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**EQUINE SARCOIDS AND BOVINE
PAPILLOMAVIRUS;
UNRAVELING THE VIRAL
PATHOGENESIS**

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BVMS

**A thesis submitted to the University of Glasgow for the degree of Doctor
of Philosophy**

**Division of Pathological Sciences
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Abstract

The equine sarcoid is the most commonly detected skin tumour in equidae worldwide and has been reported in horses, donkeys, mules and zebra. Sarcoids can be defined as locally aggressive, fibroblastic, benign tumours of the equine skin and although they do not metastasise, they exhibit variable clinical presentations ranging from small alopecic areas to rapidly growing, ulcerated fleshy masses. Clinical behaviour may also vary, from aggressive infiltrative growth to spontaneous regression. Diverse treatment modalities have been reported, and these have been used separately, or have been combined, with variable efficacy. No single therapeutic approach has been found to be universally successful. Although rarely life-threatening, equine sarcoids can have important welfare and economic implications. There is a large body of evidence now supporting the hypothesis that bovine papilloma virus (BPV) is the aetiological agent of equine sarcoids and our understanding of the general pathophysiology of the disease continues to progress; however, several fundamental aspects of the disease remain unclear.

In the first chapter, the clinical aspects of the disease are discussed and evidence to support a viral aetiology is presented, along with background information regarding papilloma virus infections in the natural host. An epidemiological overview of the disease that considers the most current theories and understanding of equine sarcoids is also given.

Chapter II describes the materials and methods used in the course of carrying out the investigations detailed in Chapters III, IV and V.

The aim of Chapter III was to investigate the potential role of flies as vectors in transmission of equine sarcoids between infected and susceptible animals. We found that BPV-1 DNA can be detected in flies trapped from areas where animals with BPV-1 infected sarcoids are housed. This study provides evidence to support the hypothesis that flies may therefore be significant in disease transmission. Such evidence will be of benefit in formulating management control strategies for fly control measures that will reduce the incidence and spread of equine sarcoids.

The work described in Chapter IV was carried out to investigate the hypothesis that the development of the sarcoid tumour arises primarily through inhibition of apoptotic pathways by viral gene products. Assessment of DNA-damage-induced apoptosis in BPV transformed equine fibroblasts revealed that sarcoid derived cells and BPV-1 transfected fibroblasts are less resistant to apoptosis than normal, BPV-negative control equine

fibroblasts, but are more likely to recover from DNA damage and continue to grow and divide. Further research was directed towards identifying the viral genes responsible for conferring resistance to apoptosis through siRNA knock-down and monitoring expression of endogenous cellular proteins known to be involved in apoptotic pathways. Using an siRNA targeted to a particular viral protein, we were able to reduce the ability of the BPV transformed cells to survive after DNA damage. Potentially, this information can now be used to develop novel therapeutic strategies.

The final chapter describes the results of a study in to the expression of the p53 protein in sarcoids *in vivo* and in BPV transformed cells *in vitro* in tissue culture. *In vivo*, analysis of 51 equine sarcoid lesions showed that 48% of tumours are associated with nuclear p53 protein expression and that high levels of p53 were associated with clinically aggressive tumour types (fibroblastic). Interestingly, we observed high levels of cytoplasmic p53 staining with no nuclear staining in one tumour *in vivo*. *In vitro*, p53 mRNA levels were assessed in BPV transformed and BPV negative fibroblasts. The half-life of the p53 protein, the cellular location and functionality were also investigated *in vitro*. The results showed that BPV transformed equine fibroblast cells also exhibit increased nuclear p53 protein expression and one cell line (EqS04b) showed a cytoplasmic staining pattern similar to that observed *in vitro*. However, the abnormal level and location of the p53 does not appear to affect the transactivational functionality of p53 since p21 expression was induced by exposing the cells to UVB irradiation.

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Declaration

I hereby declare that the work carried out in this thesis is original and was carried out by either myself or with due acknowledgement. All additional sources of information have likewise been acknowledged. This work has not been presented for the award of a degree at any other university.

Signed..........

Date: 16th November, 2010

Publications

Articles

Virus Research (2009);144(1-2):315-7

THE DETECTION OF BOVINE PAPILLOMAVIRUS TYPE 1 DNA IN FLIES.

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BOVINE PAPILLOMAVIRUS AND EQUINE SARCOIDS: APOPTOSIS AND CELL SURVIVAL FOLLOWING TREATMENT WITH UVB

M. Finlay, L. Nasir, I. Morgan, M.S. Campo

1 Chapter I:

Introduction to Equine Sarcoids and a Review of the Literature.

1.1 GENERAL INTRODUCTION

Over 70 years after their first description, equine sarcoids remain a frustrating and challenging entity for veterinary practitioners and research scientists alike. These common tumours of the skin in equidae are varied in their clinical presentation and unpredictable in their response to treatment. The widely embraced hypothesis that bovine papilloma virus (BPV) is the aetiological agent of equine sarcoids makes this disease entity a fascinating addition to the arena of papilloma virus research. Papilloma virus research began in earnest in the 1930's with the discovery that filtered extracts from cutaneous papillomas of wild cottontail rabbits could induce lesions with malignant potential in cottontail and domestic rabbits. In the 1970s a 'technical revolution' began, and the advances in molecular understanding that followed eventually led to the identification of Human Papilloma Virus (HPV) as a major public health problem. So significant were these developments that the 2008 Nobel Prize in Physiology or Medicine went to Harald zur Hausen, who discovered the role that HPV plays in the pathogenesis of cervical cancer. He demonstrated novel properties of HPV that have led to an understanding of mechanisms for papilloma virus-induced carcinogenesis and the predisposing factors for viral persistence and cellular transformation. His discovery that specific 'high-risk' HPVs are most commonly involved in cervical cancer development led to the development of a now widely used vaccine that will potentially save thousands of lives worldwide, every year.

The pathogenesis of equine sarcoids does not conform precisely to many of the currently understood models of papilloma virus infection. This makes the study of equine sarcoids particularly intriguing and challenging.

1.2 CLINICAL PRESENTATION

Equine sarcoids are locally invasive, fibroblastic skin tumours and represent the most common skin tumour in equidae worldwide (Jackson 1936; Ragland *et al.*, 1970; Pascoe and Summers 1981). They can occur as single lesions or, more commonly, multiple lesions in different forms, ranging from small wart-like protuberances to large ulcerated fibrous growths. There is an established system of clinical classification which was originally proposed by Professor D.C. Knottenbelt (2005) and is based on the physical appearance and behaviour of the tumour;

1.2.1 Occult

These tumours are persistent, grey and hairless; often circular or roughly circular (Fig 1.1a), and probably represent the earliest form of the disease. Although they are early forms it does not necessarily mean that they have been a recent occurrence. Some of these lesions remain static for years without any clinical implication. Careful palpation of the lesion will usually reveal one, several or occasionally many tiny nodules in the skin of the affected area. The skin may also feel slightly thickened and lacking in its normal elasticity. They are most commonly found on the face, axilla, inguinal and groin areas but can occur at any body site, although they are rarely identified on the distal limb region. Interference with occult lesions (either accidentally or intentionally during biopsy or ill advised treatments) usually results in a dramatic deterioration and transformation to one of the more serious forms. In many such cases, the lesion will develop into a fibroblastic lesion within weeks.

In addition to being mistaken for common and benign abrasions such as rub marks from tack, fence-posts or other solid objects, occult sarcoids may be confused with several other skin conditions such as ringworm (dermatophytosis), alopecia areata and pemphigus foliaceus.

1.2.2 Verrucose

Verrucose tumours are grey, scabby or warty in appearance (hence the term verrucous or verrucose or wart-like sarcoid). The lesions can be small and 'papilloma-like' extensions from the skin, but often they coalesce to cover large, ill-defined areas (Fig. 1.1b). Usually there is alopecia and the skin lacks normal flexibility, cracking easily when manipulated, particularly if the skin is folded over on itself. The surface of most verrucous lesions is hyperkeratinised and flakes and scale are easily rubbed from the surface. There may be some ulceration of the surface exposing red, fleshy tissue underneath. They are most

commonly identified in the face, groin, sheath, and axilla areas. Interference with verrucous lesions (either accidentally or intentionally during biopsy or inadequate treatments) usually results in a dramatic deterioration and transformation to one of the more serious forms. In many cases the lesion will develop into a fibroblastic lesion within weeks and some cases will deteriorate dramatically into a malignant form.

1.2.3 Nodular

These can be single (Fig. 1.1c) or multiple and are often present in large numbers. Some have interlocking nodules giving a 'bunch of grapes'- like appearance. Some have no skin involvement (the skin can be moved freely over the surface) but some are firmly attached to the overlying skin (the skin cannot be moved over the surface of the nodule). Some ulcerate and form bleeding exudative masses. Two types are recognised based upon the extent of skin involvement:

(i) Type A nodules

Type A nodules are characterised as nodular tumours that have no skin involvement (Fig. 1.1d). They lie entirely under the skin so that the skin can be moved freely over the lesion and the lesion can be moved freely over the deeper structures. Some type A nodules can be very deep beneath the skin (especially common in the girth and brisket regions and in the sides of the sheath). These sites have quite thick skin overlying the sarcoid and this may be both an explanation for their depth and for their common failure to erupt through the skin or to influence the skin in any secondary fashion. The overlying skin may become hairless and thinned and eventually the skin may break open (often spontaneously or during minor handling) and the lesion then either presents as an ulcerated nodule, or the nodule may fall away spontaneously leaving a haemorrhagic cavity. Extensive groups of nodules that may or may not be linked together may develop in one or more locations. Apart from the eyelid, a type A nodule can be highly dangerous in some other locations such as the sheath and groin. Some can be very extensive despite having no outward skin involvement. Histopathologically, the Type A nodule has an encapsulated nature with a loose fibrocellular surrounding that is responsible for its mobility.

(ii) Type B nodules

These sarcoids are those with visible or palpable skin involvement so that the skin cannot be moved independently. Sometimes the overlying skin is alopecic and of a verrucous nature (Fig1.1e). This type of sarcoid can develop from a type A nodule (especially if these

are interfered with or those that are neglected for long periods). The affected overlying skin is usually hairless, thickened and verrucous in appearance until the skin becomes ulcerated when a fleshy bleeding surface will be exposed. Once these lesions become ulcerated (for example, after interference / trauma) they develop rapidly into fibroblastic lesions. Eyelid nodules of this type are particularly dangerous – it has been shown that they have major propensity to deep and extensive “roots” that permeate the muscles of the eyelids (Knottenbelt, 2005). Histopathologically, Type B nodules have poorly defined margins both in the skin and the deeper tissues – this is consistent with their “fixed” nature and lack of independent mobility.

1.2.4 Fibroblastic

These tumours comprise fleshy masses, either with thin pedicle or a wide flat base that commonly bleed easily (Fig. 1.1f(i) and (ii)). They may have a wet, haemorrhagic surface. They are common at almost every site and may develop quickly from a milder form following even slight damage. They also commonly develop at the site of skin injuries on the limbs.

1.2.5 Mixed

The term ‘mixed tumour’ is used to describe lesions which contain variable mixtures of two or more of the other types (Fig. 1.1g). They can develop at any site but are commonest on the head, axilla and groin. It has been observed clinically, that many lesions are actually mixtures of the other types and so mixed sarcoid is a common diagnosis.

1.2.6 Malevolent

This is the most recently classified form (Knottenbelt 2005) and is applied to any aggressive lesion in which there is extensive local or wider spread through the skin and the subcutaneous tissues (Fig. 1.1h). These tumours can infiltrate local lymphatic vessels resulting in the development of multiple nodular or fibroblastic type growths along these vessels. Local lymph nodes may also be involved. The skin need not be broken and there may be no superficial ulceration. There are often some overlying verrucous and occult changes in the skin; there are some cases that develop large areas of verrucous sarcoid with underlying smaller cords of nodular or fibroblastic sarcoids. Usually affected horses have multiple sarcoids at other sites but isolated malignant lesions can develop; this is commonest at the point of the elbow, the medial thigh and the side of the face. This form is a possible consequence of repeated and unsuccessful treatment attempts but some cases develop spontaneously. These are also classified as malignant. There is no recorded case in

which the sarcoid has spread to internal organs and so the term malevolent is used to try to differentiate it from the metastatic, disseminating tumours of other neoplastic diseases that are also commonly referred to as 'malignant'.

Figure 1.1

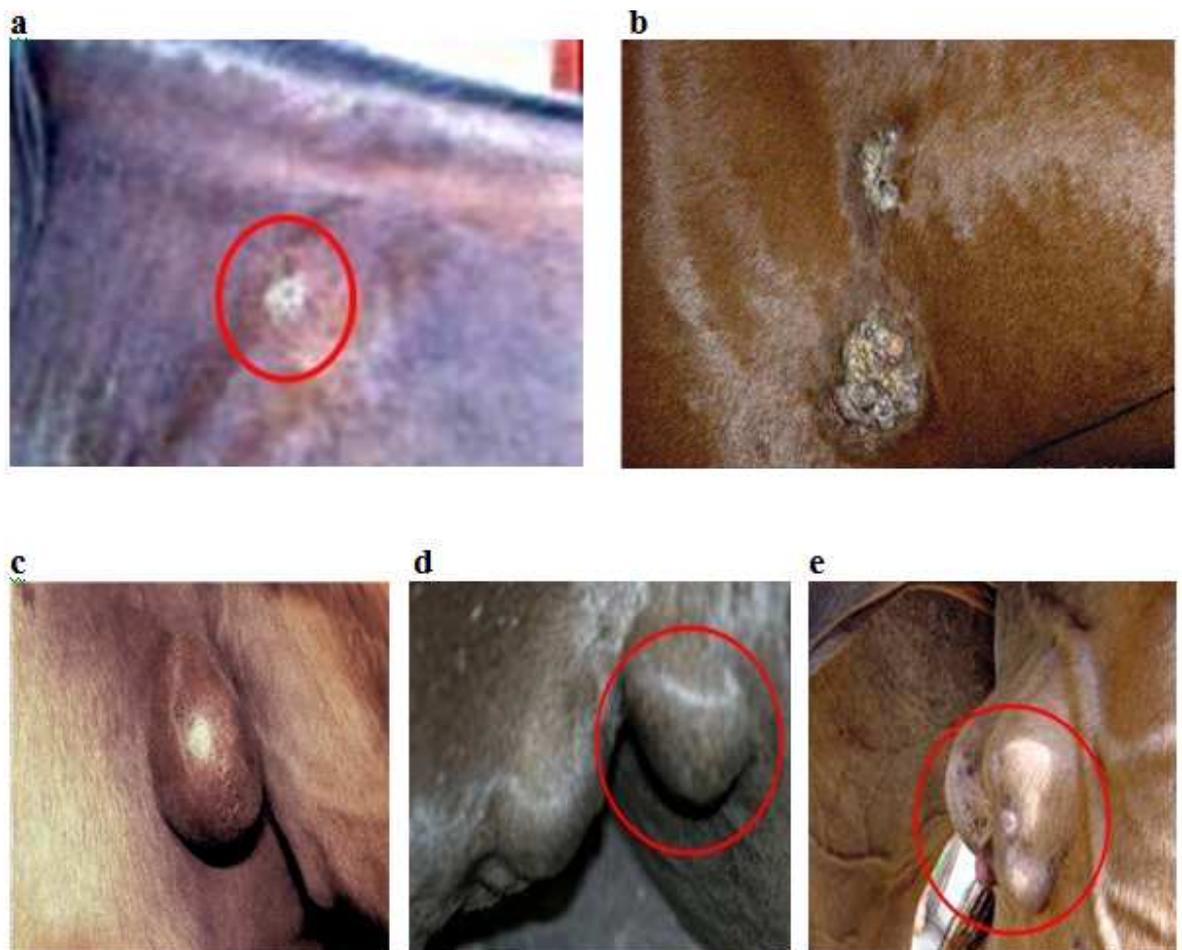


Figure 1.1 Equine sarcoids exhibit variable clinical presentation (Knottenbelt, 2005)

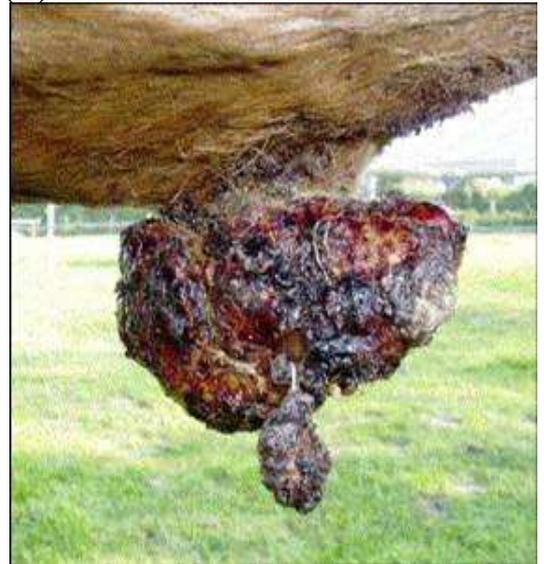
There is an established system of clinical classification which was originally proposed by Professor D.C. Knottenbelt (2005) and is based on the physical appearance and behaviour of the tumour. Occult sarcoids appear as circular, hairless lesions (Fig. 1.1a). Verrucous ('wart-like') sarcoids can range from small well defined warts, to large areas of thickened, rough and warty skin (Fig 1.1b). Nodular sarcoids (Fig 1.1c) are further divided into two sub-groups. Nodular Type A sarcoids (Fig 1.1d) are classified as those nodular sarcoids with no skin involvement, so that the skin covering the sarcoid shows no gross pathological change, and can be freely moved independently of the tumour mass. Nodular Type B sarcoids (Fig 1.1e) are those nodular sarcoids in which there is an obvious involvement of the overlying skin, such as hair-loss and ulceration, and the skin cannot be moved independently of the tumour mass.

Figure 1.1 cont.

f(i)



(ii)



g



h



Figure 1.1 (cont.) Equine sarcoids exhibit variable clinical presentation

Fibroblastic sarcoids often have a wet, ulcerated, haemorrhagic surface and tend to be large, aggressive type tumours. They can have a wide tumour base (Fig 1.1f(i)) often referred to as sessile type fibroblastic sarcoids, or, they may have a distinct 'stalk' (Fig 1.1f(ii)) and are referred to as pedunculated fibroblastic sarcoids. Mixed sarcoids are a combination of two or more of the previously mentioned types. Figure 1.1g shows a mixed tumour mass consisting of nodular and fibroblastic sarcoid types. Malevolent sarcoids (Fig 1.1h) are comparatively rare and are the only tumour type shown to infiltrate local lymphatic vessels and lymph nodes.

(Pictures from Knottenbelt, 2005)

1.3 HISTOLOGY

On histological examination, in common with observations of gross pathology, equine sarcoids continue to lack predictable and consistent characteristics. Jackson (1936) originally described the equine sarcoid as a biphasic tumour composed of epidermal and dermal components (Fig. 1.2a). However, occasionally the epidermal component can be normal, atrophic or even absent (Marti *et al.*, 1993; Lepage *et al.*, 1998; Martens *et al.*, 2000). Sections obtained from whole tumours allow for the most accurate histopathological assessment, however, surgical excision for the purpose of diagnosis may lead to a high rate of recurrence (50 - 64%) (Tarwid *et al.*, 1985; Knottenbelt *et al.*, 1995; Martens *et al.*, 2001b). Even taking a small biopsy of small and flat lesions may trigger rapid growth and ulceration. Typical changes include dermal proliferation of fusiform or spindle-shaped fibroblasts, forming whorls, interlacing bundles and haphazard arrays with one another (Goodrich *et al.*, 1998). Indeed, put simply, histological diagnosis is made on the presence of a capillary poor, fibroblastic proliferation of moderate to high cell density. Fibroblast morphology varies from slender with elongated, pointed nuclei, to plump cells with large, irregular nuclei. The cytoplasmic boundaries are often ill-defined. When present, the epidermis is reported to show hyperplasia and hyperkeratosis as well as rete peg formation in the majority of cases (Fig 1.2b). At the dermo-epidermal junction, a perpendicular orientation of fibroblasts towards the basement membrane, commonly referred to as a 'picket fence' pattern, is considered characteristic (Ragland *et al.*, 1970; Tarwid *et al.*, 1985; Scott 1988; Pulley and Stannard 1990; Marti *et al.*, 1993). The mitotic rate is usually low, there is variable amounts of collagen and rarely infiltration of inflammatory cells (Goodrich *et al.*, 1998; Martens *et al.*, 2000; Tarwid *et al.*, 1985). As alluded to above, there are few consistent histological changes that will lead to a confident diagnosis of sarcoid tumour. This is particularly relevant in the occult and nodular sarcoids as these types most commonly exhibit minimal histological changes (Martens *et al.*, 2000) and helps explain why occult sarcoids are often overlooked histologically and why nodular sarcoids are sometimes identified as fibromas, neurofibromas or fibrosarcomas. Epithelial changes seen in sarcoids are likely the result of growth-promoting factors expressed by the neoplastic fibroblasts that stimulate proliferation of surrounding epithelial cells (Carr *et al.*, 2001b).

Figure 1.2

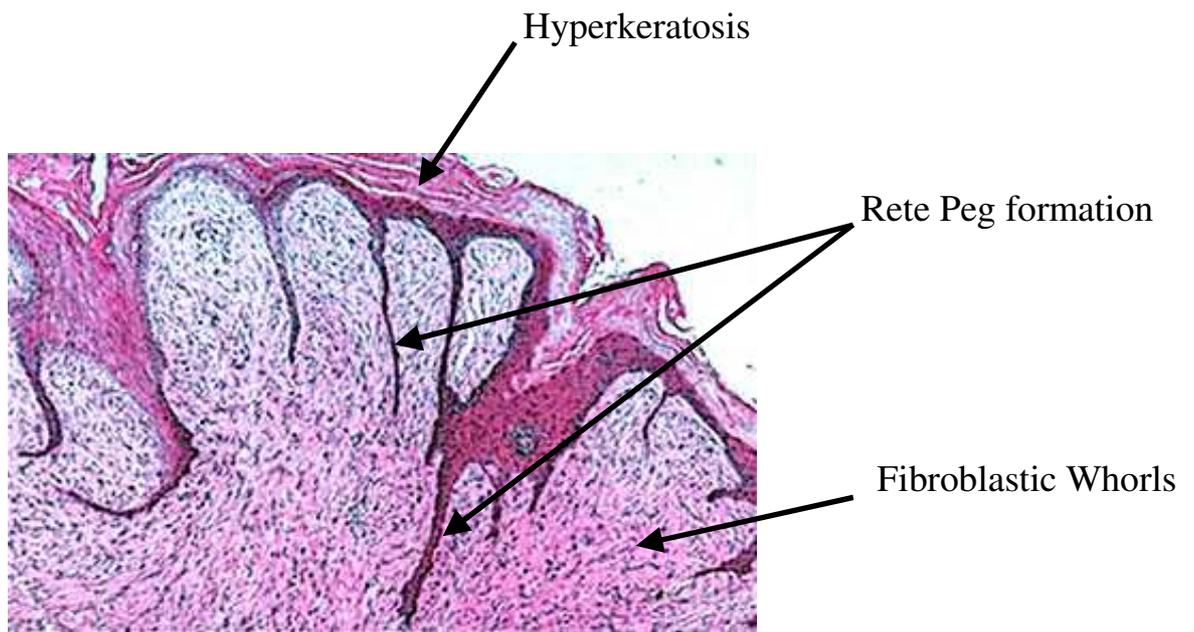


Figure 1.2 Histological findings in equine sarcoids

Histopathological section of an equine sarcoid tumour illustrating some of the more commonly observed morphological changes in the skin.

1.4 DIFFERENTIAL DIAGNOSES

There are several alternative diagnoses to consider when investigating a sarcoid-like lesion (Tarwid *et al.*, 1985; McConaghy *et al.*, 1994; Knottenbelt *et al.*, 1995). In order of prevalence, the most likely differentials are;

Exuberant granulation tissue ('proud flesh') with epidermal hyperplasia is grossly indistinguishable from fibroblastic sarcoids (Fig 1.3a) and may also present a problem on histological examination. However, exuberant granulation tissue sections are generally more vascular than sarcoids, and lack any 'picket fence' pattern.

Equine Papillomas usually develop as multiple, cauliflower-like nodules on the nose and lips of young horses (Fig. 1.3b; Sullins *et al.*, 1986). On histological examination, there is no dermal component. Equine papillomas often spontaneously regress, but this is rarely seen in sarcoids (Brostrom *et al.*, 1979).

Squamous Cell Carcinoma occur most commonly at muco-cutaneous junctions, particularly of the ocular tissue and external genitalia (Fig 1.3c). They can readily be differentiated from sarcoids on the basis of histological features.

Neurofibromas may resemble peri-ocular sarcoids but are found to be associated with nerve fibres.

Fibromas may have ulcerated surfaces which can make differentiation between sarcoids particularly challenging. They may be recognised by their different growth pattern as they expand by compressing the surrounding tissue, rather than infiltration.

Fibrosarcomas can be more challenging again, as they are more locally invasive, vary in size, and are usually irregular and poorly demarcated. Histological examination is the only way to confirm the diagnosis (Fig. 1.3d). The fibrosarcoma is a structure usually formed by fibroblasts, collagen and reticulin fibres. Tumor cells are fusiform, but they can also be stellate, separated by scarce collagen bundles. Mitoses are usually numerous, nuclei show prominent nucleoli, sometimes with the presence of multinucleated giant cells. The cell cytoplasm is poorly coloured, varying in shape and size, cell margins are difficult to distinguish (van Roggen, 1999).

Figure 1.3



Figure 1.3 Common differential diagnoses for sarcoid like lesions.

There are several alternative diagnoses to consider when investigating a sarcoid-like lesion. Proud flesh (Fig 1.3a; www.hideawayhillfarm.com) often forms at wound sites on the distal limbs – a not uncommon site of equine sarcoid development. Squamous cell carcinomas (Fig 1.3b; www.aht.org.uk) are occur most commonly at muco-cutaneous junctions, particularly of the ocular tissue and external genitalia – anatomical predilections for sarcoids. Figure 1.3c (www.homepage.usask.ca) shows the result of equine papilloma virus infection – the common wart. Fibrosarcomas can only be differentiated from sarcoids on the basis of histological examination (Fig 1.3d; www.pathologyatlas.ro).

1.5 TREATMENT OPTIONS

Currently, there is no universally effective therapy for the treatment of sarcoids. Factors that owners and veterinary practitioners must consider in the treatment of equine sarcoids include availability of treatment, location of tumour, cosmetic considerations, cost of treatment, potential complications and the risks to both the patient and the owner. Commonly employed treatments include cryotherapy, excision and local immune modulation (Goodrich *et al.*, 1998). A number of unconventional treatments have also enjoyed some popularity as owners seek cheaper and, possibly anecdotally, more successful alternatives. These include homeopathy, acupuncture and various topical ointments and feed supplements. Despite the lack of any scientific evidence, anecdotal evidence suggests tumour regression can occasionally be seen after application of these alternative therapies. Efficacy of different treatments is difficult to assess because most studies have not been designed to include controls and are frequently based on referral populations of horses treated at veterinary hospitals due to the hazardous nature of some of the treatment options (Goodrich *et al.*, 1998; Martens *et al.*, 2001a; McCauley *et al.*, 2002). These populations are more likely to represent a subset of fast-growing, recurrent or multiple tumours that first opinion veterinary practitioners have been unable to treat successfully. There are also reports of practitioners having treated sarcoids successfully with a policy of non-intervention, but again, these may represent a specific population of sarcoids that remain quiescent or those that spontaneously regress (up to 32%; Bromstrom, 1995; Martens *et al.*, 2001a). It is generally accepted that the prognosis for treatment is significantly worse if one or more unsuccessful attempts have been made previously (Knottenbelt and Walker, 1994). The number of sarcoid treatments available continues to increase on a regular basis and many therapeutic approaches have been investigated including surgical, intra-lesional, topical and immunomodulatory. Rather than merely list the many treatment options here, a selection of the more frequently employed modalities and their relative success rates, will be discussed below.

1.5.1 Surgical Excision

Ragland *et al.* (1970) reported that approximately 50% of surgically excised tumours recurred within 3 years, with most re-growths occurring within 6 months of the surgery. Other studies have reported recurrence rates from 40 to 72% when surgical excision has been employed as the sole therapeutic approach (McConaghy *et al.*, 1994; Knottenbelt and Kelly, 2000; Martens *et al.*, 2001b). Small, well-defined tumours carry the best prognosis for surgical removal, while extensive areas of poorly defined verrucose and mixed sarcoid

may result in rapid re-growth of a more aggressive sarcoid type (Knottenbelt *et al.*, 1995). Other major factors influencing the outcome of surgical intervention have recently been identified. If the surgery is performed on a horse under general anaesthesia (as opposed to a standing, sedated animal), wide surgical margins are taken (which may negatively influence wound healing), and a ‘non-touch’ surgical approach is used to ensure auto-inoculation with tumour cells and viral material is avoided, the success rates of 80% can be achieved (Howart, 1990; Brostrom, 1995; Martens *et al.*, 2001b).

Because sarcoid tumours frequently recur if treated by surgical excision, it was hypothesised that this could be due to activation of latent BPV in apparently normal tissue surrounding the lesion. Martens *et al.*, (2001c) used PCR to test for BPV in sarcoids removed by surgery and also tested apparently normal skin around the sarcoids. They found BPV in all of the sarcoids and also in the surrounding normal skin. The frequency of detection of BPV in the normal skin decreased as the resection margin was increased. They also found that in animals with a surgical margin containing BPV, there was a greater probability that these animals would show local recurrence. The role of BPV in equine sarcoid pathogenesis is discussed more fully in Section 1.4 Aetiology.

1.5.2 Cryosurgery

Cryosurgery alone, or in combination with debulking of larger sarcoids has been employed with some success (Laursen, 1987). Approximately 70% of horses treated appeared to be free from recurrences in most studies, although rates as high as 100% and as low as 60% have been reported (Lane, 1977; Goodrich *et al.*, 1998; Fretz and Barber, 1980). Cell death (cryonecrosis) is achieved by repeated freeze-thaw cycles and complications of the technique arise due to damage of adjacent or underlying structures. These include scarring, leucotrichia, facial paralysis, haemorrhage and decreased strength of cortical bone (Fretz and Barber, 1980; Genetzky *et al.*, 1983, McConaghy *et al.*, 1994). Such potential complications make cryosurgery less suitable for periocular sarcoids and those tumours located on larger vessels, nerves, ligaments or joints.

1.5.3 Laser Surgery

The carbon dioxide (CO₂) laser is the preferred instrument for excision of sarcoids due to its minimal degree of collateral thermal injury and it is used to cut and evaporate tumour tissue (Vingerhoets *et al.*, 1988; Palmer, 1989; Carstanjen *et al.*, 1997). Vingerhoets *et al.* (1988) reported that 81% of 59 horses treated by CO₂ laser were free from recurrence after 12 months. Laser ablation minimises contamination of the surgical site with tumour cells

and viral material compared to sharp surgical excision (Carr, 2006). Post-resection, there is minimal swelling and discomfort for the animal, and although there is considered to be superior cosmetic results, there is a risk of scar tissue formation and alopecia/leucotrichia.

1.5.4 Hyperthermia

Hyperthermia is a radiofrequency (orthovoltage) current-induced method of heating sarcoids to 50 °C for 30 seconds, at intervals of 1 – 3 weeks. There are limited reports in the literature, so the true efficacy of this treatment option is unclear. One study reported induced regression with no recurrence at 7 to 12 months after the last treatment, in just 3 cases of equine sarcoid (Hoffman *et al.*, 1983). Hyperthermia is often combined with radiotherapy, immunotherapy and chemotherapy (Scott and Miller, 2003).

1.5.5 Immunotherapy

Bacillus of Calmette and Guerin (BCG), an attenuated strain of *Mycobacterium bovis* is the immunomodulator most commonly employed for immunotherapy of equine sarcoids. BCG activates mononuclear cells resulting in the release of interleukin-1. Interleukin-1 non-specifically amplifies the immune response to antigens and malignant cells and has an immunoregulatory function (Vaneslow *et al.*, 1988; Martens *et al.*, 2001a). Optimal results are obtained if BCG is used in an immunocompetent host with a limited tumour burden, or post-debulking in the periocular region. An 83.5% rate of remission has been achieved with periocular sarcoids, while use in other body regions, resulted in a 48.4% rate of remission (Webster and Webster, 1985; Owen and Jagger, 1987; Marti *et al.*, 1993). There are several potential complications using this method of treatment, ranging in severity from general inflammation and malaise to severe anaphylactic reactions and death after inoculation. Advantages include tumour specificity whereby only sarcoid cell necrosis is noted on histopathologic post-BCG evaluation (Owen and Jagger, 1987).

1.5.6 Radiotherapy

The gold standard for treatment of the equine sarcoid is radiation but the availability of teletherapy and interstitial brachytherapy is very limited. Expense and practicality are the main limiting factors of this treatment as the absolute requirements for protective measures to ensure the safety of the operators and the need for patient isolation in a specialised facility post-treatment, all add to the considerable costs. Isotopes of various origin (e.g. permanently implanted seeds of radon-222 or gold-198; removable needles of radium-226, cobalt-60 or iridium-192) are placed in the tissue to deliver a continuous, high, local dose of ionising radiation (Wyn-Jones, 1983; Turrel *et al.*, 1985; Theon and Pascoe, 1995).

Responses range from 50 – 100% sarcoid-free for 1 year, where radiotherapy is used alone or in combination with hyperthermia and/or surgical debulking (Walker *et al.*, 1991; Knottenbelt and Kelly, 2000; Byam-Cook *et al.*, 2006).

1.5.7 Chemotherapy

Chemotherapy can be applied topically or intralesionally. Injection of cytotoxic drugs directly into the tumour has the benefit of achieving high drug concentration for a sustained period of time in the tumour, while sparing surrounding normal tissue (Mair and Couto, 2006). The drugs can be mixed with viscous fluids such as sesame oil prior to injection – this prolongs the persistence of the drug within the tumour tissue (Theon *et al.*, 1993). Chemotherapy drugs can be administered as a sole treatment to small sarcoids, but it is recommended that larger lesions are surgically debulked prior to drug treatment (Theon *et al.*, 1993; Theon, 1997). Cisplatin is currently the most commonly used intratumoural chemotherapeutic, although there are reports of intratumoural 5-fluorouracil (5FU) being used in a similar manner, but reports suggest it is less effective than cisplatin (Theon, 1997; Stewart *et al.*, 2006). Both cisplatin and 5-FU require strict safety precautions to be employed by those treating and handling treated animals, due to the potential carcinogenicity and teratogenicity of the drugs. Disadvantages of intratumoural injection of these drugs include potential for secondary peri-injectional infections, some degree of tissue sloughing and peri-lesional swelling.

Fluorouracil (5FU)

As a pyrimidine analogue, 5-fluorouracil is transformed inside the cell into different cytotoxic metabolites which are then incorporated into DNA and RNA, finally inducing cell cycle arrest and apoptosis by inhibiting the cell's ability to synthesize DNA. It has been shown that the use of intratumoural 5-fluorouracil compares favourably with other treatment modalities for sarcoids, with a long term successful resolution rate of 61.5%. Resistant sarcoids and sarcoids larger than 13.5 cm³ have a poorer prognosis for resolution and more aggressive therapeutic options should be considered (Stewart *et al.*, 2006). 5FU is commonly used as a topical treatment, but due to poor diffusion from the skin surface, its use is limited to flat lesions (Theon, 1998).

Cisplatin

The current understanding regarding the mechanism of action of cisplatin, is that the drug induces its cytotoxic properties through binding to nuclear DNA and subsequent

interference with normal transcription, and/or DNA replication mechanisms (Alderden *et al.*, 2006). However, there is now much evidence suggesting that the cytotoxic effects induced by binding of cisplatin to non-DNA targets (especially proteins) may contribute to its biochemical mechanism of action (Fuertesa *et al.*, 2003). Several studies have shown cisplatin to be a useful chemotherapeutic option in the treatment of equine sarcoids with one particular study observing complete regression in 78% of the tumours. It was also stated that no systemic side-effects were encountered (Spoormakers *et al.*, 2002). Most recently, cisplatin containing biodegradable beads have been developed for use in solid tumours. A retrospective study by Hewes and Sullins (2006) reported an 85% success rate in equine sarcoids treated with these beads, after a 2 year follow-up. Additional developments in the application of intralesional cisplatin include combining cisplatin with adjuvant therapies to try and improve responses. The addition of a single high-dose of interleukin-2 has not improved efficacy over cisplatin injections alone (Spoormakers *et al.*, 2003). However, cisplatin intralesional injections followed by electropulsation of sarcoids (a technique that improves the diffusion of chemotherapeutic through the tumours), resulted in regression after only 2 or 3 electrochemotherapies in 100% of the treated lesions. No adverse effect from the electric pulses was observed and no regrowth was observed in the 18 months follow-up period (Rols *et al.*, 2002).

1.6 EPIDEMIOLOGY

Sarcoids account for 35-90% of dermatological neoplasms of equidae (Goodrich *et al.*, 1998; Pascoe and Knottenbelt, 1999; Foy *et al.*, 2002; Scott and Miller, 2003). A variable prevalence ranging from 1% to 12% is reported across the world (Mohammed *et al.*, 1992; Goodrich *et al.*, 1998; Studer *et al.*, 2007) but studies on the epidemiology of the sarcoid have been hampered by a lack of population data and the low prevalence of the disease in individual, or small groups of animals. In addition, prevalence calculations are most likely to be based on populations of animals referred for sarcoid treatment, and do not include those animals that are successfully treated by first-opinion practitioners, or indeed, those cases which required no intervention. Most reports suggest that there is no apparent gender, coat-colour, seasonal, geographic or breed predilection for the occurrence of sarcoids in horses (Genetzky *et al.*, 1983; Scott and Miller, 2003). Horses of all ages may be affected but reports show the majority of affected horses are younger than 6 years, with a peak incidence between 3 and 6 years (Sullins *et al.*, 1986; Torrontegui and Reid, 1994; Foy *et al.*, 2002; Scott and Miller, 2003).

1.6.1 Anatomical predilection

Lesions can occur all over the body but show sites of predilection particularly in the paragenital/inguinal region, the thorax–abdomen and head, and the lower limbs (Sullins *et al.*, 1986; Teifke *et al.*, 1994; Goodrich *et al.*, 1998). Lesions frequently occur at sites of previous injury and scarring, or at sites that are predisposed to trauma (Torrontegui & Reid, 1994; Goodrich *et al.*, 1998; Foy *et al.*, 2002). They can occur as single or, more commonly, multiple lesions in different forms, ranging from small wart-like lesions to large ulcerated fibrous growths. Horses with multiple lesions tend to have larger sized tumours compared to horses with a single sarcoid lesion (Brostrom, 1995).

1.6.2 Breed associations and genetic factors

Evidence for a genetic basis for sarcoid susceptibility comes from family and population studies. The accumulation of cases of sarcoids in equine families has been reported, and more recently, an association between sarcoid susceptibility and the major histocompatibility complex (MHC)-encoded class-11 allele ELA W13 was reported at the population level in several breeds (James, 1968; Lazary *et al.*, 1985; Meredith *et al.*, 1986; Angelos *et al.*, 1988; Brostrom *et al.*, 1988). A long-term epidemiological study into the association of sarcoid development with breed, carried out in the USA, showed that quarter horses were nearly twice as likely to develop sarcoids compared to thoroughbreds. The

standardbred breed were found to be least likely to develop sarcoids, with the frequency of sarcoids in standardbred horses being less than half that of thoroughbreds (Angelos *et al.*, 1988). A higher incidence of sarcoid has also been reported in Arabians and Appaloosas (Miller and Campbell, 1982; Mohammed *et al.*, 1992). In Arabian horses, a statistically significant link has been identified between sarcoid development and heterozygosity for the defective DNA protein kinase catalytic subunit (DNA-PKcs) allele responsible for severe combined immunodeficiency (SCIDS) (Ding *et al.*, 2002).

Research has shown a strong association between risk of sarcoid development and certain alleles of the equine major histocompatibility complex (MHC). In horses, these proteins are called the equine leucocyte antigens (ELA). The MHC is comprised of three different classes of genes with different locations and functions – all code for proteins involved in the immune response and for protein components of the complement system (Piscopo, 1999). The Class I genes code for proteins expressed in most nucleated cells; they have a role in the recognition and killing of virus infected cells. Class II genes code for proteins found on the surface of lymphocytes and macrophages which act as receptors for foreign antigens (Cresswell, 1994). There are 17 internationally recognised Class I alleles (A1-10, A14-15, W12, W16-18, W20) and 3 Class II alleles (W13, W22-23), in addition, several regional/local variants are recognised in both classes (Lazary, 1988; Hesford *et al.*, 1989).

When the frequency of equine MHC haplotypes was examined in Thoroughbred and Standardbred horses in the USA, it was found that the equine leukocyte antigen W12 occurred more frequently in sarcoid affected horses than unaffected horses. The MHC antigen ELA W12 was found to be rare in the standardbred breed (Meredith *et al.*, 1986). It was subsequently found that the W13 haplotype is associated strongly with sarcoids in Swedish halfbreds (Brostrom *et al.*, 1988) and Swiss Warmbloods (Gerber *et al.*, 1988). A further Swedish study showed that there is an association between increased recurrence of sarcoids following surgery with the W13 haplotype and association between early onset of sarcoids and the A5 haplotype (Brostrom, 1995). However, the ELA specificities described so far are probably not directly involved in the pathogenesis of the equine sarcoid. The strong, breed-dependent association between certain ELA specificities and the prevalence of the disease might be explained by as yet undefined histocompatibility antigens that map within the MHC and/or by other genes (Brostrom *et al.*, 1988). Further work is needed in this area to elucidate the exact role of genetics in the sarcoid pathogenesis. This would potentially allow development of novel immunotherapeutic approaches and informed breeding policies to help control the disease.

1.6.3 Transmission

Details of how equine sarcoids may be transmitted between susceptible animals, is discussed at the end of the aetiology section (Section 1.6.6).

1.7 AETIOLOGY

The results of many studies have suggested the involvement of an infectious agent in the development of equine sarcoid tumours. The first report describing the equine sarcoid suggested a virus origin for the tumours based on their appearance and pattern of spread (Jackson, 1936). Outbreaks have been described in closed herds of horses and zebra, suggestive of an infectious agent (Ragland *et al.*, 1966; Nel *et al.*, 2006). In 1951, Olson and Cook demonstrated that intra-dermal inoculation of cell-free extracts from bovine skin tumours containing bovine papillomavirus into horses, resulted in the development of lesions resembling the equine sarcoid (Olson and Cook, 1951). Subsequently, Voss (1969) provided more substantial evidence when he observed that inoculation with either sarcoid tissue or cell-free extracts from equine sarcoid tumours onto the scarified skin of sarcoid-free horses, resulted in the appearance of sarcoid-like tumours at the inoculation site (Voss, 1969). However, these experimentally induced sarcoid tumours showed spontaneous regression, which is only rarely encountered in naturally occurring sarcoids. Nevertheless, there is now a large body of evidence supporting the hypothesis that Bovine Papillomavirus (BPV) is the aetiological agent of equine sarcoids. DNA of BPV 1 or BPV 2 has been detected in most of the equine sarcoids examined (Amtmann *et al.*, 1980; Müller, 1991; Goodrich *et al.*, 1998; Campo, 2002; Chambers *et al.*, 2003; Yuan *et al.*, 2007). Viral genes and proteins have also been detected in sarcoids, indicative of an active infection (Nasir and Reid, 1999; Carr *et al.*, 2001; Chambers *et al.*, 2003). Most work looking at BPV gene expression in equine sarcoid tissue has focussed on the early genes, namely E2, E5, E6 and E7 (Nasir and Reid, 1999; Carr *et al.*, 2001; Chambers *et al.*, 2003). Nasir and Reid (1999) also detected expression of the L1 gene in 50% of the tumours they studied. Little other work has been done looking specifically at the L1 and L2 capsid genes. Whilst viral particles have not been detected, Brandt *et al.*, (2008) have demonstrated BPV-1 virus-like structures in a subset of sarcoids using immunocapture PCR. This is the first evidence for the presence of potential intralesional BPV virions in the disease.

1.7.1 Papillomaviruses

Papillomaviruses (PVs) were first identified in the early 20th century, when it was shown that skin warts, or papillomas, could be transmitted between individuals by a filterable infectious agent. In 1935 Francis Peyton Rous, who had previously demonstrated the existence of a cancer-causing sarcoma virus in chickens, went on to show that a papillomavirus could cause skin cancer in infected rabbits. This was the first demonstration

that a virus could cause cancer in mammals. PVs comprise a heterogeneous group of viral agents with a double stranded DNA genome of approximately 8 kb (Lowy & Howley, 2002). Several hundred types of papillomaviruses have been identified, including the more than 100 PV types found in humans. PVs are normally strictly species-specific and have been found to infect all birds and mammals but also a small number of reptiles, in particular turtles and tortoises (de Villiers *et al.*, 2004). Infection by most papillomavirus types, depending on the type, is either asymptomatic or causes benign hyperproliferations of the skin or of mucosal tissues (Lowy & Howley, 2002). Papillomas caused by some types, however, such as human papillomaviruses (HPV) 16 and 18, carry a risk of the development of malignant disease, in particular cancer of the cervix uteri (Munoz *et al.*, 2006). PV infection of animals can also induce mucosal or skin warts, some of which can subsequently progress into malignant disease (Breitburd *et al.*, 1996; Goodrich *et al.*, 1998; Campo, 1997a; Chambers *et al.*, 2003).

1.7.2 Bovine Papillomavirus (BPV)

Like other papillomaviruses, BPVs are small non-enveloped viruses with an icosahedral capsid around 50–60 nm in diameter. Figure 1.4a shows the BPV virion. The capsid is formed of the L1 and L2 structural proteins (discussed later in this chapter), with the L1 C-terminus exposed. Ten BPV types (BPV 1-10) have been characterised and have recently been re-classified (de Villiers *et al.*, 2004) following the Greek letter nomenclature used for other virus families. According to the new nomenclature, the epitheliotropic BPV-3, BPV-4 and BPV-6 are defined as Xipapillomaviruses and BPV-1 and BPV-2 as Deltapapillomaviruses. BPV-5 has been designated an Epsilonpapillomavirus. In addition, 16 novel putative BPV have been characterised by sequence determination of the highly conserved L1 region and by phylogenetic analysis (Antonsson and Hansson, 2002).

Some types of papillomavirus, including BPV types 1 and 2, can infect fibroblasts and induce fibro-epithelial tumours, which cause benign fibropapillomas in cattle. BPV is highly prevalent, with around 50% of cattle being estimated to bear lesions in the UK (Campo, 1995). Cutaneous warts are most common in younger animals and usually spontaneously regress as they age, due to the animal's immune response. Chronically immunosuppressed animals may develop extensive papillomatosis in the upper gastrointestinal tract, which can cause difficulties with eating and breathing (Campo, 2006).

BPVs have a genome of up to 7945 bp of double-stranded DNA, with at least nine potential reading frames. Like other papillomaviruses, the genome can be split into two

principal regions as shown in Figure 1.4b. The early (E) region, encodes the transforming proteins E5, E6 and E7, and the replication and transcription regulatory proteins E1 and E2. The late (L) region encodes the structural proteins of the virus L1 and L2. The early and late regions are separated by a stretch of non-transcribed DNA, called the long control region (LCR), which contains the transcriptional promoters and enhancer, the origin of DNA replication and binding sites for numerous cellular transcription factors (Chen *et al.*, 1982). Seven promoters and alternative splicing produces more than 20 mRNA transcripts (Zheng and Baker, 2006; Jia and Zheng, 2009).

The expression of BPV-1 and -2 early and late genes is highly regulated in the natural host, at both transcription and post-transcriptional levels and strictly tied to the differentiation of keratinocytes. For example, in papilloma formation, the virus initially infects the basal keratinocytes. Papillomaviruses gain access to keratinocyte stem cells through small wounds in the skin or mucosal surface. Interactions between L1 and sulphated sugars on the cell surface promote initial attachment of the virus (Joyce *et al.*, 1999; Giroglou *et al.*, 2001). The virus is then able to enter from the cell surface via interaction with a specific receptor, likely to be the alpha-6 beta-4 integrin, and is transported to endosomes (Evander *et al.* 1997; McMillan *et al.*, 1999). The capsid protein L2 disrupts the membrane of the endosome, allowing the viral genome to escape and transit, along with L2, to the cell nucleus (Kamper *et al.*, 2006). The early region genes are then expressed in the undifferentiated basal and suprabasal layers. Viral DNA is replicated in the differentiating spinous and granular layers and, in the upper, terminally differentiated layers of the host epithelium, the late genes L1 and L2 are transcribed/translated and serve as structural proteins which encapsidate the amplified viral genomes. The new viral particles are released into the environment as the cells die (Figs. 1.4c and 1.4d).

Malignant transformation is linked to a disruption in the regulated expression of the early virus genes, the consequences of which are an uncontrolled proliferation and loss of differentiation of the infected cells. Persistent viral infection is required for neoplastic progression and failure of virus clearance is attributed to a poor immunological response (Campo, 1997a).

Figure 1.4

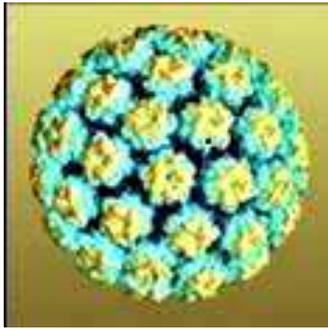


Figure 1.4a

Figure 1.4 The classification, genome organisation and life cycle of the bovine papilloma virus (BPV). BPVs are small non-enveloped DNA viruses (Fig 1.4a; www.bristol.ac.uk). BPV has a genome of 7945 bp of double stranded DNA containing 6 early (E1, E2, E4, E5, E6 and E7) and 2 late (L1 and L2) genes (Fig 1.4b). Figure 1.4c (www.ncbi.nlm.nih.gov) illustrates the life cycle of BPV in the natural host, where the virus life cycle is strictly linked to the differentiation of the infected cell. Figure 1.4d (www.lab.anhb.uwa.edu.au) shows the corresponding skin section in an H and E stained slide.

Figure 1.4b

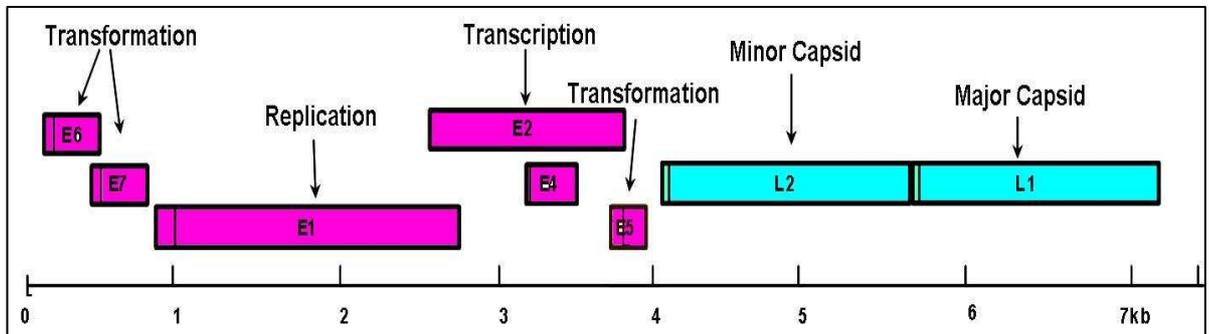


Figure 1.4c

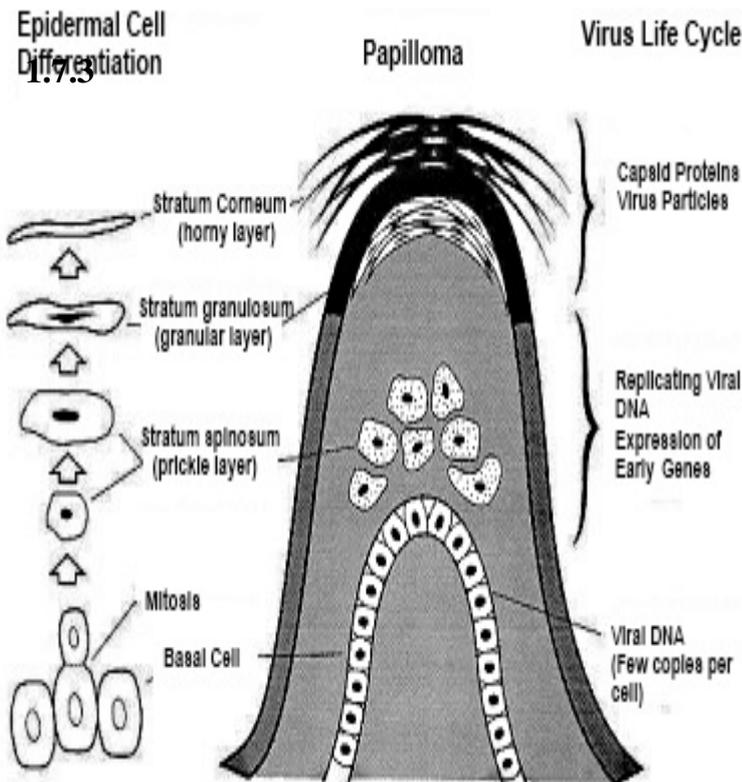
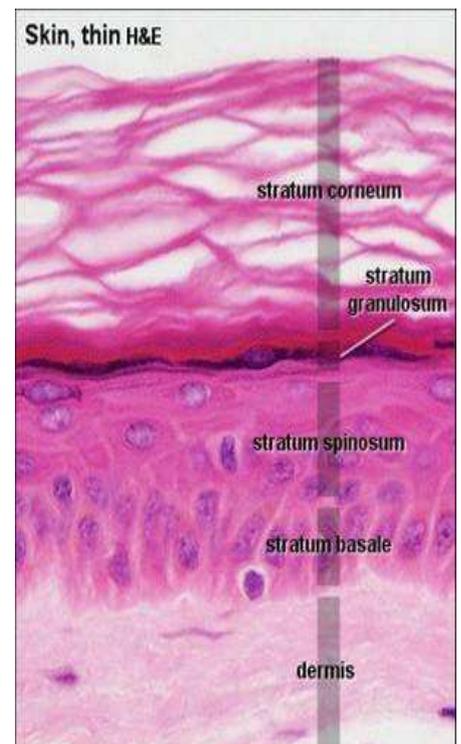


Figure 1.4d



BPV -1 and -2 and neoplastic transformation

Cancer, including PV-associated cancer, is a multi-factorial disease and several steps are required before full neoplastic transformation is achieved. Bracken fern (*Pteridium aquilinum*) has been identified as a major environmental co-factor in BPV-induced carcinogenesis in cattle. Bracken fern contains immunosuppressants and a number of mutagens. Bracken-induced immunosuppression is associated with two marked haematological changes. The first of these is a dramatic fall in polymorph nuclear leucocytes. If unchecked, this leads to severe acute immunosuppression with invasion of the bloodstream by alimentary bacteria and death from septicaemia. This is the well described veterinary syndrome of Acute Bracken Poisoning (ABP). The second effect of bracken feeding is a chronic drop in circulating lymphocytes. Even during periods of bracken withdrawal the lymphocyte count remains very low. Field cases of upper and lower GI tract cancer are found to occur at a high frequency in areas such as the Nasampolai Valley of Kenya and the Western Highlands of Scotland where cattle graze on bracken-infested land (Plowright *et al.*, 1971; Jarrett, 1980). In addition, bracken-eating cattle develop chronic enzootic haematuria, urinary bladder cancers and chromosomal abnormalities (Jarrett *et al.*, 1978; Moura *et al.*, 1988; Stocco dos Santos *et al.*, 1998; Borzacchiello *et al.*, 2001; Borzacchiello *et al.*, 2003; Lioi *et al.*, 2004). The involvement of bracken and BPV-1 or BPV-2 in bladder carcinogenesis has been recognised for a long time (Olson *et al.*, 1959), and the virus-bracken interaction has been reproduced experimentally (Campo *et al.*, 1992). BPV-1/2 infects the epithelium of the urinary bladder and establishes an abortive infection, with no production of virions. The viral DNA in the bladder lesions is still infectious and capable of initiating a replicative cycle in the permissive environment of the skin, as extracts from urinary bladder cancers could induce skin warts (Olson *et al.*, 1965). The immunosuppression induced by bracken fern prevents tumour rejection and the fern mutagens contribute to genome destabilisation.

Viruses and their hosts are in a delicately balanced relationship. Viruses must be able to overcome the host immune response to replicate and produce infectious progeny. The immune response of cattle to BPV is surprisingly poor (Campo, 1998). Animals may carry massive tumours, actively producing virus in large quantities, but cattle do not respond easily to BPV antigens during the course of infection and anti-BPV antibodies are seldom detected. The poor immune response to BPV is likely to be the main reasons for the persistence of infection: even in immunocompetent hosts, the papillomas persist for many months before regression takes place. In addition to a passive evasion of the immune system (by localisation inside epithelial cells), papillomaviruses have evolved active ways

of hiding from the host immune system (O'Brien and Campo, 2002). Among these there is down-regulation of the Major Histocompatibility Complex class I (MHC I) by expression of BPV E5 protein. MHC I plays a critical role in immune surveillance as it is responsible for the presentation of antigenic peptides to effector T-cells. Once the heavy chain of MHC I associates with β 2-microglobulin and peptides, the complex is transported from the endoplasmic reticulum through the Golgi apparatus to the plasma membrane for recognition by T-cells (Cresswell, 2000). E5 is one of the main transforming proteins of BPV (the other being E6), and is a short hydrophobic membrane protein localizing to the Golgi apparatus and other intracellular membranes. Down-regulation of MHC I by BPV E5 takes place at multiple levels: transcription of the MHC I HC gene is reduced, the MHC I HC peptide is degraded (Ashrafi *et al.*, 2002) and the MHC I complex is sequestered in the Golgi cisternae and is prevented from reaching the cell surface (Marchetti *et al.*, 2002). The absence of MHC class I from the cell surface would help the infected cells evade host immunosurveillance. In addition, recent research has shown that the E5-induced reduction of MHC I has a functional impact by reducing recognition of E5 expressing cells by HPV specific CD8+ T cells (Campo *et al.*, 2010). Furthermore, E5 has been shown to induce activation of the platelet-derived growth factor β (PDGF- β) receptor by binding to it (Borzacchiello *et al.*, 2008). Activation of PDGF- β receptors of the dermal fibroblasts results in mitogenesis in nearby epithelial cells (Carr *et al.*, 2001), contributing to the pseudoepitheliomatous hyperplasia that is typically observed in BPV infected lesions. E5 also activates numerous kinases, possibly by signalling cascades triggered by PDGF- β receptor activation, including cyclin A-cdk2, MAP, JNK, PI3 and c-Src, thus interfering with proper cell-cycle control and further signal transduction cascades (Venuti & Campo, 2002).

The E6 protein is found localized in membrane and nuclear fractions and contains two highly conserved zinc finger domains typical of DNA-binding transcription activator proteins. However, cell transformation by E6 appears to be independent of its transcription transactivation function (Ned *et al.*, 1997). While HPV E6 binds and stimulates degradation of p53, BPV E6 does not (Scheffner *et al.*, 1990; Rapp *et al.*, 1999). BPV-1 E6 (BE6) has been described as interacting with three cellular proteins: E6AP (Ned *et al.*, 1997), E6BP (also known as ERC-55) (Chen *et al.*, 1995), and paxillin (Tong and Howley, 1997; Vande Pol *et al.*, 1998). BE6 binds to similar peptide sequences found on each of these three proteins (Vande Pol *et al.*, 1998). Unlike 16E6, where the binding to E6AP induces the degradation of p53, BE6 binding to E6AP has not been shown to induce the

degradation of p53, and the in vivo interactions of E6 with paxillin or E6BP have as yet unknown consequences.

E7 protein cooperates with E5 and E6 in inducing cell transformation and once E7 is co-expressed with E5 and E6, its transformation capacity increases many-fold (Bohl *et al.*, 2001). The transformation activity is partially mediated by its ability to bind p600. In a recent study, the ability of E7 to complex with p600 correlated with its ability to enhance anchorage independence of BPV-1 E6-expressing cells (DeMasi *et al.*, 2005).

The L1 protein is thought to be exposed on the viral surface, thus suggesting it has a role in infection and immunogenicity (Modis *et al.*, 2002). L1 also mediates virus binding to the cell surface, although it is thought that the other late protein, L2, is also involved in infection (Day and Schiller, 2006). L2 is also responsible for virion assembly by binding to DNA. It is most abundant in the differentiated layers of the epidermis, particularly in mature papillomas (Anderson *et al.*, 1997)

The E1 protein functions in viral DNA replication (Lambert, 1991) and has been shown to be involved in a number of activities; (i) DNA helicase activity, (ii) BPV origin-containing DNA-specific binding activity, (iii) DNA-dependent ATPase activity, and (iv) BPV origin-specific unwinding of superhelical DNA (Seo *et al.*, 1993).

E2 binding to LCR activates or represses transcription of the viral genes and is also required to segregate episomal BPV genomes to daughter cells by tethering them to mitotic chromosomes, thus ensuring equal division and retention of the viral DNA (Baxter *et al.*, 2005).

The E4 ORF encodes for small proteins which are very abundant only in the cytoplasm of keratinocytes supporting the productive phase of viral DNA replication (Anderson *et al.*, 1997).

In situ hybridization for BPV DNA of tissue sections from sarcoids reveals viral DNA in the dermal layer within the fibroblasts but not within the epithelial cells (Lory *et al.*, 1993; Teifke *et al.*, 1994; von Tscharner *et al.*, 1996).

1.7.4 BPV latency

Like many viruses, BPVs can establish a latent infection. The viral genome can be often found in normal epithelia with no clinical sign of disease (Ogawa *et al.*, 2004) both in tumour-bearing and in clinically normal hosts. Normal epithelia are the accepted site of

latent infection, and indeed the reactivation of BPV at sites of trauma suggests that viral DNA is present in these sites in latent form, and that damage of the epithelium, possibly through production of inflammatory cytokines and stimulation of cell proliferation, induces expression of viral genes leading to papilloma formation (Campo *et al.*, 1994).

In one recent study, the presence and activity of BPV in normal skin and peripheral blood of 4 groups of horses was evaluated; (i) sarcoid-affected horses, (ii) horses living in contact with sarcoid-affected horses, (iii) horses living in contact with papilloma-affected cattle and (iv) control horses. BPV DNA was found in the normal skin of 24 of 42 horses (57%). Mainly sarcoid-affected horses and horses living in contact with cattle were carriers (73%), but BPV DNA was also detected in 50% of the horses living in contact with sarcoid-affected horses and in 30% of the control population. BPV mRNA was detected in 58% of the samples positive for BPV DNA, although in a much lower quantity compared to sarcoids (Bogaert *et al.*, 2007). Although the results of this study could not demonstrate the presence of BPV DNA in any of the blood samples taken, a more recent report demonstrates that peripheral blood mononuclear cells (PBMCs) can represent a reservoir of BPV DNA in sarcoid-affected animals (Brandt *et al.*, 2008). Researchers in South Africa recently demonstrated the presence of BPV-1 and -2 DNAs in zebra sarcoid tumours. and demonstrated that BPV-1 and -2 DNA (either single or mixed infections) are present in sarcoid tumour, healthy skin and blood of sarcoid-affected and healthy zebras from sarcoid-affected parks as well as in the blood of zebras from parks where no sarcoid has been observed before (van Dyk *et al.*, 2009). These results support the hypothesis that PBMCs may serve as host cells for BPV-1/-2 DNA and both contaminated blood and skin of infected and uninfected animals could contribute to virus latency.

1.7.5 Equine Specific Variants

Recently, it has emerged that sequence variation occurs within papillomavirus types, which can influence the cellular location and function of the oncoproteins and consequently affect the pathogenesis and transforming ability of the virus (Giannoudis & Herrington, 2001). Using sequence analysis of BPV DNA isolates extracted from sarcoids, the presence of distinct equine sarcoid-specific variants of BPV has been detected (Otten *et al.*, 1993; Reid *et al.*, 1994; Chambers *et al.*, 2003; Nasir *et al.*, 2007). The sequence changes in the E5 protein reported by Reid *et al.* (1994) and Chambers *et al.* (2003) suggest the possibility that these changes are contributing factors to the pathogenesis of the disease. Variants particular to sarcoid BPV-1 were also identified in both the long control region (LCR) and E2 sequence. The LCR of papilloma viruses (PVs) plays an important part in the

pathogenesis of PV infections as the LCR functions as the transcriptional control unit of the virus. Transcriptional control from the LCR is regulated by a range of cellular factors and also by the virally encoded early gene product E2, which is also required for viral DNA replication (McBride *et al.*, 1991). Previous studies on PVs have shown that intra-type sequence variation within the LCR and some LCR variants exhibit altered transcriptional control, which may in turn alter the pathogenesis and transforming effects of the virus (Kammer *et al.*, 2000; Kurvinen *et al.*, 2000; Veress *et al.*, 2001). Using BPV DNA extracted from sarcoid tumours, the functionality of the most common LCR variant was examined in equine and bovine cells by Nasir *et al.*, (2007). These studies showed that the activity of the variant LCR was higher in equine cells than bovine cells; the activity of the variant LCR in the presence of the E2 variant was similar to the reference/wild-type sequences in equine cells, whereas in bovine cells the variant function was reduced by 50%. These data suggest the viral BPV variants commonly detected in sarcoids have an enhanced function in equine cells compared to their function in bovine cells. As found for HPV, these sequence changes could affect the expression and function of the early virus proteins and may explain the different pathogenesis of the equine sarcoid compared to papillomas induced by BPV in cattle. However, this remains to be established. Furthermore, if, as mentioned earlier, horses are commonly asymptotically infected with 'equine-adapted' BPVs (Bogaert *et al.*, 2008), entry of the virus into the dermis, due to, for example, trauma, may be more important in sarcoid development than exposure to the PV.

1.7.6 Transmission

Although there is convincing evidence that BPV types 1 and 2 are the principal causative agent of sarcoids, there is currently no definitive evidence to support any particular hypothesis of how the disease may be transmitted. As mentioned earlier, there is a predilection for sarcoid development at wound sites and it has been proposed this may be due to flies acting as a vector as they move between wound sites on different horses. BPV DNA sequences have been detected in face flies, which are commonly seen around wounds. The same viral DNA sequences were detected in the sarcoid tissue samples taken from the horses from which the flies were removed (Kemp-Symonds, 2000). BPV infection may also be transmitted via stable management practices, such as the sharing of contaminated tack, or passed into existing wounds from contaminated pasture. A recent study by Boegart *et al.*, (2005) demonstrated the presence of BPV DNA on the normal skin of horses affected by equine sarcoids and, to a lesser degree, on the normal skin of unaffected horses living in contact with affected animals. It seems plausible that virus

present on the normal skin may gain access to the dermis through any abrasion that may breach the epidermal layers, and so result in the development of a sarcoid lesion in a susceptible animal.

1.8 AIMS AND OBJECTIVES

The aims of this PhD thesis were to:

1. Evaluate the potential role of flies as vectors in transmission of equine sarcoids between infected and susceptible animals.
2. Investigate the consequences of BPV transformation in cultured equine fibroblasts with regard to cellular response to DNA damage; apoptosis and long term cell survival.
3. Identify the viral gene(s) responsible for conferring resistance to apoptosis and/or enhanced cell survival and evaluate the use of viral-gene targeted siRNA as a potential therapeutic strategy
4. Investigate the activity, functionality and cellular location of the tumour suppressor p53 gene in equine sarcoids.

2 Chapter II:

Materials and Methods

2.1 MATERIALS

Materials in regular use, such as equipment, general reagents and solutions are detailed in this section.

2.1.1 Plasticware

Tissue culture flasks and Falcon conical centrifuge tubes (15 and 50ml) were supplied by Greiner (Gloucestershire, UK). 6-well plates and pipettes (5, 10, 25, 50ml) were supplied by Corning (Fisher, UK).

2.1.2 Solutions, media and supplements

All solutions and media for cell culture were supplied by Invitrogen, UK, unless otherwise stated.

2.1.2.1 Media

All media were supplied as sterile solutions and stored at 4 °C.

- Dulbecco's Modified Eagle's Medium (DMEM) with Glutamax-1 with sodium pyruvate, glucose and pyridoxine.
- Minimal Essential Media (MEM- α).
- Opti-MEM

2.1.2.2 Supplements

- Foetal Bovine Serum (FBS): virus and mycoplasma screened. FBS was heat inactivated at 56 °C for 30 minutes, then stored in 50ml aliquots at -20 °C.
- Penicillin/streptomycin (P/S): supplied as a 100x stock solution of 10,000 units penicillin and 10,000 units streptomycin per ml and stored in 5 ml aliquots at -20 °C.
- Fungizone; Amphotericin B (Sigma, UK): supplied as a 100x stock solution and stored in 5 ml aliquots at -20 °C.
- Trypsin-EDTA: supplied as a 100x stock solution and stored in 5 ml aliquots at -20 °C.

- Sodium pyruvate: supplied as a 100mM stock solution and stored at 4 °C.
- Non Essential Amino Acids (NEAA): supplied as a 100x stock solution and stored at 4 °C.
- Essential Amino Acids (MEM EAA): supplied as a 50x stock solution and stored at 4 °C.

All cell lines were cultured in DMEM (Invitrogen, UK) supplemented with 10% foetal calf serum, 1% Non Essential Amino Acids (NEAA), 1% Essential Amino Acids MEM EAA), 100 U/ml Penicillin/Streptomycin and 1.25 ug/ml Fungizone; Ampotericin B (Sigma, UK).

2.1.2.3 Freeze Medium

Medium for cryopreservation of cells was prepared by mixing 9 ml growth medium (appropriate to the cell line being stored) with 9 ml foetal calf serum and 2 ml DMSO (45% medium/45% foetal calf serum/10% DMSO).

2.1.3 Cell lines

Previous work done at the University of Glasgow has successfully generated equine cell lines harbouring BPV-1 genomes and expressing viral genes (Yuan *et al.*, 2007).

- EqS cell lines (EqS01, EqS02, EqS03 and EqS04b): These cell lines were derived from explants of sarcoid tumour biopsies.
- S6 cell lines (S6-1, S6-2 and S6-3): These cell lines were generated in vitro by transfection of primary fibroblasts with BPV-1 DNA (S6 cell lines).
- EqPalF cell line: BPV-1-negative foetal fibroblast cells cultured from tissue from the foetal palate.

The EqS and S6 equine cell lines are morphologically transformed, proliferate faster than parental cells, have an extended life span and can grow independently of substrate.

2.1.4 Complete kits

- Qiagen DNeasy Blood and Tissue Kit (QIAGEN, UK)
- Qiagen RNeasy Mini Kit (QIAGEN, UK)
- QIAquick® PCR Purification Kit (QIAGEN, UK)

- QIAquick® Gel Extraction Kit (QIAGEN, UK)
- ECL-PLUS Western Botting Detection Reagents (GE Healthcare, Amersham, UK)
- DNA Sequencing Kit Big Dye™ Terminator Version 3.0 Cycle Sequencing Ready Reaction (ABI Applied Biosystems, UK)
- Annexin V-FITC Apoptosis Detection Kit (Calbiochem, UK)
- SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, UK)
- Templi Phi 100 Amplification Kit (GE Healthcare, Amersham, UK)
- MycoAlert™ Mycoplasma Detection Kit (Cambrex Bio Science, UK)

2.1.5 DNA

Plasmid, molecular weight markers and oligonucleotide DNAs were stored at -20 °C.

2.1.5.1 Molecular Size Standards

All markers were supplied by Invitrogen, UK and include 1 kb DNA Ladder (size range: 75- 12,216 bp), 1 Kb Plus DNA Ladder™ (size range: 100- 12,000 bp), and 100 bp DNA Ladder (size range: 100-2072 bp).

2.1.5.2 Oligonucleotide primers

Oligonucleotides for use in polymerase chain reactions (PCR) and cycle sequencing were synthesised by IDT, UK. Primers were either reverse phase, desalted or Poly Acrylamide Gel electrophoresis (PAGE) purified and supplied as lyophilised DNA. Primers were reconstituted in dH₂O and stored at -20 °C in 500 µl aliquots at 10µM.

2.1.6 Enzymes

All enzymes were stored at -20 °C, being removed immediately before use.

2.1.6.1 Platinum Taq DNA Polymerase

Platinum Taq DNA Polymerase is a recombinant Taq DNA polymerase complexed with a proprietary antibody that inhibits polymerase activity at ambient temperatures. Activity is restored after the denaturation step in PCR cycling at 94 °C, thereby providing an

automatic “hot start” for Taq DNA polymerase in PCR, which provides increased sensitivity, specificity and yield while allowing assembly of reactions at room temperature.

2.1.6.2 Platinum Taq DNA Polymerase High Fidelity

Platinum Taq DNA polymerase High Fidelity, provided by Invitrogen, is an enzyme mixture composed of recombinant Taq DNA polymerase, *Pyrococcus* species GB-D polymerase, and Platinum Taq antibody. *Pyrococcus* species GB-D polymerase possesses a proofreading ability by virtue of its 3' to 5' exonuclease activity. Mixture of the proofreading enzyme with Taq DNA polymerase increases fidelity approximately six times over that of Taq DNA polymerase alone and allows amplification of simple and complex DNA templates over a large range of target sizes. The enzyme mixture is provided with an optimised buffer that improves enzyme fidelity and amplification of templates.

2.1.6.3 Superscript III Reverse Transcriptase

Superscript III-RT, provided by invitrogen, is a version of MMLV-RT that has been engineered to reduce RNase H activity and provide increased thermal stability.

2.1.6.4 RNaseOUT Recombinant Ribonuclease Inhibitor

RNaseOUT Recombinant Ribonuclease Inhibitor, provided by Invitrogen, is an acidic protein with a very high binding affinity for ribonucleases such as RNase A, B, C and is a non-competitive inhibitor.

2.1.6.5 Alkaline Phosphatase

Calf Intestinal alkaline phosphatase provided by Promega catalyses the hydrolysis of 5'-phosphate groups from DNA.

2.1.6.6 DNase I: DNA-free

DNase I, provided by Invitrogen, is a deoxyribonuclease I enzyme that cleaves double-stranded or single stranded DNA. Cleavage preferentially occurs adjacent to pyrimidine (C or T) residues, and the enzyme is therefore an endonuclease. Major products are 5'-phosphorylated di, tri and tetranucleotides. In the presence of magnesium ions, DNase I hydrolyzes each strand of duplex DNA independently, generating random cleavages. In the presence of manganese ions, the enzyme cleaves both strands of DNA at approximately the

same site, producing blunt ends or fragments with 1-2 base overhangs. DNase I does not cleave RNA.

2.1.7 Equipment

2.1.7.1 Major Equipment

- Benchtop centrifuge: CPR Centrifuge (Beckman)
- Microcentrifuges: Centrifuge 5402, 5415R and Minispin (Eppendorf)
- Water baths: Sub 36, and W6 (Grant)
- Spectrophotometer: GeneQuant pro RNA/DNA calculator
- Automatic Sequencing Apparatus: ABI 3010
- Pipettes: Finnipipette Techpette (0.5-10, 5-40, 40-200, 200-1000 µl)
- Ultraviolet trans-illuminator: T2201 (Sigma Chemical Company)
- Gel documentation system: Uvi tec (Thistle Scientific)
- Luminometer: Dynex MLX
- Horizontal orbital shaker: 4628-1CE Labline Instruments inc (IL)
- Incubator: B5042 (Heraeus)
- Gel systems: Hoefer HE 33 Mini Horizontal Submarine Unit
- Power packs: PAB 35-0.2 (Kikusui electronics corp, Biorad, UK)
- Balance: Precisa 100A-300M (Precisa Balances Ltd, Bucks, UK)
- Stirrer: Magnetic Stirrer Hotplate (Stuart Scientific, UK)
- PCR Machines: GeneAmp PCR System 2400, 2700 and DNA Thermal Cycler 480 (Perkin Elmer)
- Flow Cytometer: Epics XL Flow Sorter (Beckman-Coulter Inc., Miami, FL)

2.1.7.2 Consumables

- Syringe top filters (0.2 μm pore size) were supplied by Nalgene™, (NY, USA) for sterilisation of ampicillin and tissue culture reagents.
- Ependorf tubes: screw top 1.5ml tubes were from Camlab, and 0.5 ml and 1.5 ml flip top tubes were supplied by Anachem.
- Pipette tips, universal fit yellow and blue tips, were supplied by Greiner.
- Filter tip pipette tips; 10 μl from Greiner, 100, 200 and 1000 μl were supplied by Starlab, UK.
- Petri dishes were supplied by Sterilin (Staffs, UK)
- Bijoux were supplied by Greiner
- Universals were supplied by Greiner
- Scalpel blades were supplied by Swan-Morton (Sheffield)
- Parafilm was supplied by Sigma

2.1.7.3 Buffers, solutions and growth media

2.1.7.3.1 Chemotherapeutic Agents

- Cisplatin: Supplied by Hospira, UK as a ready to use sterile solution (10 mg/ml).
- 5-Fluorouracil: Supplied by Hospira, UK as a ready to use sterile solution (50 mg/ml).

2.1.7.3.2 Buffers and solutions

- 50 x TAE Buffer Solution: Tris base 484.5g, NaOAc 272.15 g, NaCl 116.8 g, Na₂EDTA 74.45 g. pH adjusted to 8.15 with glacial acetic acid and made up to 2 L volume.
- TBE Buffer Solution: 0.09M Tris Borate, and 0.002M EDTA

- Lysis Buffer: 25mM Tris HCL pH 8.0 with glacial acetic acid and made up to 2 L volume
- Lysis Buffer for Western Blotting: 0.5% NP40, 150mM NaCl, 50mM TrisHCL adjusted to pH 8.0, protease inhibitor tablet (1 per 10 ml of lysis buffer)
- 1M Tris HCL: 121g Tris base, 800ml dH₂O. Adjusted to the desired pH with concentrated HCL and made up to 1L.
- TE Buffer: 10mM Tris-HCL (pH 8.0), 1mM EDTA
- 1 x PBS: 140mM NaCl, 2.7mM KCL, 10mM Na₂HPO₄, 1.8 mM KH₂PO₄ (pH 7.3)
- 0.2M (2x) Phosphate Buffered Saline (PBS): 0.2M Na₂HPO₄, 0.2M NaH₂PO₄, 1.8% w/v NaCl, pH 7.4
- 10x DNA Gel Loading Buffer: 30% w/v glycerol, 0.25% bromophenol blue, 70% TE buffer. Stored at room temperature and used at a 1:10 dilution.
- Ethidium bromide: made to a working dilution of 10mg/ml with dH₂O in a fume cupboard. Stored away from light.
- DEPC water: 0.5ml of Diethylpyrocarbonate in 500ml water, overnight at room temperature in the fume cupboard and autoclaved.
- Lysis mix: 0.32M sucrose, 10mM Tris, 5mM MgCl, 1% Triton X
- Nuclei lysis mix: 10mM Tris, 0.4 NaCl, 2mM EDTA
- TRIS-EDTA: (10/1 pH 7.4)
- TRIS-EDTA: (10/0.1 pH7.5)
- NU-PAGE Transfer Buffer: supplied by Invitrogen and diluted 1:20
- NU-PAGE Running Buffer: supplied by Invitrogen and diluted 1:20
- 10x Ponceau's Red Stain: 2 g Ponceau S, 30 g Trichloroacetic acid (TCA), 30 g Sulphopalicylic acid. Made up to 100 ml by addition of dH₂O

- TBS-Tween: 150 mM NaCl (8.77 g), 50 mM Tris (6.06 g) and 0.05% Tween (0.5 ml). Made up to 1L by addition of dH₂O
- Blocking Buffer: 1 g non-fat skimmed milk powder in 20 ml TBS-Tween
- Antibody diluent: 0.2 g non-fat skimmed milk powder in 20 ml TBS-Tween.
- Bio-Rad Reagent: supplied Bio-Rad Laboratories (Hemel Hempstead, UK)
- Bovine Serum Albumin (BSA): supplied by Sigma Fraction V, UK.

2.2 METHODS

Common methods used throughout the thesis are described in this chapter, whilst techniques specific to one area are described in later chapters. Many of the methods described are based on standard techniques, which are detailed in Current Protocols in Molecular Biology Volumes 1 & 2 (1994).

2.2.1 Growth and manipulation of mammalian cells

2.2.1.1 Basic techniques

Techniques used in the culture and manipulation of mammalian cells were performed under aseptic conditions. Where possible, all procedures were performed in a laminar flow hood.

2.2.1.1.1 Cell lines

All cell lines were grown as an adherent monolayer in culture, in either 25 or 75 cm² tissue culture flasks stored in an incubator at 37°C with 5% CO₂

Primary foetal palate cells, EqPalF cells, and sarcoid derived cell lines from tumour were generated and established as described by Yuan ZQ (2008). Generation and establishment of EqPalF-BPV-1 transformed cells are also described (S6 cell lines). Briefly, tissue samples from foetal palate and sarcoid tumours were finely dissected and washed with Hanks Buffered Salt Solution (HBSS) (Invitrogen Ltd, Paisley, UK) and incubated overnight at 4 °C in 0.25% trypsin/HBSS. After removal of the trypsin solution, samples were incubated at 37 °C for 30 min followed by the addition of warm Dulbeccos Modified Eagles medium (DMEM) supplemented with 10% FCS, 100 U/ml penicillin/streptomycin, 1.25 µg/ml fungizone, Ampotericin B and Ciproxin (Sigma, UK), 1% non-essential amino acids and 1% essential amino acids (complete DMEM). The cell suspensions were passed through a 100 µm nylon cell strainer (BD Falcon™) and centrifuged. Resuspended cells were then seeded into 25 cm² tissue culture flasks at 1×10^6 cells per flask in 10 ml of complete DMEM and incubated in a 37 °C humidified atmosphere of 5% CO₂ in air until cells reached confluence. Cells were passaged by trypsinization and this stage was regarded as the first passage. Equine sarcoid tumor-derived fibroblasts were termed EqS cells and the fetal palate fibroblasts termed EqPalFs. All cells were maintained in culture in complete DMEM in a 37 °C humidified atmosphere of 5% CO₂ in air.

Cells were routinely tested for mycoplasma using the MycoAlert kit from Cambrex Bio Science. Briefly, 5 ml of cell culture supernatant was removed and spun down to remove floating cells. 100 µl of this supernatant was placed in a 96 well white plate. 100 µl of Mycoalert reagent was added and the mixture left for 5 minutes at room temperature. The luminescence was measured to determine Reading A. 100 µl of Mycoalert substrate was then added and after 10 minutes a second luminescence measurement was made (Reading B). Calculate ratio = Reading B/Reading A. A positive control was included as a sample in the 96 well plate and negative control of fresh media was also included.

If the Reading B/Reading A ratio was less than 1, the sample was considered negative. A result of 1-1.3 was considered a borderline result and a retest was conducted. A result of more than 1.3 means the sample is positive for mycoplasma. All tests carried out on all the cell lines used were consistently negative.

2.2.1.1.2 Cell cryopreservation and storage

For long term storage of cell line stocks, cells were stored in liquid nitrogen. Prior to storage, cells were grown to between 70 and 90% confluency and trypsinised with trypsin-EDTA before being transferred to a sterile 15 ml centrifuge tube. The cells were centrifuged at 1000 g for 5 minutes, the supernatant discarded and cells re-suspended in freezing medium (appropriate culture medium supplemented with foetal calf serum at 45% and DMSO at 10%) to a concentration of 2-4 x 10⁶ cells/ml. The cell suspension was transferred in 1 ml aliquots to labelled cryovials (NALGENE) and brought to -70 °C at a controlled rate (-1 °C/min) using a NALGENE Cryo 1 °C Freezing Container (NALGENE, USA) transferred to a -70 °C freezer overnight. Finally, vials were transferred to a liquid nitrogen freezer for long term storage. Cell stocks were revived by rapid thawing in a 37 °C water bath and subsequently used following standard techniques as described below.

2.2.1.1.3 Cell Counting

Cells were counted using a haemocytometer. Following trypsinisation or revival from frozen stocks, cells were transferred to a 15 ml centrifuge tube and centrifuged at 1200g for 5 minutes. Supernatant was discarded and cells were resuspended in 5 ml of complete medium. A 25 µl sample was withdrawn and added to 25 µl trypan blue (Sigma) resulting in a two-fold dilution. A sample of this mixture was then introduced to the haemocytometer chamber and counted using an inverted microscope at 40x magnification. Cells lying on the top and right hand perimeter of each large (1 ml) square were included whilst those on the bottom and left hand perimeter were excluded. Cell concentrations

(cells/ml) were calculated by multiplying the mean number of cells per large square by 10^4 and 2 to correct for the dilution factor.

2.2.2 DNA techniques

2.2.2.1 Extraction of DNA

DNA was extracted from a range of source materials. These included whole flies, whole blood, cell cultures, buccal swabs and tumour tissue.

2.2.2.1.1 Preparation of equine genomic DNA from peripheral blood mononuclear cells

Equine whole blood samples (5 ml) were mixed with lysis mix (45 ml) and incubated on ice for 10 mins. The mixture was then centrifuged at 2800g for 10 mins at 4 °C and the pellet re-suspended in nuclei lysis mix (3 ml). After the addition of 10% SDS (200µl) and 75µl of proteinase K (10mg/ml) the mixture was incubated at 55° C for 2 hours or at 37 °C overnight. Two extractions with 3 ml phenol/chloroform (50:50) were then performed, each centrifuged for 10 mins at 2800g at room temperature. The upper aqueous phase was then transferred to a fresh tube and the DNA precipitated by addition of 10% sodium acetate (pH 5.2) and then 3 volumes of 96% ethanol. The DNA was re-suspended in 250µl of TE Buffer and the quantity and quality of genomic DNA was assessed using the Thermo Scientific NanoDrop 2000. The NanoDrop 2000 is a micro-volume spectrophotometer that can assess the concentration and quality of DNA in a particular sample.

2.2.2.1.2 Extraction of DNA from whole flies, cell cultures, buccal swabs and tumour tissue.

DNA was extracted from all other sources using the Qiagen DNeasy kit. Although initial preparation of the source material varied slightly between cell culture, buccal swabs, flies and tumour tissue, the overall process was basically the same. Firstly, cellular components were lysed and histones and other proteins associated with the DNA are digested with the enzyme proteinase K. The DNA is then bound to a mini-column holding a silica-gel membrane that selectively binds DNA. The cellular debris is washed off using a series of buffers containing salt and ethanol and the centrifuge. The resulting extract was assessed for DNA yield and quality using nanodrop .

2.2.2.2 Determination of DNA and RNA concentration and quality

2.2.2.2.1 Determination by spectrophotometry

The optical density was measured at 260 nm and 280 nm, in comparison to a reference of TE buffer. An optical density (OD) reading of 1.0 at 260 nm corresponds to an approximate concentration of 50 µg/ml for double stranded DNA, 40 µg/ml for RNA, or 33 µg/ml for single stranded oligonucleotides. The ratio of the OD readings at 260 nm and 280 nm (OD₂₆₀/OD₂₈₀) was used to estimate sample purity, with pure preparations of DNA and RNA producing an OD₂₆₀/OD₂₈₀ of 1.8 and 2.0, respectively. A lower value suggests possible protein or phenol contamination of the sample.

2.2.2.2 Estimation of double stranded DNA concentration via gel electrophoresis

In some instances, insufficient concentration of (ribo)nucleic acid or verification of the purity of DNA fragments of a particular size did not allow quantification via spectrophotometry. In these cases, the concentration of double stranded DNA was determined by running the sample on a 1% agarose gel along with a marker of known quantity and size. The intensity of the sample fluorescence was compared to that of a mass marker (Low DNA Mass™ Ladder) following staining with ethidium bromide and visualisation by UV transillumination.

2.2.2.3 Electrophoresis of DNA

DNA fragments of 0.1-10 kb were separated and identified by agarose gel electrophoresis using a Hoefer HE 33 Mini Submarine Electrophoresis Unit (Amersham Pharmacia Biotech, San Francisco, CA). Typically, 0.5 – 0.1 g of agarose was added to 50 ml of 1x TBE buffer, melted in the microwave (medium for 1.5 minutes), and mixed to produce a 1 to 2% gel. Once the gel mix had cooled to 55° C, 1.5 µl of ethidium bromide (100mg/ml) was added and the gel poured into a gel support (100 x 65 mm) in its casting tray and an appropriate gel comb (twelve or twenty well) inserted. The gel was allowed to solidify before transferring to an electrophoresis tank; the gel was immersed in 1x TBE buffer and the comb carefully removed. DNA samples were prepared by the addition of an appropriate volume of 10x gel loading buffer. Molecular size standard DNA was prepared similarly and the samples loaded into the wells using a micropipette. Gels were run at 100 volts for 20 to 30 minutes depending on the agarose percentage, then removed from the gel apparatus and visualised on a UV transilluminator (UVi tec, Thistle Scientific) and photographed using a Mitsubishi P91 photographic unit.

2.2.3 RNA techniques

Clean, full-length RNA is essential as the starting material for molecular techniques, hence when isolating the RNA it was necessary to ensure that all the preparation material is free from ribonuclease (RNase) activity. This is a very stable, active and ubiquitous enzyme that degrades RNA requiring no cofactors for function. Inhibition of this enzyme was instigated by wiping all equipment with RNaseZAP (Invitrogen) followed by a thorough rinse with DEPC treated water. All plastic-ware was treated by soaking overnight in DEPC treated water followed by autoclaving and drying. All solutions were prepared using DEPC treated water. Gloves were worn and changed frequently.

2.2.3.1 Extraction and Purification of RNA

2.2.3.1.1 RNA extraction using Qiagen RNeasy Mini Kit

Various methods have been used for the isolation of undegraded RNA (Chirgwin *et al.*, 1979). Several procedures have now been elucidated which allow RNA isolation by a single-step procedure (Chomczynski and Sacchi 1987).

In this project, RNA was extracted from a variety of sources: from tissues snap-frozen in liquid nitrogen and stored at -80°C , from cells grown as a monolayer in tissue culture flasks, and from cell pellets stored at -80°C . In order to extract RNA from frozen tissue, tissue was firstly ground in liquid nitrogen using a mortar and pestle.

2.2.3.1.2 DNase Treatment of RNA

Contaminating DNA was removed from RNA samples using DNA-free™ (Ambion) methods. Briefly, 0.1 volume of 10x DNase I buffer (final concentration: 10 mM Tris-HCl pH7.5, 2.5 mM MgCl₂, 0.1 mM CaCl₂) and 2 units of DNase I were added directly to the RNA samples, mixed and incubated at 37 °C for approximately 60 minutes. The enzyme reaction was stopped by the addition of DNase Inactivation Reagent (5µl) for two minutes at room temperature. Because of the nature of the DNase Inactivation Reagent, tubes were periodically agitated to ensure resuspension in the RNA sample. Samples were then centrifuged at 10,000g for one and a half minutes to pellet the Inactivation Reagent and the supernatant containing the pure RNA was then transferred to a clean ependorf tube.

2.2.3.1.3 Assessment of RNA quality using agarose gel electrophoresis

RNA samples were assessed by agarose gel electrophoresis on a 1% agarose TBE gel. The quality of the RNA samples was assessed by examining the integrity of the 18S and 28S ribosomal RNA bands and comparing the bands to a 100 bp molecular weight standard (Invitrogen, UK). The 18S ribosomal unit runs at approximately 4.5 Kb and the 28S at approximately 1.2 Kb. The 18S subunit should appear at twice the intensity of the 28S subunit and mRNA appears as a smear from 0.5 through to 12 Kb.

2.2.3.1.4 First strand cDNA synthesis

The synthesis of complementary DNA (cDNA) requires the Reverse Transcriptase enzyme, initially identified as the enzyme responsible for forming DNA-RNA hybrids in retrovirus replication. Reverse transcriptase utilises an RNA template to synthesise a complementary DNA strand from a primer base-paired to the RNA that contains a free hydroxyl group at the 3' end. Primers that can be used are either random primers, designed to prime at multiple sites throughout the RNA, gene specific primers (GSPs) designed to prime at the gene of interest, or oligo-dT primers which pair with the poly-A sequence at the 3' end of most eukaryotic mRNA molecules. All instigate the synthesis of cDNA strands in the presence of the four dNTPs. The RNA-DNA hybrid is subsequently hydrolysed by raising the pH (unlike RNA, DNA is resistant to alkaline hydrolysis) or by using a ribonuclease. The 3'- end of the newly-synthesised DNA strand then forms a hairpin loop which enables priming of the synthesis of the opposite DNA strand. S1 nuclease, which recognises unpaired nucleotides, then removes the hairpin loop.

Superscript III reverse transcriptase was used for first strand cDNA synthesis. Superscript III is a version of MMLV-RT that has been engineered to reduce RNase H activity and provide increased thermal stability. Briefly, total RNA samples (up to 5 µg total RNA) were added to 1 µl of primer (either oligo(dT), GSP or random primers) and 1µl of 10mM dNTPs, and DEPC water was added to 10µl total volume. This mixture was then denatured to remove any RNA secondary structure by heating for 5 minutes at 65° C and before quenching on ice. First strand synthesis reactions were performed using the heat-treated RNA in a reaction mix containing reverse transcription buffer (10x), reverse transcriptase (200 units), DTT (0.1 M), RNase inhibitor (RNaseOUT™ 40 units) and MgCl₂ (25 mM). The reaction was incubated at 50° C for 50 minutes if oligo(dT) primer was used, or 25° C for 10 minutes followed by 50° C for 50 minutes if other primers were used, and was incubated at 85-95° C for 5 minutes to terminate the reaction before being placed on ice. Finally, RnaseH (1 µl) was added and the mixture incubated at 37° C for 20 minutes to remove the RNA template from the DNA-RNA hybrid. A Perkin-Elmer (PE)

thermal cycler 480 was used for the reaction. cDNA was stored at 4° C before immediate use or –20° C for long term storage.

2.2.4 Amplification of DNA by the polymerase chain reaction

The polymerase chain reaction (PCR) is a powerful technique for amplification of specific DNA sequences from a complex mixture of DNA. The procedure was developed by Mullis and co-workers in the mid 1980's (Mullis *et al.* 1986; Mullis and Faloona 1987) enabling large amounts of a single copy gene to be generated from genomic (Saiki *et al.*, 1985; 1986) or extra-genomic DNA (Kwok *et al.*, 1987). The initial method used the Klenow fragment of DNA polymerase I, which had to be replenished during each cycle as it is readily denatured by the amplification conditions used. The substitution of the thermostable Taq polymerase, isolated from *Thermus aquaticus*, circumvented this problem and allowed the automation of thermal cycling (Saiki *et al.*, 1988). PCR enables the amplification of any unknown DNA sequence by the simultaneous extension of primer pairs flanking the unknown sequence, each complementary to opposite strands of DNA. The uses of PCR are many although it has been superseded by more conventional molecular biological methods in many areas, including sequencing, cloning and detection and analysis of RNA. An extensive overview of PCR and its applications is available (Innis and Gelfand 1990). An overview of the procedure is given below, with more detail in the appropriate chapters.

2.2.4.1 Primer design

Primer design was aided by some basic guidelines (Innis & Gelfand 1990). An optimal primer pair should hybridise efficiently to the sequence of interest with negligible hybridisation to other sequences present in the sample. The distance between primers is rather flexible, ranging up to 30 kbp, however the PCR reaction is considerably less efficient in the amplification of products > 3kbp (Jeffreys *et al.*, 1988). Smaller distances between primers lessen the ability to obtain much sequence information and to reamplify with nested internal oligonucleotides, if required. For any given pair, the annealing temperatures and GC % were balanced.

For many applications, primers are designed to be exactly complementary to the template. For others, however, such as engineering for mutagenesis or new restriction endonuclease sites or for efforts to clone or detect gene homologues where sequence information is lacking, base-pair mismatches will be intentionally or unavoidably created. It is best to

have mismatches, such as in a restriction endonuclease linker, at the 5' end of the primer; the closer to the 3' end of the primer, the more likely a mismatch will prevent extension.

The annealing portion of primers should generally be 18-30 nucleotides in length; it is unlikely that longer primers will help to increase specificity significantly. Additional sequences such as restriction enzyme sites, epitope tags or other desired motifs can be added to the 5' end of the primer, and may effect specificity of primer binding at low temperatures on complex templates. Accordingly, these primers are successfully used when amplifying from a single template vector. Primers should be designed with an optimum GC content, avoiding unusual sequence distributions such as stretches of polypurines or polypyrimidines if possible. The formation of a secondary structure such as hairpin loops greatly effects efficiency of annealing.

Primer-dimers are a common artifact most frequently observed when small amounts of template are taken through many amplification cycles. They form when the 3' end of one primer anneals to the 3' end of the other primer and polymerase then extends each primer to the end of the other. Therefore, these can be avoided by using primers without complementary sequences, especially in the 3' end (this can be minimised by optimising the $MgCl_2$ concentration).

Table 2.1 Primer sets used over the course of the investigation

Gene I.D.	Target For Amplification	Primer Sequence 5' to 3'
1	BPV-1 E5	GCT ACG AGA ACT GCA CCA CC TGG ACA TGT GCC CGC TTG C
2	BPV-1 E6	TCC ATT CTC AGG GTT GGA TTG CAC AGT AGC AGC ATC TTA TGC
3	BPV-1 E7	CGT TGC TGA TTT TAA GTC CAT GTG GGG ACC TCG CTT CCT AGT AGG A
4	BPV-1 L1	CAT GTG GAC GCT GGA CTT CT CGT AGG CCG CCT TCA AAT AC
5	BPV-1 L2	TCC ACG GAG ACC CTC ATT ACT C GAT TTA GAG GCA ATA CTG CGG G
6	BPV-1 LCR (short)	CGG TAC ACA TCC TGT CCA GCA GAT GGT GTG ATT ATT GTT AAC
7	BPV-1 LCR (full)	CAG AAG GTA AGT CAA CTG AAA ACC GGG GTC TG
8	Fly 16s rRNA	TTA CGC TGT TAT CCT AA CAC CTG TTT AAC AAA AAC

2.2.4.2 Preparation of PCR reactions

As PCR is such a sensitive procedure it is essential to take stringent precautions to avoid PCR contamination from tube to tube or carry over of PCR products (Saiki, *et al.*, 1988). All PCR reactions were set up in a designated separate room, at the side of the main laboratory where PCR products were handled. A set of micropipettes was kept for the sole purpose of setting up PCR reactions. Filter tip pipette tips were used to decrease the risk of reaction components passing from one tube to the next. A bulk reaction mix was used in

order to minimise the number of pipetting steps. Reaction components (including primers) were aliquoted prior to use and stored at -20°C .

2.2.4.3 PCR Amplification Profiles

Table 2.2 Commonly used PCR amplification profiles

Amplification profile I.D.	Profile details
1	94°C for 4 minutes initial denaturation; 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. This cycle was repeated 35 times after which samples were held at 4°C until required.
2	94°C for 4 minutes initial denaturation; 94°C for 30 seconds, 55°C for 50 seconds, and 72°C for 30 seconds. This cycle was repeated 35 times after which samples were held at 72°C for ten minutes then held at 4°C until required.
3	94°C for 2 minutes initial denaturation; 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds. This cycle was repeated 40 times after which samples were held at 72°C for ten minutes then held at 4°C until required.
4	94°C for 2 minutes initial denaturation; 94°C for 30 seconds, 52°C for 45 seconds, and 72°C for 45 seconds. This cycle was repeated 45 times after which samples were held at 72°C for five minutes then held at 4°C until required.

Table 2.3 Primer sets with PCR conditions and expected product size

Gene I.D.	Amplification Profile I.D.	Expected Product Size
1. BPV-1 E5	3	600 base pairs
2. BPV-1 E6	2	230 base pairs
3. BPV-1 E7	2	215 base pairs
4. BPV-1 L1	1	86 base pairs
5. BPV-1 L2	2	286 base pairs
6. BPV-1 LCR (short)	1	780 base pairs
7. BPV-1 LCR (full)	1	800 base pairs
8. Fly 16s rRNA	4	415 base pairs

2.2.4.4 Purification and assessment of PCR products

Single PCR products were purified following the QIAquickPCR purification kit protocol, (QIAGEN). Briefly, the DNA adhered to the filter within the column, separating it from all other components of the PCR reaction which were washed away with various buffer solutions. The PCR products were finally eluted in 30-50 μl of sterile water, 4 μl of which were assessed by gel electrophoresis on a 1% agarose TBE gel by comparing the bands created to a 100 bp molecular weight standard (Invitrogen, UK).

2.2.5 Real-time relative quantitative PCR (RQ-PCR)

Real-time Relative Quantitative PCR (RQ-PCR) was performed on 0.1 µg cDNA or DNA samples using the specific primer probe set (Table 3) for the gene in question. Primer and probe sets were designed using Primer Express software where necessary. PCR reaction mixes contained 0.2 µM forward and reverse primers, 0.1 µM probe, 25 µl of 2× Platinum Quantitative PCR SuperMix-UDG (Invitrogen, UK), 1 µl ROX Reference Dye and water to a final volume of 50 µl. Reactions were performed on a 7500 Real Time PCR System (Applied Biosystems) with amplification profiles specific to each primer and probe set. For analysis of equine fibroblast derived cDNA/DNA, a GAPDH primer and probe set was used as an endogenous control. For analysis of cDNA/DNA derived from fly tissue, a fly actin primer and probe set was used as an endogenous control. In both cases, all reactions were performed in triplicate. The $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001) was used to compare the relative expression or DNA content levels.

Table 2.4 Primer and probe sets used in RQ-PCR experiments

Target Gene	Forward Primer 5'-3'	Reverse Primer 5'-3'	Probe (FAM - TAMRA)
GAPDH	GGT GGA GCC AAA AGG GTC AT	TTC ACG CCC ATC ACA AAC AT	ATC TCT GCT CCT TCT GCT GAT GCC CC
Fly Actin	AGG AAT GGA AGC TTG CGG TA	AAT TTT CAT GGT GGA TGG TGC	ATG CCA ACA CTG TCC TTT CTG GAG GTA
BPV-1 L1	GGC TCA CTA GTC TCA ACT GAT AAT CAA	ATG CAA TTC CAT TGT TCA TG	AAT CGG CCC TAC TGG CTA TTC CGT GC
BPV-1 L2	AGG AGG GCT AGG AAT AGG AAC ATG	TGT TCG GAG TGG TGT GTA CCT T	CTG GAA GGG TTG CTG CAG GTG GAT C
BPV-1 E6	TGG CAA GGT GTT CCA GTA ACA G	GCA TCT TAT GCA AAG CCT ATC AAG	TGA GGA AGC TGA ATT ATT GCA TGG CAA AA
BPV-1 E7	CGT TGC TGA TTT TAA GTC CAT GTG	GGG ACC TCG CTT CCT AGT AGG A	TTT CAG ACT TCC GTG CCA TTT CGG C

Amplification profiles of 2 min at 50 °C, 2 min at 95 °C and 45 cycles of 15 s at 95 °C and 45 s at 60 °C.

2.2.6 DNA sequence analysis

2.2.6.1 Automated sequencing

During sample preparation, DNA fragments in a sample are chemically labelled with fluorescent dyes. The dyes allow the detection and identification of the DNA and typically each nucleotide is labelled with one dye molecule. More specifically, PCR reactions were performed using plasmid DNA samples (200-500 ng) in a total volume of 20 µl containing 0.5µM of primers, 40mM Tris-HCl, 1mmol/L MgCl₂ and 4µl of Big Dye Terminator Cycle Sequencing Ready Reaction (ABI Prism). Samples were prepared in the PE 2400 thermal cycler incorporating 25 cycles of amplification, each cycle consisting of a denaturation step 96° C for 10 seconds followed by an annealing temperature of 50° C for 5 seconds, and an elongation step of 60° C for four minutes. DNA was then purified by precipitation methods using a 10% volume of 3M sodium acetate (Sigma) and 2.5 volumes of ethanol (95%), at room temperature for 15 minutes. Pelleted DNA (14,000 rpm for 20 minutes) was washed in ethanol (70%) and re-pelleted (14,000 rpm for 5 minutes) before all ethanol was removed and the pellets were dried at 90 °C for 1 minute. Template Suppression Reagent (25 µl) was then added and the mixture heated to 95 °C for 5 minutes and chilled before transfer to genetic analyser sample tubes.

2.2.6.1.1 Sample sequencing

Samples were finally loaded and run on the ABI PRISM 3010 Genetic Analyzer (PE Applied Biosystems, UK) under standard sequencing conditions for generation of automated sequence data. The ABI PRISM 3010 Genetic Analyzer is a multi-colour fluorescence-based DNA analysis system using the proven technology of capillary electrophoresis with 16 capillaries operating in parallel. The 3010 Genetic Analyzer is fully automated from sample loading to data analysis.

2.2.6.1.2 Sequence evaluation

The length of read is 750 bases at the 98.5% base calling accuracy with less than 2% ambiguity. The output is in the form of a chromas file. A series of different computational software programs were utilised for sequence analysis including the 'Blast' search engine contained within the NCBI database, VectorNTI (Invitrogen), ClustalW (<http://www.ch.embnet.org/software/ClustalW.html>), Transfac and Motif Search.

2.2.7 Rolling Circle Amplification (RCA)

RCA was used to evaluate samples for the presence of circular BPV episomes. This amplification method preferentially amplifies circular DNA templates with ϕ 29 DNA polymerase (Rector *et al.*, 2004). RCA will generate linear double stranded, high-molecular-weight repeated copies of the complete circular BPV genomes and digestion of this RCA product with a restriction enzyme that has only one recognition site in the BPV genome, will result in multiple double-stranded, linear copies of the BPV complete genomic DNA, which can be visualised as a single band of approximately 8kb by agarose gel electrophoresis.

Reactions were carried out using a modified procedure based on that described in the TempliPhi 100 amplification kit (Amersham Biosciences). The only change to the original protocol was to supplement the reaction with additional dNTPs to enhance the sensitivity of the reaction as reported previously (Rector *et al.*, 2004). In brief, an 11.6 μ l reaction system was composed of 5 μ l of sample buffer, 5 μ l of reaction buffer, 0.2 μ l (1U) ϕ 29 DNA polymerase enzyme, 0.4 μ l extra dNTP mixture (25mM) and 1 μ l of template DNA (100ng). Firstly, template DNA was mixed with sample buffer and denatured at 95°C for 3 minutes, then cooled to room temperature. Then reaction buffer, extra dNTPs and enzyme were mixed with the denatured template DNA and incubated at 30°C for 16 hours. The reaction mixture was then heated to 65°C for 10 minutes to inactivate the ϕ 29 DNA polymerase. 3 μ l of the RCA product was digested with 10 U of HindIII as this is a single cutter of the closed BPV-1/2 complete genome. The same amount of non-amplified sample DNA that was used as input material for the RCA was also digested with HindIII. Restriction digests were separated on a 0.6% agarose gel and visualised with ethidium bromide staining.

2.2.8 Preparation of small interfering RNA molecules (siRNAs)

SiRNAs targeting BPV-1 E5, E6 and E7 ORFs were generated. Gene sequence information for E5, E6 and E7 was obtained from the National Centre for Biotechnology Information (NCBI) and this was then used to generate potential target sequences for siRNAs. Based on recommendations from various online siRNA design software (www.imgenex.com, www.rnaidesigner.invitrogen.com, www.ambion.com), the following sense and antisense sequences were chosen:

2.2.8.1 E6 siRNA sequence

Target sequence (214 – 232) - 5' GGTGAGGAAGCTGAATTAT 3'

siRNA sequence - 5' GGUGAGGAAGCUGAAUUAU UU 3'

Scramble control sequence - 5' GGAAGGAGUAUUGACGUAU UU 3'

All RNA oligonucleotides were synthesized by Dharmacon Research (Lafayette, CO).

2.2.8.2 Introduction of siRNA into cells

Delivery of the siRNA and scramble control sequences into the cell was achieved by using Lipofectamine 2000 (Invitrogen, UK) in accordance with the manufacturer's protocol. As recommended by the manufacturer, cells were transfected when they were 30-50% confluent. Because gene knockdown levels are generally assayed at a minimum of 24 – 72 hours following transfection, transfecting cells at a lower density allows a longer interval between transfection and assay time, and minimises the loss of cell viability due to cell over-growth. In addition, anti-biotic free DMEM medium was used, as presence of antibiotics during transfection results in cell death. Opti-Mem I Reduced Serum Medium (Invitrogen, UK) was used to dilute the Lipofectamine 2000 prior to complexing with the siRNA.

2.2.8.3 Establishing efficiency of gene silencing

RQ-PCR using E5, E6 and E7 primer probe sets were used as described previously (section 2.2.5, Table 2.4) to establish the level of knockdown achieved after treatment with siRNAs. Cell populations were only used in subsequent apoptosis and cell survival analysis if the level of knockdown was determined to be at least 75%.

2.2.9 Immunohistochemistry (IHC)

Immunohistochemistry (IHC) is a technique that allows identification of cellular or tissue antigens by means of interaction between a specific antigen-antibody combination. For this project, staining was performed by Lynn Stevenson, Diagnostic Department, University of Glasgow, Department of Veterinary Medicine. Both paraffin sections of sarcoid tumours and cultured cell lines were analysed for presence and location of p53. Cells were grown on 8-well chamber slides (Lab-Tek II chamber slide system, Thermo Scientific, UK) and expression of p53 was evaluated using a two-step immunohistochemical technique using Dako EnVision kit (K4007) following the manufacturer's instructions. Briefly, once the 8

chamber frame had been removed, slides were placed in a Shandon Sequenza Staining System where the cells were fixed in acetone, rinsed with wash buffer and the endogenous biotin activity quenched by a 5 minute application of Dako Real Peroxidase blocking solution. The glass slides were incubated for 1 hour at room temperature in the presence of primary antibody (DO-7 p53 clone; Novocastra Laboratories, UK) diluted 1:200 in 10mM Tris Buffered Saline (TBS). Glass slides were then rinsed in 0.5% Tween20 in 10 mM TBS and incubated with polymer-HRP anti-mouse solution applied with the the Dako EnVision kit (Dako, UK) for 30 minutes. Slides were developed with Dako K5007 DAB (3,4,3',4'-tetra aminobiphenyl hydrochloride) for 5 minutes at 37°C, washed thoroughly with distilled water and counterstained with Gill's Haematoxylin (1 dip). The glass slides were washed and a large glass coverslip mounted over the cell culture surface. For negative controls, duplicate slides were incubated with a non-related serum instead of the p53 primary antibody. Positive controls for p53 protein consisted of equine squamous cell carcinoma tissue sections.

For sarcoid tumour sections, 3-4 µm thick paraffin-embedded tissue section were cut and mounted on histogrip (Zymed Laboratories Inc) coated slides, then dried at 56°C for 30 minutes. Specimens were deparaffinized with Xylene, rehydrated in serial graded (100%, 90%, 70%, and 50%), water-ethanol solutions and rinsed in deionized water. Target retrieval was performed using the Menarini Access Retrieval Unit, Buffer Sodium Citrate pH6, incubated for 1 min 40 sec at 125°C, full pressure. After antigen retrieval endogenous peroxidase activity was blocked by immersion of slides in peroxidase blocking solution (Provided in the Envision system) for 10 minutes. After washing with Tris Buffered Saline with Tween 20 (TBS-T) sections were incubated with primary antibody against p53 (mouse monoclonal anti-p53, clone DO-7, Novocastra, UK), for 60 minutes at room temperature. The dilution used was 1:400 in Dako Real Antibody Diluent. The slides were then rinsed in TBS-T buffer and wiped to remove excess buffer, before being incubated with peroxidase labelled polymer complex (provided in the Envision system) for 35 minutes. All slides were washed with 1X TBST buffer and then with distilled water. Sections were developed with Dako K5007 DAB (3,4,3',4'-tetra aminobiphenyl hydrochloride) for 5 minutes at 37°C, washed thoroughly with distilled water and counterstained with Gill's Haematoxylin (1 dip). For negative controls, duplicate slides were incubated with a non-related serum instead of the p53 primary antibody. Positive controls for p53 protein consisted of equine squamous cell carcinoma tissue sections.

2.2.9.1 Evaluation of Slides

Slides were examined for the presence of brown nuclear and/or cytoplasmic staining within the tumour cells and tumour sections. The proportion of positive cells per high power field was calculated for each cell line and each tumour section. Assessment of each cell line or tumour section required analysis of 3 separate high-powered fields, and the resulting numbers were averaged to give a final score. Tumours and cell lines with p53 positive cytoplasm were noted.

2.2.10 Protein Analysis

2.2.10.1 Estimating Protein Concentration

Protein was extracted from cultured cell lines and stored at -70°C . The amount of protein in cell lysates was quantified to ensure equal loading in western blot gels. Here the Bio-Rad Laboratories (Hemel Hempstead, UK) Protein Assay was used in which known bovine serum albumin (BSA, Promega Corporation, Southampton, UK) concentrations, namely 0.1 - 2 mg/ml, were used as reference values. The method is based on the Bradford assay (Bradford, 1976). Briefly, the 5x dye reagent concentrate was diluted 1 in 5 with deionised water. The standard samples were prepared as a 1:10 dilution of 10 mg/ml stock solutions of BSA. The stock solutions were diluted in 1x protein assay solution in sterile water. Samples were prepared by adding 1 μl of lysate sample to 1 ml of 1x protein assay solution. Both samples and BSA controls were mixed by inversion and left at room temperature for 5 minutes. Prepared standard BSA concentration samples were read at absorbance 595 nm and a standard curve was drawn. Protein concentrations in unknown samples were extrapolated from the linear part of this curve.

2.2.10.2 Western Blotting

Western blotting was performed using the Nupage Bis-Tris Electrophoresis System (Invitrogen, UK). The Nu-Page system uses a neutral pH, discontinuous Sodium Dodecyl Sulphate (SDS)-PAGE pre-cast gel. The pre-cast gel is formulated with a varying gradient of polyacrylamide (4% - 12%) to allow separation of a range of proteins from 1-200 kDa. Samples are treated with a reducing agent (500 mM dithiothreitol - DTT) to prevent oxidation of samples and allow sharper resolution of bands during electrophoresis. Following electrophoresis, the protein is transferred to a nylon membrane and stained with the primary antibody of interest, followed by detection with a secondary antibody which is directed against the immunoglobulin molecule of the species in which the primary

antibody was generated. This secondary antibody is conjugated to a molecule of horseradish peroxidase which produces a colour change when a chromagen is added. This system produces amplification of the original signal sufficient to allow visualisation by light microscopy.

2.2.10.2.1 Sample preparation

Cell pellets containing approximately 2×10^5 cells were resuspended in 60 μ l of lysis buffer (0.5% NP40, 150 mM NaCl, 50 mM TrisHCl (pH 8) with an added protease inhibitor cocktail (Roche, UK)) and incubated on ice for 30 minutes. Samples were then centrifuged at 13,000 rpm for 30 minutes at 4 °C. Protein quantification was performed as previously described. 10 – 30 μ g of total protein extract was added to 2 μ l NuPage™ sample reducing agent (10x, Invitrogen) along with 5 μ l NuPage™ sample buffer (4x) (Invitrogen). Total volume was made up to 25 μ l using deionised water. The solution was heated to 70 °C for 10 minutes to denature the proteins.

2.2.10.2.2 Polyacrylamide Gel Electrophoresis (PAGE)

The pre-cast gel placed in the tank (Xcell SureLock Mini-Cell (Invitrogen, UK) and an opposing gel or buffer dam was used to seal the system. 1 x MES (50 ml NuPage 20x MES Running Buffer Stock Solution (Invitrogen, UK) in 1 litre of de-ionised water) was added to the tank and 500 μ l antioxidant agent was added to the buffer in the tank adjacent to the gel(s). The antioxidant is added to perform electrophoresis under reducing conditions and migrates with the proteins during electrophoresis in order to prevent reoxidation and maintain the proteins in a reduced state. The antioxidant also protects sensitive amino acids such as methionine and tryptophan from oxidising. 25 μ l protein samples and 10 μ l marker (Seeblue Plus 2 pre-stained standard, Invitrogen, UK) were loaded onto the gel and run at 200 V for 60 minutes.

2.2.10.2.3 Membrane Transfer and protein staining

Following electrophoresis, proteins were transferred to a nitrocellulose membrane. The membrane was then briefly stained with Ponceau S solution to confirm the protein transfer. Ponceau S solution was added to the surface of the membrane and rinsed off after thirty seconds using deionised water. Successful transfer was confirmed by the presence of multiple red bands in each lane.

2.2.10.2.4 Blocking

Non-specific bands were blocked in 5% dried skimmed-milk powder solution (Marvel, UK) in Phosphate Buffered Saline (PBS) containing 0.1% Tween 20 (PBS-T) for 1 hour at room temperature or overnight at 4°C.

2.2.10.2.5 Antibody staining

Following blocking, the blocking solution was discarded and the membrane was washed in PBS-T for 1 hour. The primary antibody used was p53 and GAPDH antibody was used as a loading control. Several different p53 antibodies are commercially available. After conducting a literature search, the p53 clone CM-1 (Abcm plc., UK) was selected. CM-1 antibody recognizes both wild type and mutant forms of p53 protein under denaturing and non-denaturing conditions. However, the CM-1 antibody consistently failed to bind with the equine p53 protein, at a wide range of dilutions. A second p53 antibody was then chosen. DO-7 also recognizes both wild type and mutant forms of p53 protein under denaturing and non-denaturing conditions. It had been shown to react with p53 from a number of different species and was recommended for use in Western blotting. It was used at a dilution of 1:500 in milk powder PBS-T and was incubated with the membrane for 16 hours at 4°C.

After staining with the primary antibody, the membrane was then washed four times in PBS-T for five minutes per wash. The secondary antibody, consisting of goat anti-mouse IgG conjugated to horseradish peroxidase (Amersham, UK) was used at a dilution of 1:5000 and incubated for 1 hour at room temperature.

The membrane was then washed four times in 0.1% PBS-T for five minutes per wash. Excess liquid was drained from the membrane and 1ml detection reagent (ECL-Plus (Amersham, UK)) was evenly distributed over the membrane and incubated at room temperature for 5 minutes. The membrane was then placed in a transparent plastic wallet and placed inside an autorad x-ray film cassette. In a dark room, x-ray film was placed inside the cassette together with the membrane and developed for 2 minutes before being removed and processed in an automatic x-ray film processor. This was repeated with exposure times of 5, 10, and 20 minutes. From these exposures, the most appropriately exposed film was selected for analysis.

2.2.11 Flow Cytometric Analysis

Flow cytometry (FCM) is a method for analysing and counting a heterogeneous mixture of biological cells one cell at a time, based upon the specific light scattering and fluorescent characteristics of each cell. During measurement a laser beam is passed through a fluid stream containing the population of interest. Excitation of the analyte within the stream (sample core) results in a signal dependant on the properties of the analyte in question. The physical properties of a given analyte can be determined by measuring scattered light of two types. Side scatter (SSC), detected at $\sim 90^\circ$ to the incident light is proportional to cell granularity and the complexity of a cell's internal structure (widely used in the differentiation of cells such as granulocytes). Forward scatter (FSC), is a measurement of diffracted light, determined via a photodiode at a small angle to the axis of the laser beam. Excluding clinical haematology, all FCM analysis employs the use of flourophores. The determination of non-physical characteristics of a cell may be elucidated through labelling with an organic flourophore (e.g. fluorescein isothiocyanate (FITC)) attached to a carefully chosen primary or secondary antibody. In FCM experiments data is generally presented as histograms or bivariate histograms either as scatter, density or contour plots.

Recently, it has been shown that cells undergoing apoptosis break up the phospholipid asymmetry of their plasma membrane and expose phosphatidylserine (PS) which is translocated to the outer layer of the membrane. This occurs in the early phases of apoptotic cell death during which the cell membrane remains intact. This PS exposure may represent a hallmark (early and widespread) in detecting dying cells. Annexin V, belonging to a recently discovered family of proteins, the annexins, with anticoagulant properties has proven to be a useful tool in detecting apoptotic cells since it preferentially binds to negatively charged phospholipids like PS in the presence of Ca^{2+} and shows minimal binding to phosphatidylcholine and sphingomyeline. Changes in PS asymmetry, which is analyzed by measuring Annexin V binding to the cell membrane, were detected before morphological changes associated with apoptosis have occurred and before membrane integrity has been lost. By conjugating FITC to Annexin V it is possible to identify and quantitate apoptotic cells on a single-cell basis by flow cytometry. Staining cells simultaneously with FITC-Annexin V (green fluorescence) and the non-vital dye propidium iodide (red fluorescence) allows the discrimination of intact, viable cells (FITC-PI-), early apoptotic (FITC+ PI-) and late apoptotic (or secondary necrotic) or necrotic cells (FITC+ PI+). The term secondary necrosis (type 2 necrosis) refers to a process in which late stage apoptotic cells that failed to be engulfed by phagocytes or neighbouring cells undergo necrosis. Secondary necrosis is a post-apoptotic event commonly seen in

cultured cells that are undergoing cell death by apoptosis in vitro, induced, for example, by the absence of survival factor signals or activation of death receptors by different lethal signals. These cells, in the absence of phagocytic cells that could engulf them, ultimately cease to be metabolically active, lose membrane integrity, and release their cytoplasmic contents into the culture medium. The Calbiochem Annexin V-FITC Apoptosis Detection Kit was used and the cells were processed 24 hours after UVB irradiation, or after 72 hours of drug exposure, according to the manufacturer's protocol; approximately 105 to 106 cells were processed per sample. Adherent cells were released from their substrate using 0.05% Trypsin-EDTA (Invitrogen, UK). Care was taken when trypsinising to prevent excessive cell damage. It was necessary to remove all EDTA by washing twice in 1x PBS prior to labelling to avoid chelating the calcium necessary for annexin binding.

Three control samples were used to calibrate the instrument. First, cells re-suspended in binding buffer only, were assessed to evaluate the level of auto-fluorescence and to adjust the instrument accordingly. Then, treated cells were stained separately with Annexin V-FITC and Propidium Iodide to define the boundaries of each population. Apoptotic cells labelled with Annexin FITC should appear in the lower half of the dot plot, with no events accumulating in the upper left or upper right quadrants. Similarly, cells labelled with propidium iodide alone should show no events in the upper or lower right quadrants.

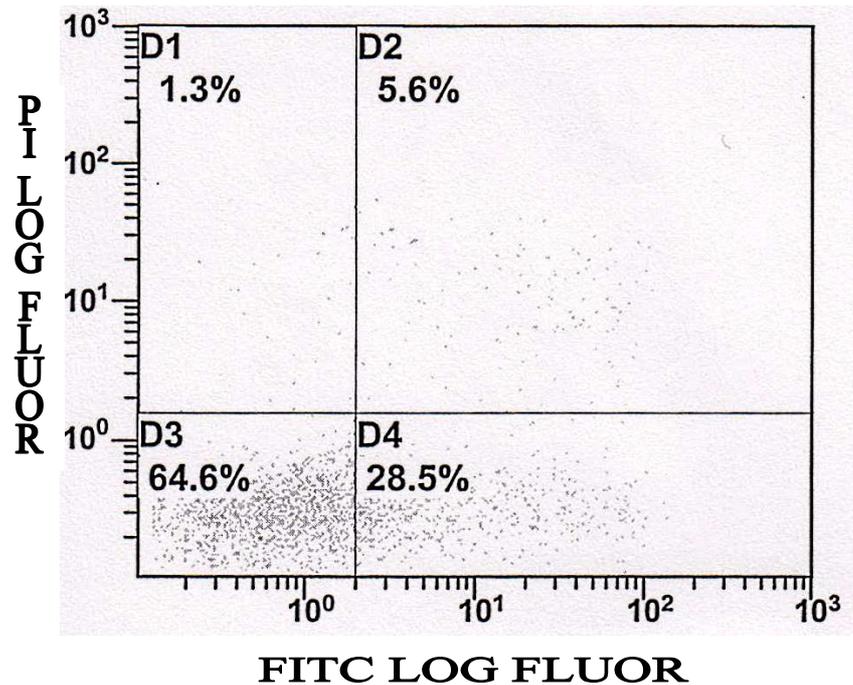


Figure 2.1 A sample cytogram (from results obtained in Chapter IV) following Annexin V-FITC and PI staining.

Each quadrant in the cytogram shown in Figure 2.1 represents a different sub-population of the cell type analysed.

D1 represents necrotic cells or cell fragments that have only been stained with propidium iodide.

D2 represents necrotic or apoptotic cells in terminal stages (secondary necrotic). These cells will be both Annexin V-FITC and propidium iodide positive.

D3 represents viable cells. These cells do not bind Annexin V-FITC or propidium iodide as reflected in the lower left-hand quadrant of the cytogram.

D4 represents early apoptotic cells with exposed PS but intact cell membranes that bind Annexin V-FITC but exclude propidium iodide.

A small percentage of normal cell death should be expected in routine cultures of untreated cells.

2.2.11.1 Evaluation of Flow Cytometry Results

In the optimisation steps of the flow cytometry investigation, it became clear that some cell lines were more sensitive to the protocol methods than others, resulting in higher than expected apoptosis levels in the untreated control cells. For example, the normal equine fibroblast (EqPalF) and the sarcoid derived fibroblast (EqS) cell populations that were not treated in any way, consistently produced apoptosis levels of less than 5%. In contrast, the BPV-1 transformed fibroblasts (S6) often displayed twice this amount. To ensure that values obtained for the different cell lines could be reliably compared, the increase in the proportion of apoptotic cells was calculated using the apoptosis level observed in each untreated cell population, as a control. This will provide a standardised value for comparing apoptosis levels both within a cell line, and between different cell lines.

2.2.12 Clonogenic Survival Assay

Protocol used was as described by Franken *et al.*, in Nature Protocols (2006). Cells were seeded at a concentration of 2000 - 4000 cells per 10cm Petri dish and maintained in 15ml of complete DMEM in a 37 °C humidified atmosphere of 5% CO₂ in air. From the results both the Plating Efficiency (PE) and Surviving Fraction (SF) of each cell line at each UVB dose was calculated. Different cell lines have different plating efficiencies and calculation of this value from the control cells is essential for establishing accurate cell survival data;

$$PE = [\text{number of colonies formed}/\text{number of cells seeded}] \times 100$$

This value is then used to calculate the Survival Fraction;

$$SF = [\text{number of colonies}/(\text{number of cells seeded} \times PE)]$$

2.2.13 Statistical Analysis

For comparison of apoptosis levels, histological grading and colony survival values, the Student t-test was used, with the level of significance set at $p < 0.05$. The t-test is a statistical test involving means of normal populations with unknown standard deviations; small samples are used, based on a variable t equal to the difference between the mean of the sample and the mean of the population divided by a result obtained by dividing the standard deviation of the sample by the square root of the number of individuals in the sample. The t-test is commonly used to show if there is a real difference between different treatments being tested in a controlled clinical trial. Simply put, the t-test determines a probability that two populations are the same with respect to the variable tested.

3 CHAPTER III:

Detection of BPV-1 DNA in flies.

3.1 INTRODUCTION

Although there is strong evidence that BPV types 1 and 2 are the principal causative agent of sarcoids, there is currently no clear evidence of a mode of transmission. As mentioned earlier in the epidemiology section (section 1.6.6), there may be a predilection for sarcoid development at wound sites and it has been proposed this may be due to flies acting as a vector as they move between wound sites on different horses (Chambers *et al.*, 2003). Sarcoids also commonly develop in the peri-ocular region and around the lips and nostrils of an animal – these areas correspond to areas of frequent congregation/feeding for some common species of equidae associated insects (Downes, 1958; Fotedar, 2001). In 2000, Kemp-Symonds demonstrated that BPV DNA could be detected in face flies, and also that the same viral DNA sequences were detected in the horses from which the flies were removed. Most recent data (both anecdotal and published) suggest that sarcoid tumours are transmitted from equid to equid. The evidence to support this includes the observations that donkeys in close contact to affected donkeys are at a higher risk for sarcoid development (Reid *et al.*, 1994a). The occurrence of epizootics of equine sarcoids in herds of horses and donkeys has been reported (Reid 1992; Ragland 1966) and more recently the reported outbreak of sarcoids in an isolated population of mountain zebras (Marais *et al.*, 2007). It was recently shown that equine sarcoids are most often associated with a specific BPV-1 variant which is not present in BPV-1 infections in cattle (Nasir *et al.*, 2007), suggesting that there is horse to horse transmission. There is also published data supporting equid to equid transmission of BPV-1 by sequence analysis of BPV-1 Long Control Region (LCR) (Nasir and Campo 2008).

The mode of natural transmission of warts in cattle caused by BPV is unknown but assumed to be direct contact between infected animals or by abrasions from inanimate objects contaminated by virus. Crops of warts have been observed appearing at sites of dehorning and tattooing, around ear tags and around the nose after using contaminated bull leads. They were also inclined to appear along lesions produced by barbed wire (Blood *et al.*, 1983). The presence of BPV DNA has subsequently been demonstrated in the blood, milk, urine, seminal fluid, and spermatozoa of BPV-infected animals (Lindsey *et al.*, 2009). The finding of BPV DNA in body fluids and tissues other than the epithelium demonstrates co-infection of other tissues or cell types by papillomavirus and shows the potential role of lymphocytes, seminal fluid and spermatozoa in BPV transmission (Lindsey *et al.*, 2009). In 2007, the presence and activity of BPV in normal skin and peripheral blood of 4 groups of horses was evaluated: sarcoid-affected horses, horses living

in contact with sarcoid-affected horses, horses living in contact with papilloma-affected cattle and control horses. BPV DNA was found in the normal skin of 24 of 42 horses (57%). Mainly sarcoid-affected horses and horses living in contact with cattle were carriers (73%), but BPV DNA was also detected in 50% of the horses living in contact with sarcoid-affected horses and in 30% of the control population. BPV mRNA was detected in 58% of the samples positive for BPV DNA, although in a much lower quantity compared to sarcoids (Bogaert *et al.*, 2007). Although the results of this study could not demonstrate the presence of BPV DNA in any of the blood samples taken, a more recent report demonstrates that peripheral blood mononuclear cells (PBMCs) can represent a reservoir of BPV DNA in sarcoid-affected animals (Brandt *et al.*, 2008). Researchers in South Africa recently demonstrated the presence of BPV-1 and -2 DNAs in zebra sarcoid tumours. They then developed a real-time PCR technique to detect and distinguish between BPV-1 and -2 infections in zebras. With this assay it was demonstrated that BPV-1 and BPV -2 DNA are present in sarcoid tumour tissue, healthy, unaffected skin and the blood of sarcoid-affected and healthy zebras (van Dyk *et al.*, 2009). These results support the hypothesis that PBMCs may serve as host cells for BPV-1/-2 DNA and both contaminated blood and skin of infected and uninfected animals could contribute to virus latency. In addition, taken together, these data support the hypothesis that insects, feeding on contaminated skin, or from the blood of wounds/biting flies, would be exposed to BPV DNA which they may in turn spread to other susceptible animals, incriminating them as likely vectors of this disease.

There are many insects that are the primary or intermediate hosts or carriers of diseases. Pathogens that are capable of being transmitted by insects include protozoa, bacteria, viruses, and such helminths as tapeworms, flukes, and roundworms (Spier *et al.*, 2004; Forster *et al.*, 2007). In the simplest terms, viruses are said to be either mechanically transmitted or biologically transmitted by their arthropod vectors. Mechanical transmission refers to the non-specific transmission of viruses by single or multiple vector species, usually on contaminated mouthparts. The viruses are unable to replicate in the vector. Biological transmission refers to the specific association of a virus with a particular arthropod species or genus, and perhaps more importantly, it refers to the fact that the virus is able to propagate within the vector (Carn, C.V., 1996). Many different species of Diptera are implicated in the mechanical transmission of animal viruses, where the insect becomes contaminated with virus during normal feeding behaviour, and virus persists on their mouthparts or body until the next feed. Some viruses are inactivated rapidly on mouthparts, whereas others survive for many days or weeks, prolonging the potential

period of transmission. Some well studied examples of common insect species being implicated in the transmission of viral disease include;

- Mosquitoes in the transmission of rabbit papilloma virus (Dalmat, H.T. 1957).
- Bushflies (*Musca vetustissima*) in the transmission of rabbit haemorrhagic disease virus (RHDV) between rabbits (McColl *et al.*, 2002).
- Blackflies (*Simulium vittatum*) in the transmission of vesicular stomatitis New Jersey virus (VSNJV) between cattle (Mead *et al.*, 2009).
- Stable flies (*Stomoxys calcitrans*) in the transmission of equine infectious anaemia virus (Issel and Foil, 1984), sheep pox virus (Kitching and Mellor, 1986), African swine fever virus (ASFV) (Mellor *et al.*, 1987), bovine viral diarrhoea virus (BVDV) (Tarry *et al.*, 1991).

Common Diptera species encountered where equines are present include the house fly (*Musca domestica*), the biting stable fly (*Stomoxys calcitrans*), the horse fly (*Tabanus* spp.) and face flies (*Musca autumnalis*). Lesser house flies (LHF - Fig. 3.1a) and stable flies (SF - Fig. 3.1b) are commonly encountered species representing both non-biting (LHF) and biting (SF) types. The stable fly has a piercing-type mouthpart and both sexes feed on the blood of warm-blooded animals. After a blood meal, the adults fly to a vertical surface to digest their food. House flies and lesser house flies do not bite because they have a sponging-type mouthpart with which they feed on semi-liquid material. Houseflies feed on excrement and may also feed on ocular or nasal secretions and on exudating wounds. The life cycles of the two species are similar, consisting of eggs, larvae (maggots), pupae, and the adult. During summer months the stable fly completes its life cycle in about three weeks and the house fly in about two weeks. Both species deposit eggs in wet, decaying organic matter. The house fly may breed during the winter in warm buildings if breeding material is present. Adult house flies are very strong fliers and can travel ¼ mile and sometimes much farther. Stable flies can move great distances, and have been shown to survive journeys of several hundred miles when carried by a weather front.

In considering these insects as potential disease vectors it is necessary to understand their internal anatomies, in particular with respect to the digestive system and salivary glands, where viruses and bacteria ingested by the fly, have been shown to accumulate (Gray and

Banerjee, 1999). These are shown in figure 3.2 a and b for the lesser house fly and the stable fly respectively.

Figure 3.1

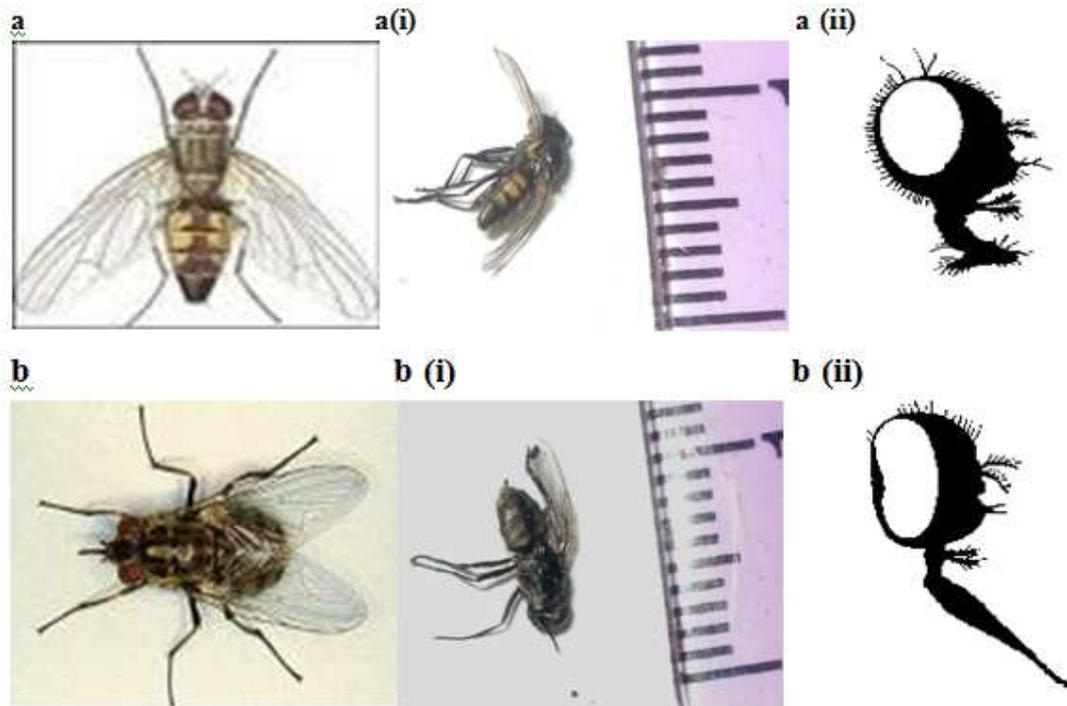
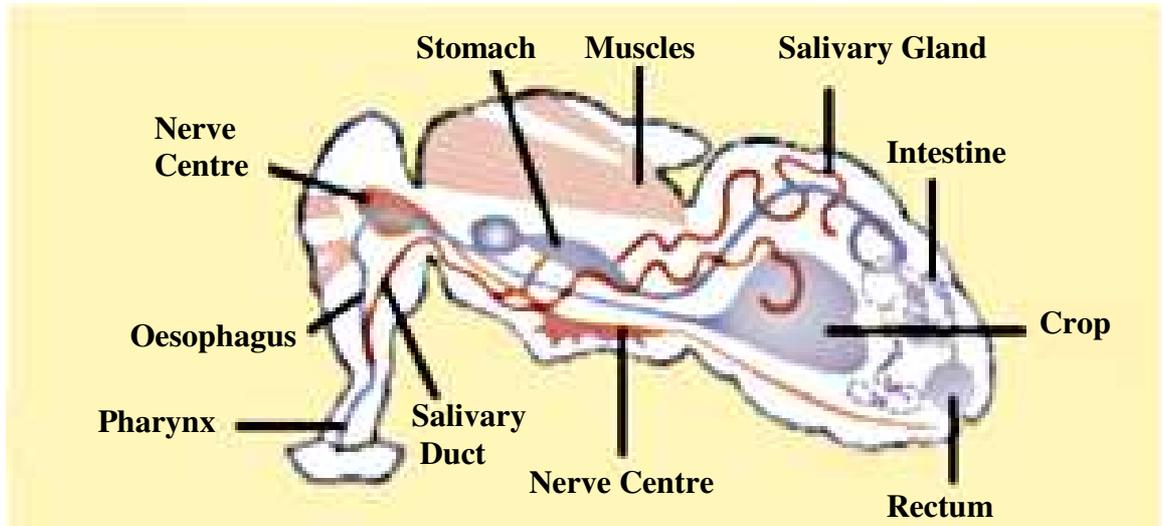


Figure 3.1 External anatomies of flies of the Diptera order.

The lesser house fly (LHF) (*Fannia canicularis*; www.ento.csiro.au) (Fig. 3.1a) and the stable fly (SF) (*Stomoxys calcitrans*; www.ces.ncsu.edu) (Fig 3.1b). Figures 3.1a(i) and 3.1b(i) show photographs taken of the LHF and stable fly after they had been removed from the paper fly traps. The LHF has a sponging mouthpart (Fig. 3.1a(ii)) and the SF has a piercing or biting mouthpart (Fig. 3.1b(iii)).

Figure 3.2

a



b

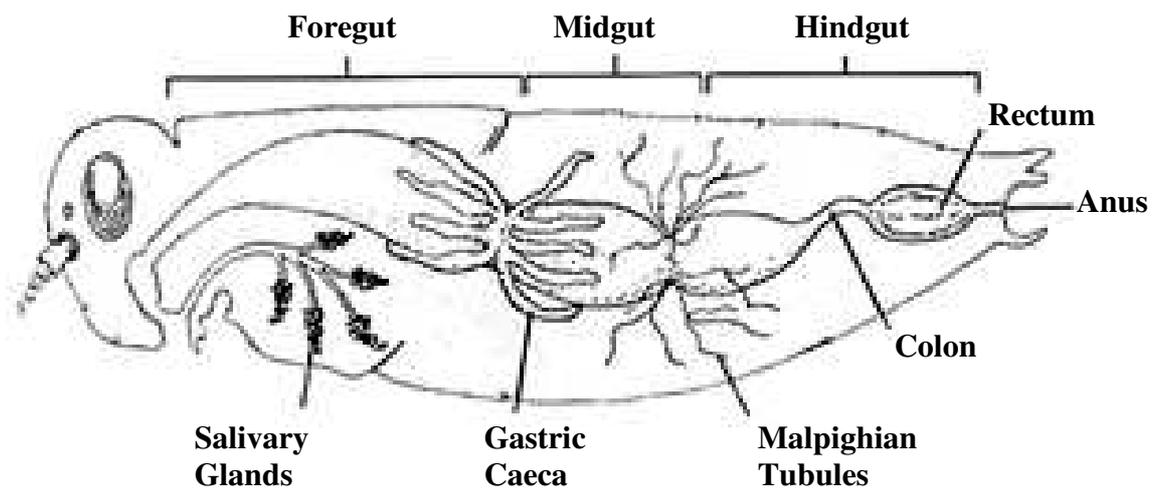


Figure 3.2 Internal anatomies of flies from the Diptera order

The internal anatomy of a typical non-biting fly is illustrated in Figure 3.2 A (www.earthlife.net), whereas a stylised diagram of the SF's internal anatomy is shown in Figure 3.2 B (www.zipcodezoo.com).

3.2 AIMS AND OBJECTIVES

The aim of the study was to investigate the potential role of flies as vectors in transmission of equine sarcoids between infected and susceptible animals.

3.3 MATERIALS AND METHODS

3.3.1 Trap Location

The Donkey Sanctuary, in Sidmouth, Devon, were collaborators for this study. The Donkey Sanctuary is a charitable organisation dedicated to the rescue and care of unwanted, neglected, and donated donkeys. The animals are maintained in dynamic groups and housed at pasture, with access to field shelters, for the summer months of the year. The fly trapping activities took place at their main premises, Slade House Farm. This farm is home to over 200 donkeys within 46 hectares. It houses the donkeys at various locations and there are usually several animals with confirmed sarcoid lesions at any time. Flies were trapped at several locations in the grounds of the Sanctuary – locations were selected on the basis of whether the stables housed sarcoid-affected (fig 3.2 a-d), or sarcoid-free animals. The traps were left in position for up to 2 weeks and then retrieved, individually bagged and sent back to the laboratory for further analysis. Several separate trapping periods took place over the course of the summer season (June, August and September, 2008). At each trapping period, traps were placed and retrieved from the same 6 places;

1. Hospital barn – sarcoid positive donkeys
2. Main office – no sarcoid donkeys within 50 metre radius
3. Buffalo barn – housing Poitou donkeys – one sarcoid positive
4. Garmston – housing one sarcoid positive donkey
5. Visitor barn – no sarcoid positive donkeys within 50 metres
6. Shelter 3 – no sarcoid positive donkeys within 200 metres

3.3.2 Trapping

From the wide range of commercially available fly traps, the adhesive, chemical-free paper-strip type traps were selected (Ref. STV 013, STV 016; dontbeapest.co.uk). These traps had been shown to preserve the insect in a relatively intact condition allowing reasonably easy identification of the species. Flies could be removed from the strips without too much difficulty and any residual glue did not appear to affect the subsequent DNA extraction and analysis. The traps could be positioned away from any possible contact with the donkeys or horses, and provided an attractive vertical surface for stable flies to move to after feeding. In addition to this method of capture, a live capture

technique was employed to catch flies at location, anaesthetise them (by freezing at -20°C) and then perform gross dissection. This involved catching the flies in a standard butterfly net (Ref. 014353; alanaecology.com), on location in two of the animal accommodations where paper traps had been previously sited (hospital barn and shelter 3), transferring them to airtight containers and placing them in -20°C for 30 minutes. Some flies from the live capture sessions were submerged in RNAlater (Qiagen, UK), and stored at 4°C for screening for BPV mRNA back at the Glasgow laboratory.

Figure 3.3

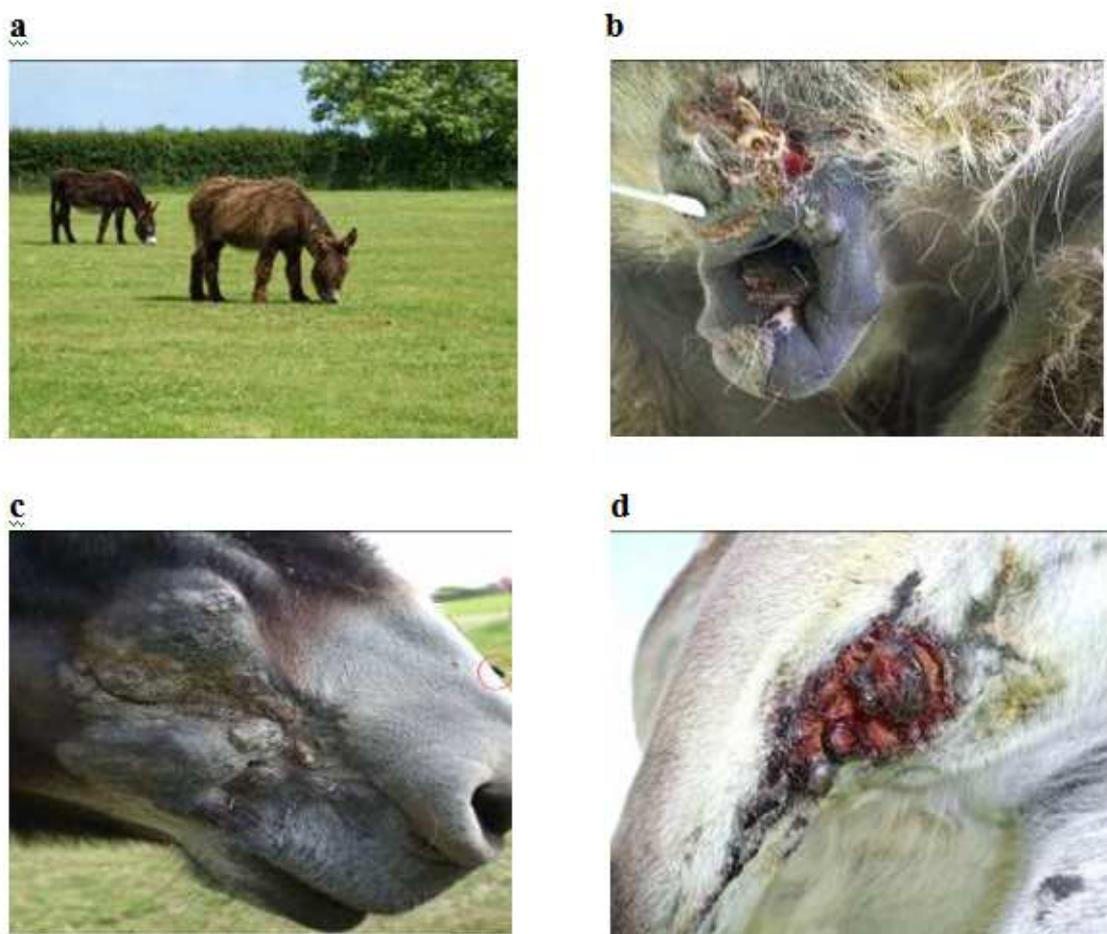


Figure 3.3 Sample collection at the Donkey Sanctuary.

The Donkey Sanctuary, Sidmouth, Devon houses over 200 donkeys, several of which had active sarcoid infections for the duration of the fly trapping period. In a group of Poitou donkeys (Fig 3.3a), there was one individual with a sarcoid tumour on its sheath. A tissue sample was collected from the surface of the tumour using the Isohelix Buccal Swab (Fig 3.3 b). Another donkey, housed in a different pasture, had a group of nodular sarcoids on the muzzle area (Fig 3.3c – house fly circled in red). A third donkey had an ulcerated fibroblastic sarcoid in its right axilla region (Fig 3.3d).

3.3.3 Fly dissection

Following acquisition of required anaesthesia levels, flies were removed from the container, their wings and legs were removed and discarded, and the body immersed in Hanks buffered saline solution (HBSS) before being bluntly dissected into head, thorax and abdomen parts using standard dissection pins. The heads, thoraces and abdomens of each different species were then stored in 100% ethanol prior to transportation back to the laboratory for further analysis.

3.3.4 Swabbing

During initial positioning of the fly traps in June 2008, Isohelix DNA Buccal Swabs from Cell Projects were used to collect surface samples from any sarcoid lesions evident on the animals housed in that location. The swabs were sealed in sterile tubes for transportation back to the laboratory. Sterile nitrile gloves were used when swabbing the surface of the sarcoid lesion, and gloves were changed between each individual donkey from which samples were collected.

3.3.5 DNA Extraction from Flies and Swabs

The flies were carefully removed from the adhesive paper, photographed, provisionally identified and batched together in separate species groups (where sufficient numbers allowed). Flies parts from the dissection study were pooled into 2 sets of 6 different samples; (i) Stable fly heads, (ii) Stable fly thoraces, (iii) Stable fly abdomens, (iv) Lesser house fly heads, (v) Lesser house fly thoraces, (vi) Lesser house fly abdomens. Each 'set' contained the body parts of 5 individual flies. DNA was extracted from the fly samples and swabs using a Qiagen DNeasy Blood and Tissue Kit in accordance with the manufacturer's instructions (Chapter II; section 2.2.2.1.2). The DNA extracts were then subject to PCR using the LCR and E5 primers as described previously in Chapter II; section 2.2.4 and Table 2.1.

3.3.6 Polymerase chain reaction (PCR)

The extracted DNA was first screened for the presence of BPV-1 DNA using a standard PCR protocol with LCR primers LCRfullF and LCRfullR (Table 2.1) used to amplify a target sequence of approximately 800 bp. Products were analysed on a 1% agarose gel containing 2 µg ethidium bromide, and those samples which were deemed to contain sufficient amounts of amplified DNA for sequencing were stored at 4 °C and the other samples subjected to a further round of PCR amplification using a nested PCR protocol with a different set of LCR primers that amplify a slightly smaller fragment of the LCR

gene sequence. The sequences of the primer sets used are listed in Chapter II; Table 2.1.

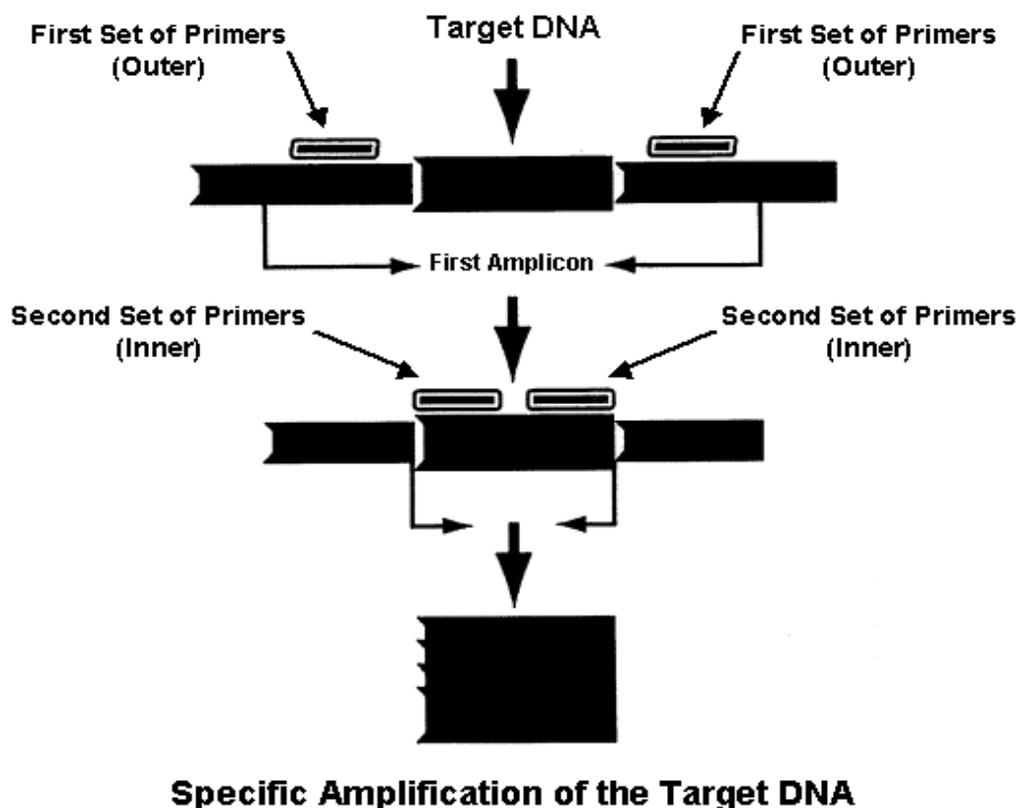


Figure 3.4 Diagrammatic representation of the nested PCR procedure (image sourced from the Institute for Viral Pathogenesis).

The target DNA sequence of one set of primers (termed 'inner' or 'nested' primers) is located within the target sequence of the second set of primers (termed 'outer' primers). Firstly, a standard PCR reaction is run using the 'outer primers'. Then a second PCR reaction is run with the 'inner primers' using the product of the first reaction as the amplification target. This procedure increases the sensitivity of the assay by reamplifying the product of the first reaction in a second reaction. The specificity of the assay is increased because the inner primers will only bind if the first PCR reaction yielded a specific product.

Briefly, 2 microlitres of the PCR product were added into a second master mix containing LCR primers LCRF and LCRRev and designed to amplify a 780bp product. The PCR cycling conditions were the same as those described previously in Chapter II; Section 2.2.4.3, Table 2.3.

After analysing these products on a gel (Fig 4) (Chapter II; section 2.2.4.4), amplified products that were positive for LCR were further processed in preparation for DNA sequencing (Chapter II; section 2.2.6).

To assess the quality of the DNA that was extracted from the fly samples, internal fly primers designed by Simon *et al.* (1994) which target the 16s rRNA gene (415 bp product), were used (Chapter II; Table 2.1). This analysis confirmed that the BPV-1 DNA was present only in samples in which fly DNA was also present. There were also samples which contained good quality fly DNA in the absence of BPV-1 LCR DNA.

3.3.7 DNA sequencing and sequencing analysis

Viral genomic DNA PCR products were purified using a Qiagen mini-prep clean-up kit and subjected to sequence analysis. Sequencing reactions were performed using the Applied Biosystems Big Dye terminal sequencing kit and sequencing performed on an ABI 3100 automated sequencer. Prior to comparative analysis with the ClustalW program provided by EMBNet, the data from all sequences were evaluated using Chromas (Version 1.44) analysis software. All PCR reactions used for sequencing were performed twice in duplicate to eliminate PCR-induced errors or sequencing errors. Full details of the method can be found in Chapter II, section 2.2.6.

3.3.8 Real-time Absolute Quantitative PCR (AQ-PCR) to determine viral load

A real-time Absolute Quantitative RT-PCR was used to assess the viral load per genome equivalent (g.e.) of fly DNA. Serial dilutions of BPV plasmid were used to generate a standard curve and insect actin primer and probe set was used as an internal control for normalisation of input DNA amount. A primer and probe set that amplifies BPV-1/2 E7 gene was used to assess the amount of viral DNA present, and AQ-PCR was performed on 0.1 µg DNA samples on a 7500 Real Time PCR system (Applied Biosystems, Warrington, UK) with an amplification profile of 2 min at 50 °C, 2 min at 95 °C and 45 cycles of 15 s at 95 °C and 45 s at 60 °C. Reactions were performed in duplicate. The primer and probe sequences used are detailed in Chapter II; Table 2. Total weight of input fly DNA was determined using a nanodrop spectrophotometer and genome equivalents of Diptera cells were calculated based on the fact that an average haploid Diptera cell nucleus contains a total DNA mass of 0.89 pg (as recorded by Gregory, T.R. (2005), in the Animal Genome Size Database, www.genomesize.com).

3.3.9 RQ-PCR to compare viral load in different anatomical locations

Real-time Relative Quantitative PCR (RQ-PCR) was performed on 1 µl of each DNA sample using E7 specific primer probe set. An insect specific actin primer and probe set was used as an endogenous control. Details of the primer and probe sequences and a full description of the method used can be found in Chapter II; Table 2.4. PCR reaction mixes contained 0.2 µM forward and reverse primers, 0.1 µM probe, 25 µl of 2× Platinum Quantitative PCR SuperMix-UDG (Invitrogen, UK), 1 µl ROX Reference Dye and water to a final volume of 50 µl. Reactions were performed on a 7500 Real Time PCR System (Applied Biosystems) with an amplification profiles specific of 2 min at 50 °C, 2 min at 95 °C and 45 cycles of 15 s at 95 °C and 45 s at 60 °C. The dissected fly parts (2 sets of 6 distinct anatomical parts) were each analysed in triplicate and the 7500 Real Time PCR System software was used to express the results relative to the viral DNA amounts in the stable fly heads.

3.3.10 RQ-PCR to screen fly samples for presence of viral mRNA

RNA was extracted from fly samples stored in RNAlater and cDNA synthesised as described in Chapter II; section 2.2.3.1.4. RQ-PCR was performed using BPV-1 E6 and E7 primer and probe sets (Table 2.4) on cDNA samples generated using the RT enzyme and on cDNA samples synthesised with no RT enzyme, as a negative control.

3.4 RESULTS

3.4.1 Both biting and non-biting flies are positive for BPV-1 DNA

The number of flies on each trap ranged from 2 or 3 to over a dozen. Traps from the June trapping period yielded the least number of flies, and those from August yielded the most. The predominant species of fly in each batch, was the lesser house fly (*Fannia carnicularis*) or regular house flies (*Musca domestica*). Both these species are non-biting flies, described as having 'sponging' mouthparts. A small number of traps had trapped sufficient numbers of stable flies (*Stomoxys calcitrans*) and these biting flies were processed as a separate batch from the non-biting house fly types. These samples are marked with an asterisk (*) on the results table (Table 3.1). At the first collection flies were collected from 3 locations which each housed one sarcoid affected donkey (sites 1, 3 and 4) and one location where there was no evidence of sarcoid affected animals (site 2).

After extraction, the fly DNA was checked for quality by screening the extracts with insect specific primers (16s RNA). Figure 3.5 shows the agarose gels obtained from running these PCR products.

Figure 3.5.

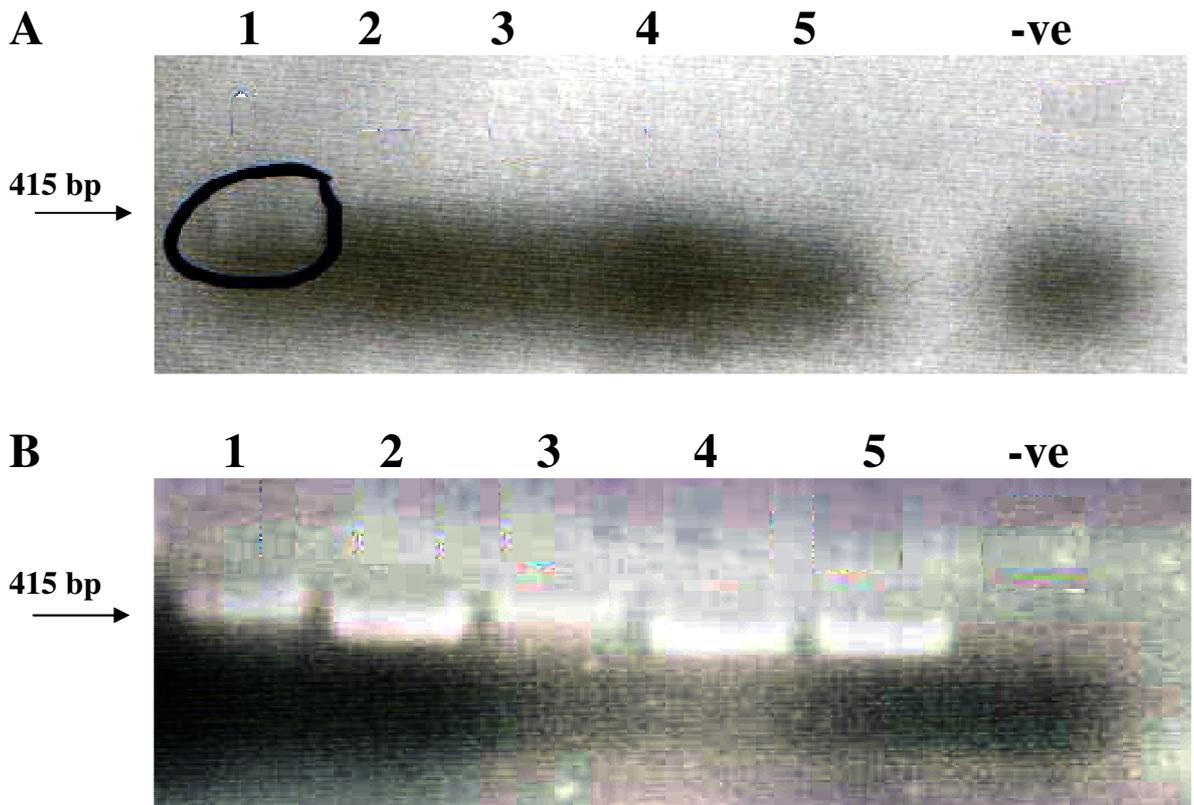


Figure 3.5 PCR amplification of DNA extracted from fly samples (1 – 5) using the internal control primers for the fly (16s RNA gene) and a re-PCR protocol (where the 'inner' and 'outer' primer sets are the same).

A – after the first thermo-cycling profile, only a very faint band is evident in sample one at the correct size (415 bp)

B – 2 μ l aliquots from the amplicons shown in figure A were subjected to a further round of amplification. A positive result was obtained from all the fly DNA samples.

In panel B, the sample in the no-template control column was an aliquot of PCR product from the no-template control sample in panel A – this methodology ensures that any product seen in the re-PCRed samples is NOT due to the contamination of the samples in panel A. These fly DNA extracts were from capture session 1 in June 2008. Although all the samples are positive for the fly internal primer target, only two of the 5 samples (shown here in lane 1 and 2) were positive for BPV-1 DNA when examined using the BPV-1 LCR primer set.

3.4.2 BPV-1 DNA recovered from fly samples contains equine specific LCR variants

Figure 3.6 lists the sarcoid specific LCR sequences variants that were detected in the comprehensive study undertaken by Nasir *et al.*, in 2007 (sequences I, II, III, IV and V) and compares them to a reference BPV-1 LCR sequence obtained from a papilloma that was removed from a Highland cow in Scotland, and the standard (STD) BPV-1 LCR sequence. Sequence data from analysis of the fly-derived BPV DNA was compared to the LCR sequence variants (I – V) and the reference BPV-1 LCR sequence.

As shown in Table 3.1, sarcoid swabs from the three donkeys at sites 1, 3 and 4 were all positive for BPV-1 LCR and sequence analysis revealed that all contained BPV-1 LCR variant IV (Accession No. DQ855068) (Nasir *et al.*, 2007). Analysis of the fly DNA samples showed that BPV-1 DNA could be detected in flies from sites 1 and 2 and that these flies harboured BPV-1 LCR variant IV. No BPV-1 DNA was detected in flies from site 3. At the second collection flies were trapped at 3 of the original locations (sites 1, 3 and 4) and one additional site (site 5). Flies from all 4 sites were positive for BPV-1 DNA and contained BPV-1 LCR variant II (Accession No. DQ855066). At the final collection in September, flies were trapped at all 5 locations; BPV-1 DNA was detected in flies from 4 sites (sites 1, 2, 4 and 5) and all contained BPV-1 LCR variant II. In summary, all fly samples that were positive for BPV-1 LCR DNA contained an equine specific LCR variant – none of the BPV-1 DNA isolated matched the reference or STD BPV-1 LCR sequence.

Figure 3.7 shows the typical results from a BPV-1 LCR screening experiment using a nested PCR protocol that utilised 2 different LCR primer sets.

Figure 3.6

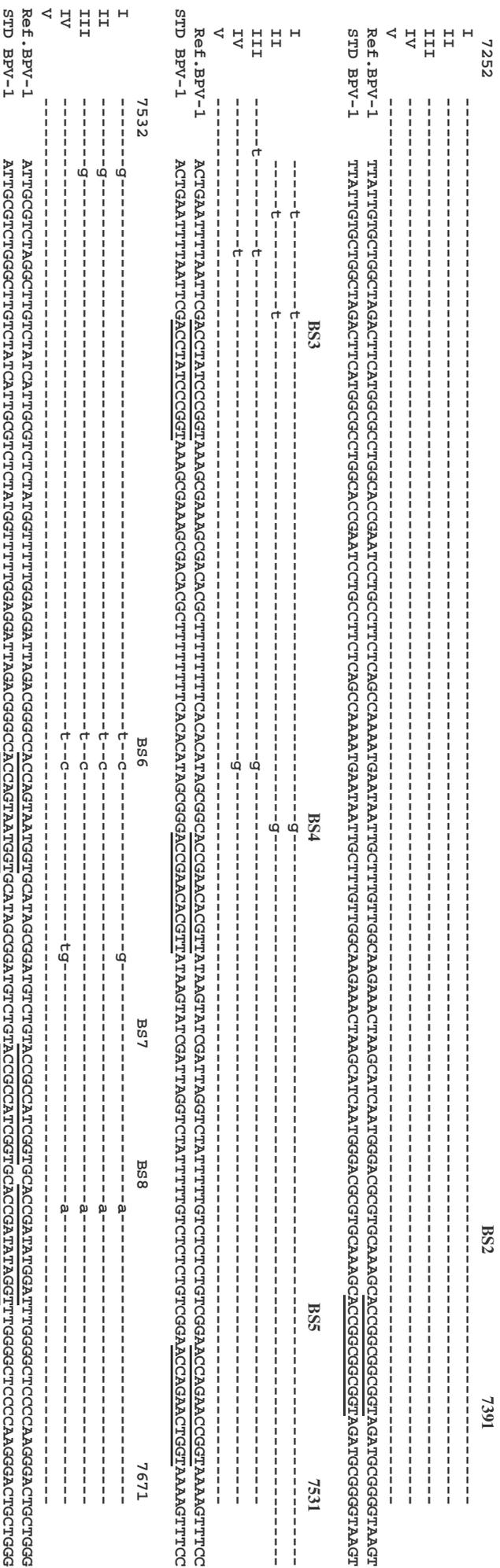


Figure 3.6 cont.

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I      7872      -t-t-      -----      -tc-t-      BS9      BS10      7811
II     -t-t-      -----      -----      -tc-t-      -----
III    -t-t-      -----      -----      -tc-t-      -----
IV     -t-t-      -----      -----      -tc-t-      -----
V      -----      -c-      -----      -g-      -----
Ref. BPV-1  ATGACAGCTCCACATTTATGTTGAATGGGCGCATTAATCACTTAAITGCTGAGACAGCTACAACTGTGTAACCTGATCTCCAAAGTACCGTTGCGGTGGGATCAAAACCGTCTTGGTCTCGAAAACCGCTTAAAC
STD BPV-1   ATGACAGCTTCATATTATATTGAATGGGCGCATTAATCACTTAAITGCTGAGACAGCTACAAAGTGTGTAACCTGATCTCCAAAGTACCGTTGCGGTGGGATCAAAACCGTCTTGGTCTCGAAAACCGCTTAAAC

7812      BS11      7947
I      -----
II     -----
III    -----
IV     -----
V      -----
Ref. BPV-1  TACACACAGGTCCACAGCCAAAGTAGGCGGATCAAAAACCTCAAAAAGCGCGGAGCCAAATCAAAATCCAGCTTTATATTTTAACTCAGCCGAAACCGGTAAAGTAACTATGATTTTTTCCAGTGAATTAATGTTG
STD BPV-1   TACACACAGGTCCACAGCCAAAGTAGGCGGATCAAAAACCTCAAAAAGCGCGGAGCCAAATCAAAATCCAGCTTTATATTTTAACTCAGCCGAAACCGGTAAAGTAAAGACTATGATTTTTTCCAGTGAATTAATGTTG

```

Figure 3.6 Sequence alignment of the five BPV-1 LCR variants (I-V), and the reference (Ref.) and STD BPV-1 LCR sequences.

E2 binding sites (BS) are underlined. Dashes (-) indicate sequence similarity with the reference LCR.

Figure 3.7.

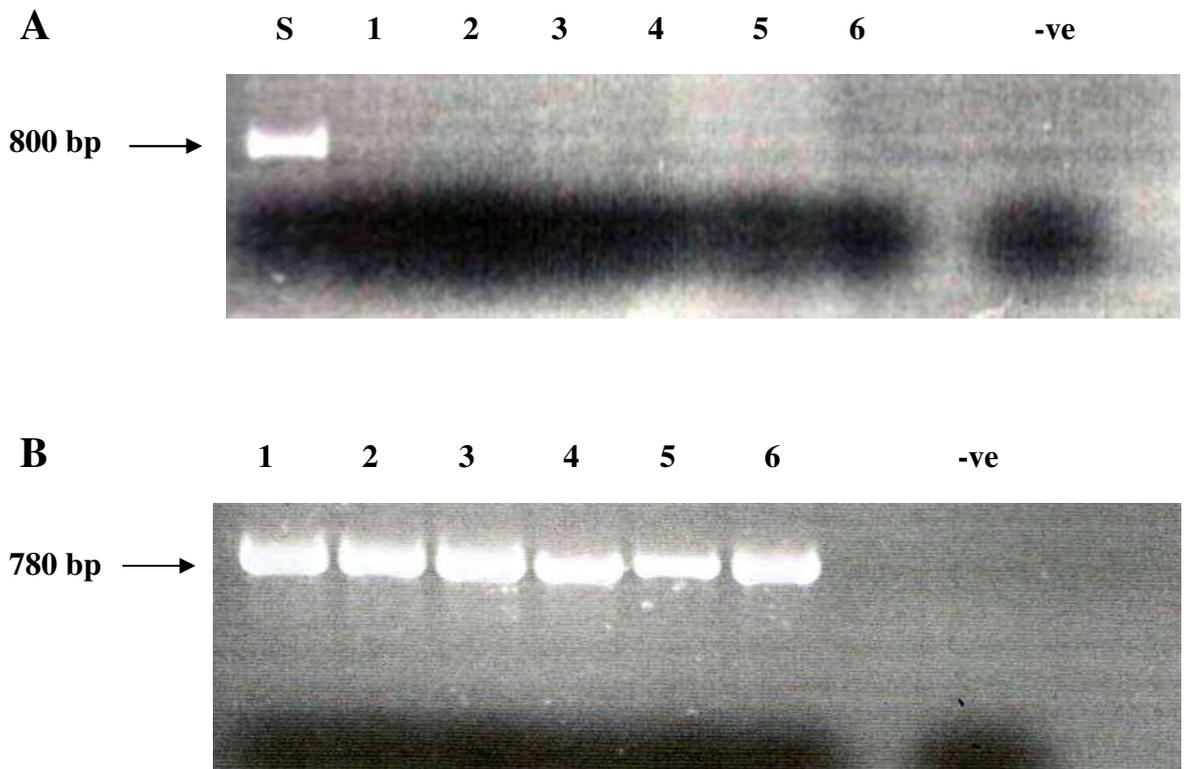


Figure 3.7 Products from PCR amplification of DNA extracted from flies using the BPV-1 LCR primer sets in a re-PCR protocol.

A – first round of PCR amplification using 6 fly DNA samples (1 – 6) and DNA extracted from a sarcoid tumour as a positive control (S). None of the fly samples show any evidence of the presence of BPV-1 DNA, but a positive result from the sarcoid DNA of the correct size (800 bp) shows the profile and primers are working correctly.

B – a second round of PCR was performed on samples 1 – 6 using the nested PCR protocol and the 'inner' LCR primer set. All samples are positive for the presence of BPV-1 DNA demonstrating the presence of a 780 bp product.

-ve = column representing no-template control sample. Sterile, de-ionised water was substituted for DNA. The -ve column in panel B contains 2 µl of the no-template control amplification product shown in panel A. In this way, if environmental contamination had given rise to the bands seen in panel B, these bands would also be seen in the no-template control sample.

Table 3.1 Results of analysis from fly samples collected in the summer fly season, 2008.

Location	Sarcoid animals present at the time of first trapping	BPV-1 in flies (June 08)	LCR variant	BPV-1 in flies (Aug 08)	LCR variant	BPV-1 in flies (Sept 08)	LCR variant
1 Hospital barn	Yes; LCR IV	+ve	LCR IV	+ve *	LCR II	+ve	LCR II
2 Office	No	+ve	LCR IV	-ve	N/A	+ve	LCR II
3 Buffalo	Yes; LCR IV	-ve	N/A	+ve	LCR II	-ve	N/A
4 Garmston	Yes; LCR IV	-ve		+ve	LCR II	+ve	LCR II
5 Visitor barn	N/A	N/A	N/A	+ve	LCR II	+ve *	LCR II

A description of each location is given in the materials and methods section of this chapter. The biting stable fly (*Stomoxys calcitrans*) samples are marked with an asterisk (*).

N/A = not analysed

1. Hospital barn – sarcoid positive donkeys
2. Main office – no sarcoid donkeys within 50 metre radius
3. Buffalo barn – housing Poitou donkeys – one sarcoid positive
4. Garmston – housing one sarcoid positive donkey
5. Visitor barn – no sarcoid positive donkeys within 50 metres
6. Shelter 3 – no sarcoid positive donkeys within 200 metres

3.4.3 Viral load is low in captured flies

Table 3.2 shows the weight of input fly DNA as determined by using a nanodrop spectrophotometer and the standard curve generated from the BPV-1 serial dilutions was used to calculate the number of viral genomes present.

Table 3.2 Analysis of fly DNA samples.

Fly Sample	Sample ID	Avg fly DNA amount ng/ul	Avg Viral Genomes/sample	Standard Deviation in viral genome number
1A	June Site 1	64.15	110	74.3
1B	June Site 2	249.6	334	131.3
2A	Aug Site 1	78.15	210.5	13.2
2B	Aug Site 3	91.25	156.5	15
2C	Aug Site 4	92.6	532	304.5
2D	Aug Site 5	138.05	129.5	28
3A	Sept Site 1	75.45	247.5	23
3B	Sept Site 2	468.55	100.5	51.8
3C	Sept Site 4	52.95	38.5	4.4
3D	Sept Site 5	72.4	83	7.85

This table shows the average amount of fly DNA in each sample that had shown to be positive for BPV DNA, along with the average number of viral genomes present in that sample as deduced from the AQ-PCR results.

BPV-1/2 viral load in Diptera cells was then determined as number of BPV-1/2 copies per genome equivalent (g.e.) of cell. For this, the C-value for Diptera species was considered. The term C-value refers to the amount of DNA contained within a haploid nucleus of a eukaryotic organism. Often, among diploid organisms, the terms C-value and genome size are used interchangeably. Genome equivalents of Diptera cells were calculated based on the fact that an average Diptera cell contains total DNA of the mass 0.89 pg (as recorded in the Animal Genome Size Database, www.genomesize.com). If one fly genome contains 0.89 pg of DNA then 1.1236 fly genomes contain 1 pg and so 1123.6 fly genomes are equivalent to 1 ng of DNA. Using the data in Table 3.2, the viral load was calculated and shown in Table 3.3.

Table 3.3 Calculation of viral load.

Sample I.D.	No. of fly genomes	Avg No. Viral Genomes	Viral load per fly cell	viral load per 1000 fly cells	SD (viral genome)	SD
1A	72078	110	0.00152	1.526	74.3	1.0
1B	280449	334	0.00119	1.190	131.3	0.5
2A	87809	210.5	0.00239	2.397	13.2	0.2
2B	102528	156.5	0.00152	1.526	15	0.1
2C	104044	532	0.00511	5.113	304.5	2.9
2D	155112	129.5	0.00083	0.834	28	0.2
3A	84775	247.5	0.00291	2.919	23	0.3
3B	526460	100.5	0.00019	0.190	51.8	0.1
3C	59494	38.5	0.00067	0.647	4.4	0.1
3D	81348	83	0.00102	1.020	7.85	0.1

Viral load was subsequently calculated and estimates of viral genome equivalent (g.e.) per 1000 diploid fly cells demonstrate that flies harbour low viral loads. SD – standard deviation.

3.4.4 The whole BPV-1 genome could not be detected as a circular genome in the DNA extracted from the captured flies

Attempts to amplify the entire BPV-1 genome using RCA techniques failed to demonstrate the presence of circular BPV-1 DNA in any of the fly samples (Fig 3.8). Using the standard protocol it was possible to obtain a positive result from the positive control supplied with the kit, and also using DNA extracted from EqS04b cells (a sarcoid derived cell line) using a protocol optimised previously in the lab by Yuan et al. 2007.

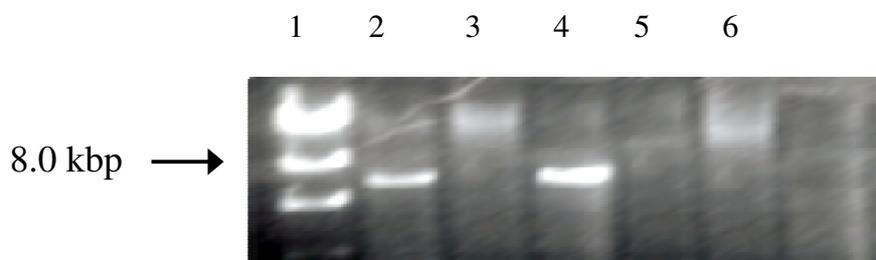


Figure 3.8 Results from rolling circle amplification (RCA) performed on fly DNA samples.

1 – positive control, 2 – water (negative control), 3 – EqS04b DNA (an additional positive control), 4, 5 and 6 – fly DNA samples.

Additional attempts were made to further optimise the RCA protocol in the hope that the right balance of input material from the fly samples could be determined and included consulting the TempliPhi manufacturer's online Troubleshooting resource. Due to the fact that the kit and protocol had been shown to be in working order and additional RCA screenings failed to demonstrate the presence of circular DNA in any of the fly samples, other options for establishing the presence of an entire BPV-1 genome were evaluated.

Full length BPV-1 primers and 'back to back' BPV-1 primers, which have previously been shown to amplify the whole BPV-1 genome (Hatama *et al.*, 2008; Brandt *et al.*, 2008) were used with Platinum Taq (Invitrogen, UK) without success. These back to back primers were also used with the Expand High Fidelity Plus system (Invitrogen, UK) with a variety of amplification profiles and MgCl₂ concentrations. This included the implementation of a touchdown thermal profile. Touchdown polymerase chain reaction or touchdown style polymerase chain reaction is a method of polymerase chain reaction by which primers will avoid amplifying nonspecific sequences. The annealing temperature during a polymerase chain reaction determines the specificity of primer annealing. The melting point of the primer sets the upper limit on annealing temperature. At temperatures just below this point, only very specific base pairing between the primer and the template will occur. At lower temperatures, the primers bind less specifically. The earliest steps of a touchdown polymerase chain reaction cycle have high annealing temperatures. The annealing temperature is decreased in increments for every subsequent set of cycles (the number of individual cycles and increments of temperature decrease is chosen by the experimenter). The primer will anneal at the highest temperature which is least-permissive of nonspecific binding that it is able to tolerate. Thus, the first sequence amplified is the one between the regions of greatest primer specificity; it is most likely that this is the sequence of interest. These fragments will be further amplified during subsequent rounds at lower temperatures, and will out-compete the nonspecific sequences to which the primers may bind at those lower temperatures. If the primer initially (during the higher-temperature phases) binds to the sequence of interest, subsequent rounds of polymerase chain reaction can be performed upon the product to further amplify those fragments. Unfortunately, none of the methods outlined above produced evidence of the presence of an entire, intact, BPV-1 genome.

Figure 3.9 shows the results of screening the fly DNA samples with the BPV-1 E5 primer set (Chapter II; Table 2.1)

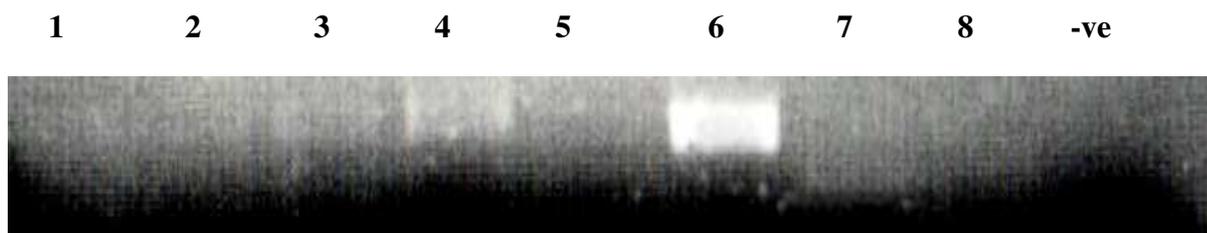


Figure 3.9 Re-PCR technique using BPV-1 E5 primers on DNA samples extracted from flies.

Despite an inability to detect the BPV-1 genome as an entire structure, standard PCR screening techniques revealed BPV-1 E5 was present in some of the fly samples.

3.4.5 All dissected fly body parts (heads, thoraces and abdomens) were positive for presence of BPV-1 DNA

The two sets of data obtained from the RQ-PCR results were combined (Table 3.4). All anatomical section samples were positive for BPV E7 and the relative amounts in each body section (head vs. thorax vs. abdomen), are shown in Figure 3.10.

Table 3.4 Results from BPV-1 screening of the grossly dissected fly body parts.

Anatomical Section	RQ value A	RQ value B	Avg RQ value	SD
SFH	1	1	1	0
SFT	2.11	3.9	3.005	1.265721
SFA	0.712	7.3	4.006	4.658419
LHFH	5.9	11	8.45	3.606245
LHFT	2	1.56	1.78	0.311127
LHFA	3.3	2.17	2.735	0.799031

All anatomical section samples were positive for BPV E7 DNA and the relative amounts present were expressed in relation to the amount present in the stable fly head samples.

- SFH – stable fly heads;
- SFT – stable fly thoraces;
- SFA – stable fly abdomens;
- LHFH – lesser house fly heads;
- LHFT – lesser house fly thoraces;
- LHFA – lesser house fly abdomens;
- SD – standard deviation.

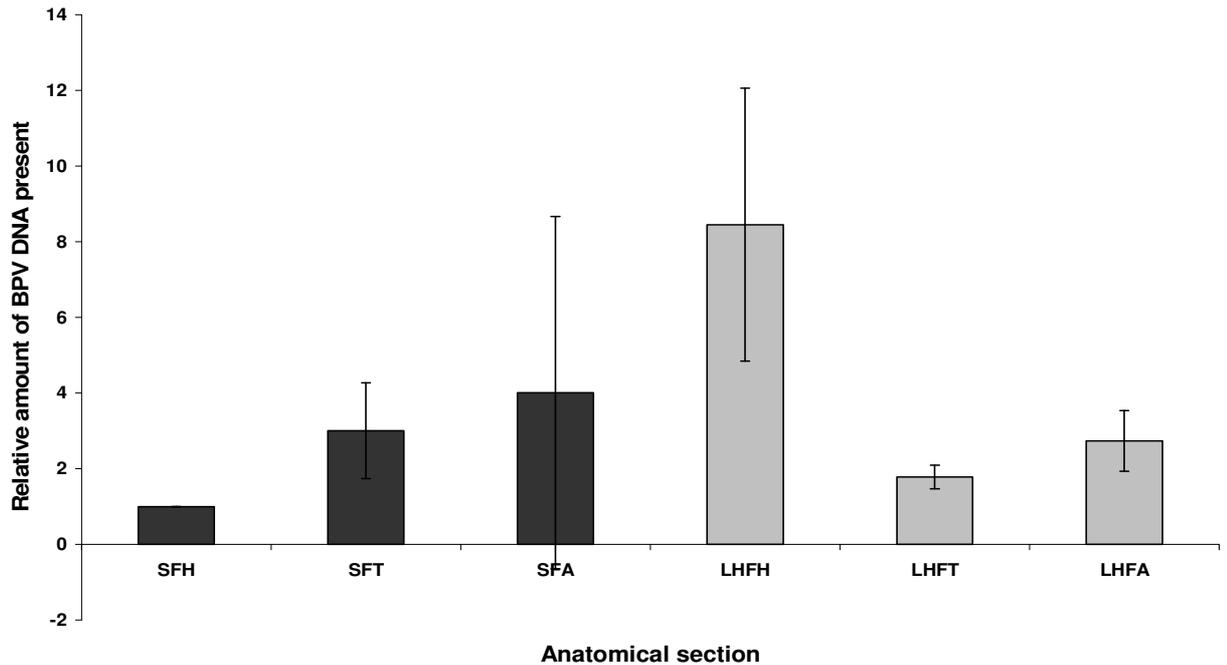


Figure 3.10 RQ-PCR analysis of the different anatomical sections for quantity of BPV-1 DNA present.

The amount of BPV-1 DNA present in each fly body section is shown relative to the amount detected in the stable fly head (SFH) sections. Values were calculated from RQ-PCR experiments using the BPV-1 E7 primer and probe set and the fly actin gene as an internal control. SFT – stable fly thoraces; SFA – stable fly abdomens; LHFH – lesser house fly heads; LHFT – lesser house fly thoraces; LHFA – lesser house fly abdomens. Lesser house flies, with their sponging mouthparts, had over an 8 fold increase in the amount of viral DNA present. In the biting stable flies, a relatively higher proportion of viral DNA was detected in the thoraces and abdomens, whereas in the lesser house flies, there was comparatively less in these body sections.

3.4.6 BPV-1 mRNA could not be detected in any fly samples

In the flies that had been stored in RNA later, viral mRNA could not be detected after screening using standard PCR, nested PCR, or the more sensitive RQ-PCR methods. After the initial extraction of RNA and generation of cDNA, PCR performed on the samples containing reverse transcription enzyme (RT) demonstrated bands of equal intensity to those seen from the samples to which no RT was added. This result suggested there was DNA contamination of the cDNA. All samples were subjected to another DNase I digest and the PCR was repeated. The results obtained were the same – both the RT positive and

the RT negative samples showed evidence of PCR amplification (LCR primers were used). A new DNase I kit was ordered and the methodology repeated, again with the same results.

At this stage, it was decided to analyse the cDNA samples in an RQ-PCR experiment. Because it seemed that there was a low level of DNA contamination that could not be removed with sequential DNase I treatments, using an RQ-PCR technique would enable a comparison of the relative amounts of BPV-1 DNA present in each sample. The theory being that samples with RT enzyme added would have both the cDNA and the contaminating DNA, whereas the RT negative samples would only have the contaminating DNA present. And so, there should be a detectable difference in the amounts of BPV-1 E7 present as assessed by RQ-PCR. The results of the RQ-PCR assessment showed there was no difference between the RT positive and RT negative samples, making it very unlikely that there was any viable RNA in the fly samples. This is perhaps not an entirely surprising result, as RNA is notoriously vulnerable to rapid degradation. Future work will require samples to be processed immediately, or for micro dissection to be performed on the specimens, prior to storage in a solution such as RNA later.

3.5 DISCUSSION

Although a causal relationship between BPV-1 and equine sarcoids is well established, how equidae become infected with BPV-1 remains unknown. The transmission between animals has been proposed to be by direct or indirect contact, however both epidemiological data and development of sarcoids in animals in the absence of contact with horses (or cattle) has led to the proposition that flies may act as vectors of disease transmission (Torreontegui and Reid 1994, Reid *et al.*, 1994, Knottenbelt 1995, Knottenbelt and Pascoe 1994). The potential role of insects in the spread of some viral diseases to and between humans and domestic animals has been confirmed in many studies (Dalmat, H.T. 1957; Issel and Foil, 1984; Mellor *et al.*, 1987; Tarry *et al.*, 1991; McColl *et al.*, 2002; Mead *et al.*, 2009). The house fly, *Musca domestica*, and the stable fly *Stomoxys calcitrans* have long been considered as potential agents for disease transmission, and pathogens have been isolated from faeces, vomitus, external surfaces, and internal organs of these species.

We have shown that all body parts of the fly tested positive for the presence of BPV-1 DNA. From the blunt dissection work in stable flies, there was found to be more viral DNA present in the thorax and abdomen sections compared to the amount detected in the head when analysed by RT-QPCR. According to the anatomy of this species, this may correlate to the salivary glands which extend through the thorax and in to the abdomen. Conversely, the lesser house fly was found to have comparatively more BPV-1 DNA present in the head sections. Because this species has large sponging mouth parts, and well developed salivary glands in both the head and thorax, this correlates with the hypothesis that the virus may accumulate in the salivary glands of both species. However, it could also be argued that due to the fact BPV-1 DNA could be detected throughout the entire body of the fly, our results demonstrate nothing more than ingestion of the viral DNA by the fly during feeding, and potentially, subsequent degradation. More precise dissection techniques would be required to confirm this hypothesis absolutely.

In this study the most commonly detected BPV-1 LCR variant in the fly samples, variant II, is the most commonly detected variant in equine sarcoids, at least in the UK accounting for approx. 66% of all sarcoid infections. Variant II is rare in cattle BPV-1 infections (Nasir *et al.*, 2007) suggesting that flies may act as vectors for BPV-1 transmission amongst equids. At the first trapping (Table 3.1), LCR variant IV was detected in fly samples. Whilst LCR variant IV is common in cattle BPV-1 infections (Nasir *et al.*, 2007),

the sarcoid tumour at the site of fly trapping also contained variant IV (Nasir *et al.*, 2007) which could account for the presence of variant IV in the flies.

While we were unable to detect BPV mRNA in the flies caught in the live capture session, it would be difficult to dismiss the possibility that BPV replication occurs in at least one of the species of flies where BPV DNA has been shown to be present. As demonstrated by the fact that BPV DNA can be detected in all body parts of dissected flies, it seems likely that some virus is internalised in the fly, and may be present in cell types permissive to virus replication. Any viral mRNA is likely to be degraded rapidly once the fly has been killed, and due to the likelihood of an internalised location, would not be preserved by immersing the fly sections in RNAlater after dissection.

Attempts to amplify the entire BPV-1 genome using RCA techniques failed. It is likely that because the viral DNA was present in such small quantities, and in such a comparatively large amount of bulky genomic DNA molecule, conditions were unsuitable for amplification of the entire circular genome. Because we managed to identify LCR DNA (Fig. 3.7.) and E5 DNA (Fig. 3.9.) via nested PCR techniques, and we were also able to detect BPV E6 and BPV E7 using RQ-PCR and AQ-PCR, we are confident that the entire viral genome was present, and, for a large part, undegraded.

The data presented in this chapter supports the hypothesis that fly species which commonly feed from the tissues around sarcoid lesions (including *M. domestica* and *S. calcitrans*) may transmit viable BPV material between infected and uninfected animals. However, the data by no means provides irrefutable evidence that flies are indeed viral vectors in equine sarcoid transmission. This study indicates that flies can harbour BPV-1 DNA, and our variant analysis implies that the common variants detected in sarcoids are the same as those we detect in flies. This, together with the distribution of lesions and the epidemiology of sarcoids, strongly suggest that flies are significant. But the ability of the fly to carry viable viral material from a sarcoid-affected animal to vulnerable areas of skin on healthy equidae has yet to be demonstrated, and how the fly and the virus are linked is another matter yet to be established.

It is worth bearing in mind that the viral load in equine sarcoid lesions has been shown to be incredibly low (Yuan *et al.*, 2007; Boegart *et al.*, 2007), often just a few viral copies per cell. The viral load analysis we carried out showed an even lower viral load in the insect cells. Before the role of these insects as vectors of disease can be confirmed, work needs to be done to establish what the infectious dose is for the development of a sarcoid lesion on a

susceptible animal. Perhaps the virus accumulates in or on the fly. Or perhaps it concentrates at certain anatomical sites within the fly allowing transference of sufficient quantities of viral material on to or in to the skin of uninfected, susceptible animals. Alternatively, the accumulation of viral material could occur on the skin of the animals on which the fly lands to feed.

Further studies are also required to establish whether these flies excrete the virus in vomitus or faeces, and if so, is the virus present in larger quantities/higher concentrations in these substances? It should also be possible to establish whether flies are purely mechanical vectors or whether the virus is active in particular insect cells. This would require micro dissection experiments to establish the existence, or otherwise, of BPV mRNA.

Additional evidence to determine the link between virus and fly and sarcoid transmission could be supplied by designing and implementing controlled transmission experiments where pathogen free insects were allowed to feed on sarcoid tissue for a certain amount of time before being incubated with a susceptible, uninfected animal. Such experiments are probably the only way to prove conclusively, that flies such as *M. domestica* and *S. calcitrans* can indeed transmit the disease from an infected animal, to an uninfected one.

4 CHAPTER IV:

Evaluation of apoptosis and cell survival in BPV-1 transformed equine fibroblasts

4.1 INTRODUCTION

Apoptosis, or programmed cell death (PCD), is a genetically programmed process of cellular destruction that is essential for normal development and homeostasis of multicellular organisms.

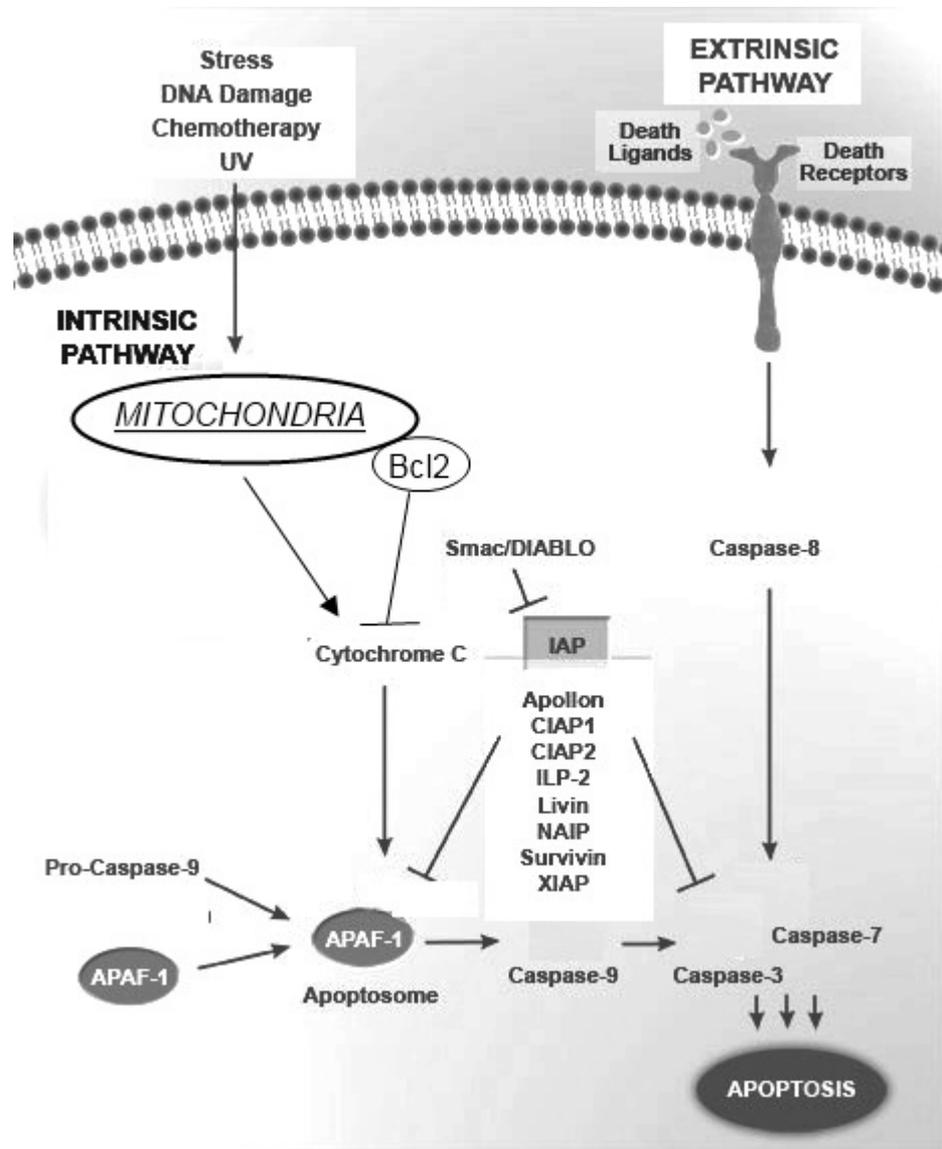


Figure 4.1 Overview of the 2 main apoptosis pathways – the INTRINSIC, and the EXTRINSIC (modified from source: www.imgenex.com).

Apoptosis can be initiated by internal events which lead to activation of the ‘Intrinsic Pathway’. This involves the disruption of mitochondria and the release of the enzyme cytochrome C, in turn leading to the downstream activation of caspases. Alternatively, cell surface receptors can be activated by specific ligands that bind to ‘death receptors’ and thus leading to activation of the ‘Extrinsic Pathway’. Death receptors are members of the tumour necrosis factor (TNF)/nerve growth factor (NGF) receptor superfamily. They make

up a subfamily characterized by the intracellular death domain (DD). The extrinsic pathway is typically mediated by immune cells, to initiate intracellular signaling and the downstream activation of relevant caspases. As illustrated in Figure 4.1, the apoptosis pathways are highly complex and there are molecules common to both the intrinsic and extrinsic pathways. In addition to pro-apoptotic proteins such as Bcl-2 and APAF-1, there are a family of proteins known as the Inhibitor of Apoptosis Proteins or IAP (lower central box, Fig. 4.1) that can inhibit both the intrinsic and extrinsic pathways once they have been activated.

Apoptosis is a term that only describes the morphological changes a cell undergoes during this mode of cell death (Kerr *et al.*, 1972). Specifically, the cell draws inward shrinking dramatically, the plasma membrane 'blebs', the endoplasmic reticulum forms vacuoles, and the chromatin is digested and condenses along the nuclear membrane, often forming spheres and/or crescent shapes (Strasser *et al.*, 2000). In contrast, necrosis, another form of programmed cell death, has distinct morphological features. During necrosis, cells first swell, then the plasma membrane collapses and cells are rapidly lysed. Biochemical hallmarks of apoptosis such as caspase activation and DNA fragmentation are usually absent in necrotic cells (Proskuryakov *et al.*, 2003).

The terms 'immediate', 'intermediate', and 'delayed' apoptosis separate the different apoptotic mechanisms into three kinetic categories, whereas the terms pre-programmed cell death (pre-PCD) and programmed cell death (PCD) describe the underlying mechanisms. Immediate apoptosis ($T < 0.5$ h post-exposure) is triggered by singlet-oxygen damage that opens the mitochondrial megachannel (Ball *et al.*, 1998), which can be mediated by photo-dynamic therapy (PDT) or ultra violet A1 (UVA1) radiation. It is a pre-PCD mechanism of apoptosis, i.e., protein synthesis is not required post-insult, because all the necessary components are constitutively synthesized and only need to be activated. Intermediate apoptosis ($0.5 \text{ h} > T < 4 \text{ h}$) is initiated by receptor cross-linking on the plasma membrane (Wang *et al.*, 1997), which can be achieved using high doses of UVB or ultra violet C (UVC) radiation. It is also a pre-PCD mechanism. Delayed apoptosis ($T > 4 \text{ h}$) is induced by DNA damage that can be caused by X-rays, UVC, UVB, UVA, and PDT. It is a PCD mechanism of apoptosis, i.e., protein synthesis is required post-insult (Godar 1996). These three apoptotic mechanisms have shared and overlapping signalling pathways and cells dying by different forms may display varying degree and combination of features (Jaattela 2004).

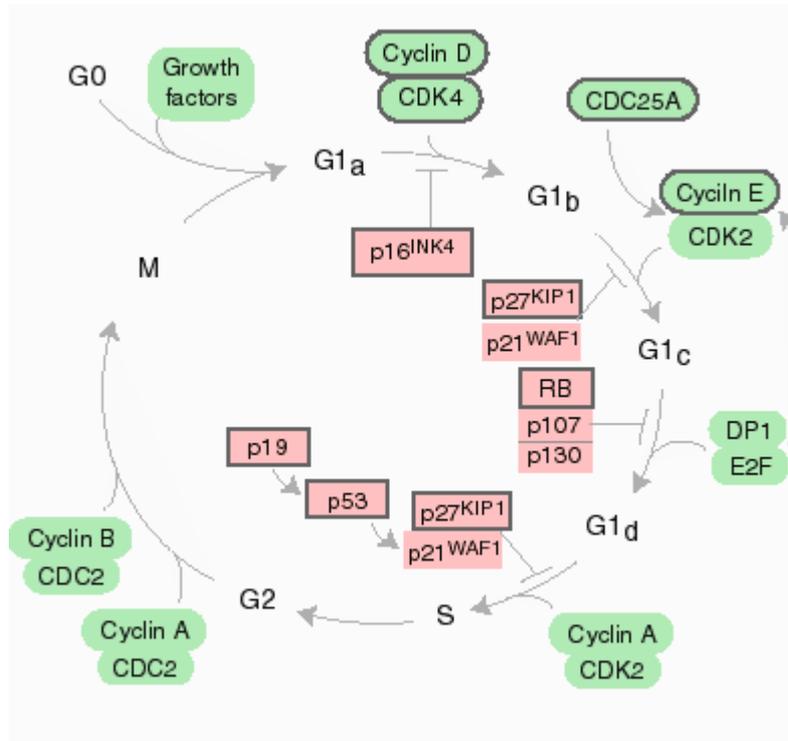


Figure 4.2 Regulation of the Cell Cycle (modified from Fred Hutchinson Cancer Research Centre website www.fhcrc.org)

Green = Positive Regulators

Pink = Negative Regulators

Dark Borders = Proteins with dark grey borders have been shown to be defective in some cancer cell lines.

Disruption of cell cycle control mechanisms is a common biological feature in human cancers, however its occurrence in sarcoid pathology remains poorly investigated. Progression through the cell cycle is mediated by protein complexes composed of cyclins, cyclin dependent kinases (CDKs) and cyclin dependent kinase inhibitors (CDKIs). The CDKIs act as inhibitors of the cell cycle and hence function as tumour suppressors, where loss of function can lead to cellular transformation (Hartwell and Kastan, 1994 and Swanton 2004). The low levels of expression of cell cycle regulatory proteins in equine sarcoids (cyclin A, CDK-2, p27^{kip1} and p53; Nixon *et al.*, 2003) suggest that sarcoid molecular pathology is generally not associated with abnormal cell cycle control mechanisms. This implies that sarcoid lesions may in fact be associated with abnormalities in apoptotic pathways, where the balance of cell growth and cell death is perturbed. Many viruses have evolved specific gene products to modulate the apoptotic process. For example, in adenoviruses several viral proteins act to regulate apoptosis in infected cells.

The adenovirus E1A protein increases cellular levels of p53 and induces p53-dependent apoptosis. This apoptosis is counteracted by the adenovirus E1B 19 kDa (inhibits the downstream pro-apoptotic actions of p53) and E1B 55 kDa proteins (interacts directly with p53 and mediates its inactivation, translocation to the cytoplasm, ubiquitination, and degradation) (McNees and Gooding, 2002).

4.1.1 BPV-1 gene products and apoptosis

Like all death-domain-containing members of the Tumour Necrosis Factor receptor (TNF-R) superfamily, TNF-R1 is involved in death signalling. Tumour Necrosis Factor (TNF) is a 17-kDa inflammatory cytokine that mediates a variety of immune and inflammatory reactions (Tracey and Cerami, 1993). Some tumour cell lines are sensitive to TNF, but most are resistant (Carswell *et al.*, 1975; Sugarman *et al.*, 1985). In 1999, it was demonstrated that BPV-1 E6 and E7 genes independently sensitise cells to TNF-induced apoptosis. BPV-1 E6 induced apoptosis independently of p53 and E7 induced apoptosis independently of the retinoblastoma protein (pRb), a tumor suppressor protein that is dysfunctional in many types of cancer (Rapp *et al.*, 1999; Liu *et al.*, 2000).

In Fas-mediated apoptosis, in contrast to TNF-mediated apoptosis, E6 and E7 demonstrated opposite effects: while E7 potentiated apoptosis triggered by an agonistic Fas antibody, E6 attenuated the effect. The Fas (CD95/APO-1) receptor is a member of the Tumour Necrosis Factor receptor (TNF-R)/ nerve growth factor receptor superfamily. Fas is expressed on the cell surface and transduces apoptotic signals upon cross-linking with the Fas ligand (FasL). Since BPV-1 exerts contrasting effects on TNF- and Fas- mediated apoptosis, E6 may target distinct components of these pathways, the most upstream of which are the death receptors Fas and TNF-R. Liu *et al.*, in 2005 used flow cytometry to show that surface Fas expression was greatly reduced in E6 expressing mouse cells, compared to E7 expressing mouse cells. The reduced Fas protein level on the cell surface is not accompanied by a decrease in total Fas levels in E6-expressing cells. Instead, considerably more Fas protein is found in the cytoplasm of cells expressing E6 (Liu *et al.*, 2005).

The papillomavirus E2 protein plays a central role in the viral life cycle as it regulates both transcription and replication of the viral genome. In 1997, a research group in France showed that transient expression of BPV-1 E2 in HeLa induced apoptosis and that the effects of E2 on cell cycle progression and cell death follow distinct pathways involving two different functions of p53 (Desaintes *et al.*, 1997).

4.1.2 Ultra Violet-B (UVB) irradiation and DNA damage

UVB-mediated apoptosis is a highly complex process in which a variety of signaling pathways are involved. Following UVB irradiation, apoptosis can be initiated through DNA damage response pathways, generation of reactive oxygen species, CD95/Fas receptor signalling, TNF-R1 signalling and TRAIL receptor signalling (Chow and Tron, 2006). Upon absorption, UVB primarily induces two types of DNA lesions, cyclobutane pyrimidine dimers and 6–4 photoproducts (Patrick, 1977). Under normal conditions these photolesions are removed by the nucleotide excision repair (NER). UVB can also affect molecular targets located in the cytoplasm or at the cell membrane; a direct and ligand-independent activation of membrane-bound cell death receptors has been demonstrated (Kulms and Schwarz, 2002). Death receptors are members of the tumor necrosis factor (TNF) receptor superfamily (Ashkenazi and Dixit, 1998). Besides the TNF receptor-1, the TNF α -related apoptosis inducing ligand (TRAIL) receptors and death receptor-3 (DR-3), CD95 (Fas, APO-1) represents one of the most potent mediators of programmed cell death. Activation of these pathways results in triggering of a cascade of cystein proteases, called caspases, the executioners of apoptosis (Peter and Kramer, 1998). The range of exposure levels of UVB for inducing apoptosis (0, 250 and 500 mJ/cm) used in this chapter, are considered 'high' levels of exposure, consistently resulting in apoptosis in treated cells. This is in contrast to the exposure level used to assess p53 function (30 mJ/cm) which is considered low, and therefore more likely to result in activation of the DNA repair response, than in cell apoptosis (Cotton and Spandau, 1997).

4.1.3 Chemotherapeutic agents and DNA damage

Cisplatin and its analogs are heavy metal complexes containing a central atom of platinum surrounded by two chloride atoms and two ammonia molecules in the 'cis' position. The current accepted theory about cisplatin mechanism of action is that the drug induces its cytotoxic properties through binding to nuclear DNA and subsequent interference with normal transcription, and/or DNA replication mechanisms. Cisplatin has biochemical properties similar to that of bifunctional alkylating agents, producing interstrand, intrastrand and monofunctional adduct cross-linking in DNA. However, there is now much evidence suggesting that the cytotoxic effects induced by binding of cisplatin to non-DNA targets (especially proteins) may contribute to its biochemical mechanism of action (Fuertesa *et al.*, 2007). Several studies have shown cisplatin to be a useful chemotherapeutic option in the treatment of equine sarcoids with one particular study

observing complete regression in 78% of the tumours. It was also stated that no systemic side-effects were encountered (Spoormakers *et al.*, 2002).

As a pyrimidine analogue, 5-fluorouracil (5-FU) is transformed inside the cell into different cytotoxic metabolites which are then incorporated into DNA and RNA. 5-FU also inhibits the nucleotide synthetic enzyme thymidylate synthase (TS), and finally induces cell cycle arrest and apoptosis by inhibiting the cell's ability to synthesize DNA. It is an S-phase specific drug and only active during certain cell cycles. In addition to being incorporated in DNA and RNA, the drug has been shown to inhibit the activity of the exosome complex, an exoribonuclease complex of which the activity is essential for cell survival. It has been shown that the use of intratumoural 5-fluorouracil compares favourably with other treatment modalities for sarcoids, with a long term successful resolution rate of 61.5%. However the study recommended that owners should be warned that resistant sarcoids and sarcoids larger than 13.5 cm³ have a poorer prognosis for resolution and more aggressive therapeutic options should be considered (Stewart *et al.*, 2006).

4.1.4 Small Interfering RNA (siRNA) technology

Small interfering RNA (siRNA), also known as short interfering RNA or silencing RNA, is a class of 20-25 nucleotide-long, double-stranded RNA molecules that play a variety of roles in biology. Most conspicuously, siRNA is involved in the RNA interference (RNAi) pathway, where it interferes with the expression of a specific gene. RNAi can trigger degradation or translation repression of homologous RNA targets in a sequence-specific manner. At least three distinct roles of RNAi have been identified;

- i) antiviral response (Mourrain *et al.*, 2000),
- ii) protection of the genome (Grishok *et al.*, 2000),
- iii) and regulation of development (Bohmert *et al.*, 1998)

The complexity of these pathways is only now being elucidated.

Figure 4.3. illustrates the mechanism of action of RNAi. Since the only RNA found in a cell should be single stranded, the presence of double stranded RNA signals is an abnormality. The cell has a specific enzyme (Dicer) that recognizes the double stranded RNA and chops it up into small fragments between 21-25 base pairs in length. These short RNA fragments (called small interfering RNA, or siRNA) bind to the RNA-induced

silencing complex (RISC). The RISC is activated when the siRNA unwinds and the activated complex binds to the corresponding mRNA using the antisense RNA. The RISC contains an enzyme to cleave the bound mRNA (Slicer) and therefore cause gene suppression. Once the mRNA has been cleaved, it can no longer be translated into functional protein.

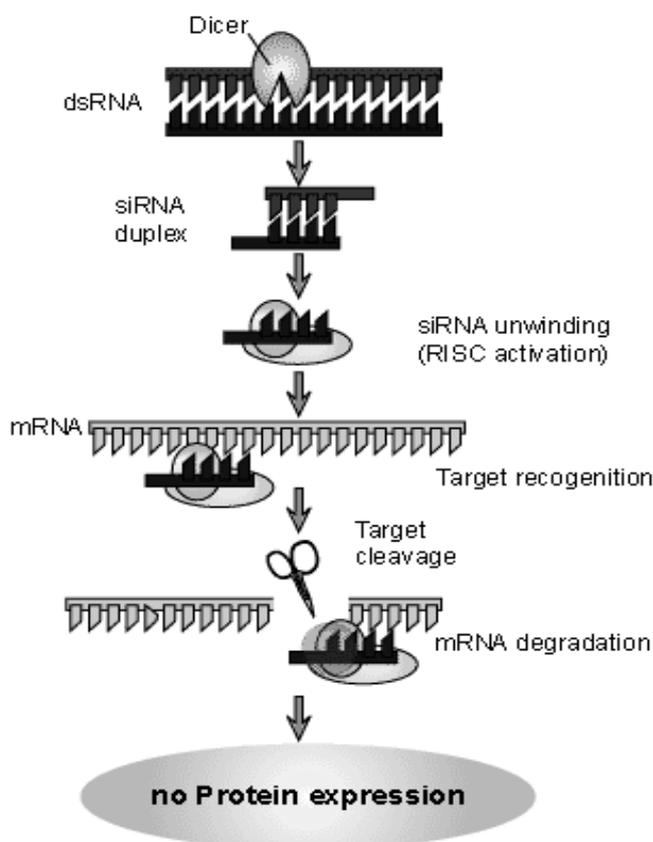


Figure 4.3 Mechanism of action of RNAi. Image sourced from The Science Creative Quaterly, Issue 4, 2009.

To date, RNAi has been extensively used as a novel and effective tool for functional genomic studies, and has exhibited great potential in treating human diseases, including human genetic and acquired disorders such as cancer and viral infections. Numerous in vitro and animal studies have provided proof-of-concept; major viral pathogens including HIV-1, respiratory viruses such as influenza A, respiratory syncytial virus (RSV), and hepatitis viruses, have been shown to be susceptible to siRNA treatment (van Rij and Andino, 2006). Indeed, several RNAi-based antiviral drugs are currently being tested in clinical trials (Hasnoot *et al.*, 2007).

In principle, RNA interference (RNAi) is a straightforward method for inducing sequence-specific silencing of one or more genes of interest with the simple introduction of small interfering RNAs. In practice, there are fundamental challenges to be considered in order

to achieve successful gene silencing. Major hurdles to therapeutic applications of RNAi include the delivery of the siRNA into the target cell and across cellular membranes into the relevant cell types, the bioavailability and stability of siRNAs, and the prevention of off-target effects and toxicity. The introduction of excessive concentrations of siRNA can result in nonspecific events due to activation of innate immune responses. Evidence to date suggests that this immune system response is most likely due to activation of the dsRNA sensor PKR, although retinoic acid inducible gene I (RIG-I) may also be involved (Sledz *et al.*, 2003; Sledz and Williams 2004). Off-targeting arises as a result of genes with incomplete complementarity being inadvertently downregulated by the siRNA. This can lead to problems with data interpretation and toxicity. Consequently, siRNA design and production is of fundamental importance, as not all siRNAs directed against a given target silence with equivalent efficiencies. A scrambled siRNA control (the same nucleotide composition as the gene-specific siRNA, but lacking significant sequence homology to the genome) is used to discount any changes to the gene expression profile that may result from the siRNA delivery method. For the purposes of this study, Lipofectamine 2000 (Invitrogen, UK) was considered to be the most suitable delivery method available. Nucleic acids carry a net negative charge under normal physiological conditions, and must come into contact with a cell membrane that also carries a net negative charge. Lipofectamine 2000 is a cationic liposome formulation that functions by complexing with nucleic acid molecules, allowing them to overcome the electrostatic repulsion of the cell membrane and to be taken up by the cell.

The efficiency, safety and long-term duration of siRNA delivery remain the major challenges to its clinical application. siRNA delivery to specific cell types is also a key challenge. A number of cell specific ligands including polysaccharide, antibody, antibody fragment, and peptide have been employed to achieve targeted delivery of siRNA. Recent studies using multilayered polyelectrolyte films (MPFs – consisting of alternate layer by layer deposition of polycations and polyanions), have shown this technique to represent a successful approach for the efficient delivery of siRNAs to target cells to combat viral infection (Dimitrova *et al.*, 2008). There is currently a great deal of work being done investigating innovative solutions for delivery of RNAi therapeutics, however it should also be kept in mind that virus-specific challenges exist such as virus-encoded suppressors of RNAi and virus escape.

To counter the antiviral RNAi response, plant and invertebrate viruses have evolved proteins that actively interfere with distinct steps of the RNAi machinery (Li and Ding,

2005). Some mammalian viruses also encode factors that exhibit RNAi suppression activity and it is currently unclear to what extent these suppressors can interfere with the activity of RNAi-based therapeutics (Andersson *et al.*, 2005; Haasnoot *et al.*, 2007).

Another hurdle is the fact that viruses readily escape from RNAi suppression by single mutations or deletions of the target sites and mutations outside the target sites. Two strategies may delay or even prevent viral escape; use siRNA designed to target a highly conserved sequence of the viral genome, and employ the simultaneous use of multiple siRNAs (Schubert *et al.*, 2005).

4.2 AIMS AND OBJECTIVES

In this chapter, the aim was to investigate the hypothesis that equine sarcoid tumours arise primarily as a result of cellular resistance to apoptosis rather than as a result of increased cellular proliferation. More specifically, to;

- i) Investigate the effects of a variety of DNA damaging agents (UVB, 5-fluoruracil and cisplatin) on apoptosis levels in BPV-1 transformed equine fibroblast cell lines.
- ii) Evaluate long-term cell survival following DNA damage in normal, and BPV-1 transformed equine fibroblast cell lines.
- iii) Identify the viral gene(s) responsible for altering the apoptotic response and long term survival of BPV-1 transformed equine fibroblast cells.

4.3 MATERIALS AND METHODS

4.3.1 Cell Culture

Normal equine fibroblasts (EqPalF cells), sarcoid derived BPV-1 transformed equine fibroblasts (EqS cells) and experimentally transformed equine fibroblasts (S6 cells) were cultured and maintained as described in Chapter II; section 2.2.1.

4.3.2 Apoptosis detection using FCM analysis

Cell culture, UVB and drug treatments were performed as previously described in Chapter II; section 2.2. FCM analysis was implemented after 24 hours (in the case of UVB exposure) or 72 hours (cisplatin and 5-fluoruracil) post-treatment to assess the levels of apoptosis present (Chapter II; section 2.2.11). FCM optimisation was carried out with each DNA damaging treatment to obtain optimal apoptosis levels of 50 – 60% per cell population and treatment. This optimisation resulted in the selection of UVB irradiation levels of 250 and 500 mJ/cm², cisplatin concentrations of 10 and 25 µg/ml and 5-FU concentrations of 10 and 15 mg/ml.

4.3.3 Clonogenic Survival Assay

Since BPV-1 positive cell lines tended to show increased apoptosis in the presence of DNA damaging agents compared to BPV-1 negative EqPalF cells, we then studied the long term survival of these cells. Once cells had been treated and harvested for FCM analysis either 24 (UVB treated cells) or 72 (cisplatin and 5-FU treated cells) hours after initial exposure to the DNA damaging agent, a small proportion of the harvested cells (5,000) were retained and used in a clonogenic survival assay. The methodology for these assays is outlined in Chapter II; section 2.2.12. After about 10 days, colonies were fixed, stained and counted and the plating efficiencies and survival fractions for each cell line and treatment level were calculated.

4.3.4 siRNA design and transfection

EqS04b cells have previously been shown to express higher levels of BPV-1 genes than the other sarcoid derived cell lines (Yuan *et al.*, 2007), so this cell line was chosen for use in the siRNA experiments. Initially, 3 BPV genes of interest were selected for evaluation; E5, E6 and E7. siRNA design software was used to generate suitable siRNA sequences targeted to each gene. These siRNA sequences are detailed in Chapter II; section 2.2.8. The E6 targeted siRNA molecule was shown to be the most effective when knock-down was

assessed by RQ-PCR (section 2.2.5). EqS04b cells were transfected with E6 targeted siRNA as described in Chapter II; section 2.2.8.4.

4.3.5 RQ-PCR to establish efficiency of siRNA knockdowns

RNA extraction from cultured cells was carried out as described in Chapter II; section 2.2.3.1. From the RNA, cDNA was synthesised (section 2.2.3.1.4) and analysed by RQ-PCR (section 2.2.5) using either an E5, E6 or E7 primer and probe set (Table 2.4). EqS04b cells that had either been left untreated or had been transformed by the siRNA scramble control sequence were used as control cells, and served as standards for the amount of BPV gene product detected in the gene specific siRNA transfected cells. By analysing both the untreated ES04b cells and the scramble sequence siRNA treated cells, any suppression of viral gene expression by merely the presence of the short RNA sequences, or the transfection reagent used, could be detected.

4.3.6 Statistical Analysis

RQ-PCR error bars were calculated as described in section 2.2.5. The differences in apoptosis and cell survival levels between the different cell lines were then analysed for statistical significance ($p < 0.05$) by using the t-test method (section 2.2.13).

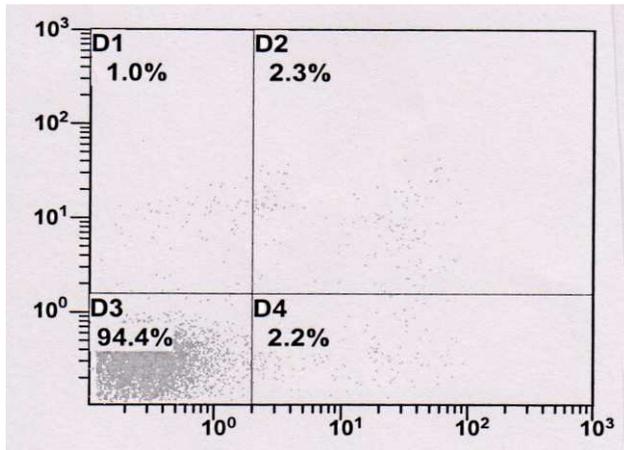
4.4 RESULTS

4.4.1 Flow cytometric analysis (FCM) reveals that both normal, and BPV-1 transformed equine fibroblast cell lines undergo apoptosis following DNA damage.

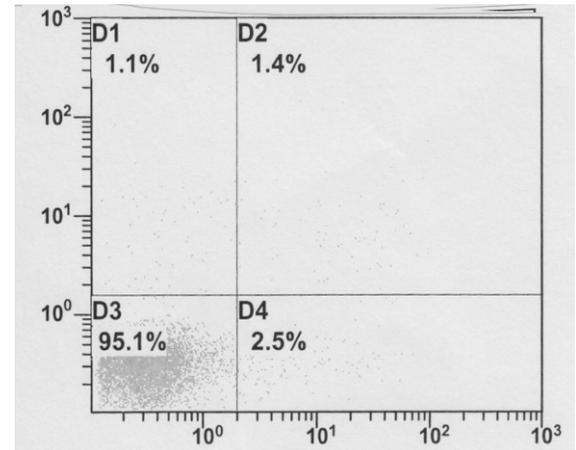
Figure 4.4 shows results obtained from FCM analysis of BPV-1 negative fibroblasts (Fig. 4.4 A (i) and B (i)) and sarcoid tumour derived, BPV-1 transformed fibroblasts (Fig. 4.4 A (ii) and B (ii)) both before and after exposure to UVB irradiation. Both cell lines show a small amount of apoptosis in the untreated cell populations which is to be expected as a result of normal cell turnover, and a response to the trypsinisation and Annexin V staining protocol. This is why it was important to keep the reactions on ice for the majority of the protocol (section 2.2.11). At the highest UVB exposure level (500 mJ/cm²), the normal equine fibroblasts show a lower percentage of apoptotic cells (27.5%) compared to the BPV-1 transformed cell lines (34.1%) 24 hours-post insult. For each DNA damaging method, all the cell lines were assessed after three independent treatment sessions and the results averaged. The differences in apoptosis levels between the different cell lines were then analysed for statistical significance ($p < 0.05$) by using the t-test method. These results are shown in Figures 4.5, 4.6 and 4.7.

Figure 4.4

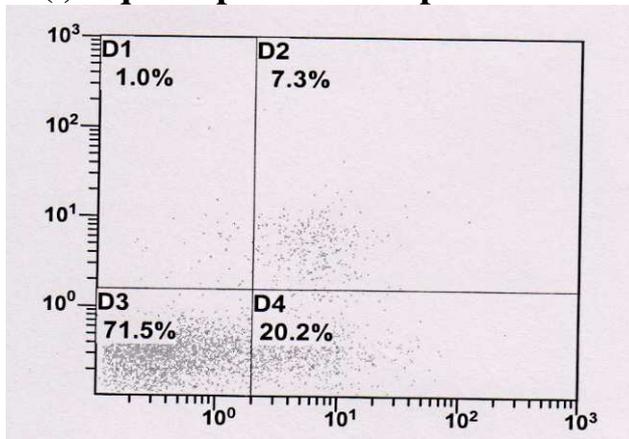
A (i) EqPalF no UVB exposure



(ii) EqS04b no UVB exposure



B (i) EqPalF post-UVB exposure



(ii) EqS04b post-UVB exposure

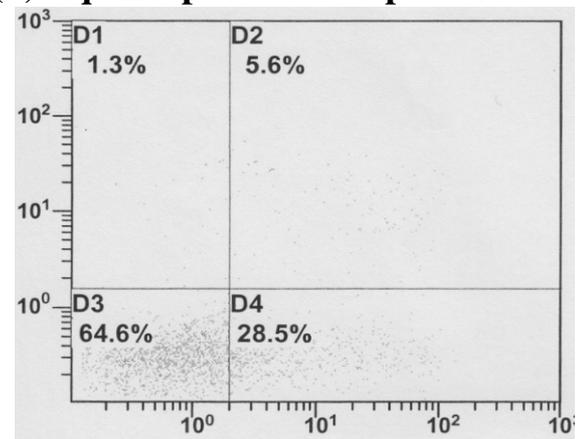


Figure 4.4 Plots from FCM analysis of normal equine fibroblast (EqPalF) cells and sarcoid derived BPV-1 transformed equine fibroblast (EqS04b) cells.

x axis: log Annexin V- FITC; **y axis:** log propidium iodide

D1 – necrotic cells (propidium iodide only)

D2 – secondary necrotic (propidium iodide AND Annexin V)

D3 – viable cells (no uptake of Annexin V or Propidium iodide)

D4 - early apoptotic (Annexin V only)

A (i) – EqPalF cells with no UVB exposure

A (ii) – EqS04b cells with no UVB exposure

B (i) – EqPalF cells 24hrs after exposure to 500 mJ/cm² UVB

B (ii) – EqS04b cells 24hrs after exposure to 500 mJ/cm² UVB

4.4.2 Sarcoid derived fibroblast cell lines consistently show significantly higher levels of apoptosis following UVB irradiation compared to normal equine fibroblast cells.

To investigate the hypothesis that equine sarcoids may be associated with abnormalities in apoptotic pathways, rather than with an increase in cell proliferation, we exposed the different cell lines to UVB irradiation as described in Chapter II; section 2.2.1 and measured the resulting levels of apoptosis by FCM analysis (section 2.2.11), 24 hours later.

As shown in Figure 4.5, both the BPV-1 transformed cell lines and sarcoid derived cell lines show increased levels of apoptosis following UVB exposure compared to uninfected equine fibroblast control cells. This increase was shown to be statistically significant in two of the experimentally transformed cell lines (S6-2 at 500 mJ/cm², and S6-3 at 250 mJ/cm²), and in all the sarcoid derived cell lines (EqS cells, Fig. 4.5), either at just one (EqS03 and EqS04b; 500 mJ/cm²), or both exposure levels (EqS03 and EqS04b).

The literature cautions that levels of apoptosis observed within 24 hours of the initial DNA damaging insult may not reflect the true extent of eventual cell death as some cells may not succumb to apoptosis for days after the DNA damage occurs (Brown and Wouters, 1999). And so perhaps the results obtained here are more a reflection of the rate of apoptosis, rather than the overall level of cell death. This is one of the reasons why we felt it important to conduct colony survival assays in combination with the short-term apoptosis experiments. However, it is worth pointing out at this stage that the literature is almost exclusively referring to data which suggested some cancer cell lines were resistant to certain treatments based on initial levels of apoptosis observed, whereas if the treated cells were cultured in a colony survival assay, the overall cell death would be much higher than the initial levels of apoptosis suggested. As illustrated in Figure 4.5, my results are the opposite of those most commonly encountered in apoptosis evaluation in cancer cell lines, with higher levels of apoptosis following UVB exposure clearly demonstrated in the transformed cell lines.

Figure 4.5.

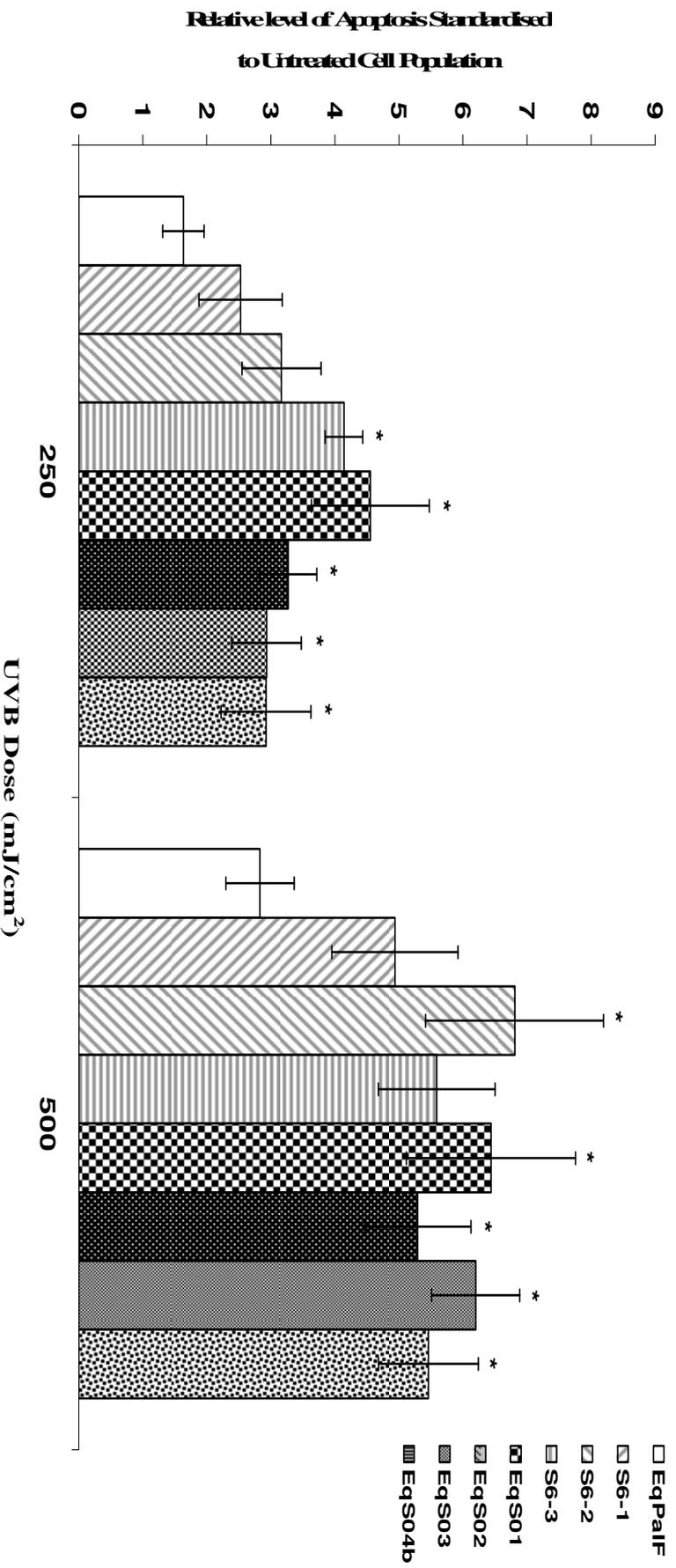


Figure 4.5 Apoptosis levels detected 24 hours post-UVB exposure using Annexin V/Propidium iodide staining and flow cytometry analysis.

Both BPV-1 transfected cell lines (S6-1, S6-2, S6-3) and sarcoid derived cell lines (EqS01, EqS02, EqS03, EqS04b) show increased levels of apoptosis following UVB exposure compared to BPV-negative foetal palate fibroblasts (EqPalF). When apoptosis levels of the BPV-1 positive cells were compared to those apoptosis levels seen in the BPV-1 negative EqPalF cells, * = statistically significant result ($p < 0.05$)

4.4.3 Sarcoid derived cell lines and control cells show similar levels of apoptosis induced by 72hrs incubation with Cisplatin.

The same panel of cell lines were then exposed to two different concentrations of cisplatin (10, 25 µg/ml) for 72 hours and apoptosis measured by FCM analysis. As shown in Figure 4.6, the increase in apoptosis levels were generally more marked in the *in-vitro* BPV-1 transformed cell lines (S6-1, S6-2, S6-3), compared to the response seen in the sarcoid derived cell lines. At the lower cisplatin concentration (10 µg/ml) there was no significant increase in apoptosis levels in any of the sarcoid derived cells lines (EqS01, EqS02, EqS03, EqS04b) compared to the BPV-negative control cells. However, all of the BPV-1 transformed cell lines (S6-1, S6-2, S6-3) showed a significant increase in apoptosis levels. The increased apoptosis at the higher concentration of cisplatin (25 µg/ml) was not statistically significant in the sarcoid derived cell lines (EqS01, EqS02, EqS03) bar one (EqS04b) and was only marginally significant ($p = 0.049$ and 0.035 respectively) for the experimentally transformed cell lines (S6-1 and S6-2).

4.4.4 Sarcoid derived cells are less sensitive to apoptosis induced by high doses of 5-Fluorouracil

To determine resistance or susceptibility to apoptosis induced by 5-FU, cells were cultured in the presence of two concentrations of 5-FU (10 and 15 mg/ml) and apoptosis rates measured by FCM analysis after 72 hours. The results have shown that in contrast to UVB and Cisplatin, sarcoid derived fibroblasts (EqS01, EqS02, EqS03, EqS04b) are more resistant to apoptosis induced by incubation with 15 mg/ml 5-FU, compared to BPV-negative control cells (Fig. 4.7). Figure 4.7 also shows that the *in vitro* BPV-1 transformed cell lines (S6-1, S6-2 and S6-3) demonstrate a similar level of apoptosis as that shown by the BPV-negative fibroblasts (bar the S6-2 cell line at the higher 5-FU concentration which shows a significantly lower apoptotic response). At the lower concentration of 5-FU (10 mg/ml), there is no statistical significance between the levels of apoptosis shown by any of the cell lines.

Figure 4.6.

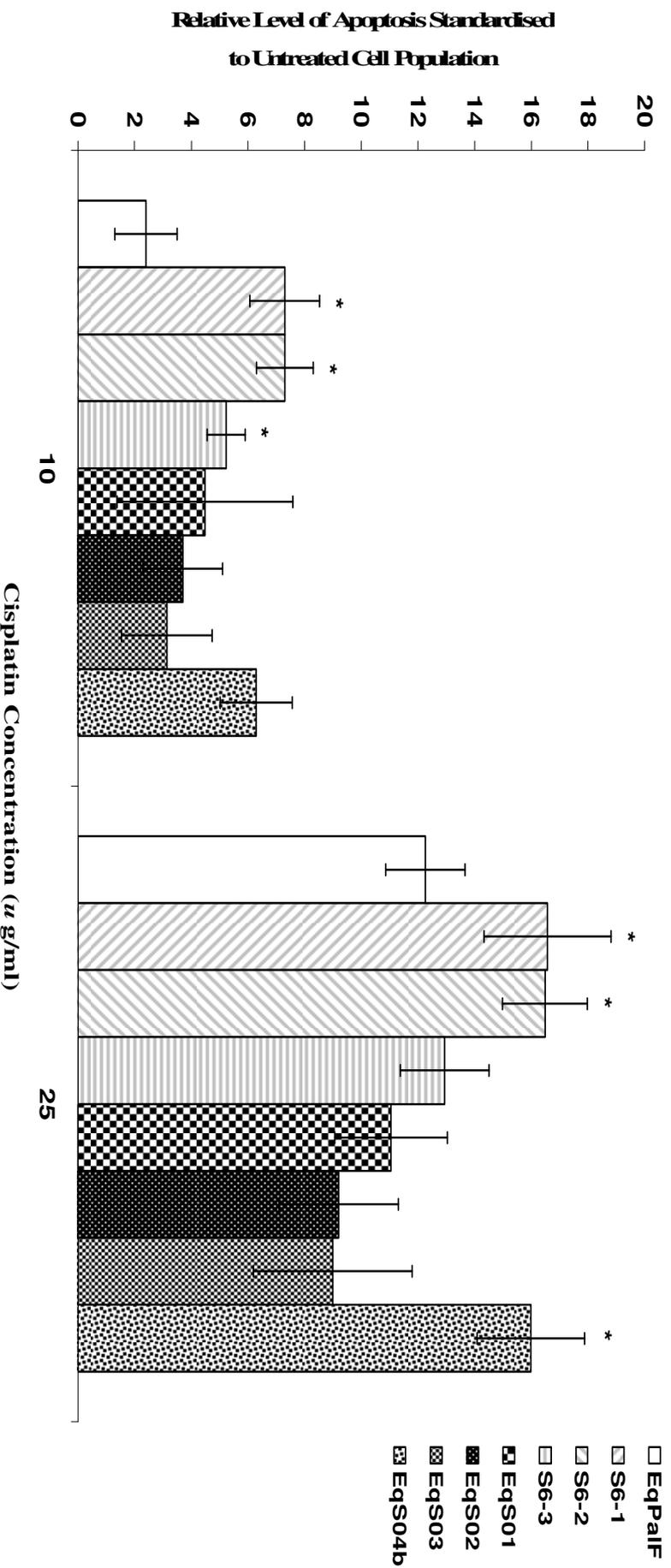


Figure 4.6 Apoptosis levels detected after 72 hours incubation with cisplatin using Annexin V/Propidium iodide staining and flow cytometry.

Only BPV-1 transfected cell lines (S6-1, S6-2, S6-3) show increased levels of apoptosis following cisplatin exposure compared to BPV-negative foetal palate fibroblasts (EqPalF) and sarcoid derived cell lines (ES01, ES02, ES03, ES04b). C0 = nothing added to media; C10 = 10 μ g/ml cisplatin; C25 = 25 μ g/ml cisplatin. * = statistically significant result ($p < 0.05$)

Figure 4.7.

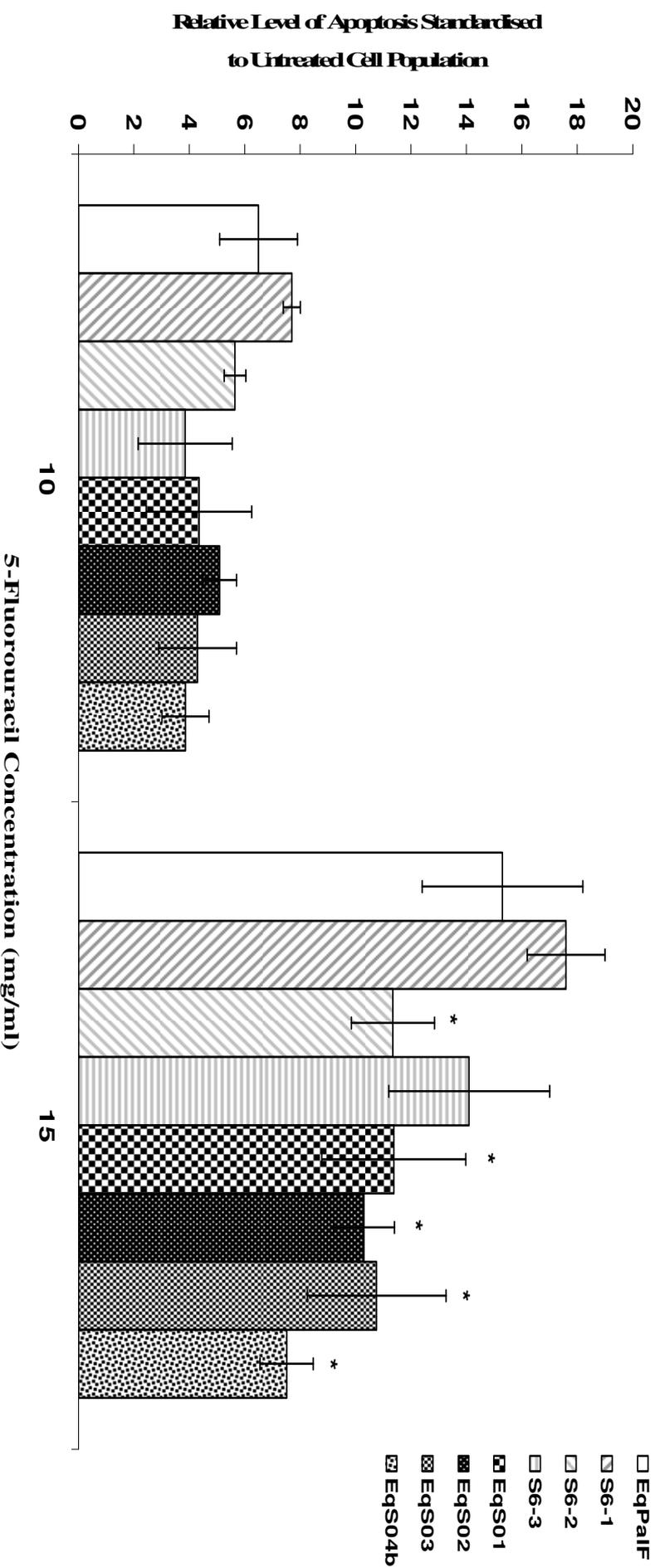


Figure 4.7 Apoptosis levels detected after 72 hrs incubation with 5-fluorouracil using Annexin V/Propidium iodide staining and flow cytometry.

There is no statistical significance between the levels of apoptosis observed in the BPV-1 transfected cell lines (S6-1, S6-2 [at lower 5-FU conc.], and S6-3) and those observed in the BPV-negative control fibroblasts (EqPalF). However, the levels of apoptosis observed in the sarcoid derived cell lines (ES01, ES02, ES03, ES04b) 72 hours after exposure to 5-fluorouracil is significantly less than in the BPV-negative control cells at the higher 5-FU concentration. F0 = nothing added to the media; F10 = 10mg/ml 5-FU; F15 = 15mg/ml 5-FU. * = statistically significant result ($p < 0.05$)

4.4.5 Sarcoid derived cell lines show enhanced cell survival rates following exposure to DNA damaging agents

The results in Figure 4.8 and Figure 4.9 show that despite having a higher percentage of apoptotic cells in the original populations, as assessed by FCM analysis, sarcoid derived and *in vitro* BPV-1 transformed cell lines exposed to either UVB or cisplatin both showed enhanced cell survival levels compared to the BPV-negative control fibroblasts.

In contrast to the results obtained with UVB and Cisplatin, when cells treated with 5 FU were subjected to clonogenic assays, all cell lines show very limited cell survival levels after exposure to the higher 5-FU concentration (15 mg/ml). At the lower 5-FU concentration, all the BPV-1 transformed cell lines bar S6-1, showed a significantly higher level of cell survival compared to the control EqPalF cells (Fig. 4.10). It is also worth noting that although the survival fractions following 5-FU treatment appear to be at least as large as the results obtained following UVB and cisplatin treatment, the scale on the y-axis reveals that the highest survival fraction achieved after 5-FU treatment is barely as high as the lowest survival fraction in the other two treatment groups. This is demonstrated to greater affect in Fig. 4.11.

As discussed in Chapter II; section 2.2.12, plating efficiencies (PE) were calculated for each cell line and from these PEs, the survival fraction (SF) for the cell line can be generated. This allows for the fact that different cell lines will have differing efficiencies in forming colonies and produces a standardised value (the SF) for comparison.

$$PE = [\text{number of colonies formed}/\text{number of cells seeded}] \times 100$$

This value is then used to calculate the Survival Fraction;

$$SF = [\text{number of colonies}/(\text{number of cells seeded} \times PE)] \times 1000$$

The average plating efficiency for each cell line is shown below in Table 4.1

Table 4.1 Average plating efficiencies for all cell lines.

Cell Line	Average Plating Efficiency
EqPalF	30
S6-1	35
S6-2	50
S6-3	40
EqS01	40
EqS02	25
EqS03	50
EqS04b	85

Figure 4.8.

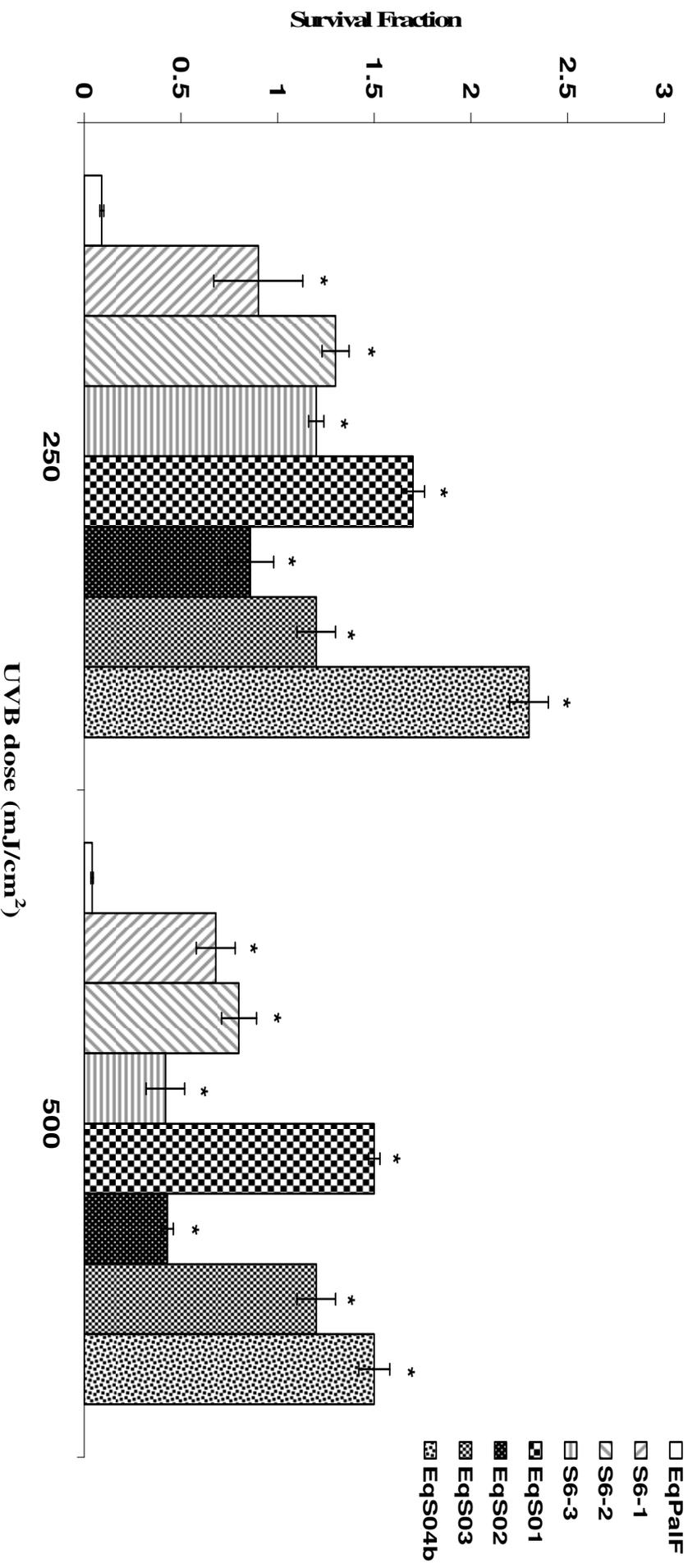


Figure 4.8 Long term cell survival of the different cell lines after UVB irradiation.

Survival Fraction was calculated from the plating efficiencies (see Table 4.1) that were determined for each individual cell line. Both sarcoid derived fibroblasts (EqS01, EqS02, EqS03, EqS04b) and BPV-1 transformed fibroblasts (S6-1, S6-2, S6-3) show statistically significant increases in proportion of cell survival after treatment. * = statistically significant result (p < 0.05)

Figure 4.9.

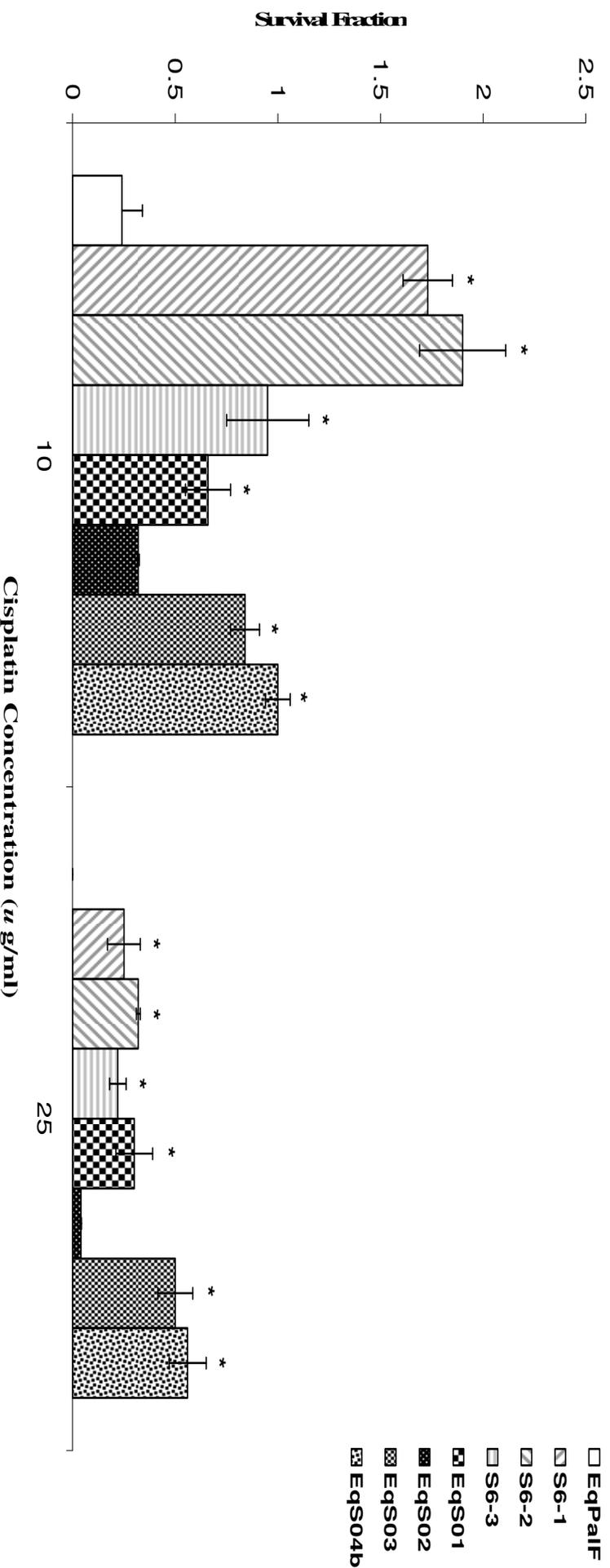


Figure 4.9 Long term cell survival of the different cell lines after 72hours incubation with cisplatin.

After 72hrs, the cisplatin containing media was replaced with cisplatin-free media and the plates were incubated for a further 10 – 14 days. Survival Fraction was calculated from the plating efficiencies that were determined for each individual cell line. Both sarcoid derived fibroblasts (EqS01, EqS02, EqS03, EqS04b) and BPV-1 transformed fibroblasts (S6-1, S6-2, S6-3) show statistically significant increases in proportion of cell survival after treatment.

* = statistically significant result ($p < 0.05$)

Figure 4.10.

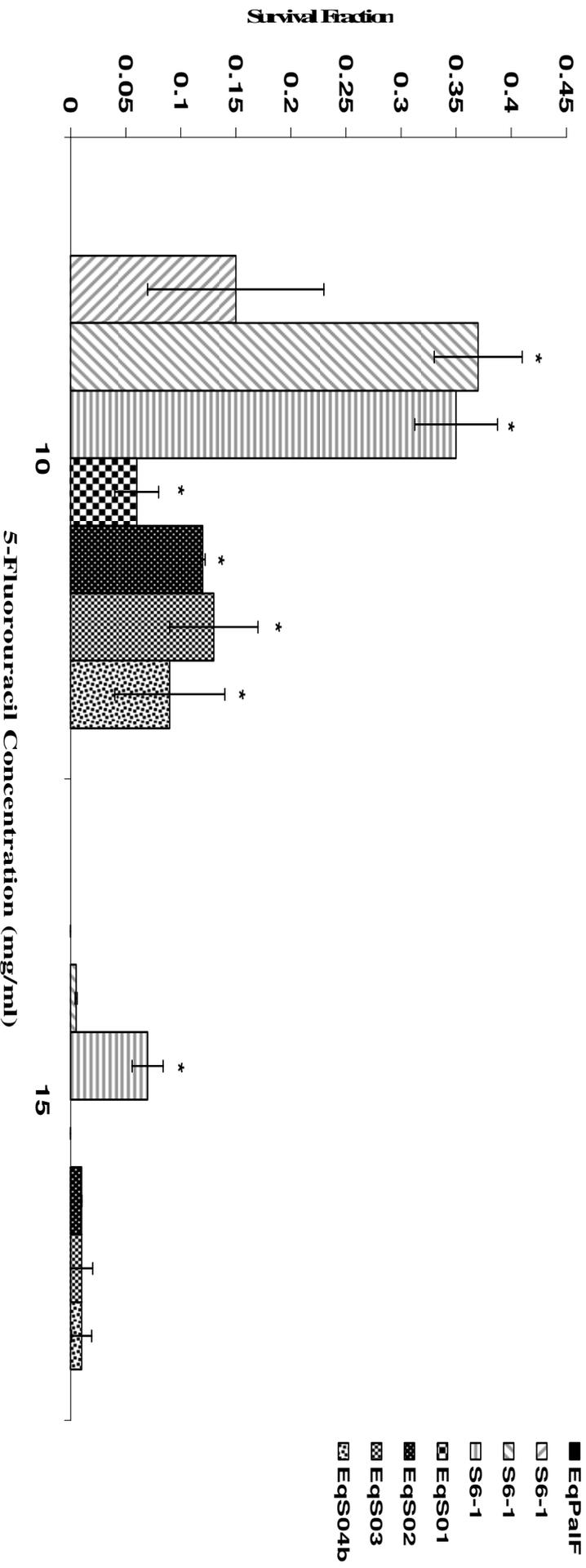


Figure 4.10 Long term cell survival of the different cell lines after 72 hours incubation with 5 - fluorouracil.

After 72hrs, the 5 - fluorouracil containing media was replaced with 5 - fluorouracil -free media and the plates were incubated for a further 10 – 14 days. Survival Fraction was calculated from the plating efficiencies that were determined for each individual cell line. Both sarcoïd derived fibroblasts (EqS01, EqS02, EqS03, EqS04b) but particularly BPV transformed fibroblasts (S6-1, S6-2, S6-3) show statistically significant increases cell survival after treatment. * = statistically significant result (p < 0.05)

Figure 4.11

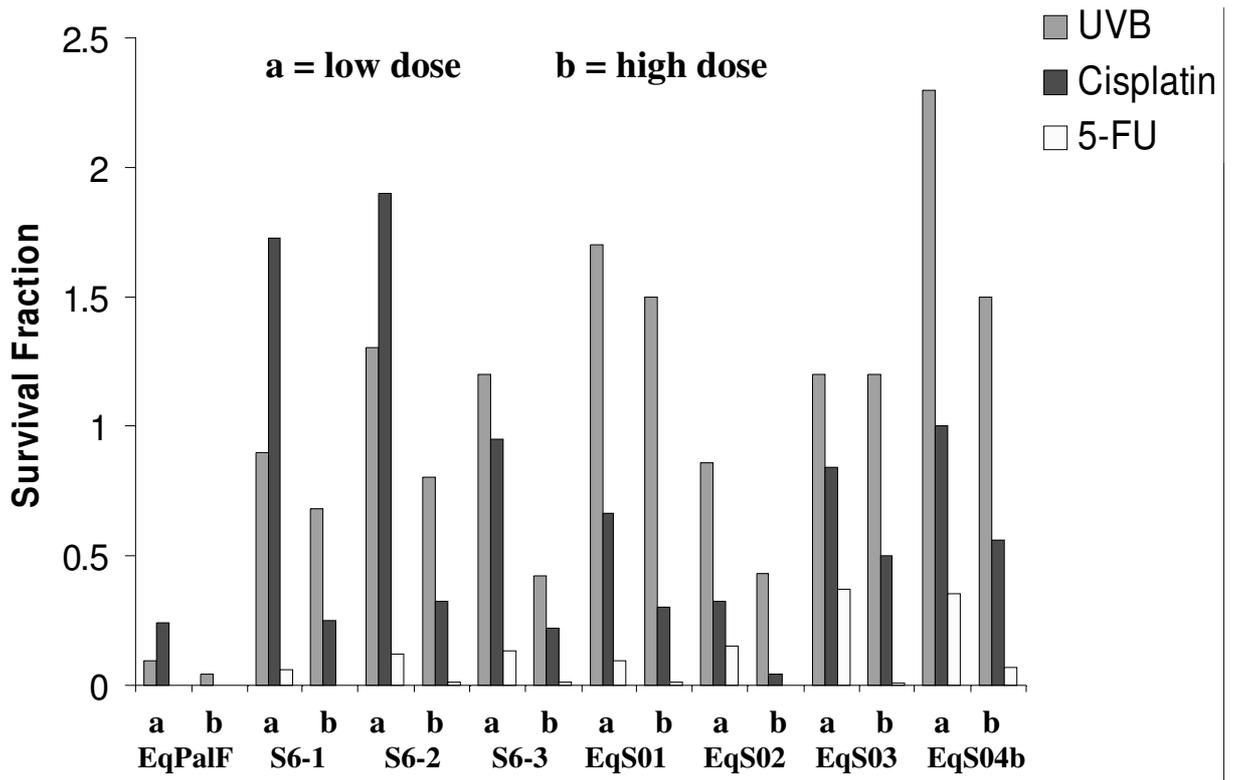


Figure 4.11 Comparison of the different DNA damaging agents and the respective survival fractions for each cell line.

This figure shows the relatively small degree of cell survival across all the cell lines following treatment of the cells with 5-FU, compared to the survival fractions for UVB and cisplatin. This figure also shows the relative inability of the BPV-negative fibroblasts (EqPalF) to survive any sort of DNA damage in the long term, even though the initial levels of apoptosis as assessed by FCM analysis in these cell lines, was generally lower than the apoptosis levels seen in the BPV-1 transformed cell lines (S6 and EqS).

4.4.6 Introduction of siRNA targeted to BPV E6 results in reduced expression of E2, E5, E6 and E7 genes.

When the E6 siRNA was introduced in to EqS04b cells it consistently resulted in reduced expression (> 80%) of the BPV-1 genes E2, E5, E6 and E7. This initial analysis work is reproduced with the kind permission of Dr Zhenguai Yuan who carried out the initial evaluation of the E6 siRNA efficacy using the RQ-PCR protocol described in section 2.2.5. For all the remaining siRNA work included in this thesis, RQ-PCR analysis of the EqS04b cells to determine the success of E6 knockdown prior to proceeding with the apoptosis/colony survival experiments, was performed as described in Chapter II, section 2.2.12, by the author. Only if the E6 knockdown was shown to be at least, 70%, did further experiments progress.

Figure 4.12 shows the RQ-PCR results obtained by Dr. Yuan after transfection of the EqS04b cells with the E6 targeted siRNA;

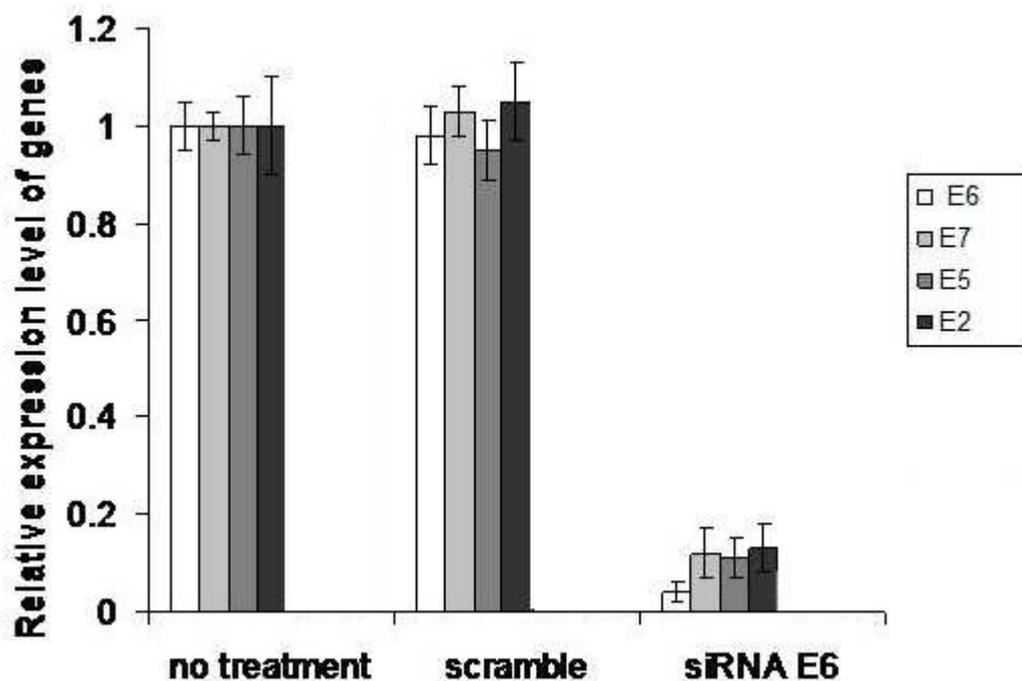


Figure 4.12 RQ-PCR analysis of expression of BPV-1 genes E2, E5, E6 and E7 in EqS04b cells 48 hours after cell transfection with E6 siRNA molecules.

4.4.7 Treatment of sarcoid derived cells with E6 siRNA prior to treatment with DNA damaging agent, does not result in altered apoptosis levels.

Figures 4.13, 4.14 and 4.15 show that there is no significant difference in the apoptotic response (as measured by FCM analysis) following insult with the DNA damaging agents UVB, cisplatin and 5-FU between untreated EqS04b cells and those treated with either the E6 siRNA sequence, or the scramble control sequence. These results show that the transfection protocol itself and the presence of the siRNA within the cell and cell nucleus does not significantly impact on the cellular response to DNA damage.

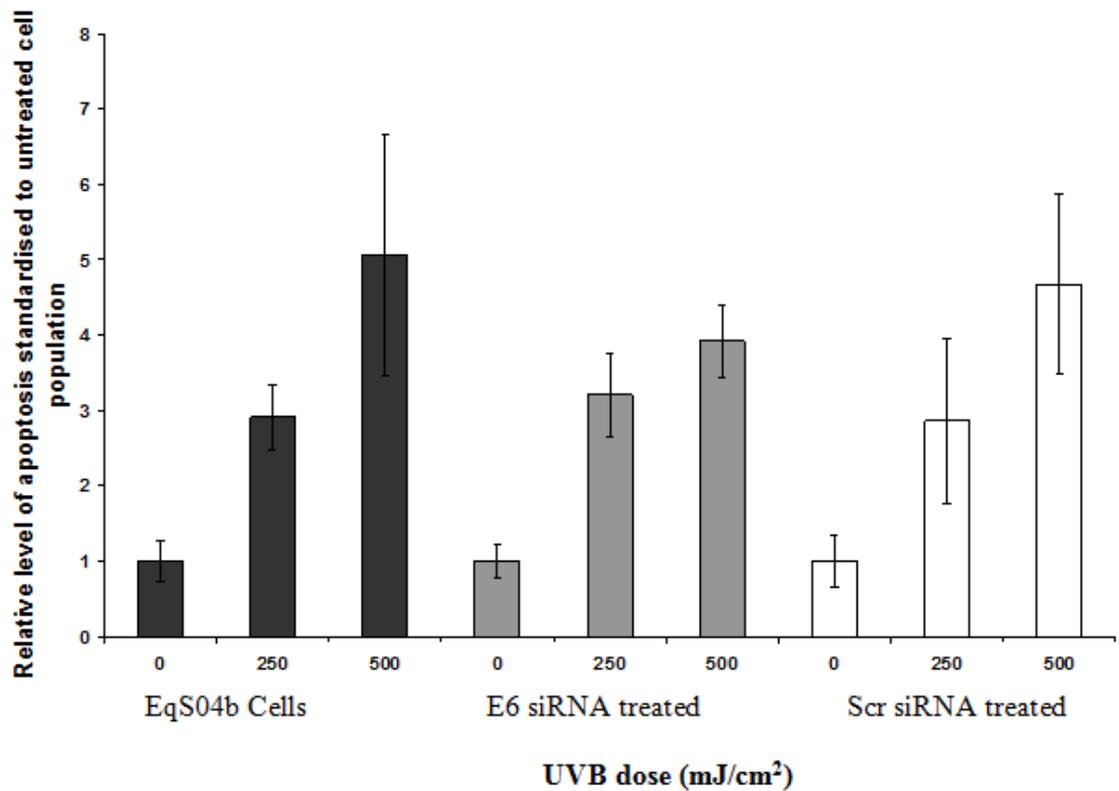


Figure 4.13 Treatment with E6 targeted siRNA prior to UVB-irradiation, does not significantly alter apoptotic response.

Apoptosis levels were measured 24 hours after exposure to UVB using Annexin V/PI FCM analysis.

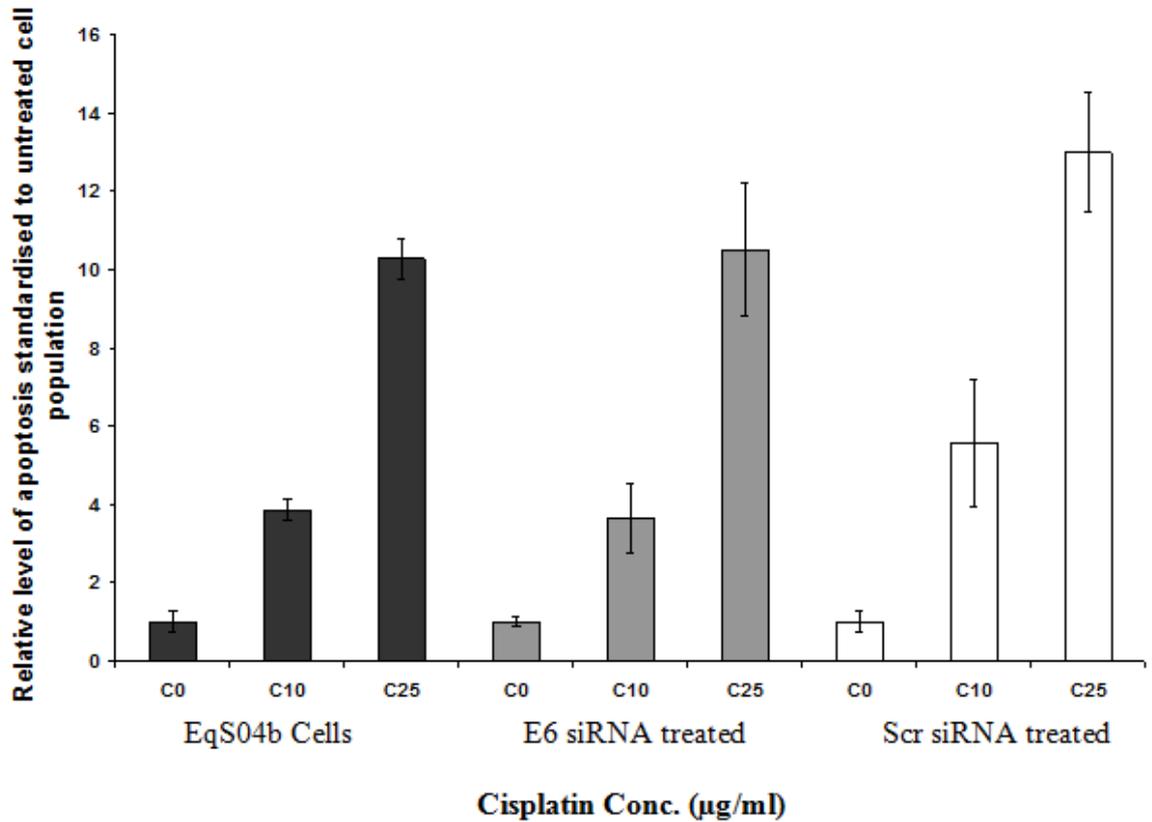


Figure 4.14 Treatment with E6 targeted siRNA prior to incubation with cisplatin, does not significantly alter apoptotic response.

Apoptosis levels were measured after 72 hours incubation with cisplatin containing media, using Annexin V/PI FCM analysis.

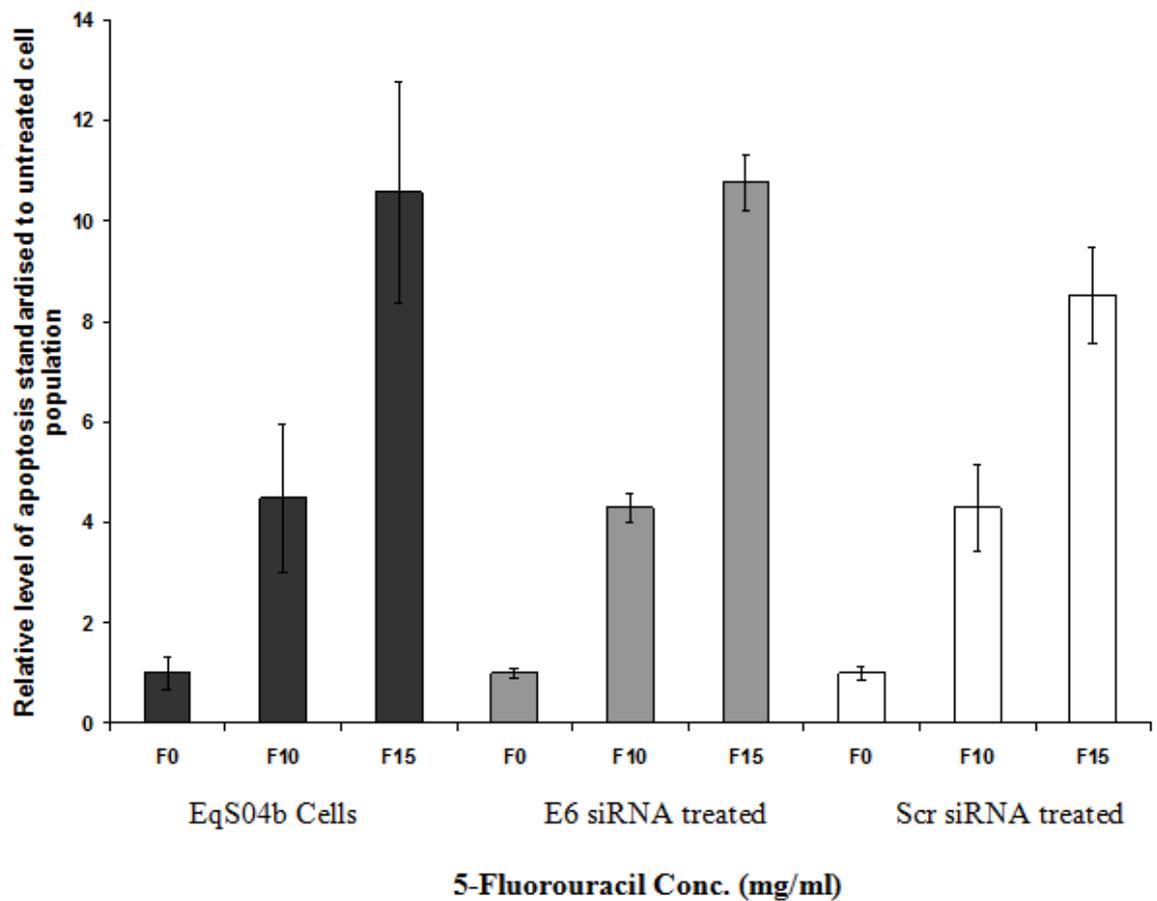


Figure 4.15 Treatment with E6 targeted siRNA prior to incubation with 5-Fluorouracil, does not significantly alter apoptotic response.

Apoptosis levels were measured after 72 hours incubation with 5 – Fluorouracil in the media, UVB using Annexin V/PI FCM analysis.

4.4.8 Treatment of sarcoid derived cells with E6 siRNA prior to treatment with a DNA damaging agent results in reduced cell survival.

Following harvesting of the different EqS04b cell populations for analysis in the FCM analysis experiments described above, a proportion of each population (EqS04b cells, EqS04b cells with Lipofectamine reagent added to the growth media, EqS04b cells incubated with the E6 siRNA and fourthly, EqS04b cells incubated with an siRNA scramble control sequence), were retained for use in colony survival assays. The clonogenic assays were performed as described in Chapter II; section 2.2.12. Briefly, 3000 cells from each EqS04b treatment population were seeded into new 10cm Petri dishes and incubated in normal growth media for 10 to 14 days until the colonies had reached a suitable size for counting. Colonies were fixed, stained and counted and the plating efficiencies and survival fractions for each cell line and treatment level were calculated.

In contrast to the results seen in assessment of apoptosis levels in E6 siRNA treated EqS04b cells, analysis of the clonogenic survival assay data revealed a significant reduction in the degree of cell survival in the E6 siRNA treated cells compared to both unmodified EqS04b cells and cells incubated with the scramble control siRNA sequence. The use of the scramble control siRNA and the inclusion of unmodified EqS04b cells in the experiment demonstrates that the reduced cell survival levels cannot be attributed merely to the presence of the E6 siRNA molecules in the cell, but must be a consequence of the targeted reduction in the expression of the BPV-1 genes.

Figure 4.16 and 4.17 show the results obtained in the clonogenic assay of the various EqS04b cell lines after UVB exposure. These images clearly show that those EqS04b cell populations that were treated with the E6 targeted siRNA molecules had significantly reduced cell survival.

Figure 4.18 contains images from the UVB clonogenic survival assay after the plates have been fixed and stained. The reduction in the number of colonies present in the plates containing the E6 siRNA cells can clearly be seen (Figure 4.18 E and F).

Figure 4.19 and 4.20 show the results obtained in the clonogenic assay of the various EqS04b cell lines after incubation with cisplatin.

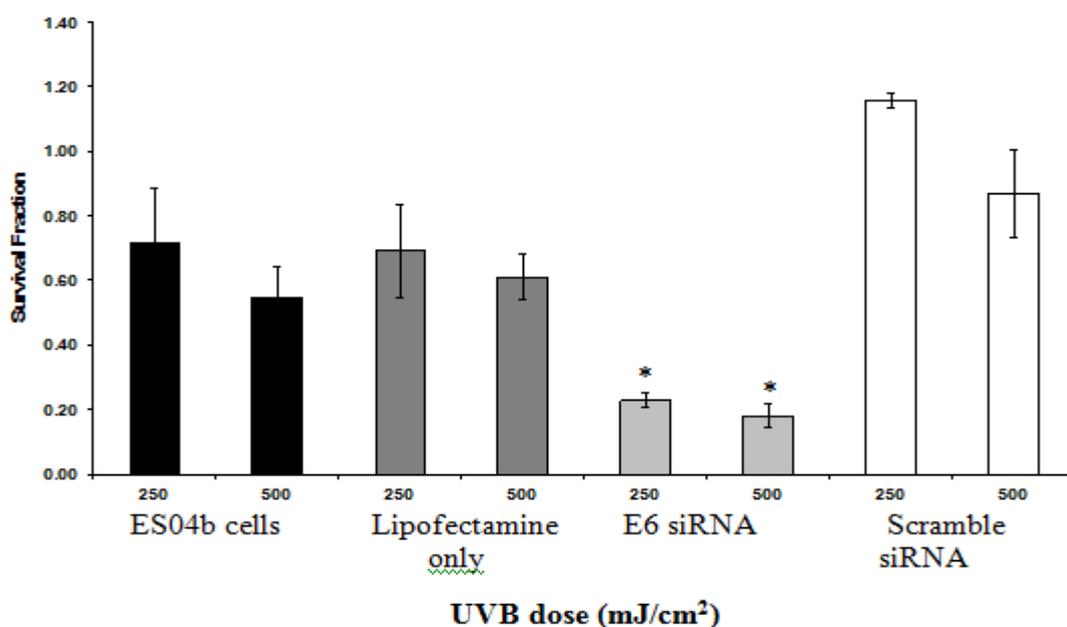


Figure 4.16 Survival Fractions calculated from colony survival data of EqS04b cells after UVB irradiation.

* = statistically significant result $p < 0.05$

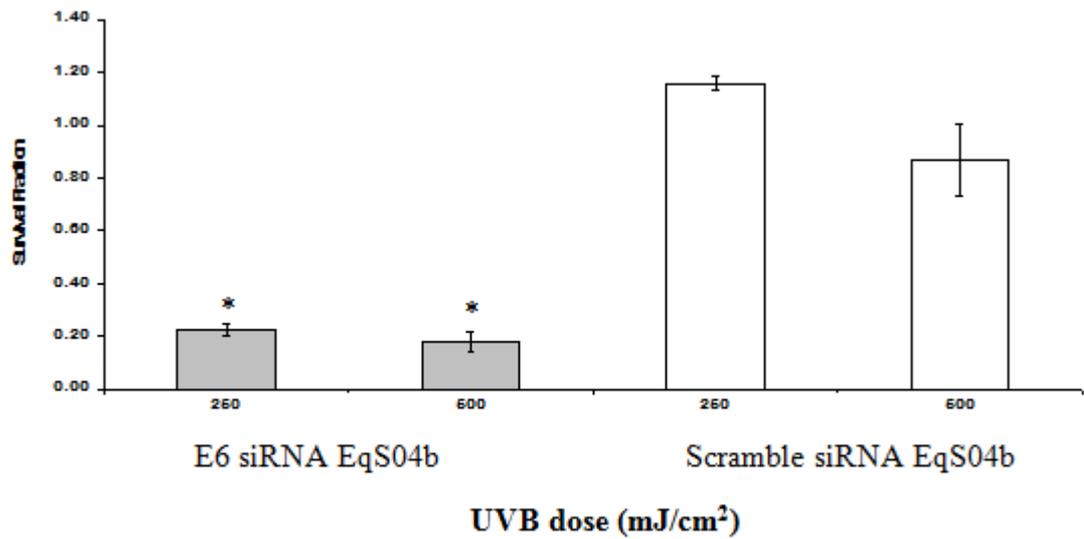


Figure 4.17 Survival Fraction of EqS04b cells treated with E6 targeted siRNA.

Shows that cells treated with E6 siRNA prior to UVB irradiation demonstrate significantly less potential for long term cell survival when compared to cells treated with a scramble siRNA sequence control.

* = statistically significant result $p < 0.05$

Figure 4.18

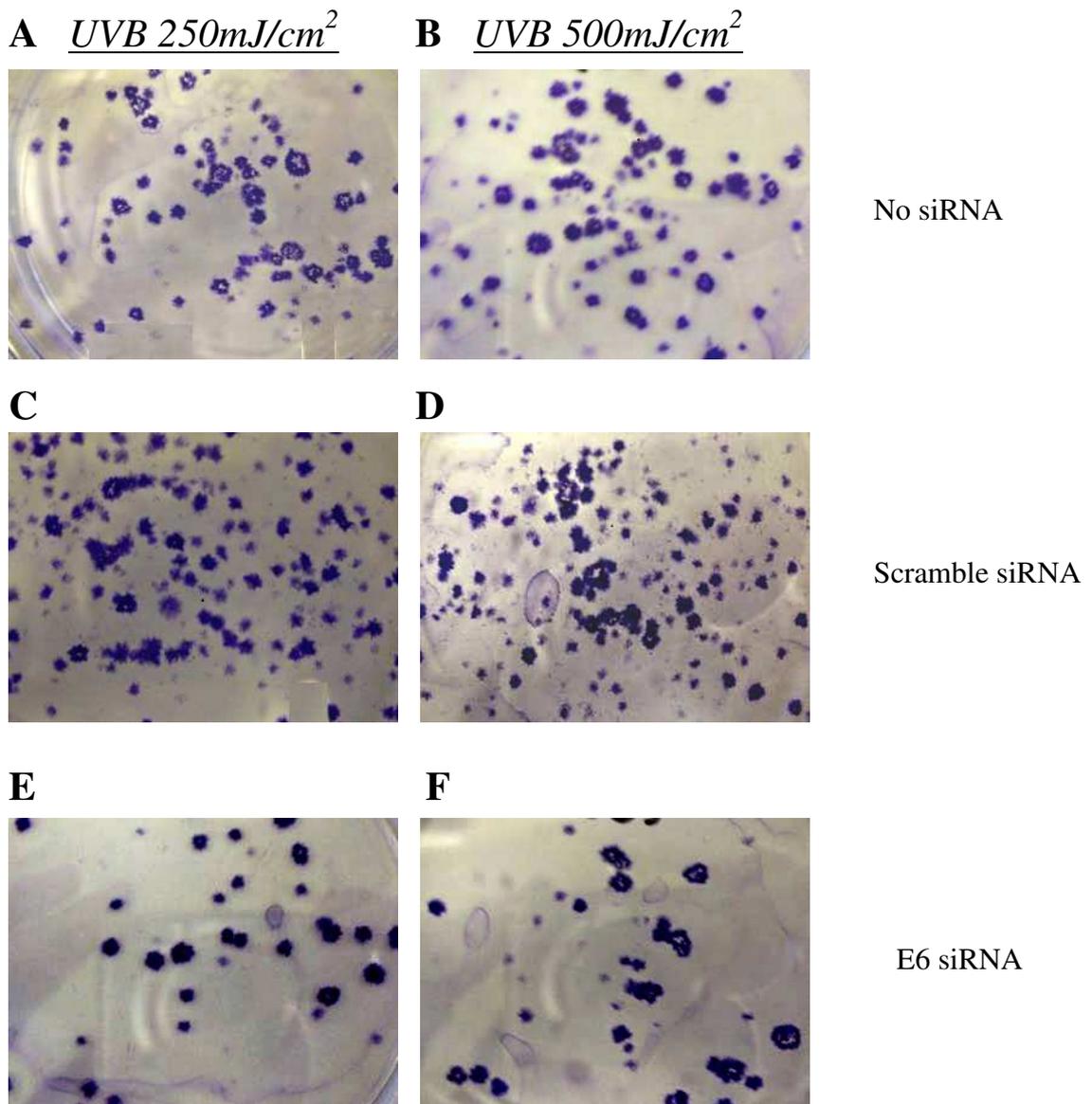


Figure 4.18 Treatment with BPV-1 E6 targeted siRNA prior to UVB exposure results in significantly reduced long term cell survival.

A – Eqs04b cells after exposure to 250 mJ/cm² UVB

B – Eqs04b cells after exposure to 500 mJ/cm² UVB

C – Eqs04b cells treated with Scramble siRNA sequence then exposed to 250 mJ/cm² UVB

D - Eqs04b cells treated with Scramble siRNA sequence then exposed to 500 mJ/cm² UVB

E - Eqs04b cells treated with E6 siRNA sequence then exposed to 250 mJ/cm² UVB

F - Eqs04b cells treated with E6 siRNA sequence then exposed to 500 mJ/cm² UVB

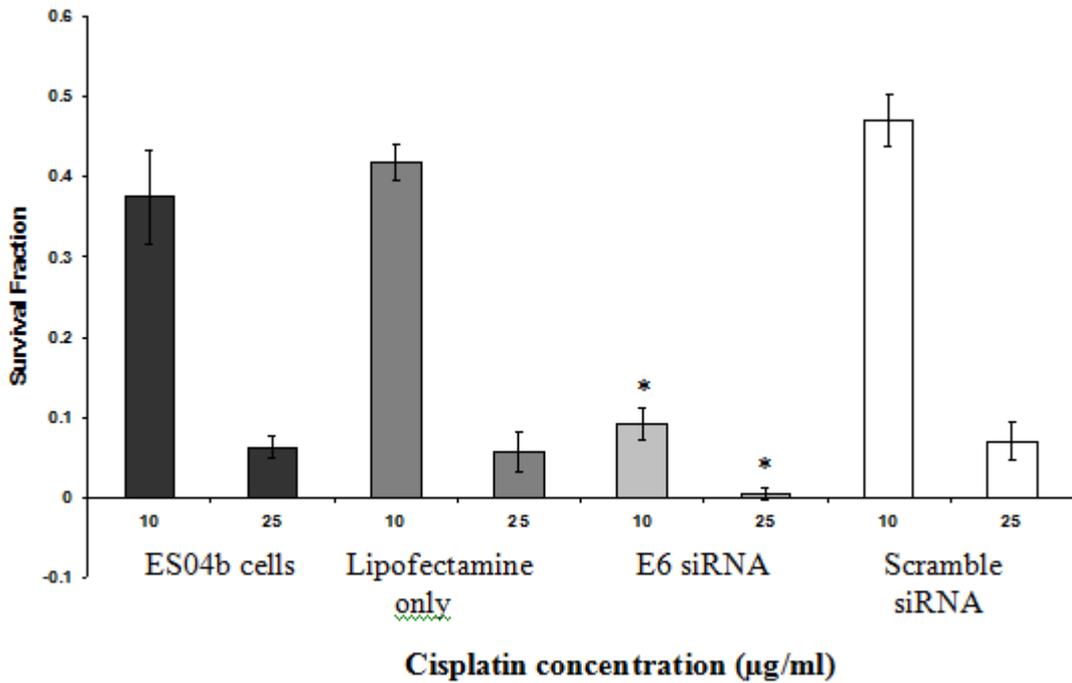


Figure 4.19 Survival Fractions calculated from colony survival data of EqS04b cells after incubation with cisplatin.

* = statistically significant result $p < 0.05$

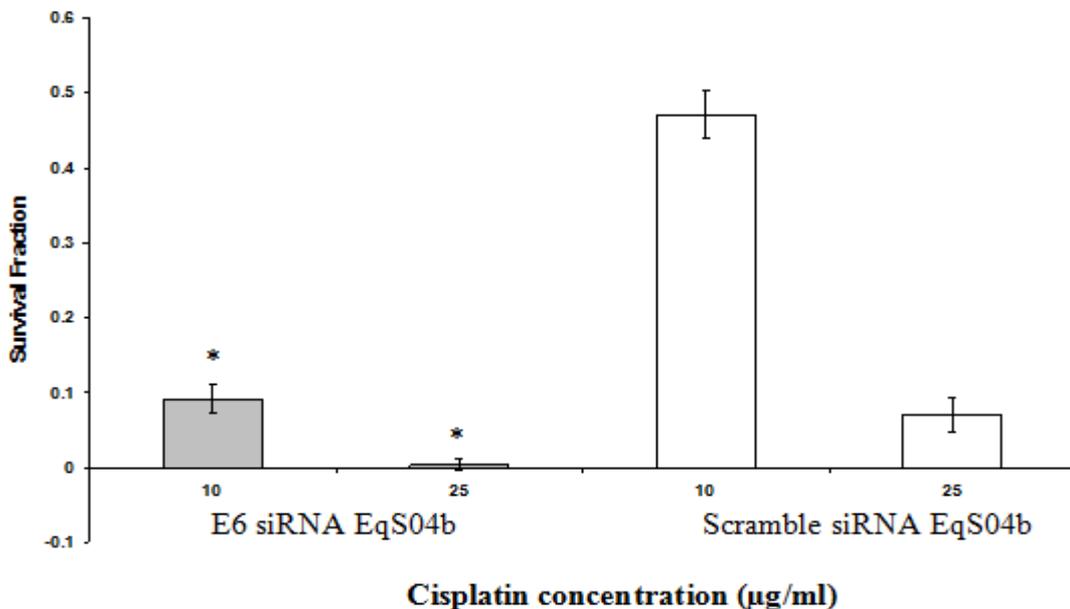


Figure 4.20 Treatment with E6 target siRNA results in reduced cell survival.

Cells treated with E6 siRNA prior to 72 hours incubation with media containing cisplatin demonstrate significantly less potential for long term cell survival when compared to cells treated with a scramble siRNA sequence control. * = statistically significant result $p < 0.05$

4.5 DISCUSSION

4.6 DISCUSSION

Throughout Chapter 4 it is assumed that the overwhelming cell response to the DNA damaging treatments used was apoptosis. It might be argued that sub-populations of the cell lines used may in fact have merely entered cell-cycle arrest rather than the programmed cell death pathway, and these populations of cells, in turn, may affect the cell survival data subsequently obtained. The exact mechanism(s) that determine the fate of a cell during the stress response are not yet fully understood. Intrinsic differences in a cell's response to a particular type of stress may be determined by which effectors are activated under particular conditions (in the case of BPV positive cells – the presence of viral proteins). BPV negative control cells may be more likely to enter cell cycle arrest after DNA damage, and while these cells would not register as apoptotic during FCM assessment, neither would they go on to form colonies in the cell survival experiments. However, during the experiment design process, the different cell populations were assessed by light microscopy for several days following DNA damage by UVB irradiation, and within 3 days of treatment, the majority of cells from all the different cell lines were found to be dead. The levels of exposure to UVB and DNA damaging pharmaceutical drugs, when compared to those previously described in the literature, were considered high enough to anticipate that the levels of DNA damage caused would overwhelm potential arrest and repair pathway options, resulting in the activation of apoptosis pathways in the majority of cases. Therefore, in light of previous work already done which suggests sarcoid molecular pathology is generally not associated with abnormal cell cycle control mechanisms (Nixon *et al.*, 2003), it seems reasonable to interpret the results obtained working under the assumption that apoptosis is the standard cell response.

Apoptosis is induced in all cell lines following treatment with the DNA damaging agents UVB, Cisplatin and 5-FU as shown by FCM analysis. Clonogenic survival assays demonstrate that even with increased apoptosis levels immediately after insult, sarcoid derived cell lines and BPV-1 transformed cell lines are more able to continue to grow and divide even with DNA damage levels that prevent the majority of normal fibroblasts from surviving.

During experiment optimisation, levels of UVB and drug exposure were selected that resulted in a maximum of 40 - 60% cells becoming apoptotic as detected by FCM analysis. In this way, it is expected that comparisons between the clonogenic survival assays can be accurately made. It is possible that the surviving colonies contain clones that are particularly resistant to treatment therapies and the presence of such resistant cells in

naturally occurring sarcoid tumours may help explain why certain tumours on individual animals remain refractory to treatment. We have observed that although the pattern of behaviour is the same for all sarcoid derived cell lines, there is an element of variation within this group. In previous studies, the viral load between cells isolated from naturally occurring sarcoid tumours has been shown to vary by as much as a four-fold difference (Bogaert *et al.*, 2007; Yuan *et al.*, 2008a). This fact may explain the variation in response seen within the sarcoid derived cell lines, and also the more uniform response observed in the experimentally transformed S6 cells where the viral load varies very little between the cell lines. Both naturally and experimentally BPV-1 transformed fibroblasts showed increased susceptibility to apoptosis induced by UVB and cisplatin, however the sarcoid derived cell lines were more resistant to apoptosis induced by 5-FU compared to the experimentally transformed cells. Previous studies looking at the expression of the viral genes E6 and E7 in various cell lines have shown that both proteins can sensitise cells to apoptosis (Liu *et al.*, 2000; Liu *et al.*, 2005). We have shown that infection of BPV-1 in equine fibroblasts sensitises these cells to apoptosis induced by UVB and cisplatin and in BPV-1 transformed fibroblasts treated with 5-FU. Experimentally transformed fibroblasts will almost certainly have a different cellular physiology in comparison to sarcoid derived fibroblasts. The cellular physiology of sarcoid derived fibroblasts will have been influenced and altered by viral gene products for a considerably longer duration than transformed cells. Microarray assessment of sarcoid derived fibroblasts have shown altered expression of over 80 genes in these cells compared to normal equine skin fibroblasts (Yuan *et al.*, 2008). In order to explain the variation between the levels of apoptosis observed with the different DNA damaging agents, it is necessary to consider the different apoptotic pathways that are activated by each individual agent. It is beyond the scope of this investigation to describe each pathway in absolute molecular detail but it is possible to examine these results in the context of one particular gene product which has been shown to be dysfunctional in sarcoid tumours, namely p53 (Nixon *et al.*, 2005). p53 will be discussed in more detail in Chapter V where the location and functionality of p53 is investigated in tumour tissue and tumour derived cell lines. For now, it is sufficient to say that both UVB and cisplatin can induce apoptosis by both p53 dependent and p53 independent pathways (Zamble *et al.*, 1998, Wang *et al.*, 1999). 5-FU however, induces apoptosis in a p53 dependent manner (Pritchard *et al.*, 1998). This basic variation between the mechanisms of apoptosis induction may explain why apoptosis levels are lowest with 5-FU which relies on p53 to induce apoptosis but p53 function is compromised in sarcoid derived cells. In addition to the potential dysregulation of p53 specifically, HMGA-1 was shown in the Yuan (2008b) microarray paper to be up-regulated by nearly 30 fold and

work by Pierantoni et al. in 2007 demonstrated that over-expression of HMGA-1 inhibited p53 apoptotic function by promoting homeodomain-interacting protein kinase 2 (HIPK2) cytoplasmic relocalisation. It seems clear from our data that the extent of apoptosis is not a reliable indicator of cellular sensitivity to these chemotherapeutic agents and so perhaps apoptosis and the genes controlling it, such as p53, appears to play little or no role in the sensitivity of sarcoid derived cells to killing by cisplatin, fluorouracil or UVB-irradiation.

What was particularly intriguing from these results, was that despite having higher initial levels of apoptosis after DNA damage as detected by flow cytometry, the BPV-1 transformed fibroblasts then went on to show significantly higher levels of cell survival in the clonogenic assays, compared to the BPV-negative control fibroblasts. The control fibroblasts generally showed lower levels of apoptosis initially, but then went on to demonstrate a much reduced cell survival potential in the clonogenic studies. Apoptosis (programmed cell death) has for a long time been considered as a one-way process – once begun, there is no turning back. In a recent paper in the British Journal of Cancer, Tang *et al.* (2008) show that apoptosis may be reversible. They had taken a panel of several cancer cell lines and treated them with an inducer of apoptosis for a few hours, and then washed it off. When they monitored the cells over the next hours, they found that many cells transiently exhibit morphological signs of apoptosis: they shrunk, the nuclei condensed, and mitochondria were degraded, but a few hours later the cells returned to their normal shape. The limiting event from which the cells had no chance of return was when degradation of the nucleus had begun.

The hallmarks of apoptosis that were investigated in the above study reflect degradation in three steps: First, the cytoskeleton, leading to cell shrinkage. Then, the mitochondria, shutting off the cell's aerobic metabolism. And finally, the nucleus, with the entire gene regulatory system. But the cytoskeleton is made to be remodelled, so it's not that surprising that the cells can cope. With the mitochondria, the situation is much more complex. Mitochondria are what remain of symbiotic bacteria that in the last couple of thousands of millions of years have become integral parts of the cells of many living beings. In contrast to normal differentiated cells, which rely primarily on mitochondrial oxidative phosphorylation to generate the energy needed for cellular processes, most cancer cells instead rely on aerobic glycolysis, a phenomenon termed 'the Warburg effect' (Kim and Dang, 2006). Aerobic glycolysis is an inefficient way to generate adenosine 5'-triphosphate (ATP), however, and the advantage it confers to cancer cells has been unclear. Theories include the speculations that, compared to healthy cells, glycolytic cells do not need

oxygen for their metabolism and although this is very inefficient it has advantages such as the capability of surviving in environments that do not have vasculature in the vicinity, and the ability to acidify the surrounding environment which may make other cells enter programmed cell death thus providing space in which the glycolytic cells can grow. What is most interesting about the function of mitochondria, in the context of the information determined by our investigations, is that it is involved in one of the principal death-signalling pathways. The mitochondrion acts as an integrating sensor of multiple insult inputs by releasing cytochrome c into the cytosol where it triggers caspase activation. It would be interesting to speculate that when the mitochondrial function is lost or impaired in cancer cells, pro-apoptotic pathways may be shut down too, so that these cells exhibit enhanced survival after an insult that would result in a complete apoptotic result in healthy cells. This theory is reinforced by the results observed in the healthy fibroblast cell line (a control group of cells that was not included in the Hong Kong study!). Low initial levels of apoptosis still translate into a much reduced cell survival outcome suggesting that the majority of cells that enter apoptosis after the initial insult, do not escape from, or reverse this process. Due to the fact the cell membrane disruption and thus exposure of phosphatidylserine has been shown to be an early event in the apoptosis pathway, it seems quite possible that the BPV-1 transformed cells have developed the ability to halt and subsequently reverse the programmed cell death pathway, after the disruption of the membrane occurs (as determined by the FCM analysis). The higher initial levels of apoptosis may be linked to the fact that cells expressing BPV-1 E6 and E7 have been shown to be more susceptible to certain types of induced apoptosis (Rapp *et al.*, 1999; Liu *et al.*, 2000; Liu *et al.*, 2005).

Researchers at the University of Harvard Medical School have created a method based on RNAi in order to restore mitochondrial function, not with the purpose of normalising the metabolism of tumour cells, but for the role they play in programmed cell death. The result of applying this therapy resulted in a surge of tumour cells undergoing apoptosis (Fantin *et al.*, 2006).

Chemotherapy is one of the major cancer treatments and the overall mechanism of action is to induce cancer cells into apoptosis. However, cancer (and sarcoids are notable for this particular trait) may recur even during repeated courses of treatment, and that has been mainly attributed into the deficiency of apoptotic pathways in these affected cells, anticancer drugs resistance of tumorigenic stem cells, and inefficiency of drug penetration into solid tumours (Coley, 2009). These new findings, involving the possibility of

mitochondrial-mediated resistance to apoptosis, uncover another mechanism that may contribute to sarcoid cell survival during therapy and, therefore, suggest potential new avenues for research and targets for therapy.

From the cell survival data, our results suggest that cisplatin is perhaps less suitable as a chemotherapeutic equine sarcoid treatment option *in vivo*. In fact, it could be postulated that using cisplatin may select for particularly resistant cells, resulting in a tumour that is refractory to continued chemotherapeutic treatment. It is interesting to consider the possible synergistic effects of combined cisplatin and 5-FU treatment and future work could assess these effects on the apoptosis levels and, more importantly from a clinical point of view, clonogenic survival levels.

Due to the fact the viral component of the aetiology of sarcoids is now widely accepted, it makes sense to focus future treatment direction towards specific anti-viral agents and/or other specific anti-viral immune response modifiers, instead of toxic chemotherapeutic agents and expensive surgical intervention. Our investigation to identify the viral protein responsible for conferring the enhanced survival ability, and then assessing this as a potential therapeutic target using siRNA technology, identified the BPV E6 as a gene of interest.

The siRNA sequence that was designed to target BPV-1 E6 showed consistent E6 knockdown levels of over 75%. The sequence used to target E6 (shown in Chapter 2; section 2.2.8.2) was also shown to consistently knock down the BPV-1 genes E2, E5 and E7 (Figure 4.12). Cells that were treated with E6 siRNA 24 hours prior to DNA damage showed very little difference in the apoptosis levels detected using flow cytometry, compared to the untreated sarcoid control cells and E6 Scramble sequence siRNA treated cells. However, once those cells were cultured in dishes for the clonogenic assay, it became evident that the E6 siRNA treated cells were unable to survive the initial DNA damage and there was significantly less colony formation with these cells, compared to untreated, and E6 Scramble sequence siRNA treated sarcoid cells.

5 CHAPTER V:

The p53 protein in equine sarcoids

5.1 INTRODUCTION

In the 1980's, the p53 tumour suppressor protein was identified as having a key role in the early stages of neoplastic disease development and progression, and today it continues to be an ever expanding area of research (with only three studies listed by Med Line in 1989 but 1027 listed in 1997). Since 1989, over 570 different mutations of p53 have been identified, in more than 8000 human cancers (Hainaut *et al.*, 1998) with the listing data base being updated twice a year.

The p53 protein has become the subject of such intense investigation for several reasons; p53 is the most commonly altered gene yet discovered in human cancer (Vogelstein and Kinzler, 1992; Hollstein *et al.*, 1991); it suppresses the growth of tumour cells containing multiple genetic changes (Baker *et al.*, 1990); germ line mutation of p53 has been linked to an inherited predisposition to cancer (Li-Fraumeni syndrome) (Malkin *et al.*, 1990; Srivastava *et al.*, 1990); increased amounts of cellular p53 protein after DNA damage have been associated with cell-cycle arrest (Kastan *et al.*, 1991) and programmed cell death (apoptosis) (Shaw, *et al.*, 1992); and mutations or losses of p53 have been associated with gene amplification (Livingstone *et al.*, 1992; Yin *et al.*, 1992) and polyploidy. Given its ability to monitor DNA damage and to promote damaged cell's responses (by repair and continued survival, or death), p53 has been called 'the guardian of the genome' (Lane, 1992). Discoveries about the biological properties of p53, and the molecular mechanisms of its functions, have given p53 much attention among tumour suppressor genes. P53 was chosen as 'molecule of the year' in 1993 by Science (Koshland, 1993).

p53 was first identified in 1979 by virtue of its association with simian virus 40 (SV40) large T-antigen and by its apparently high expression in chemically induced tumours or spontaneously transformed cells (Lane and Crawford, 1979; Linzer and Levine 1979). For the next decade, p53 was thought to be a transforming oncogene whose expression resulted in increased DNA synthesis, a necessary feature of cellular proliferation. As early as 1984 Maltzman and Czyzyk observed that exposure of non-transformed cells to UV radiation stimulated p53 production, on the basis of a post translational stabilization (Maltzman and Czyzyk 1984) and by 1987 p53 association with cellular heat shock protein had been observed in transformed cells (Hinds *et al.*, 1989). By 1989 several lines of evidence were beginning to suggest that the wild-type p53 gene was actually a tumour suppressor gene instead of an oncogene. Over the past several years, studies have begun to clarify the molecular basis for tumour suppression by p53, as well as provide insight into the role of

p53 in normal growth control. An ever increasing number of p53-associated proteins as well as p53-regulated genes have been identified. Scores of studies have catalogued mutations of p53 in human tumours. The value of early detection of p53 gene and gene product alterations, and their utility in assessing prognosis, are currently being explored.

5.1.1 Molecular Structure of the p53 Gene

The term 'p53' was originally given to the phosphoprotein of molecular weight 53 kDa (Lane and Crawford 1979). The protein was discovered before the gene, but both gene and its protein are called p53. The p53 gene is comprised of 11 exons within a chromosomal domain of 20 kilobases (Reisman *et al.*, 1988). Reisman *et al.* (1988) identified 2 promoters; the first is located 100 to 250 bp upstream of the non-coding first exon and is responsible for transcription of the major p53 mRNA species. The second (p53p2), is a stronger promoter than the first and is located within the 10,738-bp first intron, approximately 1,000 bp downstream of exon 1.

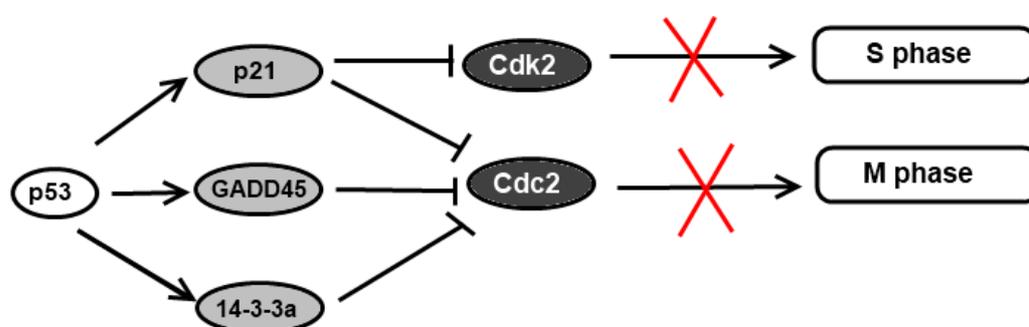
5.1.2 Role of p53 in Normal Cells

In normal cells, an equilibrium is established between growth, as determined by cell progression through the cell cycle, cellular differentiation, and restriction of cell growth (Evan and Vousden, 2001). Apoptosis is a mechanism commonly employed in normal cells to control cellular numbers (Elmore, 2007). Although it is often a common feature of tumour development, escape from apoptosis is not essential for tumour growth and progression. Tumour cell accumulation will occur if an increase in cell proliferation exceeds cell loss produced by apoptosis (Darnton, 1998). The central influence of p53 on cell proliferation is via control of cell cycle with wild-type p53 primarily involved with this control, and in the control of apoptosis. Studies of p53 have shown that it is not essential for normal growth, differentiation, and function because transgenic mice, with both copies of p53 deleted, develop normally but multiple tumours appear after a few months of life (Donehower *et al.*, 1992). After DNA damage, there is a possibility that cells with oncogenic mutations will survive and continue dividing if no measures were in place to prevent their cell cycle progression. However, in normal cells following DNA damage such as that caused by UV light, ionising radiation, or chemotherapeutic agents, levels of p53 protein rise rapidly (Maltzman and Czyzyk, 1984; Kastan *et al.*, 1991; Linke *et al.*, 1996). The wild-type p53 protein binds to p53 binding sites on DNA and activates the expression of downstream genes that negatively control growth and/or invasion. Cell entry into the S-phase (DNA replication) is blocked by expression of these downstream targets, most notably p21, resulting in arrest of the cell cycle in the G1 phase. This is achieved

primarily through inhibition of cyclin dependent kinases (CDKs) and proliferating cell nuclear antigen (PCNA) (Harper *et al.*, 1993; El-Deiry *et al.*, 1993; Xiong *et al.*, 1993). The resulting growth arrest allows time for DNA repair or, if repair is unsuccessful, then wild-type p53 can trigger apoptosis (Vogelstein and Kinzler 1992).

Details of the wild-type p53 response to DNA damage are still not fully known. What is known, is that recently discovered p53 pathways and interactions are making the picture ever more complex. p53 is known to act as a cell cycle checkpoint and to induce apoptosis by downstream trans-activation of a multiplicity of genes (Ruley, 1996). A simplified summary of these cellular interactions is given in Figure 5.1.

(a) Growth Arrest



(b) Apoptosis

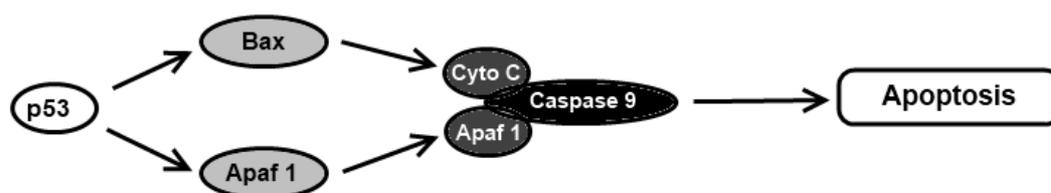


Figure 5.1 Cellular interactions of p53 [Source: Adapted from www.web-books.com].

(a) The cell cycle progression into the S phase requires the cyclin-dependent kinase enzyme Cdk2, which can be inhibited by p21. The progression into the M phase can also be inhibited by p21, GADD45 (a DNA damage responsive gene) or 14-3-3 (a protein that binds to other regulatory proteins to regulate cell cycle progression). P53 regulates the expression of these inhibitory proteins to induce growth arrest.

(b) Apoptosis can be induced by the binding of Caspase 9 (an initiator caspase in the mitochondrial death pathway) to cytochrome c (an essential part of the electron transfer chain) and Apaf1 (Apoptotic protease activating factor 1). P53 may activate the expression of Apaf1 and Bax (pro-apoptotic member of the Bcl-2 family). The latter can then initiate the release of cytochrome c from mitochondria.

5.1.3 P53 Dependent Apoptosis

In solution, the p53 protein aggregates as a tetramer and has a short half-life of approximately 20 minutes in non-stressed cells. This is due to its association with a protein, Mdm2, which targets it for degradation by virtue of its E3-ubiquitin ligase activity. Triggers for p53 dependent apoptosis include DNA damage, inappropriate oncogene activation, certain cytokines (Eizenberg *et al.*, 1995) or cytokine deprivation (Canman *et al.*, 1995), hypoxia (Canman *et al.*, 1995) and heat shock (Graeber *et al.*, 1994). In response to stress, p53 becomes post-translationally modified, initially by phosphorylation and later by acetylation. These modifications cause an increase in p53 half-life, and activate p53 as a transcription factor. Mutated p53 also has an increased half-life resulting in the positive immunostaining of p53 in cells containing mutated copies and in S-phase cells (Momand *et al.*, 2000). While much remains to be learnt about the mechanisms by which p53 senses damage there is evidence that the cell cycle checkpoint kinase, Chk2/hCds1, when in contact with damaged DNA becomes activated and phosphorylates p53 on serine 20. This results in dissociation of preformed complexes of p53 with MDM2. The activated form of Chk2/hCds1, but not inactive mutants, causes G1 arrest in cells containing wild type but not mutant p53 (Chehab *et al.*, 2000). These results are consistent with other reports that the inactivation of Chk2/hCds1 in knockout mice renders them unable to mount a p53 response after irradiation (Hirao *et al.*, 2000), and that merely increasing the amount of p53 within the cell (which may be considered to be comparable to stabilization of the protein) was insufficient to increase transcription of p53 activated genes. Transcriptional activation only occurs after post-translational modification induced by DNA damage (Xiao *et al.*, 2000). At present it is not clear if the checkpoint kinase itself senses DNA damage or responds to unknown additional signals. Activation of p53 as a transcription factor results in increased transcription of several genes involved in DNA repair, G1 arrest and apoptosis. Events concerned with DNA repair and G1 arrest are the best understood. Transcription of GADD45 by activated p53 produces a protein which can recognise an altered chromatin state and cause destabilisation of histone DNA interactions by interacting directly with the four core histones. The expected result is increased accessibility of damaged DNA to proteins involved in repair processes (Carrier *et al.*,

1999). The role of GADD45 in DNA repair is further suggested from studies in mice in which this gene was deleted; these mice have increased genome instability and increased incidence of carcinogenesis (Hollander *et al.*, 1999). Cells from these knockout animals were also deficient in ultraviolet repair (Smith *et al.*, 2000). GADD45 also appears to have a second function in causing G2 arrest, probably by its known association with the Cdc2-cyclin B1 protein complex, causing its dissociation as a result of direct interaction between the GADD45 AND CDC2 proteins (Jin *et al.*, 2000). P53 also transcriptionally activates expression of p21, which as discussed above, binds to a number of cyclin/Cdk kinases so as to inhibit their phosphorylation of retinoblastoma protein (Rb) and thus inhibit G1/S-phase transit (el Deiry *et al.*, 1993; el Deiry *et al.*, 1994). Thus activation of p53 induces genes which cause cell cycle arrest, thereby allowing time for DNA repair, and also modifies the state of chromatin so as to allow access of repair proteins to DNA. Mechanisms by which p53 stimulate apoptosis are poorly understood as are the conditions that determine whether p53 should signal cell cycle arrest, a reversible phenomena, or apoptosis which is historically understood to be irreversible (although this understanding has been challenged recently, as discussed in Chapter IV). One of the problems appears to be that different cells respond differently to stressful stimuli making creation of an overall model difficult. There is also evidence that p53 can trigger apoptosis by mechanisms requiring transcription and also by transcription-independent mechanisms. Apoptosis is triggered by changes in mitochondrial membrane potential leading to release of cytochrome c, calcium and other factors. Recent studies have shown that p53 localises to the mitochondria at the onset of p53-dependent apoptosis but not during p53-independent apoptosis or p53-mediated cell cycle arrest. Moreover, targeting of p53 to mitochondria using peptide leader sequences is sufficient to induce apoptosis, strongly suggesting a central role for this translocation (Marchenko *et al.*, 2000). In summary, p53 induces cell death by a multitude of molecular pathways involving activation of target genes and transcriptionally independent direct signalling, and it is clear that much remains to be learnt about the mechanisms of p53 induced apoptosis.

5.1.4 Papillomaviruses and the p53 protein

In Human Papillomavirus (HPV) infections, E6 is expressed early after viral infection and facilitates viral production by implementing several cellular changes. It can prolong the cellular lifespan via inhibition of apoptosis and by increasing telomerase activity. HPV E6 interacts with an ubiquitin-3 ligase and the E6 associated protein (E6-AP) to form complexes with p53. The p53 is then degraded by way of a ubiquitin-dependent proteolytic mechanism (Huibregtse *et al.*, 1993; Scheffner 1998). E6 can also block translocation of

p53 into the nucleus (Mantovani and Banks 1999) and thereby inhibit the gene regulatory functions of p53. The consequence of p53 degradation and blocking of p53 transport into the nucleus is disruption of p53 mediated cell cycle control and the cell can continue to divide in spite of DNA damage. In contrast, BPV E6 does not appear to bind to p53 (Scheffner *et al.*, 1990; Rapp *et al.*, 1999). However, BPV-1 E6 has been shown to interact with CBP/p300 in the same way as described for the E6 proteins of oncogenic human papillomaviruses. This interaction results in an inhibition of the transcriptional co-activator function of CBP/p300 required by p53 and probably by other transcription factors (Zimmermann *et al.*, 2000).

The loss of the normal tumour suppressive properties of the p53 protein has been reported in a wide variety of human cancers (Hollstein *et al.*, 1994) and in some tumours of domestic animals (Teifke and Lohr, 1996). Several investigators have examined the role of the tumour suppressor gene p53 in equine sarcoids. Bucher *et al.* (1996) failed to detect p53 gene mutations in equine sarcoid tumours, suggesting that p53 does not play a significant role in the pathogenesis of sarcoids. However, more recently, aberrant perinuclear localization of p53 has been demonstrated in 44 % of equine sarcoid lesions (Martens *et al.*, 2000), suggesting that mutational independent inactivation of p53 occurs commonly in sarcoids. p53, a key protein in tumourogenesis, has previously been shown to be dysfunctional and abnormally located in a subset of sarcoids, and the transactivation function of p53 was shown to be compromised in cells with cytoplasmic p53 (Nixon *et al.* 2005).

5.2 AIMS AND OBJECTIVES

The aims of this chapter were to;

- i) To evaluate p53 protein expression in equine sarcoid tumour tissue, cultured tumour derived equine fibroblasts and BPV-1 transformed equine fibroblast cell lines.
- ii) Determine what, if any, correlation exists between p53 protein expression and the clinical presentation of tumours.
- iii) To examine the functionality of p53 protein in equine sarcoid derived equine fibroblasts and BPV-1 transformed equine fibroblast cell lines

5.3 MATERIALS AND METHODS

5.3.1 Cell Culture

Normal equine fibroblasts (EqPalF cells), sarcoid derived BPV-1 transformed equine fibroblasts (EqS cells) and experimentally transformed equine fibroblasts (S6 cells) were cultured and maintained as described in Chapter II; section 2.2.1.

5.3.2 RQ-PCR to determine p53 transcription levels

RNA extraction from cultured cells was carried out as described in section 2.2.3.1. From the RNA, cDNA was synthesised (section 2.2.3.1.4) and analysed by RQ-PCR (section 2.2.5) using the p53 primer and probe set (Table 2.4).

5.3.3 Immunohistochemistry (IHC)

Immunohistochemistry (IHC) protocols for the staining of p53 in cultured cells and sarcoid tumour sections have been described in section 2.2.9. The sarcoid tumours were sent to our research lab from the University of Liverpool, Department of Veterinary Medicine. The tumours had been histopathologically confirmed as sarcoid tumours. An equine squamous cell carcinoma tumour was also sent from the University of Liverpool to be used as a positive control. There were 52 tumour pieces altogether; 51 sarcoid tumours and 1 squamous cell carcinoma.

5.3.4 Western Blot (WB)

A standard Western blot (WB) protocol is also described in section 2.2.10.2. For assessment of p53 response to UVB, cells were irradiated with 250 mJ/cm² UVB (process described in section 2.2.1) and then left for 4 hours before being harvested for WB. For assessment of p53 transactivation activity, cells were irradiated with a much lower dose of UVB (30 mJ/cm²), incubated overnight (~ 10 hours) and then harvested to look for the presence of p21 protein. The p21 protein was initially identified as a cell cycle regulatory protein that can cause cell cycle arrest. Expression of p21 has been shown to be up-regulated by p53 in response to DNA-damaging agents (Vouden and Lu, 2002; Liu *et al.*, 2003). Due to the difference in molecular weight of the two target proteins, p53 and p21, it was possible to probe for both proteins on the same single membrane without having to resort to stripping the membrane between looking at each protein. After protein transfer from the gel to the membrane (section 2.2.10.2.3), the membrane was cut midway between the positions of the expected bands, and after blocking, the two halves of the membrane were separated and incubated with their respective specific antibodies as described in the

WB methodology (section 2.2.10.2.5) GAPDH was used as a loading control in all WB analysis. GAPDH has a molecular weight of ~ 40 kDa and splitting of the membrane when simultaneously probing for p53 (band of ~ 50 kDa) and GAPDH needs to be done carefully.

5.3.5 Half life determination

Cycloheximide is an inhibitor of protein biosynthesis in eukaryotic organisms, produced by the bacterium *Streptomyces griseus*. Cycloheximide exerts its effect by interfering with the translocation step in protein synthesis (movement of two tRNA molecules and mRNA in relation to the ribosome) thus blocking translational elongation. Cycloheximide is widely used in biomedical research to inhibit protein synthesis in eukaryotic cells studied in vitro (en.wikipedia.org).

Due to significant toxic side effects, including DNA damage, teratogenesis, and other reproductive effects, including birth defects and toxicity to sperm (Department of Health Services, Hazard Evaluation System and Information services, California), cycloheximide is generally used only in in vitro research applications, and is not suitable for human use as a therapeutic antibiotic compound. Although it has been used as a fungicide in agricultural applications, this application is now decreasing as the health risks have become better understood.

Cycloheximide can be used as an experimental tool in molecular biology to determine the half-life of a protein. Treating cells with cycloheximide in a time-course experiment followed by Western blotting of the cell lysates for the protein of interest can show differences in protein half-life. Cycloheximide treatment provides the ability to observe the half-life of a protein without confounding contributions from transcription or translation.

For p53 half-life experiments, fibroblasts cell monolayers in 10cm Petri dishes were treated with 40 µg cycloheximide per ml of medium approximately 48 hours after the fibroblasts were seeded. Zero time points were harvested immediately using Trypsin - EDTA, and subsequent time points were incubated in medium containing cycloheximide at 37 °C for 30, 60, 120 or 300 min as indicated. Cells were then processed as described in section 2.2.10.2. Images were digitally acquired using an HP ScanJet 5200C Scanner (Hewlett-Packard) and quantified using NIH ImageJ Software analysis (NIH). The levels of p53 were normalized against the corresponding GAPDH level.

5.4 RESULTS

5.4.1 Equine sarcoid tumours show abnormal nuclear/cytoplasmic staining

To evaluate p53 expression in equine sarcoids, 51 tumour samples (Chapter V; section 5.5.3) were stained for p53 as described in Chapter II; section 2.2.9. Figure 5.2 A and B shows a tumour section of an equine squamous cell carcinoma to demonstrate the usefulness of this tumour type as a positive control in p53 IHC. Figure 5.2C illustrates the importance of having a corresponding negative control slide for each sarcoid section that was stained. Figure 5.2C is a normal equine skin section with evidence of extensive brown staining throughout. This is not p53 however, but the natural pigment melatonin which may be misinterpreted as a positive result, if there was no negative control slide to compare it to. The melanin would appear as the brown staining in a positive slide section, and the negative control slide, but p53 would only be present in the positive slide.

Having established from the positive control slides (equine squamous cell carcinoma; Figure 5.2 A and B) that the D0-7 p53 antibody is reliable in detecting equine p53 in equine tumour tissue sections, all 51 tumour samples from the University of Liverpool were submitted to the University of Glasgow Pathology Department for IHC processing and staining with D0-7. Figure 5.3 shows the range of p53 staining observed in analysis of the sarcoid tumour sections. Some sections showed no positive staining at all (Fig. 5.3A), others showed a low percentage (1 – 10%) of p53 positive nuclei (Fig. 5.3B), a medium percentage (10 – 20%; Fig. 5.3C), or a high percentage (> 20%; Fig. 5.3D). The percentage of p53 positive cells were calculated from 3 high-power fields of each tumour and the results are tabulated in Table 5.1. A more detailed description of the IHC slide analyses is given in section 2.2.9.1. Table 5.1 shows that different tumours removed from the same animal, can demonstrate different degrees of p53 positivity. For example, animal D had tumours which were mostly p53 negative, but it was also the source of one sarcoid tumour with a low percentage of p53 staining, and another tumour with a medium percentage of p53 staining.

The majority of tumour sections stained revealed exclusively nuclear localisation of the p53 protein. However one tumour, ID 51, demonstrated abnormal peri-nuclear staining as shown in Figure 5.4.

Figure 5.2

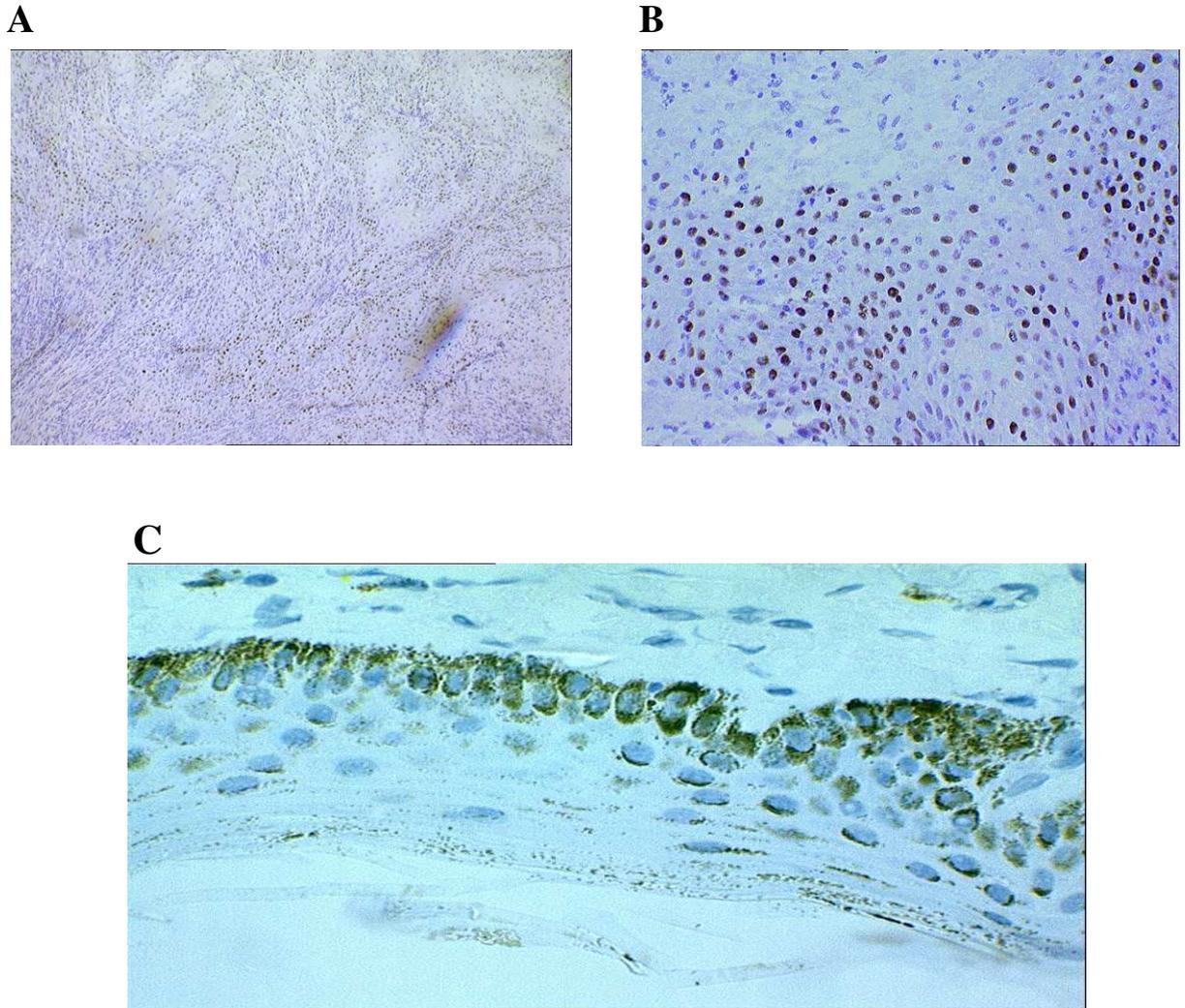


Figure 5.2 Tissue sections following immunohistochemical staining with the D0-7 p53 antibody.

A – equine squamous cell carcinoma (x 40).

B – equine squamous cell carcinoma (x 100). Note the nuclear accumulation of p53.

C – cross section through normal equine skin (x 200). The brown staining on this slide is due to the presence of the naturally occurring skin pigment, melanin. To avoid confusing melanin staining with p53 positive staining in the tumour sections, each tumour section was stained with a p53 negative control solution and any p53 positive tumour slides were compared to the corresponding negative control section to prevent false positives being recorded.

Figure 5.3

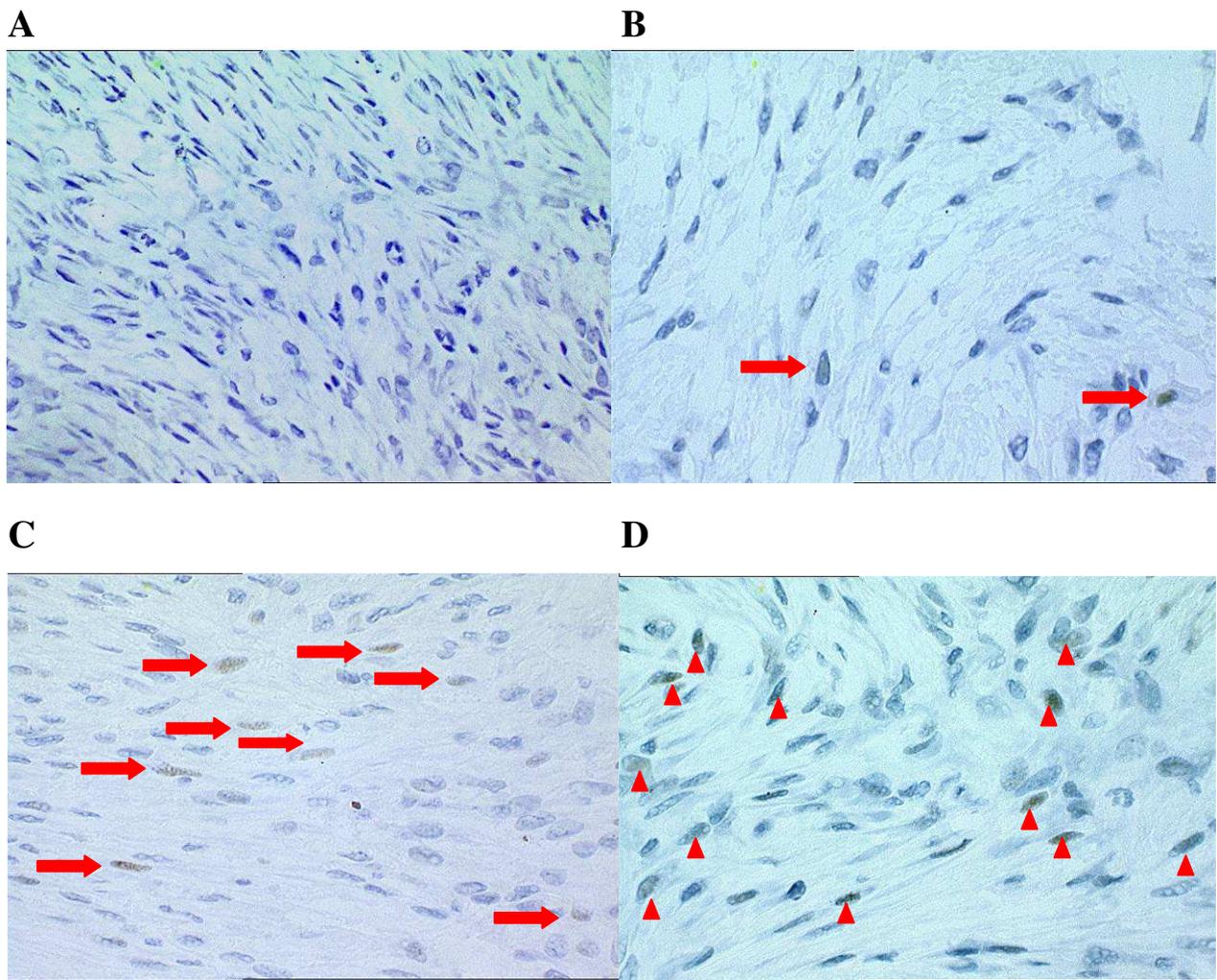


Figure 5.3 Sarcoid tumour tissue sections stained with D0-7 p53 antibody.

Some tumours showed no staining at all (A). Others were found to show a range of percentages of p53 positive nuclei. P53 positive nuclei are indicated in the images above by the red arrows. Figure B demonstrates a tumour with a 'low' percentage of positive nuclei (1 – 10%), figure C is a tumour with a 'medium' percentage of positive nuclei (10 – 20%) and tumours such as that shown in figure D were categorised as having a 'high' percentage of positive nuclei (> 20%). The numbers of tumours determined to be in each category are shown in Table 5.1.

Table 5.1 p53 positivity and clinical classification of the tumour samples.

Tumour ID	Classification	Animal ID	Clinical Type
1	0	A	Pendulous fibroblastic (Fig 1.1f(ii))
2	0	A	Pendulous fibroblastic
3	0	B	Exterior of tumour A; A = nodular/fibroblastic (mixed)
4	0	B	Margin of tumour A (mixed)
5	0	B	Base of tumour A (mixed)
6	LOW	B	Exterior of tumour B; B = nodular/fibroblastic (mixed)
7	LOW	B	Margin of tumour B (mixed)
8	0	B	Base of tumour B (mixed)
9	MED	C	Isolated nodular lesion
10	LOW	C	Fibroblastic lesion
11	MED	C	Fibroblastic lesion
12	SCC	D	SCC
13	LOW	D	Nodular
14	0	D	Nodular
15	MED	D	Nodular
16	0	D	Nodular
17	0	D	Verrucose
18	0	D	Mixed
19	MED	D	Fibroblastic
20	0	D	Occult
21	MED	E	Data unavailable
22	MED	E	Data unavailable
23	MED	E	Data unavailable
24	HIGH	E	Data unavailable
25	HIGH	F	Fibroblastic
26	MED	F	Fibroblastic
27	0	F	Fibroblastic
28	HIGH	F	Fibroblastic

29	0	F	Nodular
30	0	F	Nodular
31	0	G	Nodular
32	0	G	Nodular
33	0	G	Nodular
34	0	G	Nodular
35	0	H	Verrucose
36	HIGH	H	Fibroblastic
37	0	I	Verrucose
38	0	I	Nodular
39	MED	I	Nodular
40	0	J	Data unavailable
41	LOW	J	Data unavailable
42	LOW	J	Data unavailable
43	0	J	Data unavailable
44	0	J	Data unavailable
45	HIGH	K	Fibroblastic
46	0	K	Mixed
47	HIGH	K	Fibroblastic
48	MED	K	Nodular
49	HIGH	K	Nodular
50	HIGH	K	Fibroblastic
51	0	K	Nodular
52	HIGH*	L	Malevolent sarcoid

Table 5.1 shows p53 positive rating and clinical presentation for all of the 51 individual sarcoid tumour samples sent from Liverpool University (tumour ID 12 was a SCC and so is not included in analysis of the p53 staining data).

* = peri-nuclear staining evident (see Fig. 5.4)

SCC = squamous cell carcinoma (confirmed by histopathology)

LOW = 0 – 10% of cells have p53 positive nuclei

MED = 10 – 20% positive

HIGH = > 20% positive

Figure 5.4 below shows the peri-nuclear nature of the p53 staining observed in tumour 51.



Figure 5.4 p53 staining of sarcoïd tumour ID 52. Granular, peri-nuclear/cytoplasmic p53 has been circled in red.

5.4.2 High Levels of p53 positivity is associated with clinically aggressive tumours

Over half the tumours (51 %) were negative for p53 and represented all clinical types of tumour (with the exception of the malevolent type; Table 5.2). Table 5.2 illustrates that the majority of tumours that exhibit high levels of p53 positive staining appear to be associated with primarily fibroblastic and mixed lesions. These lesions are generally thought of as more aggressive in their clinical behaviour than other types.

Table 5.2 Association of p53 positivity with clinical type (n = 42).

p53 score (A)	No. of tumours (B) (%)	No. of (A) that are occult (%)	No. of (A) verrucose (%)	No. of (A) nodular (%)	No. of (A) fibroblastic (%)	No. of (A) mixed (%)
0	23 (54)	1 (5)	2 (9)	10 (44)	5 (21)	5 (21)
LOW	4 (9.5)	0 (0)	0 (0)	1 (25)	1 (25)	2 (50)
MED	8 (20)	0 (0)	0 (0)	2 (25)	6 (75)	0 (0)
HIGH	7 (16.5)	0 (0)	0 (0)	1 (15)	6 (85)	0 (0)

5.4.3 Sarcoid derived cell lines also exhibit abnormal nuclear/cytoplasmic protein expression.

To compare and contrast *in vivo* p53 staining results with *in vitro* p53 staining of BPV-1 transformed fibroblasts, normal and BPV-1 transformed equine fibroblast cells were grown and maintained in monolayer cell culture as described in section 2.2.1. To evaluate p53 expression in these cell lines, IHC staining for p53 was carried out as described in section 2.2.9. Having consulted the literature regarding the use of p53 antibodies in investigating p53 in equine cells, the D0-7 clone is a monoclonal antibody raised in mice that binds to human p53 wild type and mutant forms under both denaturing and non-denaturing conditions. The D0-7 clone has been successfully implemented in several equine p53 studies (Martens *et al.*, 2000; Bogaert *et al.*, 2007). As shown in Figure 5.5 and subsequent immunohistochemistry and Western blot images, this antibody works well in both IHC and Western blot applications.

Figures 5.5 to 5.8 show the staining patterns observed in all of the cell lines studied. As expected, no p53 staining was detected in the control equine fibroblasts (EqPalF cells; Fig.5.5). As seen in figures 5.6 and 5.7, the experimentally transformed fibroblasts and the sarcoid derived fibroblasts (EqS01 – 03) all show p53 positive nuclei to varying degrees. The percentage of p53 positive cells present in each individual cell line was calculated from the average seen in 3 different high powered fields. The results of this analysis are shown in Figure 5.9. The EqS04b cells show a different pattern of staining to the other BPV transformed fibroblasts (Fig. 5.8) exhibiting an entirely peri-nuclear expression of the p53 protein. Due to this peri-nuclear expression, the EqS04b cells were excluded from the quantitative analysis summarised in Figure 5.9.

Figure 5.5.

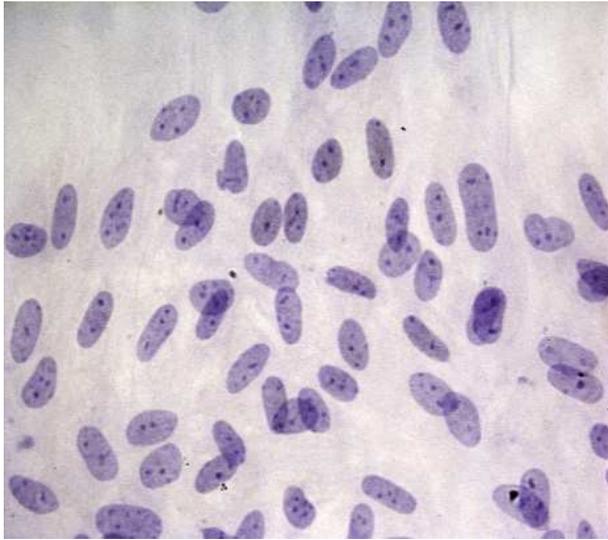


Figure 5.5 Staining of control fibroblasts (EqPalF) with D0-7 p53 antibody.

Figure 5.6.

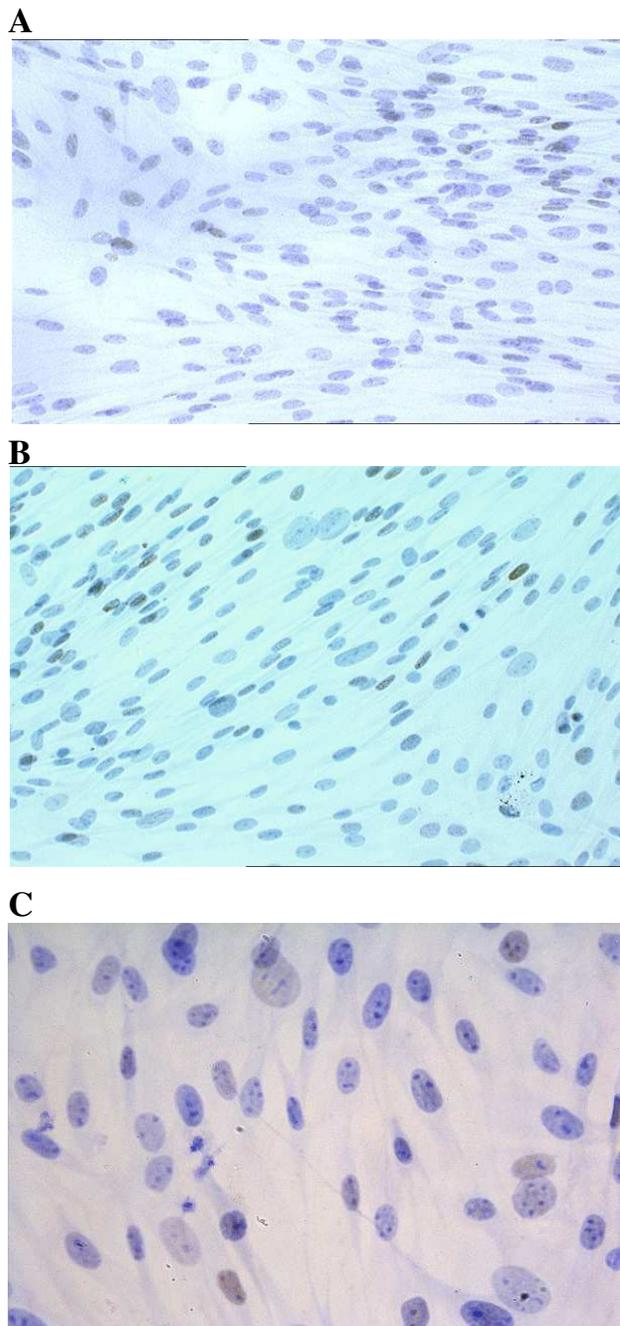


Figure 5.6 p53 staining of experimentally transformed equine fibroblasts (S6 cells).

A – S6-1 cells, untreated;

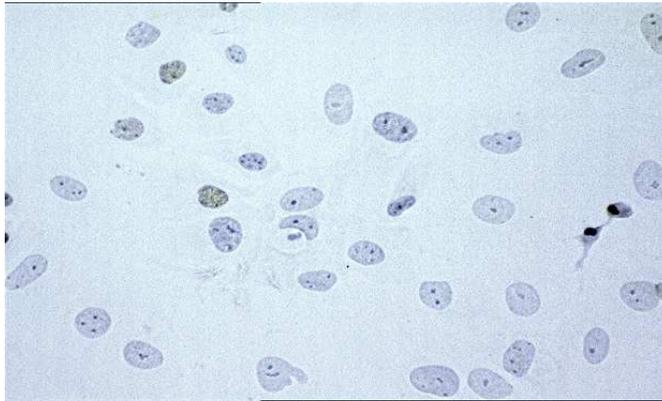
B - S6-2 cells, untreated;

C - S6-3 cells, untreated;

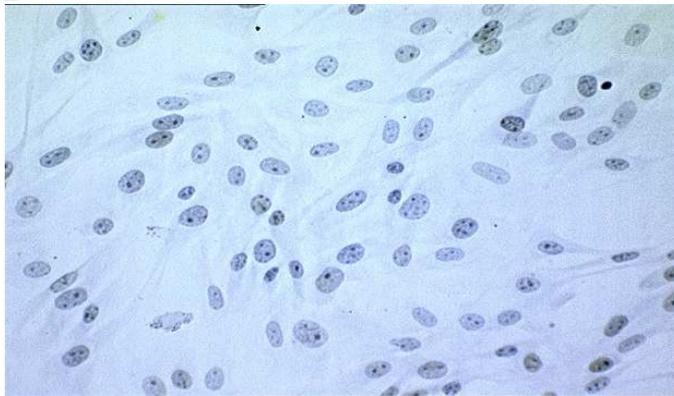
There is a relatively higher percentage of p53 positive nuclei in these cells. The differences in p53 positivity between the different cell lines is quantified in Figure 5.9

Figure 5.7.

A



B



C

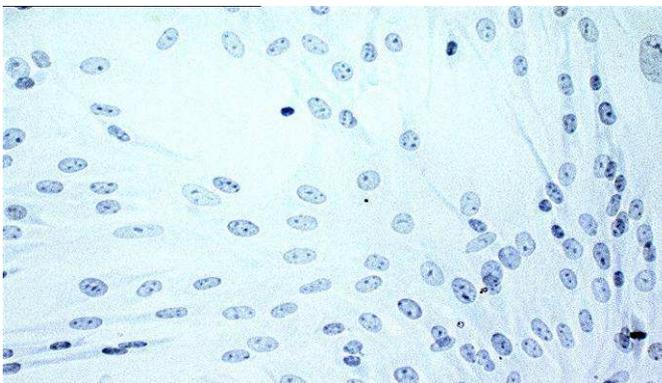


Figure 5.7 p53 staining of sarcoid tumour derived fibroblast cell cultures (EqS cells).

A – EqS01 cells, untreated;

B – EqS02 cells, untreated;

C – EqS03 cells, untreated;

As observed in the experimentally transformed fibroblasts (S6 cells), there is also a higher percentage of p53 positive nuclei present in the EqS cell lines (summarised in Figure 5.9).

Figure 5.8.

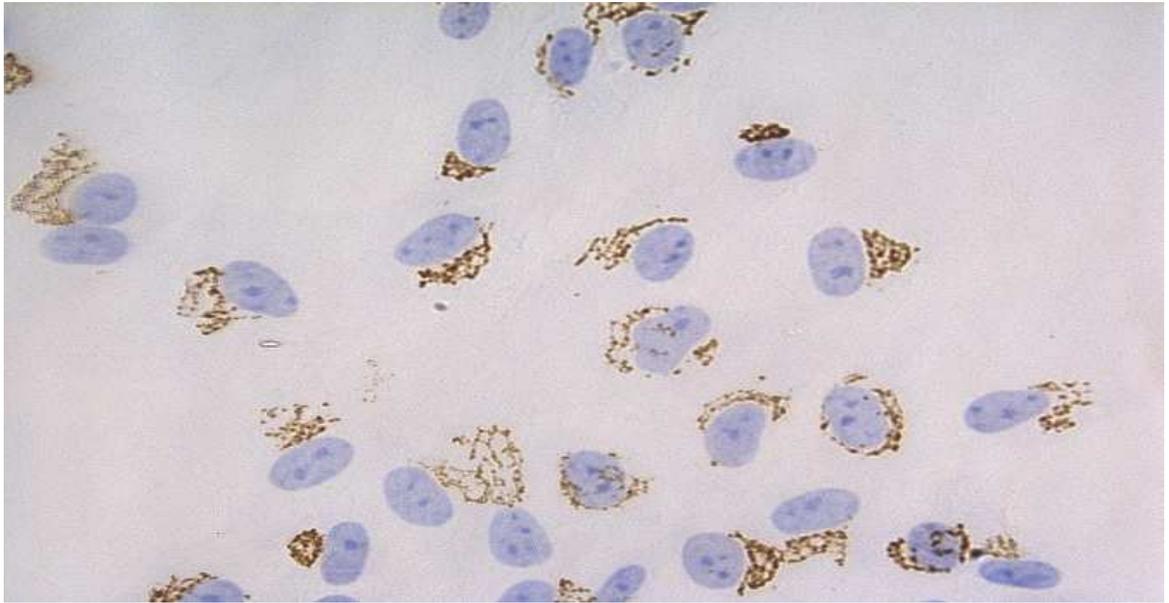


Figure 5.8 p53 staining in sarcoid derived fibroblast cell line EqS04b.

In the equine sarcoid derived fibroblast cell line EqS04b, p53 staining reveals that *all* the cells present exhibit peri-nuclear expression of the p53 protein.

5.4.4 BPV-1 transformed fibroblasts exhibit a significantly higher percentage of p53 positive nuclei compared to the control fibroblasts as determined by IHC

Analysis of the p53 stained fibroblast culture involved counting the number of p53 positive nuclei present per high powered field, and expressing this as percentage of the total number of cells present in that field. A total of 3 different images for each cell population were analysed. Figure 5.9 shows the results obtained from this analysis.

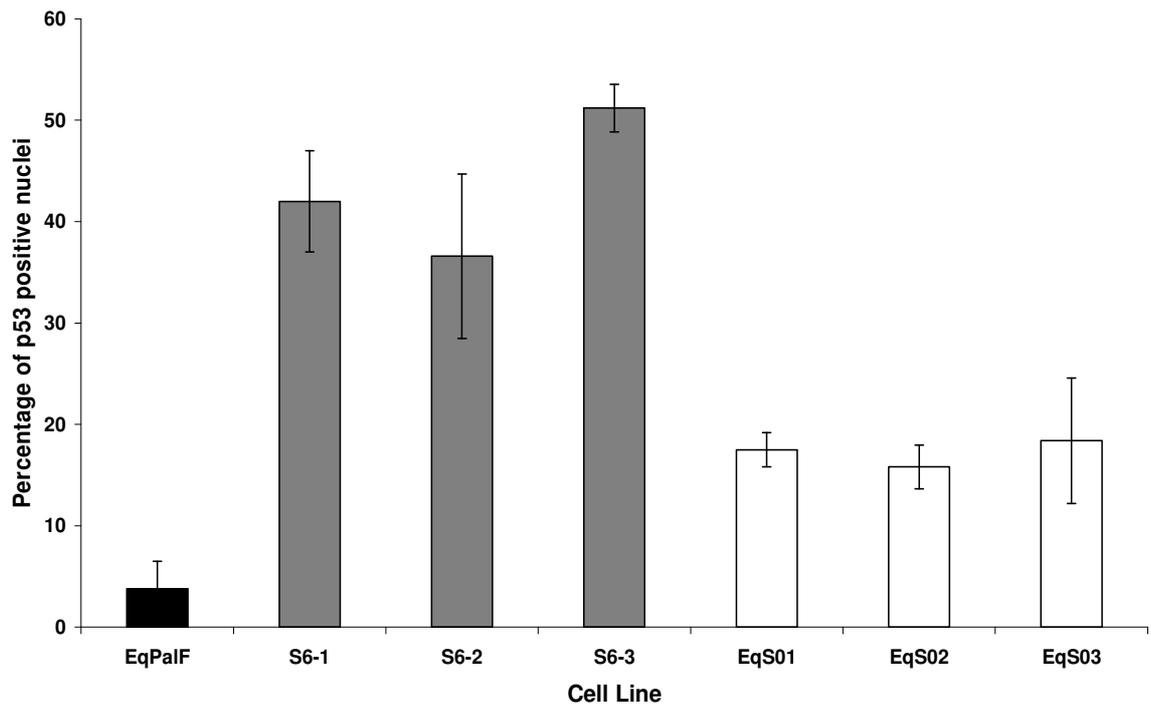


Figure 5.9 The average percentage of p53 positive nuclei present in the different untreated cell populations.

The normal equine fibroblast cell line (EqPalF) demonstrated a significantly lower percentage of p53 positive nuclei compared to both the BPV-1 transformed cells (S6-1, S6-2, S6-3) and the sarcoid derived cell lines (EqS01, EqS02, EqS03) ($p < 0.05$). The BPV-1 transformed cell lines (S6 cells) were found to have a significantly higher proportion of p53 positive cells compared to the naturally transformed, sarcoid derived fibroblasts (EqS cells). All four cell lines that have been successfully cultured from sarcoid tumour samples (the EqS cell lines), showed abnormal p53 expression. Three of them (EqS01, 02 and 03) showed a nuclear accumulation of p53 compared to BPV-negative control cells (Fig. 5.9) and the fourth, EqS04b, showed peri-nuclear accumulation of p53.

5.4.5 *In vitro* p53 staining results correspond to results obtained *in vivo*

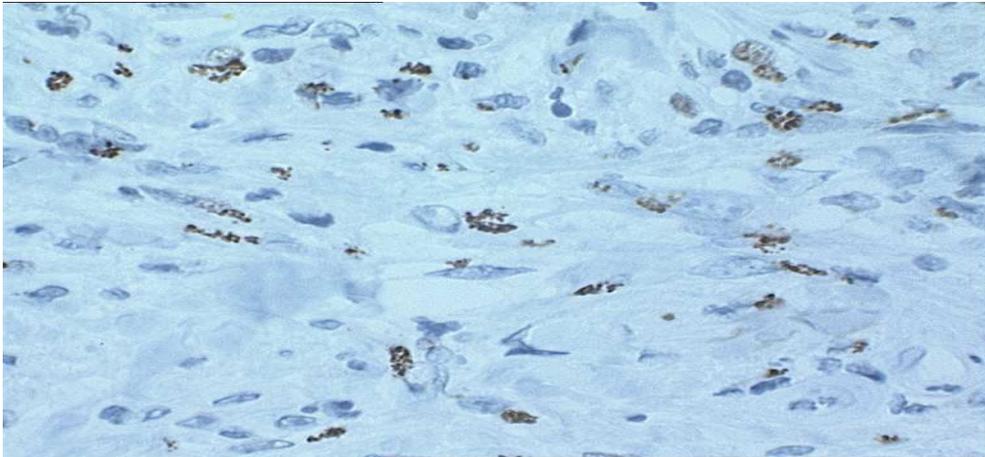
Following analysis of the 51 tumour samples, p53 staining of equine fibroblast cell lines cultured at the University of Glasgow was carried out by IHC. The tumours from which the cell lines established by Yuan in 2007 were cultured, EqS01, EqS02, EqS03 and EqS04b, were not available for IHC staining and so a direct comparison between the parent tumour and its derived cell line could not be performed. Therefore, in order to compare *in vitro* (tissue culture) results and *in vivo* (tumour section) results, a case study was performed on a horse presented to the Weipers Centre for Equine Welfare, University of Glasgow, for treatment of a chronic sarcoid problem. Images of the tumour prior to surgery are shown in Figure 5.10A. Following removal of the tumours, histopathology sections were obtained and stained with the results shown in Figure 5.10B. Figure 5.10C shows the results of p53 staining on tissue culture established from the same tumour (tissue samples were removed from the tumour prior to its histology processing). It can quite clearly be seen that the extensive peri-nuclear staining of p53 in the tumour tissue section (Fig. 5.10B) is replicated in the p53 staining of the tissue culture obtained from that same tumour. This result confirms that the cell lines cultured from sarcoid tissue samples represent a valuable resource in the study of sarcoid pathogenesis.

Figure 5.10.

A



B



C

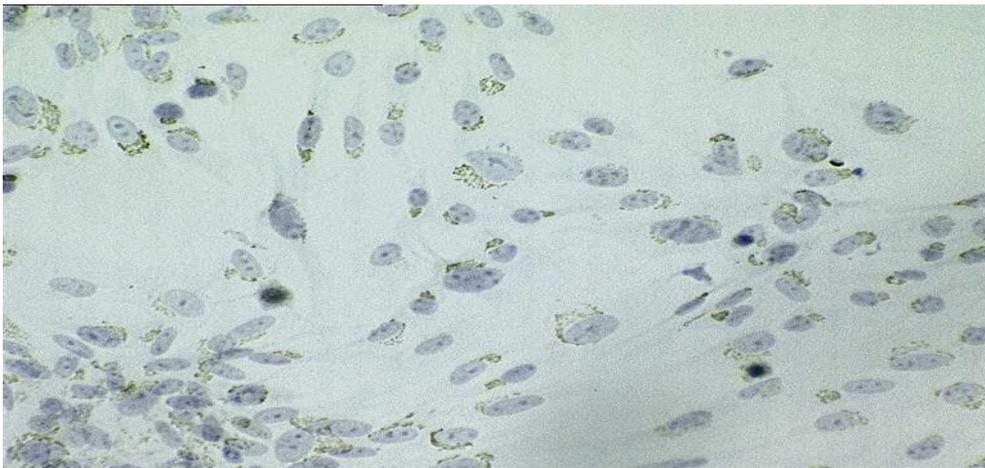


Figure 5.10 Equine sarcoid in situ (A), p53 staining of sarcoid tumour section (B) removed from horse in A. Primary cell culture established from a sarcoid removed from horse A and stained for p53 (C). Note the abnormal peri-nuclear/cytoplasmic p53 staining evident in both the tumour section and the cell culture slide.

5.4.6 p53 is not transcriptionally upregulated in BPV-1 transformed cell lines

It has previously been shown that equine sarcoids harbour the wild-type p53 (Bucher *et al.*, 1996). Therefore, in order to begin examining the functionality of p53 in equine sarcoids, we first examined the baseline expression of p53 by determining levels of p53 mRNA using RQ-PCR (section 2.2.5, p53 primer and probe set Table 2.4). The amount present in the BPV-1 transformed equine fibroblasts was compared to the mRNA levels in normal equine fibroblasts (EqPalFs). Figure 5.11 shows that there was no significant up-regulation of p53 mRNA in any of the BPV-1 transformed fibroblast cell lines.

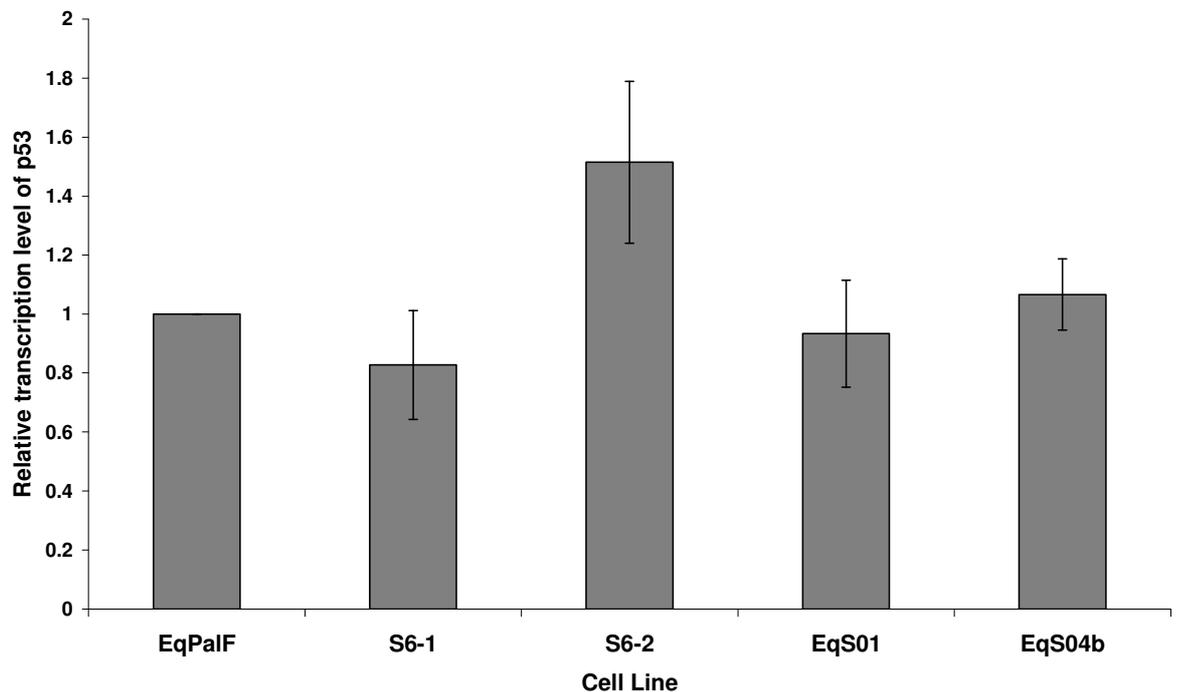


Figure 5.11 Analysis of p53 mRNA levels by RQ-PCR in the different cell types revealed no significant difference in p53 transcription levels between the control fibroblasts (EqPalF) and the BPV-1 transformed fibroblasts (S6 and EqS cell lines).

5.4.7 BPV-1 transformed equine fibroblast cells, p53 expression increases following UVB irradiation indicating a normal p53 response

p53 accumulation in response to DNA damage, amongst other cell stressors, is a well documented event (Albrechsten *et al.*, 1999; Vogelstein *et al.*, 2000). Irradiation by UVB is known to cause DNA damage and induce p53 (Yarosh *et al.*, 2002) and so, to examine whether the p53 protein in BPV transformed fibroblasts was functional, monolayer fibroblast cultures were stained for p53 before, and 4 hours after, UVB irradiation. The experiment was carried out on all cell lines (S6-1, S6-2, S6-3, EqS01, EqS02, EqS03, EqS04b), which were then stained for p53 in accordance to the IHC method outlined in section 2.2.9. In all cell lines, an increase in p53 following exposure of the cells to UVB was observed. Evidence of an increase in the amount of p53 present was determined by counting the number of p53 positive nuclei observed before, and after irradiation. This is shown in Figures 5.12 and 5.13. The fact that elevated levels of p53 are detected in all cell lines following UVB irradiation, indicates a normal function of this protein in these cell lines.

The cell lines shown in Figure 5.12 are representative of the 3 different cell types used throughout this investigation; normal (EqPalF), experimentally transformed by BPV-1 (S6) and sarcoid tumour derived fibroblasts (EqS). As can clearly be seen here, all three cell lines demonstrate an increase in p53 following UVB irradiation. This response was quantified in terms of actual percentages of p53 positive nuclei before and after irradiation and is shown in Figure 5.14.

As alluded to previously, the EqS04b cell line represents somewhat of an anomaly in terms of p53 expression as the p53 present in the untreated EqS04b cells appears to be exclusively located in a peri-nuclear arrangement in the cytoplasm of the cell. Figure 5.13 shows the results obtained when the EqS04b cells were irradiated and then stained for p53 4 hours later.

After UVB irradiation, there was no significant difference between the BPV-1 transformed S6 and EqS cell lines (compare this to the result seen in the untreated cells (Fig. 5.9), where a significant difference between these two cell lines was observed). The control cell line (EqPalF) did, on average, show a lower proportion of p53 positive nuclei after UVB irradiation compared to both the S6 and EqS cells, but the results were only found to be statistically significant in comparing the EqPalF and S6 cells lines.

Figure 5.12

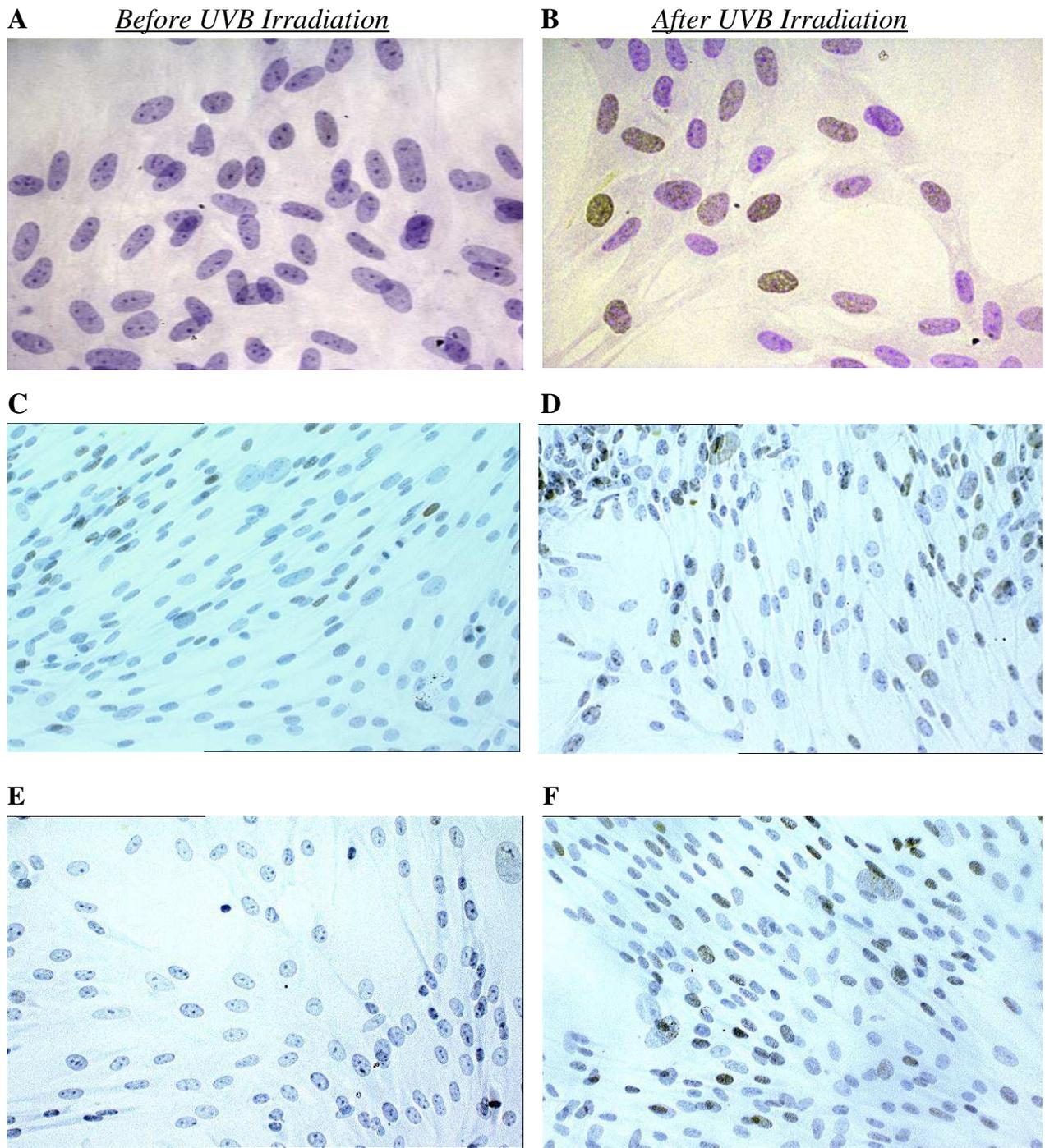


Figure 5.12 p53 staining of cell lines before, and 4 hours post-UVB irradiation.

A and B – normal equine fibroblasts (EqPalF)

C and D – experimentally transformed fibroblasts (S6-2)

E and F – sarcoid derived fibroblasts (EqS03)

Figure 5.13.

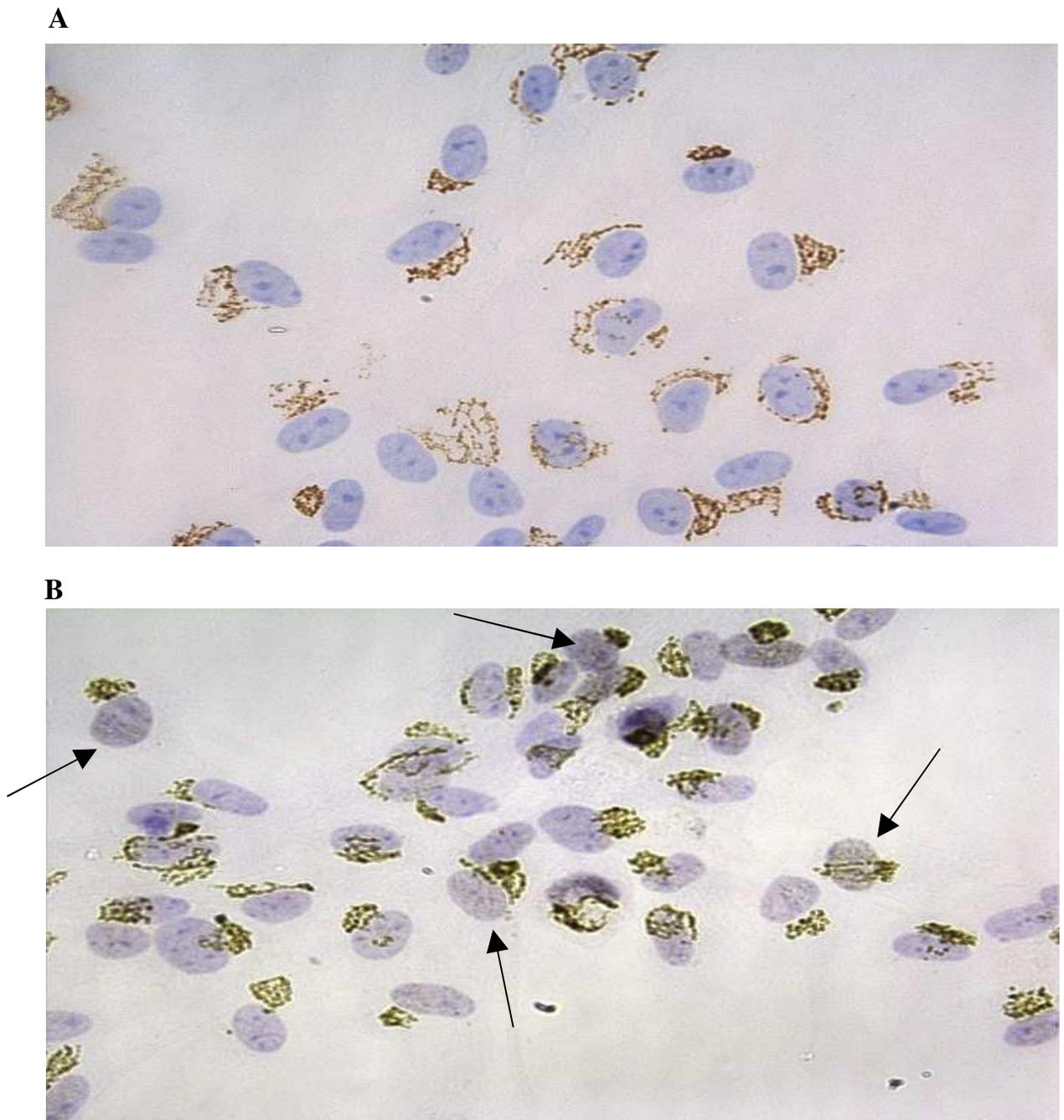


Figure 5.13 p53 staining in sarcoid derived fibroblast cell line EqS04b.

A – EqS04b cells, untreated

B – EqS04b cells, 3 hours after exposure to 250 mJ/cm² UVB radiation

Note that although the majority of the p53 detected in the untreated EqS04b cells is cytoplasmic (Fig 5.14 A), after UVB irradiation, there is evidence of nuclear p53 (arrows), suggesting an intact, at least partially, p53 response pathway.

Figure 5.14

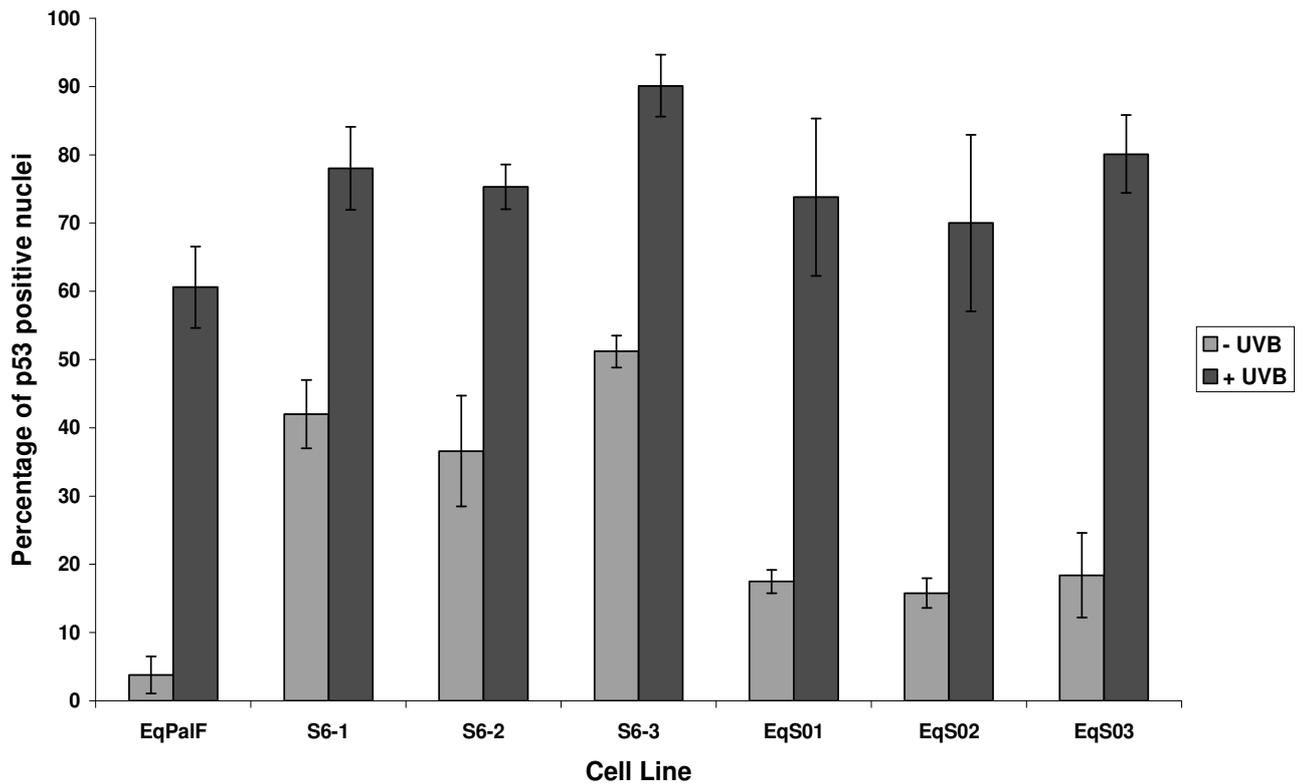


Figure 5.14 Analysis of p53 positivity before and after UVB irradiation across all the cell lines.

This chart clearly shows a marked difference in all cell lines between the proportion of p53 positive cells seen before UVB irradiation, and the proportion observed after UVB irradiation. The difference of the before and after values was determined to be significant ($p < 0.05$) across all the cell lines using the statistical analysis method outlined in Chapter II; section 2.2.13.

5.4.8 Western blot analysis demonstrates that p53 protein levels increases in all cell lines in response to DNA damage by UVB irradiation

To confirm, particularly in regard to the BPV transformed cells, that the increase in positivity detected by IHC was specifically due to an increase in the presence of p53 protein, Western blot analysis was performed on these cell lines. Cells were UVB irradiated and subsequently harvested and prepared for Western blot analysis as described in section 2.2.10.2.

Figure 5.15 shows the results of western blot analysis of BPV-1 negative (EqPalF) equine fibroblasts and BPV-1 transformed equine fibroblasts (S6 and EqS cell lines) both before (Fig. 5.15 A) and after UVB irradiation (Fig 5.15 B). Following transfer of the protein bands from the WB gel to the membrane, the membrane was cut horizontally at the 45 kDa level and the top half of the membrane probed for p53, while the bottom half was probed for GAPDH, which was used as a loading control. All cell lines show an increase in p53 amount 3 hours after exposure to 150 mJ/cm² UVB irradiation. The BPV-1 transformed cell lines (A2, A3, A4 and A5) show the presence of higher levels of p53 prior to UVB exposure compared to the BPV-negative fibroblasts (A1). This was confirmed by scanning the image and using NIH ImageJ Software analysis (NIH – data not shown). The levels of p53 were normalized against the corresponding GAPDH level and the amount of p53 in normal fibroblasts was found to be 3 – 4 times lower than in the BPV-1 transformed cells. This result correlates with the results from immunohistochemical staining where there was a significantly higher percentage of p53 positive nuclei in the BPV-1 transformed cells, compared to the BPV-negative fibroblasts (Figure 5.9).

Figure 5.15.

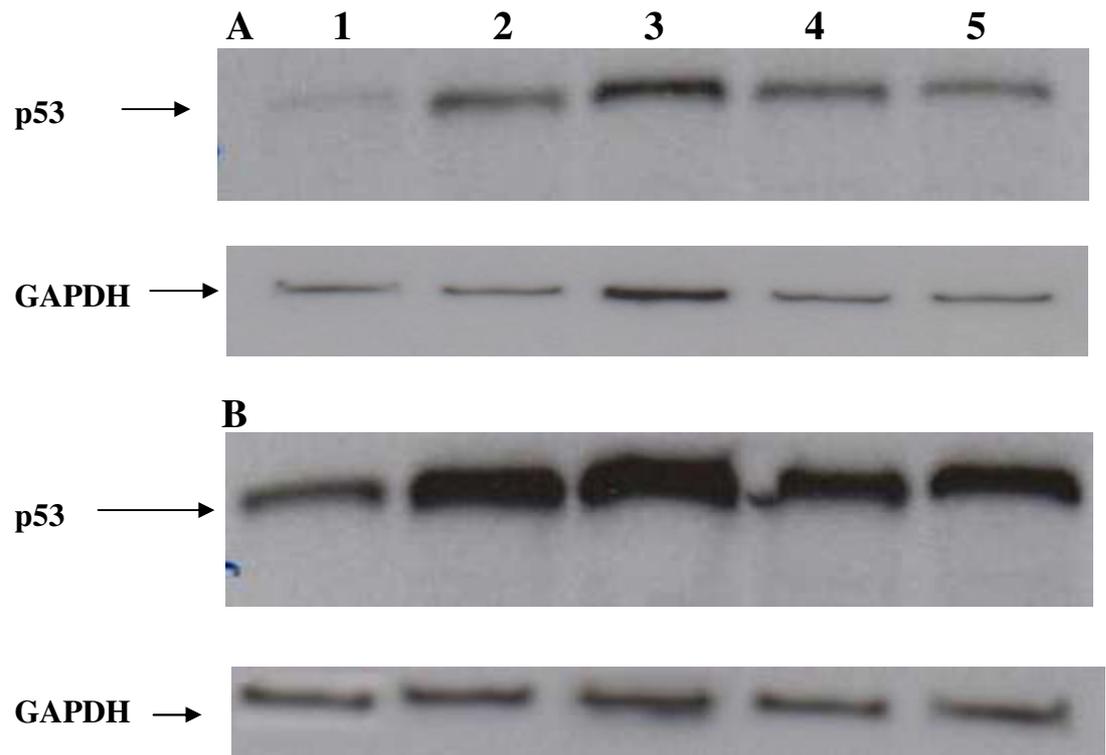


Figure 5.15 Western Blot analysis of control fibroblasts and BPV-1 transformed fibroblasts, pre- and post- UVB irradiation.

- 1 – normal equine fibroblast cells (EqPalF)
- 2 – BPV-1 transfected fibroblast cells (S6-2)
- 3 – BPV-1 transfected fibroblast cells (S6-3)
- 4 - sarcoid derived fibroblast cells (EqS02)
- 5 - sarcoid derived fibroblast cells (EqS04b)
- A – prior to UVB irradiation
- B – post-UVB irradiation (6 hours)

5.4.9 p53 protein in BPV-1 transformed fibroblasts transactivates p21 expression indicating p53 protein is transcriptionally active in these cells.

For assessment of p53 transactivation activity, cells were irradiated with a low dose of UVB (30 mJ/cm²), incubated overnight (~ 10 hours) and then harvested to look for the presence of p21 protein. As mentioned earlier, p21 expression is up-regulated by p53 as part of the DNA damage response pathway (Vousden and Lu, 2002; Liu *et al.*, 2003). Figure 5.16 shows that p21 levels in all the cell lines studied increase after exposure to UVB irradiation. This suggests that the transactivation function of p53 is intact in BPV-1 transformed equine fibroblasts.

Figure 5.16

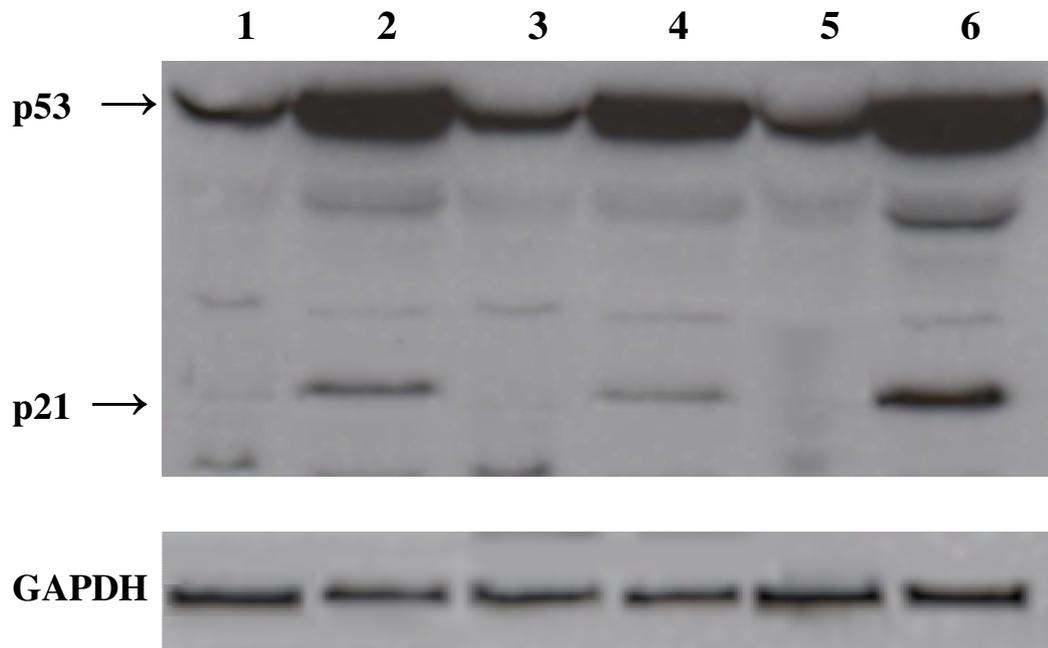


Figure 5.16 Western Blot analysis of p53 function in BPV-1 transformed cells by evaluation of p21 transactivation.

- 1 – Normal equine fibroblasts (EqPalF) prior to UVB exposure
- 2 – Normal equine fibroblasts 9 hours post-exposure to a low dose of UVB
- 3 – BPV-1 transfected fibroblasts (S6-2) prior to UVB exposure
- 4 – BPV-1 transfected fibroblasts 9 hours post-exposure to a low dose of UVB
- 5 – sarcoid derived fibroblasts (EqS04b) prior to UVB exposure
- 6 – sarcoid derived fibroblasts 9 hours post-exposure to a low dose of UVB

This Western Blot figure clearly shows both the increase of p53 in each cell line, plus the activation of p21 in each cell lines, following exposure of the cells to a low dose (30 mJ/cm²) of UVB irradiation. These results suggest that p53 is functionally active in BPV-1 transformed equine fibroblasts.

5.4.10 The half life of p53 is increased in sarcoid derived and BPV-1 transformed cells

To investigate the hypothesis that the p53 accumulation observed in BPV-1 transformed cells is as a result of an increased half life of the protein in these cells, cycloheximide was added to the media as described in Chapter V; section 5.3.5. Figure 5.18. shows the results of treating BPV-negative and BPV-1 transformed fibroblast cell cultures with cycloheximide and the harvesting the cells at different time points (0, 0.5, 1, 2.5 and 5 hours) post-addition of the drug to the media.

Figure 5.17.

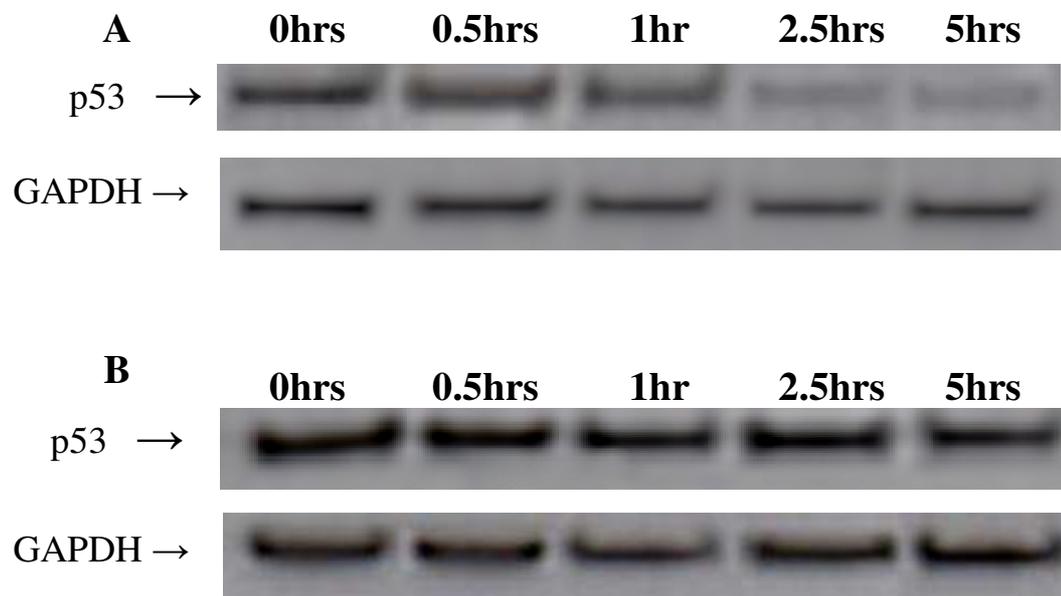


Figure 5.17 Half-life study of p53 in control equine fibroblasts (EqPalF - A) and sarcoid derived equine fibroblasts (EqS04b - B).

Cycloheximide was added to the media of the different cell lines, and the cells were harvested at the time intervals indicated. The p53 in the BPV-1 transformed cell lines is clearly evident up to at least 5 hours after cycloheximide was added. In contrast, the amount of p53 in the BPV-negative fibroblasts decreases markedly from 1 hour post-incubation with cycloheximide.

5.5 DISCUSSION

These results reveal some important information regarding the cellular location and functionality of p53 in equine sarcoids. Before this information is interpreted it is probably worthwhile stating that in the interpretation of these data, we are operating under the assumption that the p53 present in equine sarcoids is wild-type p53. This assumption is based on evidence from a number of studies (Bucher *et al.*, 1996; Nasir *et al.*, 1999; Mayr *et al.*, 2000) that looked for mutations in the equine p53 gene sequenced from equine sarcoid affected animals. In all these studies, there was no evidence of p53 mutations, which makes it likely, although does not exclude entirely the possibility of the presence of mutations, that the p53 present in equine sarcoid tumours is wild-type p53.

5.5.1 p53 Accumulation in the Nucleus

Our data demonstrates that BPV-1 transformed cells, *in vitro* and *in vivo*, show abnormal nuclear accumulation of p53. In the ordinary cellular response to DNA damage, p53 accumulates in the nucleus due to its phosphorylation by ATM/Chk2 or ATR/Chk1, and subsequent stabilisation. There, it then activates transcription of proteins involved in either; cell cycle arrest and DNA repair, or proteins involved in activating apoptosis. Whether or not a cell enters cell cycle arrest or undergoes apoptosis after activation of p53 depends on myriad factors:

- i) The type of cellular stress that caused the p53 activation in the first place. For example, in some types of cell, hypoxic conditions result in p53 activation and transcription of a hypoxia-specific target (Bnip3L) which encodes a protein that promotes apoptosis (Fei *et al.*, 2004).
- ii) The cell type can control the multiple functions of p53 by preferentially activating p53 pathways associated with cell arrest and DNA repair, OR those pathways that lead to apoptosis. For example, cells of haematological origin will tend to enter cell cycle arrest and DNA repair after DNA damage, whereas mature B-cells, have evolved to evade both p53-dependent DNA repair AND apoptosis (Phan and Dalla-Favera, 2004; Wu *et al.*, 2005).
- iii) The presence of co-factors that can also lead to the preferential activation of either pathway. For example BRCA1 suppresses the transcription of

apoptosis-related genes whilst promoting the transcription of those genes involved in DNA repair (MacLachlan *et al.*, 2002).

- iv) Recently, different isoforms of p53 have been discovered and have been shown to affect the activity of wild-type p53 (Qu *et al.*, 2004; Harms and Chen, 2005).

Accumulation of p53 in the nucleus without an initiating DNA damage event is most commonly associated with mutated p53, and is seen in some types of cancer (Esrig *et al.*, 1994; Ranzani *et al.*, 1995; Quinn *et al.*, 2000). Research in the area has also shown that the nature of the p53 mutation can determine whether or not p53 accumulation in the nucleus will occur (Tsuda and Hirohashi, 1994). It is generally accepted that the reason for mutant p53 accumulation is that it has an increased half life compared to wild-type p53, as the mutation seems to confer stability and/or a resistance to degradation. Accumulation of wild-type p53 in the nucleus is a less common occurrence, but has been observed in some examples of neoplastic disease (Rubio *et al.*, 1993; Ellison *et al.*, 1995). The reasons for this accumulation are not well understood (Jaquet *et al.*, 1999).

It is reasonable to theorise that an increase in levels of p53 in the BPV-1 transformed cells is due to an increase in p53 transcription. However, as shown in Figure 5.3, we have demonstrated here that this is not the case.

Another possibility is that the p53 protein has been stabilised in the nucleus, leading to an increased half-life. This hypothesis is supported by the results of the cycloheximide experiments (Fig. 5.13) which show that p53 is detectable for significantly longer in BPV-1 transformed fibroblasts after protein transcription has been halted by the addition of cycloheximide (CHX) to the cell growth media, compared to the untransformed control fibroblasts. The explanation for this increase in p53 half-life is less simple to elucidate and there are several possibilities to consider. It could be as a result of lower proteasomal degradation activity as also shown in adenovirus E1A-infected cells, in which inhibition of proteasomal activities by targeting the proteasomal regulatory subunit S2, extended p53 half-life and increased the level of p53 without inhibiting the p53 ubiquitination process (Turnell *et al.*, 2000; Zhang *et al.*, 2004).

It may also be caused by a dysregulated p53 ubiquitination process, specifically; the regulation of p53 by the murine double minute protein (mdm2) is compromised. The mdm2 protein regulates the activity of the p53 protein with more than one mechanism. It can block the transcriptional activity of the p53 protein, export p53 from the nucleus to the

cytoplasm and promote the degradation of p53. To promote degradation of p53, the mdm2 protein functions as a ubiquitin ligase and can ubiquitinate p53 (Honda *et al.*, 1997, Fuchs *et al.*, 1998). Ubiquitination targets the p53 protein for degradation by the proteasome. Additionally, through binding to p53, mdm2 shuttles p53 out of the nucleus to the cytoplasm for degradation (Freedman and Levine 1998). DNA damage induces phosphorylation of the p53 protein at multiple sites, including those overlapping with the mdm2-binding sites, thus preventing the association between mdm2 and p53 (Shieh *et al.*, 1997), resulting in an increase in the amount of p53 present in the cell (Buschmann *et al.*, 2000, Buschmann *et al.*, 2001). Mdm2 is p53 responsive so that when p53 is stabilised, and p53 levels increase, so too do mdm2 levels. Thus there is a negative feedback loop relationship between p53 and mdm2. Limited details regarding the status of mdm2 in equine sarcoid makes it difficult to speculate on the theory of mdm2/p53 imbalance without further work in that area.

A final possibility is that the shuttling of p53 from the nucleus to the cytoplasm is impaired, resulting in an accumulation of p53 in the nucleus, even in the presence of normally functioning mdm2. As a shuttling protein, p53 is constantly transported through the nuclear pore complex. p53 nucleocytoplasmic transport is carried out by a bipartite nuclear localisation signal (NLS) located at its C-terminal domain and two nuclear export signals (NESs), located in its N- and C-terminal regions. Mutations disrupting the NLS block p53 export and prevent mdm2-mediated cytoplasmic degradation (O'Keefe *et al.*, 2003). So, theoretically, BPV-1 proteins may interfere with the shuttling ability of the p53 molecule.

5.5.2 p53 in BPV-1 transformed cell lines is transcriptionally functional

This is evident from the Western blot analysis of the cell lines after they were analysed 9 hours post-exposure to low levels of UVB irradiation and then probed with p21 antibody (Fig 5.15). The p21 protein functions as a cyclin-dependent kinase inhibitor (CKI). It binds to and inhibits the activity of cyclin-CDK2 or -CDK4 complexes, and thus functions as a regulator of cell cycle progression at G1 (He *et al.*, 2005). The expression of the p21 gene is controlled by p53, through which p21 mediates the p53-dependent cell cycle G1 phase arrest in response to a variety of stress stimuli.

The Western blot evidence from probing with the p53 antibody also supports the hypothesis of an intact p53 response to DNA damage (Fig. 5.15 and 5.16) as levels of p53 protein in all cell lines significantly increase after a DNA damaging insult. The immunohistochemical staining of the cultured fibroblast cell lines also supports this theory,

as there is clearly an increase in p53 positive nuclei in all cell lines after the cells have been exposed to a DNA damaging stimuli (Figs. 5.12, 5.13 and 5.14). Even the sarcoid-derived cell line that exhibited cytoplasmic p53 staining (EqS04b) showed evidence of nuclear p53 accumulation after DNA damage (Fig. 5.13).

5.5.3 Cytoplasmic sequestration of p53

Cytoplasmic sequestration of p53 has been commonly observed in certain tumours, such as neuroblastomas, and breast and colon cancer (Bosari *et al.*, 1995; Lilling *et al.*, 2002). In at least a subset of these tumours, MDM2 is responsible for the cytoplasmic accumulation of p53 (Lu *et al.*, 2000), and other proteins have also been implicated in cytoplasmic retention (Truant *et al.*, 1995; Takada *et al.*, 1997; Zhan *et al.*, 1998; Weber *et al.*, 1999). Several viral proteins also influence p53 localisation, such as human papilloma virus (HPV) E6 protein (Thomas *et al.*, 1999), adenoviral E1B 55 kDa protein and the HBV HBx protein (Querido *et al.*, 1997; Ahn *et al.*, 2001; Ahn *et al.*, 2002). In addition, defects in p53 import/export have also been reported in different tumour types (Fabbro and Henderson, 2003).

In the IHC staining of cell culture and tumour tissue sections, the cytoplasmic localization of p53 did not appear to form a punctate pattern or be localised in a distinct p53 cytoplasmic body as frequently identified in cells infected with adenovirus Type-12 E1B-oncoprotein (Zhao and Liao, 2003). Neither did it resemble finely dispersed constitutive cytoplasmic p53 as observed in cells productively infected by human herpesvirus-6 (Takemoto *et al.*, 2004). Both hyperactive nuclear export and existence of tethering mechanisms should also be considered as contributing to the cytoplasmic sequestration of p53 in some BPV-1 transformed fibroblasts, but these mechanisms were not investigated in this study.

What was interesting from our results, is that although p53 was present at abnormally high levels in all the BPV-1 transformed cell lines as determined by the percentage of p53 positive nuclei detected in these cells (Fig. 5.14.), in the EqS04b and EqS013 (EqS013 is the cell line established from a sarcoid removed from the equid in Figure 5.10) cell lines, which demonstrated cytoplasmic localisation of p53, ALL the cells in the images captured showed the presence of p53 (Fig. 5.13, fig. 5.10 C). I.e. these sarcoid derived cell lines demonstrated 100% positivity for p53. It is also interesting to note that even though the p53 in untreated EqS04b cells appears to be located exclusively in the cytoplasm (Fig. 5.13), after UVB irradiation, p53 appears to be present in the cytoplasm AND the nuclei of these cells (Fig 5.13). Together with the data that demonstrates the transactivation

functionality of p53 in this cell line is intact (Fig. 5. 15 – detection of p21) this could be interpreted as evidence supporting the theory that p53 is not, in fact, inactivated in BPV-1 transformed fibroblast cells.

5.5.4 What conclusions can be drawn?

5.5.4.1 In vitro

- All BPV-1 transformed fibroblasts exhibit abnormal p53 expression, most commonly manifested as an increase in the amount of p53 positive cells present, but also seen as cytoplasmic accumulation in a number of sarcoid derived cell lines.
- The sarcoid derived cell lines where cytoplasmic accumulation of p53 was evident originated from sarcoid tumours that have previously been shown to harbour a higher than average viral load/level of viral gene expression (EqS04b cells: Yuan *et al.*, 2007) or to come from clinically aggressive and persistent tumours (EqS013 cells: From clinical case featured in figure 5.16).
- EqS013 cells in vitro, matched the pattern of p53 expression seen in the same sarcoid tumour in vivo (Fig. 5.10)

5.5.4.2 In vivo

- Just over 50% of the tumours stained for p53 did not show any abnormal p53 expression. Of the tumours that did show abnormal p53 expression, there was a greater degree of abnormality detected in those tumour types considered to be more clinically aggressive (Table 5.2).

It has been reported in the study of squamous cell carcinomas (SCC), that p53 expression increased as the degree of dysplasia and the grade of SCC advanced. In 1994, Sauter *et al.*, reported an increasing number of positive cases from 28% in mild dysplasia, to 45% in moderate dysplasia, to 54% in severe dysplasia and 50% in carcinoma in-situ. Our results demonstrate a lower overall level of p53 positivity – even in those sarcoid tumours that had over 20% of nuclei being p53 positive and were graded as ‘HIGH’, the highest percentage in this group was under 30%. Back to the work done in SCC, one study found a statistically significant increase in p53 expression in cases presenting with nodal metastasis and linked a higher p53 expression to a poorer prognosis (Jain *et al.*, 2008).

It seems reasonable, given the nature of our own findings, to speculate that higher levels of p53 expression in sarcoid tumours indicates a tendency to aggressive clinical behaviour, and the presence of cytoplasmic p53 suggests a malevolent tumour type that is very likely to recur after treatment. How useful these findings are in clinical context is unclear, although knowledge of p53 status in some instances may influence treatment approach, stable management practices and potential breeding risks.

Although we have shown that the half-life of p53 is considerably longer in BPV-1 transformed cells it is not possible, from our results, to determine how and why this has occurred. Unlike p53 anomalies that develop in cells infected by the adenovirus or the human cytomegalovirus (HCMV), there appears to be no consistency in the pattern of p53 alterations in BPV-1 infected cells. Research into adenoviruses and HCMV has determined the exact viral proteins responsible for interfering with, and inactivating, p53, and in the case of HCMV, the exact stage of infection at which the changes to p53 occur. So with those viruses, it is possible to confidently state that infection of a cell with either of those viruses, will result in inactivation of p53 by means of a reasonably well understood mechanism(s). With our in vitro results, it is possible to state with confidence, that transformation of an equine fibroblast by BPV-1 results in an increase in p53 expression. However, the in vivo analysis of the tumour sections (confirmed histologically to be sarcoid tumours) revealed that in some cases, there was no evidence at all of an increase in p53 expression. Additionally, a minority of the sarcoid derived cell lines (EqS04b and EqS013) showed total cytoplasmic localisation of p53 – if this was indeed caused by the presence of BPV-1, why wasn't it evident in all BPV-1 transformed cell lines? Further work is required to elucidate the mechanisms responsible for nuclear p53 accumulation and, in some cases, export to, and accumulation in, the cytoplasm.

5.5.4.3 In summary

- i) p53 expression is higher in BPV-1 transformed cells
- ii) p53 transcription is not upregulated in BPV-1 transformed fibroblasts
- iii) half-life of p53 in BPV-1 transformed fibroblasts is increased
- iv) p53 in BPV-1 transformed cells is transcriptionally active
- v) increased levels of p53 abnormality (high nuclear p53 positivity and cytoplasmic p53) correlate with clinical appearance/behaviour of sarcoid tumour

6 General Discussion

6.1.1 Transmission

- i) Flies harbour BPV-1 DNA
- ii) BPV-1 DNA has been found to be equine-specific variant most commonly isolated from sarcoid tumours

Further work is needed to confirm absolutely that flies can act as mechanical vectors of BPV-1/sarcoid tumours between affected and susceptible animals. This would involve designing experiments where a sarcoid affected animal was kept isolate in a room with pathogen-free flies. The BPV DNA from the sarcoid of the affected horse would be isolated and sequenced. After a certain period of time, the animal would be exchanged for another, sarcoid-free animal, which would also be kept isolated with the flies. If and when that animal developed a sarcoid or sarcoids, these could be removed and analysed and the DNA sequence compared to that isolated from the original animal. Care would need to be taken to remove any BPV contamination of the environment before the second animal was introduced, and thus minimise the likelihood that this could be a source of infection for the second animal. Once the second animal had successfully be infected, capture and analysis of the flies should be done to confirm that they harbour the same sequence/sequences of BPV DNA as the original animal. In the interests of efficiency, it might be necessary to introduce several animals into the fly room after the first animal has been removed. This would address the problem of the fact that some animals are genetically more resistant to developing sarcoids than are others.

6.1.2 Apoptosis, Cell Survival and p53

This chapter provides evidence that BPV-1 transformed fibroblasts are NOT more resistant to apoptosis compared to BPV-negative equine fibroblasts following UVB irradiation, and to a lesser extent, cisplatin treatment. The reasons for an increased sensitivity to apoptosis could be the presence of BPV proteins E6 and E7, which have previously been shown to sensitise infected cells to certain types of apoptosis. Additionally, the results from analysis of p53 in the final chapter could be applied here to help explain the significantly higher levels of apoptosis seen in BPV-1 transformed cells, soon after certain types of DNA damage. It has been shown that over-expression of wild-type p53 results in cells that are more sensitive to apoptosis induced by DNA damage (Tak *et al.*, 2000; Gu *et al.*, 2004), and so it seems reasonable to suggest that the accumulation of functioning p53 in BPV

transformed cells, would contribute to the higher apoptotic index seen in these cells following DNA damage. This also fits with the observation that the experimentally transformed fibroblasts (the S6 cells) showed a considerably higher apoptotic index in the untreated control populations when assessed by FCM analysis, compared to the sarcoid derived (EqS) cells, and the BPV-negative control fibroblasts. Chapter 5, Figure 5.14 illustrates this perfectly. The S6 cells demonstrate by far the highest proportion of p53 positive nuclei, the sarcoid derived cells are more p53 positive than the control fibroblasts (by 4 – 6 fold), but to a lesser degree than the S6 cell lines, which demonstrate a 10 – 12 fold increase compared to the control BPV-negative fibroblast cell line. That is why the FCM analysis data was presented as relative levels of apoptosis, to compensate for this factor.

Cell survival was enhanced in BPV-1 transformed cells, despite higher initial levels of apoptosis after DNA damage. A review of the p53 literature in 1999 by Brown and Wouters, states that “We find that because wild-type p53 predisposes cells to a more rapid rate of cell death after DNA damage, particularly with normal or minimally transformed cells, that short-term assays have led to the conclusion that mutations in p53 confer resistance to genotoxic agents. On the other hand, if clonogenic survival is used to assess killing in cells derived from actual solid human tumours, then apoptosis and the genes controlling it, such as p53 and bcl-2, appear to play little or no role in the sensitivity of these cells to killing by anticancer drugs and radiation.” (Brown and Wouters, 1999). This information, coupled with the very recent observations that some cancer cells are able to ‘reverse’ the apoptotic process, even after morphological cellular changes (associated with apoptosis) have taken place, provides a convincing argument to explain the data collected in this thesis.

Despite the fact that the exact mechanisms and pathways that underpin the events described above are not fully understood in BPV transformed cells, what is useful to consider, is the results that were obtained following E6 siRNA treatment of the equine sarcoid derived cell line EqS04b. Very little change in apoptosis levels was detected by FCM analysis, and yet, very significant change was observed in the clonogenic survival assay. By ‘silencing’ BPV E6 in these cells, the cell survival potential following DNA damage was greatly reduced. This has exciting implications for the development of a universally successful sarcoid treatment. Already topical siRNA therapies are in clinical trials in the treatment of a range of virally-induced human diseases. Given the encouraging results detailed in this thesis, it seems that a sensible area of future research into the

treatment of equine sarcoids, resides in the development of a technique that will introduce BPV targeted siRNA molecules into tumour cells and either, allow the animals own immune system to then recognise and remove the virally infected cells, or, enhance the efficacy of more traditional sarcoid treatments, such as excision, by being used in conjunction with them. The electropulsation technique currently being used for delivering cisplatin or 5-fluorouracil deep into tumour tissue, would be a useful approach to delivering the siRNA molecules to the optimal depth.

Further research in to the suitability of siRNA therapy as a treatment option should focus on several issues;

- i) Additional tests are required to confirm the in vitro silencing is viral specific.
- ii) In vitro effects of siRNA on normal equine fibroblasts should be determined.
- iii) The safety and tolerability of topical/intradermal siRNA delivery needs to be assessed in vivo.
- iv) The efficacy of intratumoral siRNA treatment needs to be established.

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