# Prophylactic Vaccination Against Papillomavirus-Induced Tumour Disease

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Sabine Brandt and Edmund Hainisch

## Contents



**Abstract**

The *Papillomaviridae* family comprises a large number of genetically heterogeneous papillomaviruses (PVs) that are the causative agents of benign lesions or cancer in humans and a wide range of animal species. Early research in animal PV systems has disclosed several important characteristics of PVs and led to the recognition of human papillomaviruses (HPVs) as carcinogenic viruses in 1995. One of the most crucial findings in animals was that in vitro generated PV major capsid proteins spontaneously self-assemble to empty viral capsids termed virus-like particles (VLPs) that are safe and highly immunogenic. This discovery paved the way for the establishment and commercial release of highly effective polyvalent VLP-based vaccines for the prevention of HPV-induced tumour disease in humans. In addition, it encouraged veterinary scientists to work on the establishment of analogous, VLP-based vaccines for the protection of horses and other equids from common PV-induced cutaneous and mucosal tumours that is bovine PV type 1/2 (BPV1/2)-associated sarcoids and equine PV type 2 (EcPV2)-induced squamous cell carcinomas (SCCs). So far, BPV1 and EcPV2 VLPs were

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shown to be safe and highly immunogenic in horses. Furthermore, immunisation of horses with BPV1 VLPs conferred complete protection from experimental BPV1 infection and associated pseudo-sarcoid formation and also elicited cross protection from BPV2 infection. Similarly, the protective potential of EcPV2 VLPs against experimental infection with EcPV2 pseudo-virions was shown in a murine model. Taken together, these findings indicate that BPV1 and EcPV2 VLPs are safe and highly effective in protecting equids from PV-induced sarcoids and SCCs.

## 10.1 Introduction

The *Papillomaviridae* family comprises a large number of human and animal viruses that are characterised by considerable genetic diversity yet adhere to common biological principles. Papillomaviruses (PVs) are relatively small non-enveloped viruses that consist of an icosahedral capsid harbouring a circular double-stranded DNA genome of up to 8 kbp in length. The capsid is composed of 72 L1 protein pentamers commonly termed capsomeres and 12 L2 protein monomers (Howley and Lowy 2001). The viral genome can be grossly divided into an early (E) and a late (L) coding region and a non-coding long control region (LCR). The early region codes for regulatory (E1, E2, E4) and transforming proteins (E5, E6 and E7), which are expressed early in the viral life cycle. The late region contains two genes encoding the major L1 and the minor L2 capsid proteins, which are not expressed until viral genome amplification has been completed. The LCR is essential in providing cis-responsive elements that are required for replication and transcription of the viral genome (Campo 2006b; Doorbar 2005).

PV virions cannot actively penetrate the skin or mucosa of their host. They gain access to basal epidermal cells through micro-abrasions. These stem cells provide the appropriate primary surface and secondary receptor molecules for virion attachment and uptake. There is evidence for surface heparan sulphate proteoglycans (HSPG) representing initial PV attachment sites (Giroglou et al. 2001; Joyce et al. 1999). Subsequent PV endocytosis possibly involves clathrin- and caveolin-mediated mechanisms (Day et al. 2003) and/or may necessitate the presence of tetraspanin-enriched microdomains (TEMs) (Spoden et al. 2008). The productive PV life cycle is described as being tightly linked to the differentiation process of keratinocytes. Following initial infection of basal cells, the early viral genes are expressed in the basal and suprabasal epithelial layers. The replication of the viral genome occurs in the differentiating cells of the spinous and the granular layers (Chow and Broker 2006). The late capsid genes are expressed in the final squamous layer, where new infectious virions are assembled and released via disintegration and shedding of dead squames (Graham 2006). Interestingly, PV virions are highly resistant to desiccation, thus opening the possibility of indirect transmission via fomites (Roden et al. 1997).

## 10.2 Human Papillomavirus-Induced Cancer Disease and Effective Prevention

An aetiological association of PV infection with tumour development was first established in rabbits (Shope and Hurst 1933). The observation that inoculation of cottontail and domestic rabbits with infectious wart extract induced papillomas that sometimes progressed to squamous cell carcinoma (SCC) led to cottontail rabbit papillomavirus (CRPV) becoming the first model for the study of PV infection-associated carcinogenesis (Rous and Beard 1935).

Experimental research in rabbits, cattle and dogs disclosed several fundamental characteristics of PVs, most notably their species specificity and their pronounced tropism for defined cellular environments, i.e. cutaneous or mucosal keratinocytes and, for some types, fibroblasts (Lowy 2010). Consequently, human papillomavirus (HPV) research had to rely on animal infection models for many decades (Campo 2002). This comparative approach brought together scientists from various fields of human and veterinary medicine. Investigations on CRPV, bovine papillomavirus types 1 and 4 (BPV1, BPV4) and canine oral papillomavirus (COPV) led to important insights into PV biology and pathogenicity (Campo 2002), thus paving the way for the official recognition of HPVs as carcinogenic viruses (IARC 1995).

Molecular biological methods and powerful in vitro and small animal systems established during the past 30 years led to a shift from animal PV to direct HPV research. To date, more than 200 HPV types have been identified. From these, about 15 types are carcinogenic and thus classified as high-risk (hr) HPV types. There is evidence for almost all diagnosed cervical cancers, 90 % of anal cancers, up to 50 % of genital tumours and 22 % of head and neck squamous cell carcinomas (HNSCC) being caused by hrHPV types (Dayyani et al. 2010; zur Hausen 1996, 2000, 2009). HPV oncoproteins E6 and E7 have been recognised as essential factors in HPV-induced carcinogenesis. Malignant transformation of infected epidermal cells is achieved by complex interactions of these oncoproteins with cellular factors involved in cell cycle regulation (Feller et al. 2010a, b). E5 has been shown to be likewise transforming and to downregulate major histocompatibility complex (MHC) class I cell surface expression, thus helping the virus to escape from immune surveillance and establish infection (Ashrafi et al. 2006). In conjunction with other carcinogenic factors such as UV-radiation, infection by cutaneous beta-HPV types may indirectly contribute to the development of cutaneous SCCs (Schiller and Buck 2011; Zur Hausen 1996, 2000, 2009).

One of the most crucial findings in animal PV models was that in vitro generated L1 capsid proteins spontaneously self-assemble into empty capsids termed virus-like particles (VLPs). The latter are morphologically and immunologically almost indistinguishable from wild-type virions in that they display conformation-dependent neutralisation epitopes and are able to induce high titres of type-restricted neutralising antibodies (Kirnbauer et al. 1992). Challenge studies conducted in rabbits and cows revealed that immunisation with homologous (i.e. CRPV and BPV4) but not heterologous VLPs conferred protection from experimental infection (Breitburd et al. 1995; Kirnbauer et al. 1996). Similarly, immunisation with COPV

VLPs induced protection from experimental COPV infection in dogs (Suzich et al. 1995). These and similar findings (Rose et al. 1993; Zhou et al. 1991) ultimately led to the establishment and commercial release of highly effective polyvalent VLP-based vaccines for the prevention of HPV-induced tumour disease in humans (Angioli et al. 2016; Villa et al. 2005).

## 10.3 Papillomavirus-Induced Tumours in Horses and Other Equids

### 10.3.1 Sarcoids

In cattle, bovine papillomaviruses of types 1 and 2 (BPV1; BPV2) are the causative agents of benign warts that usually regress spontaneously. Infection is productive, with cow warts harbouring millions of infectious particles (Campo 2006a). As the rare example of a cross-species infection, BPV1/2 can also infect equids, e.g. horses, donkeys, mules and zebras, and lead to the development of usually persistent, locally aggressive skin tumours termed sarcoids (Chambers et al. 2003a). The latter constitute the most commonly encountered tumour disease in horses, with a morbidity of 5.8 % in the UK (Ireland et al. 2013). Sarcoids are typically diagnosed in young adult individuals with a peak incidence at the age of seven. Depending on their gross appearance, sarcoids are classified as occult, verrucose (Fig. 10.1a), nodular, fibroblastic (Fig. 10.1b), mixed or malevolent (Knottenbelt 2005). Disease may present as single tumour or multiple lesions of various types at different sites of the body. Sarcoids have a high propensity to progress to a more severe and multiple form of disease, especially upon accidental or iatrogenic trauma, i.e. ineffective therapy (Hainisch and Brandt 2015). Disease may also impair the use of affected animals, entail considerable treatment costs and pronouncedly decrease the resale value of affected animals. As a consequence, sarcoids are the number one skin-related cause for euthanasia (Scott and Miller 2003a). Taking into consideration the high prevalence of the disease, the lack of universally effective therapeutic approaches and the fact that sarcoids affect relatively young horses, it is clear that equine sarcoids also have an important negative impact on the horse industry.

First evidence for an aetiological association of BPV1/2 with equine sarcoid disease has been obtained by inoculation experiments. In 1937, Montpellier et al. (Montpellier et al. 1937) reported the successful auto-transmission of sarcoids in a mule. In 1951 and 1969, two research groups succeeded in inducing sarcoid-like lesions by intradermal injection of horses with cow wart extract. Experimental lesions were morphologically and histologically indistinguishable from naturally acquired sarcoids, yet regressed spontaneously (Olson and Cook 1951; Ragland and Spencer 1969). Importantly, Voss was able to induce persistent sarcoids by inoculation of scarified skin with sarcoid extract, but not by intradermal injection of this inoculum (Voss 1969). The suspected causative involvement of BPV1/2 in sarcoid pathogenesis was further supported by in situ hybridization (ISH) experiments revealing the presence of viral DNA in the nuclei of tumour fibroblasts (Lancaster

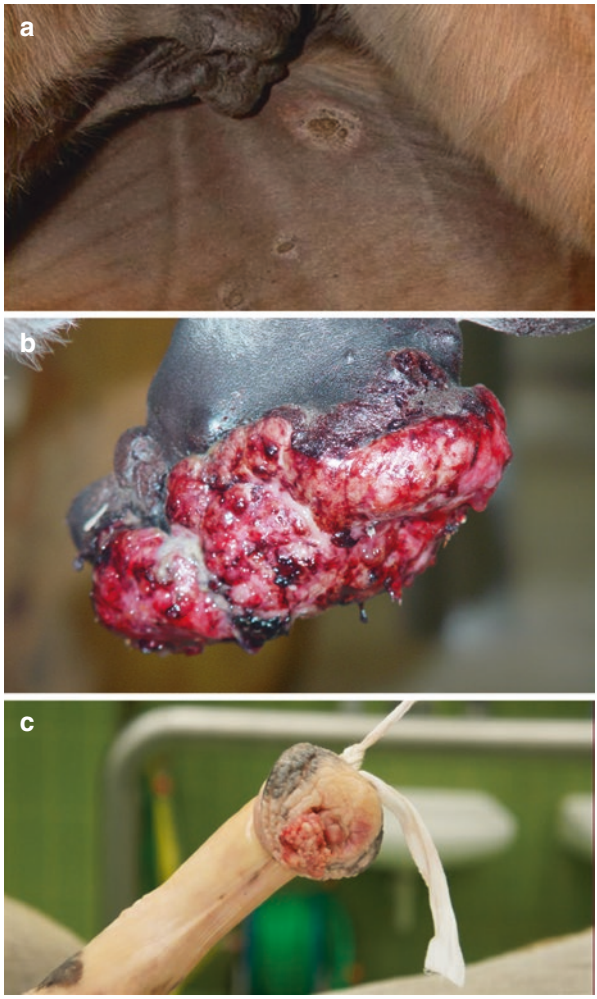


**Fig. 10.1** Sarcoids in equine species: low- to high-grade lesions. (**a**) Sarcoids on the inside of the thigh of a horse. Example of low-grade lesions. Several sarcoids are present. The lesions are characterised by a hyperkeratotic, verrucose centre and a surrounding area of alopecia with mildly thickened skin; (**b**) example of a high-grade lesion in a donkey: fibroblastic sarcoid on the prepuce; (**c**) SCC on the penile glans of an aged gelding. The picture was taken immediately before surgery to amputate the distal 15 cm of the penis

et al. 1979). However, ISH failed to demonstrate BPV1/2 DNA in sarcoid epidermis, and no virion has been detected by electron microscopy at that time. Accordingly, BPV1/2 infection was assumed to be abortive in equids, with virus exclusively residing in sarcoid fibroblasts in an episomal form (Amtmann et al. 1980; Lancaster 1981). These experiments were the first in a long row of investigations leading to the recognition of BPV1 and BPV2 as the major causative agents of equine sarcoids along with trauma (Chambers et al. 2003a; Hainisch and Brandt 2015; Nasir and Reid 2006; Nasir and Brandt 2013).

With the advent of modern molecular biological and immunological methods, many important aspects of BPV1/2 infection in equids and associated tumour development have been elucidated (Hainisch and Brandt 2015; Nasir and Brandt 2013). However, it is still unclear how BPV1/2 is transmitted to equids. Infection

is still thought to be abortive, with sarcoid-affected animals thus representing a dead-end host. As a consequence, it has been assumed that infection is directly acquired from cow wart-affected bovines or, indirectly, from contaminated fomites or may be achieved by infected cells without the need of infectious virions (Bogaert et al. 2005; Chambers et al. 2003a). Contaminated fomites may include trees and fence posts on which horses scratch their body or tack and grooming kits. BPV1/2 DNA has also been found in insects caught in the vicinity of sarcoid-affected equids (stable flies, horse flies), leading to the theory that insect vectors may have a role in BPV1/2 transmission (Finlay et al. 2009; Kemp-Symonds 2000). This concept is substantiated by the fact that sarcoids often develop at insect-infested sites of the body such as the belly, the groin and the external genitals (Hainisch and Brandt 2015).

There are several lines of evidence that refute the theories of an abortive BPV1/2 infection in equids and the transmission of infection without virion. First, it has been demonstrated that intracranial injection of hamsters with BPV1 virion resulted in the development of sarcoid-like intracranial and cutaneous lesions, whereas injection of heat-denatured virion had no apparent effect (Robl et al. 1972). In analogy, intradermal inoculation of foals with BPV1 virion led to the formation of pseudo-sarcoids, whilst inoculation with naked BPV1 genome or primary sarcoid fibroblasts containing viral episomes produced no overt skin malignancies (Hainisch et al. 2012; Hartl et al. 2011). Gobeil et al. (2007) have likewise demonstrated that sarcoids are not inducible by an infectious cell line. Second, BPV1 L1 mRNA and capsid protein were shown to be intralesionally expressed (Brandt et al. 2011; Nasir and Reid 1999). Given that PV L1 expression and subsequent virion assembly is confined to the upper epidermal layer, this finding indicates that, contrary to previously reported data, BPV1 infection may also involve equine epidermis and be productive in this skin layer. Indeed, analysis of micro-dissected sarcoid epidermis revealed the presence of viral DNA and L1 protein in a subset of tumour samples (Bogaert et al. 2010; Brandt et al. 2011). Using an approach that combines an antibody capture step for selective virion isolation with highly sensitive BPV1/2 PCR, presence of L1 capsomeres in a complex with viral genome was shown for about 58 % of tested sarcoids with maximum concentrations of 125 complexes per 50 µl of cell-free sarcoid extract (Brandt et al. 2008). In accordance with this observation, Wilson et al. have visualised BPV1 virions in sarcoid sections by transmission electron microscopy (TEM) (Wilson et al. 2013). These laboratory findings are corroborated by a field study where co-stabling of sarcoid affected with healthy donkeys resulted in the latter developing sarcoids (Nasir and Campo 2008). In addition, sarcoids of donkeys and horses were shown to contain equid-specific variants of BPV1 that are not found in bovine warts (Brandt et al. 2008; Chambers et al. 2003b; Nasir et al. 2007; Trewby et al. 2014). Taken together, it appears more realistic that intact virions are needed for initial infection of equids, which is also productive, at least in some equids and/or at some stages of sarcoid disease.

In humans, many years can elapse between initial HPV infection and associated tumour development (Bosch et al. 2006). In equids, the time span between initial infection and sarcoid development appears to be relatively short, since BPV1/2

DNA is commonly detected in lesions, intact skin and peripheral blood mononuclear cells (PBMC) of sarcoid-bearing animals, but usually not in sarcoid-free individuals (Carr et al. 2001; Chambers et al. 2003a; Nasir and Brandt 2013). The high incidence of disease especially in younger horses further supports the assumption of a relatively short incubation period (Scott and Miller 2003a). Although cases of spontaneous tumour regression have been reported, sarcoids are usually persistent tumours because equids are unable to mount a measurable immune response upon natural BPV1/2 infection. The mechanisms underlying BPV1/2 immune escape are not yet understood. However, the E5 protein may provide a direct way of immune evasion by downregulation of MHC I, which in turn compromises viral antigen presentation by this complex to immune cells (Marchetti et al. 2009).

### 10.3.2 Squamous Cell Carcinoma

Squamous cell carcinomas (SCCs) represent the most common malignant epithelial tumour in equids (Scott and Miller 2003b). They can develop anywhere on the skin, yet predominate at mucocutaneous transitions, i.e. the ocular region and external genitalia (Scott and Miller 2003b; Sundberg et al. 1977). Given the invasiveness of SCCs, surgical excision is the current therapy of choice. In severe cases, this may necessitate the exenteration of affected eyes or the en bloc resection of affected external genitalia, which in turn may lead to postsurgical complications and euthanasia of the equid patient (Mair et al. 2000; van den Top et al. 2008).

Genital SCCs (gSCCs) account for 50–85 % of all genital tumours in hospital populations. The typical patient is a gelding older than 15 years. Lesions initially present as papillomatous plaques or papillomas on the penile glans or shaft. When left untreated, lesions progress to carcinoma in situ and SCC (Fig. 10.1c) that metastasises in about 12–15 % of cases. SCCs can spread through contact to the prepuce or via lymphatics to local lymph nodes and the abdomen and in rare cases to the vertebral bodies and lungs. Mares can be likewise affected, with disease mostly involving the clitoris or vulva (Scott and Miller 2003b; van den Top et al. 2008).

Over the past decade, evidence for an active involvement of papillomavirus infection in the development of equine SCCs has substantially increased. A novel PV termed equine papillomavirus type 2 (EcPV2) was identified from a case of genital SCC and its genome fully characterised (Scase et al. 2010). Subsequent screening of a series of genital and ocular SCCs/SCC precursor lesions (plaque, papilloma, carcinoma in situ) revealed the consistent presence of EcPV2 DNA and mRNA in the genital lesions, whilst SCCs of the nictitating membrane and conjunctiva scored negative for EcPV2 (Kainzbauer et al. 2012; Scase et al. 2010; Sykora et al. 2012). Screening of ocular and genital swabs as well as milk and semen from apparently healthy horses resulted in an EcPV2 DNA detection rate of 2.6 % (Sykora et al. 2012). Consistent presence of EcPV2 in genital SCC and the low incidence of infection in tumour-free individuals were confirmed by several independent studies (Bogaert et al. 2012; Fischer et al. 2014; Knight et al. 2013; Lange et al. 2013a). Taken together, this body of evidence indicates that EcPV2 infection is causally

associated with the development of genital plaques, papillomas, carcinoma in situ and SCC. EcPV2 DNA was also detected in a subset of equine oropharyngeal SCCs and ocular SCC metastases (Kainzbauer et al. 2012; Knight et al. 2013). The significance of these findings is subject to current investigations. Since the discovery of EcPV2 in 2010, five novel EcPV types termed EcPV3–7 have been identified from genital (EcPV4) and aural plaques (EcPV5–6) and penile lesions (EcPV3, 7). However, an aetiological association of these EcPV types with tumour disease remains to be established (Lange et al. 2013b; van den Top et al. 2015).

## 10.4 Protecting Horses from Papillomavirus-Induced Tumour Disease

Depending on the location and severity of the lesions, sarcoids and SCCs are treated by topical application of antiviral ointments or chemotherapeutics, cryo- and radiotherapy, total removal of the lesion by ligation or (laser) surgery or by combinations of these modalities. The earlier disease is diagnosed and treated, the better is the chance of successful therapy (Pascoe and Knottenbelt 1999). However, early detection of gSCCs and precursor lesions can be problematic, especially in geldings, where the development of penile lesions often remains unnoticed by the owner until they have progressed to massive malodorous bleeding masses, because the penis is usually retracted in the prepuce. At such a late stage, the prognosis is poor or hopeless (Hainisch and Brandt 2015; Pascoe and Knottenbelt 1999).

Given that sarcoids and gSCCs constitute highly relevant diseases in equids and that immunisation of humans with VLP-based vaccines has proven highly effective in preventing HPV-induced cancers, an attempt was made to establish a vaccine for protection of equids from sarcoids and gSCCs. Safety and immunogenicity of BPV1 L1 VLPs were assessed in a phase I dose-escalation trial, showing that intramuscular immunisation of horses with 50, 100 and 150 μg of BPV1 L1 VLP in alum was well tolerated and induced high titres of neutralising antibodies irrespective of the dose (Hainisch et al. 2012). On the basis of inoculation experiments conducted in the 1950s and 1960s (Olson and Cook 1951; Ragland and Spencer 1969; Voss 1969), four horses were intradermally inoculated with cow wart-derived BPV1 virions, naked BPV1 genome and sarcoid cells on the neck and then monitored (Fig. 10.2). Pseudo-sarcoids developed exclusively at sites inoculated with virions. Tumours became palpable 11–32 days after inoculation, reached maximum sizes of 2 cm in diameter and then resolved spontaneously within 6 months, although no neutralising anti-BPV1 serum antibodies were detectable throughout the trial. Interestingly, viral DNA and mRNA were not only detected from lesions but also from PBMCs already before lesions were first palpable. Immunofluorescent staining revealed the presence of the E5 protein in tumour fibroblasts, but not in the apparently normal epidermis overlying the lesions. Taken together, intradermal inoculation of horses with BPV1 virions reliably resulted in the formation of transient pseudo-sarcoids, thus constituting a robust challenge model (Hartl et al. 2011).

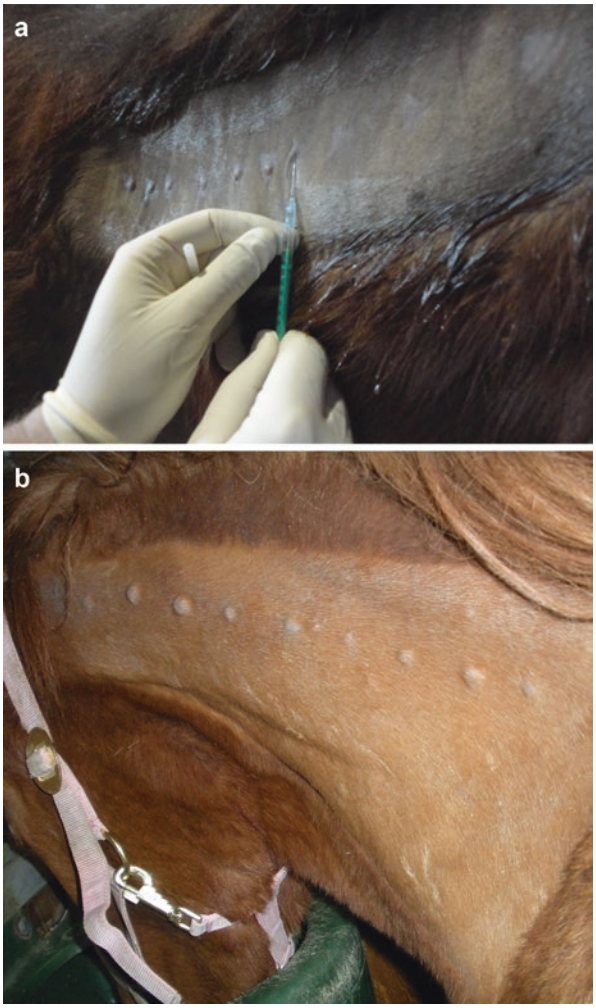


**Fig. 10.2** Skin inoculation in horses with papillomaviruses. (**a**) Intradermal injection of cow wart extract in a horse. This is a robust in vivo method to test whether a papillomavirus causes infection. In this case the horse is inoculated with BPV1 to produce pseudo-sarcoids. Note the six sites to left of the needle which have already been injected. Intradermal injection results in a small wheal as fluid cannot readily disperse in the dermis. Subcutaneous injection does not result in wheal formation. Wheal formation is therefore a control for the proper use of the technique; (**b**) intradermal inoculation of the neck with BPV1 has resulted in the development of ten pseudo-sarcoids at all ten inoculation sites approximately 5 weeks after inoculation. The lesions at this point in time are at the peak of their growth and measure about 1 cm in diameter. Five months after inoculation, regression of all the lesions was complete in this horse

This model was used to address the protective potential of BPV1 L1 VLPs. To this aim, horses were immunised with BPV1 L1 VLPs or left unvaccinated and then challenged intradermally with BPV1 virions (Fig. 10.2a). Whilst

control horses developed pseudo-sarcoids at all inoculation sites (Fig. 10.2b), vaccinated horses showed complete protection from tumour formation. Because BPV1 and BPV2 were shown to be closely related serotypes (Shafti-Keramat et al. 2009), horses were vaccinated or not vaccinated with a bivalent BPV1/EcPV2 L1 VLP vaccine and then challenged with BPV2 virions. The rationale of this trial was to study the cross-protective potential of BPV1 L1 VLP-induced antibodies against BPV2 in vivo and to address the safety and immunogenicity of EcPV2 L1 VLPs. As anticipated, intramuscular administration of the bivalent vaccine was well tolerated and induced a robust antibody response that was however significantly lower than the response to the monovalent vaccine. As a conceivable consequence, vaccination resulted in incomplete protection from BPV2-induced pseudo-sarcoid formation. Given that extremely high virion concentrations were used for horse challenge, i.e. a minimum of $10^6$ BPV2 virions per inoculation site, it can be speculated that BPV1 L1 VLPs as monovalent or component of a polyvalent vaccine will protect from natural BPV2 infection (Hainisch et al. 2015). Importantly, horses challenged with BPV1 more than 5 years after immunisation with three different doses of BPV1 L1 VLPs (Hainisch et al. 2012) were completely protected from infection. Surprisingly, protection did neither correlate with the vaccine dose nor with BPV1-neutralising serum antibody titres which were generally low and had dropped below detection level in one animal (Hainisch et al. 2015). Taken together, immunisation of horses with BPV1 L1 VLPs was safe, induced long-lasting protection from experimental BPV1 infection and confined partial protection from BPV2 challenge (Hainisch et al. 2015).

Immunisation of horses with BPV1/EcPV2 L1 VLPs proved safe and immunogenic. Therefore efforts were made to address the prophylactic potential of EcPV2 L1 VLPs in vivo. To this aim, rabbits were immunised with EcPV2 L1 or control VLPs, and then respective rabbit pre-immune or immune sera were transferred to mice. Subsequent intravaginal challenge of mice with EcPV2 L1 pseudo-virions (PsV), i.e. capsids harbouring a luciferase reporter plasmid, resulted in complete and exclusive protection from PsV infection in mice passively transferred with EcPV2 L1 VLP immune serum (Schellenbacher et al. 2015).

Provided that a causal association of EcPV2 infection with gSCCs and possibly SCC at other sites of the body can be conclusively demonstrated, these findings recommend EcPV2 L1 VLPs as prophylactic vaccine against EcPV2 infection and associated disease in equids.

## 10.5 Synopsis

Research in natural animal PV models including rabbits, dogs and cattle has chiefly contributed to today's knowledge regarding the mechanisms underlying PV infection and tumour formation. It has led to the recognition of HPVs as oncogenic viruses and ultimately to the establishment of effective vaccines for prevention of HPV-induced tumour disease in humans.

To date highly sophisticated molecular biologic and immunological methods and powerful in vitro and in vivo models are available for the direct study of HPV infection and associated human disease. This has led to important scientific and clinical insights, some of which have motivated veterinarians and virologists to attempt the establishment of vaccines for equid PV tumour prophylaxis and treatment. Whilst studies on sarcoid immunotherapy are still ongoing, a large body of evidence that BPV1 L1 and EcPV2 L1 VLPs constitute an effective vaccine for protection of equids from sarcoid and gSCC disease is available today and will be hopefully implemented into practice.

PV research in animals and humans, and particularly the establishment of PV VLPs as prophylactic vaccines, represents a good example for highly successful comparative research that merits to be encouraged for the benefit of the animal and the human patient.

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