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Investigation Into The Early Events Of Epithelial Wound Healing and HPV16 Infection

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BY

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For my family: Mike, Ellen, Brooke, Ben and Grammie. Without their support, love, humor and encouragement I certainly would not have reached this point.

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ABSTRACT

HPV16 infection evidently occurs within wounded epithelial tissue, but the cellular and molecular events that culminate in infection establishment remain poorly understood. While HPV is exposed to a multitude of cells in the wound environment, only hyperproliferative keratinocytes are believed to support productive infection. Keratinocytes acquire mobility during wound repair by undergoing prominent phenotypic alterations through an epithelial-to-mesenchymal transition (EMT). In this study, cell- and tissue-based models were employed to characterize HPV infection during epithelial wound healing, using both physical injury and growth factor treatment to induce keratinocyte migration. In a tissue-based model of wounding and infection using NIKS-derived organotypic “raft” culture, HPV infection did not occur without epithelial wounding, consistent with animal models of wounding and HPV16 infection. Using cell monolayer, keratinocytes induced into an EMT were evaluated for their ability to support HPV16 early infection, which encompasses viral binding, entry, trafficking and nuclear delivery of encapsidated genes. It was found that keratinocytes in an EMT allowed reduced levels of virions to bind the cell surface

but HPV16 infection was not supported. A subset of HPV virions appeared to enter cells during EMT, indicating that infection may be compromised post-entry. Interestingly, HPV16 infection of keratinocytes induced into EMT resumed coincident with the return of epithelial characteristics. So while an initial refractory period to infection exists in cells undergoing an EMT, HPV16 infects migrating cells during later stages of wound healing. Based on these results, migrating keratinocytes may serve as an additional reservoir of cellular HPV infection not previously identified.

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Chapter 1: An Introduction To HPV16 Replication and Epithelial Wound Healing

1. Overview

This report explores HPV16 infection during epithelial wound repair, using cell- and tissue-based models of infection to investigate how discrete wound healing events impact infection establishment. Even though wound healing and HPV infection appear to coincide during transmission, models of HPV infection oftentimes overlook wound healing. Separately, HPV infections and epithelial wound healing are complex, multistep processes that can be difficult to study; in combination, they become inherently more complex. In an attempt to provide clarity to a study that merges early events in HPV infection with early events in epithelial wound healing, Chapter 1 introduces background information on HPV biology and transmission as well as the phases and events of epithelial wound repair. To establish rationale for the experiments performed, emphasis is placed upon keratinocyte migration and current evidence for HPV infection in the wound environment. Chapter 2 describes experiments that evaluate HPV16 infection of keratinocytes undergoing migratory processes brought about by an epithelial-to-mesenchymal transition (EMT). Chapter 3 introduces the advent of a new tissue-based model that incorporates wounding and HPV16 infection using organotypic "raft" tissue culture. Finally, in Chapter 4, perspective is given on the implications of the data in Chapters 2 and 3, as well as future directions.

2. HPV Epidemiology

Human papillomaviruses (HPVs) comprise a large family of small DNA tumor viruses whose infections are largely commensal but can cause hyperplastic cell growth. As the world's most prevalent sexually transmitted infection (STI), an estimated 75% of women and 65% of men will contract a genital HPV infection in their lifetime, typically amongst the newly sexually active (12, 67, 186). Rates of genital HPV transmission are substantially higher from female to male, indicating that men may possibly experience even greater HPV prevalence (68, 79, 200). Although readily contracted, most genital HPV infections are innocuous and do not warrant medical attention. Approximately 80-90% of HPV STIs will clear spontaneously (127) and global HPV incidence hovers around 11-12% (12). The Centers for Disease Control (CDC) report that nearly 14 million of the approximately 20 million new STIs reported each year in the United States are attributed to HPV. However, a subset of carcinogenic HPV types is sufficiently widespread that it causes 4.8% of global cancer cases (177).

Each year approximately 500,000 new cervical cancer cases are diagnosed worldwide (173) and, importantly, HPV is found in essentially 100% of cervical cancer biopsies (194). Cervical cancer is the third leading cause of female cancer morbidity worldwide (57). In the developed world, cervical screening by Pap smear has led to ~50% reduction in cervical cancer incidence (69). But even without cervical screening by Pap smear, persistent HPV infections pose a lifetime cervical cancer risk of only 3.7% (13). HPV is also

associated with 5 other malignancies, including carcinomas of the vagina, vulva, penis, anus and head and neck (60).

HPVs are classified into types based upon their order of discovery and sequence differences in the L1 gene; more than 10% L1 difference from any other type designates a new HPV type (16). HPVs are further categorized according to disease-generating potential as 'low-risk' (LR) or 'high-risk' (HR). LR HPV infections are cleared within 5-6 months and cause such maladies as genital warts (176). Conversely, HR HPV infections persist for 8-14 months and can lead to several malignancies within mucosal epithelium (30, 176). Of the 200 HPVs to be identified, 40 types (both LR and HR) infect the anogenital tract, and 13-18 of these are HR types and carcinogenic (186, 187). HPV16 and -18, in particular, are responsible for 70% of cervical cancer incidence (60).

Two vaccines, Gardasil and Cervarix, have been approved as prophylaxes against most HPV infections associated with cervical cancer and genital warts. Despite such measures, vaccine uptake is suboptimal and HPV infection remains a significant public health concern. The most effective way to curb cancer caused by a viral infection is to prevent infection in the first place (77). Accordingly, this report explores HPV infection at its earliest stages, when HPV virions first make contact with tropic epithelial cells, in the hopes of developing a greater understanding of HPV infection to aid in the design of antiviral medications.

3. Features of HPVs

The principal components of the HPV virion are the capsid and genomic material, each of which is explained below.

Capsid: The HPV virion is composed of a non-enveloped outer protein shell, or capsid (Fig 1). The ~55 nm capsid has icosahedral symmetry and assembles from late proteins, L1 and L2. L1, the major capsid protein, forms the capsid shell. Within each virion, 360 copies of L1, arranged into 72 pentameric capsomeres, harbor 12-72 molecules of L2 per virion (22). L1 is a multifunctional protein that mediates virion integrity, attachment and trafficking (23). L2, or the minor late protein, is similarly multifunctional and contributes to virion assembly, trafficking and other infection steps (140). HPV contacts host heparan sulfonated proteoglycans (HSPGs) through L1.

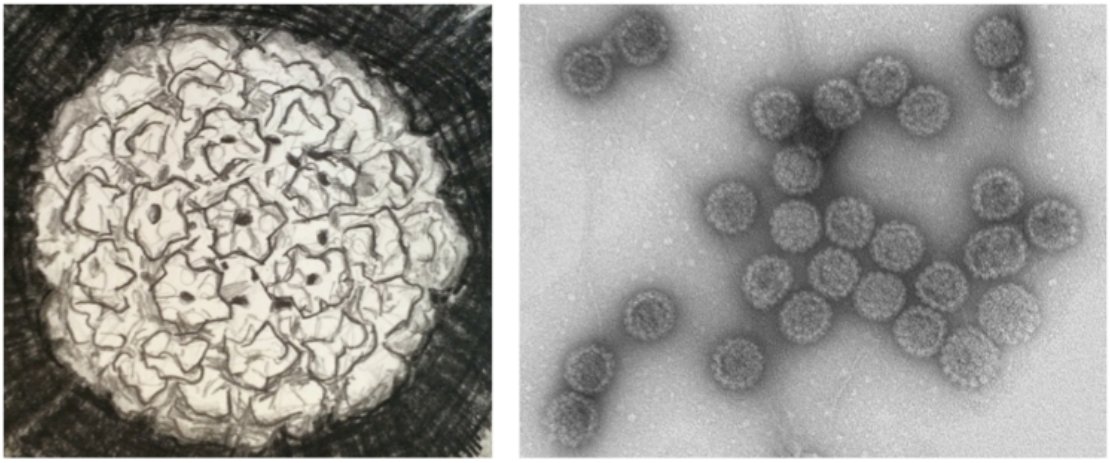


Figure 1: **(Left)** Illustration of an individual HPV16 virion. Pentameric arrangements of L1 form the capsid shell, which is roughly 55 nm in diameter. Original artwork by M. Kivitz. **(Right)** Electron micrograph of HPV16 pseudovirions (PsVs), generated by M. Kivitz. Image acquired by A. Griego.

Genome: The HPV genome, a circular, double-stranded DNA molecule roughly 8 kbp in length, resides within the capsid and is condensed by cellular histones (Fig. 2). Only one strand of the genome is transcribed during infection. Because

different viral promoters become activated as the host keratinocyte differentiates, HPV gene expression is separated into early (E) and late (L) phases (16). While early genes regulate HPV genome replication and expression, late genes encode viral structural elements. Conserved across all PVs, the minimal genetic constituents of the HPV genome include: a genome transcription and replication regulatory element, the long-control region (LCR) or upstream regulatory region (URR); early genes, E1 and E2, which bind the LCR to instigate genome replication; and structural genes, L1 and L2, for capsid formation (Fig. 2) (190). E1 and E2 are important for genome amplification and are first active when infection is established; they direct cellular replication factors to the viral origin of replication in the URR and cause rapid and transient replication of the viral genome. They are most active in the upper spinous cells just before late gene expression, amplifying the genome in preparation for packaging and virion assembly. Some HPV types encode accessory genes, E4, E5, E6 and E7, that subvert innate immunity and promote cell cycle progression. E4 is expressed in the upper epithelium and disrupts the keratin cytoskeleton in cornified cells (49). E5, E6 and E7 dramatically alter the cell cycle by causing hyperproliferation and can therefore be considered oncogenic; their expression is greatest in suprabasal cells (73, 75, 190). E6 and E7 in HPV16 are particularly oncogenic (57, 60, 186).

4. The HPV replication cycle

Overview

HPVs replicate in tissue, unlike many other viruses, which require only a single cell to generate progeny virions. HPV infects keratinocytes, specifically basal cells in the bottommost epithelial layer of the epithelium (Fig. 2). As keratinocytes differentiate, the HPV genome is spatiotemporally expressed so that early gene expression is chiefly confined to basal and lower suprabasal cells, while late genes are expressed in upper suprabasal cells and granular cells. It is not until infected cells have reached the topmost epithelial layers that structural genes are expressed and viral progeny can be assembled. Since HPV replication coincides with keratinocyte differentiation, interfering with differentiation circumvents expression of structural viral genes, preventing formation of progeny virions (73). HPVs that become too proficient at promoting cell cycle progression induce hyperproliferation to the detriment of virion production. HPV-associated cancer, a disease of unregulated cell cycle progression and invasion, is an extreme form of hyperproliferation caused by HPV-induced cell division and chromosomal instability (73, 75, 77, 127, 194, 211).

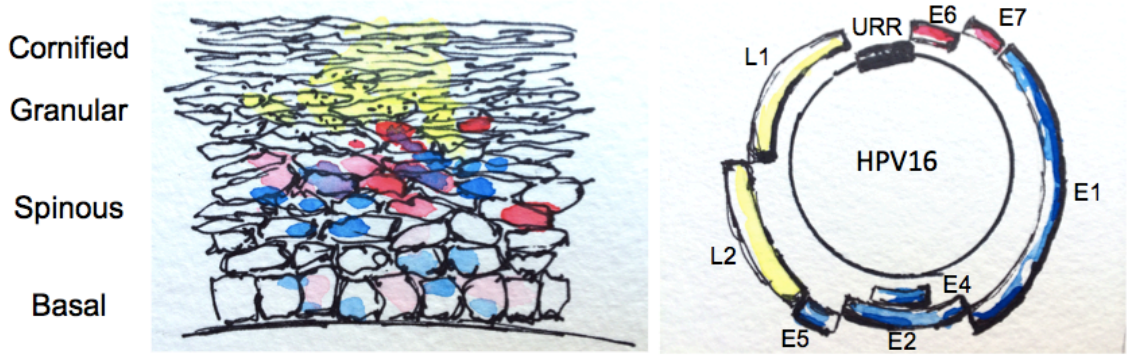


Figure 2: HPV gene expression is tightly linked to keratinocyte differentiation. Basal cells, the only mitotically active cells in epithelial tissue, differentiate and give rise to the spinous, granular and cornified layers. Differentiating, stratifying epithelial tissue (left) spatiotemporally expresses the complete HPV16 genome, a double-stranded DNA molecule with multiple open

reading frames (right). HPV infection is established in basal cells, wherein early genes (blue) direct host cell replication machinery to amplify the viral genome. When oncogenic early genes are expressed (red), the cell cycle becomes activated outside of its normal context and hyperproliferation occurs, which may lead to tumorigenesis. The viral genome is extensively amplified in the upper spinous layers just prior to late gene expression. Structural proteins (yellow) are expressed in granular cells, and assemble around the viral genome to form progeny virions, which will be shed alongside cornified cells during desquamation. Original artwork by M. Kivitz.

HPV replication, which does not induce cytolysis, necrosis or viremia, culminates in viral shedding as infected keratinocytes desquamate from the uppermost epithelial layers (Fig. 2) (178, 193). HPV transmission occurs *via* direct contact between tissues, such as skin-skin apposition or, in the case of genital infections, intimate sexual contact. Disruption of the epithelial barrier, which uncovers underlying basal cells and attachment factors, is thought to be a necessary step in HPV infection.

While HPV infects tissues throughout the female genital tract (FGT), including keratinocytes of the external genitalia, vagina, ectocervix and endocervix, cancer tends to arise from only a portion of the FGT, known as the squamocolumnar junction (SCJ) (78). Several SCJs exist in the human body, including the larynx, nasal sinuses, urethra, uterine cervix and the anal/rectal junction. The female genital SCJ, or transformation zone, is the boundary between two different tissue types, stratified squamous epithelium and simple columnar epithelium (104, 198). The female genital transformation zone, composed stratifying squamous ectocervical and simple columnar endocervical cells, is particularly prone to HPV infections (30, 78).

Binding

Through L1, infection is initiated as incoming HPV virions attach to glycoproteins displayed by host cells and the extracellular matrix (ECM). Heparan sulfonated proteoglycans (HSPGs) serve as primary attachment receptors for a range of HPV types (1, 21, 66, 88, 167). The most common HSPG in epithelial tissue, syndecan-1, serves as the predominant attachment receptor for HPV16 (167). When HPV-HS interactions are blocked, infection is prevented (66). *In vitro*, HPV16 uses only heparan sulfate (HS) to bind HaCaT cells, but binds to the ECM using both HS and laminin-332 (LN-332, formerly laminin-5) (21). HPV16 also interacts with non-human cells and tissues *via* HS (88, 97), and HPV-HS interactions are so well established that they have also been characterized by crystallography (36).

Entry

After binding HSPGs, HPV enters basal keratinocytes, potentially by a mechanism related to macropinocytosis (160). Internalization is slow, asynchronous and requires several hours for virions to cross the plasma membrane. Although the putative HPV internalization receptor has not been identified (147), various entry mechanisms have been reported, including clathrin- and caveolin-dependent endocytosis (107), as well as clathrin- and caveolin-independent endocytosis (175). The variability in HPV entry in these studies may be a result of using a multitude of epithelial and non-epithelial cell types to study viral internalization.

Several molecules implicated in HPV entry are activated when virions contact the cell surface. HPV16 binding induces intracellular signaling that is mediated in part by membrane-bound tyrosine kinase receptors like epidermal growth factor receptor (EGFR) and keratinocyte growth factor receptor (KGFR) (180). EGFR signaling instigates several cellular changes, including cytoskeletal rearrangement, which has ramifications for actin-based plasma membrane extensions such as filopodia (191, 204). Interestingly, HPV31 utilizes filopodial-based cytoskeletal rearrangement for viral uptake (172), incorporating the same tyrosine kinase and PI3-kinase signaling used by HPV16 for filopodia induction (1). Furthermore, HPV16 binding inhibits autophagy in an EGFR-dependent manner during viral entry (181).

Trafficking

After crossing the plasma membrane, HPV enters the endo-lysosomal pathway (84). HPV entry and trafficking take both infectious and non-infectious routes. Successful infection culminates in nuclear delivery of the viral genome, and HPV that does not reach the nucleus is non-infectious. Drugs that interfere with HS-HPV interactions route HPV16 into non-infectious entry pathways in HaCaT cells (166). During infectious trafficking, the low pH encountered by virions in the endo-lysosomal pathway causes conformational changes that allow the L1-based capsid to disassemble. While lysosomes retain and degrade L1, L2-genome complexes escape degradation by exiting late endosomes (94). L2-vDNA complexes travel from late endosomes to perinuclear organelles such as

the Golgi apparatus and the ER. L2 has a dynein-binding motif that facilitates vDNA trafficking along microtubules, allowing transport between organelles and cytoskeletal elements (162). Transport to the Golgi uses the retromer, a multi-subunit cytoplasmic transport complex that travels along microtubules to return Golgi factors to the trans-Golgi network (TGN) (44). L2-vDNA complexes also traffic through the endoplasmic reticulum (107).

Cell division is a critical event in HPV replication, potentially because nuclear envelope dissolution during mitosis allows L2-vDNA access inside the nucleus. L2-vDNA complexes may also enter the nucleus using karyopharyns, as L2 contains a C-terminal nuclear localization signal (NLS) (140). Cyclins, or cyclin-dependent kinases (CDKs), regulate the cell cycle but import E1 into the nucleus (47). Once inside the nucleus, the HPV genome and L2 colocalize with ND10/PML domains (38), concluding viral trafficking and likely facilitating vDNA transcription.

Genome expression

The viral genome undergoes low-level amplification once infection is established in basal cells. Because HPV uses cellular DNA replication machinery to copy its genome, mitotic progression is a necessary event for establishing HPV infections (146). The HPV genome is spatially and temporally regulated, and viral gene expression is dependent upon the differentiation status of the infected keratinocyte (Fig. 2). Early genes are predominantly expressed in basal and spinous cells, while late gene expression is restricted to terminally

differentiating granular cells. The genome is further amplified when cells reach the granular layer in preparation for packaging and viral assembly.

Assembly and shedding

Infected keratinocytes that differentiate into granular cells activate the late promoter, leading to expression of viral structural genes. As viral mRNAs are translated by cytoplasmic ribosomes, L1 and L2 enter the nucleus, the site of virion assembly. L1 assembles into the capsid and concurrently interacts with L2 to encapsidate vDNA and form infectious particles. Since HPV is not a lytic virus, progeny virions rely on keratinocyte desquamation for release. E4, expressed in the granular layer, may promote desquamation by disrupting the keratin-based cytoskeleton (49).

5. HPV16 transmission

Genital HPV exposure results from behavior common to all STIs, namely: frequency of sex or other intimate contact; number of lifetime sexual partners; recent or present number of sexual partners; and the sexual history and behavior of partners (193). Mathematical models predict, per sexual partnership, a 40% probability of HPV16 transmission (13). Since HPV is transmitted by skin-skin contact, and HPV attachment factors – ECM components and basal cells – are inaccessible in undamaged tissue, epithelial disruption appears necessary for infection. Genital tissue is wounded by sexual congress, and studies have shown that genital mucosal wound healing is closely related to as cutaneous

wound healing (15, 198). Given that HPV transmission occurs most commonly by penetrative sexual contact (25, 79, 161, 193, 199), genital injury likely facilitates HPV transmission.

Accordingly, the same behavior that increases chances of HPV transmission also increases chances of epithelial microtrauma. HPV's high rate of transmission is evident during non-penetrative sexual contact, wherein genital HPV types are detected in fingertips of newly sexually active female college students (203). Furthermore, measures taken to diminish transmission likely confer protection not only by limiting intimate contact, but also by minimizing epithelial disruption. For example, regular condom use provides roughly 60% protection against HPV16 transmission (202).

Evidence for HPV in the wounded tissue

During transmission, HPV virions appear to be introduced to tissue undergoing wound healing in response to injury, and are therefore exposed to enzymes and diverse cell types in the wound environment. Several enzymes that are active post-injury have also been shown to play a role in cell-based models of HPV early infection. Ɣ-secretase and furin/PCs, which both promote wound healing (132), are likewise implicated in HPV internalization (40, 42, 95). Other molecules implicated in HPV attachment and entry include HSPGs, like syndecan-1 (21, 66, 167), as well as LN-332 (34), α6 integrin (1, 62, 208), EGFR and KGFR (181), and AnxA2 (51, 205). Syndecan-1, EGFR and KGFR are important for HPV infection (180) and up-regulated by wound edge keratinocytes

(3, 54, 108, 112, 122). As wound healing progresses, a diverse range of cells conducting wound repair processes encounter incoming HPV virions and, in addition to hyperproliferative keratinocytes (146), HPV has been found to bind and enter dendritic cells, like Langherhans cells (20, 35, 117), which infiltrate the wound site during inflammation.

Epithelial disruption in animal models of papillomavirus infection

Support for the necessity of wounding to HPV transmission is found amongst rabbit, mouse and rhesus macaque models of HPV infection. Animal models can be used to evaluate compatible and incompatible PV infections across host (rabbit PV with rabbits; murine PV with mice, etc.) and non-host cells and tissues (HPV with mice). Although productive HPV infection cannot occur in non-human cells, tissues and animal models, studies of viral binding and entry remain possible. This block presents challenges for *in vitro* studies but early events of infection can be facilitated by measurements employing HPV pseudovirions (PsVs), which encapsidate a reporter plasmid in place of the wild type viral genome.

In the absence of epithelial disruption, HPV infection, by and large, does not occur. The cottontail rabbit PV (CRPV)/rabbit infection model demonstrates that wounded and inoculated rabbit skin leads to enhanced papillomatous growths, or warts, while intact epithelium does not. Wounding was performed by sandpaper, intradermal injection or tattooing, wherein tattooing and infection generated warty outgrowths to the highest degree (155). Inoculated and tarred

rabbit skin similarly led to enhanced papilloma growth, compared to scarification with sandpaper (63), possibly because tarring caused greater damage and therefore greater access to epithelial cells. Considering that the CRPV/rabbit model of infection is the only system available that recreates PV infection up to malignant progression (31, 32), the link between wounding and infection is particularly noteworthy.

A mouse model of HPV16 infection demonstrates that genital epithelial disruption by chemical (nonoxynol-9, a non-ionic detergent) or physical means (abrasive scraping), prior to inoculation with HPV16 PsVs augmented infection (151). It is believed that epithelial wounding allows for basement membrane access by incoming virions, and the basal membrane serves as an initial site of HPV16 attachment in the epithelium (97).

A rhesus macaque model of HPV16 infection has proven useful for studying the impact of Pap smears on HPV16 infection. Pap smears, the scraping of the female genital tract to collect cells for microscopy, were demonstrated to disrupt the genital epithelium sufficiently enough to permit HPV16 infection (152).

6. The Stages of Epithelial Wound Healing

Overview

When compromised by injury, the epithelium cannot function properly and must be repaired. Diverse cell types, communicating through soluble signaling molecules, coordinate wound repair *via* complex, tightly regulated interactions.

Keratinocytes, fibroblasts, endothelial cells, immune cells and platelets conduct wound healing *via* differentiation, migration, proliferation and apoptosis (156). Collectively, epithelial wound healing takes place by way of sequentially overlapping phases that include inflammation, reepithelialization and tissue remodeling (Table 1). Keratinocyte-mediated reepithelialization is the primary focus of this report.

	Inflammation	Reepithelialization	Tissue remodeling
Duration	0-2 days	Starts 1-2 d post-injury, lasts for several days	2-3 weeks or longer
Cells involved	• Neutrophils • Monocytes/ macrophages • Lymphocytes • Endothelial cells • Platelets	• Keratinocytes • Fibroblasts	• Keratinocytes • Fibroblasts • Myofibroblasts • Endothelial cells
Activities performed	• Hemostasis and clot formation • Immune cell infiltration	• Keratinocyte migration and hyperproliferation • Fibroblast proliferation • Granulation tissue formation	• Wound closure • Collagen secretion • Contraction

Table 1: Epithelial wound healing is a complex process carried out by multiple cell types and various signaling molecules. Although conveniently categorized into progressive stages of inflammation, reepithelialization and tissue remodeling, the stages of wound healing overlap. For the purposes of HPV infection, reepithelialization may be the most impactful event.

Characteristics of Epithelial Tissue

Epithelial tissue serves a variety of functions as the boundary between an organism and its environment. It maintains homeostasis by preventing dehydration and solute loss, protects against external threats such as invading microbes and UV exposure and, importantly, performs self-repair in the event of injury (144). Most surfaces of the body are covered with cutaneous skin, also

known as the epidermis, while contiguous internal surfaces are composed of mucosal epithelium that is characterized by mucosal secretions (33). The epithelium is made up of an underlying dermis that is collagen-rich, and an overlying, highly specialized epidermis that is keratin-rich. While the dermis derives from embryonic mesoderm and provides physical and mechanical support, epithelial tissue derives from embryonic ectoderm and confers protection. Dermal tissue is composed of several cell types, the foremost being dermal fibroblasts, which secrete and maintain much of the basement membrane, or basal lamina, that separates dermal from epidermal tissues (101). Epithelial tissue is predominantly composed of keratinocytes, but melanocytes, Langerhans cells and Merkel cells as well. Epithelial cells reside atop the basal lamina, or basement membrane, which provides mechanical support.

Because the epithelium is organized into distinct layers of cells that change their appearance as cells divide, differentiate and migrate, each layer serves a distinct function. The four categories of human epithelia are the stratum basale, stratum spinosum, stratum granulosum and stratum corneum, also known as the basal layer, spinous layer, granular layer and cornified layer, respectively (Fig. 2). These layers can be generally separated into basal and suprabasal layers, wherein each epithelial layer found above the basal layer is termed suprabasal; suprabasal cell strata therefore include the spinous, granular and cornified layers. Basal cells are the only mitotically active keratinocytes in epithelial tissue and, upon detaching from the basal lamina, begin differentiating. Keratinocytes change their appearance as they differentiate and progress from

one layer to the next, expressing a sequence of different genes that endow each strata with specialized functionality.

The stratum spinosum serves as a boundary between underlying, mitotically active basal cells and overlying, terminally differentiating cells. The stratum spinosum is made up of spinous cells, also known as prickle cells, which are inundated with desmosomal connections. Atop the stratum spinosum is the stratum granulosum, or granular layer, that forms a waterproof layer in part by extensive lipid-protein cross-linking (144). The most important physical barrier, though, is the outermost epithelial layer, the stratum corneum. Cornified cells, or squames, slough off through cornification, a specialized form of apoptosis that enables epithelial tissue to perpetually renew (27). Squames, which are mostly protective and do not contain organelles, can be considered dead. Indeed, while cornified cells are anucleate and devoid of organelles, all layers aside from the stratum corneum are made up of cells with nuclei and form the nucleated epidermis.

Basal cells adhere to the basal lamina *via* hemidesmosomes, junctional complexes connecting cells to extracellular matrix components. Keratinocytes adhere to one another through various junctional complexes, namely gap junctions (GJs), tight junctions (TJs), adherens junctions (AJs) and desmosomes (129, 144). TJs prevent solute loss and their expression is restricted to basal cells. Desmosomes provide toughness to keratinocytes throughout epithelial strata, not only because they link adjacent keratinocytes to one another but also because they connect to the keratin cytoskeleton within cells. Upon injury,

various cells become activated to help keratinocytes perform wound repair and reestablish the epithelial barrier.

Inflammation

Epithelial repair is initiated and regulated by signaling molecules during inflammation, which lasts for roughly 0-2 days post-injury (170). When soluble mediators from one cell instigate a series of events in another cell, cell behavior is altered, as brought about by differential gene expression and protein translation. Growth factor (GF) activity is modulated by morphogen gradients, activation by matrix metalloproteases (MMPs) and the availability of GF receptors (GFRs) (11).

GFs and other signaling molecules originate from multiple sources, such as damaged keratinocytes, serum released from injured blood vessels, degranulating platelets and incoming immune cells (98). Immediately post-injury, damaged keratinocytes release cytokines and GFs to alert surrounding cells to tissue damage (196). Ensuing immune cell infiltration happens as neutrophils, monocytes/macrophages, mast cells and lymphocytes accumulate at the wound site within a few minutes post-injury. Concurrently, platelet aggregation brings about hemostasis and forms a fibrin clot. The fibrin clot acts as a reservoir for GFs to bind and concentrate, and serves as a provisional matrix for migrating cells in addition to providing a temporary barrier to impede microorganisms.

Damaged keratinocytes recruit platelets and immune cells by secreting IL-1 (210), TNF-α (137, 170) and ATP (206). Platelets, in turn, secrete platelet-

derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factor beta 1 (TGFβ1), vascular-endothelial growth factor (VEGF) and fibroblast growth factor (FGF), prompting further immune cell infiltration; in the absence of hemorrhage, platelets are dispensable for wound healing (170). In response to platelet-derived VEGF and FGF, endothelial cells proliferate and perform angiogenesis. Macrophages prompt cell-mediated repair by secreting IL-1, IL-6, FGF, EGF, TGFβ1 and PDGF, and the fibrin clot is soon replaced by granulation tissue (153). Granulation tissue, named due to the granular appearance of numerous capillaries, is temporary wound connective tissue that accumulates at the wound site approximately 4 days post-injury (170). TGFβ1 converts monocytes into macrophages, which phagocytize cell debris and harm invading microbes (116, 153) by releasing reactive oxygen species (ROS) (207). Neutrophils, recruited by IL-1 and PDGF, cleanse the wound site of foreign particles and bacteria. Although neutrophils secrete PDGF and VEGF to stimulate granulation tissue synthesis, macrophages are the critical cells for transitioning from inflammation to tissue regeneration (153).

Reepithelialization

Keratinocyte-mediated reepithelialization resurfaces damaged tissue with new epithelium. During the course of inflammation, keratinocytes are exposed to a multitude of cytokines and GFs to direct migration, proliferation and differentiation, depending on the type, amount and duration of GFs.

Keratinocytes respond to extracellular signals by displaying GFRs, which initiate intracellular signaling upon binding cognate GFs (98, 128).

Leading edge keratinocytes begin migrating 1-2 days post-injury in response to both a denuded epithelial surface and GFs (54, 139, 156, 170). To migrate, keratinocytes substitute epithelial characteristics, such as polarity and immobility, for features conducive to motility (Fig. 3). They remodel adhesive contacts, activate migratory genes and undergo cytoskeletal rearrangement. Collectively, these changes are known as keratinocyte activation, or an epithelial-to-mesenchymal transition (EMT). The migrating front of cells that invade the denuded epithelial surface is supplied by hyperproliferative cells immediately behind the wound margin.

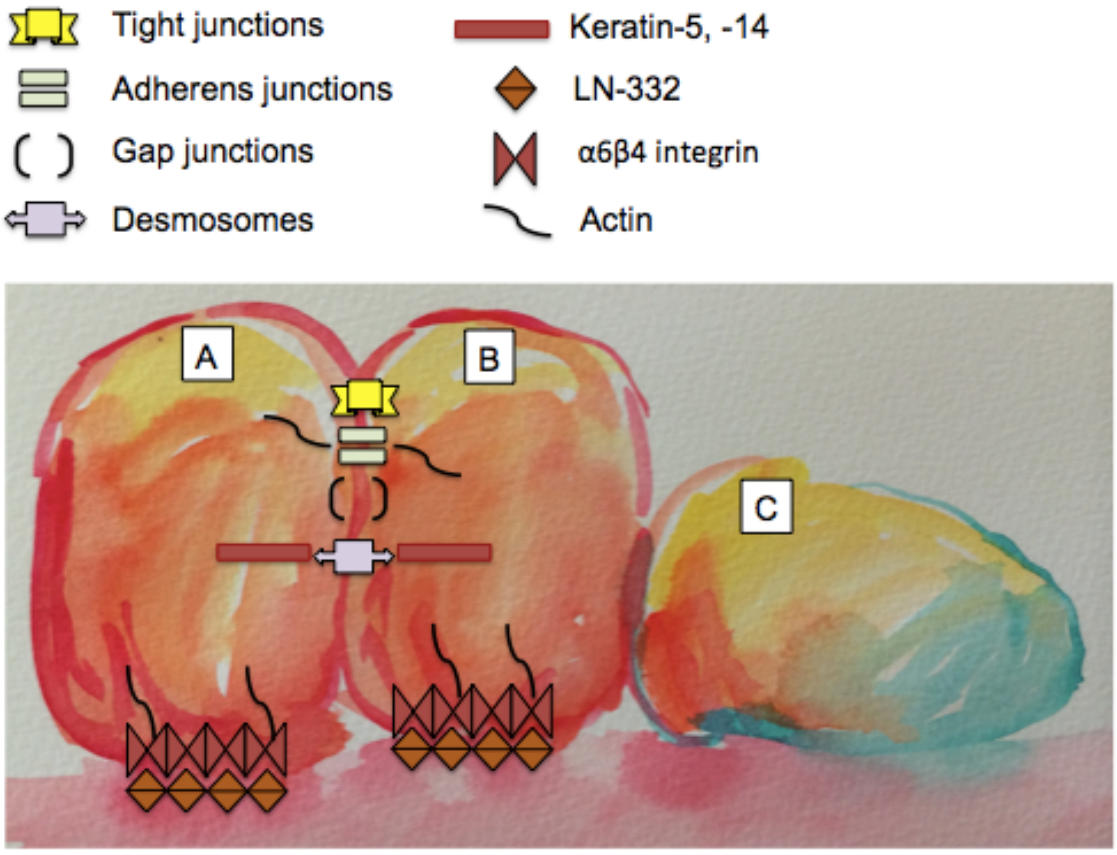


Figure 3: Keratinocyte migration and the loss of epithelial traits. For a keratinocyte to begin migrating (cell C), the adhesive connections that normally contribute to the epithelial barrier must be altered or disrupted. In stationary cells (A and B), desmosomes and adherens junctions are the principle mediators of keratinocyte adhesion, connecting to the cytoskeleton through keratins and actin, respectively. Desmosomes and adherens junctions are down-regulated in the event of cell locomotion. Tight junctions are expressed by basal cells and restrict solute diffusion across the epithelium, but during movement tight junction proteins such as ZO-1 are reorganized and may relocate from the plasma membrane to the nucleus. Gap junctions permit solute diffusion between neighboring cells but do not contribute significantly to adhesion; nonetheless, as a connection between adjacent cells, their expression is diminished upon motility. Basal cells adhere to the underlying basement membrane through hemidesmosomes composed of such receptors as α6β4 integrin, which binds extracellular matrix components like laminin-332.

Wound healing progresses as migrating keratinocytes separate desiccating eschar from viable tissue. Concurrently, the provisional ECM is extensively remodeled by fibroblasts and keratinocytes. Fibroblasts remodel the ECM by synthesizing and depositing fibronectin and collagen to generate a provisional matrix upon which keratinocytes migrate. Paracrine signaling between keratinocytes and fibroblasts propagates wound healing; keratinocytes release PDGF and IL-1, which bind receptors displayed by underlying fibroblasts, while fibroblasts, in turn, produce KGF and IL-6, which bind receptors displayed by overlying keratinocytes (164, 197). Fibroblasts respond to other inflammatory signals like bFGF, which promotes fibroblast proliferation, migration and infiltration of the wound site. TGFβ1 and PDGF likewise promote fibroblast infiltration, as do matrix metalloproteases (MMPs). Migrating keratinocytes alter cell-ECM connections by expressing different integrin receptors. Keratinocytes secrete LN-332 during migration, bolstering ECM integrity. Integrin receptors displayed by keratinocytes bind LN-332 in the ECM, forming hemidesmosomal connections. When stationary, α6β4 integrin binds LN-332 in its processed form.

During migration, however, α6β4 integrin is replaced by α3β2 integrin, which displays a higher binding affinity for unprocessed LN-332.

Tenants of keratinocyte migration

During wound closure, leading edge keratinocytes undergo intracellular and intercellular modifications. To gain mobility, they activate migratory genes, dissolve cell-cell and cell-ECM contacts and perform cytoskeletal rearrangement. Collectively, such changes enable lateral migration and wound closure during repair, and occur through a Type II EMT (Fig. 4). EMT types are discussed in the Introduction of Chapter 2.

Within intact tissue, epithelial cells are stationary and form a barrier using numerous cell-cell connections. Keratinocytes do not migrate unless provoked, be it by mechanical disruption or inflammatory signals. Migratory stimuli for keratinocytes include a denuded epithelial surface, sensed by wound edge keratinocytes, and growth factors (GFs), which contact cells throughout the wound region (83). But regardless how keratinocytes are provoked to migrate, mobility is acquired by losing epithelial traits and taking on mesenchymal characteristics. Furthermore, proteolytic cascades, MMPs and ECM-degrading enzymes are activated during migration, allowing cells to invade local tissue.

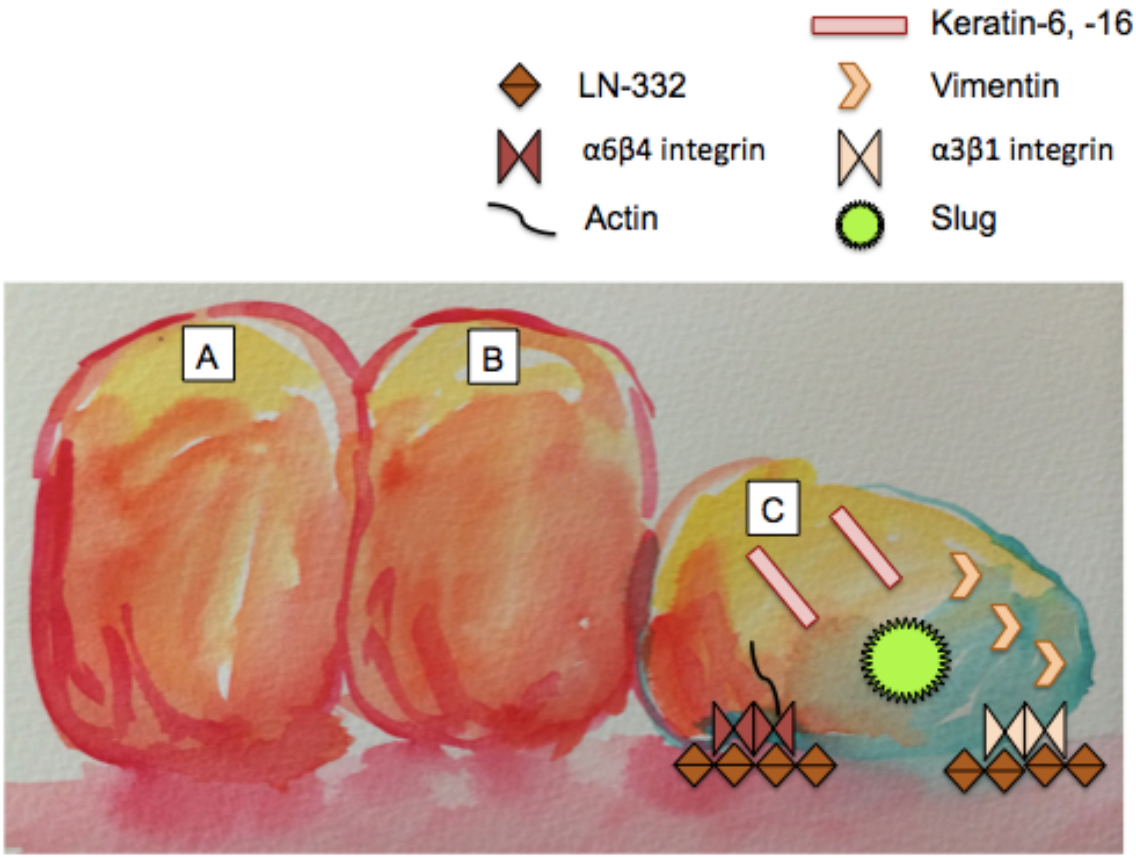


Figure 4: Keratinocyte migration and the gain of mobility. Keratinocytes take on a cobblestone appearance through aggregation, establishing a vast network of cellular connections (cells A and B). When provoked, stationary keratinocytes dissolve cell-cell and cell-ECM connections, activate migratory genes and rearrange the cytoskeleton (cell C). Collectively, these changes are known as an epithelial-to-mesenchymal transition (EMT), and they are first evident by a change in cell morphology from cobblestone to spindle-shape. As migration progresses, epithelial characteristics like apical-basal polarity are replaced by mesenchymal ones, coincident with the activation of transcription factors such as slug. Slug is a transcriptional repressor that down-regulates epithelial genes, represses proliferation and stimulates migration. Concurrently, hemidesmomal connections are altered to permit keratinocyte migration across a provisional basement membrane, as α3β1 integrin replaces α6β4 integrin to bind laminin-332.

During the inflammatory phase of wound healing, GFs, cytokines and other soluble mediators accumulate in abundance at the wound site. Whether cells migrate, divide, differentiate or die is determined by the cell's response to wound ligands during inflammation. Of the numerous wound ligands that inundate a wound, EGF, TGF-α, KGF, bFGF, IL-1 and TGFβ1 are implicated in

keratinocyte motility (89). These GFs induce intracellular signaling upon binding cognate surface receptors, and can be used to recreate and model discrete wound healing events *in vitro* (98, 139). EMT-inducing signals activate growth factor receptors (GFRs) (128) and transcription factors (TFs) (14), causing a multitude of cellular changes that can be tracked to gauge the progression of an EMT. Changes amongst major cellular structures and genes are interdependent and typically occur in tandem.

TGFβ1 is considered the most important wound ligand because it plays a role in proliferation, differentiation and migration (156). Activation of the TGFβ1 receptor (TβR) simultaneously blocks proliferation (85) and promotes migration (148). TGFβ1 induces such migratory TFs as slug (82, 174), MAPK (37) and AP-1 (14), and signals through Smad proteins (115, 119), which are implicated in EMT (82). Smads are signaling proteins that transduce extracellular signals to the nucleus in response to TβR activation (82, 115). Slug is a transcription factor that mediates cellular motility by repressing adhesive genes and up-regulating genes involved in migration (9). Slug represses E-cadherin (137) and several classes of integrins (189). Importantly, slug expression increases in migrating wound margin keratinocytes (86, 158), particularly basal cells (189). E-cadherin is a critical component of the epithelial barrier (188) and contributes to signal transduction through EGFR (129). Because migration and proliferation are mutually exclusive, TGFβ1 applied to keratinocytes *in vitro* recreates keratinocyte behavior as happens at the physiological wound edge.

EGF contributes to keratinocyte motility by activating the EGF receptor (EGFR) (195). EGFR expression increases amongst migrating wound edge keratinocytes (122). EGF binding to EGFR causes receptor dimerization and intracellular signaling (195). Upon wounding, ATP released from damaged cells activates EGFR (206), while EGF binds HSPGs and is solubilized by enzymes to activate EGFR, stimulating migration at the wound edge (185). EGF activates MAPKs, like Erk5 (121), to induce slug (7, 105).

Combined TGFβ1 and EGF exposure prompts keratinocytes to migrate (183). TGF-β1 alone is sufficient to generate an EMT (148), but when combined with EGF a robust EMT response is generated (37, 201). TGFβ1 and EGF signaling converges at slug to activate an EMT during wound healing (158, 174). TGFβ1 and EGF alter HaCaT cell behavior by enhancing migration post-wounding (148), increasing MMP expression (201), down-regulating E-cadherin and inducing vimentin (37). Vimentin is the most common intermediate filament in mesenchymal cells (55), and aids cell migration by attaching to myosin to slide the endoplasm forward on the actin cortex (74). Furthermore, TGFβ1 and EGF cause enhanced proteolytic activity and loss of E-cadherin (201). TGFβ1 causes LN-332 expression, which promotes hemidesmosome dissolution and epithelial cell motility (89).

Beside TGFβ1 and EGF, keratinocytes are exposed to and influenced by other common inflammatory signals over the course of wound healing, such as KGF, bFGF and PDGF. Notably, these GFs and their receptors both increase post-wounding (11, 98, 128, 196). Although KGF and bFGF are implicated in

keratinocyte motility (89), PDGF is not (5, 154). KGF, secreted by dermal fibroblasts, binds epithelial cells through KGFR (FGFR2IIIb), which is expressed by suprabasal cells during differentiation (142). bFGF is secreted by macrophages, fibroblasts and endothelial cells, and influences keratinocyte motility. PDGF is produced by keratinocytes during wound healing, as well as platelets and macrophages, but HaCaT cells do not express PDGF receptor (PDGFR) (154). Using TGFβ1, EGF, PDGF, KGF and bFGF, an array of wound ligands are available to alter HaCaT cell behavior and perform HPV infections.

Tissue remodeling

As reepithelialization progresses, the reestablished epithelial barrier next undergoes tissue remodeling, which lasts 2-3 weeks. Tissue remodeling entails dermal and epidermal maturation, wound contraction and continued ECM reorganization and remodeling. Wound contraction is exerted by myofibroblasts, which derive from fibroblasts in response to TGFβ1 and PDGF (5, 99). Migrating keratinocytes revert back into stationary cells and reestablish intercellular connections, for example adherens junctions, by extending filopodia to make contact with E-cadherin displayed on adjacent cell surfaces (191). Additionally, migratory cells undergo mesenchymal-to-epithelial transition (MET), the converse of an EMT, upon wound closure. The return of epithelial characteristics by wound keratinocytes reestablishes the epithelial barrier and promotes stratification by way of re-differentiation. Within granulation tissue, cells undergo selective apoptosis so that excessive numbers of fibroblasts and endothelial cells

are eliminated. Finally, granulation tissue, having originally replaced the fibrin clot, will itself be replaced by scar tissue, completing wound healing.

7. Gaps in the field and hypothesis

Current *in vitro* models of HPV early infection likely do not mimic HPV infections *in vivo*. Although epithelial disruption appears to be a critical event for establishing HPV infections, little is known about how discrete wound healing events contribute to the initiation of HPV16 infection outside of animal models. Animal-, tissue- and cell-based models of HPV replication have contributed to our current understanding of HPV biology, but each has benefits and limitations. Studies using animal models have evaluated the necessity of epithelial disruption for papillomavirus (PV) infections, finding that intact epithelium does not support infection (31, 32, 63, 88, 97, 151, 152, 155). Although animal models permit PV infection to be evaluated in a physiological setting, they are poorly suited for reductionist studies. Tissue-based models of HPV replication classically focus on events downstream of infection, such as virion production and carcinogenesis. However, there is not yet a tissue-based model to study HPV early infection. Cell-based models of HPV infection are particularly amenable to reductionist experiments and are commonly used to evaluate discrete steps in infection such as virion binding, entry and trafficking. Using monolayer cell culture, the cellular events responsible for HPV infection during wound healing have been limited to analysis of mitotically active keratinocytes (146), which aid wound healing by replenishing dead or damaged keratinocytes post-injury. Several studies

postulate that HPV infects migrating keratinocytes (39, 43), but whether this is the case has yet to be examined.

Since genital tissues *in vivo* appear to be particularly susceptible to HPV transmission (79), but *in vitro* infection models require tremendous virion amounts to achieve detectable, robust infection, marked discrepancies exist between clinical observations and *in vitro* models of infection. This study was performed to determine the impact of epithelial wounding and keratinocyte migration on HPV16 infection establishment. By physically disrupting and then infecting keratinocyte cell monolayers and organotypic raft tissue, this study simulated *in vitro* the events that occur in animal models of HPV16 infection. We hypothesize that scratch wounding enhances HPV16 infection of both keratinocyte monolayers as well as raft epithelial tissue. Additionally, we hypothesize that migrating keratinocytes, having undergone an EMT, support HPV16 infection establishment.

Chapter 2: Characterizing HPV16 Infection Of Monolayer Keratinocytes Undergoing An Epithelial-To-Mesenchymal Transition

Abstract

HPV appears to require epithelial disruption to establish infection in genital tissue, likely by microwounding during sexual activity. Wound healing progresses as cells, signaling molecules and enzymes become activated post-

injury. Although dividing keratinocytes are the host cells for HPV, the virus nonetheless encounters other keratinocytes within a wound, including keratinocytes having already engaged in stratification and differentiation, as well keratinocytes activated to migrate for wound closure. HPV is thought to infect migrating keratinocytes, but this has yet to be rigorously tested. In this chapter, keratinocytes induced by various methods into a migratory phenotype were exposed to HPV16 pseudovirions (PsVs) and measured for infection. Keratinocytes are stationary by nature; to migrate, they must undergo marked phenotypic alterations, referred to as an epithelial-to-mesenchymal transition (EMT). To assess whether migrating keratinocytes support infection, EMT was induced in HaCaT cell monolayers by scratch wounding or treatment with growth factors. We confirmed the induction of EMT by confocal microscopy and then exposed the cells to HPV16 PsV. Despite the finding that HPV PsV could bind and apparently enter HaCaT cells undergoing EMT (albeit at reduced levels compared to untreated HaCaT cells), these cells did not support HPV PsV marker gene expression. HPV16 infection was diminished when HaCaT cells were pre-treated with common wound healing growth factors, including those not involved in EMT. Mesenchymal features alone did not block infection, as murine fibroblasts, a type of mesenchymal cell in connective tissue, were readily infected by HPV16 PsVs. This indicates that EMT-associated cellular changes, and not just the mesenchymal phenotype, compromise infection. Interestingly, HPV16 infection of keratinocytes in an EMT resumed coincident with the return of epithelial characteristics. Taken together, we found that migrating keratinocytes,

which down-regulate epithelial features and instead express mesenchymal ones, do not support HPV16 infection. However, when their epithelial phenotype is restored at the end stage of reepithelialization, migrating keratinocytes become capable of infection by HPV16. This implies that during wound healing, keratinocytes undergoing an EMT are transiently impervious to HPV16 infection, and that HPV16 infection is reliant upon keratinocytes displaying the epithelial phenotype.

Introduction

The majority of cells in adult tissues are differentiated, specialized and immobile, with limited ability to self-repair in the event of injury. Epithelial tissue, however, demonstrates a marked plasticity following injury. Epithelial wound healing relies upon both cell division and migration to achieve wound closure, wherein cells use tightly organized, sequential and overlapping stages of cellular activation for wound repair and tissue regeneration (17). Keratinocytes in cutaneous and mucosal surfaces, both sites of HPV infection, are responsible for reepithelialization following tissue injury (102, 170, 198). During wound healing, cellular activities may extend to migration, division or death to restore the epithelial barrier (98, 139), each of which may impact the initiation of HPV16 infection in keratinocytes.

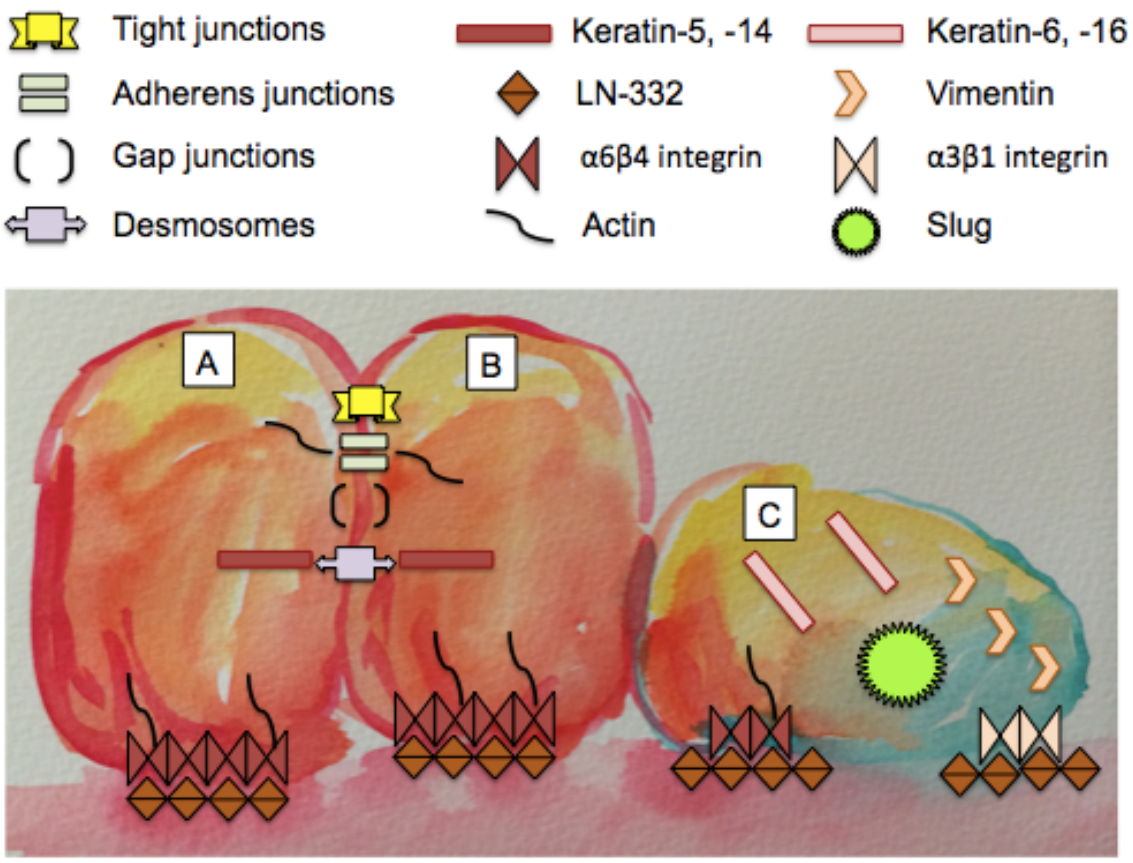


Figure 5: Keratinocyte migration at the wound margin occurs through EMT. In undamaged tissue (cells A and B), basal epithelial cells are stationary and adhere to one another through tight junctions, gap junctions, adherens junctions and desmosomes, and to laminin-332 in the basement membrane through α6β4 integrin. Upon injury, keratinocytes migrate to cover the denuded epithelial surface, using α3β1 integrin to bind provisional laminin-332 at the leading edge (cell C). Through activation of intracellular signaling cascades and altered gene expression, epithelial traits are diminished while migratory ones are up-regulated, as keratinocytes undergo an epithelial-to-mesenchymal transition (EMT). With the expression of migratory proteins such as slug and vimentin, cellular architecture is remodeled to promote migration.

The inherent regenerative ability of epithelial tissue is conferred by stem cells in the basal layer (18, 61, 109, 125). When intact and undamaged, multipotent adult epithelial stem cells supply the overlying keratinocyte layers with replacement cells, thereby enabling epithelial tissue to perpetually self-renew (17). Upon differentiating, basal keratinocytes migrate upwards and stratify to assemble into epithelial tissue. During wound healing, however, basal

keratinocytes migrate laterally at the wound margin. Keratinocyte migration, also known as keratinocyte activation, occurs by way of an epithelial-to-mesenchymal transition, or EMT (Fig. 5) (111, 126, 130).

When stimulated to migrate, epithelial cells undergo prominent phenotypic changes, including EMTs. EMTs happen in response to specific stimuli and allow multipotent cells to change phenotype in the absence of cell division. During wound healing, mechanical injury or inflammatory growth factors prompt keratinocytes to acquire lateral mobility through an EMT (28, 91, 130). Multiple types of EMTs exist and, while each type of EMT is unique, they share common signaling and transcriptional pathways (9, 45, 209). EMT types differ mostly in a context-dependent manner and are defined as primary (Type I), secondary (Type II) or tertiary (Type III) EMTs, which make possible embryogenesis, wound healing and carcinogenesis, respectively (17, 91, 93, 111, 184).

Primary EMTs are restricted to embryogenesis and development. Totipotent cells in the primitive mesoderm – primary, undifferentiated connective tissue – change phenotype and disperse throughout the emerging embryo using a Type I EMT (93). Type II EMTs happen throughout the human lifetime and always in reaction to provocation, generally injury or prolonged inflammation (130). During wound healing, cutaneous and mucosal epithelia are repaired, in part, by Type II EMTs, wherein tissue damage and inflammatory signals prompt a temporary change in phenotype (7, 86). Finally, Type III EMTs are associated with carcinogenesis and cause pathological cell growth and spreading (100,

118). They generate populations of invasive, dysregulated cells, that lead to malignancy (71, 100, 183).

Rationale And Study Hypothesis

Friction generated by intimate contact during sexual congress compromises the epithelial barrier, exposing underlying cells and HPV attachment factors (15, 198). HPV16 infection *in vivo* is augmented by epithelial disruption (151, 152) and, in the event of genital wounding, infection commences when HPV16 attaches to basal cells and ECM components (88, 97). HPV16 infects basal keratinocytes (161), the same cells that perform reepithelialization during wound repair either by migrating at the wound edge or hyperproliferating behind it (102, 156, 170). Productive HPV replication occurs when infected keratinocytes differentiate and produce progeny virions in cells of the uppermost epithelial layers. In animal models, tissue damage is necessary for the establishment of HPV infection in basal keratinocytes. Rhesus monkeys subjected to Pap smears – which disrupt the female genital tract epithelium to collect cytology specimens – were readily infected by HPV16, while monkeys not subjected to cytology specimen collection were uninfected (152). Similar studies in mouse models of HPV infection confirm the link between genital wounding and HPV infection (88, 97, 151).

Testing whether scratch wounding augments HPV16 infection of keratinocyte monolayers is the focus of investigation in this chapter, as is evaluating the potential for HPV16 infection of migrating keratinocytes *in vitro*.

Prior studies provide ample evidence that HPV contacts migrating keratinocytes (39, 43, 161). Building on previous studies that examined the importance of wounding for HPV infection, we hypothesize that 1) scratch wounding enhances HPV16 infection of HaCaT cells, and 2) migratory keratinocytes displaying EMT phenotypes in damaged epithelial monolayers support HPV16 infection. To test these hypotheses, keratinocytes in cell culture were infected with HPV16 PsVs under several wound healing conditions. These included both scratch wounding and growth factor stimulation to simulate the EMT phenotypic changes that occur during wound healing. A detailed understanding of the initiating events of HPV16 infection in the wound environment is essential for developing novel treatments and preventative measures, in addition to aiding in the design of more effective models of HPV infection.

Materials and Methods

Cell culture, media and growth conditions
HaCaT cells (a gift of Nobert Fusenig, DKFZ) are a non-tumorigenic, spontaneously immortalized human keratinocyte cell line derived from skin near the distant periphery of a melanoma on the back of a 62-year old male (19). They were maintained in DMEM/Ham's F-12 medium (Irvine Scientific), supplemented with 10% FBS (Invitrogen), amino acids (Invitrogen) and 1X glutamine-penicillin-streptomycin (GPS) (Invitrogen). HaCaT cells were used to study both HPV infection and EMT. 293T cells are a cell line derived from human embryonic kidney (HEK) tissue, which express SV40 large T antigen as a transcriptional regulator for various plasmids upon transfection (171). 293T cells were maintained in 10% fetal bovine serum (FBS) and 1X GPS, and were used for pseudovirion production, described below. NIH 3T3 "J2" murine fibroblasts were maintained in DMEM/Ham's F-12 medium (Irvine Scientific), supplemented with 10% newborn calf serum (NCS) and gentamycin. J2 cells were used for HPV16 infection.

Virion production and purification
HPV16 virions were generated in 293T cells as previously described (145, 171). Briefly, after growing 293T cells to ~70% confluence in 10 cm dishes, cells were

$Ca_3(PO_4)_2$ cotransfected with 15 µg each pXULL (an HPV16 L1 and L2 expression plasmid) and a reporter plasmid, either pClNeo-GFP (SOURCE) for GFP expression or pGL3-control (Promega) for luciferase expression. Cells were harvested 48 h post transfection by trypsinization, then were pelleted and resuspended in PBS+9.5 mM $MgCl_2$ at 100 ml/10 cm plate. Cells were then lysed, using Brij58 at a concentration of 0.35%. Unpackaged DNA was digested by adding exonuclease V (Epicentre plasmid-safe) to 20 U/ml and Benzonase (Sigma) to 0.3%. Lysates were then incubated overnight at 37°C for maturation (24), after which 0.17 volumes of 5 M NaCl was added and lysates were freeze/thawed at -80/37°C to further disrupt cellular structures. Lysates were cleared by centrifugation at 3000Xg and supernatants were loaded onto discontinuous CsCl gradients, made using 4 mL light (1.25 g/ml) CsCl underlaid with 4 mL heavy (1.4 g/ml) CsCl. Virions were purified by 18 h ultracentrifugation at 20,000 rpm and 4°C in a Sorvall TH-641 rotor/buckets. Viral bands were visible slightly above the gradient interface and were collected by side puncture with a 1.5'' 18 gauge needle and 5 ml syringe. Virions were washed and concentrated in HSB (25 mM HEPES pH 7.5, 0.5 M NaCl, 1 mM $MgCl_2$) using Amicon Ultra-4 100,000 MWCO centrifugation filter units (Millipore) and stored at -80°C. Purity and L1 protein content were determined by SDS-PAGE and Coomassie Brilliant Blue staining against bovine serum albumin (BSA) standards.

Reagents:
Growth factors: HaCaT cells were exposed to various growth factors in serum-free HaCaT media, devoid of 10% fetal calf serum (FCS). In such experiments, all growth factors were prepared in a concentration of 5 ng/mL, and exposed to cells for the indicated lengths of time, typically 24, 48 or 72 h. The growth factor panel included TGFβ1 (Gibco, #PHG9214), EGF (SOURCE), PDGF (SOURCE), KGF (SOURCE) and bFGF (SOURCE), and were applied to cells in isolation or various combinations.

Antibodies: To detect various proteins, primary antibodies were used, followed by incubation with secondary antibodies with associated fluorophores during immunofluorescent (IF) microscopy and flow cytometry, or horse radish peroxidase (HRP) during Western blotting. Primary antibodies used for IF microscopy included L1 (mouse, Santa Cruz #sc-47699), L1 (Abcam, #30809?), L1 (mouse mAb, DakoCytomation #M3528), slug (rabbit mAb, Cell Signaling #9585), vimentin (mouse mAb, DakoCytomation #M7020), EGFR (rabbit, Cell Signaling #4267S), α-SMA (rabbit pAb, Abcam #ab5694), E-cadherin (mouse mAb, DakoCytomation #M3612, clone NCH-38), β-catenin (mouse mAb, Santa Cruz #sc-7693), ZO-1 (rabbit, Cell Signaling #5406). Additional reagents included phalloidin-AF594 to detect actin and DAPI to detect nuclei. Western blotting was performed with primary antibodies to EGFR (rabbit, Cell Signaling #4267S), annexin-A2 (rabbit, Santa Cruz #sc-9061), L1 and heparan sulfate (mouse IgM, Seikagaku/amsbio #370255-1). GADPH (mouse, Millipore #MAB374) was quantified in Western blots as a loading control. HRP-conjugated

secondary antibodies included anti-rabbit HRP-IgG (GE Healthcare #NA9340V), anti-mouse HRP-IgG (GE Healthcare #NXA931) and goat anti-mouse IgM-HRP (Santa Cruz #sc-2064).

EMT induction

Scratch assays were performed as described (113, 126). Briefly, HaCaT cells were grown to near confluency in 6-well plates. Scratches were performed using a yellow P200 pipette tip, first by first making 3 vertical scratches, then 3 additional perpendicular scratches, for a total of 6 wounds per sample. For immunofluorescence and microscopy, HaCaT cells were seeded atop glass coverslips, allowed to adhere overnight, then wounded with a cell scraper to remove a large swath of cells. After scratch wounding, cells were washed with 1X PBS to remove cell debris, then stained for EMT markers or infected with HPV16 pseudovirions (PsVs), as indicated.

To treat cells with growth factors (GFs), cells were sparsely seeded in 6-well plates and allowed to adhere overnight. The next morning, cells were serum-starved for 3 h to remove endogenous GFs, washed with PBS, then placed in serum-free media (SFM), which is normal media devoid of fetal calf serum (FCS). To prepare media for GF treatment, 5 ng/mL of corresponding GFs were prepared in SFM. Cells were placed in GF media for different lengths of time, typically 24, 48 or 72 h, prior to HPV16 infection or other analyses, as indicated. To induce an epithelial-to-mesenchymal transition (EMT), HaCaT cells were routinely exposed to 5 ng/mL TGFβ1 and 5 ng/mL EGF for 24, 48 or 72 h.

Infections

Infections were performed in 6-well plates using HaCaT cells seeded to various confluencies. After preparing cells for infection by scratch wounding or GF treatment, HPV16 pseudovirions (PsVs) were added to cells, given as viral genome equivalents (vges) per cell or capsids per cell. HPV16-eGFP PsVs encapsidate the plasmid pClNeo-GFP, which encodes the gene for enhanced green fluorescent protein (eGFP). Infection using HPV16-eGFP PsVs was determined by eGFP expression, using confocal immunofluorescence microscopy for visualization or flow cytometry for quantification. HPV16-luc PsVs, harboring the pGL3-control plasmid, encode luciferase and enable infection to be measured by luciferase assay. After binding PsVs to cells for 1 h at 4°C, infections proceeded at 37°C until eGFP detection or luciferase quantification.

Immunofluorescence microscopy

For detecting eGFP expression during infection with HPV16-eGFP, as well as various markers of an EMT, HaCaT cells were seeded on glass coverslips and cultured overnight. After preparing cells by scratch wounding or GF treatment, cells were mock or HPV16 exposed. Infection proceeded for the indicated periods of time, followed by PBS washing to remove unbound virions. Cells were fixed with 3.7% paraformaldehyde (PFA) for 10 min. Cell surface proteins were

immunolabeled under non-permeabilizing conditions; to stain intracellular markers, cells were permeabilized with 0.1% TX-100. Nuclei were stained with DAPI. Images were taken on a Zeiss LSM510 Meta confocal microscope at the University of New Mexico (UNM) Cancer Center Fluorescence Microscopy Facility.

PAGE and immunoblotting

For denaturing/reducing (D/R) polyacrylamide gel electrophoresis (PAGE), cell lysates were measured for protein content using the Bradford assay, then mixed with reducing/denaturing SDS-PAGE loading buffer (62.5 mM Tris pH 6.8, 10% glycerol, 2% SDS, 0.05% 2-mercaptoethanol, 0.05% bromophenol blue) and heated to 95°C for 10 min prior to electrophoresis. For each sample, 15 ug was loaded on pre-cast 10% SDS-PAGE gels. Bradford assays were performed to determine protein concentration. Samples were combined with D/R buffer and boiled for 10 min, then 15 ug/sample resolved by SDS-PAGE. Following transfer of samples from the SDS-PAGE gel to a nitrocellulose membrane, immunoblotting was performed using the indicated antibodies.

Binding assays

To assess HPV16 binding to cell surfaces, HaCaT cells were seeded at 1e5 cells/well in 6-well plates. Control cells were maintained in normal HaCaT media, while experimental cells were serum-starved just prior to treatment with 5 ng/mL TGFβ1 and 5 ng/mL EGF to induce an EMT. After 24, 48 and 72 h, cells were exposed to HPV16 PsVs/cell for 1 h at 4°C with gentle rocking. After, cells were washed immediately with 1X PBS 4-5 times to remove unbound PsV, then lysed with radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris HCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA) supplemented with 1X Halt protease and phosphatase inhibitor cocktail (Thermo Scientific). Protein content was determined by Bradford assay and 15 ug/sample was combined with denaturing/reducing buffer. Samples were analyzed by SDS-PAGE and immunoblotting for HPV16 L1.

Entry assays

Trypsin-entry assay: HaCaT cells were seeded at 6e5 cells/well in 6-well plates then induced into an EMT for 24 h with TGFβ1 and EGF. HPV16 PsVs were bound to cells at 20,000 capsids/cell for 1 h at 4°C, then washed thoroughly with PBS (3 washes) to remove unbound virions. Samples were collected by trypsinization at 0, 4 h to capture infection under binding conditions (0 h) or entry conditions (4 h). Alternatively, samples were collected by cell scraper as a control for trypsin efficiency in removing surface-resident virions. In samples collected by trypsinization, trypsin was neutralized by adding an equal volume of cell media, after which cells were pelleted and supernatant removed. A second trypsinization was performed to remove residual surface-bound HPV, followed again by cell media addition, pelleting and aspiration, then two PBS washes. At this point, any HPV present in the samples should have already been internalized. Cells were then lysed using RIPA buffer supplemented with 1X Halt

protease and phosphatase inhibitor cocktail for 10 min. Samples were sheared using an 18-gauge needle 4-5 times to further break apart genomic material, then were centrifuged for 10 min at 4°C at 15,000 rpm to pellet large cellular debris. Supernatants were collected and subjected to a Bradford assay to determine protein concentration. Samples were combined with D/R buffer and boiled for 10 min, then 15 ug/sample resolved by SDS-PAGE. Following transfer of samples from the SDS-PAGE gel to a nitrocellulose membrane, L1 was blotted.

Microscopy: HaCaT cells were seeded to 2.5e4 cells/well atop glass coverslips in 6-well plates, then induced into an EMT for 24, 48 and 72 h with TGFβ1 and EGF. HPV16 PsVs were bound to cells at 10,000 vge/cell for 0 h or 6 h to observe binding and entry, respectively. Cells were then permeabilized, fixed and stained for L1 and indicated EMT markers, slug or vimentin. HPV16 was detected with a mouse mAb against L1 (Millipore #CBL402; CamVir-1) and visualized with anti-mouse AF-488 secondary antibody. Slug was detected with a rabbit mAb, followed by incubation with FITC-conjugated anti-rabbit secondary antibody. Likewise, HPV16 was detected using rabbit anti-sera to L1, then incubated with goat anti-rabbit AF-594 secondary antibody. Vimentin was detected using a mouse mAb (DakoCytomation #M7020) and visualized by incubation with donkey anti-mouse DyLight-488 secondary antibody. Nuclei are stained with DAPI.

Results

1. Scratch wounding of HaCaT cells does not enhance HPV infection

To test the hypothesis that HPV16 infection is enhanced by injury, scratch wounded HaCaT cells were exposed to HPV16 PsVs encapsidating an eGFP reporter genome (HPV16-eGFP). Previously, the scratch wounding and infection model has been used with herpes simplex virus type 1 (HSV1) infection of keratinocytes, wherein infection occurred amongst wound margin cells while undamaged areas of cells were uninfected (159). Here, we adapted the scratch wounding model in order to assess the impact of injury on HPV infection *in vitro*. Infection in this model is defined as HPV16 PsVs achieving cellular entry and expression of the encapsidated reporter genome. HPV16-eGFP was added to cells immediately post-wounding or after 2 h, to give a brief recovery period in

which cells could start migrating. As a control, undamaged HaCaT cells were also infected by HPV16-eGFP. After 48 h, the time needed to achieve robust, detectable eGFP expression, cells were trypsinized and assessed for eGFP expression by flow cytometry. Concurrently, scratch wounded HaCaT cells were infected with HPV16-eGFP and analyzed by confocal microscopy.

Somewhat surprisingly, scratch wounding did not markedly enhance HPV16-eGFP infection (Fig. 6A). Compared to control cells that were left intact and undamaged, the percentage of wounded cell infection only increased by 3.5%. Cells given a brief 2 h recovery period were infected at a similar rate. By microscopy, wounded HaCaT cells did not support infection, as only a small number of HPV-infected cells were detected (Fig. 6B). Since this experiment does not distinguish migrating from dividing cells, keratinocyte behavior post-wounding was next analyzed.

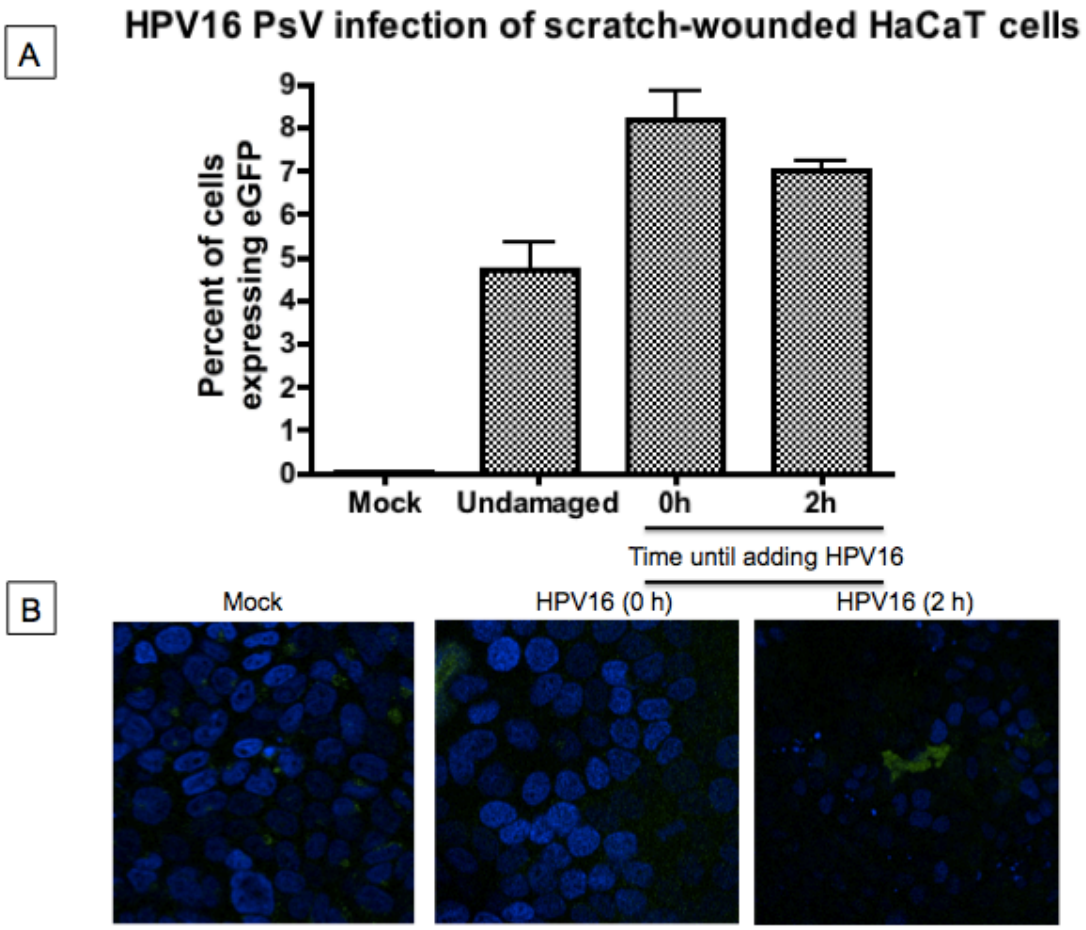


Figure 6: HPV16 infection is not markedly enhanced by scratch wounding HaCaT cells. (A) HaCaT cell monolayers were grown to confluence, left intact or scratch wounded, then exposed to 1,000 vge/cell HPV16-eGFP PsVs immediately or after 2 h. Cells were maintained in growth medium for 48 h, after which cells were harvested by trypsinization and analyzed by flow cytometry. Infection was determined by measuring eGFP expression per cell, divided by the total number of cells counted. Data is representative of 3 independent experiments, each performed in triplicate. Error bars indicate standard error of the mean. **(B)** HaCaT cells were scratch wounded and infected using 1000 vge/cell HPV16-eGFP PsVs for 48 h. Positive infection is given by eGFP detection, which can be seen at 2 h but is otherwise unapparent. Nuclei are stained with DAPI.

In order to better understand the cellular and molecular changes brought about by scratch wounding, HaCaT cells were grown to confluence on glass coverslips, scratched with a pipette tip, then fixed and visualized at different times post-injury. Cell migration was evident after 12 h and wound closure was completed by 24 h (Fig. 7A). Because EMT phenotypic changes in cells are a

consequence of wounding, confocal microscopy and indirect immunofluorescence were used to assess the expression of molecular markers of EMT (126, 209). Since scratch wounds made by a pipette tip closed after 24 h, cells were injured with a cell scraper to remove a large region of cells and create a distinct wound margin that would not close during the experiment. As expected, cells along the wound margin displayed features of keratinocyte migration, including increased internuclear distance, diminished epithelial protein expression and activation of migratory genes (Fig. 7B). The junctional proteins E-cadherin and β-catenin, components of adherens junctions, were present amongst intact cells but diminished in wound edge keratinocytes (Fig. 7B). Additionally, ZO-1, a tight junction protein, was disrupted in leading edge cells, although staining was variable and somewhat difficult to discern (Fig. 7B). To visualize cytoskeletal changes, vimentin was stained. Vimentin was not expressed by undamaged cells, but after injury, it appeared in punctate form (Fig. 7B). Slug, a transcription factor and master regulator of several events during an EMT, was expressed by keratinocytes at the wound edge (Fig. 7B). These data show that EMT occurs during keratinocyte migration in this scratch wounding model.

Although scratch wounding elicits the appropriate EMT phenotype in cells, this model was not useful for investigating HPV infection dynamics *in vitro*. Detectable eGFP expression does not occur until after migrating cells close the wound margin (Fig. 7A), making it difficult to identify infected cells. Because scratch wounded HaCaT cells did not support HPV16-eGFP infection (Fig. 6)

when undergoing EMT (Fig. 7B), alternative means of assaying HPV16 infection dynamics in the wounded environment were explored.

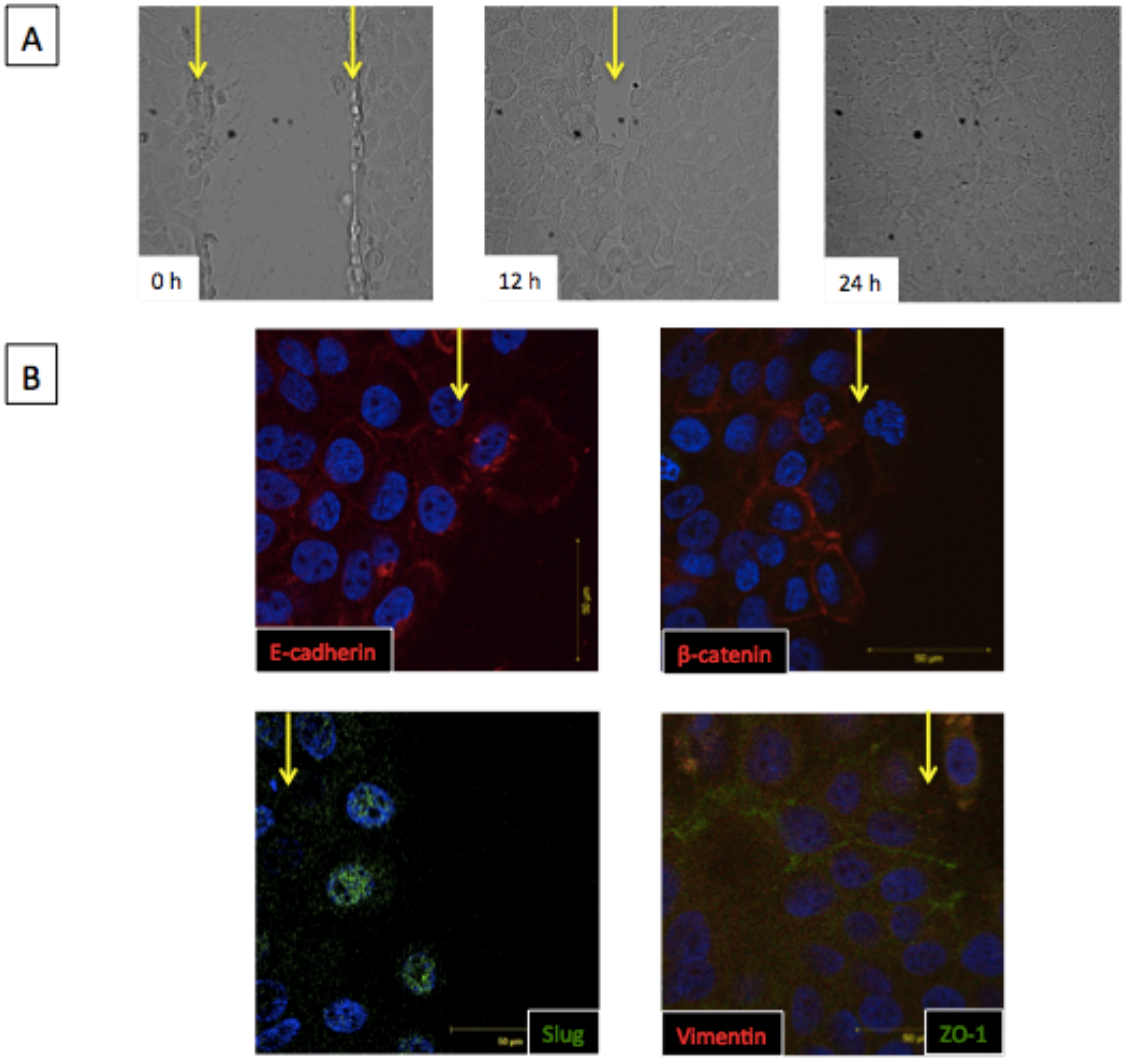


Figure 7: Scratch wounded HaCaT cells migrate and express EMT phenotypic markers. (A) HaCaT cells were grown to confluence then scratch-wounded with a pipette tip. After giving cells 24 h to recover, the wound margin was visualized by light microscopy. **(B)** Scratch wounded cells were analyzed 24 h post-injury by fixation and immunofluorescent staining, followed by confocal microscopy to visualize EMT progression. E-cadherin staining was performed with mouse anti-E-cadherin monoclonal antibody and AF594-conjugated goat anti-mouse IgG; β-catenin staining was performed with mouse anti-β-catenin monoclonal antibody and AF594-conjugated goat anti-mouse IgG. Slug was visualized with rabbit anti-slug monoclonal antibody and FITC-conjugated goat anti-rabbit IgG. Vimentin staining used mouse anti-vimentin monoclonal antibody and AF-594-conjugated chicken anti-mouse IgG, while ZO-1 staining was performed with rabbit anti-ZO-1 polyclonal antibody and FITC-conjugated goat anti-rabbit IgG. Nuclei are stained blue with DAPI; scale bar = 50 μM; yellow arrows indicate wound edge.

2. TGFβ1- and EGF-induced EMT in HaCaT cells prevents HPV16 infection

The induction of phenotypic changes in keratinocytes by the addition of GFs is a common model system for exploring the wound environment (98, 128, 139). The addition of TGFβ1 and EGF to serum-free HaCaT cell culture medium is often used to stimulate cell migration and EMT (37, 148, 201). Since initial attempts to model HPV16 infection in the wound environment using scratch wounding were unsuccessful, HaCaT cells treated with TGFβ1 and EGF were tested for their potential to be used as a surrogate for wounding-induced EMT.

After treatment with 5 ng/mL TGFβ1 and 5 ng/mL EGF for 24, 48 or 72 h, HaCaT cells displayed morphological changes characteristic of EMT (Fig. 8). Whereas cells maintained in control media (containing 10% FCS) displayed cobblestone morphology, TGFβ1- and EGF-treated cells were spindle shaped and exhibited increased internuclear distance (Fig. 8, top row). Furthermore, slug and vimentin were expressed in TGFβ1- and EGF-treated cells but not in control cells. Following GF treatment, slug was robustly detected after 24 h and sustained through 48 and 72 h (Fig. 8, middle row). Vimentin appeared in punctate form after 24 h of GF treatment, but by 48 h and especially by 72 h it had assembled into filaments (Fig. 8, bottom row). Even though not all cells treated with TGFβ1 and EGF expressed slug and vimentin, these GFs provided a more effective means of generating the activated EMT phenotype than did scratch wounding. Compared to scratch wounded HaCaT cells, which expressed slug along the leading edge (Fig. 7B), TGFβ1- and EGF-treated HaCaT cells exhibited substantial slug activation, which intensified over time (Fig. 8).

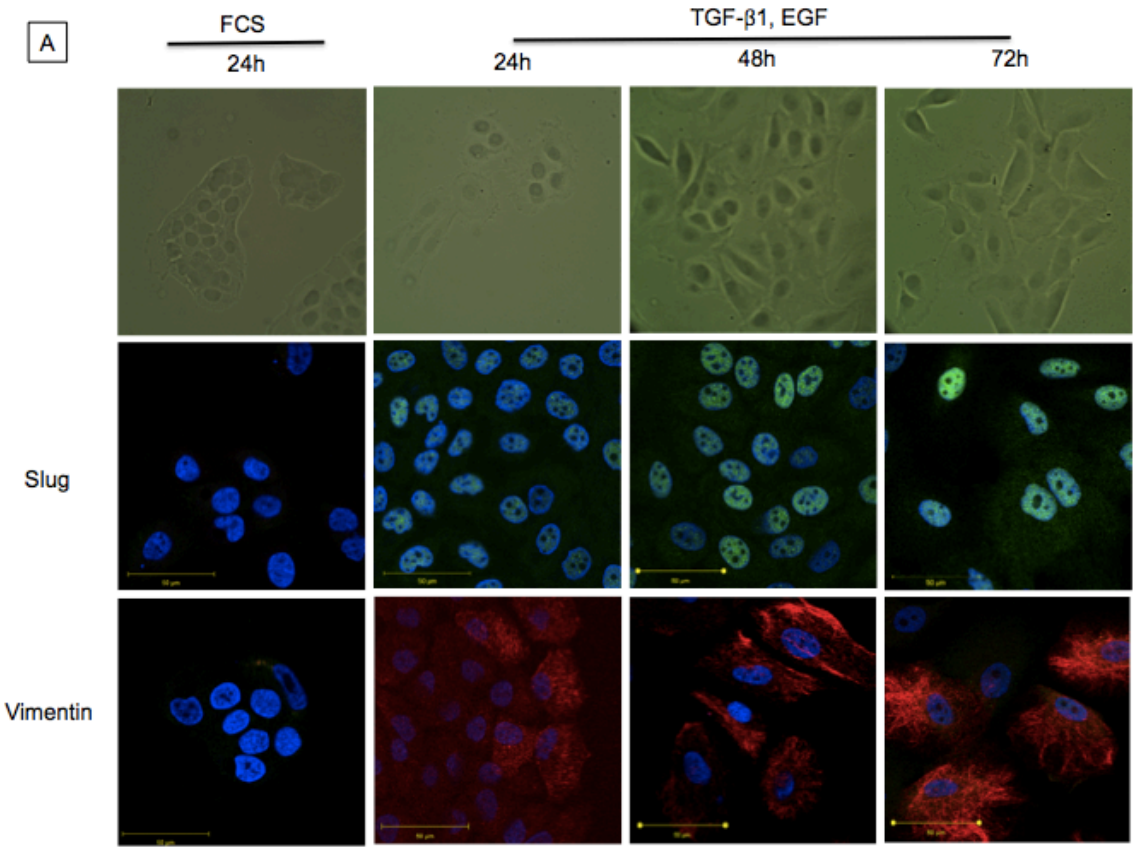


Figure 8: TGFβ1 and EGF induce an EMT in HaCaT cells. HaCaT cells were treated with 5 ng/mL TGFβ1 and 5 ng/mL EGF in combination for 24, 48 and 72 h, then fixed, mounted and imaged. Top row: Light microscopy images of HaCaT cell morphology. Middle row: Permeabilized cells were stained for slug with rabbit anti-slug monoclonal antibody and FITC-conjugated goat anti-rabbit IgG. Bottom row: Permeabilized cells were stained for vimentin using a mouse anti-vimentin monoclonal antibody and AF594-conjugated chicken anti-mouse IgG. Nuclear DNA was stained with DAPI. Images were taken with a confocal microscope. Scale bar = 50 μM.

Other proteins involved in EMT were evaluated after treatment with TGFβ1 and EGF, including EGFR, β-catenin and actin (Fig. 9). After 48 h of TGFβ1 and EGF exposure, EGFR was detected on the cell surface of non-permeabilized cells, especially on plasma membrane projections that resembled filopodia (Fig. 9A). As measured by immunoblot, EGFR expression diminished over time following TGFβ1 and EGF treatment (Fig. 9A). In TGFβ1- and EGF-

treated cells, the staining pattern of β-catenin was more diffuse than in control cells (Fig. 9B). β-catenin was not observed to translocate to the nucleus following TGFβ1 and EGF treatment, as has been observed in other studies of EMT (143). Actin, which drives cellular locomotion *via* actin-myosin contraction, was also expressed by cells in an EMT (Fig. 9B).

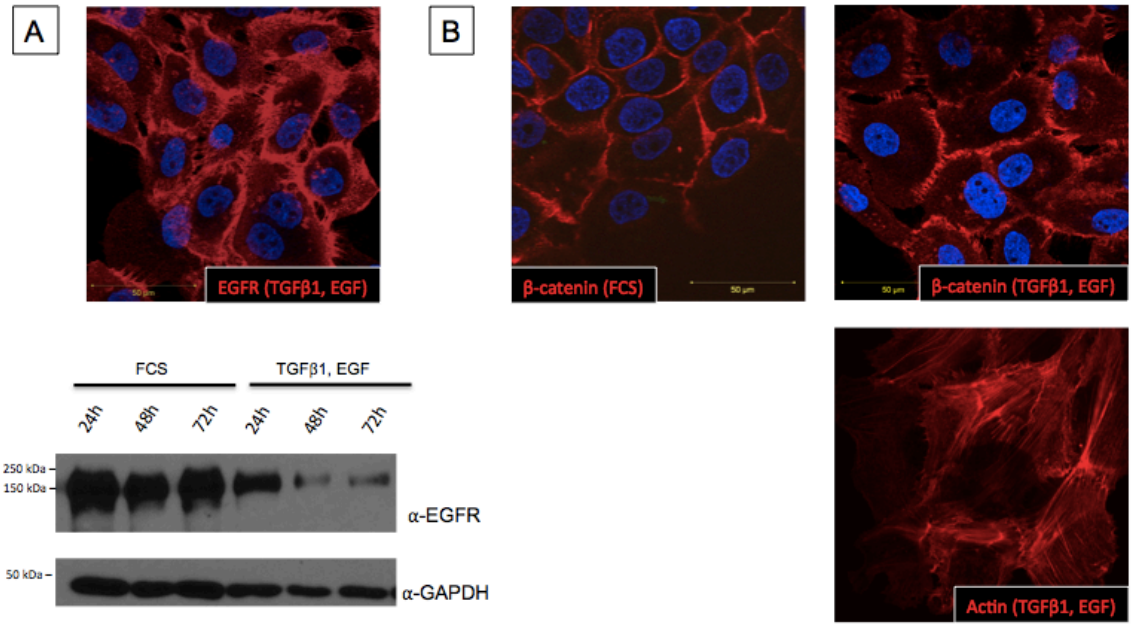


Figure 9: (A) HaCaT cells undergoing an EMT after TGFβ1 and EGF treatment express epithelial markers. (Top) HaCaT cells induced into an EMT with 5 ng/mL TGFβ1 and 5 ng/mL EGF for 48h were stained under non-permeablizing conditions to detect cell surface EGFR. EGFR staining used a mouse anti-EGFR monoclonal antibody and DyLight549-conjugated anti-mouse IgG secondary antibody. (Bottom) Cell lysates from EMT and control cells were collected at 24, 48 and 72 h and analyzed for total EGFR expression by immunoblotting, using a rabbit anti-EGFR monoclonal antibody and HRP-conjugated anti-rabbit IgG secondary antibody. **(B)** β-catenin expression was visualized amongst wounded cells maintained in 10% FCS media (left) *versus* cells treated with 5 ng/mL TGFβ1 and 5 ng/mL EGF for 48h (right). β-catenin staining was performed using a mouse anti-β-catenin monoclonal antibody and an AF594-conjugated goat anti-mouse secondary antibody. Actin, which links to β-catenin by α-catenin, was visualized using phalloidin-AF594 (bottom). Nuclei were visualized with DAPI.

We next assessed the infectability of keratinocytes in GF-induced EMT to HPV16. In these experiments we exposed TGFβ1- and EGF-treated HaCaT cells with HPV16 PsVs harboring a luciferase-expressing plasmid (HPV16-luc

PsVs) and measured infection by luciferase assay, which provides a quantifiable measure of infection for the population of cells. After treatment with TGFβ1 and EGF for 24, 48 or 72 h, cells were infected with HPV16-luc PsVs and, 24 h post-infection, cell lysates were collected and assayed. Compared to control cells kept in complete, serum-containing media, cells pre-treated with TGFβ1 and EGF did not support HPV16-luc infection (Fig. 10A). When cells were treated with TGFβ1 and EGF individually there was modest infection (Fig. 10A). TGFβ1 by itself is sufficient to generate an EMT (37, 183), but EGF also causes keratinocyte migration (10, 121). This finding demonstrates that treatment with either TGFβ1 or EGF dramatically diminishes HPV16 infection, and they are even more potent at preventing infection in combination.

To assess HPV16 infection during an EMT on a per-cell basis, cells were treated with TGFβ1 and EGF for 24 h, infected with HPV16-eGFP and analyzed by flow cytometry. Whereas control cells were infected in a dose-dependent manner, TGFβ1- and EGF-treated cells were not infected over background levels (Fig. 10B). This finding is consistent with luciferase assay data (Fig. 10A) and indicates that TGFβ1/EGF-treated HaCaT cells undergoing EMT fail to support HPV16 infection.

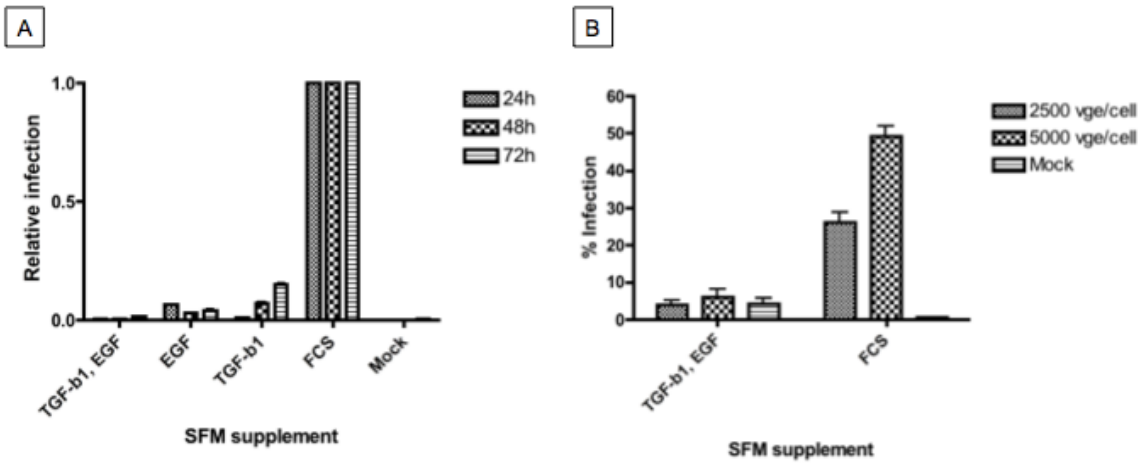


Figure 10: HaCaT cells treated with TGFβ1 and EGF do not support HPV16 infection. (A) HaCaT cells were serum-starved for 3 h to deplete endogenous growth factors, then treated with TGFβ1 (5 ng/mL) and EGF (5 ng/mL), alone or in combination, for 24, 48 or 72 h. At each time point, cells were infected with HPV16-luc at 100 vge/cell and analyzed 24h later by luciferase assay. Graph is representative of 2 experiments, performed in triplicate. Percent infection is displayed relative to control cells (kept in 10% FCS media), where infection is set at 100%. **(B)** After 24 h treatment with TGFβ1 (5ng/mL) and EGF (5ng/mL) in combination, HaCaT cells were infected with HPV16-eGFP at 2500 or 5000 vge/cell and analyzed after 48 h by flow cytometry. Graph is representative of 2 (5000 vge/cell) or 4 experiments (2500 vge/cell), performed in triplicate. Percent infection represents the number of eGFP-expressing cells divided by the total cell number. Error bars represent standard error of the mean.

3. HaCaT cells exposed to additional growth factors display reduced levels of HPV infection

In the course of wound healing, keratinocytes are exposed to a number of growth factors besides TGFβ1 and EGF (Table 2). Specifically, the expression of PDGF, bFGF and KGF and their receptors increases post-wounding (11, 98, 128, 196). TGFβ1, EGF, bFGF and KGF are implicated in keratinocyte motility (89) whereas PDGF mediates fibroblast motility (5, 110). To investigate the effects of these growth factors on the ability of keratinocytes to support HPV16 infection, HaCaT cells were pre-treated with various combinations of PDGF, bFGF and KGF (with or without TGFβ1 and EGF) for 24 h and then exposed to HPV16-luc for an additional 24 h. Exposure of cells to individual growth factors

reduced infection, albeit to varying degrees. Compared to control, TGFβ1 completely inhibited infection, EGF reduced infection 90%, and PDGF, bFGF and KGF reduced infection by 71%, 67% and 71%, respectively (Fig. 11A). Consistent with previous results (Fig. 10), TGFβ1 and EGF in combination nullified infection (Fig. 11A). Furthermore, TGFβ1 and EGF combined with other growth factors still nullified HPV16 infection (Fig. 11A). This suggests that whatever cellular changes KGF, bFGF and PDGF may have caused, HPV16 infection of HaCaT cells remained compromised after TGFβ1 and EGF brought about an EMT.

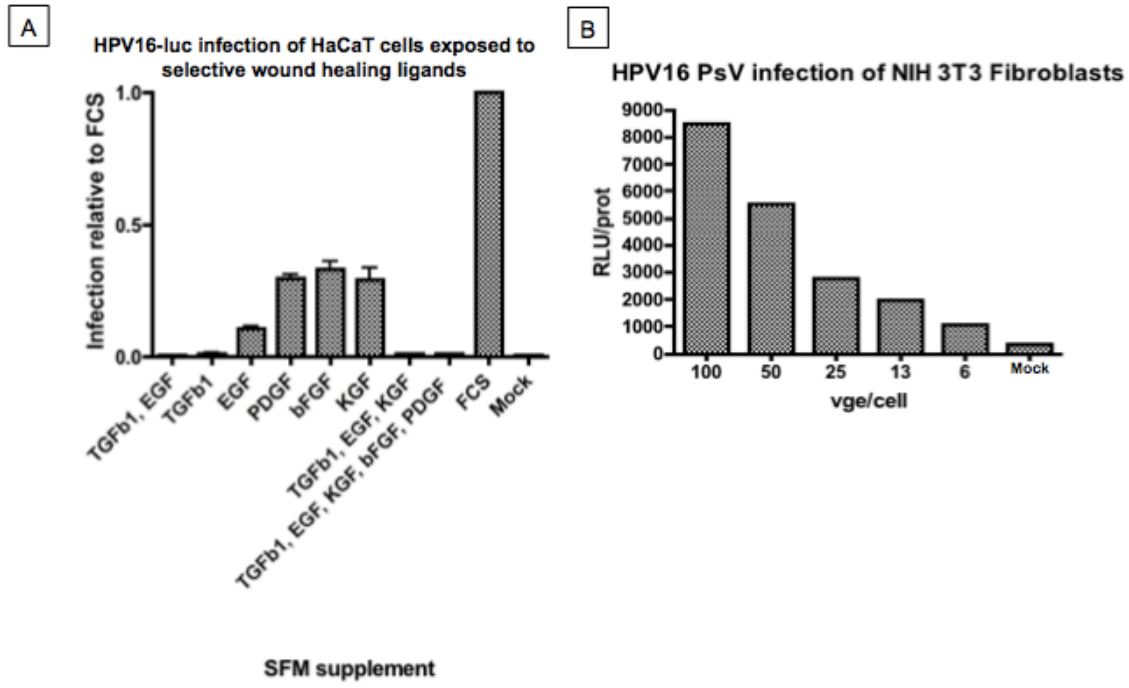


Figure 11: Keratinocytes exposed to selective wound response growth factors exhibit disparate responses to HPV16-luc infection, while murine fibroblasts support HPV16-luc infection. (A) HaCaT cells were serum-starved for 3h, then treated with TGFβ1 (5 ng/mL), EGF (5 ng/mL), PDGF (5 ng/mL), bFGF (5 ng/mL) or KGF (5 ng/mL) as indicated for 24 h. Cells were infected with HPV16 PsVs harboring a reporter genome expressing luciferase at 200 vge/cell and analyzed 24h later by luciferase assay. Graph is representative of 2 experiments, performed in triplicate. Percent infection is displayed relative to the 10% FCS infection group, where infection is set to 100%. Cells were maintained in SFM supplements as noted for the duration of the infection. Graph is the combination of 2 independent experiments, performed in triplicate. Error bars equal standard error of the mean. **(B)** Murine fibroblasts, NIH 3T3 "J2" cells, support

HPV16-luc infection in a dose-dependent manner. Graph is representative of a single experiment, with vge/cell amounts performed once each.

Although keratinocytes undergoing an EMT are oftentimes referred to as mesenchymal cells, they differ from prototypical mesenchymal cells like dermal fibroblasts because their mesenchymal features are temporary and reversible. To study HPV16 infection of mesenchymal cells of a different origin, NIH 3T3 murine fibroblasts, strain J2, were exposed to HPV16-luc and measured for infection by luciferase assay. Compared to the lack of HPV16-luc infection in keratinocytes in an EMT, (i.e. with a transitory mesenchymal phenotype) (Figs. 10, 11A), mesenchymal J2 cells readily supported HPV16-luc infection in a dose-dependent manner (Fig. 11B). This finding demonstrates that while fibroblasts and keratinocytes in an EMT share many mesenchymal characteristics, they are starkly different with regard to HPV16 infection, possibly because fibroblasts readily divide in culture but keratinocytes in an EMT do not.

Wound ligand	TGFβ1	EGF	KGF (FGF7)	bFGF (FGF2)	PDGF
Cellular source	Platelets, macrophages, fibroblasts, keratinocytes, T lymphocytes	Eosinophils, platelets, fibroblasts, macrophages, keratinocytes	Fibroblasts, dendritic epidermal T cells	Macrophages, fibroblasts and endothelial cells	Platelets, macrophages, endothelial cells, keratinocytes
Receptor display on keratinocytes	TβR	EGFR: detected in hyperproliferative cells	KGFR (FGFR2IIIb) is confined to cells committed to differentiation, therefore found mostly on suprabasal cells, but has low expression on basal cells (46)	FGFR (46) present mostly on suprabasal cells, but found in small amounts on basal cells (157)	PDGFR: absent from keratinocytes, but present on cells of mesenchymal origin: fibroblasts, smooth muscle cells (76, 150, 154)
Expression at wound site	Latent TGFβ1 is sequestered in the wound matrix, allowing sustained release by	Present on leading epithelial margins following an EMT (122)	Upregulated 100X 24 h post-wounding	Increased post-wounding	Ligand expressed in epidermis, but receptor present within

	proteolytic enzymes (MMPs)				dermis and granulation tissue (110)
Role in wound healing (for non-keratinocytes)	Reepithelialization; the most important ligand for starting migration of skin epithelial cells during wound healing; affects matrix metabolism; causes neutrophil and macrophage infiltration	Fibroblast proliferation	Fibroblast proliferation	Causes angiogenesis, matrix deposition, fibroblast chemotaxis and proliferation	Chemotactic for incoming migratory cells: neutrophils, monocytes and fibroblasts; induces fibroblast proliferation and expression of proteoglycans by fibroblasts
Keratinocyte behavior (migration, proliferation, differentiation, stratification)	Inhibit proliferation, enhance migration, alters ECM synthesis, apoptosis and differentiation	Keratinocyte proliferation, migration and granulation tissue formation; regulates keratinocyte survival, migration and proliferation	Induce proliferation during wound healing but differentiation in intact tissue	Indirect: causes fibroblast proliferation, which in turn interact with keratinocytes	Indirect: recruits other cells, which will in turn secrete keratinocyte-responsive growth factors
Impact HPV16 PsV infection	Nullify (~99%)	Reduce (~90%)	Reduce (~71%)	Reduce (~67%)	Reduce (~71%)

Table 2: Sources and activities of common growth factors during wound healing. Growth factors known to be motogenic for keratinocytes include: EGF, TGF-α, KGF, bFGF, TGFβ1 and IL-1.

4. HPV16 binding to HaCaT cells is reduced during EMT

HPV16 was not infecting keratinocytes undergoing an EMT, but the step at which infection became blocked was unclear. Because the assay for infection requires gene expression to take place, HPV binding, entry, and trafficking must all happen in order for "infection" to be determined. To explore the impact of EMT on binding, entry and trafficking of HPV16, experiments were next carried out to investigate these steps. HPV16 infection initiates when virions bind heparan sulfonated proteoglycans (HSPGs) displayed on the ECM and basal cell surface (1, 21, 66, 88, 166, 167). To determine the nature of HPV16 attachment to keratinocytes undergoing EMT processes, HaCaT cells treated with TGFβ1 and EGF for 24 h were exposed to HPV16-luc. Cells were exposed to virions under binding conditions, extensively washed to remove unbound virions, and

then lysates were collected. After resolving protein in an SDS-PAGE gel and transferring to a nitrocellulose membrane, cell-bound virions were detected by immunoblotting for the HPV major capsid protein, L1. Despite the fact that keratinocytes undergoing an EMT do not support HPV16 infection (Fig. 10, 11A), HPV16 bound to these cells, albeit at lower levels than cells kept in control media (Fig. 12A). In order to investigate the cause of this decreased binding, control HaCaT cells and HaCaT cells in an EMT were evaluated for heparan sulfate (HS) expression. HS expression was markedly altered during an EMT (Fig. 12B), although the blot is somewhat difficult to interpret. It appears that both control and TGFβ1/EGF-treated HaCaT cells express high molecular weight HS isoforms. However, they differ in that control HaCaT cells expressed a range of differently sized HS, similar to commercial HS, but TGFβ1/EGF-treated HaCaT cells did not. These data suggest that the decreased infection of HaCaT cells undergoing an EMT is at least partially due to altered HS expression and decreased HPV16 virion binding.

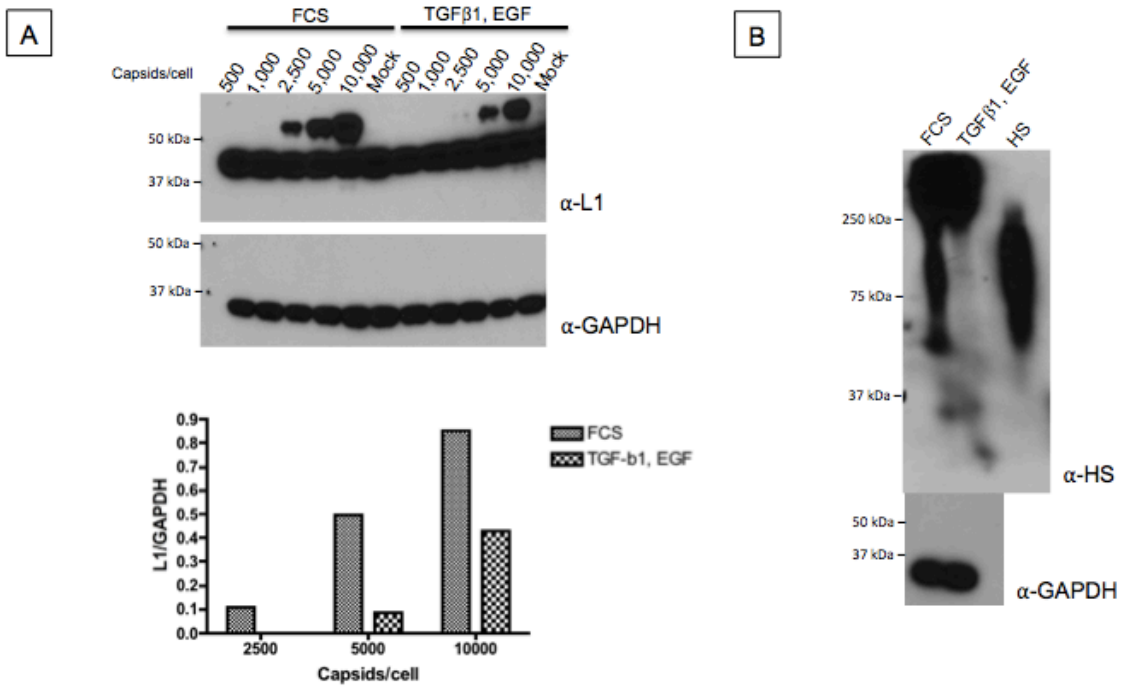


Figure 12: Keratinocytes displaying TGFβ1/EGF-induced EMT bind less HPV16 than control while exhibiting altered heparan sulfate expression. (A) Western blot analysis of L1 levels following binding of HPV16 PsVs to cells as indicated for 1h at 4°C. Cells were induced into an EMT for 24h as in Fig. 7. After binding, unbound HPV was removed by thorough PBS washing and then lysates collected immediately in RIPA buffer. Protein was resolved by loading 15ug under denaturing/reducing conditions on an SDS-PAGE gel, then transferred to a PVDF membrane. L1 was detected with a mAb against L1 (Millipore #CBL402; CamVir-1), followed by incubation with an HRP-conjugated anti-rabbit secondary antibody. Blots were re-probed with GAPDH to confirm equal loading. Control cells were kept in 10% FCS-containing media throughout the experiment. A non-specific band present at 42 kDa when blotting mammalian lysates is listed by manufacturer. **(B)** Heparan sulfate (HS) was immunobloted in HaCaT cell lysates from cells maintained in FCS or treated with TGFβ1 and EGF. One microgram of commercial HS was loaded as a positive control for the HS antibody. Blots were re-probed with GAPDH to confirm equal loading.

5. Evidence for HPV16 entry into HaCaT cells during EMT

Since HaCaT cells in an EMT retain the ability to bind virions (albeit at a decreased level) but do not support HPV16 infection, viral entry was next examined using two different assays: the “trypsin-entry assay” and confocal microscopy. The trypsin-entry assay detects internalized L1 by immunoblot. Briefly, cells induced into an EMT were exposed to HPV16 under binding

conditions and harvested immediately, or after 4 h to permit viral entry. During collection, cells were harvested either by trypsin or cell scraper and then lysed. Trypsin cleaves surface factors implicated in HPV16 attachment and entry, like EGFR (141). Subsequent SDS-PAGE and immunoblotting for L1 detects only those virions that have entered cells prior to harvest.

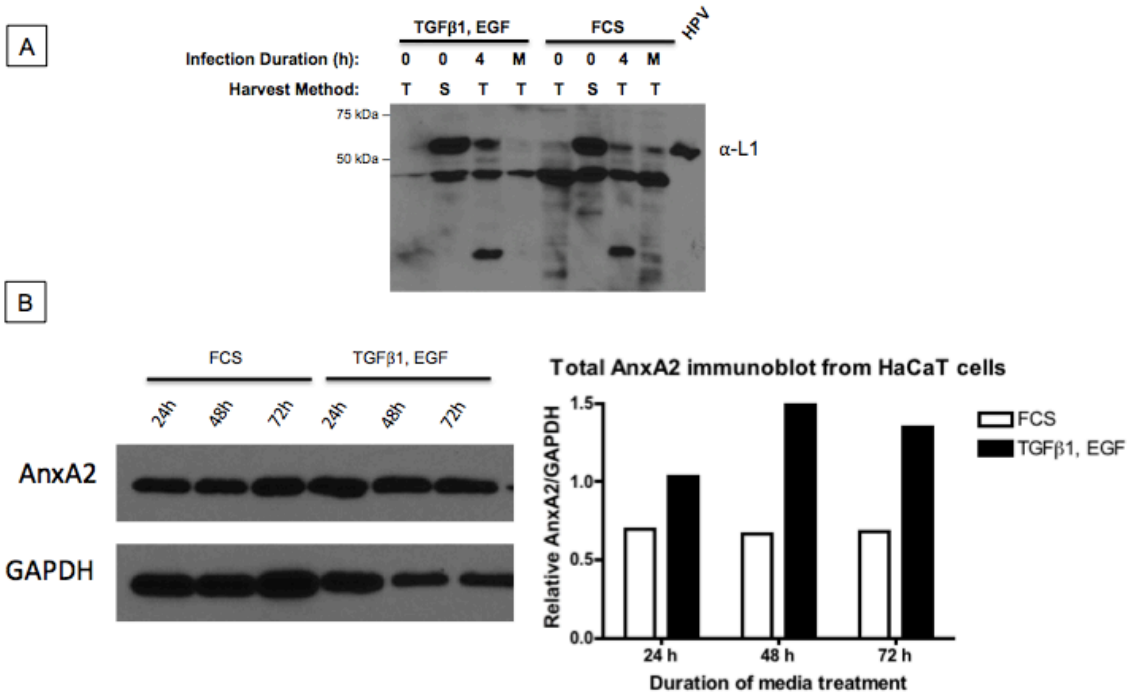


Fig. 13: HaCaT cells exposed to TGFβ1/EGF support HPV16 PsV entry, in conjunction with diminished annexin II. (A) HaCaT cells were induced into an EMT for 48 h or kept in 10% FCS media, then HPV16 was bound at 20,000 capsids per cell for 1 h at 4°C. Following viral binding (0 h) or entry for 4 h, unbound virions were removed by thorough washing with 1x PBS, then cells were collected by trypsinization (T) or by cell scraping (S). Cells were then lysed in RIPA buffer to collect cell lysates, then sheared with an 18-gauge needle. Protein was resolved by loading 15 µg under denaturing/reducing conditions on an SDS-PAGE gel, followed by transfer to a PVDF membrane. L1 was detected with a mAb against L1 (sc-47699; CamVir-1), then incubated with an HRP-conjugated anti-rabbit secondary antibody. Manufacturer lists a non-specific band at 42kDa when blotting mammalian cell lysates. M = Mock. Image is representative of two independent experiments, performed in duplicate. **(B)** Lysates from HaCaT cells in an EMT were immunoblotted for annexin II, as were control cells for comparison.

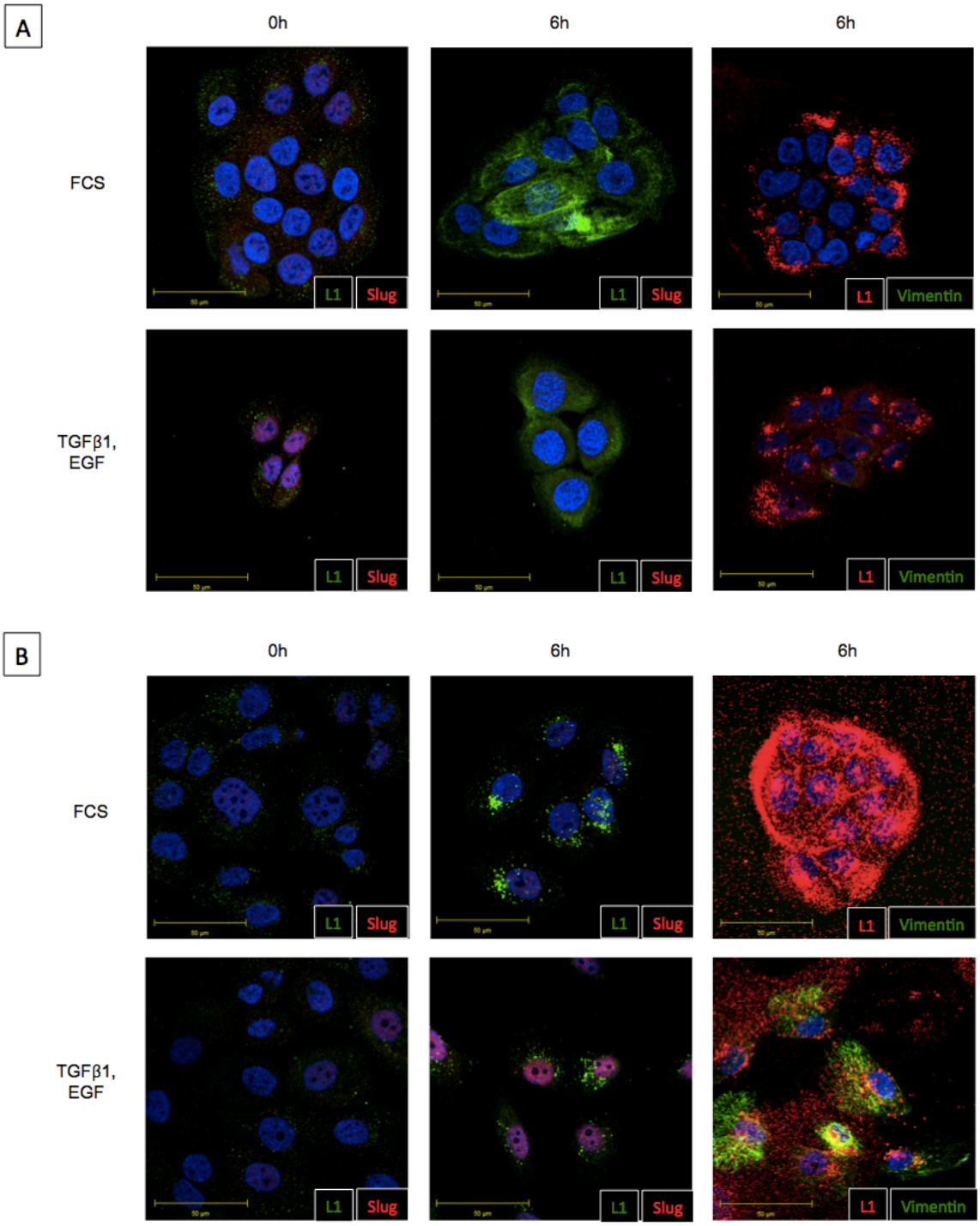
Per the L1 immunoblot, HPV appears to enter HaCaT cells during an EMT in the "trypsin-entry assay" (Fig. 13A). Importantly, trypsin was effective at

removing surface-bound HPV under binding conditions in both groups, while HPV remained bound to cells collected by scraping. After 4 h, a time sufficient for HPV to cross the plasma membrane and enter the cytoplasm, HPV was detected in both control and cells induced into an EMT. Additionally, a low molecular weight L1 cleavage product was observed in both groups after 4 h, which could be a result of L1 degradation after entry into the endo-lysosomal pathway. Although it is not possible to know if this is the case in this experiment, the fact that L1 appeared to be similarly cleaved in both control and cells in EMT indicates that HPV underwent the same processing after 4 h. It is not encouraging, however, that there was a faint L1 band in the mock-exposed cells of the control group, as this could invalidate the L1 blotting under binding and entry conditions. However, this band was absent in repeats of this experiment.

Next, HaCaT cells in an EMT were analyzed for expression of annexin, which is implicated in HPV entry. Annexins are a multifunctional family of Ca^{2+}-regulated membrane phospholipid binding proteins (80). When two Annexin A2 monomers (AnxA2) are linked by an S100A10 dimer they form an annexin heterotetromer. Of the many functions conducted by the annexin heterotetromer is endocytosis, which regulates HPV16 entry and trafficking during early infection (51). Additionally, phosphorylation of annexin A2 at Tyr-23 is implicated in loss of cell adhesion and acquisition of the mesenchymal phenotype (149). To determine whether changes in annexin expression contribute to HPV16 entry during an EMT, annexin A2 was immunoblotted. Interestingly, compared to control cells, HaCaT cells treated with TGFβ1 and EGF experienced increased

annexin A2 expression (Fig. 13B). Because this blot detected total AnxA2 expression from cell lysates and not just membrane-resident AnxA2, it is difficult to know whether AnxA2 impacted HPV16 entry during an EMT. Nonetheless, because AnxA2 expression increased, it is possible that if HPV entry was affected during an EMT, it was not because of changes to AnxA2.

We next used confocal microscopy to visually assess the interaction of HPV16 virions with keratinocytes in an EMT. HaCaT cells treated with TGFβ1 and EGF for 24, 48 and 72 h (shown in Fig. 14A, B and C, respectively) were exposed to HPV16 for 0 or 6 h, then fixed and stained to detect both HPV16 L1 and EMT markers (slug and vimentin). As shown already in Fig. 8, TGFβ1/EGF-treated cells expressed both slug and vimentin. This correlates with findings in the present experiment, where slug expression was detected at 24 h (Fig. 14A) and vimentin at 48 h (Fig. 14B). HPV16 binding and trafficking were similar in both control and EMT HaCaT cells after 24, 48 and 72 h of TGFβ1 and EGF treatment. Immediately after HPV infection, L1 was detected on the surface of cells (Fig. 14, left column). After 6 h, L1 accumulated in perinuclear regions (Fig. 14, middle and right columns). Thus, these data indicate that HPV is able to bind and enter GF-treated HaCaT cells, albeit at reduced levels compared to control cells.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Vimentin

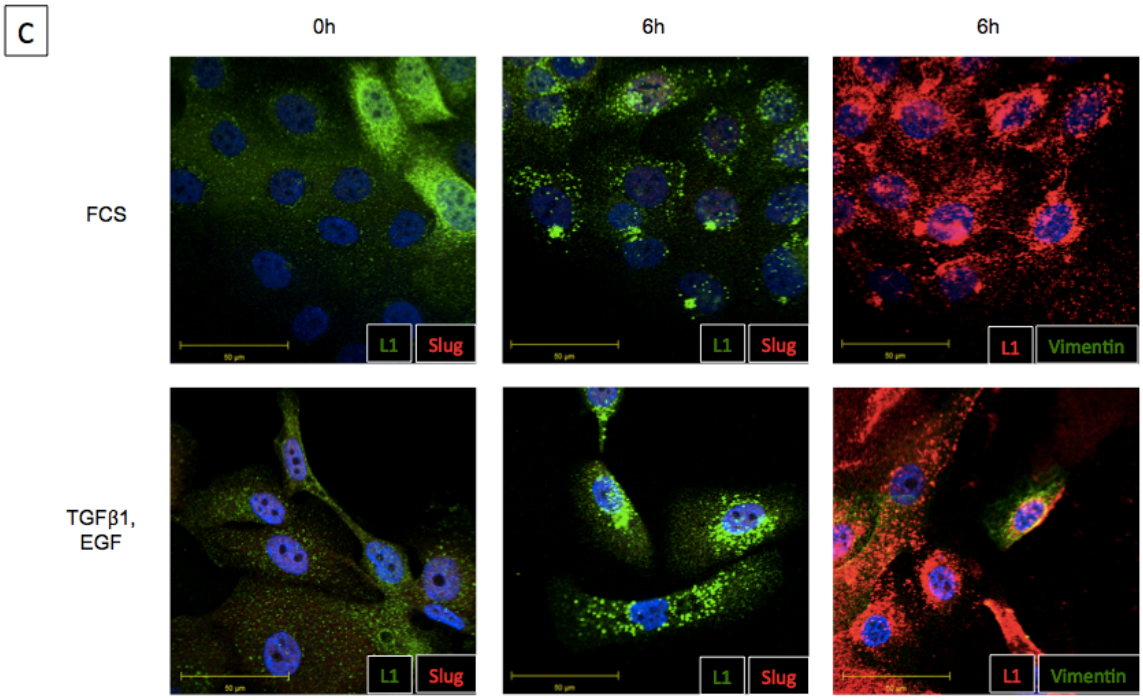

Figure 14: HPV16 entry into HaCaT cells is not prevented by TGFβ1/EGF-induced EMT. HaCaT cells induced into EMT with TGFβ1 and EGF (top), or kept in 10% FCS HaCaT media (bottom), were exposed to HPV16 PsVs (10,000 vge/cell) to visualize initial binding (0h) or entry (6h). Cells were kept in the indicated media for 24 h **(A)**, 48 h **(B)** or 72 h **(C)**. Cells were then permeabilized, fixed and stained for L1 and indicated EMT markers, slug or vimentin. HPV16 was detected with a mouse anti-HPV16 L1 monoclonal antibody (Millipore #CBL402; CamVir-1) and AF488-conjugated anti-mouse IgG secondary antibody. Slug was detected with a rabbit anti-slug monoclonal antibody and FITC-conjugated anti-rabbit IgG secondary antibody. Alternatively, HPV16 was detected using rabbit anti-sera to L1, then incubated with AF594-conjugated goat anti-rabbit secondary antibody. Vimentin was detected with a mouse anti-vimentin monoclonal antibody and DyLight-488-conjugated donkey anti-mouse IgG secondary antibody. Nuclei are stained with DAPI. Scale bar = 50 μM.

Taken together, these data suggest that there are post-entry blocks to HPV infection of activated keratinocytes. Presumably, this is due to a defect in viral trafficking or nuclear delivery.

6. HPV16 infection of HaCaT cells in an EMT resumes upon restoration of epithelial traits

Once the wound healing process is completed, mesenchymal cells can revert back to an epithelial phenotype by way of a mesenchymal-to-epithelial transition (MET) (131). The process of MET allows activated, migratory keratinocytes to cease migrating and resume epithelial duties, and can be modeled *in vitro* when EMT HaCaT cells are returned to cell culture media containing 10% FCS (92). To test whether EMT cells infected with HPV16-luc could express luciferase upon MET, HaCaT cells were induced into an EMT with TGFβ1 and EGF for 24 h, exposed to HPV16-luc under binding conditions, and then cultured in complete media for 48, 72 or 96 h. Control cells were maintained in normal growth media for the duration of the experiment. After measuring infection by luciferase assay, it was found that reversion of an EMT restores HPV16 PsV infection (Fig. 15A). Notably, luciferase expression increased over time, coincident with the reappearance of epithelial morphology (Fig. 15B). HPV16 infection of control HaCaT cells was much greater and diminished over time, possibly because contact inhibition prevented division as cells grew to confluence. These data indicate that the intracellular blockage to HPV16 infection of mesenchymal cells is reverted when MET occurs.

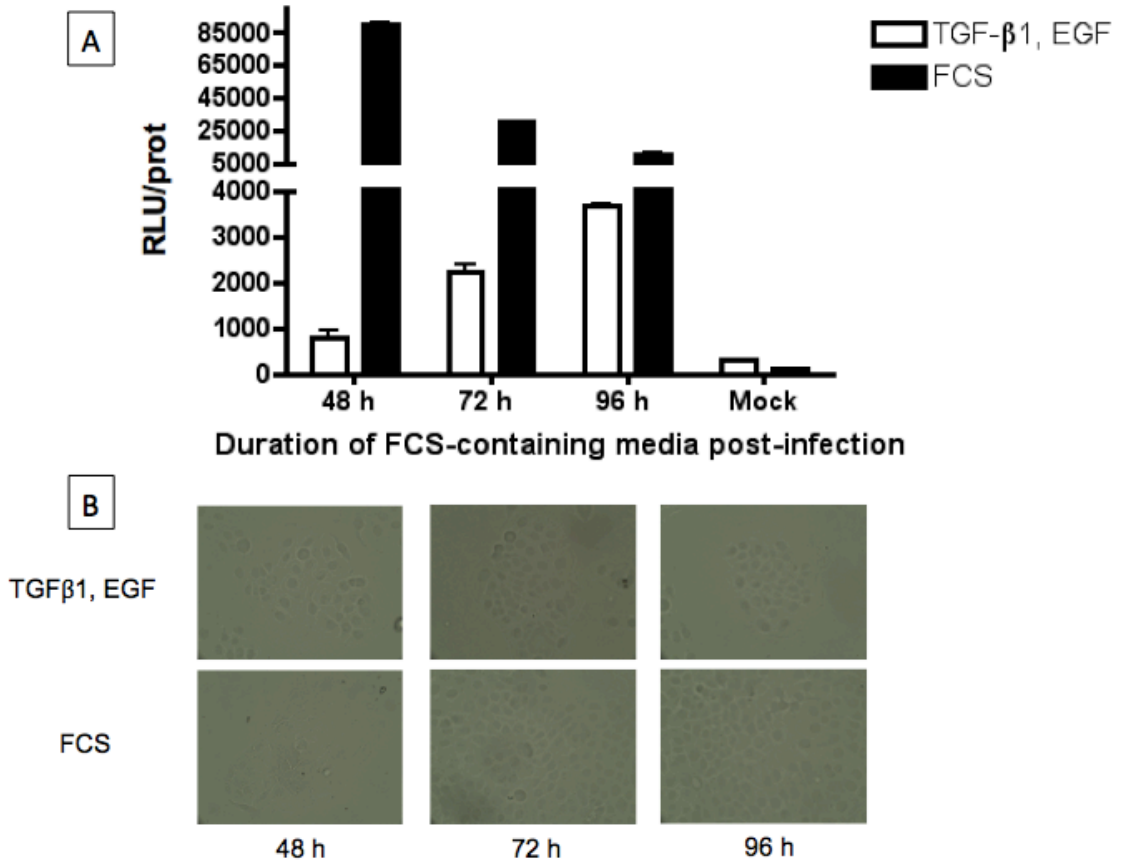

Figure 15: Reversion of an EMT restores HPV16 PsV infection. (A) After 24 h combination TGFβ1 and EGF treatment, HaCaT cells were exposed to HPV16-luc PsVs at 200 vge/cell, followed by return of 10% FCS media to bring about an MET. Infection was determined by luciferase assay after 48, 72 and 96 h in 10% FCS media, and is displayed in relative luciferase units per protein to standardize. HaCaT cells kept in 10% FCS media to maintain the epithelial phenotype for the duration of the experiment were likewise infected. **(B)** HaCaT cells induced into an EMT experienced a reversion of EMT traits upon incubation for 48, 72 and 96 h in complete, FCS-containing HaCaT media.

Discussion

Access to basal keratinocytes is a necessary step for HPV transmission and infection. Although microabrasions during sexual activity are thought to be necessary for the initiation of HPV16 infection, the molecular mechanisms that allow this to occur are unclear. Here, we developed an *in vitro* model of the wound environment and investigated if HPV infection is enhanced by epithelial injury and whether HPV infects migrating keratinocytes during the

reepithelialization phase of wound healing. To investigate the dynamics of HPV infection in this model system, HaCaT cells were induced to migrate by both scratch wounding and growth factor treatment. Initial attempts to develop a scratch wounding model were unsuccessful, but growth factor-induced epithelial-to-mesenchymal transition (EMT) of keratinocytes proved to be a useful tool for investigating HPV16 infection during this critical stage of wound healing. Upon induction of EMT, HPV16 infection does not occur (Fig. 6, 10, 11A). HPV16 binding to keratinocytes during EMT is reduced compared to control cells maintaining the epithelial phenotype (Fig. 12A), and viral entry appears to take place (Fig. 13A, 14). Most interestingly, however, is that keratinocytes undergoing an EMT regain the ability to support HPV16 infection during later stages of wound healing, namely the reversion of EMT through a mesenchymal-to-epithelial transition (MET). This leads us to propose an HPV infection model of keratinocytes undergoing an EMT, wherein infection is blocked when keratinocytes migrate and acquire a mesenchymal phenotype but resumes upon restoration of the epithelial phenotype through MET (Fig. 16).

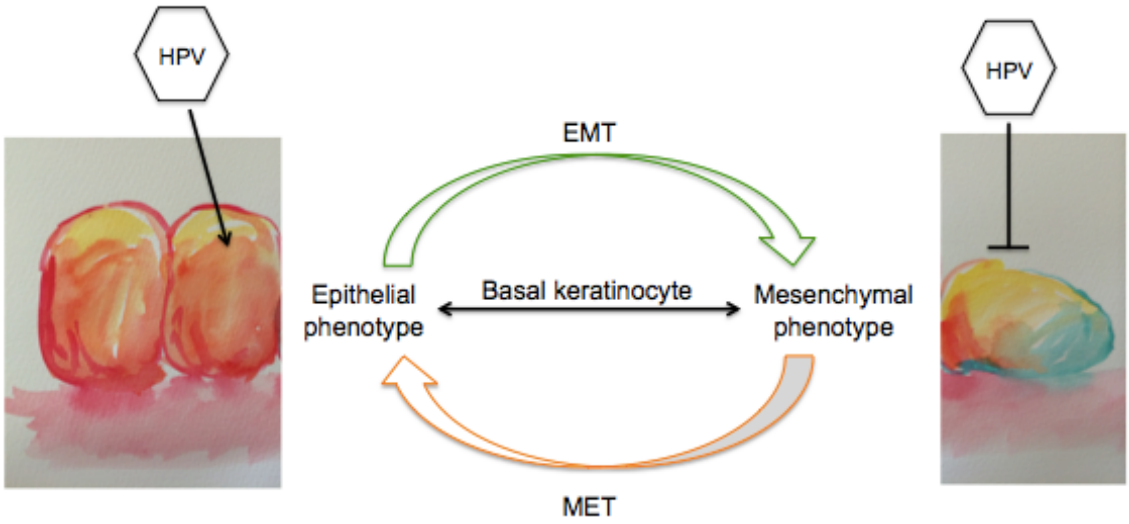

Figure 16: Proposed model of HPV16 infection of keratinocytes transitioning between epithelial and mesenchymal phenotypes during wound healing. HPV16 infects keratinocytes displaying the epithelial phenotype, but is incapable of infecting keratinocytes induced to migrate through an EMT. Because EMT is reversed by MET during late stages of wound healing, HPV16 infection of migrating keratinocytes takes place when MET occurs and epithelial characteristics are restored.

The loss of HPV16 infection during an EMT can be explained, in part, by reduced binding to the cell surface (Fig. 12A). In virology, the ability of virions to bind the cell surface is a reflection of cellular susceptibility, meaning whether or not a given cell expresses viral attachment factors on the cell surface. It is peculiar that binding was reduced during EMT because HPV16 attachment factors like syndecan-1 – which display heparan sulfate (HS) chains – are reported to have increased expression by cells at the wound margin (54, 90). In our study, however, HS expression was altered in EMT cells compared to control cells (Fig. 12B), which could contribute to reduced binding of HPV16. But due to the inherent unreliability of HS antibodies for immunoblotting, it was difficult to compare HS expression between control and EMT HaCaT cells conclusively. Nonetheless, the finding that HPV16 bound to HaCaT cells in an EMT, even though it was reduced compared to control cells, indicates that these cells retained susceptibility.

HPV16 appeared to enter keratinocytes during an EMT. While HPV was observed by confocal microscopy to enter both control and EMT HaCaT cells (Fig. 14), other data was not as convincing (Fig. 13A). Using microscopy, HPV localized to the cell periphery under binding conditions and after 6 h appeared to enter both control HaCaT cells as well as HaCaT cells in an EMT (Fig. 14). In timing and location, HPV16 trafficked to perinuclear regions in HaCaT cells in an EMT, which corresponds with experiments by Day *et al.*, who observed HPV16

trafficking to the trans-Golgi network after 6 h in HaCaT cells (44). Without staining for the plasma membrane, however, it is unclear if HPV16 PsVs truly did enter into HaCaT cells during EMT. Attempts were made to image HPV entry during EMT by performing triple staining during confocal immunofluorescence, using wheat germ agglutinin to stain the plasma membrane and immunolabeling to stain HPV16 PsVs and slug (data not shown). Unfortunately, due to technical issues this experiment was unsuccessful. Moreover, LAMP1, a lysosomal marker that HPV16 has been shown to colocalize with during trafficking (26), was stained and imaged in HaCaT cells induced into an EMT. LAMP1 expression and HPV16 trafficking exhibited similar patterns of cellular localization during EMT (data not shown). This supports the hypothesis that HPV16 enters HaCaT cells undergoing an EMT, but additional experiments are necessary.

The trypsin-entry assay revealed that HPV16 was similarly affected by both HaCaT cells treated with TGFβ1 and EGF and control cells. A limitation to the trypsin-entry assay is that HPV adhering to ECM components may have been present, despite attempts to remove surface-resident virions. However, because L1 was absent from samples harvested by trypsin immediately post-binding, it lends credence that the L1 (and its degradation product) observed after 4 h underwent internalization.

We initially hypothesized that HaCaT cells undergoing an EMT would support HPV16 infection, but to our surprise experimental results confirmed that HPV16 infection did not occur when HaCaT cells were induced into an EMT phenotype (Fig. 6, 10, 11A). Although scratch wounding of keratinocytes has

proven to be a successful model for other viruses, we were unable to translate this model to HPV. In one study, scratch wounded keratinocytes were infected by herpes simplex virus type 1 (HSV1) at wound margins while intact regions of cells were uninfected (159). This was observed by immunolabeling an early HSV1 gene product. Using antibodies to HPV early proteins is not possible because HPV early proteins are expressed at low levels and also have low immunogenicity. As an alternative, we used HPV16 pseudovirions (PsVs) encapsidating a reporter genome as a surrogate of infection. However, the scratch wounding model was revealed to be unsuitable for testing HPV PsV infection, as cells closed the wound margin during the lengthy time required to achieve robust marker gene expression (Fig. 7A).

We predicted HPV16 infection of scratch wounded HaCaT cells to increase compared to unscratched control cells, but instead there was no appreciable difference (Fig. 6A) and infection at the wound edge, where cells migrated through an EMT (Fig. 7), was unapparent (Fig. 6B). In the scratch assay, growing cells to confluency just prior to scratch wounding was necessary to synchronize the cell cycle, but this could have prevented HPV PsV infection because an active cell cycle is critical to HPV16 infection establishment (146). If cells were subconfluent prior to wounding it would have been difficult to discern whether infection occurred in wound margin cells or the expanding colonies of dividing cells. Additionally, reproducibility in wounding subconfluent cells is difficult and highly variable. Since scratch assays generate cell migration, and migration and division are mutually exclusive amongst keratinocytes, it is

possible that HPV16 infection was impeded on two fronts: 1) lack of mitotic activity amongst non-wounded, contact-inhibited cell regions, and 2) lack of mitotic activity in migrating cells at wound margins.

Further validation for the lack of HPV16 infection of HaCaT cells in an EMT was found when cells induced into EMT with TGFβ1 and EGF did not support infection while control cells were readily infected (Fig. 10). Because J2 murine fibroblasts, which display mesenchymal characteristics, were successfully infected by HPV16 (Fig. 11B), this suggests that HPV16 infection of keratinocytes was hindered not by the expression of mesenchymal genes but instead by the collective changes that happen during EMT, when epithelial proteins are down-regulated.

The discovery that keratinocytes in an EMT are not infected by HPV16 is supported by other research, where epithelial cells in an EMT no longer support viral infections. Using a model of measles virus infection with a lung adenocarcinoma cell line, Shirogane *et al.* found that infection was nullified in the presence of an EMT (169). In this instance, infection was prevented because viral entry factors were epithelial cell markers that were lost during EMT. Likewise, Lacher *et al.* found that oncolytic adenoviruses, which lyse cancer cells upon entry using the Coxsackie and Adenovirus Receptor (CAR) – a tight junction protein and epithelial marker – were non-infectious when CAR expression was lost during EMT (106). Furthermore, Strauss *et al.*, also using an oncolytic adenovirus, discovered that infection of ovarian cancer cells occurred only when cells exhibited the epithelial phenotype, but not during EMT (179).

These studies demonstrate that viral infection can be impacted by EMT through altered expression of viral entry receptors. Although the putative entry receptor for HPV is unknown, if it turns out to be an epithelial marker it may explain why HPV16 was not infectious for HaCaT cells during EMT. However, compared to these other viruses, the block in HPV infection during EMT appears unique because of the evidence that HPV entry may have occurred. Alternatively, because EMT during wound healing is a partial event, some HPV internalization machinery may be retained and functional at the cell surface, which would account for the subset of HPV particles that appeared to enter keratinocytes during EMT.

Given the promiscuous nature of HPV entry into various cell types and cell lines, it is plausible that HPV enters keratinocytes in an EMT. HPV16 readily enters non-keratinocyte and non-human cell lines, including immune cells (20, 35, 56, 117), HEK 293T kidney cells (146), cancer cells (44, 114) and even spermatozoa (59, 65), as well as COS-7 cells (66), NIH 3T3 murine fibroblasts (strain J2) (Fig. 11B), Chinese hamster ovary (CHO) cells (180), pgsA-745 cells (41), cells of the murine female genital tract (88, 97, 151) and cells of the rhesus macaque female genital tract (152). If HPV were incapable of entering HaCaT cells during an EMT, it implies that EMT compromised the viral entry machinery. Furthermore, several molecules implicated in HPV entry of epithelial HaCaT cells are expressed during an EMT. EGFR (180) and AnxA2 (51) are implicated HPV internalization and are expressed during EMT (Fig. 9A, 13B). Further

experiments are needed to ascertain whether EGFR and AnxA2 are functionally expressed for HPV16 entry in the GF-induced EMT model.

Additional attempts were made to test whether HPV16 entered HaCaT cells in an EMT, including labeling the capsid with an entry marker. CFDA is a molecule that can be attached to L1 and, upon crossing the plasma membrane and exposure to cytoplasmic enzymes, is cleaved and emits a fluorescent signal that is measurable by flow cytometry (50). Although preliminary experiments showed that CFDA-labeled HPV16 entered control and EMT HaCaT cells (data not shown), follow-up experiments are needed to confirm these preliminary results.

In summary, the scratch wounding model of HPV16 infection of cell monolayers proved unsuccessful and there appears to be a transitory loss of HPV16 infection when keratinocytes undergo an EMT during wound healing. Because models of HPV infection are oftentimes reductionist, only a single cell type is evaluated, which may or may not be physiologically relevant. In the wound environment, where HPV encounters myriad cell types, dividing keratinocytes are believed to be the sole cells in which HPV can establish infection. Although HPV PsVs can still bind and possibly enter keratinocytes in an EMT, they cannot transduce marker gene expression until keratinocytes revert back into the epithelial phenotype through MET. If keratinocytes displaying mesenchymal features support productive HPV infection, a heretofore unknown reservoir of tropic cells has been overlooked in *in vitro* models of HPV infection.

CHAPTER 3: DEVELOPING A TISSUE-BASED MODEL OF HPV16 EARLY INFECTION USING ORGANOTYPIC 'RAFT' TISSUE CULTURE

Abstract

The contribution of epithelial wounding is an understudied aspect of HPV infection models. We used organotypic "raft" culture, a model that recapitulates the structure and function of the differentiating, stratified epithelium, to evaluate the role of wounding on HPV infection. Raft tissues were generated using multiple cell types, including NIKS, HaCaT cells and immortalized cells of the female genital tract (FGT). Epithelial markers were evaluated by immunohistochemistry analyses to verify proper tissue differentiation and stratification. While all of the cell types evaluated produced epithelial tissue, analysis of proliferation and differentiation markers indicated that NIKS cells most faithfully produced fully differentiated epithelial tissue. NIKS-derived raft tissue was injured by scratch wounding with a scalpel blade and then exposed to HPV16 PsVs transducing a luciferase reporter genome. For the first time, raft tissue grown from these HPV-negative keratinocytes was infected by HPV16 PsV. This marks the advent of a tissue-based model of HPV early infection, which may potentially be used to evaluate inhibitors of HPV infection of keratinocytes assembled into tissue.

Introduction

Several experimental systems exist to study HPV infection, including cell monolayer, tissue and animal models of infection. Animal models indicate that epithelial disruption is a necessary event for the initiation of papillomavirus (PV) infection (31, 32, 63, 88, 97, 151, 152, 155). However, as described in Chapter 2, epithelial disruption of confluent HaCaT cell monolayers revealed that scratch wounding does not appreciably enhance HPV16 infection. Because animal models of infection demonstrate that epithelial disruption is required for PV infection, but *in vitro* scratch wounding models do not support this notion, there is a need to reconcile this disparity to gain a better understanding of the contribution of epithelial wounding to HPV infection.

Tissue-based models are an appealing platform to study how wound healing impacts HPV infection because they represent a compromise between cell monolayers and animal models of infection. The organotypic "raft" culture system grows fully differentiating and stratified epithelial tissue, which recapitulates the tissue biology and 3D architecture of physiological epithelium (53, 135). Raft culture is named for the manner in which tissue is developed: by placing undeveloped tissue equivalents on a metal grid at the air-liquid interface and allowing them to grow into full thickness epithelium over a period of days to weeks. Epithelial raft cultures differentiate and stratify as media and nutrients diffuse upwardly, similar to epithelial tissue development *in vivo*.

Raft cultures are useful for studying wound healing (53) and how individual growth factors and their receptors influence reepithelialization in full thickness epithelium. The reepithelialization phase of wound healing restores the

keratinocyte-derived epithelial surface, as keratinocytes migrate and divide in response to inflammatory signals generated from epithelial trauma (102, 156). A distinct advantage of raft culture over cell monolayers for studying wound healing is the presence of underlying dermal tissue, which allows for fibroblast-keratinocyte interactions (101, 197). Physiological wound healing is modulated by bidirectional crosstalk, or paracrine signaling, between fibroblasts and keratinocytes in the dermis and epithelium, respectively (182, 197). Upon injury, fibroblasts secrete chemotactic factors that prompt keratinocytes to migrate (164, 165, 182, 197). The same wound ligands used in Chapter 2 to induce migration and epithelial-to-mesenchymal transition (EMT) in HaCaT cells, TGFβ1 and EGF, have been studied in wounded raft tissue. Rafts that were scratch-wounded and treated with TGFβ1 experienced altered reepithelialization because TGFβ1 simultaneously promotes keratinocyte migration but inhibits proliferation (64). EGFR, a master regulator of keratinocyte division, migration and survival, has been found to be a key contributor to wound healing in raft culture (124). Furthermore, raft culture can be used to study EMT (192).

Rationale and study hypothesis

Raft cultures have been used to study HPV biology, but these studies focus upon events in HPV replication after infection has been established. By growing raft cultures from HPV-positive keratinocytes, raft cultures have been instrumental for studying HPV-induced tumorigenesis (120, 134), which is not possible in cell monolayer models of HPV infection. Importantly, raft cultures

grown from HPV-positive keratinocytes produce infectious virions because they satisfy differentiation-dependent HPV replication (48, 123). This indicates that cells grown using raft culture support HPV gene expression in a spatiotemporal manner, making raft culture a powerful and physiologically relevant system to study HPV biology. Although raft culture has been adapted to study HPV infection – from infected basal cells up to progeny virion formation in granular and cornified cells – it is unknown whether raft culture similarly permits early events in HPV infection, such as infection establishment post-wounding.

Epithelial wounding is an understudied component of HPV16 infection *in vivo*. In an attempt to merge the physiological relevance of animal models of HPV16 infection with reductionist approaches, a model of HPV16 early infection was developed using organotypic "raft" culture. To test the hypothesis that physical disruption of epithelium grown using raft culture permits HPV16 infection, tissue equivalents from various keratinocyte cell lines were grown, characterized and subjected to wounding and infection.

Materials and Methods

Cell lines

Normal immortal keratinocytes (NIKS) cells are a non-tumorigenic, spontaneously immortalized cell line (4). NIKS cells were grown in monolayer cell culture in the presence of mitomycin C-treated J2 3T3 mouse fibroblast feeder cells with E. medium containing 10% FBS and 100 U/mL nystatin (Sigma-Aldrich) as previously described (133). HaCaT cells (a gift of Nobert Fusenig, DKFZ) are a non-tumorigenic, spontaneously immortalized human keratinocyte cell line derived from skin near the distant periphery of a melanoma on the back of a 62-year old male (19). They were maintained in DMEM/Ham's F-12 medium (Irvine Scientific), supplemented with 10% FBS (Invitrogen), amino acids (Invitrogen) and 1X glutamine-penicillin-streptomycin (GPS) (Invitrogen).

Growing organotypic "raft" culture
To grow epithelial tissue in a raft culture, a dermal equivalent, composed of collagen and fibroblasts and collagen, was first created. J2 murine fibroblasts were mixed with type I rat tail collagen (BD-Biosciences) and reconstituted in media and buffer containing $NaHCO_3$, HEPES and NaOH. The dermal equivalent, prepared on ice to prevent polymerization, was placed in 6-well plates and incubated at 37°C until it solidified. Various epithelial cell lines were then seeded at 3e6 cells/raft atop the dermal equivalent and grown to confluence, remaining submerged in cell media. Using sterile technique, the rafts were lifted and placed on top of stainless metal grids at the air-liquid interface. Media was added up to the level of the metal grid and, by capillary action, rafts received nutrients, serum and other factors. Rafts were grown to days 13-16, and media was changed every other day with care taken to keep the epithelial surface dry so as not to disrupt the bottom-to-top diffusion that generates epithelial differentiation and stratification.

Tissue sectioning, immunohistochemistry (IHC) and immunofluorescence (IF)
To harvest raft tissues for immunohistochemical analyses, raft tissues, including the dermal equivalent, were fixed in 4% formalin, then paraffin-embedded and cut into 4 μm-thick cross-sections. Tissue sections were deparaffinized in xylene, then rehydrated using a series of alcohol rinses, then endogenous peroxidase activity quenched using 3% H_2O_2 in methanol.

Infections
Upon reaching day 14 of growth post-lift, rafts were scratch-wounded with a fresh scalpel blade to introduce a total of 8 cross-hatched wounds per raft, then infected with 2e9 vge/raft HPV16-luc PsVs for 4 days. Control rafts were left intact and undamaged. HPV16 PsV were diluted in 50 μL of complete cell media (10% FCS) then applied directly atop the raft epithelial surface. Mock-infected rafts received only complete cell media. After infection, rafts were incubated at 37°C for 4 d. Typically, raft media contains octanoic acid (C8:0) to stimulate differentiation (87). During the 4-day infection period, however, EGF was substituted for C8:0 to promote proliferation over differentiation. Concurrently, 1e5 J2 cells were seeded below raft grids post-infection to provide additional fibroblasts and augment fibroblast-keratinocyte cross-talk. Infection was evaluated by luciferase assay. To perform the luciferase assay, raft tissue was processed by detaching the keratinocyte-based epithelium from the dermal plug using tweezers. Collected tissue was digested overnight in 5X (0.25%) trypsin at 4°C to disrupt cell-cell contacts. Samples were then centrifuged for 30 seconds with a bench microfuge to collect cell pellets. Cell pellets were washed in PBS and spun again, after which they were subjected to the luciferase assay using the same luciferase assay kit as in assays of monolayer HPV16-luc infection. Infection was standardized against protein content, which was determined by Bradford assay.

Results

1. Fully stratified, differentiating epithelial tissue is grown in organotypic "raft" culture from NIKS cells and cells of the female genital tract, but not HaCaT cells

Organotypic "raft" culture is a technique that generates stratifying squamous epithelium *in vitro* (53, 64, 135). As described in Chapter 1, "Characteristics of Epithelial Tissue," stratifying epithelium is produced from basal cell amplification, upward migration and differentiation into distinct layers of cells. Each layer of epithelial tissue performs unique functions and can be characterized by differentiation markers (Fig. 17). Basal cells express proliferation markers such as Ki67 (2), in addition to keratins-5, -14 and -15 (18) and tight junction proteins (29, 144). Suprabasal and spinous cells express keratins-1 and -10 (136) and small amounts of involucrin, which is predominantly expressed by cornified cells (27, 52). Epithelial markers like E-cadherin are expressed throughout epithelial strata (209).

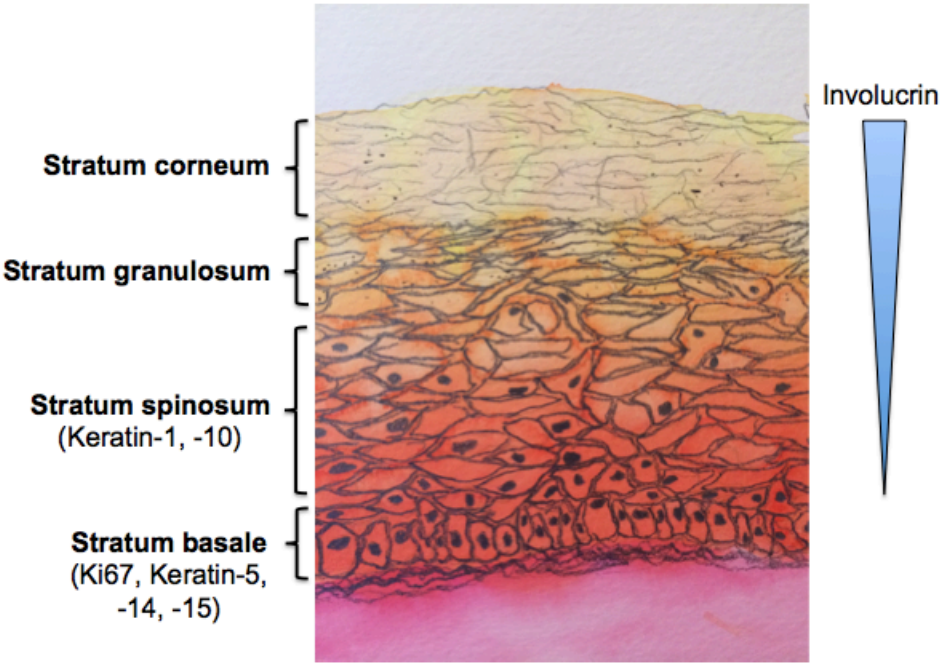

Figure 17: An illustration of keratinocyte differentiation and stratification into epithelial tissue. Basal cells in the stratum basale adhere to the basement membrane and undergo tightly regulated proliferation. Upon detachment from the basement membrane, differentiation occurs as cells join the stratum spinosum, then the stratum granulosum and finally the stratum corneum. Epithelial cells in the stratum corneum are continually shed and replaced by upwardly differentiating cells from below. The stratum basale, stratum spinosum, stratum granulosum and stratum corneum are commonly known as the basal layer, spinous layer, granular layer and cornified layers, respectively. Original artwork by M. Kivitz.

To determine the suitability of different sources of keratinocytes for evaluating tissue wounding in the context of HPV16 infection in the raft culture system, rafts were established using normal immortal keratinocytes (NIKS), HaCaT cells and keratinocytes of the female genital tract (FGT). NIKS cells are an HPV-negative, non-tumorigenic, spontaneously immortalized cell line that produces stratified squamous epithelium in organotypic culture (4). HaCaT cells, which are also HPV-negative, are a common epithelial cell line used to study HPV infection *in vitro* (26, 51, 138, 172, 180), but can also be used to make rafts (19, 163). Cell lines established from FGT tissue, including vaginal, ectocervical and endocervical keratinocytes, were previously immortalized by the HPV16 oncoproteins E6 and E7 and display tissue-specific patterns of differentiation and stratification (58). Using each of these cell types, raft tissue was generated and characterized for markers of epithelial differentiation.

Upon generating NIKS-derived rafts, fixed sections were prepared and stained. Hematoxylin and eosin (H&E) staining revealed fully stratified epithelial growth and prominent cornification (Fig. 18A). Multiple epithelial layers were distinguishable by light microscopy using immunohistochemistry (IHC) staining, but high background was apparent when this H&E-stained tissue was viewed by confocal fluorescence microscopy (Fig. 18B). Involucrin staining indicated that

appropriate differentiation occurred, as basal cells lacked involucrin but upper epithelial strata steadily increased its expression (Fig. 18C). E-cadherin was robustly detected by IHC and light microscopy (Fig. 18E); by fluorescence microscopy, however, E-cadherin was largely indistinguishable (Fig. 18F). Finally, Ki-67 was present only amongst basal cells (Fig. 18D). Collectively, these data demonstrate the feasibility of NIKS cells in organotypic "raft" culture to produce fully differentiating epithelial tissue *in vitro*.

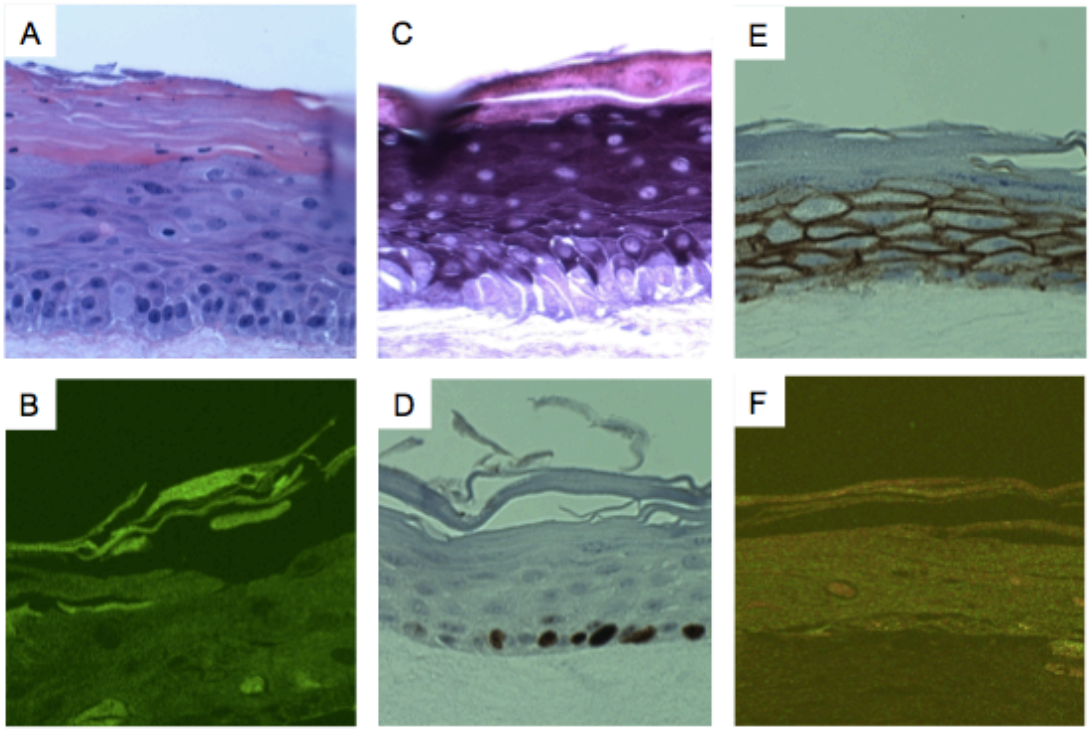

Figure 18: NIKS cells form complete epithelial tissue equivalents and display differentiation markers when grown in organotypic "raft" culture. (A) H&E by light microscopy. **(B)** H&E by immunofluorescent microscopy. **(C)** Involucrin staining by light microscopy. **(D)** Ki-67 staining by light microscopy. **(E)** E-cadherin staining by light microscopy. **(F)** E-cadherin staining by immunofluorscent microscopy. Images are shown at 400X magnification.

NIKS-derived raft culture was further characterized by staining the keratin cytoskeleton. To identify basal and suprabasal epithelial strata, keratin-14 (K14)

and keratin-1 (K1), respectively, were stained in wounded and intact raft tissue. Upon sectioning and IHC staining, K14 expression was restricted to the basal layer, while K1 was expressed by differentiating, suprabasal strata (Fig. 19).

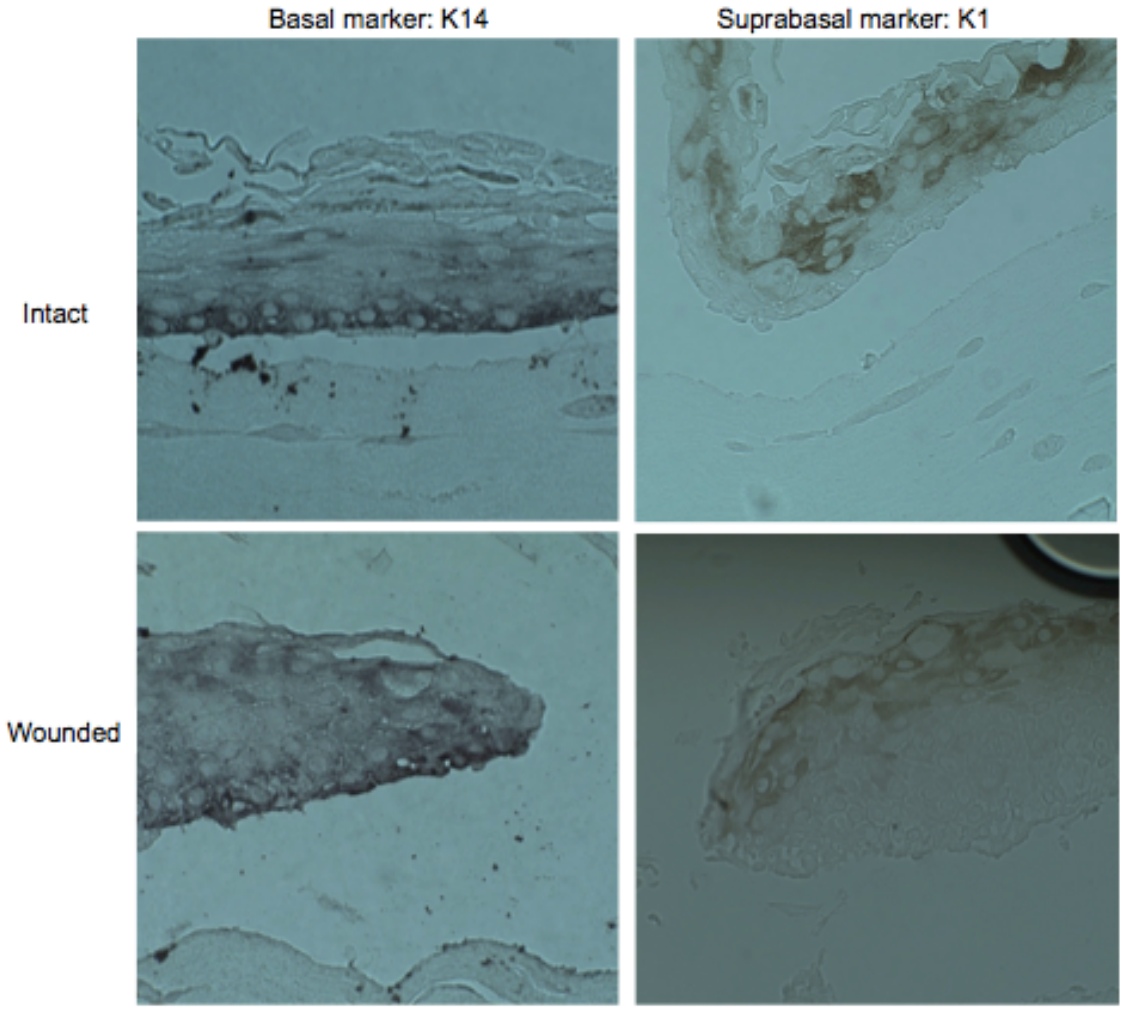

Figure 19: Intact and wounded raft tissue stained for keratin markers. NIKS-derived rafts, grown to day 14 as in Fig. 18, were wounded or left intact, then fixed, sectioned and stained for keratin markers using IHC. Keratin-14, a basal cell marker, and keratin-1, a marker of differentiation, were stained in intact and wounded tissue. Images are shown at 400X magnification.

While NIKS cells exhibited prominent differentiation and stratification (Figs. 18, 19), HaCaT cells grown under similar conditions in raft culture displayed limited differentiation and stratification (Fig. 20). A basal layer was

evident, but cells did not appear to differentiate beyond the spinous layer and cornification was not evident. Because HaCaT cells did not accommodate tissue growth in organotypic culture, they were abandoned as a tissue-based model of HPV16 infection.

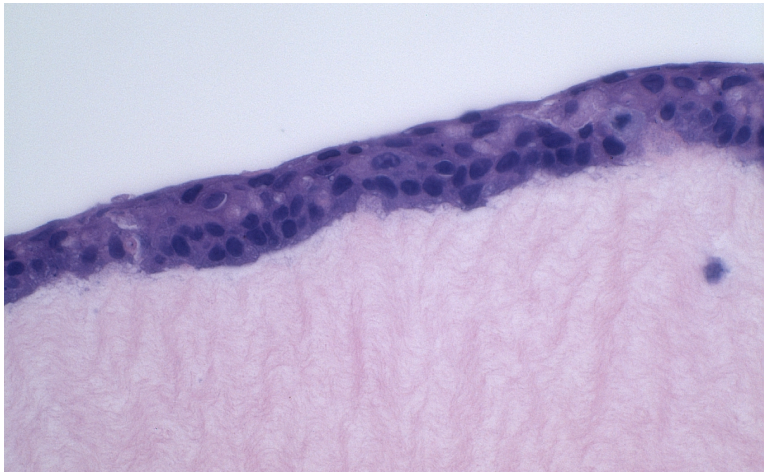

Figure 20: HaCaT cells grown in organotypic "raft" culture display limited differentiation and incompletely stratify. Tissues were harvested, fixed and sectioned on day 14 post-lift, then H&E-stained and visualized by light microscopy. Image is shown at 400X magnification.

To assess the ability of FGT cell lines to develop organotypic tissue, vaginal keratinocytes (VK2), ectocervical keratinocytes (Ect1) and endocervical keratinocytes (End1) were seeded in organotypic culture and grown to day 14. These cells lines were immortalized by transduction with a lentiviral vector stably expressing HPV16 E6 and E7 (58, 72). *In vivo*, both vaginal and ectocervical keratinocytes produce stratified squamous epithelium while endocervical keratinocytes assemble into simple columnar epithelium (198). Interestingly, fully differentiated tissue was generated from all three types of cells of the FGT (Fig. 21). Only vaginal (Fig. 21A) and ectocervical (Fig. 21B) keratinocytes were expected to differentiate and stratify, but endocervical keratinocytes also

displayed multilayered tissue growth (Fig. 21C). Cornification was apparent in rafts derived from vaginal keratinocytes (Fig. 21A), which is interesting because these cells are of mucosal origin and therefore expected to lack a cornified layer. The multilayered epithelium grown from all three FGT cell lines is possibly a reflection of tissue-growth at the air-liquid interface, outside of the physiological mucosal environment. Or, it could be a result of E6 and E7 expression in FGT cells that caused tissue to grow as observed and not as was anticipated based upon their physiological behavior.

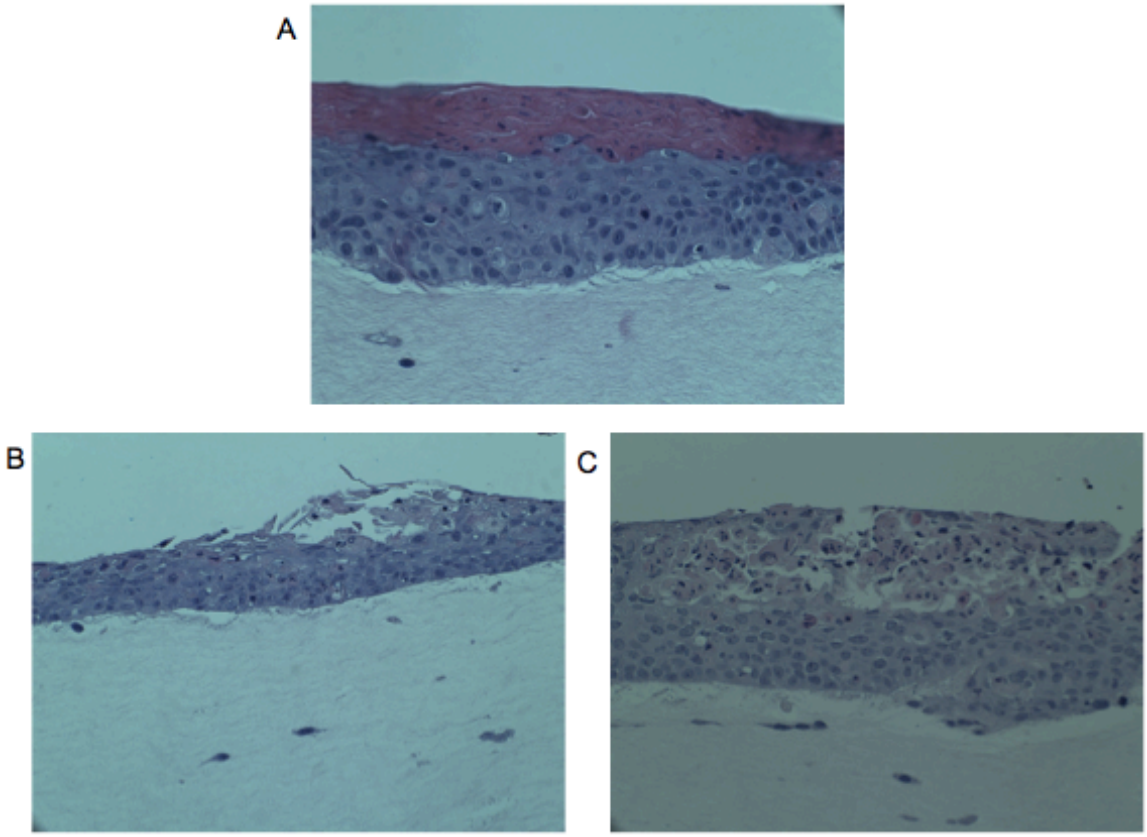

Figure 21: Growth of epithelial tissue from various cells of the female genital tract (FGT). Rafts were prepared from FGT cells, which have previously been immortalized with HPV16 E6 and E7 oncoproteins (58), and are termed VK2 (vaginal keratinocyte-2) **(A)**, Ect1 (ectocervical keratinocyte-1) **(B)** and End1 (endocervical keratinocyte-1) **(C)**. Rafts were grown to day 14, then fixed, sectioned and H&E-stained for visualization by light microscopy. Images are shown at 200X magnification.

Because NIKS cells exhibited differentiation and stratification that most resembled squamous epithelium, and because they are HPV-negative, NIKS-derived rafts were utilized for subsequent wounding and HPV16 infection studies.

2. NIKS-derived raft epithelial tissue supports HPV16 PsV infection upon scratch wounding

To study HPV16 infection in wounded epithelial tissue, NIKS-derived rafts were again generated. HPV16-luc was used to infect rafts that had been wounded using a scalpel or, as a control, intact rafts. Wounding was performed by applying 8 cross-hatched scratches to raft surfaces with a fresh scalpel blade (Fig. 22A). Subsequently, wounded or intact rafts were challenged with HPV16-luc by applying the inoculum directly atop the raft surface.

After HPV16-luc inoculation, raft media was altered to create a more favorable environment for infection. When HPV16 was introduced to intact tissue, no luciferase signal was detected above background levels, while infection was readily detected in scalpel-wounded raft tissue (Fig. 22B).

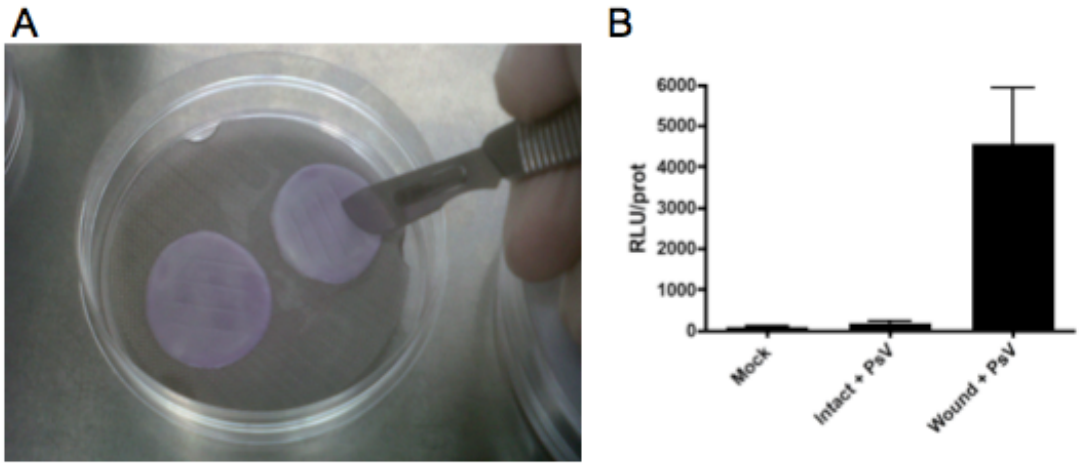

Figure 22: HPV16 infection of scratch-wounded raft tissue made from NIKS cells. (A) Picture of NIKS-derived raft tissue, injured on day 14 post-lift by scratch-wounding with a fresh scalpel blade. **(B)** Organotypic "raft" cultures derived from NIKS cells grown to day 14 were scratch-wounded with a scalpel blade, then subjected to HPV16-luc infection, using 2e9 vge/raft. During infection, EGF was substituted for C8:0 to stimulate epithelial proliferation and minimize differentiation, and J2 cells were seeded below the raft grid to augment fibroblast activity and promote keratinocyte-fibroblast interactions. Infection proceeded for 4 days, after which epithelial tissues were separated from dermal plugs, trypsinized, then analyzed by luciferase assay. Control rafts included mock-infection, as well as rafts left intact yet still exposed to HPV16-luc PsVs; N=4.

In parallel experiments incorporating epithelial disruption and HPV16-luc PsV infection, NIKS-derived rafts were injured by chemical disruption with the nonionic detergent nonoxynol-9 (N9), as was performed in a mouse model of HPV16 infection (151), and then challenged with HPV16-luc PsV. Counter to the finding that scratch-wounded rafts support HPV16 infection, rafts injured by N9 gave infection levels comparable to mock infection (data not shown). It is possible that N9 compromised cellular integrity, nullifying infection because cells were damaged beyond viability. This finding indicates that NIKS-derived raft tissue can withstand physical injury by scratch wounding and still support HPV16 PsV infection, but that chemical injury using N9 causes cellular damage that precludes HPV16 PsV infection.

Discussion

For the first time, organotypic “raft” cultures were infected by HPV16. In contrast to prior studies of HPV replication using raft culture, which evaluated late events in HPV replication using HPV-positive keratinocytes, our study examined how epithelial wounding impacts HPV infection establishment of raft tissue developed from HPV-negative keratinocytes. Rafts were grown using NIKS cells, HaCaT cells and cells of the female genital tract (FGT), but NIKS-based rafts exhibited the best growth and were used to study wounding and HPV infection establishment. Wounding with a scalpel blade was observed to be necessary for HPV16 infection, as unwounded, intact rafts were uninfected (Fig. 22). This is the first instance of fully differentiating, stratified epithelial tissue being capable of supporting early events in HPV infection. The fact that HPV infection occurred in wounded raft tissue indicates that post-wounding, virions properly bound to exposed attachment factors, entered susceptible keratinocytes, trafficked to the nucleus and achieved expression of virally encapsidated genes. While this does not tell us which cells within the differentiating, multilayered epithelium support infection, it does reveal that there are tropic cells present in raft tissue capable of being infected by HPV.

There are several implications to the finding that raft tissue supports HPV infection. First, it is now possible to evaluate HPV infection establishment outside of the *in vivo* context, where a variety of confounding variables may impact infection. In animal models, it is possible that HPV infection is influenced

by physiological responses to epithelial wounding, including inflammation, hormonal fluctuations and systemic immune responses. Because tissue grown using raft culture is composed solely of a dermal and epidermal equivalent (135), it is devoid of the vasculature and immune components that contribute to physiological wound healing (168, 170) and microbial immunity *in vivo* (96, 104). Moreover, raft tissue lacks the hormonal profile of the female genital tract which, by progressively thickening and thinning in response to hormonal changes, potentially impacts susceptibility to HPV infection. Animal studies of HPV infection commonly use hormones (e.g. Depo-Provera) to thin the female genital tract epithelium and augment infection (88, 151, 152). Because wounded raft tissue supports HPV infection, it indicates that access to tropic keratinocytes is a critical determinant of infection and that, post-wounding, the contributions of vasculature, hormones, inflammation and immunity are expendable to infection.

The second implication of the raft wounding and HPV infection model is the potential for prophylactic drug discovery, using raft culture as a tractable system to study HPV infection inhibition. Raft culture is already used to test therapeutic medications for established HPV infections, but also for other pathogens, including herpesviruses, adenoviruses, parvoviruses and poxviruses (6). With the advent of the raft wounding and HPV infection model described in this report, preventative medications may be tested in organotypic culture to curb novel HPV infections. There is precedent for this, as raft culture grown from vaginal epithelial cells has been used to test tissue responses to candidate microbicides like nonoxynol-9 (81). Using raft culture to test inhibitors of HPV

infection would allow microbicides to be gauged for toxicity, dosing and effectiveness in susceptible tissue prior to testing in animal models. Also, compounds shown to block HPV infection of HaCaT cell monolayers may be similarly evaluated in raft tissue, such as KGFR and EGFR inhibitors (180). Because KGFR expression in epithelial tissue is differentiation-dependent and begins at the spinous layer, if rafts in treated with a KGFR inhibitor supported HPV infection it would provide circumstantial evidence that basal cells were infected in a tissue-based model.

A limitation of this study is that the cells used to grow raft culture have varying degrees of physiological relevance to HPV infection. NIKS cells, which support HPV infection (70), are derived from normal human neonatal foreskin keratinocytes (4), making them pertinent for studying genital HPV infection. HaCaT cells are commonly used to study cell monolayer HPV infection, but they were isolated from adult epidermal tissue at the periphery of a malignant melanoma (19), which is related to, but different from, male and female genital epithelium. HaCaT cells readily support HPV infection as a monolayer, but they exhibited limited stratification (Fig. 20) and therefore were not used to evaluate tissue-infection by HPV. Although prior studies have shown that HaCaT cells can successfully develop into differentiating epithelial tissue (19, 163), we were unable to reproduce these results. It is possible that HaCaT cells needed more dermal fibroblasts to properly differentiate and stratify (163).

Raft epithelial cultures generated from cells of the FGT, including vaginal, ectocervical and endocervical cells, appear amenable to studies of HPV

infection. Interestingly, all three cell lines developed differentiating, stratified epithelium when grown in raft culture (Fig. 21). However, a confounding variable in using these cells to study HPV infection is that they were immortalized by HPV16 E6 and E7 (58). The goal in this study was to develop a tissue-based model of HPV16 infection establishment. Furthermore, HPV16-immortalized cells behave differently under wounding conditions than HPV-negative cell lines, as E6 and E7 contribute to EMT and influence keratinocyte migration (8, 103). Although raft tissue grown using FGT cells may support HPV16 PsV infection, they were not evaluated for their infection potential because, by expressing E6 and E7, subsequent wounding and HPV16 infection would represent a re-infection model.

Attempts were made to optimize HPV16 infection of raft tissue by varying two infection parameters: 1) the time between wounding and infection, and 2) the duration of infection. Rafts were infected 0, 4 and 8 h post-wounding, after which tissue was harvested 3 and 6 days post-infection and analyzed by luciferase assay. There were not marked differences in infection when HPV16-luc PsVs were added immediately post-wounding (0 h) or after a brief recovery period (4 or 8 h), indicating that the events occurring between injury and HPV16-luc PsV exposure do not dramatically impact infection (data not shown). Furthermore, no infection above background was seen at day 3, while at day 6 there was detectable infection (data not shown). The highest levels of HPV infection were observed at day 4 post-infection (Fig. 22).

An additional limitation of this study was the variability of raft growth, which possibly impacted detection of luciferase expression. Sometimes raft epithelium readily detached from the fibroblast-collagen dermal plug, breaking apart and lysing during the luciferase assay, while at other times epithelial tissue was resistant to dermal separation and difficult to break apart. Epithelial tissues that did not cleanly separate from the dermal plug gave lower levels of infection, likely because excess cellular material reduced the effectiveness of the luciferase assay. To overcome this limitation in future experiments, greater numbers of raft cultures should be tested to increase sample size and accommodate the inherent variability of *in vitro* tissue growth.

In summary, while intact raft tissue was resistant to infection, wounded raft tissue was readily infected (Fig. 22). This marks the development of a model of tissue infection with HPV16 that recapitulates the establishment phase of papillomavirus infection. HPV PsVs are commonly used to overcome species- and tissue-restrictions, as well as to gauge infection in cell monolayer, which represent abortive infection because progeny virions cannot be produced outside of differentiating, stratified epithelium. In this study, HPV16-luc PsVs were instrumental in demonstrating that raft tissue supports initial phases of papillomavirus infection. Furthermore, the cell line used to generate raft tissue, NIKS cells, provides physiological relevance for studying HPV16 infection because they originate from genital tissue.

Chapter 4: Discussion

Implications

HPV infections are exclusively epitheliotropic, meaning that productive infection does not involve viremia because HPV replication is restricted to differentiating, stratified epithelial tissue. Because the epithelium acts as the first line of defense against microbial invasion, HPV cannot access and infect its tropic cells, basal keratinocytes, unless the barrier integrity of skin is breached. To gain a better understanding of the contribution of epithelial disruption to HPV16 infection establishment, this study incorporated wound healing events into cell- and tissue-based models of HPV infection. Because animal models have shown that HPV infection is enhanced by physical disruption of epithelial tissues, epithelial disruption was hypothesized to be necessary for the initiation of HPV infection *in vitro*. Using organotypic "raft" culture, HPV-negative keratinocytes were grown into full thickness epithelium that differentiated and stratified similar to epithelium *in vivo*. Subsequent wounding and exposure to HPV16 pseudovirions (PsVs) revealed that infection occurred only following disruption by scratch wounding, as unwounded raft epithelial cultures were uninfected. While the hypothesis was supported in this model, HPV16 infection of scratch wounded keratinocyte monolayers indicated otherwise. Using HaCaT cells, HPV16 PsV infection was not enhanced when confluent keratinocyte monolayers were injured by scratch wounding, as infection between unwounded and wounded cells was comparable. Additionally, HPV16 infection of migrating keratinocytes, which become mobile through an epithelial-to-mesenchymal

transition (EMT), was investigated. We hypothesized that keratinocytes displaying a mesenchymal phenotype support HPV16 infection, but experimental results revealed that HPV is unable to infect such cells. Subsequent experiments investigating HPV binding and entry indicate that although viral binding to cells in an EMT is reduced, a subset of HPV virions appear to achieve cellular entry. Interestingly, HPV16 infection of keratinocytes in an EMT resumed when the epithelial phenotype was restored, an event that happens at the end of reepithelialization during wound healing. Collectively, these results indicate that epithelial wounding is a necessary event in HPV infection establishment, and that keratinocytes migrating through an EMT are only temporarily refractive to HPV infection. This implies that HPV transmission may be more complex than previously thought, and that models of HPV infection, by not accounting for wound healing, are inherently limited and only partially representative of physiological HPV infection.

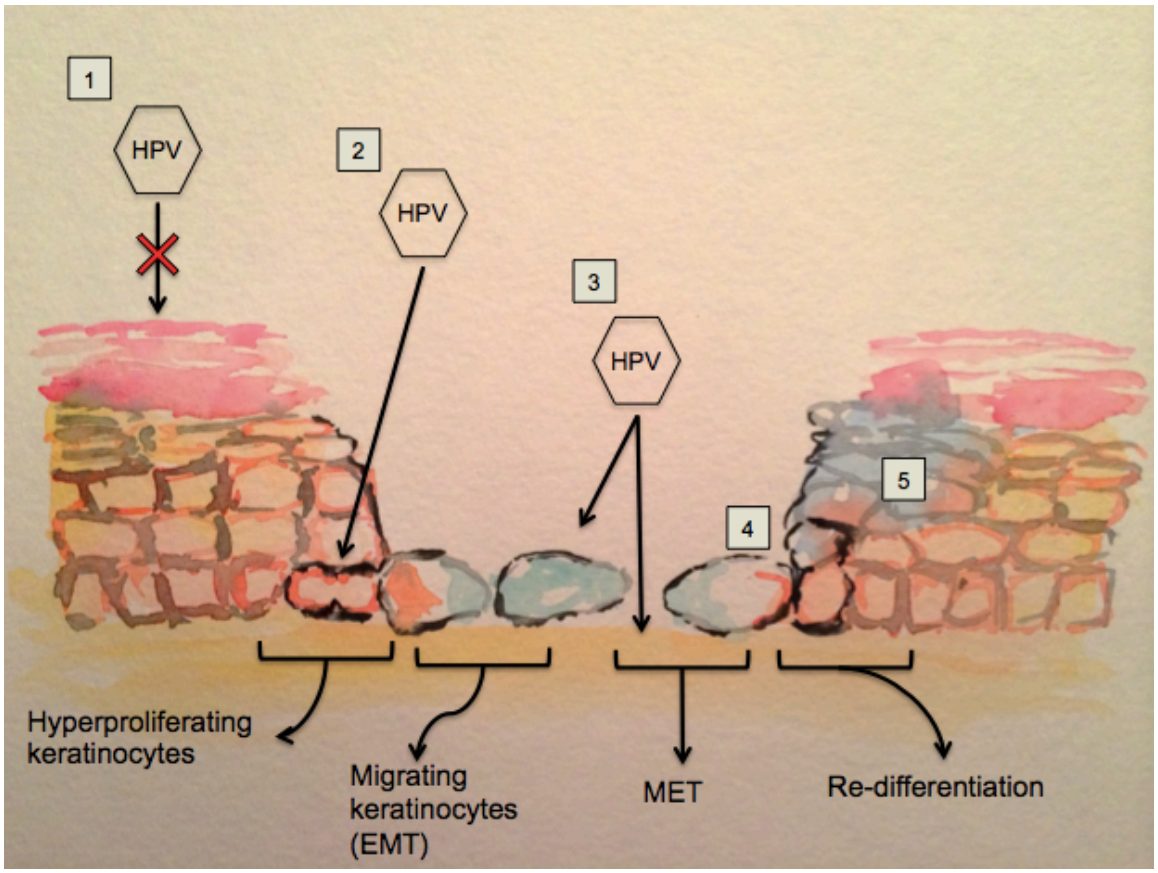

Figure 23: Proposed model of HPV16 infection in the healing epithelium. 1) HPV cannot infect intact epithelium. **2)** In wounded epithelial tissue, HPV infects mitotically active, hyperproliferative keratinocytes that supply the migratory front. **3)** HPV adheres to heparan sulfate displayed by the basement membrane and migrating cells. Migrating cells undergo EMT and express slug and vimentin, but also allow reduced numbers of HPV virions to bind. **4)** HPV enters but does not infect migrating cells, which undergo MET upon contact inhibition and the end of inflammation. **5)** Formerly migratory keratinocytes re-differentiate after wound closure, and in so doing express the HPV genome.

Future Directions

To build upon the findings in this report, I propose two sets of experiments. In the first, the effects of TGFβ1, EGF, KGF, bFGF and PDGF on HaCaT cell infectability by HPV16 will be further evaluated. TGFβ1, EGF, KGF and bFGF are each implicated in keratinocyte motility (Table 2), but only TGFβ1 and EGF were tested for markers of EMT. To understand whether KGF and bFGF similarly cause an EMT response, keratinocytes need to be evaluated for

the effects they bring about, as each caused HPV16 infection to be reduced (Fig. 11). PDGF caused a similar reduction in HPV16 infection, but how it did so is unknown. It may be that HaCaT cells, as a pre-neoplastic cell line, responded to PDGF by activating intracellular signaling pathways that undermined HPV infection.

In the second set of experiments, NIKS cells would be induced into an EMT similar to how TGFβ1 and EGF induced an EMT in HaCaT cells, then exposed to HPV16 under binding conditions. However, in place of HPV16 PsVs, authentic, infectious HPV16 would be used. Subsequently, EMT would be reverted and cells would be used in raft culture. After allowing raft tissue to differentiate and stratify, they would be used for both sectioning and staining for L1, as well as harvested for progeny HPV16. If L1 was expressed in upper epithelial strata, it would indicate that NIKS cells in an EMT support HPV16 entry and subsequent infection in a physiologically relevant tissue system. Additionally, if progeny HPV16 could be recovered in this experiment, it would support the model proposed in Fig. 23, wherein migrating cells exposed to HPV16 re-differentiation and go on to support the end stages of HPV replication.

Conclusion

Here, I have expanded upon our understanding of HPV infection in wounded epithelium, finding that HPV appears unique regarding EMT and early events in viral infection. Whereas EMTs alter expression of epithelial proteins that are entry receptors for other viruses (106, 169, 179), HPV16 infection

appears to be compromised post-internalization during a Type II EMT (Figs. 13A, 14). HPV16 infected keratinocytes in an EMT once MET occurred, meaning that a heretofore unknown reservoir of susceptible cells was identified. Prior studies have postulated that ECM-resident HPV is encountered by migrating keratinocytes to initiate infection. While the results in this report support this assertion, they do so by a more complicated mechanism than initially thought, because instead of migrating keratinocytes supporting HPV infection right away, other cellular events in wound healing, such as mesenchymal-to-epithelial transition (MET), appear to be crucial for expression of viral genes. Furthermore, a tissue-based model of HPV16 early infection was developed using scratch wounding of NIKS cells grown in raft culture. Based on the finding that HPV infection of tissue occurs only in the presence of epithelial disruption, it can be concluded that they key determinants to HPV infection do not involve inflammation, vasculature, systemic immune responses or hormonal changes.

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