An Investigation into the Synergism between Bovine Papillomavirus Type 4 and the Flavonoid Quercetin in the Transformation of Primary Bovine Palate Fibroblasts

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Disclaimer:

'Unless stated otherwise all work contained within this thesis has been completed personally'

I wished to add a quote at the start of the thesis as some sort of summation of the experience of not only the past three years but also the training to get this far through university and work. Many quotes came to mind including the joke of '*Lasciate ogni speranza voi ch'entrate*' from Alighieri Dante but this just stuck in my mind and seemed to fit so well:

'He wrought at one great work for years;
The world passed by with lofty look;
Sometimes his eyes were dashed with tears;
Sometimes his lips with laughter shook.'

Davidson, John
A Ballard of Heaven.

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Abbreviations

AT	Ataxia telangiectasia
ATP	Adenosine triphosphate
BF	Bracken Fern (*Pteridium aquilinum*)
bp	Base pair
BPV	Bovine papillomavirus
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophophate
CIAP	Calf intestinal alkaline phosphotase
CIN	Cervical intraepithelial neoplasia
CKI	Cyclin dependent kinase inhibitor
cm	Centimetres
CML	Chronic myeloid leukaemia
CRPV	Cottontail rabbit papillomavirus
DEPC	Diethyl pyrocarbonate
DMBA	7-12-dimethylbenz[α]anthracene
DMEM	Dulbecco's modified Eagle's medium
DMEM-10	Dulbecco's modified Eagle's medium plus FCS (10% v/v)
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	3' deoxyribonucleoside 5' triphosphate
dsDNA	Double stranded deoxyribonucleic acid
EBV	Epstein barr virus
E. Coli	Escherichia coli
ECL	Enhanced chemilluminesence
EDTA	Ethylenediamine tetra-acetic acid
EtBr	Ethidium bromide
EtoH	Ethanol
FCS	Foetal calf serum
FITC	Fluorescein-isothiocyanate
g	Gram
G418	Geneticin, G418-sulphate
GJIC	Gap junctional intercellular communication
HBS	HEPES buffered saline
HBV	Hepatitus B virus
HEPES	N-[2-Hyroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HIV	Human immuno-deficiency virus
HPV	Human papillomavirus
HRP	Horseradish peroxidase
HTLV-1	Human T cell leukaemia virus
hr	Hours
IP_3	Inositol 1,4,5-triphosphate
J	Joule
kb	Kilobase pairs
kD	KiloDalton

kg	Kilogram
l	Litre
LCR	Long control repeat
LOH	Loss of heterozygosity
M	Molar
MAPK	Mitogen activated protein kinase
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
MMTV	Mouse mammary tumour virus
MoLV	Moloney murine leukemia virus
mRNA	Messenger ribonucleic acid
nA	Nanoamp
NAD	Nicotinamide adenine dinucleotide
neo	Neomycin
NRK	Normal rat kidney
nts	Nucleotides
°C	Degree centigrade
OD	Optical density (light absorbance)
ORF	Open reading frame
PalF	Foetal Bovine Palate Fibroblasts
PBS	Phosphate-buffered saline
PARP	Poly(ADP-ribose) polymerase
PBT	PBS plus tween 20
PCR	Polymerase chain reaction
PE	PBS plus EDTA
PI	1-phosphotidylinositol
PIP	1-phosphotidylinositol phosphate
pg	picogram
pM	picomolar
RFLP	Restriction enzyme fragment length polymorphism
RI	Refractive Index
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase - polymerase chain reaction
sec	second
Ser	Serine
SLM	Special liquid medium
ssDNA	Single stranded deoxyribonucleic acid
SV40	Semian virus 40
TEMED	N,N,N',N'-tetramethylethylenediamine
TGN	Trans Golgi network
Thr	Threonine
TPA	12-o-tetradecanoylphorbol-13-acetate
TPCK	Tosyl-phenylalanine-chloromethyl ketone
Tris	Tris (hydroxymethyl) aminomethane
Tween 20	Polyoxyethylene sorbitan monolaurate

UV	Ultraviolet
V	Volts
v/v	Volume per unit volume
W	Watts
w/v	Weight per unit volume
μg	Microgram
μl	Microlitre
μM	Micromolar
%	percentage
^{32}P	Phosphorous isotope 32 atom

Single letter amino acid code

Alanine	Ala (A)
Argenine	Arg (R)
Asparagine	Asn (N)
Aspartic acid	Asp (D)
Cysteine	Cys (C)
Glutamic acid	Glu (E)
Glutamine	Gln (Q)
Glycine	Gly (G)
Histidine	His (H)
Isoleucine	Ile (I)
Leucine	Leu (L)
Lysine	Lys (K)
Methionine	Met (M)
Phenylalanine	Phe (F)
Proline	Pro (P)
Serine	Ser (S)
Threonine	Thr (T)
Tryptophan	Trp (W)
Tyrosine	Tyr (Y)
Valine	Val (V)

ABSTRACT

Bovine papillomavirus type 4 (BPV 4) infects the upper alimentary canal of cattle causing benign papillomas which can progress to squamous carcinomas in cattle grazing on bracken fern (BF). A single treatment with quercetin, a well characterised and potent mutagen found in BF, can cause full oncogenic transformation of cells partially transformed by BPV-4. Quercetin elevates the activity of the BPV-4 enhancer/promoter element (LCR) by up to four fold but this cannot fully explain the observed effect as the timing of quercetin exposure is critical for full transformation of the cells. We show that quercetin exposure arrests normal PalF cells in the G1 phase of the cell cycle, and this G1 arrest correlates with an increase in p53 protein levels and transcriptional activity.

Cells transformed by expression of either BPV 4 E7 and *Ha-ras* or the BPV4 genome and *Ha-ras*, fail to arrest in G1 after subsequent quercetin treatments. In these cells which are transformed but non-tumorigenic, p53 protein is elevated and transcriptionally activated in response to quercetin exposure. Yet the lack of cell cycle arrest is probably due to the viral protein E7 inhibiting $p21^{Waf1/Cip1}$.

In the transformed tumorigenic cells the failure to arrest in the G1 phase of the cell cycle is also evident. p53 protein is still present and even its stabilisation in response to quercetin can be observed in some cell lines, however p53 transcriptional activity is inhibited, probably as a result of p53 mutation. Additionally the protein which mediates p53 dependent cell cycle arrest, $p21^{Waf1/Cip1}$, is also absent from all the tumorigenic cells lending further evidence to the loss of p53 as a transcriptional activator.

Here we propose a model in which in normal cells quercetin induces G1 arrest, mediated by p53. Abrogation of this arrest by BPV-4 E7 allows the cell to proliferate allowing the accumulation of inheritable damage, including mutations

of the p53 gene at later stages. The net effect of this is full tumorigenic transformation of the cell.

CHAPTER ONE
INTRODUCTION

1.1 Multistage Nature of Cancer

Along with heart disease and strokes, cancer is one of the major causes of death in the western world, indeed 1 in 5 humans will develop cancer (Franks and Teich, 1991). There is a considerable international effort to seek understanding of the nature and causes of this disease in a hope to discover means to cure and prevent the different types of cancer.

It is believed that cancer arises through a complex series of interactions between environmental and genetic factors (Pike and Forman, 1991; Henderson *et al.,* 1991, for review), resulting in genetic lesions in the cell. As the cell accumulates successive genetic lesions the regulation and function of more key cellular regulator genes is altered. The cell will then develop the ability to override the normal checkpoints and the ability to penetrate the basement membrane and to invade surrounding tissues and resulting in an expanding population of cells which has the ability to metastasise to other regions of the organism.

The hypothesis of clonal expansion (Nowell, 1976) puts forward the notion that a single progenitor cell begins to proliferate abnormally. Further damage is then caused which permits further clonal expansion and so the cycle continues. Thus after sufficient genetic lesions have occurred the checkpoints that regulate cellular proliferation are over-ridden and a malignant tumour is formed.

The number of genetic lesions required were estimated to be anywhere from three to seven independent lesions (Renan, 1993). Recent work in human primary cells has demonstrated that as little as 3 initial changes to the cell (impacting on multiple cellular signalling pathways) can cause full transformation (Hahn *et al.,* 1999).

Evidence for this multi-stage nature of cancer comes from initial epidemiological analysis of colon and cervical cancer. There is clear evidence in the case of colon cancer that the condition comes from an evolution of benign adenomas which progress to become malignant tumours (Sugerbaker *et al.,* 1985). This kind of morphological evolution is also seen in cancer of the cervix which is classified according to the degree of cellular disruption (CIN 1-3, in increasing severity) (Richart, 1968).

The features taken into account when making a diagnosis of CIN grade are:

1. Differentiation (maturation, stratification)
    a) Present or absent
    b) Proportion of epithelium showing differentiation
2. Nuclear abnormalities
    a) Nucleus : Cytoplasmic ratio
    b) Hyperchromasia
    c) Nuclear pleomorphism and anisokaryosis
3. Mitotic activty
    a) Number of mitotic figures
    b) Height of mitosis in the epithelium
    c) Abnormal configurations

The different grade of CIN may be characterised in the following way:

CIN 1:

Maturation in upper two thirds of epithelium

Slight nuclear atypia to surface

Most nuclear abnormalities and mitotic figure in basal third and are slight

CIN 2: (As CIN1 except)

Nuclear atypia persists to surface but is more marked

Maturation present in upper half of epithelium

Mitotic figures found in basal half of epithelium

Abnormal configurations may also be seen

CIN3:

No maturation, or confined to superficial third of epitheliumm

Marked nuclear abnormalities and mitotic figure throughout

Abnormal mitotic figures are frequent

This morphological evolution of this cancer was backed up by molecular analysis discovering the activation of cellular genes during malignant progression (Roiu *et al.,* 1985).

There are thought to be 3 main stage of carcinogenesis: Initiation, promotion and progression. This model was observed easily in the mouse skin model (Hecker *et al.,* 1982) which proved useful for study due to it being well documented and easily controlled.

The initiation step involves the accumulation of a series of genetic lesions caused by genotoxic agents. This DNA damage was triggered by treatment of the mouse skin, with the drug DMBA (7-12-dimethylbenz[α]anthracene). The drug causes DNA damage and leads to mutation in the c-Ha-ras gene (Quintanilla *et al.,* 1986). In the initiation stage the resultant DNA damage causes genetic lesions and cell proliferation. In the second stage of carcinogenesis, promotion, the transformed cells proliferate resulting in benign tumours (papillomas). In the mouse skin model multiple treatments of the papillomas with another chemical agent TPA (12-o-tetradecanoylphorbol-13-acetate), which does not cause DNA damage, allows growth and persistence of the altered cells. This may be due to the control of epigenetic mechanisms such as activation of protein kinase C (Castagna *et al.,* 1982), a Ser/Thr kinase involved in signal transduction.

The final stage is progression. This stage involves the accumulation of further genetic lesions. This was evidenced by the fact that genotoxic agents increase the rate at which malignant tumours develop, yet non-genotoxic agents have no affect on the rate of tumour formation (Hennings *et al.,* 1983).

1.2 Risk Factors

These can be broadly classified into three main categories.

1. Physical Agents e.g. UV and X-rays
2. Chemical Agents (carcinogens and mutagens)
3. Infectious Agents e.g. tumour viruses

Exposure to ultraviolet light from the sun has been demonstrated to have a causal relationship with cancers of the lip and skin. However studies carried out generally involved exposure to sunlight, rather than ultraviolet light alone. However UV is presumed to be the active component due to its known tissue damaging and mutagenic effects (Cole *et al.,* 1986).

Ionising radiation, classed as ionising when it has the capacity to accelerate electrons in matter directly or indirectly, is ubiquitous. The most important natural sources are radioactive nucleotides in the environment and cosmic rays. The discovery of X-rays and nuclear fission have led to further exposure to ionising radiation, however natural sources still provide the greatest contribution to the radiation exposure of the human population. A key study into the effects of ionising radiation exposure upon cancer risk was carried out after the atomic bombing of 1945 by the Atomic Bomb Casualty Commission. Studies showed that survivors were at higher risk of leukaemia, multiple myelomas and cancers of the lung, breast, thyroid and gasterointestinal tract (Tomatis *et al.,* 1990 and references therein)

A good example of the risk conferred by exposure to chemical agents is that of cigarette smoking. Many studies over a past few decades have proven that smokers are well known to be at high risk of lung cancer. Doll & Hill in 1952 proved beyond doubt that cigarette smoking increased the chance of developing lung cancer. Smokers are also at higher risk of cervical neoplasia (Trevethan *et al.,* 1983; Reeves *et al.,* 1987; Cuzick *et al.,* 1990; Basu *et al.,* 1991). Different carcinogens contained within the smoke, some of which are proven to be more carcinogenic after metabolic activation and have electrophilic properties would

cause them to react with and damage DNA, RNA and protein e.g. Polycyclic aromatic hydrocarbons and nitrosamines (Miller, 1978; Pasquini *et al.,* 1988). Carcinogens in the diet may also play a role in the development of cancer such as alchohol consumption which is linked to increased risk of oro-pharyngeal cancer, oesophagus cancer and primary liver cancer (IARC, 1988).

In the category of infectious agents many different agents have been implicated in various cancers including viruses, parasitic animals, fungi and bacteria. Whilst viruses have been the most studied in their relation to tumour formation, examples of other infectious agents being correlated to cancer have been noted. One such example is that of *Helicobacter pylori* which is thought to be a co-factor in the development of stomach cancer (Forman *et al.,* 1990; Nomura *et al.,* 1991 and Parsonnet *et al.,* 1991).

Viruses these been shown to have both a direct effect on the development of cancer e.g. Hepatitis B virus (HBV) or Epstein Barr Virus (EBV) (see later) or a more indirect effect e.g. Human Immuno-deficiency Virus (HIV). This latter case can been seen by the fact that HIV patients have an increased risk of developing B-cell lymphomas (Zur Hausen *et al.,* 1991b) and an increased risk of developing other rare cancers such as Kaposi's sarcoma (Fauci, 1988).

Apart from these environmental factors there are of course genetic factors involved. Genetic predisposition is also a risk factor in such diseases as ataxia telangiectasia (AT), breast cancer, Retinoblastoma and Wilms tumour. In such diseases there would be a displayed family history of occurrence of the cancer. This family history would be due to inheritance of cell regulator genes with an altered activity.

1.3 Oncogenes and Tumour Suppresser Genes

1.3.1 Oncogenes

Oncogenes are activated cellular proto-oncogenes. These proto-oncogenes are genes involved in the proliferation and growth of the cell. Normally the

activity of said genes is tightly controlled only permitting the cell to undergo DNA synthesis and mitosis, along with cellular growth, under specific signals.

Oncogenes were first described in rapidly transforming retroviruses such as the Rous sarcoma virus, where genes were discovered that were not required for viral replication but were involved in viral pathogenesis (Weiss *et al.,* 1985). These genes were likely to have been picked up from cellular DNA sometime during their evolution (Bishop and Varmus, 1982). This cellular DNA includes gene controlling growth factors (e.g. *c-sis*) and their receptors (e.g. *c-erb-B, c-fms*), signal transduction protein (e.g. *c-src, ras, c-abl*), and transcription factors (e.g. *c-myc, c-fos, c-myb*). Hence oncogenes can be derived from a range of proto-oncogenes that are involved in the normal growth factor related signalling (Teich, 1991).

The proto-oncogenes will have undergone alterations in order to render them abnormally active, such as:

1. Point Mutation
2. Amplification
3. Chromosomal translocation
4. Viral transduction

For example the ras proto-oncogene can suffer point mutation by exposure to carcinogens such as DMBA in codons 12, 13 and 61 (Balmain and Brown, 1988; Brown *et al.,* 1990) in mammalian cells and animal models. *Neu* (a transmembrane protein with homology to the epidermal growth factor receptor) is also frequently activated in tumours of the nervous system in rats by a single point mutation resulting in a valine to glutamic acid causing the transforming properties of the protein product (Bargmann *et al.,* 1986).

N-*myc* can suffer amplification in many neuroblastomas (Brodeur *et al.,* 1984). There is also increasing evidence to suggest that amplification of *c-erb-B* may be a useful prognostic marker for the development of breast cancer (Barnes, 1989) .

An example of translocation is that of c-*myc* in Burkitts's lymphoma (Dalla-Favera *et al.,* 1983) which occurs between chromosomes 2 and 8. The condition of Chronic myeloid leukaemia (CML) results from the generation of a specific chromosomal abnormality called the Philadelphia chromosome which normally results from a translocation between chromosome 9 (which contains the *c-abl* gene) and 22 (at 9q34 and 22q11). The breakpoint region on chromosome 22 involved in this translocation is quite small (5.8Kb) and therefore this region was termed the breakpoint cluster region (*bcr*). This results in a protein derived from the fusion between *c-abl* and *bcr* genes (Hermans *et al.,* 1987; Dreazen *et al.,* 1988).

The transforming gene of the Rous sarcoma virus (*v-src*) is an example of viral transduction, where the virus at one point in its history acquired the *c-src* gene which encodes a receptor tyrosine kinase (Schwartzberg, 1998 for review). In fact *c-src* was first isolated as the cellular homologue of the transforming gene of the Rous sarcoma virus *v-src* (Stehelin *et al.,* 1976). The v-src transforming protein is mutated and encodes a truncated version of c-src (lacks the COOH terminus) resulting in the loss of a critical amino acid residue (Y527) causing constitutive activation of the kinase

1.3.2 Tumour Suppresser Genes

In contrast to the oncogenes, this class of genes suppress cellular proliferation. These genes contribute to cell transformation by being de-activated. Evidence for these genes first came from when tumour cell to normal cell fusions were carried out. These fusions lead to non-tumourigenic hybrids (Harris *et al.,* 1969 and Harris, 1988) indicating that the normal cells contained genetic information capable of suppressing the transformed phenotype of their tumor cell partner. However it was also observed that these hybrids had unstable karyotypes and would revert to tumorigenicity upon loss of specific chromosomes. These lost chromosomes therefore were presumed to contain critical growth regulatory genes.

Development of single chromosome studies by microcell transfer (Fournier & Ruddle, 1977) allowed a more detailed analysis of the chromosomes. This technique showed that introduction of chromosome 11 to HeLa cells and Wilms tumour cells (Saxon *et al.,* 1986 and Weissman *et al.,* 1987 respectively) suppressed growth. Since these initial discoveries other chromosomes have been associated with tumour suppresser genes.

The use of techniques such as cytogenetic chromosome banding and restriction enzyme fragment length polymorphism (RFLP) analysis has allowed researchers to home in on specific chromosome regions of interest, due to the fact that areas of the chromosome that contain tumour suppresser gene(s) suffer a phenomenon known as loss of heterozygosity (LOH). This is where these regions suffer large scale chromosome re-arrangements and/or deletions resulting in only one copy of the tumour suppresser gene at that locus.

The use of LOH studies has identified several tumour suppresser genes over the years of study such as those involved in Retinoblastoma and Wilms tumour.

Retinoblastoma is a cancer where ocular tumours form in young children (Knudson, 1971). Both sporadic and hereditary forms of the disease occur with a world wide incidence of about 1 in 20,000 and 10% of cases prove to be inheritable (Evans, 1993). Knudson in 1971 postulated that this cancer of the eye whether associated with development by familial or sporadic means, was due to two successive lesions in the cell genome. In sporadic retinoblastoma, where there is no family history, there would be lesions within the same somatic cell. However in familial retinoblastoma one of the lesions would be inherited during gametogenesis and the second would occur somatically. Karyotype analysis displayed that some retinoblastomas had deletions in chromosome 13q14 suggesting that this area may be important (Godbout *et al.,* 1983; Sparkes *et al.,* 1983). The ultimate isolation and identification of the Rb-1 gene showed that it encoded a 105 KDa protein (p105Rb) (Friend *et al.,* 1986; Lee *et al.,* 1987). This protein may be one of the most critical tumour suppressers involved in the cell

cycle (Wang *et al.,* 1994; Dyson, 1998; Mittnancht, 1998 for reviews). Hypophosphorylated, the p105Rb protein associates with the E2F family of transcription factors that promote expression of genes required for DNA synthesis (Helin, 1998 for review). Hyperphosphorylation of the p105Rb protein by the family of cyclin dependent kinases causes the release of free E2F which is then capable of activating transcription and allowing entry in S phase of the cell cycle. In addition there have also been associations of this protein to differentiation in a study where the induction of differentiation in several leukemic cell lines by treatment with phobol esters or retinoic acid resulted in dephosphorylation of the p105Rb protein which preceded the cell cycle arrest associated with differentiation (Chen *et al.,* 1989).

Wilms Tumour is a cancer that is responsible for 85% of all childhood kidney cancers (Matsungaga, 1981). This cancer also develops sporadically and more rarely familial. Studies of Wilms tumour cells isolated an area on chromosome 11p13 with LOH (Koufas *et al.,* 1984). However the situation may be more complex than that as there is also an area at position p15 on chromosome 11 which may be involved independently in Wilms tumour (Mannes *et al.,* 1988; Reeve *et al.,* 1989).

Possibly one of the best examples of a tumour suppresser protein is that of p53. p53 is negative regulator of cell proliferation as with other tumour suppressers and has been mapped to chromosome 17q31.1 (Mcbride, 1986). In addition to its control on the cell cycle, by causing arrest in response to a plethora of signals such as DNA damage and hypoxia, it is important in apoptosis and activation of DNA repair (for reviews see Ko & Prives, 1996; Agarwal *et al.,* 1998; Giacca & Kastan, 1998). Its importance to the control of the cell is underpinned by the fact that it is estimated to have lost activity in half of all cancers. In addition the germline mutation of p53 results in the Li-Fraumeni Syndrome. This syndrome results in pre-disposition to a variety of cancers such as breast carcinoma, soft tissue sarcoma, osteosarcoma and leukaemia at a young age (Malkin *et al.,* 1990; Srivasatva *et al.,* 1990).

Whilst the study of the role of the oncogenes and tumour suppresser genes in the context of a whole animal remains difficult due to the nature of the myriad complex interactions involved in a whole organism, the development of tissue culture systems has permitted easily controlled assays to be developed and provided detailed insight into the cellular functions of these regulator genes.

Tissue culture assays have again demonstrated the multi-stage nature of cancer with the expression of oncogenes or tumour-suppresser genes causing transformation of the cell but not conversion to full tumourigenicity, e.g. conferring the ability to grow in low serum conditions or independent of substrate but not form tumours in nude mice. However the results obtained from such *in vitro* systems are only ever an approximation of the proteins function and must be backed up with *in vivo* studies where possible.

One such *in vivo* study is the use of transgenic mice. The gene of interest is introduced, often by injection, into the pronuclei of fertilised mouse eggs. Then the DNA is incorporated into the mouse genome and expressed in every cell. Such studies permit the analysis of this expression pattern in the whole transgenic organism. One such experiment involved the over-expression of the *c-myc* gene under the control of a strong inducible promoter of the mouse mammary tumour virus (MMTV) long terminal repeat (Stewart *et al.,* 1984). The transgenic mice developed mammary carcinomas after a latent period of several months, thus confirming the role of *myc* as an oncogene. However as not all the mammary glands of the mouse developed carcinomas (1 out of 10 on average) and there was the long latency period before the cancer developed it indicated that the *myc* expression was not the only factor involved in the carcinoma development.

Interest in the Rb and p53 genes increased upon the discovery that virally encoded oncoproteins can bind to these proteins. Human adenovirus, Human papillomavirus and SV40 all encode proteins, namely E1A, E7 and large T antigen respectively, that bind and inactivate Rb, mimicking hyperphosphorylation of the tumour suppresser. The same viruses also encode protein that result in abrogation of p53 function, namely E1B, E6 and large T antigen (same protein that binds Rb).

The evolution of the viruses to overcome the cellular controls carried out by these tumour suppresser proteins implicates their important role in maintaining normal cell proliferation.

1.4 Viruses and Virally Associated Cancer

Viruses are thought to be associated with 15% of all human cancers world-wide (Zur Hausen, 1991b) and as such represent a major target for cancer prevention. As mentioned earlier viruses can be implicated with cancer risk by indirect effects, such as those described for HIV compromising the immune system, or direct effects which probably account for the majority of virally associated cancer. Examples of direct effects viruses can have on the development of cancer include those of viruses such as Hepatitus B virus (HBV) which is associated with hepatocellular carcinoma as evidenced by epidemiological (Trichopoulos *et al.,* 1976) and DNA analysis (Nagaya *et al.,* 1987) of liver tumours. The human T cell leukaemia virus (HTLV-1), a retrovirus, is associated with adult T cell leukaemia and lymphomas (Zur Hausen, 1991b) and the Epstein Barr Virus (EBV) is linked to several cancers including Burkitt's lymphoma, nasopharyngeal cancer and Hodgkins disease (Zur Hausen 1991b). However it is the papillomaviruses and their contribution to cancer that will be focused on in this thesis.

1.4.1 Papillomaviruses

Papillomaviruses, member of the papovavirus family, are widespread in the animal kingdom and there are examples of many different types that infect a wide range of species including dogs, sheep, birds, cattle and humans (Sundberg, 1987). They have a small double stranded circular genome of roughly 8 kilobases. The DNA is condensed into nucleosomes and encapsulated into a icosahedral virion without a lipid containing membrane envelope.

All the open reading frames (ORF) overlap to a large extent and are located one strand of the genome. Their position and sequence though are

conserved between papillomavirus species. Viral transcription is uni-directional and the mRNA is derived from splicing mechanisms. The genome can be divided into roughly three main regions. The long control region (LCR) which controls the expression of the viral genes with the combination of cellular and viral transcription factors. The early region which encodes the proteins involved in viral replication and transcription and the late region which encodes the structural proteins (Fuchs & Pfister, 1996).

Despite their genome organisation which is roughly comparable between species of papillomaviruses, they are normally highly species and anatomical site specific (Broker & Botchan, 1986). Papillomaviruses infect either the mucosal surfaces or the skin. Virus replication occurs in generally benign proliferations (papillomas) which form upon viral infection. Some types of papillomas will remain benign, with no invasion of the basement membrane, however others can progress to malignancy (Broker & Botchan, 1986).

The most commonly studied papillomaviruses are the cottontail rabbit (CRPV), human (HPV) and the bovine (BPV) viruses, and as such will be discussed below.

1.4.1.1 Cottontail Rabbit Papillomavirus (CRPV)

CRPV was the first model used to examine viral oncogenesis in mammals (Shope, 1933; Rous & Beard, 1935). The virus normally infects the hairy epithelium (Kidd & Parsons, 1936) inducing papillomas which can regress. However in 25% of rabbits the papillomas progress to squamous skin carcinoma (Kidd & Rous, 1940; Syverton, 1952), several months after viral infection (Syverton *et al.,* 1950a.b; Syverton 1952) implying the requirement for other factors. CRPV was demonstrated to synergise with chemical carcinogens e.g. tar (Rous *et al.,* 1936; Kidd & Rous, 1937) to induce malignant conversion of the papilloma in a much shorter time than with either carcinogen or virus alone (Rous & Beard, 1935; Rous & Friedewald, 1941; 1944).

1.4.1.2 Human Papillomavirus (HPV)

The HPVs are epitheliotropic agents that infect the mucosal surfaces or the skin. Infection by HPV induce warts, localised lesions with different morphological and histological manifestations (Croissant *et al.,* 1985). Some types of wart remain benign, with the dividing cells failing to invade the basal membrane. However some infections can progress and become dysplastic to varying degrees. Some of the papillomavirus lesions can develop to carcinoma in situ. Of the mucosotropic HPVs there is a subset strongly linked with the development of various forms of genital cancer especially HPVs 16 and 18. These types are classed as 'high risk' due to their association with carcinoma. Other high risk HPVs include types 31, 33, 35 and 39 all of which display high correlation to genital and oral carcinomas and therefore have received the most study. Other HPV types such as 6 and 11 (best studied in this group) produce genital warts, these lesions are usually benign and are only rarely associated with malignant cancer and as such are termed 'low risk'.

As stated above infection of the anogenital tract by HPVs results in genital warts. These warts can be of two distinct types. First they may be exophytic (condylomata acuminata) or flat (condylomata plana). The former is normally found around the penis and anus in males and occasionally on the scrotum. In females they are located to the entrance of the vagina and on the vulva, anus, perineum and occasionally on the cervix. The flat type of wart can be located to the cervix in women and is the type of wart that can progress to carcinoma which first suggested the link between papillomaviruses and genital cancer (Zur Hausen, 1977)

The first evidence between papillomaviruses and cervical cancer was due to the detection of HPV DNA in tumour cells (Gissmann & Schneider, 1986). Samples world-wide were then assayed and in each study HPV DNA was detected in the majority of the cancer biopsies (Ikenburg, 1990). The most common HPV type was HPV 16 which was found in roughly 50-70% of biopsies, with HPV 18 being the next most prevalent with 5-20% of the biopsies. This

correlation between HPV and cervical cancer has been strengthened by analysis of yet more invasive squamous cervical carcinomas, cervical adenocarcinomas and cell lines derived from these tumours for presence of HPV specific sequences. These studies have demonstrated that greater than 90% of these cases display presence of HPV DNA (Munoz & Bosch, 1996; Zur Hausen, 1991a for reviews). In fact in the majority of cases the HPV DNA has integrated into the host genome and is actively transcribed (Wettstein, 1990). This integration often occurs within the 3' part of the early region of the genome, and may cause disruption of the E2 reading frame (see section 1.4.2 for further details on E2). Therefore removal of the transcriptional repressor E2 would presumably lead to elevated levels of the viral transforming proteins E6 and E7. In support of this notion it was reporter that elevated levels of E6 and E7 expression were detected, by in situ hybridisation, in high grade cervical lesion when compared to undifferentiated keratinocytes of low grade cervical lesions (Stoler *et al.,* 1992; Durst *et al.,*1992).

Further indirect supportive evidence for the association between papillomavirus and genital cancer is due to the fact that the disease displays the characteristics of a sexually transmitted disease. For example nuns and virgins did not display the disease (Rigoni-Stern, 1842) and women whose partners had first wives with the disease were at higher risk (Gross *et al.,* 1985; Kessler, 1986).

Risk factors for the development of cervical cancer include (Broker & Botchan, 1986 and references therein):

1. Early onset of sexual activity
2. Sexual promiscuity
3. Use of oral contraceptives
4. Lower socio-economic class
5. Smoking
6. History of infection and of different HPV types

With just cervical cancer alone accounting for around 450,000 cases per annum (Broker & Botchan, 1986) with a 45% mortality even with medical

intervention there is an obvious need for research to attempt discover means to reduce this figure.

1.4.1.3 Bovine Papillomavirus (BPV)

Due to the infection of human beings with HPV for the purpose of experimentation being unethical, the study of animal papillomaviruses as a model for their action is a useful substitute. Both bovine and human papillomaviruses share certain features such as:

1. Specificity of anatomical site of infection.
2. Certain viral types associated with malignant progression.
3. Requirement of interaction between viral, cellular and environmental co-factors

There are six types of BPV which are divided into 2 sub-groups (Campo et al. 1980; 1981; Jarret *et al.,* 1984). Group A includes BPV 1, 2 and 5 which cause fibropapillomas. Group B includes BPV 3, 4 and 6 which are purely epitheliotropic.

Of these six types BPV-1 was one of the first to be studied and a lot of the early work on the papillomaviruses used this virus to dissect the ORF functions (see earlier). BPV 2 and 4 are associated with urinary bladder cancer (Campo *et al.,* 1992) and alimentary canal cancer (Campo *et al.,* 1994b). In both cases bracken consumption by the animals was identified as a co-factor. Bracken fern (*Pteridium aquilinum*) which contains carcinogens (Evans I. A. *et al.,* 1982) and immunosuppressants (Evans W.C. *et al.,* 1982) are thought to allow persistence of the papillomas through the immunosupression, and the carcinogens working with viral transforming proteins to cause conversion of the lesions to squamous carcinomas.

Note though that the BPV DNA is often lost on the progression to malignancy (Campo *et al.,* 1985), additionally when bovine palatine tissue was infected with BPV 4 DNA *in vitro* and placed in the renal capsule of nude mice, malignant cells developed. However those same cells when assayed for the

presence of the viral DNA were found to be negative (Gaukroger *et al.,* 1991). Hence a 'hit and run' hypothesis was generated (Smith and Campo, 1988) for the transformation of the cell by BPV 4. This is in contrast to the transformation incurred by HPV 16 or 18 where the viral DNA persists in the transformed cell.

1.4.2 Papillomavirus Transforming Genes

A lot of the early work on the identification of the papillomavirus ORF functions was carried out using BPV 1. In the case of this virus the 8Kb genome consists of:

1. LCR

Region of roughly 1000bp containing the sequences which bind viral and cellular factors to control transcription and replication.

2. Early region.

Originally defined as a 69% fragment of the genome sufficient for transformation in rodent cells *in vitro* (Lambert *et al.,* 1988). The region encodes the transforming proteins E5, E6 and E7 (DiMaio, 1991). In addition this region encodes the E1 protein which is involved in DNA replication (Lambert, 1991). The E1 protein binds an AT rich palindrome sequence at the region of replication (Chow & Broker, 1994; Howley, 1996). The E2 ORF encodes for 3 proteins: E2TA (full length), E2TR (carboxy terminus) and E8/E2TR (carboxy terminus of E2 ORF fused to 10 amino acids from the E8 ORF), the former acts as transcriptional activator and the latter two repress (Choe *et al.,* 1989; Lambert *et al.,* 1987; Spalholz *et al.,* 1985). In fact subsequent work has shown that the E2TA protein binds the E1 protein (Mohr, 1990) and a DNA helicase (Seo *et al.,* 1993; Yang *et al.,* 1993) and due to this is thought to aid viral replication by increasing E1's occupancy at the BPV 1 origin. In comparison the E2 protein in the high risk HPV's is a repressor of transcription (Romanczuk *et al.,* 1990) and in BPV 4 the E2 protein acts to activate transcription at low dosage levels and repress transcription at high dosage levels (Jackson & Campo, 1995; Morgan *et al.,* 1998).

The control of the expression of the viral transforming genes in BPV 1 is further complicated by the discovery that expression of E1 has been shown to repress E2 transactivated transcription from the BPV 1 major early promoter (Sandler *et al.,* 1993).

The E4 ORF is though to be involved in particle maturation (Doorbar *et al.,* 1991; Doorbar, 1996). This may be due to altering keratinocyte differentiation in order to favour viral particle production and release.

3. Late region

Encodes the 2 structural capsid proteins L1 and L2. L1 is the major capsid protein and L2 the minor capsid protein. They form icosahedral virions into which the viral DNA is packaged.

The general function of several of the BPV 1 proteins will likely be very similar to that in other papillomavirus types, even if the mechanism in which the functions are carried out do vary slightly e.g. E2 is involved in transcriptional control but in differing ways in the different viral types.

However it is the viral transforming proteins that have probably received the most attention in order to underpin the mechanisms by which they over-ride normal cellular controls on proliferation. This study in turn has shed alot of understanding on these controls in normal cell biochemistry.

The mechanism by which each of the viruses causes transformation in the cell is likely to differ as not all the viruses share the same complement of transforming genes as can be seen in table 1.1.

Table 1.1 **List of Oncogenes Found in Different Papillomaviruses**

	E5	*E6*	*E7*	*E8*
BPV 1	Yes	Yes	No	No
BPV 4	No	No	Yes	Yes
CRPV	Yes	Yes	Yes	Yes
HPV 16	Yes	Yes	Yes	No
HPV 8	No	Yes	No	No

(adapted from Campo, 1992)

The properties and functions of these transforming proteins will be discussed in further detail below from studies mostly of the high risk HPV proteins.

1.4.2.1 E7

The E7 protein is a nuclear protein of roughly 18 kilodaltons (Howley, 1996). The expression of the E7 protein alone in the absence of other transforming viral gene products leads to the immortalisation of rodent fibroblasts

There have been several domains identified in the E7 protein: a pocket protein binding domain, a casein kinase II domain and two zinc finger motifs (Cys-X-X-Cys) (Dyson *et al.,* 1989; Zerfass *et al.,* 1995; Barbosa *et al.,* 1989;1990). The ability of the E7 protein to bind to the pocket protein, p105Rb is central to the viral protein's ability to transform cells as defined by mutational analysis (Vousden, 1994). In normal cells the retinoblastoma gene product, p105Rb, is in a hypophosphorylated state in early G1 phase of the cell cycle. Hypophosphorylated it forms a complex with the E2F family of transcription factors. This results in transcriptional repression of genes that contain E2F binding sites in their promoters. Entry into S phase is associated with cyclin/cdk complexes which phosphorylate p105Rb, namely cyclin D/cdk4/6 and cyclin E/cdk2. This phosphorlyation results in p105 assuming a hyperphosphorylated state and causing the release of the transcription factor E2F, this in turn promotes transcription from E2F dependent promoters. The ability of E7 to bind to the

pocket proteins and cause dissociation of E2F from hypophosphorylated p105Rb causes constitutive expression from the E2F responsive promoters (Chellappan *et al.,* 1992). In addition it has been recently shown that p105Rb binds a histone deacetylase through its conserved pocket domain, and this association is important for its ability to repress E2F regulated transcription (Magnaghi-Jaulin *et al.,* 1998; Brehm *et al.,* 1998). E7 also interferes with this association between the histone deacetylase and p105Rb (Brehm *et al.,* 1998). One further effect on p105Rb by E7 is to reduce the stability of the protein (Boyer *et al.,* 1996; Jones *et al.,* 1997). Thus through the ability to directly bind and inactivate p105Rb, to reduce the stability of the p105Rb protein and interference with the association between the histone deacetylase and p105Rb E7 efficiently abrogates the p105Rb growth suppression ability.

In addition to this central role of E7 other interactions with cellular proteins have been reported. E7 has been shown to bind to cyclin E via interaction with the p105Rb related pocket protein p107 (McIntyre *et al.,* 1996) and also to cyclin A although at this point it is unclear whether this is a direct interaction with the cyclin or not (Arroyo *et al.,* 1993; Tommasino *et al.,* 1993). Interestingly the first E7 responsive gene *B-Myb,* which plays an essential role in cell cycle progression, has had its de-regulated expression correlated to E7's interactions with p107/E2F complexes (Lam *et al.,* 1994). In addition to binding these cyclins E7 has also been shown to deregulate expression of cyclin E (Martin *et al.,* 1998; Vogt *et al.,* 1999) and cyclin A in suspension cells (Schulze *et al.,* 1998).

Another family of protein involved in the regulation of the G1/S checkpoint the cyclin dependent kinase inhibitor (CKIs), has also been implicated as targets of E7. The CKIs function by binding and inhibiting cyclin/cdk activity. They fall into two categories: Kip/Cip family which affect the activities of cyclin D, E and A complexes e.g. $p21^{Waf1/Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ and the INK family which inhibit only D cyclin complexes e.g. $p16^{INK4a}$, $p15^{INK4b}$ and $p18^{INK4c}$ (Sherr & Roberts, 1995; 1999). Note that the Kip/Cip family are potent inhibitors of

cyclin E and A complexes but recent work suggests that they are in fact positive regulators of cyclin D complexes (Sherr & Roberts, 1999 for review).

It was shown that E7 can bind p21 directly and abrogate the inhibitor. This binding not only inhibits p21's effect on the cyclin/cdk complexes but also p21's ability to inhibit DNA replication by binding PCNA (Jones *et al.,* 1997; Funk *et al.,* 1997). The related CKI, p27, is also bound by E7 which results in abrogation of its inhibitory activity on cyclin/cdk complexes (Zerfass-Thome *et al.,* 1996).

E7 has also been shown to interact with and *trans*-activate the AP-1 family of transcription factors, including c-Jun, JunB, JunD and c-fos. The ability of E7 to bind to c-Jun was demonstrated by immunoprecipitation and yeast two hybrid systems and locates to amino acids 224 to 286 of c-Jun. Additionally the ability of E7 to *trans*-activate c-Jun induced transcription was observed using a reporter construct responsive to c-Jun (Antinore *et al.,* 1996).

As well as interacting with the retinoblastoma gene product there have been reports of an indirect interaction between E7 and the tumour suppresser protein p53. E7 was reported to repress p53 transcriptional activity via binding of the TATA box binding protein (TBP), which in turn is elevated following caesin kinase II phosphorylation (Massimi *et al.,* 1997; Massimi & Banks, 1997).

These interactions are the probable cause of G1/S checkpoint abrogation in cells expressing E7 (Hickman *et al.,* 1994; Slebos *et al.,* 1994; Demers *et al.,* 1994). In these cells although DNA damage may induce p53 to initiate a cell cycle arrest, the combination of the afore-mentioned events would permit the transcription of genes required for DNA synthesis.

In addition to the abrogation of the G1/S checkpoint there have been reports of an ability of E7 to disrupt the mitotic spindle checkpoint at the G2/M boundary. In the presence of mitotic spindle inhibitors expression of E7 allowed multiple rounds of DNA synthesis in a p53 independent manner (Di Leonardo *et al.,* 1997; Thomas &Laimins, 1998).

1.4.2.2 E6

E6 primarily alters the normal regulation of the cell cycle through its interaction with the p53 protein. E6, via the association of a cellular protein (a component of the ubiquitin proteolytic pathway), termed E6-AP, causes the rapid turnover of the p53 protein (Scheffner *et al.,* 1994). Additionally E6 can inhibit p53's ability to bind DNA, therefore inhibiting the protein's capacity as a transcriptional regulator (Vousden, 1994). E6 in complex with the E6-AP protein can also targets the transcriptional co-activator CBP/p300 which involves the C terminal zinc finger of E6 and amino acids 1808 to 1826 in CBP. E6 does not require E6-AP to bind CBP/p300 as an E6 mutant incapable of binding E6-AP is still capable of binding CBP/p300 (Zimmermann *et al.,* 1999)

As p53 is involved in G1 cell cycle arrest (Agarwal *et al.,* 1995) due to DNA damage caused by chemical agents or γ-irradiation (Kastan *et al.,* 1991; Kuerbitz *et al.,* 1992) or due to ribonucleotide depletion (Linke *et al.,* 1996) loss of function due to E6 expression results in loss of G1 arrest in response to the DNA damaging stimuli (Vousden, 1994). As p53 is also involved in the mitotic spindle checkpoint (Di Leonardo *et al.,* 1997; Taylor *et al.,* 1999) E6 also impacts on this function. Expression of the E6 protein results in abrogation of this checkpoint (Di Leonardo *et al.,* 1997; Thomas & Laimins, 1998; Thompson *et al.,* 1997). Additionally another function of p53 is the regulation of apoptosis in certain cell types e.g. those of hematopoietic origin (Oren, 1994 and references therein). Stimuli such as DNA damage (Clarke *et al.,* 1993), adenovirus E1A expression (Debbas & White, 1993) and withdrawal of growth factors (Johnson *et al.,* 1993) can induce p53 dependent apoptosis. The ability of E6 to promote degradation of p53 was shown to establish resistance to such p53 mediated apoptosis (Haupt *et al.,* 1995). E6 has also been implicated in resistance to apoptosis in cells lacking p53 (Steller *et al.,* 1996) and E6, through interaction with E6-AP, is able to inhibit Bak induced apoptosis, in a p53 independent manner, by mediating degradation of the Bak protein (Thomas & Banks, 1998).

Cancers which contain p53 mutation often contain amplifications and aneuploid chromosomes (Hollstein *et al.,* 1991), which leads to the hypothesis that p53 plays a role in the genomic integrity maintenance of the cell. Loss of genomic stability in E6, but not E7, immortalised human uroepithelial cells was observed (Reznikoff *et al.,* 1994).

Other cellular proteins observed to bind to E6 include E6-BP (Chen *et al.,* 1995). E6-BP sequence is identical to a putative calcium binding protein, however as of yet its normal function has not been elucidated. Another E6 interacting protein is paxillin, a focal adhesion protein (Tong & Howley, 1997). Both E6-BP and paxillin would help explain the nuclear and cytoplasmic distribution of E6 in the cell (Scheffner *et al.,* 1994). Additionally it was shown that only E6 from BPV 1 and HPV 16, but not low risk types bound paxillin.

Another protein observed to interact with E6 is the disc large tumour suppresser protein (hDLG) (Kiyono *et al.,* 1997). In common with paxillin and E6-BP, mutants of E6 which were unable to bind to the hDLG protein lost transformation activity. Again in common with paxillin, only E6 from high risk types bound the hDLG protein. These results suggest that as binding to these proteins correlates to transformation ability they may play a functional role in the transforming ability of the papillomaviruses.

In another study, another interaction of an E6 protein was demonstrated, between the BPV-1 E6 protein and AP-1, the TGN (trans-Golgi network)-specific clathrin adapter complex. AP-1 is a four-subunit protein complex required for clathrin-mediated cellular transport from the TGN. It was proposed that BPV-1 E6, through its interaction with AP-1, could affect cellular processes involving clathrin-mediated trafficking pathway (Tong *et al.,* 1998).

Other functions of the E6 protein include inhibition of human keratinocyte differentiation (Sherman & Shlegel, 1996) and the activation of telomerase (Klingelhutz *et al.,* 1996) an enzyme involved in the maintenance of the structures at the end of the chromosome called telomeres, which under normal conditions shorten with each round of cell division (Engelhardt & Martins, 1998 for review).

Telomerase activation in somatic cells, where it is normally inactive, is linked to cell immortalisation. This feature of the E6 protein may be linked to the observations that expression of the E6 protein led to immortalisation of p53 null mouse fibroblasts (Scobie *et al.,* 1997) and primary mouse fibroblasts (independent of any interaction with p53) (Pim *et al.,* 1994).

1.4.2.3 E5 & E8

The BPV 4 E8 protein shows a significant resemblance to the E5 protein of BPV 1, in so far as they both are very hydrophobic and have a high leucine content in addition to similar hydrophobicity profiles (Jackson *et al.,* 1991; Banks & Matlashewski, 1993). The E8 ORF encodes a polypeptide 42 amino acids in length which is located, like E5, in the Golgi and endoplasmic reticulum (occasionally in the plasma membrane) of transformed cells (Campo, 1992; Pennie *et al.,* 1993; Faccini *et al.,* 1996). It is also known that BPV 4 E8 is only expressed in the basal and superbasal regions of the papilloma (Anderson *et al.,* 1997)

E8 expression, along with E7 and *ras*, results in anchorage independent growth of primary bovine fibroblasts. E8 expression together with *ras* is lethal to the cell, unless expressed with E7, or in the context of the BPV 4 genome (Campo, 1992; Pennie *et al.,* 1993). Recent work has shown that E8 expression can also induce anchorage independent growth of the murine cell line NIH3T3 (O'Brien & Campo, 1998).

In common with the BPV 1 and HPV 16 E5 proteins, BPV 4 E8 can complex with the 16K ductin protein *in vitro* (Banks & Matlashewski, 1993; Faccini *et al.,* 1996; Oelze *et al.,* 1995). The ductin protein has been identified as a component of the gap junctions and of the vacuolar ATP-ase (Finbow *et al.,* 1991) and this may explain the observation that HPV 16 E5 or BPV 4 E8 expressing cells have reduced levels of gap junction intercellular communication (Faccini *et al.,* 1996; Oelze *et al.,* 1995). This may contribute to the virus transforming ability as cells released from the control of the surrounding environment (i.e. of non-

transformed cells) would be permissive for proliferation and consequently form an expanding transformed clone (Campo, 1992). The complexing of the E5/E8 with the vacuolar ATP-ase could result in prolonged receptor signalling due to an alteration in pH of the endosomes reducing the efficiency at which the receptors are deactivated and recycled (Straight *et al.,* 1995).

BPV 1 E5 has also been shown to bind to and activate the membrane receptors EGF and PDGF (Martin *et al.,* 1989; Petti *et al.,* 1991; Cohen *et al.,* 1993) thus likely to contribute to a proliferative signal in the transformed cell.

However it has been observed that mutations in the transmembrane domain of E5, which still permit binding to ductin, cause loss of transforming ability (Sparkowski *et al.,* 1996) and BPV1 E5 that remains in the endoplasmic reticulum can still activate the PDGF receptor but not transform cells (Sparkowski *et al.,* 1995). Hence maybe both functions of the protein are required in order to facilitate transformation, or indeed neither of these activities may be necessary for transformation and other, as yet unidentified, functions are necessary.

The HPV 16 E5 gene is less likely to play an important role in the maintenance of cell transformation, as it is often lost as a consequence of viral integration (Howley, 1996). This also applies to the BPV 4 E8 gene whose expression is also often lost due to the loss of the BPV 4 genome during progression to malignancy (Campo *et al.,* 1985). The role for the E5 and E8 protein functions in the early stages of transformation of the cell and the viral life cycle however may be more significant i.e. by promoting cell proliferation and reducing cell-cell communication.

The studies of the E5 and E8 ORFs in CRPV have also demonstrated their transforming function (Han *et al.,* 1998). Both E8 and E5 stimulated C127 and BALB/c A31 (A31) cell proliferation and affected cell cycle transition. The E8 and E5 transfectants lost cell contact inhibition. E8-C127 transfectants formed colonies in soft agar in the presence of platelet-derived growth factor (PDGF) while E5-C127 transfectants formed colonies without the requirement for PDGF. E8-C127 transfectants were highly tumorigenic whereas E5-C127 transfectants

showed weak tumorigenicity in nude mice. However both E5 and E8 A31 transfectants failed to display anchorage independent growth or form tumours in nude mice with or without the presence of PDGF. These results showed that CRPV E8 and E5 may well be oncoproteins and that the PDGF beta-receptor signalling pathway may be involved in E8-mediated C127 cell transformation. The difference observed with the two cell lines indicates there may be additional factors involved in E5 or E8 expressing cells to progress to full transformation.

1.4.3 Bovine Papillomavirus Type 4 (BPV 4)

BPV 4 is a transforming papillomavirus, which induces papillomas of the mucosal epithelium of the upper gastrointestinal tract of cattle (Campo *et al,* 1994). In healthy cattle, the papillomas develop and persist for approximately one year and are then rejected by a cell mediated immune response (Knowles *et al,* 1996). However, in cattle grazing on bracken fern the papillomas can progress to squamous cell cancer (Campo and Jarrett, 1986). Bracken fern contains immunosuppressants (Evans W.C. *et al.,* 1982) and mutagens (Evans I. A. *et al.,* 1982). Bracken-eating cattle become chronically immunosuppressed and incapable of mounting an appropriate immune response against the virus or virus infected cells (Campo, 1997b). The progression of papillomas to carcinomas has also been experimentally reproduced in bracken-fed cattle (Campo *et al.,* 1994b) thus confirming the epidemiological studies conducted in the field.

Activation of *ras* (Campo *et al,* 1990), mutation of *p53* (Scobie, 1996) and increase in epidermal growth factor receptor (Smith *et al.,* 1987) have all been found in naturally occurring bovine alimentary cancers which may be due to the exposure of the papillomas to the bracken carcinogens. These multiple independent events support the hypothesis of the multistage nature of cancer again.

Another interesting note, as mentioned earlier, on the BPV 4 associated carcinogenesis is that unlike HPV 16 or 18, the BPV 4 DNA is commonly lost on the progression to malignancy (Campo *et al.,* 1985) which has also been

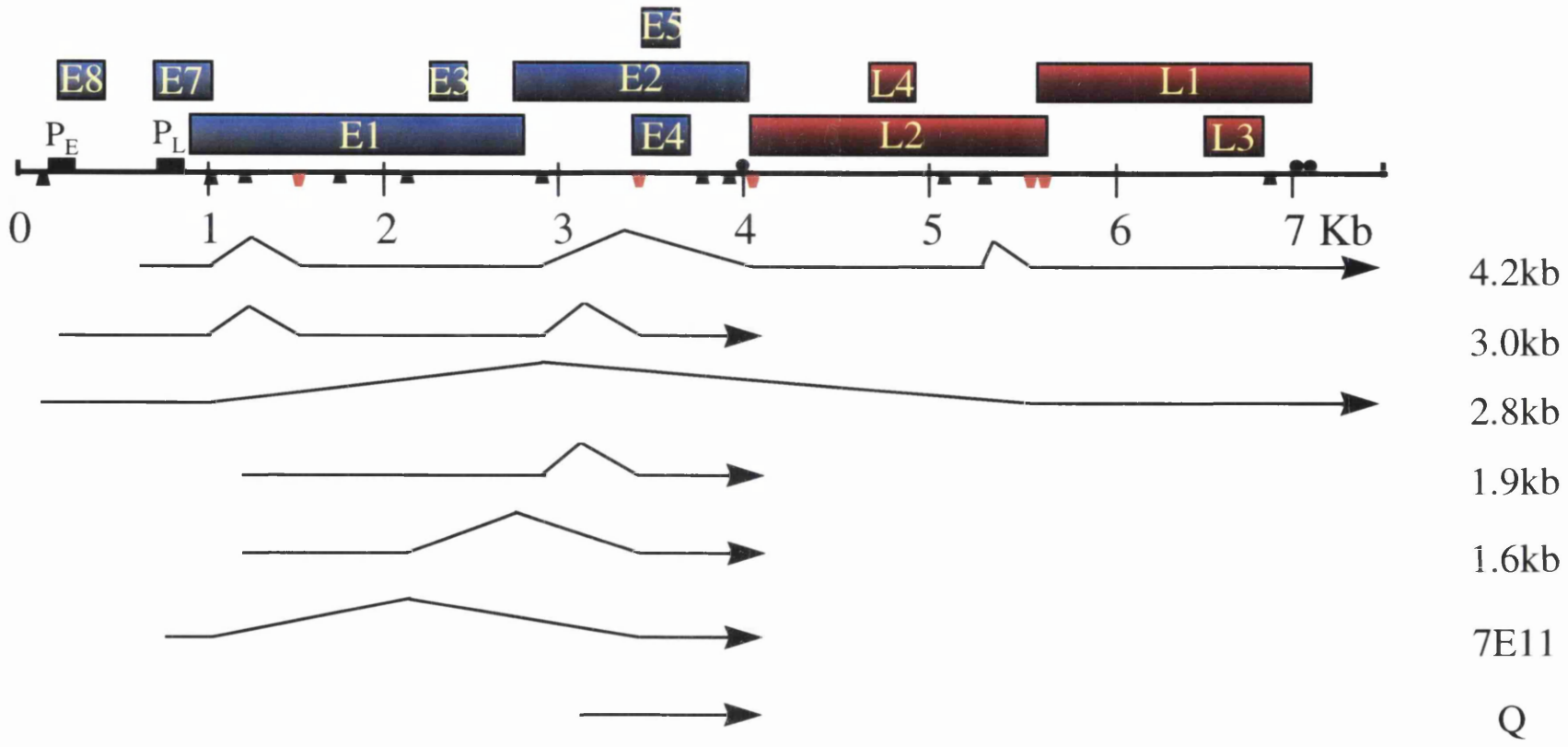


Figure 1: Genomic Organisation of BPV 4

Genome is linerised and ORFs are represented by shaded boxes P_E and P_L represent early and late promoters respectivly

Identified RNA transcripts are detailed as arrows underneath genome

▼ = Splice acceptor site ▲ = Splice donor site ● = Polyadenylation signal

adapted from Campo *et al.*,1996

demonstrated to occur in xenografts of BPV 4 infected bovine tissue (Gaukroger *et al.*, 1991).

1.4.3.1 BPV Genome

The BPV 4 genome is a double stranded DNA circle of 7.265Kb (Jackson & Campo, 1995). The genome can, as with the other papillomaviruses, be divided into 3 main sections (see figure 1):

1. Long Control Region (LCR)
2. Early Region which encode protein for replication and transcription
3. Late Region which encodes the structural capsid proteins

DNaseI footprinting of the BPV 4 LCR (nucleotides 6710-331) revealed 16 sites which were protected from the DNAase by binding of nuclear factors (Jackson & Campo, 1991). Among these site were 3 consensus E2 binding sites at nucleotides 7050 [E2(1)], 175 [E2(2)] and 267 [E2(4)], with an additional degenerate (one base pair difference from consensus site) fourth site termed E2(3) at nucelotide 252. Mutational analysis of these E2 sites showed that E2(1) and E2(3) are involved in transcriptional activation and E2(2) and E2(4) led to transcriptional repression (Jackson & Campo, 1995). The E2(2) is an important *cis*-regulatory element that may bind several nuclear factors which includes PEBP2, in addition to the E2 protein (Jackson & Campo, 1995), implying that there is competition for this site. There are likely to be other factors that may bind to this region of the LCR as deletion or mutation result in a 60 to 85% drop in LCR activity (Jackson & Campo, 1991, 1995). Analysis of the LCR has shown the presence of three, E2 independent, positive (CE 1-3) and negative regulatory (NR 1-3) elements (Jackson & Campo, 1991). These are arranged so that each positive element is paired with a negative element, also each of these elements (except NR2) has binding sites for one or more nuclear factors as determined by DNAase I footprinting. However the NR2 site is in fact bound by a nuclear factor, C/EBP capable of positive and negative activities (Pei &Shih, 1990)

At low concentrations E2 transactivates the LCR but at higher concentrations repression occurs (Jackson & Campo, 1995). The stability of the E2 complexes to E2(1) and E2(2) is greater than that of E2(3) and E2(4), so that at low E2 concentrations only the E2(1) site is occupied but at higher E2 concentrations all the E2 sites become occupied and consequently less positive cellular factors may be able to bind or become displaced (Jackson & Campo, 1995)

Note that there is a pair of E2 sites separated by 3 bp that lie 3bp upstream of the TATA box which is an arrangement found in the E6/E7 promoter of HPVs such as 6b, 11, 16, 18, 31 and 33 (Gloss & Bernard, 1990) implying that there may be a common mechanism of E2 action upon these sites. The binding of E2 to the promoter proximal E2 site in HPV 18 has been shown to interfere with the preinitiation complex (Dostanti *et al.,* 1991)

The two major transcriptional promoters have been identified: P_L (late promoter at nucleotides 777 to 902) and P_E (early promoter at the TATA box nucleotide 283) (Stamps & Campo, 1988; Jackson & Campo, 1995). The viral transcripts all are generated from one strand of the genome and several transcripts have been identified, which show a complex pattern of splicing between the ORFs (Stamps &Campo, 1988; Smith *et al.,* 1986) (see figure 1). These and the individual ORF functions will be sequentially detailed below:

1. E1. Has several different mRNA species, however the one that actually encodes the functional protein is not known. The E1 protein in BPV-1 is a phosphoprotein that is found located in the nucleus of infected cells (Sun *et al.,* 1990). It binds the viral origin of replication (Ustav *et al.,* 1991) and has helicase and DNA unwinding activities. It also interacts with the DNA polymerase alpha (Lentz *et al.,* 1993).

2. E2. The E2 ORF is transcribed into large mRNAs that cover several of the BPV-4 ORFs. Another viral transcript termed Q, possesses sequences from the 3'parts of the E2 and E4 ORFs, hence it may encode an N terminally truncated E2

protein which could act as a transcriptional repressor. As in BPV 1, this protein is a transcriptional regulator in BPV 4 (Jackson and Campo, 1991) and binds to the viral LCR to activate viral transcription along with other cellular factors. There are four E2 binding sites located in the BPV 4 LCR. Mutational analysis displayed that two E2 binding sites (located at nucleotides 7050 and 252) caused activation of the LCR when E2 was bound, and the other two sites (located at nucleotides 175 and 267) mediated repression of the LCR under conditions of high level E2 expression (Jackson and Campo, 1995; Morgan *et al.,* 1998).

3. E4. Again this ORF has several mRNA species including it (Stamps and Campo, 1988). The transcript known as the 7Ell transcript (initiated at multiple sites between 777 and 902) is the most likely to encode the functional protein, an E1^E4 fusion protein analogous to that found in HPV 1 (described by Doorbar *et al.,* 1988) with the 5 N-terminal residues of E1 fused to 118 amino acids of E4. However the 1.6Kb transcript also encodes another potential E1-E4 fusion protein, using a different part of E1. The majority of E4 is located the in differentiating layers of the papilloma, coincident in time and in location with the vegetative replication of viral DNA (Anderson *et al.,* 1997; Campo *et al.,* 1994a). Exact function of the E4 ORF has not been identified in BPV-4 but it is likely to be similar to the homologue in HPVs, where the protein interferes with the cytokeratin assembly (Doorbar *et al.,* 1991). This could result in upsetting the differentiation programme of the cell and favouring the production of the viral progeny instead.

4. E3 & **E5**. In BPV-4 they have no ATG codon and as such are assumed to have no function.

5. E7 & **E8**. These are the primary transforming genes of BPV-4 and are transcribed into a 3.0Kb RNA. These will be described in greater detail later.

6. L1 & **L2**. These are the structural proteins of the virus where L1 is the major capsid protein and L2 the minor capsid protein. They are encoded by a 2.8Kb RNA capable of encoding the L1 protein, and by a 4.2Kb RNA that has the potential to encode both the L1 and the L2 protein. The L1 and L2 capsid proteins

are found in papillomas but not transformed cells. These proteins are effective prophylactic vaccines which can prevent infection (Campo *et al.,* 1997; Campo, 1997a, b)

7. L3 & **L4**. These have an ATG codon but have unknown function in BPV 4.

1.4.3.2 BPV 4 Transformation Studies in Established and Primary cells

Initial studies of the transforming properties of the virus were carried out using murine fibroblast cell lines, namely NIH3T3 and C127 cells. These cells were transfected with the virus genome *in vitro* which transformed both cell lines. Additionally several subclones were capable of inducing tumours in nude mice (Campo & Spandidos, 1983; Smith & Campo, 1988). However complete transformation of the C127 cells required co-operation with the tumour promoter 12-o-tetradecanoylphorbol-13-acetate (TPA).

Work on the C127 cell line also lead to the discovery that a fragment of the viral genome which contained the E7 and E8 ORFs retained the ability to transform (Smith & Campo, 1988). This provided the first evidence that these two genes were the main transforming genes of BPV 4. Subsequent work on E8 in the NIH3T3 cells has shown that this protein is capable of inducing anchorage independent growth in these cells (O'Brien & Campo, 1998). Continued work on the E7 and E8 ORFs in primary cells has confirmed their role as the major transforming genes of BPV 4 (see below).

As with observations of the *in vivo* tumours the experiments with the C127 cells again showed loss of the viral genome (Smith and Campo, 1988). Of the 60 cell line tested only 9 retained the viral genome, thus reinforcing the notion that the expression of the viral genome, while required for initial transformation of the cell, is not required for the maintenance of the transformed state.

However work in the established murine cell line is removed from the cell type of the natural site of infection which is the mucous epithelium of the alimentary canal. Therefore work with cells established from this site was undertaken to examine the transforming properties of the virus under which

conditions. BPV 4 was able to transform primary bovine fibroblasts only in association with another oncogene such as *ras* (Jaggar *et al.,* 1990). Note that although keratinocytes are the natural target for BPV infection primary bovine fibroblasts (PalF) still remain a closer experimental system to study BPV than the murine established fibroblasts, and are easier to culture than the keratinocytes *in vitro*. The transfection of the BPV 4 genome together with an activated ras oncogene causes morphological transformation, anchorage independent growth and extended lifespans of the PalF cells (Jaggar *et al.,* 1990). However these cells are neither immortal or tumorigenic (Jaggar *et al.,* 1990; Pennie *et al.,* 1993).

Subgenomic fragment of the BPV 4 genome were also transfected into the PalF cells with activated *ras* in order to determine the transforming properties of the ORFs. Transfection of the E7 ORF resulted in morphological transformation of the cell resulting in a more 'spindle-like' cell morphology (Pennie *et al.,* 1993). The E7 protein is located in the cytoplasm and the nucleus (Pennie *et al.,* 1993; Campo *et al.,* 1994b) and *in vivo* E7 is expressed in all layers of the papilloma (Anderson *et al.,* 1997). In common with other E7 proteins it contains two Cys-X-X-Cys zinc binding fingers and a potential p105Rb binding domain (Jaggar *et al.,* 1990). Note though that this study also demonstrated that the protein lacked the casein kinase II site present in the E7 proteins of the high risk HPV 16 and 18. Mutation of either the zinc binding domains or the pocket protein binding site however did abolish the transforming properties of the protein (Campo *et al.,* 1994b; Jackson *et al.,* 1996) showing that these domains where necessary for the transforming properties of the protein.

Addition of the E8 ORF to the transfection caused the cells to be morphologically transformed as with the E7 transfectants but also conferred anchorage independent growth (Pennie *et al.,* 1993). These cells are still not immortal nor tumorigenic in nude mice. It is not known whether the E8 ORF, with *ras*, alone can confer anchorage independent growth as this combination proved lethal to the transfected cells. As detailed earlier though, E8 alone can confer substrate independence to the NIH3T3 cells (O'Brien & Campo, 1998). In

addition to this function, E8 also downregulates gap junction intercellular communication in PalF cells (Faccini *et al.,* 1996) thought to be due to binding of E8 to ductin, a component of the gap junctions (Finbow *et al.,* 1991)

E8 is localised to the nuclear membrane, endoplasmic reticulum, Golgi apparatus and sometime to the plasma membrane (Pennie *et al.,* 1993). The protein could be classed as a true early protein due to its expression pattern *in vivo.* It is only expressed in the basal and superbasal areas of the papilloma and therefore separated from region of vegetative viral DNA replication (Anderson *et al.,* 1997)

As with the other group B BPVs, the BPV 4 genome does not contain an E6 ORF (Jackson *et al.,* 1991). E6 is a crucial transforming gene for the HPV 16 and 18 viruses, and it is as yet unknown whether the proteins encoded by BPV 4 can replace the E6 protein either singly or in combination. However the addition of the HPV 16 E6 ORF to the transfection of PalF cells, causes the cells to become immortal (Pennie *et al.,* 1993). This result does suggest that BPV 4 does not support this phenotype with its own encoded proteins.

These studies showed that even with the addition of the HPV 16 E6 and activated *ras*, the transforming proteins of BPV 4, namely E7 and E8, still could not fully transform the PalF cells to achieve tumorigenic status (Pennie *et al.,* 1993). Further addition to the transfection of mutant p53 was required to allow full transformation (Scobie *et al.,* 1997). Interestingly though the addition of the chemical co-factor quercetin to the cells containing the BPV 4 genome, or sub-genomic fragments, could cause full transformation of the cell (Pennie & Campo, 1992; Cairney & Campo, 1995).

1.4.3.3 Co-Factors Associated with BPV 4 Transformation

BPV 4 can co-operate with the tumour promoter TPA or the initiator DMBA to induce carcinomas. BPV 4 infected palate tissue was implanted into the renal capsule of nude mice with pellets which released TPA or DMBA (Gaukroger

et al., 1993). Papillomas formed from the implants (11/33 for virus and TPA, 10/20 for virus and DMBA), with neoplasias forming from implants with the virus and either of the chemicals (4/33 for virus and TPA, 13/20 for virus and DMBA). However TPA or DMBA alone failed to induce either papillomas or neoplasias in any of the implants. The fact that the virus can synergise with either chemical suggests that it can induce transformation of the cell by working with a variety of different cellular pathways i.e. those involved in proliferation or DNA damage.

As briefly mentioned before in section 1.4.3 and 1.4.1.3, the co-factor for BPV 4 in causing alimentary canal cancer is the bracken fern (*Pteridium aquilinum).* Naturally occurring cases of alimentary canal cancer were found to occur at high frequencies in specific areas such as the Nasampolai Valley of Kenya and the Western Highlands of Scotland where the cattle were grazing on bracken infested lands (Jarret *et al.,* 1978; Plowright *et al.,* 1971). Cattle feeding on bracken fern become immunosuppressed, probably due to the sesquiterpene pterosins and pterosides found in bracken (Evans W.C. *et al.,* 1982), develop enzootic hematuria and cancer of the bowels and upper alimentary canal (Campo *et al.,* 1992; Campo *et al.,* 1994a). Cattle also show signs of a high level of chromosomal abnormalities (Moura *et al.,* 1988).

The experimental reproduction of the association between BPV 4 and bracken in the alimentary canal cancer confirmed their involvement (Campo *et al.,* 1994b). Cattle infected in the palate with the BPV 4 virus and kept on a bracken free diet developed papillomas but none of these progressed to carcinomas. Cattle fed on bracken fern without BPV 4 exposure developed neither papillomas or carcinomas of the alimentary canal. However those animals exposed to BPV 4 and bracken diets did develop cancer of the upper alimentary canal and lower bowels (2 out of 6 animals). An additional group of animals that were immunosuppressed with the drug azathioprine, with BPV 4 infection in addition, developed papillomas but not cancer. Thus this latter group indicated that other co-factors in the bracken must be required to cause the progression of the papilloma to carcinoma. The immunosuppression which is important for the persistence of the

papilloma is likely to give a greater target pool of partially transformed cells that could be acted upon by the carcinogens. In addition the immunosuppression would allow a greater duration of exposure to permit accumulation of genetic lesions and would increase the probability of transformed cells to evade the immune surveillance

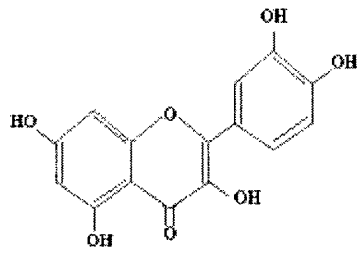
The synergism between BPV 4 and bracken is not an isolated case of this form of interaction. Another example would be smoking and HPV in a similar interaction. Smokers are more at risk of laryngeal papillomatosis (Lindeburg *et al.,* 1986), or cervical neoplasia (Trevethan *et al.,* 1983; Reeves *et al.,* 1987; Cuzick *et al.,* 1990; Basu *et al.,* 1991). This is thought to be because there are several different carcinogens contained within the smoke some of which are proven to be more carcinogenic after metabolic activation e.g. Polycyclic aromatic hydrocarbons and nitrosamines (Miller, 1978; Pasquini *et al.,* 1988). Indeed the cervical mucus of smokers was shown to be mutagenic in the S.typhimurium microsomal test system (Kier *et al.,* 1974) at an elevated frequency when compared to the cervical mucus of non smokers (Holly *et al.,* 1986), thus the mutagens are probable present throughout the body. In addition smoking has been shown to reduce the immune response of smokers causing reduced killer cell activity and suppression of T-cell function as well as reduced levels of IgA, IgG and IgM (review, Holt 1987)

1.4.3.4 Quercetin as a Co-Factor for BPV 4 Transformation

Quercetin (3, 3', 4', 5, 7-pentahydroxyflavone) (see figure 2) is a ubiquitous bioflavonoid found in a range of edible plants e.g. fruits, vegetables, wine and tea (Herrmann, 1976).

Figure.2

Chemical Structure of Quercetin



It has been shown to exhibit a wide range of biological effects on the cell. Quercetin has been demonstrated to induce cell cycle arrest in various stages of the cell cycle in different cell lines including a variety of tumorigenic cell lines (Yoshida *et al,*. 1990, Hosokawa *et al.,* 1990, Gong *et al.,* 1994, Scambia *et al.,* 1990, Ranelletti *et al.,* 1992, Avila *et al.,* 1994, Plaumann *et al.,* 1996). Quercetin also has the ability to scavenge free radicals (Bors & Saran, 1987; Negre-Salvayre & Salvayre, 1992). However in addition to some of these potentially beneficial functions of quercetin there has been a wealth of literature providing evidence for more deleterious functions of quercetin. The flavonoid can induce the formation of DNA adducts (Rahman *et al.,* 1990) and is mutagenic in both prokaryotic and eukaryotic cells (Bjieldanes & Chang, 1977; Amacher *et al.,* 1979; Maruta *et al.,* 1979; Nakayasu *et al.,* 1986; Ishikawa *et al.,* 1987). It also causes clastogenic damage (Ishidate *et al.,* 1988) and can act as an initiator in a two stage transformation protocol *in vitro* with TPA as a promoter (Sakai *et al.,* 1990). Quercetin induces recombinational mutations in cultured cells (mouse mammary tumour cells and mouse fibrosarcoma cells) as detected by DNA fingerprinting (Suzuki *et al.,* 1991). Also the activity of quercetin in the mouse lymphoma L5178Y $TK^{+}/^{-}$ mutation assay, the DNA single strand break assay (also in the L5178Y cells) and the Balb/c 3T3 chemical transformation assay shows that it can form single-strand DNA breaks (Meltz & Mac Gregor, 1981).

This single strand scission damage caused to DNA by quercetin in itself requires co-factors, namely Cu^{2+} and oxygen (Rahman *et al.,* 1990). It seems that quercetin reduces oxygen to superoxide, and in the presence of Cu^{2+} a hydroxyl radical is formed. Strand scission experiments of DNA were done with quercetin,

oxygen or hydrogen peroxide and Cu^{2+} (Fazal *et al.,* 1990). They showed that the superoxide was not a necessary intermediate, but the reaction did involve the hydroxyl radical and a radical intermediate.

In the presence of Cu^{2+}, quercetin also leads to the fragmentation of BSA (Ahmed *et al.,* 1994) which involves the binding to tryptophan residues in the protein, however this effect is not universal for all tryptophan containing proteins.

Quercetin may inhibit kinase activity by competitive inhibition of ATP binding (Graziani *et al.,* 1983). This would lead to gross overall alterations in the control of the signal transduction pathways evidenced by the fact that quercetin inhibits various cellular enzymes such as Calmodulin (Nishino *et al.,* 1984), $pp60^{v\text{-}src}$, cAMP independent protein kinase, cAMP or cGMP Phosphodiesterases, Phosphorylase kinase, Ca^{2+}/phospholipid-dependent kinase, ATP-ases, 5' nucleotidase (Glossman *et al.,* 1981; Graziani *et al.,* 1983; Graziani *et al.,* 1981; Gschwendt *et al.,* 1984; Srirastava, 1985; Lang & Racker, 1974; Shoshan & MacLennan, 1981; Beretz *et al.,* 1978; Ruckstuhl *et al.,* 1979). Further evidence of this impact on the signalling pathways within the cell comes from the study where Tosyl-phenylalanine-chloromethyl ketone (TPCK) and quercetin were shown to act synergistically, with vanadate, to increase protein-tyrosine phosphorylation in avian cells (Van Wart-Hood *et al.,* 1989).

Singhal *et al.,* in 1995, discovered that quercetin also impacted on the signal transduction activity in the human breast cancer cell line MDA-MB-435, as well as causing cytotoxicity and growth inhibition. The signal transduction activity was elevated in cancer cells as measured by increased activity of the enzymes utilising 1-phosphotidylinositol (PI), for the production of the second messenger inositol 1,4,5-triphosphate (IP_3). In these breast cancer cells quercetin inhibited PI kinase activity with minor inhibition of PIP kinase. IP_3 concentration dropped accordingly to 6% of the control level.

Another second messenger, cAMP, was shown to increase in level, after quercetin treatment (Graziani *et al.,* 1979) and of course as already mentioned quercetin affects several cAMP substrate enzymes. This sum of experimental data

proves that quercetin has a significant capacity for affecting the signalling pathways in cells as well as being capable of inducing damage to DNA and proteins.

Due to the role that bracken fern plays in carcinogenesis in cattle, the study of potential factors involved from the bracken and their cellular targets is of considerable importance. One of the factors from the bracken fern likely to play an important role is quercetin. Previous studies have shown its ability to synergise with the transforming genes of BPV 4 in transformation of PalF cells *in vitro*.

With an exposure to quercetin close to the time of transfection (20μM for 48 hours initiated 24 hours after transfection), BPV 4 E7 and *Ha-ras* alone (or BPV 4 genome and *Ha-ras*) can induce full transformation of the cell including the ability to grow independent of anchorage and tumorigenicity in nude mice. Thus the exposure of the cells to quercetin substitutes for BPV 4 E8, HPV 16 E6 and mutant p53 (Pennie & Campo, 1992; Cairney & Campo, 1995; Scobie *et al.*, 1997). BPV 4 E7 and E8 along with *Ha-ras* however fail to form clones, displaying some sort of antagonism between E8 and quercetin in this system. However under the control of the viral LCR in the whole BPV 4 genome this antagonism is not observed suggesting the control of expression of E8 may be critical.

Recent studies of the LCR have demonstrated that quercetin exposure, again just after transfection of an reporter gene under the control of the LCR, causes a 3 to 4 folds elevation of its activity (Connolly *et al.*, 1998) which was mapped to an initiator like sequence downstream of the TATA box. Thus one of the effects of quercetin action on the cells may be elevated expression of the viral oncogenes.

Other effects of quercetin on the PalF cell have also been observed which may have an impact on the synergy between virus and chemical. Exposure of the PalF cells to quercetin caused an alteration in the phosphostyrosine status in several proteins (Connolly *et al.*, 1998) indicating that quercetin is affecting the signalling pathways. This may of course lead to critical alterations in gene

expression, or responses to stimuli depending on the protein involved. Exposure to quercetin was also demonstrated to induce cell cycle arrest in the PalF cells in both the G1 and G2/M phases of the cell cycle (Connolly *et al.,* 1998) maybe due to quercetin's impact on the signalling pathways and/or induced DNA damage.

1.5 Project Aims

Previous work has demonstrated the synergy between the oncogenes of BPV 4 and quercetin in the transformation of PalF cells. Quercetin exposure in close connection to transfection of BPV 4 E7 and *Ha-ras* results in full transformation of the cell without the need for the expression of BPV 4 E7, HPV 16 E6 or mutant p53 (Pennie & Campo, 1992; Cairney & Campo, 1995; Scobie *et al.,* 1997). Additionally quercetin also elevates the activity of the viral LCR which would lead to an increase in the expression of the viral proteins and as such may contribute to the transformation of the cell (Pennie & Campo, 1992; Connolly *et al.,* 1998). The elevated expression of the BPV 4 E7 oncogene would be then hypothesised to increase the inhibition of the cyclin dependent kinase inhibitors $p21^{Waf1/Cip1}$ and $p27^{Kip1}$, thus permitting greater levels of p105Rb phosphorlyation and therefore it's inactivation, additionally to the inactivation caused by direct binding of the p105Rb protein by E7. This would therefore lead to a greater level of release from the negative effect of the p105Rb protein on the cell cycle.

However as the timing of quercetin exposure is critical to the chemical's effectiveness in the transformation of PalF cells this is unlikely to be the only function of quercetin important in the process.

Thus the initial aim of the project was to establish cell lines derived from the transfection of BPV 4 E7 and *Ha-ras* with or without quercetin exposure. Subsequent analysis of the resultant cell lines would permit a study of alterations in the tumorigenic and non-tumorigenic transformed cells. This would then lead to greater insight as to the nature of the transformation of the primary cell and the potential targets of quercetin exposure.

Additionally, study of the quercetin induced cell cycle arrest observed in PalF cells (Connolly *et al.,* 1998) and the effect of BPV 4 E7 and *Ha-ras*, on this arrest of the cell, was to be undertaken. The cell cycle arrest caused by quercetin may seem contradictory to the hypothesis of synergy between quercetin and virus. However there are reasons why this may in fact be important to the transformation of the PalF cell. Under normal conditions the cells would arrest due to quercetin exposure, however under conditions where E7 is expressed the signal to arrest could be abrogated and the cells allowed to cycle. Precedent for this would be the evidence displaying that HPV 16 E7 can abrogate p53 induced cell cycle arrest resultant from DNA damage (Demers *et al.,* 1994; Hickman *et al.,* 1994). This abrogation of arrest would then permit accumulation of genetic lesions which may promote the cells progression to full transformation.

Cell cycle analysis would allow testing of the hypothesis that abrogation of the cell cycle arrest, induced by quercetin, by the transfected genes would be an important step in the progression to tumorigenic status.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

2.1.1 Antibodies

SUPPLIER	ANTIBODY
CalbioChem- Novabiochem corporation San Diego, USA	Anti-mouse IgM horseradish peroxidase linked whole antibody (raised in goat)
CalbioChem- Novabiochem corporation San Diego, USA	Anti-Actin (Ab-1). Mouse monoclonal IgM antibody (clone JLA20).
Amersham International plc, Amersham, Bucks, England	Anti-rabbit IgG horseradish peroxidase linked whole antibody (raised in sheep)
Amersham International plc, Amersham, Bucks, England	Anti-mouse IgG horseradish peroxidase linked whole antibody (raised in sheep)
Dako, Bucks, England	Anti-CD20. FITC conjugated whole mouse monoclonal IgG antibody (clone B-Ly1)
Dako, Bucks, England	Anti-BrdU. Mouse monoclonal IgG antibody. (clone Bu20a)
Sigma, Poole, Dorset, England	Anti-mouse IgG. FITC conjugated whole antibody (raised in goat)
Transduction Laboratories, Lexington, USA	Anti-Ha-ras. Mouse monoclonal IgG antibody (clone 18)
Santa Cruz Biotechnology, Inc. California, USA	Anti-p21. Rabbit polyclonal IgG antibody (clone C-19).
Santa Cruz Biotechnology, Inc. California, USA	Anti-p53. Mouse monoclonal IgG antibody (clone Bp53.12).

2.1.2 Bacterial Hosts

SUPPLIER	BACTERIAL HOSTS
Gibco Europe Life Technologies Ltd., Paisley, Scotland	E. coli DH5α competent cells

2.1.3 Buffers

TE	10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0
TEG	25mM Tris-HCl pH 8.0, 10mM EDTA pH 8.0, 50mM Glucose
5× TBE buffer	40mM Tris base, 16mM acetic acid, 1mM EDTA, pH8.0
1 × ligase buffer	50mM Tris HCL pH7.6, 10mM $MgCl_2$, 1mM ATP, 1mM DTT, 5% (w/v) polyethylene glycol-8000
Phosphate buffered saline (PBS)	137mM NaCl, 44mM KCl, 1.4 mM KH_2PO_4, 8.5 mM Na_2HPO_4
10 × loading buffer	0.45% (w/v) Bromophenol blue, 1% (w/v) SDS, 100mM EDTA, 2.5% (w/v) Ficoll 400 in TE
SDS-PAGE Lysis buffer	1M Tris-HCl (pH 6.8), 10% (w/v) SDS, 20% (v/v) glycerol
SDS-PAGE Resolution gel buffer	0.5M Tris (pH8.8), 0.4% (w/v) SDS
SDS-PAGE Stacking gel buffer	0.5M Tris (pH6.8), 0.4% (w/v) SDS
Tris-glycine electrophoresis buffer	25mM Tris, 250mM glycine and 0.1% (w/v) SDS
2x SDS gel loading buffer	4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol and 100mM Tris, pH6.8
Hypotonic Buffer (Buffer A)	10mM HEPES/ KOH (pH7.9), 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT, 0.2mM PMSF.

High Salt Buffer (Buffer B)	20mM HEPES/ KOH (pH7.9), 25% Glycerol, 420mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.5mM DTT, 0.2mM PMSF
Dephosphorylation buffer	50mM Tris HCl, 0.1mM EDTA. pH 8.0

2.1.4 Cells

Name	Transfected Plasmids	Quercetin Exposure	Population
PalF	None	No	n.a.
E7R	pZipneo E7 and pT24Ras	No	pool
E7Q	pZipneo E7 and pT24Ras	Yes	clone
E7Qb	pZipneo E7 and pT24Ras	Yes	clone
E7QT2	pZipneo E7 and pT24Ras	Yes	clone
E7QPα	pZipneo E7 and pT24Ras	Yes	pool
E7QPβ	pZipneo E7 and pT24Ras	Yes	pool
E7QPγ	pZipneo E7 and pT24Ras	Yes	pool
Q0D	pBV4 and pT24Ras	No	clone
Q2D	pBV4 and pT24Ras	Yes	clone
Raji	None	n.a.	n.a
88529B	None	n.a.	n.a.

Note:

1. 88529B was derived from an *in vivo* transforming papilloma (Campo et al., 1985)
2. Raji cells were derived from a Human Burkitt Lymphoma (Positive from CD20 antigen expression)

2.1.5 Cell Culture Materials

SUPPLIER	MATERIAL
Cadisch and Sons, Finchley, UK	70μm filter nylon gauze

(i).Globerpharm Ltd., Esher, Surrey, England (ii). Harlan Sera-Lab ltd., Crawley Down, England	Foetal Calf Serum Foetal Calf Serum
Gibco Europe Life technologies Ltd., Paisley, Scotland	10% Dulbecco's Modified Eagles Medium 10x F10 (Ham) Medium 200 mM glutamine Geneticin. G418 sulphate MEM amino acids solution (50x) 7.5% sodium bicarbonate 100 mM sodium pyruvate 2.5% Trypsin

2.1.6 Chemicals & Enzymes

Supplier- Amersham International plc, Amersham, Bucks, England

ECL Western detection agent

Redivue [α^{32}P]dATP

Supplier- BDH Chemicals Ltd., Poole, Dorset, England.

Calcium chloride

D-glucose

Glycerol

Repelcote silicone treatment

Supplier- Beta Lab., East Mosley, Surrey, England.

Yeast Extract

Supplier- Biogenesis Ltd., Bournemouth, England.

RNAzol B

Supplier- Boehringer Mannheim UK Ltd., Lewes, East Sussex, England.

Caesium chloride

DOTAP (N-[1-(2, 3-Dioleoyloxy) propyl]-N, N, N-trimethylammoniummethylsulfate)

DNase 1, RNase-free

Protease K

Supplier- Difco laboratories, Detroit, Michigan, USA.

Bacto-Agar

Bactotryptone

Supplier- Fisons Scientific Equipment, Loughborough, England.

Acetic acid

Butan-1-ol

Chloroform

di-potasium hydrogen orthophosphate anhydrous

Ethylene diamine tetra acetate (EDTA) disodium salt

Dimethyl sulfoxide (DMSO)

Hydrochloride acid

Magnesium chloride

Magnesium sulphate

Methanol

Potassium chloride

Potassium dihydrogen orthophosphate

Propan-2-ol

Sodium acetate

Sodium carbonate

Sodium chloride

Sodium dodecyl sulphate (SDS)

Sodium hydroxide

Supplier- Fluka AG, Chemisch Fabrik CH-9470 Buchs.

Methocel MC 4000

Supplier- Gibco Europe Life technologies Ltd., Paisley, Scotland.

All DNA restriction enzymes and appropriate buffer concentrates were obtained from Gibco Life Technologies (BRL) unless otherwise stated. The following reagents were also obtained from Gibco:

Agarose (ultrapure electrophoresis grade)

Tris Base

Supplier- James Burrough Ltd., Witham, Essex, England.

Ethanol

Supplier- Kramel Biotech Ltd., Cramlington, Northumberland, England.

Ribonuclease A

Supplier- NBL Gene Sciences

Alkaline phosphatase

T4 DNA ligase

Supplier- Pharmacia-Biotech Ltd

poly(dI-dC)

poly(dA-dT)

Supplier- Promega, Southampton, England

Ribonuclease Inhibitor

Supplier-Severn Biotech Ltd., Kidderminster, Worchester, England.

30% (w/v) acrylamide:0.8% (w/v) bis-acrylamide

40% (w/v) acrylamide:2.1% (w/v) bis-acrylamide

Supplier- Sigma Chemical Co., Ltd., Poole, Dorset, England.

β-mercaptoethanol

Acetly Coenzyme A

Benzamidine

Bicinchoninic Acid solution

Bleomycin Sulphate

Bovine Serum Albumin

Bromophenol Blue

Coomassie Brilliant Blue R

Copper(II) sulphate (pentahydrate 4% (w/v) solution)

DEPC

Dithiothreitol (DTT)

Ethidium Bromide

Ficoll (type 4000)

HEPES

Leupeptin

Lysozyme

Nonidet P-40 (NP40)

ONPG

Phenol:Chloroform:Isoamyl Alcohol (25:24:1 (v/v))

PMSF

Ponceau S solution

Quercetin (3,3',4',5,7-pentahydroxyflavone)

Salmon testes DNA (sodium salt)

TEMED (N,N,N',N'-tetramethylethylenediamine)

Tween-20 (Polyoxyethylene sorbitan nonolaurate)

Supplier-Qiagen, Dorking, Surrey, England

Superfect Transfection Reagent™

Supplier- Vector Laboratories, Burlingame, USA.

VECTASHEILD™ Mounting Medium

2.1.7 Equipment and Plasticware

SUPPLIER	EQUIPMENT
Alpha Laboratories Ltd., Eastleigh,	Microfuge tube

Hampshire, England	Pastettes
Amersham International plc, Amersham, Bucks, England	Hybond-C extra
Becton Dickinson Labware, Plymouth, England	Falcon 1059 polypropylene tubes Falcon 2059 polypropylene tubes Falcon 2097 polypropylene tubes Falcon 2098 polypropylene tubes Sterile Plastipak syringes 18 gauge sterile syringe needles 60, 90 and 140 mm tissue culture dishes
Bibby sterilin Ltd., Stone, Staffs, England.	60 and 90 mm bacteriological petri dishes Sterile plastic universal containers
Costar Corporation, High Wycombe, Bucks, England	24 well tissue culture plates 96 well tissue culture plates Disposable Cell scrapers
Dupont Uk Ltd., Stevenage, Hertz, England	Polyallomer ultracentrifuge tubes
Eastman Kodak Co., Rochester, New york, USA	X-ray film (XAR-5)
Gelman Sciences, Northampton, England	Sterile 0.2 μm acrodisc filters
Ilford Ltd., Mobbrrley, Cheshire, England	Ilford PANF 50 black and white film
Nunc, Roskilde, Denmark	T25, 80, and 175 cm^2 tissue culture flasks Cryotubes
Sartorius AG, 37070 Goettingen, Germany	Collodium Bags
Technical Photo Systems., Cumbernauld, Scotland	Fuji RX medical X-ray film
Whatman International Ltd., Maidstone, Kent, England	Whatman 3MM filter paper

2.1.8 Kits

SUPPLIER	KIT
Perkin Elmer Cetus, Norwalk, USA.	GeneAmp PCR core kit GeneAmp thinwalled reaction tubes BigDye™ Terminator Cycle Sequencing
Promega ltd., Chilworth Research Centre, Southampton, England	Luciferase Assay System Reporter Lysis 5 X Buffer
Qiagen ltd., Dorking, Surrey, England	QIA prep Spin plasmid miniprep kit QIA quick gel extraction kit RNeasy Mini RNA purification kit Omniscript RT Kit HotStar Taq PCR Kit
Invitrogen, Groningen, Netherlands	S.N.A.P Total RNA Isolation Kit

2.1.9 Molecular Weight Markers

SUPPLIER	MARKER
Amersham International plc, Amersham, Bucks, England	Rainbow™ coloured protein molecular weight markers
Gibco Europe Life Technologies Ltd., Paisley, Scotland	Bacteriophage λ DNA (HindIII digested) 100bp DNA ladder 1Kb DNA Ladder

2.1.10 Other Materials

SUPPLIER	MATERIALS
Beatson Institute Central Services	Amphotericin B LB-Medium (Luria-Bertani Medium) Kanamycin Penicillin Sterile distilled water Sterile glycerol

	Sterile phosphate-buffered saline (PBS) Sterile phosphate-buffered saline + EDTA (PE)
Merck Ltd., Poole, England	Silicone grease
Johnson and Johnson Medical Limited, Berks, UK	PRESEPT* effervescent disinfectant tablets
Premier Beverages., Adbaston, Stafford, UK	Marvel (Dried Skimmed milk)

2.1.11 Plasmids

pBV4 contains the whole BPV-4 genome (7.265 kb) cloned into the Bam H I site of pAT153 (Campo & Coggins, 1982).

pT24 is a pUC13 derived plasmid containing the 6.6 kb activated human c-Ha-ras oncogene from the T24 human bladder carcinoma line originally cloned in pBR322 (Santos *et al.,* 1982). This plasmid construct was a gift from M. O'Prey (Beatson Institute, Glasgow).

pZipneoSV (XI) (referred to as **pZipneo** throughout the text) consists of a Moloney murine leukaemia virus (MoLV) transcriptional unit, including the long terminal repeats (LTRs), and pBR322 sequences. This construct has a unique BamH I cloning site and also contains DNA sequences derived from the transposon Tn5, which encodes G418-resistance (neomycin resistance) in mammalian cells (Cepko *et al.,* 1984).

pZipneoE7 contains nucleotides (nts) 652-1250 of the BPV-4 genome cloned into the BamH I site of pZipneo SV (XI) (Pennie *et al.,* 1993). In pZipneoE7 the BPV-4 E7 gene is under the transcriptional control of the Moloney leukaemia virus 5′ long terminal repeat (MoLV LTR).

pRGCfosLuc carries two p53-binding sites from the ribosomal gene cluster promoter upstream of the murine c-fos promoter linked to a firefly luciferase reporter gene.. This was a kind gift from Dr J.D.Zhu, (Cyclacell)

pFosLuc This is the control plasmid for pRGCfosLuc. It lacks the p53-binding sites, thus only contains the murine c-fos promoter linked to the firefly luciferase reporter gene. This was also a kind gift from Dr J.D.Zhu, (Cyclacell)

pCB6wtp53 This plasmid expresses wild type human p53 from the CMV promoter in the pCB6+ plasmid. This was a kind gift from Dr Kevin Gaston (University of Bristol)

pCB6mtp53 This plasmid expresses a mutant form of human p53 from the CMV promoter in the pCB6+ plasmid. This was a kind gift from Dr Kevin Gaston (University of Bristol)

pGL2NA(mdm2) This plasmid utilises the human mdm2 gene promoter linked to a firefly luciferase reporter gene. This was a kind gift from Dr Kevin Gaston (University of Bristol)

WWP-Luc This plasmid utilises the human $p21^{Waf1/Cip1}$ gene promoter linked to a firefly luciferase reporter gene. This was a kind gift from Dr Bert Vogelstein (John Hopkins Hospital, Baltimore)

pCH110 is a control vector for eukaryotic transfection assays, which contains a functional *lac*Z gene which is expressed either from the SV40 early promoter in eukaroytes or from the *E.coli gpt* promoter in prokaroytes. This vector was commercially obtained from Pharmacia..

pCMVCD20 is an eukaryotic expression plasmid, pCMV-neo-BamH1 (Baker *et al.,* 1990), of the cell surface epitope CD20. Expression is driven by the CMV promoter. This plasmid was a kind gift of Dr D.Johnson (Beatson Institute, Glasgow)

pEGFPN1 is a eukaryotic expression plasmid for the Green Flourescent Protein (GFP). Expression is driven by the CMV promoter. This plasmid was a kind gift from Dr R.Nibbs (Beatson Institute, Glasgow)

2.1.12 Water

Distilled water for the preparation of buffer stocks was obtained from a Millipore MilliRQ 15 system, and for protein, enzyme, RNA or recombinant DNA procedures was further purified on a Millipore MilliQ System to 18MΩ/cm. Sterile distilled water for making up tissue culture media was supplied by the Beatson Institute for Cancer Research Technical Service.

2.2 Methods

2.2.1 Molecular biology

2.2.1.1 Oligonucleotide Synthesis and Purification

Oligonucleotides were synthesised by Beatson Institute technical services staff on an applied Biosystems model 381A DNA Synthesiser or 392 DNA/RNA Synthesiser using the manufactures protocols and Cruachem reagents. The final primers were synthesised with or without trityl group protection. All primers were firstly deprotected after synthesis by incubating in a 55°C water bath overnight.

"Trityl on" primers were detritylated using a Cruachem oligonucleotide purification (COP) cartridge according to manufacturers instructions. Each oligonucleotide was ultimately eluted from COP cartridge minus the trityl group using 1 to 2ml of 20% (v/v) acetonitrile. The acetonitrile was evaporated off and the primer dissolved in 0.5ml sterile distilled water or TE pH 8.0. Primers were stored at -20°C.

"Trityl off" oligonucleotides were provided in ammonia. The oligonucleotides were deprotected by heating to 55°C overnight then purified by precipitation with butan-1-ol. 1ml butan-1-ol was added to 150µl oligonucleotide solution and microcentrifuged at 14000 for 20 minutes at room temperature.

Excess butanol was removed by centrifugation under vacuum and the primer dissolved in an appropriate volume of sterile distilled water or TE pH 8.0. Primer concentration was determined as described in section 2.2.1.4.

2.2.1.2 Denaturation of Double-Stranded DNA Template

The double stranded DNA (dsDNA) was alkali-denatured by using the following alkaline denaturation reaction. The reaction was carried out in a final volume of 20µl comprising of 0.5pmol of dsDNA template, 0.2M NaOH, 0.2mM EDTA and sterile, deionized water to final volume 20µl. This was incubated for 5 minutes at room temperature

The DNA was precipitated by adding one tenth of 2M ammonium acetate, pH 4.6 and 3.5 volume of ethanol and standing at -70°C for 30 minutes followed by centrifugation (14000 rpm) in a microcentrifuge for 15 minutes at 4°C. The pellet was washed with 70% ethanol before drying under vacuum for 5 minutes to remove all traces of ethanol. The pellet was dissolved in appropriate volume of sterile distilled water and stored at -20°C.

2.2.1.3 DNA Extraction with Organic Solvent and Ethanol Precipitation

DNA samples were purified by extraction with phenol:chloroform in order to remove contaminants, such as residual enzyme activities from a restriction reaction or detergent which might otherwise interfere with subsequent cloning steps. In the first round of extraction the DNA sample was mixed with an equal volume of phenol:chloroform. Phenol:chloroform was freshly prepared from an equal volume of 1M Tris-HCl pH8.0, saturated phenol and chloroform:isoamyl alcohol (24:1 v/v). The aqueous DNA and organic phase were mixed thoroughly by vortexing, then separated by centrifugation in a microcentrifuge at 14000 rpm for 5 minutes at room temperature. The upper aqueous phase was transferred in a clean eppendorf tube, care was taken not to transfer any of the interphase to the tube, and the extraction process repeated. The aqueous phase was then extracted with an equal volume of chloroform (chloroform:isoamyl alcohol, 24:1 v/v) by vortexing and centrifugation as described above. This was repeated to remove any traces of phenol from the

aqueous phase. The aqueous phase was transferred to a fresh eppendorf for ethanol precipitation.

Ethanol precipitation was used to concentrate DNA samples and also to remove solute contaminants such as salt. The aqueous DNA solution was mixed with one tenth volume of 3M sodium acetate pH 5.2 and 2-2.5 volumes of ice cold ethanol. The sample was then mixed well by inversion several times and then stored at -20°C or, alternatively, placed on dry ice for 15-30 minutes to facilitate DNA precipitation. The precipitated DNA was collected by centrifugation in a microcentrifuge at 14000 rpm for 15 minutes at 4°C. The supernatant was discarded, and the pellet was washed with 70 % ethanol to remove any trace of salt, dried under vacuum before resuspension in distilled water at an appropriate concentration. The DNA concentration was determined as described below.

2.2.1.4 Quantitation of Nucleic Acids

The concentration of nucleic acid in a solution was determined spectrophotometrically in a Beckman DU 650 spectrophotometer. Samples were diluted in TE and transferred to a quartz cuvette with a pathway of 1cm. The spectrophotometer was initially calibrated using TE buffer only as a blank. The optical density reading were obtained at 260nm and 280nm; an O.D. reading of 1 at 260nm (A_{260} = 1) corresponds approximately to a concentration of 50μg/ml of double stranded DNA, for oligonucleotides an A_{260} of 1 was taken to correspond to ~35μg/ml, and for RNA an A_{260} of 1 was taken to correspond to 40μg/ml. The ratio between readings at 260nm and 280nm (A_{260}:A_{280}) provided an estimate of the sample purity; a ratio of ~1.8 indicated that preparations contained essentially no protein or phenol contamination.

2.2.1.5 Restriction Enzyme Digestion of DNA

Restriction digests were carried out in small reaction volumes using enzymes and their appropriate concentrated buffer solutions according to the manufacturers.

Plasmid DNA was incubated with 5-10 units enzyme/μg DNA in a buffered solution ensuring that the total volume of enzyme added did not exceeded one tenth of the final reaction volume. Small quantities of plasmid DNA (<5μg) were routinely digested in a 20μl reaction volume as specified by the manufacturer for 2 hours at 37°C. Large digests were carried out in proportionally larger reaction volumes. The digestion fragments were analysed by agarose gel electrophoresis as described below.

2.2.1.6 Agarose Gel Electrophoresis

Horizontal gel cast apparatus from Pharmacia was used. In general, 1% (w/v) agarose gels were used, but smaller fragments (100-400) were separated on 2-4% gels. Low melting point agarose was used at a concentration of 1% (w/v) in order to isolate and purify required DNA restriction fragments. Gel mixes containing the appropriate amount of agarose were dissolved in 0.5 × TBE buffer by heating the solution in a glass conical flask in a microwave until all the particles of agarose gel had dissolved. The gel was poured when the agarose was hand hot and a comb with the required number and size of teeth placed immediately into the gel to form the sample wells. The gel was submerged in 0.5 × TBE buffer. The samples containing 1 × loading buffer were loaded in each well along with an appropriate size marker (e.g. 100b ladder, 1Kb ladder) into the first and/or last well in the gel and run at 70-100 constant voltage usually until the samples' blue dye front was 1-3 cm from the end of the gel. Once run, the DNA fragments were visualised by staining the gel in running buffer containing 0.5μg/ml ethidium bromide with gentle agitation for 10 minutes at room temperature. The separated DNA was visualised by illumination with short wave (312nm) UV light and photographed through a red filter onto videoprint paper using an Appligene Imager.

2.2.1.7 Isolation and Purification of DNA Restriction Fragment from Agarose Gel

The DNA fragment to be used for cloning was recovered from low melting point agarose gel and visualised as described in section 2.2.1.6. The fragment was

cut out of the gel with a clean scalpel blade and the gel slice placed in an eppendorf tube. Extraction of the DNA fragment from the agarose was achieved using a Qiagen Qiaquick gel extraction kit following the manufacturer's instructions.

2.2.1.8 Ligation of DNA Fragments

Both vector DNA and the DNA fragment to be inserted into the vector were separately digested and purified as described above and then isolated by gel electrophoresis as described in section 2.2.1.6.

The vector DNA was dephosphorylated at its termini to prevent re-ligation. After the vector DNA had been linearized by digestion, the reaction mixture was adjusted by adding dephosphorylation buffer and 1 unit of Calf Intestinal Alkaline Phosphatase (CIAP) was added to the reaction mixture and incubated at 37°C for 30 minutes. The reaction was stopped by heating to 90°C for a further 5 minutes. Another 1 unit of CIAP was added to the reaction mixture and incubated at 37°C for 15 minutes. All enzyme activity in the reaction was finally stopped by heating to 90°C for further 5 minutes.

The DNA was phenol:chloroform extracted, ethanol precipitated and then resuspended in appropriate volume of distilled water and stored at -20°C.

The DNA fragment was inserted into dephosphorylated vector (100ng) at a ratio of 3:1 respectively. The vector and inserted DNA were incubated together in a reaction containing 1 × ligase buffer and 1 unit of T4 ligase at 22°C for 1 hour or overnight. Dilutions of this reaction volume were used to transform competent bacterial cells (section 2.2.1.9).

2.2.1.9 Transformation of Bacterial Hosts

All plasmids were propagated in commercially available E. coli DH5α competent cells supplied as frozen stocks (Gibco) kept at -70°C until use. Bacteria were transformed following manufacturer's instructions. Competent cells were thawed slowly on ice, and 100 µl of aliquots put into a prechilled polypropylene culture tube (Falcon 2059) and 1-2 ng of the appropriate plasmid DNA added and mixed by gently moving the pipette tip trough the cells while dispensing. The cells were then incubated on ice for 30 min before being heat

shocked for 60 seconds at 42°C. The tube was then immediately placed on ice for 5 min. 100 µl of room temperature SOC Media (2% Bactotryptone, 0.3% Yeast Extract, 10mM NaCl, 2.5mM KCl, 20mM Mg^{2+} Stock (equimolar ratio of $MgCl_2.6H_2O$ & $MgSO_4.7H_2O$) and 20mM Glucose) was then added to each transformation reaction. The tube was then transferred to a shaking 37°C incubator (approximately 225rpm) for 1 hr to allow expression of the antibiotic resistant marker. Following this, cells were spread on an L-agar plate containing the appropriate antibiotic. The plate was inverted and incubated overnight at 37°C to allow colony formation.

2.2.1.10 Glycerol Stocks

Host strains, and their derivatives containing useful plasmids, were stored as glycerol stocks for future retrieval. 850 µl of an overnight culture was mixed gently with 150 µl sterile glycerol in a 1.5 ml Nunc Cryotubes and stored at -70°C. A sterile plastic loop was used to retrieve an aliquot of cells as and when required.

2.2.1.11 Small Scale Preparation of Plasmid DNA (Miniprep)

Small amounts of plasmid DNA were extracted from transformed bacterial colonies to identify correct clones.

Single colonies of bacteria carrying the required plasmid were picked using a sterile yellow pipette tip and grown in 5 ml culture of L-Broth (1% w/v Bactotryptone, 0.5% w/v yeast extract, 1% w/v NaCl) containing antibiotic (100µg/ml Ampicillin) at 37°C in a shaking incubator (225rpm) overnight. 10 separate colonies were generally picked for screening at any one time. Bacteria were pelleted from 1.5ml of overnight culture by spinning in a microcentrifuge (14000rpm) for 30 seconds at room temperature. DNA was prepared using the QIA prep Spin plasmid miniprep kit following the manufacture's instructions.

2.2.1.12 Large Scale Preparation of Plasmid DNA

Bacteria containing the plasmid of interest were streaked onto an L-agar plate containing the appropriate antibiotic and the plate inverted and incubated overnight at 37°C to allow colony formation. A single colony was picked, using a

sterile yellow tip, from this plate and used to inoculate a sterile universal tube containing 5 ml of L-Broth medium and the appropriate antibiotic (100μg/ml Ampicillin) which was then put in a shaking incubator at 225rpm overnight at 37°C. This culture was then added to 500 ml of Superbroth, containing 100μg/ml Ampicillin in a 1 litre glass conical flask (to allow good aeration), then returned to the shaking incubator for 48 hours. Superbroth is composed of two solution, A and B. Solution A consists of 12 g of bactotrypton, 24 g of yeast extract, and 5 ml of glycerol made up to final volume of 900 ml with distilled water. Solution B consists of 12.5 g of di-potasium hydrogen orthophosphate (K_2HPO_4) and 3.8 g of potassium dihydrogen orthophosphate (KH_2PO_4) made up to a final volume of 100 ml. Both solution were autoclaved separately and combined just prior to use.

Bacterial cells were pelleted by centrifugation in a Sorvall RC-5B centrifuge (Sorvall GS-3 rotor) at 5,000 rpm for 10 min at 4°C and the supernatant was removed. The pellets were resuspended in 18 ml of TEG (50 mM glucose, 10mM Tris-HCl (pH8.0), 10 mM EDTA). Resuspended pellets were pooled into one centrifuge bottle and 2 ml of lysozyme (10 mg/ml) were added. The suspension was mixed gently and allowed to stand on ice for 10 min. 40 ml of freshly prepared alkaline SDS (0.2 M NaOH, 1% SDS) were added and the suspension mixed by gentle inversion and placed on ice for 5 min. 30 ml of ice cold 5M potassium acetate (490.7 g KAc and 115 ml glacial acetic acid made up to 1 litre in water) were added, the whole solution inverted sharply five times and then returned on ice for 20 min. The flocculate was centrifuged at 8,000 rpm for 5 min at 4°C in a Sorvall GS-3 rotor and the supernatant filtered through gauze into a 250ml measuring cylinder. 0.6 volumes of room temperature propan-2-ol was added, then the whole solution transferred to 250ml centrifuge bottle and mixed by inversion several times and left at room temperature for 10min. The nucleic acid in this cleared lysate was precipitated and pelleted by centrifugation at 8,000 rpm for 5 min at 4°C. The supernatant was removed and the nucleic acid pellet washed with 50ml room temperature 70% ethanol to remove any salt. The solution was centrifuged for a further 5 min at 8,000 rpm at 4°C. After discarding the supernatant, the pellet was allowed to dry at room temperature for 10 min before being resuspended in 9ml of TE.

Ultracentrifugation through a caesium chloride (CsCl) density gradient was then carried out to further purify the plasmid DNA. 10 g of caesium chloride was added to the solution and allowed to dissolve at room temperature. 500µl of a 10mg/ml ethidium bromide solution was added to visualise the DNA band after centrifugation. The refractive index of this solution adjusted to 1.395. The solution was transferred to a sealable centrifuge tube (11.5ml Dupont disposable tube) with a protective metal cap over the top of each tube. Samples were spun in a balanced Beckman ultracentrifuge at 55,000 rpm for 16 to 24hr at 18°C in a T1270 rotor. The tube was carefully removed from the centrifuge rotor and placed securely in a clamp on a rotor stand. After centrifugation, any contaminating RNA was found to have pelleted at the bottom of the tube. Two distinct bands were observed; the upper band contains sheared linear plasmid DNA and residual bacterial chromosomal DNA while the lower band contains closed circular plasmid DNA. An 18 gauge needle was first inserted into the top of the tube to act as an air inlet and the lower band was gently withdrawn by similarly piercing the side of the tube ~ 1cm below the lower band with an 18 gauge needle connected to a syringe. The plasmid DNA band was then transferred to a clean ultracentrifuge tube. The tube was filled with CsCl/TE (RI=1.395) as before and underwent further centrifugation prior to plasmid extraction.

The band removed as described above was transferred to a 5 ml Bijoux tube. Ethidium bromide was removed from the solution by extracting with an equal volume of water saturated butan-2-ol. The solution was mixed and ethedium bromide separated with the upper organic phase which was carefully aspirated off and discarded into appropriate bottles. This extraction process was repeated until the lower aqueous phase was clear and colourless. The CsCl was removed by dialysing the plasmid DNA into a dialysis tube (Collodion Bag) and placed in a large beaker containing 2 litres of TE. Plasmid DNA was dialysed against TE on a magnetic stirrer for 4 hours at room temperature. The TE buffer was changed and dialysis continued for a further 4 hours at room temperature or overnight at 4°C. DNA was then ethanol precipitated and the DNA pellet was finally resuspended in 0.5-1 ml TE (pH 8.0), depending on the size of the pellet. The DNA concentration

was determined as described in section 2.2.1.4. The plasmid DNA was aliquoted and stored at -20°C.

2.2.2 Cell Culture and Transfection

2.2.2.1 Cell Culture

All cell culture work was performed following strict aseptic techniques inside a laminar flow hoods (Class II Microbiological safety Cabins; Medical Air Technology Ltd., Manchester, England). Cells were incubated in dry 37°C incubators containing 5% (v/v) CO_2 (Heraeus, Essex, England) and were routinely screened for mycoplasma infection using a fluorescent dye technique (M. Freshney, Beatson Institute, Glasgow).

2.2.2.2 Isolation of Primary Bovine Fibroblasts

The fibroblasts from foetal bovine palate were isolated as described previously by Jaggar et al., (1990). A small section of soft palate tissue was taken from bovine foetuses of less than 5 months gestation obtained from the veterinary post-mortem room at the Glasgow University Veterinary School. The palate tissue was sterilized by a 30 second wash in 70% ethanol and then dissected into small pieces approximately $2mm^2$ in size using crossed scalpels and placed into a 90mm dish being well spaced apart. The tissue was allowed to adhere to plastic by placing the dishes in a dry 37°C incubator containing 5% (v/v) CO_2 for 5 minutes. Culture medium was then added slowly to each dish so as not to disturb the adherent samples. The samples were fed twice weekly over a period of two weeks in which time fibroblasts and keratinocytes grew out of tissue mass. The medium used for both isolation and subsequent routine growth of PalF cells was DMEM supplemented with 10% foetal calf serum, 2mM glutamine, 1mM pyruvate, 0.375% sodium bicarbonate (DMEM-10). This medium selectively favoured the outgrowth of fibroblasts and, as expected, keratinocytes died. After four passages in culture the newly extracted fibroblasts were trypsinised and reseeded into large (T175 cm^2) flasks. Cells were expanded and stocks of PalF cells were frozen down in liquid nitrogen for further experiments.

2.2.2.3 Maintenance of Primary Bovine Fibroblasts in Culture

Cells were fed twice weekly, old medium was aspirated from sub-confluent flasks and fresh medium added. PalF cells were grown until just sub-confluent whereupon they were passaged approximately 1 in 4. Replating was performed as follows: for T80 cm^2 tissue culture flask medium was aspirated off and the cells washed twice with 5 ml phosphate-buffered saline (PBS). The PBS was removed and 1 ml of trypsin solution (0.25% trypsin in 1x PE buffer; PBS with the addition of EDTA to 1 mM), which had been pre-warmed to 37°C, was added to cells. Flasks were transferred to the 37°C hot room until the cells had detached from the flasks. Complete medium was added and the cell suspension transferred to a sterile universal tube. The cells were pelleted by centrifugation at 1000rpm for 5 mins at room temperature. The pellet was then resuspended in fresh growth medium and the cells reseeded at an appropriate density.

2.2.2.4 Long Term Cell Storage

To freeze cells stocks for storage, confluent cultures were trypsinised, and pelleted as described above (section 2.2.3.3). The pellet was then resuspended at a concentration of approximately 10^6 cells/ml in growth medium containing 10% (v/v) DMSO. The DMSO in the medium acts as a cryoprotectant but all solution must be chilled as DMSO is toxic to cells at room temperature. Suspensions were divided into 1 ml aliquots in 1-2 ml Nunc cryotubes and placed in a polystyrene box and frozen, well insulated, at -70°C overnight to ensure a slow rate of cooling. The ampoules were then transferred to a liquid nitrogen bank containing labelled storage rack until required. Frozen stocks were recovered by removing the ampoules from liquid nitrogen and placed into a small, covered bucket of water at 37°C. Once thawed, the cells were added to 10ml of the appropriate pre-warmed growth medium, centrifuged, resuspended in fresh growth medium and transferred to 80 cm^2 flasks.

2.2.2.5 Mycoplasma Screening

A 60mm dish of NRK (Newborn Rat Kidney) cells was provided by Marion Lacey (Beatson Institute, Glasgow). 2ml of growth medium, taken from cells being tested for the presence of mycoplasma, was added to the dish of NRK cells containing 2ml fresh medium (SLM supplemented with 10% (v/v) FCS and 2mM glutamine) and incubated for 3-4 days in a humidified incubator at 37°C in an atmosphere of 5% CO_2. The growth medium from cells being screened must have been in contact with the cells for a minimum of 2 days. At the end of the incubation period all medium was removed and cells washed twice with PBS. 2.5ml of PBS followed by 2.5ml of fixative (75% (v/v) methanol, 25% (v/v) glacial acetic acid) was added to the dish. This was removed and 5ml fixative only added. Fixative was aspirated off and a second 5ml volume of fixative added and then incuated at room temperature for 10 minutes. Fixative was poured off and the dish placed at 37°C for 15 minutes to dry completely. 5ml of Heochst 33458 stain (0.05μg/ml in PBS) was added and the cells allowed to stain for 5 minutes at room temperature. When the staining solution was removed the plate was rinsed twice with distilled water and left to air dry. A drop of distilled water was added to the centre of the dish and covered with a clean glass cover slip. Cells were finally examined using a water immersion lens on a fluorescence microscope (Leitz Wetzlar) with a Mercury vapour lamp light source. Mycoplasma, if present, can be identified as extranuclear fluorescent specs against the dark background.

2.2.2.6 Transient Transfection of Primary Bovine Fibroblasts (PalFs)

PalF cells were transfected with a range of plasmid DNAs (section 2.1.11) using the cationic lipid N-[1-(2, 3-Dioleoyloxy) propyl]-N, N, N-trimethylammoniummethylsulfate (DOTAP; Boehringer Mannheim BCL) following the manufacturer's recommendations. Briefly, cells ($2x10^5$) were plated in each 60mm plate, in duplicate, containing 3ml of appropriate growth medium (DMEM-10) the day before transfection. For each plate of cells to be transfected the following transfection mixture was set up.

DNA, DOTAP and serum free medium were mixed at a ratio of 1:4:25 respectively at room temperature and incubated for 15 minutes at room temperature.

This mixture was then added directly into the medium above the cells. The plates were moved slowly to disperse the transfection solution throughout the culture medium, and were then incubated at 37°C for 16-18 hours. After withdrawal of the medium, cells were washed twice with 2ml of pre-warmed PBS and incubated in DMEM-10 medium, until quercetin or other treatments were initiated. Typically cells were left for 12 hours after transfection before quercetin treatment began, 32 hours before UV treatment and 48 hours before bleomycin sulphate exposure.

2.2.2.7 Luciferase Assays

$2x10^5$ cells were seeded in DMEM-10 for each 60mm plate, with 3ml of medium per plate, the day before transfection. Transfection was performed using the standard DOTAP method (section 2.2.3.6). After 16-18hr., the cells were washed twice with 2ml PBS and incubated in DMEM-10 for a further 8 hrs, after which time 20-100μM quercetin or an equivalent volume of its diluent, ethanol, was added to the growth medium of the PalF cells. Incubation typically continued for a further 36 hours, with application of fresh quercetin/solvent medium every 12 hours. If required transfectant plates were exposed to either UV irradiation (50 or $120J/m^2$) 16 hours before harvesting, or bleomycin sulphate exposure (15μU) for 6 hours before harvesting.

To harvest cells were then washed twice with PBS, the PBS was completely removed by aspiration and 300μl of 1 × reporter lysis buffer (Promega) added to each well. Following 15 minutes incubation at room temperature, cells were scraped off the culture well and each lysate transferred to a 1.5ml eppendorf. Cell debris was pelleted by spinning lysates at 4°C in a microcentrifuge at 14000 for 5 minutes. The supernatant was transferred to a second eppendorf tube taking care not to disrupt the cell pellet. The lysate were either assayed for reporter enzyme activity immediately or stored at -20°C.

Luciferase activity was determined using a luminometer with automatic injection (BioOrbit, model 1251). For each sample, 80μl of lysate and 120μl of

Luciferase assay buffer (Promega) were used. Luciferase activity was normalised for protein content determined using the BCA assay (Pierce), and β-galactosidase activity as a measure of transfection efficiency.

2.2.2.8 Stable DNA Transfection of Primary Bovine Fibroblasts

PalF cells were transfected with a range of plasmid DNAs (section 2.1.11) using the cationic lipid N-[1-(2, 3-Dioleoyloxy) propyl]-N, N, N-trimethylammoniummethylsulfate (DOTAP; Boehringer Mannheim BCL) following the manufacturer's recommendations. Each reaction contained 5 μg of each relevant plasmid DNA plus 2 μg of a plasmid construct containing the selectable marker gene for neomycin resistance (pZipneo; Chapter 2.1.11).

Transfection classes are described in Chapter three in the Results section. Reactions were made up to 20 μg with sonicated salmon sperm DNA (Sigma). PalF cells were plated at a density of 5×10^5 into an 80 cm^2 flask 24 hr prior to transfection. The cells were fed with 13.5mls of growth medium on the day of transfection, the transfection mix was as follows: 80 μl of DOTAP was diluted up to 250μl with serum free DMEM medium in a separate reaction vial. DNA (20μg) was also diluted up to 250μl with serum free DMEM medium in a separate reaction vial. Both solution were mixed together and incubated for 15 min at room temperature. This mixture was then slowly added to the flask of cells and incubated at 37°C overnight. After withdrawal of the medium, cells were washed twice in PBS and then fresh complete medium was added to the flask. If appropriate, transfected cells were treated with 20μM quercetin one day after transfection for 48 hours. The next day cells were split at a dilution of 1:2 and allow to settle for 24 hr prior to selection.

2.2.2.9 Selection of Transfected Cells

Cell were selected in medium containing 500 μg/ml G418 for 21-28 days, being fed twice weekly. After this time, G418-resistant colonies were scored. Where appropriate, several colonies were picked from each transfection class in order to expand them clonally.

2.2.2.10 Isolation of Clonal Populations

Single neomycin resistant colonies were identified and their position marked using a microscope ring marker attachment. The cells were then washed twice in sterile PBS. A sterile 6 mm stainless steel cloning rings coated with sterile silicon grease (Merck, England) at the base was then placed over the identified colony thus providing a waterproof seal round each isolated colony. A total volume of 100 µl trypsin solution, which had been pre-warmed to 37°C, was pipetted within each cloning ring. After 1-2 min an equal volume of complete medium was added and the cell suspension transferred to a 24-well plate (Costar) along with 2ml medium and returned to the 37°C incubator for expansion into cell lines.

2.2.2.11 Transformation Assays

2.2.2.11.1 Anchorage Independent Growth

The ability of a cell line to form colonies in semi-solid media is taken as a phenotypic measure of its degree of transformation. The extend of transformation of cell populations was assayed by plating cells in Methocel based medium.

Efficiency of methocel colony formation was determined by adding $3.75x10^5$ cells to 15 ml of 1% methocel including 30% Foetal Calf Serum (FCS).

The mix was plated in bacterial petri dishes. Bacteriological petri dishes were used to discourage cells from adhering to the bottom of the dishes. Cells, tested in duplicate, were left at 37°C for 12 days before being scored and photographed with PanF 50 technical film. Methocel medium was made up as follows:

3g of Methocel MC 4000 (Fluka) was added to 200 mls of distilled water and autoclaved. The Methocel was left to dissolve with stirring for 2-3 days at 4°C. Following dissolution, 22 mls of 10 × F10-HAM medium (Gibco), 4 mls of 50 × minimum essential amino acids (Gibco), 4 mls of 0.1M sodium pyruvate, 5 mls of 7.5% sodium bicarbonate, 100 mls of foetal calf serum (FCS) and 2% penicillin and streptomycin were added.

2.2.2.11.2 Tumorigenicity Assay in Nude Mice

The ability to form tumours is an indicator of full cellular transformation. The transformed cells were assayed in nude mice. Cells were removed from selection and expanded at identical cell density. The cells were suspended in sterile PBS at a concentration of 10^8 cells/ml. 0.1 ml of this suspension (10^7 cells) were injected subcutaneously into a four-week old female athymic nude mice, strain MF1 nu/nu (Harlan-Olac, Bicester, England) at a single injection site. Typically five mice were injected per cell line tested and examined for tumour growth weekly up to 15-20 weeks post injection. If no tumour had developed by then the cells were considered to be non-tumorigenic.

2.2.2.12 B-Galactosidase Assay

The plasmid pCH110 was used in all transient transfections as an internal control against which the efficiency of transfection could be normalised. β-galactosidase can catalytically convert colourless o-nitrophenyl-β-D-galactopyranoside (ONPG) to yellow o-nitrophenol. The level of activity of this enzyme can be assayed by measuring changes in light absorbance at 420nm.

Cells were lysed as detailed in section 2.2.3.7. To 80μl of each cell lysate, 1ml of solution I (60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgCl_2$, 50mM β-mercaptoethanol) and 0.2ml of solution II (60mM Na_2HPO_4, 40mM NaH_2PO_4, 2mg/ml) was added. After mixing, all samples were incubated at 37°C for 30-60 minutes or until a yellow colour change could be seen. Samples were transferred to plastic disposable cuvettes and the reactions stopped by the addition of 0.5ml 1M sodium carbonate. The absorbance was read at 420nm using a Beckman DU 650 spectrophotometer.

2.2.2.13 Fluorescence Activated Cell Sorting (FACS) Analysis

Fluorescence Activated Cell Sorting was used to analyse the cell cycling status of the cell lines in a particular population. Cells were grown in the presence or absence of 0-100μM quercetin according to the experimental conditions for up to 48 hours in DMEM-10 medium with medium changes every 12 hours.

Cells were harvested by trypsinisation and pelleted at 4°C in growth medium. Each cell pellet was washed twice with ice cold PBS spinning cells, as before, between each wash. The second volume of PBS was aspirated off and the pellet was resuspended in a small residual volume of PBS (~ 500µl) before cells were fixed by the slow, drop by drop addition of 4.5ml ice cold 70% ethanol. Samples were left on ice for 1 hour. Cells were either stored at -20°C under 70% ethanol for no longer than 1 week or stained immediately.

To stain cells for analysis, each cell suspension in 70% ethanol was pelleted at 4°C for 5 minutes at 2000rpm. Ethanol was aspirated off and each cell pellet was washed once with ice cold PBS cells to remove any residual ethanol. Cells were then again pelleted at 4°C for 5 minutes at 2000rpm then resuspended in 500µl of staining solution (PBS containing 250µg/ml RNAse A, 20µg/ml propidium iodide). Samples were left covered, at room temperature for at least 30 minutes, or for longer staining times cells were then placed at 4°C, before analysis.

Stained cell samples were filtered through 70µm nylon gauze into Falcon 2054 polystyrene round bottom tubes. Samples were assayed using a Becton Dickinson FACScan machine and analysed using the 'Modfit v2.0' software package.

2.2.2.14 CD20 Tagging of Transient BPV-4 E7 Transfected PalF Cells

$1x10^6$ cells were seeded into 140m Nunc culture plates in 25 mls of DMEM-10 medium, and left to adhere to the substratum. The following day cells were transfected with 5µg of pZipneo E7 and 25µg of pCMVCD20 plasmid using the Superfect™ lipofection reagent as per manufacturers guidelines. Briefly plasmid DNA was diluted with serum free DMEM and mixed before addition of the Superfect™ reagent at 4µl per µg of plasmid DNA used. The mixture was incubated at room temperature for 15 to 20 minutes., before addition of full DMEM-10 to a final volume of 10 mls.

Cells were washed with pre-warmed PBS during the incubation period, then the transfection medium was added to the cells, ensuring full coverage of the

plate. The transfection plate was then incubated at 37°C, 5%CO_2 for 5 hours before a single wash with pre-warmed PBS and refeeding of the plate with 25 ml of DMEM-10.

Transfected cells were then treated with either 0 or 50μM quercetin over 36 hours with fresh medium being applied every 12 hours. These cultures were then harvested by trypsinisation.

Cells were then resuspended in 5ml of DMEM-10 and spun at 1000g for 5 minutes at room temperature. The pellet was then taken up in 100μl of medium containing 20μl of FITC-conjugated anti CD20 antibody (DAKO) and incubated on ice for 30 minutes. Following the incubation cells were spun down at 1000g for 5 minutes and washed twice with PBS containing 1% calf serum.

The cells were then fixed overnight at 4°C in 70% ethanol. Before analysis the cells were then washed once in PBS and stained in 20μg per ml propidium iodide and 200μg per ml RNAase A in PBS for 30 minutes at room temperature.

Samples were then assayed the using a Becton Dickinson FACScan machine and analysed using the 'CellQuest' software package. CD20 positive cells were classed as those exhibiting FITC fluorescence 20 time that of background PalF cells.

2.2.3 DNA & RNA analysis

2.2.3.1 Total RNA Extraction from Cell Lines

Cells were grown in a 175cm^2 (T175) flask to approximately 80% confluency. Total RNA was then extracted by either the RNAzol B method of extraction (Biogenesis Ltd, England) or by use of the RNeasy Kit (Qiagen). For the RNeasy kit method of purification cells were washed once with pre warmed sterile PBS and trypsinised and counted. $3x10^6$ tumorigenic or $1x10^7$ cells of all other cell lines were subjected to the RNA extraction method as per manufacturers details.

For the RNAzolB method cells were washed twice with ice cold PBS and 10mls of RNAzol B was added directly to the flask. The lysate was transferred to a Falcon 2059 polypropylene centrifuge tube and 1 ml of chloroform was added with vigorous pipeting. The top of the tube was then covered with Parafilm

(American National Can, USA) and the tube was left on ice for 15 minutes to allow phase separation to take place. The tube was then centrifuged in a sorval RC-5B (HB6 rotor) at 10,000rpm for 15 minutes at 4°C. The upper, aqueous phase was transferred to a fresh tube and an equal volume of isopropanol added. The samples were mixed and stored overnight at -20°C to allow precipitation of RNA and the RNA pelleted by centrifugation as before. The pellet was resuspended in 5 mls of 75% ice cold ethanol (made with diethylpyrocarbonate (DEPC) -treated RNase-free water), and transferred to an eppendorf tube. The RNA was pelleted in a microfuge at full speed at 4°C for 30min, dried on a speedivac and then resuspended in DEPC-treated RNase free water. If required RNA extracted by the RNAzolB protocol was given a DNA-ase treatment. This was carried out using a S.N.A.P Total RNA Isolation Kit (Invitrogen) as per manufacturers guidelines.

For all RNA extractions the concentration of RNA was measured spectrophotometrically as described in section 2.2.1.4. RNA samples were aliquoted and stored at -70°C.

2.2.3.2 Polymerase Chain Reaction (PCR)

2.2.3.2.1 Amplification of DNA

All reagents were provided in the HotStarTaq PCR Kit with the exception of dNTP's which were obtained from the Perkin-Elmer Core DNA PCR kit. Primer sequences are described in table 2.1.

Table 2.1 Oligonucleotide PCR primers

Primer Name	Primer Nucleotide Sequence
Forward E7	gctgaccttccagtcttaat
Reverse E7	tgaagaggagattgaaactg

The reaction mixture comprised 200μM of each dATP, dGTP, dCTP and dTTP, 1 × PCR kit buffer, 0.2μM of each primer, 2.5 units *HotStarTaq* polymerase (a modified thermolabile DNA polymerase from *Thermus aquaticus*)

and 1μg of DNA sample (controls included distilled water, pZipneo E7 plasmids, and 1μg of DNA from parental PalF cells). It was aliquoted into 0.5ml GeneAmp PCR reaction microfuge tubes in a final volume of 100μl. The tubes were placed into the PCR machine (Perkin-Elmer Cetus type 9600) and heated to 95°C for 15 minutes to inactivate DNase and ensure all DNA duplexes were melted, in addition to activating the HotStarTaq. The DNA was then amplified (using Perkin-Elmer Cetus type 9600 thermocycler) for 35 cycles at 94°C for 60 seconds, 59°C for 60 seconds, to allow the primers to anneal to the template DNA, followed by 72°C for 60 seconds, for E7, to allow extension of the amplimer sequences. After completion of the cycles, the reaction was incubated at 72°C for a further 7 minutes to ensure full extension and then cooled to 4°C. 5μl of each sample was analysed by agarose gel electrophoresis (as described in section 2.2.1.6) to check the correct product was amplified.

2.2.3.2.2 Amplification from RNA Reverse Transcriptase-PCR (RT-PCR)

RNA was prepared (see section 2.2.4.2) and used as the template for reverse transcription and PCR amplification of cDNA. Firstly cDNA was synthesised from RNA by reverse transcription using the Omniscript RT kit (Qiagen). The reaction was carried out according to the manufacturers instructions, to the following final concentrations: 1× RT buffer, 5mM of each of dATP, dGTP, dTTP, dCTP, 10 unit RNase inhibitor,1μg RNA, 4 units Omniscript reverse transcriptase, 1μM of Oligo-dT primer, and DEPC-treated water to a final volume of 20 μl. Control reactions using no Omnicript reverse transcriptase were carried out, in addition to assaying suitable negative cell lines for each experiment.

All samples were placed in the thermocycler and further incubated at 37°C for 60 minutes, and then incubated at 4°C for 5 minutes. The above reaction was then stored at -20°C until use. Typically 2μl of the RT reaction was carried forward to the amplification step. PCR reactions were carried out as per manufacturers guidelines (Qiagen) for HotStarTaq amplification. Briefly final reaction volumes were 100μl consisting of 2μl of RT reaction mixture combined with a final concentration of 1x PCR Buffer, 200μM of each dNTP, 0.2μM of the forward and reverse primers, 2.5 Units of the HotStarTaq made up to 98μl with

distilled water. To activate the HotStarTaq an initial incubation of 95°C was carried out for 15 minutes. Amplification proceeded for 35 cycles of 94°C for 60 seconds, 59°C for 60 seconds, plus a 60 second extension at 72 °C for E7, or 3 minute extension of p53. PCR was carried out in a Perkin-Elmer Cetus 9600 thermocycler. The samples were then analysed by agarose gel electrophoresis as described in section 2.2.1.6 to ensure correct amplification.

2.2.3.3 DNA Sequencing

The sequence of all new plasmids was checked using Taq terminator sequencing on an Applied Biosystems 373A automated DNA sequencer which was performed by Beatson Institute technical services staff.

The region to be sequenced first underwent PCR amplification. 0.5μg of template DNA was added to 12μl RQ grade H_20 plus 3.2pmoles the appropriate primer. 8μl of dye terminator cycle sequencing ready reaction premix (Perkin Elmer) was then added to each reaction volume contained in 250μl thin walled eppendorf tubes. The samples were placed in a PTC-100 programmable thermal controller (Genetic Research Instrumentation Ltd) and exposed to 25 cycles of 95° C for 30secs, 50°C for 15secs and 60°C for 4min. The PCR products were ethanol precipitated as detailed in section 2.3.2.4, washed with 70% ethanol and finally dried under vacuum before being given to a member of technical services for loading onto the sequencing gel. Details of the primers, and their sequences, used for the p53 cDNA sequencing reaction are listed in table 2.2:

Table 2.2

Primer Name	*Position*	*Primer Nucleotide Sequence*
Bp53FF	1-18	5' - tcgaaagcttatggaagaatcacaggca -3'
Bp53RRR	1140-1161	5' - tcgatctagatcagtctgagtcaggccc -3'
Bp53F2	303-318	5' - ttccgtctagggttcctg -3'
Bp53F3	637-654	5' - tatgagtcccccgagatc -3'
Bp53F4	880-898	5' - cctaggagcactaagcga -3'
Bp53R2	303-318	5' -caggaaccctagacggaa -3'
Bp53R3	637-654	5' -gatctcgggggactcata -3'
Bp53R4	880-898	5' -tcgcttagtgctcctagg -3'

2.2.3.4 End Labelling Double Stranded Oligonucleotide

Single stranded oligonucleotides were synthesised using Cruachem chemicals and an Applied Biosystems 392 DNA/RNA automated oligonucleotide synthesising machine by Beatson Institute technical services. Oligonucleotides were deprotected by incubating at 55°C overnight, ethanol precipitated and resuspended in TE buffer. The concentration of oligonucleotide solutions was measured spectophotometrically at described in section 2.3.2.3. However, an optical density reading of 1 at 260nm (Abs_{260nm}) for a single stranded oligonucleotide is roughly equivalent to a solution of 33μg/ml compared to 50μ g/ml for double stranded DNA.

Complementary single stranded oligonucleotides were mixed together in equimolar amounts, heated to 95°C for 5 minutes and then allowed to cool slowly to room temperature which enabled the two strands to anneal together into double stranded molecules. The concentration of the double stranded oligonucleotide solution was measured spectrophotometrically and diluted with TE buffer to give a final concentration of 0.1mg/ml. Oligonucleotide solutions were stored at -20°C.

Double stranded oligonucleotides with sticky ends were end labelled as follows. Using a random primed labelling kit (Boehringer Mannheim), 1μl (0.5nmol) dCTP, 1μl (0.5nmol) dATP and 1μl (0.5nmol) dGTP solutions were

added to 200ng of double stranded oligonucleotide. The reaction volume was made up to 12µl with distilled water. 2µl of 10x Klenow buffer (0.5M Tris-Cl (pH7.2), 0.1M $MgSO_4$, 1mM DTT, 0.5mg/ml bovine serum albumin (BSA - fraction V)) was added followed by 5µl [α-^{32}P]dATP and 1µl (2 units) Klenow enzyme (also taken from the random primed labelling kit). The contents of the reaction were mixed and incubated at 37°C for 30 to 60 minutes. The radiolabelled oligonucleotide was separated from unincorporated nucleotides by polyacrylamide gel electrophoresis as described in section 2.3.2.15.

2.2.3.5 Purifying Radioactive Probe Using Polyacrylamide Gel Electrophoresis

A 6% non-denaturing polyacrylamide gel was made by adding 15% (v/v) 40% (w/v) acrylamide:2.1% (w/v) bisacrylamide solution to 0.5x TBE buffer (89mM Tris base, 89mM orthoboric acid, 2mM EDTA (pH8.0)). Polymerization of the gel was catalysed by the addition of 0.07% APS and 0.08% TEMED. After mixing thoroughly, the solution was poured between two glass plates which had been siliconized with repelcote, washed with detergent and cleaned with 70% ethanol prior to use. A comb forming sample wells was placed in the top of the gel immediately after pouring. The gel, 2mm thick, was left to polymerize in a vertical position for 1 hour before use.

The gel spacer was removed and the gel, formed between the two glass sheets, was placed vertically into the electrophoresis tank (ATTO) containing 0.5x TBE and secured in place. 0.5x TBE buffer was added to the top reservoir of the tank to cover the top of the gel. The comb was removed carefully from the gel and each well rinsed out with 0.5x TBE buffer using a 20ml syringe. The gel was run for 15 minutes at 150 volts to equilibrate the gel before any samples were loaded.

The radioactively labelled sample(s) was loaded into a single gel well. No loading dye was added to the sample, however 10µl of bromophenol blue loading buffer was added to the extreme wells to monitor the gels progress. The sample(s) was electrophoresed at 150 volts at room temperature until the blue dye front was approximately 5cm from the end of the gel. The position of each radiolabelled oligonucleotide was determined by exposing the gel, covered with cling film, to a

sheet of Fuji RX medical X-ray film for 1-2 minutes. Using the X-ray film as a guide, the band containing the labelled probe was excised from the gel using a clean scalpel blade. The gel slice was transferred to a clean eppendorf tube and TE buffer was added to the tube sufficient to cover the gel slice. The tube was placed in a lead pot, sealed and left at room temperature overnight during which time the oligonucleotide eluted from the gel slice into the TE buffer. The polyacrylamide gel slice was removed from the solution and the radioactive oligonucleotide solution stored at -20°C.

2.2.3.6 Electrophoretic Mobility Shift Assay (EMSA)

Cells were harvested for their nuclear extract as described in section 2.2.4.2. Samples of nuclear extract were removed from storage at -70°C and thawed on wet ice. To 5µl (10-15µg) of nuclear protein extract, still on ice, 10µl 5x binding buffer (25mM Tris-HCl (pH7.3),5% Glycerol, 50mMMgCl_2, 175mM NaCl, 50µg/ml BSA, 28µg/ml polydAdT and 50mM DTT) was added followed by 1µl (~1ng) of radioactively labelled double stranded oligonucleotide DNA (section 2.3.2.14). 100ng of various unlabelled (cold) double stranded oligonucleotide was added to selected samples for competition analysis. The volume of each reaction mix was made up to 50µl with distilled water, mixed and incubated on ice for 30 minutes.

Each sample was loaded into a separate well on a 5% non-denaturing polyacrylamide gel prepared as described in section 2.3.2.17. 5µl loading dye mixed with 45µl 1x binding buffer was added to the end wells on the gel to allow migration of the samples down the gel to be monitored. Samples were electrophoresed at 150 volts at 4°C until the blue dye front in the tracking wells was ~3cm from the end of the gel. The gel was transferred onto Whatman 3MM filter paper and dried down on a Biorad 583 gel drier at 80°C for 2 hours using a slow rise in temperature cycle. The dried gel was placed in a lead, light tight casette with intensifying screens, covered with a sheet of Fuji RX medical X-ray film and placed at -70°C overnight.

2.2.3.7 Non-Denaturing Polyacrylamide Gel Electrophoresis

A pair of 2mm ATTO glass plates were siliconized with repelcote, washed with detergent and cleaned with 70% ethanol prior to use. A 5% polyacrylamide gel was made by adding 12.6% (v/v) of 40% (w/v) acrylamide:2.1% (w/v) bisacrylamide solution to 0.5x TBE buffer (89mM Tris base, 89mM orthoboric acid, 2mM EDTA (pH8.0)). Polymerization of the gel was catalysied by the addition of 0.07% APS and 0.08% TEMED. After mixing thoroughly, the solution was poured between two glass plates held securely together in the gel forming apparatus (ATTO). A comb to make the samples wells was placed in the top of the gel immediately after pouring. The gel was left to polymerize in a vertical position for 1 hour before use.

The gel spacer was removed and the gel, formed between the two glass sheets, was placed vertically into the electrophoresis tank (ATTO) containing 0.5x TBE and secured in place. The upper reservoir was filled with 0.5x TBE buffer, the comb was removed carefully from the gel and each well rinsed out with 0.5x TBE buffer using a 20ml syringe. The gel was run for 15 minutes at 150 volts prior to use to equilibrate the gel.

2.2.4 *Protein Analysis*

2.2.4.1 Protein Preparations from Cells for Western Blot Analysis

Cells were lysed by aspirating the culture medium off, washing the cell monolayer twice with ice-cold PBS, the PBS was completely removed by aspiration. 1.5ml of ice-cold PBS was added, cells were scraped off the dish and transferred to a 1.5 ml microcentrifuge tube. Cells were pelleted by spinning at 4°C in a microcentrifuge at 5,000rpm for 5 minutes. the supernatant was removed and 300µl of boiled lysis buffer was added to the pellet. Cells were lysed following a 5 minutes boiling, and then sonicating the resulting cell suspension using an MSE Soniprep 150 sonicator. Cell debris was pelleted at 14000 rpm and the supernatant transferred to a new microcentrifuge tube.

2.2.4.2 Protein Preparations from Cells for EMSA Analysis

Approximately $1x10^7$ cells were used in each preparation. Cells were trypsinised, pelleted and washed twice with ice cold PBS, with the second PBS wash including transfer to 1.5ml eppendorf tube. Pelleted cells were then gently resuspended in 400µl of Buffer A (Hypotonic Buffer) by flicking the tube. The mixture was then left on ice for 10 minutes before a 10 second vortex. Cells were then pelleted by a 10 second spin in a microfuge. The supernatant was then discarded and the pellet was resuspended in 20-100µl of Buffer B (High Salt Buffer). This was incubated on ice for 20 minutes then the cellular debris was pelleted by centrifugation in a microfuge for 2 minutes at 4°C. Supernatant was then removed, aliquoted and stored at -70°C. Protein concentration was determined as described in section 2.2.5.2.

2.2.4.3 Protein Concentration Assays

The BCA/CuSO4 Protein assay was used to spectrometrically determine the protein concentration of dilute solutions following the manufacturer's instructions. Protein reduce alkaline Cu(II) to Cu(I) in a concentration-dependent manner. Bicinchoninic acid is a highly specific chromogenic reagent for Cu(I), forming a purple complex with an absorbance maximum at 562nm. 10µl of protein solution was placed in separate wells in 96 well plate. 200µl of developing solution (5ml BCA(Biocinchoninic acid) solution, 100µl of 4% (w/v) CuSO4 (copper II sulphate pentahydrate solution) was added to the protein samples and incubated at 37°C for 30 minutes.

The absorbance of each sample was read at 590nm using a Dynatech MR7000 automatic plate reader. The absorbance reading was converted to concentration in µg/ml for each sample using a standard curve generated from a series of control BSA solutions of known concentration. The actual concentration of each protein sample was calculated after multipling by the relevant dilution factor.

2.2.4.4 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein samples were resolved according to the molecular weight using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). 15cm gels were used, containing gels of varying polyacrylamide content, depending on the molecular weight of the proteins being resolved, but typically 10% polyacrylamide resolving gel was used. For a single 10% gel of 15cm the following solutions were prepared:

30% acrylamide	16.67ml
Water	20.83ml
SDS-PAGE Resolution Gel Buffer	12.5ml
10% ammonium persulphate (freshly prepared)	0.2ml
TEMED	0.008m

This resolving gel was poured between two glass plates (cleaned with 70% ethanol) sealed on three sides with a gasket, then overlaid with isopropanol and left to polymerise at room temperature. Once the gel was set, the isopropanol was removed using Whatman 3MM filter paper and the stacking gel consisting of 5% polyacrylamide was poured on top of the resolving gel, a comb inserted and left to polymerise for at least 30 minutes before use. The stacking gel was prepared as follows:

30% acrylamide	2.67ml
Water	12.33ml
SDS-PAGE Stacking gel buffer	5ml
10% ammonium persulphate (freshly prepared)	0.04ml
TEMED	0.004ml

Once the stacking gel had polymerised the whole gel was transferred to an electrophoresis tank. The tank reservoirs were then filled with Tris-glycine electrophoresis buffer. After the removal of the combs, the wells were flushed with electrophoresis buffer to remove any excess stacking buffer.

Prior to loading, equivalent amount of each protein samples (80μg) was mixed with an equal volume of 2x SDS gel loading buffer. The prepared protein samples were then loaded into consecutive wells and 10μl Rainbow™ protein molecular weight marker mix (molecular weight range 14.3KD-200KD) added to

the first and/ or last well on the gel. The gel was run by electrophoresis at a constant current ~ 35mA/gel for 2-3 hours. Once the dye front was approximately 5-10cm from the bottom of the gel, the gel was removed and used for western blot analysis.

2.2.4.5 Western Blotting

Separated protein samples were transferred to a nitro-cellulose membrane by semi-dry blotting. For this purpose the gel was removed from the electrophoresis tank, then excess gel was cut away. The dimensions of the remaining gel was measured and 2 pieces of Whatman 3MM filter paper were cut to an equal size, as was one piece of nitro-cellulose (ECL-hybond). The transfer of the protein from the gel to the nitro-cellulose was performed as follows:

1. Two sheets of Whatman paper were soaked in transfer buffer (122mM Glycine, 25 mM Tris, 20% Methanol) and placed neatly on the centre of the blotting apparatus, avoiding any air bubbles.

2. Onto these was laid the nitro-cellulose membrane, the gel, then a further 6 sheets of Whatman paper, all soaked in transfer buffer.

3. The stack of sheets was rolled with a glass pipette to eliminate any air bubbles

4. 200mA was applied across the blot for approximately 60 mins, the time taken for the pre-stained marker proteins to be completely transferred

Once the transfer was completed, the membrane was blocked by shaking for a minimum of 2 hours in 50 ml of block buffer (5% Marvel (dried milk) in PBS or 3% BSA in PBS) at room temperature. The nitro-cellulose filter was washed in wash buffer (PBS) for 4 x 10 minutes. The filter was then placed in 7ml blocking buffer containing suitable primary antibody and incubated at room temperature for 1 hour with gentle shaking. The primary antibody solution was removed and the filter rinsed in blocking buffer then washed 4 times, each for 10 minutes, in 100ml volumes of fresh washing buffer. The filter was then incubated in 50 ml blocking buffer containing a 1/5000 dilution of the applicable HRP linked secondary antibody for 1 hour at room temperature with gentle shaking. The filter was washed 3 x 10 minutes with washing buffer. Excess surface liquid

was removed from the filter by briefly blotting with a piece of Whatman 3MM paper. The detection consisted of incubating the filter in an equal volume of an Amersham Enhanced chemilluminescence (ECL) detection reagents I and 2 for 1 minute at room temperature. The excess detection solution was drained off the nitro-cellulose filter and this was then wrapped in Saran wrap and exposed to Fuji-XR film for 30 second and up to 30 minutes (depending on the strength of the signal).

2.2.4.6 Stripping Western Blot Membranes

If a western blot membrane was required for multiple analyses with different probe antibodies then the initial primary and secondary antibodies were removed from the membrane by stripping.

The membrane post exposure to the Fuji-XR film was washed for 10 minutes three times in PBS before being incubated for 1 hour at room temperature in Stripping Buffer (0.2M Glycine pH 2.5, 0.2% SDS). The membrane was then washed with copious amounts of PBS until all traces of the SDS were safely removed. The membrane was then blocked as per protocol for the next probing primary antibody.

2.2.4.7 Immunofluorescence

8000 cells were seeded into single well glass chamber slides and left overnight at 37°C with DMEM-10 media, under humid conditions to minimise evaporation. Quercetin exposure was initiated on the following day as described in section 2.2.2.13. Post quercetin exposure tissue culture media was aspirated off and the cells were washed once with PBS and fixed in ice cold methanol/ acetone (2:1 v/v) for 30 mins.

The acetone was removed and each chamber was washed three times with PBS before a 10% FCS (in PBS) blocking solution applied to the cells for 1 hour at 4°C. After removing the blocking solution the primary antibody was applied at the appropriate concentration in the blocking solution and left in a moist chamber at 4°C for 16 hours.

The cells were then washed 3 times with the blocking solution and incubated with the secondary FITC-conjugated antibody for 1 hour at 4°C . The

cells received a final wash in blocking solution and were then mounted under Vectashield™ solution. Cells were analysed under UV illumination using a Leitz vario orthomate microscope (Confocal microscope). Counter staining with propidium iodide was also undertaken to visualise the cell nuclei. After washing off any residual secondary antibody if applicable cells were incubated with the staining solution (PBS containing 250μg/ml RNAse A, propidium iodide (20μ g/ml) for at least 30 minutes at room temperature before mounting.

CHAPTER THREE

RESULTS

3.1 Introduction

Cancer is a multi-stage process in which the cell accumulates a series of genetic alterations. These alterations will in turn allow the cell to overcome the normal checkpoints that regulate cellular proliferation. The resultant phenotype of these successive genetic lesions that will affect the regulation and function of oncogenes and tumour suppresser genes is invariably advanced malignancy of the tumour.

The study of the processes that contribute to the control of cell proliferation, and the factors that affect them is invaluable to achieve cures and/or preventions of this disease.

Many viruses can be critical factors in the abrogation of cell cycle control. Such viruses require their host's cellular machinery for the replication of their own genome for their life cycle, and as such often express proteins that are capable of over-riding cell cycle control. This is to permit the host cell to enter DNA synthesis and consequently express the factors the virus requires for the replication of its genome. One example relevant to this study are the papillomaviruses.

Papillomaviruses belong to the family *papovavirus* and are oncogenic viruses (zur Hausen, 1991b) which infect cutaneous and mucosal epithelia, giving rise to benign hyperproliferative lesions, commonly called warts. In the great majority of cases, these lesions are under host immunological surveillance and regress spontaneously. However, if other factors intervene, the lesions can undergo malignant transformation and develop into squamous cell carcinomas. While the co-factors that promote neoplastic progression of warts are ill-defined in humans, they have been identified in animal systems (IARC, 1995). One of such system is the papilloma-carcinoma syndrome of cattle (Campo, 1997).

Cattle are infected in their upper GI tract by bovine papillomavirus type 4 (BPV-4) which induces squamous epithelial papillomas (Campo and Jarrett, 1986). In healthy cattle, the papillomas develop and persist for approximately one

year and are then rejected by a cell mediated immune response (Knowles *et al*, 1996). However, in cattle grazing on bracken fern the papillomas can progress to squamous cell cancer (Campo and Jarrett, 1986). Bracken fern contains immunosuppressants and mutagens. Bracken-eating cattle become chronically immunosuppressed and are incapable of mounting an appropriate immune response against the virus or virus infected cells (Campo, 1997). The bracken mutagens are probably responsible for the observed activation of *ras* (Campo *et al*, 1990), mutation of *p53* (Scobie, 1996) and increase in epidermal growth factor receptors (Smith *et al.,* 1987) observed in cancers as well as the increased level of chromosomal damage observed in cattle fed on bracken fern (Moura *et al.,* 1988).

The progression of papillomas to carcinomas has been experimentally reproduced in bracken-fed cattle (Campo *et al.,* 1994b) thus confirming the epidemiological studies conducted in the field.

The multistep nature of BPV-4 cell transformation has been studied *in vitro* by use of primary bovine fibroblast cells (PalFs) from the foetus palate. E7 and E8, the transforming proteins of BPV-4, are not capable of fully transforming these cells (Pennie *et al.,* 1993). However in co-operation with Ha-ras, E7 induces morphological transformation of PalF cells and E8 is required for their anchorage independent growth but, although the cells have an extended lifespan and are capable of growing independently of adhesion to the substrate, they are neither immortal nor tumorigenic (Pennie *et al.,* 1993; O'Brien & Campo, 1998).

Although BPV-4, or E7, can transform PalFs only partially, exposure of the cells to a single dose of quercetin (3,3',4',5,7-pentahydroxyflavone, see figure 2 for the structure), a flavonoid found in bracken, can achieve oncogenic transformation and the cells can induce tumours in nude mice (Pennie and Campo 1992; Cairney and Campo, 1995). These *in vitro* observations reflect the involvement of bracken chemicals in BPV-4 associated carcinogenesis.

Quercetin is a well known mutagenic flavonoid present in bracken fern (Bjieldanes and Chang, 1977; Nakayasu *et al.,* 1986); it binds DNA causing single strand DNA breaks (Fazal *et al.,* 1990), DNA rearrangements (Suzuki *et al.,* 1991) and chromosomal damage (Ishidate, 1988).

Quercetin has also been shown to inhibit a plethora of cellular enzymes (Nishino *et al.,* 1984; Glossman *et al.,* 1981; Graziani *et al.,* 1983; Graziani *et al.,*

1981; Gschwendt *et al.,* 1984; Srirastava, 1985; Lang & Racker, 1974; Shoshan & MacLennan, 1981; Beretz *et al.,* 1978; Ruckstuhl *et al.,* 1979). Much of this inhibition may well be through quercetin's ability to competitively inhibit ATP binding (Graziani *et al.,* 1983).

One resulting effect of the properties of quercetin is cell cycle arrest. This has been experimentally shown in a variety of cell lines, where quercetin exposure has arrested the cell at different stages of the cell cycle (Yoshida *et al,*. 1990, Hosokawa *et al.,* 1990, Gong *et al.,* 1994, Scambia *et al.,* 1990, Ranelletti *et al.,* 1992, Avila *et al.,* 1994, Plaumann *et al.,* 1996). Most studies on quercetin's effect on the cell cycle have been carried out on tumorigenic cell lines, or immortalised cell lines. This causes some difficulty in the analysis due to the presence of unidentified genetic alterations that have occurred in these cells which might have affected the cell cycle checkpoints already. Hence, work to examine cell cycle control on primary cells where one can assume that there have been no alterations to the proteins controlling cellular proliferation is desirable in order to examine quercetin's effect on the cell in the context of full checkpoint capability.

Previous work demonstrated that quercetin induces G1 arrest in normally cycling PalF cells (Connolly *et al.,* 1998). Speculation that this abrogation of the G1 arrest may play a significant role in the progression to a fully transformed state of the PalF cells came from the amino acid similarity between BPV-4 E7 and the HPV-16 E7 protein, the latter being well characterised as an oncoprotein capable of over-riding G1 arrest induced by DNA damage (Hickman *et al.,* 1994; Slebos *et al.,* 1994; Demers *et al.,* 1994). Additionally the BPV-4 E7 protein contains a putative pRb105 binding site, and has two cys-X-X-cys zinc binding domains. Mutations of these domains abolished the E7 ability to cause morphological transformation with Ha-ras (Campo *et al.,* 1994a; Jackson *et al.,* 1996). Hence it was hypothesised that the expression of the BPV-4 E7 and Ha-ras proteins would allow the cell to overcome a quercetin induced G1 arrest. This would allow the cells to pass through at least one round of DNA replication in the presence of quercetin. This would then lead to generation of inheritable damage, and progression to full transformation.

3.2 Generation of Cell Lines

To study the effects of quercetin in the *in vitro* model of transformation, cell lines had to be derived. This would allow an analysis of changes that had occurred within the cell to permit full transformation. Limitation on this form of analysis though would rest on the fact that only tumourigenic derived clones would be likely to be suitable for continued passage in tissue culture, and thus for comparison to the parental PalF cells.

To overcome this problem pooled populations, rather than single clonal populations, were used for certain transfection classes to permit passage in tissue culture for a duration suitable for study.

3.2.1 Experimental Method

Stable transfection of the parental PalF cells was carried out as described in section 2.2.2.8. See table 3.1 for a description of the transfection classes carried out:

Table 3.1

Transfection Class	Plasmids Transfected	Quercetin Treatment (*)	Resultant Cell Lines
1	Salmon Sperm DNA alone	N/A	None
2	pZipneo & Salmon Sperm DNA	0μM (24 hrs)	None
3	pZipneo & Salmon Sperm DNA	20μM (24 hrs)	None
4	pZipneo & Salmon Sperm DNA	20μM (240 hrs)	None
5	pZipneo, pZipneo E7 & Salmon Sperm DNA	0μM (24 hrs)	None
6	pZipneo, pZipneo E7 & Salmon Sperm DNA	20μM (24 hrs)	None
7	pZipneo, pZipneo E7 & Salmon Sperm DNA	20μM (240 hrs)	None
8	pZipneo, pT24 & Salmon Sperm DNA	0μM (24 hrs)	None
9	pZipneo, pT24 & Salmon Sperm DNA	20μM (24 hrs)	None
10	pZipneo, pT24 & Salmon Sperm DNA	20μM (240 hrs)	None
11	pZipneo, pZipneo E7, pT24 & Salmon Sperm DNA	0μM (24 hrs)	11A,11Aα,11T,11X, 11Aβ
12	pZipneo, pZipneo E7, pT24 & Salmon Sperm DNA	20μM (24 hrs)	E7Q,E7Qb,E7QPα^{P}, E7QPβ^{P},E7QPγ^{P} 12F$^{\#}$
13	pZipneo, pZipneo E7, pT24 & Salmon Sperm DNA	20μM (240 hrs)	13B,13I,13J,13N
14	pZipneo, pZipneo E7 & Salmon Sperm DNA	n.d.	E7R^{P}

Key:

* = Quercetin treatment indicates the concentration used for 24 hours exposure. Number in parenthesis indicates the time after transfection the quercetin exposure initiated.

^{P} = Indicates the cell line was derived from a pooled population.

The last column indicates cell lines that were successfully expanded to allow injection into nude mice for tumorigenicity assay.

$^{\#}$ = Cell line generated, but lost due to contamination.

Cells were quercetin treated after transfection as indicated and selected in G418 medium for 2-3 weeks. The resultant clones were either pooled or if deemed large enough were isolated by ring cloning. Clones and pooled cells were

expanded to a minimum of $4x10^7$ cells for assay in nude mice and for generation of long term frozen stocks: 3 mice per cell line at $1x10^7$ cells per mouse and 10 vials of $1x10^6$ cells per vial for storage in liquid nitrogen as a master stock. An aliquot of the master stock was then thawed and expanded to generate a further 10 vials of $1x10^6$ cells per vial for storage as a working stock. Subsequent analysis was conducted on frozen stocks thawed and grown in tissue culture as described for parental PalF cells, namely passaged twice per week being split 1 in 4, but maintained under G418 selection.

Cells were expanded up to the required numbers for subsequent analysis. Different lines grew at different rates (see below) so the time intervals between passages varied. PalF cells were not maintained in culture for more than 3 weeks and the transformed cells for more than eight weeks, after which new stocks were thawed.

3.2.2 Experimental Results & Discussion

As can be seen from table 3.1 no clones isolated from transfection classes 2-10 were successfully expanded in tissue culture to permit analysis of their tumorigenic state in nude mouse, or other studies. This in itself would strongly suggest that the cells were poorly transformed with a limited lifespan. No clones of salmon sperm DNA transfectants developed due to the lack of a selective resistance plasmid being inserted into the cells, thus ensuring that the selective process was successful. Transfection classes 11-13 were the conditions that generated stable cell lines for further study. Clones were picked from all 3 classes, however only a few were capable of the required expansion for the nude mouse assay. Listed below in table 3.2 are the figures for the number of clones picked per class and the number of successfully expanded single clones.

Table 3.2 **Clone Isolation and Expansion Numbers**

Transfection Class	Total Number of Cells Used in Transfections	Number of Clones >1mm diameter	Number of Expanded Clones	Number of Tumorigenic Cell Lines
11	$1.6x10^6$	30	5	0
12	$2.4 x10^6$	17	3* (2)	1 (E7Q)
13	$1.6x10^6$	18	4	0

* = Three clones were capable of the required expansion level although one clone (12F) was lost through contamination before injection.

As can be seen in table 3.2 the number of clones was decreased considerably in quercetin treated cells, suggesting that exposure to the chemical could be a significant cellular stress to the cell. Thus quercetin exposure in combination with transfection could induce a high stress level for the cell, probably causing a greater number of cells to commit to apoptosis in response to this combination of insults.

The number of clones successfully expanded to at least $4x10^7$ cells was also significantly lower than the number originally isolated as well, suggesting that the transfection of E7 & Ha-ras alone was generally insufficient to confer an extended lifespan upon the transfected cell. In addition all clones isolated by this method with one exception (tumorigenic cell line from transfection class 12) proved very slow to passage, and exhibited cellular morphology indicative of senescence on continued passage in tissue culture. Hence these cell lines were deemed unsuitable for further studies after limited preliminary analysis.

One complicating factor for the generation of the clonal cell lines was likely to be the initial low number of cells post transfection. This may well have had a deleterious effect on the proliferation capability, thus resulting in clones with limited expansion potential. To facilitate studies with suitable cell lines, pooled populations were used to allow easier expansion of the transfected cells. Transfected cells were selected as per standard protocol, then resultant clones were trypsinised and pooled into tissue culture containers to ensure at least an initial 50% confluency.

This approach was undertaken for transfection class 12 and resulted in E7QPα, E7QPβ and E7QPγ (from two different transfections), which proved to be

able to survive continued passage in tissue culture and suitable for experimentation. Additionally E7R cells were also derived in this manner due to the failure of any suitable clones derived from transfection classes 5-10, suggesting ring cloning would be ineffective as a source of material for this class.

Due to the low number of cell lines obtained, previously generated cell lines (Q0D and Q2D see section 2.1.4, and table 3.3) were also studied in order to widen the breadth of analysis. These contained the whole BPV-4 genome rather than just pZipneoE7, thus allowing analysis of E7 expression under the control of the BPV-4 LCR.

Cell lines under analysis did not all grow at the same rate. Although no quantitative analysis of cell proliferation was conducted, the frequency at which cells needed passaging gave an indication of their growth rate. Thus parental PalF, E7QPβ and E7QPγ grew at approximately equivalent rates and were passaged twice per week being split 1 in 4. E7R, E7Qb and Q0D grew more slowly and were passaged only once per week on average. On the contrary E7Q, E7QPα, E7QT2 Q2D and 88529B grew more rapidly and were passaged roughly 3 times per week. Growth curves could have been established by measurement of the number of viable cells at each passage in culture, and would have given a more detailed knowledge of the proliferation rates of the cell lines. Nevertheless it is interesting to note that the faster growing cells proved to be tumourigenic in the nude mouse assay (see below).

3.3 Phenotypic Analysis

To allow proper evaluation of the stable cell lines generated presently or previously a characterisation of the lines was carried out. The phenotype of the transformed lines was analysed by assessing their ability to grow independently of substrate and to induce tumours in nude mice (for summary see table 3.3), in addition to any morphological alterations.

3.3.1 Experimental Method

Cell lines were expanded in culture as described in section 2.2.2.3 and section 3.2.2 under continuous G418 selection (500μg/ml) and allowed to proliferate to sufficient cell number to permit injection into nude mice.

Prior to injection aliquots of cells were frozen in liquid nitrogen at an early passage in order to facilitate long term usage (see section 2.2.2.4), and screened for mycoplasma infection in order to ensure cell line purity (see section 2.2.2.5).

Typically at least 3 mice were used per clone for transfection classes 11 & 13. This initial number was decided due to the potentially large number of clones to be assayed. To ensure any observed developing tumours were not a result of the injection protocol a negative control of PalF cells were used. Additionally to ensure that any lack of observed tumour was not due to a vestigial immune response in the mice a positive control cell line was used. This was either Q2D, previously demonstrated to be tumorigenic in the nude mouse assay (Cairney & Campo, 1995), or another cell line '15.6' which contains the BPV-4 genome, activated ras, HPV-16 E6 and a mutant p53. This latter cell line had also shown to be tumorigenic in the nude mouse assay (Scobie *et al.,* 1997). Later injections using the cell lines E7Q, E7QPα,β,γ and E7R used a greater number of mice (5 to 8 per clone, see table 3.3 for summary).

For each injection $1x10^7$ cells were used per mouse in a volume of 100µl of PBS. Cells were kept on ice until the point of injection. See section 2.2.2.11.2 for full details.

In addition to analysis of the cell lines capability to form solid tumours in nude mice, they were also assayed for their ability to grow independent of substrate. In brief, 5mls of 1% Methocel medium containing $1.25x10^5$ cells (taken from a stock of $5x10^6$ cells grown out from frozen stocks) were plated into 60mm bacterial grade petri dishes and stored at 37°C, 5% CO_2 in a humid atmosphere for 10 days. After the incubation colonies, of 0.1mm or greater diameter, were scored in 3 random field of $16mm^2$. The average efficiency colony formation was then calculated on the basis of number of cells seeded to number of colonies formed.

3.3.2 Experimental Results & Discussion

3.3.2.1 Cell Morphology

In addition to the analysis of the cell lines ability to grow in methocel medium and to form tumours in nude mice a more basic level of transformation, that of morphological alteration, was observed in all the cell lines generated. Figures 3 to 5 show the non tumourigenic, transformed cell lines exhibit a spindle like cell morphology when compared to PalF cells. Additionally they also adopt a 'criss-cross' growth pattern over each other. If left to grow to confluence these cell lines will form foci of cells piling on top of each other which can elongate to form long foci. Comparison between the E7R and Q0D cell lines shows little difference in the cell morphologies indicating that E7 and Ha-ras expression alone is sufficient for the cellular morphology alteration and overcome contact inhibition. Other viral proteins are unlikely to play a part in gross alteration as evidenced by the similarity of Q0D to E7R.

The quercetin exposure protocol used to induce full transformation does not always confer additional changes to the cell morphology or contact inhibition behaviour as can been seen in E7Qb and 12F. Both cell lines resemble those of transfection class 11, and both display the ability to form foci in confluent flasks, but show no obvious other morphological alterations.

The progression of the cells to full transformation however is associated with a morphology differing from both wild type and transformed cells. Q2D and E7Q were shorter and more rounded. This may be due to the faster proliferation rates of these cell lines, not allowing cells to increase in size as compared to the non tumorigenic cell lines. In addition to their altered appearance these cell lines and their derivatives showed a high level of cell debris during normal tissue culture passaging, as deduced by the excessive level of cell debris formed in the culture medium and that still attached to the growing monolayer. While some apoptosis was seen in the non tumorigenic transformed cell lines during passaging the level was much lower than that observed in E7Q and Q2D. In the main apoptosis in these non tumorigenic cell lines was associated with the lines entering senescence.

3.3.2.2 Anchorage Independent Growth

Upon analysis of whether the cell lines could grow independent of substrate or not, only those cells that had also proven to be tumorigenic were capable of growth in methocel medium. All other cell lines tested were incapable of anchorage independent growth.

Figure 6 shows the results of the initial set of methocel assays. PalF cells were used as the negative control cell line. These cells were incapable of forming colonies in methocel, the small clumps of cells seen are most likely to be the result of a few rounds of cell division before loss of integrin signalling for example (Giancotti &Ruoslahti, 1990) would result in cell cycle arrest possibly via loss of cyclin A expression (Guadagno *et al.,* 1993). As a positive control the cell line 15.6 (Scobie *et al.,* 1997) was used as it had been previously proven to show anchorage independent growth.

In this assay E7Qb, 11Aβ, 11X, 13B and 13I were tested. The cell line 12F was not tested due to loss in tissue culture. The cell lines 13N, 13J, 11T, 11Aα and 11A were not tested due to their low growth rates at the time of experimentation. Their inability to proliferate on tissue culture plastic made them poor candidates for growth independent of substrate. A likely explanation is that these cell lines were entering senescence upon prolonged culturing. As figure 6 demonstrates however those cell lines tested were also incapable of anchorage independent growth.

In the second round of assays (figure 7) again PalF cells were used for comparison as the negative control cell line, with Q2D used as the positive control cell line (Pennie and Campo, 1992). All the cell lines in culture at this point were capable of normal rates of proliferation in comparison to PalF cells and all were assayed.

Colonies only formed in those cell lines proven to be tumorigenic (see section 3.3.2.3), namely E7Q and its derivative E7QT2, E7QPα and 88529B. The cell lines E7R, Q0D and E7QPβ and E7QPγ all failed to form colonies of 0.1mm or greater diameter in methocel. Hence this indicates that not only expression of BPV-4 E7 and Ha-ras does not confer anchorage independent growth, but the stable transfection of the

Cell Line Morphology

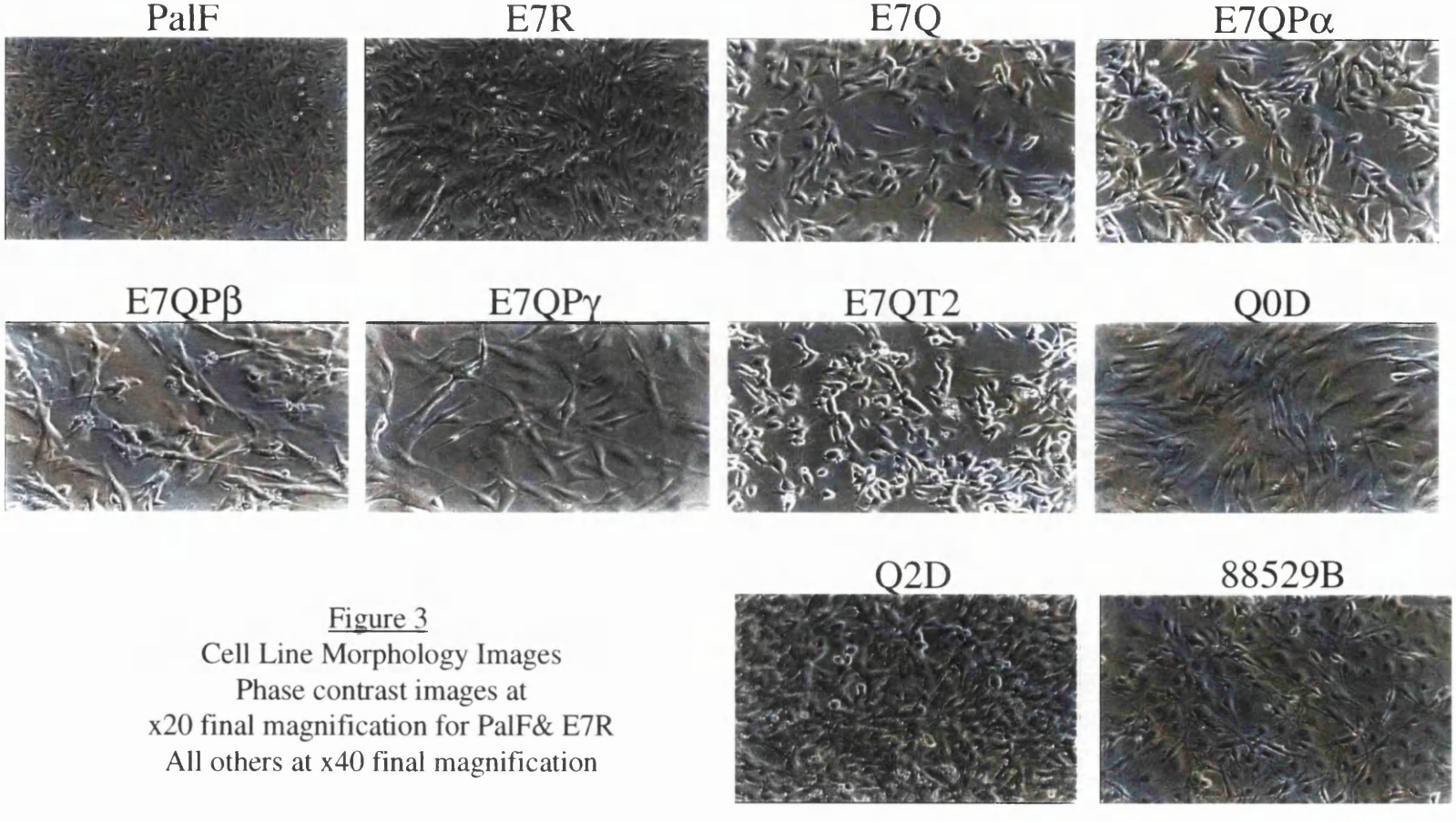


Figure 3
Cell Line Morphology Images
Phase contrast images at
x20 final magnification for PalF& E7R
All others at x40 final magnification

Cell Line Morphology

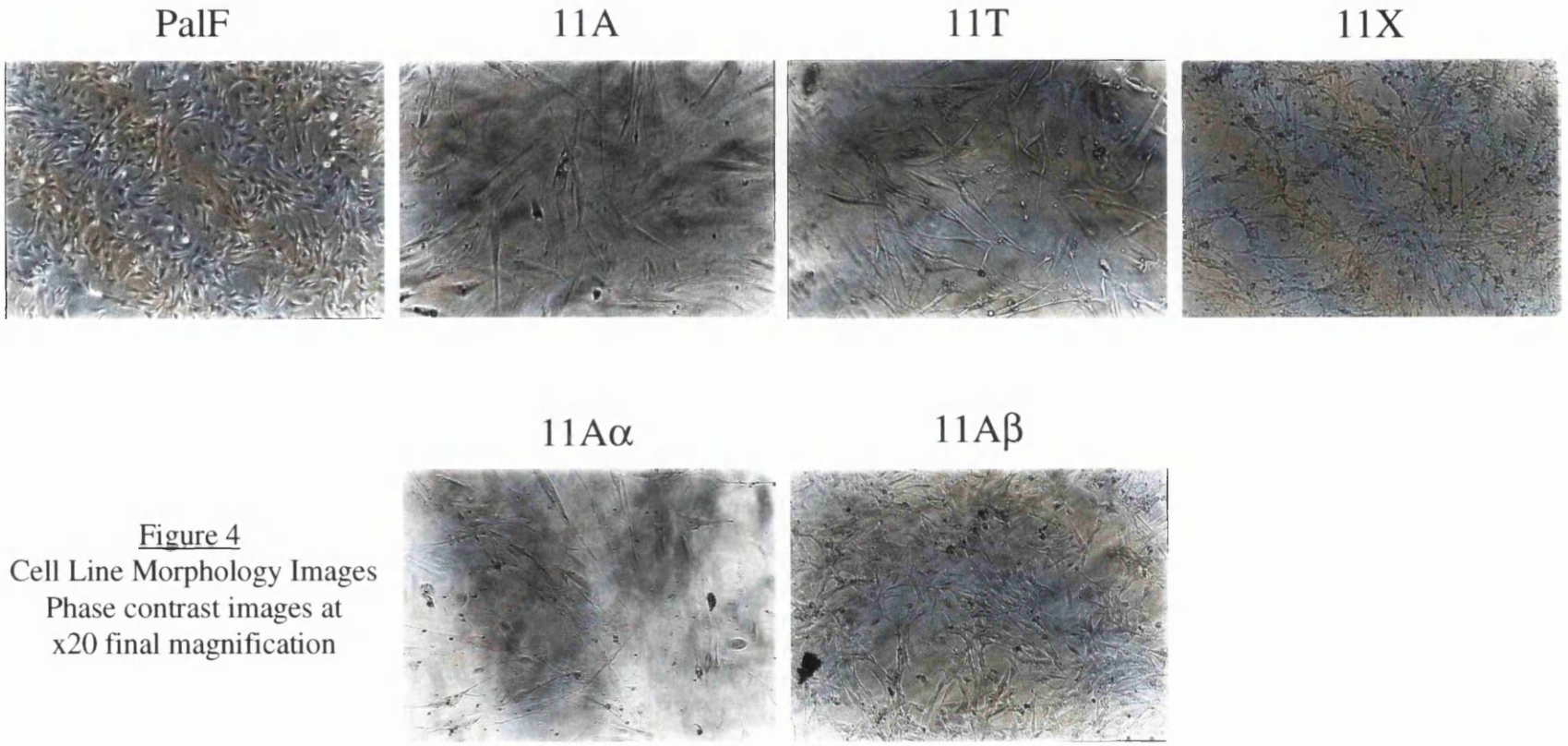


Figure 4
Cell Line Morphology Images
Phase contrast images at
x20 final magnification

Cell Line Morphology

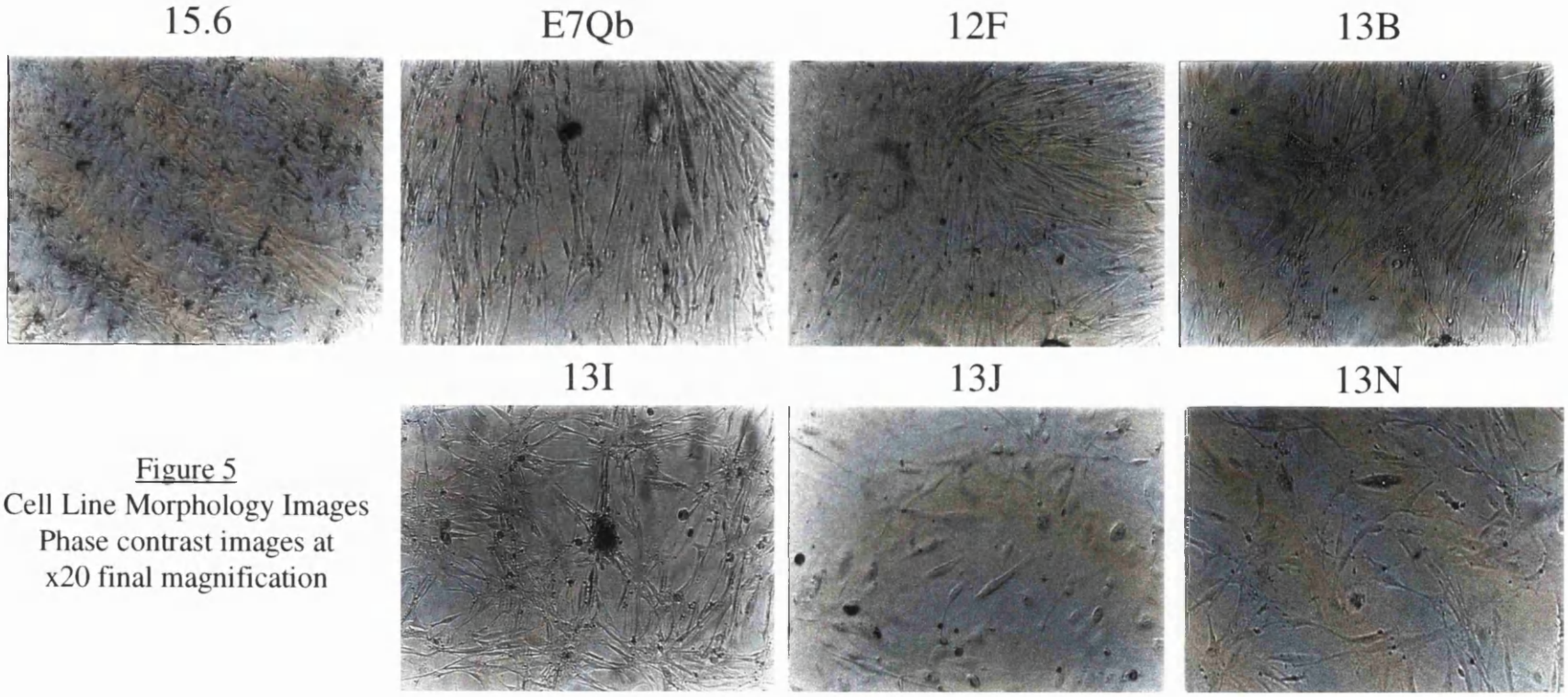


Figure 5
Cell Line Morphology Images
Phase contrast images at
x20 final magnification

Anchorage independence Colony

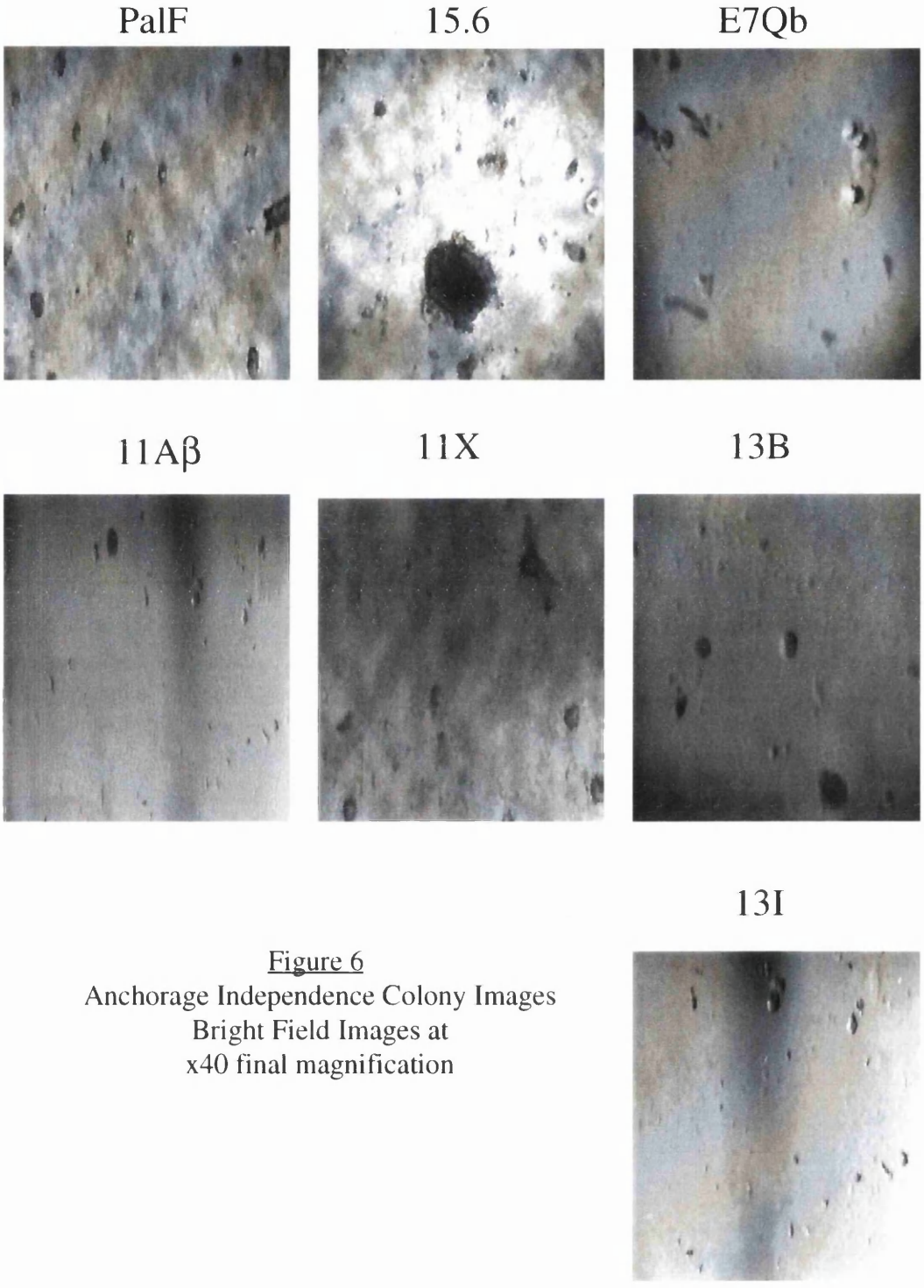


Figure 6
Anchorage Independence Colony Images
Bright Field Images at
x40 final magnification

Anchorage independence Colony Photos

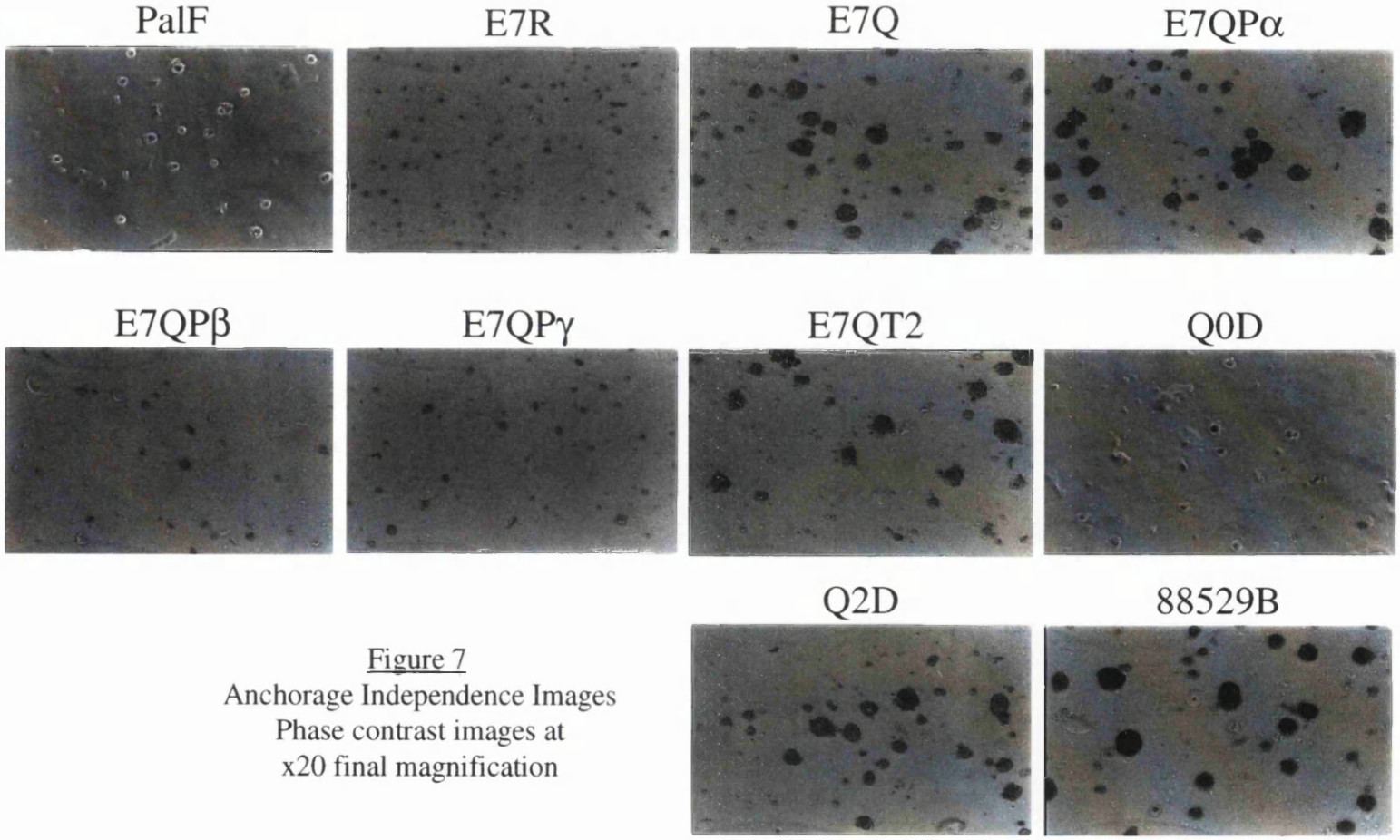


Figure 7
Anchorage Independence Images
Phase contrast images at
x20 final magnification

full genome with Ha-ras into PalF cells (Q0D) did not confer this capability either, in this system, although Pennie *et al.,* in 1993 did show that the full genome and Ha-ras were capable of conferring anchorage independent growth to PalF cells. However the expression level of the other viral protein which is implicated in anchorage independent growth, BPV-4 E8, is not known. Lack of growth may be attributable to loss of expression of this protein.

The ability of the tumorigenic cell lines, E7Q, E7QPα and Q2D, to form anchorage independent colonies in contrast to the non-tumorigenic cell lines, E7Qb and Q0D, shows that quercetin treatment can permit transformation of the cell to be independent of substrate. However this is not an obligatory result of quercetin treatment of the cells as E7QPβ, E7QPγ fail to form anchorage independent colonies. This is further reinforced by the results of 13B & 13J in which exposure of the transfected cells to quercetin, 10 days after transfection, does not establish this phenotype either.

3.3.2.3 Tumour Formation in Nude Mice

All cell lines capable of culturing to sufficient levels were assayed for tumour formation in nude mice, this representing full transformation of the cell. For a full summary see table 3.3.

PalF cells were used as a negative control with either Q2D or 15.6 used as a positive control as previously these cells were shown to be tumorigenic (Pennie and Campo 1992; Scobie *et al.,* 1997). The cell line Q0D was previously shown to be non tumorigenic (Pennie and Campo 1992) whereas 88529B was capable of forming tumours (Campo *et al.,* 1985).

Injection of the cell lines derived from transfection protocol 11 or 13 resulted in no tumour formation or observed differences from injection with PalF cells. The same was true for injection with E7R or the quercetin treated cell line E7Qb.

Injection with Q2D,E7Q or E7QPα resulted in rapid tumour formation. The mice had to be sacrificed due to tumour load within 3 weeks of injection of the cells. However there was one exception, where Q2D cells were injected in a different mouse strain (ICRF1). In this case Q2D injected mice survived the

course of the assay (2 months) with only 4 out of the 5 mice developing tumours. At this point the cells were used as a positive control for the cell lines E7QPβ and E7QPγ. These latter two cell lines formed lumps at the point of injection which regressed and failed to induce tumours beyond reasonable doubt. With concern over whether these observations over E7QPβ and E7QPγ had been adversely affected by a vestigal immune response in these mice, a repeat injection was carried out using the original strain of mice. In this experiment again all the cohort of Q2D mice had to be sacrificed after 17 days due to tumour load. E7QPβ and E7QPγ derived lumps which began regressing after 23 days, but not tumours. These 'lumps' were likely to be cysts generated from the injection of the cells (personal communication, T.Hamilton, Beatson Institute). However the PalF or E7R cohorts carried out in the same experiment showed no such lumps.

Tumours derived from injections with Q2D, E7QPα or E7Q were explanted and cultured *in vitro*. Of the tumours derived there were two successful explantations from each of the cell lines E7QPα and E7Q. These resulted in the cell lines E7QPT1 and E7QPT2 from E7QPα tumours, and E7QT1 together with E7QT2 from E7Q tumours. There were three successful explantations of Q2D tumours resulting in the cell lines Q2DT1, Q2DT2 and Q2DT3. The Q2D cell lines derived from explantations were not used for further experimentation and will not be discussed further.

In summary, where expression of the BPV-4 E7 protein in conjunction with activated Ras morphologically transformed the cell, only cells treated with quercetin 24 hours post transfection were capable of growth in semi-solid media (Table 3.3 & Figure 7), in agreement with previous results (Pennie and Campo 1992; Cairney and Campo, 1995). However of these five quercetin-treated lines, only two induced tumours in nude mice (Table 3.3), indicating that quercetin treatment does not inevitably leads to a fully transformed phenotype.

It is interesting to note that the ability to proliferate independent of substrate also correlates with whether the cell is fully tumorigenic. This anchorage independent growth ability may well be a critical late event in the progression to full transformation.

In order to address this situation a more detailed analysis, possibly down to single cell seeding in methocel to ensure no aggregation, then analysis of the resultant substrate independent clones could allow determine expression levels of key proteins e.g. by quantitative RT-PCR.

3.4 Determination of Transfected Gene Expression

3.4.1 Expression of BPV-4 E7 in Cell Lines

3.4.1.1 Experimental Method

To relate the observations of the cell lines capabilities in nude mice and methocel, to the E7 expression of the cells, analysis of the viral gene expression was undertaken. This would ensure any anomalous results were not due to loss of E7 expression in the cell lines or indeed gross over expression.

Due to the lack of a suitable antibody for the detection of E7, RT-PCR approach was taken to check not only for stable retention of the plasmid but also its expression, to ensure that there had been no silencing on integration into the host genome for example. The limit to this approach was of course that there would be no definitive answer to expression levels, only a rough guide by the PCR product level. However due to time constraints making antibody generation unfeasible, RT-PCR was considered a more suitable option.

Primers to an internal portion of the BPV-4 E7 message were kindly donated by G.Sibbet (Beatson Institute), which generated a 170bp product upon PCR amplification (5'-gctgaccttccagtcttaat (642-661) and 3'-tgaagaggagattgaaactg (793-812)). As a positive control, pZipneoE7 was used with the same primers; negative controls were reactions without RT or without template (dH_2O).

Initially total RNA was isolated using the RNAzolB method with a DNA-ase treatment step to ensure removal of DNA contamination. However this proved to be unreliable in generating RNA of high enough quality for RT-PCR, hence the extraction and purification protocol was switched to using the RNeasy Kit (Qiagen). Total RNA was extracted from the cell lines and purified according to the manufacturers guidelines, quantified for RNA concentration, and stored at -70°C until use (see sections 2.2.3.1 and 2.2.1.4).

For full details see section 2.2.3.2 on methodology of the RT-PCR protocol. In brief 1μl of total RNA was used for the reverse transcriptase reaction with an Oligo dT primer. The enzyme and buffers were from the Omniscript RT kit (Qiagen). Reactions were made up according to the manufacturers recommendations and incubated at 37°C for 60 minutes to allow extension from the primer along the template.

For the tumorigenic cell lines 10% of the RT reaction was carried forward to the PCR step, whilst with the transformed, non-tumorigenic cell lines 50% of the RT reaction was carried over to the PCR stage.

PCR was carried out using the HotStarTaq Kit (Qiagen) as per manufacturer guidelines. At this point the BPV-4 internal primers were used for amplification. 35 rounds of PCR were carried out for each reaction. Note other DNA Polymerases were tried, but failed to give consistent results and often lacked specificity. Thus conversion to a hot start protocol proved to increase specificity and reliability, in conjunction with an improved RNA isolation and RT stage.

3.4.1.2 Experimental Results & Discussion

As can be seen in figure 8 (a) and (b) E7 mRNA was detected in all cell lines except the negative control line PalF and the transforming papilloma cell line 88529B. This latter result is in agreement with the absence of BPV-4 DNA in this cell line (Campo *et al.,* 1985).

It is interesting that although the RT-PCR was not performed under quantitative conditions, increased E7 RNA amplification was obtained from the quercetin-treated cell lines and particularly from the tumorigenic lines E7Q, E7QT2 and also possibly Q2D, since only 10% of the RT reaction was used for the PCR step for E7Q, E7QT2 and Q2D, whereas 50% of the RT reaction was needed for the PCR step for the other cell lines (Figure 8 (a) and (b)).

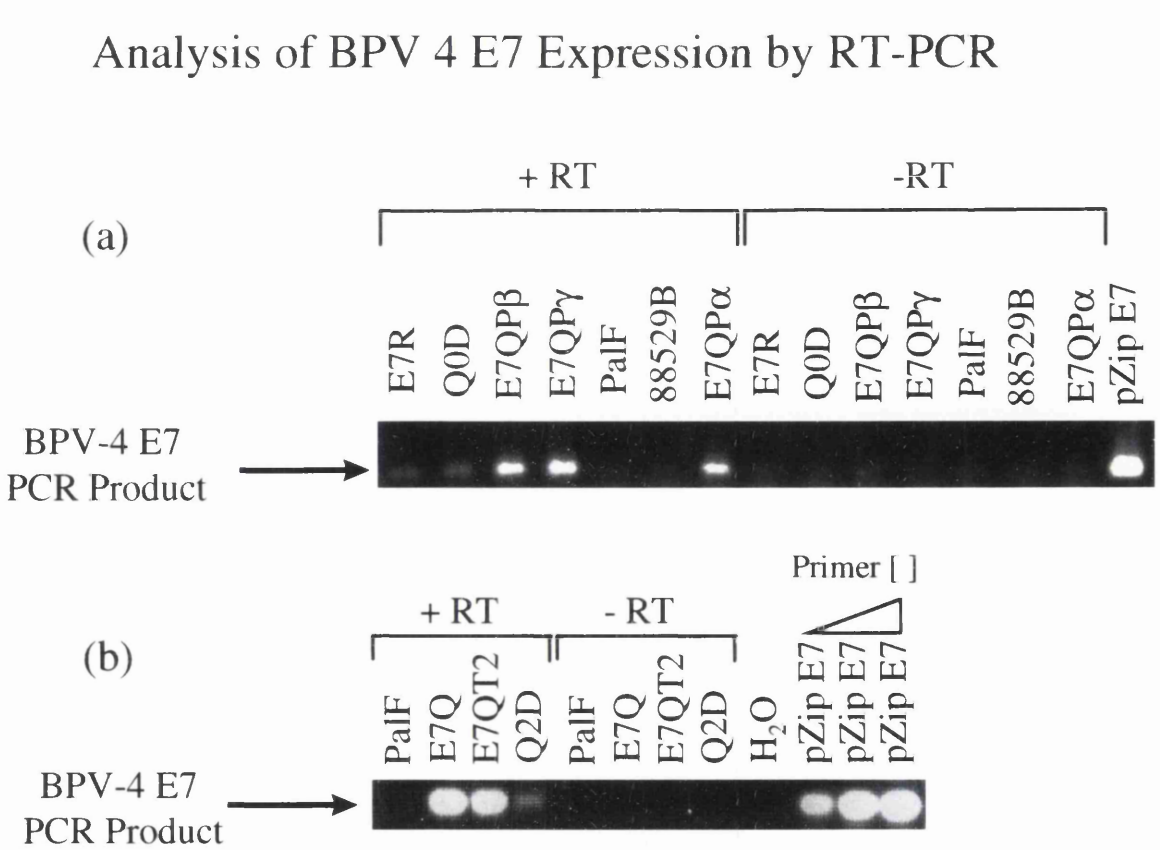


Figure 8. ***Detection of BPV-4 E7 in cell lines***

(a) Detection of E7 RNA by RT-PCR in non-tumorigenic cell lines. E7QPα, E7QPβ and E7QPγ are quercetin-treated pooled lines. PalF and 88529B cells were used as a negative control. pZipE7 is pZipneoE7, used as a positive control.

(b) Detection of E7 RNA by RT-PCR in tumorigenic cell lines.

3.4.2 Expression of Ras in cell lines

3.4.2.1 Experimental Method

Western blot analysis was undertaken to analyse expression of Ras, using an antibody raised against human Ha-Ras. See section 2.2.4 for full experimental methodology on protein analysis. In brief, cycling cells in normal tissue culture conditions were harvested and lysed. E7QPα, E7QPβ and E7QPγ cell lines were treated with a variety of conditions prior to harvesting. 80μg of each cell lines lysate was then loaded onto an SDS-PAGE gel (4% Stacking gel, 10% Resolution gel) to facilitate separation. The gel was then blotted to nitrocellulose, and non specific protein interaction were blocked using 5% Marvel milk in PBS for at least 1 hour at room temperature. Post blocking, the membrane was probed with the anti-Ha-ras antibody then visualised using an HRP conjugated secondary antibody. As a control for expression levels both normal PalF cells and HeLa protein extracts were used. The latter ensures that any band detected in the bovine samples can be matched for mobility with a human ras protein. PalF cells allowed determination of whether the antibody would detect bovine Ras, and to allow estimation of the wild type expression level, in comparison to the other cell lines.

3.4.2.2 Experimental Results and Discussion

Ras was elevated in the transformed cell lines and markedly so in the tumorigenic cell lines (Figure 9 (a) to (d)). Whether the Ras protein detected in the transformed cells is human Ha-ras, endogenous bovine Ras or both is not known as the antibody also detected Ras in PalF and 88529B cells (Figure 9 (a)), indicating that the antibody could react with both bovine and human Ha-ras protein.

Figure 9 (b) &(c) also shows the ras expression in response to different stimuli in the E7QPα, E7QPβ and E7QPγ cell lines. There is no evident alteration of Ras expression in the three cell lines when exposed to quercetin, UV or bleomycin sulphate in comparison to untreated cells. However E7QPβ and E7QPγ both show an elevated expression of the Ras protein in comparison to parental PalF cells (figure 9 (d)).

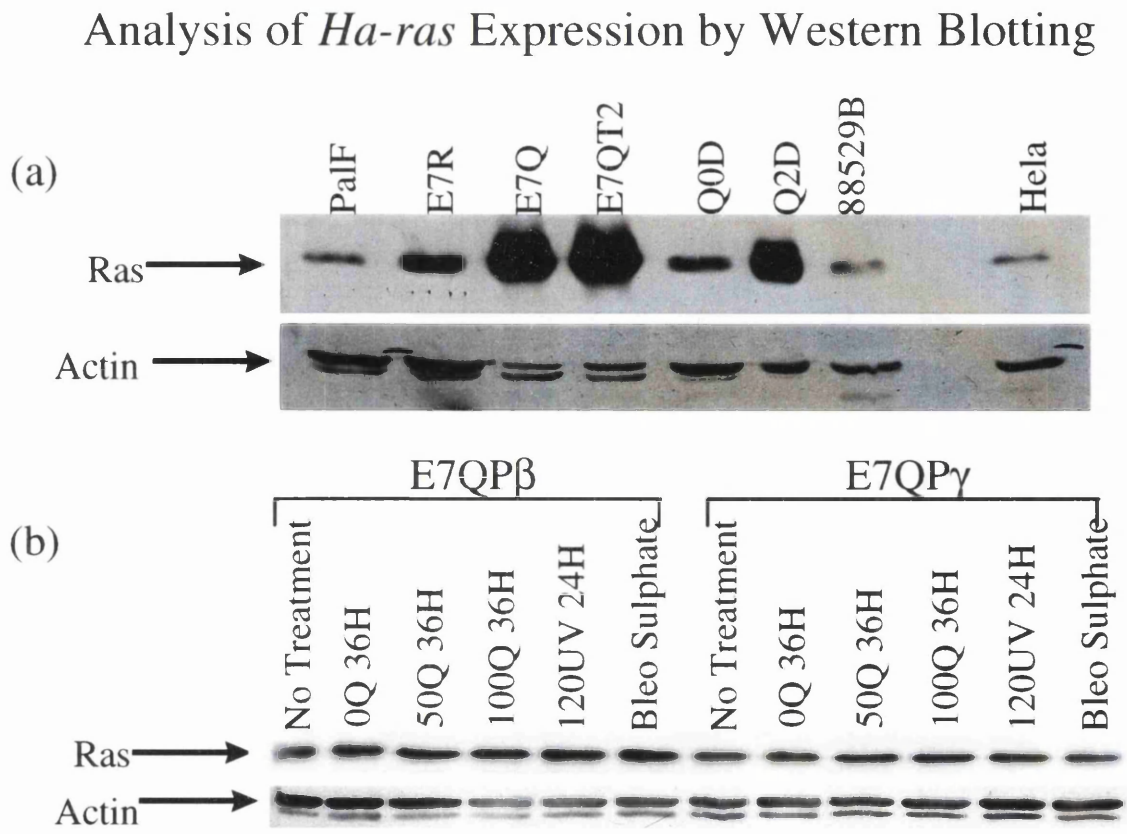


Figure 9. ***Detection of Ras expression in cell lines***
Western Blot analysis of Ras expression. (a) The membranes were probed with an anti-Ras antibody and an anti-actin antibody as a loading control. A lysate from HeLa cells was used as positive control. (b) Ras expression in E7QPβ and E7QPγ cells was also monitored in cells exposed to quercetin (Q) at the indicated μM concentrations, to UV light or to bleomycin sulphate.

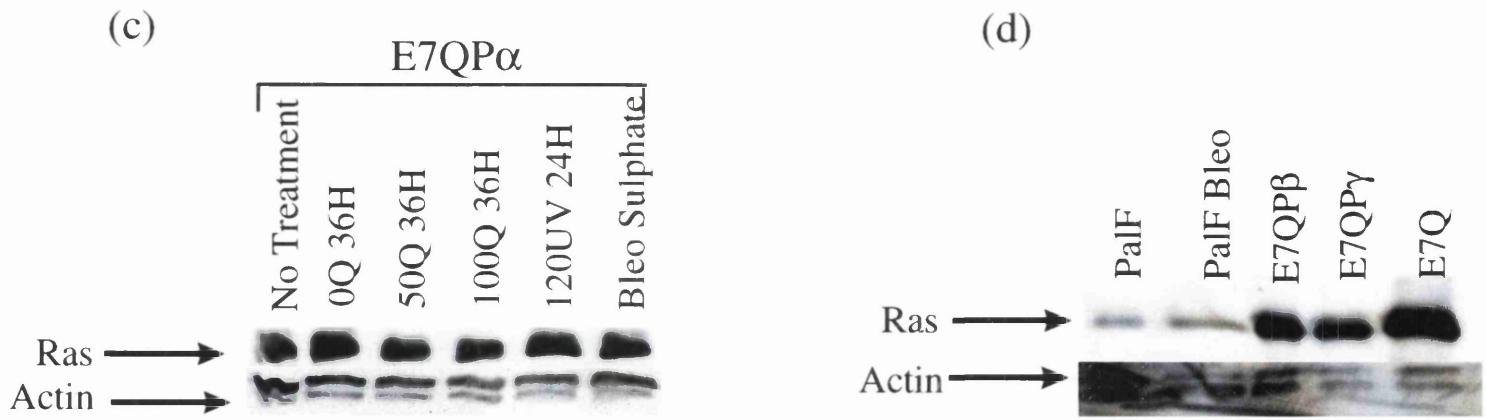


Figure 9. ***Detection of Ras expression in cell lines***
Western Blot analysis of Ras expression. (c) Ras expression in E7QPα cells was monitored in cells exposed to quercetin (Q) at the indicated μM concentrations, to UV light or to bleomycin sulphate. (d) The membranes were probed with an anti-Ras antibody and an anti-actin antibody as a loading control.

CHAPTER FOUR

RESULTS

4.1 Cell Cycle Analysis

Following from the earlier results showing the G1 and G2/M arrest in PalF cells in response to quercetin, it was hypothesised that the abrogation of the arrest may play a part in the transformation of the cell. Under normal conditions quercetin exposure would induce cell cycle arrest by causing cellular stresses and DNA damage (see section 1.4.3.4). The cell would pause and either decide to repair the damage or if this is excessive commit to apoptosis. Under conditions of expression of the E7 protein and activated *Ha-ras* cell cycle arrest would be abrogated due to the activation of the MAPK pathway by *Ha-ras* providing a proliferative growth signal to the cell, and inhibition of the cell cycle inhibitor p105Rb by E7. This latter effect would aid the proliferative signal generated by the release of the transcription factor E2F, driving gene expression required for DNA synthesis. This drive to enter DNA synthesis would then overcome the G1 arrest occuring in normal PalF cells.

Upon entry into S phase, cells would accumulate DNA damage, caused by quercetin exposure, which would result in additional genetic lesions contributory to full transformation of the cell. These genetic lesions may be due to direct mutation of key oncogene or tumour suppressor genes, or indeed may result in a temporary 'mutator' phenotype. A result of this temporal state of instability again would further permanent genetic lesions allowing full transformation.

Focus is retained on the G1 transition due to several factors. The major reason is due to the timing of quercetin exposure. Quercetin exposure in the generation of the tumourigenic cells is only a single dose for 24 hours, whereas the quercetin exposure required for cells to significantly start accumulating in G2/M is 36 to 48 hours. Although when treated with quercetin in the transformation schedule some cells will have already traversed G1 and will therefore stop in G2/M, the majority of the cells would be upstream of the G1 checkpoint. In addition to this factor, the E7 oncoprotein of papillomaviruses has

been well documented to be able to overcome G1 arrest (Hickman *et al.,* 1994; Slebos *et al.,* 1994; Demers *et al.,* 1994) and thus the abrogation of the G1 arrest by BPV 4 E7 is likely. There are also reports of E7 induced endoreduplication of the DNA under mitotic arrest (Di Leonardo *et al.,* 1997; Thomas &Laimins, 1998). However this fails to indicate a true ability to override G2/M control and subsequent normal entry into G1, more of an ability to trigger the cellular mechanisms for DNA synthesis under unsuitable conditions.

4.1.1 Experimental Method

See section 2.2.2.13 for full methodology of the cell cycle analysis by FACS. In brief cells were plated in 90mm culture dishes and treated with either 0, 20, 50 or 100μM quercetin over 48 hours with fresh medium being applied every 12 hours. Some cell lines were assayed with only 0 or 50μM over 48 hours as 50μM caused a significant cell cycle arrest. Cells were then harvest and fixed using EtOH. Fixed cells were then stained using propidium iodide and RNAase A (the latter to remove any staining of RNA which would cause errors in the resultant FACS analysis) to stain the DNA. The stained samples were then analysed on the FACS scan to determine the percentage of the cells in each of the phases of the cell cycle. Sample data were then modelled on 'Modfit v2.0' software for detailed cell cycle population distribution.

4.1.2 Experimental Results and Discussion

The initial observation from the cell cycle data is that all transformed lines except one, are capable of overriding the G1 cell cycle arrest. In addition to this it is also plain that none of the cell lines are capable of overriding the G2/M cell cycle arrest.

Confirming earlier observations, the quercetin treatment of the PalF cells resulted in a large decrease in the S phase population concomitant with an increase in the G2/M population, indicating the cells moving out of S phase into, and arresting in, G2/M. The decrease in the S phase population paralleled by a stable G1 population, provides evidence of the G1 arrest. There is normally a slight decrease over 48 hours in the G1 population which would likely be due to cells

bypassing the G1 checkpoint before a sufficient signal from the quercetin exposure would have triggered the cell cycle arrest response.

Examination of the E7Q cell line showed a dramatic alteration in response to quercetin. Over 48 hours of 20μM to 100μM quercetin, there only remained a very small percentage of the cells left in G1 (see figure 10 and table 4.1), and also resulted in a significant reduction in the S phase population. There was also a large accumulation of cells in the G2/M phase. This was a clear example of the loss of G1 checkpoint but retention of the G2/M checkpoint.

The cell line Q2D showed a similar, if less dramatic, response. Again there was a drop in G1 population together with an increase in G2/M population indicating G1 arrest abrogation. There was also a decrease in S phase, as in E7Q. However as under propidium iodide staining this is only a 'snapshot' of the cell cycle, it is unknown whether the observed decrease is due to cells progressing into and through the S phase slower then normal or due to increased rates of entry into G2. The former argument could be postulated to be caused by quercetin's effect on the kinases and phosphotases within the cell (see section 1.4.3.4). The signalling pathways required for entry, and progression through DNA synthesis may therefore fail to induce correct phosphorylation cascades or alter gene expression, for example, sufficiently for commitment to S phase. Another aspect could be DNA adducts inducing stalls in the replication machinery, whilst repair was carried out. Alternatively accelerated exit from S phase could come from the same mechanisms, kinases and phospohatases may be affected in order to shift bias towards a faster progression rather than inhibition in the signalling pathways e.g. Abrogation of cell cycle inhibitor proteins or inhibition of DNA repair complexes hence no signalling for the DNA Polymerase to pause during synthesis.

The fact that E7Q and Q2D show abrogation of the G1 arrest could be due to one of two main mechanisms. Firstly one possible explanation is that expression of E7 and activated *Ha-ras* could lead as described earlier to a significant growth proliferation signal and inhibition of cell cycle inhibitor proteins such as p105Rb. This would then permit passage through the G1 checkpoint. The second could be due to inheritance of an alteration in the cell due to previous exposure to quercetin. This resistance to quercetin G1 arrest may work upon the cell cycle regulation directly such as loss of function of the cell cycle

inhibitor proteins or be due to a selection of some more classical method of resistance e.g. enzymatic detoxification of quercetin, increased export, enhanced DNA repair complex activity.

Studies of the cell lines E7R and Q0D were carried out to resolve this question. Both of these cell lines expressed E7 and activated *Ha-ras*, without having been previously exposed to quercetin. Test results for Q0D showed a loss of G1 arrest. The overall change in cell cycle population was equivalent to Q2D. There was a drop in G1 and S phase with a concomitant increase in G2/M. Hence this experiment suggested that it was the expression of the viral proteins in conjunction with activated *Ha-ras* that was inducing the bypass of the G1 checkpoint. In support of this conclusion the cell line expressing only BPV-4 E7 and activated *Ha-ras*, E7R, also failed to arrest in G1. Figure 12 shows that there was a drop in G1 phase and increase in G2/M phase after 48 hours of quercetin exposure. In addition there was also an increase in S phase. This is likely to be due to cells moving through the S phase, slower than the PalF cells, to arrest in G2/M. Evidence supporting this hypothesis is the fact that the E7R cells proliferate at a slower rate in tissue culture (data not shown), and by the lower basal percentage of cells in S phase of an E7R population than a PalF population (figures 11 and 12).

The abrogation of G1 arrest in E7R cells appeared be dependent on the number of passages in tissue culture. Early passage E7R cells were capable of abrogating the G1 arrest but not the G2/M arrest (figure 12). However at later passages they reverted to a response more closely resembling wild type PalF cells arresting in G1 and G2/M (see table 4.3 and figure 12 labelled E7R(HP)). In addition the alteration in cell cycle response to quercetin correlates to an alteration in the morphology of the cell line. High passage E7R cells lose their spindle like morphology and closely resemble PalF cells (data not shown).

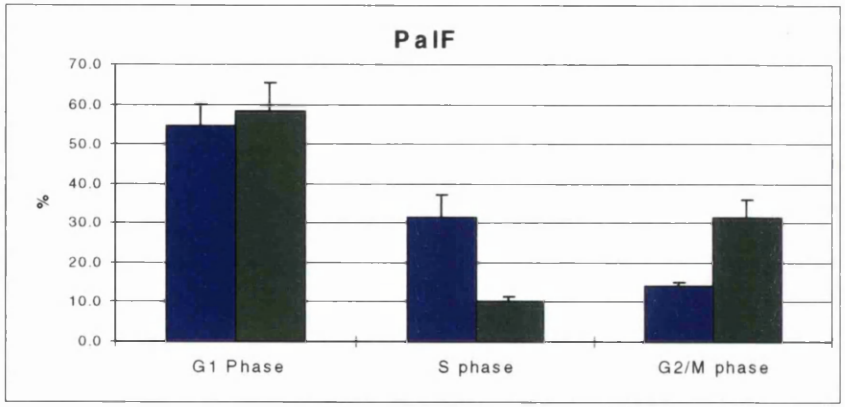
The mechanism for this shift is unknown and requires further study. Possible reasons for the change in behaviour could be a selection against high levels of BPV-4 E7 expression and/or Ha-ras in stable cultures. Thus at low passage there would be sufficient levels of E7 to bind and inhibit p105Rb and cdk inhibitors $p27^{Kip1}$ and $p21^{Waf1/Cip1}$, and/or Ha-ras to drive MAPK pathway to

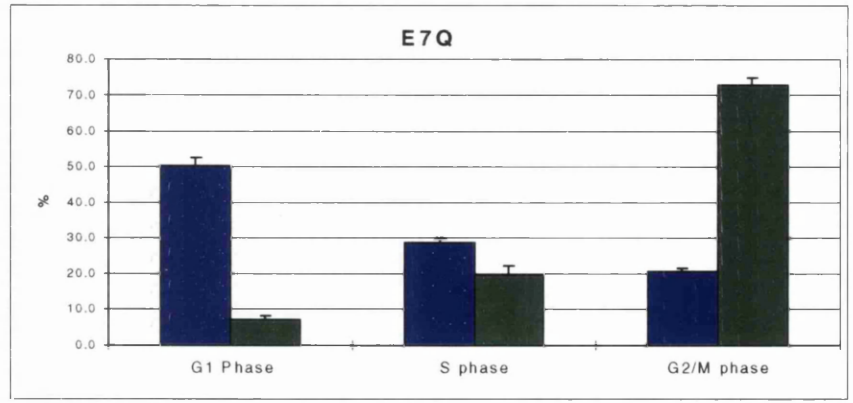
Cell Cycle Response to Quercetin in Various Cell Lines

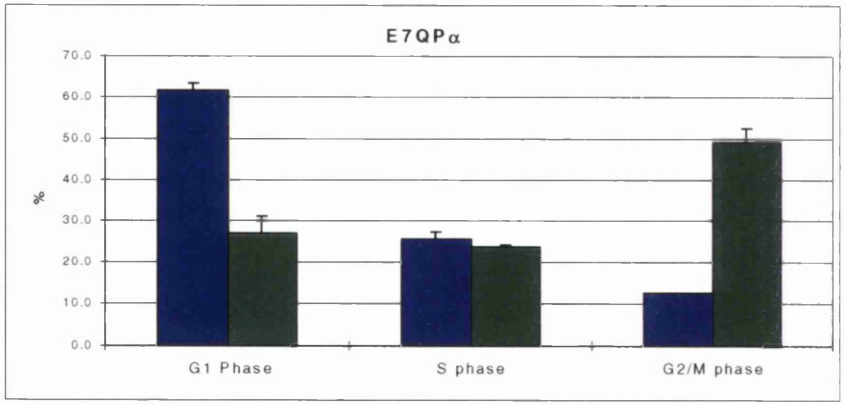
Figure 10. ***Cell cycle profiles of normal and transformed PalF cells***

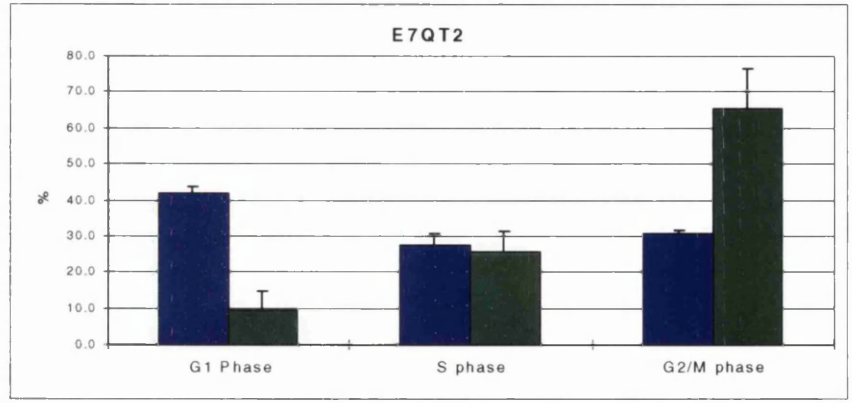
Cells grown in 20μM quercetin (■) or equivalent volume of EtOH (■) for 48 hours were analysed by FACS.

The histograms represent percentage of cells in the G1, S or G2/M phases of the cell cycle.







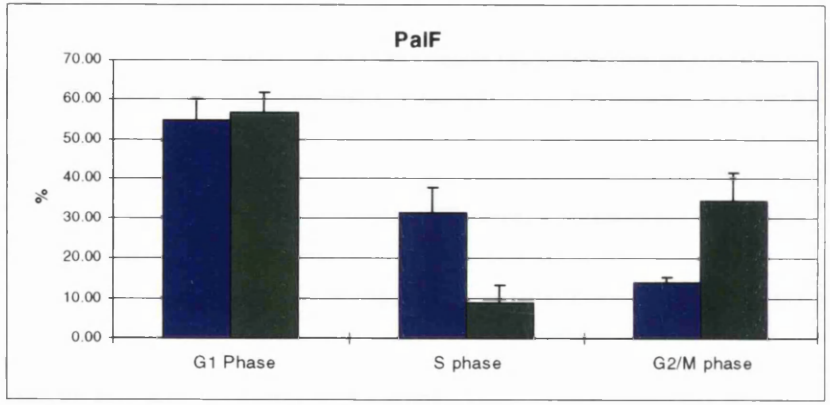


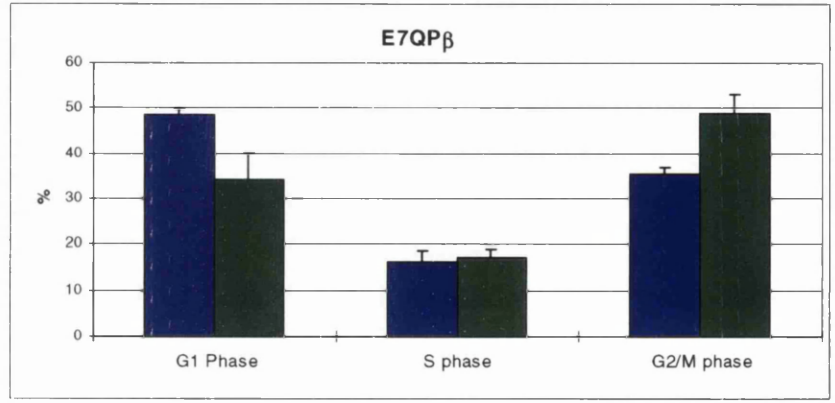
Cell Cycle Response to Quercetin in Various Cell Lines

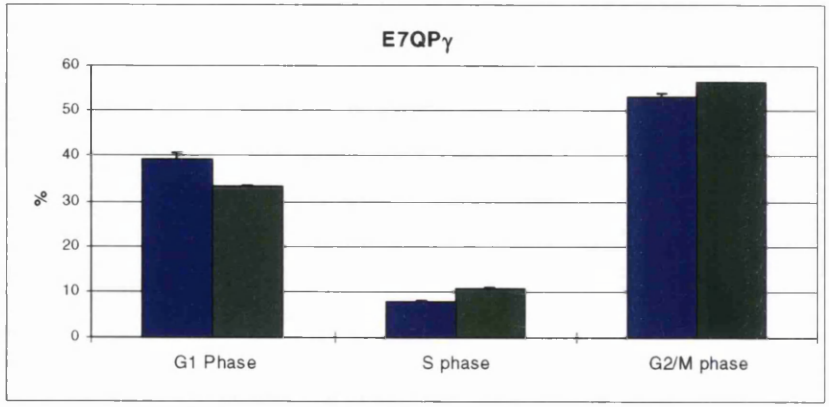
Figure 11. ***Cell cycle profiles of normal and transformed PalF cells***

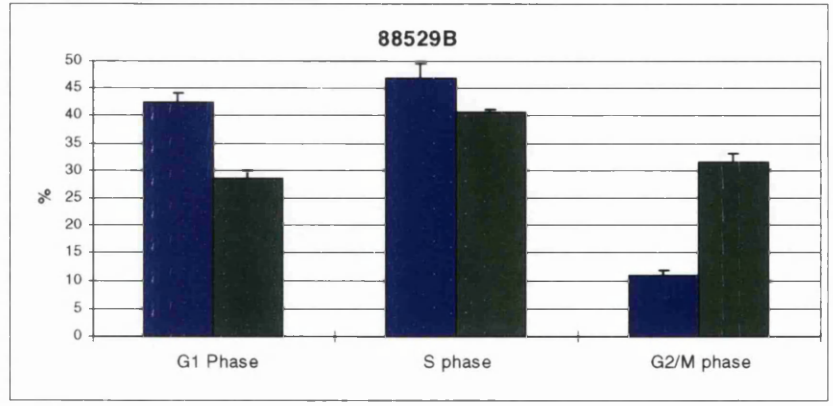
Cells grown in 50μM quercetin (■) or equivalent volume of EtOH (■) for 48 hours were analysed by FACS.

The histograms represent percentage of cells in the G1, S or G2/M phases of the cell cycle.







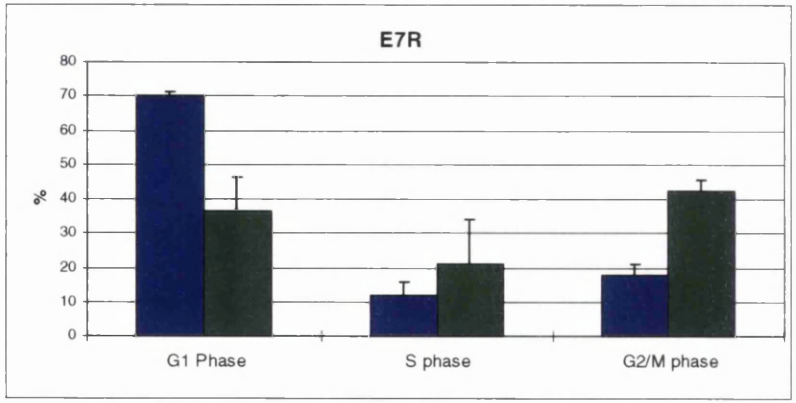


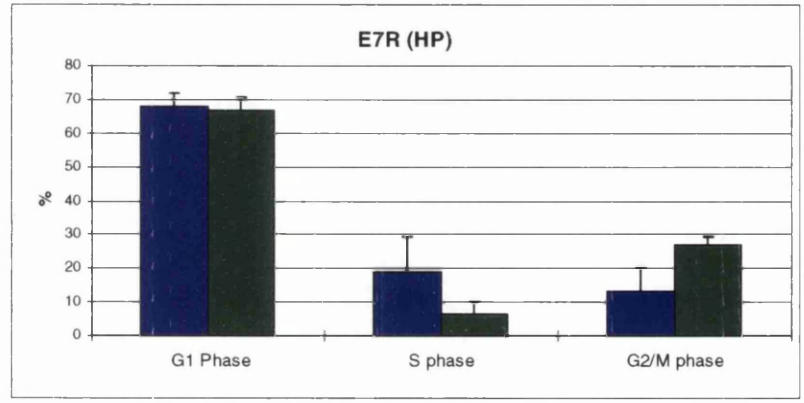
Cell Cycle Response to Quercetin in Various Cell Lines

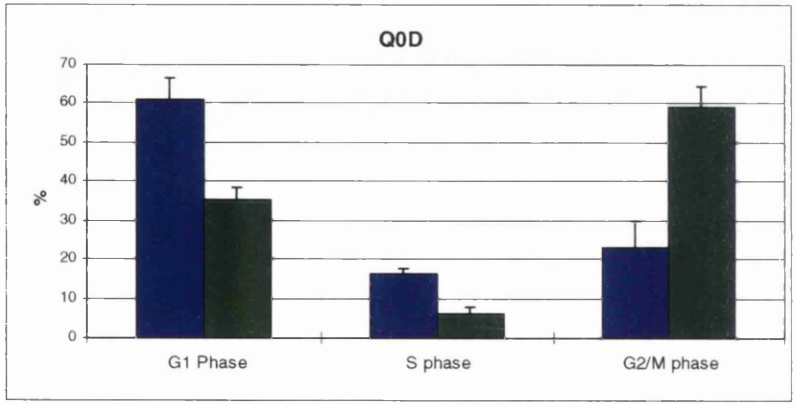
Figure 12. ***Cell cycle profiles of normal and transformed PalF cells***

Cells grown in 50μM quercetin (■) or equivalent volume of EtOH (■) for 48 hours were analysed by FACS.

The histograms represent percentage of cells in the G1, S or G2/M phases of the cell cycle.







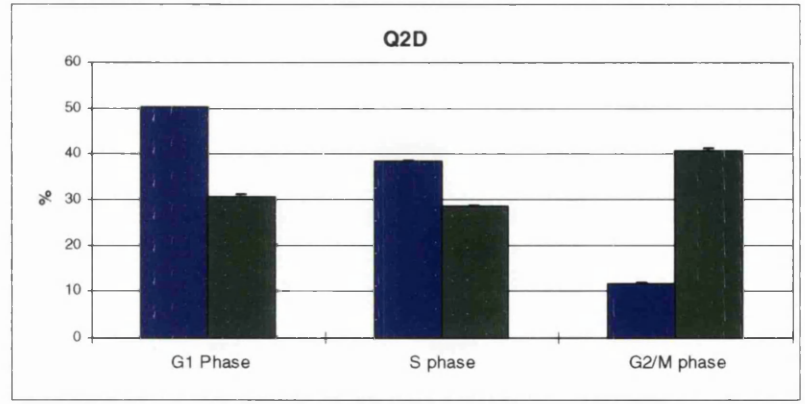


Table 4.1

Summary of Cell Cycle Response to Quercetin in PalF & E7Q cells

PalF

Exposure Time (hours)	Quercetin [ ] μ M	G1 Phase Average		S Phase Average		G2/M Phase Average	
0	0	**58.40**	+/- 5.6	**28.96**	+/- 7.4	**12.63**	+/- 2.4
12	0	**55.25**	+/- 5.9	**32.60**	+/- 7.1	**12.15**	+/- 3.4
24	0	**54.01**	+/- 7.2	**33.99**	+/- 9.3	**12.07**	+/- 3.7
36	0	**55.43**	+/- 5.5	**30.59**	+/- 6.4	**13.98**	+/- 1.9
48	0	**54.60**	+/- 5.5	**31.33**	+/- 6.0	**14.07**	+/- 1.1
0	20	**59.60**	+/- 1.4	**26.65**	+/- 3.1	**13.76**	+/- 3.6
12	20	**64.08**	+/- 6.3	**19.99**	+/- 3.1	**15.89**	+/- 2.5
24	20	**57.62**	+/- 2.8	**19.52**	+/- 3.3	**22.86**	+/- 0.4
36	20	**59.59**	+/- 2.0	**13.09**	+/- 0.7	**27.33**	+/- 2.2
48	20	**58.34**	+/- 6.9	**10.19**	+/- 1.4	**31.47**	+/- 4.5
0	50	**61.26**	+/- 4.0	**24.95**	+/- 5.1	**13.79**	+/- 3.3
12	50	**61.12**	+/- 7.1	**15.11**	+/- 1.9	**23.76**	+/- 5.2
24	50	**59.29**	+/- 3.6	**16.10**	+/- 1.5	**24.61**	+/- 2.6
36	50	**58.65**	+/- 5.8	**10.08**	+/- 3.7	**31.27**	+/- 7.0
48	50	**56.84**	+/- 4.7	**8.94**	+/- 4.1	**34.22**	+/- 6.4
0	100	**57.30**	+/- 2.9	**29.95**	+/- 1.4	**12.76**	+/- 1.2
12	100	**67.98**	+/- 1.9	**7.93**	+/- 1.7	**24.10**	+/- 2.9
24	100	**67.40**	+/- 3.8	**9.18**	+/- 1.2	**23.43**	+/- 4.1
36	100	**61.27**	+/- 5.4	**5.76**	+/- 2.6	**32.98**	+/- 6.6
48	100	**52.03**	+/- 6.1	**4.14**	+/- 1.6	**43.84**	+/- 6.3

E7Q

Exposure Time (hours)	Quercetin [ ] μ M	G1 Phase Average		S Phase Average		G2/M Phase Average	
0	0	**49.10**	+/- 0.56	**24.55**	+/- 0.43	**26.35**	+/- 0.99
12	0	**46.00**	+/- 1.20	**29.57**	+/- 0.74	**24.44**	+/- 0.45
24	0	**48.82**	+/- 1.80	**26.62**	+/- 1.22	**24.57**	+/- 0.59
36	0	**50.58**	+/- 1.52	**28.29**	+/- 2.19	**21.13**	+/- 3.71
48	0	**50.48**	+/- 1.97	**28.78**	+/- 1.19	**20.75**	+/- 0.78
0	20	**47.77**	+/- 1.42	**26.17**	+/- 0.61	**26.06**	+/- 0.81
12	20	**39.75**	+/- 8.31	**34.63**	+/- 4.69	**25.63**	+/- 3.62
24	20	**15.51**	+/- 5.13	**25.95**	+/- 1.54	**58.54**	+/- 3.59
36	20	**9.43**	+/- 2.67	**25.40**	+/- 0.66	**65.17**	+/- 3.33
48	20	**7.25**	+/- 0.75	**19.73**	+/- 2.54	**73.03**	+/- 1.78
0	50	**45.99**	N/A	**25.28**	N/A	**28.73**	N/A
12	50	**43.22**	N/A	**31.54**	N/A	**25.24**	N/A
24	50	**10.64**	N/A	**24.18**	N/A	**65.18**	N/A
36	50	**10.13**	N/A	**27.24**	N/A	**62.63**	N/A
48	50	**5.01**	N/A	**15.56**	N/A	**79.43**	N/A
0	100	**50.10**	N/A	**24.77**	N/A	**25.13**	N/A
12	100	**39.55**	N/A	**33.68**	N/A	**26.77**	N/A
24	100	**11.72**	N/A	**22.46**	N/A	**65.82**	N/A
36	100	**10.32**	N/A	**26.81**	N/A	**62.87**	N/A
48	100	**6.37**	N/A	**19.04**	N/A	**74.59**	N/A

Table 4.2

Summary of Cell Cycle Response to Quercetin in E7QPα, β and γ cells

E7QPα

Exposure Time (hours)	Quercetin [ ] μ M	G1 Phase Average		S Phase Average		G2/M Phase Average	
0	0	**59.92**	+/- 2.04	**24.91**	+/- 1.25	**15.18**	+/- 0.79
12	0	**57.17**	+/- 0.44	**25.60**	+/- 0.07	**17.24**	+/- 0.50
24	0	**60.08**	+/- 1.83	**24.64**	+/- 1.46	**15.28**	+/- 0.37
36	0	**58.42**	+/- 0.50	**29.28**	+/- 0.36	**12.32**	+/- 0.15
48	0	**61.75**	+/- 1.65	**25.69**	+/- 1.60	**12.56**	+/- 0.06
0	20	**59.45**	+/- 1.72	**24.17**	+/- 1.44	**16.39**	+/- 0.28
12	20	**56.78**	+/- 1.61	**24.78**	+/- 2.73	**18.45**	+/- 1.11
24	20	**32.97**	+/- 6.25	**31.27**	+/- 0.83	**35.76**	+/- 5.43
36	20	**27.29**	+/- 1.16	**27.11**	+/- 1.94	**45.61**	+/- 3.10
48	20	**27.19**	+/- 3.95	**23.78**	+/- 0.49	**49.04**	+/- 3.47
0	50	**59.26**	N/A	**22.84**	N/A	**17.90**	N/A
12	50	**53.81**	N/A	**28.20**	N/A	**17.99**	N/A
24	50	**32.75**	N/A	**33.89**	N/A	**33.36**	N/A
36	50	**20.88**	N/A	**31.96**	N/A	**47.17**	N/A
48	50	**22.79**	N/A	**23.86**	N/A	**53.35**	N/A
0	100	**58.39**	N/A	**26.20**	N/A	**15.42**	N/A
12	100	**47.38**	N/A	**30.26**	N/A	**22.35**	N/A
24	100	**31.84**	N/A	**28.94**	N/A	**39.22**	N/A
36	100	**21.30**	N/A	**33.25**	N/A	**45.45**	N/A
48	100	**20.42**	N/A	**25.17**	N/A	54.41	N/A

E7QPβ

Exposure Time (hours)	Quercetin [ ] μ M	G1 Phase Average		S Phase Average		G2/M Phase Average	
0	0	**53.4**	+/- 1.3	**14.4**	+/- 0.7	**32.3**	+/- 2.0
12	0	**52.7**	+/- 2.6	**14.6**	+/- 1.0	**32.7**	+/- 1.6
24	0	**51.8**	+/- 1.5	**14.2**	+/- 3.3	**34.1**	+/- 2.0
36	0	**50.4**	+/- 2.6	**14.6**	+/- 1.9	**35.1**	+/- 1.0
48	0	**48.5**	+/- 1.4	**16.2**	+/- 2.5	**35.4**	+/- 1.5
0	50	**52.6**	+/- 1.2	**14.1**	+/- 0.3	**33.3**	+/- 1.0
12	50	**49.3**	+/- 3.7	**16.9**	+/- 3.4	**33.8**	+/- 0.8
24	50	**42.3**	+/- 3.6	**20.5**	+/- 3.4	**37.2**	+/- 2.0
36	50	**34.0**	+/- 4.0	**19.5**	+/- 3.0	**46.5**	+/- 4.0
48	50	**34.1**	+/- 6.0	**17.1**	+/- 1.8	**48.8**	+/- 4.3

E7QPγ

Exposure Time (hours)	Quercetin [ ] μ M	G1 Phase Average		S Phase Average		G2/M Phase Average	
0	0	**36.1**	+/- 0.3	**8.4**	+/- 0.3	**55.6**	+/- 0.0
12	0	**34.4**	+/- 0.3	**8.4**	+/- 0.8	**57.1**	+/- 0.5
24	0	**35.5**	+/- 0.9	**7.6**	+/- 0.6	**57.0**	+/- 0.3
36	0	**37.4**	+/- 0.5	**8.2**	+/- 0.0	**54.4**	+/- 0.5
48	0	**39.3**	+/- 1.3	**7.7**	+/- 0.4	**52.9**	+/- 0.9
0	50	**35.4**	+/- 0.8	**8.2**	+/- 0.1	**56.4**	+/- 0.9
12	50	**35.7**	+/- 0.7	**4.6**	+/- 1.4	**59.8**	+/- 0.8
24	50	**33.5**	+/- 0.5	**7.6**	+/- 0.6	**58.9**	+/- 0.1
36	50	**29.0**	+/- 1.4	**10.0**	+/- 0.3	**61.0**	+/- 1.1
48	50	**33.2**	+/- 0.3	**10.4**	+/- 0.4	**56.4**	+/- 0.2

Table 4.3

Summary of Cell Cycle Response to Quercetin in E7R and 88529B Cells

E7R

Exposure	Quercetin [ ]	G1 Phase		S Phase		G2/M Phase	
Time (hours)	μ M	Average		Average		Average	
0	0	**62.6**	+/- 3.9	**13.0**	+/- 1.8	**24.4**	+/- 2.1
12	0	**69.7**	+/- 0.0	**9.8**	+/- 1.2	**20.4**	+/- 1.2
24	0	**69.4**	+/- 2.4	**8.0**	+/- 2.6	**22.6**	+/- 5.0
36	0	**70.2**	+/- 2.3	**5.9**	+/- 1.2	**23.9**	+/- 1.2
48	0	**70.3**	+/- 1.0	**11.8**	+/- 4.1	**18.0**	+/- 3.1
0	50	**65.1**	+/- 3.2	**15.0**	+/- 0.4	**19.9**	+/- 2.8
12	50	**72.7**	+/- 2.9	**7.0**	+/- 2.2	**20.4**	+/- 5.1
24	50	**68.8**	+/- 4.2	**16.3**	+/- 5.6	**14.9**	+/- 9.8
36	50	**67.4**	+/- 4.3	**14.0**	+/- 9.7	**18.7**	+/- 5.4
48	50	**36.6**	+/- 9.8	**21.0**	+/- 13.1	**42.4**	+/- 3.2

E7R (HP)

Exposure	Quercetin [ ]	G1 Phase		S Phase		G2/M Phase	
Time (hours)	μ M	Average		Average		Average	
0	0	**74.9**	+/- 5.3	**8.9**	+/- 2.0	**16.2**	+/- 3.9
12	0	**65.0**	+/- 8.7	**19.2**	+/- 7.4	**15.9**	+/- 3.0
24	0	**66.9**	+/- 4.3	**15.1**	+/- 4.0	**17.9**	+/- 2.5
36	0	**67.4**	+/- 3.6	**16.2**	+/- 6.8	**16.4**	+/- 3.7
48	0	**67.9**	+/- 4.0	**18.9**	+/- 10.3	**13.2**	+/- 7.2
0	50	**74.2**	+/- 4.8	**9.9**	+/- 3.7	**15.9**	+/- 2.0
12	50	**68.6**	+/- 6.8	**11.7**	+/- 6.3	**19.7**	+/- 9.7
24	50	**65.2**	+/- 6.8	**15.6**	+/- 5.9	**19.2**	+/- 6.5
36	50	**72.7**	+/- 3.9	**5.9**	+/- 3.8	**21.4**	+/- 1.7
48	50	**66.7**	+/- 4.0	**6.3**	+/- 4.1	**27.0**	+/- 2.5

88529B

Exposure	Quercetin [ ]	G1 Phase		S Phase		G2/M Phase	
Time (hours)	μ M	Average		Average		Average	
0	0	**43.8**	+/- 6.5	**43.5**	+/- 6.2	**12.7**	+/- 0.3
12	0	**42.9**	+/- 2.2	**44.6**	+/- 1.7	**12.5**	+/- 0.5
24	0	**42.3**	+/- 2.3	**46.1**	+/- 5.1	**11.5**	+/- 2.8
36	0	**38.8**	+/- 3.0	**50.5**	+/- 5.2	**10.7**	+/- 2.1
48	0	**42.3**	+/- 1.7	**46.9**	+/- 2.7	**10.9**	+/- 1.0
0	50	**47.3**	+/- 4.6	**36.7**	+/- 2.9	**16.0**	+/- 1.7
12	50	**22.7**	+/- 3.6	**71.8**	+/- 9.0	**5.4**	+/- 5.4
24	50	**42.7**	+/- 4.5	**50.1**	+/- 6.5	**7.2**	+/- 2.0
36	50	**35.6**	+/- 1.0	**30.2**	+/- 0.9	**34.2**	+/- 1.9
48	50	**28.4**	+/- 1.5	**40.6**	+/- 0.6	**31.4**	+/- 1.8

Table 4.4

Summary of Cell Cycle Response to Quercetin in Q0D and Q2D Cells

Q0D

Exposure Time (hours)	Quercetin [ ] μ M	G1 Phase Average		S Phase Average		G2/M Phase Average	
0	0	**61.67**	+/- 4.2	**16.74**	+/- 1.0	**21.61**	+/- 5.2
12	0	**55.30**	+/- 4.5	**22.83**	+/- 1.1	**21.88**	+/- 5.5
24	0	**59.08**	+/- 3.0	**19.59**	+/- 0.8	**21.33**	+/- 3.8
36	0	**59.89**	+/- 5.1	**17.08**	+/- 3.2	**23.04**	+/- 8.3
48	0	**60.99**	+/- 5.6	**16.14**	+/- 1.5	**22.88**	+/- 7.1
0	50	**63.01**	+/- 1.8	**17.64**	+/- 2.2	**19.31**	+/- 3.9
12	50	**57.11**	+/- 9.6	**8.36**	+/- 1.3	**34.54**	+/- 10.9
24	50	**58.42**	+/- 4.3	**11.06**	+/- 0.4	**30.53**	+/- 4.7
36	50	**49.03**	+/- 8.2	**9.38**	+/- 1.5	**41.59**	+/- 6.7
48	50	**35.16**	+/- 3.2	**5.81**	+/- 2.1	**59.04**	+/- 5.3

Q2D

Exposure Time (hours)	Quercetin [ ] μ M	G1 Phase Average		S Phase Average		G2/M Phase Average	
0	0	**54.23**	+/- 3.3	**33.29**	+/- 6.4	**12.49**	+/- 3.1
12	0	**53.42**	+/- 1.7	**32.15**	+/- 1.9	**14.45**	+/- 0.2
24	0	**51.94**	+/- 3.0	**34.05**	+/- 4.6	**14.02**	+/- 1.6
36	0	**51.07**	+/- 2.1	**34.99**	+/- 2.0	**13.94**	+/- 0.1
48	0	**50.08**	+/- 0.2	**38.38**	+/- 0.1	**11.55**	+/- 0.3
0	50	**55.69**	+/- 2.0	**34.35**	+/- 1.8	**9.97**	+/- 0.2
12	50	**57.03**	+/- 0.6	**20.31**	+/- 0.9	**22.67**	+/- 0.4
24	50	**54.92**	+/- 0.4	**22.05**	+/- 0.3	**23.04**	+/- 0.1
36	50	**41.43**	+/- 0.0	**33.65**	+/- 0.1	**24.93**	+/- 0.1
48	50	**30.73**	+/- 0.4	**28.62**	+/- 0.2	**40.66**	+/- 0.6

Table 4.5

Summary of Cell Cycle Response to Quercetin in E7OT1 and E7OT2 Cells

E7OT1

Exposure Time (hours)	Quercetin [ ] μ M	G1 Phase Average		S Phase Average		G2/M Phase Average	
0	0	**41.2**	+/- 0.8	**26.3**	+/- 1.8	**32.5**	+/- 1.0
12	0	**44.0**	+/- 1.8	**28.0**	+/- 0.1	**27.9**	+/- 1.9
24	0	**43.0**	+/- 3.0	**28.4**	+/- 0.2	**28.7**	+/- 2.8
36	0	**44.5**	+/- 1.5	**29.2**	+/- 0.6	**26.4**	+/- 0.9
48	0	**46.5**	+/- 4.5	**28.9**	+/- 1.1	**24.6**	+/- 3.3
0	20	**41.5**	+/- 0.9	**25.7**	+/- 0.7	**32.7**	+/- 1.6
12	20	**43.8**	+/- 6.7	**24.8**	+/- 4.4	**31.4**	+/- 2.3
24	20	**31.3**	+/- 4.3	**26.0**	+/- 0.2	**42.7**	+/- 4.0
36	20	**26.3**	+/- 8.7	**29.2**	+/- 0.5	**44.5**	+/- 8.2
48	20	**24.0**	+/- 5.9	**29.4**	+/- 4.4	**46.7**	+/- 1.5
0	50	**46.3**	N/A	**26.7**	N/A	**27.1**	N/A
12	50	**51.7**	N/A	**21.4**	N/A	**27.0**	N/A
24	50	**31.7**	N/A	**26.0**	N/A	**42.3**	N/A
36	50	**38.6**	N/A	**29.3**	N/A	**32.1**	N/A
48	50	**25.7**	N/A	**23.3**	N/A	**51.0**	N/A
0	100	**45.9**	N/A	**24.4**	N/A	**29.7**	N/A
12	100	**50.2**	N/A	**19.3**	N/A	**30.5**	N/A
24	100	**35.3**	N/A	**25.4**	N/A	**39.4**	N/A
36	100	**37.5**	N/A	**30.1**	N/A	**32.4**	N/A
48	100	**34.9**	N/A	**21.8**	N/A	**43.4**	N/A

E7OT2

Exposure Time (hours)	Quercetin [ ] μ M	G1 Phase Average		S Phase Average		G2/M Phase Average	
0	0	**40.6**	+/- 4.6	**23.9**	+/- 1.0	**35.5**	+/- 3.7
12	0	**39.3**	+/- 3.1	**25.6**	+/- 0.8	**35.1**	+/- 2.3
24	0	**39.3**	+/- 2.2	**27.5**	+/- 1.6	**33.2**	+/- 3.8
36	0	**45.2**	+/- 2.8	**28.5**	+/- 1.5	**26.3**	+/- 1.3
48	0	**41.9**	+/- 1.8	**27.6**	+/- 3.0	**30.6**	+/- 1.2
0	20	**41.1**	+/- 2.5	**24.3**	+/- 0.1	**34.6**	+/- 2.5
12	20	**48.6**	+/- 4.0	**19.9**	+/- 1.4	**31.5**	+/- 2.7
24	20	**32.1**	+/- 6.9	**30.7**	+/- 6.1	**37.2**	+/- 0.9
36	20	**15.7**	+/- 5.8	**32.5**	+/- 2.1	**51.8**	+/- 3.7
48	20	**9.3**	+/- 5.2	**25.4**	+/- 5.8	**65.3**	+/- 11.0
0	50	**43.1**	N/A	**24.0**	N/A	**32.9**	N/A
12	50	**47.6**	N/A	**17.8**	N/A	**34.7**	N/A
24	50	**24.2**	N/A	**39.1**	N/A	**36.7**	N/A
36	50	**10.2**	N/A	**38.6**	N/A	**51.2**	N/A
48	50	**13.1**	N/A	**27.0**	N/A	**59.9**	N/A
0	100	**45.8**	N/A	**19.3**	N/A	**34.9**	N/A
12	100	**43.3**	N/A	**19.0**	N/A	**37.7**	N/A
24	100	**24.6**	N/A	**32.7**	N/A	**42.7**	N/A
36	100	**10.3**	N/A	**43.1**	N/A	**46.6**	N/A
48	100	**4.7**	N/A	**22.2**	N/A	**73.1**	N/A

Table 4.6

Summary of Cell Cycle Response to Quercetin in E7QPT1 and E7QPT2 Cells

E7Q PT1

Exposure	Quercetin [ ]	G1 Phase		S Phase		G2/M Phase	
Time (hours)	μ M	Average		Average		Average	
0	0	**49.0**	+/- 0.0	**25.5**	+/- 2.8	**25.4**	+/- 2.8
12	0	**52.5**	+/- 1.7	**25.9**	+/- 0.8	**21.6**	+/- 2.5
24	0	**57.4**	+/- 1.5	**24.0**	+/- 1.0	**18.6**	+/- 2.6
36	0	**52.2**	+/- 0.6	**27.3**	+/- 0.6	**20.5**	+/- 0.1
48	0	**55.7**	+/- 1.0	**25.1**	+/- 0.3	**19.2**	+/- 0.7
0	20	**46.3**	+/- 0.0	**27.1**	+/- 2.6	**26.6**	+/- 2.6
12	20	**46.6**	+/- 12.1	**26.3**	+/- 3.0	**27.1**	+/- 9.2
24	20	**29.9**	+/- 6.8	**24.0**	+/- 0.5	**46.2**	+/- 6.3
36	20	**23.1**	+/- 3.2	**24.0**	+/- 0.5	**53.0**	+/- 2.7
48	20	**15.3**	+/- 2.3	**20.9**	+/- 0.6	**63.8**	+/- 1.7
0	50	**47.6**	N/A	**26.3**	N/A	**26.1**	N/A
12	50	**31.7**	N/A	**25.9**	N/A	**42.4**	N/A
24	50	**19.6**	N/A	**20.5**	N/A	**59.9**	N/A
36	50	**16.5**	N/A	**19.6**	N/A	**64.0**	N/A
48	50	**10.6**	N/A	**23.0**	N/A	**66.4**	N/A
0	100	**44.6**	N/A	**21.9**	N/A	**33.5**	N/A
12	100	**34.2**	N/A	**25.7**	N/A	**40.1**	N/A
24	100	**21.8**	N/A	**22.7**	N/A	**55.5**	N/A
36	100	**14.7**	N/A	**21.6**	N/A	**63.8**	N/A
48	100	**15.6**	N/A	**21.8**	N/A	**62.6**	N/A

E7Q PT2

Exposure	Quercetin [ ]	G1 Phase		S Phase		G2/M Phase	
Time (hours)	μ M	Average		Average		Average	
0	0	**48.6**	+/- 3.1	**29.7**	+/- 3.4	**21.7**	+/- 0.3
12	0	**53.2**	+/- 2.5	**28.7**	+/- 0.4	**18.1**	+/- 2.9
24	0	**49.1**	+/- 1.2	**30.9**	+/- 0.7	**20.0**	+/- 0.4
36	0	**49.3**	+/- 1.0	**32.8**	+/- 0.9	**18.0**	+/- 0.1
48	0	**51.0**	+/- 0.1	**30.4**	+/- 0.3	**18.7**	+/- 0.2
0	20	**52.7**	+/- 1.2	**28.0**	+/- 2.6	**19.4**	+/- 1.3
12	20	**46.2**	+/- 6.1	**30.9**	+/- 6.8	**23.0**	+/- 0.8
24	20	**29.4**	+/- 1.3	**33.0**	+/- 2.2	**37.6**	+/- 1.0
36	20	**18.0**	+/- 1.4	**26.8**	+/- 2.0	**55.2**	+/- 0.6
48	20	**16.4**	+/- 1.4	**25.6**	+/- 0.1	**58.1**	+/- 1.4
0	50	**47.7**	N/A	**31.1**	N/A	**21.2**	N/A
12	50	**42.6**	N/A	**33.4**	N/A	**24.0**	N/A
24	50	**21.9**	N/A	**34.6**	N/A	**43.5**	N/A
36	50	**16.5**	N/A	**28.4**	N/A	**55.1**	N/A
48	50	**14.7**	N/A	**26.7**	N/A	**58.5**	N/A
0	100	**45.9**	N/A	**31.1**	N/A	**21.6**	N/A
12	100	**55.7**	N/A	**33.4**	N/A	**26.4**	N/A
24	100	**42.9**	N/A	**34.6**	N/A	**34.7**	N/A
36	100	**38.4**	N/A	**28.4**	N/A	**29.5**	N/A
48	100	**39.4**	N/A	**26.7**	N/A	**37.8**	N/A

stimulate passage into S phase. Higher passage cultures could have selected against expression of the proteins and thus have insufficient levels to create a strong enough proliferative drive into DNA synthesis. This selection may be through activation of the bovine homologue of $p19^{ARF}$ (see chapter six for more details) Alternatively counteracting cellular proteins, such as the cdk inhibitors for example, may be elevated in response to E7 and Ha-ras. This would then balance the action of the oncoproteins. Possible requirements of other viral proteins to eliminate this selection/counter-action would explain why this process does not occur in the cell line Q0D.

Analysis of the cell lines E7QPβ and E7QPγ was also undertaken. E7QPβ displayed loss of G1 arrest with a drop in G1 and increase in G2/M populations (see figure 11 and table 4.2). Note though this cell line did not display a significant drop in S phase. Again this maybe due to the balance of the oncogene expression in the cell lines, with those that displayed high levels of Ha-ras protein and the strongest E7 RT-PCR product levels being able to maintain a significant population undergoing DNA synthesis.

E7QPγ displayed two unusual features. Firstly under normal passage conditions it maintained a large G2/M population in relation to wild type PalF cells, indeed any of the other cell lines (see figure 11 and table 4.2). Additionally under quercetin exposure, no significant shift in cell cycle population distribution was observed. Likely explanations for this lack of shift in cell cycle profile could be that there is no cell cycle arrest in these cells. This is unlikely as none of the other cell lines expressing E7 and Ha-ras were capable of overcoming the G2/M block. Inheritance of a genetic alteration allowing inhibition of the G2/M block is not unfeasible and cannot be ruled out as a possible explanation. Another hypothesis would stem from the observation that the base cell cycle profile resembles the other cell lines once arrested i.e. low G1 and S phase and high G2/M. This would indicate that during the period of experimentation these cells were undergoing a cellular crisis and were already in an arrested state. Hence quercetin treatment would have no effect. One argument against this point was the fact that the cells were tested over a period of three weeks in which time they were able to be passaged normally indicating no state of arrest. The final explanation is that this is the normal distribution of cycling E7QPγ cells. The low G1 and S

phase may well reflect the cells rapid passage through these stages due to the oncogene expression. Again further analysis with techniques suitable to a dynamic view of the cell cycle would help elucidate these issues.

In addition to E7QT2, other cell lines derived from explanted tumors were also examined for loss of G1 arrest. When exposed to quercetin E7QT1, E7QPT1 and E7QPT2 all demonstrated the loss of G1 arrest, and the maintenance of the G2/M arrest (see tables 4.5 & 4.6).

The study of the transforming papilloma cell line, 88529B, continued the demonstration of cells transformed by BPV-4 to bypass G1 arrest. As figure 11 shows there is a characteristic drop in the percentage of cells in G1 and an increase in the number of cells in G2/M, with only a small alteration in the S phase population. As an additional note, examination of table 4.3 also demonstrates this cell line exhibits a prominent elevation in S phase, 12 hours after initiation of quercetin exposure. This would significantly increase the number of cells exposed to quercetin undergoing DNA synthesis. It would be interesting to examine whether this would also be a feature of primary keratinocytes.

Overall in summary it can be seen that the loss of the G1 arrest in response to quercetin exposure does not automatically indicate tumorigenic status of the cell. Although the abrogation of the G1 arrest may be necessary for the full transformation of the cell, indicated by the fact that all the tumorigenic cell lines tested in the study have lost the G1 arrest in response to quercetin, it is not sufficient to confer tumorigenic status. This latter point is proven by the ability of the transformed non-tumorigenic cell lines to bypass the G1 arrest. Also the ability to bypass the G2/M arrest is not required for tumorigenic status as none of the tumorigenic cell lines were able to bypass this arrest.

The ability to overcome the G1 arrest requires the expression of BPV 4 E7 and activated Ha-ras as far as can be determined by this series of studies. Whether E7 or Ha-ras alone could overcome the arrest is unknown. Although the fact that no clones expressing only E7, or an activated Ha-ras, were able to be isolated and outgrown, let alone be tumorigenic in combination with quercetin treatment, suggests that these oncogenes are insufficient to abrogate the arrest in isolation.

Examination of a pooled population of cells expressing either gene in isolation would help determine this question, if such a population could be established.

4.2 Reversibility of the Quercetin Induced Cell Cycle Arrest

Based on the hypothesis that the abrogation of the quercetin induced arrest of the cell is pivotal to the progression of the cell to full transformation investigation into the nature of the cell cycle arrest was required.

To address this question PalF cells or E7QT2 cells were treated with quercetin for 48 hours and then maintained in normal DMEM-10 medium. At 24 hours intervals cells were harvested, fixed in ethanol and stained with propidium iodide. Cell cycle distributions were then examined on the FACS machine as per cell cycle analysis as described in section 2.2.2.13.

4.2.1 Experimental Method

PalF or E7QT2 cells were exposed to 0μM or 50μM quercetin for 48 hours as per cell cycle analysis protocols (section 2.2.2.13). Cells were then maintained in normal DMEM-10 medium for up to a further 96 hours post arrest with fresh medium being applied every 12 hours. In addition quercetin treated cell samples were also maintained in 0μM or 50μM quercetin for up to a further 96 hours to determine cell cycle profiles under prolonged exposure to quercetin.

Cells were harvested every 24 hours post arrest, by trypsinization, and fixed in 70% EtOH at 4°C for at least 1 hour. Samples were then stained with propidium iodide and cell cycle distribution determined by analysis on the FACS machine.

Cell Cycle Analysis of Cells released from Quercetin Induced Arrest

PalF Cells

E7QT2 Cells

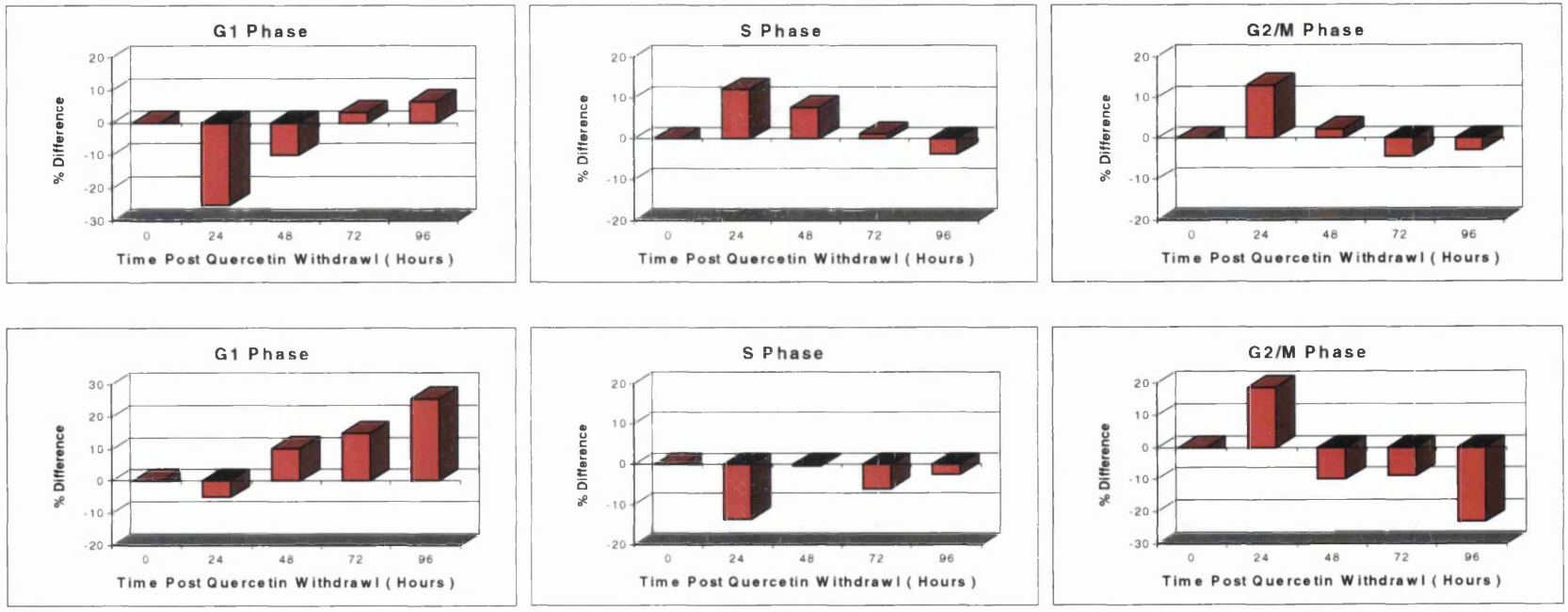


Figure 13. ***Cell cycle profiles of normal and transformed PalF cells released from quercetin induced arrest***

Cells grown in 50μM quercetin for 48 hours then placed in normal DMEM-10 for up to 96 hours were analysed by FACS. The histograms represent percentage of cells in the G1, S or G2/M phases of the cell cycle.

Table 4.7 Cell Cycle Analysis of Cells released from Quercetin Induced Arrest

Time Post Arrest (hours)	Media Applied	PalF % in G1 / G0	PalF % in S	PalF % in G2 /M	E7QT2 % in G1 / G0	E7QT2 % in S	E7QT2 % in G2 /M
0	DMEM-10	60.14	12.58	27.28	7.16	24.76	68.08
24	DMEM-10	35	24.71	40.29	2.12	11.12	86.76
48	DMEM-10	50.25	20.28	29.47	17.37	24.36	58.27
72	DMEM-10	63.38	13.85	22.77	22.08	18.83	59.09
96	DMEM-10	66.81	8.78	24.4	32.88	22.15	44.97
0	0Q	59.37	10.35	30.28	9.07	25.97	64.96
24	0Q	37.92	25.44	36.64	4.34	8.45	87.2
48	0Q	53.5	17.52	28.99	21.07	16.51	62.42
72	0Q	62.46	12.51	25.03	28.94	24.07	47
96	0Q	69.73	11.74	18.53	34.02	26.28	39.7
0	50Q	60.9	11.67	27.43	8.32	26.41	65.27
24	50Q	57.23	9.01	33.76	2.71	12.18	85.11
48	50Q	57.27	9.22	33.5	2.11	8.39	89.5
72	50Q	63.47	7.59	28.94	1.32	5.63	93.05
96	50Q	59.44	10.16	30.4	2.92	0	97.08
0#	DMEM-10	63.52	23.18	13.3	45.9	26.88	27.22
24#	DMEM-10	75.04	14.01	10.95	50.07	27.89	22.04
48#	DMEM-10	82.93	10.23	6.84	54.49	25.78	19.73
72#	DMEM-10	86.11	7.47	6.42	54.08	24.63	21.3
96#	DMEM-10	85.6	7.3	7.1	52.25	30.97	16.78

= Cells maintained throughout experiment in DMEM-10 medium and not undergone quercetin induced arrest.

4.2.2 Experimental Results and Discussion

Upon removal of quercetin both cell lines rapidly re-entered the cell cycle. Figure 13 demonstrates that in PalF cells, that had undergone G1 and G2/M arrest, within 24 hours of quercetin withdrawal there was a large decrease in the percentage of cells in the G1 phase of the cell cycle. There is of course a concomitant increase in the percentage of cells in S and G2/M phase. This indicates a rapid release from the G1 arrest in these cells and the movement of the released cells through to G2/M. The evidence of the reversibility of the quercetin induced G2/M arrest comes from the fact that from the time points 24 hours to 72 hours the G2/M population decreases, indicating cells moving out of the G2/M phase back into cell cycle. Thus for the PalF cells arrest induced by quercetin is rapidly reversible.

Examination of the cell line E7QT2 indicates also the reversible nature of the G2/M arrest. Figure 13 shows that after 24 hours of quercetin withdrawal this cell line actually continues to arrest, with a further decrease in G1 and S phase as the cells empty into the G2/M phase of the cell cycle. However 48 hours post withdrawal re-entry into the cell cycle initiates as the percentage of cells in G2/M decrease and cells re-enter G1. This indicates that also in this cell line the arrest is reversible.

4.3 Analysis of the NIH3T3 cell line as a Possible Alternative Cell Line

4.3.1 Cell Cycle Analysis

Due to the shortage of suitable antibodies available against bovine antigens , and the possibility of analysis of E7 only expressing cell lines, an investigation was carried out to determine whether another cell line could be used for analysis.

As an initial choice, due to immediate availability of both parental and stable cell lines expressing BPV-4 oncogenes, NIH3T3 cells were examined.

Following from previous work displaying the ability of quercetin to induce a G1 and G2/M cell cycle arrest in PalF cells, it was decided, as an initial parameter, to examine whether the NIH3T3 cell line would behave in a similar manner. This feature would be important to ensure that the mechanisms through

which quercetin would be acting in the primary bovine fibroblasts would also likely elicit the same responses in the murine cell line.

4.3.2 Experimental Method

The essential details of the FACS analysis are in section 2.2.2.13. Briefly either PalF or NIH3T3 Zipneo (NIH3T3 cells stably transfected with pZipneo) cells were plated in 90mm culture dishes and treated with either 0, 20, 50 or 100μM Quercetin over 48 hours with fresh medium being applied every 12 hours. Cells were then harvest and fixed using EtOH. Fixed cells were then stained using propidium iodide and RNAase A (the latter to remove any staining of RNA which would cause errors in the resultant FACS analysis) to stain the DNA. The stained samples were then analysed on the FACS scan to determine the percentage of the cells in each of the phases of the cell cycle.

4.3.3 Experimental Results and Discussion

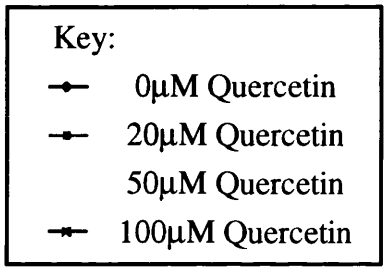
Upon treatment with quercetin PalF cells arrest in G1 and G2/M phases of the cell cycle. This can be observed in Figure 14, by the stable G1 percentage upon quercetin exposure occurring with a concomitant decrease in S phase population and an increase in the G2/M population. Exposure to increasing levels of quercetin resulted in a more prominent shift in the populations in each phases of the cell cycle e.g. 20μM quercetin for 48 hours results in a decrease of the cells in S phase from 23.55% to 11.58%, however 100μM quercetin for 48 hours causes a decrease from 31.37% to 2.52%, displaying a dose dependent effect on the cell cycle distribution of the PalF cells.

Examination of the cell cycle profiles of the NIH3T3 Zipneo cells, under quercetin exposure, showed a different response. Quercetin exposure caused an arrest in G2/M evidenced by the increasing percentage of G2/M, but failed to cause a G1 arrest. Figure 14 shows that even after 48 hours of quercetin treatment at 50 or 100μM NIH3T3 Zipneo cells showed little decrease in the S phase percentage. Additionally to this they also showed evidence of a decrease in the G1 population rather than the stable population seen in PalF cells, suggesting that cells were moving out of G1 and S phase and accumulating only in G2/M.

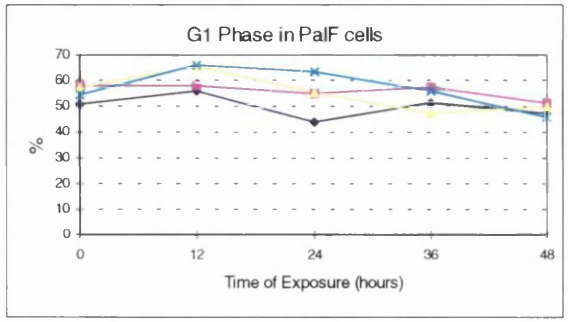
Figure 14 (facing page). ***Cell cycle profiles of normal and transformed PalF cells***

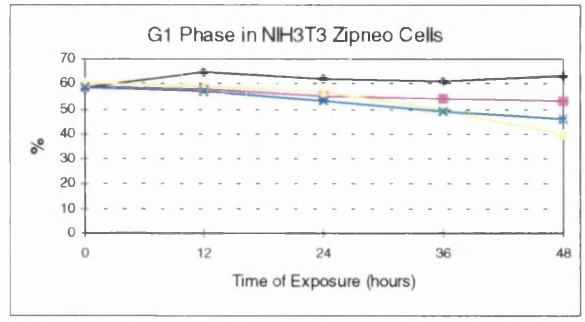
PalF or NIH3T3 cells grown in 20-100μM quercetin or equivalent volume of EtOH for 48 hours were analysed by FACS.

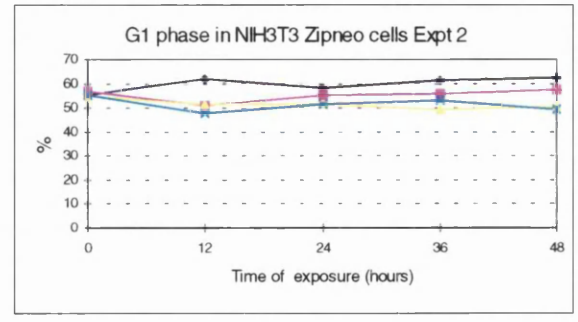
The lines represent percentage of cells in the G1, S or G2/M phases of the cell cycle.

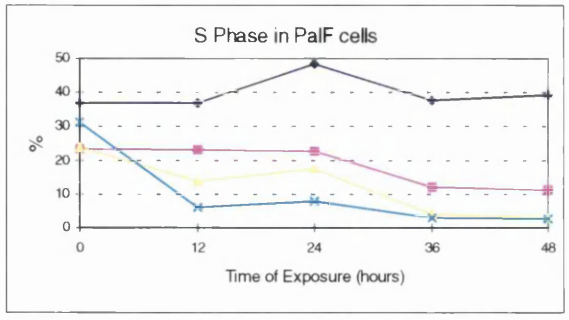


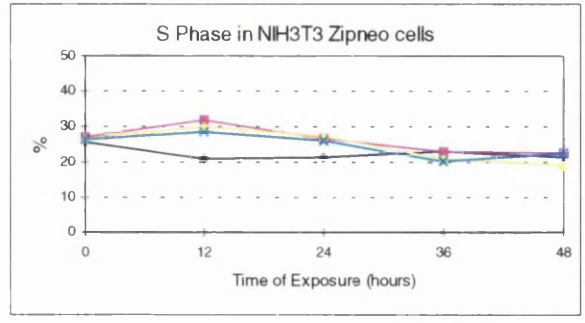
Cell Cycle Response to Quercetin Exposure in PalF and NIH3T3 Zipneo Cells

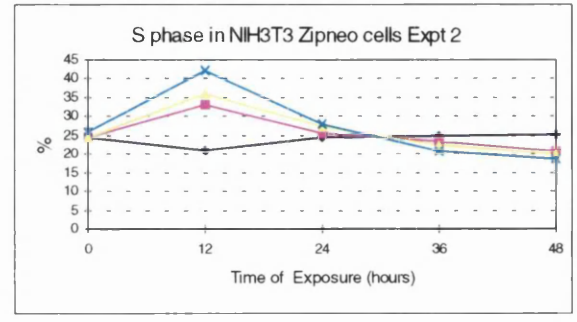


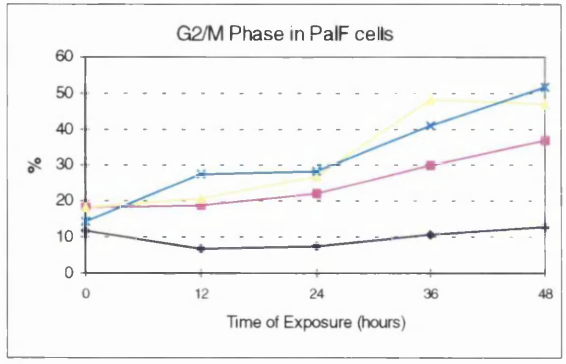


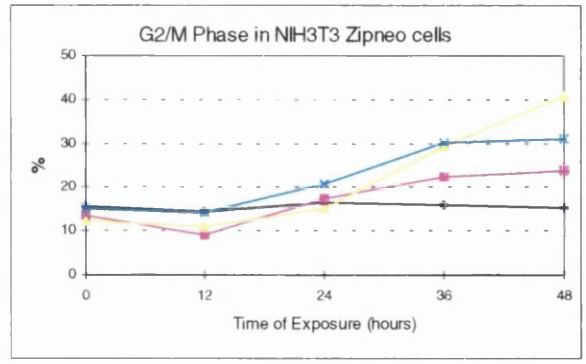


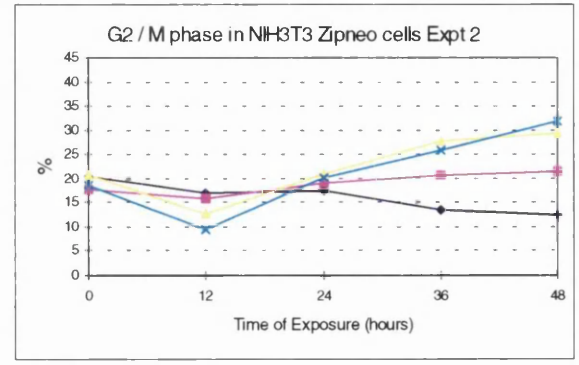












This lack of G1 arrest in response to quercetin exposure in the NIH3T3 cells meant this cell line was not a suitable alternative for study.

4.4 Analysis of Transient BPV-4 E7 Transfectants

To attempt to determine wheter BPV 4 E7 expression alone could cause abrogation of the quercetin induced arrest, transient transfections of BPV-4 E7 were examined.

Ideally a study of a transfected population in which all cells expressed the protein of interest would be desirable. This would commonly be attained through use of retroviral vectors which are capable of very high transfection rates, or by the generation of stable transfected cell lines.

Unfortunately both of these techniques at the time of study were unsuitable for use. The retroviral system of transfection had been established but required extensive initial set up time, and as detailed earlier cell lines expressing only E7 were unable to be cultured *in vitro*.

Hence in order to tackle the problem of examining E7-only transfectants a system of tagging was used.

4.4.1 Experimental Method

See section 2.2.2.14. In brief, $1x10^6$ PalF cells were transfected with a combination of 5μg of pZipneo E7 with 20μg of the transfectant tag plasmid pCMVCD20, or as a control 4μg of pZipneo with the pCMVCD20 plasmid. This plasmid expresses a cell surface marker, the CD20 antigen, which is used to identify the transfected population from the background untransfected cells.

In these cases DOTAP transfection was substituted with the lipofection reagent Superfect™ (Qiagen) but otherwise was carried out essentially as in section 2.2.2.6. Transfected cells were then treated with either 0μM or 50μM quercetin over 36 hours with fresh medium every 12 hours, 24 hours post transfection.

Cell were then harvested by trypsination and probed with the primary FITC tagged antibody that recognises the CD20 epitope. Once unbound antibody was washed away cells were then stained with propidium iodide.

Samples were analysed in the FACS machine and cells expressing FITC florescence were gated, and propidium iodide staining of DNA content measured in this population to determine cell cycle distribution.

4.4.2 Experimental Results and Discussion

Transient transfection levels were obtained using the Superfect™ reagent by examination of cells transfected with a green fluorescent protein (GFP) expression plasmid, pEGFPN1, under the florescent microscope. This allowed quick and easy determination of the approximate number of transfected cells in the population Florescence was assayed 48 hours after transfection to allow expression of the GFP as this would be close to the time of harvesting of cells in the actual experiments. Roughly 10% of the population was transfected at this point, indicating a good efficiency of transfection.

Positive control experiments were carried out to ensure the ability of the system to work, by using a cell line which constitutively expresses the CD20 antigen (Raji cells). The antibody bound to the cells and induced a shift in the FITC florescence profile of the cell population in comparison to control treated populations (data not shown).

Despite greater than the requisite 5000 E7-only transfected cells being collected and assayed, background noise made meaningful determination of the cell cycle profile of these cells difficult. Overall this meant an inability to clarify any change in cell cycle behaviour in the transfected population in comparison to the parental PalF cells with this technique at present.

CHAPTER FIVE

RESULTS

5.1 Analysis of p53 Protein in Cell Lines

Understanding the nature of the cell cycle arrest in the PalF cells would be invaluable in the study of the transformation process by BPV-4 E7 and Ha-ras.

As quercetin induced a G1 and G2/M arrest in the PalF cells, yet only a G2/M arrest in the transformed cell lines, the determination of the mechanism of the G1 arrest was paramount. It has been shown by a wealth of literature that p53, the so called 'guardian of the genome', is involved in mediating cell cycle arrest in G1 (for reviews see Ko & Prives, 1996; Agarwal *et al.,* 1998; Giacca & Kastan, 1998), in response to many stimuli, and indeed the involvement of p53 in the G2/M arrest is also likely (Di Leonardo *et al.,* 1997) and is the subject of much study.

A good initial candidate for the cell cycle regulator of the quercetin induced cell cycle arrest was the p53 protein. At the time of the investigation there was not an antibody that was known to react against the bovine p53. However as bovine p53 cDNA had been sequenced, the amino acid sequence was derived and comparison of the bovine protein to the human protein was undertaken. This analysis showed several regions of complete homology between the human and bovine amino acid sequences, mainly in the conserved regions of the DNA binding domains, in addition to a region in the N terminus. This region also corresponded to the epitope of a commercially available antibody, Bp53.12, which recognises residues 18-30 in human p53 (see figure 15) and the antibody was suitable for western blotting.

Most other commercially available antibodies were unsuitable due to poor sequence homology between bovine and human p53 protein sequences in the corresponding epitope regions. However two other antibodies were also tested against bovine p53 due to promising sequence homology in the recognised epitopes. These were clone PAB240 (residues 213-217) and clone DO-1 (residues

21-25). However both of these antibodies failed to cross react with bovine p53 on western blot membranes.

The Bp53.12 antibody was successful in detecting bovine p53 in western blots even though it overlapped the region recognised by clone DO-1. The DO-1 antibody failed presumably due to the single amino acid alteration between bovine and human p53 in the epitope. Bp53.12 may well have succeeded in binding to the region as it binds a larger epitope in which homology between human and bovine p53 is maintained. This fact would then permit strong enough binding despite the single amino acid difference between the two species.

5.1.1 Experimental Method

Cell lines were treated with various concentrations of quercetin for 36 hours with fresh medium being used every 12 hours as per the treatment protocol for cell cycle assays (section 4.1.1). This was in order to allow comparison between the cell cycle studies and the protein levels investigated. In addition to quercetin treatments, control treatments of UV exposure ($120J/m^2$ 24 hours before harvesting) or in some instances bleomycin sulphate (15μU for 6 hours before harvesting) were used to induce p53 in the cell lines. Additional samples from untreated cells were also taken.

Protein samples were then derived from these treated cell lines, and protein concentration estimated. 80μg of total cell lysate was used per well, ran out on an SDS-PAGE gel and blotted on to nitrocellulose membrane. The membrane was then probed using Bp53.12 and a HRP-linked secondary antibody. In order to double check loading, a control probing for actin levels was carried out, by stripping the blot and re probing with the anti actin antibody (see section 2.2.4 for full details).

5.1.2 Experimental Results and Discussion

As figure 16 indicates the exposure of PalF cells to quercetin induces an increase in the p53 level. Additionally exposure to UV irradiation also causes an increase in p53 protein levels. This figure also displays that in E7Q cells p53 protein levels increase upon exposure to quercetin or UV. The descendent of E7Q, E7QT2, shows an elevation of p53 in response to UV but not to quercetin This

Alignment of p53 protein Sequence (a.a 1-39)

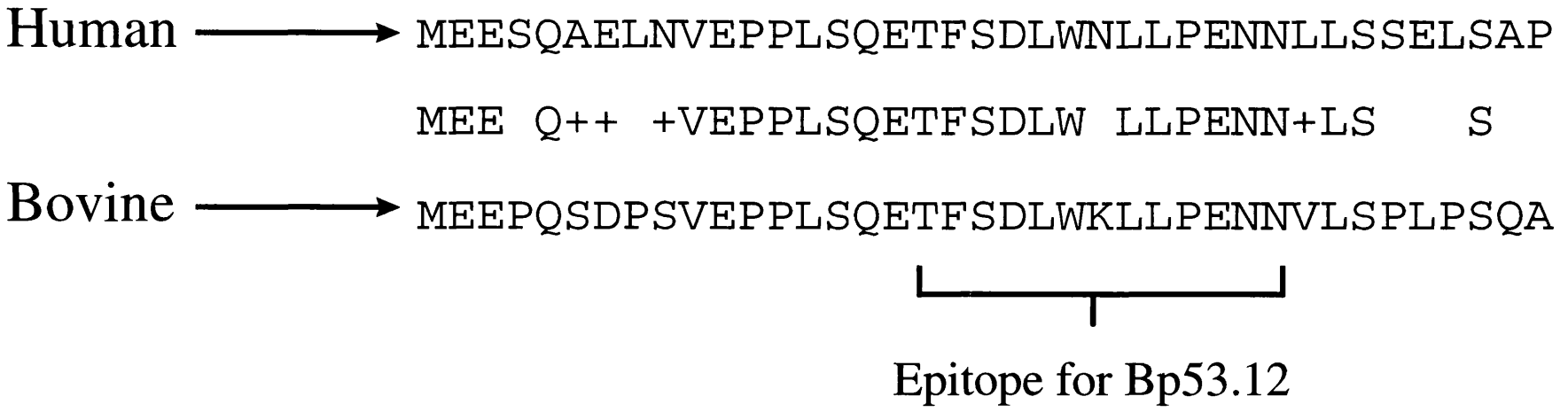


Figure 15. ***Alignment of First 39 Amino Acids of Human and Bovine p53***
Comparison of human and bovine p53 protein sequences carried out in order to determine regions of homology which also corresponded to regions recognised by commercially available antibodies.
Epitope of the antibody Bp53.12 is indicated by the sequence covered by the bar gate
+ = Conservative amino acid changes

may be due to the high levels of p53 in the absence of quercetin. Importantly however it is evident that the E7Q cell line not only retains expression of p53 but also retains responsiveness to DNA damaging agents.

One way to examine whether the p53 protein was functioning normally in the tumorigenic cells was to assay the expression of $p21^{Waf1/Cip1}$, a known target of p53 transcriptional activation (El-Deiry et al., 1993).

Reprobing the blot with a $p21^{Waf1/Cip1}$ reactive antibody shows differences between the cell lines. As can been seen in figure 16, $p21^{Waf1/Cip1}$ is elevated in PalF cells in response to quercetin or UV as one would expect from an elevation of p53 in these cells, thus reinforcing the hypothesis that quercetin induces the cell cycle arrest via p53 and $p21^{Waf1/Cip1}$. Figure 16 also shows no $p21^{Waf1/Cip1}$ staining in the E7Q and E7QT2 sample lanes. This indicates that there may be dysfunction in the p53 up-regulation of $p21^{Waf1/Cip1}$.

In E7R, E7QPβ, E7QPγ and Q0D p53 is elevated in response to quercetin and thus is p21(figures 17 and 18). E7QPα and Q2D show variation from the wild type response. E7QPα shows a response to UV and bleomycin sulphate in p53 protein levels indicating functional response pathways/mechanisms and expression of the protein. However no visible difference is observed with quercetin treatments in comparison to ethanol treated cells. This may due to the high background of the blot rather than a dysfunctional response due to the protein level elevation to the other DNA damaging agents. As in E7Q no p21 protein is present in these cells as detected by western blot analysis, thus although present the p53 protein seems incapable of inducing expression of the p21 protein.

Q2D provided further evidence of dysregulation in the p53 response pathway. As seen in figure 18, Q2D displays a constitutive overexpression of p53, and no response to DNA damaging agents such as quercetin, UV or bleomycin sulphate. However despite high levels of the p53 protein in these cells there was no evidence of $p21^{Waf1/Cip1}$ expression.

The cell line 88529B although assayed for p53 protein levels in response to UV exposure or quercetin exposure, failed to generate a signal with the Bp53.12 antibody. Note though due to low protein concentrations of the cell lysates derived for the western blot analysis only 60μg rather than 80μg of total cell lysate was loaded per well. This may be in part an explanation however some

detection of p53 protein levels should have been visible at this loading level. Thus at this point no information is known about p53 response to quercetin or UV exposure in this cell line, apart from a presumed low level of expression.

In summary the non tumorigenic transformed cell lines display a normal p53 protein level elevation in response to quercetin. However the tumorigenic cell lines although showing expression of the p53 protein show no expression of the $p21^{Waf1/Cip1}$ protein, the inhibitor of the cdk2 protein kinase activity.

5.2 Analysis of p53 Transcriptional Activity in PalF

Following on from the observations obtained from the western blot analysis, investigations into determining the status of p53 function in the cell lines was started. Reporter assay systems were used to elucidate whether the loss of p21 expression in the tumorigenic cell lines was likely due to loss of p53 transcriptional function, indicating that although p53 may be present it was inhibited as a transcriptional activator.

The main reporter consttruct used in the analysis of the p53 protein was pRGCfosLuc. This contains two p53 consensus binding sites derived from the ribosomal gene cluster promoter upstream of a minimal murine c-fos promoter. This was linked to a firefly luciferase reporter gene. A control reporter, pfosLuc was the same as pRGCfosLuc with the exception that it lacked the two p53 binding sites. Any activity difference between the two plasmids in a reporter assay could be attributed to the binding of p53 to the DNA binding site and induced transcription of the reporter gene.

In addition to this system two other reporters were used on a more limited scale. These were WWP-Luc and pGL2NA(mdm2). The former links the human $p21^{Waf1/Cip1}$ gene promoter to the firefly luciferase gene, and the latter uses the human Mdm2 gene promoter, another known target of p53 (Barak *et al.,* 1993) linked to the firefly luciferase gene. These two additional reporter systems allowed examination of a more 'natural' target of p53 binding and transcriptional activation.

As with the FACS and western blot analysis cells containing the reporters were treated with quercetin and/or UV in order to ascertain the response of the p53 protein to these agents.

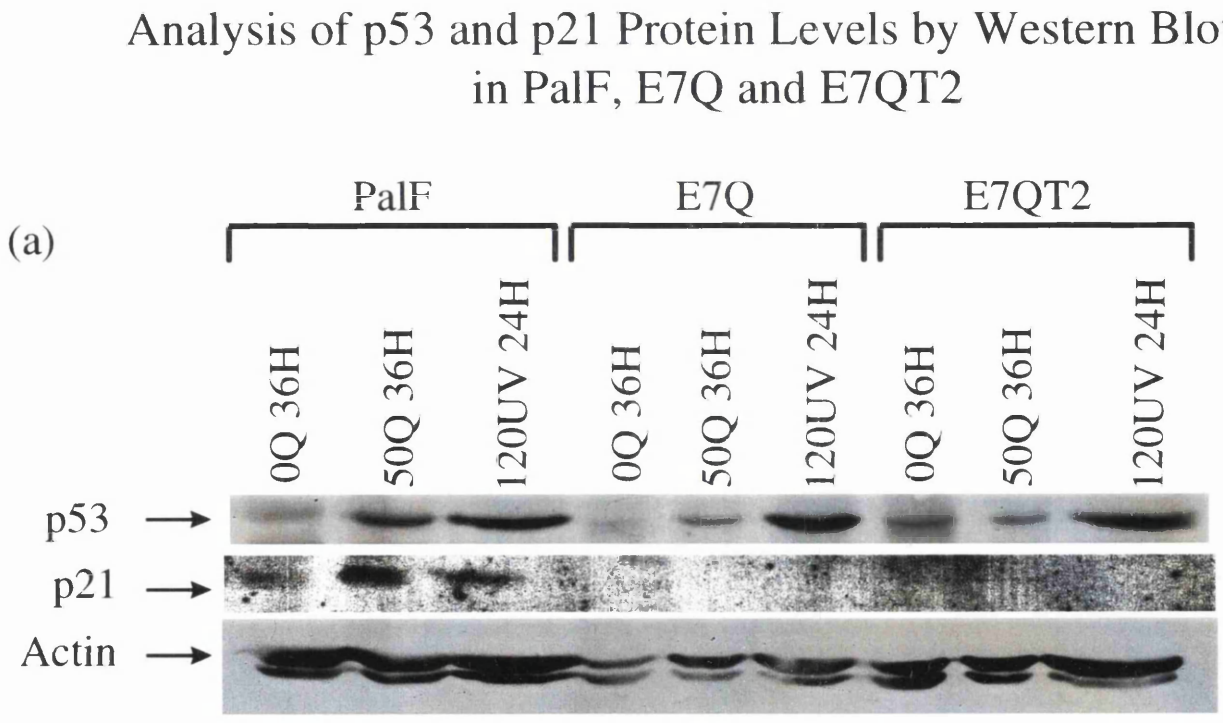


Figure 16. ***p53 protein is stabilised in cells exposed to DNA damaging stimuli***

Cells were treated with quercetin at 50µM or 100µM or equivalent volume of EtOH for 36 hours. Where indicated cells were treated with 120J/m^2 of UV and harvested 24 hours later, or bleomycin sulphate at 15µU for 6 hours and harvested immediately after. Membranes were probed for p53 (top row), p21 (middle row) and actin as a loading control (bottom row).

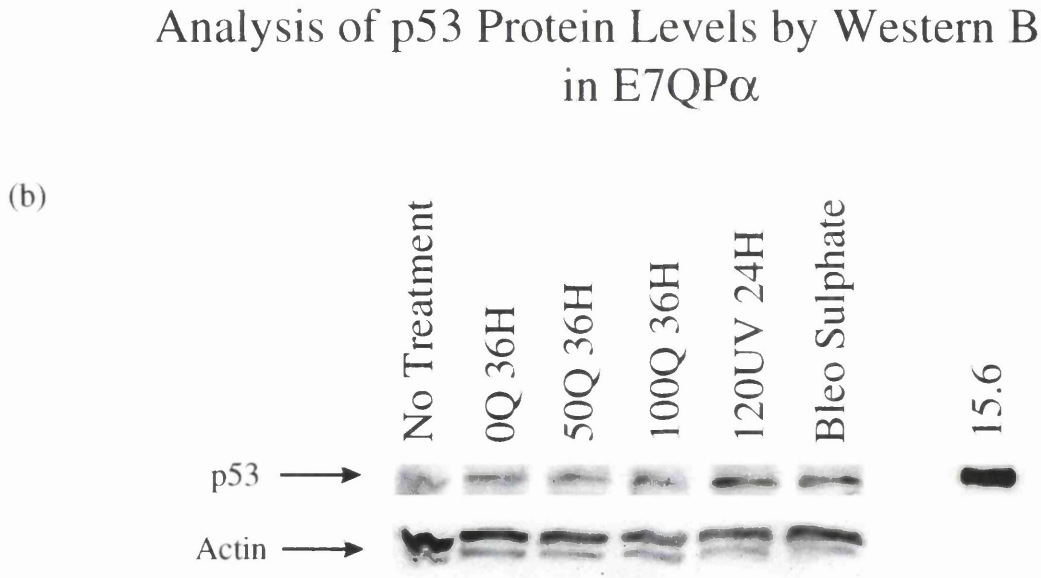


Figure 16. ***p53 protein is stabilised in cells exposed to DNA damaging stimuli***

E7QPα cells were treated with quercetin at 50μM or 100μM or equivalent volume of EtOH for 36 hours. Where indicated cells were treated with 120J/m^2 of UV and harvested 24 hours later, or bleomycin sulphate at 15μU for 6 hours and harvested immediately after. Membranes were probed for p53 (top row) and actin as a loading control (bottom row). 15.6 used as a positive control (expresses mutant human p53) (p21 data not shown for E7QPα as protein was undetected in blot)

Analysis of p53 and p21 Protein Levels by Western Blotting inE7QPβ, E7QPγ and E7R

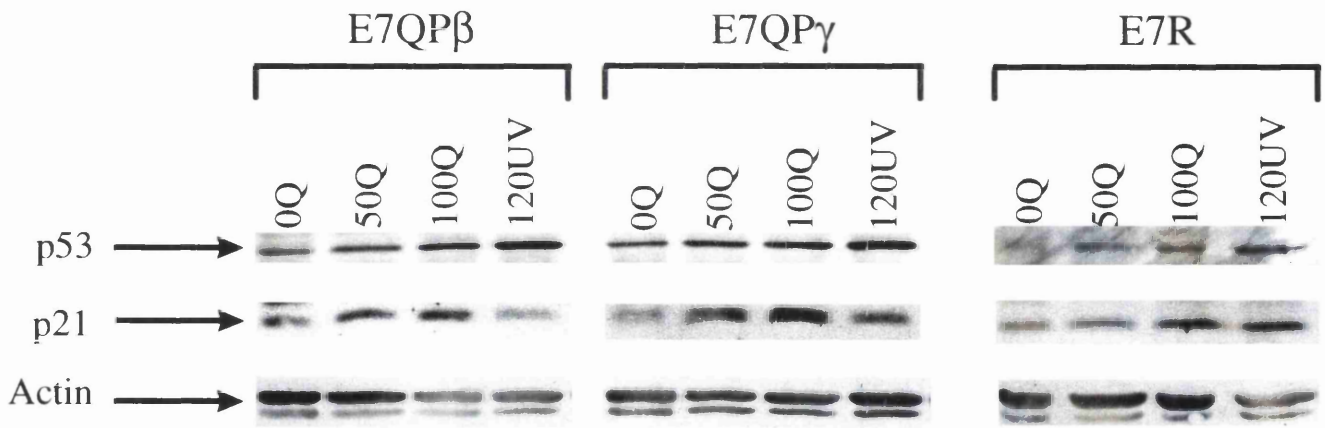


Figure 17. ***p53 protein is stabilised in cells exposed to DNA damaging stimuli***

Cells were treated with quercetin at 50μM or 100μM or equivalent volume of EtOH for 36 hours. Where indicated cells were treated with 120J/m^2 of UV and harvested 24 hours later, or bleomycin sulphate at 15μU for 6 hours and harvested immediately after. Membranes were probed for p53 (top row), p21 (middle row) and actin as a loading control (bottom row)

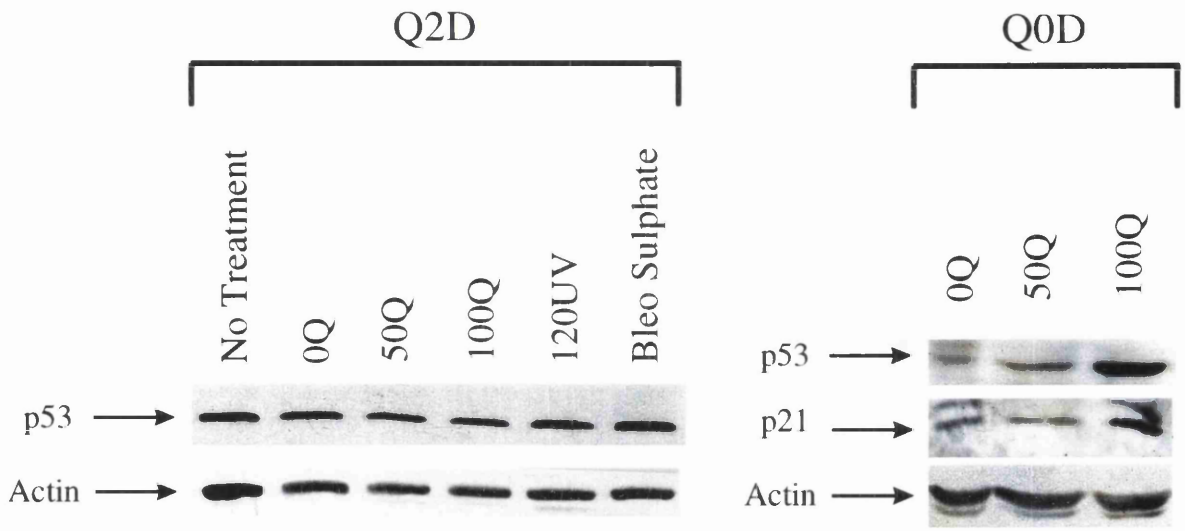


Figure 18. ***p53 protein is stabilised in Q0D cells exposed to DNA damaging stimuli,and constitutively stabilised in Q2D***
Cells were treated with quercetin at 50μM or 100μM or equivalent volume of EtOH for 36 hours. Where indicated cells were treated with 120J/m^2 of UV and harvested 24 hours later, or bleomycin sulphate at 15μU for 6 hours and harvested immediately after. Membranes were probed for p53 (top row), p21 (middle row in Q0D) and actin as a loading control (bottom row). p21 data not shown for Q2D as protein was undetected in blot

5.2.1 Experimental Method

PalF cells were transfected with 4μg of the pRGCfosLuc reporter, or pFosLuc control, as described in section 2.2.2.6. The transfected cells were then treated with quercetin (0, 20,50 or 100μM) initiating 8 hours post transfection and lasting for 16 hours before harvesting. Control treatment of UV exposure was also carried out 16 hours pre-harvesting with an exposure of $50J/m^2$.

Cells were then harvested in the luciferase lysis buffer (Promega) and asssayed for luciferase activity as described in section 2.2.2.7, and normalised to protein concentration as described in section 2.2.4.3

5.2.2 Experimental Results and Discussion

As can be seen in figure 19 the exposure of the transfected cells to quercetin or UV induced a 3-4 fold elevation of the p53 transcriptional activity, in comparison to control treatment of ethanol alone in the medium. The DNA damaging agent UV also induced a 3 fold transcriptional activity.

This increase in transcriptional activity is attributable to the p53 protein rather than through the c-fos minimal promoter as the luciferase activity of the control reporter does not significantly alter with the quercetin treatments. This is visualised in the figure by the control plasmid bar. The standard deviation bars indicate total variation seen in the range of quercetin treatments.

5.3 Analysis of p53 Transcriptional Activity in all Cell lines

The result of p53 transcriptional activity elevation, in response to quercetin exposure, re-enforced the hypothesis that p53 was at least in part responsible for the cell cycle arrest observed in the PalF cells. Following from this the determination of the p53 status of the transformed and tumourigenic cell lines was undertaken, the hypothesis being that whilst p53 protein may be present in these cells, it may be dysfunctional and unable to induce transcription.

5.3.1 Experimental Method

Cells of the various cell lines were transfected with 4μg of the reporter with 4μg of pCH110 as a transfection control as described in section 2.2.2.6. The

transfected cells were then treated with quercetin (0μM or 50μM), initiating 8 hours after transfection, over 36 hours with fresh medium being applied every 12 hours. Control treatments of UV exposure were also carried out at $50J/m^2$ and/or $120J/m^2$, 16 hours before harvesting.

Cells were then harvested in the luciferase lysis buffer (Promega) and asssayed for luciferase activity as described in section 2.2.2.7. Luciferase activity was then normalised to specific β-galactosidase activity (ascertained by measuring the β-galactosidase activity as described in section 2.2.2.12, and protein concentration as described in section 2.2.4.3)

5.3.2 Experimental Results

From examination of the parental PalF cells, it can be seen that the exposure of 50μM quercetin over 36 hours elevates the p53 transcriptional activity of the cells 2-3 fold over ethanol alone in the medium (figure 20). The initial observation with the short quercetin exposure normalised to protein concentration alone, is reproducible in this system of longer quercetin exposure controlled for transfection efficiency.

This activity increase of the pRGCfosLuc reporter can be ascribed to the p53 protein as the pFosLuc control reporter did not respond to quercetin exposure. Also in support of this fact, figure 20 shows that the activity of the pRGCfosLuc reporter was at least 20 to 120 fold higher than the pFosLuc control reporter construct. This shows that PalF cells even 36 hours after transfection, with ethanol only added to the medium, display a significant level of background p53 transcriptional activity.

Treatment of E7Q, or the descendent cell line E7QT2, with quercetin over 36 hours showed a very different response. As can been seen in figures 21 and 22, not only do the cell lines display a very low level of p53 transcriptional activity in comparison to the PalF cells, indeed there was no significant difference between the activity of pRGCfosLuc and of pFosLuc. Also a lack of a functional response to quercetin exposure was observed, with no significant difference between the quercetin treated cells and the ethanol treated cells.

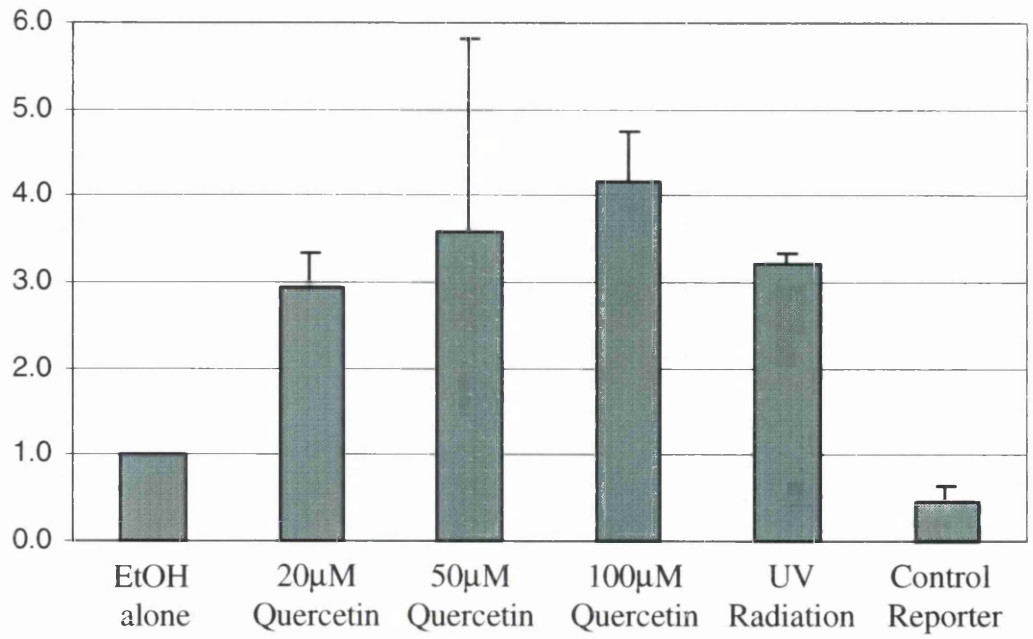


Figure 19. ***Quercetin stimulates p53 transcriptional activity***

Cells were transfected with 4µg of pRGCfosLuc. Transfections were then treated with 20-100 µM quercetin over 16 hours or with an equivalent volume of EtOH. UV of 50J/m^2 was used as a postive control. Luciferase activity was normalised to protein concentration. The results are expressed as fold induction over EtOH alone. Control transfections with pfosLuc (control reporter) were also carried out and total variation in the control reporter with the different quercetin concentration is shown.

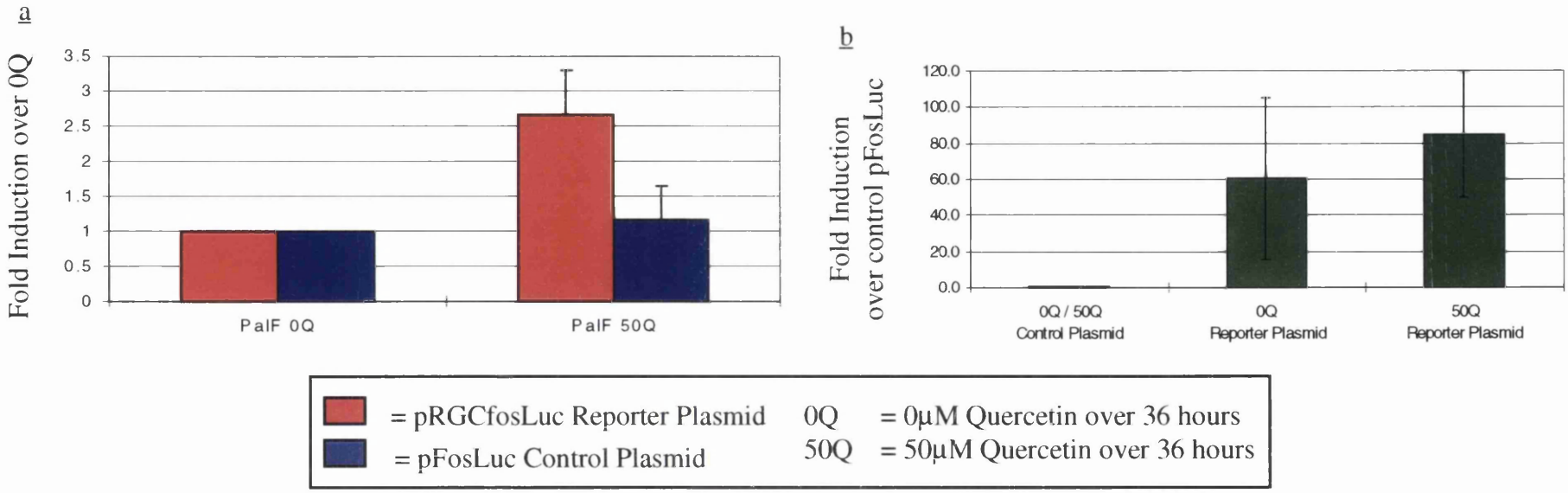


Figure 20. ***Quercetin stimulates p53 transcriptional activity in PalF***

Cells were transfected with 4µg of pRGCfosLuc together with 4µg of pCH110 as a control for transfection efficiency. Control transfections with pFosLuc were also carried out. Transfections were then treated with 50µM quercetin over 36 hours or with an equivalent volume of EtOH. Luciferase activity was normalised to β-galactosidase specific activity.

The results are expressed as fold induction over EtOH alone (a) or over average activity of pFosLuc (b).

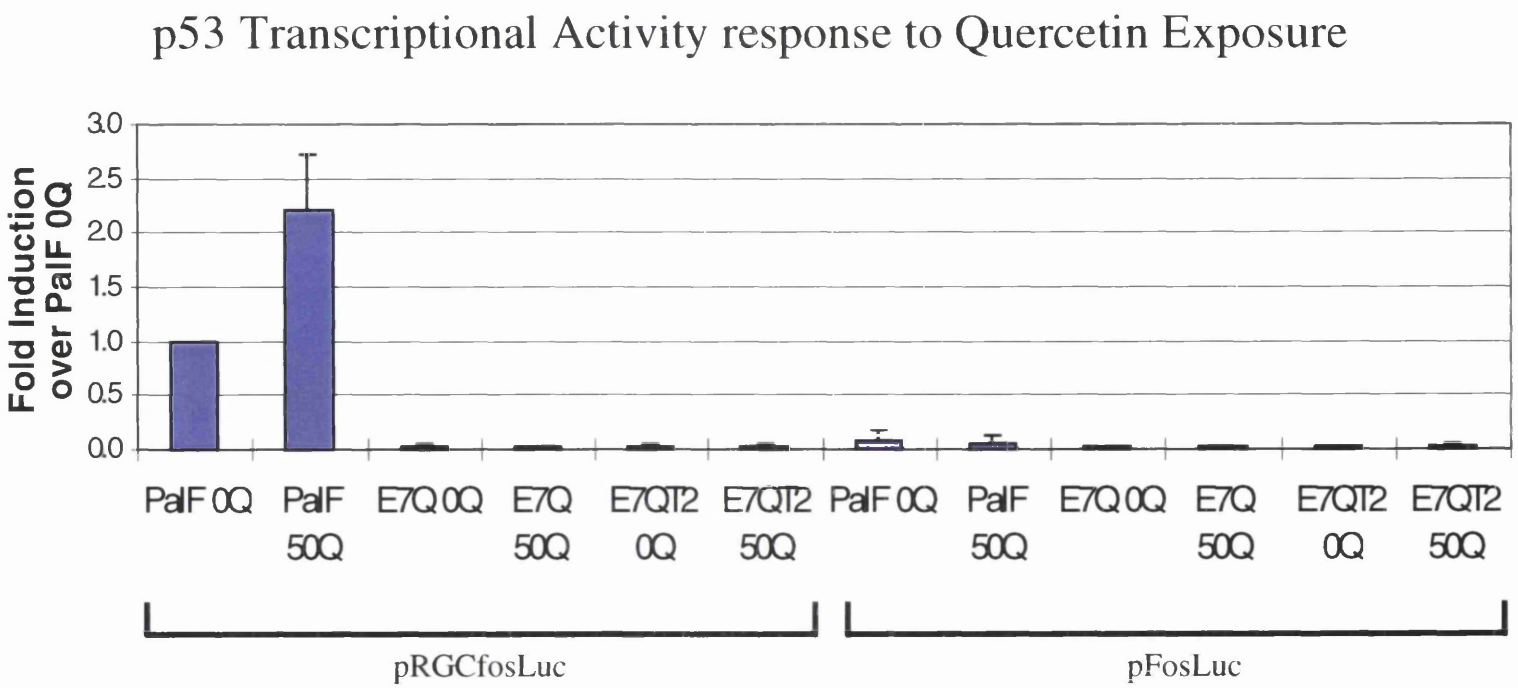


Figure 21. ***Quercetin does not stimulate p53 transcriptional activity in E7Q and E7QT2***

Cells were transfected with 4µg of pRGCfosLuc together with 4µg of pCH110 as a control for transfection efficiency. Transfections were then treated with 50µM quercetin over 36 hours or with an equivalent volume of EtOH. Luciferase activity was normalised to β-galactosidase specific activity. The results are expressed as fold induction over EtOH alone in PalF. Control transfections with pFosLuc were also carried out

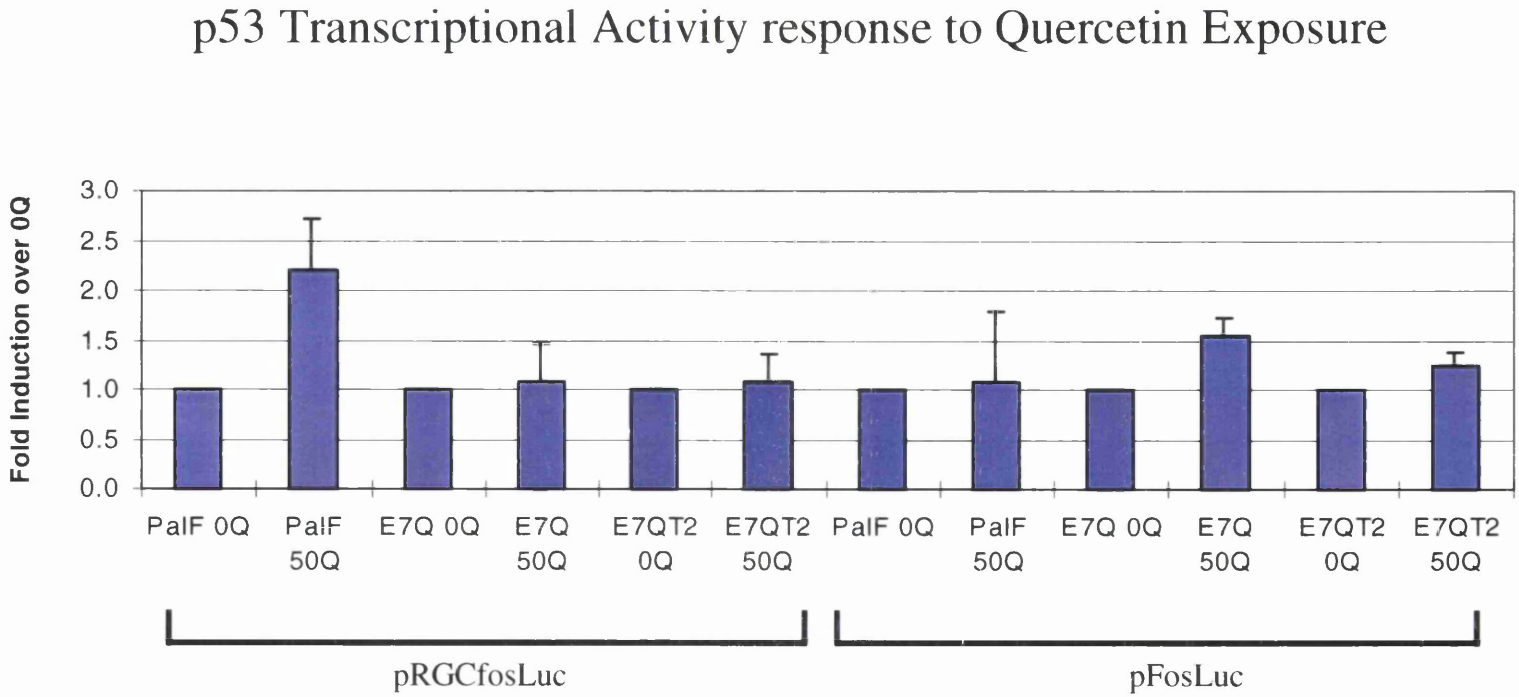


Figure 22. ***Quercetin does not stimulate p53 transcriptional activity in E7Q and E7QT2***

Cells were transfected with 4µg of pRGCfosLuc together with 4µg of pCH110 as a control for transfection efficiency. Transfections were then treated with 50µM quercetin over 36 hours or with an equivalent volume of EtOH. Luciferase activity was normalised to β-galactosidase specific activity. The results are expressed as fold induction over EtOH alone in PalF. Control transfections with pFosLuc were also carried out

An essentially equivalent picture emerged from analysis of the E7QPα cell line (see figures 23 and 24). In this assay UV exposure was also tested as to whether it would induce a p53 response. Again there proved to be no response and no luciferase activity attributable to the p53 protein i.e. no difference between pRGCfosLuc and pFosLuc.

This pattern of no p53 transcriptional activity response was repeated in the other tumorigenic cell line Q2D. Again this showed no response to quercetin exposure (see figures 25 and 26). There may be a slight elevation of p53 transcriptional activity upon UV exposure of the cells, however this may be due to experimental variation due to low activities seen with this reporter in this cell line. It is however, also arguable whether any alteration of p53 transcriptional activity would be relevant *in vivo* due to the very low levels of p53 transcriptional activity in comparison to the PalF cells. This includes luciferase activity of PalF cells treated with ethanol alone, or by the lack of difference between reporter and control plasmids transfected into the tumorigenic cells.

One hypothesis to explain the loss of function by the p53 protein could be the expression of the BPV-4 E7 protein in these cell lines inhibiting p53 transcriptional activity via TBP, as has been reported with HPV E7 (Massimi *et al.,* 1996; Massimi & Banks, 1997). To address this question analysis of the other non tumorigenic cell lines was undertaken.

Initially the transformed E7R cell line was tested with quercetin exposure and UV exposure. As can been seen in comparison to PalF cells, the E7R cell line exhibits an equivalent level of p53 transcriptional activity (figure 27). Indeed when examined in comparison to E7R cells treated with ethanol alone the quercetin treated cells exhibited a normal p53 response, elevating the luciferase activity 2-3 fold (figure 28). It is interesting to note the lack of a functional response to UV. Whether the cell line exhibits a lack of response to UV, a resistance, or indeed a sensitivity is not known. Further testing of the E7R cells with a greater variation of UV exposures would answer this point. However in the context of the study the results of a normal p53 response, in the reporter assay, to quercetin exposure indicates that the loss of p53 transcriptional activity is not a requirement for the cells to bypass the G1 arrest. In addition it may well be likely

that the loss of this function of the p53 protein is an important step in the progression to full transformation of the cell.

The Q0D, E7QPβ and E7QPγ cell lines showed a level of p53 transcriptional activity comparable to, or slightly higher than, that of the PalF cells (see figures 29 to 31). Quercetin treatment of these cell lines induced a functional p53 transcriptional response of 2-3 fold, equivalent to that of PalF cells. UV exposure of these cells displayed a slightly different result. E7QPβ and E7QPγ displayed a doubling of p53 transcriptional activity in response to a UV exposure of 50J/m^2. However increased dosages resulted in a decrease of the response. Excessive exposure of UV to these cells may result in enough DNA damage not to permit normal functioning of the DNA damage response pathway.

Q0D cells showed no response to UV exposure, which may be due to a resistance to UV induced damage. Such a resistance could be from mutation of p53 resulting in the inability to phosphorylate the protein at specific serine residue(s) associated with UV but not some other DNA damaging agents e.g. serine 392 in human p53 (Giaccia & Kastan, 1998 and references therein). Another possible mechanism is a sensitivity to UV induced damage, by loss of a DNA repair protein function for example. The cells would be unable to mount a full response due to the high level of DNA damage affecting transcription, resulting in no visible p53 transcriptional activity elevation even though the gene and protein may still be functional.

Analysis of the transforming papilloma cell line 88529B displayed an unusual response to the stimuli. These cells exhibited a level of p53 transcriptional activity roughly comparable to the PalF cells (figure 31) when taking the luciferase activity of the PalF cells treated with ethanol alone as a base figure. Comparison of the 88529B cells treated with 50μM quercetin showed that the reporter activity did not significantly vary from the control cells. However UV exposure at 50 or 120J/m^2 resulted in an elevation. Again this may be due to varying tolerances of the different cell lines to the different agents. As the 88529B cell line is derived from keratinocytes this may suggest a cell lineage feature where the keratinocytes display a normal UV DNA damage response, yet require a greater exposure to quercetin than the fibroblasts to elicit a response. Analysis of primary keratinocytes would shed light on this question. Failure of the 88529B

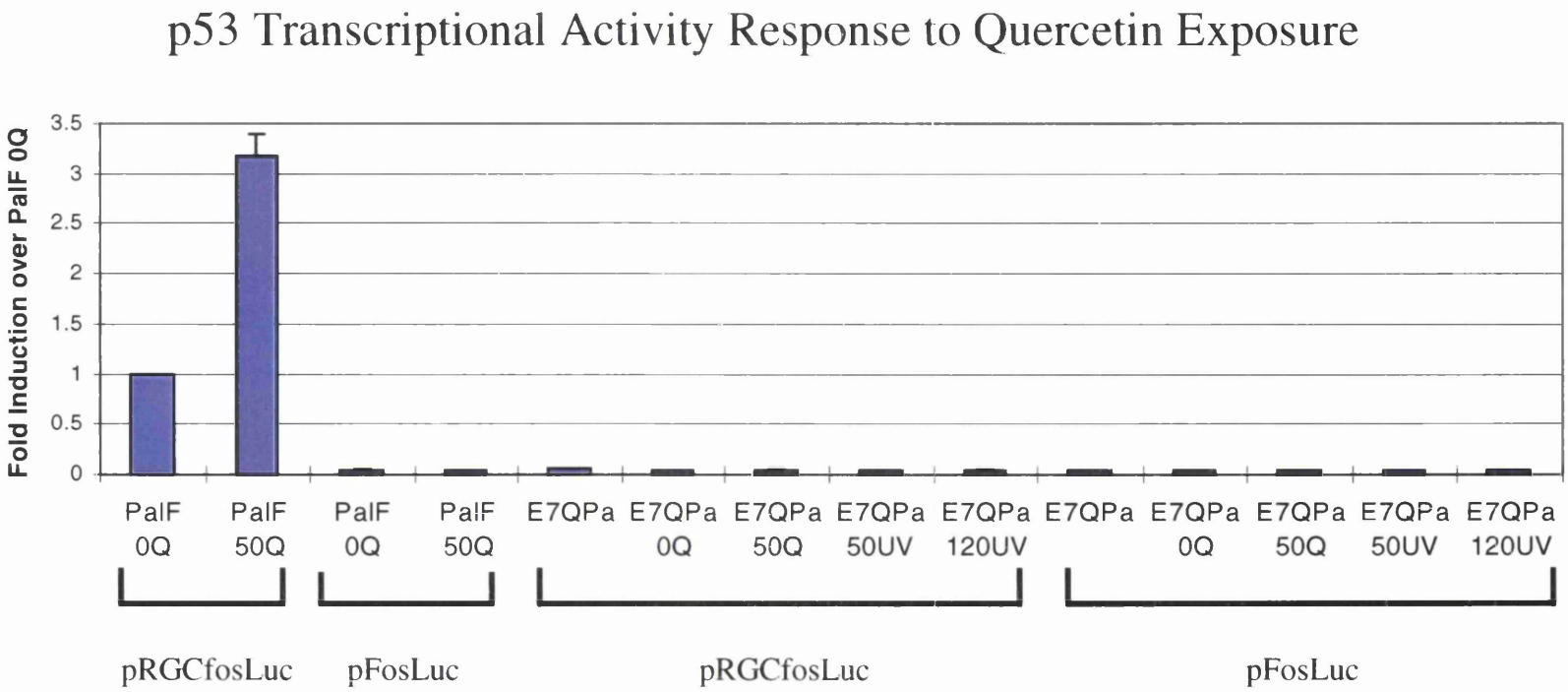


Figure 23. ***Quercetin does not stimulate p53 transcriptional activity in E7QPα (labelled as E7QPa)***

Cells were transfected with 4µg of pRGCfosLuc together with 4µg of pCH110 as a control for transfection efficiency. Transfections were then treated with 50µM quercetin over 36 hours or with an equivalent volume of EtOH. Control treatments with UV at 50 or 120 J/m^{2}were carried out. Luciferase activity was normalised to β-galactosidase specific activity. The results are expressed as fold induction over EtOH alone in PalF. Control transfections with pFosLuc were also carried out

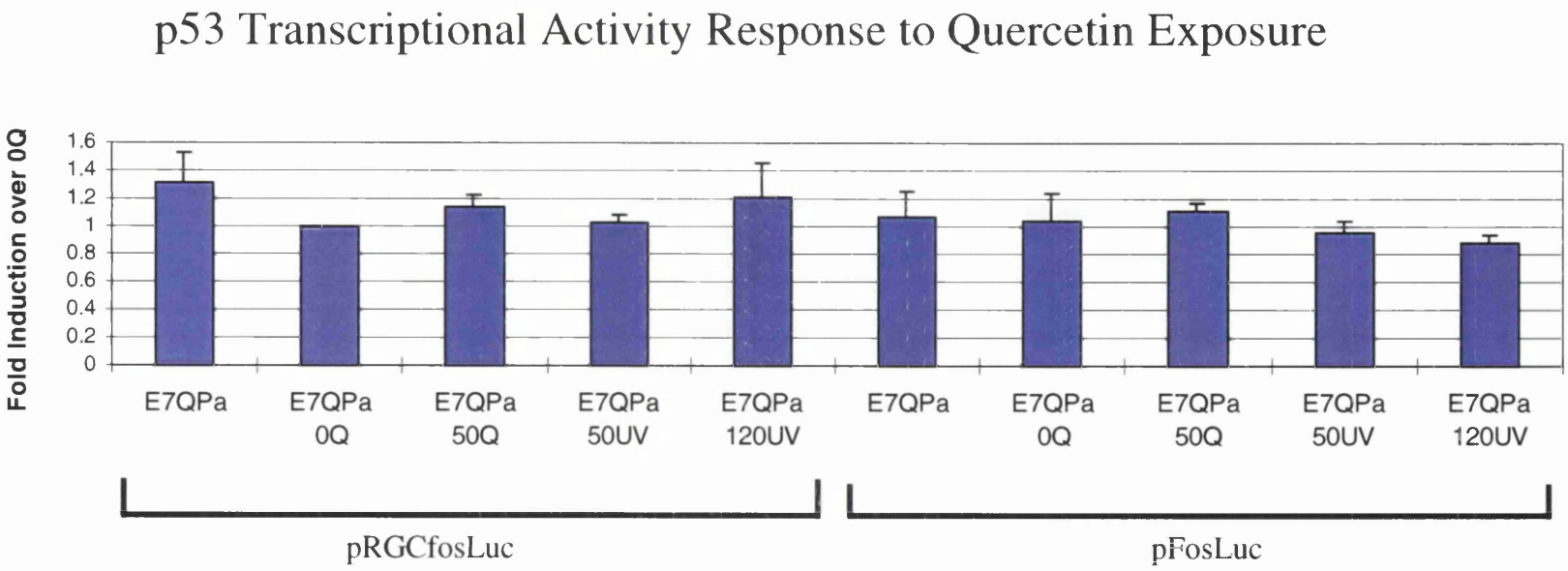


Figure 24. ***Quercetin does not stimulate p53 transcriptional activity in E7QPα (labelled as E7QPa)***

Cells were transfected with 4µg of pRGCfosLuc together with 4µg of pCH110 as a control for transfection efficiency. Transfections were then treated with 50µM quercetin over 36 hours or with an equivalent volume of EtOH. Control treatments with UV at 50 or 120 J/m^{2}were carried out. Luciferase activity was normalised to β-galactosidase specific activity. The results are expressed as fold induction over EtOH alone. Control transfections with pFosLuc were also carried out

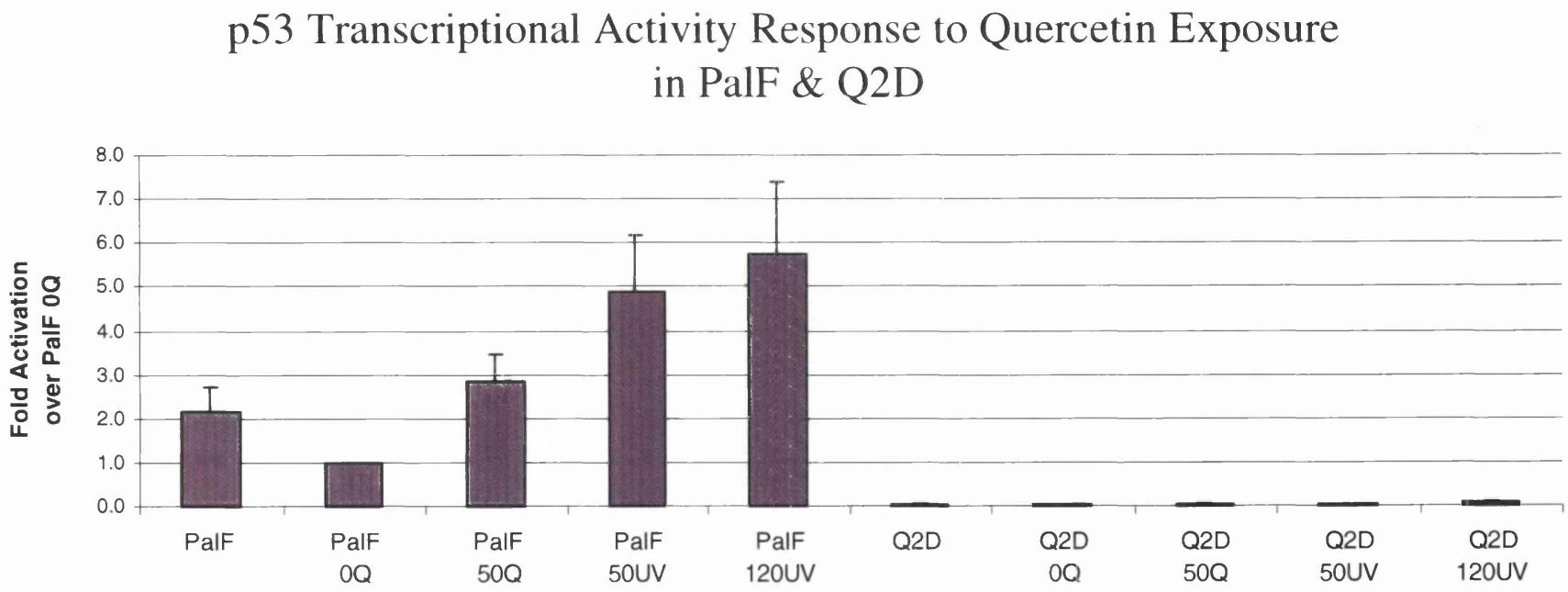


Figure 25. ***Quercetin does not stimulate p53 transcriptional activity in Q2D***

Cells were transfected with 4µg of pRGCfosLuc together with 4µg of pCH110 as a control for transfection efficiency. Transfections were then treated with 50µM quercetin over 36 hours or with an equivalent volume of EtOH. Control treatments with UV at 50 or 120 J/m^2 were carried out. Luciferase activity was normalised to β-galactosidase specific activity. The results are expressed as fold induction over EtOH alone in PalF. Control transfections with pFosLuc were also carried out (not shown)

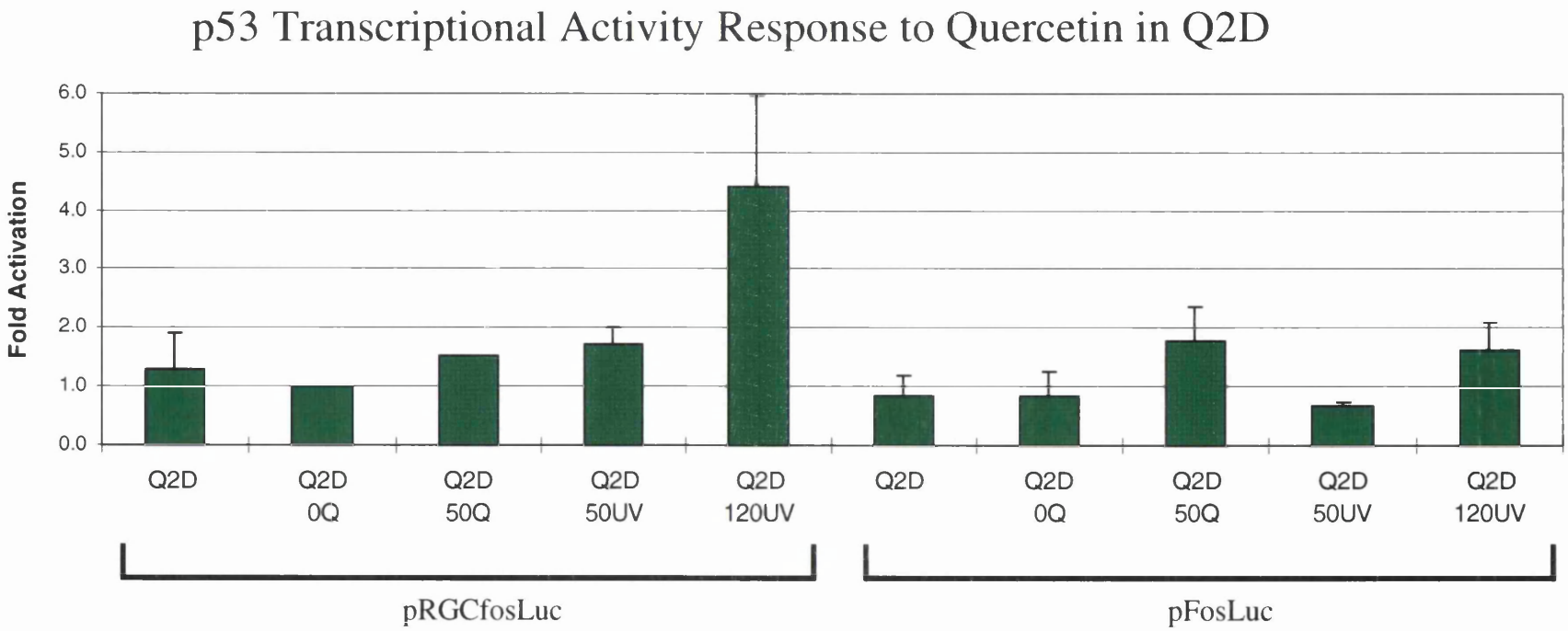


Figure 26. ***Quercetin does not stimulate p53 transcriptional activity in Q2D***

Cells were transfected with 4μg of pRGCfosLuc together with 4μg of pCH110 as a control for transfection efficiency. Transfections were then treated with 50μM quercetin over 36 hours or with an equivalent volume of EtOH. Control treatments with UV at 50 or 120 J/m^2 were carried out. Luciferase activity was normalised to β-galactosidase specific activity. The results are expressed as fold induction over EtOH alone. Control transfections with pFosLuc were also carried out

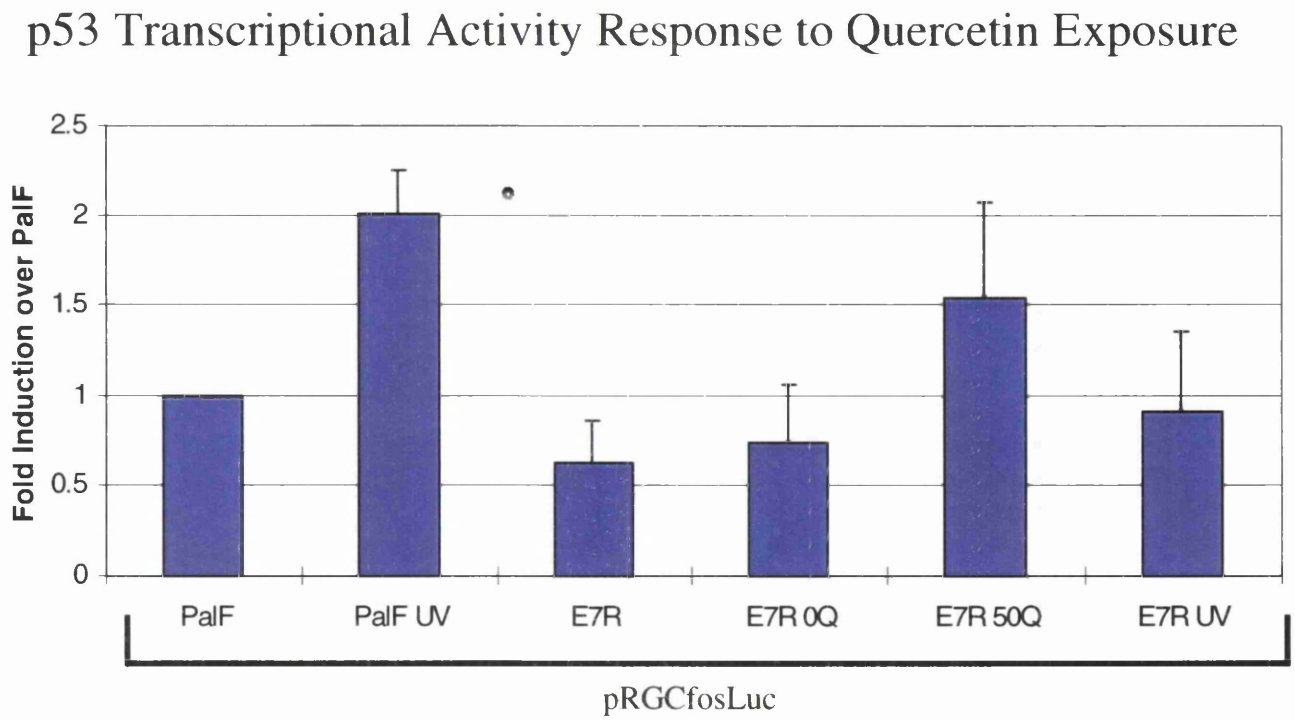


Figure 27. ***Quercetin stimulates p53 transcriptional activity in E7R***

Cells were transfected with 4µg of pRGCfosLuc together with 4µg of pCH110 as a control for transfection efficiency. Transfections were then treated with 50µM quercetin over 36 hours or with an equivalent volume of EtOH. Control treatments with UV at 50 J/m^2 were carried out. Luciferase activity was normalised to β-galactosidase specific activity. The results are expressed as fold induction over EtOH alone in PalF. Control transfections with pFosLuc were also carried out (not shown)

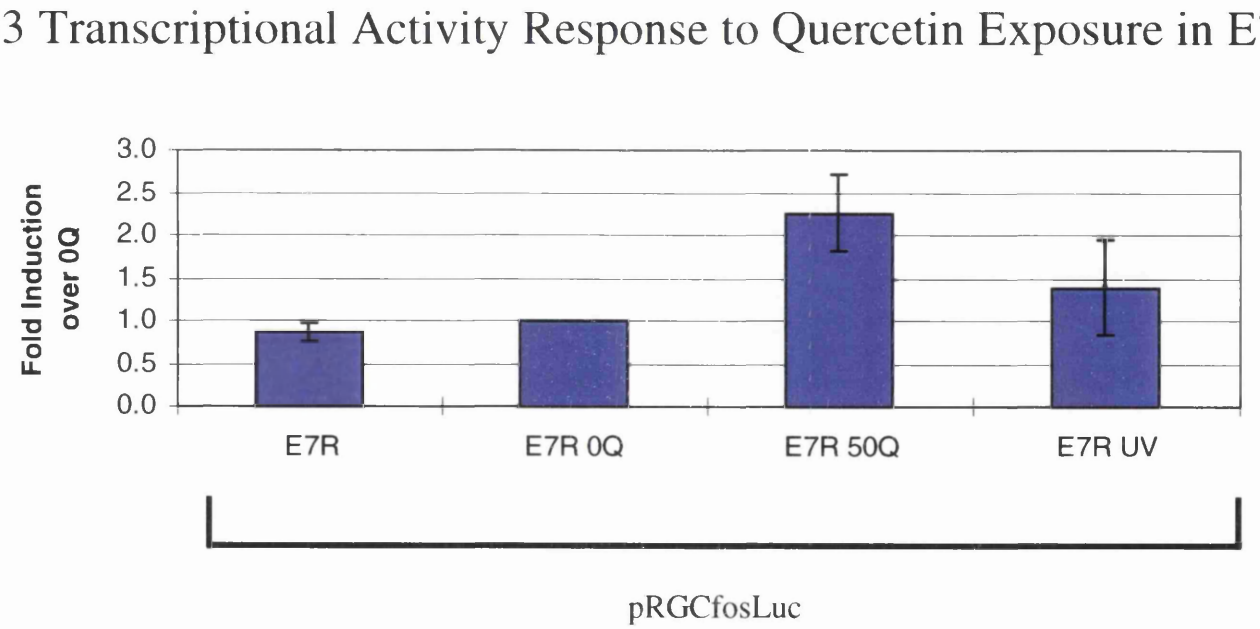


Figure 28. ***Quercetin stimulates p53 transcriptional activity in E7R***

Cells were transfected with 4µg of pRGCfosLuc together with 4µg of pCH110 as a control for transfection efficiency. Transfections were then treated with 50µM quercetin over 36 hours or with an equivalent volume of EtOH. Control treatments with UV at 50 J/m^2 were carried out. Luciferase activity was normalised to β-galactosidase specific activity. The results are expressed as fold induction over EtOH alone. Control transfections with pFosLuc were also carried out (not shown)

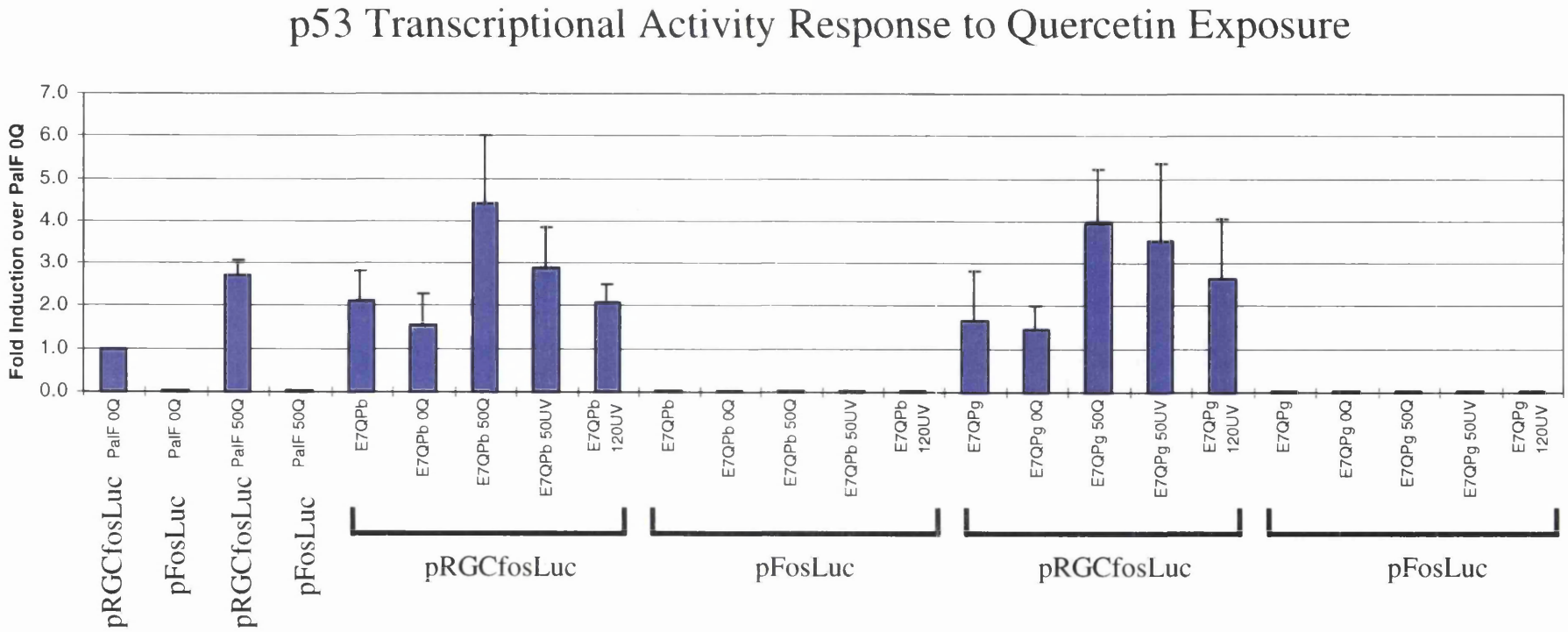


Figure 29. ***Quercetin stimulates p53 transcriptional activity in E7QPβ and E7QPγ (labelled E7QPb and E7QPg respectivily)*** Cells were transfected with 4µg of pRGCfosLuc together with 4µg of pCH110 as a control for transfection efficiency. Transfections were then treated with 50µM quercetin over 36 hours or with an equivalent volume of EtOH. Control treatments with UV at 50 or 120J/m^2 were carried out. Luciferase activity was normalised to β-galactosidase specific activity. The results are expressed as fold induction over EtOH alone in PalF. Control transfections with pFosLuc were also carried out

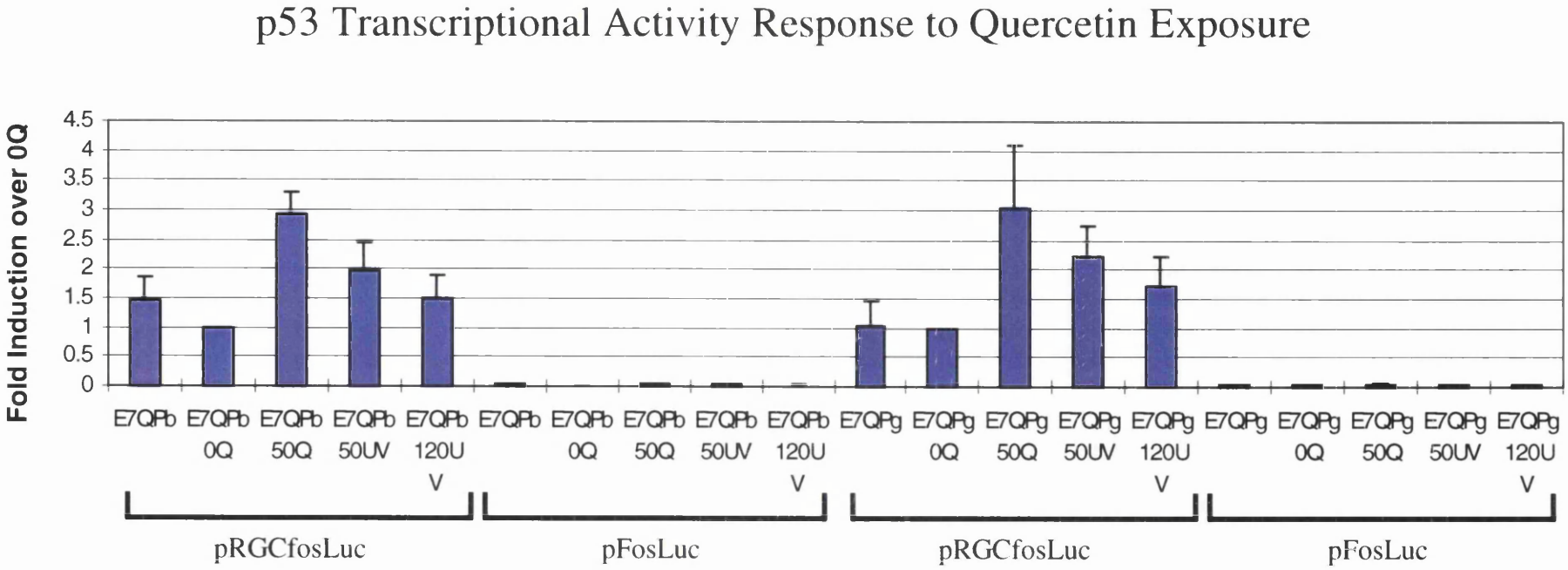


Figure 30. ***Quercetin stimulates p53 transcriptional activity in E7QPβ and E7QPγ (labelled E7QPb and E7QPg respectivily)***
Cells were transfected with 4µg of pRGCfosLuc together with 4µg of pCH110 as a control for transfection efficiency. Transfections were then treated with 50µM quercetin over 36 hours or with an equivalent volume of EtOH. Control treatments with UV at 50 or 120J/m^2 were carried out. Luciferase activity was normalised to β-galactosidase specific activity. The results are expressed as fold induction over EtOH alone. Control transfections with pFosLuc were also carried out

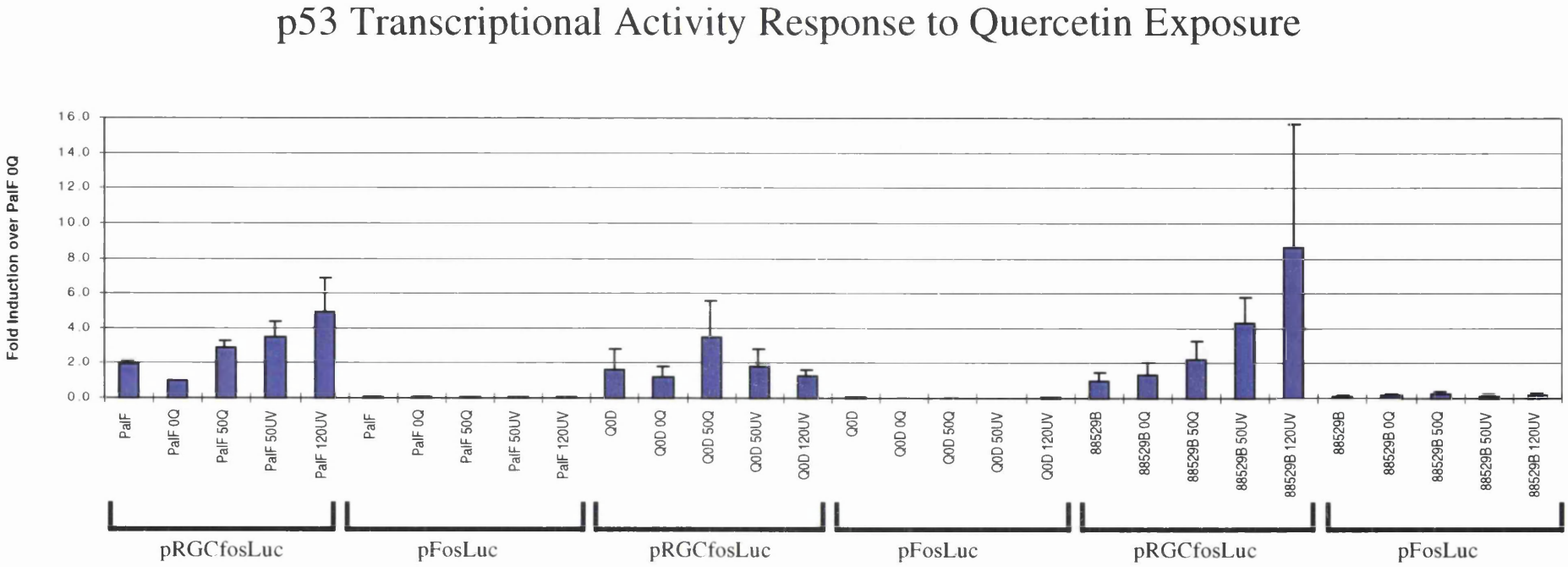


Figure 31. ***Quercetin stimulates p53 transcriptional activity in Q0D amd 888529B***

Cells were transfected with 4µg of pRGCfosLuc together with 4µg of pCH110 as a control for transfection efficiency. Transfections were then treated with 50µM quercetin over 36 hours or with an equivalent volume of EtOH. Control treatments with UV at 50 or 120J/m^2 were carried out. Luciferase activity was normalised to β-galactosidase specific activity. The results are expressed as fold induction over EtOH alone in PalF. Control transfections with pFosLuc were also carried out

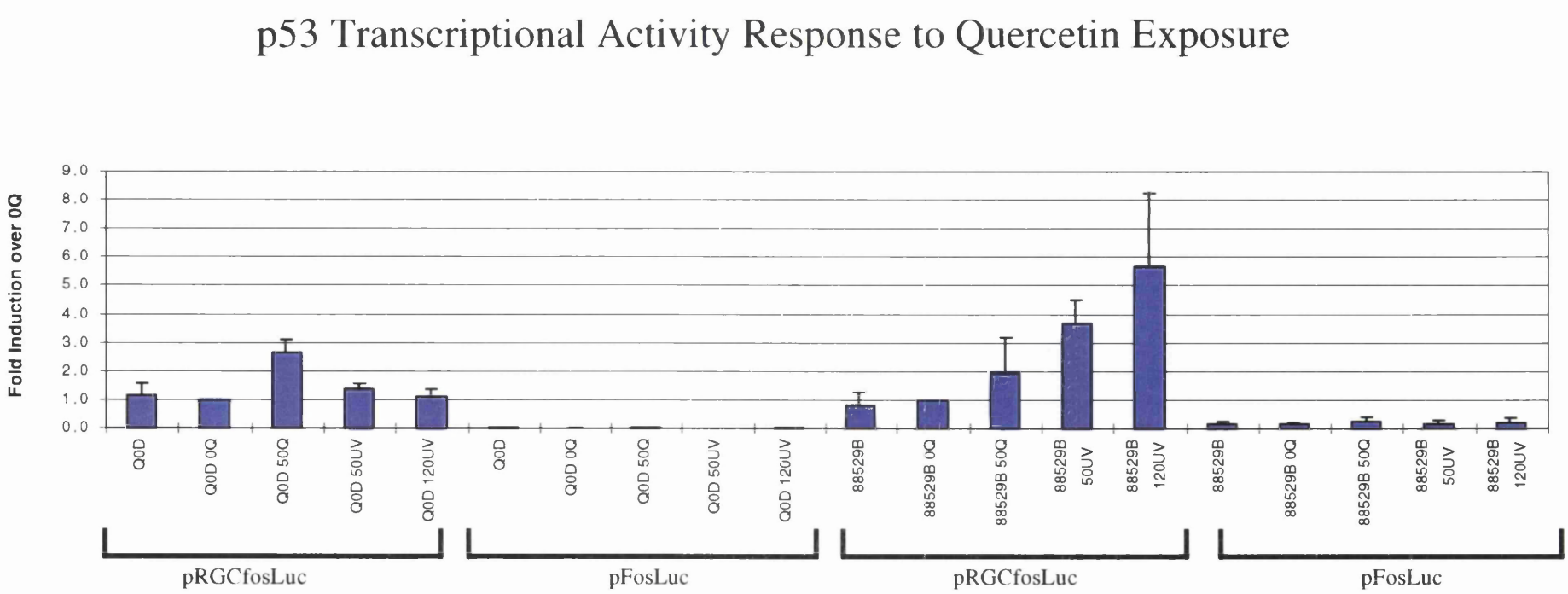


Figure 32. ***Quercetin stimulates p53 transcriptional activity in Q0D and 88529B***

Cells were transfected with 4µg of pRGCfosLuc together with 4µg of pCH110 as a control for transfection efficiency. Transfections were then treated with 50µM quercetin over 36 hours or with an equivalent volume of EtOH. Control treatments with UV at 50 or 120J/m^2 were carried out. Luciferase activity was normalised to β-galactosidase specific activity. The results are expressed as fold induction over EtOH alone. Control transfections with pFosLuc were also carried out

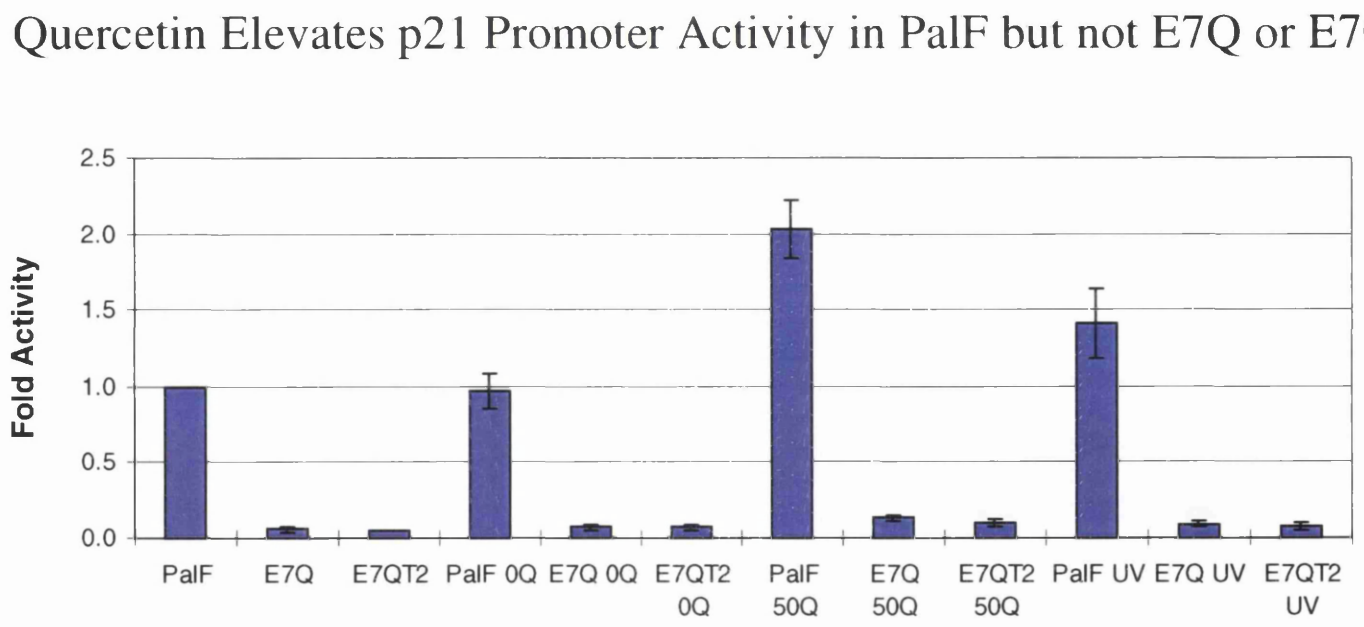


Figure 33. ***Quercetin does not stimulate p53 transcriptional activity in E7Q or E7QT2***

Cells were transfected with 4µg of WWP-LUC together with 4µg of pCH110 as a control for transfection efficiency. Transfections were then treated with 50µM quercetin over 36 hours or with an equivalent volume of EtOH. Control treatments with UV at 50J/m^2 were carried out. Luciferase activity was normalised to β-galactosidase specific activity. The results are expressed as fold induction over reporter transfection alone in PalF.

cells to generate a reliable p53 transcriptional activity response to quercetin may be a factor in the cell line's progression to full transformation.

In summary we find a correlation between the tumorigenic status of the cell and p53 transcriptional activity status, with the tumorigenic cells showing no response to quercetin. This suggests that loss of transcriptional activity of p53 may be a critical event in the progression to tumorigenic status. However it is also obvious that the abrogation of p53 transcriptional activity does not correlate with the ability to bypass G1 arrest in the cell lines as indicated by E7R, Q0D, E7QPβ and E7QPγ. This shows that the abrogation of the p53 transcriptional activity is not a requisite for the cell to bypass the quercetin induced G1 arrest.

5.3.3 Experimental Results and Discusion Part 2

In addition to the reporter studies carried out with the pRGCfosLuc reporter, limited further study was also carried out with two other reporters WWP-Luc and pGL2NA(mdm2). The latter was also used in conjunction with exogenus p53 as detailed later.

Transfection of the PalF, E7Q and E7QT2 cell lines with the WWP-Luc reporter provided further evidence of the loss of p53 transcriptional activity in those cell lines. Taking the PalF cells with the WWP-Luc reporter transfected with no treatment as a base level of luciferase activity, figure 33 demonstrates that quercetin treatment doubles the p53 transcriptional activity also in this system as well. UV exposure elicited a more modest elevation in p53 transcriptional activity.

Importantly with the use of this reporter it is also evident that the E7Q and E7QT2 cell lines again display a very low level of p53 transcriptional activity in comparison to the PalF cells. However close inspection of the luciferase activities shows that the E7Q (but not E7QT2) cell line does display a doubling of p53 transcriptional activity in response to quercetin treatment. This may be due to experimental error due to the low activities observed with the tumorigenic cell line or due to possible quercetin responsive sites in the human p21 promoter other than the p53 responsive site. However it is unlikely that any elevation seen in the E7Q cell line would be relevant *in vivo* due to the low level of p53 transcriptional activity, in relation to PalF cells, even after any putative elevation.

5.4 Analysis of Exogenus p53 Transcriptional Activity in E7Q and E7QT2

In a preliminary study the transcriptional activity of exogenus p53 was assayed in these cell lines. This would help determine whether the loss of p53 transcriptional activity in the tumorigenic cell lines was due to mutation or post translational modifications to the protein.

5.4.1 Experimental Method

The various cell lines were transfected with 4μg of the reporter (pRGCfosLuc, pGL2NA(mdm2) or WWP-Luc) and 20μg of the relevant expression plasmid (pCB6wtp53 or pCB6mtp53) or vector control (pcDNA or pZipneo). 4μg of pCH110 was also co-transfected as a transfection control as described in section 2.2.2.6 in the experiment with WWP-Luc or the pRGCfosLuc experiment in figure 36.

Cells were harvested 36 hours after transfection in the luciferase lysis buffer (Promega) and assayed for luciferase activity as described in section 2.2.2.7. Luciferase activity was then normalised to specific β-Galactosidase activity (ascertained by measuring the β-Galactosidase activity as described in section 2.2.2.12, and protein concentration as described in section 2.2.4.3) in the experiment using the WWP-Luc reporter construct or the experiment with pRGCfosLuc shown in figure 36. The other experiments with either pRGCfosLuc or pGL2NA(mdm2) were normalised to protein concentration alone.

5.4.2 Experimental Results and Discussion

Figure 34 shows the results of the first experiment using the pRGCfosLuc reporter. The luciferase activity in the PalF cells is 3 fold greater than that of the E7QT2 cells. This initial observation suggested a decrease in the exogenus human p53 transcriptional activity when expressed in the tumorigenic cell line.

This initial observation was also supported by use of the mdm2 promoter reporter construct, pGL2NA(mdm2). As figure 35 shows, the transfection of this reporter into the PalF cells elicited a base level of luciferase activity in itself. The addition of pcDNA to the transfection elevated the luciferase activity 5 fold over

Exogenous p53 Transcriptional Activity in PalF, E7Qand E7QT2

Figure 34 pRGCfosLuc Reporter

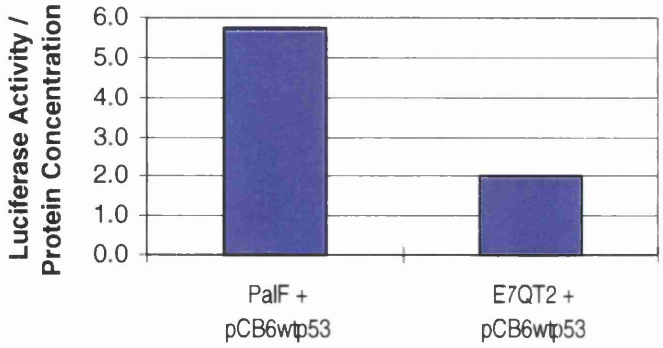


Figure 35 pGL2NA(mdm2) Reporter

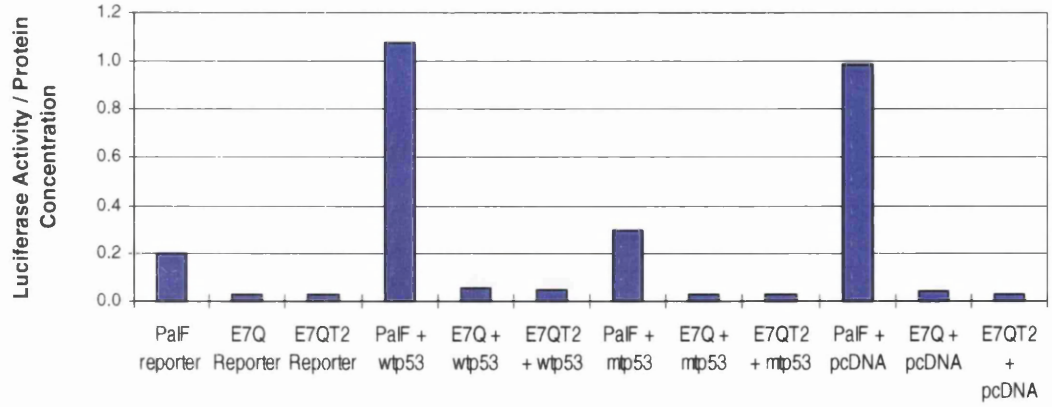


Figure 36 & 37. ***Exogenous p53 transcriptional activity in PalF, E7Q and E7QT2***

Cells were transfected with 4µg of reporter together with 20µg of pCB6wtp53, pCB6mutp53 or pcDNA. Transfected cells were harvested 36 hours later and assayed for luciferase activity and protein concentration. Luciferase activity was normalised to protein concentration.

the transfection of reporter alone indicating that the addition of the extra DNA to the transfection combination induced an elevation of p53 transcriptional activity in the cells. It is also evident that as with the pRGCfosLuc and WWP-Luc reporters the E7Q and E7QT2 cell lines exhibited a much lower level of luciferase activity demonstrating again the loss of the endogenous p53 function in the cells. Additionally it is also evident that when pcDNA or pCB6wtp53 is added to the transfection, whilst luciferase (i.e. p53 transcriptional activity) activity is increased in PalF cells, it fails to be elevated in the E7Q and E7QT2 cells.

Thus this second experiment also supports the initial observation that the transcriptional activity of exogenously added p53 is inhibited in these cells The results with the exogenous wild type p53 leave the possibility of either transcriptional repression in the tumorigenic cells, post translational modification or increased protein degradation as possible mechanisms for this inhibition of p53 activity.

However one fault of the initial two experiments was that the luciferase activities were not controlled for transfection efficiency, a factor likely to have an impact when expressing a potent promoter of apoptosis and cell cycle arrest in the transfected cells for 36 hours and comparing to the control vector alone. Hence a third experiment was carried out using the pRGCfosLuc reporter. As can be seen in figure 36, co-transfection of the pCB6wtp53 plasmid rather than either pZipneo or pCDNA results in a large increase in reporter. Additionally although there is still a significant level of p53 transcriptional activity in the E7Q and E7QT2 cells upon co-transfection with pCB6wtp53 it is roughly one fifth that seen in PalF cells. This confirms that the exogenous wild type p53 is inhibited in the cell lines E7Q and E7QT2.

Finally a series of assays was carried out using the WWP-Luc reporter, in the presence of pCB6wtp53, in the PalF, E7Q and E7QT2 cells again with pCH110 used as a transfection efficiency control. Figure 37 illustrates the activity of the reporter as fold induction over the reporter transfected alone. Reduction of the reporter activity was seen with transfection into the E7Q and E7QT2 cells as before due to low endogenous p53 transcriptional activity in response to the exogenus DNA. Additionally elevation of reporter activity was seen upon co-transfection with either pcDNA or pCB6wtp53. However although transfection

with pCB6wtp53 into the E7Q or E7QT2 cells induced an elevation of p53 transcriptional activity, the activity was significantly lower than that of the PalF cells, again repeating earlier findings with the other reporter constructs.

It is evident that co-transfection of WWP-Luc and pGL2NA(mdm2) with either pcDNA or pCB6wtp53 results in comparable reporter activities. In the case of the experiment utilising the pGL2NA(mdm2) this may be ascribed to lack of controlling for transfection efficiency thus artificially lowering the activity of cells expressing p53 but this cannot explain the lack of difference observed with WWP-Luc. One possible explanation of this phenomenon is that the reporter was sensitive to the transfection of the greater amounts of exogenous DNA, such p53 response to transfected DNA has been reported before (Siegal et al., 1995). Reporter activity may well have been close to maximum from the levels of endogenous p53 induced by the transfection of pcDNA. It was observed that the co-transfection of greater amounts of DNA with the two reporters utilising p53 binding in a the context of a whole gene promoter resulted in high background reporter activities in comparison to other p53 stimuli such as quercetin or UV.

5.5 Detection of p53 Protein Localisation by Immunofluorescence

In order to attempt to determine whether the p53 transcriptional activity in the E7Q and E7QT2 cells was due to an aberrant cellular localisation of the p53 protein in these cells immunofluoresence was carried out. Examination of both endogenous bovine and exogenous human p53 was performed.

5.5.1 Experimental Method

PalF, E7Q or Q2D cells were seeded into chamber slides and either treated with 0μM or 50μM quercetin for 36 hours before fixation and antibody binding as described in section 2.2.4.7. Alternatively cells were transfected with 4μg of pCB6wtp53 or pcDNA as control vector essentially as described in section 2.2.2.6 with the exception of 8000 cells being seeded into 16 well chamber slides and 300μl of medium used at each refeeding step. Transfected cells were then left for 24 hours to permit expression of the p53 protein before fixation and antibody probing.

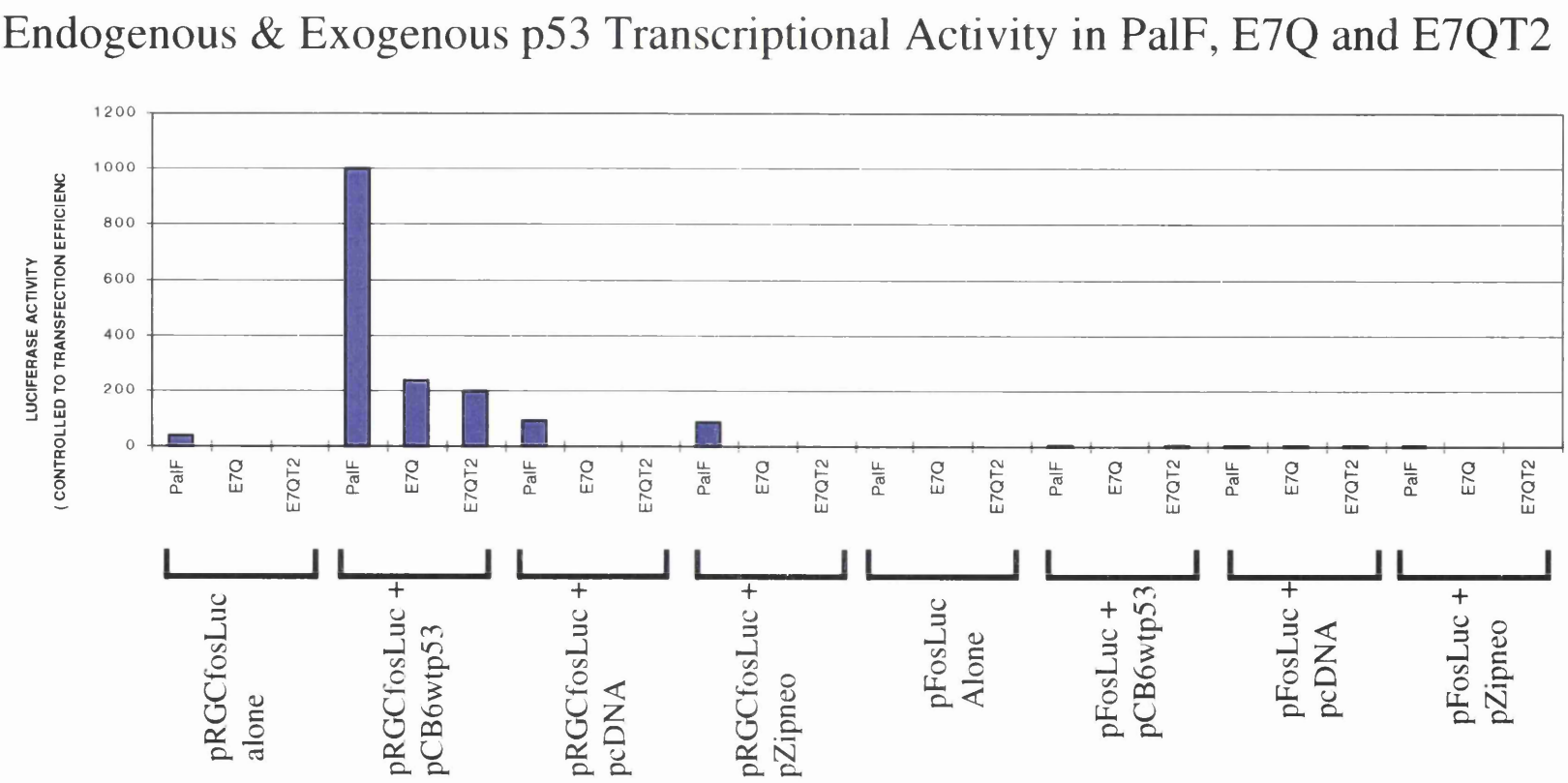


Figure 36. ***p53 transcriptional activity in PalF, E7Q and E7QT2***

Cells were transfected with 4µg of pRGCfosLuc and 20µg of either pCB6wtp53, pcDNA or pZipneo together with 4µg of pCH110 as a control for transfection efficiency. Transfections were then left for 36 hours. Luciferase activity was normalised to β-galactosidase specific activity. Control transfections with pFosLuc were also carried out

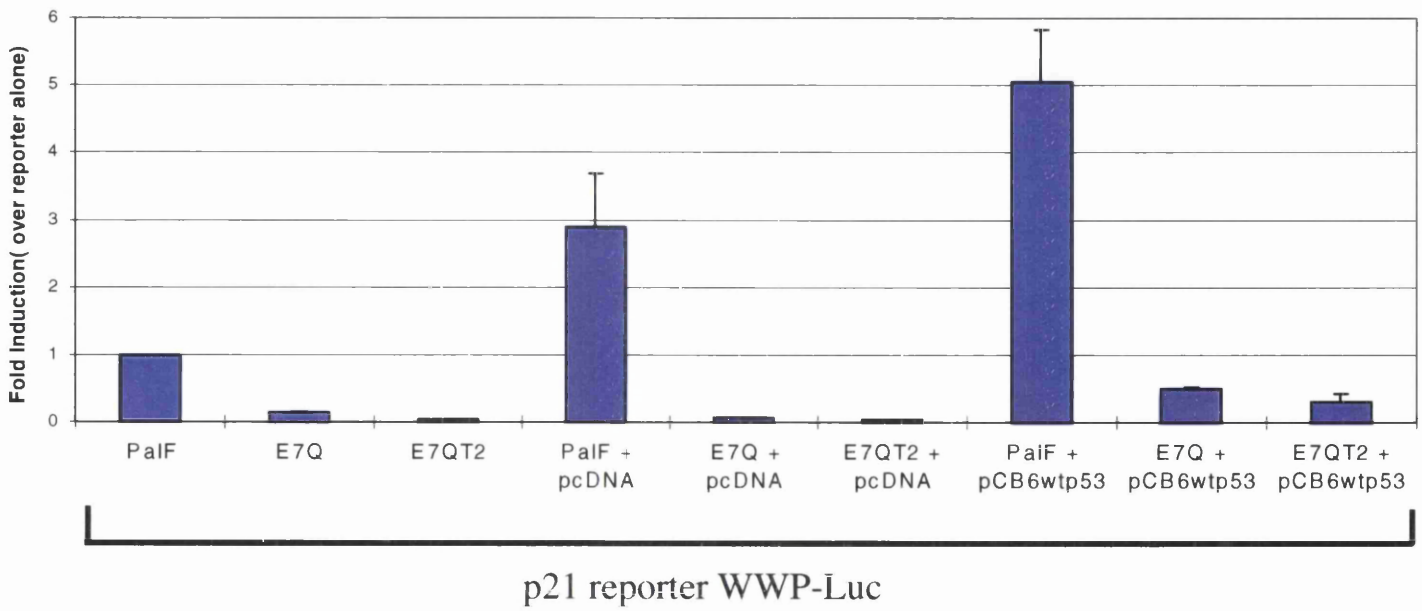


Figure 37. ***p53 transcriptional activity in PalF, E7Q and E7QT2***

Cells were transfected with 4µg of WWP-Luc and 20µg of either pCB6wtp53, or pcDNA, together with 4µg of pCH110 as a control for transfection efficiency. Transfections were then left for 36 hours. Luciferase activity was normalised to β-galactosidase specific activity.

5.5.2 Experimental Results and Discussion

There was successful propidium iodide staining showing cells present throughout the protocol. However despite prolonged incubations with antibody no visible signal was detected from either endogenous or exogenous p53. Use of other antibodies may prove more effective at detection of p53 under these experimental conditions. The range of commercially available antibodies raised against human p53 would make the study of the localisation of the exogenous wild type human p53 possible. Reduction of the timing of expression of the exogenous p53 may also favour localisation due to the probability that fewer cells would have committed to apoptosis under a shorter time frame.

5.6 Detection of p53 Protein Binding by EMSA

Electromobility shift assays were carried out on the cell extracts of PalF, E7Q and E7QT2 cell lines in order to determine whether the endogenous p53 protein was capable of binding to a target DNA sequence. Inability to successfully bind to the DNA would be an explanation for the proteins failure to induce transcription.

5.6.1 Experimental Method

Cell extracts were harvested from the cell lines assayed as described in section 2.2.4.2, and protein concentration estimated as in section 2.2.4.3.

Probe was then radioactivily end labelled with ^{32}P and purified on a non-denaturing polyacrylamide gel as described in sections 2.2.3.4 and 2.2.3.5. The probe used was the RGC (Ribosomal Gene Cluster) sequence as the p53 reporter pRGCfosLuc. The exact sequence is as follows:

Probe Name	***Sequence***
RGC	5'- gatccgattgccttgcctggacttgcctcgccttgccttttg -3'
AP-1	5'- agctgtgtcctcatgct - 3'

Cell extracts were then mixed with the probe and competed with 100 fold excess of cold unlabelled probe for self competition or a cold AP-1 probe for non-specific competition (see above for sequence) then run out on a non-denaturing

polyacrylamide gel, dried down and exposed to film as described in section 2.2.3.6 and 2.2.3.7

5.6.2 Experimental Results and Discussion

Although not conclusive the work does suggest that the p53 protein of the E7Q and E7QT2 cell lines is still capable of binding DNA.

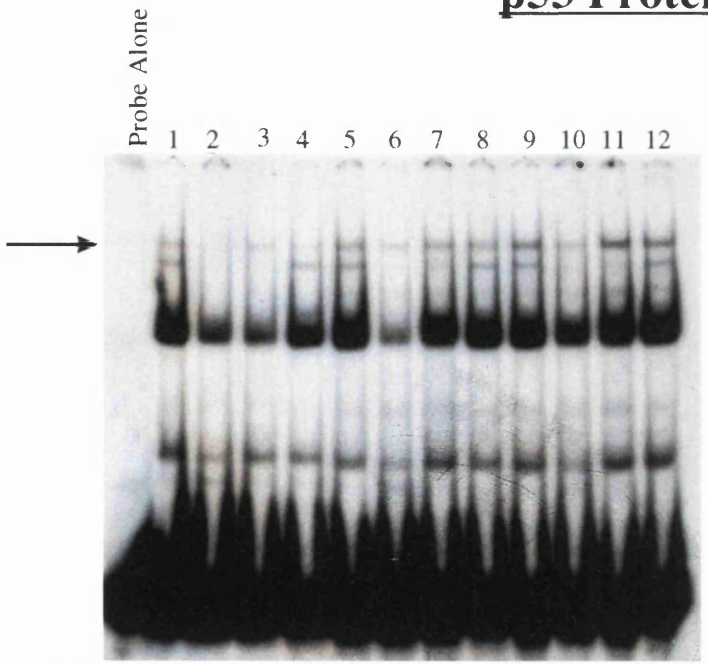
As figures 38 to 41 demonstrate several candidate bands were detected upon incubation of the RGC probe with the PalF cell lysate. However the band indicated with the arrow was determined to be the prime candidate for study, because it was competed by cold RGC probe and not by a non-specific probe, AP-1. This experiment also shows that this putative p53 band is also present in the cell lysates from E7Q and E7QT2.

The analysis continued with work on cell extracts derived from cells treated with bleomycin sulphate. This was in an attempt to elevate the cellular levels of the p53 protein and thus to generate a greater signal in the bandshift assays. Figure 39 again displays the candidate band, marked by an arrow, competed by self oligonucleotide but not by the AP-1 probe. Figure 41 displays the results of the E7R cell line, with the candidate band seen in the cells treated with 0μM quercetin for 36 hours. The comparatively lower band signal generated in the 50μM quercetin cell extracts could be due to protein degradation in these samples rather than any true effect of quercetin on p53 binding.

The stronger lower band seen in figures 38 to 41 is also a candidate band, due to the strength of the signal generated. However this band is sometimes competed by a non-specific competitor indicating that this band maybe the result of non-specific interaction. One possibility is that this band is monomeric p53, as there are 3 higher bands (with the highest band of this quartet being the prime candidate band) with could correspond to dimers, trimers and tetramers respectively.

However this is by no means definite evidence due to the lack of a suitable candidate supershift band, by use of the Bp53.12 antibody. In addition a non-specific competitor more closely resembling the RGC probe such as a mutant form of the RGC sequence would permit greater confidence in the assays.

p53 Protein Binding Assays

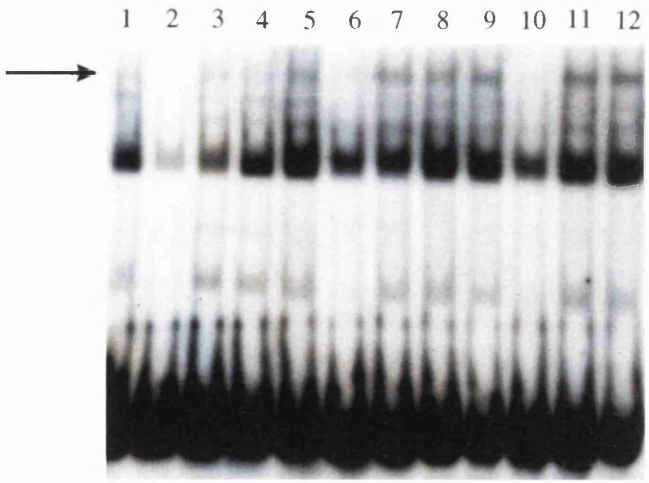


KEY:
1 = PalF cell extract + RGC probe
2 = PalF cell extract + RGC probe + cold RGC Competitor
3 = PalF cell extract + RGC probe + cold AP-1 Competitor
4 = PalF cell extract + RGC probe + Bp53.12
5 = E7Q cell extract + RGC probe
6 = E7Q cell extract + RGC probe + cold RGC Competitor
7 = E7Q cell extract + RGC probe + cold AP-1 Competitor
8 = E7Q cell extract + RGC probe + Bp53.12
9 = E7QT2 cell extract + RGC probe
10 = E7QT2 cell extract + RGC probe + cold RGC Competitor
11 = E7QT2 cell extract + RGC probe + cold AP-1 Competitor
12 = E7QT2 cell extract + RGC probe + Bp53.12

Figure 38. ***p53 DNA binding activity in PalF, E7Q and E7QT2***

Cell extracts were incubated with the ^{32}P end labelled RGC probe and competed for 30 minutes on ice by 100 fold excess of either cold RGC probe for specific inhibition or cold AP-1 probe for non specific inhibition. Where indicated reaction also included the antibody Bp53.12 to attempt to supershift the p53/DNA complex. Prime candidate band for the p53/DNA complex is indicated by the arrow

p53 Protein Binding Assays

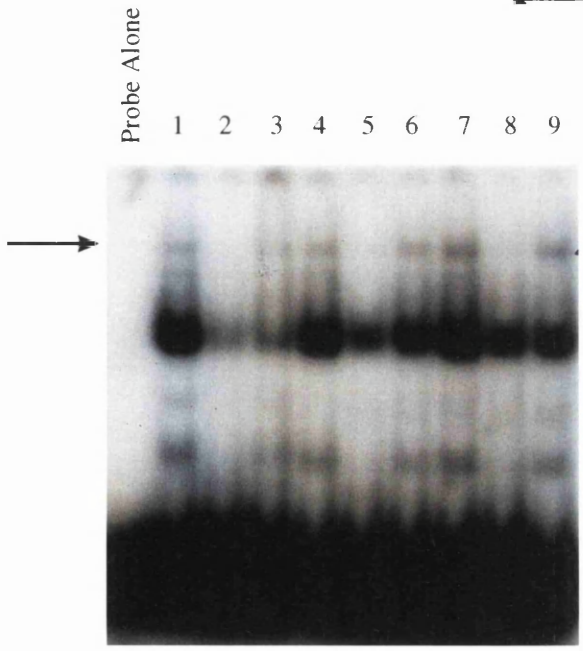


KEY:
1 = PalF cell extract + RGC probe
2 = PalF cell extract + RGC probe + cold RGC Competitor
3 = PalF cell extract + RGC probe + cold AP-1 Competitor
4 = PalF cell extract + RGC probe + Bp53.12
5 = E7Q cell extract + RGC probe
6 = E7Q cell extract + RGC probe + cold RGC Competitor
7 = E7Q cell extract + RGC probe + cold AP-1 Competitor
8 = E7Q cell extract + RGC probe + Bp53.12
9 = E7QT2 cell extract + RGC probe
10 = E7QT2 cell extract + RGC probe + cold RGC Competitor
11 = E7QT2 cell extract + RGC probe + cold AP-1 Competitor
12 = E7QT2 cell extract + RGC probe + Bp53.12

Figure 39. ***p53 DNA binding activity in PalF, E7Q and E7QT2***

Cell extracts were incubated with the ^{32}P end labelled RGC probe and competed for 30 minutes on ice by 100 fold excess of either cold RGC probe for specific inhibition or cold AP-1 probe for non specific inhibition. Where indicated reaction also included the antibody Bp53.12 to attempt to supershift the p53/DNA complex. Prime candidate band for the p53/DNA complex is indicated by the arrow

p53 Protein Binding Assays



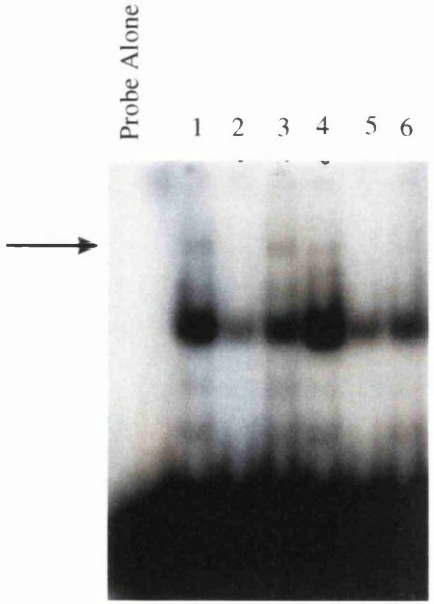
KEY:
1 = PalF cell extract + RGC probe
2 = PalF cell extract + RGC probe + cold RGC Competitor
3 = PalF cell extract + RGC probe + cold AP-1 Competitor
4 = E7Q cell extract + RGC probe
5 = E7Q cell extract + RGC probe + cold RGC Competitor
6 = E7Q cell extract + RGC probe + cold AP-1 Competitor
7 = E7QT2 cell extract + RGC probe
8 = E7QT2 cell extract + RGC probe + cold RGC Competitor
9 = E7QT2 cell extract + RGC probe + cold AP-1 Competitor

N.B. All cell extracts derived from bleomycin treated cells (15μU for 6 hours pre harvesting)

Figure 40. ***p53 DNA binding activity in PalF, E7Q and E7QT2***

Bleomycin treated cell extracts were incubated with the^{32}P end labelled RGC probe and competed for 30 minutes on ice by 100 fold excess of either cold RGC probe for specific inhibition or cold AP-1 probe for non specific inhibition. Prime candidate band for the p53/DNA complex is indicatedby the arrow

p53 Protein Binding Assays



KEY:
1 = E7R cell extract + RGC probe
2 = E7R cell extract + RGC probe + cold RGC Competitor
3 = E7R cell extract + RGC probe + cold AP-1 Competitor
5 = E7R cell extract + RGC probe
6 = E7R cell extract + RGC probe + cold RGC Competitor
7 = E7R cell extract + RGC probe + cold AP-1 Competitor

N.B.
1-3 = cell extracts derived from cells treated with 0μM Quercetin for 36 hours
4-6 = cell extracts derived from cells treated with 50μM Quercetin for 36 hours

Figure 41. ***p53 DNA binding activity in E7R***

Cell were treated with either 0mM or 50mM Quercetin for 36 hours before harvesting. Cell extracts were incubated with the ^{32}P end labelled RGC probe and competed for 30 minutes on ice by 100 fold excess of either cold RGC probe for specific inhibition or cold AP-1 probe for non specific inhibition. Prime candidate band for the p53/DNA complex is indicated by the arrow

5.7 p53 cDNA Sequencing

As a further investigation into the nature of the loss of p53 transcriptional activity in the tumourigenic cell lines, sequencing of the p53 cDNA of E7Q, E7QPα, E7QT2 and Q2D was undertaken. The loss of p53 transcriptional activity in these cell lines may then be attributable to mutations in the sequence resulting in alterations in the amino acid sequence of the protein.

5.7.1 Experimental Method

Total RNA was extracted from the tumourigenic cell lines and the parental PalF cells as described in section 2.2.3.1. 1μg of total RNA was then used to synthesize cDNA and subsequent amplification (section 2.2.3.2.2).

The resultant RT-PCR product was then cloned into pcDNA3+ by digestion with HindIII and XbaI (restriction sites included in the 5' and 3' RT-PCR primers) of the PCR product and vector. Following dephosphorylation of the vector the product was ligated into the pcDNA3+ vector.

DH5α bacterial hosts were then transformed with the ligation products and selected by ampicillin resistance. Plasmid DNA was extracted by use of Qiagen miniprep kits (see section 2.2.1).

Plasmids were then sequenced as described in section 2.2.3.3. Two separate clones were sequenced of each cell type cDNA with a series of primers to sequence the entire cDNA.

5.7.2 Experimental Results and Discussion

The entire p53 cDNAs of the parental PalF, E7Q, E7QT2 and E7QPα were successfully sequenced. p53 cDNA from Q2D was not obtained despite repeated RT-PCR attempts.

Analysis of the sequences showed that the p53 gene in parental PalF cells had undergone no mutations when compared to the published p53 cDNA sequence (Dequiet et al., 1995); however in E7Q and E7QPα two adjacent point mutations, gga to gtt (Position 835 and 836) were detected. These mutations were detected in both DNA strands and in both clones of each cell type. Additionally overlapping sequences over the mutated region consistently confirmed the alteration. These

changes result in an amino acid alteration from Glycine to Valine at codon 278 in bovine p53 (equivalent to codon 271 in human) in conserved domain V, part of the DNA binding domain of the protein (see figure 42).

This may explain, at least in these cell lines, the loss of transcriptional activity of the p53 protein. The mutation being within the conserved DNA binding domains could result in the p53 protein being unable to bind to the DNA, and as such be inhibited in the transcriptional activation of downstream effectors.

5.8 Chromosome Karyotype Analysis

Karyotype analysis was done to determine whether the tumorigenic cells had undergone gross chromosomal abnormalities. This then would provide information on whether the cells had decreased genomic stability due to the inhibition of the p53 protein. In addition identification of chromosomal damage site may identify further possible gene mutations contributing to the transformed phenotype.

Live cell cultures of the E7Q cell line together with the parental PalF cells were sent to collaborators in the laboratory of Dr Stocco dos Santos (Butantan Institute, San Paulo, Brazil) for chromosome analysis as part of a continuing collaboration.

Figure 43 shows the preliminary results. The PalF cells have sustained minor changes possibly due to the isolation of the line, indicated by the marker chromosomes. E7Q however seems to have sustained significantly greater levels of chromosomal abnormalities shown on the spreads labelled E7Q1 to E7Q3.

This initial evidence suggests that the loss of p53 function in these cells may have resulted in a decrease in chromosomal stability. The elevated level of chromosomal damage in these cells could contribute to further alterations in gene expression and ultimately transformed phenotype. Continued passages *in vitro* would be expected to result in more alterations to the karyotype and altered cell phenotype. Further characterisation of the karyotypes of these cells however is required for more support of this hypothesis.

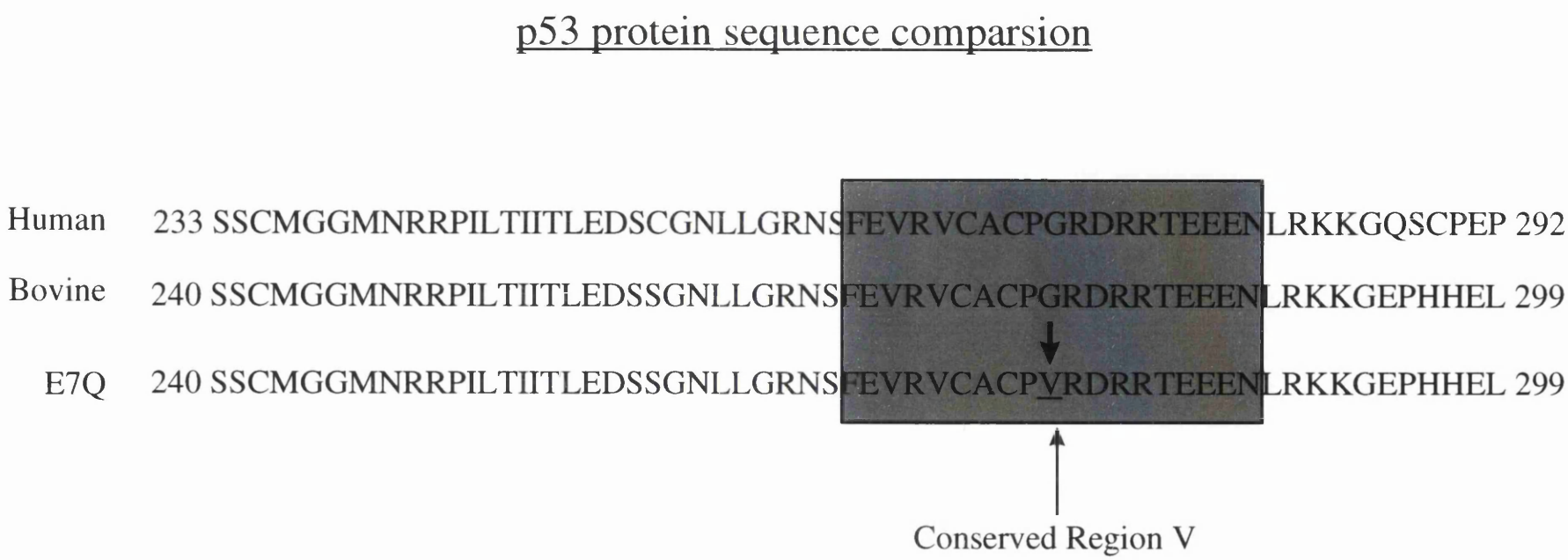


Figure 42. ***Protein sequence alignment of Human and Bovine p53 covering conserved region V***
p53 amino acid sequence. The conserved region V is boxed and the mutated amino acid residue is indicated

Chromosome Spreads of E7Q and Parental PalF Cells

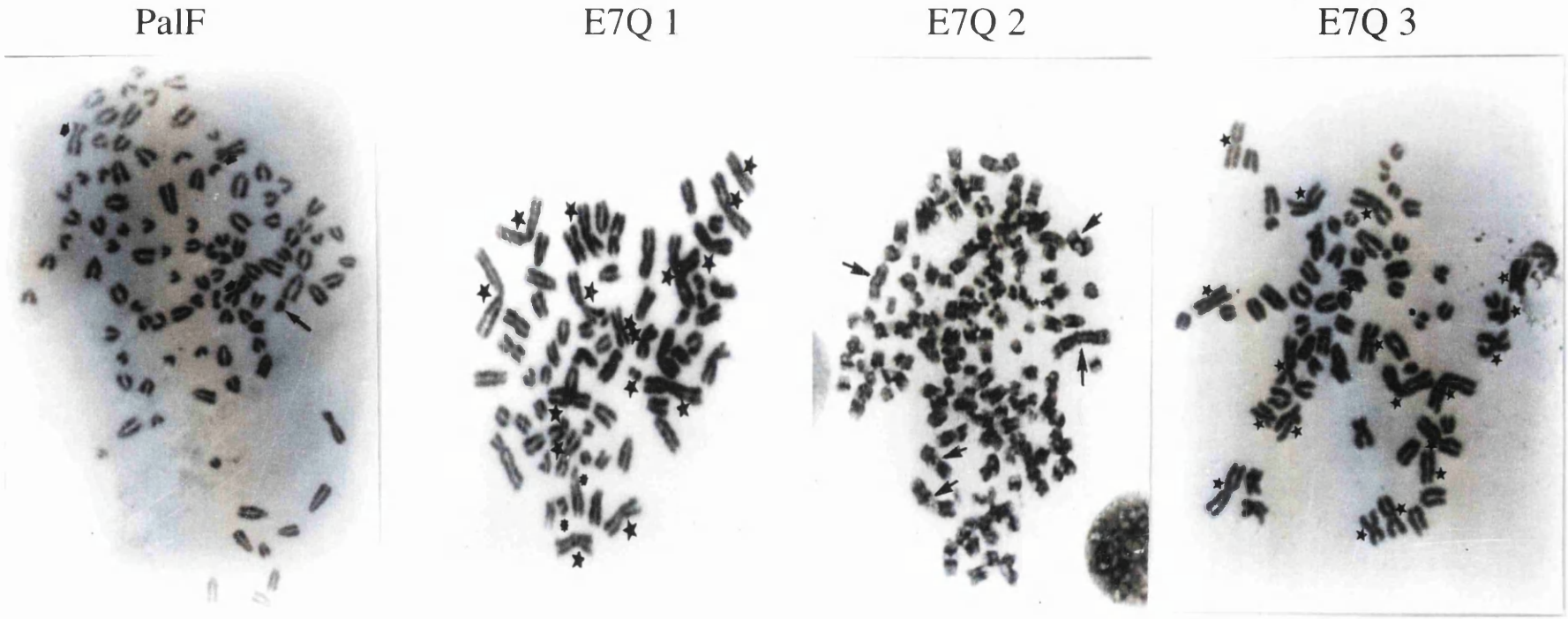


Figure 43. ***Chromosome analysis of E7Q and parental PalF cells***
PalF: Arrows indicate presence of 2 X chromosomes and the Y chromosome along with two marker chromosomes
E7Q 1: 5 point stars indicate chromosomes markers, 8 point starts indicate acentric fragments and breaks
E7Q 2: Arrows indicate regions with no identificable patterns of G banding
E7Q 3: 5 point stars indicate chromosome markers, 8 point starts indicate acentric fragments and breaks

CHAPTER SIX
DISCUSSION

6.1 Introduction

BPV 4 infects the mucosal epithelium of the upper gastrointestinal tract of cattle which results in the development of papillomas (Campo *et al.,* 1981). In cases of immuno-competent animals these papillomas are generally benign and will regress after roughly one year (Knowles *et al.,* 1997).

In the cases of bracken grazing cattle these papillomas persist and can progress to squamous carcinoma (Campo & Jarret, 1986). The persistence of the papilloma is ascribed to the immunosuppressants of the bracken fern (Campo, 1997; Evans W. C. *et al.,* 1982) which make the animals incapable of mounting an adequate immune response against the virally infected cells.

Progression of the persistent papillomas to squamous carcinomas is then likely due to the continued exposure to the mutagens contained in the bracken fern (Evans I. A. *et al.,* 1982) which induce successive genetic lesions. Support for this hypothesis comes from the observation that activation of *ras* (Campo *et al.,* 1990), mutation of p53 (Scobie, 1996) and elevation in EGF receptor levels (Smith *et al.,* 1987) have been observed in naturally occurring alimentary cancers in cattle. These data again support the hypothesis of the multi-stage nature of cancer.

This synergy between the virus and the bracken fern observed in field cases has also been experimentally reproduced (Campo *et al.,* 1994) further supporting the association of the two elements in the carcinogenesis of alimentary canal cancer of cattle.

Of the mutagens found in bracken fern, quercetin has been demonstrated to synergise with the virus in transformation of primary bovine palate fibroblasts *in vitro* (Pennie & Campo *et al.,* 1992; Cairney & Campo, 1995). A single 48 hour exposure of 20μM quercetin 24 hours after transfection of the BPV 4 genome and *Ha-ras* (or BPV 4 E7 and *Ha-ras*) can induce full tumorigenic transformation of

the cell. The alteration of the activity of the viral LCR and phosphotyrosine status of proteins in the cell have been postulated to be involved in the process of transformation of the cell. However it is the ability of quercetin to induce cell cycle arrest that has received the most attention in the studies detailed here.

6.2 Generation and Characterisation of Cell Lines

This work generated a panel of cell lines from normal primary fibroblasts to fully transformed tumorigenic cells. These lines were extensively characterised biologically and molecularly and these studies have yielded a valuable insight into the synergy between viral and chemical factors in cell transformation. However further analysis of the proliferation rates of the different lines would be required for a deeper understanding of their biology and for a more accurate interpretation of the cell cycle profiles. Different numbers of population doubling may have affected the observed results but given that cells were maintained in culture for short periods only, any variation due to culturing would be limited.

The inability to derive clones expressing BPV 4 E7 or activated *Ha-ras* alone is evidence that neither oncogene is sufficient to confer an extended lifespan. The expression of both oncogenes permitted selection of clones for further expansion. However these clones proliferated slowly and most failed to grow *in vitro* for more than a few population doublings. Those clones that underwent sufficient passages for further analysis proved to be morphologically transformed but not anchorage independent or tumorigenic (see below). The expression of both BPV 4 E7 and *Ha-ras* therefore permitted morphologically transformed cells to survive in culture for a short period of time.

Due to the lack of a suitable cell line (i.e. capable of continued tissue culture passage) expressing BPV4 E7 and *Ha-ras* alone without quercetin exposure the generation of a pooled cell populations was then undertaken. This cell line (E7R) in common with other E7 and *Ha-ras* only cell lines proved to be morphologically transformed but incapable of anchorage independent growth. The change of morphology and cell cycle phenotype (see chapters three and four) of

the E7R cell line was hypothesised to be due to decreased expression of the oncogenes. Expression of E7 alone without other viral oncogenes, or other alterations, may result in the triggering of cellular mechanisms designed to detect abnormal regulation of the cell cycle machinery This hypothesis would suggest that persistence of E7, and *Ha-ras,* expression in PalF cells may require further changes to the cell. This is supported by the observation that the cell lines Q0D, E7QPβ and E7QPγ did not change in morphological appearance over the course of *in vitro* culturing. Q0D has E7 expression under the control of the viral LCR and may have a lower basal E7 expression than that derived from the pZipneo plasmid promoter (MoLV). This lower level may be permitted by the cell, or alternatively other viral proteins may compensate for the cellular responses to E7 expression. The fact that neither E7QPβ or E7QPγ changed phenotype may be a consequence of quercetin exposure immediately after transfection. Although not causing the genetic lesions required for the full transformation of the cell, other inheritable events such as gene mutation, or epigenetic mechanisms e.g. altered methylation patterns, perturbation of autoregulatory loops (Macleod, 1996 for review) may have occurred to cause the cell to be permissive to high E7 expression levels, indeed quercetin has been shown to cause hypermethylation (Ishikawa *et al.,* 1987). Such alterations, that are non-transforming, may also be in effect in the Q0D cells to permit E7 expression.

Studies have shown that the locus encoding the tumour suppresser protein $p16^{INK4a}$ also encodes another protein termed $p19^{ARF}$ (in mice, $p14^{ARF}$ in humans) (Quelle *et al.,*1995). Oncogenic signals such as overexpression of Myc, E1A or E2F-1 induce expression of the $p19^{ARF}$ protein and result in an increase in p53 protein levels by binding and inactivation of Mdm2 (a protein which binds p53 and targets it for degradation and is in turn regulated by p53 resulting in a feedback loop) (Sherr, 1998 for review). Elevation of p53 would then in turn result in cell cycle arrest and/or apoptosis. With the case of E7, high levels of E2F due to p105Rb inhibition may also act to induce $p19^{ARF}$ expression. Whilst p53 mediated cell cycle arrest may be ineffective in cells expressing high levels of E7,

apoptosis would probably be unaffected based on the evidence that p53 mediated apoptosis occurs in HPV E7 expressing cells normally (Wang *et al.,* 1996; Iglesias *et al.,* 1998; Stoppler *et al.,* 1998; Jones *et al.,* 1997). However in cells also expressing HPV 16 or 18 E6, p53 would be targeted for degradation and as such inhibited in its capacity to induce apoptosis (Puthenveettil *et al.,* 1996; Thomas *et al.,* 1996). In the case of E7R, this mechanism would explain the change of morphology and cell cycle phenotype, however analysis of not only the E7 expression levels in the cell line over a period of time after transfection would have to be assayed, but also levels of the bovine homologue of $p19^{ARF}$, Mdm2 and p53 would provide information on whether this response was undertaken by the cell. As stated above the cell lines Q0D, E7QPβ and E7QPγ may have compensatory features such as one of the other viral genes or an alterations induced by quercetin to inhibit this $p19^{ARF}$ response to E7 and/or Ha-ras expression. In addition the expression of mutant p53 in the tumorigenic cell lines (see later) may protect these cells from $p19^{ARF}$ mediated apoptosis.

However the activation of $p19^{ARF}$ is not the only mechanism of selection against E7 expression by p53 elevation. Work by Seavey *et al.,* 1999 demonstrated that HPV 16 E7 could stabilise p53 by inhibiting interaction of p53 and Mdm2, through a $p19^{ARF}$ independent mechanism.

The tumorigenic cell lines generated by expression of BPV 4 E7 and *Ha-ras* combined with quercetin exposure and studied, namely E7Q, E7QPα and E7QT2, are morphologically transformed, appear immortal, and grow independent of substrate whilst lacking expression of E8. BPV 4 E8 is associated with conferring anchorage independent growth as, along with E7 and *ras*, expression results in anchorage independent growth of primary bovine fibroblasts. (Pennie *et al.,* 1993) and recent work has shown that E8 expression can also induce anchorage independent growth of NIH3T3 (O'Brien & Campo, 1998). O'Brien and Campo also showed that in the NIH3T3 cells E8 was able to transactivate the cyclin A promoter and to elevate cyclin A levels. Cyclin A is likely to be the major target of adhesion signalling (Guadagno *et al.,* 1993) as expression was

found to be dependent on cell adherence in NRK cells and hence loss of adherence resulted in G1 arrest. Additionally transfection of cyclin A expression vectors resulted in restoration of cyclin A protein levels and cyclin A/cdk2 activity together with entry into S phase and multiple rounds of cell division whilst in suspension.

As mentioned earlier, consistent with previous findings the expression of E7 and *Ha-ras* alone did not confer anchorage independence in the cell line E7R. The failure of E7QPβ, E7QPγ and E7Qb to successfully grow independent of adherence suggested the expression of E7, *Ha-ras* combined with quercetin treatment does not automatically confer anchorage independence. However the inhibition of the transcriptional activity of p53 (evident in the tumorigenic cell lines), as a result of quercetin exposure, may be pivotal to the development of the anchorage independent phenotype in the absence of E8 expression. Supportive evidence for this are the experiments carried out with a human mutant p53 in PalF cells demonstrating that the mutant p53 was capable of conferring anchorage independent growth (Scobie *et al.*,1997).

The tumorigenic cell line Q2D expresses E8 and has an inhibited p53. This cell line is morphologically transformed, immortal and grows independent of substrate. It is unknown whether one or both of these events result in the loss of anchorage independence. E8 may cause upregulation of cyclin A whilst loss of function by p53 may result in loss of activation of other genes required for cell cycle arrest due to anchorage loss. A possibility would be a role for $p21^{Waf1/Cip1}$, which is elevated by p53, to inhibit the cyclin A/cdk2 complex under conditions of anchorage loss. Also the effect of p53 may be more indirect and contribute to the phenotype due to increased levels of genomic instability, resulting in further genetic lesions impacting upon the adhesion dependence signalling pathway.

The failure of the cell line Q0D to grow independent of substrate contradicts previous findings of Pennie *et al.,* 1993. This may be due to loss, or decrease, of E8 expression in this cell line upon continued passaging or other

undetermined alterations. The cell line however maintains a transformed morphology due to expression of BPV 4 E7 and Ha-ras.

Interestingly the analysis of the ability to proliferate independent of substrate correlated well with tumorigenic status whilst morphological transformation did not. This may well represent the fact that anchorage independence is a feature of cells at a later stage of transformation. The causative agent in the tumorigenic cells that permits anchorage independent growth is not yet pinned down and there may be different factors in each of the cell lines.

The highest *Ha-ras*, and possibly E7, expression was obtained with the tumorigenic cell lines with the exception of 88529B. The lack of high level *Ha-ras* expression in 88529B may be a cell type specific effect or due to other compensating alterations to the cell such as over expression of cyclin D or a constitutively active Raf. The fact that this cell line also has lost the BPV4 genome, and consequently E7 expression, suggests that the cell may well have undergone a greater number of genetic lesions than the cells derived *in vitro* in order to remain transformed and tumorigenic without the effects of the two oncoproteins E7 and Ha-ras.

The high level *Ha-ras* expression, in fibroblasts, may be required for tumorigenicity in order to supply a significant proliferative drive to the cell combined with a high E7 expression to permit efficient inhibition of the p105Rb tumour suppresser protein. Such a high level of Ha-ras may well have been selected for as it exceeds that of cells normally expressing *Ha-ras* from the pT24 plasmid e.g. E7R and Q0D (figure 9). Evidence for elevation of E7 expression comes from the RT-PCR product level (figure 8) related to the initial template level used in each reaction i.e. tumorigenic cells generated greater product level with less starting template. This evidence however is circumstantial and further investigation by more quantitative mechanisms is required to prove whether E7 expression is elevated in tumorigenic cells.

The activated Ras oncoprotein induces DNA synthesis and cell proliferation (Downward, 1997) and in cells with a functional Rho GTPase

suppresses $p21^{Waf1/Cip1}$ (Olson *et al.,* 1998). Ras expression is highest in the tumorigenic cells and undoubtedly activated Ras contributes to PalF transformation, with BPV 4 E7, maybe through further inhibition of $p21^{Waf1/Cip1}$. Additionally Ras activates the MAPK pathway causing upregulation of cyclin D (Downward, 1997; Kerkhoff & Rapp, 1998) and there are reports that cyclin E/cdk2 complexes are activated by Ras/Raf signalling via *c-myc* (Kerkhoff & Rapp, 1998 and references therein). Supportive of this was the report that Cyclin E/cdk2 complexes are normally inactive in cells that have lost adhesion signalling. However in *Ha-ras* transformed NRK cells the cyclin E/cdk2 complexes remained active (Carstens *et al.,* 1996). Therefore the activation of both the cyclin D and E complexes would also cause an increase in the inactivation of p105Rb by phosphorylation. It is worth stressing that neither activated Ras alone nor E7 alone are capable of PalF cell transformation, even partial. Hence it is likely that both oncogenes need to alter the control of the p105Rb protein in order to achieve sufficient inhibition to permit cell cycle passage. Cumulative inhibition of the $p21^{Waf1/Cip1}$ CDKI by both *Ha-ras* signalling and directly by E7 would also be hypothesised to be more efficient that by one of the oncogenes alone.

As can be seen in table 3.2 (chapter three) the number of clones isolated decreased significantly upon treatment of the transfectants with quercetin. This drop in clone frequency could be due to quercetin's action(s) causing the elevation of p53 and consequently cell cycle arrest (see later also). This cell cycle arrest may then progress to become apoptosis if the cell 'decides', possibly via a mechanism dependent on p53 level, that the cumulative stress of the transfection of DNA, expression of the oncogenes and quercetin exposure makes the cell non-viable for continued controlled proliferation (Chen *et al.,* 1996; Ko & Prives, 1996 for review). In those cells with sufficient BPV 4 E7 and *Ha-ras* expression at the time of quercetin exposure, cell cycle arrest would fail to occur in G1 due to the inhibition of p105Rb and $p21^{Waf1/Cip1}$ by E7 and *Ha-ras* signalling. *Ha-ras* signalling would also generate a proliferative drive by activation of the MAPK pathway (see later). Whether BPV 4 E7 and/or *Ha-ras* would impact upon the

apoptotic functions of p53 (or p53 independent mechanism) is unknown. However due to the lower level of clone formation upon quercetin exposure of the cells this would argue against an inhibition of apoptosis by BPV 4 E7 and *Ha-ras*. For the transfected cells to avoid entering apoptosis the heritable damage that occurs under quercetin exposure could not exceed a certain threshold. Beyond this threshold the cellular insult would be too great and apoptosis would ensue. However below the threshold, although genetic lesions may occur due to the abrogation of the G1 cell cycle arrest, apoptosis would not be initiated and the alterations would be inherited.

The alterations generated from the quercetin exposure would be largely untransforming, however mutations to key cell regulators i.e. oncogenes and tumour suppresser genes would promote progression to full transformation. The balance of damage sustainable by the cell for continued proliferation and the target requirement of the genes to promote transformation would explain the low frequency of tumourigenic clones resulting from this *in vitro* system.

Note though that the inheritable alterations that occur in the tumorigenic cells need not to have happened in the time of quercetin exposure. Due to quercetins wide ranging effects on the cell (see section 1.4.3.4) it would be feasible for exposure to the chemical to generate a 'mutator' phenotype for a limited duration. This maybe due to a variety of epigenetic mechanisms (Macleod, 1996 for review) and enzymatic inhibition sufficient to permit accumulation of genetic lesions.

6.3 Quercetin-Induced G1 Arrest is Abrogated in E7 Expressing Cells.

Quercetin arrests primary bovine palate fibroblasts in both the G1 and G2/M phases of the cells. Upon quercetin exposure there was a noticeable increase in the percentage of cells in S phase 12 hours after initiation of the chemical treatment. Whether this was due to an elevation of entry into DNA synthesis or a lag of cells exiting DNA synthesis is unknown. Due to the potential for quercetin to induce DNA damage (see section 1.4.3.4) this may well suggest a stall in DNA

synthesis as the DNA adducts induced by quercetin have to be repaired. Additionally quercetin has been shown to be able to inhibit DNA polymerases (Ono & Nakane, 1990) by competition with the template.primer complex. Quercetin has also been shown to inhibit RNA polymerase II (Nose, 1984) which may well contribute to the putative stall in S phase by slowing expression of essential genes. The general lower level of DNA synthesis after 48 hours of quercetin exposure may favour this hypothesis. However also possible could be the accelerated entry of cells into S phase. This could be envisaged by quercetin altering the phosphorlyation states of key proteins such as transcription factors and signalling molecules also by altering enzyme activities as described in section 1.4.3.4 resulting in the expression of genes required for DNA synthesis.

The IC50 for other cells exposed to quercetin i.e. the concentration of quercetin required for 50% inhibition of growth, has been reported to be between 1nM to 1μM (Scambia *et al.,* 1990b; Ranelletti *et al.,* 1992; Larocca *et al.,* 1995; Piantelli *et al.,* 1995) although some cell lines have been reported to require a greater concentration of 20μM or more (Hosokawa *et al.,* 1990; Avila *et al.,* 1994). For those cell lines which undergo cell cycle arrest at low concentrations of quercetin this may be linked to the presence of high levels of type II oestrogen binding receptors (Ranelletti *et al.,* 1992) which are also elevated by quercetin exposure (Scambia *et al.,* 1993). This link with type II oestrogen binding receptors is reinforced by studies showing that the affinity of a flavonoid for the type II oestrogen binding receptors correlates with the ability to induce cell cycle arrest (Ferrandina *et al.,* 1998; Scambia *et al.,* 1990; 1990b) Those cell lines which require high quercetin levels may therefore contain low levels of the type II oestrogen binding receptors and arrest due to quercetin's other effects such as enzymatic inhibition (see section 1.4.3.4). With quercetin arresting PalF cells at a concentration of 20μM or more it is evidence that these cells contain low levels of type II oestrogen binding receptors. Quercetin exposure has also been documented to cause reversible cell cycle arrest (Scambia *et al.,* 1990b; Ranelletti *et al.,* 1992)

which has been corroborated in these findings on both the PalF cells and the tumorigenic cell line E7QT2.

Contrary to normal PalF cells which arrest in G1 and G2/M in the presence of quercetin (Connolly *et al.,* 1998), all the transformed cells failed to arrest in G1. These cells however maintained the G2/M check point. This observation was also paralleled by the data demonstrating that the tumorigenic cells transformed by transfection of the BPV 4 genome and *Ha-ras* with quercetin exposure also failed to arrest in G1 upon re-exposure to quercetin yet maintained the ability to arrest in G2/M.

The cell line E7QPβ also demonstrated an abrogated G1 arrest and a maintained G2/M arrest. The fact that the line was not tumorigenic yet failed to arrest in G1 demonstrated that alteration of the cell cycle regulation of the cell was in itself not sufficient for the tumorigenic state.

In the cells transformed by transfection with the BPV 4 genome and *Ha-ras,* i.e. Q0D, the G1 arrest in response to exposure to quercetin also failed to occur. The importance of this data was that it displayed that the expression of the activated oncogene in addition to the viral oncogenes was sufficient for the abrogation of the G1 arrest.

The prime candidate thought to be responsible for the abrogation of this arrest was the BPV 4 E7 oncoprotein, due to the protein's shared features with the HPV 16 E7, namely the putative p105Rb and the zinc binding fingers (Jaggar *et al.,* 1990). These domains are known to be important to the transforming properties of the protein as determined by mutation analysis (Campo *et al.,* 1994b; Jackson *et al.,* 1996). Additionally to this, the evidence from the experimental data on HPV E7 proteins (see section 1.4.2.1 for details) would also support the hypothesis that the BPV 4 E7 protein could be capable of abrogating a G1 arrest.

The data from the studies of the cell line E7R which expresses the BPV 4 E7 oncoprotein in the context of an activated *Ha-ras* gene was an initial approach to the analysis of this question due to the lack of E7 or *Ha-ras* only expressing cell lines.

Exposure to quercetin resulted in the cells accumulating in G2/M only, thus abrogating the G1 arrest. However this phenotype was only present in low passage E7R cells. Higher passages resulted in the cells arresting in G1 and G2/M like the parental PalF cells. While unsubstantiated, the reversion of the cells was hypothesised to be due to alteration in levels of the E7 oncoprotein (see later).

An attempt to test cells expressing only BPV 4 E7 was undertaken by use of tagging transiently transfected cells using the CD20 cell surface marker. However whilst significant numbers of cells were able to be detected as CD20 positive, cell cycle profile distribution was unattainable. Further experimentation to increase transfection efficiency together with reduction of non specific antibody binding may result in successful analysis of E7-only transfected cells by this mechanism.

The G2/M check point is reached after 36-48 hours of quercetin-treatment, well beyond the quercetin-exposure period used for cell transformation (24 hours). Although when treated with quercetin in the transformation schedule some cells will have already traversed G1 and will therefore stop in G2/M, the majority of the cells would be upstream of the G1 check point. Therefore our study concentrated on what mechanisms allow the transformed cells to overcome the quercetin-induced G1 arrest and to traverse through G1 to complete the cell cycle.

In summary the data presented shows that the BPV 4 E7 oncogene in the context of an activated *Ha-ras* confers the ability to bypass quercetin induced G1 arrest. The retained ability to bypass the G1 arrest over continued passages may also rely on expression of other viral genes as evidenced by the cell line Q0D which retained the ability to overcome quercetin induced G1 arrest even after prolonged passaging in cell culture. Alternatively further genetic lesions may also aid retention of high E7 expression.

6.4 p53 Malfunction in Transformed Cells.

In normal PalF cells, quercetin-induced G1 arrest is accompanied by a stabilisation and an increase in transcriptional activity of p53, consonant with

DNA damage induced by quercetin (Fazal *et al.,* 1990; Suzuki *et al.,* 1991) and subsequent activation of p53 (Levine, 1997). In transformed cells, the behaviour of p53 differs depending on whether or not the cells are tumorigenic. In both quercetin-untreated and quercetin-treated non tumorigenic cells, p53 is stabilised as well as transcriptionally activated upon re-exposure to quercetin, whereas in quercetin-treated tumorigenic cells p53 is transcriptionally dead, although still capable of being stabilised or, as in the case of Q2D, constitutively stable.

How can the abrogation of G1 arrest in non tumorigenic cells be reconciled with an activated, transcriptionally functional p53? In other systems, E7 has been shown to bind and inhibit $p21^{Waf1/Cip1}$, thus preventing DNA damage-induced cell cycle arrest (Funk *et al.,* 1997) and additionally Ha-ras signalling has also been implicated in $p21^{Waf1/Cip1}$ inhibition (Olson *et al.,* 1998). $p21^{Waf1/Cip1}$ is an inhibitor of G1 cyclin-dependent kinases (Harper *et al.,* 1993) and its expression is transcriptionally regulated by p53 (El-Deiry *et al.,* 1993). Inhibition of p105Rb by E7 (Vousden, 1994) and Ha-ras signalling causing upregulation of cyclin D (Downward, 1997; Kerkhoff & Rapp, 1998) and activation of cyclin E/cdk2 complexes (Kerkhoff & Rapp, 1998; Carstens *et al.,* 1996) would also serve to promote progression through the G1 checkpoint by elevation of the levels of free E2F allowing expression of the genes required for entry into DNA synthesis. Accordingly, in our non tumorigenic transformed cells, where p53 transcriptional activity is unaltered, $p21^{Waf1/Cip1}$ is elevated but fails to cause a G1 arrest and is likely to be inhibited by E7 and possibly Ha-ras signalling. The quercetin-induced increase in LCR transcription activity (Connolly *et al.,* 1998) is likely to lead to increased expression of E7 and thus further inhibition of $p21^{Waf1/Cip1}$ and p105Rb.This would explain how p53, apparently wild-type in its behaviour, fails to arrest the cells in G1.

In contrast, in tumorigenic cells p53 is transcriptionally non functional and accordingly there is no production of $p21^{Waf1/Cip1}$. The lack of p53 transcriptional activity in E7Q, E7QPα and E7QT2 can be ascribed to the mutation detected in their p53 sequence. The glycine to valine substitution has taken place in conserved

region V, in the DNA binding domain (Ko and Prives, 1996). Although not tested directly, the mutation is the most likely reason for the malfunction of p53 in the tumorigenic cells. The report of inhibition of p53 transcriptional activity by E7 (Massimi *et al.,* 1996; Massimi & Banks, 1997) indicates this function may also be responsible for the inhibition. The low transcriptional activity of the wild type human p53 in the tumorigenic cells as compared to that seen in PalF cells suggested that there may be inhibition of the p53 transcriptional activity. However the possibility of the mutant bovine p53 forming complexes with the human p53 or competing for binding sites, and therefore inhibiting is a complication.

The fact that E7Q and E7QPα have exactly the same mutation raises the possibility that they are in fact two populations of the same cell line. However despite this similarity the two cell lines show different western blot profiles for p53 protein levels upon treatment with quercetin or other DNA damaging agents. The mutation found in the E7Q, E7QPα and E7QT2 cell lines may be characteristic of quercetin induced mutation. Sequencing of Q2D would help to ascertain whether this is the case.

p53 mutations have been detected in two naturally occurring cancers in bracken fed cattle (Scobie, 1997). In these cases the same mutation, CCC to ACC, was found at codon 243 in conserved region IV, leading to a proline to threonine substitution. Further analysis of p53 mutations in both naturally occurring cancers and *in vitro* transformed cells will be needed to determine whether the mutations detected so far are particular to bracken/quercetin-exposed cells.

It is interesting to note that transformation by BPV 4, a papillomavirus lacking the E6 ORF, requires the inhibition of p53 for PalF cells to become tumorigenic. In essence the resultant effects of quercetin exposure are replacing the requirement of some, but not necessarily all, of the E6 protein functions for full transformation.

Further evidence for the loss of p53 function in the tumorigenic cells comes from the karyotype analysis of the E7Q cell line. This cell line contains a series of chromosome alterations in comparison to parental PalF cells suggesting a

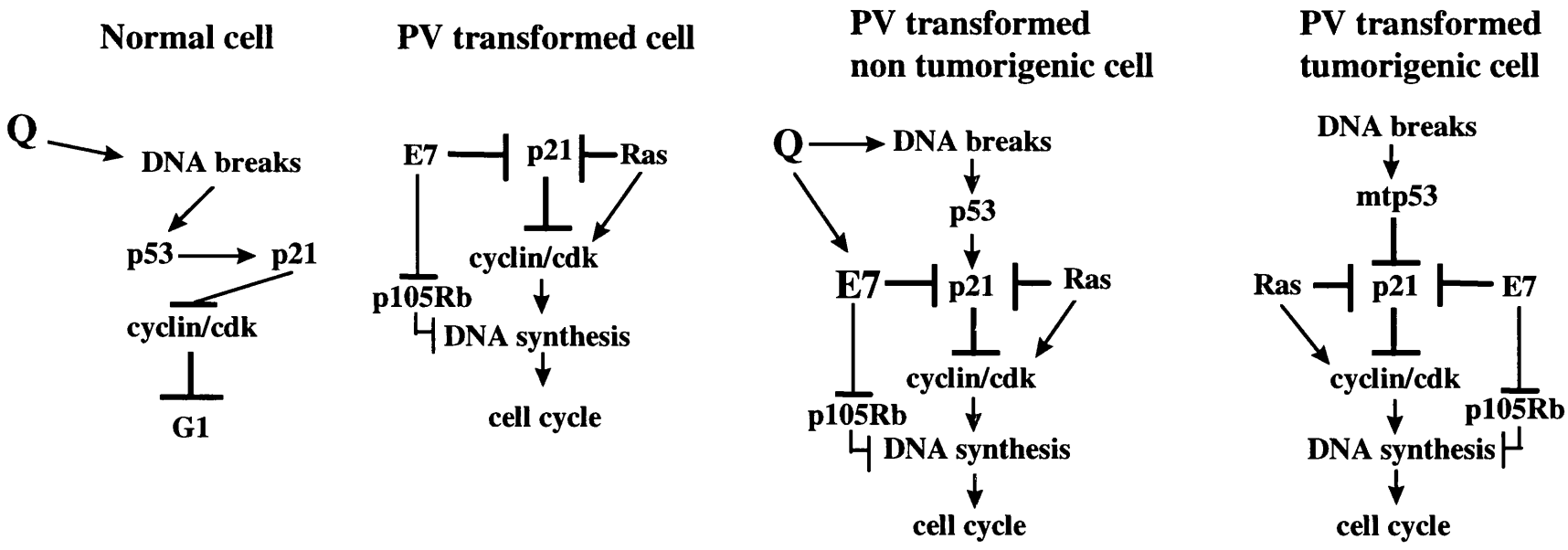


Figure 44. ***Synergy between quercetin and papillomavirus E7 transforming protein.***
In a normal cell, quercetin induces DNA breaks, with consequent activation of p53 and $p21^{Waf1/Cip1}$, inhibition of cyclin/cdks and cell cycle arrest in G1. In a papillomavirus (and Ras) transformed cell, E7 binds and inhibits p105Rb and $p21^{Waf1/Cip1}$, G1 cyclin/cdks are active and the cell goes into cycle. Exposure of this cell to quercetin results in DNA breaks and activation of the viral LCR with increased expression of E7; p53 is activated but $p21^{Waf1/Cip1}$ is inhibited by E7 and Ras resulting in G1 arrest abrogation. During the proliferation of this cell, p53 gene mutations take place and the cell is fully transformed and tumorigenic.

decrease of genomic stability, a phenotype associated with loss of p53 function (Hollstein *et al.,* 1991)

6.5 Conclusions.

Considering all the above observations together, we propose the following series of events as being the most likely to account for the synergism between virus and quercetin resulting in full tumorigenic cell transformation (figure 44): quercetin induces DNA damage and in normal cells this causes G1 arrest through the activation of p53 and $p21^{Waf1/Cip1}$.

In cells containing the viral genome, quercetin trans-activates the LCR with consequent elevated expression of the viral transforming protein E7. G1 arrest is abrogated as p105Rb and $p21^{Waf1/Cip1}$ are inhibited by E7, together with the putative inhibition by Ha-ras signalling, and the traversing of G1 by damaged cells allows their expansion into transformed clones. During clonal expansion, subsequent p53 mutations render the cells fully tumorigenic. This critical step will further increase the genomic instability of the cell and thus lead to further damage. Indeed chromosomal analysis of quercetin-treated fully transformed cells has revealed numerous abnormalities (see figure 43) (Stocco dos Santos, Beniston and Campo, unpublished observations), in agreement with previous results (Ishidate, 1988) and with the observed chromosomal instability in cells with non functional p53 (Ko and Prives, 1996).

The requirement for activated Ras for BPV-4 *in vitro* cell transformation and for quercetin exposure and mutant p53 for cell progression to tumorigenicity parallels the activation of Ras and the mutations of p53 observed in naturally occurring cancers in bracken-feeding cattle (Campo *et al.,* 1990; Scobie, 1996). This supports the view that the interplay between E7 and quercetin observed *in vitro* cell transformation may well play a vital role in carcinogenesis of the upper GI tract. What is the relevance of the synergy between BPV-4 and bracken to human cancer? In humans too, exposure to bracken fern, whether in the diet or as spores, has been epidemiologically linked to oesophageal and gastric cancer in

several parts of the world (Galpin *et al.,* 1990; Marliere *et al.,* 1995; Alonso-Amelot *et al.,* 1996; Hirayama, 1979; Villalobos-Salazar *et al.,* 1995) and quercetin has been shown to be absorbed by humans into the blood stream (Paganga & Rice-Evans, 1997). All forms of culinary bracken have been shown to be carcinogenic in experimental animals (Santos *et al.,* 1987, 1992) and DNA adducts have been found in the upper GI tissue of mice fed bracken extracts or bracken spores (Povey *et al.,* 1996). Human papillomavirus (HPV) type 16 has been found in approximately 50% of cancers and pre-cancers of the upper GI tract (Chang *et al.,* 1990; Togawa *et al.,* 1994; Suzuk *et al.,* 1996) and Campo and colleagues have detected HPV-16 DNA in biopsies of oesophageal cancer from Brazil (unpublished observations). These findings suggest that some cases of cancer of the upper GI tract in humans may have the same aetiology as in cattle, i.e. papillomavirus and bracken.

The unpublished observation that, like the LCR of BPV-4, also the LCR of HPV-16 is trans-activated by quercetin, opens up the possibility that the molecular mechanisms we have started elucidating for cell transformation and cancer in cattle operate also in humans.

6.6 Future Work

A more indepth analysis of cell doubling and growth rates should have been made for each individual cell line. This would have controlled for any variation in behaviour due solely to cell age. The avoidance of continued culturing and the periodic use of fresh stocks, lend confidence that the conclusions presented here are justified.

To continue the work detailed here the initial experiments would focus on the p53 status of the tumorigenic cells and the expression of E7. Analysis of the p53 sequence in Q2D would demonstrate whether the inhibition of the protein is likely to be by mutation rather than through the action(s) of E7. Also the type and position of mutations detected, if any, would show whether quercetin caused characteristic mutations.

Determination of the expression levels of BPV 4 E7 in the PalF cells with an activated Ha-ras by either accurate western blotting, post development of a suitable antibody, or quantitative RT-PCR would help ascribe loss of the phenotype in the E7R to the putative loss of E7 expression. Additionally the use of a retroviral transfection system would be invaluable to the study of E7 in the ability to bypass p53 induced G1 arrest. Transient transfection with a retroviral E7 vector followed by quercetin exposure would allow cell cycle analysis of E7-only expressing cells. Alternatively an inducible system of E7 expression from a mammalian expression vector would allow an equivalent analysis.

Continuing the further analysis of BPV 4 E7 action in the cells could include the analysis of p105Rb phosphorylation status and $p19^{ARF}$ expression levels to prove experimentally whether the BPV 4 E7 is capable of inhibiting the p105Rb protein and activating the $p19^{ARF}$ response. This would also open the possibility to determine whether cells expressing high levels of E7 and/or Ha-ras selected for loss of p53 and/or $p19^{ARF}$ in order to sustain high expression of the oncogene(s).

A greater number of tumorigenic clones to study, as well as an efficient form of transient assays for oncogene expression would permit a broader scope of analysis. This would determine with greater clarity whether p53 inhibition by mutation was a consistent event in progression to full transformation. In addition the study of expression and function of other candidate genes, such as $p19^{ARF}$ or $p21^{Waf1/Cip1}$, for transformation could be studied with greater statistical backing.

The further analysis of quercetins action upon the cell is also desirable. The determination of whether quercetin is capable of causing DNA damage in the PalF cells at the concentrations used in these studies is required to prove that the chemical agent could be directly responsible for the p53 mutation. Also this would give a causative link between quercetin exposure and p53 protein stabilisation and subsequent cell cycle arrest. The use of the Comet and Tunel assays to detect single strand DNA breaks *in vivo* would be invaluable in this study. However additional safeguards would have to be carried out in order to ensure that the DNA

damage observed was due to quercetin and not through induction of apoptosis. This could be undertaken by examination of poly(ADP-ribose) polymerase (PARP) cleavage levels and the apoptotic promoting protein, Bak, levels. PARP causes a burst of poly-ADP-ribosylation of nuclear proteins required for onset of apoptosis in some cell lines (Simbulan-Rosenthal *et al.,* 1998). PARP is then cleaved, which is catalysed by caspase 3, and is proposed to prevent depletion of NAD and ATP (both are PARP substrates) which are thought to be required later on in apoptosis (Berger, 1985). Bak is one of the Bcl2 family of proteins which influence the activation of the family of caspase proteases. These caspases then cleave a number of cellular proteins to bring out cell death (Rao &White, 1997)

The generation of the bovine p53 expression plasmids in this study also permits further analysis. One experiment would be to re-introduce the bovine p53 expression into the tumorigenic cell lines. This would help determine whether re-introduction of the protein was capable of reverting the cell to a non tumorigenic phenotype. However experiments with the human p53 expression plasmid suggested that although there may be inhibition of the transcriptional activity of the exogenous p53, apoptosis was still activated due to the low transfection efficiencies. Also the observations that the tumorigenic cell lines seem to undergo a significant amount of cell death whilst proliferating in cell culture and exposure to DNA damaging agents reduce transfection efficiencies (personal observations) suggests that the apoptotic pathways is still functional in these cells. Whether these pathways are p53 dependent or not is unknown.

In addition to re-introduction of the wild type p53 into the tumorigenic cells, a more interesting experiment would be to introduce the mutant p53 into cells expressing E7 and Ha-ras such as E7QPβ, E7QPγ or E7R (low passage), or indeed carrying out transfections with pZipneo E7, pT24 and pBov53mut (mutant p53 cDNA from E7Q cloned into pcDNA3+). The transfection of PalF cells with the pBov53mut plasmid by itself would also have to be carried out to ensure that in isolation this was not able to cause full transformation. All of these would help

determine whether these three alterations of the cell were capable of causing full transformation.

The study by Scobie *et al.,* 1997 showed that a mutant human p53 by itself or in conjunction with the BPV 4 genome was unable to generate tumorigenic clones, and that this required the addition of HPV 16 E6. These experiments suggest that further changes to the cell would be required to induce tumorigenicity, however the use of a different mutant p53 may be a crucial difference in the system.

The comparison of E7QPβ and E7QPγ to E7R, upon the addition of the mutant bovine p53, would also shed light into whether quercetin exposure generates other inheritable changes required for transformation apart from p53 mutation. Treatment of the pZipneo E7, pT24 and pBov53mut PalF transfectants with quercetin would eliminate possible errors induced by examination of already established cell lines.

As described earlier introduction of human p53 into the tumorigenic cell lines indicated that there may be inhibition of the protein by E7 in reporter assays. Further experimentation could be carried out with the bovine p53 to determine whether the E7 oncoprotein was capable of inhibiting it. However due to the likelihood of inhibition by the mutant p53 in the tumorigenic cell lines this would have to be carried out in other cells with do not contain mutant p53 but have high E7 expression levels. Alternatively the use of *in vitro* translated proteins in an *in vitro* cell free reporter system could help determine whether this inhibition occurred *in vivo.*

Analysis of whether HPV could synergise with quercetin exposure in this *in vitro* system using primary human cell lines would be invaluable to determine quercetins contribution to oesophageal and gastric cancer. Also other chemicals such as ptaquiloside and α-ecdysone which are found in the bracken fern could be assayed singly, or in combination, for any synergy with the papillomavirus. The use of primary human cell lines would also permit a broader scope of analysis due to reagent availability for human systems.

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