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Quercetin Elevates $p27^{Kip1}$ and Arrests Both Primary and HPV16 E6/E7 Transformed Human Keratinocytes in G1

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ABSTRACT

Our previous work with primary bovine fibroblasts demonstrated that quercetin, a potent mutagen found in high levels in bracken fern (*Pteridium aquilinum*), arrested cells in G1 and G2/M, in correlation with p53 activation. The expression of bovine papillomavirus type 4 (BPV-4) E7 overcame this arrest and lead to the development of tumourigenic cells lines (Beniston *et al.,* 2001). Given the possible link between papillomavirus infection, bracken fern in the diet and cancer of the upper gastrointestinal (GI) tract in humans, we investigated whether a similar situation would occur in human cells transformed by human papillomavirus type 16 (HPV-16) oncoproteins. Quercetin arrested primary human foreskin keratinocytes in G1. Arrest was linked to an elevation of the cyclin dependent kinase inhibitor (cdki) $p27^{Kip1}$. Expression of the HPV16 E6 and E7 oncoproteins in transformed cells failed to abrogate cell cycle arrest. G1 arrest in the transformed cells was also linked to an increase of $p27^{Kip1}$ with a concomitant reduction of cyclin E associated kinase activity. This elevation of $p27^{Kip1}$ was due not only to increased protein half-life, but also to increased mRNA transcription.

INTRODUCTION

Human papillomaviruses are small DNA tumour viruses, which can infect mucosal and cutaneous epithelia, giving rise to benign hyperproliferative lesions. These lesions are under host immune surveillance and can regress spontaneously. Of the greater than 100 papillomaviruses now typed, roughly 30 types preferentially infect the mucosa of the anogenital tract. These HPVs can be grouped into high and low risk types. The high-risk types are those inducing lesions that can progress to squamous cell carcinoma. The rate of progression may be low, but nevertheless infection with high risk HPVs accounts for nearly all-human cervical cancers (for review see zur Hausen, 1996). The HPV types most commonly linked with genital cancer are 16 and 18, and less frequently types 31, 33, 35 and 39. As well as genital cancer these high-risk viruses have been linked to oropharyngeal carcinoma (Gillison *et al.*, 2000)

High risk HPV encode at least three different growth stimulating and transforming proteins: E5, E6 and E7. These proteins have an expanding number of cellular targets and functions (for review zur Hausen, 2000). E6 targets several tumour suppressor and anti-apoptotic proteins, perhaps the most important of which is p53 (Mantovani and Banks, 2001). These interactions cause the rapid degradation of the target proteins with consequent cell immortalisation. The E5 protein from HPV interacts with the 16K subunit c protein in mammalian cells and yeast (Conrad *et al.*, 1993, Ashby et al, 2001) and, through this interaction, down-regulates gap junction intercellular communication (Oelze *et al.*, 1995) and alters the acidification of endosomes (Straight *et al.*, 1995) leading to continuous signalling from growth factors receptors. The E7 oncoprotein has many roles (for review see Munger *et al.*, 2001). One main function of E7 is to bind the pocket proteins causing the dissociation of the transcription factor E2F. Additionally E7 causes the destabilization of the p105Rb protein (Boyer *et al.*, 1996; Jones *et al.*, 1997b) and interferes with the association between histone deacetylase and p105Rb (Brehm *et al.*, 1998). Among the other cellular factors with which E7 is thought to interact are the cyclin dependent kinase inhibitors $p21^{Waf1}$ and $p27^{Kip1}$. These proteins are potent inhibitors of cyclin E and A complexes, and may be positive regulators of the cyclin D complexes (Sherr and Roberts, 1999 for review). E7 directly binds to $p21^{Waf1}$ and inhibits both its effect on the cyclin/cdk complexes

and ability to inhibit DNA replication by binding PCNA (Jones *et al.,* 1997a; Funk *et al.*, 1997). The related cdki, $p27^{Kip1}$, is also bound by E7, resulting in abrogation of the inhibitory activity on cyclin/cdk complexes (Zerfass-Thome *et al.,* 1996). The combination of the HPV16 E6 and E7 oncoproteins has been shown to be sufficient to immortalise primary human keratinocytes (Hawley-Nelson *et al.,* 1989).

Progression of PV induced lesions to carcinoma requires co-factors. In the case of cervical carcinoma, some of the co-factors have been identified e.g. immunosuppression, smoking, oestrogen and progesterone (Dell and Gaston, 2001 and references therein). A detailed study of the nature of the co-carcinogens and their interaction with papillomavirus has been conducted in cattle. Infection of the upper GI tract of cattle with BPV-4 causes benign papillomas. These papillomas can progress to carcinoma in cattle feeding on bracken fern. During progression, the *Ha-ras* oncogene becomes activated and the *p53* gene undergoes mutations, highlighting the interaction between the mutagens present in the fern and the virus (Campo *et al.,* 1994). Quercetin is a potent mutagen widespread in nature but at its highest levels in bracken fern (Bjieldanes and Chang, 1977; Nakayasu *et al.,* 1986). It causes oxidative DNA damage (Yamashita and Kawanishi, 2000), single strand DNA breaks (Fazal *et al.,* 1990), DNA rearrangements (Suzuki *et al.,* 1991) and chromosomal aberrations (Ishidate *et al.,* 1988). Additionally quercetin interferes with several kinases (Jackson and Campo, 1995; Davies *et al.,* 2000). In our *in vitro* experimental model of BPV-4 associated oesophageal cancer, we found that quercetin could cause full oncogenic transformation of primary bovine fibroblasts partially transformed by BPV-4 and activated *Ha-ras* (Pennie and Campo, 1992; Cairney and Campo, 1995). This transformation was ascribed to the expression of BPV-4 E7 overcoming a quercetin-induced G1 arrest mediated by p53, leading to the accumulation of mutations, including, as *in vivo*, mutations of the p53 gene (Beniston *et al.,* 2001).

HPV16 has been associated with up to 50% of oesophageal pre-cancers and cancers particularly in developing countries (Chang *et al.,* 1990; Togawa *et al.,* 1994; Suzuk *et al.,* 1996; Cooper *et al.,* 1995; de Villiers *et al.,* 1999; Syrjanen, 2002). The highly variable geographical incidence of oesophageal cancer and of HPV detection in the cancer suggests a multi-factorial aetiology and the involvement of environmental dietary co-factors (Syrjanen, 2002). Some human populations are exposed to bracken

fern and this exposure has been linked epidemiologically to cancer of the upper GI tract in several parts of the developed and developing world (Galpain *et al.,* 1990; Marliere *et al.,* 2000; Alonso-Amelot *et al.,* 1996; Hirayama, 1979; Villalobos-Salazar *et al.,* 1995).

Given the possibility that papillomavirus infection and bracken fern are associated with oesophageal cancer in humans, as they are in cattle, and that quercetin promotes oncogenic transformation of BPV transformed cells, we decided to assay whether quercetin played a role in transformation of human keratinocytes by HPV.

MATERIALS AND METHODS

Plasmids

pCH110 carries the LacZ reporter gene driven by the SV40 early promoter (Pharmacia, Herefordshire, UK). pλ34-11 was a kind gift from Dr G. Sonenshein (Boston, USA) and has the human $p27^{Kip1}$ promoter upstream of the firefly luciferase gene. pLXSN, pLXSN16E6, pLXSN16E7 and pLXSN16E6E7 are retroviral vectors containing the HPV16 E6, E7 or E6 and E7 genes (kind gift from Dr D.Galloway (Seattle, USA).

Tissue Culture

The retroviral vector constructs (pLXSN) were transfected in the packaging cell line Phoenix Ampho, cultured using DMEM supplemented with 10% foetal calf serum at 37°C, 5%CO_2 (Invitrogen Carlsbad, CA). 24 hours after transfection the supernatant was taken and cell debris was spun out. The virus containing supernatant was stored at -70°C until further use. Primary human foreskin keratinocytes (HFKs) (Bio-Whittaker, Berks, UK) were routinely cultured in KGM-2 medium (Bio-Whittaker) at 37°C, 5%CO_2 being split 1 in 4 when 80-90% confluent. HFKE6/E7 were generated from HFKs being infected with recombinant retrovirus. To infect primary HFK cells 24 hours before infection $2x10^5$ cells were seeded into 25cm^2 flasks with 5ml of media. 1.5ml of infection mix was overlaid on the cells for 6 hours at 37°C before being removed and replaced with 15ml of media. G418 selection of cells was started 24 hours after infection at 500μg/ml for 14-21 days. Stable HFKE6/E7 cells were routinely cultured in KGM-2 medium (Bio-Whittaker) with the addition of 500μg/ml G418 (Invitrogen,) at 37°C, 5%CO_2 and split 1 in 4 when 80-90% confluent. HaCaT cells were gown in DMEM without $CaCl_2$, supplemented with 1mM Sodium Pyruvate and 2mM Glutamine and 10%FCS at 37°C, 5%CO_2 and split 1 in 6 when 80-90% confluent.

Cell proliferation was assayed by cell counts. HFKE6/E7 cells were seeded into 100mm tissue culture plates ($8x10^5$/plate) and then left for 24 hours before being treated with 0, 5, 10 or 20μM quercetin. Cells were treated for 24, 48 or 72 hours, with new medium being added with fresh quercetin or EtOH every 24 hours. Cells

were trypsinzed, resuspended in 6ml of 5%FCS in PBS and counted in duplicate in a haemocytometer.

Cell Cycle Analysis

$5x10^5$ cells were plated into 100mm dishes and 24 hours later treated with 5, 10, 20 or 50μM quercetin in EtOH (Sigma-Aldrich) for 36 hours. Control cells (0 μM) were treated with an equal volume of solvent (EtOH). Cells were trypsinised and centrifuged at 1000rpm at room temperature for 10 minutes. Supernatant was then removed before cells were resuspended in 0.5ml of IFA buffer (150mM Tris-HCl pH 7.6, 500mM NaCl, 0.5% NaN_3, 5% Foetal Calf Serum) and fixed with 70% EtOH. Fixed cells were stained using 20μg/ml propidium iodide (Sigma-Aldrich) and 200μg/ml RNA-aseA (Kramel Biotech, Northumberland, UK), before being assayed on a Coulter Epics XL-MCL FACS machine.

Immunoblotting and Immunoprecipitation

Cells were seeded into 100mm dishes ($5x10^5$ per plate) and then left for 24 hours before being treated with 0, 5, 10 or 20μM quercetin. Cells were treated for 36 hours with new medium being added with fresh quercetin or EtOH after 24 hours, before being harvested. Cells were harvested in 100μl NP40 lysis buffer (0.1% NP40, 150mM NaCl and 50mM Tris-HCl pH 7.6 with protease inhibitor cocktail (Roche, Mannheim, GER) per plate and scraped into eppendorf tubes on ice. Cells were lysed by being passed through an 18-gauge needle seven times and debris was pelleted by centrifugation at 14K rpm for 10 minutes at 4°C. Supernatant was then taken and assayed for protein concentration by the BCA method (Sigma-Aldrich) and stored at -20°C until use.

For western blotting 25-100μg of cell lysate were first run on Tris-Glycine buffered SDS-PAGE gels utilising MOPS pH buffer (Invitrogen), before being transferred to nitrocellulose membrane for probing. Membranes were blocked in 5% Marvel milk in PBS for 1 hour at room temperature before being probed with the primary antibody for 1½ hours in 5% Marvel /PBS at room temperature. Membranes were washed three times for 10mins in PBS before the secondary antibody was added for 1 hour at room temperature in 5% Marvel/PBS. Membranes were again thoroughly washed in PBS

before being developed with ECL reagent (Amersham-Pharmacia, Bucks, UK). Primary Antibodies used were: p105Rb= IF8 (Santa Cruz, Santa Cruz, CA), p53= D0-1 (Serotec), $p21^{Waf1}$= Ab3 (Oncogene Research Products, La Jolla, CA), $p27^{Kip1}$= Clone 57 (Transduction Laboratories, Heidelberg, GER), $p16^{INK4a}$ = DCS50 (Sigma-Aldrich), β-Actin= AB1 (Oncogene Research Products), Skp2(p45)= N19 (Santa Cruz), Cyclin E= HE12 (Santa Cruz). Secondary Antibodies were anti mouse IgG-HRP (Amersham-Pharmacia), anti mouse IgM-HRP and anti goat IgG-HRP (Santa Cruz).

For immunoprecipitation 250μg of lysate was pre-cleared using 4μg of a non-specific mouse monoclonal antibody (Santa Cruz) and 100μl of Protein A-Sepharose beads (Sigma-Aldrich) for a total of 3 hours in a total volume of 500μl.
Supernatants were collected and cyclin E was immunoprecipitated using 1μg of cyclin E antibody HE111 (Santa Cruz) for 1.5 hours before addition of 30μl of Protein A-Sepharose beads. Lysates were incubated, with agitation, for a further 1.5 hours before the beads were pelleted and washed 3 times with ice-cold lysis buffer. 20μl of x2 sample buffer (100mM Tris-HCl pH 6.8, 4% SDS, 0.2% Bromophenol Blue, 20% Glycerol, 200mM DTT) was mixed with the beads. Beads were heated for 10 minutes at 70°C. Samples were then assayed by immunoblotting as above.

Cyclin E Associated Kinase Assay

Cells were seeded into 100mm dishes ($5x10^5$ per plate) and then left for 24 hours before being treated with 0, 5, 10 or 20μM quercetin. Cells were treated for 36 hours with new medium being added with fresh quercetin or EtOH after 24 hours, before being harvested. Cells were lysed in E/K2 activity lysis buffer (50mM Hepes pH7.5, 150mM NaCl, 1% NP40, 2.5mM EGTA, 10mM NaF, 10mM β-Glycerophosphate, 1mM Sodium Orthovanadate, 1mM DTT), by 3 freeze/thaw cycles. Lysate was spun at 14K rpm for 10 minutes to remove cell debris and the supernatant was assayed for protein concentration and stored at -70°C. 500μg of cell lysate were used per assay, and cyclin E was precipitated with 4μg of HE111 (Santa Cruz). Kinase activity was assayed by transfer of ^{32}P to Histone H1 as substrate at 30°C for 30 minutes. Complexes were released from the beads by heating at 70°C for 10 minutes before

being run on a 10% Acrylamide SDS-PAGE gel, dried and exposed to film (Kodak, Rochester, NY).

Reporter Gene Analysis

$2x10^5$ HaCaT cells were plated into 60mm dishes 24 hours before transfection. Cells were transfected with 2μg of pλ34-11, or pGL3TK, and 2μg of pCH110 as a transfection efficiency control using Lipofectamine (Invitrogen) for 5 hours at 37°C, 5%CO_2 in serum free medium. Quercetin treatment started 24 hours after transfection with 0 to 50μM quercetin for 36 hours with fresh medium every 24 hours. Cells were harvested and assayed for luciferase activity with luciferase assay buffer (Promega, Madison, WI) in a Dynex-MLX luminometer. Protein concentration was determined by BCA assay (Sigma-Aldrich) and β-galactosidase activity was measured by colour conversion of ONPG (Sigma-Aldrich) monitored at 420nm.

Real Time RT-PCR and Standard RT-PCR

Total RNA was isolated from HFKE6/E7 cells using the RNeasy Minikit (Qiagen, Sussex, UK), and residual DNA was removed by DNA-aseI treatment according to the manufacturers guidelines (Invitrogen). Real time RT-PCR for $p27^{Kip1}$, HPV16E7 and β-Actin mRNA was carried out using the TaqmanÆ EZ RT-PCR Kit (Applied Biosystems, Foster City, CA) with gene specific primers and FAM/TAMRA probe designed by Primer Express v1.7 software. 10ng of RNA was used per reaction, with each condition being carried out in triplicate. For $p27^{Kip1}$ primers were: p27FOR 5'-CCGGTGGACCACGAAGAGT-3' and p27REV 5'-GCTCGCCTCTTCCATGT CTC-3' with probe 5'-AACCCGGGACTTGGAGAAGCACTGC-3'. For HPV16E7 primers were: E7FOR 5'-AAGTGTGACTCTACGCTTCGGTT-3' and E7REV 5'-CATGTATAGTTGTTTGCAGCTCTGTG-3' with probe 5'-TGCGTACAAAGCACACACGTAGACATTCGTA-3'. For β-Actin the primers and probe were commercially available (Applied Biosystems). RT-PCR was performed using an ABI Prism 7700 Sequence detector. In each experiment additional reactions with 8 to 10 10-fold serial dilutions of template DNA were performed with each set of primers and probes on the same 96 well plates to generate standard curves. All samples were amplified in duplicate. The relative amounts of $p27^{Kip1}$, HPV16E7 or β-Actin mRNA were determined by using the standard curve method. Levels of

$p27^{Kip1}$ and HPV16 E7 mRNA were normalised to β-Actin mRNA expression levels. Standards, β-Actin control samples and either $p27^{Kip1}$ or HPV16 E7 test samples were analysed on the same 96 well plate for each individual experiment.

In addition each RNA preparation used was tested for DNA contamination by using β-Actin primers and Taq polymerase instead of RT and Taq in standard RT-PCR reactions. Reverse transcription and subsequent PCR of 200ng of total RNA was carried out using gene specific primers with OneStep RT-PCR Kit (Qiagen) according to the manufacturers guidelines. β-actin mRNA was amplified using the primers: ACTINFOR 5'-GCGTCTGGACCTGGCTGGCCGGGACCT-3' and ACTINREV 5'-GGAAGGCTGGAAGAGTGCCTCAGGGCAG-3'. HPV16 E6 and E7 mRNA was detected in expressing cell lines using standard RT-PCR reactions. The OneStep RT-PCR Kit (Qiagen) was used according to the manufacturers guidelines with gene specific primers and 200ng of total RNA. As with the Real time RT-PCR DNA contamination was assayed by use of Taq polymerase instead of RT and Taq. HPV16 E6 was amplified using the primers: E6 DETECT FOR: 5' gcgacccagaaagttaccac 3' and E6 DETECT REV: 5' gcaacaagacatacatcgaccgg 3'. HPV16 E7 was amplified using the primers: E7 DETECT FOR: 5' gcaaccagagacaactgatctc 3' and E7 DETECT REV: 5' tggggcacacaattcctagtg 3'.

Cyclohexamide Analysis of protein half-life

Protein half-life analysis of $p27^{Kip1}$ in HFKE6/E7 cells was carried out using cyclohexamide (CHX) (Sigma-Aldrich). $5x10^5$ cells were seeded in 100mm plates; then left for 24 hours before being treated with 0, 5, 10 or 20μM quercetin. Cells were treated for 36 hours, with new medium being added with fresh quercetin or EtOH after 24 hours, before being harvested. CHX in DMSO was added to a final concentration of 100μg/ml to each plate, DMSO only was used as a negative control. Cells were harvested in NP40 lysis buffer (see above) at regular time intervals after CHX/DMSO addition. Lysates were then run on Tris-Glycine buffered SDS-PAGE gels utilising MOPS pH buffer (Invitrogen), before being transferred to nitrocellulose membrane for probing for $p27^{Kip1}$. Bands were then analysed by densitometry using a UMAX Powerlook III Flatbed scanner and ImageQuant v5.2 software.

RESULTS

1. Quercetin induces cell cycle arrest in HFK cells.

Previous work in the bovine system showed that quercetin was capable of arresting primary fibroblasts in both G1 and G2/M phases of the cell cycle. The expression of the BPV-4 E7 oncoprotein with activated *Ha-ras* was capable of overcoming the G1, but not the G2/M, arrest (Beniston *et al.*, 2001). We reasoned that primary HFK cells might also undergo cell cycle arrest after quercetin exposure and that the HPV16 E6 and E7 oncoproteins might be able to abrogate this arrest.

Primary HFK cells were infected with a retrovirus expressing HPV16 E6 and/or E7. Cells were selected in G418 before being exposed to quercetin and assayed. To ensure that the HPV16 E6 and E7 oncogenes were expressed, RT-PCR was carried out to detect E6 and E7 mRNA. As a positive control two HaCaT cell clones expressing tagged versions of the oncoproteins were used. DNA Polymerase only (Taq) reactions without reverse transcriptase were carried out to detect contaminating DNA. Figure 1A shows that both E6 and E7 are expressed in the HFK E6, E7 and E6/E7 cell lines. The reduction in p53 and p105Rb, the main targets of E6 and E7 respectively (Mantovani and Banks, 2001; Munger et al., 2001), confirmed the expression of the viral proteins (Figure 1B).
Only the HFKE6/E7 cells will be discussed here.

Primary HFK cells were exposed to 0 to 10μM quercetin for 36 hours, and HFKE6/E7 cells up to 20μM. Cells were then fixed, stained with propidium iodide and assayed by FACS. Figure 2a shows that the primary HFK cells underwent a distinct increase in the G1 population after 36 hours with 5 or 10μM quercetin with a corresponding decrease in S and G2/M phases. Higher concentrations resulted in no further increase in the number of cells in G1 or decrease in the number of cells in G2/M (data not shown), leading to the conclusion that quercetin arrested the cells in G1. HFKE6/E7 transformed cells displayed an equivalent response to quercetin with an increase in G1 and decrease in S phase (figure 2b). Untreated HFKE6/E7 cells continued to expand over 72 hours whereas quercetin treated HFKE6/E7 cells stopped proliferating (figure 2c). This showed that the cells were in fact arresting and not simply proliferating

more slowly. Hence we can conclude that both primary and E6/E7 transformed human keratinocytes arrest in G1 upon quercetin treatment.

2. $p27^{Kip1}$ is elevated in HFK and HFKE6/E7 cells upon quercetin exposure.

Earlier work had linked quercetin exposure to p53 elevation with an increase in $p21^{Waf1}$ (Plaumann et al., 1996; Beniston *et al.,* 2001), therefore both p53 and $p21^{Waf1}$ levels were assayed by immunoblotting. In addition as quercetin caused G1 arrest in HFKE6/E7 two other cdk inhibitors associated with G1 cell cycle arrest, $p16^{INK4a}$ and $p27^{Kip1}$, were also examined. Figure 3a shows that in primary HFKs p53 was only slightly elevated following quercetin exposure of 5 to 20μM. However the increase in p53 protein levels did not correlate to an increase of $p21^{Waf1}$ expression, and there was a noticeable decrease in the level of $p21^{Waf1}$at 20μM quercetin. At 50μM quercetin p53 protein levels raised still further, while $p21^{Waf1}$ levels dropped below the level of detection (data not shown). The related cdki $p27^{Kip1}$ however increased at 5 to 20μM quercetin, suggesting that $p27^{Kip1}$ mediated the G1 arrest. HFKE6/E7 cells showed no change in p53 and $p21^{Waf1}$ levels in response to quercetin, with the exception of a decrease in $p21^{Waf1}$ levels at 20μM quercetin (figure 3b). In HFKE6/E7cells, as in primary cells, $p27^{Kip1}$ increased with quercetin exposure up to 20μM, again suggesting its role in G1 arrest. Additionally in both primary and E6/E7 transformed HFK cells $p16^{INK4a}$ decreased with quercetin exposure indicating that this cdk4 inhibitor is unlikely to play an important role in quercetin induced G1 arrest in these cells. These changes in cell cycle regulators following quercetin treatment were consistently observed over at least three experiments.

3. Quercetin causes an increase in $p27^{Kip1}$ protein half-life

In agreement with the increase in the $p27^{Kip1}$ protein, quercetin caused an increase in the half-life of $p27^{Kip1}$ protein. HFKE6/E7 cells treated with DMSO only after 36 hours of 0μM quercetin treatment showed a continuous elevation of $p27^{Kip1}$, whereas cells treated with 100μg/ml cyclohexamide (in DMSO) displayed a reduction in $p27^{Kip1}$ protein levels over 24 hours (top two rows, figure 4a). This demonstrated that the cyclohexamide treatment was successful in inhibiting protein translation. Cells treated with 5-20μM quercetin for 36 hours and with cyclohexamide, displayed an increase in $p27^{Kip1}$ half-life. Protein bands were analysed by densitometry and data

were normalised to the 0 hour time point. After 36 hours of 0μM quercetin $p27^{Kip1}$ half-life was calculated to be approximately 1.5 hours, whereas after treatment with 5μM quercetin, $p27^{Kip1}$ half-life was approximately 4 hours. After treatment with 10 or 20μM quercetin, $p27^{Kip1}$ half-life was as long as 24 hours (figure 4a). A half-life of 1.5 hours in normally cycling cells seemed high as $p27^{Kip1}$ half-life has been predicted to be in the region of around 30 to 40 minutes (Reinstein *et al.*, 2000). However E6/E7 transformed cells do have a constitutively higher level of $p27^{Kip1}$ expression than primary cells (figure 4b) indicating some degree of base level activation. Indeed it has been observed that $p27^{Kip1}$ levels are elevated in cervical lesions (Zehbe *et al.*, 1999).

To further investigate $p27^{Kip1}$ half-life in quercetin treated HFK E6/E7 cells Skp2(p45) levels were assayed by western blot analysis. Skp2(p45) is the F-box protein, which specifically recognises $p27^{Kip1}$ (Carrano *et al.*, 1999; Malek et al., 2001) and recruits it to the SCF core complex, thus promoting its ubiquitination and degradation, although there are suggestions that a Skp2(p45)-independend degradation of $p27^{Kip1}$ in G1 exists (Hara et al., 2001). Analysis of Skp2(p45) levels in HFKE6/E7 showed that Skp2(p45) levels decreased as the concentration of quercetin increased (figure 4c). This effect is unlikely to be due to alterations in Skp2(p45) levels during the cell cycle, as in normally cycling cells Skp2(p45) is low in early/mid G1 and high in late G1, concomitant with the normal decrease in $p27^{Kip1}$ protein.

4. **Reduction in Cyclin E levels and increased association of $p27^{Kip1}$ with Cyclin E correlate with decreased Cyclin E associated kinase activity**

As quercetin treatment elevated $p27^{Kip1}$ protein levels and increased the protein's half-life, it was hypothesized that the cause of the G1 arrest in the cells was due to the activation of the cdki. To confirm this, cyclin E-associated kinase (E/K2) assays were carried out. Cyclin E was immunoprecipitated and activity was assayed by transfer of ^{32}P to Histone H1. Non-specific IgG control immunoprecipitations showed no associated kinase activity (data not shown). Figure 5a shows that control cells had a detectable E/K2 activity, whereas quercetin significantly decreased or completely abolished E/K2 activity even at 5μM. Cyclin E was immunoprecipitated under

identical conditions and immunoblotting revealed that its amount was approximately constant up to 10 μM quercetin (Figure 5a). The observation was supported by separate western blot analysis (figure 5b). The initial increase of cyclin E with 5μM quercetin seen during immunoprecipitations (figure 5a) was not observed consistently (figure 5b).

To determine whether the loss of E/K2 activity was due to inhibition by $p27^{Kip1}$, membranes were stripped and re-probed for $p27^{Kip1}$. As can be seen in figure 5a the level of $p27^{Kip1}$ associated with cyclin E increased with increasing quercetin concentration. We interpret these results as an indication that quercetin raised the $p27^{Kip1}$ level associated with cyclin E , and this abolished cyclin E-associated kinase activity.

5. **Quercetin treatment activates the $p27^{Kip1}$ promoter and results in increased mRNA levels**

A key control method for $p27^{Kip1}$ is post translation control through its half-life (Lloyd *et al.,* 1999; Nakayama *et al.,* 2001 for reviews). However other methods of control may also play a part. To examine possible transcriptional control we used the human $p27^{Kip1}$ promoter directing the transcription of the firefly luciferase gene (pλ34-11). The plasmid was transfected into HaCaT cells, which then were treated for 36 hours with 0 to 20μM quercetin before being harvested. Transfection efficiency was checked by co-transfection with the pCH110 β-galactosidase plasmid. Quercetin had no effect on expression of β-galactosidase from the SV40 promoter (data not shown). Figure 6 shows the average of 4 transcription assays each done in duplicate, expressed as fold activation over control. The activity of the $p27^{Kip1}$ promoter increased significantly with increasing quercetin concentrations, from 3 fold at 5μM to 6 fold at 20μM quercetin. Higher quercetin concentrations did not result in further increase of the $p27^{Kip1}$ promoter activity (data not shown). The pGL3TK plasmid in which the tk promoter directs expression of luciferase (Vance *et al.,* 1999) was used as a control to examine whether any possible effect observed was specific for the $p27^{Kip1}$ promoter or common to other promoters. There was no significant increase in the transcriptional activity of the tk promoter with increasing quercetin concentration (figure 6). These results indicate that quercetin-dependent trans-activation of the

$p27^{Kip1}$ gene promoter acts via one or more elements specific to the promoter, and not on components of the basic transcription machinery.

Real-time RT-PCR was carried out to assay whether the increase in transcription activity of the p27 promoter resulted in elevated levels of $p27^{Kip1}$mRNA. Figure 7a shows the average of 4 individual experiments each consisting of triplicate samples. The results confirmed that quercetin exposure did increase the level of $p27^{Kip1}$mRNA in HFKE6/E7 by approximately 3 fold at 20μM quercetin.

6. **Quercetin treatment results in no alteration in HPV16 E7 mRNA levels**

Another key factor in the arrest of the HFKE6/E7 cells could be not only the elevation of the cdki $p27^{Kip1}$, but also the reduction of the viral oncoprotein hypothesized to abrogate its function i.e. E7. As attempts to detect E7 in the HFKE6/E7 cells by immunoblotting were unsuccessful with available antibodies, a more indirect assessment method had to be undertaken. Real time RT-PCR was carried out to determine whether quercetin treatment of these cells had any impact upon E7 mRNA levels. Figure 7b shows the average of four independent experiments each consisting of triplicate samples. Quercetin treatment resulted in no significant alteration in E7 mRNA levels. This would indicate that there is no variation in the level of HPV16E7 due to quercetin exposure.

DISCUSSION

Considerable evidence points to a possible co-operation or synergy between HPV infection and bracken fern in carcinogenesis of the upper GI tract in humans, as reported in cattle. People exposed to bracken fern, whether as spores or in the diet, are at greater risk of developing oesophageal and gastric carcinoma (Galpain *et al.,* 1990; Marliere *et al.,* 2000; Alonso-Amelot *et al.,* 1996; Hirayama, 1979 and Villalobos-Salazar *et al.,* 1995; Alonso-Amelot and Avendano, 2001). Moreover HPV-16 has been found in up to approximately 50% of oesophageal cancers and pre-cancers in developing countries (Chang et al., 1990; Togawa et al., 1994; Suzuk et a., 1996; Cooper et al., 1995; de Villiers et al., 1999; Syrjanen, 2002). There is a geographical overlap between consumption of bracken fern, infection of the alimentary canal mucosa by HPV and upper GI tract cancer particularly in Asia and South America.

Our study aimed to determine whether quercetin treatment would cause cell cycle arrest in primary human keratinocytes, the natural host of HPV, as it does in a large variety of primary and transformed cell lines (Beniston *et al.,* 2001; Casagrande and Darbon, 2001; Choi *et al.,* 2001; Plaumann *et al.,* 1996). We hypothesised that expression of the HPV16 E6 and E7 oncogenes would abrogate the quercetin-induced cell cycle arrest thereby leading, as in the bovine model, to inheritable genetic alterations, the accumulation of which, together with the action of the viral oncoproteins, would increase the risk of full transformation of the cell.

Quercetin arrested both HFK and HFKE6/E7 cells in the G1 phase of the cell cycle. The data presented do not conclusively prove or disprove a G2/M arrest in either cell type in response to quercetin, and further investigation is yet to be done. Hence here we have concentrated on the molecular mechanisms of the G1 arrest. In bovine cells quercetin caused elevation of both p53 and $p21^{Waf1}$, however in the HFKE6/E7 cells there was no p53 response over the full range of quercetin concentrations, though this may be due in part to E6-mediated degradation of p53. HFK cells did demonstrate a modest increase in p53 levels at high quercetin concentration, but in these conditions, a number of cell cycle regulators were noticeably decreased or absent, possibly due to apoptosis induced by quercetin (Yamashita and Kawanishi, 2000; Rong et al., 2000;

Iwashita et al., 2000). These observations make it unlikely that p53 or $p21^{Waf1}$ play a necessary role in quercetin-induced G1 arrest in HFK cells.

Expression of E7 has been shown to overcome the effects of high levels of $p16^{INK4a}$, a member of the INK4 family of cdki (Martin *et al.,* 1998), and high expression of $p16^{INK4a}$ has been correlated to E7 expression in cervical cancer samples (Sano *et al.,* 2002). Quercetin treatment lead to a reduction of $p16^{INK4a}$ levels in both HFK and HFKE6/E7 cells, strongly indicating that $p16^{INK4a}$ is unlikely to be the cause of the observed G1 arrest.

Quercetin caused a dose dependent increase in $p27^{Kip1}$ levels in both primary and transformed cells. This was due to an increase in transcriptional activity of the $p27^{Kip1}$ gene and the concomitant increase in mRNA. In addition the half-life of the protein increased too and this was accompanied by the reduction of the F box protein Skp2(p45) that promotes the turnover of $p27^{Kip1}$ (Carrano *et al.,* 1999). Increasing quercetin concentrations lead to increased association of $p27^{Kip1}$ with cyclin E and a corresponding decrease in cyclin E-associated kinase activity. Altogether these observations provide evidence that quercetin causes G1 arrest in HFK and HFKE6/E7 cells through $p27^{Kip1}$.

HPV16E7 has been reported to bind directly to, and inhibit both $p21^{Waf1}$ and $p27^{Kip1}$ (Funk *et al.,* 1997; Jones *et al.,* 1997a; Zerfass-Thome *et al.,* 1996). Additionally high levels of $p21^{Waf1}$ and $p27^{Kip1}$ have been found in cervical samples containing HPV16 (Zehbe *et al.,* 1999). While our study does not examine the effect of E7 on $p21^{Waf1}$, it is interesting that in HFKE6/E7 cells $p27^{Kip1}$ is the most likely cause of G1 arrest upon quercetin exposure. In agreement with others we noted an increased expression of $p27^{Kip1}$ in HFKE6/E7 cells (figure 4b). This high level of $p27^{Kip1}$ may be tolerated by the E7-expressing cells causing a significant level of cdk2 activity to be retained. Upon quercetin treatment however the amount of $p27^{Kip1}$ raises still further and possible alterations in post-translational modification of $p27^{Kip1}$ could promote an inhibitory role on cell cycle progression, such as alleviation of PKB phosphorylation (Liang *et al.,* 2002).

The failure of HFK16E6/E7 cells to overcome quercetin-induced G1-arrest does not disprove the role of either HPV or bracken fern in the aetiology of human oesophageal carcinoma. There are several reasons to account for the different outcome of quercetin treatment of PV-transformed bovine and human cells: bovine fibroblasts may intrinsically respond to quercetin differently from human keratinocytes. This would be supported by the observation that quercetin induces both p53 and $p21^{Waf1}$ in bovine fibroblasts (Beniston et al., 2001) but $p27^{Kip1}$ in HFK (Figure 3). Moreover, the major difference between our bovine and human *in vitro* systems is the absence of activated *Ha-ras* in the HFKE6/E7 cells. Activated *Ha-ras*, seen in BPV-4 associated carcinoma, was required for immortalisation of BPV-4 transformed cells, due to the lack of the E6 gene in BPV-4 (Jackson *et al.*, 1991). Members of the *ras* gene family are often over-expressed or amplified (Galiana et al., 1995; Sorsdahl et al., 1994) in human oesophageal cancers, implicating the ras proteins in oesophageal carcinogenesis, although activating point mutations are rare (Arber et al., 2000; Casson et al., 1997). It would be informative to ascertain what effects quercetin would have in HFKE6/E7 overexpressing ras. Furthermore, bracken fern contains numerous mutagens and carcinogens in addition to quercetin, including the sesquiterpene ptaquiloside. Ptaquiloside has been shown to be one of the active chemicals in bracken fern in Salmonella and in rats (Matoba et al., 1987; Hirono et al., 1987; Potter and Baird, 2000). It will be important to test ptaquiloside in HFK16E6/E7 cells.

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FIGURE LEGENDS

Figure 1: A, Detection of E6 and E7 in established cell lines. Gene Specific RT-PCR was carried out for HPV16E6 (lanes 1-7) and HPV16E7 (lanes 8-14). Vector only (lanes 1, 4, 8 &11), pLXSNE6 (lane 2 & 5), pLXSNE7 (lane 9 &12), pLXSNE6/E7 (lane 3,6, 10 & 13). HaCaT HAE6 cell line RNA was positive control for E6 (lane 7) and HaCaT FLAGE7 cell line RNA was positive control for E7 (lane 14). DNA contamination controls were reactions using Taq only (lanes 4-6 & 11-13). B, reduction in p53 and p105Rb protein levels in HFKE6/E7. membranes were probed with D0-1 for p53 and IF8 for p105Rb (top row) and AB1 for β-Actin (bottom row)..

Figure 2: Cell cycle profiles of primary and transformed cells. Cells were grown in 0 to 10 or 20μM quercetin for up to 36 hours. (A) Histograms show the differences in cell cycle phases between control cells grown in EtOH and cells grown in quercetin. Representative cell cycle profiles of primary HFK cells grown in 0, 5 or 10μM quercetin for 36 hours are shown. (B) Representative cell cycle profiles of HFK E6/E7 cells grown in 0, 5, 10 or 20μM quercetin for 36 hours are shown. Percentage population of cells stated under graphs were determined using PI staining by Expo 32 Multicomp software. (C) Cell proliferation assay. HFKE6/E7 cells were treated with 0 to 20μM quercetin up to 72 hours before being trypsinized and counted. The experiment was repeated three times and the average number of cells is shown with error bars (SD).

Figure 3: $p27^{Kip1}$ protein is elevated in response to quercetin exposure in (A) primary HFK and (B) HFKE6/E7 cells. Cells were treated with 0 to 20μM quercetin for 36 hours before harvesting. Membranes were probed for p53 (top tow), $p21^{Waf1}$ (second row), $p27^{Kip1}$ (third row), $p16^{INK4a}$ (fourth row) and β-Actin used as a loading control (bottom row). The experiment was repeated three times at least with identical results.

Figure 4: $p27^{Kip1}$ protein half-life is elevated in response to quercetin exposure in HFK E6/E7. (A) Cells were treated with 0μM quercetin for 36 hours before being treated with 100μg/ml cyclohexamide (CHX) or DMSO control for up to 24 hours. Samples were immunoblotted and membranes were probed for $p27^{Kip1}$ (upper two

panels) to ensure CHX activity. Immunoblot assay of HFKE6E7 cells treated with 0 to 20μM quercetin for 36 hours then 100μg/ml CHX for up to 24 hours. Samples were immunoblotted and membranes were probed for $p27^{Kip1}$ (lower four panels). Densitometry was carried out on bands, normalised to protein levels at time point 0 hour, and shown by graphical representation. (B) Comparison of $p27^{Kip1}$ levels in both primary and HFKE6E7 cells. Cells were treated with 0,5 or 20μM quercetin for 36 hours before harvesting. Membranes were probed for $p27^{Kip1}$ (top row) with Actin used as a loading control (bottom row). (C) Immunoblot assay of Skp2 (p45) protein levels (top row) with actin as a loading control (bottom row). Cells were treated with 0 to 20μM quercetin for 36 hours before harvesting.

Figure 5: Quercetin reduces cyclin E associated kinase activity in HFKE6/E7 cells. Cells were treated with 0 to 10μM quercetin for 36 hours before harvesting. (A) Cyclin E associated kinase activity was determined by radiolabelling of histone H1 by phosphorylation (top). Cyclin E immunoprecipitation was carried out in parallel as a loading control (middle). Membrane was then re-probed for $p27^{Kip1}$ (bottom row). (B) Membranes were probed for Cyclin E (top tow) with Actin used as a loading control (bottom row).

Figure 6: Quercetin increases transcription from the $p27^{Kip1}$ promoter. Cells were transfected with 2μg of either pλ34-11 (human $p27^{Kip1}$ promoter reporter) or pGL3TK (TK basal promoter reporter) together with 2μg of pCH110 (constitutive β-galactosidase expression construct) as a control for transfection efficiency. Cells were then treated with 0 to 20μM quercetin for 36 hours. Luciferase activity was normalized to β-galactosidase specific activity. Results are shown as fold induction over control treatment (0μM).

Figure 7: A. Quercetin elevates $p27^{Kip1}$ mRNA levels. Cells were treated with 0 to 20μM quercetin for 36 hours before total RNA was extracted. Real Time RT-PCR was carried out to determine $p27^{Kip1}$ mRNA levels and were normalised to β-Actin mRNA levels as a loading control. Results are shown as fold increase over 0μM quercetin. **B**. Quercetin does not affect HPV16E7 mRNA levels. Cells were treated

with 0 to 10μM quercetin for 36 hours before total RNA was extracted. Real Time RT-PCR was carried out to determine HPV16E7 mRNA levels and were normalised to β-Actin mRNA levels as a loading control. Results are shown as fold change over 0μM quercetin.

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Figure 1.

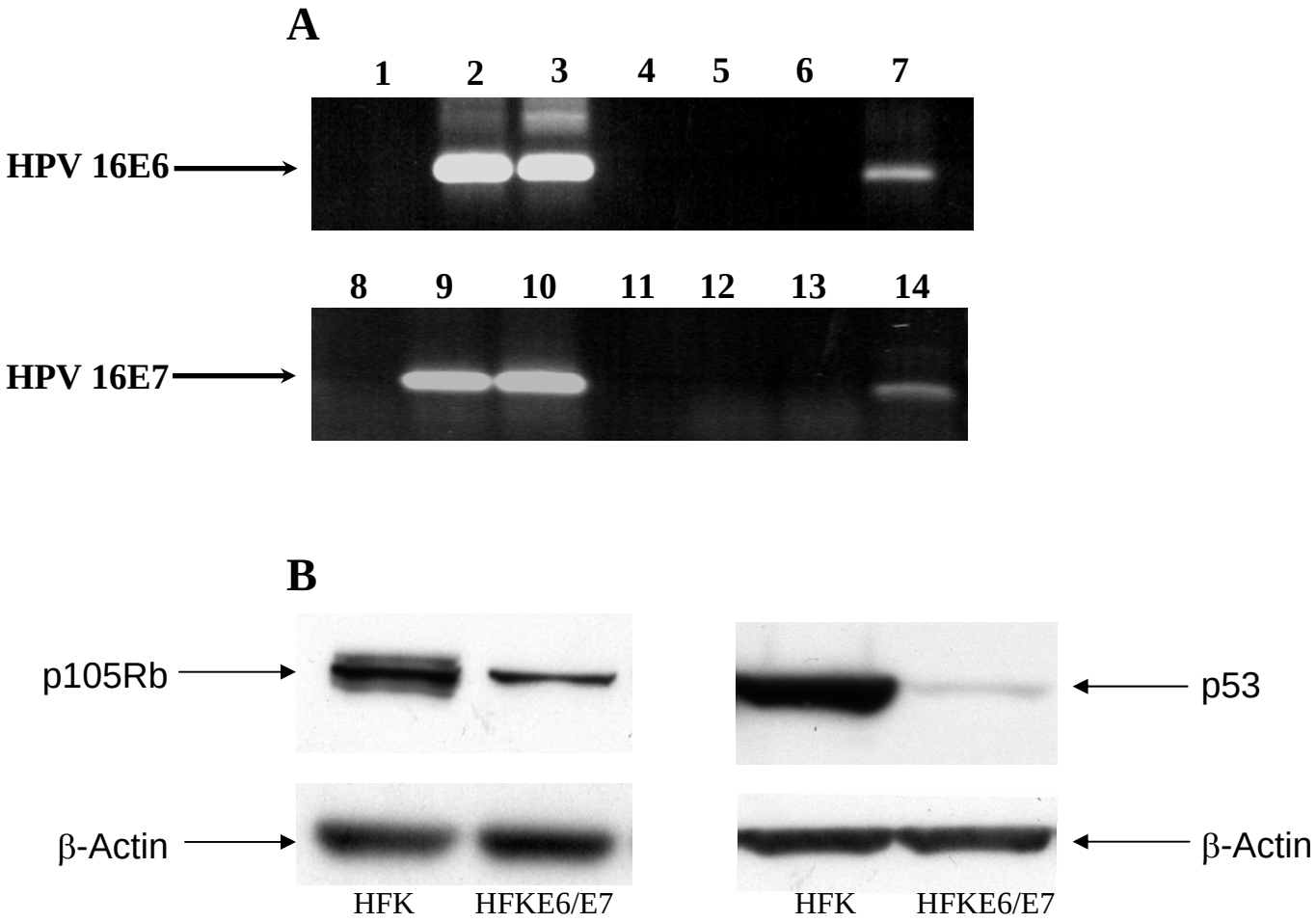
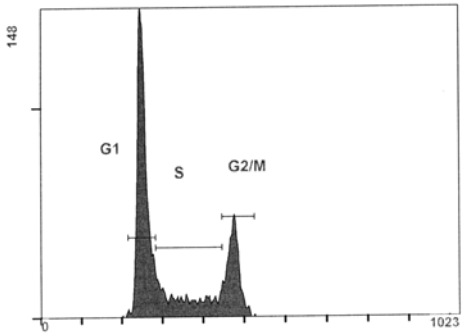

A
1 2 3 4 5 6 7
HPV 16E6
8 9 10 11 12 13 14
HPV 16E7
B
p105Rb
β-Actin
HFK HFKE6/E7
p53
β-Actin
HFK HFKE6/E7

Figure 2.

A

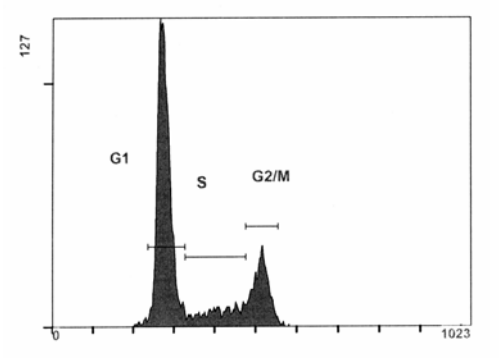
0μM Quercetin



DNA Content

G1= 53.9% **S**= 23.6% **G2/M**= 22.5%

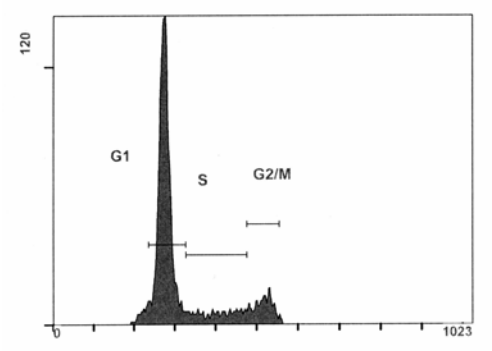
5μM Quercetin



DNA Content

G1= 64.7% **S**= 15.3% **G2/M**= 20.0%

10μM Quercetin



DNA Content

G1= 70.6% **S**= 16.0% **G2/M**= 13.4%

Figure 3.

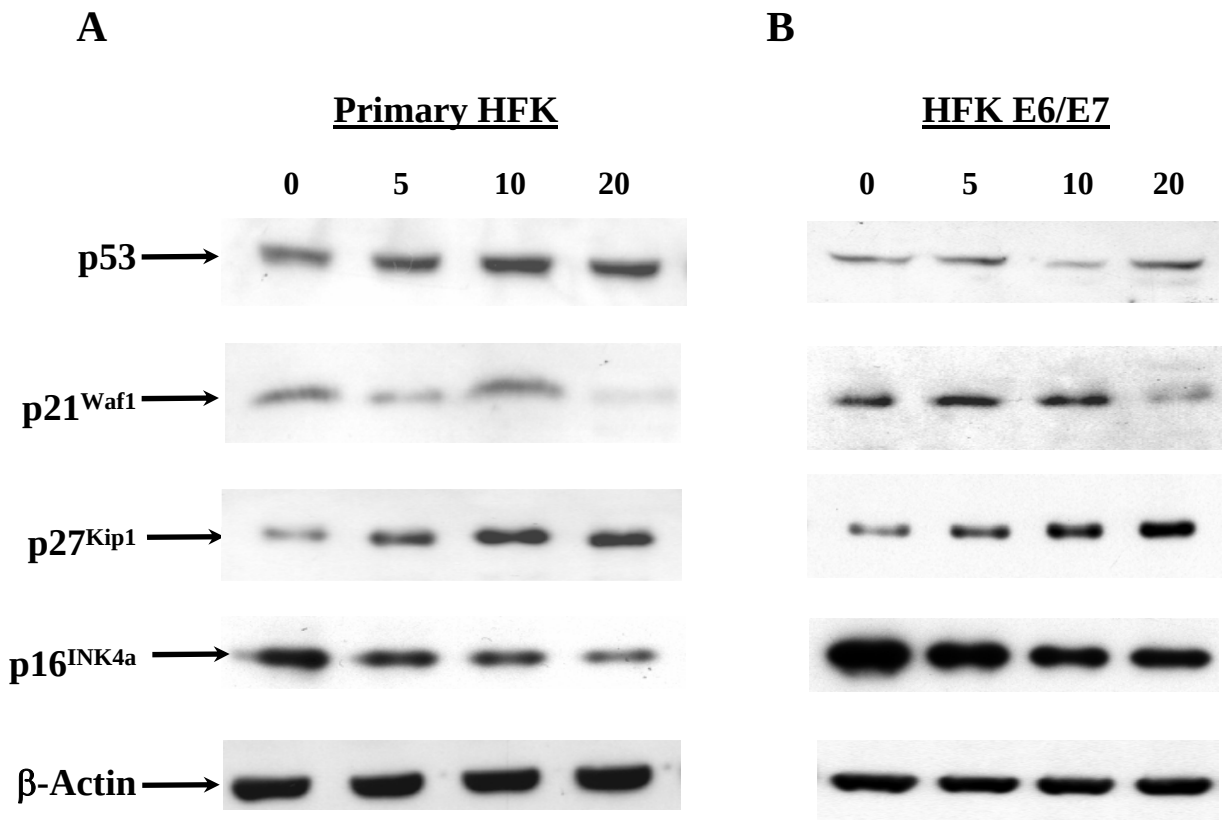

A
B
Primary HFK
HFK E6/E7
0
5
10
20
0
5
10
20
p53
p21Waf1
p27Kip1
p16INK4a
β-Actin

Figure 4.

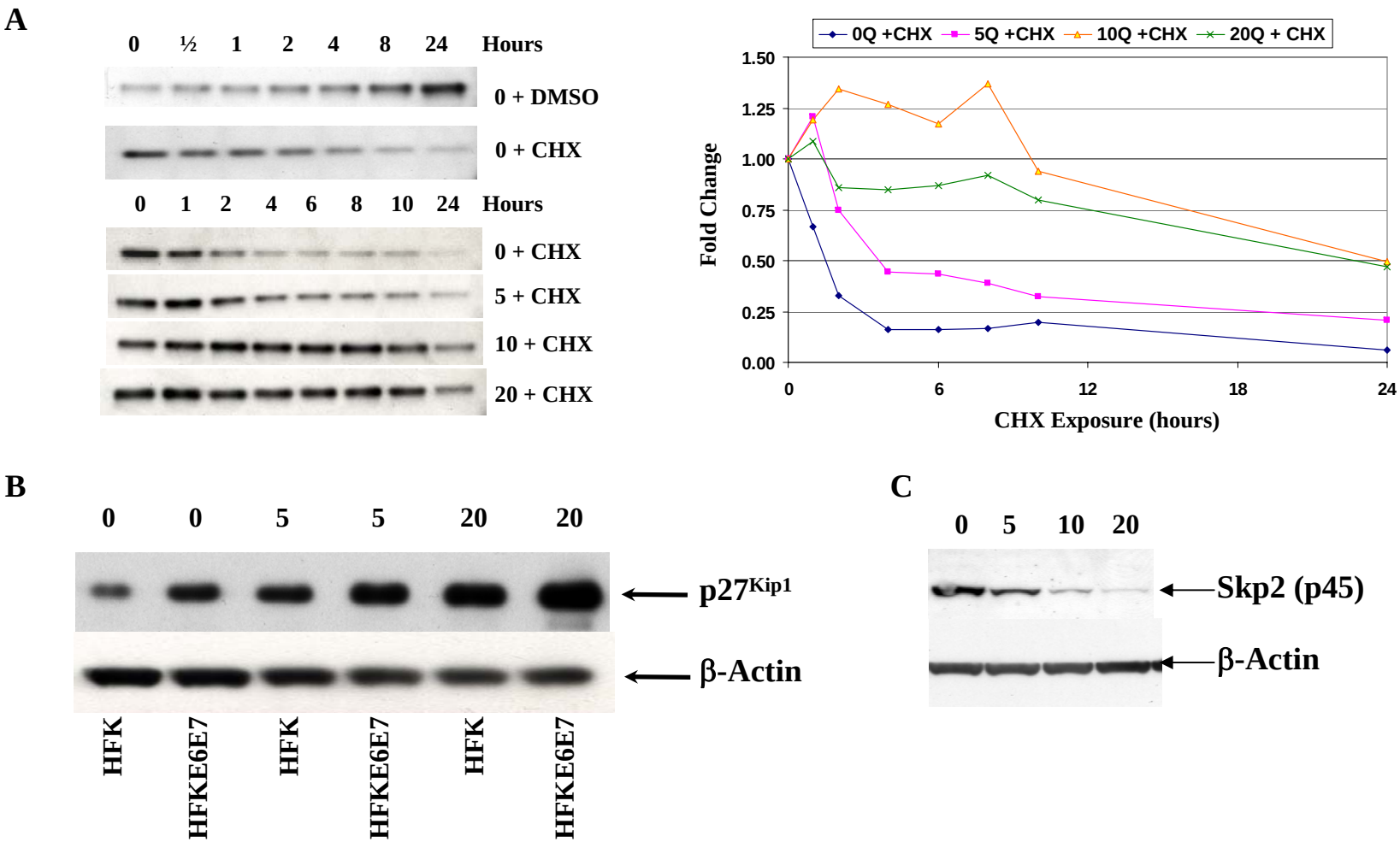

A
0 ½ 1 2 4 8 24 Hours
0 + DMSO
0 + CHX
0 1 2 4 6 8 10 24 Hours
0 + CHX
5 + CHX
10 + CHX
20 + CHX
0Q +CHX
5Q +CHX
10Q +CHX
20Q + CHX
Fold Change
1.50
1.25
1.00
0.75
0.50
0.25
0.00
0
6
12
18
24
CHX Exposure (hours)
B
0 0 5 5 20 20
p27Kip1
β-Actin
HFK
HFKE6E7
HFK
HFKE6E7
HFK
HFKE6E7
C
0 5 10 20
Skp2 (p45)
β-Actin

Figure 5.

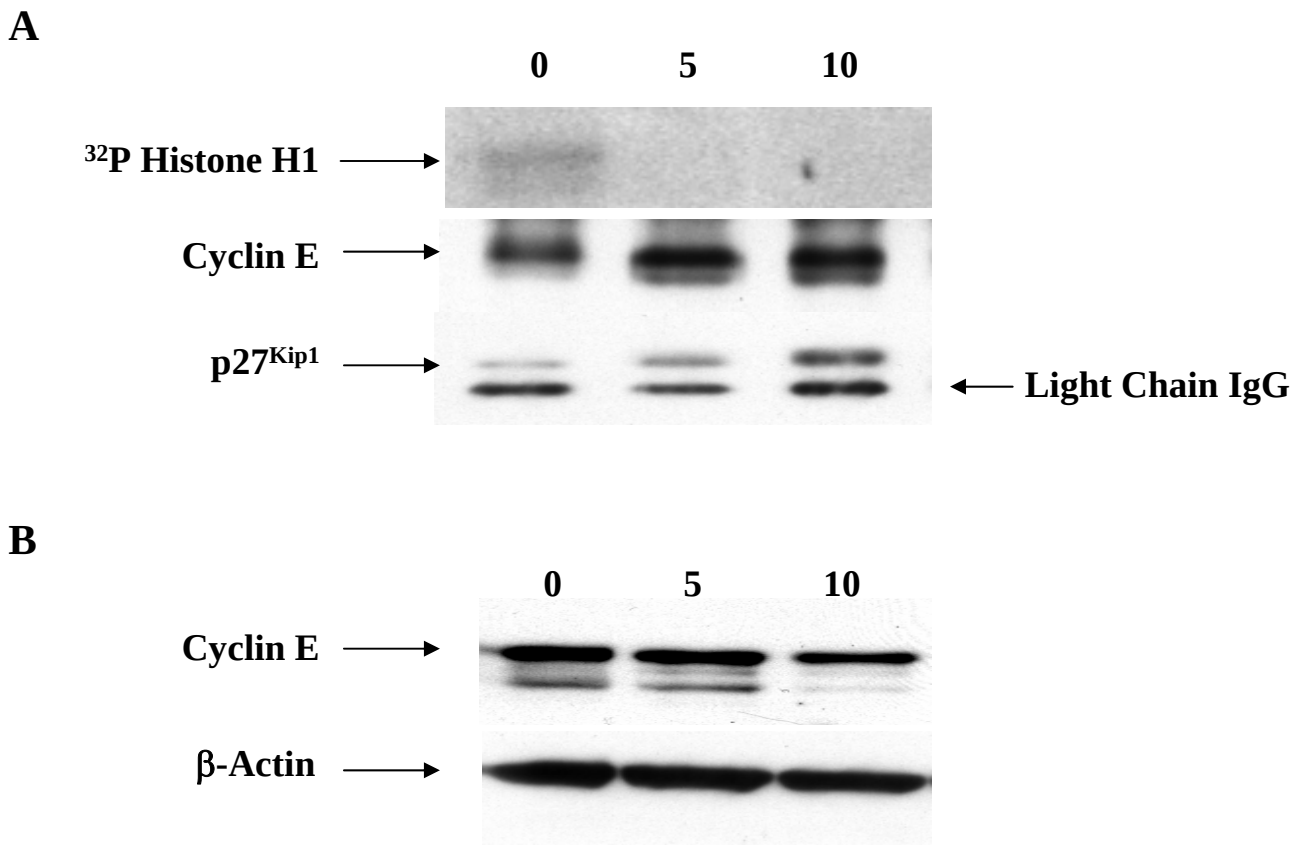

A
0
5
10
32P Histone H1
Cyclin E
p27Kip1
Light Chain IgG
B
0
5
10
Cyclin E
β-Actin

Figure 6

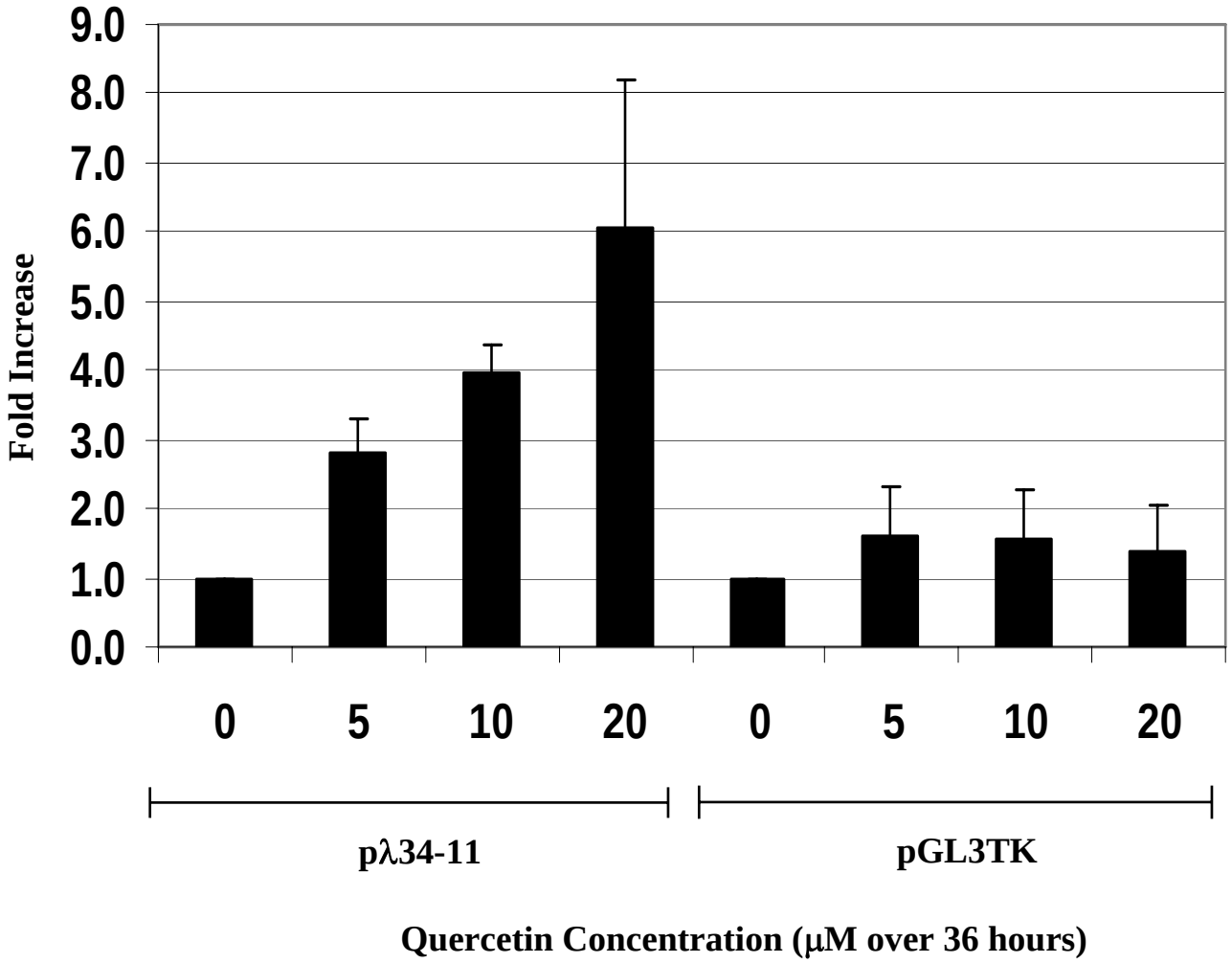
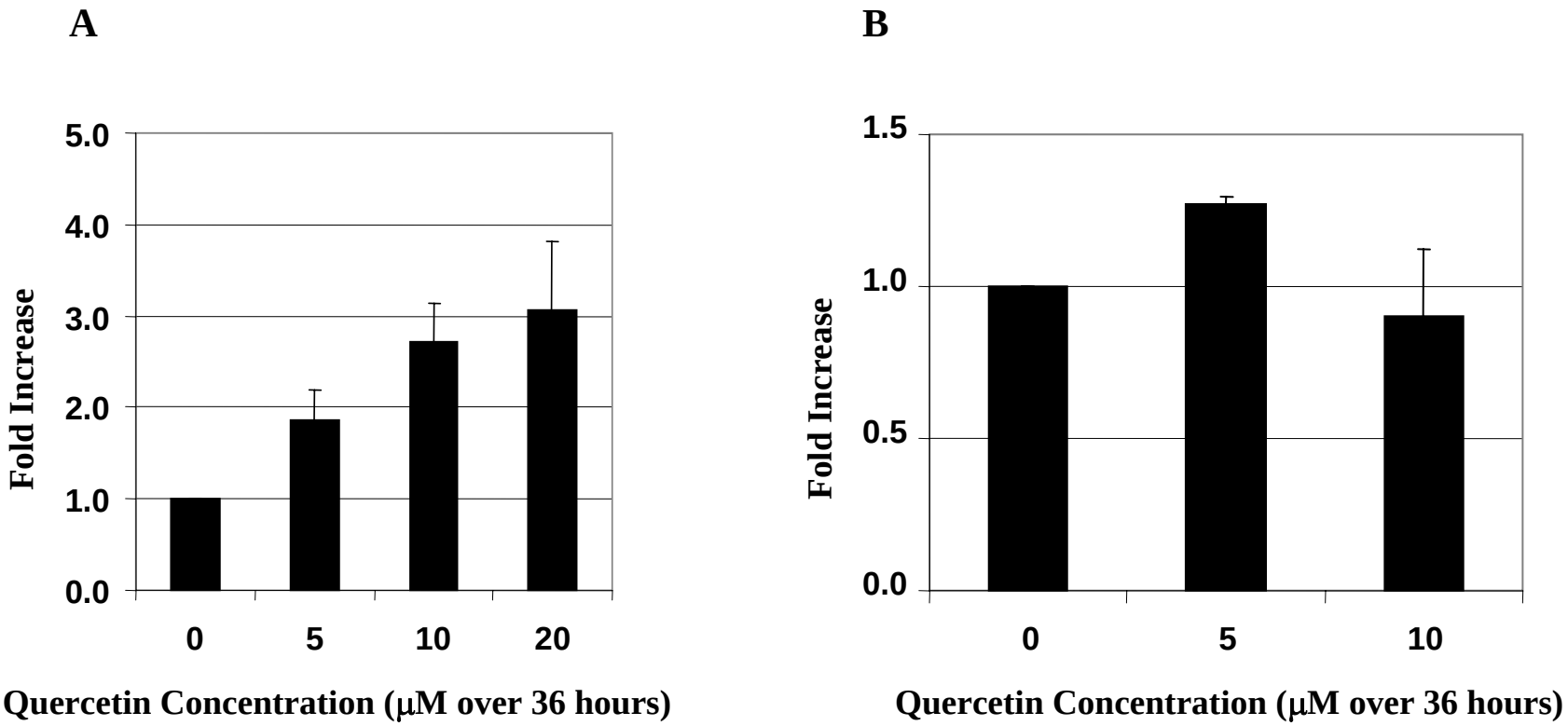

9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Fold Increase
0
5
10
20
0
5
10
20
pλ34-11
pGL3TK
Quercetin Concentration (μM over 36 hours)

Figure 7


A
5.0
4.0
3.0
2.0
1.0
0.0
Fold Increase
0
5
10
20
Quercetin Concentration (μM over 36 hours)
B
1.5
1.0
0.5
0.0
Fold Increase
0
5
10
Quercetin Concentration (μM over 36 hours)