Functional Demonstration of a Mortality Phenotype Associated with 4cen-q23

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Declaration

I am the sole author of this thesis. All the references have been consulted by myself in the preparation of this manuscript. Unless otherwise acknowledged, all the work presented in this thesis was performed personally.

ACF	Aberrant crypt foci
ADPKD	Autosomal dominant polycystic kidney disease
ALT	Alternative lengthening of telomeres
APC	Adenomatous polyposis coli
ATM	Mutated in ataxia telangiectasia
BAC	B1 artificial chromosome
bHLH	basis helix-loop-helix
BICR	Beatson Institute for Cancer Research
BRCA	Breast cancer-associated
CC	Cervical carcinoma
CEA	Carcinoembryonic antigen
CGH	Comparative genome hybridisation
CIN	Cervical intraepithelial neoplasia
DBD	DNA-binding domain
DCC	Deleted in colorectal cancer
EA	Esophageal adenocarcinoma
EC	Esophageal carcinoma
EGFR	Epidermal growth factor receptor
ERC	Extrachromosomal rDNA circle
ESCC	Esophageal squamous cell carcinoma
EST	Expressed sequence tag
FAP	Familial adenomatous polyposis
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HDF	Human diploid fibroblasts
HNSCC	Squamous cell carcinoma of the head and neck
HPV	Human papillomavirus
IBD	Irritable bowel disease
LFS	Li-Fraumeni syndrome
LOH	Loss of Heterozygosity
M1	Mortality stage 1
M2	Mortality stage 2

MEF	Mouse embryo fibroblast
MMCT	Microcell-mediated monochromosome transfer
MPD	Mean population doublings
MZF-2	Myeloid-specific zinc finger protein 2
NHEJ	Nonhomologous end joining
NIMA	Never in mitosis A
NSCLC	Non-small cell lung cancer
OD	Oligomerisation domain
ORF	Open reading frame
OSCC	Oral squamous cell carcinoma
PCNA	Proliferating cell nuclear antigen
PIN2	Protein interacting with NIMA 2
PML	Promyelocytic leukaemia
PP1	Protein phosphatases (type 1)
QPCR	Quantitative PCR
pRB	retinoblastoma
RCC	Renal cell carcinoma
RDA	Representational difference analysis
RFLP	Restriction fragment length polymorphisms
ROH	Retentions of heterozygosity
SAGE	Serial analysis of gene expression
SAM	Sterile alpha motif
SCC	Squamous cell carcinoma
SCLC	Small cell lung cancer
SIR	Silent information regulator
SV40	Simian virus 40
TBP	TATA-binding protein
TCC	Transitional cell carcinoma
TD	Transactivation domain
TEP1	Telomerase protein component 1
TIN2	TRF1-interacting nuclear protein 2
TRF	Telomere factor
tsg	Tumour suppressor gene
TUNEL	Terminal uracil nucleotide end labelling

XMMCT	X-irradiated microcell-mediated monochromosome transfer
XP	Xeroderma pigmentosum
YAC	Yeast artificial chromosome

Reagents

BrdU	5-Bromo-2'-deoxy-uridine
BSA	Bovine serum albumin
DCS	Donor calf serum
DH_2O	De-ionised water
DMEM	Dulbeccos modified eagles medium
DMSO	dimethylsulphoxide
EDTA	Ethylenediaminetetra-acetic acid
FBS	Foetal bovine serum
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulphonic acid)
MES	2-(N-morpholino) ethansulfonic acid
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PHA-P	Phytohemaglutinin
SSC	sodium chloride, sodium citrate
SST	sodium chloride, sodium citrate, tris
TAE	tris, acetic acid, ethylendiaminetetra-acetic acid
TBE	tris, boric acid, ethylendiaminetetra-acetic acid
TE	tris, ethylendiaminetetra-acetic acid
TEMED	Tetramethylenediamine
Tris	2-amino-2-(hydroxymethyl)propane-1, 3-diol

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Units

bp	base pair
Bq	becquerel
c	centi
g	gram
g	gravity
Gy	Grey
hr	hour
k	kilo
l	litre
M	mega
μ	micro
m	milli
m	metre
M	molar
min	minute
n	nano
°C	degree celsius
PD	population doublings
rpm	revolutions per minute
RT	room temperature
s	second
U	unit
V	volts
v/v	volume for volume
W	watts
w/v	weight for volume

Abstract

Normal human keratinocytes possess a finite replicative lifespan whereas most advanced squamous cells carcinomas are immortal. The mechanisms, whose abrogation's can be considered necessary to achieve immortality, include those involving the negative cell cycle regulators p53, $p16^{INK4A}$, and the telomere repair reverse-transcriptase enzyme, telomerase. Other specific chromosomes have also been demonstrated to carry functions whose loss is necessary for the development of immortality, through immortal phenotype reversion upon reintroduction into an immortal cell line.

We demonstrate here the phenotypic reversion of immortal HNSCC-derived keratinocyte cell lines to a mortal growth-arrest upon reintroduction of a resistance marker tagged wild-type human chromosome 4. We further demonstrate that this phenotypic reversion occurs only in cell lines, which display LOH on 4q (BICR6 and BICR31), and not in those with intact endogenous chromosome 4 copies (BICR3 and BICR19), and that it is chromosome 4-specific, chromosomes 6, 11, and 15 having no effect on proliferative lifespan following introduction into BICR6 by MMCT. Through XMMCT-based truncated chromosomal fragment generation the functional complementation was localised to 4cen-q23, whilst fine mapping in segregants arising from the MMCT experiments identified an approximate 1.5Mb locus containing a minimal number of candidate genes.

Through biological assay we have further determined the growth-arrest to have characteristics of crisis. This was determined through low BrdU incorporation balanced with high levels of apoptosis to statistically significance levels (<0.05). We found no evidence for involvement of telomeric attrition in the observed phenotype, through insufficient phenotypic lag (3-10 MPD) and growth-arrest in the presence of ectopic hTERT expression, suggesting the operation of an alternative mechanism. This suggests the presence of gene(s) at 4cen-q23 whose loss is advantageous to the development of immortality in advanced tumours including HNSCC.

Chapter 1: Introduction

1) Cancer as a Multistage Process

1.1) Theories of Tumourigenesis

The initiation and progression of a normal cell to the molecular dysfunctionality of a malignant cell is brought about through the accumulation of genetic alterations that deregulate controlling pathways. Tumour progression and its development in a qualitative manner was first proposed by Foulds (1957) such that multiple rather than single specific changes were required for malignancy to develop. This multistep deregulation brings about the activation of oncogenes accompanied by, although not in every instance, the inactivation of tsg's (Bishop, 1987). The malignant threshold however is defined by the accumulation of genetic aberrations rather than the sequence of events. The paradigm that genes were implicated in malignant progression was first provided by the isolation of dominantly acting transforming genes, oncogenes, from both avian and mammalian retroviruses followed by the identification of cellular homologs, proto-oncogenes. The observation that these oncogenes could induce the neoplastic transformation of rodent cells in culture demonstrated the dominance over their cellular counterparts and provided molecular evidence for the involvement of genes in neoplastic progression.

The concept that biological events in tumour progression represented the effects of genetic instability and the resultant sequential selection of variant subpopulations based on that instability was proposed by Nowell (1976). However, intrinsic to this theory was the idea that the genetic instability was due to the activation of specific gene loci rather than the loss of regulatory loci whereas it is now recognised to be due to both effects. The idea that two copies of a 'recessive' gene must be lost or inactivated to allow neoplastic development was first postulated in the Knudsons 'two hit' hypothesis' (1971) developed in reference to childhood retinoblastoma. This is reinforced now by observations that the loss of one copy of a 'recessive' gene can increase the potential for the second copies inactivation. A unifying

somatic mutation theory of cancer was also proposed by Comings where loss of regulatory genes and activation of proto-oncogenes had a role in tumourigenesis drawing on both Nowells earlier proposition and Knudsons hypothesis. This developed a unifying thread common to both earlier theories where tumour progression through loss of function mutations characterised by point mutations, frameshift mutations and deletion of chromosomal regions was accompanied by amplification of chromosomal regions encoding oncogenes. Therefore mutational events in the development of a tumour can be largely characterised as either a gain of function (oncogenes) or a loss of function (tsg's).

1.2) Functional Demonstrations

Early studies into tumourigenicity suggested that it was a dominant trait in murine fusion experiments (Barski *et al.,* 1962), although this interpretation did not incorporate the initial suppression of tumourigenicity, which had occurred in hybrids. This initial observation was refuted in studies, which showed that in fusions between non-malignant and malignant cells the malignancy was recessive to the normal phenotype (Harris *et al.,* 1969; Stanbridge, 1976) illustrating a non-malignant dominance. This was followed by the suggestion that specific chromosomes were involved in the suppression of malignancy whose loss was required for malignancy to develop (Harris *et al.,* 1971). The observed early dominance feature (Barski *et al.,* 1962) was most likely therefore to be due to the *in situ* loss of chromosomes from the hybrid populations allowing the outgrowth of revertant segregants through positive selection pressures (Peehl and Stanbridge, 1981). The genesis of the idea that specific chromosomes could in some form regulate a cells predisposition to form a tumour was found some 60-80 years earlier. The pair of Von Hansemann (1890) and Boveri (1914) were perhaps the first to propose the idea of chromosomal imbalance leading to malignant progression. Both their earlier theories were reinforced by the postulation that the balance between specific chromosomes would determine controlling factors, which would either potentiate or suppress malignant

development (Hitotsumachi *et al.*, 1972). Further reinforcement was provided by the demonstration that an excess of 5_7 over 7_3 (hamster chromosome nomenclature; Yamamoto *et al*, 1973) led to an expression of malignancy in tumour clones (Benedict *et al.*, 1975). In a refinement of the earlier works and theories the first tentative evidence that specific chromosomal loss could lead to a re-emergence of tumourigenicity was shown in fusions between normal diploid fibroblasts and HeLa cells where the loss of an unassigned copy of chromosome 11 or 14 led to the re-expression of the tumourigenic phenotype (Stanbridge *et al.*, 1981). This work was reinforced with the further observation that a single chromosome loss could be responsible for the re-emergence of tumourigenic variants (Stoler and Bouck, 1985; Saxon *et al.*, 1986). Therefore over an approximate 100-year period the idea of chromosomal change being key to progressive tumour development proceeded from hypothesis to an evidenced fact.

Although loss of function mutations and oncogene amplifications are perhaps the best characterised mechanisms of gene expression deregulation a further non-mutagenic form of gene silencing is demonstrated by DNA methylation. This process involves the covalent modification at the fifth carbon position of cytosine residues within CpG dinucleotides that cluster in CpG islands at the 5' ends of many genes. Methylation can affect gene expression by preventing the binding of transcription factors to promoter sequences, through preferential binding of repression factors to the methylated sites, and through methylation perhaps changing chromatin structure to an inactive form. The cell cycle factors; PCNA, $p16^{INK4A}$, and $p21^{WAF1}$, have been linked to methylation regulation suggesting possible regulation of the cell cycle in this manner. Transcriptional silencing has been shown to occur by hypermethylation of promoter regions in *pRB* and the von Hippel-Lindau tsg in retinoblastoma and renal cancer respectively (Ohtani-Fujita *et al.*, 1993; Herman *et al.*, 1994).

The progression of tumourigenesis is due to perhaps three key mechanisms of genetic dysfunction, which exert their functions largely on deregulation of the cell cycle. These can be characterised as discussed

above as loss of function through LOH or mutation removing an intrinsic highly regulated tsg and therefore in effect losing important cell cycle regulatory blocks, as gain of function amplifications inappropriately leading to a constitutive activation of otherwise induction-controlled oncogenes driving cell cycle machinery in an uncontrolled manner, and undergoing inactivation through promoter silencing by methylation or conversely activation by silencing of a repressor.

1.3) Colorectal Carcinoma Model

An early demonstration of tumourigenic development occurring in a multistep process was provided by studies into colon cancer. The rare dominantly inherited susceptibility to colon cancer, familial adenomatous polyposis (FAP) gene, adenomatous polyposis (*APC*), was identified initially through microdeletion of the 5q21 locus in a patient with developmental disorders and *APC* (Herrera *et al.,* 1986). The analysis of rare cancer predisposition syndromes like FAP (<1% of colorectal carcinomas) can lead to the discovery of genes involved in sporadic cancers through abrogation of mutual pathways. Linkage analysis suggested that the 5q21 locus was tightly linked to the disorder (Bodmer *et al.,* 1987; Leppert *et al.,* 1987) whilst LOH evidence suggested that 5q21 allelic loss occurred in up to 40% of all cases of colorectal carcinoma (Bodmer *et al.,* 1989). Further analysis demonstrated the *APC* gene had not only undergone somatic alteration in tumours of sporadic colorectal cancer patients but also showed germline mutations in FAP patients (Groden *et al.,* 1991; Nishisho *et al.,* 1991). Germ-line mutations and LOH or somatic mutation of the remaining allelic copy meets the minimal requirements of a tsg as outlined by Knudson (1971). However, germline mutations of *APC* were not sufficient for tumour formation, as evidenced by FAP patients (Kinzler and Vogelstein, 1996), although mutation of the parental wild-type allele is the rate-limiting step in colorectal carcinoma development (Ichii *et al.,* 1992; Levy *et al,* 1994; Luongo *et al.,* 1994). Studies into FAP led to the identification of the gene *APC* which itself is the rate-limiting factor in colorectal carcinoma development. *APC* inactivation has

been shown in dysplastic aberrant crypt foci (ACF), which are the earliest examinable form of neoplastic lesion believed to be adenoma precursors (Jen *et al.,* 1994). This led to the suggestion that APC was a 'gatekeeper' of colonic epithelial proliferation whose mutation led to an imbalance of cell division over cell death (Kinzler and Vogelstein, 1996), this suggestion came in some part from the observation where introducing wild-type expression in *APC* mutant epithelial cells resulted in apoptotic cell death (Morin *et al.,* 1996) and an evidenced involvement in the *WNT* signalling pathway (Erdmann *et al.,* 2000). Further evidence suggested that in the development of colorectal carcinoma recessive alterations occurred on chromosome 17 and 18; the chromosome 17 alteration site, *p53*, was altered in >80% of tumours analysed (Baker *et al.,* 1990). However, p53 cannot induce colorectal carcinoma in an APC-independent manner (Garber *et al.,* 1991). The chromosome 18 gene has several candidates although *Deleted in Colorectal Cancer* (*DCC*) and *SMAD* may be good candidates for this abrogation site (Hahn *et al.,* 1996; Cho and Vogelstein, 1992). Changes that were implicated in the further development of colorectal carcinoma included dominant mutations of the *ras* oncogene and expression alterations of human leukocyte antigen (HLA)-A, B, and C determinates (Bodmer *et al.,* 1989). A similar situation to that seen with *p53* is observed with *ras* such that although mutations occur frequently in colonic tumour progression, *APC* mutation is still required for colorectal neoplastic development (Jen *et al.,* 1994). Through further analysis a pattern of progression in a multistep fashion had began to emerge, this showed a sequential pattern of LOH which correlated well with tumour stage (Boland *et al.,* 1995) which ultimately is thought to require a minimum of four changes. The transition from normal colonic epithelium to benign adenoma is characterised by the inactivation of APC, and the loss of proliferation control; this is followed by the dominant activation of ras, and inappropriate mitogenic stimuli; the accumulation of LOH on 18q, presumably inactivating DCC; followed by LOH on 17p, p53, at the transition from adenoma to carcinoma undoubtedly accompanied by further genetic changes. Therefore the analysis of the rare FAP disorder led to the unravelling of a multistep neoplastic developmental pathway with a rate-limiting step (APC) requirement for

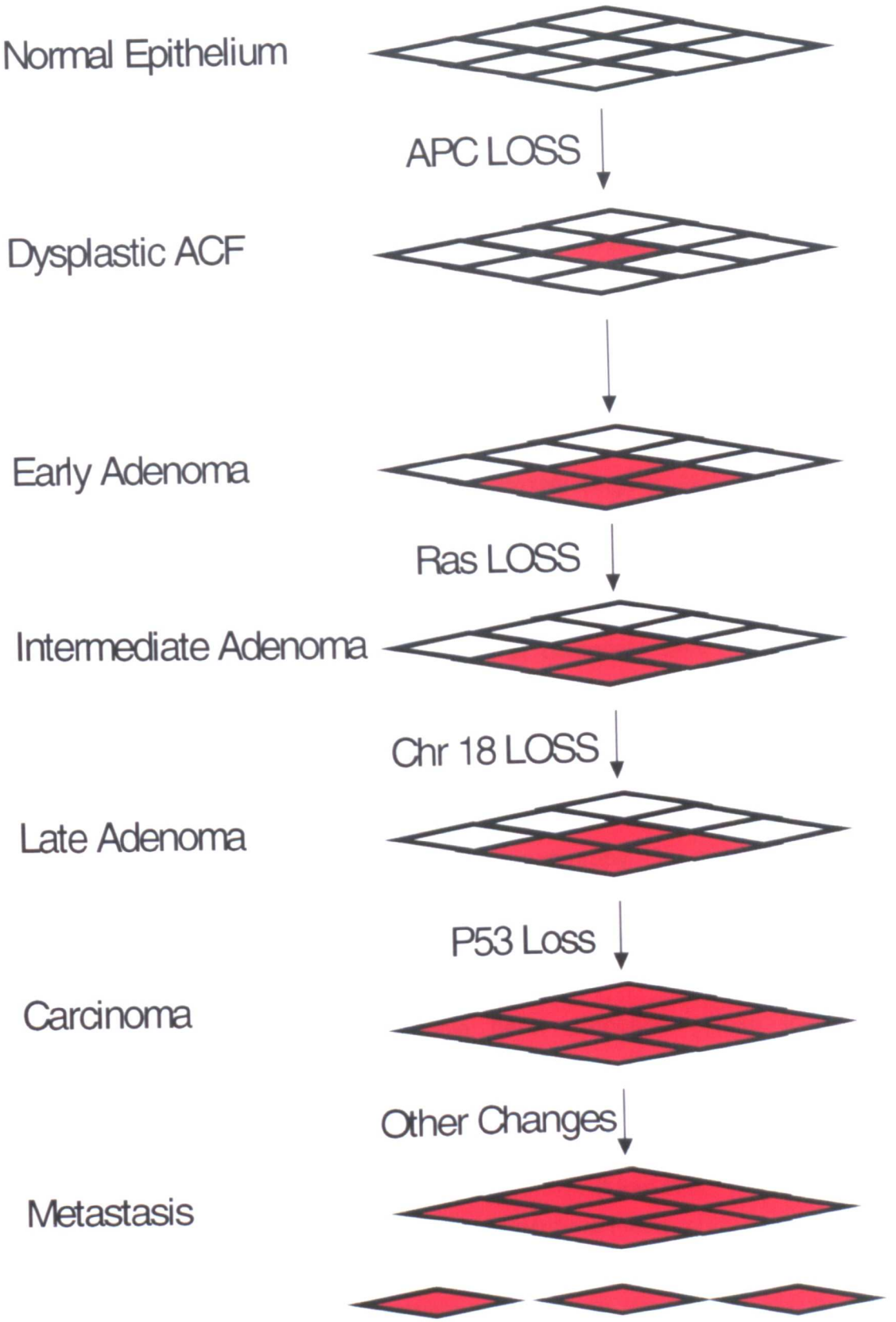
colorectal carcinoma development. A representation of events in colorectal cancer development is shown in Figure 1.1.

1.4) Multistage Progression in Other Cancers

The proposition that progressive tumourigenesis was the result of the accumulation of genetic changes proposed by theories such as those described above has been suggested for a number of further tumour types. Although chromosomal amplifications occur frequently in tumourigenesis, demonstrated in regions containing oncogenes, in this instance we are concerned with the loss of chromosomal sequences and their accumulation in the advanced stages of tumourigenesis. Studies into the genetics of bladder cancer development have shown LOH in a number of common sites many of which are necessary for tumourigenic progression at 3p, 4p, 4q, 8p, 9p21, 9q22, 9q32-q33, 9q34, 10q23, 11p, 11q, 13q14, 14q, and 17p13 although not necessarily thought to occur in that order (Knowles, 1999). Cervical carcinoma has been shown to demonstrate losses repeatedly on a number of different chromosomes on 3p14-p22, 4*p16*, 5p15, 6p21-p22, 11q23, and 17p13.3 again indicative of a multiple step requirement for tumourigenesis (Lazo, 1999). Malignant mesothelioma accumulates losses on chromosomes 1p, 3p, 6q, 9p, 13q, 15q and 22q although in the development of this aggressive tumour the order of losses does not seem to be as critical as their occurrence (Murthy and Testa, 1999). Poor prognosis in prostate cancer has been shown to be accompanied by LOH at 5q, 6q, 7q, 8p, 8q, 10q, 13q, 16q, 17p, 17q, and 18q although the order of these accumulative losses occur has not been determined (Verma *et al.,* 1999). Fundamentally, these observations serve to highlight that not only do similar chromosomal regions undergo LOH in the development of a wide range of tumour types but that they may also occur in an undetermined order where tumour advancement is reliant on a minimal change. This suggests that loss of the same genetic loci at similar

Figure 1.1. Colorectal carcinoma model of tumour progression through multiple changes

Schematic demonstrating the sequential genetic changes, that occur in the progression from normal epithelium to metastatic colorectal carcinoma.


Normal Epithelium
APC LOSS
Dysplastic ACF
Early Adenoma
Ras LOSS
Intermediate Adenoma
Chr 18 LOSS
Late Adenoma
P53 Loss
Carcinoma
Other Changes
Metastasis

stages are required in a number of diverse tumours indicative of common pathways undergoing abrogation. This is therefore in agreement with the idea of accumulative changes adding to the tumourigenic potential of neoplastic development.

1.5) HNSCC as an Example of Multistage Cancer.

The development of HNSCC is associated with overexposure to the common carcinogenic factors tobacco and alcohol as well as other environmental and occupational factors (Landrigan and Baker, 1991. The standard treatment regime as described by Zatterstrom *et al.* (1991) was in brief; patients with T1 tumours underwent primary surgery, patients with T2 and resectable T3 and T4 tumours received preoperative radiotherapy (50Gy) followed by surgery, laryngeal T1-3 carcinomas and non-resectable T3 and T4 underwent radiotherapy (64-70Gy) and followed by selective salvage surgery, patients with regional metastasis at diagnosis were treated with radiotherapy followed by neck dissection. The 'T' system of tumour classification represents the stage of the growth, i.e. T1 is the smallest tumour size with no local invasion whereas T4 is the largest classification with invasion of local tissues. Generally there is an increasing tendency to follow organ preservation protocols where chemotherapy is combined with radiotherapy for advanced lesions in place of that described above with a reversion to surgery where treatment fails (Collins, 1998).

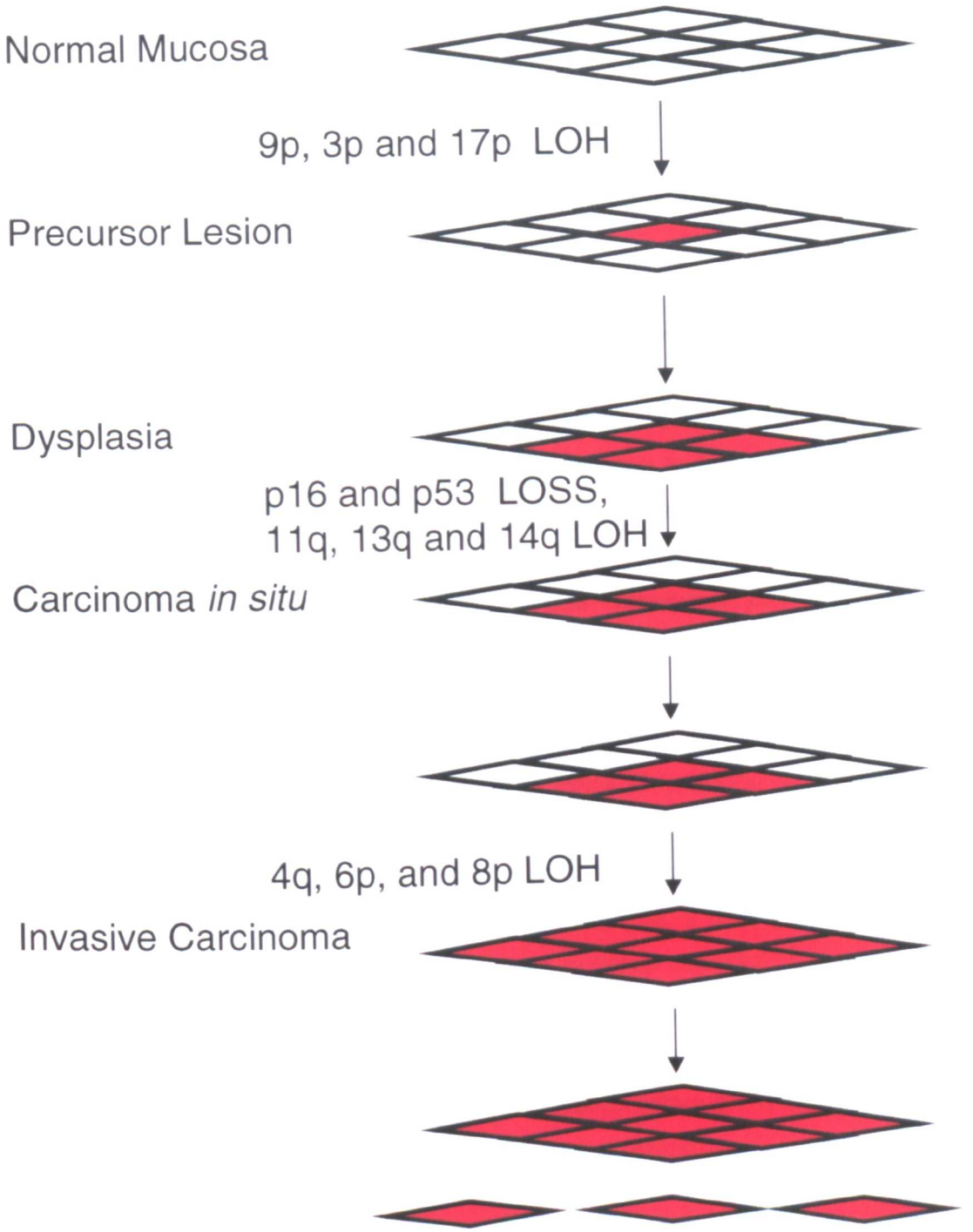
Several candidate genes have been proposed whose loss or amplification is essential for SCC development including *carcinoembryonic antigen* (*CEA*) (Kimura *et al.*, 2000); $p16^{INK4A}$ (Lydiatt *et al.*, 1998), *p21* (Erber *et al.*, 1997), and *cyclin D1* (Kyomoto *et al.*, 1997; Capaccio *et al.*, 2000), *p53* (Cabelguenne *et al.*, 2000) and *pRB*; *ras* and *c-myc* (Field *et al.*, 1989); *EGFR* and *HER-2/neu*; *collagenase* and *matrix metalloproteinase*; and *SCC antigen* (*SCCA*) *1* and *2* (Kimura *et al.*, 2000). None of these have been conclusively demonstrated as successful prognostic indicators of HNSCC (Villaret *et al.*, 2000), indeed some have been demonstrated not to be e.g. *ras* (Yarbrough *et*

al., 1994), and *CEA* (Kimura *et al.,* 2000). As the number of genes whose deregulation is linked to tumourigenesis increases the idea of a single gene whose loss is predictive of therapeutic response and patient mortality is perhaps slightly optimistic although the development of microarrays may provide patterns of alterations in HNSCC providing a global expression change pattern.

In keeping with the nature of multistep development LOH is seen frequently on a number of chromosomes in HNSCC although these events do not occur simultaneously but rather are ordered in an approximate sequential series of events as expected in the progression of tumourigenesis. Allelic losses were demonstrated in HNSCC on chromosome 3p14 (Mao *et al.,* 1996), 3p13-cen, 3p21, and 3p25-pter (Maestro *et al.,* 1993; Gotte *et al.,* 2000; Schantz *et al.,* 2000), 6p21.3 (Feenstra *et al.,* 1999, 2000), 7 (Berker-karauzum *et al.,* 1999), 8p (Li *et al.,* 1994), 9p21 (Mao *et al.,* 1996; Reed *et al.,* 1996; Waber *et al.,* 1997; Lydiatt *et al.,* 1998), and 9q22.3 (Loughran *et al.,* 1994; Van der Riet *et al.,* 1994; Ah-See *et al.,* 1994), 10q (Ah-See *et al.,* 1994), 10q22-q26 (Gasparotto *et al.,* 1999), 11p (Ah-See *et al.,* 1994), 13q14 (Van der Riet *et al.,* 1994), and 13q34 (Maestro *et al.,* 1996; Gunduz *et al.,* 2000; Sanchez-Cespedes *et al.,* 2000), 14q (Lee *et al.,* 1997), 15q (Feenstra *et al.,* 1999), 16q24 (Wang *et al.,* 1999), 17p (Li *et al.,* 1994), 18q (Pearlstein *et al.,* 1998), 18q21.1-q23 (Takebayashi *et al.,* 2000) and 22q (Poli-Frederico *et al.,* 2000). In the published data outlined above and elsewhere there are over 19 separately identified loci of which 4 may represent known tsg's, this would imply there are at least a further 14 genes whose deregulation can proffer a growth advantage onto the developing HNSCC tumour. It is not clear however which loss events are critical and which are consequential and therefore which losses represent the true rate-limiting steps in the development of HNSCC. An approximate ordering is thought to occur from normal mucosa with an early 9p loss followed by 3p and 17p loss in precursor lesions, dysplasia loss of p16, 11q, 13q and 14q accompanied by p53 mutation leading to carcinoma *in situ* followed by loss of 6p, 4q and 8p leading to invasive carcinoma (Califano *et al.,* 1996) (Figure 1.2). A number of case studies (discussed in more detail below and in Chapter 3) have described

Figure 1.2. HNSCC model of tumour progression through multiple changes

Schematic demonstrating an approximation of the sequential genetic changes, that are thought to occur in the progression from normal mucosa to invasive carcinoma.


Normal Mucosa
9p, 3p and 17p LOH
Precursor Lesion
Dysplasia
p16 and p53 LOSS,
11q, 13q and 14q LOH
Carcinoma *in situ*
4q, 6p, and 8p LOH
Invasive Carcinoma

LOH on chromosome 4 (Nawroz *et al.*, 1994; Loughran *et al.*, 1994, 1997; Pershouse *et al.*, 1997) and have contributed to the observation that these allelic losses are a late event in HNSCC tumourigenesis (Califano *et al.*, 1996, Lee *et al.*, 1997, Rosin *et al.*, 2000). These observed losses implicate a region on the long arm of chromosome 4 but provide no evidence for a locus on 4p in HNSCC (Loughran *et al.*, 1994).

The loss of p53 may represent a relatively early event in HNSCC neoplastic development. The overexpression of p53 in dysplastic lesions and CIS was shown to occur before the development of invasive carcinoma (Pavelic *et al.*, 1994), which may suggest a response to tumour progression and accumulating DNA damage by the gene. The implication here is that p53 loss correlates with a more advanced, aggressive, and less amenable to treatment tumour phenotype. Mutation of a *p53* homologue, *p63*, was found in a HNSCC line of epidermal origin although p53 was also mutated in the line preventing determination of whether the mutation was relevant to the development of the tumour (Osada *et al.*, 1998). Conversely, the *p63* locus, 3q28, was shown to be over-represented in HNSCC tumours, which itself is generally indicative of gene amplification, although both p53 status and stage were unknown (Yamaguchi *et al.*, 2000). Clearly the role of p63 in HNSCC requires further demonstration to determine what role, if any; it plays in this cancer progression (see Section 3.2). The putative tsg *p33^{ING1}* which has been functionally linked into the p53 pathway did not demonstrate any somatic mutations in a HNSCC study suggesting it may not have a role in these tumours (Sanchez-Cespedes *et al.*, 2000) although a further study found 8% (3/34) of HNSCC analysed possessed missense mutations of the gene (Gunduz *et al.*, 2000). The low number of mutations demonstrated would suggest that the position of *p33^{ING1}* in HNSCC development is far from clear although does not seem to be a critical factor. Furthermore in studies of HNSCC lines normal pRB activity was present (Loughran *et al.*, 1996) although LOH was shown frequently at 9p21 suggesting inactivation of the G1/S phase transition checkpoint mechanisms through abrogation of p16^{INK4A} (Loughran *et al.*, 1994; Reed *et al.*, 1996). The *p16^{INK4A}* gene has been shown to be deficient in a number of HNSCC lines through mutation (Yeudall

et al., 1994; Zhang *et al.*, 1994; Reed *et al.*, 1996) and homozygous deletion (Loughran *et al.*, 1994; Cairns *et al.*, 1995; Munro *et al.*, 1999). The oncogene *c-myc* was shown to be frequently amplified in HNSCC however this is at odds with later studies and therefore should be interpreted with caution (Yokota *et al.*, 1986). The overexpression of EGFR has been associated with SCC tumourigenicity (Santon *et al.*, 1986) and suggested to be necessary for SCC derived cell lines (Modjtahedi *et al.*, 1993) however a study using 10 HNSCC lines found no obvious correlation between elevated levels of EGFR and tumour progression (Stanton *et al.*, 1994) and no link with EGFR expression. Analysis of oncogenes in the same HNSCC lines found no *ras* mutations (Clark *et al.*, 1993) and no *c-myc* amplifications (Agochiya *et al.*, 1999) although a consistent increase in cyclin D1 was observed (Nikolic, 1993) which had no obvious relationship with EGFR expression. From these observations it is clear that the same critical pathways are subject to abrogation in HNSCC tumour lines as would be expected in any advanced tumour, that is a deregulation of the cell cycle through mutation and silencing of the prominent tsg's that control the key regulatory pathways.

2) Role of Cellular Immortality in Human Cancer

2.1) Biological Examples

The phenomenon of cellular immortality has been described for a number of advanced tumours, although not in every instance. Tumour immortality has obvious clinical implications, as a consequence of the accumulated genetic changes, which lead to greater likelihood of recurrence following therapy and a general decrease in responsiveness to treatment. The genetic changes, which occur in association with the development of immortality, vary from cell type to cell type, although there are similarities, which in some instances reflect the same critical genetic events.

2.1.1) Melanoma

Normal melanocyte proliferation and differentiation disruption is thought to arise and progress through the accumulation of genetic defects that lead ultimately to the development of melanoma (Albino, 1995). The development of malignant and immortal melanoma has been characterised through accompaniment by key genetic changes, where although the exact order of changes have not been characterised, which can be categorised into approximate groupings; those necessary for neoplastic development, and those necessary for immortal development. Immortal melanomas themselves characteristically arise from the dermal infiltrating 'vertical growth phase' rather than the 'radial growth phase' lesion. The reasons for this are not completely clear although may be due to forced clonal selection through immune response leading to increasing rates of mutation (Prehn, 1996). Initial analysis suggested LOH at 9p and 10q were losses associated with early stage melanoma lesion development whereas 6q, 18q, 1p, 11q, and 17q were associated with melanoma progression (Walker *et al.*, 1995). Paired sample analysis went on to show that whereas LOH on 9p, 6q, 8p, and 18q was found on both primary tumours and their respective metastasis in a number of cases LOH of 6q, 11q and 7q was detected in the metastasis and not in the primary tumours (Morita *et al.*, 1998). Further evidence from primary tumours and their metastases showed LOH at 9p21 was a common event in neoplastic development and found additional allelic losses on 14q and Xq which also reflected an early change (Rubben *et al.*, 2000). Loss of either p16INK4A or less frequently pRB accompanies sporadic melanomas and cell lines derived thereof indicating the importance of this pathway loss for the development of deregulated proliferation (Bartkova *et al.*, 1996). The association of the *p16^{INK4A}* gene with poor prognosis in 'vertical growth phase' melanoma and the early-stage losses of 9p21 suggest that in melanoma this gene acts as a 'gatekeeper' (see Section 3.1) (Straume *et al.*, 2000). This is further reinforced by the frequent inactivation of p16^{INK4A} in familial melanoma kindreds (Hussussian *et al.*, 1994). Interestingly, virtually all immortal melanoma lines have defects in the p16/pRB pathway (Walker *et al.*, 1998).

Conversely, 'vertical growth phase' tumours lacking p53 expression delivered better patient prognosis indicating that mutation of this gene is likely to be a later event in advanced tumourigenic development (Straume *et al.,* 2000). This evidence of accumulative LOH is indicative of a positive selection within the tumour biased towards a propensity for accumulation of genetic alterations. In immortal tumours this clearly may be a reflection of immune response exposure along with $p16^{INK4A}$ loss allowing advanced neoplastic progression to occur. Although not all metastases are immortal these changes must reflect the minimal possible changes accompanied by the reactivation of telomerase (see Section 4.2.2) to achieve immortality.

2.1.2) Colorectal Carcinoma

The model of sequential alterations checked by the existence of biological rate-limiting steps is illustrated in Section 1.2 by the well-understood mechanisms behind the development of colorectal carcinoma. However, further genetic events underpin the progression to immortality by the carcinoma once developed. Through the development of an immortalised non-tumourigenic epithelial cell line from a large pre-malignant colorectal adenoma and subsequent observations the loss of chromosome 1 was implicated in immortal development suggesting it may have been a rate-limiting event in the development of this phenotype (Paraskeva *et al.,* 1988). A further implication from this study was that the acquirement of immortality was a late event as the cell line was derived from a large adenoma and no immortal cell line generation was seen from smaller adenomas. Further karyotypic analysis detected abnormalities of chromosomes 1, 7, 14, 17, 18, and 22, which had occurred in premalignant adenoma cell lines. The observation that colon adenomas of $<1cm^3$ are rarely capable of infinite growth *in vitro* whereas adenomas of $>1cm^3$ are often immortal (Paraskeva *et al,* 1988; 1989) confirms the idea that the accumulation of changes through lifespan can be sufficient to achieve immortality.

2.1.3) Bladder

The process to immortalisation is well characterised as resulting from recessive changes, through loss of gene function, leading to deregulation of proliferation and DNA-repair control. However, dominant alterations can also contribute to immortalisation as evidenced in bladder cells where amplification of 20q in SV40 E7-infected cells resulted in a mortality bypass and the development of the immortal phenotype (Savelieva *et al.,* 1997; Cuthill *et al.,* 1999). Therefore in this instance a dominantly acting oncogene has enabled a bypass of mortality into immortality. Further analysis of bladder cell lines showed that certain losses; 3p14, 8p21, 11p15, 13q14, and 17p13 (Presti *et al.,* 1991; Hubuchi *et al.,* 1993; Knowles *et al.,* 1994), correlated with late-stage disease whereas others were grade and stage independent; 9q (Ross *et al.,* 1996). Inactivation of tsg's in TCC pathogenesis is best characterised for *pRB* (13q14), *p16* (9p21) and *p53* (17p13) (Presti *et al.,* 1991; Xu *et al.,* 1993; Esrig *et al.,* 1994; Spruck *et al.,* 1994; Cairns *et al.,* 1995). Interestingly, where immortal TUC lines do not contain a $p16^{INK4A}$ alteration, they show a *pRB* alteration (Yeager *et al.,* 1995), which demonstrates that abrogation of the same pathway results from these separate alterations. The critical alteration in TCC immortalisation can be either pRB or p16 loss accompanied by one additional alteration, such as amplification of 20q11-q12, or loss of either 3p13-p14 or 8p21-pter (Yeager *et al.,* 1998). Recent data also suggests most immortal TCCs also carry defects in the p53/ARF pathway (Sarkar *et al.,* 2000).

2.1.4) HNSCC

In line with the studies described earlier it was shown that immortality of HNSCC is recessive to a mortal phenotype (Berry *et al.,* 1994) suggesting the inactivation of tsg's in the immortal lines. Mutation of *p53* has been shown to be a common (45-90%) feature of immortal HNSCC lines (Hollstein *et al.,* 1991; Sakai and Tsuchida, 1992; Boyle *et al.,* 1993; Burns *et al.,* 1993) although non-immortal neoplastic cultures usually possess an unaltered copy

of the gene (Burns *et al.,* 1994). Immortalisation is usually accompanied by loss of p53 activity therefore, perhaps unsurprising was the demonstration that *p53* mutation, but not overexpression, correlated with decreased median patient survival (Mineta *et al.,* 1998), as this is indicative of a replicative senescence bypass. The *p16^{INK4A}* gene was shown to be expressed normally in senescent neoplastic cultures (Loughran *et al.,* 1996) demonstrating the necessity of its inactivation for cellular immortality of these cells. The generation of an immortal phenotype is considered to be a late event in the progression of HNSCC that may be indicative of a late, rather than an early, acquirement of a genetic change in malignant progression. LOH was not seen at 3p13-cen, 3p21, 3p25-ptel, 9p21, 9q22.3 and 13q14 in senescent cultures demonstrating these losses as being a later event in tumourigenesis (Edington *et al.,* 1995). The implication in the development of immortality in HNSCC is the necessary abrogation of the 'gatekeepers', p16^{INK4A} and p53, along with deregulation of the enzyme telomerase (see Section 4.2.2) accompanied by accumulative changes through advanced progression. Mutation of *pRB* is an infrequent event in HNSCC development with G1/S phase dysregulation tending to occur through p16^{INK4A} (Lang *et al.,* 1998). In the presence of constitutive p53, E2F-1, and p21^{CIP1} expression telomerase was not sufficient to rescue HNSCC lines from apoptosis further demonstrating the necessity of these pathways abrogation's (see Section 3) in HNSCC immortality (Henderson *et al.,* 2000). However, unlike melanoma, bladders, and colon cancer where immortality is a late event, some HNSCC that pass through a pre-malignant dysplastic stage, show immortality (Dr. F. McGregor, BICR, unpublished data). This may suggest two distinct forms of HNSCC where the timing of immortality is different (Edington *et al.,* 1995)

3) Tumour Suppressor Genes Inactivated in Immortalisation

3.1) Gatekeepers and Caretakers

The observations that similar genes were undergoing inactivation in neoplastic development led to the development of the theoretical grouping proposed by Kinzler and Vogelstein (1997). The main proposition of this theory was that genes which were involved in proliferation control would operate as 'gatekeepers' whereas those concerned with maintaining genetic integrity could be classified as 'caretakers'. The inactivation of a 'gatekeeper' would result in inappropriate proliferation and tumourigenic development whereas caretaker inactivation would lead to an increasing propensity to acquire new mutation and hence the potential for increasing neoplastic potential. The idea of 'gatekeepers' providing a rate-limiting step to tumourigenesis is illustrated in colon cancer and retinoblastoma by APC (see Section 1.2) and pRB (see Section 3.3) respectively. Through tumourigenic development other mutations are clearly accumulated although they are not sufficient to initiate the process (Garber *et al.*, 1991; Jen *et al.*, 1994). Loss of caretaker gene function on the other hand is not sufficient for tumourigenesis although it can increase the likelihood of this occurring through acquirement of further changes. Examples of 'caretaker' genes include the nucleotide-excision repair genes responsible for xeroderma pigmentosum (*XP*), *ATM* which is responsible for ataxia telangiectasia, and *BRCA1* and *-2* (Breast cancer) which are responsible for familial breast cancer susceptibility (Sharan *et al.*, 1997).

Regulatory genes whose functions are commonly lost through LOH, mutation, and promoter silencing have frequently gone on to be characterised as tsg's whose loss has deregulated cell cycle control and hence allowed uncontrolled proliferation of a cancer cell. These genes therefore fit the designated assignment of 'gatekeepers'. The best-characterised tsg's are perhaps *p53*, *pRB* and $p16^{INK4A}$, although, due to the increasing complexity of

cross talk, in many senses they can be described as being on the same pathway although functioning in distinctly different manners. p53 itself acts as a transcription factor in response to DNA damage, induced by a number of different agents leading to a block in the cell cycle until the damage is repaired, or alternatively, driving an unrepairable cell into apoptosis (Lane, 1992). pRB acts as both a transcriptional activator and repressor dependant on its phosphorylation state and through this function exerts control on G1/S phase progression. $p16^{INK4A}$ acts as an inhibitor of the cyclin/cdk complexes that phosphorylate pRB turning it into a transcriptional activator and primarily functioning as a negative regulator of the cell cycle. Therefore all three proteins exert a control on the G1/S phase of the cell cycle and are sufficient to induce a growth arrest at this stage. That abrogation of some or all of these three genes is important in tumourigenesis is evidenced by the observation that where DNA tumour virus infection has taken place no abnormalities are found in these genes although in all other cell lines a degree of inactivation has occurred (Whitaker *et al.,* 1995).

3.2) p53; 'Guardian of the Genome'

3.2.1) p53 as a Gatekeeper

One of the first two tsg's cloned *p53* reinforces many of the ideas concerning the classical tsg. The evidence that a *p53*-germline mutation resulted in Li-Fraumeni syndrome (LFS), an autosomal-dominant inherited predisposition to multiple cancer types, suggested that *p53* fitted the definition of the 'gatekeeper' gene (Li and Fraumeni, 1969; Lustbader *et al.,* 1992). Before cancer progression in kindreds the second *p53* allele must be lost in an approximation of Knudsons model (see Section 1.1) i.e. through LOH or somatic mutation. Situated on 17p13 the *p53* gene encodes a nuclear phosphoprotein transcription factor with a plethora of target sites and interacting molecules. However, the two major functions of p53 are the negative regulation of the cell cycle and the induction of apoptosis. The p53 negative regulation response is seen due to DNA-damage caused by γ-

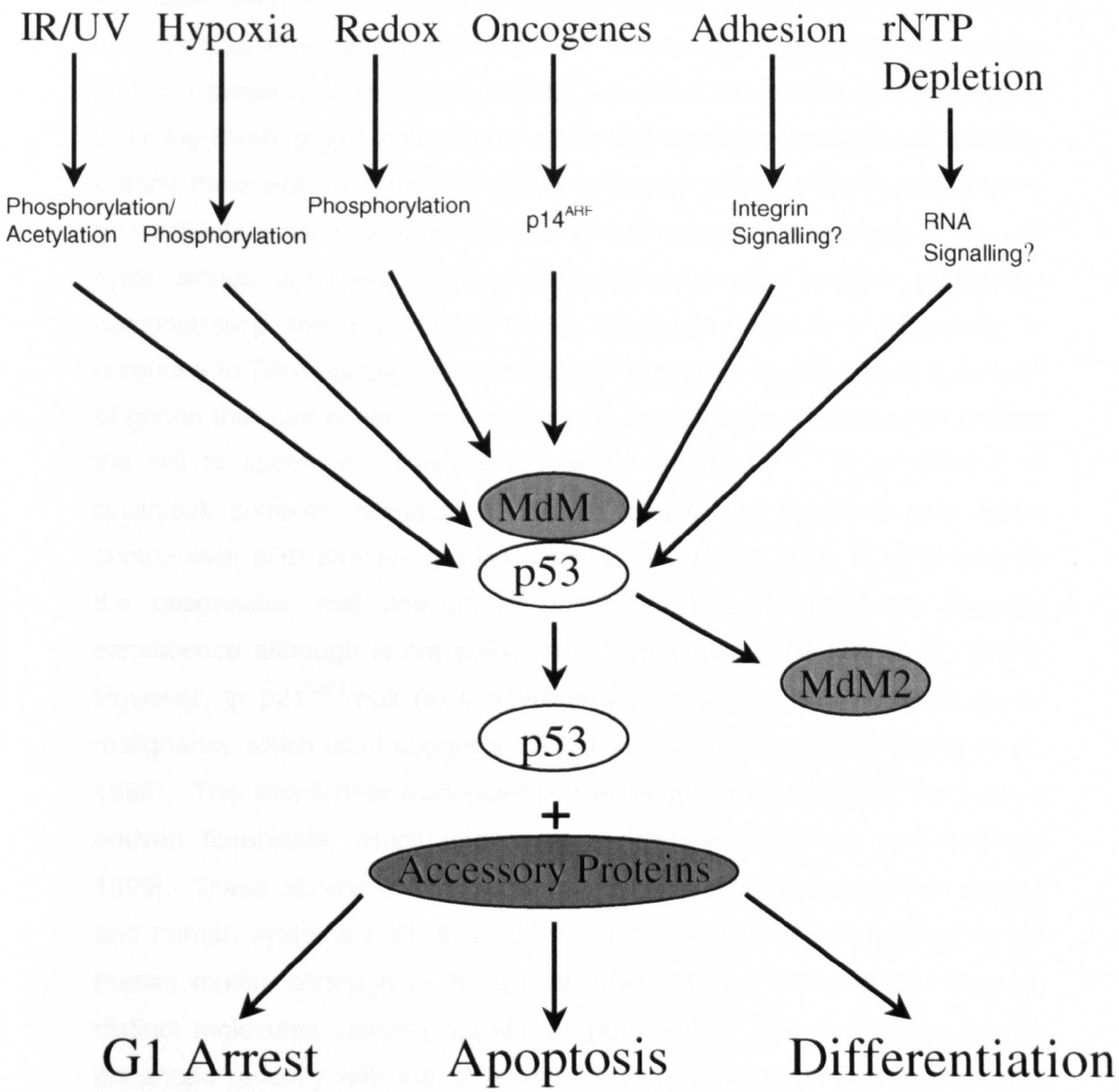
irradiation, u.v-irradiation, hypoxia, or carcinogens. The molecule itself can detect and bind to damaged DNA through its C-terminal domain resulting in further p53 activation, increase in p53 protein levels and activation of downstream target genes by the transcription factor activity of the protein. The C-terminal domain itself can also interact with a number of DNA repair proteins including the XP-B and XP-D components of TFIIH (Wang and Prives, 1995) and the Rad51 protein (Buchhop *et al.,* 1997) demonstrating a great utility of domain function. Many of the target genes are components of cell cycle arrest machinery at both the G1/S and G2/S checkpoints further evidenced by p53-dependant arrests at both of these phase transitions. This suggests that loss of p53 function would lead to compromised cell cycle checkpoints allowing survival of DNA-damaged cells and leading to accumulation of sufficient genetic 'hits' to potentially initiate tumourigenesis. Cells lacking a functional p53 fail to arrest in G1 following induced DNA damage (Kastan *et al.,* 1991; Kuerbitz *et al.,* 1992; Bond *et al.,* 1994) although re-introduction of wild-type p53 leads to G1 arrest in such cells (Baker *et al.,* 1990) clearly demonstrating p53-dependance. The role of p53 is less clear in G2 arrest as cells lacking a functional copy can still undergo this form of growth arrest (Passalaris *et al.,* 1999) although the various isoforms of p53 may have some role in this ability (see below) and therefore redundancy cannot be ruled out. It is perhaps unsurprising then that cells from individuals suffering from the rare inherited heterozygous $p53^{+/-}$ Li-Fraumeni syndrome (Li and Fraumeni, 1969) which carries a predisposition to several cancer types occasionally undergo spontaneous immortalisation with loss of the wild-type *p53* allele (see Knudson's model) (Rogan *et al.,* 1995; Shay *et al.,* 1995) having lost both regulatory control of the cell cycle and the key mechanism to initiate repair genetic damage or remove critically damaged cells.

3.2.2) p53; Structure and Effector Molecules

A feature of p53 is its multiple, unique, functional domains including the NH2-terminal transactivation domain (TD), the sequence-specific DNA-binding domain (DBD) necessary for transcription transactivation, and the COOH-terminal domain containing the oligomerisation domain (OD) and the apoptotic domain (Wang *et al.,* 1996); or DNA-damage domain (Reed *et al.,* 1995). Covalent regulation of p53 occurs by phosphorylation, O-glycosylation, and acetylation at multiple sites throughout the gene in all domain structures (Prives and Hall, 1999). Stimuli for covalent regulation are shown in Figure 1.3. Transcriptional activation targets of p53 include those associated with growth control and cell cycle checkpoints; $p21^{WAF1/CIP1/SDI1}$ (El-Deiry et al., 1993), WIP1, MDM2, GADD45, EGFR, PCNA, cyclin D1, cyclin G, TGF-β, and 14-3-3σ; DNA repair; GADD45, PCNA, and $p21^{WAF1/CIP1/SDI1}$; and apoptosis; BAX (Miyashita and Reed, 1995), BCL-XL, FAS, IGF-BP3 (Rajah et al., 1997), PAG608, XPB, XPD and DR5 (Nagane et al., 1997; Admundson et al., 1998; Levrero et al., 2000). Although these transcriptional targets have been targeted into appropriate functional groupings it should be noted that redundancy of molecules exists and in inappropriate situations will perform atypical roles. Further mutual control exists with the tsg $p33^{ING1}$ where the presence of both tsg's is required for either to function properly (Garkavtsev *et al.,* 1998). Clearly $p33^{ING1}$ cannot be said to be under p53 transcriptional control but more accurately transcriptional targets of both are under a form of unspecified mutual control. DNA binding by p53 is optimal when the protein is in a tetrameric state where binding occurs via the OD of four p53 molecules (Jeffrey *et al.,* 1995). Although the tetrameric state is the optimal configuration wild type protein complexed with mutant protein can be sufficient for inactivation. The binding of p53 to p300/CBP regulates its ability to bind cognate sequences and activate transcription and also provides a platform for the MDM2 degradation complex to form and is therefore necessary for normal function. The ubiquitin-like protein, SUMO, has also been shown recently to have p53 binding and activation properties (Lohrum and Vousden, 2000), which suggests that it may form part of the p300/CBP

Figure 1.3. Signals leading to the modification and activation of p53

Schematic of p53 activation signals, accessory proteins and molecular endpoints.



Adapted from Giaccia and Kastan, 1998

platform. Transcriptional repression targets of p53 feature both cellular and viral promoters, which do not necessarily contain p53-binding sites, include bcl-2 (Miyashita et al., 1994), c-fos and SV40 large T antigen. Conversely p53 is a target for inactivation itself by a number of virally encoded proteins including SV40 large T antigen, HPV E6, HBV X antigen, and adenovirus E1a. Clearly there are a number of further cascades of activation in response to p53 activation although viral removal of p53 negative regulation of the cell cycle allows unchecked cell proliferation and virus particle production demonstrating the importance of its functional removal. Therefore in response to DNA damage recognition p53 regulates expression of a number of genes that can either arrest a cell until the damage is repaired or prompt the cell to apoptose. The p53-responsive gene *p21^{CIP1}* is an inhibitor of cyclin/cdk complex formation at the G1 phase and therefore also exerts control over pRB phosphorylation (Dimri *et al.,* 1994). This is evidenced by the observation that deletion of *p21^{CIP1}* in human fibroblasts prevents senescence although is not sufficient to bypass crisis (Brown *et al.,* 1997). However, in p21^{CIP1}-null mice development is normal with no evidence of malignancy which itself suggests no prevention of senescence (Deng *et al.,* 1995). This was further evidenced by the demonstration of p21^{CIP1}-null mice derived fibroblasts, which underwent senescence (Pantoja and Serrano, 1999). These observations indicate mechanistic differences between murine and human systems such that p21^{CIP1} is an effector of senescence in the human model, although in the murine model it may be redundant through distinct molecules carrying out similar processes. This model was greatly enhanced recently with the observations that G1-arrest in senescence was p21^{CIP1}-dependent and that in p21^{CIP1}-null MEFs (Mouse embryo fibroblasts) the cells had lost the ability to instigate a G1-arrest senescence and instead underwent a crisis-like death instigated through continued divisions whilst assuming the appearance of a senescent culture (Dulic *et al.,* 2000). Therefore, both in the murine and human system p21^{CIP1} is required for appropriate senescence although in humans the p53-induced response is stronger than that seen in the murine model. The oncoprotein MDM2 can inhibit both p53 and pRB although under p53 transcriptional control it operates in an autoregulatory feedback loop (Ko and Prives, 1996). Once transcribed

MDM2 binds to the transcriptional activation domain of p53 and blocks the regulatory function whilst also promoting the rapid reduction of protein levels through promotion of degradation by ubiquitin complexes (Prives, 1998). In fact MDM2 acts to transport p53 from the nucleus to the cytoplasm (Honda et al., 1997) where MDM2 functions as an E3 ubiquitin ligase catalysing p53 degradation (Roth *et al.,* 1998). Discussed in more detail below is the further example of cross talk provided by the observation that $p14^{ARF}$ regulates p53 and is encoded by a sequence overlapping with $p16^{INK4A}$ which negatively regulates pRB. Over 5000 published p53 mutations in both tumours and cell lines have been identified indicating the importance of the gene to cellular integrity (Souza *et al.,* 1997; Hainaut *et al.,* 1998; Lu, 2000).

3.2.3) p53 Homologues

For a number of years *p53* was thought, perhaps surprisingly given the important role it performs, not to belong to a gene family unlike the situation seen with both $p16^{INK4A}$ and pRB. However that situation is now clearer with the identification of a number of homologues with some conservation of function between members. Evolutionarily the identification of an invertebrate p53 homologue was indicative of an adaptive history even though there is very little functional data to accompany the much longer mollusc version (Soussi and May, 1996). The remaining family members, *p73* (Kaghad *et al.,* 1997) and *p63* (Schmale and Bamberger, 1997), both encode multiple polypeptides (p73 has 6 variants, p63 has 14 variants) all of which share functional domains with p53 although not every domain in every instance (Lohrum and Vousden, 2000) (Figure 1.4). All members of the p53 family do contain the TD, a DBD, and OD. The highest levels of homology are found in the DBD suggestive of common targets although demonstrating homology in the OD only homo-oligomers have been shown to form with no hetero-oligomers observed (Levrero *et al.,* 2000). An observation from SAGE analysis suggests that 6/36 p53-induced transcripts are induced by a p73 isoform (Yu *et al.,* 1999), which is suggestive of homologue–specific targeting

Figure 1.4. Structural isoforms of p53 share similar domain structures and possess distinct motifs

Cartoon illustrating similar domain structures of p53 and isoforms p63 and p73.

Adapted from Lohrum and Vousden, 2000

although it is not clear what the activated transcripts function as. Additionally these homologues have considerably longer C-terminal domains that contain a protein-protein interaction domain called a sterile alpha motif (SAM) (Chi *et al.,* 1999) this domain is also found in mollusc p53. The SAM domain is found in proteins that regulate development although those found in p63 and p73 do not mediate homo- or hetero-dimerisation. Although these homologues share a number of features of the perhaps evolutionarily refined p53 no convincing mutations of either homologue has been identified and therefore they are not likely to be bone fide tsg's as first postulated (Prives and Hall, 1999). The role of p63 in fact may be more concerned with correct development, whilst p73 may also be concerned with this function and with a slight tumour suppressive capability. Furthermore, viral oncoproteins known to inhibit p53; SV40 Large T, HPV-16 E6, and E1B 55kDa; do not inhibit p73 although adenovirus E4ORF6 does though through a conserved domain and therefore not specifically targeting it, this would suggest that p53 is the primary cellular target (Levrero *et al.,* 2000). Of great interest is the observation that a particular p53 sequence polymorphism which seems to play a role in the ability of p53 to inactivate p73 is more frequently altered in some cancers (Di Como *et al.,* 1999) which is suggestive of a preferential inactivation although the specifics behind this are not clear. Further functions of p73 were highlighted recently with the demonstrations that where p53 was abrogated E2F-1 could still induce apoptosis although through activation of p73, whereas in T-cells E2F-1 could induce apoptosis in a p53-independent manner which operated through the activation of p73 (Lissy *et al.,* 2000; Irwin *et al.,* 2000). This is perhaps unsurprising as both molecules share a great deal of structural homology and familial conservation, clearly a great deal is still to be learnt about this family structure. There are two human homologues which were identified simultaneously; p40, was identified which shared strong conservation with the DBD although functionally its role remains unclear (Trink *et al.,* 1998) and p63 (p51), which shares greater homology with a p73 homologue in the key functional domains (Osada *et al.,* 1998). Although p63 shares greater homology with p73 it shares to a lesser degree characteristics of p53 such as suppression of colony formation, apoptotic death, and p21 promoter transcriptional activation. There is now no doubt that p53 belongs to

a family of similar molecules of which examples are found further down the 'evolutionary tree'. However these identified homologues do not fulfil the majority of the p53 functions and may therefore be earlier models of the protein, which through progressive 'gain of function' mutation and recombination have given rise to the current version of the negative regulator of the cell cycle. Clearly work remains to be done in this area that should provide a great deal of further information on the degrees of redundancy that exist between family members.

3.3) p16 and pRB; G1/S Checkpoint Control

Although p53 exerts control over both G1/S and G2/S, although not absolutely in the case of the latter, a further control exists at the G1/S checkpoint prior to the initiation of DNA synthesis. The pathway central to the regulation of the G1/S phase transition of the cell cycle is characterised by four genes, at least one of which is mutated in many forms of cancer, *p16-cyclin D1-CDK4-pRB*. In some tumour forms, $p16^{INK4}$ dysfunction is the common mechanism leading to inactivation of the pathway, i.e. NSCLC, although pRB inactivation is preferred in others, i.e. SCLC, but generally both are not inactivated in immortal cell lines (Whitaker *et al.*, 1995). It is also clear that other members of this pathway can contribute to or provide the dysfunctional step in the pathway.

3.3.1) Cloning of pRB

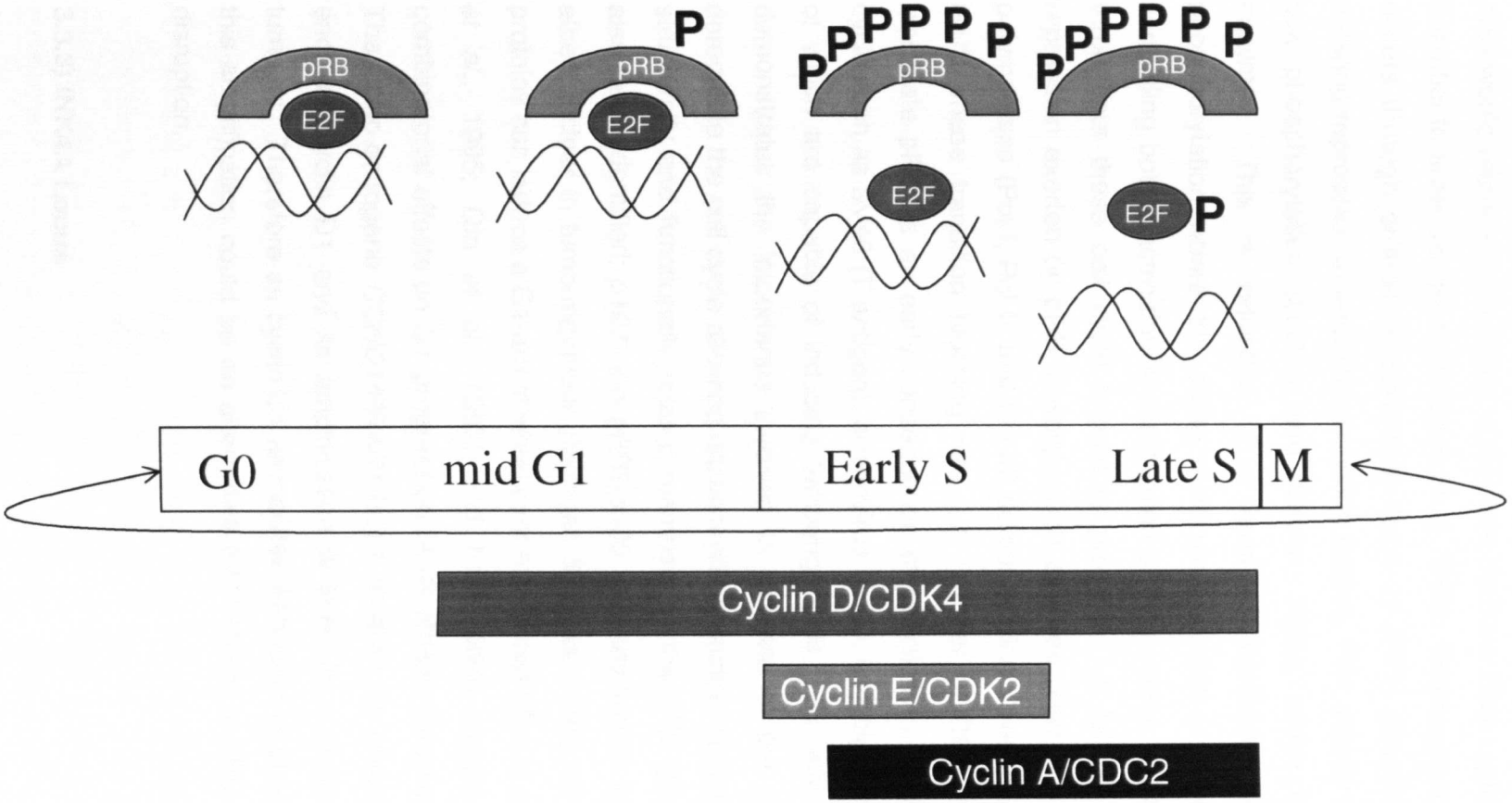
The path to the identification and cloning of the proto-tsg, pRB, provides an interesting insight into the scientific process. Retinoblastoma is an autosomal dominant hereditary ocular tumour that occurs in childhood. The postulate made by Knudson (1971) was to suggest that this heritable cancer was due to recessive, loss of function mutations occurring in a putative tsg. This theory proposed that the familial transition of a germ line mutation complemented by a further somatic mutation would be sufficient to initiate tumourigenesis. Knudson hypothesised that in instances were no familial

precedence existed two separate somatic mutations would be necessary to initiate tumourigenesis. This formulated the basis behind the now famous Knudson 'two hit' hypothesis, which is now accepted as the paradigmatic model behind tsg silencing. The discovery of individuals with inheritable retinoblastoma and deletions at 13q14 provided the evidence required to demonstrate the first 'hit' (Yunis and Ramsay, 1978), this was followed by the demonstration of LOH at 13q14 arising through chromosome loss, deletion, mitotic recombination or gene conversion in both familial and sporadic cases which provided the necessary evidence for the second 'hit' (Cavenee *et al.,* 1983; Knudson, 1978). The isolation of pRB was undertaken after the discovery of a homozygous deletion on 13q14 enabled the identification of sequences expressed in normal retinoblasts but missing from retinoblastomas (Friend *et al.,* 1986). Through this identification, and demonstration of reversion of tumourigenic properties in cell culture by the normal cDNA, *pRB* was identified as a bone fide tsg (Friend *et al.,* 1986). Aside from retinoblastoma tumours mutations of *pRB* have now been found in a number of different tumours including EC, sarcomas, SCLC, bladder carcinomas and breast carcinomas (Horowitz et al., 1993; Lee et al., 1999; Lu, 2000).

3.3.2) pRB Function and Effectors

The pRB protein is a ubiquitously expressed, 105 kDa nuclear phosphoprotein which when hypophosphorylated binds to and controls a number of cellular proteins and transcription factors (E2F family) which are essential for G1/S phase transition, pRB also antagonises transcription factor complexes essential for S phase entry (see Figure 1.5). The phosphorylation of pRB results in an inactivation of its binding ability to transcription factors allowing release and appropriate transcription to occur prior to entry into S phase. The phosphorylation of pRB is due to cyclin D1/cdk4 and other cyclin/cdk complexes (Sherr, 1994; Lundberg & Weinberg, 1998) that lift the inhibition of G1/S transition progression. Therefore a failure to phosphorylate

Figure 1.5. Regulation of pRB during the mitotic cell cycle through phosphorylation (P) by cyclin/cdk complexes releasing transcription factors (*E2F*)


pRB
E2F
P
pRB
E2F
P P P P P P
pRB
E2F
P P P P P P
pRB
E2F
P
G0
mid G1
Early S
Late S
M
Cyclin D/CDK4
Cyclin E/CDK2
Cyclin A/CDC2

pRB would result in a G1 block, a block on *de novo* DNA replication, and the potential to enter senescence (Stein *et al,* 1990). Dephosphorylation of pRB occurs through protein phosphatases (type 1) *(*PP1*)* (Berndt *et al.,* 1997) allowing repression of target transcription factors, PP1 activity is regulated by cdk phosphorylation (Ludlow and Nelson, 1995), which inactivates the enzyme. This is indicative of a level of feedback control in pRB phosphorylation where the same molecule operates in a regulatory loop controlling both inactivation and activation of the protein, which is evidenced throughout these cell cycle regulatory proteins. A further transcriptional repression exerted by pRB is weighed on all three classes of nuclear RNA polymerases (Pol I, Pol II, and Pol III) demonstrating obvious relevance to its G1/S phase transition blocking role. A number of DNA tumour viruses inactivate pRB as an early consequence of infection to deregulate the cell cycle such as SV40 (T antigen), adenovirus (E1A), and high risk HPV (E7) all of which are capable of inducing tumourigenesis. As seen with p53 this demonstrates the importance involved in removal of the pRB protein to deregulate the cell cycle allowing unchecked production of viral particle. Two structurally and functionally related members of the *pRB* gene family have also been identified; *p107* and pRB2/*p130*, and implicated to have an role, albeit unclear in tumourigenesis (Mulligan & Jacks, 1998) although all three proteins can induce a G1-arrest when over expressed (Zhu *et al.,* 1993; Lukas *et al.,* 1995; Qin *et al.,* 1992) and have been observed to display combinatorial effects on G1 progression block effects (Classon *et al.,* 2000). The proto-oncogene *CCND1/PRAD1/BCL1* located on chromosome 11q13 encodes cyclin D1 and its amplification is seen in a number of types of tumours. Therefore as cyclin D1 associates with cdk4 to phosphorylate pRB this amplification could be an alternative mechanism to pRB growth control disruption.

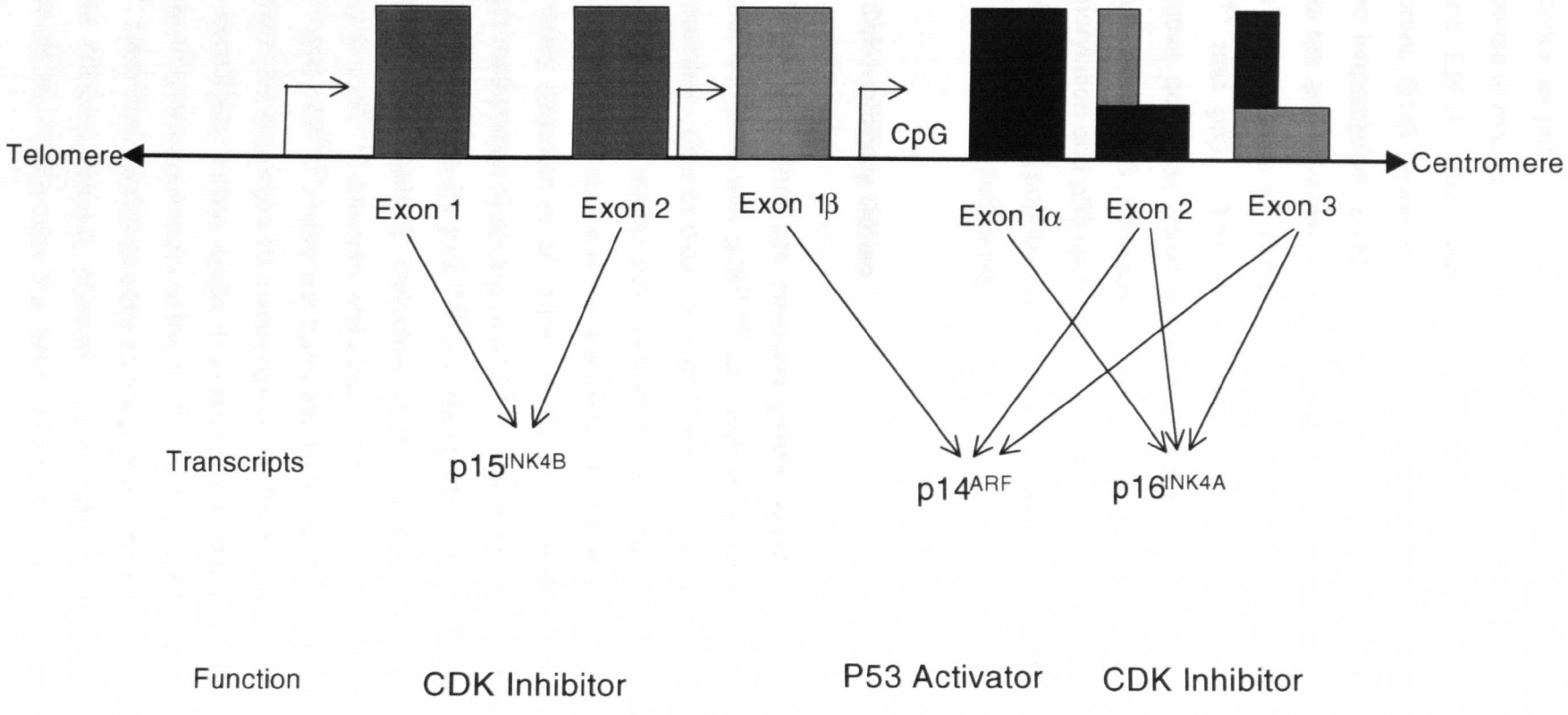
3.3.3) INK4A Locus

Situated in chromosome 9p21 is the negative cell cycle regulator $p16^{INK4A/CDKN2/MTS1}$, not only is 9p21 allele loss frequently observed but

mutations in the gene and transcriptional silencing through promoter methylation are common in a wide range of cancers (Okamoto *et al.,* 1994; Loughran *et al.,* 1994; Cairns *et al.,* 1995; Loughran *et al.,* 1996; Reed *et al.,* 1996; MacGrogan and Bookstein, 1997; Wong *et al.,* 1997; Sharpless and DePinho, 1999; Lu, 2000). The *p16^{INK4A}* gene is encoded by three exons (1α, 2, and 3) however at the same locus a second gene *p14^{ARF}*, murine *p19^{ARF}*, (Quelle *et al.,* 1995) is transcribed using an alternative first exon which lies 5' to exon 1α and then spliced onto exon 2. A second reading frame is used on exon 2 so that p14^{ARF} shares no sequence homology with p16^{INK4A} and does not share any cdk-inhibitory activities; the locus structure is shown in Figure 1.6. The protein itself is a cell cycle modulator that regulates pRB function by inhibiting cyclin D1/cdk4 activity; through inhibition of cdk4 kinase activity (Serrano *et al.,* 1993; Koh *et al.,* 1995), and thereby preventing the alleviation of the hypophosphorylated pRB block on G1/S phase transition. Transcriptional repression of p16^{INK4A} has been shown to occur through pRB suggesting a regulatory feedback loop also involving the cyclin-dependant kinases (Li *et al.,* 1994), which operates in the normal cellular environment. Where LOH of 9p21 occurs methylation of the 5' CpG island on a remaining allele is sufficient to down regulate the gene thereby preventing activity providing an alternative mechanism to Knudsons 'two-hit' paradigm. Mutations have been demonstrated in several tumour types, which specifically affect p16^{INK4A} and not p14^{ARF} (Munro *et al.,* 1999; Sharpless and DePinho, 1999) in fact no reported point mutations of *p14^{ARF}* have occurred which are predicted to affect its functional domains. A solitary silent mutation in *p16^{INK4A}*, which alters *p14^{ARF}*, has been reported, although this does not occur in a conserved region and as such is probably a polymorphism. Further observations suggest that exon 1β contributes most if not all of the domain functions of p14^{ARF}. A keratinocyte study found no detectable p14^{ARF} protein in senescent cells suggesting that its involvement in this process may be minimal (Munro *et al.,* 1999). However p14^{ARF} does possess tumour suppressor characteristics in murine models and therefore may reveal relevance to the human system although perhaps will not have such

Figure 1.6. Structure of the INK4 locus

Cartoon illustrating the structure of the INK4A locus at 9p21, the organisational relationship of *p14*^{ARF}, *p15*^{INK4B}, and p16INK4A, and the functions of their gene products.


Telomere
CpG
Centromere
Exon 1
Exon 2
Exon 1β
Exon 1α
Exon 2
Exon 3
Transcripts
p15INK4B
p14ARF
p16INK4A
Function
CDK Inhibitor
P53 Activator
CDK Inhibitor

significance as p53, pRB, and $p16^{INK4A}$. $p14^{ARF}$ itself undergoes upregulation in response to mitogenic stimuli response genes such as E1A, myc, ras, V-abl, and E2F-1, which leads to p53 stabilisation and therefore permits appropriate G1/S arrest or apoptosis to occur in the instance of mitogenic-induced inappropriate proliferation (Palmero *et al.,* 1998). Mitogenic stimuli such as ras and raf when constitutively activated have been demonstrated to induce senescence in human fibroblasts with a correlatory production of $p16^{INK4A}$ and p53. This is perhaps an unsurprising result given that constitutive activation could cause a deregulation of the cell cycle and a resultant drive into senescence caused by $p16^{INK4A}$ inhibition of pRB phosphorylation and p53 upregulation in response to increased sequestration of MDM2 by the hypophosphorylated pRB (see below) rather than a direct $p14^{ARF}$-mediated growth arrest.

3.3.4) Cdk-inhibitory Genes

There are other cdk inhibitory genes, which share some degree of functional similarity with $p16^{INK4A}$ although most work has focused on that family member. One of these is $p15^{INKB}$ that lies centromeric to $p16^{INK4A}$ and shares about 70% amino acid similarity and although co-deletion of these genes frequently occurs events specifically targeting $p15^{INK4B}$ were shown to occur rarely (Izumoto *et al.,* 1995; Kamb *et al.,* 1994; Flores *et al.,* 1996), although methylation silencing of $p15^{INK4B}$ has been recorded in HCC (Wong *et al.,* 2000). A role for $p15^{INK4B}$ was demonstrated in G1-arrest in response to TGF-β, which itself is indicative of a response through an alternative pathway to $p16^{INK4A}$ (Hannon and Beach, 1994). Two other family members, $p18^{INK4C}$ and $p19^{INK4D}$, have not been shown to be mutated and therefore are not likely to be important for tumourigenesis (Batova *et al.,* 1997). All of this family specifically inhibit cyclin D-associated kinases (Parry *et al.,* 1995). Similarly, only rare mutations of the $p21^{CIP1/Waf1/Sdi1}$ family members, $p27^{KIP1}$ or $p57^{KIP2}$, have been demonstrated although the former may be downregulated in some cancers (breast, prostate, colon, gastric, lung, and esophageal (Tsihlias *et al.,* 1999) and the latter, situated at 11p15, was found to be

maternally imprinted and downregulated through LOH of one allele copy in cancer predisposition cases studied (Nijjar *et al.,* 1999; Lee *et al.,* 1999). This kinase inhibitory protein family bind and inhibit cyclin E/cdk2 and cyclin A/cdk2 complexes (Sherr and Roberts, 1995) suggesting an active role in late G1 and S-phase regulation.

3.4) Cross talk

As alluded to above pRB can form a trimeric complex *in vivo and in vitro* with p53 and MDM2 (Hsieh *et al.,* 1999), which is a clear example of the cross-talk complexity that exists at the molecular level. The antiapoptotic function of MDM2 on p53-induced apoptosis is overcome by pRB in this instance. This occurs through pRB binding to MDM2 through its conserved C pocket domain (Xiao *et al.,* 1995), the same domain which interacts with E2F1 (Weinberg, 1995) and *c-abl* (Welch and Wang, 1993) which in turn prevents MDM2-mediated p53 degradation although it does not de-repress p53-mediated transactivation or alleviate p53-mediated transrepression suggesting important differences to these two mechanisms. Interestingly MDM2 can stimulate the transactivation function of E2F1, which could in turn be due to the MDM2–pRB interaction (Xiao et al., 1995). E2F1 itself has been shown to be an upstream mediator of p53-mediated apoptosis (Pan *et al.,* 1998) and a transcriptional activator of $p14^{ARF}$ suggesting the transcription factor has a wide range of activities, which may be under dual control of both pRB and p53-mediated MDM2 transcription. MDM2 itself binds to the hypophosphorylated form of pRB (Xiao et al., 1995) suggesting binding occurs in early G1 sequestering MDM2 and allowing p53-mediated apoptosis to progress. MDM2 amplification has been shown in primary tumours (Oliner et al., 1992), which could be beneficial to tumourigenesis through preventing the p53 apoptotic response by elevating the p53-degradation. MDM2 and the alternative transcript from INK4A gene provide a further example of cross talk again. $p14^{ARF}$ itself can bind to MDM2 through its exon 1β-encoded domain and thereby prevent the cytoplasmic degradation of p53 (Pomerantz et al., 1998) through promoting the degradation of MDM2 (Zhang et al., 1998). This

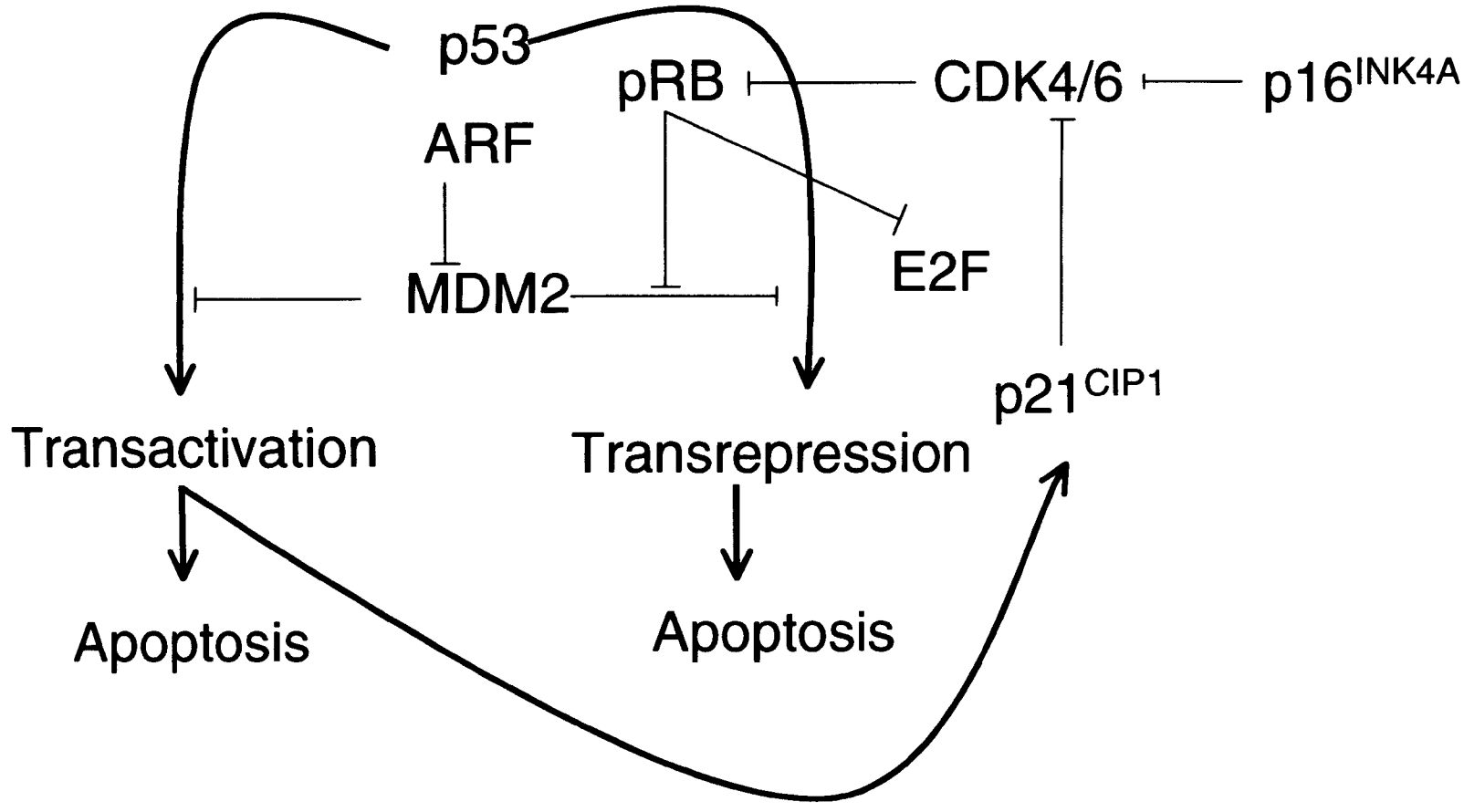
action permits stabilisation and p53-mediated apoptosis or growth arrest to occur depending on the cellular situation. Therefore, two genes encoded from the same locus can separately effect the regulation of two important tsg's (Figure 1.7). The $p21^{CIP1}$ cdk inhibitor can also provide some interaction between the pRB and p53 regulatory pathways due to its inhibitory effects on pRB phosphorylation and its status as a transcriptional target of p53 (Weinberg, 1995). The underlying basis of regulatory cell cycle progression control could therefore be described as being dependant on two key tsg's, pRB and p53, and the molecular cross-talk which exists between them and its mediation by a number of key molecules.

4) Biological Mechanisms of Replicative Senescence

Replicative senescence and crisis (alternatively known as M1 and M2) are both examples of potential intrinsic tumour suppressor mechanisms as both provide blocks to the proliferative lifespan of divisive cells. Replicative senescence differs from crisis in that it is an obligatory default end-point of replicative lifespan in response to progressively shortening telomere ends and perhaps other undefined signals. Replicative senescence therefore acts to prevent the cell accumulating sufficient genetic damage through unrestrained proliferation coupled with genetic instability to instigate tumourigenesis. Crisis is characterised as an apoptotic response to appropriate signals, such as critically short telomeres. Crisis is perhaps not a tumour suppressor mechanism as such in that unless the cell has bypassed replicative senescence and carried on dividing its telomeres can't shorten sufficiently to induce apoptosis. The proposition that the end-point replication problem of telomeres and hence their progressive shortening could be linked to the behaviour of cells in replicative senescence and crisis was first postulated by Cooke and Smith (1986). Although crisis is a response to a senescence bypass it occurs as a by-product of the uncontrolled proliferation, rather than as a controlled response to the prolonged lifespan. However replicative senescence and crisis, although referred to as unassociated processes, in physiological terms, overlap significantly as evidenced by the appearance of

Figure 1.7. Figurative description of cross-talk described in section 3.4

Flow diagram describing molecular cross-talk between p53 and pRB mediated by effector molecules. Lines ending in horizontal bars indicate a repression by the molecule from which the line originates.


p53
pRB
CDK4/6
p16INK4A
ARF
E2F
MDM2
p21CIP1
Transactivation
Transrepression
Apoptosis
Apoptosis

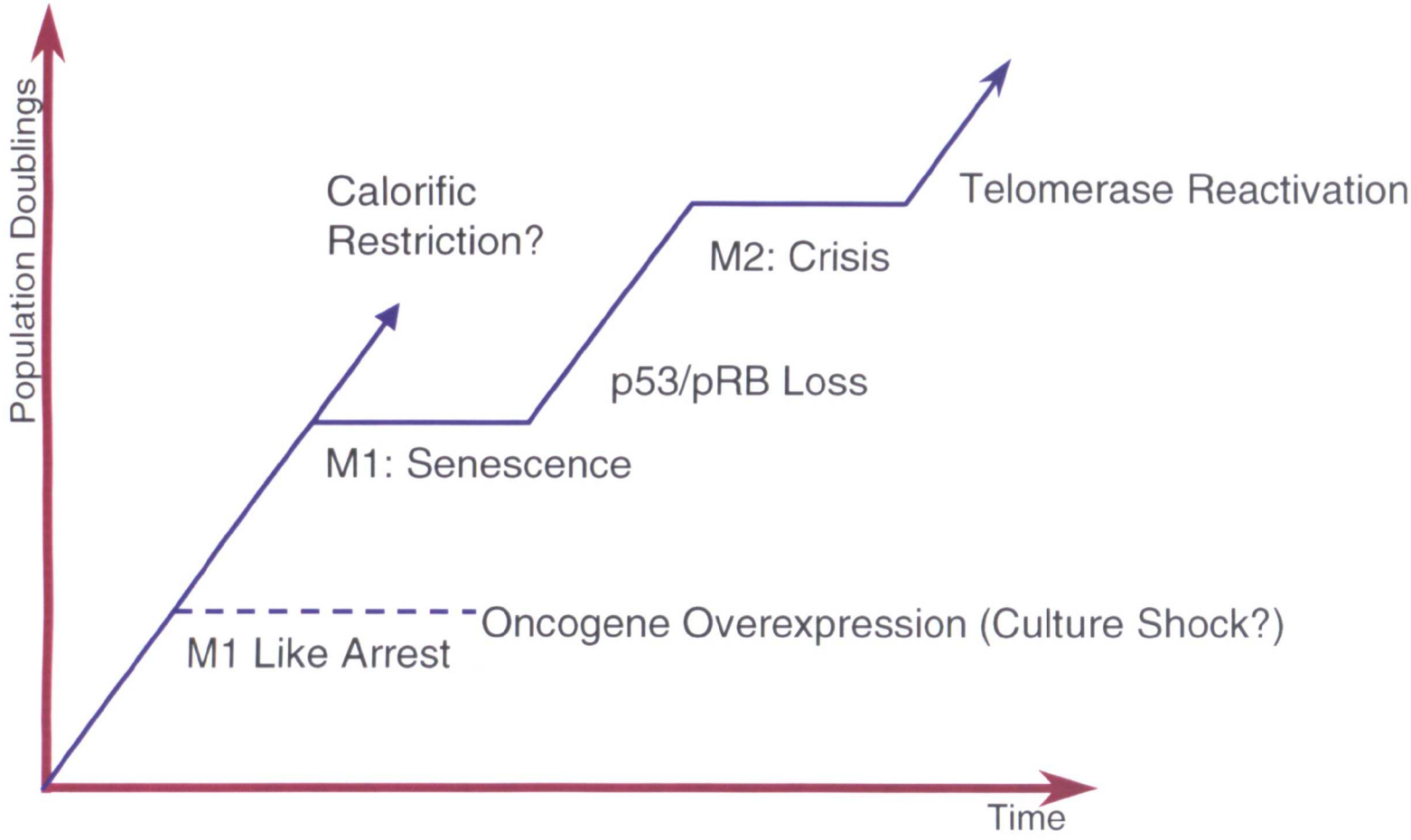
dicentric chromosomes, chromosomal fragments and endoreduplication in the later stages of senescence (Saksela and Moorehead, 1963; Yanishevsky and Carrano, 1975; Benn, 1976; Bayreuther *et al.,* 1992). This is perhaps unsurprising, given the asynchronous nature of cell populations leading to different growth potentials in different sub-populations. There are other mechanisms, which are proposed to induce replicative senescence in mammalian organisms such as calorific restriction and 'culture shock' although neither has been demonstratively proven and accordingly may not be immediately relevant to the human system. Models of senescence and abrogation points are shown in Figure 1.8.

4.1) Replicative Senescence

The phenomenon of replicative senescence can be characterised succinctly as a non-replicating, viable state where a block is present in the cell cycle preventing progression from G1 to S phase therefore preventing *de novo* DNA synthesis. Whilst held in the GO/G1 phase cells assume an enlarged, flattened morphology with an altered gene expression and a general increase in protein, RNA, glycogen, lipid, and lysozymes with no increase in DNA content (Harley *et al.,* 1980). Senescent cells are therefore distinct from terminally differentiated cells in that they undergo further differentiation without division. The metabolic activity retained by senescent cells is such that they can remain in culture for up 2 years in their non-divisive state (Matsumura *et al.,* 1979) and are therefore resistant to apoptosis. One of the triggers for entry into senescence is probably due to the shortening of a subset of telomeres such that they are no longer indistinguishable from DNA damage prompting upregulation of cell cycle inhibitors p53 and $p16^{INK4A}$ (Harley, 1991; Shay *et al.,* 1991; Robles and Adami, 1998; Shapiro *et al.,* 1998) leading to a block prior to S phase entry. It is precisely this block in the replicative lifespan that has led to the development of the hypothesis that senescence may be a cellular tumour suppressor mechanism controlled by a number of dominantly acting genes (Sager, 1989).

Figure 1.8. Models of senescence and immortalisation

Diagram illustrating the growth-arrest checkpoints, M1 (replicative senescence) and M2 (crisis), in relation to cell population doublings and time. Molecular events allowing escape from replicative blocks are indicated.


Population Doublings
Calorific
Restriction?
M2: Crisis
Telomerase Reactivation
p53/pRB Loss
M1: Senescence
M1 Like Arrest
Oncogene Overexpression (Culture Shock?)
Time

Senescence of normal human fibroblasts was described initially as a recessive trait overcome by fusion with the dominant immortal HeLa cell line (Gey *et al.,* 1952) although malignancy was recessive to the normal phenotype in the same study (Stanbridge, 1976). However later studies showed that senescence was the dominant phenotype and that recessive changes in the abnormal cell resulted in cellular immortality (Pereira-Smith and Smith, 1983). Senescence was also shown to be due to active mechanisms rather than random 'weathering' effects in these studies, perhaps most clearly where in cell-cell fusions between senescent cells with a protein synthesis block and young cells the dominance of senescence was alleviated indicating a probable mediation of senescence by proteins (Norwood *et al.,* 1974). Replicative senescence of cells in culture was first documented in the studies of Hayflick and Moorehead (1961) where cells were shown to enter a viable non-replicative state after approximately 60 population doublings in an asynchronous population. Furthermore, the number of cell doublings remaining in a population was shown to be linked to the sample donor age (Schneider and Mitsui, 1976), indicating some form of replicative potential which was fundamentally intrinsic to the culture and not to the culture system. Senescence itself was shown to be an intrinsic mechanism of tumour suppression in normal cells (O'Brien *et al.,* 1986) although a mechanism which itself seems fairly open to abrogation. Sasaki *et al* (1994) discuss the propriety of senescence as a tumour suppressive mechanism by drawing parallels to the situation as it would occur *in vivo.* They postulate that all of the changes necessary to accumulate for neoplastic development, without loss or gain of lifespan potential, would turn an individual cell into an approximate maximum of 5.4×10^8 (2^{29} cells or 29 doublings). Devita *et al* (1975) suggest that a tumour comprising 2^{30} cells would be approximately $1 cm^3$ in size which itself would not exert an immediate impact on lifestyle in a large organism.

4.1.1) Replicative Senescence: Effector Molecules

An important mechanistic approach to study alterations in senescent cultures was to examine them alongside serum-starved quiescent cultures. Quiescent cultures will reinitiate DNA synthesis and re-enter the cell cycle when serum stimulated whereas senescent cultures will not. From studies such as these it became clear that senescent cultures had impaired c-fos expression and therefore lacked full the S-phase requirement transcription factor AP-1 expression (Seshadri and Campisi, 1990; Riabowol *et al.*, 1992a). Senescent cultures are also deficient in PKC and phospholipase D activation that is likely to be due to elevated levels of ceramide (Riabowol *et al.*, 1992b; Venable *et al.*, 1994). Senescent cells demonstrate repression of cell cycle regulators cyclin A and cdc2 (Stein *et al.*, 1991), DNA synthesising enzymes such as PCNA, dihydrofolate reductase and histones (Pang and Chen, 1994) E2F-1 (Dimri et al., 1994, E2F-5 (Good *et al.*, 1996), and pRB remains constitutively underphosphorylated (Stein *et al.*, 1990). The bHLH (basis helix-loop-helix) transcription factors Id1 and Id2 are also repressed in senescent cells which combined with the observed immortalisation of keratinocytes with a crisis bypass through telomerase reactivation stimulated by Id family members (Alani *et al.*, 1999) suggests a critical role in proliferation control by this family (Hara *et al.*, 1994). Functionally senescent human fibroblasts and endothelial cells have both been shown to overexpress interleukin-1a whilst levels of TIMP-1 and TIMP-3 (Tissue inhibitors of metalloproteinases) have been shown to rise in the former (West *et al.*, 1989; Millis *et al.*, 1992; Wick *et al.*, 1994) and ICAM (intercellular adhesion molecule) overexpression has been shown in the latter (Maier *et al.*, 1990; 1993), additionally, senescent mammary epithelial cells, normal fibroblasts, and keratinocytes overexpress RARβ (Retinoic acid receptor β) (Swisshelm *et al.*, 1994; Si *et al.*, 1995; Dr F. McGregor, BICR, personal communication). Further changes in senescent cultures are demonstrated by the presence of large amounts of the G1 and G1/S phase cyclins complexed with the respective cdk's, Cyclin D1/Cdk4 and Cyclin E/Cdk2, although lacking catalytic activity (Dulic *et al.*, 1993) probably due to elevated expression of the

ubiquitous cyclin/Cdk inhibitors $p16^{INK4A}$ and $p21^{CIP1}$ (Noda *et al.*, 1994; Alcorta *et al.*, 1996) although $p16^{INK4A}$ accumulates gradually whereas $p21^{CIP1}$ undergoes rapid accumulation followed by a decline. This could be indicative of $p21^{CIP1}$ initiating and $p16^{INK4A}$ maintaining a senescent growth arrest state (Stein, 1999). The candidate tsg $p33^{ING1}$ has also been shown to be upregulated in senescent cells and to be an inducer of senescence (Garkavtsev *et al.*, 1998). The p53 growth inhibition induction cannot function without the presence of $p33^{ING1}$ and vice versa demonstrating a critical pathway to senescent arrest. The repression of $p33^{ING1}$ expression has been observed in a number of metastatic breast cancers although it is very rarely mutated in this and other tumour types (Toyama *et al.*, 1999; Gunduz *et al.*, 2000; Sanchez-Cespedes *et al.*, 2000), which would not rule out transcriptional silencing in advanced tumours. The changes which occur in senescent cells v's normal cells encompass a number of different pathways and functional roles including the previously characterised negative regulators of the cell cycle, mitogenic response elements, and structural elements demonstrating a global cellular response once committed to the senescent growth-arrest.

4.1.2) Replicative Senescence: Viral Abrogation

One clear mechanism to overcome senescence was demonstrated in senescent HDF (Human diploid fibroblasts) where after infection with the SV40 virus DNA synthesis was reinitiated (Gorman and Cristafolo, 1985). The real observation behind this demonstration was that the cellular machinery required for DNA synthesis was still intact although not functioning during senescence. When the SV40 T antigen was placed under a steroid-inducible promoter and infected cells nurtured carefully through crisis to immortality upon removal of the steroid the immortal cells arrested in G1 (Wright *et al.*, 1989). This demonstrated that although the T antigen could sequester the proteins required for senescence growth arrest once released p53 and pRB were still sufficient to block unrestrained proliferation. This would suggest that other mechanisms aside the key genes for the senescent

arrest to occur are p53 and pRB (and genes contained in the same pathway) as both are functionally abrogated in tumours and targeted by specific proteins from DNA tumour viruses.

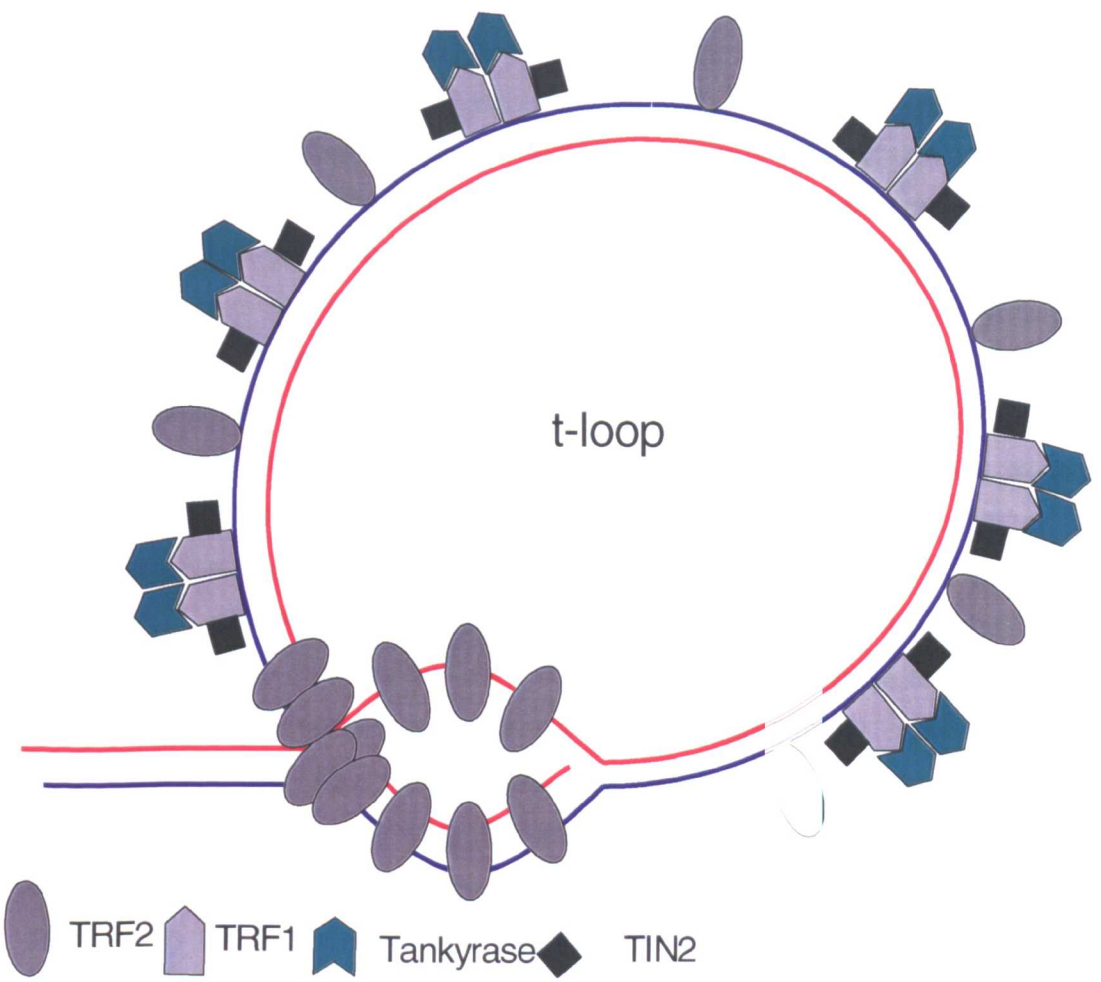
4.1.3) Telomeric Basis of Replicative Senescence

Telomeres are composed of guanine-rich nucleotides that serve to cap the ends of eukaryotic chromosomes when bound by a complex of proteins and are maintained by the enzyme telomerase, itself a specialised reverse transcriptase. The hexanucleotide repeat TTAGGG represents the telomeric sequence in humans and constitutes approximately 10 Kb at the end of each telomere. The genome contains a number of intrachromosomal repeats which are frequently associated with chromosomal fragile sites, these are almost certainly evolutionary remnants of chromosomal fusions, but have also been proposed as hotspots for recombination (Azzalin *et al.*, 1997; Hastie and Allshire, 1989). The telomere end replication problem is such that the unidirectional DNA polymerases are not able to replicate the 3'-end of the telomere leading to their progressive shortening through divisive lifespan (Levy *et al.*, 1992). Telomere end 3' overhangs generally consist of a variable stretch of approximately 50-100 nucleotides (Greider, 1999). Early observations suggested that the progressive shortening of telomere ends might have a role in senescence (Olovnikov, 1973; Harley *et al.*, 1990; Allsopp *et al.*, 1992) with the demonstrations that telomeres shortened by 50-60bp per population (Harley *et al.*, 1990; Counter *et al.*, 1992) and that they therefore over the course of a 60 population doubling lifespan they would shorten by 3.6Kb. Senescent cell telomere has been reported to decline to approximately 6Kb before precipitating growth arrest although this may not be a ubiquitous cellular property (Allsopp *et al.*, 1992), as this would assume a common starting telomere length of approximately 10Kb, which may not be a common feature of all telomeres. The demonstration that ectopic expression of hTERT led to a halt and stabilisation of telomere degradation and length and an immortalisation of primary human fibroblasts was perhaps the first direct proof that replicative senescence was due, at least in part, to telomere

shortening (Bodnar *et al.*, 1998; Vaziri and Benchimol, 1998). The short telomere recognition mechanism remains to be unravelled although decreasing telomere length may not be sufficient to prevent chromosome ends resembling a double-strand DNA break through failure in forming a 't-loop' structure. Intriguingly, where the transient expression of hTERT prolonged normal human fibroblast lifespan by 50% the telomeres at senescence were shorter than those found in matched untreated senescent cells (Steinert *et al.*, 2000). This is indicative of either an elongation of the shortest telomere to a matched average, or alternatively, of hTERT assisting in the assembly of a stabilising telomeric 'scaffold'. The telomere end is buried in a 't-loop' structure (Griffith *et al.*, 1999) bound by a complex of proteins, including TRF1 (TERF1 locus) (Chong *et al.*, 1995), PIN2 (Protein interacting with NIMA) (alternative splice product from TERF1) (Shen *et al.*, 1997), TRF2 (Chong *et al.*, 1995; Broccoli *et al.*, 1997), Tankyrase (Smith *et al.*, 1998) and TIN2 (TRF1-interacting nuclear protein 2) (Kim *et al.*, 1999) (Figure 1.9). Tankyrase and TIN2 both bind to TRF1 at the telomere end and acts to regulate its activity in distinct manners, which serve to regulate telomere length (van Steensel and de Lange, 1997) whilst TRF2 protects the 3' chromosome end which is buried in the 't-loop' structure (van Steensel *et al.*, 1998) and also plays a role in regulating telomere length (Smogorzewska *et al.*, 2000). TRF1 homodimerises prior to telomere binding where it induces a shallow bend in the telomeric sequence, which could define the degree of curve at the telomere end (Bianchi *et al.*, 1999). The demonstration that cells deficient in TRF2 underwent apoptosis in an experimental manner reminiscent of a DNA-damage response reinforces the critical role of telomere end protection within the cell (Karlseder *et al.*, 1999). Shortened telomere ends may not be able to successfully invade the loop, although may still be protected by TRF2 preventing DNA-damage recognition, which could in itself provide a senescence signal through inappropriate structure allowing chromatin-condensation dependant transcription to occur. PIN2 is virtually identical to TRF1 and forms both homo- and hetero-dimers with it and demonstrates an upregulation in G2/M and a decrease in G1 (Shen *et al.*, 1997) suggesting it is linked into the mitotic phase of the cycle perhaps assisting in telomere stabilisation and 't-loop' formation prior to this point.

Figure 1.9. Telomeric 't-loop' and binding proteins

Model of hypothesised structure of the protective chromosomal end structure, the telomere. Telomere structure is stabilsed by binding proteins TRF1, TRF2, Tankyrase, and TIN2. PIN2 is not included in this diagram although can be presumed to belong to the complex formed by TRF1, Tankyrase, and TIN2.


t-loop
TRF2
TRF1
Tankyrase
TIN2

4.1.4) Oncogene-induction of Replicative Senescence (Culture Shock)

There is evidence to suggest that the senescence programme can be activated in response to certain oncogenes. The demonstration that the H-RAS-V12 could activate the senescence program in primary mouse and human cells and that this response was associated with accumulation of p53 and $p16^{INK4A}$ is perhaps mechanistically unsurprising (Serrano *et al.,* 1997). A further demonstration showed that H-RAS-V12 activated $p19^{ARF}$ blocking p53 degradation, presumably through MDM2 degradation and allowing the p53 senescence response to occur. However, as *p14*^{ARF} specific targeting has not been shown in human cancers it may be that this observation has more relevance to the murine system where the senescence response may be more dependent on the mitogen response system. Indeed there is evidence to suggest that mouse and human senescence are both mechanistically different. Murine telomeres are approximately five times longer than human (40-60Kb v's 10Kb) and murine telomerase is expressed in far more tissues than its human counterpart (Greenberg *et al.,* 1998; Martin-Rivera *et al.,* 1998). After 20 population doublings in culture MEFs enter a state likened to senescence with appropriate gene expression such as p53, $p16^{INK4A}$, $p19^{ARF}$, and $p21^{CIP1}$. However given the extremely long telomeres, the constitutive MEF telomerase expression, and the insufficient divisions for telomeric attrition to occur it seems very unlikely that telomere length is the basis for MEF senescence. As the emergence of any telomere-based senescence would likely not arise until a large number of divisions had passed a tumour cell could have formed a life threatening mass in a smaller mammal by that point which is indicative of a necessity for distinct cellular failsafe mechanisms between organisms. The senescence induced by H-RAS-V12 in MEFs (Pantoja and Serrano, 1999) greatly resembles that which occurs normally suggesting that MEF senescence may be oncogene induced, or perhaps linked into the RAS/MAP kinase signalling pathway, and that $p19^{ARF}$, not $p16^{INK4A}$, may be pivotal in the murine system. This was further underlined by the earlier demonstration that mouse cultured growth arrest was not dependant on the pRB pathway (Zalvide and DeCaprio, 1995) and that $p16^{INK4A}$ was not therefore critical to the mouse senescent arrest.

4.2) Crisis and Immortality

Crisis is the final checkpoint preventing the progression of the errant cell within the population from accumulating sufficient genetic changes to achieve cellular immortality. Escape from the M1 checkpoint, achieved most commonly through abrogation by loss of function mutations, promoter silencing, or viral sequestration of p53, pRB and other cell cycle inhibitors results in progressive telomere shortening and resultant chromosomal instability. The observed chromosomal instability is due to end-to-end (Robertsonian) chromosomal fusions and non-disjunctions during mitosis that ultimately lead to cell death induced by massive genetic catastrophe. Escape from crisis requires that the errant cell reactivate the mechanisms designed to protect telomere ends and chromosomal integrity in subpopulations of cells. Escape from senescence itself is not sufficient to immortalise a tumour although it may predispose to this phenotype, however not all tumours are immortal demonstrating that the required genetic events have not yet occurred (Barrett and Wiseman, 1987). Therefore to escape from the massive genetic damage incurred in crisis and achieve cellular immortalisation the re-establishment of chromosomal end integrity may be the minimal positive selection requirement. The potential targets of inactivation, even given the accumulation of genetic instability, which can provide an escape from crisis however may not be that great on a genome scale given that this escape is based on telomeric stabilisation. This would suggest that as a tumour suppressor mechanism crisis might provide an important role in the telomere-biased human system although conventionally would not be expected to fill the same role in the murine system.

4.2.1) Telomeric Basis of Crisis

The first demonstration that broken chromosomes without telomere ends led to aberrant recombination's, end-to-end fusions, and 'breakage-fusion-bridge' cycles were undertaken in *Zea mays* (McClintock, 1941) although broken chromosomes do not strictly resemble critically short telomere ends both instigate a DNA-damage response and therefore can be considered sufficiently similar. With the observation that the mean telomere length in fibroblasts passaged *in vitro* decreased by approximately 2kb before the cultures ceased division, a mean 40 population doublings (Harley *et al,* 1990) and the previous observations of limited replicative lifespan in normal diploid fibroblasts (Hayflick, 1961) this led to the hypothesis that telomere length was intrinsic to the replicative capacity of ordinary cells. Indeed chromosomal instability and telomere end-to-end fusions are seen in the last few doublings of senescent fibroblast cultures (Sherwood *et al.,* 1988; Benn, 1976) providing evidence that telomeres may act as 'mitotic clocks' capable of defining an endpoint to replicative lifespan. The last few divisions prior to senescence could therefore be the point where sufficient genetic instability occurs to inactivate tsg's allowing further divisions and the development of further accumulatory genetic damage prior to crisis. Genetic instability itself has therefore been defined as a hallmark of crisis highlighted by chromosomal fusions and aneuploidy (Counter *et al.,* 1992) where the mean telomere length was 1.5Kb in a crisis population suggestive of approximately 70 populations beyond senescence arrest, this assumes no increase in telomere degradation beyond senescence. The emergence of immortalised clones from a culture in crisis is a very rare event that is invariably accompanied by re-established telomere maintenance that can be due to either telomerase (Counter *et al.,* 1992, 1998) or the ALT (Alternative lengthening of telomeres) mechanism (Lundblad and Blackburn, 1993; Bryan *et al.,* 1995; 1997) (see Section 4.2.4) as evidenced by the re-expression of telomerase in post-crisis immortal cells and by the fact that the ectopic expression of hTERT enables cells to bypass crisis (Counter *et al.,* 1998; Zhu *et al.,* 1999). More rarely human cancer cells use the ALT mechanism, discussed in Section 4.2.4.

Although capable of driving cells beyond the M1 checkpoint and extending life span by 20-30 population doublings through sequestration of the checkpoint genes pRB and p53, infection with SV40 is not generally sufficient to drive cells beyond crisis and therefore not solely sufficient for immortalisation (Stein, 1985; Wright and Shay, 1992). Post-infection with DNA viruses such as SV40, adenovirus, and human papillomaviruses (HPV) the extended lifespan prior to crisis would provide the opportunity for further genetic events to occur. The observation that immortalised cells emerge post-infection with DNA viruses is therefore in keeping with theories of genetic event accumulation being a prerequisite for immortalisation of neoplastic tumours (Chang, 1986). Human cells expressing SV40 Large T antigen which have bypassed senescence immortalise upon transfection with hTERT indicating the inability of T antigen to rescue telomere ends alone and again providing further evidence for a telomere-directed crisis (Counter *et al.,* 1998; Zhu *et al.,* 1999). In a similar manner hTERT can complement HPV E7 to drive cells beyond crisis, which in part could be a response to deregulated cell cycle mechanics (Kiyono *et al.,* 1998) as E7 constitutively activates pRB alleviating its block on G1/S progression. HPV E6 expression in pre-crisis human keratinocytes was itself sufficient to activate telomerase (Klingelhutz *et al.,* 1996) through a mechanism where c-myc deregulation occurs prompting upregulation of hTERT mRNA (Wang *et al.,* 1998) although this result shows some variance across cell types as seen with hTERT-induced immortalisation.

4.2.2) Telomerase

The enzyme telomerase has been described previously to be sufficient to rescue cells from replicative senescence and furthermore as sufficient for immortalisation through crisis escape (Montalto *et al.,* 1999). The holoenzyme consists primarily of the hTERT catalytic subunit (Kilian *et al.,* 1997; Meyerson *et al.,* 1997; Nakamura *et al.,* 1997) and the hTR RNA subunit (Feng *et al.,* 1995). A number of other proteins are bound to components of the holoenzyme such as TEP1 (Telomerase protein component 1) (Harrington *et al.,* 1997), molecular chaperone proteins p23 and

Hsp90 (Holt *et al.,* 1999), and to hTR such as hStau (double-stranded RNA-binding protein) and L22 (nucleolus-localised RNA-binding protein) (Le *et al.,* 2000). TEP1 has also been shown to be a component of the large cytoplasmic ribonucleoprotein complexes termed 'vaults' which are suggested to associate with nuclear pore complexes, although this may reflect a utility of function for this RNA-binding protein (Kickhoefer *et al.,* 1999). Telomerase has been demonstrated to be inappropriately active at high levels in >80% of human cancers (Shay and Bacchetti, 1997). This activity allows the tumour to escape the crisis stage through stabilisation of telomere ends and the cancer cells to continue dividing indefinitely and essentially achieve cellular immortality. Regulation of telomerase at the transcriptional level is exercised through the gene encoding the catalytic subunit hTERT (Meyerson *et al.,* 1997) such that most normal human somatic cells lack hTERT mRNA and undergo telomere shortening as a function of cell division (Harley *et al.,* 1990; Allsopp *et al.,* 1992). The RNA template component of telomerase, hTR, is ubiquitously expressed although where endogenous hTERT is expressed the hTR mRNA levels and half-life rise considerably (Yi *et al.,* 1999) further demonstrating the importance of hTERT regulation. Telomerase activity has been reported in normal keratinocytes although this is postulated to arise from a subpopulation of basal stem cells, which are thought to display base activity levels not sufficient to prevent telomeric shortening and senescence (Yasumoto *et al.,* 1996; Harle-Bachor and Boukamp, 1996). This is suggestive of a minimal threshold telomerase level for effective telomere stability below which insufficient telomeric reconstruction occurs. Using a catalytically active C-term hemagluttinin-tagged hTERT it was demonstrated that this was not sufficient to avert crisis (Feng *et al.,* 1995) suggesting the possibility of a stabilising role (not simply an elongatory role) performed by the telomerase enzyme although the C-term hemagluttinin-tag has been reported to interfere with the catalytic activity of hTERT on its substrate (Ouellette *et al.,* 1999) perhaps intimating an existence of an experimental artefact. The observation that the restoration of telomerase activity and stabilisation of telomere ends led to immortalisation of human fibroblasts (Bodnar *et al.,* 1998; Counter *et al.,* 1998; Vaziri and Benchimol, 1998; Halvorsen *et al.,* 1999; Zhu *et al.,* 1999) provided proof that immortalisation provided an

escape from crisis and therefore crisis was due to critically shortened telomeres. Conversely when telomerase activity was disrupted in human cancer cells the telomeres underwent drastic shortening with continuing division and entered crisis (Hahn *et al.,* 1999; Zhang *et al.,* 1999). Conflictingly a study showed that the telomeres were elongated and maintained well above crisis lengths whereas a similar study found the telomeres were stabilised and maintained well below the crisis threshold (Counter *et al.,* 1998; Zhu *et al.,* 1999). Although this seems at odds the critical message is the stabilisation of the telomere ends is required for crisis escape and therefore the minimal requirement is hTERT deregulation. Although the ectopic expression of hTERT was sufficient for immortalisation of presenescent fibroblasts and retinal pigment epithelial cells (Bodnar *et al.,* 1998) and normal mesothelial cells (Dickson *et al.,* 2000) this is not the case for every cell type analysed.

The catalytic subunit of telomerase hTERT has been characterised as the site of enzyme repression in normal cells. In telomerase-expressing tumours the deregulation of expression control is likely to occur as a consequence of repression control rather than 'gain of function' mutation. A number of candidate transcriptional repressors have come to the fore in recent times, which act, directly on the hTERT promoter sequences. The myeloid-specific zinc finger protein 2 (MZF-2) has been shown to interact directly with a sequence 400 bp silencer sequence upstream of the hTERT proximal core promoter (Fujimoto *et al.,* 2000) whilst the Mad transcription factor was also shown to repress the hTERT promoter although this repression was removed by ectopic c-myc expression (Oh *et al.,* 2000) which is in agreement with other findings where the transcription factors Sp1 and c-myc cooperate to activate hTERT transcription (Kyo *et al.,* 2000). The Wilms' tsg (WT1) has also been shown to repress hTERT transcription although it has been described as interacting with the promoter sequences immediately upstream from the MZF-2 interaction sites (Oh *et al.,* 1999). Further evidence for a repressor of hTERT comes from functional studies where introduction of chromosome 3 into cell lines with functional telomerase and LOH on 3p resulted in telomerase repression and growth-arrest entry (Ohmura *et al.,*

1995; Cuthbert *et al.,* 1999), deletion analysis in this study identified 3p21.3-p22 and 3p12-21.1 as potential chromosomal locations of this gene, the latter region was also identified as a putative telomerase repressor gene location in an earlier study (Tanaka *et al.,* 1998; Cuthbert *et al.,* 1999). The catalytic subunit of telomerase may be under the repressional control of a number of transcription factors although some of these exert an inappropriate positive control on the promoter sequences and there would seem to be further repressors still to be cloned.

4.2.3) Keratinocyte Immortalisation

Normal human keratinocytes and mammary epithelial cells have been reported to require inactivation of the pRB/$p16^{INK4A}$ pathway alongside ectopic hTERT expression to achieve immortalisation in culture (Kiyono *et al.,* 1998; Dickson *et al.,* 2000). These findings suggest furthermore that keratinocyte senescence is reliant on $p16^{INK4A}$ but can operate independently of telomere length. Although differences clearly exist between different cell types this stochastic mechanism of senescence, which is independent of telomere length, clearly requires more in depth research. The bHLH protein Id-1 which functions as a negative regulator of basic HLH transcription factors was found to be sufficient for immortalisation of primary human keratinocytes (Alani *et al.,* 1999) with no crisis stage observed. Ectopic expression of Id-1 led to the activation of telomerase, phosphorylation of pRB and a decrease in the p53-mediated DNA-damage response through repression of cell cycle regulatory transcription factors enabling bypass of both M1 and M2. Immortal human cervical carcinoma cell lines which were p53 and pRB wild-type through HPV18 E6 and E7 presence underwent rapid senescence following abrogation of the viral proteins demonstrating the need for further genetic changes beside telomerase deregulation in this instance for keratinocyte immortalisation (Goodwin *et al.,* 2000). From these lines of evidence it is clear that the minimal requirements for immortality in keratinocytes may differ from other cell types although clearly the same basic mechanisms are utilised in each instance demonstrating their importance to normal cell regulation.

4.2.4) Alternative Lengthening of Telomeres (ALT)

An alternative pathway to telomerase-based telomere maintenance was proposed following the identification of telomerase-negative human cell lines which exhibited telomere maintenance at lengths greatly exceeding those expected from the observed replicative lifespans (Bryan *et al.,* 1995; Bryan and Reddel, 1997). This mechanism was termed ALT and was postulated to function via a recombination basis, based on observations in yeast (Lundblad and Blackburn, 1993; Lundblad, 1997). Interestingly, immortal cell lines, which maintain their telomeres by the ALT mechanism, have been shown to contain a novel form of PML body that colocalises to the telomeric ends in a subset of cell populations (Yeager *et al.,* 1999). The novel PML bodies contain promyelocytic leukaemia protein (PML), telomeric DNA, TRF1, TRF2 and additionally replication factor A, RAD51 and RAD52 which are involved in recombination and DNA metabolism (New *et al.,* 1998). The presence of these proteins at the telomere ends in the novel PML bodies led the authors to suggest that they could either be sites of telomeric maintenance or staging platforms for said process (Yeager *et al.,* 1999). Of further interest was the observation that Ras-induced premature senescence in human fibroblasts involved an upregulation of PML protein (Ferbeyre *et al.,* 2000) which may be evidence of a 'culture shock' pathway occurring through telomere ends in certain cell lines. However in cell-cell fusions between ALT immortal cells and normal fibroblasts the hybrids quickly senesced, whereas cell-cell fusions between ALT immortal and telomerase-positive immortal cells resulted in a repression of the ALT pathway (Perrem *et al.,* 1999). This is suggestive of ALT repression pathways existing in both normal fibroblasts and telomerase-positive cells. The ALT mechanism is still relatively poorly understood although it does not appear to be the dominant pathway in cellular immortalisation especially in immortal cells from naturally-occurring human tumours (Kim *et al.,* 1994; Bryan *et al.,* 1997).

4.2.5) Telomeric-dependence?

As discussed above there are differences between human and murine senescence programmes insofar as they are not both dependent on telomere signals. The demonstration that after 5 generations telomerase-deficient mice started to exhibit growth and proliferative organ defects (Lee *et al.*, 1998) and late generation spontaneous tumour formations which harboured end-to-end chromosome fusions (Rudolph *et al.*, 1999) showed that although much longer than their human counterparts if shortened significantly murine chromosomes would behave in the same manner after a significant latency. Further experimentation with $mTR^{-/-}$ $p53^{+/-}$ mice showed after 5-7 generations the development of epithelial carcinomas and lesions of the large intestine (Artandi *et al.*, 2000) rather than the characteristic murine soft tissue sarcomas and lymphomas. This may be due to the shortening of the telomeres to the point where they resemble the situation as seen in human tumours allowing the development of these characteristic tumours. This would seem to indicate that out with the laboratory situation there are key and intrinsic differences to the murine and human forms of growth arrest and as such this might reflect different evolutionary priorities in each respective system. Importantly the frequency of escape from crisis in murine cells ($mTR^{-/-}$ $p53^{-/-}$) approaches 100% compared to 10^{-7} in SV40 infected telomerase negative human cells (Shay *et al.*, 1993; Wright and Shay; 2000). Fundamentally crisis may not have a role in the murine system although its role as an inhibitor of further tumour progression in the human system should not be presumptively underestimated.

4.3) Calorific Dependency

The demonstration that calorific restriction can lead to an extension of lifespan was provided in a number of model organisms. Observations in *S.cerevisiae* showed that a family of proteins, the SIR (silent information regulator) genes were determinants of lifespan. The family is composed of SIR1, SIR2, SIR3, and SIR4, which were all identified initially through silencing of mating type loci (Ivy *et al.,* 1986; Rine and Herskowitz; 1987), which in itself provided a good clue as to their function. Further lines of evidence suggested involvement in nonhomologous end joining (NHEJ), which is a mechanism utilised to repair breaks in DNA (Critchlow and Jackson, 1998). Mammalian homologues of yeast proteins involved in this process also carry out NHEJ although in yeast efficient functioning requires SIR2, SIR3, and SIR4 (Tsukamoto *et al.,* 1997; Boulton and Jackson; 1998) this process has implications when considered along with potential telomere recognition as double-strand breaks in aging mammalian cells. The aging process in yeast has been demonstrably shown to be due to the accumulation of extrachromosomal rDNA circles (ERCs), which arise through inappropriate expression, replication block and DNA-double strand repair, by homologous recombination (Sinclair and Guarente; 1997). However, the demonstration that NHEJ did not affect aging and that *sir2*, *sir3*, or *sir4* haploids had shortened lifespans through increase in ERC formation suggested a mechanism with very little in common with a mammalian system (Kaeberlein *et al.,* 1999). The increase in ERC formation was shown to be due to *sir2* mutation whilst deletion of *FOB1* (gene required for ERC formation (Kobayashi and Horiuchi; 1996)) alleviated the observed lifespan shortening. This demonstrated that not only was ERC formation responsible for lifespan shortening but also that SIR2 acted to repress ERC formation. *SIR2* is the only member of the SIR gene family conserved in higher eukaryotes. SIR2 and the mouse homologue were both then shown to be nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylases, the enzymatic processes were driven by NAD which itself suggests that the protein could respond to the cellular levels of NAD, and where high exert a deacetylation

silencing control over chromatin, repressing transcription and thereby controlling the divisive potential (Imai *et al.,* 2000). The demonstration of SIR2 lifespan extension in an NAD-dependent manner showed that calorie restriction, at least in yeast, extends lifespan and thereby postpones senescence in a seemingly telomere-independent pathway based on the inhibition of toxic free radical formation (Lin *et al.,* 2000). Calorific restriction in mouse models has led to an extension of lifespan, although obvious difficulties lie in extrapolating this data to the human system. The presence of SIR2 homologues may imply that these pathways still exist to some degree in higher eukaryotes, although under the control of a much larger set of molecules than evidenced in *S.cerevisiae.*

4.4) Culture Shock

The actions of carcinogens have been shown to be sufficient for the immortalisation of normal cells in culture (Newbold *et al.,* 1982) where Syrian hamster cultures became immortal at a frequency of 0.9-7 X 10-5 and 1.6-6 X 10-7 for embryo and dermal cells respectively. It was proposed that this effect was an early event that enhanced the cells for further malignant transformation by oncogenes (Newbold and Overell, 1983). This carcinogen–induced immortalisation demonstrates features of a multistep pathway (Thomassen *et al.*, 1985; Barrett and Fletcher, 1987; Bols *et al.,* 1991), which although greatly accelerated clearly has similarities with the neoplastic development *in vivo*. That repeated treatments with 4-Nitroquinoline 1-oxide were required before immortalisation of normal human fibroblasts further evidenced the necessity for multiple mutational events for this phenotype to develop (Bai *et al.,* 1993). However, later studies showed that the immortalisation of Syrian hamster fibroblasts was not telomere-based, as they were telomerase-positive throughout culture lifespan and senescence, but rather SV40-T-antigen was sufficient to bypass senescence and crisis in a single-step immortalisation (Russo *et al.,* 1998). This was furthered by the demonstration of Syrian hamster embryo cells which underwent senescence after approximately 30 population doublings with no telomeric shortening and

high telomerase expression (Carman *et al.,* 1998). This has characteristics of the 'culture shock', described in Section 4.5, where inappropriate mitogenic signalling overrides the cellular transcription machinery driving a premature senescence. Therefore, the repetitive carcinogen exposure, which immortalises rodent cultures, is likely to occur through eventual abrogation of the p53 and $p16^{INK4A}$-signalling pathways although not through a telomeric maintenance process. The MMCT of chromosome 9 into rodent cells to some extent supports this idea as this led to the deletion of the INK4A locus whereas the $p16^{INK4A}$ cDNA led to growth-arrest of hybrids (England *et al.,* 1996).

The observed differences in senescence induction pathways has led to the proposition that murine cells a undergo growth-arrest based on the acquirement of damage through tissue culture conditions (Sherr and DePinho, 2000) leading to 'culture shock'. Murine cells paced in culture proliferate beyond *in vivo* levels (Toldaro and Green, 1963) but eventually undergo a cessation of division accompanied by upregulation of $p16^{INK4A}$, p21CIP1, $p19^{ARF}$, and p53. MEFs undergo growth arrest after approximately 30 doublings although immortal variants do frequently emerge that possess mutant p53 alleles and loss of the INK4 locus. However MEFs derived from p53-null or ARF-null mice can be propagated indefinitely whereas those lacking pRB or p21CIP1 undergo growth-arrest (Kamijo *et al.,* 1997; Zindy *et al.,* 1998). Therefore, the disruption of p53 regulation is the critical mechanism in the mouse for senescence. However, overexpression of ras in MEFs led to a premature senescence accompanied by accumulation of $p16^{INK4A}$, $p15^{INK4B}$ and p53 (Malumbres *et al.,* 2000). Indeed, in the absence of $p16^{INK4A}$ and p53 the ras-induced premature senescence pathway does not function in rodent cells (Serrano *et al.,* 1997). The ras induction of $p16^{INK4A}$ and $P15^{INK4B}$ was shown to operate through the Raf-Mek-Erk pathway suggesting inappropriate mitogenic pathway signalling, whilst constitutive MEK mitogen signalling reinforces this idea by inducing premature senescence in a p53- and $p16^{INK4A}$-dependent manner (Lin *et al.,* 1998). The oncogenic Ras stimulated accumulation of p53 was shown to occur through an activation of $p19^{ARF}$ (Palmero *et al.,* 1998), which suggests that at least in

the murine system Ras can activate both the p53 and pRB systems of growth-arrest. Explant of MEFs into culture is associated with the accumulation of $p19^{ARF}$, and hence stabilisation of p53, which could explain the shorter, than expected, lifespan of these cells in culture (Zindy *et al.,* 1998). The observation that the p53-induced late G1/S phase, kinase inhibitory, p21CIP1 is not an effector of ras-based premature senescence in MEFs and that p21CIP1-null cells could still undergo proliferative arrest (Pantoja *et al.,* 1999) is suggestive of an elevated mitogenic signalling pathway in these cells driving a senescent response in reply to a recognition of dysfunction. Similar ras-induced premature-senescence phenotypes have been observed in human models operating through the same molecules. In human non-immortal fibroblasts activation of Raf-1 also led to premature onset of senescence in a p16-dependent manner (Zhu *et al.,* 1998), whilst Ras also induced promyelocytic leukaemia (PML) protein (see Section 4.2.5) which itself promoted premature senescence (Ferbeyre *et al.,* 2000) (see Section 4.2.5). Overexpression of E2F-1 and $p14^{ARF}$ also led to a premature growth-arrest in human fibroblasts (Dimri *et al.,* 2000). These observations that in response to the overexpression of components of the MEK pathway a cell can be driven into premature senescence through regular proliferative-arrest components could be due to a number of factors. An earlier report suggested that MEFs could be maintained in culture for over 200 population doublings (Loo *et al.,* 1987), this is at variance with other reports where 30 doublings are more commonly observed (Toldaro and Green, 1963). A key difference between these figures is that the longer lifespans were achieved in serum-free situations as compared to the shorter lifespans observed with serum. If indeed the presence of serum is responsible for the drastic shortening of lifespan it is conceivable that in a similar fashion to those situations described above, the mitogenic signalling pathways are forcing the cells into the premature senescence. Forced expression of mitogen signalling pathways could in effect shorten lifespan through a overloading of transcription machinery, forcing errors, leading to activation of negative cell cycle regulators. However, human cells grow for longer in culture, which suggests that mitogen pathways may be under greater regulation in these cells

whereas mouse cells are more susceptible to the deregulation of these pathways resulting in the premature senescence of 'culture shock'.

5) Methods of Tumour Suppressor Gene Detection

A number of powerful techniques are used to clone novel tsg's. These techniques are used in combination rather than reliance on a sole mechanism thereby aligning complementary data sets to provide clarity in a frequently confusing situation. Additionally due to the development of new technologies methods can change and acquire new characteristics at a rapid rate.

5.1) Comparative Genome Hybridisation

The fundamental basis behind CGH is that in tumour progression chromosomal regions, which contain oncogenes, are likely to be over-represented and therefore the oncogenes amplified. Conversely, chromosomal regions in which tsg's are located are likely to show a decreased copy number through deletion of one allele and mutation of the other leading to deregulation of growth control. The basis of the technique is the simultaneous hybridisation of immunohistochemically-labelled normal (driver) and tumour (tester) DNA is hybridised to normal human metaphase chromosomes. Conventionally the driver and tester DNAs are labelled with green and red flourochromes respectively. The resultant ratio of green to red fluorescence specifically decreases and increases, provides an indication of relative copy number in the tumour line (Kallioniemi *et al.,* 1992). Although a powerful technique CGH has its limitations it cannot be used to detect point mutations, small deletions or amplifications, or translocations. Realistically it can be used to detect 10 megabase copy number increases or decreases or smaller high-level DNA copy number increases.

5.2) Expression Microarrays

The technology behind expression microarrays has come as a direct result of the vast increase in public domain data available of cDNA clones and their sequences (reviewed in 'The Chipping Forecast', Nature Supplement, 1998. The fundamental approach to microarrays is the hybridisation of mRNA samples to arrays of cDNA (or oligonucleotide sequence derivatives) clones. The cDNA samples are chemically attached to slides or chips upon which the hybridisations take place. Both species are labelled with different coloured flourochromes (red (tumour) or green (normal)) and following the hybridisation the slide (or chip) is analysed using appropriate software to develop an expression pattern, for instance where a sample chamber is red then this would indicate an overexpression in the tumour, green would suggest an expression reduction in the tumour, and yellow would suggest no change in expression from tumour to normal mRNA. This technique is particularly well suited to large-scale analysis containing up to 40,000 different clones and would be expected to provide some exciting results in the future.

5.3) Linkage Analysis

The principle behind genetic mapping is to discover how often two loci are separated by meiotic recombination. Familial linkage analysis has led to the identification of a number of tsg's whose mutation creates a predisposition to cancer susceptibility and therefore is thought of by many as the 'gold standard' of cancer gene identification. In the first instance families are sought who demonstrate disease susceptibility and are then subject to whole chromosome screens using a variety of restriction fragment length polymorphisms (RFLPs), minisatellites, microsatellites, and single nucleotide polymorphisms (SNPs). This screen would be performed at a density of approximately one marker per 10cM. Following typing by polymorphic markers the likelihood that the markers are linked (or not) is calculated through the lod (logarithm of odds) score, where +3 and –2 define linkage and exclusion respectively. The lod score is defined as the logarithm of the

likelihood ratio. Scores, which are greater than 3 are then refined through finer mapping of, identified locus and candidate genes identified. Linkage analysis has lead to the identification of the genes responsible for Li-Fraumeni syndrome (*p53*), retinoblastoma (*pRB*), FAP (*APC*), familial breast and ovarian cancer (*BRCA1* and *2*), and familial melanoma ($p16^{INK4A}$) (Malkin *et al.,* 1990; Srivastava *et al.,* 1990; Yunis and Ramsay, 1978; Bodmer *et al.,* 1987; Leppert *et al.,* 1987; Easton *et al.,* 1993; Hussussian *et al.,* 1994)

5.4) Loss of Heterozygosity

The ability to inactivate tumour suppressor genes through loss of a copy of an endogenous chromosome accompanied by mutation on the remaining gene copy has been proposed as an important mechanism in the development of carcinogenesis (Knudson, 1971). This manifestation of allelic loss has been termed LOH and has been implicated in a variety of tumour types and has been instrumental in the positioning of a number of loci implicated in tumourigenesis. Defects in components of the mitotic machinery have been implicated in the development of this chromosomal instability allowing aneuploidy to develop (Pihan *et al.,* 1999). The study of LOH is based on the PCR amplification of highly polymorphic endogenous microsatellite repeats (Weber and May, 1989; Loius *et al.,* 1992; Weissenbach *et al.,* 1992; Gyapay *et al.,* 1994) and resolving the two alleles using gel electrophoresis. In this manner it can be clear if the tumour has lost an allele copy as compared to the matched normal tissue. LOH results from approximately 50% of second events that lead to inactivation of a tsg. Large deletions of chromosomal arms, whole chromosome loss, and mitotic recombination result in LOH whereas point mutations do not. The LOH technique has provided the major pathway to the finding of a number of new tsg's. Amongst these are *Smad4/DPC4* (Hahn *et al.,* 1996), *PTEN/MMAC1* (Steck *et al.,* 1997), and *PPP2R1B* (Wang *et al.,* 1999).

5.5) Representational Difference Analysis

This technique and CGH operate on intrinsically the same principles although representational difference analysis (RDA) operates at the nucleic acid level rather than chromosomal (Lisitsyn and Wigler, 1993; Diatchenko *et al.*, 1996, 1999). Technically the two genomes to be analysed undergo restriction enzyme digestion and are ligated to genome specific adaptor/linker oligonucleotides. This generates the representations of both genomes to be analysed, which can then be PCR amplified using specific adaptor/linker oligonucleotide primers. These representations are then denatured and hybridised to each other, i.e. one representation forms the 'tester' (conventionally tumour DNA) and the other the 'driver' (conventionally normal DNA). DNA sequences that are common to both genomes form heteroduplexes whilst unique tester sequences form homoduplexes, which can then be enriched through PCR amplification using the specific adaptor/linker oligonucleotide primers. In this manner changes in the DNA representation changes resulting from deletion, amplification or mutation can be identified and sequenced in an attempt to identify novel genes important to the tumourigenic phenotype. A further variation on this technique exists which operates on the same principles although mRNA is extracted, reverse-transcribed and the cDNA amplified and hybridised in the same manner from both 'tester' and 'driver' populations although affinity separation is used to remove driver-driver and tester-driver sequences (Lopez-Fernandez and del Mazo, 1993). This enables differences in expression to be analysed and novel or missing mRNA's to be identified.

6) Evidence for a Tumour Suppressor Gene and Mortality Gene on Chromosome 4

6.1) Functional Evidence

The first evidence of a link between chromosome 4 and tumourigenicity was provided by cell-cell fusions between a fibrosarcoma cell line and normal fibroblasts (Benedict *et al.,* 1984) followed by injection into athymic mice. The reappearance of tumourigenicity in the mice correlated with the loss of chromosome 4. This was suggestive of chromosome 4 carrying a gene whose loss was beneficial to the progression of tumourigenesis. A variety of cell-cell fusions between normal and immortal cell lines where senescence was the dominant phenotype (Pereira-Smith and Smith, 1988) led to the suggestion that similar pathways may be inactivated in tumourigenesis in differing cell types. This study led to the assignment of a group of cells to the same phenotypic grouping; GM2096SV9 (Origin-defective SV40-transformed XP skin fibroblasts), T98G (Glioblastoma), HeLa (Cervical carcinoma), and J82 (Bladder carcinoma). Further evidence of this grouping was provided by the MMCT of chromosome 4 into the same subset of cells, with the exception of GM2096SV9, causing the reversion of the immortal phenotype to a phenotype characteristic of replicative senescence (Ning *et al.,* 1991). Therefore the observed senescent phenotype was due to the introduced chromosome 4 and the phenotype was characterised by a flattening cellular morphology and a resemblance to typical senescent cells. Clearly this is an ambiguous definition that could be due to effects other than senescence. However in all of the flattened colonies retention of the exogenous chromosome 4 copy was demonstrated showing that the phenotype was due to the introduced chromosome. This demonstrated that the functional complementation that was occurring was likely replacing a component in a commonly lost pathway, which enhanced the tumourigenic potential in the target cell lines.

6.2) Locus Identification

Losses on chromosome 4 are seen in a wide variety of tumour types using powerful techniques such as CGH, LOH, and RDA (as described above). The identified loci on both 4p and 4q are discussed at length in Chapter 3 therefore we will provide a defined overview of those findings in this section.

6.2.1) Comparative Genome Hybridisation

CGH studies in breast cancer demonstrated frequent losses on 4q (Schwendel *et al.,* 1998). Further CGH studies on colorectal carcinogenesis found chromosome 4 copy number reduction to be a frequent event in advanced tumours (Ried *et al.,* 1996) and losses of a locus on *4p16* were implicated to be beneficial to malignant development (Paredes-Zaglul *et al.,* 1998). SCLC has shown common losses using CGH on 4p15-p16, 4q11-q23, 4q24-q26, and 4q32 (Levin *et al.,* 1994; Petersen *et al.,* 1997; Schwendel *et al.,* 1997; Petersen *et al.,* 2000). One locus was suggested through CGH analysis at 4q28 in RCC (Renal cell carcinoma) although no further definition was provided (Jiang *et al.,* 1998). Although a powerful technique CGH does not allow definition between allelic copies and instead simply provides an overall amplification or reduction of generalised chromosomal areas. From all the available CGH data however, chromosomal regions 4p15-p16, 4q11-q23, 4q24-q26, 4q28, and 4q32 have shown copy number reductions, which are suggestive of allelic loss.

6.2.2) Loss of Heterozygosity

LOH studies in bladder cancer revealed two areas of common deletion at 4p15 and 4q34, which correlated with an advanced tumour stage (Knowles *et al.,* 1994; Polascik *et al.,* 1995). Further LOH studies in breast cancer revealed losses at the same loci identified in bladder and two additional loci at 4p16.3 and 4q25-q26 (Shivapurkar *et al.,* 1999). CC (Cervical carcinoma)

LOH studies showed losses at 4q21-q23 (Mitra *et al.,* 1994) whilst CIN (Cervical intraepithelial neoplasia) LOH studies demonstrated losses at 4ptel and 4qtel (Larson *et al.,* 1997. In HNSCC four loci have been identified using LOH including one at 4p11-p12, 4q21, 4q25, and 4q32-q34 (Loughran *et al.,* 1997; Pershouse *et al.,* 1997). A number of LOH studies in HCC have shown losses at 4q21-q22, 4q25, 4q26-q27, 4q31, 4q32, 4q33, and 4q34 (Chou *et al.,* 1998; Piao *et al.,* 1998; Rashid *et al.,* 1999; Bando *et al.,* 1999). OSCC (Oral squamous cell carcinoma) LOH studies showed a locus at 4q25 was frequently lost (Wang *et al.,* 1999) whilst EA (Esophageal adenocarcinoma) LOH studies revealed three regions of common LOH at 4q21.1-q22, 4q32-q33, and 4q35 (Rumpel *et al.,* 1999) and ESCC (Esophageal squamous cell carcinoma) LOH studies revealed three regions of LOH at 4p16, 4p12-p14, and 4q21.3-q22 (Hu *et al.,* 2000). LOH analysis based on linkage studies in two syndromes, which predispose to neoplastic development revealed two loci at 4q23 (Irritable Bowel Disease (IBD); Cho *et al.,* 1998) and 4q21 (Scleroatrophic Syndrome; Lee *et al.,* 2000). Fundamentally therefore although a number of different loci are identified through these described LOH studies these define regions of common loss at 4p16, 4p15, 4p12-p14, 4p11-p12, 4q21-q22, 4q23, 4q25, 4q26-q27, 4q31, 4q32, 4q33, and 4q34. It is extremely unlikely however that chromosome 4 harbours 12 separate tsg although it is likely to harbour a tsg on both of its chromosomal arms. Additionally a number of intrachromosomal telomere-like repeats have been identified on chromosomes 4, which are proposed to be fragile sites, at 4cen, 4q13, 4q25, and 4q28 (Azzalin *et al.,* 1997). These do not account for all the LOH observed on chromosome 4 however a number of regions could be interpreted as resulting from fragile site breakpoints. Precedence for multiple targets of tumour suppression on a single chromosome arm has been documented on 18q where the tsg Smad4/DPC4 is inactivated by deletions in colorectal carcinoma (Hahn *et al.,* 1996) although some of these deletions also involve the putative tsg, DCC (Hahn *et al.,* 1996) indicating that there may be other targets.

6.2.3) Representational Difference Analysis

RDA analysis in osteosarcomas identified a region from 4q32-qtel although no suitable candidate genes were found (Simons *et al.,* 1999). There have been no further published RDA studies concerning chromosome 4 although this technique does rely on changes in expression and as such would perhaps not detect mutations with any effect on expression but solely on function.

6.4) Chromosome 4 Losses May Represent a Late Stage Event

A significant number of lines of evidence have suggested that LOH on chromosome 4 occurs as a late event in tumourigenesis perhaps as a key event for neoplastic immortality. Indeed gynaecological carcinoma showed 4q LOH was the fourth most frequent site of loss after 17p (*p53*), 9p ($p16^{INK4A}$), and 13q (*pRB*) indicating its advantage as a late event loss (Fujii *et al.,* 2000) coming after deregulation of the three classical tsg described above. A correlation with LOH on chromosome 4 and advanced tumour stage was shown in bladder cancer (Polascik *et al.,* 1995) and again where CGH losses were shown in muscle invasive TCC (transitional cell carcinomas) with no corresponding losses in superficial TCCs (Yeager *et al.,* 1998). A CGH study in colorectal carcinomas found no chromosome 4 copy number reduction in less advanced samples (Ried *et al.,* 1996) and that in colon cancer metastases there was an increasing frequency of losses (Paredes-Zaglul *et al.,* 1998). Epithelial hyperplasia and low-grade dysplasia have also shown an associated loss of chromosome 4 to cases that have shown progression (Rosin *et al.,* 2000). Therefore the loss of chromosome 4 may occur at a later stage of tumour progression, perhaps beyond the replicative senescence checkpoint as *p53* and $p16^{INK4A}$, mutations have been cited to occur prior in tumourigenesis (Loughran *et al.,* 1997).

7) Aims

The fundamental aim of this project was to test for the existence of a HNSCC mortality gene on chromosome 4. Further aims were to map chromosomal losses in an attempt to define the mortality gene locus on chromosome 4. Previous work had demonstrated LOH of chromosome 4 in immortal HNSCC lines and tumours. Functional evidence however, was limited to earlier by studies by Benedict *et al.,* (1984) which suggested the presence of a tsg, and Ning *et al.,* (1991) which suggested the presence of a mortality-associated gene. However, no functional studies had been undertaken in immortal HNSCC to demonstrate immortal reversion. Therefore, in the course of this project we aimed to test for a mortality function associated with chromosome 4 through utilisation of MMCT, to undertake to determine the nature of this mortality effect through biological assay, and to map the position of the gene(s) responsible for this phenotype.

Chapter 2

1) Materials

1.1) Cell lines

Source	Cell line	Origin
ATCC, 12301 Parklawn Drive, Rockville, Maryland, 20852. USA	3T3	Swiss Mouse Embryos Cat. No. CCL92.
	HT1080	Fibrosarcoma, Cat. No. CCL121.
	NRK	Mycoplasma Indicator, Cat. No. CRL 6509
	A92	Mouse Fibrosarcoma.
Dr R F Newbold Brunel University, Middlesex, UK	A9HYTK4 A9HYTK6 A9HYTK11 A9HYTK15	

Mouse A92 cells containing a single resistance marker tagged human chromosome (Cuthbert *et al.*, 1995).

Dr Olivia Periera-Smith, Baylor College of Medicine, Houston, Texas, USA	HeLa A9F4	Cervical Carcinoma

Mouse A92 cell containing a fragment of Neomycin resistance tagged chromosome 4. The fragment was generated from an intact chromosome 4 introduced into HT1080 and then transferred back into A9 cells.

Dr G. P. Nolan, Stanford University Medical Centre, San Francisco, California, USA	Pheonix A	Retroviral Packaging Cell Line

Cell Lines obtained from Dr. E. K. Parkinson are listed in Table 2.1.

1.2) General cell culture media and supplies

Supplier	Material
A/S Nunc Botolph Claydon, UK	1ml Cryotubes
Autogen Bioclear Holly Ditch Farm, Mile Elm, Calne, Wiltshire, UK	Fetal Bovine Serum Cat. No. S011S
Becton Dickinson Labware Plymouth, UK	Falcon tissue culture dishes, full range
Calbiochem-Novabiochem UK Ltd Boulevard Industrial Park, Padge Road, Beeston, Nottingham, NG9 2JR, UK	Hygromycin B, Cat. No. 400051.

Table 2.1. Cell lines obtained from Dr. E. K. Parkinson.

Description of immortal keratinocyte cell lines used in experimental processes. The table indicates the site of each tumour along with TNM staging and proliferative fate. Table adapted from Edington *et al.*, 1995.

Keratinocyte	Site	Tumour Stage (TNM)	Proliferative Fate
BICR 3	Alveolus	$T_2N_OM_O$	Immortal
BICR 6	Hypopharynx	$T_4N_1M_O$	Immortal
BICR 18	Larynx	Metastasis	Immortal
BICR 19	Epidermis	Not Applicable	Immortal
BICR 22	Tongue	Metastasis	Immortal
BICR 31	Tongue	$T_4N_{2B}M_O$	Immortal
BICR 56	Tongue	$T_4N_1M_O$	Immortal

Fisher Scientific UK Bishop Meadow Road, Leicestershire, LE11 5RG, Loughborough, UK	Dimethylsulfoxide ($CH_3.SO.CH_3$) Cat. No. BPE231-1.
Gibco BRL Life Technologies Paisley, UK	DMEM, Cat No. 21969-035 HEPES Buffer, Cat No. 15630-056 L-Glutamine, Cat. No. 25030-032. Geneticin (G418 Sulphate), Cat. No. 11811-098.
Sigma Chemical Company Fancy Road Poole, Dorset, UK	Penicillin G (benzylpenicillin) sodium salt, Cat No. P3032 Streptomycin Sulphate BP, Cat. No. S9137. Hydrocortisone 21Hemisuccinate, Cat No. H2270. Bovine Donor Calf Serum, Cat No. C-9676
Worthington Biochemical Corporation Reading, UK	Trypsin, Cat. No. 39J3128.

1.3) Specialized Cell Culture Techniques

Supplier	Material
Costar Bucks, UK	5 mm filter membranes
Gibco BRL Life Technologies, Paisley, UK	$25cm^2$ (40ml) Straight Sided Flasks, Cat. No. 52094A.
Nalge Nunc International Corp., 2000 North Aurora Road, Naperville, IL. 60563-1796, USA	8 Well Permanox® Slide, Cat. No. 177445.
Sigma Chemical Company Poole, UK	Chloroquinine, Cat. No. C6628. Cytochalasin B, Cat. No. C6762. Demecolcine, Cat. No. D6165. Phytohemaglutinin, Cat. No. C9017. Polybrene (Hexadimethrine), Cat. No. H9268 Polyethylene Glycol-1000, Cat. No. P3515. Hoeschst 33258, Cat. No. B1155.

1.4) Immunocytochemistry antibodies and materials

Supplier	Material
Autogen Bioclear UK Calne, UK	Mortalin C-19 [GRP 75] Goat Polyclonal Antibody, Cat. No. sc-1058
Boehringer Mannhiem UK (Diagnostics & Biochemicals) Ltd Bell Lane, Lewes, East Sussex, BN7 1LG, UK	5-Bromo-2'-deoxy-uridine Labelling and Detection Kit 1, Cat. No. 1296 736.
Calbiochem-Novabiochem (U.K.) Ltd Boulevard Industrial Park, Padge Road, Beeston, Nottingham, NG9 2JR, UK	Normal Goat Serum, Cat. No. 566380.
Intergen Company The Magdalene Centre, The Oxford Science Park, Oxford, OX4 4GA, UK	Apoptag® Fluorescein Direct *In Situ* Apoptosis Detection Kit. Cat. No. S7160

Lab Vision (UK) Ltd The Pines, Fordham Road, Newmarket, Suffolk, CB8 7LG UK	Tubulin Ab-4 (Clone DM1-A + DM1-B) Mouse Monoclonal Antibody, Cat. No. #MS-719-P1
Sigma Chemical Company Poole, UK	Nonidet P-40 (NP-40), Cat. No. N-3516. Bovine Serum Albumin (BSA), Cat. No. A9647. Donkey Anti-goat IgG-FITC Cat. No. sc-2024.
Vector Laboratories Inc. 16 Wulfric Square, Bretton, Peterborough, PE3 8RF, UK	Vectashield With DAPI, Cat. No. H-1200. Texas Red Anti-Mouse IgG, Cat. No. TI-2000.

1.5) Molecular Biology Kits

Supplier	Material
QIAGEN Ltd Boundary Court, Gatwick Rd, Crawley, West Sussex, RH10 2AX, UK	Rneasy® Mini Kit, Cat No. 74104 QIAamp® DNA Mini Kit, Cat No. 51304 QIAshredder, Cat No. 79654 Plasmid Midi Kit, Cat No. 12143

1.6) Chemicals

Solutions and buffers were prepared using dH_20 from a Millipore MilliRO 15 System.

Supplier	Chemical
Agar Scientific Ltd 66a Cambridge Road, Stanstead, Essex, CM24 BDA, UK	Paraformaldehyde ($(CH_2O)_nH{\cdot}OH$)), Cat. No. R1018.
Amersham International Little Chalfont, UK	Redivue [a^{32}P]dCTP~ 3000Ci/mmol. Cat No. AA0005.
BioFine Ltd London, UK	X-Gal
James Burrough (F.A.D) Ltd 70 Eastways Industrial Park, Witham, Essex, CM8 3YE, UK	Ethanol (C_2H_5OH) Cat. No. SIN 1170.
Fisher Scientific UK Bishop Meadow Road, Loughborough, Leicestershire, LE11 5RG, UK	D (+) Glucose ($C_6H_{12}O_6$), Cat. No. G/0500/53 Glycine ($NH_2CH_2{\cdot}COOH$), Cat. No. BPE381-1. Magnesium Chloride ($MgCl_2{\cdot}6H_2O$),

	Cat. No. M/0600/53. Methanol (CH_3OH), Cat. No. BPE1105-1. Sodium Chloride (NaCl), Cat. No. BPE358-1.
Sigma Chemical Company Poole, UK	Ethidium bromide, Cat. No. E1510, Giemsa's Stain, Cat. No. GS500 Glacial Acetic Acid ($C_2H_4O_2$), Cat. No. A6283; MES (2- [N-Morpholino] ethanesulfonic acid), Cat No. M8250, TEMED (N,N,N',N'-Tetramethyl-ethylenediamine), Cat. No. T8133; EDTA (Ethylenediaminetetra – acetic acid, $C_{10}H_{14}N_2O_8Na_2{\cdot}2H_2O$), Cat. No. ED2SS; Potassium ferricyanide ($K_3Fe(CN_6)$), Cat No. P3667; Potassium ferrocyanide ($K_4Fe(CN)_6{\cdot}H_2O$), Cat No. P9387; Rhodamine B, Cat No. R6626.

1.7) PCR Reagents

Supplier	Enzyme
PE Applied Biosystems Warrington, Cheshire, UK	Taqman Gold RT-PCR Kit, Cat No. N808-0232; AmpliTaq® DNA Polymerase, Cat. No. N801-0060; 10X PCR Buffer & $MgCl_2$ Solution, Cat. No N808-0010.

1.8) Laboratory plasticware

Supplier	Material
Becton Dickinson Labware Plymouth, UK	Falcon tubes
Bibby-Sterilin Stone, UK	5 ml Bijous; 20ml Universals
Elkay Galway, Eire	Microcentrifuge tubes; Pipette tips.
Greiner Labortechnik Stonehouse, UK	Filter pipette tips

PE Applied Biosystems Warrington, Cheshire, UK	MicroAmp® Optical 96-well Reaction Plate and Optical Caps. Cat No. P/N 403012

1.9) Electrophoresis gels

Supplier	Material
Gibco BRL Life Technologies Paisley, UK	Agarose, electrophoresis grade;
National Diagnostics Hull, UK	SequaGel Buffer, Cat No. EC-835 Sequagel Concentrate, Cat No. EC-830 Sequagel Diluent, Cat No. EC-840

1.10) Paper and X-ray film

Supplier	Material
Sigma Chemical Company Poole, UK	Kodak X-OMAT Film, Cat. No. F5263.
Whatman International Maidstone, UK	3mm Chromatography paper, Cat. No. 3030 917.

1.11) Microbial host, media and supplies

Supplier	Material
Difco, Central Avenue, West Molesey, Surrey, KT8 2SE, UK	Tryptone, Bacto Cat. No. 0123-15 Yeast extract, Bacto Cat. No. 0127-15
Bibby-Sterilin Stone, UK	Petri dishes
Gibco BRL Life Technologies Paisley, UK	Library Efficiency DH5α^{TM} Competent Cells Cat. No. 18263-012 S.O.C. Medium Cat. No. 15544-018
Sigma Chemical Company Poole, UK	Ampicillin, Cat. No. A9393.

1.12) Plasmids

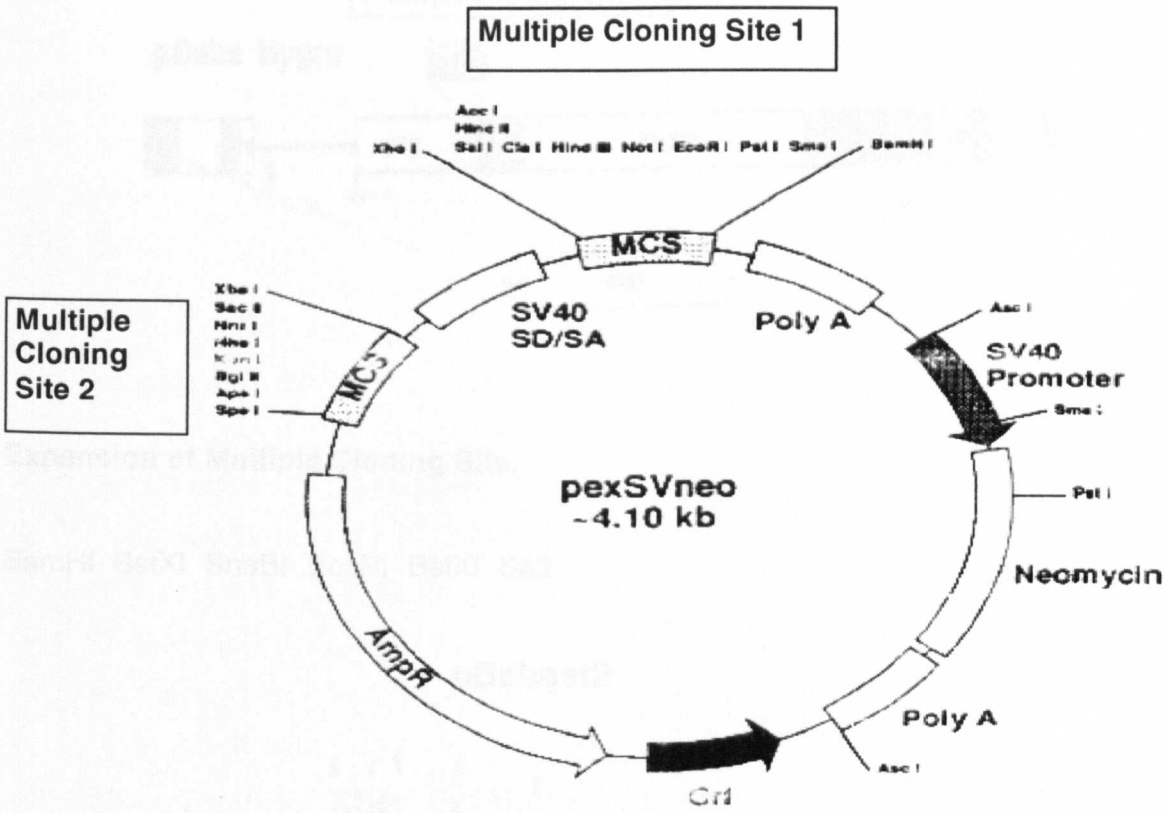
See Figure 2.1 for schematics of. plasmids

pBabe Hygro obtained from Dr S. Barnett, Department of Neurology. University of Glasgow. U.K.

Figure 2.1. Plasmids used in transfections

Cartoon representing the structures of pexSVneo, plasmid used as a vector in MORF4 transfections (see Chapter 6, Section 6.2), pBabeHygro, plasmid used to generate Hygromycin resistant 3T3 feeder cells, and pBabest2, retroviral cassette used in keratinocyte infections.

pexSVneo



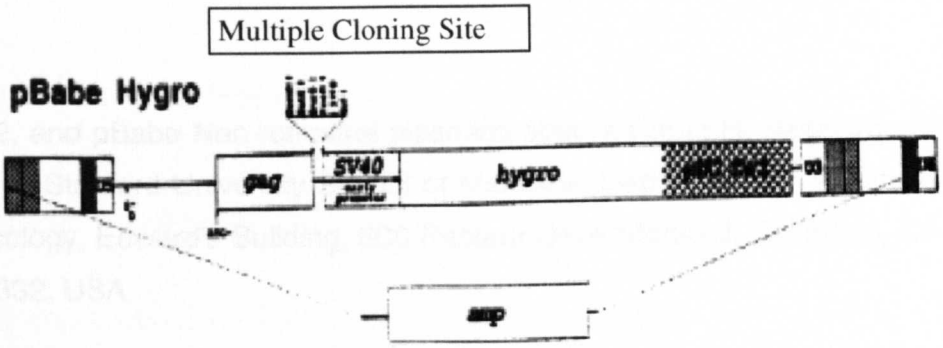
Expansion of Multiple Cloning Site 1.

XhoI AccI/HincII/SalI ClaI HindIII NotI EcoR1 PstI SmaI BamHI.

Expansion of Multiple Cloning Site 2.

XbaI SacII NraI NheI KpnI BglII ApaI SpeI

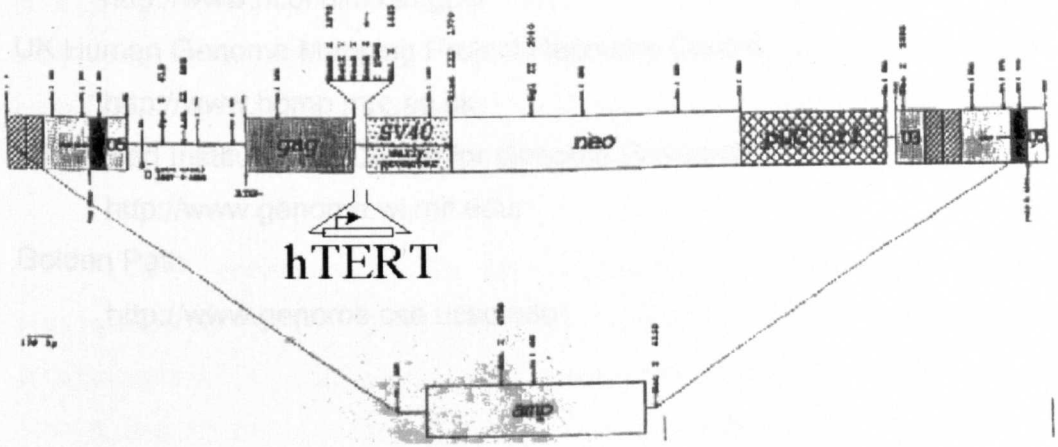
pBabe Hygro



Expansion of Multiple Cloning Site.

BamHI BstXI SnaBI EcoRI BstXI SalI.

pBabest2



pexSVneo, pexSVneoMORF4FS, pexSVneoMORF4 plasmids were obtained from Dr Olivia Periera-Smith, Baylor College of Medicine, Houston, Texas, USA.

pBabest2, and pBabe Neo retroviral plasmids obtained from H. Vaziri and S. Benchimol. Stanford University School of Medicine, Department of Molecular Pharmacology, Edward's Building, 300 Pasteur Drive Stanford, California, CA 94305-5332, USA.

1.13) Internet Links

Centre d'Etude du Polymorphisme Humain (CEPH)

http://www.cephb.fr/

Cooperative Human Linkage Centre (CHLC)

http://lpg.nci.nih.gov/CHLC

National Centre for Biotechnology Information (NCBI)

http://www.ncbi.nlm.nih.gov/

UK Human Genome Mapping Project Resource Centre

http://www.hgmp.mrc.ac.uk

Whitehead Institute/MIT Center for Genome Research

http://www.genome.wi.mit.edu/

Golden Path

http://www.genome.cse.ucsc.edu

2) Methods

2.1) Cell culture techniques

2.1.1) Culture of cell lines

All keratinocyte cell lines except when stated otherwise were cultured in DMEM supplemented with 10% FBS (see Table 2.2), 20mM HEPES Buffer, 2 mM L-glutamine, 50U/ml Penicillin, 20ng/ml Streptomycin, and 0.4ng/μl Hydrocortisone. All other tumour-derived lines were cultured as above excepting the Hydrocortisone supplement. The Swiss 3T3 fibroblasts were cultured in DMEM supplemented with 10% DCS, 40mM HEPES Buffer, 2 mM L-glutamine, 50U/ml Penicillin, 20ng/ml Streptomycin, All chromosome donor cell lines were cultured in DMEM supplemented with 10% FBS, 40mM HEPES Buffer, 2 mM L-glutamine, 50U/ml Penicillin, 20ng/ml Streptomycin, containing 800 U/ml Hygromycin B. The donor line A9F4 was cultured as for those previously stated with a 1mg/ml G418 supplement replacing the Hygromycin B. All cell lines were maintained in a humid 37°C/5% CO_2 incubator.

Cryopreserved cell stocks were prepared from early-passage cell lines and hybrids as follows. Cells were maintained in culture until they reached approximately 75% confluence. They were then trypsinised, neutralized with normal growth medium and pelleted. The cell pellet was resuspended in freezing medium (10% DMSO in normal growth medium) to an appropriate density and the suspension transferred into cryotubes (1 ml per vial). The cryotubes were wrapped in cotton wool and placed into a -70 °C freezer overnight to slow freeze, and the following day were immersed in liquid nitrogen for long-term storage. Cryotubes were recovered from liquid nitrogen by immediate immersion in a large volume of water at 37°C. The cell suspension was then made up to a volume of 10 ml with normal growth

Table 2.2. Optimal culture conditions for all keratinocyte lines

Supplemented FBS concentrations at which immortal keratinocytes were judged to grow optimally. Derived from Edington *et al.*, 1995.

Keratinocyte Line	Optimal Serum
BICR 3	10%
BICR 6	10%
BICR 18	2%
BICR 19	10%
BICR 22	2%
BICR 31	10%
BICR 56	10%

medium and the cells pelleted by gentle centrifugation, 1000rpm for 5min. Cells were resuspended and plated at an appropriate density.

2.1.2) Mycoplasma Testing

Media of at least 48hrs contact with cells to be tested was added to 10^4 NRK cells and incubated for 96hrs. Media was then removed and cells were washed twice with PBS followed by fixing in Methanol / Acetic Acid (3:1) twice consecutively and finally for 10min. After fixative removal 0.05μg/ml Hoechst 33258 (Chen, 1977; fluorescent DNA stain) was added and left for 10min followed by two consecutive washes in distilled water. Cells were mounted in McIlvaines's Buffer (0.2M Na_2HPO_4, 0.1M Citric Acid) and examined using fluorescence microscopy.

2.1.3) Selection Determination

Kill curves were used to determine the appropriate level of antibiotic selection to use in MMCT and Transfections. Cells were plated at 10^5 per 60mm dish and placed under selection media after 48hr. Thereafter selection media was changed twice weekly. Complete cell death after 2 weeks was taken to be the appropriate antibiotic selection. Appropriate cell line selection determination values are listed in Table 2.3.

2.1.4) Transfection

Cells to be stably transfected were seeded 24hr previously in 60mm dishes at an appropriate density to reach approximately 60% confluence. 5μg plasmid DNA dissolved in TE, pH7.4 was diluted with DMEM containing no serum or antibiotics to a total volume of 150μl. The solution was then mixed, spun down briefly, 20μl of Superfect Transfection Reagent added and vortexed for 10sec prior to incubation for 10min at room temperature to allow complex formation. Whilst complex formation was taking place the recipient plates

Table 2.3. Selection determination for cell lines

Selection agent (Hygromycin and Neomycin) sensitivity for immortal cell lines determined through kill curve construction (see Section 2.1.3).

Cell Line	Hygromycin B concentration (U/ml)	Neomycin concentration (G418 Sulphate) (mg/ml)
HeLa	300	1.2
HT1080	200	N.d
BICR 3	60	0.6
BICR 6	125	0.6
BICR 18	60	N.d
BICR 19	80	N.d
BICR 22	25	N.d
BICR 31	60	N.d
3T3	150	1.0

were washed once with PBS. After complex formation 1ml normal growth medium was added to the reaction tube, mixed, and transferred to the recipient plate and incubated for 2-3hrs. Following incubation cells were washed 4 times with PBS, fresh growth medium added and incubated for 48hrs. Finally cells were passaged 1:8 into appropriate selection media and selected until the appearance of clones. Table 2.4 outlines the plasmids used for transfections.

2.1.5) Microcell-mediated monochromosome transfer

Donor cells (murine A92 cells containing an individual *Hytk*-tagged human chromosome) were plated into six straight-edged 25 cm^2 flasks at a donor specific density in the range of 1.3×10^6 to 1.45×10^6 cells per flask. After 24 hr Demecolcine was added to a final concentration of 75 ng/ml, and the flasks were incubated at 37°C for a further 48 hr. Following this incubation the medium was then replaced with 30 ml of 10 mg/ml Cytochalasin B in serum-free medium supplemented with 40mM HEPES Buffer and 2 mM L-glutamine and incubated for 30 minutes. The flasks were then placed in a GSA rotor (cushioned with 75gm mQH_2O) and spun in a pre-heated Sorvall RC 28S centrifuge, programmed with the following parameters: slow start, 15 min; 7600 rpm/9500g; 75 min; slow stop 10 min; 37 °C; brake off. Following the run the pellets (consisting of micro cells and cell debris) were resuspended in a small amount of supernatant and transferred into a single 15 ml sterile Falcon tube. The suspension was centrifuged (3470 rpm, RT, 5 min), and the pellet resuspended in 10 ml of serum-free medium. The microcell suspension was then passed through three sterile 5 mm filters, and the final suspension centrifuged as before. Meanwhile the recipient cells, approximately 80% confluent on 10 cm plates (seeded 48hrs previously), were rinsed twice with serum-free medium. The microcell pellet was resuspended in 3 ml of 20 mg/ml PHA-P in serum-free medium, and the whole suspension added to a plate of recipient cells, the control plate was treated with PHA-P/serum-free medium only. The cells were then incubated at 37 °C for approximately 2 hours. Following this incubation 3 ml of 45% PEG-1000 in serum-free

Table 2.4. Plasmids used in transfections

Table listing plasmid names and their sources. See Figure 2.1 for schematic of plasmids

Plasmid	Source
pbabeHygro	Dr. Sue Barnett
pexSVneo	Dr.Olivia Periera-Smith
pexSVneoMORF4FS	Dr.Olivia Periera-Smith
pexSVneoMORF4	Dr.Olivia Periera-Smith

medium with 17% DMSO was added gently to the cells, to give a final concentration of 22.5% PEG and 8.5% DMSO, and moved continuously for 45s at RT. After this incubation the cells were consecutively rinsed five times with serum-free medium, (1 x 30 s, 1 x 45 s and 3 x 60 s rinses), and incubated at 37 °C overnight in ordinary media. The cells were then trypsinised and re-plated at $1x10^6$ per 10 cm dish. After 72 hrs Hygromycin B or G418 selection at a suitable predetermined concentration (see Table 2.3) was added to the plates. The selection medium was changed twice weekly and colony formation monitored. A schematic of this procedure is shown in Figure 2.2.

2.1.6) Assay for endogenous β-galactosidase activity

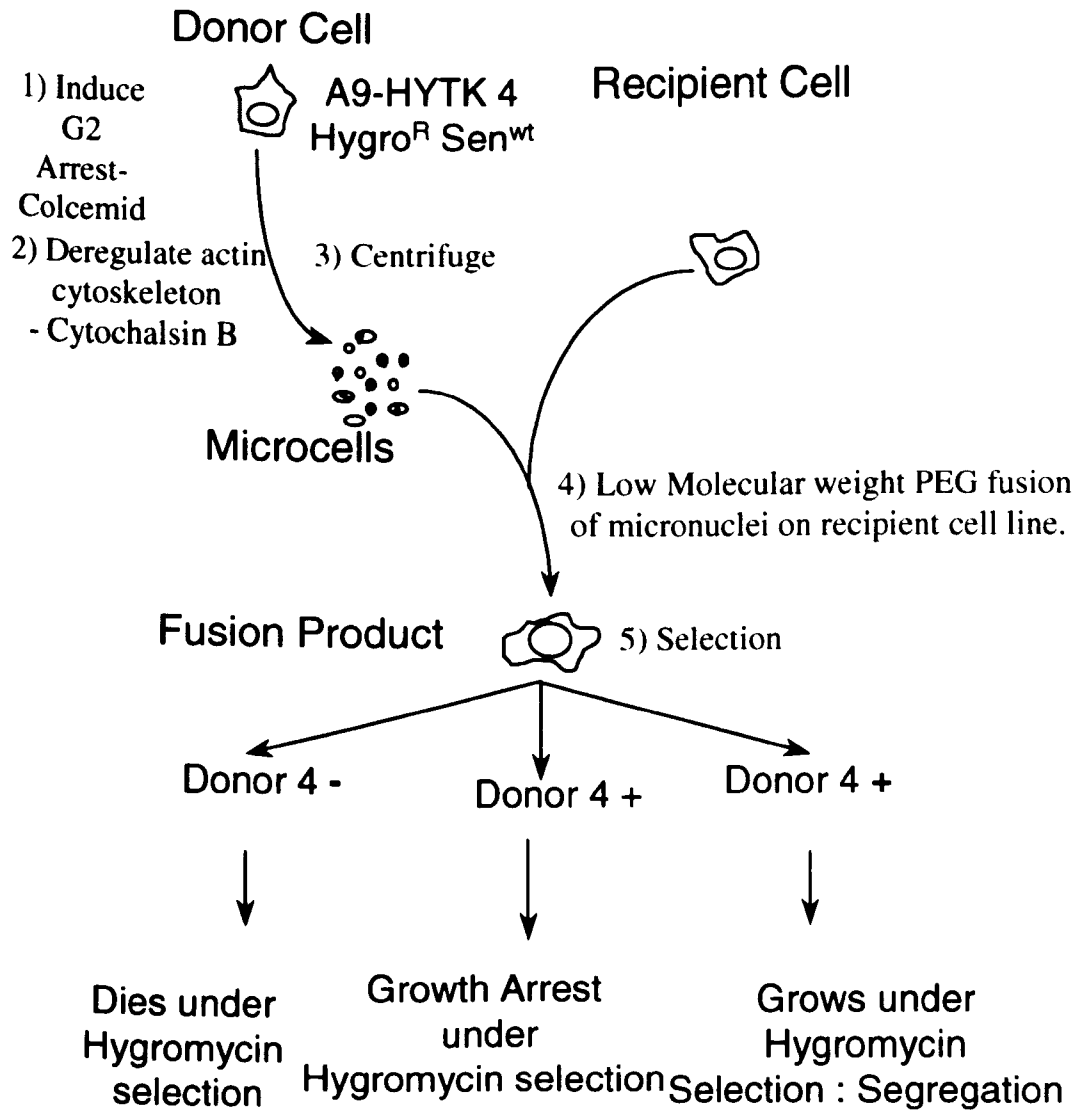
Adhered cells were rinsed twice with PBS, and fixed by incubating in 3% (v/v) formaldehyde in PBS for 5 min at RT. The cells were then twice rinsed again with PBS. 5 ml of X-gal staining solution (50 mM MES, 150 mM NaCl, 2mM $MgCl_2$, 5mM $K_4Fe(CN)_6 \cdot H_2O$ (potassium ferrocyanide), 5mM $K_3Fe(CN_6)$ (potassium ferricyanide) and 1mg/ml X-Gal) at pH 6 was added to each 10 cm plate. The dishes were incubated in a humid box for 16 hr at 37 °C. Following this incubation the cells were rinsed with mQH_2O and assessed histochemically for blue staining.

2.1.7) Rhodamine Stain for Keratinocytes

Adherent cells were first washed with PBS and then overlaid with 10% Formaldehyde/PBS and agitated on a shaker for 10 min. Following this the cells were washed once with PBS and overlaid with 1% Rhodamine B stain and agitated for 30 min on a shaker. The cells were then rinsed with tap water until all surplus stain was removed, air dried and observed under a low power microscope

Figure 2.2. Microcell-mediated monochromosome transfer technique

For description of experimental procedure see Section 2.1.5.


Donor Cell
A9-HYTK 4
Hygro^{R} Sen^{wt}
Recipient Cell
1) Induce G2 Arrest- Colcemid
2) Deregulate actin cytoskeleton - Cytochalsin B
3) Centrifuge
Microcells
4) Low Molecular weight PEG fusion of micronuclei on recipient cell line.
Fusion Product
5) Selection
Donor 4 -
Donor 4 +
Donor 4 +
Dies under Hygromycin selection
Growth Arrest under Hygromycin selection
Grows under Hygromycin Selection : Segregation

2.1.8) Giemsa Stain

Cells were washed twice with PBS and fixed in 10% Formaldehyde/PBS and fixed for 30 min. Following fixation cells were washed in H_2O and then stained with 10% Giemsa's/H_2O for 30 min. Following stain cells were washed in H_2O and air-dried. Cells were observed under a low power microscope.

2.1.9) Determination of population doublings

The number of mean population doublings (MPD) that hybrids from MMCT and Transfections had achieved was calculated at each passage according to the formula of Paul *et al.*, 1975.

$$PD = (\log N_b - \log N_a) \times 3.32$$

where N_a = initial cell number

N_b = final cell number

2.1.10) Metaphase Spreads

Cells were seeded and cultured to approximately 70% confluence and Demecolcine added to a final concentration of 100ng/ml and incubated at 37°C for 1 hr. The cells were then trypsinised as normal, pelleted and resuspended in a small amount of media by tapping. Through dropwise addition, over a gentle vortex, 10ml of prewarmed (37°C) 75mM KCl was added to the cell suspension and then the mixture incubated for 15 min at 37°C. Following this 1ml of ice-cold fix (80% methanol, 20% acetic acid) was added to the suspension, incubated on ice for 5min and centrifuged at 1000-2000 rpm for 10 min. Remove supernatant carefully and add dropwise 10ml ice cold fix, incubate on ice for 5 min and centrifuge remove supernatant and repeat. Metaphases were dropped onto ice-cold slides and washed with 1ml

ice-cold fix and allowed to air dry. Metaphase spreads were carried out by Mrs H. Ireland, BICR.

2.1.11) Production of Retrovirus by Transient Infection

Pheonix A cells were seeded at a density of 2 X 10^6/6cm dish 24 hours prior to transfection. The cells were medium changed 5min before transfection and replaced with medium containing 25μM chloroquinine. During this incubation the transfection mixture was prepared by combining 8μg DNA, 300mM $CaCl_2$ and H_2O to 500ml. A further 500ml 2 X HBS (50mM HEPES pH 7.05, 10mM KCL, 12mM Dextrose, 280mM NaCl, 1.5mM $Na_2HPO_4 \cdot 2H_2O$, pH 7.05) was added and the whole solution bubbled for 15sec to mix thoroughly. This mixture was then immediately added to the cells and incubated at 37°C for 8.5 hrs after which time the cells were medium changed and again after a further 24 hrs. Supernatant was collected 50 hrs post-transfection and filtered once through a 0.45μm membrane prior to freezing and titrating.

2.1.12) Viral Titre Assessment

NIH3T3 cells were seeded at a density of 10^5 cells/6cm dish 24hrs prior to transfection. 100μl virus was serially diluted (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) and added to sterile 4μg/ml polybrene containing 10% DCS to a total volume of 4ml. Dishes of NIH3T3 were infected with 1.5ml of appropriate dilutions; controls contained no virus, and incubated for 4 hrs at 37°C. After this time 10%DCS/DMEM was added to bring the volume to 5ml and left overnight. Dishes were then medium changed and following 24 hrs incubation this was replaced with 1mg/ml G418 selection. Selection was performed for 10 days followed by staining with Giemsa and titre calculated.

2.1.13) Viral Infection of Keratinocytes

Cells to be infected were seeded at a density of 10^5 cells/6cm dish 72hrs prior to infection. Viral supernatant was added to 8mg/ml polybrene in 10H to a total volume of 5ml containing at least 1.35 X 10^4 viral particles and incubated at 37°C for 4 hrs. Following the incubation the media was replaced with fresh 10H and the cells left overnight to recover. The cells were then split and seeded at a density of 3 X 10^5/9cm dish allowed to recover for a further 72 hrs and then placed under appropriate selection until resistant colonies had appeared. Following selection the resistant colonies were pooled and stored in liquid nitrogen for further use.

2.2) Immunocytochemistry

2.2.1) Immunofluorescence

Cells were plated into 8-well chamber slides at a density of 280 cells per well and incubated for an appropriate time i.e. 6 hrs or 6 weeks. The cells were then washed briefly with PBS, and fixed for 5 min in precooled –10°C methanol and allowed to air dry. All incubations were carried out at room temperature in a humidified chamber. Once air-dried the cells were permeablized with 0.1% NP-40 for 20min followed by blocking with 10% goat serum for 20 min to suppress non-specific binding of immunoglobin. The cells were then washed twice with PBS and incubated with the primary antibody (in 2% BSA/PBS) for 1 hr. Following the primary antibody incubation the cells were washed three times with PBS and incubated with the directly conjugated secondary antibody (1μg/ml Fluorescein/Texas Red®). The cells were then washed five times with PBS; gaskets removed, blotted dry and mounted in Vectashield with DAPI. Mounted cells were sealed with coverslips and clear nail varnish and stored at 4°C for future viewing.

2.2.2) 5-Bromo-2'-deoxy-uridine Incorporation Analysis

5-Bromo-2'-deoxy-uridine (BrdU) incorporation analysis was undertaken using the 5-Bromo-2'-deoxy-uridine Labelling and Detection Kit 1 (Boehringer Mannheim). Reagents were supplied as kit components. Cells were plated in 8 chamber slides at minimal density (280 cells/chamber) and maintained in media for an appropriate time i.e. 6 days or 6 weeks). BrdU labelling reagent was added to the sample at a final concentration of 10 μmol/ml diluted in sterile cell culture medium. The cells were then incubated for an appropriate period of time (1hr, 4hrs, or 48hrs) at 37°C in a 5% incubator. After this incubation the slides were washed three times with PBS and then fixed with 70% Ethanol (in 50mM Glycine buffer, pH2.0) for at least 20 min at –20°C. The slides were then washed again with PBS as described above before covering the cells with anti-BrdU (mouse monoclonal antibody containing nucleases, in PBS/glycerine) diluted in Incubation Buffer (66mM Tris-buffer, 0.66mM $MgCl_2$, and 1mM 2-mercaptoethanol) and incubating for 30 min at 37°C. Following on from this incubation the slides were again washed as above and then covered with anti-mouse-Ig-flourescein and incubated for 30 min at 37°C. Slides were then washed as above, dried, and gaskets removed followed by mounting in Vectashield/DAPI. Slides were sealed using clear nail varnish and coverslips and stored at 4°C for future viewing

2.2.3) TUNEL Analysis

TUNEL analysis was undertaken using the Apoptag® Fluorescein Direct *In Situ* Apoptosis Detection Kit (Intergen Company). Reagents were supplied as kit components. Cells were plated in 8 chamber slides at minimal density (280 cells/chamber) and maintained in media for an appropriate time i.e. 6 days or 6 weeks). Fixation of cells was undertaken using 1% Paraformaldehyde in PBS, pH7.4, for 10 min at room temperature followed by 2x 5 min washes in PBS. Cells were then post-fixed in precooled Ethanol/Acetic Acid for 5 min at –20°C, drained, and washed as above. Excess liquid was then removed and 75μl Equilibration Buffer added directly into each chamber and incubated for at least 10sec. Following this incubation

and aspiration of the Equilibration Buffer 55μl of Working Strength TdT Enzyme (70% Reaction Buffer/30% TdT Enzyme) was added to each specimen and incubated for 1hr at 37°C in a humidified chamber. To stop the reaction the slides were placed in a coplin jar containing Working Strength Stop/Wash Buffer (3% Stop/Wash Buffer/97% Distilled H_2O) and agitated for 15 s followed by 10 min incubation at room temperature. Slides were then dried, gaskets removed, followed by mounting in Vectashield/DAPI. Slides were sealed using clear nail varnish and coverslips and stored at 4°C for future viewing.

2.2.4) Chromosome Painting

The previously prepared metaphase spread slides were immersed in 2 X SSC at 37°C for 20min and then briefly in H_2O. The slides were then rapidly denatured for 1min exactly in 0.4M NaOH / 0.6M NaCl and then quickly washed 3 times in neutralization solution (1M NaCl / 0.5M Tris-HCl pH7.2) agitating whilst washing. 3 X 1min washes in neutralization solution whilst moving continuously followed this. Slides were treated with 30μg/ml pepsin in 1mM HCl for 5 min and then washed in 4 X SSC for 3min followed by 1 X PBS for 3 min. Metaphases were then serially dehydrated in increasing concentrations of ethanol (70%, 90%, absolute) for 3 min per dilution and air dried prior to denaturation. Spreads were denatured in 70% formamide in 2 X SST for exactly 90 s at 68°C in a coplin jar and then transferred into ice-cold 70% ethanol for 3 min, serially dehydrated as above and allowed to air dry. Prior to the above step the probe was denatured at 70-72°C for 10 min and then maintained at 37°C for 30min whilst denaturing the metaphase spread. Hybridise 15μl probe to metaphase slide, seal with evostick and incubate for 48-72 hrs at 37°C in the dark. The probe consisted of 1μl of each paint, 1μl Cot1 DNA and hybridisation buffer (50% deionised formamide, 10% dextran sulphate, 10% 20 X SSC, 8% 0.5M Na_2HPO_4, 1% SDS, 1% 100xDenhardt's, and 10% H_2O to 15μl total volume. To visualize slides, they were washed for 5 min in prewarmed (37°C) 2 X SSC for 5 min and coverslips removed followed by washes at 45°C (2 x 50% formamide, 1 X SSC, and 2 x 0.1xSSC)

of 5 min each. Slides are then washed 4 X SST, 3 min per wash. Block slides using 100µl 3 X BSA in 4 X SST, cover with paraffin slip and incubate at 37°C for 20 min in humidified chamber, followed by a 3 min wash in 4 X SST and paraffin removal. Appropriate antibody dilution's prepared in 4 X SST, to 100µ l, used to cover metaphase areas and incubated at 37°C. Once complete wash twice in 4 X SST add DAPI mountant, coverslip and seal with nail varnish. Slides must be kept in the dark as much as possible through the denaturation and detection procedures. Dr. S. Dowens (Dept. of Pathology, University of Cambridge) and Mrs Hazel Ireland (BICR) carried out chromosome Painting.

2.2.5) Visualization by Fluorescence Microscopy

Visualization of slides was undertaken using a Zeiss Axioskop Fluorescent Microscope using the appropriate filters for colour detection. Images were captured using a CCD Camera System (Princeton Instruments) and analysed using IPLab Spectrum 10, Version 3.0 (Signal Analyticals).

2.3) Extraction, Synthesis and Quantitation of Nucleic Acids

2.3.1) Extraction of Genomic DNA from Mammalian Cell Lines

Genomic DNA was extracted from mammalian cell lines and hybrids using the QIAamp® DNA Mini Kit (QIAGEN). All reagents and plasticware were supplied as kit components. Cells were first harvested by trypsinisation, pelleted and washed twice in PBS followed by resuspension in 200µl PBS. Lysis of cells was performed by first adding 1/10 volume Proteinase K followed by an equal volume Buffer AL, vortexing the sample for 15sec and incubating for 10 min at 56°C. Following incubation ½ volume Ethanol was added to the sample, vortexed again for 15sec, transferred into a QIAamp spin column and centrifuged at 15300rpm for 1min. The flowthrough was discarded and the column was first washed with 500µl Buffer AW1 and then

with 500µl of BufferAW2, both wash steps are centrifuged as above. Eluting with an appropriate volume of Buffer AE eluted the DNA from the column.

2.3.2) Extraction of Total RNA from Mammalian Cell Lines

Total RNA was extracted from mammalian cell lines and hybrids using the Rneasy® Mini Kit (QIAGEN). All reagents and plasticware were supplied as kit components. Cultured cells were first trypsinised and pelleted at 300 x g and lysed by addition of an appropriate volume of Buffer RLT (containing β-Mercaptoethanol), vortexed to mix, and the lysate transferred onto a QIAshredder column followed by centrifugation at 15300rpm for 2 min. The homogenized lysate was mixed with an equal volume of 70% Ethanol and applied onto an Rneasy mini spin column and centrifuged for 15 s at 8000 X g. The column was then washed first with Buffer RW1 and then Buffer RPE with the flowthrough being discarded at each step, centrifugation carried out at 8000 X g for 15 s. The final wash step used Buffer RPE at 15300rpm for 2 min before elution, into a 1.5ml collection tube, with an appropriate volume of Rnase-free H_2O using centrifugation at 8000 X g for 1 min.

2.3.3) Synthesis of Oligonucleotides

Oligonucleotides were synthesized at the BICR as a core service on a 394 RNA/DNA synthesizer (Applied Biosystems) using Cruachem reagents. Oligonucleotides were deprotected and lyophilized using the Hybaid Proligo system and supplied in this form. The supplied oligonucleotide was resuspended in 500µl of mQH_2O and the concentration determined as described below.

2.3.4) Quantitation of Nucleic Acid Concentrations

Nucleic acid was quantified by spectrophotometric determination of its UV light absorbency. 5 ml of sample was added to 495 m1 of de-ionised water

and the absorbency of the solution measured at 260 nm and 280 nm in a quartz cuvette, using dH_2O as a blank. The concentration of the solution was calculated using de Beer's law on the basis that an optical density of 1.0 at 260 nm corresponds to a concentration of 50ng/μl for double-stranded DNA, 40ng/μl for total RNA, and 30ng/μl for single-stranded oligonucleotides. Pure preparations of DNA and RNA have a ratio of A_{260}/A_{280} readings between 1.5 and 1.9.

2.4) PCR and analysis of amplification products

2.4.1) PCR

Standard PCR was generally undertaken using 100ng Template DNA, 10μM Forward Primer, 10μM Reverse Primer, 1x Reaction Buffer II (Perkin-Elmer), 25mM $MgCl_2$, 2mM each dNTP, 0.04U Amplitaq Polymerase (Perkin-Elmer), and mQH_2O to a total volume of 23μl. Occasional slight alterations to the annealing temperature or concentration of magnesium ions were required for optimal results. The PCR conditions consisted of a 3min denaturation at 95°C, followed by 30 rounds of 30 s denaturation at 94 °C, 30 s annealing at 60 °C, and 1 min extension at 72 °C, finally the reactions were held for a final 7 min extension at 72°C before ramping down to 4°C. Occasional alterations were made to the annealing temperatures or to the extension time to optimise PCR conditions. Reactions were carried out using a GeneAMP 9600 (Perkin-Elmer Cetus). Prior to sequencing or quantification (see below) PCR product was purified using the QIAquick PCR Purification Kit (QIAgen). A full list of all primers used is outlined in Appendix 1.

2.4.2) PCR Product Purification

This was undertaken using the QIAquick PCR Purification following the manufacturers protocol as briefly described. Add 5 volumes of Buffer PB to 1 volume of PCR reaction mix and apply mixture to QIAquick spin column in a

2ml collection tube and centrifuge at 10,000rpm for 60 s. Following this discard flow-through and wash column by adding 0.75ml Buffer PE and centrifuging as above, discard eluant and spin again as above. To elute purified PCR product 30μl elution buffer was applied to the column and incubated at room temperature for 1 min and centrifuged as above. The manufacturer as part of the kit supplied all reagents and plasticware. Centrifugation was performed on a conventional tabletop microcentrifuge. The concentration of the sample was determined by running 10% of total volume against an appropriate DNA ladder, for example the 1kb ladder (GibcoBRL).

2.4.3) Sequencing

Completed sequencing reactions were ran out in-house at the BICR on an ABI 373 sequencer (Applied Biosystems). Prior to this 40-80ng of purified PCR product and 3.2pmol primer oligonucleotide was added to BigDye Terminator cycle sequencing reagents (Perkin-Elmer Applied Biosystems). Reactions were performed on a PCT-100 thermal cycler (Genetic Research Instrumentation Ltd) using 25 cycles of 10 s at 95°C, 5 s at 55°C, and 4 min at 60°C. Completed sequencing reactions were analysed using DNAStar software. A full list of primers is outlined in Appendix 2.

2.4.3) Real-Time Quantitative PCR

All two-step Real Time-QPCR reactions were carried out using 96-well reaction plates. 50ng of total cell RNA was added to Reverse Transcription reaction mix comprising 1xTaqman RT Buffer, 5.5mM $MgCl_2$, 500μM of each dNTP, 2.5μM Random Hexamer, 0.4 U/μl Rnase Inhibitor, 1.25 U/μl Multiscribe Reverse Transcriptase. The total reaction volume was made up to 5μl with Rnase-free H_2O. The cycling parameters for the Real Time-PCR step were 10 min Incubation Hold at 25°C, 30 min Reverse Transcription at 48°C, followed by a 5 min Reverse Transcriptase Inactivation at 95°C. Transfer 20μl

of PCR reaction mix into each tube; comprising 1x Taqman Buffer A, 5.5 mM $MgCl_2$, 200μM deoxyATP, 200μM deoxyCTP, 200μM deoxyGTP, 400μM deoxyUTP, 100nM MyoD1 Fluorigenic Probe, 200nM MyoD1 Forward Primer, 200nM Myod1 Reverse Primer, 0.01 U/μl, and 0.025 U/μl Amplitaq Gold DNA Polymerase. The PCR reaction mix volume was made up to 20μl using Rnase-free H_2O. Cycling steps for the PCR step were 2 min UNG Incubation at 50°C, 10 min Amplitaq Gold Activation at 95°C, and 40 cycles of a 15 s denature at 95°C and 1min Anneal/Extend at 60°C. Reactions and product analysis were carried out upon an ABI PRISM 7700 Sequence Detection System. Primers designed using Primer Express™ software (see Table 2.5.), MyoD1 intron/exon structure obtained from Zingg *et al,* 1991.

2.4.4) PCR Analysis of Microsatellites

Microsatellite Analysis was undertaken using 40ng of genomic template DNA extracted from matched HNSCC fibroblasts and tumour lines and hybrids resulting from the MMCT procedure. Total PCR Reaction Mix comprised 40ng DNA, 1x Buffer 1 (Perkin-Elmer), 200μM dNTP's, 10% DMSO, 1μM Forward Primer, 1μM Reverse Primer, 10kBq α-^{32}P-dCTP, and 0.5U Taq Polymerase. Cycling steps comprised 3 min at 95°C, followed by 30 cycles of (30 s at 94°C, 30 s at 55°C, and 30 s at 72°C), and finally 7 min at 72°C. The Microsatellite markers were derived from Genethon, a full list of polymorphic marker specific primers is described in Appendix 3. Radiolabelled Microsatellite PCR products were visualized using Denaturing Polyacrylamide Gel Electrophoresis.

2.4.5) Denaturing Polyacrylamide Gel Electrophoresis

The radiolabelled PCR products of polymorphic markers were resolved on a range of polyacrylamide gels under denaturing conditions. This range was dependent on the length of the PCR product; see Table 2.6. A gel solution was prepared from Sequagel stock solutions using 7.5M Urea (Sequagel Diluent), 25% $^{w}/_{v}$ Acrylamide solution 19:1 Acryl:Bisacryl and 7.5M Urea

Table 2.5. MyoD1 primers used in Real Time-QPCR

See Section 2.4.3 for description of technique and description of primer construction.

	Length	Tm	%GC	Primer
Forward Primer	19	59	63	ACAGTGGCGACTCAGACGC
Taqman Probe	20	69	70	CAGCCCGCGCTCCAACTGCT
Reverse Primer	22	59	45	GCTGTAATCCATCATGCCATCA
Amplicon	65	84	60	

Table 2.6. Microsatellite PCR product size and appropriate gel acrylamide concentration

Concentration of acrylamide was optimised to PCR product size to ensure seperation of differing microsatellite bands by gel migration.

Product Size	Gel
100-130bp	8%
130-170bp	7%
170-220bp	6%
220-300bp	5%
300-500bp	4%

(Sequagel Concentrate), and Sequagel Buffer comprising 7.5M Urea, 1M Tris-Borate and 20mM EDTA pH8.3. The solution was polymerised by the addition of 650ml of 10% $^{w}/_{v}$ ammonium persulphate and 100ml of TEMED per 100ml of gel solution. This solution was then poured between glass plates separated by 0.4 mm spacers and allowed to set at room temperature for approximately 1 hr. Gels were pre-run at 60W for 45 min in 1 x TBE (90 mM Tris-borate and 2 mM EDTA pH 8.0) to warm the gels to approximately 50 °C before the loading of samples. Denaturing Gel Loading Buffer (98% $^{v}/_{v}$ Formamide, 10mM EDTA pH 8.0, 0.1% $^{w}/_{v}$ Xylene Cyanol and 0.1% $^{w}/_{v}$ Bromophenol Blue) was added to the completed PCR reactions (5μl added to 10μl reaction) and this mixture denatured by heating at 95°C for 5 min and immediately quenched on ice. 5ml of this mixture was subjected to electrophoresis at 50W for approximately 4 hr depending upon the size of the PCR product. The gel was then transferred to a sheet of Whatman 3M paper and dried under vacuum at 80 °C for 45 min. The PCR products were detected by autoradiography using X-ray film.

2.4.6) Agarose gel electrophoresis of unlabelled PCR products

Non-radioactive PCR products were separated on non-denaturing agarose gels and visualized by staining with Ethidium bromide and UV transillumination. Gels were prepared by dissolving electrophoresis grade agarose in I X TAE (40 mM Tris-acetate and 2 mM EDTA pH 8) through microwaving on a medium heat setting for 2-3min. Ethidium Bromide to a final concentration of 0.5μg/ml was added to the dissolved agarose before being poured into an appropriate gel former and left to set for approximately 20 min. Once solid, gels were placed into electrophoresis tanks containing I x TAE. Samples to run were combined with 1/5 volume Running Buffer (50% Glycerol, 100mM EDTA, 1x TAE, 0.1% Bromophenol Blue) and loaded into the appropriate well. Electrophoresis was performed for 1 hr at 100V to ensure separation of bands. In order to estimate the size of fragments resolved by electrophoresis, samples were run alongside known molecular

weight markers, such as a 1kb ladder. DNA was visualized by UV transillumination and the gel analysed using Biorad software.

2.5) Microbiological techniques

2.5.1) Plasmid DNA Extraction

Plasmid DNA was extracted using the QIAGEN Midi Kit. A single colony was picked from a selective plate (see Section 2.5.2) and used to inoculate a 5ml starter culture of LB medium containing 100μg/ml Ampicillin. The culture was incubated for 8 hrs at 37°C in a shaker, diluted 1/1000 into selective LB medium and incubated for a further 16 hrs at 37°C in a shaker. The bacterial cells were harvested by centrifugation at 6000 X g for 15 min, all supernatant removed and resuspended completely in 10ml Buffer P1. An equal volume of Buffer P2 was added and gently mixed by inversion followed by incubation at room temperature for 5 min. A further incubation of 20 min on ice followed the addition of 10ml Buffer P3 (chilled). Centrifugation at 20,000 X g for 30 min at 4°C separated the plasmid DNA from cell debris, the supernatant from this spin was removed promptly and respun at 20,000 X g for 15 min at 4°C. Whilst the final spin was underway applying 10ml Buffer QBT and allowing it to empty by gravity flow equilibrated a QIAGEN-tip 500. On completion of the spin the supernatant was removed and applied to the QIAGEN-tip and allowed to enter the resin by gravity flow. The QIAGEN-tip was washed twice with 30ml Buffer QC allowing gravity flow to move the buffer in both cases. Elution of DNA from the resin was done with 15ml Buffer QF and collected in a 30ml tube followed by precipitation by adding 0.7 volumes of Isopropanol to the eluted DNA. This mixture was then centrifuged at 15,000 X g for 30 min at 4°C and the supernatant decanted leaving the DNA pellet in the tube. The DNA pellet was washed with 5ml of 70% Ethanol by centrifugation at 15,000 X g for 10 min and the supernatant again decanted leaving the DNA precipitate. The pellet was then air-dried for 10 min and resuspended in TE, pH8.0. Plasmid DNA concentration was determined as described for genomic DNA

2.5.2) Transformation of bacteria with plasmid DNA

DH5αcompetent strain of E.coli was thawed on ice and 20μl transferred into a prechilled 1.5ml microcentrifuge tube. Plasmid DNA diluted to a final concentration of 10ng/μl-20ng/μl was added to the DH5α cells, mixed and incubated on ice for 30 min. The cells were then heat-shocked for 40 s in a 42°C water bath and then placed on ice for 2min. 80μl S.O.C Medium (GibcoBRL) was added to the microcentrifuge tube and agitated in a shaker at 225rpm at 1hr at 37°C. All of the cells were then spread on LB medium (Luria-Bertani Medium; 10g bacto-tryptone, 5g bacto-yeast extract, 10g NaCl in 1ltr deionised H_2O pH7.0. LB Medium prepared in-house at the BICR) plates containing 1.5% (w/v) agar supplemented with the appropriate antibiotic and poured in advance. Plates were incubated in an inverted position at 37 °C, and colonies transferred into LB medium the following day.

Chapter 3

3.1) Combined Analysis Suggest Areas of Deletion on Chromosome 4

The ability to inactivate tumour suppressor genes through loss of a copy of an endogenous chromosome accompanied by mutation on the remaining gene copy has been proposed as an important mechanism in the development of carcinogenesis. This manifestation of allelic loss has been termed Loss of Heterozygosity (LOH) and has been implicated in a variety of tumour types. Defects in components of the mitotic machinery have been implicated in the development of this chromosomal instability allowing aneuploidy to develop (Pihan *et al.*, 1999). The study of LOH is based on the PCR amplification of highly polymorphic, ubiquitous, and abundant endogenous microsatellite repeats (Weber and May, 1989; Loius *et al.*, 1992; Weissenbach *et al.*, 1992; Gyapay *et al.*, 1994) and resolving the two alleles using gel electrophoresis. Microsatellites themselves consist of repeating units of 1-6bp that are highly polymorphic due to their high mutation rate (10^{-3}-10^{-4} per generation) (Weber and Wong, 1993). In this manner it can be clear if the tumour has lost an allele copy as compared to the matched normal tissue. The polymorphic markers are continuously placed in order at both Genemap 2000 and Marshfield (Broman *et al.*, 1998), see list of websites for more details.

Chromosome 4 LOH has been observed in multiple tumour types although the tsg's undergoing inactivation have yet to be cloned or identified. Indeed the LOH observed occurs on both the short p arm and the long q arm identifying several potential tsg sites on this chromosome. An illustration of the findings of these studies is outlined below.

An allelotypic study in bladder cancer revealed LOH on 4p in 22% (13/60) of samples analysed although no LOH on 4q was observed (Knowles *et al.*, 1994). Using a large number of primary bladder carcinoma samples two common regions of lost were identified (Polascik *et al.*, 1995). These regions were localised to a 3.9cM region on 4p15 (29%, 82/282) and a 12cM

region on 4q34 (24.1%, 68/282) and correlated well with an advanced tumour stage.

Breast cancer studies have also implicated LOH on chromosome 4 as important for tumour progression. A CGH study on 39 invasive ductal breast carcinomas found loss of material on 4q in 51% (20/39) of samples analysed making it the second most frequent loss seen (Schwendel *et al.,* 1998), whereas a further allelotyping of breast carcinomas revealed four regions of common loss (Shivapurkar *et al.,* 1999). These regions comprised two from both the p and q arms, namely, a locus on 4p15.1-p15.3 of 3cM length (63%, 26/41), a locus on 4p16.3 of 750-kb long (58%, 22/38), a locus on 4q33-q34 of 14cM length (79%, 27/34), and a locus on 4q25-q26 (67%, 26/39).

A study utilising CGH in consecutive stages of colorectal carcinogenesis found that chromosome 4 was lost in 31% (5/16) of colorectal carcinomas (Ried *et al.,* 1996). A chromosome 4 copy number reduction was not seen in either low-grade adenomas or high-grade adenomas suggesting the reduction was a late event. CGH studies in colon cancer found increasing frequency of losses at 4p16 were present in metastases as compared to primary tumours (Paredes-Zaglul *et al.,* 1998). This suggests that although not sufficient the loss of 4p16 may in this instance be advantageous to malignant development.

Gynaecological carcinosarcoma cases studied revealed 4q losses to be the fourth most frequent site of LOH (29.4%, 5/17) after 17p (*p53*), 9p (*p16*), and 13q (*pRB*) although no specific locus was identified (Fujii *et al.,* 2000). CCs studied allelotypically revealed LOH on 4q (46%) that specifically mapped to the 4q21-q23 region (Mitra *et al.,* 1994), although this study did not define any specific locus. A semi-automated fluorescence-based LOH detection system was used to identify two preliminary regions (Hampton *et al.,* 1996). These regions mapped to a 63cM region distal to D4S1627 on 4p and a 67cM region distal to D4S1625 on 4q. CIN demonstrated LOH on both 4p and 4q and identified a 24.4cM region at the telomeric end of 4p and a 1.1 cM region at the telomeric end of 4q (Larson *et al.,* 1997).

HNSCC cases studied have also been shown to demonstrate LOH on chromosome 4. Importantly findings would indicate that LOH at 4q is a later event in HNSCC tumourigenesis in agreement with the work described previously (Califano *et al.,* 1996; Koch and McQuone, 1997; Rosin *et al.,* 2000). Allelotypic analysis of advanced HNSCC demonstrated loss of both chromosome 4 arms without specifying any definite locus (Nawroz *et al.,* 1994). This study showed 4p loss in 38% (8/21) and 4q loss in 37% (9/24) of samples analysed, whilst a further study utilised CGH to show frequent losses of the chromosome in approximately 50% of samples from a panel of 133 primary HNSCC (Bockmuhl *et al.,* 2000). A study using immortal HNSCC derived cell lines implicated a locus at 4q32-q34 between D4S1535 and D4S408 where 75% (6/8) of samples showed losses (Loughran *et al.,* 1997, Parkinson *et al.,* 1997). This correlated well with an earlier study where losses were seen on 4q and not on 4p although a lack of informativity prevented locus definition (Loughran *et al.,* 1994). However a similar study found LOH on 4q in 81% (22/27) and 4p in 41% (11/27) of HNSCC tumours analysed (Pershouse *et al.,* 1997). This implicated a small region on 4q21 between D4S400 and D4S423, a region on 4q25 between D4S1616 and D4S407, and a third region on 4p11-p12 of 7cM.

An early study using a panel of RFLPs against hepatocellular carcinoma (HCC) samples found evidence of 4q allele loss in 58% (7/12) of cases which localised to 4q11-q25 (Buetow *et al.,* 1989). Further use of RFLP showed LOH on chromosome 4 in 28% (4/14) of cases (Fujimoto *et al.,* 1994). In hyperploid HCC LOH of 4q was observed in 42% (20/48) of samples suggestive of an alternative mechanism in the tumour progression (Legoix *et al.,* 1999) although no specific locus was identified in this study. Three minimally lost regions were identified in 15 HCCs analysed by LOH on 4q26-q27 of 7cM (67%, 10/15), 14cM (67%, 10/15), and 6cM (73%, 11/15) although there was no data to distinguish between these areas (Chou *et al.,* 1998). Seven regions on 4q were demonstrated to have undergone a high frequency of LOH in HCCs in an additional study (Piao *et al,* 1998). These were 1) 7cM region centred on 4q25 at D4S1615 (47%, 16/34), 2) 5cM centred on 4q31 at D4S1598 (50%, 17/34), 3) 3cM centred on 4q31 at

D4S620 (59%, 20/34), 4) 5cM on 4q31 between D4S1566 and D4S2979 (56%, 19/34), 5) 4cM on 4q32 defined by D4S1545 and D4S1617 (47%, 16/34), 6) 2cM on 4q33 defined by D4S1537 (56%, 19/34), and 7) 7cM on 4q34 between D4S3041 and D4S1535 (62%, 21/34). A separate study identified two regions of LOH centred on 4q12-q21 and 4q22-q24 which were lost in 50% (12/24) of HCCs with informative alleles (Rashid *et al,* 1999). The 4q21 lost was region defined to a deleted region of 1cM on 4q21-q22 based on common LOH in 48% (12/25) of HCCs analysed and delineated by D4S1534 and D4S2929 (Bando *et al.,* 1999).

SCLC has been shown to undergo a high incidence of deletions on 4q. Using the CGH technique losses were seen on 4q in 55% (10/18) at 4q24-q26 (Levin *et al.,* 1994), and 86% (19/22) of SCLCs examined with two common regions lost at 4q11-q23 and 4q32 (Petersen *et al.,* 1997). CGH analyses of primary SCLC and their metastases found over 60% of cases showed losses on chromosome 4 with deletions at 4q11-q23 and 4q32 present (Schwendel *et al.,* 1997), however no single aberration was shown to be significantly associated with metastatic phenotype. A further CGH study in clonal evolution of metastatic SCLC of the lung found that 4p and 4q losses were present in over 50% of samples however a deletion at 4p15-p16 occurred more frequently in the metastatic stage (Petersen *et al.,* 2000).

CGH analyses of both adenocarcinoma and SCC of the lung showed frequent losses of 4q were seen in both entities (Petersen *et al.,* 1997). This indicates that although both tumours have distinct originating tissues they can be characterised by common genetic losses suggestive of common pathways being deregulated.

The findings in epithelial hyperplasia and low-grade dysplasia which demonstrated progression showed LOH on 4q in 30% (8/27) of cases whereas those with no progression showed LOH on 4q in 5% (4/79) (Rosin *et al.,* 2000) suggesting that LOH on 4q may be advantageous for tumour progression. A localization study in OSCC revealed a frequency of LOH on the q arm of 66% (21/32) compared to 25% (8/32) on the shorter p arm

however the common region of loss was centred at D4S1573 on 4q25 with a minimal distance of 4cM lost (Wang *et al.,* 1999). Allelotypic analysis in EA revealed an involvement of 4q losses, although no specific locus was identified in this study, with 54.5% (12/22) of samples showing long arm loss (Hammoud *et al.,* 1996). Further examination found LOH on 4q in EA at three regions of common loss (Rumpel *et al.,* 1999). These were centred on D4S1534 at 4q21.1-q22 (56%, 13/23), on D4S620 at 4q32-q33 (48%, 13/27), and on D4S426 at 4q35 (62%, 16/26). A genome wide screen in ESCC found LOH on 4p in 65% (24/37) and 4q in 67% (62/93) of cases studied (Hu *et al.,* 2000). In this same study three regions with a very high frequency of LOH were identified at 4p16 with D4S2366 (78%, 7/9), 4p12-p14 with D4S2632 (90%, 9/10), and 4q21.3-q22 with D4S2361 (100%, 7/7) although the size of the regions are all approximately 20cM due to density of microsatellite markers used in the screen.

Representational difference analysis (RDA) in osteosarcomas, where normal DNA acted as the tester and tumour DNA as the driver therefore highlighting any deleted sequences, identified a region from 4q32-qtel of approximately 26.2cM although a preliminary known gene screen did not identify any suitable candidates (Simons *et al.,* 1999). LOH analysis in dedifferentiated chondrosarcoma demonstrated that deletion of a chromosome 4 allele had occurred in both the anaplastic sarcoma and the low-grade malignant cartilage-forming tumour (Bovee *et al.,* 1999). Furthermore the chromosome 4 losses had occurred as separate events in each component as both had lost a different copy indicative of separate deleterious events.

CGH studies revealed that 4q losses were also present in RCCs with 50% (6/12) showing 4q deletions (Jiang *et al.,* 1998). The minimal region of overlap in this study was suggestive of minimal loss locus on 4q28 although no further definition of the region was reported.

There has also been an implication in other non-cancerous disease states that LOH on chromosome 4 is important for their progression. Linkage

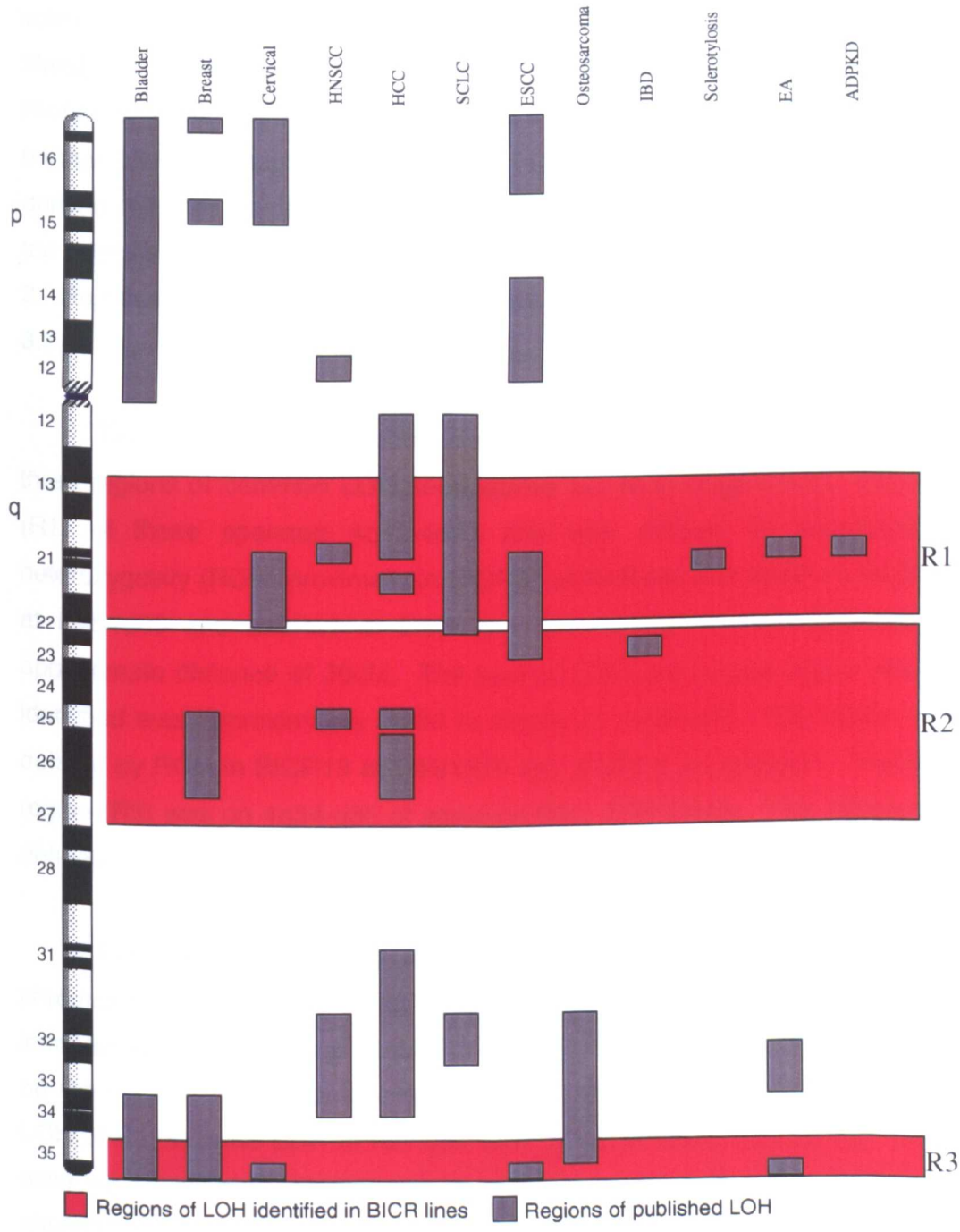
studies on mixed families suffering from forms of IBD identified a locus centred on D4S1647 and D4S2623 (Mlod 1.71, P = 2.5 X 10^{-3}) at 4q23 (Cho *et al.*, 1998). The human autosomal polycystic kidney disease (ADPKD) has at least three loci that account for the disease, one of these is localised to 4q21 where 10% (3/30) of samples showed LOH whilst a further 27% (8/30) demonstrated somatic mutations (Torra *et al.*, 1999). A further linkage study involved in identifying the gene responsible for an autosomal dominant scleroatrophic syndrome which also predisposes to aggressive early onset SCC identified a locus on 4q21 centred on D4S2380 (Mlod 8.69) of approximately 3.1cM (Lee *et al.*, 2000).

3.2) LOH Analysis Reveals Three Regions of Loss in Immortal HNSCC Lines

Given the wide variety of informative LOH from the large variety of different tumour types it is clear that there are a number of different regions being identified as sites for potential tsg's. An unbiased view at the available discrete data sources reveals that there are probably 9 different common regions of LOH on chromosome 4 shared to differing degrees between tumour types (see Figure 3.1). Given the varying nature of these studies we decided to further our interest in HNSCC by carrying out an initial LOH screen

Figure 3.1. Regions of chromosome 4 LOH identified in previous studies

Numerous regions of LOH have been identified and published as putative sites of tsg localisation. Figure 3.1 illustrates these identified regions (grey boxes) and the tumour type or disease of origin (listed along the top of the diagram). The representation of chromosome 4 on the left hand side delivers an approximation of the chromosomal position of the identified regions. The areas boxed in red illustrate the regions of LOH identified in Figure 3.3 from the panel of 10 immortal HNSCC lines.


Bladder
Breast
Cervical
HNSCC
HCC
SCLC
ESCC
Osteosarcoma
IBD
Sclerotylosis
EA
ADPKD
p
q
16
15
14
13
12
12
13
21
22
23
24
25
26
27
28
31
32
33
34
35
R1
R2
R3
Regions of LOH identified in BICR lines
Regions of published LOH

across chromosome 4 using an approximate 10cM microsatellite repeat spacing which spanned both arms of the chromosome, all microsatellite details are described in Appendix 1. The ordering of the markers were verified using the CEPH/Genethon chromosome 4 linkage map, the Marshfield chromosome 4 sex averaged linkage map, and the Stanford Human Genome Centre YAC STS-content map. For this study we analysed normal DNA and matched tumour DNA from a panel of 10 immortal HNSCC derived cell lines as described in Chapter 2. The study was performed using the microsatellite PCR and gel electrophoresis methods described in Chapter 2. Representative gel images illustrating LOH and ROH are shown in Figure 3.2.

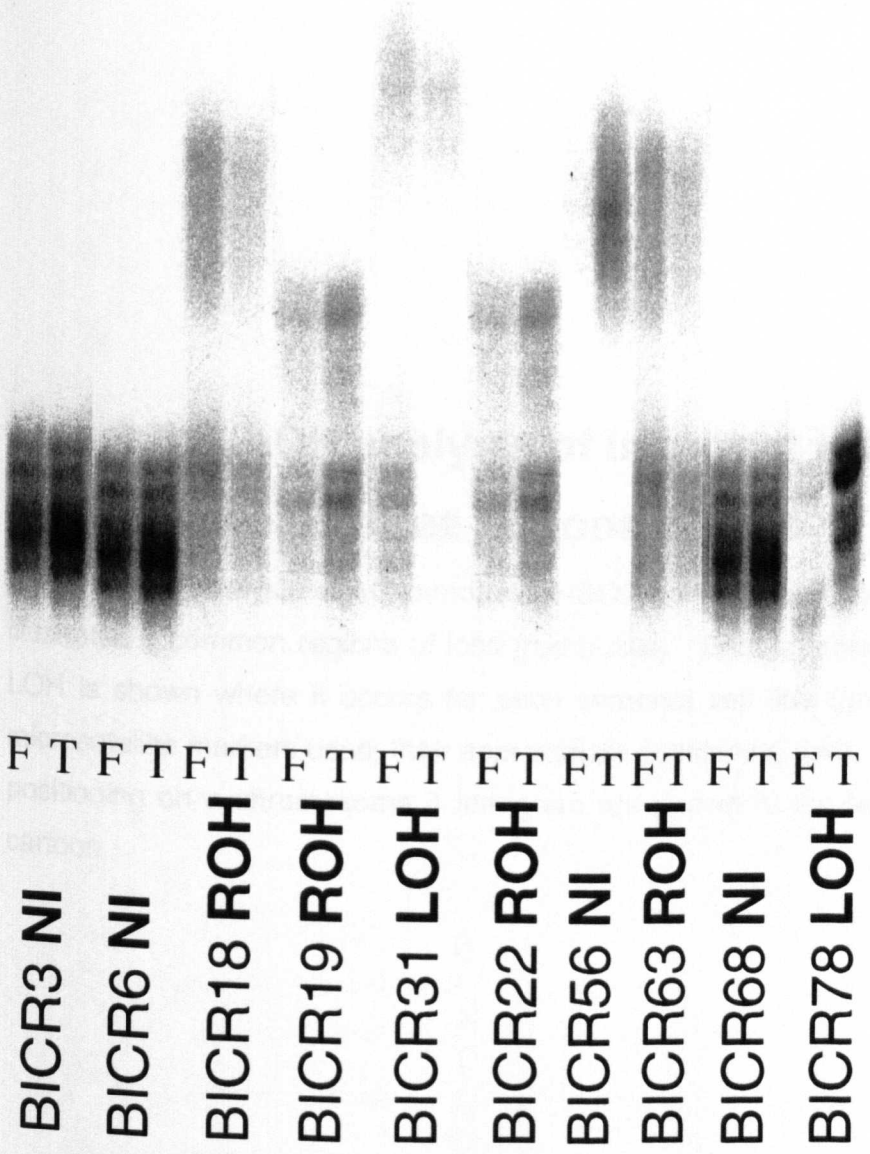
The results of our broad screen across the chromosome identified three regions of common LOH seen across our lines (Figure 3.2). The first (R1) of these spanned 4q12-4q23 and was defined by retentions of heterozygosity (ROH) proximally in BICR31 at D4S409 and distally in BICR18 at D4S1089 and BICR22 at D4S423. This region of LOH spanned an approximate distance of 30cM. The second (R2) and largest region of LOH identified was approximately 36cM in length on 4q22-q28. This region was defined by ROH in BICR18 at D4S1570 and BICR22 at D4S2981. The third region (R3) was on 4q34-q35 of approximately 9cM length. This region was defined by ROH in BICR68 at D4S3032 and in BICR68 at D4S2930.

From the analysis of our lines we observed complete ROH of chromosome 4 in 30% (3/10) of cases, BICR3, BICR19, and BICR63. In the immortal keratinocyte lines studied 70% (7/10) showed some degree of LOH on the endogenous chromosome copy. Of these lines 50% (5/10) showed LOH of R1, showed LOH of R2, and 60% (6/10) showed LOH of R3. There were no observable discrete areas of LOH on 4p with 30% (3/10) lines showing losses. In every case of 4p loss the entire 4q arm was also lost whereas 40% (4/10) lines with an intact p arm had deletions on the q arm.

From our analysis then we had identified three regions of LOH on our lines however we had no basis on which to distinguish which area was likely

Figure 3.2. Microsatellite marker D4S409 illustrates both ROH and LOH

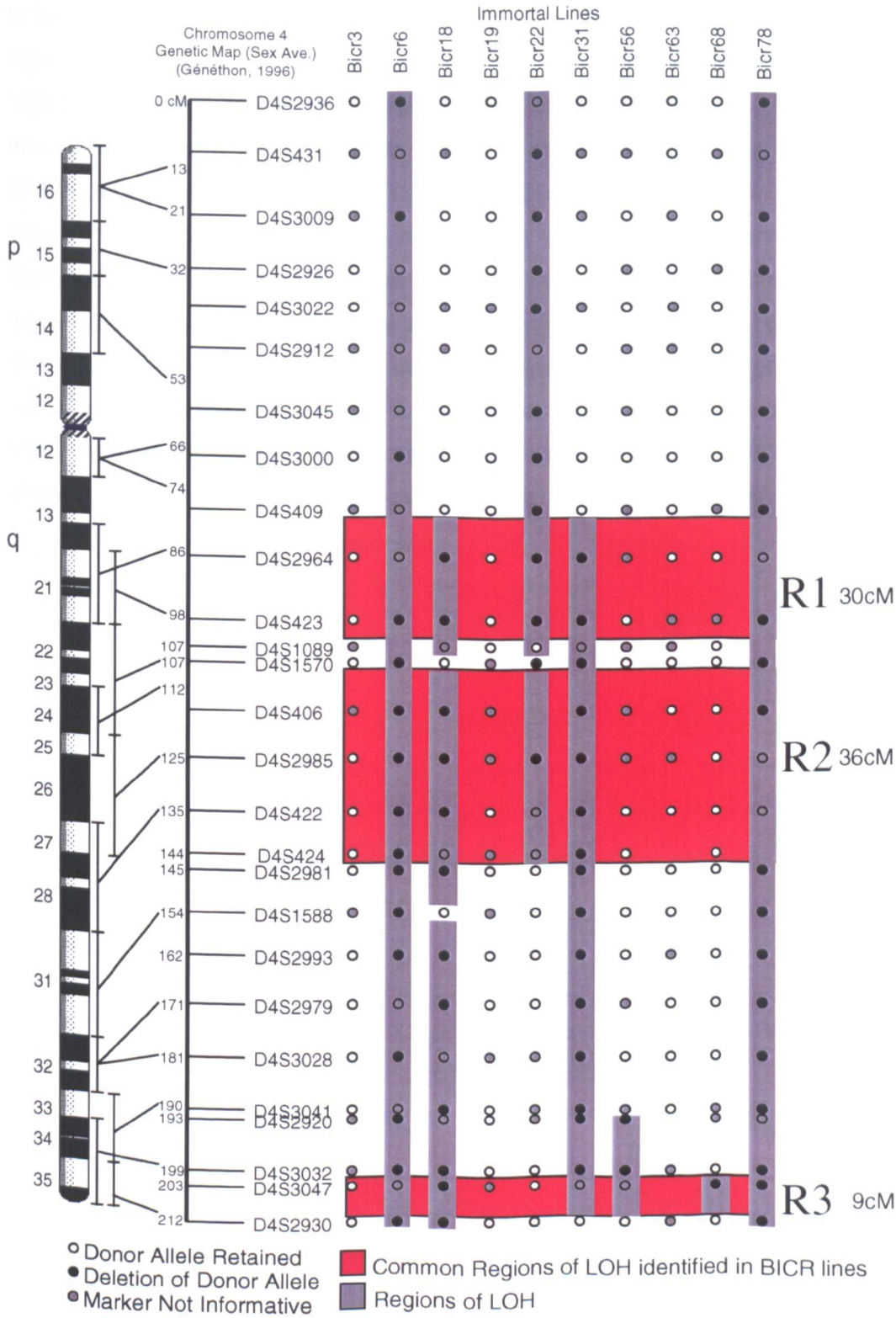
The image of D4S409 microsatellite PCR products following seperation on a 5% denaturing polyacrylamide gel (see Chapter 2, Section 2.4.5) serves to illustrate LOH and ROH. D4S409 delineates the upper margin of Region 1 through ROH of BICR 18 and BICR31. For each cell line the left hand band indicates normal patient fibroblast DNA whilst the right hand band indicates patient tumour DNA.



F = Fibroblast DNA
LOH = Loss of Heterozygosity
NI = Not Informative
ROH = Retention of Heterozygosity
T = Tumour DNA

Figure 3.3. LOH analysis of immortal HNSCC lines reveals three regions of loss

Microsatellite analysis of 10 immortal HNSCC-derived keratinocyte cell lines illustrates 3 common regions of loss (red boxes). Endogenous chromosome LOH is shown where it occurs for each immortal cell line (grey box). The microsatellite markers used, their approximate positioning (cM), and cytogenic positioning on a chromosome 4 ideogram are shown to the left of the LOH cartoon.


Immortal Lines
Chromosome 4
Genetic Map (Sex Ave.)
(Généthon, 1996)
Bicr3
Bicr6
Bicr18
Bicr19
Bicr22
Bicr31
Bicr56
Bicr63
Bicr68
Bicr78
0 cM
D4S2936
D4S431
D4S3009
D4S2926
D4S3022
D4S2912
D4S3045
D4S3000
D4S409
D4S2964
D4S423
D4S1089
D4S1570
D4S406
D4S2985
D4S422
D4S424
D4S2981
D4S1588
D4S2993
D4S2979
D4S3028
D4S3041
D4S2920
D4S3032
D4S3047
D4S2930
p
q
R1 30cM
R2 36cM
R3 9cM
Donor Allele Retained
Deletion of Donor Allele
Marker Not Informative
Common Regions of LOH identified in BICR lines
Regions of LOH

to be a good candidate for an intensive screen to identify any potential novel tsg on chromosome 4. Indeed our three identified regions agreed with every region previously identified in the studies described above. The more advanced a tumour the greater likelihood of observing increasing regions of loss through genomic instability. This instability would be reflected in random losses throughout the genome however in the regions, which we have identified, there is a repetitive loss occurring in a number of different tumour types increasing confidence in the identified regions. LOH analysis has been described as a useful source of circumstantial evidence whilst providing no formal proof for the presence of a tsg (Casey *et al.,* 1993). To extend our study then we decided to carry out MMCT experiments to identify both the phenotype and the location of the gene through segregation events.

Chapter 4

4.1) MMCT of Exogenous Chromosome 4 Induces Growth Arrest in Lines Exhibiting LOH on the Endogenous Copy

From the LOH study, which we undertook on our 10 HNSCC immortal keratinocyte lines, we were able to identify three regions on the endogenous chromosome as potential tsg sites. These regions also agreed with that previously discussed in the literature (see Chapter 3) so clearly we needed to select between our regions before launching a focused gene hunt. These identified regions also concur with observations previously made in the literature although without any bias towards any favoured locus. To distinguish between these three regions of LOH we used MMCT to ascertain whether any functional complementation would occur followed by the development of truncated chromosomes through XMMCT to determine where the complementing locus lay. We also hoped to develop loss maps from resultant hybrids to confirm our regional findings from above.

MMCT has been previously demonstrated to be of use in identifying genes lost in tumour progression in a number of studies through functional complementation of an inactivated copy on the endogenous chromosome by the introduced exogenous copy. This technique has been used to identify potential tsg's on Chromosome 1 (Yamada *et al.*, 1990; Sasaki *et al.*, 1994; Miele *et al.*, 1996), 1q (Hensler *et al.*, 1994, Karlsson *et al.*, 1996), 2 (Uejima *et al.*, 1995), 2p22-p25 (Mashimo *et al.*, 2000), 3p (Rimessi *et al.*, 1994, Tanaka *et al.*, 1998), 3p21-p22 (Killary *et al.*, 1992), 4 (Ning *et al.*, 1991), 6 (Yamada *et al.*, 1990; Welch *et al.*, 1994; Miele *et al.*, 1996)), 6q (Sandhu *et al.*, 1994), 6q14-q21 (Sandhu *et al.*, 1996), 6q16.3-q23 (Miele *et al.*, 2000), 7q (Ogata *et al.*, 1993), 8 (Gustafson *et al.*, 1996), 9 (Yamada *et al.*, 1990b), 9p21 (England *et al.*, 1996), 11 (Weissman *et al.*, 1987; Oshimura *et al.*, 1990), 11p15 (Dowdy *et al.*, 1990, Koi *et al.*, 1993), 17 (Casey *et al.*, 1993), 18 (Sasaki *et al.*, 1994), and X (Klein *et al.*, 1991, Wang *et al.*, 1992, Trott *et al.*, 1995).

The first evidence of a link between chromosome 4 and tumourigenicity was provided by cell-cell fusions between a fibrosarcoma cell line and normal fibroblasts (Benedict *et al.*, 1984) followed by injection into athymic mice. The reappearance of tumourigenicity in the mice correlated with the loss of chromosome 4. This was suggestive of chromosome 4 carrying a gene whose loss is beneficial to the progression of tumourigenesis. Further evidence was provided by the MMCT of chromosome 4 into a subset of cells (HeLa, J82, and T98G) causing the reversion of the immortal phenotype to a phenotype characteristic of replicative senescence (Ning *et al.*, 1991). This demonstrated that the functional complementation that was occurring was likely replacing a component in a commonly lost pathway enhancing the tumourigenic potential.

We used MMCT (see Chapter 2, Section 2.1.5) to introduce an exogenous copy of chromosome 4, which was resistant to both neomycin and hygromycin (McNeill and Brown, 1980; Santerre *et al.*, 1984; Saxon *et al.*, 1985; Ning *et al*, 1991; Cuthbert *et al*, 1995) into a preselected panel of our cell lines (see Table 4.1). Chromosome painting of the A92 donor murine line and the A9HYTK4 line with a single copy of chromosome 4 is shown in Figure 4.1. Dr. S. Dowen, Dept. of Pathology, University of Cambridge, and Mrs H Ireland, BICR undertook all chromosome painting described throughout. The exogenous chromosome 4 was tagged using an amphotropic pseudotype of the replication-defective retrovirus vector tgLS(+)HYTK, the HYTK is a fusion gene from the bacterial *hph* (conferring resistance to Hygromycin B, hygromycin phosphotransferase) and herpes simplex virus thymidine kinase (Lupton *et al.*, 1991, Trott *et al.*, 1995), followed by secondary tagging using LN(neo) retroviral infection (Newbold and Cuthbert, 1996). For our purposes in this instance we utilised the Hygromycin resistance marker for hybrid selection. The selected cell lines were matched for a number of characteristics genetically (see Table 4.1) but differed critically in whether they showed LOH (BICR6, BICR31) or ROH (BICR3, BICR19) of the endogenous chromosome 4 copy. We found that we observed a growth arrest like phenotype in BICR6 (39%, 24/62)) and additionally in the second

Table 4.1. Characteristics of cell lines used for MMCT

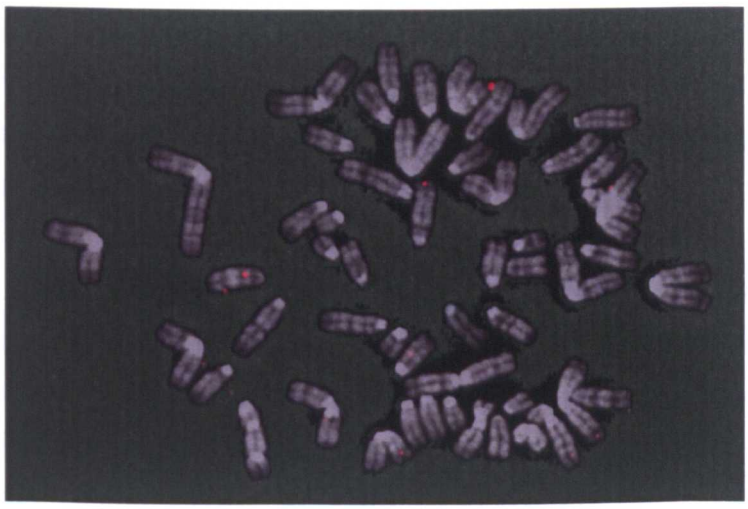
The criteria used for cell line selection are tabulated overleaf. These criteria are defined as the absence of *ras* mutations, the inactivation of $p16^{INK4A}$, the mutation of p53, consistent levels of EGFR number per cell, positive hTERT expression, LOH on 3p, and the presence or absence of LOH on 4q.

Cell Line Characteristic	BICR3	BICR6	BICR19	BICR31
Harvey *ras* mutation (Clark et al., 1993)	No	No	No	No
p16INK4A (Loughran et al., 1994)	Mutated	Deleted	Mutated	Deleted
p14ARF (Munro et al., 1999)	Yes	No	Yes	No
p53 (Burns et al., 1993)	Mutated	Mutated	Exon 10 Deleted	Mutated
EGFR number/cell (Stanton et al., 1994)	1.4×10^6	1.8×10^6	0.2×10^6	1.2×10^6
Telomerase (Loughran et al., 1994)	Positive	Positive	Positive	Positive
LOH on 3p (Loughran et al., 1997)	Yes	Yes	Yes	Yes
LOH on 4q (This Study)	No	Yes	No	Yes

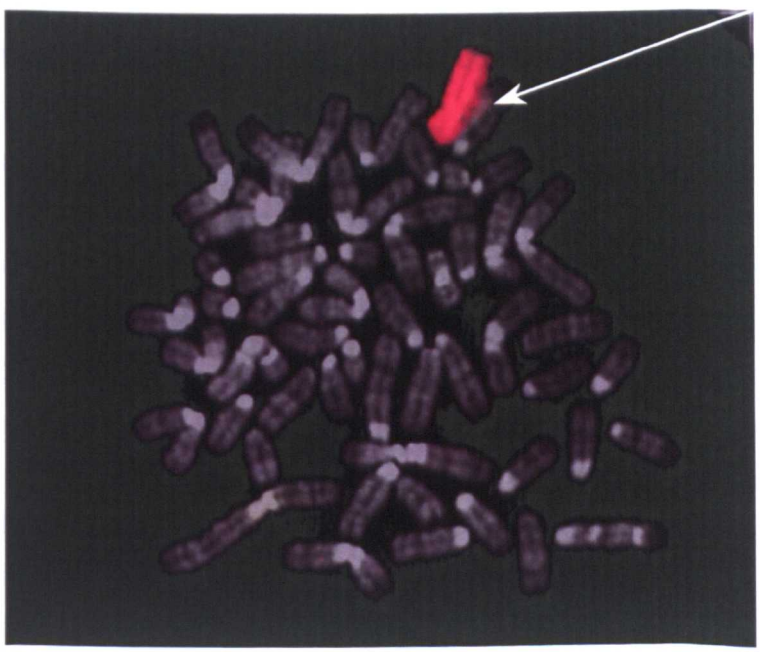
Figure 4.1. Chromosome painting images

Metaphase spreads of murine line A92 (a) which does not show hybridisation to human chromosome 4-specific paint, smaller red specks in this image are background staining rather than non-specific annealing. Metaphase spread of A9HYTK4 murine donor line with a single human chromosome 4 (b) illustrated by the annealing of the paint to the introduced contigous chromosomal element.

(a)



(b)



cell line BICR31 (27%, 3/11). We did not observe any form of growth arrest in either BICR3 (0%, 0/13) or BICR19 (0%, 0/6). These results suggested primarily that in agreement with the findings of Ning *et al.* (1991) we could ascribe a phenotype associated with chromosome 4 that was capable of reversing an immortal phenotype. These results are shown in tabular format in Table 4.2. We had also shown that that the chromosome 4-associated growth arrest phenotype did not occur in those lines with no LOH on the endogenous copy suggestive of a novel functional complementation occurring in those lines with LOH on 4q.

To determine the specificity of the phenotype we used MMCT to transfer a panel of chromosomes into the recipient cell lines BICR3, BICR6 and BICR31. We introduced chromosome 6 into BICR3 and BICR6, BICR19 and BICR31 were not suitable recipients as both display LOH on the endogenous chromosome 6 copy suggesting deletion of a gene advantageous to tumourigenesis. Transfer of chromosome 6 into BICR3 (0%, 0/1) and BICR6 (0%, 0/32, work also carried out by Dr. S. Fitzsimmons, BICR) resulted in no reversion of immortality demonstrating that the growth-arrest phenotype was specific to chromosome 4 and was not due to a non-specific drug-resistance effect. The low hybrid yield observed with BICR3 was indicative of the lines poor hybrid forming ability in agreement with the chromosome 4 transfers rather than a reduction in hybrid forming ability due to the donor chromosome. The further transfer of chromosomes 11 (0%, 0/4) and 15 (0%, 0/33, work also carried out by Dr. S. Fitzsimmons, BICR) into BICR6 resulted in no observable growth arrest further demonstrating the specificity of the phenotype to the donor chromosome 4 in complementing the loss on the endogenous 4q in BICR6. Transfer of chromosome 11 into BICR31 resulted in no observable growth-arrest demonstrating a specificity of phenotype to abrogated pathways. Transfer of chromosome 15 into BICR19 (0%, 0/1) and BICR31 (10%, 1/10) demonstrated a further growth arrest pathway potentially inactivated in BICR31 tumourigenesis but out-with the scope of this investigation. These results are shown in tabular format in Table 4.3.

Table 4.2. Transfer of Chromosome 4 into immortal HNSCC keratinocyte cell lines

The introduction of chromosome 4 into our preselected panel of immortal keratinocyte lines (see Table 4.1) demonstrates a specific growth-arrest phenotype related to a display of LOH on the endogenous 4q arm (BICR6 and BICR31). Immortal lines with apparently intact endogenous 4q arms do not display a phenotype (BICR3 and BICR19). The observed growth-arrest occurred in 27% and 39% of hybrids from BICR6 and BICR31 respectively.

Chromosome	Line	LOH on 4q	Growth-Arrested Hybrids	Total Growth-Arrested Hybrids (%)
A9HYTK4	BICR3	No	0/13	0%
A9HYTK4	BICR6	Yes	24/62	39%
A9HYTK4	BICR19	No	0/6	0%
A9HYTK4	BICR31	Yes	3/11	27%

Table 4.3. Transfer of control chromosomes into immortal HNSCC keratinocyte cell lines

Experimental controls to ascertain the specificity of the growth-arrest observed following introduction of an exogenous chromosome 4 in specific lines. Introduction of chromosomes 6, 11, and 15 does not illicit a growth-arrest response in BICR6 demonstrating the specificity orf the phenotype to chromosome 4 introduction. N.d.= Not defined.

Chromosome	Line	LOH on recipient chromosome	Growth-Arrested Hybrids	Total Growth-Arrested Hybrids (%)
A9HYTK6	BICR6	No	0/32	0%
A9HYTK6	BICR3	No	0/1	0%
A9HYTK11	BICR6	N.d.	0/4	0%
A9HYTK 15	BICR6	N.d.	0/33	0%
A9HYTK11	BICR31	N.d.	1/10	10%

4.2) XMMCT Generates Chromosomal Fragments Which Define a Phenotypic Locus

The principle employed in with XMMCT is the same as in MMCT with the exceptions being that the microcells are treated with low doses of X-irradiation before being fused back onto a suitable donor background (i.e. A92) (Dowdy *et al.,* 1990) (see Figure 4.2). The truncated chromosomes can then be characterised quickly using human specific microsatellite marker sequences and those suitable for transfer selected and introduced into the appropriate recipient using MMCT. This technique has been used successfully to identify the position of a metastasis suppressor on 2p22-p25 (Mashimo *et al.,* 2000), a telomerase repressor on 3p14.2-p21.1 (Tanaka *et al.,* 1998), a metastasis suppressor on 8p12-p21 (Nihei *et al.,* 1996) and a rhabdomyosarcoma specific senescent growth arrest to 11p15 (Koi *et al.,* 1993).

To generate truncated chromosome 4 copies we used a dose exposure range of 50 – 200 rads. Previous attempts using higher doses (300-600 rads) resulted in a shattering of the human chromosome 4 suggesting a lower dose requirement. From the panel of truncated hybrids wide ranges of deletions were observed ranging from small interstitial deletions to whole chromosome arm losses. The hybrids were screened using microsatellite markers by Dr. S. Bryce, BICR, and the results are shown in Figure 4.3. In this panel there were four hybrids that would allow us to determine which of our characterised regions were responsible for the phenotype. These fragments are shown in Figure 4.4. MMCT of fragment A92i24 into BICR6 would rule out any locus on 4p. Fragment A92i30 would rule out Regions 2 and 3 if we observed a phenotype following transfer into BICR6, if not it would rule out Region 1. No phenotype following transfer with A92i32 into BICR6 would rule out Regions 1 and 2 whilst ruling in Region 3. The smallest selected fragment, A92i33, with retention of activity would rule out Region 3 and the telomeric end of Region 2 whilst ruling in the remaining regions. Chromosome fragment painting was performed by Dr. S. Dowen to confirm the fragments were not recombined

Figure 4.2. X-Irradiated microcell-mediated monochromosome transfer

In XMMCT the microcells are generated in the manner described previously (Chapter 2, Section 2.1.5). Prior to fusion back onto the mouse background (A92) the microcells are treated with low doses of radiation to induce double-strand breaks and truncated chromosomes.

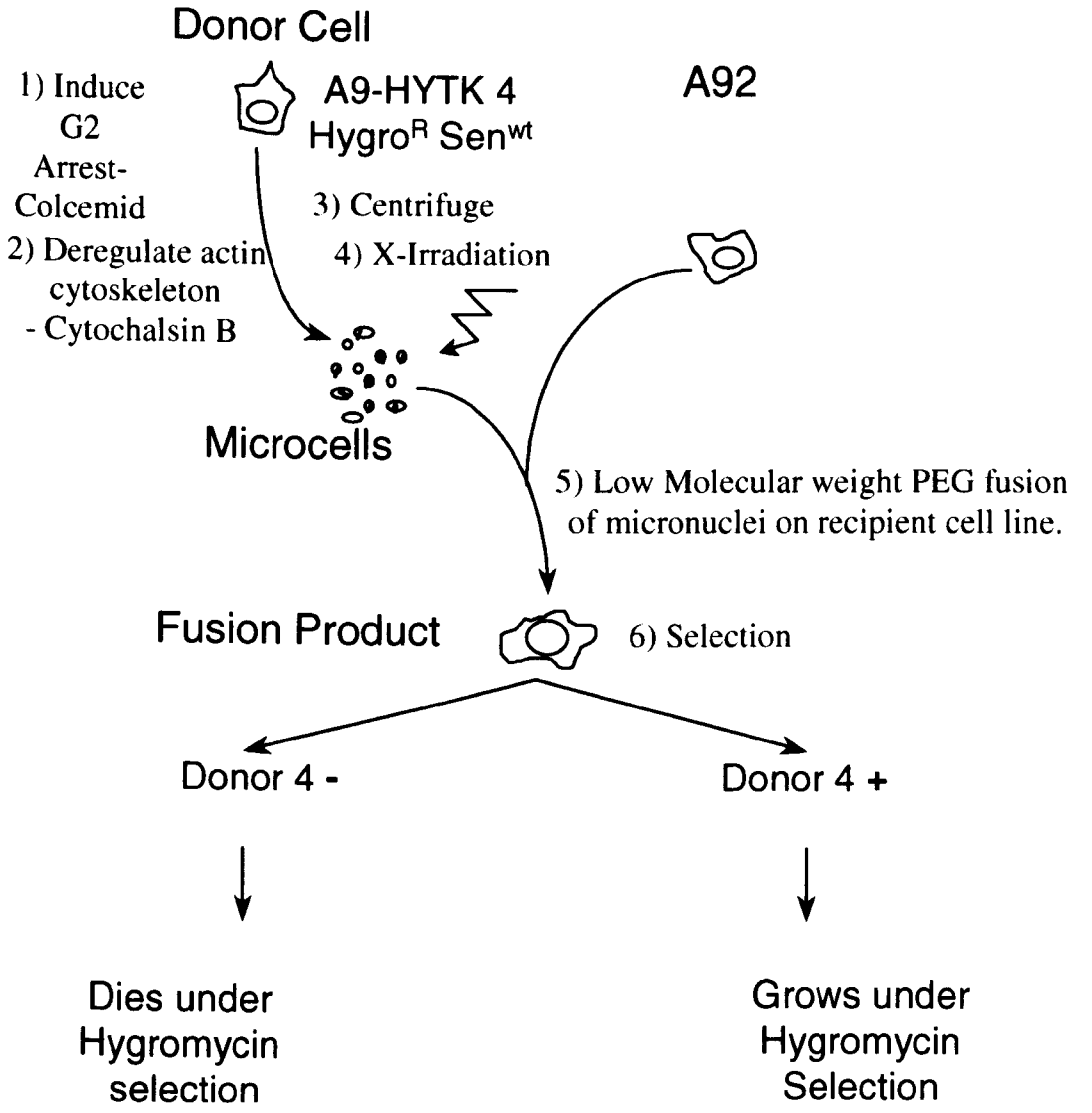

Donor Cell
1) Induce G2 Arrest- Colcemid
2) Deregulate actin cytoskeleton - Cytochalsin B
A9-HYTK 4
Hygro^R Sen^wt
A92
3) Centrifuge
4) X-Irradiation
Microcells
5) Low Molecular weight PEG fusion of micronuclei on recipient cell line.
Fusion Product
6) Selection
Donor 4 -
Donor 4 +
Dies under Hygromycin selection
Grows under Hygromycin Selection

Figure 4.3. Panel of irradiated hybrids generated by XMMCT

Illustration of the range of truncated chromosome 4 copies generated by the XMMCT technique (see Figure 4.2). Regions lost are shown in grey boxes, regions identified in Chapter 3 are shown in red boxes. The microsatellite markers used, their approximate positioning (cM), and cytogenic positioning on a chromosome 4 ideogram are shown to the left of the LOH cartoon.

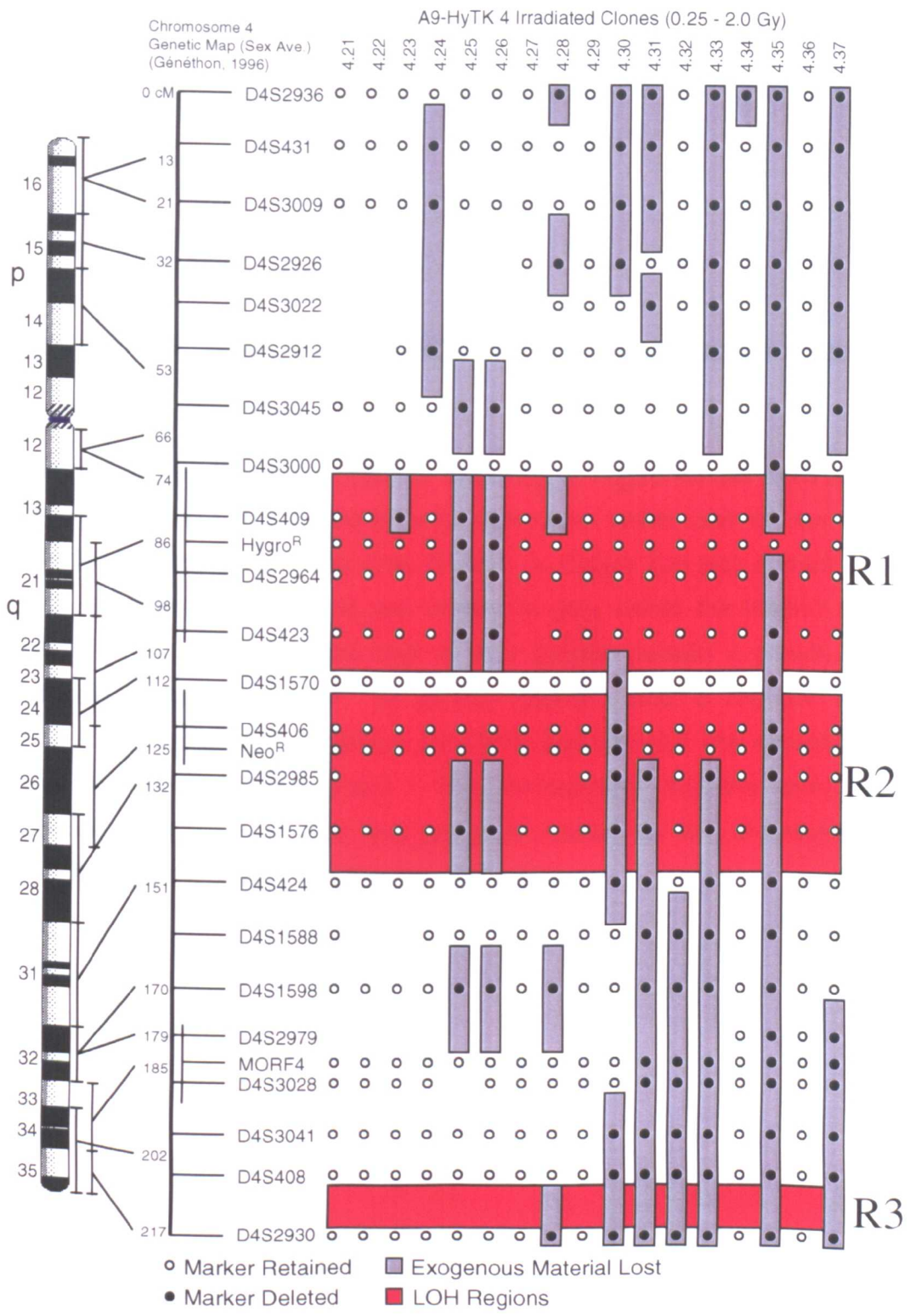
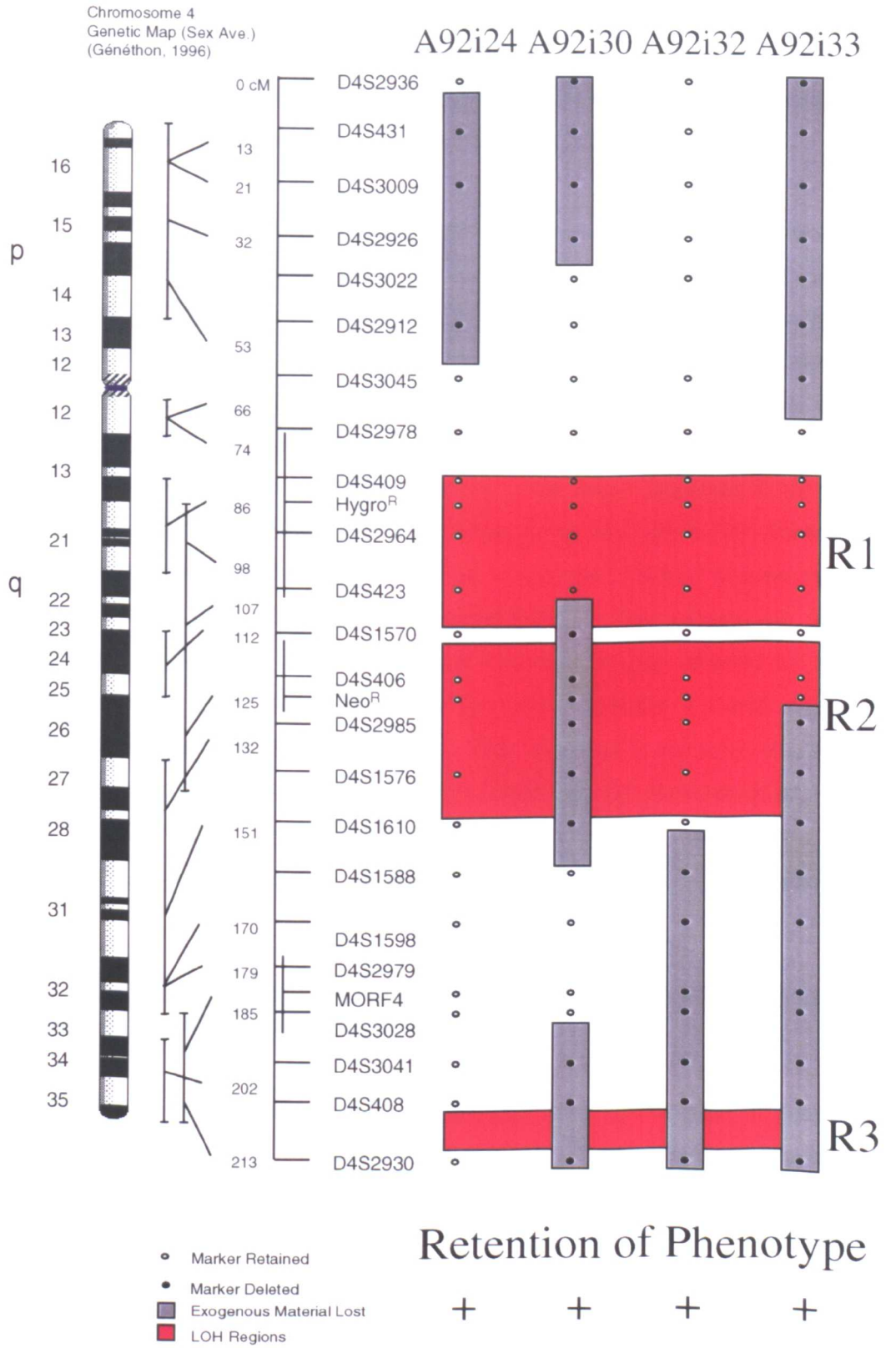

A9-HyTK 4 Irradiated Clones (0.25 - 2.0 Gy)
Chromosome 4
Genetic Map (Sex Ave.)
(Généthon, 1996)
4.21
4.22
4.23
4.24
4.25
4.26
4.27
4.28
4.29
4.30
4.31
4.32
4.33
4.34
4.35
4.36
4.37
0 cM
D4S2936
D4S431
D4S3009
D4S2926
D4S3022
D4S2912
D4S3045
D4S3000
D4S409
Hygro^R
D4S2964
D4S423
D4S1570
D4S406
Neo^R
D4S2985
D4S1576
D4S424
D4S1588
D4S1598
D4S2979
MORF4
D4S3028
D4S3041
D4S408
D4S2930
13
21
32
53
66
74
86
98
107
112
125
132
151
170
179
185
202
217
p
q
16
15
14
13
12
12
13
21
22
23
24
25
26
27
28
31
32
33
34
35
R1
R2
R3
Marker Retained
Marker Deleted
Exogenous Material Lost
LOH Regions

Figure 4.4. Selected A92HYTK4 irradiated clones (0.25 - 2.0 Gy)

From the panel of truncated hybrids illustrated in Figure 4.3 we were able to select four which would functionally determine which one of our three identified regions (red boxes) (see Chapter 3, Figure 3.3) was correct. The areas of chromosome 4 loss are shown by grey boxes the legend at the bottom of the page indicates that all four hybrids caused a growth-arrest phenotype following transfer. As all four hybrids shared a common region defined by microsatellite markers D4S2978 and D4S423 the growth-arrest locus should lie in this region. The microsatellite markers used, their approximate positioning (cM), and cytogenic positioning on a chromosome 4 ideogram are shown to the left of the LOH cartoon.


Chromosome 4
Genetic Map (Sex Ave.)
(Généthon, 1996)
A92i24 A92i30 A92i32 A92i33
p
q
16
15
14
13
12
12
13
21
22
23
24
25
26
27
28
31
32
33
34
35
0 cM
13
21
32
53
66
74
86
98
107
112
125
132
151
170
179
185
202
213
D4S2936
D4S431
D4S3009
D4S2926
D4S3022
D4S2912
D4S3045
D4S2978
D4S409
Hygro^R
D4S2964
D4S423
D4S1570
D4S406
Neo^R
D4S2985
D4S1576
D4S1610
D4S1588
D4S1598
D4S2979
MORF4
D4S3028
D4S3041
D4S408
D4S2930
R1
R2
R3
Marker Retained
Marker Deleted
Exogenous Material Lost
LOH Regions
Retention of Phenotype
+ + + +

with mouse material (see Chapter 2, Section 2.2.4 for method). This revealed that with the exception of A92i32 all truncated copies of chromosome 4 were maintained stably on the murine background, A92i32 had recombined with mouse material. The painting is shown in Figure 4.5 and Figure 4.6.

Transfer of our selected panel of truncated chromosomes was performed with the recipient BICR6. The results of this series of transfers are displayed in Table 4.4. Every fragment tested demonstrated a growth-arrested phenotype, at a similar ratio of 33%-50% of hybrids, and due to a shared minimal common region this enabled us to define our minimal region. In each instance an intact chromosome 4 was introduced simultaneously into a separate recipient and the growth arrest ratios were observed to be similar to the fragments. The result with A92i32 supports the conclusion drawn from the other fragments although due to the presence of fused mouse material caution should be exercised in interpretation. The phenotype will be examined in detail in Chapter 5. The common region shared by all our fragments was found within Region 1 and encompasses a 22cM region on 4q21 defined by D4S409 and D4S423. This region is in agreement with LOH studies carried out on EA (Rumpel *et al.,* 1999), ESCC (Rumpel *et al.,* 1999), HCC (Rashid *et al.,* 1999, Bando *et al.,* 1999), HNSCC (Pershouse *et al.,* 1997), SCLC (Petersen *et al.,* 2000), and sclerotylosis (Lee *et al.,* 2000).

4.3) Allelic Pattern Loss in Immortal Segregant Hybrids Define Minimal Region Centred on D4S423

A recognized feature of the MMCT technique is the generation of deleted or rearranged exogenous chromosomes (Leach *et al.,* 1989; Newbold and Cuthbert, 1996; Zhu *et al.,* 1998). In monochromosome hybrids which do not demonstrate the functionally complementated phenotype these segregated hybrids can be used to map which regions of the exogenous chromosome are missing and therefore where the complementing locus may

Figure 4.5. Chromosome painting of XMMCT generated truncated fragments A9i24 and A9i30

Metaphase spreads of A9i24 (a) and A9i30 (b) were hybridised to a human chromosome 4-specific paint to highlight the presence of independent, contiguous chromosomal elements. Figure 4.5 (b) has some degree of background hybridisation although this does not correspond to DAPI stained regions and is not therefore chromosomal material. In each instance the arrows indicate the truncated human chromosome 4 in the DAPI and painted image.

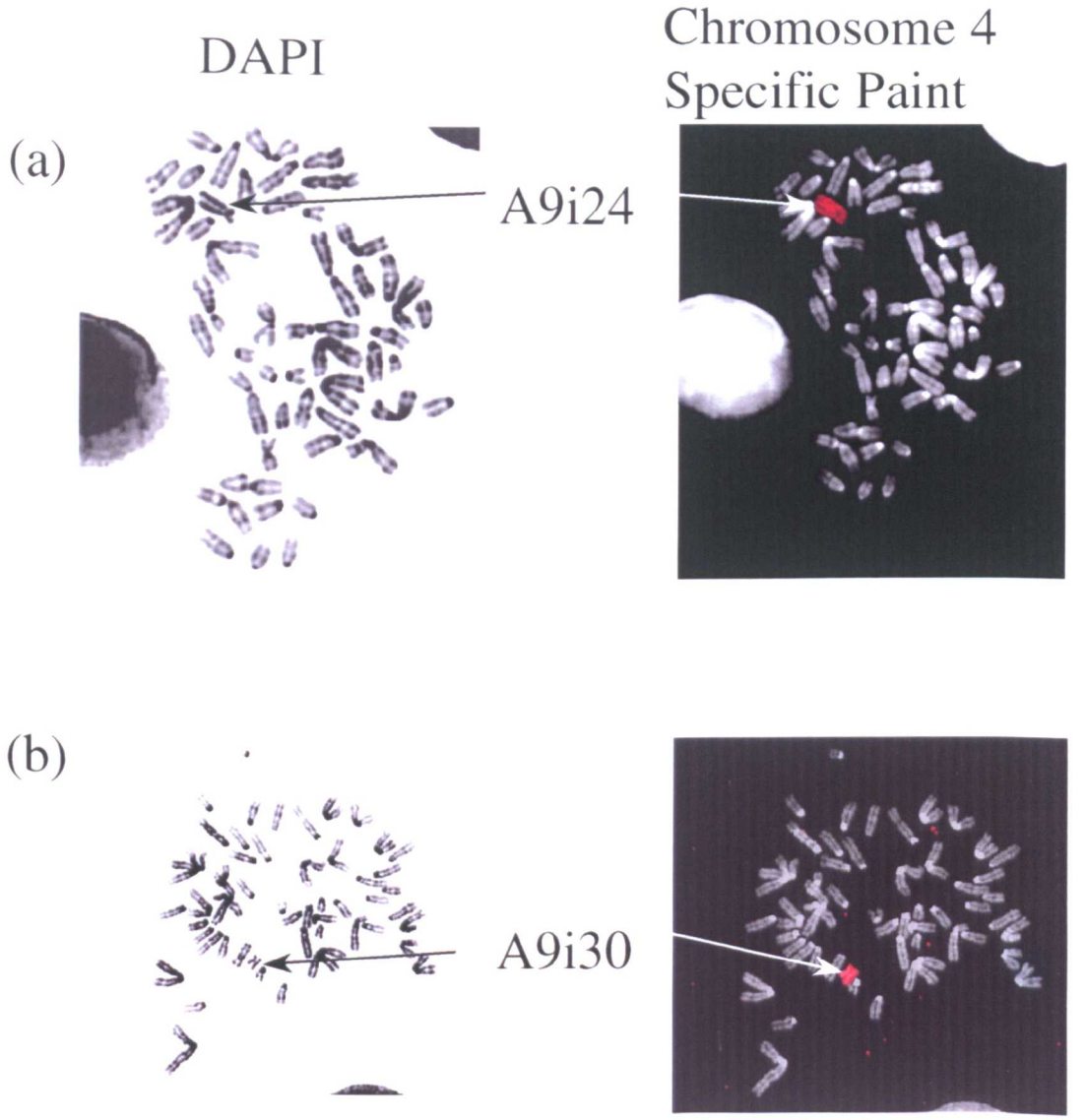

DAPI
Chromosome 4
Specific Paint
(a)
A9i24
(b)
A9i30

Figure 4.6. Chromosome painting of XMMCT generated truncated fragments A9i32 and A9i33

Metaphase spreads of A9i32 (a) and A9i33 (b) were hybridised to a human chromosome 4-specific paint to highlight the presence of independent, contiguous chromosomal elements. Figure 4.6 (a) illustrates that the human chromosome 4 fragment has recombined with a mouse telomere. In each instance the arrows indicate the truncated human chromosome 4 in the DAPI and painted image.

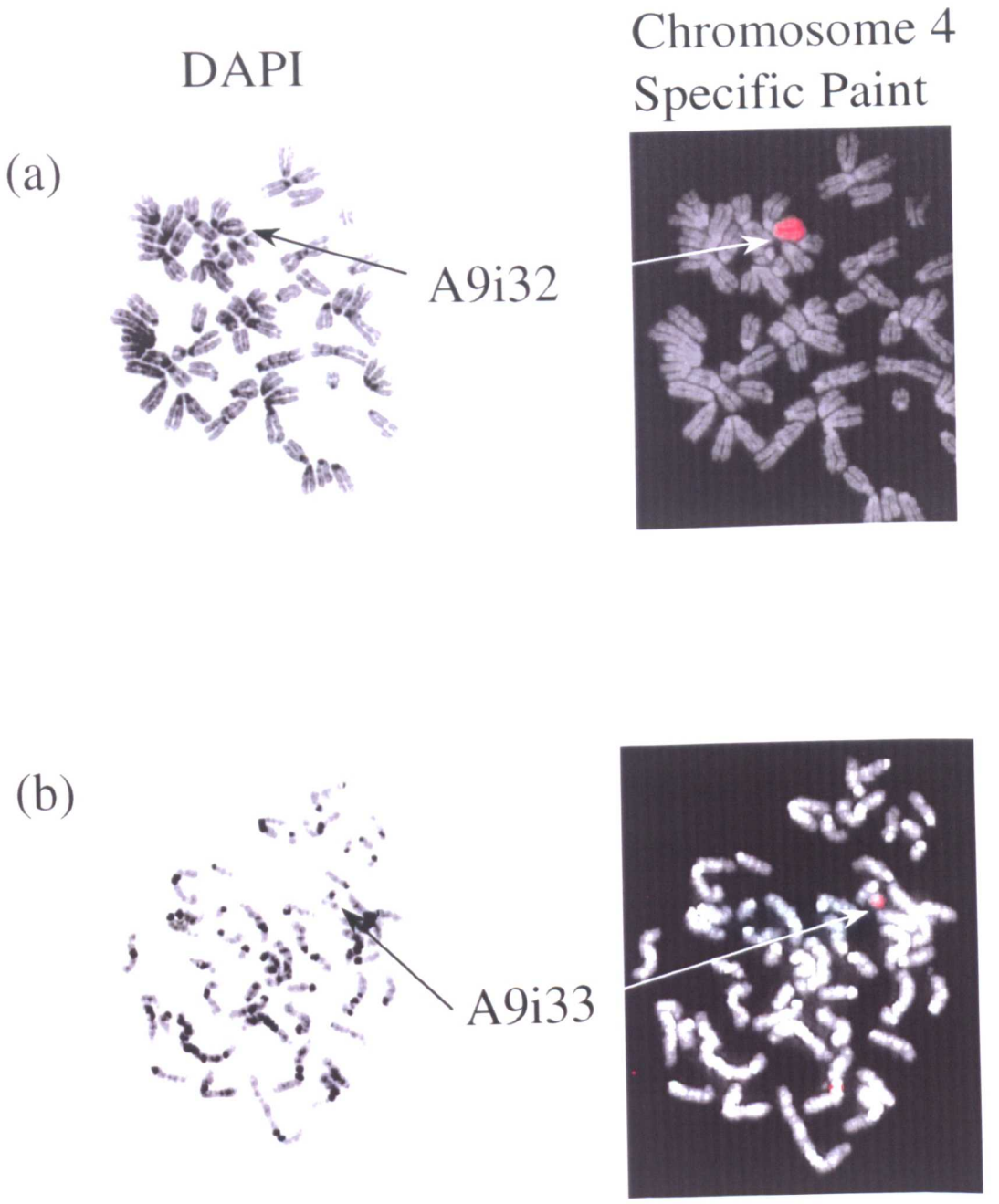

DAPI
Chromosome 4
Specific Paint
(a)
A9i32
(b)
A9i33

Table 4.4. Results from chromosome 4 fragment MMCT experiments

Transfer of the panel of truncated chromosomes identified in Figure 4.4 demonstrates retention of the growth-arrest phenotype in all instances. This indicates that the region of common retention spanning D4S2978 to D4S423 retains the growth-arrest phenotype. The observed phenotype occurs in the range of 33%-50%, this similar value to that seen in Table 4.2 suggests the growth-arrest mechanisms are the same.

Fragment	Recipient	Hybrids	Growth-Arrested Hybrids	Total Growth-Arrested Hybrids (%)
A9i32	BICR6	17	7	41.2%
Control	BICR6	16	6	37.5%
A9i24	BICR6	30	15	50%
Control	BICR6	26	7	27%
A9i33	BICR6	22	9	40.9
Control	BICR6	33	13	39.3%
A9i30	BICR6	30	10	33%
Control	BICR6	41	8	19.5%

lie. This approach has led to the successful confirmation of p16INK4A at 9p21 (England *et al,* 1996), SURF1 at 9p34 (Zhu *et al.,* 1998), a potential tsg on 3p (Rimessi *et al.,* 1994), a genetic locus at 3p21-p22 involved in tumour suppression (Killary *et al.,* 1992), a senescence-associated gene on 6q14-q21 (Sandhu *et al.,* 1996), a metastasis suppressor on 6q16.3-q23 (Miele *et al.,* 2000), a metastasis suppressor on 8p12-p21 (Nihei *et al.,* 1996) and a tsg on 8p22-p23 (Gustafson *et al.,* 1996).

To take advantage of this feature of the MMCT technique we opted to analyse the segregants from which we had recovered genomic DNA using the panel of polymorphic microsatellite markers described in Chapter 2 to give a general 10cM spacing across the whole chromosome span. In BICR6 where we had lost an entire chromosome copy we would expect a high proportion of markers to be informative due to the homozygous nature of the endogenous copy. In each instance we PCR amplified the microsatellite in the A92 murine line (Cuthbert *et al.,* 1995), the A9HYTK4 donor line (Lupton *et al.,* 1991, Cuthbert *et al.,* 1995), the respective BICR line, and H_2O as a blank. The microsatellite PCR methodology is described in Chapter 2, Section 2.4.4. Completed reactions were loaded onto and run on polyacrylamide gels until the bands resolved: they were then visualized using autoradiography (see Chapter 2, Section 2.4.5).

To determine which hybrids were segregants, hybrids which demonstrated partial exogenous allele loss, we carried out a microsatellite screen across this minimal region in hybrids resulting from transfers with A92i24, i32, and i33 using the markers D4S2978, D4S409, D4S2964, D4S423, and D4S1570. The Microsatellite PCR and gel electrophoresis used to resolve the polymorphic alleles are described in Chapter 2. The hybrids resulting from the smallest fragment transfer, BICR6/4i33 showed that intact hybrids accounted for 11% (2/19) of the total whilst 63% (12/19) of hybrids had deleted all exogenous material, 26% (5/19) displayed segregation of the introduced chromosome fragment in the minimal region tested. Hybrids resulting from transfers with A92i24 demonstrated 73% (16/22) of all hybrids were intact, 5% (1/22) had deleted all material and the remaining 22% (5/22)

had segregated exogenous material. From this screen we determined that 64% (9/14) of BICR6/4i32 hybrids had retained all exogenous material across this region, 21% (3/14) of hybrids had deleted all material with a remaining 15% (2/14) displaying segregation of exogenous material in the minimal region. Characteristically, the larger fragments displayed a greater propensity to remain intact (A92i24) than the smaller fragments (A92i33), which is a likely reflection of chromosomal stability through the transfer procedure. The segregated hybrids are shown in Figure 4.7. A strikingly high percentage of these segregants had lost D4S423 as shown in Figure 4.8, which is suggestive of a locus in this area.

Analysis of BICR6/Chromosome 4 immortal hybrids revealed that 23% (8/35) had lost all exogenous material with the exception of the Hygromycin resistance marker. A further 46% (16/35) of immortal hybrids revealed a completely intact exogenous chromosome 4 copy, however when a panel of these were cultured without the lethally irradiated feeder (see Chapter 2) 86% (6/7) underwent growth arrest contrasted with 0% (0/5) immortal segregants cultured under the same conditions (Figure 4.9). This indicates that the minimal requirement for the growth-arrest phenotype is an intact exogenous chromosome 4 although the growth-arrest phenotype can be overcome by other mechanisms, which could be related to culture conditions or epigenetic mechanisms such as promoter methylation. Chromosome painting of a BICR6/Chromosome 4 hybrid with an intact exogenous chromosome as judged by microsatellite analysis was demonstrated to have an intact solitary extra copy of Chromosome 4 through painting on metaphase spreads, as shown in Figure 4.10. This was suggestive of a favourable culture system enabling epigenetic silencing of the locus responsible for the growth arrest. A total of 31.5% (11/35) of immortal hybrids revealed under microsatellite analysis that they had segregated exogenous chromosome 4 material, either *in situ* or as an artefact of the experimental system. These segregants are shown diagrammatically in Figure 4.11 where for added clarity the three regions previously identified in our LOH study are shown. The ratio of marker loss in the minimally identified region for all BICR6/chromosome 4 hybrids is shown in Figure 4.12. Observable losses are not as high as those seen with

Figure 4.7. Microsatellite analysis of fragment segregants

Microsatellite analysis of immortal segregants arising from MMCT of truncated fragments into BICR6 (see Table 4.4). The minimal region of functional complementation identified in Figure 4.4 demonstrates a high frequency of loss at D4S423. The red box indicates Region 1, identified in Chapter 3, which is the minimal region of functional complementation. Grey boxes indicate regions of exogenous chromosome loss. Microsatellite marker D4S409 displays retention in 100% of segregants analysed indicating its proximity to the exogenous chromosome Hygromycin resistance cassette.

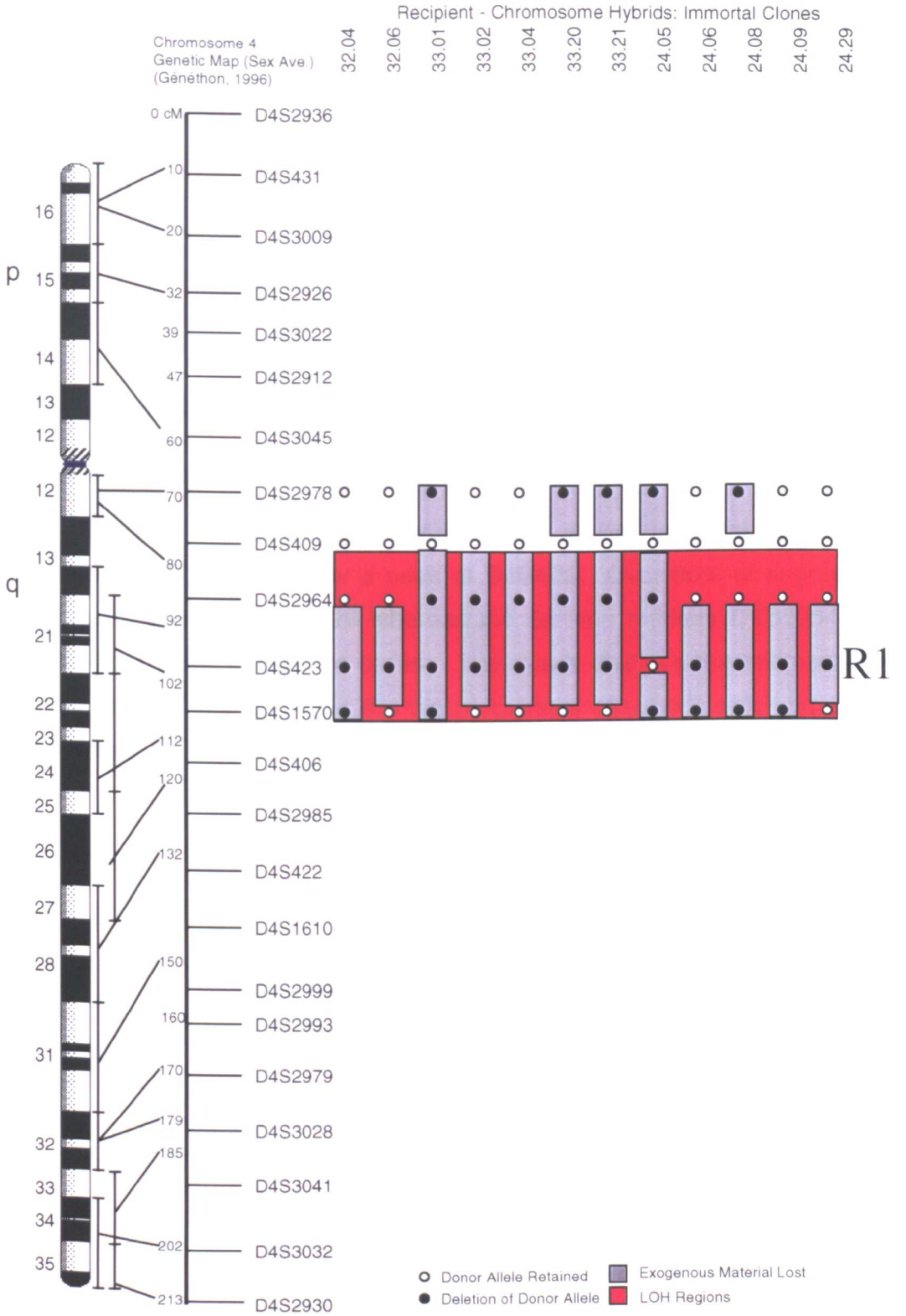

Recipient - Chromosome Hybrids: Immortal Clones
32.04
32.06
33.01
33.02
33.04
33.20
33.21
24.05
24.06
24.08
24.09
24.29
Chromosome 4
Genetic Map (Sex Ave.)
(Généthon, 1996)
0 cM
D4S2936
10
D4S431
16
20
D4S3009
p
15
32
D4S2926
39
D4S3022
14
47
D4S2912
13
12
60
D4S3045
12
70
D4S2978
D4S409
13
80
q
D4S2964
92
21
D4S423
102
R1
22
D4S1570
23
112
D4S406
24
120
25
D4S2985
26
132
D4S422
27
D4S1610
28
150
D4S2999
160
D4S2993
31
170
D4S2979
179
D4S3028
32
185
33
D4S3041
34
202
D4S3032
35
213
D4S2930
Donor Allele Retained
Deletion of Donor Allele
Exogenous Material Lost
LOH Regions

Figure 4.8. Microsatellite marker loss suggests locus close to D4S423

The frequency of microsatellite losses seen in the immortal segregants described in Figure 4.7 show a peak at D4S423. Over 90% of segregants have lost the marker D4S423 whereas the flanking markers D4S2964 and D4S1570 are lost in 50% of cases. The x-axis displays the microsatellite marker name, the y-axis indicates the percentage of hybrids which have lost the marker.

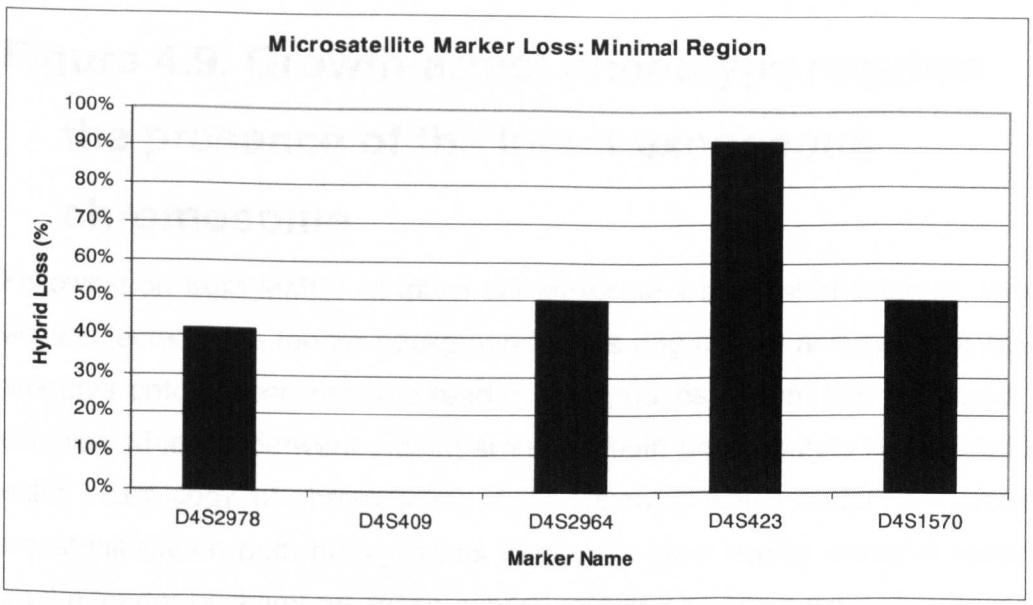

Microsatellite Marker Loss: Minimal Region
Hybrid Loss (%)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
D4S2978
D4S409
D4S2964
D4S423
D4S1570
Marker Name

Figure 4.9. Growth-arrest phenotype requires the presence of the intact exogenous chromosome

Following on from MMCT of intact chromosome 4 a panel of colonies, which were selected on a feeder background, were ring cloned and split into equal amounts onto feeder free and feeder backgrounds. From this we found; 1) colonies which underwent growth-arrest on both backgrounds possessed an extra intact copy of chromosome 4, 2) Colonies which underwent growth-arrest initially on both backgrounds frequently gave rise to immortal variants on the feeder background, these hybrids retained an intact extra chromosome 4 copy, and 3) Colonies which formed immortal variants had either lost the entire exogenous chromosome 4, had undergone segregation of the exogenous chromosome 4 material, or in some instances had also retained an intact exogenous chromosome 4 copy. This is suggestive of an ability to silence the locus responsible for the growth-arrest phenotype on the feeder background but not in the absence of the feeder layer. This indicates a likelihood of a complex mechanism behind the observed phenotype

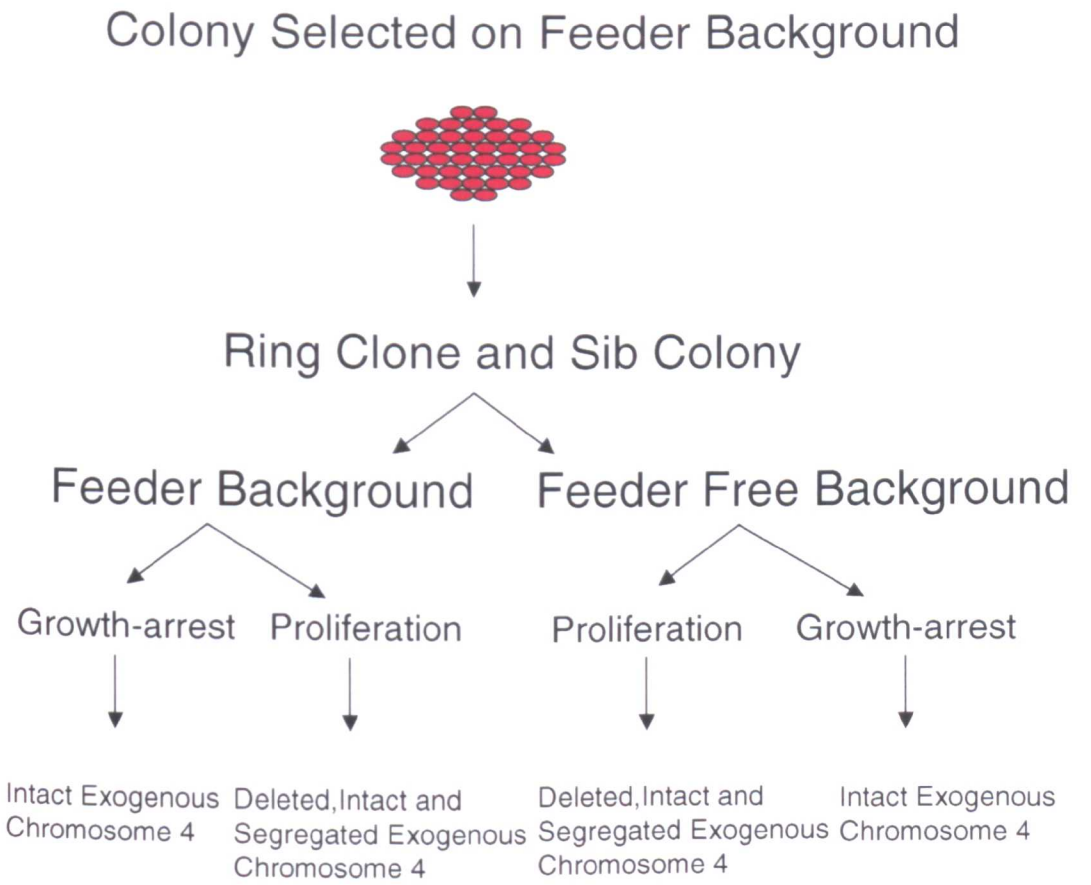
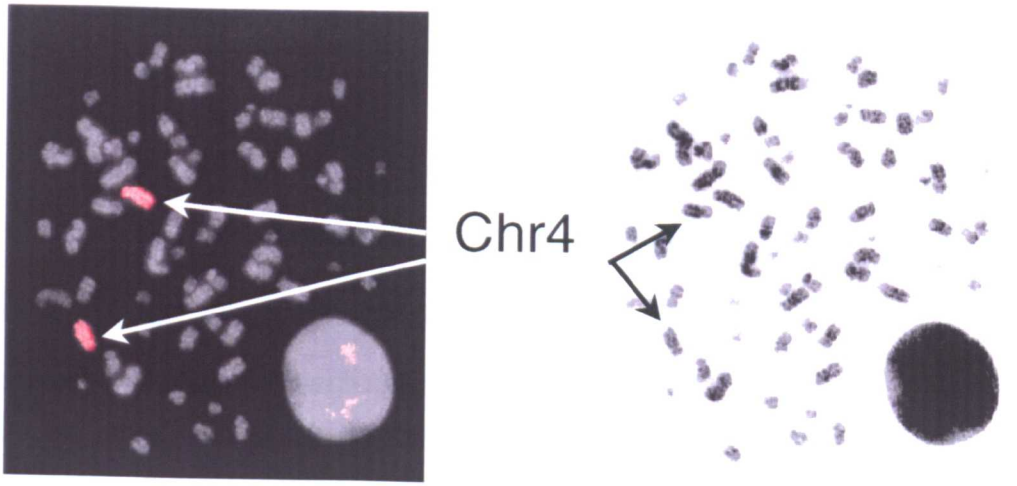

Colony Selected on Feeder Background
Ring Clone and Sib Colony
Feeder Background
Feeder Free Background
Growth-arrest
Proliferation
Proliferation
Growth-arrest
Intact Exogenous Chromosome 4
Deleted,Intact and Segregated Exogenous Chromosome 4
Deleted,Intact and Segregated Exogenous Chromosome 4
Intact Exogenous Chromosome 4

Figure 4.10. Chromosome painting reveals an intact extra copy of chromosome 4 in BICR6/chromosome 4 hybrid

(a) Metaphase spreads of the immortal BICR6 cell line hybridised with a chromosome 4-specific paint illustrate the presence of two endogenous chromosome 4 copies. The unspread nuclei in the bottom right hand of the diagram also indicates what may be two endogenous copies of chromosome 4. (b) The metaphase spread of a BICR6/Chromosome 4 hybrid displays an extra copy of chromosome 4, this was also confirmed using microsatellite marker analysis (data not shown). Arrows indicate chromosome 4 copies in both painted and DAPI metaphase images.

(a)



(b)

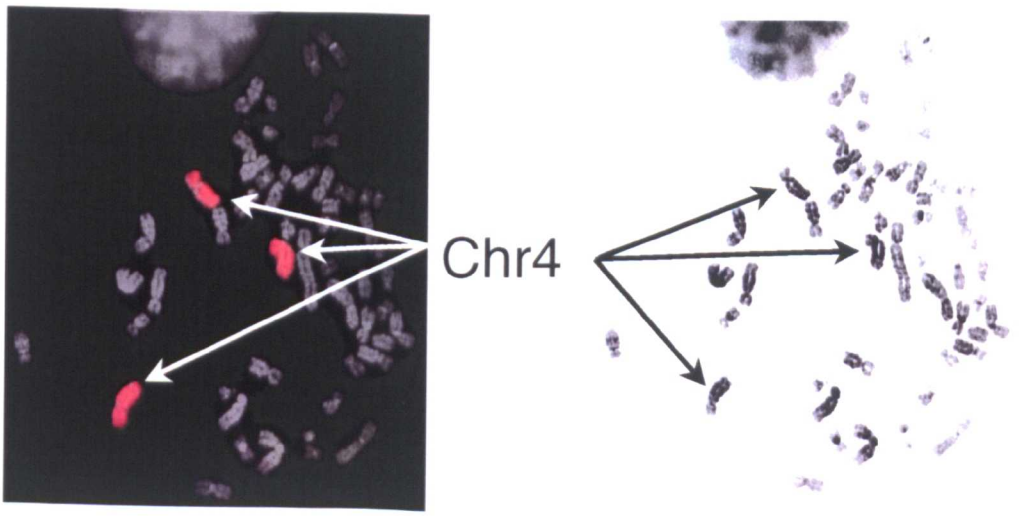


Figure 4.11. Exogenous material loss in BICR6/chromosome 4 segregants

Microsatellite analysis of segregants arising from MMCT of chromosome 4 into BICR6, show losses across the whole exogenous chromosome. Losses of exogenous chromosomal material (grey boxes) occur in all three regions identified in Chapter 3 (red boxes). Hybrid number is listed along the top axis. The microsatellite markers used, their approximate positioning (cM), and cytogenic positioning on a chromosome 4 ideogram are shown to the left of the LOH cartoon.

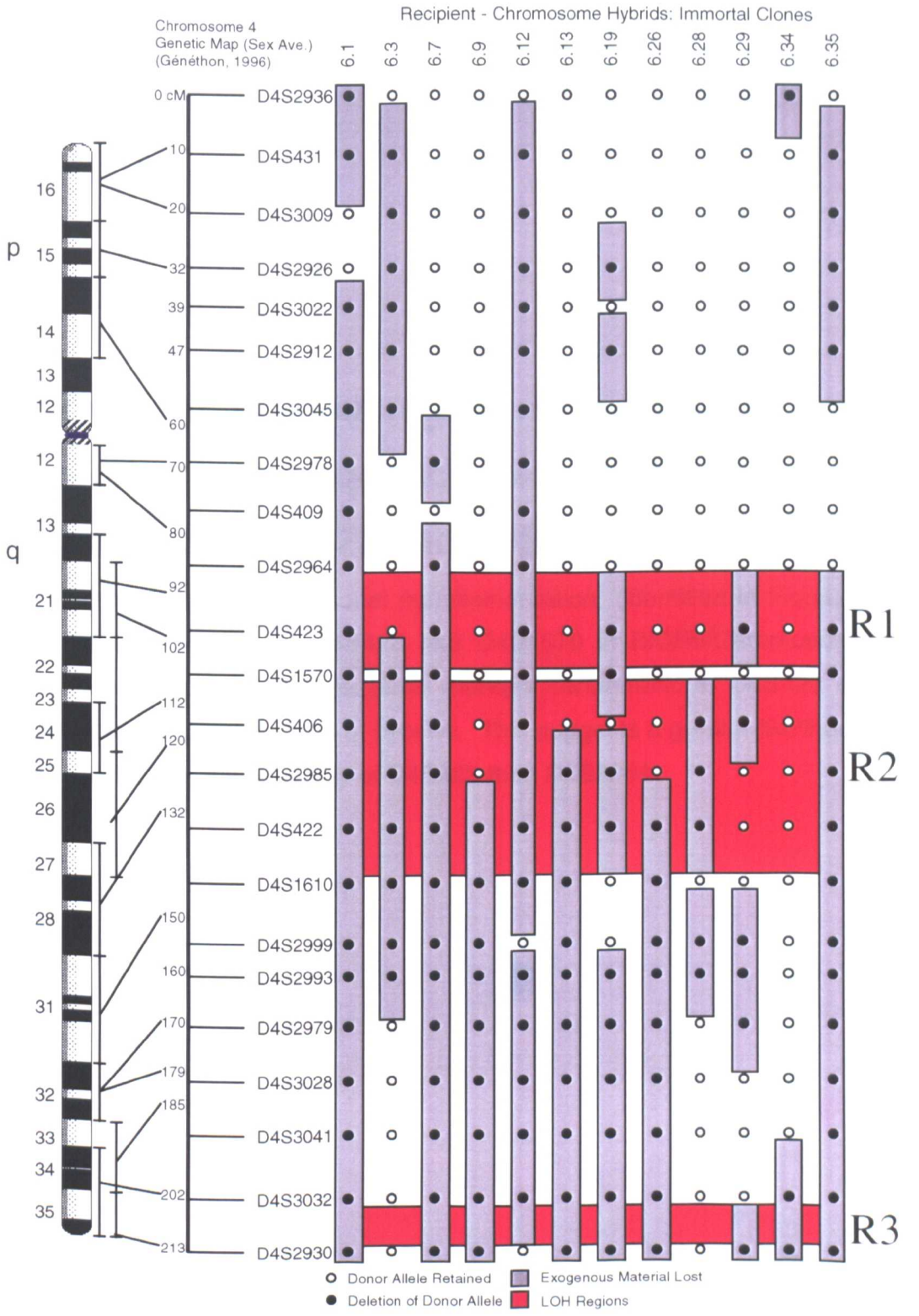
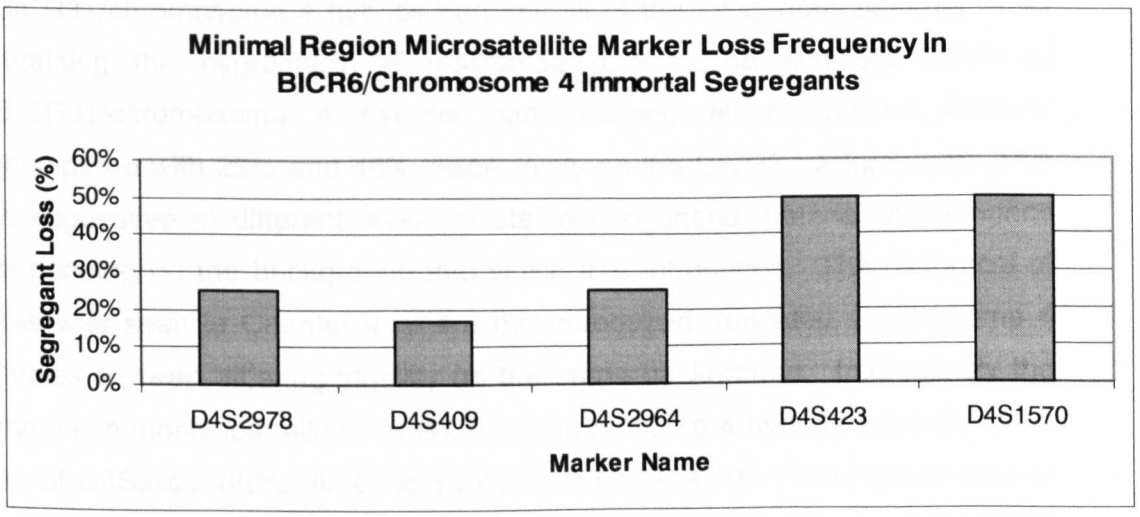

Recipient - Chromosome Hybrids: Immortal Clones
Chromosome 4
Genetic Map (Sex Ave.)
(Généthon, 1996)
6.1
6.3
6.7
6.9
6.12
6.13
6.19
6.26
6.28
6.29
6.34
6.35
0 cM
10
20
32
39
47
60
70
80
92
102
112
120
132
150
160
170
179
185
202
213
D4S2936
D4S431
D4S3009
D4S2926
D4S3022
D4S2912
D4S3045
D4S2978
D4S409
D4S2964
D4S423
D4S1570
D4S406
D4S2985
D4S422
D4S1610
D4S2999
D4S2993
D4S2979
D4S3028
D4S3041
D4S3032
D4S2930
p
q
16
15
14
13
12
12
13
21
22
23
24
25
26
27
28
31
32
33
34
35
R1
R2
R3
Donor Allele Retained
Deletion of Donor Allele
Exogenous Material Lost
LOH Regions

Figure 4.12. Minimal region microsatellite marker loss frequencies suggest region spanning D4S423 and D4S1570

The minimal region of functional complementation, (identified in Figure 4.4) show losses of 50% for D4S423 and D4S1570 in BICR6/Chromosome 4 immortal segregants. Microsatellite markers centromeric to D4S423 show losses in approximately 25% of hybrids. This suggests a greater likelihood of the locus lying in the proximity of D4S423 than D4S2964.


Minimal Region Microsatellite Marker Loss Frequency In BICR6/Chromosome 4 Immortal Segregants
Segregant Loss (%)
60%
50%
40%
30%
20%
10%
0%
D4S2978
D4S409
D4S2964
D4S423
D4S1570
Marker Name

the fragment hybrids in the minimal region although this does not rule out microdeletions or gene silencing though other epigenetic mechanisms.

Consequently we examined the BICR31/chromosome 4 hybrids to determine whether their status matched that seen previously in Chapter 4 and that seen in the BICR6/chromosome 4 hybrid panel. BICR31, as was shown in Chapter 3, has ROH on the short p arm whilst showing LOH on the majority of the long q arm from D4S409 at 4q13 to D4S2930 at the telomeric end of 4q35. Unlike the BICR6/chromosome 4 examination none of the BICR31/chromosome 4 hybrids had lost all of the exogenous material whilst retaining the hygromycin B resistance gene alone and 9% (1/11) of BICR31/chromosome 4 hybrids had retained all exogenous material (compared with 23% and 46% respectively on the BICR6 background). This is suggestive of different levels of stable exogenous material maintenance depending on the background into which it is introduced. The reciprocal of this was seen in Chapter 4 where the introduced truncated chromosome 4 copies showed differing stability on the same background. Interestingly the most commonly lost alleles in BICR31/chromosome 4 hybrids were on 4p14-15 at D4S3022 (80%, 8/10) and D4S2912 (80%, 8/10). The highest ratio of long arm losses was on 4q33 at D4S3028 (70%, 7/10) and 4q34 at D4S3032 (70%, 7/10). However, the majority of the BICR31/chromosome 4 hybrids showed losses at the D4S423 (60%, 6/10) locus, which has been suggested through BICR6 segregant analysis. Furthermore as BICR31 does not display LOH on the 4p arm this would argue against the gene responsible for the similar phenotypes being located on that chromosomal arm. Truncated chromosome transfers into BICR6 excluded the chromosomal regions 4pcen-16 and 4q25-35, and as both cell lines display a similar phenotype (see Chapter 5) it is likely that the identified locus is the same for both cell lines. The BICR31 hybrids also display complex break patterns, which can provide misleading information when presented in the absence of complementary functional data. The stability of exogenous chromosomes has been shown to vary depending on the recipient line following MMCT and may simply reflect a difference in toleration of such material (Dr. S. Bryce, BICR, personal communication). The deletion map of all BICR31/Chromosome 4 segregants

is shown in Figure 4.13. The ratio of marker loss in the minimally identified region in BICR31/4 hybrids is shown in Figure 4.14. A substitute informative polymorphic marker for D4S409 was not identified for BICR31 and therefore no loss ratio is present in this column, although a low loss ratio would be expected through retention of the hygromycin resistance cassette.

The immortal hybrids resulting from monochromosome transfer into recipients BICR3 and BICR19, which had both displayed full ROH on the endogenous chromosome 4 copies, were examined to ascertain the loss patterns on the introduced chromosome in this unreactive background. In the BICR19/chromosome 4 hybrids 67% (4/6) had retained a completely intact exogenous chromosome 4 copy when tested with microsatellite markers at a 20cM spacing. There were two hybrids with deletions of material in the panel, one of these had deleted all exogenous material (17%, 1/6), whilst the other had deleted all but from 4q32-tel (17%, 1/6). The BICR3/chromosome 4 hybrids examined had deleted all exogenous material transferred (100%, 5/5) with no microsatellite markers present in a 20cM screen. This corroborates the findings with BICR6 and BICR31 where different stability of exogenous material was dependent on the recipient background.

A study of hybrids resulting from preliminary chromosome 4 transfers into lines BICR18 and BICR56 revealed that 100% (1/1) and 100% (1/1) respectively had retained all markers tested however as stated previously due to the low hybrid yield this result can not be used to discriminate between our identified regions.

Given the strikingly high ratio of D4S423 loss (92%) in the hybrids resulting from the fragmented chromosome transfers the lower ratios observed with the intact chromosome transfers (BICR6/Chromosome 4, 50%, and BICR31/Chromosome 4, 40%) suggest that the loss map data alone can be misleading and requires complementation by other techniques such as XMMCT. However total losses of the minimal region in the fragment experiments as described are 51% (28/55), whilst BICR6 hybrids show 52%

Figure 4.13. Exogenous material loss in BICR31/chromosome 4 segregants

Microsatellite analysis of segregants arising from MMCT of chromosome 4 into BICR31, show losses across the whole exogenous chromosome. Losses of exogenous chromosomal material (grey boxes) occur in all three regions identified in Chapter 3 (red boxes). Hybrid number is listed along the top axis. The microsatellite markers used, their approximate positioning (cM), and cytogenic positioning on a chromosome 4 ideogram are shown to the left of the LOH cartoon.

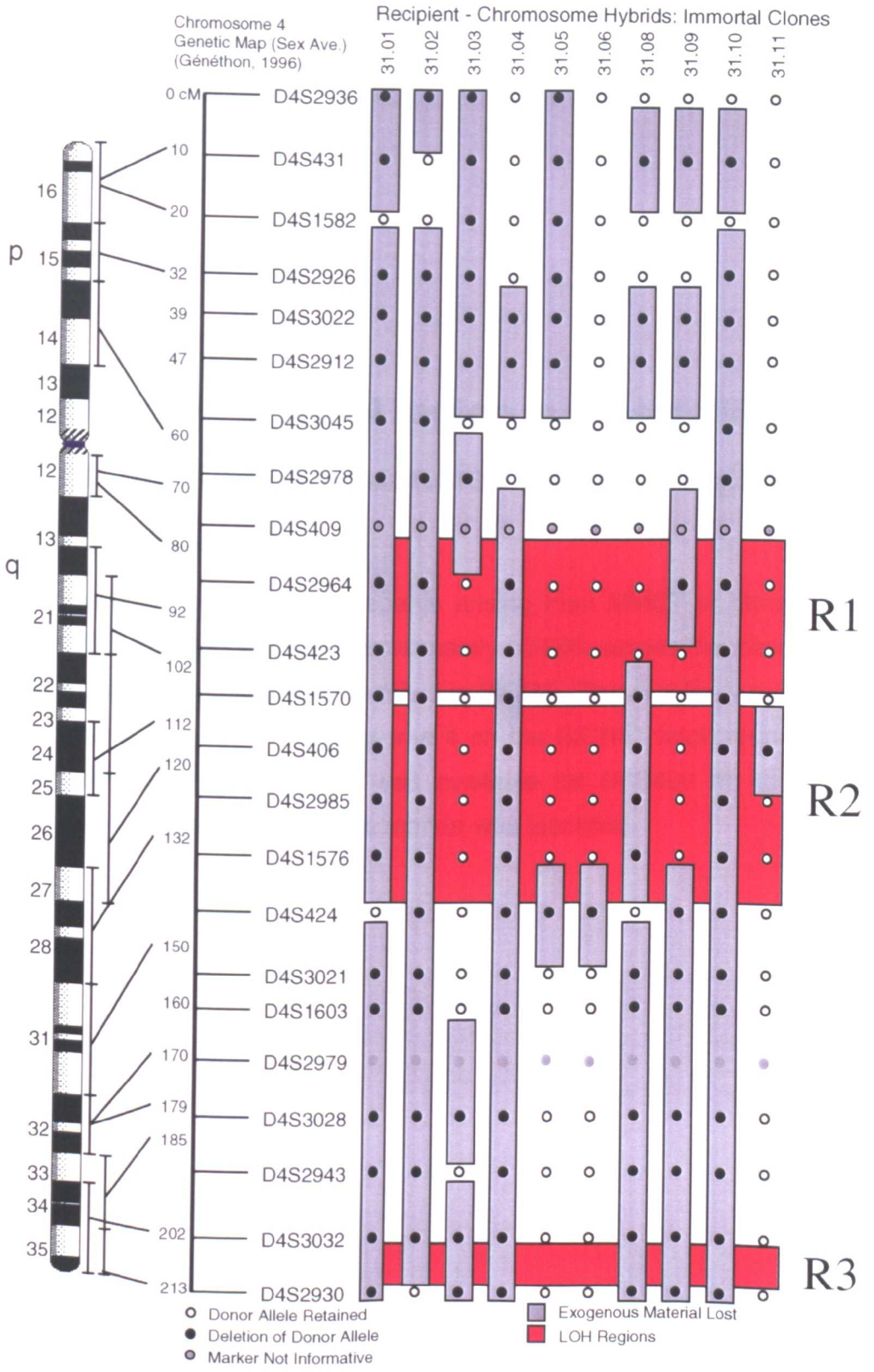
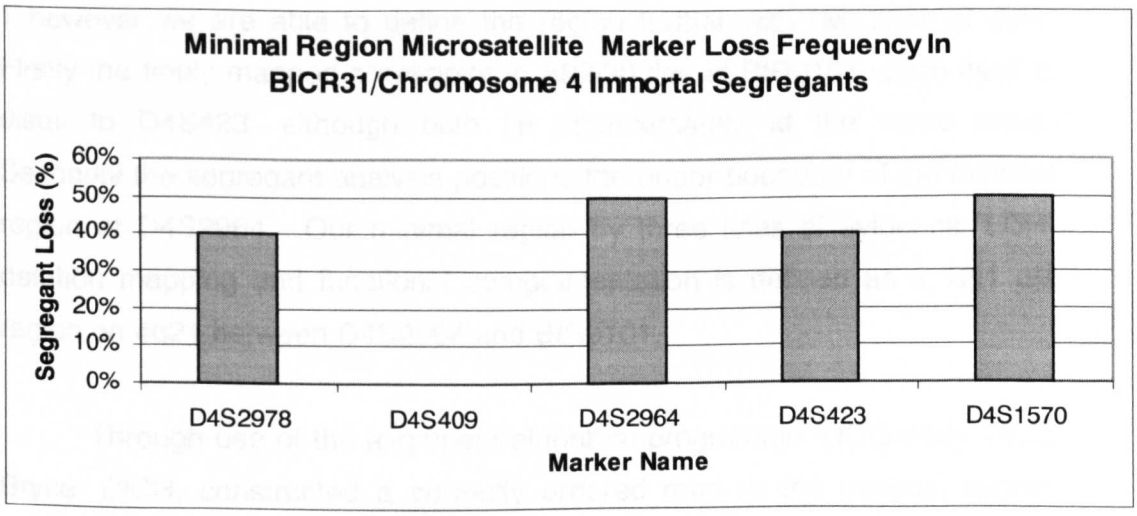

Chromosome 4
Genetic Map (Sex Ave.)
(Généthon, 1996)
Recipient - Chromosome Hybrids: Immortal Clones
31.01
31.02
31.03
31.04
31.05
31.06
31.08
31.09
31.10
31.11
0 cM
D4S2936
10
D4S431
20
D4S1582
32
D4S2926
39
D4S3022
47
D4S2912
60
D4S3045
70
D4S2978
80
D4S409
92
D4S2964
102
D4S423
112
D4S1570
120
D4S406
132
D4S2985
D4S1576
D4S424
150
D4S3021
160
D4S1603
170
D4S2979
179
D4S3028
185
D4S2943
202
D4S3032
213
D4S2930
p
q
16
15
14
13
12
12
13
21
22
23
24
25
26
27
28
31
32
33
34
35
R1
R2
R3
Donor Allele Retained
Deletion of Donor Allele
Marker Not Informative
Exogenous Material Lost
LOH Regions

Figure 4.14. Minimal region microsatellite marker loss frequencies suggest equivalence across 4q21

Microsatellite analysis of segregants arising from MMCT of chromosome 4 into BICR31 show losses of approximately 40-50% across the minimal area of functional complementation identified in BICR6. This is a likely reflection of a less stable exogenous chromosome 4 on the BICR31 background than that seen with BICR6. No data was available for D4S409 do to a lack of informative alleles, no surrogate marker was identified.


Minimal Region Microsatellite Marker Loss Frequency In BICR31/Chromosome 4 Immortal Segregants
Segregant Loss (%)
60%
50%
40%
30%
20%
10%
0%
D4S2978
D4S409
D4S2964
D4S423
D4S1570
Marker Name

(15/29) and BICR31 hybrids show 50% (6/12). This demonstrates a similar frequency of loss of the minimal region in all lines of evidence. Furthermore Table 4.5. shows the losses in the minimal region at D4S423 are most statistically significant as compared to the other markers in the region by both Fishers Exact Test and Chi Squared (X^2).

With an initial degree of caution we have successfully defined the region of chromosome 4 responsible for the observed growth-arrest phenotype. The region identified correlates with our initially identified Region 1 however we are able to define the region further with two lines of data. Firstly the finely mapped breakpoint in A92i30 lies at BIR0101 which itself is distal to D4S423, although both lie approximately at the same locus. Secondly the segregant analysis positions the upper boundary of the minimal region at D4S2964. Our minimal region by three lines of evidence; LOH, deletion mapping and functional complementation is defined as a 12.1 cM region on 4q21 between D4S2964 and BIR0101.

Through use of the alignment algorithm programme 'OOGreedy' Dr. S Bryce, BICR, constructed a correctly ordered map of the minimal region, which we had identified previously. The alignment algorithm programme OOGreedy represents an automated system product sequence contig builder from draft sequence data. Through this a contig spanning the minimal region was constructed allowing definition of the physical map to parallel that of the previously defined genetic map (Genethon, 1996). This map is shown in Figure 4. 15.

Microsatellite analysis of a panel of our segregants from both our intact chromosome 4 transfers and the fragmented chromosome transfer experiments is shown in Figure 4.16. From this data we can demonstrate a preferentially lost locus at D4S3037 and D4S2404 on 4q21-q23 with 94% of segregants analysed having lost these markers (Figure 4.17). This loss defines a minimal region on the long arm of chromosome 4 of approximately 1.5Mb bordered by the markers D4S423 and BIR0101. This minimal region

Table 4.5. Statistical relevance of losses in the identified minimal region demonstrate weighting towards D4S423

Statistical analysis using both Fishers Exact Test and Chi^2 demonstrate that the losses observed at D4S423 are the most statistically significant by both tests. The Chi^2 value shows the losses at D4S423 to be highly significant.

Microsatellite Marker	Fishers Exact Test (Probability)	X^2 (Probability)
D4S2978	0.19	0.81
D4S409	0.18	0.40
D4S2964	0.19	0.65
D4S423	0.10	0.04
D4S1570	0.17	0.32

Figure 4.15. Minimal region marker order and locations

Illustration of the marker order and physical distance across the minimal region identified from LOH analysis (Chapter 3), segregant exogenous material loss analysis (this Chapter), and functional complementation (this Chapter). The equivalence of the genetic and physical distances (18Mb/18cM) suggests an area of low recombination. Figure produced by Dr. S. Bryce

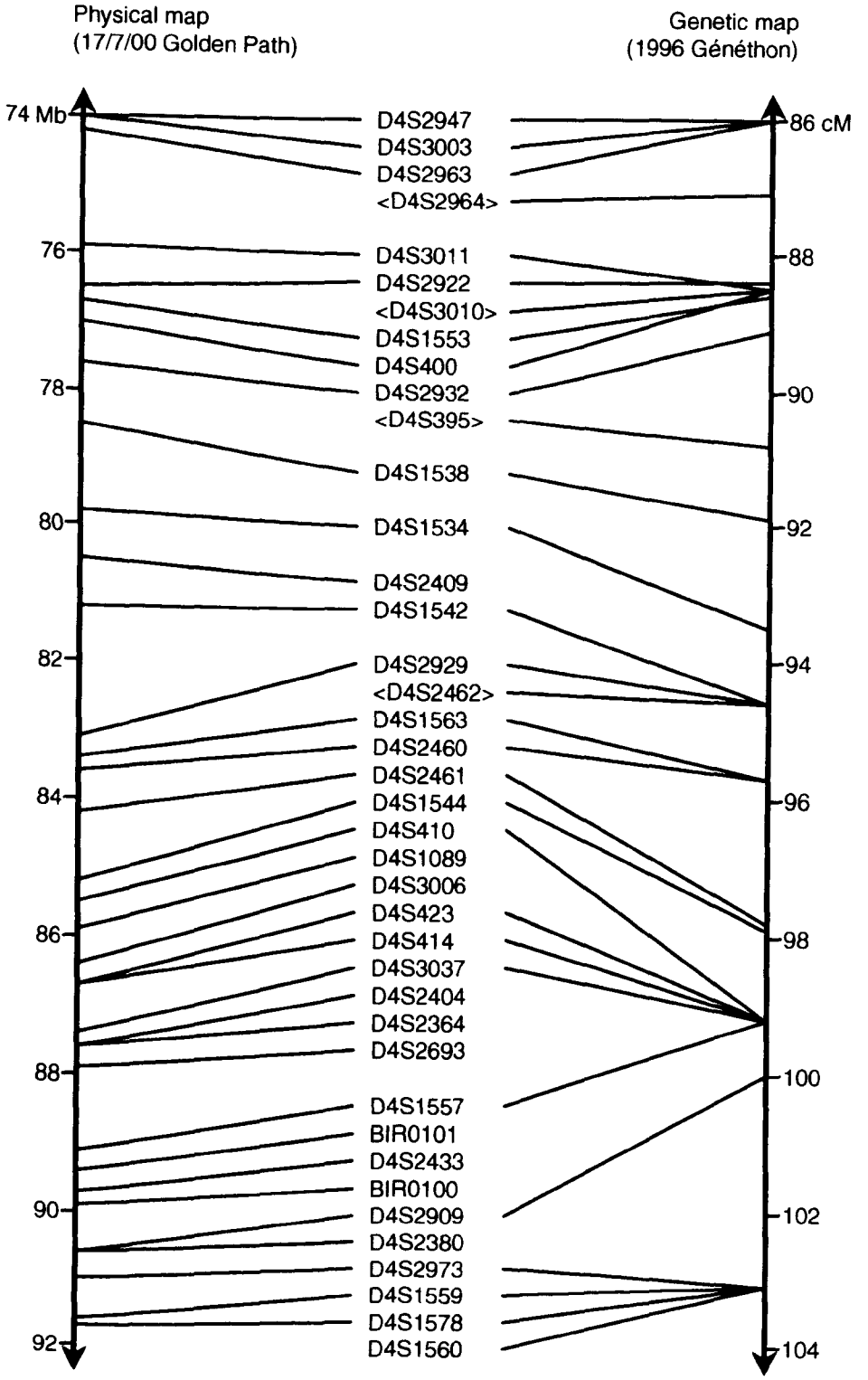

Physical map
(17/7/00 Golden Path)
Genetic map
(1996 Généthon)
74 Mb
76
78
80
82
84
86
88
90
92
86 cM
88
90
92
94
96
98
100
102
104
D4S2947
D4S3003
D4S2963
<D4S2964>
D4S3011
D4S2922
<D4S3010>
D4S1553
D4S400
D4S2932
<D4S395>
D4S1538
D4S1534
D4S2409
D4S1542
D4S2929
<D4S2462>
D4S1563
D4S2460
D4S2461
D4S1544
D4S410
D4S1089
D4S3006
D4S423
D4S414
D4S3037
D4S2404
D4S2364
D4S2693
D4S1557
BIR0101
D4S2433
BIR0100
D4S2909
D4S2380
D4S2973
D4S1559
D4S1578
D4S1560

Figure 4.16. Minimal region exogenous material loss in BICR6/chromosome 4 segregants

Microsatellite analysis of segregants arising from MMCT of intact and truncated copies of chromosome 4 into BICR6 show losses across the whole minimally identified region on the exogenous chromosome (grey boxes). The minimal region of loss is defined by microsatellite markers D4S423 and BIR0101, which is illustrated by the red box. Hybrid number is listed along the top axis. The microsatellite markers used and their physical separation are shown to the left of the LOH cartoon.

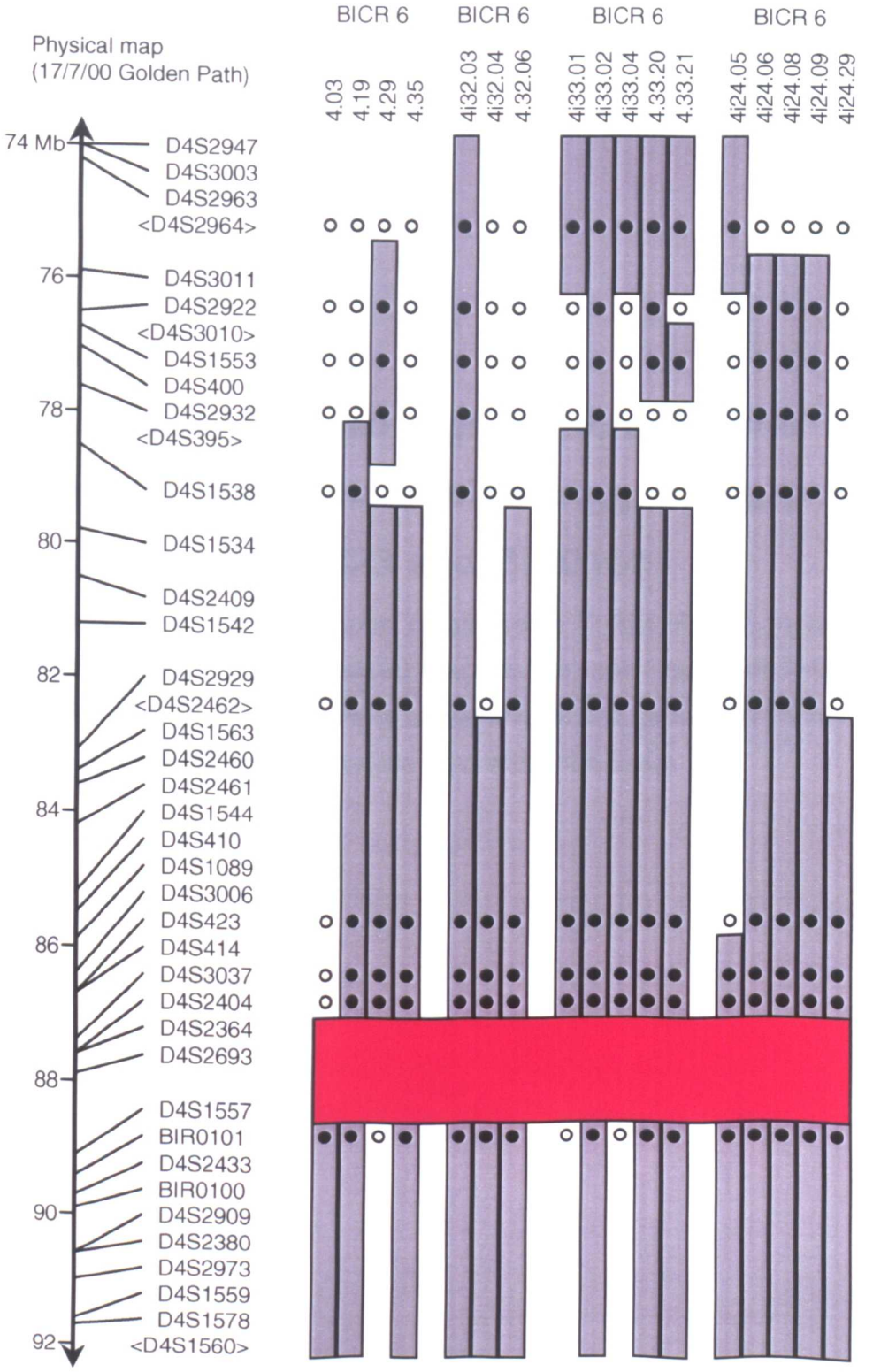
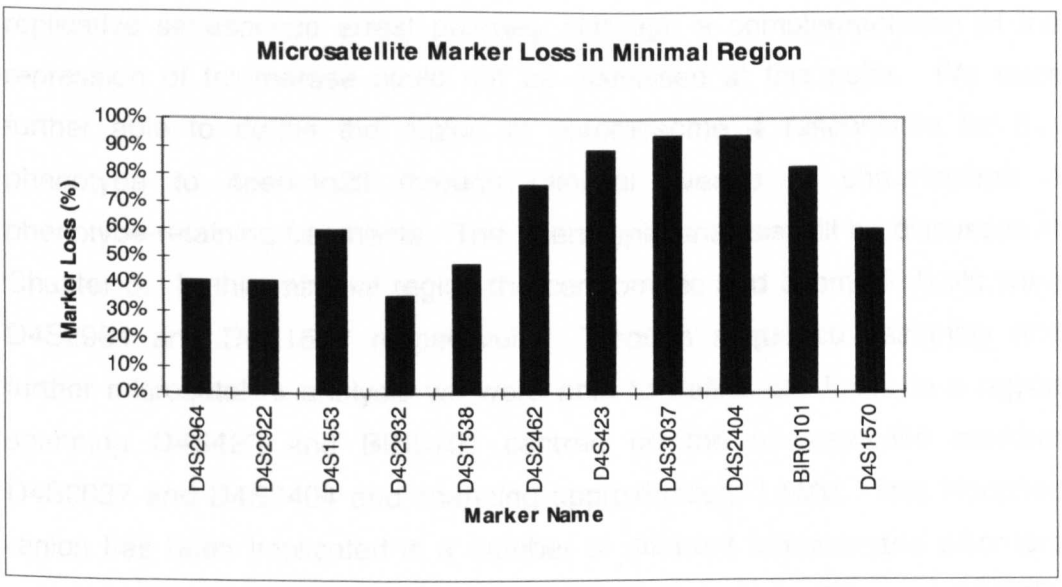

Physical map
(17/7/00 Golden Path)
74 Mb
76
78
80
82
84
86
88
90
92
D4S2947
D4S3003
D4S2963
<D4S2964>
D4S3011
D4S2922
<D4S3010>
D4S1553
D4S400
D4S2932
<D4S395>
D4S1538
D4S1534
D4S2409
D4S1542
D4S2929
<D4S2462>
D4S1563
D4S2460
D4S2461
D4S1544
D4S410
D4S1089
D4S3006
D4S423
D4S414
D4S3037
D4S2404
D4S2364
D4S2693
D4S1557
BIR0101
D4S2433
BIR0100
D4S2909
D4S2380
D4S2973
D4S1559
D4S1578
<D4S1560>
BICR 6
4.03
4.19
4.29
4.35
BICR 6
4i32.03
4i32.04
4.32.06
BICR 6
4i33.01
4i33.02
4i33.04
4.33.20
4.33.21
BICR 6
4i24.05
4i24.06
4i24.08
4i24.09
4i24.29

Figure 4.17. Minimal region microsatellite marker loss frequencies suggest region spanning D4S423 and BIR0101

The frequencies of exogenous chromosome 4 loss arising in segregants reaches levels of greater than 90% across the region spanning D4S423 and BIR0101 (Figure 4.16). This is suggestive of the gene responsible for the observed growth-arrest phenotype lying within this locus.

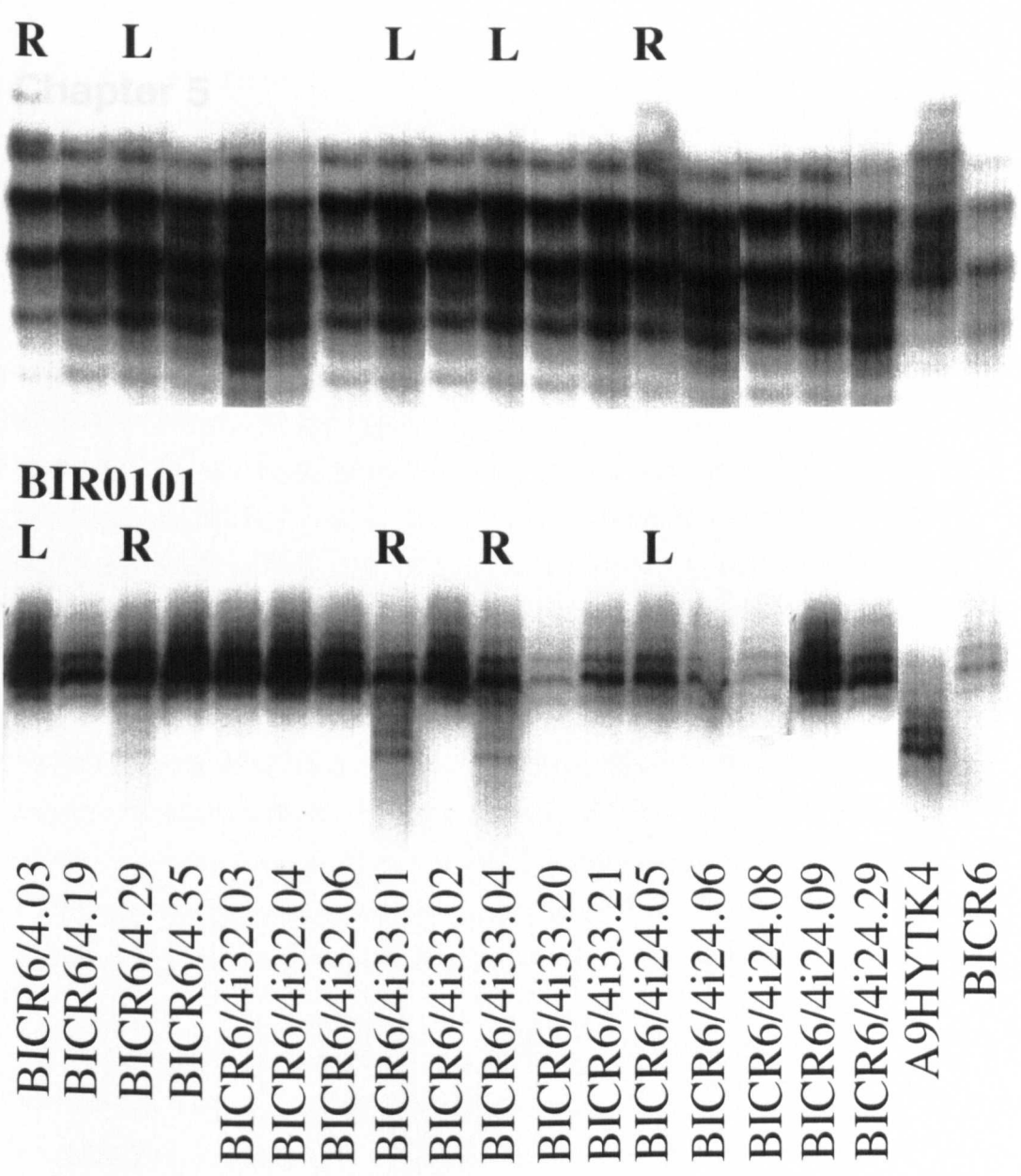

Microsatellite Marker Loss in Minimal Region
Marker Loss (%)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
D4S2964
D4S2922
D4S1553
D4S2932
D4S1538
D4S2462
D4S423
D4S3037
D4S2404
BIR0101
D4S1570
Marker Name

analysis was undertaken with the assistance of Dr. N. Craig and, Dr. V. Morrison. Microsatellite markers, which serve to define the minimal locus, are shown in Figure 4.18.

Through use of the MMCT and the adaptive XMMCT we were able to reproducibly identify a growth-arrest phenotype following specifically transfer with chromosome 4. The phenotype in question occurred on cell line backgrounds with deregulated p53, p16INK4A, and positive telomerase expression. This would suggest that the growth-arrest was not linked into the replicative senescence arrest pathway although a complementation of the repression of telomerase could not be dismissed at this point. We were further able to define the region of chromosome 4 responsible for this phenotype to 4cen-4q23 through minimal overlap of chromosome 4 phenotype-retaining fragments. The phenotypic analysis will be discussed in Chapter 5. In this minimal region the centromeric and telomeric limits were D4S2964 and D4S1557 respectively. Through sequence assembly and further microsatellite analysis we were able to define our locus to a region spanning D4S423 and BIR0101 centred on the microsatellite markers D4S3037 and D4S2404 and spanning approximately 1.5Mb. This identified region has been implicated in a number of different tumours and disorders such as CC, HNSCC, HCC, SCLC, ESCC, Sclerotylosis, EA, and ADPKD. However although implicated in the locus we previously described (D4S2964-D4S1557) we know of no study, which has concisely identified a minimal locus from this region. Given the size of the region we were able to identify a limited number of candidates, these will be discussed in Chapter 6.

Figure 4.18. Microsatellite markers D4S423 and BIR0101 define the minimal locus.

Primary data representing the microsatellite markers loss pattern as described in Figure 4.16. Immortal hybrids BICR6/4.03 and BICR6/4i24.05 define the upper boundary with retentions of D4S423. The lower boundary is defined by retentions of BIR0101 in BICR6/4.29, BICR6/4i33.01, and BICR6/4i33.04. In each instance where one boundary marker is retained the other is lost.



L = Loss of Exogenous Allele
R = Retention of Exogenous Allele

Chapter 5

5.1) Growth-Arrested Phenotype Has Characteristics Of Crisis

The functional evidence to suggest the presence of a tsg on chromosome 4 was first provided with the demonstration that immortal variants arising from cell-cell fusions between the fibrosarcoma line HT1080 and normal human fibroblasts correlated with the loss of chromosome 4 (Benedict *et al.,* 1984). This was suggestive of the dominant suppression of tumourigenicity provided by a gene(s) on chromosome 4 being selected against allowing the development of the tumourigenic phenotype. Following on from this study, on the basis of a subset of cells (GM2096SV9, T98G, J82) identified to undergo growth-arrest resembling replicative senescence following cell-cell fusion with the cervical carcinoma cell line HeLa (Pereira-Smith and Smith, 1988), the introduction of an exogenous chromosome 4 by MMCT into members of the same subset of cells and HeLa produced the same growth-arrest features (Ning *et al.,* 1991) therefore ascribing the phenotype to the introduced chromosome. A further observation showed a reduced colony forming ability in the murine A9 line however this report was not investigated further (England *et al.,* 1996). The reported functional evidence would suggest that the activity associated with chromosome 4 is involved in the reversion of the immortal phenotype observed in tumour lines to that of a growth-arrest resembling replicative senescence.

As stated in Chapter 4 we observed a growth-arrest phenotype in hybrids resulting from transfer of chromosome 4 into the immortal keratinocyte lines BICR6 and BICR31. The BICR6/Chromosome 4 hybrid growth-arrest phenotype, which was reproducibly observed, is shown in Figure 5.1. The BICR31/Chromosome 4 hybrid growth-arrest phenotype is shown in Figure 5.2. The observed growth-arrest displayed some clonal variety with a display of phenotype occurring after 3 and 10 population doublings. Growth-arrested hybrids from both lines are greatly enlarged with massive nuclei and cytoplasmic regions both of which are recognised features of cellular senescence. We next examined

Figure 5.1. Immortal and growth-arrested BICR6 hybrids

(a) Phase contrast image of immortal keratinocyte line BICR6, (b) Growth-arrested BICR6/Chromosome 4 hybrid showing characteristic features described below, (c) Photomicroscopy image of SA-βGal stained BICR6/Chromosome 4 hybrid showing tightly packed keratinocytes with an absence of specific nuclear staining, (d) SA-βGal stained growth-arrested BICR6/Chromosome 4 hybrid demonstrating flattened cells with massive nuclei and a positive blue stain, (e) Multinucleate cells in image (b), (f) Attempted cytokinesis which ultimately gives rise to multinucleate cell labelled in (b). Scale= 100 microns

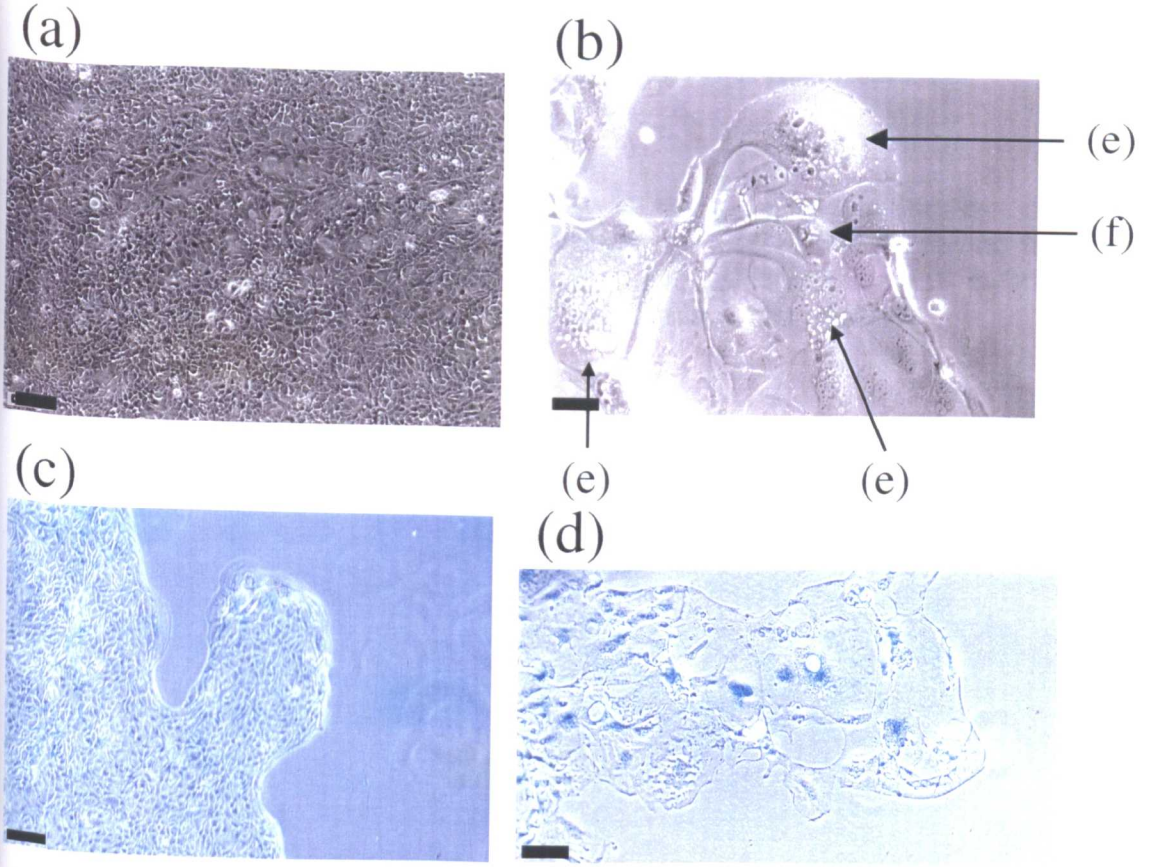
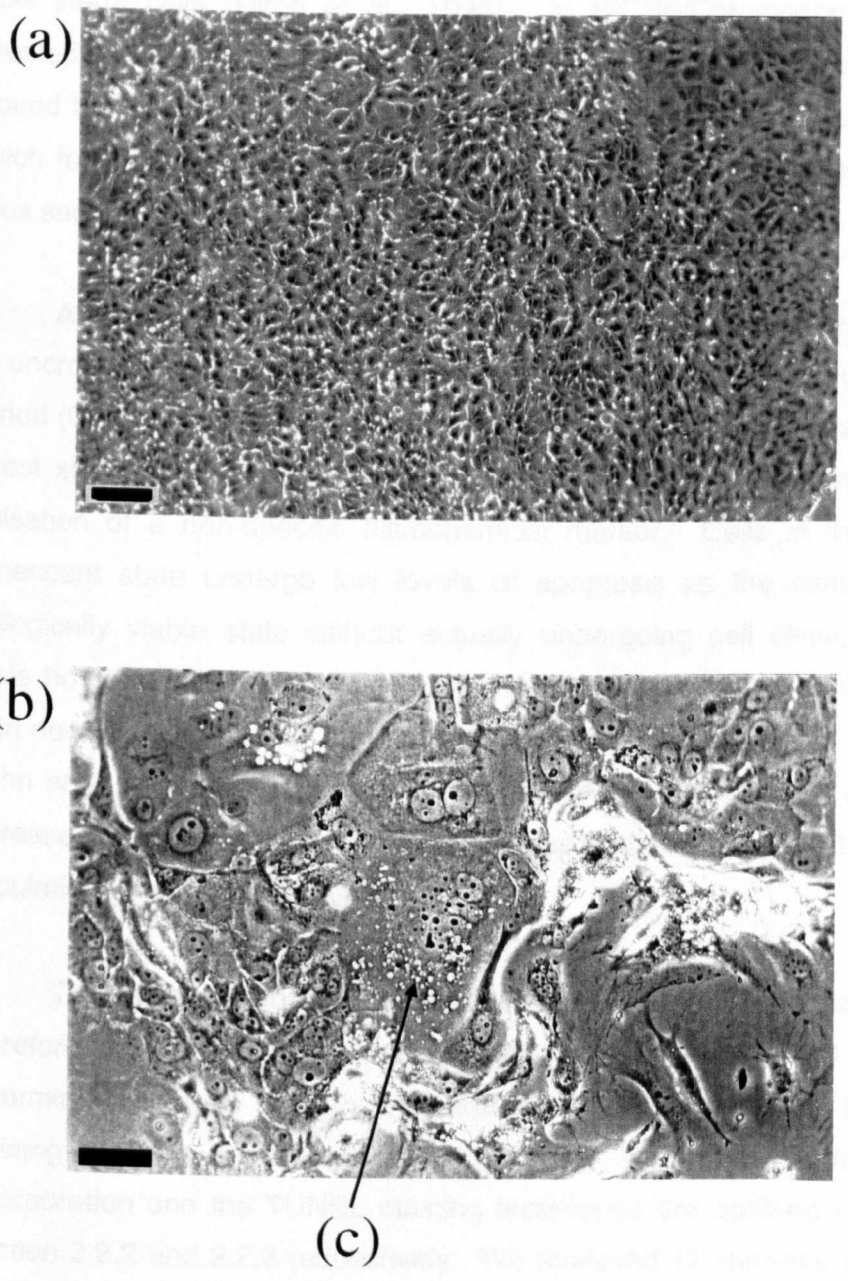

(a)
(b)
(e)
(f)
(e)
(e)
(c)
(d)

Figure 5.2. Immortal and growth-arrested BICR31 hybrids

(a) Phase microscopy image of immortal keratinocyte line BICR31, and, (b) Image of growth-arrested BICR31/chromosome 4 hybrid displaying features seen in Figure 5.1 of growth-arrested BICR6/chromosome 4 hybrids, (c) Multinucleate cell as seen in Figure 5.1. Scale = 100 microns


(a)
(b)
(c)

BICR6/Chromosome 4 hybrids for the presence of the biomarker of growth-arrest, Senescence-Associated β-galactosidase (SA-βGal) at pH 6, which has been shown to be detectable histochemically in replicatively senescent and crisis stage cells (Dimri *et al.,* 1995). In BICR6/Chromosome 4 growth-arrested hybrids we found a detectable SA-βGal activity that was localised around the nuclear region (Figure 5.1 (d)). Although this is not indicative of which form of growth-arrest is occurring (replicative senescence or crisis) it does serve as an important biomarker of this induced phenotype.

A recognised definition of the senescent state is when less than 5% of an uncrowded cell population incorporate a nucleic acid marker during a 24hr period (Cristofalo and Sharf, 1973). With this approach the senescent growth-arrest state is quantifiable in a biologically valid assay system rather than utilisation of a non-specific histochemical marker. Cells in the replicative senescent state undergo low levels of apoptosis as the cells maintain a biologically viable state without actually undergoing cell division. Cells in crisis however still incorporate nucleic acid markers, albeit at a lower level than normal counterparts, but exhibit a higher level of apoptosis (Stein, 1985, Hahn *et al.,* 1999). Crisis cells are still in cycle but the levels of cell death increase with a constant level of cell birth resulting in an apparent cessation of population growth (Stein, 1985).

To define the form of growth arrest, which we were observing, we therefore carried out BrdU incorporation staining and TUNEL staining to determine the levels of both in mortal and immortal hybrids. Examples of staining patterns are shown in Figure 5.3. The protocols for the BrdU incorporation and the TUNEL staining techniques are outlined in Chapter 2, Section 2.2.2 and 2.2.3 respectively. We analysed 12 immortal and 8 mortal BICR6/Chromosome 4 hybrids to determine the rate of BrdU label incorporation after 48hrs, BICR6 and BICR6/HYTK were included as labelling controls, results are shown in Figure 5.4. We found that mortal BICR6/Chromosome 4 hybrids had a mean labelling index after 48hrs of

Figure 5.3. BrdU incorporation and TUNEL staining in mortal and immortal BICR6 hybrids

(a) 48hr BrdU staining in mortal and immortal BICR6/Chromosome 4 hybrids shows incorporation of the nucleic acid after 6 weeks in culture, (b), TUNEL staining in mortal and immortal BICR6/Chromosome 4 hybrids shows an elevation of apoptosis in mortal hybrids after 6 weeks in culture, (c) Mulinucleate cell, (d) Apoptotic cell.

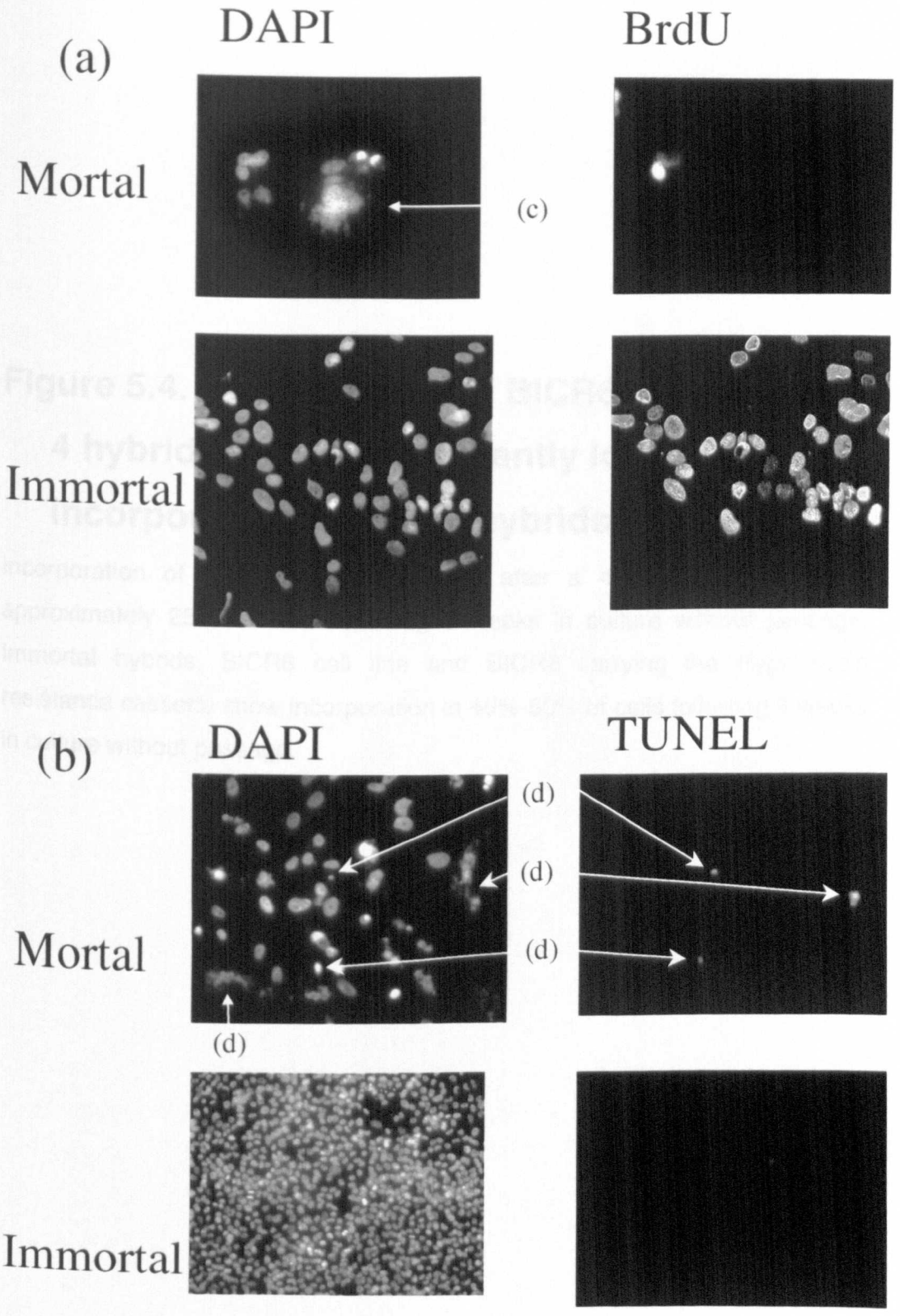
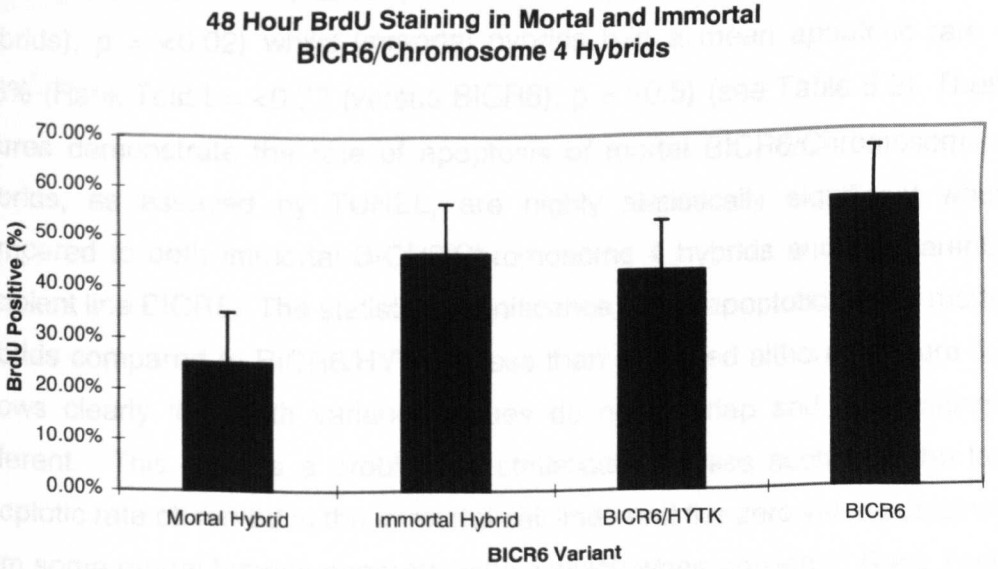

(a)
DAPI
BrdU
Mortal
(c)
Immortal
(b)
DAPI
TUNEL
(d)
(d)
(d)
Mortal
(d)
Immortal

Figure 5.4. BrdU staining of BICR6/chromosome 4 hybrids shows significantly lower levels of incorporation in mortal hybrids

Incorporation of BrdU in mortal hybrids after a 48hr pulse is seen in approximately 25% of cells following 6 weeks in culture without passage. Immortal hybrids, BICR6 cell line and BICR6 carrying the Hygromycin resistance cassette show incorporation in 45%-50% of cells following 6 weeks in culture without passage.


48 Hour BrdU Staining in Mortal and Immortal BICR6/Chromosome 4 Hybrids
BrdU Positive (%)
70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
Mortal Hybrid
Immortal Hybrid
BICR6/HYTK
BICR6
BICR6 Variant

25.2% (Rank Test, t = >2.0 (versus BICR6, BICR6/HYTK, and immortal hybrids), p = <0.05) whilst immortal hybrids had a mean labelling index after 48hrs of 46.6% (Rank Test, t = <1.0 (versus BICR6 and BICR6/HYTK), p = >0.5). Both probabilities suggested that the result for the mortal hybrids were statistically significant as compared to the immortal hybrids, probabilities are shown in Table 5.1. We then analysed 15 immortal and 7 mortal BICR6/Chromosome 4 hybrids to determine the level of apoptosis present in both sets, results are shown in Figure 5.5. No population of less than 80 cells as included in this analysis. Mortal hybrids showed a mean apoptotic rate of 2.5% (Rank Test t = /<2.52 (versus BICR6, BICR6/HYTK, and immortal hybrids), p = <0.02) whilst immortal hybrids had a mean apoptotic rate of 0.6% (Rank Test t = <0.77 (versus BICR6), p = >0.5) (see Table 5.2). These figures demonstrate the rate of apoptosis of mortal BICR6/Chromosome 4 hybrids, as assayed by TUNEL, are highly statistically significant when compared to both immortal BICR6/Chromosome 4 hybrids and the parental recipient line BICR6. The statistical significance of the apoptotic rate of mortal hybrids compared to BICR6/HYTK is less than expected although Figure 5.5 shows clearly that both variance values do not overlap and are strikingly different. This reflects a problem in statistical analyses such that the low apoptotic rate observed in the immortal cell lines and the zero values obtained from some mortal hybrids generate values which when applied to Rank Tests tend to skew the probabilities to a less significant value than that expected from observed differences. The variation in apoptotic rate between immortal hybrids and BICR6 was not statistically significant although a similar situation to that described previously existed for comparison of BICR6/HYTK and immortal hybrids where the large number of zero score values skewed the test to the levels of significance whereas in Figure 5.5 both data sets visibly overlap and fall within visible error bar values. Although the data sets present some degree of statistical variance this is likely a reflection of a number of factors. Keratinocytes are highly resistant to apoptotic cell death and those, which do undergo apoptosis do so relatively rapidly, the TUNEL assay can only provide an indication of apoptotic death at that point in time and so does not record earlier or later events. The hygromycin resistance gene induces

Table 5.1. Statistical values for 48hr BrdU staining

The non-parametric rank test displays the observed differences in BrdU labelling between mortal and immortal hybrid populations to be statistically significant to the $p = <0.05$ level. Differences in incorporation seen between immortal populations are not statistically significant ($p = >0.5$). When $p = <0.05$ this is taken as indicating a significant difference.

BICR6 Variants	Rank Test (t)	Probability
BICR6/HYTK and Mortal BICR6/Chromosome4 Hybrid	2.19	<0.05
BICR6/Mortal	2.19	<0.05
BICR6/HYTK and Immortal	0.47	>0.8
BICR6/Immortal	0.75	>0.5
Immortal/Mortal	2.08	<0.05

Figure 5.5. TUNEL positive staining in mortal BICR6/chromosome 4 hybrids is greatly increased compared to immortal hybrids

The levels of apoptosis seen in mortal hybrid cells rise to approximately 2.5% after 6 weeks in culture without passage. The levels of apoptosis seen in immortal cells peaks at approximately 0.5% of cells after 6 weeks in culture without passage. This is indicative of a greatly increased apoptotic rate in the mortal hybrids.

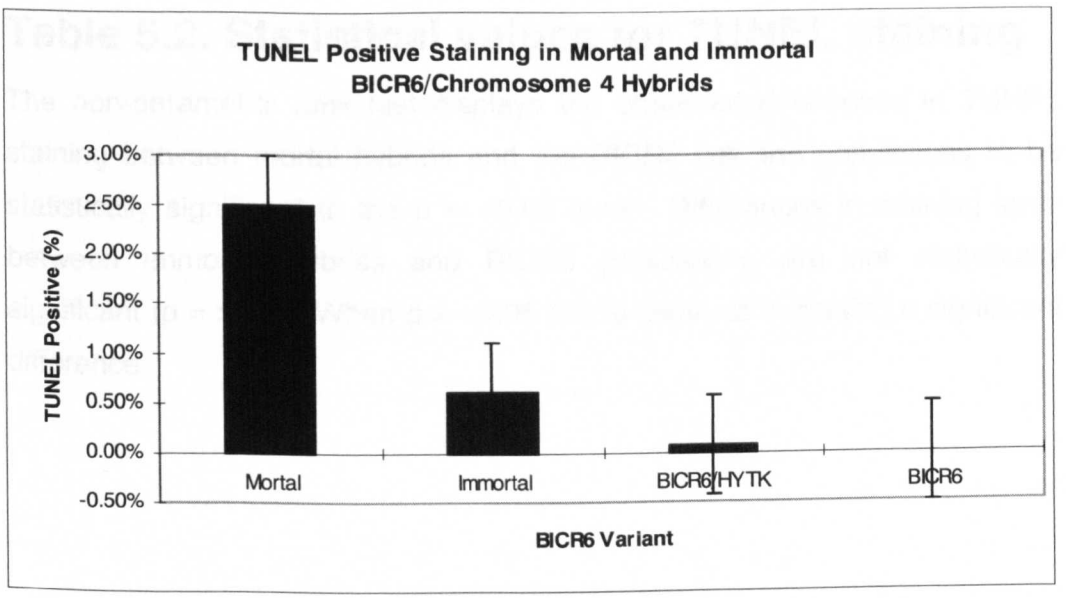

TUNEL Positive Staining in Mortal and Immortal BICR6/Chromosome 4 Hybrids
TUNEL Positive (%)
3.00%
2.50%
2.00%
1.50%
1.00%
0.50%
0.00%
-0.50%
Mortal
Immortal
BICR6/HYTK
BICR6
BICR6 Variant

Table 5.2. Statistical values for TUNEL staining

The non-parametric rank test displays the observed differences in TUNEL staining between mortal hybrids and the BICR6 cell line populations to be statistically significant to the $p = <0.05$ level. Differences in staining seen between immortal hybrids and BICR6 populations are not statistically significant ($p = >0.5$). When $p = <0.05$ this is taken as indicating a significant difference.

BICR6 Variants	Rank Test (t)	Probability
BICR6/HYTK and Mortal BICR6/Chromosome4 Hybrid	1.57	<0.2
BICR6/Mortal	2.21	<0.05
BICR6/HYTK and Immortal	1.97	<0.05
BICR6/Immortal	0.77	>0.5
Immortal/Mortal	2.52	<0.02

cell death through apoptotic mechanisms and therefore a minimal background could be expected in both the immortal hybrids and the BICR6/HYTK cells as shown in Figure 5.5. These factors will tend to mask the significance of observations although the mortal hybrids do maintain a high level of statistical relevance as compared to the controls.

From the previously cited definitions of growth arrest we could now therefore increasingly define the form that we could repeatedly observe. We had observed SA-βGal staining which is indicative of growth arrest along with a lower rate of nucleotide incorporation and an increased rate of apoptosis. From these three lines of evidence we have defined the growth arrest occurring in the mortal hybrids as a form of crisis (M2 arrest).

Crisis is thought to occur by the shortening of telomeres to a critical length where end-to-end chromosomal fusions occur leading to non-disjunction of chromosomes, formation of anaphase bridges, incomplete cytokinesis and resultant cell death (Counter *et al.,* 1998) although there may also be telomere-independent crisis mechanisms (Artandi and DePinho, 2000, Zhu *et al.,* 1999). To determine if critically short telomeres were responsible for the observed crisis-like phenotype we measured the average telomere lengths of a panel of BICR lines, Dr. K. Gordon, BICR, carried out this work (data not shown). This showed that the telomeres of BICR6 were maintained at a low level whereas the telomeres of BICR31 were maintained at a much longer length. The two lines BICR6 and BICR31 had both reproducibly demonstrated the crisis-like phenotype however both clearly had greatly different telomere lengths, although this does not rule out a critically short BICR31 telomere being masked by the presence of longer ones. The two control lines BICR3 and BICR19 both also had differing telomere lengths. In BICR3 they were maintained at a short length whereas in BICR19 had much longer telomeres. This result would suggest that the crisis-like arrest might not be telomere-dependant.

The cells lines used in this study all demonstrated deregulated telomerase activity detectable through standard detection kits and also showed LOH on 3p (putative site of telomerase repressor). The determined variation in MMCT target line telomere length was cautiously suggestive of a telomere-independent crisis mechanism. The ends of telomeres are regenerated and maintained stably by the telomerase complex. To determine if the crisis-like growth arrest was due to blocking the effect of the telomerase enzyme BICR6 and BICR31 both underwent infection with a retrovirus carrying an ectopic copy of the limiting catalytic component of the telomerase enzyme (hTERT). Dr. E. K. Parkinson, BICR, carried out viral infections and Dr. N. Barr, BICR, prepared viral titre (see Chapter 2, Section 2.1.11-13) for methods). MMCT of Chromosome 4 into BICR6, BICR6neo (infected with the retrovirus alone), BICR6hTERT, BICR31, BICR31neo, and BICR31hTERT were carried out to determine if there was a blocking of the phenotype by the ectopic telomerase hTERT subunit. Results of these transfer experiments are shown in Table 5.3. We were still able to observe the crisis-like phenotype in both BICR6hTERT and BICR31hTERT thus indicating that the observed reversion to the mortal phenotype did not seem to be caused by any obvious blocking of the endogenous telomerase enzyme. This interpretation is supported by preliminary data from experiments in collaboration with Dr. K. Gordon, BICR, where we showed the mortal BICR6/Chromosome 4 hybrids still expressed TRAP activity (data not shown). Furthermore, the fact that the telomeres of both BICR6 and BICR31 were greatly extended by the ectopic expression of hTERT, suggests that the chromosome 4 mortality effect does not involve telomeric attrition either. This data along with the variation in telomere length suggests that the locus responsible for the phenotype may be operating without directly acting on the telomerase enzyme.

CpG island methylation has been shown to transcriptionally silence a number of tsgs in cancers from all stages. Furthermore, the unknown senescence gene(s) on chromosome X is known to be silenced by methylation through several passages of the donor A9 cells (Klein *et al.*, 1991; Wang *et al.*, 1992). As described in Chapter 4 we had observed on several

Table 5.3. Transfer of chromosome 4 into stably expressing hTERT BICR6 and BICR31 lines

Ectopic hTERT expressing BICR6 and BICR31 both undergo growth-arrest following MMCT of chromosome 4 into these recipient cell lines. This is indicative of the observed growth-arrest phenotype not occurring through the telomerase-based telomere maintenance pathway.

Recipient Line	Chromosome	Growth-Arrested Hybrids	Total Growth-Arrested Hybrids (%)
BICR6	A9HYTK4	5/21	23.8%
BICR6neo	A9HYTK4	4/26	15.4%
BICR6tert	A9HYTK4	3/12	25%
BICR31neo	A9HYTK4	0/2	0%
BICR31tert	A9HYTK4	3/5	60%

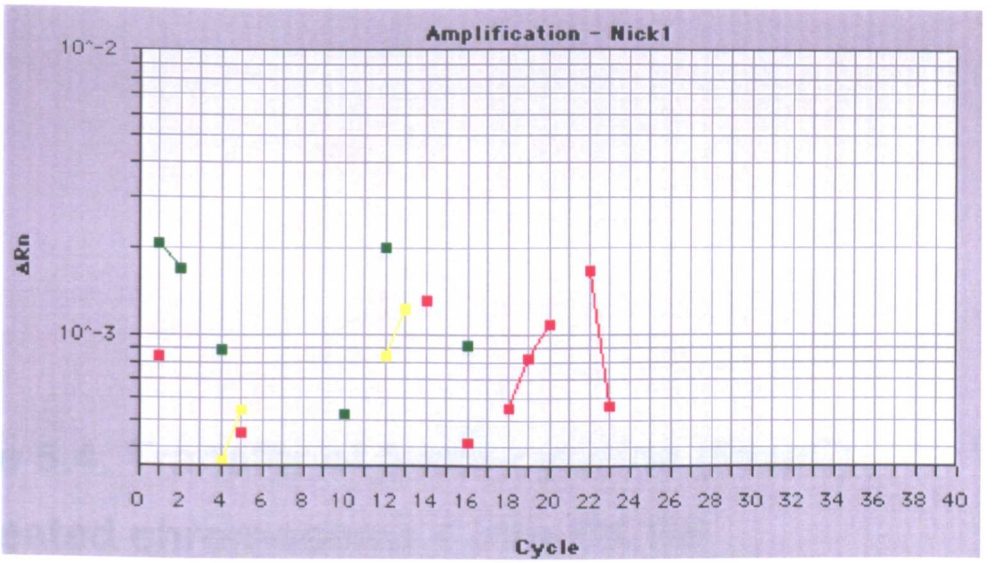
occasions that hybrids with a seemingly intact exogenous chromosome 4 underwent growth arrest when plated in minimal conditions whereas in optimal conditions continued dividing indefinitely. To assess whether this rescue was occurring through an epigenetic silencing of the mortality-associated locus we treated our donor line A9HYTK4 with the demethylating agent 5-aza-cytidine. To determine whether our line had been successfully demethylated we performed real-time PCR on RNA extracted from both treated and untreated cells using primers that spanned an intron of the mouse MyoD1 gene. MyoD1, a muscle differentiation gene, had been shown previously to be silenced through methylation at an early stage of mouse development. The ubiquitously expressed GAPDH gene was used as a control to assess the relative values. From this we determined that although GAPDH was expressed in both samples the MyoD1 gene had been transcriptionally reactivated in the 5-aza-cytidine treated sample alone suggesting that demethylation had occurred. The MyoD1 amplification plots are shown in Figure 5.6. We then introduced the demethylated chromosome into BICR6 using MMCT to determine whether we would see a greater rate of growth arrest than that seen with the untreated donor line. Results shown in Table 5.4. We did not see any significant increase in the number of colonies that underwent growth arrest with the demethylated chromosome vs. the untreated chromosome. However we did see a significant improvement in colony yield (4-5 fold) which may suggest either a subpopulation of donor cells carrying silenced resistance markers or alternatively the demethylation of a gene(s) beneficial to colony formation.

Through further experimentation we were able to more rigorously define the phenotype, which we repeatedly observed following transfer of a minimal region of chromosome 4 in various contexts. Through SA-βgal staining, BrdU labelling, and apoptotic rate determination we were able to classify the growth-arrested phenotype as a delayed crisis. As the immortal target cell line was both p53 and p16INK4A negative and telomerase positive a replicative senescence and crisis bypass had clearly occurred and therefore in a reversion to mortality the cell line could be expected to re-enter growth-

Figure 5.6. Real-time PCR analysis of MyoD1

MyoD1 expression in 5-aza-cytidine untreated, (a), and treated, (b), A9HYTK4 donor line. Yellow, red and green lines represent amplification curves for three independent reactions. No amplification of amplicon is seen in untreated reactions (a) whereas in treated reactions recordable amplification of the amplicon is seen after 27-30 reactions (b)

(a)


Amplification - Nick1
10^-2
10^-3
ΔRn
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Cycle

(b)

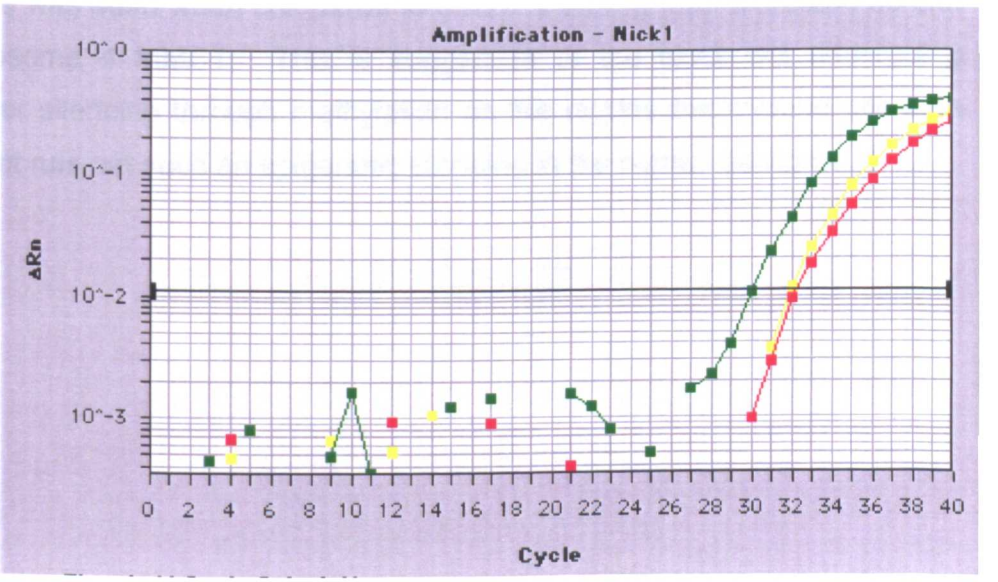

Amplification - Nick1
10^0
10^-1
10^-2
10^-3
ΔRn
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Cycle

Table 5.4. Transfer of 5-aza-cytidine (5azaC) treated chromosome 4 into BICR6

Following MMCT of chromosome 4, which had been pre-treated with the demethylating agent 5azaC, no increase in the levels of growth-arresting colonies was seen when compared to colonies arising from untreated control chromosome 4 MMCT. This is suggestive of the locus not undergoing promoter silencing through methylation on the murine background although does not rule out such an epigenetic silencing in the human background.

Recipient Line	Chromosome	Growth-Arrested Hybrids	Total Growth-Arrested Hybrids
BICR6	A9HYTK4	1/7	15%
BICR6	A9HYTK4-5azaC	7/31	23%
BICR6	A9HYTK4-5azaC	8/37	22%

arrest by the most recently abrogated pathway. However crisis is thought to occur through critically short telomeres and we could find no direct correlation between the telomere size of our recipient cells and the phenotypic effect of chromosome 4. Telomerase acts to stabilise and elongate the telomeres according to the cellular situation however when our endogenous hTERT expressing recipient lines were supplemented with an exogenous ectopic copy we saw no ablation of the phenotype in the recipient lines which responded. The donor chromosome when treated with demethylating agents did not demonstrate any overall increase or reduction in the number of colonies, which underwent growth-arrest although this does not determine whether promoter repression forms a basis for transcriptional control over the observed phenotype. Therefore the observed growth-arrest phenotype is not mediated through a direct telomerase repression and does not appear to be telomere-linked, although the observed delay prior to arrest, mitotic failures, and multinucleate cells (see Figure 5.1) would imply a telomere involvement. In Chapter 4 we described the definition of a 1.5Mb minimal region of loss in our segregants therefore in Chapter 6 we shall discuss these in terms of candidate potential.

Chapter 6

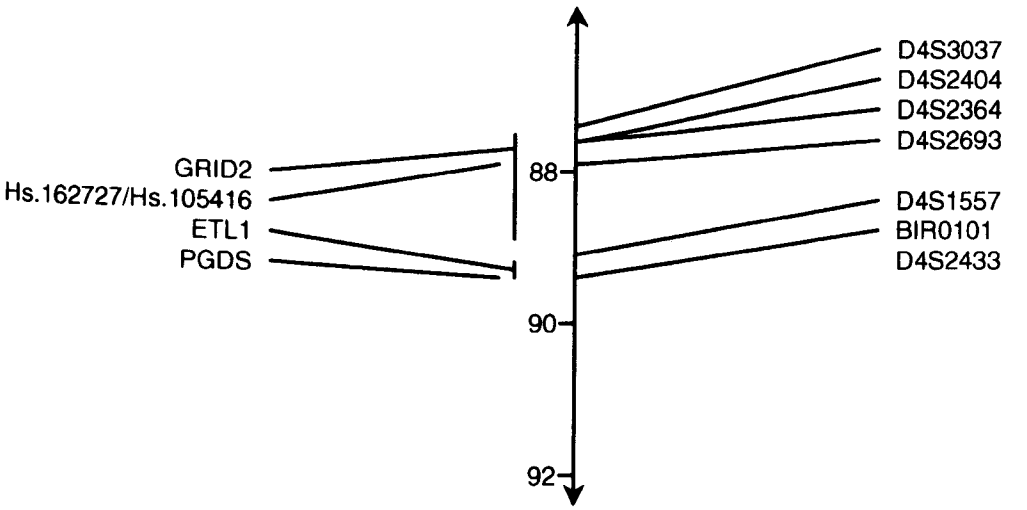
6.1) Locus Definition Reveals Four Candidate Genes

Through fine mapping of the minimal region of loss identified in Chapter 4 we were able to localise a region of 1.5Mb of chromosome 4 located on 4q22-q23 where we had evidence to suggest a cell mortality-related cancer gene may be located. Through the recent progress in genome sequencing the majority of the sequence of this chromosomal region was available although not in an assembled consensus form. Using the Golden Path algorithm Dr. S. Bryce (BICR) was able to assemble a contig covering this identified region and to identify candidate genes on this region. This approach has led to the identification of three (possibly four) candidate genes within our region; GRID2, ETL1, PGDS, and possibly a fourth novel gene. The ordering of these genes in relation to the microsatellite markers used to map the minimal region are shown in Figure 6.1

6.1.1) Candidate 1: GRID2; δ2 Glutamate Receptor

The GRID2 gene (Hs.248130) maps to 4q22 and has been implicated in a large number of neurodegenerative disorders where cell death results from inappropriate activation of the glutamate receptor system (Hu *et al.,* 1998). Studies in GRID2 functions have been performed largely in the murine system where a number of mutations have been characterised as GRID2-based. The binding ligand for GRID2 is however unknown as it does not seem to bind to glutamate. *Hotfoot* is a recessive mouse mutation which was characterised by cerebral ataxia associated with mild cerebellar abnormalities, a further mutation *Lurcher* is semi-dominant and affects postnatal cerebellar development such that homozygous mice die promptly due to massive loss of neurons in the mid- and hindbrain (Lalouette *et al.,* 1998). *Lurcher* and *hotfoot* were both discovered to be due to mutations in the GRID2 gene, although the lurcher mutation led to a dysfunction of the ion channel and a constitutive inward current which may lead to Purkinje cell death by an excitotoxic process, whilst the hotfoot mutation leads to a loss or

Figure 6.1. Microsatellite marker order and gene sequence in minimal region


GRID2
Hs.162727/Hs.105416
ETL1
PGDS
88
90
92
D4S3037
D4S2404
D4S2364
D4S2693
D4S1557
BIR0101
D4S2433

abolishment of GRID2 activity. The neurodegeneration in *lurcher* mice does not seem to occur in a p53-dependant manner but in both Purkinje and cerebellar granule cells to lessening degrees is via a Bax-dependant pathway although in Purkinje cells this is caspase-3-independant (Doughty *et al.*, 2000). The association of GRID2 predominantly to neurodegenerative disorders does not necessarily preclude it from candidate potential as we witnessed apoptotic death of growth-arrested hybrids in a p53-independant manner and therefore have a potential phenotypic match. Unfortunately GRID2 studies in human cell lines are minimal and therefore complementation effects of the gene in a cell line are unknown. SAGE library analysis shows GRID2 expression in two brain libraries, Duke 1273 and BB542 whitematter, but no keratinocyte libraries.

6.1.2) Candidate 2: Novel Genes?

Within the minimal identified region there are two unigene clusters represented as Hs.162727 and Hs.1112936. Hs.162727 consists of two EST sequences, which combine to form a single contig of 438 nucleotides. BLAST searches with these two EST sequences do not however reveal any matching sequences in the databases. Sage libraries show variable expression levels, which are highest in one normal cerebellum sample and approximately 3-fold less expression in ovarian carcinoma, microvascular endothelial cells and a further brain sample. Hs.105416 consists of a single EST of 333 nucleotides, which does not match with any other sequences by BLAST search. Sage libraries show variable expression levels again although a breast cancer line (PTEN null) shows a greatly elevated tag count (although from a small tag set), normal epithelium shows a two-fold reduction in tags from PTEN null whilst colorectal carcinoma, ovarian carcinoma, primary colon tumour, and a breast ductal carcinoma show a four-fold reduction from PTEN null levels. Therefore both EST clusters do not seem to be obviously promising candidates although both Hs.162727 and Hs.105416 are expressed in normal tissue with a general reduction in tumour lines. Hs.105416 is found in PTEN null breast cancer lines at high levels whilst showing an overall expression

reduction in different tumour types (colorectal, ovarian, and breast carcinomas, and primary colon tumour). This may be indicative of the Hs.105416 locus gene product being under the repressional control of the PTEN tsg. Hs.162727 and Hs.105416 may therefore have candidate potential although more sequence data on these two clusters would be required before any further studies could take place.

6.1.3) Candidate 3: KIAA1122

The unigene cluster Hs.21356 consists of 87 EST sequences which represent the protein KIAA1122 (Hirosawa *et al.,* 1999). This protein is expressed in a large number of tissue types; adipose, brain, breast, CNS, colon, ear, germ cell, heart, kidney, lung, pancreas, skin, testis, tonsil, uterus, embryo, connective tissue, lung, ovary although to differing degrees. PTEN null breast cancer demonstrates the greatest tag count (although from a low total tag count, normal cerebellum has approximately ¼ less tags, normal kidney cells and 293-IND have an approximate 2-fold reduction of tags whilst medullablastoma, prostate carcinoma, normal epithelia, and ovarian carcinoma display an approximate 4-fold reduction, and adenocarcinoma, and Duke H54 lacZ have a 5-fold less count. This wide and varied distribution of expression makes any immediate judgements difficult to formulate and must await further data. The KIAA1122 protein shares homology with a number of proteins from different species and belongs to the highly conserved SWI/SNF family of ATPase proteins with putative helicase function. Members of this family are seen in *H.sapiens, M.musculus, G.Gallus, D.melanogaster, C.elegans, S.cerevisiae, S.pombe, A.thaliana,* and *P.falciparum.* The greatest homology to KIAA1122 is shared with the *M.musculus* Enhancer Trap Locus-1 (ETL-1) gene, which may therefore share functional similarities with KIAA1122.

Enhancer trap locus-1 (ETL-1) shares the greatest sequence homology with KIAA1122 with approximately 92% aa sequence similarity. ETL-1 demonstrates widespread expression particularly in the CNS and epithelia

and also shows homology to members of the SWI/SNF (yeast mating type switching/sucrose non-fermenting) ATPase/helicase family including *Brahma*, *D.melanogaster* trithorax homeotic gene regulator, SNF2/SWI2, yeast transcriptional activator (Tamkun *et al.,* 1992), RAD54 (recombination repair), STH-1 and MOT1, yeast helicase-related proteins. No family members display observable helicase activity suggesting this may not be a functional domain. SWI/SNF proteins form complexes of approximately 2MDa in both yeast and mammals where many components share homology (Wang *et al.,* 1996). ETL-1 was the first mammalian member of this now large (over 100 members) group of proteins which are implicated in gene regulation, chromatin structure remodelling (Soininen *et al.,* 1992) and mitotic chromosome segregation, all containing a conserved region required for binding and hydrolysis of ATP (Henikoff, 1993). ETL-1 belongs to a distinct subfamily also containing FUN30p, neither however is essential for cell viability or is ETL-1 required for DNA repair (double-strand breaks or pyrimidine dimer excision) both therefore having relatively undefined biological activity (Ouspenski *et al.,* 1999; Schoor *et al.,* 1999). ETL-1 knockout mice do demonstrate a number of post-embryonic developmental defects, which would suggest a role in transcriptional switching as development proceeds (Schoor *et al.,* 1999). SNF2 and *Brahma* are strikingly similar being of both a similar size (194 v's 185 kDa), sharing 57% homology and containing patterns of motifs typical of helicases (Laurent *et al.,* 1992). The SNF2 family member MOT1 acts to dissociate TATA-binding protein (TBP) from DNA in an ATP-driven manner with a minimum of two conformational changes and hence regulate transcription, which may provide a model behind the SNF2 ATPase domain functional mechanism (Auble *et al.,* 1997). This mechanism led to the theory that SWI/SNF complexes acted as ATP-driven DNA translocases, which disassociated proteins from DNA thereby disrupting nucleosome structure and facilitating transcription factor binding to DNA. However, the observation that MOT1 did not act as DNA translocase but rather dissociated TBP in a transient DNA-binding ATP-driven manner suggests a more complex situation. Further clarification of the mode of SWI/SNF complex action should reveal a great deal about chromatin remodelling and transcriptional control. As a candidate mortality gene

KIAA1122 may display a number of desirable characteristics although its expression pattern may not fit an obvious expected pattern with no ubiquitous down-regulation in tumour lines. However, further biological data on functional roles of the protein should provide useful insight into transcriptional mechanics.

6.1.4) Candidate 3: Prostaglandin D Synthase

The glutathione requiring hematopoietic prostaglandin D synthase (H-PGDS) catalyses the isomerisation of PGH2, a common prostanoid precursor, to PGD_2 (Urade *et al.,* 1995) and is the key enzyme for D and J prostanoid production which are involved in the immune system and mast cells. H-PGDS has functions in various peripheral tissues such as platelet aggregation prevention, induction of vasodilation, and bronchoconstriction (Giles and Leff, 1988). PGD_2 is further converted to 9α, 11β-PGF_2, which has various pharmacological actions and to PGJ_2, which is then further converted in the presence of serum albumin. The J series of PGs were shown to have an antiproliferative effect on tumour cells through induction of G1 arrest in neuroblastoma cell lines by N-myc suppression (Marui *et al.,* 1990) and a growth rate reduction in transformed epithelial cells and melanoma (Bregman *et al.,* 1986; Ikai *et al.,* 1991). *H-PGDS* spans approximately 41kb and consists of six exons divided by five introns and was the first recognised vertebrate homologue of sigma-class glutathione S-transferase (Kanaoka *et al.,* 1997, 2000). In SAGE expression libraries there is no differentiation between normal and tumour tissues with both demonstrating relatively low levels. H-PGDS has recently been shown to undergo transcriptional control through an OCT-1 element in the 5'-flanking region and an AP-2 site within the untranslated first exon (Fujimori *et al.,* 2000). The differential expression patterns observed with H-PGDS do not immediately suggest candidate gene potential although a number of other factors could potentially suggest otherwise. Downstream molecules have been shown to have an antiproliferative effect on tumour growth and furthermore the H-PGDS promoter and non-translated regions have been shown to be recognition sites

for at least two transcription factors, which suggests a tightly regulated control of H-PGDS expression. Abrogation of H-PGDS could then conceivably have a role in tumourigenesis although only further biological experimentation will reveal what this may be.

6.1.5) Candidacy Potential

A danger lies in identifying novel genes or identifying a candidate to fill a role, in that it can be easy to assume potential candidacy from the greatest volume of available data and hence disregard others for whom the data sources remain sparse. Therefore from the four potential candidate genes in the identified loci in the absence of further biological data only tentative assumptions should be made.

GRID2 induces apoptotic cell death in a p53-independent manner which itself fits with our observed phenotype. GRID2 expression has not been shown in keratinocyte libraries which would infer against a required abrogation for tumourigenesis, although would not rule out inappropriate expression in keratinocyte cell lines causing the crisis-like growth-arrest.

The potentially novel genes represented by Hs.162727 and Hs.105416 unigene clusters do not have any direct biological evidence to suggest candidacy. Tissue expression analyses suggest that they have appropriate profiles for genes with down-regulated functions in tumourigenesis through silencing or mutation by their high and low expression levels in normal and tumour tissues respectively.

KIAA1122 has a degree of biological characterisation through assumptions based on homology studies with the *M.musculus* homologue ETL-1. The studies with ETL-1 suggest it is part of a larger SWI/SNF complex, which is comprised of multiple proteins and conserved through to yeast. These complexes are involved in chromatin remodelling, transcriptional control and mitotic chromosome separation all of which have

obvious potential roles in tumourigenesis. The expression patterns observed with KIAA1122 do not fit an obvious role in tumourigenesis although tumour stage and specificity may account for this.

H-PGDS does not assume obvious candidate potential, it has relatively ubiquitous low expression in both normal and tumour tissue. It functions mainly in the peripheral tissues and in the immune system although down stream products have been shown to induce an uncharacterised G1 growth-arrest in neuroblastomas and to inhibit growth in transformed epithelial cells and melanomas which may be indicative of a loss advantageous to tumourigenesis. As such, H-PGDS can also be considered a potential candidate although further work is clearly required.

From the four candidates perhaps *GRID2* and *KIAA1122* emerge as potentially the most inviting genes to account for the observed phenotype. However each of the potential candidate genes requires a biological assay in the previously used cell types to determine whether a similar phenotype can be observed and whether mutations or methylation silencing are present in those immortal lines that show LOH in the region in question, thereby fitting Knudson's 'two-hit' model for an abrogated in cancer gene.

6.2) Mortality Factor from chromosome 4

The early characterisation of a potential gene on chromosome 4 with the ability to reverse an immortal phenotype was undertaken in cell-cell fusions and then by MMCT into responsive cell lines (Pereira-Smith and Smith, 1988; Ning *et al.,* 1991). In early 1998 a report suggested that through a serendipitous event the gene responsible for the earlier observed growth-arrest phenotype had been cloned and was called Mortality Factor from Chromosome 4 (*MORF4*) (Ehrenstein, 1998). MORF4 was observed to induce growth-arrest in two cell lines (HeLa and T98G) which had responded in a similar manner to the intact chromosome 4 and a cell line (HeLa) which

responded to an 800kb fragment of chromosome 4 containing MORF4 (Bertram *et al.*, 1999, 1999b).

Through collaborative ties we were granted access to the 800kb fragment of chromosome 4 containing *MORF4* (A9-F4) and to plasmids containing the full length *MORF4* construct, a *MORF4* frameshift mutant (*MORF4*-FS), and the empty vector, pexSVneo. We first performed MMCT of A9-F4 into the recipient cell lines BICR6 and HeLa to ascertain whether the subchromosomal fragment mimicked the whole chromosome effect. The A9-F4 fragment had no effect on BICR6 and did not cause any growth-arrest phenotype in 9 hybrids and furthermore the exogenous *MORF4* gene was retained (Bryce *et al.*, 1999). Upon introduction of A9-F4 into HeLa we observed growth-arrest in 64% (9/14) of hybrids. Although we could reproduce the previously reported effect in HeLa cells we could observe no discernible effect on BICR6, results are shown in Table 6.1. We then undertook a series of transfections into HeLa, BICR6, and BICR3 to determine if the full-length construct would have any effect. The full length construct caused 86% (6/7) Hela clones to undergo growth-arrest in a similar manner to that observed with A9-F4, flattened and positively staining for SA-βgal. In BICR6 we observed 0/12 colonies undergo growth-arrest with the full-length construct, 0/12 colonies undergo growth-arrest with MORF4-FS, and 0/10 with pexSVneo. The full-length construct caused 0/3 BICR3 clones to undergo growth-arrest. These results are shown in Table 6.2. To determine that the transfections had been successful we PCR amplified the plasmid Neomycin resistance cassette, result shown in Figure 6.2, and were able to show that all clones, with one exception, had uptaken a copy of the plasmid. We were unable to pursue expression analysis through RT-PCR of MORF4 in the transfectant clones as the *MORF4* and *MRG15* sequences (see below) are indistinguishable and the ubiquitous expression of *MRG15* would have made such detection highly unlikely. We were unable to amplify the *MORF4* cassette from the plasmid pexSVneo through difficulties encountered in plasmid sequencing which demonstrated that the insert had been ligated into an incorrect site, XbaI rather than NheI, which placed

Table 6.1. MMCT with A9-F4 does not induce growth-arrest BICR 6. MMCT with chromosome 15 does not induce growth-arrest in HT1080 or HeLa

Due to the lack of growth-arrest observed following monochromosome transfer of A9-F4 into BICR6 we were able to conclude that MORF4 was not the keratinocyte mortality gene. Chromosome 15 also failed to induce growth-arrest in both HeLa and HT1080 immortal cell lines demonstrating an inability to functionally complement any dysfunctional pathways.

Chromosome	Cell Line	Total Growth-Arrested Hybrids	Total Growth-Arrested Hybrids (%)
A9-F4	BICR6	0/9	0%
A9-F4	HeLa	9/14	64%
A9HYTK15	HT1080	0/11	0%
A9HYTK15	HeLa	1/11	9%

Table 6.2. Transfection of MORF4 into keratinocyte lines suggests it is not the chromosome 4 mortality gene

The failure of MORF4, following transfection into BICR6, to induce a growth-arrest phenotype similar to that seen following MMCT of chromosome 4 into the same cell line implies that it is not the keratinocyte mortality gene. The growth-arrest phenotype observed in HeLa may be a consequence of plasmid-driven MORF4 overexpression.

Transfection	Cell Line	Total Growth-Arrested	Total Growth-Arrested (%)
MORF4	HeLa	6/7	86%
MORF4	BICR6	0/12	0%
MORF4FS	BICR6	0/12	0%
pexSVneo	BICR6	0/10	0%
MORF4	BICR3	0/3	0%

Figure 6.2. Amplified vector Neo^r cassette in MORF4 transfectants

BICR3, BICR6, and HeLa MORF4 transfectants all demonstrated amplification of the plasmid Neomycin resistance cassette and therefore their transfectant status.

100kB DNA Ladder
Vector + MORF4
Vector + MORF4-FS
Vector Only
Blank
BICR6
MORF4.1
MORF4.2
MORF4.3
MORF4.4
MORF4.5
MORF-FS.1
MORF-FS.2
MORF-FS.3
MORF-FS.4
MORF-FS.5
MORF-FS.6
Vector.1
Vector.2
Vector.3
Vector.4
Vector.5
BICR3
BICR3.1
BICR3.2
BICR3.3
HeLa
HeLa.1
Neo^r

MORF4 in an alternate multiple cloning site (see Chapter 2, Figure 2.1). This therefore raised concerns surrounding plasmid integrity and as such we did not pursue this line of investigation. We concluded from the MMCT introduction of A9-F4, and tentatively from the MORF4 transfections, that MORF4 was not the keratinocyte growth-arrest gene although we did see a phenotype in HeLa.

MORF4 is a processed pseudogene whose direct lineage ancestor is MRG15, which is located on chromosome 15. We introduced chromosome 15 into BICR 6, as described in Chapter 4, and observed 0/33 growth-arrested hybrids. We then introduced chromosome 15 into two further cell lines HT1080, and HeLa. HT1080 is a cell line, which was described as not belonging to the same subset of cell lines that HeLa was part of and therefore not thought to have a mutation of the growth-arrest gene on chromosome 4. Chromosome 15 did not have any noticeable effect on HT1080 in 11 hybrids examined although did induce growth-arrest in 9% (1/11) of HeLa hybrids. HeLa has a background clonal attenuation rate of approximately 10% (Bertram *et al.,* 1999), which may be due to a down-regulation of telomerase in these cells (Bryan *et al.,* 1998) and this can therefore be taken as an artifact of the experimental conditions.

We concluded from the above experiments that MORF4 was not the keratinocyte mortality gene. Furthermore in MMCT experiments with the irradiated fragments, A92i32 and i33 both induced a growth-arrest phenotype on BICR6 and neither chromosomal fragment contains MORF4 (see Chapter 4). Furthermore MORF4 did not lie within our minimal identified region and was not one the four candidate genes, which we had identified.

Chapter 7

7.1) Evidence for a Gene Abrogated in Cancer on Chromosome 4

Evidence for a locus on chromosome 4 whose loss was beneficial to tumourigenesis was first provided by subcutaneous injections of hybrids resulting from cell-cell fusions between normal human fibroblasts and the human fibrosarcoma cell lines into athymic mice (Benedict *et al.,* 1984). The tumourigenic potential of the HT1080 cell line was suppressed following these fusions although in instances where tumours did emerge this was correlated with the loss of both chromosomes 1 and 4 implying a loss beneficial to tumour formation. A further implication from these studies is that on the remaining copies of the lost chromosomes 1 and 4 the loci responsible for suppression had undergone silencing through mutation or methylation. These studies were reinforced through further cell-cell fusions between subsets of immortal tumour lines which were matched on the basis of whether they ceased proliferation following fusion, implying the complementation of a gene lost during tumourigenesis by one of the fused lines, or continued proliferating indefinitely, suggesting no complementation had occurred and the fused lines had achieved immortality through abrogation of distinct mechanisms (Pereira-Smith and Smith, 1988). MMCT based introduction of chromosome 4 into a grouped subset of these lines (HeLa, J82, and T98G) identified chromosome 4 as capable of inducing the previously observed growth-arrest and therefore of complementing the pathway which had undergone abrogation during the progression to immortality (Ning *et al.,* 1991). The growth-arrested hybrids resulting from MMCT introduction into HeLa were predominantly shown to contain an intact exogenous chromosome 4 through the use of polymorphic markers, which showed the necessity of the locus complementation for the phenotype. The line, HT1080, previously observed to escape from suppression to form tumours in athymic mice was, perhaps surprisingly, not reported to undergo growth-arrest upon introduction of chromosome 4. However in later studies HT1080 was observed to undergo growth arrest following MMCT introduction of chromosome 4 whereas T98G did not display any observable cessation of proliferation (Dr. S. Bryce, BICR, personal

communication). The further observation that HeLa did not undergo growth-arrest following cell-cell fusion with a complementing cell line (Berrington, 1999) and that suggests that the earlier subset assignments may have been an oversimplification and that a great deal of complexity surrounds the abrogated growth-arrest mechanisms in immortal tumour lines (Whitaker *et al.,* 1992; Duncan *et al.,* 1993). The evidence provided by these observed cell-cell fusions and MMCT experiments led to the conclusion that chromosome 4 contained a locus which was capable of inducing a replicative senescence-like growth arrest although no further locus identification was performed in these studies.

7.2) Chromosome 4 Induces a Mortal Growth-Arrest in Immortal HNSCC

Through MMCT of chromosome 4 into immortal keratinocyte cell lines (BICR6, BICR31) we have been able to agree with earlier observations and demonstrate an exogenous chromosome 4-specific growth-arrest. We have added to this demonstration by showing that this only occurred in lines demonstrating LOH on the endogenous chromosome 4q arm. This would suggest that the shorter p arm of chromosome 4 does not carry the gene responsible for this phenotype but does not rule out the existence of genes involved in growth-arrest on this chromosomal arm, which may be abrogated in other pathways to immortality. The specificity of the chromosome 4-induced growth-arrest was further defined through the generation of truncated fragments of chromosome 4 and consequent introduction into an immortal keratinocyte cell line (BICR6), which defined a region on chromosome 4 defined by 4cen and 4q23. As growth-arrest was observed following MMCT of truncated chromosomes, which lacked 4cen-p16 and 4q23-q35 this, implies that the gene responsible for the phenotype, which we observed, did not lie in these chromosomal regions. This is significant as no specific LOH was observed in the HNSCC lines on 4p (see Section 3.) although it had been implicated by other studies. Furthermore, a number of studies had implicated regions within 4q25-q35 as potential sites for cancer genes although these

were not implicated in this study. The specificity of this growth-arrest was further demonstrated by the MMCT introduction of a subset of chromosomes (A9-F4, A9HYTK6, A9HYTK11, A9HYTK15 (see Chapter 2, Section 2.1) into the immortal keratinocyte cell line, BICR6, which demonstrated that the growth-arrest phenotype was specific to the 4cen-q23 locus and was not an artifact of the experimental procedure such as the resistance gene selection or murine material. Introduction by MMCT of chromosomes 11 and 15 into the immortal keratinocyte line BICR31 showed that although 11 had no effect a growth-arrest induction was observed following transfer with 15. This is suggestive of a further pathway abrogation present in the BICR31 immortal keratinocyte line, which is restored by the introduction of chromosome 15. The recent demonstration of the chromosome 15 Pif1p helicase, yeast homologue, causing inhibition of telomerase and telomeric shortening perhaps provides a candidate for this phenotype (Zhou *et al.*, 2000). Furthermore, in immortal keratinocyte cell lines which did not display LOH on the endogenous chromosome 4 copy (BICR3, BICR19) we did not observe any growth-arrest following on from MMCT introduction of an exogenous copy of the chromosome. The lack of observed phenotype in those lines which displayed ROH of the endogenous chromosome 4 copy further demonstrate the specificity of the phenotype to those cells displaying LOH and rule out the phenotype occurring as both a consequence of introduction of any exogenous chromosome, through the selection agent, as a dosage effect, and through other artefacts of the experimental system such as contamination of the human chromosome 4 with mouse material. Similar lines of study in HeLa using MMCT to introduce a subset of truncated chromosomal fragments suggest that the growth-arrest phenotype in these cells is located minimally to 4cen-q25 (Dr. S. Bryce, BICR, personal communication). This suggests that the earlier functional complementation observed with HeLa and HT1080 may be the same as that observed with the immortal keratinocyte lines. These lines of evidence suggest that 4cen-q23 carries a locus which is functionally abrogated in immortal HNSCC lines which show LOH in this region although further pathways may exist represented by loci on other chromosomes as the complementation did not occur in HNSCC cell lines which displayed intact exogenous chromosome 4 copies.

7.3) LOH Analysis in Immortal HNSCC lines Demonstrates Losses in the Locus Identified Through Functional Complementation

LOH on chromosome 4 has been shown in a number of studies and in a number of different forms of cancer. 4qcen-25, as described in Section 7.2, represents the region of chromosome 4 we identified through functional complementation. Studies which show LOH or copy number decrease in this region come from 4q21-q23 (CC), 4q21.1-q22 (EA), 4q21.3-q22 (ESCC), 4q21 (HNSCC), 4q21-q22, 4q11-q25 (HCC), 4q11-q23 (SCLC), 4q21 (Sclerotylosis), 4q23 (IBD), and 4q21 (ADPKD) (Buetow *et al.,* 1989; Mitra *et al.,* 1994; Pershouse *et al.,* 1997; Bando *et al.,* 1999; Petersen *et al.,* 1997; Cho *et al.,* 1998; Rumpel *et al.,* 1999; Torra *et al.,* 1999; Hu *et al.,* 2000; Lee *et al.,* 2000). Although studies of chromosome 4 LOH have revealed other regions on the chromosome as implicit in tumour development we had demonstrated that the functional complementation was due to a locus in the 4cen-q25 area by demonstrating a retention of phenotype where all other regions where missing. We carried out an LOH study against a panel of immortal HNSCC keratinocyte cell lines and demonstrated loss at 4cen-q25 in half of those lines tested. This suggested that although there was a clear correlation between LOH of this region and a functionally complemented growth-arrest this was clearly not the case in all the cell lines which may have abrogated other pathways on other chromosomes. Difficulties lie in the interpretation of LOH studies where a small number of polymorphic markers have been used to analyse the endogenous chromosomal heterozygosity. Where a small number of markers have been utilised and interpreted as whole chromosomal losses this can clearly lead to inaccurate reporting as any interstitial losses are undiscovered or alternatively retentions are missed through smaller losses.

The microsatellite analysis of BICR31/Chromosome 4 hybrids revealed complex break patterns and a general exogenous chromosomal instability. This itself may be a response to the exogenous chromosome 4 specifically as in studies involving the introduction of chromosome 6 into BICR31 through

MMCT the majority of hybrids (63%, 12/19) retained intact exogenous chromosomes (Dr. S. Fitzsimmons, BICR, personal communication). With chromosome 4 the number of intact chromosomes was extremely minimal (9%, 1/11) implying a different response of the recipient cell line to particular exogenous materials. The observed phenotypes in both BICR6/ and BICR31/Chromosome 4 hybrids were both sufficiently similar to appear to be due to the same mechanism (Figure 5.1 and Figure 5.2). This was further evidenced, as the ectopic hTERT catalytic subunit was not sufficient to block the phenotype in either cell line. These lines of evidence suggest that a similar mechanism is operating in both BICR6 and BICR31 hybrids which, when functionally restored, drives the cells into a crisis-like growth-arrest hall-marked by multinucleation and unsuccessful cytokinesis followed by apoptosis in an apparently telomere-independent manner. This further suggests that the restored mechanism recognises the aberrant nature of the immortal keratinocyte HNSCC-derived cell lines and drives them into the apoptotic pathway.

The strategy of segregant hybrid loss map construction to highlight regions whose deletion is advantageous to tumour growth or disorder progression has met with mixed success in previous studies (Leach *et al.*, 1989; Killary *et al.*, 1992; Rimessi *et al.*, 1994; England *et al.*, 1996; Newbold and Cuthbert, 1996; Nihei *et al.*, 1996; Sandhu *et al.*, 1996; Zhu *et al.*, 1998; Berrington, 1999; Miele *et al.*, 2000). This approach led to the successful confirmation of $p16^{INK4A}$ and cloning of SURF1, a tsg and a factor involved in Leigh syndrome (neurological disorder) respectively. In our study we have used this approach to identify a minimal region within 4cen-q25 containing a discrete number of potential candidate genes. Analysis of segregants arising from whole and truncated chromosome 4 MMCT experiments has defined a locus on 4q22.3-q23 of approximately 1.5Mb defined by the microsatellite markers D4S423 and BIR0101. This locus has been implicated in studies on CC, EA, ESCC, HCC, HNSCC, SCLC, and Sclerotylosis (see Chapter 3, Section 3.). This locus could carry a gene whose function is abrogated in a number of different tumours and disorders although only further biological analysis can determine this. A number of hybrids with seemingly intact

exogenous chromosomes have presumably silenced the target gene through microdeletions on the introduced copy or methylation silencing. Further analysis has revealed the existence of interstitial losses within the target locus in at least one hybrid which otherwise appears completely intact (data not shown). The benefits of this mapping strategy have limitations and the density and informative nature of these markers provide two such examples. Figure 4.15 demonstrates that across the minimal identified locus spanning D4S2964 and BIR0101, 34% (10/29) of markers were not informative as judged under denaturing sequence gels. These non-polymorphic markers cause increasing difficulty in locus determination. The non-polymorphic marker problem and the minimal physical distance drastically limit this mapping strategy. The position we have arrived at may in fact represent the endpoint of this strategy, albeit a relatively successful endpoint. Further deletions are likely to be within the coding sequence of the target gene itself, these will not be discovered until further sequencing and exon analysis strategies are introduced. Precedence for microdeletions exist such as those evidenced for $p16^{INK4A}$ where deletion of a single exon was shown after MMCT introduction of chromosome 9 into murine cells (England *et al.*, 1996).

The hygromycin resistance cassette is thought to be located in the proximity of D4S409 which itself is an explanation for the number of hybrid segregants which retained this region. Due to the close proximity of our identified locus to the resistance cassette this suggests that the generation of a truncated chromosome 4 fragment through XMMCT, which did not carry the locus, was extremely unlikely although not impossible. This technical problem goes some way to explaining our inability to generate a truncated chromosome, which did not carry the growth-arrest phenotype. The majority of the generated truncated nuclei did exist as independent chromosomes on the murine background, with the exception of A9i32 and possibly A9i33, and therefore discredit the possibility of a murine effect. Previous MMCT control experiments demonstrated the absence of a non-specific murine chromosome-effect. In the situation of a fused human-mouse chromosome entering a recipient cell there would be no pressure for the maintenance of the murine material, which would result in rapid loss through segregation or

deletion. However the retention of markers telomeric to the identified locus in BICR6 segregants demonstrates the specificity of the observed losses to the abrogation of the growth-arrest phenotype. The control lines analysed had no complex deletion patterns in the minimally lost region, which is further suggestive of specificity to those cell lines with demonstrable LOH on 4q22.3-q23. Analysis of HeLa segregants arising from whole and truncated chromosome 4 transfers also implicate the identified region on 4q22.3-q23 as being lost at greater frequencies than other sites and therefore likely to be where the locus responsible for the phenotype lies (Dr. S. Bryce, BICR, personal communication).

Through the approach outlined above we have identified the locus spanning 4q22.3-q23 as the target site of deletion in immortal HNSCC variants. The possibility exists that the strategy had successfully mapped a recombinational hotspot characterised by a common breakpoint. However this is unlikely as evidenced by the genetic distance being much less than the physical distance (see Chapter 4, Figure 4.15). In the instance of a recombinational hotspot the reverse would be expected with genetic distance exceeding the physical distance through frequent recombinations with other sites. However, as the genetic distance is the lesser of the two values (0.1cM v's 1.5Mb) this would indicate a stable region suggesting against a breakpoint. Statistical analyses of the marker losses also argue against these occurring in a non-specific manner. Analysis of markers spanning 4cen–q23 demonstrates that losses in the region identified are the most significant by both Fishers Exact and Chi^2, this demonstrates that these losses are occurring at a far greater frequency than background losses.

7.4) 4q23 is linked to human SCC

Sclerotylosis is an autosomal dominant genodermatosis characterised by congenital scleroatrophy of the distal extremities and early onset aggressive SCC in the third to fourth decade of life followed sharply by metastasis formation (Huriez *et al.*, 1968; Hamm *et al.*, 1996). The

pathogenetic mechanism behind tumourigenesis in sclerotylosis is still however, unknown. Linkage analysis of familial syndromes has lead to the discovery of a number of important cancer genes (see Chapter1, Section 5.3). A linkage study of two families in the north of France revealed a locus on chromosome 4 displayed a maximum lod score of 12.22, which is highly significant (Lee *et al.,* 2000). The locus identified in this study ranges from D4S414 to D4S1591, which lies on 4q21-q24 and therefore also contains the locus, which we have identified. As sufferers of this syndrome develop early-onset SCC of the extremities, the gene responsible for this syndrome could act in a rate-limiting manner, as described previously for the prototypic 'gatekeeper' gene *APC* in colorectal carcinoma (see Chapter 1, Section 1.2). These lines of evidence are intriguing, and it remains possible that the autosomal dominant sclerotylosis gene could be the same gene responsible for the conversion of immortal HNSCC to a mortal phenotype.

7.5) Nature of the Chromosome 4 Mortal Phenotype

Previous definitions of the growth-arrest phenotype induced by chromosome 4 suggested a flattening of cells with a concomitant cessation of growth potential, both features reminiscent of replicative senescence (Pereira-Smith and Smith, 1988; Ning *et al.,* 1991). Through analysis of growth-arrested BICR6/chromosome 4 hybrids we were able to define the observed phenotype as a delayed crisis rather than a senescent arrest. Inappropriate expression of SA-βgal at pH6 has been described as a biomarker of cells in growth arrest (Dimri *et al.,* 1995). Growth-arrested hybrids following MMCT of chromosome 4 into BICR6 and HeLa (Dr. S. Bryce, BICR, personal communication) both stain positively for this biomarker, this does not however distinguish between senescence and crisis. A biological definition of a senescent growth-arrest is a less than 5% nucleic acid analogue incorporation after 24hrs, or rather 95% senescent cells remaining out of cycle (Cristofalo and Sharf, 1973) and remaining resistant to apoptosis (Stein and Dulic, 1995). Cells approaching crisis have an initial nucleic acid analogue labelling index similar to that seen in controls although as crisis approaches the incorporation

plummets to levels similar to those seen in senescence but with a concomitant increase in levels of cell death through apoptosis (Stein and Dulic, 1995). We observed a nucleic acid analogue, BrdU, incorporation at a rate approximately 25% that of the parental immortal cell line controls after a 48hrs labelling pulse and an increased rate of apoptosis of approximately 4-fold that of parental immortal cell line controls as measured by the TUNEL assay. This enables us to conclude that the growth-arrested hybrids resulting from MMCT of chromosome 4, or fragments derived thereof, are undergoing a form of crisis with a mean nucleic acid incorporation of 25% further suggesting that these hybrids have a lifespan in culture following MMCT of approximately 14 weeks (Stein and Dulic, 1995) prior to apoptosis. The apoptotic and nucleic acid incorporation analyses were performed after 12 weeks in culture; through extrapolation to the values of Stein and Dulic this enables us to make this conclusion regarding culture lifespan. The TUNEL method of apoptosis detection has demonstrated a lack of sensitivity in previous studies (Labat-Moleur *et al.,* 1998), which raises the possibility that the actual rate of apoptosis occurring was higher than that observed. The above described apoptotic rates have also been observed in growth-arrested HeLa hybrids following introduction of chromosome 4 by MMCT (Dr. S. Bryce and Mrs H. Ireland, BICR, personal communication) which further suggests that the same mechanism is undergoing functional complementation in the immortal HNSCC lines with LOH on chromosome 4cen-q25 as in the HeLa cells. From the available biochemical data we were able to conclude that the growth-arrested hybrids were undergoing a form of crisis rather than senescent growth-arrest and that this phenotype was similar in both HNSCC immortal keratinocyte lines and the ovarian carcinoma line, HeLa. The immortal keratinocyte line BICR6 has silenced the *p53* gene through mutation, which suggests that apoptosis is induced in a p53-independent manner in an as yet unclear mechanism which has not yet been determined. However, recent data suggests that one route may operate through the p53 homologue, p73 (see Chapter 1, Section 3.2.3), which cannot be mechanistically ruled out at this stage.

7.6) Chromosome 4 does not Cause Crisis by Precipitating Renewed Telomeric Attrition

A common mechanism of crisis is through telomeric attrition through repeated proliferative divisions leading to chromosomal instability, an inability to prevent the telomeric end being recognised as a double-strand break, chromosomal fusions, and non-disjunction's during mitosis which ultimately lead to cell death through apoptosis. The immortal cell lines used all possessed positive telomerase activity which has been shown to maintain telomeres in a stable manner and whose activity is generally required to escape from crisis. Measurement of the telomere lengths of the lines used in the MMCT experiments did not show a clear correlation between the observed growth-arrest and recipient line telomere length. The cell lines, which responded to the exogenous chromosome 4, BICR6 and BICR31, had mean telomere lengths of less than 4kb and 4-7kb respectively (Dr. K. Gordon, BICR, personal communication). Assuming a maximal telomeric loss of 100bp per division and an observed growth-arrest following approximately 10 population doublings then the telomeres would decrease to a mean length of 3kb in BICR6 and 6kb in BICR31. Although we were not able to measure the telomere length in growth-arrested hybrids it is unlikely that this shortening would be sufficient to induce crisis. The telomeres in cells have been reported to shorten to 1.5kb prior to the induction of crisis (Counter *et al.*, 1998), which would suggest that the apoptosis must have been occurring in a telomere-independent manner. However the presence of a critically short telomere or exposed telomere end within the growth-arrested hybrid cannot be ruled out and this would possibly be sufficient to induce apoptotic death. The telomere lengths of BICR3 and BICR19 are 4-5kb and 15kb respectively (Dr. K. Gordon, BICR, personal communication) and neither of these lines responded to the exogenous chromosome 4 copy following MMCT. The implication from these findings is that the crisis growth-arrest which we characterised in BICR6 and observed in BICR31 is not telomere-based through a consequence critical shortening as insufficient doublings exist for sufficient telomeric attrition to occur to drive the cell into apoptosis. However, images of growth-arrested hybrids from both BICR6 and BICR31 suggest

cellular multinucleation resulting from failed mitotic separation and failed cytokinesis, which is suggestive of telomeric dysfunction (see Figure 5.1 and 5.2).

The catalytic subunit of the enzyme telomerase is a minimal requirement for escape from crisis and cellular immortality. This is achieved through the rescue of telomere ends above the minimal threshold length and stabilisation of the telomere ends through further unlimited cellular divisions. Both the cell lines, which responded to the exogenous chromosome 4, and those that did not, had positive telomerase expression and demonstrated LOH at chromosome 3p. When we introduced chromosome 4 using MMCT into the recipient lines BICR6 and BICR31, which had been stably infected with the a retrovirus plasmid construct carrying an ectopic hTERT catalytic component, we saw no abrogation of the crisis growth-arrest phenotype, which suggested further that the apoptosis was not telomere-linked. Although the telomere ends would theoretically be maintained by the ectopic hTERT if the locus on 4cen-q23 interfered with the endogenous hTERT and thereby prevent apoptosis we did not observe any growth-arrest rescue. Furthermore, measurement of the telomere lengths in BICR6hTERT and BICR31hTERT demonstrated that the ectopic hTERT had catalysed the addition of numerous telomeric repeats onto the ends of the chromosomes. The telomeres on BICR6 had elongated to approximately 20kb whereas those on BICR31 had reached approximately 25kb, from starting lengths of less than 4kb and 4-7kb respectively. We can conclude from this that the lengths of the telomeric repeats on the end of the chromosome are not obviously involved in the mortal phenotype, which we observed. Although the digestion of the telomere ends of the hTERT-infected BICR6hTERT and BICR31hTERT may be incomplete, this does not dissuade from both the observed phenotype and the overall general appearance of telomeric extension in these cells. This does not rule out an effect on a downstream component of the telomerase pathway, or alternatively an element involved in telomeric stabilisation, or t-loop formation. Through observations of growth-arrested hybrids the phenotype looks to act at mitosis causing inappropriate mitotic separation which suggests that the mechanism behind the growth-arrest may be a novel

pathway or an uncharacterised mechanism carried out by an existing pathway involved in this process.

As the observed growth-arrest phenotype does not occur through disruption of the telomerase hTERT catalytic subunit this leaves the obvious question, what is the mechanism behind the crisis-like phenotype in these immortal cells? An alternative telomeric mechanism cannot be ruled out at this stage as a number of different factors associate with and stabilise telomere ends and assist in t-loop formation. The potential mechanism could act through negative regulation of these telomere-associated proteins such as TRF-1, TRF-2. Negative regulation of TRF-1 (or PIN2 (Shen *et al.,* 1997)) could lead to an inhibition of 'telomeric curve' thereby preventing t-loop formation and masking the telomeric 3'-overhang allowing its recognition as DNA-damage, albeit in a p53-independent manner, leading to apoptotic cell death. Similarly negative regulation of TRF-2, which has been shown to lead to a DNA-damage apoptotic response (Karlseder *et al.,* 1999), would expose the 3'-overhang to damage-recognition factors. Both of these mechanisms could conceivably occur in the presence of telomerase activity, as telomeric extension would not prevent their recognition as DNA-damage due to the exposed telomere ends. A similar phenotype to that which we described; multinucleation, micronuclei, flattening, enlarged cytoplasm, and apoptotic death, has been described in Hela cells following introduction of chromosome 4 by MMCT (Dr. S. Bryce, BICR, personal communication). This phenotype has been induced in neonatal foreskin fibroblasts and HeLa cells through azelaic bishydroxamic acid (ABHA) (histone deacetylase inhibitor) treatment (Qiu *et al.,* 2000). This treatment leads to a loss of a G2 checkpoint resulting in aberrant mitosis and the previously described phenotype. Intriguingly the locus candidate gene KIAA1122 belongs to the SWI/SNF family, which has been implicated in chromatin remodelling and transcriptional control. The histone deacetylase inhibitor ABHA could conceivably cause the same phenotype as the reintroduction of a chromatin-remodelling gene could induce a retrospective silencing of inappropriate gene expression leading to the observed phenotype.

Evidence obtained by monitoring hybrids in limiting conditions suggested that the locus responsible for the crisis-like phenotype had the potential to undergo silencing in favourable growth conditions. Through treatment of chromosome donor cells with demethylating agents followed by introduction into the chromosome 4-responsive line BICR6 we did not see any alteration in the number of hybrids which displayed the characteristic growth-arrest phenotype. This would imply that the locus responsible does not undergo promoter silencing through methylation on the murine background. The hybrids in this experiment were not picked and seeded at minimal density in stringent conditions and so we cannot draw any conclusions on phenotypic rescue through promoter methylation in these hybrids. However, as silencing did seem to occur in hybrids with apparently intact exogenous chromosomes this would suggest that promoter methylation or microdeletions have occurred in these hybrids.

7.7) Candidate Genes

The dual approach of functional complementation and exogenous loss map construction in this instance has been a relative success in terms of this project. We have identified a minimal locus, strongly supported by several lines of evidence, which contains a number of candidate genes. These genes have been identified through construction of EST clusters and sequence alignment. However, this does not rule out the possibility of inappropriate expression by an otherwise silent locus for which no EST exists. This situation has precedence, as no EST exists for $p14^{ARF}$ due to its lack of expression in ordinary situations. The three candidate genes, which we have identified; *KIAA1122*, *GRID2*, *PGDS* and the two EST clusters could all potentially be the target of silencing in advanced HNSCC and other tumour types. However the lack of expression of GRID2 and PGDs in normal keratinocytes does argue strongly against the likelihood of either of these genes being responsible for the observed phenotype.

The *KIAA1122* gene has a number of characteristics, all derived through family member homologs, which do not preclude it from candidacy. The immediate homologue of *KIAA112* is the *M.musculus ETL-1*, which is a member of the larger family of SWI/SNF chromatin ATP-dependent remodelling complexes. ETL-1 shares homology with a number of members of this large family although may function in a distinct manner. The *brahma D.melaongaster* gene is one of these homologs and its direct homologue BRG1 (Khavari *et al.,* 1993) has been shown to be directly associated with the RNA polymerase II holoenzyme suggesting an involvement in transcription. *Brahma* itself has been shown in *D.melanogaster* to be a requirement for E2F transcriptional activity suggesting a conservation of function between these two species (Staehling-Hampton *et al.,* 1999). Furthermore, when BRG1 was overexpressed in mammalian cell lines it induced a senescence-like growth-arrest, which was overridden by the ectopic expression of cyclin D and cyclin E (Shanahan *et al.,* 1999). These lines of evidence all suggest a role played by SNF2 family member, BRG-1, in transcriptional regulation at the G1/S-phase of the cell cycle. A further SWI/SNF family member SNF5 was demonstrably shown to be a leading candidate for an initiating event in malignant rhabdoid tumours (MRTs), which occur early in childhood (Versteege *et al.,* 1998). These paediatric tumours demonstrated frequent deletions of one copy of the *SNF5* gene and frequent somatic mutations of the other thereby fitting the stringent characteristics of a tsg. Further mutational studies revealed mutations in most rhabdoids, choroids plexus carcinomas, primitive neuroectodermal tumours, and medulloblastomas. Mutations were not demonstrated in breast cancers, Wilms' tumours, glioblastomas, ependymomas, or sarcomas although most analysed cases showed LOH at the *SNF5* loci. Family members of the SWI/SNF multiprotein complexes have been demonstrated to exert a control of transcriptional regulation through chromatin remodelling and of being mutational targets in tumours. These are family members, however, this does not necessitate the involvement of KIAA1122 in tumourigenesis as distinct functions are demonstrated within protein family groupings. As a member of a large multiprotein complex forming family this gene may represent a novel target of mutation, downstream effects could lead to dysregulation of transcriptional control and

therefore, proliferation. Intriguingly the prototypic family members in yeast were termed SWI/SNF, for yeast mating type switching (SWI) and sucrose non-fermenting (SNF). As described in Chapter 1 (Section 4.5) calorific restriction has been proposed as a mechanism to prolong lifespan in yeast. Although this has not been conclusively demonstrated in mammals it remains possible that the mutation of these genes could represent the abrogation of an evolutionarily conserved mechanism whose disruption allows a lifting of metabolic control and the potential to accumulate damaging mutations. A further SWI/SNF2 family member is the RAD54 protein, which is involved in DNA double-strand break homologous repair (Dasika *et al.,* 1999) RAD52 epistasis group. Correct functioning of the repair complex requires the presence of a number of group members including RAD54 (Tan *et al.,* 1999). RAD54 could therefore fit the 'caretaker' classification, as mutation of this gene would allow the accumulation of unrepaired DNA double-strand breaks, which could stimulate an apoptotic cellular response. There are therefore a number of possible roles the ETL-1 homologue, KIAA1122, could have in tumourigenesis through sharing common domain structures with SNF2 family members. KIAA1122 could have a unique role however and homologue analysis can only serve as an indicator of possible function.

7.8) Future Plans

Differentiation between these candidates will require a number of further analytical strategies. Candidate BACs and cDNAs will have to be introduced into target cell lines, which have shown a response to the intact and truncated chromosomes and observed for phenotypic alterations i.e. BICR6 and HeLa. Conversely these will have to be introduced into cell lines which do not show a response to the intact chromosome i.e. BICR19 and the osteosarcoma line, 143B (Dr. S. Bryce, BICR, personal communication). Once phenotypic alteration has been established mutation screening would be required throughout immortal HNSCC lines where LOH has been established in this region along with methylation-specific PCR, where appropriate, to establish if alternative silencing has occurred. These analyses will determine if the candidate genes fit the criteria of Knudson's model for genes abrogated in cancer. Further determination will of course be required if a candidate fulfils the criteria to establish pathways to which the gene belongs and mechanisms through which it exerts its functions. This can be determined through protein-protein interaction assays such as yeast two hybrid screens, immunoaffinity chromatography, and immunoprecipitation, and also generating an epitope-flagged molecule to determine cellular localisation. Double-knock-out mice could be generated eventually (p53-null as the cell lines respond in a p53-independent manner), if the mechanism operates through destabilisation of the t-loop and 3'-overhang masking then presumably a phenotype would be observable in a murine system as this would bypass the situation encountered in telomerase-deficient mice.

7.9) Conclusions

The importance of a gene whose abrogation presumably can assist in the immortalisation of advanced tumours and whose reintroduction can revert the cells to a mortal phenotype could be of clinical significance. Mortal tumours can undergo growth-arrest through replicative senescence although abrogation of key gene pathways, p53 and pRB, can afford escape from this

growth-arrest allowing, through abrogation of hTERT expression and escape from crisis, progression into cellular immortality. Advanced cancers are frequently aggressive and immortal which minimises both patient survival rates and quality of life. Through three lines of evidence we have identified a locus on chromosome 4, which is responsible for a mortal growth-arrest in immortal HNSCC lines. This has been achieved through LOH analysis, functional complementation, and segregant loss map construction. Others have also implicated the identified locus through linkage analysis, in familial early-onset SCC, which suggests an involvement in tumourigenesis. The chromosome 4 mortality gene may therefore provide an opportunity to identify a novel cellular mortality mechanism, which induces both growth-arrest and apoptosis in immortal cells regardless of genetic status or hTERT expression. This could provide a novel mechanism to target advanced tumours in a p53-independant, hTERT-independent manner and therefore minimise non-specific cell toxicity's, which occur as a by-product of conventional therapies.

References

Agochiya, M., Brunton, V. G., Owens, D. W., Parkinson, E. K., Paraskeva, C., Keith, W. N., and Frame, M. C. (1999). Increased dosage and amplification of the focal adhesion kinase gene in human cancer cells, Oncogene *18*, 5646-53.

Ah-See, K. W., Cooke, T. G., Pickford, I. R., Soutar, D., and Balmain, A. (1994). An allelotype of squamous carcinoma of the head and neck using microsatellite markers, Cancer Res *54*, 1617-21.

Alani, R. M., Hasskarl, J., Grace, M., Hernandez, M. C., Israel, M. A., and Munger, K. (1999). Immortalization of primary human keratinocytes by the helix-loop-helix protein, Id-1, Proc Natl Acad Sci U S A *96*, 9637-41.

Albino, A. P. (1995). Genes involved in melanoma susceptibility and progression [see comments], Curr Opin Oncol *7*, 162-9.

Alcorta, D. A., Xiong, Y., Phelps, D., Hannon, G., Beach, D., and Barrett, J. C. (1996). Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts, Proc Natl Acad Sci U S A *93*, 13742-7.

Allsopp, R. C., Vaziri, H., Patterson, C., Goldstein, S., Younglai, E. V., Futcher, A. B., Greider, C. W., and Harley, C. B. (1992). Telomere length predicts replicative capacity of human fibroblasts, Proc Natl Acad Sci U S A *89*, 10114-8.

Artandi, S. E., Chang, S., Lee, S. L., Alson, S., Gottlieb, G. J., Chin, L., and DePinho, R. A. (2000). Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice [see comments], Nature *406*, 641-5.

Artandi, S. E., and DePinho, R. A. (2000). Mice without telomerase: what can they teach us about human cancer?, Nat Med *6*, 852-5.

Auble, D. T., Wang, D., Post, K. W., and Hahn, S. (1997). Molecular analysis of the SNF2/SWI2 protein family member MOT1, an ATP- driven enzyme that dissociates TATA-binding protein from DNA, Mol Cell Biol *17*, 4842-51.

Azzalin, C. M., Mucciolo, E., Bertoni, L., and Giulotto, E. (1997). Fluorescence in situ hybridization with a synthetic (T2AG3)n polynucleotide detects several intrachromosomal telomere-like repeats on human chromosomes, Cytogenet Cell Genet *78*, 112-5.

Bai, L., Mihara, K., Kondo, Y., Honma, M., and Namba, M. (1993). Immortalization of normal human fibroblasts by treatment with 4-nitroquinoline 1-oxide, Int J Cancer *53*, 451-6.

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

Baker, S. J., Preisinger, A. C., Jessup, J. M., Paraskeva, C., Markowitz, S., Willson, J. K., Hamilton, S., and Vogelstein, B. (1990b). p53 gene mutations occur in combination with 17p allelic deletions as late events in colorectal tumorigenesis, Cancer Res *50*, 7717-22.

Bando, K., Nagai, H., Matsumoto, S., Koyama, M., Kawamura, N., Onda, M., and Emi, M. (1999). Identification of a 1-cM region of common deletion on 4q35 associated with progression of hepatocellular carcinoma, Genes Chromosomes Cancer *25*, 284-9.

Barrett, J. C., and Wiseman, R. W. (1987). Cellular and molecular mechanisms of multistep carcinogenesis: relevance to carcinogen risk assessment, Environ Health Perspect *76*, 65-70.

Barski, G., and Cornefert, F. (1962). J Natl Cancer Inst *28,* 801-21.

Bartkova, J., Lukas, J., Guldberg, P., Alsner, J., Kirkin, A. F., Zeuthen, J., and Bartek, J. (1996). The p16-cyclin D/Cdk4-pRB pathway as a functional unit frequently altered in melanoma pathogenesis, Cancer Res *56*, 5475-83.

Batova, A., Diccianni, M. B., Yu, J. C., Nobori, T., Link, M. P., Pullen, J., and Yu, A. L. (1997). Frequent and selective methylation of p15 and deletion of both p15 and p16 in T-cell acute lymphoblastic leukemia, Cancer Res *57*, 832-6.

Bayreuther, K., Francz, P. I., Gogol, J., and Kontermann, K. (1992). Terminal differentiation, aging, apoptosis, and spontaneous transformation in fibroblast stem cell systems in vivo and in vitro, Ann N Y Acad Sci *663*, 167-79.

Benedict, W. F., Rucker, N., Mark, C., and Kouri, R. E. (1975). Correlation between balance of specific chromosomes and expression of malignancy in hamster cells, J Natl Cancer Inst *54*, 157-62.

Benedict, W. F., Weissman, B. E., Mark, C., and Stanbridge, E. J. (1984). Tumorigenicity of human HT1080 fibrosarcoma X normal fibroblast hybrids: chromosome dosage dependency, Cancer Res *44*, 3471-9.

Benn, P. A. (1976). Specific chromosome aberrations in senescent fibroblast cell lines derived from human embryos, Am J Hum Genet *28*, 465-73.

Berker-Karauzum, S., Luleci, G., Ozbilim, G., Erdogan, A., Kuzucu, A., and Demircan, A. (1998). Cytogenetic findings in thirty lung carcinoma patients, Cancer Genet Cytogenet *100*, 114-23.

Berndt, N., Dohadwala, M., and Liu, C. W. (1997). Constitutively active protein phosphatase 1alpha causes pRB-dependent G1 arrest in human cancer cells, Curr Biol *7*, 375-86.

Berrington, M. (1999). Investigation of the role of chromosome 7 in human cell immortalisation and cancer, Thesis, University of Glasgow.

Berry, I. J., Burns, J. E., and Parkinson, E. K. (1994). Assignment of two human epidermal squamous cell carcinomas cell lines to more than one complementation group for the immortal phenotype, Mol Carcinog *9*, 134-42.

Bertram, M. J., Berube, N. G., Hang-Swanson, X., Ran, Q., Leung, J. K., Bryce, S., Spurgers, K., Bick, R. J., Baldini, A., Ning, Y., *et al.* (1999a). Identification of a gene that reverses the immortal phenotype of a subset of cells and is a member of a novel family of transcription factor-like genes, Mol Cell Biol *19*, 1479-85.

Bertram, M. J., Berube, N. G., Swanson, X. H., and Pereira-Smith, O. M. (1999b). Assembly of a BAC contig of the complementation group B cell senescence gene candidate region at 4q33-q34.1 and identification of expressed sequences, Genomics *56*, 353-4.

Bianchi, A., Stansel, R. M., Fairall, L., Griffith, J. D., Rhodes, D., and de Lange, T. (1999). TRF1 binds a bipartite telomeric site with extreme spatial flexibility, Embo J *18*, 5735-44.

Bishop, J. M. (1987). The molecular genetics of cancer, Science *235*, 305-11.

Bockmuhl, U., Schluns, K., Kuchler, I., Petersen, S., and Petersen, I. (2000). Genetic imbalances with impact on survival in head and neck cancer patients, Am J Pathol *157*, 369-75.

Bodmer, W. F., Bailey, C. J., Bodmer, J., Bussey, H. J., Ellis, A., Gorman, P., Lucibello, F. C., Murday, V. A., Rider, S. H., Scambler, P., and et al. (1987). Localization of the gene for familial adenomatous polyposis on chromosome 5, Nature *328*, 614-6.

Bodmer, W. F., Cottrell, S., Frischauf, A. M., Kerr, I. B., Murday, V. A., Rowan, A. J., Smith, M. F., Solomon, E., Thomas, H., and Varesco, L. (1989). Genetic analysis of colorectal cancer, Princess Takamatsu Symp *20*, 49-59.

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

Boland, C. R., Sato, J., Appelman, H. D., Bresalier, R. S., and Feinberg, A. P. (1995). Microallelotyping defines the sequence and tempo of allelic losses at tumour suppressor gene loci during colorectal cancer progression, Nat Med *1*, 902-9.

Bols, B. L., Naaktgeboren, J. M., and Simons, J. W. (1991). Immortalization of Syrian hamster embryo cells is in itself a multistep event, Cancer Res *51*, 1177-84.

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

Boulton, S. J., and Jackson, S. P. (1998). Components of the Ku-dependent non-homologous end-joining pathway are involved in telomeric length maintenance and telomeric silencing, Embo J *17*, 1819-28.

Bovee, J. V., Cleton-Jansen, A. M., Rosenberg, C., Taminiau, A. H., Cornelisse, C. J., and Hogendoorn, P. C. (1999). Molecular genetic characterization of both components of a dedifferentiated chondrosarcoma, with implications for its histogenesis [see comments], J Pathol *189*, 454-62.

Boveri, T. (1914). *Zur frage der erstehung maligner tumoren* (On the problems of the origins of human tumours). Gustav Fischer, Jena.

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

Bregman, M. D., Funk, C., and Fukushima, M. (1986). Inhibition of human melanoma growth by prostaglandin A, D, and J analogues, Cancer Res *46*, 2740-4.

Broccoli, D., Smogorzewska, A., Chong, L., and de Lange, T. (1997). Human telomeres contain two distinct Myb-related proteins, TRF1 and TRF2, Nat Genet *17*, 231-5.

Broman, K. W., Murray, J. C., Sheffield, V. C., White, R. L., and Weber, J. L. (1998). Comprehensive human genetic maps: individual and sex-specific variation in recombination, Am J Hum Genet *63*, 861-9.

Bryan, T. M., Englezou, A., Dunham, M. A., and Reddel, R. R. (1998). Telomere length dynamics in telomerase-positive immortal human cell populations, Exp Cell Res *239*, 370-8.

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

Bryan, T. M., Marusic, L., Bacchetti, S., Namba, M., and Reddel, R. R. (1997). The telomere lengthening mechanism in telomerase-negative immortal human cells does not involve the telomerase RNA subunit, Hum Mol Genet *6*, 921-6.

Bryan, T. M., and Reddel, R. R. (1997). Telomere dynamics and telomerase activity in in vitro immortalised human cells, Eur J Cancer *33*, 767-73.

Buchhop, S., Gibson, M. K., Wang, X. W., Wagner, P., Sturzbecher, H. W., and Harris, C. C. (1997). Interaction of p53 with the human Rad51 protein, Nucleic Acids Res *25*, 3868-74.

Buetow, K. H., Murray, J. C., Israel, J. L., London, W. T., Smith, M., Kew, M., Blanquet, V., Brechot, C., Redeker, A., and Govindarajah, S. (1989). Loss of heterozygosity suggests tumor suppressor gene responsible for primary hepatocellular carcinoma, Proc Natl Acad Sci U S A *86*, 8852-6.

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

Burns, J. E., Clark, L. J., Yeudall, W. A., Mitchell, R., Mackenzie, K., Chang, S. E., and Parkinson, E. K. (1994). The p53 status of cultured human premalignant oral keratinocytes, Br J Cancer *70*, 591-5.

Cabelguenne, A., Blons, H., de Waziers, I., Carnot, F., Houllier, A. M., Soussi, T., Brasnu, D., Beaune, P., Laccourreye, O., and Laurent-Puig, P. (2000). p53 alterations predict tumor response to neoadjuvant chemotherapy in head and neck squamous cell carcinoma: a prospective series, J Clin Oncol *18*, 1465-73.

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

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

Capaccio, P., Pruneri, G., Carboni, N., Pagliari, A. V., Quatela, M., Cesana, B. M., and Pignataro, L. (2000). Cyclin D1 expression is predictive of occult metastases in head and neck cancer patients with clinically negative cervical lymph nodes, Head Neck *22*, 234-40.

Carman, T. A., Afshari, C. A., and Barrett, J. C. (1998). Cellular senescence in telomerase-expressing Syrian hamster embryo cells, Exp Cell Res *244*, 33-42.

Casey, G., Plummer, S., Hoeltge, G., Scanlon, D., Fasching, C., and Stanbridge, E. J. (1993). Functional evidence for a breast cancer growth suppressor gene on chromosome 17, Hum Mol Genet *2*, 1921-7.

Cavenee, W. K., Dryja, T. P., Phillips, R. A., Benedict, W. F., Godbout, R., Gallie, B. L., Murphree, A. L., Strong, L. C., and White, R. L. (1983). Expression of recessive alleles by chromosomal mechanisms in retinoblastoma, Nature *305*, 779-84.

Chang, P. L., Gunby, J. L., Tomkins, D. J., Mak, I., Rosa, N. E., and Mak, S. (1986). Transformation of human cultured fibroblasts with plasmids carrying dominant selection markers and immortalizing potential, Exp Cell Res *167*, 407-16.

Chi, S. W., Ayed, A., and Arrowsmith, C. H. (1999). Solution structure of a conserved C-terminal domain of p73 with structural homology to the SAM domain, Embo J *18*, 4438-45.

Cho, J. H., Nicolae, D. L., Gold, L. H., Fields, C. T., LaBuda, M. C., Rohal, P. M., Pickles, M. R., Qin, L., Fu, Y., Mann, J. S., *et al.* (1998). Identification of novel susceptibility loci for inflammatory bowel disease on chromosomes 1p, 3q, and 4q: evidence for epistasis between 1p and IBD1, Proc Natl Acad Sci U S A *95*, 7502-7.

Cho, K. R., and Vogelstein, B. (1992). Suppressor gene alterations in the colorectal adenoma-carcinoma sequence, J Cell Biochem Suppl, 137-41.

Chong, L., van Steensel, B., Broccoli, D., Erdjument-Bromage, H., Hanish, J., Tempst, P., and de Lange, T. (1995). A human telomeric protein, Science *270*, 1663-7.

Chou, Y. H., Chung, K. C., Jeng, L. B., Chen, T. C., and Liaw, Y. F. (1998). Frequent allelic loss on chromosomes 4q and 16q associated with human hepatocellular carcinoma in Taiwan, Cancer Lett *123*, 1-6.

Clark, L. J., Edington, K., Swan, I. R., McLay, K. A., Newlands, W. J., Wills, L. C., Young, H. A., Johnston, P. W., Mitchell, R., Robertson, G., and et al. (1993). The absence of Harvey ras mutations during development and progression of squamous cell carcinomas of the head and neck, Br J Cancer *68*, 617-20.

Classon, M., Salama, S., Gorka, C., Mulloy, R., Braun, P., and Harlow, E. (2000). Combinatorial roles for pRB, p107, and p130 in E2F-mediated cell cycle control [In Process Citation], Proc Natl Acad Sci U S A *97*, 10820-5.

Collins, S. L. (1998). Controversies in multimodality therapy for head neck cancer: clinical and biological perspectives. In: Thawley, S. E., Panje, W. R., Batsikas, J. G., and Lindberg, R. D, eds. Comprehensive Management of Head Neck Tumours. Philadelphia: W. B. Saunders.

Comings, D. E. (1973). A general theory of carcinogenesis, Proc Natl Acad Sci U S A *70,* 3324-8.

Cooke, H. J., and Smith, B. A. (1986). Variability at the telomeres of the human X/Y pseudoautosomal region, Cold Spring Harb Symp Quant Biol *51*, 213-9.

Counter, C. M., Avilion, A. A., LeFeuvre, C. E., Stewart, N. G., Greider, C. W., Harley, C. B., and Bacchetti, S. (1992). Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity, Embo J *11*, 1921-9.

Counter, C. M., Meyerson, M., Eaton, E. N., Ellisen, L. W., Caddle, S. D., Haber, D. A., and Weinberg, R. A. (1998). Telomerase activity is restored in human cells by ectopic expression of hTERT (hEST2), the catalytic subunit of telomerase, Oncogene *16*, 1217-22.

Cristofalo, V. J., and Sharf, B. B. (1973). Cellular senescence and DNA synthesis. Thymidine incorporation as a measure of population age in human diploid cells, Exp Cell Res *76*, 419-27.

Critchlow, S. E., and Jackson, S. P. (1998). DNA end-joining: from yeast to man, Trends Biochem Sci *23*, 394-8.

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

Cuthbert, A. P., Trott, D. A., Ekong, R. M., Jezzard, S., England, N. L., Themis, M., Todd, C. M., and Newbold, R. F. (1995). Construction and characterization of a highly stable human: rodent monochromosomal hybrid panel for genetic complementation and genome mapping studies, Cytogenet Cell Genet *71*, 68-76.

Cuthill, S., Agarwal, P., Sarkar, S., Savelieva, E., and Reznikoff, C. A. (1999). Dominant genetic alterations in immortalization: role for 20q gain, Genes Chromosomes Cancer *26*, 304-11.

Dasika, G. K., Lin, S. C., Zhao, S., Sung, P., Tomkinson, A., and Lee, E. Y. (1999). DNA damage-induced cell cycle checkpoints and DNA strand break repair in development and tumorigenesis, Oncogene *18*, 7883-99.

DeVita, V. T., and Young, R. C. (1975). Combination versus single agent chemotherapy: a review of the basis for selection of drug treatment of cancer., Cancer *35*, 98-110.

Di Como, C. J., Gaiddon, C., and Prives, C. (1999). p73 function is inhibited by tumor-derived p53 mutants in mammalian cells, Mol Cell Biol *19*, 1438-49.

Diatchenko, L., Lau, Y. F., Campbell, A. P., Chenchik, A., Moqadam, F., Huang, B., Lukyanov, S., Lukyanov, K., Gurskaya, N., Sverdlov, E. D., and Siebert, P. D. (1996). Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries, Proc Natl Acad Sci U S A *93*, 6025-30.

Diatchenko, L., Lukyanov, S., Lau, Y. F., and Siebert, P. D. (1999). Suppression subtractive hybridization: a versatile method for identifying differentially expressed genes, Methods Enzymol *303*, 349-80.

Dickson, M. A., Hahn, W. C., Ino, Y., Ronfard, V., Wu, J. Y., Weinberg, R. A., Louis, D. N., Li, F. P., and Rheinwald, J. G. (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*, 1436-47.

Dimri, G. P., Hara, E., and Campisi, J. (1994). Regulation of two E2F-related genes in presenescent and senescent human fibroblasts, J Biol Chem *269*, 16180-6.

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

Dimri, G. P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E. E., Linskens, M., Rubelj, I., Pereira-Smith, O., 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*, 9363-7.

Doughty, M. L., De Jager, P. L., Korsmeyer, S. J., and Heintz, N. (2000). Neurodegeneration in Lurcher mice occurs via multiple cell death pathways, J Neurosci *20*, 3687-94.

Dowdy, S. F., Scanlon, D. J., Fasching, C. L., Casey, G., and Stanbridge, E. J. (1990). Irradiation microcell-mediated chromosome transfer (XMMCT): the generation of specific chromosomal arm deletions, Genes Chromosomes Cancer *2*, 318-27.

Dulic, V., Drullinger, L. F., Lees, E., Reed, S. I., and Stein, G. H. (1993). Altered regulation of G1 cyclins in senescent human diploid fibroblasts: accumulation of inactive cyclin E-Cdk2 and cyclin D1-Cdk2 complexes, Proc Natl Acad Sci U S A *90*, 11034-8.

Duncan, E. L., Whitaker, N. J., Moy, E. L., and Reddel, R. R. (1993). Assignment of SV40-immortalized cells to more than one complementation group for immortalization, Exp Cell Res *205*, 337-44.

Easton, D. F., Bishop, D. T., Ford, D., and Crockford, G. P. (1993). Genetic linkage analysis in familial breast and ovarian cancer: results from 214 families. The Breast Cancer Linkage Consortium, Am J Hum Genet *52*, 678-701.

Edington, K. G., Loughran, O. P., Berry, I. J., and Parkinson, E. K. (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*, 254-65.

Ehrenstein, D. (1998). Immortality gene discovered [news], Science *279*, 177.

England, N. L., Cuthbert, A. P., Trott, D. A., Jezzard, S., Nobori, T., Carson, D. A., and Newbold, R. F. (1996). Identification of human tumour suppressor genes by monochromosome transfer: rapid growth-arrest response mapped to 9p21 is mediated solely by the cyclin-D-dependent kinase inhibitor gene, CDKN2A (p16INK4A), Carcinogenesis *17*, 1567-75.

Erber, R., Klein, W., Andl, T., Enders, C., Born, A. I., Conradt, C., Bartek, J., and Bosch, F. X. (1997). Aberrant p21(CIP1/WAF1) protein accumulation in head-and-neck cancer, Int J Cancer *74*, 383-9.

Erdmann, K. S., Kuhlmann, J., Lessmann, V., Herrmann, L., Eulenburg, V., Muller, O., and Heumann, R. (2000). The Adenomatous Polyposis Coli-protein (APC) interacts with the protein tyrosine phosphatase PTP-BL via an alternatively spliced PDZ domain, Oncogene *19*, 3894-901.

Esrig, D., Elmajian, D., Groshen, S., Freeman, J. A., Stein, J. P., Chen, S. C., Nichols, P. W., Skinner, D. G., Jones, P. A., and Cote, R. J. (1994). Accumulation of nuclear p53 and tumor progression in bladder cancer [see comments], N Engl J Med *331*, 1259-64.

Feenstra, M., Veltkamp, M., van Kuik, J., Wiertsema, S., Slootweg, P., van den Tweel, J., de Weger, R., and Tilanus, M. (1999). HLA class I expression and chromosomal deletions at 6p and 15q in head and neck squamous cell carcinomas, Tissue Antigens *54*, 235-45.

Feenstra, M., Verdaasdonk, M., van der Zwan, A. W., de Weger, R., Slootweg, P., and Tilanus, M. (2000). Microsatellite analysis of microdissected tumor cells and 6p high density microsatellite analysis in head and neck squamous cell carcinomas with down-regulated human leukocyte antigen class I expression, Lab Invest *80*, 405-14.

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

Ferbeyre, G., de Stanchina, E., Querido, E., Baptiste, N., Prives, C., and Lowe, S. W. (2000). PML is induced by oncogenic ras and promotes premature senescence, Genes Dev *14*, 2015-27.

Field, J. K., Spandidos, D. A., Stell, P. M., Vaughan, E. D., Evan, G. I., and Moore, J. P. (1989). Elevated expression of the c-myc oncoprotein correlates with poor prognosis in head and neck squamous cell carcinoma, Oncogene *4*, 1463-8.

Flores, J. F., Walker, G. J., Glendening, J. M., Haluska, F. G., Castresana, J. S., Rubio, M. P., Pastorfide, G. C., Boyer, L. A., Kao, W. H., Bulyk, M. L., *et al.* (1996). Loss of the p16INK4A and p15INK4b genes, as well as neighboring 9p21 markers, in sporadic melanoma, Cancer Res *56*, 5023-32.

Foulds, L. (1957). Tumor progression, Cancer Res *17,* 355-356.

Friend, S. H., Bernards, R., Rogelj, S., Weinberg, R. A., Rapaport, J. M., Albert, D. M., and Dryja, T. P. (1986). A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma, Nature *323*, 643-6.

Fujii, H., Yoshida, M., Gong, Z. X., Matsumoto, T., Hamano, Y., Fukunaga, M., Hruban, R. H., Gabrielson, E., and Shirai, T. (2000). Frequent genetic heterogeneity in the clonal evolution of gynecological carcinosarcoma and its influence on phenotypic diversity, Cancer Res *60*, 114-20.

Fujimori, K., Kanaoka, Y., Sakaguchi, Y., and Urade, Y. (2000). Transcriptional activation of human hematopoietic prostaglandin D synthase gene in megakaryoblastic cells: Roles of OCT-1 element in the 5'-flanking region and AP-2 element in the untranslated exon 1, J Biol Chem.

Fujimoto, K., Kyo, S., Takakura, M., Kanaya, T., Kitagawa, Y., Itoh, H., Takahashi, M., and Inoue, M. (2000). Identification and characterization of negative regulatory elements of the human telomerase catalytic subunit (hTERT) gene promoter: possible role of MZF-2 in transcriptional repression of hTERT, Nucleic Acids Res *28*, 2557-62.

Fujimoto, Y., Hampton, L. L., Wirth, P. J., Wang, N. J., Xie, J. P., and Thorgeirsson, S. S. (1994). Alterations of tumor suppressor genes and allelic losses in human hepatocellular carcinomas in China [see comments], Cancer Res *54*, 281-5.

Garber, J. E., Goldstein, A. M., Kantor, A. F., Dreyfus, M. G., Fraumeni, J. F., Jr., and Li, F. P. (1991). Follow-up study of twenty-four families with Li-Fraumeni syndrome, Cancer Res *51*, 6094-7.

Garkavtsev, I., Grigorian, I. A., Ossovskaya, V. S., Chernov, M. V., Chumakov, P. M., and Gudkov, A. V. (1998). The candidate tumour suppressor p33ING1 cooperates with p53 in cell growth control [see comments], Nature *391*, 295-8.

Gasparotto, D., Vukosavljevic, T., Piccinin, S., Barzan, L., Sulfaro, S., Armellin, M., Boiocchi, M., and Maestro, R. (1999). Loss of heterozygosity at 10q in tumors of the upper respiratory tract is associated with poor prognosis, Int J Cancer *84*, 432-6.

Gey, G. O., Coffman, W. D., and Kubicek, N. T. (1952). Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. Cancer Res *12,* 264-5.

Giles, H., and Leff, P. (1988). The biology and pharmacology of PGD2, Prostaglandins *35*, 277-300.

Good, L., Dimri, G. P., Campisi, J., and Chen, K. Y. (1996). Regulation of dihydrofolate reductase gene expression and E2F components in human diploid fibroblasts during growth and senescence, J Cell Physiol *168*, 580-8.

Goodwin, E. C., Yang, E., Lee, C. J., Lee, H. W., DiMaio, D., and Hwang, E. S. (2000). Rapid induction of senescence in human cervical carcinoma cells [In Process Citation], Proc Natl Acad Sci U S A *97*, 10978-83.

Gorman, S. D., and Cristofalo, V. J. (1985). Reinitiation of cellular DNA synthesis in BrdU-selected nondividing senescent WI-38 cells by simian virus 40 infection, J Cell Physiol *125*, 122-6.

Gotte, K., Hadaczek, P., Coy, J. F., Wirtz, H. W., Riedel, F., Neubauer, J., and Hormann, K. (2000). Fhit expression is absent or reduced in a subset of primary head and neck cancer, Anticancer Res *20*, 1057-60.

Greenberg, R. A., Allsopp, R. C., Chin, L., Morin, G. B., and DePinho, R. A. (1998). Expression of mouse telomerase reverse transcriptase during development, differentiation and proliferation, Oncogene *16*, 1723-30.

Greider, C. W. (1999). Telomeres do D-loop-T-loop [comment], Cell *97*, 419-22.

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

Groden, J., Thliveris, A., Samowitz, W., Carlson, M., Gelbert, L., Albertsen, H., Joslyn, G., Stevens, J., Spirio, L., Robertson, M., and et al. (1991). Identification and characterization of the familial adenomatous polyposis coli gene, Cell *66*, 589-600.

Gunduz, M., Ouchida, M., Fukushima, K., Hanafusa, H., Etani, T., Nishioka, S., Nishizaki, K., and Shimizu, K. (2000). Genomic structure of the human ING1 gene and tumor-specific mutations detected in head and neck squamous cell carcinomas, Cancer Res *60*, 3143-6.

Gustafson, C. E., Wilson, P. J., Lukeis, R., Baker, E., Woollatt, E., Annab, L., Hawke, L., Barrett, J. C., and Chenevix-Trench, G. (1996). Functional evidence for a colorectal cancer tumor suppressor gene at chromosome 8p22-23 by monochromosome transfer, Cancer Res *56*, 5238-45.

Gyapay, G., Morissette, J., Vignal, A., Dib, C., Fizames, C., Millasseau, P., Marc, S., Bernardi, G., Lathrop, M., and Weissenbach, J. (1994). The 1993-94 Genethon human genetic linkage map [see comments], Nat Genet *7*, 246-339.

Hahn, S. A., Schutte, M., Hoque, A. T., Moskaluk, C. A., da Costa, L. T., Rozenblum, E., Weinstein, C. L., Fischer, A., Yeo, C. J., Hruban, R. H., and Kern, S. E. (1996). DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. [see comments], Science *271*, 350-3.

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

Hainaut, P., Hernandez, T., Robinson, A., Rodriguez-Tome, P., Flores, T., Hollstein, M., Harris, C. C., and Montesano, R. (1998). IARC Database of p53 gene mutations in human tumors and cell lines: updated compilation, revised formats and new visualisation tools, Nucleic Acids Res *26*, 205-13.

Halvorsen, T. L., Leibowitz, G., and Levine, F. (1999). Telomerase activity is sufficient to allow transformed cells to escape from crisis, Mol Cell Biol *19*, 1864-70.

Hamm, H., Traupe, H., Brocker, E. B., Schubert, H., and Kolde, G. (1996). The scleroatrophic syndrome of Huriez: a cancer-prone genodermatosis, Br J Dermatol *134*, 512-8.

Hampton, G. M., Larson, A. A., Baergen, R. N., Sommers, R. L., Kern, S., and Cavenee, W. K. (1996). Simultaneous assessment of loss of heterozygosity at multiple microsatellite loci using semi-automated fluorescence-based detection: subregional mapping of chromosome 4 in cervical carcinoma, Proc Natl Acad Sci U S A *93*, 6704-9.

Hannon, G. J., and Beach, D. (1994). p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest [see comments], Nature *371*, 257-61.

Hara, E., Yamaguchi, T., Nojima, H., Ide, T., Campisi, J., Okayama, H., and Oda, K. (1994). Id-related genes encoding helix-loop-helix proteins are required for G1 progression and are repressed in senescent human fibroblasts, J Biol Chem *269*, 2139-45.

Harle-Bachor, C., and Boukamp, P. (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*, 6476-81.

Harley, C. B. (1991). Telomere loss: mitotic clock or genetic time bomb?, Mutat Res *256*, 271-82.

Harley, C. B., Futcher, A. B., and Greider, C. W. (1990). Telomeres shorten during ageing of human fibroblasts, Nature *345*, 458-60.

Harley, C. B., Pollard, J. W., Chamberlain, J. W., Stanners, C. P., and Goldstein, S. (1980). Protein synthetic errors do not increase during aging of cultured human fibroblasts, Proc Natl Acad Sci U S A *77*, 1885-9.

Harrington, L., Zhou, W., McPhail, T., Oulton, R., Yeung, D. S., Mar, V., Bass, M. B., and Robinson, M. O. (1997). Human telomerase contains evolutionarily conserved catalytic and structural subunits, Genes Dev *11*, 3109-15.

Harris, H. (1971). Cell fusion and the analysis of malignancy, Proc R Soc Lond B Biol Sci *179*, 1-20.

Harris, H., Miller, O. J., Klein, G., Worst, P., and Tachibana, T. (1969). Suppression of malignancy by cell fusion, Nature *223*, 363-8.

Hastie, N. D., and Allshire, R. C. (1989). Human telomeres: fusion and interstitial sites, Trends Genet *5*, 326-31.

Hayflick, L., and Moorehead, P. S. (1961). The limited *in vitro* lifetime of human diploid cell strains, Exp Cell Res *25,* 585-621

Henderson, Y. C., Breau, R. L., Liu, T. J., and Clayman, G. L. (2000). Telomerase activity in head and neck tumors after introduction of wild- type p53, p21, p16, and E2F-1 genes by means of recombinant adenovirus, Head Neck *22*, 347-54.

Henikoff, S. (1993). Transcriptional activator components and poxvirus DNA-dependent ATPases comprise a single family, Trends Biochem Sci *18*, 291-2.

Hensler, P. J., Annab, L. A., Barrett, J. C., and Pereira-Smith, O. M. (1994). A gene involved in control of human cellular senescence on human chromosome 1q, Mol Cell Biol *14*, 2291-7.

Herman, J. G., Latif, F., Weng, Y., Lerman, M. I., Zbar, B., Liu, S., Samid, D., Duan, D. S., Gnarra, J. R., Linehan, W. M., and et al. (1994). Silencing of the VHL tumor-suppressor gene by DNA methylation in renal carcinoma, Proc Natl Acad Sci U S A *91*, 9700-4.

Herrera, L., Kakati, S., Gibas, L., Pietrzak, E., and Sandberg, A. A. (1986). Gardner syndrome in a man with an interstitial deletion of 5q, Am J Med Genet *25*, 473-6.

Hirosawa, M., Nagase, T., Ishikawa, K., Kikuno, R., Nomura, N., and Ohara, O. (1999). Characterization of cDNA clones selected by the GeneMark analysis from size-fractionated cDNA libraries from human brain, DNA Res *6*, 329-36.

Hitotsumachi S, Rabinowitz Z, Sachs L. (1972). Chromosomal control of chemical carcinogenesis, Int J Cancer *9*, 305-15.

Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. C. (1991). p53 mutations in human cancers, Science *253*, 49-53.

Holt, S. E., Aisner, D. L., Baur, J., Tesmer, V. M., Dy, M., Ouellette, M., Trager, J. B., Morin, G. B., Toft, D. O., Shay, J. W., *et al.* (1999). Functional requirement of p23 and Hsp90 in telomerase complexes, Genes Dev *13*, 817-26.

Horowitz, J. M. (1993). Regulation of transcription by the retinoblastoma protein, Genes Chromosomes Cancer *6*, 124-31.

Hsieh, J. K., Chan, F. S., O'Connor, D. J., Mittnacht, S., Zhong, S., and Lu, X. (1999). pRB regulates the stability and the apoptotic function of p53 via MDM2, Mol Cell *3*, 181-93.

Hu, N., Roth, M. J., Polymeropolous, M., Tang, Z. Z., Emmert-Buck, M. R., Wang, Q. H., Goldstein, A. M., Feng, S. S., Dawsey, S. M., Ding, T., *et al.* (2000). Identification of novel regions of allelic loss from a genomewide scan of esophageal squamous-cell carcinoma in a high-risk Chinese population, Genes Chromosomes Cancer *27*, 217-28.

Hu, W., Zuo, J., De Jager, P. L., and Heintz, N. (1998). The human glutamate receptor delta 2 gene (GRID2) maps to chromosome 4q22, Genomics *47*, 143-5.

Huriez, C., Deminatti, M., Agache, P., and Mennecier, M. (1968). [A gene dysplasia not previously known: frequently degenerative sclero- atrophying and keratodermic genodermatosis of the extremities], Sem Hop *44*, 481-8.

Hussussian, C. J., Struewing, J. P., Goldstein, A. M., Higgins, P. A., Ally, D. S., Sheahan, M. D., Clark, W. H., Jr., Tucker, M. A., and Dracopoli, N. C. (1994). Germline p16 mutations in familial melanoma [see comments], Nat Genet *8*, 15-21.

Ichii, S., Horii, A., Nakatsuru, S., Furuyama, J., Utsunomiya, J., and Nakamura, Y. (1992). Inactivation of both *APC* alleles in an early stage of colon adenomas in a patient with familial adenomatous polyposis (FAP), Hum Mol Genet *1*, 387-90.

Ikai, K., Sone, R., and Fukushima, M. (1991). Inhibitory effects of prostanoids on the proliferation of transformed human epidermal cells in culture, Skin Pharmacol *4*, 9-13.

Imai, S., Armstrong, C. M., Kaeberlein, M., and Guarente, L. (2000). Transcriptional silencing and longevity protein Sir2 is an NAD- dependent histone deacetylase, Nature *403*, 795-800.

Irwin, M., Marin, M. C., Phillips, A. C., Seelan, R. C., Smith, D. I., Liu, W., Flores, E. R., Tsai, K. Y., Jacks, T., Vousden K. H., and Kaelin Jr., W. G. (2000). Role for the p53 homologue p73 in E2F-1-induced apoptosis, Nature *407,* 645-8.

Israeli, D., Tessler, E., Haupt, Y., Elkeles, A., Wilder, S., Amson, R., Telerman, A., and Oren, M. (1997). A novel p53-inducible gene, PAG608, encodes a nuclear zinc finger protein whose overexpression promotes apoptosis, Embo J *16*, 4384-92.

Ivy, J. M., Klar, A. J., and Hicks, J. B. (1986). Cloning and characterization of four SIR genes of Saccharomyces cerevisiae, Mol Cell Biol *6*, 688-702.

Izumoto, S., Arita, N., Ohnishi, T., Hiraga, S., Taki, T., and Hayakawa, T. (1995). Homozygous deletions of p16INK4A/MTS1 and p15INK4B/MTS2 genes in glioma cells and primary glioma tissues, Cancer Lett *97*, 241-7.

Jeffrey, P. D., Gorina, S., and Pavletich, N. P. (1995). Crystal structure of the tetramerization domain of the p53 tumor suppressor at 1.7 angstroms, Science *267*, 1498-502.

Jen, J., Powell, S. M., Papadopoulos, N., Smith, K. J., Hamilton, S. R., Vogelstein, B., and Kinzler, K. W. (1994). Molecular determinants of dysplasia in colorectal lesions, Cancer Res *54*, 5523-6.

Jiang, F., Moch, H., Richter, J., Egenter, C., Gasser, T., Bubendorf, L., Gschwind, R., Sauter, G., and Mihatsch, M. J. (1998). Comparative genomic hybridization reveals frequent chromosome 13q and 4q losses in renal carcinomas with sarcomatoid transformation, J Pathol *185*, 382-8.

Kaeberlein, M., McVey, M., and Guarente, L. (1999). The SIR2/3/4 complex and SIR2 alone promote longevity in Saccharomyces cerevisiae by two different mechanisms, Genes Dev *13*, 2570-80.

Kaghad, M., Bonnet, H., Yang, A., Creancier, L., Biscan, J. C., Valent, A., Minty, A., Chalon, P., Lelias, J. M., Dumont, X., *et al.* (1997). Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers, Cell *90*, 809-19.

Kallioniemi, A., Kallioniemi, O. P., Sudar, D., Rutovitz, D., Gray, J. W., Waldman, F., and Pinkel, D. (1992). Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors, Science *258*, 818-21.

Kamb, A., Shattuck-Eidens, D., Eeles, R., Liu, Q., Gruis, N. A., Ding, W., Hussey, C., Tran, T., Miki, Y., Weaver-Feldhaus, J., and et al. (1994). Analysis of the *p16* gene (*CDKN2*) as a candidate for the chromosome 9p melanoma susceptibility locus, Nat Genet *8*, 23-6.

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

Kanaoka, Y., Ago, H., Inagaki, E., Nanayama, T., Miyano, M., Kikuno, R., Fujii, Y., Eguchi, N., Toh, H., Urade, Y., and Hayaishi, O. (1997). Cloning and crystal structure of hematopoietic prostaglandin D synthase [published erratum appears in Cell 1999 Feb 5;96(3):449], Cell *90*, 1085-95.

Kanaoka, Y., Fujimori, K., Kikuno, R., Sakaguchi, Y., Urade, Y., and Hayaishi, O. (2000). Structure and chromosomal localization of human and mouse genes for hematopoietic prostaglandin D synthase. Conservation of the ancestral genomic structure of sigma-class glutathione S-transferase, Eur J Biochem *267*, 3315-22.

Karlseder, J., Broccoli, D., Dai, Y., Hardy, S., and de Lange, T. (1999). p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2, Science *283*, 1321-5.

Karlsson, C., Stenman, G., Vojta, P. J., Bongcam-Rudloff, E., Barrett, J. C., Westermark, B., and Paulsson, Y. (1996). Escape from senescence in hybrid cell clones involves deletions of two regions located on human chromosome 1q, Cancer Res *56*, 241-5.

Kastan, M. B., Onyekwere, O., Sidransky, D., Vogelstein, B., and Craig, R. W. (1991). Participation of p53 protein in the cellular response to DNA damage, Cancer Res *51*, 6304-11.

Khavari, P. A., Peterson, C. L., Tamkun, J. W., Mendel, D. B., and Crabtree, G. R. (1993). BRG1 contains a conserved domain of the SWI2/SNF2 family necessary for normal mitotic growth and transcription, Nature *366*, 170-4.

Kickhoefer, V. A., Stephen, A. G., Harrington, L., Robinson, M. O., and Rome, L. H. (1999). Vaults and telomerase share a common subunit, TEP1, J Biol Chem *274*, 32712-7.

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

Killary, A. M., Wolf, M. E., Giambernardi, T. A., and Naylor, S. L. (1992). Definition of a tumor suppressor locus within human chromosome 3p21-p22, Proc Natl Acad Sci U S A *89*, 10877-81.

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

Kim, S. H., Kaminker, P., and Campisi, J. (1999). TIN2, a new regulator of telomere length in human cells [see comments], Nat Genet *23*, 405-12.

Kimura, Y., Fujieda, S., Takabayashi, T., Tanaka, T., Sugimoto, C., and Saito, H. (2000). Conventional tumor markers are prognostic indicators in patients with head and neck squamous cell carcinoma, Cancer Lett *155*, 163-8.

Kinzler, K. W., and Vogelstein, B. (1996). Lessons from hereditary colorectal cancer, Cell *87*, 159-70.

Kinzler, K. W., and Vogelstein, B. (1997). Cancer-susceptibility genes. Gatekeepers and caretakers [news; comment] [see comments], Nature *386*, 761, 763.

Kiyono, T., Foster, S. A., Koop, J. I., McDougall, J. K., Galloway, D. A., and Klingelhutz, A. J. (1998). Both pRB/p16INK4A inactivation and telomerase activity are required to immortalize human epithelial cells [see comments], Nature *396*, 84-8.

Klein, C. B., Conway, K., Wang, X. W., Bhamra, R. K., Lin, X. H., Cohen, M. D., Annab, L., Barrett, J. C., and Costa, M. (1991). Senescence of nickel-transformed cells by an X chromosome: possible epigenetic control, Science *251*, 796-9.

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

Knowles, M. A. (1999). Identification of novel bladder tumour suppressor genes, Electrophoresis *20*, 269-79.

Knowles, M. A., Elder, P. A., Williamson, M., Cairns, J. P., Shaw, M. E., and Law, M. G. (1994). Allelotype of human bladder cancer, Cancer Res *54*, 531-8.

Knudson, A. G., Jr. (1971). Mutation and cancer: statistical study of retinoblastoma, Proc Natl Acad Sci U S A *68*, 820-3.

Knudson, A. G., Jr. (1978). Retinoblastoma: a prototypic hereditary neoplasm, Semin Oncol *5*, 57-60.

Kobayashi, T., and Horiuchi, T. (1996). A yeast gene product, Fob1 protein, required for both replication fork blocking and recombinational hotspot activities, Genes Cells *1*, 465-74.

Koch, W. M., and McQuone, S. (1997). Clinical and molecular aspects of squamous cell carcinoma of the head and neck in the nonsmoker and nondrinker, Curr Opin Oncol *9*, 257-61.

Koh, J., Enders, G. H., Dynlacht, B. D., and Harlow, E. (1995). Tumour-derived *p16* alleles encoding proteins defective in cell-cycle inhibition, Nature *375*, 506-10.

Koi, M., Johnson, L. A., Kalikin, L. M., Little, P. F., Nakamura, Y., and Feinberg, A. P. (1993). Tumor cell growth arrest caused by subchromosomal transferable DNA fragments from chromosome 11, Science *260*, 361-4.

Kuerbitz, S. J., Plunkett, B. S., Walsh, W. V., and Kastan, M. B. (1992). Wild-type p53 is a cell cycle checkpoint determinant following irradiation, Proc Natl Acad Sci U S A *89*, 7491-5.

Kyo, S., Takakura, M., Taira, T., Kanaya, T., Itoh, H., Yutsudo, M., Ariga, H., and Inoue, M. (2000). Sp1 cooperates with c-myc to activate transcription of the human telomerase reverse transcriptase gene (hTERT), Nucleic Acids Res *28*, 669-77.

Kyomoto, R., Kumazawa, H., Toda, Y., Sakaida, N., Okamura, A., Iwanaga, M., Shintaku, M., Yamashita, T., Hiai, H., and Fukumoto, M. (1997). Cyclin-D1-gene amplification is a more potent prognostic factor than its protein over-expression in human head-and-neck squamous-cell carcinoma, Int J Cancer *74*, 576-81.

Labat-Moleur, F., Guillermet, C., Lorimier, P., Robert, C., Lantuejoul, S., Brambilla, E., and Negoescu, A. (1998). TUNEL apoptotic cell detection in tissue sections: critical evaluation and improvement critical evaluation and improvement, J Histochem Cytochem *46*, 327-34.

Lalouette, A., Guenet, J. L., and Vriz, S. (1998). *Hotfoot* mouse mutations affect the delta 2 glutamate receptor gene and are allelic to *lurcher*, Genomics *50*, 9-13.

Landrigan, P. J., and Baker, D. B. (1991). The recognition and control of occupational disease [see comments], Jama *266*, 676-80.

Lane, D. P. (1992). Cancer. p53, guardian of the genome [news; comment] [see comments], Nature *358*, 15-6.

Lang, J. C., Tobin, E. J., Knobloch, T. J., Schuller, D. E., Bartynski, K. J., Mountain, R. E., Nicholson, R., DeYoung, B. R., and Weghorst, C. M. (1998). Frequent mutation of *p16* in squamous cell carcinoma of the head and neck, Laryngoscope *108*, 923-8.

Larson, A. A., Liao, S. Y., Stanbridge, E. J., Cavenee, W. K., and Hampton, G. M. (1997). Genetic alterations accumulate during cervical tumorigenesis and indicate a common origin for multifocal lesions, Cancer Res *57*, 4171-6.

Laurent, B. C., Yang, X., and Carlson, M. (1992). An essential Saccharomyces cerevisiae gene homologous to SNF2 encodes a helicase-related protein in a new family, Mol Cell Biol *12*, 1893-902.

Lazo, P. A. (1999). The molecular genetics of cervical carcinoma, Br J Cancer *80*, 2008-18.

Le, S., Sternglanz, R., and Greider, C. W. (2000). Identification of two RNA-binding proteins associated with human telomerase RNA, Mol Biol Cell *11*, 999-1010.

Leach, R. J., Thayer, M. J., Schafer, A. J., and Fournier, R. E. (1989). Physical mapping of human chromosome 17 using fragment-containing microcell hybrids, Genomics *5*, 167-76.

Lee, D. J., Koch, W. M., Yoo, G., Lango, M., Reed, A., Califano, J., Brennan, J. A., Westra, W. H., Zahurak, M., and Sidransky, D. (1997). Impact of chromosome 14q loss on survival in primary head and neck squamous cell carcinoma, Clin Cancer Res *3*, 501-5.

Lee, E. Y., Huang, S., Shew, J. Y., Donoso, L. A., and Lee, W. H. (1991). Diverse mutations lead to inactivation of the retinoblastoma gene, Prog Clin Biol Res *362*, 221-40.

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

Lee, M. P., DeBaun, M. R., Mitsuya, K., Galonek, H. L., Brandenburg, S., Oshimura, M., and Feinberg, A. P. (1999). Loss of imprinting of a paternally expressed transcript, with antisense orientation to KVLQT1, occurs frequently in Beckwith-Wiedemann syndrome and is independent of insulin-like growth factor II imprinting, Proc Natl Acad Sci U S A *96*, 5203-8.

Lee, Y. A., Stevens, H. P., Delaporte, E., Wahn, U., and Reis, A. (2000). A gene for an autosomal dominant scleroatrophic syndrome predisposing to skin cancer (Huriez syndrome) maps to chromosome 4q23 [letter], Am J Hum Genet *66*, 326-30.

Legoix, P., Bluteau, O., Bayer, J., Perret, C., Balabaud, C., Belghiti, J., Franco, D., Thomas, G., Laurent-Puig, P., and Zucman-Rossi, J. (1999). Beta-catenin mutations in hepatocellular carcinoma correlate with a low rate of loss of heterozygosity, Oncogene *18*, 4044-6.

Leppert, M., Dobbs, M., Scambler, P., O'Connell, P., Nakamura, Y., Stauffer, D., Woodward, S., Burt, R., Hughes, J., Gardner, E., and et al. (1987). The gene for familial polyposis coli maps to the long arm of chromosome 5, Science *238*, 1411-3.

Levin, N. A., Brzoska, P., Gupta, N., Minna, J. D., Gray, J. W., and Christman, M. F. (1994). Identification of frequent novel genetic alterations in small cell lung carcinoma, Cancer Res *54*, 5086-91.

Levrero, M., De Laurenzi, V., Costanzo, A., Gong, J., Wang, J. Y., and Melino, G. (2000). The p53/p63/p73 family of transcription factors: overlapping and distinct functions, J Cell Sci *113*, 1661-70.

Levy, D. B., Smith, K. J., Beazer-Barclay, Y., Hamilton, S. R., Vogelstein, B., and Kinzler, K. W. (1994). Inactivation of both *APC* alleles in human and mouse tumors, Cancer Res *54*, 5953-8.

Levy, M. Z., Allsopp, R. C., Futcher, A. B., Greider, C. W., and Harley, C. B. (1992). Telomere end-replication problem and cell aging, J Mol Biol *225*, 951-60.

Li, F. P., and Fraumeni, J. F., Jr. (1969). Rhabdomyosarcoma in children: epidemiologic study and identification of a familial cancer syndrome, J Natl Cancer Inst *43*, 1365-73.

Li, X., Lee, N. K., Ye, Y. W., Waber, P. G., Schweitzer, C., Cheng, Q. C., and Nisen, P. D. (1994a). Allelic loss at chromosomes 3p, 8p, 13q, and 17p associated with poor prognosis in head and neck cancer, J Natl Cancer Inst *86*, 1524-9.

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

Lin, A. W., Barradas, M., Stone, J. C., van Aelst, L., Serrano, M., and Lowe, S. W. (1998). Premature senescence involving p53 and p16 is activated in response to constitutive MEK/MAPK mitogenic signaling, Genes Dev *12*, 3008-19.

Lin, S. J., Defossez, P. A., and Guarente, L. (2000). Requirement of NAD and SIR2 for life-span extension by calorie restriction in saccharomyces cerevisiae [In Process Citation], Science *289*, 2126-8.

Lisitsyn, N., and Wigler, M. (1993). Cloning the differences between two complex genomes, Science *259*, 946-51.

Lissy, N. A., Davis, P. K., Irwin, M., Kaelin, W. G., and Dowdy, S. F. (2000). A common E2F-1 and p73 pathway mediates cell death induced by TCR activation, Nature *407*, 642-44.

Lohrum, M. A., and Vousden, K. H. (2000). Regulation and function of the p53-related proteins: same family, different rules, Trends Cell Biol *10*, 197-202.

Loo, D. T., Fuquay, J. I., Rawson, C. L., and Barnes, D. W. (1987). Extended culture of mouse embryo cells without senescence: inhibition by serum, Science *236*, 200-2.

Lopez-Fernandez, L. A., and del Mazo, J. (1993). Construction of subtractive cDNA libraries from limited amounts of mRNA and multiple cycles of subtraction, Biotechniques *15*, 654-6, 658-9.

Loughran, O., Clark, L. J., Bond, J., Baker, A., Berry, I. J., Edington, K. G., Ly, I. S., Simmons, R., Haw, R., Black, D. M., *et al.* (1997). Evidence for the inactivation of multiple replicative lifespan genes in immortal human squamous cell carcinoma keratinocytes, Oncogene *14*, 1955-64.

Loughran, O., Edington, K. G., Berry, I. J., Clark, L. J., and Parkinson, E. K. (1994). Loss of heterozygosity of chromosome 9p21 is associated with the immortal phenotype of neoplastic human head and neck keratinocytes, Cancer Res *54*, 5045-9.

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

Louis, D. N., von Deimling, A., and Seizinger, B. R. (1992). A (CA)n dinucleotide repeat assay for evaluating loss of allelic heterozygosity in small and archival human brain tumor specimens, Am J Pathol *141*, 777-82.

Ludlow, J. W., and Nelson, D. A. (1995). Control and activity of type-1 serine/threonine protein phosphatase during the cell cycle, Semin Cancer Biol *6*, 195-202.

Lukas, J., Parry, D., Aagaard, L., Mann, D. J., Bartkova, J., Strauss, M., Peters, G., and Bartek, J. (1995). Retinoblastoma-protein-dependent cell-cycle inhibition by the tumour suppressor p16, Nature *375*, 503-6.

Lundberg, A. S., and Weinberg, R. A. (1998). Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes, Mol Cell Biol *18*, 753-61.

Lundblad, V. (1997). The end replication problem: more than one solution [news; comment], Nat Med *3*, 1198-9.

Lundblad, V., and Blackburn, E. H. (1993). An alternative pathway for yeast telomere maintenance rescues est1- senescence, Cell *73*, 347-60.

Luongo, C., Moser, A. R., Gledhill, S., and Dove, W. F. (1994). Loss of *APC+* in intestinal adenomas from Min mice, Cancer Res *54*, 5947-52.

Lupton, S. D., Brunton, L. L., Kalberg, V. A., and Overell, R. W. (1991). Dominant positive and negative selection using a hygromycin phosphotransferase-thymidine kinase fusion gene, Mol Cell Biol *11*, 3374-8.

Lustbader, E. D., Williams, W. R., Bondy, M. L., Strom, S., and Strong, L. C. (1992). Segregation analysis of cancer in families of childhood soft-tissue-sarcoma patients, Am J Hum Genet *51*, 344-56.

Lydiatt, W. M., Davidson, B. J., Schantz, S. P., Caruana, S., and Chaganti, R. S. (1998). 9p21 deletion correlates with recurrence in head and neck cancer, Head Neck *20*, 113-8.

MacGrogan, D., and Bookstein, R. (1997). Tumour suppressor genes in prostate cancer, Semin Cancer Biol *8*, 11-9.

Maestro, R., Gasparotto, D., Vukosavljevic, T., Barzan, L., Sulfaro, S., and Boiocchi, M. (1993). Three discrete regions of deletion at 3p in head and neck cancers, Cancer Res *53*, 5775-9.

Maier, J. A., Statuto, M., and Ragnotti, G. (1993). Senescence stimulates U937-endothelial cell interactions, Exp Cell Res *208*, 270-4.

Maier, J. A., Voulalas, P., Roeder, D., and Maciag, T. (1990). Extension of the life-span of human endothelial cells by an interleukin- 1 alpha antisense oligomer, Science *249*, 1570-4.

Malkin, D., Li, F. P., Strong, L. C., Fraumeni, J. F., Jr., Nelson, C. E., Kim, D. H., Kassel, J., Gryka, M. A., Bischoff, F. Z., Tainsky, M. A., and et al. (1990). Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms [see comments], Science *250*, 1233-8.

Malumbres, M., Perez De Castro, I., Hernandez, M. I., Jimenez, M., Corral, T., and Pellicer, A. (2000). Cellular response to oncogenic ras involves induction of the Cdk4 and Cdk6 inhibitor p15(INK4b), Mol Cell Biol *20*, 2915-25.

Mao, L., Lee, J. S., Fan, Y. H., Ro, J. Y., Batsakis, J. G., Lippman, S., Hittelman, W., and Hong, W. K. (1996). Frequent microsatellite alterations at chromosomes 9p21 and 3p14 in oral premalignant lesions and their value in cancer risk assessment, Nat Med *2*, 682-5.

Martin-Rivera, L., Herrera, E., Albar, J. P., and Blasco, M. A. (1998). Expression of mouse telomerase catalytic subunit in embryos and adult tissues, Proc Natl Acad Sci U S A *95*, 10471-6.

Marui, N., Sakai, T., Hosokawa, N., Yoshida, M., Aoike, A., Kawai, K., Nishino, H., and Fukushima, M. (1990). N-myc suppression and cell cycle arrest at G1 phase by prostaglandins, FEBS Lett *270*, 15-8.

Mashimo, T., Goodarzi, G., Watabe, M., Cuthbert, A. P., Newbold, R. F., Pai, S. K., Hirota, S., Hosobe, S., Miura, K., Bandyopadhyay, S., *et al.* (2000). Localization of a novel tumor metastasis suppressor region on the short arm of human chromosome 2, Genes Chromosomes Cancer *28*, 285-93.

Matsumura, T., Zerrudo, Z., and Hayflick, L. (1979). Senescent human diploid cells in culture: survival, DNA synthesis and morphology, J Gerontol *34*, 328-34.

McClintock, B. (1941). the stability of broken ends of chromosomes in *Zea mays.* Genetics *26,* 234-282,

McNeill, C. A., and Brown, R. L. (1980). Genetic manipulation by means of microcell-mediated transfer of normal human chromosomes into recipient mouse cells, Proc Natl Acad Sci U S A *77*, 5394-8.

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

Miele, M. E., Jewett, M. D., Goldberg, S. F., Hyatt, D. L., Morelli, C., Gualandi, F., Rimessi, P., Hicks, D. J., Weissman, B. E., Barbanti-Brodano, G., and Welch, D. R. (2000). A human melanoma metastasis-suppressor locus maps to 6q16.3-q23, Int J Cancer *86*, 524-8.

Miele, M. E., Robertson, G., Lee, J. H., Coleman, A., McGary, C. T., Fisher, P. B., Lugo, T. G., and Welch, D. R. (1996). Metastasis suppressed, but tumorigenicity and local invasiveness unaffected, in the human melanoma cell line MelJuSo after introduction of human chromosomes 1 or 6, Mol Carcinog *15*, 284-99.

Millis, A. J., McCue, H. M., Kumar, S., and Baglioni, C. (1992). Metalloproteinase and TIMP-1 gene expression during replicative senescence, Exp Gerontol *27*, 425-8.

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

Mitra, A. B., Murty, V. V., Li, R. G., Pratap, M., Luthra, U. K., and Chaganti, R. S. (1994). Allelotype analysis of cervical carcinoma, Cancer Res *54*, 4481-7.

Modjtahedi, H., Eccles, S., Box, G., Styles, J., and Dean, C. (1993). Immunotherapy of human tumour xenografts overexpressing the EGF receptor with rat antibodies that block growth factor-receptor interaction, Br J Cancer *67*, 254-61.

Montalto, M. C., Phillips, J. S., and Ray, F. A. (1999). Telomerase activation in human fibroblasts during escape from crisis, J Cell Physiol *180*, 46-52.

Morin, P. J., Vogelstein, B., and Kinzler, K. W. (1996). Apoptosis and *APC* in colorectal tumorigenesis, Proc Natl Acad Sci U S A *93*, 7950-4.

Morita, R., Fujimoto, A., Hatta, N., Takehara, K., and Takata, M. (1998). Comparison of genetic profiles between primary melanomas and their metastases reveals genetic alterations and clonal evolution during progression, J Invest Dermatol *111*, 919-24.

Mulligan, G., and Jacks, T. (1998). The retinoblastoma gene family: cousins with overlapping interests, Trends Genet *14*, 223-9.

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

Murthy, S. S., and Testa, J. R. (1999). Asbestos, chromosomal deletions, and tumor suppressor gene alterations in human malignant mesothelioma, J Cell Physiol *180*, 150-7.

Nakamura, T. M., Morin, G. B., Chapman, K. B., Weinrich, S. L., Andrews, W. H., Lingner, J., Harley, C. B., and Cech, T. R. (1997). Telomerase catalytic subunit homologs from fission yeast and human [see comments], Science *277*, 955-9.

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

New, J. H., Sugiyama, T., Zaitseva, E., and Kowalczykowski, S. C. (1998). Rad52 protein stimulates DNA strand exchange by Rad51 and replication protein A [see comments], Nature *391*, 407-10.

Newbold, R. F., and Cuthbert, A. P. (1996). Mapping human senescence genes using interspecific monochromosome transfer. In: Freshney, R. I., and Freshney, M. E, eds. Culture of immortalised cells. Wiley-Liss.

Newbold, R. F., and Overell, R. W. (1983). Fibroblast immortality is a prerequisite for transformation by EJ c-Ha- ras oncogene, Nature *304*, 648-51.

Newbold, R. F., Overell, R. W., and Connell, J. R. (1982). Induction of immortality is an early event in malignant transformation of mammalian cells by carcinogens, Nature *299*, 633-5.

Nihei, N., Ichikawa, T., Kawana, Y., Kuramochi, H., Kugoh, H., Oshimura, M., Hayata, I., Shimazaki, J., and Ito, H. (1996). Mapping of metastasis suppressor gene(s) for rat prostate cancer on the short arm of human chromosome 8 by irradiated microcell-mediated chromosome transfer, Genes Chromosomes Cancer *17*, 260-8.

Nijjar, T., Wigington, D., Garbe, J. C., Waha, A., Stampfer, M. R., and Yaswen, P. (1999). p57KIP2 expression and loss of heterozygosity during immortal conversion of cultured human mammary epithelial cells, Cancer Res *59*, 5112-8.

Nikolic, M. (1993). CCND1 a cell cycle regulator and proto-oncogene, Thesis. University of Glasgow.

Ning, Y., Weber, J. L., Killary, A. M., Ledbetter, D. H., Smith, J. R., and Pereira-Smith, O. M. (1991). Genetic analysis of indefinite division in human cells: evidence for a cell senescence-related gene(s) on human chromosome 4, Proc Natl Acad Sci U S A *88*, 5635-9.

Nishisho, I., Nakamura, Y., Miyoshi, Y., Miki, Y., Ando, H., Horii, A., Koyama, K., Utsunomiya, J., Baba, S., and Hedge, P. (1991). Mutations of chromosome 5q21 genes in FAP and colorectal cancer patients, Science *253*, 665-9.

Noda, A., Ning, Y., Venable, S. F., Pereira-Smith, O. M., and Smith, J. R. (1994). Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen, Exp Cell Res *211*, 90-8.

Norwood, T. H., Pendergrass, W. R., Sprague, C. A., and Martin, G. M. (1974). Dominance of the senescent phenotype in heterokaryons between replicative and post-replicative human fibroblast-like cells, Proc Natl Acad Sci U S A *71*, 2231-5.

Nowell, P. C. (1976). The clonal evolution of tumor cell populations, Science *194*, 23-8.

O'Brien, W., Stenman, G., and Sager, R. (1986). Suppression of tumor growth by senescence in virally transformed human fibroblasts, Proc Natl Acad Sci U S A *83*, 8659-63.

Ogata, T., Ayusawa, D., Namba, M., Takahashi, E., Oshimura, M., and Oishi, M. (1993). Chromosome 7 suppresses indefinite division of nontumorigenic immortalized human fibroblast cell lines KMST-6 and SUSM-1, Mol Cell Biol *13*, 6036-43.

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

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

Ohmura, H., Tahara, H., Suzuki, M., Ide, T., Shimizu, M., Yoshida, M. A., Tahara, E., Shay, J. W., Barrett, J. C., and Oshimura, M. (1995). Restoration of the cellular senescence program and repression of telomerase by human chromosome 3, Jpn J Cancer Res *86*, 899-904.

Ohtani-Fujita, N., Fujita, T., Aoike, A., Osifchin, N. E., Robbins, P. D., and Sakai, T. (1993). CpG methylation inactivates the promoter activity of the human retinoblastoma tumor-suppressor gene, Oncogene *8*, 1063-7.

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

Oliner, J. D., Kinzler, K. W., Meltzer, P. S., George, D. L., and Vogelstein, B. (1992). Amplification of a gene encoding a p53-associated protein in human sarcomas [see comments], Nature *358*, 80-3.

Olovnikov, A. M. (1973). A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon, J Theor Biol *41*, 181-90.

Osada, M., Ohba, M., Kawahara, C., Ishioka, C., Kanamaru, R., Katoh, I., Ikawa, Y., Nimura, Y., Nakagawara, A., Obinata, M., and Ikawa, S. (1998). Cloning and functional analysis of human p51, which structurally and functionally resembles p53 [see comments] [published erratum appears in Nat Med 1998 Sep;4(9):982], Nat Med *4*, 839-43.

Oshimura, M., Kugoh, H., Koi, M., Shimizu, M., Yamada, H., Satoh, H., and Barrett, J. C. (1990). Transfer of a normal human chromosome 11 suppresses tumorigenicity of some but not all tumor cell lines, J Cell Biochem *42*, 135-42.

Ouellette, M. M., Aisner, D. L., Savre-Train, I., Wright, W. E., and Shay, J. W. (1999). Telomerase activity does not always imply telomere maintenance, Biochem Biophys Res Commun *254*, 795-803.

Ouspenski, II, Elledge, S. J., and Brinkley, B. R. (1999). New yeast genes important for chromosome integrity and segregation identified by dosage effects on genome stability, Nucleic Acids Res *27*, 3001-8.

Palmero, I., Pantoja, C., and Serrano, M. (1998). p19ARF links the tumour suppressor p53 to Ras [letter], Nature *395*, 125-6.

Pan, H., Yin, C., Dyson, N. J., Harlow, E., Yamasaki, L., and Van Dyke, T. (1998). Key roles for E2F1 in signaling p53-dependent apoptosis and in cell division within developing tumors, Mol Cell *2*, 283-92.

Pang, J. H., and Chen, K. Y. (1994). Global change of gene expression at late G1/S boundary may occur in human IMR-90 diploid fibroblasts during senescence, J Cell Physiol *160*, 531-8.

Pantoja, C., and Serrano, M. (1999). Murine fibroblasts lacking p21 undergo senescence and are resistant to transformation by oncogenic Ras, Oncogene *18*, 4974-82.

Paraskeva, C., Finerty, S., and Powell, S. (1988). Immortalization of a human colorectal adenoma cell line by continuous in vitro passage: possible involvement of chromosome 1 in tumour progression, Int J Cancer *41*, 908-12.

Paraskeva, C., Harvey, A., Finerty, S., and Powell, S. (1989). Possible involvement of chromosome 1 in in vitro immortalization: evidence from progression of a human adenoma-derived cell line in vitro, Int J Cancer *43*, 743-6.

Paredes-Zaglul, A., Kang, J. J., Essig, Y. P., Mao, W., Irby, R., Wloch, M., and Yeatman, T. J. (1998). Analysis of colorectal cancer by comparative genomic hybridization: evidence for induction of the metastatic phenotype by loss of tumor suppressor genes, Clin Cancer Res *4*, 879-86.

Parkinson, E. K., Newbold, R. F., and Keith, W. N. (1997). The genetic basis of human keratinocyte immortalisation in squamous cell carcinoma development: the role of telomerase reactivation, Eur J Cancer *33*, 727-34.

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

Passalaris, T. M., Benanti, J. A., Gewin, L., Kiyono, T., and Galloway, D. A. (1999). The G(2) checkpoint is maintained by redundant pathways, Mol Cell Biol *19*, 5872-81.

Pavelic, Z. P., Li, Y. Q., Stambrook, P. J., McDonald, J. S., Munck-Wikland, E., Pavelic, K., Dacic, S., Danilovic, Z., Pavelic, L., Mugge, R. E., and et al. (1994). Overexpression of p53 protein is common in premalignant head and neck lesions, Anticancer Res *14*, 2259-66.

Pearlstein, R. P., Benninger, M. S., Carey, T. E., Zarbo, R. J., Torres, F. X., Rybicki, B. A., and Dyke, D. L. (1998). Loss of 18q predicts poor survival of patients with squamous cell carcinoma of the head and neck, Genes Chromosomes Cancer *21*, 333-9.

Pereira-Smith, O. M., and Smith, J. R. (1983). Evidence for the recessive nature of cellular immortality, Science *221*, 964-6.

Pereira-Smith, O. M., and Smith, J. R. (1988). Genetic analysis of indefinite division in human cells: identification of four complementation groups, Proc Natl Acad Sci U S A *85*, 6042-6.

Perrem, K., Bryan, T. M., Englezou, A., Hackl, T., Moy, E. L., and Reddel, R. R. (1999). Repression of an alternative mechanism for lengthening of telomeres in somatic cell hybrids, Oncogene *18*, 3383-90.

Pershouse, M. A., El-Naggar, A. K., Hurr, K., Lin, H., Yung, W. K., and Steck, P. A. (1997). Deletion mapping of chromosome 4 in head and neck squamous cell carcinoma, Oncogene *14*, 369-73.

Petersen, I., Langreck, H., Wolf, G., Schwendel, A., Psille, R., Vogt, P., Reichel, M. B., Ried, T., and Dietel, M. (1997). Small-cell lung cancer is characterized by a high incidence of deletions on chromosomes 3p, 4q, 5q, 10q, 13q and 17p, Br J Cancer *75*, 79-86.

Petersen, S., Aninat-Meyer, M., Schluns, K., Gellert, K., Dietel, M., and Petersen, I. (2000). Chromosomal alterations in the clonal evolution to the metastatic stage of squamous cell carcinomas of the lung, Br J Cancer *82*, 65-73.

Piao, Z., Park, C., Park, J. H., and Kim, H. (1998). Deletion mapping of chromosome 4q in hepatocellular carcinoma, Int J Cancer *79*, 356-60.

Pihan, G. A., and Doxsey, S. J. (1999). The mitotic machinery as a source of genetic instability in cancer, Semin Cancer Biol *9*, 289-302.

Polascik, T. J., Cairns, P., Chang, W. Y., Schoenberg, M. P., and Sidransky, D. (1995). Distinct regions of allelic loss on chromosome 4 in human primary bladder carcinoma, Cancer Res *55*, 5396-9.

Poli-Frederico, R. C., Bergamo, N. A., Reis, P. P., Kowalski, L. P., Zielenska, M., Squire, J. A., and Rogatto, S. R. (2000). Chromosome 22q a frequent site of allele loss in head and neck carcinoma [In Process Citation], Head Neck *22*, 585-90.

Pomerantz, J., Schreiber-Agus, N., Liegeois, N. J., Silverman, A., Alland, L., Chin, L., Potes, J., Chen, K., Orlow, I., Lee, H. W., *et al.* (1998). The Ink4a tumor suppressor gene product, p19Arf, interacts with MDM2 and neutralizes *MDM2*'s inhibition of p53, Cell *92*, 713-23.

Prehn, R. T. (1996). The paradoxical association of regression with a poor prognosis in melanoma contrasted with a good prognosis in keratoacanthoma, Cancer Res *56*, 937-940.

Presti, J. C., Jr., Reuter, V. E., Galan, T., Fair, W. R., and Cordon-Cardo, C. (1991). Molecular genetic alterations in superficial and locally advanced human bladder cancer, Cancer Res *51*, 5405-9.

Prives, C., and Hall, P. A. (1999). The p53 pathway, J Pathol *187*, 112-26.

Qin, X. Q., Chittenden, T., Livingston, D. M., and Kaelin, W. G., Jr. (1992). Identification of a growth suppression domain within the retinoblastoma gene product, Genes Dev *6*, 953-64.

Qiu, L., Burgess, A., Fairlie, D. P., Leonard, H., Parsons, P. G., and Gabrielli, B. G. (2000). Histone deacetylase inhibitors trigger a G2 checkpoint in normal cells that is defective in tumor cells, Mol Biol Cell *11*, 2069-83.

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

Rashid, A., Wang, J. S., Qian, G. S., Lu, B. X., Hamilton, S. R., and Groopman, J. D. (1999). Genetic alterations in hepatocellular carcinomas: association between loss of chromosome 4q and p53 gene mutations, Br J Cancer *80*, 59-66.

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

Reed, M., Woelker, B., Wang, P., Wang, Y., Anderson, M. E., and Tegtmeyer, P. (1995). The C-terminal domain of p53 recognizes DNA damaged by ionizing radiation, Proc Natl Acad Sci U S A *92*, 9455-9.

Riabowol, K., Schiff, J., and Gilman, M. Z. (1992). Transcription factor AP-1 activity is required for initiation of DNA synthesis and is lost during cellular aging, Proc Natl Acad Sci U S A *89*, 157-61.

Riabowol, K. T. (1992)b. Transcription factor activity during cellular aging of human diploid fibroblasts, Biochem Cell Biol *70*, 1064-72.

Ried, T., Knutzen, R., Steinbeck, R., Blegen, H., Schrock, E., Heselmeyer, K., du Manoir, S., and Auer, G. (1996). Comparative genomic hybridization reveals a specific pattern of chromosomal gains and losses during the genesis of colorectal tumors, Genes Chromosomes Cancer *15*, 234-45.

Rimessi, P., Gualandi, F., Morelli, C., Trabanelli, C., Wu, Q., Possati, L., Montesi, M., Barrett, J. C., and Barbanti-Brodano, G. (1994). Transfer of human chromosome 3 to an ovarian carcinoma cell line identifies three regions on 3p involved in ovarian cancer, Oncogene *9*, 3467-74.

Rine, J., and Herskowitz, I. (1987). Four genes responsible for a position effect on expression from HML and HMR in Saccharomyces cerevisiae, Genetics *116*, 9-22.

Robles, S. J., and Adami, G. R. (1998). Agents that cause DNA double strand breaks lead to p16INK4A enrichment and the premature senescence of normal fibroblasts, Oncogene *16*, 1113-23.

Rogan, E. M., Bryan, T. M., Hukku, B., Maclean, K., Chang, A. C., Moy, E. L., Englezou, A., Warneford, S. G., Dalla-Pozza, L., and Reddel, R. R. (1995). Alterations in p53 and p16INK4 expression and telomere length during spontaneous immortalization of Li-Fraumeni syndrome fibroblasts, Mol Cell Biol *15*, 4745-53.

Rosin, M. P., Cheng, X., Poh, C., Lam, W. L., Huang, Y., Lovas, J., Berean, K., Epstein, J. B., Priddy, R., Le, N. D., and Zhang, L. (2000). Use of allelic loss to predict malignant risk for low-grade oral epithelial dysplasia [see comments], Clin Cancer Res *6*, 357-62.

Ross, R. K., Jones, P. A., and Yu, M. C. (1996). Bladder cancer epidemiology and pathogenesis, Semin Oncol *23*, 536-45.

Rubben, A., Babilas, P., Baron, J. M., Hofheinz, A., Neis, M., Sels, F., and Sporkert, M. (2000). Analysis of tumor cell evolution in a melanoma: evidence of mutational and selective pressure for loss of p16ink4 and for microsatellite instability, J Invest Dermatol *114*, 14-20.

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

Rumpel, C. A., Powell, S. M., and Moskaluk, C. A. (1999). Mapping of genetic deletions on the long arm of chromosome 4 in human esophageal adenocarcinomas, Am J Pathol *154*, 1329-34.

Russo, I., Silver, A. R., Cuthbert, A. P., Griffin, D. K., Trott, D. A., and Newbold, R. F. (1998). A telomere-independent senescence mechanism is the sole barrier to Syrian hamster cell immortalization, Oncogene *17*, 3417-26.

Sager, R. (1989). Tumor suppressor genes: the puzzle and the promise, Science *246*, 1406-12.

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

Saksela, E., and Moorehead, P. S. (1963). Proc Natl Acad Sci USA *50,* 390.

Sanchez-Cespedes, M., Okami, K., Cairns, P., and Sidransky, D. (2000). Molecular analysis of the candidate tumor suppressor gene ING1 in human head and neck tumors with 13q deletions, Genes Chromosomes Cancer *27*, 319-22.

Sandhu, A. K., Hubbard, K., Kaur, G. P., Jha, K. K., Ozer, H. L., and Athwal, R. S. (1994). Senescence of immortal human fibroblasts by the introduction of normal human chromosome 6, Proc Natl Acad Sci U S A *91*, 5498-502.

Sandhu, A. K., Kaur, G. P., Reddy, D. E., Rane, N. S., and Athwal, R. S. (1996). A gene on 6q 14-21 restores senescence to immortal ovarian tumor cells, Oncogene *12*, 247-52.

Santerre, R. F., Allen, N. E., Hobbs, J. N., Jr., Rao, R. N., and Schmidt, R. J. (1984). Expression of prokaryotic genes for hygromycin B and G418 resistance as dominant-selection markers in mouse L cells, Gene *30*, 147-56.

Santon, J. B., Cronin, M. T., MacLeod, C. L., Mendelsohn, J., Masui, H., and Gill, G. N. (1986). Effects of epidermal growth factor receptor concentration on tumorigenicity of A431 cells in nude mice, Cancer Res *46*, 4701-5.

Sarkar, S., Julicher, K. P., Burger, M. S., Della Valle, V., Larsen, C. J., Yeager, T. R., Grossman, T. B., Nickells, R. W., Protzel, C., Jarrard, D. F., and Reznikoff, C. A. (2000). Different combinations of genetic/epigenetic alterations inactivate the p53 and pRB pathways in invasive human bladder cancers, Cancer Res *60*, 3862-71.

Sasaki, M., Honda, T., Yamada, H., Wake, N., Barrett, J. C., and Oshimura, M. (1994). Evidence for multiple pathways to cellular senescence, Cancer Res *54*, 6090-3.

Savelieva, E., Belair, C. D., Newton, M. A., DeVries, S., Gray, J. W., Waldman, F., and Reznikoff, C. A. (1997). 20q gain associates with immortalization: 20q13.2 amplification correlates with genome instability in human papillomavirus 16 E7 transformed human uroepithelial cells, Oncogene *14*, 551-60.

Saxon, P. J., Srivatsan, E. S., Leipzig, G. V., Sameshima, J. H., and Stanbridge, E. J. (1985). Selective transfer of individual human chromosomes to recipient cells, Mol Cell Biol *5*, 140-6.

Saxon, P. J., Srivatsan, E. S., and Stanbridge, E. J. (1986). Introduction of human chromosome 11 via microcell transfer controls tumorigenic expression of HeLa cells, Embo J *5*, 3461-6.

Schantz, S. P., Huang, Q., Shah, K., Murty, V. V., Hsu, T. C., Yu, G., Andersen, P. E., Huvos, A. G., and Chaganti, R. S. (2000). Mutagen sensitivity and environmental exposures as contributing causes of chromosome 3p losses in head and neck cancers, Carcinogenesis *21*, 1239-46.

Schmale, H., and Bamberger, C. (1997). A novel protein with strong homology to the tumor suppressor p53, Oncogene *15*, 1363-7.

Schneider, E. L., and Mitsui, Y. (1976). The relationship between in vitro cellular aging and in vivo human age, Proc Natl Acad Sci U S A *73*, 3584-8.

Schoor, M., Schuster-Gossler, K., Roopenian, D., and Gossler, A. (1999). Skeletal dysplasias, growth retardation, reduced postnatal survival, and impaired fertility in mice lacking the SNF2/SWI2 family member ETL1, Mech Dev *85*, 73-83.

Schwendel, A., Langreck, H., Reichel, M., Schrock, E., Ried, T., Dietel, M., and Petersen, I. (1997). Primary small-cell lung carcinomas and their metastases are characterized by a recurrent pattern of genetic alterations, Int J Cancer *74*, 86-93.

Schwendel, A., Richard, F., Langreck, H., Kaufmann, O., Lage, H., Winzer, K. J., Petersen, I., and Dietel, M. (1998). Chromosome alterations in breast carcinomas: frequent involvement of DNA losses including chromosomes 4q and 21q, Br J Cancer *78*, 806-11.

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

Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D., and Lowe, S. W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4A, Cell *88*, 593-602.

Seshadri, T., and Campisi, J. (1990). Repression of c-fos transcription and an altered genetic program in senescent human fibroblasts, Science *247*, 205-9.

Shanahan, F., Seghezzi, W., Parry, D., Mahony, D., and Lees, E. (1999). Cyclin E associates with BAF155 and BRG1, components of the mammalian SWI-SNF complex, and alters the ability of BRG1 to induce growth arrest, Mol Cell Biol *19*, 1460-9.

Shapiro, G. I., Edwards, C. D., Ewen, M. E., and Rollins, B. J. (1998). p16INK4A participates in a G1 arrest checkpoint in response to DNA damage, Mol Cell Biol *18*, 378-87.

Sharan, S. K., Morimatsu, M., Albrecht, U., Lim, D. S., Regel, E., Dinh, C., Sands, A., Eichele, G., Hasty, P., and Bradley, A. (1997). Embryonic lethality and radiation hypersensitivity mediated by Rad51 in mice lacking Brca2 [see comments], Nature *386*, 804-10.

Sharpless, N. E., and DePinho, R. A. (1999). The INK4A/ARF locus and its two gene products, Curr Opin Genet Dev *9*, 22-30.

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

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

Shay, J. W., Tomlinson, G., Piatyszek, M. A., and Gollahon, L. S. (1995). Spontaneous in vitro immortalization of breast epithelial cells from a patient with Li-Fraumeni syndrome, Mol Cell Biol *15*, 425-32.

Shay, J. W., Van Der Haegen, B. A., Ying, Y., and Wright, W. E. (1993). The frequency of immortalization of human fibroblasts and mammary epithelial cells transfected with SV40 large T-antigen, Exp Cell Res *209*, 45-52.

Shen, M., Haggblom, C., Vogt, M., Hunter, T., and Lu, K. P. (1997). Characterization and cell cycle regulation of the related human telomeric proteins Pin2 and TRF1 suggest a role in mitosis, Proc Natl Acad Sci U S A *94*, 13618-23.

Sherr, C. J., and DePinho, R. A. (2000). Cellular senescence: mitotic clock or culture shock?, Cell *102*, 407-10.

Sherr, C. J., Kato, J., Quelle, D. E., Matsuoka, M., and Roussel, M. F. (1994). D-type cyclins and their cyclin-dependent kinases: G1 phase integrators of the mitogenic response, Cold Spring Harb Symp Quant Biol *59*, 11-9.

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

Sherwood, S. W., Rush, D., Ellsworth, J. L., and Schimke, R. T. (1988). Defining cellular senescence in IMR-90 cells: a flow cytometric analysis, Proc Natl Acad Sci U S A *85*, 9086-90.

Shivapurkar, N., Sood, S., Wistuba, II, Virmani, A. K., Maitra, A., Milchgrub, S., Minna, J. D., and Gazdar, A. F. (1999). Multiple regions of chromosome 4 demonstrating allelic losses in breast carcinomas, Cancer Res *59*, 3576-80.

Si, S. P., Tsou, H. C., Lee, X., and Peacocke, M. (1995). Effect of cellular senescence and retinoic acid on the expression of cellular retinoic acid binding proteins in skin fibroblasts, Exp Cell Res *219*, 243-8.

Simons, A., Schepens, M., Forus, A., Godager, L., van Asseldonk, M., Myklebost, O., and van Kessel, A. G. (1999). A novel chromosomal region of allelic loss, 4q32-q34, in human osteosarcomas revealed by representational difference analysis, Genes Chromosomes Cancer *26*, 115-24.

Sinclair, D. A., Mills, K., and Guarente, L. (1998). Molecular mechanisms of yeast aging, Trends Biochem Sci *23*, 131-4.

Smith, S., Giriat, I., Schmitt, A., and de Lange, T. (1998). Tankyrase, a poly(ADP-ribose) polymerase at human telomeres [see comments], Science *282*, 1484-7.

Smogorzewska, A., van Steensel, B., Bianchi, A., Oelmann, S., Schaefer, M. R., Schnapp, G., and de Lange, T. (2000). Control of human telomere length by TRF1 and TRF2, Mol Cell Biol *20*, 1659-68.

Soininen, R., Schoor, M., Henseling, U., Tepe, C., Kisters-Woike, B., Rossant, J., and Gossler, A. (1992). The mouse Enhancer trap locus 1 (Etl-1): a novel mammalian gene related to Drosophila and yeast transcriptional regulator genes, Mech Dev *39*, 111-23.

Soussi, T., and May, P. (1996). Structural aspects of the p53 protein in relation to gene evolution: a second look, J Mol Biol *260*, 623-37.

Spruck, C. H., 3rd, Ohneseit, P. F., Gonzalez-Zulueta, M., Esrig, D., Miyao, N., Tsai, Y. C., Lerner, S. P., Schmutte, C., Yang, A. S., Cote, R., and et al. (1994). Two molecular pathways to transitional cell carcinoma of the bladder, Cancer Res *54*, 784-8.

Srivastava, S., Zou, Z. Q., Pirollo, K., Blattner, W., and Chang, E. H. (1990). Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome. Nature *348*, 747-9.

Staehling-Hampton, K., Ciampa, P. J., Brook, A., and Dyson, N. (1999). A genetic screen for modifiers of E2F in Drosophila melanogaster, Genetics *153*, 275-87.

Stanbridge, E. J. (1976). Suppression of malignancy in human cells, Nature *260*, 17-20.

Stanbridge, E. J., Flandermeyer, R. R., Daniels, D. W., and Nelson-Rees, W. A. (1981). Specific chromosome loss associated with the expression of tumorigenicity in human cell hybrids, Somatic Cell Genet *7*, 699-712.

Stanton, P., Richards, S., Reeves, J., Nikolic, M., Edington, K., Clark, L., Robertson, G., Souter, D., Mitchell, R., Hendler, F. J., 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*, 427-33.

Steck, P. A., Pershouse, M. A., Jasser, S. A., Yung, W. K., Lin, H., Ligon, A. H., Langford, L. A., Baumgard, M. L., Hattier, T., Davis, T., *et al.* (1997). Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers, Nat Genet *15*, 356-62.

Stein, G. H. (1985). SV40-transformed human fibroblasts: evidence for cellular aging in pre- crisis cells, J Cell Physiol *125*, 36-44.

Stein, G. H., Beeson, M., and Gordon, L. (1990). Failure to phosphorylate the retinoblastoma gene product in senescent human fibroblasts, Science *249*, 666-9.

Stein, G. H., Drullinger, L. F., Robetorye, R. S., Pereira-Smith, O. M., and Smith, J. R. (1991). Senescent cells fail to express cdc2, cycA, and cycB in response to mitogen stimulation, Proc Natl Acad Sci U S A *88*, 11012-6.

Stein, G. H., Drullinger, L. F., Soulard, A., and Dulic, V. (1999). Differential roles for cyclin-dependent kinase inhibitors p21 and p16 in the mechanisms of senescence and differentiation in human fibroblasts, Mol Cell Biol *19*, 2109-17.

Stein, G. H., and Dulic, V. (1995). Origins of G1 arrest in senescent human fibroblasts, Bioessays *17*, 537-43.

Steinert, S., Shay, J. W., and Wright, W. E. (2000). Transient expression of human telomerase extends the life span of normal human fibroblasts, Biochem Biophys Res Commun *273*, 1095-8.

Stoler, A., and Bouck, N. (1985). Identification of a single chromosome in the normal human genome essential for suppression of hamster cell transformation, Proc Natl Acad Sci U S A *82*, 570-4.

Straume, O., Sviland, L., and Akslen, L. A. (2000). Loss of nuclear p16 protein expression correlates with increased tumor cell proliferation (Ki-67) and poor prognosis in patients with vertical growth phase melanoma, Clin Cancer Res *6*, 1845-53.

Swisshelm, K., Ryan, K., Lee, X., Tsou, H. C., Peacocke, M., and Sager, R. (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*, 133-41.

Takebayashi, S., Ogawa, T., Jung, K. Y., Muallem, A., Mineta, H., Fisher, S. G., Grenman, R., and Carey, T. E. (2000). Identification of new minimally lost regions on 18q in head and neck squamous cell carcinoma, Cancer Res *60*, 3397-403.

Tamkun, J. W., Deuring, R., Scott, M. P., Kissinger, M., Pattatucci, A. M., Kaufman, T. C., and Kennison, J. A. (1992). brahma: a regulator of Drosophila homeotic genes structurally related to the yeast transcriptional activator SNF2/SWI2, Cell *68*, 561-72.

Tan, T. L., Essers, J., Citterio, E., Swagemakers, S. M., de Wit, J., Benson, F. E., Hoeijmakers, J. H., and Kanaar, R. (1999). Mouse Rad54 affects DNA conformation and DNA-damage-induced Rad51 foci formation, Curr Biol *9*, 325-8.

Tanaka, H., Shimizu, M., Horikawa, I., Kugoh, H., Yokota, J., Barrett, J. C., and Oshimura, M. (1998). Evidence for a putative telomerase repressor gene in the 3p14.2-p21.1 region, Genes Chromosomes Cancer *23*, 123-33.

Thomassen, D. G., Gilmer, T. M., Oshimura, M., Annab, L. A., and Barrett, J. C. (1985). Multistage neoplastic transformation of normal and preneoplastic Syrian hamster embryo cells by viral oncogenes, Carcinog Compr Surv *9*, 41-9.

Todaro, G. J., and Green H. (1963). J Cell Biol *17*, 299-313.

Torra, R., Badenas, C., San Millan, J. L., Perez-Oller, L., Estivill, X., and Darnell, A. (1999). A loss-of-function model for cystogenesis in human autosomal dominant polycystic kidney disease type 2, Am J Hum Genet *65*, 345-52.

Toyama, T., Iwase, H., Watson, P., Muzik, H., Saettler, E., Magliocco, A., DiFrancesco, L., Forsyth, P., Garkavtsev, I., Kobayashi, S., and Riabowol, K. (1999). Suppression of ING1 expression in sporadic breast cancer, Oncogene *18*, 5187-93.

Trink, B., Okami, K., Wu, L., Sriuranpong, V., Jen, J., and Sidransky, D. (1998). A new human p53 homologue [letter] [see comments] [published erratum appears in Nat Med 1998 Sep;4(9):982], Nat Med *4*, 747-8.

Trott, D. A., Cuthbert, A. P., Overell, R. W., Russo, I., and Newbold, R. F. (1995). Mechanisms involved in the immortalization of mammalian cells by ionizing radiation and chemical carcinogens, Carcinogenesis *16*, 193-204.

Tsihlias, J., Kapusta, L., and Slingerland, J. (1999). The prognostic significance of altered cyclin-dependent kinase inhibitors in human cancer, Annu Rev Med *50*, 401-23.

Tsukamoto, Y., Kato, J., and Ikeda, H. (1997). Silencing factors participate in DNA repair and recombination in Saccharomyces cerevisiae [see comments], Nature *388*, 900-3.

Uejima, H., Mitsuya, K., Kugoh, H., Horikawa, I., and Oshimura, M. (1995). Normal human chromosome 2 induces cellular senescence in the human cervical carcinoma cell line SiHa, Genes Chromosomes Cancer *14*, 120-7.

Urade, Y., Watanabe, K., and Hayaishi, O. (1995). Prostaglandin D, E, and F synthases, J Lipid Mediat Cell Signal *12*, 257-73.

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

van Steensel, B., and de Lange, T. (1997). Control of telomere length by the human telomeric protein TRF1 [see comments], Nature *385*, 740-3.

van Steensel, B., Smogorzewska, A., and de Lange, T. (1998). TRF2 protects human telomeres from end-to-end fusions, Cell *92*, 401-13.

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

Venable, M. E., Blobe, G. C., and Obeid, L. M. (1994). Identification of a defect in the phospholipase D/diacylglycerol pathway in cellular senescence, J Biol Chem *269*, 26040-4.

Verma, R. S., Manikal, M., Conte, R. A., and Godec, C. J. (1999). Chromosomal basis of adenocarcinoma of the prostate, Cancer Invest *17*, 441-7.

Versteege, I., Sevenet, N., Lange, J., Rousseau-Merck, M. F., Ambros, P., Handgretinger, R., Aurias, A., and Delattre, O. (1998). Truncating mutations of hSNF5/INI1 in aggressive paediatric cancer, Nature *394*, 203-6.

Villaret, D. B., Wang, T., Dillon, D., Xu, J., Sivam, D., Cheever, M. A., and Reed, S. G. (2000). Identification of genes overexpressed in head and neck squamous cell carcinoma using a combination of complementary DNA subtraction and microarray analysis, Laryngoscope *110*, 374-81.

Von Hansemann, D. (1890). Uber asymmetrische Zellteilung in Epithelkrebsen und deren biologische Bedeutung, Virchows Arch Pathol Anat Physiol *119,* 298-307.

Waber, P., Dlugosz, S., Cheng, Q. C., Truelson, J., and Nisen, P. D. (1997). Genetic alterations of chromosome band 9p21-22 in head and neck cancer are not restricted to p16INK4A, Oncogene *15*, 1699-704.

Walker, G. J., Flores, J. F., Glendening, J. M., Lin, A. H., Markl, I. D., and Fountain, J. W. (1998). Virtually 100% of melanoma cell lines harbor alterations at the DNA level within CDKN2A, CDKN2B, or one of their downstream targets, Genes Chromosomes Cancer *22*, 157-63.

Walker, G. J., Palmer, J. M., Walters, M. K., and Hayward, N. K. (1995). A genetic model of melanoma tumorigenesis based on allelic losses, Genes Chromosomes Cancer *12*, 134-41.

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

Wang, S. S., Virmani, A., Gazdar, A. F., Minna, J. D., and Evans, G. A. (1999a). Refined mapping of two regions of loss of heterozygosity on chromosome band 11q23 in lung cancer, Genes Chromosomes Cancer *25*, 154-9.

Wang, T. H., and Wang, H. S. (1996). p53, apoptosis and human cancers, J Formos Med Assoc *95*, 509-22.

Wang, W., Xue, Y., Zhou, S., Kuo, A., Cairns, B. R., and Crabtree, G. R. (1996a). Diversity and specialization of mammalian SWI/SNF complexes, Genes Dev *10*, 2117-30.

Wang, X., Gleich, L., Pavelic, Z. P., Li, Y. Q., Gale, N., Hunt, S., Gluckman, J. L., and Stambrook, P. J. (1999b). Cervical metastases of head and neck squamous cell carcinoma correlate with loss of heterozygosity on chromosome 16q, Int J Oncol *14*, 557-61.

Wang, X. L., Uzawa, K., Imai, F. L., and Tanzawa, H. (1999c). Localization of a novel tumor suppressor gene associated with human oral cancer on chromosome 4q25, Oncogene *18*, 823-5.

Wang, X. W., Lin, X., Klein, C. B., Bhamra, R. K., Lee, Y. W., and Costa, M. (1992). A conserved region in human and Chinese hamster X chromosomes can induce cellular senescence of nickel-transformed Chinese hamster cell lines, Carcinogenesis *13*, 555-61.

Wang, X. W., Vermeulen, W., Coursen, J. D., Gibson, M., Lupold, S. E., Forrester, K., Xu, G., Elmore, L., Yeh, H., Hoeijmakers, J. H., and Harris, C. C. (1996b). The XPB and XPD DNA helicases are components of the p53-mediated apoptosis pathway, Genes Dev *10*, 1219-32.

Wang, Y., and Prives, C. (1995). Increased and altered DNA binding of human p53 by S and G2/M but not G1 cyclin-dependent kinases, Nature *376*, 88-91.

Weber, J. L., and May, P. E. (1989). Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction, Am J Hum Genet *44*, 388-96.

Weber, J. L., and Wong, C. (1993). Mutation of human short tandem repeats, Hum Mol Genet *2*, 1123-8.

Weinberg, R. A. (1995). The retinoblastoma protein and cell cycle control, Cell *81*, 323-30.

Weissenbach, J., Gyapay, G., Dib, C., Vignal, A., Morissette, J., Millasseau, P., Vaysseix, G., and Lathrop, M. (1992). A second-generation linkage map of the human genome [see comments], Nature *359*, 794-801.

Weissman, B. E., Saxon, P. J., Pasquale, S. R., Jones, G. R., Geiser, A. G., and Stanbridge, E. J. (1987). Introduction of a normal human chromosome 11 into a Wilms' tumor cell line controls its tumorigenic expression, Science *236*, 175-80.

Welch, D. R., Chen, P., Miele, M. E., McGary, C. T., Bower, J. M., Stanbridge, E. J., and Weissman, B. E. (1994). Microcell-mediated transfer of chromosome 6 into metastatic human C8161 melanoma cells suppresses metastasis but does not inhibit tumorigenicity, Oncogene *9*, 255-62.

Welch, P. J., and Wang, J. Y. (1993). A C-terminal protein-binding domain in the retinoblastoma protein regulates nuclear c-Abl tyrosine kinase in the cell cycle, Cell *75*, 779-90.

West, M. D., Pereira-Smith, O. M., and Smith, J. R. (1989). Replicative senescence of human skin fibroblasts correlates with a loss of regulation and overexpression of collagenase activity, Exp Cell Res *184*, 138-47.

Whitaker, N. J., Bryan, T. M., Bonnefin, P., Chang, A. C., Musgrove, E. A., Braithwaite, A. W., and Reddel, R. R. (1995). Involvement of pRB-1, p53, p16INK4 and telomerase in immortalisation of human cells, Oncogene *11*, 971-6.

Whitaker, N. J., Kidston, E. L., and Reddel, R. R. (1992). Finite life span of hybrids formed by fusion of different simian virus 40-immortalized human cell lines, J Virol *66*, 1202-6.

Wick, M., Burger, C., Brusselbach, S., Lucibello, F. C., and Muller, R. (1994). A novel member of human tissue inhibitor of metalloproteinases (TIMP) gene family is regulated during G1 progression, mitogenic stimulation, differentiation, and senescence, J Biol Chem *269*, 18953-60.

Wong, D. J., Barrett, M. T., Stoger, R., Emond, M. J., and Reid, B. J. (1997). p16INK4A promoter is hypermethylated at a high frequency in esophageal adenocarcinomas, Cancer Res *57*, 2619-22.

Wong, I. H., Lo, Y. M., Yeo, W., Lau, W. Y., and Johnson, P. J. (2000). Frequent p15 promoter methylation in tumor and peripheral blood from hepatocellular carcinoma patients [In Process Citation], Clin Cancer Res *6*, 3516-21.

Wright, W. E., Pereira-Smith, O. M., and Shay, J. W. (1989). Reversible cellular senescence: implications for immortalization of normal human diploid fibroblasts, Mol Cell Biol *9*, 3088-92.

Wright, W. E., and Shay, J. W. (1992). The two-stage mechanism controlling cellular senescence and immortalization, Exp Gerontol *27*, 383-9.

Wright, W. E., and Shay, J. W. (2000). Telomere dynamics in cancer progression and prevention: fundamental differences in human and mouse telomere biology, Nat Med *6*, 849-51.

Xiao, Z. X., Chen, J., Levine, A. J., Modjtahedi, N., Xing, J., Sellers, W. R., and Livingston, D. M. (1995). Interaction between the retinoblastoma protein and the oncoprotein MDM2, Nature *375*, 694-8.

Xu, H. J., Cairns, P., Hu, S. X., Knowles, M. A., and Benedict, W. F. (1993). Loss of pRB protein expression in primary bladder cancer correlates with loss of heterozygosity at the pRB locus and tumor progression, Int J Cancer *53*, 781-4.

Yamada, H., Horikawa, I., Hashiba, H., and Oshimura, M. (1990a). Normal human chromosome 1 carries suppressor activity for various phenotypes of a Kirsten murine sarcoma virus-transformed NIH/3T3 cell line, Jpn J Cancer Res *81*, 1095-100.

Yamada, H., Wake, N., Fujimoto, S., Barrett, J. C., and Oshimura, M. (1990b). Multiple chromosomes carrying tumor suppressor activity for a uterine endometrial carcinoma cell line identified by microcell-mediated chromosome transfer, Oncogene *5*, 1141-7.

Yamaguchi, K., Wu, L., Caballero, O. L., Hibi, K., Trink, B., Resto, V., Cairns, P., Okami, K., Koch, W. M., Sidransky, D., and Jen, J. (2000). Frequent gain of the p40/p51/p63 gene locus in primary head and neck squamous cell carcinoma, Int J Cancer *86*, 684-9.

Yamamoto, T., Rabinowitz, Z., and Sachs, L. (1973). Identification of the chromosomes that control malignancy, Nat New Biol *243*, 247-50.

Yanishevsky, R., and Carrano, A. V. (1975). Prematurely condensed chromosomes of dividing and non-dividing cells in aging human cell cultures, Exp Cell Res *90*, 169-74.

Yarbrough, W. G., Shores, C., Witsell, D. L., Weissler, M. C., Fidler, M. E., and Gilmer, T. M. (1994). ras mutations and expression in head and neck squamous cell carcinomas, Laryngoscope *104*, 1337-47.

Yasumoto, S., Kunimura, C., Kikuchi, K., Tahara, H., Ohji, H., Yamamoto, H., Ide, T., and Utakoji, T. (1996). Telomerase activity in normal human epithelial cells, Oncogene *13*, 433-9.

Yeager, T., Stadler, W., Belair, C., Puthenveettil, J., Olopade, O., and Reznikoff, C. (1995). Increased p16 levels correlate with pRB alterations in human urothelial cells, Cancer Res *55*, 493-7.

Yeager, T. R., Neumann, A. A., Englezou, A., Huschtscha, L. I., Noble, J. R., and Reddel, R. R. (1999). Telomerase-negative immortalized human cells contain a novel type of promyelocytic leukemia (PML) body, Cancer Res *59*, 4175-9.

Yeager, T. R., and Reznikoff, C. A. (1998). Methotrexate resistance in human uroepithelial cells with p53 alterations, J Urol *159*, 581-5.

Yeudall, W. A., Crawford, R. Y., Ensley, J. F., and Robbins, K. C. (1994). MTS1/CDK4I is altered in cell lines derived from primary and metastatic oral squamous cell carcinoma, Carcinogenesis *15*, 2683-6.

Yi, X., Tesmer, V. M., Savre-Train, I., Shay, J. W., and Wright, W. E. (1999). Both transcriptional and posttranscriptional mechanisms regulate human telomerase template RNA levels, Mol Cell Biol *19*, 3989-97.

Yokota, J., Tsunetsugu-Yokota, Y., Battifora, H., Le Fevre, C., and Cline, M. J. (1986). Alterations of myc, myb, and ras^{Ha} proto-oncogenes in cancers are frequent and show clinical correlation, Science *231*, 261-5.

Yu, J., Zhang, L., Hwang, P. M., Rago, C., Kinzler, K. W., and Vogelstein, B. (1999). Identification and classification of p53-regulated genes, Proc Natl Acad Sci U S A *96*, 14517-22.

Yunis, J. J., and Ramsay, N. (1978). Retinoblastoma and subband deletion of chromosome 13, Am J Dis Child *132*, 161-3.

Zalvide, J., and DeCaprio, J. A. (1995). Role of pRB-related proteins in simian virus 40 large-T-antigen- mediated transformation, Mol Cell Biol *15*, 5800-10.

Zatterstrom, U. K., Wennerberg, J., Ewers, S. B., Willen, R., and Attewell, R. (1991). Prognostic factors in head and neck cancer: histologic grading, DNA ploidy, and nodal status, Head Neck *13*, 477-87.

Zhang, S. Y., Klein-Szanto, A. J., Sauter, E. R., Shafarenko, M., Mitsunaga, S., Nobori, T., Carson, D. A., Ridge, J. A., and Goodrow, T. L. (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*, 5050-3.

Zhang, X., Mar, V., Zhou, W., Harrington, L., and Robinson, M. O. (1999). Telomere shortening and apoptosis in telomerase-inhibited human tumor cells, Genes Dev *13*, 2388-99.

Zhang, Y., Xiong, Y., and Yarbrough, W. G. (1998). ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the pRB and p53 tumor suppression pathways, Cell *92*, 725-34.

Zhou, J., Monson, E. K., Teng, S., Schulz, V. P., and Zakian, V. A. (2000). Pif1p helicase, a catalytic inhibitor of telomerase in yeast, Science *289*, 771-4.

Zhu, J., Wang, H., Bishop, J. M., and Blackburn, E. H. (1999). Telomerase extends the lifespan of virus-transformed human cells without net telomere lengthening [see comments], Proc Natl Acad Sci U S A *96*, 3723-8.

Zhu, J., Woods, D., McMahon, M., and Bishop, J. M. (1998). Senescence of human fibroblasts induced by oncogenic Raf, Genes Dev *12*, 2997-3007.

Zhu, L., van den Heuvel, S., Helin, K., Fattaey, A., Ewen, M., Livingston, D., Dyson, N., and Harlow, E. (1993). Inhibition of cell proliferation by p107, a relative of the retinoblastoma protein, Genes Dev *7*, 1111-25.

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

Zingg, J. M., Alva, G. P., and Jost, J. P. (1991). Characterisation of a genomic clone covering the structural mouse MyoD1 gene and its promoter region, Nucleic Acids Res *19*, 6433-9.

Appendices

Appendix 1

PCR Primers

Primers used to amplify a portion of the hygromycin B resistance cassette from pBabe Hygro:

F: CTGGGGCGCCCTCTGGTAAG

R: GCGTGGATATGTCCTGCGGG

product size: 209 bp

Primers used to amplify a portion of the neomycin resistance cassette from psp72 containing a corrected PMC1 neo polA cassette:

F: ATGCTCTTCGTCCAGATCAT

R: AAAGTATCCATCATGGCTGAT

product size: 150 bp

Primers used to amplify the MORF4 gene:

F: AAATGGGCTAAATGCCGTAG

R: CACTTTACAGCATATCCCTG
Product size: 1526

Appendix 2

Sequencing Primers

MORF4 Sequencing Primers

Forward:	S1-F	CAGTGTGCTGTATTCAGGAG
	S2-F	ATTGGGATGATGGGGTTCCG
	S3-F	GCTCTTCTATCTTCCTGCCG
Reverse:	S1-R	CAGACCAGATGTCTTCTTTCC
	S2-R	CCCAACATTAGGTTGAAGTA
	S3-R	CTAACCTCTTCAAAGCACATCG

Appendix 3: Microsatellite Markers

Marker Name	Forward Primer	Reverse Primer	Min/Max Length
D4S2936	cactcaagcctggggg	tggcacatcaccaacaac 1	70/178
D4S431	aggcatactaggccgtatt	ttcccatcagcgtcttc	252/256
D4S3009	atggcctgtgaatcaaccc	aatcctttgaagacggccc	267/275
D4S1582	atcagggttctccacacaaa	ttggttgaaacttgtggatataaa	114/122
D4S2926	ttaaccagtggaggccagt	ctcttaaagaacagggtgtctgata	185/197
D4S3022	gccaagatagcgccac	atagtctgtcccgaaccc	137/149
D4S2912	agctaaaccattatggcat	tctagttaattctccgtttcat	222/234
D4S3045	tatgcgtcaggcactg	ggcaaggacttccagg	117/121
D4S2978	acaaacaatgccaaagaa	gatctggtgtaggtaaggg	267/271
D4S409	cttgccgtcagactcaaact	caacctttcaatgttagggc	279/293
D4S2964	aagctaagacccaacttcttt	tcatgcaatccacacag	177/189
D4S2922	cattggtccactccagttct	ataaagggcagttagggatg	258-268
D4S1553	gctatgaaatgcttctgctt	aaaagactgttagttaaacatcagg	202-206
D4S2932	gagcaaaactctgtctcaaaaataa	ggcttacttggaaaggtctctt	209-221
D4S1538	tgagaattgcttggaaccga	ccacacagcacggagacatt	149-161
D4S2462	agctcatataggtgtnctattca	gtgggcctgtcctgtt	280-294
D4S423	ttgagtagttcctgaagcagc	caaagtcctccatcttgagtg	111/119
D4S3037	gtttagttttccattcctgaattac	ccaactcaaatgcctgtc	202-208
D4S2404	atatatatgatgcatgtcaaaatgg	ctgggcaaactcatttcaac	131/N.d
BIR0101	ttgcccatcttttgatcagc	tgtactttgtgagatccacc	N.d/N.d
D4S1591	ggggagttcgaggctg	agancttttttagggaagagtca	116/118
D4S1570	caagatgaaagcacaacgta	cctggcccattatattcctt	187/187
D4S1564	agcccaggaggtgaag	gagatttctaggaaacattgag	222/238
D4S406	ctggttttaaggcatgtttg	tcctcagggaggtctaatca	242/250
D4S2985	ttacactgaagaatgtgagagcc	ggccttggaactactgatgg	252/252
D4S2938	tgcaaattcgtgggac	tggtgtagtattccatcgtg	164/170
D4S429	ggtgatccacctgcct	aagccactgaccttcact	197/205

Marker Name	Forward Primer	Reverse Primer	Min/Max Length
D4S3039	gacagcctattgtagtaacttgtgg	tagtcagggtgctctagggg	167/169
D4S2959	agcttccatggtcattagagt	tagggtcctccaaagaacaga	126/126
D4S422	ggcaagantccgtctcaa	tgaagtaaaatttgggagattgt	85/87
D4S1576	attgtncatatatcatcacctgg	acagcataaactaaaatttgggg	239/243
D4S1579	cccccaccttcctgac	ctggagcatccgtgtg	146/148
D4S1610	gcatgggggtagaaatacac	ttacatcgtcctaaaatcaaacat	169/169
D4S3008	taactgaatggatttgagactacc	gcttgatcttgccttgga	267/270
D4S1548	tgccataaacaaggtgaaac	ttacccaactgctacaccat	261/265
D4S1588	ccggacatctgaggtcttat	tccagaaatggctagagaga	234/238
D4S2999	gtttgttgcctgaatttcc	catgtccatttcctcaagtc	208/212
D4S2982	ccagtcaaccccaacac	gaaatgaaacatgatgacacc	187/199
D4S2993	aagttggatgaaacacagc	tttacctgtccctgactcttag	156/160
D4S1597	tatagtggcccctagtgttacat	ttaggcttctcaaagcataagac	275/275
D4S2979	tttctgcagctaccatgaat	tagtttgccaacccctagtc	145/157
D4S3028	tatccctaaactttaatgccttt	gtctacacctcctacacccc	232/232
D4S3041	aatccctaggcaaataccat	tcttgagtggctgaaactacat	131/133
D4S2943	ccttatgaaccaggtaaccc	ctttcccagatatgtcgatttag	210/218
D4S2920	acacagcacagtttgtttga	gacctgcctaagcctttg	110/112
D4S1554	cttgtttcctgttgagcact	gaatgatgtactgatcacccagat	196/196
D4S2954	ccatttcagtgtctgtgacta	ggaagccaattcctcata	127/131
D4S1535	acttgtgatatatacctgccg	tgtgagagcagaatgttgag	183/183
D4S408	ggtctgatgaaaatgttctcaagc	tagactgggttgttagggactctc	231/243
D4S3047	agaaggcccttgaagtgata	tgacgacaggtcggtt	236/242
D4S3032	tgaaattctattgaccaatgatgtg	tagcacctggatttaccatgac	139/139
D4S1540	tgaacccgtgaagtgg	ggtgggatatgttaattgc	187/191
D4S2930	cctcatggtaggttaatcccacg	tattgaatgcccgccatttg	217/219

N.d = Not Defined.