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The role of furin in human papillomavirus infection

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THE ROLE OF FURIN IN HUMAN PAPILLOMAVIRUS INFECTION

by

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THESIS

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DEDICATION

To my children: Vidalia, Isaias and Andres. Without their unending love and patience I would not have made it through. Thank you.

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The Role of Furin in Human Papillomavirus Infection

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Abstract

Human papillomaviruses (HPVs) are the causative agent of cervical cancer and infect skin and mucosal membranes. Through wounding the virus establishes infection in the basal layer of human keratinocytes and requires differentiating squamous epithelium to complete the viral life cycle. HPV virions contain two structural viral proteins: the major capsid protein L1 that self-assembles into capsid structures, and the minor capsid protein, L2, that is essential for infection. It is believed that HPV cellular entry occurs via receptor-mediated endocytosis and that the primary attachment factors used by the virus are heparan sulfonated proteoglycans (HSPGs). Most current HPV literature follows an entry model whereby L1 binds to HSPGs, causing a conformational change in the capsid, thus allowing access to a furin/proprotein convertase (PC) cleavage site on L2. Once L2 is furin-cleaved, the virus binds to a yet unknown secondary internalization receptor(s). There is, however, a body of experimental evidence that does not support this entry model. For example, L1-only virus like particles (VLPs) are able to enter cells with similar kinetics as L1/L2 VLPs and mutagenesis of the L2 furin cleavage site does not abrogate the virion's ability to enter the cell. Furthermore, recent research in our

laboratory indicates an alternative mechanism of HPV infection, whereby the virus binds to cell-anchored HSPGs that are normally in complex with a variety of ligands including growth factors (GF), cytokines, extracellular matrix (ECM) proteins, and proteinases. Ectodomain shedding of syndecan-1 and HSPG by matrix metalloproteinases (MMPs) allows the HPV-HSPG-GF complexes to become soluble and move to secondary GF receptors, thereby allowing the virus to gain access to the cell. While MMPs are known to activate many biological molecules, they themselves are activated by furin/PCs. Due to these new observations and the existence of experimental evidence that does not support the current model of HPV infection, we postulate that the function of furin has been misinterpreted. Our data suggest that a cellular component important for the infection process is also a target of furin/PC activity. Furthermore, we have found that multiple PC members play roles at different steps of HPV infection.

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Abbreviations

Ab: antibody
BPV: bovine papillomavirus
CIN: cervical intraepithelial neoplasia
ECM: extracellular matrix
EGFR: epidermal growth factor receptor
FM-PsV(s): furin matured pseudovirion(s)
GF: growth factor
GFR: growth factor receptor
h: hour(s)
HiP: high yield production
HK: human keratinocyte
HMW: high molecular weight
HPVs: human papillomaviruses
HS: heparan sulfate
HSPG(s): heparan sulfate proteoglycans
kb: kilobases
MPV: murine polyomavirus
MMP(s): matrix metalloproteinase(s)
mM: millimolar
μM: micromolar
nm: nanometers
nt-PsV(s): non-treated pseudovirion(s)
PC: proprotein convertase
PsV(s): pseudovirion(s)
PV(s): papillomavirus
RTK: receptor tyrosine kinase
$t_{1/2}$: entry half time
vge: viral genome equivalents
VLP: virus-like particle
WGA: wheat germ agglutinin

CHAPTER 1: INTRODUCTION

Papillomavirus background

Human papillomaviruses (HPVs) are non-enveloped DNA tumor viruses belonging to the family *Papillomaviridae*, and infect hosts in species-specific manners. Papillomaviruses (PVs) have been isolated from a wide range of avian and mammalian species and are grouped into phylogenies based on the pathologies induced by different viruses. Over 200 genotypes of HPV have been discovered, with the majority of the 16 distinct HPV genera belonging to the alpha or beta genera. HPVs have been further categorized into high-risk and low-risk types based on their oncogenic potential (38).

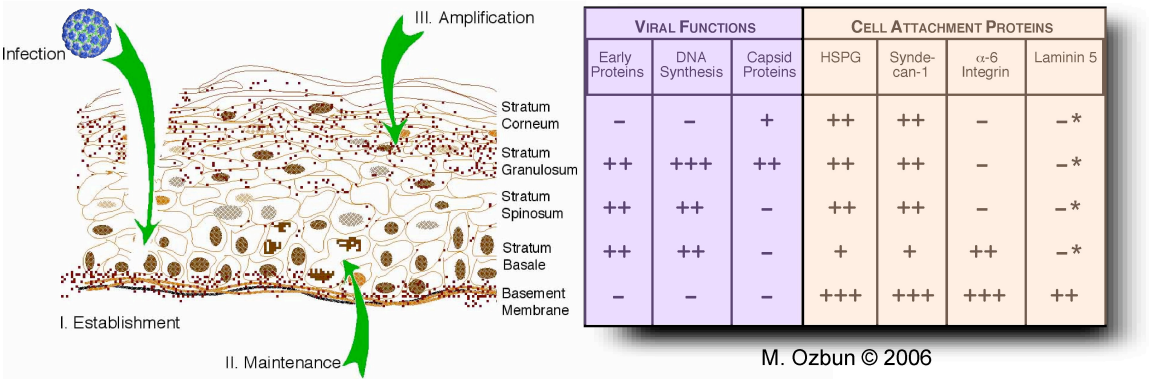
The capsid of HPV is non-enveloped and has a T=7 icosahedral structure. The viral particles are 50-60 nm in diameter and are formed through the association of disulfide bonds among capsid proteins. L1, the major capsid protein, contributes 360 molecules arranged into 72 pentamers to the virion while L2, the minor capsid protein, may exist within the capsid in as few as 12 and at most 72 molecules (21). Within the virion capsid resides an 8-kb double stranded circular DNA genome that is associated with histones. The genome encodes for six early proteins (E1, E2, E4, E5, E6 and E7) and the two late proteins (L1 and L2).

The HPV Life Cycle

HPV replication and infection are unique, requiring a stratified squamous epithelium to complete the full viral life cycle (Fig. 1). It is thought that the virus gains access to the basal layer of keratinocytes through wounds or micro-abrasions, establishing infection only in mitotically active cells (72). Early proteins, such as E1 and

E2, are expressed only in the lower epidermal layers and are responsible for episomal genome maintenance. As cells derived from the infected basal layer differentiate, they ascend through the stratum with increased genome amplification occurring and other early proteins, including E1^E4 and E5, being expressed.

The Differentiation-Dependent Life Cycle of Papillomaviruses



Viral Functions			Cell Attachment Proteins			
Early Proteins	DNA Synthesis	Capsid Proteins	HSPG	Synde-can-1	α-6 Integrin	Laminin 5
–	–	+	++	++	–	–*
++	+++	++	++	++	–	–*
++	++	–	++	++	–	–*
++	++	–	+	+	++	–*
–	–	–	+++	+++	+++	++

Figure 1.1. Model for PV infection in a stratified epithelium. Three stages of viral genome replication are indicated; major viral functions and presence of potential cellular attachment moieties in the differentiated epithelial tissues are noted at the right. In **Stage I** the viral genome is established at low copy number (10-50 copies/cell). In **Stage II** viral genomes are replicated along with cellular DNA. **Stage III** is the vegetative DNA replication phase occurring in suprabasal cells. Late gene expression restricted to the upper, differentiated epithelial layers is concurrent with viral DNA amplification leading to virion morphogenesis. Virion image from ref. (54). Many HSPGs including syndecans and glypicans are expressed on keratinocyte membranes throughout the epidermis and mucosa (52, 58). Alpha 6-integrin expression is generally restricted to basal keratinocytes where it can pair with beta 4-integrin attaching the keratin cytoskeleton to the basement membrane, in some cases by binding to laminin 5 (LN5: also known as laminin 32) (57). LN5 is an extracellular molecule found in the basement membrane where it anchors cells and is secreted into the leading edge of the wound (indicated by *) (2).

In neoplastic lesions high-risk HPV genomes may be found integrated into the host cell chromosomes where they induce expression of E6 and E7 at high levels. These early proteins lend to the oncogenic potential of HPV, as they deregulate the cell cycle and induce long term uncontrolled cell proliferation by binding to and altering the function of tumor suppressor proteins: E6 binds to and facilitates the degradation of p53,

while E7 binds to and inhibits pRb to push the infected cell through critical cell cycle checkpoints (48). Over time these events lead to an accumulation of mutations that can eventually cause cells to become malignant (8).

HPVs and Disease

HPVs cause both benign and malignant tumors in cutaneous and mucosal epithelium. HPVs cause most common sexually transmitted disease globally and are the etiological agent of 99.7% of cervical cancer cases worldwide (87). In addition to causing cervical cancer, HPVs are linked to other anogenital as well as head-and-neck cancers. The high-risk types most commonly associated with malignancy are HPV16, 18 and 31. The most common low-risk types, HPV6 and 11, are also found in the anogenital tract and although they cause warts, they are uncommonly associated with malignancy. It is also known that infection with a high-risk HPV type is a necessary, albeit insufficient, step in the onset of cervical cancer. Other factors such as, parity, smoking, immune function, disease state and age can all contribute to an individual's cancer progression (17).

Over 90% of women with low levels of cervical intraepithelial neoplasia (CIN) will have spontaneous regression without treatment. Due to high levels of screening and early treatment, the mortality rate related to cervical cancer in the developed world (3%) is relatively low compared to other cancers. Screening in the form of the Papanicolaou (Pap) smear, which detects cellular abnormalities, has caused the rate of cervical cancer in the U.S. to steadily decline over the last 50 years (8). In the developing world however, the relative death rate from cervical carcinoma is still the highest among cancer types

(15%), primarily due to a lack of screening, which leads to late detection, causing most cases to be fatal (13).

HPV Vaccines

Recently, two prophylactic VLP-based HPV vaccines targeting the L1 protein have become commercially available: Cervarix® is a bivalent vaccine targeting HPV16 and 18 (GSK); and Gardasil® is a quadrivalent vaccine designed against HPV6, 11, 16 and 18 (Merck). They are safe, highly immunogenic and nearly 100% effective in protecting against infection of the HPV types included within the respective vaccine (15). However, there are several drawbacks with these vaccines. For example, poor vaccine uptake exists as the target group for vaccination is pre-pubescent girls and there has been much social resistance to vaccinate against a viral agent that is sexually transmitted. Additionally, the cost of manufacturing the vaccine remains high, as it is VLP-based and must therefore be produced in eukaryotic cells (15). The high cost poses yet another obstacle in lessening the cervical cancer burden in developing countries, where screening levels are low. The currently available vaccines also provide no therapeutic benefit; those already infected with HPV are still at risk for malignant progression. Finally, current L1-based vaccines provide little to no cross protection for other HPV types; many high-risk HPV types, in particular 31 and 45, are not fully protected against with these vaccines.

This has led researchers to begin developing L2 peptide based vaccines, which are being designed to confer cross protection amongst multiple HPV types. Several animal studies in both rabbit and bovine models have confirmed that immunization against L2 provides cross protection between PV types (28, 34). Although, L2 vaccines would

provide cross protection and are less expensive as they can be produced in bacteria (15), they are not highly immunogenic and provide no therapeutic value. Even with the creation of improved HPV vaccine platforms, there will still be a need for additional therapeutic strategies, as there are currently over 20 million infected individuals in the U.S. alone (8). However, in order to develop more effective therapeutic approaches, ambiguous data for some areas of HPV biology must be fully elucidated. Additionally, if initial keratinocyte entry by the virus is better understood, better prophylaxis can be achieved.

HPV Entry

Historically, investigating the mechanism of HPV cellular entry has been challenging, as the number of HPV particles recovered from actual *in vivo* infections is so low that it has been nearly impossible to conduct infectivity studies (65). Additionally, because HPVs require epithelial differentiation to complete their life cycle, producing virus stocks in a laboratory setting has proven technically difficult. Not until the development and implementation of viral manufacturing techniques such as the organotypic-raft tissue culture system and the high-yield production (HiP) method were meaningful viral entry and infection studies possible. The organotypic-raft tissue culture system facilitates the completion of the viral life cycle because it recapitulates stratified epithelium (53). The high-yield production (HiP) method generates pseudovirions (PsVs) with viral or reporter genome packaged within self-assembling L1 and L2 capsids in transiently transfected mammalian cells. This method is advantageous because it produces 100X more infectious virus per cell than the organotypic-raft system,

independent of viral replication (10, 66). Although, these methods have permitted more thorough investigation of HPV cellular entry, conflicting data exist about the receptor mediated endocytic pathway used by HPVs. Moreover, the technique used to produce virus in these studies may influence the resulting route of cell entry.

HPV viral entry is a multi-step process with a number of reported attachment factors and suspected internalization receptors at the cell surface, a topic to be addressed at length in the subsequent paragraphs. Once viral entry is initiated, there are several endocytic pathways by which viruses gain access to the cell. These include clathrin- or caveolin-mediated entry, macropinocytosis and even pathways termed non-clathrin and non-caveolin mediated, all of which uptake extracellular matter into the cell by the formation of discrete vesicles that fuse with early endosomes where viral uncoating takes place (61, 62). HPVs have been reported to use several types of endocytosis; for example, several studies have demonstrated that HPV16 and 33 use a clathrin-mediated pathway (25, 76), whereas our lab using organotypic-derived virus showed a caveolin-mediated pathway for HPV31 (78). There is an additional study, reporting a clathrin and caveolae independent pathway with the involvement of tetraspanin-enriched microdomains (80). Although, the aforementioned studies have demonstrated disparate entry routes for different HPV types, the vast majority of HPV studies agree that the primary attachment factor used by the virus is heparan-sulfonated proteoglycans (HSPGs).

HSPGs and MMPs

HSPGs are ubiquitously present on the surface of animal cells as well as within the extracellular matrix (ECM) (5). They are categorized into three subfamilies: the

membrane-spanning proteoglycans, including syndecan-1, (abundant in epithelial cells), the glycophosphatidyl inositol (GPI) linked proteoglycans, and the secreted extracellular matrix proteoglycans. These highly negatively charged moieties consist of a core protein attached to heparan sulfate (HS) glycosaminoglycan (GAG) side chains, made up of interchanging N-acetylglucosamine (GlcNac) or N-sulphoglucosamine (GlcNS) units and uronic acids (29). As HSPGs are synthesized, a series of post-translational modifications occur in the Golgi leading to different degrees of acetylation and sulfation on the HS side chains (7). Due to this, HSPGs exist in a wide array of conformations, allowing the moieties to interact with a diverse population of protein binding partners (47). The large repertoire of ligands able to interact with HSPGs allows these molecules to play many roles within cell biology, such as in wound healing, organogenesis, growth factor binding and sequestration, as well as chemokine transportation and presentation (5).

HSPGs, including syndecan-1, are the primary attachment factors used by the majority of HPV types tested thus far (35, 42), and are involved in the infection process of many different viruses including those from the *Flaviviridae*, *Retroviridae*, and *Herpesviridae* families (50). Extensive experimental evidence demonstrates the requirement of HS for HPV cellular binding and entry. For example, PsV treatment with heparin, functioning as a competitive inhibitor to the virus, inhibits infection as does removal of cell surface HS using heparinase I or sodium chlorate (35). Additionally, HS-binding drugs inhibit HPV infection (75) and an *in vivo* model demonstrated that heparinase III instillation into the murine genital tract prior to challenge, severely reduces HPV infection (41). In cell culture, HS binding to the HPV L1 capsid protein occurs through charged interactions [Dasgupta, #3267] and it is believed that HPV initially

binds to HSPGs on both the cell surface and deposited ECM. However, in the *in vivo* murine female cervicovaginal model, HPV-HS binding first occurs on the basement membrane and the virus particles then transfer to a cell surface receptor (41, 46).

Although, the majority of studies indicate that HSPGs are the primary attachment factor in HPV infection, there is clear experimental evidence that a secondary non-HSPG internalization receptor exists for HPV. For example, viral particles treated post attachment with HS chemical inhibitors or neutralizing antibodies enter cells *via* a non-infectious pathway (75) and infection of HS null cells is possible when virions are modified by the addition of furin during the maturation process (27). Additionally, some studies indicate that the HPV interaction with HS at the cell surface is not absolute for infection, for instance organotypic-raft tissue-derived HPV31 infection of a number of human keratinocyte cell lines is HS independent (63) and HPV5 infection is not inhibited when virions are pre-treated with a highly sulfated form of heparin (41).

Another important aspect of HSPG biology to consider while investigating their function in HPV infection, is the process of cell surface “shedding”. Shedding is the process by which the ectodomains of HSPGs such as syndecan and glypican are proteolytically cleaved at the cell surface. These “shed” ectodomains function as soluble effectors and form matrices with ECM proteins that act as physical barriers as well as repositories for growth factors (GF) (7). The enzymes responsible for syndecan ectodomain shedding include members of the matrix metalloproteinase (MMP) family, specifically MMP-9, MMP-7, MT1-MMP, ADMTS1, and ADAM17 (18). In addition to their role in HSPG ectodomain shedding, MMPs function to both degrade ECM proteins and to activate a myriad of extracellular proteins. These diverse interactions allow MMPs

to wield effects on many cellular events, including cell proliferation and migration, angiogenesis, host immunity, as well as chronic inflammation, tumor invasion and metastasis. While MMPs activate many effector proteins, they too must be enzymatically converted from the zymogen into an active form. This activation occurs through several mechanisms including the activity of members of the proprotein convertase (PCs) family. Intracellularly, proMMPs are processed in the Golgi by the proteolytic cleavage of PCs and are then shuttled to the cell surface (67). Up to 40% of MMPs contain the basic sequence motif targeted by PCs (RXKR or RRKR) and activation of several types of MMPs has been directly measured. For example, furin (the most well characterized PC) is responsible for the activation of MT1-MMP and MT-MMP3 (45, 88). ADAM17 and ADAM 10 disintegrin metalloproteinases are also likely activated by furin (1, 51).

Proprotein Convertases

PCs, including furin, are serine endoproteases that activate a wide array of proproteins by cleavage at specific basic sequence motifs, the consensus cleavage site being just downstream from RXKR or RRKR (where X = any amino acid). Furin, a type-I trans-membrane protein, is ubiquitously expressed across many cell types including keratinocytes. It is most abundant in the trans-Golgi network but also functions within endosomes and at the cell surface. The activity of furin is strictly calcium dependent and important in many homeostatic cellular events, such as activation of signaling receptors, GFs, and, as aforementioned, MMPs (59, 83). Since furin modifies such an extensive spectrum of growth and ECM-remodeling factors, it is also associated with cancer metastasis and tumor progression. Furin over-expression in tumor cells of head-and-neck,

breast, and lung cancers is documented, and it has been referred to as a "master switch" in the regulation of tumor progression and growth (3). Moreover, research indicates that treatment of cancer cells with a selective furin inhibitor reduces their invasive potential, indicating that furin inhibition could be a viable treatment for cancer (4). In addition to its well-established role in cancer, furin is also implicated in the cleavage and activation of many different pathogens. Several bacterial toxins such as anthrax toxin protective antigen, Pseudomonas exotoxin A, and diphtheria toxin are each processed by furin during entry into the host cell (36, 37, 55). Similarly, furin activates a number of viral coat proteins during intracellular virus assembly (33). These include influenza A virus (69), Ebola and Marburg viruses (85), (86) as well as HIV (6). The role of furin in HPV infection however, is distinct for viral pathogens, in that HPV is one of the first viruses described requiring furin activity during viral entry (68). Although Semliki Forest and dengue virus entry have been reported to involve furin activity as well (89, 90).

Furin and HPV Infection

The Day-Schiller group at the U.S. National Institutes of Health has developed the widely accepted model of HPV entry. They propose that L1 binds to HSPG receptors on the cell surface and the ECM (*in-vitro*) or basement membrane (*in-vivo*). This initial binding to HSPGs causes an L1 capsid conformational change that allows an otherwise masked cleavage site on the minor capsid protein, L2 to become accessible. This site is then cleaved by extracellular furin allowing the virus to detach from the HS moiety and interact with the yet unknown secondary internalization receptor (26). Several studies provide experimental evidence for this theory. For instance, Richards *et al.* (68)

demonstrated that HPV infection was blocked when HeLa and CHO-K1 derivative cell lines were treated with a broadly-active PC inhibitor, decanoyl-RVKR-chloromethyl-ketone (dec-RVKR-CMK), and that the N-terminus of L2 of phylogenetically distinct papillomavirus types contains a furin cleavage consensus site (amino acids 8-12) not present on L1. Additionally, this study revealed that L2 is a furin substrate in *in-vitro* assays and PsVs carrying L2 that is mutated in the furin cleavage site have reduced infectivity. In another study conducted by this research group, the investigators exposed viral particles to furin during particle maturation in an attempt to access and pre-cleave L2 before full capsid formation took place. These virions, termed furin "pre-cleaved" were able to infect primary keratinocyte and CHO-K1 derived cells lines, pgsa-745 (HSPG null), FD11 (furin null) and FD11+ (transfected with cDNA expressing furin). These cells also were efficiently infected when exposed to viral particles in the presence of conditioned medium containing exogenous furin. This study also demonstrated that virions matured in the presence of furin bound more readily to the plasma membrane of HSPG null cells (24). By using a murine challenge model, these same investigators confirmed these *in-vitro* findings; instillation of the PC inhibitor, dec-RVKR-CMK, into the murine genital tract significantly decreased infection, and particles exposed to furin during the maturation process were again able to overcome this inhibition (46). Another piece of experimental evidence consistent with the Day HPV entry model is that the monoclonal L2 neutralizing antibody (RG-1) recognizing an epitope (amino acids 17-36) just downstream of the furin cleavage site, causes viral capsid binding to the cell surface to be perturbed (23). Additionally, binding of the RG-1 antibody to L2 is only detectable by immunofluorescence confocal microscopy, after 4 hours post attachment in the mouse

challenge model (46). This experimental evidence has allowed researchers to postulate the current Day model of HPV entry: L2 becomes exposed after virus attachment at the cell surface, revealing the L2 antibody-binding site. This model is attractive because it explains how RG-1 and other L2 antibodies that confer cross protection between HPV types [Gambhira, 2007 #3281;Rubio, #3282] could be neutralizing. Even though L2 resides within the viral capsid covered by L1, a capsid conformational change induced by cell binding could reveal the furin cleavage site and subsequent to this cleavage, L2 antibody binding could occur.

Inconsistencies in the Day entry model

Although there are a large amount of data providing evidence in support of the Day model of HPV entry, there are several experimental findings that the Day model fails to explain. For instance, L1 only VLPs are able to enter cells (56) and the entry kinetics for L1 only & L1/L2 VLPs are markedly similar (77). Additionally, BrdU-labeled PsV genomes, only detectable after uncoating, are present within cells infected with PsVs containing L2 mutated at the furin cleavage site (68), indicating that cellular entry by the virus is not deterred even when the L2 furin cleavage-site is lost. A 2006 study also observed that L2 was not specifically needed for viral uncoating, but demonstrated that a peptide located on the C terminus of L2 was required for egress from the endosome (44). Another experimental finding, not in full agreement with the current entry model is the ability of "mature" virus particles treated with exogenous furin to infect HS null cells (24). This research group had previously shown that only "immature" virus particles were susceptible to furin pre-cleavage, a finding that led them to hypothesize that mature virus

particles must undergo a conformational change before the L2 furin cleavage site is exposed (68). In cells that are devoid of HS, the attachment factor reportedly needed to cause the L1 conformational change in mature PsV is not available; therefore, it is unclear how the addition of exogenous furin to an intact viral particle would permit infection in HS null cells. We propose these data could rather indicate that the activity of furin has an effect on L2 intracellularly, once a decrease in endosomal pH allows the virus to uncoat and the L2 cleavage site is exposed (79). Additionally, we postulate that furin acts on a cellular factor that once activated, confers the virus infection potential in an environment lacking HS. Furthermore, the source of exogenous furin used in experiments to treat mature PsVs was conditioned medium, from a CHO derived (Δ fur) cell line secreting a functional truncated form of furin. Although a control experiment was carried out demonstrating that the addition of conditioned medium from furin null cells to untreated virus did not increase infection, it cannot be ruled out that components of the conditioned medium that are also targets of furin such as MMPs or GFs, could have aided the PsV's ability to infect HS null cells. Even though viral particles matured in the presence of furin are able to infect HS null cells, they too can be rendered non-infectious by heparinase treatment as seen in the murine genital tract infection model (46). Taken together, these experimental observations are inconsistent with the Day model of entry. Alternative interpretations of the Day group's findings along with current data from our lab (summarized below) provide evidence for a novel model of HPV infection.

New Model of HPV-Cell Binding & Entry

Recent data from our lab provide evidence for an alternative model of HPV binding and entry, whereby infection requires the formation and release of soluble high molecular weight (HMW) complexes consisting of HPV, HS and GFs (HPV-HS-GFs). As part of the normal physiology of the cell, MMPs initiate the release or "shedding" of the ectodomains of HSPGs like syndecan-1 and membrane bound GFs (Fig. 2) (31). Whether attached to the cell or in the soluble form, HSPG moieties interact with these soluble growth factors and function to serve as repositories for the molecules. Once HPVs are introduced into the cellular environment, they attach to HSPGs also bound to GFs. This complex is then cleaved off the membrane by MMPs, and the resulting HMW complex can interact with a growth factor receptor (GFR), such as epidermal growth factor receptor (EGFR) triggering cell signaling necessary for infection and gaining access to the cell. The experimental evidence consistent with this model includes that HPV, HS and GFs are detected within HMW complexes present in the supernatant of PsV-exposed HaCaT cells; MMP inhibitors prevent the release of HPV from the cell surface and reduce infectivity; HPV exposure induces GFR activation and EGFR inhibitors hinder HPV infection (81). As noted above, furin activates MMPs, GFs and GFRs; therefore, it is quite possible that the necessity of furin during HPV infection is due not solely to L2 cleavage but also to aid in the formation and release of HPV-HS-GF complexes.

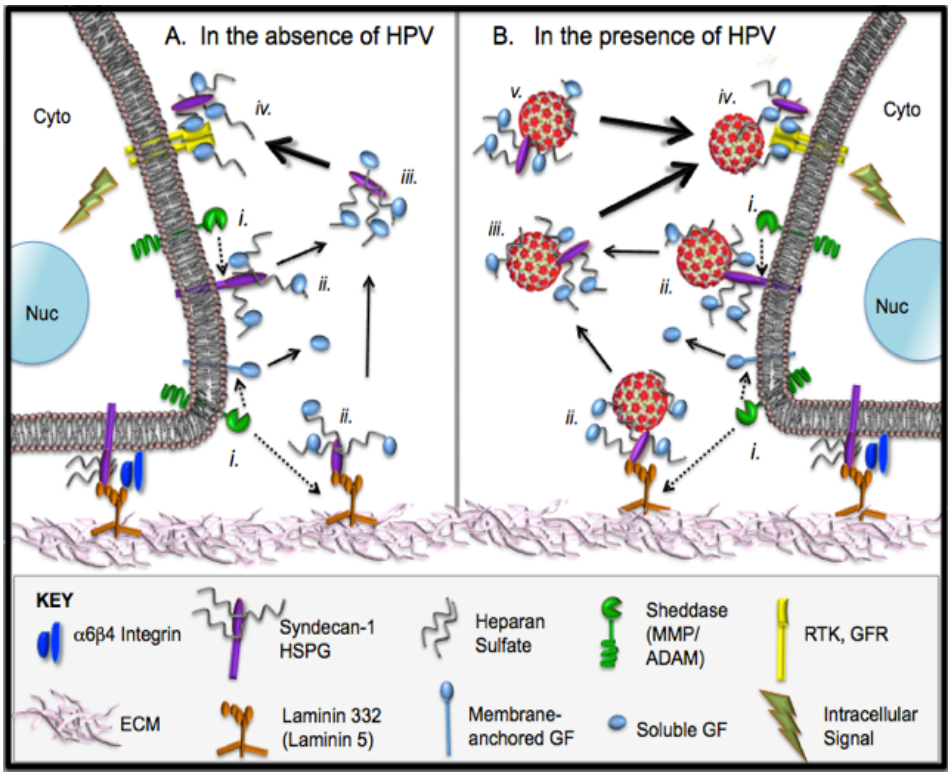


Figure 1.2. Normal HSPG biology and proposed model for extracellular interactions of HPVs in the context HS-GF complexes. **(A)**. Natural processes of HSPG shedding that occur in the absence of HPV. The lower edges of epithelial cell lipid bilayers are depicted interacting with the ECM. The ECM consisting of collagens, elastins, fibronectins, laminins is shown in pink. Laminin 332 (formerly laminin 5; orange) interacts with syndecan-1 (purple) and alpha-6 beta-4 integrin (dark blue) on the cell surface to provide cell anchorage to the ECM/basement membrane. Notably, these three molecules have been identified as HPV attachment factors. (i.) Sheddases including MMPs and ADAM sheddases (green) normally catalyze the release or ‘‘shedding’’ (dotted arrows) of membrane-bound GFs (light blue) and other bioactive molecules, the protein ectodomains of HSPGs like syndecan-1, and ECM residents like laminin 332 (31). (ii.) HSPGs in the plasma membrane and ECM act as local depots for soluble GFs and other bioactive molecules. The HS-GF and bioactive compounds can interact with their cognate receptors laterally, via soluble form after release (iii), or in the ECM when cells migrate over the HSPG-complexes. (iii.) Sheddases including MMPs and heparanases and proteolytic processing of laminin 332 liberate soluble complexes containing GFs and HS/syndecan-1. (iv.) Soluble HS-GF complexes bind to GFR/RTK (yellow) and activate intracellular signaling cascades. **(B).** The natural processes of HSPG decoration and release from the cells also occur in the presence of HPV particles (red). The virion image is based on the atomic structure from Modis et al. (54). By virtue of interaction with HS, HPV can join the complex at each stage where HSPG is involved (i–iv). HPV could associate with soluble HS-GF in a naive infection site or during release from infected cells (v.). HPV association with syndecan-1 via HSPG and binding of syndecan-1 to laminin 332 and alpha-6 beta-4 integrin are consistent with the fact that HPV particles colocalize and interact with each of these extracellular molecules (Figure from Surviladze *et al.* (81)).

Gaps in knowledge and rationale of this work

Owing to these new observations and the flaws of the current model of HPV infection, it is possible that the role of furin in HPV infection has been misinterpreted or not fully elucidated. Although L2 does posses a furin consensus cleavage site, the presence of this site is not required for cellular entry; cleavage could take place either at an extracellular locale or downstream in the infectious pathway, (e.g. during endosomal residence). The enzyme could however be important for both L2 cleavage and MMP activation during HPV infection, or have a yet undefined function. Without further investigation, furin's role(s) in HPV infection remains poorly defined. It is therefore **the goal of this study to more closely examine the role of furin and proprotein convertases (PCs) in HPV early infection.** *The central hypothesis of the study is that, in addition to L2 cleavage, PCs have functions that regulate HPV infection of HKs.* The specific aims set forth for the study are: 1) to investigate the infectious phenotype and presence of HS in "furin matured" PsV preparations; and 2) to determine how the absence of furin/PCs affects HPV early infection.

CHAPTER 2: MATERIALS AND METHODS

Cell Culture

Cell lines used in this work are summarized in Table 1. HEK-293T cells are derived from a human embryonic kidney cell line immortalized with SV40 large T antigen and were grown in DMEM high glucose (Irvine Scientific) supplemented with 10% FCS (Sigma or Atlas Biologicals), 1% glutamine-penicillin-streptomycin (Sigma). HaCaT cells, a spontaneously immortalized epithelial line derived from adult skin (9), were maintained in DMEM/Ham's F-12 medium (Sigma) supplemented with 10% FCS (Sigma or Atlas Biologicals), 4X amino acids (Sigma) and 10% glutamine-penicillin-streptomycin (Sigma). CHO-K1 and their derivative pgsd-677 (49), and FD11 (36) cells were maintained in DMEM/Ham's F-12 medium supplemented with 10% FCS (Sigma or Atlas Biologicals), 4X amino acids (Sigma), 1% glutamine-penicillin-streptomycin (Sigma), 2% nystatin (Invitrogen) and 1% Glutamax (Invitrogen). FD11+furin cells (37) were maintained in same medium as CHO-K1 cells with the addition of 200μg/ml G418 (Gibco BRL Life Technologies).

Table 1. Cell Lines used in this Study.

Cell line	Source	Known Deficiency	PC Expression	Reference
293T	Human embryonic kidney	N/A	N/A	(11)
HaCaT	Spontaneously immortalized HK	N/A	N/A	(9)
CHO-K1	Chinese hamster ovaries	N/A	furin, PC5/6, PACE4, PC7/8	(84)
pgsd-677	CHO-K1 parent	HSPG, Cell surface PC5/6	furin, PACE4, PC7/8	(49)
FD11	CHO-K1 parent	furin	PC5/6, PACE4, PC7/8	(36)
FD11+furin	FD11 parent	N/A	furin, PC5/6, PACE4, PC7/8	(37)

HPV Pseudovirion (PsV) Production and Purification

HPV PsVs were generated in 293T cells as previously described (16, 78). The transfection-based method was modified from that previously published (11, 12). 293T cells were transfected by the calcium phosphate method with codon optimized HPV16-L1/L2 expressing plasmid, pXULL (11), and a luciferase reporter (pGL3-control, Promega) genome. Cells were then trypsinized, pelleted, and resuspended at $1x10^8$ cells/ml in Dulbecco's PBS/9.5mM $MgCl_2$ at 48h post-transfection. Cells were lysed with 0.35% Brij58 and freeze-thawed 3X. Lysates were treated with 20U/ml exonuclease V (Epicentre plasmid-safe) and 0.3% Benzonase (Sigma) in order to digest any non-encapsidated DNA. Preparations were allowed to mature overnight at 37°C. For non-treated PsV (nt-PsV) and furin matured PsV (FM-PsV) preparations, cell lysate was divided into two portions and furin (10U/100µl of cell pellet volume, Enzo Life Sciences) was added to one portion (FM-PsV). After maturation, preparations were clarified by low speed centrifugation, supernatants were layered on top of a 1.25g/ml-1.4g/ml CsCl gradient. The viral band was captured by side puncture after 16-18h of centrifugation at 20,000xg. Using Amicon Ultra-4 centrifugal 100K filters (Millipore), virions were washed and concentrated in HSB (25mM HEPES pH 7.5, 0.5 M NaCl, 1mM $MgCl_2$). SDS-PAGE and Coomassie staining assessed virion stocks for purity and L1 protein content. Viral genome equivalents (vge) of packaged reporter plasmids were determined by qPCR.

Infections

Cells were seeded at 1.0-2.5 x 10^5 cells per well in 4 or 9cm^2 wells and were allowed to attach overnight. Cells were 60% to 80% confluent at the time of infection. PsVs stocks were thawed from -80°C to room temperature (RT) and sonicated for 20s. Virions were added to cells at (100-200vge/cell), and allowed to attach with gentle rocking at 4°C for 1h. The cells were washed once with an excess of medium and then incubated at 37°C with fresh medium. Infections were allowed to proceed for 20-30h at which time infection levels were quantified by luciferase assay. Cells were washed 2X in PBS and then lysed with 1X cell culture lysis reagent (Promega) for 15m at RT. To remove large cell debris, cell samples were then subjected to centrifugation (10s at 13,000xg). Luciferase activity was measured by using the Dual luciferase assay kit (Promega) and a Lumat LB 9501 luminometer (Berthold Technologies) or a Glowmax 1000 luminometer (Promega). Sample protein content was measured by Bradford assay and used to normalize the RLU raw data (RLU/protein concentration). Infections were carried out in duplicate or triplicate and the experiments were replicated 2-5 times. Error bars represent standard error of the mean. For heparinase pretreated infections, nt-PsV or FM-PsV were diluted in complete medium (CM) or heparinase III buffer (20mM Tris-HCl pH 7.5, 0.1mg/ml BSA and 4mM $CaCl_2$) treated for 2h at 37°C with heparinase I (3U, Sigma) or heparinase III (1.5U, Sigma) before exposure to pgsd-677 cells for 1h at 4°C. The entry half time ($t_{1/2}$) of nt-PsV and FM-PsV was assessed using a post-attachment neutralizing antibody time course experiment. H16.V5 monoclonal IgG2b antibody to HPV16 VLP (19) was diluted 1:2000 in medium and added to HaCaT cells at times 0, 4, 8, 12, 16 and 20h post attachment. Infections in the presence of furin

inhibitors were performed after HaCaT cells were pretreated with each inhibitor for 30m at 37°C. Inhibitors were present during viral attachment and as the infection proceeded at 37°C for 24h. Dec-RVKR-CMK (Furin Inhibitor I, Calbiochem) was used at 1μM or 5μM. H-(D)RRRRRR-NH_2 (Furin Inhibitor II, Calbiochem) was used 1μM or 5μM. Alpha-1 PDX (Pierce) was used at 5μM. For the furin inhibited time course, HaCaT, CHO-K1 or FD11+furin cells were treated or mock treated with dec-RVKR-CMK (1μM) for 1 or 2h at 37°C. At 0,1, 2, 4, 8, and 20 or 26h post attachment dec-RVKR-CMK (1μM) was added to respective wells and infection was allowed to proceed at 37°C for 30h. For furin rescued time course infections, FD11 cells wells were treated or mock treated with furin (1U, Sigma) for 1 or 2h at 37°C. At 0,1, 2, 4, 8, and 26h post attachment furin (1U) was added to respective wells and infection was allowed to proceed at 37°C for 30h.

SDS-PAGE and Immunoblotting

Nt-PsV or FM-PsV preparations were diluted in Heparinase III buffer, left untreated, treated for 10m or 2h with Heparinase III (1U). Samples were then solubilized in 6X Laemmli buffer with ß-mercaptoethanol with or without heat for 10m and subjected to SDS-PAGE using a pre-cast 10% polyacrylamide gel (BioRad). Proteins were then transferred to PVDF membranes and probed with primary antibodies: mouse monoclonal HPV16 L1 (Abcam) or mouse monoclonal HS (Millipore). Antigen-antibody interactions were detected using horseradish peroxidase-labeled secondary antibody (Pierce).

Immunofluorescence Staining and Microscopy

HaCaT cells were cultured overnight on cover slips in 4 cm^2 wells. Cells were treated with dec-RVKR-CMK (5μM) or H-(D)RRRRRR-NH_2 (5μM) for 30min at 37°C or left untreated. HPV16 PsVs were allowed to attach (6500 vge/cell) for 1 hr at 4°C. Unbound virus was washed off and furin inhibitors were added to appropriate wells. Virus internalization was initiated by a shift to 37°C and infection was allowed to proceed overnight. At 20h post infection, cells were treated with a low pH solution (0.2M NaAcetate, 0.5M NaCl pH 4.6) at 4°C or left untreated. Cells were washed 2X in PBS, fixed in 3.7% PFA for 30m at RT and then stained with wheat germ agglutinin-Alexa Fluor® 488 (Invitrogen) for 18m. After 3 PBS washes, cells were permeabilized in 0.1% Triton X for 5m at RT, and again PBS washed 3X before being blocked in 1% BSA in PBS for 1h at RT. Cells were then incubated with mouse monoclonal antibody (1:200) against HPV16L1 (Millipore) for 1h at RT. Following 3X PBS washes, cells were incubated with DyLight™ 594-conjugated affinityPure donkey Anti-mouse IgG (1:200; Jackson Immunochem) for 1h at RT. Cells were again washed 4X in PBS and coverslips were inverted onto Vectashield mounting medium with DAPI (Vector Laboratories). Images were acquired with a Zeiss LSM 510 META confocal system using a DAPI/FITC/Rhodamine filter configuration. Laser intensities were kept constant during imaging. For transmission electron microscopy (TEM), viral stocks were diluted in HSB or Heparinase III buffer just prior to imaging and then visualized by TEM (Hitachi 7500) at 80kV following binding to carbon-coated, plasma discharged treated electron microscopic grids and negative staining with 2% uranyl acetate.

CHAPTER 3: AIM 1 RESULTS

The first aim of the study was to investigate the infectious phenotype and presence of HS in "furin matured" PsV (FM-PsV) preparations. During PsV production in the 293T cell system, virions recovered from cell lysates are in a "loose" or immature capsid conformation. In order for the virions to reach a tighter conformation through inter-L1 disulfide bonding they are allowed to mature overnight at 37°C (11, 12). It is during this step, where presumably L2 is accessible, that furin is added to the virion preparation. Day *et al.* (24) demonstrated that HPV16 PsVs allowed to mature in the presence of furin could infect HSPG null cells, and concluded that this was due to the pre-cleavage of L2 conferring the virion the ability to bypass the initial HSPG interaction needed for a capsid conformational change. However, the researchers acknowledge that only 35% of L2 was cleaved within the virion preparation, and in another study from the same group the infectious potential of FM-PsVs was perturbed when virions were heparinase-treated in the murine genital tract infection model (46). Due to the fact that PsV maturation takes place in the presence of cell lysates, there is an alternative interpretation of the reported findings by Day *et al*. In light of the new model of HPV entry proposed by our lab, whereby a complex of HPV-HS-GF is released from the cell surface prior to engagement of a secondary internalization receptor (81), we thought it possible that during the viral production process PsVs may become "decorated" with HS-GF and possibly other factors to some extent. In fact, Surviladze *et al.* showed that released HPV-HS-GF complexes are able to infect HSPG deficient cells (81). Several other furin substrates and binding partners, such as growth factors and HSPGs, are present within cell lysates, and it is known that furin activates MT1-MMP as well as

several growth factors (83). It is possible that these bioactive molecules, if coupled to the virus could confer the potential to infect HS null cells. We therefore hypothesized that in the process of generating FM-PsV, wherein immature virus particles are exposed to furin in 293T lysates, the virus particles become "decorated" with cellular factors essential for infection. Thus, my goal for this aim of the study was to generate particles allowed to mature in the presence of furin and determine if these particles became associated with HSPG during the production process.

In order to verify that in our laboratory FM-PsV did indeed infect cells lacking HS, a CHO-K1 derivative cell line pgsd-677, lacking the enzymes required for heparan sulfate polymerization was used (49). FM-PsVs and non-treated PsVs (nt-PsVs) were allowed to attach to the cells for 1h at 4°C, cells were then washed, refed with growth medium and incubated at 37°C to allow viral entry. At 24h infection levels were quantified by luciferase assay. As expected, nt-PsVs had a very low infectious potential in pgsd-677 cells lacking HS, but FM-PsVs did infect almost 7 times better than nt-PsVs (Fig. 3.1). This confirmed the findings of Day *et al.* (24) and again demonstrated the fact that virions allowed to mature in the presence of furin have the ability to infect cells lacking HS. In the parental CHO-K1 cells that express HSPG, FM-PsVs infected 2.5 times more than nt-PsVs. Although we expected FM-PsVs would infect HS null cells due to their proposed decoration with HS, the finding that FM-PsVs also better infect CHO-K1 cells is noteworthy. CHO-K1 cells are much more readily infected than HS null cells, by both nt-PsVs and FM-PsVs (66 and 25 times greater, respectively).

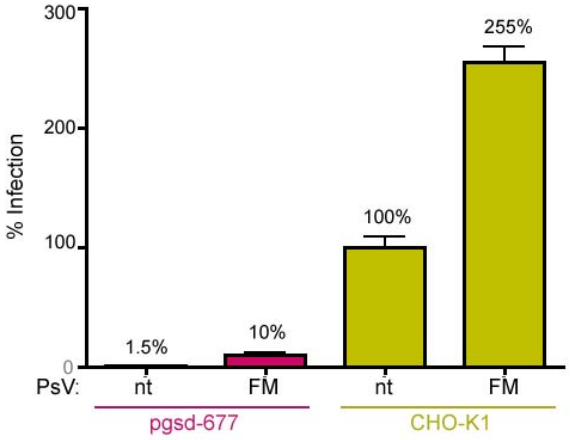


Figure 3.1. FM-PsV has a greater infectious potential regardless of HS status of cell. Nt-PsVs or FM-PsVs (150 vge/cell) were allowed to attach for 1h at 4°C to subconfluent CHO-K1 (wild type HSPG) or derivative pgsd-677 (HSPG null) cells. Unbound PsVs were washed off and internalization was initiated by a shift to 37°C. Infection levels were quantified at 24h post infection by luciferase activity. Each experiment was carried out in triplicate and the error bars represent the standard error of the mean (n=6). Values were normalized to nt-PsV infection in CHO-K1 cells. The mock level for CHO-K1 cells was 0.29% of nt-PsV infection and the mock of pgsd-677 cells was 12% of nt-PsV infection.

The results in Fig. 3.1 suggest that CHO-K1 cells provide additional important factor(s) for infection aside from what the FM-PsVs provide in HSPG-null cells. This also indicates that the factor(s) important for infection is not just increased levels of HSPG at the cell surface because presumably, FM-PsVs don't require HS. This could be due to either FM-PsVs containing pre-cleaved L2 as the Day model suggests, thereby abolishing the need for HS primary attachment or alternatively (or in addition) by HS-FM-PsV association during virus production and maturation, as we hypothesize. Additionally, although HSPG synthesis is the main difference between wild type CHO-K1 cells and pgsd-677 cells, the HS deficient pgsd-677 cells also lack cell surface PC5/6 (73). This could potentially affect the ability of HPV to infect these cells because, without this PC localized at the plasma membrane, furin/PC dependent processes, whether they be L2 cleavage or activation of a factor important for infection, will be severely hindered. Together, these results suggest that FM-PsVs are modified in such a way that their infectious potential is greater, regardless of the presence or absence of HS on the cell surface.

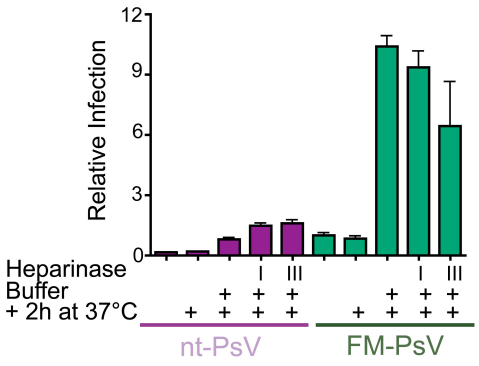


Figure 3.2. **Heparinase pretreatment of FM-PsV does not hinder infection in HSPG null cells**. Nt-PsVs or FM-PsVs were treated for 2h at 37°C with heparinase I (3U) or heparinase III (1.5U) in heparinase III buffer (20mM Tris-HCl pH 7.5, 0.1mg/ml BSA and 4mM $CaCl_2$). Treated and untreated virions (150vge/cell) were diluted in CM and allowed to attach to subconfluent pgsd-677 (HSPG null) cells for 1h at 4°C. Unbound PsVs were washed off and internalization was initiated by a shift to 37°C. Infection levels were quantified at 24h post infection by luciferase activity. Each experiment was carried out in triplicate and the error bars represent the standard error of the mean (n=3). Mock level was 75% of nt-PsV infection and infection is relative to FM-PsV set to 1.

To further test the hypothesis that the ability of FM-PsV to infect cells lacking HSPG is due to a greater association of FM-PsV with HS, virions were pretreated with heparinase I or III and exposed to pgsd-677 cells lacking HS. We expected that if FM-PsVs were associated with HS, PsV treatment with heparinase I or III would disrupt this association and infection of pgsd-677 cells would be inhibited. Surprisingly, this did not occur as pre-treatment of both nt-PsVs and FM-PsVs with heparinase I (3U), or heparinase III (1.5U) for 2h at 37°C did not cause a significant reduction in infection (Fig. 3.2). As per the manufacturer's recommendations (Sigma product sheet) heparinase enzymatic reactions require the presence of heparinase III buffer (20mM Tris-HCl pH 7.5, 0.1mg/ml BSA and 4mM $CaCl_2$). As a control, infection of pgsd-677 cells with nt-PsVs and FM-PsVs pre-treated for 2h at 37°C, in the presence of heparinase III buffer alone was assessed. FM-PsVs and nt-PsVs were diluted in complete medium (CM) or heparinase III buffer and placed at 37°C for 2h. CM was then added to each dilution and added to cells for 1h at 4°C. Cells were washed, refed with growth medium and incubated at 37°C to allow viral entry. At 24h infection levels were again quantified by luciferase assay. As compared to virions incubated for 2h at 37°C in the presence of (CM), the

treatment of virions with heparinase III buffer alone augmented infection 5 fold for nt-PsV and 10 fold for FM-PsVs. This was also an unexpected result, but we suspect that the likely constituent of heparinase III buffer responsible for the increased levels of infection is Ca^{2+}. In this experiment the final Ca^{2+} concentration in CM is 0.4mM whereas PsV pre-treated with heparinase III buffer were in 4mM Ca^{2+} at cell exposure. Ca^{2+} has been reported to enhance viral capsomer association in murine polyomavirus (MPV) [Chuan, #3292], and bovine papillomavirus (BPV) (60); allowing the virus to remain stable in solution could increase the number of viral particles available to infect the cells. Additionally, Ca^{2+} is required for furin/PC activity (83), if these enzymes are working at their maximum potential HPV infection levels will likely rise since infection is incontrovertibly furin/PC dependent.

Although, Fig. 3.2 suggests a trend in infection decrease for heparinase-treated FM-PsVs, no statistically significant change in infection levels was reproducibly seen when virions were exposed to heparinase. Therefore, we strove to directly measure HS associated with FM-PsVs, and determine if heparinase cleavage was active during the reaction conditions. Previous findings in our lab indicate that HS present in association with PsV is released from cells and "shed" into CM post exposure to cells (81). Although, detection of HS is challenging due to the dearth of effective antibodies, Surviladze *et al*. showed that PsVs released from cells were present in a HMW complex of $>10^4$ kDa. This complex was shown to be detergent resistant and heat sensitive and could be further dissociated by treatment with heparinase III (81). As detection of HS in these HMW HPV-HS-GF complexes from CM post exposure to cells was challenging, we chose to first test whether HS was present in our nt-PsV and FM-PsV preparations

using a combination of heparinase III and heat treatment followed by immunoblot for L1 and HS. We reasoned that such treatments would cause the shift in size of L1 proteins by SDS-PAGE if HS molecules were present at substantial levels in association with the PsVs. We also suspected that if HS was present in high enough quantities within the virion preparations, it might be detectable by immunoblotting for HS directly when the virions were treated with heparinase. Equal amounts of the nt-PsV or FM-PsV preparations were either heated, treated with heparinase III for 10m or 2h, or subjected to a combination of heat and heparinase III treatment (Fig. 3.3A).

The immunoblots allowed for a number of important observations. First, although 10 μg of total L1 protein (PsV preparation) was loaded into each well of the SDS polyacrylamide gel, differing amounts of L1 protein were detected by immunoblot depending upon the treatment of the PsVs prior to loading on the gel. There was a small amount of HPV16 L1 detected in lanes where PsV preparations were not heated or heparinase-treated (Fig. 3.3A, lanes 1 and 8). This might be due to an inability of the stable PsV-HMW complexes to enter the gels, which have an upper size restriction of ≈ 350 kDa (BioRad product sheet). The results could also be due to lack of immuno-recognition by the L1 antibody on the blot (i.e., the epitopes are not readily available without heparinase treatment or heating). Thus, our data indicate that heating of the PsVs

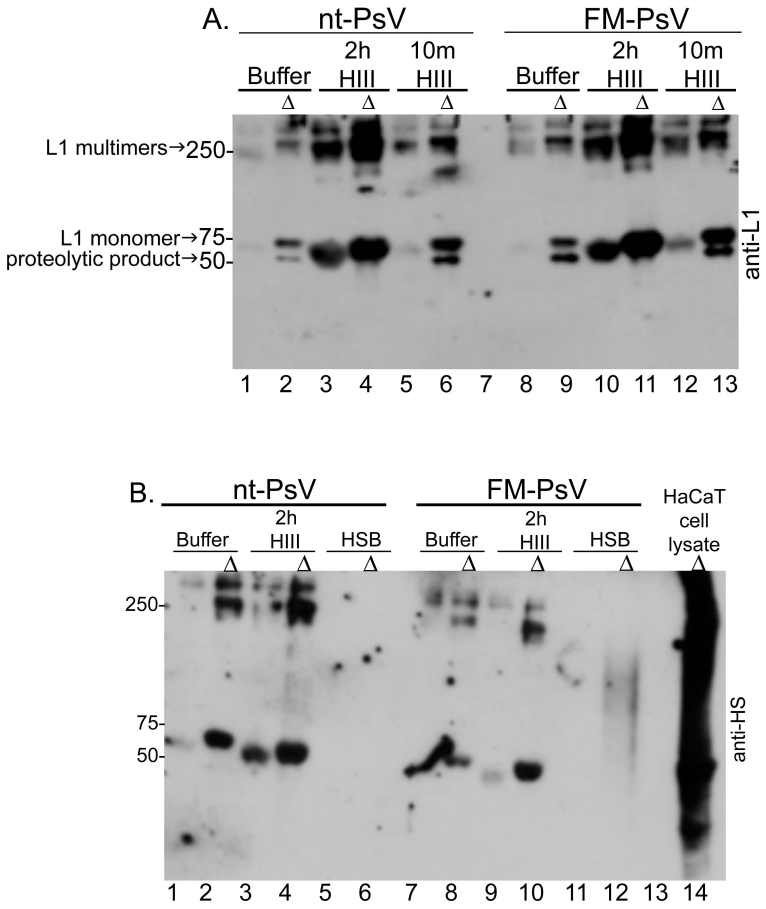


Figure 3.3. Heparan sulfate is present in nt-PsV & FM-PsV preparations. Nt-PsV or FM-PsV preparations (10µg) were diluted in Heparinase III buffer and left untreated, treated for 2h or 10 m with Heparinase III. Samples were subjected to SDS-PAGE under reducing conditions (ß-mercaptoethanol) with heat (Δ) or without for 10m followed by an immunoblot for HPV16 L1 (Abcam) (A) or HS (Millipore) (B). Arrows at the left side of panel A indicate the species of multimers, monomers and proteolytic products of L1 (12).

affects the size and/or conformation of the PsV in permitting entry into the gel. The amount of immuno-reactive L1 detected increased when the PsVs were heated (Fig. 3.3A, lanes 2, 9). Similarly, heparinase III treatment of PsVs yielded more L1 detection (Fig. 3.3A lanes 3-6, 10-13), and longer heparinase III treatment resulted in even higher detection of L1 (compare 2h *versus* 10m treatments). Furthermore, heating and heparinase III treatments were synergistic in their effects (compare Fig. 3.3A lanes 1 and 8 to lanes 4 and 11). Additionally, we can surmise that the heparinase III enzyme was functioning in these reactions (10m or 2h treatment of the virus at 37°C in heparinase III buffer) due to the difference in L1 detection observed (Fig. 3.3A lanes 3-6, 10-13). HS

presence within the virion preparations was further confirmed by immunoblotting for HS directly (Fig. 3.3B). HaCaT cell lysate was used as a positive control for HS detection in this experiment, and appears as a large smear on the membrane (Fig 3.3B. lane 14), expected due to the numerous proteins modified with HS of varying sulfonation levels present in cell lysate, all able to react with the HS antibody used. As seen with the immunoblot for L1 (Fig. 3.3A), the amount of immuno-reactive HS detected increased when the PsVs were heated and treated with heparinase III (Fig. 3.3B lanes 2-4, 8-10). It is unlikely that heparan or HS disassociation from the virus was complete, as evidenced by the detection of HS at essentially the same molecular weights as L1. This is an indication that L1 and HS are associated, but also suggests that under the experimental treatment conditions, the binding partners are so stably complexed that they cannot be fully disassociated. This may explain why heparinase treatment of FM-PsVs did not significantly reduce infection in HS null cells (Fig. 3.2) if HS was still coupled to the virions allowing infection. Another noteworthy aspect of the HS immunoblot is that HS was only detected in the PsV preparations when virions were diluted in heparinase III buffer (Fig. 3.3B lanes 1-4, 7-10) No immuno-recognition was noted when virus was diluted in HSB (the normal diluent and storage buffer for pseudovirion preparations) (Fig 3.3B lanes 5-6, 11-12). This indicates that not only does the heparinase III buffer augment infection, it also modifies the virus complex in such a way that PsVs can enter the gel and/or the epitopes for HS are recognizable.

Another strategy utilized to investigate the infectious phenotype of FM-PsV was to assay the entry half time ($t_{1/2}$) of the virus population by determining the time needed for half the infectious population to escape antibody mediated neutralization at the cell

surface. Based on our working hypothesis that FM-PsV virions are more extensively pre-decorated than nt-PsVs, we expected the internalization time of FM-PsVs to be quicker because presumably those virions would be able to bypass the initial HSPG attachment factor and go straight to a secondary internalization receptor. Figure 3.4 depicts a neutralizing antibody time course experiment, which measures infection levels after 24h. The H16.V5 antibody neutralizes infection by interfering with viral endocytosis; the antibody does not hinder virus-cell surface association but does cause HPV16 virions to remain at the cell surface, as measured by immunofluorescent confocal microscopy (27). In order to calculate the $t_{1/2}$ time of HPV16 PsV preparations, the H16.V5 antibody was added at successive time points post PsV binding to and initiation of entry in HaCaT cells. The infection data were plotted over time, and FM-PsV infection levels were normalized to nt-PsV that were not exposed to H16.V5. The plot shows that FM-PsVs had a faster internalization time ($t_{1/2} \approx 6$h) than nt-PsVs ($t_{1/2} \approx 10$h). These data indicate that FM-PsVs are modified in such a way that they can escape the plasma membrane faster than nt-PsV. Similar to the greater infection potential in CHO-K1 and pgsd-677

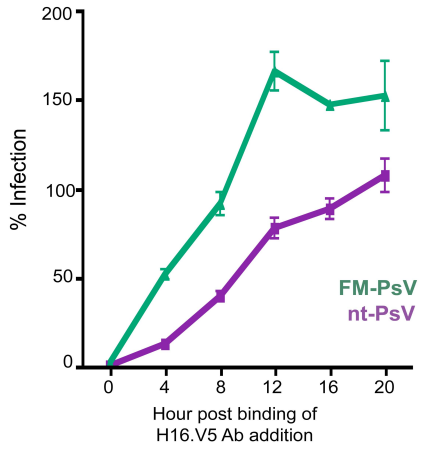


Figure 3.4. FM-PsVs are internalized more rapidly than nt-PsVs. Post attachment antibody mediated neutralization time course. Nt-PsVs or FM-PsVs (150 vge/cell) were allowed to attach for 1h at 4°C to subconfluent HaCaT cells. Unbound PsVs were washed off and internalization was initiated by a shift to 37°C. At the indicated times post attachment, half the media in each respective well were replaced with a dilution of 1:1000 H16.V5 Ab in fresh media (final Ab dilution 1:2000). Infection levels were quantified at 24h post infection by luciferase activity and values were normalized to untreated nt-PsVs at 0h. Each experiment was carried out in duplicate and the error bars represent the standard error of the mean (n=4). Mock level was 0.012% of nt-PsV infection.

cells, FM-PsVs also demonstrated a 1.5 fold higher infectivity in HaCaT cells than did nt-PsVs. Although, this experiment does not measure HS association with PsVs, it does support the idea that FM-PsV particles are associated with a factor(s) that allows faster and higher overall infectivity.

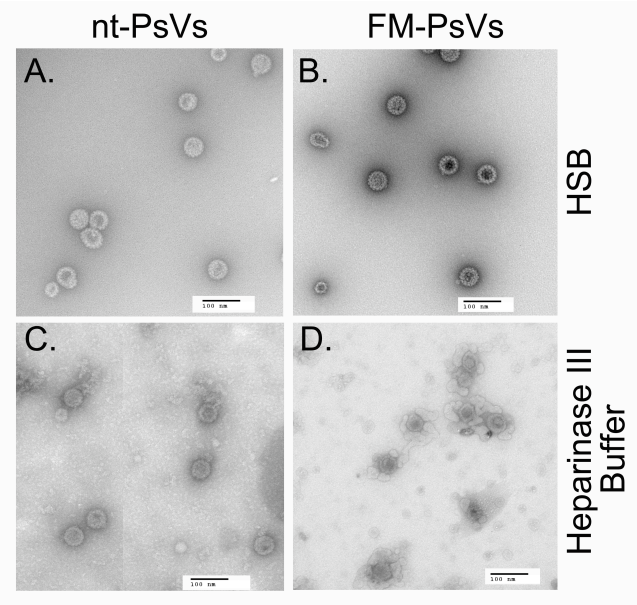


Figure 3.5. Electron micrographs of nt-PsVs and FM-PsVs. Nt-PsVs and FM-PsVs were diluted in HSB (A, B) or Heparinase III buffer (C, D) just prior to imaging and then visualized by transmission electron microscopy (TEM) (Hitachi 7500) at 80kV following binding to carbon-coated, plasma discharged treated electron microscopic grids and negative staining with 2% uranyl acetate. Black bars equal 100 nm.

To visualize the capsid structure of nt-PsVs and FM-PsVs, and more directly assess the association of virions with potential binding partners, high-resolution transmission electron microscopy (TEM) images of the preparations were attained. We suspected heparinase III buffer modified capsid structure based on our findings that HS associated PsVs were only resolved by immunoblot when PsVs were diluted in heparinase III buffer. Thus, additional images in the presence of buffer were also acquired. Both the nt-PsV and FM-PsV preparations contained intact capsid structures; however, additional associated molecules were not visualized (Fig. 3.5 A, B). Furthermore, when virions were incubated for 2h at 37° C in heparinase III buffer

immediately prior to imaging, viral particles became associated with aggregates contained within the heparinase III buffer and took on a less regular form (Fig. 3.5 C, D). However, because of the associating material, it was difficult to discern specific effects on capsid structure.

Taken together, these data indicate that both nt-PsVs and FM-PsVs are associated with HS upon isolation from cells, yet the infectious potential of FM-PsVs is greater in all of the cell lines tested thus far and FM-PsVs posses a quicker $t_{1/2}$ internalization time than nt-PsVs. Additionally, we found that heparinase III buffer dramatically augments PsVs' ability to infect HS null cells and increases the ability of HS to be detected by immunoblot on the viral particles. Furthermore, virions imaged by TEM displayed an irregular conformation in the presence of the buffer.

CHAPTER 4: AIM 2 RESULTS

The second aim of this study was to determine how the absence of furin, either through the use of pharmaceutical inhibitors or a furin mutant cell line, affects early steps in HPV16 PsV infection. In addition, experiments supporting this aim have been designed to determine the time point at which furin is crucial in early infection, specifically with the goal of addressing whether furin activity is necessary before or after viral internalization.

It is well established that the use of dec-RVKR-CMK ("Furin Inhibitor I") hinders HPV infection in HeLa cells as well as in the murine female genital tract (46, 68). To assess if this same inhibition was seen in HaCaT cells, as well as with other small molecule furin inhibitors, we carried out infections in the presence of Furin Inhibitor I, hexa-D-arginine ("Furin inhibitor II") and a serpine furin inhibitor, alpha-1 PDX. Table 2 compares the properties of the furin inhibitors used in the study.

Table 2. Furin/PC Inhibitors used in this Study.

	Mode of Inhibition	Cell Permeable	Dissociation Constant	Refs.
Furin Inhibitor I dec-RVKR-CMK	Blocks activity by irreversibly binding to catalytic site	Yes	furin (K_i~1nM) PC5/PC6, PC7/PC8 (K_i=0.12nM) PACE4 (K_i=3.6nM)	(33)
Furin Inhibitor II H-(D)RRRRRR-NH_2	Competitive Inhibitor	Poorly	furin (K_i~106nM) PACE4 (580nM)	(14, 55, 71)
alpha-1 PDX	Slow tight binding mechanism/ suicide substrate inhibitor	Yes	furin (K_i ~0.6nM) PC5/6 (K_i~2.3nM) PACE4 (NO inhibition) PC7 (NO inhibition)	(39, 40)

Infection of HaCaT cells with nt-PsVs in the presence of Furin inhibitor I at either concentration (1μM or 5μM) was almost completely abrogated (by 96%). Whereas Furin Inhibitor II, at a low concentration (1μM), reduced nt-PsV infection by 75%, and at a higher concentration (5μM) near complete inhibition was noted (Fig. 4.1A). Furthermore, FM-PsV infections were abolished by either inhibitor at the higher concentration (5μM), but at 1μM, Furin Inhibitor I treatment decreased infection by 44% and the presence of hexa-D-arginine at 1μM reduced infection by only 11%. Interestingly, FM-PsVs were not immune to furin inhibition, as would be expected per the Day model of entry. If L2 was truly pre-cleaved in these preparations, there should be no reason furin inhibition would hinder infection by FM-PsVs.

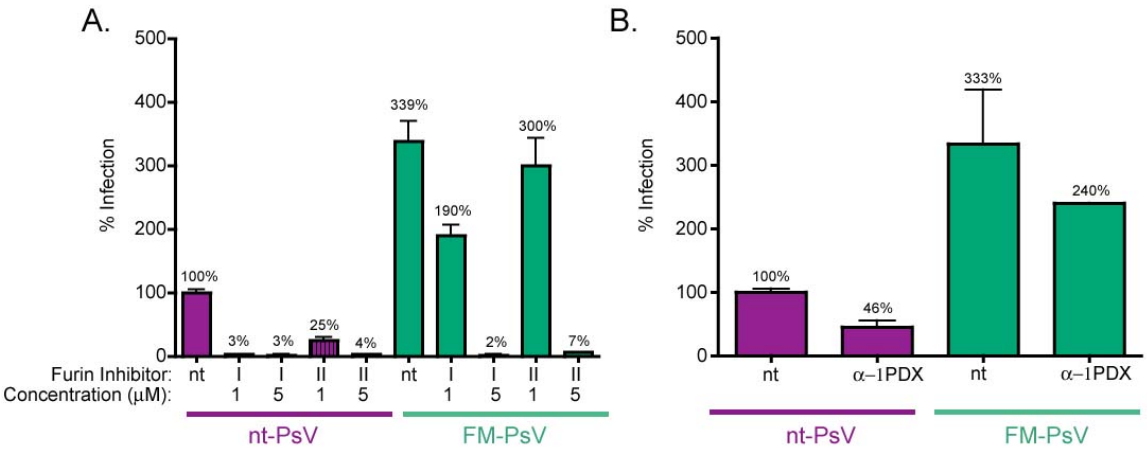


Figure 4.1. Furin inhibitors hinder FM-PsV infections. Subconfluent HaCaT cells were exposed to Furin Inhibitor I, II (at 1 or 5μM) or alpha-1PDX (5μM) for 30m at 37°C or left untreated. Nt-PsVs or FM-PsVs (150 vge/cell) were allowed to attach for 1 hr at 4°C. Unbound PsVs were washed off and inhibitors were again added to their respective wells. Internalization was initiated by a shift to 37°C. Infection levels were quantified at 24 h post infection by luciferase activity. Each experiment was carried out in duplicate and the error bars represent the standard error of the mean (n=4). Values were normalized to untreated nt-PsV infection. A. Mock level was 1% of nt-PsV infection. B. Mock level was 5% of nt-PsV infection.

Day's group determined that furin or PC5/6 were the PCs likely required during HPV infection (68). Keratinocytes express furin, PACE4, PC5/6 and PC7/8 (64) and experiments in LoVo and FD11 furin null cells lines indicated that PACE4 and PC7/8 could not functionally substitute for furin or PC5/6 (68). Although the Day group's findings suggest furin or PC5/6 to be the likely candidates important in HPV infection, our data indicated that nt-PsVs and FM-PsVs were differentially inhibited by dec-RVKR-CMK *versus* the poly-arginine -- the former having a broad specificity for all PC family members and the later inhibiting at least furin and PACE4, while its effect on PC5/6 and PC7 is not established in the literature. Owing to these issues, we tested the effects of alpha-1 PDX, a serpin molecule that selectively inhibits only furin and PC5/6A (39). This inhibitor reduced infection with nt-PsVs by over 50% but only decreased infection with FM-PsVs by 28% (Fig. 4.1B). These results again confirm that FM-PsVs are less susceptible to furin inhibition than nt-PsVs but also indicate that another PC family member could play a role in HPV keratinocyte infection, because even when furin and PC5/6 are specifically targeted, infection of either virus type is not fully abrogated at 5μM.

To investigate the ability of FM-PsVs to infect furin-deficient cells as established by Day *et al.* (24), nt-PsVs and FM-PsVs were used to infect two CHO-K1 derivative cells lines, furin deficient FD11 cells and FD11+ cells transfected with cDNA expressing furin (36, 37). FM-PsVs infected FD11 cells 13X more efficiently than did nt-PsVs (Fig. 4.2). This pattern was also observed in the FD11+ cells, as FM-PsVs infected 2X as well as did nt-PsVs. Interestingly, not only did the infectious potential of nt-PsVs rise dramatically in the FD11+ cells, so did that of the FM-PsVs; 7X better for FM-PsVs and

50X better for nt-PsVs. These results further support the likelihood of a cellular component that requires furin function during HPV infection, because even when PsVs are allowed to mature in the presence of furin and may well contain pre-cleaved L2, their infectious potential is even more pronounced when furin-expressing cells are infected.

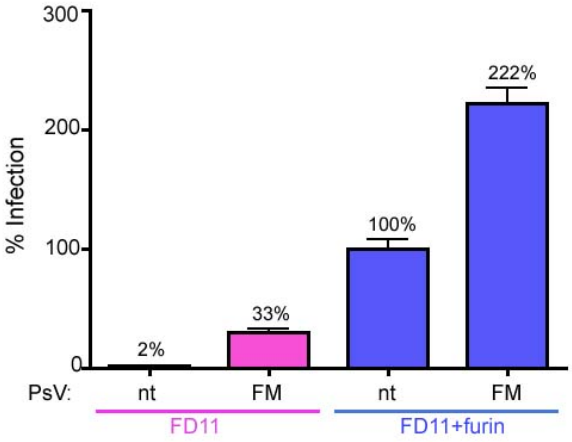


Figure 4.2. FM-PsVs infect furin null cells. Nt-PsVs or FM-PsVs (150 vge/cell) were allowed to attach to subconfluent FD11 or FD11+furin cells for 1h at 4°C. Unbound PsVs were washed off and internalization was initiated by a shift to 37°C. Infection levels were quantified at 24h post infection by luciferase activity. Each experiment was carried out in triplicate and the error bars represent the standard error of the mean (n=6). Values were normalized to nt-PsV infection in FD11+furin cells. FD11 cells mock level was 0.02% of nt-PsV infection and FD11+furin cells mock level was 0.0005% of nt-PsV infection.

Another goal for this aim of the study was to determine the time point at which furin is crucial for infection. The entire infection process is characterized as cell binding, entry, uncoating, transcription and translation followed by viral assembly and release. Early infection, however is described as comprising those events that occur prior to viral gene transcription including, binding, entry, uncoating and viral genome translocation to the nucleus, for a DNA virus, such as HPV. Although, the internalization time for a population of HPV particles is remarkably protracted and has been reported to vary among viral types, ($t_{1/2}$ time of HPV16 ≈ 4h, (25) and HPV31 ≈ 14h (78)), for the purpose of this study, early infection is being designated as those events that occur within the first 8h. This investigation is specifically using HPV16 PsVs, and our neutralizing

antibody time course data ($t_{1/2}$ time of nt-PsV ≈ 10h) (Fig. 3.4) supports a slower internalization time for HPV16 as compared to other studies (25). It is therefore likely, if furin is acting on a cellular factor or the virus prior to entry, furin inhibition at a very early time point such as ≤ 4h should hinder infection. If furin were not important until later in the infectious cycle we would expect significant reductions in infection levels only when furin inhibition is initiated after 4h. To test this possibility a time course inhibition assay was utilized, whereby Furin Inhibitor I, the cell permeable compound, was added to HaCaT cells at sequential time points throughout the infectious cycle (Fig. 4.3A). The addition of the furin inhibitor, at every time point up to 4h post infection severely inhibited the infection readout of PsV after 24h, and when added at 4h infection levels were only 50% compared to the 8h time point where infection was completely restored. These results indicate that furin is predominantly needed early during infection when the majority of the PsVs are binding and entering the cell. The reduced infection levels seen at the 4h mark could be due to the protracted internalization time of the virus, where some PsVs could be inhibited at an extracellular location at this late time point. Similar results were seen when this same time course inhibition assay was performed in CHO-K1 cells (Fig. 4.3B). The only notable difference being that full infection in this cell line was restored earlier, at the 4h time point. The same assay executed in FD11+ furin cells also produced similar results but full infection was again restored earlier, at 2h (Fig. 4.3C). As was seen in Fig. 4.2, the infection levels of FD11+ furin cells were well beyond those seen in HaCaT cells or parental CHO-K1 cells. Again, this demonstrates that HPV infection is augmented in cells expressing furin at elevated levels. Another noteworthy, but albeit, unexpected result from these experiments was that in CHO-K1

cells, when the inhibitor was added at 4 and 8h post binding, infection levels rose well above non-treated cells (the standard of 100% infection). This same result was even more pronounced in the FD11+furin cell line where at 4 and 8h addition of the furin inhibitor caused infection levels to double and almost triple, respectively. Without further investigation it is unclear as to why the addition of Furin Inhibitor I, a broadly acting PC inhibitor, at a late time point would aid the infectious process so dramatically. Nevertheless, these time course inhibition experiments indicate that furin/PC activity is necessary before 4h, very early in HPV infection.

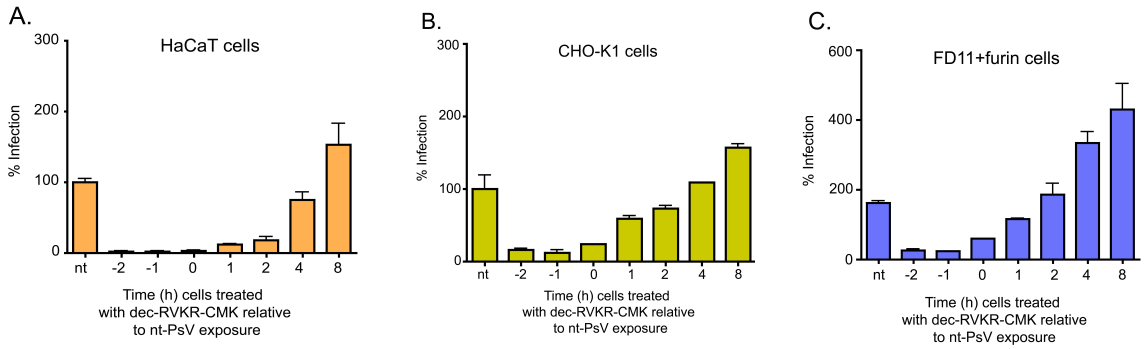


Figure 4.3. Furin Inhibitor I time course assay demonstrates that furin is necessary early in HPV infection. Time course inhibition assay wherein subconfluent (A) HaCaT, (B) CHO-K1 or (C) FD11+furin cells were exposed to dec-RVKR-CMK (1μM). Nt-PsVs (150vge/cell) were allowed to attach for 1h at 4°C in the presence or absence of the inhibitor. dec-RVKR-CMK was added to pre-treated, attachment treated and 0h wells. Unbound PsVs were washed off and internalization was initiated by a shift to 37°C. At 1, 2, 4 or 8h cells were exposed to inhibitor. Infection levels were quantified at 24h post infection by luciferase activity. Each experiment was carried out in duplicate and the error bars represent the standard error of the mean (n=4). A. HaCaT cells mock level was 0.026% and values were normalized to untreated control at 0hr, B. CHO-K1 cells mock level was 0.004% and values were normalized to untreated control at 0hr, C. FD11+ furin mock level was 0.001% and values were normalized to untreated control of CHO-K1 cells.

To further investigate the requirement of furin in early infection, the furin-null cell line, FD11, was exposed to exogenous furin at various time points before and after

the addition of nt-PsVs (Fig. 4.4). Based on the results from the previous time course inhibition assay we expected that addition of furin up to the 4h time point would cause the most significant enhancement of infection. As expected, when FD11 cells were treated with furin prior to HPV16 attachment (-2h), at attachment (-1h), and at the 0h time point, infection levels more than doubled. This trend however, was not observed at the 1, 2 and 4h time points, where infection levels were comparable to untreated cells. These results indicate that furin's activity on the cells themselves likely effect the infection outcome. The only times at which furin activity in the presence of virus enhanced infection was at the -1h mark of infection, when the virus was binding to the cells and at the 0h time point, when viral entry initially occurs. Taken together, these data point toward an early role of furin/PC activity in HPV infection and further support the notion that furin, possibly in conjunction with another PC family member, is specifically involved in HPV infection because when furin is exogenously added to the cellular environment of FD11 cells infection levels increase.

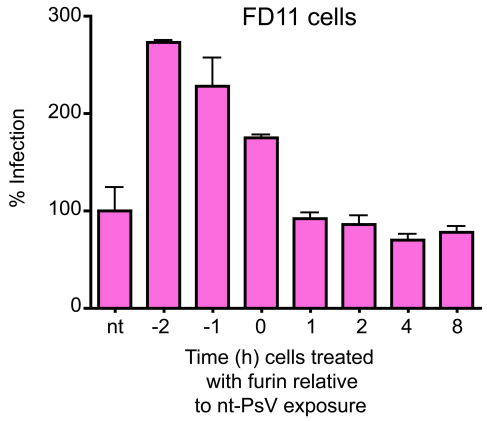


Figure 4.4. Furin is necessary early in HPV infection. Time course of furin rescue of infection wherein subconfluent FD11 cells were exposed to furin (1U). Furin was added prior to (-2, -1h), at the time of (0h) and post (1-8h) nt-PsV exposure. Nt-PsVs (150vge/cell) were allowed to attach for 1 hr at 4°C. Unbound PsVs were washed off and internalization was initiated by a shift to 37°C. Infection levels were quantified at 24h post infection by luciferase activity. Each experiment was carried out in duplicate and the error bars represent the standard error of the mean (n=4). Values were normalized to untreated control and mock level was 0.048%.

The results from the time course experiments demonstrate that furin/PC activity is necessary early in infection and suggest furin has an effect on a cellular component. However, these experiments cannot definitively reveal whether the enzymatic activity of a PC is required specifically before or after virus internalization. Richards *et al.* demonstrated that treatment with Furin Inhibitor I did not completely block BPV1 entry in C127 cells (68). In these experiments the investigators used immunofluorescent confocal microscopy to show co-localization of HA-tagged L2 and BrdU labeled viral psuedogenome with an endosomal marker Lamp-1, in cells treated with furin inhibitor. From this observation the researchers concluded that furin inhibition induced retention of genome and L2 protein in the endosome. However, the mouse mammary epithelial cell line used is not naturally infected by HPV and this piece of experimental data does not fit the research group's "Day model of entry," where furin activity is required at the plasma membrane. Therefore, in an attempt to resolve the ambiguity of the entry model and determine whether furin/PC activity is necessary prior to virus internalization, we examined if the use of different furin inhibitors (Table 2) would block virus entry into HaCaT cells. In the presence of Furin Inhibitor I, virus internalization was completely abrogated as visualized by immunofluorescent confocal microscopy. The virus appears to be trapped on the outside of the cell and co-localized with the plasma membrane (Fig. 4.5A), suggesting PC inhibition blocks the majority of viral entry. Additionally, in order to remove any residual virus present on the outside of the cell membrane, an acid wash procedure commonly used to remove ligands from cellular receptors was employed (43). Cells were acid washed with a low pH solution after 20h of infection in the presence of Furin Inhibitor I (Fig. 4.5B). In this image there is no PsV present, further indicating that

the broadly acting PC inhibitor blocked virus internalization. However, this phenotype was not seen when the cells were treated with Furin Inhibitor II. In this case, internalized virus was seen in both images where cells had been acid washed or not (Fig. 4.5C, D). Since Furin Inhibitor I does inhibit all PC members and Furin Inhibitor II targets only furin and PACE 4, these results suggest that, not only furin, but also other members of the PC family contribute to the internalization process of HPV into keratinocytes.

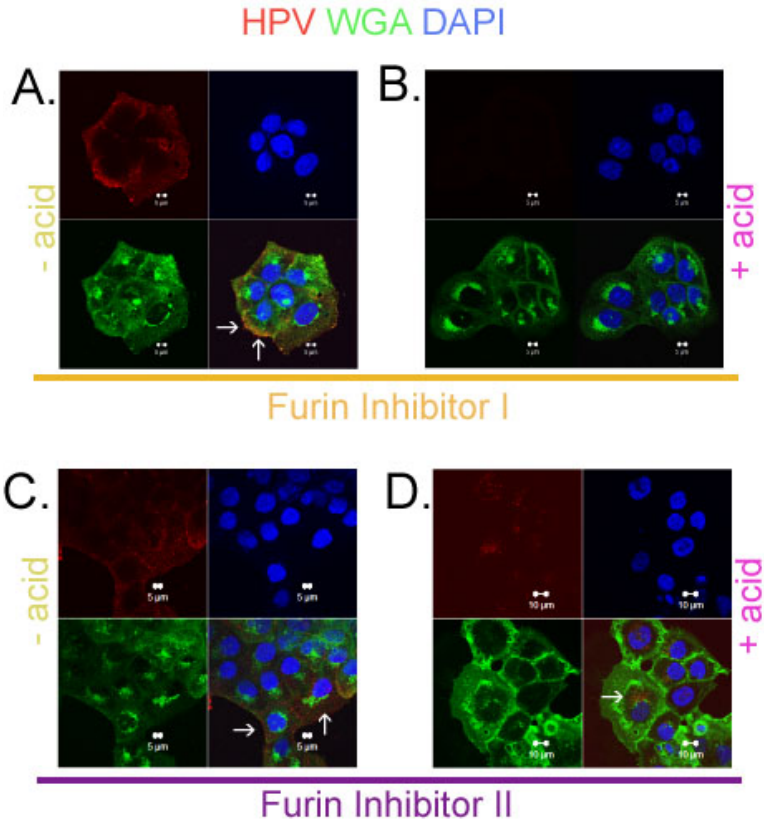


Figure 4.5. Furin Inhibitor I blocks HPV internalization. HaCaT cells were cultured overnight on cover slips. Cells were exposed to Furin inhibitor I (A, B) or II (C, D) (5μM) for 30m at 37°C or left untreated. Nt-PsVs (6500 vge/cell) were allowed to attach for 1h at 4°C. Unbound PsVs were washed off and inhibitors were added to respective wells. Internalization was initiated by a shift to 37°C. After 24h cells were washed at 4°C in a low pH solution (B, D) or left untreated (A, C). Cells were stained with WGA (to visualize the plasma membrane), exposed to mouse monoclonal anti-HPV16 (CamVir-1, Millipore) and then incubated with DyLight™ 594-conjugated affinityPure donkey anti-mouse IgG for 1hr at RT. Vectashield with DAPI mounting solution was used to visualize nuclei.

In summary, the experiments used to determine how the absence of furin/PCs affect HPV infection demonstrate that furin as well as another PC family member are important early in the HPV infection process. However, some of our experimental

evidence does suggest furin activity is specifically required in HPV infection because FD11 cells are poorly infected by nt-PsV and when exogenous furin is added to FD11 cells at early time points, infection levels double. On the other hand, when only furin and PC5/6 are specifically inhibited, infection is not fully perturbed in HaCaT cells, suggesting activity of another PC present in keratinocytes could functionally substitute for furin/PC5/6. Additionally, these experiments reveal that FM-PsVs carry a factor that renders the PsVs to be less susceptible to furin/PC inhibition and more readily infectious in FD11 cells. Importantly, FM-PsV particles are not immune to furin inhibition signifying furin must play another role in HPV infection. If adding furin during the maturation of PsVs does indeed result in simply the "pre-cleavage" of L2 and this is an obligatory step for viral entry, then FM-PsV infection should be immune to furin inhibition. Furthermore, the fact that nt-PsVs and FM-PsVs both infect FD11+ cells much better than they infect FD11 cells supports our conclusion from Aim 1, that a cellular component important for the infection process could be the target of furin as it is highly expressed in this cell type. The findings of this aim of the study also illustrate that the activity of furin/PC is needed very early in infection, prior to 4h. The fact that broadly inhibiting the entire family of PCs with Furin Inhibitor I blocks viral internalization, whereas using a more specific inhibitor, Furin Inhibitor II does not, indicates an additional PC member, other than furin, perhaps PC5/6, is specifically needed for initial virus internalization.

CHAPTER 5: DISCUSSION AND FUTURE STUDIES

The prevailing model for HPV infection set forth by Day and Schiller proposes that L1 binds to HSPG receptors on the cell surface and basement membrane, thus causing a L1 capsid conformational change that allows the masked furin cleavage site on L2 to become accessible. Once L2 cleavage by furin occurs, the virus supposedly detaches from HS and moves to the secondary internalization receptor (26). Yet, a number of experimental observations are inconsistent with this model. These include that L1 only VLPs are able to enter cells with similar entry kinetics as L1/L2 VLPs (56, 77), and cellular entry of PsVs is not deterred even when the L2 furin cleavage-site is mutated (68). L2 was also shown to be required for egress from the endosome, but not needed for viral entry or uncoating (44). Additionally, "mature" virus particles treated with exogenous furin are able to infect HS null cells better than immature particles exposed to furin during the maturation process (24). This result also does not support the Day model of HPV entry, because if HS is not present for interaction with mature PsVs, then the cells should lack the ability to trigger the conformational change and L2 accessibility. Thus, it is not clear how the addition of exogenous furin to an intact viral particle permits L2 cleavage by furin enhancing infection of HS null cells. An alternative or additional possibility is that furin is acting on a cellular factor aside from L2, which is important for infection of HS null cells.

Therefore, the central goal of this study was to more closely examine the role(s) of furin/PCs in HPV infection. Recent data from our lab has established that HPV infection is initiated by the formation and release of infectious HMW complexes containing HPV-HS-GFs from the cellular membrane. This release is dependent on the

activity of MMPs and once these HPV-HMW "decorated" complexes are "shed" from the cell, they interact with GFRs and are subsequently internalized (81). In light of this new model of HPV entry, furin activity could be important in a number of steps during the "shedding" process. For instance, furin/PCs activate growth factors like TGF-β, enhance expression of MMPs, as well as induce collagen processing important in ECM remodeling (3), each of which could promote better decoration of the virus. Hence, the overall hypothesis of this study is that furin and other PCs have additional roles in HPV infection, aside from L2 cleavage. The experimental evidence we provide supporting this hypothesis is summarized below.

In order to understand how furin maturation of PsVs as described by Day *et al.* (24) functioned, we compared the infectivity of nt-PsVs and FM-PsVs in furin- and HS-deficient cell lines. We found several important results. First, FM-PsV infects every cell line tested, HaCaT, CHO-K1 wild type, FD11+furin, FD11, as well as pgsd-677 cells. Our nt-PsVs infected these cells poorly if at all, indicating FM-PsVs do carry a modification that confers them with a greater infectious potential. Secondly, FM-PsVs infect CHO-K1 wild type cells and FD11+furin cells at much higher levels than they infect the counterpart mutant cells.

The Day model of infection that suggests L2 cleavage is a HS and furin-dependent process, explains why FM-PsV (if L2 pre-cleaved) could infect cells lacking HS (pgsd-677s) or furin (FD11s). However, it fails to clarify why FM-PsV infection is so much greater in HSPG proficient CHO-K1 wild type cells or FD11+furin cells compared to their counterpart mutant cells. If the addition of furin during the maturation process of PsVs simply pre-cleaves the L2 capsid protein, then one would expect FM-PsV, if

expressing a L2 pre-cleaved phenotype, to infect parental and mutant derivative cell lines at similar levels. This poses the question, why does the presence of cellular factors such as furin or HS augment these infections so greatly? Day and co-workers fail to account for additional related deficiencies that the mutant cells are likely to have. It is probable that because the mutant cell lines lack furin and HS they are also deficient in the ability to properly "shed" fully decorated HMW infectious complexes. Without furin, MMP activity and GF processing will be hindered (3) and without HS and GFs, formation of HPV-HS-GF complexes will not occur.

An additional facet of PC biology important to consider in HPV infection is the fact that PC5/6 is not present on the surface of pgsd-677 cells. PC5/6 is expressed as two different splice variants, A and B. PC5/6B contains a trans membrane domain but is only expressed in the kidney and small intestines, whereas PC5/6A (also referred to as just PC5/6) lacks a transmembrane domain and is expressed in every other tissue type (74). Although, PC5/6 lacks a transmembrane domain, HS can tether it to the cell surface; thus pgsd-677 cells lack PC5/6 and CHO-K1 and FD11+furin cells have an abundance of PC5/6 on their surface (73). Therefore, the low infectivity of pgsd-677 cells could be due not just to their lack of HS, but also due to the absence of PC5/6 at the cell surface. Conversely, the large quantity of PC5/6 present at the surface of CHO-K1 and FD11+furin cells could be one reason that infection levels in these cells are so high. One could test this postulate by simply adding exogenous furin and/or PC5/6 to HS null cells during HPV infection, if infection levels increased this would indicate that it is the lack of PC activity and not only the lack of HS at the cell surface that obstructs infection of pgsd-677 cells.

FM-PsVs have a faster internalization $t_{1/2}$ time than nt-PsVs, which indicates FM-PsVs escape antibody-mediated neutralization faster and thus enter HaCaT cells quicker. We postulated that this could be due to the association of FM-PsVs with a component such as HS that allows FM-PsVs to bypass the need for primary receptor engagement and go straight to a secondary receptor. We did indeed demonstrate by immunoblot that HS was present within the virion preparation of FM-PsV, but also found HS associated with nt-PsV.

Although, HS was present in both preparations this does not rule out the possibility that FM-PsVs are "decorated" with another bioactive molecule, such as a particular growth factor, which could confer the virion the increased ability to infect at high levels. The association of specific GFs with FM-PsV should be further examined by immunoblot, ELISA or Luminex® assays. Additionally, although heparinase treatment of these preparations did not hinder their infectious potential in pgsd-677 cells, this could be due to a very stable HS-virion complex, such that heparinase activity is inadequate. This explanation is supported by the fact that the preparations must be reduced, heated and diluted in Heparinase III buffer before the PsVs can disassociate enough to enter the gel and/or be recognized by immunoblot for HS.

The results of our immunoblots as well as experiments using PsVs pre-treated with heparinase revealed that a component of Heparinase III buffer, likely Ca^{2+} augmented infection and modified the capsid structure of the virion preparations, allowing the epitope of HS to be unmasked. Ca^{2+} could potentially affect HPV infection in a number of ways. Foremost, furin/PC function is strictly Ca^{2+} dependent, increased concentration of the ion could boost convertase function and in turn augment HPV

infection, as any furin dependent process would increase. Secondly, Ca^{2+} is known to affect the function of several known HPV binding partners, for instance Ca^{2+} is required for the association of HPV with annexin A2, a potential secondary HPV receptor (Dziduszko and Ozbun manuscript in preparation).

It is also known that Ca^{2+} is required for HSPG cell surface recycling in rat parathyroid cells (82). If Ca^{2+} does indeed affect HSPG function, this could play a role in HPV infection as well. Although, this would need to be tested in a relevant cell line as our experiments demonstrating the effect of heparinase III buffer on infection were carried out in HSPG null cells. Lastly, Ca^{2+} is known to stabilize the capsid structure of murine polyomavirus (MPV), a virus with similar capsid structure to HPV [Chuan, #3292] and is also required for capsid reassembly after capsomer disruption of BPV (60). Stabilizing viral capsomer structure could improve the viruses' ability to bind to necessary receptors and/or binding partners during infection. Although, our TEM images did not reveal a "tighter" capsid structure in the presence of heparinase III buffer, this could be due to other interfering complexes within the buffer, such as BSA. Nonetheless, further work is necessary to determine how Ca^{2+} alters capsid structure and augments HPV infection.

The TEM images however, did reveal that the FM-PsVs appear to contain more "loosely" associated capsids than did nt-PsVs. Studies have suggested that particles with a darker core are less electron dense as they take up more negative stain (30). Our FM-PsV TEM images do contain more particles appearing to have a "looser" or less mature capsid structure as more of them have a darker core. Perhaps then, the greater infectious potential of FM-PsVs could be attributed to the preparation containing more particles that

were “loose” or leaky; this type of structure could allow the capsid to readily un-coat and nuclear genome delivery to occur more efficiently. Furthermore, it is known that immature HPV particles are infectious and it is thought that they are likely involved in naturally occurring infections (12). However, further examination of FM-PsV structure is required to determine if an immature conformation confers these virions with a greater infectious potential.

The use of pharmacological inhibitors with different specificities revealed three important aspects of PC functions in HPV early infection. The varied furin/PC inhibitors used in the study (Table 2) affected PsV internalization and infection in different ways, at different times and at different cellular locations of the early infection process. Furin inhibitors affected nt-PsV and FM-PsV slightly differently and importantly FM-PsVs were not immune to pharmaceutical furin inhibition. Both the chloro-methyl-ketone and the poly-arginine at 5μM inhibit FM-PsV infection. If allowing PsVs to mature in the presence of furin only modifies the virus by pre-cleaving L2, and this is the only furin dependent process in HPV infection, then FM-PsV virus should be able to bypass the need for PC activity and infect when the inhibitors are applied. That FM-PsVs were not immune to furin inhibition provides powerful evidence for our underlying hypothesis that furin or another PC must play an additional role during HPV infection.

Additionally, FM-PsV infections were less affected by the inhibitors at the lower concentration tested (1μM) than nt-PsV infection. The difference in inhibition seen between these concentrations (1μM versus 5μM) could be attributed to several different mechanisms. Foremost, the poly-arginine (Furin Inhibitor II) is a competitive inhibitor of furin, whereas the chloro-methyl ketone (Furin Inhibitor I) blocks the activity of furin by

irreversibly binding to the catalytic site of the enzyme, a much stronger mode of inhibition (14, 33); at a low concentration Furin Inhibitor II could simply not outcompete furin. Secondly, Furin Inhibitor II has been reported to be poorly cell permeable (55). Consequently the intracellular concentration of the poly-arginine could be too low to wield an inhibitory intracellular effect on furin, necessary if the endosomal release of the virus is furin dependent, as initially proposed by Day's group (68). This would also be expected if the translocation of furin to the cell surface is a required intracellular early step that is also susceptible to furin inhibition. Additionally, MMP activation within the Golgi, a furin dependent process, could also be perturbed. Lastly, at higher concentrations, the inhibitor may affect other PC family members (14), which could play a yet undetermined role in HPV entry and infection.

FM-PsVs are modified in such a way that allows them to be less susceptible to furin/PC5/6 inhibition as demonstrated by the fact that administration of alpha-1 PDX, the serpine molecule that only selectively inhibits furin and PC5/6, causes FM-PsV infection to be perturbed by only 28% as compared to over 50% for nt-PsVs. This result coupled with the fact that FM-PsVs can also better infect pgsd-677 and FD11 cells, than nt-PsVs, indicates FM-PsVs are associated with an additional component that could account for its high infectious potential.

In using Furin Inhibitor I, the chloro-methyl ketone, which broadly inhibits all PC members, we were also able to determine that furin/PC activity is crucial before the 4h mark of infection, suggesting early events such as initial cell entry are furin/PC dependent. HaCaT cell infection was still significantly inhibited when Furin Inhibitor I was added 4h post binding, but CHO-K1 and FD11+furin cells were more resistant to PC

inhibition at this time point and only showed pronounced susceptibility to PC inhibition before 2h post binding. Intriguingly, in both of these cell types (CHO-K1 and FD11+furin) the addition of Furin Inhibitor I at the 8h mark increased infection substantially, suggesting blocking PC activity at a late time point benefits the infection process. However, since the average entry time of HPV particles is protracted and varies among viral types, it is difficult to fully interpret time course assay data. Although, we have demonstrated that PC activity is crucial in the first 4h of infection, this does not pinpoint an exact time when furin/PC activity is necessary. To differentiate if furin/PC activity was required before or after viral internalization, viral entry in the presence of furin/PC inhibition was examined using immunofluorescent confocal microscopy. Broadly inhibiting all PCs with Furin Inhibitor I blocked viral entry, whereas the poly-arginine (Furin Inhibitor II) did not hinder virus internalization. This indicates that more than one PC member likely plays a role in HPV infection and they act at different steps. Moreover, our data indicate that infection levels rise in FD11 cells only when exogenous furin is added before and during viral attachment, indicating that specifically furin is required very early in infection, as the virus initially binds the cell. However, a further distinction between how furin and other PC members are specifically involved in viral internalization versus downstream steps of infection requires additional investigation. Prior to postulating the full model of HPV entry (26), the Day-Schiller research group demonstrated that furin L2 cleavage was necessary for viral endosomal escape, as L2 cleavage-site mutants entered and uncoated but remained in the endosomal compartment (68). In comparing these findings with our own data it is likely that furin as well as another PC member is necessary for multiple steps of HPV infection, possibly one for

entry, such as PC5/6, by modifying a cellular component that acts as a viral binding partner and another, such as furin, for actual L2 cleavage either before or after the virus is internalized. Additional immunofluorescent confocal microscopy experiments using nt-PsV and FM-PsV in the presence of furin inhibitors, including alpha-1 PDX, along with endosomal markers could help further reveal the nature of which PC(s) activity is necessary in HPV infection.

Although, the specific mechanism of furin/PC activity has not been fully elucidated in this study, several important findings have been made. Furin is likely not the only PC member involved in HPV infection and cleavage of the L2 minor capsid protein is not the only furin dependent process required for HPV early entry. Furin/PC activities affect so many diverse processes now known to be involved in HPV infection, including growth factor processing and MMP activation (3). Therefore, it is essential that future studies examine if furin inhibition, either by the use of furin null cell lines or pharmaceutical inhibitors, hinders the release of HPV-HS-GF HMW viral complexes from the cell surface. Additionally, further research addressing which PC member is required for what step of infection is necessary. However, such studies will be challenging, due to the fact that within the cell culture model of infection, aside from immunofluorescent confocal microscopy, there is currently no definitive method to distinguish between furin/PC dependent processes that occur at the cell surface *versus* those within the cell. The use of neutralizing monoclonal antibodies or other inhibitors specific to different PC members, that do not cross the cell membrane, would be one way to discriminate extracellular versus intracellular furin/PC function. However, as of yet these types of reagents are not available. Until such time as these activities can be

differentiated, revealing the specific points at which furin/PC activity is crucial during HPV infection will remain elusive.

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