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The Genetic Basis of Human Keratinocyte Immortalisation in Squamous Cell Carcinoma Development: the Role of Telomerase Reactivation

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Normal human keratinocytes have a finite replicative lifespan which culminates in senescence. Chromosomal telomere length may act as a mediator of replicative senescence, signalling cell cycle arrest in G1 when one or more telomeres become too short. Telomeric attrition in normal keratinocytes may be due to inadequate levels of telomerase activity and possibly also to oxidative damage. In advanced squamous cell carcinoma replicative senescence breaks down to yield immortal variants, in which several dominantly acting genes are functionally compromised, including p53 and the cyclin D-Cdk4/6 inhibitor CDKN2A/p16. The increased activity of both of these proteins would be expected to contribute to the G1 arrest in senescence and we have shown that levels of p16 are dramatically increased in senescent keratinocytes. In addition, two other genes which control a cell cycle G1 checkpoint independently of p53 and pRb appear dysfunctional. These genes are uncloned but map to chromosome 4q and 7q31.1 and appear to represent senescence complementation groups B and D, respectively. In immortal neoplastic keratinocytes, telomerase is strongly upregulated and there is evidence for a suppressor of the enzyme on the short arm of chromosome 3 mapping to 3p21.2–p21.3. We have also mapped the human telomerase RNA gene to 3q26.3 and found it to be overrepresented or amplified in a proportion of squamous cell tumours and cell lines. These observations may explain why isochromosome 3q is so common in human squamous carcinoma. None of these genetic alterations are seen in carcinomas which senesce and suggest that multiple genetic alterations are required for keratinocyte immortality. © **1997 Elsevier Science Ltd. All rights reserved.**

Key words: telomerase, CDKN2, senescence, immortality, squamous cell carcinoma

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INTRODUCTION

ALL NORMAL human somatic cells, including keratinocytes, are known to have a finite replicative lifespan *in vitro* which culminates in senescence [1–3] and more recent data suggest that a similar process occurs as keratinocytes age *in vivo* [4, 5]. In the later stages of squamous cell carcinoma development and in recurrent tumours, replicative senescence breaks down to yield immortal cells which can proliferate essentially indefinitely [6]. These results suggest that the selection pressure for cellular immortality occurs when the replicative capacity of the tumour cells becomes exhausted and that cellular immortality may facilitate tumour progression and tumour recurrence [6].

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THE RELATIONSHIP OF THE TELOMERE THEORY TO KERATINOCYTE REPLICATIVE SENESCENCE

The lagging strand of DNA molecules is incompletely replicated by conventional DNA polymerases because of the end replication problem [7] and unless other mechanisms intervene, the DNA at the chromosome ends, the telomeres (Figure 1), will shorten by 30–150 bp per cell division. Unicellular organisms and the cells of the human germline solve this problem by means of the enzyme telomerase [8] which can perfectly restore the gap in the lagging strand. It

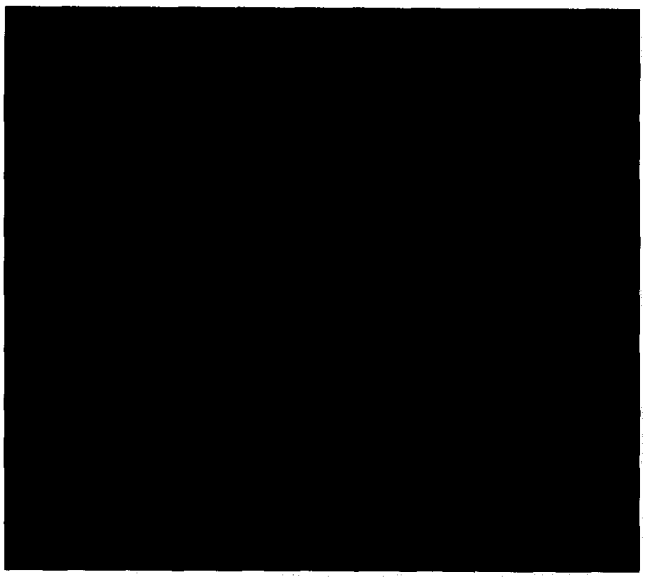

Figure 1. Visualisation of normal human telomeres by peptide nucleic acid labelling. The telomeres are labelled in green which may sometimes appear yellow on the photograph.

has been suggested that when telomerase is inactivated during development [9], telomeric attrition begins and forms the basis of an intrinsic replicative senescence clock [10], which when exhausted, signals the cell cycle to arrest in G1 [11]. This contention is supported by the observation that the telomeres of all somatic cells and tissues tested, including those of the haemopoietic stem cells [12] and the epidermis [13], do indeed shorten with replicative age *in vitro* [12, 14] and donor age *in vivo* [13, 14]. Also, experimental extension of telomere length can lengthen replicative lifespan in somatic cell hybrids [15] suggesting that at least in human cells the telomere may be instrumental in the control of proliferative lifespan. However, more recent data suggest that telomerase activity is present in the basal layer of the human epidermis *in situ* [16] and that one source of the activity may be the keratinocytes of the greatest proliferative power [17] which may include the epidermal stem cells *in vivo* [18]. If so, it is necessary to explain why a telomerase positive stem cell of the epidermis still suffers telomeric attrition with increasing age of the donor [13]. One explanation is that the telomerase activity in epidermal stem cells is insufficient to sustain the telomeres efficiently [19] and the lack of quantification in the epidermal telomerase studies [16, 17] render this a strong possibility. An alternative or additional explanation is that the intrinsic telomeric clock is subject to environmental modulation. It has recently been suggested that oxidative damage may induce deletion of telomeric sequences at a faster rate than the end replication problem [20] (Figure 2), and this hypothesis is expanded below. There is a wealth of evidence to suggest that the manipulation of oxidative stress can influence the lifespan and fitness of whole organisms [21, 22]. Hyperoxia reduces the replicative capacity of human fibroblasts and leads to accelerated telomeric attrition [20] whereas hypoxia and several antioxidants have been reported to extend proliferative lifespan (see [23] and references therein). Heat shock shortens both yeast [24] and human fibroblast [25] replicative lifespan and in the former case by a mechanism involving oxidative damage [24]. Furthermore, human individuals and cells with defects in genes that are responsible for the DNA integrity following

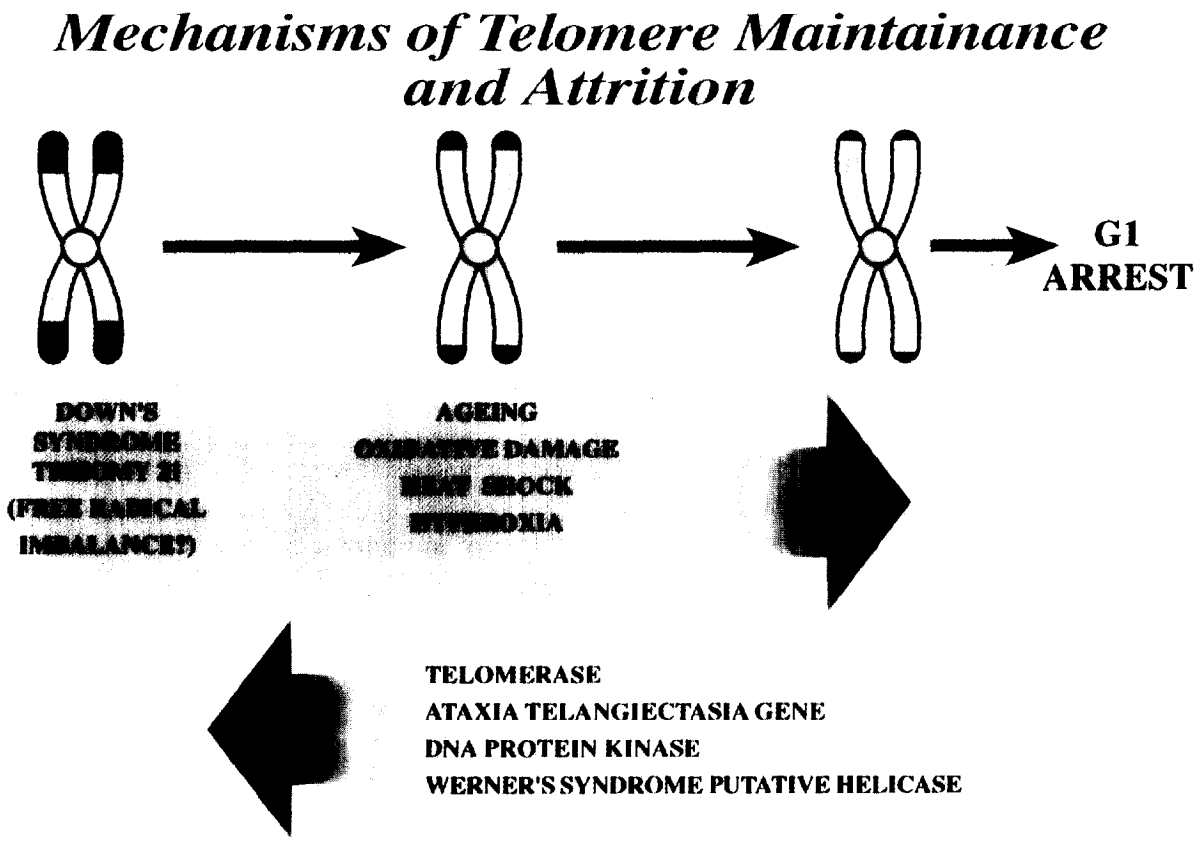

Figure 2. Intrinsic and extrinsic controls in the regulation of telomere length.

oxidative damage, such as ataxia telangiectasia (ATM-1), show premature ageing, a reduced replicative lifespan and accelerated telomeric attrition [26, 27]. The dysfunction of the ATM-like gene, TEL-1, in yeast also results in telomeric attrition [28]. Down's syndrome cells which are trisomic for all of the chromosome 21, also exhibit accelerated telomeric attriton [29] and a reduced replicative lifespan [30]. It has been speculated that an extra copy of the superoxide dismutase 1 gene on chromosome 21 may result in an imbalance in free radical metabolism and increased peroxidation to cause these phenotypes [31]. Fibroblasts from individuals carrying the classical premature ageing Werner's syndrome gene show accelerated telomeric attrition and a reduced replicative lifespan [32, 33] and interestingly the Werner's gene shows homology to the helicases [32], yet another gene involved in the repair or maintainance of DNA. The DNA repair efficiency of telomeric repeats is lower than of coding sequences [33], so the sudden truncation of one telomere following oxidative damage may be more frequent than supposed. A rapid subthreshold loss of telomeric sequences would not be lethal [12–14] but would be expected to condemn the cell suffering rapid loss to premature senescence [15] and in fact, such premature loss of proliferative capacity is seen stochastically in normal cultures of fibroblasts [34] and keratinocytes [4]. There is no evidence that the loss of one telomere can arrest proliferation in human cells, largely due to technical difficulties, but the experimental amputation of a yeast telomere has been shown to cause proliferation arrest via a DNA damage cell cycle checkpoint [35]. Furthermore, recent evidence indicates that senescent human cells have permanently activated signalling mechanisms that are shared by those produced by DNA double strand breaks (see below and [36]).

TELOMERIC ATTRITION AND G1 ARREST

If telomeric attrition is the major instrument of the human replicative sensescence programme then there must be a mechanism by which one or more critically shortened telomeres signal to effect a G1 arrest. One mechanism that has been proposed is that the shortening telomeric DNA is no longer able to bind its associated proteins and, as a result, the free ends of the DNA are recognised as a double strand break by genes responsible for signalling a G1 arrest in such situations (Figure 3). These would include the ATM gene discussed above, p53, GAD45, and the cyclin-Cdk inhibitor p21 (for a review see [37]) and their activity would be expected to be constituitive in senescent cells. It is now known that the activity of p53 as a transcription factor increases following DNA double strand breaks and in senescent human fibroblasts [38] and that one of its targets, the cyclin-Cdk inhibitor CDKN1/p21, also increases [39]. The high levels of p21 in senescent cells probably explains the inactive state of its major target, the cyclin E/Cdk2 kinase [40] and partially, the underphosphorylated state of pRb [41] and the lack of S-phase entry [3, 42]. However, we and others have shown that another cyclin-Cdk inhibitor, CDKN2/p16, accumulates in senescent fibroblasts [43] and keratinocytes [44] and this inhibitor may also contribute to the G1 senescence checkpoint by inhibiting the cyclin D/Cdk4 and cyclin D/Cdk6 kinases whose major target is pRb [45]. There is currently no evidence that CDKN2/p16

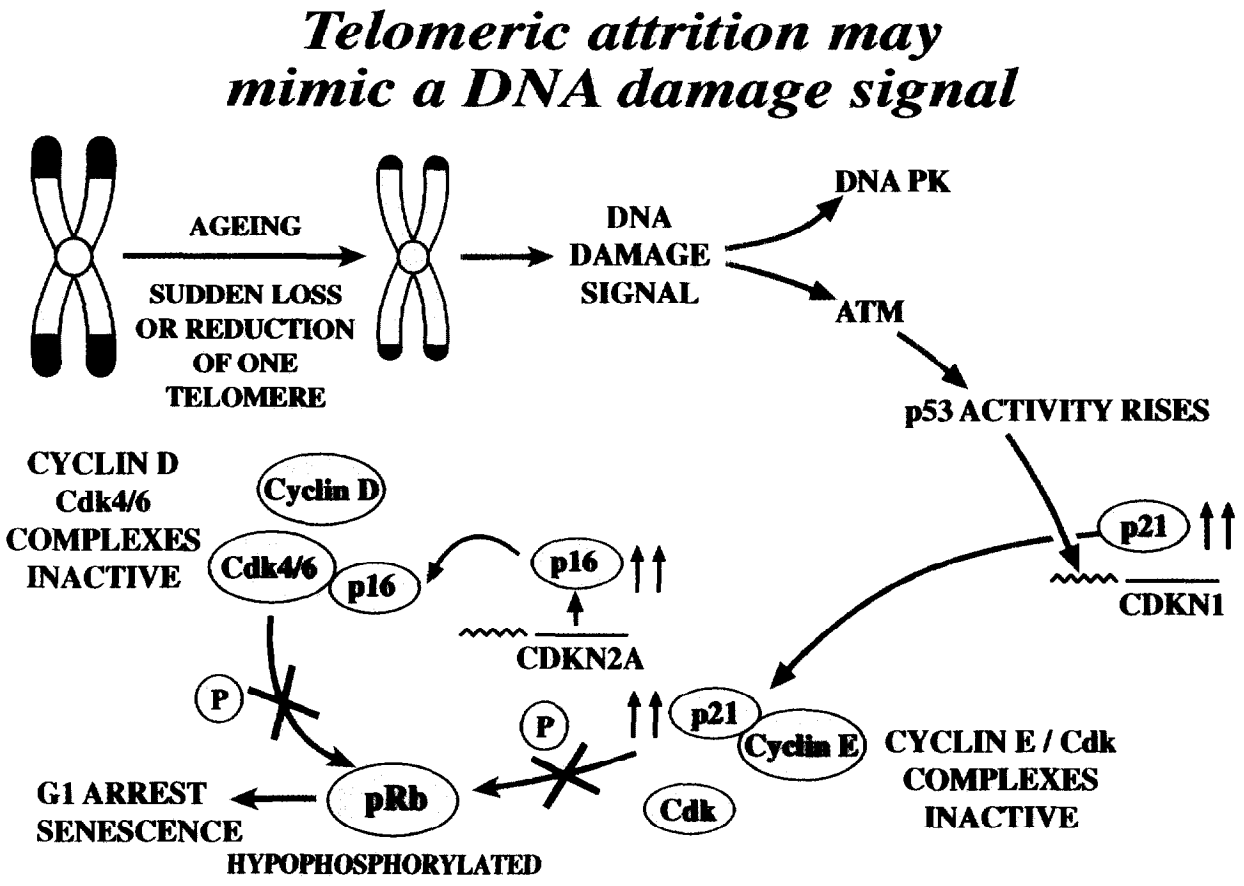

Figure 3. The shortened telomere as a DNA double strand break: possible signalling mechanisms towards G1 arrest.

is upregulated by DNA damage [46] but this matter has still to be investigated intensively.

Another mechanism by which telomeric attrition may regulate gene expression is by the lack of binding of the repressor activator protein RAP-1 to the telomere [47, 48]. As a result of this, the silencing molecules SIR 3 and SIR 4 are inefficiently recruited and increase their concentration elsewhere; the silencing of genes close to the telomere is relaxed [47] but is increased at the other sites in the genome. Mutations of SIR 4 can inhibit yeast cell senescence even in the presence of telomeric attrition [47] supporting the above model, at least in yeast. Although it should be stressed that there is no evidence for such a mechanism in human cells, a similar mechanism could contribute to the permanent transcriptional silencing of some late G1 genes such as *C-FOS* [49].

EXPERIMENTAL MODELS OF HUMAN CELLULAR IMMORTALISATION

When human fibroblasts are transfected with Simian virus 40 (SV40) or its large-T antigen (SV40LT), cell cultures with an extended proliferative lifespan are generated and there is persuasive evidence that the main function of SV40LT is to abrogate both the *TP53* and *RB* tumour suppressor gene pathways [50, 51]. During this extended proliferative lifespan the transformed cells have undetectable telomerase activity and the telomeres continue to shorten, to the point where the telomeres are too short to prevent chromosome fusions [52]. Frequently dicentric chromosomes then form, there is considerable loss of genetic material, and the cells enter crisis where cell production is counterbalanced by cell death [53, 54]. Rare immortal cells emerge from crisis and have high levels of telomerase [53], and it has been suggested that this is the result of one or more endogenous cellular mutations [53]. Using the conditional expression of SV40LT antigen, two groups were able to show that, even after a cell mutation had rendered the cells immortal, the expression of the LT antigen was still required for the maintenance of the immortal phenotype [53, 54]. Based on these observations, Wright and associates suggested that there were two "mortality" mechanisms: M1 and M2. M1 was suggested to be overidden by LT antigen and M2 function to be abolished by the mutation(s) of cellular gene(s), at least one of which would be predicted to regulate telomerase [52].

THE DEVELOPMENT OF KERATINOCYTE CULTURES AMENABLE TO MOLECULAR GENETIC ANALYSIS AND THE ROLE OF IMMORTALITY IN THE PATHOGENESIS OF SCC-HN

In order to understand the genetic changes underpinning the immortal phenotype in a naturally occuring human tumour, we established keratinocyte cultures from all stages of head and neck squamous cell carcinoma development (SCC-HN) together with normal fibroblasts from the same individual [6]. We used techniques which had previously been shown to preserve the phenotype of SCC-HN faithfully [55] with the aim of identifying the *in vivo* genetic alterations responsible for the immortal phenotype. The cultures were characterised as neoplastic by virtue of their relatively slow rate of controlled cell death in suspension culture and their karyotype, which was usually aneuploid in carcinomas [6]. Papillomas, leukoplakias and most erythoplakias senesce *in vitro* and are genetically stable, but the frequency of immortal variants rises with the stage of tumour progression [6]. Mortal metastatic carcinoma cultures occur but are rare, and recurrent carcinomas, which have probably undergone the most cell divisions, having regrown following resection and radiotherapy, are invariably immortal [6]. These data therefore support the notion that tumours may form without the cells comprising them being intrinsically immortal, but immortality seems greatly to aid tumour progression and may be essential for tumour recurrence and consequently treatment failure. It has indeed been suggested that immortality would increase the pool of cells available for further mutation and would accelerate clonal evolution and progression [56]. Immortality would also expand the pool of cells capable of regenerating the tumour following therapy. The breakdown of senescence in neoplastic keratinocytes invariably involves the loss of p53 function and loss of heterozygosity at multiple loci, including some that harbour genes with possible roles in senescence [6]. This observation is consistent with the known role of p53 in the monitoring of genetic damage and protecting the cell against gene amplification [57], recombination [58] and certain types of aneuploidy [59]. It has also been suggested that p53 may have a more direct role in replicative senescence (see above) and that telomeric attrition may select for cells lacking a functional p53 protein [11, 60]. If this is true then the very short telomeres of the senescent cells combined with the loss of p53 could lead to the rapid evolution of immortal cell clones by the deletion of other genes involved in cellular senescence [19, 56, 61]. The ageing process could take place prior to neoplastic conversion by generating a pool of senescent target cells in the epithelium as part of a field effect or following neoplastic conversion. It is unclear which of these mechanisms predominates in human SCC-HN.

THE ROLE OF THE CYCLIN D-Cdk INHIBITOR CDKN2A/p16 IN KERATINOCYTE SENESCENCE

When DNA tumour viruses immortalise human cells, they eliminate the function of p53 and pRb [50, 51] and p53 is ubiquitously dysfunctional in immortal but not senescent neoplastic keratinocyte cultures [6]. However, preliminary investigations into the pRb status of immortal SCC-HN keratinocytes revealed no obvious abnormalities in protein location or phosphorylation [62]. Subsequently, we found that all immortal SCC-HN but not the senescent ones showed LOH at 9p21 which harbours the *CDKN2A/p16* gene, a negative regulator of cyclin D_1 [62]. When we derived antibodies to p16 we showed that all nineteen immortal keratinocyte lines tested lacked a functional p16 protein whereas five of six senescent cultures expressed a normal p16 protein [44]. The mechanism of inactivation was homozygous deletion or transcriptional silencing by methylation, rather than point mutation, and very similar results have been reported for SCC-HN *in vivo* [63, 65]. The re-introduction of p16 into immortal keratinocytes resulted in proliferation arrest, suggesting that p16 could be instrumental in keratinocyte senescence [44]. Further evidence for this comes from the observation that p16 levels rise dramatically when keratinocytes senesce *in vitro* but not when they differentiate [44] or are cultured to high density (O. Loughran and E.K. Parkinson, Beatson Institute,

Glasgow, U.K.). Furthermore, p16 does not appear to accumulate when cells are held quiescent for up to 16 weeks in culture (O. Loughran and E.K. Parkinson, Beatson Institute, Glasgow, U.K.) illustrating that p16 accumulation, like replicative senescence, is related to the number of times a cell divides and not to the length of time spent in culture [1].

THE SENESCENCE COMPLEMENTATION GROUP GENES

Somatic cell genetic experiments have shown that many immortal cell lines can be placed into one of four complementation groups for senescence [66] and subsequent monochromosome transfer studies have shown that the genes responsible for complementation groups B, C and D mapped to chromosomes 4q, 1q25 and 7q31, respectively [67–69]. The complementation group A locus is not yet known. The genes concerned cause a G1 arrest in the appropriate target cells and do not seem to require p53 or pRb to function [67], and therefore it might be expected that the complementation group pathway(s) would also be dysfunctional in immortal neoplastic kerinocytes. We found that LOH at the complementation group B locus on 4q was common in SCC-HN, LOH at 7q31 (group D) was less common and LOH at 1q25 (group C) was never observed [70]. Furthermore, the mutually exclusive nature of the LOH on 4q and 7q tentatively suggests that the genes reside on the same pathway to senescence and may be functionally linked [70]. The isolation of the genes mapping to the above genetic loci should further illuminate the mechanism of replicative senescence.

GENETIC ALTERATIONS REGULATING TELOMERASE IN SCC-HN

It has now been shown that many human tumours *in vivo* are composed of cells with very short telomeres and high levels of telomerase [71] and the inhibition of telomerase activity in immortal human cancer results in renewed telomeric attrition, crisis and death [72]. The original interpretation of these results was that either telomerase was reactivated at some stage of tumour progression [71] (Figure 4a) or it failed to be switched off during development [73] (Figure 4b). However, because telomerase activity may be present in keratinocyte stem cells [16, 17], it is possible that as tumours become progressively less differentiated with progression, the fraction of pre-existing positive cells may become so large that high levels of telomerase are detected in most advanced tumours [74] (Figure 4c). It

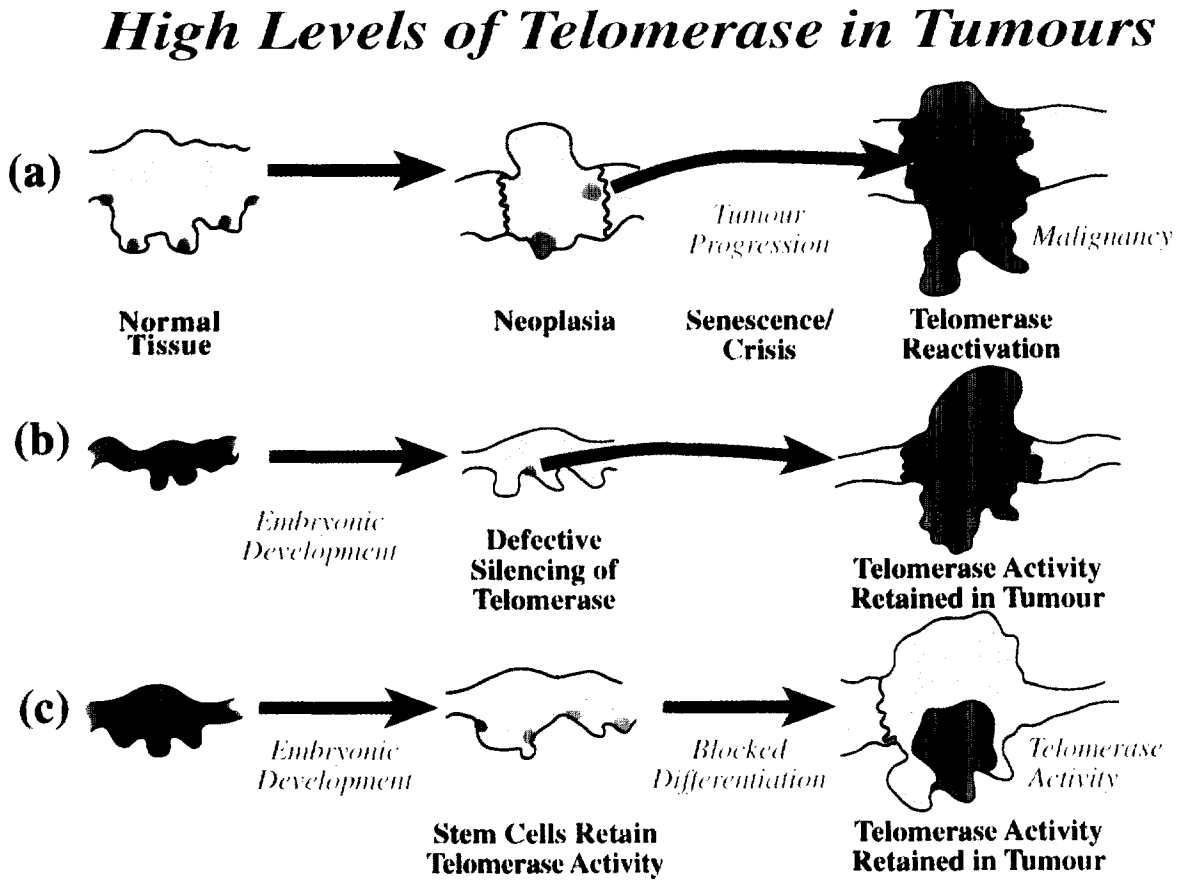

Figure 4. High telomerase levels in human tumours: selection versus reactivation. (a) The tumour arises from a telomerase-negative target cell and telomerase becomes reactivated following the exhaustion of proliferative capacity to yield immortal tumour cell clones. Such tumours are genetically unstable and have dysfunctional p53. (b) The tumour arises from a pre-existing telomerase positive cell in the embryo which fails to inactivate the enzyme at the appropriate stage of development. Such tumours are genetically stable and have functional p53. (c) The tumour arises from a pre-existing telomerase positive stem cell which at first differentiates at high frequency into a telomerase negative transit cell. As differentiation becomes increasingly blocked with tumour progression, the proportion of telomerase positive stem cells rises, until it reaches the point where the tumour is classified telomerase positive. Such tumours may be genetically stable and have wild type p53.

is likely that all of these situations are relevent to different forms of human cancer in varying proportions, but the prevailing mechanism in SCC-HN or indeed other tumours is not established. The reactivation hypothesis predicts that telomerase negative tumours exist and that telomerase positive tumour cells possess heritable genetic alterations in positive and negative regulators of the enzyme complex. Approximately 20% of the human SCC are telomerase negative [71, 75, 76] and somatic cell fusion experiments between telomerase positive immortal cells and normal cells results in a 100-fold downregulation of enzyme activity, although not complete suppression [15]. These results suggest that suppressors of telomerase activity exist, but may also indicate an increase in the activity of positive components of the enzyme, since the complete replacement of a normal genome in the hybrid does not suppress telomerase completely. Ohmura and associates recently provided evidence for a suppressor of kidney carcinoma telomerase activity on human chromosome 3 [77]. Telomerase was suppressed 20-fold following monochromosome transfer, telomeric attrition was resumed and the clones containing an intact chromosome 3 ceased proliferating. Since immortal SCC-HN lines show consistent LOH on the short arm of chromosome 3 [77], we introduced a normal copy of this chromosome into three telomerase-positive SCC-HN lines and monochromosome hybrids of one line showed a marked reduction in telomerase activity. Markers of keratinocyte differentiation were not induced in the hybrids, so an indirect suppression of the enzyme as a consequence of terminal differentiation [16] is not a likely explanation for the results. Hybrids which retained telomerase activity had lost a fragment of the exogenous chromosome in the 3p21.2–p21.3 region (unpublished data). Experiments are underway to map this locus more accurately using a larger panel of telomerase positive segregants as a first step to positional cloning. Cloning of the gene responsible for this effect will be necessary if its direct effect on the telomerase enzyme is to be tested. The availability of the gene should also reveal whether loss of function mutations in the gene contribute to the pathogenesis of SCC-HN.

In addition to the loss of possible suppressor genes on chromosome 3p, there are numerous reports of gains of 3q material in SCC-HN [78] and often both occur simultaneously in the form of an isochromosome 3q [79]. It is possible that the telomerase components themselves, when upregulated, could function as oncogenes. Furthermore, the RNA component of the enzyme hTR (human telomerase RNA) is essential for the immortal phenotype of human cells and has been mapped to the distal portion of chromosome 3q [72]. We recently mapped the *hTR* gene more accurately to 3q26.3 and showed that *hTR* was amplified and, more frequently, over-represented in a number of tumour types *in vivo*, including SCC-HN [80]. Tumours showing amplification of *hTR* also show considerable overexpression of the gene [80], so *hTR* is a candidate for the oncogene mapping to the 3q26–q27 amplicon in SCC-HN [78]. Although telomerase activity does not always correlate with *hTR* mRNA levels [81], it is possible that an increased *hTR* copy number contributes to the increased telomerase activity in a subset of tumours where hTR levels become rate-limiting. We are currently trying to manipulate *hTR* copy number in cell lines, so that the causal link between *hTR* amplification and increased telomerase activity can be established. The loss of a telomerase suppressor on the short arm of chromosome 3 whilst, at the same time, causing a duplication of the *hTR* gene itself, would be an attractive explanation for the frequent reports of isochromosome 3 in a wide variety of human cancers [79] (Figure 5) but further evidence is required to support this idea.

Figure 5. Visualisation of isochromosome 3q in a human head and neck cell line. Note the duplication of the *hTR* gene detected by fluorescence *in situ* hybridisation and labelled in green.

CONCLUSION

In summary, there is now considerable evidence to support the existence of replicative senescence in human squamous epithelia *in vivo* [4, 5]. This process breaks down in SCC-HN to yield immortal variants which in turn ensures rapid tumour progression, recurrence and consequent lethality [6]. We have begun to identify some of the genetic defects that may lead to keratinocyte immortality [6, 44, 62, 70] and our results indicate that at least four separate pathways need to be disrupted if a human keratinocyte is to attain an immortal phenotype, namely, p53, pRb, telomerase and an unknown pathway controlled by the complementation group genes. Possible genetic alterations responsible for their dysfunction are summarised in Table 1. The challenge ahead is to understand how these pathways contribute to replicative senescence, ageing pathology and human dis-

Table 1. Possible mechanisms of dysfunction in senescence pathways in SCC-HN

	Mechanisms of dysfunction/senescence pathway
p53	Mutation or loss of expression
pRb	Dysfunction of *CDKN2/p16* (deletion/methylation/point mutation) plus cyclin D_1 amplification in advanced SCC-HN
Complementation group pathway	LOH on chromosome 4q (complementation group B) or LOH on chromosome 7q31 (complementation group D)
Telomerase	Inactivation of a suppressor on chromosome 3p21.2-p21.3 and/or increased copy number or amplification *hTR*

eases. The prospect of manipulating proliferative lifespan offers exciting new possibilities in the treatment of human disease including those of squamous epithelia.

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