Comparing Canonical and Dicer Substrate Small Interfering RNA and Their Effects on the Silencing of Two Common Human Papillomavirus 16 E6 Variants

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Abstract

High-risk human papillomaviruses (HPVs), such as HPV16, cause nearly all cases of cervical cancer as well as a number of head and neck cancers. Over expression of the E6 oncoprotein helps drive the malignant phenotype, therefore making it a promising therapeutic target. There are two common HPV16 E6 variants, Asian-American (AA) and European Prototype (EP), that differ by six single nucleotide polymorphisms (SNPs) resulting in AA having greater oncogenic potential than EP. Oncogene-specific down regulation mediated by RNA interference (RNAi) is an efficient approach that uses synthetic small interfering RNA (siRNA) to target and degrade E6 mRNA. Several E6 siRNAs were designed based on previously validated data, the Rosetta siRNA Design Algorithm, and Dicer substrate siRNA Design Algorithm. After transfection with the oligonucleotides in CaSki cells, Rosetta siRNA and Rosetta dicer substrate siRNA (DsiRNA) showed the highest E6 knockdown, having IC_{50} values of 7 ± 13 nM and 3 ± 2 nM, respectively. However, the differences between siRNA and DsiRNA were not statistically significant. Rosetta siRNA and Rosetta DsiRNA were then transfected into primary human foreskin keratinocytes (PHFKs) previously transduced with AA and EP, resulting in IC_{50} values of 3 ± 2 pM and 6 ± 3 pM respectively, for AA, and 72 ± 69 pM and 38 ± 2 pM respectively, for EP. E6 protein levels in both variants after treatment with either Rosetta siRNA or Rosetta DsiRNA were no longer detectable so to validate downstream process restoration once the E6 protein was knocked down, human telomerase reverse transcriptase (hTERT) mRNA and p53 protein levels were measured. Throughout this study, there was no significant difference between cells transfected with Rosetta DsiRNA and Rosetta siRNA when E6 mRNA, E6 protein, p53, and hTERT

levels were measured by either RT-qPCR or western blot. After transfection with 6.75 nM of either Rosetta siRNA or Rosetta DsiRNA, p53 levels increased and hTERT levels decreased compared to negative controls. These findings suggest that Rosetta DsiRNA and Rosetta siRNA do not differ in downstream process restoration when targeting HPV16 E6 *in vitro* but may have an effect on duration of knockdown or stability of E6 mRNA *in vivo*.

Lay Summary

Human papillomavirus (HPV) commonly infects skin cells and the E6 protein of high risk HPV types, such as HPV16, can cause malignant changes in infected cells. There are several HPV16 E6 variants commonly found in North American populations and our research group focuses on the Asian-American (AA) and European Prototype (EP) variants. AA is better at causing these malignant changes than EP and the reason is yet to be determined. To aid in the completion of these studies, a molecule needs to be made that will stop the E6 protein from being produced in each of the variants. E6 protein production can be blocked by using small interfering RNAs (siRNAs). However, these molecules have several limitations, including quick degradation and the potential to interact with the wrong targets. First, a literature search was completed determining what design algorithms are available and several molecules were chosen to determine the best target site on the E6 gene. A comparison between regular siRNAs and Dicer Substrate siRNAs (DsiRNAs) was made and an investigation to see how these molecules worked in both variants was analyzed. Both siRNA and DsiRNA were able to knockdown E6 protein levels, restore downstream processes, and do so with a low concentration thus reducing any potential unwanted side effects. However, there was no difference in cells treated with either siRNA or DsiRNA or between the E6 variants throughout this study. These molecules show tremendous promise as a tool for targeted gene silencing and optimizing these molecules will be very helpful which can be employed for functional and mechanistic HPV studies.

Dedication

To my family for always being there for me.

Acknowledgments

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List of Abbreviations

27 mers	27 bp Oligonucleotides
19-22 mers	19-22 bp Oligonucleotides
AA	Asian American HPV16 E6 Variant
ANOVA	Analysis of Variance
bp	Base Pairs
BSA	Bovine Serum Albumin
Ct	Cycle Threshold
DAPI	4',6-diamidino-2-phenylindole fluorescent stain
DC	Detergent Compatible
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
dNTP	Deoxynucleotide
DPBS	Dulbecco's Phosphate Buffered Saline
DsiRNA	Dicer Substrate siRNA
dsRNA	Double-Stranded RNA
dsRBD	dsRNA-Binding Domain
dT	Deoxy Thymidine
E2F	Transcription Factor
E6AP	Ubiquitin Ligase
EDTA	Ethylenediaminetetraacetic acid
EP	European Prototype HPV16 E6 Variant
FBS	Fetal Bovine Serum

HA	Hemagglutinin
HECT Domain	Homologous to the E6-AP Carboxyl Terminus Domain
HPRT-1	Hypoxanthine Phosphoribosyltransferase 1
HPV	Human Papillomavirus
HRP	Horseradish Peroxidase
HSD	Honestly Significant Difference
hTERT	Human Telomerase Reverse Transcriptase
IC_{50}	Concentration Resulting in 50% Inhibition
IC_{95}	Concentration Resulting in 95% Inhibition
IDT	Integrated DNA Technologies
kDa	KiloDalton
KGM	Keratinocyte Growth Medium
ORF	Open Reading Frame
NC-1	DsiRNA Negative Control
p53	Tumour Suppressor Protein
p97	E6/E7 Promoter
PAZ Domain	Piwi Argonaute and Zwille Domain
PCR	Polymerase Chain Reaction
PHFK	Primary Human Foreskin Keratinocytes
PMSF	Phenylmethylsulfonyl Fluoride
pRb	Retinoblastoma Protein
PSSM	Position-Specific Scoring Matrices
PVDF Membrane	Polyvinylidene Difluoride Membrane

RIPA Buffer	Radioimmunoprecipitation Assay Buffer
RISC	RNA Induced Silencing Complex
RNAi	RNA interference
RQI	RNA Quality Indicator
RT-qPCR	Real-Time Reverse Transcription Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	SDS - Polyacrylamide Gel Electrophoresis
SEM	Standard Error of Mean
shRNA	Short Hairpin RNA
siRNA	Small Interfering RNA
SNP	Single Nucleotide Polymorphism
S Phase	Synthesis Phase
ssRNA	Single-Stranded RNA
TBRRI	Thunder Bay Regional Research Institute
TBS	Tris-Buffered Saline
TBST	Tris-Buffered Saline With 0.05% TWEEN® 20

1. Introduction

1.1 Human Papillomavirus and Cancer

Human papillomavirus (HPV) is a double-stranded DNA virus that infects the keratinocytes of skin and mucosal membranes (zur Hausen 2002). There are more than 200 HPV types known to date (Papillomavirus Episteme (PaVE); http://pave.niaid.nih.gov/#home) divided into low-risk and high-risk HPVs. Low-risk types, such as HPV6 and HPV11, are associated with benign lesions (Korzeniewski et al. 2011). HPV16, which is phylogenetically clustered within the *Aplphapapillomavirus 9* species group (Bernard et al. 2010), is the most common high-risk type, being responsible for more than half of HPV related cancers (Crow 2012), and, with constant infection, can cause the malignant transformation of infected cells through the cellular changes induced by the viral E6 protein (Niccoli et al. 2012). Therefore, HPV16 is the focus of this study.

1.1.1 HPV16 Genome and Viral Life Cycle

The HPV16 genome is 7905 base pairs (bp) long and encodes six early proteins (E1, E2, E4, E5, E6, E7) mainly expressed in the lower and mid epithelial layers, and two late proteins (L1, L2) (Figure 1) expressed in the upper layers of infected epithelium (Doorbar 2005). The two late proteins compose the viral capsid, E1 and E2 proteins initiate viral replication, control viral gene expression and help maintain the virus episomally, E4 and E5 proteins indirectly aid viral genome amplification, and E6 and E7 proteins are oncoproteins or cancer causing proteins (Doorbar 2012). The E7 protein is most well-characterized for its ability to bind to and inactivate the retinoblastoma protein (pRb). This leads to the release of the transcription factor E2F which causes entry into the

synthesis phase (S phase) of the cell cycle and proliferation in the absence of normal growth factors (Klingelhutz and Roman 2012) (Figure 2). The key oncoprotein produced by the virus is E6. Among a variety of effects, E6 is best known for causing degradation of the tumour suppressor protein p53, and immortalization through an increase in human telomerase reverse transcriptase (hTERT) expression (Figure 2) (Doorbar 2012). The E6 protein forms a complex with the ubiquitin ligase E6AP and binds to the tumour suppressor protein p53 (Scheffner et al. 1993). The E6-E6AP-p53 complex requires the ability of E6 to dimerize through self-association of the amino-terminal domain of E6 to initiate the transfer of ubiquitin from a carboxy-terminal thioester in the HECT (Homologous to the E6-AP Carboxyl Terminus) domain of E6AP to p53. This then leads to the degradation of p53 through the proteasome (Vande Pol and Klingelhutz 2013). This loss of p53 prevents the cell from entering apoptosis allowing the accumulation of mutations which can result in malignant transformations. The E6 protein of high risk HPVs activates the hTERT promoter which induces its expression (Van Doorslaer and Burk 2012). Increasing hTERT expression allows telomerase to synthesize telomeres at the ends of chromosomes (Klingelhutz et al. 1996). In the absence of telomerase activity most human cells undergo a progressive shortening of telomeres with each cell division, ultimately leading to chromosomal instability and senescence (Harley et al. 1990). By replenishing telomeres on the ends of chromosomes, as well as contributions from other processes, the cells can become immortal (Kim et al. 1994).

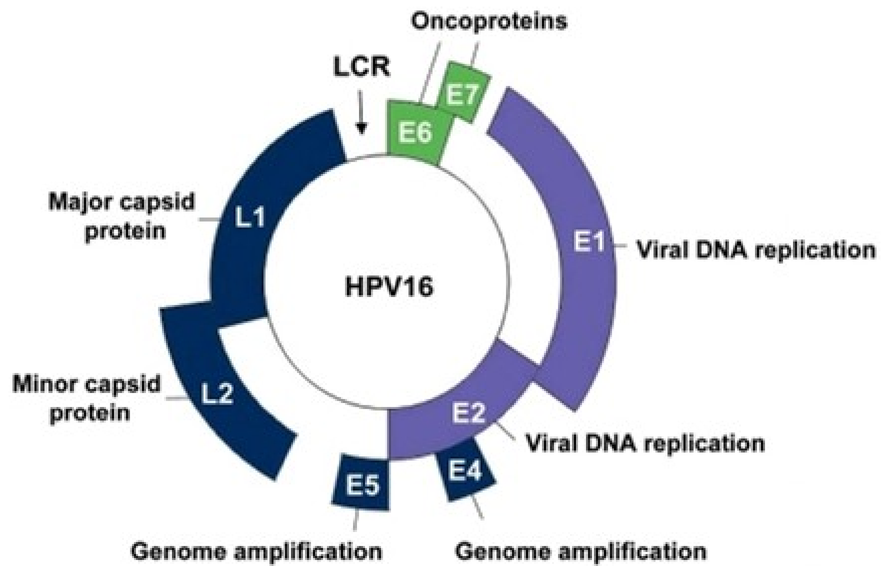

Figure 1. The HPV16 genome. A diagram representing the HPV16 genome and the location of the Long Control Region (LCR), the six early genes (E1, E2, E4, E5, E6 and E7), the two late genes (L1 and L2) and their major roles. Image modified from http://openi.nlm.nih.gov/detailedresult.php?img=3137155_TOVJ-5-80_F1&req=4.

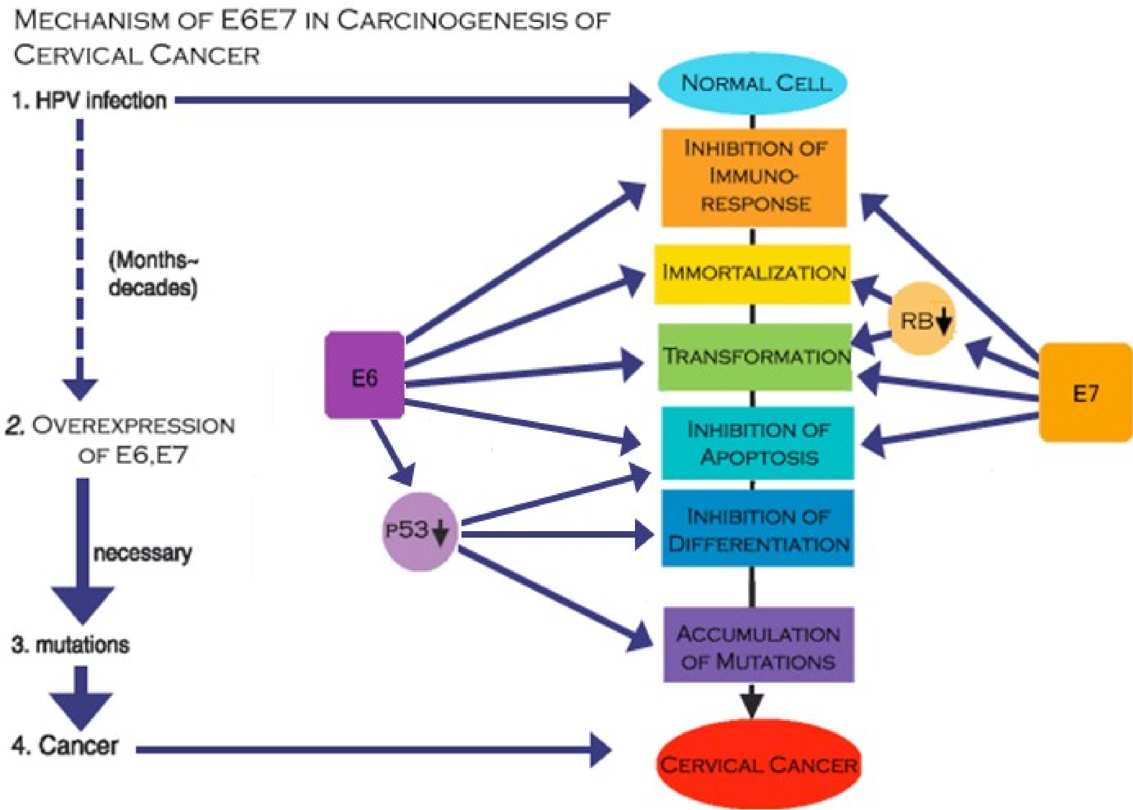

Figure 2. HPV16 E6 and E7 oncoproteins and carcinogenesis. A simplified representation of the progression from HPV infection to cancer caused by the over expression of E6 and E7 (left). E6 and E7 are involved in many cellular processes, with binding to p53 and pRb respectively being two common processes that have been extensively studied (right). Image modified from http://oncohealthcorp.com/technology.html.

The HPV life cycle is dependent on the differentiation of the host epithelial cells (Flores et al. 1999, Doorbar 2012). The viral particles can gain access to the basal cells through micro-abrasions in the epithelium of mucosal membranes. The viral genome is initially amplified as low-copy number extrachromosomal cellular elements and when amplifying is increased to high-copy number, capsid proteins are synthesized and viral particles are released with the shedding of differentiated keratinocytes (Flores et al. 1999, zur Hausen 2002). While the majority of HPV infections are cleared by the host's immune system, persistent infection and integration of the virus into the host genome may lead to malignant transformations (Figure 3) (zur Hausen 2002).

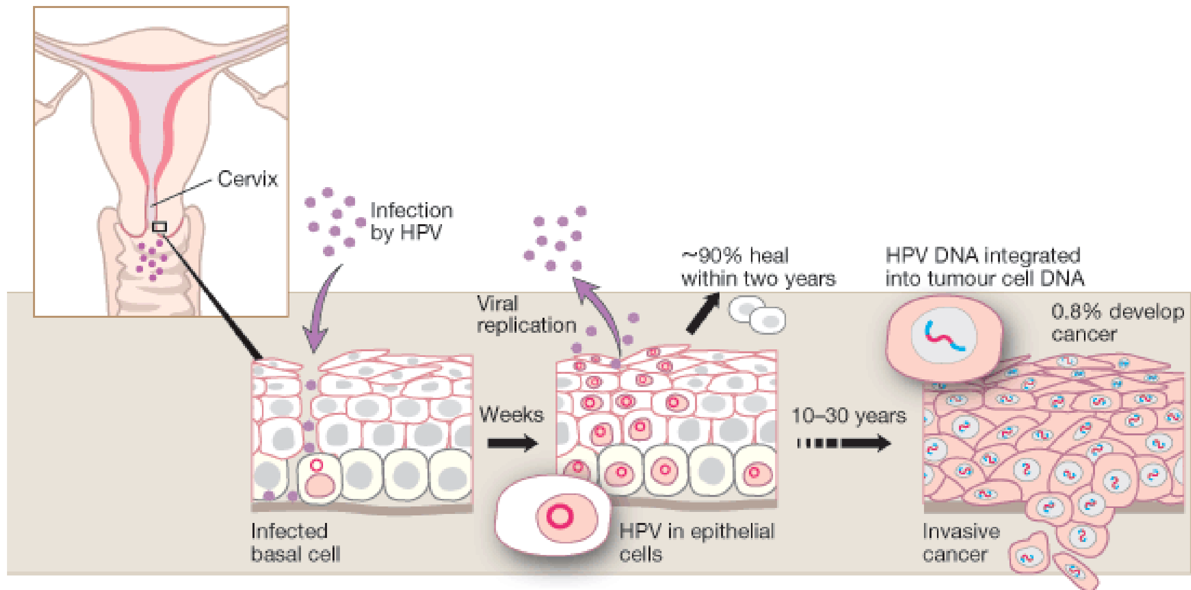

Figure 3. HPV16 viral life cycle. The progression of infection by HPV to cervical cancer. HPV infects the basal cells where viral replication occurs. Within two years most of the infection is cleared by the hosted immune system. If infection is persistent and the virus integrates itself into the host genome, the resulting upregulation of E6 and E7 expression can lead to cancer. Image modified from The Nobel Committee for Physiology or Medicine 2008- Illustrated by: Annika Röhl.

1.2 Targeting HPV16 E6

Since E6 is the main oncoprotein responsible for immortalization, and disruption of other cellular processes (Figure 2) results in the maintenance of the malignant phenotype (Klingelhutz et al. 1996, Singhania et al. 2012), specifically disrupting the activity of this oncoprotein is of particular interest. Given that E6 is only found in cells infected with HPV and not in normal tissues, E6 targeted treatments have the potential to reverse the malignant phenotype of infected keratinocytes while leaving healthy cells unharmed.

E6 expression and activity can be blocked at the mRNA and protein levels, respectively. Some research groups target the E6 protein specifically using small molecules, most of which resemble a flavonoid structure, to interfere with E6 protein-protein interactions (Cherry et al. 2013,Yuan et al. 2012, Malecka et al. 2014). Other groups have been using E6 specific antibodies (Courtête et al. 2007, Togtema et al. 2012) for radioimmunotherapy or in combination with cisplatin (Harris et al. 2013, Harris et al. 2011). Although E6 can be found within the cytoplasm of cells, the majority of this protein is located within the nucleus (Jackson et al. 2013), making it difficult to directly block using molecules which are often too large to passively diffuse into the nucleus (Pante et al. 2002, Togtema et al. 2012). Certain proteins can be transported through the nuclear membrane when they are tagged with a specific amino acid sequence, called a nuclear localization signal (Cokol et al. 2000).

Other molecules capable of selectively targeting and degrading E6 activity focus on its mRNA transcript. E6 mRNA is found within the cytoplasm so molecules that target the transcript only need to reach the perinuclear region of the cytoplasm and do not

require the addition of a nuclear localization signal. This makes small interfering RNAs (siRNAs) a more feasible and effective alternative. HPV16 E6 mRNA is bicistronic, sharing one transcript together with E7 (Smotkin et al. 1989) under the control of promoter p97. Some research groups target this E6/E7 promoter which prevents the transcription of the E6/E7 mRNA from occurring (Zhou et al. 2012, Zhou et al. 2013, Hong et al. 2009). There are two splice variants of the E6/E7 transcript known as E6*I-E7 and E6*II-E7 where E6* indicates a portion of the E6 open reading frame (ORF) being spliced out (Figure 4) (Smotkin et al. 1989). Since E6 induces cellular changes that result in malignant transformations of infected cells (Niccoli et al. 2012), effectively suppressing E6 while maintaining a minimal effect on E7 is the focus of this study. E6 can only be translated from the full-length transcript, E6/E7, whereas E7 can be translated from all three transcripts (Schneider-Gädicke and Schwarz 1986, Smotkin et al. 1989, Vaeteewoottacharn et al. 2005) so in order to suppress E6 only, siRNAs need to be designed to target the splice regions of the transcript. Some groups have designed siRNAs targeting the segment of E6 that falls along the splice site (Jiang and Milner 2002, Yamato et al. 2008, Courtête et al. 2007, Togtema MSc Thesis 2013). Not all research groups target E6 alone and there are many groups that use designed siRNAs that target the E6/E7 mRNA transcript outside of this splice site (Chang et al. 2010, Bai et al. 2006, Yamato et al. 2008) some of which are combined with chemotherapy (Jung et al. 2012, Tan et al. 2012).

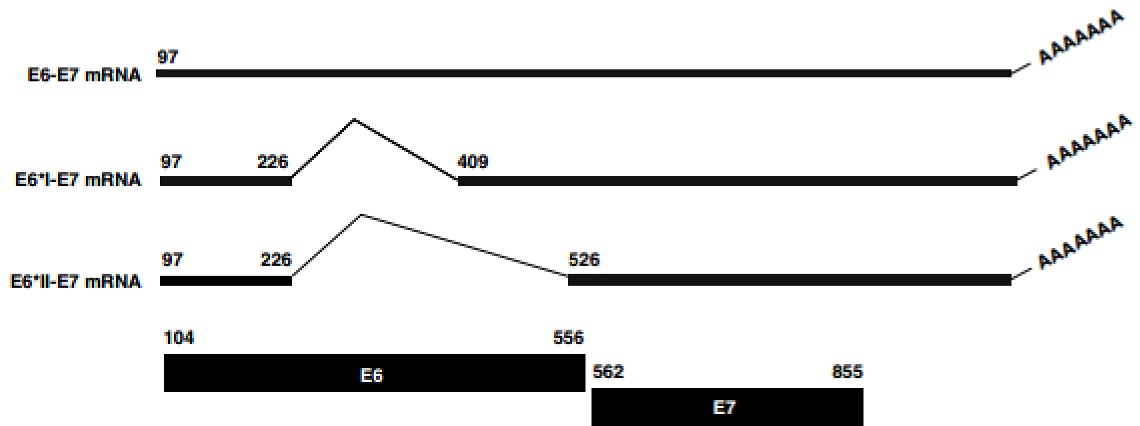

Figure 4. HPV E6 mRNA splice variants. A depiction of the HPV16 E6/E7 bicistronic transcript and two splice variants. E6*I and E6*II are truncated, preventing E6 translation. E6 can only be translated from the full length transcript, unlike E7 which can be translated from all three. Image modified from Yamato et al. 2008.

1.3 RNA Interference Pathway

An efficient approach to selectively eliminate E6 expression is by utilizing the RNA interference (RNAi) pathway. RNAi is a mechanism by which plants and invertebrates are able to defend against viral infections (Fire et al. 1998) (Figure 5). The general mechanism of RNAi involves the cleavage of double-stranded RNA (dsRNA) to short 21-23nt siRNAs. This processing event is catalyzed by Dicer, a highly conserved, dsRNA-specific endonuclease that is a member of the RNase III family (Hammond et al. 2000; Bernstein et al. 2001). The siRNAs then become incorporated into a group of proteins within the cell known as the RNA induced silencing complex (RISC) (Pecot et al. 2011). Active RISC promotes the unwinding of the siRNA through an ATP-dependent process, and the unwound antisense strand guides RISC to the complementary mRNA (Nykanen et al. 2001). The targeted mRNA is then cleaved by Argonaute 2, an endonuclease contained within the RISC, at a single site that is defined with regard to where the 5'-end of the antisense strand is bound to the mRNA target sequence (Hammond et al. 2000; Elbashir et al. 2001b, Meister et al. 2004). For RNAi-mediated mRNA cleavage and degradation to be successful, 5'-phosphorylation of the antisense strand must occur, and the double helix of the antisense-target mRNA duplex must be in the A form (Chiu and Rana 2002). The RISC is a multiple-turnover complex that does not require ATP hydrolysis and has a variable half-life (Chiu and Rana 2002, Gregory et al. 2005, Applied Biosystems Inc. 2008). Finding methods for maintaining the longevity of siRNAs in human cells is fundamental for applying RNAi to laboratory and therapeutic applications.

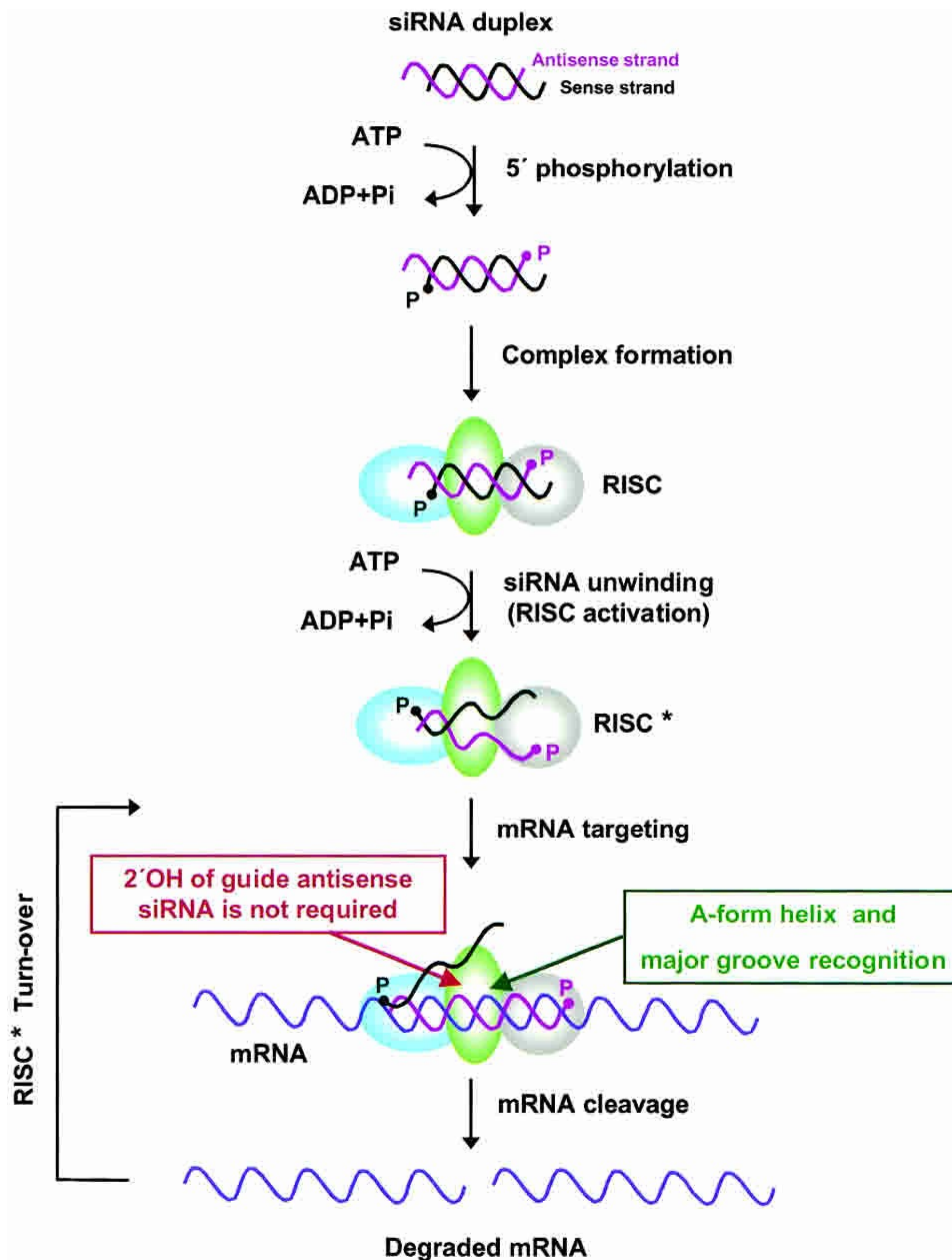

Figure 5. RNA interference pathway in human cells. Model for RNAi starting from the siRNA duplex and ending with degraded target mRNA. This highlights the requirement of the A-form helix and major groove for mRNA cleavage. Image taken from Chiu and Rana 2003.

1.3.1 Role of Dicer

Dicer is a monomeric endonuclease that belongs to the RNaseIII family. This enzyme contains an N-terminal RNA helicase domain, a Piwi Argonaute and Zwille (PAZ) domain, two RNase III domains, and a C-terminal dsRNA-binding domain (dsRBD) (Elbashir et al. 2002, Lau et al. 2009). The PAZ domain is an RNA-binding domain, which binds specifically to the 3' end of a single-stranded RNA (ssRNA) (Lingel et al. 2003, Vermeulen et al. 2005) On substrate binding, the enzyme rearranges such that the dsRBD clamps the dsRNA substrate over the surface of the catalytic domain dimer (Jinek and Doudna 2009). Four conserved acidic amino-acid residues in the active site of each RNaseIII domain coordinate two metal cations, suggesting that Dicer uses a two-metal-ion mechanism to catalyze RNA cleavage (Jinek and Doudna 2009). One metal ion binds and activates the water nucleophile, and the second metal ion facilitates departure of the 3′-oxygen atom (Nicholson 2014). The 17.5 Å distance between the two metal-ion pairs in the two active sites matches the width of the major groove in a dsRNA duplex. Modelling the binding of a dsRNA substrate to the enzyme reveals that the duplex runs along a flat surface formed by the platform domain and makes electrostatic interactions with a number of positively charged residues (Macrae et al. 2006). Processing by Dicer results in siRNA duplexes, typically 21-25 nucleotides in length that have 5'-phosphate and 3'-hydroxyl termini, and subsequently, these siRNAs are recognized by the RISC (Bernstein et al. 2001, Hammond et al. 2000). It is thought that Dicer is also required to introduce the siRNA into RISC and that Dicer is involved in RISC assembly (Lee et al. 2004; Tomari et al. 2004).

1.3.2 Function of RISC

The incorporation of siRNAs into the RISC requires the presence of 5'-phosphate groups and 3'-dinucleotide overhangs at the termini (Jinek and Doudna 2009, Elbashir et al. 2001a, Elbashir et al. 2001b). The PAZ domain specifically recognizes the 3' ends of ssRNAs, suggesting that it might function as a molecule for anchoring the 3' end of the guide strand within the RISC (Lingel et al. 2003). The 5' phosphate group is essential for slicing reliability due to the position of the cleavage site in the target RNA strand, which is determined by its distance from the 5' phosphate group of the guide strand (Elbashir et al. 2001a, Elbashir et al. 2001b). The control of RNA unwinding is an important step in the specificity of siRNA-triggered RNAi. siRNAs generally enter the RISC assembly pathway in a double-stranded form but only one strand is present in the activated RISC. This uptake is not random and is largely dictated by the relative thermal stability of base pairing at the two ends of the siRNA duplex (Khvorova et al. 2003, Schwarz et al. 2003). The strand with the less stable 5' end is selected by Argonaute and integrated into the RISC (Khvorova et al. 2003). The seed region of the guide strand (nucleotides 2-8) engages in Watson-Crick base-pairing interactions with the target RNA, assuming an A-form helical conformation (Pratt and MacRae 2009). After target mRNA cleavage, the mRNA strand is released and the RISC is recycled. In silencing pathways that rely on RNA silencing, the Argonaute protein carries out multiple cycles of target binding, cleavage and product release, while the guide strand remains bound to the protein (Jinek and Doudna 2009).

1.3.3 Short Interfering RNA (siRNA)

1.3.3.1 siRNA Design Databases

The success of RNAi depends on specific interactions between the siRNA and the target mRNA. However, the silencing ability of different siRNAs varies widely (Liu et al. 2012). Shifting siRNAs by a base or two along the mRNA sequence can change their potency by ten-fold or more (Kim et al. 2005). Several rules for rational design of siRNAs were proposed by numerous research groups, some of which can be found in Table 1. These design rules had a strong impact on the design methods of siRNA, and were mostly based on the information of GC content, preference or avoidance of specific nucleotides at specific positions, and siRNA sequence motifs. These design rules were proposed in the early 2000s and relied on very limited siRNA efficacy data at the time to help validate the design efficiency. More siRNA design development continued into the mid-2000s and new research groups became involved with developing more advanced statistical and machine-learning methods which led to more robust siRNA design rules. These design rules were incorporated into siRNA design tools that were available for public use and some are summarized by Liu et al. 2012 and references therein.

One software system, siDirect (Naito et al. 2004), provides highly effective, target-specific siRNAs directed for mammalian RNA interference. These siRNA sequences are selected using novel guidelines that were established through an extensive study of the relationship between siRNA sequences and RNAi activity (Ui-Tei et al. 2004, Reynolds et al. 2004, Amarzguioui and Prydz 2004).

While dedicated viral siRNA databases are lacking, there are a few siRNA databases reported in the literature. For example HuSiDa (Truss et al. 2005 and

references therein) and siRNAdb (Chalk et al. 2005 and references therein) provide sequences of published functional siRNA targeting human genes, while siRecords (Ren et al. 2009 and references therein) focuses on siRNA data of mammalian RNAi experiments and DSTHO (Dash et al. 2006 and references therein) on human oncogenes. A siRNA/shRNA database, known as VIRsiRNAdb, provides information on 1358 experimentally validated siRNAs pertaining to 42 important human viruses belonging to 19 different virus families and targeting as many as 150 different viral genome regions (Thakur et al. 2012 and references therein). VIRsiRNAdb provides comprehensive details of experimentally validated viral siRNAs targeting the diverse genome regions at one platform to help researchers working in the field of siRNA-based antiviral therapeutic development. While approximately 21 sequences targeting HPV16 E6 expression have been developed and are available in VIRsiRNAdb, there is high variability with the experimental designs which makes it difficult to determine the best strategy to follow for effective E6 silencing.

Although there are many siRNA design paradigms, there are still several problems, including inconsistencies among different siRNA design rules and incomplete feature sets of siRNAs, improper integration of the cross-platform siRNA data, inadequate consideration of the specificity of target mRNAs and, possible ways to effectively reduce off target effects. These four problems are explained in further detail by Liu et al. 2012 and references therein.

Table 1. A Summary of Classic siRNA Design Rules.

Rule Name	Design Rules	References
Ui-Tei Rules	• A/U at the 5' end of the antisense strand • G/C at the 5' end of the sense strand • At least five A/U residues in the 5' terminal one-third of the antisense strand • Absence of any GC stretch of >9nt in length	Ui-Tei et al. 2004
Amarzguioui Rules	• 32-58% G/C content • A higher A/U content in the 3' end than that in the 5' end of the sense strand • A G/C a position 1 in the sense strand • A base other than U at position 1 in the sense strand • An A at position 6 in the sense strand • An A/U at position 19 in the sense strand	Amarzguioui and Prydz 2004
Reynolds Rules	• 30-52% G/C content • At least 3 A/U at positions 15-19 in the sense strand • Absence of internal repeats • AN A at positions 3 and 19 in the sense strand • A U at position 10 in the sense strand • A base other than G or C at position 19 in the sense strand • A base other than A at position 13 in the sense strand	Reynolds et al. 2004
Tuschl Rules	• Select the target region preferably 50-100nt downstream of the start codon • Search for sequences 5'-AA(N19)UU in antisense strand (N is any nucleotide) • Search for sequences 5'-(N'19)TT in sense strand (N is any nucleotide) • 32-79% G/C content	Elbashir et al. 2001a

1.3.3.2 Rosetta siRNA Design Algorithm

Recommendations for the design of siRNAs are constantly being improved as knowledge of the RNAi process continues to expand. Sigma-Aldrich has a major intellectual portfolio in RNAi. They have committed to the rapidly developing area of RNAi through collaborations with many research organizations. One of the collaborations is with Rosetta Inpharmactics LLC who has a proprietary and patent pending bioinformatics design tool for siRNA research and development purposes. They apply the Rosetta siRNA Design Algorithm which bases its predictions on knowledge of the siRNA seed region and uses Position-Specific Scoring Matrices (PSSM). Additionally, the Rosetta siRNA Design Algorithm was developed utilizing data from over three years of gene-silencing experiments, ensuring that the algorithm's *in silico* rules are guided and reinforced by experimental evidence (Jackson et al. 2003, Jackson et al. 2006, Majercak et al. 2006, Espeseth et al. 2006).

1.3.4 Dicer Substrate siRNA

Integrated DNA Technologies (IDT) is the world's largest supplier of custom nucleic acids, serving the areas of academic research, biotechnology, clinical diagnostics, and pharmaceutical development. Their primary business is the manufacturing of custom DNA and RNA oligonucleotides for research applications. It has recently been described (Kim et al. 2005) that chemically synthesized RNA duplexes in the 25-30 bp length range can have as much as a 100-fold increase in potency compared with 21 bp siRNAs at the same location. Unfortunately, not all 27 bp oligonucleotides show this kind of increased potency. It is well known that shifting a 21 bp siRNA by a base or two along the mRNA

sequence can change its potency by ten-fold or more (Kim et al. 2005) which may be due to target accessibility.

Dicer substrate siRNAs (DsiRNAs) are processed by Dicer into 21bp siRNAs and designed so that cleavage results in a single desired product. This is possible due to a novel asymmetric design where the RNA duplex has a single 2-base 3′ overhang on the antisense strand and is blunt on the other end; the blunt end is modified with DNA bases (Rose et al. 2005). The incorporation of a 3' overhang at one end introduces a preference for processing to start from that end, while DNA nucleotides at the opposite blunt end enforce this asymmetry while blocking processing events involving the terminal two phosphodiester linkages. Dicer cannot cleave DNA residues since ssRNAs bind to the PAZ domain of the enzyme. This design provides Dicer with a single favorable binding site that helps direct the cleavage reaction. The crystal structure of Dicer from the unicellular eukaryote *Giardia intestinalis* revealed that the ability of Dicer enzymes to produce dsRNA fragments of a specific length originates from a unique spatial arrangement of the PAZ domain and the RNAseIII domains (Macrae et al. 2006).

Functional polarity is introduced by this processing event, which favors antisense strand loading into RISC. Increased antisense loading will result in increased mRNA cleavage. The observed increased potency obtained using longer dsRNAs to trigger RNAi is thought to result from providing Dicer with a substrate instead of a product and that this improves the rate or efficiency of entry of the RNA duplex into RISC as facilitated by Dicer (Figure 6).

IDT devised design rules that allow for intelligent designs of 27 bp DsiRNAs where the actual diced product can be predicted. IDT bases their design of DsiRNAs on

the combination of elements from both historical 21 bp oligonucleotide design criteria and new 27 bp oligonucleotide design criteria which are constantly being developed. Using this DsiRNA approach, sustained knockdown has been regularly achieved using sub-nanomolar concentrations (Hefner et al. 2008).

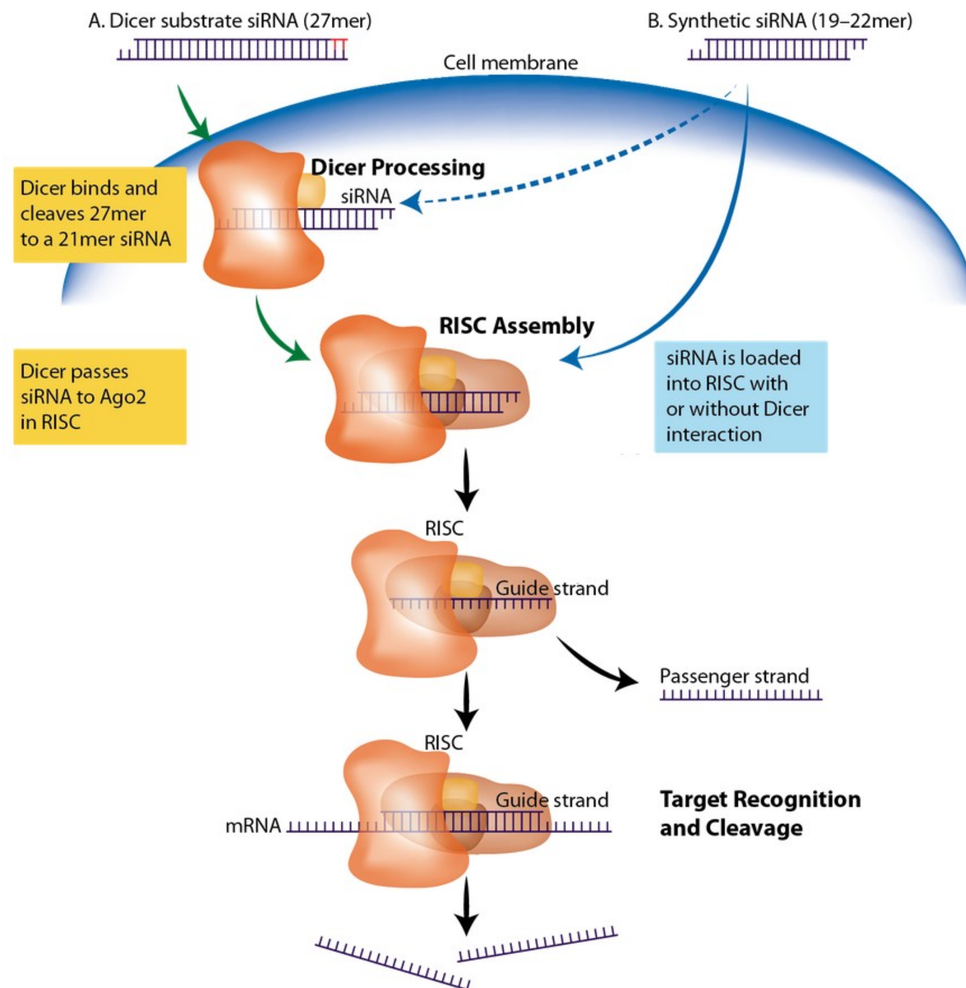

Figure 6. Comparison of DsiRNAs and siRNAs in the RNAi pathway. A. Dicer-substrate 27 bp oligonucleotides (27 mers) follow a two step process before being part of the RISC. **B.** Synthetic siRNAs, which are smaller than Dicer-substrates bypass the first step and only require one step before being part of the RISC. DsiRNAs and siRNAs follow the same steps once they are part of the RISC leading to target mRNA degradation. Image taken from http://www.vwmin.org/idt-dna-custom-dsirna.html.

1.4 Research Rationale

When considering treatment with E6 siRNA, there are several factors that need to be addressed. Identifying an optimal target sequence and determining how accessible that target sequence is are both critical to the success of RNA interference experiments. Gene silencing using unmodified siRNAs for both *in vitro* and *in vivo* experiments has several limitations that need to be overcome in order to achieve high target gene knockdown. Unmodified siRNAs are vulnerable to nuclease activity, resulting in easy degradation and short half-lives when in circulation. These molecules are capable of triggering an innate immune response *in vitro* and *in vivo* and are also known for non-specific binding which can cause uncontrolled off-target effects. In order to overcome these limitations, modifications to the siRNA duplex may improve or solve these limitations while still retaining RNAi activity.

While DsiRNAs have been shown to increase RNAi potency compared to regular siRNAs (Hefner et al. 2008, Kim et al. 2005), no previous study has compared DsiRNAs and siRNAs that target the same region of the HPV16 E6 transcript and the impact they have on the AA and EP variants. As mentioned previously, these two variants differ due to 6 SNPs (Yamada et al. 1995, Zehbe et al. 1998), resulting in the AA E6 variant having a higher risk factor in cervical cancer than EP E6 (Berumen et al. 2001) and both enhanced immortalization and transformation abilities compared to the EP E6 variant (Richard et al. 2010, Niccoli et al. 2012). These differential functional abilities lead to speculations that downstream cellular process may not be affected in the same way by HPV16 E6 variants (Jackson et al. 2014). To determine the best approach of RNAi when targeting HPV16 E6, several design algorithms need to be considered and tested *in vitro*

by not only looking at E6 mRNA and protein levels but also downstream processes to confirm that the E6 knockdown seen results in their restoration.

1.5 Hypotheses

1. Modifications to the siRNA backbones, as seen with DsiRNAs, will allow us to:
    a. Achieve increased HPV16 E6 knockdown compared to regular siRNAs in the E6 variants
    b. Lower the concentrations of siRNA needed to do this in the E6 variants
2. The increased knockdown of E6 expression will cause greater restoration of two main drivers for immortalization (*i.e.* increase p53 expression to induce apoptosis and decrease hTERT expression to prevent telomere elongation) compared to regular siRNAs

1.6 Research Aims

1. Conduct a literature search for different siRNA design algorithms and choose the best recommended target sites along the HPV16 E6 transcript to test their ability to affect E6 gene expression
2. Calculate the IC_{50} values for each of the target sites chosen in HPV16+ CaSki cells to determine the best siRNA and DsiRNA
3. Choose the siRNA and DsiRNA that gives the lowest IC_{50} value in CaSki cells and calculate the IC_{50} values in the AA and EP cells lines
4. Measure molecules downstream to E6 (such as p53 and hTERT) to confirm their restoration

2. Materials and Methods

2.1 Cell Culture

2.1.1 Cell Lines

CaSki (ATCC Cat. #CRL-1550) cells were cultured in complete growth medium, which contained Dulbecco's Modified Eagle Medium (DMEM; Fisher Scientific, Cat. # SH3024301 supplemented with 10% (v/v) fetal bovine serum (FBS; Fisher Scientific, Cat. # SH3039603) and 1X antibiotic/antimycotic (Fisher Scientific, Cat. # SV3007901). The cells were grown at 37°C in a humidified incubator with 95% air and 5% carbon dioxide gas. The medium was changed every second to third day. Once the cells reached a confluence of 70-80% they were passaged. This was achieved by removing and discarding the culture medium. The flask was then washed once with sterile Dulbecco's phosphate buffered saline (DPBS; Fisher Scientific, Cat. # SH3002802) to remove any cells that were suspended in the flask and any residual serum that would inhibit cell detachment. Trypsin (Fisher Scientific, Cat. # SH3023602) was then added to the flask and placed back into the 37°C incubator to facilitate cell detachment. Once the cells were detached, the trypsin was inactivated by the addition of complete growth medium that was at least three times the volume of the trypsin. The trypsin was removed by centrifugation at 25 x g (GS-6KR Centrifuge; Beckman, Mississauga, ON, Canada) for 5 minutes at 4°C. The supernatant was removed from the pellet and discarded followed by resuspension of the pellet with fresh medium. The cell number was determined by using a TC10™ Automated Cell Counter (BioRad, Mississauga, ON, Canada) and an appropriate number of cells were seeded back into the flask and placed back into the humidified incubator. Aseptic techniques were strictly followed.

Primary human foreskin keratinocytes (PHFKs; Cell Applications Inc, Cat. # 102-05n) were used. The PHFKs were previously retrovirally transduced with either the EP or AA variant of HPV16 E6 carrying a hemagglutinin (HA) tag on the C terminus (Niccoli et al. 2012). In this study both variants were passaged over 70 times, confirming their immortality.

The cells were cultured in Serum-Free Keratinocyte Growth Medium (Cedarlane, Cat. # 131-500) which was stored the same way as mentioned above. The trypsin was neutralized with Neutralizing Solution (Cedarlane, Cat. # 080-100) and the Neutralizing Solution was removed by centrifugation at 25 x g (GS-6KR Centrifuge; Beckman, Mississauga, ON, Canada) for 5 minutes. The supernatant was removed from the pellet and discarded followed by the re-suspension of the pellet with fresh medium. The cell number was determined as described above.

2.1.2 Mycoplasma Testing

Cells were routinely tested for *Mycoplasma* contamination every second to third passage. Approximately 100,000 cells were seeded onto a sterile glass coverslip (Fisher Scientific, Cat. # 12-23 541A) inside a 35 mm dish (Fisher Scientific, Cat. # 08772A) containing the appropriate growth medium and placed into the humidified incubator for 2-3 days. After the incubation, the medium was removed and discarded. The glass coverslip was then fixed with 1 mL of 3 parts methanol (Fisher Scientific, Cat. # A4544) and 1 part glacial acetic acid (Sigma-Aldrich, Cat. # 695092 2.5L) for 5 minutes. The fixative was removed and discarded and another 1 mL of the fixative was then added to the glass coverslip for 10 minutes. The coverslips were mounted using Vectashield

medium with DAPI (Vector Laboratories, Cat. # H-1200), to stain the nuclei fluorescent blue. The slides were examined using an inverted Zeiss Axiovert 200 microscope (Carl Zeiss Canada Ltd., North York, ON, Canada) for the presence of *Mycoplasma* DNA in the cytoplasm of the cells.

2.1.3 Cryopreservation of Cells

Cells were detached from the flask using trypsin and counted as mentioned in the section above. Aliquots of 900 μL of the suspended cells were pipetted into Nalgene 1.5 mL cryogenic vials (Fisher, Cat. #03-337-7Y) that contained 100 μL of the cryoprotective agent dimethylsulfoxide (DMSO, Sigma-Aldrich, Cat. #34869). The 1 mL cryogenic vials were frozen at -80°C in a Nalgene controlled rate freezing container (Fisher, Cat. #5100-0001) for a minimum of 24 hours and a maximum of two weeks. The vials were then transferred into a liquid nitrogen tank for long term storage.

2.1.4 Thawing Cells

Cryovials were carefully removed from the liquid nitrogen tank and placed into a 37°C bath to thaw. Once thawed, the 1 mL contents were transferred to a sterile 15 mL conical tube containing 9 mL of the appropriate medium. The tube was then placed in a centrifuge and spun at 25 x g (GS-6KR Centrifuge; Beckman, Mississauga, ON, Canada) for 5 minutes to separate the toxic DMSO from the cells. The supernatant was removed and discarded leaving a cell pellet which was re-suspended in the desired volume of the appropriate medium. The re-suspended cells in their fresh medium were then seeded into a growing flask and placed into a 37°C humidified incubator.

2.2 siRNA and Dicer Substrate siRNA

siRNA duplexes were custom synthesized by Sigma-Aldrich containing no base pair mismatches with either variant but targeting a region of the transcript expected to be structurally different between EP and AA E6. One siRNA duplex chosen was an experimentally validated E6 siRNA sequence (Courtête et al. 2007, Togtema, 2013) and another siRNA duplex was chosen based on the proprietary Rosetta algorithm (Table 2). Once the dried pellets were received, they were dissolved in nuclease-free dH_2O to a stock concentration of 2 μM. Aliquots were made to prevent multiple freeze-thaw cycles and these were stored at -20°C. A scrambled version of the E6 siRNA, having no target transcript within the cell, was used as a negative treatment control.

Dicer substrate siRNA (DsiRNA) duplexes were custom synthesized by Integrated DNA Technologies and the dried pellets were re-suspended the same way as mentioned above. A negative treatment control of the E6 DsiRNA (NC1), which had been validated by IDT to not target any sequences in human transcriptomes, was used (Table 2).

Table 2. siRNA Sequences Used in This Study.

Sequence	Location on HPV16 E6 Transcript	Sense strand (5' –> 3')	Antisense strand (5' –> 3')
MT siRNA**	386-404	CCGUUGUGUGAUUUGUUAA[dT][dT]	UUAACAAAUCACACAACGG[dT][dT]
MT DsiRNA*	386-410	CCGUUGUGUGAUUUGUUAAUUAG**GT**	ACCUAAUUAACAAAUCACACAACGGUU
Rosetta siRNA	247-265	GGAUUUAUGCAUAGUAUAU[dT][dT]	AUAUACUAUGCAUAAAUCC[dT][dT]
Rosetta DsiRNA*	247-271	GGAUUUAUGCAUAGUAUAUAGAG**AT**	AUCUCUAUAUACUAUGCAUAAAUCCCG
DsiRNA A*	293-317	GAUAAAUGUUUAAAGUUUUAUUC**TA**	UAGAAUAAAACUUUAAACAUUUAUCAC
DsiRNA B*	261-285	AUUAGAACAGCAAUACAACAAAC**CG**	CGGUUUGUUGUAUUGCUGUUCUAAUGU
DsiRNA C*	312-336	AUUCUAAAAUUAGUGAGUAUAGA**CA**	UGUCUAUACUCACUAAUUUUAGAAUAA
Scrambled siRNA	N/A	UAUGUGCUAUGUAUUAUUG[dT][dT]	CAAUAAUACAUAGCACAUA[dT][dT]
NC-1 negative control*	N/A	CGUUAAUCGCGUAUAAUACGCGU**AT**	AUACGCGUAUUAUACGCGAUUAACGAC

* Each strand contains RNA bases except for the sense strand of the DsiRNAs and the NC-1 negative control which are modified to have two DNA bases (shown underlined and bolded) at the end of the sense strands.
** This sequence has been previously validated by Courtête et al. 2007 and Togtema, 2013.

2.3 Relative mRNA Expression

2.3.1 Chemical Transfection to Determine IC_{50} Values

CaSki, EP E6, and AA E6 cells were seeded into 6-well plates 24 hours before transfection with siRNA at a concentration of 200,000 cells/well for the AA E6 cell line, 150,000 cells/well for the EP E6 cell line, and 100,000 cells/well for the CaSki cell line. After 24 hours the culture medium was removed and discarded and was replaced with 3 mL of fresh culture medium.

Transfection complexes were made for each well containing HiPerFect chemical transfection reagent (a mixture of cationic and neutral lipids) (Qiagen, Cat. # 301705), siRNA or DsiRNA, and culture medium (Refer to Table 3 for exact amounts). Before the complexes were added to the wells, they were mixed briefly by vortexing and incubated at room temperature for 10 minutes to allow complex formation. After the incubation period, the 600 μL transfection complexes were added drop-wise to their respective wells. The 6-well plates were gently tilted back and forth to ensure even distribution of the complexes. The plates were then placed into the humidified incubator for 48 hours. Untransfected cells (i.e. treated with culture medium only) were used to determine baseline E6 mRNA levels. All transfection experiments were independently repeated in triplicate (n=3).

Table 3. Transfection Complex Volumes Used for IC_{50} Experiments.

Desired siRNA and DsiRNA Concentrations	Cell Types		
	AA	**EP**	**CaSki**
0.00025 nM	3.6 µL of 0.25 nM siRNA 578.4 µL of medium 18 µL HiPerFect	3.6 µL of 0.25 nM siRNA 578.4 µL of medium 18 µL HiPerFect	N/A
0.0025 nM	36 µL of 0.25 nM siRNA 546 µL of medium 18 µL HiPerFect	36 µL of 0.25 nM siRNA 546 µL of medium 18 µL HiPerFect	N/A
0.025 nM	360 µL of 0.25 nM siRNA 222 µL of medium 18 µL HiPerFect	360 µL of 0.25 nM siRNA 222 µL of medium 18 µL HiPerFect	N/A
0.25 nM	0.45 µL of 2 µM siRNA 581.55 µL of medium 18 µL HiPerFect	0.45 µL of 2 µM siRNA 581.55 µL of medium 18 µL HiPerFect	0.45 µL of 2 µM siRNA 581.55 µL of medium 18 µL HiPerFect
2.5 nM	4.5 µL of 2 µM siRNA 577.5 µL of medium 18 µL HiPerFect	4.5 µL of 2 µM siRNA 577.5 µL of medium 18 µL HiPerFect	4.5 µL of 2 µM siRNA 577.5 µL of medium 18 µL HiPerFect
25 nM	45 µL of 2 µM siRNA 537 µL of medium 18 µL HiPerFect	45 µL of 2 µM siRNA 537 µL of medium 18 µL HiPerFect	45 µL of 2 µM siRNA 537 µL of medium 18 µL HiPerFect
250 nM	450 µL of 2 µM siRNA 132 µL of medium 18 µL HiPerFect	450 µL of 2 µM siRNA 132 µL of medium 18 µL HiPerFect	450 µL of 2 µM siRNA 132 µL of medium 18 µL HiPerFect

*All complexes in this table add up to 600 µL and were added dropwise into 6-well plates containing 3 mL of fresh medium.

2.3.2 RNA Extraction

The cells were harvested from the 6-well plates 48 hours following transfection using trypsin as described above. Complete growth medium for CaSki and neutralizing solution for AA E6 and EP E6 was added to the cells to inactivate the trypsin and the samples were centrifuged for 5 minutes at 25 x g (GS-6KR Centrifuge; Beckman). The supernatant was removed and discarded while the pellet was re-suspended in 1mL DPBS. Each sample was centrifuged for 5 min at 25 x g (GS-6KR Centrifuge; Beckman) and the DPBS was removed and discarded from each cell pellet. The dry cell pellets were frozen at -80 °C until RNA extraction.

RNA extraction was performed using the Arctus PicoPure RNA Isolation Kit (Applied Biosystems, Cat. # KIT0204). The optional DNase treatment (RNase-free DNase Set; Qiagen, Cat. # 79254) step was included and all samples were eluted in 30 μL of elution buffer. All samples were stored at -80 °C until conversion to cDNA.

The quantity of the RNA in ng/μL as well as its integrity, as assessed by the 28S:18S ratio, was determined using the Experion Automated Electrophoresis System with the StdSens Analysis chip kit (BioRad, Cat. # 700-7111). All samples with an RNA quality indicator (RQI) value of less than 7 were potentially degraded and were not used. All samples in this study achieved an RQI of greater than 7.

2.3.3 Conversion of RNA to cDNA

Each RNA sample was reverse transcribed into complementary DNA (cDNA) using the High Capacity cDNA Archive Kit (Applied Biosystems, Cat. # 4322171) in a 60 μL reaction. The volume of eluted RNA was brought up to 30 μL using nuclease-free

dH2O and added to 30 µL of 2X master mix. The 2X master mix contained 2X RT Buffer, 2X random primers, 2X dNTPs, multiscribe enzyme, and nuclease-free dH2O to raise the final volume to the correct amount. The reactions were run in a 2720 Thermal Cycler (Applied Biosystems) using the following parameters: 10 minutes at 25 °C, 120 minutes at 37 °C, 5 minutes at 85 °C and an infinite hold at 4 °C. All cDNA samples were stored at -20 °C until real-time reverse transcription polymerase chain reaction (RT-qPCR) analysis.

2.3.4 Real-Time Reverse Transcription Polymerase Chain Reaction Analysis of Relative mRNA Expression

RT-qPCR analysis (7500 Real-Time PCR System; Applied Biosystems) was used to determine the change in the relative expression of HPV16 E6, in response to treatment of the cells with E6 siRNA, E6 DsiRNA, scrambled siRNA and the negative DsiRNA control NC1. Hypoxanthine phosphoribosyltransferase 1 (HPRT1) was used as the reference gene, as previous studies by our group have shown its expression to remain unchanged in response to HPV infection (DeCarlo et al. 2008).

Reactions of 90 µL were mixed containing 45 µL of Taqman® Universal PCR Master Mix (Applied Biosystems, Cat. # 4364338), 4.5 µL of 20X Taqman® Gene Expression Assay (Applied Biosystems, Table 4), 150 ng cDNA, and nuclease-free dH2O to raise the final volume to the correct amount. Twenty-five µL of the reaction mixture was loaded into each of 3 wells on a MicroAmp® Optical 96-Well Reaction Plate (Applied Biosystems, Cat. # 4306737). Positive CaSki cell controls and negative nuclease-free dH2O controls containing the HPRT1 Gene Expression Assay were run as

technical controls on each plate. Cycle threshold (Ct) data was analyzed relative to HPRT1 and calibrated to cells treated with medium only or HiPerFect only ($2^{-\Delta\Delta Ct}$, Livak and Schmittgen 2001).

2.4 Relative Protein Expression

2.4.1 Chemical Transfection with siRNA and DsiRNA

EP E6 and AA E6 were grown in T25 (25 cm^2) flasks in keratinocyte growth medium (KGM) (Fisher Scientific, Cat. # 101269) until they reached ~35% confluence. The culture medium was aspirated and replaced with 5 mL of fresh culture medium. Transfection complexes of 1 mL were mixed for each flask. To give a final concentration of 6.75 nM (D)siRNA in the flask, each complex consisted of 30 µL HiPerFect chemical transfection reagent, 20.25 µL of 2 µM Rosetta siRNA, Rosetta DsiRNA, NC-1 or scrambled siRNA stock, and 949.75 µL culture medium. The complex including HiPerFect only contained of 30 µL HiPerFect chemical transfection reagent and 970 µL culture medium. The complexes were mixed by vortexing, incubated at room temperature for 10 minutes at room temperature and then added drop-wise to the flasks. The flasks were gently tilted to ensure even distribution of the complexes and were returned to the incubator for forty-eight hours.

Table 4. Gene Expression Assays Used in this Study.

TaqMan® Gene Expression Assay	Assay ID number
HPRT-1	Hs99999909_m1
HPV16 Full Length E6*	AI0IW1V
HPV16 Full Length AA E6*	AIWR2XO
hTERT	Hs00162669_m1

*custom

2.4.2 Protein Extraction

Forty-eight hours following transfection, the cells were harvested from the flasks using trypsin as described above. Following removal of the Neutralizing Solution, each cell pellet was resuspended in 4 mL DPBS (Fisher, Cat. # SH3002802). The cells were split into two tubes: 1 mL of the suspended cells was placed into a 15 mL tube for RNA extraction and the remaining 3 mL were left in the tubes for protein extraction. Each sample was centrifuged for 5 min at 25 x g (GS-6KR Centrifuge; Beckman) and the DPBS was removed from each cell pellet. The cell pellets for the RNA extraction were placed in the -80°C freezer until ready for RNA extraction (see Section 2.3). For protein extraction, lysis buffer composed of 1 mL cold RIPA buffer (50 mM Tris-HCl (pH 7.5), 150 mM sodium chloride (Fisher, Cat. # S2713), 0.1 % SDS, 1 % sodium deoxycholate, 1 % Triton X-100 (Fisher, Cat. # BP151-100), 5 mM EDTA, and 10 mM sodium fluoride (Fisher, Cat. # S299-100)), 10 mM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich, Cat. # P7626), a 1:100 dilution of protease inhibitor cocktail (Sigma-Aldrich, Cat. # P8340), and 2 mM of sodium orthovanadate (Sigma-Aldrich, Cat. # S6508-50G) was mixed fresh and chilled at 4 °C. Each fresh pellet was resuspended in 110 µL of the chilled lysis buffer and left on ice and in the 4 °C fridge for 20 minutes. The solutions were centrifuged for 10 minutes at 21,000 x g in a microcentrifuge pre-chilled to 4 °C (Centrifuge 5415C; Eppendorf, Mississauga, ON, Canada) to remove cell debris and obtain the protein containing supernatant.

A Detergent Compatible (DC) protein assay (BioRad, Cat. # 500-0112) was completed to determine protein concentration. A 2 % volume of each sample, 10 % of Reagent A' (2 % of Reagent S mixed with 98 % of Reagent A) and 87 % of Reagent B

were mixed in that exact order. Various concentrations of bovine serum albumin (BSA) were used as the standards. The reactions were incubated for 15 minutes at room temperature and transferred to a 96-well plate (Fisher, Cat. # 087722c) where the protein concentration was analyzed using a plate reader (PowerWave XS; BioTek).

2.4.3 Gel Electrophoresis

Thirty µg of protein was mixed with 6X SDS loading buffer (375 nM Tris-HCl pH 6.8, 10 % SDS, 50 % glycerol, 0.03 % bromophenol blue) with freshly added dithiothreitol (Fisher Scientific, Cat. #BP1725) to a final concentration of 600 nM. All samples were brought up to the highest sample volume using 1X SDS loading buffer. The SDS containing samples were heated at 95 °C for 5 minutes to denature the protein. The BioRad Prestained SDS-PAGE Low Range Standards (BioRad, Cat. # 161-0305) (heated for 2 minutes) as well as the BioRad Precision Plus Protein Dual Colour Standards (BioRad, Cat. # 161-0374) (not heated) were used for protein ladders. All samples and standards were centrifuged for 1 minute at 21,000 x g (Centrifuge 5415C; Eppendorf) and loaded onto a Mini-Protean TGX Precast Gel (4-20 % gradient) (BioRad, Cat. # 456-1094). The gel was run in 1X running buffer (25 mM Tris Base, 190 mM glycine (Fisher, Cat. # BP381-5), and 0.5 % SDS) at 120 V for 1 hour and 10 minutes, until the dye front ran off the bottom of the gel.

2.4.4 Protein Transfer

Following the completion of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were transferred to PVDF membrane (Fisher, Cat. # PI88518), which had been activated by soaking in 100 % methanol (Fisher, Cat. # A4544) for 15 minutes. The transfer was done at 100 V for 1 hour in chilled 1X transfer buffer (25 mM Tris Base, 190 mM glycine, and 20 % methanol 100 mL) with an ice pack and a stir bar in the container to keep the temperature cool. Following the transfer, the membrane was soaked in 1X TBS (20 mM Tris base pH 7.5, 150 mM sodium chloride) with 0.05 % TWEEN® 20 (Fisher, Cat. # 337-100) (TBST) for 10 minutes. The membrane was then cut between the 25 and 37 kDa markers on the Precision Plus ladder and between the 34 and 47 kDa markers on the Low Range ladder allowing for p53/actin (top half of membrane) and HA (bottom half of membrane) to be detected simultaneously.

2.4.5 p53 and HA Detection

Both membrane halves were blocked with 5 % non-fat milk in 0.05 % TBST for 1 hour at room temperature on a rocker. The top half of the membrane was then incubated with a mouse monoclonal p53 primary antibody (DAKO, Cat. # M700101) diluted to 1:1000 in 50 mL tubes (Fisher, Cat. #1443222) on a tube roller overnight at 4 °C. The bottom half of the membrane was incubated with a mouse monoclonal HA primary antibody (Abcam, Toronto, ON, Canada, Cat. # ab18181) diluted to 1:500 in 50 mL tubes (Fisher, Cat. #1443222) on a tube roller overnight at 4 °C.

The following morning, the membrane halves were rinsed once in TBST and then were washed 3 x 5 minutes in TBST on a rocker. Both the bottom and the top half of the membrane were incubated with a goat anti-mouse +HRP secondary antibody (Jackson ImmunoResearch, Cat. # 115-035-062) diluted 1:2000 in 5 % non-fat milk in TBST. The incubation was for 1 hour at room temperature on a rocker. The membranes were then rinsed again in TBST and washed 4 x 5 minutes in TBST on a rocker. Chemiluminescence was done using the Western Lightening®-ECL kit (PerkinElmer, Waltham, MA, USA, Cat. #NEL100001EA). One mL of each of the two solutions was mixed together and applied to each membrane half for 1 minute with agitation. The membrane halves were then immediately imaged using the Biospectrum 410 Imaging System (UVP) with a 10 minute exposure for HA with 4x4 binning and a 1 minute exposure for p53 with 4x4 binning. Densitometry was performed using the density analysis tool provided with VisionWorks software using actin as a loading control.

2.4.6 Stripping and Re-probing for Actin

Since both actin and p53 are very close in size (~46 and 53 kD respectively), they could not be imaged at the same time. After the top half of the membranes were imaged for p53, the membranes were washed with a mild stripping buffer (1.5 % glycine, 0.1 % SDS, 1 % Tween 20, pH adjusted to 2.2 and final volume brought up to 500 mL) twice for 7 minutes at room temperature. The membranes were then washed twice more with TBST for 5 minutes. The membranes were blocked with 5 % non-fat milk powder in TBST for 1 hour at room temperature on a rocker. The top half of the membrane was then incubated with a goat polyclonal actin primary antibody (Santa Cruz, Dallas, TX, USA,

Cat. # SC-1616) diluted to 1:1000 in 5 % non-fat milk powder in 50 mL tubes (Fisher, Cat. #1443222) on a tube roller overnight at 4 °C.

The following morning, the membranes were rinsed once in TBST and then were washed 3 x 5 minutes in TBST on a rocker. The membranes were incubated with a donkey anti-goat +HRP secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA, Cat. # 705-035-147) diluted 1:2000 in 5 % non-fat milk powder for 1 hour at room temperature on a rocker. The membranes were rinsed and washed, and chemiluminescence was done as described above. The membranes were then immediately imaged as mentioned aboved with a 2 minute exposure for actin with 2x2 binning.

2.5 Statistical Analyses

Statistical analyses and the creation of graphs were done using the open source programming language R, version 3.2.1 (R Development Core Team 2015). Data were determined to be parametric based on independence, normality (using Shapiro-Wilk's test) and homogeneity of variance (using Bartlett's test). Based on these results, one or two-way ANOVAs were used to determine global differences between the means. If significant differences were found, Tukey's HSD post hoc analyses were performed. All IC_{50} values were determined using R based on the LL.4 algorithm. The significance level (α) was set, a priori, at 0.05. Unless indicated otherwise, data represent mean +/- SEM.

3. Results

3.1 HPV 16 E6 Transcript Targets

Identifying an optimal target sequence is critical to the success of RNA interference experiments. Since it is difficult to predict the optimal siRNA sequence for a given target, multiple siRNAs need to be evaluated. A literature search was completed determining which siRNA resulted in the highest HPV16 E6 knockdown that targeted the HPV16 E6 splice site. A database of experimentally validated siRNA/shRNAs, VIRsiRNAdb (Thakur et al. 2012 and references therein), summarizes approximately 21 sequences targeting HPV16 E6 expression. Of these 21 sequences only 5 siRNA targets fall within the E6 splice site (Jiang and Milner 2002, Courtête et al. 2007, Yamato et al. 2008). These sequences were transfected in either CaSki or SiHa, and had varying levels of success (36 – 70 % E6 silencing efficacy). They targeted different regions along the E6 splice site, used varying concentrations of complexes (10 – 50 nM), and used different transfection strategies (Oligofectamine, HiPerFect, and Lipofectamine). Given the variability in past experimental designs, it was difficult to determine the best strategy to follow.

3.1.1 MT siRNA Target Has Been Previously Shown to Knockdown E6 Expression and Restore p53 Levels

The siRNA that used the lowest amount of RNAi complex and achieved high E6 knockdown was the one published by Courtête et al. (2007) and was thus used in this study. The chosen siRNA had no base pair mismatches with either variant but targeted a region of the transcript expected to be structurally different between the AA and EP

variants (Togtema, 2013). This target site achieved approximately 90 % knockdown at the mRNA level with 25 nM of the siRNA complex in both variants (Togtema, 2013). The sequence of this siRNA, labeled as MT siRNA, on the CaSki, AA, and EP cell types can be found in Table 2.

Since the other four siRNA duplexes listed in the VIRsiRNAdb database that targeted the E6 splice site used higher concentrations and achieved varying levels of knockdown, they were not considered for this study (Jiang and Milner 2002, Yamato et al. 2008). In order to evaluate several siRNAs to determine the optimal siRNA sequences, new siRNAs need to be designed based on established design algorithms.

3.1.2 Rosetta Algorithm

Recommendations for the design of siRNAs are constantly being improved upon as knowledge of the RNAi process continues to expand. We previously ordered custom siRNAs from Sigma-Aldrich and determined that they have their own proprietary algorithm in choosing an optimal target site along the mRNA transcript. The Rosetta siRNA Design Algorithm was developed utilizing data from over three years of gene-silencing experiments, ensuring that the algorithm's *in silico* rules are guided and reinforced by experimental evidence (Jackson et al. 2003, Jackson et al. 2006, Majercak et al. 2006, Espeseth et al. 2006). To my knowledge, no siRNA has been previously designed by this algorithm that targets HPV16 E6 splices sites. Based on the HPV 16 E6 transcript, Sigma-Aldrich provided their top three target sites. However, only their top target site aligned within the E6 splice site. As mentioned previously, effectively suppressing E6 while most likely rendering E7 intact is possible by only targeting the E6

splice site, the siRNA that only aligned with the splice site was used. The sequence of this siRNA, labeled as Rosetta siRNA, on the CaSki, AA, and EP cell types can be found in Table 2.

3.1.3 Dicer Substrate siRNAs (DsiRNAs)

IDT recommended using Dicer-substrate RNAs (DsiRNAs) instead of the canonical siRNAs based on previous experiments (Hefner et al. 2008, Kim et al. 2005). DsiRNAs are chemically synthesized 27 bp duplex RNAs that have increased potency in RNA interference compared to traditional siRNAs (Kim et al. 2005). Using this approach, IDT states that sustained knockdown has been regularly achieved using sub-nanomolar concentrations. New design rules specific to DsiRNAs are constantly being developed. To my knowledge, no siRNA has been previously designed by this algorithm that targets HPV16 E6 splices sites. Based on the HPV 16 E6 transcript, IDT selected three target sites for their DsiRNAs along the splice site of the two E6 variants. These DsiRNAs are labeled as DsiRNA A, DsiRNA B, and DsiRNA C. The sequences of these three DsiRNAs on the CaSki, AA, and EP cell types can be found in Table 2.

3.2 Relative E6 mRNA Expression in CaSki, AA, and EP Cell Types

The relative E6 mRNA expression between the three cell types was determined to see if there is was difference baseline between these cell types. The expression was determined relative to HPRT-1. The E6 expression ratio at the mRNA level between AA, CaSki, and EP was found to be significantly different comparing these three cell types ($p=9.49e-10$) (Figure 7). Since there was a difference in E6 expression, the E6

knockdown percentages were calibrated to the cells treated with media only for the IC_{50} experiments and their corresponding negative controls (NC-1 for DsiRNA and scrambled for siRNA) for any downstream experiments. Both AA and EP showed higher E6 expression levels compared to CaSki which make these cell lines more appropriate for E6 specific *in vitro* experiments which is why downstream restoration assays in this study focused on these two variants. No issues with *Mycoplasma* contamination were noted throughout the study.

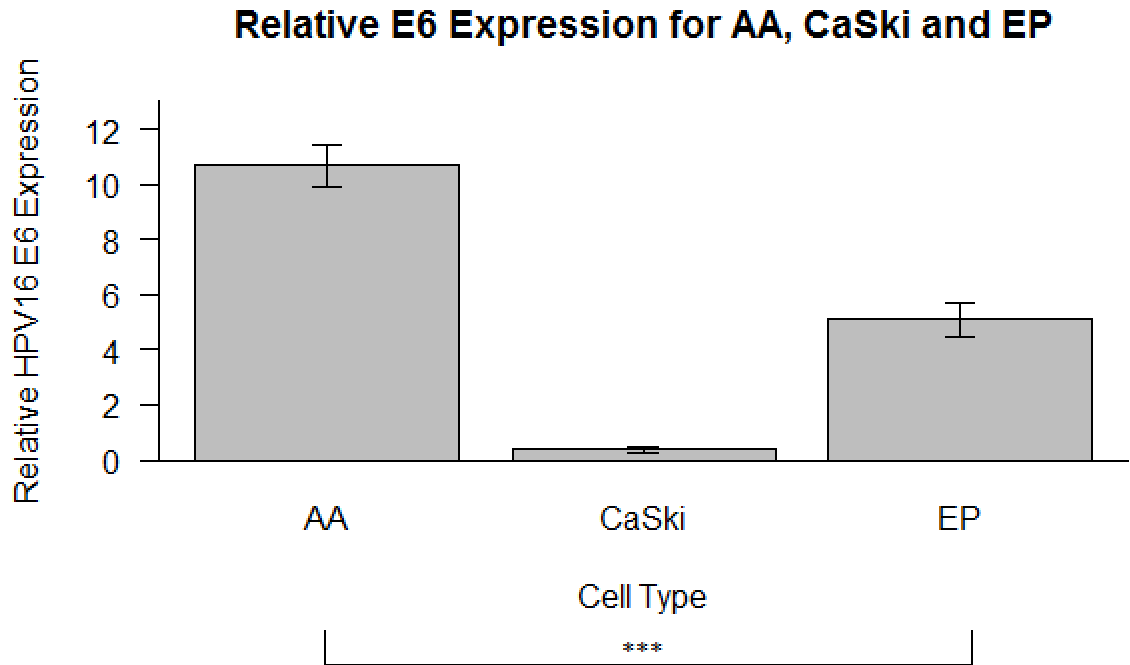

Figure 7. Relative E6 mRNA expression ratio comparing AA, CaSki, and EP cells. The E6 expression ratio at the mRNA level between AA, CaSki, and EP was found to be significantly different comparing these three cell types (p=9.49e-10). Expression was determined relative to HPRT-1. Statistical analysis was performed using a one-way ANOVA followed by Tukey's HSD contrasts post hoc. Data represents mean ± SEM, *n*=9 for all. ***denotes p<0.001.

3.3 CaSki IC_{50} Results

3.3.1 DsiRNA A, B, and C Showed Similar E6 Knockdown

To determine the best DsiRNA, based on the relative E6 mRNA expression, using the top three DsiRNAs recommended from IDT, an IC_{50} value was calculated for each DsiRNA. Treatment with higher concentrations of all three DsiRNAs significantly reduced E6 expression in CaSki cells (p=0.0256). There was no significant difference between the three DsiRNA target sites (p=0.8187) (Figure 8). The expression was determined relative to HPRT-1 and calibrated to cells in medium only. The IC_{50} values could only be calculated by the R software for DsiRNA C to be 71 ± 166 nM. Since the E6 knockdown only reached approximately 50 % and the averages varied with many samples, it was difficult for the software to permit a curve analysis for all three DsiRNAs investigated.

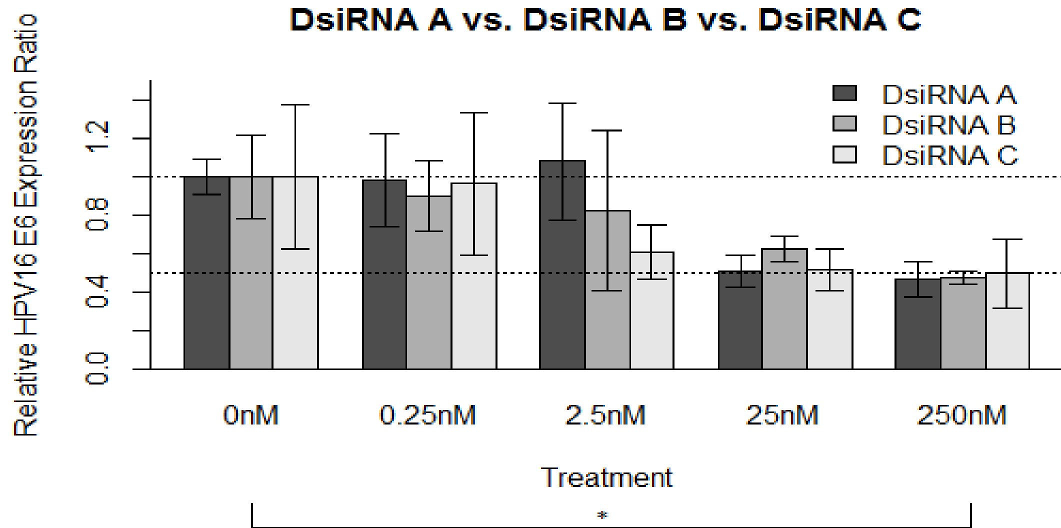

Figure 8. Relative E6 mRNA expression ratio comparing DsiRNA A, B and C in CaSki cells. Treatment with higher concentrations of all three DsiRNAs significantly reduced E6 expression in CaSki cells (p=0.0256). There was no significant difference between the DsiRNA target sites (p=0.8187). Expression was determined relative to HPRT-1 and calibrated to cells treated with growth medium only. Statistical analysis was performed using a two-way ANOVA followed by Tukey's HSD contrasts post hoc. Data represents mean ± SEM, *n*=3 for all. * denotes p=0.05-0.01.

3.3.2 Rosetta siRNA Showed More E6 Knockdown than MT siRNA

Based on my DsiRNA IC_{50} values, the lowest IC_{50} value obtained was 71 ± 166 nM. The Rosetta siRNA and MT siRNA were transfected into CaSki cells to determine if their IC_{50} values were better than the previously determined values. Treatment with higher concentrations of both siRNAs reduced E6 expression in CaSki cells but not significantly ($p=0.0888$). Rosetta siRNA showed more E6 knockdown than MT siRNA but this difference was not statistically significant ($p=0.0636$) (Figure 9). The expression was determined relative to HPRT-1 and calibrated to cells with growth medium only. The IC_{50} value for MT siRNA could not be determined by the software since the E6 knockdown did not reach the 50 % mark making it difficult for the software to permit a curve analysis. However, the IC_{50} value for Rosetta siRNA was determined to be 7 ± 13 nM which is at least 10 times lower than the three DsiRNAs measured previously in this study. Since the Rosetta target site achieves the lowest IC_{50} value when compared to MT siRNA and the three DsiRNAs, it was used for downstream experiments in this study.

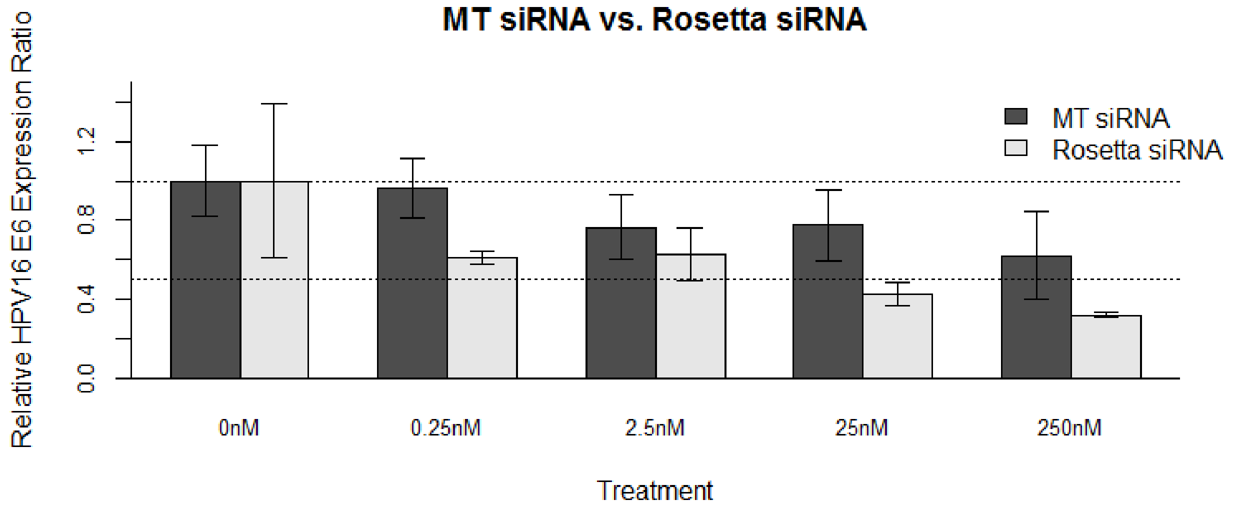

Figure 9 Relative E6 mRNA expression ratio comparing MT siRNA and Rosetta siRNA in CaSki cells. Treatment with higher concentrations of both siRNAs reduced E6 expression in CaSki cells but not significantly (p=0.0888). There was no significant difference between the siRNA target sites (p=0.0636). Expression was determined relative to HPRT-1 and calibrated to cells treated with growth medium only. Statistical analysis was performed using a two-way ANOVA followed by Tukey's HSD contrasts post hoc. Data represents mean ± SEM, *n*=3 for all.

3.3.3 siRNA Converted into DsiRNA

Traditionally, siRNAs are chemically synthesized as 21 bp oligonucleotides whereas DsiRNAs are chemically synthesized as 27 bp oligonucleotides which are then cleaved by Dicer into 21 mer siRNAs. According to IDT, their DsiRNAs were recommended over the classic siRNAs, which mimic the dicer cleavage product. To determine if there was a difference in knockdown based on the presence or absence of Dicer at the first step in the RNAi pathway the two siRNAs, Rosetta siRNA and MT siRNA were converted to their corresponding DsiRNAs (Figure 10). Since MT siRNA has been previously used by this research group (Togtema, 2013) as well as another research group (Courtête et al. 2007) which showed promising results, we decided to convert it into a DsiRNA, even though an IC_{50} value was unable to be calculated, to determine if changes in the RNA structure influenced efficacy. Since Rosetta siRNA gave the best IC_{50} values, we wanted to compare that to its DsiRNA form. The sequences of these two new DsiRNAs on the CaSki, AA, and EP cell types can be found in Table 2.

Rosetta siRNA:

5′- GGAUUUAUGCAUAGUAUAUAG-3′
3′- CCUAAAUACGUAUCAUAUAUC-5′

Rosetta DsiRNA:

5′- GGAUUUAUGCAUAGUAUAUAG′ AG**AT**-3′
3′-GCCCUAAAUACGUAUCAUAUA′ UCUCUA-5′

MT siRNA:

5′- CCGUUGUGUGAUUUGUUAAUU-3′
3′- GGCAACACACUAAACAAUUAA-5′

MT DsiRNA:

5′- CCGUUGUGUGAUUUGUUAAUU′ AG**GT**-3′
3′-UUGGCAACACACUAAACAAUU′ AAUCCA-5′

Figure 10. Conversion of Rosetta siRNA and MT siRNA into their corresponding DsiRNAs. The siRNAs were converted into DsiRNAs based on the predicted cleavage sites by Dicer shown by the " ′ " symbol within the sequence of the DsiRNAs. The DsiRNA has a single 2-base 3' overhang on the antisense strand and a blunt end modified with DNA bases (shown underlined and bolded). This is processed by Dicer into siRNAs with symmetric 2-base 3' overhangs on each end.

3.3.4 MT DsiRNA Showed More E6 Knockdown than MT siRNA

While the MT siRNA target site along the E6 mRNA transcript did not produce a "hit" as a good target site for DsiRNA, since this target site was previously validated it was still converted into a DsiRNA and compared to its siRNA form. Treatment with higher concentrations of the siRNA and the DsiRNA reduced E6 expression in CaSki cells significantly (p=0.00659). MT DsiRNA showed more E6 knockdown than MT siRNA but this difference was not statistically significant (p=0.07622) (Figure 11). The expression was determined relative to HPRT-1 and calibrated to cells with growth medium only. The IC_{50} value for MT siRNA, as stated previously, could not be determined by the software but the IC_{50} value for MT DsiRNA was determined to be 23 ± 12 nM which is lower than DsiRNA A, B and C but not as low as Rosetta siRNA.

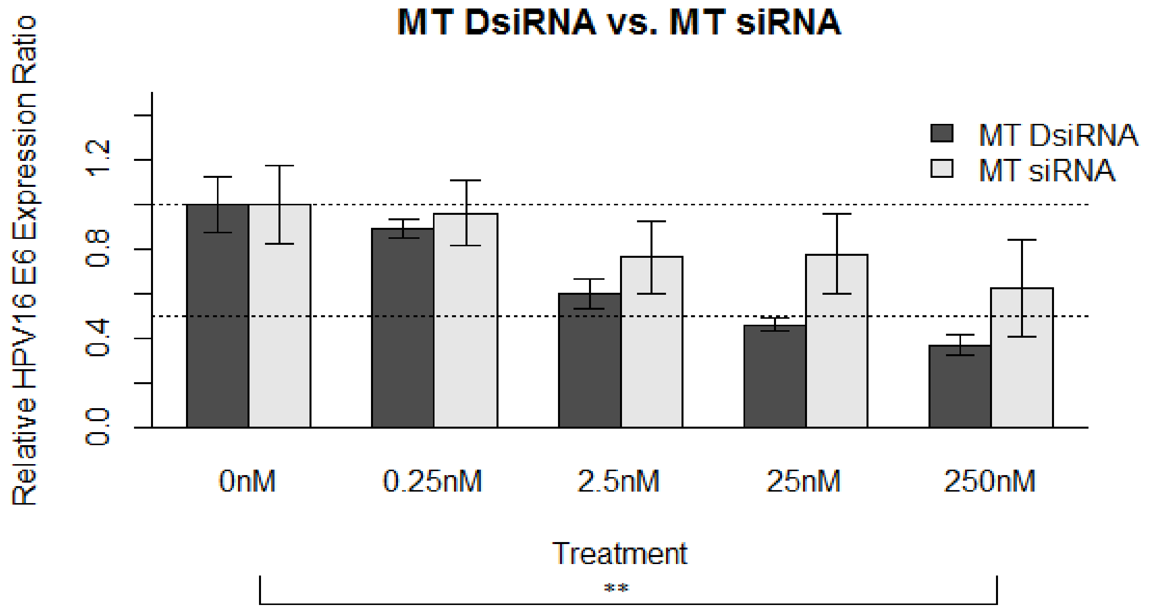

Figure 11. Relative E6 mRNA expression ratio comparing MT DsiRNA and MT siRNA in CaSki cells. Treatment with higher concentrations of both MT DsiRNA and MT siRNA significantly reduced E6 expression in CaSki cells (p=0.00659). There was no significant difference between the DsiRNA and siRNA target sites (p=0.07622). Expression was determined relative to HPRT-1 and calibrated to cells treated with growth medium only. Statistical analysis was performed using a two-way ANOVA followed by Tukey's HSD contrasts post hoc. Data represents mean ± SEM, *n*=3 for all. ** denotes p=0.01-0.001.

3.3.5 Rosetta DsiRNA Showed More E6 Knockdown than Rosetta siRNA

Unlike MT siRNA, Rosetta siRNA did come up as a potential target site for DsiRNA. However, it was ranked lower than the top three that IDT recommended. Treatment with higher concentrations in the siRNA and the DsiRNA reduced E6 expression in CaSki cells significantly ($p=0.00197$). Rosetta DsiRNA showed more E6 knockdown than Rosetta siRNA but this difference was not statistically significant ($p=0.567$) (Figure 12). The expression was determined relative to HPRT-1 and calibrated to cells with growth medium only. The IC_{50} value for Rosetta siRNA was previously determined to be 7 ± 13 nM and the IC_{50} value for Rosetta DsiRNA was determined to be 3 ± 2 nM. Since the Rosetta target site achieves the lowest IC_{50} value when compared to the other four DsiRNAs tested, it will be used for downstream experiments in this study.

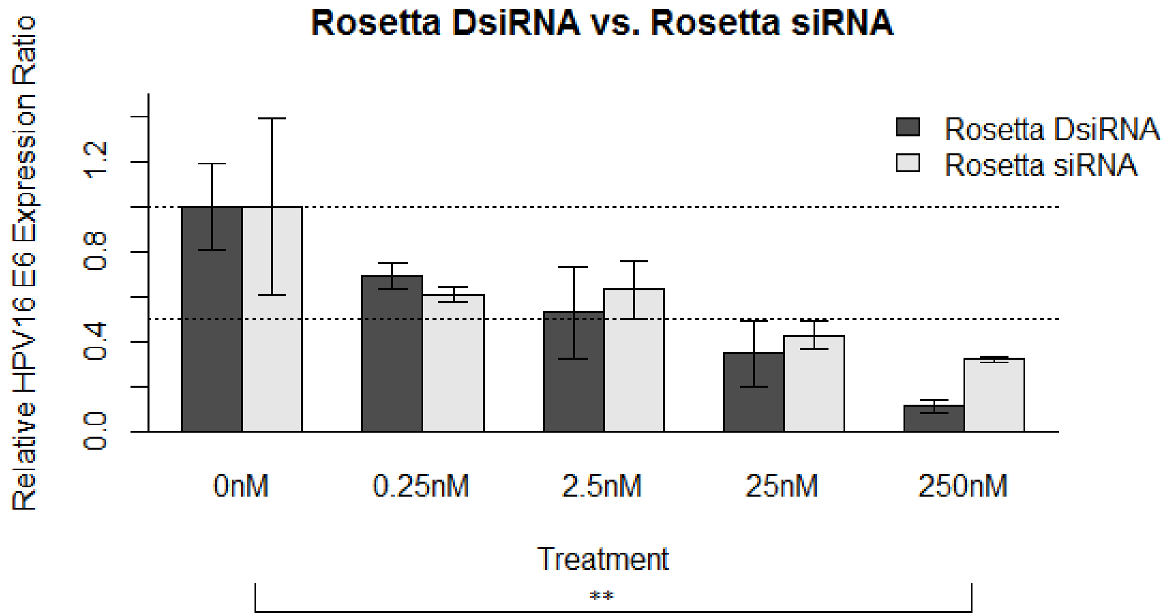

Figure 12. Relative E6 mRNA expression ratio comparing Rosetta DsiRNA and Rosetta siRNA in CaSki cells. Treatment with higher concentrations of both Rosetta DsiRNA and Rosetta siRNA significantly reduced E6 expression in CaSki cells (p=0.00197). There was no significant difference between the DsiRNA and siRNA target sites (p=0.567). Expression was determined relative to HPRT-1 and calibrated to cells treated with growth medium only. Statistical analysis was performed using a two-way ANOVA followed by Tukey's HSD contrasts post hoc. Data represents mean ± SEM, *n*=3 for all. **denotes p=0.01-0.001.

3.4 AA IC_{50} Results

3.4.1 Rosetta DsiRNA Showed Similar E6 Knockdown Compared to Rosetta siRNA

Since the Rosetta target site worked the best for both siRNA and DsiRNA in CaSki cells, these two oligonucleotides were used for targeting the PHFKs previously transduced with the AA variant. To determine if by bypassing the initial Dicer cleavage of the oligonucleotide, as seen with siRNAs, gives better E6 knockdown than using DsiRNAs that target the E6 transcript the IC_{50} values were calculated and compared. Treatment with higher concentrations in the siRNA and the DsiRNA reduced E6 expression in AA cells significantly (p=8.87e-15). Rosetta DsiRNA showed similar E6 knockdown compared to Rosetta siRNA (p=0.897) (Figure 13). The expression was determined relative to HPRT-1 and calibrated to cells with growth medium only. The IC_{50} value for Rosetta siRNA was determined to be 3 ± 2 pM and the IC_{50} value for Rosetta DsiRNA was determined to be 6 ± 3 pM. These IC_{50} values are approximately 1000 times lower than those calculated when transfected into CaSki cells.

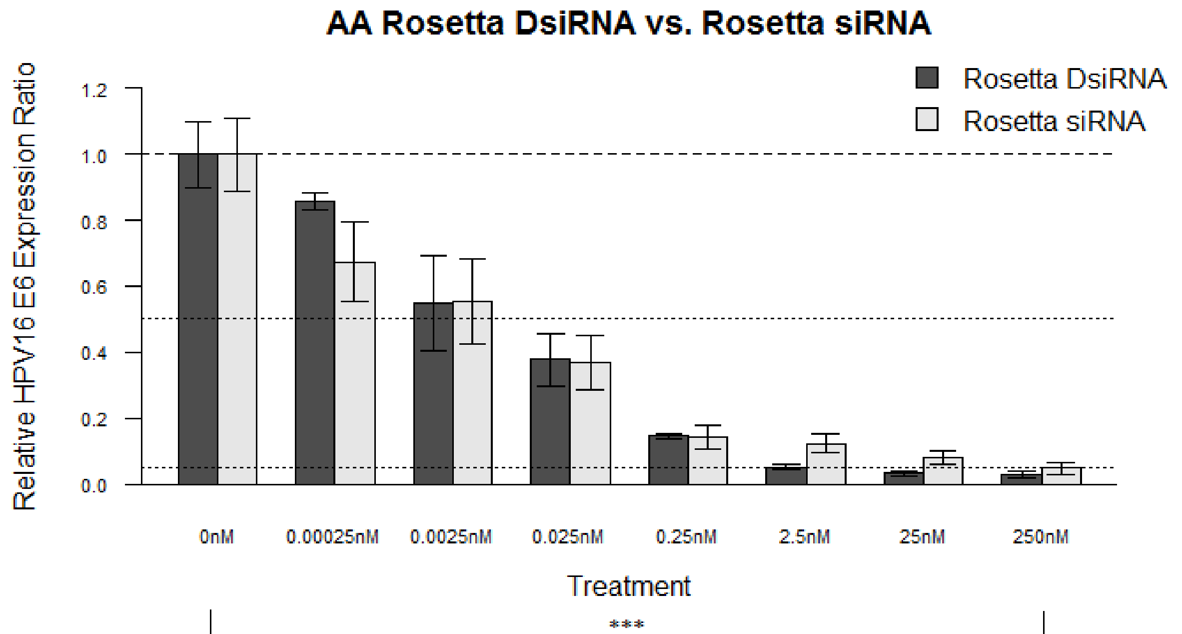

Figure 13. Relative E6 mRNA expression ratio comparing Rosetta DsiRNA and Rosetta siRNA in the AA variant. Treatment with higher concentrations of both Rosetta DsiRNA and Rosetta siRNA significantly reduced E6 expression in the AA variant (p=8.87e-15). There was no significant difference between the DsiRNA and siRNA sites (p=0.897). Expression was determined relative to HPRT-1 and calibrated to cells treated with growth medium only. Statistical analysis was performed using a two-way ANOVA followed by Tukey's HSD contrasts post hoc. Data represents mean ± SEM, *n*=3 for all. ***denotes p<0.001.

3.5 EP IC_{50} Results

3.5.1 Rosetta DsiRNA Showed More E6 Knockdown at Higher Concentrations Compared to Rosetta siRNA in EP Cells

Since the difference between Rosetta siRNA and DsiRNA was minimal in the HPV16 AA variant, an IC_{50} was calculated for these two oligonucleotides in PHFKs previously transduced with the EP variant to see if a similar trend was followed. Treatment with higher concentrations of the siRNA and the DsiRNA reduced E6 expression in EP cells significantly (p=1.84e-09). Rosetta DsiRNA showed similar E6 knockdown at lower concentrations but showed more knockdown at higher concentrations compared to Rosetta siRNA (p=0.223) (Figure 14). The expression was determined relative to HPRT-1 and calibrated to cells with growth in medium only. The IC_{50} value for Rosetta siRNA was determined to be 72 ± 69 pM and the IC_{50} value for Rosetta DsiRNA was determined to be 38 ± 2 pM. While these IC_{50} values are approximately 100 times lower than those seen when transfected into CaSki cells, they are not as low as those determined with the AA variant.

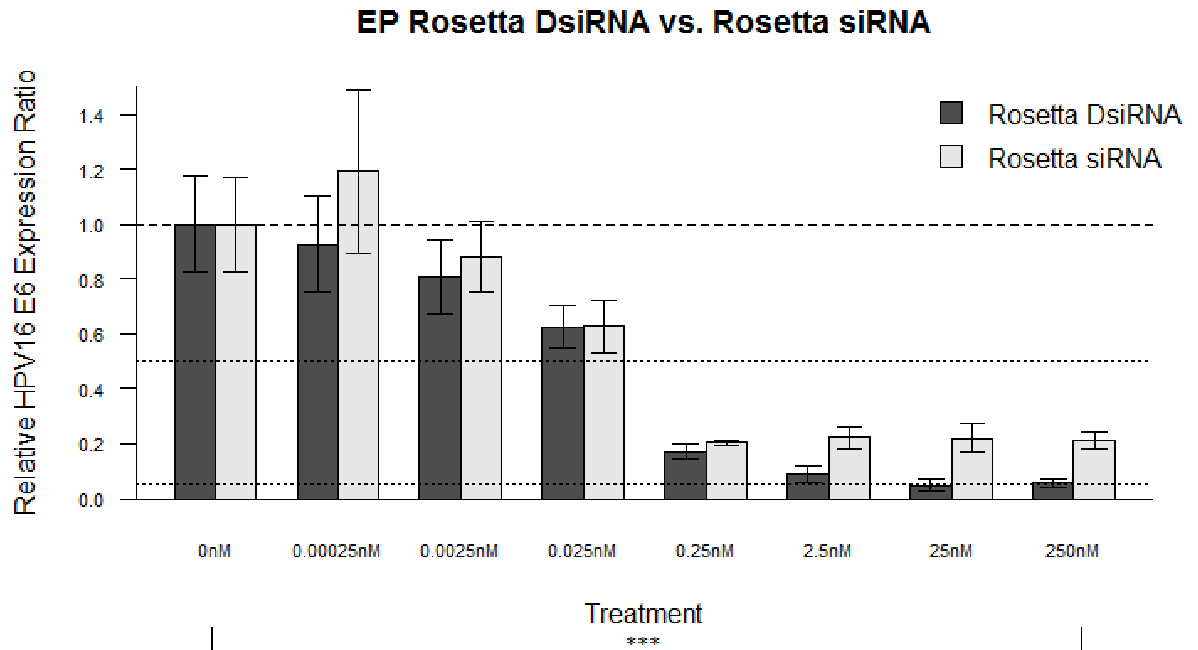

Figure 14. Relative E6 mRNA expression ratio comparing Rosetta DsiRNA and Rosetta siRNA in the EP variant. Treatment with higher concentrations of both Rosetta DsiRNA and Rosetta siRNA significantly reduced E6 expression in the EP variant (p=1.84e-09). There was no significant difference between the DsiRNA and siRNA (p=0.223). Expression was determined relative to HPRT-1 and calibrated to cells treated with growth medium only. Statistical analysis was performed using a two-way ANOVA followed by Tukey's HSD contrasts post hoc. Data represents mean ± SEM, *n*=3 for all. ***denotes p<0.001.

3.6 Downstream Process Restoration

To determine if there is a difference between DsiRNA and siRNA in the HPV16 E6 variants, E6 levels were measured at both protein and mRNA levels. Since the E6 protein is involved with p53 degradation and hTERT knockdown, these two downstream processes were also measured to check for their restoration on the same group of cells. By checking these downstream processes, we can validate that the E6 knockdown achieved by transfection with either siRNA or DsiRNA results in their restoration. The concentration of both DsiRNA and siRNA used was 6.75 nM since this was the average IC_{95} of both variants (AA IC_{95} = 4.5 nM and EP IC_{95}= 9 nM). Using 6.75 nM, it was expected that AA would have slightly greater E6 mRNA knockdown than 95 % and EP would have slightly less E6 mRNA knockdown than 95 %.

3.6.1 E6 mRNA Levels Decreased in Both AA and EP When Treated with Rosetta DsiRNA and Rosetta siRNA

To determine the relative E6 mRNA expression in cells treated with DsiRNA and siRNA targeting the same region of the transcript, 6.75 nM of the transfection complexes were used and incubated in AA and EP for 48 hours. In both the AA and the EP variants there was a significant difference between the E6 specific oligonucleotides relative to their negative controls (p=2.84e-07 and p=2.85e-06 respectively). However, there was no significant difference between DsiRNA and siRNA (p=0.412 and p=0.516 respectively) (Figure 15 A and B). In the AA variant, there was 95 % and 90 % E6 knockdown with E6 specific DsiRNA and siRNA, respectively. There was lower knockdown in the EP variant which only had 87 % and 71 % knockdown with the DsiRNA treated and siRNA treated

cells, respectively. Higher level of knockdown was achieved in the AA variant but the differences between the variants were not significant (p=0.077) (Figure 15 C). The expression was determined relative to HPRT-1 and calibrated to cells with HiPerFect only.

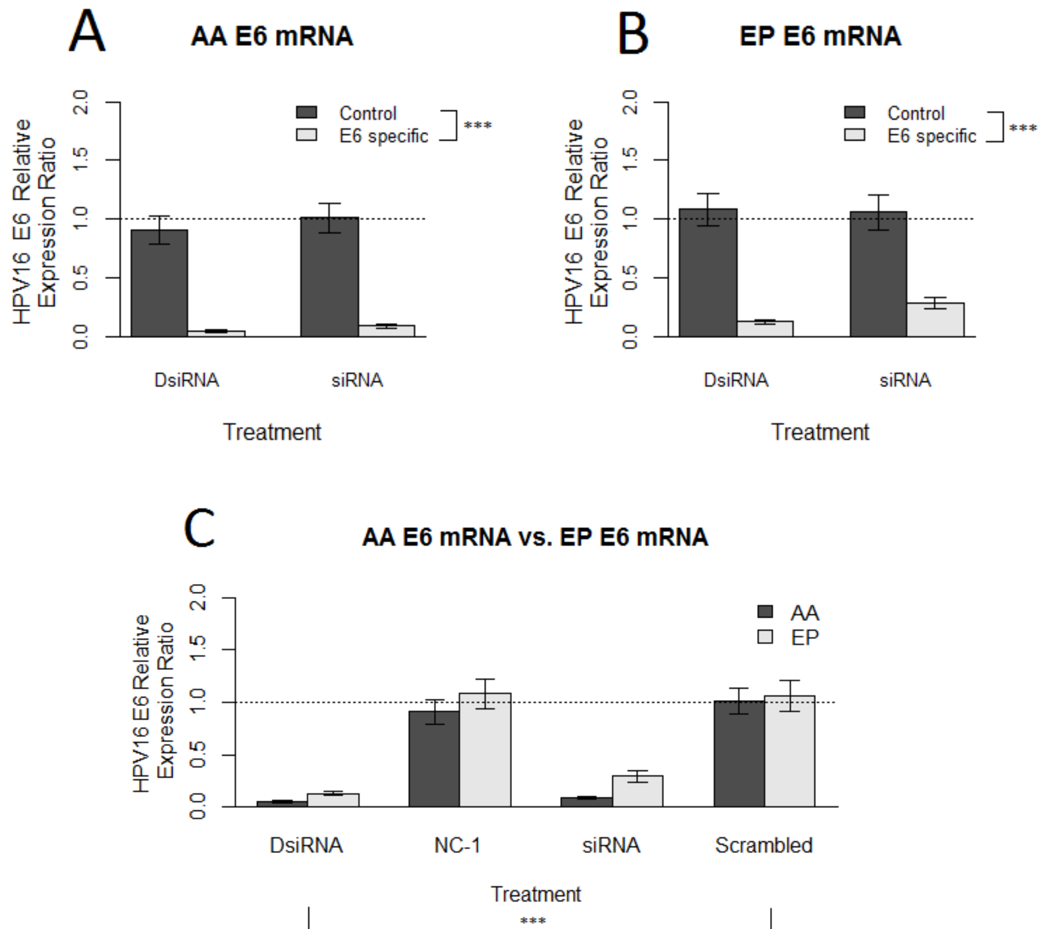

Figure 15. Relative E6 mRNA expression ratio comparing Rosetta DsiRNA and Rosetta siRNA in the HPV16 E6 variants. A. Treatment with Rosetta DsiRNA and Rosetta siRNA in AA significantly reduced E6 expression compared to the corresponding negative controls (p=2.84e-07) but were not significant between Rosetta siRNA and DsiRNA (p=0.412). **B.** Treatment with Rosetta DsiRNA and Rosetta siRNA in EP significantly reduced E6 expression compared to the corresponding negative controls (p=2.85e-06) but were not significant between Rosetta siRNA and DsiRNA (p=0.516). **C.** There was no statistical difference between the two HPV16E6 variants (p=0.077). Expression was determined relative to HPRT-1 and calibrated to cells treated with HiPerFect only (shown as the dotted line at 1.0). Scrambled siRNA is the control for Rosetta siRNA and NC-1 negative DsiRNA is the control for Rosetta DsiRNA. Statistical analysis was performed using a two-way ANOVA followed by Tukey's HSD contrasts post hoc. Data represents mean ± SEM, *n*=4 for all. ***denotes p<0.001.

3.6.2 E6 Protein Levels Were Knocked Down Similarly with Rosetta DsiRNA and Rosetta siRNA in Both Variants

There was no significant difference between the Rosetta DsiRNA and the Rosetta siRNA mRNA levels in both variants ($p=0.412$ for AA and $p=0.516$ for EP) (Figure 15 A and B), so E6 expression was also examined at the protein level to see if a similar pattern was observed. Indirect detection of the E6 protein via Western blot was done by detecting the human influenza hemagglutinin (HA) tag located on the transduced E6 protein. This approach is beneficial because it eliminates any potential detection biases which could be caused if the E6 antibody had different binding affinities for each of the E6 variants. The Western blot indicated that treatment with 6.75 nM Rosetta DsiRNA or Rosetta siRNA both resulted in elimination of a visibly detectable amount of protein for AA and EP E6 (Figure 16 A and B). Since E6 protein levels were reduced to an undetectable level, densitometry could not be applied. Each replicate ($n=4$) showed complete knockdown and any differences calculated from these Western Blots may have been a result of slightly different actin densities instead of E6 protein densities. No non-specific bands were present on the membrane.

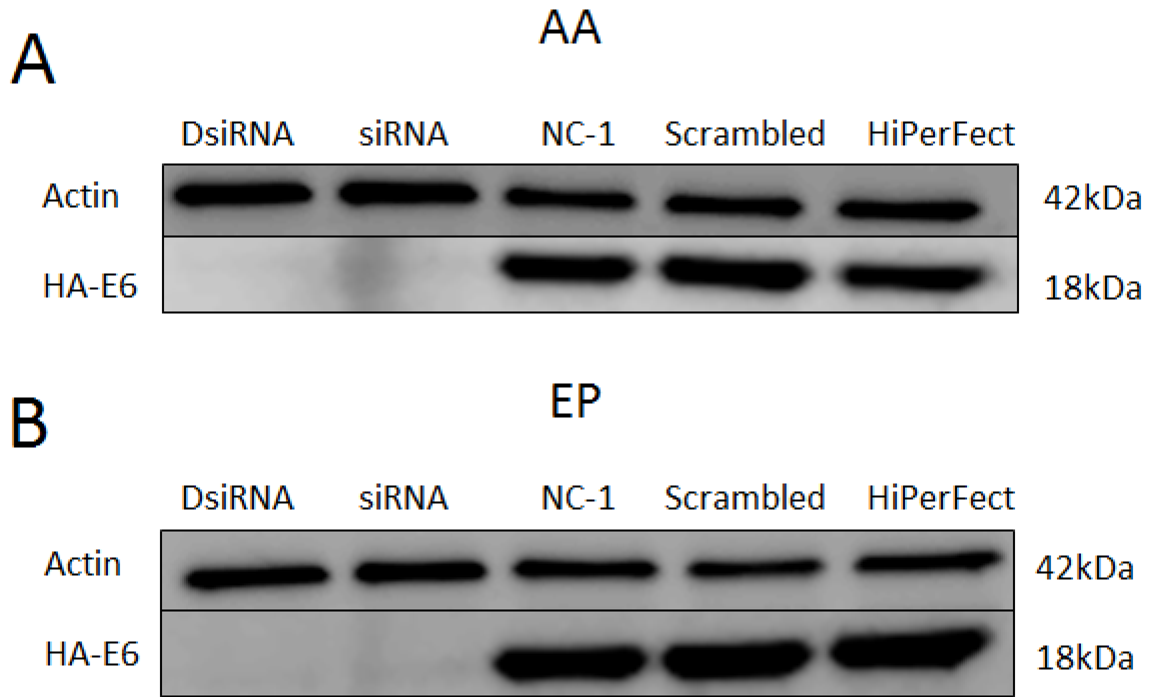

Figure 16. Western blot of the E6 protein HA tag after treatment with 6.75 nM of Rosetta siRNA and Rosetta DsiRNA. Treatment with Rosetta siRNA and DsiRNA reduced E6-HA protein below visibly detectable levels in **A.** AA and **B.** EP cells. DsiRNA refers to cells treated with Rosetta DsiRNA, siRNA refers to cells treated with Rosetta siRNA, NC-1 refers to cells treated with DsiRNA negative control, Scrambled refers to cells treated with siRNA scrambled control, and HiPerFect refers to cells treated with HiPerFect only. Representative images from a set of four independent experiments.

3.6.3 hTERT Levels Decreased Equally in both HPV16 E6 Variants after Treatment with Rosetta DsiRNA and Rosetta siRNA

HPV16 E6 is capable of activating the catalytic subunit of the enzyme telomerase, hTERT, by activating the promoter which induces hTERT expression. Once expressed it adds telomere repeats to the ends of chromosomes (Klingelhutz et al. 1996, Van Doorslaer and Burk 2012). By replenishing telomeres on the ends of chromosomes, the cells may become immortal (Kim et al. 1994, and Klingelhutz et al. 1996) which is why this was one of the cellular processes downstream of E6 investigated. In both the AA and the EP HPV16 E6 variants there was a significant difference in hTERT mRNA between the E6 specific DsiRNA and siRNA to their relative negative controls (p=3.07e-08 and p=9.32e-10 respectively). In the AA variant, there was 79 % and 83 % hTERT knockdown with E6 specific DsiRNA and siRNA, respectively. A similar trend was followed in the EP variant which had 61 % and 89 % knockdown with the DsiRNA treated and siRNA treated cells, respectively. In both variants there was more hTERT mRNA knockdown when Rosetta siRNA was used compared to Rosetta DsiRNA, but this was not statistically significant (p=0.492 for AA and p=0.05395 for EP) (Figure 17 A and B). When comparing the two variants, there was no statistical difference between them (p=0.509) (Figure 17 C). The expression was determined relative to HPRT-1 and calibrated to cells with HiPerFect only.

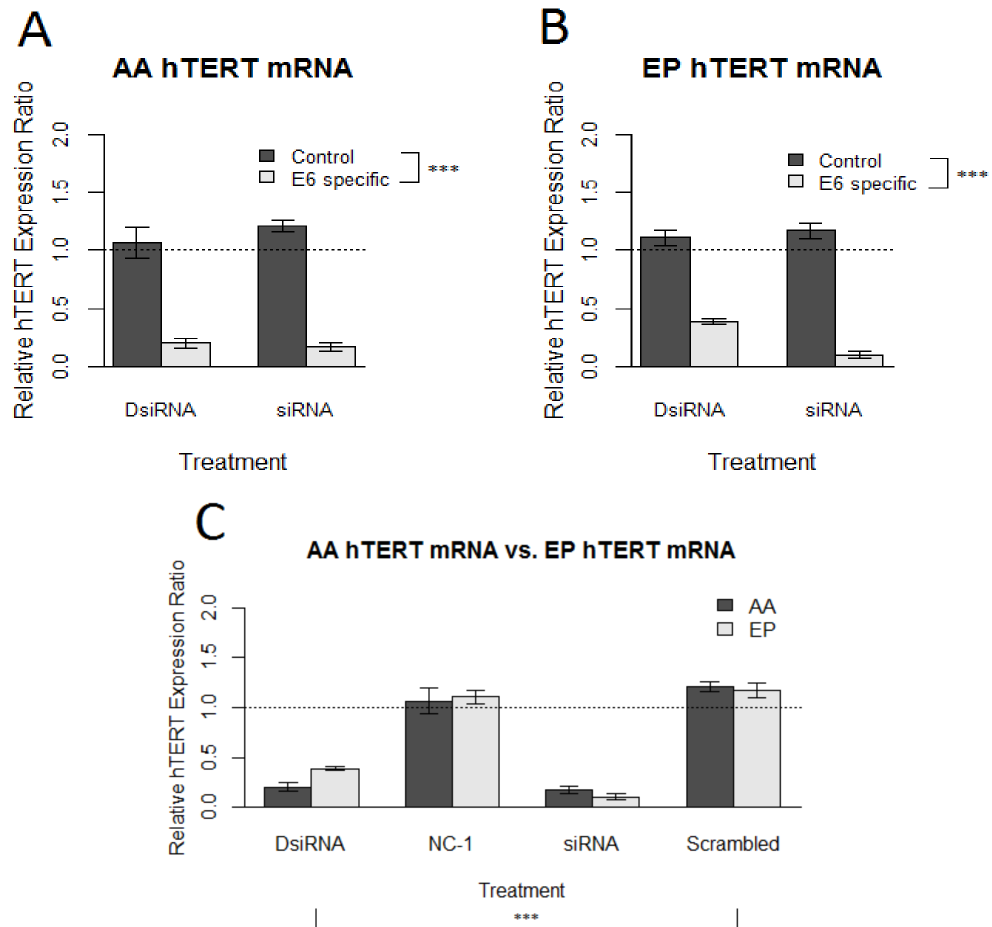

Figure 17. Relative hTERT mRNA expression ratio comparing Rosetta DsiRNA and Rosetta siRNA in the HPV16 E6 variants. A. Treatment with Rosetta DsiRNA and Rosetta siRNA in AA significantly reduced hTERT expression compared to the corresponding negative controls (p=3.07e-08) but were not significant between Rosetta siRNA and DsiRNA (p=0.492). **B.** Treatment with Rosetta DsiRNA and Rosetta siRNA in EP significantly reduced hTERT expression compared to the corresponding negative controls (p=9.32e-10) but were not significant between Rosetta siRNA and DsiRNA (p=0.05395). **C.** There was no statistical difference between the two HPV16E6 variants (p=0.509). Expression was determined relative to HPRT-1 and calibrated to cells treated with HiPerFect only (shown as the dotted line at 1.0). Scrambled siRNA is the control for Rosetta siRNA and NC-1 negative DsiRNA is the control for Rosetta DsiRNA Statistical analysis was performed using a two-way ANOVA followed by Tukey’s HSD contrasts post hoc. Data represents mean ± SEM, *n*=4 for all. ***denotes p<0.001.

3.6.4 p53 Levels Were Restored Equally When Treated with Rosetta siRNA and Rosetta DsiRNA in Both HPV16 E6 Variants

Since E6 plays a major role in p53 degradation, p53 protein levels were investigated to determine its restoration following treatment with either DsiRNA or siRNA. The Western blot indicated that treatment with 6.75 nM E6 DsiRNA or E6 siRNA both resulted in an increase of p53 protein for AA and EP E6 (Figure 18 A and B). When densitometry was applied to the membrane images, very high levels were detected and these values were made relative to the corresponding actin and calibrated to the HiPerFect only samples. Both variants showed a statistically significant increase in p53 protein when treated with either DsiRNA or siRNA ($p=0.00234$ for AA and $p=1.11e-06$ for EP) (Figure 19 A and B). There was no significant difference between DsiRNA and siRNA in AA and EP ($p=0.99001$ and $p=0.237$ respectively) and when comparing the two variants, there was no significant difference in p53 restoration ($p=0.616$) (Figure 19 C). No non-specific bands were present on the membrane.

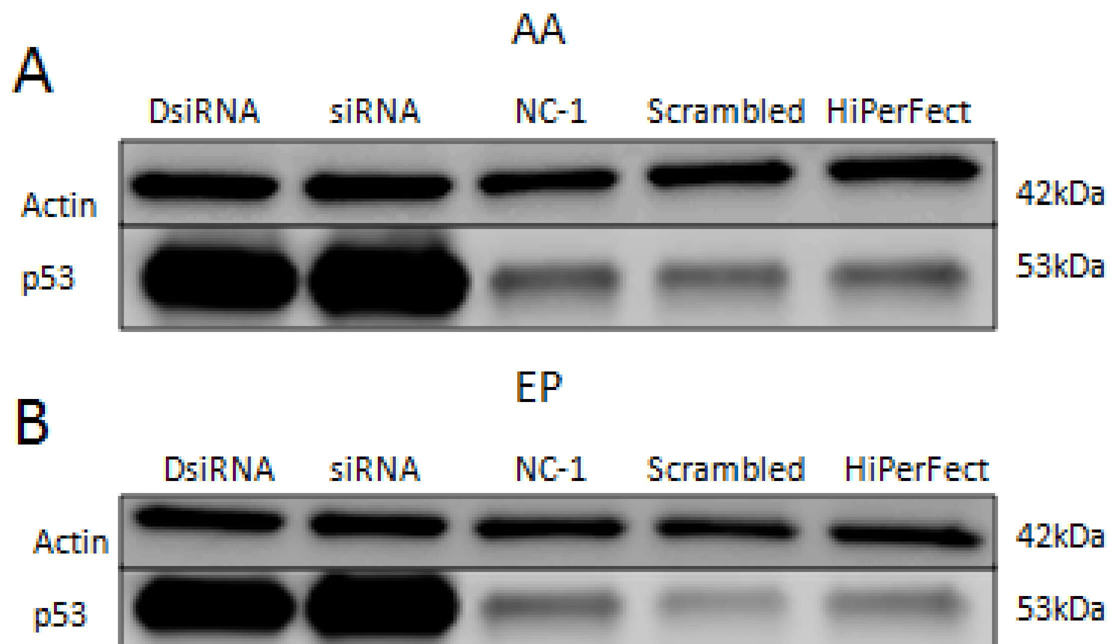

Figure 18. Western blot of the p53 protein after treatment with 6.75 nM of Rosetta siRNA and Rosetta DsiRNA. Treatment with Rosetta siRNA and DsiRNA increased p53 protein levels in **A.** AA and **B.** EP cells. DsiRNA refers to cells treated with Rosetta DsiRNA, siRNA refers to cells treated with Rosetta siRNA, NC-1 refers to cells treated with DsiRNA negative control, Scrambled refers to cells treated with siRNA scrambled control, and HiPerFect refers to cells treated with HiPerFect only. Representative images from a set of four independent experiments.

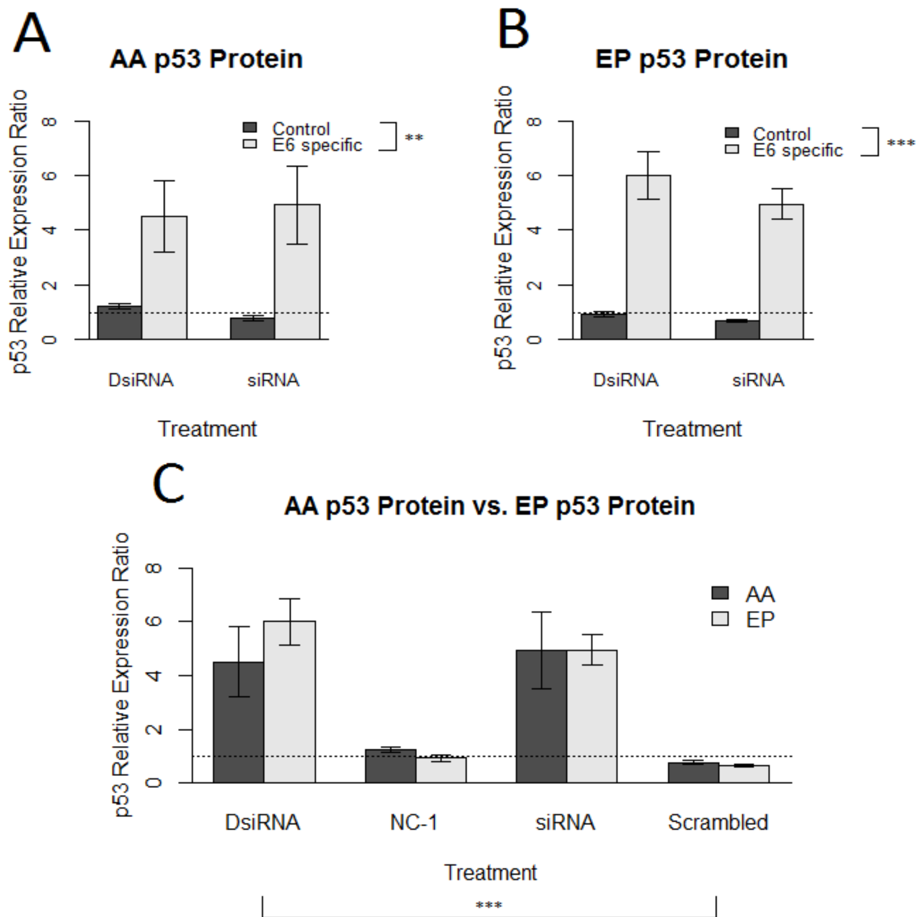

Figure 19. Relative p53 protein expression ratio comparing Rosetta DsiRNA and Rosetta siRNA in the HPV16 E6 variants. A. Treatment with Rosetta DsiRNA and Rosetta siRNA in AA significantly increased p53 expression compared to the corresponding negative controls (p=0.00234) but were not significant between Rosetta siRNA and DsiRNA (p=0.99001). **B.** Treatment with Rosetta DsiRNA and Rosetta siRNA in EP significantly increased p53 expression compared to the corresponding negative controls (p=1.11e-06) but were not significant between Rosetta siRNA and DsiRNA (p=0.237). **C.** There was no statistical difference between the two HPV16E6 variants (p=0.616). Expression was determined relative to actin and calibrated to cells treated with HiPerFect only (shown as the dotted line at 1.0). Scrambled siRNA is the control for Rosetta siRNA and NC-1 negative DsiRNA is the control for Rosetta DsiRNA Statistical analysis was performed using a two-way ANOVA followed by Tukey's HSD contrasts post hoc. Data represents mean ± SEM, *n*=4 for all. ***denotes p<0.001.

4. Discussion

4.1 Differential HPV16 E6 mRNA Knockdown after Targeting Various Regions of the E6 Transcript

It is critical to identify an optimal target sequence to achieve successful RNAi experiments but these target sequences are difficult to predict. It is well known that shifting siRNAs by a base or two along the mRNA sequence can change its potency by ten-fold or more (Rose et al. 2005,Holen et al. 2002, Reynolds et al. 2004). This requires researchers to choose siRNAs that have either been previously validated, as with the MT siRNA (Courtête et al. 2007), or designed based on established design algorithms, such as the Rosetta siRNA algorithm and the DsiRNA algorithm that both have been used by many researchers (Jackson et al. 2003, Jackson et al. 2006, Majercak et al. 2006, Espeseth et al. 2006, Kim et al. 2005, Hefner et al. 2008).

Based on this study and by using CaSki cells as the initial cell model, each target site showed variable E6 knockdown. The observed differences in potency of siRNAs targeting different regions of the E6 mRNA suggest that target accessibility plays an important role in governing a siRNA response (Mysara et al. 2011). Surprisingly, the MT siRNA used in this study did not perform as well as it had been validated to do so. The Courtête group (2007) used 10 nM of this siRNA and achieved approximately 60 % E6 knockdown whereas in this study, knockdown below 50 % was not attained even with 250 nM of the siRNA complex. However, when this siRNA sequence was converted into a DsiRNA, the knockdown levels were closer to those published (Courtête et al. 2007) achieving an IC_{50} value of 23 ± 12 nM.

The lowest IC_{50} values achieved in this study in CaSki cells were by siRNA target sites designed by the Rosetta algorithm. The IC_{50} values for Rosetta siRNA and DsiRNA were 7 ± 13 nM and 3 ± 2 nM respectively and these values fall within the highest percent knockdown achieved by siRNAs that target the HPV16 E6 splice site (Jiang and Milner 2002, Courtête et al. 2007, Yamato et al. 2008). Rosetta DsiRNA achieves slightly more E6 knockdown than Rosetta siRNA in CaSki (Figure 12), AA (Figure 13), and EP (Figure 14) cells but this difference was not statistically significant. This slight difference may be explained by the fact that Rosetta siRNA has two deoxythymidine overhangs (Table 2) which have been shown to be poor substrates for the PAZ domains *in vitro* (Ma et al. 2004, Snead et al. 2013). Since it is thought that Dicer is required to introduce the siRNA into RISC, providing Dicer with a substrate instead of a product may also improve the rate or efficiency of entry of the siRNA into RISC (Lee et al. 2004; Tomari et al. 2004). These results stress the importance of locating the best target site along the specific gene of interest and show how difficult it is to design a universal siRNA algorithm.

4.2 Comparing DsiRNAs and siRNAs that Target the Same Region

By comparing the E6 knockdown at both the mRNA level and the protein level, there was no observed difference when using 6.75 nM of Rosetta DsiRNA or Rosetta siRNA in either of the HPV16 E6 variants. Since IDT claimed that DsiRNAs are better than siRNAs for RNAi (Kim et al. 2005, Hefner et al. 2008), both MT siRNA and Rosetta siRNA were converted into DsiRNAs. Even though there was no significant difference between the siRNAs and their corresponding DsiRNAs, there was a slight

difference showing that the DsiRNAs achieved more E6 mRNA knockdown than their respective siRNAs in CaSki, AA and EP cells (Figure 11, 12, 13, and 14). This trend was also shown by another group that targeted β-catenin and they concluded that DsiRNAs showed high potencies and in many cases induced strong mRNA knockdown even when the corresponding siRNA functioned relatively weakly (Dudek et al. 2014).

Another research group compared multiple DsiRNA and siRNA pairs targeting PTEN (67 DsiRNA-siRNA pairs) and Factor VII (63 DsiRNA-siRNA pairs). This group selected the top 10 DsiRNAs and siRNAs targeting PTEN and FVII and determined that they were not all sequence matched (Foster et al. 2012). Chemical modifications were applied to the duplex strands composing of 2'-OMe, phosphorothioate, and deoxythymidine (dT) residues in various combinations and locations. Once modifications were applied, no modified DsiRNA was more active than its matched siRNA which could be explained by the locations of the modifications interfering with Dicer cleavage or handling. Based on their findings this group concluded that siRNAs are better suited for use as therapeutics due to better tolerance of chemical modifications, reduced immunostimulation, and smaller size (Foster et al. 2012). However, their results varied and for the most part, no consistent differences between DsiRNA and siRNA were recorded. There were no significant differences between DsiRNA and siRNA when the complexes were unmodified, which is consistent with the results obtained in this study. If chemical modifications were to be added to the DsiRNA and siRNA duplexes in this study, it would be difficult to determine where they should be placed. Placing certain modifications in one position of the DsiRNA duplex may be better or worse when placed in that same position on the siRNA duplex since DsiRNAs need to be processed by Dicer

before being incorporated into RISC. Since the Rosetta siRNA and Rosetta DsiRNA in this study follow a different initial processing step (Figure 6) but both target the same E6 mRNA transcript, not seeing a statistically significant difference in E6 knockdown, p53 restoration, and hTERT knockdown is not surprising.

4.3 Restoration of Downstream Processes

Once statistically similar E6 mRNA knockdown was achieved using Rosetta siRNA and DsiRNA in both variants (Figure 13 and 14), it was possible to study E6 at the protein level as well as check the restoration of hTERT and p53. In RNAi experiments, when focussing on protein-coding RNAs, it is beneficial to monitor both mRNA and protein levels of the target, in this case E6. If mRNA reduction is seen without a corresponding reduction in protein levels this may indicate that protein turnover is slow (Doench et al. 2003). In this study, both AA and EP, treated with either Rosetta siRNA or DsiRNA, reduced E6 protein levels to undetectable amounts based on western blotting (Figure 16). Since both RNAi complexes yielded undetectable levels of E6 protein, p53 protein and hTERT mRNA levels were analyzed to validate that once E6 protein is eliminated, downstream processes become restored and to determine whether this restoration is equal in both.

The mRNA expression of hTERT is estimated to be less than 1 to 5 copies per normal cell (Yi et al. 1999, Cong et al. 2002) and is closely associated with telomerase activity. The levels of hTERT are generally increased in immortal cells, suggesting that hTERT is the primary determinant for the enzyme activity (Cong et al. 2002). Treatment with DsiRNA or siRNA targeting the E6 mRNA transcript resulted in significant hTERT

knockdown when compared to cells treated with the negative DsiRNA and negative siRNA controls in both of the AA and EP variants (Figure 17 A and B). Shown by another group, siRNA knockdown of either E6 or E7 in HPV-immortalized cells reduced hTERT transcription and telomerase activity (Lui et al. 2008). Since hTERT mRNA levels, protein levels, and telomere length do not always follow a linear trend, it is difficult to conclude that the down regulation of hTERT mRNA determined in this study has halted telomere elongation.

The ubiquitin-mediated degradation of p53 prevents the cell from entering apoptosis, allowing the accumulation of mutations which can result in malignant transformations. By targeting the E6 transcript with siRNA, p53 protein levels are expected to increase. Treatment with DsiRNA or siRNA targeting the E6 mRNA transcript resulted in a significant increase in p53 protein expression (Figure 19). Surprisingly, there are low levels of p53 in the negative controls and the HiPerFect only cells (Figure 18). This suggests that not all p53 is degraded in PHFKs previously transduced with the HPV16 E6 variants. This low p53 protein level detected in the negative controls contradicts what a previous student obtained using these cell lines (Niccoli et al. 2012). Our Western blotting procedures have improved since then, having optimized the type and amount of protease inhibitors. A previous student, using immunocytochemistry instead of Western Blot, saw sporadic p53 staining in negative controls resulting in low level averages of p53 in control samples (Togtema, 2013). Once treated with either DsiRNA or siRNA there was no difference in p53 expression between these RNAi complexes nor was there a difference between the two variants (Figure 19).

5. Conclusion and Future Directions

DsiRNAs and siRNAs show tremendous promise as a tool for targeted gene silencing. However, their utility depends on their ability to specifically knock down the target gene without interfering with the expression or function of other genes or proteins. To our knowledge, this was the first study to compare HPV16 E6 targeted DsiRNAs and siRNAs in the AA and EP variants. Throughout this study, there was no significant difference between using Rosetta DsiRNA or Rosetta siRNA in AA and EP based on E6 mRNA, protein, p53 protein restoration, and hTERT mRNA knockdown. Since this study was solely based on *in vitro* experiments future studies need to be done to determine if similar trends are seen *in vivo*. However, safe and efficient delivery of the therapeutic siRNAs has been a major hurdle in RNAi for *in vivo* studies. Chemical modifications can be applied to the duplexes to improve their stability and there have been many studies on types and locations of modifications applied to the siRNA duplexes (Dutta et al. 2011, Shukla et al. 2009). Packaging of the siRNA into liposomes or nanoparticles can also add protection and improve cellular uptake (Wang et al. 2011). Since one of IDT's claims is that DsiRNAs have a longer duration of knockdown (Kim et al. 2005, Hefner et al. 2008), this should be reconfirmed at both the mRNA and protein levels *in vitro* and *in vivo* for both E6 variants. This study chose the best target site based on the full HPV16 genome of CaSki cells but only looked at E6 protein, p53 protein and hTERT mRNA levels in AA and EP. A future study needs to determine whether similar differences will persist in the context of the full length HPV16 genome and if the presence of the other viral proteins affects E6 knockdown, hTERT knockdown, or p53 restoration. Other cellular effects may

also be looked at such as interferon responses when either siRNA or DsiRNA is added to the cells.

It has been mentioned in this study that mRNA levels and protein levels do not always follow a linear relationship. With only checking both mRNA and protein levels of AA and EP E6 with 6.75 nM of either Rosetta siRNA or Rosetta DsiRNA incubated for 48 hours, it is difficult to determine how quickly knockdown is achieved as well as the duration of knockdown. Future studies should be done looking at shorter and longer incubation times measuring both mRNA and protein levels. If hTERT mRNA restoration is analyzed again, the telomerase protein level should be analyzed via western blot as well as telomere length since it is unknown if they all show a linear relationship. Since both AA and EP originated from PHFKs, these cells should also be included in downstream experiments to get a more exact baseline value for each experiment in this study.

Since there was no statistical difference determined between Rosetta siRNA and Rosetta DsiRNA when comparing p53 restoration or hTERT knockdown nor were there any statistically significant difference between AA and EP. These findings may suggest that even though Rosetta DsiRNA and Rosetta siRNA do not differ in downstream process restoration when targeting HPV16 E6 *in vitro* they may have an effect on restoration, duration of knockdown, and oligonucleotide stability *in vivo*. Since there was no significant difference between the two variants when analyzing E6 mRNA, E6 protein, p53 protein, and hTERT mRNA this may suggest that the greater carcinogenicity seen with the AA variant is not due to p53 or hTERT confirming what was previously shown by Zehbe et al. (2009), Richard et al. (2010), and Niccoli et al. (2012).

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