ROLE OF MICRORNA-363 IN HUMAN PAPILLOMAVIRUS-ASSOCIATED SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK

by

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Bachelor of Science, University of Wisconsin – Madison, 2006

Submitted to the Graduate Faculty of

the School of Medicine in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2012

UNIVERSITY OF PITTSBURGH

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Squamous cell carcinoma of the head and neck (SCCHN) ranks sixth among cancers worldwide. Despite recent advances in the detection and therapy of SCCHN, the five-year survival rate remains low. Human papillomavirus (HPV) has been detected in a new class of SCCHN that has emerged in younger patients without the common risk factors of alcohol and/or tobacco. HPV-positive and HPV-negative SCCHN have different clinical characteristics, leading some to classify them as distinct diseases. High-risk HPVs have been implicated in a number of cancers including cervical, anogenital, and head and neck cancers. Micro (mi) RNAs are a recently discovered class of endogenously encoded small RNAs that most commonly function as negative regulators of gene expression. MiRNA expression profiles are often altered in cellular stress conditions, including cancers and viral infections. We hypothesized that HPV disrupts cellular miRNA expression in HPV-positive SCCHN and contributes to the development of this cancer.

MiRNA microarray analysis of HPV-positive and HPV-negative SCCHN cell lines revealed the dysregulation of several miRNAs, including the upregulation of miR-363 and downregulation of miR-181a, miR-218, and miR-29a. Exogenous expression of the HPV-16 E6 oncogene in normal primary human keratinocytes showed similar changes in the above miRNAs, implicating HPV E6 in this up/downregulation. Tissues from SCCHN patients in the western Pennsylvania area showed an HPV-positivity rate of 59 percent. HPV-positive SCCHN cases were seen only in the tonsil and base of tongue, and patients with HPV-positive SCCHN were

diagnosed on average nine years younger than those with HPV-negative SCCHN. Additionally, miR-363 was upregulated in HPV-16-positive SCCHN tissues compared to the HPV-negative SCCHN tissues.

We utilized gene expression data and spectral counting proteomics to narrow the possible cellular targets of miR-363 in SCCHN. As confirmed via luciferase assays, miR-363 targets MYO1B, a myosin protein involved in cell motility. Functional assays showed that overexpression of miR-363 in HPV-negative SCCHN cells or siRNA knockdown of MYO1B in HPV-positive SCCHN cells independently reduced cell migration. Furthermore, the addition of miR-363 to HPV-negative SCCHN cells also caused an increase in cell proliferation and colony formation.

Based on our studies, we envision that high-level expression of the E6 oncogene in HPV-positive SCCHN results in an increase in miR-363 levels. Increased expression of miR-363 may then reduce MYO1B levels, thereby reducing the migratory ability of these cells. Furthermore, high levels of miR-363 may also promote cellular proliferation and colony formation.

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PREFACE

The work presented in this dissertation could not have been accomplished without the help of many people. I would like to thank my mentor, Dr. Saleem Khan, for his support and guidance during my time in his lab. Saleem has encouraged me in many opportunities that helped me learn and develop as an independent scientist. I would also like to thank the past and present members of the Khan lab for all of their advice and support. I thank my committee members, Dr. Ferris, Dr. Okada, Dr. Robbins, and Dr. Sarkar for their time, insight, and expert advice. I would like to thank my friends and family, especially my parents, my sister, my grandmother, and my husband, for their unconditional support throughout my entire education.

COMMONLY USED ABBREVIATIONS

bp	base pair
cDNA	complementary DNA
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
FACS	Fluorescence-Activated Cell Sorting
FISH	Fluorescence *in situ* Hybridization
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
HFK	Human Foreskin Keratinocyte
HPV	Human Papillomavirus
kb	kilo base
LB media	Luria-Bertani media
mRNA	messenger RNA
miRNA	microRNA
MS	Mass Spectrometry
MYO1B	Mysoin 1B
NOK	Normal Oral Keratinocyte
nt	Nucleotide
OPSCC	Oropharyngeal Squamous Cell Carcinoma
PCR	Polymerase Chain Reaction
PSCC	Pharyngeal Squamous Cell Carcinoma
qRT-PCR	Real-Time quantitative RT-PCR
RPMI	Roswell Park Memorial Institute medium

RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SCCHN	Squamous Cell Carcinoma of the Head and Neck
siRNA	Short Interfering RNA
UTR	Untranslated Region
wt	wildtype

1.0 INTRODUCTION

1.1 SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK

Squamous cell carcinoma of the head and neck (SCCHN) arises in the mucosal linings of the larynx, nasal cavity, oral cavity, paranasal sinuses, pharynx, or oropharynx and ranks sixth among cancers worldwide (Kamangar et al., 2006). In the United States, population-based studies estimate there will be 40,250 new cases of oral cavity and pharynx cancers and 12,360 new cases of laryngeal cancers, resulting in 7,850 and 3,650 deaths in 2012, respectively (Siegel et al., 2012). The majority of SCCHN patients are diagnosed with stage III or stage IV tumors, often involving regional lymph nodes (Jemal et al., 2010). The SCCHN 5-year survival rate of ~60 percent has not improved in the past ten years, indicating a great need for understanding the mechanisms of SCCHN pathogenesis (Howlader et al., 2011).

Well-known risk factors for SCCHN include alcohol consumption and tobacco use (Blot et al., 1988), which over time induce mutations in essential genetic pathways that regulate the cell cycle. Reactivation of telomerase is seen in up to 90 percent of SCCHN (McCaul et al., 2002), the loss of 9p21, which encodes p16, is seen in 70-80 percent of SCCHN (Mao et al., 1996; Reed et al., 1996), the cyclin D1 gene CCND1 located on chromosome 11q13 is amplified in up to 80 percent of SCCHN (Smeets et al., 2006), and mutations in the p53 tumor suppressor gene are seen in 60-80 percent of SCCHN (Balz et al., 2003; Poeta et al., 2007; van Houten et al., 2002). Additionally, high expression of epidermal growth factor receptor (EGFR) is seen in 90 percent of SCCHN cases (Grandis and Tweardy, 1993) and is associated with a poor prognosis (Rubin Grandis et al., 1998).

There has been a decrease in the overall number of SCCHN cases in the past ten years, however there has been an increase in the cases of oropharyngeal SCCHN, where patients are being diagnosed at a younger age compared to the other SCCHN sites (Chaturvedi et al., 2008).

The increase in the subset of SCCHN arising in the oropharynx is due to infection with high-risk human papillomavirus (HPV), and often occurs in individuals without the risk factors of alcohol consumption or tobacco use, leading some to classify HPV-positive SCCHN as a distinct tumor entity (Vidal and Gillison, 2008).

1.2 HUMAN PAPILLOMAVIRUS

Papillomaviruses are members of the *Papillomaviridae* family of viruses and can infect a variety of animals including mammals and birds. Human papillomaviruses (HPVs) are common wart-causing viruses, with over 150 types identified to date (de Villiers et al., 2004). HPVs are small double-stranded DNA, nonenveloped viruses that infect the basal lamina of skin and mucous membranes. They contain a circular genome of approximately 8 kb, that can be divided into three major regions: early (coding for E1, E2, E4, E5, E6, and E7), late (coding for L1 and L2), and a long control region (LCR, non-coding) (Hebner and Laimins, 2006). Due to their small genome size, HPVs do not encode most enzymes that are required for their replication. Therefore, the HPV life cycle depends upon the ability of the infected host cells to differentiate and proliferate. HPV infects the basal layer of squamous epithelial cells, usually through abrasions or lesions in the skin or mucosa. In basal cells, the HPV genome is maintained in low-copy episomal form, and early genes are turned on. The early HPV promoter activates transcription of the E1 replicative helicase and E2 transcription/replication factor. Then, expression of the E6 and E7 oncogenes in the infected cells promotes cell cycle progression. As the infected cell progresses through the differentiation pathway, the late HPV promoter is activated and the viral structural genes L1 and L2 are expressed. As the infected cell reaches

terminal differentiation, the early and late promoters work to increase production of new virions that are shed and can reinfect the surrounding cells (Hebner and Laimins, 2006).

HPVs have been associated with several types of cancers, including cervical, anogenital and oral cancers (De Vuyst et al., 2009; Frisch et al., 1997; Pascual et al., 2007; Tran et al., 2007b; Walboomers et al., 1999). This correlation has led to the classification of HPVs based on the likelihood of cellular progression to malignancy (Munger et al., 2004). Most HPVs such as types 6 and 11 are low-risk and maintain their genome in an episomal form. Low-risk HPV infections are either cleared by the host immune system or persist in recurrent warts. However, the genomes of the high-risk HPVs, most prevalent of which are types 16 and 18, are commonly found integrated into the host genome, often disrupting the viral E1 and E2 genes that function to regulate the viral E6 and E7 oncogenes. This integration is usually followed by over-expression of E6 and E7, which can begin the cascade toward carcinogenesis (Munger et al., 2004).

1.2.1 Human Papillomavirus Oncogenes

High-risk HPVs have two potent oncogenes, E6 and E7, which help to transform healthy, normal cells into cancer cells. These oncogenes cooperatively work to disrupt host cell cycle control mechanism. High-risk HPV E6 targets many cellular proteins to aid in cellular proliferation, transformation, and immortalization (Figure 1). E6 prevents p53-mediated growth arrest by the ubiquitination and subsequent proteasome-mediated degradation of p53 by the E6-E6AP-p53 tripartate complex (Huibregtse et al., 1991; Scheffner et al., 1990; Werness et al., 1990). Although E6 reduces the net levels of p53, additional p53 may be activated in response to stress including DNA damage. E6 binds to the histone acetyltransferases p300 and CBP and prevents their ability to acetylate and stabilize p53 (Kumar et al., 2002; Patel et al., 1999; Zimmermann et

al., 1999). E6 also targets ADA3, another histone acetyltransferase, for ubiquitin-mediated degradation thereby preventing it from acetylating and stabilizing p53 (Kumar et al., 2002; Patel et al., 1999; Zimmermann et al., 1999). High-risk E6 prevents cell death by directly binding to the TNF receptor 1 (Filippova et al., 2002) and by interacting with FADD, caspase 8, BAK, BAX, and IRF3 (Filippova et al., 2004; Garnett et al., 2006; Ronco et al., 1998; Thomas and Banks, 1998, 1999). To help immortalize cells, E6 binds E6AP and activates the telomerase promoter via interacting with repressors (USF1/2, NFX1-91) and activators (c-myc/max, Sp1, NFX1-123, and histone acetyltransferase complexes) (Howie et al., 2009). The high-risk E6 protein interacts with and causes the proteasome-mediated degradation of PDZ domain-containing proteins, including hDlg and hScrib, which disrupt cell adhesion and polarity (Thomas et al., 2008).

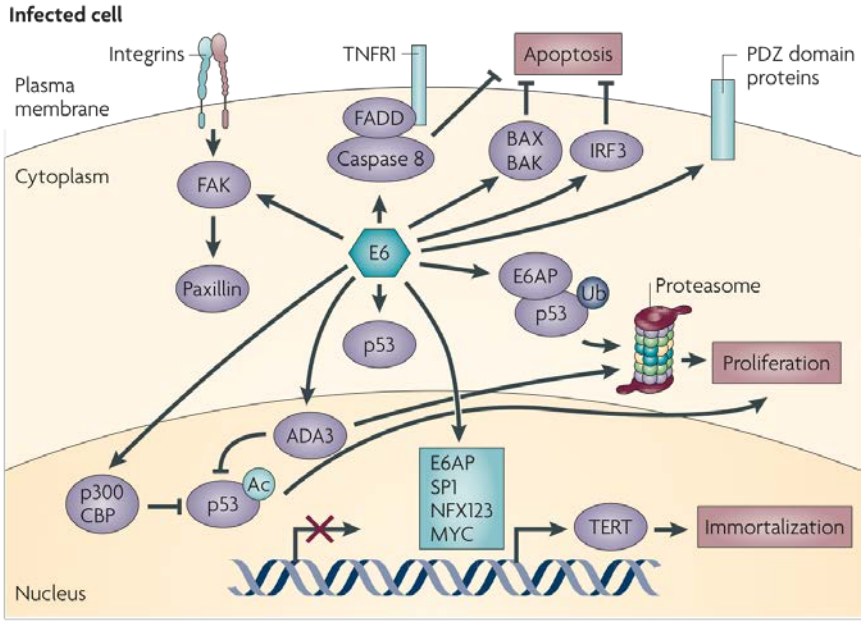

Figure 1: HPV-16 E6 Cellular Interactions

(Moody and Laimins, 2010, with permission)

High-risk HPV E7 functions in collaboration with E6 by targeting additional cellular proteins and further promoting cellular proliferation and transformation (Figure 2). The Retinoblastoma protein (pRb) is one of the main regulators of cellular entry into the S-phase. E7 binds to hypophosphorylated pRb, as well as to the other related pocket proteins p107 and p130. The E7 binding mediates the degradation of pRb, thereby releasing the E2F transcription factors to act on promoters of S-phase-specific genes (Boyer et al., 1996; Chellappan et al., 1992; Jones et al., 1997b; Zerfass et al., 1995). High-risk E7 binds to and neutralizes the CDK inhibitors p21 and p27, thereby increasing the levels of cyclin A and cyclin E, which increases cellular levels of CDK2 and promotes the entry of the cells into the S-phase (Funk et al., 1997; Jones et al., 1997a; Zerfass-Thome et al., 1996). Furthermore, E7 binds to histone deacetylases (HDACs) which results in increased E2F2-mediated transcription and entry of the cells into the S-phase (Longworth et al., 2005). E7 also acts to prevent the interferon response by binding to IRF1 and p48, thereby preventing activation of the Stat1-Stat2 transcription factors (Barnard and McMillan, 1999; Park et al., 2000).

High-risk HPV E5 has emerged as a potential oncoprotein, and has functions that can potentiate E6 and E7 mediated cellular transformation (Bouvard et al., 1994; Valle and Banks, 1995). The HPV-16 E5 gene can transform murine fibroblasts and human keratinocytes, and contributes to skin carcinogenesis in mice (Maufort et al., 2007; Straight et al., 1993; Valle and Banks, 1995). E5 activates the EGFR signaling pathway, which results in increased angiogenesis and cell proliferation, and decreased apoptosis (DiMaio and Mattoon, 2001; Genther Williams et al., 2005). Most studies of E5 have been in an overexpression model, and its functional *in vivo* role is less understood.

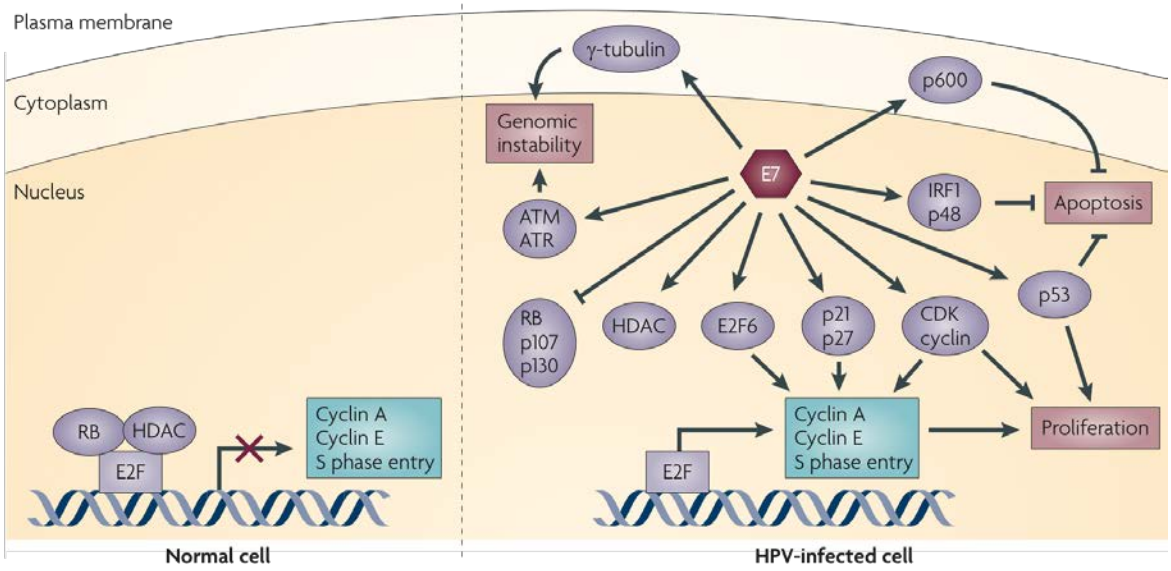

Figure 2: HPV-16 E7 Cellular Interactions

(Moody and Laimins, 2010, with permission)

1.2.2 Human Papillomavirus-Positive Squamous Cell Carcinoma of the Head and Neck

HPV is considered a major cause of oropharyngeal cancer in developed countries because it is detected in 45-90 percent of these cancers (D'Souza et al., 2007; Kreimer et al., 2005; Nasman et al., 2009). HPVs have also been detected in 24 percent of laryngeal and 23 percent of oral cavity cancers (Hobbs et al., 2006; Kreimer et al., 2005). High-risk HPV-16 accounts for 90-95 percent of HPV-positive oropharyngeal cancers, while the remaining five percent are caused by high-risk HPV-18, -31, -33, and -35 (Gillison et al., 2012; Gillison et al., 2000; Mork et al., 2001; Snijders et al., 1996; Zhang et al., 2004). In the United States, oropharyngeal cancer rates are on the rise despite a general decline in tobacco use, consistent with an increase in the number of HPV-associated SCCHN cases (Chaturvedi et al., 2008; Marur et al., 2010; Nasman et al., 2009).

HPV-positive and HPV-negative SCCHN show different clinical and demographic characteristics, leading some to classify them as distinct diseases (Vidal and Gillison, 2008). As summarized in Table 1, HPV-positive SCCHN typically arise in the oropharynx area with detection rates of up to 50 percent or more, while HPV-negative SCCHN arise in all head and neck sites (Hammarstedt et al., 2006; Klussmann et al., 2001; Paz et al., 1997; Venuti et al., 2004; Vidal and Gillison, 2008). Patients with HPV-positive SCCHN are diagnosed at a younger age (40-60 years) compared to patients with HPV-negative SCCHN (> 60 years) (Cruz et al., 1996; D'Souza et al., 2007; Fakhry et al., 2008; Mellin et al., 2000; Smith et al., 2004). SCCHN is predominantly a male disease with a 3:1 ratio of men:women, and most often occurs in white educated men who are frequently of higher socioeconomic status (D'Souza et al., 2007; Fakhry et al., 2008; Gillison et al., 2008).

The emergence of and increase in HPV-associated SCCHN has led to new risk factors for SCCHN. While the risk factors for HPV-negative SCCHN include alcohol consumption and/or tobacco use, the risk factors for HPV-positive SCCHN include oral HPV infection caused by sexual behavior (Table 1). A case-control study showed that a high number of vaginal or oral sex partners was associated with high oropharyngeal cancer rates, with the majority of those cancers testing positive for HPV (D'Souza et al., 2007). HPV-positive SCCHN tumors have infrequent p53 mutations, but p53 expression is usually low due to inactivation of p53 by the HPV E6 protein (Moody and Laimins, 2010; Poeta et al., 2007; van Houten et al., 2002). The E7 oncoprotein expressed in HPV-positive SCCHN reduces pRb and cyclin D levels. Since pRb negatively regulates p16, the reduction/loss of pRb in HPV-positive SCCHN causes an increase in p16 levels. However in HPV-negative SCCHN, p16 expression is reduced, usually due to

mutations, deletions, or methylation of the p16 gene (Table 1) (Olshan et al., 1997; Rischin et al., 2010).

Table 1: HPV-positive and HPV-negative SCCHN characteristics

	HPV-positive	**HPV-negative**
Anatomic site	Oropharynx (tonsil, base of tongue)	All sites
Age	Younger	Older
Gender	3:1 men	3:1 men
Risk factors	Sexual behavior	Alcohol and tobacco
Incidence	Increasing	Decreasing
p53 mutations	Rare	$\geq$ 50 percent
p16 expression	High	Low
Response to treatment	Better	Worse
Survival	High	Low

A large meta-analysis of several published reports of SCCHN showed that patients with HPV-positive SCCHN have a lower risk of recurrence and mortality compared to patients with HPV-negative SCCHN (Ragin and Taioli, 2007). Several studies have shown that tumors positive for HPV DNA responded better to chemotherapy, radiation, and surgery compared to tumors lacking the HPV DNA (Fakhry et al., 2008; Tran et al., 2007b; Vidal and Gillison, 2008; Worden et al., 2008). Additionally, individuals with HPV-positive SCCHN have a better prognosis compared to those with HPV-negative SCCHN, with 95 percent of patients with HPV-positive SCCHN still alive after two years, compared to only 62 percent of patients with HPV-negative SCCHN (Fakhry et al., 2008).

1.3 MICRORNAS

MicroRNAs (miRNAs) are endogenously encoded single-stranded RNAs that most commonly function as negative regulators of gene expression. MiRNAs were discovered in *Caenorhabditis elegans* in 1993 by Victor Ambrose (Lee et al., 1993) and are predicted to regulate the expression of up to a third of all genes (Lewis et al., 2005).

1.3.1 MicroRNA Biogenesis

The majority of miRNAs are transcribed by RNA polymerase II into a primary miRNAs (pri-miRNA) containing a stem-loop structure (Figure 3) (Lee et al., 2004). Pri-miRNAs are capped and polyadenylated (Cai et al., 2004) and then processed into a 60-80 bp imperfectly paired stem-loop precursor (pre) miRNA by the RNase III enzyme Drosha and its cofactor Pasha/DGCR8 (Gregory et al., 2004). The pre-miRNA is actively transported out of the nucleus to the cytoplasm by Ran-GTP and Exportin 5 (Lund et al., 2004) where the RNase III enzyme Dicer recognizes the two-nucleotide 3' overhang of the pre-miRNA and cleaves it into a 21-24 nucleotide miRNA duplex (Hutvagner et al., 2001). The miRNA duplex is loaded into the RNA-Induced Silencing Complex (RISC), and only the mature miRNA strand is retained (Kawamata et al., 2009). The RISC contains a member of the Argonaute protein family, a double-stranded RNA binding protein TRBP, and Dicer (Chendrimada et al., 2005). The mature miRNA guides the RISC to complementary sites in the 3' untranslated region (UTR) of the target mRNA where depending upon the degree of complementarity, the miRNA-RISC will either repress the translation of the target mRNA or target the mRNA for degradation (Eulalio et al., 2008). The 5′ 2-8 nt of the miRNA, known as the seed sequence, is usually complementary to the mRNA

target, and the remaining miRNA-mRNA interaction contains many mismatches. MiRNAs with low complementarity to the target mRNA generally promote translational repression of the mRNA whereas miRNAs with greater complementarity to the mRNA generally target the mRNA for degradation (see Figure 3) (Eulalio et al., 2008; Martinez et al., 2002). Since miRNAs do not have to be fully complementary to the target mRNA, one miRNA can regulate many genes, and one gene can be regulated by many miRNAs (John et al., 2004; Kiriakidou et al., 2004; Krek et al., 2005; Lewis et al., 2003; Lim et al., 2005).

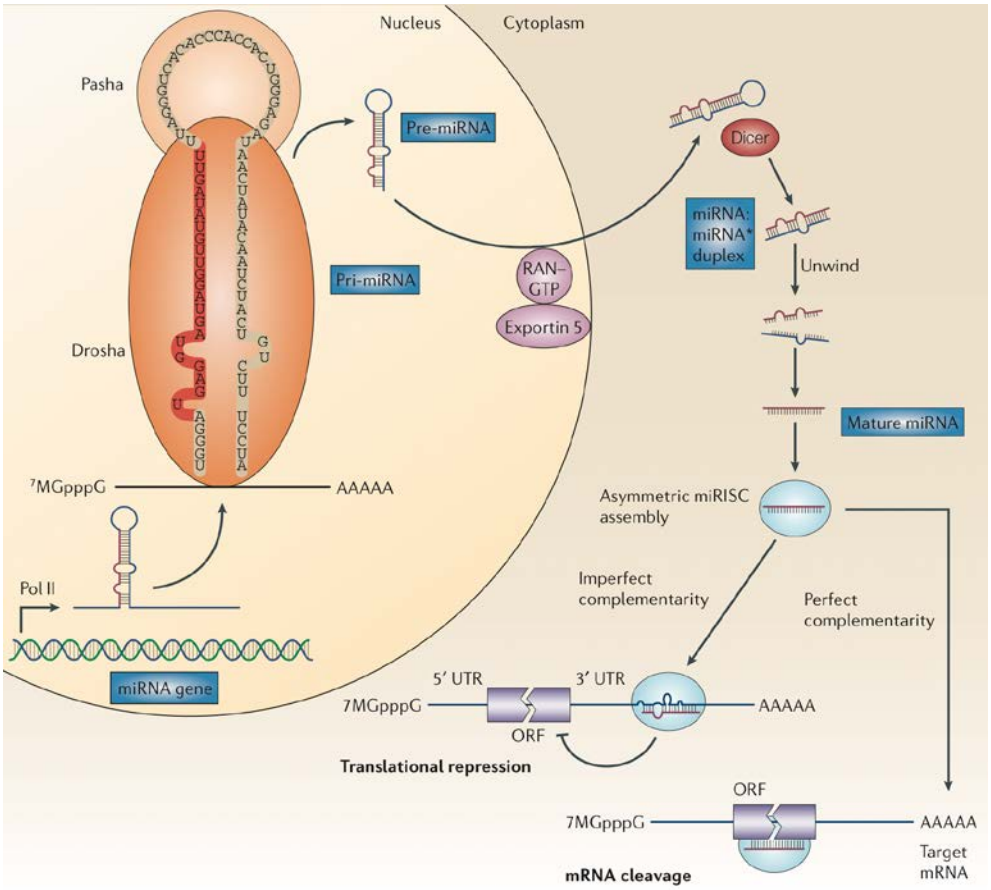

Figure 3: MicroRNA Biogenesis

(Esquela-Kerscher and Slack, 2006, with permission)

1.3.2 MicroRNA Involvement in Cancer

The small percentage of miRNAs that have been characterized function in many cellular regulatory processes. Recently, many miRNAs have been shown to function as tumor suppressors or oncogenes, and the dysregulation of such miRNAs has been shown to correlate with tumor progression (Figure 4) (Esquela-Kerscher and Slack, 2006). Up to one-half of well-studied miRNA genes are located in fragile sites of the genome, where genomic disruption often leads to cancer (Calin et al., 2004). Low expression of a tumor suppressive miRNA can lead to lack of repression of its target oncogenes, whereas high expression of an oncogenic miRNA can lead to repression of its target tumor suppressor genes. Both types of such events can then lead to cancer (Figure 4) (Esquela-Kerscher and Slack, 2006). Global miRNA dysregulation as well as dysregulation of miRNA processing machinery is seen in many tumors (Caldas and Brenton, 2005; Esquela-Kerscher and Slack, 2006; Lu et al., 2005).

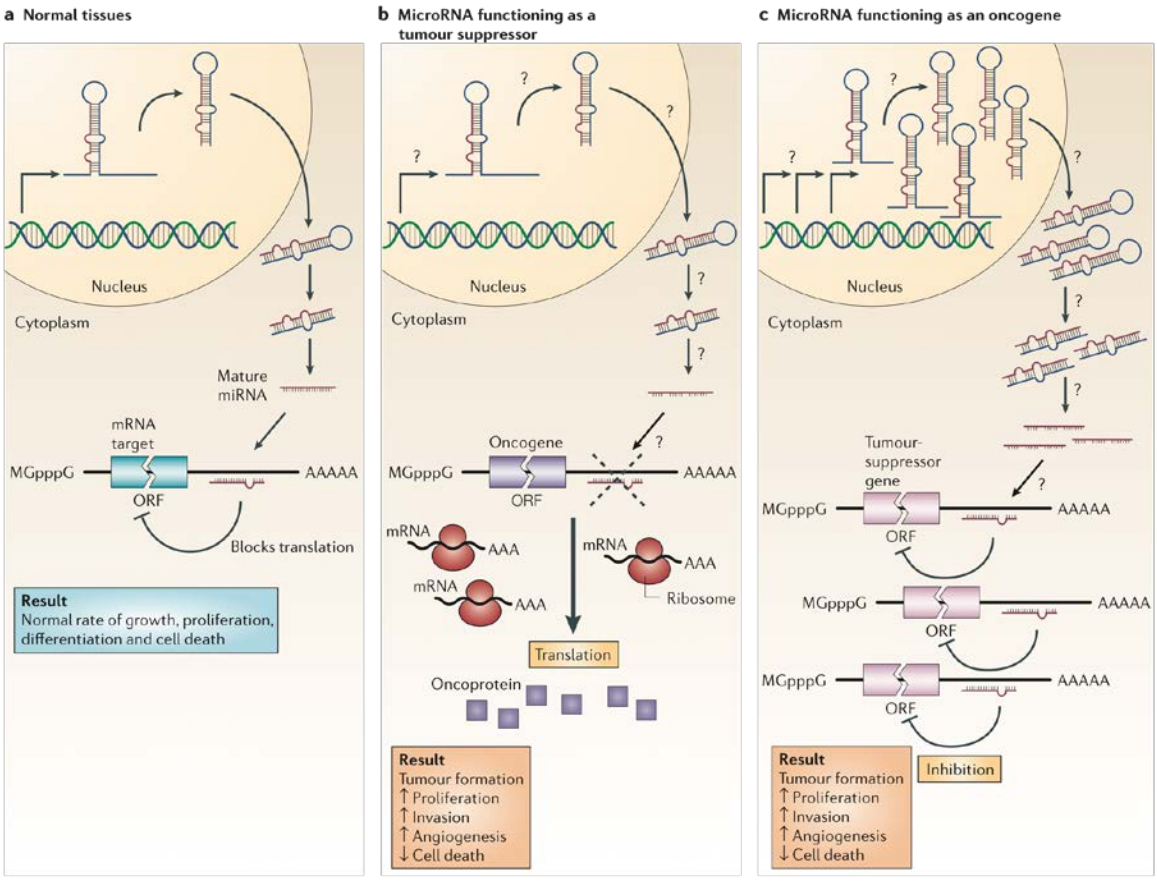

Figure 4: Tumor suppressive and Oncogenic MicroRNAs

(Esquela-Kerscher and Slack, 2006, with permission)

The first miRNA shown to act as a tumor suppressor was the miR-15a-miR-16-1 cluster in B-cell chronic lymphocytic leukemia (CLL) (Calin et al., 2002). More than half of all CLL cases have deletions in the miR-15a and miR-16 chromosomal regions, and these miRNAs are downregulated or deleted in several other types of cancers. These tumor suppressor miRNAs are known to target Bcl-2, cyclin D1, and Wnt3a (Bandi et al., 2009; Bonci et al., 2008; Cimmino et al., 2005). MiR-143 and miR-145 are downregulated in colorectal and bladder tumors and breast, cervical, lymphoid, and prostate cancer cell lines (Esquela-Kerscher and Slack, 2006; Michael et al., 2003; Villadsen et al., 2012). The genes encoding the let-7 family of miRNAs are

located in fragile sites that are linked to breast, cervical, lung, and urothelial cancers (Calin et al., 2004). The downregulation of these let-7 family members in lung cancer correlates with poor prognosis and survival (Takamizawa et al., 2004). Furthermore, miRNAs in the let-7 family target the Ras oncogene; therefore decreased expression of these miRNAs leads to increased cell survival and cancer (Johnson et al., 2005).

One of the most well studied oncogenic miRNAs is miR-21. This was one of the first miRNAs to be identified in humans and is overexpressed in almost all cancers, where it promotes cell survival (Asangani et al., 2008; Chan et al., 2005; Fujita et al., 2008; Park et al., 2009a; Si et al., 2007; Zhu et al., 2008). High levels of miR-21 have been found in breast, cervical, colon, head and neck, hepatocellular, lung, ovarian, pancreatic, prostate, stomach, and thyroid cancers, as well as glioblastoma and leukemia (Asangani et al., 2008; Frankel et al., 2008; Krichevsky and Gabriely, 2009; Lu et al., 2008b; Meng et al., 2007; Si et al., 2007). The miR-17~92 cluster, known as “oncomiR-1”, is often amplified and overexpressed in B cell lymphomas and small cell lung cancer (Hayashita et al., 2005; Matsubara et al., 2007; Ota et al., 2004). The c-Myc oncogene is often upregulated in many cancers, and it serves as a transcription factor for the miR-17~92 miRNA cluster (He et al., 2005; O'Donnell et al., 2005; Sylvestre et al., 2007). The c-Myc protein also represses miRNA expression by directly binding to the promoters of many miRNAs, including let-7a/f/d, miR-15a, miR-16, miR-22, miR-26a/b, miR-29a/b, miR-30c/e, miR-34a, and miR-146a (Chang et al., 2008b; Kleine-Kohlbrecher et al., 2006). There are two other miRNA clusters (miR-106a~363 and miR-106b~25) that belong to the miR-17~92 family of miRNA clusters, and these miRNA clusters are thought to have arisen through a series of deletions and/or duplications, as many of the miRNAs have the same seed sequence and are

thought to target similar genes (Ventura et al., 2008). Thus, since miR-17~92 is an oncogenic miRNA cluster, miR-106a~363 and miR-106b~25 are also potential oncogenic miRNA clusters.

MiRNAs are also involved in signaling pathways that are commonly disrupted in cancer. The tumor suppressor protein p53 is mutated in up to 50 percent of cancers, and dysfunctional p53 signaling is found in up to 80 percent of tumors (Levine et al., 2004; Levine et al., 2006; Olivier et al., 2004). While p53 regulates several miRNAs, it is also targeted by some miRNAs. MiR-504 and miR-125b directly regulate p53 (Hu et al., 2010). Additionally, miR-29, miR-34a, and miR-122 positively regulate p53 through their targets p85α, SIRT1, and cyclin G1, respectively (Fornari et al., 2009; Park et al., 2009b; Yamakuchi et al., 2008). The p53 protein is a direct activator of the members of the miR-34 family through its binding to the promoters of the miR-34a and miR-34b/c genes (Chang et al., 2007; Corney et al., 2007; He et al., 2007; Raver-Shapira et al., 2007; Tazawa et al., 2007). The miR-34 family members target many proteins that regulate the cell cycle, including cyclin E2, CDK4, CDK6, and BCL2. In cancers where p53 is mutated/disrupted, the miR-34 family members are not expressed, and this leads to cellular proliferation (Bommer et al., 2007; Chang et al., 2007; Tazawa et al., 2007). Additionally, miR-34a is downregulated in cells expressing high-risk HPV E6, which targets p53 for ubiquitin-mediated degradation (Wang et al., 2009a). The p53 protein also regulates hypoxia-induced signaling by activating miR-107, which targets the hypoxia inducible factor-1β (Yamakuchi et al., 2010). The p53 protein activates the promoter of miR-145, which negatively regulates the c-Myc oncogene (Sachdeva et al., 2009) and p53 activates the transcription of miR-192 and miR-215, which target genes that regulate the cell cycle (Georges et al., 2008). The miR-17~92, miR-106b~25, and miR-106a~92 clusters are regulated by p53 through its repression of E2F1 (Brosh et al., 2008). Finally, p53 interacts with Drosha to enhance Drosha-

mediated primary miRNA maturation and enhances the expression of miR-16, miR-143, miR-145, and miR-206 (Suzuki et al., 2009).

High-risk HPV E6 and E7 proteins are known to interact with many cellular proteins and promote cellular transformation (Moody and Laimins, 2010). Among these interactions, c-Myc and p53 are known to be involved in complex networks that involve miRNAs. Thus, disruption of c-Myc and/or p53 by the HPV oncogenes may alter the expression of miRNAs that are regulated by or involved in the c-Myc or p53 networks, (Chang et al., 2008b; O'Donnell et al., 2005; Suzuki et al., 2009).

1.3.3 MicroRNA Involvement in SCCHN

There have been many reports on dysregulated miRNAs in SCCHN cell lines and tissues. Although miRNA expression can vary by site (tonsil, tongue, etc.), there are several commonly dysregulated miRNAs in all SCCHN sites. Some commonly downregulated miRNAs in head and neck cancer compared to adjacent normal tissue include miR-375, miR-133a, and miR-133b (Avissar et al., 2009; Childs et al., 2009; Hui et al., 2010; Lajer et al., 2011; Tran et al., 2007a; Wong et al., 2008a; Wong et al., 2008b). Known as a tumor suppressive miRNA, low expression of miR-375 is associated with low survival of oral cancer patients, reduced cellular apoptosis, and increased proliferation (Harris et al., 2012; Nohata et al., 2011b). The tumor suppressive miR-133a is often downregulated in several cancers, including SCCHN, where it targets genes that are involved in cell migration and invasion (Kinoshita et al., 2012a; Kinoshita et al., 2012b; Nohata et al., 2011a).

One commonly overexpressed miRNA in head and neck cancer compared to adjacent normal tissue is miR-155 (Chang et al., 2008a; Hui et al., 2010; Lajer et al., 2011; Ramdas et al., 2009; Wong et al., 2008b). This miRNA is overexpressed in many cancers, but in Epstein-Barr-virus-negative nasopharyngeal carcinoma, high expression of miR-155 is associated with poor prognosis (Du et al., 2011).

1.4 PROJECT HYPOTHESIS

We hypothesized that infection with high-risk HPV would lead to overexpression of the HPV E6 and E7 oncogenes. Expression of these oncogenes in SCCHN is expected to disrupt cellular miRNA expression differently than in HPV-negative SCCHN. The altered miRNA expression in HPV-positive and HPV-negative SCCHN would lead to alterations in the expression of the target gene(s), thus altering pathways involved in HPV-positive and HPV-negative SCCHN. The altered pathways may provide insight into the different characteristics seen in HPV-positive and HPV-negative SCCHN.

2.0 ALTERATION OF MICRORNA PROFILES IN SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK CELL LINES BY HUMAN PAPILLOMAVIRUS

Work described in this section was published in Head & Neck (Head Neck. 2011 Apr;33(4):504-12) with authors Abigail I. Wald, Elizabeth E. Hoskins, Susanne I. Wells, Robert L. Ferris, and Saleem A. Khan.

2.1 INTRODUCTION

Squamous cell carcinoma of the head and neck (SCCHN) ranks sixth among cancers worldwide (Tran et al., 2007b). Many of these cases are associated with heavy consumption of alcohol and/or tobacco use, which over time induce mutations in essential genetic pathways that regulate the cell cycle. However, human papillomavirus (HPV) type 16 DNA has been found in up to 30 percent of these cancers, most often in the oropharynx region, and such cases of SCCHN are often found in individuals without the risk factors of alcohol and tobacco use (Chaturvedi et al., 2008; Tran et al., 2007b). The HPV-positive SCCHN subset have increased in the past 10 years (Chaturvedi et al., 2008). Because of this demographic shift and distinct clinical behavior, the association and relevance of HPV in SCCHN is under intense investigation.

Characteristics of HPV-associated SCCHN are very different from HPV-negative SCCHN, causing disputes whether these cancers should be classified as distinct tumors (Vidal and Gillison, 2008). HPV-positive oral tumors often exhibit loss of cell cycle control proteins, including pRb and cyclin D1, whereas these two proteins are commonly overexpressed in HPV-negative oral tumors (Tran et al., 2007b; Vidal and Gillison, 2008). One of the most common tumor suppressor proteins, p53, is mutated in up to half of oral cancers, but is very rarely mutated in HPV-positive SCCHN, and tumors with a high viral load have a better prognosis compared to tumors with a low viral load or tumors that are HPV-negative (Tran et al., 2007b; Vidal and Gillison, 2008). Patients with HPV-positive oral tumors have a better response to chemotherapy, radiation, and surgery (Vidal and Gillison, 2008), and have evidence of immune

activation against viral antigens (Albers et al., 2005), despite having frequent metastasis to regional lymph nodes (Vidal and Gillison, 2008). The biological basis for the differential behavior of HPV-positive SCCHN is not understood.

Micro (mi) RNAs are small, ~22 nt long, chromosome-encoded single-stranded RNAs that are commonly associated with negative regulation of gene expression (Bartel, 2004). MiRNAs are transcribed and exported to the cytoplasm where further processing takes place, and the mature miRNA strand is incorporated into the RNA-induced silencing complex (Bartel, 2004). The miRNA guides the RNA-induced silencing complex to the 3′ untranslated region of its target mRNA where, depending upon the degree of complementarity, the miRNA either translationally represses the mRNA or targets it for degradation (Bartel, 2004). MiRNA dysregulation has been implicated in many different types of human cancers (Esquela-Kerscher and Slack, 2006; Tong and Nemunaitis, 2008).

Previous reports have shown altered miRNA profiles in head and neck cancers compared to the normal oral tissue (Childs et al., 2009; Hui et al., 2010; Ramdas et al., 2009; Wong et al., 2008a). MiRNAs with high expression in the tumors compared to the normal oral tissue included miR-21, whereas miR-125b was downregulated (Childs et al., 2009; Hui et al., 2010; Ramdas et al., 2009). Basal miRNA expression in 9 head and neck cancer cell lines found that 33 miRNAs were expressed at a high level and 22 miRNAs were expressed at a low level (Tran et al., 2007a). Interestingly, one of these cell lines UM-SCC47, is HPV-16-positive (Bradford et al., 2003). In all 9 cell lines, let-7a, miR-16, miR-21, and miR-205 were highly expressed, and miR-342, miR-346, and miR-373* were expressed at low levels (Tran et al., 2007a). Although these studies show alterations in miRNA levels in head and neck cancer, they do not address the role of HPVs. Because the number of cases of HPV-16-positive SCCHN have been increasing in

the past 10 years (Chaturvedi et al., 2008), and the characteristics of HPV-positive and HPV-negative SCCHN support distinction between these cancers (Vidal and Gillison, 2008), we sought to analyze the miRNA profiles in HPV-positive and HPV-negative SCCHN cell lines.

In this study, we demonstrate that miRNA expression profiles in HPV-16-positive SCCHN cells are distinctly different from those in HPV-negative SCCHN cells and in normal oral keratinocytes (NOKs) that have been immortalized by activation of h-TERT. Using human foreskin keratinocytes (HFKs) expressing either the HPV-16 E6 or E7 oncogene, we also demonstrate that expression of the E6 oncogene results in upregulation of miR-363 and downregulation of miR-181a, mR-218 and miR-29a. Furthermore, siRNA knockdown of HPV-16 E6 in the HPV-positive SCCHN cell line SCC2 reduces the expression of miR-363.

2.2 MATERIALS AND METHODS

2.2.1 Cell Lines

The cell lines used in this study are described in Table 2. Two HPV-16-positive SCCHN cell lines, UD-SCC-2 (gift from Dr. Henning Bier, University of Dusseldorf) (Gwosdz et al., 2005), and UPCI:SCC90 (Ferris et al., 2005), and two HPV-negative SCCHN cell lines, PCI-13 and PCI-30 (gifts from Dr. Theresa Whiteside, UPCI) (Chikamatsu et al., 1999) were used for miRNA expression profile analysis. The HPV-16-positive SCCHN cell lines UM-SCC47 (gift from Dr. Thomas Carey, University of Michigan) (Bradford et al., 2003; Brenner et al., 2010) and 93-VU-147T (gift from Dr. Hans Joenje, VU Medical Center Van der Boechorststraat 7, The Netherlands) (Steenbergen et al., 1995) were used with the above cells lines for validation of the

miRNA microarrays. The UPCI:SCC90, UM-SCC-47, ad 93-VU-147T cell lines contain integrated HPV-16 DNA, whereas the HPV-16 status (integrated vs. episomal) of the UD-SCC-2 cell line is not known (Ferris et al., 2005; Gupta et al., 2009; Steenbergen et al., 1995). However, all the HPV-16-positive cell lines were shown to express the viral E6 and E7 genes (Figure 5B). The UD-SCC-2, UPCI:SCC90, UM-SCC47, PCI-13 and PCI-30 cell lines were grown in Dulbecco's modified Eagle's medium (Lonza, Walkersville, MD) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 2% L-glutamine at 37°C in the presence of 5% CO_2. The 93-VU-147T cell line was grown in Dulbecco's modified Eagle's medium/F12 medium (MediaTech, Manassas, VA) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 2% L-glutamine at 37°C in the presence of 5% CO_2. NOKs that were immortalized by activation of h-TERT (Piboonniyom et al., 2003) were grown in defined keratinocytes serum-free medium (Gibco, Grand Island, NY) supplemented with bovine pituitary extract and 1% penicillin/streptomycin at 37°C in the presence of 5% CO_2. Primary HFKs that were transduced with an LXSN-based retroviral vector expressing high-risk HPV-16 E6 or E7 were grown in EpiLife medium (Invitrogen, Carlsbad, CA) supplemented with human keratinocytes growth supplements (Invitrogen) and 1% penicillin/streptomycin at 37°C in the presence of 5% CO_2.

Table 2: SCCHN cell line characteristics

SCCHN Sample	TNM Classification	Specimen Site	Sex	HPV-status	p53 gene
PCI-13	T4N1M0	Oral Cavity	Male	HPV-negative	E286K
PCI30	T3N1M0	Oral Cavity	Male	HPV-negative	wt
UD-SCC2	T1N2M0	Hypopharynx	Male	HPV-16	wt
UPCI:SCC90	T2N1M0	Base of Tongue	Male	HPV-16	wt
UM-SCC47	T3N1M0	Lateral Tongue	Male	HPV-16	wt
93-VU-147T	T4N2	Floor of Mouth	Male	HPV-16	wt

2.2.2 Human Papillomavirus Status of Samples

The SCCHN cell lines were confirmed to be either HPV-positive or HPV-negative by polymerase chain reaction (PCR) analysis using the MY09/MY11 primer set, which amplifies a conserved region of the HPV L1 gene (Ferris et al., 2005). Although the HPV-positive SCCHN cell lines have previously been characterized (Bradford et al., 2003; Ferris et al., 2005; Gwosdz et al., 2005), we confirmed the HPV status of these cells. UD-SCC-2, UPCI:SCC90, UM-SCC47, and 93-VU-147T were further confirmed to contain HPV-16 DNA by PCR using primers that amplify a 477-bp region of the HPV-16 E6 gene using 5′-ATGCACCAAAAGAGAACTGC-3′ as the forward primer and 5′-TTACAGCTGGGTTTCTCTAC-3′ as the reverse primer. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as a loading control using 5′-AGGGGAGATTCAGTGTGGTG-3′ as the forward primer and 5′-GGCCTCCAAGGAGTAAGACC-3′ as the reverse primer, amplifying a 122-bp region. All PCR reactions were performed as described previously (Ferris et al., 2005). The PCR amplified DNA was analyzed by agarose gel electrophoresis.

2.2.3 RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction Analysis

Total RNA was isolated from all the cell lines grown to 90% confluency using the Ultraspec™ RNA Isolation System (Biotecx, Houston, TX, USA). DNase-I-treated total RNA (1 μg) of UD-SCC-2, UPCI:SCC90, UM-SCC47, and 93-VU-147T was subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) analysis for expression of the HPV-16 E6 and E7 oncogenes using the Advantage® Clontech RT-for-PCR Kit (Clontech, Mountain View, CA, USA) according to the manufacturer's instructions.

The HPV-16 E6 gene was amplified using the primer set described above, and expression of the HPV-16 E7 gene was done using the forward primer 5′-CAGCTCAGAGGAGGAGGATG-3′ and the reverse primer 5′-GCACAACCGAAGCGTAGAGT-3′, amplifying a 115-bp region. Expression of the GAPDH gene was used as a control, using the primer set described above. The PCR products were analyzed by agarose gel electrophoresis (Fig. 5B). The HFK-16E6 and HFK-16E7 cell lines were also confirmed to be expressing the intended oncogene via RT-PCR as described above.

2.2.4 MicroRNA Microarray Analysis

Small RNAs (<200 nt) were enriched from total RNA from 2 HPV-positive SCCHN cell lines (UD-SCC-2 and UPCI:SCC90) and 2 HPV-negative SCCHN cell lines (PCI-13 and PCI-30) using the RNeasy Mini Kit and the RNeasy MinElute Clean Up Kit (Qiagen, Valencia, CA). The small RNA fractions were validated on a 15% acrylamide gel. Small RNA fractions obtained from 5 μg of total RNA were labeled with AlexaFluor 647 (Invitrogen) using the *mir*Vana Labeling Kit and hybridized (in duplicate) to the *mir*Vana miRNA Bioarrays V2

(Ambion, Austin, TX). These bioarrays contained 662 antisense oligonucleotides, spotted in quadruplicate, which included 328 known human miRNAs, 152 predicted miRNAs (ambi-miRNAs), 266 mouse miRNAs (114 unique miRNAs), and 238 rat miRNAs (46 unique miRNAs). The arrays were hybridized with labeled miRNAs at 42°C overnight. Each array was subsequently washed once in low stringency was solution followed by washing twice in high stringency was solution, and dried by centrifugation. The arrays were immediately scanned using the GenePix 4000B scanner and the median fluorescent intensities, minus the background fluorescence, were obtained using the GenePix Pro 6.0 software. The median fluorescent intensities of each spot on the bioarrays were $\log_2$ transformed and normalized using the mean intensities within the array and the global mean adjustment between the arrays by the GEDA program (http://bioinformatics.upmc.edu/GE2/GEDA.html). Significance Analysis of Microarray program version 1.21 (http://www-stat.stanford.edu/~tibs/SAM/) was used to perform a *t* test to obtain the differential miRNA expression patterns of each sample. MiRNAs with at least a 2-fold change in expression with a q-value (false discovery rate) of zero were considered to have significant changes in their expression between the samples.

2.2.5 SiRNA Knockdown of Human Papillomavirus-16 E6 and Transfection Assays

The role of HPV-16 E6 in altered miRNA expression in HPV-positive SCCHN cell lines was analyzed using double-stranded siRNA against HPV-16 E6 (siRNA 209 complementary to E6 positions 277 to 298, sense sequence 5'-UCCAUAUGCUGUAUGUGAUTT-3'; Dharmacon, Lafayette, CO) (Jiang and Milner, 2002; Tang et al., 2006). The HPV-positive SCCHN cell line SCC2 was seeded (1.5×10^5) into 6-well plates, and after 24 hours transfected with 125 nM siRNA using Lipofectamine 2000 Reagent (Invitrogen) and OPTI-MEM I (Gibco). BLOCK-iT

fluorescent oligo (Invitrogen) was used as a negative control siRNA (it has no human homologous sequences) and a transfection efficiency control. Cells were harvested after 72 hours, and RNA extractions were done as previously described.

2.2.6 Real-Time Quantitative Reverse Transcriptase Polymerase Chain Reaction

Array data were confirmed by real-time quantitative RT-PCR (qRT-PCR) using the TaqMan MicroRNA Reverse Transcription Kit and the TaqMan MicroRNA Assays (Applied Biosystems, Foster City, CA) and the Real-Time Thermocycler iQ5 (Bio-Rad, Hercules, CA). These assays use stem-loop primers designed to amplify only the mature miRNA. DNase I-treated total RNA (5 ng) was used for each reaction, and all reactions were done in triplicate. Assays were performed in accord with the manufacturer's instructions and the miRNA levels were normalized to the small nucleolar RNU43 levels. Relative expression levels of the miRNAs were calculated using the $2^{-\Delta\Delta CT}$ values (Livak and Schmittgen, 2001). Statistical analysis was done via a 2-tailed *t* test.

HPV-16 E6 expression in siRNA knockdown experiments was confirmed via qRT-PCR using the QuantiTect SYBR Green PCR kit (Qiagen) in accord with the manufacturer's instructions. The E6 gene was amplified using the forward primer 5′-AGCGACCCAGAAAGTTACCA-3′ and the reverse primer 5′-GCATAAATCCCGAAAAGCAA-3′, amplifying a 134-bp region. The E6 mRNA levels were normalized to the GAPDH gene, using the primer set described above. DNase I-treated total RNA (1 μg) was used for each reaction, and all the reactions were done in triplicate. The E6 mRNA levels were normalized to the GAPDH levels, and relative expression levels were

calculated using the $2^{-\Delta\Delta CT}$ values (Livak and Schmittgen, 2001). Statistical analysis was done via a 2-tailed *t* test.

2.3 RESULTS

2.3.1 MicroRNA Expression in Squamous Cell Carcinoma of the Head and Neck Cell Lines

The UD-SCC-2, UPCI:SCC90, UM-SCC47, and 93-VU-147T cell lines were confirmed to be HPV-positive by PCR analysis using the MY09/MY11 primers (Figure 5A). These cell lines were further confirmed to contain HPV-16 DNA by PCR using E6 gene primers (Figure 5A). Finally, the expression of the HPV-16 E6 and E7 genes in these cell lines was confirmed by RT-PCR analysis (Figure 5B). We then analyzed miRNA expression in 2 HPV-16-positive (UD-SCC-2 and UPCI:SCC90) and 2 HPV-negative (PCI-13 and PCI-30) SCCHN cell lines utilizing *miR*Vana miRNA Bioarrays V2. MiRNA analysis showed that 129 human miRNAs were expressed in both of the HPV-negative cell lines (PCI-13 and PCI-30), with miR-21, miR-16, and miR-29a being the most highly expressed (Supplementary Table 1). The HPV-16-positive cell lines (UD-SCC-2 and UPCI:SCC90) both expressed 216 human miRNAs, indicating a general upregulation of miRNA expression in the presence of HPV-16 DNA. The miRNAs with high basal expression included miR-205, miR-16, and miR-21 (Supplementary Table 2).

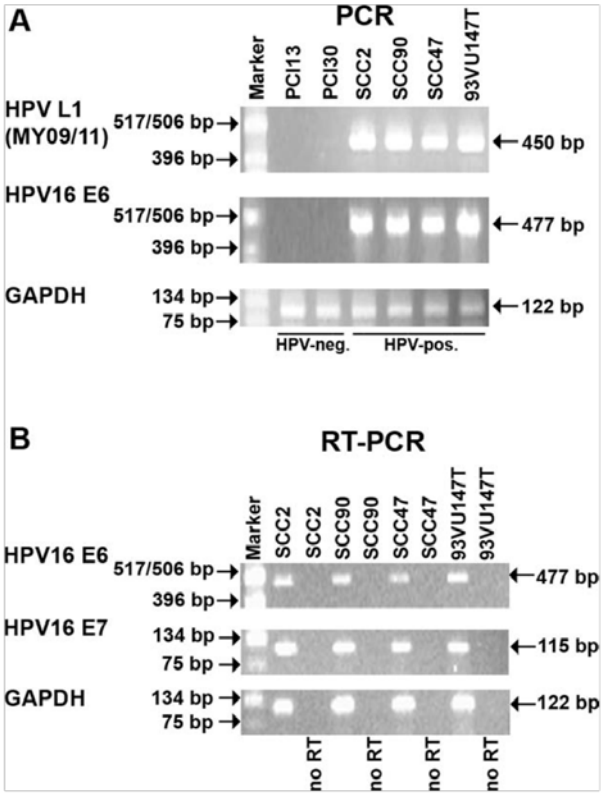

Figure 5: HPV status of SCCHN cell lines

(A) PCR of all SCCHN cell lines to check HPV status. (B) RT-PCR for HPV-16 E6 and E7 expression

2.3.2 MicroRNA Expression is Altered in Human Papillomavirus-16-Positive Squamous Cell Carcinoma of the Head and Neck Cell Lines

We compared the miRNA expression profiles in 2 HPV-16-positive cell lines, SCC2 and SCC90, with that of 2 HPV-negative cell lines, PCI13 and PCI30. Two human miRNAs, miR-363 and miR-33, and the rat miRNA miR-497 (which differs from human miR-497 by just 1 nucleotide), were upregulated in HPV-positive cell lines compared to HPV-negative cell lines (Table 3). Eight human miRNAs and 1 predicted human miRNA were downregulated in HPV-positive cell lines (Table 3). A comparison of miRNA expression between individual cell lines showed that 2 miRNAs were downregulated in SCC2 compared to PCI13 (Supplementary Table 3), whereas 6 miRNAs were overexpressed in SCC2 compared to PCI30 (Supplementary Table 4). When SCC90 was compared to PCI13, 4 miRNAs were overexpressed and 3 miRNAs were underexpressed (Supplementary Table 5). On the other hand, 10 miRNA were overexpressed in SCC90 compared to PCI30 (Supplementary Table 6). MiR-363 was upregulated in SCC90 compared to both PCI13 and PCI30 cell lines, and in SCC2 compared to PCI30 (Supplementary Tables 4-6). We also found that miR-181a was downregulated in both SCC2 and SCC90 compared to PCI13 (Supplementary Tables 3 and 5). Although there were differences in miRNA expression in individual SCCHN cell lines, several miRNAs including miR-363 and miR-181a were similarly altered in both the HPV-positive cell lines in individual and pair-wise comparisons with the 2 HPV-negative cell lines.

Table 3: MiRNAs differentially expressed in HPV-16-positive SCCHN cell lines compared to HPV-negative SCCHN cell lines

MiRNA	Fold Change *
Overexpressed	
hsa_miR_363	5.16
rno_miR_497	3.20
hsa_miR_33	1.99
Underexpressed	
hsa_miR_155	-7.60
hsa_miR_181a	-7.34
hsa_miR_181b	-6.76
hsa_miR_29a	-4.68
hsa_miR_218	-4.22
hsa_miR_222	-3.69
hsa_miR_221	-3.38
hsa_miR_142_5p	-3.20
ambi_miR_13232	-3.10

Abbreviations: MiRNA, microRNA; HPV, human papillomavirus; SCCHN, squamous cell carcinoma of the head and neck; miR, microRNA; hsa, human; rno, rat; ambi, Ambion predicted. *Mean fold changes in HPV-16-positive SCCHN cell lines UD-SCC-2 and UPCI:SCC90 compared to HPV-negative SCCHN cell lines PCI-13 and PCI-30. The q-values of all miRNAs were 0.

2.3.3 Human Papillomavirus-16-Positive Squamous Cell Carcinoma of the Head and Neck Cell Lines have Altered MicroRNA Expression as Compared to Both Human Papillomavirus-Negative Squamous Cell Carcinoma of the Head and Neck Cell Lines and Immortalized Normal Oral Keratinocytes

To validate the microarray data, we carried out qRT-PCR analysis for selected miRNAs that were found to be differentially expressed in SCC2 and SCC90 (HPV-positive) cell lines compared to PCI13 and PCI30 (HPV-negative). For this, we used 4 HPV-16-positive SCCHN cell lines (2 that were included in the array analysis and 2 that were not) and 2 HPV-negative SCCHN cell lines. To exclude miRNA profiles only associated with squamous differentiation or immortalization, we also used an NOK cell line that has been immortalized by activation of h-TERT (Piboonniyom et al., 2003). The most overexpressed miRNA in the HPV-positive cells

based on the array analysis was miR-363. The qRT-PCR results showed higher expression of miR-363 in HPV-positive cell lines SCC2 (6.3-fold), SCC90 (28.9-fold), SCC47 (1.3-fold), and 93-VU-147T (5.5-fold) compared to NOK cells (Figure 6A). The expression of miR-363 in the HPV-negative cell lines was reduced 12-fold in PCI13 and 7-fold in PCI30 cells compared to the NOK cells (Figure 6A). MiR-363 was upregulated in the above 4 HPV-positive SCCHN cell lines by 61.7-fold, 283.1-fold, 12.8-fold, and 54.4-fold, respectively, compared to the average expression in PCI13 and PCI30, with $p < .01$ (Figure 6A). Because miR-363 is part of a cluster of miRNAs, we carried out qRT-PCR analysis for 2 other miRNAs in the cluster, miR-106a and miR-92a. There was no difference in expression of these miRNAs in the HPV-positive SCCHN cell lines compared to the HPV-negative SCCHN cell lines (data not shown). The Ambion *miR*Vana miRNA microarray includes a probe for both rat and human miR-497. The rat miR-497 differs from the human miR-497 by the addition of 1 adenosine at its 3′ end (Griffiths-Jones et al., 2008). Because the rat miR-497, but not the human miR-497, was significantly altered in the HPV-positive samples as compared to the HPV-negative samples in our array analysis, we also carried out qRT-PCR analysis for this miRNA. These results showed that human miR-497 was upregulated in 3 HPV-positive cell lines, SCC2 (17.2-fold), SCC90 (6.8-fold), and 93-VU-147T (1.61-fold) compared to the NOK cells (Figure 6B). This miRNA was slightly downregulated in HPV-negative PCI13 (1.8-fold) and PCI30 (2.0-fold) cells relative to the NOK cells (Figure 2B). MiR-497 was upregulated in the above 3 HPV-positive SCCHN cell lines by 32.6-fold, 13.0-fold, and 3.1-fold, respectively, compared to the average expression in PCI13 and PCI30, with $p < .01$ (Figure 6B).

The array results also showed that 8 human miRNAs, and 1 predicted human miRNA were downregulated in 2 HPV-positive cell lines, SCC2 and SCC90, compared to the HPV-

negative cell lines (Table 3). The downregulation of a few selected representative miRNAs in 4 HPV-positive cell lines as compared to both the HPV-negative cell lines and NOK cells was confirmed by qRT-PCR. MiR-155 was downregulated in SCC2 (36.7-fold), SCC90 (87.4-fold), SCC47 (2.9-fold), and 93-VU-147T (1.4-fold) compared to the NOK cells (Figure 6C). This miRNA was downregulated in the 4 HPV-positive cell lines by 42.7-fold, 101.7-fold, 3.4-fold, and 1.6-fold, respectively, compared to the average expression in the PCI13 and PCI30 cell lines, with $p < .01$ for SCC2, SCC90, and SCC47, and $p < .05$ for 93-VU-147T (Figure 6C). MiR-181a was downregulated in SCC2 (212.3-fold), SCC90 (8.7-fold), SCC47 (7.3-fold), and 93-VU-147T (3.4-fold) compared to the NOK cells (Figure 6D). This miRNA was downregulated in the above 4 HPV-positive cell lines by 91.4-fold, 3.7-fold, 3.1-fold, and 1.6-fold, respectively, compared to the average expression in the PCI13 and PCI30 cell lines, with $p < .01$ for SCC2, SCC90, and SCC47 (Figure 6D). Similarly, miR-218 was downregulated in SCC2 (43.3-fold), SCC90 (28.5-fold), SCC47 (1.4-fold), and 93-VU-147T (35.1-fold) compared to the NOK cells (Figure 6E). This miRNA was downregulated in the above 4 HPV-positive cell lines by 141.9-fold, 93.4-fold, 4.6-fold, and 114.7-fold, respectively, compared to the average expression in the PCI13 and PCI30 cell lines, with $p < .01$ for SCC2, SCC90, and 93-VU-147T, and $p < .05$ for SCC47 (Figure 6E). Finally, miR-29a was downregulated in SCC2 (144.8-fold), SCC90 (59.9-fold), SCC47 (26.5-fold), and 93-VU-147T (22.3-fold) compared to the NOK cells (Figure 6F), whereas this miRNA was downregulated 18.3-fold, 7.6-fold, 3.4-fold, and 2.8-fold, respectively, compared to the average values of PCI13 and PCI30 cell lines, with $p < .01$ for SCC2, SCC90, and SCC47, and $p < .05$ for 93-VU-147T (Figure 6E).

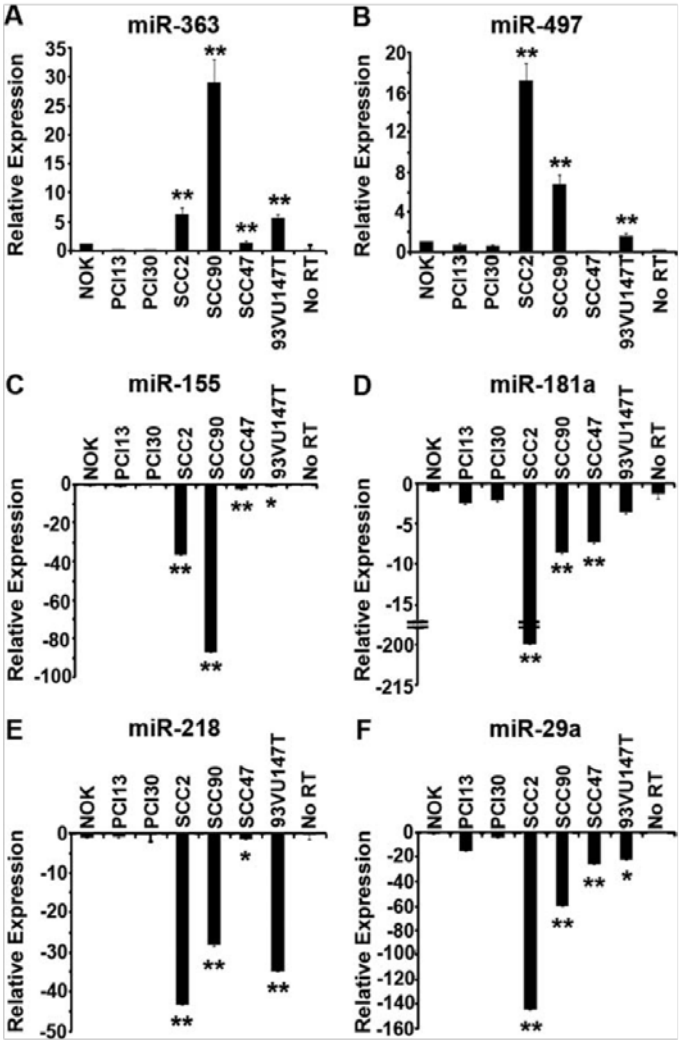

Figure 6: QRT-PCR validation of microRNA expression data in 4 HPV-positive and 2 HPV-negative SCCHN cell lines, and NOK cells

No RT, no reverse transcriptase added. Intensity values are relative to the NOK cells, which were arbitrarily assigned a value of 1 or -1. The *p* values for the HPV-positive cell lines compared to the 2 HPV-negative SCCHN cell lines are indicated by * ($p < .05$) and ** ($p < .01$).

2.3.4 Human Papillomavirus-16 E6 Oncogene Alters MicroRNA Expression

To test whether the altered miRNA expression in the HPV-positive SCCHN cell lines was due to the expression of the HPV-16 oncogenes, we used primary HFKs transduced with either the empty vector (LXSN) or vectors expressing the high-risk HPV-16 E6 or E7. Analysis of RNA samples from HFKs containing the empty LXSN vector by RT-PCR showed that it did not express the HPV-oncogenes while the HFK-16E6 and HFK-16E7 cell lines expressed the appropriate oncogene (Figure 7A). We then tested the relative expression levels of 4 miRNAs, miR-363, miR-181a, miR-218, and miR-29a that were significantly affected in HPV-positive cell lines (Figure 6). QRT-PCR analysis showed that miR-363 (upregulated in HPV-positive SCCHN cell lines compared to both HPV-negative SCCHN cell lines and NOK cells) was upregulated in the HFK-16E6 cell line, with $p < .01$, but not in the HFK-16E7 cell line (Figure 7B). Similarly, miR-181a, miR-218, and miR-29a (downregulated in HPV-positive SCCHN cell lines compared to both HPV-negative SCCHN cell lines and NOK cells) were downregulated in the HFK-16E6 cell line, with $p < .01$. These miRNAs were either not affected or affected to a lesser extent in the HFK-16E7 cell line (Figure 7B). These results showed that expression of the E6 oncogene of HPV-16 is associated with upregulation of miR-363 and downregulation of miR-181a, miR-218, and miR-29a.

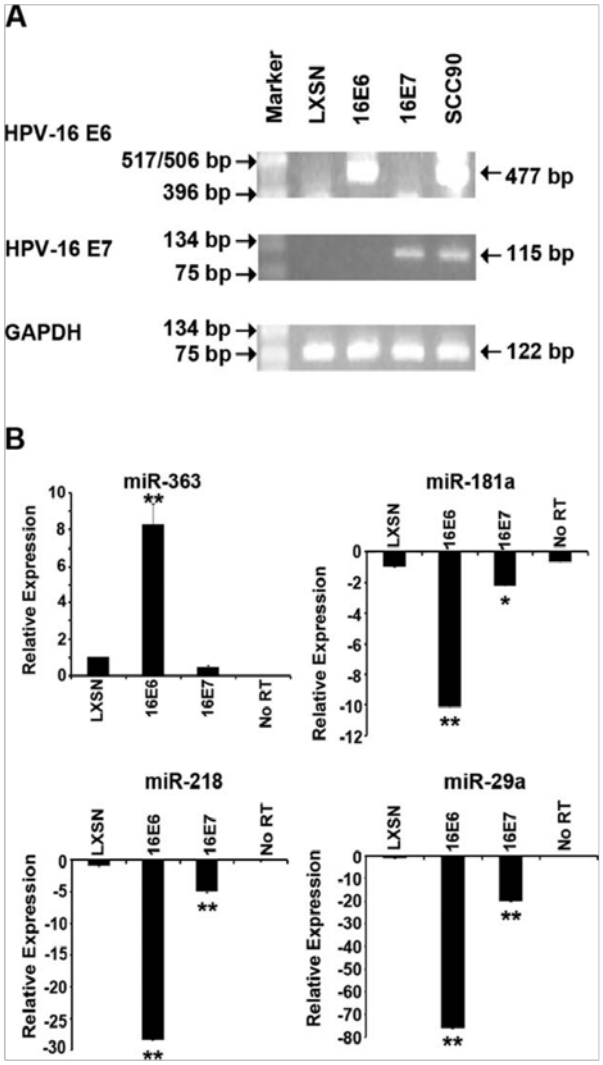

Figure 7: MiRNA expression in HFK cells expressing HPV-16 E6 or E7

(A) RT-PCR analysis of the human papillomavirus (HPV)-16 E6 and E7 oncogene expression in the respective HFK cell line. The UPCI:SCC90 cell line, known to express the HPV-16 E6 and E7 genes was used as a positive control. (B) QRT-PCR analysis of miR-363, miR-181a, miR-218, and miR-29a in the HFK cell lines. No RT, no reverse transcriptase added. Intensity values are relative to the HFK-LXSN cells, which were arbitrarily assigned a value of 1 or -1. The *p* values for the HPV-16 E6 and E7 expressing HFK cell lines compared to the HFK-LXSN cell line are indicated by ** ($p<0.01$) and * ($p<0.05$).

Because the expression of the HPV-16 E6 oncogene altered miRNA expression in the HFK cells, siRNA knockdown of HPV-16 E6 was done in the HPV-positive SCCHN cell line SCC2. The qRT-PCR results showed that reduction in the levels of E6 were accompanied by a reduction in miR-363 levels (Figure 8). These results suggest that E6 is involved in the upregulation of miR-363 in HPV-positive cell lines.

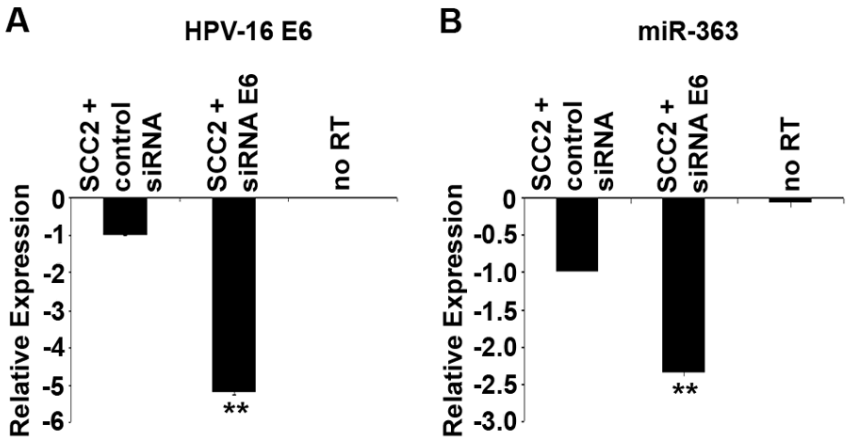

Figure 8: qRT-PCR analysis of the HPV-positive SCCHN cell line SCC2 transfected with siRNA against HPV-16 E6

(A) QRT-PCR analysis of HPV-16 E6 oncogene expression in the HPV-positive cell line SCC2 upon transfection with a negative control siRNA or with siRNA against E6. (B) QRT-PCR analysis of miR-363 expression in cells transfected with a negative control siRNA or with siRNA against E6. No RT, no reverse transcriptase added. Intensity values are relative to the SCC2 cells transfected with a negative control siRNA, which were arbitrarily assigned a value of -1. The *p* values for the SCC2 + siRNA E6 cells compared to the SCC2 with the control siRNA are indicated by ** ($p<0.01$).

2.4 DISCUSSION

HPV-positive and HPV-negative SCCHN have distinctly different clinical outcomes, and the expression and mutation status of important cell cycle control proteins are very different (Tran et al., 2007b; Vidal and Gillison, 2008). Our data show that several cellular miRNAs are also differentially expressed in HPV-positive SCCHN cell lines as compared with HPV-negative SCCHN cell lines. Many of these miRNAs were also found to be differentially expressed between the HPV-positive SCCHN cell lines and transformed oral keratinocytes lacking HPV DNA. Similarly, expression of the high-risk HPV-16 E6 oncogene in HFKs was strongly associated with changes in miRNA expression similar to that seen in the HPV-positive SCCHN cell lines, whereas siRNA knockdown of HPV-16 E6 reversed this effect for miR-363. These results suggest that HPV-16, and in particular the E6 oncogene, may be involved in altering the levels of several cellular miRNAs.

Altered regulation of cellular miRNAs has been observed in several types of human cancers (Esquela-Kerscher and Slack, 2006; Lui et al., 2007; Tong and Nemunaitis, 2008) and upon oncogenic viral infections, including hepatitis B and C (Jiang et al., 2008), Epstein-Barr virus (Godshalk et al., 2008), human T-cell lymphotrophic virus 1 (Yeung et al., 2008), and HPV-16 (Lui et al., 2007; Wang et al., 2008; Wang et al., 2009a). Recent studies have analyzed miRNA expression in SCCHN (Chang et al., 2008a; Kozaki et al., 2008; Ramdas et al., 2009; Tran et al., 2007a; Wong et al., 2008a; Wong et al., 2008b) and found that miRNA profiles in SCCHN are different compared to normal oral tissue. However, currently there is no information available on differential miRNA expression between HPV-positive and HPV-negative SCCHN. Because HPV infection has been shown to play a significant role in the

etiology and prognosis of SCCHN (Tran et al., 2007b; Vidal and Gillison, 2008), we wanted to study the effect of HPV-16 infection on cellular miRNA dysregulation in SCCHN.

Very few HPV-positive SCCHN cell lines have been described in the literature. Of 4 such cell lines that are available, we used 2 (UD-SCC-2 and UPCI:SCC90) to compare their miRNA expression profiles to that of 2 HPV-negative SCCHN cell lines (PCI13 and PCI30) by microarray analysis. We further used the 2 additional HPV-16-positive SCCHN cell lines (UM-SCC47 and 93-VU-147T) to validate the miRNA data obtained in the above comparison. MiRNA microarray analysis showed that 3 miRNAs (human miR-363 and miR-33, and rat miR-497) were upregulated and 8 known and 1 predicted miRNAs were downregulated in the HPV-positive SCCHN cell lines compared to the HPV-negative SCCHN cell lines (Table 3 and Figure 6). The miRNA microarray analysis was used as a screening tool, and the data were subsequently validated via qRT-PCR. Similar to our current results, we have previously found that qRT-PCR analysis is much more sensitive than the miRNA microarrays and the fold-difference in qRT-PCR assays is usually much greater (Martinez et al., 2008). MiR-363 was specifically upregulated in HPV-16-positive SCCHN cell lines compared to the HPV-negative SCCHN cell lines and NOK cells (Table 3 and Figure 6A), suggesting a possible role of HPV-16 in altering the levels of this miRNA. Furthermore, experiments with HFKs showed that expression of the HPV-16 E6 oncogene increased the levels of miR-363 (Figure 7B) and siRNA knockdown of E6 reversed this effect (Figure 8). Interestingly, miR-363 is part of the oncogenic miR-17~92 family of clusters, which is composed of 3 clusters of miRNAs, miR-17~92, miR-106a~363, and miR-106b~25 and thought to have evolved through a series of deletions and duplications (Ventura et al., 2008). Other members of this family of miRNA clusters have been implicated in cancers, including small cell lung cancer (Hayashita et al., 2005), B-cell lymphoma

(Ota et al., 2004), and T-cell leukemia (Landais et al., 2007). MiRNAs in this family have similar or identical seed sequences (Ventura et al., 2008), and because the seed sequence of a mature miRNA contributes significantly to its specificity for its target mRNA (Bartel, 2004), it has been hypothesized that miRNAs in the miR-17~92 family may have similar functions (Ventura et al., 2008). MiR-363 has identical seed sequences to miR-92-1, miR-92-2, and miR-25 (Griffiths-Jones et al., 2008). MiR-92-2 and miR-25 are also overexpressed in pancreatic, prostate, and stomach cancers (Volinia et al., 2006). Recently, miR-25 has been shown to be upregulated in gastric cancers where it targets p57, an essential tumor suppressor (Kim et al., 2009). Because miR-363 and miR-25 have the same seed sequence, and miR-25 is involved in cell cycle disruption (Kim et al., 2009), it is possible that miR-363 may also be involved in the dysregulation of the cell cycle in HPV-associated SCCHN. The qRT-PCR analysis for miR-106a and miR-92a did not show any differences in expression between the HPV-positive and HPV-negative SCCHN cell lines (data not shown). This is not surprising because many miRNAs in a cluster have independent promoters. Landais *et. al.* (Landais et al., 2007) has shown that the miR-106a~363 cluster of miRNAs in mice is located downstream of the *Kis2* gene. This gene has 3 different transcription start sites and it seems to encode the primary miRNAs of the miR-106a~363 cluster. Also, the radiation leukemia virus is commonly integrated close to the *Kis2* locus. In mice, radiation leukemia virus-induced tumors had varied expression of miRNAs in the miR-106a~363 cluster, indicating that they may not be transcribed from the same promoter (Landais et al., 2007). Also, in gastric cancer, miR-363 was shown to be downregulated compared to the normal tissue, whereas all of the other miRNAs in the miR-106a~363 cluster were upregulated (Kim et al., 2009). Thus, while miR-363 is overexpressed in

HPV-positive SCCHN cells compared to HPV-negative SCCHN cells, it is not surprising that we did not see a difference in expression of miR-106a and miR-92a between these cell lines.

Our results also show downregulation of several miRNAs in HPV-associated SCCHN cell lines as compared to both HPV-negative SCCHN and NOK cell lines, including miR-155, miR-181a, miR-218, and miR-29a (Table 3, Figure 6, and Figure 7B). We have recently demonstrated that the HPV-16 E6 oncogene downregulates miR-218 expression in HPV-16 positive cervical carcinomas (Martinez et al., 2008). Furthermore, we showed that miR-218 targets *LAMB3,* and downregulation of miR-218 by the E6 oncogene results in overexpression of *LAMB3* in cervical carcinoma cells (Martinez et al., 2008). We found that expression of HPV-16 E6 in HFK cells also reduced the levels of miR-218 (Figure 7B). The downregulation of miR-218 in both HPV-positive cervical and oropharyngeal cancer cell lines suggests that HPV-16 may target cellular pathways common to these 2 types of cancers. Although it is documented that p53 expression activates miR-34a (He et al., 2007) and miR-34a levels are reduced in HPV-16-positive cervical cancer (Wang et al., 2009a), we did not find a statistically significant difference between miR-34a levels between HPV-positive and HPV-negative SCCHN cell lines. Whereas all the HPV-positive cell lines used in our study are p53 wild-type (wt), the HPV-negative cell line PCI-13 has a p53 mutation while PCI-30 has wt p53 (Table 2). There are several possible reasons for our observations of miR-34a. For example, because the p53 pathway is complex, it is possible that a single miRNA may be subject to multiple regulatory mechanisms.

Viral infections have been implicated in altered cellular miRNA expression. In human B lymphocytes infected with the Epstein-Barr virus, elevated levels of miR-155 help in viral persistence by reducing NF-κB signaling (Lu et al., 2008a). It is intriguing that miR-155 was

found to be downregulated in the presence of HPV-16 in our studies (Table 3, Figure 6C, and Figure 7B). There have been other studies on miRNA expression in head and neck cancers that have found miR-155 and miR-181a to be upregulated in oral cancer compared to normal oral tissue (Chang et al., 2008a; Wong et al., 2008a; Wong et al., 2008b). However, when we compared HPV-positive and HPV-negative SCCHN cell lines, these miRNAs were downregulated in the presence of HPV-16 DNA. Future studies should define the relationship between reduced levels of these miRNAs in HPV-positive SCCHN.

Our studies showed that miR-181a and miR-29a were downregulated in HPV-positive SCCHN cells compared to HPV-negative SCCHN and NOK cells (Table 3, Figure 6D and 6F, and Figure 7B). The levels of these 2 miRNAs also decreased upon expression of the HPV-16 E6 oncogene in HFKs (Figure 7B), suggesting a role for E6 in downregulation of these miRNAs. The miR-181 family is known to be highly expressed in the brain (Miska et al., 2004) and is involved in thymocyte development (Chen et al., 2004), but its role in other tissues is less well-understood. Our data are the first to show a downregulation of miR-181a and miR-181b in HPV-positive SCCHN cell lines compared to HPV-negative SCCHN cell lines (Table 3, Figure 6D, and Figure 7B). Overexpression of miR-181a and miR-181b have been shown to induce apoptosis and inhibit growth and invasion in glioma cells (Shi et al., 2008). Further studies on the roles of the miR-181 family may elucidate roles of these miRNAs in the different characteristics seen in HPV-positive and HPV-negative SCCHN. MiR-29a has been shown to interact with viral proteins. For example, miR-29a targets the HIV-1 Nef protein and interferes with viral replication (Ahluwalia et al., 2008). MiR-29a also targets p85 and CDC42, which are negative regulators of p53 (Park et al., 2009b). Because the HPV-16 E6 protein promotes the degradation of the p53 protein (zur Hausen, 2002), it is possible that the downregulation of miR-

29a by E6 may further reduce p53 levels upon persistent HPV infection. The precise role of HPV infection in cellular miRNA dysregulation, and the role of HPVs in SCCHN development, which also contributes to a better prognosis for these cancers as compared to their HPV-negative counterpart, will be the subject of future studies.

3.0 INCREASED EXPRESSION OF MICRORNA-363 IN HUMAN PAPILLOMAVIRUS-ASSOCIATED SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK TISSUES

3.1 INTRODUCTION

Squamous cell carcinoma of the head and neck (SCCHN) is the most common head and neck malignancy and ranks sixth among cancers worldwide (Tran et al., 2007b). There are more than 35,000 new cases of SCCHN in the United States and 500,000 new cases worldwide each year (Greenlee et al., 2001). Although there have been advances in the detection and therapy of SCCHN, the prognosis still remains poor, with a 5-year survival of approximately 50 percent (Greenlee et al., 2001). The majority of SCCHN cases are associated with heavy alcohol consumption and/or tobacco use, which over time induce mutations in essential genetic pathways that regulate the cell cycle (Tran et al., 2007b). However, an emerging class of SCCHN has been associated with high-risk human papillomavirus (HPV), where up to 30 percent of SCCHN contain HPV-16 DNA, most often in the oropharynx region (oropharynx, base of tongue, and tonsil), and often occurs in individuals without the major risk factors of alcohol and tobacco use (D'Souza et al., 2007; Tran et al., 2007b).

HPVs are small double-stranded DNA viruses that infect the basal lamina of the skin or mucous membranes (zur Hausen, 2002). High-risk HPVs have been associated with several types of cancer, including cervical, anogenital, and oral cancers. In HPV-associated cancers, expression of the E6 and E7 oncoproteins is essential for cellular transformation (Munger et al., 2004). The high-risk HPV E6 and E7 proteins target many cellular proteins to induce cellular transformation (for review on HPV oncogenes, see (Moody and Laimins, 2010)).

HPV-positive and HPV-negative SCCHN have different characteristics, leading many to consider HPV-positive SCCHN a distinct tumor entity (Vidal and Gillison, 2008). The tumor

suppressor protein p53 is mutated in up to half of oral cancers, but is rarely mutated in HPV-positive SCCHN (Agrawal et al., 2011; Stransky et al., 2011; Vidal and Gillison, 2008). Patients with HPV-positive oral tumors have a much better prognosis compared to HPV-negative SCCHN, with better response to chemotherapy, radiation, and surgery (Vidal and Gillison, 2008). It is also known that HPV-positive SCCHN cases have improved immune system activation to viral antigens (Albers et al., 2005) and have a lower likelihood of metastasis than patients with HPV-negative oral tumors (Fakhry and Gillison, 2006). A recent study (Gillison et al., 2008) reported the association of HPV and oropharyngeal cancers with and without the established risk factors of alcohol and tobacco use, suggesting that HPV-positive and HPV-negative SCCHN are discrete molecular, clinical, and pathological diseases. Because of a recent increase in HPV-positive SCCHN cases (Chaturvedi et al., 2008) and their distinct clinical behavior, the molecular basis for the differences between HPV-positive and HPV-negative SCCHN is under intense investigation.

Micro (mi) RNAs are small, endogenously encoded single-stranded RNAs that are most commonly associated with post-transcriptional negative regulation of gene expression (Bartel, 2004). Some miRNAs have been shown to function as tumor suppressors or oncogenes, and the dysregulation of such miRNAs have been observed in various cancers, including head and neck cancers (Childs et al., 2009; Esquela-Kerscher and Slack, 2006; Hui et al., 2010; Ramdas et al., 2009; Tran et al., 2007a; Volinia et al., 2006; Wong et al., 2008a).

We have previously reported differences in miRNA expression in HPV-positive and HPV-negative SCCHN cell lines, and miR-363 was found to be the most overexpressed miRNA in HPV-positive SCCHN cell lines (Wald et al., 2011). In this study, we sought to investigate

whether miR-363 is also overexpressed in HPV-positive SCCHN tumors compared to the HPV-negative SCCHN tumors in patients from the western Pennsylvania area.

3.2 MATERIALS AND METHODS

3.2.1 SCCHN Cell Lines

Two SCCHN cell lines were used as positive and negative controls for HPV status and HPV gene expression analysis. PCI-30 (gift from Dr. Theresa Whiteside, University of Pittsburgh Cancer Institute) is an HPV-negative SCCHN cell line and UPCI:SCC90 is an HPV-16-positive SCCHN cell line (previously described in (Wald et al., 2011)). These cell lines were maintained in Dulbecco's modified Eagle's medium (Lonza, Walkersville, MD) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 2% L-glutamine at 37°C plus 5% CO_2.

3.2.2 SCCHN Tissues

41 SCCHN tissues were obtained from patients according to Institutional Review Board protocol 99-069. Upon surgical removal, a portion of all tissues was sent to pathology for official tumor staging and the remainder of all tissues was flash frozen until further processing.

3.2.3 DNA Isolation

DNA was isolated from all SCCHN tissues and cell lines using the DNeasy Blood & Tissue kit (Qiagen, Valencia, CA) according to the manufacturer's protocol.

3.2.4 RNA Isolation

Total RNA was isolated from all SCCHN tissues and cell lines using the Ultraspec RNA Isolation System (Biotecx, Houston, TX) according to the manufacturer's protocol.

3.2.5 PCR and RT-PCR

HPV status of all SCCHN tissues was analyzed using the MY09/MY11 primer set, which amplifies a conserved region of the HPV L1 gene (Ferris et al., 2005). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as a loading control, using 5′-CGACCACTTTGTCAAGCTCA-3′ as the forward primer and 5′-AGGGGTCTACATGGCAACTG-3′ as the reverse primer, amplifying a 332-bp region. All PCR reactions were performed using 20ng template DNA, 200μM of each deoxynucleoside triphosphate (dNTP), 0.5μM of each primer, and 0.5 units of Taq polymerase and the associated buffer (Promega, Madison, WI). Thermocycler conditions for all PCR reactions were 94°C for 5 minutes; 35 cycles of 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds; and 72°C for 10 minutes. The PCR amplified DNA was visualized by agarose gel electrophoresis. HPV-positive samples were tested for HPV type by amplifying the HPV-16 gene. HPV-16 E6

primers were forward 5′-AATGTTTCAGGACCCACAGG-3′ and 5′-CAGCTGGGTTTCTCTACGTG-3′, amplifying a 454-bp region.

RT-PCR was done on HPV-16 E6 and E7 oncogenes to show expression in HPV-positive SCCHN tumors. The HPV-16 E6 gene was amplified using the primer set described above and the HPV-16 E7 gene was amplified using the forward primer 5′-CATGGAGATACACCTACATTGCAT-3′ and the reverse primer 5′-GAACAGATGGGGCACACAAT-3′, amplifying a 281-bp region. cDNA synthesis was done using the iScript™ Select cDNA Synthesis Kit (Bio-Rad, Hercules, CA) according to the manufacturer's protocol using random primers. DNase I-treated RNA (60ng of total RNA) was used for each cDNA synthesis reaction, and the cDNA was PCR amplified for HPV-16 E6, HPV-16 E7, and GAPDH.

3.2.6 Determination of HPV DNA and p16 Expression in Tumor Tissue

Fluorescence *in situ* hybridization (FISH) for HPV-16 DNA was performed with a proprietary pan selective probe set (Dako Cytomation, Carpinteria, CA). Immunohistochemical (IHC) evaluation of the remaining deparaffinized sections was performed using immunoperoxidase staining for p16 (p16INK4 mAb, BD Pharmingen, dilution 1:200) and scored semiquantitatively for each core. Nuclear staining by HPV-16 FISH and diffuse p16 IHC (>80% cells) was considered HPV-positive.

3.2.7 Real-Time Quantitative Reverse Transcriptase PCR (qRT-PCR)

Relative levels of miR-363 were measured using the TaqMan MicroRNA Reverse Transcription Kit and the TaqMan MicroRNA Assay (Applied Biosystems, Foster City, CA) and the iQ5 Real-Time Thermocycler (Bio-Rad). Twenty-five nanograms of total RNA was used for each reaction, and all reactions were performed in triplicate. Assays were done according to the manufacturer's instructions, and the miRNA levels were normalized to the RNU43 small nucleolar RNA levels. Relative miR-363 expression was calculated using the $2^{-\Delta\Delta CT}$ values (Livak and Schmittgen, 2001). Statistical analysis of miR-363 qRT-PCR expression was done via a 2-tailed *t* test.

3.3 RESULTS AND DISCUSSION

3.3.1 HPV is Prevalent in Western PA SCCHN Cases

We studied a cohort of 41 SCCHN patients treated at the University of Pittsburgh Cancer Institute between the years 2006 and 2009. The clinical and demographic characteristics of the patients are detailed in Table 4 and summarized in Table 5. Upon receipt, all tissues were tested for HPV positivity via PCR with the MY09/MY11 primers as described previously (Figure 9) (Wald et al., 2011). Although HPV FISH was done for some SCCHN samples, DNA PCR was used for all samples because of its high sensitivity of amplifying HPV DNA and to complete the HPV testing on all samples. Of the 41 SCCHN samples, 24 (59%) were HPV-positive and 17 (41%) were HPV-negative. Fifteen of the 18 samples tested for HPV via FISH were HPV-

positive, confirming that testing for HPV via PCR has higher sensitivity than HPV testing via FISH. All samples that were tested clinically for p16 (16 of the 24 HPV-positive samples) were positive for p16. Only six HPV-negative SCCHN samples were tested for p16, two of which were p16 positive, indicating that some HPV-negative SCCHN samples had high p16 expression.

Table 4: Detailed demographics of SCCHN tissues

Specimen Site	TNM Stage	Gender	Race	Age at diagnosis	HPV status	Tobacco history	Alcohol history	Status	Metastasis
Gum	T1N0M0	Male	Caucasian	78	HPV-negative	never smoker	never drinker	Deceased	cheek mucosa, mouth, tongue
Cheek	T2N1M0	Male	Caucasian	68	HPV-negative	former smoker	current drinker	Deceased	cheek mucosa, lip, soft palate, oropharynx
Mouth (1)	T1N1M0	Male	Caucasian	80	HPV-negative	never smoker	never drinker	Alive	none
Mouth (2)	T2N2CM0	Male	Caucasian	56	HPV-16	former smoker	former drinker	Alive	none
Tongue (1)	T3N2M0	Male	Caucasian	64	HPV-16	current smoker	current drinker	Deceased	mouth, lung
Tongue (2)	T3N2BM0	Male	Caucasian	64	HPV-negative	current smoker	former drinker	Deceased	none
Tongue (3)	T4N2BM0	Female	Caucasian	48	HPV-negative	current smoker *	current drinker *	Deceased	none
Tongue (4)	T4AN2CM0	Male	Caucasian	65	HPV-negative	former smoker	current drinker	Deceased	none
Vallecula	T2BN2M0	Male	African-American	51	HPV-16	never smoker	never drinker	Alive	none
Base of tongue (1)	T1N2BM0	Female	Caucasian	84	HPV-negative	current smoker *	current drinker	Deceased	none
Base of tongue (2)	T1N2BM0	Male	Caucasian	50	HPV-16	current smoker *	former drinker	Alive	none
Base of tongue (3)	T2N0	Female	Caucasian	57	HPV-16	former smoker	never drinker	Deceased	lymph node (tongue)
Base of tongue (4)	T2N0M0	Male	Caucasian	60	HPV-16	former smoker	never drinker	Deceased	none
Base of tongue (5)	T2N0M0	Male	Caucasian	60	HPV-negative	former smoker	current drinker *	Deceased	base of tongue
Base of tongue (6)	T2N1M0	Male	Caucasian	51	HPV-16	current smoker *	former drinker	Alive	none
Base of tongue (7)	T2N1M0	Female	Caucasian	82	HPV-negative	current smoker	current drinker *	Alive	lung
Base of tongue (8)	T2N2M0	Male	Caucasian	70	HPV-16	unknown	unknown	Deceased	recurrence - vallecula
Base of tongue (9)	T2N2BM0	Male	Caucasian	62	HPV-16	never smoker	never drinker	Alive	none
Base of tongue (10)	T2N2CM0	Male	Caucasian	48	HPV-16	current smoker	current drinker	Deceased	lymph nodes (head/face/neck)
Base of tongue (11)	T2N3M0	Male	African-American	68	HPV-16	current smoker *	current drinker	Deceased	lymph nodes (intrathoracic)
Base of tongue (12)	T3N0M0	Female	Caucasian	59	HPV-negative	never smoker	never drinker	Alive	none
Oropharynx (1)	T2N0M0	Male	Caucasian	48	HPV-negative	current smoker	former drinker	Alive	none
Oropharynx (2)	T4BN2BM0	Male	Caucasian	54	HPV-negative	former smoker	current drinker	Deceased	tongue, lymph nodes (head/face/neck) mouth

Oropharynx (3)	recurrent - stage unknown	Male	Caucasian	59	HPV-negative	current smoker *	current drinker *	Alive	primary - gum, 2 recurrences oropharynx
Tonsil (1)	T1N1M0	Male	Caucasian	46	HPV-16	never smoker	current drinker	Alive	met via PET scan - Level II LN
Tonsil (2)	T2N0M0	Female	Caucasian	54	HPV-16	unknown	unknown	Alive	none
Tonsil (3)	T2N0M0	Male	Caucasian	64	HPV-16	former smoker	never drinker	Alive	recurrence - base of tongue
Tonsil (4)	T2N0M0	Male	Caucasian	60	HPV-16	never smoker	never drinker	Alive	none
Tonsil (5)	T2N1M0	Male	Caucasian	49	HPV-16	snuff *	current drinker	Deceased	bones in skull/face, lung, bones in pelvic/sacrum
Tonsil (6)	T2N2	Male	Caucasian	50	HPV-16	former smoker	never drinker	Alive	none
Tonsil (7)	T2N2M0	Male	Caucasian	48	HPV-16	current smoker	current drinker	Deceased	none
Tonsil (8)	T2N2AM0	Male	Caucasian	61	HPV-16	former smoker	former drinker	Alive	none
Tonsil (9)	T2N2BM0	Male	Caucasian	50	HPV-16	current smoker	current drinker	Alive	none
Tonsil (10)	T2N2BM0	Female	Caucasian	56	HPV-16	current smoker *	current drinker	Alive	none
Tonsil (11)	TXN2BM0	Female	Caucasian	48	HPV-16	former smoker	current drinker	Alive	none
Tonsil (12)	T2N2BM0	Female	Caucasian	55	HPV-negative	current smoker *	current drinker *	Alive	none
Tonsil (13)	T2N2CM0	Male	Caucasian	63	HPV-16	current smoker *	current drinker *	Alive	none
Tonsil (14)	T2N3M0	Male	Caucasian	49	HPV-16	current smoker *	current drinker *	Alive	none
Tonsil (15)	T4N2BM0	Male	Caucasian	65	HPV-negative	current smoker *	current drinker *	Deceased	none
Tonsil (16)	T4N3M0	Male	Caucasian	65	HPV-negative	current smoker *	former drinker	Deceased	none
Tonsil (17)	T4AN2BM0	Male	Caucasian	57	HPV-negative	current smoker	current drinker	Deceased	none

Abbreviations: * unknown if quit

Table 5: Summary of SCCHN tissue characteristics

	HPV-16-positive	**HPV-negative**
Age, mean (SD)	55.6 (7.1)	64.2 (11.2)
Gender, *n* (%)		
Males	20 (83%)	12 (71%)
Females	4 (17%)	5 (29%)
Tumor location		
Mouth*	1	3
Tongue	1	3
Vallecula	1	0
Base of tongue	8	4
Oropharynx	0	3
Tonsil	13	4
Tumor stage		
T1NXMX	2	3
T2NXMX	21	5
T3NXMX	1	2
T4NXMX	0	6
Unknown	0	1

* Mouth samples include mouth, gum, and cheek.

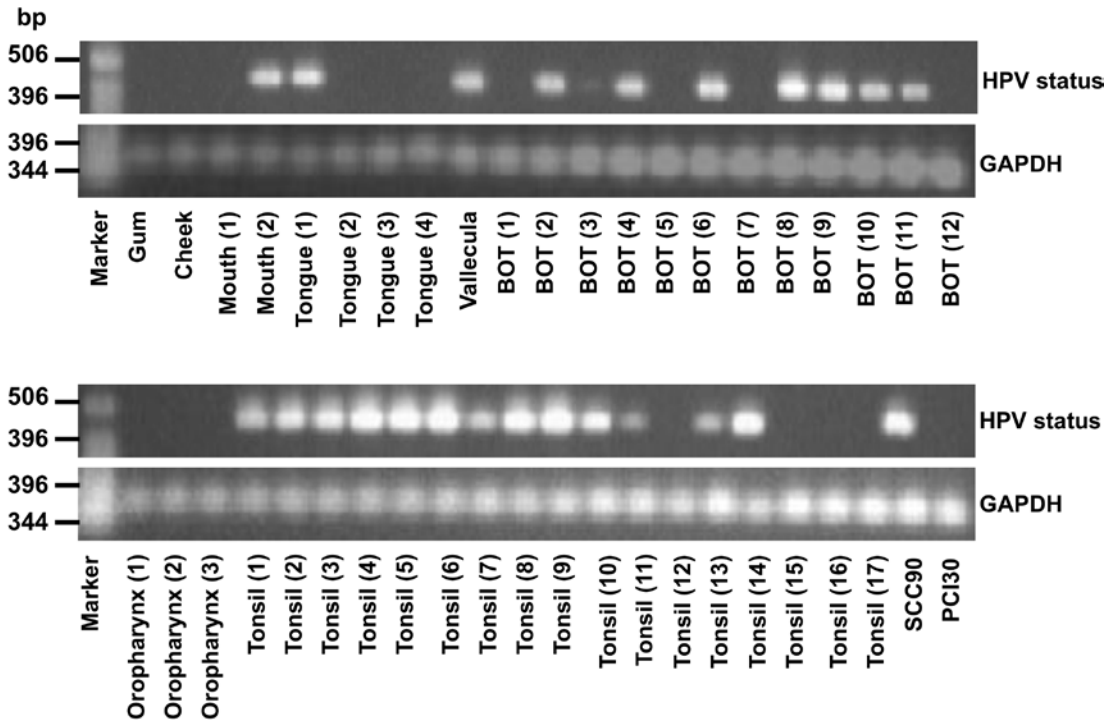

Figure 9: HPV status in SCCHN tissues

PCR analysis of all SCCHN tissues using the MY09/MY11 primer set to detect HPV positivity. SCC90, an HPV-positive SCCHN cell line control. PCI30, an HPV-negative SCCHN cell line control.

All HPV-positive samples were subsequently tested for HPV type via DNA PCR for the HPV-16 and HPV-18 E6 gene. All HPV-positive samples were found to contain HPV-16 DNA (Figure 10A). All of the HPV-positive tumor tissues were found to express the HPV-16 E6 and E7 oncogenes as determined by RT-PCR (Figure 10B).

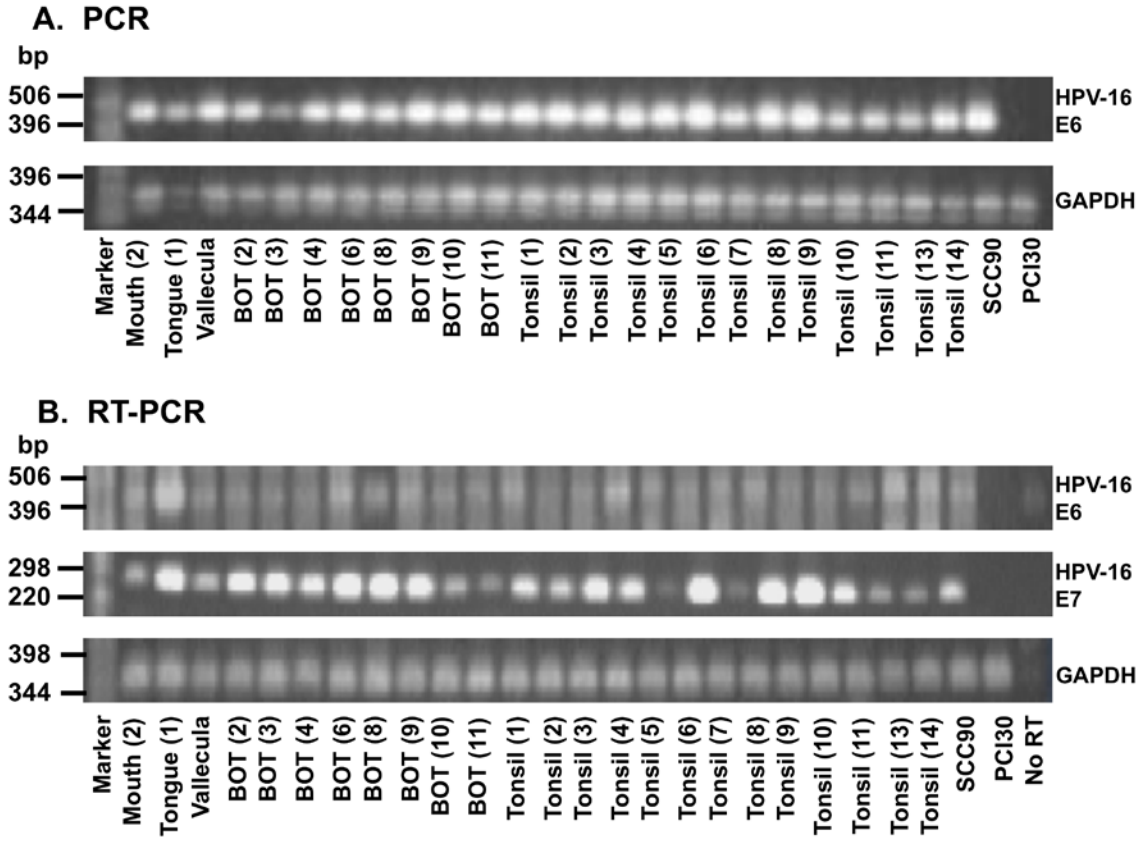

Figure 10: HPV-16 PCR and RT-PCR in SCCHN tissues

(A) PCR of HPV-16 E6 gene in HPV-positive SCCHN tissues. (B) RT-PCR of HPV-16 E6 and E7 in HPV-positive SCCHN tissues. SCC90, an HPV-16-positive SCCHN cell line positive control; PCI30, an HPV-negative SCCHN cell line negative control; No RT, no reverse transcriptase added (used SCC90 sample).

As expected, the site with the highest HPV positivity rate (via PCR) in our cohort was the tonsil, with 76 percent being HPV-positive, followed by tumors of the base of tongue, with 67 percent being HPV-positive (Table 5). The HPV-positive SCCHN cases of the tonsil and base of tongue represented approximately 88 percent of all HPV-positive tumors. The HPV-negative SCCHN cases had a more even distribution of the tumor sites, including the base of tongue, oropharynx, tonsil, mouth, and tongue (Table 5). The average age at diagnosis for patients with HPV-positive SCCHN was 55.6 years, or approximately nine years younger than for patients with HPV-negative SCCHN tumors, similar to other studies (Chaturvedi et al., 2008; Tran et al., 2007b). Ninety-six percent of HPV-positive SCCHN cases were classified as T1 or T2, whereas only 50 percent of HPV-negative SCCHN cases manifested small primary tumors (Table 5). These results are consistent with demographics and clinopathologic features reported for HPV-positive disease, supporting the reflection of our cohort with those reported in the literature (Chaturvedi et al., 2008).

3.3.2 MicroRNA-363 is Overexpressed in HPV-Positive SCCHN Tissues

We have previously reported that several miRNAs have altered expression in HPV-positive SCCHN cell lines compared to HPV-negative SCCHN cell lines, including the upregulation of miR-363 and downregulation of miR-181a, miR-218, and miR-29a (Wald et al., 2011). In that study, we also showed that differential expression of these miRNAs was dependent upon the expression of the HPV-16 E6 oncogene (Wald et al., 2011). We carried out qRT-PCR analysis of miR-363 expression in the HPV-positive and HPV-negative SCCHN samples. HPV-positive SCCHN samples expressed higher miR-363 levels compared to the HPV-negative SCCHN samples, with $p<0.01$ (Figure 11). These data are consistent with those obtained with the

SCCHN cell lines (Wald et al., 2011), indicating that our work with SCCHN cell lines has clinical relevance.

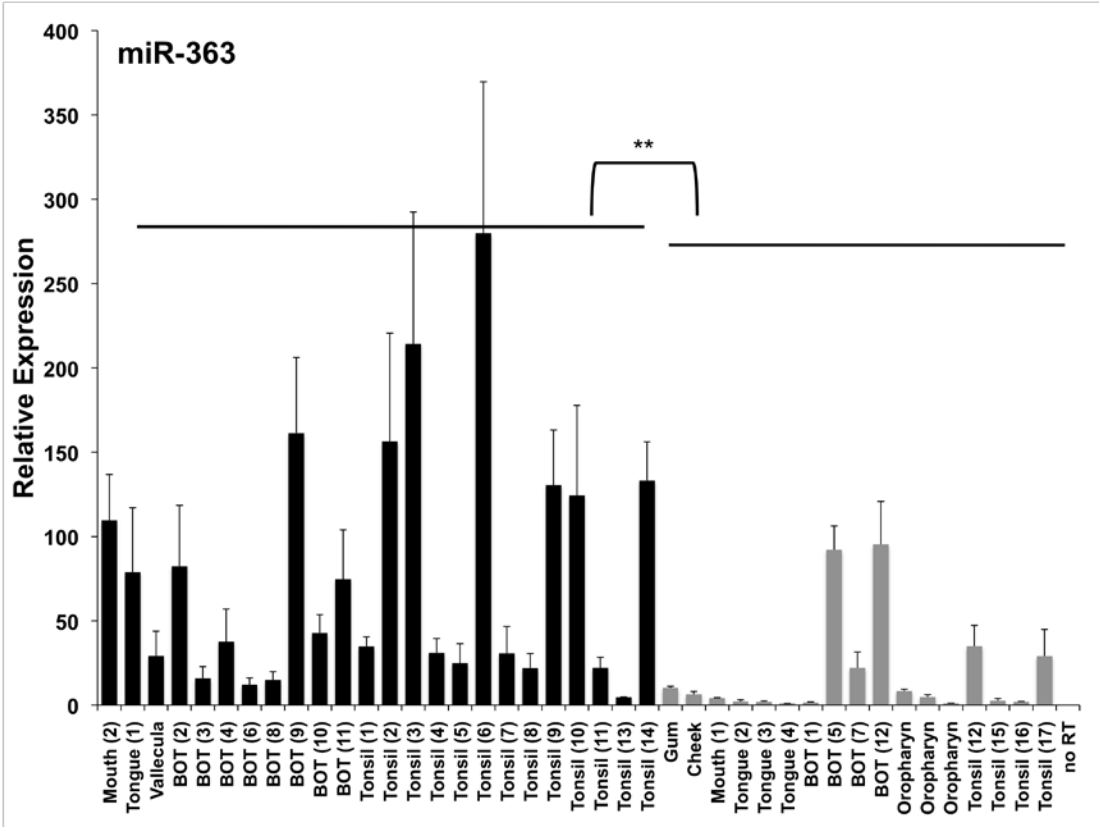

Figure 11: MicroRNA-363 expression in SCCHN tissues

QRT-PCR analysis of miR-363 in HPV-positive and HPV-negative SCCHN tissues. Black bars, HPV-16-positive SCCHN samples; gray bars, HPV-negative SCCHN samples. Intensity values are relative to the HPV-negative SCCHN sample with the lowest miR-363 expression, which was arbitrarily assigned a value of 1. The *p*-value for the HPV-positive samples compared to the HPV-negative samples is indicated by ** ($p<0.01$). BOT, base of tongue; no RT, no reverse transcriptase added.

MiR-363 has recently been shown to be overexpressed in HPV-positive pharyngeal squamous cell carcinoma (PSCC) tissues in a Danish population (Lajer et al., 2011). Lajer and colleagues found miR-363 to be the most overexpressed miRNA in 9 HPV-positive PSCC compared to 11 HPV-negative PSCC (Lajer et al., 2011). Interestingly, in their study, PSCC had an HPV-positivity rate of 42 percent, while OSCC (oral squamous cell carcinoma) had an HPV-positivity rate of only 3 percent. The PSCC tumors came from the tonsil, base of tongue, and other pharyngeal sites, whereas the OSCC samples came from the tongue, bucca, floor of mouth, gingiva, and other oral cavity sites (Lajer et al., 2011). This supports our finding that HPV-positivity rates are highest among the tonsil and base of tongue.

The association between high miR-363 expression in HPV-positive head and neck cancers can be further explored for possible targets of therapeutic intervention or biomarkers for prognosis.

4.0 MICRORNA-363 TARGETS MYSOIN 1B AND INCREASES THE PROLIFERATION AND COLONY FORMATION OF SCCHN CELL LINES

4.1 INTRODUCTION

Squamous cell carcinoma of the head and neck (SCCHN) is the sixth most common cancer in the United States (Jemal et al., 2010) and one of the leading cancers in developing countries (Parkin et al., 1999). Along with the well-established risk factors of alcohol and tobacco use, infection with high-risk human papillomavirus (HPV), most notably type-16, has emerged as a significant risk factor for SCCHN (Chaturvedi et al., 2008; Tran et al., 2007b). Characteristics of HPV-positive SCCHN differ from those of HPV-negative SCCHN, supporting the notion that these cancers should be classified as distinct tumor entities (Vidal and Gillison, 2008). HPV-positive SCCHN most often occurs in the oropharynx region (upwards of 50 percent or more), and is diagnosed in younger patients compared to HPV-negative SCCHN. Moreover, SCCHN tumors that are HPV-positive have infrequent p53 mutations while up to half of HPV-negative SCCHN tumors have p53 mutations (Vidal and Gillison, 2008). Patients with HPV-positive SCCHN tumors respond better to chemotherapy, radiation, and surgery, and have an overall 60 percent reduction in death from SCCHN than patients with HPV-negative SCCHN tumors (Tran et al., 2007b; Vidal and Gillison, 2008).

We have previously shown that micro (mi) RNA profiles in HPV-positive SCCHN cell lines differ from HPV-negative SCCHN cell lines, and some miRNAs are dysregulated as a direct result of the HPV-16 E6 and E7 oncogene expression (Wald et al., 2011). Furthermore miR-363, which was the most highly overexpressed miRNA in HPV-positive SCCHN cell lines compared to HPV-negative SCCHN cell lines, also shows this trend in a subset of HPV-positive and HPV-negative SCCHN tumors from the western Pennsylvania population. In this study, we

sought to identify the possible mechanisms by which miR-363 may contribute to SCCHN pathogenesis. We show that miR-363 targets myosin 1b, which reduces the migratory ability of SCCHN cells. Additionally, miR-363 increases the proliferation and colony formation of SCCHN cells.

4.2 MATERIALS AND METHODS

4.2.1 Cell Culture and Transfections

The HPV-negative SCCHN cell line PCI-30 was grown in Dulbecco's modified Eagle's medium (Lonza, Walkersville, MD) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 2% L-glutamine at 37°C in the presence of 5% CO_2. The HPV-negative SCCHN cell line JHU-028 was grown in Roswell Park Memorial Institute medium (Lonza) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 2% L-glutamine at 37°C in the presence of 5% CO_2.

HPV-negative SCCHN cells PCI-30 or JHU-028 (1.5×10^5) were seeded in 6-well plates in antibiotic-free media. The following day, cells were transfected with 50 nM pre-miR (Applied Biosystems, Foster City, CA) or 50 nM siRNA against MYO1B (ThermoFisher Scientific, San Jose, CA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and Opti-mem (Gibco, Grand Island, NY). A FAM-labeled negative control pre-miR (Applied Biosystems) or the Block-it fluorescent oligo (Invitrogen) was used as the negative controls and to measure transfection efficiency. Cells were harvested 48 hours after transfection and RNA and proteins were isolated for various assays.

4.2.2 RNA Isolation

Total RNA was isolated from cell lines using the Ultraspec RNA Isolation System (Biotecx, Houston, TX) according to the manufacturer's instructions.

4.2.3 Quantitative Real Time RT-PCR

To confirm the efficiency of miR-363 transfections, miRNA expression was measured using the TaqMan MicroRNA Reverse Transcription Kit and the TaqMan MicroRNA Assays (Applied Biosystems) and the Real-Time Thermocycler iQ5 (Bio-Rad, Hercules, CA). Total RNA (50 μg) was used for each reaction, and all of the reactions were performed in triplicate according to the manufacturer's instructions. MiRNA levels were normalized to small nucleolar RNU43 levels and relative expression was calculated using the $2^{-\Delta\Delta CT}$ values (Livak and Schmittgen, 2001). Gene expression (mRNA) levels were measured using the iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad). The MYO1B gene was amplified using the forward primer 5′-GGTCTGGTGTGGAGGTCCTA-3′ and the reverse primer 5′-CGTTGCTTCCTCAGGTCTTC-3′, amplifying a 127 bp region. The MYO1B mRNA levels were normalized to the GAPDH mRNA levels, using the forward primer 5′-CAGCCTCAAGATCATCAGCA-3′ and the reverse primer 5′-TGTGGTCATGAGTCCTTCCA-3′ which amplifies a 106 bp region. DNase-I-treated total RNA (50 ng) was used for each reaction, and all the reactions were done in triplicate. MYO1B mRNA was normalized to GAPDH, and the relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ values (Livak and Schmittgen, 2001)

4.2.4 Spectral Counting Proteomics

The HPV-negative SCCHN cell line PCI-30 was transfected as described above with a negative control pre-miR or pre-miR-363. After 48 hours, cells were lysed in 50 mM Tris-HCl, pH 7.4, 0.5% SDS, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF) by boiling for 10 minutes, cooling to ambient temperature and then sonicating three times. Protein quantitation was performed using the BCA Assay (Pierce, Rockford, IL) and 30 mg of protein was loaded onto a 10% Bis-Tris gel and run until the proteins entered the stacking portion of the gel. The gel bands for each sample were excised and diced into approximately 1 mm^2 sections and destained in 25 mM NH_4HCO_3/50% acetonitrile (ACN) with shaking. Gel bands were dehydrated by addition of neat ACN and then dried in a 37°C incubator.

Proteins in the gel bands were reduced in 25 mM NH_4HCO_3, 10 mM dithiothreitol (DTT) at 56°C for 1 hour followed by dehydration with ACN as before, and then alkylation was carried out in 25 mM NH_4HCO_3, 55 mM iodoacetamide for 45 minutes in the dark at ambient temperature followed by dehydration. Gel bands were rehydrated with trypsin (Promega, porcine sequencing grade, 20 ng/ml) on ice for 45 minutes. Supernatant was removed and the gel bands were washed once with 500 μl of 25 mM NH_4HCO_3, and then the gel bands were covered with 25 mM NH_4HCO_3, (~100 μl) and incubated at 37°C overnight. The digest solution was transferred into a clean microcentrifuge tube and peptides were extracted from the gel pieces with sonication in a water bath for 10 minutes in 75 μl of 50% ACN/5% formic acid. The supernatant was removed and added to the previous solution and this was repeated two additional times. The combined extract was lyophilized to dryness and stored at -80°C until analysis.

Samples were analyzed by nanoflow reverse-phase liquid chromatography (Ultimate 3000, Dionex, Sunnyvale, CA) coupled online via electrospray ionization to a hybrid linear ion trap-Orbitrap mass spectrometer (LTQ-Orbitrap, ThermoFisher Scientific, San Jose, CA). Five replicate injections of peptide extracts were resolved on 100 μm i.d. by 360 μm o.d. by 200 mm long fused silica capillary columns (Polymicro Technologies, Phoenix, AZ) slurrypacked in-house with 5 μm, 300 Å pore size C-18 silica-bonded stationary phase (Jupiter, Phenomenex, Torrance, CA). After sample injection, peptides were eluted from the column using a linear gradient of 2% mobile phase B (0.1% formic acid in ACN) to 40% mobile phase B over 125 minutes at a constant flow rate of 200 nl/minutes followed by a column wash consisting of 95% B for an additional 30 minutes at a constant flow rate of 400 nl/minute. The column was then re-equilibrated in mobile phase A (0.1% formic acid in water) for 20 minutes prior to the next injection. Eluting peptides were ionized using an electrospray voltage of 1.8 kV and analyzed in the LTQ-Orbitrap MS which was configured to collect high resolution (R=60,000 at *m/z* 400) broadband mass spectra (*m/z* 375-1800) from which the seven-most abundant peptide molecular ions dynamically determined from the MS scan were selected for tandem MS using a relative collision-induced dissociation (CID) energy of 30%. Dynamic exclusion was utilized to minimize redundant selection of peptides for CID.

Peptide identifications were obtained by searching the LC-MS/MS data utilizing SEQUEST (BioWorks, v3.2, ThermoFisher Scientific) on a 72-node Beowulf cluster against a UniProt-derived human proteome database (11/2009 release) obtained from the European Bioinformatics Institute (EBI) using the following parameters: trypsin (KR); full enzymatic cleavage; two missed cleavage sites; 20 ppm peptide mass tolerance peptide tolerance, 0.5 amu fragment ion tolerance; and variable modifications for methionine oxidation (*m/z* 15.99492) and

cysteine carboxyamidomethylation (*m/z* 57.02146). Resulting peptide identifications were filtered according to specific SEQUEST scoring criteria: delta correlation (DCn) ^{3} 0.08 and charge state dependent cross correlation (XCorr) scores of ^{3} 1.9 for $[M+H]^{1+}$, ^{3} 2.2 for $[M+H]^{2+}$, and ^{3} 3.5 for $[M+H]^{3+}$. These criteria resulted in a false discovery rate of <1% for all peptides identified as determined by searching the data against a decoy human database where the protein sequences were reversed. The differences in protein abundance between the samples were derived by spectral counting.

4.2.5 Western Blotting

Proteins (25 μg) from total cell lysates from the HPV-negative SCCHN cell line PCI-30 transfected with pre-miR-363 were separated on a 10% SDS polyacrylamide gel and transferred to Immobilion-P PVDF membrane (Millipore, Billerica, MA). The membrane was incubated with a primary rabbit polyclonal antibody against MYO1B (Santa Cruz Biotechnology, Santa Cruz, CA), and subsequently incubated with a 1:10,000 dilution of the secondary anti-rabbit horseradish peroxidase antibody (Amersham Biosciences, Piscataway, NJ). Blots were visualized using chemiluminescence with the ECL Plus Western Blotting Detection Reagents (Amersham Biosciences), followed by film exposure. A murine monoclonal antibody against GAPDH (Chemicon, Billerica, MA) was used to show equal sample loading.

4.2.6 Cloning of the MYO1B 3′ UTR into pMiR-Report Luciferase Vector

The MYO1B 3′ UTR (1429 bp) was PCR amplified from the HPV-positive SCCHN cell line SCC90 using the forward primer 5′-GGACTAGTAACCGTCTCCTTGAAGTTGC-3′ and the

reverse primer 5′-GGAAGCTTGGCACAAGGCAAGAAGAATC-3′, amplifying a 1,725 bp region. These primers were designed with an added *Spe*I restriction site on the forward primer and a *Hin*dIII restriction site on the reverse primer to aid in directional cloning. PCR products were separated on a 1% agarose gel, and the DNA was isolated using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). Three micrograms of each of the purified PCR products and the pMiR-Report luciferase vector (Applied Biosystems) were digested with *Spe*I and *Hin*dIII at 37°C for two hours. The digested DNA was separated on a 1% agarose gel, purified using the QIAquick Gel Extraction Kit (Qiagen), and quantified using the Nanodrop. The digested MYO1B 3′ UTR and pMiR-Report vector were ligated at a 1:3 (vector:insert) ratio at 16°C for 16 hours. DNA was precipitated and the ligated plasmid was electroporated into competent DH5α cells. After a one-hour recovery, cells were plated on LB + ampicillin plates and incubated at 37°C overnight. Ten colonies were patched on LB + ampicillin plates and incubated at 37°C overnight. A lysates gel was run to determine the presence of the pMiR-Report:MYO1B 3′ UTR plasmid. Selected colonies that contained the plasmid were grown in LB broth + ampicillin at 37°C overnight and the pMiR-Report:MYO1B 3′ UTR plasmids were extracted using the Wizard Plus Minipreps DNA Purification System (Promega, Madison, WI). The plasmids were subsequently digested with *Hin*dIII and *Bam*HI for 2 hours at 37°C and run on a 1% agarose gel to confirm the insertion of the appropriate DNA fragment.

4.2.7 Luciferase Assays

HPV-negative JHU-028 SCCHN cells (10,000 cells per well) were seeded in a 96-well plate in antibiotic free media. After 24 hours, cells were transfected using Lipofectamine 2000

(Invitrogen) and Opti-mem (Gibco) with the following nucleic acids: (A) 0.1 μg pMiR-Report + 2.5 ng phRL-TK, (B) 0.1 μg pMiR-Report + 2.5 ng phRL-TK + 50 nM negative pre-miR, (C) 0.1 μg pMiR-Report + 2.5 ng phRL-TK + 50 nM pre-miR-363, (D) 0.1 μg pMiR-Report:MYO1B 3′ UTR + 2.5 ng phRL-TK, (E) 0.1 μg pMiR-Report:MYO1B 3′ UTR + 2.5 ng phRL-TK + 50 nM negative pre-miR, or (F) 0.1 μg pMiR-Report:MYO1B 3′ UTR + 2.5 ng phRL-TK + 50 nM pre-miR-363. After 48 hours, luciferase activity was measured using the Dual-Luciferase Reporter Assay (Promega) and the Synergy 2 Luminometer.

4.2.8 Transwell Migration Assays

The HPV-negative SCCHN cell line JHU-028 was transfected with pre-miR-363 or an siRNA against MYO1B as described above. Forty-eight hours after transfection, cells were harvested and re-seeded into 24-well Transwell inserts, 8 μm pore size (Corning) in media depleted of serum. The bottom chambers of the transwell plate were filled with media containing 20% serum to serve as a chemoattractant. Transwells were stained with 0.1% crystal violet at 1, 3, and 5 hours post-plating and the cells were counted and photographed under a microscope. The mean of eight fields from four separate assays was used to determine the average number of migratory cells.

4.2.9 Apoptosis Assay

The HPV-negative SCCHN cell line JHU-028 was transfected with pre-miR-363 as described above. Forty-eight hours after transfection, live cells, dead cells, and apoptotic cells were analyzed using the AlexaFluor 488 Annexin V/Dead Cell Apoptosis Kit with AlexaFluor 488

annexin V and PI for Flow Cytometry (Invitrogen) according to the manufacturer's instructions on the BD FACS Canto (BD Biosciences, San Jose, CA).

4.2.10 Cell Cycle Analysis by Flow Cytometry

The HPV-negative SCCHN cell line JHU-028 was transfected with pre-miR-363 as described above. Forty-eight hours after transfection, cells were harvested and fixed in 70% ethanol overnight at 4°C. Subsequently, the cells were washed once with 1X PBS and stained with propidium iodide for four hours and subjected to flow cytometry using the BD FACS Canto (BD Biosciences).

4.2.11 MTT Assay

The HPV-negative SCCHN cell line JHU-028 was transfected with pre-miR-363 as described above. Twenty-four hours post-transfection, cells were harvested and re-seeded at 2,000 cells per well of a 96-well plate. After an additional 24 hours (48 hours post-transfection), cells were incubated with 20 μl of the MTT reagent (5mg/ml) at 37°C for 3 hours. Subsequently, growth media and MTT reagent were removed from the wells, and 100 μl dimethyl sulfoxide was added and the plate was incubated at 37°C for 5 minutes on a shaker. Absorbance was immediately measured at 490 nm.

4.2.12 Cell Counting

The HPV-negative SCCHN cell line JHU-028 was transfected with pre-miR-363 as described above. Cells were harvested and counted at 0, 24, 48, and 72 hours post-transfection. The mean of three wells of cells (per condition) was used to determine the average number of live cells. The number of live cells is relative to the zero hour time-point, which was set at 100%.

4.2.13 *In-vitro* Wound Healing Assay

The HPV-negative SCCHN cell line JHU-028 was transfected with pre-miR-363 as described above. After 24 hours, the transfected cells were harvested and re-seeded to confluency in a 24-well plate. After an additional 24 hours (48 hours post-transfection), a scratch/wound was made using a sterile pipet tip that cleared ~500 μm wide gap in the confluent cells. The wound width was measured and photographed every four hours until closed.

4.2.14 Colony Formation Assay

The HPV-negative SCCHN cell line PCI-30 was transfected with pre-miR-363 as described above. Cells were harvested forty-eight hours post transfection and re-suspended at 20,000 cells/ml in 0.3% agarose/media solution. Two milliliters of the cell suspension was plated on top of 2 ml of solidified 0.5% agarose/media solution in a 6-well plate. The 0.3% agarose/media/cell suspension was incubated at room temperature for one hour to allow the agarose to solidify, and then the 6-well plate was incubated at 37°C in 5% CO_2. Every two days, 500 μl of media was added to each well to prevent the gel from drying. After 21 days, cells were stained with 0.5%

crystal violet and visualized under a dissecting microscope. The mean number of colonies of three wells of a six-well plate was used to determine the average number of colonies formed per condition.

4.3 RESULTS

4.3.1 MicroRNA-363 Targets Myosin 1B in HPV-Positive SCCHN

Since a miRNA can potentially regulate dozens of genes (Lim et al., 2005), we used gene expression profiling data (Martinez et al., 2007), spectral counting proteomics, and computationally predicted miRNA target databases to identify the potential gene target(s) of miR-363 in SCCHN. Since we have previously shown that miR-363 is overexpressed in HPV-positive SCCHN compared to HPV-negative SCCHN ((Wald et al., 2011) and unpublished data), the list of computationally predicted targets of miR-363 was compared with genes down-regulated in HPV-positive oropharyngeal squamous cell carcinoma (OPSCC) compared to HPV-negative OPSCC (Martinez et al., 2007), and with proteins downregulated by more than two-fold in HPV-negative SCCHN cells overexpressing miR-363 compared to HPV-negative SCCHN cells expressing a negative control miRNA.

The HPV-negative SCCHN cell line PCI-30 had 35 proteins downregulated by at least 50 percent upon exogenous overexpression of miR-363 compared to the same cell line expressing a negative control miRNA (Table 6). These proteins were considered potential miR-363 targets from the proteomics data, along with the 150 downregulated genes in HPV-positive OPSCC compared to HPV-negative OPSCC that were identified in a previously published study

(Martinez et al., 2007), for a total of 185 potential miR-363 targets. Interestingly, only the myosin 1b gene was found to be similarly downregulated between the proteomics and gene expression data.

Table 6: Proteins downregulated in cells overexpressing miR-363 via spectral counting proteomics

Protein	Number of peptides identified in PC1-30 + neg control	Number of peptides identified in PC1-30 + miR-363	Ratio
Isoform 1 of Myosin-Ic	26	52	0.50
Enoyl-CoA hydratase, mitochondria	11	23	0.49
Isoform Alpha-3B of Integrin alpha-3	8	17	0.49
Peroxisomal multifunctional enzyme type 2	7	15	0.48
Isoform A of Bifunctional 3'-phosphoadenosine 5'-phosphosulfate synthetase 2	4	9	0.47
Isoform Alpha of Signal transducer and activator of transcription 1-alpha/beta	13	28	0.47
Isoform 1 of Hepatocyte growth factor-regulated tyrosine kinase substrate	3	7	0.47
Isoform 1 of Myosin-Ib	3	7	0.47
CAAX prenyl protease 1 homolog	3	7	0.47
ADP/ATP translocase 2	10	22	0.47
Guanine nucleotide-binding protein G(k) subunit alpha	3	7	0.47
Ras-related protein Ral-A	3	7	0.47
Acetyl-CoA acetyltransferase, mitochondrial	10	22	0.47
60S ribosomal protein L27	3	7	0.47
Protein transport protein Sec23A	3	7	0.47
Isoform 1 of Myb-binding protein 1A	3	7	0.47
Isoform 1 of Arylacetamide deacetylase-like 1	12	27	0.45
Splicing factor 3B subunit 1	6	14	0.45
Isoform 1 of Neural cell adhesion molecule L1	5	12	0.44

Isoform 1 of ATPase family AAA domain-containing protein 3A	5	12	0.44
Isoform 2 of Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5	7	17	0.43
Isoform 1 of Nuclear pore complex protein Nup155	3	8	0.41
Aconitate hydratase, mitochondrial	3	8	0.41
Probable ATP-dependent RNA helicase DDX47	3	8	0.41
Protein tyrosine phosphatase-like protein PTPLAD1	3	8	0.41
FACT complex subunit SPT16	7	18	0.41
Isoform 1 of Cytoplasmic FMR1-interacting protein 1	4	11	0.39
Isoform 1 of MAP7 domain-containing protein 1	3	9	0.37
Tyrosine-protein phosphatase non-receptor type 1	5	16	0.33
Isoform 1 of Fragile X mental retardation syndrome-related protein 1	4	13	0.33
Interferon-induced protein with tetratricopeptide repeats 1	3	12	0.28
Guanine nucleotide-binding protein G(i), alpha-1 subunit	4	16	0.27
Isoform 1 of CUB domain-containing protein 1	3	13	0.26
Inositol 1,4,5-trisphosphate receptor type 3	5	21	0.26
Isoform 3 of Ribosome-binding protein 1	5	25	0.22

The 35 proteins from Table 6 and 150 downregulated genes from our previous study (Martinez et al., 2007) were cross-referenced with computationally predicted miR-363 targets obtained from the miRanda and TargetScan databases. This cross-comparison resulted in six potential targets based upon proteomics data and ten potential targets based upon the gene expression data, with one potential miR-363 target in common, myosin 1b (MYO1B, Figure 12).

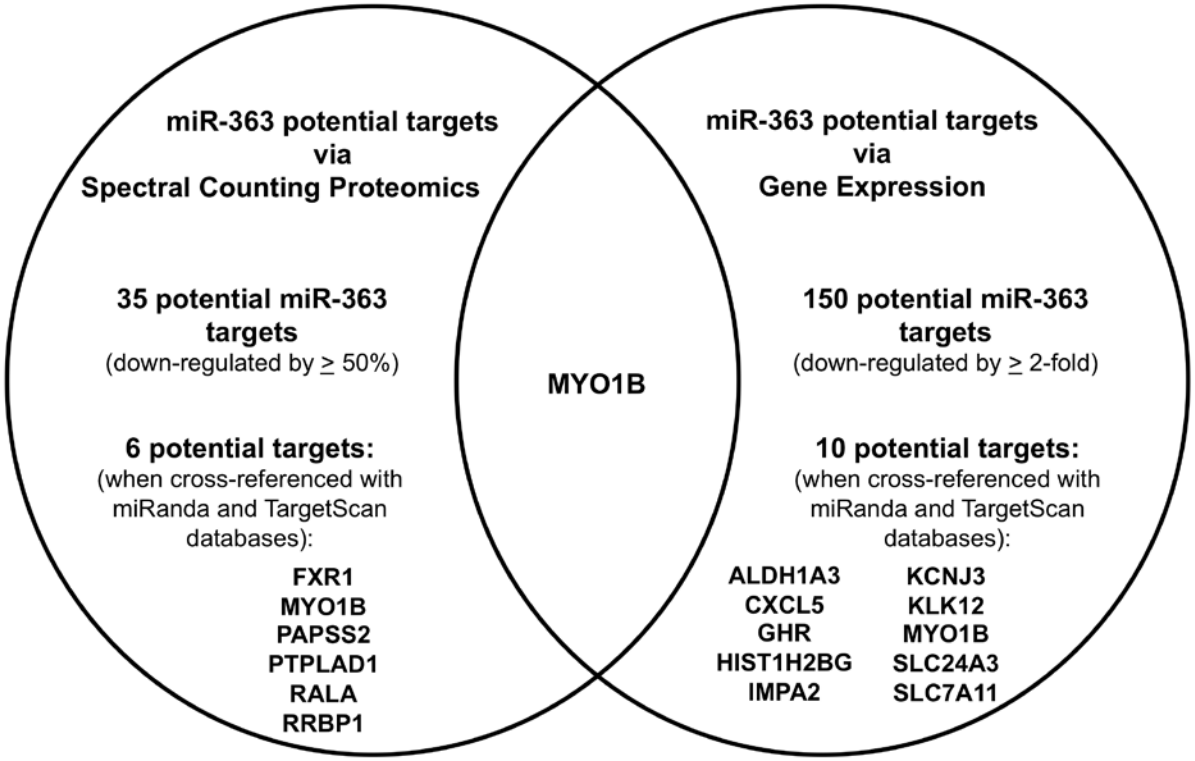

Figure 12: Venn diagram of potential miR-363 targets

The 3′ UTR of MYO1B contains two predicted binding sites for miR-363, and since MYO1B expression at both the mRNA and protein levels was reduced upon miR-363 overexpression (Figure 13), it was considered a likely target of miR-363.

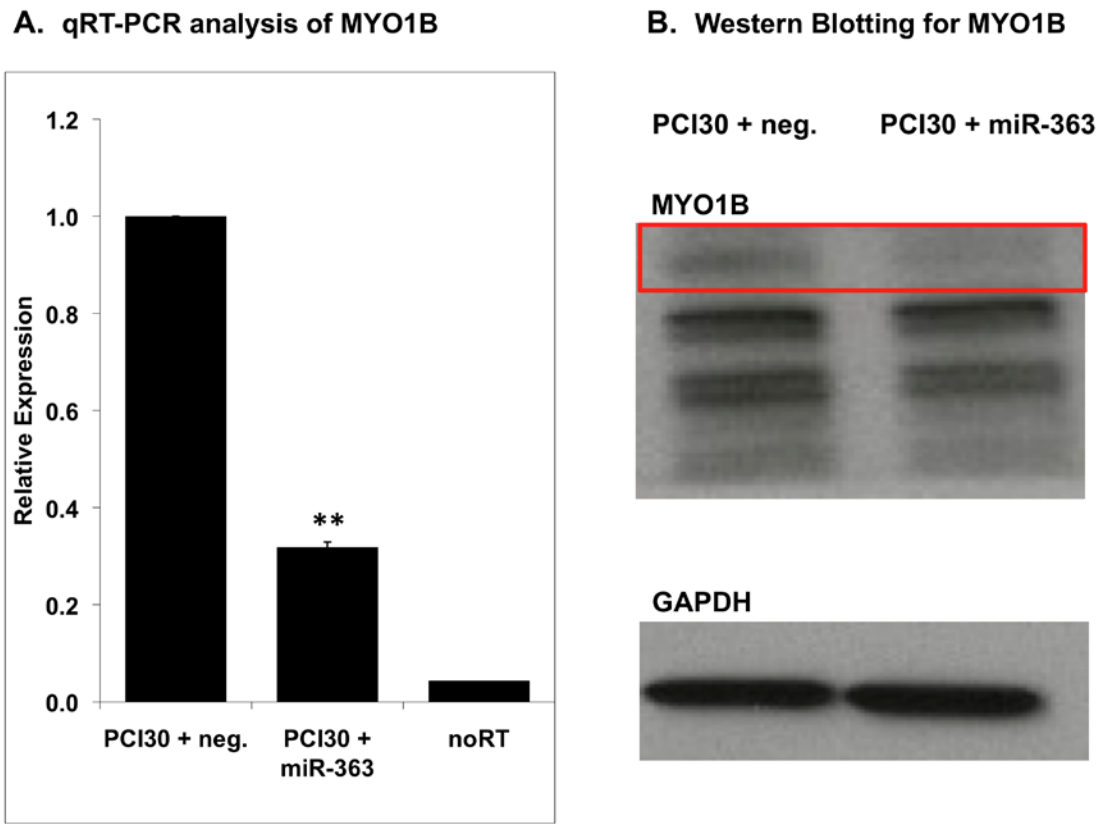

Figure 13: MYO1B expression is reduced upon miR-363 expression

HPV-negative SCCHN cell line PCI30 transfected with either a negative control miRNA or miR-363. (A) qRT-PCR for MYO1B at mRNA level. (B) Western blot for MYO1B at protein level. The *p* value for PCI30 + miR-363 compared to PCI30 + neg. is indicated by ** ($p < 0.01$).

To test whether MYO1B was a direct target of miR-363, we cloned the 3′ UTR of MYO1B downstream of the firefly luciferase gene in the pMiR-Report vector. A dual luciferase assay, where the firefly luciferase was normalized to the renilla luciferase, showed that the firefly luciferase activity was reduced when miR-363 was overexpressed but not when a control miRNA that has no binding sites in the MYO1B 3′ UTR was expressed (Figure 14). These results suggest that miR-363 binds directly to the 3′ UTR of MYO1B and represses its expression.

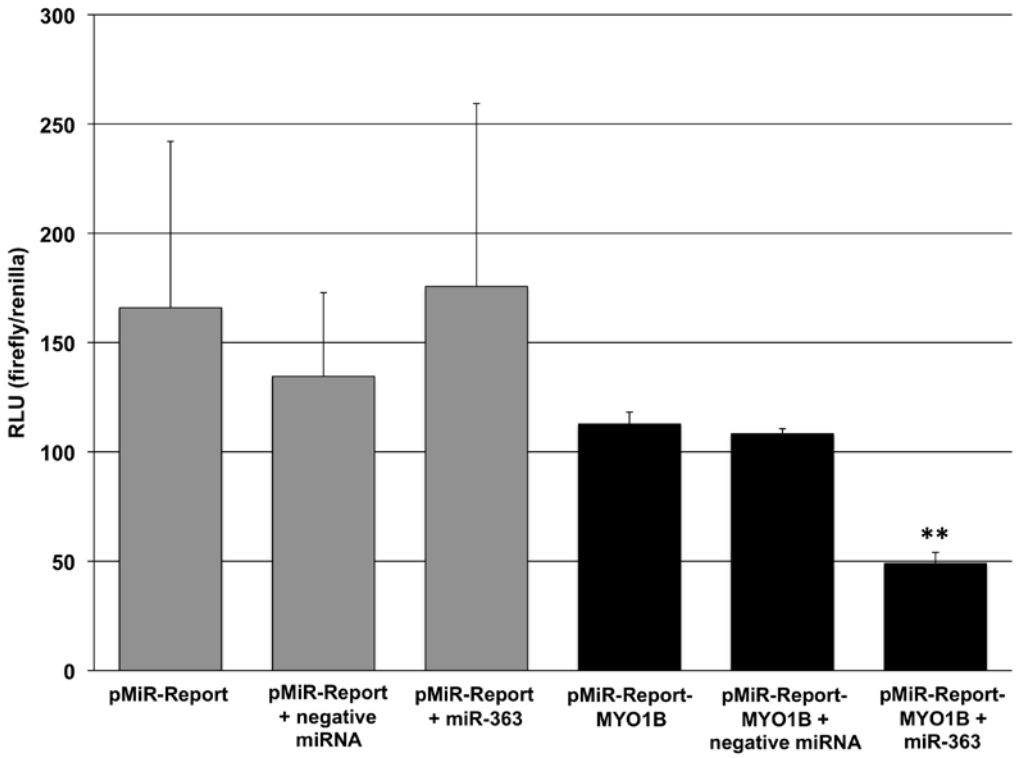

Figure 14: MYO1B is a direct target of miR-363 in SCCHN

Dual luciferase assay of HPV-negative SCCHN cells transfected with reporter vectors with or without the MYO1B 3′UTR cloned downstream of firefly luciferase. The *p* value for pMiR-Report-MYO1B + miR-363 compared to pMiR-Report-MYO1B or pMiR-Report-MYO1B + miR-363 compared to pMiR-Report-MYO1B + negative miRNA is indicated by ** ($p < 0.01$).

4.3.2 MicroRNA-363 Reduces the Migratory Ability of HPV-Negative SCCHN Cells

Since myosin proteins are associated with cellular migration amongst other functions (Edgar et al., 1996; Evangelista et al., 2000), we wished to investigate the effect of miR-363 on cellular migration. Transwell migration assays showed that there was a significant decrease in the number of migratory HPV-negative SCCHN cells when miR-363 was overexpressed compared to the same cells that do not express miR-363 (Figure 15). Since miR-363 targets MYO1B in SCCHN (Figure 14) and MYO1B is involved in cell migration (Edgar et al., 1996; Evangelista et al., 2000), we wanted to test whether reduction in MYO1B levels directly reduce cell migration. Knockdown of MYO1B via siRNA in HPV-negative SCCHN cells showed reduced cell migration compared to cells expressing a negative control siRNA (Figure 16). These results indicate that high miR-363 expression (which would reduce MYO1B levels) as well as reduction in MYO1B levels by siRNA knockdown reduce cell migration (Figures 15 and 16).

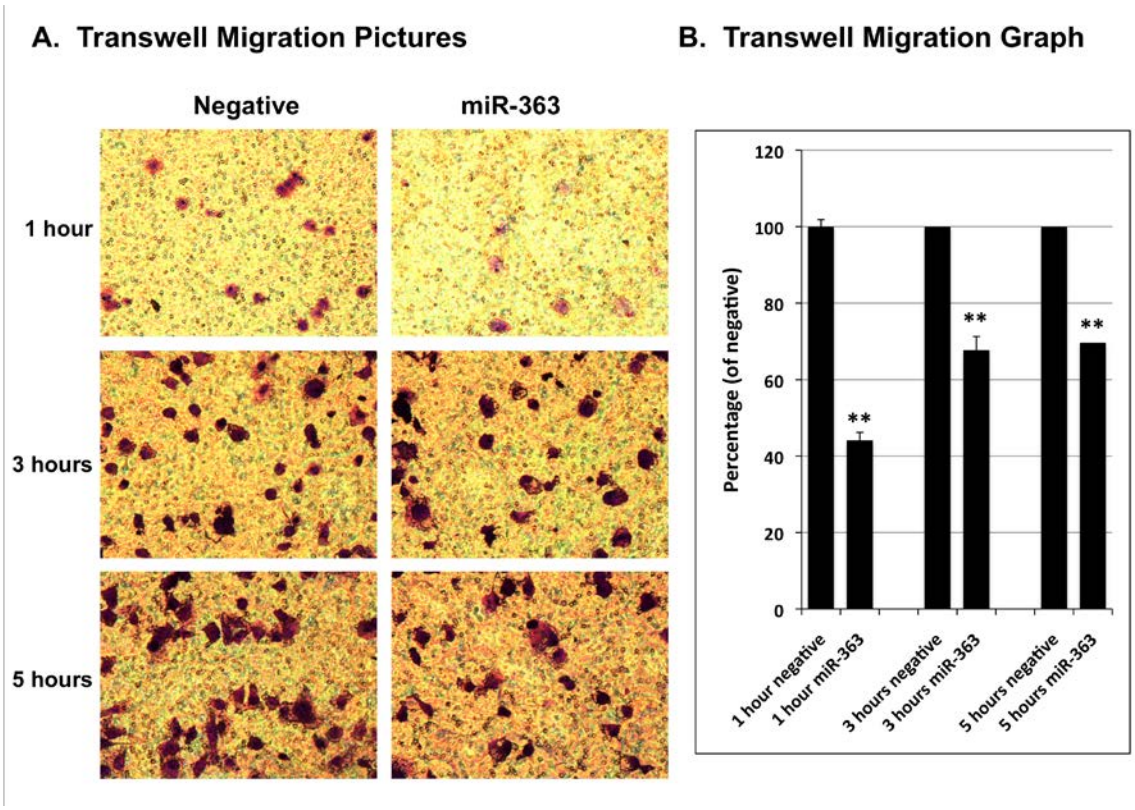

Figure 15: MiR-363 reduces the migratory ability of HPV-negative SCCHN cells

HPV-negative SCCHN cell line JHU-028 transfected with either a negative control miRNA or miR-363. (A) Transwell migration pictures at specified time points. (B) Graph of migratory cells. The *p* value for cells expressing miR-363 compared to cells expressing a negative miRNA is indicated by ** ($p < 0.01$).

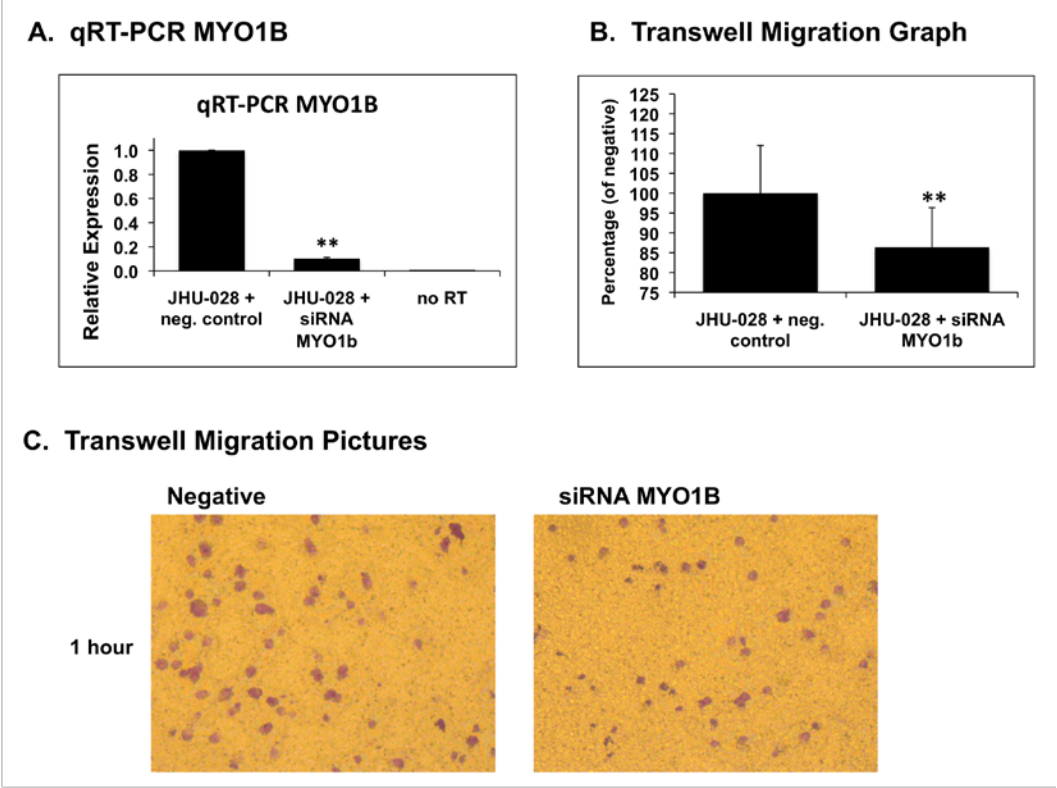

Figure 16: siRNA knockdown of MYO1B reduces cell migration in HPV-negative SCCHN cells

HPV-negative SCCHN cell line JHU-028 transfected with either a negative control miRNA or miR-363. (A) qRT-PCR of MYO1B expression post-transfection. (B) Graph of migratory cells. (C) Transwell migration pictures. The *p* value for cells expressing miR-363 compared to cells expressing a negative miRNA is indicated by ** ($p < 0.01$).

4.3.3 MicroRNA-363 Increases Proliferation of HPV-Negative SCCHN Cells

In order to determine the functional role of miR-363 in SCCHN, we carried out apoptosis, cell cycle, and proliferation assays. We observed no difference in the apoptosis of HPV-negative SCCHN cells (JHU-028 and PCI-30) overexpressing miR-363 compared to cells lacking miR-363 expression (data not shown). There was a slight, but not statistically significant, increase in the percentage of cells in the S-phase of the cell cycle when HPV-negative SCCHN cells (JHU-

028 and PCI-30) overexpressed miR-363 compared to the same cells expressing a negative control miRNA (data not shown). However, miR-363 overexpression in HPV-negative SCCHN cells (JHU-028) increased the proliferation capacity of these cells as measured via MTT assay and cell counting (Figure 17). Furthermore, growth of miR-363-transfected cells in serum-depleted media either had the same effect as cells grown in the normal media, or exacerbated the effect seen in cells grown in the normal media (Figure 17).

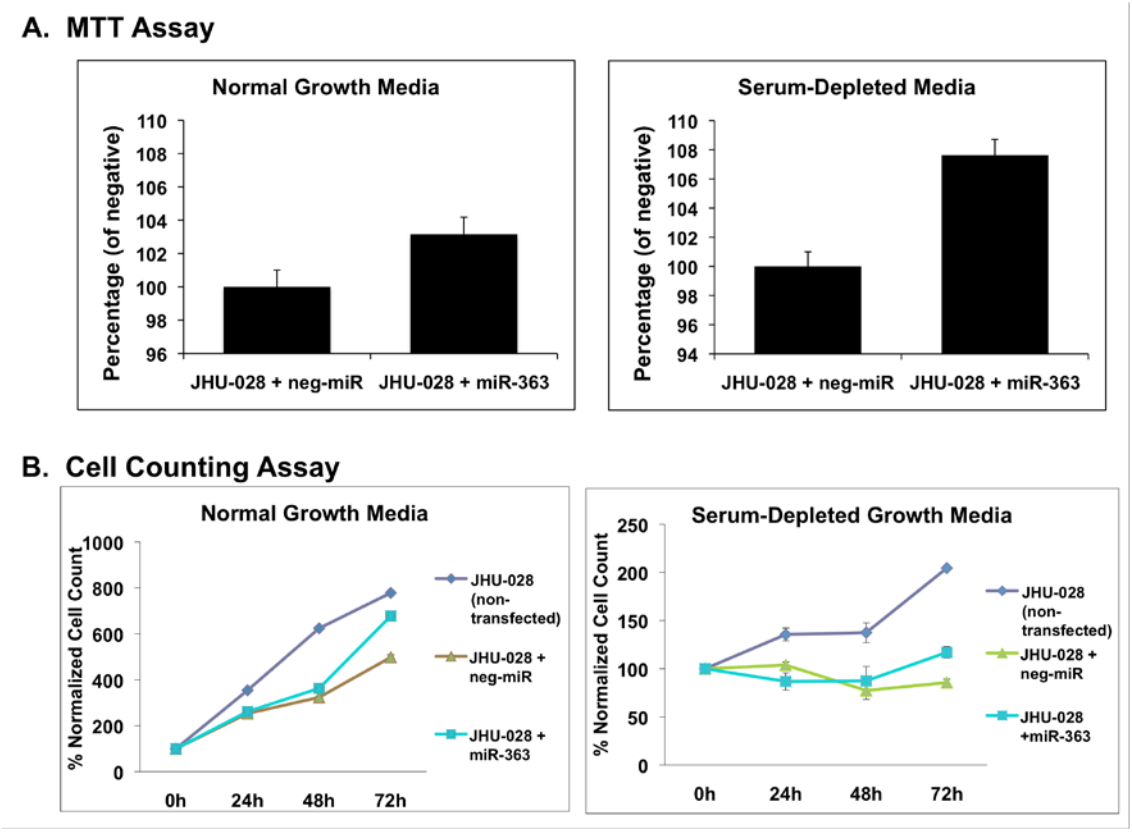

Figure 17: MicroRNA-363 increases the proliferation of HPV-negative SCCHN cells

HPV-negative SCCHN cell line JHU-028 transfected with either a negative control miRNA or miR-363. (A) MTT assay of transfected cells grown in normal media (left) or serum-depleted media (right). (B) Cell counting assay of transfected cells grown in normal media (left) or serum-depleted media (right).

Furthermore, an *in vitro* wound healing assay showed that miR-363 enhanced the ability of HPV-negative SCCHN cells to close the gap/lesion at a faster rate than in the presence of a control miRNA (Figure 18).

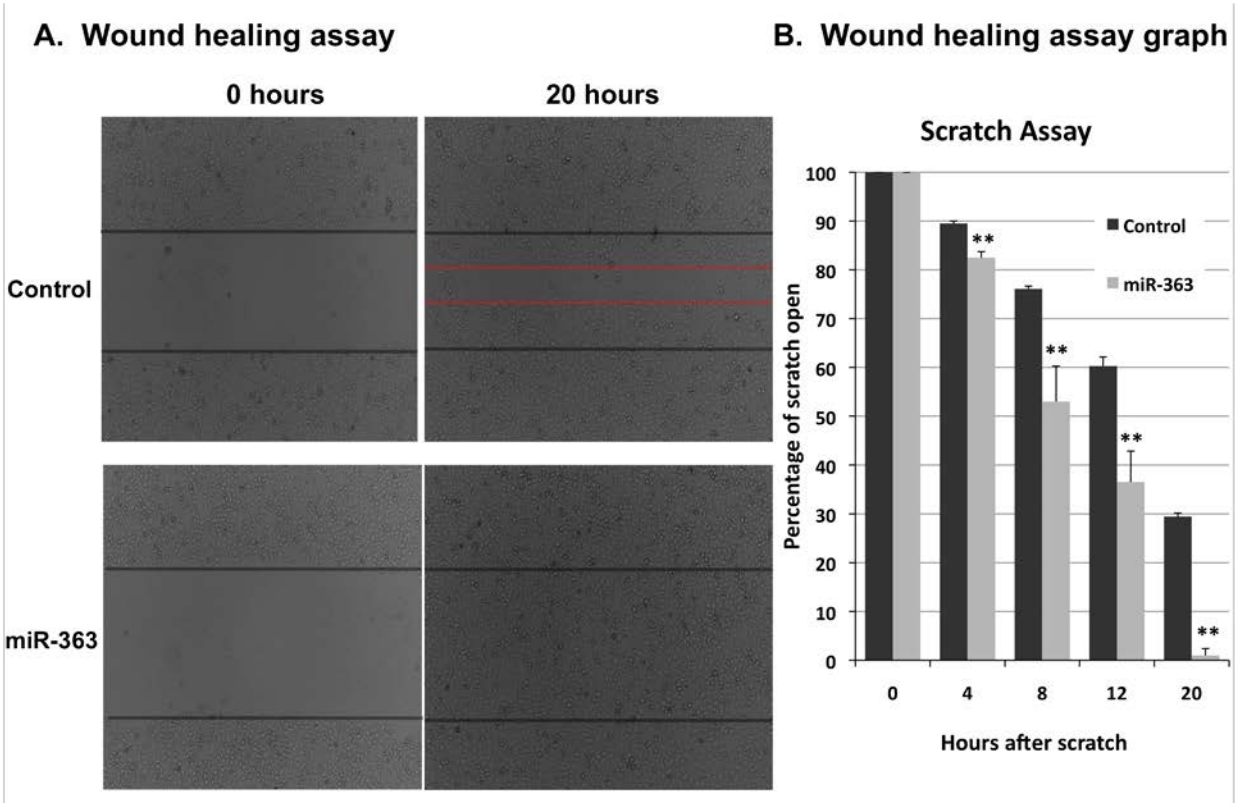

Figure 18: MicroRNA-363 enhances the ability of HPV-negative SCCHN cells to close a gap/wound

HPV-negative SCCHN cell line JHU-028 transfected with either a negative control miRNA or miR-363. (A) Wound healing assay pictures taken at 0 hours and 20 hours. (B) Graph of wound healing assay at designated time points. The *p* value for cells expressing miR-363 compared to cells expressing a negative miRNA is indicated by ** ($p < 0.01$).

4.3.4 MicroRNA-363 Increases Colony Formation by HPV-Negative SCCHN Cells

Overexpression of miR-363 in HPV-negative SCCHN cell line PCI-30 showed an increase in the number of colonies formed by cells expressing miR-363 compared to cells expressing a negative control miRNA in a soft agar assay (Figure 19). In addition, the colony size was significantly greater in cells expressing miR-363 compared to those expressing a negative control miRNA (Figure 19). These results suggest that miR-363 may promote cell growth and colony formation.

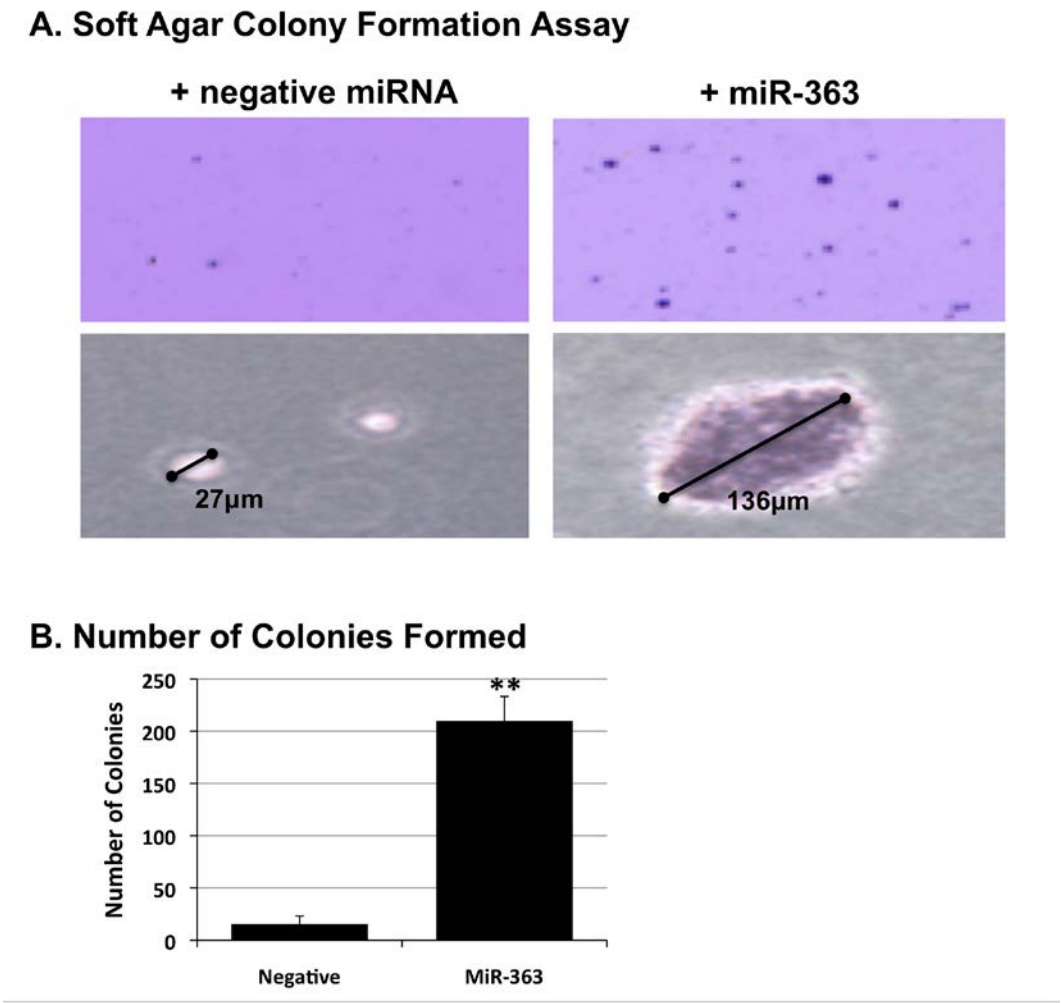

Figure 19: MicroRNA-363 increases colony formation in HPV-negative SCCHN cells

HPV-negative SCCHN cell line PCI-30 transfected with either a negative control miRNA or miR-363. (A) Picture of a representative well of a six-well plate, showing colonies formed, including representative diameter. (B) Graph representing the number of colonies formed per condition. The *p* value for cells expressing miR-363 compared to cells expressing a negative miRNA is indicated by ** ($p < 0.01$).

4.4 DISCUSSION

We have shown that miR-363 is overexpressed in HPV-positive SCCHN cell lines and tissue samples compared to HPV-negative SCCHN cell lines and tissues (Wald et al., 2011). Furthermore, expression of the HPV-16 E6 oncogene increases the expression of miR-363 while siRNA knockdown of HPV-16 E6 reduces miR-363 expression (Wald et al., 2011). We sought to characterize the functional role of miR-363 in SCCHN.

MiR-363 is part of the miR-106a~363 cluster of miRNAs, which is one of three miRNA clusters in the oncogenic miR-17~92 family of clusters (Ventura et al., 2008). Several miRNAs in these clusters are upregulated in other cancers, including T-cell leukemia (Landais et al., 2007), colon cancer (Bandres et al., 2006; Tsuchida et al., 2011; Volinia et al., 2006; Yu et al., 2011), and lung cancer (Hayashita et al., 2005; Osada and Takahashi, 2011). MiR-363, miR-25, and miR-92 all belong to the miR-17~92 family and have identical seed sequences (Ventura et al., 2008). Since the seed sequence of a mature miRNA helps determine its target mRNA (Bartel, 2004), it is hypothesized that these three miRNAs could target similar (if not the same) genes (Ventura et al., 2008). MiR-25 is overexpressed in bile duct, gastric, and ovarian cancers where it targets the TRAIL Death Receptor 4 (Razumilava et al., 2012), p57 (Kim et al., 2009), and BIM (Zhang et al., 2012), respectively. Additionally, miR-92 levels are increased in colon cancer and small cell lung cancer where it targets BIM (Tsuchida et al., 2011) and RAB14 (Kanzaki et al., 2011), respectively. Moreover, in myeloid cells miR-92 targets p63 (Manni et al., 2009). Because miR-363 has the same seed sequence as miR-25 and miR-92, and miR-25 and miR-92 have been implicated in cell cycle regulation, it is possible that miR-363 has a similar function.

Since miRNAs can either target mRNA transcripts for degradation or promote their translational repression, a decrease in the levels of their target mRNA is not always observed (Eulalio et al., 2008). We were successfully able to narrow the potential targets of miR-363 by utilizing spectral counting proteomics (Table 6), gene expression profiling data previously generated in our laboratory (Martinez et al., 2007), and computationally predicted miRNA target databases. Myosin 1B (MYO1B) showed a significant decrease at the mRNA and protein levels when miR-363 was overexpressed (Figure 13), and was confirmed to be a direct target of miR-363 by the luciferase assay (Figure 14). Myosins are common motor proteins involved in many cellular processes, including cell motility (Edgar et al., 1996; Evangelista et al., 2000). MiR-363 overexpression caused by HPV-16 E6 in HPV-positive SCCHN cell lines results in a decrease in MYO1B expression (Figure 13) and reduces cell migration (Figure 15). Additionally, when MYO1B levels were reduced via siRNA knockdown, cell migration also decreased (Figure 16). These data show that miR-363 expression causes a decrease in cell migration in SCCHN by reducing MYO1B expression.

To test a more comprehensive role for miR-363 in SCCHN, we analyzed its possible roles via several functional assays. Addition of exogenous miR-363 to HPV-negative SCCHN cells that do not express miR-363 caused an increase in cell proliferation, as measured by cell counting, MTT, wound healing, and colony formation assays (Figures 17-19). The HPV E6 and E7 oncogenes are known to promote cell proliferation as well as viral replication (Moody and Laimins, 2010). Overexpression of miR-363 by the HPV-16 E6 oncogene may be an additional mechanism by which HPVs could promote cell proliferation. We were unable to significantly knock-down miR-363 levels in HPV-positive SCCHN cell lines by using either an antagomir to

miR-363 or by siRNA knockdown of E6 (data not shown). Therefore, the direct effect of reducing miR-363 expression in HPV-positive SCCHN cell lines could not be tested.

We also carried out colony formation assays in soft agar using the HPV-negative SCCHN cell line PCI-30. The colony forming ability of these cells has not been previously characterized. We found that overexpression of miR-363 in this cell line increased both the number and size of the colonies (Figure 19). This was particularly striking because in these cells, very few colonies were observed when a negative control miRNA was expressed but many colonies were seen in the presence of miR-363 (Figure 19). Thus, our observation that HPV-negative SCCHN cells form larger colonies when expressing miR-363 compared to a negative control miRNA supports our results that miR-363 promotes the proliferative ability of SCCHN cells.

The data presented in this study show that miR-363 targets MYO1B which reduces cell migration. Furthermore, miR-363 increases proliferation and colony formation of HPV-negative SCCHN cells. This data could give more insight and help lead to a better understanding of the differences between HPV-positive and HPV-negative SCCHN. Based on our study, we envision that infection with HPV-16 causes overexpression of the E6 and E7 oncogenes and E6 expression results in an increase in miR-363 levels. High expression of miR-363 may then reduce MYO1B levels, thereby reducing the migratory ability of these cells. Furthermore, high levels of miR-363 may also promote cellular proliferation and colony formation.

5.0 SUMMARY, CONCLUSION, AND FUTURE DIRECTIONS

5.1 GENERAL SUMMARY AND CONCLUSIONS

High-risk HPV infection is associated with several cancers, including cervical, anogenital and a subset of head and neck cancer (De Vuyst et al., 2009; Frisch et al., 1997; Pascual et al., 2007; Tran et al., 2007b; Walboomers et al., 1999). The number of HPV-positive SCCHN cases has been increasing in the past ten years, and the characteristics of HPV-positive and HPV-negative SCCHN are different, leading some to treat them as distinct tumor entities (Chaturvedi et al., 2008; D'Souza et al., 2007). The role of miRNAs in the regulation of gene expression has provided insights into the dysregulation of miRNA expression profiles in human diseases, including cancers (Esquela-Kerscher and Slack, 2006). We hypothesized that overexpression of the E6 and E7 oncogenes in HPV-positive SCCHN would alter miRNA profiles in SCCHN differently than in HPV-negative SCCHN. Differences between miRNA profiles in HPV-positive and HPV-negative SCCHN would lead to specific alterations in the targets of these miRNAs, giving rise to the different characteristics observed for HPV-positive and HPV-negative SCCHN.

These studies are amongst the first to show a role for HPV in altering cellular miRNA profiles in SCCHN. The miRNA microarray analysis showed dysregulation of several miRNAs in the HPV-positive SCCHN cell lines compared to the HPV-negative SCCHN cell lines. Several of these miRNAs were altered specifically in the presence of HPV-16 E6 and/or E7, including the upregulation of miR-363, and the downregulation of miR-181a, miR-218, and miR-29a. Additionally, siRNA knockdown of HPV-16 E6 in HPV-positive SCCHN cell lines showed reduced expression of miR-363, indicating that miR-363 expression was dependent upon HPV-16 E6 expression (Wald et al., 2011).

We further showed that in addition to the HPV-positive SCCHN cell lines, miR-363 was also overexpressed in tissue samples from HPV-positive SCCHN patients from Western Pennsylvania compared to the HPV-negative SCCHN tissues. These results suggest that miR-363 overexpression may have clinical relevance. Additionally, the characteristics of our SCCHN tissues were similar to a previously published study (Chaturvedi et al., 2008). The tonsil and base of tongue had the highest rate of HPV-positivity, and represented 88% of HPV-positive SCCHN tissues in our cohort, while the HPV-negative SCCHN tumors were evenly distributed between the various head and neck sites. Patients with HPV-positive SCCHN were about nine years younger than those with HPV-negative SCCHN, and a vast majority of the HPV-positive SCCHN tissues compared to only one-half of the HPV-negative SCCHN tissues were classified as T1 or T2 tumors.

Since miRNAs act to repress their target mRNAs (Bartel, 2004), we utilized gene expression data, spectral counting proteomics, and computationally predicted miRNA target databases to show that miR-363 targets MYO1B in SCCHN. Furthermore, high levels of miR-363, and low levels of MYO1B reduced cell migration in SCCHN cells. To further elucidate the role of miR-363 in SCCHN, we carried out several studies which suggested that miR-363 increases proliferation and colony formation of SCCHN cells.

It is not surprising that miRNA expression profiles are different in HPV-positive and HPV-negative SCCHN. HPV alters miRNA profiles in HaCaT cells that were transfected with the HPV-11, -16, or -45 whole genomes (Dreher et al., 2011), as well as HaCaT cells transfected with only the HPV-16 E5 gene (Greco et al., 2011). Although transfection with one gene or the entire HPV genome does not mimic natural HPV infection, it confirms that the HPV viral genes can alter cellular miRNA profiles. There are many reports on dysregulated miRNAs in cervical

cancer (99% of which are caused by high-risk HPV) and head and neck cancer, but our studies are amongst the first to show that miRNAs are dysregulated differently in HPV-positive and HPV-negative SCCHN.

5.2 FUTURE DIRECTIONS

The data presented here show that miRNA profiles in HPV-positive and HPV-negative SCCHN are different. Furthermore, miR-363 is overexpressed in HPV-positive SCCHN compared to HPV-negative SCCHN, targets MYO1B to reduce cellular migration, and increases cellular proliferation and colony formation. Additional studies are necessary to further analyze the role of miRNAs in the development of HPV-associated SCCHN. For example, identification of the mechanism by which the E6 oncogene of HPV-16 promotes the overexpression of miR-363 would be of great interest. The cellular functions and domains of HPV E6 are well characterized. We have used three HPV-16 E6 mutants (F2V, L50G, and L110Q) that are defective in their interaction with specific cellular targets, but we were unable to identify a direct mechanism by which E6 regulates cellular miRNAs. Future studies would include the use of additional E6 mutants to study the mechanism of upregulation of miR-363 by the E6 oncogene.

MiR-363 is part of a cluster of miR-106a~363 miRNAs, however only miR-363 was found to be overexpressed in our studies. A cluster of miRNAs is defined by the proximity to other miRNAs and can either have one promoter for the transcription of polycistronic miRNAs, or a miRNA cluster can have multiple promoters (Monteys et al., 2010; Ozsolak et al., 2008; Wang et al., 2009b). The promoter(s) of the miR-106a~363 cluster are not known, and since only miR-363 is overexpressed in HPV-positive SCCHN, it is likely that either miR-363 has its

own promoter or it is processed differently from a polycistronic RNA than other miRNAs in the cluster. Identifying the miR-363 (or cluster) promoter, and the mechanism of its upregulation in HPV-positive SCCHN should provide valuable insight into the development of this disease. A previous report predicted the transcription start site for miR-363 (Ozsolak et al., 2008), and provides a good starting point for such studies. The cloning of such regions into a promoter-less luciferase vector should lead to the identification of the miR-363 promoter. Once a promoter region for miR-363 has been identified, chromatin immunoprecipitation (ChIP) assays could be done to investigate whether E6 (or one of its downstream targets) binds to the miR-363 promoter. The E6 protein could also be targeted to the miR-363 promoter through its interaction with a cellular transcription factor that acts at this promoter. In addition, epigenetic mechanisms may also be involved in the regulation of miR-363 expression. There have been previous reports demonstrating silencing of miRNAs by DNA methylation in oral cancer (Kozaki et al., 2008; Langevin et al., 2011). *In vitro* analysis of SCCHN cells treated with 5-azacytidine which inhibits DNA methylation, or trichostatin A which inhibits histone deacetylation, may provide insight into the possible regulation of miR-363 by epigenetic mechanisms.

Additionally, since we have shown that expression of HPV-16 E6 in primary human keratinocytes increases miR-363 levels (Figure 7), gene expression analysis of the E6-expressing keratinocytes may provide clues to cellular factors which may be involved in the upregulation of miR-363.

Another direction for future experiments could involve the ability of miR-363 to induce or prevent tumor formation. We have shown that HPV-negative SCCHN cells expressing exogenous miR-363 form more colonies (via soft agar assay, Figure 19). This initial finding

could be further explored in animal model systems, which may give insight into the *in vivo* role of miR-363 in HPV-positive SCCHN.

This dissertation details the role of one altered miRNA, miR-363, in HPV-positive SCCHN. There were several other miRNAs whose levels were altered in HPV-positive compared to HPV-negative SCCHN cells that could be investigated for their role in the development of HPV-positive SCCHN as well as for diagnosis and developing new treatment approaches for SCCHN.

SUPPLEMENTARY TABLES

Supplementary Table 1 Basal expression of miRNAs in HPV-16 positive SCCHN cell lines

Name	Median		Name	Median
hsa_miR_21	15166.0		hsa_let_7f	807.0
hsa_miR_16	9969.5		hsa_miR_181b	791.0
hsa_miR_29a	9183.5		hsa_miR_99a	791.0
hsa_miR_23b	5818.5		hsa_miR_19b	719.5
hsa_miR_221	5180.5		hsa_miR_20a	682.5
hsa_miR_24	5157.0		hsa_miR_15a	598.0
hsa_miR_23a	4930.0		hsa_let_7g	592.5
hsa_let_7a	4129.5		hsa_miR_20b	562.5
hsa_miR_31	4102.0		hsa_miR_30b	560.0
hsa_miR_222	3579.5		hsa_miR_155	540.5
hsa_miR_30a_5p	2689.0		hsa_miR_191	508.5
hsa_miR_26a	2684.5		hsa_miR_210	405.5
hsa_miR_29b	2591.5		hsa_miR_30e_5p	394.0
hsa_miR_107	2116.5		hsa_miR_200b	324.0
hsa_miR_15b	2051.5		hsa_miR_125a	318.5
hsa_miR_103	2039.5		hsa_miR_361	313.0
hsa_miR_106a	2014.5		hsa_miR_320	278.0
hsa_miR_30d	1795.5		hsa_miR_203	207.5
hsa_miR_27b	1665.5		hsa_miR_138	202.5
hsa_let_7d	1655.0		hsa_miR_185	183.0
hsa_miR_93	1534.5		hsa_miR_130b	170.0
hsa_miR_106b	1533.5		hsa_miR_181d	138.0
hsa_miR_205	1515.5		hsa_miR_7	123.0
hsa_miR_125b	1407.5		hsa_miR_96	122.0
hsa_miR_100	1324.0		hsa_miR_92	121.5
hsa_miR_182	1264.0		hsa_miR_422b	110.5
hsa_miR_17_5p	1250.5		hsa_miR_99b	107.5
hsa_miR_130a	1176.0		hsa_miR_18a	98.0
hsa_miR_30c	1086.0		hsa_miR_494	97.0
hsa_let_7c	1054.5		hsa_miR_193b	91.5
hsa_miR_22	1054.0		hsa_miR_224	85.5
hsa_let_7i	1031.0		hsa_miR_151	82.5
hsa_miR_181a	991.0		hsa_miR_152	80.5
hsa_miR_200c	965.5		hsa_miR_128a	76.0
hsa_let_7b	862.0		hsa_miR_196a	69.0
hsa_miR_27a	856.0		hsa_miR_200a	68.5
hsa_miR_25	842.5		hsa_miR_128b	67.0
hsa_let_7e	826.0		hsa_miR_423	63.5
			hsa_miR_136	63.0

hsa_miR_26b	58.5
hsa_miR_29c	55.5
hsa_miR_28	55.0
hsa_miR_342	50.0
hsa_miR_193a	43.0
hsa_miR_10a	42.5
hsa_miR_18b	37.0
hsa_miR_183	36.0
hsa_miR_30a_3p	35.0
hsa_miR_301	34.5
hsa_miR_146b	31.0
hsa_miR_362	26.5
hsa_miR_146a	25.0
hsa_miR_98	23.5
hsa_miR_196b	22.5
hsa_miR_218	19.0
hsa_miR_339	18.5
hsa_miR_189	18.0
hsa_miR_452	17.5
hsa_miR_324_3p	17.0
hsa_miR_142_5p	16.5
hsa_miR_503	16.0
hsa_miR_194	15.5
hsa_miR_491	15.5
hsa_miR_455	14.5
hsa_miR_19a	14.0
hsa_miR_17_3p	12.5
hsa_miR_195	11.5
hsa_miR_141	11.0
hsa_miR_30e_3p	10.0
hsa_miR_148a	9.0
hsa_miR_186	9.0
hsa_miR_345	8.0
hsa_miR_500	8.0
hsa_miR_335	7.0
hsa_miR_126	6.5
hsa_miR_143	6.5
hsa_miR_213	6.5
hsa_miR_331	5.0
hsa_miR_424	5.0
hsa_miR_198	4.5
hsa_miR_181c	4.0
hsa_miR_9_AS	3.5
hsa_miR_192	2.5
hsa_miR_363_AS	2.5

hsa_miR_422a	2.5
hsa_miR_429	2.5
hsa_miR_145	1.5
hsa_miR_485_5p	1.5
hsa_miR_489	1.0
hsa_miR_18a_AS	0.5
hsa_miR_513	0.5
hsa_miR_1	0.0
hsa_miR_101	0.0
hsa_miR_105	0.0
hsa_miR_10b	0.0
hsa_miR_122a	0.0
hsa_miR_124a	0.0
hsa_miR_126_AS	0.0
hsa_miR_127	0.0
hsa_miR_129	0.0
hsa_miR_132	0.0
hsa_miR_133a	0.0
hsa_miR_133b	0.0
hsa_miR_134	0.0
hsa_miR_135a	0.0
hsa_miR_135b	0.0
hsa_miR_137	0.0
hsa_miR_139	0.0
hsa_miR_140	0.0
hsa_miR_142_3p	0.0
hsa_miR_144	0.0
hsa_miR_147	0.0
hsa_miR_148b	0.0
hsa_miR_149	0.0
hsa_miR_150	0.0
hsa_miR_153	0.0
hsa_miR_154	0.0
hsa_miR_154_AS	0.0
hsa_miR_182_AS	0.0
hsa_miR_184	0.0
hsa_miR_187	0.0
hsa_miR_188	0.0
hsa_miR_190	0.0
hsa_miR_191_AS	0.0
hsa_miR_197	0.0
hsa_miR_199a	0.0
hsa_miR_199a_AS	0.0
hsa_miR_199b	0.0
hsa_miR_200a_AS	0.0

hsa_miR_202	0.0
hsa_miR_202_AS	0.0
hsa_miR_204	0.0
hsa_miR_206	0.0
hsa_miR_208	0.0
hsa_miR_211	0.0
hsa_miR_212	0.0
hsa_miR_214	0.0
hsa_miR_215	0.0
hsa_miR_216	0.0
hsa_miR_217	0.0
hsa_miR_219	0.0
hsa_miR_220	0.0
hsa_miR_223	0.0
hsa_miR_296	0.0
hsa_miR_299_3p	0.0
hsa_miR_299_5p	0.0
hsa_miR_302a	0.0
hsa_miR_302a_AS	0.0
hsa_miR_302b	0.0
hsa_miR_302b_AS	0.0
hsa_miR_302c	0.0
hsa_miR_302c_AS	0.0
hsa_miR_302d	0.0
hsa_miR_32	0.0
hsa_miR_323	0.0
hsa_miR_324_5p	0.0
hsa_miR_325	0.0
hsa_miR_326	0.0
hsa_miR_328	0.0
hsa_miR_329	0.0
hsa_miR_33	0.0
hsa_miR_330	0.0
hsa_miR_337	0.0
hsa_miR_338	0.0
hsa_miR_340	0.0
hsa_miR_346	0.0
hsa_miR_34a	0.0
hsa_miR_34b	0.0
hsa_miR_34c	0.0
hsa_miR_363	0.0
hsa_miR_365	0.0
hsa_miR_367	0.0
hsa_miR_368	0.0
hsa_miR_369_3p	0.0
hsa_miR_369_5p	0.0
hsa_miR_370	0.0
hsa_miR_371	0.0
hsa_miR_372	0.0
hsa_miR_373	0.0
hsa_miR_373_AS	0.0
hsa_miR_374	0.0
hsa_miR_375	0.0
hsa_miR_376a	0.0
hsa_miR_376a_AS	0.0
hsa_miR_376b	0.0
hsa_miR_377	0.0
hsa_miR_378	0.0
hsa_miR_379	0.0
hsa_miR_380_3p	0.0
hsa_miR_380_5p	0.0
hsa_miR_381	0.0
hsa_miR_382	0.0
hsa_miR_383	0.0
hsa_miR_384	0.0
hsa_miR_409_3p	0.0
hsa_miR_409_5p	0.0
hsa_miR_410	0.0
hsa_miR_412	0.0
hsa_miR_425	0.0
hsa_miR_431	0.0
hsa_miR_432	0.0
hsa_miR_432_AS	0.0
hsa_miR_433	0.0
hsa_miR_448	0.0
hsa_miR_449	0.0
hsa_miR_450	0.0
hsa_miR_451	0.0
hsa_miR_452_AS	0.0
hsa_miR_453	0.0
hsa_miR_483	0.0
hsa_miR_484	0.0
hsa_miR_485_3p	0.0
hsa_miR_486	0.0
hsa_miR_487a	0.0
hsa_miR_487b	0.0
hsa_miR_488	0.0
hsa_miR_490	0.0
hsa_miR_492	0.0
hsa_miR_493_3p	0.0

hsa_miR_493_5p	0.0
hsa_miR_495	0.0
hsa_miR_496	0.0
hsa_miR_497	0.0
hsa_miR_498	0.0
hsa_miR_499	0.0
hsa_miR_501	0.0
hsa_miR_502	0.0
hsa_miR_504	0.0
hsa_miR_505	0.0
hsa_miR_506	0.0
hsa_miR_507	0.0
hsa_miR_508	0.0
hsa_miR_509	0.0
hsa_miR_510	0.0
hsa_miR_511	0.0
hsa_miR_512_3p	0.0
hsa_miR_512_5p	0.0
hsa_miR_514	0.0
hsa_miR_515_3p	0.0
hsa_miR_515_5p	0.0
hsa_miR_516_3p	0.0
hsa_miR_516_5p	0.0
hsa_miR_517_AS	0.0
hsa_miR_517a	0.0
hsa_miR_517b	0.0
hsa_miR_517c	0.0
hsa_miR_518a	0.0
hsa_miR_518a_2_AS	0.0
hsa_miR_518b	0.0
hsa_miR_518c	0.0
hsa_miR_518c_AS	0.0
hsa_miR_518d	0.0
hsa_miR_518e	0.0
hsa_miR_518f	0.0
hsa_miR_518f_AS	0.0
hsa_miR_519a	0.0

hsa_miR_519b	0.0
hsa_miR_519c	0.0
hsa_miR_519d	0.0
hsa_miR_519e	0.0
hsa_miR_519e_AS	0.0
hsa_miR_520a	0.0
hsa_miR_520a_AS	0.0
hsa_miR_520b	0.0
hsa_miR_520c	0.0
hsa_miR_520d	0.0
hsa_miR_520d_AS	0.0
hsa_miR_520e	0.0
hsa_miR_520f	0.0
hsa_miR_520g	0.0
hsa_miR_520h	0.0
hsa_miR_521	0.0
hsa_miR_522	0.0
hsa_miR_523	0.0
hsa_miR_524	0.0
hsa_miR_524_AS	0.0
hsa_miR_525	0.0
hsa_miR_525_AS	0.0
hsa_miR_526a	0.0
hsa_miR_526b	0.0
hsa_miR_526b_AS	0.0
hsa_miR_526c	0.0
hsa_miR_527	0.0
hsa_miR_539	0.0
hsa_miR_542_3p	0.0
hsa_miR_542_5p	0.0
hsa_miR_544	0.0
hsa_miR_545	0.0
hsa_miR_9	0.0
hsa_miR_95	0.0

The basal expression of miRNAs in the HPV-negative cell lines PCI-13 and PCI-30 was identified using miRNA microarrays. We obtained the median fluorescent intensity for each miRNA, and miRNAs having values of zero in at least four of their corresponding spots (4 spots per array and two arrays per sample) were considered to be non-expressing.

Supplementary Table 2 Basal expression of miRNAs in HPV-16 positive SCCHN cell lines

Name	Median	hsa_let_7g	1005.5
hsa_miR_205	25541.5	hsa_miR_20a	981.0
hsa_miR_16	22308.0	hsa_miR_494	944.5
hsa_miR_24	7394.0	hsa_miR_30e_5p	933.0
hsa_miR_23b	6441.5	hsa_miR_191	814.0
hsa_miR_141	6103.0	hsa_let_7e	802.5
hsa_miR_26a	5640.0	hsa_miR_30c	765.5
hsa_miR_21	5434.0	hsa_miR_222	744.0
hsa_let_7a	5364.5	hsa_miR_422b	678.0
hsa_miR_23a	4327.0	hsa_miR_361	659.5
hsa_miR_27b	4024.0	hsa_miR_363	636.5
hsa_miR_203	3831.5	hsa_miR_125a	572.0
hsa_miR_200b	3692.0	hsa_miR_92	517.0
hsa_miR_106a	3677.0	hsa_miR_320	471.5
hsa_miR_106b	3634.0	hsa_miR_18a	441.0
hsa_miR_107	3458.0	hsa_miR_210	423.0
hsa_miR_93	3143.5	hsa_miR_195	414.5
hsa_let_7i	3062.0	hsa_miR_99b	377.5
hsa_miR_15b	2664.5	hsa_miR_148a	282.5
hsa_miR_17_5p	2634.0	hsa_miR_342	274.5
hsa_miR_200c	2559.5	hsa_miR_130b	273.0
hsa_let_7b	2537.5	hsa_miR_151	269.0
hsa_let_7c	2512.5	hsa_miR_224	259.0
hsa_let_7d	2493.5	hsa_miR_18b	245.5
hsa_miR_103	2413.0	hsa_miR_128a	226.0
hsa_miR_19b	2231.5	hsa_miR_152	213.0
hsa_miR_200a	2214.0	hsa_miR_96	183.5
hsa_miR_25	2114.0	hsa_miR_128b	182.5
hsa_miR_31	1904.5	hsa_miR_100	169.0
hsa_miR_99a	1839.5	hsa_miR_423	156.0
hsa_miR_30a_5p	1630.5	hsa_miR_7	151.5
hsa_miR_130a	1534.5	hsa_miR_19a	146.0
hsa_let_7f	1483.5	hsa_miR_29b	145.0
hsa_miR_27a	1469.0	hsa_miR_183	140.5
hsa_miR_15a	1458.5	hsa_miR_185	139.5
hsa_miR_30d	1436.0	hsa_miR_26b	125.5
hsa_miR_30b	1379.0	hsa_miR_28	114.0
hsa_miR_221	1369.5	hsa_miR_301	111.0
hsa_miR_125b	1243.0	hsa_miR_429	99.0
hsa_miR_182	1236.5	hsa_miR_29c	98.0
hsa_miR_20b	1213.0	hsa_miR_193a	84.0
hsa_miR_34a	1188.0	hsa_miR_17_3p	83.5
hsa_miR_29a	1083.0	hsa_miR_339	79.5
hsa_miR_22	1010.5	hsa_miR_362	78.5

hsa_miR_138	78.0	hsa_miR_126	9.5
hsa_miR_196a	72.5	hsa_miR_188	9.5
hsa_miR_189	63.0	hsa_miR_425	9.5
hsa_miR_98	53.0	hsa_miR_330	8.5
hsa_miR_324_3p	42.0	hsa_miR_213	8.0
hsa_miR_335	42.0	hsa_miR_373_AS	8.0
hsa_miR_422a	41.5	hsa_miR_9_AS	8.0
hsa_miR_331	38.0	hsa_miR_374	7.5
hsa_miR_186	35.5	hsa_miR_485_5p	7.5
hsa_miR_491	35.5	hsa_miR_505	7.0
hsa_miR_136	32.5	hsa_miR_542_5p	7.0
hsa_miR_194	31.5	hsa_miR_181d	6.5
hsa_miR_30e_3p	30.5	hsa_miR_518c_AS	6.0
hsa_miR_345	29.5	hsa_miR_155	5.5
hsa_miR_452	29.0	hsa_miR_182_AS	5.5
hsa_miR_181b	26.5	hsa_miR_324_5p	5.5
hsa_miR_192	26.0	hsa_miR_409_5p	5.5
hsa_miR_484	23.5	hsa_miR_122a	5.0
hsa_miR_363_AS	23.0	hsa_miR_181c	5.0
hsa_miR_503	22.5	hsa_miR_184	5.0
hsa_miR_196b	21.5	hsa_miR_302c_AS	5.0
hsa_miR_497	21.5	hsa_miR_346	5.0
hsa_miR_193b	20.0	hsa_miR_34b	5.0
hsa_miR_378	19.0	hsa_miR_492	4.5
hsa_miR_149	18.5	hsa_miR_299_3p	4.0
hsa_miR_511	18.5	hsa_miR_129	3.5
hsa_miR_379	18.0	hsa_miR_142_3p	3.5
hsa_miR_202	17.5	hsa_miR_365	3.5
hsa_miR_146b	16.5	hsa_miR_483	3.5
hsa_miR_500	16.5	hsa_miR_519e_AS	3.5
hsa_miR_198	16.0	hsa_miR_10a	3.0
hsa_miR_132	15.5	hsa_miR_206	3.0
hsa_miR_513	15.5	hsa_miR_212	3.0
hsa_miR_370	15.0	hsa_miR_424	3.0
hsa_miR_432	15.0	hsa_miR_134	2.5
hsa_miR_521	14.5	hsa_miR_140	2.5
hsa_miR_148b	14.0	hsa_miR_368	2.5
hsa_miR_18a_AS	14.0	hsa_miR_373	2.5
hsa_miR_197	13.0	hsa_miR_450	2.5
hsa_miR_200a_AS	13.0	hsa_miR_452_AS	2.5
hsa_miR_453	13.0	hsa_miR_455	2.5
hsa_miR_181a	12.0	hsa_miR_517a	2.5
hsa_miR_101	11.5	hsa_miR_519d	2.5
hsa_miR_30a_3p	11.5	hsa_miR_544	2.5
hsa_miR_33	11.5	hsa_miR_10b	2.0

hsa_miR_127	2.0	hsa_miR_135b	0.0
hsa_miR_143	2.0	hsa_miR_137	0.0
hsa_miR_328	2.0	hsa_miR_139	0.0
hsa_miR_499	2.0	hsa_miR_142_5p	0.0
hsa_miR_518a_2_AS	2.0	hsa_miR_144	0.0
hsa_miR_145	1.5	hsa_miR_147	0.0
hsa_miR_187	1.5	hsa_miR_150	0.0
hsa_miR_199a	1.5	hsa_miR_153	0.0
hsa_miR_220	1.5	hsa_miR_154	0.0
hsa_miR_371	1.5	hsa_miR_154_AS	0.0
hsa_miR_381	1.5	hsa_miR_190	0.0
hsa_miR_431	1.5	hsa_miR_191_AS	0.0
hsa_miR_485_3p	1.5	hsa_miR_199a_AS	0.0
hsa_miR_493_3p	1.5	hsa_miR_202_AS	0.0
hsa_miR_495	1.5	hsa_miR_204	0.0
hsa_miR_501	1.5	hsa_miR_208	0.0
hsa_miR_519c	1.5	hsa_miR_211	0.0
hsa_miR_9	1.5	hsa_miR_214	0.0
hsa_miR_32	1.0	hsa_miR_215	0.0
hsa_miR_486	1.0	hsa_miR_216	0.0
hsa_miR_487a	1.0	hsa_miR_217	0.0
hsa_miR_489	1.0	hsa_miR_218	0.0
hsa_miR_502	1.0	hsa_miR_219	0.0
hsa_miR_515_5p	1.0	hsa_miR_223	0.0
hsa_miR_520d	1.0	hsa_miR_296	0.0
hsa_miR_520d_AS	1.0	hsa_miR_299_5p	0.0
hsa_miR_526b	1.0	hsa_miR_302a	0.0
hsa_miR_527	1.0	hsa_miR_302a_AS	0.0
hsa_miR_542_3p	1.0	hsa_miR_302b	0.0
hsa_miR_133b	0.5	hsa_miR_302b_AS	0.0
hsa_miR_146a	0.5	hsa_miR_302c	0.0
hsa_miR_199b	0.5	hsa_miR_302d	0.0
hsa_miR_380_3p	0.5	hsa_miR_323	0.0
hsa_miR_449	0.5	hsa_miR_325	0.0
hsa_miR_487b	0.5	hsa_miR_326	0.0
hsa_miR_510	0.5	hsa_miR_329	0.0
hsa_miR_517b	0.5	hsa_miR_337	0.0
hsa_miR_518f	0.5	hsa_miR_338	0.0
hsa_miR_525	0.5	hsa_miR_340	0.0
hsa_miR_1	0.0	hsa_miR_34c	0.0
hsa_miR_105	0.0	hsa_miR_367	0.0
hsa_miR_124a	0.0	hsa_miR_369_3p	0.0
hsa_miR_126_AS	0.0	hsa_miR_369_5p	0.0
hsa_miR_133a	0.0	hsa_miR_372	0.0
hsa_miR_135a	0.0	hsa_miR_375	0.0

hsa_miR_376a	0.0	hsa_miR_517c	0.0
hsa_miR_376a_AS	0.0	hsa_miR_518a	0.0
hsa_miR_376b	0.0	hsa_miR_518b	0.0
hsa_miR_377	0.0	hsa_miR_518c	0.0
hsa_miR_380_5p	0.0	hsa_miR_518d	0.0
hsa_miR_382	0.0	hsa_miR_518e	0.0
hsa_miR_383	0.0	hsa_miR_518f_AS	0.0
hsa_miR_384	0.0	hsa_miR_519a	0.0
hsa_miR_409_3p	0.0	hsa_miR_519b	0.0
hsa_miR_410	0.0	hsa_miR_519e	0.0
hsa_miR_412	0.0	hsa_miR_520a	0.0
hsa_miR_432_AS	0.0	hsa_miR_520a_AS	0.0
hsa_miR_433	0.0	hsa_miR_520b	0.0
hsa_miR_448	0.0	hsa_miR_520c	0.0
hsa_miR_451	0.0	hsa_miR_520e	0.0
hsa_miR_488	0.0	hsa_miR_520f	0.0
hsa_miR_490	0.0	hsa_miR_520g	0.0
hsa_miR_493_5p	0.0	hsa_miR_520h	0.0
hsa_miR_496	0.0	hsa_miR_522	0.0
hsa_miR_498	0.0	hsa_miR_523	0.0
hsa_miR_504	0.0	hsa_miR_524	0.0
hsa_miR_506	0.0	hsa_miR_524_AS	0.0
hsa_miR_507	0.0	hsa_miR_525_AS	0.0
hsa_miR_508	0.0	hsa_miR_526a	0.0
hsa_miR_509	0.0	hsa_miR_526b_AS	0.0
hsa_miR_512_3p	0.0	hsa_miR_526c	0.0
hsa_miR_512_5p	0.0	hsa_miR_539	0.0
hsa_miR_514	0.0	hsa_miR_545	0.0
hsa_miR_515_3p	0.0	hsa_miR_95	0.0
hsa_miR_516_3p	0.0		
hsa_miR_516_5p	0.0		
hsa_miR_517_AS	0.0		

The basal expression of miRNAs in the HPV-16 positive cell lines UD-SCC-2 and UPCI:SCC90 was identified using miRNA microarrays. We obtained the median fluorescent intensity for each miRNA, and miRNAs having values of zero in at least four of their corresponding spots (4 spots per array and two arrays per sample) were considered to be non-expressing.

Supplementary Table 3 MiRNAs Differentially Expressed in HPV-16-positive SCCHN cell line UD-SCC-2 compared to HPV-negative SCCHN cell line PCI-13

MiRNA	Fold Change
Underexpressed	
hsa_miR_181a	-13.72
hsa_miR_181b	-13.37

miR, microRNA; hsa, human; The *q*-values of all miRNAs were 0.

Supplementary Table 4 MiRNAs Differentially Expressed in HPV-16-positive SCCHN cell line UD-SCC-2 compared to HPV-negative SCCHN cell line PCI-30

MiRNA	Fold Change
Overexpressed	
hsa_miR_205	31.66
hsa_miR_141	14.18
hsa_miR_200c	11.39
hsa_miR_34a	8.60
hsa_miR_200b	7.69
hsa_miR_363	4.85

miR, microRNA; hsa, human; The *q*-values of all miRNAs were 0.

Supplementary Table 5 MiRNAs Differentially Expressed in HPV-16-positive SCCHN cell line UPCI:SCC90 compared to HPV-negative SCCHN cell line PCI-13

MiRNA	Fold Change
Overexpressed	
hsa_miR_148a	5.56
hsa_miR_363	5.47
hsa_miR_186	4.69
hsa_miR_101	3.54
Underexpressed	
hsa_miR_155	-11.36
hsa_miR_181a	-5.63
ambi_miR_13143	-5.43

miR, microRNA; hsa, human; ambi, Ambion predicted; The *q*-values of all miRNAs were 0.

Supplementary Table 6 MiRNAs Differentially Expressed in HPV-16-positive SCCHN cell line UPCI:SCC90 compared to HPV-negative SCCHN cell line PCI-30

MiRNA	Fold Change
Overexpressed	
hsa_miR_205	22.92
hsa_miR_203	15.85
hsa_miR_141	15.34
hsa_miR_200c	11.47
hsa_miR_34a	10.50
hsa_miR_200b	9.47
hsa_miR_200a	7.79
hsa_miR_429	6.29
hsa_miR_363	5.41
hsa_miR_200a_AS	2.68

miR, microRNA; hsa, human; The *q*-values of all miRNAs were 0.

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