

Use of an *in vivo* animal model for assessing the role of integrin $\alpha_6\beta_4$ and Syndecan-1 in early steps in papillomavirus infection

Hao-Shun Huang, Paul F. Lambert*

McArdle Laboratory for Cancer Research, Department of Oncology, University of Wisconsin School of Medicine and Public Health, Madison, WI, USA

ARTICLE INFO

Article history:

Received 18 August 2011
 Returned to author for revisions
 16 September 2011
 Accepted 21 August 2012
 Available online 18 September 2012

Keywords:

Human papillomavirus (HPV)
 Integrin $\alpha_6\beta_4$ (Int $\alpha_6\beta_4$)
 Heparin sulfate proteoglycans (HSPGs)
 Syndecan-1 (Sdc-1)

ABSTRACT

Human papillomaviruses (HPV) are small DNA tumor viruses. HPV infection requires entry of virions into epithelial host cells that support the viral life cycle. Here, we used an *in vivo* mouse model, in which HPV pseudoviruses (PVs) are scored for their ability to transduce reporter genes, to test the role of various cellular proteins in entry. We initially investigated the role of integrin $\alpha_6\beta_4$ in mediating early steps of HPV infection. Deficiency of integrin $\alpha_6\beta_4$ is modestly but significantly suppressed reporter-gene transduction by PVs in conditional integrin β_4 knockout mice. We also investigated the role of syndecan 1, a heparin sulfate proteoglycan (HSPG) for its role in HPV infection. We did not see a significant reduction in reporter-gene transduction by PVs in syndecan-1 null mice. This indicates that this HSPG is not essential for early steps in HPV infection, but does not discount a need of other HSPGs in mediating HPV infection.

© 2012 Elsevier Inc. All rights reserved.

Introduction

Human papillomaviruses (HPVs) are the most common sexually transmitted (STD) pathogens. They mainly cause benign lesions called papillomas or warts on skin and mucosa. Approximately a dozen mucosotropic HPV genotypes (the ‘high risk’ HPVs), including HPV16, are etiologically linked to the development of multiple malignancies, such as cervical, vaginal, anal, penile and a subset of head and neck cancers (zur Hausen, 2009). In the currently held model for the HPV life cycle, HPVs enter cells within the basal layer of stratified squamous epithelia at sites of wounding, establish their genomes as nuclear plasmids in these cells, and only upon differentiation of daughter cells, produce progeny virus that are released into the environment to infect the next host (Sapp and Bienkowska-Haba, 2009; Schiller et al., 2010). Despite considerable research regarding the biology and oncogenicity of HPVs, the earliest steps of HPV infection responsible for the binding/entry of virus particles to the natural host cells, their internalization and delivery of the HPV genomes to the host nucleus are not yet well defined. In this study, we examined the cellular requirements for binding and entry.

The current literature on the cellular requirements for HPV infection is confusing. Different proteins have been identified to mediate HPV infection by different groups, including integrin $\alpha_6\beta_4$ (Evander et al., 1997; McMillan et al., 1999) and its natural ligand

laminin 5 located in the extracellular matrix that forms the basement membrane to which basal epithelial cells bind (Culp et al., 2006a, 2006b, HSPGs on cell surface (Giroglou et al., 2001; Joyce et al., 1999; Knappe et al., 2007; Shafti-Keramat et al., 2003; Surviladze et al., 2012) and in extracellular matrix (Selinka et al., 2007), as well as cyclophilins (Bienkowska-Haba et al., 2009, 2012). While integrin $\alpha_6\beta_4$ is debated as to its role in binding and entry of HPVs, there has arisen equally inconclusive data regarding the role of alternative cellular receptors in mediating HPV entry. Because papillomaviral VLPs have long been known to bind to a broad range of cell lines (Muller et al., 1995), this has led to the hypothesis that papillomaviral receptors must be highly conserved in their expression amongst different cell types. Several laboratories have described studies in tissue culture that support a role of HSPGs in HPV infection. A fairly well conserved motif in the carboxyl region of the major capsid protein, L1, which is conserved among different HPV genotypes, is similar to known heparin binding motifs (Joyce et al., 1999). HSPGs are reported in mediating entry of HPV16 L1/L2 and HPV33L1/L2 PVs in COS-7 and HeLa cells (Giroglou et al., 2001); whereas, other studies refute a role of integrin $\alpha_6\beta_4$ in entry of HPV33L1/L2 PVs to these same cells (Selinka et al., 2002). The amino acid residues of HPV16 L1 that mediate binding to HSPGs have been further defined (Knappe et al., 2007), and this binding was found to cause a conformational change in L1 that exposes a furin cleavage site in the minor capsid protein, L2, that was found to be critical for infection (Richards et al., 2006). Carrageenan, a class of sulfated polysaccharides extracted from algae, was found to inhibit HPV PVs infections *in vitro* (Buck et al., 2006) and *in vivo* (Roberts et al., 2007). Syndecan-1, a predominant HSPG in epithelial

* Corresponding author.

E-mail address: plambert@wisc.edu (P.F. Lambert).

cells has a high binding affinity for HPV16 VLPs and increases susceptibility to infection by HPV11 (Shafti-Keramat et al., 2003), suggesting it may function as a HPV receptor. However, HPV31 infection of HaCaT cells was not inhibited by heparin or heparinase, indicating that the role of HSPGs might be cell type if not HPV genotype specific (Patterson et al., 2005). In contrast, syndecan-1 was argued to complex with HPV16 and may play a crucial role in infection (Surviladze et al., 2012). The loss of the activity of cyclophilins (CyP), especially Cyclophilin B (CyPB) which colocalizes with syndecan-1, blocks HPV16/18 infection in cells (Bienkowska-Haba et al., 2009). An interesting finding indicates that integrin $\alpha_6\beta_4$ interacts and colocalizes with syndecan-1 (Wang et al., 2010). These findings complicate the interpretation of a role of integrin $\alpha_6\beta_4$ and/or HSPGs in HPV infection process. In sum, there is considerable confusion as to what cellular proteins are important for HPV infection.

We posit that the differing and in many cases mutually exclusive conclusions reported in the literature at least in part reflect the choice of cells and culturing conditions used in past studies. We hypothesize that infection by HPV is mediated by multiple cellular proteins including integrin $\alpha_6\beta_4$ and syndecan-1. To avoid the confusion rendered by cell culture studies, we tested our hypothesis in an animal model that provides us the means for monitoring early steps of binding and entry by HPV pseudoviruses (PVs) in a relevant tissue. If multiple proteins facilitate HPV infection *in vivo*, we may not observe a complete disruption of HPV infection when only a single cellular gene is deleted. In such circumstances, the efficiency of transduction by an HPV PS must be accessed in a dose-dependent manner to evaluate the relative importance of candidate proteins. To achieve the goal, we adapted a mouse model that permits one to monitor transduction by HPV PVs in a relevant tissue, the female reproductive tract (Roberts et al., 2007). We modified this *in vivo* model to allow us to quantify transduction by HPV16 pseudoviruses carrying the reporter gene Luciferase. This refined mouse model permitted us to quantify *in vivo* reporter gene transduction by HPV pseudoviruses in a dose-dependent manner, thereby providing us the means to access cellular requirements using genetically engineered mouse strains deficient for individual cellular genes and their gene products that have been previously implicated in HPV entry in tissue culture studies. Mice conditionally deficient for integrin $\alpha_6\beta_4$, or nulligenic for syndecan-1 were evaluated for their susceptibility to reporter gene transduction by HPV pseudoviruses.

Results

Modification of a mouse model for quantifying the efficiency of reporter gene transduction by HPV PVs: a model for assessing genetic requirements for early steps in papillomavirus infection

To test our hypothesis that multiple cellular proteins mediate HPV binding/entry together or independently, we first modified a previously described mouse model for *in vivo* transduction of reporter genes by HPV PVs (Roberts et al., 2007). HPV16 PVs (HPV16:LucF) containing both firefly luciferase and green fluorescent protein genes were generated, the titers of these stocks measured by flow cytometric measurement of GFP-positive cells exposed to serial dilutions of the PV stock, and then known titers of PV used to infect reproductive tracts of experimental mice. The luminescence (luciferase assay) from vaginocervical tissue lysates were subsequently measured quantitatively as readout for HPV infection. The relative luminescence units (RLU) per μg total protein (Bradford protein assay) corresponded to the amount of HPV16:LucF challenged in mice reproductive tracts (Fig. 1). Our

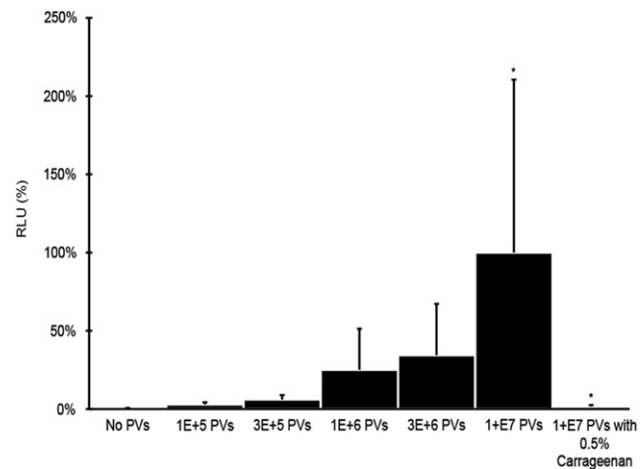


Fig. 1. HPV16 infection *in vivo* in a dose dependent. Each group of mice reproductive tracts ($N=6$) were challenged with different amount of PVs (HPV16:LucF) as indicated. An additional group of mice were challenged with $1\text{E}+07$ PVs and 0.5% carrageenan. 48 h after infection, mouse female reproductive tracts were harvested, protein lysates made, and luciferase assays were carried out to quantify the infectivity. Concentrations of total proteins were measure by Bradford protein assay. The infectivity level (relative luminescence units, RLU/ μg) of mice treated with $1\text{E}+07$ PVs was set as 100%. * $P=0.004$ (Wilcoxon rank sum test, 2 sided).

refined mouse model provides an alternative means to quantify the efficiency of HPV PV gene transduction *in vivo*, as a means for assessing genetic requirements for early steps in papillomavirus infection.

Infectivity of HPV, as scored by PV gene transduction, is significant reduced in $K14\text{Cre}/\text{Int}\beta_4^{\text{fl}}$ mice

Integrin $\alpha_6\beta_4$, restricted primarily to the basal layer of epithelia (Wilhelmsen et al., 2006), has been argued by some but not other investigators to mediate binding to and possibly the uptake of papillomavirus particles by cells (Evander et al., 1997; McMillan et al., 1999). The finding that laminin 5, the ECM partner to integrin $\alpha_6\beta_4$, may serve to trap HPV particles to the ECM (Culp et al., 2006a, 2006b), provides another potential link to this integrin. This led us to hypothesize that integrin $\alpha_6\beta_4$ in the relevant tissue may behave differently from what has been indicated in monolayer tissue culture. It has been proposed that a non-HSPG receptor is essential for HPV infection (Kines et al., 2009; Surviladze et al., 2012). That there are interactions between integrin $\alpha_6\beta_4$ and syndecan-1 and others HSPGs (Wang et al., 2010) raises the possibility that integrin $\alpha_6\beta_4$ may act independently or in conjunction with other cellular proteins in facilitating HPV infection *in vivo*. We applied our mouse model to access the role of integrin $\alpha_6\beta_4$ in HPV infection *in vivo* by testing whether the efficiency of reporter gene transduction by HPV PVs is diminished significantly in mice deficient for integrin $\alpha_6\beta_4$.

Because of the perinatal lethality resulting from loss of both alleles of integrin α_6 or integrin β_4 in the germ line (Raymond et al., 2005), we examine the requirement of integrin $\alpha_6\beta_4$ for HPV PV gene transduction *in vivo*, making use of genetically engineered mice conditionally deficient for integrin β_4 ($K14\text{Cre}/\text{Int}\beta_4^{\text{fl}}$) (Raymond et al., 2005) (integrin α_6 conditional mice were not available for study). Cells of skin epithelia from $K14\text{Cre}/\text{Int}\beta_4^{\text{fl}}$ mice that have undergone Cre-loxP mediated disruption of the conditional allele for integrin β_4 also have undetectable levels of integrin α_6 . (Raymond et al., 2005). Importantly, there was no compensatory increase in expression of other integrins in the integrin β_4 deficient cells *in vivo* (Raymond et al., 2005). We observed that the efficiency of HPV16 PVs gene transduction was modestly but significantly

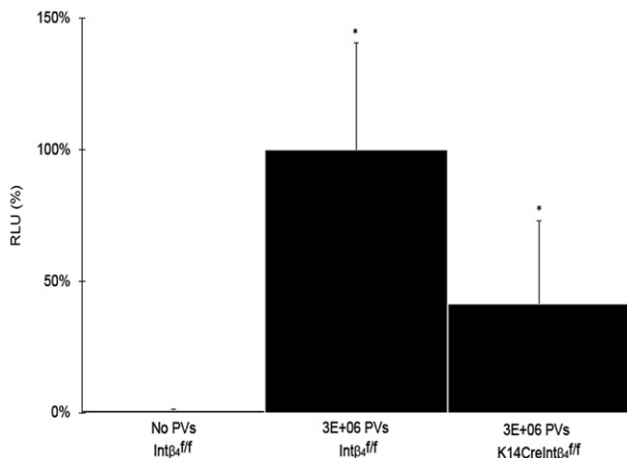


Fig. 2. HPV16 infectivity in integrin $\alpha_6\beta_4$ sufficient versus deficient mice. *Intβ₄^{fl/fl}* and *K14CreIntβ₄^{fl/fl}* mice reproductive tracts ($N=6$) were challenged with or without $3E+06$ PVs (HPV16:LucF) as indicated. 48 h after infection, mouse female reproductive tracts were harvested, protein lysates made, and luciferase assays were carried out to quantify the infectivity. Concentrations of total proteins were measured by Bradford protein assay. The infectivity level (relative luminescence units, RLU/ μ g) of *Intβ₄^{fl/fl}* mice treated with $3E+06$ PVs was set as 100%. * $p=0.04$ (Wilcoxon rank sum test, 2 sided).

reduced in *K14CreIntβ₄^{fl/fl}* mice 48 h post infection when compared to that in *Intβ₄^{fl/fl}* mice (Fig. 2). This same reduction in infection was observed over 96 h indicating that the reduction in infection was not simply due to a delay in infection (Fig. S1 supplementary material). In both the wild type and the integrin conditional null mice, the luciferase signal decreased over the time course with maximal expression seen at 48 h. This was as expected because the rapid turnover of epithelial cells in the female reproductive tract results in sloughing off of infected cells by 72–96 h. By 120 h a difference in luciferase signal between the wild type and integrin conditional null mice was no longer observed and the level of luciferase activity was low in both strains, presumably reflective of the epithelial cell turnover. These data support the hypothesis that integrin $\alpha_6\beta_4$ contributes quantitatively, but perhaps not qualitatively, to the efficiency of HPV infection *in vivo*.

HPV PVs transduce reporter genes in portions of mouse female reproductive tract epithelia having little-to-no detectable levels of integrin α_6

In the original study describing the phenotypes of the *K14CreIntβ₄^{fl/fl}* mice in skin epithelia, it was observed that not all epithelial cells lost expression of integrin β_4 , and this correlated with retention of expression of integrin α_6 (Raymond et al., 2005). To learn whether this is also the case in the female reproductive tract and to learn if HPV pseudoviruses selectively transduce those portions of the reproductive tract deficient for integrin expression, we performed integrin α_6 -specific immunofluorescence on *K14CreIntβ₄^{fl/fl}* mice.

In the female reproductive tract epithelia, *Intβ₄^{fl/fl}* mice express integrin α_6 consistently in basal cells (Fig. 3A); whereas, *K14CreIntβ₄^{fl/fl}* mice lose expression of integrin α_6 with patches of integrin α_6 -negative tissue interdigitated between patches of integrin α_6 -positive tissue (Fig. 3B). This is consistent with what was observed in the skin of this same mouse genotype (Raymond et al., 2005). To look at susceptibility of integrin α_6 -positive versus integrin α_6 -negative tissue to HPV PV-mediated gene transduction, *K14CreIntβ₄^{fl/fl}* and *Intβ₄^{fl/fl}* mice were exposed to HPV16:TWB PVs carrying the tdTomato gene and 72 h post-exposure reproductive tracts were snap frozen, sectioned, stained with antibodies to integrin α_6 and analyzed by fluorescence microscopy. We observed that most epithelial cells in

the reproductive tract of the *Intβ₄^{fl/fl}* mice express integrin α_6 in basement membrane and displayed robust tdTomato signal indicative of infection (Fig. 3A). The tdTomato-positive cells were confirmed to be of epithelial origin by staining for Keratin 14, a marker for epithelial cells (Fig. 3C). In the *K14CreIntβ₄^{fl/fl}* mice, we observed that most of the tdTomato positive epithelia accompany positive staining for integrin α_6 ; however, there were clearly tdTomato red positive cells in regions without detectable levels of integrin α_6 . From these observations, we conclude that cellular integrin $\alpha_6\beta_4$ is not absolutely required for HPV infection *in vivo*, though it appears to influence the efficiency of infection.

Syndecan-1, the predominant HSPG in epithelial cells, is not necessary for HPV infection in vivo, based upon PV gene transduction studies

To address whether syndecan-1 is required for HPV infection, we first confirmed the binding of HPV16 PVs to mouse syndecan-1 by immunoprecipitation in mouse C127 cells (Fig. S2 supplementary material), and then monitored the efficiency of HPV PV-mediated gene transduction in syndecan-1 null mice. The efficiency of HPV PV-mediated gene transduction was not significantly different in syndecan-1-sufficient versus -null mice by monitoring the level of luciferase expression in extracts of female reproductive tracts of mice exposed to HPV16:LucF PVs (Fig. 4). We also observed the same results when using HPV31 and HPV45 PVs (Fig. S3 supplementary material). These results argue that syndecan-1 is not essential for HPV infection in this mouse model. However, because carrageenan did inhibit *in vivo* HPV PV-mediated gene transduction in our hands (Fig. 1) as reported by others (Roberts et al., 2007), this raises the likelihood that other HSPGs can mediate infection in the absence of syndecan-1.

Discussion

Based upon our *in vivo* studies, we conclude that neither integrin $\alpha_6\beta_4$ nor syndecan-1, two putative cell surface receptors for papillomaviruses are absolutely required for gene transduction of the mouse female reproductive tracts by HPV pseudoviruses. These results provide a strong rationale for pursuing the identification of alternative cell surface receptors for HPVs. One obvious possibility, in the case of syndecan-1 null mice, is that other HSPGs can serve as replacement receptors for syndecan-1. Earlier studies documented compensatory increases in other HS-modified proteins (Liu et al., 2003), and the surface expression of integrins (Stepp et al., 2007) in syndecan-1 null keratinocytes. Thus an obvious route of further study is to look at the possible role of these proteins that might also be increased in their expression in the female reproductive tracts of syndecan-1 null mice. That carrageenan is an effective inhibitor of HPV pseudovirus infection in the mouse female reproductive tract argues that HSPGs are important for HPV infection in this mouse model (Fig. 1) (Roberts et al., 2007).

On the other hand, compensation is not likely to be an explanation for the retention of infectivity in areas of the female reproductive tract of *K14CreIntβ₄^{fl/fl}* mice that fail to express detectable integrin α_6 , as no compensatory increases in other integrins have been detected in these mice (Raymond et al., 2005). Also, the fact that integrin α_6 was lost in the female reproductive tract of *K14CreIntβ₄^{fl/fl}* mice would argue that no alternative integrin α_6 partner is induced, for, were a partner induced in its expression, then levels of integrin α_6 would not have decreased. This leaves us to question the absolute necessity for any integrin in mediating HPV infection *in vivo* in this mouse model, even though there clearly is a quantitative reduction in infection in the *K14CreIntβ₄^{fl/fl}* mice.

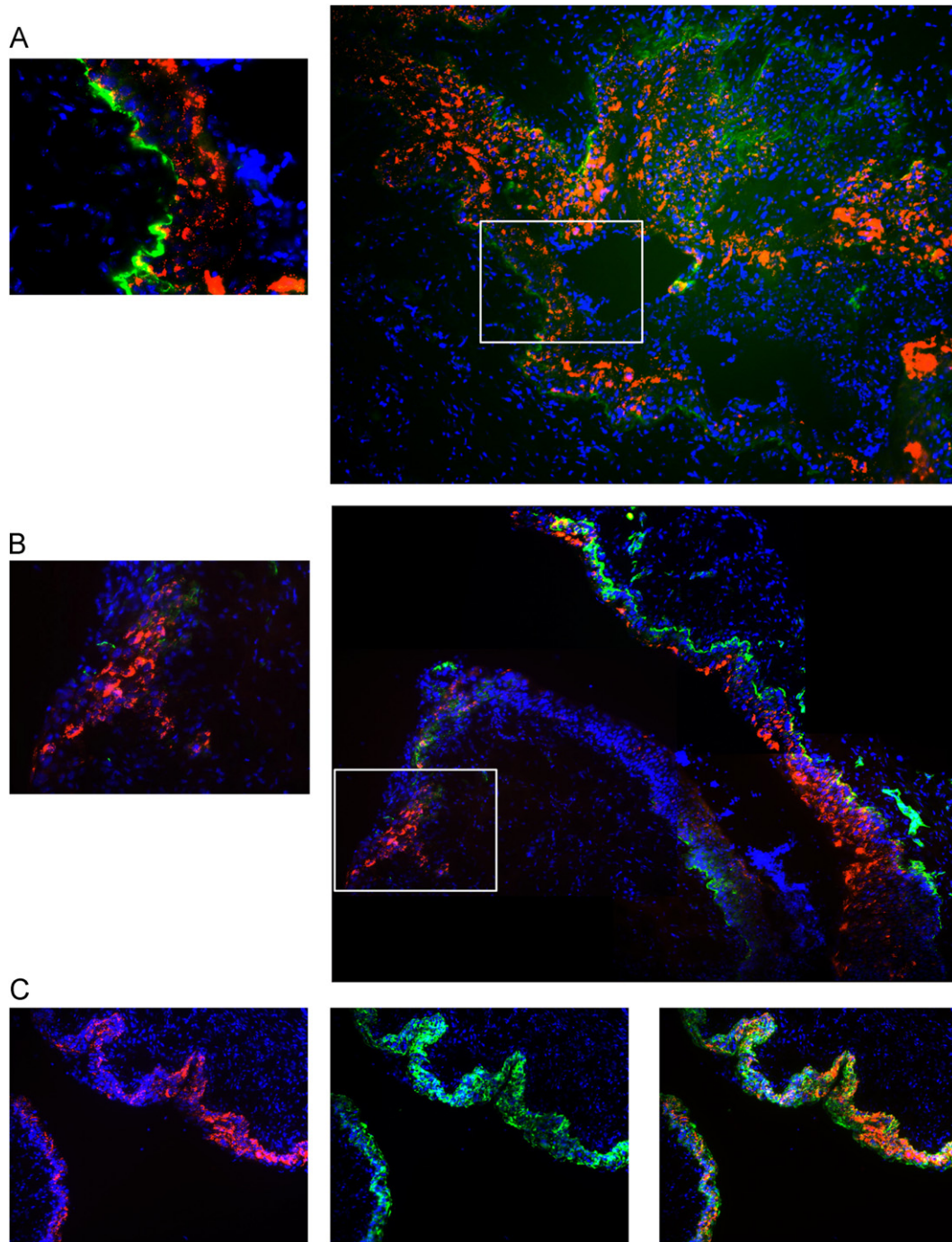


Fig. 3. HPV16 PVs infection in the reproductive tract epithelia of integrin $\alpha_6\beta_4$ sufficient versus deficient mice. *Int\beta_4^{fl/fl}* and *K14CreInt\beta_4^{fl/fl}* mice reproductive tracts were challenged with $1E+07$ PVs (HPV16:TWB). 72 h after infection, mouse female reproductive tracts were harvested, snap frozen, cryosection made, DAPI staining (blue) for cell nuclei and tdTomato red fluorescence (red) indicated as viral infection were monitored by microscopy. (A) HPV16 PVs infection in *Int\beta_4^{fl/fl}* mice; FITC (green) indicates the immunohistochemistry staining for integrin α_6 (B) HPV16 PVs infection in *K14CreInt\beta_4^{fl/fl}* mice; FITC (green) indicates the immunohistochemistry staining for integrin α_6 (C) HPV16 PVs infection in *Int\beta_4^{fl/fl}* mice; FITC (green) indicates the immunohistochemistry staining for Keratin14.

Our study demonstrates the utility of mice in monitoring the cellular requirements for HPV infection, and points to their use in assessing the role of other cellular factors in mediating HPV infection. Our and others' previous studies (Huang et al., 2010; Karanam et al., 2010) demonstrated that the strong antiviral activity of gamma secretase inhibitors both *in vitro* and *in vivo*. Gamma secretase is known to cleave a number of cell surface molecules including growth factor receptors (Kopan and Ilagan, 2004). One or more of these cell surface targets of gamma secretase may prove to be essential cell surface receptors for HPV infection.

The availability of genetically engineered strains of mice deficient in these cellular genes provides a basis for definitive future studies.

Material and methods

Plasmids

p16SheLL, p31SheLL, and p45SheLL (~10.8 kilo base pair plasmids and too large to be packaged in HPV VLPs) that express

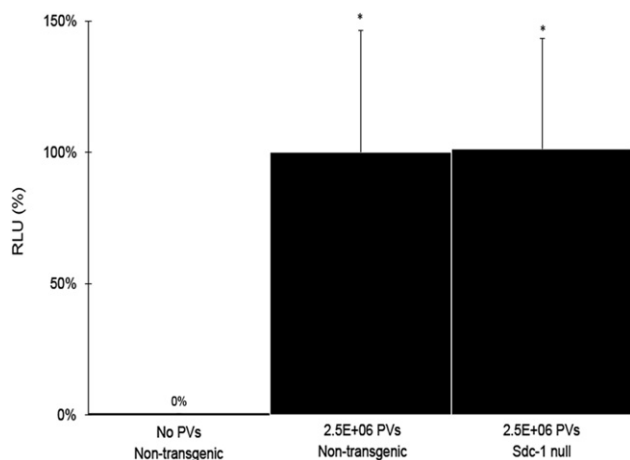


Fig. 4. HPV16 infectivity in int-1-sufficient versus -null mice. Non-transgenic (syndecan-1-sufficient) and syndecan-1 null mice reproductive tracts ($N=6$) were challenged with or without $2.5E+06$ PVs (HPV16:LucF) as indicated. 48 h after infection, mouse female reproductive tracts were harvested, protein lysates made, and luciferase assays were carried out to quantify the infectivity. Concentrations of total proteins were measured by Bradford protein assay. The infectivity level (relative luminescence units, RLU/ μg) of non-transgenic mice treated with $2.5E+06$ PVs was set as 100%. * $P=0.87$ (Wilcoxon rank sum test, 2 sided).

the HPV major capsid protein, L1, and minor capsid protein, L2. pLucF, that encodes both firefly luciferase (Luc) and green fluorescent protein (GFP). pTWB, that encodes red fluorescent protein (tdTomato). These plasmids are gifts from Dr. Christopher Buck in National Cancer Institute.

Cell cultures

293FT cells expressing enhanced SV40 large T antigen (Invitrogen) were maintained in DMEM medium (high glucose) (Invitrogen) containing 10% fetal bovine serum (Harlan) supplemented with 0.1 mM MEM non-essential amino acids (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 2 mM L-glutamine (Invitrogen) and 500 $\mu\text{g}/\text{ml}$ Geneticin. Mouse C127 cells were cultured in DMEM medium containing 10% fetal bovine serum.

Antibodies

Rat anti-human integrin α_6 antibody conjugated with fluorescein isothiocyanate (FITC), GoH3 (Serotec), Rabbit-antimouse keratin14 antibody conjugated with FITC (Covance/BAbCO), Mouse anti-HPV16 L1 antibody (Abcam, ab69), Mouse anti-syndecan-1 antibody (Santa Cruz) and Goat anti-mouse HRP light chain (Jackson ImmunoResearch).

Generation of HPV16 pseudoviruses (PVs)

HPV virus-like particles (VLPs) carrying encapsidated reporter plasmids are referred in the papillomavirus field as HPV pseudoviruses (PVs). Reporter pseudovirions were produced using previously-described methods (Buck et al., 2004; Buck and Thompson, 2007). Briefly, two plasmids expressing HPV16 L1/L2 capsids proteins and reporter genes respectively were cotransfected into 293FT cells to generate HPV16 pseudovirions, HPV16:LucF or HPV16:TWB. Pseudovirions were harvested by detergent lysis of the cells 48 h after transfection. Particles were allowed to mature overnight (Buck et al., 2005) and were purified by ultracentrifugation through OptiPrep Density Gradient Medium (Sigma). The titers of HPV16 PVs were determined by flow cytometric analysis of 293FT cells treated with various dilutions

of purified pseudovirion stock. Alternatively, HPV31 and HPV45 PVs were generated as described as above.

Mouse model for quantifying the in vivo efficiency of HPV infection

HPV:LucF pseudoviruses were used to establish a quantitative mouse infection model for HPV in the female reproductive tract. The method is a modified version of a previously published model (Roberts et al., 2007). Indicated numbers of HPV:LucF PVs were delivered intravaginally in 4% carboxyl methyl cellulose (Sigma Cat. #C4888) using 6–8 weeks old virgin female mice. The mice were prepared by subcutaneous injection with 3 mg medroxyprogesterone acetate (Sicor, Depo-Provera) 4 day prior pseudovirus challenge to induce diestrus. 6 h prior to PVs instillation, the mice were pre-treated vaginally with Conceptrol (Caldwell Consumer Health), an over the counter spermicide product containing 4% nonoxynol-9. Administration of nonoxynol-9 has previously been shown to potentiate HPV infection due to chemical injury of the vaginal/cervical epithelium (Roberts et al., 2007). 48 h after delivery of the PVs, mouse female reproductive tracts were harvested, homogenized in lysis buffer for luciferase assay by a tissue homogenizer (PowerGen 125, Fisher Scientific), and soluble tissue lysates were used to quantify the efficiency of infection by luciferase assay as per the manufacturer (Promega, Cat. #1501 and #1531).

Bradford protein assay

The amount of total proteins in reproductive tissue lysates used for luciferase assay was measured by Bradford protein assay (Bio-Rad Protein Assay Dye reagent Concentrate, Cat. #500-0006). The infectivity of PVs is indicated as relative luminescence units (RLU) per μg .

A time course of HPV16 infection in integrin α_6 sufficient versus deficient mice

$Int\beta_4^{ff}$ and $K14CreInt\beta_4^{ff}$ mice reproductive tracts were challenged as described as above with or without $3E+06$ HPV16:LucF PVs. Mouse female reproductive tracts were harvested at 48, 72, 96, and 120 h post infection, protein lysates made, and luciferase assays were carried out to quantify the infectivity. Concentrations of total proteins were measured by Bradford protein assay.

Cell susceptibility for HPV16 infection in integrin $\alpha_6\beta_4$ sufficient versus deficient mice

Alternatively, experiments described above were repeated using $1E+07$ HPV16:TWB PVs in $Int\beta_4^{ff}$ and $K14CreInt\beta_4^{ff}$ mice. 72 h after delivery of the PVs, mouse female reproductive tracts were harvested, snap frozen, cryosection made, and tdTomato red fluorescence expression indicated as viral infection were monitored by microscopy (Axiovert 200M, Carl Zeiss Microimaging).

Immunohistochemistry staining for integrin and keratin 14

Mouse female reproductive tracts infected with HPV16:TWB were collected, embedded in cryoprotectant (Tissue-Tek O.C.T., Sakura Finetek Europ, Zoeterwoude, The Netherlands). Cryosections were prepared, fixed in ice-cold acetone, and blocked in PBS with 5% Bovine Serum Albumin (BSA, A2153, Sigma) or 5% horse serum (H0146, Sigma) for one hour at room temperature. Cryosections are then incubated with 1:100 dilution of primary Ab conjugated with FITC in blocking solution at 4 °C overnight. Slides mounting medium with 4'-6-Diamidino-2-phenylindole (DAPI) (Vectashield, hard set, H-1500, Vector Laboratories) was used for

cell nuclei staining. Fluorescence images were taken by Carl Zeiss microscopy.

Immunoprecipitation (IP)/Westerns

1E+07 mouse C127 cells were exposed to 1E+08 HPV16 PVs (10 M.O.I.) for 4 h, and then treated or untreated (Mock Infection) with water-soluble cross-linker (1 mM DTSSP, Thermo Scientific) in cold PBS and incubated for 30 min at 4 °C. Cells were washed three times with cold PBS and quenched with 50 mM Tris pH 7.5 for 15 min at 4 °C. Cell were washed three times with cold PBS and solubilized with 1ml cold lysis buffer (1% TX100, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 10 ng/ml leupeptin, 10 ng/ml aprotinin). Save soluble proteins in supernatants by centrifugation at 13,000 rpm for 10 min. Add mouse anti HPV16 L1 antibody with protein A-agarose (Thermo Scientific, Pierce) beads for 1 h at 4 °C. Beads were washed twice with cold lysis buffer, resuspended in 20 ul Laemmli sample buffer and boiled for 5 min. Solubilized proteins were resolved by 12% SDS-PAGE (Boi-Rad) and were electroblotted onto PVDF membranes. Membranes were blocked, and probed with primary antibodies (mouse anti-HPV16 L1 or anti-syndecan-1) and anti-mouse secondary antibody. Antibody/protein complexes on blots were detected using enhanced chemiluminescence (Amersham Biosciences).

HPV infections in syndecan-1 sufficient versus null mice

Alternatively, experiments describe above were repeated using 2.5E+06 HPV16:LucF/HPV31:LucF/HPV45:LucF PVs in non-transgenic (syndecan-1 sufficient) and syndecan-1 null mice. Mouse female reproductive tracts were harvested at 48 h post infection, protein lysates made, and luciferase assays were carried out to quantify the infectivity. Concentrations of total proteins were measure by Bradford protein assay.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.virol.2012.08.032>.

References

- Bienkowska-Haba, M., Patel, H.D., Sapp, M., 2009. Target cell cyclophilins facilitate human papillomavirus type 16 infection. *PLoS Pathog* 5, e1000524.
- Bienkowska-Haba, M., Williams, C., Kim, S.M., Garcea, R.L., Sapp, M., 2012. Cyclophilins facilitate dissociation of the HPV16 capsid protein L1 from the L2/DNA complex following virus entry. *J. Virol.* 86, 9875–9887.
- Buck, C.B., Pastrana, D.V., Lowy, D.R., Schiller, J.T., 2004. Efficient intracellular assembly of papillomaviral vectors. *J. Virol.* 78, 751–757.
- Buck, C.B., Thompson, C.D., 2007. Production of papillomavirus-based gene transfer vectors. *Curr. Protoc. Cell Biol.* Unit 26, 21. (Chapter 26).
- Buck, C.B., Thompson, C.D., Pang, Y.Y., Lowy, D.R., Schiller, J.T., 2005. Maturation of papillomavirus capsids. *J. Virol.* 79, 2839–2846.
- Buck, C.B., Thompson, C.D., Roberts, J.N., Muller, M., Lowy, D.R., Schiller, J.T., 2006. Carrageenan is a potent inhibitor of papillomavirus infection. *PLoS Pathog.* 2, e69.
- Culp, T.D., Budgeon, L.R., Christensen, N.D., 2006a. Human papillomaviruses bind a basal extracellular matrix component secreted by keratinocytes which is distinct from a membrane-associated receptor. *Virology* 347, 147–159.
- Culp, T.D., Budgeon, L.R., Marinkovich, M.P., Meneguzzi, G., Christensen, N.D., 2006b. Keratinocyte-secreted laminin 5 can function as a transient receptor

- for human papillomaviruses by binding virions and transferring them to adjacent cells. *J. Virol.* 80, 8940–8950.
- Evander, M., Frazer, I.H., Payne, E., Qi, Y.M., Hengst, K., McMillan, N.A., 1997. Identification of the alpha6 integrin as a candidate receptor for papillomaviruses. *J. Virol.* 71, 2449–2456.
- Giroglou, T., Florin, L., Schafer, F., Streeck, R.E., Sapp, M., 2001. Human papillomavirus infection requires cell surface heparan sulfate. *J. Virol.* 75, 1565–1570.
- Huang, H.S., Buck, C.B., Lambert, P.F., 2010. Inhibition of gamma secretase blocks HPV infection. *Virology* 407, 391–396.
- Joyce, J.G., Tung, J.S., Przysiecki, C.T., Cook, J.C., Lehman, E.D., Sands, J.A., Jansen, K.U., Keller, P.M., 1999. The L1 major capsid protein of human papillomavirus type 11 recombinant virus-like particles interacts with heparin and cell-surface glycosaminoglycans on human keratinocytes. *J. Biol. Chem.* 274, 5810–5822.
- Karanam, B., Peng, S., Li, T., Buck, C., Day, P.M., Roden, R.B.S., 2010. Papillomavirus infection requires gamma secretase. *J. Virol.* 84, 10661–10670.
- Kines, R.C., Thompson, C.D., Lowy, D.R., Schiller, J.T., Day, P.M., 2009. The initial steps leading to papillomavirus infection occur on the basement membrane prior to cell surface binding. *Proc. Natl. Acad. Sci. USA* 106, 20458–20463.
- Knappe, M., Bodevin, S., Selinka, H.C., Spillmann, D., Streeck, R.E., Chen, X.S., Lindahl, U., Sapp, M., 2007. Surface-exposed amino acid residues of HPV16 L1 protein mediating interaction with cell surface heparan sulfate. *J. Biol. Chem.* 282, 27913–27922.
- Kopan, R., Ilagan, M.X., 2004. Gamma-secretase: proteasome of the membrane? *Nat. Rev. Mol. Cell Biol.* 5, 499–504.
- Liu, B.Y., Kim, Y.C., Leatherberry, V., Cowin, P., Alexander, C.M., 2003. Mammary gland development requires syndecan-1 to create a beta-catenin/TCF-responsive mammary epithelial subpopulation. *Oncogene* 22, 9243–9253.
- McMillan, N.A., Payne, E., Frazer, I.H., Evander, M., 1999. Expression of the alpha6 integrin confers papillomavirus binding upon receptor-negative B-cells. *Virology* 261, 271–279.
- Muller, M., Gissmann, L., Cristiano, R.J., Sun, X.Y., Frazer, I.H., Jenson, A.B., Alonso, A., Zentgraf, H., Zhou, J., 1995. Papillomavirus capsid binding and uptake by cells from different tissues and species. *J. Virol.* 69, 948–954.
- Patterson, N.A., Smith, J.L., Ozbun, M.A., 2005. Human papillomavirus type 31b infection of human keratinocytes does not require heparan sulfate. *J. Virol.* 79, 6838–6847.
- Raymond, K., Krefl, M., Janssen, H., Calafat, J., Sonnenberg, A., 2005. Keratinocytes display normal proliferation, survival and differentiation in conditional beta4-integrin knockout mice. *J. Cell Sci.* 118, 1045–1060.
- Richards, R.M., Lowy, D.R., Schiller, J.T., Day, P.M., 2006. Cleavage of the papillomavirus minor capsid protein, L2, at a furin consensus site is necessary for infection. *Proc. Natl. Acad. Sci. USA* 103, 1522–1527.
- Roberts, J.N., Buck, C.B., Thompson, C.D., Kines, R., Bernardo, M., Choyke, P.L., Lowy, D.R., Schiller, J.T., 2007. Genital transmission of HPV in a mouse model is potentiated by nonoxynol-9 and inhibited by carrageenan. *Nat. Med.* 13, 857–861.
- Sapp, M., Bienkowska-Haba, M., 2009. Viral entry mechanisms: human papillomavirus and a long journey from extracellular matrix to the nucleus. *FEBS J.* 276, 7206–7216.
- Schiller, J.T., Day, P.M., Kines, R.C., 2010. Current understanding of the mechanism of HPV infection. *Gynecol. Oncol.* 118, S12–S17.
- Selinka, H.C., Florin, L., Patel, H.D., Freitag, K., Schmidtke, M., Makarov, V.A., Sapp, M., 2007. Inhibition of transfer to secondary receptors by heparan sulfate-binding drug or antibody induces noninfectious uptake of human papillomavirus. *J. Virol.* 81, 10970–10980.
- Selinka, H.C., Giroglou, T., Sapp, M., 2002. Analysis of the infectious entry pathway of human papillomavirus type 33 pseudovirions. *Virology* 299, 279–287.
- Shafti-Keramat, S., Handisurya, A., Kriehuber, E., Meneguzzi, G., Slupetzky, K., Kirnbauer, R., 2003. Different heparan sulfate proteoglycans serve as cellular receptors for human papillomaviruses. *J. Virol.* 77, 13125–13135.
- Stepp, M.A., Liu, Y., Pal-Ghosh, S., Jurjus, R.A., Tadwalkar, G., Sekaran, A., Losicco, K., Jiang, L., Larsen, M., Li, L., Yuspa, S.H., 2007. Reduced migration, altered matrix and enhanced TGFbeta1 signaling are signatures of mouse keratinocytes lacking Sdc1. *J. Cell Sci.* 120, 2851–2863.
- Surviladze, Z., Dziduszko, A., Ozbun, M.A., 2012. Essential roles for soluble virion-associated heparan sulfonated proteoglycans and growth factors in human papillomavirus infections. *PLoS Pathog.* 8, e1002519.
- Wang, H., Leavitt, L., Ramaswamy, R., Rapraeger, A.C., 2010. Interaction of syndecan and alpha6beta4 integrin cytoplasmic domains: regulation of ErbB2-mediated integrin activation. *J. Biol. Chem.* 285, 13569–13579.
- Wilhelmsen, K., Litjens, S.H., Sonnenberg, A., 2006. Multiple functions of the integrin alpha6beta4 in epidermal homeostasis and tumorigenesis. *Mol. Cell Biol.* 26, 2877–2886.
- zur Hausen, H., 2009. Papillomaviruses in the causation of human cancers—a brief historical account. *Virology* 384, 260–265.