Dysregulation of Host Cellular microRNA Expression by the Human Papillomavirus E6 and E7 Oncoproteins

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Dysregulation of Host Cellular microRNA Expression by the Human

Papillomavirus E6 and E7 Oncoproteins

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by

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ABSTRACT

Human papillomaviruses (HPVs) are small DNA viruses with a tropism for squamous epithelia. Nearly all cervical cancers are caused by a small group of high-risk HPVs and these viruses are also associated with anal, vaginal, vulvar, penile and oropharyngeal cancers. The high-risk HPV16 E6 and E7 proteins are the major drivers of cell transformation and HPV-carcinogenesis. The most well known oncogenic activities of HPV16 E6 and E7 are degradation of the p53 and retinoblastoma tumor suppressors, respectively. However, HPV16 E6/E7 also drive transformation via other mechanisms, and this dissertation investigates how HPV16 E6 and E7 dysregulate expression of non-coding RNAs, with a focus on microRNAs (miRs).

Although high-risk HPVs are not known to encode miRs, these viruses have been shown to alter the expression of host cellular miRs. In many previous HPV miR-profiling studies, differentiating cells were analyzed. Given HPVs alter epithelial cell differentiation, it is unclear whether reported changes in miRs are directly caused by HPV gene expression or represent sequelae of HPV-induced changes in epithelial cell differentiation. This dissertation examines cellular miRs modulated by expression of HPV16 E6/E7 in undifferentiated primary human epithelial cells.

In **Chapter 2**, we perform a comprehensive analysis of miRs and mRNAs altered by HPV16 E6/E7 using deep sequencing. Pairing miRs with potential targets, our data show that many observed changes in mRNA expression may be due, in part, to perturbation of miRs by HPV16 E6/E7. In **Chapter 3**, we investigate the alteration of miR biogenesis enzymes by HPV16 E6/E7. Expression of the HPV16 oncoproteins increases levels of DROSHA and DICER. Furthermore, manipulation of DROSHA levels may be one mechanism by which HPV16 E6/E7 expression dysregulates cellular miR expression. In **Chapter 4**, we examine how expression of HPV16 E6/E7 alters the expression of miRs in extracellular vesicles. Some miRs are similarly regulated by HPV16 E6/E7 in cells and extracellular vesicles whereas others are not. Overall, this dissertation shows that modulation of miRs is an important part of HPV16 E6/E7 mediated reprogramming of cellular gene expression and may contribute to the transforming activities of these proteins.

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CHAPTER ONE

Introduction

This chapter is adapted from a previously published manuscript:

Contributions: I wrote the manuscript and Karl Munger aided in writing and editing the manuscript.

1.1 Human Papillomavirus Molecular Biology

With a prevalence of 70 million cases and an incidence of 14 million new transmissions each year, human papillomavirus (HPV) infections of the anogenital tract are the most common sexually transmitted diseases in the US (CDC, 2015). High-risk (HR) HPVs are the causative agents of cervical cancer and, worldwide, it is estimated that 500,000 cases of cervical cancer occur each year, which result in over 250,000 deaths (Schiffman *et al.*, 2007). Cervical cancer is the 4th most common cancer in women and the 7th most common cancer overall (Bray *et al.*, 2013). The burden of cervical cancer is disproportionately high in low-income countries due to a scarcity in resources to implement widespread screening, vaccination and treatment programs (Forman *et al.*, 2012). While safe and efficacious vaccines for the prevention of HPV infection are available, they do not protect those already infected with HPV and they do not protect against all HPV types. Therefore, continued studies of the molecular biology of HPV are necessary to develop improved screening techniques and prophylactic vaccines for the prevention of HPV infection, as well as better therapeutic options, including vaccines, for the treatment of HPV infection.

1.1.1 HPV Classification

HPVs are members of the distinct virus family, the *Papillomaviridae*. The *Papillomaviridae* family is divided into 39 genera (Bernard *et al.*, 2010; de Villiers *et al.*, 2004), based on L1 sequence identity of 60% or greater, with each genus designated by a letter of the Greek alphabet. PVs within a genus that share 60-70% L1 sequence identity are termed a species (Bernard *et al.*, 2010; de Villiers *et al.*, 2004). Additionally,

within a species, PVs with 71-89% L1 sequence identity are considered a type (Bernard *et al.*, 2010; de Villiers *et al.*, 2004). As of 2017, 205 different HPV types have been identified, which have been categorized into five genera including the following: 65 *Alphapapillomaviruses*, 51 *Betapapillomaviruses*, 84 *Gammapapillomaviruses*, 4 *Mupapillomaviruses* and a single *Nupapillomavirus* (Van Doorslaer *et al.*, 2013). At least 25 more additional types have been identified however, these viruses are currently pending classification. HPVs with 90-98% L1 sequence identity are termed subtypes and those with >98% L1 sequence identity are considered variants (Bernard *et al.*, 2010). Arguably, HPVs in the alpha genus are of the greatest medical importance given they are associated with oral and mucosal cancers, as well as cancers of the anogenital tract. Table 1.1 includes a summary of main HPV genotypes and their associated diseases.

1.1.2 Virion and Genome Structure and Organization

HPVs are non-enveloped DNA viruses with a tropism for the squamous epithelium. Each virus particle consists of an icosahedral capsid of about 60 nm in diameter, containing a single molecule of double stranded circular DNA of approximately 8,000 base pairs (Knipe and Howley, 2013). Only one strand of the double stranded DNA genome is used as a template for transcription and this coding strand contains three genomic regions, including approximately ten open reading frames (ORFs) shown in Figure 1.1. Many viral proteins are expressed from polycistronic mRNAs (Favre *et al.*, 1975; Zheng and Baker, 2006). The early region (E)

Table 1.1. Main HPV genotypes and their associated diseases.

Genus	Species	Representative HPV types	Tropism	Associated Diseases
Alpha-PV	α1	32	mucosal	Heck's disease
	α2	3, 10, 28	cutaneous	flat warts
	α4	2, 27, 57	cutaneous	common warts
	α7	18, 39, 45, 59, 68	mucosal	intraepithelial neoplasia, invasive carcinoma
	α9	16, 31, 33, 35, 52, 58	mucosal	intraepithelial neoplasia, invasive carcinoma
	α10	6, 11	mucosal	condylomata acuminata
		13		Heck's disease
Beta-PV	β1c	5, 8, 12, 14, 19, 20, 21, 24, 25, 36, 47	cutaneous	Epidermodysplasia verruciformis
	β2	9, 15, 17, 22, 23, 37, 38	cutaneous	Epidermodysplasia verruciformis
	β3	49	cutaneous	Epidermodysplasia verruciformis
Gamma-PV	γ1	4, 65	cutaneous	warts
	γ4	60	cutaneous	warts
Mu-PV	μ1	1	cutaneous	plantar warts
	μ2	63	cutaneous	warts
Nu-PV	ν	41	cutaneous	warts

This table summarizes information on the main HPV genotypes, their tropism and associated diseases. Information in this table was gathered from several sources including pave.niaid.nih.gov (Cubie, 2013; de Villiers *et al.*, 2004; Van Doorslaer *et al.*, 2013). Heck's disease, also known as focal epithelial hyperplasia (FEH), is a rare, benign mucosal proliferation that is strongly associated with HPV infection (Said *et al.*, 2013). Other details on HPV classification can be found in the text in section 1.1.1.

ORF	HPV16 PROTEIN FUNCTION
E1	origin binding protein, ATPase-dependent helicase involved in genome replication
E2	regulator of viral gene transcription, association with E1 (origin binding), viral genome partitioning
E4	expressed abundantly as E1^E4 fusion protein, cytokeratin network destabilization, virus release and transmission
E5	small transmembrane protein, interacts with EGF receptor activating mitogenic pathways
E6	drives cell cycle allowing genome amplification in upper epithelial layers, association with E6AP and degradation of p53, PDZ-protein binding, hTert activation
E7	drives cell cycle allowing genome amplification in upper epithelial layers, association with and degradation of pRB, mitotic mutator
E8	expressed as E8^E2 fusion protein, acts as a repressor of transcription and replication during the viral life cycle
L1	major capsid protein, assembles into pentameric capsids forming the icosahedral virion (prophylactic vaccines)
L2	minor capsid protein, involved in viral DNA encapsidation, facilitates viral entry and trafficking

EARLY REGION: proteins necessary for viral replication

LATE REGION: viral capsid proteins

LONG CONTROL REGION: sequences controlling viral replication & transcription

Figure 1.1. HPV16 genome organization and protein function.

On the left, the HPV16 genomic map of 7906 base pairs is shown. Only the coding strand is included and transcription occurs in the clockwise direction. The early promoter (P_{97}) is indicated by an arrow at the approximate position of the RNA initiation site in the long control region LCR. The late promoter (P_{670}) is also indicated by an arrow at its initiation site in the E7 ORF. The early region is depicted in blue and contains proteins necessary for viral replication including E1, E2, E3, E4, E5, E6, E7

Figure 1.1 (Continued)

and E8. The late region is shown in purple and contains the viral capsid proteins L1

and L2. The LCR is shown in green and contains sequences controlling viral replication

and transcription. On the right, a table of the HPV16 ORFs and a brief description of

their corresponding viral functions is shown. More details can be found in section 1.1.2

of the text.

contains up to seven ORFs encoding viral regulatory proteins and the late (L) region

encodes the two viral capsid proteins. Each ORF in the early region is designated "E"

followed by a numeral, indicative of the length of the ORF. The third region of the

genome has been referred to as the long control region (LCR), the upstream regulatory

region (URR) or the noncoding region (NCR). This genomic region contains the origin

of DNA replication, as well as transcription control sequences (Knipe and Howley,

2013).

Viral Proteins

The early HPV ORFs include E1, E2, E4, E5, E6, E7 and E8 (Doorbar *et al.*,

1986; Zheng and Baker, 2006) (see Figure 1.1). E1 codes for an ATP dependent viral

DNA helicase (Bergvall *et al.*, 2013) that can bind to the AT-rich origin of replication and

E2 proteins function in viral transcription, replication and genome partitioning. The full

length E2 protein encodes a transcriptional activator. In contrast, a truncated form of E2

transcribed from an internal ATG and the E8^E2 fusion protein repress transcription

(McBride, 2013). E4 is embedded within the E2 gene and is primarily expressed as an

E1^E4 fusion protein during the late stages of the viral life cycle. E4 binds to cytokeratin

filaments, disrupting their structure, and is thought to play a role in viral escape from

cornified epithelial layers (Doorbar, 2013). E5 is a small transmembrane protein, which

has been best studied with bovine papillomavirus type 1 (BPV1). BPV1 E5 is an

oncogenic small, hydrophobic, single pass transmembrane protein that forms dimers

and interacts with and activates receptor tyrosine kinase receptors, including the EGF

and PDGF receptors. Similar activities have also been ascribed to HPV E5, which

encodes multi pass transmembrane proteins that share only limited sequence similarity

with BPV1 E5 (DiMaio and Petti, 2013). HPV E5 proteins have also been reported to

play a role in apoptosis and in evasion of the immune response (DiMaio and Petti,

2013). HPV E6 and E7 both drive cell cycle entry to allow genome amplification in

upper epithelial layers. HR HPV E6 proteins have oncogenic activities. They bind and

degrade p53, as well as cellular PDZ proteins, and they activate telomerase (Vande Pol

and Klingelhutz, 2013). HR HPV E7 proteins bind and degrade the retinoblastoma

tumor suppressor, pRB, and contribute to malignant progression by inducing genomic

instability (Dyson *et al.*, 1989; Roman and Munger, 2013). HPV E8 is expressed as an

E8^E2 fusion protein, in which E8 replaces the E2 activation domain responsible for

transcriptional control and activation of DNA replication, thereby functioning as a

repressor of transcription and replication (Ammermann *et al.*, 2008). The late region

encodes the major (L1) and minor (L2) capsid proteins (see Figure 1.1). Given the L1

ORF is the most conserved among PVs, it is used for phylogenetic organization and

HPV classification. Notably, L1 will spontaneously assemble into virus like particles,

which is the basis for the currently available prophylactic vaccine formulations

discussed in greater detail in section 1.1.7 (Buck *et al.*, 2013).

1.1.3 HPVs and Cancer

The first HPVs that were associated with cancer were beta HPV5 and 8. They

were detected in warts and cancers arising in patients with a rare genetic disorder,

Epidermodysplasia verruciformis (EV) (Orth *et al.*, 1978; Pass *et al.*, 1977). Most of

these patients carry mutations in one of the two EVER genes, EVER1 and EVER2 (also

referred to as TMC6 and TMC8, respectively), located on chromosome17q25.3 (Ramoz *et al.*, 2002). Although the molecular basis remains enigmatic, EV patients cannot efficiently clear beta HPV infections and develop warts all over their bodies. These warts can undergo malignant progression at sun-exposed areas of the body. Beta HPVs may contribute to non-melanoma skin cancers that arise as a frequent complication in long-term immunosuppressed organ transplant patients. It remains unclear whether beta HPVs contribute to non-melanoma skin cancer formation in patients that are not systemically immunosuppressed (reviewed in (Meyers and Munger, 2014; Orth, 2008; Pfister, 2003)). Of note, HPV sequences are not detected in every tumor cell, suggesting that these viruses may contribute to tumor induction but may not be necessary for maintenance of the transformed state. Studies with transgenic mice have shown that E6, E7 and, quite uniquely E2, each have oncogenic activities.

The approximately 40 alpha HPVs that infect mucosal epithelia are classified as low-risk (LR) or HR based on their clinical association with generally benign warts or lesions that have a propensity for malignant progression. LR HPVs, for example HPV6 and HPV11 or HPV13 cause genital warts and oral focal epithelial hyperplasia (Heck's disease), respectively (Padayachee and van Wyk, 1991). HR HPVs, such as HPV16 and HPV18, cause intraepithelial neoplasia, which can progress to invasive carcinoma. HR HPVs are the causative agents of approximately 5.2% of all human cancers worldwide and HR HPV associated cervical carcinoma is the 4th most common cancer among women globally (Chaturvedi, 2010; de Martel *et al.*, 2012; Tota *et al.*, 2011). Almost all cervical cancers are caused by HR HPVs and two types, HPV16 and HPV18, are detected in up to 70% of all cervical cancers (Winer *et al.*, 2006b). HR HPV

infections also account for 95% of anal cancers, 70% of oropharyngeal cancers (Chaturvedi *et al.*, 2011), 60% of vaginal cancers, 50% of vulvar cancers and 35% of penile cancers (Gillison *et al.*, 2008). HPV16 is by far the most prevalent HPV type detected in these cancers. HPV associated cancers generally represent non-productive infections, as described in more detail in section 1.1.5.

1.1.4 HPV Productive Infection and Lifecycle

A hallmark of the HPV lifecycle is its close association with the differentiation program of the infected host squamous epithelium (see Figure 1.2). HPVs firstly infect undifferentiated basal epithelial cells and then viral progeny are produced in differentiated daughter cells in the uppermost epithelial layers (Hong and Laimins, 2013a).

Methods for Studying the Viral Lifecycle

Historically, it has been difficult to study the PV life cycle in the laboratory. Organotypic raft cultures, in which epithelial cells are grown on a fibroblast-containing matrix at the air liquid interface where they form a stratified, skin-like structure, provided the necessary breakthrough to recapitulate the full viral life cycle and to produce infectious HPV in tissue culture (Dollard *et al.*, 1992; Meyers *et al.*, 1992).

Virus like particles (VLPs) are produced by ectopic expression of L1 either alone or in combination with L2 in mammalian cells. Pseudoviruses are VPLs that contain either viral genomes or reporter plasmids and can be produced similarly. Given their relative ease of production, VLPs and pseudoviruses have been the main tools used to
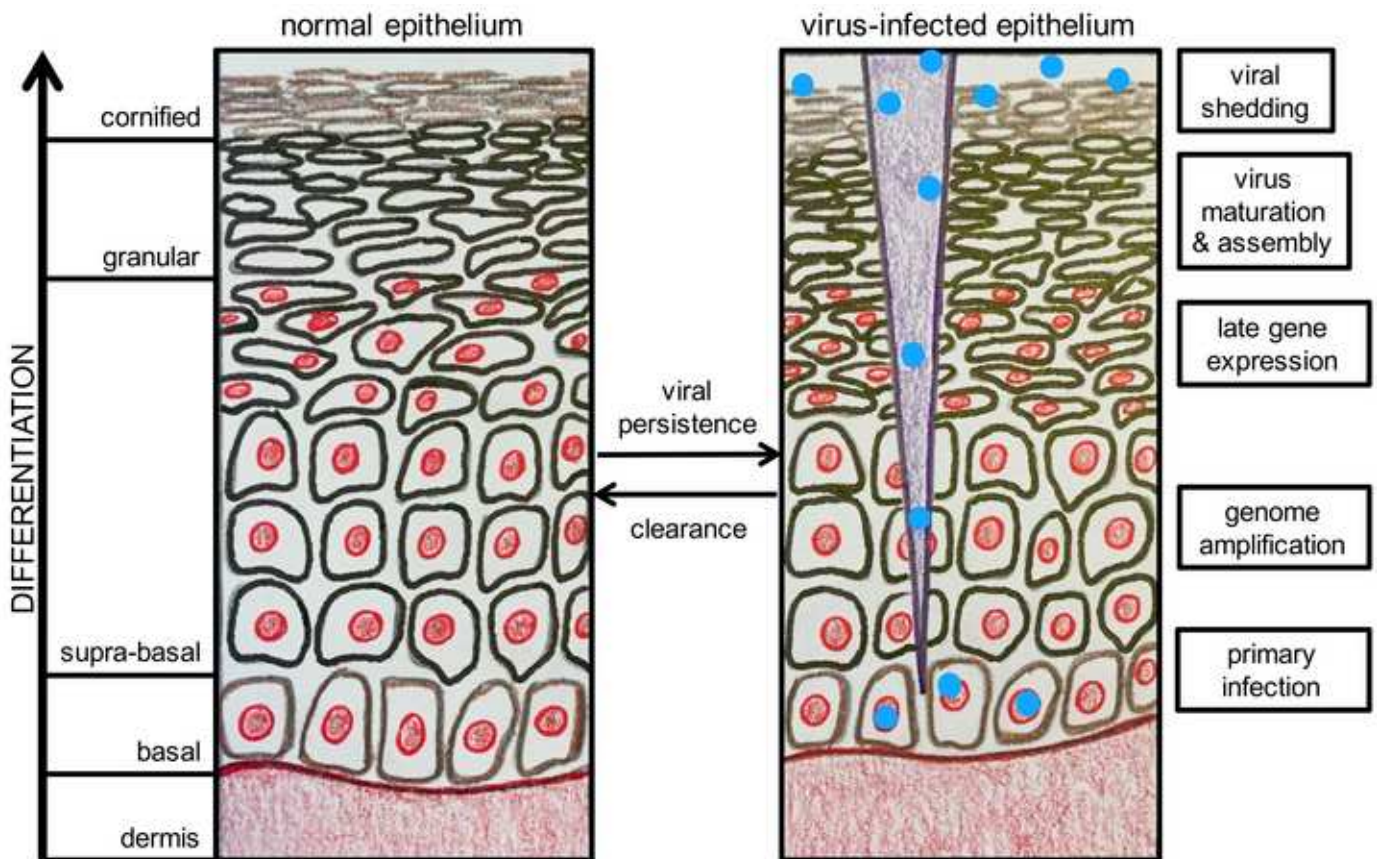
Figure 1.2. The HPV lifecycle in differentiating squamous epithelium.

An illustration of normal differentiating squamous epithelium is shown on the left with the layers of the differentiating epithelium noted. On the right, a brief description of the HPV life cycle stage occurring in the corresponding epithelial layer is shown. Greater detail on HPV productive infection and the viral life cycle is included in section 1.1.4. This figure was illustrated by M. E. Harden and adapted from a figure by C. L. Nguyen.

study HPV structure, assembly, entry and infectivity. Due to the exquisite species specificity of PVs, an animal model that closely recapitulates HPV infection and disease in humans has not been established. In 2010, a new papillomavirus (MmuPV) was identified in the common house mouse (*Mus musculus*), allowing studies of PVs in laboratory mice for the first time (Ingle *et al.*, 2011). MmuPV infection of mice most closely resembles human infection by cutaneous PVs and information gleaned from studies of MmuPV infections of laboratory mice may be useful to better understand HPV associated human pathogenesis.

HPV infection

The only cells capable of undergoing cell division in the squamous epithelium are basal cells. Consequently, PVs must specifically infect these cells to allow establishment of a persistent infection. Cells in the basal epithelial layer consist of stem cells and transit amplifying cells. In order for an HPV infection to be persistently maintained, the epithelial stem cells in the basal layer must become infected (Egawa, 2003; Kaur and Li, 2000). However, given basal epithelial cells are shielded by several layers of differentiated cells, they are not easily accessible and the virus must infect these protected cells through micro wounds that expose lower epithelial layers (Knipe and Howley, 2013). Additionally, cells located in the squamocolumnar transformation zone in the cervix and anus have been shown to be particularly accessible and vulnerable to HPV infection (Herfs *et al.*, 2012).

Viral Attachment and Entry

Virions bind initially to heparan sulfate proteoglycans (HSPGs), which serve as primary attachment receptors on basal cells or exposed basement membrane resulting from epithelial trauma or permeabilization (Schafer *et al.*, 2015). Initial L1 attachment to HSPGs induces conformational changes in the virus capsid ultimately resulting in loss of affinity for the primary receptor and transfer of the virus to an as of yet poorly characterized entry receptor. Whether or not these conformational changes may be caused by cyclophilin B (Bienkowska-Haba *et al.*, 2009) remains a subject of controversy. Ultimately, the L2 amino terminus is exposed, making it susceptible to cleavage by furin-related proteases, which is necessary for infection by some HPVs (Cruz *et al.*, 2015; Day and Schiller, 2009; Richards *et al.*, 2006). Internalization of capsids from the cell surface is asynchronous and can take from two to four hours, with some capsids remaining on the surface much longer than others. Capsids on the surface are propelled by directed motion (surfing) from filopodia to the cell body via actin retrograde flow (Schelhaas *et al.*, 2008; Smith *et al.*, 2008). A delay of one to three days can occur between cell surface binding and viral genome transcription (Day *et al.*, 2003; Roberts *et al.*, 2007). However, if the infected cells are close to mitosis, nuclear entry and detection of viral gene expression can occur at much earlier time points post infection (Broniarczyk *et al.*, 2015).

The virus is endocytosed through a potentially novel mechanism, similar to macropinocytosis, that is clathrin, caveolin and lipid raft independent (Schelhaas *et al.*, 2012). Virions are then trafficked through the endosomal system where they undergo further structural changes that result in partial uncoating (Day and Schelhaas, 2014).

During viral uncoating in acidified endosomes, cyclophilin B aids in the dissociation of L1 from the L2/viral genome complex and L1 is targeted to lysosomes for degradation (Bienkowska-Haba *et al.*, 2012). The minor capsid protein, L2, mediates delivery of the viral genome from the early endosome to the trans Golgi network through direct interactions with the retromer complex (Bonifacino and Hurley, 2008; Popa *et al.*, 2015). Specifically, L2 associates with sorting nexin 17 to allow escape of the L2/viral genome complex from late endosomal compartments (Bergant Marusic *et al.*, 2012). This interaction is conserved across multiple HPV types and is essential for viral infection (Bergant and Banks, 2013). L2 also directly interacts with sorting nexin 27, another member of the host retromer complex, to aid in viral trafficking (Pim *et al.*, 2015). Movement of the virus through the cytoplasm to the nucleus likely occurs along microtubules through the association of L2-associated vesicles with the motor protein dynein light chains (Florin *et al.*, 2006; Schneider *et al.*, 2011). Entry of the viral genome into the nucleus requires mitosis (Pyeon *et al.*, 2009) and this process is mediated by L2. Following nuclear entry, L2 and the viral genome colocalize at ND10 domains, which is a critical step in the establishment of infection and allows transcription of the viral genome (Day *et al.*, 2004).

Genome Replication and Gene Expression

After infection, initial genome amplification occurs prior to maintenance of the viral genome in the nuclei of infected basal epithelial cells. Viral DNA is maintained in basal epithelial cells as a stable multicopy plasmid or episome. E1 and E2 are among the first viral proteins to be expressed and, while initial amplification is thought to require

E1 and E2, E1 may not be necessary once viral copy numbers have reached a threshold of 50-100 copies (Maglennon *et al.*, 2011). Based on studies of cell lines, episomal copy number is thought to be approximately 200 copies per cell (Doorbar *et al.*, 2015). However, using laser capture methods, 50-100 copies per cell have been detected in the basal layer of productive warts (Maglennon *et al.*, 2011). Viral genomes replicate once per cell cycle, on average, during S phase, ensuring persistent infection of basal cells. In this "latent" phase of the viral lifecycle, HPV genomes are thought to persist in basal epithelial cells for years to decades. However, at some point, a switch from stable replication (genome maintenance) to vegetative viral DNA replication must occur to allow the production of genomes for packaging into virions. Little is known about the mechanism regulating this switch.

Vegetative viral DNA replication occurs in differentiating cells of the squamous epithelium. Two HPV proteins, E1 and E2, are actively involved in viral genome replication. E1 is the only virally encoded enzyme and functions as an ATP dependent helicase (Bergvall *et al.*, 2013). E1 binds AT rich sequences at the origin of replication with weak affinity and is required for initiation and elongation of viral DNA synthesis. E2 stabilizes E1 binding to the origin of replication, by interacting with $ACCN_6GGT$ sequences adjacent to the origin, resulting in high affinity binding of the E1/E2 complex to the origin of replication (McBride, 2013). HPVs do not encode any other replication enzymes and must hijack the host DNA synthesis machinery to accomplish replication of the viral genome. E1 and E2 recruit cellular DNA polymerases and other essential accessory enzymes to enable viral genome replication. Normally, differentiating cells would not be capable of supporting DNA synthesis given they have withdrawn from the

cell cycle upon exiting the basal layer of the epithelium. However, HPVs are able to activate cellular DNA replication machinery to allow vegetative viral DNA synthesis through the actions of E6 and E7.

Another mechanism of vegetative viral DNA synthesis has been proposed based on data suggesting that viral DNA is replicated after host DNA, possibly in the G2 phase of the cell cycle (Banerjee *et al.*, 2011; Nakahara *et al.*, 2005). This indicates that the virus would need other resources to replicate viral genomes and it is thought that the virus may use the DNA damage response, in particular recombination-dependent replication (RDR), to produce genomes in differentiated cells (Sakakibara *et al.*, 2013). Infection by HPVs is known to induce a DNA damage response (DDR) and HPVs hijack both the ATR and ATM DDR signaling pathways for their differentiation-dependent life cycles (reviewed in (Hong and Laimins, 2013b)). The ATM pathway is particularly important for differentiation-dependent genome amplification. HPV E1 and E7 activate the ATM DDR and E2 can also modulate this pathway through binding to E1 (Fradet-Turcotte *et al.*, 2011; Sakakibara *et al.*, 2011). Additionally, the ATR pathway appears to play a role in HPV replication in undifferentiated cells by affecting episomal maintenance (Edwards *et al.*, 2013; Reinson *et al.*, 2013).

E6 and E7 contribute to the viral lifecycle by modifying the cellular environment to allow viral genome amplification in growth arrested, terminally differentiated cells, which would normally be incompetent for DNA replication. In particular, HR E6 and E7 drive cell proliferation in the basal and parabasal layers causing an increase in the size of the initial infected area. Many papillomavirus E7 proteins target the retinoblastoma tumor suppressor pRB and the related "pocket proteins" p107 and p130 (Barrow-Laing *et al.*,

2010; Dyson *et al.*, 1989; Felsani *et al.*, 2006; Klingelhutz and Roman, 2012; Roman, 2006). HR HPV E7 proteins target the pocket proteins for degradation through the ubiquitin/proteasome system. By binding and/or triggering degradation of pocket proteins, E7 causes release of E2F family members from pocket protein bound transcriptional repressor complexes. This results in constitutive activation of E2F modulated gene expression programs that control DNA synthesis and cell proliferation (Munger and Howley, 2002). Additionally, some HPV E7 proteins avoid triggering G1 arrest during epithelial cell differentiation by inactivating the CDK2 inhibitors, CDKN1A and CDKN1B (reviewed in (Moody and Laimins, 2010)).

HR HPV E6 proteins inactivate the p53 tumor suppressor by targeting it for proteasomal degradation through the associated E3 ubiquitin ligase, UBE3A (E6AP). This action blocks the anti proliferative and pro apoptotic activities of p53 in response to DNA damage and cellular stress caused by aberrant S-phase entry (Scheffner *et al.*, 1993). HR HPV E6 expression also upregulates telomerase activity, allowing the maintenance of telomere integrity despite repeated cell divisions (Galloway *et al.*, 2005; Gewin and Galloway, 2001; Klingelhutz *et al.*, 1996). Additionally, HR HPV E6 proteins target cellular PDZ domain containing proteins that regulate cell contact and signaling pathways (Kiyono *et al.*, 1997; Lee *et al.*, 1997). LR HPV E6 proteins, while able to interact with UBE3A, do not directly bind p53, but may target p53 transcriptional activity indirectly by binding to p300 and/or TIP60 (Thomas and Chiang, 2005) (Jha *et al.*, 2010). Moreover, LR HPV E6 proteins do not activate telomerase activity and lack the C-terminal PDZ binding domain. Beta HPV E6 proteins do not appear to target any of these pathways but have been reported to inhibit NOTCH and TGFß signaling by

associating with MAML and SMAD proteins, respectively (Brimer *et al.*, 2012; Mendoza *et al.*, 2006; Rozenblatt-Rosen *et al.*, 2012; Tan *et al.*, 2012).

Assembly, Maturation and Viral Release

Completion of the viral lifecycle involves cell cycle exit and expression of L1 and L2 to allow genome packaging. Virion assembly occurs in the nuclei of terminally differentiated keratinocytes, in which viral genome replication and expression of viral proteins has occurred (Knipe and Howley, 2013). Nuclear entry of L1 and L2 is mediated by cellular karyopherins (Darshan *et al.*, 2004; Merle *et al.*, 1999), which transport molecules between the nucleus and the cytoplasm. L1 can assemble into VLPs and L2 may increase the efficiency of this reaction (Kirnbauer *et al.*, 1993; Zhou *et al.*, 1991). Packaging of the viral genome is not thought to be sequence specific (Buck *et al.*, 2004) and may involve a size determination mechanism (Knipe and Howley, 2013). Maturation of viral particles occurs in the upper layers of terminally differentiated squamous epithelia, where particles are exposed to an oxidizing environment. This maturation process involves the accumulation of disulfide bonds between L1 proteins, resulting in condensation of the capsid, thereby increasing its stability and resistance to proteolytic digestion (Buck *et al.*, 2005). HPVs are non lytic and viral shedding occurs due to normal loss of nuclear and cytoplasmic integrity during terminal differentiation of the infected keratinocyte (Knipe and Howley, 2013). E4 may also contribute to virion release by binding to cytokeratin filaments and disrupting their structure (Doorbar, 2013).

1.1.5 Non-Productive HPV Infection and Transformation

HR HPV associated cancers frequently represent non-productive infections, in which viral proteins are expressed but no infectious virus is produced. Deregulation of viral gene expression can be caused by viral genome integration in high-grade premalignant lesions, often resulting in expression of just two viral proteins, E6 and E7. Such integration events frequently cause disruption or deletion of the E2 ORF, which encodes a transcriptional repressor of E6/E7 expression (Bernard *et al.*, 1989; Thierry and Yaniv, 1987). Hence, E6/E7 mRNA expression may be higher from integrated HPV subgenomes. Additionally, E6/E7 mRNAs produced from integrated genomes have been reported to be more stable than mRNAs expressed from episomal genomes (Jeon and Lambert, 1995). A genome wide analysis of HPV genomes in cervical lesions and cancers showed that HPV integration sites frequently directly flank chromosomal aberrations that include focal amplifications, rearrangements, deletions and/or translocations (Akagi *et al.*, 2014). Based on a "looping" model, these host genomic alterations are triggered when HPV integrant mediated DNA replication and recombination form viral host DNA concatemers. This also results in amplification of E6 and E7 containing viral genome fragments (Akagi *et al.*, 2014). It is interesting to note, however, that some cervical cancers retain viral genomes in episomal form (Matsukura *et al.*, 1989; Pett and Coleman, 2007; Vinokurova *et al.*, 2008). In these cases, viral gene expression is likely deregulated by aberrant epigenetic modifications of the viral genome (reviewed in (Johannsen and Lambert, 2013)).

Therefore, the expression of E6 and E7 is deregulated in cervical carcinomas on multiple levels and the expression of cellular genes flanking sites of viral genome

integration may also be compromised, at least in those cases where viral genome integration has occurred (Ojesina *et al.*, 2014). Furthermore, in cervical disease, it is thought that expression of E6 and E7 underlies the distinctive neoplastic phenotypes (reviewed in (Doorbar *et al.*, 2015)). E6 and E7 expression is thought to increase during progression from cervical intraepithelial neoplasia 1 (CIN1) to CIN3 and, given the ability of HR, but not LR HPV E6 and E7 proteins, to trigger genomic instability, E6 and E7 expression importantly contribute to malignant progression (Isaacson Wechsler *et al.*, 2012; Munger *et al.*, 2006). A recent study has shown that cervical carcinomas contain recurrent mutations in cellular genes including EP300, FBXW7, PIK3CA, HLA-B, TP53, MAPK1, PTEN, ERBB2, NFE2L2 and STK11 (Ojesina *et al.*, 2014).

1.1.6 Prevention of HPV- Associated Diseases and Cancer

Routine screening is critical for the early detection of HPV and prevention of associated diseases and cancer. The Papanicolaou smear, or the Pap test, has been the method of choice for cervical cancer screening for over 60 years (Tambouret, 2013). Since its widespread implementation, cervical cancer deaths have decreased dramatically. However, the Pap test has a relatively high rate of false positives, as well as false negatives. In 2014, the U.S. Food and Drug Administration (FDA) approved a PCR based HPV test (the cobas HPV test) that detects HR types and genotypes HPV16 and 18, as well as 12 other HR HPVs, for primary screening in cervical cancer (Stoler *et al.*, 2015). Similar to many other countries, screening guidelines were updated in the US to recommend HPV primary screening as an alternative to cytology based screening strategies (Huh *et al.*, 2015). Routine screening options now include cytology alone,

cytology in conjunction with HPV testing with or without genotyping or HPV primary

screening with genotyping (Saslow *et al.*, 2012). The incorporation of HPV genotyping

into cervical cancer screening is thought to decrease the incidence of cervical cancer by

improving the detection of CIN (Wright *et al.*, 2016). While condoms have been shown

to reduce the risk of HPV infection (Hippelainen *et al.*, 1994; Winer *et al.*, 2006a), as

well as a lubricant, carrageenan (Roberts *et al.*, 2007) and a carrageenan-based

microbicide, Carraguard (Marais *et al.*, 2011), the best and most efficacious method of

prevention is vaccination.

1.1.7 HPV Vaccination

Vaccination is the only effective measure to prevent HPV infection, and

development of a prophylactic HPV vaccine was widely hailed as a historic

achievement. Vaccination against HPVs has been in effect since 2006 and the three

currently available prophylactic vaccines are composed of recombinant HPV L1 capsid

proteins that self assemble into VLPs and induce the production of high level,

neutralizing, type specific antibodies by eliciting a strong B cell mediated immune

response (Knipe and Howley, 2013). All three vaccines are administered as three

injections over a time period of six months and the immune response to vaccination is

superior to the response to natural infection, thus providing long-term immunity (Naud *et

al.*, 2014; Nygard *et al.*, 2015).

Gardasil® was the first approved HPV vaccine and includes VLPs of the most

prevalent LR (HPV6, HPV11) and HR (HPV16, HPV18) HPVs. Another HPV vaccine,

Cervarix®, was developed by GlaxoSmithKline (GSK) and is a bivalent vaccine targeting

HPV16 and HPV18. In 2014, the FDA approved the newest HPV vaccine, Gardasil 9®,

which protects against 9 HPVs, including HPV16, 18, 6, 11, 31, 33, 45, 52 and 58. The

five additional HPV genotypes Gardasil 9® protects against account for an additional 15-

20% of cervical cancer cases (Joura *et al.*, 2015). As a result, vaccination has the

potential to prevent ~90% of cervical cancer cases (Beavis and Levinson, 2016). The

Advisory Committee on Immunization Practices (ACIP) currently recommends one of

the three HPV vaccines for routine vaccination at 11-12 years of age (Markowitz *et al.*,

2014). The ACIP also recommends vaccination for females age 13-26, males age 13-

21 and men who have sex with men through age 26, as well as immunocompromised

individuals not previously vaccinated (Markowitz *et al.*, 2014).

Cervarix® and Gardasil® both have excellent safety profiles and have been

shown to be highly efficacious against infections with their respective HPV types

(Garland *et al.*, 2007; Group, 2007; Harper *et al.*, 2006; Munoz *et al.*, 2010; Paavonen

et al., 2009). Both vaccines also show some limited cross protection against HPV types

not targeted by the vaccines (Brown *et al.*, 2009; Harper *et al.*, 2006; Paavonen *et al.*,

2009; Wheeler *et al.*, 2012). In vaccination programs with high coverage rates, these

vaccines have been shown to induce herd immunity (Ali *et al.*, 2013; Brotherton *et al.*,

2011; Mesher *et al.*, 2013).

Although the effects of the vaccines on the incidence of cervical and other HPV

related cancers are not likely to be realized for several decades (Barr and Tamms,

2007), a decrease in the incidence of HPV infections, precancerous lesions and genital

warts has already been demonstrated in multiple studies (reviewed in (Bonanni *et al.*,

2015)). However, given these vaccines are prophylactic, they are designed to prevent

HPV infection from occurring and are no longer effective once HPV infection has already been established (Hildesheim *et al.*, 2007). As a result, several therapeutic HPV vaccines, designed to treat patients with established HPV infection or even those with CIN, are currently in development (reviewed in (Yang *et al.*, 2016)). The discovery of immunological checkpoint inhibitors has also greatly re-energized these efforts. However, no therapeutic HPV vaccines are currently available on the market.

1.2 HPV and microRNAs

HR HPV E6 and E7 also manipulate the expression of non-coding RNAs and this dissertation will focus on the dysregulation of microRNAs (miRs) by E6 and E7. HPVs may not encode their own miRs (Cai *et al.*, 2006) but it is clear from many studies that HPVs manipulate expression of host cellular miRs (Gunasekharan and Laimins, 2013; McKenna *et al.*, 2010; Melar-New and Laimins, 2010; Wang *et al.*, 2014; Yablonska *et al.*, 2013). These small RNAs are endogenously expressed, noncoding, RNA molecules that regulate gene expression at the post-transcriptional level. They target mRNAs via complementarity with the miR seed region (nt 2-8) and repress their expression by inhibition of translation or mRNA degradation (reviewed in (Huntzinger and Izaurralde, 2011)). The small size of miRs, along with their ability to inhibit expression of multiple targets, makes them the perfect tool by which viruses, like HPVs, can modulate gene expression to promote viral replication or carcinogenesis.

While miR expression signatures have been examined in HR HPV-expressing cancer tissues and cells, in most of these studies, differentiating cells were analyzed. Given HPVs alter epithelial cell differentiation, it is unclear whether reported changes in

miRs are directly caused by HPV gene expression or represent outcomes of HPV-induced changes in epithelial cell proliferation and differentiation. In an effort to tease apart these two different possibilities, this dissertation examines miRs modulated by expression of HPV16 E6/E7 in undifferentiated primary human epithelial cells. A more in-depth introduction to previous studies of HPV-associated miRs is provided in Chapter 2. As one might expect, the genesis of miRs is a highly regulated process and this process has been found to be disrupted in many cancers (reviewed in (Hata and Lieberman, 2015)). The details of miR biogenesis and what is known about this process in HPV-associated cancers is discussed below and in Chapter 3. Additionally, a few studies have investigated how the HPV oncoproteins alter the expression of miRs in exosomes (Chiantore *et al.*, 2016; Honegger *et al.*, 2015) and this will be further introduced below and in Chapter 4. Overall, the focus of this dissertation is to understand in more depth how HPV16 E6/E7 modulation of miRs may contribute to the transforming activities of these proteins.

1.2.2 microRNA Biogenesis

It is well known that miRs are aberrantly expressed in tumors compared to matched normal tissues (Lu *et al.*, 2005). While some studies have suggested a general repression of overall miR expression in cancers (Chang *et al.*, 2008), this is not universally true. The expression of miRs is altered in HPV-associated cancers and, our work (see Chapter 2), as well as studies by others (Wang *et al.*, 2014; Yablonska *et al.*, 2013), reveals that expression of the HPV16 E6 and/or E7 oncoproteins importantly contribute to many of the changes in miR expression observed in HPV-associated

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cancers. A key question that emerges from these observations is, "through what mechanism(s) are miRs misexpressed by expression of the HPV16 E6/E7 oncoproteins?"

Perturbation of microRNA Expression in Cancer

There are many mechanisms by which miR expression may be altered in cancers. The primary miR transcript can be subject to transcriptional activation or repression and miRs can also be silenced epigenetically via DNA methylation of the miR promoter. The stability of the mature miR, and its corresponding half-life, can also be enhanced or reduced, thereby altering miR expression in tumors. Additionally, RNA binding proteins can manipulate miR levels by interacting with the miR secondary structure or through sequence specific mechanisms (reviewed in (Adams *et al.*, 2014)). RNA editing has also been observed in tumors (Skarda *et al.*, 2009) and miRs, at various stages in their biogenesis, can be the target of editing as well (Lagana *et al.*, 2012), resulting in changes in miR expression, among other things, depending on the specific sequence edited. Competing endogenous RNAs (ceRNAs) can also act as "sponges", competing the miRs away from their intended target RNAs (Salmena *et al.*, 2011). Lastly, single nucleotide polymorphisms (SNPs) in tumors can disturb miR-target interactions and miR processing, resulting in downstream effects on miR expression (reviewed in (Adams *et al.*, 2014)).

While there are many possible mechanisms by which the activity of miRs may be altered in HPV-associated cancers, as well as by expression of HPV16 E6/E7, Chapter 3 of this dissertation examines perturbation of miR biogenesis as a potential mechanism

to alter miR expression. The miR biogenesis pathway is frequently deregulated in

cancers (reviewed in (Adams *et al.*, 2014; Hata and Lieberman, 2015)) and our focus on

the miR biogenesis pathway is based on previously published data on DROSHA and

DICER in the context of cervical cancer.

Canonical Biogenesis of microRNAs

Given the widespread effects of miR regulation on gene expression in a variety of

human diseases, including cancers, the possibility of experimentally manipulating the

miR biogenesis pathway to alter pathologic miR expression has been a subject of

extensive research. Through these studies, an improved understanding of the process

by which these small RNAs are generated has emerged.

Canonical human miR biogenesis begins with transcription of primary miR (pri-

miR) transcripts, which may include one or multiple clustered hairpin structures

containing mature miRs. Most pri-miRs are transcribed by RNA polymerase (pol) II (Cai

et al., 2004; Lee *et al.*, 2004), while a small subset of pri-miRs are transcribed by RNA

pol III (Borchert *et al.*, 2006; Canella *et al.*, 2010; Ozsolak *et al.*, 2008). Following

transcription, similar to mRNAs, pri-miRs are capped and polyadenylated. MiR hairpins

can be found in introns or exons of mRNAs or long non-coding RNAs, as well as in

intergenic regions. Primary miR transcripts are recognized and cleaved in the nucleus

by the Microprocessor complex (Denli *et al.*, 2004; Gregory *et al.*, 2004; Han *et al.*,

2004). The Microprocessor complex is minimally comprised of the RNase III enzyme,

DROSHA (Lee *et al.*, 2003), and the RNA binding protein, DGCR8 (DiGeorge critical

region 8)(Landthaler *et al.*, 2004). Microprocessor cleaves pri-miRs 10-11 base pairs

from the base of the stem loop, resulting in 55-70 nt hairpins, known as precursor-miRs (pre-miR)(Xie and Steitz, 2014). Exportin-5 (XPO5) binds pre-miRs in the nucleus, together with the co-factor Ran-GTP, followed by nuclear export to the cytoplasm via the nuclear pore complex (Bohnsack *et al.*, 2004; Lund *et al.*, 2004; Yi *et al.*, 2003). GTP-hydrolysis in the cytoplasm results in dissociation of the pre-miR from XPO5. DICER, another RNase III enzyme, recognizes structural features of the pre-miR and subsequently cleaves off the loop of the pre-miR hairpin producing the mature miR duplex of ~22 nt (Grishok *et al.*, 2001; Hutvagner *et al.*, 2001; Ketting *et al.*, 2001; Knight and Bass, 2001). The mature miR duplex is then incorporated into the RNA-induced silencing complex (RISC) containing DICER, an Argonaute protein (AGO) and the RNA binding proteins TRBP (TAR RNA binding protein)(Chendrimada *et al.*, 2005; Haase *et al.*, 2005) and PACT (protein activator of PKR)(Lee *et al.*, 2006). All four human AGO proteins (AGO1-4) are functional in miR binding and subsequent target regulation; however, the only AGO with catalytic slicer activity is AGO2 (Liu *et al.*, 2004; Meister *et al.*, 2004). Following loading of the miR duplex into the RISC complex, one strand of the miR duplex is selected as the guide strand (miR), based on the strength of base-pairing at its 5' end, to form the functional miR-RISC complex (miRISC)(Khvorova *et al.*, 2003; Schwarz *et al.*, 2003). The other strand, known as the passenger strand (miR*), is displaced and degraded. The miRISC, the final product of this carefully orchestrated maturation process, can then function to silence miR targets via mRNA degradation/destabilization, translational repression, or a combination of both mechanisms.

Non-Canonical microRNA Biogenesis

Shortly after the canonical process of human miR biogenesis was initially

described, alternative mechanisms of miR processing, involving bypass of RNAse III

cleavage steps, were discovered. These non-canonical mechanisms of miR processing

are thought to allow expression of particular miRs in different cell states or

developmental stages, resulting in differential gene expression. While non-canonical

miRs represent only a small fraction of all human miRs, the alternative mechanisms by

which these miRs are processed are conserved in different organisms (Xie and Steitz,

2014). Given our focus in Chapter 3 is on key enzymes that are part of the canonical

miR biogenesis pathway, a detailed discussion of non-canonical biogenesis pathways is

beyond the scope of this work but is reviewed in (Xie and Steitz, 2014).

Alterations of Key microRNA Biogenesis Enzymes in Cervical Cancer

Although we (see Chapter 2), and others (Wang *et al.*, 2014; Yablonska *et al.*,

2013), have shown that expression of the HPV16 oncoproteins E6 and E7 alters human

miR expression, an investigation into whether or not the HPV16 oncoproteins

manipulate the miR biogenesis machinery to alter human miR expression has not been

initiated. In Chapter 3, we investigate how expression of HPV16 E6 and/or E7 may alter

the canonical miR biogenesis pathway, with a focus on the two key enzymes in this

pathway, DROSHA and DICER. Expression of these enzymes is altered in cervical

cancers, almost all of which are caused by high-risk HPVs, in particular HPV16.

DROSHA and *DICER* are both frequently genomically altered in cancers, with

DROSHA regularly amplified or mutated and *DICER* most often mutated (Cerami *et al.*,

2012; Gao *et al.*, 2013). Specifically, the gain of chromosome 5p is observed in more than 50% of advanced cervical squamous cell carcinomas (SCC) and *DROSHA* is the most significantly upregulated transcript associated with this chromosomal gain (Muralidhar *et al.*, 2007). *DROSHA* copy number gain and overexpression is associated with differences in miR expression, increasing the levels of some miRs and decreasing the levels of others. Another study corroborated these results suggesting that 5p gain is the most frequent genetic alteration in invasive cervical cancer (Scotto *et al.*, 2008). Additionally, *DROSHA* overexpression was observed in all tumors with 5p gain, suggesting that *DROSHA* may be a critical target with a potential role in tumor progression. *DROSHA* mRNA levels were reported to be upregulated in HPV positive cervical cancer cell lines but to a lesser extent in an HPV negative cervical cancer cell line C33A (Zhou *et al.*, 2013). To determine the functional consequences of *DROSHA* copy number gain and overexpression, another study depleted DROSHA in cervical cancer cell lines and observed reduced cell migration and invasiveness (Muralidhar *et al.*, 2011). Additionally, the effect of altered DROSHA levels on miR profiles was investigated and a subset of 45 out of 319 miRs examined showed a significant association with DROSHA levels (Muralidhar *et al.*, 2011). Interestingly, they also found that increases in DROSHA expression must exceed a certain threshold before there are downstream consequences on miR expression.

DICER RNA levels have been reported to be lower in cervical cancer tissues; however, this particular study was limited by the small number of tissue samples available for analysis (Zhao *et al.*, 2014). Another group reported varied *DICER* mRNA expression among cervical cancer specimens, with low *DICER* mRNA expression in

36.7% of samples and higher *DICER* mRNA expression in 63.3% of samples (He *et al.*, 2014). An additional analysis showed that DICER protein expression was much higher in cervical cancer samples than in matched non-cancerous samples (He *et al.*, 2014).

While these data support the notion that DROSHA and DICER levels are altered in cervical tissues and cells, given HPV16 E6/E7 are the causative agents of cervical cancer at the molecular level, we hypothesize that these effects on DROSHA and DICER may be through expression of HPV16 E6/E7. Chapter 3 of this dissertation investigates the perturbation of DROSHA and DICER expression by the HPV16 oncoproteins

1.2.3 microRNAs in Extracellular Vesicles

In 1983, the observation of membranous vesicles released by exocytosis of vesicular endosomes in mammalian cells was first reported (Harding *et al.*, 1983). Initially dismissed as cellular debris, extracellular vesicles (EVs) have now emerged as important vehicles of biological signals. EVs can influence the microenvironment through transfer of various cargo, including proteins, lipids, DNA and RNA (Kanada *et al.*, 2015; Turturici *et al.*, 2014). A variety of physiological and pathological processes have been linked to EVs, including inflammation, immune disorders, neurological diseases and cancer (Cossetti *et al.*, 2014; Hazan-Halevy *et al.*, 2015; Zomer *et al.*, 2015). EVs can be isolated from numerous human body fluids including blood (Caby *et al.*, 2005), urine (Pisitkun *et al.*, 2004), semen (Vojtech *et al.*, 2014), saliva (Ogawa *et al.*, 2011), cerebral spinal fluid (Baraniskin *et al.*, 2011) and breast milk (Admyre *et al.*, 2007). This, and the fact that EVs provide information about their cell of origin, has lead

to the notion that they may serve as biomarkers for early and non-invasive disease detection. Also, since they originate from intracellular material and are not recognized by the immune system as foreign, the potential use of EVs as packaging tools for the delivery of therapeutics is being explored (Arslan *et al.*, 2013; Deregibus *et al.*, 2007; Katsuda *et al.*, 2013; Ohno *et al.*, 2013; Sahoo *et al.*, 2011).

Types of Extracellular Vesicles

Cells release multiple types of EVs, which differ in size, biogenesis and molecular composition. The term "EV" refers to distinct subpopulations of membrane-enclosed vesicles, which have been found to frequently co-purify together when current EV isolation methods are utilized. Exosomes are some of the smallest EVs (40-150 nm), which originate in endosomes. Exosomes are exocytosed into the extracellular space following accumulation in multivesicular bodies (MVBs). Vesicles of various sizes (50-1,000 nm) can also be shed from the plasma membrane via actin dependent processes and these are referred to by several names including microvesicles, ectosomes or microparticles (Akers *et al.*, 2013). Additionally, small EVs (50-500 nm) and larger apoptotic bodies (50-5,000 nm) can be released from apoptotic cells (Ihara *et al.*, 1998).

While efforts have been made to reach a consensus on vesicle nomenclature and classification, it has become clear that there is some overlap in function and composition among EV populations, making this task difficult (Klein-Scory *et al.*, 2014). Moreover, current isolation methods cannot definitively distinguish exosomes from other EVs, as the characterization of physical properties and molecular markers is still lacking

(Choi *et al.*, 2012; Haqqani *et al.*, 2013; Muralidharan-Chari *et al.*, 2009). Regardless, it is clear that cells release a variety of EVs, which all likely contribute to the biology of the diseases they are associated with, including cancer.

Cancer-Associated Extracellular Vesicles

Tumor cells utilize EVs to communicate with each other at the paracrine level but they also exchange information with the tumor microenvironment though activation of molecular pathways that are distinct from those mediated by small molecules, cytokines and growth factors (Webber *et al.*, 2015). Uptake of cancer-associated EVs by immune cells can drastically alter the immune microenvironment, leading either to escape of the immune response or activation of immune suppression. Tumor EVs can also contain pro-angiogenic factor cargo, which directly promotes endothelial cell migration and the formation of new blood vessels. This is critical for cancer cell proliferation, as the blood supplies oxygen and nutrients, as well as allows for waste removal. The tumor vascular network is even more imperative during metastatic invasion, facilitating access of tumor cells to the bloodstream (Nishida *et al.*, 2006). Depending on their cargo, cancer-associated EVs can promote an oncogenic or tumor suppressive environment.

Extracellular Vesicles and microRNAs

The discovery of miRs in EVs has greatly expanded their potential utility, allowing for new avenues of communication, additional biomarkers for detection and, since EVs are non-immunogenic, the opportunity for innovative therapeutics (Kim *et al.*, 2017). In particular, the ability of EV-associated miRs to alter gene expression, both in local and

distant environments, enhances their potential for therapeutic applications. The

packaging of miRs into EVs is also advantageous, as it has been shown to protect

these small RNAs from degradation while in transit to their targets (Hunter *et al.*, 2008;

Valadi *et al.*, 2007). It is still uncertain whether specific miRs or groups of miRs are

selectively packaged into EVs. Some miRs do appear to be actively targeted to EVs

and various mechanisms to explain this have been proposed (discussed in (Kim *et al.*,

2017)). Another unknown is the stoichiometry of miRs in EVs. It is not understood

whether all EVs in a population contain the same amount of a particular miR or whether

only specific EVs contain a particular miR.

These questions and others must be answered to fully understand the true

potential of miRs in EVs. As studies of EV-associated miRs continue, it is certain that

more will be learned about the targeted sorting, enrichment and packaging of miRs into

EVs, as well as new functions of miRs in EVs.

Extracellular Vesicles and HPVs

The first report investigating HPVs and EVs involved the silencing of HPV18

E6/E7 in HeLa cells and examination of the contents and amounts of EVs release from

HeLa cells utilizing a Survivin as a model molecule (Honegger *et al.*, 2013). This study

found that silencing of HPV18 E6/E7 in HeLa cells led to reduced protein levels of

Survivin in exosomes, as well as significantly increased the overall amount of exosomes

released from HeLa cells (Honegger *et al.*, 2013). Whether or not the HPV

oncoproteins E6 and/or E7 are present in exosomes is unclear. One study was not able

to detect either of the two proteins in exosomes from HeLa cells (Honegger *et al.*,

2013). However, a more recent study detected E6 and E7 mRNAs in exosomes

released from HFKs transduced with the HPV16 oncoproteins (Chiantore *et al.*, 2016).

Only a handful of studies have examined miRs in exosomes released from HPV

containing cells. One study examined miRs in exosomes from HPV16 E6/E7

expressing HFKs utilizing an RT-qPCR array. Interestingly, they found only 8 of the 384

miRs that were included in the array in exosomes released from HPV16 E6/E7

expressing HFKs, with miR-222 being the most highly abundant (Chiantore *et al.*, 2016).

Another study, utilized siRNAs against HPV18 E6/E7 in HPV18 positive HeLa cells and

examined the effects on miRs in exosomes by deep sequencing (Honegger *et al.*,

2015). Their data showed that expression of HPV18 E6/E7 determined a signature of

seven miRs in exosomes released from HeLa cells and these miRs possess pro-

proliferative or anti-apoptotic potential (Honegger *et al.*, 2015). The concentration of

these miRs in exosomes appeared to be dependent on expression of the viral

oncoproteins. They also found, using HeLa and SiHa cells, that there is overlap in the

exosome-associated miRs regulated by E6/E7 in HPV18 and HPV16 positive cervical

cancer cells, respectively (Honegger *et al.*, 2015).

Towards identifying miRs in exosomes with potential for non-invasive cervical

cancer screening, another study found elevated levels of exosomal miRs in

cervicovaginal lavage specimens of cervical cancer patients (Liu *et al.*, 2014). In

particular, levels of miR-21 and miR-146a were significantly higher in vesicles from HPV

positive cervical cancer patients (Liu *et al.*, 2014). Studies examining the utility of

circulating exosomal miRs in other cancers and diseases have shown promising results

(Kuwabara *et al.*, 2011; Rabinowits *et al.*, 2009; Taylor and Gercel-Taylor, 2008) but

more work is necessary to improve the reproducibility of these results between studies. While many EV biomarkers have been associated with various diseases, results from individual studies are dissimilar and this may be due to differences in EV purification methods (Taylor and Shah, 2015). Chapter 4 of this dissertation further examines how HPV16 E6 and E7 oncoprotein expression alters miR expression in EVs.

Summary and Significance

Historically, the field of PV research has experienced many exciting breakthroughs since the discovery of the first animal PV by Richard Shope in the 1930s (Shope and Hurst, 1933). Widespread implementation of the Pap smear in the 1960s greatly reduced the incidence and mortality of cervical cancer (Tambouret, 2013). The incidence of cervical cancer declined by half from 1975 to 2012 in the US and, similarly, the death rate from cervical cancer in the US in 2012 was less than half of what it was in 1975 (Society, 2016). These dramatic declines are due to early detection of cervical cancer with the Pap test and provide the most compelling validation of the concept that early detection can dramatically decrease cancer incidence and mortality. While a similar cytological test is used for early detection of anal cancer in high-risk populations (Chiao *et al.*, 2006), it is important to note that there are currently no similar procedures for the early detection of HPV-associated oropharyngeal cancers.

Before the introduction of HPV vaccines in 2006, it was estimated that one woman died every 10 minutes as a result of cervical cancer globally (Bryan *et al.*, 2016). A decline in vaccine type HPV prevalence of 56% was observed over a four year period in the US following implementation of Cervarix® and Gardasil® (Markowitz *et al.*, 2013).

These numbers are impressive given the vaccination rate was relatively low at the time of the study (~32%) (Markowitz *et al.*, 2013). While the complete benefit of vaccine protection again HPV-associated cancers and diseases will not be observed for several decades, the results thus far are extremely promising. With the advent of Gardasil 9® in 2014, and the promise of other prophylactic and therapeutic vaccines currently in development, it is likely that the burden of HPV-associated diseases and cancer will decrease even more dramatically.

Despite excellent screening methods for some HPV-associated cancers and several efficacious vaccines, there are still many open questions regarding the biology of these carcinogenic viruses and it is critically important to continue studying these viruses at the molecular level. The focus of this dissertation is on the molecular biology of HPV16 E6 and E7 and how these viral oncoproteins perturb the expression of miRs. In **Chapter 2**, we perform a systematic analysis of miRs and mRNAs altered by HPV16 E6/E7 in primary, undifferentiated human foreskin keratinocytes using deep sequencing. Pairing miRs with potential targets via bioinformatic analysis, our data show that many observed changes in mRNA expression may be due, in part, to perturbation of miRs by HPV16 E6/E7. In **Chapter 3**, we investigate perturbation of the miR biogenesis machinery by HPV16 E6/E7. Expression of the HPV16 oncoproteins increases levels of two key enzymes in the miR biogenesis pathway, DROSHA and DICER. Furthermore, manipulation of DROSHA levels may be one of many mechanisms by which HPV16 E6/E7 expression dysregulates cellular miR expression. In **Chapter 4**, we examine how expression of HPV16 E6/E7 alters the expression of miRs in EVs released from primary, undifferentiated human foreskin keratinocytes. Some miRs appear to be

similarly regulated by HPV16 E6/E7 in cells and EVs whereas others are not. These data suggest that some miRs in EVs may be promising biomarkers for expression of HPV16 E6/E7 and other miRs may be selectively packaged into EVs. Overall, this dissertation shows that modulation of miRs is an important part of HPV16 E6/E7 mediated reprogramming of cellular gene expression and may contribute to the transforming activities of these proteins.

In the long-term, we are confident that our characterization of miRs in cells and exosomes may be useful as biomarkers of HPV16 E6/E7 expression. Of equal importance, the results of this dissertation will surely be the basis of future studies further investigating the consequences of HPV16 E6/E7 manipulation of host cellular miRs.

REFERENCES

1.   **Adams, B.D., Kasinski, A.L., Slack, F.J.** (2014). Aberrant regulation and function of microRNAs in cancer. Curr Biol: 24, R762-776.

2.   **Admyre, C., Johansson, S.M., Qazi, K.R., Filen, J.J., Lahesmaa, R., Norman, M., Neve, E.P., Scheynius, A., Gabrielsson, S.** (2007). Exosomes with immune modulatory features are present in human breast milk. J Immunol: 179, 1969-1978.

3.   **Akagi, K., Li, J., Broutian, T.R., Padilla-Nash, H., Xiao, W., Jiang, B., Rocco, J.W., Teknos, T.N., Kumar, B., Wangsa, D., He, D., Ried, T., Symer, D.E., Gillison, M.L.** (2014). Genome-wide analysis of HPV integration in human cancers reveals recurrent, focal genomic instability. Genome Res: 24, 185-199.

4.   **Akers, J.C., Gonda, D., Kim, R., Carter, B.S., Chen, C.C.** (2013). Biogenesis of extracellular vesicles (EV): exosomes, microvesicles, retrovirus-like vesicles, and apoptotic bodies. J Neurooncol: 113, 1-11.

5.   **Ali, H., Donovan, B., Wand, H., Read, T.R.H., Regan, D.G., Grulich, A.E., Fairley, C.K., Guy, R.J.** (2013). Genital warts in young Australians five years into national human papillomavirus vaccination programme: national surveillance data. BMJ (Clinical research ed): 346, f2032.

6.   **Ammermann, I., Bruckner, M., Matthes, F., Iftner, T., Stubenrauch, F.** (2008). Inhibition of transcription and DNA replication by the papillomavirus E8-E2C protein is mediated by interaction with corepressor molecules. J Virol: 82, 5127-5136.

7.   **Arslan, F., Lai, R.C., Smeets, M.B., Akeroyd, L., Choo, A., Aguor, E.N., Timmers, L., van Rijen, H.V., Doevendans, P.A., Pasterkamp, G., Lim, S.K., de Kleijn, D.P.** (2013). Mesenchymal stem cell-derived exosomes increase ATP levels, decrease oxidative stress and activate PI3K/Akt pathway to enhance myocardial viability and prevent adverse remodeling after myocardial ischemia/reperfusion injury. Stem Cell Res: 10, 301-312.

8.   **Banerjee, N.S., Wang, H.K., Broker, T.R., Chow, L.T.** (2011). Human papillomavirus (HPV) E7 induces prolonged G2 following S phase reentry in differentiated human keratinocytes. J Biol Chem: 286, 15473-15482.

9.   **Baraniskin, A., Kuhnhenn, J., Schlegel, U., Chan, A., Deckert, M., Gold, R., Maghnouj, A., Zollner, H., Reinacher-Schick, A., Schmiegel, W., Hahn, S.A., Schroers, R.** (2011). Identification of microRNAs in the cerebrospinal fluid as marker for primary diffuse large B-cell lymphoma of the central nervous system. Blood: 117, 3140-3146.

10.   **Barr, E., Tamms, G.** (2007). Quadrivalent human papillomavirus vaccine. Clin Infect Dis: 45, 609-607.

11.   **Barrow-Laing, L., Chen, W., Roman, A.** (2010). Low- and high-risk human papillomavirus E7 proteins regulate p130 differently. Virology: 400, 233-239.

12.   **Beavis, A.L., Levinson, K.L.** (2016). Preventing Cervical Cancer in the United States: Barriers and Resolutions for HPV Vaccination. Front Oncol: 6, 19.

13.   **Bergant, M., Banks, L.** (2013). SNX17 facilitates infection with diverse papillomavirus types. J Virol: 87, 1270-1273.

14.   **Bergant Marusic, M., Ozbun, M.A., Campos, S.K., Myers, M.P., Banks, L.** (2012). Human papillomavirus L2 facilitates viral escape from late endosomes via sorting nexin 17. Traffic: 13, 455-467.

15.   **Bergvall, M., Melendy, T., Archambault, J.** (2013). The E1 proteins. Virology: 445, 35-56.

16.   **Bernard, B.A., Bailly, C., Lenoir, M.C., Darmon, M., Thierry, F., Yaniv, M.** (1989). The human papillomavirus type 18 (HPV18) E2 gene product is a repressor of the HPV18 regulatory region in human keratinocytes. J Virol: 63, 4317-4324.

17.   **Bernard, H.U., Burk, R.D., Chen, Z., van Doorslaer, K., zur Hausen, H., de Villiers, E.M.** (2010). Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments. Virology: 401, 70-79.

18.   **Bienkowska-Haba, M., Patel, H.D., Sapp, M.** (2009). Target cell cyclophilins facilitate human papillomavirus type 16 infection. PLoS Pathog: 5, e1000524.

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

20.   **Bohnsack, M.T., Czaplinski, K., Gorlich, D.** (2004). Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. RNA: 10, 185-191.

21.   **Bonanni, P., Bechini, A., Donato, R., Capei, R., Sacco, C., Levi, M., Boccalini, S.** (2015). Human papilloma virus vaccination: impact and recommendations across the world. Ther Adv Vaccines: 3, 3-12.

22.   **Bonifacino, J.S., Hurley, J.H.** (2008). Retromer. Curr Opin Cell Biol: 20, 427-436.

23.   **Borchert, G.M., Lanier, W., Davidson, B.L.** (2006). RNA polymerase III transcribes human microRNAs. Nat Struct Mol Biol: 13, 1097-1101.

24.   **Bray, F., Ren, J.S., Masuyer, E., Ferlay, J.** (2013). Global estimates of cancer prevalence for 27 sites in the adult population in 2008. Int J Cancer: 132, 1133-1145.

25.   **Brimer, N., Lyons, C., Wallberg, A.E., Vande Pol, S.B.** (2012). Cutaneous papillomavirus E6 oncoproteins associate with MAML1 to repress transactivation and NOTCH signaling. Oncogene: 31, 4639-4646.

26.   **Broniarczyk, J., Massimi, P., Bergant, M., Banks, L.** (2015). Human Papillomavirus Infectious Entry and Trafficking Is a Rapid Process. J Virol: 89, 8727-8732.

27.   **Brotherton, J.M.L., Fridman, M., May, C.L., Chappell, G., Saville, A.M., Gertig, D.M.** (2011). Early effect of the HPV vaccination programme on cervical abnormalities in Victoria, Australia: an ecological study. Lancet (London, England): 377, 2085-2092.

28.   **Brown, D.R., Kjaer, S.K., Sigurdsson, K., Iversen, O.E., Hernandez-Avila, M., Wheeler, C.M., Perez, G., Koutsky, L.A., Tay, E.H., Garcia, P., Ault, K.A., Garland, S.M., Leodolter, S., Olsson, S.E., Tang, G.W., Ferris, D.G., Paavonen, J., Steben, M., Bosch, F.X., Dillner, J., Joura, E.A., Kurman, R.J., Majewski, S., Munoz, N., Myers, E.R., Villa, L.L., Taddeo, F.J., Roberts, C.,**

Tadesse, A., Bryan, J., Lupinacci, L.C., Giacoletti, K.E., Sings, H.L., James, M., Hesley, T.M., Barr, E. (2009). The impact of quadrivalent human papillomavirus (HPV; types 6, 11, 16, and 18) L1 virus-like particle vaccine on infection and disease due to oncogenic nonvaccine HPV types in generally HPV-naive women aged 16-26 years. J Infect Dis: 199, 926-935.

29.   **Bryan, J.T., Buckland, B., Hammond, J., Jansen, K.U.** (2016). Prevention of cervical cancer: journey to develop the first human papillomavirus virus-like particle vaccine and the next generation vaccine. Curr Opin Chem Biol: 32, 34-47.

30.   **Buck, C.B., Day, P.M., Trus, B.L.** (2013). The papillomavirus major capsid protein L1. Virology: 445, 169-174.

31.   **Buck, C.B., Pastrana, D.V., Lowy, D.R., Schiller, J.T.** (2004). Efficient intracellular assembly of papillomaviral vectors. J Virol: 78, 751-757.

32.   **Buck, C.B., Thompson, C.D., Pang, Y.Y., Lowy, D.R., Schiller, J.T.** (2005). Maturation of papillomavirus capsids. J Virol: 79, 2839-2846.

33.   **Caby, M.P., Lankar, D., Vincendeau-Scherrer, C., Raposo, G., Bonnerot, C.** (2005). Exosomal-like vesicles are present in human blood plasma. Int Immunol: 17, 879-887.

34.   **Cai, X., Hagedorn, C.H., Cullen, B.R.** (2004). Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. RNA: 10, 1957-1966.

35.   **Cai, X., Li, G., Laimins, L.A., Cullen, B.R.** (2006). Human papillomavirus genotype 31 does not express detectable microRNA levels during latent or productive virus replication. J Virol: 80, 10890-10893.

36.   **Canella, D., Praz, V., Reina, J.H., Cousin, P., Hernandez, N.** (2010). Defining the RNA polymerase III transcriptome: Genome-wide localization of the RNA polymerase III transcription machinery in human cells. Genome Res: 20, 710-721.

37.   **CDC**, 2015. Epidemiology and Prevention of Vaccine-Preventable Diseases, 13 ed. Public Health Foundation, Washington, D.C.

38. **Cerami, E., Gao, J., Dogrusoz, U., Gross, B.E., Sumer, S.O., Aksoy, B.A., Jacobsen, A., Byrne, C.J., Heuer, M.L., Larsson, E., Antipin, Y., Reva, B., Goldberg, A.P., Sander, C., Schultz, N.** (2012). The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov: 2, 401-404.

39. **Chang, T.C., Yu, D., Lee, Y.S., Wentzel, E.A., Arking, D.E., West, K.M., Dang, C.V., Thomas-Tikhonenko, A., Mendell, J.T.** (2008). Widespread microRNA repression by Myc contributes to tumorigenesis. Nat Genet: 40, 43-50.

40. **Chaturvedi, A.K.** (2010). Beyond cervical cancer: burden of other HPV-related cancers among men and women. J Adolesc Health: 46, S20-26.

41. **Chaturvedi, A.K., Engels, E.A., Pfeiffer, R.M., Hernandez, B.Y., Xiao, W., Kim, E., Jiang, B., Goodman, M.T., Sibug-Saber, M., Cozen, W., Liu, L., Lynch, C.F., Wentzensen, N., Jordan, R.C., Altekruse, S., Anderson, W.F., Rosenberg, P.S., Gillison, M.L.** (2011). Human papillomavirus and rising oropharyngeal cancer incidence in the United States. J Clin Oncol: 29, 4294-4301.

42. **Chendrimada, T.P., Gregory, R.I., Kumaraswamy, E., Norman, J., Cooch, N., Nishikura, K., Shiekhattar, R.** (2005). TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. Nature: 436, 740-744.

43. **Chiantore, M.V., Mangino, G., Iuliano, M., Zangrillo, M.S., De Lillis, I., Vaccari, G., Accardi, R., Tommasino, M., Columba Cabezas, S., Federico, M., Fiorucci, G., Romeo, G.** (2016). Human papillomavirus E6 and E7 oncoproteins affect the expression of cancer-related microRNAs: additional evidence in HPV-induced tumorigenesis. J Cancer Res Clin Oncol: 142, 1751-1763.

44. **Chiao, E.Y., Giordano, T.P., Palefsky, J.M., Tyring, S., El Serag, H.** (2006). Screening HIV-infected individuals for anal cancer precursor lesions: a systematic review. Clin Infect Dis: 43, 223-233.

45. **Choi, D.S., Choi, D.Y., Hong, B.S., Jang, S.C., Kim, D.K., Lee, J., Kim, Y.K., Kim, K.P., Gho, Y.S.** (2012). Quantitative proteomics of extracellular vesicles derived from human primary and metastatic colorectal cancer cells. J Extracell Vesicles: 1.

46.   **Cossetti, C., Iraci, N., Mercer, T.R., Leonardi, T., Alpi, E., Drago, D., Alfaro-Cervello, C., Saini, H.K., Davis, M.P., Schaeffer, J., Vega, B., Stefanini, M., Zhao, C., Muller, W., Garcia-Verdugo, J.M., Mathivanan, S., Bachi, A., Enright, A.J., Mattick, J.S., Pluchino, S.** (2014). Extracellular vesicles from neural stem cells transfer IFN-gamma via Ifngr1 to activate Stat1 signaling in target cells. Mol Cell: 56, 193-204.

47.   **Cruz, L., Biryukov, J., Conway, M.J., Meyers, C.** (2015). Cleavage of the HPV16 Minor Capsid Protein L2 during Virion Morphogenesis Ablates the Requirement for Cellular Furin during De Novo Infection. Viruses: 7, 5813-5830.

48.   **Cubie, H.A.** (2013). Diseases associated with human papillomavirus infection. Virology: 445, 21-34.

49.   **Darshan, M.S., Lucchi, J., Harding, E., Moroianu, J.** (2004). The l2 minor capsid protein of human papillomavirus type 16 interacts with a network of nuclear import receptors. J Virol: 78, 12179-12188.

50.   **Day, P.M., Baker, C.C., Lowy, D.R., Schiller, J.T.** (2004). Establishment of papillomavirus infection is enhanced by promyelocytic leukemia protein (PML) expression. Proc Natl Acad Sci U S A: 101, 14252-14257.

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

52.   **Day, P.M., Schelhaas, M.** (2014). Concepts of papillomavirus entry into host cells. Curr Opin Virol: 4, 24-31.

53.   **Day, P.M., Schiller, J.T.** (2009). The role of furin in papillomavirus infection. Future microbiology: 4, 1255-1262.

54.   **de Martel, C., Ferlay, J., Franceschi, S., Vignat, J., Bray, F., Forman, D., Plummer, M.** (2012). Global burden of cancers attributable to infections in 2008: a review and synthetic analysis. Lancet Oncol: 13, 607-615.

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

56. **Denli, A.M., Tops, B.B., Plasterk, R.H., Ketting, R.F., Hannon, G.J.** (2004). Processing of primary microRNAs by the Microprocessor complex. Nature: 432, 231-235.

57. **Deregibus, M.C., Cantaluppi, V., Calogero, R., Lo Iacono, M., Tetta, C., Biancone, L., Bruno, S., Bussolati, B., Camussi, G.** (2007). Endothelial progenitor cell derived microvesicles activate an angiogenic program in endothelial cells by a horizontal transfer of mRNA. Blood: 110, 2440-2448.

58. **DiMaio, D., Petti, L.M.** (2013). The E5 proteins. Virology: 445, 99-114.

59. **Dollard, S.C., Wilson, J.L., Demeter, L.M., Bonnez, W., Reichman, R.C., Broker, T.R., Chow, L.T.** (1992). Production of human papillomavirus and modulation of the infectious program in epithelial raft cultures. OFF. Genes Dev: 6, 1131-1142.

60. **Doorbar, J.** (2013). The E4 protein; structure, function and patterns of expression. Virology: 445, 80-98.

61. **Doorbar, J., Campbell, D., Grand, R.J., Gallimore, P.H.** (1986). Identification of the human papilloma virus-1a E4 gene products. EMBO J: 5, 355-362.

62. **Doorbar, J., Egawa, N., Griffin, H., Kranjec, C., Murakami, I.** (2015). Human papillomavirus molecular biology and disease association. Rev Med Virol: 25 Suppl 1, 2-23.

63. **Dyson, N., Howley, P.M., Munger, K., Harlow, E.** (1989). The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science: 243, 934-937.

64. **Edwards, T.G., Helmus, M.J., Koeller, K., Bashkin, J.K., Fisher, C.** (2013). Human papillomavirus episome stability is reduced by aphidicolin and controlled by DNA damage response pathways. Journal of virology: 87, 3979-3989.

65. **Egawa, K.** (2003). Do human papillomaviruses target epidermal stem cells? Dermatology: 207, 251-254.

66. **Favre, M., Orth, G., Croissant, O., Yaniv, M.** (1975). Human papillomavirus DNA: physical map. Proc Natl Acad Sci U S A: 72, 4810-4814.

67. **Felsani, A., Mileo, A.M., Paggi, M.G.** (2006). Retinoblastoma family proteins as key targets of the small DNA virus oncoproteins. Oncogene: 25, 5277-5285.

68. **Florin, L., Becker, K.A., Lambert, C., Nowak, T., Sapp, C., Strand, D., Streeck, R.E., Sapp, M.** (2006). Identification of a dynein interacting domain in the papillomavirus minor capsid protein l2. J Virol: 80, 6691-6696.

69. **Forman, D., de Martel, C., Lacey, C.J., Soerjomataram, I., Lortet-Tieulent, J., Bruni, L., Vignat, J., Ferlay, J., Bray, F., Plummer, M., Franceschi, S.** (2012). Global burden of human papillomavirus and related diseases. Vaccine: 30 Suppl 5, F12-23.

70. **Fradet-Turcotte, A., Bergeron-Labrecque, F., Moody, C.A., Lehoux, M., Laimins, L.A., Archambault, J.** (2011). Nuclear accumulation of the papillomavirus E1 helicase blocks S-phase progression and triggers an ATM-dependent DNA damage response. Journal of virology: 85, 8996-9012.

71. **Galloway, D.A., Gewin, L.C., Myers, H., Luo, W., Grandori, C., Katzenellenbogen, R.A., McDougall, J.K.** (2005). Regulation of telomerase by human papillomaviruses. Cold Spring Harb Symp Quant Biol: 70, 209-215.

72. **Gao, J., Aksoy, B.A., Dogrusoz, U., Dresdner, G., Gross, B., Sumer, S.O., Sun, Y., Jacobsen, A., Sinha, R., Larsson, E., Cerami, E., Sander, C., Schultz, N.** (2013). Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Signal: 6, pl1.

73. **Garland, S.M., Hernandez-Avila, M., Wheeler, C.M., Perez, G., Harper, D.M., Leodolter, S., Tang, G.W.K., Ferris, D.G., Steben, M., Bryan, J., Taddeo, F.J., Railkar, R., Esser, M.T., Sings, H.L., Nelson, M., Boslego, J., Sattler, C., Barr, E., Koutsky, L.A., Females United to Unilaterally Reduce Endo/Ectocervical Disease, I.I.** (2007). Quadrivalent vaccine against human papillomavirus to prevent anogenital diseases. The New England journal of medicine: 356, 1928-1943.

74. **Gewin, L., Galloway, D.A.** (2001). E box-dependent activation of telomerase by human papillomavirus type 16 E6 does not require induction of c-myc. J Virol: 75, 7198-7201.

75. **Gillison, M.L., Chaturvedi, A.K., Lowy, D.R.** (2008). HPV prophylactic vaccines and the potential prevention of noncervical cancers in both men and women. Cancer: 113, 3036-3046.

76. **Gregory, R.I., Yan, K.P., Amuthan, G., Chendrimada, T., Doratotaj, B., Cooch, N., Shiekhattar, R.** (2004). The Microprocessor complex mediates the genesis of microRNAs. Nature: 432, 235-240.

77. **Grishok, A., Pasquinelli, A.E., Conte, D., Li, N., Parrish, S., Ha, I., Baillie, D.L., Fire, A., Ruvkun, G., Mello, C.C.** (2001). Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing. Cell: 106, 23-34.

78. **Group, F.I.S.** (2007). Quadrivalent vaccine against human papillomavirus to prevent high-grade cervical lesions. The New England journal of medicine: 356, 1915-1927.

79. **Gunasekharan, V., Laimins, L.A.** (2013). Human papillomaviruses modulate microRNA 145 expression to directly control genome amplification. J Virol: 87, 6037-6043.

80. **Haase, A.D., Jaskiewicz, L., Zhang, H., Laine, S., Sack, R., Gatignol, A., Filipowicz, W.** (2005). TRBP, a regulator of cellular PKR and HIV-1 virus expression, interacts with Dicer and functions in RNA silencing. EMBO Rep: 6, 961-967.

81. **Han, J., Lee, Y., Yeom, K.H., Kim, Y.K., Jin, H., Kim, V.N.** (2004). The Drosha-DGCR8 complex in primary microRNA processing. Genes Dev: 18, 3016-3027.

82. **Haqqani, A.S., Delaney, C.E., Tremblay, T.L., Sodja, C., Sandhu, J.K., Stanimirovic, D.B.** (2013). Method for isolation and molecular characterization of extracellular microvesicles released from brain endothelial cells. Fluids Barriers CNS: 10, 4.

83. **Harding, C., Heuser, J., Stahl, P.** (1983). Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. J Cell Biol: 97, 329-339.

84. **Harper, D.M., Franco, E.L., Wheeler, C.M., Moscicki, A.-B., Romanowski, B., Roteli-Martins, C.M., Jenkins, D., Schuind, A., Costa Clemens, S.A., Dubin, G., group, H.P.V.V.S.** (2006). Sustained efficacy up to 4.5 years of a bivalent L1 virus-like particle vaccine against human papillomavirus types 16 and 18: follow-up from a randomised control trial. Lancet (London, England): 367, 1247-1255.

85. **Hata, A., Lieberman, J.** (2015). Dysregulation of microRNA biogenesis and gene silencing in cancer. Sci Signal: 8, re3.

86. **Hazan-Halevy, I., Rosenblum, D., Weinstein, S., Bairey, O., Raanani, P., Peer, D.** (2015). Cell-specific uptake of mantle cell lymphoma-derived exosomes by malignant and non-malignant B-lymphocytes. Cancer Lett: 364, 59-69.

87. **He, L., Wang, H.Y., Zhang, L., Huang, L., Li, J.D., Xiong, Y., Zhang, M.Y., Jia, W.H., Yun, J.P., Luo, R.Z., Zheng, M.** (2014). Prognostic significance of low DICER expression regulated by miR-130a in cervical cancer. Cell Death Dis: 5, e1205.

88. **Herfs, M., Yamamoto, Y., Laury, A., Wang, X., Nucci, M.R., McLaughlin-Drubin, M.E., Munger, K., Feldman, S., McKeon, F.D., Xian, W., Crum, C.P.** (2012). A discrete population of squamocolumnar junction cells implicated in the pathogenesis of cervical cancer. Proc Natl Acad Sci U S A: 109, 10516-10521.

89. **Hildesheim, A., Herrero, R., Wacholder, S., Rodriguez, A.C., Solomon, D., Bratti, M.C., Schiller, J.T., Gonzalez, P., Dubin, G., Porras, C., Jimenez, S.E., Lowy, D.R., Costa Rican, H.P.V.V.T.G.** (2007). Effect of human papillomavirus 16/18 L1 viruslike particle vaccine among young women with preexisting infection: a randomized trial. JAMA: 298, 743-753.

90. **Hippelainen, M.I., Hippelainen, M., Saarikoski, S., Syrjanen, K.** (1994). Clinical course and prognostic factors of human papillomavirus infections in men. Sex Transm Dis: 21, 272-279.

91. **Honegger, A., Leitz, J., Bulkescher, J., Hoppe-Seyler, K., Hoppe-Seyler, F.** (2013). Silencing of human papillomavirus (HPV) E6/E7 oncogene expression affects both the contents and the amounts of extracellular microvesicles released from HPV-positive cancer cells. Int J Cancer: 133, 1631-1642.

92. **Honegger, A., Schilling, D., Bastian, S., Sponagel, J., Kuryshev, V., Sultmann, H., Scheffner, M., Hoppe-Seyler, K., Hoppe-Seyler, F.** (2015). Dependence of intracellular and exosomal microRNAs on viral E6/E7 oncogene expression in HPV-positive tumor cells. PLoS Pathog: 11, e1004712.

93. **Hong, S., Laimins, L.A.** (2013a). Regulation of the life cycle of HPVs by differentiation and the DNA damage response. Future Microbiol: 8, 1547-1557.

94. **Hong, S., Laimins, L.A.** (2013b). Regulation of the life cycle of HPVs by differentiation and the DNA damage response. Future microbiology: 8, 1547-1557.

95. **Huh, W.K., Ault, K.A., Chelmow, D., Davey, D.D., Goulart, R.A., Garcia, F.A., Kinney, W.K., Massad, L.S., Mayeaux, E.J., Saslow, D., Schiffman, M., Wentzensen, N., Lawson, H.W., Einstein, M.H.** (2015). Use of primary high-risk human papillomavirus testing for cervical cancer screening: interim clinical guidance. Obstet Gynecol: 125, 330-337.

96. **Hunter, M.P., Ismail, N., Zhang, X., Aguda, B.D., Lee, E.J., Yu, L., Xiao, T., Schafer, J., Lee, M.L., Schmittgen, T.D., Nana-Sinkam, S.P., Jarjoura, D., Marsh, C.B.** (2008). Detection of microRNA expression in human peripheral blood microvesicles. PLoS One: 3, e3694.

97. **Huntzinger, E., Izaurralde, E.** (2011). Gene silencing by microRNAs: contributions of translational repression and mRNA decay. Nat Rev Genet: 12, 99-110.

98. **Hutvagner, G., McLachlan, J., Pasquinelli, A.E., Balint, E., Tuschl, T., Zamore, P.D.** (2001). A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science: 293, 834-838.

99. **Ihara, T., Yamamoto, T., Sugamata, M., Okumura, H., Ueno, Y.** (1998). The process of ultrastructural changes from nuclei to apoptotic body. Virchows Arch: 433, 443-447.

100. **Ingle, A., Ghim, S., Joh, J., Chepkoech, I., Bennett Jenson, A., Sundberg, J.P.** (2011). Novel laboratory mouse papillomavirus (MusPV) infection. Vet Pathol: 48, 500-505.

101. **Isaacson Wechsler, E., Wang, Q., Roberts, I., Pagliarulo, E., Jackson, D., Untersperger, C., Coleman, N., Griffin, H., Doorbar, J.** (2012). Reconstruction of human papillomavirus type 16-mediated early-stage neoplasia implicates E6/E7 deregulation and the loss of contact inhibition in neoplastic progression. J Virol: 86, 6358-6364.

102. **Jeon, S., Lambert, P.F.** (1995). Integration of human papillomavirus type 16 DNA into the human genome leads to increased stability of E6 and E7 mRNAs: implications for cervical carcinogenesis. Proc Natl Acad Sci U S A: 92, 1654-1658.

103. **Jha, S., Vande Pol, S., Banerjee, N.S., Dutta, A.B., Chow, L.T., Dutta, A.** (2010). Destabilization of TIP60 by human papillomavirus E6 results in attenuation of TIP60-dependent transcriptional regulation and apoptotic pathway. Mol Cell: 38, 700-711.

104. **Johannsen, E., Lambert, P.F.** (2013). Epigenetics of human papillomaviruses. Virology: 445, 205-212.

105. **Joura, E.A., Giuliano, A.R., Iversen, O.E., Bouchard, C., Mao, C., Mehlsen, J., Moreira, E.D., Jr., Ngan, Y., Petersen, L.K., Lazcano-Ponce, E., Pitisuttithum, P., Restrepo, J.A., Stuart, G., Woelber, L., Yang, Y.C., Cuzick, J., Garland, S.M., Huh, W., Kjaer, S.K., Bautista, O.M., Chan, I.S., Chen, J., Gesser, R., Moeller, E., Ritter, M., Vuocolo, S., Luxembourg, A., Broad Spectrum, H.P.V.V.S.** (2015). A 9-valent HPV vaccine against infection and intraepithelial neoplasia in women. N Engl J Med: 372, 711-723.

106. **Kanada, M., Bachmann, M.H., Hardy, J.W., Frimannson, D.O., Bronsart, L., Wang, A., Sylvester, M.D., Schmidt, T.L., Kaspar, R.L., Butte, M.J., Matin, A.C., Contag, C.H.** (2015). Differential fates of biomolecules delivered to target cells via extracellular vesicles. Proc Natl Acad Sci U S A: 112, E1433-1442.

107. **Katsuda, T., Tsuchiya, R., Kosaka, N., Yoshioka, Y., Takagaki, K., Oki, K., Takeshita, F., Sakai, Y., Kuroda, M., Ochiya, T.** (2013). Human adipose tissue-derived mesenchymal stem cells secrete functional neprilysin-bound exosomes. Sci Rep: 3, 1197.

108. **Kaur, P., Li, A.** (2000). Adhesive properties of human basal epidermal cells: an analysis of keratinocyte stem cells, transit amplifying cells, and postmitotic differentiating cells. J Invest Dermatol: 114, 413-420.

109. **Ketting, R.F., Fischer, S.E., Bernstein, E., Sijen, T., Hannon, G.J., Plasterk, R.H.** (2001). Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. Genes Dev: 15, 2654-2659.

110. **Khvorova, A., Reynolds, A., Jayasena, S.D.** (2003). Functional siRNAs and miRNAs exhibit strand bias. Cell: 115, 209-216.

111. **Kim, K.M., Abdelmohsen, K., Mustapic, M., Kapogiannis, D., Gorospe, M.** (2017). RNA in extracellular vesicles. Wiley Interdiscip Rev RNA.

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

113. **Kiyono, T., Hiraiwa, A., Fujita, M., Hayashi, Y., Akiyama, T., Ishibashi, M.** (1997). Binding of high-risk human papillomavirus E6 oncoproteins to the human homologue of the Drosophila discs large tumor suppressor protein. Proc Natl Acad Sci U S A: 94, 11612-11616.

114. **Klein-Scory, S., Tehrani, M.M., Eilert-Micus, C., Adamczyk, K.A., Wojtalewicz, N., Schnolzer, M., Hahn, S.A., Schmiegel, W., Schwarte-Waldhoff, I.** (2014). New insights in the composition of extracellular vesicles from pancreatic cancer cells: implications for biomarkers and functions. Proteome Sci: 12, 50.

115. **Klingelhutz, A.J., Foster, S.A., McDougall, J.K.** (1996). Telomerase activation by the E6 gene product of human papillomavirus type 16. Nature: 380, 79-82.

116. **Klingelhutz, A.J., Roman, A.** (2012). Cellular transformation by human papillomaviruses: lessons learned by comparing high- and low-risk viruses. Virology: 424, 77-98.

117. **Knight, S.W., Bass, B.L.** (2001). A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in Caenorhabditis elegans. Science: 293, 2269-2271.

118. **Knipe, D.M., Howley, P.M.**, 2013. Fields virology, 6th ed. Wolters Kluwer/Lippincott Williams & Wilkins Health, Philadelphia, PA.

119. **Kuwabara, Y., Ono, K., Horie, T., Nishi, H., Nagao, K., Kinoshita, M., Watanabe, S., Baba, O., Kojima, Y., Shizuta, S., Imai, M., Tamura, T., Kita, T., Kimura, T.** (2011). Increased microRNA-1 and microRNA-133a levels in serum of patients with cardiovascular disease indicate myocardial damage. Circ Cardiovasc Genet: 4, 446-454.

120. **Lagana, A., Paone, A., Veneziano, D., Cascione, L., Gasparini, P., Carasi, S., Russo, F., Nigita, G., Macca, V., Giugno, R., Pulvirenti, A., Shasha, D., Ferro, A., Croce, C.M.** (2012). miR-EdiTar: a database of predicted A-to-I edited miRNA target sites. Bioinformatics: 28, 3166-3168.

121. **Landthaler, M., Yalcin, A., Tuschl, T.** (2004). The human DiGeorge syndrome critical region gene 8 and Its D. melanogaster homolog are required for miRNA biogenesis. Curr Biol: 14, 2162-2167.

122. **Lee, S.S., Weiss, R.S., Javier, R.T.** (1997). Binding of human virus oncoproteins to hDlg/SAP97, a mammalian homolog of the Drosophila discs large tumor suppressor protein. Proc Natl Acad Sci U S A: 94, 6670-6675.

123. **Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Radmark, O., Kim, S., Kim, V.N.** (2003). The nuclear RNase III Drosha initiates microRNA processing. Nature: 425, 415-419.

124. **Lee, Y., Hur, I., Park, S.Y., Kim, Y.K., Suh, M.R., Kim, V.N.** (2006). The role of PACT in the RNA silencing pathway. EMBO J: 25, 522-532.

125. **Lee, Y., Kim, M., Han, J., Yeom, K.H., Lee, S., Baek, S.H., Kim, V.N.** (2004). MicroRNA genes are transcribed by RNA polymerase II. EMBO J: 23, 4051-4060.

126. **Liu, J., Carmell, M.A., Rivas, F.V., Marsden, C.G., Thomson, J.M., Song, J.J., Hammond, S.M., Joshua-Tor, L., Hannon, G.J.** (2004). Argonaute2 is the catalytic engine of mammalian RNAi. Science: 305, 1437-1441.

127. **Liu, J., Sun, H., Wang, X., Yu, Q., Li, S., Yu, X., Gong, W.** (2014). Increased exosomal microRNA-21 and microRNA-146a levels in the cervicovaginal lavage specimens of patients with cervical cancer. Int J Mol Sci: 15, 758-773.

128. **Lu, J., Getz, G., Miska, E.A., Alvarez-Saavedra, E., Lamb, J., Peck, D., Sweet-Cordero, A., Ebert, B.L., Mak, R.H., Ferrando, A.A., Downing, J.R., Jacks, T., Horvitz, H.R., Golub, T.R.** (2005). MicroRNA expression profiles classify human cancers. Nature: 435, 834-838.

129. **Lund, E., Guttinger, S., Calado, A., Dahlberg, J.E., Kutay, U.** (2004). Nuclear export of microRNA precursors. Science: 303, 95-98.

130. **Maglennon, G.A., McIntosh, P., Doorbar, J.** (2011). Persistence of viral DNA in the epithelial basal layer suggests a model for papillomavirus latency following immune regression. Virology: 414, 153-163.

131. **Marais, D., Gawarecki, D., Allan, B., Ahmed, K., Altini, L., Cassim, N., Gopolang, F., Hoffman, M., Ramjee, G., Williamson, A.L.** (2011). The effectiveness of Carraguard, a vaginal microbicide, in protecting women against high-risk human papillomavirus infection. Antivir Ther: 16, 1219-1226.

132. **Markowitz, L.E., Dunne, E.F., Saraiya, M., Chesson, H.W., Curtis, C.R., Gee, J., Bocchini, J.A., Jr., Unger, E.R., Centers for Disease, C., Prevention**. (2014). Human papillomavirus vaccination: recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Recommendations and reports : Morbidity and mortality weekly report Recommendations and reports / Centers for Disease Control: 63, 1-30.

133. **Markowitz, L.E., Hariri, S., Lin, C., Dunne, E.F., Steinau, M., McQuillan, G., Unger, E.R.** (2013). Reduction in human papillomavirus (HPV) prevalence among young women following HPV vaccine introduction in the United States, National Health and Nutrition Examination Surveys, 2003-2010. J Infect Dis: 208, 385-393.

134. **Matsukura, T., Koi, S., Sugase, M.** (1989). Both episomal and integrated forms of human papillomavirus type 16 are involved in invasive cervical cancers. Virology: 172, 63-72.

135. **McBride, A.A.** (2013). The papillomavirus E2 proteins. Virology: 445, 57-79.

136. **McKenna, D.J., McDade, S.S., Patel, D., McCance, D.J.** (2010). MicroRNA 203 expression in keratinocytes is dependent on regulation of p53 levels by E6. J Virol: 84, 10644-10652.

137. **Meister, G., Landthaler, M., Patkaniowska, A., Dorsett, Y., Teng, G., Tuschl, T.** (2004). Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. Mol Cell: 15, 185-197.

138. **Melar-New, M., Laimins, L.A.** (2010). Human papillomaviruses modulate expression of microRNA 203 upon epithelial differentiation to control levels of p63 proteins. J Virol: 84, 5212-5221.

139. **Mendoza, J.A., Jacob, Y., Cassonnet, P., Favre, M.** (2006). Human papillomavirus type 5 E6 oncoprotein represses the transforming growth factor beta signaling pathway by binding to SMAD3. J Virol: 80, 12420-12424.

140. **Merle, E., Rose, R.C., LeRoux, L., Moroianu, J.** (1999). Nuclear import of HPV11 L1 capsid protein is mediated by karyopherin alpha2beta1 heterodimers. J Cell Biochem: 74, 628-637.

141. **Mesher, D., Soldan, K., Howell-Jones, R., Panwar, K., Manyenga, P., Jit, M., Beddows, S., Gill, O.N.** (2013). Reduction in HPV 16/18 prevalence in sexually active young women following the introduction of HPV immunisation in England. Vaccine: 32, 26-32.

142. **Meyers, C., Frattini, M.G., Hudson, J.B., Laimins, L.A.** (1992). Biosynthesis of human papillomavirus from a continuous cell line upon epithelial differentiation. Science: 257, 971-973.

143. **Meyers, J.M., Munger, K.** (2014). The viral etiology of skin cancer. J Invest Dermatol: 134, E29-32.

144. **Moody, C.A., Laimins, L.A.** (2010). Human papillomavirus oncoproteins: pathways to transformation. Nat Rev Cancer: 10, 550-560.

145. **Munger, K., Hayakawa, H., Nguyen, C.L., Melquiot, N.V., Duensing, A., Duensing, S.** (2006). Viral carcinogenesis and genomic instability. EXS, 179-199.

146. **Munger, K., Howley, P.M.** (2002). Human papillomavirus immortalization and transformation functions. Virus Res: 89, 213-228.

147. **Munoz, N., Kjaer, S.K., Sigurdsson, K., Iversen, O.-E., Hernandez-Avila, M., Wheeler, C.M., Perez, G., Brown, D.R., Koutsky, L.A., Tay, E.H., Garcia, P.J., Ault, K.A., Garland, S.M., Leodolter, S., Olsson, S.-E., Tang, G.W.K., Ferris, D.G., Paavonen, J., Steben, M., Bosch, F.X., Dillner, J., Huh, W.K., Joura, E.A., Kurman, R.J., Majewski, S., Myers, E.R., Villa, L.L., Taddeo, F.J., Roberts, C., Tadesse, A., Bryan, J.T., Lupinacci, L.C., Giacoletti, K.E.D., Sings, H.L., James, M.K., Hesley, T.M., Barr, E., Haupt, R.M.** (2010). Impact of human papillomavirus (HPV)-6/11/16/18 vaccine on all HPV-associated genital diseases in young women. Journal of the National Cancer Institute: 102, 325-339.

148. **Muralidhar, B., Goldstein, L.D., Ng, G., Winder, D.M., Palmer, R.D., Gooding, E.L., Barbosa-Morais, N.L., Mukherjee, G., Thorne, N.P., Roberts, I., Pett, M.R., Coleman, N.** (2007). Global microRNA profiles in cervical squamous cell carcinoma depend on Drosha expression levels. J Pathol: 212, 368-377.

149.   **Muralidhar, B., Winder, D., Murray, M., Palmer, R., Barbosa-Morais, N., Saini, H., Roberts, I., Pett, M., Coleman, N.** (2011). Functional evidence that Drosha overexpression in cervical squamous cell carcinoma affects cell phenotype and microRNA profiles. J Pathol: 224, 496-507.

150.   **Muralidharan-Chari, V., Clancy, J., Plou, C., Romao, M., Chavrier, P., Raposo, G., D'Souza-Schorey, C.** (2009). ARF6-regulated shedding of tumor cell-derived plasma membrane microvesicles. Curr Biol: 19, 1875-1885.

151.   **Nakahara, T., Peh, W.L., Doorbar, J., Lee, D., Lambert, P.F.** (2005). Human papillomavirus type 16 E1circumflexE4 contributes to multiple facets of the papillomavirus life cycle. J Virol: 79, 13150-13165.

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

153.   **Nishida, N., Yano, H., Nishida, T., Kamura, T., Kojiro, M.** (2006). Angiogenesis in cancer. Vasc Health Risk Manag: 2, 213-219.

154.   **Nygard, M., Saah, A., Munk, C., Tryggvadottir, L., Enerly, E., Hortlund, M., Sigurdardottir, L.G., Vuocolo, S., Kjaer, S.K., Dillner, J.** (2015). Evaluation of the Long-Term Anti-Human Papillomavirus 6 (HPV6), 11, 16, and 18 Immune Responses Generated by the Quadrivalent HPV Vaccine. Clin Vaccine Immunol: 22, 943-948.

155.   **Ogawa, Y., Miura, Y., Harazono, A., Kanai-Azuma, M., Akimoto, Y., Kawakami, H., Yamaguchi, T., Toda, T., Endo, T., Tsubuki, M., Yanoshita, R.** (2011). Proteomic analysis of two types of exosomes in human whole saliva. Biol Pharm Bull: 34, 13-23.

156.   **Ohno, S.I., Takanashi, M., Sudo, K., Ueda, S., Ishikawa, A., Matsuyama, N., Fujita, K., Mizutani, T., Ohgi, T., Ochiya, T., Gotoh, N., Kuroda, M.** (2013). Systemically Injected Exosomes Targeted to EGFR Deliver Antitumor MicroRNA to Breast Cancer Cells. Mol Ther: 21, 185-191.

157.   **Ojesina, A.I., Lichtenstein, L., Freeman, S.S., Pedamallu, C.S., Imaz-Rosshandler, I., Pugh, T.J., Cherniack, A.D., Ambrogio, L., Cibulskis, K., Bertelsen, B., Romero-Cordoba, S., Trevino, V., Vazquez-Santillan, K.,**

Guadarrama, A.S., Wright, A.A., Rosenberg, M.W., Duke, F., Kaplan, B., Wang, R., Nickerson, E., Walline, H.M., Lawrence, M.S., Stewart, C., Carter, S.L., McKenna, A., Rodriguez-Sanchez, I.P., Espinosa-Castilla, M., Woie, K., Bjorge, L., Wik, E., Halle, M.K., Hoivik, E.A., Krakstad, C., Gabino, N.B., Gomez-Macias, G.S., Valdez-Chapa, L.D., Garza-Rodriguez, M.L., Maytorena, G., Vazquez, J., Rodea, C., Cravioto, A., Cortes, M.L., Greulich, H., Crum, C.P., Neuberg, D.S., Hidalgo-Miranda, A., Escareno, C.R., Akslen, L.A., Carey, T.E., Vintermyr, O.K., Gabriel, S.B., Barrera-Saldana, H.A., Melendez-Zajgla, J., Getz, G., Salvesen, H.B., Meyerson, M. (2014). Landscape of genomic alterations in cervical carcinomas. Nature: 506, 371-375.

158. **Orth, G.** (2008). Host defenses against human papillomaviruses: lessons from epidermodysplasia verruciformis. Curr Top Microbiol Immunol: 321, 59-83.

159. **Orth, G., Jablonska, S., Favre, M., Croissant, O., Jarzabek-Chorzelska, M., Rzesa, G.** (1978). Characterization of two types of human papillomaviruses in lesions of epidermodysplasia verruciformis. Proc Natl Acad Sci U S A: 75, 1537-1541.

160. **Ozsolak, F., Poling, L.L., Wang, Z., Liu, H., Liu, X.S., Roeder, R.G., Zhang, X., Song, J.S., Fisher, D.E.** (2008). Chromatin structure analyses identify miRNA promoters. Genes Dev: 22, 3172-3183.

161. **Paavonen, J., Naud, P., Salmeron, J., Wheeler, C.M., Chow, S.N., Apter, D., Kitchener, H., Castellsague, X., Teixeira, J.C., Skinner, S.R., Hedrick, J., Jaisamrarn, U., Limson, G., Garland, S., Szarewski, A., Romanowski, B., Aoki, F.Y., Schwarz, T.F., Poppe, W.A.J., Bosch, F.X., Jenkins, D., Hardt, K., Zahaf, T., Descamps, D., Struyf, F., Lehtinen, M., Dubin, G., Group, H.P.S., Denham, I., Garland, S., Mindel, A., O'Sullivan, M., Skinner, S.R., Waddell, R., de Carvalho, N., Naud, P., Teixeira, J.C., De Sutter, P., Poppe, W.A.J., Tjalma, W., Aoki, F.Y., Diaz-Mitoma, F., Dionne, M., Ferguson, L., Miller, M., Papp, K., Ramjattan, B., Romanowski, B., Somani, R., Apter, D., Astikainen, S., Karppa, T., Kekki, M., Keranen, H., Kudjoi, N., Kuortti, M., Kupari, M., Kyha-Osterlund, L., Isaksson, R., Lehtinen, M., Levanen, H., Liljamo, T., Loonberg, K., Niemi, L., Paavonen, J., Palmroth, J., Petaja, T., Rekonen, S., Romppanen, U., Siitari-Mattila, M., Tuomivaara, L., Vilkki, M., Belling, K.H., Gent, T., Grubert, T., Harlfinger, W., Holst, A., Hopker, W.D., Jensen-El Tobgui, S., Merder, G., Peters, K., Schoenian, S., Schulze, K., Schwarz, T., Wackernagel, C., Boselli, F., Mojana, G., Salmeron, J., Benitez, G., Crisostomo, C., Del Rosario-Raymundo, R., Germar, M.J., Limson, G., Raymundo, J., Remollino, M.C., Villanueva, G., Villanueva, S., Zamora, J.D., Zamora, L., Bajo, J., Bayas, J., Campins, M., Castellsague, X., Castro, M., Centeno, C., Cruzet, F., Rodriguez, L., Torne, A., Vidart, J.A., Chow, S.N., Yu, M.H., Yuan, C.C., Huang, S.C., Ho, H.N., Chen, R.J., Lin, H.H., Chu, T.Y.,

Angsuwathana, S., Jaisamrarn, U., Wilawan, K., Abdulhakim, E., Cruickshank, M., Kitchener, H., Lewis, D., Pavel, I., Robinson, J., Szarewski, A., Ackerman, R., Ault, K., Bennett, N., Caldwell, M., Chambers, C., Chatterjee, A., Civitarese, L., Demars, L., De Santis, T., Downs, L., Ferris, D., Fine, P., Gall, S., Harper, D., Hedrick, J., Herzig, W., Hiraoka, M., Huh, W., Kamemoto, L., Klein, T., Koltun, W., Kong, A., Lalezari, J., Lee, P., Leeman, L., Luber, S., Martens, M., Michelson, J., Nebel, W., Peterson, C., Pitts, K., Rosen, J., Rosenfeld, W., Scutella, M., Seidman, L., Sperling, M., Sperling, R., Stager, M., Stapleton, J., Swenson, K., Thoming, C., Twiggs, L., Waldbaum, A., Wheeler, C.M., Yardley, M., Zbella, E., Kiviat, N., Klugman, K.P., Nieminen, P., Bergeron, C., Eisenstein, E., Karron, R., Marks, R., Nolan, T., Tay, S.K., Albers, S., Bollaerts, P., Camier, A., Colau, B., De Breyne, A., Genevrois, S., Issaka, Z., Martens, N., Peeters, P., Smoes, N., Spiessens, B., Tavares, F., Tonglet, A., Vanden-Dunghen, S., Vilain, A.S., Ward, K.R., Alt, E., Iskaros, B., Limaye, A., Liu-Jarin, X., Luff, R.D., McNeeley, M., Provenzano, C., Winkler, B., Molijn, A., Quint, W., Struijk, L., Van de Sandt, M., Van Doorn, L.J., Greenacre, M., Baronikova, S., Zahaf, T., David, M.P., Declerck, L., Dupin, J., Maroye, J.L. (2009). Efficacy of human papillomavirus (HPV)-16/18 AS04-adjuvanted vaccine against cervical infection and precancer caused by oncogenic HPV types (PATRICIA): final analysis of a double-blind, randomised study in young women. Lancet (London, England): 374, 301-314.

162. **Padayachee, A., van Wyk, C.W.** (1991). Human papillomavirus (HPV) DNA in focal epithelial hyperplasia by in situ hybridization. Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology: 20, 210-214.

163. **Pass, F., Reissig, M., Shah, K.V., Eisinger, M., Orth, G.** (1977). Identification of an immunologically distinct papillomavirus from lesions of epidermodysplasia verruciformis. J Natl Cancer Inst: 59, 1107-1112.

164. **Pett, M., Coleman, N.** (2007). Integration of high-risk human papillomavirus: a key event in cervical carcinogenesis? J Pathol: 212, 356-367.

165. **Pfister, H.** (2003). Chapter 8: Human papillomavirus and skin cancer. J Natl Cancer Inst Monogr, 52-56.

166. **Pim, D., Broniarczyk, J., Bergant, M., Playford, M.P., Banks, L.** (2015). A Novel PDZ Domain Interaction Mediates the Binding between Human Papillomavirus 16 L2 and Sorting Nexin 27 and Modulates Virion Trafficking. J Virol: 89, 10145-10155.

167. **Pisitkun, T., Shen, R.F., Knepper, M.A.** (2004). Identification and proteomic profiling of exosomes in human urine. Proc Natl Acad Sci U S A: 101, 13368-13373.

168. **Popa, A., Zhang, W., Harrison, M.S., Goodner, K., Kazakov, T., Goodwin, E.C., Lipovsky, A., Burd, C.G., DiMaio, D.** (2015). Direct binding of retromer to human papillomavirus type 16 minor capsid protein L2 mediates endosome exit during viral infection. PLoS Pathog: 11, e1004699.

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

170. **Rabinowits, G., Gercel-Taylor, C., Day, J.M., Taylor, D.D., Kloecker, G.H.** (2009). Exosomal microRNA: a diagnostic marker for lung cancer. Clin Lung Cancer: 10, 42-46.

171. **Ramoz, N., Rueda, L.A., Bouadjar, B., Montoya, L.S., Orth, G., Favre, M.** (2002). Mutations in two adjacent novel genes are associated with epidermodysplasia verruciformis. Nat Genet: 32, 579-581.

172. **Reinson, T., Toots, M., Kadaja, M., Pipitch, R., Allik, M., Ustav, E., Ustav, M.** (2013). Engagement of the ATR-dependent DNA damage response at the human papillomavirus 18 replication centers during the initial amplification. Journal of virology: 87, 951-964.

173. **Richards, R.M., Lowy, D.R., Schiller, J.T., Day, P.M.** (2006). Cleavage of the papillomavirus minor capsid protein, L2, at a furin consensus site is necessary for infection. Proc Natl Acad Sci U S A: 103, 1522-1527.

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

175. **Roman, A.** (2006). The human papillomavirus E7 protein shines a spotlight on the pRB family member, p130. Cell Cycle: 5, 567-568.

176. **Roman, A., Munger, K.** (2013). The papillomavirus E7 proteins. Virology: 445, 138-168.

177. **Rozenblatt-Rosen, O., Deo, R.C., Padi, M., Adelmant, G., Calderwood, M.A., Rolland, T., Grace, M., Dricot, A., Askenazi, M., Tavares, M., Pevzner, S.J., Abderazzaq, F., Byrdsong, D., Carvunis, A.R., Chen, A.A., Cheng, J., Correll, M., Duarte, M., Fan, C., Feltkamp, M.C., Ficarro, S.B., Franchi, R., Garg, B.K., Gulbahce, N., Hao, T., Holthaus, A.M., James, R., Korkhin, A., Litovchick, L., Mar, J.C., Pak, T.R., Rabello, S., Rubio, R., Shen, Y., Singh, S., Spangle, J.M., Tasan, M., Wanamaker, S., Webber, J.T., Roecklein-Canfield, J., Johannsen, E., Barabasi, A.L., Beroukhim, R., Kieff, E., Cusick, M.E., Hill, D.E., Munger, K., Marto, J.A., Quackenbush, J., Roth, F.P., DeCaprio, J.A., Vidal, M.** (2012). Interpreting cancer genomes using systematic host network perturbations by tumour virus proteins. Nature: 487, 491-495.

178. **Sahoo, S., Klychko, E., Thorne, T., Misener, S., Schultz, K.M., Millay, M., Ito, A., Liu, T., Kamide, C., Agrawal, H., Perlman, H., Qin, G., Kishore, R., Losordo, D.W.** (2011). Exosomes from human CD34(+) stem cells mediate their proangiogenic paracrine activity. Circ Res: 109, 724-728.

179. **Said, A.K., Leao, J.C., Fedele, S., Porter, S.R.** (2013). Focal epithelial hyperplasia - an update. Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology: 42, 435-442.

180. **Sakakibara, N., Chen, D., McBride, A.A.** (2013). Papillomaviruses use recombination-dependent replication to vegetatively amplify their genomes in differentiated cells. PLoS Pathog: 9, e1003321.

181. **Sakakibara, N., Mitra, R., McBride, A.A.** (2011). The papillomavirus E1 helicase activates a cellular DNA damage response in viral replication foci. Journal of virology: 85, 8981-8995.

182. **Salmena, L., Poliseno, L., Tay, Y., Kats, L., Pandolfi, P.P.** (2011). A ceRNA hypothesis: the Rosetta Stone of a hidden RNA language? Cell: 146, 353-358.

183. **Saslow, D., Solomon, D., Lawson, H.W., Killackey, M., Kulasingam, S.L., Cain, J., Garcia, F.A., Moriarty, A.T., Waxman, A.G., Wilbur, D.C., Wentzensen, N., Downs, L.S., Jr., Spitzer, M., Moscicki, A.B., Franco, E.L., Stoler, M.H., Schiffman, M., Castle, P.E., Myers, E.R., Committee, A.-A.-A.C.C.G.** (2012). American Cancer Society, American Society for Colposcopy and Cervical Pathology, and American Society for Clinical Pathology screening guidelines for the prevention and early detection of cervical cancer. CA Cancer J Clin: 62, 147-172.

184. **Schafer, G., Blumenthal, M.J., Katz, A.A.** (2015). Interaction of human tumor viruses with host cell surface receptors and cell entry. Viruses: 7, 2592-2617.

185. **Scheffner, M., Huibregtse, J.M., Vierstra, R.D., Howley, P.M.** (1993). The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell: 75, 495-505.

186. **Schelhaas, M., Ewers, H., Rajamaki, M.L., Day, P.M., Schiller, J.T., Helenius, A.** (2008). Human papillomavirus type 16 entry: retrograde cell surface transport along actin-rich protrusions. PLoS Pathog: 4, e1000148.

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

188. **Schiffman, M., Castle, P.E., Jeronimo, J., Rodriguez, A.C., Wacholder, S.** (2007). Human papillomavirus and cervical cancer. Lancet: 370, 890-907.

189. **Schneider, M.A., Spoden, G.A., Florin, L., Lambert, C.** (2011). Identification of the dynein light chains required for human papillomavirus infection. Cell Microbiol: 13, 32-46.

190. **Schwarz, D.S., Hutvagner, G., Du, T., Xu, Z., Aronin, N., Zamore, P.D.** (2003). Asymmetry in the assembly of the RNAi enzyme complex. Cell: 115, 199-208.

191. **Scotto, L., Narayan, G., Nandula, S.V., Subramaniyam, S., Kaufmann, A.M., Wright, J.D., Pothuri, B., Mansukhani, M., Schneider, A., Arias-Pulido, H., Murty, V.V.** (2008). Integrative genomics analysis of chromosome 5p gain in cervical cancer reveals target over-expressed genes, including Drosha. Mol Cancer: 7, 58.

192. **Shope, R.E., Hurst, E.W.** (1933). Infectious Papillomatosis of Rabbits : With a Note on the Histopathology. J Exp Med: 58, 607-624.

193. **Skarda, J., Amariglio, N., Rechavi, G.** (2009). RNA editing in human cancer: review. APMIS: 117, 551-557.

194. **Smith, J.L., Lidke, D.S., Ozbun, M.A.** (2008). Virus activated filopodia promote human papillomavirus type 31 uptake from the extracellular matrix. Virology: 381, 16-21.

195. **Society, A.C.**, 2016. Cancer Facts & Figures 2016. American Cancer Society, Atlanta.

196. **Stoler, M.H., Austin, R.M., Zhao, C.** (2015). Point-Counterpoint: Cervical Cancer Screening Should Be Done by Primary Human Papillomavirus Testing with Genotyping and Reflex Cytology for Women over the Age of 25 Years. J Clin Microbiol: 53, 2798-2804.

197. **Tambouret, R.H.** (2013). The evolution of the Papanicolaou smear. Clin Obstet Gynecol: 56, 3-9.

198. **Tan, M.J., White, E.A., Sowa, M.E., Harper, J.W., Aster, J.C., Howley, P.M.** (2012). Cutaneous beta-human papillomavirus E6 proteins bind Mastermind-like coactivators and repress Notch signaling. Proc Natl Acad Sci U S A: 109, E1473-1480.

199. **Taylor, D.D., Gercel-Taylor, C.** (2008). MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. Gynecol Oncol: 110, 13-21.

200. **Taylor, D.D., Shah, S.** (2015). Methods of isolating extracellular vesicles impact down-stream analyses of their cargoes. Methods: 87, 3-10.

201. **Thierry, F., Yaniv, M.** (1987). The BPV1-E2 trans-acting protein can be either an activator or a repressor of the HPV18 regulatory region. EMBO J: 6, 3391-3397.

202. **Thomas, M.C., Chiang, C.M.** (2005). E6 oncoprotein represses p53-dependent gene activation via inhibition of protein acetylation independently of inducing p53 degradation. Mol Cell: 17, 251-264.

203. **Tota, J.E., Chevarie-Davis, M., Richardson, L.A., Devries, M., Franco, E.L.** (2011). Epidemiology and burden of HPV infection and related diseases: implications for prevention strategies. Prev Med: 53 Suppl 1, S12-21.

204. **Turturici, G., Tinnirello, R., Sconzo, G., Geraci, F.** (2014). Extracellular membrane vesicles as a mechanism of cell-to-cell communication: advantages and disadvantages. Am J Physiol Cell Physiol: 306, C621-633.

205. **Valadi, H., Ekstrom, K., Bossios, A., Sjostrand, M., Lee, J.J., Lotvall, J.O.** (2007). Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol: 9, 654-659.

206. **Van Doorslaer, K., Tan, Q., Xirasagar, S., Bandaru, S., Gopalan, V., Mohamoud, Y., Huyen, Y., McBride, A.A.** (2013). The Papillomavirus Episteme: a central resource for papillomavirus sequence data and analysis. Nucleic Acids Res: 41, D571-578.

207. **Vande Pol, S.B., Klingelhutz, A.J.** (2013). Papillomavirus E6 oncoproteins. Virology: 445, 115-137.

208. **Vinokurova, S., Wentzensen, N., Kraus, I., Klaes, R., Driesch, C., Melsheimer, P., Kisseljov, F., Durst, M., Schneider, A., von Knebel Doeberitz, M.** (2008). Type-dependent integration frequency of human papillomavirus genomes in cervical lesions. Cancer Res: 68, 307-313.

209. **Vojtech, L., Woo, S., Hughes, S., Levy, C., Ballweber, L., Sauteraud, R.P., Strobl, J., Westerberg, K., Gottardo, R., Tewari, M., Hladik, F.** (2014). Exosomes in human semen carry a distinctive repertoire of small non-coding RNAs with potential regulatory functions. Nucleic Acids Res: 42, 7290-7304.

210. **Wang, X., Wang, H.K., Li, Y., Hafner, M., Banerjee, N.S., Tang, S., Briskin, D., Meyers, C., Chow, L.T., Xie, X., Tuschl, T., Zheng, Z.M.** (2014). microRNAs are biomarkers of oncogenic human papillomavirus infections. Proc Natl Acad Sci U S A: 111, 4262-4267.

211. **Webber, J.P., Spary, L.K., Sanders, A.J., Chowdhury, R., Jiang, W.G., Steadman, R., Wymant, J., Jones, A.T., Kynaston, H., Mason, M.D., Tabi, Z., Clayton, A.** (2015). Differentiation of tumour-promoting stromal myofibroblasts by cancer exosomes. Oncogene: 34, 290-302.

212. **Wheeler, C.M., Castellsague, X., Garland, S.M., Szarewski, A., Paavonen, J., Naud, P., Salmeron, J., Chow, S.N., Apter, D., Kitchener, H., Teixeira, J.C., Skinner, S.R., Jaisamrarn, U., Limson, G., Romanowski, B., Aoki, F.Y., Schwarz, T.F., Poppe, W.A., Bosch, F.X., Harper, D.M., Huh, W., Hardt, K., Zahaf, T., Descamps, D., Struyf, F., Dubin, G., Lehtinen, M., Group, H.P.S.**

(2012). Cross-protective efficacy of HPV-16/18 AS04-adjuvanted vaccine against cervical infection and precancer caused by non-vaccine oncogenic HPV types: 4-year end-of-study analysis of the randomised, double-blind PATRICIA trial. Lancet Oncol: 13, 100-110.

213. **Winer, R.L., Hughes, J.P., Feng, Q., O'Reilly, S., Kiviat, N.B., Holmes, K.K., Koutsky, L.A.** (2006a). Condom use and the risk of genital human papillomavirus infection in young women. N Engl J Med: 354, 2645-2654.

214. **Winer, R.L., Hughes, J.P., Feng, Q., O'Reilly, S., Kiviat, N.B., Holmes, K.K., Koutsky, L.A.** (2006b). Condom use and the risk of genital human papillomavirus infection in young women. The New England journal of medicine: 354, 2645-2654.

215. **Wright, T., Huang, J., Baker, E., Garfield, S., Hertz, D., Cox, J.T.** (2016). The budget impact of cervical cancer screening using HPV primary screening. Am J Manag Care: 22, e95-e105.

216. **Xie, M., Steitz, J.A.** (2014). Versatile microRNA biogenesis in animals and their viruses. RNA Biol: 11, 673-681.

217. **Yablonska, S., Hoskins, E.E., Wells, S.I., Khan, S.A.** (2013). Identification of miRNAs dysregulated in human foreskin keratinocytes (HFKs) expressing the human papillomavirus (HPV) Type 16 E6 and E7 oncoproteins. Microrna: 2, 2-13.

218. **Yang, A., Jeang, J., Cheng, K., Cheng, T., Yang, B., Wu, T.C., Hung, C.F.** (2016). Current state in the development of candidate therapeutic HPV vaccines. Expert Rev Vaccines, 1-19.

219. **Yi, R., Qin, Y., Macara, I.G., Cullen, B.R.** (2003). Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev: 17, 3011-3016.

220. **Zhao, H., Jin, X., Su, H., Deng, X., Fang, Y., Shen, L., Xie, C.** (2014). Down-regulation of Dicer expression in cervical cancer tissues. Med Oncol: 31, 937.

221. **Zheng, Z.M., Baker, C.C.** (2006). Papillomavirus genome structure, expression, and post-transcriptional regulation. Front Biosci: 11, 2286-2302.

222. **Zhou, J., Cai, J., Huang, Z., Ding, H., Wang, J., Jia, J., Zhao, Y., Huang, D., Wang, Z.** (2013). Proteomic identification of target proteins following Drosha knockdown in cervical cancer. Oncol Rep: 30, 2229-2237.

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

224. **Zomer, A., Maynard, C., Verweij, F.J., Kamermans, A., Schafer, R., Beerling, E., Schiffelers, R.M., de Wit, E., Berenguer, J., Ellenbroek, S.I., Wurdinger, T., Pegtel, D.M., van Rheenen, J.** (2015). In Vivo imaging reveals extracellular vesicle-mediated phenocopying of metastatic behavior. Cell: 161, 1046-1057.

CHAPTER TWO

Modulation of microRNA-mRNA Target Pairs by Human Papillomavirus 16

Oncoproteins

This chapter is adapted from a previously published manuscript:

Harden ME, Prasad N, Griffiths A, and Munger K. (2017). Modulation of microRNA-mRNA Target Pairs by Human Papillomavirus 16 Oncoproteins. MBio. 8:e02170-16.

Contributions: I performed all experiments unless otherwise indicated and wrote the manuscript. Nripesh Prasad prepared the libraries for RNA sequencing (RNAseq), ran the RNAseq experiments, performed the initial RNAseq data analysis and edited the manuscript. Anthony Griffiths aided in small RNA sequencing (miRseq) experimental design and provided reagents and instrumentation to perform the miRseq experiments. Karl Munger aided in experimental design and edited the manuscript.

SUMMARY

The E6 and E7 proteins are the major oncogenic drivers encoded by high-risk human papillomaviruses (HPVs). While many aspects of the transforming activities of these proteins have been extensively studied, there are fewer studies that have investigated how HPV E6/E7 expression affects expression of cellular noncoding RNAs. The goal of our study was to investigate HPV16 E6/E7 modulation of cellular microRNA (miR) levels and to determine the potential consequences on cellular gene expression.

We performed deep sequencing of small and large cellular RNAs in primary, undifferentiated cultures of human foreskin keratinocytes (HFKs) with stable expression of HPV16 E6/E7 or a control vector. After integration of the two data sets we identified 51 differentially expressed cellular miRs associated with modulation of 1,456 potential target mRNAs in HPV16 E6/E7 expressing HFKs. We discovered that the degree of differential miR expression in HFKs expressing HPV16 E6/E7 was not necessarily predictive of the number of corresponding mRNA targets or the potential impact on gene expression. Additional analyses of the identified miR-mRNA pairs suggest modulation of specific biological activities and biochemical pathways. Overall, our study supports the model that perturbation of cellular miR expression by HPV16 E6/E7 importantly contributes to the rewiring of cellular regulatory circuits by the high-risk HPV E6 and E7 proteins that contribute to oncogenic transformation.

INTRODUCTION

Human papillomaviruses (HPVs) are small, double-stranded DNA viruses that infect undifferentiated basal epithelial cells of stratified epithelia (reviewed in (McLaughlin-Drubin *et al.*, 2012)). A subset of HPVs classified as "high-risk" are the causative agents of almost all cervical cancers, as well as many other anogenital tract and oral carcinomas. The E6 and E7 proteins are consistently expressed in high-risk HPV+ lesions and cancers and are the main drivers of cell transformation (reviewed in (McLaughlin-Drubin and Munger, 2009; Mesri *et al.*, 2014)). HPV E6 and E7 are small proteins with no intrinsic enzymatic or DNA-binding activities that function through targeting host pathways that modulate multiple downstream effectors (reviewed in (McLaughlin-Drubin *et al.*, 2012)), thereby causing alterations in critical physiological processes deemed "hallmarks of cancer" (Hanahan and Weinberg, 2000; Mesri *et al.*, 2014). Most notably, high-risk HPV E6 and E7 proteins bind and target the TP53 and retinoblastoma tumor suppressor protein RB1 (Dyson *et al.*, 1989) for proteasomal degradation (Boyer *et al.*, 1996; Jones *et al.*, 1997) (Scheffner *et al.*, 1990). In addition, high-risk HPV E6 and E7 also interact with many other multifunctional, non-redundant proteins, including transcription factors and epigenetic regulators, which in turn, cause alterations in cellular gene expression. In addition to coding genes, high-risk HPV E6 and E7 also cause alterations in expression of non-coding RNAs, including microRNAs (miRs) (Yablonska *et al.*, 2013).

miRs are small (~22 nucleotide), non-coding RNAs that regulate target mRNAs at the post-transcriptional level. Most mammalian mRNAs are miR targets (Friedman *et*

al., 2009). Targeting involves binding of the miR seed (nucleotides 2-7) to

complementary sequences in target mRNAs, with most miR target sites mapping to 3'

untranslated regions (Bartel, 2009). Regulation of target mRNAs can occur via mRNA

destabilization or translational repression or a combination of both mechanisms.

Specifically, mRNA destabilization accounts for the majority of miR-mediated repression

(Eichhorn *et al.*, 2014; Guo *et al.*, 2010), while only 10%-25% of overall miR repression

is due to inhibition of translation (Hendrickson *et al.*, 2009). Each individual miR can

alter the expression of hundreds of targets (Lim *et al.*, 2005) and mRNAs can be

regulated by multiple miRs. Typically, miRs impart modest effects on any single target

and are thought to balance or "fine-tune" gene expression. However, the additive effect

of multiple miRs targeting a particular pathway or one miR targeting several

components of a specific pathway can result in substantial biological consequences.

Therefore, through manipulation of host miRs, HPV E6 and E7 may modulate many

downstream mRNA targets involved in various biological processes.

At least one HPV type, HPV31, does not encode miRs (Cai *et al.*, 2006).

However, it cannot be ruled out that some other HPVs may encode miRs. Regardless,

by altering host miR expression, HPVs can reap many of the benefits achieved through

viral miRs without encoding their own. To date, only few studies (Gunasekharan and

Laimins, 2013; Wang *et al.*, 2014) have used small RNA sequencing (miRseq) to

investigate alterations in host miRs in the context of high-risk HPV infection and these

studies utilized organotypic raft cultures comprised of epithelial cells undergoing

differentiation. Given HPVs, particularly the HPV E6 and E7 proteins, can alter

epithelial cell differentiation and/or sustain cellular proliferation in differentiated cells

(McCance *et al.*, 1988), it is unclear whether the reported changes in miRs levels are directly caused by HPV gene expression or whether they represent the consequence of HPV-induced changes in epithelial cell proliferation and differentiation.

To circumvent this complication, we aimed to investigate how expression of high-risk HPV E6 and E7 modulates miR levels in homogenous populations of undifferentiated primary human foreskin keratinocytes (HFKs). We performed deep sequencing of miRs from HFK populations with stable, low-level HPV16 E6/E7 expression and donor and passage matched control vector transduced HFKs. To comprehensively capture the potential impact of miR regulation on cellular mRNA abundance, we performed deep sequencing of cellular RNAs (RNAseq) that were simultaneously isolated from the identical HFKs populations used for miR profiling. After pairing the miR expression data with the RNA expression data, we identified miRs that are likely to be functionally important in HPV16 E6/E7 expressing HFKs. Additional bioinformatic analyses revealed key canonical pathways that are specifically enriched in the identified miR-mRNA target pairs in comparison to the entire RNAseq data set. Taken together, our study shows that modulation of cellular miR expression plays a substantial role in the HPV16 E6/E7 mediated reprogramming of cellular gene expression and may importantly contribute to the oncogenic activities of these proteins.

MATERIALS AND METHODS

Cell Culture

HFKs were isolated from a pool of de-identified newborn foreskin samples and transduced with LXSN based recombinant retroviruses encoding HPV16 E6 or E7 or both oncogenes or control LXSN vector as previously described (Halbert *et al.*, 1991). The two, independent HFK populations utilized in this study were generated from two, distinct pools of human foreskin samples. Donor, passage and density matched HFK populations were used in all experiments.

RNA Isolation

Large (≥200 nt) and small (<200 nt) RNAs were prepared for sequencing using the *mir*Vana™ miRNA Isolation Kit (Ambion, Life Technologies) according to the manufacturer's protocol. For RT-qPCR experiments, total RNA was isolated using the miRNeasy Mini Kit (Qiagen) as described in the manufacturer's instructions.

microRNA Sequencing (miRseq)

Small RNA libraries were prepared from small RNA utilizing the TruSeq® Small RNA Library Preparation Kit (Illumina) as described in the manufacturer's sample preparation guide. Gel purified small RNA cDNA libraries were quantified using the Qubit® 2.0 Fluorometer (Invitrogen, Life Technologies), diluted to a final concentration of 10 nM and pooled in equimolar amounts prior to cluster generation. Paired-End (PE)

sequencing (1-2 million, 50 bp paired-end reads) was performed using the Illumina MiSeq® Sequencing System (Illumina).

miRseq Data Analysis

Post-processing of the miRseq reads from each sample was performed according to the HudsonAlpha Genomic Services Laboratory (GSL) unique in-house pipeline as previously described (Ning *et al.*, 2014). The differential expression of miRs was calculated on the basis of their fold change (cut-off ≥ ±3.0) observed between different groups (control HFKs versus HFKs + HPV16 E6/E7). The p-value of differentially expressed miRs was estimated via z-score using the Benjamini Hochberg FDR correction of 0.05 (Klipper-Aurbach *et al.*, 1995).

RNA Sequencing (RNAseq)

The concentration and integrity of the isolated large RNA (≥200 nt) was estimated using a Qubit® 2.0 Fluorometer (Invitrogen, Life Technologies) and Agilent 2100 Bioanalyzer (Applied Biosystems, Life Technologies), respectively. Five hundred ng of large RNA was utilized for downstream RNAseq processing. First, ribosomal RNA (rRNA) was removed using Ribo-Zero™ Magnetic Gold (Yeast) Kit (Epicenter, Illumina) according to the manufacturer's recommended protocol. The RNA was then fragmented and primed for first strand synthesis using the NEBNext® RNA First Strand Synthesis Module (New England BioLabs). Second strand synthesis was performed using the NEBNext® RNA Second Strand Synthesis Module.

Samples were prepared using the NEBNext® DNA Library Prep Master Mix Set for Illumina®, with slight modifications. Briefly, end-repair was performed followed by A-tailing and custom adapter ligation. Samples were then individually barcoded with GSL primers and amplified by 12 cycles of PCR. Library quantity was assessed by Qubit® 2.0 Fluorometer and library quality was estimated utilizing a DNA 1000 chip on an Agilent 2100 Bioanalyzer. Further quantification of the final libraries for downstream sequencing applications was determined using the qPCR-based KAPA Biosystems Library Quantification Kit (Kapa Biosystems). Each library was diluted to a final concentration of 12.5 nM and pooled in equimolar amounts prior to clustering. PE sequencing (50 million, 100 bp PE reads) was performed using the Illumina HiSeq® 2500 Sequencing System (Illumina).

Processing and Analysis of RNAseq Reads

Downstream analysis of the sequenced reads from each sample was performed per a unique in-house pipeline designed by GSL. Briefly, quality control checks on raw sequence data from each sample were performed using FastQC (Babraham Bioinformatics). Raw reads were then mapped to the reference human genome hg19 using TopHat v2.0 (Langmead *et al.*, 2009; Trapnell *et al.*, 2009) with two mismatches allowed and other default parameters. The alignment metrics of the mapped reads was estimated using SAMtools (Li *et al.*, 2009). Aligned reads were then imported into the commercial data analysis platform, Avadis NGS (Strand Scientifics). After quality inspection, the aligned reads were filtered on the basis of read quality metrics where reads with a base quality score less than 30, alignment score less than 95 and mapping

quality less than 40 were removed. Remaining reads were then filtered on the basis of

read statistics, where missing mates, translocated, unaligned and flipped reads were

removed. The reads list was then filtered to remove duplicates. Samples were grouped

and quantification of transcript abundance was performed on this final read list using

Trimmed Means of M-values (TMM) (Robinson and Oshlack, 2010) as the normalization

method. Differential expression of RNAs was calculated on the basis of fold change

(cut-off ≥ ±2.0) observed between defined conditions. The p-value of the differentially

expressed RNAs was estimated by z-score calculations utilizing the Benjamini

Hochberg FDR correction of 0.05 (Klipper-Aurbach *et al.*, 1995). Ingenuity Pathway

Analysis (IPA) (Qiagen) software was used to analyze the unique canonical pathways,

biological functions and networks affected.

Integration of RNAseq and miRseq data

Differentially expressed miRs identified via miRseq that met threshold cutoffs

(fold change ≥ ±3.0, FDR ≤ 0.05) were uploaded into Ingenuity® Pathway Analysis

(Qiagen) and analyzed using the microRNA Target Filter. This filter displays

experimentally validated and predicted mRNA targets from TargetScan, TarBase,

miRecords and the Ingenuity® Knowledge Base for each miR in the dataset.

Differentially expressed RNAs identified by RNAseq that met threshold cutoffs (fold

change ≥ ±2.0, FDR ≤ 0.05) were then uploaded using the "add/replace mRNA dataset"

function. Using the "expression-pairing" feature, only potential targets differentially

expressed in the RNAseq data are shown, all other potential targets are filtered out. To

further refine the data, the "inverse correlation" filter was employed to focus on changes in potential targets that are inversely correlated with changes in the corresponding miR.

RT-qPCR

For miR RT-qPCR, total RNA was reverse transcribed with the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Life Technologies) as described in the manufacturer's protocol, utilizing miR-specific, stem loop primers (Applied Biosystems, Life Technologies). TaqMan MicroRNA Assays (Applied Biosystems, Life Technologies) were employed to detect mature miRs using the comparative Ct method with the StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific). Assay IDs 000390, 000391, 002367 and 000507 were utilized to detect miR-15b-5p, -16-5p, -193b-3p and -203a-3p, respectively. RT-qPCR assays were performed in triplicate and the non-coding small nuclear RNA (snRNA) U6 (Assay ID: 001973) was utilized as an endogenous, small RNA control.

For RT-qPCR of miR targets, following RNA isolation, total RNA was DNAase treated with the TURBO DNA-*free*™ kit. DNAse treated total RNA was then reverse transcribed utilizing TaqMan® Reverse Transcription Reagents (Life Technologies, Applied Biosystems). TaqMan Assays for TP63 (Assay ID: Hs00978343_m1) and BMI1 (Assay ID: Hs00995536_m1) were employed to detect targets using the comparative Ct method with the StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific). RT-qPCR assays were performed in triplicate and 18S ribosomal RNA was utilized as an internal control.

microRNA Mimics and Inhibitors

Overexpression of miR-203a-3p was achieved using a miRCURY™ LNA microRNA Mimic (472239-001, Exiqon). A negative control miR mimic (miRCURY™ LNA microRNA Mimic Negative Control 479903-001, Exiqon), with the same design features of the miRCURY™ LNA microRNA Mimics and no homology to any known miR or mRNA sequences in mice, rats or humans, were used as a negative control for overexpression. Inhibition of miR-203a-3p was accomplished utilizing a miRCURY™ LNA microRNA Power Inhibitor (4100339-101, Exiqon). As a negative control for miR inhibition, a miRCURY™ LNA microRNA inhibitor control (199006-101, Exiqon) was used, which is similar in sequence length and LNA™ design, with no homology to any known microRNA or mRNA sequence in the mouse, rat or human genome.

Transfection of microRNA Mimics and Inhibitors

HFKs were transfected with miR mimics and inhibitors using Lipofectamine® 2000 (Invitrogen, Life Technologies) as described in the manufacturer's instructions with some modifications. To achieve optimal overexpression of miRs using a miRCURY™ LNA microRNA Mimic (Exiqon), 0.05 nM of mimic was transfected and samples were harvested 24 hours post-transfection. To achieve optimal knockdown of miRs using a miRCURY™ LNA microRNA Power Inhibitor (Exiqon), 20 nM of inhibitor was transfected and samples were harvested 48 hours post-transfection. The same amount of control mimic or inhibitor was transfected and control samples were harvested at 24 and 48 hours post-transfection, respectively. As an additional control, HFKs were

treated with the transfection reagents alone. RT-qPCR for known miR targets was

utilized to confirm successful miR overexpression or knockdown.

RESULTS

Expression of HPV16 E6/E7 in HFKs alters host microRNA expression profiles

Alterations in miR levels in response to high-risk HPV16 E6/E7 expression in undifferentiated human epithelial cells has not been extensively studied. We employed miRseq to investigate the modulation of miR expression in two independent, donor and passage matched HFK populations, each with stable expression of HPV16 E6/E7 or a control vector. For the purpose of this analysis, we applied threshold cut-offs of ≥ 10 miR reads, ≥ ±3.0 times fold change in expression, and an FDR ≤ 0.05. The results from the two control samples and the two samples with expression of HPV16 E6/E7 were averaged and only miRs with changes consistent in both samples were considered in downstream analyses.

A total of 2,104 out of the 2,588 (81%) human miRs compiled in miRBase release 21 (Griffiths-Jones, 2004; Griffiths-Jones *et al.*, 2006; Griffiths-Jones *et al.*, 2008; Kozomara and Griffiths-Jones, 2011, 2014) were detected. Applying the threshold cut-offs, 78 miRs were differentially expressed in HPV16 E6/E7 expressing HFKs as compared to the control vector transduced populations (Figure 2.1A). Of the 78 differentially expressed miRs, 62 miRs were up-regulated and 16 miRs were down-regulated. The top 15 most up and down-regulated miRs are shown in Figure 2.1B. Additionally, while the most suppressed miR (miR-1249) was decreased 9.2 fold, five miRs were up-regulated more than 9.2 fold. The expression of several miRs was confirmed via TaqMan miR assay in multiple additional HFK populations (Figure 2.2A-D).
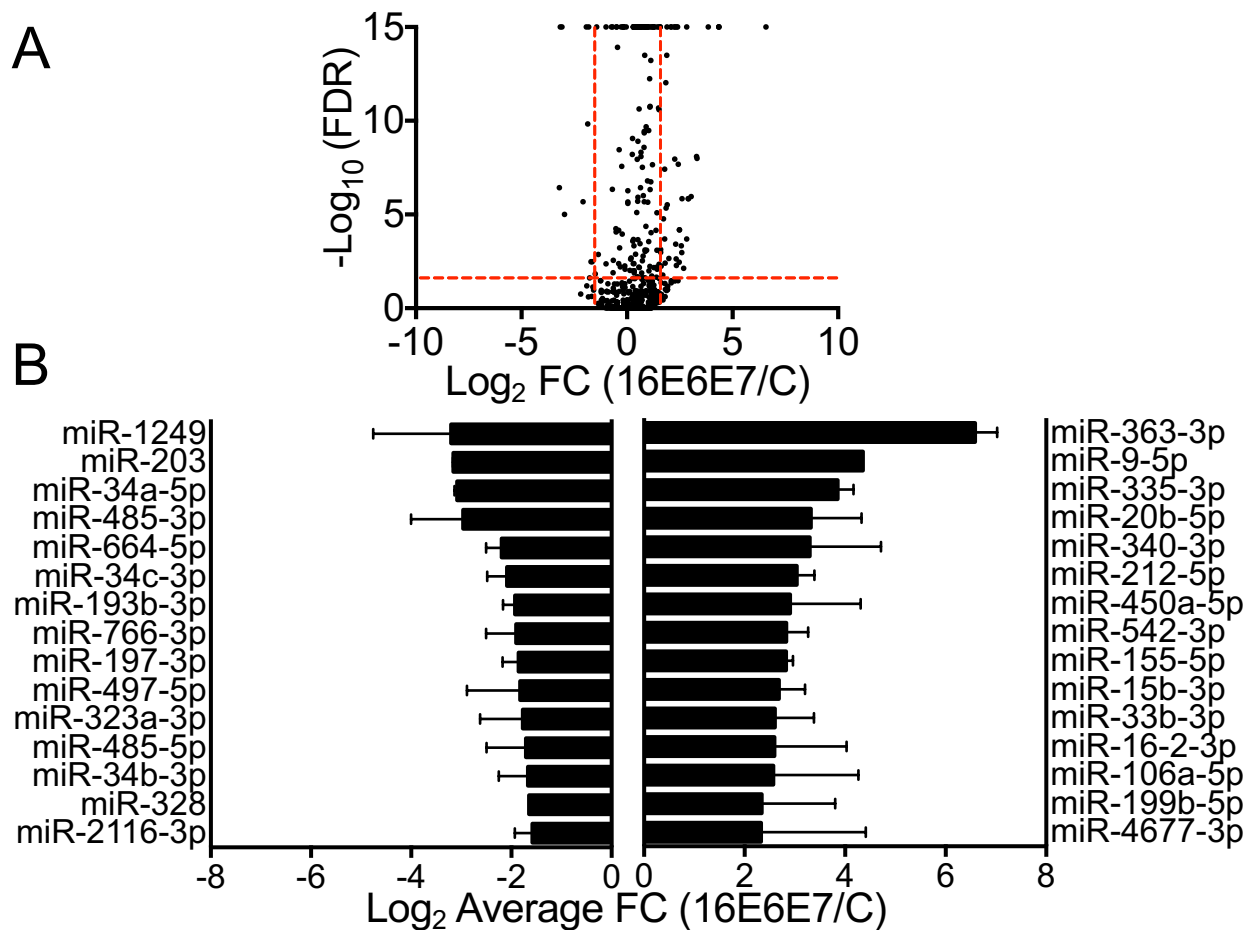
Figure 2.1. miRseq of HPV16 E6/E7 expressing or control vector transduced HFKs

(A) Volcano plot of the miRseq data with each black dot depicting the average expression of individual miRs in two HFK populations. The $-\log_{10}$ transformed false discovery rate (FDR) is plotted on the y-axis and the $\log_2$ transformed fold change (FC) in HPV16 E6/E7 expressing HFKs compared to controls on the x-axis. Vertical red lines indicate the FC thresholds ($-3 \geq$ FC ≥ 3) and horizontal red lines the FDR threshold (FDR ≤ 0.05). (B) Graph of the top 15 up and down-regulated miRs in HPV16 E6/E7 expressing HFKs. Error bars indicate standard error of the mean (SEM).

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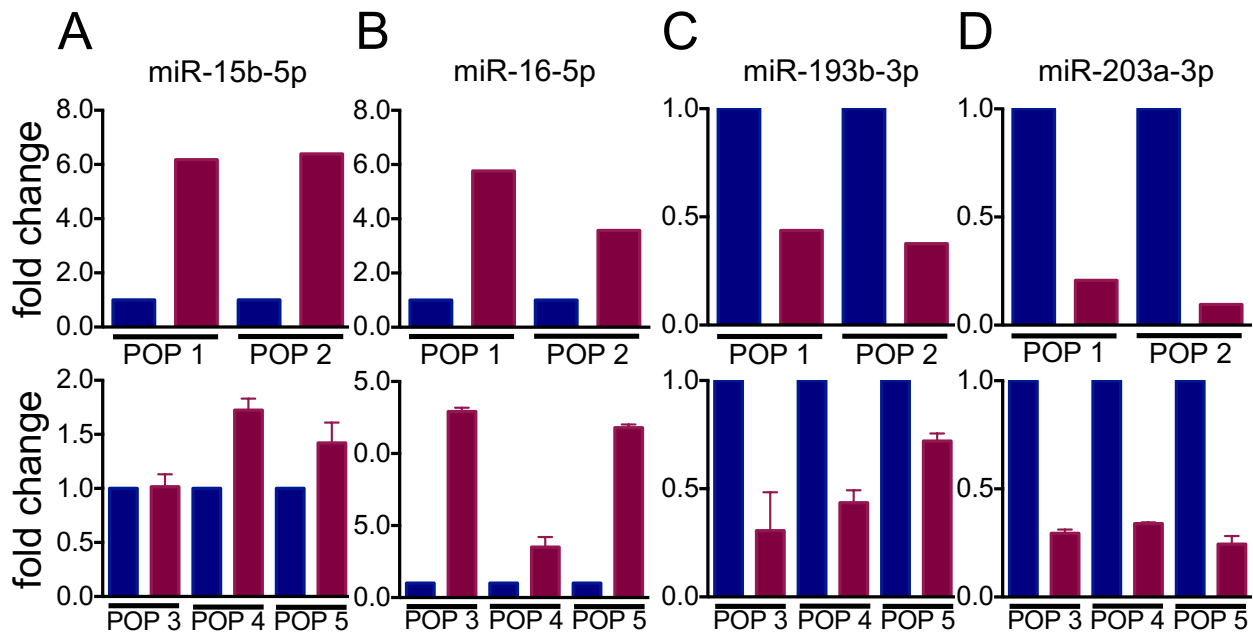
Figure 2.2. Validation of selected miRs identified by miRseq. Validation of (A) miR-15b-5p, (B) miR-16-5p, (C) miR-193b-3p and (D) miR-203a-3p levels in HPV16 E6/E7 expressing- (red) and control vector transduced HFKs (blue). The top graphs show miR expression from two HFKs populations determined by miRseq. The bottom graphs show expression of the corresponding miRs in three additional HFK populations via RT-qPCR. Expression of the non-coding small nuclear RNA U6 spliceosomal RNA (U6) was used as an internal control in TaqMan miR assays. Results represent averages and standard deviations of at least three independent experiments.

To understand the contributions of the individual oncoproteins to changes in miR expression, we investigated the expression of miRs in two matched HFK populations with expression of HPV16 E6 or HPV16 E7 alone. Supplemental Table 2.1 shows lists of the top miRs that were consistently up or down regulated in both HFK populations. Six miRs are up-regulated by both HPV16 E6 and E7 (Supplemental Table 2.1). Similarly, five miRs are down-regulated by both HPV16 E6 and E7 (Supplemental Table 2.1). Several miRs, miR-33b-3p, -542-3p and -335-3p, are up-regulated in HFKs expressing both HPV16 E6/E7, as well as in HFKs expressing HPV16 E6 and E7 alone. Similarly, miR-193b-3p is down-regulated in HPV16 E6/E7 expressing HFKs, as well as in HFKs expressing HPV16 E6 or E7 alone. However, the expression of some HPV16 E6/E7 modulated miRs are driven by one specific oncoprotein. Up-regulation of miR-16-2-3p in HPV16 E6/E7 expressing HFKs is driven by E7 expression (Supplemental Table 2.1) whereas up-regulation of miR-363-3p, -9-5p and -450a-5p is driven by E6 (Supplemental Table 2.1). Down-regulation of miR-197-3p and -1249 in HPV16 E6/E7 expressing HFKs is driven by E7 (Supplemental Table 2.1) whereas down-regulation of miR-34a-5p and -34c-3p is driven by E6 (Supplemental Table 2.1). Hence, some miRs altered in HPV16 E6/E7 expressing HFKs are driven by expression of both HPV16 E6 and E7, whereas others are independently modulated by E6 or E7 alone.

Expression of HPV16 E6/E7 in HFKs alters human microRNA clusters

Approximately 20% of all known human miRs are genomically clustered. Based on the miRBase (Griffiths-Jones, 2004; Griffiths-Jones *et al.*, 2006; Griffiths-Jones *et al.*,

2008; Kozomara and Griffiths-Jones, 2011, 2014) definition of a miR cluster, there are

153 genomic clusters made up of 465 human miRs (Chaulk *et al.*, 2016). Many miR

clusters have been shown to be co-expressed from the same primary miR transcript

(Chaulk *et al.*, 2016; Lee *et al.*, 2002; Sempere *et al.*, 2004; Ventura *et al.*, 2008). Since

the evolution of miR clusters is thought to have involved gene duplications, clustered

miRs are often members of the same seed family (Bentwich *et al.*, 2005; Chen *et al.*,

2005; Giraldez *et al.*, 2005; Houbaviy *et al.*, 2003; Suh *et al.*, 2004). Of functional

importance, altering expression of multiple miRs in a gene cluster may result in

coordinated regulation of multiple biological processes (Guo *et al.*, 2014).

Therefore, we assessed whether some of the differentially expressed miRs that

met our threshold cut-offs were part of miR clusters. Additionally, for any HPV16 E6/E7

regulated miR associated with a cluster, we re-examined the expression of other miRs

belonging to that cluster. This analysis showed that 35 out of the 78 differentially

expressed miRs were members of a genomic cluster and of these 35 miRs, 13 were

found to be part of larger clusters (≥3 miRs) whereas the rest were members of small

clusters containing only 2 miRs. Seven miRs, including miR-362, -106a, -20b, -363, -

542, -450a-1 and -450a-2, were part of a cluster of ≥6 miRs, and miR-485 and -323a

were part of a cluster of ≥13 miRs. As shown in Supplemental Table 2.2, in some

clusters, all of the miRs within the cluster show the same trend in expression. However,

in other clusters, miRs within the cluster show a mixed trend in expression, with

expression of some miRs up-regulated and some down-regulated as a result of HPV16

E6/E7. In total, expression of 26 miR clusters was altered in response to HPV16 E6/E7

expression, suggesting that HPV16 E6/E7 expression modulates individual miRs and miR clusters.

RNAseq analysis of HPV16 E6/E7 expressing HFKs

To comprehensively assess the effects of the observed miR expression changes on potential target RNAs, we also performed RNAseq with large RNAs (≥200 nt) that were simultaneously isolated from the same two independent populations of HFKs from which miR expression was analyzed. Similar to the miRseq data, threshold cut-offs of ≥ 10 reads, ≥ ±2.0 times fold change in expression and an FDR ≤ 0.05 were employed for analysis of the RNAseq data. A volcano plot of the RNAseq data is shown in Figure 2.3A. In total, 3,471 protein-coding RNAs, corresponding to 16% of all human protein-coding RNAs and 8.7% of HGNC approved genes (which include non-protein coding genes), were significantly altered in HPV16 E6/E7 expressing HFKs (Supplemental Table 2.3). More RNAs were down-regulated than up-regulated and Figure 2.3B shows the top 15 most up and down-regulated RNAs.

High-risk HPV E6 proteins are known to increase expression of the catalytic protein subunit of human telomerase, TERT (McMurray and McCance, 2003), and TERT was up-regulated in our HPV16 E6/E7 expressing HFKs. Similarly the high-risk HPV biomarker CDKN2A (p16^{INK4A}) was expressed at higher levels in HPV16 E6/E7 expressing HFKs than control HFKs. Consistent with TP53 inactivation by HPV16 E6 (Werness *et al.*, 1990), lower levels of TP53 transcriptional targets including CDKN1A (p21^{CIP1}), BAX, GADD45A and MDM2 were detected in HPV16 E6/E7 expressing HFKs compared to controls. These observations suggest that our data agree with previously
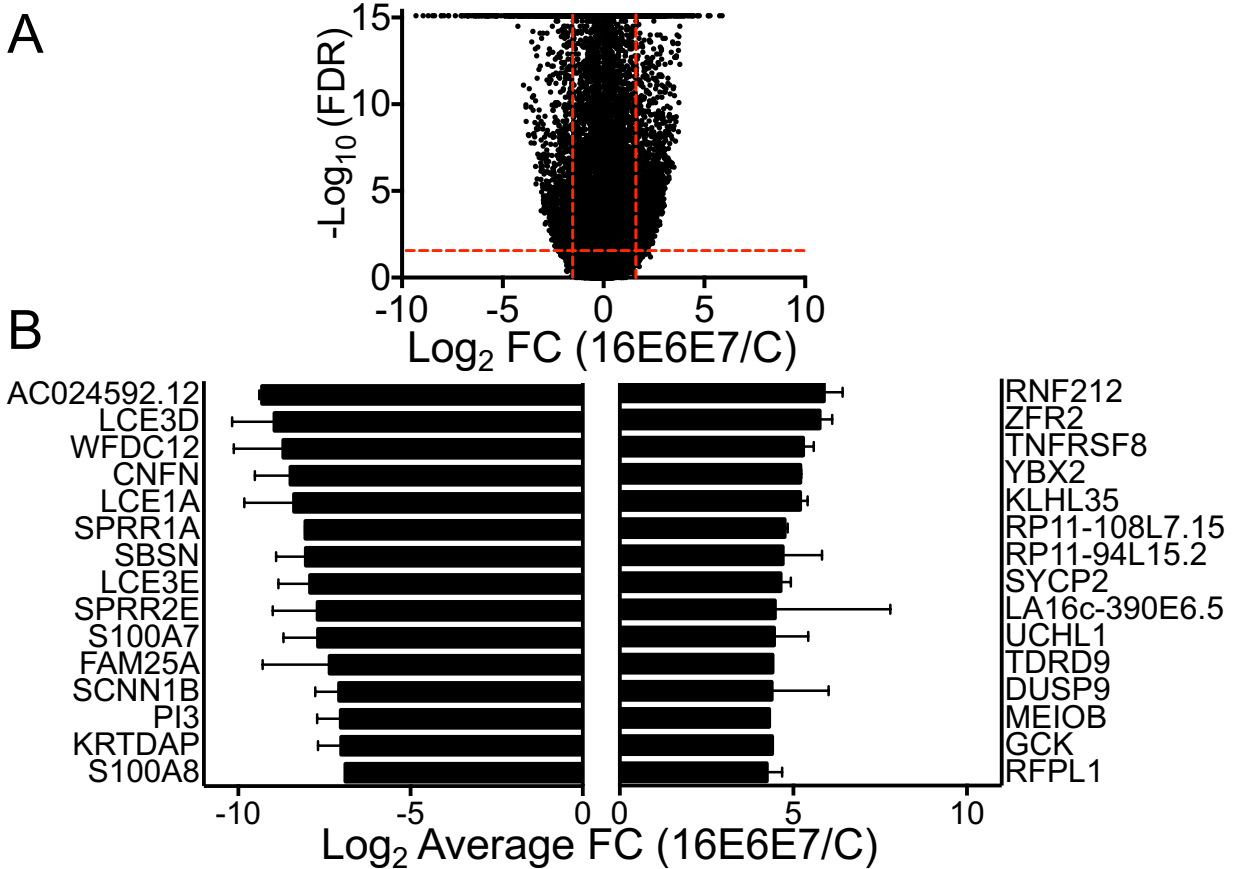
Figure 2.3. RNAseq of large RNAs from HPV16 E6/E7 expressing or control vector transduced HFKs. (A) Volcano plot of the RNAseq data with each black dot depicting the average expression of individual RNAs in two HFK populations. The −$\log_{10}$ transformed false discovery rate (FDR) is plotted on the y-axis and the $\log_2$ transformed fold change (FC) in HPV16 E6/E7expressing HFKs compared to controls on the x-axis. Vertical red lines indicate the FC thresholds (-2 ≥ FC ≥ 2) and horizontal red lines the FDR threshold (FDR ≤ 0.05). (B) Graph of the top 15 up and down-regulated RNAs in HPV16 E6/E7 expressing HFKs. Error bars indicate standard error of the mean (SEM).

observed HPV-associated gene expression changes.

Integration of miRseq and RNAseq data to identify potential microRNA -mRNA target pairs

The Ingenuity Pathway Analysis (IPA) microRNA Target Filter was used to predict mRNA targets. IPA contains ~1.5 million miR targeting interactions and incorporates experimentally validated miR interactions from TarBase and miRecords, predicted mRNA targets from TargetScan and miR-related findings manually curated from the published literature. Targeting information was available for 52 miRs of the 78 differentially expressed miRs, and yielded 13,217 potential mRNA targets. To restrict the potential targets to just those RNAs detected by RNAseq, we incorporated the RNAseq data set into the miR Target Filter analysis pipeline. To specifically identify mRNAs inversely correlated in expression with corresponding miRs, we then utilized an expression-pairing filter. Integration of the RNAseq data, along with the inverse correlation expression-pairing filter, reduced the number of potential mRNA targets to 1,456 potential targets for 51 differentially expressed miRs. This corresponds to an average of 29 potential mRNA targets per individual miR. A schematic of the miR-mRNA expression-pairing pipeline is shown in Figure 2.4. The top 10 most up and down-regulated miRs resulting from the miR-mRNA pairing analysis are shown in Table 2.1 and the full results of this analysis are shown in Supplemental Table 2.4.

Of the 1,456 potential miR targets identified, 711 mRNAs (49%) are potentially targeted by more than miR. Of these, 349 mRNAs are potentially targeted by two, 182 by three, 90 by four, 46 by five, and 22 by six miRs. All mRNAs potentially targeted by

85
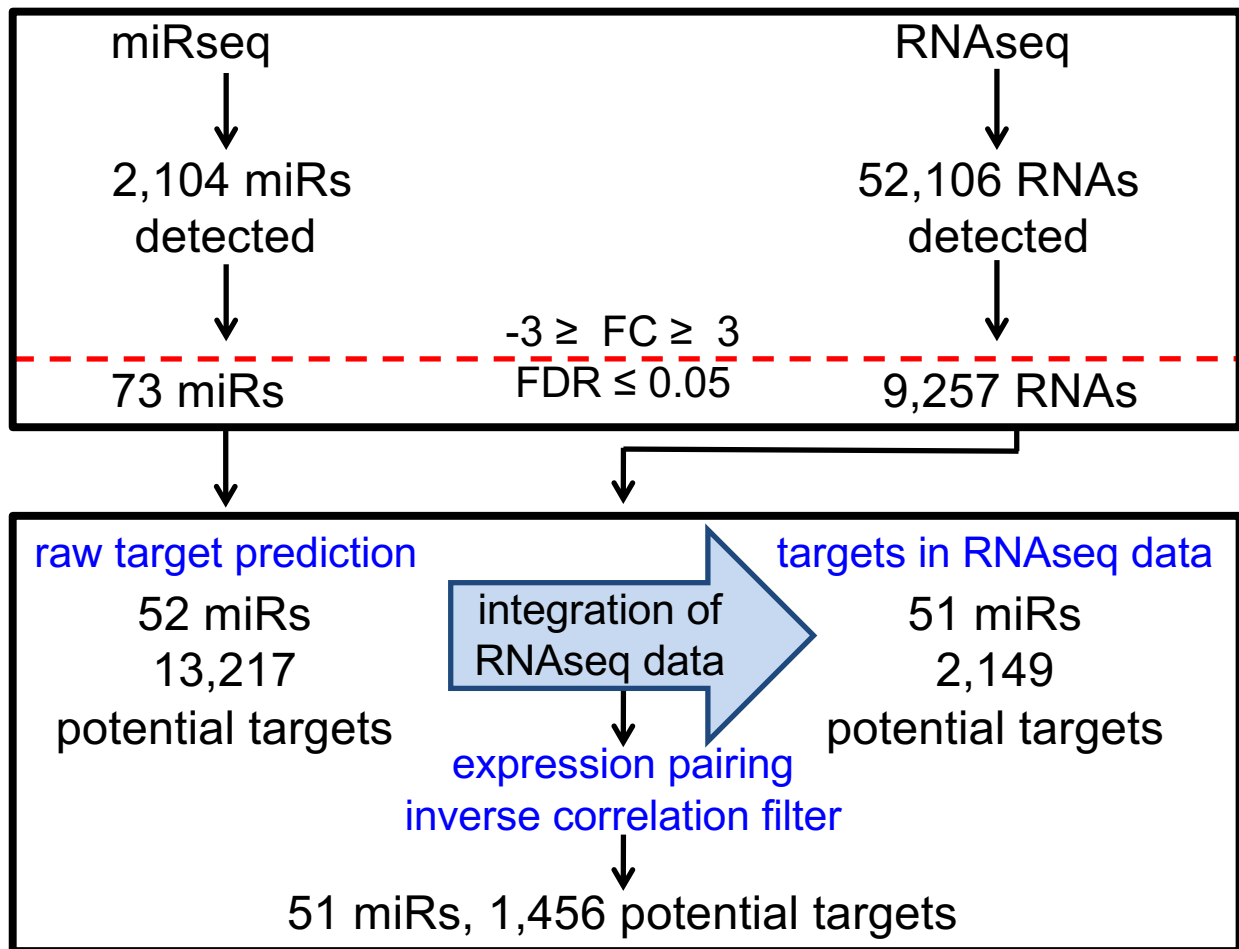
Figure 2.4. Schematic of the miR-mRNA expression-pairing pipeline. The miR Target Filter in Qiagen's Ingenuity Pathway Analysis (IPA) software was used for the pairing of the miRseq and RNAseq data sets. The horizontal red line indicates where threshold cutoffs were employed and blue text denotes key steps in the analysis process. See text for details.

Table 2.1. Top 10 up and down-regulated miRs identified in the miR-mRNA pairing analysis with the potential for functional importance in HPV16 E6/E7 expressing HFKs

Up-Regulated miRs	Down-Regulated Targets			
miR	FC (E6E7/C)	P-value	# Target Genes	Top 5 Target Genes
miR-363-3p	96.11	≥1.00E-15	92	KLK12, SLC6A14, STEAP4, GRHL1, ACAN
miR-9-5p	20.46	≥1.00E-15	103	WFDC12, SMPD3, CALB2, TNNT2, MUC15
miR-20b-5p	9.99	1.02E-08	103	KRT23, CRCT1, ATP12A, KLK7, SPACA4
miR-450a-5p	7.48	1.42E-06	8	PCDHGB7, ZNF365, IGLON5, ZNF385A, DUSP10
miR-542-3p	7.10	2.02E-04	39	ALDH3B2, CST6, MUC15, PPP2R2C, SPSB4
miR-155-5p	7.08	≥1.00E-15	50	MAFB, CSF2RB, INPP5D, SHANK2, GJA5
miR-33b-3p	6.07	1.42E-06	46	LCE3D, WFDC12, LCE3E, TMPRSS13, TGM5
miR-4435	5.59	6.62E-05	75	CNFN, SCNN1B, RNF222, KLK11, TMPRSS13
miR-195-5p	5.56	3.54E-03	123	CEACAM6, HMOX1, ZNF750, RASGEF1B, LYPD5
miR-30b-3p	5.34	3.37E-02	113	PI3, PLA2G4E, ALDH3B2, KLK7, HOPX
miR-335-5p	5.27	≥1.00E-15	40	KPRP, XKRX, INPP5D, CTSV, SLC15A1
miR-199b-5p	5.07	5.21E-03	53	CRCT1, KLK7, A2ML1, RPS10-NUDT3, TSPYL6
Down-Regulated miRs	Up-Regulated Targets			
miR	FC (E6E7/C)	P-value	# Target Genes	Top 5 Target Genes
miR-1249	-9.19	3.66E-07	24	ICAM5, FGFBP3, CRIP2, CERS1, CNTD2
miR-203	-8.93	≥1.00E-15	85	TNFRSF8, GLYATL2, GABRA5, NEK2, INA
miR-34a-5p	-8.47	≥1.00E-15	111	TLX2, MCIDAS, NUP210, FOXR2, IL21R
miR-485-3p	-7.78	9.87E-06	64	LY75, PRRX1, SHISA2, RIPPLY3, HMMR
miR-34c-3p	-4.25	2.07E-06	37	LY75-CD302, SMIM10, PEG3, WDR76, TMEM56
miR-193b-3p	-3.80	≥1.00E-15	58	KLRG2, TAF7L, SPATA31D1, PNMA3, CAMK2N2
miR-197-3p	-3.62	1.46E-10	65	MEIOB, ZNF853, BTNL9, GABRA5, TFR2
miR-323a-3p	-3.41	2.40E-02	18	PLA2G3, HENMT1, PLPP2, ANKRD20A4, ZFPM2
miR-485-5p	-3.27	3.28E-03	120	CLDN11, PNMAL2, THY1, TMEM200B, GOLGA6L1
miR-328	-3.13	3.28E-03	118	GCK, SYNGR3, LRRC10B, ISM2, LCK

≥7 miRs are listed in Supplemental Table 2.5. In particular, Transcriptional Repressor GATA Binding 1 (TRPS1) is potentially targeted by 10 miRs and the ABL Proto-Oncogene 2 non-receptor tyrosine kinase (ABL2) is potentially targeted by 12 miRs. The average fold change in miRs potentially targeting ABL2 is 14.34 and the range in miR expression is between 3 fold and 96 fold. We also examined TRPS1, targeted by 10 miRs, and observed an average fold change in targeting miRs of 4.7 with a range from 3 fold to 10 fold. Based on these data, it does not appear that all miRs targeting a particular mRNA are increased or decreased to a similar degree.

Overall, this data-driven integration of the miRseq and RNAseq data sets revealed that expression of 67.8% (1,456/2,149) potential target mRNAs are inversely correlated with expression of the respective miRs, suggesting that these mRNAs may represent biologically relevant targets of the corresponding miRs.

Identification of microRNAs with the potential to regulate targets in HFKs expressing HPV16 E6/E7

Based on the integrative analysis of miRseq and RNAseq data described above, we next generated a list of miRs that may have functional importance in HPV16 E6/E7 expressing HFKs (Table 1 and Supplemental Table 2.4). This list was curated based on our initial miRseq data and incorporates the miR-mRNA pairing analysis described above. Some highly differentially expressed miRs had a large number of potential targets identified via the miR-mRNA pairing analysis. An example is miR-9-5p, which is up-regulated in HFKs expressing HPV16 E6/E7 by 20 fold and has 102 potential targets. Likewise, some miRs are less dramatically differentially expressed in HFKs

expressing HPV16 E6/E7 and have few potential targets. For example, miR-577 is up-regulated 3.5 fold in HPV16 E6/E7 expressing HFKs and has 8 potential targets. However, in other cases the extent of differential miR expression did not correlate with the number of potential mRNA targets modulated by a given miR. Some highly differentially expressed miRs were paired with very few potential mRNA targets in HPV16 E6/E7 expressing HFKs. In particular, miR-450a-5p was up-regulated 7 fold in HFKs expressing HPV16 E6/E7 but only had 8 potential targets. In contrast, other less dramatically differentially expressed miRs were paired with a large number of potential targets. For example, miR-4532 was only 3 fold upregulated in HPV16 E6/E7 expressing HFKs but could be paired with 90 potential mRNA targets. Hence, our integration of the miRseq and RNAseq data sets allowed for the identification of miRs with the greatest potential for miR-mediated mRNA target regulation, rather than just a set of differentially expressed miRs.

One example of a well-studied miR that we validated from our curated list is miR-203a-3p, which is thought to act as a "switch" between epithelial proliferation and differentiation by targeting the TP53 related TP63 (Yi *et al.*, 2008). The Laimins laboratory first showed that HPVs block induction of miR-203a-3p during differentiation through E7-mediated interference of the MAPK/PKC pathway and that miR-203a-3p inhibition was necessary for HPV genome amplification upon differentiation, as well as for long-term maintenance of HPV episomes (Melar-New and Laimins, 2010). The McCance laboratory also investigated miR-203a-3p, reporting that miR-203a-3p levels are reduced by E6 via abrogation of TP53 (McKenna *et al.*, 2010). Our miRseq and RT-qPCR data suggest that miR-203a-3p levels are decreased by both HPV16 E6 and E7

(Figure 2.5). Integration of the miRseq and RNAseq data revealed 85 potential targets

of miR-203a-3p in HPV16 E6/E7 expressing HFKs. We examined two canonical miR-

203a-3p targets, TP63 and BMI1 (Chen *et al.*, 2015). Using a miR mimic to

overexpress miR-203a-3p, we restored miR-203a-3p levels in HPV16 E6/E7 expressing

HFKs and observed decreased TP63 and BMI1 steady-state mRNA levels. When we

inhibited miR-203a-3p in HFKs expressing HPV16 E6/E7 via a locked nucleic acid

(LNA) inhibitor, we were able to further decrease miR-203a-3p levels, which resulted in

higher TP63 and BMI1 mRNA levels (Figure 2.5). Taken together, our data show that

both HPV16 E6 and E7 function to reduce miR-203a-3p levels.

Potential microRNA targets are involved in unique pathways compared to overall

gene expression changes in HPV16 E6/E7 expressing HFKs

　　　To categorize pathways relevant to the observed changes in miR expression as

a result of HPV16 E6/E7 expression, we utilized the core analysis function of IPA. This

analysis identifies relationships, mechanisms, functions and pathways of relevance to a

particular dataset. We compared core analyses of the miR-modulated mRNAs

identified in the miR-mRNA pairing analysis with the mRNA expression changes

identified by RNAseq. Both data sets were found to be associated with "Cancer" and

"Reproductive System Disease", as well as "Cellular Movement" and "Cell Morphology".

However, some predicted molecular and cellular functions, including "Cellular

Development", "Molecular Transport" and "Growth and Proliferation" were specifically

associated with changes in miR targeted mRNAs (Supplemental Table 2.6).

Additionally, the highest scoring predicted upstream regulators were different between
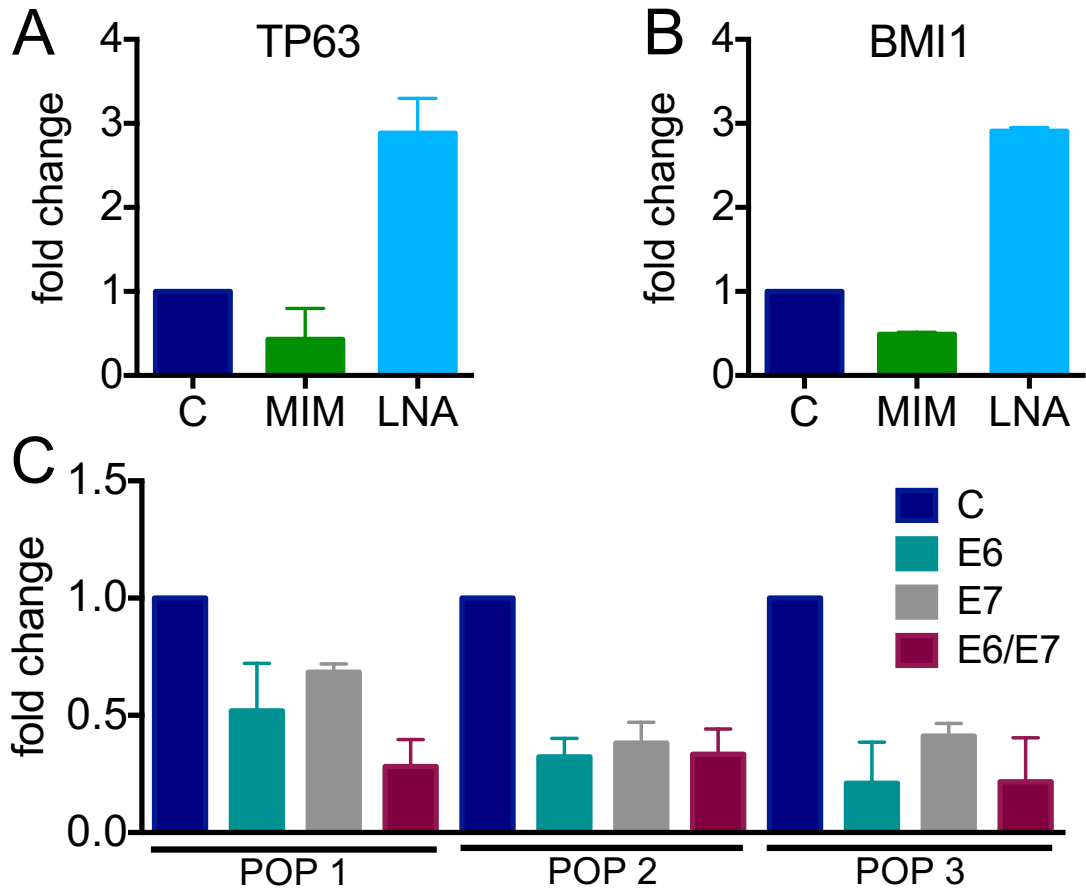
Figure 2.5. Modulation of miR-203a-3p targets and analysis of miR-203a-3p expression. Effects of a miR-203a-3p mimic (MIM, green) or a locked nucleic acid inhibitor (LNA, light blue) on (A) TP63 and (B) BMI1 levels in HPV16 E6/E7 expressing HFKs. Expression of 18S ribosomal RNA (18S) was utilized as an internal control and values were normalized to a negative control (C) mimic or LNA. TP63 and BMI1 expression was assessed by RT-PCR. (C) Expression of miR-203a-3p in three independently derived HFK populations expressing HPV16 E6, E7, E6/E7 or a control vector via TaqMan miR assay. Expression of the non-coding small nuclear RNA U6 spliceosomal RNA (U6) was used as an internal control. Results represent averages of at least three independent experiments.

the two data sets (Supplemental Table 2.7). Overall, these data show that HPV16

E6/E7 regulated mRNAs that are candidate targets of miR modulation are associated

with some biological activities and biochemical pathways that are distinct from

expression changes that are not directly modulated by miR expression.

DISCUSSION

The high-risk HPV E6 and E7 proteins reprogram the infected host cell to allow

for viral genome replication in growth arrested, terminally differentiated epithelial cells

and are the main drivers of cell transformation that ultimately lead to HPV-associated

cancers. Since miRs modulate levels and/or translation of multiple host mRNAs that

regulate a variety of biological activities, they are particularly attractive targets for the

HPV E6 and E7 proteins. In this study, we utilized deep sequencing to examine miR

expression and also examined changes in RNA expression as a result of HPV16 E6/E7

in parallel. Integrating the two data sets, we identified miRs modulated by expression of

HPV16 E6/E7 that may have functional implications in high-risk HPV biology.

We observed 67.8% of potential target RNAs inversely correlated with

expression of their respective miRs, suggesting the potential for miR-mediated

regulation of these RNAs. This number agrees closely with estimations that 60% of all

mRNAs are controlled by miRs (Bartel, 2009), consistent with the notion that miR

regulation is the most abundant mode of posttranscriptional regulation of gene

expression (Jansson and Lund, 2012).

Bioinformatic analyses identified several cellular processes that were significantly

targeted by miR-modulated mRNAs. Additional analyses utilizing IPA revealed

canonical pathways including cyclins, cell cycle regulation (z-score= 2.33) and estrogen-

mediated S-phase entry (z-score= 2.24) to be significantly activated and aryl

hydrocarbon reception signaling to be significantly inhibited (z-score= -2.45) in the

RNAseq data set of all RNAs altered by HPV16 E6/E7 expression. In contrast, ATM

signaling (z-score= 2.12) was significantly activated in HPV16 E6/E7 expressing HFKs based on analysis of miR modulated RNAs. These results suggest that miR modulated RNAs in HPV16 E6/E7 HFKs are involved in distinct canonical pathways that are relevant in the context of HPV biology and imply that HPV16 E6/E7 regulation of cellular miRs contributes to the biological activities of these two proteins.

A total of 49% of potential target RNAs are potentially modulated by multiple miRs. Analysis of RNAs targeted by more than miR indicates that endothelian-1 signaling (z-score= -2.11), p38 MAPK signaling (z-score= -2.12) and G1/S checkpoint regulation (z-score= -2.24) are significantly inhibited and that the ATM signaling pathway is significantly activated (z-score= 2.24). RNAs potentially targeted by just one miR in our study were up-regulated 3.9 fold and down-regulated 13.0 fold, on average, whereas RNAs potentially targeted by more than one miR were up-regulated 3.4 fold and down-regulated 5.7 fold, on average. This suggests that many miR modulated RNAs may also be regulated by other mechanisms, consistent with the notion that miRs act to "fine tune" gene expression.

Our analysis showed that more miRs are up-regulated rather than down-regulated in response to expression of HPV16 E6/E7. While reduced levels of miRs are often observed in tumors due to genetic loss, epigenetic silencing, defects in miR biogenesis or widespread transcriptional repression (Chang *et al.*, 2008; Lu *et al.*, 2005), our results may be explained by the fact that our experimental system more closely mimics an HPV associated premalignant lesion, rather than a late-stage invasive carcinoma. Nevertheless, many of the miR expression changes detected in HPV16 E6/E7 expressing HFKs were also observed in HPV+ head and neck squamous cell

carcinoma cell lines (Wald *et al.*, 2011) and tumors (Chapman *et al.*, 2015). We also observed changes in miR expression that have been detected in HPV+ anal carcinomas (Myklebust *et al.*, 2011), vulvar cancers (de Melo Maia *et al.*, 2013), and penile squamous cell carcinoma (Barzon *et al.*, 2014). A comparison of our data to miR expression in HPV-associated human biopsies are detailed in Supplemental Table 2.8. These results suggest that miR alterations in HPV-associated tumors are likely caused by HPV E6/E7 expression and that these miR-mRNA pairs may be potential "drivers" of HPV carcinogenesis. Our results also indicate that a core set of miRs may be altered in all HPV-associated epithelial cancers as a result of HPV16 E6/E7 expression, whereas some miRs may be specific to an HPV-associated cancer of a particular anatomical site.

Modulation of cellular miR levels by HPV gene expression has been previously investigated by other groups (Gunasekharan and Laimins, 2013; Wang *et al.*, 2014). We examined miR expression in more uniform populations of undifferentiated HFKs, allowing us to identify miRs that are likely modulated directly as a consequence of HPV16 E6/E7 expression rather than representing the expansion of proliferating, undifferentiated cells in E6/E7 expressing raft cultures. Simultaneous miRseq and RNAseq enabled us to investigate in detail the potential influence of miR regulation on overall gene expression in HPV16 E6/E7 expressing HFKs. Supplemental Tables 2.8, 2.9 and 2.10 compare our data with other studies of HPV-associated miRs in the literature. We hypothesize that many of the differences in miR expression that we observe are the result of analyzing undifferentiated human epithelial cells, whereas most other studies analyzed differentiating cells. Additional differences may be due to

differences in HPV type or analysis of the effect of whole HPV genomes compared to our study, which focused only on effects of HPV16 E6 and/or E7 on miRs.

While the focus of our study was on alterations in miRs resulting from expression of both HPV16 E6/E7, we also performed miRseq of HFKs expressing HPV16 E6 or E7 alone to understand the consequences of individual oncoproteins on miR expression. Given that the TP53 and the E2F pathways are key targets of HPV16 E6 and E7, respectively, we considered the possibility that some of the miRs regulated by HPV16 E6 or E7 may be TP53 or E2F responsive miRs. The miR-106b~25 cluster is known to be regulated by E2F family members (Emmrich and Putzer, 2010) and a member of that cluster, miR-25-5p, is one of the top miRs up-regulated by HPV16 E7. Additionally, the miR-15b~16-2 cluster is an E2F target (Bueno *et al.*, 2010) and all three members of that cluster, miR-15b-5p, -16-5p and -16-2-3p, are on our list of HPV16 E7 up-regulated miRs. The TP53 tumor suppressor can transcriptionally activate miR genes, as is the case for the miR-34 family and others (Braun *et al.*, 2008; Chang *et al.*, 2007; Raver-Shapira *et al.*, 2007; Tarasov *et al.*, 2007). Both miR-34a-5p and -34c-3p are down-regulated in HPV16 E6 expressing HFKs. Additionally, TP53 can activate processing of specific miRs, such as miR-143-3p (Boominathan, 2010; Suzuki *et al.*, 2009) and miR-143-3p was on our list of HPV16 E6 down-regulated miRs. Other HPV16 E6 and/or E7 modulated miRs have not been identified to be TP53 or E2F responsive, suggesting that HPV16 E6 and E7 may also alter miR expression through other mechanisms.

We compared our list of top up-regulated and down-regulated miRs in HPV16 or E7 expressing HFKs to a miR analysis performed by the Khan laboratory (Yablonska *et al.*, 2013). Several miRs, for example, miR-100-3p, were found to be up-regulated by

HPV16 E7, whereas other miRs showed different trends of expression. We also compared our results to a study examining miR expression resulting from expression of HPV18 E6 or E7 (Wang *et al.*, 2014). Our data agree with their observation of down-regulation of miR-34a-5p by E6 and up-regulation of miR-25-5p by E7, as well as the finding that modulation of expression of some miRs can be attributed to one or both oncoproteins.

Our study showed that expression of HPV16 E6/E7 in HFKs not only changes expression of individual miRs, but also alters expression entire groups of genomically clustered miRs. Of interest, we observe some of the same miR clusters altered by HPV16 E6/E7 expression as has been observed in studies of cervical cancer (Servin-Gonzalez *et al.*, 2015). HPV16 E6/E7 modulates both tumor suppressive and oncogenic miR clusters. For example, HPV16 E6/E7 up-regulates all miRs of the oncogenic miR-106b~25 cluster (Hudson *et al.*, 2013; Poliseno *et al.*, 2010) and down-regulates all miRs of the tumor suppressive miR-34b~34c cluster (reviewed in (Hermeking, 2010)).

Most of the early studies on miRs in cancer have focused on a single miR and modulation of a single target mRNA. While these studies were useful, this paradigm of research in the miR field has now been mostly replaced with studies that analyze the global landscape of miR expression and utilize integrative methods to investigate the potential effects of these alterations on cellular processes. Additionally, human cells encode ≥ 2,500 mature miRs and a single miR can regulate expression and/or translation of hundreds of RNA targets. Therefore, aberrant miR expression will

influence a multitude of target transcripts, causing alteration in multiple signaling pathways. Moreover, many mRNAs are subject to regulation by multiple miRs.

Our study shows that high-risk HPV E6/E7 expression in normal human cells causes a dramatic rewiring of cellular gene expression and that modulation of cellular miR expression plays an important role in this process. A large percentage of RNAs expressed in HPV16 E6/E7 expressing keratinocytes are potentially targeted by miRs that are modulated by E6/E7 expression. Genes involved in specific cellular processes and pathways, including cell cycle regulation and ATM signaling seem to be selectively regulated by miRs. Moreover, our study has also identified some miRs that have been previously reported to be dysreguated in HPV associated lesions and cancers as targets of the HPV E6 and E7 oncoproteins.

REFERENCES

1.   **Bartel, D.P.** (2009). MicroRNAs: target recognition and regulatory functions. Cell: 136, 215-233.

2.   **Barzon, L., Cappellesso, R., Peta, E., Militello, V., Sinigaglia, A., Fassan, M., Simonato, F., Guzzardo, V., Ventura, L., Blandamura, S., Gardiman, M., Palu, G., Fassina, A.** (2014). Profiling of expression of human papillomavirus-related cancer miRNAs in penile squamous cell carcinomas. Am J Pathol: 184, 3376-3383.

3.   **Bentwich, I., Avniel, A., Karov, Y., Aharonov, R., Gilad, S., Barad, O., Barzilai, A., Einat, P., Einav, U., Meiri, E., Sharon, E., Spector, Y., Bentwich, Z.** (2005). Identification of hundreds of conserved and nonconserved human microRNAs. Nat Genet: 37, 766-770.

4.   **Boominathan, L.** (2010). The tumor suppressors p53, p63, and p73 are regulators of microRNA processing complex. PLoS One: 5, e10615.

5.   **Boyer, S.N., Wazer, D.E., Band, V.** (1996). E7 protein of human papilloma virus-16 induces degradation of retinoblastoma protein through the ubiquitin-proteasome pathway. Cancer Res: 56, 4620-4624.

6.   **Braun, C.J., Zhang, X., Savelyeva, I., Wolff, S., Moll, U.M., Schepeler, T., Orntoft, T.F., Andersen, C.L., Dobbelstein, M.** (2008). p53-Responsive micrornas 192 and 215 are capable of inducing cell cycle arrest. Cancer Res: 68, 10094-10104.

7.   **Bueno, M.J., Gomez de Cedron, M., Laresgoiti, U., Fernandez-Piqueras, J., Zubiaga, A.M., Malumbres, M.** (2010). Multiple E2F-induced microRNAs prevent replicative stress in response to mitogenic signaling. Mol Cell Biol: 30, 2983-2995.

8.   **Cai, X., Li, G., Laimins, L.A., Cullen, B.R.** (2006). Human papillomavirus genotype 31 does not express detectable microRNA levels during latent or productive virus replication. J Virol: 80, 10890-10893.

9.   **Chang, T.C., Wentzel, E.A., Kent, O.A., Ramachandran, K., Mullendore, M., Lee, K.H., Feldmann, G., Yamakuchi, M., Ferlito, M., Lowenstein, C.J.,**

Arking, D.E., Beer, M.A., Maitra, A., Mendell, J.T. (2007). Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. Mol Cell: 26, 745-752.

10. **Chang, T.C., Yu, D., Lee, Y.S., Wentzel, E.A., Arking, D.E., West, K.M., Dang, C.V., Thomas-Tikhonenko, A., Mendell, J.T.** (2008). Widespread microRNA repression by Myc contributes to tumorigenesis. Nat Genet: 40, 43-50.

11. **Chapman, B.V., Wald, A.I., Akhtar, P., Munko, A.C., Xu, J., Gibson, S.P., Grandis, J.R., Ferris, R.L., Khan, S.A.** (2015). MicroRNA-363 targets myosin 1B to reduce cellular migration in head and neck cancer. BMC Cancer: 15, 861.

12. **Chaulk, S.G., Ebhardt, H.A., Fahlman, R.P.** (2016). Correlations of microRNA:microRNA expression patterns reveal insights into microRNA clusters and global microRNA expression patterns. Mol Biosyst: 12, 110-119.

13. **Chen, P.Y., Manninga, H., Slanchev, K., Chien, M., Russo, J.J., Ju, J., Sheridan, R., John, B., Marks, D.S., Gaidatzis, D., Sander, C., Zavolan, M., Tuschl, T.** (2005). The developmental miRNA profiles of zebrafish as determined by small RNA cloning. Genes Dev: 19, 1288-1293.

14. **Chen, T., Xu, C., Chen, J., Ding, C., Xu, Z., Li, C., Zhao, J.** (2015). MicroRNA-203 inhibits cellular proliferation and invasion by targeting Bmi1 in non-small cell lung cancer. Oncol Lett: 9, 2639-2646.

15. **de Melo Maia, B., Lavorato-Rocha, A.M., Rodrigues, L.S., Coutinho-Camillo, C.M., Baiocchi, G., Stiepcich, M.M., Puga, R., de, A.L.L., Soares, F.A., Rocha, R.M.** (2013). microRNA portraits in human vulvar carcinoma. Cancer Prev Res (Phila): 6, 1231-1241.

16. **Dyson, N., Howley, P.M., Munger, K., Harlow, E.** (1989). The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science: 243, 934-937.

17. **Eichhorn, S.W., Guo, H., McGeary, S.E., Rodriguez-Mias, R.A., Shin, C., Baek, D., Hsu, S.H., Ghoshal, K., Villen, J., Bartel, D.P.** (2014). mRNA destabilization is the dominant effect of mammalian microRNAs by the time substantial repression ensues. Mol Cell: 56, 104-115.

18.   **Emmrich, S., Putzer, B.M.** (2010). Checks and balances: E2F-microRNA crosstalk in cancer control. Cell Cycle: 9, 2555-2567.

19.   **Friedman, R.C., Farh, K.K., Burge, C.B., Bartel, D.P.** (2009). Most mammalian mRNAs are conserved targets of microRNAs. Genome Res: 19, 92-105.

20.   **Giraldez, A.J., Cinalli, R.M., Glasner, M.E., Enright, A.J., Thomson, J.M., Baskerville, S., Hammond, S.M., Bartel, D.P., Schier, A.F.** (2005). MicroRNAs regulate brain morphogenesis in zebrafish. Science: 308, 833-838.

21.   **Griffiths-Jones, S.** (2004). The microRNA Registry. Nucleic Acids Res: 32, D109-111.

22.   **Griffiths-Jones, S., Grocock, R.J., van Dongen, S., Bateman, A., Enright, A.J.** (2006). miRBase: microRNA sequences, targets and gene nomenclature. Nucleic Acids Res: 34, D140-144.

23.   **Griffiths-Jones, S., Saini, H.K., van Dongen, S., Enright, A.J.** (2008). miRBase: tools for microRNA genomics. Nucleic Acids Res: 36, D154-158.

24.   **Gunasekharan, V., Laimins, L.A.** (2013). Human papillomaviruses modulate microRNA 145 expression to directly control genome amplification. J Virol: 87, 6037-6043.

25.   **Guo, H., Ingolia, N.T., Weissman, J.S., Bartel, D.P.** (2010). Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature: 466, 835-840.

26.   **Guo, L., Yang, S., Zhao, Y., Zhang, H., Wu, Q., Chen, F.** (2014). Global analysis of miRNA gene clusters and gene families reveals dynamic and coordinated expression. Biomed Res Int: 2014, 782490.

27.   **Halbert, C.L., Demers, G.W., Galloway, D.A.** (1991). The E7 gene of human papillomavirus type 16 is sufficient for immortalization of human epithelial cells. J Virol: 65, 473-478.

28.   **Hanahan, D., Weinberg, R.A.** (2000). The hallmarks of cancer. Cell: 100, 57-70.

29.	**Hendrickson, D.G., Hogan, D.J., McCullough, H.L., Myers, J.W., Herschlag, D., Ferrell, J.E., Brown, P.O.** (2009). Concordant regulation of translation and mRNA abundance for hundreds of targets of a human microRNA. PLoS Biol: 7, e1000238.

30.	**Hermeking, H.** (2010). The miR-34 family in cancer and apoptosis. Cell Death Differ: 17, 193-199.

31.	**Houbaviy, H.B., Murray, M.F., Sharp, P.A.** (2003). Embryonic stem cell-specific MicroRNAs. Dev Cell: 5, 351-358.

32.	**Hudson, R.S., Yi, M., Esposito, D., Glynn, S.A., Starks, A.M., Yang, Y., Schetter, A.J., Watkins, S.K., Hurwitz, A.A., Dorsey, T.H., Stephens, R.M., Croce, C.M., Ambs, S.** (2013). MicroRNA-106b-25 cluster expression is associated with early disease recurrence and targets caspase-7 and focal adhesion in human prostate cancer. Oncogene: 32, 4139-4147.

33.	**Jansson, M.D., Lund, A.H.** (2012). MicroRNA and cancer. Mol Oncol: 6, 590-610.

34.	**Jones, D.L., Thompson, D.A., Munger, K.** (1997). Destabilization of the RB tumor suppressor protein and stabilization of p53 contribute to HPV type 16 E7-induced apoptosis. Virology: 239, 97-107.

35.	**Klipper-Aurbach, Y., Wasserman, M., Braunspiegel-Weintrob, N., Borstein, D., Peleg, S., Assa, S., Karp, M., Benjamini, Y., Hochberg, Y., Laron, Z.** (1995). Mathematical formulae for the prediction of the residual beta cell function during the first two years of disease in children and adolescents with insulin-dependent diabetes mellitus. Med Hypotheses: 45, 486-490.

36.	**Kozomara, A., Griffiths-Jones, S.** (2011). miRBase: integrating microRNA annotation and deep-sequencing data. Nucleic Acids Res: 39, D152-157.

37.	**Kozomara, A., Griffiths-Jones, S.** (2014). miRBase: annotating high confidence microRNAs using deep sequencing data. Nucleic Acids Res: 42, D68-73.

38.	**Langmead, B., Trapnell, C., Pop, M., Salzberg, S.L.** (2009). Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol: 10, R25.

39. **Lee, Y., Jeon, K., Lee, J.T., Kim, S., Kim, V.N.** (2002). MicroRNA maturation: stepwise processing and subcellular localization. EMBO J: 21, 4663-4670.

40. **Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R., Genome Project Data Processing, S.** (2009). The Sequence Alignment/Map format and SAMtools. Bioinformatics: 25, 2078-2079.

41. **Lim, L.P., Lau, N.C., Garrett-Engele, P., Grimson, A., Schelter, J.M., Castle, J., Bartel, D.P., Linsley, P.S., Johnson, J.M.** (2005). Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature: 433, 769-773.

42. **Lu, J., Getz, G., Miska, E.A., Alvarez-Saavedra, E., Lamb, J., Peck, D., Sweet-Cordero, A., Ebert, B.L., Mak, R.H., Ferrando, A.A., Downing, J.R., Jacks, T., Horvitz, H.R., Golub, T.R.** (2005). MicroRNA expression profiles classify human cancers. Nature: 435, 834-838.

43. **McCance, D.J., Kopan, R., Fuchs, E., Laimins, L.A.** (1988). Human papillomavirus type 16 alters human epithelial cell differentiation in vitro. Proc Natl Acad Sci U S A: 85, 7169-7173.

44. **McKenna, D.J., McDade, S.S., Patel, D., McCance, D.J.** (2010). MicroRNA 203 expression in keratinocytes is dependent on regulation of p53 levels by E6. J Virol: 84, 10644-10652.

45. **McLaughlin-Drubin, M.E., Meyers, J., Munger, K.** (2012). Cancer associated human papillomaviruses. Curr Opin Virol: 2, 459-466.

46. **McLaughlin-Drubin, M.E., Munger, K.** (2009). Oncogenic activities of human papillomaviruses. Virus Res: 143, 195-208.

47. **McMurray, H.R., McCance, D.J.** (2003). Human papillomavirus type 16 E6 activates TERT gene transcription through induction of c-Myc and release of USF-mediated repression. J Virol: 77, 9852-9861.

48. **Melar-New, M., Laimins, L.A.** (2010). Human papillomaviruses modulate expression of microRNA 203 upon epithelial differentiation to control levels of p63 proteins. J Virol: 84, 5212-5221.

49. **Mesri, E.A., Feitelson, M.A., Munger, K.** (2014). Human viral oncogenesis: a cancer hallmarks analysis. Cell Host Microbe: 15, 266-282.

50. **Myklebust, M.P., Bruland, O., Fluge, O., Skarstein, A., Balteskard, L., Dahl, O.** (2011). MicroRNA-15b is induced with E2F-controlled genes in HPV-related cancer. Br J Cancer: 105, 1719-1725.

51. **Ning, M.S., Kim, A.S., Prasad, N., Levy, S.E., Zhang, H., Andl, T.** (2014). Characterization of the Merkel Cell Carcinoma miRNome. J Skin Cancer: 2014, 289548.

52. **Poliseno, L., Salmena, L., Riccardi, L., Fornari, A., Song, M.S., Hobbs, R.M., Sportoletti, P., Varmeh, S., Egia, A., Fedele, G., Rameh, L., Loda, M., Pandolfi, P.P.** (2010). Identification of the miR-106b~25 microRNA cluster as a proto-oncogenic PTEN-targeting intron that cooperates with its host gene MCM7 in transformation. Sci Signal: 3, ra29.

53. **Raver-Shapira, N., Marciano, E., Meiri, E., Spector, Y., Rosenfeld, N., Moskovits, N., Bentwich, Z., Oren, M.** (2007). Transcriptional activation of miR-34a contributes to p53-mediated apoptosis. Mol Cell: 26, 731-743.

54. **Robinson, M.D., Oshlack, A.** (2010). A scaling normalization method for differential expression analysis of RNA-seq data. Genome Biol: 11, R25.

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

56. **Sempere, L.F., Freemantle, S., Pitha-Rowe, I., Moss, E., Dmitrovsky, E., Ambros, V.** (2004). Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. Genome Biol: 5, R13.

57. **Servin-Gonzalez, L.S., Granados-Lopez, A.J., Lopez, J.A.** (2015). Families of microRNAs Expressed in Clusters Regulate Cell Signaling in Cervical Cancer. Int J Mol Sci: 16, 12773-12790.

58. **Suh, M.R., Lee, Y., Kim, J.Y., Kim, S.K., Moon, S.H., Lee, J.Y., Cha, K.Y., Chung, H.M., Yoon, H.S., Moon, S.Y., Kim, V.N., Kim, K.S.** (2004). Human

embryonic stem cells express a unique set of microRNAs. Dev Biol: 270, 488-498.

59.   **Suzuki, H.I., Yamagata, K., Sugimoto, K., Iwamoto, T., Kato, S., Miyazono, K.** (2009). Modulation of microRNA processing by p53. Nature: 460, 529-533.

60.   **Tarasov, V., Jung, P., Verdoodt, B., Lodygin, D., Epanchintsev, A., Menssen, A., Meister, G., Hermeking, H.** (2007). Differential regulation of microRNAs by p53 revealed by massively parallel sequencing: miR-34a is a p53 target that induces apoptosis and G1-arrest. Cell Cycle: 6, 1586-1593.

61.   **Trapnell, C., Pachter, L., Salzberg, S.L.** (2009). TopHat: discovering splice junctions with RNA-Seq. Bioinformatics: 25, 1105-1111.

62.   **Ventura, A., Young, A.G., Winslow, M.M., Lintault, L., Meissner, A., Erkeland, S.J., Newman, J., Bronson, R.T., Crowley, D., Stone, J.R., Jaenisch, R., Sharp, P.A., Jacks, T.** (2008). Targeted deletion reveals essential and overlapping functions of the miR-17 through 92 family of miRNA clusters. Cell: 132, 875-886.

63.   **Wald, A.I., Hoskins, E.E., Wells, S.I., Ferris, R.L., Khan, S.A.** (2011). Alteration of microRNA profiles in squamous cell carcinoma of the head and neck cell lines by human papillomavirus. Head Neck: 33, 504-512.

64.   **Wang, X., Wang, H.K., Li, Y., Hafner, M., Banerjee, N.S., Tang, S., Briskin, D., Meyers, C., Chow, L.T., Xie, X., Tuschl, T., Zheng, Z.M.** (2014). microRNAs are biomarkers of oncogenic human papillomavirus infections. Proc Natl Acad Sci U S A: 111, 4262-4267.

65.   **Werness, B.A., Levine, A.J., Howley, P.M.** (1990). Association of human papillomavirus types 16 and 18 E6 proteins with p53. Science: 248, 76-79.

66.   **Yablonska, S., Hoskins, E.E., Wells, S.I., Khan, S.A.** (2013). Identification of miRNAs dysregulated in human foreskin keratinocytes (HFKs) expressing the human papillomavirus (HPV) Type 16 E6 and E7 oncoproteins. Microrna: 2, 2-13.

67.   **Yi, R., Poy, M.N., Stoffel, M., Fuchs, E.** (2008). A skin microRNA promotes differentiation by repressing 'stemness'. Nature: 452, 225-229.

CHAPTER THREE

Perturbation of DROSHA and DICER Expression by Human Papillomavirus 16

Oncoproteins

This chapter is adapted from a previously published manuscript:

Contributions: I performed all experiments and wrote the manuscript. Karl Munger aided in experimental design and helped to write and edit the manuscript.

SUMMARY

Many tumors, including cervical carcinomas, show dysregulated expression of the microRNA (miR) processing machinery, specifically DROSHA and DICER. Some cervical cancers exhibit chromosome 5p amplifications, with *DROSHA* being the most significantly upregulated transcript, and this is observed in all tumors with 5p gain. *DROSHA* and *DICER* mRNA levels, however, are higher in HPV positive cancer lines than in an HPV negative cervical carcinoma line. We show that high-risk HPV E6/E7 expression in HPV negative C33A cervical carcinoma cells and primary human foreskin keratinocytes causes increased expression of DROSHA and DICER mRNA and protein. Most importantly, many DROSHA regulated miRs are dysregulated in HPV16 E6/E7 expressing cells. These results suggest that increased DROSHA levels contribute to HPV16 E6/E7 dysregulation of cellular miR expression.

INTRODUCTION

DROSHA is a double-stranded RNA-specific ribonuclease (RNAse III) and the catalytic component of the microprocessor protein complex, which is rate limiting for the initial processing of primary microRNA transcripts (pri-miRs) into precursor miRs (pre-miRs) in the nucleus (Denli *et al.*, 2004; Han *et al.*, 2004; Lee *et al.*, 2003; Xie and Steitz, 2014). After export to the cytoplasm, pre-miRs are processed by another RNAse III enzyme, DICER, into the mature miR duplex (Grishok *et al.*, 2001; Hutvagner *et al.*, 2001; Ketting *et al.*, 2001; Knight and Bass, 2001). The miR duplex is then incorporated into the RNA-induced silencing complex (RISC), containing DICER, an AGO (Argonaute) protein (Liu *et al.*, 2004; Meister *et al.*, 2004), and the RNA binding proteins TRBP (TAR RNA binding protein) (Chendrimada *et al.*, 2005; Haase *et al.*, 2005) and PACT (protein activator of PKR) (Lee *et al.*, 2006). After RISC binding, one strand of the miR duplex is selected as the guide strand (miR), based on the strength of base-pairing at its 5' end, to form the functional miR-RISC complex (miRISC) (Khvorova *et al.*, 2003; Schwarz *et al.*, 2003). The other strand, known as the passenger strand (miR*), is displaced and degraded. The miRISC, the final product of this carefully orchestrated maturation process, can then function to silence miR targets via mRNA degradation/destabilization, translational repression, or a combination of both mechanisms (Djuranovic *et al.*, 2012).

The miR biogenesis pathway, including DROSHA and DICER expression, is frequently dysregulated in human cancers (reviewed in (Adams *et al.*, 2014; Hata and Lieberman, 2015). *DROSHA* is frequently amplified or mutated whereas *DICER* is most often mutated (Cerami *et al.*, 2012; Gao *et al.*, 2013). Altered *DROSHA* and *DICER*

expression has also been noted in cervical carcinoma. Specifically, a gain of chromosome 5p has been observed in most advanced cervical squamous cell carcinomas. *DROSHA* is the most significantly upregulated transcript associated with this chromosomal gain and *DROSHA* overexpression has been observed in all tumors with 5p gain, suggesting that *DROSHA* may be a critical 5p target with a potential role in cervical cancer progression (Muralidhar *et al.*, 2007; Scotto *et al.*, 2008). *DROSHA* mRNA levels were also shown to be increased in the HPV positive SiHa and HeLa cervical cancer cell lines and to a lesser extent in the C33A HPV negative cervical cancer cell line (Muralidhar *et al.*, 2007; Zhou *et al.*, 2013).

Interestingly, modulation of DROSHA expression in cervical carcinoma lines did not result in global alterations of miR levels and only a small subset of 45 out of 319 miRs examined showed changes in expression upon modulation of DROSHA levels (Muralidhar *et al.*, 2011). Modulation of these miRs was linked to alterations in cell migration and invasiveness (Muralidhar *et al.*, 2011), two important hallmarks of cancer progression.

Reports on *DICER* mRNA levels in cervical cancers are not consistent. A small study reported lower *DICER* mRNA levels in cervical cancer tissues (Zhao *et al.*, 2014), whereas another study reported varied *DICER* mRNA expression among cervical cancer specimens, with low *DICER* mRNA expression in 36.7% of samples and higher *DICER* mRNA expression in 63.3% of samples (He et al., 2014).

We have previously investigated modulation of cellular miR levels in response to HPV16 E6/E7 expression in primary human foreskin keratinocytes (Harden *et al.*, 2017). Given that the high-risk HPV E6 and E7 proteins are consistently expressed in cervical

carcinoma lines, that HPV positive cervical carcinoma lines expressed *DROSHA* at

higher levels than the HPV negative C33A line and the fact that DROSHA expression

was shown to only affect expression of a small subset of miRs in cervical cancer lines,

we set out to determine whether HPV E6 and/or E7 expression may cause altered

DROSHA and/or DICER levels. Additionally, we aimed to investigate whether some of

the alterations in cellular miR expression that we previously observed may potentially be

caused by altered DROSHA expression.

MATERIALS AND METHODS

Cell Culture

Primary human foreskin keratinocytes (HFKs) were isolated from a pool of de-identified newborn foreskins and cultured as previously described (Harden *et al.*, 2017). CaSki, C33A, HeLa and SiHa cells (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin and 50 mg/ml streptomycin. HFKs were transduced with LXSN based recombinant retroviruses encoding HPV16 E6 and/or E7 or a control LXSN vector (Halbert *et al.*, 1991) as previously described (Harden *et al.*, 2017). C33As were transiently transfected with pCMV HPV16 E6 and/or pCMV HPV16 E7 plasmids, a control pCMV plasmid as a control utilizing FuGENE 6 Transfection Reagent (Promega) according to the manufacturer's protocol. HFKs were grown to 80% confluence prior to passaging and passaged up to 8 times. In all experiments, donor and passage matched HFK populations were used.

Reverse Transcription quantitative PCR (RT-qPCR)

Total RNA was isolated using the miRNeasy Mini Kit (Qiagen) per the manufacturer's instructions. For RT-qPCR of *DROSHA* and *DICER*, following RNA isolation, total RNA was DNAse-treated with the TURBO DNA-*free* kit (Ambion/Thermo Fisher Scientific). DNAse-treated total RNA was then reverse transcribed utilizing TaqMan Reverse Transcription Reagents (Applied Biosystems/Thermo Fisher Scientific). TaqMan Assay IDs Hs00203008_m1 and Hs00229023_m1 (Applied

Biosystems/Thermo Fisher Scientific) were employed to detect *DROSHA* and *DICER*,

respectively, using the comparative Ct method with the StepOnePlus Real-Time PCR

System (Thermo Fisher Scientific). RT-qPCR assays were performed in triplicate and

18S ribosomal RNA was utilized as an internal control.

Western Blotting

Protein lysates were prepared by incubating the cells in ML buffer (300 mM NaCl,

0.5% Nonidet P-40 [NP-40], 20 mM Tris-HCl [pH 8.0], 1 mM EDTA) supplemented with

one Complete EDTA-free Protease Inhibitor Cocktail tablet (Roche) per 50 ml lysis

buffer. The cells were incubated on ice for 20 minutes, scraped and rotated at 4°C for

20 minutes and then cleared by centrifugation at 16,000 × g for 20 min. Protein

concentrations were determined via the Bradford method (Bradford, 1976). Samples

containing 200 µg of protein were boiled in NuPAGE® LDS Sample Buffer (4X)

(Invitrogen), separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and

transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore).

The membranes were blocked for 2 h in 5% nonfat dry milk in TBST buffer (137 mM

NaCl, 2.7 mM KCl, 25 mM Tris [pH 7.4], 0.1% Tween 20). Primary antibodies were

used as follows: DROSHA (ab12286; Abcam) at 1:500, DICER (3363; Cell Signaling

Technology) at 1:1,000 and β-actin (MAB1501; Millipore) at 1:1,000. Secondary anti-

mouse IgG and anti-rabbit IgG horseradish peroxidase-conjugated antibodies

(Amersham) were used at 1:10,000 dilutions. Proteins were visualized by enhanced

chemiluminescence (Luminata™ Crescendo Western HRP Substrate; Millipore) and

electronically acquired with a Syngene G:BOX image station (Syngene) equipped with

GeneSys software, v1.5.6.0. Loading was assessed using β-actin immunoblots.

RESULTS

Increased *DROSHA* and *DICER* mRNA levels in HPV positive cervical cancer cell lines

DROSHA is frequently expressed at high levels in cervical carcinomas. Therefore, we examined *DROSHA* mRNA levels by RT-qPCR in the HPV16 positive CaSki and SiHa, the HPV18 positive HeLa and the HPV-negative C33A cervical cancer cell lines. Compared to primary human foreskin keratinocytes (HFKs), *DROSHA* mRNA was significantly upregulated in CaSki (24.4 fold; $P<0.0001$), SiHa (26.8 fold; $P<0.0001$), HeLa (7.2 fold; $P<0.0001$) but was not significantly modulated in C33A cells (1.1 fold; $P=0.72$) (Figure 3.1A). These results partially confirm and extend a previous report that documented increased *DROSHA* mRNA expression in HeLa, SiHa and C33A cervical cancer cell lines (Muralidhar *et al.*, 2007; Zhou *et al.*, 2013).

Reports in the literature on *DICER* mRNA expression in cervical cancers are inconsistent (He *et al.*, 2014; Zhao *et al.*, 2014). Therefore, we next determined *DICER* mRNA expression in cervical carcinoma lines. Our analysis revealed that compared to HFKs, *DICER* mRNA levels were significantly higher in the HPV16 positive CaSki (4.9 fold; $P=0.0005$) and SiHa (2.7 fold; $P=0.0136$) but not significantly altered in HPV18 positive HeLa (-2.7 fold; $P=0.0978$) or the HPV negative C33A (-4.9 fold; $P=0.0946$) lines (Figure 3.1B). In summary, our data are partially consistent with a previous report (Zhou *et al.*, 2013), and show that *DROSHA* mRNA levels are significantly increased in all HPV positive cervical cancer cell lines, whereas *DICER* mRNA levels were only significantly increased in the HPV16 positive CaSki and SiHa, but not in the HPV18
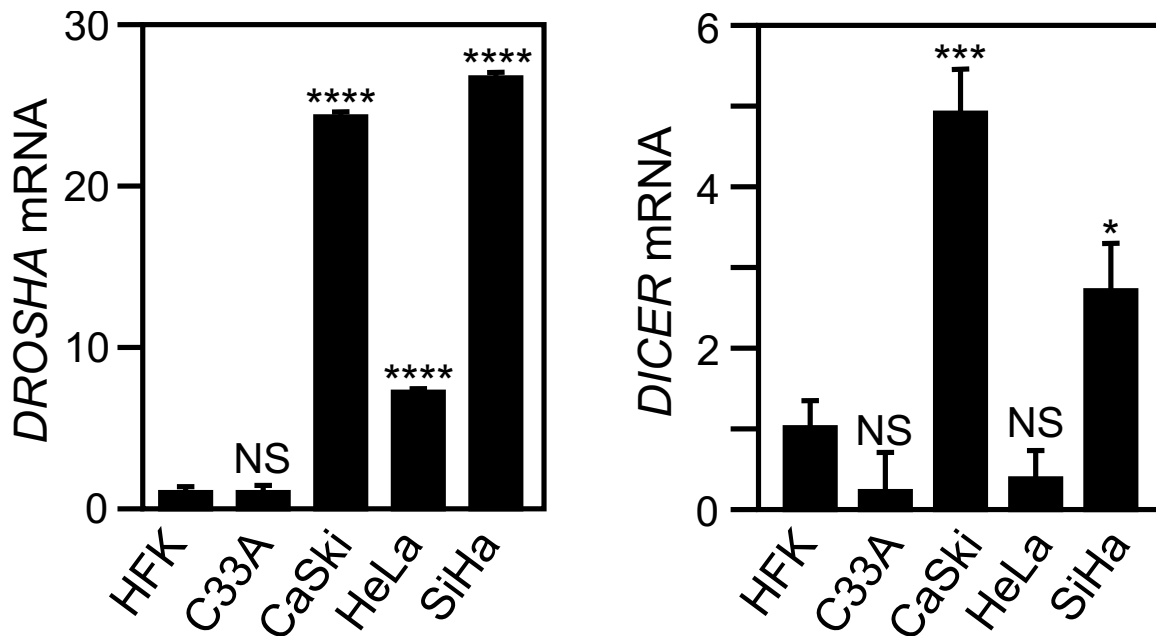
Figure 3.1. Levels of *DROSHA* and *DICER* mRNA in cervical cancer cell lines.

Total RNA was harvested from HFKs and C33A (HPV negative), CaSki (HPV16), SiHa (HPV16) and HeLa (HPV18) cells. (A) *DROSHA* and (B) *DICER* mRNA levels were determined by RT-qPCR. Expression of 18S ribosomal RNA (18S) was utilized as a control and results were normalized to *DROSHA* and *DICER* expression in HFKs. Results show averages of three independent experiments and error bars depict standard deviation. Unpaired, two-tailed t-tests with a 95% confidence level were performed to determine statistical significance. *, **, *** and **** indicate statistical significance at a $P<0.05$, $P<0.01$, $P<0.001$ and $P<0.0001$ respectively. "NS" indicates not statistically significant.

positive HeLa or the HPV negative C33A cervical carcinoma lines.

HPV16 E6/E7 expression in HPV negative C33A cells increases *DROSHA* mRNA

Our results suggested that *DROSHA* mRNA levels in cervical cancer lines may correlate with HPV status (Figure 3.1). Hence, we investigated whether HPV16 E6 and/or E7 expression in C33A cells may increase *DROSHA* mRNA levels. We transiently transfected C33A cells with CMV based E6 or E7 expressing vectors either alone or in combination and determined *DROSHA* mRNA levels by RT-qPCR. *DROSHA* mRNA levels were most upregulated upon HPV16 E6/E7 co-transfection (2.3 fold; $P=0.0335$), followed by transfection with HPV16 E7 alone (2.0 fold; $P=0.0073$) but were not significantly altered by HPV16 E6 expression alone (1.3 fold; $P=0.2372$) (Figure 3.2). These results suggest that HPV16 E6/E7 expression causes increased *DROSHA* mRNA expression in C33A cells. *DROSHA* mRNA levels in HPV16 E6/E7 expressing C33A cells were lower than in HPV16 positive cervical cancer cell lines. However, it is difficult to directly compare the various cell lines and the effect of HPV16 E6/E7 on *DROSHA* mRNA levels in C33A cells depends on several experimental parameters, including E6/E7 expression levels and the cellular background of C33A cells.

HPV16 E6/E7 expression in primary human foreskin keratinocytes increases *DROSHA* and *DICER* mRNA levels

Since our results from cervical cancer cell lines suggested a possible correlation between *DROSHA* and *DICER* mRNA levels and HPV16 status, we hypothesized that
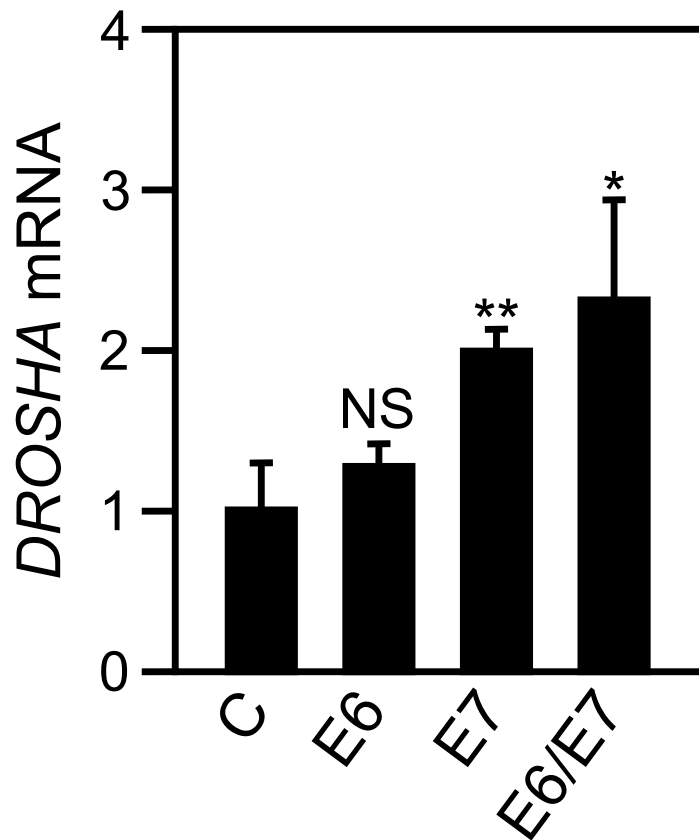
Figure 3.2. *DROSHA* mRNA expression in HPV negative C33A cervical cancer cells with transient expression of HPV16 E6/E7. C33A cells were transiently transfected with empty vector (C), HPV16 E6 alone (E6), HPV16 E7 alone (E7) or both HPV16 E6/E7 (E6/E7). *DROSHA* mRNA levels were analyzed by RT-qPCR. Expression of 18S ribosomal RNA (18S) was utilized as a control and values were normalized to control vector transduced C33A cells. Results represent averages of three independent experiments and error bars show standard deviation. * and ** indicate statistical significance at a P<0.05 and P<0.01, respectively. "NS" indicates not statistically significant.

expression of the HPV E6 and/or E7 oncoproteins in primary human foreskin keratinocytes may be sufficient to alter *DROSHA* and *DICER* mRNA levels. To test this hypothesis, we analyzed *DROSHA* and *DICER* mRNA levels by RT-qPCR in three independent, donor and passage matched HFK populations each with stable expression of HPV16 E6 and/or E7 or a control vector. In two of the three HFK populations tested, HPV16 E6 expression caused a significant increase in *DROSHA* mRNA levels compared to control HFKs. In contrast, HPV16 E7 expressing HFKs had *DROSHA* mRNA levels that were similar to or slightly lower than control HFKs. HPV16 E6/E7 expressing HFKs, however, consistently exhibited significantly increased *DROSHA* mRNA levels that exceeded those in HPV16 E6 expressing HFKs (Figure 3.3A). Similarly, *DICER* mRNA levels were higher in HPV16 E6 expressing HFKs. Except in population 2, HPV16 E7 expressing HFKs also had significantly higher *DICER* mRNA levels. *DICER* mRNA levels were consistently and significantly increased in HPV16 E6/E7 co-expressing cells (Figure 3.3B).

HPV16 E6/E7 expression in primary epithelial cells increases DROSHA and DICER protein levels

We next examined by immunoblot experiments, whether the observed modulation of *DROSHA* and *DICER* mRNA levels resulted in similar alterations in DROSHA and DICER protein levels. DROSHA protein levels were consistently increased in HPV16 E6 and/or E7 expressing HFKs although the magnitude of the effect varies between the different HFK populations (Figure 3.4A). DICER protein levels are also increased in HPV16 E6 and/or E7 expressing HFKs although only the HPV16
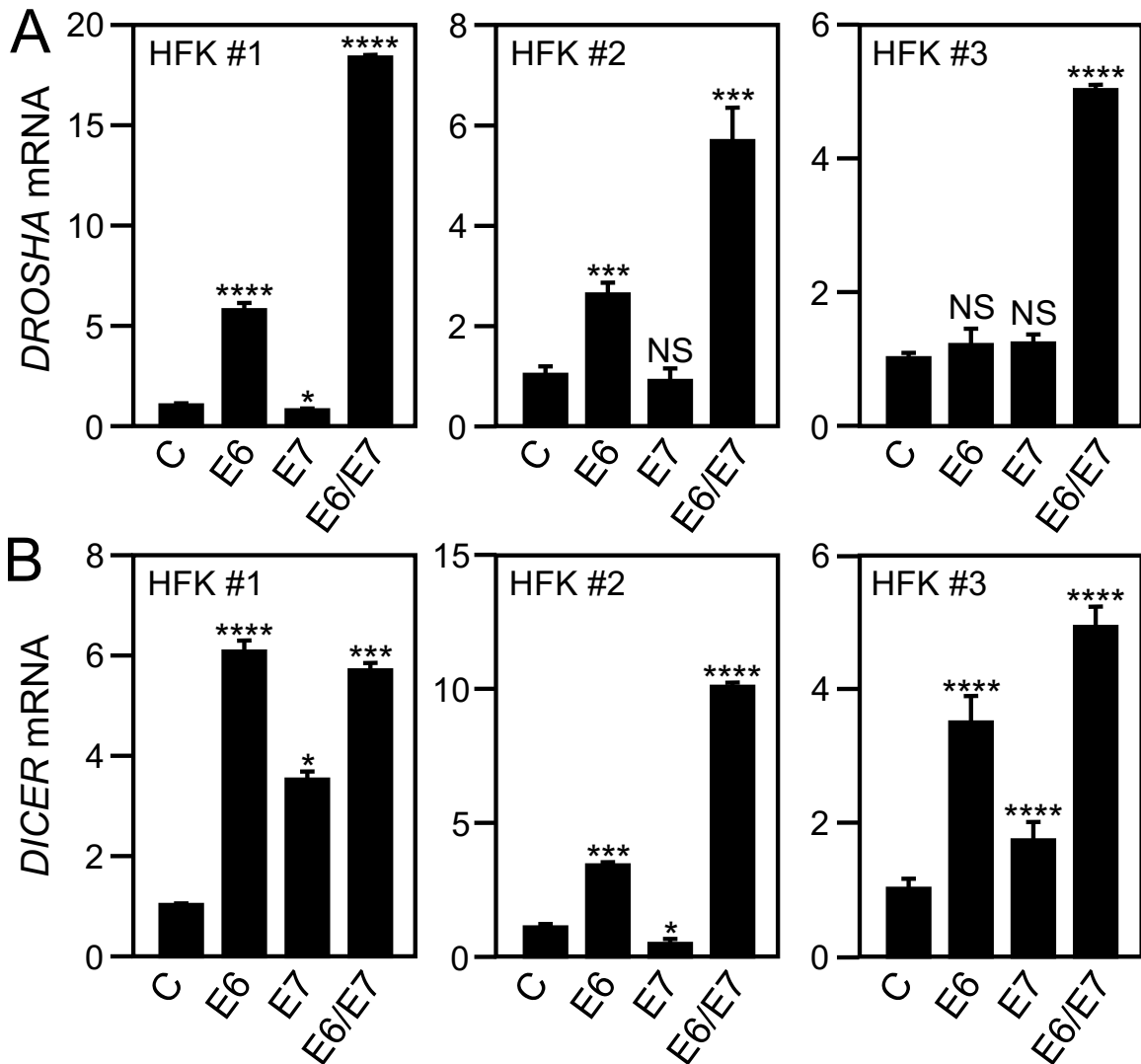
Figure 3.3. *DROSHA* and *DICER* mRNA levels in HPV16 E6 and/or E7 expressing

primary human foreskin keratinocytes. Three donor and passage matched HFK

populations with stable expression of HPV16 E6 (E6), HPV16 E7 (E7), HPV16 E6/E7

(E6/E7) or an empty vector (C) were analyzed for (A) *DROSHA* and (B) *DICER* mRNA

expression by RT-qPCR. Expression of 18S ribosomal RNA (18S) was utilized as a

control and values were normalized to control vector transduced HFKs. Similar results

were obtained with three additional HFK populations tested. Error bars show standard

Figure 3.3 (Continued)

deviation. Unpaired, two-tailed t-tests with a 95% confidence level were performed to determine statistical significance. *, **, *** and **** indicate statistical significance at a $P<0.05$, $P<0.01$, $P<0.001$ and $P<0.0001$ respectively. "NS" indicates not statistically significant. Please note that the HFK populations tested in these experiments are distinct from those shown in Figure 3.4.
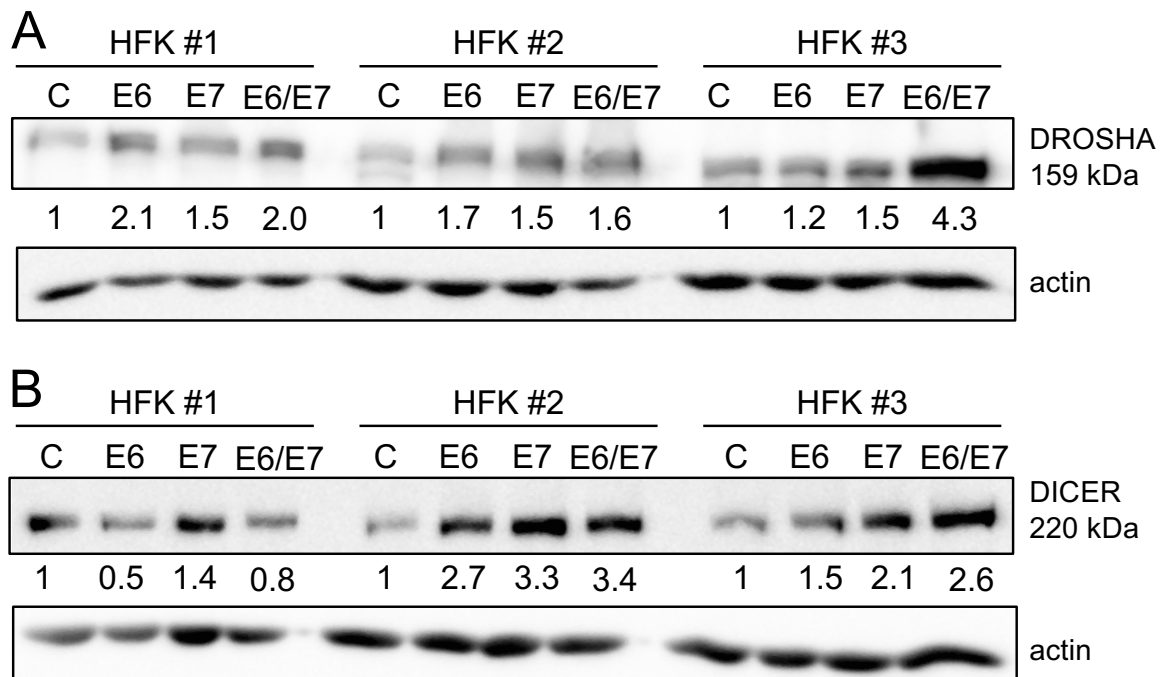
Figure 3.4. Protein levels of DROSHA and DICER in HPV16 E6 and/or E7 expressing primary human foreskin keratinocytes. Three donor and passage matched HFK populations with stable expression of HPV16 E6 (E6), HPV16 E7 (E7), HPV16 E6/E7 (E6/E7) or an empty vector (C) were analyzed for (A) DROSHA and (B) DICER protein expression by immunoblot analysis. Steady state levels of actin served as a loading control and quantifications normalized to actin levels are indicated underneath the blots. Similar results were obtained in several additional experiments. Please note that the HFK populations tested in these experiments are distinct from those shown in Figure 3.3.

E7 expressing HFKs expressed higher DICER protein in population 1 (Figure 3.4B).

In combination with the data shown in Figure 3.3, these results show that HPV16 E6/E7

expression in primary human foreskin keratinocytes can cause increased DROSHA and

DICER levels and these increases are, at least in part, due to increased mRNA levels.

Expression of DROSHA-regulated microRNAs is altered in HPV16 E6/E7

expressing primary human foreskin keratinocytes

A previous study showed that modulation of DROSHA expression in cervical

carcinoma cells levels significantly altered expression of only 45 out of 319 miRs tested

(Muralidhar *et al.*, 2011). Since we observed increased DROSHA levels in HPV16

E6/E7 expressing HFKs, we wanted to determine if the levels of DROSHA-regulated

miRs were correspondingly altered in HPV16 E6/E7 expressing HFKs. We previously

performed a comprehensive analysis of HPV16 E6/E7 induced alterations of cellular

miR expression (Harden *et al.*, 2017). Hence, we assessed expression of the 45

DROSHA-regulated miRs (Muralidhar *et al.*, 2011) in our data set (Table 3.1). A total of

37 out of 45 (82%) of DROSHA-regulated miRs are regulated in HPV16 E6/E7

expressing HFKs consistent with increased DROSHA expression. This analysis

suggests that at least some of the HPV16 E6/E7 induced alterations in cellular miR

expression that we observed may be a consequence of the increased DROSHA levels

in HFKs expressing HPV16 E6/E7.

We also compared the DROSHA-regulated miRs to our list of the 15 most up- or

down regulated miRs identified by a comprehensive target pairing analysis in HPV16

E6/E7 expressing HFKs (Harden *et al.*, 2017). Only one DROSHA-regulated miR, miR-

Table 3.1. Expression of DROSHA regulated miRs in cervical squamous cell carcinoma lines.

DROSHA-regulated miR	Regulation (Observed)*	Regulation (Literature)**	Overlap
miR-193a-3p	down-regulated	down-regulated	
miR-138-5p	down-regulated	down-regulated	
miR-370	up-regulated	down-regulated	■
miR-380-3p	down-regulated	down-regulated	
miR-520e	down-regulated	down-regulated	
miR-7-5p	up-regulated	up-regulated	
miR-148b-3p	up-regulated	up-regulated	
miR-98-5p	up-regulated	up-regulated	
miR-378a-3p	up-regulated	up-regulated	
miR-141-3p	up-regulated	up-regulated	
miR-107	down-regulated	up-regulated	■
let-7c	up-regulated	up-regulated	
let-7a-5p	up-regulated	up-regulated	
miR-30c-5p	up-regulated	up-regulated	
let-7f-5p	up-regulated	up-regulated	
miR-125b-5p	up-regulated	up-regulated	
miR-23a-3p	up-regulated	up-regulated	
miR-125a-5p	up-regulated	up-regulated	
let-7d-5p	up-regulated	up-regulated	
miR-23b-3p	down-regulated	up-regulated	■
miR-100-5p	up-regulated	up-regulated	
miR-185-5p	up-regulated	up-regulated	
let-7e-5p	up-regulated	up-regulated	
let-7b-5p	up-regulated	up-regulated	
miR-200c-3p	down-regulated	up-regulated	■
miR-7i-5p	up-regulated	up-regulated	
miR-15b-5p	up-regulated	up-regulated	
miR-26a-5p	up-regulated	up-regulated	
miR-106b-5p	up-regulated	up-regulated	
miR-22-3p	down-regulated	up-regulated	■

Table 3.1 (Continued)

DROSHA-regulated miR	Regulation (Observed)*	Regulation (Literature)**	Overlap
let-7g-5p	up-regulated	up-regulated	
miR-24-3p	up-regulated	up-regulated	
miR-16-5p	up-regulated	up-regulated	
miR-130a-3p	up-regulated	up-regulated	
miR-151a-3p	up-regulated	up-regulated	
miR-27b-3p	up-regulated	up-regulated	
miR-27a-3p	up-regulated	up-regulated	
miR-203a-3p	down-regulated	up-regulated	■
miR-338-3p	up-regulated	up-regulated	
miR-99b-5p	up-regulated	up-regulated	
miR-330-3p	down-regulated	up-regulated	■
miR-31-5p	down-regulated	up-regulated	■
miR-342-3p	up-regulated	up-regulated	
miR-191-5p	up-regulated	up-regulated	
miR-10b-5p	up-regulated	up-regulated	

*Data from (Harden *et al.*, 2017).

**Data from (Muralidhar *et al.*, 2011).

203a-3p, was on that list. We, and others (Harden *et al.*, 2017; McKenna *et al.*, 2010; Melar-New and Laimins, 2010), however, had shown that this miR is downregulated in HPV16 E6/E7 expressing HFKs, the opposite of what would have been expected based on increased DROSHA expression (Muralidhar *et al.*, 2011). Hence, DROSHA-regulated miRs are not amongst the most highly modulated miRs in HPV16 E6/E7 expressing HFKs and DROSHA-regulated miRs must also be modulated by HPV16 E6/E7 through other pathways.

DROSHA-regulated microRNAs and microRNAs altered by HPV16 E6/E7 are involved in modulating similar cellular pathways

To examine pathways relevant to the DROSHA-regulated miRs, we utilized the core analysis function of Ingenuity Pathway Analysis (IPA). This type of analysis identifies relationships, mechanisms, functions and pathways of relevance to a particular dataset. Out of the 45 DROSHA-responsive miRs, 30 were associated with "cancer", 26 were associated with "organismal injury and abnormalities" and 25 were associated with "reproductive system disease." Top molecular and cellular functions affiliated with these miRs were "cell movement, development, growth and proliferation" as well as "cell cycle" and "DNA replication, recombination and repair." Interestingly, the top five molecular and cellular functions associated with the DROSHA responsive miRs were the same as those associated with all miRs altered by HPV16 E6/E7 expression. However, the order of significance of association, based on p-value, is different. For miRs associated with DROSHA levels "cell movement" was the top molecular and cellular function followed by "cell development, growth and proliferation"

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and lastly "cell cycle." For all miRs perturbed by HPV16 E6/E7 expression "cell cycle" was the top molecular and cellular function followed by "cell movement", and "cell development, growth and proliferation." These results suggest that DROSHA regulated miRs participate in similar, cellular processes compared to miRs previously identified to be modulated by HPV16 E6/E7 expression.

DISCUSSION

Similar to other cancers, expression of the miR biosynthesis machinery is dysregulated in cervical carcinomas. *DROSHA* expression is frequently upregulated and 5p gene amplification involving the *DROSHA* gene has also been reported in cervical cancers. Our experiments partially confirm and extend a previous publication (Zhou *et al.*, 2013) and document higher *DROSHA* mRNA levels in HPV16 positive SiHa and CaSki, as well as HPV18 positive Hela, but not in the HPV negative C33A cervical cancer line. We show that HPV16 E6/E7 expression in C33A cells causes an increase in *DROSHA* expression although not to the levels observed in HPV positive cervical cancer lines.

In our experimental system, we introduce expression of HPV16 E6/E7 into low passage primary HFKs that have not yet acquired the amplification of chromosome 5p, carrying the *DROSHA* gene, which is frequently observed in cervical cancers. Given the relatively high frequency of chromosome 5p amplification, one may hypothesize that high level DROSHA expression is important for HPV positive cervical cancers. Therefore, we hypothesize that increased DROSHA expression by HPV16 E6/E7 may be a mechanism to establish higher DROSHA expression at very early stages of HPV carcinogenesis until the gain of chromosome 5p can occur.

Our results with HFKs suggest that HPV16 E6 is the main driver of *DROSHA* expression. While E6 expression also causes increased *DROSHA* expression in HPV-negative C33A cervical carcinoma cells, HPV16 E7 is the major driver of *DROSHA* mRNA upregulation in this cell line. Given C33A cells express mutant p53, this E6-

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mediated increase in C33A cells is p53 independent. However, since E6 generally

caused a more marked increase in *DROSHA* expression in HFKs that express wild type

p53, it is conceivable that p53 inactivation by E6 may contribute to increased DROSHA

expression. Co-expression of HPV16 E6/E7 in primary human foreskin keratinocytes

resulted in the most dramatic increases in DROSHA and DICER mRNA and protein

expression. This may be the result of the well-known functional cooperativity of the

HPV16 E6 and E7 oncoproteins (Moody and Laimins, 2010).

We also noted differences in the HPV16 E6/E7-dependent regulation of

DROSHA/DICER at the mRNA and protein levels. This suggests that DROSHA/DICER

expression by HPV16 E6/E7 may also be regulated post-transcriptionally, which has

been previously observed for both DROSHA (Han et al., 2009) and DICER (Wiesen and

Tomasi, 2009).

It was previously noted that modulation of DROSHA levels in cervical cancer

lines altered expression of only 45 of the 319 miRs that were tested (Muralidhar *et al.*,

2011). Since not all miRs were evaluated in this study, there are likely additional

DROSHA-regulated miRs to be discovered. While most (40/45) of the "DROSHA-

regulated" miRs were expressed at higher levels, some (5/45) were expressed at lower

levels. We showed that 37 out of 45 (82%) of the DROSHA-regulated miRs are

expressed in HPV16 E6/E7 expressing primary human foreskin keratinocytes consistent

with DROSHA regulation. Hence, at least some of the HPV16 E6/E7 mediated changes

in cellular miR expression might be through a mechanism involving increased DROSHA

expression. Nonetheless, DROSHA was not the major driver of expression of some

other DROSHA-regulated miRs, including miR-203-3p, in HPV16 E6/E7 expressing

cells.

To directly test whether increased DROSHA expression in E6 and E6/E7 expressing cells contributes to increased expression of these miRs, we attempted to silence DROSHA expression in our engineered HFK populations. We used both lentiviral transduction with multiple shRNAs and transfections with siRNAs, however, we were unable to decrease *DROSHA* levels sufficiently to examine the effects of DROSHA silencing on HPV16 E6/E7 expressing HFKs. We were only able to decrease *DROSHA* levels by ~50% in HFKs using transient transfection of several different siRNAs. HPV16 E6/E7 expressing HFKs transduced with DROSHA shRNAs did not survive selection post-transduction and, perhaps, this is due to other non-miR related functions of DROSHA (Johanson *et al.*, 2013) that may be important in primary human foreskin keratinocytes. This, unfortunately, precluded us from directly and conclusively determining whether and how increased DROSHA expression by E6 and E7 contributes to modulation of cellular miR levels.

To determine whether DROSHA-regulated miRs affect specific cellular signaling pathways, we compared a core analysis of these miRs to the entire miRseq dataset (-1 ≥ FC ≥ 1). We utilized less stringent fold change cutoffs since the reported fold changes for the DROSHA-regulated miRs ranged from 1.8-4.2 fold (Muralidhar *et al.*, 2011). This comparative analysis revealed that many of the same molecular and cellular functions are associated with both DROSHA regulated and HPV16 E6/E7 modulated miRs.

In summary, our study shows that HPV16 E6/E7 oncoprotein expression alters RNA and protein levels of DROSHA and DICER, two critical enzymes in the canonical

miR biogenesis pathway. Examination of known DROSHA-regulated miRs suggests

that HPV16 E6/E7 perturbation of DROSHA levels may be one mechanism by which

HPV16 E6/E7 expression perturbs cellular miR expression. In addition, given the miR

biogenesis independent activities of DROSHA and DICER, their increased expression

may also contribute to cervical carcinogenesis through miR independent mechanisms.

Further studies will be necessary to arrive at a mechanistic understanding of DICER

and DROSHA modulation by HPV16 E6/E7.

REFERENCES

1.   **Adams, B.D., Kasinski, A.L., Slack, F.J.** (2014). Aberrant regulation and function of microRNAs in cancer. Curr Biol: 24, R762-776.

2.   **Boyerinas, B., Park, S.M., Hau, A., Murmann, A.E., Peter, M.E.** (2010). The role of let-7 in cell differentiation and cancer. Endocr Relat Cancer: 17, F19-36.

3.   **Bradford, M.M.** (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem: 72, 248-254.

4.   **Brueckner, B., Stresemann, C., Kuner, R., Mund, C., Musch, T., Meister, M., Sultmann, H., Lyko, F.** (2007). The human let-7a-3 locus contains an epigenetically regulated microRNA gene with oncogenic function. Cancer Res: 67, 1419-1423.

5.   **Cerami, E., Gao, J., Dogrusoz, U., Gross, B.E., Sumer, S.O., Aksoy, B.A., Jacobsen, A., Byrne, C.J., Heuer, M.L., Larsson, E., Antipin, Y., Reva, B., Goldberg, A.P., Sander, C., Schultz, N.** (2012). The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov: 2, 401-404.

6.   **Chendrimada, T.P., Gregory, R.I., Kumaraswamy, E., Norman, J., Cooch, N., Nishikura, K., Shiekhattar, R.** (2005). TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. Nature: 436, 740-744.

7.   **Denli, A.M., Tops, B.B., Plasterk, R.H., Ketting, R.F., Hannon, G.J.** (2004). Processing of primary microRNAs by the Microprocessor complex. Nature: 432, 231-235.

8.   **Djuranovic, S., Nahvi, A., Green, R.** (2012). miRNA-mediated gene silencing by translational repression followed by mRNA deadenylation and decay. Science: 336, 237-240.

9.   **Gao, J., Aksoy, B.A., Dogrusoz, U., Dresdner, G., Gross, B., Sumer, S.O., Sun, Y., Jacobsen, A., Sinha, R., Larsson, E., Cerami, E., Sander, C., Schultz, N.** (2013). Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Signal: 6, pl1.

10. **Grishok, A., Pasquinelli, A.E., Conte, D., Li, N., Parrish, S., Ha, I., Baillie, D.L., Fire, A., Ruvkun, G., Mello, C.C.** (2001). Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing. Cell: 106, 23-34.

11. **Haase, A.D., Jaskiewicz, L., Zhang, H., Laine, S., Sack, R., Gatignol, A., Filipowicz, W.** (2005). TRBP, a regulator of cellular PKR and HIV-1 virus expression, interacts with Dicer and functions in RNA silencing. EMBO Rep: 6, 961-967.

12. **Halbert, C.L., Demers, G.W., Galloway, D.A.** (1991). The E7 gene of human papillomavirus type 16 is sufficient for immortalization of human epithelial cells. J Virol: 65, 473-478.

13. **Han, J., Lee, Y., Yeom, K.H., Kim, Y.K., Jin, H., Kim, V.N.** (2004). The Drosha-DGCR8 complex in primary microRNA processing. Genes Dev: 18, 3016-3027.

14. **Harden, M.E., Prasad, N., Griffiths, A., Munger, K.** (2017). Modulation of microRNA-mRNA Target Pairs by Human Papillomavirus 16 Oncoproteins. MBio: 8.

15. **Hata, A., Lieberman, J.** (2015). Dysregulation of microRNA biogenesis and gene silencing in cancer. Sci Signal: 8, re3.

16. **He, L., Wang, H.Y., Zhang, L., Huang, L., Li, J.D., Xiong, Y., Zhang, M.Y., Jia, W.H., Yun, J.P., Luo, R.Z., Zheng, M.** (2014). Prognostic significance of low DICER expression regulated by miR-130a in cervical cancer. Cell Death Dis: 5, e1205.

17. **Hutvagner, G., McLachlan, J., Pasquinelli, A.E., Balint, E., Tuschl, T., Zamore, P.D.** (2001). A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science: 293, 834-838.

18. **Johanson, T.M., Lew, A.M., Chong, M.M.** (2013). MicroRNA-independent roles of the RNase III enzymes Drosha and Dicer. Open Biol: 3, 130144.

19. **Ketting, R.F., Fischer, S.E., Bernstein, E., Sijen, T., Hannon, G.J., Plasterk, R.H.** (2001). Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. Genes Dev: 15, 2654-2659.

20. **Khvorova, A., Reynolds, A., Jayasena, S.D.** (2003). Functional siRNAs and miRNAs exhibit strand bias. Cell: 115, 209-216.

21. **Knight, S.W., Bass, B.L.** (2001). A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in Caenorhabditis elegans. Science: 293, 2269-2271.

22. **Lawrie, C.H., Chi, J., Taylor, S., Tramonti, D., Ballabio, E., Palazzo, S., Saunders, N.J., Pezzella, F., Boultwood, J., Wainscoat, J.S., Hatton, C.S.** (2009). Expression of microRNAs in diffuse large B cell lymphoma is associated with immunophenotype, survival and transformation from follicular lymphoma. J Cell Mol Med: 13, 1248-1260.

23. **Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Radmark, O., Kim, S., Kim, V.N.** (2003). The nuclear RNase III Drosha initiates microRNA processing. Nature: 425, 415-419.

24. **Lee, Y., Hur, I., Park, S.Y., Kim, Y.K., Suh, M.R., Kim, V.N.** (2006). The role of PACT in the RNA silencing pathway. EMBO J: 25, 522-532.

25. **Liu, J., Carmell, M.A., Rivas, F.V., Marsden, C.G., Thomson, J.M., Song, J.J., Hammond, S.M., Joshua-Tor, L., Hannon, G.J.** (2004). Argonaute2 is the catalytic engine of mammalian RNAi. Science: 305, 1437-1441.

26. **Lu, L., Katsaros, D., de la Longrais, I.A., Sochirca, O., Yu, H.** (2007). Hypermethylation of let-7a-3 in epithelial ovarian cancer is associated with low insulin-like growth factor-II expression and favorable prognosis. Cancer Res: 67, 10117-10122.

27. **McKenna, D.J., McDade, S.S., Patel, D., McCance, D.J.** (2010). MicroRNA 203 expression in keratinocytes is dependent on regulation of p53 levels by E6. J Virol: 84, 10644-10652.

28. **Meister, G., Landthaler, M., Patkaniowska, A., Dorsett, Y., Teng, G., Tuschl, T.** (2004). Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. Mol Cell: 15, 185-197.

29. **Melar-New, M., Laimins, L.A.** (2010). Human papillomaviruses modulate expression of microRNA 203 upon epithelial differentiation to control levels of p63 proteins. J Virol: 84, 5212-5221.

30. **Moody, C.A., Laimins, L.A.** (2010). Human papillomavirus oncoproteins: pathways to transformation. Nat Rev Cancer: 10, 550-560.

31. **Muralidhar, B., Goldstein, L.D., Ng, G., Winder, D.M., Palmer, R.D., Gooding, E.L., Barbosa-Morais, N.L., Mukherjee, G., Thorne, N.P., Roberts, I., Pett, M.R., Coleman, N.** (2007). Global microRNA profiles in cervical squamous cell carcinoma depend on Drosha expression levels. J Pathol: 212, 368-377.

32. **Muralidhar, B., Winder, D., Murray, M., Palmer, R., Barbosa-Morais, N., Saini, H., Roberts, I., Pett, M., Coleman, N.** (2011). Functional evidence that Drosha overexpression in cervical squamous cell carcinoma affects cell phenotype and microRNA profiles. J Pathol: 224, 496-507.

33. **Schwarz, D.S., Hutvagner, G., Du, T., Xu, Z., Aronin, N., Zamore, P.D.** (2003). Asymmetry in the assembly of the RNAi enzyme complex. Cell: 115, 199-208.

34. **Scotto, L., Narayan, G., Nandula, S.V., Subramaniyam, S., Kaufmann, A.M., Wright, J.D., Pothuri, B., Mansukhani, M., Schneider, A., Arias-Pulido, H., Murty, V.V.** (2008). Integrative genomics analysis of chromosome 5p gain in cervical cancer reveals target over-expressed genes, including Drosha. Mol Cancer: 7, 58.

35. **Wiesen, J.L., Tomasi, T.B.** (2009). Dicer is regulated by cellular stresses and interferons. Mol Immunol: 46, 1222-1228.

36. **Xie, M., Steitz, J.A.** (2014). Versatile microRNA biogenesis in animals and their viruses. RNA Biol: 11, 673-681.

37. **Zhao, H., Jin, X., Su, H., Deng, X., Fang, Y., Shen, L., Xie, C.** (2014). Down-regulation of Dicer expression in cervical cancer tissues. Med Oncol: 31, 937.

38. **Zhou, J., Cai, J., Huang, Z., Ding, H., Wang, J., Jia, J., Zhao, Y., Huang, D., Wang, Z.** (2013). Proteomic identification of target proteins following Drosha knockdown in cervical cancer. Oncol Rep: 30, 2229-2237.

CHAPTER FOUR

Human Papillomavirus 16 E6 and E7 Oncoprotein Expression Alters microRNA

Expression in Extracellular Vesicles

This chapter is adapted from a previously published manuscript:

Harden ME and Munger K. (2017). Human Papillomavirus 16 E6 and E7 oncoprotein expression alters microRNA expression in extracellular vesicles. Virology: 508, 63-69.

Contributions: I performed all experiments and wrote the manuscript. Karl Munger aided in experimental design and helped to write and edit the manuscript.

SUMMARY

Extracellular vesicles released by cancer cells are mediators of intercellular communication that have been reported to contribute to carcinogenesis. Since they are readily detected in bodily fluids they may also be used as cancer biomarkers. The E6/E7 oncoproteins drive human papillomavirus (HPV)-associated cancers, which account for approximately 5% of all human cancers worldwide. Here, we investigate how HPV16 E6/E7 oncogene expression in primary human epithelial cells alters microRNA (miR) expression in extracellular vesicles and compare these to changes in intracellular miR expression. Examining a panel of 68 cancer related miRs revealed that many miRs had similar expression patterns in cells and in extracellular vesicles, whereas some other miRs had different expression patterns and may be selectively packaged into extracellular vesicles. Interestingly, the set of miRs that may be selectively packaged in HPV16 E6/E7 extracellular vesicles is predicted to inhibit necrosis and apoptosis.

INTRODUCTION

Extracellular vesicles (EVs) are lipid bilayer surrounded structures, ranging from 40 nm to several µm in size, that are released by a variety of cells, including tumor cells. There are multiple types of EVs, which differ in size, biogenesis and molecular composition. EVs are classified into three main groups based on their biogenesis and size: membrane shedding-, multivesicular body- and apoptotic-derived (Kim *et al.*, 2017). Membrane shedding EVs are derived from budding of the plasma membrane, range from 0.2 to 1 µm in diameter and are referred to as microvesicles or microparticles (Muralidharan-Chari *et al.*, 2010; Ratajczak *et al.*, 2006). Exosomes are small EVs (40 to 150 nm) that originate from the late endosomal trafficking machinery (Yanez-Mo *et al.*, 2015). Their biogenesis involves accumulation in multivesicular bodies and release through fusion with the plasma membrane (Pan *et al.*, 1985). Apoptotic derived EVs, or apoptotic bodies, have diameters ranging from 0.5 to 2 µm, and are released via blebbing of the plasma membrane of apoptotic cells (Hristov *et al.*, 2004; Kranich *et al.*, 2008).

Exosomes have recently been the focus of intense interest in cancer research. Exosomes released from cancer cells can promote tumorigenesis through multiple mechanisms including influencing the tumor microenvironment, providing immune system regulation and stimulating angiogenesis (reviewed in (Ciardiello *et al.*, 2016)). In fact, the cargo of cancer-derived exosomes, comprised of functional proteins, microRNAs (miRs), DNA and/or mutated mRNA, can have oncogenic or tumor suppressive activities. Furthermore, the ability of exosomes to condition the pre-metastatic niche has been shown *in vivo* (Peinado *et al.*, 2012). Some miRs packaged

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in exosomes may regulate the expression of target RNAs in recipient cells but other functions have also been uncovered. For example, exosome-associated miRs can be ligands for Toll-like receptors (TLRs), resulting in induction of the immune response or inhibition of macrophage activation through suppression of TLR signaling (Alexander *et al.*, 2015; Fabbri *et al.*, 2012; Phinney *et al.*, 2015; Tsvetkova *et al.*, 2013).

The first report investigating exosomes in HPV18 positive HeLa cervical carcinoma cells showed that silencing of E6/E7 expression in HeLa cells led to reduced Survivin levels in exosomes and an increase in the overall amount of exosomes released from HeLa cells (Honegger *et al.*, 2013). The E6 and E7 proteins were not detected in HeLa exosomes by this group (Honegger *et al.*, 2013) but a more recent study detected E6 and E7 mRNAs in exosomes released from HPV16 E6/E7 expressing primary human foreskin keratinocytes (HFKs) (Chiantore *et al.*, 2016).

Just these few studies have examined miRs in exosomes released from HPV containing cells. An RT-PCR based study with HPV16 E6/E7 expressing HFKs detected only 8 of the 384 miRs that were included in their assay, with miR-222 being the most highly abundant (Chiantore *et al.*, 2016). In another study, the authors silenced HPV18 E6/E7 expression in HeLa cells and showed that expression of HPV18 E6/E7 determined expression of seven exosomal miRs, and that these miRs possess pro-proliferative or anti-apoptotic potential (Honegger *et al.*, 2015). Silencing E6/E7 expression in the HPV16 positive SiHa cervical cancer line identified a similar set of HPV16 E6/E7 regulated miRs in exosomes (Honegger *et al.*, 2015). Additionally, determination of exosomal miRs in cervicovaginal lavage specimens of cervical cancer

patients showed that miR-21 and miR-146a levels were significantly higher in vesicles from HPV positive cervical cancer patients (Liu *et al.*, 2014).

Understanding the results of some studies and comparisons between studies has been difficult since various methods have been used for exosome isolation. It is now clear that all the existing isolation methods for exosomes also yield various amounts of other EVs (Choi *et al.*, 2012; Haqqani *et al.*, 2013; Muralidharan-Chari *et al.*, 2009) and it may be prudent to refer to these preparations as exosome-enriched EVs (exo-EVs). Here, we investigated expression of a panel of 68 cancer-related miRs in cells, and in exosome-enriched EVs (exo-EVs) released by HPV16 E6/E7 expressing HFKs and matched control vector transduced HFKs. We show that most miRs analyzed are similarly regulated by E6/E7 expression in cells and in exo-EVs. Some miRs, however, are expressed differently in cells and in exo-EVs, suggesting that several miRs may be selectively packaged in exo-EVs secreted by HPV16 E6/E7 expressing cells. Interestingly, these selectively packaged miRs are predicted to inhibit apoptosis and necrosis. Our results, therefore, agree with and extend previous studies (Honegger *et al.*, 2015) that suggest expression of the high-risk HPV oncoproteins alters the expression of miRs secreted in EVs.

MATERIALS AND METHODS

Cell Culture

HFKs were isolated and maintained in keratinocyte-serum-free media (KSFM) as previously described (Harden *et al.*, 2017). HFKs were transduced with LXSN based recombinant retroviruses encoding both HPV16 E6 and E7 (Halbert *et al.*, 1991) or a control LXSN vector as previously described (Harden *et al.*, 2017). Retroviral transduction of HFKs was validated by immunoblotting and RT-qPCR to assess the protein and RNA levels of HPV16 E6 and E7, respectively. HFKs were grown to 80% confluence prior to passaging and only passaged up to 8 times. In all experiments, donor and passage matched HFK populations were used.

Isolation of Exosome-enriched Extracellular Vesicles

For the isolation of exo-EVs, HFK media was cleared of endogenous exosomes present in bovine pituitary extract (Riches *et al.*, 2014), which is a component of KSFM, by ultracentrifugation at 100,000 x g for 24 hours at 4°C. The cleared media was then added to HPV16 E6/E7 expressing and matched control vector transduced HFKs for 24 hours and then used for exo-EV isolation using Invitrogen's Total Exosome Isolation Reagent (from cell culture media) according to the manufacturer's instructions.

RNA methods

Total RNA was harvested from cells and exo-EVs using the mirVana miR Isolation Kit (Ambion) according to the manufacturer's instructions. Cellular RNA

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sample concentrations were determined using a NanoDrop2000c spectrophotometer (Thermo Fisher Scientific). The quantity and quality of RNA from exo-EVs was determined by Agilent Bioanalyzer 2100 with a Small RNA Chip.

For miR RT-qPCR, total RNA was reverse transcribed with the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Life Technologies) as described in the manufacturer's protocol, utilizing miR-specific, stem loop primers (Applied Biosystems, Life Technologies). TaqMan® MicroRNA Assays (Applied Biosystems, Life Technologies) were employed to detect miR-16-5p and miR-34a-5p using the comparative Ct method with the StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific). Assay IDs 000391 and 000426 were used to quantify miR-16-5p and miR-34a-5p, respectively. RT-qPCR assays were performed in triplicate and the non-coding small nuclear RNA (snRNA) U6 (Assay ID: 001973) was utilized as an endogenous, small RNA control.

Protein methods

Protein lysates from cells and exo-EVs were prepared in ML buffer (300 mM NaCl, 0.5% Nonidet P-40 [NP-40], 20 mM Tris-HCl [pH 8.0], 1 mM EDTA) supplemented with one Complete EDTA-free Protease Inhibitor Cocktail tablet (Roche) per 50 ml lysis buffer. Protein concentrations were determined via the Bradford method (Bradford, 1976). Samples from cells and exo-EVs containing 60 μg of protein were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Primary antibodies were as follows: RIG-I, AG-20B-0009-C100 (Adipogen), 1:1,000; HSC70, sc-7298 (Santa Cruz Biotechnology), 1:200; CD9, C9993 (Sigma-

Aldrich), 1:500; Rab5B, sc-598 (Santa Cruz Biotechnology) 1:100; Histone H3, 17-10046 (EMD Millipore), 1:1,000; actin, MAB1501 (Millipore) 1:1,000. Secondary anti-mouse IgG and anti-rabbit IgG horseradish peroxidase-conjugated antibodies (Amersham) were used at 1:10,000 dilutions. Proteins were visualized by enhanced chemiluminescence (Luminata™ Crescendo Western HRP Substrate; Millipore) and electronically acquired with a Syngene G:BOX image station (Syngene) equipped with GeneSys software, v1.5.6.0.

Nanoparticle Tracking Analysis (NTA)

Determination of exo-EV concentration and size was performed using a NanoSight NS300 with a high sensitivity sCMOS camera and a green 532 nm laser (Malvern). NTA allows the visualization and analysis of extracellular particles by relating Brownian motion to particle size. Exo-EV samples were analyzed at 1:1,000 dilutions to achieve the optimal particle number (20-100 particles) in the field of view.

FirePlex® miR profiling

To examine the expression of miRs in exo-EVs, we utilized the FirePlex® miR oncology assay to detect a panel of 68 cancer-related miRs (see Supplemental Table 4.1). This assay utilizes three-dimensional hydrogel particles encoded with unique miR "barcodes" made by optical liquid stamping (Chapin *et al.*, 2011; Chapin and Doyle, 2011). RNAs isolated from exo-EVs released from two, independent passage and donor matched populations of HPV16 E6/E7 expressing and control vector transduced HFKs were submitted for FirePlex® miR profiling. Data analysis was performed using

the Firefly® Analysis Workbench software (http://www.abcam.com/kits/firefly-analysis-workbench-software-for-multiplex-miR-assays). Unpaired, two-tailed t-tests with a 95% confidence level were performed to determine statistical significance between cell and exo-EV samples.

RESULTS

Characterization of exosome-enriched extracellular vesicles released from human foreskin keratinocytes

To validate our exo-EV preparations, we examined several marker proteins. Our preparations scored positive for the exosome markers HSC70, CD9 and RAB5. In contrast, RIG-I, Actin and Histone H3, which should not be present in exosomes, were not detected in our EV preparations (Figure 4.1A). These results are consistent with the isolation of exosomes. However, since we cannot rule out that other EVs may be also present in our samples, we refer to these preparations as exosome-enriched EVs (exo-EVs).

Analysis of the size and concentration of exosome-enriched extracellular vesicles released from human foreskin keratinocytes

To analyze the size and concentration of exo-EVs released from HFKs, we performed nanoparticle tracking analysis (NTA). NTA tracks the movement of single particles, as small as 10 nm in diameter, in a suspension under Brownian motion. The particles scatter light when illuminated by a laser, which is captured by a high sensitivity camera. Software is then used to track the motion of each particle from frame to frame and the rate of particle movement is related to a sphere equivalent hydrodynamic radius as calculated through the Stokes-Einstein equation (Dragovic *et al.*, 2011; Hole *et al.*, 2013). A representative size distribution profile of exo-EVs released from HPV size expressing HFKs as determined by NTA in shown in Figure 4.1B. The 16 E6/E7
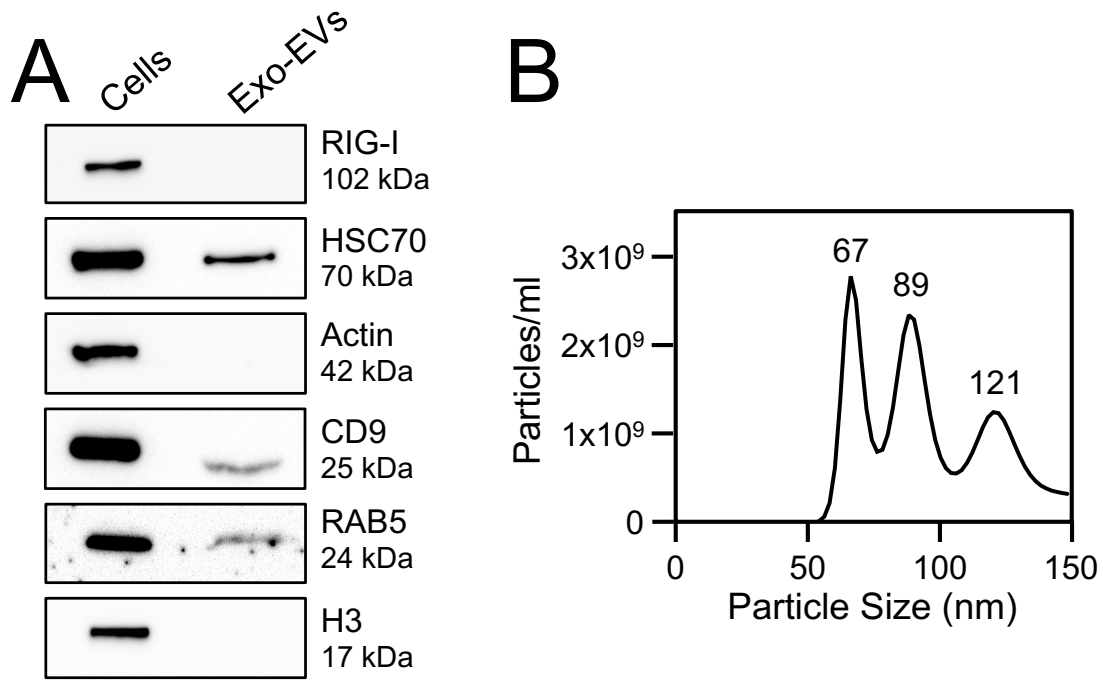
Figure 4.1. Biochemical and biophysical characterization of extracellular vesicles isolated from HPV16 E6/E7 expressing primary human foreskin keratinocytes. (A) Immunoblot analysis of exosome markers HSC70, CD9 and RAB5 and non-exosome associated proteins RIG-I, actin and histone H3. **(B)** Size determination and quantification of extracellular vesicles by Nanoparticle Tracking Analysis. See text for details.

representative distribution profile shows several peaks, one at 67 nm, one at 89 nm and

one at 121 nm. This analysis shows that HPV16 E6/E7 expressing HFKs release EVs

in the size range of exosomes and allowed us to determine the concentration of exo-

EVs in each sample for downstream analysis.

Validation of the FirePlex® microRNA Assay

We compared intracellular expression of miR-16-5p and miR-34a-5p by miR

sequencing, quantitative RT PCR and FirePlex® miR Assay (Figure 4.2). These

analyses show that the FirePlex® miR Assay yields expression data that are consistent

with other detection methods. In addition, there was excellent agreement of intracellular

miR expression in control and HPV16 E6/E7 expressing HFKs with our previously

published miR sequencing data (Harden *et al.*, 2017).

Expression of HPV16 E6/E7 alters the expression of microRNAs in exosome-enriched extracellular vesicles released from human foreskin keratinocytes

Total RNA was harvested from cells and exo-EVs released from two,

independent populations of HPV16 E6/E7 expressing HFKs and matched control HFKs.

We used the FirePlex® miR oncology assay to assess levels of a panel of 68 cancer

related miRs. As a control, we also utilized the same method to analyze intracellular

miR expression in total RNA from the same HFK samples. The Firefly® Analysis

Workbench was used to analyze the expression of cellular and exo-EV-associated

miRs. Unfiltered expression data for all 68 miRs examined in the oncology panel are
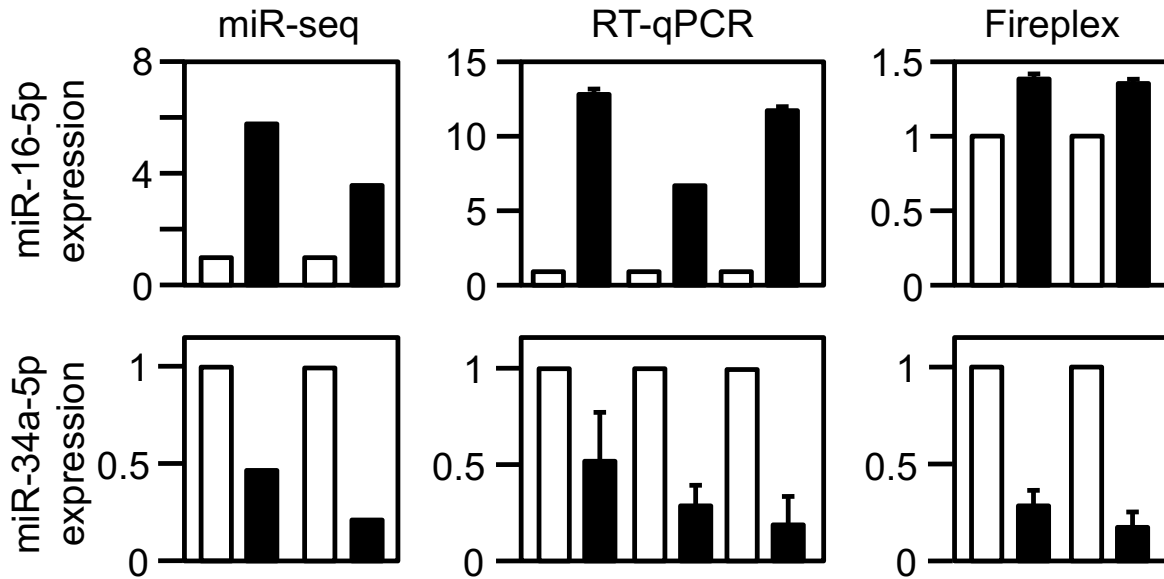
Figure 4.2. Comparison of miR quantification methods. Quantification of intracellular miR-16-5p (top) and miR-34a-5p (bottom) expression in different populations of HPV16 E6/E7 expressing primary human foreskin keratinocytes (black bars) and matched control vector transduced primary human foreskin keratinocytes (white bars) by miR sequencing (miRseq) (Harden *et al.* 2017), reverse transcription quantitative PCR (RT-qPCR) and Fireplex® miR assays. Y-axis values are relative to matched control vector transduced primary human foreskin keratinocytes.

shown in Supplemental Table 4.2. Each of the two matched keratinocyte populations is prepared from three or more foreskin samples from a different donor and it is not unusual to observe considerable variation between different keratinocyte preparations. Our analysis included all miRs with expression above the limit of detection of the assay and those that showed consistent results in expression (HPV16 E6E7/C) in both HFK populations tested. This resulted in 31 differentially expressed miRs in exo-EVs and 48 intracellular miRs above the limit of detection of the assay with consistent results in expression in both HFK populations tested (Table 4.1). Of the differentially expressed miRs in exo-EVs, 19 were upregulated and 12 were downregulated and, of the differentially expressed intracellular miRs, 31 were upregulated and 17 were downregulated (Table 4.1). Overall, these results show that HPV16 E6/E7 expression in HFKs alters the expression of miRs in exo-EVs released from these cells compared to control vector transduced HFKs.

Comparing intracellular microRNA expression and microRNAs in exosome-enriched extracellular vesicles

We then compared the expression of miRs in cells and in exo-EVs. As before, we only included miRs with expression above the limit of detection of the assay and those that showed consistent modulation of expression (HPV16 E6E7/C) in both HFK populations tested. There were 23 miRs that met these criteria, both intracellularly and in exo-EVs (Table 4.2). To determine miRs with expression patterns that were

Table 4.1. Expression of miRs in two independently derived populations of HPV16 E6/E7 expressing HFKs (cells) and in exosome-enriched extracellular vesicles (exo-EVs) as compared to control HFKs.

miR	FC (E6E7/C) cells	SD (E6E7/C) exo-EVs	FC (E6E7/C) cells	SD (E6E7/C) exo-EVs
let-7d-5p	1.31	0.091	-	-
let-7g-5p	1.52	0.244	-	-
let-7i-5p	-	-	0.47	0.225
miR-9-5p	2.13	0.076	-	-
miR-10b-5p	1.22	0.212	-	-
miR-15b-5p	1.28	0.176	-	-
miR-16-5p	1.37	0.02	1.04	0.037
miR-17-5p	1.15	0.201	-	-
miR-18a-5p	-	-	2.24	1.089
miR-19a-3p	1.77	0.195	1.84	1.068
miR-20a-5p	1.35	0.081	-	-
miR-21-5p	0.91	0.097	0.58	0.106
miR-22-3p	-	-	0.44	0.158
miR-25-3p	2.21	0.647	3.2	2.335
miR-29a-3p	0.78	0.059	-	-
miR-29b-3p	0.76	0.097	-	-
miR-29c-3p	0.96	0.017	-	-
miR-34a-5p	0.24	0.074	0.37	0.081
miR-92a-3p	1.1	0.029	-	-
miR-93-5p	1.29	0.098	1.68	0.624
miR-103a-3p	-	-	1.6	1.635
miR-106a-5p	1.28	0.126	-	-
miR-106b-5p	1.85	1.113	2.35	1.635
miR-107	0.89	0.029	1.51	0.634
miR-125b-5p	0.95	0.065	-	-
miR-127-3p	0.64	0.171	-	-
miR-130a-3p	-	-	1.54	0.59
miR-141-3p	0.94	0.008	-	-
miR-146a-5p	0.48	0.202	-	-
miR-148a-3p	1.34	0.271	-	-
miR-148b-3p	1.47	0.274	1.51	0.311

Table 4.1 (Continued)

miR-150-5p	0.79	0.224	-	-
miR-151a-3p	-	-	0.76	0.158
miR-155-5p	2.84	0.814	2.31	1.643
miR-181a-5p	1.21	0.084	-	-
miR-182-5p	0.91	0.08	4.23	4.179
miR-187-3p	0.81	0.239	-	-
miR-192-5p	-	-	0.84	0.145
miR-195-5p	1.71	0.994	1.3	0.125
miR199a-3p	1.46	0.481	-	-
miR-199a-5p	3.07	2.107	-	-
miR-200b-3p	1.39	0.2	0.92	0.053
miR-200c-3p	0.96	0.026	-	-
miR-205-5p	1.14	0.098	0.71	0.193
miR-210-3p	0.8	0.045	-	-
miR-218-5p	1.45	0.402	2.04	1.36
miR-221-3p	-	-	0.63	0.368
miR-222-3p	0.85	0.004	1.65	0.362
miR-320a	1.47	0.03	0.47	0.284
miR-335-5p	2.44	0.239	2.87	1.939
miR-375	1.41	0.04	2.96	1.485
miR-376c-3p	0.56	0.295	0.71	0.137
miR-378a-3p	1.68	0.213	0.61	0.307
miR-574-3p	1.81	0.633	-	-
miR-625-3p	1.43	0.46	2.59	0.705
miR-652-3p	1.2	0.225	1.68	0.139

FC, Fold Change; **SD**, Standard Deviation. Only miRs that showed the same trend in expression in both populations are listed.

Table 4.2. Expression of miRs detected in HPV16 E6/E7 expressing HFKs (cells) and the corresponding exosome-enriched extracellular vesicles (exo-EVs) as compared to control HFKs.

miR	FC (E6E7/C) cells	SD (E6E7/C) cells	FC (E6E7/C) exo-EVs	SD (E6E7/C) exo-EVs	p-value cells vs exo-EVs
miR-16-5p	1.37	0.02	1.04	0.037	0.0002
miR-19a-3p	1.77	0.195	1.84	1.068	ns
miR-21-5p	0.91	0.097	0.58	0.106	0.0176
miR-25-3p	2.21	0.647	3.2	2.335	ns
miR-34a-5p	0.24	0.074	0.37	0.081	ns
miR-93-5p	1.29	0.098	1.68	0.624	ns
miR-106b-5p	1.85	1.113	2.35	1.635	ns
miR-107	0.89	0.029	1.51	0.634	ns
miR-148b-3p	1.47	0.274	1.51	0.311	ns
miR-155-5p	2.84	0.814	2.31	1.643	ns
miR-182-5p	0.91	0.08	4.23	4.179	ns
miR-195-5p	1.71	0.994	1.3	0.125	ns
miR-200b-3p	1.39	0.2	0.92	0.053	0.0159
miR-205-5p	1.14	0.098	0.71	0.193	0.0255
miR-218-5p	1.45	0.402	2.04	1.36	ns
miR-222-3p	0.85	0.004	1.65	0.362	0.0191
miR-320a	1.47	0.03	0.47	0.284	0.0038
miR-335-5p	2.44	0.239	2.87	1.939	ns
miR-375	1.41	0.04	2.96	1.485	ns
miR-376c-3p	0.56	0.295	0.71	0.137	ns
miR-378a-3p	1.68	0.213	0.61	0.307	0.0077
miR-625-3p	1.43	0.46	2.59	0.705	ns
miR-652-3p	1.2	0.225	1.1	0.139	ns

FC, Fold Change; **SD**, Standard Deviation. Only miRs above the limit of detection of the assay, that showed the same trend in expression in both HFK populations tested and with data from cells and exo-EVs are listed.

statistically different between cells and exo-EVs, we utilized unpaired, two-tailed t-tests with a 95% confidence level, comparing the expression of each miR in cells to expression of the same miR in exo-EVs. We found that 16 miRs were similarly regulated intracellularly and in exo-EVs, with most miRs upregulated intracellularly and in exo-EVs, and only a few miRs downregulated intracellularly and in exo-EVs. In contrast, seven miRs showed a different abundance in exo-EVs than intracellularly. Together, our data show that HPV16 E6/E7 expression alters the expression of many miRs in a similar manner intracellularly and in exo-EVs. However, the expression pattern of some miRs is different intracellularly compared to exo-EVs.

Analysis of differentially expressed microRNAs in exosome-enriched extracellular vesicles released from HPV16 E6/E7 expressing human foreskin keratinocytes

To uncover potential functions of miRs in exo-EVs, we utilized the core analysis function of Ingenuity Pathway Analysis (Qiagen), which identifies relationships, mechanisms, functions and pathways of relevance to a particular dataset. Specifically, we compared core analyses of miRs that were expressed similarly intracellularly and in exo-EVs and those miRs that were differentially expressed intracellularly and in exo-EVs.

The molecular/cellular functions the two sets of miRs had in common were "cell cycle, development and cell to cell signaling." For the miRs that were similarly regulated by expression of HPV16 E6/E7 intracellularly and in exo-EVs, the top molecular and cellular functions that were unique to that dataset were "cell growth and proliferation" and "cell movement". In contrast, for the miRs regulated differently by

HPV16 E6/E7 intracellularly and in exo-EVs, distinct molecular and cellular functions

were "cell death and survival" and "cellular compromise." "Cellular compromise" refers

to any process that may compromise the function of the cell, as well as functions

associated with damage or degeneration of cells, including cellular atrophy, damage,

disruption and swelling. When we examined the miRs regulated differently by HPV16

E6/E7 intracellularly and in exo-EVs more closely, we found that within the "cell death

and survival" category, "necrosis" and "apoptosis" were predicted to be inhibited by this

group of miRs, although the z-scores were not significant (-1.170 and -1.053,

respectively) (Table 4.3). Nonetheless, these results suggest that miRs that may be

selectively packaged into exo-EVs of HPV16 E6/E7 expressing HFKs may inhibit cell

death.

Table 4.3. miRs that are differentially expressed (16E6E7/C) intracellularly and in exosome-enriched extracellular vesicles are predicted to inhibit cell death and promote survival

cell death/ survival	activation z-score	p-value	associated miRs
necrosis	-1.17	0.00699	miR-16-5p, -200b-3p, -222-3p, -320a, -378a-3p
apoptosis	-1.053	0.00673	miR-16-5p, -200b-3p, -222-3p, -320a, -378a-3p
cell viability	0.262	0.00328	miR-16-5p, -200b-3p, -222-3p, -378a-3p,

DISCUSSION

Our study examined how EV-associated miRs are altered by expression of the high-risk HPV E6 and E7 oncoproteins, the major drivers of HPV-associated cancers. As there is no consensus on a gold standard method for exosome isolation, it cannot be claimed that there is an optimal method that should be used and the commercial reagent we utilized in this study has been thoroughly compared to other methods for the isolation of exosomes (Helwa *et al.*, 2017). Specifically, the reagent utilized in our study interacts with water molecules in the sample, thereby forcing less soluble sample components, like EVs, out of solution. The EVs can then be collecting by a short, low-speed centrifugation rather than a long, high-speed ultracentrifugation.

To biochemically analyze our exosome preparations, we followed recommendations from the International Society for Extracellular Vesicles (Lotvall *et al.*, 2014). While there are no true exosomes-specific markers, there are proteins that are exosomes-enriched, and it is recommended to examine three or more of these proteins from the following categories: 1) transmembrane/lipid-bound extracellular proteins; 2) cytosolic proteins; 3) intracellular proteins (Lotvall *et al.*, 2014). We analyzed six different proteins: RIG-I, HSC70, actin, CD9, RAB5 and H3. RIG-I is a cytosolic RNA sensor of the innate immune system, is not packaged into exosomes (Boelens *et al.*, 2014) and we did not observe this protein in our samples. HSC70 is a chaperone protein found in exosomes from most cell types (Geminard *et al.*, 2004; Thery *et al.*, 2001) and we detected HSC70 in our samples. We did not detect actin in our samples, indicating that our preparations were free of cellular debris (Angeloni *et al.*, 2016). The

CD9 tetraspanin is expected to be present in exosomes (Lotvall *et al.*, 2014) and we detected this protein in our samples. Endosome or membrane binding proteins, such as RAB5, are expected to be present in exosomes (Lotvall *et al.*, 2014), and we detected RAB5 in our samples. Nuclear histones, such as H3, should be absent in exosomes, but are present in some other EV types (Lotvall *et al.*, 2014), and we did not observe H3 in our preparations. Overall, our biochemical characterization of our exo-EV preparations meets and exceeds the requirements recommended by the International Society for Extracellular Vesicles for the characterization of exosomes.

We also utilized NTA to assess the size of exo-EVs, and to determine the concentration of exo-EVs in our samples. We found that HPV16 E6/E7-expressing HFKs release three distinct populations of exo-EVs of 67, 89 and 121 nm in diameter. This might seem surprising, as it is generally assumed that exosomes are a homogeneous population. However, a recent study revealed that multiple cell types release more than one subpopulation of exosomes (Willms *et al.*, 2016). These subpopulations were shown to carry different protein and RNA cargoes, suggesting that they may have distinct biological activities on recipient cells (Willms *et al.*, 2016). Hence, HPV16 E6/E7 expressing HFKs may also release several exo-EV subpopulations and it will be interesting to determine whether they each carry unique cargoes, and have different activities on neighboring cells. Due to the amount of sample required for NTA, we were only able to investigate exo-EV size distributions from HPV16 E6/E7 expressing HFKs and it will be fascinating to elucidate whether or not HPV16 E6/E7 expressing cells induce alterations in the abundance of specific exo-EV populations.

There are only a few studies of miRs contained in exosomes secreted from HPV expressing cells and clinical lesions (Chiantore *et al.*, 2016; Honegger *et al.*, 2013; Honegger *et al.*, 2015). One previous study found miR-222-3p to be significantly expressed in exosomes from HPV16 expressing HFKs (Chiantore *et al.*, 2016). We also found miR-222-3p to be upregulated in our HPV16 E6/E7 exo-EV samples. Interestingly, this miR is downregulated in HPV16 E6/E7 expressing HFKs but upregulated in exo-EVs released from the same cells. The same study (Chiantore *et al.*, 2016) also detected miR-320a in exosomes and we found miR-320a to be expressed in exosomes released from HPV16 E6/E7 expressing HFKs as well. This miR was upregulated in HPV16 E6/E7 HFKs but downregulated in exosomes released from these cells.

We also observed some overlap with another study, which utilized deep sequencing to examine exosomes released from HeLa cells in which expression of HPV18 E6/E7 was silenced (Honegger *et al.*, 2015). Consistent with this study, we also detected miR-21-5p, -222-3p, -320a and -378a-3p in our exo-EVs. Two of these miRs, miR-378a-3p and miR-21-5p, are part of the seven miR signature associated with HPV18 E6/E7 oncogene expression that was identified in this study (Honegger *et al.*, 2015).

An important finding from our analysis of miR expression in exo-EVs released by HPV16 E6/E7 expressing HFKs was that some miRs are expressed similarly in exo-EVs and intracellularly whereas others are not. This is consistent with what has been previously reported for cancer-associated EVs. In some cases, the miR content of exosomes mirrors miR expression in the tumor (Rabinowits *et al.*, 2009; Taylor and

Gercel-Taylor, 2008). However, in other cases, some miRs are much more highly abundant in exosomes than within tumor cells, suggesting that these miRs are preferentially packaged in exosomes (Jaiswal *et al.*, 2012; Pigati *et al.*, 2010). Any miRs that are expressed at lower or higher levels in exosomes released from HPV16 E6/E7 expressing cells compared to control HFKs (Table 4.1) may potentially serve as biomarkers for HPV-associated diseases and cancers. Candidates include miR-21-5p, identified in our study and one other (Honegger *et al.*, 2015), as well as miR-222-3p, -320a and -378a-3p that were observed in our experiments and two other studies (Chiantore *et al.*, 2016; Honegger *et al.*, 2015).

Pathway analysis of the miRs that are differentially expressed in HPV16 E6/E7 HFK secreted exo-EVs than intracellularly suggest that these miRs inhibit necrosis and apoptosis. While the z-scores associated with necrosis and apoptosis were not significant, this is likely due to the small list of miR analyzed. The transfer of exosomes by other cell types to recipient cells has been previously linked to effects on apoptosis (Rivoltini *et al.*, 2016; Yang *et al.*, 2015) and necrosis (Nong *et al.*, 2016) and our data suggest that expression of HPV16 E6/E7 inhibits apoptosis and necrosis in neighboring normal cells through miRs secreted in exo-EVs. Supporting this finding, one of the few previous studies of HPV-associated miRs in exosomes reported that several of E6/E7-dependent exosomal miRs were linked to control of cell proliferation and apoptosis (Honegger *et al.*, 2015).

REFERENCES

1.   **Alexander, M., Hu, R., Runtsch, M.C., Kagele, D.A., Mosbruger, T.L., Tolmachova, T., Seabra, M.C., Round, J.L., Ward, D.M., O'Connell, R.M.** (2015). Exosome-delivered microRNAs modulate the inflammatory response to endotoxin. Nat Commun: 6, 7321.

2.   **Angeloni, N.L., McMahon, K.M., Swaminathan, S., Plebanek, M.P., Osman, I., Volpert, O.V., Thaxton, C.S.** (2016). Pathways for Modulating Exosome Lipids Identified By High-Density Lipoprotein-Like Nanoparticle Binding to Scavenger Receptor Type B-1. Sci Rep: 6, 22915.

3.   **Boelens, M.C., Wu, T.J., Nabet, B.Y., Xu, B., Qiu, Y., Yoon, T., Azzam, D.J., Twyman-Saint Victor, C., Wiemann, B.Z., Ishwaran, H., Ter Brugge, P.J., Jonkers, J., Slingerland, J., Minn, A.J.** (2014). Exosome transfer from stromal to breast cancer cells regulates therapy resistance pathways. Cell: 159, 499-513.

4.   **Bradford, M.M.** (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem: 72, 248-254.

5.   **Chapin, S.C., Appleyard, D.C., Pregibon, D.C., Doyle, P.S.** (2011). Rapid microRNA profiling on encoded gel microparticles. Angew Chem Int Ed Engl: 50, 2289-2293.

6.   **Chapin, S.C., Doyle, P.S.** (2011). Ultrasensitive multiplexed microRNA quantification on encoded gel microparticles using rolling circle amplification. Anal Chem: 83, 7179-7185.

7.   **Chiantore, M.V., Mangino, G., Iuliano, M., Zangrillo, M.S., De Lillis, I., Vaccari, G., Accardi, R., Tommasino, M., Columba Cabezas, S., Federico, M., Fiorucci, G., Romeo, G.** (2016). Human papillomavirus E6 and E7 oncoproteins affect the expression of cancer-related microRNAs: additional evidence in HPV-induced tumorigenesis. J Cancer Res Clin Oncol: 142, 1751-1763.

8.   **Choi, D.S., Choi, D.Y., Hong, B.S., Jang, S.C., Kim, D.K., Lee, J., Kim, Y.K., Kim, K.P., Gho, Y.S.** (2012). Quantitative proteomics of extracellular vesicles

derived from human primary and metastatic colorectal cancer cells. J Extracell Vesicles: 1.

9. **Ciardiello, C., Cavallini, L., Spinelli, C., Yang, J., Reis-Sobreiro, M., de Candia, P., Minciacchi, V.R., Di Vizio, D.** (2016). Focus on Extracellular Vesicles: New Frontiers of Cell-to-Cell Communication in Cancer. Int J Mol Sci: 17, 175.

10. **Dragovic, R.A., Gardiner, C., Brooks, A.S., Tannetta, D.S., Ferguson, D.J., Hole, P., Carr, B., Redman, C.W., Harris, A.L., Dobson, P.J., Harrison, P., Sargent, I.L.** (2011). Sizing and phenotyping of cellular vesicles using Nanoparticle Tracking Analysis. Nanomedicine: 7, 780-788.

11. **Fabbri, M., Paone, A., Calore, F., Galli, R., Gaudio, E., Santhanam, R., Lovat, F., Fadda, P., Mao, C., Nuovo, G.J., Zanesi, N., Crawford, M., Ozer, G.H., Wernicke, D., Alder, H., Caligiuri, M.A., Nana-Sinkam, P., Perrotti, D., Croce, C.M.** (2012). MicroRNAs bind to Toll-like receptors to induce prometastatic inflammatory response. Proc Natl Acad Sci U S A: 109, E2110-2116.

12. **Geminard, C., De Gassart, A., Blanc, L., Vidal, M.** (2004). Degradation of AP2 during reticulocyte maturation enhances binding of hsc70 and Alix to a common site on TFR for sorting into exosomes. Traffic: 5, 181-193.

13. **Halbert, C.L., Demers, G.W., Galloway, D.A.** (1991). The E7 gene of human papillomavirus type 16 is sufficient for immortalization of human epithelial cells. J Virol: 65, 473-478.

14. **Haqqani, A.S., Delaney, C.E., Tremblay, T.L., Sodja, C., Sandhu, J.K., Stanimirovic, D.B.** (2013). Method for isolation and molecular characterization of extracellular microvesicles released from brain endothelial cells. Fluids Barriers CNS: 10, 4.

15. **Harden, M.E., Prasad, N., Griffiths, A., Munger, K.** (2017). Modulation of microRNA-mRNA Target Pairs by Human Papillomavirus 16 Oncoproteins. MBio: 8.

16. **Helwa, I., Cai, J., Drewry, M.D., Zimmerman, A., Dinkins, M.B., Khaled, M.L., Seremwe, M., Dismuke, W.M., Bieberich, E., Stamer, W.D., Hamrick, M.W., Liu, Y.** (2017). A Comparative Study of Serum Exosome Isolation Using Differential Ultracentrifugation and Three Commercial Reagents. PLoS One: 12, e0170628.

17. **Hole, P., Sillence, K., Hannell, C., Maguire, C.M., Roesslein, M., Suarez, G., Capracotta, S., Magdolenova, Z., Horev-Azaria, L., Dybowska, A., Cooke, L., Haase, A., Contal, S., Mano, S., Vennemann, A., Sauvain, J.J., Staunton, K.C., Anguissola, S., Luch, A., Dusinska, M., Korenstein, R., Gutleb, A.C., Wiemann, M., Prina-Mello, A., Riediker, M., Wick, P.** (2013). Interlaboratory comparison of size measurements on nanoparticles using nanoparticle tracking analysis (NTA). J Nanopart Res: 15, 2101.

18. **Honegger, A., Leitz, J., Bulkescher, J., Hoppe-Seyler, K., Hoppe-Seyler, F.** (2013). Silencing of human papillomavirus (HPV) E6/E7 oncogene expression affects both the contents and the amounts of extracellular microvesicles released from HPV-positive cancer cells. Int J Cancer: 133, 1631-1642.

19. **Honegger, A., Schilling, D., Bastian, S., Sponagel, J., Kuryshev, V., Sultmann, H., Scheffner, M., Hoppe-Seyler, K., Hoppe-Seyler, F.** (2015). Dependence of intracellular and exosomal microRNAs on viral E6/E7 oncogene expression in HPV-positive tumor cells. PLoS Pathog: 11, e1004712.

20. **Hristov, M., Erl, W., Linder, S., Weber, P.C.** (2004). Apoptotic bodies from endothelial cells enhance the number and initiate the differentiation of human endothelial progenitor cells in vitro. Blood: 104, 2761-2766.

21. **Jaiswal, R., Luk, F., Gong, J., Mathys, J.M., Grau, G.E., Bebawy, M.** (2012). Microparticle conferred microRNA profiles--implications in the transfer and dominance of cancer traits. Mol Cancer: 11, 37.

22. **Kim, K.M., Abdelmohsen, K., Mustapic, M., Kapogiannis, D., Gorospe, M.** (2017). RNA in extracellular vesicles. Wiley Interdiscip Rev RNA.

23. **Kranich, J., Krautler, N.J., Heinen, E., Polymenidou, M., Bridel, C., Schildknecht, A., Huber, C., Kosco-Vilbois, M.H., Zinkernagel, R., Miele, G., Aguzzi, A.** (2008). Follicular dendritic cells control engulfment of apoptotic bodies by secreting Mfge8. J Exp Med: 205, 1293-1302.

24. **Liu, J., Sun, H., Wang, X., Yu, Q., Li, S., Yu, X., Gong, W.** (2014). Increased exosomal microRNA-21 and microRNA-146a levels in the cervicovaginal lavage specimens of patients with cervical cancer. Int J Mol Sci: 15, 758-773.

25. **Lotvall, J., Hill, A.F., Hochberg, F., Buzas, E.I., Di Vizio, D., Gardiner, C., Gho, Y.S., Kurochkin, I.V., Mathivanan, S., Quesenberry, P., Sahoo, S., Tahara, H., Wauben, M.H., Witwer, K.W., Thery, C.** (2014). Minimal

experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles. J Extracell Vesicles: 3, 26913.

26. **Muralidharan-Chari, V., Clancy, J., Plou, C., Romao, M., Chavrier, P., Raposo, G., D'Souza-Schorey, C.** (2009). ARF6-regulated shedding of tumor cell-derived plasma membrane microvesicles. Curr Biol: 19, 1875-1885.

27. **Muralidharan-Chari, V., Clancy, J.W., Sedgwick, A., D'Souza-Schorey, C.** (2010). Microvesicles: mediators of extracellular communication during cancer progression. J Cell Sci: 123, 1603-1611.

28. **Nong, K., Wang, W., Niu, X., Hu, B., Ma, C., Bai, Y., Wu, B., Wang, Y., Ai, K.** (2016). Hepatoprotective effect of exosomes from human-induced pluripotent stem cell-derived mesenchymal stromal cells against hepatic ischemia-reperfusion injury in rats. Cytotherapy: 18, 1548-1559.

29. **Pan, B.T., Teng, K., Wu, C., Adam, M., Johnstone, R.M.** (1985). Electron microscopic evidence for externalization of the transferrin receptor in vesicular form in sheep reticulocytes. J Cell Biol: 101, 942-948.

30. **Peinado, H., Aleckovic, M., Lavotshkin, S., Matei, I., Costa-Silva, B., Moreno-Bueno, G., Hergueta-Redondo, M., Williams, C., Garcia-Santos, G., Ghajar, C., Nitadori-Hoshino, A., Hoffman, C., Badal, K., Garcia, B.A., Callahan, M.K., Yuan, J., Martins, V.R., Skog, J., Kaplan, R.N., Brady, M.S., Wolchok, J.D., Chapman, P.B., Kang, Y., Bromberg, J., Lyden, D.** (2012). Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. Nat Med: 18, 883-891.

31. **Phinney, D.G., Di Giuseppe, M., Njah, J., Sala, E., Shiva, S., St Croix, C.M., Stolz, D.B., Watkins, S.C., Di, Y.P., Leikauf, G.D., Kolls, J., Riches, D.W., Deiuliis, G., Kaminski, N., Boregowda, S.V., McKenna, D.H., Ortiz, L.A.** (2015). Mesenchymal stem cells use extracellular vesicles to outsource mitophagy and shuttle microRNAs. Nat Commun: 6, 8472.

32. **Pigati, L., Yaddanapudi, S.C., Iyengar, R., Kim, D.J., Hearn, S.A., Danforth, D., Hastings, M.L., Duelli, D.M.** (2010). Selective release of microRNA species from normal and malignant mammary epithelial cells. PLoS One: 5, e13515.

33. **Rabinowits, G., Gercel-Taylor, C., Day, J.M., Taylor, D.D., Kloecker, G.H.** (2009). Exosomal microRNA: a diagnostic marker for lung cancer. Clin Lung Cancer: 10, 42-46.

34. **Ratajczak, J., Wysoczynski, M., Hayek, F., Janowska-Wieczorek, A., Ratajczak, M.Z.** (2006). Membrane-derived microvesicles: important and underappreciated mediators of cell-to-cell communication. Leukemia: 20, 1487-1495.

35. **Riches, A., Campbell, E., Borger, E., Powis, S.** (2014). Regulation of exosome release from mammary epithelial and breast cancer cells - a new regulatory pathway. Eur J Cancer: 50, 1025-1034.

36. **Rivoltini, L., Chiodoni, C., Squarcina, P., Tortoreto, M., Villa, A., Vergani, B., Burdek, M., Botti, L., Arioli, I., Cova, A., Mauri, G., Vergani, E., Bianchi, B., Della Mina, P., Cantone, L., Bollati, V., Zaffaroni, N., Gianni, A.M., Colombo, M.P., Huber, V.** (2016). TNF-Related Apoptosis-Inducing Ligand (TRAIL)-Armed Exosomes Deliver Proapoptotic Signals to Tumor Site. Clin Cancer Res: 22, 3499-3512.

37. **Taylor, D.D., Gercel-Taylor, C.** (2008). MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. Gynecol Oncol: 110, 13-21.

38. **Thery, C., Boussac, M., Veron, P., Ricciardi-Castagnoli, P., Raposo, G., Garin, J., Amigorena, S.** (2001). Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles. J Immunol: 166, 7309-7318.

39. **Tsvetkova, I.B., Cheng, F., Ma, X., Moore, A.W., Howard, B., Mukhopadhyay, S., Dragnea, B.** (2013). Fusion of mApple and Venus fluorescent proteins to the Sindbis virus E2 protein leads to different cell-binding properties. Virus Res: 177, 138-146.

40. **Willms, E., Johansson, H.J., Mager, I., Lee, Y., Blomberg, K.E., Sadik, M., Alaarg, A., Smith, C.I., Lehtio, J., El Andaloussi, S., Wood, M.J., Vader, P.** (2016). Cells release subpopulations of exosomes with distinct molecular and biological properties. Sci Rep: 6, 22519.

41. **Yanez-Mo, M., Siljander, P.R., Andreu, Z., Zavec, A.B., Borras, F.E., Buzas, E.I., Buzas, K., Casal, E., Cappello, F., Carvalho, J., Colas, E., Cordeiro-da**

Silva, A., Fais, S., Falcon-Perez, J.M., Ghobrial, I.M., Giebel, B., Gimona, M., Graner, M., Gursel, I., Gursel, M., Heegaard, N.H., Hendrix, A., Kierulf, P., Kokubun, K., Kosanovic, M., Kralj-Iglic, V., Kramer-Albers, E.M., Laitinen, S., Lasser, C., Lener, T., Ligeti, E., Line, A., Lipps, G., Llorente, A., Lotvall, J., Mancek-Keber, M., Marcilla, A., Mittelbrunn, M., Nazarenko, I., Nolte-'t Hoen, E.N., Nyman, T.A., O'Driscoll, L., Olivan, M., Oliveira, C., Pallinger, E., Del Portillo, H.A., Reventos, J., Rigau, M., Rohde, E., Sammar, M., Sanchez-Madrid, F., Santarem, N., Schallmoser, K., Ostenfeld, M.S., Stoorvogel, W., Stukelj, R., Van der Grein, S.G., Vasconcelos, M.H., Wauben, M.H., De Wever, O. (2015). Biological properties of extracellular vesicles and their physiological functions. J Extracell Vesicles: 4, 27066.

42. **Yang, J., Liu, X.X., Fan, H., Tang, Q., Shou, Z.X., Zuo, D.M., Zou, Z., Xu, M., Chen, Q.Y., Peng, Y., Deng, S.J., Liu, Y.J.** (2015). Extracellular Vesicles Derived from Bone Marrow Mesenchymal Stem Cells Protect against Experimental Colitis via Attenuating Colon Inflammation, Oxidative Stress and Apoptosis. PLoS One: 10, e0140551.

CHAPTER FIVE

Discussion

Overall Implications of Our Studies

This dissertation investigates the perturbation of host cellular miRs by the high-risk HPV16 oncoproteins E6 and E7. Through these studies, we have advanced our knowledge of HPV-associated miRs. A visual diagram depicting the subject matter of each of the three data chapters of this dissertation and how they are related is shown in Figure 5.1.

In **Chapter 2**, we found that expression of HPV16 E6/E7 alters the expression of individual miRs and whole clusters of miRs. Integration of miRseq and RNAseq data allowed us to observe that miR regulation is an important mode of posttranscriptional regulation of gene expression in HPV16 E6/E7 expressing HFKs. Bioinformatic pathway analysis of these results also revealed that miR-modulated RNAs are involved in distinct canonical pathways in HFKs expressing HPV16 E6/E7 and that regulation of miRs by HPV16 E6/E7 contributes to the biological activity of these oncoproteins. Around half of RNAs targeted by miRs in HPV16 E6/E7 expressing HFKs may be targeted by more than one miR and, as one would anticipate, miR-modulated RNAs are also regulated by other mechanisms. Our data indicate that miR alterations in HPV-associated tumors are likely caused by HPV E6/E7 expression and some of the miR-mRNA pairs identified in this study may be potential "drivers" of HPV carcinogenesis. Additionally, our results suggest that a core set of miRs may be altered in all HPV-associated epithelial cancers as a result of HPV16 E6/E7 expression, whereas some miRs may be specific to an HPV-associated cancer of a particular anatomical site. Some miRs modulated by expression of HPV16 E6/E7 are TP53 or E2F responsive and other miRs are altered by HPV16 E6/E7 through other mechanisms. Our findings also
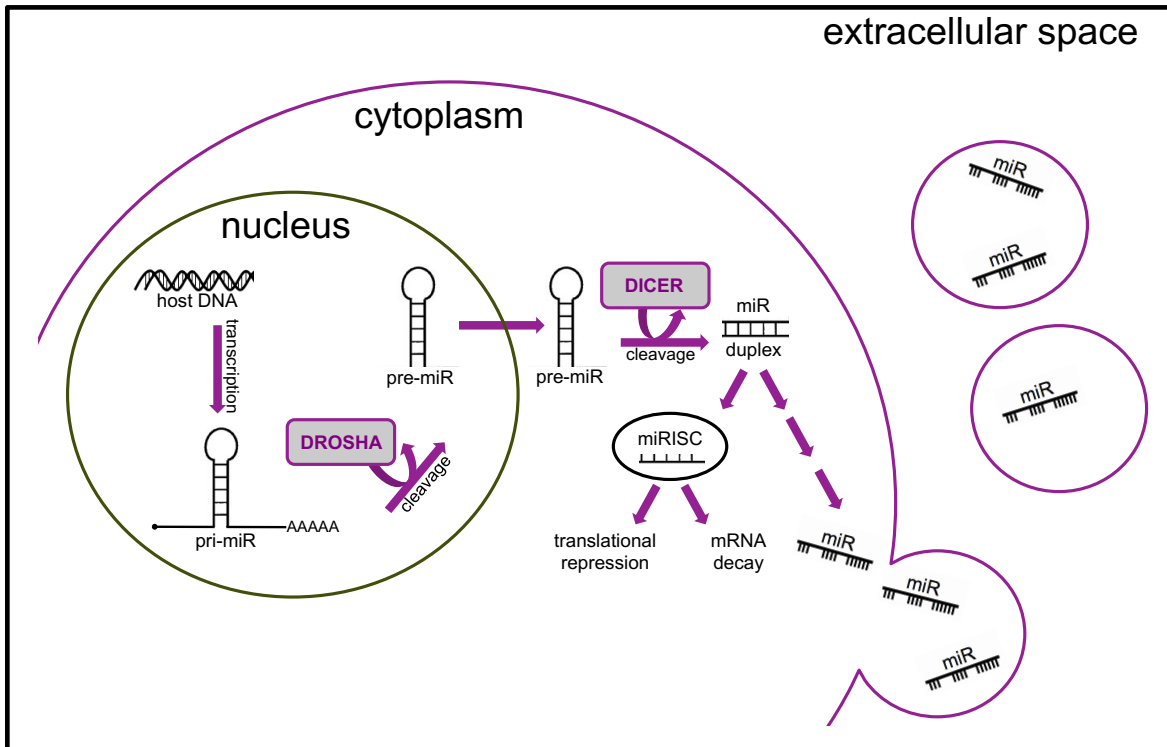
Figure 5.1 Visual dissertation overview. This diagram depicts the subject matter of each of the three data chapters of this dissertation and how they are connected. Chapter 1 investigates HPV16 E6/E7 modulation of miR-mRNA target pairs. Chapter 2 examines a mechanism HPV16 E6/E7 may utilize to alter miR expression, through perturbation of the miRNA biogenesis enzymes DROSHA and DICER. Chapter 3 analyzes the modulation of miRs in extracellular vesicles resulting from HPV16 E6/E7 expression. More details are found in the text in Chapters 2 to 5.

show that expression of miRs can be perturbed by one or both oncoproteins. Ultimately, modulation of miRs by HPV16 E6/E7 plays an important role in the dramatic rewiring of cellular gene expression that results from expression of the two oncoproteins, eventually leading to cellular transformation and HPV-associated cancers.

In **Chapter 3**, we investigate the effect of HPV16 E6/E7 expression on the miR biogenesis machinery. Our results show that expression of HPV16 E6/E7 alter expression of two key enzymes in the canonical miR biogenesis pathway, DROSHA and DICER. Examining known DROSHA responsive miRs, we found that some HPV16 E6/E7 mediated changes in cellular miR expression may be through a mechanism involving increased DROSHA expression. A comparative pathway analysis also revealed that many similar molecular and cellular functions are associated with both DROSHA regulated and HPV16 E6/E7 modulated miRs. Lastly, taking into account miR biogenesis independent activities of DROSHA and DICER, increases in their expression as a result of HPV16 E6/E7 may also contribute to cervical carcinogenesis through additional miR independent mechanisms.

In **Chapter 4**, we examine the expression of miRs in exo-EVs released from HPV16 E6/E7 expressing HFKs. Investigation of the size of exo-EVs revealed that expression of HPV16 E6/E7 may result in release of several exo-EV subpopulations and these subpopulations may carry distinct cargoes and thus, have different effects on neighboring cells. A key finding from our analysis of miR expression in exo-EVs released by HPV16 E6/E7 expressing HFKs was that some miRs are expressed similarly in exo-EVs and intracellularly whereas others are not. This implies that some miRs expressed in exo-EVs released from HPV16 E6/E7 expressing HFKs may be

candidate biomarkers of HPV16 E6/E7 expressing cells whereas other miRs may be selectively packaged into exo-EVs released from HPV16 E6/E7 expressing HFKs. Any miR expressed at higher or lower levels in exo-EVs released from HPV16 E6/E7 expressing cells compared to normal HFKs may serve as a biomarker for HPV-associated diseases and cancers. Finally, pathway analysis of differentially expressed miRs in HPV16 E6/E7 HFK secreted exo-EVs suggest that these miRs may inhibit necrosis and apoptosis. These data imply that expression of HPV16 E6/E7 may inhibit apoptosis and necrosis in neighboring normal cells through miRs secreted in exo-EVs.

Ultimately, our studies show that HPV16 E6/E7 manipulate the expression of cellular and extracellular miRs and one mechanism HPV16 E6/E7 may use to accomplish this is through perturbation of the miRNA biogenesis enzymes DROSHA and DICER. Our RNAseq data of potential targets and pathway analysis indicates that miR alterations by HPV16 E6/E7 are important for a multitude of cellular processes. The following sections discuss the results of each dissertation chapter in more depth.

Chapter 2: Modulation of microRNA-mRNA Target Pairs by Human Papillomavirus 16 Oncoproteins

The high-risk HPV E6 and E7 oncoproteins are the drivers of cell transformation that ultimately lead to HPV-associated cancers. In this chapter, we aim to systematically identify miRs modulated by expression of HPV16 E6/E7 that may have functional implications in high-risk HPV biology. Towards this goal, we utilized deep sequencing to examine miR expression in undifferentiated HFKs to understand how miRs are perturbed as a result of HPV16 E6/E7 expression. Importantly, we also

examined changes in RNA expression as a result of HPV16 E6/E7 by deep sequencing

in parallel, to understand how potential target RNAs may be regulated by miRs.

There are four particularly unique aspects that differentiate our study from others.

First, we examine miR expression in undifferentiated HFKs, which are biologically

relevant given HPV-associated cancers typically arise from undifferentiated basal

epithelial cells. Additionally, given HPVs alter epithelial cell differentiation, it is unclear

whether previously reported changes in miRs are directly caused by HPV gene

expression or represent outcomes of HPV-induced changes in epithelial cell

proliferation and differentiation. While these studies are useful, particularly for the

discovery of potential biomarker miRs (Wang *et al.*, 2014), we aimed to concentrate our

efforts on identifying miR changes resulting from HPV16 E6/E7 expression in

undifferentiated HFKs.

Second, we focus specifically on changes in miRs resulting from expression of

the two viral oncoproteins given they are consistently expressed in HPV-associated

cancers rather than utilizing the whole genome or HPV-associated tissues. Third, we

employ deep sequencing to examine miR expression changes, a superior and powerful

method of miR analysis, which has only been used in a few studies of HPV-associated

miRs to date (Gunasekharan and Laimins, 2013; Wang *et al.*, 2014).

Lastly, we utilized RNAseq combined with a miR-mRNA pairing analysis to

concurrently examine changes in potential miR targets, to begin to understand the

potential impact of miR-mediated regulation in HFKs expressing HPV16 E6/E7. We feel

our study is comprehensive given that it combines miRseq, RNAseq and a miR-mRNA

pairing analysis to assess both miR and RNA expression, culminating in a view of the

potential influence of miR regulation on overall gene expression in HFKs expressing HPV16 E6/E7.

Comparing our results to key findings that have been previously published in the field of miRs, high-risk HPVs and cervical cancer, we observe some similarities and some differences. It is not surprising that we observe both similarities and differences between our data and others given the variety in experimental conditions utilized, cell types assayed and method of analyzing miR expression. Our study is one of few which used deep sequencing to analyze miR expression (Gunasekharan and Laimins, 2013; Wang *et al.*, 2014) and unique, given it was combined with RNAseq to examine the regulation of potential miR targets.

Two key studies also used miRseq to analyze miR expression in raft cultures infected with HPV18 (Wang *et al.*, 2014) or HPV31 (Gunasekharan and Laimins, 2013). Comparing trends in regulation of miR expression to miRs identified in these key studies, which used a similar method of miR analysis, we observe 54-70% overlap between our data and these previously published results. These differences are to be expected given both studies analyze miRseq data of small RNA from other high-risk HPV (HPV18 or HPV31) infected and uninfected rafts cultures of differentiated epithelial cells. Our results are from HPV16 E6/E7 oncoprotein expressing undifferentiated HFKs and miR expression is known to be cell type and tissue-specific (Ludwig *et al.*, 2016).

Almost all cervical cancers are caused by high-risk HPVs (Winer *et al.*, 2006), however, high-risk HPV infections also account for 95% of anal cancers, 70% of oropharyngeal cancers (Chaturvedi *et al.*, 2011), 60% of vaginal cancers, 50% of vulvar cancers and 35% of penile cancers (Gillison *et al.*, 2008). Given HPV16 is by far the

most prevalent HPV type detected in these cancers, we also compared our data to miR

profiling studies of other HPV-associated cancers. A study of HPV positive anal

carcinomas identified the up-regulation of miR-15b as strongly associated with the

expression of several E2F-regulated genes (Myklebust *et al.*, 2011). The expression of

miR-15b was also up-regulated in HFKs expressing HPV16 E6/E7 in our study. We

observe overlap in differentially expressed miRs from HPV positive squamous cell

carcinomas of the head and neck (HNSCC), in particular, overexpression of miR-363,

miR-33 and decreased expression of miR-181a and miR-142-5p (Wald *et al.*, 2011).

Our results also agree with another HNSCC study showing up-regulation of miR-20b

and miR-9 (Hui *et al.*, 2010), which were significantly associated with p16 status, a

surrogate marker for HPV infection. There is also some overlap of our results with miR

expression data from HPV positive vulvar carcinoma. In particular, two miRs correlated

with clinical, anatomical and pathologic features in vulvar cancers, miR-223-5p and

miR-19b-1-5p (de Melo Maia *et al.*, 2013), which were also decreased in HPV16 E6/E7

expressing HFKs. Additionally, our results agree with a study of a HPV positive penile

squamous cell carcinoma, which showed a decrease in miR-23b and miR-145 levels, as

well as an increase in expression of miR-196a (Barzon *et al.*, 2014).

   Interestingly, some of the key changes in miR expression as a result of HPV16

E6/E7 in HFKs are in close agreement with changes observed in HPV positive HNSCC.

In particular, the miR we observe to be most up-regulated, miR-363-3p, has been

shown to up-regulated in HPV+ HNSCC cell lines (Wald *et al.*, 2011) and tumors

(Chapman *et al.*, 2015). This up-regulation has been shown to be the result of specific

expression of E6 (Wald *et al.*, 2011), which we also confirm in our study. Additionally,

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two other miRs that are highly up-regulated in response to HPV16 E6/E7 expression in HFKs, miR-20b and miR-9, were also found to be up-regulated in HPV positive HNSCC (Hui *et al.*, 2010). Others have reported that HPV positive HNSCC is significantly more similar to HPV positive cervical squamous cell carcinoma (CSCC) than HPV negative HNSCC or CSCC based on miR (Lajer *et al.*, 2012) and mRNA profiles (Pyeon *et al.*, 2007). Our results appear to suggest this as well, perhaps supporting the idea that a core set of miRs may be altered in all HPV-associated cancers as a result of HPV16 E6/E7 expression, whereas some miRs may be specific to an HPV-associated cancer of a particular anatomical site.

Our study showed that expression of HPV16 E6/E7 in HFKs results in changes in expression of individual miRs but also entire groups of genomically clustered miRs. By altering expression of miRs that may function cooperatively, changes in expression of downstream targets may be more sensitive to small changes in miR expression. Therefore, the ability of HPV16 E6/E7 to alter expression of miR clusters, in addition to individual miRs, greatly enhances the ability of HPV16 E6/E7 to regulate miR targets and pathways they are involved in.

Comparing to a literature review study, which compiled information on the expression of miR clusters in cervical cancer (Servin-Gonzalez *et al.*, 2015), we observed some of the same miR clusters altered by expression of HPV16 E6/E7 in our study. Specifically, expression of the miR-106a~363, -106b~25, -29a~29b-1, -34b~34c and -181a-1~181b-1 clusters was perturbed both by HPV16 E6/E7 and in cervical cancers. Changes in the expression of multiple miRs by HPV16 E6/E7 through manipulation of miR clusters may promote an environment that is more suitable for the

viral lifecycle or viral oncogenesis. As an example, HPV16 E6/E7 up-regulates all miRs that are part of the oncogenic miR-106b~25 cluster (Hudson *et al.*, 2013; Poliseno *et al.*, 2010) and down-regulates all miRs in the miR-34b~34c cluster, which is known to be tumor suppressive (reviewed in (Hermeking, 2010)). However, since HPV16 E6/E7 perturbs the expression of many miR clusters, both oncogenic and tumor suppressive, the overall outcome of altering miR cluster expression is unclear.

Overall, our data show agreement with other studies of high-risk HPV associated miRs, as well as, studies of alteration of miRs in several HPV-associated cancers, in particular HNSCC. While we would not expect our data to perfectly overlap with other studies due to differences in experimental conditions and analysis methods, the similarities we observe are encouraging and provide validation for the results of our study.

While previously conventional studies of single miRs and their targets were useful, this paradigm of research in the miR field has now been mostly replaced with studies of many miRs and their targets at once. This is, in part, due to advances in methods for the detection of miRs and identification of their targets, as well as decreases in cost associated with these methods. However, a more compelling reason is the fact that humans encode ≥ 2,500 mature miRs and we know that miRs do not function alone in the human body. A single miR can regulate hundreds of targets, but aberrant miR expression can influence a multitude of target transcripts, as well as signaling pathways. For these reasons, we aimed to investigate global changes in miRs and their potential target RNAs as a result of HPV16 E6/E7 expression in HFKs.

Interestingly, our results showed more miRs up-regulated rather than down-regulated in response to expression of HPV16 E6/E7.

While reduced levels of miRs are often observed in tumors due to genetic loss, epigenetic silencing, defects in miR biogenesis or widespread transcriptional repression (Chang *et al.*, 2008; Lu *et al.*, 2005), our results are unsurprising, as our experimental system may more closely mimic a low grade cervical intraepithelial neoplasia, rather than a late-stage malignant tumor. Our experimental system includes expression of HPV16 E6/E7 in early passage undifferentiated HFKs, which are the primary target of HPV infection in males. While undifferentiated basal epithelial cells may be the cells from which a cervical cancer arises, cervical cancers arise years or decades after initial infection. Perhaps, over time, the host miR expression profile in the presence of continued HPV16 E6/E7 expression would change to a more repressed phenotype, as miR expression is known to be context-specific. Further studies are necessary to understand why more miRs are up-regulated in response to HPV16 E6/E7 expression and the biological consequences of this phenomenon.

There are many methods of identification of potential miR targets and no method is necessarily superior, each has caveats associated with it. An important consideration when selecting methods is whether the focus of the study is on a single or select few miRs or a large group of miRs. Since the focus of our study was how expression of HPV16 E6/E7 globally perturbed host miR expression, we chose a method that would allow us to examine potential targets of many miRs.

Our method combined miRseq and RNAseq from the same set of samples, followed by miR-mRNA target pairing utilizing predicted and experimentally verified miR

targeting information from numerous sources. This well-controlled experimental method

allowed us to study the endogenous expression of miRs, without the need for artificial

overexpression of miRs, which is necessary for some target identification methods.

Non-natural overexpression of miRs can result in experimental artifacts, including

increased off-target effects and/or false positives (reviewed in (Eulalio and Mano,

2015)). While some effects of overexpression can be countered by miR inhibition, the

inhibition of highly expressed miRs or miRs from the same family can prove technically

challenging. Additionally, our method, unlike other methods based on AGO crosslinking

and immunoprecipitation (reviewed in (Hausser and Zavolan, 2014)), did not depend on

the strength of interaction between a miR and the RNA-induced silencing complex

(RISC) or on miR abundance. Our method also allowed us to examine potential miR

targets in a biologically relevant cell environment to detect potential cell-specific natural

targets.

However, our method, like other methods, was not without caveats. Our method

yielded both direct and indirect potential miR targets and any changes in potential miR

targets are part of a pool of indirect changes in transcript abundance as a result of

HPV16 E6/E7 expression. Given this information, we only focused on RNAs that were

inversely correlated in expression with their respective miR. As a result, we likely

overlooked the effects of miR regulation that results in "fine tuning" or balancing of gene

expression, as well as miRs whose effects are masked by the effects of other more

potent miRs or other upstream regulators altogether. As an example, miR-203a-3p has

1,032 potential predicted or experimentally validated targets revealed via the miR

Target Filter analysis in IPA. When we limit potential targets to just those present in our

RNAseq data, the number of potential targets decreases dramatically to 151 potential targets. As mentioned in the Chapter 2 results, when we employ the expression-pairing filter and focus only on RNAs with inverse expression to the miR, the resulting number is 85 potential targets. However, there are 66 other potential targets of miR-203a-3p with expression that is not inversely correlated to expression of the miR. It is possible that miR-203a-3p is still regulating these RNAs but, the outcome of gene expression in the presence of HPV16 E6/E7 is likely regulated via other mechanisms, which overcome or negate the regulatory effect of miR-203a-3p. Overall, it is important to emphasize that not all of the RNA expression changes observed in our study are due to miR regulation, further experimental validation will be required to conclusively determine which expression changes can be linked to miR expression.

Additionally, our method did not allow detection of the precise location of miR binding sites, as other methods can. More importantly, some miR targeting occurs at the level of translation repression and our method of detecting potential targets would overlook those potential targets. Despite missing some targets regulated by miRs at the level of translation, it has been shown that the majority of miR-mediated repression (~84%) is attributed to decreased mRNA abundance (Guo *et al.*, 2010). Therefore, it is likely that the majority of potential miR targets would be detected via our method. Ultimately, our large scale, unbiased method allowed us to uncover gene networks potentially regulated by miRs, rather than individual miR: target interactions, which was the goal of our study.

A strength of our study is the ability to identify miRs based on their potential to regulate gene expression in HFKs expressing HPV16 E6/E7. Most studies have

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identified miRs of importance based on miR abundance alone. This method is practical, given the need for detection of the miR in various downstream assays, which sometimes require high amounts of starting material. However, the possibility exists that some miRs of lower abundance may be overlooked. Our method of combining miRseq and RNAseq data, along with the target pairing analysis, allowed us to identify both high and low abundance miRs with the potential to regulate gene expression. Importantly, we were able to identify miRs of low abundance but with high potential to regulate gene expression as evidenced by a high number of potential targets. For example, miR-4532, upregulated only 3 fold in HFKs expressing HPV16 E6/E7, was paired with 90 potential targets. This miR, and others, likely would not have been considered particularly important had our study been based on miR abundance alone.

Another strength of our study is the ability to examine the potential effect of miR-mediated regulation on overall gene expression in HFKs expressing HPV16 E6/E7. In particular, our study provides clues on how HPV16 E6/E7 alteration of miRs may impact gene expression. We observed 67.8% of potential target RNAs inversely correlated with expression of their respective miRs, suggesting the potential for miR-mediated regulation of these RNAs. It is possible that this number could be even higher, as we utilized relatively strict fold change cut-offs for miR expression ($-3 \geq FC \geq 3$). It has been estimated by others that 60% of all mRNAs are controlled by miRs and our results agree closely with these estimations (Bartel, 2009). Based on this observation, it has been suggested that miR regulation is the most abundant mode of posttranscriptional regulation (Jansson and Lund, 2012) and the results of our study supports this notion as well.

As expected, we observed many RNAs regulated by more than one miR. In fact, employing our threshold cut-offs, we observed 49% of potential targets to be regulated by more than one miR. The percentage may be depressed due to our stringent cut-off values for the miRseq and RNAseq data. Interestingly, if we examine the average fold change of RNAs potentially targeted by one miR versus more than one miR, we observe that RNAs targeted by just one miR, on average, are slightly more highly up-regulated/down-regulated compared to RNAs targeted by more than one miR. Specifically, RNAs potentially targeted by just one miR in our study were up-regulated 3.9 fold and down-regulated 13.0 fold, on average. However, RNAs potentially targeted by more than one miR were up-regulated 3.4 fold and down-regulated 5.7 fold, on average. While one might hypothesize that RNAs targeted by more than one miR might be more highly up-regulated or down-regulated, the changes observed in RNA expression are not solely the result of miR regulation and could be the result of indirect changes in RNA expression as a result of HPV16 E6/E7 expression.

If we compare RNAs targeted by just one miR to RNAs targeted by more than one miR utilizing IPA, we notice some significant differences and similarities based on activation z-score values ($-2 \geq$ z-score ≥ 2). Analysis of RNAs targeted by more than miR indicates that the following pathways are significantly inhibited compared to RNAs targeted by just one miR: endothelian-1 signaling (z score= -2.11), p38 MAPK signaling (z score= -2.12) and G1/S checkpoint regulation (z score= -2.24). In contrast, the ATM signaling pathway is significantly activated (z score= 2.24) upon analysis of RNAs targeted by just one miR compared to RNAs targeted by more than one miR.

When we compared a pathway analysis of the miR-mRNA pairing data to an analysis of the entire RNAseq data set, we observed some similarities and some differences. Both data sets were found to be associated with cancer and reproductive system disease. Several molecular and cellular functions were also the same between the data sets, including cellular movement and cell morphology. However, some molecular and cellular functions appeared to be specifically associated with changes in mRNAs that may be targets of miRs. In particular, cellular development, morphology, growth and proliferation were identified to uniquely involve RNAs that are potential targets of miRs in HFKs expressing HPV16 E6/E7. Additional analyses utilizing IPA revealed canonical pathways that significantly differ between the two datasets based on activation z-score values (-2 ≥ z-score ≥ 2). These analyses reveal cyclins, cell cycle regulation (z score= 2.33) and estrogen-mediated S-phase entry (z score= 2.24) to be significantly activated and aryl hydrocarbon reception signaling to be significantly inhibited (z score= -2.45) in the RNAseq data set of all RNAs altered by expression of HPV16 E6/E7. In contrast, ATM signaling (z score= 2.12) was significantly activated based on analysis of RNAs with the potential to be miR targets.

These results suggest that RNAs with the potential to be targets of miRs altered by expression of HPV16 E6/E7 are involved in distinct canonical pathways, molecular and cellular functions that may be relevant in the context of HPV biology. Additionally, these results imply that HPV16 E6/E7 regulation of cellular miRs and their potential downstream targets is an important function of these two transforming oncoproteins.

Contributions to the Field

Our study is the first comprehensive, combined analysis of miRseq and RNAseq data, together with a target pairing analysis, in the field of HPV-associated miRs. These data provide a glimpse of how expression of HPV16 E6/E7 may be altering gene expression on a global scale through miRs. We have validated the expression of miRs previously reported in the literature, as well as identified new miRs regulated by HPV16 E6/E7 that may subjects of future studies. While validating previously reported miRs may not seem like an important contribution, there is so much variation in miR profiling in HPV associated cells and tissues due to differences in samples and experimental techniques. Therefore, it is imperative to compare miR profiling studies in the field to get a sense of key miRs involved in HPV biology that show similar trends in expression across many studies. It is expected to observe similarities and differences when comparing studies but there may be core groups of miRs that are similarly altered across high-risk and low-risk HPV types, for example. Importantly, the information generated from these studies we consider to be a "treasure trove" of data that will fuel many future studies in our laboratory, as well as other laboratories.

Future Work

There are many future experiments that could evolve from the large miRseq and RNAseq datasets generated in these studies. A valuable addition to our study would be large scale validation of miR regulated mRNAs. With unlimited resources, the most comprehensive method would likely be performing RNAseq in HFKs in the presence of individual or multiple miR inhibitors and controls. We have also been interested in

determining functional consequences of some of the miRs identified in the miRseq experiments that, based on our miR-mRNA pairing data, have potential to regulate large numbers of target mRNAs. Previously, we have examined cell viability, wound healing, growth in soft agar and senescence using miR inhibitors to look for functional effects of individual miRs on these processes. We saw very little effect on these processes when inhibiting expression of individual miRs, likely given the majority of miRs participate in "fine-tuning" of gene expression. Since we observed HPV16 E6/E7 alters the expression of miRs in clusters, it would be interesting to knockdown whole miR clusters and then determine by process of elimination which individual miR(s) are responsible for an observed phenotype. Additionally, our pathway analysis data could provide clues on other functional assays to pursue. We could also utilize various mutant of HPV16 E6 and/or E7 to determine regions of the oncoproteins that appear to be important for miR regulation, as well as compare miR alterations by other high-risk or low-risk HPV E6 and E7 oncoproteins.

Chapter 3: Perturbation of DROSHA and DICER Expression by Human Papillomavirus 16 Oncoproteins

It is clear from our studies and others that expression of the HPV16 oncoproteins E6 and E7 alters expression of host miRs. A key question in the field that has not been addressed is the mechanism(s) through which HPV16 E6 and E7 affect expression of a multitude of human miRs. While there are many known ways in which miR expression can be altered in cancers, and it is likely that expression of HPV16 E6 and E7 alter miR expression through multiple mechanisms, in Chapter 3 we address the possibility that

E6 and E7 may manipulate key enzymes in the miR biogenesis pathway to perturb host miR expression.

We focused our efforts on examining the two major enzymes involved in the canonical miR biogenesis pathway, DROSHA and DICER. It is clear from large-scale cancer genomics data sets that *DROSHA* and *DICER* are commonly altered in cancers, with *DROSHA* most frequently amplified and *DICER* most often mutated across multiple cancer types. Of interest to this study, *DROSHA* and *DICER* are also genomically altered in cervical cancers and RNA and protein levels are elevated in cervical cancer tissue samples. We observed elevated RNA levels of *DROSHA* and *DICER* in the HPV16 positive CaSki and SiHa cervical cancer cell lines. *DROSHA* levels were only slightly increased in HPV18 positive HeLa cells and *DICER* levels were lower than the HFK control in this cell line. *DROSHA* and *DICER* levels were decreased compared to HFK controls in HPV negative C33A cells in our study. Our data differ from a previous report examining *DROSHA* RNA levels in cervical cancer cell lines including HeLa, SiHa and C33A (Zhou *et al.*, 2013). *DROSHA* was most upregulated in HeLa cells, least upregulated in C33A cells and an intermediate level of *DROSHA* upregulation was observed in SiHa cells.

Our results may differ due to the use of different controls in our study compared to the previous study. In our study, HFKs were utilized as a control and in the previous study the authors utilize CRL2614 cells, which they refer to as a normal cervical epithelial cell line, as well as three, independent primary cultures of normal cervical epithelial cells were utilized. Utilizing CRL2614 cells as a control is not ideal for these experiments, as these cells are transformed by expression of HPV16 E6/E7 (ATCC)

and thus, should not be considered "normal." Even the normal cervical epithelial cells

were harvested from hysterectomy samples from women with diseases unrelated to the

cervix, which also may not be an ideal (Muralidhar *et al.*, 2007). While it is difficult to

find a perfect control, we feel HFKs are a good control as they are freshly harvested

from neonatal foreskin, the target tissue of HPV infection of males. These primary cells

are kept at low passage and are not transformed or derived from diseased patients.

Ultimately, these important differences in controls may account for the differences we

observe in *DROSHA* and *DICER* levels in cervical cancer cell lines.

We hypothesize that the differences in *DROSHA* and *DICER* levels observed in

the HPV18 positive HeLa cell line may be due to the difference in high-risk HPV type,

although we do not directly test this in this chapter. An important finding that served as

an impetus for the additional experiments in this chapter was that *DROSHA* and *DICER*

RNA levels were not elevated in the HPV negative C33A cervical cancer cell line. This

result led us to question if the HPV status of the cervical cancer cell line studied was

related to alterations in *DROSHA* and *DICER* RNA levels and our data from cervical

cancer cell lines appeared to fit this pattern. It is important to point out that C33A cells

are also deficient for BRG-1(Dunaief *et al.*, 1994), a component of the SWI/SNF

complex, and alterations like this may account for the discrepancies we observed in

DROSHA and DICER RNA levels we observed in C33A cells.   Given the high-risk HPV

oncoproteins are the causative agents of HPV associated cancers at the molecular

level, we wondered if differences in *DROSHA* and *DICER* levels were correlated with

expression of HPV16 E6/E7. To address this hypothesis, we examined DROSHA and

DICER levels in HPV16 E6/E7 expressing HFKs and in the HPV negative C33A cell

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line.

Our data in HPV16 E6E7 expressing HFKs showed that DROSHA and DICER

RNA and protein levels are elevated compared to control HFKs. The observed

upregulation in DROSHA and DICER RNA and protein levels appears to mediated

mainly through HPV16 E6. Given expression of the high-risk HPV oncoproteins alone

results in changes in DROSHA and DICER levels suggests that the elevated levels we

observed in HPV positive cervical cancer cell lines may be due to expression of the

HPV oncoproteins. To further test this, we also introduced the HPV oncoproteins into

the HPV negative C33A cell line through both transient transfection and stable

transduction. Expression of HPV16 E6/E7 in C33As results in an increase in *DROSHA*

RNA levels, although not to the same extent as in HFKs or in HPV positive cervical

cancer cell lines. The data for stably transduced C33As showed an increase in

DROSHA RNA levels, however, it was not a statistically significant increase so these

data were not included in this chapter. Nevertheless, expression of HPV16 E6/E7 in

HFKs and in C33As results in elevated DROSHA levels, suggesting the alterations in

DROSHA may be due to expression of HPV16 E6/E7. Additionally, these data may

indicate that DROSHA upregulation is independent of TP53 or pRB given both tumor

suppressors are mutated in C33A cells (Scheffner *et al.*, 1991) and HPV16 E6/E7 are

expressed.

One explanation for increased DROSHA levels mainly being mediated by HPV16

E6 may be related to degradation of the tumor suppressor TP53 by HPV16 E6, which

blunts the TP53 transcriptional response. TP53 has been shown to enhance the

biogenesis of several growth-suppressive miRs in response to DNA damage (Suzuki *et*

al., 2009). In particular, TP53 interacts with the DROSHA processing complex through p68 (DDX5), facilitating the processing of primary miR to precursor miR in some cell types. Transcriptionally inactive TP53 mutants interfere with assembly of the DROSHA complex and p68, resulting in impaired miR processing (Suzuki *et al.*, 2009). Perhaps in HFKs with expression of HPV16 E6 alone or HPV16 E6/E7, DROSHA RNA and protein levels increase to overcome this attenuation of miR processing due to a blunted TP53 transcriptional response. DICER RNA and protein levels may then increase to compensate for increased DROSHA RNA and protein levels upstream in the miR biogenesis pathway.

A comparison of previously identified DROSHA associated miRs (Muralidhar *et al.*, 2011) with miR expression changes identified in HPV16 E6/E7 expressing HFKs in Chapter 2, revealed that expression of the majority of DROSHA-associated miRs are similarly altered in HFKs expressing HPV16 E6/E7. This may indicate that HPV16 E6/E7 alteration of DROSHA levels may have functional effects on downstream miR expression. Additionally, it has been suggested from studies of SCC that increases in DROSHA must exceed a threshold of ~2-4.5 fold change over control and our data from HPV16 E6/E7 expressing HFKs exceeds that threshold, further supporting the notion that alterations in DROSHA by HPV16 E6/E7 have functional effects on downstream miR expression.

For the few DROSHA associated miRs that show different trends in expression in HFKs expressing HPV16 E6/E7, it is important to note that these DROSHA responsive miRs were not originally identified in HFKs. Given miRs are known to be cell type specific, it is possible that some DROSHA associated miRs may be cell type specific.

Additionally, in the previous study, a limited panel of 319 miRs were analyzed (Muralidhar *et al.*, 2011) so it is also possible that additional miRs not evaluated in this study may be DROSHA responsive.

Given that only a subset of 45 miRs were found to be associated with DROSHA levels (Muralidhar *et al.*, 2011), we investigated whether any of these miRs were part of a cluster or miR family since this was not addressed in the previous study. We found that 18 out of 45 miRs were part of a miR cluster and these 18 miRs made up 8 different miR clusters. Interestingly, 7 miRs out of 45 miRs were part of the let-7 family of miRs, which are known to be frequently deregulated in cancers. Let-7 is most well known as a tumor suppressor miR and, as a result, let-7 family members are frequently downregulated in many cancer types (Boyerinas *et al.*, 2010). However, in some cancers upregulation of let-7 family members has been observed (Brueckner *et al.*, 2007; Lawrie *et al.*, 2009; Lu *et al.*, 2007) and, in our study and in (Muralidhar *et al.*, 2011), all DROSHA associated let-7 family members are upregulated. The presence of miR clusters and families in the group of miRs associated with DROSHA levels may hint at a mechanism by which DROSHA is associated with this particular subset of miRs.

To identify pathways unique to the DROSHA-associated miRs, we compared a core analysis of these miRs to the entire miRseq dataset ($-1 \geq FC \geq 1$). In this analysis, we utilized less stringent fold change cutoffs since the reported fold changes for the DROSHA-responsive miRs ranged from 1.8-4.2 fold (Muralidhar *et al.*, 2011). This comparative analysis revealed that many of the same molecular and cellular functions are associated with both groups of miRs, DROSHA responsive and HPV16 E6/E7 altered. These data suggest that the "dual targeted" molecular and cellular

functions are important for HPV16 E6/E7 to manipulate both through miRs and through DROSHA, ultimately leading to effects on downstream miR expression.

Additionally, we compared the previously identified DROSHA-associated miRs identified to our list of miRs identified in the miR-mRNA pairing analysis in HPV16 E6/E7 expressing HFKs described in Chapter 2. We found there was little overlap between the DROSHA-associated miRs and miRs of potential functional importance in HPV16 E6/E7 expressing HFKs. This may indicate that DROSHA-responsive miRs are distinct from miRs identified to be of potential functional importance in HPV16 E6/E7 expressing HFKs. It is also important to note that the fold change cutoffs utilized to identify miRs of potential functional importance in Chapter 2 were fairly stringent (-3 ≥ FC ≥ 3) and this may explain the lack of overlap between these two groups of miRs. It is possible that if less stringent fold change cutoffs were employed the overlap between these two groups of miRs may be greater. However, it is not expected that all miRs of functional importance will be DROSHA responsive and vice versa.

In summary, data from this chapter suggest that the HPV16 oncoproteins may alter RNA and protein levels of DROSHA and DICER, two critical enzymes in the canonical miR biogenesis pathway. Examination of miRs that are known to be DROSHA responsive suggests that HPV16 E6/E7 perturbation of DROSHA levels may have functional effects on miR expression. Further studies will be necessary to full understand how HPV16 E6/E7 manipulates the miR biogenesis pathway but this may be one mechanism through which the high-risk HPV oncoproteins function to alter host miR expression.

Contributions to the Field

This chapter reveals a new mechanism by which HPV16 E6/E7 may manipulate expression of multiple miRs at once. Although this is likely just one of several mechanisms by which HPV16 E6/E7 perturb miR expression, this chapter uniquely focuses on alterations in upstream miR regulators, which has been the subject of little investigation in the field of HPV-associated miRs. Most studies of HPV-associated miRs have focused solely on miR profiling in HPV-associated cells and tissues or followed up on specific, individual miRs and it is important to investigate the key players that orchestrate the processes that ultimately result in changes in miR expression.

Future Work

There are several key experiments that will be important to complete to solidify our observations and these would include knockdown and overexpression of DROSHA and DICER. As discussed in Chapter 3, we attempted these experiments using shRNAs and siRNA pools in HFKs with little to no success. Based on our experience with transfection and transduction of primary cells and observations gleaned from the literature, we feel that this experimental failure is due to the sensitive nature of the primary cells we work with. A method to try next might utilize electroporation using a protocol for difficult to transfect, primary cells. If we could successfully knockdown or overexpress DROSHA and/or DICER, it would be interesting to perform miRseq in HFKs with overexpression or knockdown of these key miR biogenesis enzymes, to define DROSHA and DICER "responsive" miRs in HFKs. It would also be worthwhile to investigate whether or not HPV16 E6/E7 manipulates other components of the miR

biogenesis machinery.

Chapter 4: Human Papillomavirus 16 E6 and E7 Oncoprotein Expression Alters microRNA Expression in Extracellular Vesicles

The study of EVs is still in its infancy and there are two key reasons these extracellular entities may be valuable in the diagnosis and treatment of human disease. First, EVs can be detected in easily accessible bodily fluids and, thus far, it appears that in many diseases that are specific patterns of EV cargo that could serve as possible prognostic or diagnostic biomarkers. Therefore, EVs have potential to facilitate earlier and less invasive detection of diseases. Second, since EVs are derived from intracellular material, they are being explored as possible packaging tools for the delivery of genetic materials and/or drugs. However, for these ideas to become reality, the basic science behind EVs must be further explored and better defined. As a result, any study that provides some additional knowledge of EV biology will be useful and contribute to the future use of EVs as biomarkers and tools for therapeutic delivery. Our study examined how EV-associated miRs are altered by expression of the two high-risk HPV oncoproteins E6 and E7, which are the causative agents of HPV-associated cancers.

Exosome isolation and characterization are still considered scientific challenges (Lotvall *et al.*, 2014; Szatanek *et al.*, 2015). Therefore, it is important to discuss the isolation method utilized in this chapter. Since there is no consensus on a "gold standard" method for exosome isolation, it cannot be claimed that there is an optimal

method that should be used (Lotvall *et al.*, 2014). Differential ultracentrifugation is a traditional and reliable method for the isolation of exosomes (Jeppesen *et al.*, 2014). However, there are some issues associated with this method and newer methods are less time consuming and more compatible with limited biological samples. Several studies have shown that centrifuging at high speeds can result in fusion of particles with contaminants and other proteins, disturbing their physical properties (Linares *et al.*, 2015; Rood *et al.*, 2010; van der Pol *et al.*, 2012). Additionally, applying centrifugal forces over multiple cycles, while advantageous for the removal of cellular debris and contaminants, can result in lower and more variable exosome yield (Alvarez *et al.*, 2012).

We employed a newer, less tedious alternative to differential ultracentrifugation using a commercial kit for the isolation of exo-EVs in our study. The commercial kit we utilized has been thoroughly compared to differential ultracentrifugation and several other methods and validated to be an excellent alternative to differential ultracentrifugation for the isolation of exosomes (Helwa *et al.*, 2017). Specifically, the reagent utilized in our study interacts with water molecules in the sample, thereby forcing less soluble sample components, like EVs, out of solution. The EVs can then be collecting by a short, low-speed centrifugation rather than a long, high-speed ultracentrifugation. However, our method, and other methods, may not selectively enrich for exosomes and may also co-isolate other contaminating factors, like extracellular RNA binding proteins (Van Deun *et al.*, 2014). For this reason, we are careful to refer to the EVs in our samples as exosome-enriched extracellular vesicles (exo-EVs) rather than exosomes.

To evaluate the presence of exo-EVs in our samples, we followed recommendations from a publication of the International Society for Extracellular Vesicles describing the minimal acceptable experimental requirements to claim the presence of EVs (Lotvall *et al.*, 2014). EVs must be isolated from extracellular fluids and we harvested our exo-EVs from the conditioned cell culture medium of HFKs expressing HPV16 E6/E7 or controls. Next, we examined the protein cargo of our exo-EVs by Western blotting analysis. While there are no true "exosome specific" markers, there are proteins that appear to be "exosome-enriched" that can be analyzed and it is recommended to examine three or more of these proteins at a minimum (Lotvall *et al.*, 2014). In particular, it is recommended to assess proteins from the following categories: 1) transmembrane/lipid-bound extracellular proteins; 2) cytosolic proteins; 3) intracellular proteins.

We monitored the steady state levels of 6 different proteins, some of which are expected to be present in exosomes and some of which are not expected to be in exosomes. RIG-I, a cytosolic RNA sensor of the innate immune system, is not packaged into exosomes (Boelens *et al.*, 2014) and we did not observe this protein in our exo-EV samples. HSC70 is a chaperone protein found in exosomes from most cell types (Geminard *et al.*, 2004; Thery *et al.*, 2001) and we observe HSC70 in our exo-EV samples. We did not detect actin in our exo-EV samples, indicating that our exo-EV preparations were free of cellular debris (Angeloni *et al.*, 2016). However, the presence of actin in exosomes seems to be cell dependent, as other studies have detected actin in exosomes from dendritic cells, enterocytes and mastocytes (Thery *et al.*, 2002). Tetraspanins, like CD9 are expected to be present or enriched in exosomes (Lotvall *et*

al., 2014) and we observe this protein in our exo-EV samples. Endosome or membrane

binding proteins, such as Rab5, are also expected to be present or enriched in

exosomes (Lotvall *et al.*, 2014), which we also identified. Nuclear histones, like H3,

should be absent or under-represented in exosomes, but present in other EV types

(Lotvall *et al.*, 2014), and we did not observe H3 in our exo-EVs. Overall, our

characterization of the protein content of our samples meets and exceeds the

requirements recommended by the International Society for Extracellular Vesicles and

indicates that our exo-EV samples are enriched for exosomes.

We utilized NTA to assess the size of exo-EVs, as well as determine the

concentration of exo-EVs in our samples. We found that expression of HPV16 E6/E7

results in release of three distinct populations of exo-EVs, a population of 67, 89 and

121nm in diameter. This might seem surprising, as the majority of the EV field assumes

exosomes to be a homogeneous population. However, a recent study revealed that

multiple cell types release more than one subpopulation of exosomes (Willms *et al.*,

2016). This study showed that exosome subpopulations are characterized by their

distinct size, protein and RNA composition, and these differences resulted in diverse

functional effects on recipient cells (Willms *et al.*, 2016). These results suggest that

HPV16 E6/E7 expressing HFKs release several subpopulations of exo-EVs and each

subpopulation may have unique cargo, which could result in different functional effects

on neighboring cells.

Only a few studies of HPV-associated exosomes have been reported (Chiantore

et al., 2016; Honegger *et al.*, 2013; Honegger *et al.*, 2015) and our study corroborates

previous findings, as well as contributes new knowledge to our understanding of miRs in

HPV-associated EVs. One previous study (Chiantore *et al.*, 2016) found miR-222-3p to be significantly expressed in exosomes from HPV16 expressing HFKs. We also found miR-222-3p to be upregulated in our HPV16 E6/E7 exo-EV samples. Interestingly, this miR is downregulated in HPV16 E6/E7 expressing cells but upregulated in exo-EVs released from the same cells. The same study (Chiantore *et al.*, 2016) also found miR-320a in exosomes and we found miR-320a to be expressed in exosomes released from HPV16 E6/E7 expressing HFKs as well. This miR was upregulated in HPV16 E6/E7 HFKs and downregulated in exosomes released from the same cells. Our experimental approach, utilizing HPV16 E6/E7 and control expressing HFKs was similar to (Chiantore *et al.*, 2016), however we analyzed the expression of miRs in EVs with a highly sensitive and specific assay tailored for the detection of miRs in circulating biofluids, which allowed us to detect more miRs in our samples.

Comparing our data to another study, which utilized deep sequencing to examine exosomes released from HeLa cells in which expression of E6/E7 was silenced, we observed some overlap with our data analyzing miR expression in exo-EVs (Honegger *et al.*, 2015). In particular, we also observed miR-21-5p, -222-3p, -320a and -378a-3p in exo-EVs from HPV16 E6/E7 HFKs. Two of these miRs, miR-378a-3p and miR-21-5p, are part of the 7 miR signature affiliated with HPV E6/E7 oncogene expression identified in this study (Honegger *et al.*, 2015). Experimentally, it is ideal to use an unbiased sequencing approach as in (Honegger *et al.*, 2015); however, since this was our first study of miRs in exo-EVs, we felt it would be most useful to focus on a panel of miRs already known to be associated with cancer. Additionally, rather than silencing expression of the oncoproteins in cervical cancer cell lines, we prefer to use primary

cells, from tissue which is the target of HPV infection of males, and express HPV16 E6/E7 in these cells at biologically relevant levels.

An important finding from our analysis of miR expression in exo-EVs released by HPV16 E6/E7 expressing HFKs was that some miRs are expressed similarly in exo-EVs and parental cells and others are not. This is consistent with what has been previously reported in the literature for other cancer-associated EVs. The miR content of some exosomes has been reported to be similar to that of the original tumor (Rabinowits *et al.*, 2009; Taylor and Gercel-Taylor, 2008), which is ideal for the use of exosome-associated miRs for early detection of disease. However, in other studies, an abundance of particular miRs not expressed or expressed at very low levels in the parental cells has been observed, suggesting that certain miRs are preferentially packaged in exosomes (Jaiswal *et al.*, 2012; Pigati *et al.*, 2010).

These findings indicate that both miRs expressed similarly and differently than parental cells are interesting for different reasons and we observe examples of both in exo-EVs released by HPV16 E6/E7 expressing HFKs. In particular, miR-21-5p, identified in our study and one other (Honegger *et al.*, 2015), may be a useful biomarker for HPV16 E6/E7 expression. Additionally, further studies of miR-222-3p, -320a and -378a-3p to determine if they are selectively packaged in response to expression of HPV16 E6/E7 would be worthwhile as these miRs were observed in our experiments and two other studies (Chiantore *et al.*, 2016; Honegger *et al.*, 2015). Nonetheless, it is important to keep in mind that the mechanism behind selective packaging and release of exosomal miRs is still a subject of debate and the reliability of exosomal miRs as markers of disease remains to be determined.

Pathway analysis of the miRs we identified to be expressed differently by HPV16 E6/E7 in exo-EVs compared to parental cells revealed that these miRs appear to uniquely inhibit pathways involved in necrosis and apoptosis. While the z-scores associated with necrosis and apoptosis were not significant, this does not make this finding uninteresting. In fact, it is likely that the z-scores are not significant simply due to the small list of miR analyzed. The transfer of exosomes by other cell types to recipient cells has been previously linked to effects on apoptosis (Rivoltini *et al.*, 2016; Yang *et al.*, 2015) and necrosis (Nong *et al.*, 2016) and perhaps, expression of HPV16 E6/E7 inhibits apoptosis and necrosis in neighboring cells through manipulation of miR expression in exo-EVs. Supporting this finding, one of the few previous studies of HPV-associated miRs in exosomes reported that several of E6/E7-dependent exosomal miRs were linked to control of cell proliferation and apoptosis.

In summary, we isolated and characterized EVs released by HPV16 E6/E7 expressing HFKs and control HFKs. Analysis of the protein content of these EVs suggests that these EVs are exosome-enriched. NTA results indicate that expression of HPV16 E6/E7 in HFKs may result in the release of several subpopulations of exo-EVs rather than a single, homogeneous exo-EV population. We also examined the miR content of exo-EVs released from HPV16 E6/E7 and control expressing HFKs. Our data agree with findings from a few previous studies indicating that HPV16 E6/E7 alters the expression of miRs in exosomes (Chiantore *et al.*, 2016; Honegger *et al.*, 2015). Through this analysis we found that some miRs are expressed similarly in exo-EVs and parental cells and other are not, which has not been previously reported for HPV-associated EVs. The miRs expressed similarly in exo-EVs and parental cells may be

useful as biomarkers of HPV16 E6/E7 expressing cells or cancers. The miRs

expressed differently in exo-EVs compared to parental HPV16 E6/E7 HFKs may be

selectively packaged into exo-EVs and perhaps have unique functions in recipient cells.

Ultimately, further studies of HPV-associated EVs and their miR cargo are warranted to

uncover their full diagnostic and therapeutic potential.

Contributions to the Field

These data confirm and expand upon the little data that currently exists on HPV-

associated miRs in EVs. Given the biology of EVs is not well understood, any study of

EVs is a contribution to the field. In particular, we have shown that HPV16 E6/E7

expression may result in release of several subpopulations of EVs of various sizes,

potentially carrying distinct cargo. We also found that HPV16 E6/E7 alters the

expression of some miRs in cells and EVs in a similar fashion and these miRs may

serve as biomarkers of HPV16 E6/E7 expression. However, HPV16 E6/E7 alters the

expression of others miRs differently in cells and in EVs, suggesting that these miRs

may be selectively packaged as a result of HPV16 E6/E7 expression. Overall, our

studies reveal some unique insights into the biology of HPV-associated miRs in EVs

and these data will be the basis of future studies.

Future Work

Since there is so little known about the biology of HPV-associated miRs in EVs,

there is much future work to be done. Given our observation of some miRs with

different patterns of expression as a result of HPV16 E6/E7 expression, this would be

worthwhile to follow up on more comprehensively using miRseq to examine all miRs in EVs released from HPV16 E6/E7 and control expressing HFKs. These data could be compared with our miRseq data in cells to understand more comprehensively miRs with similar and different patterns of expression in cells and EVs. More importantly, it will be necessary to determine if miRs in EVs released from HPV16 E6/E7 expressing HFKs have effects on recipient cells. We are currently in the process of collaborating with the laboratory of Dr. Paul Lambert to examine the effects of EVs from HPV16 E6/E7 and control HFKs on fibroblasts using RNAseq. These *in vitro* data will then be compared to stromal gene expression changes previously observed by their laboratory *in vivo* (mouse). Our hypothesis is that miRs in EVs released from HPV16 E6/E7 expressing cells may be responsible for some of the gene expression changes observed in the stroma *in vivo*.

The Potential Far-Reaching Impact of Our Studies on Human Health

It is important to envision how the results of these studies may benefit human health in the future. These studies have described miRs associated with HPV16 E6/E7 expression in cells and in extracellular vesicles (EVs) and this could be potentially useful in the clinical management of HPV-associated cancers. In the longer-term, it is also possible that our studies, together with data from other studies, could contribute valuable information towards the development of miR-base therapeutics for HPV-associated diseases and cancers. These subjects are discussed in more detail below.

miR as biomarkers

The expression patterns of miRs are thought to be applicable to several aspects of the clinical management of cancers including diagnosis, prognosis, remission, release and metastasis (reviewed in (Hayes *et al.*, 2014)). It is thought that miRs may be even more useful than mRNAs as prognostic indicators as they are more stable within clinical samples and exhibit robust expression patterns (Lu *et al.*, 2005). As such, the investigation of miR signatures for prognostic purposes is currently underway in multiple clinical trials. Of interest in cervical cancer diagnosis, one study examined the expression of miRs in cervical exfoliated cells collected for Pap testing (Tian *et al.*, 2014). Their results showed that they were able to detect miRs in cervical exfoliated cells and found that expression of miR-424 or miR-375 individually or a multimarker panel of miR-424/miR-375/miR-218 showed superior performance over the traditional Pap test (Tian *et al.*, 2014). These results are exciting and provide proof of concept data that miR expression could serve as a biomarker in samples that are already normally collected for the Pap smear, thus potentially improving the assay without altering the sample collection protocol.

Interestingly, several studies have also associated miRs with biomarkers for treatment therapy decisions and this may eventually be incorporated into clinical decision making. For instance, treatment of chronic myeloid leukemia with imatinib decreases the levels of cells with the BCR-ABL rearrangement, an oncogenic gene fusion that characterizes this disease, and miR-451 levels correlate with BCR-ABL levels at the time of diagnosis and during treatment (Scholl *et al.*, 2012). There is also some evidence that SNPs in miR target sites may be predictors of treatment responses

(Pardini *et al.*, 2013; Sebio *et al.*, 2013; Wynendaele *et al.*, 2010). SNPs in miR target

sites may also be markers of genetic susceptibility in cancer, predicting subsets of

patients at risk for poor outcomes or lack of treatment response (Ziebarth *et al.*, 2012).

With regards to HPV-associated cancers, in particular, cervical cancer, there is some

evidence that miRs may be utilized to predict responses to treatment with radiotherapy,

a standard treatment for invasive cervical cancer (Liu *et al.*, 2015; Pedroza-Torres *et al.*,

2014; Song *et al.*, 2015). The ability to predict radioresistance, prior to a course of

radiotherapy, is extremely valuable to allow the design of an optimal treatment strategy

for high-risk HPV positive cervical cancers.

As mentioned in Chapter 4, the potential for circulating miRs to serve as non-

invasive biomarkers of various cancer types is an area of extensive research. Released

from tumor cells and protected in EVs, these miRs are stably detected in bodily fluids

even after multiple freeze-thaw cycles and exposure to room temperature conditions

(Mitchell *et al.*, 2008). There is still much to learn about the biology of miRs in EVs but

the ease of detection and stability of miR expression in bodily fluids makes miRs in EVs

an exciting and promising avenue for the future development of non-invasive cancer

biomarkers.

miR for disease classification

Given miR expression frequently reflects the embryonic or developmental origin

of tumors, miRs can also be useful for the classification of tumors. In fact, in a blinded

study of 22 different tumor types, miR expression identified cancer tissue origin to a

high accuracy rate (greater than 90%)(Rosenfeld *et al.*, 2008). Incorrect classification of

tumors is also an issue, even in cancers with well-defined classification, such as in breast cancer (Andorfer *et al.*, 2011). Therefore, analysis of miR expression could improve on current methods for the classification of tumors. MiRs may also be useful for the prediction of metastatic outcomes in patients, as they have been shown to have a role in cancer progression (Pencheva and Tavazoie, 2013). For example, in breast cancer metastases, specific miRs have been observed to support endothelial recruitment to metastatic sites and thus, miRs could serve as predictive markers of this kind of event (Png *et al.*, 2011).

Extrapolating to HPV-associated cervical cancers, cervical intraepithelial neoplasias (CIN) develop slowly and progression to cervical cancer is preventable and can be cured if detected early enough (Holowaty *et al.*, 1999). However, with current screening methods, including cervical cytology and HPV testing, the ability to accurately determine the CIN grade or predict regression or progression of CINs is difficult. An HPV-associated miR with consistent expression results across multiple studies is miR-203a-3p. We (Chapter 2), and two others (McKenna *et al.*, 2010; Melar-New and Laimins, 2010), have shown that this miR is decreased upon expression of high-risk HPV E6/E7 and this miR may be a good candidate for an HPV-associated miR biomarker. In fact, a recent study examined expression of this miR as a biomarker to differentiate different CIN stages and cervical cancer (Coimbra *et al.*, 2016). Although their results are preliminary, and further evidence is needed in larger sample sizes and/or in multiple cohorts, expression of this miR appears to be useful in the diagnosis of cervical cancer stages. Other miRs identified in this dissertation could be useful as indicators of HPV16 E6/E7 expression for diagnostic purposes in the future.

microRNA-based therapeutics

In addition to studies of miRs as biomarkers, miR and miR inhibitor constructs are under investigation as potential therapeutic agents for cancer, as well as other diseases. These therapies may be designed to directly target tumor cells or to enhance other therapies by reducing drug resistance (Liu *et al.*, 2014; Xiao *et al.*, 2014). Currently, there are eight miR-based therapeutics in US clinical trials, spanning a variety of disease areas including one for oncology, as well as others for endocrine, respiratory, renal and infectious diseases (Biomedtracker). Of these therapies, five are in Phase I and three are in Phase II clinical development, with many other miR-based therapeutics still in preclinical testing. In particular, MRG-106, a locked nucleic acid inhibitor of miR-155-5p, is currently in a Phase I clinical study for cutaneous T-cell lymphoma, in which miR-155-5p is known to be an oncomiR with a strong mechanistic link to the disease. MRG-106 showed strong pharmacodynamic activity in preclinical models (Seto *et al.*, 2015) and preliminary data from their small Phase I trial of six patients was just released at the end of last year. MRG-106 is delivered via intratumoral injection and, thus far, it is well tolerated and promising therapeutic improvements have been observed in patients (Querfeld *et al.*, 2016).

The most advanced miR-based therapy in clinical development, Miravirsen, targets miR-122 and is being developed for the treatment of hepatitis C virus infection (Janssen *et al.*, 2013). Miravirsen is a locked nucleic acid inhibitor of miR-122 that has been shown to reduce viral RNA with no evidence of resistance. Interestingly, while the intended target is the mature miR-122, it was found to also have affinity for the pri- and

pre-miR-122, resulting in reduced processing to the mature miR form and enhancement of the therapeutic effect (Gebert *et al.*, 2014).

There are still some concerns with miR-based therapeutics, including targeted delivery of these kinds of molecules in humans. Resistance to therapy may also become an issue, however, this may be resolved through utilization of combinatorial therapies. In fact, some anti-miR therapies are being designed to target whole miR families (Obad *et al.*, 2011), thereby avoiding the problem of resistance altogether. Additionally, the potential side effects of miR-based therapeutics in humans are unknown and since miR can be exported in exosomes, systemic side effects may be possible, which may only become apparent in clinical trials. Also, therapies that involve synthetic miRs may overwhelm the endogenous miR processing machinery. As a result, the processing of endogenous miRs may be reduced and the side effects of this in humans are unknown.

While our data did not include functional assays in the presence or absence of miR mimics and inhibitors to assess specific functional effects of individual miRs, our data do provide a wealth of information to serve as a basis for the identification of functionally important miRs. Our miR-mRNA pairing analysis revealed many miRs that appear to be targeting RNAs in HPV16 E6/E7 expressing HFKs and these may be excellent candidates in future functional studies. However, we can compare our data to others studies to identify HPV-associated miRs that may have potential as future drug targets in high risk HPV infections or HPV-associated cancers.

As mentioned previously, we, and two others (McKenna *et al.*, 2010; Melar-New and Laimins, 2010), observe a decrease in miR-203a-3p expression in the presence of

HPV16 E6/E7 expression. One study examined the functional implications of decreasing this miR in cells with expression of HPV16 E6/E7 and found that when this miR was artificially overexpressed, HPV genome amplification and long-term maintenance of HPV episomes are perturbed (Melar-New and Laimins, 2010). We also observe decreased levels of miR-145-5p and these data agree with a study in HPV-31 positive raft cultures. This study showed that forced overexpression of miR-145-5p resulted in reduced episomal viral DNA and reduced genome amplification in differentiated cells (Gunasekharan and Laimins, 2013). These examples showcase miRs with potential to be therapeutic targets for the treatment of high-risk HPV infections or HPV-associated cancers in the future.

REFERENCES

1.   **Alvarez, M.L., Khosroheidari, M., Kanchi Ravi, R., DiStefano, J.K.** (2012). Comparison of protein, microRNA, and mRNA yields using different methods of urinary exosome isolation for the discovery of kidney disease biomarkers. Kidney Int: 82, 1024-1032.

2.   **Andorfer, C.A., Necela, B.M., Thompson, E.A., Perez, E.A.** (2011). MicroRNA signatures: clinical biomarkers for the diagnosis and treatment of breast cancer. Trends Mol Med: 17, 313-319.

3.   **Angeloni, N.L., McMahon, K.M., Swaminathan, S., Plebanek, M.P., Osman, I., Volpert, O.V., Thaxton, C.S.** (2016). Pathways for Modulating Exosome Lipids Identified By High-Density Lipoprotein-Like Nanoparticle Binding to Scavenger Receptor Type B-1. Sci Rep: 6, 22915.

4.   **Bartel, D.P.** (2009). MicroRNAs: target recognition and regulatory functions. Cell: 136, 215-233.

5.   **Barzon, L., Cappellesso, R., Peta, E., Militello, V., Sinigaglia, A., Fassan, M., Simonato, F., Guzzardo, V., Ventura, L., Blandamura, S., Gardiman, M., Palu, G., Fassina, A.** (2014). Profiling of expression of human papillomavirus-related cancer miRNAs in penile squamous cell carcinomas. Am J Pathol: 184, 3376-3383.

6.   **Boelens, M.C., Wu, T.J., Nabet, B.Y., Xu, B., Qiu, Y., Yoon, T., Azzam, D.J., Twyman-Saint Victor, C., Wiemann, B.Z., Ishwaran, H., Ter Brugge, P.J., Jonkers, J., Slingerland, J., Minn, A.J.** (2014). Exosome transfer from stromal to breast cancer cells regulates therapy resistance pathways. Cell: 159, 499-513.

7.   **Boyerinas, B., Park, S.M., Hau, A., Murmann, A.E., Peter, M.E.** (2010). The role of let-7 in cell differentiation and cancer. Endocr Relat Cancer: 17, F19-36.

8.   **Brueckner, B., Stresemann, C., Kuner, R., Mund, C., Musch, T., Meister, M., Sultmann, H., Lyko, F.** (2007). The human let-7a-3 locus contains an epigenetically regulated microRNA gene with oncogenic function. Cancer Res: 67, 1419-1423.

9.   **Chang, T.C., Yu, D., Lee, Y.S., Wentzel, E.A., Arking, D.E., West, K.M., Dang, C.V., Thomas-Tikhonenko, A., Mendell, J.T.** (2008). Widespread microRNA repression by Myc contributes to tumorigenesis. Nat Genet: 40, 43-50.

10.   **Chapman, B.V., Wald, A.I., Akhtar, P., Munko, A.C., Xu, J., Gibson, S.P., Grandis, J.R., Ferris, R.L., Khan, S.A.** (2015). MicroRNA-363 targets myosin 1B to reduce cellular migration in head and neck cancer. BMC Cancer: 15, 861.

11.   **Chaturvedi, A.K., Engels, E.A., Pfeiffer, R.M., Hernandez, B.Y., Xiao, W., Kim, E., Jiang, B., Goodman, M.T., Sibug-Saber, M., Cozen, W., Liu, L., Lynch, C.F., Wentzensen, N., Jordan, R.C., Altekruse, S., Anderson, W.F., Rosenberg, P.S., Gillison, M.L.** (2011). Human papillomavirus and rising oropharyngeal cancer incidence in the United States. J Clin Oncol: 29, 4294-4301.

12.   **Chiantore, M.V., Mangino, G., Iuliano, M., Zangrillo, M.S., De Lillis, I., Vaccari, G., Accardi, R., Tommasino, M., Columba Cabezas, S., Federico, M., Fiorucci, G., Romeo, G.** (2016). Human papillomavirus E6 and E7 oncoproteins affect the expression of cancer-related microRNAs: additional evidence in HPV-induced tumorigenesis. J Cancer Res Clin Oncol: 142, 1751-1763.

13.   **Coimbra, E.C., M, D.A.C.G.L., Junior, M.R., TH, D.E.O., J, D.A.C.S.N., AC, D.E.F.** (2016). Expression Profile of MicroRNA-203 and its DeltaNp63 Target in Cervical Carcinogenesis: Prospects for Cervical Cancer Screening. Anticancer Res: 36, 3939-3946.

14.   **de Melo Maia, B., Lavorato-Rocha, A.M., Rodrigues, L.S., Coutinho-Camillo, C.M., Baiocchi, G., Stiepcich, M.M., Puga, R., de, A.L.L., Soares, F.A., Rocha, R.M.** (2013). microRNA portraits in human vulvar carcinoma. Cancer Prev Res (Phila): 6, 1231-1241.

15.   **Dunaief, J.L., Strober, B.E., Guha, S., Khavari, P.A., Alin, K., Luban, J., Begemann, M., Crabtree, G.R., Goff, S.P.** (1994). The retinoblastoma protein and BRG1 form a complex and cooperate to induce cell cycle arrest. Cell: 79, 119-130.

16.   **Eulalio, A., Mano, M.** (2015). MicroRNA Screening and the Quest for Biologically Relevant Targets. J Biomol Screen: 20, 1003-1017.

17. **Gebert, L.F., Rebhan, M.A., Crivelli, S.E., Denzler, R., Stoffel, M., Hall, J.** (2014). Miravirsen (SPC3649) can inhibit the biogenesis of miR-122. Nucleic Acids Res: 42, 609-621.

18. **Geminard, C., De Gassart, A., Blanc, L., Vidal, M.** (2004). Degradation of AP2 during reticulocyte maturation enhances binding of hsc70 and Alix to a common site on TFR for sorting into exosomes. Traffic: 5, 181-193.

19. **Gillison, M.L., Chaturvedi, A.K., Lowy, D.R.** (2008). HPV prophylactic vaccines and the potential prevention of noncervical cancers in both men and women. Cancer: 113, 3036-3046.

20. **Gunasekharan, V., Laimins, L.A.** (2013). Human papillomaviruses modulate microRNA 145 expression to directly control genome amplification. J Virol: 87, 6037-6043.

21. **Guo, H., Ingolia, N.T., Weissman, J.S., Bartel, D.P.** (2010). Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature: 466, 835-840.

22. **Hausser, J., Zavolan, M.** (2014). Identification and consequences of miRNA-target interactions--beyond repression of gene expression. Nat Rev Genet: 15, 599-612.

23. **Hayes, J., Peruzzi, P.P., Lawler, S.** (2014). MicroRNAs in cancer: biomarkers, functions and therapy. Trends Mol Med: 20, 460-469.

24. **Helwa, I., Cai, J., Drewry, M.D., Zimmerman, A., Dinkins, M.B., Khaled, M.L., Seremwe, M., Dismuke, W.M., Bieberich, E., Stamer, W.D., Hamrick, M.W., Liu, Y.** (2017). A Comparative Study of Serum Exosome Isolation Using Differential Ultracentrifugation and Three Commercial Reagents. PLoS One: 12, e0170628.

25. **Hermeking, H.** (2010). The miR-34 family in cancer and apoptosis. Cell Death Differ: 17, 193-199.

26. **Holowaty, P., Miller, A.B., Rohan, T., To, T.** (1999). Natural history of dysplasia of the uterine cervix. J Natl Cancer Inst: 91, 252-258.

27. **Honegger, A., Leitz, J., Bulkescher, J., Hoppe-Seyler, K., Hoppe-Seyler, F.** (2013). Silencing of human papillomavirus (HPV) E6/E7 oncogene expression affects both the contents and the amounts of extracellular microvesicles released from HPV-positive cancer cells. Int J Cancer: 133, 1631-1642.

28. **Honegger, A., Schilling, D., Bastian, S., Sponagel, J., Kuryshev, V., Sultmann, H., Scheffner, M., Hoppe-Seyler, K., Hoppe-Seyler, F.** (2015). Dependence of intracellular and exosomal microRNAs on viral E6/E7 oncogene expression in HPV-positive tumor cells. PLoS Pathog: 11, e1004712.

29. **Hudson, R.S., Yi, M., Esposito, D., Glynn, S.A., Starks, A.M., Yang, Y., Schetter, A.J., Watkins, S.K., Hurwitz, A.A., Dorsey, T.H., Stephens, R.M., Croce, C.M., Ambs, S.** (2013). MicroRNA-106b-25 cluster expression is associated with early disease recurrence and targets caspase-7 and focal adhesion in human prostate cancer. Oncogene: 32, 4139-4147.

30. **Hui, A.B., Lenarduzzi, M., Krushel, T., Waldron, L., Pintilie, M., Shi, W., Perez-Ordonez, B., Jurisica, I., O'Sullivan, B., Waldron, J., Gullane, P., Cummings, B., Liu, F.F.** (2010). Comprehensive MicroRNA profiling for head and neck squamous cell carcinomas. Clin Cancer Res: 16, 1129-1139.

31. **Jaiswal, R., Luk, F., Gong, J., Mathys, J.M., Grau, G.E., Bebawy, M.** (2012). Microparticle conferred microRNA profiles--implications in the transfer and dominance of cancer traits. Mol Cancer: 11, 37.

32. **Janssen, H.L., Reesink, H.W., Lawitz, E.J., Zeuzem, S., Rodriguez-Torres, M., Patel, K., van der Meer, A.J., Patick, A.K., Chen, A., Zhou, Y., Persson, R., King, B.D., Kauppinen, S., Levin, A.A., Hodges, M.R.** (2013). Treatment of HCV infection by targeting microRNA. N Engl J Med: 368, 1685-1694.

33. **Jansson, M.D., Lund, A.H.** (2012). MicroRNA and cancer. Mol Oncol: 6, 590-610.

34. **Jeppesen, D.K., Hvam, M.L., Primdahl-Bengtson, B., Boysen, A.T., Whitehead, B., Dyrskjot, L., Orntoft, T.F., Howard, K.A., Ostenfeld, M.S.** (2014). Comparative analysis of discrete exosome fractions obtained by differential centrifugation. J Extracell Vesicles: 3, 25011.

35. **Lajer, C.B., Garnaes, E., Friis-Hansen, L., Norrild, B., Therkildsen, M.H., Glud, M., Rossing, M., Lajer, H., Svane, D., Skotte, L., Specht, L., Buchwald, C., Nielsen, F.C.** (2012). The role of miRNAs in human papilloma virus (HPV)-

associated cancers: bridging between HPV-related head and neck cancer and cervical cancer. Br J Cancer: 106, 1526-1534.

36. **Lawrie, C.H., Chi, J., Taylor, S., Tramonti, D., Ballabio, E., Palazzo, S., Saunders, N.J., Pezzella, F., Boultwood, J., Wainscoat, J.S., Hatton, C.S.** (2009). Expression of microRNAs in diffuse large B cell lymphoma is associated with immunophenotype, survival and transformation from follicular lymphoma. J Cell Mol Med: 13, 1248-1260.

37. **Linares, R., Tan, S., Gounou, C., Arraud, N., Brisson, A.R.** (2015). High-speed centrifugation induces aggregation of extracellular vesicles. J Extracell Vesicles: 4, 29509.

38. **Liu, M.X., Siu, M.K., Liu, S.S., Yam, J.W., Ngan, H.Y., Chan, D.W.** (2014). Epigenetic silencing of microRNA-199b-5p is associated with acquired chemoresistance via activation of JAG1-Notch1 signaling in ovarian cancer. Oncotarget: 5, 944-958.

39. **Liu, S., Song, L., Zhang, L., Zeng, S., Gao, F.** (2015). miR-21 modulates resistance of HR-HPV positive cervical cancer cells to radiation through targeting LATS1. Biochem Biophys Res Commun: 459, 679-685.

40. **Lotvall, J., Hill, A.F., Hochberg, F., Buzas, E.I., Di Vizio, D., Gardiner, C., Gho, Y.S., Kurochkin, I.V., Mathivanan, S., Quesenberry, P., Sahoo, S., Tahara, H., Wauben, M.H., Witwer, K.W., Thery, C.** (2014). Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles. J Extracell Vesicles: 3, 26913.

41. **Lu, J., Getz, G., Miska, E.A., Alvarez-Saavedra, E., Lamb, J., Peck, D., Sweet-Cordero, A., Ebert, B.L., Mak, R.H., Ferrando, A.A., Downing, J.R., Jacks, T., Horvitz, H.R., Golub, T.R.** (2005). MicroRNA expression profiles classify human cancers. Nature: 435, 834-838.

42. **Lu, L., Katsaros, D., de la Longrais, I.A., Sochirca, O., Yu, H.** (2007). Hypermethylation of let-7a-3 in epithelial ovarian cancer is associated with low insulin-like growth factor-II expression and favorable prognosis. Cancer Res: 67, 10117-10122.

43. **Ludwig, N., Leidinger, P., Becker, K., Backes, C., Fehlmann, T., Pallasch, C., Rheinheimer, S., Meder, B., Stahler, C., Meese, E., Keller, A.** (2016).

Distribution of miRNA expression across human tissues. Nucleic Acids Res: 44, 3865-3877.

44.   **McKenna, D.J., McDade, S.S., Patel, D., McCance, D.J.** (2010). MicroRNA 203 expression in keratinocytes is dependent on regulation of p53 levels by E6. J Virol: 84, 10644-10652.

45.   **Melar-New, M., Laimins, L.A.** (2010). Human papillomaviruses modulate expression of microRNA 203 upon epithelial differentiation to control levels of p63 proteins. J Virol: 84, 5212-5221.

46.   **Mitchell, P.S., Parkin, R.K., Kroh, E.M., Fritz, B.R., Wyman, S.K., Pogosova-Agadjanyan, E.L., Peterson, A., Noteboom, J., O'Briant, K.C., Allen, A., Lin, D.W., Urban, N., Drescher, C.W., Knudsen, B.S., Stirewalt, D.L., Gentleman, R., Vessella, R.L., Nelson, P.S., Martin, D.B., Tewari, M.** (2008). Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A: 105, 10513-10518.

47.   **Muralidhar, B., Goldstein, L.D., Ng, G., Winder, D.M., Palmer, R.D., Gooding, E.L., Barbosa-Morais, N.L., Mukherjee, G., Thorne, N.P., Roberts, I., Pett, M.R., Coleman, N.** (2007). Global microRNA profiles in cervical squamous cell carcinoma depend on Drosha expression levels. J Pathol: 212, 368-377.

48.   **Muralidhar, B., Winder, D., Murray, M., Palmer, R., Barbosa-Morais, N., Saini, H., Roberts, I., Pett, M., Coleman, N.** (2011). Functional evidence that Drosha overexpression in cervical squamous cell carcinoma affects cell phenotype and microRNA profiles. J Pathol: 224, 496-507.

49.   **Myklebust, M.P., Bruland, O., Fluge, O., Skarstein, A., Balteskard, L., Dahl, O.** (2011). MicroRNA-15b is induced with E2F-controlled genes in HPV-related cancer. Br J Cancer: 105, 1719-1725.

50.   **Nong, K., Wang, W., Niu, X., Hu, B., Ma, C., Bai, Y., Wu, B., Wang, Y., Ai, K.** (2016). Hepatoprotective effect of exosomes from human-induced pluripotent stem cell-derived mesenchymal stromal cells against hepatic ischemia-reperfusion injury in rats. Cytotherapy: 18, 1548-1559.

51.   **Obad, S., dos Santos, C.O., Petri, A., Heidenblad, M., Broom, O., Ruse, C., Fu, C., Lindow, M., Stenvang, J., Straarup, E.M., Hansen, H.F., Koch, T., Pappin, D., Hannon, G.J., Kauppinen, S.** (2011). Silencing of microRNA families by seed-targeting tiny LNAs. Nat Genet: 43, 371-378.

52. **Pardini, B., Rosa, F., Barone, E., Di Gaetano, C., Slyskova, J., Novotny, J., Levy, M., Garritano, S., Vodickova, L., Buchler, T., Gemignani, F., Landi, S., Vodicka, P., Naccarati, A.** (2013). Variation within 3'-UTRs of base excision repair genes and response to therapy in colorectal cancer patients: A potential modulation of microRNAs binding. Clin Cancer Res: 19, 6044-6056.

53. **Pedroza-Torres, A., Lopez-Urrutia, E., Garcia-Castillo, V., Jacobo-Herrera, N., Herrera, L.A., Peralta-Zaragoza, O., Lopez-Camarillo, C., De Leon, D.C., Fernandez-Retana, J., Cerna-Cortes, J.F., Perez-Plasencia, C.** (2014). MicroRNAs in cervical cancer: evidences for a miRNA profile deregulated by HPV and its impact on radio-resistance. Molecules: 19, 6263-6281.

54. **Pencheva, N., Tavazoie, S.F.** (2013). Control of metastatic progression by microRNA regulatory networks. Nat Cell Biol: 15, 546-554.

55. **Pigati, L., Yaddanapudi, S.C., Iyengar, R., Kim, D.J., Hearn, S.A., Danforth, D., Hastings, M.L., Duelli, D.M.** (2010). Selective release of microRNA species from normal and malignant mammary epithelial cells. PLoS One: 5, e13515.

56. **Png, K.J., Halberg, N., Yoshida, M., Tavazoie, S.F.** (2011). A microRNA regulon that mediates endothelial recruitment and metastasis by cancer cells. Nature: 481, 190-194.

57. **Poliseno, L., Salmena, L., Riccardi, L., Fornari, A., Song, M.S., Hobbs, R.M., Sportoletti, P., Varmeh, S., Egia, A., Fedele, G., Rameh, L., Loda, M., Pandolfi, P.P.** (2010). Identification of the miR-106b~25 microRNA cluster as a proto-oncogenic PTEN-targeting intron that cooperates with its host gene MCM7 in transformation. Sci Signal: 3, ra29.

58. **Pyeon, D., Newton, M.A., Lambert, P.F., den Boon, J.A., Sengupta, S., Marsit, C.J., Woodworth, C.D., Connor, J.P., Haugen, T.H., Smith, E.M., Kelsey, K.T., Turek, L.P., Ahlquist, P.** (2007). Fundamental differences in cell cycle deregulation in human papillomavirus-positive and human papillomavirus-negative head/neck and cervical cancers. Cancer Res: 67, 4605-4619.

59. **Querfeld, C., Pacheco, T., Foss, F.M., Halwani, A.S., Porcu, P., Seto, A.G., Ruckman, J., Landry, M.L., Jackson, A.L., Pestano, L.A., Dickinson, B.A., Sanseverino, M., Rodman, D.M., Gordon, G., Marshall, W.** (2016). Preliminary Results of a Phase 1 Trial Evaluating MRG-106, a Synthetic microRNA Antagonist (LNA antimiR) of microRNA-155, in Patients with CTCL. Blood: 128, 1829.

60. **Rabinowits, G., Gercel-Taylor, C., Day, J.M., Taylor, D.D., Kloecker, G.H.** (2009). Exosomal microRNA: a diagnostic marker for lung cancer. Clin Lung Cancer: 10, 42-46.

61. **Rivoltini, L., Chiodoni, C., Squarcina, P., Tortoreto, M., Villa, A., Vergani, B., Burdek, M., Botti, L., Arioli, I., Cova, A., Mauri, G., Vergani, E., Bianchi, B., Della Mina, P., Cantone, L., Bollati, V., Zaffaroni, N., Gianni, A.M., Colombo, M.P., Huber, V.** (2016). TNF-Related Apoptosis-Inducing Ligand (TRAIL)-Armed Exosomes Deliver Proapoptotic Signals to Tumor Site. Clin Cancer Res: 22, 3499-3512.

62. **Rood, I.M., Deegens, J.K., Merchant, M.L., Tamboer, W.P., Wilkey, D.W., Wetzels, J.F., Klein, J.B.** (2010). Comparison of three methods for isolation of urinary microvesicles to identify biomarkers of nephrotic syndrome. Kidney Int: 78, 810-816.

63. **Rosenfeld, N., Aharonov, R., Meiri, E., Rosenwald, S., Spector, Y., Zepeniuk, M., Benjamin, H., Shabes, N., Tabak, S., Levy, A., Lebanony, D., Goren, Y., Silberschein, E., Targan, N., Ben-Ari, A., Gilad, S., Sion-Vardy, N., Tobar, A., Feinmesser, M., Kharenko, O., Nativ, O., Nass, D., Perelman, M., Yosepovich, A., Shalmon, B., Polak-Charcon, S., Fridman, E., Avniel, A., Bentwich, I., Bentwich, Z., Cohen, D., Chajut, A., Barshack, I.** (2008). MicroRNAs accurately identify cancer tissue origin. Nat Biotechnol: 26, 462-469.

64. **Scheffner, M., Munger, K., Byrne, J.C., Howley, P.M.** (1991). The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. Proc Natl Acad Sci U S A: 88, 5523-5527.

65. **Scholl, V., Hassan, R., Zalcberg, I.R.** (2012). miRNA-451: A putative predictor marker of Imatinib therapy response in chronic myeloid leukemia. Leuk Res: 36, 119-121.

66. **Sebio, A., Pare, L., Paez, D., Salazar, J., Gonzalez, A., Sala, N., del Rio, E., Martin-Richard, M., Tobena, M., Barnadas, A., Baiget, M.** (2013). The LCS6 polymorphism in the binding site of let-7 microRNA to the KRAS 3'-untranslated region: its role in the efficacy of anti-EGFR-based therapy in metastatic colorectal cancer patients. Pharmacogenet Genomics: 23, 142-147.

67. **Servin-Gonzalez, L.S., Granados-Lopez, A.J., Lopez, J.A.** (2015). Families of microRNAs Expressed in Clusters Regulate Cell Signaling in Cervical Cancer. Int J Mol Sci: 16, 12773-12790.

68. **Seto, A.G., Beatty, X.T., Pestano, L.A., Dickinson, B.A., Warren, M.S., Rodman, D.M., Jackson, A.L.** (2015). Preclinical Results Supporting Therapeutic Development of Mrg-106, an Oligonucleotide Inhibitor of Mir-155, in CTCL. Blood: 126, 2758.

69. **Song, L., Liu, S., Zeng, S., Zhang, L., Li, X.** (2015). miR-375 Modulates Radiosensitivity of HR-HPV-Positive Cervical Cancer Cells by Targeting UBE3A through the p53 Pathway. Med Sci Monit: 21, 2210-2217.

70. **Suzuki, H.I., Yamagata, K., Sugimoto, K., Iwamoto, T., Kato, S., Miyazono, K.** (2009). Modulation of microRNA processing by p53. Nature: 460, 529-533.

71. **Szatanek, R., Baran, J., Siedlar, M., Baj-Krzyworzeka, M.** (2015). Isolation of extracellular vesicles: Determining the correct approach (Review). Int J Mol Med: 36, 11-17.

72. **Taylor, D.D., Gercel-Taylor, C.** (2008). MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. Gynecol Oncol: 110, 13-21.

73. **Thery, C., Boussac, M., Veron, P., Ricciardi-Castagnoli, P., Raposo, G., Garin, J., Amigorena, S.** (2001). Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles. J Immunol: 166, 7309-7318.

74. **Thery, C., Zitvogel, L., Amigorena, S.** (2002). Exosomes: composition, biogenesis and function. Nat Rev Immunol: 2, 569-579.

75. **Tian, Q., Li, Y., Wang, F., Li, Y., Xu, J., Shen, Y., Ye, F., Wang, X., Cheng, X., Chen, Y., Wan, X., Lu, W., Xie, X.** (2014). MicroRNA detection in cervical exfoliated cells as a triage for human papillomavirus-positive women. J Natl Cancer Inst: 106.

76. **van der Pol, E., Boing, A.N., Harrison, P., Sturk, A., Nieuwland, R.** (2012). Classification, functions, and clinical relevance of extracellular vesicles. Pharmacol Rev: 64, 676-705.

77. **Van Deun, J., Mestdagh, P., Sormunen, R., Cocquyt, V., Vermaelen, K., Vandesompele, J., Bracke, M., De Wever, O., Hendrix, A.** (2014). The impact

of disparate isolation methods for extracellular vesicles on downstream RNA profiling. J Extracell Vesicles: 3.

78. **Wald, A.I., Hoskins, E.E., Wells, S.I., Ferris, R.L., Khan, S.A.** (2011). Alteration of microRNA profiles in squamous cell carcinoma of the head and neck cell lines by human papillomavirus. Head Neck: 33, 504-512.

79. **Wang, X., Wang, H.K., Li, Y., Hafner, M., Banerjee, N.S., Tang, S., Briskin, D., Meyers, C., Chow, L.T., Xie, X., Tuschl, T., Zheng, Z.M.** (2014). microRNAs are biomarkers of oncogenic human papillomavirus infections. Proc Natl Acad Sci U S A: 111, 4262-4267.

80. **Willms, E., Johansson, H.J., Mager, I., Lee, Y., Blomberg, K.E., Sadik, M., Alaarg, A., Smith, C.I., Lehtio, J., El Andaloussi, S., Wood, M.J., Vader, P.** (2016). Cells release subpopulations of exosomes with distinct molecular and biological properties. Sci Rep: 6, 22519.

81. **Winer, R.L., Hughes, J.P., Feng, Q., O'Reilly, S., Kiviat, N.B., Holmes, K.K., Koutsky, L.A.** (2006). Condom use and the risk of genital human papillomavirus infection in young women. The New England journal of medicine: 354, 2645-2654.

82. **Wynendaele, J., Bohnke, A., Leucci, E., Nielsen, S.J., Lambertz, I., Hammer, S., Sbrzesny, N., Kubitza, D., Wolf, A., Gradhand, E., Balschun, K., Braicu, I., Sehouli, J., Darb-Esfahani, S., Denkert, C., Thomssen, C., Hauptmann, S., Lund, A., Marine, J.C., Bartel, F.** (2010). An illegitimate microRNA target site within the 3' UTR of MDM4 affects ovarian cancer progression and chemosensitivity. Cancer Res: 70, 9641-9649.

83. **Xiao, F., Bai, Y., Chen, Z., Li, Y., Luo, L., Huang, J., Yang, J., Liao, H., Guo, L.** (2014). Downregulation of HOXA1 gene affects small cell lung cancer cell survival and chemoresistance under the regulation of miR-100. Eur J Cancer: 50, 1541-1554.

84. **Yang, J., Liu, X.X., Fan, H., Tang, Q., Shou, Z.X., Zuo, D.M., Zou, Z., Xu, M., Chen, Q.Y., Peng, Y., Deng, S.J., Liu, Y.J.** (2015). Extracellular Vesicles Derived from Bone Marrow Mesenchymal Stem Cells Protect against Experimental Colitis via Attenuating Colon Inflammation, Oxidative Stress and Apoptosis. PLoS One: 10, e0140551.

85. **Zhou, J., Cai, J., Huang, Z., Ding, H., Wang, J., Jia, J., Zhao, Y., Huang, D., Wang, Z.** (2013). Proteomic identification of target proteins following Drosha knockdown in cervical cancer. Oncol Rep: 30, 2229-2237.

86. **Ziebarth, J.D., Bhattacharya, A., Cui, Y.** (2012). Integrative analysis of somatic mutations altering microRNA targeting in cancer genomes. PLoS One: 7, e47137.

APPENDIX

Supplementary Material

Table S2.1. miRs modulated in HFKs with expression of individual viral oncoproteins (threshold cut-offs: ≥ 10 reads; FDR ≤ 0.05)

16E7: Up-Regulated miRs	
miR	FC
miR-542-3p[a]	7.86
miR-100-3p	5.55
miR-345-5p	5.37
miR-7974[a]	5.11
miR-181b-3p	5.10
miR-30b-3p	4.74
miR-224-3p[a]	4.43
miR-25-5p	4.22
miR-129-5p	4.06
miR-16-2-3p[b]	4.06
miR-15b-5p[a]	3.96
miR-335-3p[a]	3.95
miR-16-5p	3.60
miR-33b-3p[a]	3.52
miR-873-3p	3.23

16E6: Up-Regulated miRs	
miR	FC
miR-363-3p[b]	44.68
miR-9-5p[b]	15.16
miR-335-3p[a]	13.57
miR-335-5p	10.13
miR-542-3p[a]	6.84
miR-450a-5p[b]	6.46
miR-1271-5p	6.06
miR-450b-5p	5.51
miR-193a-5p	4.91
miR-7974[a]	4.78
miR-548w	4.48
miR-224-3p[a]	4.42
miR-362-5p	4.26
miR-33b-3p[a]	4.26
miR-15b-5p[a]	4.10

16E7: Down-Regulated miRs	
miR	FC
miR-197-3p[b]	-2.01
miR-10a-3p	-1.97
miR-485-3p	-1.96
miR-203a-3p	-1.75
miR-328-3p	-1.73
miR-193b-3p	-1.63
miR-1249[b]	-1.54
miR-3607-3p	-1.54
miR-3065-5p	-1.50
miR-136-3p	-1.49
miR-873-5p	-1.43
miR-3065-3p	-1.30
let-7e-3p	-1.30
miR-132-3p	-1.25
miR-221-3p	-1.10

16E6: Down-Regulated miRs	
miR	FC
miR-34a-5p[b]	-3.58
miR-487b-3p	-2.62
miR-485-3p	-2.18
miR-328-3p	-2.08
miR-642a-5p	-2.03
miR-34c-3p[b]	-1.83
miR-758-3p	-1.75
miR-410-3p	-1.72
miR-143-3p	-1.71
miR-31-3p	-1.69
let-7d-3p	-1.51
miR-193b-3p	-1.46
miR-3065-3p	-1.42
miR-136-3p	-1.41
miR-3614-5p	-1.30

Table S2.1. (Continued)

[a]miRs that are up or down-regulated by both HPV16 E6 and E7 individually and in

HPV16 E6/E7 expressing HFKs

[b]miRs that are up or down-regulated by one oncoprotein and also up or down-regulated

in HPV16 E6/E7 expressing HFKs

Table S2.2. miR clusters modulated by expression of HPV16 E6/E7

Clustered miRs[a]	Chromosome Location	FC (E6E7/C)
miR-374c-5p	chrX: 74218549-74218618 [+]	-1.0
miR-374b-3p	chrX: 74218547-74218618 [-]	3.5
miR-374b-5p	chrX: 74218547-74218618 [-]	1.6
miR-421	chrX: 74218377-74218461 [-]	2.1
miR-532-5p	chrX: 50003148-50003238 [+]	-1.2
miR-532-3p	chrX: 50003148-50003238 [+]	-1.0
miR-188-5p	chrX: 50003503-50003588 [+]	-1.4
miR-500a-5p	chrX: 50008431-50008514 [+]	1.7
miR-362-5p	chrX: 50008964-50009028 [+]	3.7
miR-362-3p	chrX: 50008964-50009028 [+]	-1.0
miR-501-5p	chrX: 50009722-50009805 [+]	1.2
miR-500b	chrX: 50010672-50010750 [+]	-1.0
miR-660-5p	chrX: 50013241-50013337 [+]	-1.4
miR-660-3p	chrX: 50013241-50013337 [+]	-1.6
miR-502-5p	chrX: 50014598-50014683 [+]	-1.0
miR-502-3p	chrX: 50014598-50014683 [+]	-1.2
miR-106a-5p	chrX: 134170198-134170278 [-]	5.9
miR-18b-5p	chrX: 134170041-134170111 [-]	2.3
miR-20b-5p	chrX: 134169809-134169877 [-]	10.0
miR-20b-3p	chrX: 134169809-134169877 [-]	1.3
miR-19b-2-5p	chrX: 134169671-134169766 [-]	-1.0
miR-92a-2-5p	chrX: 134169538-134169612 [-]	1.3
miR-363-3p	chrX: 134169378-134169452 [-]	96.1
miR-424-5p	chrX: 134546614-134546711 [-]	1.0
miR-503	chrX: 134546328-134546398 [-]	1.3
miR-542-5p	chrX: 134541341-134541437 [-]	2.1
miR-542-3p	chrX: 134541341-134541437 [-]	7.1
miR-450a-2-5p	chrX: 134540508-134540607 [-]	7.5
miR-450a-1-5p	chrX: 134540341-134540431 [-]	7.5
miR-450b-5p	chrX: 134540185-134540262 [-]	2.7
hsa-let-7a-5p	chr9: 94175957-94176036 [+]	1.0
hsa-let-7a-3p	chr9: 94175957-94176036 [+]	1.6
hsa-let-7f-1	chr9: 94176347-94176433 [+]	-1.0
hsa-let-7d-5p	chr9: 94178834-94178920 [+]	1.2
hsa-let-7d-3p	chr9: 94178834-94178920 [+]	-3.5

Table S2.2 (Continued)

miR-3154	chr9: 128244947-128245030 [-]	-1.0
miR-199b-5p	chr9: 128244721-128244830 [-]	5.1
miR-199b-3p	chr9: 128244721-128244830 [-]	1.6
miR-30d-5p	chr8: 134804876-134804945 [-]	1.2
miR-30d-3p	chr8: 134804876-134804945 [-]	1.6
miR-30b-3p	chr8: 134800520-134800607 [-]	5.3
miR-550a-5p	chr7: 30289794-30289890 [+]	4.8
miR-550a-3p	chr7: 30289794-30289890 [+]	2.0
miR-550b-1	chr7: 30289794-30289890 [-]	-1.0
miR-106b-5p	chr7: 100093993-100094074 [-]	2.4
miR-106b-3p	chr7: 100093993-100094074 [-]	1.4
miR-93-5p	chr7: 100093768-100093847 [-]	1.9
miR-93-3p	chr7: 100093768-100093847 [-]	1.6
miR-25-5p	chr7: 100093560-100093643 [-]	5.1
miR-25-3p	chr7: 100093560-100093643 [-]	2.7
miR-29b-1-5p	chr7: 130877459-130877539 [-]	5.4
miR-29b-1-3p	chr7: 130877459-130877539 [-]	1.6
miR-29a-5p	chr7: 130876747-130876810 [-]	1.6
miR-29a-3p	chr7: 130876747-130876810 [-]	1.6
miR-15b-5p	chr3: 160404588-160404685 [+]	4.2
miR-15b-3p	chr3: 160404588-160404685 [+]	6.4
miR-16-2-3p	chr3: 160404745-160404825 [+]	6.0
miR-191-5p	chr3: 49020618-49020709 [-]	1.8
miR-191-3p	chr3: 49020618-49020709 [-]	4.8
miR-425-5p	chr3: 49020148-49020234 [-]	1.5
miR-425-3p	chr3: 49020148-49020234 [-]	1.4
miR-642a-5p	chr19: 45674928-45675024 [+]	-3.1
miR-642b-5p	chr19: 45674932-45675008 [-]	-1.0
miR-642b-3p	chr19: 45674932-45675008 [-]	-1.0
miR-212-5p	chr17: 2050271-2050380 [-]	8.2

Table S2.2 (Continued)

miR-212-3p	chr17: 2050271-2050380 [-]	2.9
miR-132-5p	chr17: 2049908-2050008 [-]	3.0
miR-33b-5p	chr17: 17813836-17813931 [-]	3.2
miR-33b-3p	chr17: 17813836-17813931 [-]	6.1
miR-6777	chr17: 17813480-17813545 [-]	not detected
miR-497-5p	chr17: 7017911-7018022 [-]	-3.5
miR-195-5p	chr17: 7017615-7017701 [-]	5.6
miR-195-3p	chr17: 7017615-7017701 [-]	1.6
miR-193b-5p	chr16: 14303967-14304049 [+]	-1.4
miR-193b-3p	chr16: 14303967-14304049 [+]	-3.8
miR-365a-5p	chr16: 14309285-14309371 [+]	-1.8
miR-365a-3p	chr16: 14309285-14309371 [+]	1.2
miR-5587-5p	chr16: 535316-535368 [+]	-1.0
miR-5587-3p	chr16: 535316-535368 [+]	-1.0
miR-3176	chr16: 543277-543366 [+]	3.1
miR-381	chr14: 101045920-101045994 [+]	-1.1
miR-487b	chr14: 101046455-101046538 [+]	-2.3
miR-539-5p	chr14: 101047321-101047398 [+]	-1.6
miR-889	chr14: 101047901-101047979 [+]	1.1
miR-544a	chr14: 101048658-101048748 [+]	-1.0
miR-655	chr14: 101049550-101049646 [+]	-1.0
miR-487a	chr14: 101052446-101052525 [+]	1.2
miR-382-5p	chr14: 101054306-101054381 [+]	-1.0
miR-382-3p	chr14: 101054306-101054381 [+]	-1.0
miR-134	chr14: 101054687-101054759 [+]	-1.4
miR-668	chr14: 101055258-101055323 [+]	-1.6
miR-485-5p	chr14: 101055419-101055491 [+]	-3.3
miR-485-3p	chr14: 101055419-101055491 [+]	-7.8
miR-323b-5p	chr14: 101056219-101056300 [+]	-1.0
miR-323b-3p	chr14: 101056219-101056300 [+]	-1.0
miR-154-5p	chr14: 101059755-101059838 [+]	-1.1
miR-154-3p	chr14: 101059755-101059838 [+]	-1.0
miR-496	chr14: 101060573-101060674 [+]	-1.0
miR-377-3p	chr14: 101062050-101062118 [+]	-1.1

Table S2.2 (Continued)

miR-541-5p	chr14: 101064495-101064578 [+]	-1.0
miR-541-3p	chr14: 101064495-101064578 [+]	-1.0
miR-409-5p	chr14: 101065300-101065378 [+]	-1.5
miR-203a	chr14: 104117405-104117514 [+]	-8.9
miR-203b-5p	chr14: 104117418-104117503 [-]	-1.0
miR-203b-3p	chr14: 104117418-104117503 [-]	2.4
miR-379-5p	chr14: 101022066-101022132 [+]	-1.0
miR-411-5p	chr14: 101023325-101023420 [+]	1.1
miR-299-5p	chr14: 101023794-101023856 [+]	-1.0
miR-380-5p	chr14: 101025017-101025077 [+]	-1.0
miR-380-3p	chr14: 101025017-101025077 [+]	-1.0
miR-1197	chr14: 101025564-101025651 [+]	-1.0
miR-323a-5p	chr14: 101025732-101025817 [+]	-1.0
miR-323a-3p	chr14: 101025732-101025817 [+]	-3.4
miR-758	chr14: 101026020-101026107 [+]	-1.1
miR-329-1	chr14: 101026785-101026864 [+]	1.3
miR-329-2	chr14: 101027100-101027183 [+]	1.3
miR-494	chr14: 101029634-101029714 [+]	-1.9
miR-1193	chr14: 101030052-101030129 [+]	-1.0
miR-543	chr14: 101031987-101032064 [+]	-1.1
miR-495	chr14: 101033755-101033836 [+]	-1.9
miR-181a-3p	chr1: 198859044-198859153 [-]	1.4
miR-181b-5p	chr1: 198858873-198858982 [-]	1.4
miR-181b-3p	chr1: 198858873-198858982 [-]	3.8
miR-30e-5p	chr1: 40754355-40754446 [+]	1.8
miR-30e-3p	chr1: 40754355-40754446 [+]	1.5
miR-30c-1-3p	chr1: 40757284-40757372 [+]	3.3
miR-100-5p	chr11: 122152229-122152308 [-]	1.8
miR-100-3p	chr11: 122152229-122152308 [-]	4.8
hsa-let-7a-2-3p	chr11: 122146522-122146593 [-]	1.5
miR-34b-5p	chr11: 111512938-111513021 [+]	-1.1
miR-34b-3p	chr11: 111512938-111513021 [+]	-3.2
miR-34c-5p	chr11: 111513439-111513515 [+]	-1.4

Table S2.2 (Continued)

miR-34c-3p	chr11: 111513439-111513515 [+]	-4.2
miR-331-5p	chr12: 95308420-95308513 [+]	3.0
miR-3685	chr12: 95309923-95309984 [+]	-1.0

[a]miRs in bold were found to be both differentially expressed by HPV16 E6/E7 and part of a miR cluster, unbolded miRs are also part of the miR clusters but did not meet threshold cutoffs

Table S2.3. Numbers and types of RNAs detected by RNAseq

(threshold cut-offs: ≥ 10 reads; FDR ≤ 0.05; -2 ≥ FC ≥ 2)

RNA type	number
protein coding RNA	3,471
pseudogene RNA	2,703
long, intervening non-coding RNA (lincRNA)	1,452
antisense RNA	1,058
long, non-coding transcripts in introns of coding genes w/no exon overlap	193
small nuclear RNA (snRNA)	110
RNA w/out ORF (processed_transcript)	87
small nucleolar RNA (snoRNA)	70
long, non-coding transcripts w/coding genes in introns on the same strand	35
immunoglobulin (Ig) variable chain & T-cell receptor (TcR) RNA	34
inactivated immunoglobulin RNA	28
polymorphic pseudogene RNA	16

Table S2.5. RNAs identified via the miR-mRNA pairing analysis to be potentially targeted by two or more miRs

number of targeting miRs	number or name of RNAs potentially targeted
2	349
3	182
4	90
5	46
6	22
7	ABCG4, ANKRD52, ATXN1, CCDC71L, CLCN5 ERBB3, FYCO1, KMT2C, MTF1, NFAT5 SH3PXD2A, SMURF1, SOX4, TANC2, ZNF365
8	CFLAR, CREBRF, SHANK2
9	CUX1, RORA
10	TRPS1
12	ABL2

Table S2.6. Molecular and cellular functions identified via IPA core analysis associated with the RNAseq and potential miR target RNA data sets

ALL RNAs	
Molecular & Cellular Functions	p-value range[a]
Cell Cycle	$5.46 \times 10^{-3} - 3.13 \times 10^{-16}$
Cellular Assembly & Organization	$4.59 \times 10^{-3} - 6.72 \times 10^{-14}$
DNA Replication, Recombination & Repair	$4.59 \times 10^{-3} - 6.72 \times 10^{-14}$
Cell Morphology	$5.48 \times 10^{-3} - 3.67 \times 10^{-7}$
Cellular Movement	$5.42 \times 10^{-3} - 8.62 \times 10^{-6}$
POTENTIAL miR TARGET RNAs	
Molecular & Cellular Functions	p-value range[a]
Cellular Development[b]	$1.60 \times 10^{-4} - 3.15 \times 10^{-16}$
Cellular Movement	$1.53 \times 10^{-4} - 7.49 \times 10^{-13}$
Cellular Growth & Proliferation[b]	$1.48 \times 10^{-4} - 1.20 \times 10^{-11}$
Molecular Transport[b]	$1.61 \times 10^{-4} - 3.67 \times 10^{-11}$
Cell Morphology	$6.78 \times 10^{-5} - 4.28 \times 10^{-10}$

[a]The p-value for a given molecular or cellular function is calculated by comparing the number of genes in the data set that participate in that process to the total number of genes that are known to be associated with a given molecular or cellular function in the reference set. The p-value range refers to the range of p-values assigned to more specific molecular and cellular functions that fall within the larger molecular and cellular function categories shown.

[b]Bolded molecular and cellular functions are unique to the data set containing RNAs that are potential targets of miRs.

Table S2.7. Predicted upstream regulators identified via IPA core analysis based on gene expression changes observed in the RNAseq and potential miR target RNA data sets

ALL RNAs		
Upstream Regulator	P-value of Overlap[a]	Predicted Activation
RABL6	1.02^{-20}	Activated
CCND1	1.59^{-20}	Activated
CDKN1A	1.75^{-19}	Inhibited
EHF	1.85^{-19}	Inhibited
dextran sulfate	2.20^{-17}	
POTENTIAL miR TARGET RNAs		
Upstream Regulator	P-value of Overlap[a]	Predicted Activation
TNF	3.35^{-17}	
TGFB1	3.92^{-17}	Inhibited
beta-estradiol	1.68^{-16}	
gefitinib	6.27^{-13}	
CREB1	1.27^{-12}	

[a]The overlap p-value is calculated using Fisher's Exact Test and determines whether there is a statistically significant overlap between genes in the dataset and genes that are regulated by a transcription factor. P-values < 0.01 are considered significant and can be utilized to identify upstream regulators that may explain the observed gene expression changes.

Table S2.8. Comparison of trends in miR expression in HFKs expressing HPV16

E6/E7 with previously described HPV-associated miRs

miR	Observed	Literature	References	Notes[a]
let-7a-5p	up	down	(1, 2)	CxCa
miR-10b-5p	up	up	(3)	CxCa, HNSCC
miR-100-5p	up	down	(4)	CxCa
miR-106b-5p	up	up	(5, 6)	OPC, VC
miR-124-3p	down	down	(7)	
miR-1246	down	down	(8)	CxCa
miR-1254	up	down	(6)	VC
miR-125a-5p	up	down	(9)	CxCa
miR-125b-5p	up	down	(10)	VC
miR-1291	down	down	(6)	VC
miR-139-5p	down	down	(3)	CxCa, HNSCC
miR-139-3p	down	down	(3)	CxCa, HNSCC
miR-143-3p	down	down	(11)	CxCa
miR-145-5p	down	down	(3, 12)	CxCa, HNSCC
miR-15a-5p	up	up	(3)	CxCa, HNSCC
miR-15b-5p	up	up	(13)	AC
miR-155-5p	up	up	(14)	OPC
miR-16-5p	up	up	(3, 15)	HNSCC, CxCa
miR-18a-5p	up	down	(14)	OPC
miR-186-5p	up	up	(6)	VC
miR-193a-5p	up	down	(6)	VC
miR-196a-5p	up	up	(16)	CxCa
miR-199a-5p	down	down	(3, 5)	CxCa, HNSCC, OPC
miR-199a-3p	up	down	(3)	CxCa, HNSCC
miR-199b-5p	down	down	(3)	CxCa, HNSCC
miR-20b-5p	up	up	(3, 17)	OPC, CxCa, HNSCC
miR-203a-3p	down	down	(18, 19)	
miR-205-5p	down	down	(20)	
miR-21-5p	up	up	(1, 2, 16, 21, 22) (6, 23)	CxCa, VC
miR-218-5p	up	down	(24-27)	CIN, OPC, PSCC
miR-22-3p	down	down	(6)	VC
miR-221-5p	up	up	(16, 28)	CxCa
miR-222-3p	up	up	(28)	
miR-223-3p	down	down	(14)	OPC
miR-23b-3p	down	down	(29)	
miR-24-3p	up	down	(20)	
miR-25-3p	up	up	(5)	OPC

Table S2.8 (Continued)

miR-26b-5p	up	down	(6)	VC
miR-27a-3p	up	up	(16)	CxCa
miR-29a-3p	up	down	(30)	CxCa
miR-29c-3p	up	up	(6)	VC
miR-31-5p	down	down	(14)	OPC
miR-3144-5p	down	down	(31)	
miR-328	down	down	(3)	CxCa, HNSCC
miR-34a-5p	down	down	(32-35)	CxCa
miR-375	down	down	(36, 37)	
miR-379-5p	down	down	(3)	CxCa, HNSCC
miR-381	down	down	(3)	CxCa, HNSCC
miR-574-3p	down	down	(3)	CxCa, HNSCC
miR-875-5p	down	down	(31)	
miR-9-5p	up	up	(5, 14, 17, 38)	CxCa, OPC
miR-9-3p	down	up	(17)	OPC
miR-93-5p	up	up	(5)	OPC

[a]notes indicate when one or more literature references includes samples analyzed from

HPV-associated cancer biopsies; AC= anal cancer; CxCa= cervical cancer; HNSCC=

head and neck squamous cell carcinoma; OPC= oropharyngeal cancer; PSCC= penile

squamous cell carcinoma; VC= vulvar cancer

REFERENCES for Table S2.8

1. **Shishodia G, Shukla S, Srivastava Y, Masaldan S, Mehta S, Bhambhani S, Sharma S, Mehrotra R, Das BC, Bharti AC.** 2015. Alterations in microRNAs miR-21 and let-7a correlate with aberrant STAT3 signaling and downstream effects during cervical carcinogenesis. Mol Cancer **14:**116.

2. **Shishodia G, Verma G, Srivastava Y, Mehrotra R, Das BC, Bharti AC.** 2014. Deregulation of microRNAs Let-7a and miR-21 mediate aberrant STAT3 signaling during human papillomavirus-induced cervical carcinogenesis: role of E6 oncoprotein. BMC Cancer **14:**996.

3. **Lajer CB, Garnaes E, Friis-Hansen L, Norrild B, Therkildsen MH, Glud M, Rossing M, Lajer H, Svane D, Skotte L, Specht L, Buchwald C, Nielsen FC.** 2012. The role of miRNAs in human papilloma virus (HPV)-associated cancers: bridging between HPV-related head and neck cancer and cervical cancer. Br J Cancer **106:**1526-1534.

4. **Li BH, Zhou JS, Ye F, Cheng XD, Zhou CY, Lu WG, Xie X.** 2011. Reduced miR-100 expression in cervical cancer and precursors and its carcinogenic effect through targeting PLK1 protein. Eur J Cancer **47:**2166-2174.

5. **Miller DL, Davis JW, Taylor KH, Johnson J, Shi Z, Williams R, Atasoy U, Lewis JS, Jr., Stack MS.** 2015. Identification of a human papillomavirus-associated oncogenic miRNA panel in human oropharyngeal squamous cell carcinoma validated by bioinformatics analysis of the Cancer Genome Atlas. Am J Pathol **185:**679-692.

6. **de Melo Maia B, Lavorato-Rocha AM, Rodrigues LS, Coutinho-Camillo CM, Baiocchi G, Stiepcich MM, Puga R, de ALL, Soares FA, Rocha RM.** 2013. microRNA portraits in human vulvar carcinoma. Cancer Prev Res (Phila) **6:**1231-1241.

7. **Wilting SM, van Boerdonk RA, Henken FE, Meijer CJ, Diosdado B, Meijer GA, le Sage C, Agami R, Snijders PJ, Steenbergen RD.** 2010. Methylation-mediated silencing and tumour suppressive function of hsa-miR-124 in cervical cancer. Mol Cancer **9:**167.

8. **Yang Y, Xie YJ, Xu Q, Chen JX, Shan NC, Zhang Y.** 2015. Down-regulation of miR-1246 in cervical cancer tissues and its clinical significance. Gynecol Oncol **138:**683-688.

9.	**Fan Z, Cui H, Xu X, Lin Z, Zhang X, Kang L, Han B, Meng J, Yan Z, Yan X, Jiao S.** 2015. MiR-125a suppresses tumor growth, invasion and metastasis in cervical cancer by targeting STAT3. Oncotarget **6:**25266-25280.

10.	**Nuovo GJ, Wu X, Volinia S, Yan F, di Leva G, Chin N, Nicol AF, Jiang J, Otterson G, Schmittgen TD, Croce C.** 2010. Strong inverse correlation between microRNA-125b and human papillomavirus DNA in productive infection. Diagn Mol Pathol **19:**135-143.

11.	**Chen Y, Ma C, Zhang W, Chen Z, Ma L.** 2014. Down regulation of miR-143 is related with tumor size, lymph node metastasis and HPV16 infection in cervical squamous cancer. Diagn Pathol **9:**88.

12.	**Gunasekharan V, Laimins LA.** 2013. Human papillomaviruses modulate microRNA 145 expression to directly control genome amplification. J Virol **87:**6037-6043.

13.	**Myklebust MP, Bruland O, Fluge O, Skarstein A, Balteskard L, Dahl O.** 2011. MicroRNA-15b is induced with E2F-controlled genes in HPV-related cancer. Br J Cancer **105:**1719-1725.

14.	**Gao G, Gay HA, Chernock RD, Zhang TR, Luo J, Thorstad WL, Lewis JS, Jr., Wang X.** 2013. A microRNA expression signature for the prognosis of oropharyngeal squamous cell carcinoma. Cancer **119:**72-80.

15.	**Zheng ZM, Wang X.** 2011. Regulation of cellular miRNA expression by human papillomaviruses. Biochim Biophys Acta **1809:**668-677.

16.	**Gocze K, Gombos K, Juhasz K, Kovacs K, Kajtar B, Benczik M, Gocze P, Patczai B, Arany I, Ember I.** 2013. Unique microRNA expression profiles in cervical cancer. Anticancer Res **33:**2561-2567.

17.	**Hui AB, Lin A, Xu W, Waldron L, Perez-Ordonez B, Weinreb I, Shi W, Bruce J, Huang SH, O'Sullivan B, Waldron J, Gullane P, Irish JC, Chan K, Liu FF.** 2013. Potentially prognostic miRNAs in HPV-associated oropharyngeal carcinoma. Clin Cancer Res **19:**2154-2162.

18.	**Melar-New M, Laimins LA.** 2010. Human papillomaviruses modulate expression of microRNA 203 upon epithelial differentiation to control levels of p63 proteins. J Virol **84:**5212-5221.

19. **McKenna DJ, McDade SS, Patel D, McCance DJ.** 2010. MicroRNA 203 expression in keratinocytes is dependent on regulation of p53 levels by E6. J Virol **84:**10644-10652.

20. **McKenna DJ, Patel D, McCance DJ.** 2014. miR-24 and miR-205 expression is dependent on HPV onco-protein expression in keratinocytes. Virology **448:**210-216.

21. **Liu S, Song L, Zhang L, Zeng S, Gao F.** 2015. miR-21 modulates resistance of HR-HPV positive cervical cancer cells to radiation through targeting LATS1. Biochem Biophys Res Commun **459:**679-685.

22. **Yao T, Lin Z.** 2012. MiR-21 is involved in cervical squamous cell tumorigenesis and regulates CCL20. Biochim Biophys Acta **1822:**248-260.

23. **Bumrungthai S, Ekalaksananan T, Evans MF, Chopjitt P, Tangsiriwatthana T, Patarapadungkit N, Kleebkaow P, Luanratanakorn S, Kongyingyoes B, Worawichawong S, Pientong C.** 2015. Up-Regulation of miR-21 Is Associated with Cervicitis and Human Papillomavirus Infection in Cervical Tissues. PLoS One **10:**e0127109.

24. **Wu DW, Chuang CY, Lin WL, Sung WW, Cheng YW, Lee H.** 2014. Paxillin promotes tumor progression and predicts survival and relapse in oral cavity squamous cell carcinoma by microRNA-218 targeting. Carcinogenesis **35:**1823-1829.

25. **Li Y, Liu J, Yuan C, Cui B, Zou X, Qiao Y.** 2010. High-risk human papillomavirus reduces the expression of microRNA-218 in women with cervical intraepithelial neoplasia. J Int Med Res **38:**1730-1736.

26. **Martinez I, Gardiner AS, Board KF, Monzon FA, Edwards RP, Khan SA.** 2008. Human papillomavirus type 16 reduces the expression of microRNA-218 in cervical carcinoma cells. Oncogene **27:**2575-2582.

27. **Barzon L, Cappellesso R, Peta E, Militello V, Sinigaglia A, Fassan M, Simonato F, Guzzardo V, Ventura L, Blandamura S, Gardiman M, Palu G, Fassina A.** 2014. Profiling of expression of human papillomavirus-related cancer miRNAs in penile squamous cell carcinomas. Am J Pathol **184:**3376-3383.

28. **Yang CJ, Shen WG, Liu CJ, Chen YW, Lu HH, Tsai MM, Lin SC.** 2011. miR-221 and miR-222 expression increased the growth and tumorigenesis of oral carcinoma cells. J Oral Pathol Med **40:**560-566.

29. **Au Yeung CL, Tsang TY, Yau PL, Kwok TT.** 2011. Human papillomavirus type 16 E6 induces cervical cancer cell migration through the p53/microRNA-23b/urokinase-type plasminogen activator pathway. Oncogene **30:**2401-2410.

30. **Li Y, Wang F, Xu J, Ye F, Shen Y, Zhou J, Lu W, Wan X, Ma D, Xie X.** 2011. Progressive miRNA expression profiles in cervical carcinogenesis and identification of HPV-related target genes for miR-29. J Pathol **224:**484-495.

31. **Lin L, Cai Q, Zhang X, Zhang H, Zhong Y, Xu C, Li Y.** 2015. Two less common human microRNAs miR-875 and miR-3144 target a conserved site of E6 oncogene in most high-risk human papillomavirus subtypes. Protein Cell **6:**575-588.

32. **Geng D, Song X, Ning F, Song Q, Yin H.** 2015. MiR-34a Inhibits Viability and Invasion of Human Papillomavirus-Positive Cervical Cancer Cells by Targeting E2F3 and Regulating Survivin. Int J Gynecol Cancer **25:**707-713.

33. **Wang X, Wang HK, McCoy JP, Banerjee NS, Rader JS, Broker TR, Meyers C, Chow LT, Zheng ZM.** 2009. Oncogenic HPV infection interrupts the expression of tumor-suppressive miR-34a through viral oncoprotein E6. RNA **15:**637-647.

34. **Wang X, Meyers C, Guo M, Zheng ZM.** 2011. Upregulation of p18Ink4c expression by oncogenic HPV E6 via p53-miR-34a pathway. Int J Cancer **129:**1362-1372.

35. **Li B, Hu Y, Ye F, Li Y, Lv W, Xie X.** 2010. Reduced miR-34a expression in normal cervical tissues and cervical lesions with high-risk human papillomavirus infection. Int J Gynecol Cancer **20:**597-604.

36. **Song L, Liu S, Zeng S, Zhang L, Li X.** 2015. miR-375 Modulates Radiosensitivity of HR-HPV-Positive Cervical Cancer Cells by Targeting UBE3A through the p53 Pathway. Med Sci Monit **21:**2210-2217.

37. **Bierkens M, Krijgsman O, Wilting SM, Bosch L, Jaspers A, Meijer GA, Meijer CJ, Snijders PJ, Ylstra B, Steenbergen RD.** 2013. Focal aberrations

indicate EYA2 and hsa-miR-375 as oncogene and tumor suppressor in cervical carcinogenesis. Genes Chromosomes Cancer **52:**56-68.

38.   **Liu W, Gao G, Hu X, Wang Y, Schwarz JK, Chen JJ, Grigsby PW, Wang X.** 2014. Activation of miR-9 by human papillomavirus in cervical cancer. Oncotarget **5:**11620-11630.

Table S2.9. Comparison of trends in miR expression in HFKs expressing HPV16 E6/E7 with study by Wang *et al.* 2014. PNAS, 111:4262-4267.

miR	HPV16 E6E7/C (Observed)	HFK18/HFK (Literature)	Regulation[a]	Notes[b]
miR-16-5p	3.0	3.2	up	CaCx
miR-25-3p	2.7	1.6	up	CaCx
miR-92a-1-5p	1.5	1.9	up	CaCx
miR-93-5p	1.9	2.3	up	
miR-106b-5p	2.4	1.9	up	
miR-210	-2.7	1.4		
miR-224-5p	-1.4	4.1		
miR-378a-5p	2.7	1.9	up	CaCx
miR-22-3p	-1.1	−2.1	down	CaCx
miR-24-3p	1.4	−1.5		
miR-27a-3p	1.0	−2.0		CaCx
miR-29a-3p	1.4	−2.5		CaCx
miR-100-5p	1.8	−2.8		CaCx

[a]shading indicates the trend in miR expression was not the same between our study and the compared study

[b]notes indicate when a miR was also detected in HPV-associated cervical cancer (CaCx) biopsies in the study by Wang *et al.* 2014. PNAS, 111:4262-4267

Table S2.10. Comparison of trends in miR expression in HFKs expressing HPV16 E6/E7 with study by Gunasekharan *et al.* 2013. J Virol, 87:6037-6043.

miR	HPV16 E6E7/C (Observed)	viv-31gen/viv (Literature)	Regulation[a]
miR-1246	-1	31.7	
miR-335-3p	14.5	10.6	up
miR-1260b	-1.4	8.7	
miR-3613-5p	-1.4	5.9	
miR-1260a	-1	4.6	
miR-1285-3p	-1.9	4.1	
miR-576-5p	2.8	4.0	up
miR-615-5p	3.7	3.9	up
miR-92b-3p	2.4	3.7	up
miR-25-5p	5.1	3.3	up
miR-582-3p	2.1	-9.2	
miR-199a-5p	-1	-8.7	down
miR-214-3p	-1	-8.4	down
miR-143-3p	-2.3	-7.3	down
miR-145-5p	-1	-7.1	down
miR-145-3p	-1	-6.9	down
miR-369-3p	-1.3	-6.5	down
miR-655	-1	-6.4	down
miR-493-3p	-1.1	-6.2	down
miR-493-5p	-1.1	-5.3	down

[a]shading indicates the trend in miR expression was not the same between our study and the compared study