasia D17, which lacks p16 and RARβ expression. We could then investigate if p53 inactivation is able to upregulate telomerase activity and/or induce immortalisation. Two approaches could be utilised to inactivate p53: infection with HPV E6 or with a retroviral vector expressing a dominant negative p53 construct. The first approach would probably be less specific, since HPV E6 is known to activate telomerase independently of its effects on p53 degradation (Klingelhutz et al. 1996). However, a large panel of well-characterized Arg175 p53 mutants (Tyr175, Trp175, Asp175 and Phe175), along with Tyr141, His175 and Trp248 mutants (these last two being mutational hotspots in human cancer) have completely lost their transactivating functions and can inhibit wild type p53 activity (Hachiya et al. 1994) (Blagosklonny et al. 2001). It has to be kept in mind that the dominant negative effects of p53 mutants are promoter selective, as a consequence of selective loss of transactivation functions (Blagosklonny et al. 2001). Therefore, it would probably be more informative to investigate the effects of a combination of different mutants using retroviruses with different selectable markers.

12.4 Relevance of these molecular changes *in vivo*

The evidence generated by our studies has shed new light on two aspects of oral cancer progression: what molecular changes are involved at different stages and in what temporal sequence they have to occur to yield to keratinocyte immortalisation. Using an *in vitro* model to study a biological process that takes place *in vivo* offers several advantages: it allows a wider range of observations by expanding the cellular population in culture; it is amenable for interventional procedures. In turn, the interpretation of *in vitro* results requires caution, as established cell cultures are different from their tissue of origin in that they are extrapolated from the natural anatomic setting and somehow inevitably modified to allow growth in tissue culture.

One of the features that makes our *in vitro* model of oral cancer progression particularly valuable is the availability of frozen biopsy material corresponding to six of the established cell cultures (3 mortal and 3 immortal dysplasias). This will allow verification of whether the observations made *in vitro* actually correspond to events that have taken place in the original tumour sample, giving a new significance to our observations regarding the process of cellular immortalisation.

At the current state of knowledge, none of the features of oral premalignant lesions that can be assessed by clinical or histologic examination have shown any sort of correlation with the development of a mortal or immortal phenotype in culture. We have found that dysplasias classified as mild or moderate at biopsy had no greater probability of giving rise to a mortal culture than those classified as severe. Similarly, the clinical subdivision of premalignant lesions into leukoplakias and erythroplakias was not predictive of their proliferative life span: combining our data with Ken Parkinson's group's data, 2/6 erythroplakias and 5/13 leukoplakias are immortal in culture.

Therefore, if a correspondence between *in vitro* and *in vivo* molecular phenotypes were to be established, it would enable us to predict which biopsies are likely to give rise to an immortal/mortal culture based solely on molecular markers. The advantages of this approach are obvious. Establishing primary cultures from oral biopsies is a time and resource consuming process, accompanied by a significant culture failure rate mainly due to bacterial/fungal contamination of the original biopsy (50% in our group's experience). In addition, the dimensions of the biopsy samples are often not sufficient to establish a culture along with DNA/RNA/protein extraction and *in situ* studies; thus being able to assign all of the available material to one or few purposes would certainly be of value.

Another approach that we propose to further refine the molecular differences between mortal and immortal dysplasias is the gene expression profiling by microarray analysis. As previously stated, our group is already undertaking a research project aimed at identifying molecular markers regulating mortality using this novel approach. The availability of biopsy material and corresponding cultures from three mortal and three immortal dysplasias will enables us to identify those molecular changes (some of which may be novel) consistently associated with keratinocyte immortalisation in culture and verify their occurrence in the original biopsies.

12.5 Prognostic value of our observations

The identification of a panel of markers consistently associated with the mortal to immortal transition in oral cancer is useful not only because it elucidates the molecular biology of cellular immortalisation, but also for its potential clinical and prognostic applications.

It is generally accepted that the acquisition of an immortal phenotype is an important step in carcinogenesis, and our earlier findings have clearly demonstrated that in oral cancer progression it most commonly occurs in premalignant lesions. The percentage of biopsies presenting an immortal phenotype in culture is significantly higher in late stage oral cancer (86%) than in dysplasias (37%). Nevertheless, there is no clear indication that the dysplasias that progress to fully developed SCC are actually a subset of the immortal dysplasias, or even occur with a higher frequency in this group.

	Mortal	Immortal	Total
Leukoplakias	**1/8**	**0/5**	**1/13**
Erythro(leuko)plakias	**4/4**	**2/2**	**6/6**
Both	**5/12**	**2/7**	

This table represent the ratio of mortal and immortal dysplasias having evolved to SCC, stratified in leukoplakias versus erythroleukoplakias. Combining our data with Ken Parkinson's group's observations, only 1/13 leukoplakias evolved into SCC, and it was mortal in culture. In contrast, all of our 6 erythroleukoplakias evolved to SCC, regardless of their proliferative fate in culture. This is in keeping with the literature reports describing a clear difference in progression rate between these two types of oral dysplasias (5% for leukoplakias and 25-50% for erythroplakias within 5-10 years). It would be interesting to investigate how these two types of premalignant lesions differ in molecular terms, by comparing the genetic profiling of a reasonable number of such two types of premalignant lesions through microarray analysis. Considering that erythroplakias and leukoplakias present a substantially different progression rate, if molecular markers typically expressed in erythroplakias were found in a subset of leukoplakias they could be indicative of a

higher chance of cancer development for these lesions and therefore be of prognostic significance.

In contrast, the acquisition of unlimited proliferative potential in culture does not appear to be a good prognostic indicator in oral premalignant lesions. In fact, 2/7 (29%) immortal dysplasias and 5/12 (42%) mortal dysplasias have evolved so far, with patients being followed up for 3-7 years. However, the statistical significance of such small numbers is debatable and will be necessary to obtain data on a wider number of samples/patients before considering this data conclusive. It could be argued that although immortality generally occurs at early stages of oral cancer progression, the acquisition of such phenotype becomes of crucial and possibly of selective importance only at later stages of carcinogenesis. We currently have no data to support or discard the hypothesis that a neoplastic lesion that generates a mortal culture when sampled at the dysplasia stage could generate an immortal culture if biopsied after malignant transformation. On these grounds, it would be extremely useful and informative to obtain serial biopsies from a neoplastic site before and after progression, and assess the proliferative potential of the corresponding cultures.

As reported in literature, the histological grading of dysplasias is not thought to be a reliable prognostic indicator but, in fact, all three of our dysplasias that evolved had been graded as moderate/severe or severe/CIS. Again, this correlation is weak in view of the small numbers currently analysed, but it could prove significant after completion of the prospective study on a larger number of patients that has been set up by our group.

12.5.1 Ultimate objective

Clearly, the most valuable end point of most *research projects is their potential* clinical and prognostic applications. As extensively discussed in previous chapters, oral cancer *still* has a low 5 year survival rate, and the medical or surgical treatment of premalignant lesions often proves ineffective because of the high percentage of patients dropping out of medical treatment, or lesions recurring after laser ablation.

It would therefore be of great advantage both for the patients and for the National Health System to selectively treat those lesions that are more likely to undergo malignant transformation. At the moment, no combination of clinical or histological criteria allows this distinction. We feel that a considerable amount of effort should be spent in identifying a panel of molecular markers that have prognostic significance in oral cancer, and probably

the most appropriate approach would be microarray analysis. Even if a rigid distinction between potentially benign and malignant lesions proves impossible, being able to classify the biopsies into high risk or low risk classes would be useful. A similar rationale has been widely used over the years for other clinical conditions, like prenatal diagnosis of trisomy 21 and neural tube defects. Having proved cost effective and clinically significant in those scenarios, an analogous method could be applied with success for prevention of oral cancer.

13 Bibliography

Aaronson, D. S. and C. M. Horvath (2002). "A road map for those who know JAK-STAT." Science **296**(5573): 1653-5.

Ashcroft, M., M. H. Kubbutat and K. H. Vousden (1999). "Regulation of p53 function and stability by phosphorylation." Mol Cell Biol **19**(3): 1751-8.

Avilion, A. A., M. A. Piatyszek, J. Gupta, J. W. Shay, S. Bacchetti and C. W. Greider (1996). "Human telomerase RNA and telomerase activity in immortal cell lines and tumor tissues." Cancer Res **56**(3): 645-50.

Bacchetti, S. and C. M. Counter (1995). "Telomeres and telomerase in human cancer." International Journal of Oncology **7**: 423-432.

Baker, S. J., S. Markowitz, E. R. Fearon, J. K. Willson and B. Vogelstein (1990). "Suppression of human colorectal carcinoma cell growth by wild-type p53." Science **249**(4971): 912-5.

Banoczy, J. (1977). "Follow-up studies in oral leukoplakia." J Maxillofac Surg **5**(1): 69-75.

Bartkova, J., J. Lukas, H. Muller, M. Strauss, B. Gusterson and J. Bartek (1995). "Abnormal patterns of D-type cyclin expression and G1 regulation in human head and neck cancer." Cancer Res **55**(4): 949-56.

Bates, S., A. C. Phillips, P. A. Clark, F. Stott, G. Peters, R. L. Ludwig and K. H. Vousden (1998). "p14ARF links the tumour suppressors RB and p53." Nature **395**(6698): 124-5.

Berard, J., F. Laboune, M. Mukuna, S. Masse, R. Kothary and W. E. Bradley (1996). "Lung tumors in mice expressing an antisense RARbeta2 transgene." Faseb J **10**(9): 1091-7.

Berenson, J. R., J. Yang and R. A. Mickel (1989). "Frequent amplification of the bcl-1 locus in head and neck squamous cell carcinomas." Oncogene **4**(9): 1111-6.

Blagosklonny, M. V., P. Giannakakou, L. Y. Romanova, K. M. Ryan, K. H. Vousden and T. Fojo (2001). "Inhibition of HIF-1- and wild-type p53-stimulated transcription by codon Arg175 p53 mutants with selective loss of functions." Carcinogenesis **22**(6): 861-7.

Blasco, M. A., H. W. Lee, M. P. Hande, E. Samper, P. M. Lansdorp, R. A. DePinho and C. W. Greider (1997). "Telomere shortening and tumor formation by mouse cells lacking telomerase RNA." Cell **91**(1): 25-34.

Blaydes, J. P., V. Gire, J. M. Rowson and D. Wynford-Thomas (1997). "Tolerance of high levels of wild-type p53 in transformed epithelial cells dependent on auto-regulation by mdm-2." Oncogene **14**(15): 1859-68.

Bodnar, A. G., M. Ouellette, M. Frolkis, S. E. Holt, C. P. Chiu, G. B. Morin, C. B. Harley, J. W. Shay, S. Lichtsteiner and W. E. Wright (1998). "Extension of life-span by introduction of telomerase into normal human cells." Science **279**(5349): 349-52.

Bond, J. A., M. F. Haughton, J. M. Rowson, P. J. Smith, V. Gire, D. Wynford-Thomas and F. S. Wyllie (1999). "Control of replicative life span in human cells: barriers to clonal expansion intermediate between M1 senescence and M2 crisis." Mol Cell Biol **19**(4): 3103-14.

Bond, J. A., F. S. Wyllie and D. Wynford-Thomas (1994). "Escape from senescence in human diploid fibroblasts induced directly by mutant p53." Oncogene **9**(7): 1885-9.

Bos, M., J. Mendelsohn, Y. M. Kim, J. Albanell, D. W. Fry and J. Baselga (1997). "PD153035, a tyrosine kinase inhibitor, prevents epidermal growth factor receptor activation and inhibits growth of cancer cells in a receptor number-dependent manner." Clin Cancer Res **3**(11): 2099-106.

Bova, R. J., D. I. Quinn, J. S. Nankervis, I. E. Cole, B. F. Sheridan, M. J. Jensen, G. J. Morgan, C. J. Hughes and R. L. Sutherland (1999). "Cyclin D1 and p16INK4A expression predict reduced survival in carcinoma of the anterior tongue." Clin Cancer Res **5**(10): 2810-9.

Bowman, T., R. Garcia, J. Turkson and R. Jove (2000). "STATs in oncogenesis." Oncogene **19**(21): 2474-88.

Boyle, J. O., J. Hakim, W. Koch, P. van der Riet, R. H. Hruban, R. A. Roa, R. Correo, Y. J. Eby, J. M. Ruppert and D. Sidransky (1993). "The incidence of p53 mutations increases with progression of head and neck cancer." Cancer Res **53**(19): 4477-80.

Boyle, P., G. J. Macfarlane, W. J. Blot, F. Chiesa, J. L. Lefebvre, A. M. Azul, N. de Vries and C. Scully (1995). "European School of Oncology Advisory report to the European Commission for the Europe Against Cancer Programme: oral carcinogenesis in Europe." Eur J Cancer B Oral Oncol **31B**(2): 75-85.

Boyle, P., G. J. Macfarlane, T. Zheng, P. Maisonneuve, T. Evstifeeva and C. Scully (1992). "Recent advances in epidemiology of head and neck cancer." Curr Opin Oncol **4**(3): 471-7.

Brennan, J. A., J. O. Boyle, W. M. Koch, S. N. Goodman, R. H. Hruban, Y. J. Eby, M. J. Couch, A. A. Forastiere and D. Sidransky (1995). "Association between cigarette smoking and mutation of the p53 gene in squamous-cell carcinoma of the head and neck." N Engl J Med **332**(11): 712-7.

Brennan, P. and I. Bray (2002). "Recent trends and future directions for lung cancer mortality in Europe." Br J Cancer **87**: 43-48.

Bryan, T. M., A. Englezou, J. Gupta, S. Bacchetti and R. R. Reddel (1995). "Telomere elongation in immortal human cells without detectable telomerase activity." Embo J **14**(17): 4240-8.

Burns, J. E., M. C. Baird, L. J. Clark, P. A. Burns, K. Edington, C. Chapman, R. Mitchell, G. Robertson, D. Soutar and E. K. Parkinson (1993). "Gene mutations and increased levels of p53 protein in human squamous cell carcinomas and their cell lines." Br J Cancer **67**(6): 1274-84.

Burns, J. E., R. McFarlane, L. J. Clark, R. Mitchell, G. Robertson, D. Soutar and E. K. Parkinson (1994). "Maintenance of identical p53 mutations throughout progression of squamous cell carcinomas of the tongue." Eur J Cancer B Oral Oncol **30B**(5): 335-7.

Cairns, P., T. J. Polascik, Y. Eby, K. Tokino, J. Califano, A. Merlo, L. Mao, J. Herath, R. Jenkins, W. Westra and et al. (1995). "Frequency of homozygous deletion at p16/CDKN2 in primary human tumours." Nat Genet **11**(2): 210-2.

Califano, J., P. van der Riet, W. Westra, H. Nawroz, G. Clayman, S. Piantadosi, R. Corio, D. Lee, B. Greenberg, W. Koch and D. Sidransky (1996). "Genetic progression model for head and neck cancer: implications for field cancerization." Cancer Res **56**(11): 2488-92.

Callender, T., A. K. el-Naggar, M. S. Lee, R. Frankenthaler, M. A. Luna and J. G. Batsakis (1994). "PRAD-1 (CCND1)/cyclin D1 oncogene amplification in primary head and neck squamous cell carcinoma." Cancer **74**(1): 152-8.

Canman, C. E., D. S. Lim, K. A. Cimprich, Y. Taya, K. Tamai, K. Sakaguchi, E. Appella, M. B. Kastan and J. D. Siliciano (1998). "Activation of the ATM kinase by ionizing radiation and phosphorylation of p53." Science **281**(5383): 1677-9.

Carlos de Vicente, J., A. Herrero-Zapatero, M. F. Fresno and J. S. Lopez-Arranz (2002). "Expression of cyclin D1 and Ki-67 in squamous cell carcinoma of the oral cavity: clinicopathological and prognostic significance." Oral Oncol **38**(3): 301-8.

Chan, T. A., H. Hermeking, C. Lengauer, K. W. Kinzler and B. Vogelstein (1999). "14-3-3Sigma is required to prevent mitotic catastrophe after DNA damage." Nature **401**(6753): 616-20.

Chan, T. A., P. M. Hwang, H. Hermeking, K. W. Kinzler and B. Vogelstein (2000). "Cooperative effects of genes controlling the G(2)/M checkpoint." Genes Dev **14**(13): 1584-8.

Chiesa, F., N. Tradati, M. Marazza, N. Rossi, P. Boracchi, L. Mariani, M. Clerici, F. Formelli, L. Barzan, A. Carrassi and et al. (1992). "Prevention of local relapses and new localisations of oral leukoplakias with the synthetic retinoid fenretinide (4-HPR). Preliminary results." Eur J Cancer B Oral Oncol **28B**(2): 97-102.

Choi, Y. and E. Fuchs (1990). "TGF-beta and retinoic acid: regulators of growth and modifiers of differentiation in human epidermal cells." Cell Regul **1**(11): 791-809.

Colgin, L. M. and R. R. Reddel (1999). "Telomere maintenance mechanisms and cellular immortalization." Curr Opin Genet Dev **9**(1): 97-103.

Colgin, L. M., C. Wilkinson, A. Englezou, A. Kilian, M. O. Robinson and R. R. Reddel (2000). "The hTERTalpha splice variant is a dominant negative inhibitor of telomerase activity." Neoplasia **2**(5): 426-32.

Cong, Y. S., J. Wen and S. Bacchetti (1999). "The human telomerase catalytic subunit hTERT: organization of the gene and characterization of the promoter." Hum Mol Genet **8**(1): 137-42.

Counter, C. M., H. W. Hirte, S. Bacchetti and C. B. Harley (1994). "Telomerase activity in human ovarian carcinoma." Proc Natl Acad Sci U S A **91**(8): 2900-4.

Crow, J. A. and D. E. Ong (1985). "Cell-specific immunohistochemical localization of a cellular retinol-binding protein (type two) in the small intestine of the rat." Proc Natl Acad Sci U S A **82**: 4707-4711.

Crowe, D. L., L. Hu, L. J. Gudas and J. G. Rheinwald (1991). "Variable expression of retinoic acid receptor (RAR beta) mRNA in human oral and epidermal keratinocytes; relation to keratin 19 expression and keratinization potential." Differentiation **48**(3): 199-208.

Cuthbert, A. P., J. Bond, D. A. Trott, S. Gill, J. Broni, A. Marriott, G. Khoudoli, E. K. Parkinson, C. S. Cooper and R. F. Newbold (1999). "Telomerase repressor sequences on chromosome 3 and induction of permanent growth arrest in human breast cancer cells." J Natl Cancer Inst **91**(1): 37-45.

David, M., L. Wong, R. Flavell, S. A. Thompson, A. Wells, A. C. Larner and G. R. Johnson (1996). "STAT activation by epidermal growth factor (EGF) and amphiregulin. Requirement for the EGF receptor kinase but not for tyrosine phosphorylation sites or JAK1." J Biol Chem **271**(16): 9185-8.

de Stanchina, E., M. E. McCurrach, F. Zindy, S. Y. Shieh, G. Ferbeyre, A. V. Samuelson, C. Prives, M. F. Roussel, C. J. Sherr and S. W. Lowe (1998). "E1A signaling to p53 involves the p19(ARF) tumor suppressor." Genes Dev **12**(15): 2434-42.

Dickson, M. A., W. C. Hahn, Y. Ino, V. Ronfard, J. Y. Wu, R. A. Weinberg, D. N. Louis, F. P. Li and J. G. Rheinwald (2000). "Human keratinocytes that express hTERT and also bypass a p16(INK4a)- enforced mechanism that limits life span become immortal yet retain normal growth and differentiation characteristics." Mol Cell Biol **20**(4): 1436-47.

Dimri, G. P., K. Itahana, M. Acosta and J. Campisi (2000). "Regulation of a senescence checkpoint response by the E2F1 transcription factor and p14(ARF) tumor suppressor." Mol Cell Biol **20**(1): 273-85.

Dimri, G. P., X. Lee, G. Basile, M. Acosta, G. Scott, C. Roskelley, E. E. Medrano, M. Linskens, I. Rubelj, O. Pereira-Smith and et al. (1995). "A biomarker that identifies senescent human cells in culture and in aging skin in vivo." Proc Natl Acad Sci U S A **92**(20): 9363-7.

Dome, J. S., S. Chung, T. Bergemann, C. B. Umbricht, M. Saji, L. A. Carey, P. E. Grundy, E. J. Perlman, N. E. Breslow and S. Sukumar (1999). "High telomerase reverse transcriptase (hTERT) messenger RNA level correlates with tumor recurrence in patients with favorable histology Wilms' tumor." Cancer Res **59**(17): 4301-7.

Ducrest, A. L., H. Szutorisz, J. Lingner and M. Nabholz (2002). "Regulation of the human telomerase reverse transcriptase gene." Oncogene **21**(4): 541-52.

Edington, K. G., O. P. Loughran, I. J. Berry and E. K. Parkinson (1995). "Cellular immortality: a late event in the progression of human squamous cell carcinoma of the head and neck associated with p53 alteration and a high frequency of allele loss." Mol Carcinog **13**(4): 254-65.

el-Deiry, W. S., T. Tokino, V. E. Velculescu, D. B. Levy, R. Parsons, J. M. Trent, D. Lin, W. E. Mercer, K. W. Kinzler and B. Vogelstein (1993). "WAF1, a potential mediator of p53 tumor suppression." Cell **75**(4): 817-25.

Elenbaas, B., L. Spirio, F. Koerner, M. D. Fleming, D. B. Zimonjic, J. L. Donaher, N. C. Popescu, W. C. Hahn and R. A. Weinberg (2001). "Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells." Genes Dev **15**(1): 50-65.

Faust, J. B. and T. C. Meeker (1992). "Amplification and expression of the bcl-1 gene in human solid tumor cell lines." Cancer Res **52**(9): 2460-3.

Feng, J., W. D. Funk, S. S. Wang, S. L. Weinrich, A. A. Avilion, C. P. Chiu, R. R. Adams, E. Chang, R. C. Allsopp, J. Yu and et al. (1995). "The RNA component of human telomerase." Science **269**(5228): 1236-41.

Field, J. K., A. Malliri, S. A. Butt, J. R. Grosney, D. Phillips, D. A. Spandidos and A. S. Jones (1993). "p53 expression in end stage squamous cell carcinoma of the head and neck prior to chemotherapy treatment: expression correlates with a very poor clinical outcome." International Journal of Oncology **3**: 431-435.

Finlay, C. A., P. W. Hinds, T. H. Tan, D. Eliyahu, M. Oren and A. J. Levine (1988). "Activating mutations for transformation by p53 produce a gene product that forms an hsc70-p53 complex with an altered half-life." Mol Cell Biol **8**(2): 531-9.

Firpo, E. J., A. Koff, M. J. Solomon and J. M. Roberts (1994). "Inactivation of a Cdk2 inhibitor during interleukin 2-induced proliferation of human T lymphocytes." Mol Cell Biol **14**(7): 4889-901.

Forrester, K., S. E. Lupold, V. L. Ott, C. H. Chay, V. Band, X. W. Wang and C. C. Harris (1995). "Effects of p53 mutants on wild-type p53-mediated transactivation are cell type dependent." Oncogene **10**(11): 2103-11.

Foster, S. A. and D. A. Galloway (1996). "Human papillomavirus type 16 E7 alleviates a proliferation block in early passage human mammary epithelial cells." Oncogene **12**(8): 1773-9.

Fuchs, E. (1990). "Epidermal differentiation: the bare essentials." J Cell Biol **111**(6 Pt 2): 2807-14.

Gallo, O., M. Santucci and A. Franchi (1997). "Cumulative prognostic value of p16/CDKN2 and p53 oncoprotein expression in premalignant laryngeal lesions." J Natl Cancer Inst **89**(15): 1161-3.

Girard, F., U. Strausfeld, A. Fernandez and N. J. Lamb (1991). "Cyclin A is required for the onset of DNA replication in mammalian fibroblasts." Cell **67**(6): 1169-79.

Gluckman, J. L., P. J. Stambrook and Z. P. Pavelic (1994). "Prognostic significance of p53 protein accumulation in early stage T1 oral cavity cancer [letter]." Eur J Cancer B Oral Oncol **30B**(4): 281.

Gonzalez-Suarez, E., E. Samper, J. M. Flores and M. A. Blasco (2000). "Telomerase-deficient mice with short telomeres are resistant to skin tumorigenesis." Nat Genet **26**(1): 114-7.

Gonzalez-Suarez, E., E. Samper, A. Ramirez, J. M. Flores, J. Martin-Caballero, J. L. Jorcano and M. A. Blasco (2001). "Increased epidermal tumors and increased skin wound healing in transgenic mice overexpressing the catalytic subunit of telomerase, mTERT, in basal keratinocytes." Embo J **20**(11): 2619-30.

Grandis, J. R. and D. J. Tweardy (1993). "Elevated levels of transforming growth factor alpha and epidermal growth factor receptor messenger RNA are early markers of carcinogenesis in head and neck cancer." Cancer Res **53**(15): 3579-84.

Grandis, J. R., M. F. Melhem, E. L. Barnes and D. J. Tweardy (1996). "Quantitative immunohistochemical analysis of transforming growth factor- alpha and epidermal growth factor receptor in patients with squamous cell carcinoma of the head and neck." Cancer **78**(6): 1284-92.

Grandis, J. R., A. Chakraborty, M. F. Melhem and D. J. Tweardy (1997). "Inhibition of epidermal growth factor receptor gene expression and function decreases proliferationof head and neck squamous cell carcinoma but not normal mucosal epithelial cells." Oncogene **15**: 409-416.

Grandis, J. R., S. D. Drenning, A. Chakraborty, M. Y. Zhou, Q. Zeng, A. S. Pitt and D. J. Tweardy (1998). "Requirement of Stat3 but not Stat1 activation for epidermal growth factor receptor- mediated cell growth In vitro." J Clin Invest **102**(7): 1385-92. (a)

Grandis, J. R., A. Chakraborty, Q. Zeng, M. F. Melhem and D. J. Tweardy (1998). "Downmodulation of TGF-alpha protein expression with antisense oligonucleotides inhibits proliferation of head and neck squamous carcinoma but not normal mucosal epithelial cells." J Cell Biochem **69**(1): 55-62. (b)

Grandis, J. R., D. J. Tweardy and M. F. Melhem (1998). "Asynchronous modulation of transforming growth factor alpha and epidermal growth factor receptor protein expression in progression of premalignant lesions to head and neck squamous cell carcinoma." Clin Cancer Res **4**(1): 13-20. (c)

Grandis, J. R., M. F. Melhem, W. E. Gooding, R. Day, V. A. Holst, M. M. Wagener, S. D. Drenning and D. J. Tweardy (1998). "Levels of TGF-alpha and EGFR protein in head and neck squamous cell carcinoma and patient survival." J Natl Cancer Inst **90**(11): 824-32. (d)

Grandis, J. R., S. D. Drenning, Q. Zeng, S. C. Watkins, M. F. Melhem, S. Endo, D. E. Johnson, L. Huang, Y. He and J. D. Kim (2000). "Constitutive activation of Stat3 signaling abrogates apoptosis in squamous cell carcinogenesis in vivo." Proc Natl Acad Sci U S A **97**(8): 4227-32.

Greenberg, R. A., R. C. O'Hagan, H. Deng, Q. Xiao, S. R. Hann, R. R. Adams, S. Lichtsteiner, L. Chin, G. B. Morin and R. A. DePinho (1999). "Telomerase reverse transcriptase gene is a direct target of c-Myc but is not functionally equivalent in cellular transformation." Oncogene **18**(5): 1219-26.

Greider, C. W. and E. H. Blackburn (1985). "Identification of a specific telomere terminal transferase activity in Tetrahymena extracts." Cell **43**(2 Pt 1): 405-13.

Griffith, J. D., L. Comeau, S. Rosenfield, R. M. Stansel, A. Bianchi, H. Moss and T. de Lange (1999). "Mammalian telomeres end in a large duplex loop." Cell **97**(4): 503-14.

Hachiya, M., A. Chumakov, C. W. Miller, M. Akashi, J. Said and H. P. Koeffler (1994). "Mutant p53 proteins behave in a dominant, negative fashion in vivo." Anticancer Res **14**(5A): 1853-9.

Hahn, W. C., C. M. Counter, A. S. Lundberg, R. L. Beijersbergen, M. W. Brooks and R. A. Weinberg (1999). "Creation of human tumour cells with defined genetic elements." Nature **400**(6743): 464-8. (a)

Hahn, W. C., S. A. Stewart, M. W. Brooks, S. G. York, E. Eaton, A. Kurachi, R. L. Beijersbergen, J. H. Knoll, M. Meyerson and R. A. Weinberg (1999). "Inhibition of telomerase limits the growth of human cancer cells." Nat Med **5**(10): 1164-70. (b)

Hainaut, P. and M. Hollstein (2000). "p53 and human cancer: the first ten thousand mutations." Adv Cancer Res **77**: 81-137.

Han, J., L. Jiao, Y. Lu, Z. Sun, Q. M. Gu and K. J. Scanlon (1990). "Evaluation of N-4-(hydroxycarbophenyl) retinamide as a cancer prevention agent and as a cancer chemotherapeutic agent." In Vivo **4**(3): 153-60.

Harle-Bachor, C. and P. Boukamp (1996). "Telomerase activity in the regenerative basal layer of the epidermis inhuman skin and in immortal and carcinoma-derived skin keratinocytes." Proc Natl Acad Sci U S A **93**(13): 6476-81.

Harper, J. W., S. J. Ellèdge, K. Keyomarsi, B. Dynlacht, L. H. Tsai, P. Zhang, S. Dobrowolski, C. Bai, L. Connell-Crowley, E. Swindell and et al. (1995). "Inhibition of cyclin-dependent kinases by p21." Mol Biol Cell **6**(4): 387-400.

Hayflick, L. (1965). "The limited in-vitro lifetime of human diploid cell strains." Exp Cell Res **37**: 614-636.

Hirao, A., Y. Y. Kong, S. Matsuoka, A. Wakeham, J. Ruland, H. Yoshida, D. Liu, S. J. Elledge and T. W. Mak (2000). "DNA damage-induced activation of p53 by the checkpoint kinase Chk2." Science **287**(5459): 1824-7.

Hong, W. K., J. Endicott, L. M. Itri, W. Doos, J. G. Batsakis, R. Bell, S. Fofonoff, R. Byers, E. N. Atkinson, C. Vaughan and et al. (1986). "13-cis-retinoic acid in the treatment of oral leukoplakia." N Engl J Med **315**(24): 1501-5.

Houle, B., C. Rochette-Egly and W. E. Bradley (1993). "Tumor-suppressive effect of the retinoic acid receptor beta in human epidermoid lung cancer cells." Proc Natl Acad Sci U S A **90**(3): 985-9.

Hu, L., D. L. Crowe, J. G. Rheinwald, P. Chambon and L. J. Gudas (1991). "Abnormal expression of retinoic acid receptors and keratin 19 by human oral and epidermal squamous cell carcinoma cell lines." Cancer Res **51**(15): 3972-81.

Ishitoya, J., M. Toriyama, N. Oguchi, K. Kitamura, M. Ohshima, K. Asano and T. Yamamoto (1989). "Gene amplification and overexpression of EGF receptor in squamous cell carcinomas of the head and neck." Br J Cancer **59**(4): 559-62.

Jares, P., P. L. Fernandez, E. Campo, A. Nadal, F. Bosch, G. Aiza, I. Nayach, J. Traserra and A. Cardesa (1994). "PRAD-1/cyclin D1 gene amplification correlates with messenger RNA overexpression and tumor progression in human laryngeal carcinomas." Cancer Res **54**(17): 4813-7.

Kamijo, T., F. Zindy, M. F. Roussel, D. E. Quelle, J. R. Downing, R. A. Ashmun, G. Grosveld and C. J. Sherr (1997). "Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF." Cell **91**(5): 649-59.

Kapoor, M. and G. Lozano (1998). "Functional activation of p53 via phosphorylation following DNA damage by UV but not gamma radiation." Proc Natl Acad Sci U S A **95**(6): 2834-7.

Kashiwazaki, H., H. Tonoki, M. Tada, I. Chiba, M. Shindoh, Y. Totsuka, R. Iggo and T. Moriuchi (1997). "High frequency of p53 mutations in human oral epithelial dysplasia and primary squamous cell carcinoma detected by yeast functional assay." Oncogene **15**(22): 2667-74.

Kastan, M. B., Q. Zhan, W. S. el-Deiry, F. Carrier, T. Jacks, W. V. Walsh, B. S. Plunkett, B. Vogelstein and A. J. Fornace, Jr. (1992). "A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia." Cell **71**(4): 587-97.

Kato, J., H. Matsushime, S. W. Hiebert, M. E. Ewen and C. J. Sherr (1993). "Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4." Genes Dev **7**(3): 331-42.

Kaur, J., A. Srivastava and R. Ralhan (1998). "Prognostic significance of p53 protein overexpression in betel- and tobacco-related oral oncogenesis." Int J Cancer **79**(4): 370-5.

Kilian, A., D. D. Bowtell, H. E. Abud, G. R. Hime, D. J. Venter, P. K. Keese, E. L. Duncan, R. R. Reddel and R. A. Jefferson (1997). "Isolation of a candidate human telomerase catalytic subunit gene, which reveals complex splicing patterns in different cell types." Hum Mol Genet *6(12): 2011-9.*

Kim, N. W., M. A. Piatyszek, K. R. Prowse, C. B. Harley, M. D. West, P. L. Ho, G. M. Coviello, W. E. Wright, S. L. Weinrich and J. W. Shay (1994). "Specific association of human telomerase activity with immortal cells and cancer." Science **266**(5193): 2011-5.

Kim, S. H., P. Kaminker and J. Campisi (2002). "Telomeres, aging and cancer: in search of a happy ending." Oncogene **21**(4): 503-11.

Kiyono, T., S. A. Foster, J. I. Koop, J. K. McDougall, D. A. Galloway and A. J. Klingelhutz (1998). "Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells." Nature **396**(6706): 84-8.

Klapper, L. N., M. H. Kirschbaum, M. Sela and Y. Yarden (2000). "Biochemical and clinical implications of the ErbB/HER signaling network of growth factor receptors." Adv Cancer Res **77**: 25-79.

Klingelhutz, A. J., S. A. Foster and J. K. McDougall (1996). "Telomerase activation by the E6 gene product of human papillomavirus type 16." Nature **380**(6569): 79-82.

Knecht, R., R. Elez, M. Oechler, C. Solbach, C. von Ilberg and K. Strebhardt (1999). "Prognostic significance of polo-like kinase (PLK) expression in squamous cell carcinomas of the head and neck." Cancer Res **59**(12): 2794-7.

Koff, A., A. Giordano, D. Desai, K. Yamashita, J. W. Harper, S. Elledge, T. Nishimoto, D. O. Morgan, B. R. Franza and J. M. Roberts (1992). "Formation and activation of a cyclin E-cdk2 complex during the G1 phase of the human cell cycle." Science **257**(5077): 1689-94.

Kolquist, K. A., L. W. Ellisen, C. M. Counter, M. Meyerson, L. K. Tan, R. A. Weinberg, D. A. Haber and W. L. Gerald (1998). "Expression of TERT in early premalignant lesions and a subset of cells in normal tissues." Nat Genet **19**(2): 182-6.

Koontongkaew, S., L. Chareonkitkajorn, A. Chanvitan, M. Leelakriangsak and P. Amornphimoltham (2000). "Alterations of p53, pRb, cyclin D(1) and cdk4 in human oral and pharyngeal squamous cell carcinomas." Oral Oncol **36**(4): 334-9.

Kramer, I. R. (1969). "Precancerous conditions of the oral mucosa. A computer-aided study." Ann R Coll Surg Engl **45**(6): 340-56.

Kramer, I. R., R. B. Lucas, J. J. Pindborg and L. H. Sobin (1978). "Definition of leukoplakia and related lesions: an aid to studies on oral precancer." Oral Surg Oral Med Oral Pathol **46**(4): 518-39.

Kropveld, A., A. D. van Mansfeld, N. Nabben, G. J. Hordijk and P. J. Slootweg (1996). "Discordance of p53 status in matched primary tumours and metastases in head and neck squamous cell carcinoma patients." Eur J Cancer B Oral Oncol **32B**(6): 388-93.

Kubbutat, M. H., S. N. Jones and K. H. Vousden (1997). "Regulation of p53 stability by Mdm2." Nature **387**(6630): 299-303.

Lazarus, P., J. Stern, N. Zwiebel, A. Fair, J. P. Richie, Jr. and S. Schantz (1996). "Relationship between p53 mutation incidence in oral cavity squamous cell carcinomas and patient tobacco use." Carcinogenesis **17**(4): 733-9.

Lee, H. W., M. A. Blasco, G. J. Gottlieb, J. W. Horner, 2nd, C. W. Greider and R. A. DePinho (1998). "Essential role of mouse telomerase in highly proliferative organs." Nature **392**(6676): 569-74.

Leid, M., P. Kastner and P. Chambon (1992). "Multiplicity generates diversity in the retinoic acid signalling pathways." Trends Biochem Sci **17**(10): 427-33.

Leonard, J. H., J. H. Kearsley, G. Chenevix-Trench and N. K. Hayward (1991). "Analysis of gene amplification in head-and-neck squamous-cell carcinoma." Int J Cancer **48**(4): 511-5.

Levine, A. J. (1997). "p53, the cellular gatekeeper for growth and division." Cell **88**(3): 323-31.

Li, Y., M. A. Nichols, J. W. Shay and Y. Xiong (1994). "Transcriptional repression of the D-type cyclin-dependent kinase inhibitor p16 by the retinoblastoma susceptibility gene product pRb." Cancer Res **54**(23): 6078-82.

Lippman, S. M., J. G. Batsakis, B. B. Toth, R. S. Weber, J. J. Lee, J. W. Martin, G. L. Hays, H. Goepfert and W. K. Hong (1993). "Comparison of low-dose isotretinoin with beta carotene to prevent oral carcinogenesis." N Engl J Med **328**(1): 15-20.

Liu, Q., S. Guntuku, X. S. Cui, S. Matsuoka, D. Cortez, K. Tamai, G. Luo, S. Carattini-Rivera, F. DeMayo, A. Bradley, L. A. Donehower and S. J. Elledge (2000). "Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint." Genes Dev **14**(12): 1448-59.

Lotan, R., X. C. Xu, S. M. Lippman, J. Y. Ro, J. S. Lee, J. J. Lee and W. K. Hong (1995). "Suppression of retinoic acid receptor-beta in premalignant oral lesions and its up-regulation by isotretinoin." N Engl J Med **332**(21): 1405-10.

Loughran, O., A. Malliri, D. Owens, P. H. Gallimore, M. A. Stanley, B. Ozanne, M. C. Frame and E. K. Parkinson (1996). "Association of CDKN2A/p16INK4A with human head and neck keratinocyte replicative senescence: relationship of dysfunction to immortality and neoplasia." Oncogene **13**(3): 561-8.

Macfarlane, G. J., T. Zheng, J. R. Marshall, P. Boffetta, S. Niu, J. Brasure, F. Merletti and P. Boyle (1995). "Alcohol, tobacco, diet and the risk of oral cancer: a pooled analysis of three case-control studies." Eur J Cancer B Oral Oncol **31B**(3): 181-7.

Mano, Y., T. Shimizu, S. Tanuma and K. Takeda (2000). "Synergistic down-regulation of telomerase activity and hTERT mRNA expression by combination of retinoic acid and GM-CSF in human myeloblastic leukemia ML-1 cells." Anticancer Res **20**(3A): 1649-52.

Martin, G. M., C. A. Sprague and C. J. Epstein (1970). "Replicative life-span of cultivated human cells. Effects of donor's age, tissue, and genotype." Lab Invest **23**(1): 86-92.

Matsushime, H., D. E. Quelle, S. A. Shurtleff, M. Shibuya, C. J. Sherr and J. Y. Kato (1994). "D-type cyclin-dependent kinase activity in mammalian cells." Mol Cell Biol **14**(3): 2066-76.

McGregor, F., A. Muntoni, J. Fleming, J. Brown, D. H. Felix, G. MacDonald, E. K. Parkinspon and P. R. Harrison (2002). "Molecular changes associated with oral dysplasia progression and acquisition of immortality: potential for its reversal by 5-azacytidine." Cancer Res **62**(August 15).

McGregor, F., E. Wagner, D. Felix, D. Soutar, K. Parkinson and P. R. Harrison (1997). "Inappropriate retinoic acid receptor-beta expression in oral dysplasias: correlation with acquisition of the immortal phenotype." Cancer Res **57**(18): 3886-9.

Merlo, A., J. G. Herman, L. Mao, D. J. Lee, E. Gabrielson, P. C. Burger, S. B. Baylin and D. Sidransky (1995). "5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers [see comments]." Nat Med **1**(7): 686-92.

Meyerson, M., C. M. Counter, E. N. Eaton, L. W. Ellisen, P. Steiner, S. D. Caddle, L. Ziaugra, R. L. Beijersbergen, M. J. Davidoff, Q. Liu, S. Bacchetti, D. A. Haber and R. A. Weinberg (1997). "hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization." Cell **90**(4): 785-95.

Michalides, R., N. van Veelen, A. Hart, B. Loftus, E. Wientjens and A. Balm (1995). "Overexpression of cyclin D1 correlates with recurrence in a group of forty-seven operable squamous cell carcinomas of the head and neck." Cancer Res **55**(5): 975-8.

Mineta, H., A. Borg, M. Dictor, P. Wahlberg, J. Akervall and J. Wennerberg (1998). "p53 mutation, but not p53 overexpression, correlates with survival in head and neck squamous cell carcinoma." Br J Cancer **78**(8): 1084-90.

Miracca, E. C., L. P. Kowalski and M. A. Nagai (1999). "High prevalence of p16 genetic alterations in head and neck tumours." Br J Cancer **81**(4): 677-83.

Miyashita, T. and J. C. Reed (1995). "Tumor suppressor p53 is a direct transcriptional activator of the human bax gene." Cell **80**(2): 293-9.

Miyoshi, Y., K. Tsukinoki, T. Imaizumi, Y. Yamada, T. Ishizaki, Y. Watanabe, Y. Sasakura, Y. Lin, M. Hosaka and Y. Kubota (1999). "Telomerase activity in oral cancer." Oral Oncol **35**(3): 283-9.

Morales, C. P., S. E. Holt, M. Ouellette, K. J. Kaur, Y. Yan, K. S. Wilson, M. A. White, W. E. Wright and J. W. Shay (1999). "Absence of cancer-associated changes in human fibroblasts immortalized with telomerase." Nat Genet **21**(1): 115-8.

Mori, T., K. Miura, T. Aoki, T. Nishihira, S. Mori and Y. Nakamura (1994). "Frequent somatic mutation of the MTS1/CDK4I (multiple tumor suppressor/cyclin-dependent kinase 4 inhibitor) gene in esophageal squamous cell carcinoma." Cancer Res **54**(13): 3396-7.

Morin, G. B. (1989). "The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats." Cell **59**(3): 521-9.

Morris, M., P. Hepburn and D. Wynford-Thomas (2002). "Sequential extension of proliferative lifespan in human fibroblasts induced by over-expression of CDK4 or 6 and loss of p53 function." Oncogene **21**(27): 4277-88.

Motokura, T., T. Bloom, H. G. Kim, H. Juppner, J. V. Ruderman, H. M. Kronenberg and A. Arnold (1991). "A novel cyclin encoded by a bcl1-linked candidate oncogene." Nature **350**(6318): 512-5.

Munro, J., K. Steeghs, V. Morrison, H. Ireland and E. K. Parkinson (2001). "Human fibroblast replicative senescence can occur in the absence of extensive cell division and short telomeres." Oncogene **20**(27): 3541-52.

Munro, J., F. J. Stott, K. H. Vousden, G. Peters and E. K. Parkinson (1999). "Role of the alternative INK4A proteins in human keratinocyte senescence: evidence for the specific inactivation of p16INK4A upon immortalization." Cancer Res **59**(11): 2516-21.

Murray, A. W. (1989). "Cyclin synthesis and degradation and the embryonic cell cycle." J Cell Sci Suppl **12**: 65-76.

Mutirangura, A., P. Supiyaphun, S. Trirekapan, V. Sriuranpong, A. Sakuntabhai, S. Yenrudi and N. Voravud (1996). "Telomerase activity in oral leukoplakia and head and neck squamous cell carcinoma." Cancer Res **56**(15): 3530-3.

Nakano, K. and K. H. Vousden (2001). "PUMA, a novel proapoptotic gene, is induced by p53." Mol Cell **7**(3): 683-94.

Nawroz, H., P. van der Riet, R. H. Hruban, W. Koch, J. M. Ruppert and D. Sidransky (1994). "Allelotype of head and neck squamous cell carcinoma." Cancer Res **54**(5): 1152-5.

Nilsson, P., C. Mehle, K. Remes and G. Roos (1994). "Telomerase activity in vivo in human malignant hematopoietic cells." Oncogene **9**(10): 3043-8.

O-charoenrat, P., P. H. Rhys-Evans, D. J. Archer and S. A. Eccles (2002). "C-erbB receptors in squamous cell carcinomas of the head and neck: clinical significance and correlation with matrix metalloproteinases and vascular endothelial growth factors." Oral Oncol **38**(1): 73-80.

Oda, E., R. Ohki, H. Murasawa, J. Nemoto, T. Shibue, T. Yamashita, T. Tokino, T. Taniguchi and N. Tanaka (2000). "Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis." Science **288**(5468): 1053-8. (a)

Oda, K., H. Arakawa, T. Tanaka, K. Matsuda, C. Tanikawa, T. Mori, H. Nishimori, K. Tamai, T. Tokino, Y. Nakamura and Y. Taya (2000). "p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53." Cell **102**(6): 849-62. (b)

Oh, S., Y. Song, J. Yim and T. K. Kim (1999). "The Wilms' tumor 1 tumor suppressor gene represses transcription of the human telomerase reverse transcriptase gene." J Biol Chem **274**(52): 37473-8.

Oh, S., Y. H. Song, J. Yim and T. K. Kim (2000). "Identification of Mad as a repressor of the human telomerase (hTERT) gene." Oncogene **19**(11): 1485-90.

Okami, K., A. L. Reed, P. Cairns, W. M. Koch, W. H. Westra, S. Wehage, J. Jen and D. Sidransky (1999). "Cyclin D1 amplification is independent of p16 inactivation in head and neck squamous cell carcinoma." Oncogene **18**(23): 3541-5.

Okamoto, A., D. J. Demetrick, E. A. Spillare, K. Hagiwara, S. P. Hussain, W. P. Bennett, K. Forrester, B. Gerwin, M. Serrano, D. H. Beach and et al. (1994). "Mutations and altered expression of p16INK4 in human cancer." Proc Natl Acad Sci U S A **91**(23): 11045-9.

Opitz, O. G., Y. Suliman, W. C. Hahn, H. Harada, H. E. Blum and A. K. Rustgi (2001). "Cyclin D1 overexpression and p53 inactivation immortalize primary oral keratinocytes by a telomerase-independent mechanism." J Clin Invest **108**(5): 725-32.

Ozanne, B., C. S. Richards, F. Hendler, D. Burns and B. Gusterson (1986). "Over-expression of the EGF receptor is a hallmark of squamous cell carcinomas." J Pathol **149**(1): 9-14.

Pagano, M., R. Pepperkok, J. Lukas, V. Baldin, W. Ansorge, J. Bartek and G. Draetta (1993). "Regulation of the cell cycle by the cdk2 protein kinase in cultured human fibroblasts." J Cell Biol **121**(1): 101-11.

Papadimitrakopoulou, V., J. Izzo, S. M. Lippman, J. S. Lee, Y. H. Fan, G. Clayman, J. Y. Ro, W. N. Hittelman, R. Lotan, W. K. Hong and L. Mao (1997). "Frequent inactivation of p16INK4a in oral premalignant lesions." Oncogene **14**(15): 1799-803.

Parkin, D. M., P. Pisani and J. Ferlay (1999). "Estimates of the worldwide incidence of 25 major cancers in 1990." Int J Cancer **80**(6): 827-41.

Parry, D., S. Bates, D. J. Mann and G. Peters (1995). "Lack of cyclin D-Cdk complexes in Rb-negative cells correlates with high levels of p16INK4/MTS1 tumour suppressor gene product." Embo J **14**(3): 503-11.

Pendino, F., M. Flexor, F. Delhommeau, D. Buet, M. Lanotte and E. Segal-Bendirdjian (2001). "Retinoids down-regulate telomerase and telomere length in a pathway distinct from leukemia cell differentiation." Proc Natl Acad Sci U S A **98**(12): 6662-7.

Pines, J. and T. Hunter (1991). "Human cyclins A and B1 are differentially located in the cell and undergo cell cycle-dependent nuclear transport." J Cell Biol **115**(1): 1-17.

Poddar, S., W. K. Hong, S. M. Thacher and R. Lotan (1991). "Retinoic acid suppression of squamous differentiation in human head-and-neck squamous carcinoma cells." Int J Cancer **48**(2): 239-47.

Polyak, K., J. Y. Kato, M. J. Solomon, C. J. Sherr, J. Massague, J. M. Roberts and A. Koff (1994). "p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest." Genes Dev **8**(1): 9-22.

Quelle, D. E., M. Cheng, R. A. Ashmun and C. J. Sherr (1997). "Cancer-associated mutations at the INK4a locus cancel cell cycle arrest by p16INK4a but not by the alternative reading frame protein p19ARF." Proc Natl Acad Sci U S A **94**(2): 669-73.

Quelle, D. E., F. Zindy, R. A. Ashmun and C. J. Sherr (1995). "Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest." Cell **83**(6): 993-1000.

Ramirez, R. D., C. P. Morales, B. S. Herbert, J. M. Rohde, C. Passons, J. W. Shay and W. E. Wright (2001). "Putative telomere-independent mechanisms of replicative aging reflect inadequate growth conditions." Genes Dev **15**(4): 398-403.

Reed, A. L., J. Califano, P. Cairns, W. H. Westra, R. M. Jones, W. Koch, S. Ahrendt, Y. Eby, D. Sewell, H. Nawroz, J. Bartek and D. Sidransky (1996). "High frequency of p16 (CDKN2/MTS-1/INK4A) inactivation in head and neck squamous cell carcinoma." Cancer Res **56**(16): 3630-3.

Rheinwald, J. G. and M. A. Beckett (1981). "Tumorigenic keratinocyte lines requiring anchorage and fibroblast support cultures from human squamous cell carcinomas." Cancer Res **41**(5): 1657-63.

Rheinwald, J. G., W. C. Hahn, M. R. Ramsey, J. Y. Wu, Z. Guo, H. Tsao, M. De Luca, C. Catricala and K. M. O'Toole (2002). "A two-stage, p16(INK4A)- and p53-dependent keratinocyte senescence mechanism that limits replicative potential independent of telomere status." Mol Cell Biol **22**(14): 5157-72.

Roed-Petersen, B. (1971). "Cancer development in oral leukoplakia: follow-up of 331 patients." Journal of Dental Research **50**: 711.

Romanov, S. R., B. K. Kozakiewicz, C. R. Holst, M. R. Stampfer, L. M. Haupt and T. D. Tlsty (2001). "Normal human mammary epithelial cells spontaneously escape senescence and acquire genomic changes." Nature **409**(6820): 633-7.

Roth, J., M. Dobbelstein, D. A. Freedman, T. Shenk and A. J. Levine (1998). "Nucleo-cytoplasmic shuttling of the hdm2 oncoprotein regulates the levels of the p53 protein via a pathway used by the human immunodeficiency virus rev protein." Embo J **17**(2): 554-64.

Rousseau, A., M. S. Lim, Z. Lin and R. C. Jordan (2001). "Frequent cyclin D1 gene amplification and protein overexpression in oral epithelial dysplasias." Oral Oncol **37**(3): 268-75.

Roussel, M. F. (1999). "The INK4 family of cell cycle inhibitors in cancer." Oncogene **18**(38): 5311-7.

Rubin, A. L. and R. H. Rice (1986). "Differential regulation by retinoic acid and calcium of transglutaminases in cultured neoplastic and normal human keratinocytes." Cancer Res **46**(5): 2356-61.

Rudolph, K. L., S. Chang, H. W. Lee, M. Blasco, G. J. Gottlieb, C. Greider and R. A. DePinho (1999). "Longevity, stress response, and cancer in aging telomerase-deficient mice." Cell **96**(5): 701-12.

Ryan, K. M., A. C. Phillips and K. H. Vousden (2001). "Regulation and function of the p53 tumor suppressor protein." Curr Opin Cell Biol **13**(3): 332-7.

Sakaguchi, K., J. E. Herrera, S. Saito, T. Miki, M. Bustin, A. Vassilev, C. W. Anderson and E. Appella (1998). "DNA damage activates p53 through a phosphorylation-acetylation cascade." Genes Dev **12**(18): 2831-41.

Sakai, E. and N. Tsuchida (1992). "Most human squamous cell carcinomas in the oral cavity contain mutated p53 tumor-suppressor genes." Oncogene **7**(5): 927-33.

Sauter, E. R., D. Cleveland, B. Trock, J. A. Ridge and A. J. Klein-Szanto (1994). "p53 is overexpressed in fifty percent of pre-invasive lesions of head and neck epithelium." Carcinogenesis **15**(10): 2269-74.

Sauter, E. R., J. A. Ridge, S. Litwin and C. J. Langer (1995). "Pretreatment p53 protein expression correlates with decreased survival in patients with end-stage head and neck cancer." Clin Cancer Res **1**(11): 1407-12.

Schantz, S. P. and G. Yu (2002). "Head and Neck cancer incidence trends in young americans, 1973-1997, with a special analysis for tongue cancer." Arch. Otolaryngol Head Neck Surg **128**: 268-274.

Schoelch, M. L., J. A. Regezi, N. P. Dekker, I. O. Ng, A. McMillan, B. L. Ziober, Q. T. Le, S. Silverman and K. K. Fu (1999). "Cell cycle proteins and the development of oral squamous cell carcinoma." Oral Oncol **35**(3): 333-42.

Serrano, M., G. J. Hannon and D. Beach (1993). "A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4." Nature **366**(6456): 704-7.

Serrano, M., H. Lee, L. Chin, C. Cordon-Cardo, D. Beach and R. A. DePinho (1996). "Role of the INK4a locus in tumor suppression and cell mortality." Cell **85**(1): 27-37.

Shahnavaz, S. A., G. Bradley, J. A. Regezi, N. Thakker, L. Gao, D. Hogg and R. C. Jordan (2001). "Patterns of CDKN2A gene loss in sequential oral epithelial dysplasias and carcinomas." Cancer Res **61**(6): 2371-5.

Shay, J. W. and S. Bacchetti (1997). "A survey of telomerase activity in human cancer." Eur J Cancer **33**(5): 787-91.

Shay, J. W., O. M. Pereira-Smith and W. E. Wright (1991). "A role for both RB and p53 in the regulation of human cellular senescence." Exp Cell Res **196**(1): 33-9.

Shay, J. W. and W. E. Wright (1996). "Telomerase activity in human cancer." Curr Opin Oncol **8**(1): 66-71.

Shay, J. W., W. E. Wright, D. Brasiskyte and B. A. Van der Haegen (1993). "E6 of human papillomavirus type 16 can overcome the M1 stage of immortalization in human mammary epithelial cells but not in human fibroblasts." Oncogene **8**(6): 1407-13.

Sherr, C. J. (1993). "Mammalian G1 cyclins." Cell **73**(6): 1059-65.

Sherr, C. J. (1994). "G1 phase progression: cycling on cue." Cell **79**(4): 551-5.

Sherr, C. J. and J. M. Roberts (1995). "Inhibitors of mammalian G1 cyclin-dependent kinases." Genes Dev **9**(10): 1149-63.

Shieh, S. Y., M. Ikeda, Y. Taya and C. Prives (1997). "DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2." Cell **91**(3): 325-34.

Shin, D. M., J. Kim, J. Y. Ro, J. Hittelman, J. A. Roth, W. K. Hong and W. N. Hittelman (1994). "Activation of p53 gene expression in premalignant lesions during head and neck tumorigenesis." Cancer Res **54**(2): 321-6.

Shin, D. M., J. Y. Ro, W. K. Hong and W. N. Hittelman (1994). "Dysregulation of epidermal growth factor receptor expression in premalignant lesions during head and neck tumorigenesis." Cancer Res **54**(12): 3153-9.

Silverman, S., Jr. and M. Gorsky (1990). "Epidemiologic and demographic update in oral cancer: California and national data--1973 to 1985." J Am Dent Assoc **120**(5): 495-9.

Silverman, S., Jr., M. Gorsky and F. Lozada (1984). "Oral leukoplakia and malignant transformation. A follow-up study of 257 patients." Cancer **53**(3): 563-8.

Slaughter, D. P., H. W. Southwick and W. Smejkal (1953). ""Field cancerisation" in oral stratified squamous epithelium: clinical implications of multicentric origin." Cancer **6**: 963-968.

Soder, A. I., J. J. Going, S. B. Kaye and W. N. Keith (1998). "Tumour specific regulation of telomerase RNA gene expression visualized by in situ hybridization." Oncogene **16**(8): 979-83.

Soder, A. I., S. F. Hoare, S. Muir, J. J. Going, E. K. Parkinson and W. N. Keith (1997). "Amplification, increased dosage and in situ expression of the telomerase RNA gene in human cancer." Oncogene **14**(9): 1013-21.

Stanton, P., S. Richards, J. Reeves, M. Nikolic, K. Edington, L. Clark, G. Robertson, D. Souter, R. Mitchell, F. J. Hendler and et al. (1994). "Epidermal growth factor receptor expression by human squamous cell carcinomas of the head and neck, cell lines and xenografts." Br J Cancer **70**(3): 427-33.

Steenbergen, R. D., D. Kramer, C. J. Meijer, J. M. Walboomers, D. A. Trott, A. P. Cuthbert, R. F. Newbold, W. J. Overkamp, M. Z. Zdzienicka and P. J. Snijders (2001). "Telomerase suppression by chromosome 6 in a human papillomavirus type 16-immortalized keratinocyte cell line and in a cervical cancer cell line." J Natl Cancer Inst **93**(11): 865-72.

Stich, H. F., A. P. Hornby, B. Mathew, R. Sankaranarayanan and M. K. Nair (1988). "Response of oral leukoplakias to the administration of vitamin A." Cancer Lett **40**(1): 93-101.

Sui, G., C. Soohoo, B. Affar el, F. Gay, Y. Shi and W. C. Forrester (2002). "A DNA vector-based RNAi technology to suppress gene expression in mammalian cells." Proc Natl Acad Sci U S A **99**(8): 5515-20.

Sumida, T., H. Hamakawa, K. Sogawa, A. Sugita, H. Tanioka and N. Ueda (1999). "Telomerase components as a diagnostic tool in human oral lesions." Int J Cancer **80**(1): 1-4.

Sundqvist, K., P. Kulkarni, S. S. Hybbinette, F. Bertolero, Y. Liu and R. C. Grafstrom (1991). "Serum-free growth and karyotype analyses of cultured normal and tumorous (SqCC/Y1) human buccal epithelial cells." Cancer Commun **3**(10-11): 331-40.

Swisshelm, K., K. Ryan, X. Lee, H. C. Tsou, M. Peacocke and R. Sager (1994). "Down-regulation of retinoic acid receptor beta in mammary carcinoma cell lines and its up-regulation in senescing normal mammary epithelial cells." Cell Growth Differ **5**(2): 133-41.

Todd, R., B. R. Donoff, R. Gertz, A. L. Chang, P. Chow, K. Matossian, J. McBride, T. Chiang, G. T. Gallagher and D. T. Wong (1989). "TGF-alpha and EGF-receptor mRNAs in human oral cancers." Carcinogenesis **10**(8): 1553-6.

Ulaner, G. A., J. F. Hu, T. H. Vu, L. C. Giudice and A. R. Hoffman (1998). "Telomerase activity in human development is regulated by human telomerase reverse transcriptase (hTERT) transcription and by alternate splicing of hTERT transcripts." Cancer Res **58**(18): 4168-72.

Unsal-Kacmaz, K., A. M. Makhov, J. D. Griffith and A. Sancar (2002). "Preferential binding of ATR protein to UV-damaged DNA." Proc Natl Acad Sci U S A **99**(10): 6673-8.

Van Craenenbroeck, K., P. Vanhoenacker, J. E. Leysen and G. Haegeman (2001). "Evaluation of the tetracycline- and ecdysone-inducible systems for expression of neurotransmitter receptors in mammalian cells." Eur J Neurosci **14**(6): 968-76.

van der Riet, P., H. Nawroz, R. H. Hruban, R. Corio, K. Tokino, W. Koch and D. Sidransky (1994). "Frequent loss of chromosome 9p21-22 early in head and neck cancer progression." Cancer Res **54**(5): 1156-8.

Vaziri, H. and S. Benchimol (1998). "Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span." Curr Biol **8**(5): 279-82.

Wan, H., N. Oridate, D. Lotan, W. K. Hong and R. Lotan (1999). "Overexpression of retinoic acid receptor beta in head and neck squamous cell carcinoma cells increases their sensitivity to retinoid-induced suppression of squamous differentiation by retinoids." Cancer Res **59**(14): 3518-26.

Wang, J., L. Y. Xie, S. Allan, D. Beach and G. J. Hannon (1998). "Myc activates telomerase." Genes Dev **12**(12): 1769-74.

Webley, K., J. A. Bond, C. J. Jones, J. P. Blaydes, A. Craig, T. Hupp and D. Wynford-Thomas (2000). "Posttranslational modifications of p53 in replicative senescence overlapping but distinct from those induced by DNA damage." Mol Cell Biol **20**(8): 2803-8.

Wei, W., R. M. Hemmer and J. M. Sedivy (2001). "Role of p14(ARF) in replicative and induced senescence of human fibroblasts." Mol Cell Biol **21**(20): 6748-57.

Wolf, G., R. Elez, A. Doermer, U. Holtrich, H. Ackermann, H. J. Stutte, H. M. Altmannsberger, H. Rubsamen-Waigmann and K. Strebhardt (1997). "Prognostic significance of polo-like kinase (PLK) expression in non- small cell lung cancer." Oncogene **14**(5): 543-9.

Woods, D. B. and K. H. Vousden (2001). "Regulation of p53 function." Exp Cell Res **264**(1): 56-66.

Wu, K. J., C. Grandori, M. Amacker, N. Simon-Vermot, A. Polack, J. Lingner and R. Dalla-Favera (1999). "Direct activation of TERT transcription by c-MYC." Nat Genet **21**(2): 220-4.

Wu, X., J. H. Bayle, D. Olson and A. J. Levine (1993). "The p53-mdm-2 autoregulatory feedback loop." Genes Dev 7(7A): 1126-32.

Wynford-Thomas, D. (1999). "Cellular senescence and cancer." J Pathol **187**(1): 100-11.

Xia, W., Y. K. Lau, H. Z. Zhang, A. R. Liu, L. Li, N. Kiyokawa, G. L. Clayman, R. L. Katz and M. C. Hung (1997). "Strong correlation between c-erbB-2 overexpression and overall survival of patients with oral squamous cell carcinoma." Clin Cancer Res **3**(1): 3-9.

Xia, W., Y. K. Lau, H. Z. Zhang, F. Y. Xiao, D. A. Johnston, A. R. Liu, L. Li, R. L. Katz and M. C. Hung (1999). "Combination of EGFR, HER-2/neu, and HER-3 is a stronger predictor for the outcome of oral squamous cell carcinoma than any individual family members." Clin Cancer Res **5**(12): 4164-74.

Xiong, Y., H. Zhang and D. Beach (1992). "D type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA." Cell **71**(3): 505-14.

Xu, D., Q. Wang, A. Gruber, M. Bjorkholm, Z. Chen, A. Zaid, G. Selivanova, C. Peterson, K. G. Wiman and P. Pisa (2000). "Downregulation of telomerase reverse transcriptase mRNA expression by wild type p53 in human tumor cells." Oncogene **19**(45): 5123-33.

Xu, X. C., J. Y. Ro, J. S. Lee, D. M. Shin, W. K. Hong and R. Lotan (1994). "Differential expression of nuclear retinoid receptors in normal, premalignant, and malignant head and neck tissues." Cancer Res **54**(13): 3580-7.

Yamamoto, T., N. Kamata, H. Kawano, S. Shimizu, T. Kuroki, K. Toyoshima, K. Rikimaru, N. Nomura, R. Ishizaki, I. Pastan and et al. (1986). "High incidence of amplification of the epidermal growth factor receptor gene in human squamous carcinoma cell lines." Cancer Res **46**(1): 414-6.

Yi, X., J. W. Shay and W. E. Wright (2001). "Quantitation of telomerase components and hTERT mRNA splicing patterns in immortal human cells." Nucleic Acids Res **29**(23): 4818-25.

Yu, J. Y., S. L. DeRuiter and D. L. Turner (2002). "RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells." Proc Natl Acad Sci U S A **99**(9): 6047-52.

Yuan, J., A. Horlin, B. Hock, H. J. Stutte, H. Rubsamen-Waigmann and K. Strebhardt (1997). "Polo-like kinase, a novel marker for cellular proliferation." Am J Pathol **150**(4): 1165-72.

Yuen, P. W., M. Man, K. Y. Lam and Y. L. Kwong (2002). "Clinicopathological significance of p16 gene expression in the surgical treatment of head and neck squamous cell carcinomas." J Clin Pathol **55**(1): 58-60.

Zakrzewska, J. M. (1999). "Fortnightly review: oral cancer." Bmj **318**(7190): 1051-4.

Zambetti, G. P. and A. J. Levine (1993). "A comparison of the biological activities of wild-type and mutant p53." Faseb J **7**(10): 855-65.

Zariwala, M., S. Schmid, M. Pfaltz, H. Ohgaki, P. Kleihues and R. Schafer (1994). "p53 gene mutations in oropharyngeal carcinomas: a comparison of solitary and multiple primary tumours and lymph-node metastases." Int J Cancer **56**(6): 807-11.

Zhang, S. Y., A. J. Klein-Szanto, E. R. Sauter, M. Shafarenko, S. Mitsunaga, T. Nobori, D. A. Carson, J. A. Ridge and T. L. Goodrow (1994). "Higher frequency of alterations in the p16/CDKN2 gene in squamous cell carcinoma cell lines than in primary tumors of the head and neck." Cancer Res **54**(19): 5050-3.

Zindy, F., C. M. Eischen, D. H. Randle, T. Kamijo, J. L. Cleveland, C. J. Sherr and M. F. Roussel (1998). "Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization." Genes Dev **12**(15): 2424-33.

Zou, C. P., J. L. Clifford, X. C. Xu, P. G. Sacks, P. Chambon, W. K. Hong and R. Lotan (1994). "Modulation by retinoic acid (RA) of squamous cell differentiation, cellular RA-binding proteins, and nuclear RA receptors in human head and neck squamous cell carcinoma cell lines." Cancer Res **54**(20): 5479-87.

14 Appendix

Publications arising from this work are included in this section:

1) McGregor[1], F., A. Muntoni[1], J. Fleming, J. Brown, D. H. Felix, G. MacDonald, E. K. Parkinson and P. R. Harrison (2002). "Molecular changes associated with oral dysplasia progression and acquisition of immortality: potential for its reversal by 5-azacytidine." Cancer Res **62**(August 15).

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2) Muntoni, A., J. Fleming, K. Gordon, K. Hunter, F. McGregor, E. K. Parkinson and P. R. Harrison. "Immortalisation of senescing oral dysplasias by ectopic expression of hTERT requires loss of INK4A and/or retinoic acid receptor β, but not p53 mutation". Submitted to Cancer Research.

Immortalization of senescing oral dysplasias by ectopic expression of hTERT requires loss of INK4A and/or retinoic acid receptor-β, but not p53 mutation[1]

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Running Title:

Molecular analysis of oral dysplasias

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[3] Abbreviations used are: HN, head and neck; hTERT, human telomerase reverse transcriptase; PD, population doubling; RAR-β, retinoic acid receptor-β; Rb, retinoblastoma gene; SCC, squamous cell carcinoma.

Keywords

Head and neck cancer; preneoplasia; immortalization; RAR-β; senescence; $p16^{INK4A}$; $p14^{ARF}$; telomerase

Abstract

Our previous work showed that acquisition of immortality at the dysplasia stage of oral cancer progression was consistently associated with four changes: loss of retinoic acid receptor (RAR)-β and $p16^{INK4A}$ expression, p53 mutations and activation of telomerase. One atypical dysplasia (D17) that senesced after an extended lifespan showed loss of RAR-β and $p16^{INK4A}/p14^{ARF}$ expression but retained wild-type p53 and telomerase was not activated. We now demonstrate that retroviral delivery of hTERT results in telomere lengthening and immortalization of D17 with no change in wild-type p53. In contrast, expression of hTERT in two other typical mortal dyplasia cultures (that retain RAR-β and $p16^{INK4A}$ expression) does not extend their lifespan, even though telomeres are lengthened.

Introduction

Normal cells undergo replicative senescence in culture due to erosion of telomeres and fail to divide further but remain viable. Although the role of senescence in vivo remains to be established, escape from senescence and acquisition of immortality are considered to be important characteristics of cancer cells (1) since most primary cancers, and virtually all recurrences, are immortal (2). Studies with various cell types have indicated that senescence can be bypassed by abrogation of the p53 and retinoblastoma gene (Rb)[3] pathways but the cells then enter a second proliferative block (crisis) involving genomic instability and cell death, from which rare immortal variants emerge due to activation of telomerase and telomere maintenance (3). Several studies have reported that some normal cell types can be immortalised by ectopic expression of the catalytic component of telomerase (hTERT) alone, whereas others, such as mammary epithelial cells and

keratinocytes, cannot without concomitant loss of $p16^{INK4A}$ expression (4). However, it remains controversial whether this loss of $p16^{INK4A}$ is due to sub-optimal culture conditions or simply passage in culture (4). Senescence can also be induced by oncogene expression in cultured cells (5). However, it is not clear whether the mechanisms of escape from senescence by normal cells are the same as those in mortal preneoplastic cells since, for example, $p16^{INK4A}$ is known to be regulated positively and negatively by oncogenes (6). This report addresses these issues in a head and neck/oral keratinocyte pre-neoplasia model of squamous carcinoma (SCC) development. In our previous work, we showed that acquisition of immortality at the dysplasia stage of oral SCC was consistently associated with loss of retinoic acid receptor (RAR)-β and $p16^{INK4A}$ expression, p53 mutations and activation of telomerase, suggesting that all are required for immortality (7, 8). None of the mortal dysplasias showed any of these changes, except one (D17) which had an extended lifespan and an intermediate molecular phenotype i.e. loss of retinoic acid receptor (RAR)-β and $p16^{INK4A}$ expression, but not p53 mutations and activation of telomerase. In this report we have tested whether ectopic activation of telomerase by transduction of a retroviral hTERT expression vector is sufficient to immortalise naturally occurring oral dysplasias, or whether other changes, such as p53 mutations and loss of RAR-β and $p16^{INK4A}$ expression are also required.

Materials and Methods

Cell Culture. The methods for isolation, establishment and culture of the primary human oral cultures used in this study have been described previously (7, 8). The irradiated 3T3 feeder cells were removed by EDTA treatment before analysis of keratinocytes.

Retrovirus production and ectopic expression of hTERT. The retroviruses were produced by infecting the amphotropic packaging line Phoenix A with pBabeNeo or pBabest2 (hTERT) retroviral constructs (9) and selecting with the minimum concentration of G418 found to kill the uninfected cultures. Dysplasia cultures were infected at about 80% confluency with 1.35×10^4 infectious units/ml in the presence of 4 μg/ml of polybrene. The dysplasia cells were plated on G418-resistant 3T3 feeder cells, selected 48h later with 200 μg/ml of G418 and cultured under constant selection. Pooled G418-resistant colonies were used in all experiments to avoid variability due to clonal heterogeneity of the population.

Senescence assays. Cells were stained for senescence-associated β-galactosidase activity (SABG) (8), DNA fragmentation measured by the TUNEL assay (10) and the proliferative index of colonies determined by BrdU labelling (11).

Telomerase Assay. Telomerase activity was measured as described previously (8).

TRF measurement. This was carried out as described by (11) except that the blot was hybridised with a radiolabeled oligonucleotide probe $(TTAGGG)_3$ at 42°C and subsequently washed in 6xSSC/1%SDS at 42°C.

p53 sequencing. DNA was isolated form hTERT transduced D17 cultures once they had reached 86 population doublings. Sequencing of exons 2-11 was performed as described previously (8).

Western blots. The levels of total p53, p53 phosphorylated at serine 15 , p14 ARF and ERKs 1&2 were determined using the following antibodies as indicated by the maunufacturers: total p53 (D01; Santa Cruz Biotechnology); ser 15 phospho-specific p53 (16G8, New England BioLabs); p14ARF (C-18, sc-8613; Santa Cruz Biotechnology); ERKs 1&2 (ERK1(C-16)-G; Santa Cruz Biotechnology).

Results and Discussion

Ecotopic expression of telomerase in mortal dysplasia cultures. In previous work (7, 8), we established a panel of primary cultures of oral dysplasias using the 3T3 feeder layer culture system of Rheinwald and coworkers which has been shown to facilitate growth of keratinocytes at all stages of cancer progression and to maintain the characteristics of the original tumors (12): it has also been reported to avoid the premature growth arrest associated with culture of epithelial cells in chemically-defined keratinocyte growth medium directly on plastic culture dishes (13). In our previous analysis, we demonstrated that about half of oral dysplasias were immortal in culture and that they showed four characteristic molecular differences from mortal dysplasias: loss of expression of RAR-β and $p16^{INK4A}$, reactivation of telomerase through hTERT upregulation and mutations in p53 (7, 8). One dysplasia culture (D17) with an atypically extended life span (45 population doublings (PDs)) (compared to 4-30 PDs for other mortal dysplasias) showed an intermediate molecular phenotype: loss of expression of RAR-β and $p16^{INK4A}$, like immortal dysplasias, but no detectable telomerase activity and wild type p53 like mortal dysplasias. The loss of RAR-β and $p16^{INK4A}$ expression in D17 is not a culture artefact since we have also shown loss of both markers in the biopsy from which D17 was derived (data not shown). All our mortal dysplasia cultures undergo

terminal growth arrest due to senescence rather than 'crisis' since the arrested colonies show no apoptosis by TUNEL staining and the percentage of proliferating cells by BrdU labelling is low (less than 20%) (Fig. 1).

In order to assess the importance of telomerase activation in cellular immortalization of our mortal dysplasias, we used the amphotropic retroviral vector pBabeEST to ectopically express hTERT into two typical mortal cultures (D25, D30) and also D17. Parallel cultures were also infected with the 'empty' retroviral vector expressing the neomycin resistance gene alone. After infection, neomycin-resistant colonies were pooled, checked for telomerase activity and telomere lengths as soon as sufficient cells were available and monitored for their life span.

The mortal dysplasia culture D30 had an expected life span of 30 PDs and was infected at 8.5, 9, 17 and 18 PDs in four separate experiments. D25 had an expected life span of 25 PDs and was infected at 7, 9 and 18 PDs in three separate experiments. In parallel experiments, the D17 dysplasia culture was infected after having reached 34.5 and 39 PDs, with an expected lifespan of 45 PDs. With D25 and D30 dyplasias, no difference in growth rate or lifespan was observed between the uninfected cultures, the cultures infected with the empty vector and those infected with the hTERT vector. However, with D17, the cells infected with the hTERT vector grew for at least 130 PDs and thus appear to be immortal, but this did not occur with uninfected cells or cells infected with the 'empty' vector, which senesced like the uninfected cells (Fig. 1).

To check whether the dysplasias infected with the hTERT vector had functional telomerase, telomerase activity was assayed as well as telomere length maintenance. In both D30 and D17, telomerase activity was undetectable in uninfected cultures and

cultures infected with the empty vector, but activity was readily detectable in cultures infected with the hTERT vector (Fig. 2). In both cases, the activation of telomerase after infection with the hTERT vector was functional in maintaining/elongating telomeres: during culture of D30 and D17 to senescence, telomeres progressively became shorter, but were increased significantly after infection with the hTERT vector but not the 'empty' vector (Fig. 3). Finally, infection with the hTERT vector, but not the 'empty' vector, abrogated the increased phosphorylation of p53 at serine 15 that otherwise occurs during dysplasia senescence (Fig. 4A): this site is known to be involved in p53-mediated DNA damage responses (14) and seems to be a sensor of DNA damage signals relayed by the ATM pathway in response to critically short telomeres (14, 15). Overall, these experiments expressing hTERT ectopically in D30/D25 and D17 strongly suggest that telomerase activation/telomere maintenance is not sufficient per se to immortalise naturally-occurring mortal dysplasias unless they have lost expression of RAR-β and/or $p16^{INK4A}$. Loss of $p16^{INK4A}$ and RAR-β in dysplasias is not an in vitro artefact since both changes have been found in large fraction of dysplasias in vivo (16, 17).

However, a recent report showed that in normal human keratinocytes, p16 dysfunction and telomerase over-expression were insufficient for immortalisation and that p53 dysfunction was also required (18). In these experiments p16 and p53 dysfunction in concert lead to an accumulation of the other protein encoded by the INK4A locus, $p14^{ARF}$. Since $p14^{ARF}$ can stabilise p53 by inhibiting the MDM2 ubiquitin ligase (see (18) and references therein), the accumulation of $p14^{ARF}$ in $p16^{INK4A}$-deficient normal keratinocytes (18) would provide a plausible explanation for the necessity to additionally disrupt the p53 pathway in their experiments. On the basis of these results, Rheinwald et

al (18) suggested that the telomere-mediated activation of p53 by short telomeres, previously shown to occur in human fibroblasts (14), might be unnecessary for the senescence of normal human keratinocytes. To address the role of $p14^{ARF}$ in the immortalisation of D17, we attempted to detect $p14^{ARF}$ by western blotting and showed that it was undetectable (Fig. 4B), consistent with the frequent co-deletion of the proteins in oral dysplasia (19). Thus in the neoplastic D17 culture the co-dysfunction of p16 and $p14^{ARF}$ (together with RARβ) allows these cells to undergo further telomeric attrition and procede to a further senescence checkpoint that involves the phosphorylation of p53 at serine 15. The current observations, and those recently reported (18), suggest that p53 can mediate two distinct mechanisms of senescence in neoplastic oral keratinocytes. Furthermore, this hypothesis is also consistent with the observed co-existence of $p14^{ARF}$ dysfunction and p53 mutation in the same immortal neoplastic keratinocyte lines (19). Since our previous work demonstrated a frequent association between p53 mutations, telomerase activation and acquisition of immortality in our series of oral dysplasia cultures (7, 8) we determined whether immortalization of our D17 culture by hTERT/telomerase activation also required p53 mutation. However, sequencing of the entire p53 coding region using DNA from the hTERT infected D17 culture at 86 PDs revealed no mutations. Thus, p53 mutation is not an obligatory requirement for immortalization of oral dysplasias provided that telomerase is activated exogenously. Wild type p53 is known to down-regulate hTERT in other contexts (20) and thus p53 mutation may be one route for hTERT/telomerase activation for acquisition of immortality during oral dysplasia progression. Alternatively, since p53 appears to sense telomere dysfunction to mediate a G1 cell cycle checkpoint (14, 15), correcting telomere

dysfunction by ectopically activating telomerase may remove any requirement for p53 mutation in this situation. Clearly, these alternatives are not mutually exclusive. Finally, although p53 dysfunction is highly correlated with the immortal phenotype of the cancer cell (8), abrogation of other functions of wild type p53 may be required for the development of other aspects of the cancer cell phenotype.

Acknowledgements

We are grateful to Dr. H. Vaziri for his generous gift of the hTERT retroviral construct.

We are grateful to Prof. J. Wyke and Prof. B. Ozanne for critically reading the manuscript.

References

1. Campisi, J. Cancer, aging and cellular senescence, In Vivo. *14:* 183-8., 2000.
2. Shay, J. W. Telomerase in human development and cancer, J Cell Physiol. *173:* 266-70., 1997.
3. Lustig, A. J. Crisis intervention: the role of telomerase, Proc Natl Acad Sci U S A. *96:* 3339-41., 1999.
4. Stewart, S. A. and Weinberg, R. A. Senescence: does it all happen at the ends?, Oncogene. *21:* 627-30., 2002.
5. Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D., and Lowe, S. W. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a, Cell. *88:* 593-602., 1997.

6. Ohtani, N., Zebedee, Z., Huot, T. J., Stinson, J. A., Sugimoto, M., Ohashi, Y., Sharrocks, A. D., Peters, G., and Hara, E. Opposing effects of Ets and Id proteins on p16INK4a expression during cellular senescence, Nature. *409:* 1067-70., 2001.

7. McGregor, F., Wagner, E., Felix, D., Soutar, D., Parkinson, K., and Harrison, P. R. Inappropriate retinoic acid receptor-beta expression in oral dysplasias: correlation with acquisition of the immortal phenotype, Cancer Res. *57:* 3886-9., 1997.

8. McGregor, F., Muntoni, A., Fleming, J., Brown, J., Felix, D. H., MacDonald, G., Parkinspon, E. K., and Harrison, P. R. Molecular changes associated with oral dysplasia progression and acquisition of immortality: potential for its reversal by 5-azacytidine, Cancer Res. *in press:*, 2002.

9. Vaziri, H. and Benchimol, S. Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span, Curr Biol. *8:* 279-82., 1998.

10. Ghose, A., Fleming, J., El-Bayoumy, K., and Harrison, P. R. Enhanced Sensitivity of Human Oral Carcinomas to Induction of Apoptosis by Selenium Compounds: Involvement of Mitogen-activated Protein Kinase and Fas Pathways, Cancer Res. *61:* 7479-7487, 2001.

11. Munro, J., Steeghs, K., Morrison, V., Ireland, H., and Parkinson, E. K. Human fibroblast replicative senescence can occur in the absence of extensive cell division and short telomeres, Oncogene. *20:* 3541-52., 2001.

12. Rheinwald, J. G. and Beckett, M. A. Tumorigenic keratinocyte lines requiring anchorage and fibroblast support cultures from human squamous cell carcinomas, Cancer Res. *41:* 1657-63., 1981.

13. Ramirez, R. D., Morales, C. P., Herbert, B. S., Rohde, J. M., Passons, C., Shay, J. W., and Wright, W. E. Putative telomere-independent mechanisms of replicative aging reflect inadequate growth conditions, Genes Dev. *15:* 398-403., 2001.

14. Webley, K., Bond, J. A., Jones, C. J., Blaydes, J. P., Craig, A., Hupp, T., and Wynford-Thomas, D. Posttranslational modifications of p53 in replicative senescence overlapping but distinct from those induced by DNA damage, Mol Cell Biol. *20:* 2803-8., 2000.

15. Pandita, T. K. ATM function and telomere stability, Oncogene. *21:* 611-8., 2002.

16. Papadimitrakopoulou, V., Izzo, J., Lippman, S. M., Lee, J. S., Fan, Y. H., Clayman, G., Ro, J. Y., Hittelman, W. N., Lotan, R., Hong, W. K., and Mao, L. Frequent inactivation of p16INK4a in oral premalignant lesions, Oncogene. *14:* 1799-803., 1997.

17. Xu, X. C., Ro, J. Y., Lee, J. S., Shin, D. M., Hong, W. K., and Lotan, R. Differential expression of nuclear retinoid receptors in normal, premalignant, and malignant head and neck tissues, Cancer Res. *54:* 3580-7., 1994.

18. Rheinwald, J. G., Hahn, W. C., Ramsey, M. R., Wu, J. Y., Guo, Z., Tsao, H., De Luca, M., Catricala, C., and O'Toole, K. M. A two-stage, p16(INK4A)- and p53-dependent keratinocyte senescence mechanism that limits replicative potential independent of telomere status, Mol Cell Biol. *22:* 5157-72., 2002.

19. Munro, J., Stott, F. J., Vousden, K. H., Peters, G., and Parkinson, E. K. Role of the alternative INK4A proteins in human keratinocyte senescence: evidence for the specific inactivation of p16INK4A upon immortalization, Cancer Res. *59:* 2516-21., 1999.

20. Xu, D., Wang, Q., Gruber, A., Bjorkholm, M., Chen, Z., Zaid, A., Selivanova, G., Peterson, C., Wiman, K. G., and Pisa, P. Downregulation of telomerase reverse transcriptase mRNA expression by wild type p53 in human tumor cells, Oncogene. *19:* 5123-33., 2000.

LEGENDS TO FIGURES

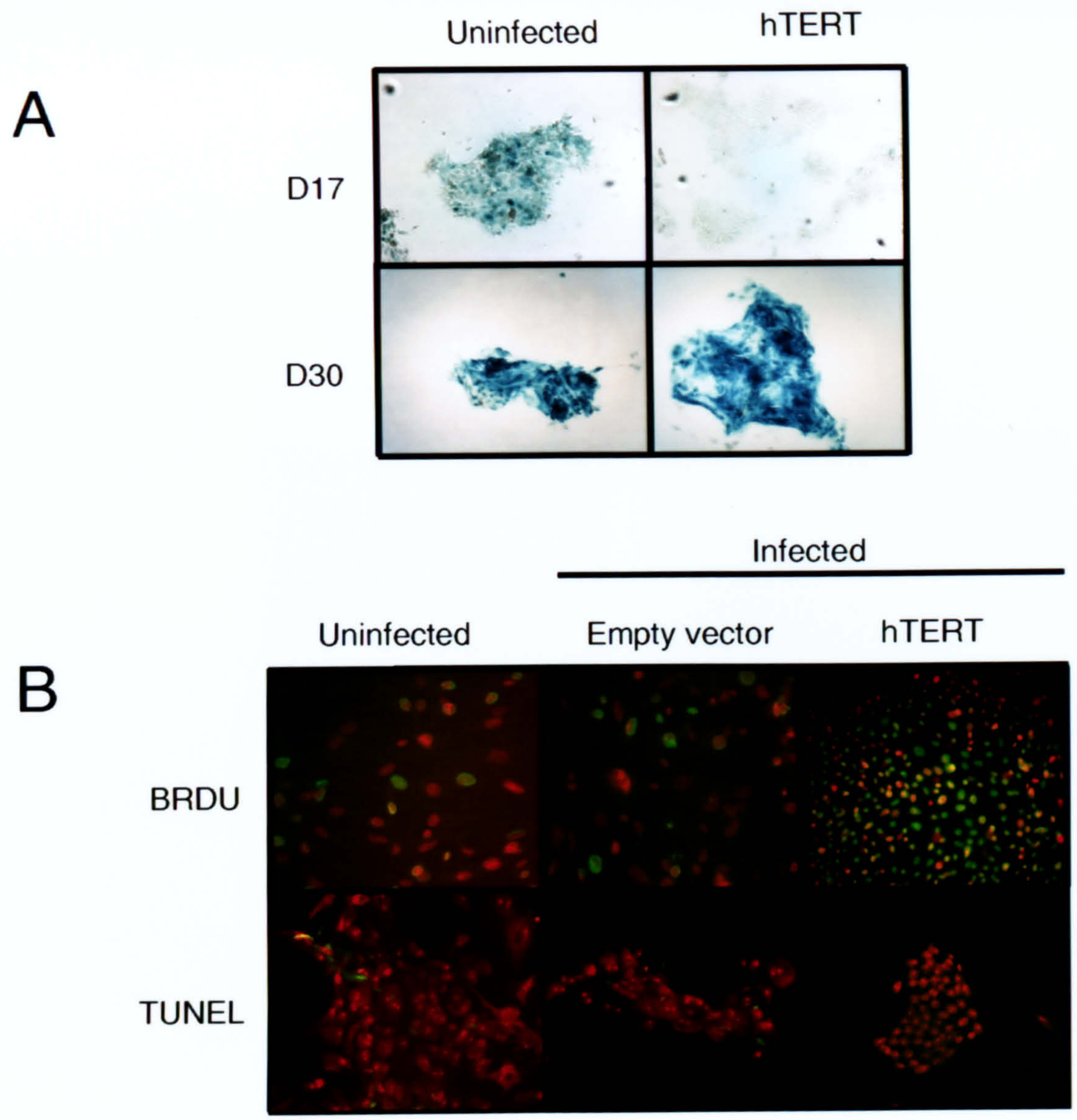
Fig.1. Oral dysplasias senescence. (A) SABG staining of uninfected D17 and D30 cultures and after infection with the hTERT retroviral vector. Colonies were stained at terminal growth arrest or at 85 PDs in the case of D17 cultures infected with the hTERT vector. (B) D17 cultures were labelled for 48 h with BrdU to measure their proliferative index or assayed for DNA breaks/apoptosis by TUNEL staining. Colonies were analysed during terminal growth arrest or at 85 PDs in the case of hTERT infected cultures.

Fig. 2. Telomerase activity in (A) D30 and (B) D17 cultures before and after infection with hTERT expression vector. TRAPeze assay was performed on 400ng (left panel) or 200 ng (right panel) of protein extract. The gels show the PCR products generated after staining with ethidium bromide. The uninfected cultures were analysed at the indicated PDs, and as soon as sufficient numbers of cells were available after infection with the empty vector or with the hTERT vector. WI38 and HeLa cells were included as telomerase negative and positive controls respectively.

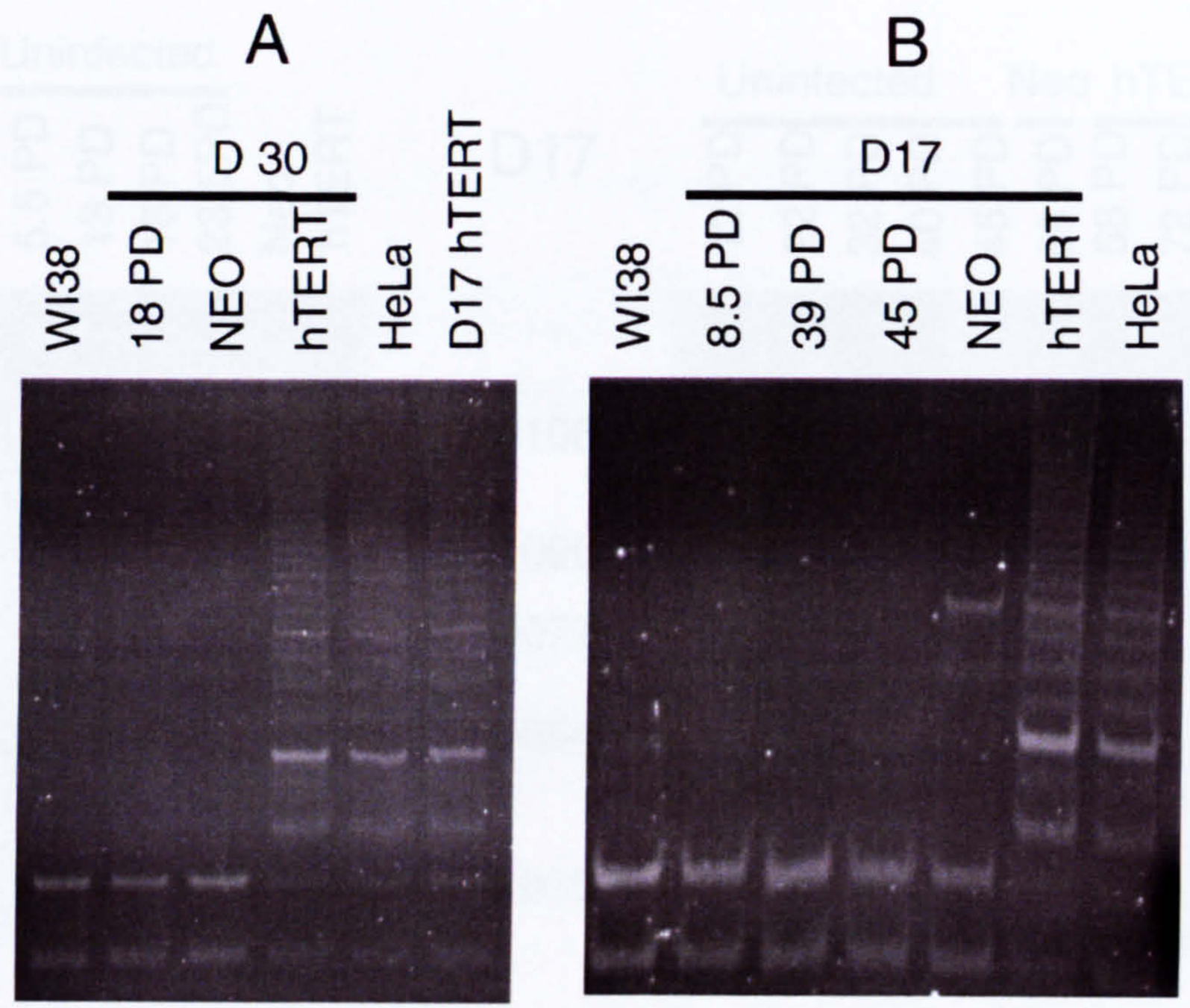
Fig. 3. Telomere lengths were measured by Southern blotting in (A) D30 and (B) D17 cultures at different stages of their life span, comprising senescence (the number of population doubling is indicated for each sample), after infection with the empty vector (NEO) and with the hTERT transducing vector (hTERT). With both D30 and D17, the intensity of signals with the 'empty'vector infected cultures is weaker than for the other

samples due to the small amounts of DNA available but show that the telomeres are significantly shorter than in cells infected with the hTERT vector.

Fig. 4. (A) Total p53 levels and p53 phosphorylation status at serine 15. D17 cultures at various stages of progression towards senescence, or after infection with the hTERT vector were analysed for the levels of p53 and phospho serine15 p53 status by Western blotting. (B) Measurement of p14 ARF in D17 cultures at various stages of progression towards senescence. A SCC previously shown to express ARF (BICR 3) and a SCC lacking p14 ARF (19) were also included as positive and negative controls. In both (A) and (B), re-probing of the blots to measure the levels of ERKs 1&2 were included as loading controls.



Muntoni et al. Fig. 1



Muntoni et al. Fig. 2