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Recent advances in preclinical model systems for papillomaviruses

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Abstract

Preclinical model systems to study multiple features of the papillomavirus life cycle have greatly aided our understanding of Human Papillomavirus (HPV) biology, disease progression and treatments. The challenge to studying HPV in hosts is that HPV along with most PVs are both species and tissue restricted. Thus, fundamental properties of HPV viral proteins can be assessed in specialized cell culture systems but host responses that involve innate immunity and host restriction factors requires preclinical surrogate models. Fortunately, there are several well-characterized and new animal models of papillomavirus infections that are available to the PV research community. Old models that continue to have value include canine, bovine and rabbit PV models and new rodent models are in place to better assess host-virus interactions. Questions arise as to the strengths and weaknesses of animal PV models for HPV disease and how accurately these preclinical models predict malignant progression, vaccine efficacy and therapeutic control of HPV-associated disease. In this review, we examine current preclinical models and highlight the strengths and weaknesses of the various models as well as provide an update on new opportunities to study the numerous unknowns that persist in the HPV research field.

Introduction

Papillomaviruses are a diverse group of DNA viruses that cause epithelial lesions of skin and mucosa (<https://pave.niaid.nih.gov/>). These viruses are found ubiquitously in the animal kingdom and contribute substantial morbidity and mortality in the form of cancers of the anogenital and oral mucosa. Oral cancers associated with human papillomavirus type 16 (HPV16) are on the increase, and the effectiveness of current prophylactic vaccines against several high-risk HPV types (hrHPV) on HPV cancerous disease await final confirmation after several decades of data collection. Animal papillomaviruses are now characterized in many mammalian species and in several preclinical laboratory models (reviewed in (Rector & Van, 2013)). In particular, rodent, lagomorph, canine, bovine and equine papillomaviruses have been studied as surrogates for HPV disease, diagnosis, treatment and vaccine assessment (reviewed in Peh, Middleton et al., 2002). New PV models in the laboratory

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mouse system are available and continue to advance our knowledge of mucosal infections in clinically important sites.

Significant advances in understanding papillomavirus biology were obtained in early studies on bovine, rabbit and dog models. From these initial studies we gained important fundamental knowledge on viral gene function, tissue tropism, cancer progression, vaccine efficacy and therapeutics. More recent models include multi-mammate rats, and a mouse papillomavirus that can infect laboratory mouse strains. Much is still to be learned regarding the role of innate immunity on control (or lack of control) during the early stages of infection, the molecular basis of tissue tropism and site-specific targeting of PV infections at non-lymphoid tissues of the anogenital and oral mucosa. In this review we discuss some recent advances in preclinical papillomavirus models that continue to improve our understanding of papillomavirus biology, virus life cycle and therapeutic control of these important human viral pathogens.

Preclinical models (in vivo)

1. Bovine papillomavirus and cattle

Bovine papillomavirus type 1 (BPV-1) produces fibropapillomas on cattle causes tumors in rodents and transforms fibroblasts in culture (Lancaster, Olson et al., 1976; Lancaster, Theilen et al., 1979; Dvoretzky, Shober et al., 1980). It was the first papillomavirus genome to be sequenced (Figure 1) (Chen, Howley et al., 1982) and the BPVs are important preclinical models to study cutaneous and mucosal infections and PV-associated cancers. BPV-1-induced papillomas can be large and produced substantial quantities of infectious virions that were subsequently used to study viral structure transforming function in cell culture and viral protein and gene function (Baker, Newcomb et al., 1991; Booy, Roden et al., 1998; Meischke, 1979; Rabson, Yee et al., 1986; DiMaio, Treisman et al., 1982; Baker & Howley, 1987). In addition, further studies with the bovine papillomaviruses revealed many different types which demonstrated different tissue specificities (Campo, 1987; Rector & Van Ranst, 2013). BPV-2 and BPV-4 were found to be associated with bladder and alimentary canal cancers respectively (Campo, Jarrett et al., 1992; Gaukroger, Bradley et al., 1993) and are important models to study PV infections and environmental co-carcinogens (Campo, 1987). Few researchers today use this model as costs and management of cattle in academic and industrial institutions are significant and other smaller preclinical models are available. Important contributions from this virus are particularly noted in the discovery of a small hydrophobic protein known as E5 (Figure 1) (Schiller, Vass et al., 1986; DiMaio, Guralski et al., 1986) which is also found in many human papillomaviruses (HPVs) and that has transforming function (reviewed in DiMaio & Petti, 2013), host immune modulating activity (Ashrafi, Brown et al., 2006), and can activate various cellular growth factors (DiMaio & Petti, 2013; Conrad, Bubb et al., 1993; Finbow, Pitts et al., 1991). Another interesting model has arisen from the observation that BPV-1 can cause equine sarcoids in horses (Chambers, Ellsmore et al., 2003). This equine model is amenable to testing immunotherapeutic approaches given that these infections are difficult to treat yet retain BPV-1 viral antigens as foreign targets for T-cell based strategies (Carr, Theon et al., 2001; Chambers, Ellsmore et al., 2003; Bogaert, Woodham et al., 2015).

Opportunities for ongoing and new studies with the bovine PV models

- Assess mechanisms of tissue restriction of various different bovine PV types. Determine the viral components that lead to a “relaxed” tissue restriction of BPV-1/2 in which both epithelial and fibroblastic cells respond to infection in vivo.
- Assess efficacy of vaccines targeting multiple types with different anatomical locations in single outbred animals.
- Determine the molecular and cellular mechanisms of BPV-associated cancer in the co-presence of plant-derived co-carcinogens.
- Test immunotherapeutic approaches to cure/prevent persistent and recalcitrant lesions caused by BPV-1 infection of horses.

2. Canine papillomavirus and dogs

Canine oral papillomavirus (COPV) or CPV1 (Figure 2) was the first canine PV studied and has relevance to clinically important HPV infections due to the mucosotropic nature of this virus (Chambers & Evans, 1959; Watrach, Small et al., 1970; Delius, Van Ranst et al., 1994). The model was used to assess virus-like particle (VLP) vaccines and DNA-based prophylactic vaccines (Suzich, Ghim et al., 1995; Kirnbauer, Chandrachud et al., 1996; Moore, Walcott et al., 2003) as well as natural host immunity to infections that predominantly regress over time (Nicholls, Moore et al., 2001). An intriguing observation arose when COPV genome was sequenced in which a non-coding region of 1500 bp was found to be located between the E2 and L2 gene (Delius, Van Ranst et al., 1994). The evolutionary origins of this sequence and its potential function in the viral life cycle remain unknown (Bravo & Felez-Sanchez, 2015). More recent studies have identified several new canine PV types in pigmented plaques, and others that cause malignant lesions between the digits of dog paws (Luff, Rowland et al., 2016; Luff, Affolter et al., 2012; Lange, Tobler et al., 2009; Lange & Favrot, 2011). Again, with the addition of new canine PV types recently discovered, the canine model continues to have value as a model to study multiple PV infections with different tissue tropisms that could infect an individual. In recent times, very few researchers continue to use the canine PV models to study aspects of PV biology, therapeutics and vaccine testing in laboratory settings.

Opportunities for ongoing and new studies with the canine PV models

- Determine the host and viral molecular and events that lead to malignant progression of lesions between digits.
- Assess mechanisms of tissue restriction of various canine PV types.
- Determine the evolutionary origins of a non-coding region between the E2 and L2 genes that is uniquely preserved in COPV.
- Assess efficacy of vaccines targeting multiple types with different anatomical locations in single outbred animals.

- Determine the molecular and cellular immune mechanisms that lead to spontaneous regression of infections.

3. Rabbit papillomaviruses and rabbits

Cottontail rabbit papillomavirus (CRPV) or SfPV1 (Figure 3) and rabbit oral papillomavirus (ROPV) or OcPV1, have been studied extensively as models for viral function and structure (Hu, Cladel et al., 2007; Belnap, Olson et al., 1996), anti-viral treatments (Christensen, 2005; Kreider, Balogh et al., 1990), vaccines (Breitburd, Kirnbauer et al., 1995; Christensen, Reed et al., 1996), tissue and species restriction (Parsons & Kidd, 1942; Munday, Aberdeen et al., 2007), latent infections (Selvakumar, Schmitt et al., 1997; Maglennon, McIntosh et al., 2011) and immunotherapy (Han, Cladel et al., 1999; Selvakumar, Borenstein et al., 1995). Experiments with these models began in the 1930s and CRPV was the first recorded papillomavirus to be associated with malignant progression (Rous & Beard, 1935). These models continue to offer valuable opportunities to study fundamental properties of papillomaviruses such as tissue restriction and viral function in the context of intact hosts with functioning immune systems. Comparative genomics and evolutionary analyses provide additional data sets that define origins of PV viral proteins and a window into a better understanding of HPV biology (Rector & Van Ranst, 2013; Bravo & Felez-Sanchez, 2015; Doorbar, 2016).

(i) Malignant progression—CRPV was the first papillomavirus to be studied experimentally (Shope & Hurst, 1933) and to demonstrate malignant potential. In the 1930s, Rous and Beard first described malignant changes to benign CRPV skin warts in infected domestic rabbits (Rous & Beard, 1935; Rous, Kidd et al., 1936). Further studies demonstrated that various carcinogenic compounds could accelerate malignant progression rates (Rous & Kidd, 1938) and provided an important preview into the subsequent connection between several high-risk HPV types and cervical cancer (reviewed in zur Hausen & de Villiers, 2015). More recent studies in the CRPV model have shown that increasing the ratio of common to rare synonymous codons in the viral oncogenes E6 and E7 can accelerate malignant progression rates (Cladel, Budgeon et al., 2013b; Cladel, Hu et al., 2008b). Earlier studies had indicated that removing an Rb consensus binding sequence in the E7 gene led to a marked reduction in papilloma growth rates but was not essential for wart formation (Defeo-Jones, Vuocolo et al., 1993). Follow-up studies to determine whether malignant progression was delayed with these modified genomes were not pursued. Note that an increased rate of malignant progression can be achieved via codon modifications as mentioned above (Cladel, Budgeon et al., 2013b), and that these latter strategies to access viral oncogene function are more approachable given that the codon-modified genomes retain infectivity.

(ii) Viral gene function in vivo—Considerable knowledge of viral gene function has been gleaned from in vitro studies using BPV-1 transformation assays (Schlegel, Wade-Glass et al., 1986; Brimer, Wade et al., 2014) and organotypic raft cultures (reviewed in Ryndock, Biryukov et al., 2015; Regan & Laimins, 2013). Transforming activities of E6, E7 and E5 were analyzed also using transfection of human cell lines with individual and dual viral expression constructs (Durst, Dzarlieva-Petrusevska et al., 1987; Münger, Phelps et al.,

1989; Barbosa & Schlegel, 1989). The requirements for PV genes in vivo however are more challenging to assess and the CRPV model has been a pioneering model for such studies. Genes found to be essential to produce papillomas include E6, E7, E1 and E2. Non-essential genes include E4, E5, E8 (renamed E10, (<https://pave.niaid.nih.gov/>)), L1 and L2 although the latter two genes are clearly needed to complete the virus life cycle (Brandsma, Yang et al., 1992; Hu, Cladel et al., 2007). New viral genes were also discovered including E10 (Han, Cladel et al., 1998; Nonnenmacher, Salmon et al., 2006) and E9^{E2} (Jeckel, Loetzsch et al., 2003) which has been renamed E8^{E2} (<https://pave.niaid.nih.gov/>) to establish equivalence with HPV types (Stubenrauch, Hummel et al., 2000). The E8 (renamed E10) protein in CRPV and ROPV appears to have a similar function to E5 in HPV and loss of this protein in vivo leads to slow-growing papillomas (Nonnenmacher, Salmon et al., 2006; Hu, Han et al., 2002). Despite these studies, there remains limited information on the role of PV viral proteins in the PV life cycle in vivo, particularly the impact of viral proteins on the innate immune system.

(iii) Tissues and Species restriction—CRPV and ROPV are excellent models to experimentally address species and tissue restriction of papillomaviruses. These studies are currently achievable in the CRPV system because viral DNA is infectious (Ito, 1970) and improved methods to infect skin with viral DNA have been described (Cladel, Hu et al., 2008a). Early experiments with CRPV demonstrated that only rabbits and hares were susceptible to infection and no other laboratory species except fetal rat skin could be infected (Rous & Beard, 1934; Kreider & Breedis, 1969). In addition, the cutaneous tropism of CRPV and the mucosal tropism of ROPV was directly tested and found to be tissue-tropic (Parsons & Kidd, 1942; Harvey, Cladel et al., 1998). Recent unpublished studies in our lab have indicated that CRPV with replacement of the ROPV E6 and E7 genes or ROPV URR lead to loss of function whereas replacement of the CRPV L1 with the ROPV L1 led to successful infection (Hu, Cladel et al., 2007) (Table 1). These studies indicate that the viral components that control tissue-tropism are likely to map to multiple elements of the PV genome. We could insert the ROPV URR into the CRPV URR and retain infectivity but we could not eliminate the CRPV URR (Hu, Cladel et al., 2007). Histological differences between several different CRPV mutant genomes can be studied in situ on the same animal so that host genetic differences are removed from the analyses (Figure 4). In this example, 3 different CRPV genomes (wild-type CRPV and two mutant genomes) were used to induce papillomas on rabbit back skin and morphological differences can be observed.

(iv) Viral latency—Another understudied area of PV biology is the concept of latent infections. At present, latent HPV infections are indirectly suggested by detection of viral DNA at low copy without detection of viral RNA activity (Doorbar, 2013). However, in the rabbit CRPV and ROPV models, latent infections can be directly tested by several strategies. Steinberg and colleagues examined a model system of subthreshold infection of skin with CRPV followed by delayed “activation” using UV light. Their studies demonstrated that UV treatments of subclinical infections could lead to active infection demonstrating the persistence of viral DNA/RNA at the infected sites that did not show clinical signs of infection (Amella, Lofgren et al., 1994; Zhang, Nouri et al., 1999). Doorbar and colleagues demonstrated that ROPV infections that have been “cured” by host immunity showed

persistent viral DNA and RNA many months after clinical resolution of the primary infection (Maglennon, McIntosh et al., 2011). Collectively, these studies provide a clear case for the existence and persistence of latent or subclinical papillomavirus infections.

(v) Natural host immunity—Both the CRPV and ROPV models have been important models to assess natural host immunity to infections (Selvakumar, Schmitt et al., 1997; Wilgenburg, Budgeon et al., 2005). There are two natural CRPV variants that induce widely different responses in lab rabbits: one variant leads to persistent infections whereas the second variant showed high levels of regression with heavy infiltrates of host immune T-cells (Salmon, Nonnenmacher et al., 2000; Hu, Cladel et al., 2002). Minor changes to the E6 gene that converted amino acids from the regressive strain into the persistent strain altered the phenotype towards regression (Salmon, Nonnenmacher et al., 2000; Hu, Cladel et al., 2002). These studies demonstrated that adaptive immunity to PV infections were influenced by either or both antigenic changes to the viral antigen and/or functional changes to the viral oncogene. These potentially different outcomes await further experimentation. In addition, the oral infections of the tongue with ROPV lead to complete regression in all infected rabbits with heavy infiltrates of various T-cell subsets, cells with DC markers and an upregulation of MHC Class II expression on the infected keratinocytes (Wilgenburg, Budgeon et al., 2005).

(vi) Host-virus interactions using modified CRPV genomes—New information can be determined using mutant CRPV genomes to test the role of various host factors that impact virus life cycle and host innate and adaptive immunity. The key to the success of such studies in the CRPV model is that the viral DNA can be modified using various molecular biological strategies and that these mutant viral genomes can be directly applied to the skin of rabbits for testing outcomes (Hu, Peng et al., 2006; Leiprecht, Notz et al., 2014). For example, an siRNA expression cassette was introduced into the CRPV genome to test the role of host proteins in the virus life cycle (Leiprecht, Notz et al., 2014). This strategy remains underexploited in the CRPV rabbit model.

(vii) Vaccine testing (prophylactic and therapeutic)—The CRPV model has been used extensively to test the efficacy of VLP vaccines, and was the first preclinical model to demonstrate the strong and persistent protection via these reagents (Breitburd, Kirnbauer et al., 1995; Embers, Budgeon et al., 2002; Christensen, Reed et al., 1996; Gambhira, Jagu et al., 2007). More recent testing has been completed using chimeric and pseudovirus technology in which the CRPV genome has been encapsidated by HPV of various types and rabbits subsequently vaccinated with various HPV VLP and L2-based vaccines (Mejia, Culp et al., 2006; Jagu, Karanam et al., 2015). Both type-specific and cross-reactive protection can be assessed using these modifications (Boxus, Fochesato et al., 2016; Kalnin, Tibbitts et al., 2014).

Considerable studies have also been conducted to test therapeutic T-cell based vaccines including recombinant viruses and bacteria (Jensen, Selvakumar et al., 1997; Brandsma, Shlyankevich et al., 2004), DNA-based vectors (Han, Cladel et al., 1999; Han, Cladel et al., 2000; Sundaram, Tigelaar et al., 1996), and synthetic long peptides (Vambutas, DeVoti et al., 2005; Hu, Budgeon et al., 2014). These studies have collectively shown that vaccines that

induce T-cell responses to E1, E2, E6, E7, E10 and L1 show strong to intermediate protection against subsequent infection (Han, Cladel et al., 1999; Hu, Cladel et al., 2006; Hu, Han et al., 2002; Leachman, Shylankevich et al., 2002). Less efficacy has been shown when post-infection vaccinations are conducted in which some responses to E1, E6 and E7 have been observed (Han, Cladel et al., 2000). Modifications to the rabbit model include the development of an HLA-A2.1 transgenic rabbit line that can assist with the activation and measurements of HLA-A2.1-restricted CD8 responses (Hu, Peng et al., 2006; Hu, Schell et al., 2010; Hu, Cladel et al., 2008).

Opportunities for ongoing and new studies with the rabbit PV models

- Determine the host and viral molecular and events that lead to malignant progression of cutaneous lesions infected with CRPV.
- Assess viral components of CRPV and ROPV that confer tissue restriction between cutaneous and mucosal sites.
- Assess impact of the evolutionary selection of codon bias on papillomavirus genes.
- Determine the impact of viral genes on host innate immune function in immunocompetent individuals.
- Test the *in situ* role of various host immune and keratinocyte factors in the CRPV life cycle.
- Determine the immunological mechanisms that lead to failure to clear CPRV infections using the persistor/regressor hybrid genomes.
- Compare the viral RNA expression profiles in active clinical infections versus latent subclinical infections.

4. Primate papillomavirus and vaginal/cervical models

Many primate-specific papillomaviruses have been isolated and sequenced (Van Doorslaer, Bernard et al., 2011; Chen, Van et al., 2009). The most important types were located in vaginal mucosa and several studies were conducted to study both infection and transmission (Ostrow, Coughlin et al., 1995; Ostrow, McGlennen et al., 1990; Bergin, Bell et al., 2013; Harari, Wood et al., 2013). The importance of these studies is that they represented the first experimental model to study vaginal and cervical disease and an opportunity to study natural (sexual) transmissions. The challenges for these studies are significant in the areas of expense and control of individual transmissions given the requirements for most primate centers to co-house individuals.

Opportunities for ongoing and new studies with primate PV models

- Systematically assess natural sexual transmission of anogenital PV infections: male-to-female and female-to-male.
- Determine whether secondary infections occur in individuals: genital-to-anal and anal-to-genital.

5. Rodent papillomaviruses and rodents

Rodent papillomavirus models have been difficult to establish due to the prolonged absence of a mouse PV that could infect laboratory strains of mice. Recently, such a virus has indeed been discovered and a number of new observations using this model have been published. The earliest observations on rodent models included the finding of a mouse PV in European harvest mouse colony (O'Banion, Reichmann et al., 1988; Van, Rector et al., 2007), although infections of lab mice were not documented. Some excellent studies were conducted on multimammate rats that are susceptible to two PVs that infect skin and mucosa (Amtmann, Volm et al., 1984; Nafz, Schafer et al., 2008). Finally, a mouse PV that was found in a colony of athymic mice and in a wild house mouse has been adapted to study in lab mouse strains (Ingle, Ghim et al., 2011; Schulz, Gottschling et al., 2012). These models, which collectively enhance our understanding of PV infections in both cutaneous and mucosal tissues, are discussed below.

Multi-mammate rat—Two papillomaviruses, McPV1 and McPV2 infect an African rodent species known as the multi-mammate rat (Reinacher, Muller et al., 1978; Amtmann, Volm et al., 1984; Nafz, Schafer et al., 2008). These two types display cutaneous and anogenital tropisms (Nafz, Schafer et al., 2008). A colony of these animals has been established in Heidelberg, Germany. Cesarean-derived, and hence PV-free animals have been obtained to develop a secondary and disease-free breeding colony to study natural transmission in a colony environment (Schafer, Neumann et al., 2011). These rodent PVs are excellent models to study natural transmission and malignant progression in outbred populations (Vinzon, Braspenning-Wesch et al., 2014), tumor progression (Nafz, Ohnesorge et al., 2007) and vaccines (Vinzon, Braspenning-Wesch et al., 2014).

MmuPV-1 (Figure 4) and laboratory mice—MmuPV-1 is a recently discovered mouse papillomavirus that infects laboratory strains of mice (Ingle, Ghim et al., 2011; Schulz, Gottschling et al., 2012). In a few short years, several groups have already established several novel observations with this model including:

- Most mouse strains are susceptible to infection in which adaptive immunity plays a major role in rapid clearance (Handisurya, Day et al., 2014; Wang, Jiang et al., 2015; Sundberg, Stearns et al., 2014).
- The virus can infect both cutaneous and mucosal epithelium (Cladel, Budgeon et al., 2013a; Cladel, Budgeon et al., 2015a; Hu, Budgeon et al., 2015)
- UV light treatments of primary infections can lead to persistence even in immunocompetent mouse strains (Uberoi, Yoshida et al., 2016)
- A role for innate immune control of infections is suggested by studies that show resistance to infection by various immunodeficient mouse strains (Sundberg, Stearns et al., 2014).

(i) Tissue tropism and site selectivity: MmuPV-1 was first isolated and identified from lesions on the muzzles of a colony of athymic mice (Ingle, Ghim et al., 2011) and from skin from a wild-caught house mouse in Germany (Schulz, Gottschling et al., 2012). Experiments

have shown that back, muzzle and tail skin are susceptible sites and led the initial investigators to conclude that this virus shows a cutaneous tropism (Ingle, Ghim et al., 2011). However, additional studies demonstrated that secondary infections could be found in mucosal tissues of the vaginal, anal and oral cavities (Cladel, Budgeon et al., 2013a; Cladel, Budgeon et al., 2015b) and that infections could be experimentally induced at these sites with ease (Cladel, Budgeon et al., 2015a; Hu, Budgeon et al., 2015). More recent studies in our lab with several strains of mice with varying levels of immune deficiency have indicated that there are unknown mechanisms of altered tissue tropism in which vaginal sites in two strains show active and persistent infections whereas direct infection of tail skin showed no signs of clinical disease. These latter observations pave the way for studies on tissue tropism, innate immunity and selective adaptive immunity to MmuPV-1 infections.

(ii) Immune privilege, Innate immunity and differential site-specific disease: The MmuPV-1 mouse model has generated opportunities to unravel the roles of host restriction factors, innate immune control, immune privileged sites and tissue tropism in this new rodent PV model. Each new publication on this virus model continues to present new information on viral-host interactions and, given the propensity of genetically-defined mouse models currently available, much will be learned on the range of topics listed in this section that is relevant to clinical disease with HPV. A number of key clinical unknowns surrounding HPV disease may be tested in this model and include:

How does the adaptive immune response track to different non-immune tissue sites to eliminate PV infections?: Note that the MmuPV-1 is capable of infecting different skin, vaginal, anal and oral sites and that these different sites are similarly targeted by various HPVs. Does the immune system respond equally to infections that initiate at these diverse locations or are there degrees of “immune privilege” that alter immune responsiveness? There is also the possibility that local microflora can influence adaptive immune responses to PV infections that would result in an apparent difference in disease outcome similar to our findings that in some mouse strains, vaginal infections persist whereas skin infections are clinically inapparent. The molecular mechanisms of T-cell based migration to these tissues and the cellular markers that are associated with trafficking, adherence, and persistence in vaginal/anal/oral epithelium are receiving increased attention in immunology studies (Shin & Iwasaki, 2012). The question as to whether these antigen-specific T-cells remain to monitor subclinical disease is also an interesting avenue of research that is possible with this mouse PV model given the site-specificity of PV infections.

Is there a different innate immune response to PV infections at different anatomical locations?: The microenvironments in oral, vaginal and anal mucosal sites are different at multiple levels including host keratinocytes, local immune tissues and resident microflora. Each of these characteristics can influence the innate immune system differentially. We also do not yet know whether the virus life cycle is identical at each of these different anatomical locations. Clinical infections with HPV16 can also be located in anal, vaginal, cervical and oral tissues and a common assumption is that the virus life cycle of HPV16 and host immunity are similar for each location. The mouse PV model has potential to address such

questions and is also amenable to genetic manipulation of the viral genome as well as components of host adaptive and innate immunity and keratinocyte function.

(iii) Environmental co-factors and malignant progression: The MmuPV-1 model has been used to assess environmental co-factors that contribute to viral persistence and malignant progression (Uberoi, Yoshida et al., 2016) following on from studies using the multi-mammate rat PV model (Siegmund, Wayss et al., 1991; Nafz, Ohnesorge et al., 2007). Lambert and co-workers have shown that UV treatments of virus-infected immunocompetent mice altered the outcome of infections in the ear pinnae that prevented adaptive immune clearance and malignant progression (Uberoi, Yoshida et al., 2016). These observations set the stage for studies with various environmental co-factors that can be directly assessed in the context of an intact immune system as well as various genetically altered mouse strains. The model can thus be used to determine the outcome on PV infections of both cutaneous and mucosal sites in the presence of hormonal changes, contraceptives, co-incident viral infections, chemical carcinogens and alcohol.

(iv) Viral gene function: MmuPV-1 (Figure 5) has a typical PV genome organization with E1, E2, E4, E6, E7, L1 and L2 open reading frames and an Upstream Regulatory Region (URR). Unique features include the lack of an E5 ORF that is a common feature of other rodent PVs in the Pi PV family (Schulz, Gottschling et al., 2012). The function of the viral proteins can be assessed experimentally and we have tested the outcome of loss of the late proteins L1 and L2 with some interesting observations (Hu, unpublished). For example, neither L1 or L2 is required for papilloma formation or viral DNA replication, however, lack of L2 suggested some alteration in tissue tropism and a change in the location of the L1 protein to include the cytoplasm of infected epithelial cells. Other testing awaits further analyses. New observations will proceed from additional studies to assess other understudied ORFs (Ajiro & Zheng, 2015), and possible impacts of viral factors on host miRNA (Zheng & Wang, 2011).

Opportunities for ongoing and new studies with the rodent PV models

- Determine the host, viral and environmental molecular and events that lead to malignant progression of lesions at cutaneous and mucosal sites.
- Determine the molecular and cellular immunological mechanisms that lead to successful clearance and “control” of PV infections at different anatomical sites.
- Assess impact of the evolutionary selection of codon bias on papillomavirus genes.
- Determine the impact of viral genes on host innate immune function in genetically-defined mouse strains
- Test the *in situ* role of various host factors in the MmuPV-1 life cycle.
- Examine wild populations of mice for additional mouse-specific PV types.

6. Other papillomavirus animal models (transgenic mouse models, tumor transplant mouse models, athymic xenograft model)

Various other models to study papillomaviruses have been developed which include transgenic mouse models, tumor transplant models and athymic xenograft models. Initial work with a transgenic mouse model containing BPV-1 and HPV-5 and HPV-18 showed the development of skin lesions and viral amplification in situ in mice with BPV-1 genomes (Kondoh, Li et al., 1995; Hanahan, Wetzel et al., 1989). Several transgenic mouse models expressing HPV viral oncogenes in cutaneous tissues have also been studied extensively (Thomas, Pitot et al., 2011; Damian-Morales, Serafin-Higuera et al., 2016; Griep, Herber et al., 1993; Akgul, Pfefferle et al., 2006; Schaper, Marcuzzi et al., 2005). Important discoveries have accrued from these models on the impact of viral oncogenes in vivo, and at important anatomic sites such as the cervix and anal epithelium (Spurgeon, Chung et al., 2014; Thomas, Pitot et al., 2011).

Tumor-transplantation mouse models have been exploited to test various immunotherapeutic approaches to cure HPV-associated disease and cancers (Lin, Guarnieri et al., 1996). In particular, mouse cell lines that express various HPV viral oncogenes have been used to examine improved antigen presentation strategies (Cheng, Hung et al., 2003), define the role of CD8 T-cells in tumor control (Hung, Cheng et al., 2003; Beyranvand, van der Sluis et al., 2016), assess check-point inhibitors that alleviate T-cell exhaustion (Liu, Zhou et al., 2016; Mkrtichyan, Chong et al., 2013) and improve vaccine efficacy (Song, Yang et al., 2014; Peng, Qiu et al., 2016; Soong, Song et al., 2014).

Mouse xenograft models were the first experimental system to grow stocks of HPV in a laboratory setting (Kreider, Howett et al., 1985). HPV11, HPV16, HPV40, HPV83 and HPV6 were successfully grown in human foreskin xenografts in athymic and SCID mice (Bonnez, DaRin et al., 1998; Brown, McCowry et al., 1998; Christensen, Koltun et al., 1997), and important observations on the tissue tropism were obtained for HPV11 using tissue fragments from different anatomical locations (Kreider, Howett et al., 1987). These models have not been studied in recent years most probably due to the development of organotypic raft culture systems (Ozbun & Patterson, 2014) and pseudovirus technologies (Buck, Pastrana et al., 2005; Pyeon, Lambert et al., 2005). Nevertheless, these models retain value in the assessment of viral tropism and the production of large quantities of native virions for structural studies.

In vitro models

We briefly mention here the important role of some in vitro model systems that have greatly improved our understanding of HPV biology. In particular, the organotypic raft culture system (reviewed in (Biryukov & Meyers, 2015; Meyers, Bromberg-White et al., 2002; Ryndock, Biryukov et al., 2015; Broker & Chow, 2001)) has been used extensively to assess viral gene function in host keratinocytes and provides opportunities to establish host markers that can be validated in preclinical animal models discussed in this review.

Conclusions

Animal PV models have paved the way for improved knowledge of many fundamental principles of PV and HPV biology. The unique tissue and species restriction and the intimate association with differentiating keratinocytes with the virus life cycle still await further elucidation at the molecular and cellular levels. A better understanding of the role of host viral restriction factors and host innate immunity will only occur with continued study of preclinical papillomavirus models that are amenable to genetic manipulations. A number of unanswered questions related to papillomavirus biology, treatment and host responses are presented below.

- How can we use preclinical models to study the role of innate immunity in HPV infections and disease?
- How can we validate markers/host restriction factors in HPV disease using preclinical models?
- How do we study host immunity to infections in situ that arise in non-immune tissues?
- How accurate are animal models of HPV disease (Peh, Middleton et al., 2002)?
- What improvements are needed for in vivo preclinical PV models to better understand HPV biology?
- What HPV clinical paradigms can be addressed using preclinical models?
- Are there any other animal model PV systems that could be developed and/or improve our understanding of HPV biology?

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Highlights

- In this review, we discuss some recent advances in preclinical papillomavirus models that continue to improve our understanding of papillomavirus biology, virus life cycle and therapeutic control of these important human viral pathogens. Model systems include bovine, canine, rabbit and mouse papillomaviruses.

Figure 1.
BPV-1 genome.

Figure 2.
CPV1 genome

Figure 3.
CRPV genome

Figure 4.

Histology of CRPV-induced rabbit back-skin papillomas on a single rabbit. A. wild-type CRPV persistor strain, B. CRPVE6rE6p mutant, and C. mE10-CRPV mutant. CRPVE6rE6p is a genome that includes the entire wtCRPV persistor genome with a fragment of the E6 gene replaced with the corresponding region of the E6 genome of the wtCRPV regressor strain. The mE10-CRPV genome is the wtCPRV persistor genome in which the ATG of the E10 ORF is mutated (70). The wtCPRV and E6r/E6p–CRPV show large papillomatous fronds whereas the mE8-CRPV mutant shows short papillomatous fronds. The CRPVE6r/E6p is highly immunogenic in contrast to the wtCPRV papillomas and there are collections of lymphocytes at the base of the papillomas (arrowed). Representative histology of papillomas from a single rabbit (one of 3 rabbits with similar infections and histopathology).

Figure 5.
MmuPV1 genome

Table 1*In vivo* outcome of CRPV/ROPV hybrid genomes^a

Construct	Hybrid CRPV/ROPV genome	Skin Papillomas	Ref
E1m#1	CRPV E1 sequence 3128–3170 replaced by corresponding ROPV sequence (42bp or 14 aa)	Yes	(Hu, Cladel et al., 2007)
E1m#2	CRPV E1 sequence 1999–2017 replaced by corresponding ROPV sequence (18bp or 6 aa)	Yes	(Hu, Cladel et al., 2007)
E2m#1	CRPV E2 sequence 4021–4283 replaced by corresponding ROPV sequence (262bp or ~87 aa)	Yes	(Hu, Cladel et al., 2007)
E2m#2	CRPV E2 sequence 3128–3382 replaced by corresponding ROPV sequence (254bp or ~82 aa)	Yes	(Hu, Cladel et al., 2007)
E6m#2	CRPV E6 and E7 replaced by ROPV E6 and E7	No	(Hu, Cladel et al., 2007)
E6m#3	CRPV E6 sequence 326–476 replaced by corresponding ROPV sequence (150bp or 50 aa)	No	(Hu, Cladel et al., 2007)
E6m#4	CRPV E6 sequence 326–973 replaced by corresponding ROPV sequence (647bp or ~214 aa)	No	(Hu, Cladel et al., 2007)
E6m#5	CRPV E6 sequence 476–973 replaced by corresponding ROPV sequence (497bp or ~132 aa)	No	(Hu, Cladel et al., 2007)
URRm#2	Replacement of CRPV URR with ROPV URR	No	(Hu, Cladel et al., 2007)
URRm#6	Insertion of ROPV URR into CRPV URR	Yes	(Hu, Cladel et al., 2007)
L1m#1	Replacement of CRPV L1 with ROPV L1	Yes	(Hu, Cladel et al., 2006)

^aCRPV constructs were made with various substitutions listed above. Each modified/mutant CRPV genome was tested *in situ* on the back of the rabbit and tested for the ability to generate papillomas in normal immunocompetent NZW rabbits using our delayed scarification technique. In most cases, the viral DNA was assessed genetically to ensure that the mutant genome was the source of the papilloma. We did not assess the infected site to determine whether subclinical infections occurred.