20 Applications of Viral Nanoparticles Based on Polyomavirus and Papillomavirus Structures

Jiřina Suchanová, Hana Španielová, and Jitka Forstová

CONTENTS

20.1 INTRODUCTION

Polyomaviruses (PyVs) and papillomaviruses (PVs) are small nonenveloped tumorigenic viruses. They share many common morphological features (Klug 1965) that led to their original coclassification as part of *Papovaviridae* family. In 1998, the detailed understanding of their distinct biology and genome organization resulted in the splitting of these viruses into two separate families: *Polyomaviridae* (containing the single genus Polyomavirus) and *Papillomaviridae* (containing the single genus Papillomavirus) (Van Regenmortel et al. 1999). Since their discovery, PyVs have served as research tools for revealing basic principles of viral capsid structure (Klug 1965; Anderer et al. 1967; Finch 1974; Rayment et al. 1982) and many important molecular processes in living cells. Moreover, since the early 1970s, the potential of empty

polyoma viral particles as carriers of genes into mammalian cells has been recognized (Osterman et al. 1970; Qasba and Aposhian 1971; Aposhian et al. 1975). Research into PVs has lagged behind due to the difficulty of their cultivation. DNA recombinant technology has helped to regain research interest in PVs, especially with the discovery of the presence of two types of human PVs (HPVs), HPV16 and HPV18, in human cervical tumors (Dürst et al. 1983). Consequently, several studies have demonstrated that more than a dozen HPV types are important etiological agents in human cancer. These findings accelerated PV research and led to the development of virus-like particle (VLP) technology, mainly for vaccine production. On the contrary, well-known human PyVs, BK virus (BKPyV) and JC polyomavirus (JCPyV) discovered in the 1970s (Zurhein and Chou 1965; Gardner et al. 1971), were never recognized as oncogenic in humans (Abend et al. 2009; Maginnis and Atwood 2009), and research focused on nonhuman PyVs as vectors for gene and immune therapy in PyV-unrelated cancers (Krauzewicz and Griffin 2000; Tegerstedt et al. 2005a). However, the Merkel cell PyV (MCPyV), the newly discovered PyV found in biopsies of the rare and aggressive human neuroendocrine skin cancer Merkel cell carcinoma (MCC) (Feng et al. 2008), changed the view of the oncogenic potential of PyVs in humans, and protective vaccines may soon become an important area of research. Conversely, the availability of two different protective vaccines against high-risk HPVs may result not only in progress toward therapeutic vaccines but also in the development of PV-based nanocarriers of drugs, diagnostic probes, or therapeutic genes, similar to those of PyV-based vectors. This chapter summarizes the important developments in PyV- and PV-based nanotechnology. It compares both systems for different applications when the biology and structure of the virus, interactions between the virus and its host cell, and vector production facilities are taken into account. The suitability of these viral nanotechnology tools as gene, protein, drug, or other compound nanocarriers as well as vaccines is discussed for both virus families together with the advantages and disadvantages connected to specific uses.

20.2 BIOLOGY OF PAPILLOMAVIRUSES AND POLYOMAVIRUSES

20.2.1 General Characteristics

PyVs and PVs share many common features in their morphology and biology, but they differ in some aspects. Table 20.1 shows some of these features.

TABLE 20.1
Properties of PyVs and PVs

Characteristics	PyV	PV
Virion		
Capsid symmetry	Icosahedral	Icosahedral
Diameter	45 nm	55 nm
Composition	VP1, VP2, VP3,[a] genome with cellular histones	L1, L2, genome with cellular histones
Genome		
Type (size)	Circular dsDNA (5 kbp)	Circular dsDNA (8 kbp)
ORFs	6–7 (encoded by both DNA strands)	8–10 (encoded by the same DNA strand)
Infection		
Hosts	Mammals, birds	Mammals, birds, reptiles
Tissue tropism	Various	Skin and mucosa (epithelia)
Result of acute infection	Unapparent	Microlesions, benign warts
Persistent/latent infection	Yes	Yes
Oncogenic potential		
Tumors in immunocompetent host	No	Yes (high-risk types)[b]
Tumors in immunocompromised or nonpermissive host	Yes	Yes
In vitro cell transformation	Yes	Rarely
Individual members		
Infect humans (representative members)	12 HPyV species (BKPyV, JCPyV, MCPyV)	170 HPV types (HPV16, HPV18—high-risk types)[b]
Representative animal isolates	MPyV (mouse), SV40 (monkey), HaPyV (hamster)	BPV (bovine), CRPV (rabbit), COPV(canine)
Summary	NCBI taxonomy database[c] (Browser ID: 151340)	NCBI taxonomy database[c] (Browser ID: 151341)

[a] MCPyV capsid does not contain VP3 protein (Schowalter and Buck 2013).

[b] The International Agency for Research on Cancer (IARC) classified 12 different HPV types as carcinogenic to humans: types 16, 18,31, 33, 35, 39, 45, 51, 52, 56, 58, and 59, with HPV16 and HPV18 types most frequently found in cervical cancers (Bouvard et al. 2009); several animal PVs (bovine, bat, feline) are etiological agents in carcinoma (Rector and Van Ranst 2013).

[c] Benson et al. (2009).

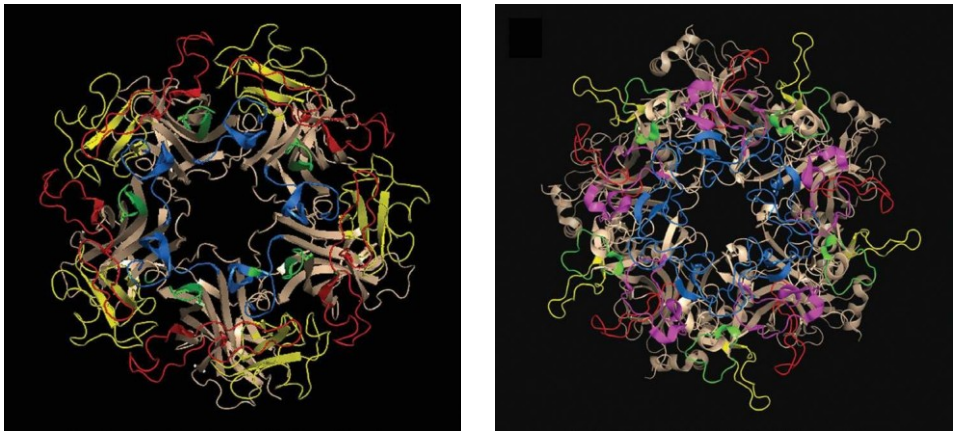
At present, the NCBI taxonomy database (Benson et al. 2009) counts almost 100 PyV species in the Polyomaviridae family, so far comprising 12 human PyVs (Ehlers and Wieland 2013). PyVs infect only mammals and birds. Most mammalian PyVs have not been directly linked to acute disease after natural infection of an immunocompetent host. Primary infection usually results in lifelong persistence, and PyVs are probably widespread benign members of the extensive flora of viruses that are associated with the body. The major sites of persistence for human PyVs are the skin, the kidney, the central nervous system, and the hematopoietic system. Under immunosuppression, however, reactivation of the viruses can occur, leading to several disease patterns (Dalianis and Hirsch 2013). PyVs are believed to have a narrow host range. Even though virus replication in permissive cells is connected with the production of tumor antigens (T antigens), the subsequent virion production leads to virus-induced cell lysis, thus preventing cell transformation. However, most mammalian PyVs are able to induce malignant tumors after inoculation of nonpermissive hosts or exhibit transforming properties in cell culture. In contrast, PyVs of birds, which are highly pathogenic especially for young animals, do not exhibit tumorigenic properties at all (Johne and Müller 2007; zur Hausen 2008).

PV isolates are traditionally described as *types*, and the taxonomy of the PVs is rapidly evolving (De Villiers 2013). For human PVs, higher-order clusters based on the sequence identity of L1 open reading frame have been established for classification in genera and species (De Villiers et al. 2004). At present, 170 HPV types are known, compared to only 12 human PyVs (De Villiers 2013). Similar to PyVs, the nonHPVs have been recovered from a vast array of mammalian species, including the mouse (Ingle et al. 2010), whose PV was not known of for a long time. PVs have been found in birds and, unlike PyVs, also in three species of reptiles (Rector and Van Ranst 2013). Depending on the type of the tissue of origin, PV types are commonly grouped as either cutaneous or mucosal. The mucosal-type HPVs differ in their carcinogenic potential, and according to this, they are sorted into three groups: high risk, intermediate risk, and low risk (Bouvard et al. 2009). The low-risk group produces benign skin lesions and includes HPV6, HPV11, HPV42, HPV43, and HPV44. HPV types from this group are not associated with carcinomas, but HPV6 and HPV11 can cause genital warts. The intermediate group is composed of HPV31, HPV33, HPV35, HPV51, HPV52, and HPV58, which are detected in benign skin lesions as well as in cancer cells. The high-risk HPVs are preferentially detected in carcinomas and encompass, for example, HPV16, HPV18, HPV45, and HPV56 (Furumoto and Irahara 2002). Because the intermediate-risk group is also present in cancer cells, it is very difficult to distinguish HPV types between these two groups, and sometimes the intermediate-risk HPVs are presented as part of the high-risk group.

Productive infection takes place only in differentiating keratinocytes, and virions are frequently found in the skin swabs of healthy humans or animals (Antonsson and Hansson 2002; Rector and Van Ranst 2013). In some cases, PVs cause benign tumors (warts, papillomas) found in the skin and mucosal epithelia in their natural host and occasionally in related species (reviewed in zur Hausen 2001). Some papillomatous proliferations induced by specific types of PVs bear a high risk for malignant progression (reviewed in zur Hausen 2002). PVs seem to coexist with their host preferentially in a latent infection over long periods of time. Similar to PyVs, immune suppression can lead to reactivation or increased susceptibility to reinfection, and immunodeficiency may also predispose humans and animals to develop papillomas and carcinomas (reviewed in Sundberg et al. [2000] and Denny et al. [2012]).

20.2.2 Virion Structure

The capsid proteins of PyVs and PVs exhibit only weak sequence homologies (Belnap et al. 1996), but the structures of monomeric VP1 and L1 are remarkably similar; they have a typical jelly roll structure comprised of an eight-stranded antiparallel barrel. Electron microscopy and image analysis of negatively stained PyVs and PVs (Klug 1965) have helped to determine that the capsids of both viruses have 72 pentameric capsomeres composed of major capsid proteins arranged with T = 7d icosahedral lattice symmetry. The atomic structures of simian virus 40 (SV40) (Liddington et al. 1991; Stehle et al. 1996), murine PyV (MPyV) (Rayment et al. 1982; Stehle et al. 1994), HPV16 (Chen et al. 2000), three other HPVs (HPV11, HPV18, and HPV35) (Bishop et al. 2007), and bovine papillomavirus 1 (BPV1) (Wolf et al. 2010) showed that capsomere arrangement and intercapsomere contacts are slightly different between the two families (Figure 20.1). The C-terminal arms of the major capsid protein always mediate interpentamer contacts. For PyVs, the C-terminal assembly domain (approximately 60 residues) invades the neighboring pentamer and terminates within the target subunit. In SV40, the C-terminal arm is anchored to the invaded pentamer by an interpentamer disulfide bond, and in MPyV, the invading arm is locked in place by an intrapentamer disulfide bond (Stehle et al. 1996). In contrast, the long C-terminal ends (approximately 90 aa residues) of BPV1 form elaborate loops to create the interpentamer contacts and reinsert into the core of the pentamer from which they emerge (Wolf et al. 2010). The formation of disulfide bonds is important for stable virion assembly in both viral families. In MPyV, disulfide bonds enable complete particle assembly and prevent capsid disassembly, but are not essential for the formation of VLPs (Schmidt et al. 2000). In SV40, transient disulfide bonding occurs during the intracellular folding and pentamerization of the major capsid protein VP1 (Li et al. 2002), and disulfide bonds that stabilize the capsid structure (Ishizu et al. 2001) are observed between SV40 VP1 pentamers (Stehle et al. 1996). For PVs, the extent of disulfide bonding can slightly differ between species and serotypes, as HPV16 L1 has been observed to dimerize and trimerize (Ishii et al. 2003) and BPV1 has more extensive cross-linking (Buck et al. 2004; Wolf et al. 2010). Moreover, the cellular DNA in recombinant VLPs increases the disulfide cross-linking of L1, indicating



(a) (b)

FIGURE 20.1 Structure of the recombinant VP1 pentamer (a) (PDB ID, 1VPN, Stehle and Harrison [1996]) and L1 pentamer (b) (PDB ID, 2R5I, Bishop et al. [2007]). Ribbon drawing of the pentamer with elaborate loop domains located on the exterior surface of the assembled pentamer. Each loop is highlighted in a different color: BC loop in red, DE loop in blue, EF loop in yellow, FG loop in magenta, and HI loop in green. Note that L1 loops are more elongated than VP1 loops and that the FG loop is not formed in a PyV pentamer. The C-termini of both, VP1 and L1, are disordered and likely extended into the interior space of the pentamer in the particle. Pentamers were visualized and colored using the PyMOL molecular graphics system. (From DeLano, W.L., *The PyMOL Molecular Graphics System*, version: v0.99, Schrödinger LCC, Cambridge, MA, 2006.)

that nucleic acid in the virion likely induces a capsid conformation that is structurally distinct from that of the VLP (Fligge et al. 2001). The recent model suggests that assembled virions of PVs undergo a slow process of disulfide bond formation and shuffling (i.e., maturation) in order to stabilize the virion by correct formation of inter-L1 disulfide bonds (Buck and Trus 2012). Interestingly, treatment of purified BPV1 with reduction agent dithiothreitol (DTT) is associated with a conformational change resulting in expansion of the capsids by approximately 10% in diameter (Li et al. 1998). This expansion allows the penetration of proteases and nucleases to the interior, which can then result in virion disruption. This structural change may correspond to the *open* capsids seen by cryoelectron microscopy in different PV species but not found in the PyVs (Belnap et al. 1996). This suggests that PyVs use additional factors for virion stabilization. Indeed, calcium ions, which are not used to stabilize the PV virions, are important for the assembly of PyV capsids. The calcium-binding sites consist mainly of acidic amino acids, which are a conserved feature in VP1 sequences across PyVs. Forming calcium salt bridges has been shown to be important for SV40 virion formation (Li et al. 2003) and MPyV capsid assembly (Haynes et al. 1993; Schmidt et al. 2000; Chuan et al. 2010).

The VP1 and L1 monomers adopt a very similar structure. The eight antiparallel strands are ordered in two β-sheets, which stick against one another in each monomer, forming the hydrophobic core of each protein. Additional β-strands align with the β-sandwiches from neighboring capsid protein molecules to form pentamers. Within the core domain, four or three predominant loops are located on the exterior surface in the assembled pentamer and particle in PyVs (Figure 20.1a; BC, DE, EF, HI loops) or PVs (Figure 20.1b; DE, EF, FG loops), respectively. L1 loops are more elongated than VP1 loops and mediate additional interpentameric contacts: the HI loop of one monomer intertwines with the FG and EF loop of the anticlockwise neighbors (Garcea and Chen 2007). The capsid protein sequences exposed on the surface of pentamers and virions represent the most variable regions among PV serotypes (Chen et al. 2000), BKPyV virus variants (Jin et al. 1993; Luo et al. 2012; Pastrana et al. 2013), and PyV species in general (Fang et al. 2010; Neu et al. 2011). This variability probably represents an immunologically driven evolution of serotypes and tissue specificity adaptation. Epitopes identified for neutralizing monoclonal antibodies for HPV16 and HPV11 can be mapped directly to surface loop domains on the capsomere (Chen et al. 2000). Similarly, it has been found that polymorphism located close to the receptor-binding site in the BC loop of VP1 in BKPyV genotypes can permit the escape from antibody-mediated neutralization and determine cellular tropisms and pathogenic potentials (Pastrana et al. 2013). Different strains of MPyV also exhibit different tropisms and pathogenic potentials depending on mutation in the surface loop of the VP1 protein (Mezes and Amati 1994; Bauer et al. 1995).

In PyV virions, the VP1 capsomeres associate with two minor capsid proteins, VP2 and VP3, which are not needed for capsid assembly but play important roles during the early steps of virus infection (Section 20.2.3). Crystallographic studies of MPyV have shown that minor proteins insert into the inward-facing cavity along the fivefold axis of a VP1 pentamer (Griffith et al. 1992). The C-terminus of VP2/VP3 inserts in an unusual, hairpin-like manner into the axial cavity of the VP1 pentamer, where it is anchored strongly by hydrophobic interactions. The sequence alignment of VP2 from eight different PyV species detected conserved amino acids in the region covering the contact structure between VP1 and VP2 (residues 269–296), thus suggesting that interaction

between the VP2 and VP1 pentamers involves a similar structure in all PyVs. The N-terminal part of the minor protein appears to be flexible and not tightly folded. Therefore, it can, under the appropriate circumstances (e.g., during cell entry), emerge from the inside of the virion through the 12.5 Å capsomere openings (Chen et al. 1998). PyV virions contain an average of one minor capsid protein (either VP2 or VP3) per capsomere (Imperiale and Major 2007), but this issue has been revised by Schowalter and Buck (2013), and the possibility that two minor capsid proteins associate with one pentameric capsomere cannot be excluded.

Analogously, biochemical analysis of HPV16 capsid preparations showed that up to 72 molecules of L2 can be incorporated per capsid, and cryoelectron microscopy and image reconstruction analysis of these capsids have revealed an icosahedrally ordered L2-specific density beneath the axial lumen of each L1 capsomere (Buck et al. 2008). L1–L2 contacts are mediated by a well-characterized hydrophobic interaction domain in the C-terminal part of L2 and probably also by the interaction domain in the N-terminal part of L2 (Finnen et al. 2003). Both termini of L2 molecules seem to be closely apposed within the capsid (Buck et al. 2008), but a small portion of the N-terminal region of L2 is thought to be exposed on the surface of mature capsids (Hagensee et al. 1993; Liu et al. 1997; Kondo et al. 2007).

20.2.3 Interactions between Virus Proteins and Host Cell

This section will focus on the interactions of PyV and PV particles or their constituents during their attachment on the surface of host cells, the internalization process, and their trafficking toward the cell nucleus where gene expression and virus replication take place.

20.2.3.1 Interactions of Virions with Receptors on Plasma Membrane

Both PyVs and PVs enter cells by receptor-mediated endocytosis. The major structural protein VP1 of PyVs or L1 of PVs is responsible for attachment of virions to the cell surface.

20.2.3.1.1 Receptors of Papillomaviruses

PVs are highly species- and tissue-specific viruses. They infect skin and mucosa epithelial cells exclusively. Differentiation of these cells is vital for the completion of PV replication. This fact impedes PV propagation in vitro. Progress was made after developing and propagation of VLPs composed of the major structural protein, L1, or both PV structural proteins, L1 and L2 (Zhou et al. 1991; Kirnbauer et al. 1992; Hagensee et al. 1993; Kirnbauer et al. 1993; Rose et al. 1993; Touzé et al. 1996; Hildesheim et al. 2007). However, particles lacking L2 and/or viral DNA complexed with host cell histones exhibit slight conformation changes that more or less affect their interactions. This problem was at least in part overcome by the development of particles more resembling native virions, known as pseudovirions (PsVs), containing reporter plasmids in complex with cell histones (Buck et al. 2004, 2005a) (Section 20.4.2). The development of in vitro organotypic epithelial *raft* cultures, permitting full differentiation of keratinocytes, provided the successful propagation of some HPVs for control experiments (Meyers et al. 1992). Entry of PVs into in vitro cultured epithelial cells is initiated by attachment of virions to the cell surface mediated by receptors. Interestingly, in vivo experiments in a mouse model revealed that prior to transfer to the basal keratinocyte cell surface, the epithelial basement membrane underlying basal keratinocytes is the primary site of HPV PsV binding during infection of the genital tract (Roberts et al. 2007; Kines et al. 2009).

Since the 1990s, several proteins of host cells have been described as receptors or molecules interacting with the virions of PVs during their internalization by cells. First, integrin-α6 in combination with β4 or β1 was found to bind VLPs in vitro (Evander et al. 1997; McMillan et al. 1999). Later, several other receptor or possible coreceptor candidates were suggested as heparan sulfate proteoglycans (HSPGs) (Joyce et al. 1999); laminin-332, formerly named laminin-5 (Culp et al. 2006a,b); tetraspanins (Spoden et al. 2008; Scheffer et al. 2013); growth factor receptors (Surviladze et al. 2012); or annexin A2 (Woodham et al. 2012).

Now it is commonly accepted that HSPGs mediate the first interaction of PVs with the cell surface (Joyce et al. 1999; Combita et al. 2001; Giroglou et al. 2001; Richards et al. 2013). HSPGs are heterogeneous population of molecules, composed of cell surface or matrix proteins with covalently bound glycosaminoglycans, modified by sulfation and acetylation, especially with heparan sulfate (HS). They are present on the surface or extracellular matrix of most cells. Interaction of HSPG was demonstrated for VLPs or PsVs of HPV5, HPV11, HPV16, HPV18, HPV31, and HPV33 and also for BPV1 (as reviewed in Raff et al. 2013). Experiments with VLPs composed of L1 only, and structural studies, proved that the PV major structural protein is responsible for the primary interaction with HSPGs (Joyce et al. 1999; Knappe et al. 2007; Dasgupta et al. 2011). The process of virus attachment and internalization was intensively studied with HPV16 PsVs. After interaction of virions with HSPG, they undergo conformation changes, affecting both L1 and L2 and leading to the exposure of the N-terminus of the minor structural protein, L2. Cyclophilin B (peptidyl-prolyl *cis*/*trans* isomerase) was suggested to facilitate L2 exposure (Bienkowska-Haba et al. 2009). The same group showed later that cyclophilins are also employed at an additional, postinternalization step for dissociation of L1 from a complex of L2 and genome DNA prior to egress from endosomes (Bienkowska-Haba et al. 2012). The exposed L2 N-terminus is cleaved by furin or by the related proprotein convertase 5/6. The consensus sequence for furin cleavage is highly conserved among PVs, and cleavage is essential for infection (Richards et al. 2006). Antibodies against the exposed L2 sequences were found to neutralize the virus (Gambhira et al. 2007; Day et al. 2008), and recently, a vaccine based on these sequences was prepared and induced protective immunity in mice (Chen et al. 2014). A neutralizing epitope of L2 was described to interact with the annexin

A2 heterotetramer and inhibition of an endogenous annexin A2 with antibody against annexin A2 reduced HPV16 infection (Woodham et al. 2012). PsVs precleaved by furin (unlike normal mature PsVs or native virions) were able to infect HSPG negative cells (Day et al. 2008). It has been hypothesized that conformation changes of virions after HSPG binding and furin cleavage expose a secondary binding site on the virus particle for a putative secondary receptor (Schiller et al. 2010). Whether it is α4-integrin, or one of other aforementioned candidates, or an as-of-yet detected cell surface molecule remains to be explored.

Single-particle tracking of fluorescently labeled HPV16 PsVs reveals that they bind preferentially to filopodia and afterward move rapidly on the surface of the cultured cells toward the cell body (Schelhaas et al. 2008). This *surfing* depends on the actin cytoskeleton. Particles then accumulate in discrete membrane areas prior to internalization (Schiller et al. 2010). It is not clear whether the movement from the filopodia is in connection with the HSPG receptor. As the movement of the virus particles on filopodia resembles the movement of epidermal growth factor receptor (EGFR) and EGFR signaling disruption reduces the infectivity of the HPV16 PsVs, participation of EGFR in a complex mediating HPV16 PsV surfing is hypothesized (Raff et al. 2013).

Binding and/or internalization of PVs is apparently connected to the transient activation of signaling pathway(s). Signaling mediated by integrins has been described, supporting a role of integrins in virus internalization. It was observed that HPV16 PsVs induce after their adsorption to HSPG receptor activation of focal adhesion kinase (FAK), necessary for virus entry into early endosomes (EEs) (Abban and Meneses 2010). The authors suggest the role of α4-integrin in FAK induction. Furthermore, by α4β6-activated phosphatidylinositol 3-kinase (PI3 kinase) pathway, early postadsorption of VLPs of HPV types 6b, 18, 31, 35, and BPV1 was observed (Fothergill and McMillan 2006). Other possible secondary receptor candidates of growth factor receptors were found to be rapidly phosphorylated and downstream effectors activated after HPV16 VLP binding (Surviladze et al. 2012). A recent study revealed that cellular entry of HPV16 PsVs into cells involves activation of the PI-3/Akt/mTOR pathway, leading to autophagy inhibition (Surviladze et al. 2013).

20.2.3.1.2 Receptors of Polyomaviruses

After years of intensive but unsuccessful searching for the protein receptor recognized by SV40 or MPyV, Tsai et al. (2003) demonstrated that hydrophilic sialylated oligosaccharide moieties connected with hydrophobic ceramide gangliosides serve as cellular receptors for these viruses, GM1 for SV40, and GD1a and GT1b for MPyV. The surface loop of the major capsid protein, VP1, interacts with the oligosaccharide parts of the gangliosides. Oligosaccharide (glycan) moieties of gangliosides were shown to be recognized also by other studied PyVs; GD1b and GT1b gangliosides are utilized by human BKPyV (Low et al. 2006), and GT1b ganglioside was described as the receptor for MCPyV (Erickson et al. 2009). Sialic acid (5-*N*-acetyl neuraminic acid; Neu5Ac) is crucial for the interaction of gangliosides with VP1 proteins of MPyV, BKPyV, and MCPyV. Therefore, MPyV, BKPyV, and MCPyV are able to hemagglutinate guinea pig, human, and sheep red blood cells, respectively. SV40 does not hemagglutinate red blood cells. Hemagglutination assays suggested, and structural studies confirmed, that the methods of interaction of different PyVs with glycans differ (Erickson et al. 2009; Neu et al. 2012). Infection by simian B-lymphotropic PyV (LPyV) also depends on the sialic acid on the surface of host cells. Glycan array screening has revealed that LPyV specifically recognizes a linear carbohydrate motif terminating in α2,3-linked Neu5Ac (Neu et al. 2013). Its closest related human PyV 9 (HPyV9), with different tropism, preferentially binds a similar linear carbohydrate motif that, however, terminates in 5-*N*-glycolyl neuraminic acid (Neu5Gc) (Khan et al. 2014).

Later studies of interaction of MCPyV with surface molecules (Schowalter et al. 2011; Neu et al. 2012) revealed that the primary interaction of the MCPyV with the cell surface is not mediated by GT1b or other sialylated glycans but by glycosaminoglycans, such as HS. The authors suggested that HS is required for the initial interaction of MCPyV with host cells, and secondary interaction with a sialylated coreceptor (which might be the glycan of GT1b or of another glycolipid or glycoprotein) is then necessary for virus internalization.

Recent high-resolution x-ray structure analysis of the major capsid proteins, VP1, from human PyVs HPyV6 and HPyV7, revealed substantial differences in virion surfaces in comparison to all other known PyV structures. The VP1 groove employed in interaction with specific sialic acid–containing glycan receptors in other PyVs is blocked, and HPyV6 and HPyV7 VP1 apparently do not interact with sialylated compounds in solution or on cultured human cells (Ströh et al. 2014).

The search for a receptor for human neurotropic PyV, JCPyV, was rather complicated. Several studies collected evidence that glycoproteins or glycolipids (possibly gangliosides), terminated by sialylated oligosaccharides, can serve as JCPyV receptors (Liu et al. 1998; Komagome et al. 2002; Dugan et al. 2008). In 2004, the serotoninergic 5-HT2A receptor that belongs to the serotonin receptor family and is a G protein-coupled receptor was described as a cellular receptor for the human neurotropic PyV, JCPyV, on human glial cells (Elphick et al. 2004).

Later, by using a glycan array screen and structure analyses, the linear 2,6-linked pentameric oligosaccharide, lactoseries tetrasaccharide c (LSTc; sequence NeuNAc-α2,6-Gal-β1,4-GlcNAc-β1,3-Gal-β1,4-Glc), the glycan which is not part of ganglioside was identified as the receptor motif for adsorption of human JCPyV on the surface of host cells (Neu et al. 2010). A recent study (Assetta et al. 2013) revealed that JCPyV infection requires both the LSTc and 5-HT2A receptors. While LSTc-VP1 interaction mediates the initial attachment of the virus to cells, the 5-HT2A receptor contributes to JCPyV infection by an as-yet unclear mechanism as a coreceptor, facilitating entry of virions into host cells.

Besides JCPyV and MCPyV, the concept of receptors and coreceptors might also be applicable to other PyVs (O'Hara et al. 2014). In earlier studies, SV40 binding to cells was shown to be blocked by antibodies directed against class major histocompatibility proteins (Breau et al. 1992), and, for the MPyV, α4β1-integrin was suggested as one of the possible coreceptors acting at the postattachment level (Caruso et al. 2003).

There are several aspects of PyV infection (and VLP pseudoinfection) that should be taken into consideration:

1. Although the majority of the PyVs studied interact with glycan moieties containing sialic acids, their interactions can be significantly different, owing to differences in VP1 surface loop sequences and virion surface conformation (Jin et al. 1993; Stehle and Harrison 1996; Stehle et al. 1996; Chen et al. 2000; Luo et al. 2012; Pastrana et al. 2013).
2. Mutations in surface loops of VP1 can markedly change virus tropism (Mezes and Amati 1994; Bauer et al. 1995).
3. Binding some glycolipids or glycoproteins containing sialic acids (pseudoreceptors) can be counterproductive and may result in virus destruction instead of productive infection. In situ hybridization of viral genomes with fluorescently labeled MPyV genomic DNA proved that, indeed, the majority of virions internalized by cells never deliver carried genomes into the cell nucleus (Mannová and Forstová 2003). Using a ganglioside-deficient cell line, Quian and Tsai showed that GD1a is the functional entry receptor for MPyV, binding to the virus on the plasma membrane, forming part of a complex that is internalized and further transported for productive infection. They also observed that, in contrast, glycoproteins acted as *decoy receptors*, restricting the productive infection of MPyV (Qian and Tsai 2010).

20.2.3.2 Internalization of Virions by Cells and Virus Trafficking toward the Nucleus

20.2.3.2.1 Papillomaviruses

Internalization of PVs by cells and their trafficking to the cell nucleus has been intensively studied. However, studies have often resulted in diverse, controversial findings, and limited consensus has been found. Differences have been ascribed to the different natures of the virus particles used (L1 VLPs versus L1/L2 VLPs, mature versus immature PsVs, or native virions), to cell types and experimental conditions, and to various PV genotypes used, although discrepancies have appeared even in experiments performed with the same PV genotype.

Endocytosis via clathrin-coated pits was described for BPV1 and HPV16 and HPV58 (Bousarghin et al. 2003; Day et al. 2003). For HPV31, caveola-mediated uptake was described (Bousarghin et al. 2003; Smith et al. 2008), while in another study clathrin-dependent endocytosis was suggested (Hindmarsh and Laimins 2007). A novel clathrin- and caveolin-independent entry of HPVs was described first for HPV16 (Spoden et al. 2008). Authors showed that the inhibition of clathrin-, caveolin-, and membrane raft–dependent endocytic pathways by dominant-negative mutants and small interfering RNA (siRNA)–mediated knockdown, as well as inhibition of dynamin function, did not impair infection. Moreover, they suggested that HPV16 associates with tetraspanin proteins on the plasma membrane and that tetraspanin-enriched membrane microdomains might act as entry platforms for HPV16. Thorough study of HPV16 entry into epithelial cells (Schelhaas et al. 2012), exploiting biochemical and various microscopy methods, combined with green fluorescent protein (GFP) expression after plasmid delivery by PsVs, confirmed clathrin-, caveolin-, cholesterol-, and dynamin-independent HPV16 entry into HeLa and HaCaT cells. The pathway exhibited some features of macropinosis. Similar methods of entry were confirmed for HPV18 and HPV31 (Spoden et al. 2013).

Internalization of PVs is, in comparison with other nonenveloped viruses, very slow and asynchronous, lasting several hours (Giroglou et al. 2001; Culp and Christensen 2004; Schelhaas et al. 2008). Half times of the internalization of HPV16 ranged from 4 to 12 h p.i. for the fastest and average particles (Spoden et al. 2013), and expression of reporter gene of the pseudogenome could not be detected until 24–48 h postinfection (Schelhaas et al. 2012).

Further trafficking of PVs also remains obscure. Relatively good consensus exists in respect to the requirement of acidic endosomal pH for productive infection. Infection can be blocked by selective inhibitors of endosomal acidification (Selinka et al. 2002; Day et al. 2003; Dabydeen and Meneses 2009; Schelhaas et al. 2012). Analysis of the average time that HPV16 requires for acid activation revealed that the half time of activation was 6 h. The virus required several hours to be exposed to low pH or to an enzyme requiring low pH (Schelhaas et al. 2012).

Internalized papillomaviral particles enter early compartments, EEs, or macropinosomes. Accordingly, PV infection depends on GTPase Rab5. Colocalization with EE has been observed for BPV1 (Day et al. 2003; Laniosz et al. 2008) and for HPV31 (Smith et al. 2008). No significant colocalization with an EEA1 marker of EEs was found for HPV16 (Schelhaas et al. 2012). However, the authors of the study detected a brief comigration of the viral particles with Rab5-positive compartments. The virus then appeared in multivesicular bodies, in late endosomes (LEs), and in the endolysosomal compartment. The combination of endosomal acidic pH and action of endosomal proteases may result in an uncoating process.

Uncoating can be detected during passage through the endosomal compartment. Previous furin cleavage of L2 and cyclophilin B–mediated separation of L2 together with the viral genome from the major capsid protein, L1, are necessary for escape from the LE (Bienkowska-Haba et al. 2012; Day et al. 2013).

Despite the appearance of virus particles in the endolysosomal compartment, surprisingly, Rab7 GTPase was found to

be dispensable for HPV16 or HPV31 infection (Smith et al. 2008; Schelhaas et al. 2012; Day and Schelhaas 2014).

Recently, it was observed that an uncoated viral pseudogenome in complex with L2 travels from LEs to the *trans*-Golgi network (TGN), while the major structural protein, L1, is retained mostly within the LE and appears to become degraded (Day et al. 2013). This traveling is dependent upon furin cleavage of L2. Infection in the presence of a furin inhibitor or with particles containing L2 furin cleavage mutants results in the accumulation of uncoated capsids, L2, and DNA in a late endosomal compartment. The traveling of PVs to TGN can also be prevented with inhibitors of anterograde and retrograde Golgi trafficking, brefeldin A, or golgicide A. GTPases Rab9a and Rab7b were determined to mediate this transit. Expression of dominant-negative versions of these GTPases (but not of Rab7a) inhibited HPV16 pseudovirus infection (Day et al. 2013). Genome-wide siRNA screening identified many retrograde transport factors required for efficient PV infection, including multiple subunits of the retromer, which were described to initiate retrograde transport from the EE (Lipovsky et al. 2013). PVs therefore seem to travel to the TGN from both EEs and LEs. Another sorting protein was found to interact with PVs on their way to the cell nucleus: nexin 17 (SNX17) was identified as an interacting partner of L2 protein, and its depletion was connected with the lysosomal degradation of L2 (Bergant Marušič et al. 2012; Bergant and Banks 2013). Thus, L2 protein is important for the escape of the genome from the LEs. Moreover, its conserved C-terminal peptide (the last 23 aa) was shown to be able to interact with and disrupt membranes (Kämper et al. 2006). The precise method of escape of the L2/genome complex from endosomes is not known.

Finally, PV genomes and L2 can be detected in the cell nucleus, predominantly localizing in promyelocytic leukemia (PML) bodies (Day et al. 2004). However, the way in which they reach the nucleus is not understood. One model suggests that an L2–genome complex released from the endosome is transported along microtubules and delivered to the nucleus via nucleopores. The hypothesis has been supported by the fact that nocodazole (tubulin-disrupting agent) inhibits PV infection (Day et al. 2003; Schelhaas et al. 2012) and, more importantly, by finding that L2 interacts directly with dynein light chains (Florin et al. 2006; Schneider et al. 2011). L2 protein also possesses a nuclear localization signal for karyopherin-mediated transport to the nucleus (Darshan et al. 2004).

Another model emerged from evidence that for the establishment of HPV infection and genome expression, cell division is required (Pyeon et al. 2009). Authors of this study suggest the possibility that nuclear envelope breakdown is necessary for the HPV genome to enter the nucleus. This model was very recently strongly supported by a systematic RNA interference (RNAi) silencing approach for the identification of host cell proteins required during HPV16 infection (Aydin et al. 2014). The screening uncovered a crucial role for mitosis in HPV16 nuclear entry and the HPV16 pseudogenome requirement of changes in nuclear envelope permeability facilitated by nuclear envelope breakdown.

20.2.3.2.2 Polyomaviruses

The entry of PyVs into host cells and trafficking from the cell membrane toward the cell nucleus has been intensively investigated on SV40 and MPyV model viruses. Unlike trafficking studies of PVs, research into PyV trafficking can be performed using native virions. However, the interpretation of observations has been complicated by the fact that only a minority of adsorbed and internalized virions successfully deliver their genomes into the cell nucleus, and it has not been easy to distinguish the productive pathway from that leading to virus destruction.

The most intensively studied PyV, SV40, was first described to exploit a unique endocytic pathway: after binding the GM1 ganglioside receptor, virions of SV40 become internalized by caveolae and then fuse with a large caveolin-rich endocytic compartment named by the authors as a *caveosome*. From this newly described nonacidic organelle, the virus was transported by unidentified vesicles that did not contain caveolin, along microtubules to the endoplasmic reticulum (ER) (Pelkmans et al. 2001). On the other hand, human JCPyV was found to enter glial cells by receptor-mediated clathrin-dependent endocytosis (Pho et al. 2000). At the same time, MPyV internalization was described as caveola and clathrin independent (Gilbert and Benjamin 2000). MPyV enters epithelial and fibroblast cells in smooth, tightly fitted monopinocytotic vesicles (Mackay and Consigli 1976; Richterová et al. 2001). However, caveolin-1 was detected in some monopinocytotic vesicles carrying MPyV virions by immunoelectron microscopy (Richterová et al. 2001), and Gilbert and Benjamin revealed that after addition of the GD1a receptor to rat glioma C6 cells deficient in complex ganglioside production, virus particles were internalized in caveolin-1-positive vesicles (Gilbert and Benjamin 2004). Furthermore, BKPyV was found to be associated with caveolin-1-positive vesicles in Vero cells or in human renal proximal tubular epithelial cells (Eash et al. 2004; Moriyama et al. 2007). Nevertheless, MPyV virions were efficiently internalized by Jurkat cells, which do not express caveolin-1, and lack of caveolae and overexpression of a caveolin-1 dominant-negative mutant in mouse epithelial cells did not prevent their productive infection. More recently, the caveosome concept described for SV40 has been disclaimed by authors (Mercer et al. 2010; Engel et al. 2011), and caveosomes have been described as artifacts of overexpression of caveolin fused with enhanced green fluorescent protein (EGFP) or as LEs. Now, it has been established that SV40, MPyV, and BKPyV become internalized by lipid raft microdomains (which may or may not contain caveolin-1) as infectivity of all three viruses is sensitive to cholesterol depletion (Pelkmans et al. 2001; Richterová et al. 2001; Eash et al. 2004; Gilbert and Benjamin 2004). Virion internalization is associated with tyrosine kinase signaling (Pelkmans et al. 2005; Swimm et al. 2010; Ewers and Helenius 2011) and transient actin disorganization (Richterová et al. 2001; Pelkmans et al. 2002).

Both MPyV and SV40 virions colocalize with an EEA1 marker of EEs and Rab5 GTPase (Mannová and Forstová

2003; Pelkmans et al. 2004), and infectivity of MPyV and SV40 was negatively affected by expression of a Rab5 dominant-negative mutant (Liebl et al. 2006; Engel et al. 2011). Then, the viruses appear in an endolysosomal compartment, LEs, and multivesicular bodies. Their infectivity is affected by overexpression of a dominant-negative Rab7 GTPase mutant (Qian et al. 2009; Engel et al. 2011; Zila et al. 2014). Qian and coworkers suggested that in endolysosome compartments, the GD1a receptor stimulates MPyV sorting from LEs and/or lysosomes to the ER. This suggestion is supported by previous observations that the addition of GD1a to cells deficient for gangliosides had no effect on the overall level of virus binding but mediated the transit of MPyV to ER (Gilbert and Benjamin 2004). There is a common consensus that the productive pathway continues by the transit of PyVs to the ER. However, the precise mechanism controlling the transport of MPyV to the ER remains to be clarified. No colocalization of SV40 or MPyV or BKPyV with Golgi apparatus has been observed (Pelkmans et al. 2001; Mannová and Forstová 2003; Moriyama and Sorokin 2008; Engel et al. 2011). Transport of SV40 to the cell nucleus was found to be inhibited by Brefeldin A, acting through inhibition of the ARF1 GTPase, which is known to regulate assembly of COPI coat complexes on Golgi cisternae (Norkin et al. 2002). Furthermore, colocalization of the virus with a βCOP subunit of the COP1 coatomer was detected (Norkin and Kuksin 2005). Moreover, a retrograde trafficking inhibitor of ricin and Shiga-like toxins inhibited infection by SV40 and human BKPyV and JCPyV (Nelson et al. 2013). However, no significant colocalization of MPyV with βCOP, and only mild sensitivity to Brefeldin A, was detected (Mannová and Forstová 2003). A common agreement exists that intact microtubules and a dynein motor are vital for PyV transport into the ER (Pelkmans et al. 2001; Ashok and Atwood 2003; Gilbert et al. 2003; Zila et al. 2014). Further studies are needed to solve the mechanism of PyV trafficking to the ER.

Even less clear is the mechanism by which PyVs deliver their genomes into the cell nucleus. Based on electron microscopy analyses, early papers suggest that SV40 (Maul et al. 1978) or MPyV (Mackay and Consigli 1976) enter the cell nucleus by fusion of vesicles carrying virions directly with the nuclear envelope, so bypassing nuclear pores. At present, a hypothesis that virions, partially disassembled in the ER, translocate by an as-yet unknown mechanism to the cytosol and travel to the nucleus through nuclear pores is commonly accepted. Although never proven, several findings supporting the hypothesis have been published. Schelhaas and collaborators described the dependence of SV40 infection on ER folding and quality control. Downregulation of thiol-disulfide oxidoreductases, ER57 and PDI-protein disulfide isomerase, and two ER membrane proteins, Derline 1 and Sel1L, involved in the export of misfolded proteins from the ER to the cytosol for proteasomal degradation, significantly inhibited virus infection (Schelhaas et al. 2007). For the MPyV, it has been found that downregulation of PDI and Derline 2 decreases the level of MPyV infection (Gilbert et al. 2006; Lilley et al. 2006). However, the nature and extent of virus disassembly in the ER and the means by which viral genomes are transported to the cytosol and subsequently to the nucleus are not known. The possibility cannot be excluded that the virus moves directly from the ER to the cell nucleus, bypassing nuclear pores.

One possibility of transit from the ER to the cytosol is the utilization of channels for the elimination of misfolded or unassembled proteins from the ER for proteasomal degradation (ER-associated degradation). The crystal structure of the ER translocon showed that the pore of the protein-conductive channel would allow the passage of a molecule with a diameter of 10–12 Å. In its open form, the diameter of the ER translocon was estimated to be 40–60 Å (Meusser et al. 2005). However, the diameter of PyV virions is approximately 45 nm, and the nucleocore without the capsid shell is about 30 nm in diameter.

In vitro studies on the minor structural proteins, VP2 and VP3, of SV40 (Daniels et al. 2006) and of MPyV (Rainey-Barger et al. 2007; Huerfano et al. 2010) showed that the proteins are able to bind, insert into, fuse, and even perforate cell membranes. Thus, the minor proteins VP2 and VP3 might be good candidates for helping the virus to deliver genomes to the cell nucleus.

Although significant progress in understanding PV and PyV trafficking from a host cell membrane to the cell nucleus has been achieved, several gaps remain to be filled, and many details must be clarified.

20.3 VIRUS PROTEIN SELF-ASSEMBLY AND VLP PRODUCTION

The major capsid proteins of PyVs and PVs rapidly oligomerize through a self-assembly process in vitro without the help of minor capsid proteins. In fact, intact purified proteins are never found in the monomeric form under physiological conditions. Both proteins have been successfully produced in various expression systems. Depending on the virus type, designed mutation, and actual conditions, recombinant proteins are purified as viral capsid protein assemblies: pentamers, polymorphic capsid structures, VLPs, or pseudovirion-like particles (PLPs) (for terminology see Table 20.6). Figure 20.2 gives an example of a highly polydispersed preparation of VLPs that can be further purified to near homogeneity. Recent reviews (Teunissen et al. 2013) give an excellent summary of PyV (Cho et al. 2011) and of PV production systems. Table 20.2 presents an overview of the expression system used for VLP production of both viral families.

Mammalian cells were historically the first system for PyV VLP production and isolation, when empty capsids (Crawford et al. 1962) and PsVs containing host cell DNA (Michel et al. 1967) were observed in routine viral preparations. Recently, it has been shown for SV40 that vector DNA with a size that does not exceed the size of a viral genome can be encapsidated if the viral capsid proteins are produced in the system *in trans*. For such PsV production, the

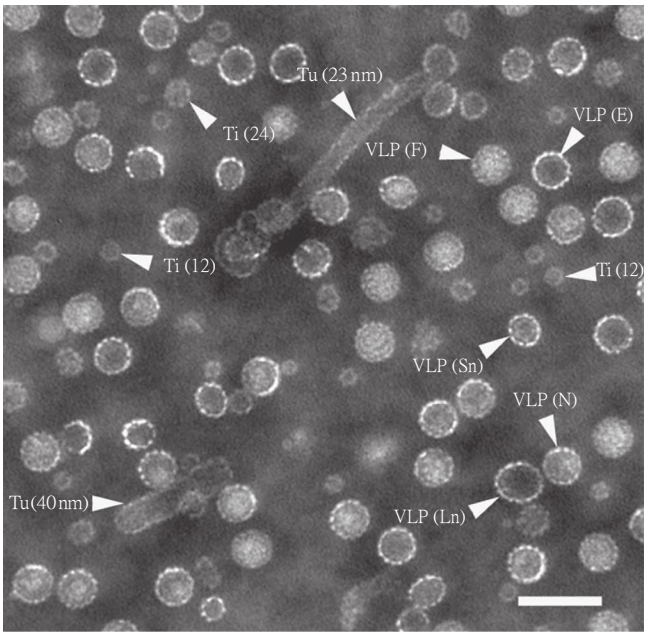


FIGURE 20.2 Electron micrograph of polymorphic structures found in MPyV VP1 VLP preparation from Sf9 cells. VLPs, which may be devoid of nucleic acid, appear as *empty* particles, VLPs (E), or may contain fragments of DNA and appear as *full* particles, VLPs (F). VLPs of various size and morphology can be formed: VLPs of normal size, VLPs (N); VLPs that are larger than normal, VLPs (Ln); VLPs that are smaller than normal, VLP (Sn); VLPs that can form tubular structures (Tu) of various diameters (in brackets) or can be composed of 12 or 24 capsomeres (labeled as *Tiny* Ti [12] or Ti [24]), respectively. Magnification 75,000×. Bar = 100 nm. Electron microscopy, Jiřina Suchanová.

wild-type virus serves as a helper (Oppenheim and Peleg 1989) to sustain high expression of capsid genes, which is difficult to achieve by expression from recombinant vectors. Due to the strictly differentiation-dependent expression of genes for capsid proteins, this approach was impossible to perform with PVs. Instead, it has been shown for PVs that several modifications of the coding sequence of capsid protein genes are needed to obtain reasonable production of capsid proteins from a heterogeneous vector (Zhou et al. 1999; Leder et al. 2001; Mossadegh et al. 2004). The genes should be optimized for codon usage and other properties known to aid protein expression, such as modification of mRNA secondary structures that might impede transcription or nuclear export. The same types of modification were proven useful for PyVs (Tolstov et al. 2009). Nowadays, the cotransfection of expression vectors encoding codon-optimized capsid protein genes with DNA that serves as a target for encapsidation is an established procedure for the production of PyV as well as PV PsVs for specialized applications (Section 20.4.2.2). For the production of VLPs devoid of target DNA, however, the mammalian system is not usually used, due to expensive transfection and cultivation conditions and a risk of potentially infectious contaminants. Interestingly, in mammalian cells, with a good transfection method and modified genes, the yield can be as high as 20 mg of the capsid protein, assembled into PsVs, per liter of media (1×10^9 cells) for PyVs (Pastrana et al. 2009; Tolstov et al. 2009) and 10 mg for PV VLPs (Buck and Thompson 2007; Buck 2012). Capsid proteins of PyVs as well as HPV PsVs have been also produced in mammalian cells with recombinant vaccinia viruses (Stamatos et al. 1987; Zhou et al. 1991; Unckell et al. 1997).

Insect cells, predominantly *Spodoptera frugiperda* (Sf9) and *Trichoplusia ni* (High Five™, H5) ovary cells and baculovirus expression systems, are a good choice for the production of most VLP types. The system is used for manufacturing one of the VLP-based vaccines against HPV Cervarix (GlaxoSmithKline). VLPs are usually assembled inside the cell nucleus. The quantity depends usually on the type or variant of the parental virus (Touzé et al. 1998) and capsid gene modification (e.g., codon optimization). The yield can be as high as 40 mg of VP1 protein assembled in VLPs per liter of cultivation media for a nonmodified VP1 gene from MPyV (our unpublished observation) and 10 mg of L1 protein per liter for PVs with optimally modified L1 genes (Xu et al. 2014). Moreover, it has been shown that a modified baculovirus-based (MultiBac) expression system can substantially improve VLP production by multiple folds (e.g., for HPV2 from 1 to 40 mg/L) for certain HPV types, whereas the conventional baculovirus expression system gives a low yield (Senger et al. 2009). VLPs can also be produced by baculoviruses in insect larvae with reasonable yield for HPV16 L1 recombinant protein produced in *T. ni* larvae; the yield was five times higher than that in cell culture and reached 21 mg/g of insect biomass (about four insect larvae) (Millán et al. 2010). For HPV6b, the VLPs were also reported to be successfully produced in larvae with a *Bombyx mori* nucleopolyhedrovirus bacmid expression system (Palaniyandi et al. 2012). The *Drosophila* inducible/secreted expression system was also used to produce VLPs. The yield of HPV16 L1 protein was 1.1 g/L of media (Zheng et al. 2008). The yield of MPyV VP1 tagged with a secretion signal for targeting to the extracellular medium was disappointingly low (2–4 mg VP1 per liter of media), and only a small fraction of the recombinant secreted protein assembled into VLP-like structures (Ng et al. 2007).

While production of VLPs from PyVs in plants has never been reported, the need for cost effective manufacturing of VLP-based vaccines against HPV led to exploration of the production of PV-based VLPs in several transgenic plant systems, using tobacco or potato plants (reviewed in Rybicki 2009). VLPs produced in these systems had correct morphology and elicited an immunological response after intravenous or oral administration, but the yields were very low. Varsani et al. (2003) reported a yield as low as 2–4 ng HPV16 L1 protein per gram of fresh tobacco leaf material, corresponding to 0.0003% (w/w) for expression from nonmodified capsid gene. Plant codon usage optimization (Warzecha et al. 2003) or usage of humanized L1 gene (Biemelt et al. 2003) has been shown to increase the yield of HPV11 L1 to 20 ng/g of fresh tuber in potato plants and to 14 μg of HPV16 L1 per gram of fresh tobacco leaves (0.5% of total soluble protein), respectively. The threshold for commercial

TABLE 20.2
Expression Systems for VLP Production

System	Virus (Protein/s)	References[a]	Structure[b]	Notes[c]
PV*s*				
Mammalian				
Vaccinia virus expression system	HPV1 (L1/L2)	Zhou et al. (1991)	VLPs	Incorrectly assembled, d = 35–40 nm
	HPV1 (L1/L2)	Hagensee et al. (1993)	VLPs	Expected morphology and size
	BPV1 (L1, L1/L2)	Zhou et al. (1993)	VLPs	L1/L2 VLPs encapsidated DNA
	HPV18 (L1, L1/L2)	Stauffer et al. (1998)	VLPs	L1/L2 VLPs encapsidated DNA
	HPV6b (L1)	Fang et al. (1999)	VLPs	
	HPV33 (L1, L1/L2)	Unckell et al. (1997)	VLPs	Empty and full capsids (PsVs), L2 not needed for encapsidation
Semliki forest virus–based expression	HPV16 (L1, L1/L2)	Heino et al. (1995)	VLPs	Correct morphology and size, no Tu
Fowlpox virus expression	HPV16 (L1)	Zanotto et al. (2011)	Prevalent Ti, some VLPs	Low expression of L1 protein, low yield of particles
Direct transfection of expression vectors	HPV16 (L1, L1/L2)	Leder et al. (2001)	VLPs	Codon-optimized L1 and L2 genes, VLPs formed abundantly in cell nucleus
	HPV11 (L1)	Mossadegh et al. (2004)	VLPs	Codon-optimized L1 gene, VLPs formed in the cell nucleus
	BPV1 (L1, L1/L2), HPV16 (L1/L2)	Buck et al. (2004)	VLPs	PsVs generated in 293TT cells
Insect				
Baculovirus expression system	BPV1 (L1), HPV16 (L1)	Kirnbauer et al. (1992)	VLPs	Correct morphology and size, also smaller particles
	HPV16 (L1/L2), HPV6 (L1), HPV11 (L1), CRPV (L1)	Kirnbauer et al. (1993)	VLPs	Better yield of VLPs from clinical sample variants and after L1/L2 coexpression
	HPV11 (L1)	Rose et al. (1993)	VLPs	VLPs formed in cell nucleus, but purification not efficient (in vitro assembly)
	HPV11 (L1)	Christensen et al. (1994)	VLPs	VLPs of variable size
	HPV11,16,18 (L1)	Rose et al. (1994)	VLPs	
	COPV(L1)	Suzich et al. (1995)	VLPs	Correct (d = 55 nm) size and morphology
	HPV33 (L1/L2)	Volpers et al. (1994)	VLPs, Tu	Spherical VLPs (d = 50–60 nm) and tubular structures (d = 25–30 nm or 50–60 nm)
	HPV6b (L1), HPV11 (L1, L1/L2), HPV16 (L1, L1/L2)	Muller et al. (1995)	VLPs	Regular VLPs
	HPV45 (L1)	Touzé et al. (1996)	VLPs	Low yield
	HPV6, HPV11, HPV16, HPV31, HPV33, HPV35, HPV18, HPV39, HPV45 (L1)	Giroglou et al. (2001)	VLPs	Regular VLPs
	HPV2, 3, 10, 27, 77B (L1) BPV5, BPV6 (L1)	Senger et al. (2009)	VLPs	MultiBac—high production system (8–40 times increase in yield)
	HPV16, HPV18 (L1)	Harper et al. (2004)	VLPs	Bivalent HPV vaccine Cervarix (GlaxoSmithKline)
	MusPV (L1, 4 variants)	Joh et al. (2014)	VLPs	
Drosophila expression system	HPV16 (L1)	Zheng et al. (2008)	VLPs	
Insect larvae/*T. ni*	HPV16 (L1)	Millán et al. (2010)	VLPs, Ti, Tu	Polymorphic structures formed
Insect larvae/*B. mori* nucleopolyhedrovirus expression system	HPV6b (L1)	Palaniyandi et al. (2012)	VLPs, Ti	Short-length and full-length L1 formed tiny or mixed population of VLP/Ti, respectively

(*Continued*)

TABLE 20.2 (*Continued*)
Expression Systems for VLP Production

System	Virus (Protein/s)	References[a]	Structure[b]	Notes[c]
Yeast				
S. cerevisiae	HPV6a (L1, L1/L2)	Hofmann et al. (1995)	VLPs	Smaller particles d = 40–50 nm, capsomeres and monomeric protein
	CRPV (L1, L1/L2)	Jansen et al. (1995)	VLPs	Spherical particles, d = 50 nm
	HPV11(L1), HPV11/6a hybrid	Neeper et al. (1996)	VLPs	VLPs detected in cell lysates d = 40–50 nm
	HPV16 (L1/L2)	Rossi et al. (2000)	VLPs	VLPs for gene transfer
	HPV11(L1)	Cook et al. (1999)	VLPs	VLPs of variable size d = 32–97 nm
	HPV16, HPV6 (L1/L2 coexpression 16/6)	Buonamassa et al. (2002)	VLPs	Coexpression of 4 proteins, chimeric VLPs
	HPV11, HPV6, HPV16 (L1)	Mach et al. (2006)	VLPs	Irregular shape, d = 30–60 nm
	HPV6, HPV11, HPV16, HPV18 (L1) (vaccine)	Markowitz et al. (2007)	VLPs	Quadrivalent HPV vaccine Gardasil® (Merck and Co., Inc., Whitehouse Station, New Jersey)
	HPV16 (L1)	Kim et al. (2007), Park et al. (2008)	VLPs	Particles d = 51 ± 15 nm, purification method described
S. pombe	HPV16 (L1, L1/L2), HPV16/HPV6 (L1/L2)	Sasagawa et al. (1995)	VLPs	L2 not incorporated in VLPs
Pichia pastoris	HPV16 (L1)	Liu et al. (2007)	VLPs	Variable size approx. 50 nm
	HPV16 (L1)	Bazan et al. (2009)	VLPs	L1 protein unstable, low yield (aggregation)
	HPV16, 18 (L1)	Rao et al. (2011)	VLPs	Variable size approx. 53 nm
Bacterial				
Escherichia coli	HPV11 (L1/L2)	Finnen et al. (2003)	Capsomeres	VLPs assembled in vitro
	HPV11 (L1, L1 mutants)	Li et al. (1997)	Capsomeres	VLPs assembled in vitro
Lactobacillus casei	HPV16 (L1)	Aires et al. (2006)	VLPs	VLPs produced intracellularly, d = 30–60 nm
Bacillus subtilis	HPV33 (L1)	Baek et al. (2012)	VLPs	Highly heterogeneous VLPs (d = 20–60 nm), problems with purification
Plant				
Tobacco and/or potato leaves	HPV11 (L1)	Warzecha et al. (2003)	VLPs	Uniform spherical particles, d = 55 nm
	HPV16 (L1)	Biemelt et al. (2003)	VLPs, capsomeres	Mainly capsomeres, VLPs, d = 55–65 nm and smaller
	HPV16 (L1)	Fernández-San Millán et al. (2008)	VLPs	VLPs assembled in the stroma of chloroplasts, high yield, d = 55–65 nm and smaller
	HPV8 (L1)	Matić et al. (2012)	VLPs, Ti	
PyV*s*				
Mammalian				
Direct transfection of expression vector	BKPyV, JCPyV, SV40, LPyV (VP1/VP2/VP3)	Nakanishi et al. (2008)	VLPs	VLPs in the form of PsVs
	SV40 (VP1/VP2/VP3)	Oppenheim and Peleg (1989)	VLPs	VLPs in the form of PsVs
	JCPyV (VP1/VP2/VP3)	Shishido et al. (1997)	VLPs	VLPs observed in the nucleus
	MPyV (VP1/VP2/VP3) MCPyV (VP1/VP2)	Tolstov et al. (2009)	VLPs	Uniform VLPs (d = 55–58 nm)
Avian				
Influenza virus expression system	APyV (VP1/VP2/VP3/VP4)	Johne and Müller (2004)	VLPs	VLP (d = 45 nm) purified

(*Continued*)

TABLE 20.2 (*Continued*)
Expression Systems for VLP Production

System	Virus (Protein/s)	References[a]	Structure[b]	Notes[c]
Insect				
Baculovirus expression system	HPyV6, HPyV7, TSPyV (VP1)	Nicol et al. (2013)	VLPs, Ti	HPyV6, predominantly Ti, some regular VLPs; HPyV7, regular VLPs; TSPyV, predominantly regular VLPs, some Ti
	MCPyV (VP1)	Touzé et al. (2010)	VLPs	Regular VLPs, d = 45 nm; different clinical isolate generates only protein aggregates
	HPyV9 (VP1)	Nicol et al. (2012)	Ti (VLPs)	Mainly tiny VLPs (d = 24 nm), few regular VLPs (d = 45 nm) in preparations
	APyV (VP1/VP2/VP3)	An et al. (1999)	Capsomeres	VLPs assembled in vitro
	JCPyV (VP1)	Chang et al. (1997)	VLPs	Regular VLPs, d = 45 nm
	MPyV (VP1)	Montross et al. (1991)	VLPs	Regular VLPs in nucleus, purified empty VLPs (d = 46 nm), smaller VLPs in minority
	MPyV (VP1/VP2/VP3)	Forstová et al. (1993)	VLPs	Regular VLPs
	LPyV (VP1)	Pawlita et al. (1996)	VLPs	VLPs of regular size (d = 45 nm) found in cell nucleus; VLPs contain DNA
	MPtV (VP1)	Tegerstedt et al. (2003)	VLPs	VLPs of regular size (d = 45 nm), antibody does not cross-react with MPyV
	SV40 (VP1, VP1/VP2/VP3)	Kosukegawa et al. (1996)	VLPs	VLPs of regular size (d = 45 nm)
	BKPyV (VP1)	Touzé et al. (2001)	VLPs	Regular empty VLPs, d = 45 nm
	TSPyV (VP1)	Chen et al. (2011)	VLPs	Regular d = 45 nm and smaller VLPs
	GHPyV (VP1)	Zielonka et al. (2006)	VLPs, capsomeres	Capsomeres prevalent in preparation, VLPs of regular size (d = 45 nm)
Drosophila expression system	HaPyV (VP1)	Voronkova et al. (2007)	VLPs	Regular VLPs, observed in cell nucleus
	MPyV (VP1)	Ng et al. (2007)	VLPs, aggregates	Secreted VP1, low yield, altered disulfide bonding, irregular deformed VLPs
Yeast				
S. cerevisiae	HaPyV (VP1)	Sasnauskas et al. (1999)	VLPs	Regular size, VLPs in nucleus and cytoplasm
	BKPyV, JCPyV, SV40, HaPyV, MPyV, BFPyV (VP1)	Sasnauskas et al. (2002)	VLPs, Ti	VLPs (except HaPyV and MPyV) heterogeneous in size, d = 45–50 nm; minor fraction of Ti VLPs, d = 20–25 nm
	BKPyV, JCPyV (VP1)	Hale et al. (2002)	VLPs, Ti	BKPyV VLPs, d = 45–50 nm and d = 20–25 nm particle. JCPyV VLPs, d = 50–55 nm
	GHPyV (VP1/VP2), APyV (VP1)	Zielonka et al. (2006)	VLPs, Ti, capsomeres	GHPyV forms exclusively VLPs, d = 20 nm, low stability of VLPs
	APyV, CPyV, FPyV, GHPyV (VP1)	Zielonka et al. (2012)	VLPs, Ti capsomeres	VLPs (d = 45 nm), Ti (d = 25 nm), variable yield of VLPs depending on virus type
	ChPyV (VP1)	Zielonka et al. (2011)	VLPs, Ti, capsomeres	Low efficiency of VLP (d = 45 nm, d = 25 nm)
	JCPyV (VP1)	Chen et al. (2001)	VLPs	Regular size of VLPs (d = 45 nm)
	MPyV (VP1)	Palková et al. (2000)	VLPs, Ti	VLPs (d = 45 nm) in the nucleus, minor fraction of Ti, VLPs containing naked DNA without histones

(*Continued*)

TABLE 20.2 (*Continued*)
Expression Systems for VLP Production

System	Virus (Protein/s)	References[a]	Structure[b]	Notes[c]
Bacterial				
E. coli	SV40 (VP1)	Wróbel et al. (2000)	VLPs	GroELS chaperone system and His-tag on N-terminus, VLPs detected in cell extract, no in vitro assembly reaction, empty capsids prevalent, some full capsids
	MPyV (VP1)	Salunke et al. (1986)	Capsomeres	In vitro assembly reaction for VLPs formation (see Table 20.3)
	JCPyV (VP1)	Ou et al. (1999)	VLPs	VLPs detected in cell extracts
	HaPyV (VP1)	Voronkova et al. (2007)	VLPs	VLPs detected in cells and cell extracts
	BFPyV (VP1)	Rodgers et al. (1994)	Capsomeres	VLPs assembled in vitro

[a] Only reports of the first production or conflicting reports are listed.
[b] Ti, tiny particles; Tu, tubular structures; see Figure 20.2.
[c] d, diameter.

production of recombinant protein in plants is considered to be 1% of total soluble protein (Fischer et al. 2004). Indeed, it has been shown for HPV16 VLP production that specific optimization of the transcriptional or translational context and chloroplast localization of expression can improve the yield manyfold (17% of total soluble protein) (Maclean et al. 2007; Fernández-San Millán et al. 2008; Lenzi et al. 2008; Matić et al. 2012), making the plant system an interesting alternative for vaccine production.

Both PyV- and PV-based VLPs can be successfully produced in yeast, and quadrivalent HPV6/HPV11/HPV16/HPV18 vaccine (Gardasil, Merck and Co., Inc.) produced in *Saccharomyces cerevisiae* has been successfully introduced into the market. The first VLPs consisting of either L1 alone or L1/L2 produced in yeast were derived from cottontail rabbit PV (CRPV) (Jansen et al. 1995). The study showed that the VLPs were morphologically indistinguishable from native virions and protected rabbits from CRPV-induced wart formation after immunization. Further reports of HPV VLP production followed (Hofmann et al. 1995; Sasagawa et al. 1995; Neeper et al. 1996; Cook et al. 1999). Some reports noted that HPV L1 VLPs produced in *S. cerevisiae* yeast display type-dependent properties of particles (Mach et al. 2006). Whereas HPV18 L1 protein forms uniformly assembled VLPs (60 nm in diameter), L1 proteins of HPV6, HPV11, and HPV16 tend to form more irregular particles of 30–50 nm in diameter, which has no effect on their immunogenic properties but limits particle stability. The authors introduced an efficient procedure of disassembling and reassembling the yeast-derived VLPs to achieve more uniform particle morphology (60 nm diameter spheres) and maximized stability. Disassembly and reassembly of particles seems nowadays to be an important step in manufacturing yeast-derived VLPs for better immunoreactivity (Zhao et al. 2012b) and morphology, decreased heterogeneity, and increased thermal stability (Zhao et al. 2012b). In fact, the limited stability and aggregation of the recombinant HPV VLPs purified from yeast (Shi et al. 2005) may result in loss of HPV VLPs during purification procedures, and buffer conditions, such as high salt and nonionic surfactants, can substantially increase the yield (Kim et al. 2007; Park et al. 2008). The yields of VLPs from yeast can be high, and the yeast production system allows cultivation in large quantities with a relatively simple culture medium. Cook et al. (1999) reported production of HPV11 VLPs from 200 L, using a galactose-inducible *S. cerevisiae* expression system, where the yield of L1 came to approximately 15% of the total soluble protein of the yeast cell lysate and 6 mg/L of media of highly purified VLPs. Alternatively, the HPV VLPs can be produced in *Pichia pastoris* (Bazan et al. 2009) with similar (9.5 and 6.4 mg/L of HPV16 and HPV18 VLPs, respectively) (Rao et al. 2011) or even higher (20 mg/L of HPV58 VLPs) (Jiang et al. 2011) yields.

Interestingly, in contrast to PyVs, PVs have the inherent capacity to replicate in yeast (Angeletti et al. 2002), and packaging of actively replicating target DNA can lead to the production of PsVs containing full-length HPV genomes (Angeletti 2005) or plasmid DNA with a reporter gene (Rossi et al. 2000). The proof of the concept of PyV-based VLPs production in yeast was assessed using hamster PyV (HaPyV) (Sasnauskas et al. 1999). VP1 was expressed in *S. cerevisiae* and VLPs were abundantly formed in the nucleus as well as in the cytoplasmic compartment. Consequently, our group showed formation of VLPs from MPyV VP1 produced by a galactose-inducible *S. cerevisiae* yeast expression system (Palková et al. 2000). We also showed that a subpopulation of VLPs carried fragments of plasmid or linear chromosomal DNA and that newly synthesized VP1 can interact with mitotic microtubules, thus inhibiting yeast growth. In agreement with this, Sasnauskas et al. (2002) reported the importance of yeast strain selection for high expression of VP1 of PyVs from humans (JCPyV and BKPyV), rhesus monkeys (SV40), hamsters (HaPyV), mice (MPyV), and birds (budgerigar fledgling disease virus

[BFPyV]), but they showed the formation of VLPs devoid of nucleic acid. The reported yields of VLPs were high: 40 mg/L for mammalian PyVs and 5 mg/L for BFPyV. Generally, the yield in purified VP1 preparations may differ remarkably between viruses and can be quite low for goose hemorrhagic PyV (GHPyV) (1.2 mg/L) (Zielonka et al. 2006) or chimpanzee PyV (ChPyV) VP1 (0.3 mg/L) (Zielonka et al. 2011).

The expression of capsid proteins in a bacterial expression system often leads to the purification of protein in a pentameric form (Salunke et al. 1986; Li et al. 1997), but HaPyV or JCPyV VP1 expressed in *E. coli* assembles directly to VLPs (Ou et al. 1999; Voronkova et al. 2007). For HPV11 and HPV16, the purification of 3–5 mg of near-homogeneous L1 protein from 1 L of cell culture was reported (Chen et al. 2001). For MPyV, the effect of host, plasmid, and culture conditions on the expression of VP1 capsid protein in *E. coli* was examined, and the expression yield of 180 mg of soluble VP1 per liter of bacterial culture was obtained (Chuan et al. 2008). Several optimizing conditions enabled the same group to achieve even higher production rates (≈0.3 g of glutathione S-transferase [GST]-VP1 protein per liter of culture) in laboratory shake-flask conditions (Lipin et al. 2008). The extremely high production of MPyV VP1 (4.38 g of GST-VP1 protein per liter) in high-cell-density fed-batch cultivation in recombinant *E. coli* has been demonstrated (Liew et al. 2010). The disadvantage of the bacterial system is that recombinant protein preparations from bacteria always bear the risk of contaminating endotoxins, which are highly toxic in humans and therefore have to be eliminated from vaccine preparations and VLPs intended to be used in clinic.

Capsid proteins can also be produced in cell-free systems. VLP production, using this system with components derived either from bacteria or yeasts, has been so far reported only for PVs for PVs (Iyengar et al. 1996; Wang et al. 2008). The latter system yielded 50–70 μg of HPV58 L1 protein per milliliter of reaction volume after optimization (Wang et al. 2008). Purified pentamers can be efficiently assembled into VLPs in vitro in high ionic strength with the addition of cal- cium (in case of PyVs) or oxidation of disulfide bonds (in case of PVs). Assembled VLPs can be further disassembled and reassembled into the desired structures by changing the buffer conditions and temperatures. Tables 20.3 and 20.4 show several reported disassembly/reassembly systems. These reaction systems are valuable tools for the study of virion assembly, which is a poorly understood phenomenon and also serves for the preparation of viral nanostructures for different biomedical applications (e.g., an increase in the stability of yeast-derived HPV vaccines).

20.4 UTILIZATION OF VLPs AS A CARGO DELIVERY SYSTEM FOR THERAPY AND DIAGNOSTICS

VLPs have been studied intensively as diagnostic and therapeutic compounds. Their structural stability, manipulation tolerance, and ability for molecule incorporation with fast and low-cost production make them an ideal tool for use in gene therapy, immunotherapy, and diagnostics. Here, VLPs serve as vehicles for the transport of therapeutic DNA, drugs, antigens, or contrast agents into target cells. In some cases, including PV and PyV, the application of VLPs can be limited by their nonspecific binding to various cell types. On the contrary, the application potential of VLPs can increase if the selectivity of VLPs for distinct cells is guaranteed. DNA technology enables the preparation of genetically modified capsids on demand, and purified VLPs can be used as the ideal polyvalent monodispersed protein nanoobjects for further chemical engineering. The exterior of the VLPs can be functionalized by the connection of targeting molecules, and the interior of the particles can encapsulate cargo molecules for cellular delivery.

20.4.1 Retargeting of VLPs

The concepts of vector targeting are well recognized throughout the gene therapy field (reviewed in Waehler et al. 2007) where current eukaryotic viral vectors can infect cells with high efficiency, but they have the disadvantage that their native tropism must be ablated to avoid the transduction of nontarget tissue. Similarly, PyV and PV VLPs, with their inherent capacity to bind to a wide array of cell types (Section 20.4.2), might need to be detargeted from primary receptor binding and retargeted to the new destination by attachment of the targeting moiety. Although in some instances the addition of the targeting ligand reduces the native tropism sufficiently, detargeting is usually achieved by the genetic mutation of several amino acids that are responsible for interaction with the primary receptor. To reprogram VLP cell binding and entry, ligands that direct targeting to specific cell types should be attached to the surface of VLPs. The rational design of VLP modification, therefore, requires knowledge of virion structure, which is fortunately known for species of PyV and PV (Section 20.2.2). The selection of the targeting moiety depends on the nature of the target cell and the actual biomedical application and usually consists of the polypeptide molecule (e.g., epidermal growth factor [EGF]) that naturally binds the receptors on target cells (EGF receptors are overrepresented in some cancer cells), monoclonal antibody (or single-chain antibody) against the target cell receptor, or small targeting peptide motifs. These small targeting peptides can be chosen either from library selection approaches or from naturally occurring motifs. For this purpose, the RGD motif (tripeptide Arg-Gly-Asp), which targets vectors to integrins overrepresented in tumors and vasculature, has most commonly been used. Furthermore, nonpeptide molecules, such as sugars, fatty acids, nonpeptide hormones, or small molecular compounds, can serve as targeting molecules (Waehler et al. 2007).

The major advantage of VLPs against other therapeutic carriers is their ability to expose a large number of targeting molecules whose number and orientation can be controlled. The attachment of a targeting moiety can be performed by genetic or chemical methods. Genetic approaches allow

TABLE 20.3
Main Conditions for In Vitro Reconstitution of VLPs from Capsomeres and the Outcome of Assembly Reaction after Dialysis

Protein (s)	System	References	Conc.	Time	T (°C)	pH	Salt	Ca	VLPs[a]	Tiny[b]	Tu[c]	Pentamers	Aggregates
VP1 (MPyV)	*E. coli*	Salunke et al. (1989)	0.5–1 mg/mL	2 days	RT	7.2	150 mM NaCl	0.5 mM	+++ N, Sn	+		+	
						8.5	150 mM NaCl	0.5 mM	Disrupted	+++		+	
						5.0	150 mM NaCl	0.5 mM	+++ N, Ln	+		+	
						7.2	2 M $(NH_4)_2SO_4$	—	++ N, Sn	++	++ (15 nm)		
						8.5	2 M $(NH_4)_2SO_4$	—	++ N, Sn	++	+ (15 nm)	+	
						5.0	2 M $(NH_4)_2SO_4$	—	++++				
VP1 (SV40)	Insect	Kanesashi et al. (2003)	0.06 mg/mL	1 day	4	7.2	150 mM NaCl	2 mM					++++
					RT	5.0	150 mM NaCl	2 mM			++++ (30 nm)		
					RT (4)	7.2	1 M NaCl	2 mM	+++ N, Sn, Ln	+	++ (45 nm)		
					4	7.2	1 M NaCl	—		++++			
					4	7.2	2 M $(NH_4)_2SO_4$	2 mM	++++ N, Sn				
HPV11	*E. coli*	Li et al. (1997)	nd	[d]	RT	7.2	1 M NaCl	—	+++				+

Abbreviations: Conc., capsid protein concentration; Ca, calcium concentration; RT, room temperature; nd, not determined; number of "+" indicating quantity of each assembly form.

[a] VLPs of various size can be formed (N, normal size; Ln, larger than normal; Sn, smaller than normal)—see Figure 20.2.

[b] Particles composed of 12 or 24 capsomeres are labeled as *Tiny*.

[c] VLPs can form tubular structures (Tu) of various diameters (in brackets).

[d] VLPs assembled during elution from the phosphocellulose column in 1 M NaCl.

TABLE 20.4
Examples of Disassembly/Reassembly Procedures

Virus/VLPs (System)	References	Disassembly[a]	Reassembly	Notes
MPyV virion	Brady et al. (1977)	10 mM Tris–HCl (pH 8.5), 150 mM NaCl, 10 mM EGTA, 3 mM DTT, 30 min	Not done	Show stabilizing effect of high salt (1 M NaCl) and calcium ions (5 mM for disassembly); pH > 8.5 increases the disruption, high concentration (50–150 mM) of EGTA decreases disruption.
MPyV virion	Brady et al. (1979)	1 mM EGTA, 0.1 M ME, 0.15 M NaCl in 0.05 M Tris–HCl (pH 7.4), 30 min, RT	Dialyzing in 10%/DMSO, 0.01% Triton X-100 in PBS (pH 7.4) with 0.5 μM $CaCl_2$	Virions dissociated in the pH range of 7.4–7.8 have higher frequency of reassembly; dissociation at pH 8.0 is harmful to the reassembly; higher concentration of $CaCl_2$ is inhibitory to the assembly.
SV40 virion	Colomar et al. (1993)	50 mM Tris–HCl (pH 7.9), 150 mM NaCl, 1 mM EGTA, 20 mM DTT, 37°C, 1 h	Gradual addition of $CaCl_2$ (5 mM final)	Disassembly not complete—disassembly/reassembly verified by infectivity assay not EM.
SV40 VP1 VLPs (insect)	Kanesashi et al. (2003)	20 mM Tris–HCl (pH 7.9), 0.1% Nonidet P-40, 25 mM EGTA, 30 mM DTT, 1 h at 37°C plus gel filtration: 20 mM Tris–HCl (pH 7.9), 150 mM NaCl, 5 mM EGTA, 5 mM DTT	Dialyzing 9 μg of the purified pentamer preparation for 24 h against the various buffers or 2 M $(NH_4)_2SO_4$, 2 mM $CaCl_2$, pH 7.2, 4°C	Reports about aggregation in physiological salt condition (150–30 mM NaCl) and acidic pH (pH < 5.0), formation of VLPs in high concentrations of $(NH_4)_2SO_4$ did not require $CaCl_2$.
HPV16 L1/2 VLPs (insect)	Kawana et al. (1998)	1 mg of VLPs incubated in 1 mL of PBS containing ME (5%), 16 h at 4°C	VLPs mixed with 2 mg of plasmid and dialyzed against 4 L PBS, 0.5 M NaCl, 2 mM $CaCl_2$, 24 h at 4°C	Electron microscopy confirmed the presence of particles; 10 μg of PLPs from 1 mg of disassembled capsids.
HPV6, HPV11, HPV16 L1 VLPs (yeast)	Mach et al. (2006)	0.15 M NaCl, 35 mM sodium phosphate, 2 mM EDTA, 0.03% polysorbate 80, 100 mM Tris (pH 8.2), 10 mM DTT	Dialyzing against a solution of high salt concentration (0.5–1 M NaCl) at a lower pH (6–7)	Aggregation during the disassembly and initial reassembly in low salt solutions; disassembly performed under high salt conditions (0.5–1.2 M NaCl) with polysorbate 80 to eliminate the aggregation.
HPV11 L1 VLPs (insect)	McCarthy et al. (1998)	PBS, 150 mM NaCl, 5% ME, 16 h, 4°C	Capsomeres (0.5–5.0 mg) dialyzed versus 4 L of PBS with 0.5 M NaCl, 4°C, 24 h	The aggregated VLPs were resistant to disassembly; 0.5 M NaCl during reassembly designed to stabilize VLPs.
BKPyV VP1 VLPs (insect)	Touzé et al. (2001)	50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA, 20 mM DTT, 30 min RT	Dilution in 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 2 mM $CaCl_2$, 1% DMSO; $CaCl_2$ molarity increased stepwise from 2 to 5 mM (1 mM/h) at 20°C to reach a final volume of 500 μL.	1μg of plasmid DNA in 50 μL of 50 mM Tris–HCl buffer (pH 7.5), 150 mM NaCl added to 50 μL (10 μg) of disrupted VLPs and further diluted.

[a] ME, 2-mercaptoethanol; DTT, dithiothreitol.

ablation of natural tropism and introduce a targeting or adaptor molecule in one step but are usually limited by the size of the ligand that can be incorporated without compromising the assembly, stability, and yield of VLPs. In addition, the peptide affinities to the receptor may be influenced by the location in the capsid. More importantly, nonpeptide molecules cannot be used as targeting moieties, despite being high-affinity ligands for different targets (Waehler et al. 2007). On the contrary, the chemical modification of VLPs allows covalent attachment of specific ligands from a large collection of protein and nonprotein compounds by the classical techniques of protein alteration and cross-linking (reviewed

in Wong [1991] and Strable and Finn [2009]). Moreover, the coupling of full-length proteins can be performed without negatively affecting the structural and biological integrity of VLPs. The techniques use acylation of the amino groups of lysine, alkylation of the sulfhydryl group of cysteine and activation of carboxylic acid residues (of aspartic and glutamic acids), and coupling with added amines. These amino acids, together with the aromatic groups of tyrosine and tryptophan, have distinct reactivity patterns and therefore predominantly serve for bioconjugation purposes (reviewed in Strable and Finn [2009]). On the other hand, the utilization of chemical approaches can be limited by the absence of these amino acids in the appropriate positions on the VLP surface or by the fact that reaction conditions promote disassembly or aggregation or severely reduce the yield of modified VLPs during purification steps. Conversely, chemical cross-linkers can, in some instances, significantly reduce the capacity of VLPs to disassemble intracellularly, which might be undesirable for specific applications.

The combination of genetic and chemical techniques for modifying the surface of viral particles seems to limit the disadvantages of both approaches. Genetic techniques can be used to introduce a specific chemical reactivity (amino acid[s]) at defined positions on the viral capsid surface. An addition of few amino acids usually has no effect on VLP stability, in contrast to extensive amino acid changes when genetic retargeting is applied. After production of the VLPs in a conventional expression system, the newly integrated amino acid(s) can be used to chemically couple ligands for targeting.

Generally, the single- or two-component systems can be used for the attachment of the targeting ligand to VLPs. The single-component system uses direct incorporation of a targeting moiety into or onto VLPs, whereas the second strategy uses the adaptor molecule to mediate the attachment of a targeting ligand to VLPs. Both of these strategies have been explored for retargeting PyV VLPs. Since the methods are essentially the same as for the preparation of VLPs designed to expose immunodominant epitopes for vaccination purposes (Section 20.5.2.2) or for peptide delivery (Section 20.4.3) application, PV VLPs are modified in the same way.

20.4.1.1 Targeting of VLPs by a Single-Component System

The single-component system, where the targeting moiety is directly attached to the VLPs, is more technically challenging and less versatile than the use of the two-component system, but might provide stable and functionally homogenous retargeted particles. In addition, this approach might simplify high-titer production since there is no need to create a separate adaptor or docking molecules. Both genetic and chemical methods are used for the creation of targeted VLPs.

Targeting by genetic modification requires the construction of genetic fusion between the capsid protein and the targeting ligand. This leads to the formation of a special variant of so-called chimeric VLPs (Table 20.6), where the targeting sequence must be exposed on the surface of the VLP. Finding the suitable positions in the surface loop of capsid proteins that allow this type of modification without affecting the physical integrity of the particles is usually the main obstacle in the construction of these VLPs. It is also crucial that the displayed ligand maintains its bridging ability toward the target after fusion. Both PyVs and PVs have been used for genetic modification and the effect on VLPs stability analyzed, but only few reports of successful targeting exist.

Both phenomenon stability and targeting were systematically analyzed for SV40 VP1 VLPs by Takahashi et al. (2008). They inserted FLAG epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys octapeptide) with short triglycine flexible linkers, which assist the molecule to achieve the most preferable conformation, in different positions of all surface loops of the VP1 protein (BC, DE, EF, and HI). For targeting, they inserted three consecutive RGD motifs. They demonstrated that only a small number of positions (one in DE and one in the HI loop) of VP1 can accommodate foreign peptides without affecting VLP formation and that at least three glycine residues flanking both sides of the foreign peptide were needed for VLP assembly. Moreover, the RGD motifs displayed on the VLPs were found to be directly involved in cell attachment, and interaction with cells was enhanced for VLPs displaying RGD in DE as well as HI loop compared to VLPs carrying the FLAG-tags at the same positions. The latter VLPs also associated with target cells only very weakly compared to wild-type VLPs, because both DE and HI loops are, together with the BC loop, involved in receptor binding (Neu et al. 2008).

The same targeting peptide has been used for the retargeting of VLPs derived from LPyV (Langner et al. 2004). The single RGD motif without a linker sequence was replaced in 11 different positions (in BC, DE, and HI loops) in the VP1 protein sequence, and only five mutant proteins (three in BC loop and one each in HI and DE loops) were found to yield VLPs. The modifications led to the loss of LPyV receptor binding of all VLPs. Specific binding to αvβ3-integrin, an important marker of angiogenesis in solid tumors, was shown for VLPs carrying RGD in the BC loop. Interestingly, no binding was observed with three other integrins, αvβ6, αIIbβ3, and αvβ5, which also recognize RGD with more restricted ligand recognition profiles than αvβ3.

MPyV VLPs are probably the VLPs most frequently modified by a genetic approach for different purposes; Table 20.5 summarizes these reports. The VP1 loops were modified to carry foreign epitopes (Sections 20.4.1 and 20.5.2.2) or adaptor sequences (Section 20.4.2.2) of various lengths, but only one study attempted to incorporate targeting molecule directly into VP1 protein (Shin and Folk 2003). In this study, VLPs were targeted to a urokinase-type plasminogen activator receptor (uPAR), which is a protein expressed by many cancer cells, where it correlates with metastasis and poor prognosis. To restrict the binding of VLPs to a natural receptor, the VP1 protein was modified by the FLAG in the HI loop before subsequent manipulation. The fragments of uPAR activator were inserted into all surface-exposed loops

TABLE 20.5
Overview of MPyV VP1 Protein Modification and Their Influence on the VLPs Assembly

Virus	Insert	Location	Utilization	Expression System	Assembled VLPs	References
MPyV	Pre-S1 phil—two hydrophilic fragments from HBV pre-S1 sequence (70 aa + 6 aa linkers)	HI loop	Immunization	*S. cerevisiae*	Yes	Skrastina et al. (2008)
MPyV	B-cell epitopes (12 and 14 aa)	BC loop	Immunization	*E. coli*	Yes	Neugebauer et al. (2006)
MPyV	Protein Z (antibody binding to a domain of protein A) (57 aa + 17 aa linkers)	HI loop	Retargeting	*E. coli*	Yes	Gleiter and Lilie (2001)
MPyV	WW domain (from murine FBP11) (38 aa)	DE loop HI loop	Retargeting	*E. coli*	No	Schmidt et al. (2001)
MPyV	Peptide with 8 glutamate and 1 cysteine residues	HI loop	Retargeting	*E. coli*	Yes	Stubenrauch et al. (2001)
MPyV	Peptide sequence binding uPAR (60 aa) or FLAG sequence (8 aa)	BC loop DE loop HI loop EF loop	Retargeting	Baculovirus (insect)	No Yes	Shin and Folk (2003)
MPyV	Peptide sequence from Bcr-Abl protein (25 aa)	HI loop	Immunization	Baculovirus (insect)	No	Španielová (unpublished results)

Abbreviations: aa, amino acids; FBP11, formin-binding protein 11; uPAR, urokinase-type plasminogen activator receptor.

(BC, DE, EF, HI) of a detargeted variant of VP1, but only the insertion of an N-terminal part of a 60 aa uPAR activator fragment into the EF loop had no detrimental effect on protein solubility and particle integrity. However, only VLPs with a diameter of 20 nm were formed. When these mutant VP1 proteins were coexpressed with a detargeted variant of VP1 protein, they formed heterotypic VLPs of a regular size. Compared to wild-type VLPs, these heterotypic VLPs did not bind cells with uninduced expression of uPAR. However, after induction of the expression of uPAR, the heterotypic VLPs bound specifically to uPAR on the surface of target cells. These data suggested that the EF loop, which is exposed laterally, far from the receptor-binding site, might still provide a targeting function and be more flexible in accommodating longer foreign sequences.

A combination of genetic and chemical techniques has been used for the preparation of SV40 VLPs targeted to the EGF receptor (Kitai et al. 2011). The reactive cysteine residue, which was introduced into the DE loop of the VP1 protein, was subsequently used for chemical conjugation of full-length human EGF to the surface of VLPs through a thiol group with heterobifunctional cross-linker SM(PEG)2, which possesses maleimide and succinimide moieties. The chemical reaction had a 44% yield of intact modified VLPs and resulted in the conjugation of approximately 40 molecules of human EGF per VLP. These EGF-VLPs were further shown to display enhanced selectivity for cells that overexpress the EGF receptor, and their internalization was 10-fold greater than that of unmodified VLPs. Moreover, the study provided the first evidence that VLPs targeted by single-component systems not only can increase the binding capacity of VLPs to specific target cells but also can enhance cellular uptake through the EGF receptor–mediated endocytosis.

20.4.1.2 Targeting of VLPs by a Two-Component System

The two-component system does not attach the targeting moiety directly to VLPs, but uses noncovalently bound adaptor molecules for this purpose. Adaptors are molecules with dual specificities: one end binds the viral protein, and the other binds the receptor on the target cell. The adaptor strategy possesses great flexibility, as different adaptors can readily be coupled to the same VLPs to allow for easy testing of several target receptors. On the other hand, the system might be limited by varying coupling efficiency and suboptimal stability of the VLP–adaptor complex, especially in vivo (Waehler et al. 2007). Nevertheless, this system was frequently used for the modification of PyV as well as PV VLPs for different applications.

In a two-component system, the VLPs and adaptor molecule can be designed in such a way that VLPs can be genetically or chemically modified to display the versatile adaptor-binding motif. This strategy has been explored primarily for MPyV VLPs, and different motifs have been used in several complementary studies.

In one study, the HI loop of VP1 protein was genetically engineered to display a 9 aa region of polyanionic peptide (Glu_8Cys, E8C) on the surface of VLPs (Stubenrauch et al. 2001). Polyionic fusion peptides are highly soluble, and their interaction does not depend on specific secondary structures. This manipulation therefore did not lead to a destabilization of VLPs (Stubenrauch et al. 2000). This region was subsequently used as a docking site for electrostatic association with a targeting molecule a tumor-specific antibody Fv fragment fused with a complementary polycationic (Arg8Cys, R8C) tag. Finally, the cysteine residues of the polyionic peptides enabled covalent cross-linking under oxidizing conditions.

Approximately 30 antibody fragments were bound to engineered E8C VLPs and the system proved to be highly specific and efficient. The VLPs were packaged with a plasmid containing the reporter gene, and the selectivity of VLPs coupled with tumor-specific antibody for the target cells was determined from transduction assays. Transduction with targeted VLPs resulted in fivefold higher β-galactosidase activity in target cells than transduction with E8C VLPs not decorated with the antibody. However, the transduction efficiency was lower than the cell-type nonspecific binding transduction of wild-type VLPs and was generally rather low (3% and 5%, respectively) (Stubenrauch et al. 2001). The complementary study (May et al. 2002) verified the specific and efficient association of E8C VLPs with cellular targets mediated by the antibody fragment and suggested that their incapacity to surmount the endosomal membrane and escape lysosomal degradation is responsible for the lack of functional transduction of the respective cells (see Section 20.4.2).

In the other study, a 38 aa domain of protein Z was inserted via short serine–glycine linkers into the HI loop (Gleiter and Lilie 2001). Protein Z is a binding domain, derived from protein A of the bacteria *Staphylococcus aureus*, which is able to specifically bind antibody immunoglobulins. This insertion did not affect VLP stability or the functional integrity of the Z domain. A humanized monoclonal antibody bound the Z domain on the surface of VLPs with high affinity and a stoichiometry of around 0.8 antibody molecules per VP1-Z monomer. This specific targeting function was confirmed with Herceptin antibody. Herceptin binds selectively to the HER2 glycoprotein, a member of the EGF receptor family, which is present on several different human tumor cells. This antibody directed the respective particles specifically toward the cells with high expression of HER2. Without the antibody coupled to the VLPs or with the cell line, which does not express HER2, no targeting was observed.

Another study investigated a strategy that uses a genetic fusion of capsid protein with 28 aa of the WW domain of the mouse formin-binding protein 11 (Schmidt et al. 2001). The WW domains are very small protein domains that bind proline-rich ligands with high affinity. They are named after two conserved tryptophan residues that are essential for the maintenance of the native fold and specific binding ligands. The WW domain was flanked by serine–glycine linkers (5 aa) and inserted in either the DE or the HI loop of the VP1 sequence. Whereas the first variant of WW-VP1 fusion yielded completely formed particles after stabilization with disulfide bonds, the latter WW-VP1 fusion protein lost the capacity to form VLPs. WW VLPs were tested for their capacity to bind a proline-rich sequence with a PPLP (Pro-Pro-Leu-Pro) consensus motif by addition of GFP with the PPLP-tag. Approximately 25 ± 5 molecules of GFP could bind to the capsid surface. Unfortunately, the coupling efficiency as well as the stability of the complex was limited by the fast dissociation reaction, and the strategy was not used further for targeting experiments. The same system was, however, successfully used for VLP-mediated intracellular delivery of protein and peptides (Günther et al. 2001) (Section 20.4.3).

Besides targeting purposes, the similar two-component systems are often adapted for application, where VLPs serve as carriers of other substances. For example, BPV-based VLPs with a polyglutamic acid–cysteine sequence inserted into a surface-exposed region of the L1 major capsid protein were successfully used for the coupling of an antigen with an N-terminal polyarginine cysteine tag for immunization purposes (Pejawar-Gaddy et al. 2010). Interestingly, the E8C region influenced the stability of BPV when inserted to a BC or DE loop of L1, but HI loop modification yielded regular E8C VLPs that conjugated with the antigen with a higher efficiency than reported (Stubenrauch et al. 2001) for MPyV VLPs (14% and 8%, respectively). Even higher coupling efficiency with the two-component system based on strong interactions between biotin and streptavidin has been reported for BPV1 L1 VLPs (Chackerian et al. 2001), where biotinylated VLPs have bound to approximately 540 streptavidin tetramers.

20.4.1.3 Concluding Remarks

The numerous studies done mainly with PyV VLPs have shown that retargeting in respect to selective cellular binding is possible, but only few studies have demonstrated selective particle uptake. The low level of unspecific internalization of VLPs is evident in all of the studies. Technically, the chemical modification of particles seems to be more efficient and less challenging than the genetic approach. Most studies have conclusively suggested that the size of a foreign sequence genetically inserted into a capsid protein is a limiting factor for self-assembly into VLPs, but this is generally hard to predict. For MPyV VP1, sequences no longer than 40 aa together with flexible linkers (e.g., glycine–serine) can usually be inserted without compromising VLP integrity, but occasionally the incorporation of the whole 18 kDa enzyme can be successful (Gleiter et al. 1999). For PV, peptides of up to 60 aa can be fused to the truncated L1 without disrupting the assembly of VLPs (Müller et al. 1997). However, the integrity of the VLPs is probably influenced by the actual position and character of the foreign sequence introduced into the capsid protein, so various reports of unsuccessful manipulation in VLPs surface loops exist (see Table 20.5 for MPyV VLPs). Accordingly, the versatility of a two-component system that requires genetic modification of VLPs might be limited by particle stability, and chemical methods for adaptor coupling might be favorable for many applications.

20.4.2 Nucleic Acid Delivery

Nucleic acids are obvious cargo molecules for VLPs. Historically, the need for safe and efficient gene delivery vehicles in the flourishing field of gene therapy led to attempts to use VLPs exclusively for the delivery of DNA for gene expression. Today, the approaches for direct delivery of mRNA or silencing molecules (antisense DNA/RNA, siRNA) (Lund et al. 2010) are a more attractive direction for research. Moreover, VLPs can serve as shielding vehicles for the delivery of DNA vaccines (Section 20.5.1.2).

TABLE 20.6
PV and PyV Particle Terminology[a]

Virion	A complete virus particle composed of all capsid proteins and viral genome in the form of minichromosome; capable of initiating infection and expressing viral genes
Virus-like particle (VLP)	A noninfectious particle composed of major capsid protein with or without minor capsid proteins (should be specified); encapsidating no specific (but may contain unspecific) nucleic acid
Chimeric VLP (CVLP)	VLP formed from capsid protein(s) fused with foreign sequence(s)
Pseudocapsid	Equivalent to VLP, reflects differences in the composition of naturally occurring capsids and heterogeneously expressed particles; usually composed exclusively of major capsid protein; encapsidating no specific (but may contain unspecific) nucleic acid
Capsoid	Pseudocapsid-like structure formed after assembly of capsid protein, which was recombinantly expressed in *E. coli*
Pseudovirion (PsV)	A virus particle composed of major and minor protein(s) capable of transducing the reporter gene expression; plasmid encapsidated in the form of a minichromosome
Pseudovirion-like particle (PLP)	Similar to PsV, but the capsid protein composition differs from virion capsid or the nucleic acid as it is encapsidated as naked and not in the form of a minichromosome.
Quasivirion (QV)	An infectious particle assembled *artificially* in vivo (in 293TT cells) by supplying the capsid proteins and genome in *trans*; it is composed of all capsid proteins and viral genome in the form of a minichromosome; it is capable of initiating infection and expressing viral genes
Quasivirion-like particle (QVLP)	An infectious particle assembled *artificially* in vitro; it is composed of all capsid proteins and viral genome in the form of naked DNA (for PyVs the term polyoma-like particles was used)

[a] Compiled from Ozbun and Kivitz (2012) and literature cited in the text.

Terminologically, several papovaviral assemblies with nucleic acid can be recognized (Table 20.6). The general term *viruslike particles* is usually used for the description of noninfectious capsid-like particles (the actual protein composition in respect to the presence of minor proteins should be specified) produced in a heterologous expression system. VLPs made only from the major capsid protein are alternatively called pseudocapsids (Forstová et al. 1995). These particles may be completely devoid of a nucleic acid (appearing as *empty* particles under an electron microscope) or may contain fragments of DNA (appearing as *full* particles under electron microscope). These two populations of particles are separated into two distinct bands with slightly different buoyant densities during CsCl gradient ultracentrifugation. For MPyV VLPs, these particles are sometimes described as *light* ($\rho = 1.290$ g/cm^3) and *heavy* ($\rho > 1.300$ g/cm^3), respectively (Palková et al. 2000). It has been demonstrated for PyV VLPs produced in a baculovirus expression system that these DNA fragments originate either from the host cells or from the baculovirus genome and are complexed with cellular histones into pseudonucleocores (Pawlita et al. 1996; Gillock et al. 1997). In contrast, MPyV VLPs produced in *S. cerevisiae* did not assemble with cellular histones and *full* particles isolated from yeast cells contained linear DNA fragments, up to 3 kbp long, encapsidated as *naked* DNA by empty VP1 particles. VLPs purified from insect cells are heavily contaminated with RNases (Forstová, unpublished observation), but the VLPs produced in a yeast or bacterial system may be contaminated with RNA (Ou et al. 1999; Sasnauskas et al. 1999; Gedvilaite et al. 2000).

VLPs can be complexed with a specific nucleic acid in vitro as well as in vivo. PsV-based technology established in a mammalian expression system (Section 20.4.2.1.1) allows the production of infectious particles that closely resemble a native virion containing the major and minor proteins and specific DNA complexed with cellular histones in the form of a minichromosome. These particles are called PsVs. The same term, however, is sometimes used for DNA-loaded particles without minor capsid proteins, for particles purified from yeast expression system where DNA is not assembled with histones, as well as for particles prepared by an in vitro disassembly/reassembly approach, which contain only naked DNA. Although these types of particles can be infectious to some extent, we recommend the definition of these particles as PLPs. The same abbreviation was originally used for polyoma-like particles (Barr et al. 1979), the particles formed from purified empty capsids after incubation with supercoiled DNA. For this type of particle, we would today select the term *quasivirion-like particle (QVLP)* reflecting the term *quasivirion (QV)*, coined by the laboratory of Neil D. Christensen (Culp et al. 2006c) to describe virions with an authentic virus genome with a nucleocore produced in vivo in 293TT (Pyeon et al. 2005).

20.4.2.1 Preparation of VLP–Nucleic Acid Complexes

Several methods for the preparation of complexes made from VLPs and nucleic acids have been developed. VLPs loaded with nucleic acids can be prepared either in vivo or in a cell-free system (in vitro). The expression of the reporter gene is often used as the readout for the successful packaging and delivery of DNA into the cells.

20.4.2.1.1 In Vivo Production of PsVs and PLPs

PsVs are usually produced in the easy-to-transfect human embryonic kidney cells, 293TT, which express a high level of SV40 large T antigen (Buck et al. 2004), but other cell lines expressing T antigen can be used (e.g., Cos cells). The 293TT

cell line is usually cotransfected with helper and target vectors. Both the helper vectors ensuring the expression of capsid proteins and the target vectors harboring the reporter gene carry the SV40 origin of replication (ori). These two SV40 regulatory elements (large T antigen and SV40 ori) are needed to achieve high-level production of capsid proteins as well as a high concentration of target DNA for encapsidation. Moreover, it has been shown for the SV40 virus that the ori sequence overlaps with the SV40 packaging signal (Oppenheim et al. 1992) but pseudovirions or QVs can be prepared in the absence of this signal (Pyeon et al. 2005) and the concentration of the target vector seems to be crucial for its encapsidation (Culp et al. 2006c; Španielová et al. 2014). The system is universal and allows the production of SV40 vectors with exchangeable capsids that exhibit differential efficiency of gene transduction to the target cells (Nakanishi et al. 2008). On the other hand, the system is also stochastic, promiscuous, and not very efficient. The efficiency of encapsidation of the reporter vector is estimated to be approximately 5% for BPV PsV (Buck et al. 2004), and the observed particle-to-infectivity ratios (expressed as reporter plasmid copies per infectious units) are highly variable for different HPV types (varies between 20 and 5000) (Handisurya et al. 2012). HPV16 and HPV18 L1/L2 PsV stocks contain substantial amounts of encapsidated cellular DNA (Buck et al. 2005b). This can complicate the subsequent PsV-based assays and generates safety issues connected with PsV production.

HPV33 PsVs were prepared with a vaccinia virus expression system with a packaging efficiency that was rather low (1 input plasmid per 25,000 particles), probably because cellular DNA fragments generated by the lytic infection of vaccinia virus competed with the target plasmid during encapsidation (Unckell et al. 1997). Based on the same principle, the BPV PsVs were prepared in insect Sf9 cells that were coinfected with L1/L2 recombinant and E2 recombinant baculoviruses and transfected with the target plasmid (Zhao et al. 2000). The packaging efficiency was estimated to be 0.01% (1 input plasmid per 10,000 particles).

PsVs and PLPs can be prepared in vivo in nonmammalian systems. Rossi et al. (2000) demonstrated the production of HPV16 PLPs containing L1 and L2 proteins and the GFP reporter plasmid in yeasts. These PLPs were successfully used for the delivery and expression of the reporter gene after in vitro infection of mammalian cells and after injection of PLPs into mice. Although promising, the approach exhibited substantial variations in yield, and finally, PLP production was lost entirely (Peiler 2004). The presence of a plasmid in VLPs derived from yeast has been demonstrated for PyVs (Palková et al. 2000), but the PyV-derived PLPs produced in yeast have never been used for gene delivery purposes. Interestingly, since JCPyV and HaPyV VLPs self-assemble into VLPs inside the bacterial cells, the PLPs composed of VP1 protein and reporter gene expression plasmid can be produced in vivo in *E. coli*. This method substantially increases the efficiency of subsequent gene transduction, from 2% recorded for JCPyV PLPs prepared in vitro to 80% for in vivo DNA packaged PLPs (Chen et al. 2010).

20.4.2.1.2 In Vitro Production of PsV and PLPs

Several procedures for in vitro complexation of VLPs with nucleic acid have been established.

During these procedures, nucleic acid is loaded to the VLPs by their exposure to osmotic shock, sequential disassembly and reassembly, chemical conjugation with nucleic acid, or simply direct mixing with nucleic acid. These cell-free methods require highly purified VLPs devoid of contaminating DNA (inside) and nuclease activity (outside). Preparation of VLPs is therefore crucial for successful encapsulation of the cargo molecule. VLPs formed during production in most expression systems (except for bacterial) contain fragments of cellular DNA, and VLPs must therefore be either purified to near homogeneity by density gradient ultracentrifugation to obtain just *empty* pseudocapsids or disassembled, exposed to nuclease treatment, and reassembled. According to our experience, the elimination of nuclease contamination requires at least one purification step by centrifugation in sucrose density gradient. Table 20.7 shows a selection of some experiments where VLPs complexed in vitro were used.

20.4.2.1.2.1 Osmotic Shock Procedure Nucleic acid loading by the passive osmotic shock procedure was originally developed for PyV PLPs (Barr et al. 1979). The loading process probably proceeds in several steps. First, a DNA/RNA molecule binds to an empty capsid to form what is designated as a DNA–capsid-binding complex. Forstová et al. (1995) showed that only empty or disrupted VLPs can form these complexes, whereas *full* virions do not interact with exogenous DNA. The second step consists of the lowering of ionic strength by the addition of distilled water, which facilitates the entrance of DNA into the capsid, as shown by Barr et al. (1979). In the third step, the added nuclease cleaves and removes the external DNA that is not able to enter the capsid. The encapsidated DNA is further protected from nuclease action. The fragment of DNA that is encapsidated does not consist of a specific sequence, and encapsidation is sequence independent. Either linear, circular, or supercoiled DNA, as well as single-stranded DNA, rRNA, and synthetic oligonucleotides, was originally used for PLP formation (Slilaty et al. 1982).

There is likely a size constraint on the amount of genetic information that might be packaged by this method. The limit appears to be between 1.8 and 2.5 kbp for dsDNA (Slilaty et al. 1982; Forstová et al. 1995) with circular DNA being packaged more efficiently than linear DNA (Forstová et al. 1995). The character of interactions between MPyV pseudocapsids and DNA during osmotic shock has been studied in detail by electron microscopy (Štokrová et al. 1999) (Figure 20.3). The study revealed that pseudocapsids form only weak interactions with internal parts of the circular form of DNA. In contrast, two pseudocapsids bound to each end of a linearized DNA molecule were found to form highly stable complexes where the DNA is partially encapsidated. The level of protection from nuclease activity was the same for linearized or circular DNA—approximately

TABLE 20.7
Selection of Reports Using In Vitro Methods of PLP Preparation for the Gene Transfer

Production System/Vector	Parental Virus (protein[s])	PLP/PsV Method	VLP/DNA Ratio	Detection System	Quantity of DNA (pg/cell)	Transduction Efficiency	References
Sf21/baculovirus	SV40 (VP1)	Nuclear extract (reassembly)	10/100	MDR, GFP (4.7 kbp)/ various cell lines	1000	70%–100%	Kimchi-Sarfaty et al. (2004)
Sf21/baculovirus	HPV16 (L1)	Disassembly/ reassembly	25/5	GFP (5 kbp) β-galactosidase (7.2 kbp), various cells	2	70% (GFP)	Touzé and Coursaget (1998)
Yeast	HPV11 and HPV16 (L1, L1/2)	Chemical conjugation	20/1.12	β-Lactamase (PCR fragment (1.8 kbp)/ C33A	20	15%–40%	Yeager et al. (2000)
Sf21/baculovirus	BKPyV (VP1)	Direct mixing (plus other)	10/1	Luciferase (7.1 kbp), β-galactosidase/Cos-7	20	50% (β-gal)	Touzé et al. (2001)
E. coli	HaPyV (VP1)	Disassembly/ reassembly	50/1	GFP(4 kbp)/Cos-7, CHO	1	Low (∼20%)	Voronkova et al. (2007)
Sf158/ baculovirus	JCPyV (VP1)	Disassembly/ reassembly	3/1	β-Galactosidase (4.5 kbp)/Cos-7	0.25	20%	Goldmann et al. (1999)
Sf9/baculovirus	HPV6, HPV11, HPV16 (L1, L1/L2)	Chemical conjugation	25/10	β-Galactosidase (8.9 kbp)/various cell lines	100	1% 20% (with adenovirus)	Muller et al. (1995)
Sf9/baculovirus	MPyV (VP1)	Direct mixing	30/1	β-Galactosidase, GFP/ Cos-7	20	Few cells (β-gal) 0.1%–0.5% (GFP)	Krauzewicz et al. (2000)
Sf21/baculovirus	MPtV (VP1)	Direct mixing	10–30/2	EGFP (4.7 kbp)/293 and Cos-1	1	0.03%	Tegerstedt et al. (2003)
Sf9/baculovirus	HPV16 (L1/L2)	Disassembly/ reassembly	1/2	β-Galactosidase (6.8 kbp)/several cell lines	0.25	0.00425%	Kawana et al. (1998)

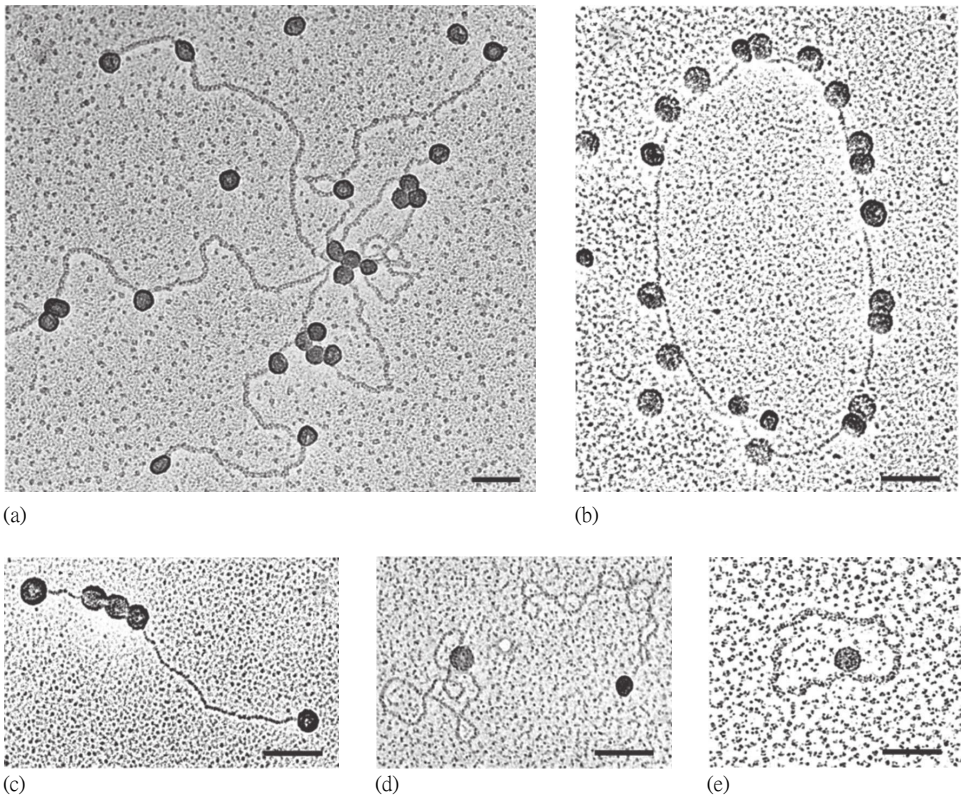


FIGURE 20.3 Electron micrographs of MPyV VLP interactions with linearized (panels a through c) or circular (panels d and e) bacterial plasmids. Aqueous spreading technique, bar = 100 nm. Electron microscopy, Jitka Štokrová.

2.5 kbp. The pentameric capsomeres exhibited high binding affinity for both linearized and circular DNAs, but the interaction did not lead to the protection of target DNA after nuclease treatment.

The procedure is widely used for the preparation of transduction-competent PyV-based PLPs (Forstová et al. 1995; Soeda et al. 1998; Henke et al. 2000; Krauzewicz et al. 2000b; Stubenrauch et al. 2001; Touzé et al. 2001), but HPV PLPs were also successfully prepared by this method (Combita et al. 2001). Empty capsids package DNA most efficiently when complexes are formed at a molar ratio of5:1 for capsids/DNA (Aposhian et al. 1975), and for maximal transduction efficiency, the optimum loading ratio seems to be the same, despite the fact that a significant portion of DNA associated with pseudocapsids appears not to be packaged when observed by electron microscopy (Krauzewicz et al. 2000b). Oligonucleotides are incorporated into the VLPs at a higher ratio than plasmid DNA (72 oligonucleotides per VLP), and the process is pH dependent; the highest oligonucleotide encapsidation capacity occurred at pH 5 (Braun et al. 1999).

To improve the packaging capacity of VLPs, the polycationic amine, poly-l-lysine, was examined as a DNA-condensing agent. The addition of poly-l-lysine to the reaction before osmotic shock increased the size of nuclease-protected plasmid DNA (7.2 kbp) and enhanced transient, but not stable, expression of genes carried into cells by MPyV VP1 pseudocapsids (Soeda et al. 1998). Interestingly, the extent of protection did not correlate with transduction efficiency, and unprotected VLP/DNA complexes could sustain a high level of transduction in vitro and in vivo (Soeda et al. 1998).

20.4.2.1.2.2 Disassembly/Reassembly Procedure Nucleic acids and other cargos can be encapsulated into VLPs during the formation of VLPs from capsomeres. The procedure was pioneered by Salunke et al. (1986). Depending on the conditions, several forms of viral assemblies can arise, and Table 20.3 gives an overview of the conditions used to form VLPs and/or other structures from capsomeres. The loading of nucleic acid is performed by its addition to capsomeres, which have been either purified from a bacterial system or prepared by a disassembly reaction from VLPs. The most widely used procedures for disassembly and reassembly are described in Table 20.4. Generally, the disassembly of VLPs requires a reducing reagent (DTT, β-mercaptoethanol) and chelating agent (ethylenediaminetetraacetic acid [EDTA], ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid [EGTA]) and a mildly basic pH (pH = 8.5). In our experience, the aggregation of material can be a complicating factor during disassembly and reassembly (see also Notes in Table 20.4), and the aggregate material should be removed either during the purification of capsomeres on column chromatography or during the centrifugation step, before the reassembly reaction is initiated. For reassembly, the capsomeres are usually dialyzed against buffers with an acidic to neutral pH, higher ionic strength, and, in the case of PyVs, the addition of calcium. Interestingly, the reassembly buffer consisting of 2 M $(NH_4)_2SO_4$ seems to give the best results even without the calcium ions (Table 20.3). Calcium ions are also not required for capsid assembly when the reconstituted bacterial chaperones are used in the reaction (Chromy et al. 2003).

It has been shown that the presence of nucleic acids can enhance the assembly reaction of SV40 PLPs or MPyV PLPs (Braun et al. 1999; Mukherjee et al. 2010) and DNA can mediate the formation of 40 nm SV40 particles (Tsukamoto et al. 2007). The latter system requires DNA longer than 250 bp at a minimum concentration of 5 mg/L, and for plasmid DNA (4729 bp), optimal concentrations appear to be 10–20 mg/L (3.1–6.1 nM), corresponding to a molar ratio of 1:1 for capsids/DNA. Similar to osmotic shock procedure, nuclease treatment of these SV40 PLPs converted plasmid DNA to fragments of less than 2 kbp, suggesting that the naked DNA was only partially packaged into VP1 VLPs (Tsukamoto et al. 2007). Other reports, however, showed that the size limit for encapsidation of DNA by this method corresponds to the size of the parental virus genome and was 8 kbp for HPV16 PLPs (Touzé and Coursaget 1998). Occasionally, VLPs were reported to accommodate and transduce, albeit with low efficiency, plasmids of a bigger size than the viral genome, as shown for BKPyV (Touzé et al. 2001).

In theory, packaging efficiency can be enhanced by the addition of DNA-condensing agent before the assembly of capsomeres is initiated. This strategy has been used for the formation of MPyV PLPs (Henke et al. 2000). Here, the authors tried to condense DNA with histone sulfate or activated dendrimers. Dendrimers are able to condense DNA by the strong interactions of their positively charged amino groups with the negatively charged phosphate groups of the DNA. Although dendrimers formed aggregates that could be used for in vitro assembly reaction, electron microscopy revealed incomplete capsid assembly, and transduction experiments showed no expression of the reporter gene (Henke et al. 2000). DNA with histone sulfate formed large aggregates (500–6200 nm) that could not be used for assembly reaction. In contrast, compact SV40 PLPs were formed when reporter plasmid was in vitro associated with purified histones and used for encapsidation (Enomoto et al. 2011). The plasmid was fully protected from nuclease treatment, but the maximum length was not longer than the size of the SV40 genome.

Significantly longer target plasmid DNA can be encapsidated into SV40 PLPs by the procedure developed by the A. Oppenheim group (Sandalon et al. 1997). This method is based on the assumption that nuclear factors (e.g., chaperones) may increase the efficiency of encapsidation. The VLPs are produced by a baculovirus expression system in *S. frugiperda* cells, and disassembly, heterologous nucleic acid addition, and reassembly take place in the nuclease-treated nuclear extracts of these cells without VLP purification. The procedure was shown to yield 10 times more transduction-competent PLPs than the disassembly/reassembly method performed with purified VLPs. The improved protocol permitted the packaging of at least 17 kbp plasmid DNA without the requirement for any viral sequences (Kimchi-Sarfaty et al. 2003). It has been suggested (Mukherjee et al. 2007) that the absence of histones provides space and facilitates the packaging of significantly larger plasmids. The optimal VP1 capsid/DNA molar

ratio was set at 1:1, which corresponds to a VP1/DNA ratio of 5:1 on a weight basis (for 5 kbp DNA molecule). The electron microscopy examination of the purified VP1/2/3-PLPs showed well-assembled particles of uniform size (45 nm) and shape. The PLPs were found to have an infectivity ratio 1:3 × 10^5 (1000-fold less than the SV40 virion) (Mukherjee et al. 2007). The same study indicated the minimal concentration of VP1 protein in nuclear extract to be 1 mg/mL (~~5%~~ 10% of nuclear extract stock). It is interesting to note that this method of PLP preparation uses a relatively large quantity of DNA, corresponding to 1 ng/cell during transduction (compared to 1–20 pg/cell used in other protocols; see Table 20.7).

20.4.2.1.2.3 Chemical Coupling Procedure It has been shown that targeted delivery of DNA can be enhanced by the coupling of DNA directly to the adenoviral capsids, which significantly reduces the number of viral particles used but still maintains high levels of gene expression (Wagner et al. 1992). A method where the viral capsid was chemically conjugated to poly(l-lysine) and bound ionically to DNA molecules (Cristiano et al. 1993) was used by Muller et al. (1995) to physically link the intact PV VLPs with the plasmid (8.9 kbp), harboring the reporter gene to study the uptake of particles into different cell lines. They showed that the VLP–DNA complexes were able to bind and penetrate into a broad range of cells, but for reporter gene expression, the coinfection with reporter constructed from a replication-defective adenovirus (facilitates lysis of lysosome membranes) was required. This suggests that (1) tropism of infection by different PV is controlled by events downstream of initial binding and uptake and that (2) this approach can be used for successful nucleic acid delivery if lysosome escape would be promoted (e.g., by arginine-rich cell-penetrating peptides) (El-Sayed et al. 2009). Another group showed even more promising results with a PCR-generated reporter gene (1.8 kbp) driven by a human cytomegalovirus promoter covalently cross-linked to the outside of the HPV VLPs. They suggested that the coupling ratio of the reporter construct to VLP might be crucial for success and reported that up to 40% of target cells can be consistently infected with these PLPs. Further, they demonstrated that L2 inclusion into the VLP dramatically improved infection efficiency for HPV16 and HPV11 PLPs (Yeager et al. 2000).

20.4.2.1.2.4 Direct Mixing Procedure Chemical coupling to the VLP exterior has clearly shown that encapsidation into the particle is unnecessary for successful delivery of nucleic acids into the cell. Some reports even suggested that physical linkage to VLPs is not needed and that VLPs can be mixed with DNA without any further treatment. Touzé et al. (2001), comparing osmotic shock, disassembly/reassembly, and direct mixing methods for BKPyV PLP preparation, found the later procedure to be the most efficient method in transduction assays. The same results were obtained for different types of HPV VLPs and indicated that a direct mixing procedure leads to the highest level of protection against nuclease (45% for osmotic shock, 31% for disassembly/reassembly, and 55% for a direct mixing method in HPV16 PLP preparations) (Combita et al. 2001). Similarly, Krauzewicz et al. (2000a) showed that this method resulted in sustained ex vivo and in vivo transfers of a reporter gene by MPyV VLPs. Other studies, however, reported conflicting results. Clark et al. (2001) showed that the transfection efficiency of MPyV VLP–DNA complexes appeared to be the same or lower than that of DNA alone. Other studies also indicated that mixing DNA and VLPs results in poor short-term in vitro transfection (Touzé and Coursaget 1998; Ou et al. 1999). The reason could be that a significant proportion of DNA remains free in the preparations (Clark et al. 2001) and unpackaged DNA might trigger cellular defense mechanisms leading to loss or silencing of the transgenes and consequent inefficiency of the vector in transduction assays (Bishop et al. 2006).

20.4.2.2 Gene Transfer

Gene delivery vehicles should efficiently penetrate the cell and facilitate gene expression in the target cell. Despite the restricted tissue tropism of some PyV as well as PV, both viruses use for the primary cell bind widespread receptors' moieties (sialic acid and HS, respectively) and therefore enter many different cell lines. Muller et al. (1995) found that 15 out of 16 different cell lines that originated from different tissues and species were able to take up the HPV VLP–reporter plasmid complexes. Moreover, constructs composed of HPV16 L1 and L2 proteins delivered the DNA into cells as efficiently as the VLP–reporter plasmid complex made of L1 alone, suggesting that the L2 protein is not necessary for PV binding and penetration. In another study, the HPV33 L1 VLPs were also found to bind to all of the cell lines tested, including insect Sf9 cells (Volpers et al. 1995). Conversely, L1 VLPs derived from the nine different HPV types (16, 18, 31, 33, 39, 45, 58, 59, and 68) were able to transfer genes into Cos-7 cells, thus showing that most HPV types can be used for gene transfer (Combita et al. 2001). The binding and uptake of PyV VLPs by various cell lines was systematically investigated only for murine pneumotropic virus (MPtV) VLPs (Tegerstedt et al. 2003), but model PyVs (MPyV, SV40) are known to enter and efficiently transform heterologous cells in culture derived from various nonpermissive hosts (Pipas 2009). Moreover, numerous studies done with PyV PLPs and PsVs have confirmed that these are able to enter a variety of cell lines and even transduce reporter genes (e.g., see Table 20.7). Compared to PVs, which require 4–12 h for cellular uptake (Volpers et al. 1995), the internalization of PyVs and VLPs is rapid (Pelkmans et al. 2001; Richterová et al. 2001), and within 40 min approximately 50% of all membrane-bound capsids are internalized (Tegerstedt et al. 2003).

All these results indicate that polyoma- and papilloma-derived VLPs are efficient for penetration into a vast variety of cells. Although this can be good for application where the widespread distribution of a vector is needed, the selectivity is usually required for therapeutic purposes. To increase therapeutic potential, modification of capsid proteins can be performed to abrogate type-specific epitopes and induce retargeting of VLPs to specific cells (see Section 20.4.1).

Interaction of a virus capsid with the cell surface receptor is often an important, but not sole, determinant of success in delivering genetic information for gene expression. There is emerging evidence that events downstream of cell surface interactions such as endocytosis, virus-induced signaling, intracellular trafficking, and transcriptional regulators may also significantly contribute to the expression of genes carried by a virus-based vector. Numerous gene transduction experiments that have been performed with PyV and PV vectors over the last 20 years have helped to identify some of these factors. The experiments that substantially contributed to identify these factors or showed potential for further applications are summarized this section.

The pioneering work of Forstová et al. (1995) proved that PLPs made from MPyV VP1 pseudocapsids loaded with DNA by osmotic shock are able to stably transduce genes for expression. They used an interesting method to assess this delivery system: the linear fragment (1.6 kbp) of the PyV middle T antigen gene, the principal oncogene of MPyV, was transduced by PLPs into the immortalized rat-2 cells, and the transformed foci were observed 3 weeks later. Although the PLPs' loading with DNA was low (only 5%–10% of input DNA was protected from nuclease treatment), the efficiency of transduction was higher than that seen in the calcium phosphate or liposome transfection method when the same amount of input DNA was used. In the complementary experiment, the whole plasmid DNA containing either the reporter gene chloramphenicol acetyltransferase (CAT) (6.2 kbp) or the p43 gene was used for transduction into human liver CCL13 cells and into human embryonic lung fibroblast cells. The loading capacity of VP1 pseudocapsids was higher for the circular plasmid (30% of input DNA was protected against nucleases after osmotic shock) and the high expression of the reporter gene as well as the p43 gene was observed after 3 days. For the CAT gene, the transduction efficiency was clearly better than with the control lipofectin transfection. Follow-up studies (Krauzewicz et al. 2000a) demonstrated much lower transduction efficiency into Cos-7 (10 times lower than the calcium phosphate method) with the same MPyV VP1 pseudocapsids, but loaded with plasmid by the direct mixing method. These PLPs were, however, able to sustain ex vivo transfer into nondividing rabbit corneal explants and stable expression (after administration of 10^{13} PLPs per animal) of the reporter gene in several tissues of nude and immunocompetent mice. Complementary study (Heidari et al. 2000) examined the persistence and tissue distribution of PyV DNA in normal and immunodeficient mice inoculated in the form of MPyV PLPs, through plasmid DNA or as a natural virus. Mice inoculated with PLPs were found to carry 10–50-fold and 50–100-fold higher copy numbers than mice inoculated with plasmid alone in immunodeficient and normal mice, respectively. The number of DNA copies found in mice inoculated with PLPs was similar to that found in mice infected with PyV, but normal mice were found be more resistant to PLP/virus treatment (DNA detected in 7 out of 11 mice) than immunocompromised animals (DNA detected in 14 out of 15 animals). Importantly, when present in animals, DNA was widely distributed to almost all tissues up to 6 months p.i. The result confirmed previous results (Krauzewicz et al. 2000a) and indicated that the immune system may influence the persistence of viral DNA introduced by pseudocapsids but does not totally eliminate it.

In vivo gene transfer was also performed with MPtV PLPs prepared by direct mixing method with a small group (two animals) of normal mice. Three weeks after intraperitoneal inoculation of pure reporter plasmid (pEGFP-C1) or PLPs loaded with plasmid, the PCR detected pEGFP-C1 DNA in many organs of mice inoculated with PLPs, but not in mice inoculated with plasmid alone (Tegerstedt et al. 2003).

In general, these studies indicated that PLP-mediated DNA delivery favored long-term expression, whereas the initial expression was rather low. This discrepancy was partly explained by the fact that DNA has been readily integrated in host genetic information and by the observation that the DNA loading method (osmotic shock) yielded large aggregates of VLPs and DNA, which could reduce the effectiveness of transfer by sequestering the material. The efficiency of gene transfer correlates with the level of nuclease protection of DNA and is apparently an important factor during gene transfer (Combita et al. 2001). As demonstrated by Enomoto et al. (2011), naked DNA protruding from VLPs might inhibit cell attachment, whereas nucleosome arrangement enhances compact particle formation and cellular uptake. Unpackaged DNA might trigger cellular defense mechanisms leading to silencing of the transgenes, and consequent inefficiency of the vector in transduction assays (Bishop et al. 2006) and hyperacetylation of histones, as found in native SV40 virions (Chestier and Yaniv 1979; Coca-Prados et al. 1980), could significantly enhance reporter gene expression (Enomoto et al. 2011).

In vivo techniques for PLP and PsV production usually yield particles that completely protect encapsidated DNA from nuclease action. Studies with JCPyV PLPs that can be made in vivo in *E. coli* even indicate that nucleosomal arrangement, which is absent in this system, is not crucial for successful gene transfer. In fact, one of the few functional studies of the gene transfer was performed with JCPyV PLPs prepared by this system (Chen et al. 2010). In this study, the expression of plasmids harboring either the reporter gene (GFP) or a gene encoding a prodrug-converting enzyme (thymidine kinase [TK]) was packaged into the self-assembled VLPs. Purified PLPs that contained the full-length plasmid were used for the transduction of human carcinoma cells (COLO-320 HSR) since JCPyV seems to have a specific tropism for colon epithelial cells and is detected in carcinoma lesions (Coelho et al. 2010). GFP gene expression was achieved in 90% of cells. The same PLPs were selectively transduced in vivo to tumor nodules in nude mice bearing human COLO 320 HSR tumors, when intravenously injected into the mouse. Treatment with PLPs containing the TK gene resulted in total tumor growth inhibition after intraperitoneal injection of ganciclovir for 3 weeks, whereas there was no inhibition in the negative control groups (PLP-TK without ganciclovir or PBS with or without ganciclovir). The work

demonstrated that JCPyV VP1 PLPs can efficiently protect genetic information and deliver it for gene expression in nude mice. The reason for high selectivity for tumor tissues is interesting, but not completely clear: the authors have suggested that human tissues may be more susceptible to JCPyV PLP infection than mouse cells.

The inconsistencies in gene transfer efficiencies during different experimental settings were also explained by the fact that two modes of entry of viral particles into the cells exist: the productive pathway, which leads to gene expression, and the nonproductive, default pathway for cargo, which enters the cell in an unspecific manner, for example, by phagocytosis. Differences in surface characteristics induced by DNA packaging or absence of VP2 and VP3 minor proteins were suggested to affect the interaction with the cell in a way that VLPs would be significantly less effective for DNA delivery than natural virions (Krauzewicz et al. 2000b).

For PV PsVs, numerous studies have clearly shown that PLPs consisting of L1 alone are infectious, but L2 enhances infectivity (Unckell et al. 1997). The same has been shown to be true for some PyV-derived PsVs. Specifically, a recent study (Schowalter and Buck 2013) indicates that infectivity of the BKPyV VP1–only PsVs is dramatically lower compared to the VP1 + VP2 + VP3 PsVs on all tested cell lines. In contrast, the effect of VP2 on MCPyV pseudovirus transduction efficiency differs dramatically between cell lines. Thus, infectious entry of the BKPyV pseudovirus appears to differ from the MCPyV pseudovirus with regard to its dependence on minor capsid proteins, indicating that differences can exist between virus types in general. Moreover, the study showed that not only the sole presence but also the ratio of minor proteins can dramatically accelerate PsV *infectivity*, which was never zero even for VP1-only particles. The same conclusion has been drawn from experiments performed with in vitro reconstituted SV40 QVs or PsVs. Although the minor capsid proteins VP2/3 strongly facilitated gene transducing activity, the gene expression level after transduction with PsVs was only 2% of that achieved by SV40 virions. The authors speculated that the slightly lower content of VP2/3 in VP1/2/3 PsVs affected postinternalization processes, such as virion disassembly, nuclear translocation, and DNA replication (Enomoto et al. 2011). This may explain why the repeated production of PsV stocks is more consistent for PV PsV than PyV PsVs. L1 and L2 genes are usually cotransfected on one plasmid, whereas for PyV PsVs, at least one capsid protein is usually encoded on a separate plasmid. The actual cotransfection efficiency then determines the yield of fully *infectious* PsV (Španiel ová et al. 2014). As demonstrated by the work of Nakanishi et al. (2008), the coexpression of all capsid proteins from one plasmid may be crucial for obtaining PsV stocks that can transduce permissive cells with efficiencies reaching 100% and similar to that of the virion. The same study also showed that capsid exchange could significantly alter the cell specificity of gene transfer.

The PsVs generated in mammalian cells, as well as in yeast systems, serve predominantly as a diagnostic tool. PsVs are used for the detection of neutralization antibodies in vaccinated or infected individuals. These neutralization assays depend on the ability of antibodies in a test serum to prevent infection of cells by a virus. Since infectious HPV virions are not readily available, the PsV-based technology is used to generate infectious particles (Buck et al. 2005a). PsVs containing an easily detectable reporter gene (alkaline phosphatase, luciferase, GFP) are purified on density gradients and are used to infect a detection cell line. To increase sensitivity, the detection cell line should express SV40 large T antigen to support replication of the reporter vector, which harbors the SV40 ori. The presence of antibodies to HPV in a test serum blocks infection of PsVs and reduces the signal from the reporter gene. These assays are more type specific than the enzyme-linked immunosorbent assays (ELISAs). This was demonstrated for HPV subtypes (Pastrana et al. 2004), as well as BKPyV subtypes, when serotypes not recognized by VLP-based ELISA could be discovered by neutralization assay (Pastrana et al. 2013). Neutralization can be used for characterizing candidate vaccines, quantification of seroresponsiveness, and diagnosis of viral subtypes.

For safety reasons, PsVs produced in mammalian cells have limited potential as vehicles for therapeutic genes. Nevertheless, some studies exist. One study using a gutless recombinant SV40 (rSV40) vector examined the feasibility of SV40 PsVs for gene therapy in cystic fibrosis (CF) (Mueller et al. 2010). The genetic defect in CF is caused by mutations in a cell membrane chloride channel, the cystic fibrosis transmembrane conductance regulator (CFTR). The human CFTR gene was cloned into a rSV40 vector under the control of the SV40 early promoter and was used as a target plasmid for production of PsVs in Cos-7. These cells carry an integrated copy of the wild-type SV40 genome that is defective at the ori. The authors noted that the SV40 capsid genes, under the control of the SV40 late promoter, are not expressed constitutively in Cos-7 cells, but that the presence of a replicating rSV40 vector that includes the late promoter is sufficient to activate Cos-7 transcription of the capsid genes in *trans* and the vector could be packaged. By this procedure, they were able to obtain high-titer stocks (approximately 10^9 infectious units [IU]/mL) of SV40 PsVs. They showed that rSV40–CFTR was able to induce the expression of CFTR protein, which localized to the plasma membrane and restored channel function to CFTR-deficient cells. When matched groups of 5 $Cftr^{-/-}$ mice were treated with 4.0×10^7 particles of either the rSV40–CFTR vector or the irrelevant rSV40–BUGT negative control via intratracheal injection, delivering rSV40–CFTR to the lungs of $Cftr^{-/-}$ mice resulted in a reduction of the pathology associated with intratracheal *Pseudomonas aeruginosa* infection. CFTR gene was stably expressed at least around 9 weeks post delivery and the authors reported that animals were free from virus revertants and no adverse effects or toxicity was evident.

Another study demonstrated targeting of SV40 PLPs to mouse liver by the use of the hydrodynamic tail-vein injection (Arad et al. 2005). The PLPs were prepared either in vivo or in vitro in nuclear extracts of Sf9 cells and contained the

luciferase reporter gene. After injection, the luciferase activity in the mouse liver was monitored with a light detection camera. In vivo transduction of hepatocytes was efficient and persistent (lasted at least 107 days). Importantly, SV40-specific antibodies were observed in mice administered with SV40 PLPs, and antibody response was dose related. The authors observed a decline in luciferase activity during long-term observation and speculated that the immune response against the vector eliminates some transgene-expressing cells early after transduction.

In spite of the widely accepted notion that minor proteins are important for high PLP transfer, the approach of producing SV40 PLPs by the disassembly/reassembly method in the nuclear extract of Sf9 cells reproducibly achieved high efficiencies of transduction (close to 100% cells transduced) in a wide variety of cells without VP2/3 proteins (Kimchi-Sarfaty and Gottesman 2004; Kimchi-Sarfaty et al. 2004). Originally, minor proteins were included in the system (Oppenheim et al. 1986; Sandalon and Oppenheim 1997; Rund et al. 1998; Kimchi-Sarfaty et al. 2002) and suggested to improve transduction substantially (Sandalon et al. 1997). At the same time, the PLPs made solely from VP1 containing longer target DNAs (17 kbp) were observed to be larger (55 nm) than normal VLPs or virions (45 nm). The study speculated that the absence of minor proteins increased the space for encapsidation and decreased the rigidity of the capsid (Kimchi-Sarfaty et al. 2003). This corresponded with the previous observation that VP2/3 containing PLPs showed better protection against nuclease treatment (Sandalon et al. 1997).

The system with or without the minor proteins was, however, successfully used for gene transfer. In particular, SV40 PLPs were shown to be very efficient for gene delivery into human hematopoietic cells, and ex vivo transduction of hematopoietic stem cells with SV40 PLP permitted expression of, for example, multidrug resistance 1 (MDR1) gene or β-globin in several studies (Rund et al. 1998; Dalyot-Herman et al. 1999; Kimchi-Sarfaty et al. 2002; Kimchi-Sarfaty et al. 2004).

The potential of SV40 PLPs in anticancer therapy was also examined (Kimchi-Sarfaty et al. 2006). Using these SV40 PLPs, a truncated *Pseudomonas* exotoxin gene (PE38) was delivered into various human cells and dramatically reduced their viability, thus showing that the toxin has a similar toxicity in cells of various origins. Interestingly, this nontargeted route of PE38 delivery was found to be effective in the treatment of human adenocarcinomas growing in nude mice, when injected either intratumorally or systemically. Controls, including daily delivery of the same DNA in the naked form, as well as empty capsid proteins or GFP encapsidated using the same PsV system, all failed to inhibit tumor growth. The authors noted that treatment that started later, on larger tumors, was less effective than treatment that started only 2 days after tumor inoculation; thus, the effectiveness of the therapy is related to the ratio of PsVs to the number of tumor cells. Treatment also caused no abnormalities in mice. Three of the PE38-treated mice stayed tumor-free after a year and a half. The authors speculate that the SV40 PsVs circulate in the blood and are taken up selectively by the tumors through the enhanced permeability and retention effect (Kimchi-Sarfaty et al. 2006).

20.4.2.3 Gene Silencing

RNAi is a sequence-specific, naturally occurring gene-silencing mechanism. A number of approaches have been developed in recent years that allow for exploitation of this process principally through the use either of (1) synthetic siRNAs or duplex RNA oligonucleotides or of (2) plasmid-expressed short hairpin RNAs (shRNAs) that must be endogenously processed to generate a siRNA. The in vivo use of RNAi therapy is limited by obstacles related to effective delivery into the cell, and PyV and PV VLPs may serve as efficient RNAi delivery vehicles.

20.4.2.3.1 RNA Transfer

SV40 PLPs produced in vitro in nuclear extracts of Sf9 cells were shown to transduce not only DNA but also siRNA (Kimchi-Sarfaty et al. 2005). The same system was used to deliver plasmid-expressed shRNAs and synthetic siRNAs into human cells. After transduction with SV40 PLPs loaded with siRNA corresponding to the GFP gene, the complete silencing of the GFP gene was observed in HeLa cells stably expressing GFP. The delivery of siRNA by SV40 PLPs was more efficient than the Lipofectamine method.

Packaging of short ssRNA (75–800 nucleotides) into SV40 PLPs was also performed by the disassembly/reassembly method (Kler et al. 2012). The encapsidation of RNA seemed to be very fast and the method yielded small particles (diameters of 24.5 nm) made from one molecule of 524 nucleotides RNA and 12 pentamers. Unfortunately, neither the packaging of longer ssRNA molecules nor the RNA transfer into cells was examined in the study. If efficient, the system can be an attractive means of ssRNA transduction for special applications.

20.4.2.3.2 Short Hairpin RNA Delivery

The utilization of PyV and PV VLPs for gene therapy application may be restricted by their packaging capacity, but they can be an efficient means of delivering RNAi effectors, such as DNA encoding the shRNA sequences.

The JCPyV PLPs were experimentally used for silencing of the IL-10 gene, which is overexpressed in patients with systemic lupus erythematosus. VP1 VLPs produced in yeasts were loaded (by osmotic shock) with a PCR fragment containing a sequence of IL-10 shRNA. After transduction into a murine macrophage cell line, IL-10 shRNA was found to reduce IL-10 expression by 85%–89%, as compared with unloaded VLPs. In BALB/c mice, IL-10 shRNA abolished 95% of IL-10 secretion (Chou et al. 2010).

More recently, JCPyV VP1–PLPs were successfully used for the silencing of BKPyV infection. PLPs produced in vivo in *E. coli* were used as a delivery system to transfer plasmid-encoding shRNA for BKPyV large T antigen into BKPyV-infected human kidney cells. PLP-mediated transduction

with the plasmid decreased the proportions of BKPyV large T antigen and VP1-expressing cells by 73% and 82%, respectively (Lin et al. 2014).

The potential of shRNA transduction mediated by PV VLPs was explored for the inhibition of cervical cancer cell growth. HPV31 PLPs were loaded by the disassembly/reassembly method with plasmids encoding the shRNA for two main oncoproteins of high-risk HPV, E6, and E7 (Bousarghin et al. 2009). The silencing of both genes was achieved after transduction in HPV-positive cells lines. E6 silencing resulted in the accumulation of cellular p53 and reduced cell viability. Cell death was observed when E7 expression was suppressed. In mice, where murine TC1 cells expressing HPV16 E6 and E7 oncogenes induced fast-growing tumors, HPV PLPs coding only for an E7 shRNA were sufficient for dramatic inhibition of tumor growth.

20.4.2.3.3 Oligonucleotide Transfer

Antisense oligonucleotides are specific drugs to inhibit gene expression at the transcriptional level. MPyV VP1 VLPs were investigated as a means of oligonucleotide delivery system into the cells. Capsoids formed after expression in *E. coli* were loaded with the fluorescently labeled oligonucleotides directed against the *N*-methyl-d-aspartate (NMDA) receptor by an osmotic shock. These PLPs were used for the transduction of mouse fibroblasts that overexpress the NMDA receptor and allow a functional antisense oligonucleotide test system based on excitotoxicity (cell death). In comparison with several other delivery methods, MPyV VP1 VLPs showed very low uptake and only a moderate effect in a functional antisense oligonucleotide test (Weyermann et al. 2004).

In contrast, JCPyV VP1 VLPs showed better potential as an oligonucleotide delivery vehicle for human neurological disorders. In the model system of SV40-transformed human fetal glial cells, VLPs generated in yeast were used to package and deliver an antisense oligodeoxynucleotide against an SV40 large T antigen, the main SV40 oncoprotein. The oligonucleotide transfer resulted in the inhibition of large T antigen expression and subsequently led to cell death. As expected from JCPyV tropism, VLPs were able to deliver oligonucleotides into human astrocytoma, neuroblastoma, and glioblastoma cells with high efficiency, and in vivo delivery of oligonucleotides into a human neuroblastoma tumor nodule by VLP was also demonstrated (Wang et al. 2004).

An SV40 PLP system was also explored for the delivery of peptide nucleic acid (PNA) into the cells (Macadangdang et al. 2011). PNA is a synthetic DNA analog in which the sugar-phosphate backbone is replaced with a polyamide backbone. PNA can bind with high affinity and sequence specificity to complementary nucleic acid sequences and can be used to suppress gene expression (Corradini et al. 2007). PNA molecules designed to bind in the region of the major transcription initiation site of the MDR1 gene were loaded in vitro to SV40 VP1 VLPs in the nuclear extract of Sf9 cells. These PLPs could effectively decrease MDR1 mRNA levels in drug-resistant KB-8-5 cells. Compared to other delivery systems, SV40 VP1 PLP transduction times were very short (2.5 h versus 48–96 h), and treatment with micromolar concentrations of antisense PNA yielded almost a 30% reduction in MDR1 mRNA levels. Importantly, it also increased the sensitivity of cells to the chemotherapeutic agent Adriamycin. The study concluded that the combination of PNA with the SV40-based delivery system is a method for suppressing a gene of interest that could be broadly applied to numerous targets (Macadangdang et al. 2011).

20.4.2.4 Concluding Remarks

Numerous studies have used the polyoma- and papilloma-derived VLPs as gene delivery vehicles with various successes but have shown their potential for gene transfer applications. Due to differences in experimental settings, these studies are impossible to compare directly even if the same type of PLPs was used. The potential pitfalls were, however, identified and can be possibly solved by exploiting or, on the contrary, manipulating the inherent biological features of these viruses.

Two factors emerged as very important aspects for successful gene delivery: (1) efficient packaging of nucleic acid with no DNA exposed to the outside space and (2) the *virus-like* composition of particles with the correct ratio of minor proteins. The latter factor seems to be more important for some virus types (e.g., BKPyV) than for others (e.g., JCPyV). Nonviral production of such a capsid may be more difficult for PyV VLPs, which have two minor proteins, than for PV VLPs. The minor proteins probably facilitate endosome/lysosome escape, and inclusion of other penetrating peptides in the system can accelerate gene expression. Similarly, nucleosomal arrangement of genetic information with hyperacetylated histones can be beneficial but probably not necessary. The main challenges in gene (and some other cargos) delivery are, however, the unspecific binding and uptake of these particles and their immunogenicity. Both challenges can at least in part be overcome by the modification of capsid structures by chemical or genetic means or by selecting the virus with specific tropism for VLP production. The repeated administration of these VLPs for some applications will probably require sequential use of VLPs from different virus types. Luckily, PyVs and PVs offer a wide selection of materials for further exploration.

Some reports of gene transfer performed in animals have clearly shown long-term expression of transgenes introduced by PyV VLPs—vehicles. The immune system was found to partly restrict the persistence of a transgene but not totally eliminate it. The system was found to be suitable for ex vivo transduction of hematopoietic cells. Its potential for anticancer gene therapies has been also explored, but experiments performed with xenografted immunocompromised animals might be misleading in regard to actual efficacy and need further verification. The utilization of these VLPs for classical gene therapy applications is therefore still far from reach, but special applications, such a gene silencing, offer another interesting possibility for their use.

20.4.3 Protein and Peptide Delivery

The use of many potent proteinaceous pharmaceutical agents might be complicated by their instability, side effects, and their need to be delivered intracellularly to exert their therapeutic action. VLPs of both PyVs and PVs are able to serve as nanocontainers for foreign peptides: the exterior can be used for labeling or targeting and the interior space for encapsulation of biologically active protein or peptide cargos. Encapsidation into a VLP has the advantage of hiding and protecting the proteins from external proteases and from recognition by the immune system. Peptide loading into the inner space of PV and PyV VLPs is usually achieved either by genetic fusion with a major capsid protein or by the utilization of minor protein in a single- or two-component attachment system (see Section 20.4.1 for description).

Although VLP-based vaccines do not usually require intracellular delivery of an antigen, its encapsulation may be important for the delivery of cytotoxic T-lymphocyte (CTL) epitopes. This strategy has been explored for peptide fusion to major capsid protein L1, since PV VLPs are widely used as transport vehicles of various antigens, including oncoproteins, for anticancer immunotherapy. An extensive study was conducted where the C-terminal 34 aa of HPV16 L1 protein were replaced with various segments of oncoprotein E7. Peptides up to 60 aa could be fused to the truncated L1 without disrupting the assembly of VLPs (Müller et al. 1997). Besides E7, many other epitopes or peptides have been subsequently added to the truncated C-terminal of L1 protein, for example, human immunodeficiency virus (HIV)-IIIB CTL epitope of gp160, gp120, reverse transcriptase, and protein Nef (Peng et al. 1998; Liu et al. 2000). Even though the C-terminal part of the PV L1 protein is preferable for peptide fusion, chimeric particles with GFP added to the C- and N-terminal part of the L1 protein prove that both ends are suitable for protein fusion without disrupting the assembly of VLPs, but the actual location of GFP molecules in VLPs has not been analyzed (Windram et al. 2008).

Genetic fusion with the major capsid protein of MPyV has been also explored for encapsidation of protein cargo—the GFP molecule (Günther et al. 2001). According to the theoretical calculation, 360 globular proteins with an average size of up to 17 kDa can be encapsidated into VP1 particles. PyV VP1 protein seems to be less flexible to peptide fusions than the L1 protein, and therefore the WW domain-based two-component system (see Section 20.4.1.2) was used for protein encapsidation. The WW domain was fused to the N-terminal part of MPyV VP1, and 260 polyproline-tagged GFP molecules were reported to have become encapsidated. The authors, however, did not attempt to verify the position of GFP in the particle but showed that these particles were able to deliver cargos to mouse fibroblasts NIH 3T3 (Günther et al. 2001).

An alternative approach to peptide delivery is conjugation of a foreign protein to a minor protein. The resulting fusion protein uses minor proteins as an anchor for noncovalent association with VP1 pentamers and, as such, should be hidden inside the particle. Even though the theoretical maximum amount of fused protein per VLP is 72, the actual number is usually lower. PV HPV16 L2 protein is mostly applied for the transfer of an immunogenic epitope of E7 oncoprotein. The C-terminal part of the L2 protein was successfully fused either with parts of E7-derived peptides (Rudolf et al. 2001; Wakabayashi et al. 2002) or with the whole oncoprotein (Greenstone et al. 1998). Another oncoprotein, E2, was also conjugated to L2 (Davidson 2003), and even together with E7 (Qian et al. 2006). The removal of the central amino acids of the L2 protein (70–390) allowed the simultaneous incorporation of three oncoproteins, E1, E2, and E7. Although this peptide sequence reached approximately 130 kDa, it did not disrupt VLP assembly and produced chimeric particles that were highly immunogenic (Tobery et al. 2003). Windram et al. (2008) proved that not only C-terminal but also N-terminal fusion of GFP to L2 protein led to chimeric VLPs without exerting an influence on their stability.

An electron density map of MPyV reveals that in VP2 amino acids between Val-269 and Tyr-296 are responsible for interaction with three VP1 monomers of the pentamer and that a 45 aa long C-terminal segment of VP2/VP3 protein comprising these residues should be sufficient for a tight association with the cavity of VP1 pentamers (Chen et al. 1998). Accordingly, the stretches of 49 aa of the C-terminal part of VP2 served as an anchor sequence for the loading of GFP. The VLPs produced were regularly shaped and were stable. The amount of encapsidated GFP did not reach the theoretical number of 72 but was still very high at 64 (Abbing et al. 2004). A similar approach was used for N-terminal fusion of EGFP to a truncated minor protein, VP3 (corresponding to VP2 residues in positions 225–324) (Bouřa et al. 2005). This construct served for the testing of various processes and immunity responses, for example, EGPF (Frič et al. 2008) and Bcr-Abl (Hrusková et al. 2009). The MPyV VP2 protein anchor was also used for sufficient transport of a 683 aa long fragment of HER2 (Tegerstedt et al. 2005b, 2007) and full-length human prostate-specific antigen (PSA) (Eriksson et al. 2011) into dendritic cells (DCs).

Analogically, the linkage of foreign protein to SV40 PyV minor capsid proteins was investigated using a series of EGFP fusions (Inoue et al. 2008). Based on the structural model of SV40, where VP2 C-terminal residues 275–302 interact with the inner surface of the conical cavity of the VP1 pentamer (Chen et al. 1998), the coding sequence for VP2 amino acids 222–352 was used as anchor for protein fusion. Interestingly, the N-terminal EGFP fusions either interfered with VLP formation or were not incorporated into the particles. The C-terminal fusions were efficiently incorporated into VLPs. The minimal C-terminal region sufficient for incorporation into VLPs was finally determined to be a 36 aa residue of VP2 protein (273–308). Furthermore, the study demonstrated that a full-length prodrug-converting enzyme, cytosine deaminase, could be incorporated through the VP2 anchor. The enzyme retained its activity (converted 5-fluorocytosine to 5-fluorouracil) in purified VLPs, thus showing that small molecules and ions can gain access to the

interior of VLPs. VLPs were also shown to deliver cytosine deaminase activity to the cells.

In conclusion, the fusion of foreign protein to minor proteins seems to have—unlike fusion to the major capsid protein—a minor impact on VLP stability, but the amount of transported protein is significantly lower (72 versus 360 molecules). Moreover, the stoichiometry of minor protein-anchored cargo in VLPs might vary between batches. Unlike for other viruses (Wen et al. 2012), the chemical conjugation of the peptides into the interior space of PV or PyV VLPs has not been reported so far. Several studies have shown that PyV and PV particles can deliver peptides intracellularly. The potential commercial utilization was immediately identified by the authors of these transport systems, and their patenting shortly followed (US 6991795 B1; US 7011968 B1).

20.4.4 Delivery of Other Molecular Compounds

Highly sensitive noninvasive imaging techniques performed in the absence of ionizing radiation are urgently needed in clinics for early diagnoses of diseases (e.g., cancer) and frequent monitoring of therapeutic progress. One of these techniques is magnetic resonance imaging (MRI), which possesses high temporal and spatial resolution. The recently recognized potential of optical imaging, especially in invisible near-infrared (NIR) fluorescent light, has led to the commercial availability of NIR fluorescence imaging systems for diagnostics and image-guided surgery (Gioux et al. 2010). Contrast agents and imaging probes are a key part of these techniques, as they allow a higher resolution and greater sensitivity in diagnostic images. Nevertheless, for enhancement of diagnostic imaging capabilities, the sensitivity, biocompatibility, and biodistribution of various contrast materials need to be improved. VLPs, as proteinaceous biodegradable nanoscale platforms, offer the opportunity to modify the chemical and physical properties of contrast materials in order to overcome these concerns. The surface modification of VLPs with targeting ligands can highly improve the accuracy of imaging techniques and signal strength. Interior and exterior engineering allow the combination of different functional imaging modalities with therapeutic compounds into a single formulation of a *theranostic* tool (Rosen et al. 2011; Cheng et al. 2012). Both PyV and PV VLPs have been used experimentally as imaging agents or drug delivery agents.

Chemical conjugation of fluorophores to virion or VLP surfaces is a routinely used technique for studying virus–host cell interactions in vitro. In applied research, these techniques can be used for validating the interaction of VLPs with target cells or tissues during in vitro tests of targeted drug delivery. A useful system for imaging the intracellular uptake of VLPs was designed for PV VLPs. HPV6 L1 or HPV16 L1/L2 VLPs were chemically coupled with *silent* fluorochrome carboxyfluorescein diacetate succinimidyl ester (CFDA SE), which fluoresces only after exposure to intracellular esterases. The labeled VLPs had the same structure and appearance as unlabeled VLPs. Importantly, the internalization of labeled VLPs into the host cells was not altered (Bergsdorf et al. 2003; Drobni et al. 2003).

Utilization of the internal space of a particle for encapsulation of a fluorescent probe leaves the exterior free for interaction with the receptor and/or for the attachment of targeting ligands. This strategy has been reported for PyV VP1 VLPs. In one study, all naturally occurring cysteines in MPyV VP1 protein were replaced by serines, and the new cysteine was reintroduced to the GH loop exposed toward the interior of the VLP. Then, fluorescent dye (fluorescein; Texas Red) was specifically conjugated to the cysteine through a maleimide linker. These VLPs retained their receptor-binding ability and entered C2C12 mouse cells (Schmidt et al. 1999).

Diagnostic or therapeutic compounds could also possibly be incorporated by a simple encapsidation during VLP assembly. This method was investigated for JCPyV VP1 VLPs and enabled the packing of fluorescent dyes Cy3 (Qu et al. 2004) and propidium iodide (PI) (Goldmann et al. 2000). The fluorescence intensity of VLPs with encapsidated PI was dependent on the initial PI concentration. Importantly, only fully formed VLPs were stably associated with PI or Cy3 dye, which was not removed from particles during the long-term dialyzing step. The authors confirmed that dyes associated with VP1 noncovalently (Qu et al. 2004), but the absence of traces of DNA inside the VLPs before reassembly reaction was not verified. This could be of some importance, since VLPs are permeable for small molecules (Inoue et al. 2008; Kitai et al. 2011) and the principle of dye stabilization inside the particle is not clear.

Fluorescence is particularly suitable for in vitro imaging applications. Fluorescence imaging probes dedicated to in vivo imaging, however, need to be highly bright fluorophores absorbing and emitting in the NIR range and provide a high signal-to-background ratio. This requires the targeting abilities of the probes to accumulate specifically in cells to be labeled, while being cleared from surrounding tissues. In vivo imaging is limited by the spectral properties of body fluids. This defines a narrow *optical window* between 650 and 900 nm, where light is able to penetrate deeper (a few centimeters' depth) and tissue autofluorescence is also reduced (Mérian et al. 2012).

Quantum dots (QDs) are ideal candidates for in vivo imaging probes. QDs are nanocrystals made of semiconductor materials that display outstanding optical properties, such as a high absorption coefficient and fluorescence quantum yields (Li and Zhu 2013). The immediate optical feature of colloidal QDs is their color. The smaller the QD, the higher the energy of the light that is emitted (and so the bluer the fluorescence spectrum). QDs are also highly dense and therefore visible by electron microscopy without negative staining. Encapsulation of QD into VLPs can provide a platform for visualization of early steps of virus infection, QD targeting, and construction of clusters with different modalities. All this has been shown for SV40 VLPs. Packing of QD was achieved by the reassembly of SV40 VP1 pentamers in the presence of an assembly buffer with QDs and resulted in the formation of particles that were homogenous in size (approximately 24 nm), mainly T = 1 particles consisting of 12 VP1 pentamers. The ratio was one QD per VLP. These particles

were highly stable under routine storage conditions. The internalization of these particles by target cells was unaltered and was similar to the early infection steps of the wild-type virus (Li et al. 2009). Recently, an interesting phenomenon of QD encapsidation was observed. Surprisingly, QDs enhanced assembly of SV40 VLPs from VP1 pentamers directly in the dissociation buffer, and a high affinity between the QD and SV40 VP1 protein was discovered (Gao et al. 2013). QDs were suggested to act as scaffolds, favoring the correct inter-pentamer contacts. Furthermore, it was established that the structural stability and integrity of these particles are absolutely dependent on disulfide bonds formed in VP1 during capsid assembly (Li et al. 2013). The same group also tested the encapsidation of QDs modified by different surface coatings. Their results indicated that positively, negatively, or neutrally charged QDs were encapsulated with comparable efficiencies, and all types of QDs–VLPs preserved their cell-entering ability (Li et al. 2010).

Another class of nanoparticles that possess—depending on their size and shape—interesting optical properties, such as strong absorption and scattering in the visible–NIR region, are gold nanoparticles (AuNPs). They have been used in various types of biomedical applications including photothermal therapy, biosensing, and gene delivery for anticancer therapy (see Khlebtsov et al. [2013] for review). AuNPs of different particle sizes and surface decoration were encapsulated within the SV40 capsids, where the encapsulation efficiency increased with the size of AuNPs modified with methoxypolyethylene glycol 750 (mPEG750) (from 10 to 30 nm). Encapsulation of AuNPs modified with negatively charged DNA ligands was also tested. The electrostatic interactions promoted the encapsidation efficiency of AuNPs with smaller diameters (10 and 15 nm). Moreover, the AuNPs encapsulated into SV40 VLPs were successfully delivered into living Vero cells, whereas the AuNPs alone were unable to enter these cells (Wang et al. 2011). Except for encapsidation, AuNPs could be conjugated to the surface of VLPs. This conjugation of AuNPs was published for the JCPyV. AuNPs of different sizes (5, 10, and 15 nm) were linked to JCPyV VLPs through sialic acid–linked lipid, which was conjugated to AuNPs by a ligand-exchange reaction. The most suitable size of AuNPs for optical detection was 15 nm (Niikura et al. 2009). This approach was combined with encapsidation of QDs or AuNPs inside the SV40 VLPs, and VNP-guided 3D hybrid nanoarchitectures were assembled (Li et al. 2011, 2012). First, the SV40 VP1 protein was genetically modified to display only one cysteine, so that five cysteines were exposed on the VP1 pentamer. These altered SV40 VLPs were used for encapsidation of QDs by self-assembly, and AuNPs were conjugated to the surface through the affinity of gold to thiol groups. As a result, 3D hybrid nanoarchitectures of SV40 VNPs with one QD per VNP and a certain number of AuNPs (from 1 to 12) were obtained (Li et al. 2011). Afterward, this group encapsidated two other types of QDs or AuNPs into SV40 VNPs. As previously, AuNPs were linked to the VNP surface, but this time the conjugation was based on electrostatic interaction between negatively charged AuNPs and positively charged amino acids on the outer surface of SV40 VNPs, and the average AuNP number per VLPs was 27 (Li et al. 2012).

For MRI, various contrast agents exist. Magnetic iron oxide–based nanoparticles (MNPs) are often considered for theranostic applications. Their superparamagnetic nature enables MRI, and particles themselves can be used for therapy through techniques such as magnetic hyperthermia (Gupta and Gupta 2005). Association of VLPs with these particles can therefore help to extend the spectra of their application, for example, by addition of cancer-targeting moieties. This was achieved by Enomoto et al. (2013): the authors encapsulated citrate-coated MNP into SV40 VLPs and then chemically conjugated EGF to the surface of the SV40 VLPs. Linking EGF to these SV40 VLPs–MNPs enabled them to enter the cell lines of human epidermoid carcinoma and human colon adenocarcinoma in a selective manner. Tumor cells are more heat sensitive than normal cells, so these MNPs can serve not only as an imaging tool, but also as a heater element for hyperthermia (Gupta and Gupta 2005). These types of particles have great potential to be applied as excellent material for directed tumor diagnostics and therapy.

The proof of principle for loading of therapeutic molecules such as low-molecular-weight drugs into VLPs was reported for PyV VLPs. Utilizing the covalent linkage of MPyV to a VP2 anchor, 462 molecules of methotrexate were loaded per VLP. Methotrexate is a well-known antifolate used in tumor therapy. The drug was delivered to methotrexate-sensitive cells, CCRF-CEM, and clearly demonstrated time- and concentration-dependent cytotoxicity (Abbing et al. 2004). Other research groups used naturally presented cysteines of the JCPyV PyV for linking the drug molecule via disulfide bonds inside the particle. The hydrophobic drug paclitaxel (PTX) was conjugated to JCPyV VLPs through modified β-cyclodextrin with a thiol-reactive group. The authors were able to encapsulate up to 12.3 PTX molecules, and these particles had a clear cytotoxic effect on NIH 3T3 cells (Niikura et al. 2013).

The essential properties required for a particle drug delivery system are not only specific targeting and cell entry but also the retention and release of the drug molecule in the target cells. First, low-pH-controlled drug release from VLPs was established (Ohtake et al. 2010). This group used a hexahistidine motif (His_6) tag fused to the N-terminus of a 225 aa VP2 anchor for noncovalent linking of fluorescent dye sulforhodamine 101 (SR). This dye was conjugated to nitrilotriacetic acid (NTA), which targets the His_6-tag sequence. The hexahistidine motif is ideal for release triggered by changes in pH because the pK_a of this motif is approximately 6.5, and a lower pH eliminates the His_6-tag affinity. The NTA-SR entered JCPyV VLPs containing the His_6-tag through simple diffusion and bound specifically to the His_6-tag at the physiologic pH (7.4) and in the presence of cobalt ions. After adjustment of the pH to 5.0, the release saturation was achieved within 20 min. Second, the redox-responsive drug release was discovered (Niikura et al. 2013). It utilizes glutathione (GSH), which has been widely recognized as a

ubiquitous stimulus for drug release in cells, and its concentration is three times higher inside than outside of the cell.

JCPyV VLPs were conjugated with thiol β-cyclodextrin ($CD\text{-}SSO_3^-$) through disulfide bonds between cysteine residues and a thiol-reactive group. The release speed was highly dependent on GSH concentration. The plateau was achieved within 5 min with the 10 mM GSH, and with 10 μm GSH, it lasted several hours. The controlled release approaches are extremely important for increasing the possibility of using VLPs as intelligent nanocarriers for therapeutic compounds.

In conclusion, numerous studies have shown that PyV VLPs can potentially serve as versatile functional hybrid nanostructures for application in the field of targeted drug delivery and diagnostic imaging. The interior of VLPs can accommodate drugs as well as various imaging compounds and increase their biocompatibility. The utilization of minor proteins as noncovalently attached drug anchors offers a unique opportunity for controllable drug release. The exterior of VLPs can display multiple imaging molecules or addressable moieties, thus creating targeted multimodal nanoparticles. PV VLPs are less extensively studied than PyV for these types of applications, but their potential utilization as theranostic agents could be very similar to PyV VLPs.

20.5 UTILIZATION OF VIRUS-LIKE PARTICLES FOR IMMUNOTHERAPY

VLPs of both viral families are suitable candidates for immunotherapy due to the high immunogenicity of their major capsid protein. VLPs are characterized by their ability to induce a humoral as well as a cellular immune response. Their structure is similar to the wild-type virus but they lack genetic material, thus providing a safe alternative to attenuated or inactive vaccines. The most important characteristic of VLPs is their ability to induce the production of neutralizing antibodies, which are critical mediators of immunity against viral challenge. These antibodies could be produced by T-helper cell-dependent or T-helper cell-independent mechanisms. Through these mechanisms, VLPs are able to induce a rapid and fulminant humoral immune response without the need for an adjuvant (Jennings and Bachmann 2008). The T-helper cell-dependent immunogenicity of VLPs is entailed by the character of their interaction with DCs. At the beginning of an effective immune reaction is a process of activation of the immature antigen receiving DC into a mature antigen-presenting DCs. The presentation of antigen by DCs expressing costimulation molecules and cytokines is necessary for induction of an effective and strong T-cell-specific immune response followed by long-lasting memory. VLPs were tested in vitro for their ability to stimulate human DCs, and the results showed that both papillomaviral and polyomaviral VLPs could induce a variety of phenotypic and functional changes: enhancement of the expression of major histocompatibility complex (MHC) glycoproteins, costimulation molecules (CD40, CD80, and CD86), and production of cytokines (IL-12, IL-6) (Lenz et al. 2001; Rudolf et al. 2001; Gedvilaite et al. 2006; Bickert et al. 2007).

However, PyV-derived VLPs appeared not to be consistent in DC activation. In contrast to rodent PyVs, BKPyV, JCPyV, and SV40-derived VLPs demonstrated only weak DC maturation and, correspondingly, low secretion of IL-12. The difference in DC maturation might be caused by the quality of antigen preparation, by the level of VLP uptake, or by the more extensive trigger of pattern recognition receptors inducing human DC maturation (Gedvilaite et al. 2006).

T-helper cell-independent humoral immunity is induced by the repetitive and highly ordered structure of VLPs. These repetitive epitopes enable the cross-linking of specific immunoglobulins that comprise the B-cell receptor, which is a critical signal for B-cell activation (Bachmann et al. 1995; Thyagarajan et al. 2003). Generation of neutralizing antibodies by both PVs and PyVs has been shown to be largely T-helper cell independent (Szomolanyi-Tsuda et al. 2001; Yang et al. 2005).

VLPs can be utilized for vaccination in two different ways: against capsid proteins or against foreign proteins that have been attached to VLPs. The main usage is the application of VLPs for vaccination against the virus from which they derived. Some VLP-based vaccines are already available on the market, such as prophylactic vaccines against HPVs (e.g., Gardasil, Merck; Cervarix, GlaxoSmithKline) or against hepatitis B (e.g., Engerix, GlaxoSmithKline; Recombivax HB, Merck). Hepatitis B virus (HBV) vaccines were already confirmed to be highly effective, with the induction of lifelong immunity and protection against the development of hepatocellular carcinoma (FitzSimons et al. 2005). The long-term efficacy of HPV vaccines is under investigation. This section will summarize the current knowledge of the utilization of polyoma- and papilloma-based VLPs for the development of prophylactic and therapeutic vaccines.

20.5.1 PAPILLOMAVIRUSES

HPV infection plays a key role in the etiology of cervical carcinoma, which is the fourth most frequent malignancy for women worldwide. Globally, there are approximately 530,000 new cervical carcinoma cases per year, with a mortality of 270,000 women per year. Around 85% of all new cases come from the developing countries (WHO 2014), and the majority are diagnosed in women older than 40 years. HPV infection is also connected to other carcinomas such as those in the anogenital region, head and neck cancers, and also benign genital warts in both men and women. It is estimated that HPV causes around 80% of anal carcinomas and 40%–60% of vulvar, vaginal, and penile carcinomas. Nevertheless, these malignancies are relatively rare and preferentially affect adults older than 50 years. HPV16 is the most common culprit in these tumors (WHO 2009).

HPV infection of the genital tract in both men and women is nowadays known as the most frequent sexually transmitted disease. The entry gate of infection is enabled by the microtrauma of the mucous membranes of the skin. Transmission is possible not only by sexual intercourse itself but also during noncoital sexual activities. The percentage of high-risk

HPV infected women after the initiation of sexual life rises up to 30%, reached within adolescence (under 25 years old). The prevalence then gradually decreases to 5% because the majority of infected women undergo spontaneous clearance by their immune system. Nevertheless, in some cases, persistent infections occur and induce malignant cell transformation. It is impossible to predict whether spontaneous clearance or tumor growth will occur following HPV infection. While HPV infection is an etiological cause of cervical carcinoma development, there are a number of cofactors that support dysplastic changes, such as smoking, promiscuity, hormonal contraception usage, an early start to sexual life, or illnesses connected to immunosuppression (Muñoz et al. 2006).

The HPV life cycle is restricted to the mucous membranes: no viremia occurs. HPV infects the basal cells of the cervical epithelium but does not induce cell death or inflammatory changes; therefore, activation of antigen-presenting cells is insufficient and the immune reaction is low (Stanley et al. 2006). Approximately 50% of women infected by HPV will not experience induction of the antibody response. The other 50% will be shown to evolve low antibody titers, but the numbers are not sufficient to develop protection against repeated infections. The amount of neutralizing antibodies in cervicovaginal secretions plays a vital role in the development of HPV protection. These antibodies bind the viral particles and thus block infection of the basal cells. So, the sufficient level of neutralizing antibodies in the epithelial region is necessary for antiviral protection. Vaccination induces high and stable level of antibodies in the serum. The level of serum antibodies strongly correlates with antibody level in the cervicovaginal secretions, proving the penetration of antibodies from serum to cervicovaginal secretions (Schwarz et al. 2010).

VLP-based vaccine development programs began on the basis of the discovery by several academic groups that the papillomaviral major coat protein, L1, can self-assemble into VLPs when expressed as a recombinant protein in a heterologous eukaryotic system. VLP-based vaccines against PVs induce a specific immune response (Fausch et al. 2003) and are formed from the major capsid protein, L1 (Koutsky et al. 2002), or both capsid proteins, L1 and L2 (Lenz et al. 2001). Chimeric vaccines also exist, where the structural protein L1 or L2 is fused to the whole or part of an oncoprotein, E2, E6, or E7 (Greenstone et al. 1998; Rudolf et al. 2001; Tobery et al. 2003).

20.5.1.1 First-Generation Vaccines

PV-based commercial prophylactic vaccines, after their approval by the FDA, became well known worldwide. The development of these vaccines was a long, demanding, and expensive research process that began with the immunization of rabbits, cattle, and canines with appropriate papillomaviral VLPs that protected the animals against papillomaviral infection (Breitburd et al. 1995; Suzich et al. 1995; Kirnbauer et al. 1996). The positive effect of papillomaviral VLPs on animal models led to their application on human volunteers. First, L1 VLPs from HPV18 were tested. Forty women took part in the vaccination study, ten of which received a placebo. It was demonstrated that the HPV18 L1 VLP vaccine was well tolerated and exhibited high immunogenicity (Ault et al. 2004). Then, HPV16 L1 VLPs were tested on 2000 women, some of whom received a placebo instead of the vaccine. The results showed that none of the vaccinated women were infected with HPV16 (Brown et al. 2004). Another study focused on L1 VLPs derived from HPV11 or HPV16 (Fife et al. 2004). This study also proved the high immunogenicity of papillomaviral VLPs, and every vaccinated woman developed antibodies against these VLPs. Both vaccine types were well tolerated, and the only adverse effect was a reaction in the site of injection.

Two marketed prophylactic vaccines contain VLPs, which consist of an L1 coat protein self-assembled into spheres mimicking the natural conformation of the virus. Both vaccines—bivalent (Cervarix, GSK) and quadrivalent (Gardasil, Merck)—are composed of two high-risk HPV16 and HPV18 VLPs. The quadrivalent vaccine has additional HPV6 and HPV11 VLPs that are responsible for nearly 90% of genital warts. The vaccines differ also in their production system and adjuvant composition. Gardasil vaccine is produced in yeast, and amorphous aluminum hydroxyphosphate sulfate is used as adjuvant. The bivalent vaccine Cervarix is produced in insect cells and adjuvanted with AS04, which combines aluminum hydroxide and monophosphoryl lipid-A, a modified endotoxin that is an antagonist of Toll-like receptor 4. Vaccines are administered intramuscularly, according to three dose protocols (0, 1, and 6 months for Cervarix; 0, 2, and 6 months for Gardasil), and the most common side effects are redness, fatigue, swelling, headache, and fever (Gonçalves et al. 2014). A comparison of observational studies revealed that Cervarix caused more side effects than Gardasil, probably due to the type of adjuvant system (Einstein et al. 2009). Vaccines protect against the HPV types included in the vaccination. Durable protection against the corresponding infection of HPV type has been observed for up to 9.4 years in the case of the bivalent vaccine (GlaxoSmithKline Vaccine HPV-007 Study Group et al. 2009; Lehtinen et al. 2012; Naud et al. 2014) and up to 5 years for the quadrivalent vaccine (Villa et al. 2006; The FUTURE I/II Study Group et al. 2010). The safety and efficacy of this vaccine has been confirmed for the vaccination of pregnant women (Goss et al. 2014).

The FDA licensed the Gardasil vaccine in 2006 for young females of 9–26 years old and the Cervarix vaccine for young females aged 10–25 years old. However, the vaccination could also be applied to women with evidence of prior HPV exposure. Although the therapeutic effect against anogenital cancer was not proven, these vaccines were able to prevent reinfection of HPV (Olsson et al. 2009; Miltz et al. 2014). The Gardasil vaccine was also, in 2009, approved by the FDA for the vaccination of 9–26-year-old males, and results from a phase III clinical trial proved a 90% reduction in genital lesions (Giuliano et al. 2011).

In a direct comparison, the quadrivalent vaccine produced lower levels of antibodies over 2 years than the bivalent vaccine, probably due to its adjuvant system being less

efficient (Einstein et al. 2009). Another reason could be the production of VLPs of the bivalent vaccine in the baculovirus expression system: routine purification procedures might not precisely discriminate between VLPs and baculoviruses, and as a consequence, the VLP preparations could be contaminated by baculoviral particles that are able to enhance the adjuvant properties (Abe et al. 2003). Moreover, commercial vaccines differ also in their ability to induce cross protection against other related HPV types. The bivalent vaccine enabled wider cross protection, and the obtained vaccine efficacy was about 78% for HPV31, 45% for HPV33, and 77% for HPV45 (Paavonen et al. 2009; Lu et al. 2011; Wheeler et al. 2012). The vaccine efficacy of the quadrivalent vaccine was only around 46% for HPV31, 29% for HPV33, and 8% for HPV45 (Brown et al. 2009; Westra et al. 2013), and thus significant cross protection was obtained only against HPV31. For both vaccines, no statistically significant cross protection against persistent infection with HPV52 or HPV58 was detected (Kemp et al. 2011; Malagón et al. 2012). More data are needed to establish the duration of cross protection.

20.5.1.2 Second-Generation Vaccines

Current prophylactic vaccination programs should have a significant impact on the reduction of HPV-related malignancies, owing to their ability to provide long-term protection against cervical intraepithelial neoplasia (Cadman 2008). However, the vaccines currently registered have some limitations affecting their worldwide application: the main problems include their low stability, costly production, no therapeutic effect, and limited effective cross protection to other HPV types. Since 85% of all newly diagnosed HPV-related carcinomas come from developing countries, the pressure on the development of a second-generation HPV vaccine rises.

20.5.1.2.1 Increase in Stability and Cost Efficiency

The low-cost and noninvasive biopharmaceuticals enabling widespread immunization are desired mostly in developing countries. The application of vaccines by injection might transmit other infectious diseases, generally due to repeated needle usage. The vaccines currently available must be stored in a low-temperature environment of around 6°C, and their production also requires advanced techniques and facilities. Thus, repeated vaccination is very impractical for low-resource countries. Edible (oral) vaccines have gained positive attention in this context. The principle is based on the contact between the antigen and the surface of a mucosal membrane—ideally of the gastrointestinal tract or respiratory system. These vaccines could be applied in the form of capsules, aerosols, or gel, with a precise dosage regarding the required antigen concentration. However, oral applications need significantly higher doses of antigen (approximately a hundred times) to induce an effective immune response. The antigen dose could be lowered by combination with the appropriate adjuvants (Rose et al. 1999; Gerber et al. 2001). There are two patents of edible vaccine vehicles: the first one is for the HPV16 L1 protein produced in the yeast *Schizosaccharomyces pombe* (US20090017063; 2009), which can induce a systemic immune response and production of HPV16 type-specific neutralizing and mucosal antibodies. The second one is for oncoprotein E7 of HPV16 carried by a lactic acid–based vehicle (WO2010079991; 2010), which induces an antigen-specific cellular immune response after oral administration in mice. In the last decade, the use of plants as a production system for vaccine antigen production has become more popular: plant expression systems offer a cheap, robust, and relatively fast alternative production system. The high potential of plant vaccines is in their utilization in third-world countries, but also in advanced countries where development is directed toward noninvasive applications that are more comfortable for patients. HPV VLPs self-assemble in the transgenic plant cell environment, and the robust plant cellulose membrane even protects VLPs against gastric low pH and allows their safe transport to the colon. The relatively high resistance of naked HPV VLPs to the gastrointestinal tract has been published (Rose et al. 1999). The production of an oral HPV vaccine is theoretically feasible; however, more research data are needed. On the other hand, the low-cost and simple application of these vaccines could potent their further research and help to introduce complex vaccine programs against HPV (Schiller and Nardelli-Haefliger 2006; Stanley et al. 2008).

20.5.1.2.2 Broadening of Cross Protection

Although immunization with current prophylactic vaccines is very efficient in inducing a protective antibody response against HPV6, HPV11, HPV16, and HPV18, such antibodies are predominantly type restricted. There is evidence of cross protection that could possibly be explained by phylogenetic similarities between the L1 genes of HPV types. The bivalent vaccine has shown cross protection against HPV31, HPV33, and HPV45, but the quadrivalent vaccine only against HPV31. However, the cross protective stimulation of antibody production represents <1% of the neutralizing activity induced by the dominant conformational epitopes. In order to prevent all HPV-related tumors, specific VLPs for each HPV type involved in cancer must be developed. Although it does not seem to be economically feasible, studies are ongoing to increase the number of HPV types to nine in the quadrivalent vaccines by adding the VLPs of HPV31, HPV33, HPV45, HPV52, and HPV58. This nonavalent vaccine is currently under development by Merck Research Laboratories, and a mathematical model predicts that this vaccine could raise protection from 70% to 90% of the infections responsible for invasive cervical cancer (Serrano et al. 2012). It is also possible that this vaccine will provide cross protection to other nonvaccinated HPV types, such as current commercial prophylactic vaccines. This nonavalent preventive vaccine is currently in a phase III clinical trial on women between 16 and 26 years old (National Cancer Institute 2014).

As an alternative approach to the increase in the number of various HPV L1 VLPs, the incorporation of L2 minor protein into VLPs has arisen as a possible means for cross protection improvement. In contrast to L1 protein, L2 protein is barely

visible for the immune system during infection because L2 protein is not exposed until the virus binds to the basement membrane. Therefore, the neutralizing antibodies directed against L2 are not produced. The pressure to evolve L2 neutralizing epitopes is low, therefore L2 sequences (especially between 20 and 38 aa) are highly preserved among various high-risk HPV types (Karanam et al. 2009). A study based on the vaccination of sheep with HPV6, HPV16, or HPV18 L1/L2 VLPs proved that L2 protein contains a subdominant, cross neutralizing epitope. The sheep antiserum from each HPV type provided cross protection to the other tested HPV types (Roden et al. 2000). However, the immune response induced by HPV VLPs composed of both L1 and L2 was predominantly directed against the L1 protein. This low immunogenicity of L2 might be caused by a higher amount of L1 protein compared to L2 protein, by the distant spacing of L2 protein, or by the fact that L2 protein is hidden inside the L1 capsid (Karanam et al. 2009). In many studies, the L2 protein peptide sequences were inserted into the surface loops of the L1 protein; these studies revealed the most potent cross neutralizing antibody sequences to be on the N-terminus of the L2 protein (Kondo et al. 2007, 2008; McGrath et al. 2013). Although L2 protein vaccination evoked broad-spectrum immunity, the efficiency was low. However, HPV16 L1/L2 VLP vaccination of rabbits together with Freund's adjuvant resulted in a strong increase in the level of neutralizing antibody production (Schellenbacher et al. 2009). This was consistent with the previous data, where conjugation of modified adjuvant mLTK63 to C-terminus of HPV16 L2 protein induced higher titers of HPV16-specific, long-lasting neutralizing antibodies and splenocyte proliferation (Xu et al. 2008). The data demonstrate the requirement of repeated immunizations and adjuvant utilization for high anti-L2 immune response induction.

20.5.1.2.3 Development of Therapeutic VLP Vaccine

Papillomaviral VLPs are intensively studied as an immunology tool for the production of therapeutic vaccines against HPV-related cancer, vaccines against other viral infections, or even vaccines against nonviral diseases such as Alzheimer's disease or arthritis. First, the current possibilities for therapeutic vaccine development against diseases connected to HPV infection will be discussed.

Prophylactic HPV vaccines induce a potent immune response that results in the production of high titers of neutralizing antibodies sufficient to prevent infection (Day et al. 2010; Naud et al. 2014). However, for anticancer immunotherapy, the cellular immune response is more important than antibody production. Although HPV L1 VLPs have been shown to cause a potent cellular immune response (Woo et al. 2007), there is no evidence of their therapeutic effect (Hildesheim et al. 2007; Olsson et al. 2007; Miltz et al. 2014). Therefore, E6 and E7—the major viral oncoproteins of high-risk HPVs—are intensively studied as interesting T-cell response targets.

The E6 and E7 proteins are produced in the early phase of infection and inactivate two tumor suppressor proteins, p53 and pRb. Protein p53 is inactivated by E6, which forms a stable complex with p53, and, in turn, this complex undergoes a proteolysis (Scheffner et al. 1990). The protein pRb is bound by E7 and this interaction results in the release of the transcriptional factor E2F that promotes the transcription of genes required for cell DNA synthesis and cell cycle progression (Dyson et al. 1989). The viral oncogenes, E6 and E7, are thought to modify the cell cycle so as to retain the differentiating host keratinocyte in a state that is favorable to the amplification of viral genomes and consequent late gene expression (Münger and Howley 2002). It has also been shown that the E2 protein of high-risk HPVs can participate in cell transformation because its expression allows chromosomal instability and promotes integration of viral genome, which has been documented as one of the major steps leading to HPV-induced transformation (for review see Bellanger et al. 2011).

Recently, early antigens have represented a frequent target for therapeutic intervention in vaccine development. Various vaccine designs have been studied, including DNA vaccines, protein/peptide-based vaccines, or vaccines based on chimeric VLPs. Several series of studies of DNA vaccines, encompassing naked DNA and viral or bacterial-based DNA vaccines, have been published. These vaccines can be easily prepared and manipulated, are low cost, and are stable. On the other hand, naked DNA vaccines are limited by low transfection efficiency, and live vectors possess a potential risk of toxicity and activation of immunosuppressive factors in humans (for review see Hung et al. [2008] and Cho et al. [2011]). The protein-/peptide-based vaccines are based on the direct transfer of synthesized HPV early antigens, are poorly immunogenic, and need strong adjuvants for the induction of a protective immune response (for review see Bijker et al. [2007] and Hung et al. [2008]). Therefore, the next therapeutic vaccines are based on the transport of early proteins through HPV VLPs. These VLPs could potentially induce both a prophylactic and therapeutic immune response, resulting in the prevention of reinfection and/or control of reactivation.

The combination of early and late HPV proteins in chimeric VLPs is a strategy with which to meet this goal (Jochmus et al. 1999). In chimeric VLPs, foreign epitopes could be coupled to VLPs either by fusion to the major capsid protein, L1, or by fusion to the minor capsid protein, L2 (see Section 20.4.3). In an initial study of Müller et al. (1997), various parts of an E7 protein were conjugated to the C-terminally truncated HPV16 L1 protein for chimeric HPV16 L1/E7 VLP construction. Only chimeric VLPs bearing up to 55 N-terminal aa of the E7 protein gave a high yield of uniform particles (Müller et al. 1997). These HPV16 L1ΔCE7$_{1–55}$ chimeric VLPs induced an E7-specific T-cell response in vitro (Kaufmann et al. 2001) and were used for the vaccination of women with high-grade cervical intraepithelial neoplasia (Kaufmann et al. 2007). However, the difference between the immune responders and placebo recipients in terms of histological improvement was only 14%. Other research groups used the minor protein, L2, as an anchor for the coupling

of early antigens. Chimeric HPV16 L1/L2E7 VLPs were obtained by conjugation of the full-length E7 protein to the C-terminus of the L2 protein and its coexpression with the L1 in a baculovirus expression system. The vaccination of mice with these VLPs induced protection against the outgrowth of an HPV E7–positive tumor (Greenstone et al. 1998). Later, the comparison of HPV16 L1 and L1/L2 chimeric particles conjugated with the first 57 aa of HPV16 E7 protein was described. The L1 chimeric particles induced a significantly higher E7-specific immune response than L1/L2 chimeric particles, where the E7 sequence was fused to the L2 protein (Wakabayashi et al. 2002). This is consistent with the amount of delivered epitope. Fusion to L1 could theoretically transfer 360 epitopes per particle, whereas fusion to an L2 protein would transfer only 72 epitopes. Afterward, another vaccine strategy for increasing the therapeutic potential by incorporating HPV16 E2 protein into VLPs was examined. Through the conjugation of full-length E7 and E2 in a row to the C-terminus of the L2 protein, chimeric HPV16 L1/L2E7E2 VLPs were prepared. Their morphology was similar to HPV16 L1 VLPs (Qian et al. 2006). Unfortunately, these chimeric particles were unable to induce a CTL response against E7 or E2 without immunomodulators. Moreover, the cost would be too high for their utilization as a commercial vaccine. The vaccination with chimeric particles could potentially induce both responses: the prophylactic one against the HPV16 virus and the therapeutic antitumor response against the cells transformed by HPV16. Nevertheless, if the patient is already infected or vaccinated and an immune response against the L1 protein is induced, the efficiency of this combined vaccine might be reduced by neutralizing anti-L1 antibodies (Da Silva et al. 2003).

HPV VLPs could be utilized also as a vaccination platform against other non-HPV-related diseases. The first option is the conjugation of immunodominant peptides to the capsid surface by genetic modification. Specific anti hepatitis B core antigen (HBc) antibody production was induced by the insertion of a hexameric DPASRE peptide from the HBV virus core antigen into different L1 surface loops, and only one (BC) loop failed to result in anti-HBc antibody induction in mice, suggesting that the epitope inserted into the BC loop was hidden inside the capsid (Sadeyen et al. 2003). BPV L1 protein was used as a platform for genetic incorporation of chemokine receptor type 5 (CCR5), which is the major coreceptor of the HIV. Inoculation of these chimeric L1-CCR5 VLPs into mice highly induced the production of anti-CCR5 autoantibodies, which bound the CCR5 receptor and inhibited the HIV infection in vitro (Chackerian et al. 1999). The other sequence inserted into BPV L1 was the first 9 aa of human amyloid-β protein, and stable chimeric VLPs were produced. Rabbits were chosen for the immunization because there exists a 100% identity match of the 9 aa sequence of amyloid-β between rabbits and humans. Chimeric VLPs generated specific autoantibodies against the amyloid-β peptide and thus broke the B-cell tolerance to this self-antigen (Zamora et al. 2006). Autoantibodies against amyloid-β were previously described as a method for decreasing cognitive decline in Alzheimer's disease (Hock et al. 2003), and thus the Zamora research group used model transgenic mice of human Alzheimer's disease for immunization with these chimeric VLPs. The deposits of amyloid-β in the brain decreased, and more amyloid-β was present in the plasma, suggesting its release from the brain. It seems that these chimeric VLPs could serve as an efficient immunotherapeutic for human Alzheimer's disease (Zamora et al. 2006). The second option connects the protein epitopes to the PV VLP surface by chemical conjugation. The most frequently used technique is the conjugation of a streptavidin-fused epitope to biotinylated BPV VLPs. Peptides derived from tumor necrosis factor alpha (TNF-α; the proinflammatory cytokine), amyloid-β, or CCR5 were displayed on the surface and induced specific antibody production against the exposed peptides in mice (TNF-α, amyloid-β) or in macaques (CCR5) (Chackerian et al. 2001, 2004; Li et al. 2004). The VLPs with TNF-α were even successful in decreasing collagen-induced arthritis development in mice (Chackerian et al. 2001). The third option is based on the ability of the L1 protein to encapsidate DNA during self-assembly. For DNA transfer, VLPs lacking L2 and nucleocores are not suitable, as L2 protein and the correct conformation of particles are necessary for infection and efficient delivery of DNA into the cell nucleus. Therefore, for this purpose, PsVs produced in packaging cells, containing L1, L2, and DNA complexed with histones, have been used. HPV PsVs were tested as a platform for the delivery of a DNA vaccine against respiratory syncytial virus (Graham et al. 2010) or against HIV (using the simian immunodeficiency virus model) (Gordon et al. 2012). Both vaccines were delivered intravaginally and induced specific CD8+ T-cell immune responses in mice (respiratory syncytial virus) or in macaques (HIV). The vaginal application was necessary because HPV does not infect intact epithelial cells. Furthermore, an experimental DNA vaccine encoding the ovalbumin antigen was encapsidated into HPV PsVs. After subcutaneous application, a strong ovalbumin-specific CD8+ immune response was evoked (Peng et al. 1998). All PsV-DNA vaccinations were more efficient when compared to the naked DNA vaccines. Thus, HPV PsVs are considered as a promising platform for DNA-based therapeutic vaccination.

20.5.1.3 Concluding Remarks

Vaccination is the most effective way for prevention of infectious diseases. Despite the great promise for cervical and other HPV-related cancers, there are several characteristics of current HPV vaccines that need to be improved. The vaccines are unaffordable in many parts of the world, even though the quadrivalent vaccine has been offered to GAVI Alliance that increased the access to vaccine in poor countries, incredibly only for $5 per dose. Second-generation prophylactic HPV vaccines, currently in clinical trials, may overcome several limitations of the current commercially used vaccines. First, they could mediate the protection against additional oncogenic HPV types by broadening of cross protection through incorporation of L2 or multivalent

vaccine production. Second, they should be less dependent on refrigerated conditions, needles, and low costs. The edible vaccines might solve all these deficiencies. Plants in particular are at the center of attention because they are cheap, can be orally administered, are grown easily in developing countries, and produce high amounts of recombinant protein. Third, the prophylactic together with the therapeutic effect is required. Therefore, the combination of late and early HPV proteins has been extensively studied. Early patents include chimeric vaccines containing HPV L1 with the E7 protein (US6649167, 2003; US7754430, 2010).

Unfortunately, only a few of the second-generation vaccine candidates discussed earlier are ready for clinical trials, their success is still uncertain, and the time for commercial use is very distant.

20.5.2 Polyomaviruses

In general, infection with mammalian PyVs is asymptomatic, and the vast majority of the population is seropositive. Representative examples of human PyVs that are intensively studied include BKPyV, JCPyV, and MCPyV. The BKPyV has the highest seropositivity: it increases with age and finally reaches almost 100%. Children up to 5 years of age are 63% seropositive, and children 10 years of age are nearly all seropositive (Stolt et al. 2003). BKPyV and JCPyV are present in cases of kidney transplantations, but only BKPyV is able to induce PyV-associated nephropathy, which is the most common reason for rejection of a renal transplant (Fishman 2002). JCPyV has significantly lower seropositivity: with 5-year-old children being 27% positive and adults reaching 72% seropositivity (Stolt et al. 2003). Reactivation of this virus needs an immunosuppressive state and induces a progressive multifocal leukoencephalopathy that is caused by the demyelination of neurons (Fishman 2002). The prevalence of MCPyV differs among the continents (Europe, 85% [Becker et al., 2009]; United States, 70%; and Australia, 25% [Garneski et al., 2009]) and gender, where women have a higher prevalence of MCPyV in MCC (aggressive neuroectodermal tumors) than men (Andres et al. 2010). MCPyV infects Merkel cells—cellular mechanoreceptors in skin—where the viral infection is one of the most leading factors for their malignant transformation (Andres et al. 2010). The MCCs are at least 80% positive for MCPyV presence, and the viral stimulator for tumor development could be the integration of the virus into the host cell genome (Feng et al. 2008).

Polyomaviral VLPs are highly immunogenic. They induce an antibody-mediated immune response, which prevents viral infection and also leads to a cell-mediated immune response that protects against the formation and evolution of virus-induced tumorigenesis. This immunogenic potential could be utilized in vaccine development against the native viral capsids as well as other illnesses, even of a nonviral origin. In this case, VLPs perform two functions: to carry the antigen and to serve as an adjuvant. As was described in Section 20.4.3, there are different ways in which antigens (obviously of protein origin) can be connected to the viral particle. The connection could result in the display of the antigen on the particle surface or lead to it being hidden inside the VLP core.

20.5.2.1 Native VLP Vaccines

The productive infection of PyVs is restricted to their natural host; therefore, the eligible and most widely used system for studying immunity and pathogenesis is the MPyV infection model. Heidari et al. (2002) reported that MPyV VLPs were successful against MPyV infection in the vaccination of normal and even T-cell-immunodeficient mice ($CD4^{-/-}$ $CD8^{-/-}$). After induced infection, approximately half of the mice from each group were MPyV DNA-free and protected. However, the titers of anti-VP1 antibodies were generally higher in the case of normal mice. This might be caused by a less-effective IgG switch in the absence of functional T cells (Heidari et al. 2002). In the following study, the authors tried to use different immunization protocols to improve the percentage of protected mice. The best results were obtained by subcutaneous rather than intraperitoneal application, irrespective of the presence of Freund's adjuvant (Vlastos et al. 2003). For comparison of immunogenicity, MPyV VLPs and VP1 protein fused to GST (in the form of pentamers) were administered as vaccines. While MPyV VLPs protected all mice, GST-VP1 protein protected 100% of normal but only 60% of T-cell-immunodeficient mice ($CD4^{-/-}CD8^{-/-}$) (Vlastos et al. 2003). The lower antibody production was probably caused by the disability of GST-VP1 fusion protein to assemble into VLPs. The repetitive structure of VLPs was proved to be necessary for inducing a strong T-cell-independent humoral response (Szomolanyi-Tsuda et al. 1998; Velupillai et al. 2006).

However, VLPs could be used not only for the prevention of viral infection but also for antitumor therapeutic vaccination. Peptides derived from MPyV T antigens were described as a target of the T-cell immune response. These peptides prevent tumor development and also mediate tumor rejection. They are presented by MHC molecules and called tumor-specific transplantation antigens (TSTAs) (reviewed in Ramqvist and Dalianis [2010]). As some MPyV tumors express VP1 protein, the hypothesis of using VP1-derived peptides as a TSTA was experimentally tested by Franzén et al. (2005). Vaccination with MPyV VLPs should prevent mice from outgrowth of three different MPyV tumors producing T antigens: hair follicle tumor (derived from ACA mouse strain), sarcoma and fibrosarcoma (derived from CBA mouse strain). ACA hair follicle tumor was completely rejected, while partial protection against the CBA fibrosarcoma and no protection against the CBA sarcoma outgrowth were achieved (Franzén et al. 2005). Although this experiment showed promising results for the application of VP1 VLPs as an antitumor agent, its widespread utilization is implausible, since VP1 production in MPyV- or MCPyV-induced tumors is minor (Talmage et al. 1992; Sanjuan et al. 2001; Holländerová et al. 2003; Haugg et al. 2014), is connected to episomal viral DNA presence (Talmage et al. 1992; Stubenrauch et al. 2001; Holländerová

et al. 2003), and disappears with passaging of tumor cell line (Talmage et al. 1992; Holländerová et al. 2003).

The human PyVs BK and JC need immunosuppression for reactivation; therefore, vaccine development for that limited population might not be cost effective. On the other hand, accumulating evidence of the role of MCPyV in cancer induction justifies investigation into the vaccine development. Since MCC patients have very high antibody titers against MCPyV, it has been suggested that humoral immunity alone would not prevent the disease and that a cell-mediated immune mechanism might be involved in protection against MCPyV-induced malignancy (Pastrana et al. 2009). However, immunization of healthy individuals (seropositive or seronegative for MCPyV) by MCPyV VP1 VLPs led to helper T-cell responses, which were highly antigen specific and concentration dependent. IFN-γ was the most readily detectable cytokine (Kumar et al. 2011). This interferon has antiviral and tumor-suppressive functions, and its production is associated with a favorable prognosis of MCC (Paulson et al. 2011). Nowadays, the mechanism based on transfer of MCPyV-specific T cells as a source of reactive antitumor immunity was published (Chapuis et al. 2014). The therapy was applied to a 67-year-old man with metastatic MCPyV-expressing MCC. The vaccination was well tolerated and evoked a durable complete response in two of three metastatic lesions. The transferred CD8+ T cells preferentially accumulated in the tumor tissue.

Another field where vaccine production might be economically attractive is the poultry industry. One of the offending diseases is hemorrhagic nephritis and enteritis of geese, which is characterized by high morbidity and mortality in geese between 3 and 10 weeks of age. This illness is caused by the GHPyV. VLPs of this PyV were successfully produced in yeast or insect cells (Zielonka et al. 2006), and VLPs from insect cells were used for the vaccination of goslings. VLPs provided protection to goslings after their vaccination at 1 day of age and boosting after 17 days, and surprisingly even without boosting. The efficacy was not influenced by the dose of the antigen (Mató et al. 2009).

20.5.2.2 Chimeric VLPs as Vaccines against Foreign Epitopes

The ability to induce an immune response against either a self-antigen or an antigen of a pathogen is very desirable during therapeutic and prophylactic vaccine development. Therefore, effort was concentrated on the development of various agents that would exhibit this effect and combine it with other important features such as minimal side effects, safety for the organism, low-cost production, and relatively easy preparation and administration. VLPs meet all of the aforementioned requirements, and after their modification, they can be used as vectors for a multimeric presentation of foreign antigens in vaccine technology. One of the essential tasks is the development of an efficient tumor treatment, which is complicated by the very individual and heterogenic tumor character. The main goal of nonviral tumor immunotherapy is the induction of immune responses to autoantigens and the breaking of an established tumor tolerance. Utilization of VLPs in this context should have a great future; however, there are still only a limited number of studies investigating this VLP potential.

20.5.2.2.1 Epitopes Displayed on the Surface of Polyomavirus-Based VLPs

In case of PyV-based VLPs, epitopes can be inserted into one of the surface loops of the major structural protein, VP1, generating VLPs with foreign epitopes exposed on the surface. This approach is limited by the inserted peptide length, as the longer peptides are able to influence the assembly and stability of VLPs.

As an alternative to the commercial HBV vaccine, chimeric VLPs carrying a hydrophilic component of the pre-S1 sequence of HBV (75 aa) in the HI loop of MPyV VP1 protein were constructed (Skrastina et al. 2008). The pre-S1 sequence is directly responsible for the binding of HBV to human hepatocytes and is not present on the 22 nm particles currently used for vaccination against HBV. The delivery of this sequence on the surface of chimeric VLPs is of the highest interest for multitarget HBV vaccine development. Chimeric VLPs were subcutaneously applied to mice and led to an induction of a strong antibody response against the inserted epitope, as well as to stimulation of IL-12 and IFN-γ production. Interestingly, the insertion of the epitope lowered the humoral response to MPyV VP1 protein (Skrastina et al. 2008). This phenomenon was not achieved in the previous study, where S1 epitopes derived from pre-S1 sequence were inserted into various loops of HaPyV VP1 protein. Assembled chimeric VLPs induced a strong anti-VP1 and anti-S1 antibody response in mice (Gedvilaite et al. 2000). As an alternative approach for influenza A vaccine production, VLPs derived from SV40 were tested. Chimeric SV40 VLPs were constructed by insertion of HLA-A*02:01 restricted CTL epitope corresponding to the influenza A virus matrix protein peptide 58–66 (FMP 58–66) into the DE or HI loop (Kawano et al. 2014). These chimeric VLPs effectively induced influenza-specific cytotoxic T cells and heterosubtypic protection against influenza A viruses without the need for an adjuvant.

HaPyV VLPs as a source for chimeric VLP production became the center of attention of the scientific group of R. G. Ulrich (Humboldt University, Berlin, Germany) and K. Sasnauskas (Institute of Biotechnology, Vilnius, Lithuania). Based on the crystal structure of SV40 VP1 protein (Chen et al. 1998), they predicted suitable insertion sites in HaPyV VP1 protein. First, a peptide (5 aa) derived from the S1 protein of HBV was inserted into four different regions of HaPyV. The positions corresponded to loops BC, EF, FG, and HI and were identified using numbers from one to four (Gedvilaite et al. 2000). In two additional constructs, the same peptide was inserted into two sites (positions 1 and 2 or 1 and 3). All recombinant VP1 proteins could self-assemble into chimeric VLPs and, together with Freund's complete adjuvant, were intraperitoneally administered to mice. The ability of chimeric VLPs to evoke specific antibodies (mainly IgG) against the S1-derived peptide was dependent on the place of insertion. The highest titers were obtained in position 1 (BC loop), and as expected, the

combination of this site with another site for epitope presentation induced a stronger antigen-specific antibody response. Additionally, splenocytes derived from immunized animals exhibited the increased production of IL-12 and IFN-γ (Gedvilaite et al. 2000). In the next study, the same group inserted a T-cell-recognized epitope derived from carcinoembryonic antigen (CEA) to positions 1 or 4 or 1 and 4 with or without the flanking linker (double glycine-serine [GS] on both sites of the insert). Furthermore, a construct with insertion into all four positions with flanking linkers was prepared. Surprisingly, all recombinant proteins were able to form chimeric VLPs irrespective of the flanking linker presence, except that with insertions in all four sites, which did not assemble into VLPs. The highest antibody response was induced by chimeric VLPs with an inserted epitope in position 1, without any influence of linker presence on immunogenicity. The CEA-specific antibodies were detectable even 6 months after immunization (Lawatscheck et al. 2007). Subsequently, the insertion of a mucin-1 (MUC-1) CTL epitope into position 1 and/or 4, with or without glycine–serine linker, or again into all four sites with linkers, was tested (Zvirbliene et al. 2006; Dorn et al. 2008). In agreement with the previous study, only the VP1 with all four insertions was unable to self-assemble into VLPs. The chimeric VLPs carrying the MUC-1 with GS linkers in sites 1 and 4 were the most potent for specific anti-MUC-1 antibody production. These particles were also able to maturate human DCs and evoke a specific CTL response in vitro (Dorn et al. 2008). In another study of CTL response, chimeric HaPyV VLPs with a GP33 CTL epitope derived from lymphocytic choriomeningitis virus (LCMV) (Pircher et al. 1990) incorporated into the BC or HI loop induced protective memory and a CTL response (Mazeike et al. 2012). T-cell proliferation was induced both in vitro and in vivo without adjuvant usage. After intravenous immunization of mice, 70% of them were fully and 30% were partially protected from LCMV infection.

Similar studies were performed with 45 aa, 80 aa, or 120 aa long segments from the N-terminal part of the nucleocapsid protein (NP) from the Puumala hantavirus (PUUV). These segments were inserted into all four positions (80 aa peptide only into positions 1 and 4) of HaPyV VP1 protein. Only positions 1 and 4 were able to tolerate insertion of the long foreign peptides without affecting VLP assembly (Gedvilaite et al. 2004). These chimeric VLPs were injected to BALB/c mice and, without any adjuvant, generated high titers of IgG antibodies against PUUV NP and stimulation of IL-2 and IFN-γ secretion. However, adjuvant usage induced a 10-fold higher antibody production. Both humoral and cellular immune responses were observed. The strongest immune response was observed after immunization with the longest 120 aa segment. Insertion of PUUV NP segments also reduced antigenicity of the HaPyV VP1 protein. The level of reduction was dependent on the size of the inserted protein; with an increased size of the inserted protein, the antigenicity of the VP1 protein decreased. These results were confirmed by a following study with chimeric HaPyV VLPs carrying a 120 aa PUUV NP segment, which promoted the generation of five antigen-specific antibodies of IgG isotypes but no VP1-specific antibodies (Zvirbliene et al. 2006).

Further effort was directed toward the production of a low-cost vaccine against group A streptococcus (GAS), which causes severe infections in low-income nations. A highly conserved 20 aa peptide (p145) from the M protein of GAS has been shown to generate specific human antibodies that are able to opsonize multiple strains of GAS (Pruksakorn et al. 1994). Later, the minimal protective epitope within the p145 protein was defined precisely as 12 aa epitope called J8 (Hayman et al. 1997). In order to recognize a small difference in the peptide sequence, a strategy published by Relf et al. (1996), based on the insertion of p145 peptides into other peptides known to form α-helix, was applied. The flanking peptides for driving the constitution of a helical structure were taken from the GCN4 protein, a DNA-binding protein in yeast. The resulting 28 aa long peptide was constructed with GCN4-derived linkers (6 aa) and a J8 epitope in the middle (Hayman et al. 1997). This epitope GCN4-J8i-GCN4 was inserted into the HI loop of MPyV VP1 protein, and stable chimeric particles were produced. The subcutaneous delivery of these particles into mice induced high titers of J8i-specific antibodies with a bactericidal effect (Middelberg et al. 2011). Afterward, the efficacy of chimeric VLPs carrying two copies of the J8i antigenic element was examined and compared to those carrying only a single copy of J8i. IgG isotypes induced by both chimeric VLPs were similar, indicating a mixed T-helper cell response (Chuan et al. 2013; Rivera-Hernandez et al. 2013). Chuan et al. (2013) also showed that the chimeric VLPs displaying J8i successfully induced high titers of J8i-specific antibodies, even in mice that were previously immunized with chimeric MPyV VLPs and exhibited high anti-VP1 antibody titers.

In most studies the vaccines were administered subcutaneously or intraperitoneally. The chimeric VLPs previously mentioned, carrying J8i peptide, were also delivered intranasally without an adjuvant. This administration induced both IgG and antigen-specific mucosal IgA antibodies. Vaccinated mice showed improved survival; however, the statistically significant level of protection was two times lower than the positive control, which was vaccinated with GCN4-J8i-GCN4 conjugated to diphtheria toxoid (Rivera-Hernandez et al. 2013).

20.5.2.2.2 Epitopes Hidden Inside Polyomavirus-Based VLPs

Internalization of foreign sequences inside VLPs can be achieved by conjugation to VLPs through the major protein, VP1, or minor proteins VP2 or VP3. This approach enables longer peptide insertions, which is usually impossible in the case of surface-exposed loops. For vaccine development, the epitopes buried within the capsid core might induce an inefficient immune response. For the induction of a CTL response, however, it was shown that the display of epitopes on the capsid surface is not necessary. The immunodominant CD8+ T-cell epitope derived from ovalbumin was fused to the C-terminal part of the VP1 protein. Chimeric VLPs induced CD8+ and CD4+ T cells specific for the ovalbumin

epitope (Bickert et al. 2007) and were able to protect mice from ovalbumin-expressing tumors (Brinkman et al. 2004). Moreover, mice vaccinated at 4 and 11 days after the melanoma tumor challenge were also protected against tumor outgrowth (Brinkman et al. 2005).

Vaccination by VLPs with antigen internalized inside the particle against tumors of nonviral origin is well described for the proto-oncogene HER2/neu model. This proto-oncogene is frequently overexpressed in breast, lung, ovarian, gastric, and pancreatic cancer. Transmembrane and extracellular domains of human HER2/neu protein were fused to the MPyV minor protein VP2, and chimeric VPLs ($HER2_{1–683}$PyVLPs) were produced by coexpression with MPyV VP1 protein in a baculovirus expression system. Two different in vivo models were used for vaccination experiments with these particles for testing the rejection of, and protection against, HER2/neu tumors. Protection was assessed in both models after a single vaccination. In the group of mice transfected with human HER2/neu, the vaccination protected mice and rejected their tumors, while in the group of BALB-neuT mice, developing spontaneous neuT-induced tumors in mammary glands, only protection against tumor growth was induced. Both models failed in the induction of HER2-specific antibodies. The protection elicited by these chimeric VLPs was provided by the cellular immune response. The presence of HER2-specific T cells was demonstrated using an enzyme-linked immunospot assay (Tegerstedt et al. 2005b). Similar results were also obtained for MPtV chimeric VLPs (Andreasson et al. 2009). The following study of chimeric MPtV VLPs revealed the stimulation of both CD4+ and CD8+ T-lymphocytes. According to the obtained results, CD4+ and CD8+ T-lymphocytes could act independently, in part, during tumor rejection after vaccination. In combination with CpG oligonucleotides, these chimeric particles induced a long-lasting immunologic memory persisting for at least 10 weeks (Andreasson et al. 2010).

The efficiency of T-cell stimulation could also be enhanced by vaccination with DCs loaded with VLPs in vitro. For the rejection of HER2/neu tumors, a dose of chimeric VP1/VP2-HER2 MPyV VLPs that was 10 times lower was sufficient, when the VLPs were not used directly but loaded onto DCs prior to administration. The vaccine efficiency was preserved, and 100% of treated animals were protected after a single immunization with DCs loaded with chimeric VP1/VP2-HER2 MPyV (Tegerstedt et al. 2005b). The same approach was useful during the vaccination against prostate cancer. Chimeric MPyV VLPs containing PSA fused to the minor structural VP2 protein protected the mice only marginally. However, vaccination with DCs loaded with VP1/VP2-PSA MPyV VLPs in the presence of CpG oligonucleotides induced the protection of mice against the outgrowth of a PSA-expressing tumor. The production of anti-VP1 antibodies was eight times lower compared to vaccination with VP1/VP2-PSA MPyV VLPs alone (Eriksson et al. 2011).

Model antigens for immunization were also conjugated to the minor protein, VP3. The C-terminal sequence (49 aa) of MPyV minor protein VP3 was used for the coupling of enhanced GFP protein. After intranasal administration, both VP1 and EGFP induced proliferation of specific T-helper cells and production of IL-12 and IFN-γ (Bouřa et al. 2005; Frič et al. 2008).

20.5.2.3 Concluding Remarks

The main advantages of PyV VLP–based vaccines can be summarized as follows: first, they are very tolerant to peptide insertions into the VP1 surface loops that generally cause the major problems of generating chimeric VLPs. Polyomaviral VLPs are capable of long peptide conjugations, as was proved by the fusion of the entire enzyme dihydrofolate reductase (Gleiter et al. 1999) or incorporation of a 120 aa long peptide from PUUV nucleoprotein (Gedvilaite et al. 2004). Second, they are highly immunogenic and stimulate the maturation of DCs irrespective of adjuvant presence. Third, they are very stable. They can tolerate relatively high temperatures (up to 70°C) or various pH levels (Nims and Plavsic 2013). The possibility of long-term storage without affecting the immunogenicity of VLPs was also confirmed (Caparrós-Wanderley et al. 2004). No reduced induction of immune responses in intranasally immunized mice was observed after storage of VLPs for 9 weeks at room temperature.

20.5.3 Conclusion

The success of prophylactic HPV vaccines has increased interest in artificial viral particles and has also led to an intensive development of new vaccine applications. Nowadays, a number of VLP-using approaches are being tested not only for vaccination purposes but also for the immunotherapeutic treatment of cancer (Kimchi-Sarfaty and Gottesman 2004; Tegerstedt et al. 2005), rheumatoid arthritis (Chackerian et al. 2001), or even smoking addiction (Maurer et al. 2005). The investigation of VLPs as carriers for protein and DNA delivery was also heightened. The immune response to a transgene carried by PsVs is stronger than the response to transgenes applied to the organism as naked DNA (Clark et al. 2001). Animal VLPs are superior for this kind of application compared to their human counterparts, which might inhibit the efficiency of the vector due to their preexisting immunity in the organism. The high immunogenicity of VLPs could also represent a problem for repeated applications; therefore, modifications of VLPs will be necessary to narrow the range of their target cells or lower VLP recognition by the immune system (Heidari et al. 2000). Recent results have shown that through the fusion of long epitopes, the immune response to the capsid protein is significantly decreased (Zvirbliene et al. 2006), suggesting the feasibility of such modifications. Although a great deal of data has been published, the generation of chimeric VLPs is largely empirical, and nowadays, it is almost impossible to predict whether the modifications will affect the assembly or whether the inserted epitopes will be immunogenic. Solving this uncertainty in the preparation of the desired VLPs will be a great challenge for the future.

ACKNOWLEDGMENTS

This work was supported by the Grant Agency of Charles University (Project GAUK/913613 (Jiřina Suchanová and Hana Španielová); the Grant Agency of the Czech Republic (Project P302/13-26115S) (Jitka Forstová and Hana Španielová); the Ministry of Education, Youth and Sports of the Czech Republic (Project SVV-2014-260081); and Charles University in Prague (Project UNCE 204013).

LIST OF ABBREVIATIONS

aa	amino acid
APyVs	avian polyomaviruses
AuNPs	gold nanoparticles
BFPyV	budgerigar fledgling disease virus, budgerigar fledgling polyomavirus
BKPyV	BK polyomavirus
BPV	bovine papillomavirus
CCR5	chemokine receptor type 5
CEA	carcinoembryonic antigen
CF	cystic fibrosis
CFDA SE	carboxyfluorescein diacetate succinimidyl ester
CFTR	cystic fibrosis transmembrane conductance regulator
ChPyV	chimpanzee polyomavirus
COPV	canine oral papillomavirus
CPyV	crow polyomavirus
CRPV	cottontail rabbit papillomavirus
CTL	cytotoxic T-lymphocyte
DC	dendritic cell
DTT	dithiothreitol
E. coli	*Escherichia coli*
E8C	polyglutamic acid–cysteine
EEs	early endosomes
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
EGFR	epidermal growth factor receptor
ER	endoplasmic reticulum
FDA	Food and Drug Administration
FLAG	octapeptide Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys
FPyV	finch polyomavirus
GAS	group A streptococcus
GFP	green fluorescent protein
GHPyV	goose hemorrhagic polyomavirus
GST	glutathione S-transferase
HaPyV	hamster polyomavirus
HBV	hepatitis B virus
HER2	human epidermal growth factor receptor 2
HPV	human papillomavirus
HPyV	human polyomavirus
HS	heparan sulfate
HSPGs	heparan sulfate proteoglycans
JCPyV	JC polyomavirus
LCMV	lymphocytic choriomeningitis virus
LEs	late endosomes
LPyV	simian B-lymphotropic polyomavirus
LSTc	lactoseries tetrasaccharide c
MCC	Merkel cell carcinoma
MCPyV	Merkel cell polyomavirus
MDR1	multidrug resistance 1
ME	2-mercaptoethanol
MHC	major histocompatibility complex
MNPs	magnetic nanoparticles
MPtV	murine pneumotropic virus
MPyV	murine polyomavirus
MRI	magnetic resonance imaging
MUC-1	mucin 1
MusPV	mouse papillomavirus
Neu5Ac	5-*N*-acetyl neuraminic acid, sialic acid
PLP	pseudovirion-like particle
PNA	peptide nucleic acid
PSA	prostate-specific antigen
PsV	pseudovirion
PUUV	Puumala hantavirus
PV	papillomavirus
PyV	polyomavirus
QD	quantum dot
QV	quasivirion
QVLP	quasivirion-like particle
R8C	polyarginine cysteine
RGD	tripeptide Arg-Gly-Asp
rSV40	recombinant SV40 vector
Sf9	cell line from *Spodoptera frugiperda*
shRNA	short hairpin RNA
siRNAs	small interfering RNAs
SV40	simian virus 40
T antigen	tumorigenic antigen
TGN	*trans*-Golgi network
TK	thymidine kinase
TNF-α	tumor necrosis factor alpha
TSPyV	Trichodysplasia spinulosa–associated polyomavirus
TSTA	tumor-specific transplantation antigen
uPAR	urokinase-type plasminogen activator receptor
VLP	virus-like particle
VNP	viral nanoparticle

REFERENCES

Abban, C.Y. and P.I. Meneses. 2010. Usage of heparan sulfate, integrins, and FAK in HPV16 infection. *Virology* 403(1) (July 20): 1–16.

Abbing, A., U.K. Blaschke, S. Grein, M. Kretschmar, C.M.B. Stark, M.J.W. Thies, J. Walter et al. 2004. Efficient intracellular delivery of a protein and a low molecular weight substance via recombinant polyomavirus-like particles. *The Journal of Biological Chemistry* 279(26) (June 25): 27410–27421.

Abe, T., H. Takahashi, H. Hamazaki, N. Miyano-Kurosaki, Y. Matsuura, and H. Takaku. 2003. Baculovirus induces an innate immune response and confers protection from lethal influenza virus infection in mice. *The Journal of Immunology* 171(3) (August 1): 1133–1139.

Abend, J.R., M. Jiang, and M.J. Imperiale. 2009. BK virus and human cancer: Innocent until proven guilty. *Seminars in Cancer Biology* 19(4) The Polyomaviruses (August): 252–260.

Aires, K.A., A.M. Cianciarullo, S.M. Carneiro, L.L. Villa, E. Boccardo, G. Pérez-Martinez, I. Perez-Arellano, M.L.S. Oliveira, and P.L. Ho. 2006. Production of human papillomavirus type 16 L1 virus-like particles by recombinant *Lactobacillus casei* cells. *Applied and Environmental Microbiology* 72(1) (January 1): 745–752.

An, K., S.A. Smiley, E.T. Gillock, W.M. Reeves, and R.A. Consigli. 1999. Avian polyomavirus major capsid protein VP1 interacts with the minor capsid proteins and is transported into the cell nucleus but does not assemble into capsid-like particles when expressed in the baculovirus system. *Virus Research* 64(2) (November): 173–185.

Anderer, F.A., H.D. Schlumberger, M.A. Koch, H. Frank, and H.J. Eggers. 1967. Structure of simian virus 40 II. Symmetry and components of the virus particle. *Virology* 32(3): 511–523.

Andreasson, K., M. Eriksson, K. Tegerstedt, T. Ramqvist, and T. Dalianis. 2010. CD4+ and CD8+ T cells can act separately in tumour rejection after immunization with murine pneumotropic virus Chimeric Her2/neu virus-like particles. ed. A. Gregson. *PLoS ONE* 5(7) (July 19): e11580.

Andreasson, K., K. Tegerstedt, M. Eriksson, C. Curcio, F. Cavallo, G. Forni, T. Dalianis, and T. Ramqvist. 2009. Murine pneumotropic virus chimeric Her2/*neu* virus-like particles as prophylactic and therapeutic vaccines against Her2/*neu* expressing tumors. *International Journal of Cancer* 124(1) (January 1): 150–156.

Andres, C., B. Belloni, U. Puchta, C.A. Sander, and M.J. Flaig. 2010. Prevalence of MCPyV in merkel cell carcinoma and non-mcc tumors. *Journal of Cutaneous Pathology* 37(1) (January): 28–34.

Angeletti, P.C. 2005. Replication and encapsidation of papillomaviruses in *Saccharomyces cerevisiae*. *Methods in Molecular Medicine* 119: 247–260.

Angeletti, P.C., K. Kim, F.J. Fernandes, and P.F. Lambert. 2002. Stable replication of papillomavirus genomes in *Saccharomyces cerevisiae*. *Journal of Virology* 76(7) (April): 3350–3358.

Antonsson, A. and B.G. Hansson. 2002. Healthy skin of many animal species harbors papillomaviruses which are closely related to their human counterparts. *Journal of Virology* 76(24) (December 15): 12537–12542.

Aposhian, H.V., R.E. Thayer, and P.K. Qasba. 1975. Formation of nucleoprotein complexes between polyoma empty capsides and DNA. *Journal of Virology* 15(3): 645–653.

Arad, U., E. Zeira, M.A. El-Latif, S. Mukherjee, L. Mitchell, O. Pappo, E. Galun, and A. Oppenheim. 2005. Liver-targeted gene therapy by SV40-based vectors using the hydrodynamic injection method. *Human Gene Therapy* 16(3) (March): 361–371.

Ashok, A. and W.J. Atwood. 2003. Contrasting roles of endosomal pH and the cytoskeleton in infection of human glial cells by JC virus and simian virus 40. *Journal of Virology* 77(2) (January): 1347–1356.

Assetta, B., M.S. Maginnis, I. Gracia Ahufinger, S.A. Haley, G.V. Gee, C.D.S. Nelson, B.A. O'Hara, S.A. Allen Ramdial, and W.J. Atwood. 2013. 5-HT2 receptors facilitate JC polyomavirus entry. *Journal of Virology* 87(24) (December): 13490–13498.

Ault, K.A., A.R. Giuliano, R.P. Edwards, G. Tamms, L.-L. Kim, J.F. Smith, K.U. Jansen et al. 2004. A Phase I study to evaluate a human papillomavirus (HPV) type 18 L1 VLP vaccine. *Vaccine* 22(23–24) (August): 3004–3007.

Aydin, I., S. Weber, B. Snijder, P. Samperio Ventayol, A. Kuhbacher, M. Becker, P.M. Day et al. 2014. Large scale RNAi reveals the requirement of nuclear envelope breakdown for nuclear import of human papillomaviruses. *PLoS Pathogens* 10(5) (May 29): e1004162. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4038628/. Accessed August 10, 2014.

Bachmann, M.F., H. Hengartner, and R.M. Zinkernagel. 1995. T Helper cell-independent neutralizing B cell response against vesicular stomatitis virus: Role of antigen patterns in B cell induction? *European Journal of Immunology* 25(12) (December 1): 3445–3451.

Baek, J.O., J.W. Seo, O. Kwon, S.M. Park, C.H. Kim, and I.H. Kim. 2012. Production of human papillomavirus type 33 L1 major capsid protein and virus-like particles from *Bacillus subtilis* to develop a prophylactic vaccine against cervical cancer. *Enzyme and Microbial Technology* 50(3) (March 10): 173–180.

Barr, S.M., K. Keck, and H.V. Aposhian. 1979. Cell-free assembly of a polyoma-like particle from empty capsids and DNA. *Virology* 96(2) (July 30): 656–659.

Bauer, P.H., R.T. Bronson, S.C. Fung, R. Freund, T. Stehle, S.C. Harrison, and T.L. Benjamin. 1995. Genetic and structural analysis of a virulence determinant in polyomavirus VP1. *Journal of Virology* 69(12) (January 12): 7925–7931.

Bazan, S.B., A. de A.M. Chaves, K.A. Aires, A.M. Cianciarullo, R.L. Garcea, and P.L. Ho. 2009. Expression and characterization of HPV-16 L1 capsid protein in *Pichia pastoris*. *Archives of Virology* 154(10) (October 1): 1609–1617.

Becker, J.C., R. Houben, S. Ugurel, U. Trefzer, C. Pföhler, and D. Schrama. 2009. MC polyomavirus is frequently present in merkel cell carcinoma of European patients. *The Journal of Investigative Dermatology* 129(1) (January): 248–250.

Bellanger, S., C.L. Tan, Y.Z. Xue, S. Teissier, and F. Thierry. 2011. Tumor suppressor or oncogene? A critical role of the human papillomavirus (HPV) E2 protein in cervical cancer progression. *American Journal of Cancer Research* 1(3): 373.

Belnap, D.M., N.H. Olson, N.M. Cladel, W.W. Newcomb, J.C. Brown, J.W. Kreider, N.D. Christensen, and T.S. Baker. 1996. Conserved features in papillomavirus and polyomavirus capsids. *Journal of Molecular Biology* 259(2): 249–263.

Benson, D.A., I. Karsch-Mizrachi, D.J. Lipman, J. Ostell, and E.W. Sayers. 2009. GenBank. *Nucleic Acids Research* 37(suppl 1) (January 1): D26–D31.

Bergant, M. and L. Banks. 2013. SNX17 facilitates infection with diverse papillomavirus types. *Journal of Virology* 87(2) (January): 1270–1273.

Bergant Marušič, M., M.A. Ozbun, S.K. Campos, M.P. Myers, and L. Banks. 2012. Human papillomavirus L2 facilitates viral escape from late endosomes via sorting nexin 17. *Traffic* (Copenhagen, Denmark) 13(3) (March): 455–467.

Bergsdorf, C., C. Beyer, V. Umansky, M. Werr, and M. Sapp. 2003. Highly efficient transport of carboxyfluorescein diacetate succinimidyl ester into COS7 cells using human papillomavirus-like particles. *FEBS Letters* 536(1–3) (February): 120–124.

Bickert, T., G. Wohlleben, M. Brinkman, C.M. Trujillo-Vargas, C. Ruehland, C.O.A. Reiser, J. Hess, and K.J. Erb. 2007. Murine polyomavirus-like particles induce maturation of bone marrow-derived dendritic cells and proliferation of T cells. *Medical Microbiology and Immunology* 196(1) (March): 31–39.

Biemelt, S., U. Sonnewald, P. Galmbacher, L. Willmitzer, and M. Müller. 2003. Production of human papillomavirus Type 16 virus-like particles in transgenic plants. *Journal of Virology* 77(17) (January 9): 9211–9220.

Bienkowska-Haba, M., H.D. Patel, and M. Sapp. 2009. Target cell cyclophilins facilitate human papillomavirus Type 16 infection. *PLoS Pathogens* 5(7) (July):e1000524. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2709439/. Accessed August 4, 2014.

Bienkowska-Haba, M., C. Williams, S.M. Kim, R.L. Garcea, and M. Sapp. 2012. Cyclophilins facilitate dissociation of the human papillomavirus type 16 capsid protein L1 from the L2/DNA complex following virus entry. *Journal of Virology* 86(18) (September): 9875–9887.

Bijker, M.S., C.J.M. Melief, R. Offringa, and S.H. van der Burg. 2007. Design and development of synthetic peptide vaccines: Past, present and future. *Expert Review of Vaccines* 6(4) (August): 591–603.

Bishop, B., J. Dasgupta, M. Klein, R.L. Garcea, N.D. Christensen, R. Zhao, and X.S. Chen. 2007. Crystal structures of four types of human papillomavirus L1 capsid proteins: Understanding the specificity of neutralizing monoclonal antibodies. *The Journal of Biological Chemistry* 282(43) (September 6): 31803–31811.

Bishop, C.L., M. Ramalho, N. Nadkarni, W. May Kong, C.F. Higgins, and N. Krauzewicz. 2006. Role for centromeric heterochromatin and PML nuclear bodies in the cellular response to foreign DNA. *Molecular and Cellular Biology* 26(7) (April): 2583–2594.

Bouřa, E., D. Liebl, R. Špíšek, J. Frič, M. Marek, J. Štokrová, V. Holáň, and J. Forstová. 2005. Polyomavirus EGFP-pseudocapsids: Analysis of model particles for introduction of proteins and peptides into mammalian cells. *FEBS Letters* 579(29) (December): 6549–6558.

Bousarghin, L., A. Touzé, G. Gaud, S. Iochmann, E. Alvarez, P. Reverdiau, J. Gaitan, M.-L. Jourdan, P.-Y. Sizaret, and P.L. Coursaget. 2009. Inhibition of cervical cancer cell growth by human papillomavirus virus-like particles packaged with human papillomavirus oncoprotein short hairpin RNAs. *Molecular Cancer Therapeutics* 8(2) (January 2): 357–365.

Bousarghin, L., A. Touzé, P.-Y. Sizaret, and P. Coursaget. 2003. Human papillomavirus types 16, 31, and 58 use different endocytosis pathways to enter cells. *Journal of Virology* 77(6) (March): 3846–3850.

Bouvard, V., R. Baan, K. Straif, Y. Grosse, B. Secretan, F.E. Ghissassi, L. Benbrahim-Tallaa, N. Guha, C. Freeman, and L. Galichet. 2009. A review of human carcinogens—Part B: Biological agents. *The Lancet Oncology* 10(4): 321–322.

Brady, J.N., J.D. Kendall, and R.A. Consigli. 1979. In vitro reassembly of infectious polyoma virions. *Journal of Virology* 32(2) (January 11): 640–647.

Brady, J.N., V.D. Winston, and R.A. Consigli. 1977. Dissociation of polyoma virus by the chelation of calcium ions found associated with purified virions. *Journal of Virology* 23(3) (January 9): 717–724.

Braun, H., K. Boller, J. Löwer, W.M. Bertling, and A. Zimmer. 1999. Oligonucleotide and plasmid DNA packaging into polyoma VP1 virus-like particles expressed in *Escherichia coli*. *Biotechnology and Applied Biochemistry* 29 (Pt 1) (February): 31–43.

Breau, W.C., W.J. Atwood, and L.C. Norkin. 1992. Class I major histocompatibility proteins are an essential component of the simian virus 40 receptor. *Journal of Virology* 66(4) (April): 2037–2045.

Breitburd, F., R. Kirnbauer, N.L. Hubbert, B. Nonnenmacher, C. Trin-Dinh-Desmarquet, G. Orth, J.T. Schiller, and D.R. Lowy. 1995. Immunization with viruslike particles from cottontail rabbit papillomavirus (CRPV) can protect against experimental CRPV infection. *Journal of Virology* 69(6): 3959–3963.

Brinkman, M., J. Walter, S. Grein, M.J.W. Thies, T.W. Schulz, M. Herrmann, C.O.A. Reiser, and J. Hess. 2005. Beneficial therapeutic effects with different particulate structures of murine polyomavirus VP1-coat protein carrying self or non-self CD8 T cell epitopes against murine melanoma. *Cancer Immunology, Immunotherapy* 54(6) (February 1): 611–622.

Brinkman, M., J. Walter, I. Jennes, M. Neugebauer, W.M. Bertling, S. Grein, M.J.W. Thies, M. Weigand, T. Beyer, and M. Herrmann. 2004. Recombinant murine polyoma virus-like-particles induce protective antitumour immunity. *Letters in Drug Design & Discovery* 1(2): 137–147.

Brown, D.R., K.H. Fife, C.M. Wheeler, L.A. Koutsky, L.M. Lupinacci, R. Railkar, G. Suhr et al. 2004. Early assessment of the efficacy of a human papillomavirus type 16 L1 virus-like particle vaccine. *Vaccine* 22(21–22) (July 29): 2936–2942.

Brown, D.R., S.K. Kjaer, K. Sigurdsson, O. Iversen, M. Hernandez- Avila, C.M. Wheeler, G. Perez et al. 2009. The impact of quadrivalent human papillomavirus (HPV; Types 6, 11, 16, and 18) L1 virus- like particle vaccine on infection and disease due to oncogenic nonvaccine HPV types in generally HPV- naive women aged 16–26 years. *The Journal of Infectious Diseases* 199(7) (April): 926–935.

Buck, C.B. 2012. Protocol for harvesting pseudovirus producer cells. http://home.ccr.cancer.gov/lco/pseudovirusproduction.htm. Accessed October 25, 2013.

Buck, C.B., N. Cheng, C.D. Thompson, D.R. Lowy, A.C. Steven, J.T. Schiller, and B.L. Trus. 2008. Arrangement of L2 within the papillomavirus capsid. *Journal of Virology* 82(11) (January 6): 5190–5197.

Buck, C.B., D.V. Pastrana, D.R. Lowy, and J.T. Schiller. 2004. Efficient intracellular assembly of papillomaviral vectors. *Journal of Virology* 78(2) (January): 751–757.

Buck, C.B., D.V. Pastrana, D.R. Lowy, and J.T. Schiller. 2005a. Generation of HPV pseudovirions using transfection and their use in neutralization assays. *Methods in Molecular Medicine* 119: 445–462.

Buck, C.B. and C.D. Thompson. 2007. Production of papillomavirus-based gene transfer vectors. *Current Protocols in Cell Biology*, Eds. J. S. Bonifacino, M. Dasso, J. B. Harford, J. Lippincott-Schwartz, and K. M. Yamada (Bethesda, MD) Chapter 26 (December): Unit 26.1.

Buck, C.B., C.D. Thompson, Y.-Y.S. Pang, D.R. Lowy, and J.T. Schiller. 2005b. Maturation of papillomavirus capsids. *Journal of Virology* 79(5) (March): 2839–2846.

Buck, C.B. and B.L. Trus. 2012. The papillomavirus virion: A machine built to hide molecular achilles' heels. In *Viral Molecular Machines*, ed. M.G. Rossmann and V.B. Rao, pp. 403–422. Advances in Experimental Medicine and Biology, Vol. 726. New York: Springer. http://link.springer.com/chapter/10.1007/978-1-4614-0980-9_18. Accessed January 2, 2014.

Buonamassa, D.T., C.E. Greer, S. Capo, T.S. BenedictYen, C.L. Galeotti, and G. Bensi. 2002. Yeast coexpression of human papillomavirus types 6 and 16capsid proteins. *Virology* 293(2): 335–344.

Cadman, L. 2008. The future of cervical cancer prevention: Human papillomavirus vaccines. *The Journal of Family Health Care* 18(4): 131–132.

Caparrós-Wanderley, W., B. Clark, and B.E. Griffin. 2004. Effect of dose and long-term storage on the immunogenicity of murine polyomavirus VP1 virus-like particles. *Vaccine* 22(3–4) (January): 352–361.

Caruso, M., L. Belloni, O. Sthandier, P. Amati, and M.-I. Garcia. 2003. Alpha4beta1 integrin acts as a cell receptor for murine polyomavirus at the postattachment level. *Journal of Virology* 77(7) (April): 3913–3921.

Chackerian, B., L. Briglio, P.S. Albert, D.R. Lowy, and J.T. Schiller. 2004. Induction of autoantibodies to CCR5 in macaques and subsequent effects upon challenge with an R5-tropic simian/human immunodeficiency virus. *Journal of Virology* 78(8) (April 15): 4037–4047.

Chackerian, B., D.R. Lowy, and J.T. Schiller. 1999. Induction of autoantibodies to mouse CCR5 with recombinant papillomavirus particles. *Proceedings of the National Academy of Sciences of the United States of America* 96(5): 2373–2378.

Chackerian, B., D.R. Lowy, and J.T. Schiller. 2001. Conjugation of a self-antigen to papillomavirus-like particles allows for efficient induction of protective autoantibodies. *The Journal of Clinical Investigation* 108(3) (August): 415–423.

Chang, D., C.Y. Fung, W.C. Ou, P.C. Chao, S.Y. Li, M. Wang, Y.L. Huang, T.Y. Tzeng, and R.T. Tsai. 1997. Self-assembly of the JC virus major capsid protein, VP1, expressed in insect cells. *Journal of General Virology* 78(6) (January 6): 1435–1439.

Chapuis, A.G., O.K. Afanasiev, J.G. Iyer, K.G. Paulson, U. Parvathaneni, J.H. Hwang, I. Lai et al. 2014. Regression of metastatic merkel cell carcinoma following transfer of polyomavirus-specific T cells and therapies capable of re-inducing HLA class-I. *Cancer Immunology Research* 2(1) (January 1): 27–36.

Chen, L.S., M. Wang, W.C. Ou, C.Y. Fung, P.L. Chen, C.F. Chang, W.S. Huang, J.Y. Wang, P.Y. Lin, and D. Chang. 2010. Efficient gene transfer using the human JC virus-like particle that inhibits human colon adenocarcinoma growth in a nude mouse model. *Gene Therapy* 17(8): 1033–1041.

Chen, P.-L., M. Wang, W.-C. Ou, C.-K. Lii, L.-S. Chen, and D. Chang. 2001. Disulfide bonds stabilize JC virus capsid-like structure by protecting calcium ions from chelation. *FEBS Letters* 500(3): 109–113.

Chen, T., P.S. Mattila, T. Jartti, O. Ruuskanen, M. Söderlund-Venermo, and K. Hedman. 2011. Seroepidemiology of the newly found trichodysplasia spinulosa-associated polyomavirus. *Journal of Infectious Diseases* 204(10) (November 15): 1523–1526.

Chen, X., H. Liu, T. Zhang, Y. Liu, X. Xie, Z. Wang, and X. Xu. 2014. A vaccine of L2 epitope repeats fused with a modified IgG1 Fc induced cross-neutralizing antibodies and protective immunity against divergent human papillomavirus types. *PLoS ONE* 9(5): e95448.

Chen, X.S., G. Casini, S.C. Harrison, and R.L. Garcea. 2001. Papillomavirus capsid protein expression in *Escherichia coli*: Purification and assembly of HPV11 and HPV16 L1. *Journal of Molecular Biology* 307(1) (March 16): 173–182.

Chen, X.S., R.L. Garcea, I. Goldberg, G. Casini, and S.C. Harrison. 2000. Structure of small virus-like particles assembled from the L1 protein of human papillomavirus 16. *Molecular Cell* 5(3): 557–567.

Chen, X.S., T. Stehle, and S.C. Harrison. 1998. Interaction of polyomavirus internal protein VP2 with the major capsid protein VP1 and implications for participation of VP2 in viral entry. *The EMBO Journal* 17(12): 3233–3240.

Cheng, Z., A.A. Zaki, J.Z. Hui, V.R. Muzykantov, and A. Tsourkas. 2012. Multifunctional nanoparticles: Cost versus benefit of adding targeting and imaging capabilities. *Science* 338(6109) (November 16): 903–910.

Chestier, A. and M. Yaniv. 1979. Rapid turnover of acetyl groups in the four core histones of simian virus 40 minichromosomes. *Proceedings of the National Academy of Sciences of the United States of America* 76(1) (January 1): 46–50.

Cho, H.-J., Y.-K. Oh, and Y.B. Kim. 2011. Advances in human papilloma virus vaccines: A patent review. *Expert Opinion on Therapeutic Patents* 21(3) (March): 295–309.

Chou, M.-I., Y.-F. Hsieh, M. Wang, J.T. Chang, D. Chang, M. Zouali, and G.J. Tsay. 2010. In vitro and in vivo targeted delivery of IL-10 interfering RNA by JC virus-like particles. *Journal of Biomedical Science* 17(1) (June 24): 51.

Christensen, N.D., R. Höpfl, S.L. DiAngelo, N.M. Cladel, S.D. Patrick, P.A. Welsh, L.R. Budgeon, C.A. Reed, and J.W. Kreider. 1994. Assembled baculovirus-expressed human papillomavirus type 11 L1 capsid protein virus-like particles are recognized by neutralizing monoclonal antibodies and induce high titres of neutralizing antibodies. *Journal of General Virology* 75(9) (January 9): 2271–2276.

Chromy, L.R., J.M. Pipas, and R.L. Garcea. 2003. Chaperone-mediated in vitro assembly of polyomavirus capsids. *Proceedings of the National Academy of Sciences of the United States of America* 100(18): 10477–10482.

Chuan, Y.P., Y.Y. Fan, L.H.L. Lua, and A.P.J. Middelberg. 2010. Virus assembly occurs following a pH- or Ca^{2+}-triggered switch in the thermodynamic attraction between structural protein capsomeres. *Journal of the Royal Society Interface* 7(44) (March 6): 409–421.

Chuan, Y.P., L.H.L. Lua, and A.P.J. Middelberg. 2008. High-level expression of soluble viral structural protein in *Escherichia coli*. *Journal of Biotechnology* 134(1–2) (March 20): 64–71.

Chuan, Y.P., T. Rivera-Hernandez, N. Wibowo, N.K. Connors, Y. Wu, F.K. Hughes, L.H.L. Lua, and A.P.J. Middelberg. 2013. Effects of pre-existing anti-carrier immunity and antigenic element multiplicity on efficacy of a modular virus-like particle vaccine. *Biotechnology and Bioengineering* 110(9) (September): 2343–2351.

Clark, B., W. Caparros-Wanderley, G. Musselwhite, M. Kotecha, and B.E. Griffin. 2001. Immunity against both polyomavirus VP1 and a transgene product induced following intranasal delivery of VP1 pseudocapsid–DNA complexes. *Journal of General Virology* 82(11): 2791–2797.

Coca-Prados, M., G. Vidali, and M.T. Hsu. 1980. Intracellular forms of simian virus 40 nucleoprotein complexes. III. Study of histone modifications. *Journal of Virology* 36(2): 353–360.

Coelho, T.R., L. Almeida, and P.A. Lazo. 2010. JC virus in the pathogenesis of colorectal cancer, an etiological agent or another component in a multistep process? *Virology Journal* 7: 42.

Colomar, M.C., C. Degoumois-Sahli, and P. Beard. 1993. Opening and refolding of simian virus 40 and in vitro packaging of foreign DNA. *Journal of Virology* 67(5) (May): 2779–2786.

Combita, A.L., A. Touzé, L. Bousarghin, P.Y. Sizaret, N. Muñoz, and P. Coursaget. 2001. Gene transfer using human papillomavirus pseudovirions varies according to virus genotype and requires cell surface heparan sulfate. *FEMS Microbiology Letters* 204(1) (October 16): 183–188.

Cook, J.C., J.G. Joyce, H.A. George, L.D. Schultz, W.M. Hurni, K.U. Jansen, R.W. Hepler et al. 1999. Purification of virus-like particles of recombinant human papillomavirus type 11 major capsid protein l1 from *Saccharomyces cerevisiae*. *Protein Expression and Purification* 17(3) (December): 477–484.

Corradini, R., S. Sforza, T. Tedeschi, F. Totsingan, and R. Marchelli. 2007. Peptide nucleic acids with a structurally biased backbone: Effects of conformational constraints and stereochemistry. *Current Topics in Medicinal Chemistry* 7(7): 681–694.

Crawford, L.V., E.M. Crawford, and D.H. Watson. 1962. The physical characteristics of polyoma virus. I. Two types of particle. *Virology* 18 (October): 170–176.

Cristiano, R.J., L.C. Smith, M.A. Kay, B.R. Brinkley, and S.L. Woo. 1993. Hepatic gene therapy: Efficient gene delivery and expression in primary hepatocytes utilizing a conjugated adenovirus–DNA complex. *Proceedings of the National Academy of Sciences of the United States of America* 90(24) (December 15): 11548–11552.

Culp, T.D., L.R. Budgeon, and N.D. Christensen. 2006a. Human papillomaviruses bind a basal extracellular matrix component secreted by keratinocytes which is distinct from a membrane-associated receptor. *Virology* 347(1) (March 30): 147–159.

Culp, T.D., L.R. Budgeon, M.P. Marinkovich, G. Meneguzzi, and N.D. Christensen. 2006b. Keratinocyte-secreted laminin 5 can function as a transient receptor for human papillomaviruses by binding virions and transferring them to adjacent cells. *Journal of Virology* 80(18) (September): 8940–8950.

Culp, T.D. and N.D. Christensen. 2004. Kinetics of in vitro adsorption and entry of papillomavirus virions. *Virology* 319(1) (February): 152–161.

Culp, T.D., N.M. Cladel, K.K. Balogh, L.R. Budgeon, A.F. Mejia, and N.D. Christensen. 2006c. Papillomavirus particles assembled in 293TT cells are infectious in vivo. *Journal of Virology* 80(22) (January 11): 11381–11384.

Dabydeen, S.A. and P.I. Meneses. 2009. The role of NH4Cl and cysteine proteases in human papillomavirus Type 16 infection. *Virology Journal* 6: 109.

Dalianis, T. and H.H. Hirsch. 2013. Human polyomaviruses in disease and cancer. *Virology* 437(2) (March 15): 63–72.

Dalyot-Herman, N., D. Rund, and A. Oppenheim. 1999. Expression of beta-globin in primary erythroid progenitors of beta-thalassemia patients using an SV40-based gene delivery system. *Journal of Hematotherapy & Stem Cell Research* 8(6) (December): 593–599.

Daniels, R., N.M. Rusan, P. Wadsworth, and D.N. Hebert. 2006. SV40 VP2 and VP3 insertion into ER membranes is controlled by the capsid protein VP1: Implications for DNA translocation out of the ER. *Molecular Cell* 24(6) (December 28): 955–966.

Darshan, M.S., J. Lucchi, E. Harding, and J. Moroianu. 2004. The L2 minor capsid protein of human papillomavirus type 16 interacts with a network of nuclear import receptors. *Journal of Virology* 78(22) (November): 12179–12188.

Dasgupta, J., M. Bienkowska-Haba, M.E. Ortega, H.D. Patel, S. Bodevin, D. Spillmann, B. Bishop, M. Sapp, and X.S. Chen. 2011. Structural basis of oligosaccharide receptor recognition by human papillomavirus. *The Journal of Biological Chemistry* 286(4) (January 28): 2617–2624.

Da Silva, D.M., J.T. Schiller, and W.M. Kast. 2003. Heterologous boosting increases immunogenicity of chimeric papillomavirus virus-like particle vaccines. *Vaccine* 21(23) (July): 3219–3227.

Davidson, E.J. 2003. Human papillomavirus type 16 E2- and L1-specific serological and T-Cell responses in women with vulval intraepithelial neoplasia. *Journal of General Virology* 84(8) (August 1): 2089–2097.

Day, P.M., C.C. Baker, D.R. Lowy, and J.T. Schiller. 2004. Establishment of papillomavirus infection is enhanced by promyelocytic leukemia protein (PML) expression. *Proceedings of the National Academy of Sciences of the United States of America* 101(39) (September 28): 14252–14257.

Day, P.M., R. Gambhira, R.B.S. Roden, D.R. Lowy, and J.T. Schiller. 2008. Mechanisms of human papillomavirus type 16 neutralization by l2 cross-neutralizing and l1 type-specific antibodies. *Journal of Virology* 82(9) (May): 4638–4646.

Day, P.M., R.C. Kines, C.D. Thompson, S. Jagu, R.B. Roden, D.R. Lowy, and J.T. Schiller. 2010. In vivo mechanisms of vaccine-induced protection against HPV infection. *Cell Host & Microbe* 8(3) (September): 260–270.

Day, P.M., D.R. Lowy, and J.T. Schiller. 2003. Papillomaviruses infect cells via a clathrin-dependent pathway. *Virology* 307(1) (March 1): 1–11.

Day, P.M., D.R. Lowy, and J.T. Schiller. 2008. Heparan sulfate-independent cell binding and infection with furin-precleaved papillomavirus capsids. *Journal of Virology* 82(24) (December): 12565–12568.

Day, P.M. and M. Schelhaas. 2014. Concepts of papillomavirus entry into host cells. *Current Opinion in Virology* 4 (February): 24–31.

Day, P.M., C.D. Thompson, R.M. Schowalter, D.R. Lowy, and J.T. Schiller. 2013. Identification of a role for the trans-golgi network in human papillomavirus 16 pseudovirus infection. *Journal of Virology* 87(7) (April): 3862–3870.

DeLano, W.L. 2006. *The PyMOL Molecular Graphics System*, version: v0.99. Cambridge, MA: Schrödinger LCC.

Denny, L.A., S. Franceschi, S. de Sanjosé, I. Heard, A.B. Moscicki, and J. Palefsky. 2012. Human papillomavirus, human immunodeficiency virus and immunosuppression. *Vaccine* 30, Supplement 5 (November 20): F168–F174.

De Villiers, E.-M. 2013. Cross-roads in the classification of papillomaviruses. *Virology* 445(1–2): 2–10.

De Villiers, E.-M., C. Fauquet, T.R. Broker, H.-U. Bernard, and H. zur Hausen. 2004. Classification of papillomaviruses. *Virology* 324(1): 17–27.

Dorn, D.C., R. Lawatscheck, A. Zvirbliene, E. Aleksaite, G. Pecher, K. Sasnauskas, M. Özel et al. 2008. Cellular and humoral immunogenicity of hamster polyomavirus-derived virus-like particles harboring a mucin 1 cytotoxic T-cell epitope. *Viral Immunology* 21(1) (March): 12–26.

Drobni, P., N. Mistry, N. McMillan, and M. Evander. 2003. Carboxyfluorescein diacetate, succinimidyl ester labeled papillomavirus virus-like particles fluoresce after internalization and interact with heparan sulfate for binding and entry. *Virology* 310(1) (May): 163–172.

Dugan, A.S., M.L. Gasparovic, and W.J. Atwood. 2008. Direct correlation between sialic acid binding and infection of cells by two human polyomaviruses (JC Virus and BK Virus). *Journal of Virology* 82(5) (March): 2560–2564.

Dürst, M., L. Gissmann, H. Ikenberg, and H. zur Hausen. 1983. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. *Proceedings of the National Academy of Sciences of the United States of America* 80(12) (January 6): 3812–3815.

Dyson, N., P.M. Howley, K. Münger, and E. Harlow. 1989. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science* (New York) 243(4893) (February 17): 934–937.

Eash, S., W. Querbes, and W.J. Atwood. 2004. Infection of vero cells by BK virus is dependent on caveolae. *Journal of Virology* 78(21) (November): 11583–11590.

Ehlers, B. and U. Wieland. 2013. The novel human polyomaviruses HPyV6, 7, 9 and beyond. *APMIS* 121(8): 783–795.

Einstein, M.H., M. Baron, M.J. Levin, A. Chatterjee, R.P. Edwards, F. Zepp, I. Carletti et al. 2009. Comparison of the immunogenicity and safety of cervarix and gardasil human papillomavirus (HPV) cervical cancer vaccines in healthy women aged 18–45 years. *Human Vaccines* 5(10) (October): 705–719.

Elphick, G.F., W. Querbes, J.A. Jordan, G.V. Gee, S. Eash, K. Manley, A. Dugan et al. 2004. The human polyomavirus, JCV, uses serotonin receptors to infect cells. *Science* (New York) 306(5700) (November 19): 1380–1383.

El-Sayed, A., S. Futaki, and H. Harashima. 2009. Delivery of macromolecules using arginine-rich cell-penetrating peptides: Ways to overcome endosomal entrapment. *The AAPS Journal* 11(1) (January 6): 13–22.

Engel, S., T. Heger, R. Mancini, F. Herzog, J. Kartenbeck, A. Hayer, and A. Helenius. 2011. Role of endosomes in simian virus 40 entry and infection. *Journal of Virology* 85(9) (May): 4198–4211.

Enomoto, T., M. Kawano, H. Fukuda, W. Sawada, T. Inoue, K.C. Haw, Y. Kita et al. 2013. Viral protein-coating of magnetic nanoparticles using simian virus 40 VP1. *Journal of Biotechnology* 167(1) (August 10): 8–15.

Enomoto, T., I. Kukimoto, M. Kawano, Y. Yamaguchi, A.J. Berk, and H. Handa. 2011. In vitro reconstitution of SV40 particles that are composed of VP1/2/3 capsid proteins and nucleosomal DNA and direct efficient gene transfer. *Virology* 420(1) (November 10): 1–9.

Erickson, K.D., R.L. Garcea, and B. Tsai. 2009. Ganglioside GT1b is a putative host cell receptor for the merkel cell polyomavirus. *Journal of Virology* 83(19) (October): 10275–10279.

Eriksson, M., K. Andreasson, J. Weidmann, K. Lundberg, K. Tegerstedt, T. Dalianis, and T. Ramqvist. 2011. Murine polyomavirus virus-like particles carrying full-length human PSA protect BALB/c mice from outgrowth of a PSA expressing tumor. ed. M.M. Rodrigues. *PLoS ONE* 6(8) (August 17): e23828.

Evander, M., I.H. Frazer, E. Payne, Y.M. Qi, K. Hengst, and N.A. McMillan. 1997. Identification of the alpha6 integrin as a candidate receptor for papillomaviruses. *Journal of Virology* 71(3) (March): 2449–2456.

Ewers, H., and A. Helenius. 2011. Lipid-mediated endocytosis. *Cold Spring Harbor Perspectives in Biology* 3(8) (August): a004721.

Fang, C.-Y., H.-Y. Chen, M. Wang, P.-L. Chen, C.-F. Chang, L.-S. Chen, C.-H. Shen et al. 2010. Global analysis of modifications of the human BK virus structural proteins by LC–MS/MS. *Virology* 402(1): 164–176.

Fang, N.X., I.H. Frazer, J. Zhou, and G.J. Fernando. 1999. Post translational modifications of recombinant human papillomavirus type 6b major capsid protein. *Virus Research* 60(2) (April): 113–121.

Fausch, S.C., D.M. Da Silva, and W.M. Kast. 2003. Differential uptake and cross-presentation of human papillomavirus virus-like particles by dendritic cells and langerhans cells. *Cancer Research* 63(13): 3478–3482.

Feng, H., M. Shuda, Y. Chang, and P.S. Moore. 2008. Clonal integration of a polyomavirus in human merkel cell carcinoma. *Science* (New York) 319(5866) (February 22): 1096–1100.

Fernández-San Millán, A., S.M. Ortigosa, S. Hervás-Stubbs, P. Corral-Martínez, J.M. Seguí-Simarro, J. Gaétan, P. Coursaget, and J. Veramendi. 2008. Human papillomavirus L1 protein expressed in tobacco chloroplasts self-assembles into virus-like particles that are highly immunogenic. *Plant Biotechnology Journal* 6(5) (June): 427–441.

Fife, K.H., C.M. Wheeler, L.A. Koutsky, E. Barr, D.R. Brown, M.A. Schiff, N.B. Kiviat et al. 2004. Dose-ranging studies of the safety and immunogenicity of human papillomavirus type 11 and type 16 virus-like particle candidate vaccines in young healthy women. *Vaccine* 22(21–22) (July 29): 2943–2952.

Finch, J.T. 1974. The surface structure of polyoma virus. *Journal of General Virology* 24(2) (January 8): 359–364.

Finnen, R.L., K.D. Erickson, X.S. Chen, and R.L. Garcea. 2003. Interactions between papillomavirus L1 and L2 capsid proteins. *Journal of Virology* 77(8) (April 15): 4818–4826.

Fischer, R., E. Stoger, S. Schillberg, P. Christou, and R.M. Twyman. 2004. Plant-based production of biopharmaceuticals. *Current Opinion in Plant Biology* 7(2) (April): 152–158.

Fishman, J.A. 2002. BK virus nephropathy—Polyomavirus adding insult to injury. *The New England Journal of Medicine* 347(7) (August 15): 527–530.

FitzSimons, D., G. François, A. Hall, B. McMahon, A. Meheus, A. Zanetti, B. Duval et al. 2005. Long-term efficacy of hepatitis B vaccine, booster policy, and impact of hepatitis B virus mutants. *Vaccine* 23(32) (July): 4158–4166.

Fligge, C., F. Schafer, H.-C. Selinka, C. Sapp, and M. Sapp. 2001. DNA-induced structural changes in the papillomavirus capsid. *Journal of Virology* 75(16) (August): 7727–7731.

Florin, L., K.A. Becker, C. Lambert, T. Nowak, C. Sapp, D. Strand, R.E. Streeck, and M. Sapp. 2006. Identification of a dynein interacting domain in the papillomavirus minor capsid protein l2. *Journal of Virology* 80(13) (July): 6691–6696.

Forstová, J., N. Krauzewicz, V. Sandig, J. Elliott, Z. Palková, M. Strauss, and B.E. Griffin. 1995. Polyoma virus pseudocapsids as efficient carriers of heterologous DNA into mammalian cells. *Human Gene Therapy* 6(3) (March): 297–306.

Forstová, J., N. Krauzewicz, S. Wallace, A.J. Street, S.M. Dilworth, S. Beard, and B.E. Griffin. 1993. Cooperation of structural proteins during late events in the life cycle of polyomavirus. *Journal of Virology* 67(3) (January 3): 1405–1413.

Fothergill, T. and N.A.J. McMillan. 2006. Papillomavirus virus-like particles activate the PI3-kinase pathway via alpha-6 beta-4 integrin upon binding. *Virology* 352(2) (September 1): 319–328.

Franzén, A.V., K. Tegerstedt, D. Holländerova, J. Forstová, T. Ramqvist, and T. Dalianis. 2005. Murine polyoma- virus-VP1 virus-like particles immunize against some polyomavirus-induced tumours. *In Vivo* (Athens, Greece) 19(2) (April): 323–326.

Frič, J., M. Marek, V. Hrušková, V. Holáň, and J. Forstová. 2008. Cellular and humoral immune responses to chimeric EGFP-pseudocapsids derived from the mouse polyomavirus after their intranasal administration. *Vaccine* 26(26) (June): 3242–3251.

Furumoto, H. and M. Irahara. 2002. Human papilloma virus (HPV) and cervical cancer. *The Journal of Medical Investigation* 49(3–4) (August): 124–133.

Gambhira, R., B. Karanam, S. Jagu, J.N. Roberts, C.B. Buck, I. Bossis, H. Alphs, T. Culp, N.D. Christensen, and R.B.S. Roden. 2007. A protective and broadly cross-neutralizing epitope of human papillomavirus L2. *Journal of Virology* 81(24) (December): 13927–13931.

Gao, D., Z.-P. Zhang, F. Li, D. Men, J.-Y. Deng, H.-P. Wei, X.-E. Zhang, and Z.-Q. Cui. 2013. Quantum dot-induced viral capsid assembling in dissociation buffer. *International Journal of Nanomedicine* 8: 2119–2128.

Garcea, R.L. and X.S. Chen. 2007. Papillomavirus structure and assembly. In *The Papillomaviruses*. Eds. R.L. Garcea and D. DiMaio. New York: Springer.

Gardner, S.D., A.M. Field, D.V. Coleman, and B. Hulme. 1971. New human papovavirus (B.K.) isolated from urine after renal transplantation. *Lancet* 1(7712) (June 19): 1253–1257.

Garneski, K.M., A.H. Warcola, Q. Feng, N.B. Kiviat, J.H. Leonard, and P. Nghiem. 2009. Merkel cell polyomavirus is more frequently present in North American than Australian merkel cell carcinoma tumors. *Journal of Investigative Dermatology* 129(1) (January): 246–248.

Gedvilaite, A., D.C. Dorn, K. Sasnauskas, G. Pecher, A. Bulavaite, R. Lawatscheck, J. Staniulis et al. 2006. Virus-like particles derived from major capsid protein VP1 of different polyomaviruses differ in their ability to induce maturation in human dendritic cells. *Virology* 354(2) (October): 252–260.

Gedvilaite, A., C. Frömmel, K. Sasnauskas, B. Micheel, M. Özel, O. Behrsing, J. Staniulis, B. Jandrig, S. Scherneck, and R. Ulrich. 2000. Formation of immunogenic virus-like particles by

inserting epitopes into surface-exposed regions of hamster polyomavirus major capsid protein. *Virology* 273(1) (July): 21–35.

Gedvilaite, A., A. Zvirbliene, J. Staniulis, K. Sasnauskas, D.H. Krüger, and R. Ulrich. 2004. Segments of puumala hantavirus nucleocapsid protein inserted into chimeric polyomavirus-derived virus-like particles induce a strong immune response in mice. *Viral Immunology* 17(1): 51–68.

Gerber, S., C. Lane, D.M. Brown, E. Lord, M. DiLorenzo, J.D. Clements, E. Rybicki, A.-L. Williamson, and R.C. Rose. 2001. Human papillomavirus virus-like particles are efficient oral immunogens when coadministered with *Escherichia coli* heat-labile enterotoxin mutant R192G or CpG DNA. *Journal of Virology* 75(10) (May 15): 4752–4760.

Gilbert, J. and T. Benjamin. 2004. Uptake pathway of polyomavirus via ganglioside GD1a. *Journal of Virology* 78(22) (November): 12259–12267.

Gilbert, J., W. Ou, J. Silver, and T. Benjamin. 2006. Downregulation of protein disulfide isomerase inhibits infection by the mouse polyomavirus. *Journal of Virology* 80(21) (November): 10868–10870.

Gilbert, J.M. and T.L. Benjamin. 2000. Early steps of polyomavirus entry into cells. *Journal of Virology* 74(18) (September): 8582–8588.

Gilbert, J.M., I.G. Goldberg, and T.L. Benjamin. 2003. Cell penetration and trafficking of polyomavirus. *Journal of Virology* 77(4) (February): 2615–2622.

Gillock, E.T., S. Rottinghaus, D. Chang, X. Cai, S.A. Smiley, K. An, and R.A. Consigli. 1997. Polyomavirus major capsid protein VP1 is capable of packaging cellular DNA when expressed in the baculovirus system. *Journal of Virology* 71(4): 2857–2865.

Gioux, S., H.S. Choi, and J.V. Frangioni. 2010. Image-guided surgery using invisible near-infrared light: Fundamentals of clinical translation. *Molecular Imaging* 9(5) (October): 237–255.

Giroglou, T., L. Florin, F. Schafer, R.E. Streeck, and M. Sapp. 2001. Human papillomavirus infection requires cell surface heparan sulfate. *Journal of Virology* 75(3) (February): 1565–1570.

Giroglou, T., M. Sapp, C. Lane, C. Fligge, N.D. Christensen, R.E. Streeck, and R.C. Rose. 2001. Immunological analyses of human papillomavirus capsids. *Vaccine* 19(13–14): 1783–1793.

Giuliano, A.R., J.M. Palefsky, S. Goldstone, E.D. Moreira, M.E. Penny, C. Aranda, E. Vardas et al. 2011. Efficacy of quadrivalent HPV vaccine against HPV infection and disease in males. *New England Journal of Medicine* 364(5) (February 3): 401–411.

GlaxoSmithKline Vaccine HPV-007 Study Group, B. Romanowski, P.C. de Borba, P.S. Naud, C.M. Roteli-Martins, N.S. De Carvalho, J.C. Teixeira et al. 2009. Sustained efficacy and immunogenicity of the human papillomavirus (HPV)-16/18 AS04-adjuvanted vaccine: Analysis of a randomised placebo-controlled trial up to 6.4 years. *Lancet* 374(9706) (December 12): 1975–1985.

Gleiter, S. and H. Lilie. 2001. Coupling of antibodies via protein Z on modified polyoma virus-like particles. *Protein Science* 10(2): 434–444.

Gleiter, S., K. Stubenrauch, and H. Lilie. 1999. Changing the surface of a virus shell fusion of an enzyme to polyoma VP1. *Protein Science* 8(12): 2562–2569.

Goldmann, C., H. Petry, S. Frye, O. Ast, S. Ebitsch, K.-D. Jentsch, F.-J. Kaup, F. Weber, C. Trebst, and T. Nisslein. 1999. Molecular Cloning and Expression of Major Structural Protein VP1 of the human polyomavirus JC virus: Formation of virus-like particles useful for immunological and therapeutic studies. *Journal of Virology* 73(5): 4465–4469.

Goldmann, C., N. Stolte, T. Nisslein, G. Hunsmann, W. Lüke, and H. Petry. 2000. Packaging of small molecules into VP1-virus-like particles of the human polyomavirus JC virus. *Journal of Virological Methods* 90(1): 85–90.

Gonçalves, A.K., R.N. Cobucci, H.M. Rodrigues, A.G. de Melo, and P.C. Giraldo. 2014. Safety, tolerability and side effects of human papillomavirus vaccines: A systematic quantitative review. *The Brazilian Journal of Infectious Diseases* 18(6) (April 27): 651–659. http://linkinghub.elsevier.com/retrieve/pii/S1413867014000695. Accessed July 14, 2014.

Gordon, S.N., R.C. Kines, G. Kutsyna, Z.-M. Ma, A. Hryniewicz, J.N. Roberts, C. Fenizia et al. 2012. Targeting the vaginal mucosa with human papillomavirus pseudovirion vaccines delivering simian immunodeficiency virus DNA. *The Journal of Immunology* 188(2) (January 15): 714–723.

Goss, M.A., F. Lievano, M.M. Seminack, and A. Dana. 2014. No adverse signals observed after exposure to human papillomavirus type 6/11/16/18 vaccine during pregnancy: 6-Year pregnancy registry data. *Obstetrics and Gynecology* 123 Suppl. 1 (May): 93S.

Graham, B.S., R. Kines, K.S. Corbett, J. Nicewonger, T.R. Johnson, M. Chen, D. LaVigne et al. 2010. Mucosal delivery of human papillomavirus pseudovirus-encapsidated plasmids improves potency of DNA vaccination. *Mucosal Immunology* 3(5) (September): 475–486.

Greenstone, H.L., J.D. Nieland, K.E. De Visser, M.L. De Bruijn, R. Kirnbauer, R.B. Roden, D.R. Lowy, W.M. Kast, and J.T. Schiller. 1998. Chimeric papillomavirus virus-like particles elicit antitumor immunity against the E7 oncoprotein in an HPV16 tumor model. *Proceedings of the National Academy of Sciences of the United States of America* 95(4): 1800–1805.

Griffith, J.P., D.L. Griffith, I. Rayment, W.T. Murakami, and D.L. Caspar. 1992. Inside polyomavirus at 25-A resolution. *Nature* 355(6361) (February 13): 652–654.

Günther, C., U. Schmidt, R. Rudolph, and G. Böhm. 2001. Protein and peptide delivery via engineered polyomavirus-like particles. *The FASEB Journal* 15(9) (May 9): 1646–1648. http://www.fasebj.org/content/early/2001/07/02/fj.00-0645fje.short. Accessed July 31, 2014.

Gupta, A.K. and M. Gupta. 2005. Synthesis and surface engineering of iron oxide nanoparticles for biomedical applications. *Biomaterials* 26(18) (June): 3995–4021.

Hagensee, M.E., N. Yaegashi, and D.A. Galloway. 1993. Self-assembly of human papillomavirus type 1 capsids by expression of the L1 protein alone or by coexpression of the L1 and L2 capsid proteins. *Journal of Virology* 67(1) (January): 315–322.

Hale, A.D., D. Bartkeviciūte, A. Dargeviciūte, L. Jin, W. Knowles, J. Staniulis, D.W.G. Brown, and K. Sasnauskas. 2002. Expression and antigenic characterization of the major capsid proteins of human polyomaviruses BK and JC in *Saccharomyces cerevisiae*. *Journal of Virological Methods* 104(1): 93–98.

Handisurya, A., P.M. Day, C.D. Thompson, C.B. Buck, K. Kwak, R.B.S. Roden, D.R. Lowy, and J.T. Schiller. 2012. Murine skin and vaginal mucosa are similarly susceptible to infection by pseudovirions of different papillomavirus classifications and species. *Virology* 433(2) (November 25): 385–394.

Harper, D.M., E.L. Franco, C. Wheeler, D.G. Ferris, D. Jenkins, A. Schuind, T. Zahaf et al. 2004. Efficacy of a bivalent L1 virus-like particle vaccine in prevention of infection with human papillomavirus types 16 and 18 in young women: A randomised controlled trial. *The Lancet* 364(9447): 1757–1765.

Haugg, A.M., D. Rennspiess, A. zur Hausen, E.-J.M. Speel, G. Cathomas, J.C. Becker, and D. Schrama. 2014. Fluorescence in situ hybridization and qPCR to detect merkel cell polyomavirus physical status and load in merkel cell carcinomas. *International Journal of Cancer* 135(12) (May 9): 2804–2815.

Hayman, W.A., E.R. Brandt, W.A. Relf, J. Cooper, A. Saul, and M.F. Good. 1997. Mapping the minimal murine T cell and B cell epitopes within a peptide vaccine candidate from the conserved region of the M protein of group A Streptococcus. *International Immunology* 9(11) (November): 1723–1733.

Haynes, J.I., D. Chang, and R.A. Consigli. 1993. Mutations in the putative calcium-binding domain of polyomavirus VP1 affect capsid assembly. *Journal of Virology* 67(5) (January 5): 2486–2495.

Heidari, S., N. Krauzewicz, M. Kalantari, A. Vlastos, B.E. Griffin, and T. Dalianis. 2000. Persistence and tissue distribution of DNA in normal and immunodeficient mice inoculated with polyomavirus VP1 pseudocapsid complexes or polyomavirus. *Journal of Virology* 74(24) (December): 11963–11965.

Heidari, S., A. Vlastos, T. Ramqvist, B. Clark, B.E. Griffin, M.-I. Garcia, M. Perez, P. Amati, and T. Dalianis. 2002. Immunization of T-cell deficient mice against polyomavirus infection using viral pseudocapsids or temperature sensitive mutants. *Vaccine* 20(11): 1571–1578.

Heino, P., J. Dillner, and S. Schwartz. 1995. Human papillomavirus type 16 capsid proteins produced from recombinant semliki forest virus assemble into virus-like particles. *Virology* 214(2) (December 20): 349–359.

Henke, S., A. Rohmann, W.M. Bertling, T. Dingermann, and A. Zimmer. 2000. Enhanced in vitro oligonucleotide and plasmid DNA transport by VP1 virus-like particles. *Pharmaceutical Research* 17(9) (September): 1062–1070.

Hildesheim, A., R. Herrero, S. Wacholder, A.C. Rodriguez, D. Solomon, M.C. Bratti, J.T. Schiller et al. 2007. Effect of human papillomavirus 16/18 L1 viruslike particle vaccine among young women with preexisting infection: A randomized trial. *The Journal of the American Medical Association* 298(7) (August 15): 743–753.

Hindmarsh, P.L. and L.A. Laimins. 2007. Mechanisms regulating expression of the HPV 31 L1 and L2 capsid proteins and pseudovirion entry. *Virology Journal* 4 (February 26): 19.

Hock, C., U. Konietzko, J.R. Streffer, J. Tracy, A. Signorell, B. Müller-Tillmanns, U. Lemke et al. 2003. Antibodies against B-amyloid slow cognitive decline in Alzheimer's disease. *Neuron* 38(4) (May 22): 547–554.

Hofmann, K.J., J.C. Cook, J.G. Joyce, D.R. Brown, L.D. Schultz, H.A. George, M. Rosolowsky, K.H. Fife, and K.U. Jansen. 1995. Sequence Determination of human papillomavirus type 6a and assembly of virus-like particles in *Saccharomyces cerevisiae*. *Virology* 209(2): 506–518.

Holländerová, D., H. Raslová, D. Blangy, J. Forstová, and M. Berebbi. 2003. Interference of mouse polyomavirus with the c-Myc gene and its product in mouse mammary adenocarcinomas. *International Journal of Oncology* 23(2) (August): 333–341.

Hrusková, V., A. Morávková, K. Babiarová, V. Ludvíková, J. Fric, V. Vonka, and J. Forstová. 2009. Bcr-Abl fusion sequences do not induce immune responses in mice when administered in mouse polyomavirus based virus-like particles. *International Journal of Oncology* 35(6) (December): 1247–1256.

Huerfano, S., V. Zíla, E. Boura, H. Spanielová, J. Stokrová, and J. Forstová. 2010. Minor capsid proteins of mouse polyomavirus are inducers of apoptosis when produced individually but are only moderate contributors to cell death during the late phase of viral infection. *The FEBS Journal* 277(5) (March): 1270–1283.

Hung, C.-F., B. Ma, A. Monie, S.-W. Tsen, and T.-C. Wu. 2008. Therapeutic human papillomavirus vaccines: Current clinical trials and future directions. *Expert Opinion on Biological Therapy* 8(4) (April): 421–439.

Imperiale, M.J. and E.O. Major. 2007. Polyomaviridae. In *Fields Virology*, eds. B.N. Fields, D.M. Knipe, and P.M. Howley, pp. 2263–2298. 5th edn. Philadelphia, PA: Wolters Kluwer Health/Lippincott Williams & Wilkins.

Ingle, A., S. Ghim, J. Joh, I. Chepkoech, A. Bennett Jenson, and J.P. Sundberg. 2010. Novel laboratory mouse papillomavirus (MusPV) infection. *Veterinary Pathology* 48(2) (August 4): 500–505.

Inoue, T., M. Kawano, R. Takahashi, H. Tsukamoto, T. Enomoto, T. Imai, K. Kataoka, and H. Handa. 2008. Engineering of SV40-based nano-capsules for delivery of heterologous proteins as fusions with the minor capsid proteins VP2/3. *Journal of Biotechnology* 134(1–2) (March 20): 181–192.

Ishii, Y., K. Tanaka, and T. Kanda. 2003. Mutational analysis of human papillomavirus type 16 major capsid protein L1: The cysteines affecting the intermolecular bonding and structure of L1-capsids. *Virology* 308(1) (March 30): 128–136.

Ishizu, K.-I., H. Watanabe, S.-I. Han, S.-N. Kanesashi, M. Hoque, H. Yajima, K. Kataoka, and H. Handa. 2001. Roles of disulfide linkage and calcium ion-mediated interactions in assembly and disassembly of virus-like particles composed of simian virus 40 VP1 capsid protein. *Journal of Virology* 75(1) (January 1): 61–72.

Iyengar, S., K.V. Shah, K.L. Kotloff, S.J. Ghim, and R.P. Viscidi. 1996. Self-assembly of in vitro-translated human papillomavirus type 16 L1 capsid protein into virus-like particles and antigenic reactivity of the protein. *Clinical and Diagnostic Laboratory Immunology* 3(6) (November): 733–739.

Jansen, K.U., M. Rosolowsky, L.D. Schultz, H.Z. Markus, J.C. Cook, J.J. Donnelly, D. Martinez, R.W. Ellis, and A.R. Shaw. 1995. Vaccination with yeast-expressed cottontail rabbit papillomavirus (CRPV) virus-like particles protects rabbits from CRPV-induced papilloma formation. *Vaccine* 13(16): 1509–1514.

Jennings, G.T. and M.F. Bachmann. 2008. The coming of age of virus-like particle vaccines. *Biological Chemistry* 389(5) (January 1): 521–536. http://www.degruyter.com/view/j/bchm.2008.389.issue-5/bc.2008.064/bc.2008.064.xml. Accessed July 30, 2014.

Jiang, Z., G. Tong, B. Cai, Y. Xu, and J. Lou. 2011. Purification and immunogenicity study of human papillomavirus 58 virus-like particles expressed in *Pichia pastoris*. *Protein Expression and Purification* 80(2) (December): 203–210.

Jin, L., P.E. Gibson, W.A. Knowles, and J.P. Clewley. 1993. BK virus antigenic variants: Sequence analysis within the capsid VP1 epitope. *Journal of Medical Virology* 39(1) (January): 50–56.

Jochmus, I., K. Schäfer, S. Faath, M. Müller, and L. Gissmann. 1999. Chimeric virus-like particles of the human papillomavirus type 16 (HPV 16) as a prophylactic and therapeutic vaccine. *Archives of Medical Research* 30(4): 269–274.

Joh, J., A.B. Jenson, A. Ingle, J.P. Sundberg, and S. Ghim. 2014. Searching for the initiating site of the major capsid protein to generate virus-like particles for a novel laboratory mouse papillomavirus. *Experimental and Molecular Pathology* 96(2) (April): 155–161. http://www.sciencedirect.com/science/article/pii/S001448001300155X. Accessed August 11, 2014.

Johne, R. and H. Müller. 2004. Nuclear localization of avian polyomavirus structural protein VP1 is a prerequisite for the formation of virus-like particles. *Journal of Virology* 78(2) (January 15): 930–937.

Johne, R. and H. Müller. 2007. Polyomaviruses of birds: Etiologic agents of inflammatory diseases in a tumor virus family. *Journal of Virology* 81(21) (January 11): 11554–11559.

Joyce, J.G., J.S. Tung, C.T. Przysiecki, J.C. Cook, E.D. Lehman, J.A. Sands, K.U. Jansen, and P.M. Keller. 1999. The L1 major capsid protein of human papillomavirus type 11 recombinant virus-like particles interacts with heparin and cell-surface glycosaminoglycans on human keratinocytes. *The Journal of Biological Chemistry* 274(9) (February 26): 5810–5822.

Kämper, N., P.M. Day, T. Nowak, H.-C. Selinka, L. Florin, J. Bolscher, L. Hilbig, J.T. Schiller, and M. Sapp. 2006. A membrane-destabilizing peptide in capsid protein L2 is required for egress of papillomavirus genomes from endosomes. *Journal of Virology* 80(2) (January): 759–768.

Kanesashi, S., K. Ishizu, M. Kawano, S. Han, S. Tomita, H. Watanabe, K. Kataoka, and H. Handa. 2003. Simian virus 40 VP1 capsid protein forms polymorphic assemblies in vitro. *Journal of General Virology* 84(7) (January 7): 1899–1905.

Karanam, B., S. Jagu, W.K. Huh, and R.B.S. Roden. 2009. Developing vaccines against minor capsid antigen L2 to prevent papillomavirus infection. *Immunology and Cell Biology* 87(4) (May): 287–299.

Kaufmann, A.M., J. Nieland, M. Schinz, M. Nonn, J. Gabelsberger, H. Meissner, R.T. Müller et al. 2001. HPV16 L1E7 chimeric virus-like particles induce specific HLA-restricted T cells in humans after in vitro vaccination. *International Journal of Cancer* 92(2): 285–293.

Kaufmann, A.M., J.D. Nieland, I. Jochmus, S. Baur, K. Friese, J. Gabelsberger, F. Gieseking et al. 2007. Vaccination trial with HPV16 L1E7 chimeric virus-like particles in women suffering from high grade cervical intraepithelial neoplasia (CIN 2/3). *International Journal of Cancer* 121(12): 2794–2800.

Kawana, K., H.Yoshikawa,Y. Taketani, K.Yoshiike, and T. Kanda. 1998. In vitro construction of pseudovirions of human papillomavirus type 16: Incorporation of plasmid DNA into reassembled L1/L2 capsids. *Journal of Virology* 72(12) (January 12): 10298–10300.

Kawano, M., K. Morikawa, T. Suda, N. Ohno, S. Matsushita, T. Akatsuka, H. Handa, and M. Matsui. 2014. Chimeric SV40 virus-like particles induce specific cytotoxicity and protective immunity against influenza A virus without the need of adjuvants. *Virology* 448 (January): 159–167.

Kemp, T.J., A. Hildesheim, M. Safaeian, J.G. Dauner, Y. Pan, C. Porras, J.T. Schiller, D.R. Lowy, R. Herrero, and L.A. Pinto. 2011. HPV16/18 L1 VLP vaccine induces cross-neutralizing antibodies that may mediate cross-protection. *Vaccine* 29(11) (March): 2011–2014.

Khan, Z.M., Y. Liu, U. Neu, M. Gilbert, B. Ehlers, T. Feizi, and T. Stehle. 2014. Crystallographic and glycan microarray analysis of human polyomavirus 9 VP1 identifies N-glycolyl neuraminic acid as a receptor candidate. *Journal of Virology* 88(11) (June): 6100–6111.

Khlebtsov, N., V. Bogatyrev, L. Dykman, B. Khlebtsov, S. Staroverov, A. Shirokov, L. Matora et al. 2013. Analytical and theranostic applications of gold nanoparticles and multifunctional nanocomposites. *Theranostics* 3(3) (February 20): 167–180.

Kim, S.N., H.S. Jeong, S.N. Park, and H.-J. Kim. 2007. Purification and immunogenicity study of human papillomavirus type 16 L1 protein in *Saccharomyces cerevisiae*. *Journal of Virological Methods* 139(1) (January): 24–30.

Kimchi-Sarfaty, C., N.S. Alexander, S. Brittain, S. Ali, and M.M. Gottesman. 2004. Transduction of multiple cell types using improved conditions for gene delivery and expression of SV40 pseudovirions packaged in vitro. *BioTechniques* 37(2) (August): 270–275.

Kimchi-Sarfaty, C., M. Arora, Z. Sandalon, A. Oppenheim, and M.M. Gottesman. 2003. High cloning capacity of in vitro packaged SV40 vectors with No SV40 virus sequences. *Human Gene Therapy* 14(2) (January 20): 167–177.

Kimchi-Sarfaty, C., S. Brittain, S. Garfield, N.J. Caplen, Q. Tang, and M.M. Gottesman. 2005. Efficient delivery of RNA interference effectors via in vitro-packaged SV40 pseudovirions. *Human Gene Therapy* 16(9) (September): 1110–1115.

Kimchi-Sarfaty, C. and M.M. Gottesman. 2004. SV40 pseudovirions as highly efficient vectors for gene transfer and their potential application in cancer therapy. *Current Pharmaceutical Biotechnology* 5(5) (October): 451–458.

Kimchi-Sarfaty, C., O. Ben-Nun-Shaul, D. Rund, A. Oppenheim, and M.M. Gottesman. 2002. In vitro-packaged SV40 pseudovirions as highly efficient vectors for gene transfer. *Human Gene Therapy* 13(2) (January 20): 299–310.

Kimchi-Sarfaty, C., W.D. Vieira, D. Dodds, A. Sherman, R.J. Kreitman, S. Shinar, and M.M. Gottesman. 2006. SV40 pseudovirion gene delivery of a toxin to treat human adenocarcinomas in mice. *Cancer Gene Therapy* 13(7) (July): 648–657.

Kines, R.C., C.D. Thompson, D.R. Lowy, J.T. Schiller, and P.M. Day. 2009. The initial steps leading to papillomavirus infection occur on the basement membrane prior to cell surface binding. *Proceedings of the National Academy of Sciences of the United States of America* 106(48) (December 1): 20458–20463.

Kirnbauer, R., F. Booy, N. Cheng, D.R. Lowy, and J.T. Schiller. 1992. Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. *Proceedings of the National Academy of Sciences of the United States of America* 89(24) (December 15): 12180–12184.

Kirnbauer, R., L.M. Chandrachud, B.W. O'neil, E.R. Wagner, G.J. Grindlay, A. Armstrong, G.M. McGarvie, J.T. Schiller, D.R. Lowy, and M.S. Campo. 1996. Virus-like particles of bovine papillomavirus type 4 in prophylactic and therapeutic immunization. *Virology* 219(1): 37–44.

Kirnbauer, R., J. Taub, H. Greenstone, R. Roden, M. Dürst, L. Gissmann, D.R. Lowy, and J.T. Schiller. 1993. Efficient self-assembly of human papillomavirus type 16 L1 and L1-L2 into virus-like particles. *Journal of Virology* 67(12) (December): 6929–6936.

Kitai, Y., H. Fukuda, T. Enomoto, Y. Asakawa, T. Suzuki, S. Inouye, and H. Handa. 2011. Cell selective targeting of a simian virus 40 virus-like particle conjugated to epidermal growth factor. *Journal of Biotechnology* 155(2) (September 10): 251–256.

Kler, S., R. Asor, C. Li, A. Ginsburg, D. Harries, A. Oppenheim, A. Zlotnick, and U. Raviv. 2012. RNA encapsidation by SV40-derived nanoparticles follows a rapid two-state mechanism. *Journal of the American Chemical Society* 134(21) (May 30): 8823–8830.

Klug, A. 1965. Structure of viruses of the papilloma-polyoma type: II. Comments on other work. *Journal of Molecular Biology* 11(2): 424–431, IN45.

Knappe, M., S. Bodevin, H.-C. Selinka, D. Spillmann, R.E. Streeck, X.S. Chen, U. Lindahl, and M. Sapp. 2007. Surface-exposed amino acid residues of HPV16 L1 protein mediating interaction with cell surface heparan sulfate. *The Journal of Biological Chemistry* 282(38) (September 21): 27913–27922.

Komagome, R., H. Sawa, T. Suzuki, Y. Suzuki, S. Tanaka, W.J. Atwood, and K. Nagashima. 2002. Oligosaccharides as receptors for JC virus. *Journal of Virology* 76(24) (December): 12992–13000.

Kondo, K., Y. Ishii, H. Ochi, T. Matsumoto, H. Yoshikawa, and T. Kanda. 2007. Neutralization of HPV16, 18, 31, and 58 pseudovirions with antisera induced by immunizing rabbits with synthetic peptides representing segments of the HPV16 minor capsid protein L2 surface region. *Virology* 358(2) (February): 266–272.

Kondo, K., H. Ochi, T. Matsumoto, H. Yoshikawa, and T. Kanda. 2008. Modification of human papillomavirus-like particle vaccine by insertion of the cross-reactive L2-epitopes. *Journal of Medical Virology* 80(5) (May): 841–846.

Kosukegawa, A., F. Arisaka, M. Takayama, H. Yajima, A. Kaidow, and H. Handa. 1996. Purification and characterization of virus-like particles and pentamers produced by the expression of SV40 capsid proteins in insect cells. *Biochimica et Biophysica Acta (BBA)—General Subjects* 1290(1): 37–45.

Koutsky, L.A., K.A. Ault, C.M. Wheeler, D.R. Brown, E. Barr, F.B. Alvarez, L.M. Chiacchierini, and K.U. Jansen. 2002. A controlled trial of a human papillomavirus type 16 vaccine. *New England Journal of Medicine* 347(21): 1645–1651.

Krauzewicz, N., C. Cox, E. Soeda, B. Clark, S. Rayner, and B.E. Griffin. 2000a. Sustained ex vivo and in vivo transfer of a reporter gene using polyoma virus pseudocapsids. *Gene Therapy* 7(13) (July): 1094–1102.

Krauzewicz, N. and B.E. Griffin. 2000. Polyoma and papilloma virus vectors for cancer gene therapy. *Advances in Experimental Medicine and Biology* 465: 73–82.

Krauzewicz, N., J. Stokrová, C. Jenkins, M. Elliott, C.F. Higgins, and B.E. Griffin. 2000b. Virus-like gene transfer into cells mediated by polyoma virus pseudocapsids. *Gene Therapy* 7(24) (December): 2122–2131.

Kumar, A., T. Chen, S. Pakkanen, A. Kantele, M. Söderlund-Venermo, K. Hedman, and R. Franssila. 2011. T-Helper cell-mediated proliferation and cytokine responses against recombinant merkel cell polyomavirus-like particles. ed. H. Tse. *PLoS ONE* 6(10) (October 3): e25751.

Langner, J., B. Neumann, S.L. Goodman, and M. Pawlita. 2004. RGD-mutants of B-lymphotropic polyomavirus capsids specifically bind to αvβ3 integrin. *Archives of Virology* 149(10) (October 1): 1877–1896.

Laniosz, V., K.A. Holthusen, and P.I. Meneses. 2008. Bovine papillomavirus type 1: From clathrin to caveolin. *Journal of Virology* 82(13) (July): 6288–6298.

Lawatscheck, R., E. Aleksaite, J.A. Schenk, B. Micheel, B. Jandrig, G. Holland, K. Sasnauskas, A. Gedvilaite, and R.G. Ulrich. 2007. Chimeric polyomavirus-derived virus-like particles: The immunogenicity of an inserted peptide applied without adjuvant to mice depends on its insertion site and its flanking linker sequence. *Viral Immunology* 20(3) (September): 453–460.

Leder, C., J.A. Kleinschmidt, C. Wiethe, and M. Müller. 2001. Enhancement of capsid gene expression: Preparing the human papillomavirus type 16 major structural gene L1 for DNA vaccination purposes. *Journal of Virology* 75(19) (January 10): 9201–9209.

Lehtinen, M., J. Paavonen, C.M. Wheeler, U. Jaisamrarn, S.M. Garland, X. Castellsagué, S.R. Skinner, D. Apter, P. Naud, and J. Salmerón. 2012. Overall efficacy of HPV-16/18 AS04-adjuvanted vaccine against grade 3 or greater cervical intraepithelial neoplasia: 4-Year end-of-study analysis of the randomised, double-blind PATRICIA trial. *The Lancet Oncology* 13(1): 89–99.

Lenz, P., P.M. Day, Y.-Y.S. Pang, S.A. Frye, P.N. Jensen, D.R. Lowy, and J.T. Schiller. 2001. Papillomavirus-like particles induce acute activation of dendritic cells. *The Journal of Immunology* 166(9): 5346–5355.

Lenzi, P., N. Scotti, F. Alagna, M.L. Tornesello, A. Pompa, A. Vitale, A.D. Stradis et al. 2008. Translational fusion of chloroplast-expressed human papillomavirus type 16 L1 capsid protein enhances antigen accumulation in transplastomic tobacco. *Transgenic Research* 17(6) (December 1): 1091–1102.

Li, F., H. Chen, L. Ma, K. Zhou, Z.-P. Zhang, C. Meng, X.-E. Zhang, and Q. Wang. 2013. Insights into stabilization of a viral protein cage in templating complex nanoarchitectures: Roles of disulfide bonds. *Small* 10(3) (September 9): 536–543.

Li, F., H. Chen, Y. Zhang, Z. Chen, Z.-P. Zhang, X.-E. Zhang, and Q. Wang. 2012. Three-dimensional gold nanoparticle clusters with tunable cores templated by a viral protein scaffold. *Small* 8(24) (December 21): 3832–3838.

Li, F., D. Gao, X. Zhai, Y. Chen, T. Fu, D. Wu, Z.-P. Zhang, X.-E. Zhang, and Q. Wang. 2011. Tunable, discrete, three-dimensional hybrid nanoarchitectures. *Angewandte Chemie International Edition* 50(18) (April 26): 4202–4205.

Li, F., K. Li, Z.-Q. Cui, Z.-P. Zhang, H.-P. Wei, D. Gao, J.-Y. Deng, and X.-E. Zhang. 2010. Viral coat proteins as flexible nano-building-blocks for nanoparticle encapsulation. *Small* 6(20) (October 18): 2301–2308.

Li, F., Z.-P. Zhang, J. Peng, Z.-Q. Cui, D.-W. Pang, K. Li, H.-P. Wei, Y.-F. Zhou, J.-K. Wen, and X.-E. Zhang. 2009. Imaging viral behavior in mammalian cells with self-assembled capsid-quantum-dot hybrid particles. *Small* 5(6) (March 20): 718–726.

Li, J. and J.-J. Zhu. 2013. Quantum dots for fluorescent biosensing and bio-imaging applications. *Analyst* 138(9) (April 2): 2506–2515.

Li, M., P. Beard, P.A. Estes, M.K. Lyon, and R.L. Garcea. 1998. Intercapsomeric disulfide bonds in papillomavirus assembly and disassembly. *Journal of Virology* 72(3) (January 3): 2160–2167.

Li, M., T.P. Cripe, P.A. Estes, M.K. Lyon, R.C. Rose, and R.L. Garcea. 1997. Expression of the human papillomavirus type 11 L1 capsid protein in *Escherichia coli*: Characterization of protein domains involved in DNA binding and capsid assembly. *Journal of Virology* 71(4) (January 4): 2988–2995.

Li, P.P., A. Nakanishi, S.W. Clark, and H. Kasamatsu. 2002. Formation of transitory intrachain and interchain disulfide bonds accompanies the folding and oligomerization of simian virus 40 Vp1 in the cytoplasm. *Proceedings of the National Academy of Sciences of the United States of America* 99(3) (May 2): 1353–1358.

Li, P.P., A. Naknanishi, M.A. Tran, K.-I. Ishizu, M. Kawano, M. Phillips, H. Handa, R.C. Liddington, and H. Kasamatsu. 2003. Importance of Vp1 calcium-binding residues in assembly, cell entry, and nuclear entry of simian virus 40. *Journal of Virology* 77(13) (January 7): 7527–7538.

Li, Q., C. Cao, B. Chackerian, J. Schiller, M. Gordon, K.E. Ugen, and D. Morgan. 2004. Overcoming Antigen Masking of Anti-Amyloidbeta Antibodies Reveals Breaking of B Cell Tolerance by Virus-like Particles in Amyloidbeta Immunized Amyloid Precursor Protein Transgenic Mice. *BMC Neuroscience* 5(1): 21.

Liddington, R.C., Y. Yan, J. Moulai, R. Sahli, T.L. Benjamin, and S.C. Harrison. 1991. Structure of simian virus 40 at 3.8-Å Resolution. *Nature* 354(6351) (November 28): 278–284.

Liebl, D., F. Difato, L. Horníková, P. Mannová, J. Stokrová, and J. Forstová. 2006. Mouse Polyomavirus Enters Early Endosomes, Requires their acidic pH for productive infection, and meets transferrin Cargo in Rab11-positive endosomes. *Journal of Virology* 80(9) (May): 4610–4622.

Liew, M.W.O., A. Rajendran, and A.P.J. Middelberg. 2010. Microbial production of virus-like particle vaccine protein at gram-per-litre levels. *Journal of Biotechnology* 150(2): 224–231.

Lilley, B.N., J.M. Gilbert, H.L. Ploegh, and T.L. Benjamin. 2006. Murine polyomavirus requires the endoplasmic reticulum protein Derlin-2 to initiate infection. *Journal of Virology* 80(17) (September): 8739–8744.

Lin, M.-C., M. Wang, C.-Y. Fang, P.-L. Chen, C.-H. Shen, and D. Chang. 2014. Inhibition of BK virus replication in human kidney cells by BK virus large tumor antigen-specific shRNA delivered by JC virus-like particles. *Antiviral Research* 103 (March): 25–31.

Lipin, D.I., L.H.L. Lua, and A.P.J. Middelberg. 2008. Quaternary size distribution of soluble aggregates of glutathione-S-transferase-purified viral protein as determined by asymmetrical flow field flow fractionation and dynamic light scattering. *Journal of Chromatography A* 1190(1–2) (May 9): 204–214.

Lipovsky, A., A. Popa, G. Pimienta, M. Wyler, A. Bhan, L. Kuruvilla, M.-A. Guie et al. 2013. Genome-wide siRNA screen identifies the retromer as a cellular entry factor for human papillomavirus. *Proceedings of the National Academy of Sciences of the United States of America* 110(18) (April 30): 7452–7457.

Liu, C.K., G. Wei, and W.J. Atwood. 1998. Infection of glial cells by the human polyomavirus JC is mediated by an N-linked glycoprotein containing terminal alpha(2–6)-linked sialic acids. *Journal of Virology* 72(6) (June): 4643–4649.

Liu, D., Y. Zhang, X. Yu, C. Jiang, Y. Chen, Y. Wu, Y. Jin et al. 2007. Assembly and immunogenicity of human papillomavirus type 16 major capsid protein (HPV16 L1) in *Pichia pastoris*. *Chemical Research in Chinese Universities* 23(2) (March): 200–203.

Liu, W.J., L. Gissmann, X.Y. Sun, A. Kanjanahaluethai, M. Müller, J. Doorbar, and J. Zhou. 1997. Sequence close to the N-terminus of L2 protein is displayed on the surface of bovine papillomavirus type 1 virions. *Virology* 227(2) (January 20): 474–483.

Liu, W.J., X.S. Liu, K.N. Zhao, G.R. Leggatt, and I.H. Frazer. 2000. Papillomavirus virus-like particles for the delivery of multiple cytotoxic T cell epitopes. *Virology* 273(2) (August 1): 374–382.

Low, J.A., B. Magnuson, B. Tsai, and M.J. Imperiale. 2006. Identification of gangliosides GD1b and GT1b as receptors for BK virus. *Journal of Virology* 80(3) (February): 1361–1366.

Lu, B., A. Kumar, X. Castellsagué, and A. Giuliano. 2011. Efficacy and safety of prophylactic vaccines against cervical HPV infection and diseases among women: A systematic review & meta-analysis. *BMC Infectious Diseases* 11(1): 13.

Lund, P.E., R.C. Hunt, M.M. Gottesman, and C. Kimchi-Sarfaty. 2010. Pseudovirions as vehicles for the delivery of siRNA. *Pharmaceutical Research* 27(3) (March): 400–420.

Luo, C., H.H. Hirsch, J. Kant, and P. Randhawa. 2012. VP-1 quasispecies in human infection with polyomavirus BK. *Journal of Medical Virology* 84(1): 152–161.

Macadangdang, B., N. Zhang, P.E. Lund, A.H. Marple, M. Okabe, M.M. Gottesman, D.H. Appella, and C. Kimchi-Sarfaty. 2011. Inhibition of multidrug resistance by SV40 pseudovirion delivery of an antigene peptide nucleic acid (PNA) in cultured cells. *PLoS ONE* 6(3): e17981.

Mach, H., D.B. Volkin, R.D. Troutman, B. Wang, Z. Luo, K.U. Jansen, and L. Shi. 2006. Disassembly and reassembly of yeast-derived recombinant human papillomavirus virus-like particles (HPV VLPs). *Journal of Pharmaceutical Sciences* 95(10): 2195–2206.

Mackay, R.L. and R.A. Consigli. 1976. Early events in polyoma virus infection: Attachment, penetration, and nuclear entry. *Journal of Virology* 19(2) (August): 620–636.

Maclean, J., M. Koekemoer, A.J. Olivier, D. Stewart, I.I. Hitzeroth, T. Rademacher, R. Fischer, A.-L. Williamson, and E.P. Rybicki. 2007. Optimization of human papillomavirus type 16 (HPV-16) L1 expression in plants: Comparison of the suitability of different HPV-16 L1 gene variants and different cell-compartment localization. *Journal of General Virology* 88(5) (January 5): 1460–1469.

Maginnis, M.S. and W.J. Atwood. 2009. JC virus: An oncogenic virus in animals and humans? *Seminars in Cancer Biology* 19(4) The Polyomaviruses (August): 261–269.

Malagón, T., M. Drolet, M.-C. Boily, E.L. Franco, M. Jit, J. Brisson, and M. Brisson. 2012. Cross-protective efficacy of two human papillomavirus vaccines: A systematic review and meta-analysis. *The Lancet Infectious Diseases* 12(10) (October): 781–789.

Mannová, P. and J. Forstová. 2003. Mouse polyomavirus utilizes recycling endosomes for a traffic pathway independent of COPI vesicle transport. *Journal of Virology* 77(3) (February): 1672–1681.

Markowitz, L.E., E.F. Dunne, M. Saraiya, H.W. Lawson, H. Chesson, E.R. Unger, Centers for Disease Control and Prevention (CDC), and Advisory Committee on Immunization Practices (ACIP). 2007. Quadrivalent human papillomavirus vaccine: Recommendations of the advisory committee on immunization practices (ACIP). *Recommendations and Reports: Morbidity and Mortality Weekly Report. Recommendations and Reports/Centers for Disease Control* 56(RR-2) (March 23): 1–24.

Matić, S., V. Masenga, A. Poli, R. Rinaldi, R.G. Milne, M. Vecchiati, and E. Noris. 2012. Comparative analysis of recombinant human papillomavirus 8 L1 production in plants by a variety of expression systems and purification methods. *Plant Biotechnology Journal* 10(4): 410–421.

Mató, T., Z. Pénzes, P. Rueda, C. Vela, V. Kardi, A. Zolnai, F. Misák, and V. Palya. 2009. Recombinant subunit vaccine elicits protection against goose haemorrhagic nephritis and enteritis. *Avian Pathology* 38(3) (June): 233–237.

Maul, G.G., G. Rovera, A. Vorbrodt, and J. Abramczuk. 1978. Membrane fusion as a mechanism of simian virus 40 entry into different cellular compartments. *Journal of Virology* 28(3) (December): 936–944.

Maurer, P., G.T. Jennings, J. Willers, F. Rohner, Y. Lindman, K. Roubicek, W.A. Renner, P. Müller, and M.F. Bachmann. 2005. A therapeutic vaccine for nicotine dependence: Preclinical efficacy, and phase I safety and immunogenicity. *European Journal of Immunology* 35(7): 2031–2040.

May, T., S. Gleiter, and H. Lilie. 2002. Assessment of cell type specific gene transfer of polyoma virus like particles presenting a tumor specific antibody Fv fragment. *Journal of Virological Methods* 105(1) (August): 147–157.

Mazeike, E., A. Gedvilaite, and U. Blohm. 2012. Induction of insert-specific immune response in mice by hamster polyomavirus VP1 derived virus-like particles carrying LCMV GP33 CTL epitope. *Virus Research* 163(1) (January): 2–10.

McCarthy, M.P., W.I. White, F. Palmer-Hill, S. Koenig, and J.A. Suzich. 1998. Quantitative disassembly and reassembly of human papillomavirus type 11 viruslike particles in vitro. *Journal of Virology* 72(1) (January 1): 32–41.

McGrath, M., G.K. Villiers, E. Shephard, I.I. Hitzeroth, and E.P. Rybicki. 2013. Development of human papillomavirus chimeric L1/L2 candidate vaccines. *Archives of Virology* 158(10) (May 1): 2079–2088.

McMillan, N.A., E. Payne, I.H. Frazer, and M. Evander. 1999. Expression of the alpha6 integrin confers papillomavirus binding upon receptor-negative B-cells. *Virology* 261(2) (September 1): 271–279.

Mercer, J., M. Schelhaas, and A. Helenius. 2010. Virus entry by endocytosis. *Annual Review of Biochemistry* 79: 803–833.

Mérian, J., J. Gravier, F. Navarro, and I. Texier. 2012. Fluorescent nanoprobes dedicated to in vivo imaging: From preclinical validations to clinical translation. *Molecules* 17(5) (May 10): 5564–5591.

Meusser, B., C. Hirsch, E. Jarosch, and T. Sommer. 2005. ERAD: The long road to destruction. *Nature Cell Biology* 7(8) (August): 766–772.

Meyers, C., M.G. Frattini, J.B. Hudson, and L.A. Laimins. 1992. Biosynthesis of human papillomavirus from a continuous cell line upon epithelial differentiation. *Science* (New York) 257(5072) (August 14): 971–973.

Mezes, B. and P. Amati. 1994. Mutations of polyomavirus VP1 allow in vitro growth in undifferentiated cells and modify in vivo tissue replication specificity. *Journal of Virology* 68(2) (February): 1196–1199.

Michel, M.R., B. Hirt, and R. Weil. 1967. Mouse cellular DNA enclosed in polyoma viral capsids (pseudovirions). *Proceedings of the National Academy of Sciences of the United States of America* 58(4): 1381.

Middelberg, A.P.J., T. Rivera-Hernandez, N. Wibowo, L.H.L. Lua, Y. Fan, G. Magor, C. Chang, Y.P. Chuan, M.F. Good, and M.R. Batzloff. 2011. A microbial platform for rapid and low-cost virus-like particle and capsomere vaccines. *Vaccine* 29(41) (September 22): 7154–7162.

Millán, A.F.-S., S. Gómez-Sebastián, M.C. Nuñez, J. Veramendi, and J.M. Escribano. 2010. Human papillomavirus-like particles vaccine efficiently produced in a non-fermentative system based on insect larva. *Protein Expression and Purification* 74(1) (November): 1–8.

Miltz, A., H. Price, M. Shahmanesh, A. Copas, and R. Gilson. 2014. Systematic review and meta-analysis of L1-VLP-based human papillomavirus vaccine efficacy against anogenital pre-cancer in women with evidence of prior HPV exposure. ed. M. Scheurer. *PLoS ONE* 9(3) (March 3): e90348.

Montross, L., S. Watkins, R.B. Moreland, H. Mamon, D.L. Caspar, and R.L. Garcea. 1991. Nuclear assembly of polyomavirus capsids in insect cells expressing the major capsid protein VP1. *Journal of Virology* 65(9) (January 9): 4991–4998.

Moriyama, T., J.P. Marquez, T. Wakatsuki, and A. Sorokin. 2007. Caveolar endocytosis is critical for BK virus infection of human renal proximal tubular epithelial cells. *Journal of Virology* 81(16) (August): 8552–8562.

Moriyama, T. and A. Sorokin. 2008. Intracellular trafficking pathway of BK virus in human renal proximal tubular epithelial cells. *Virology* 371(2) (February 20): 336–349.

Mossadegh, N., L. Gissmann, M. Müller, H. Zentgraf, A. Alonso, and P. Tomakidi. 2004. Codon optimization of the human papillomavirus 11 (HPV 11) L1 gene leads to increased gene expression and formation of virus-like particles in mammalian epithelial cells. *Virology* 326(1) (August 15): 57–66.

Mueller, C., M.S. Strayer, J. Sirninger, S. Braag, F. Branco, J.-P. Louboutin, T.R. Flotte, and D.S. Strayer. 2010. In vitro and in vivo functional characterization of gutless recombinant SV40-derived CFTR vectors. *Gene Therapy* 17(2) (February): 227–237.

Mukherjee, S., M. Abd-El-Latif, M. Bronstein, O. Ben-nun -Shaul, S. Kler, and A. Oppenheim. 2007. High cooperativity of the SV40 major capsid protein VP1 in virus assembly. *PLoS ONE* 2(8) (August 22):e765 http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1942081/. Accessed September 19, 2013.

Mukherjee, S., S. Kler, A. Oppenheim, and A. Zlotnick. 2010. Uncatalyzed assembly of spherical particles from SV40 VP1 pentamers and linear dsDNA incorporates both low and high cooperativity elements. *Virology* 397(1): 199–204.

Muller, M., L. Gissmann, R.J. Cristiano, X.Y. Sun, I.H. Frazer, A.B. Jenson, A. Alonso, H. Zentgraf, and J. Zhou. 1995. Papillomavirus capsid binding and uptake by cells from different tissues and species. *Journal of Virology* 69(2) (February): 948–954.

Müller, M., J. Zhou, T.D. Reed, C. Rittmüller, A. Burger, J. Gabelsberger, J. Braspenning, and L. Gissmann. 1997. Chimeric papillomavirus-like particles. *Virology* 234(1) (July 21): 93–111.

Münger, K. and P.M. Howley. 2002. Human papillomavirus immortalization and transformation functions. *Virus Research* 89(2) (November): 213–228.

Muñoz, N., X. Castellsagué, A.B. de González, and L. Gissmann. 2006. Chapter 1: HPV in the etiology of human cancer. *Vaccine* 24 (August): S1–S10.

Nakanishi, A., B. Chapellier, N. Maekawa, M. Hiramoto, T. Kuge, R. Takahashi, H. Handa, and T. Imai. 2008. SV40 vectors carrying minimal sequence of viral origin with exchangeable capsids. *Virology* 379(1) (September 15): 110–117.

National Cancer Institute. 2014. Broad spectrum HPV (human papillomavirus) vaccine study in 16- to 26-year-old women (V503-001) 2014 (January 16). http://www.cancer.gov/clinicaltrials/search/view. Accessed July 16, 2014.

Naud, P.S., C.M. Roteli-Martins, N.S. De Carvalho, J.C. Teixeira, P.C. de Borba, N. Sanchez, T. Zahaf, G. Catteau, B. Geeraerts, and D. Descamps. 2014. Sustained efficacy, immunogenicity, and safety of the HPV-16/18 AS04-adjuvanted vaccine: Final analysis of a long-term follow-up study up to 9.4 years post-vaccination. *Human Vaccines & Immunotherapeutics* 10(8) (June 19): 2147–2162

Neeper, M.P., K.J. Hofmann, and K.U. Jansen. 1996. Expression of the major capsid protein of human papillomavirus type 11 in *Saccharomyces cerevisiae*. *Gene* 180(1–2) (November21): 1–6.

Nelson, C.D.S., D.W. Carney, A. Derdowski, A. Lipovsky, G.V. Gee, B. O'Hara, P. Williard, D. DiMaio, J.K. Sello, and W.J. Atwood. 2013. A retrograde trafficking inhibitor of ricin and shiga-like toxins inhibits infection of cells by human and monkey polyomaviruses. *mBio* 4(6): e00729–e00713.

Neu, U., H. Hengel, B.S. Blaum, R.M. Schowalter, D. Macejak, M. Gilbert, W.W. Wakarchuk et al. 2012. Structures of merkel cell polyomavirus VP1 complexes define a sialic acid binding site required for infection. *PLoS Pathogens* 8(7): e1002738.

Neu, U., Z.M. Khan, B. Schuch, A.S. Palma, Y. Liu, M. Pawlita, T. Feizi, and T. Stehle. 2013. Structures of B-lymphotropic polyomavirus VP1 in complex with oligosaccharide ligands. *PLoS Pathogens* 9(10) (October): e1003714.

Neu, U., M.S. Maginnis, A.S. Palma, L.J. Ströh, C.D.S. Nelson, T. Feizi, W.J. Atwood, and T. Stehle. 2010. Structure-function analysis of the human JC polyomavirus establishes the LSTc pentasaccharide as a functional receptor motif. *Cell Host & Microbe* 8(4) (October 21): 309–319.

Neu, U., J. Wang, D. Macejak, R.L. Garcea, and T. Stehle. 2011. Structuresofthemajorcapsidproteinsofthe Human Karolinska Institutet and Washington University Polyomaviruses. *Journal of Virology* 85(14) (July 15): 7384–7392.

Neu, U., K. Woellner, G. Gauglitz, and T. Stehle. 2008. Structural basis of GM1 ganglioside recognition by simian virus 40. *Proceedings of the National Academy of Sciences of the United States of America* 105(13) (April 1): 5219–5224.

Neugebauer, M., B.Walders, M. Brinkman, C. Ruehland,T. Schumacher, W.M. Bertling, E. Geuther et al. 2006. Development of a vaccine marker technology: Display of B cell epitopes on the surface of recombinant polyomavirus-like pentamers and capsids induces peptide-specific antibodies in piglets after vaccination. *Biotechnology Journal* 1(12) (December): 1435–1446.

Ng, J., O. Koechlin, M. Ramalho, D. Raman, and N. Krauzewicz. 2007. Extracellular self-assembly of virus-like particles from secreted recombinant polyoma virus major coat protein. *Protein Engineering, Design & Selection* 20(12) (December): 591–598. Nicol, J.T.J., R. Robinot, A. Carpentier, G. Carandina, E. Mazzoni, M. Tognon, A. Touzé, and P. Coursaget. 2013. Age-specific seroprevalences of merkel cell polyomavirus, human polyomaviruses 6, 7, and 9, and trichodysplasia spinulosa-associated polyomavirus. *Clinical and Vaccine Immunology* 20(3) (January 3): 363–368.

Nicol, J.T.J., A. Touzé, R. Robinot, F. Arnold, E. Mazzoni, M. Tognon, and P. Coursaget. 2012. Seroprevalence and Cross-Reactivity of Human Polyomavirus 9. *Emerging Infectious Diseases* 18(8) (August): 1329–1332.

Niikura, K., K. Nagakawa, N. Ohtake, T. Suzuki, Y. Matsuo, H. Sawa, and K. Ijiro. 2009. Gold nanoparticle arrangement on viral particles through carbohydrate recognition: A non-cross-linking approach to optical virus detection. *Bioconjugate Chemistry* 20(10) (October 21): 1848–1852.

Niikura, K., N. Sugimura, Y. Musashi, S. Mikuni, Y. Matsuo, S. Kobayashi, K. Nagakawa et al. 2013. Virus-like Particles with Removable Cyclodextrins Enable Glutathione-Triggered Drug Release in Cells. *Molecular BioSystems* 9(3): 501.

Nims, R.W. and M. Plavsic. 2013. Polyomavirus inactivation—A review. *Biologicals: Journal of the International Association of Biological Standardization* 41(2) (March): 63–70.

Norkin, L.C., H.A. Anderson, S.A. Wolfrom, and A. Oppenheim. 2002. Caveolar endocytosis of simian virus 40 is followed by Brefeldin A-Sensitive transport to the endoplasmic reticulum, where the virus disassembles. *Journal of Virology* 76(10) (May): 5156–5166.

Norkin, L.C. and D. Kuksin. 2005. The Caveolae-mediated sv40 entry pathway bypasses the golgi complex En Route to the endoplasmic reticulum. *Virology Journal* 2: 38.

O'Hara, S.D., T. Stehle, and R. Garcea. 2014. Glycan receptors of the polyomaviridae: Structure, function, and pathogenesis. *Current Opinion in Virology* 7C (June 28): 73–78.

Ohtake, N., K. Niikura, T. Suzuki, K. Nagakawa, S. Mikuni, Y. Matsuo, M. Kinjo, H. Sawa, and K. Ijiro. 2010. Low pH-triggered model drug molecule release from virus-like particles. *ChemBioChem* 11(7) (May 3): 959–962.

Olsson, S.-E., S.K. Kjaer, K. Sigurdsson, O.-E. Iversen, M. Hernandez-Avila, C.M. Wheeler, G. Perez et al. 2009. Evaluation of quadrivalent HPV 6/11/16/18 vaccine efficacy against cervical and anogenital disease in subjects with serological evidence of prior vaccine type HPV infection. *Human Vaccines* 5(10) (October): 696–704.

Olsson, S.-E., L.L. Villa, R.L.R. Costa, C.A. Petta, R.P. Andrade, C. Malm, O.-E. Iversen et al. 2007. Induction of immune memory following administration of a prophylactic quadrivalent human papillomavirus (HPV) types 6/11/16/18 L1 virus-like particle (VLP) vaccine. *Vaccine* 25(26) (June): 4931–4939.

Oppenheim, A. and A. Peleg. 1989. Helpers for efficient encapsidation of SV40 pseudovirions. *Gene* 77(1) (April 15): 79–86.

Oppenheim, A., A. Peleg, E. Fibach, and E.A. Rachmilewitz. 1986. Efficient introduction of plasmid DNA into human hemopoietic cells by encapsidation in simian virus 40 pseudovirions. *Proceedings of the National Academy of Sciences of the United States of America* 83(18): 6925–6929.

Oppenheim, A., Z. Sandalon, A. Peleg, O. Shaul, S. Nicolis, and S. Ottolenghi. 1992. A cis-acting DNA signal for encapsidation of simian virus 40. *Journal of Virology* 66(9) (January 9): 5320–5328.

Osterman, J.V., A. Waddell, and H.V. Aposhian. 1970. DNA and gene therapy: Uncoating of polyoma pseudovirus in mouse embryo cells. *Proceedings of the National Academy of Sciences of the United States of America* 67(1): 37–40.

Ou, W.C., M. Wang, C.Y. Fung, R.T. Tsai, P.C. Chao, T.H. Hseu, and D. Chang. 1999. The major capsid protein, VP1, of human JC virus expressed in *Escherichia coli* is able to self-assemble into a capsid-like particle and deliver exogenous DNA into human kidney cells. *Journal of General Virology* 80(1) (January 1): 39–46.

Ozbun, M.A. and M.P. Kivitz. 2012. The art and science of obtaining virion stocks for experimental human papillomavirus infections. In *Small DNA Tumour Viruses*, ed. K. Gaston, pp. 19–35. Norfolk, U.K.: Horizon Scientific and Caister Academic Press.

Paavonen, J., P. Naud, J. Salmeron, C.M. Wheeler, S.N. Chow, D. Apter, H. Kitchener, X. Castellsague, J.C. Teixeira, and S.R. Skinner. 2009. Efficacy of human papillomavirus (HPV)-16/18 AS04-adjuvanted vaccine against cervical infection and precancer caused by oncogenic HPV types (PATRICIA): Final analysis of a double-blind, randomised study in young women. *The Lancet* 374(9686): 301–314.

Palaniyandi, M., T. Kato, and E.Y. Park. 2012. Expression of human papillomavirus 6b L1 protein in silkworm larvae and enhanced green fluorescent protein displaying on its virus-like particles. *SpringerPlus* 1 (October 4). http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3725899/. Accessed July 14, 2014.

Palková, Z., T. Adamec, D. Liebl, J. Štokrová, and J. Forstová. 2000. Production of polyomavirus structural protein VP1 in yeast cells and its interaction with cell structures. *FEBS Letters* 478(3) (August 4): 281–289.

Park, M.-A., H.J. Kim, and H.-J. Kim. 2008. Optimum conditions for production and purification of human papillomavirus type 16 L1 protein from *Saccharomyces cerevisiae*. *Protein Expression and Purification* 59(1) (May): 175–181.

Pastrana, D.V., C.B. Buck, Y.-Y.S. Pang, C.D. Thompson, P.E. Castle, P.C. FitzGerald, S. Krüger Kjaer, D.R. Lowy, and J.T. Schiller. 2004. Reactivity of human sera in a sensitive, high-throughput pseudovirus-based papillomavirus neutralization assay for HPV16 and HPV18. *Virology* 321(2) (April 10): 205–216.

Pastrana, D.V., U. Ray, T.G. Magaldi, R.M. Schowalter, N. Çuburu, and C.B. Buck. 2013. BK polyomavirus genotypes represent distinct serotypes with distinct entry tropism. *Journal of Virology* 87(18) (September 15): 10105–10113.

Pastrana, D.V., Y.L. Tolstov, J.C. Becker, P.S. Moore, Y. Chang, and C.B. Buck. 2009. Quantitation of human seroresponsiveness to merkel cell polyomavirus. ed. R.L. Garcea. *PLoS Pathogens* 5(9) (September 11): e1000578.

Paulson, K.G., J.G. Iyer, A.R. Tegeder, R. Thibodeau, J. Schelter, S. Koba, D. Schrama et al. 2011. Transcriptome-wide studies of merkel cell carcinoma and validation of intratumoral CD8+ lymphocyte invasion as an independent predictor of survival. *Journal of Clinical Oncology* 29(12) (April 20): 1539–1546.

Pawlita, M., M. Müller, M. Oppenländer, H. Zentgraf, and M. Herrmann. 1996. DNA encapsidation by viruslike particles assembled in insect cells from the major capsid protein VP1 of B-lymphotropic papovavirus. *Journal of Virology* 70(11) (January 11): 7517–7526.

Peiler, T. 2004. Pseudovirionen zur Simulation von Infektionen mit humanpathogenen Papillomaviren. Dissertation. http://archiv.ub.uni-heidelberg.de/volltextserver/4831/. Accessed December 22, 2013.

Pejawar-Gaddy, S., Y. Rajawat, Z. Hilioti, J. Xue, D.F. Gaddy, O.J. Finn, R.P. Viscidi, and I. Bossis. 2010. Generation of a tumor vaccine candidate based on conjugation of a MUC1 peptide to polyionic papillomavirus virus-like particles. *Cancer Immunology, Immunotherapy* 59(11) (July 21): 1685–1696.

Pelkmans, L., T. Bürli, M. Zerial, and A. Helenius. 2004. Caveolin-stabilized membrane domains as multifunctional transport and sorting devices in endocytic membrane traffic. *Cell* 118(6) (September 17): 767–780.

Pelkmans, L., E. Fava, H. Grabner, M. Hannus, B. Habermann, E. Krausz, and M. Zerial. 2005. Genome-wide analysis of human kinases in clathrin- and caveolae/raft-mediated endocytosis. *Nature* 436(7047) (July 7): 78–86.

Pelkmans, L., J. Kartenbeck, and A. Helenius. 2001. Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER. *Nature Cell Biology* 3(5) (May): 473–483.

Pelkmans, L., D. Püntener, and A. Helenius. 2002. Local actin polymerization and dynamin recruitment in SV40-induced internalization of caveolae. *Science* (New York) 296(5567) (April 19): 535–539.

Peng, S., I.H. Frazer, G.J. Fernando, and J. Zhou. 1998. Papillomavirus virus-like particles can deliver defined CTL epitopes to the MHC class I pathway. *Virology* 240(1) (January 5): 147–157.

Pho, M.T., A. Ashok, and W.J. Atwood. 2000. JC virus enters human glial cells by clathrin-dependent receptor-mediated endocytosis. *Journal of Virology* 74(5) (March): 2288–2292.

Pipas, J.M. 2009. SV40: Cell Transformation and Tumorigenesis. Small Viruses, Big Discoveries: The Interwoven Story of the Small DNA Tumor Viruses. *Virology* 384(2): 294–303.

Pircher, H., D. Moskophidis, U. Rohrer, K. Bürki, H. Hengartner, and R.M. Zinkernagel. 1990. Viral escape by selection of cytotoxic T cell-resistant virus variants in vivo. *Nature* 346(6285) (August 16): 629–633.

Pruksakorn, S., B. Currie, E. Brandt, D. Martin, A. Galbraith, C. Phornphutkul, S. Hunsakunachai, A. Manmontri, and M.F. Good. 1994. Towards a vaccine for rheumatic fever: Identification of a conserved target epitope on M protein of group A streptococci. *Lancet* 344(8923) (September 3): 639–642.

Pyeon, D., P.F. Lambert, and P. Ahlquist. 2005. Production of infectious human papillomavirus independently of viral replication and epithelial cell differentiation. *Proceedings of the National Academy of Sciences of the United States of America* 102(26) (June 28): 9311–9316.

Pyeon, D., S.M. Pearce, S.M. Lank, P. Ahlquist, and P.F. Lambert. 2009. Establishment of human papillomavirus infection requires cell cycle progression. *PLoS Pathogens* 5(2) (February): e1000318.

Qasba, P.K. and H.V. Aposhian. 1971. DNA and gene therapy: Transfer of mouse DNA to human and mouse embryonic cells by polyoma pseudovirions. *Proceedings of the National Academy of Sciences of the United States of America* 68(10) (October): 2345–2349.

Qian, J., Y. Dong, Y.-Y.S. Pang, R. Ibrahim, J.A. Berzofsky, J.T. Schiller, and S.N. Khleif. 2006. Combined prophylactic and therapeutic cancer vaccine: Enhancing CTL responses to HPV16 E2 using a chimeric VLP in HLA-A2 mice. *International Journal of Cancer* 118(12) (June 15): 3022–3029.

Qian, M., D. Cai, K.J. Verhey, and B. Tsai. 2009. A lipid receptor sorts polyomavirus from the endolysosome to the endoplasmic reticulum to cause infection. *PLoS Pathogens* 5(6) (June): e1000465.

Qian, M. and B. Tsai. 2010. Lipids and proteins act in opposing manners to regulate polyomavirus infection. *Journal of Virology* 84(19) (October): 9840–9852.

Qu, Q., H. Sawa, T. Suzuki, S. Semba, C. Henmi, Y. Okada, M. Tsuda, S. Tanaka, W.J. Atwood, and K. Nagashima. 2004. Nuclear entry mechanism of the human polyomavirus JC virus-like particle: Role of importins and the nuclear pore complex. *The Journal of Biological Chemistry* 279(26) (June 25): 27735–27742.

Raff, A.B., A.W. Woodham, L.M. Raff, J.G. Skeate, L. Yan, D.M. Da Silva, M. Schelhaas, and W.M. Kast. 2013. The evolving field of human papillomavirus receptor research: A review of binding and entry. *Journal of Virology* 87(11) (June): 6062–6072.

Rainey-Barger, E.K., B. Magnuson, and B. Tsai. 2007. A chaperone-activated nonenveloped virus perforates the physiologically relevant endoplasmic reticulum membrane. *Journal of Virology* 81(23) (December): 12996–13004.

Ramqvist, T. and T. Dalianis. 2010. Lessons from immune responses and vaccines against murine polyomavirus infection and polyomavirus-induced tumours potentially useful for studies on human polyomaviruses. *Anticancer Research* 30(2): 279–284.

Rao, N.H., P.B. Babu, L. Rajendra, R. Sriraman, Y.-Y.S. Pang, J.T. Schiller, and V.A. Srinivasan. 2011. Expression of codon optimized major capsid protein (L1) of human papillomavirus type 16 and 18 in *Pichia pastoris*; purification and characterization of the virus-like particles. *Vaccine* 29(43) (October 6): 7326–7334.

Rayment, I., T.S. Baker, D.L. Caspar, and W.T. Murakami. 1982. Polyoma virus capsid structure at 22.5 A resolution. *Nature* 295(5845) (January 14): 110–115.

Rector, A. and M. Van Ranst. 2013. Animal papillomaviruses. *Virology* 445(1–2): 213–223.

Relf, W.A., J. Cooper, E.R. Brandt, W.A. Hayman, R.F. Anders, S. Pruksakorn, B. Currie et al. 1996. Mapping a conserved conformational epitope from the M protein of group A streptococci. *Pept Res.* 9(1) (Jan–Feb): 12–20.

Richards, K.F., M. Bienkowska-Haba, J. Dasgupta, X.S. Chen, and M. Sapp. 2013. Multiple heparan sulfate binding site engagements are required for the infectious entry of human papillomavirus type 16. *Journal of Virology* 87(21) (November): 11426–11437.

Richards, R.M., D.R. Lowy, J.T. Schiller, and P.M. Day. 2006. Cleavage of the papillomavirus minor capsid protein, L2, at a furin consensus site is necessary for infection. *Proceedings of the National Academy of Sciences of the United States of America* 103(5) (January 31): 1522–1527.

Richterová, Z., D. Liebl, M. Horák, Z. Palková, J. Stokrová, P. Hozák, J. Korb, and J. Forstová. 2001. Caveolae are involved in the trafficking of mouse polyomavirus virions and artificial VP1 pseudocapsids toward cell nuclei. *Journal of Virology* 75(22) (November): 10880–10891.

Rivera-Hernandez, T., J. Hartas, Y. Wu, Y.P. Chuan, L.H.L. Lua, M. Good, M.R. Batzloff, and A.P.J. Middelberg. 2013. Self-adjuvanting modular virus-like particles for mucosal vaccination against group A streptococcus (GAS). *Vaccine* 31(15) (April 8): 1950–1955.

Roberts, J.N., C.B. Buck, C.D. Thompson, R. Kines, M. Bernardo, P.L. Choyke, D.R. Lowy, and J.T. Schiller. 2007. Genital transmission of HPV in a mouse model is potentiated by nonoxynol-9 and inhibited by carrageenan. *Nature Medicine* 13(7) (July): 857–861.

Roden, R.B.S., W.H. Yutzy, R. Fallon, S. Inglis, D.R. Lowy, and J.T. Schiller. 2000. Minor capsid protein of human genital papillomaviruses contains subdominant, cross-neutralizing epitopes. *Virology* 270(2) (May): 254–257.

Rodgers, R.E., D. Chang, X. Cai, and R.A. Consigli. 1994. Purification of recombinant budgerigar fledgling disease virus VP1 capsid protein and its ability for in vitro capsid assembly. *Journal of Virology* 68(5): 3386–3390.

Rose, R.C., W. Bonnez, C. Da Rin, D.J. McCance, and R.C. Reichman. 1994. Serological differentiation of human papillomavirus types 11, 16 and 18 using recombinant virus-like particles. *The Journal of General Virology* 75(Pt 9) (September): 2445–2449.

Rose, R.C., W. Bonnez, R.C. Reichman, and R.L. Garcea. 1993. Expression of human papillomavirus type 11 L1 protein in insect cells: In vivo and in vitro assembly of viruslike particles. *Journal of Virology* 67(4) (April): 1936–1944.

Rose, R.C., C. Lane, S. Wilson, J.A. Suzich, E. Rybicki, and A.L. Williamson. 1999. Oral vaccination of mice with human papillomavirus virus-like particles induces systemic virus-neutralizing antibodies. *Vaccine* 17(17) (April 23): 2129–2135.

Rosen, J.E., S. Yoffe, A. Meerasa, M. Verma, and F.X. Gu. 2011. Nanotechnology and diagnostic imaging: New advances in contrast agent technology. *Journal of Nanomedicine & Nanotechnology* 02(05) (October 21):e1000115. http://www.omicsonline.org/2157-7439/2157-7439-2-115.digital/2157-7439-2-115.html. Accessed August 4, 2014.

Rossi, J.L., L. Gissmann, K. Jansen, and M. Müller. 2000. Assembly of human papillomavirus type 16 pseudovirions in *Saccharomyces cerevisiae*. *Human Gene Therapy* 11(8) (May 20): 1165–1176.

Rudolf, M.P., S.C. Fausch, D.M. Da Silva, and W.M. Kast. 2001. Human dendritic cells are activated by chimeric human papillomavirus type-16 virus-like particles and induce epitope-specific human T cell responses in vitro. *The Journal of Immunology* 166(10): 5917–5924.

Rund, D., M. Dagan, N. Dalyot-Herman, C. Kimchi-Sarfaty, P.V. Schoenlein, M.M. Gottesman, and A. Oppenheim. 1998. Efficient transduction of human hematopoietic cells with the human multidrug resistance gene 1 via SV40 pseudovirions. *Human Gene Therapy* 9(5) (March 20): 649–657.

Rybicki, E.P. 2009. Plant-produced vaccines: Promise and reality. *Drug Discovery Today* 14(1–2) (January): 16–24.

Sadeyen, J.-R., S. Tourne, M. Shkreli, P.-Y. Sizaret, and P. Coursaget. 2003. Insertion of a foreign sequence on capsid surface loops of human papillomavirus type 16 virus-like particles reduces their capacity to induce neutralizing antibodies and delineates a conformational neutralizing epitope. *Virology* 309(1) (April 25): 32–40.

Salunke, D.M., D.L. Caspar, and R.L. Garcea. 1989. Polymorphism in the assembly of polyomavirus capsid protein VP1. *Biophysical Journal* 56(5) (November 1): 887–900.

Salunke, D.M., D.L.D. Caspar, and R.L. Garcea. 1986. Self-assembly of purified polyomavirus capsid protein VP1. *Cell* 46(6) (September): 895–904.

Sandalon, Z., N. Dalyot-Herman, A.B. Oppenheim, and A. Oppenheim. 1997. In vitro assembly of SV40 virions and pseudovirions: Vector development for gene therapy. *Human Gene Therapy* 8(7) (May 1): 843–849.

Sandalon, Z. and A. Oppenheim. 1997. Self-assembly and protein–protein interactions between the SV40 capsid proteins produced in insect cells. *Virology* 237(2): 414–421.

Sanjuan, N., A. Porras, J. Otero, and S. Perazzo. 2001. Expression of major capsid protein VP-1 in the absence of viral particles in thymomas induced by murine polyomavirus. *Journal of Virology* 75(6) (March 15): 2891–2899.

Sasagawa, T., P. Pushko, G. Steers, S.E. Gschmeissner, M.A. Nasser Hajibagheri, J. Finch, L. Crawford, and M. Tommasino. 1995. Synthesis and assembly of virus-like particles of human papillomaviruses type 6 and type 16 in fission yeast *Schizosaccharomyces pombe*. *Virology* 206(1) (January 10): 126–135.

Sasnauskas, K., A. Bulavaite, A. Hale, L. Jin, W.A. Knowles, A. Gedvilaite, A. Dargeviciūte et al. 2002. Generation of recombinant virus-like particles of human and non-human polyomaviruses in yeast *Saccharomyces cerevisiae*. *Intervirology* 45(4–6): 308–317.

Sasnauskas, K., O. Buzaite, F. Vogel, B. Jandrig, R. Razanskas, J. Staniulis, S. Scherneck, D.H. Krüger, and R. Ulrich. 1999. Yeast cells allow high-level expression and formation of polyomavirus-like particles. *Biological Chemistry* 380(3) (March): 381–386.

Scheffer, K.D., A. Gawlitza, G.A. Spoden, X.A. Zhang, C. Lambert, F. Berditchevski, and L. Florin. 2013. Tetraspanin CD151 mediates papillomavirus type 16 endocytosis. *Journal of Virology* 87(6) (March): 3435–3446.

Scheffner, M., B.A. Werness, J.M. Huibregtse, A.J. Levine, and P.M. Howley. 1990. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell* 63(6) (December 21): 1129–1136.

Schelhaas, M., H. Ewers, M.-L. Rajamäki, P.M. Day, J.T. Schiller, and A. Helenius. 2008. Human papillomavirus type 16 entry: Retrograde cell surface transport along actin-rich protrusions. *PLoS Pathogens* 4(9): e1000148.

Schelhaas, M., J. Malmström, L. Pelkmans, J. Haugstetter, L. Ellgaard, K. Grünewald, and A. Helenius. 2007. Simian virus 40 depends on ER protein folding and quality control factors for entry into host cells. *Cell* 131(3) (November 2): 516–529.

Schelhaas, M., B. Shah, M. Holzer, P. Blattmann, L. Kühling, P.M. Day, J.T. Schiller, and A. Helenius. 2012. Entry of human papillomavirus type 16 by actin-dependent, clathrin- and lipid raft-independent endocytosis. *PLoS Pathogens* 8(4): e1002657.

Schellenbacher, C., R. Roden, and R. Kirnbauer. 2009. Chimeric L1–L2 virus-like particles as potential broad-spectrum human papillomavirus vaccines. *Journal of Virology* 83(19) (July 29): 10085–10095.

Schiller, J.T., P.M. Day, and R.C. Kines. 2010. Current understanding of the mechanism of HPV infection. *Gynecologic Oncology* 118(1 Suppl.) (June): S12–S17.

Schiller, J.T. and D. Nardelli-Haefliger. 2006. Chapter 17: Second generation HPV vaccines to prevent cervical cancer. *Vaccine* 24 (August): S147–S153.

Schmidt, U., J. Kenklies, R. Rudolph, and G. Böhm. 1999. Site-specific fluorescence labelling of recombinant polyomavirus-like particles. *Biological Chemistry* 380(3) (March): 397–401.

Schmidt, U., R. Rudolph, and G. Böhm. 2000. Mechanism of assembly of recombinant murine polyomavirus-like particles. *Journal of Virology* 74(4) (February 15): 1658–1662.

Schmidt, U., R. Rudolph, and G. Böhm. 2001. Binding of external ligands onto an engineered virus capsid. *Protein Engineering* 14(10) (January 10): 769–774.

Schneider, M.A., G.A. Spoden, L. Florin, and C. Lambert. 2011. Identification of the dynein light chains required for human papillomavirus infection. *Cellular Microbiology* 13(1) (January): 32–46.

Schowalter, R.M. and C.B. Buck. 2013. The merkel cell polyomavirus minor capsid protein. *PLoS Pathogens* 9(8) (August 22): e1003558.

Schowalter, R.M., D.V. Pastrana, and C.B. Buck. 2011. Glycosaminoglycans and sialylated glycans sequentially facilitate merkel cell polyomavirus infectious entry. *PLoS Pathogens* 7(7) (July): e1002161.

Schwarz, T.F., M. Kocken, T. Petäjä, M.H. Einstein, M. Spaczynski, J.A. Louwers, C. Pedersen et al. 2010. Correlation between levels of human papillomavirus (HPV)-16 and 18 antibodies in serum and cervicovaginal secretions in girls and women vaccinated with the HPV-16/18 AS04-adjuvanted vaccine. *Human Vaccines* 6(12) (December 1): 1054–1061.

Selinka, H.-C., T. Giroglou, and M. Sapp. 2002. Analysis of the infectious entry pathway of human papillomavirus type 33 pseudovirions. *Virology* 299(2) (August 1): 279–287.

Senger, T., L. Schädlich, L. Gissmann, and M. Müller. 2009. Enhanced papillomavirus-like particle production in insect cells. *Virology* 388(2): 344–353.

Serrano, B., L. Alemany, S. Tous, L. Bruni, G.M. Clifford, T. Weiss, F.X. Bosch, and S. de Sanjosé. 2012. Potential impact of a nine-valent vaccine in human papillomavirus related cervical disease. *Infect Agent Cancer* 7(1): 38.

Shi, L., G. Sanyal, A. Ni, Z. Luo, S. Doshna, B. Wang, T.L. Graham, N. Wang, and D.B. Volkin. 2005. Stabilization of human papillomavirus virus-like particles by non-ionic surfactants. *Journal of Pharmaceutical Sciences* 94(7) (July): 1538–1551.

Shin, Y.C. and W.R. Folk. 2003. Formation of polyomavirus-like particles with different VP1 molecules that bind the urokinase plasminogen activator receptor. *Journal of Virology* 77(21) (October 13): 11491–11498.

Shishido, Y., S. Nukuzuma, J. Mukaigawa, S. Morikawa, K. Yasui, and K. Nagashima. 1997. Assembly of JC virus-like particles in COS7 cells. *Journal of Medical Virology* 51(4) (April): 265–272.

Skrastina, D., A. Bulavaite, I. Sominskaya, L. Kovalevska, V. Ose, D. Priede, P. Pumpens, and K. Sasnauskas. 2008. High immunogenicity of a hydrophilic component of the hepatitis B virus preS1 sequence exposed on the surface of three virus-like particle carriers. *Vaccine* 26(16) (April): 1972–1981.

Slilaty, S.N., K.I. Berns, and H.V. Aposhian. 1982. Polyoma-like particle: Characterization of the DNA encapsidated in vitro by polyoma empty capsids. *The Journal of Biological Chemistry* 257(11) (October 6): 6571–6575.

Smith, J.L., S.K. Campos, A. Wandinger-Ness, and M.A. Ozbun. 2008. Caveolin-1-dependent infectious entry of human papillomavirus type 31 in human keratinocytes proceeds to the endosomal pathway for pH-dependent uncoating. *Journal of Virology* 82(19) (October): 9505–9512.

Soeda, E., N. Krauzewicz, C. Cox, J. Stokrová, J. Forstová, and B.E. Griffin. 1998. Enhancement by polylysine of transient, but not stable, expression of genes carried into cells by polyoma VP1 pseudocapsids. *Gene Therapy* 5(10) (October): 1410–1419.

Španielová, H., M. Fraiberk, J. Suchanová, J. Soukup, and J. Forstová. 2014. The encapsidation of polyomavirus is not defined by a sequence-specific encapsidation signal. *Virology* 450–451: 122–131.

Spoden, G., K. Freitag, M. Husmann, K. Boller, M. Sapp, C. Lambert, and L. Florin. 2008. Clathrin- and caveolin-independent entry of human papillomavirus type 16—Involvement of tetraspanin-enriched microdomains (TEMs). *PLoS ONE* 3(10): e3313.

Spoden, G., L. Kühling, N. Cordes, B. Frenzel, M. Sapp, K. Boller, L. Florin, and M. Schelhaas. 2013. Human papillomavirus types 16, 18, and 31 share similar endocytic requirements for entry. *Journal of Virology* 87(13) (July): 7765–7773.

Stamatos, N.M., S. Chakrabarti, B. Moss, and J.D. Hare. 1987. Expression of polyomavirus virion proteins by a vaccinia virus vector: Association of VP1 and VP2 with the nuclear framework. *Journal of Virology* 61(2) (February): 516–525.

Stanley, M., L. Gissmann, and D. Nardelli-Haefliger. 2008. Immunobiology of human papillomavirus infection and vaccination—Implications for second generation vaccines. *Vaccine* 26 (August): K62–K67.

Stanley, M., D.R. Lowy, and I. Frazer. 2006. Chapter 12: Prophylactic HPV vaccines: Underlying mechanisms. *Vaccine* 24 (August): S106–S113.

Stauffer, Y., K. Raj, K. Masternak, and P. Beard. 1998. Infectious human papillomavirus type 18 pseudovirions. *Journal of Molecular Biology* 283(3): 529–536.

Stehle, T., S.J. Gamblin, Y. Yan, and S.C. Harrison. 1996. The structure of simian virus 40 refined at 3.1 Å resolution. *Structure* 4(2): 165–182.

Stehle, T. and S.C. Harrison. 1996. Crystal structures of murine polyomavirus in complex with straight-chain and branched-chain sialyloligosaccharide receptor fragments. *Structure* (London, England: 1993) 4(2) (February 15): 183–194.

Stehle, T., Y. Yan, T.L. Benjamin, and S.C. Harrison. 1994. Structure of murine polyomavirus complexed with an oligosaccharide receptor fragment. *Nature* 369(6476) (May 12): 160–163.

Štokrová, J., Z. Palková, L. Fischer, Z. Richterová, J. Korb, B.E. Griffin, and J. Forstová. 1999. Interactions of heterologous DNA with polyomavirus major structural protein, VP1. *FEBS Letters* 445(1): 119–125.

Stolt, A., K. Sasnauskas, P. Koskela, M. Lehtinen, and J. Dillner. 2003. Seroepidemiology of the human polyomaviruses. *The Journal of General Virology* 84(Pt 6) (June): 1499–1504.

Strable, E. and M.G. Finn. 2009. Chemical modification of viruses and virus-like particles. *Current Topics in Microbiology and Immunology* 327: 1–21.

Ströh, L.J., U. Neu, B.S. Blaum, M.H.C. Buch, R.L. Garcea, and T. Stehle. 2014. Structure analysis of the major capsid proteins of the human polyomavirus 6 and 7 reveals an obstructed sialic acid binding site. *Journal of Virology* 88(18) (July 9): 10831–10839.

Stubenrauch, K., A. Bachmann, R. Rudolph, and H. Lilie. 2000. Purification of a viral coat protein by an engineered polyionic sequence. *Journal of Chromatography B: Biomedical Sciences and Applications* 737(1–2) (January 14): 77–84.

Stubenrauch, K., S. Gleiter, U. Brinkmann, R. Rudolph, and H. Lilie. 2001. Conjugation of an antibody Fv fragment to a virus coat protein: Cell-specific targeting of recombinant polyoma-virus-like particles. *The Biochemical Journal* 356(Pt 3) (June 15): 867–873.

Sundberg, J.P., M.V. Ranst, R. Montali, B.L. Homer, W.H. Miller, P.H. Rowland, D.W. Scott et al. 2000. Feline papillomas and papillomaviruses. *Veterinary Pathology Online* 37(1) (January 1): 1–10.

Surviladze, Z., A. Dziduszko, and M.A. Ozbun. 2012. Essential roles for soluble virion-associated heparan sulfonated proteoglycans and growth factors in human papillomavirus infections. *PLoS Pathogens* 8(2): e1002519.

Surviladze, Z., R.T. Sterk, S.A. DeHaro, and M.A. Ozbun. 2013. Cellular entry of human papillomavirus type 16 involves activation of the phosphatidylinositol 3-Kinase/Akt/mTOR pathway and inhibition of autophagy. *Journal of Virology* 87(5) (March): 2508–2517.

Suzich, J.A., S.J. Ghim, F.J. Palmer-Hill, W.I. White, J.K. Tamura, J.A. Bell, J.A. Newsome, A.B. Jenson, and R. Schlegel. 1995. Systemic immunization with papillomavirus L1 protein

completely prevents the development of viral mucosal papillomas. *Proceedings of the National Academy of Sciences of the United States of America* 92(25) (December 5): 11553–11557.

Swimm, A.I., W. Bornmann, M. Jiang, M.J. Imperiale, A.E. Lukacher, and D. Kalman. 2010. Abl family tyrosine kinases regulate sialylated ganglioside receptors for polyomavirus. *Journal of Virology* 84(9) (May): 4243–4251.

Szomolanyi-Tsuda, E., J.D. Brien, J.E. Dorgan, R.L. Garcea, R.T. Woodland, and R.M. Welsh. 2001. Antiviral T-cell-independent type 2 antibody responses induced in vivo in the absence of T and NK cells. *Virology* 280(2) (February): 160–168.

Szomolanyi-Tsuda, E., Q.P. Le, R.L. Garcea, and R.M. Welsh. 1998. T-cell-independent immunoglobulin G responses in vivo are elicited by live-virus infection but not by immunization with viral proteins or virus-like particles. *Journal of Virology* 72(8): 6665–6670.

Takahashi, R., S. Kanesashi, T. Inoue, T. Enomoto, M. Kawano, H. Tsukamoto, F. Takeshita et al. 2008. Presentation of functional foreign peptides on the surface of SV40 virus-like particles. *Journal of Biotechnology* 135(4) (July): 385–392.

Talmage, D.A., R. Freund, T. Dubensky, M. Salcedo, P. Gariglio, L.M. Rangel, C.J. Dawe, and T.L. Benjamin. 1992. Heterogeneity in state and expression of viral DNA in polyoma virus-induced tumors of the mouse. *Virology* 187(2) (April): 734–747.

Tegerstedt, K., K. Andreasson, A. Vlastos, K.O. Hedlund, T. Dalianis, and T. Ramqvist. 2003. Murine pneumotropic virus VP1 virus-like particles (VLPs) bind to several cell types independent of sialic acid residues and do not serologically cross react with murine polyomavirus VP1 VLPs. *Journal of General Virology* 84(12) (January 12): 3443–3452.

Tegerstedt, K., A. Franzén, T. Ramqvist, and T. Dalianis. 2007. Dendritic cells loaded with polyomavirus VP1/VP2Her2 virus-like particles efficiently prevent outgrowth of a Her2/neu expressing tumor. *Cancer Immunology, Immunotherapy* 56(9) (June 26): 1335–1344.

Tegerstedt, K., A.V. Franzén, K. Andreasson, J. Joneberg, S. Heidari, T. Ramqvist, and T. Dalianis. 2005a. Murine polyomavirus virus-like particles (VLPs) as vectors for gene and immune therapy and vaccines against viral infections and cancer. *Anticancer Research* 25(4) (January 7): 2601–2608.

Tegerstedt, K., J.A. Lindencrona, C. Curcio, K. Andreasson, C. Tullus, G. Forni, T. Dalianis, R. Kiessling, and T. Ramqvist. 2005b. A single vaccination with polyomavirus VP1/VP2Her2 virus-like particles prevents outgrowth of HER-2/neu-expressing tumors. *Cancer Research* 65(13): 5953–5957.

Teunissen, E.A., M. de Raad, and E. Mastrobattista. 2013. Production and biomedical applications of virus-like particles derived from polyomaviruses. *Journal of Controlled Release* 172(1) (November): 305–321.

The FUTURE I/II Study Group, J. Dillner, S.K. Kjaer, C.M. Wheeler, K. Sigurdsson, O.E. Iversen, M. Hernandez-Avila et al. 2010. Four year efficacy of prophylactic human papillomavirus quadrivalent vaccine against low grade cervical, vulvar, and vaginal intraepithelial neoplasia and anogenital warts: Randomised controlled trial. *BMJ* 341 (July 20): c3493.

Thyagarajan, R., N. Arunkumar, and W. Song. 2003. Polyvalent antigens stabilize B cell antigen receptor surface signaling microdomains. *The Journal of Immunology* 170(12) (June 15): 6099–6106.

Tobery, T.W., J.F. Smith, N. Kuklin, D. Skulsky, C. Ackerson, L. Huang, L. Chen, J.C. Cook, W.L. McClements, and K.U. Jansen. 2003. Effect of vaccine delivery system on the induction of HPV16L1-specific humoral and cell-mediated immune responses in immunized rhesus macaques. *Vaccine* 21(13–14) (March 28): 1539–1547.

Tolstov, Y.L., D.V. Pastrana, H. Feng, J.C. Becker, F.J. Jenkins, S. Moschos, Y. Chang, C.B. Buck, and P.S. Moore. 2009. Human merkel cell polyomavirus infection II. MCV is a common human infection that can be detected by conformational capsid epitope immunoassays. *International Journal of Cancer* (*Journal International Du Cancer*) 125(6) (September 15): 1250–1256.

Touzé, A., L. Bousarghin, C. Ster, A.-L. Combita, P. Roingeard, and P. Coursaget. 2001. Gene transfer using human polyomavirus BK virus-like particles expressed in insect cells. *Journal of General Virology* 82(12) (January 12): 3005–3009.

Touzé, A. and P. Coursaget. 1998. In vitro gene transfer using human papillomavirus-like particles. *Nucleic Acids Research* 26(5) (March 1): 1317–1323.

Touzé, A., C. Dupuy, M. Chabaud, P. Le Cann, and P. Coursaget. 1996. Production of human papillomavirus type 45 virus-like particles in insect cells using a recombinant baculovirus. *FEMS Microbiology Letters* 141(1) (July 15): 111–116.

Touzé, A., J. Gaitan, F. Arnold, R. Cazal, M.J. Fleury, N. Combelas, P.-Y. Sizaret et al. 2010. Generation of merkel cell polyomavirus (MCV)-like particles and their application to detection of MCV antibodies. *Journal of Clinical Microbiology* 48(5) (January 5): 1767–1770.

Touzé, A., S.E. Mehdaoui, P.-Y. Sizaret, C. Mougin, N. Muñoz, and P. Coursaget. 1998. The L1 major capsid protein of human papillomavirus type 16 variants affects yield of virus-like particles produced in an insect cell expression system. *Journal of Clinical Microbiology* 36(7) (January 7): 2046–2051.

Tsai, B., J.M. Gilbert, T. Stehle, W. Lencer, T.L. Benjamin, and T.A. Rapoport. 2003. Gangliosides are receptors for murine polyoma virus and SV40. *The EMBO Journal* 22(17) (September 1): 4346–4355.

Tsukamoto, H., M. Kawano, T. Inoue, T. Enomoto, R. Takahashi, N. Yokoyama, N. Yamamoto et al. 2007. Evidence that SV40 VP1–DNA interactions contribute to the assembly of 40-Nm spherical viral particles. *Genes to Cells* 12(11): 1267–1279.

Unckell, F., R.E. Streeck, and M. Sapp. 1997. Generation and neutralization of pseudovirions of human papillomavirus type 33. *Journal of Virology* 71(4) (January 4): 2934–2939.

Van Regenmortel, M.H.V., D.H.L. Bishop, and C.M. Fauquet. 1999. *Virus Taxonomy: Seventh Report of the International Committee on Taxonomy of Viruses*. San Diego, CA: Academic.

Varsani, A., A.-L. Williamson, R.C. Rose, M. Jaffer, and E.P. Rybicki. 2003. Expression of human papillomavirus type 16 major capsid protein in transgenic Nicotiana tabacum cv. Xanthi. *Archives of Virology* 148(9) (September 1): 1771–1786.

Velupillai, P., R.L. Garcea, and T.L. Benjamin. 2006. Polyoma virus-like particles elicit polarized cytokine responses in APCs from tumor-susceptible and-resistant mice. *The Journal of Immunology* 176(2): 1148–1153.

Villa, L.L., R.L.R. Costa, C.A. Petta, R.P. Andrade, J. Paavonen, O.-E. Iversen, S.-E. Olsson et al. 2006. High sustained efficacy of a prophylactic quadrivalent human papillomavirus types 6/11/16/18 L1 virus-like particle vaccine through 5 years of follow-up. *British Journal of Cancer* 95(11) (December 4): 1459–1466.

Vlastos, A., K. Andreasson, K. Tegerstedt, D. Holländerová, S. Heidari, J. Forstová, T. Ramqvist, and T. Dalianis. 2003. VP1 pseudocapsids, but not a glutathione-S-transferase VP1 fusion protein, prevent polyomavirus infection in a T-cell immune deficient experimental mouse model. *Journal of Medical Virology* 70(2) (June): 293–300.

Volpers, C., P. Schirmacher, R.E. Streeck, and M. Sapp. 1994. Assembly of the major and the minor capsid protein of human papillomavirus type 33 into Virus-like particles and tubular structures in insect cells. *Virology* 200(2) (May 1): 504–512.

Volpers, C., F. Unckell, P. Schirmacher, R.E. Streeck, and M. Sapp. 1995. Binding and internalization of human papillomavirus type 33 virus-like particles by eukaryotic cells. *Journal of Virology* 69(6) (January 6): 3258–3264.

Voronkova, T., A. Kazaks, V. Ose, M. Özel, S. Scherneck, P. Pumpens, and R. Ulrich. 2007. Hamster polyomavirus-derived virus-like particles are able to transfer in vitro encapsidated plasmid DNA to mammalian cells. *Virus Genes* 34(3) (June 1): 303–314.

Waehler, R., S.J. Russell, and D.T. Curiel. 2007. Engineering targeted viral vectors for gene therapy. *Nature Reviews Genetics* 8(8) (August): 573–587.

Wagner, E., K. Zatloukal, M. Cotten, H. Kirlappos, K. Mechtler, D.T. Curiel, and M.L. Birnstiel. 1992. Coupling of adenovirus to transferrin-polylysine/DNA complexes greatly enhances receptor-mediated gene delivery and expression of transfected genes. *Proceedings of the National Academy of Sciences of the United States of America* 89(13) (July 1): 6099–6103.

Wakabayashi, M.T., D.M. Da Silva, R.K. Potkul, and W.M. Kast. 2002. Comparison of human papillomavirus type 16 L1 chimeric virus-like particles versus L1/l2 chimeric virus-like particles in tumor prevention. *Intervirology* 45(4–6): 300–307.

Wang, M., T.-H. Tsou, L.-S. Chen, W.-C. Ou, P.-L. Chen, C.-F. Chang, C.-Y. Fung, and D. Chang. 2004. Inhibition of simian virus 40 large tumor antigen expression in human fetal glial cells by an antisense oligodeoxynucleotide delivered by the JC virus-like particle. *Human Gene Therapy* 15(11) (November 1): 1077–1090.

Wang, T., Z. Zhang, D. Gao, F. Li, H. Wei, X. Liang, Z. Cui, and X.-E. Zhang. 2011. Encapsulation of gold nanoparticles by simian virus 40 capsids. *Nanoscale* 3(10): 4275.

Wang, X., J. Liu, Y. Zheng, J. Li, H. Wang, Y. Zhou, M. Qi, H. Yu, W. Tang, and W.M. Zhao. 2008. An optimized yeast cell-free system: Sufficient for translation of human papillomavirus 58 L1 mRNA and assembly of virus-like particles. *Journal of Bioscience and Bioengineering* 106(1): 8–15.

Warzecha, H., H.S. Mason, C. Lane, A. Tryggvesson, E. Rybicki, A.-L. Williamson, J.D. Clements, and R.C. Rose. 2003. Oral immunogenicity of human papillomavirus-like particles expressed in potato. *Journal of Virology* 77(16) (August 15): 8702–8711.

Wen, A.M., S. Shukla, P. Saxena, A.A.A. Aljabali, I. Yildiz, S. Dey, J.E. Mealy et al. 2012. Interior engineering of a viral nanoparticle and its tumor homing properties. *Biomacromolecules* 13(12) (December 10): 3990–4001.

Westra, T.A., I. Stirbu-Wagner, S. Dorsman, E.D. Tutuhatunewa, E.L. de Vrij, H.W. Nijman, T. Daemen, J.C. Wilschut, and M.J. Postma. 2013. Inclusion of the benefits of enhanced cross-protection against cervical cancer and prevention of genital warts in the cost-effectiveness analysis of human papillomavirus vaccination in the Netherlands. *BMC Infectious Diseases* 13(1): 75.

Weyermann, J., D. Lochmann, and A. Zimmer. 2004. Comparison of antisense oligonucleotide drug delivery systems. *Journal of Controlled Release* 100(3) (December 10): 411–423.

Wheeler, C.M., X. Castellsagué, S.M. Garland, A. Szarewski, J. Paavonen, P. Naud, J. Salmerón, S.-N. Chow, D. Apter, and H. Kitchener. 2012. Cross-protective efficacy of HPV-16/18 AS04-adjuvanted vaccine against cervical infection and precancer caused by non-vaccine oncogenic HPV types: 4-Year end-of-study analysis of the randomised, double-blind PATRICIA trial. *The Lancet Oncology* 13(1): 100–110.

WHO. 2009. Human papillomavirus vaccines. WHO position paper. Weekly Epidemiological Record no. 84, 15, pp. 117–132. http://www.who.int/wer/2009/wer8415.pdf?ua = 1. Accessed July 14, 2014.

WHO. 2014. WHO | Human Papillomavirus (HPV). WHO. http://www.who.int/immunization/diseases/hpv/en/. Accessed July 14, 2014.

Windram, O.P., B. Weber, M.A. Jaffer, E.P. Rybicki, D.N. Shepherd, and A. Varsani. 2008. An investigation into the use of human papillomavirus type 16 virus-like particles as a delivery vector system for foreign proteins: N- and C-terminal fusion of GFP to the L1 and L2 capsid proteins. *Archives of Virology* 153(3) (January 4): 585–589.

Wolf, M., R.L. Garcea, N. Grigorieff, and S.C. Harrison. 2010. Subunit interactions in bovine papillomavirus. *Proceedings of the National Academy of Sciences of the United States of America* 107(14) (June 4): 6298–6303.

Wong, S.S. 1991. *Chemistry of Protein Conjugation and Cross-Linking*. Boca Raton, FL: CRC Press.

Woo, M.-K., S.-J. Hur, S. Park, and H.-J. Kim. 2007. Study of cell-mediated response in mice by HPV16 L1 virus-like particles expressed in *Saccharomyces cerevisiae*. *Journal of Microbiology and Biotechnology* 17(10) (October): 1738–1741.

Woodham, A.W., D.M. Da Silva, J.G. Skeate, A.B. Raff, M.R. Ambroso, H.E. Brand, J.M. Isas, R. Langen, and W.M. Kast. 2012. The S100A10 subunit of the annexin A2 heterotetramer facilitates L2-mediated human papillomavirus infection. *PLoS ONE* 7(8): e43519.

Wróbel, B., Y. Yosef, A.B. Oppenheim, and A. Oppenheim. 2000. Production and purification of SV40 major capsid protein (VP1) in *Escherichia coli* strains deficient for the GroELS chaperone machine. *Journal of Biotechnology* 84(3) (December 28): 285–289.

Xu, X., T. Zhang, Y. Xu, and D. Fan. 2014. Virus-like particles of capsid proteins from human papillomavirus type 16/58/18/6/11 and the method for preparation and the uses thereof. Patent no. CN101148661 B 2013-01-02.

Xu, Y., H. Zhang, and X. Xu. 2008. Enhancement of vaccine potency by fusing modified LTK63 into human papillomavirus type 16 chimeric virus-like particles. *FEMS Immunology & Medical Microbiology* 52(1) (January): 99–109.

Yang, R., F.M. Murillo, M.J. Delannoy, R.L. Blosser, W.H. Yutzy, S. Uematsu, K. Takeda, S. Akira, R.P. Viscidi, and R.B. Roden. 2005. B lymphocyte activation by human papillomavirus-like particles directly induces Ig class switch recombination via TLR4-MyD88. *The Journal of Immunology* 174(12): 7912–7919.

Yeager, M.D., M. Aste-Amezaga, D.R. Brown, M.M. Martin, M.J. Shah, J.C. Cook, N.D. Christensen et al. 2000. Neutralization of human papillomavirus (HPV) pseudovirions: A novel and efficient approach to detect and characterize HPV neutralizing antibodies. *Virology* 278(2) (December 20): 570–577.

Zamora, E., A. Handisurya, S. Shafti-Keramat, D. Borchelt, G. Rudow, K. Conant, C. Cox, J.C. Troncoso, and R. Kirnbauer. 2006. Papillomavirus-like particles are an effective platform for amyloid-B immunization in rabbits and transgenic mice. *The Journal of Immunology* 177(4): 2662–2670.

Zanotto, C., E. Pozzi, S. Pacchioni, M. Bissa, C.D.G. Morghen, and A. Radaelli. 2011. Construction and characterisation of a recombinant fowlpox virus that expresses the human papilloma virus L1 protein. *Journal of Translational Medicine* 9(1) (November 4): 190.

Zhao, K.-N., K. Hengst, W.-J. Liu, Y.H. Liu, X.S. Liu, N.A.J. McMillan, and I.H. Frazer. 2000. BPV1 E2 protein enhances packaging of full-length plasmid DNA in BPV1 pseudovirions. *Virology* 272(2): 382–393.

Zhao, Q., M.J. Allen, Y. Wang, B. Wang, N. Wang, L. Shi, and R.D. Sitrin. 2012a. Disassembly and reassembly improves morphology and thermal stability of human papillomavirus type 16 virus-like particles. *Nanomedicine: Nanotechnology, Biology and Medicine* 8(7): 1182–1189.

Zhao, Q., Y. Modis, K. High, V. Towne, Y. Meng, Y. Wang, J. Alexandroff et al. 2012b. Disassembly and reassembly of human papillomavirus virus-like particles produces more virion-like antibody reactivity. *Virology Journal* 9 (February 22): 52.

Zheng, J., X. Yang, Y. Sun, B. Lai, and Y. Wang. 2008. Stable high-level expression of truncated human papillomavirus type 16 L1 protein in Drosophila schneider-2 cells. *Acta Biochimica et Biophysica Sinica* 40(5) (January 5): 437–442.

Zhou, J., W.J. Liu, S.W. Peng, X.Y. Sun, and I. Frazer. 1999. Papillomavirus capsid protein expression level depends on the match between codon usage and tRNA availability. *Journal of Virology* 73(6) (January 6): 4972–4982.

Zhou, J., D.J. Stenzel, X.-Y. Sun, and I.H. Frazer. 1993. Synthesis and assembly of infectious bovine papillomavirus particles in vitro. *Journal of General Virology* 74(4) (January 4): 763–768.

Zhou, J., X.Y. Sun, D.J. Stenzel, and I.H. Frazer. 1991. Expression of vaccinia recombinant HPV 16 L1 and L2 ORF proteins in epithelial cells is sufficient for assembly of HPV virion-like particles. *Virology* 185(1) (November): 251–257.

Zielonka, A., A. Gedvilaite, J. Reetz, U. Rösler, H. Müller, and R. Johne. 2012. Serological cross-reactions between four polyomaviruses of birds using virus-like particles expressed in yeast. *Journal of General Virology* 93(Pt 12) (January 12): 2658–2667.

Zielonka, A., A. Gedvilaite, R. Ulrich, D. Lüschow, K. Sasnauskas, H. Müller, and R. Johne. 2006. Generation of virus-like particles consisting of the major capsid protein VP1 of goose hemorrhagic polyomavirus and their application in serological tests. *Virus Research* 120(1–2) (September): 128–137.

Zielonka, A., E.J. Verschoor, A. Gedvilaite, U. Roesler, H. Müller, and R. Johne. 2011. Detection of chimpanzee polyomavirus-specific antibodies in captive and wild-caught chimpanzees using yeast-expressed virus-like particles. *Virus Research* 155(2): 514–519.

Zila, V., F. Difato, L. Klimova, S. Huerfano, and J. Forstova. 2014. Involvement of microtubular network and its motors in productive endocytic trafficking of mouse polyomavirus. *PLoS ONE* 9(5): e96922.

zur Hausen, H. 2001. Proliferation-inducing viruses in non-permissive systems as possible causes of human cancers. *The Lancet* 357(9253): 381–384.

zur Hausen, H. 2002. Papillomaviruses and cancer: From basic studies to clinical application. *Nature Reviews Cancer* 2(5) (May): 342.

zur Hausen, H. 2008. Novel human polyomaviruses—Re-Emergence of a well known virus family as possible human carcinogens. *International Journal of Cancer* 123(2): 247–250.

Zurhein, G. and S.M. Chou. 1965. Particles resembling papova viruses in human cerebral demyelinating disease. *Science* (New York) 148(3676) (June 11): 1477–1479.

Zvirbliene, A., L. Samonskyte, A. Gedvilaite, T. Voronkova, R. Ulrich, and K. Sasnauskas. 2006. Generation of monoclonal antibodies of desired specificity using chimeric polyomavirus-derived virus-like particles. *Journal of Immunological Methods* 311(1–2) (April): 57–70.