

In vitro biology of fibropapilloma-associated turtle herpesvirus and host cells in Hawaiian green turtles (*Chelonia mydas*)

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Fibropapillomatosis (FP) of green turtles has a global distribution and causes debilitating tumours of the skin and internal organs in several species of marine turtles. FP is associated with a presently non-cultivable alphaherpesvirus Chelonid fibropapilloma-associated herpesvirus (CFPHV). Our aims were to employ quantitative PCR targeted to *pol* DNA of CFPHV to determine (i) if DNA sequesters by tumour size and/or cell type, (ii) whether subculturing of cells is a viable strategy for isolating CFPHV and (iii) whether CFPHV can be induced to a lytic growth cycle *in vitro* using chemical modulators of replication (CMRs), temperature variation or co-cultivation. Additional objectives included determining whether non-tumour and tumour cells behave differently *in vitro* and confirming the phenotype of cultured cells using cell-type-specific antigens. CFPHV *pol* DNA was preferentially concentrated in dermal fibroblasts of skin tumours and the amount of viral DNA per cell was independent of tumour size. Copy number of CFPHV *pol* DNA per cell rapidly decreased with cell doubling of tumour-derived fibroblasts in culture. Attempts to induce viral replication in known CFPHV-DNA-positive cells using temperature or CMR failed. No significant differences were seen in *in vitro* morphology or growth characteristics of fibroblasts from tumour cells and paired normal skin, nor from CFPHV *pol*-DNA-positive intestinal tumour cells. Tumour cells were confirmed as fibroblasts or keratinocytes by positive staining with anti-vimentin and anti-pancytokeratin antibodies, respectively. CFPHV continues to be refractory to *in vitro* cultivation.

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INTRODUCTION

Fibropapillomatosis (FP) is a neoplastic disease of marine turtles; it has a global presence and causes significant external (skin) and internal (organ) tumours (Herbst, 1994). FP is a major cause of stranding of green turtles (*Chelonia mydas*) in Hawaii (Aguirre *et al.*, 1998; Chaloupka *et al.*, 2008; Work *et al.*, 2004) and Florida (Foley *et al.*, 2005), but prevalence of the disease in Hawaii has been decreasing in recent years for unknown reasons. FP is transmissible using cell-free filtrates (Herbst *et al.*,

1995), and molecular studies consistently show alphaherpesviral DNA associated with tumour tissue in green, olive ridley (*Lepidochelys olivacea*) (Quackenbush *et al.*, 2001) and loggerhead (*Caretta caretta*) (Lackovich *et al.*, 1999) turtles in the Pacific and Atlantic oceans.

Both spontaneous and experimentally induced fibropapillomas have been characterized extensively on the basis of gross and microscopic morphology, revealing that lytic viral production, as evidenced by intranuclear inclusions with viral particles, is rare and limited to epidermal layers of the tumour (Herbst *et al.*, 1999). Greenblatt *et al.* (2004) found higher levels of Chelonid fibropapilloma-associated herpesvirus (CFPHV) *pol* mRNA expression in the

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superficial portion of some skin tumours; however, whether the virus sequesters in a particular cell layer is unknown. Knowing which cell types contain the highest levels of viral DNA may help target sites of viral replication for *in vitro* isolation studies.

CFPHV continues to be refractory to *in vitro* cultivation despite several attempts at virus isolation using green turtle cells and classical cell culture methods (Lu *et al.*, 1999; Moore *et al.*, 1997). This inability to culture the virus makes confirming the role of CFPHV as a cause of tumours difficult. In this context, CFPHV is similar to other tumorigenic herpesviruses such as Marek's disease virus (MDV) that causes lymphoid tumours in chickens (Venugopal, 2000), Lucké frog herpesvirus (LFHV) that causes adenocarcinoma in frogs (Skinner & Mizell, 1972) or Kaposi's sarcoma virus (KSV) (Ganem, 2006) and Epstein-Barr virus (EBV) (Young & Rickinson, 2004) that are associated with skin tumours and nasopharyngeal carcinomas of humans, respectively. In contrast with CFPHV, various strategies have been used to culture these viruses, including induction with chemical modulators of replication (CMR) in the case of MDV (Nazerian, 1975), EBV (Nutter *et al.*, 1987) or KSV (Renne *et al.*, 1996), co-cultivation of duck embryo fibroblasts with infected lymphoid cells in the case of MDV (Churchill & Biggs, 1967) or incubation of infected cells at varying temperatures in the case of LFHV (Skinner & Mizell, 1972).

Other than incubation of infected green turtle tissues with embryo fibroblasts (Moore *et al.*, 1997), these strategies have not been systematically evaluated to culture CFPHV, partly because of the lack of available virus-infected cell lines. In Hawaii, turtles severely afflicted with FP occasionally strand and, for humane reasons, are sometimes killed because of poor prognosis for recovery or survival. We took advantage of these specimens to gain further understanding of the *in vitro* biology of CFPHV in the hope that we could provide guidance on strategies that could affect success or failure in virus isolation attempts. Our objectives were to (i) see if CFPHV DNA sequesters by tumour size and cell type, (ii) determine if subculture of the virus is a viable strategy for isolating CFPHV, (iii) see if CFPHV can be induced to lytic growth using CMR, temperature variation or co-cultivation, (iv) determine if non-tumour and tumour cells behave differently *in vitro* and (v) confirm the connective and epithelial origin of cultured tumour cells.

METHODS

Tumours and viral DNA. Skin tumours originated from green turtles stranded in Hawaii with terminal FP (Work *et al.*, 2004). Tumours were cleaned with soap and water, rinsed extensively and held for 2–4 h at 4 °C in antibiotic solution (PBS, pH 7.2, 20 units penicillin ml⁻¹, 0.2 mg streptomycin sulfate ml⁻¹, 10 µg gentamicin sulfate ml⁻¹ and 5 µg amphotericin B ml⁻¹; all Sigma-Aldrich). Tumours were rinsed twice in PBS and minced; 4–6 tissue pieces of 1–2 mm were placed in 12.5 cm² tissue culture flasks to establish

primary cultures. Tissues were incubated in Leibowitz's media (L-15; Gibco) supplemented with 10% fetal calf serum (FCS; HyClone) 2 mM L-glutamine, 1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 units penicillin ml⁻¹, 0.1 mg streptomycin sulfate ml⁻¹, 50 µg gentamicin sulfate ml⁻¹ and 2.5 µg amphotericin B ml⁻¹. Flasks were incubated at 30 °C in ambient air and media was changed twice a week. As monolayers approached confluence (75%), cells were subcultured at 1:2 to 1:4 split ratios using a 0.25% trypsin/versene solution (Invitrogen) to detach and disaggregate cells. Turtle keratinocytes were obtained from skin tumours by incubating with 50% dispase (BD Bioscience), mincing the epidermis and plating in 12.5 cm² flasks. Primary subculture keratinocytes were harvested with trypsin/versene and used for co-cultivation attempts.

DNA was extracted from cells and tissues using DNEasy kits following the manufacturer's instructions (Qiagen). Quantitative PCR (qPCR) for turtle *pol* DNA was carried out as described by Quackenbush *et al.* (2001) without modification using a Bio-Rad iCycler and 100 ng DNA template per reaction. Standard curves of CFPHV *pol* DNA ranging in concentration from 10² to 10⁶ copies were run in duplicate (Quackenbush *et al.*, 2001) and the following controls were included: known CFPHV-negative fibroblasts originating from skin biopsies of captive-raised hatchling or normal skin from green turtles, known CFPHV-*pol*-positive tissue from tumours, AE buffer (Qiagen) and sterile nuclease-free water. Results were expressed as copies of CFPHV *pol* DNA per cell, assuming a DNA concentration of 5 pg per cell for green turtles (Gregory *et al.*, 2007).

Viral DNA versus tumour size and subculture. To correlate tumour size with levels of CFPHV *pol* DNA, tumours were measured on the two largest axes with callipers (cm²) and placed into three size categories: small <2 cm² (seven tumours), medium 2–40 cm² (12 tumours) and large >40 cm² (nine tumours). Size categories were based on a frequency histogram plot of previously published sizes of 254 green turtle tumours (Work *et al.*, 2004). Tumour sections were incubated overnight in 50% dispase (BD Bioscience) at 4 °C, and the epidermal and dermal layers were isolated and frozen separately for subsequent qPCR analysis for CFPHV *pol* DNA. Copy load per cell of viral DNA in dermal cells was compared against tumour size using linear regression, and the mean viral load of epidermal cells versus dermal cells was compared using Student's *t*-test (Daniel, 1987).

To evaluate the effect of subculture on levels of CFPHV *pol* DNA, fibroblasts were harvested from tumours of the skin, heart, spleen, lung and fascia and cultured as described above. Original tissues and cell pellets obtained from subcultures 1, 2, 3, 4, 5, 10 and 15 were stored frozen (–70 °C) for subsequent qPCR analyses.

Evaluation of CMR and temperature on viral growth. To evaluate whether various CMRs induced lytic growth of the virus, we used early subculture (<subculture 2) tumour fibroblasts that were CFPHV DNA-positive. Cells were seeded in triplicate in 24-well culture plates at 10⁵ cells per well or 3 × 10⁴ cells per well for 12-well plates as pure fibroblast cultures. For co-cultivations, fibroblasts were plated with green turtle keratinocytes (20 000–40 000 cells per well). When cells reached 75% confluence (4–5 days), cultures were incubated with 1 mM sodium butyrate for 1, 3 and 5 days (Yu *et al.*, 1999), 20 ng phorbol 12-myristate 13-acetate (TPA) ml⁻¹ for 1, 3 and 5 days (Yu *et al.*, 1999), 50 µM forskolin for 4 days (Smith *et al.*, 1992), 10 ng tumour necrosis factor alpha (TNF-α) ml⁻¹ for 4 days (Kitano *et al.*, 1993), 5 µM trichostatin A for 18 h (Countryman *et al.*, 2008), 20 µg iododeoxyuridine (IUDR) ml⁻¹ for 48 h (Nazerian, 1975), 20 µg IUDR ml⁻¹ for 24 h plus 10 nM dexamethasone for 24–72 h (Paran *et al.*, 1973), 10 mM valproic acid for 18 h (Countryman *et al.*, 2008), 10 µM norepinephrine or 1 nM epinephrine for 24 h (Chang *et al.*, 2005), 1% DMSO for 4 days (Tanaka *et al.*, 1985), 5 µM hexamethylenedisacetamide for 4 days (Bernstein & Kappes, 1988),

10 μM 5-azacytidine for 4 days (Chen *et al.*, 2001) and 1 μM dexamethasone for 24–72 h (Paran *et al.*, 1973). All chemicals were from Sigma, except for dexamethasone and epinephrine, which were from Phoenix Pharmaceutical and MP Biomedicals, respectively. Cells were observed every 1–3 days for presence of cytopathic effects (CPE) compared with non-treated controls, and were harvested and frozen at 20 days.

To assess the effects of temperature, CFPHV-DNA-positive cells were incubated at 24 or 30 °C for 20–30 days, observed every 1–3 days for presence of CPE, harvested and frozen. To evaluate the utility of heat shock to induce virus (Moriya *et al.*, 1994), cells incubated at 24 or 30 °C were incubated for 3 h at 37 °C, returned to their original temperature, observed for CPE and harvested 20 days later. To assess whether virus can be reactivated with cryopreservation in DMSO (Reid & Bridgen, 1991), CFPHV-DNA-positive cells (primary culture) were incubated at 30 °C for 30 days, then a portion of those cells were resuspended in FCS containing 10% DMSO and stored in liquid nitrogen for 5 days. The cells were then thawed, subcultured, examined for the presence of CPE, compared with non-treated controls and harvested after the second and seventh subcultures. CFPHV *pol* DNA levels for cells at day 0 (prior to treatment) and day 20 (after treatment) were measured and compared.

Effects of virus infection on cell growth and morphology. To compare growth characteristics and morphology of fibroblasts from tumours versus normal skin, the following cells were established in culture: dermal fibroblasts from normal skin of two immature FP-free captive-bred green turtles that had been raised in captivity, and fibroblasts from paired normal and tumorous skin from two free-range green turtles afflicted with FP. A seventh cell line consisting of chronically infected CFPHV-*pol*-positive intestinal tumour cells was used for comparison to assess the effects of viral infection on cell growth kinetics. Cells were evaluated for growth characteristics between subcultures 8 and 16. All assays were done in 24-well plates using triplicate wells. We determined seeding density by seeding at the following densities: 2, 8, 40, 200, 1000, 5000 and 25 000 cells cm^{-2} . Cells were incubated at 30 °C and media was changed every 3 days. After 10 days, adherent cells were detached from culture flasks using trypsin/versene buffer and counted with a haemocytometer. To determine plating efficiency, cells were seeded at a density of 50 000 cells cm^{-2} and the percentage of non-adhered cells was determined after 30, 60 and 120 min and 24 h. To determine growth response, cells were seeded at 10 000 cells cm^{-2} , trypsinized at 3, 7, 10, 14, 17, 21, 24 and 28 days, and counted using a haemocytometer. Population doublings were calculated as described previously (Pollard & Walker, 1997). Serum dependency was assessed by seeding cells at 1000 cells cm^{-2} , incubating with 0, 5 and 10% FCS for 12 days and counting with a haemocytometer.

Cell morphology was assessed using light microscopy, karyotyping and immunohistochemistry. For karyotyping, cells at exponential growth phase at subculture 20 were incubated overnight at 30 °C with 0.5 μg demecolcine ml^{-1} (Sigma-Aldrich) and chromosomes were prepared as described previously (Pollard & Walker, 1997). Immunohistochemistry was used to confirm that cells in culture were fibroblasts or keratinocytes. Cells were grown at 30 °C to 75–80% confluence, washed three times in PBS, fixed with paraformaldehyde for 15 min at room temperature and then washed again with PBS. Cells were then permeabilized with 0.5% Triton X-100 (Sigma-Aldrich) for 10 min at room temperature followed by a wash with PBS. Cells were blocked with PBS with 2% milk (PBS-M) for 30 min, followed by incubation with monoclonal mouse IgG2a/ κ anti-vimentin (clone Vim 3B4; LabVision) and monoclonal mouse IgG1 anti-pan cytokeratin (clone PCK-26; Sigma) diluted 1:50 and 1:5000, respectively, in PBS-M for 30 min at room temperature. Cells were washed three times in PBS-M and incubated for 30 min with

horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody (Sigma-Aldrich) diluted 1:1000 in PBS-M. After three rinses in PBS, diaminobenzidine (DAB) was added to the cells according to the manufacturer's instructions (Pierce) for colorimetric analysis.

To confirm the nature of binding of monoclonal mouse IgG2a/ κ anti-vimentin to turtle vimentin, fibroblasts or keratinocytes, 2×10^6 cells ml^{-1} were homogenized in PBS, homogenates were mixed 1:2 in Laemmli buffer and proteins were separated on a 10% polyacrylamide gel with a 4% stacking gel using a Mini-Protean II electrophoresis system (Bio-Rad). Proteins were transferred onto a nitrocellulose membrane using a mini transblot electrotransfer cell (Bio-Rad). The membrane was blocked with PBS-M and treated with mouse anti-vimentin and rabbit anti-mouse conjugate as described above for immunohistochemistry. Positive control antigen for anti-vimentin and anti-pancytokeratin antibodies included vimentin Ab-2 and cytokeratin 20 (both LabVision), respectively. Negative control antigens for anti-vimentin and anti-pancytokeratin antibodies were keratinocyte and fibroblast homogenates, respectively.

RESULTS

There was no significant relationship between tumour size and levels of CFPHV *pol* DNA (Fig. 1a). CFPHV *pol* DNA copy number in the dermis of tumours were significantly higher ($t=7.07$, $P<0.0001$) than those in the epidermis (Fig. 1b). For both external and internal tumours, viral DNA decreased rapidly to less than 10% of original tissue

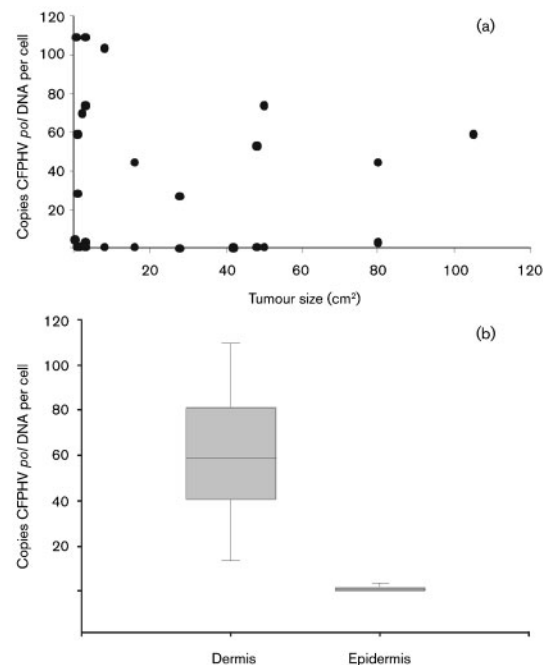


Fig. 1. (a) Scatterplot of CFPHV *pol* DNA load in dermis versus tumour size ($n=28$). Note, two tumours at what appears to be 0 actually measured 0.2 cm^2 . (b) Box plot of CFPHV *pol* DNA load in dermis and epidermis from paired samples ($n=28$) of tumours from green turtles afflicted with FP. Whiskers indicate SD, upper and lower bounds of the boxes indicate the 5 and 95 percentiles, and horizontal lines in the middle of the boxes indicate the means.

burdens by subculture 1 and continued decreasing to essentially undetectable levels by subculture 10 (Fig. 2). No CPE was seen in cells at any time during the experiment. One cell line (intestine tumour) remained consistently CFPHV-*pol*-DNA-positive through to subculture 46, whereupon viral DNA disappeared with subsequent subcultures after the cells were frozen and thawed.

Induction of known infected cells with CMR, at various temperatures, by cryopreservation with DMSO or keratinocyte-fibroblast co-cultivations uniformly failed to induce viral replication. No CPE was seen in any cultures for any treatment and DNA levels in known CFPHV-*pol*-DNA-positive cells decreased by 21–99% (Table 1).

Cells grew from primary explants in 4–10 days and formed confluent monolayers in 20–29 days in the case of normal skin, 30–32 days for paired tumour-affected skin from FP-afflicted turtles and 43 days for skin from FP-free turtles. Optimal plating density for all cell types ranged from 40 to 1000 cells cm^{-2} and, based on this, 1000 cells cm^{-2} were used for all subsequent studies except plating efficiency. Fibroblasts from skin tumours or intestinal tumour cells that were CFPHV-*pol*-DNA-positive for 46 subcultures generally grew more slowly than those from paired normal skin or normal skin from FP-free turtles; however, these differences were not significant for serum dependency, growth kinetics, seeding density or plating efficiency (Fig. 3).

Modal karyotype for all six cell lines was 55–56. Morphologically, fibroblasts had a fibroblast-like appearance (Fig. 4a), whereas keratinocytes had a distinct cobblestone pattern (Fig. 4b). Fibroblasts stained with vimentin whereas keratinocytes did not (Fig. 4c), and keratinocytes stained with pancytokeratin whereas fibroblasts did not (Fig. 4d). Under phase-contrast or when stained with Wright's Giemsa, cells from normal and tumour-affected skin were morphologically indistinguishable. Mouse anti-vimentin antibodies recognized several proteins in fibroblast homogenates in the 50–60 kDa range,

but failed to stain any bands for keratinocyte homogenates. Mouse anti-pancytokeratin antibodies recognized proteins in keratinocyte homogenates in the 45–60 kDa range but failed to stain any bands for fibroblast homogenates (Fig. 5).

DISCUSSION

Most herpesviruses undergo intermittent lytic replication followed by latency (Young & Rickinson, 2004). In herpesviral diseases that induce tumours, latent viral proteins are thought to be responsible for tumorigenesis (Young & Rickinson, 2004). Alternatively, the virus infects cells and then disappears after inducing irreversible genetic damage that leads to cell transformation and generation of tumours (the hit-and-run hypothesis) (Ambinder, 2000). In turtles, larger tumours are presumably older, so if a hit-and-run mechanism is responsible for tumour formation, larger tumours should have little to no viral DNA. The lack of a relationship between tumour size and levels of CFPHV viral DNA suggests otherwise and indicates that CFPHV somehow plays a role in tumour formation *in vivo*.

Skin tumours in green turtles affected by FP are characterized by the presence of a sessile to arborizing collagen matrix mixed with pleomorphic haphazardly arranged fibroblasts overlaid by markedly acanthotic epidermis (Herbst *et al.*, 1999). On occasion, rare clumps of herpesvirus-like intranuclear inclusions are present within the keratinized epidermis (Jacobson *et al.*, 1989) but not fibroblasts. The presence of higher levels of CFPHV *pol* DNA in dermis versus epidermis suggests that while vegetative viral production occurs in keratinocytes, a majority of the CFPHV viral genomes resides in fibroblasts integrated either into host DNA or as episomes (Ganem, 2006). Given that less than 2% of skin tumours have intranuclear inclusions visible on histology (Herbst *et al.*, 1999), the lack of viral DNA in epidermal cells is not surprising. However, other investigators have occasionally found higher levels of CFPHV *pol* DNA in superficial tumour tissues versus deeper tissues (Greenblatt *et al.*, 2004), seemingly contradicting our findings. In the Greenblatt study, superficial tumour tissue included biopsies of both epidermis and dermis. In contrast, in the present study, we analysed epidermal and dermal cells separately, thereby providing a more accurate picture of the distribution of viral DNA in turtle tumours. Two potential explanations for the inconsistently higher levels of viral DNA in superficial tumours found by Greenblatt *et al.* (2004) exist: (i) superficial fibroblasts contain more viral DNA than deep fibroblasts or (ii) some superficial tumour biopsies may have contained actively replicating virus within the epidermis.

Treatment with CMR has been successfully used to induce replication of retroviruses (Paran *et al.*, 1973) and herpesviruses (Countryman *et al.*, 2008); however, the mechanisms by which they do this are unclear. For herpesviruses, the ability to induce replication depends

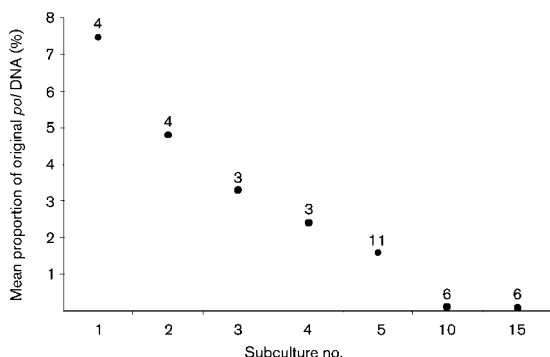


Fig. 2. Mean percentage of CFPHV *pol* DNA load per cell remaining in cultured fibroblasts relative to viral DNA loads in original tissue (100%) versus subculture number. The number above each dot indicates the number of replicate experiments.

Table 1. Effects of various treatments on copies of CFPHV *pol* DNA in cultured cells from green turtles afflicted with FP

FB indicates the number of attempts on fibroblasts only, while FBKC indicates the number of attempts on co-cultures of fibroblasts and keratinocytes. Mean (\pm SD) number of copies of CFPHV *pol* DNA per cell at the start (DNA-Start) and end (DNA-End) of the experiment along with percentage decrease of CFPHV *pol* DNA are shown.

Treatment	FB	FBKC	DNA-Start	DNA-End	% Decrease
5-Azacytidine	3	2	101 (69)	20 (30)	80
Dexamethasone	3	2	101 (69)	19 (33)	80
DMSO	2	2	135 (20)	11 (16)	91
Epinephrine	2	2	105 (44)	34 (57)	67
Forskolin	2	2	105 (44)	77 (149)	26
Hexamethylenebisacetamide	3	2	101 (69)	15 (28)	85
Isobutylmetaxanthine	1	2	105 (44)	42 (81)	60
IUDR	3	2	101 (36)	21 (35)	78
IUDR + dexamethasone	2	2	115 (28)	16 (25)	86
<i>n</i> -Butyrate	3	0	1825 (283)	1239 (338)	32
Norepinephrine	1	2	105 (44)	32 (58)	68
TNF- α	3	2	101 (69)	11 (18)	88
Phorbol 12-myristate 13-acetate	3	0	1825 (283)	1432 (238)	21
Trichostatin A	2	2	105 (44)	50 (90)	52
Valproic acid	1	2	129 (22)	49 (83)	62
Heat shock (37 °C, 3 h)	3	2	28 (4)	3 (3)	88
DMSO and freezing (30 °C)	1	0	15.1	3.4	77
DMSO and freezing (24 °C)	1	0	15.1	0.08	99

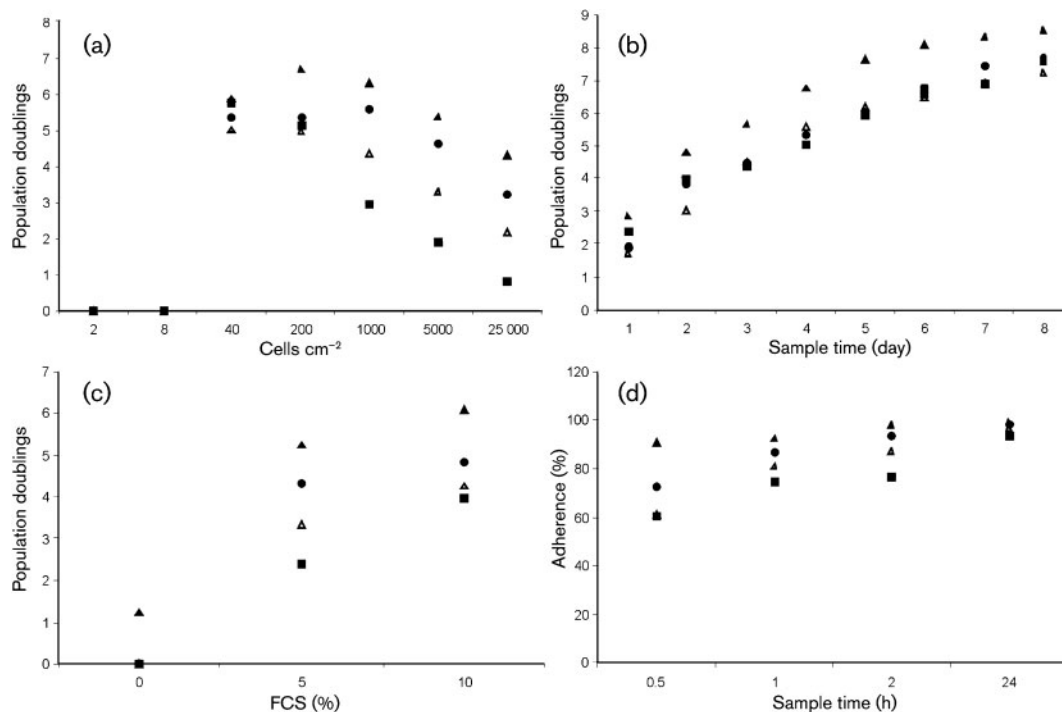


Fig. 3. Cultured fibroblasts from paired normal (▲) and tumourous (△) skin, skin from tumour-free turtles (●) and CFPHV-positive intestinal tumour cells (■). Except for intestinal tumour cells, each symbol represents the mean of duplicates (two turtles each). (a) Seeding density versus population doublings, (b) growth kinetics, (c) serum dependency and (d) plating efficiency.

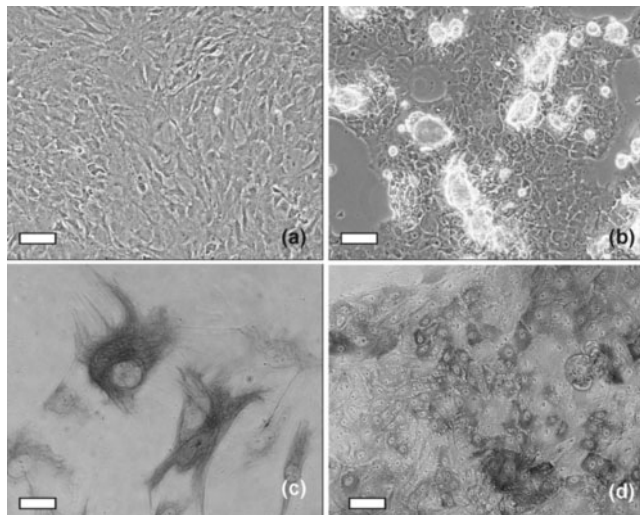


Fig. 4. Cells from skin tumours of Hawaiian green turtles with FP. (a) Fibroblasts. Note the stellate appearance. Bar, 60 μ m. (b) Keratinocytes. Note the cobblestone appearance. Bar, 60 μ m. (c) Fibroblasts stained with anti-vimentin antibodies. Bar, 20 μ m. (d) Keratinocytes stained with anti-pancytokeratin antibodies. Bar, 30 μ m.

on not only the type of CMR but also the type of cell line being induced (Gradoville *et al.*, 2002). The lack of persistently infected cell lines does not help attempts to isolate CFPHV, so we tried to get around this problem by using early subculture cells known to contain CFPHV *pol* DNA. CMRs work through various mechanisms that

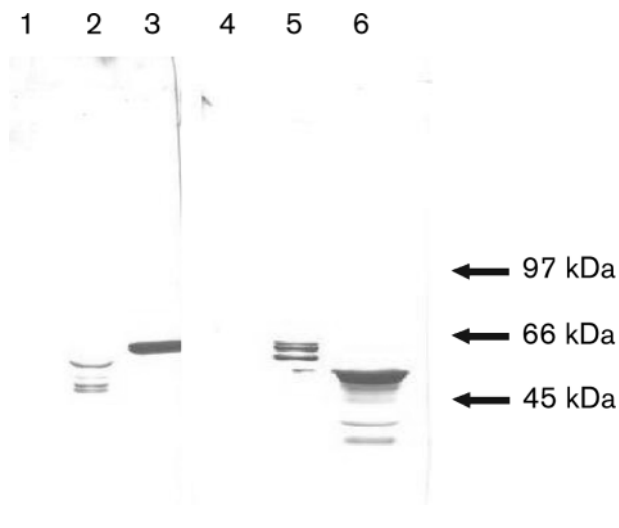


Fig. 5. Western blot containing green turtle fibroblast (FB) or keratinocyte (KC) homogenates. Lanes: 1, FB + anti-pancytokeratin antibody (APCKab); 2, FB + anti-vimentin antibody (AVab); 3, Vimentin + AVab; 4, KC + AVab; 5, KC + APCKab; 6, cytokeratin 20 + APCKab.

inhibit or facilitate DNA replication. Butyrate, trichostatin A and valproic acid all inhibit histone deacetylase, phorbol esters are protein kinase activators and 5 azacytidine is a DNA methyltransferase inhibitor; all of these in various combinations have been successfully used to induce replication of KSV and EBV from persistently infected cell lines (Countryman *et al.*, 2008). Forskolin (an adenylate cyclase activator) and isobutylmetaxanthine (a phosphodiesterase inhibitor) have been used successfully to reactivate herpes simplex virus type 1 (HSV-1) from cell cultures (Smith *et al.*, 1992). Granulocyte precursors latently infected with human cytomegalovirus can be induced to lytic replication by TNF- α because these compounds induce cellular differentiation (Streblow & Nelson, 2003). Cells latently infected with MDV have been induced to lytic replication by administering the DNA replication inhibitor 5 IUDR (Nazerian, 1975). KSV has been induced to replicate from latently infected primary effusion lymphomas by epinephrine and norepinephrine that act through β 1-adrenoreceptors (Chang *et al.*, 2005). Finally, dexamethasone alone has successfully been used to increase lytic replication of cytomegalovirus from fibroblasts (Thiele & Woods, 1988) and dexamethasone plus IUDR has induced viral replication of murine leukaemia virus C (Paran *et al.*, 1973). None of these mechanisms appear to be viable strategies for inducing lytic replication of CFPHV from infected turtle cells *in vitro*.

Other strategies to successfully induce replication of latent viruses have included incubation at varying temperatures, submitting cells to cryopreservation with DMSO and co-cultivation of cells. LFHV grows optimally at approximately 11 $^{\circ}$ C (Skinner & Mizell, 1972). Our rationale for incubating tissues at 24 and 30 $^{\circ}$ C was based on seasonal mean ocean temperatures in Hawaii which seldom deviate beyond the range of 23–28 $^{\circ}$ C. While 30 $^{\circ}$ C may seem on the high side, sea turtles will haul out on shore and bask, whereupon their core temperature can easily exceed 30 $^{\circ}$ C (Rice *et al.*, 2000). Cultures of known infected turtle cells at both 24 and 30 $^{\circ}$ C failed to induce viral replication under the conditions of this study. Likewise, heat shock, which has been used successfully to induce replication of HSV-1 from neuronal cultures (Halford *et al.*, 1996) also failed to induce viral replication. Co-cultivation of duck embryo fibroblasts with lymphoid cells infected with MDV has been used to rescue virus from latently infected cells (Churchill & Biggs, 1967). Although lytic replication of CFPHV appears to be limited to keratinocytes, co-cultivation of this cell type with known infected fibroblasts failed to induce viral replication. In this context, CFPHV may be more akin to papillomaviruses, in which maturation of keratinocytes is needed to induce viral replication (Wang *et al.*, 2009).

Tumour cells *in vitro* lose herpesviral DNA with subculturing. A similar phenomenon occurs in Kaposi's sarcoma, a human tumour associated with a herpesvirus. In that system, sarcoma cells lose episomal viral genomic DNA over time *in vitro* because the pressures that lead to

maintenance of viral DNA *in vivo* are absent (Ganem, 2006). Thus, there are two possible reasons why CFPHV DNA in culture is lost over time in cultured green turtle cells: (i) loss of episomal DNA due to a lack of pressures needed to maintain viral DNA or (ii) competitive survival advantage of virus-free cells in culture.

Neoplastic cells typically have lower adherence and serum dependency for growth (Eagle *et al.*, 1970). In this study, we saw no differences in plating efficiency and serum dependency between primary cultures derived from tumorous and normal skin. The lack of differences in morphology and *in vitro* behaviour of fibroblasts derived from tumorous and normal skin suggests that cells from tumours cultured *in vitro* lose neoplastic characteristics found *in vivo*. Comparisons between cell types were done between subcultures 8 to 14, long after viral genomes disappeared from cells; however, the growth characteristics of an infected intestinal tumour cell line also did not differ from those of normal cells. Others have reported that a comparison of *in vitro* characteristics of tumour and normal cells yields highly variable results (Eagle *et al.*, 1970).

The skin fibroblasts used in this study behaved similarly to turtle skin fibroblasts used by other investigators (Lu *et al.*, 1999; Moore *et al.*, 1997); explants grew out of tissues within 10 days and optimal growth was at 30 °C. A karyotype of 55–56 matched that found by Moore *et al.* (1997) for green turtles but was less variable than that seen by Lu *et al.* (1999). The plating efficiencies seen in our study were higher than those reported by either Lu *et al.* (1999) or Moore *et al.* (1997), and this may be attributable to media; the aforementioned studies used RPMI 1640 or M199 with CO₂ incubation, whereas we used L-15 with no CO₂.

Green turtle fibroblasts in cultures were stained for vimentin with commercially available anti-vimentin antibodies, and keratinocytes in mixed fibroblast–keratinocyte cultures were stained with commercially available anti-pancytokeratin antibodies. Vimentin is a 57 kDa intermediate filament protein considered as diagnostic for mesenchymal cells such as fibroblasts (Fuchs & Weber, 1994). The presence of vimentin and cytokeratin in green turtle fibroblasts and keratinocytes, respectively, was confirmed by Western blotting, in which mouse monoclonal anti-vimentin antibodies bound protein bands at the appropriate molecular mass range for these proteins. In addition to morphology, positive staining of cultured cells with anti-vimentin antibodies and anti-pancytokeratin antibodies confirmed the identity of cultured cells. Future strategies to isolate CFPHV might consider establishment of organotypic raft cultures (Wang *et al.*, 2009). However, successful implementation of such a strategy awaits cultures of fibroblast-free turtle keratinocytes.

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Use of trade, product or firm names does not imply endorsement by the US Government.

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