Investigation of HSV-1 Interaction with the miRNA Pathway

During Lytic Infection in Keratinocytes

Meaghan Flagg

Department of Molecular, Cellular, and Developmental Biology
University of Colorado at Boulder

April 5, 2013

Thesis Advisor:
Dr. Rui Yi Department of Molecular, Cellular, and Developmental Biology

Defense Committee:
Dr. Rui Yi Department of Molecular, Cellular, and Developmental Biology
Dr. Jennifer Martin Department of Molecular, Cellular, and Developmental Biology
Dr. Debra Goldberg Department of Computer Science
Herpes Simplex Virus type 1 (HSV-1) infects nearly 60% of the U.S. population, and is a significant cause of viral encephalitis and neuroinflammatory diseases. HSV-1 actively replicates in stratified epithelia, and then migrates to sensory neurons where it establishes a life-long, latent infection. It was recently demonstrated that HSV-1 can suppress exogenous RNA silencing, and viral replication is enhanced in the absence of Ago2. I investigated this further in a physiologically relevant in vitro epidermal cell culture model to determine if suppression of RNAi pathway components had any effect on HSV-1 replication, and if HSV-1 modulated endogenous miRNA silencing and expression. I found that deletion of Ago2 enhanced HSV-1 viral titers two to six-fold, and that the virus is capable of suppressing expression of mature miRNAs as well as their silencing function. These findings contribute to the nascent body of literature examining antiviral RNAi functions in mammals, and further our understanding of the epidermal response to one of the most common viral infections in the U.S.
INTRODUCTION

EPIDERMAL IMMUNITY

The epidermis is one of the most immunogenic organs in the body. Large numbers of hematopoietic-derived cells populate the skin, including 20 billion T cells residing in the human epidermis (Clark et al. 2006). Numerous other cell types, of both myeloid and lymphoid lineages, make up the remaining epidermal resident immune cell population. The skin, along with other forms of stratified epithelia, forms the primary barrier to microbial infections. Keratinocytes, the cell type that makes up the epidermis, secrete anti-microbial peptides and express immune surveillance molecules. In addition to the sensing carried out by immune sentinels, keratinocytes play a major role in activating and promoting inflammatory responses.

Epidermal immune responses are critical for combating many bacterial infections, as well as extremely common viral infections such as Herpes Simplex Virus type 1 (HSV-1) and Human Papillomavirus (HPV). HPV infects many types of stratified epithelium, including the epidermis, and is a major cause of cervical cancers (Vandermark et al. 2012). HSV-1 carries out its lytic infection cycle in keratinocytes, which serve as the exclusive environment for HSV-1 viral production. Despite the number of human diseases involving immune responses in the skin, very little is known about how keratinocytes function to mount an effective adaptive response.

The skin is a highly organized tissue, composed primarily of keratinocytes in stratified layers of sequential differentiation states (Figure 1). Throughout the life of an organism, skin is constantly shed and regenerated, and therefore has a remarkable capacity for self-renewal and regulation of homeostasis. The mesenchymal-epithelial junction is defined by the basement membrane, to which basal keratinocytes are attached. Basal keratinocytes make up the interfollicular epidermal stem cell population, and are characterized by the expression of keratins 5 and 14 (Blanpain and Fuchs 2009). These cells undergo asymmetric cell division to form the suprabasal layer, marked by keratin 10 and loricrin expression (Lechler and Fuchs
Suprabasal cells have permanently exited the cell cycle, but are still capable of performing metabolic processes. Beyond the suprabasal layer, keratinocytes begin to cornify and form the granular and stratum corneal layers of the epidermis. These tough, cornified layers function as a barrier, protecting the organism from microbes and other environmental hazards.

Keratinocytes form the organism’s primary barrier against microbial infection, and therefore are highly equipped to detect the presence of microbes. This is carried out at the cellular level through a class of molecules known as pattern recognition receptors (PRRs). These protein complexes are located on the plasma membrane, in the endosome, or occasionally in the cytosol, and serve to detect the presence of viruses, bacteria, and parasites. PRRs recognize molecular signatures specific to pathogens, termed pathogen-associated molecular patterns (PAMPs). These molecular signatures are motifs that are not found on the organism’s host cells, and therefore signify the presence of a microbe. Some of these signatures include double-stranded RNA (dsRNA), a hallmark of RNA virus infection; lipopolysaccharide, a component of
gram-negative bacterial membranes; flagellin and peptidoglycan, other components of bacterial membranes; and CpG-unmethylated DNA, which is found in viruses and bacteria, but not eukaryotes. Signaling through these receptors is triggered by ligand binding, and leads to activation of pro-inflammatory transcription factors, including NF-κB, NFAT, and AP-1 (c-Jun-Fos heterodimer). These proteins induce transcription of pro-inflammatory cytokines and chemokines, as well as interferons (IFNs) in the case of viral infection. PRRs are critical for not only detecting the presence of an infection, but also assisting the immune system in fine-tuning its response to the specific type of invading microbe.

Numerous immune cells of hematopoietic lineage reside within the interfollicular epidermis (IFE). These cells include dendritic cells, Langerhans cells, macrophages, and T cell lineages. Langerhans cells, dendritic cells, and macrophages are descendants of the myeloid lineage, whereas T cell populations are derived from lymphoid progenitors. Langerhans cells (LCs) are a distinct subset of dendritic cells, found primarily in the skin and other forms of stratified epithelium. These cells are characterized by the presence of distinctive Birbeck granules (Valladeau et al. 2000). LCs are critical for immunoregulation in the skin, and have been shown to have both pro- and anti-inflammatory properties (Kaplan et al. 2010; Lutz et al. 2010; Stoitzner 2010). Non-langerin expressing dendritic cells (DCs), termed dermal dendritic cells (dDCs), also contribute to adaptive epidermal immune responses. They are professional antigen presenting cells (APCs), meaning they take up antigen from the surrounding tissue and present it to naïve T cells on MHC class II complexes. There is also some evidence that dDCs, excluding LCs, are responsible for priming naïve CD8+ T cells in the lymphoid tissue (Allan et al. 2003). The skin is home to a diverse repertoire of T cells, including CD4+ and CD8+ effector, memory, and regulatory subtypes (Clark et al. 2006; Seneschal et al. 2012; Jiang et al. 2012). These cells reside permanently in the epidermis, providing long-lasting protective immunity and
INTRODUCTION

are distinct from their lymphatic relatives (Seneschal et al. 2012, Jiang et al. 2012). These skin-resident immune cells are important for translating intracellular innate immune responses in keratinocytes to an effective, leukocyte-mediated adaptive response.

Keratinocytes also assist with systemic immune activation by signaling to resident APCs including DCs, macrophages, and epidermal-specific LCs. DCs and LCs make up the dominant population of APCs in the skin. Several populations of CD4+ or CD8+ resident memory T cells (T\(_{RM}\)) also reside in the skin, but their interaction with keratinocytes has not been documented. LCs are an important bridge between innate and adaptive immunity in the skin. LCs physically interact with neighboring keratinocytes though E-cadherin, suggesting that keratinocytes provide a niche for LCs (Tang et al. 1993). The keratinocyte-generated niche is essential for maturation of LCs, as they develop from hematopoietic precursors located in the epidermis, rather than migrating to the skin after maturation. Evidence suggests that growth factors essential for LC development, such as TGFβ1 and M-CSF, are produced by bulge stem cells in the hair follicle (Merad et al. 2008) (Figure 1). This suggests that the bulge region of the hair follicle may provide the environment for maturation of epidermal myeloid cells, but this has not yet been tested. In addition to physical interactions between keratinocytes and LCs, there is also evidence that keratinocytes are essential for LC function. It has been demonstrated in vitro that keratinocytes are required for activation of LCs in response to artificial stimuli (Sugita et al. 2007). Following stimulation with TLR9 ligand (CpG dsDNA), only LCs co-cultured with keratinocytes or treated with keratinocyte cell culture media were able to stimulate T cell proliferation in a mouse model (Sugita et al. 2007). Based on these findings, keratinocytes are clearly active participants in the epidermal immune response by activating LCs to perform their antigen-presenting functions.
The epidermis is well established to serve a critical barrier function, protecting organisms from the surrounding microbe-dominated environment. It is one of the most immunogenic organs in the body, containing numerous types of myeloid and lymphoid lineages. However, there is no consensus regarding the role of keratinocytes in the epidermal immune response. A thorough investigation of keratinocyte-mediated response to pathogens is critical for advancing human health, particularly for the numerous infections of stratified epithelia. In order to gain a clear picture of how keratinocytes function in the systemic immune response, a detailed understanding of the molecular regulation of the intracellular innate immune response is essential.

RNA SILENCING

RNA interference (RNAi) is a post-transcriptional regulatory mechanism that is broadly conserved across most eukaryotes. The RNAi pathway has grown to encompass a family of small, non-coding RNA molecules that function to specifically repress gene expression at the RNA level. The discovery of RNA interference was groundbreaking in that it uncovered an entirely novel function for RNA molecules as regulators within the cell. RNAi was first discovered and characterized as an exogenous gene silencing tool in C. Elegans (Fire et al. 1998). Since its discovery, RNAi has continually been developed as a synthetic gene silencing mechanism to be used as a molecular biology tool and eventually as a therapeutic.

In 1993, the first endogenous small RNA duplexes were discovered. In C. Elegans, the lin-4 gene was found to encode a small RNA with antisense complementarity to developmental regulator LIN-14. Lin-4 was widely known to spatially and temporally control developmental regulator LIN-14 in a repressive fashion. However, the lin-4 gene was found not to encode a protein, but rather two small RNA products with complementarity to regions in the LIN-14 3’
un-translated region (UTR) (Lee et al. 1993). At the same time, it was revealed that these complementary regions in the 3’ UTR were necessary and sufficient for lin-4 regulation of LIN-14 (Wightman et al. 1993). These discoveries were revolutionary in that they established RNAi as a cellular-encoded gene regulation tool and suggested a mechanism for its function. More of these molecules were subsequently identified, and the term “microRNA” was coined in 2001 (Lee et al. 2001; Lau et al. 2001). MicroRNAs (miRNAs) are present in most eukaryotic organisms, ranging from yeast (Drinnenberg et al. 2009) to mammals (Elbashir et al. 2001a).

miRNA BIOGENESIS AND FUNCTION

miRNAs are short, 21-22 nucleotide, single-stranded RNA molecules. They serve as fine tuners of gene expression during development and a variety of stress conditions. miRNAs repress gene expression of their targets, acting to prevent translation of specific messenger RNAs (mRNAs). miRNAs cause destabilization, translational inhibition, or in unique cases, cleavage of specific mRNAs. During the process of targeting, miRNAs are incorporated into a complex termed RISC (RNA-induced silencing complex). The miRNA guide strand is directly bound by an argonaute protein, and directs RISC to a set of target mRNAs. miRNA targeting is determined by sequence complementarity. A 7-mer within the mature miRNA, referred to as the seed sequence, directs the vast majority of miRNA targeting. This seed sequence base pairs with complementary motifs in the 3’ UTR of the mRNA, resulting in repression of the message mediated by the RISC proteins.

Most miRNAs are encoded similarly to protein-coding genes. Most miRNAs characterized to date are transcribed by RNA polymerase II (Pol II), forming a primary transcript that is processed and exported from the nucleus (Figure 2). miRNAs are encoded both in independent transcripts and intronic regions, although many miRNAs are generated from a
distinct locus with a unique promoter (Bartel 2004). The subset of intronic miRNAs are generally encoded on the same strand as the mRNA, and processed from introns in a similar manner as snoRNAs. (Bartel 2004). A large subset of miRNAs, particularly in Drosophila, occur in clusters. Multiple miRNAs are co-transcribed and subsequently processed from poly-cistronic primary transcripts. Clustered miRNAs often share related sequences, although this is not a requirement or determinant for clustering (Lagos-Quintana et al. 2001; Lau et al. 2001). Transcription of
miRNA genes by Pol II produces a long, primary transcript that remains in the nucleus (Lee et al. 2002). Within these primary transcripts (pri-miRNA), there is a conserved stem-hairpin-loop structure (Lagos-Quintana et al. 2001). The hairpin is cleaved by the nuclear RNase III enzyme drosha, resulting in a 70 nt precursor miRNA (pre-miRNA) (Figure 2). Drosha cleaves near the base of the stem, leaving at 2 nt overhang on the 3’ arm (Lee et al. 2003). Drosha acts in concert with pasha/DGCR8 as part of the microprocessor complex. DGCR8 is essential for miRNA processing, as its ablation results in accumulation of primary transcript and loss of mature miRNA (Denli et al. 2004). Transport of this precursor out of the nucleus requires active export by exportin-5 (Yi et al. 2003) (Figure 2). Once in the cytosol, pre-miRNAs undergo a final cleavage step to yield the 22 nt mature form. Because of its previously identified involvement in cleavage of long, dsRNA into siRNAs, the RNase III-type endonuclease dicer was predicted by many groups to be responsible for cleavage of 70 nt pre-miRNA into the 22 nt mature form. Conclusive evidence was provided when dicer was knocked out in a mouse model, and ablation of mature miRNAs was observed (Grishok et al. 2001; Hutvágner et al. 2001). Dicer recognizes the double-stranded pre-miRNA at the stem-loop region. It cleaves the dsRNA stem proximal to the loop, once again leaving the 3’ end 2 nt overhang typical of RNase III-type endonucleases (Figure 2). The resulting mature miRNA duplex contains two 2 nt overhangs at the 3’ end of each strand, derived from cleavage events by both dicer and drosha. Recently, a dicer-independent miRNA pathogenesis pathway was discovered (Cheloufi et al. 2010; Cifuentes et al. 2010). miR-451, which was observed in the mature form in the absence of dicer, was found to be cleaved by argonaute 2 (ago2). Ago2 performs catalytic function, referred to as “slicer” activity, in order to cleave dsRNA during siRNA (perfect match) targeting. In mammals, ago2 is the only argonaute family member that maintains this catalytic activity. Dicer-independent pre-
miRNA is loaded into the RNA binding groove in ago2, where it is cleaved twice to produce the mature form (Cheloufi et al. 2010).

Following the miRNA’s maturation, miRNA silencing is mechanistically similar to the RNAi pathway. Once the mature miRNA is released into the cytosol, it is loaded onto an argonaute protein and becomes associated with RISC (Martinez et al. 2002) (Figure 2). Argonaute is essential for RISC function, and is present in RISCs purified from many different species (Hammond et al. 2001). miRNAs exist briefly in a duplex form, immediately following processing by dicer but prior to incorporation into RISC. The strand responsible for targeting, referred to as the guide strand, is biochemically identical to the complementary (star) strand. It is hypothesized that argonautes distinguish between these two strands by sensing the strength of base pairs at the 5’ end of each strand. The strand with the loosest 5’ base pairing becomes bound to argonaute, and the other strand is stripped away and degraded (Bartel 2004). Once the miRNA guide strand has been bound by an argonaute, it becomes fully associated with RISC. Human RISC is composed of an argonaute protein in complex with Gemin3 and Gemin4 (Mourelatos et al. 2002). The functions of Gemin3 and 4 in RISC are largely unknown.

Argonautes are the best-studied component of the mammalian RISC complex. In mammals, there are four members of the argonaute family (ago1-4). They all contain similar structures, and all are capable of incorporating into the miRNA-induced RISC. miRNAs appear to be nonspecifically loaded onto different argonaute family members. miRNA loading generally correlates with argonaute abundance. In epidermal keratinocytes, ago2 was found to bind 60% of miRNAs, followed by ago1 and ago3. In the absence of one argonaute family member, other members are able to compensate and bind additional miRNAs (Wang et al. 2012). The argonautes consist of two major conserved domains. The PAZ domain, near the N-terminus, resembles a single and double-stranded RNA binding domain, and is thought to be responsible
for binding the miRNA guide strand, and possibly the miRNA-mRNA duplex. The downstream PIWI domain contains an RNaseH-like endonuclease site, which is catalytically active only in ago2. This site is responsible for mRNA cleavage in perfect-match targeting scenarios (Filipowicz et al. 2008). After becoming bound by argonaute, the single-stranded miRNA guides RISC to mRNA targets based on sequence complementarity.

Both miRNAs and siRNAs recognize target mRNAs via complementary base pairing (Figure 2). However, miRNAs and siRNAs recognize targets in slightly different manners, and result in different mechanisms of translational repression. siRNAs pair completely with their target mRNAs, interacting along the full length of the siRNA. A perfect match between the siRNA and the mRNA triggers a conformational change in argonaute, bringing the catalytic domain of ago2 in close proximity with the mRNA and facilitating cleavage. Unlike siRNAs, miRNAs pair with targets through a 7-mer motif referred to as a seed sequence. The seed sequence is located in the 5’ end of the miRNA, spanning nucleotides 2-8 (Lai 2002). This 5’ region preferentially determines targeting by miRNAs, and to some extent in siRNAs. This conclusion was reached following evidence of enriched sequence conservation in the seed region of related miRNAs or orthologs (Bartel 2004). Additionally, bioinformatic predictions of miRNA targets were found to significantly increase accuracy by scanning for sites complementary to the seed region, compared to other 7-mers within the miRNA (Jackson et al. 2013; Lewis et al. 2003). A possible contributing factor to preferential 5’ end targeting may be the structure of the miRNA when it is bound by argonaute. Argonaute appears to display the miRNA’s 5’ end in an exposed, A-form helix structure, which is optimized for base pairing interactions with the mRNA target (Bartel 2004). miRNAs target mRNAs predominantly in the 3’ UTR of the transcript (Lai 2002). Often, a single mRNA will have multiple target sites in its 3’ UTR, and can be regulated by many different miRNAs.
Once a miRNA recognizes a target, it can prevent protein synthesis by several different mechanisms. It was originally thought that the origin of the small RNA in RISC determined its ability to cleave an mRNA target. However, in rare cases where the miRNA has significant complementarity to the mRNA extending beyond the seed sequence, it can also trigger cleavage of the mRNA (Hutvager and Zamore 2002). The site of cleavage is extremely precise, and appears to be directed between bases 10-11 of the miRNA (Elbashir et al. 2001b). mRNA cleavage is mediated by RISC-incorporated ago2, which possesses endonuclease activity in its PIWI domain (Filipowicz et al. 2008). Ago2 is the only argonaute family member involved in the cleavage-based RNAi pathway, as it is the only homolog that retains catalytic activity in its endonuclease domain.

The most common mechanism of miRNA targeting is mRNA deadenylation and destabilization. Global inhibition of the miRNA pathway results in broad and significant changes in levels of mRNA transcripts detected by micro array, suggesting that miRNAs act significantly at the level of mRNA stability. The proposed mechanism of action begins with deadenylation at the poly-A tail. Argonaute recruits GW182 to RISC through its PIWI domain. GW182 then synergizes with the CCR4-NOT deadenylating complex to shorten the poly-A tail (Behm-Ansmant et al. 2006). This initiates a cascade of mRNA degradation, starting with exosome-mediated 3’-5’ degradation. Decapping soon follows at the 5’ end, catalyzed by DCP1 and DCP2. Once decapping has occurred, the exonuclease XRN1 begins 5’-3’ degradation. miRNA-mediated repression is relieved in the absence of DCP1 and DCP2, indicating a requirement for deadenylation and decapping in miRNA-mediated mRNA decay (Behm-Ansmant et al. 2006). High throughput screening methods, such as micro array and quantitative PCR, enable rapid detection of miRNA targets that are repressed via mRNA decay.
miRNA regulation also occurs through translational repression in the absence of mRNA degradation or cleavage. This canonical distinction from the RNAi pathway was first elucidated in the study of *C. Elegans* miRNA *lin-4*. *Lin-4* was known to downregulate expression of the LIN-14 protein product, but had no effect on mRNA levels (Whightman et al. 1993). The precise mechanism of translational repression has not yet been confirmed, although it appears to be mediated by argonaute. miRNA-independent attachment of Ago2 to the 3’ UTR of reporter transcripts mimicked miRNA-mediated repression at the protein level, but had no effect on mRNA stability (Pillai et al. 2004). Mechanisms of Ago2-mediated repression were further investigated, leading to the current hypothesis that RISC prevents translation initiation. mRNA circularization is essential for translation initiation by promoting binding of initiation factor eIF4E to the m7G cap on the mRNA. Circularization occurs via interaction of cap-binding protein eIF4G with PABP1 (polyadenylate-binding protein 1). RISC appears to inhibit translation initiation through some form of interaction with the mRNA cap structure. This is supported by data demonstrating that miRNAs were unable to repress expression of reporters translated from an internal ribosome entry site (IRES) or bi-cistronic mRNA (Pillai et al. 2005). Ago2 was also implicated as the catalyst for m7G cap-dependent repression, as artificial tethering of Ago2 to the 3’ UTR of reporter transcripts exhibited identical repression patterns (Pillai et al. 2005). It is likely that Ago2 prevents cap-dependent translation by preventing eIF4E from interacting with the m7G cap. Ago2 contains a region in between the PAZ and PIWI domains that is similar to the cap-binding region of eIF4E, and binds m7G caps via a group of aromatic residues. Mutation of these residues abolishes translational repression by Ago2, suggesting that it acts as a competitive inhibitor for eIF4E (Kiriakidou et al. 2007). Interaction with eIF4E proposes a potential driving force behind 3’ UTR restricted miRNA targeting, as it brings argonautes into close proximity with eIF4E, eIF4G, and PABP1 during mRNA circularization. miRNAs may also act
to repress translation during the elongation phase. This is mainly supported by evidence that target mRNAs, as well as ago-miRNA complexes, co-precipitate with polysomes (Filipowicz et al. 2008).

RNAi AS AN ANTIVIRAL DEFENSE MECHANISM

The RNAi pathway has been theorized to be an archaic anti-viral defense mechanism. Because of its ability to cleave or repress RNA, it is poised to act against foreign nucleic acids. RNAi has already been established as an innate antiviral defense in plants (Baulcombe 2004; Voinnet 2005). The RNAi pathway can function in several ways to restrict viral replication. Firstly, dicer’s ability to non-specifically cleave long dsRNAs into fragments has the potential to destroy viral nucleic acid replication intermediates in RNA viruses. This theory is supported by studies in Drosophila demonstrating increased susceptibility to viral infection in the absence or inhibition of dicer (van Rij et al. 2006; Wang et al. 2006). In Drosophila, dicer function is split between two proteins. Dicer-1 is responsible for maturation of miRNAs, while dicer-2 produces siRNAs by cleavage of long dsRNA. Genetic ablation of dicer-2 increased susceptibility to Flock House Virus (FHV) and Cricket Paralysis Virus (CrPV), both single stranded RNA (ssRNA) viruses (Wang et al. 2006). In the absence of dicer-2 or Ago-2, viral RNA was greatly increased, and insect mortality was dramatically accelerated (Wang et al. 2006). Interestingly, 22 nt siRNAs accumulated during CrPV infection in vitro, potentially suggesting a mechanism for dicer as a producer of anti-viral siRNAs (Wang et al. 2006). Perhaps the strongest evidence for the importance of RNAi in the antiviral response is that both FHV and CrPV encode an RNA silencing suppressor (RSS). Viruses are under great selection pressure, and dispensable nucleic acids are rapidly lost from the genome. The presence of a conserved gene product in many viruses designed to counteract RNA silencing indicates the importance of this pathway in the host
response. FHV and CrPV are both extremely small viruses, containing only two open reading frames (ORFs). In each virus, one open reading frame is responsible for counteracting the effects of host-mediated RNA silencing. The RSS function was identified by determining which genomic region was specifically dispensable for infection in dicer-2 or Ago-2 null flies but required for infection in WT animals (Wang et al. 2006). The B2 protein of FHV was identified to possess RSS activity, and was used in future studies to specifically inhibit dicer-mediated cleavage of long dsRNA (van Rij et al. 2006, Wang et al. 2006).

A second potential mechanism for viral restriction by the RNAi pathway is the incorporation of virus-specific siRNAs processed by dicer into RISC. This is supported by the finding that Ago-2, the core catalytic component of RISC, is required for antiviral defense in Drosophila. Ago-2 null flies were challenged with both CrPV and Drosophila C Virus (DCV). In both cases, Ago-2 null animals exhibited increased and accelerated mortality, as well as significantly higher loads of viral RNA. Loss of Ago-2 produced a more severe phenotype during infection than loss of dicer-2, suggesting a critical role for RISC in viral restriction (van Rij et al. 2006). An RSS was also identified in DCV. Using a luciferase reporter system, DCV infection was found to block silencing of the reporter by a long (~500 nt) dsRNA. Silencing mediated by transfection of 21 nt siRNAs was unaffected, suggesting that the DCV RSS acts at the level of dicer processing. Its target was further narrowed to dicer-2, by the finding that ratios of mature to precursor miRNAs were unchanged during infection (van Rij et al. 2006). While RNA silencing restriction acts across different virus families, the insect viruses tested all share a commonality in that they are single stranded RNA viruses. RNAi-based mechanisms of RNA virus restriction are relatively easy to conjure, as both dicer and ago2 are designed to cleave dsRNA, a universal replication intermediate for RNA viruses. The RNAi pathway has been firmly established as an innate antiviral mechanism in both plants and Drosophila, but has yet to be studied in
mammalian systems or outside of RNA virus challenge. Additionally, RNAi-mediated antiviral studies to date have been restricted to siRNAs, with the potential involvement of the endogenous miRNA remaining elusive. While dicer-mediated cleavage of replication intermediates is an unlikely restriction mechanism for non-RNA viruses, it is certainly possible that virus-specific siRNAs or miRNAs act through RISC to repress viral mRNAs.

Recently, it was reported that HSV-1 suppressed exogenous siRNA silencing in human cells. A hairpin was designed to result in production of a miRNA (through the canonical biogenesis pathway) with perfect complementarity to eGFP. The synthetic miRNA effectively caused degradation of eGFP mRNA upon co-transfection. However, HSV-1 infection reversed this degradation, resulting in eGFP mRNA stabilization (Wu et al. 2009). These data suggest that HSV-1 encodes a gene product with RSS function. To determine the relevance for RNA silencing suppression for HSV-1 replication, human embryonic kidney 293 cells transformed with SV-40 large T antigen (HEK293T) knocked down for ago2 via siRNA were infected with HSV-1, and viral titers were compared to virus produced from wild-type (WT) cells. Viral titers were significantly enhanced in the absence of ago2, suggesting an antiviral restriction mechanism mediated by RISC (Wu et al. 2009). The evidence of action by human RISC against a dsDNA virus provides novel insight into the potential antiviral functions of RNAi in mammals, and necessitates further investigation in this area.

HERPES SIMPLEX VIRUS

Herpes Simplex Virus Type 1 (HSV-1) is a large, double-stranded DNA virus belonging to the herpesviridae family. Along with HSV-2, pseudorabies virus, varicella-zoster virus, and equine herpesvirus 1, it forms the subfamily of alphaherpesvirinae. Alphaherpesvirinae carry out lytic infections in stratified epithelia including mucosal and cornified tissues. The hosts of
viral replication are keratinocytes residing in the skin or genital tract. HSV-1 predominantly replicates in the skin, at the site of cutaneous-mucosal epithelial junctions such as areas around the mouth. Recently, however, HSV-1 has been detected with increasing frequency in the genital tract of individuals diagnosed with genital herpes (Xu et al. 2006). HSV-2, the canonical causative agent of genital herpes, results in ano-genital lesions. HSV-1 infections are commonly acquired during childhood due to direct skin contact, while the vast majority of HSV-2 infections are sexually transmitted. Aside from their distinct tissue tropisms, HSV-1 and HSV-2 carry out very similar life cycles. Initial exposure to Herpes Simplex viruses results in a productive lytic replication phase, with sites of active virus replication producing visible sores. The virus then enters sensory ganglia, and spreads through the neural network by traveling along the axons of individual neurons. In the neuron, the virus adopts a latent life cycle- it becomes a quiescent episome, producing low levels of a non-coding transcript. This allows the virus to evade immune detection and persist indefinitely in the host. Reactivation of the virus to produce a lytic “outbreak” intermittently occurs, likely driven by stress. During reactivation, the virus reverses its travel route along neuronal axons back to its tissue of origin, where it once again enters a lytic replication phase in keratinocytes.

Herpes Simplex viruses are the most prevalent members of the herpesviridae family. Data from the 1999-2004 U.S. National Health and Nutrition Surveys (NHANES) found 57.7% of the U.S. population to be infected with HSV-1, and 16.2% infected with HSV-2, based on seroprevalence analysis (Xu et al. 2006). The CDC reported similar findings in their analysis of updated 2005-2008 NHANES data, determining the fraction of HSV-2 positive individuals between 14 and 49 years of age to be 17% (CDC, 2006). While most Herpes Simplex virus infections are benign and often asymptomatic, they can pose significant risks to human health. Because of their neural tropism, infections may lead to encephalitis or meningitis. Additionally,
the presence of genital sores caused by HSV-1 or HSV-2 greatly increases the risk of HIV transmission. This is due in part to the potential for direct, blood-to-blood contact, and also the recruitment of lymphocytes to the site of HSV replication, which serve as hosts for HIV (Ogawa et al. 2013). Since cutaneous and mucosal stratified epithelia are the major reservoir for active HSV replication in humans, and serve as the site of primary infection, it is imperative to understand how keratinocytes respond to infection in order to limit disease.

HSV-1 infection begins in the epidermis, following contact with an infected individual. The virus actively replicates in keratinocytes, performing a lytic life cycle. The virus exhibits a preference for proliferative basal keratinocytes, although this is not correlated with receptor expression (Schelhaas et al. 2003). Virus entry can proceed by either endocytosis, or fusion at the plasma membrane, depending on cell type. In both cases, interaction with viral glycoprotein gD with one of its receptors, HVEM, nectin-1, or nectin-2, is required for initiation. The prominent method of HSV-1 entry in keratinocytes is endocytosis (Nicola et al. 2005). When the virus is endocytosed, it follows a terminal, lysosome-destined pathway. The virus uses low endosomal pH to facilitate capsid opening and effective entry. Lysomotropic agents that raise endosomal pH were found to prevent HSV-1 entry in both primary and immortalized human keratinocytes (Nicola et al. 2005). Subsequently, evidence of viral fusion at the plasma membrane was revealed in keratinocytes. Both plasma membrane fusion and endocytosis require the presence of dynamin, which is involved in vesicle cleavage (Rahn et al. 2011). Additionally, cholesterol inhibitor MβCD also disrupted viral entry, suggesting a requirement for lipid raft-dependent endocytosis (Rahn et al. 2011). Inside the endosome, a second gD-receptor interaction is required for final escape into the cytosol (Nicola et al. 2004). Once the virus enters the cytosol, the envelope falls apart and releases naked capsid. The capsid then travels to the nuclear periphery, and releases its DNA through a pore. Viral proteins gD, gB, and the gH-gL
dimer are required for effective trafficking to the nucleus (Nicola et al. 2004). The viral genome remains as an episome in the nucleus, where transcription begins in a highly organized temporally controlled manner. Viral gene products are produced in three distinct phases. First, the \( \alpha \) genes are produced, followed by the \( \beta \), and then \( \gamma \) products. Synthesis of \( \beta \) and \( \gamma \) genes is dependent on the presence of the preceding group. The \( \alpha \) set of gene products consists of primarily non-structural genes, and is synthesized between 3 and 4 hours post infection. \( \beta \) genes are also primarily non-structural, although their production requires both the presence of \( \alpha \) genes as well as ongoing RNA synthesis. The majority of structural genes are located within the \( \gamma \) group. \( \gamma \) gene synthesis continues up to 12 hours after initial infection, nearing the time point of the first progeny virion release (Honess and Roizman, 1974).

**VIRAL miRNA**

Recently, the search for novel miRNAs expanded to include viral genomes. The first viral miRNAs were predicted and validated in dsDNA virus families, including simian polyomaviruses (SV-40) and herpesviruses. Most of the viral miRNAs validated to date are encoded by gamma herpesviruses, such as Epstein Barr Virus (EBV) and Kaposi’s sarcoma-Associated herpesvirus (KSHV) (Pfeffer et al. 2004; Cai et al. 2005). The \( \beta \) herpesvirus, human Cytomegalovirus (HCMV) has also been demonstrated to encode functionally expressed miRNAs (Pfeffer et al. 2005). HSV-1 also encodes several miRNAs, both during lytic and latent infection. These miRNAs were initially predicted in 2006 using a novel virus-specific miRNA prediction algorithm (Cui et al. 2006). Since currently identified viral miRNAs are typically not conserved in related viral species, bioinformatic prediction algorithms for viral miRNAs have had to rely predominantly on secondary structure to filter through genomes and identify potential miRNAs. The HSV-1 strain 17 genome was scanned for 21 nucleotide stretches that are contained within regions predicted
to form stem-loop structures according to RNA folding algorithms. Hits were then filtered according to GC content (between 30-75%), and their location relative to viral coding regions. This computational approach predicted 24 mature miRNAs from 13 potential precursors, encoded at 11 different loci (Cui et al. 2006). One of these miRNAs, denoted miR-H1, was functionally validated for expression during lytic infection in Vero cells. Antisense probes against the 5’ arm of the pre-miRNA sequence were able to detect both 60 nt precursor and a 21 nt mature transcript by northern blot hybridization (Cui et al. 2006). The mature sequence of miR-H1 is conserved to later-isolated HSV-1 and HSV-2 mature miR-H6, suggesting a potential common function during viral infection (Umbach et al. 2008; Jurak et al. 2012).

HSV-1 also expresses a long, non-coding RNA transcript termed latency-associated transcript (LAT). LAT is the only viral transcript produced during latency, and is thought to help maintain the virus in a quiescent state. LAT contains a stable 2 kb intron, and an unstable 6.3 kb exon, although it is not predicted to encode a protein (Ferrel et al. 1991; Kang et al. 2006). LAT was found to encode four functionally expressed miRNAs. miR-H2, miR-H3, miR-H4, and miR-H5 are expressed from four distinct miRNA precursors within LAT (Umbach et al. 2008). Expression of these miRNAs was validated in vitro by small RNA sequencing from complementary DNA (cDNA) libraries derived from 293T cells transfected with a LAT-encoding plasmid. These results were validated in vivo in latently infected mice, where miR-H2, miR-H3, and miR-H5 were detected by deep sequencing (Umbach et al. 2008). A sixth viral miRNA, miR-H6, was identified in the cDNA libraries of latently infected mouse ganglia. miR-H6 is transcribed from the antisense strand in the LAT promoter. The stem-loop precursor for miR-H6 is also encoded at the same loci and in the antisense direction of miR-H1. Using q-RT-PCR analysis, all five HSV-1 encoded miRNAs were also detected during lytic infection in Vero cells (Umbach et al. 2008). The mature sequences of miR-H1 and miR-H6 share significant sequence identity (Umbach et al. 2008).
Several of these miRNAs have functional implications due to seed sequence complementarity with viral gene products. miR-H2 is transcribed in the antisense direction to ICP0, and was found to repress its translation. miR-H6 is capable of repressing ICP4 (Umbach et al. 2008). Both ICP0 and ICP4 are required for expression of viral gene products and are suppressed during latency, suggesting a potential role for LAT-encoded miRNAs in promoting latency by suppressing factors required for reactivation. Recently, miR-H1 was found to repress host cell protein ATRX during lytic infection, in cooperation with viral host-cell shutoff protein vhs. miR-H1 was able to repress a reporter containing only the 3’ UTR of ATRX, strongly supporting evidence that miR-H1 is a functional miRNA during infection, acting through the canonical mammalian RNA silencing pathway (Jurak et al. 2012).

HSV-1 is fundamentally interconnected with the RNAi pathway due to both its functionally expressed viral miRNAs and its newly demonstrated RSS activity. My study aimed to characterize HSV-1 interaction with the endogenous miRNA pathway, including both its RNA and protein components. In 2009, Wu et al. suggested an interaction between HSV-1 and the RNAi pathway by demonstrating that inhibition of ago2 enhanced viral replication, and disrupted exogenous RNA silencing. As a result of these findings, I decided to investigate potential interactions of HSV-1 with the miRNA pathway, as miRNAs comprise most of the endogenous small RNAs in mammals. Using a physiologically relevant keratinocyte in vitro culture system, I assessed the result of suppression of RNAi pathway components on viral production, and investigated the effects of HSV-1 infection on miRNA silencing and expression. In accordance with the findings of Wu et al. in 2009, we hypothesized that the endogenous RNAi pathway can restrict HSV-1 infection in keratinocytes, and the virus counteracts this restriction by blocking miRNA silencing. Our findings confirmed this hypothesis, and suggested ablation of mature miRNA suppression as a potential mechanism for HSV-1 RSS activity.
Transgenic mice: Mice expressing floxed alleles of ago2 (ago2\(^{fl/fl}\)) or DGCR8 (DGCR8\(^{fl/fl}\)) were used to generate keratinocytes knocked out for Ago2 or DGCR8, respectively. Ago2\(^{fl/fl}\) mice were obtained from A. Tarakhovsky (Rockefeller University). These animals were generated via knock-in insertion of LoxP sites flanking exons 9-11 of the Eif2c2 gene (ago2). Deletion of these exons produces a non-functional gene product by eliminating the mid domain of ago2, resulting in loss of expression (O’Carroll et al. 2007). Ago2\(^{fl/fl}\) mice were generated on a C57BL/6 background, but were subsequently bred into a mixed background. DGCR8\(^{fl/fl}\) animals were created by inserting LoxP sites flanking exon 3. Deletion of exon 3 results in a frame shift mutation and a truncated, non-functional protein product (Yi et al. 2009). Wild-type animals, lacking LoxP sites, are denoted as Ago2\(^{+/+}\) or DGCR8\(^{+/+}\). Ago2 was deleted in ago2\(^{fl/fl}\) cells by in vitro cre delivery by a cre-expressing adenovirus (Ad-Cre-GFP). The adeno-viral vector contains cre recombinase fused to GFP, as a marker of efficient transduction. To control for any potential effects due to adenoviral infection, cells denoted as ago2 WT were infected with a GFP-only expressing adenovirus (Ad-GFP). Adenoviruses were obtained from the University of Iowa Gene Transfer Vector Core. Mice expressing cre recombinase under the control of the keratin-14 promoter were obtained from E. Fuchs (Rockefeller University). These animals were generated by injection of k14-driven cre into oocytes, followed by random genomic integration (Vasioukhin et al. 1999). Multiple founder lines were generated, and screened for potential phenotypes due to insertional mutagenesis. DGCR8\(^{fl/+}\) animals were mated with k14-cre mice to generate DGCR8\(^{fl/+}\) k14-cre progeny. These mice were heterozygous for functional DGCR8, but maintained viability. DGCR8\(^{fl/+}\) k14-cre animals were subsequently bred with each other to produce a distribution of animals with DGCR8\(^{+/+}\) k14-cre (WT), DGCR8\(^{fl/+}\) k14-cre (het), or DGCR8\(^{fl/fl}\) k14-cre (KO) genotypes. DGCR8 knockout animals are viable up to postnatal day 6 (p6), allowing ample time for isolation of primary keratinocytes.
**Primary Keratinocyte Isolation and Culture:** Primary keratinocytes were isolated from animals sacrificed at postnatal day 1 (p1). Total back skin was harvested and placed on a dispase enzyme solution overnight at 4°C, to separate the epidermis from the dermis. The next day, the epidermis was manually removed and placed in a 0.5% trypsin solution for five minutes at 37°C. Trypsin digestion releases living basal and suprabasal keratinocytes from the dead stratum corneal layers. Basal keratinocytes are the dominant population isolated by this method, and are selected for in culture because of their proliferative potential. Trypsin digestion generates a single-cell suspension, which can subsequently be plated to give rise to an immortalized culture, or be directly plated for primary cell analyses. Immortalized cultures were obtained by plating primary keratinocytes onto a monolayer of murine fibroblast cells and cultured until spontaneously immortalized colonies arose (MEKs). Keratinocytes were maintained in E medium with 0.05mM calcium. For primary cell assays, freshly isolated keratinocytes were directly plated into culture dishes. Cells were grown in E medium supplemented with 0.2mM calcium for 24 hours post isolation, after which the calcium concentration was lowered to 0.05mM.

**HSV-1 Plaque titer assay:** Viral supernatant was collected and used to make serial tenfold dilutions in phosphate-buffered saline (PBS). Each dilution was used to infect one well of a confluent monolayer (1 million cells) of African Green Monkey kidney (Vero) cells in a six well plate. Four hours post infection, medium was replaced with low-serum DMEM (2% FBS) with 0.44% agarose and allowed to solidify at room temperature for five minutes. Plates were incubated at 37°C for two days, and then fixed overnight in 4% PFA. Cells were stained with 0.2% crystal violet in 70% ethanol to visualize plaque formation. Dilutions containing between ten and one hundred plaques were used to calculate titer. The numbers of plaques were
counted, multiplied by the dilution factor and the volume used to prepare the dilution. Titers are reported in plaque forming units per milliliter (PFU/mL).

**HSV-1 viral stocks:** HSV-1 KOS strain was purchased from ATCC. Viruses were plaque-purified to isogenic stocks using the plaque assay technique described above. Individual plaques obtained during the plaque assay were assumed to contain a clonal virus population derived from one infectious unit. Three days post infection, in the absence of fixation, agarose above a single plaque was removed and re-suspended in PBS. This stock was expanded in Vero cells, and purified through two additional plaque assays resulting in a total of three rounds of plaque purification. Purified virus was then grown on a monolayer of Vero cells. Supernatant was collected 48 hours post infection, at 100% CPE (cytopathic effect). Supernatant was stored at -80°C for use in future experiments. Two distinct strains were isolated during this process: one capable of forming syncytia (syn+) and one negative for syncytia formation (syn-). Syn+ virus was selected for use in further experiments. UV-inactivated HSV-1 was derived from plaque-purified syn+ viral stocks, which were exposed to UV-C light at a dosage of 2.0 mJ/cm².

**RNA extraction and q-RT-PCR:** Total RNA was harvested from keratinocytes by Trizol® extraction, followed by precipitation in 100% isopropanol, and 80% ethanol wash. RNA pellets were dried in a 37°C heat block for 7 min to remove residual ethanol. Dried RNA was dissolved in RNase free DEPC-treated water on ice for 30 min. RNA concentration was determined by spectroscopy on a Nanodrop-2000 instrument. 500ng of RNA was used in the reverse transcriptase reaction (RT) to make cDNA libraries (miScript kit, Qiagen). The reaction was held at 37°C for 60 minutes, followed by a 5 minute denaturation at 95°C. No-RT (NRT) controls were also generated from each RNA sample during the reverse transcription procedure, and were used to evaluate possible DNA contamination during q-RT-PCR reactions. Quantitative PCR was performed using the SYBR green master mix (Biorad), according to the manufacturer-provided
instructions. 10μL reactions were performed in 384 well plates, and SYBR green intensity was measured using a Biorad CFX384 1000 series real-time PCR machine. No-RT and no-template (water) controls were run alongside all samples to assess any nucleic acid contamination. miScript primers were used in conjunction with a proprietary universal primer for miRNA and small RNA q-RT-PCR (Qiagen). Relative quantity and standard error of the mean (SEM) calculations were performed with BioRad CFX software.

**Western blotting:** Protein lysates were prepared from cultured or primary keratinocytes. Cells were removed from culture dishes by trypsinization for 10 minutes at 37°C, followed by quenching with an equal volume of E medium. Cells were then pelleted and washed in PBS. Cell pellets were lysed in SET buffer (2% SDS, 20mM Tris pH 6.8, 1mM EDTA pH 8.0) and boiled for 30 minutes. Total protein was quantified using a BCA assay (Pierce). 20μg of protein was mixed with Laemmli buffer (4% SDS, 10% β-mercaptoethanol, 20% glycerol, 0.02% bromophenol blue, 125mM Tris-HCl) and loaded onto a 10% acrylamide SDS-PAGE gel. Fisher PagerulerPlus® prestained protein ladder was loaded alongside samples to guide size determination. Following electrophoresis, proteins were transferred onto PVDF membranes. The transfer was performed at 4°C and 100V, for 90 minutes in a solution of 20% methanol, 25mM Tris-base, and 192mM glycine. Following the transfer, membranes were blocked in a solution of 5% milk in PBS-T (1X PBS plus 0.2% tween-20). Membranes were washed in PBS-T three times for 20 minutes, and placed in primary antibody overnight at 4°C. The following antibodies were used: Ago2 (1:5,000, MBL RN029PW), β-tubulin (1:5,000, Cell Signaling #2146S), DGCR8 (1:1,000, Aviva Systems Biology #ARP40984_P050). Membranes were washed again after primary antibody incubation in PBS-T three times for 10 minutes. HRP-conjugated goat anti-rabbit secondary antibody was diluted 1:20,000 in 5% milk in PBS-T. Secondary antibody incubations were done for 2 hours at room temperature. Membranes were then subjected to a final PBS-T wash three times for 20
minutes. Blots were incubated in ECL prime chemiluminescent substrate (GE) for two minutes, and then exposed to CL-Xposure film (Kodak) for varying times.

**Plasmid construction:** Luciferase expression plasmid pGL3 control was obtained from Promega. It drives expression of firefly luciferase from an SV-40 promoter, and downstream enhancer. Several cloning sites have been placed between the stop codon and poly-A site, allowing insertion of 3’UTR elements or miRNA target sites. For miR-203 and miR-205 reporters, two serial perfect match target sites were inserted into pGL3 downstream of luciferase, separated by spacer nucleotides (pGL3-2x203, and pGL3-2x205). Perfect match target sites bear 100% sequence complementarity to the full-length mature miRNA, and catalyze cleavage of luciferase mRNA and prevent translation. The miR-200b reporter was constructed to contain two matches to the 2-8 seed region, separated by a spacer. These bulged target sites mediate cleavage-independent translational repression of luciferase.

**Luciferase assays:** Immortalized keratinocytes were plated in 24-well plate format, at a density of 50,000 cells per well. 24 hours after plating, cells were transfected using the lipid-based reagent trans-it LT-1 (mirus bio). The following concentrations were used (per well): 50μL opti-MEM media, 380ng MIGR-GFP (fluorescence marker indicating successful transfection), 20ng firefly luciferase reporter (pGL3, 2x203, 2x205, or 2x200b), 2ng renilla luciferase, and 1.5μL LT-1. Transfection mixes were incubated for 20 minutes at room temperature before being added drop-wise to cell cultures. All assays were designed in triplicate in 24-well plate format. At 36 hours post-transfection, cells were infected with wild type HSV-1, or DMEM (noninfected control) at an MOI of 1 PFU per cell. Cells were lysed directly in the plate with 1x passive lysis buffer in PBS (Promega) 12 hours post infection. The Dual Luciferase Assay kit (Promega) was used to quantify luciferase levels. Each independent experiment was conducted in biological triplicate, to account for inherent variation in the assay. Relative luciferase units were
determined from the average of firefly:renilla (internal transfection control) ratios. Data was then normalized within each reporter to noninfected controls, to visualize fold change among reporters with varied baseline expression. Error was calculated from standard deviation of the mean, and propagated across data normalization.
**RESULTS**

**Increased titers of HSV-1 are produced from keratinocytes in the absence of ago2.** It was previously reported that siRNA knockdown of ago2 in 293T cells produced four-fold higher titers of HSV-1 from those cells (Wu et al. 2009). However, there are several caveats in this study. Since ago2 is a critical component of RISC, it is unable to be efficiently silenced with RNAi-based techniques. Additionally, 293T (human embryonic kidney) cells are a poor model for lytic infection, since productive replication of HSV-1 in vivo occurs in keratinocytes. Using a murine genetic knockout system, I sought to test the effect of ago2 on viral production in a physiologically relevant keratinocyte culture system. Ago2 was deleted *in vitro* from immortalized murine keratinocytes derived from an ago2\(^{fl/fl}\) mouse. Cre recombinase was delivered to keratinocytes in an adenoviral vector, which also included a fluorescent GFP marker fused to cre (Ad-Cre-GFP; ago2 KO). As a control, ago2\(^{fl/fl}\) cells were treated with a GFP-only expressing adenovirus (Ad-GFP), to ensure that adenoviral infection had no effect on HSV-1 production (ago2 WT). Keratinocytes were infected with Ad-Cre-GFP or Ad-GFP at an MOI of 25, which was the minimum required to efficiently deplete ago2 protein (Figure 3B). Four days after adenoviral infection, ago2 WT and ago2 WT keratinocytes were infected with HSV-1 at MOI’s of 0.1 and 0.01. At the time of infection, protein lysate was collected to verify loss of ago2 protein by western blot (Figure 3B). After 36 hours, virus-containing supernatant was collected and titer was determined by plaque assay on Vero cells. Keratinocytes lacking ago2 produced six-fold higher titers of HSV-1 when infected at MOI=0.01, and two-fold higher titers at MOI=0.1 (Figure 3A). I hypothesized that infection at the higher MOI was successful in infecting the vast majority of ago2 WT and ago2 WT cultures by 36 hours post infection and approaching saturated conditions, thereby masking the effect of ago2 loss-of-function on HSV-1 production.
HSV-1-mediated cytopathic effect is accelerated in keratinocytes deficient for ago2. After observing increased viral titers in the absence of ago2, I hypothesized that ago2 KO cells had lost a form of viral restriction. Certain strains of HSV-1 form large, multicellular fusions in vitro, called syncytia. It is unknown why some viruses form syncytia, but it is thought to be mediated by viral membrane fusion protein gD. The particular sub-strain of HSV-1 KOS we have purified is capable of syncytia formation in keratinocytes and Vero cells, and serves as an easily distinguishable mark of cytopathic effect (CPE). To determine if ago2 KO cells were less able to control HSV-1 replication than their wild-type counterparts, we infected keratinocytes with HSV-1 at an MOI of 0.01. At 24 hours post-infection, visible cytopathic effect was assessed in each population. Ago2 WT cultures showed little, if any, signs of viral CPE. Ago2 KO cells,
however, had numerous clearly formed syncytia and substantially more cell death (Figure 3).

Uninfected cells displayed no difference in viability after loss of ago2.

**HSV-1 relieves endogenous miRNA silencing.** In 2009, Wu and colleagues reported that HSV-1 infection suppressed exogenous shRNA-mediated silencing of a GFP reporter (Wu et al. 2009). A mechanism for this RSS activity was not discussed. To explore this effect further, I decided to determine if HSV-1 infection could suppress silencing by endogenous miRNAs. Using a luciferase assay system, firefly luciferase reporter constructs were designed to contain target sites for several miRNAs that are highly expressed in the skin. I selected miR-203, miR-205, and miR-200b. miR-203, miR-205, and miR-200b represent 24%, 12%, and 2% of all miRNAs present in the skin, respectively (Zhang et al., unpublished data). The pGL3 control vector was used as the backbone for construction of reporters, and was also used as an experimental control. pGL3
control expresses firefly luciferase in mammalian cells, driven from an SV-40 promoter. It also contains an SV-40 poly-A site and enhancer element downstream of luciferase. Two perfect-match (miR-203, miR-205) or bulged (miR-200b) were inserted into the pGL3 control vector, in the 3’ UTR region of luciferase (2x203, 2x205, 2x200b). This results in miRNA-specific repression of luciferase by these miRNAs. Immortalized MEKs were transiently transfected with pGL3 control, 2x203, 2x205, or 2x200b. Renilla luciferase was co-transfected along with each firefly reporter, as a control for transfection efficiency. 36 hours post transfection, keratinocytes were infected with HSV-1 at MOI=1, or noninfected (media) control. Infection progressed for 12 hours, allowing expression of all α, β, and γ genes. Cells were then lysed, and firefly and renilla luminescence was quantified using the Promega Dual Luciferase Assay. As expected, the addition of miRNA target sites to the 3’ UTR of pGL3 luciferase greatly reduced reporter expression in cells expressing those miRNAs, compared to pGL3 control (Figure 5A).
RESULTS

However, in HSV-1 infected cells, miRNA target sites had no repressive effects compared to noninfected controls, and reporter expression was elevated up to two-fold above pGL3 control levels (Figure 5B).

**HSV-1 infection abolishes expression of mature host miRNAs.** After observing HSV-1-mediated suppression of endogenous miRNA silencing, I sought to investigate the mechanism. I hypothesized that HSV-1 could either prevent expression of mature miRNAs, blocking their ability to silence targets, or disrupt the formation or action of RISC. To determine if miRNA expression was disrupted by HSV-1, I examined levels of mature miRNA transcripts by quantitative real-time PCR in keratinocytes infected with HSV-1 or media control. MEKs were infected with HSV-1 at an MOI of 1. After 12 hours, cells were lysed and total RNA was extracted. cDNA libraries were generated by reverse transcription, and used in q-RT-PCR reactions to compare levels of miR-200b, miR-203, and miR-205 between infected and noninfected samples. Small nuclear and small nucleolar RNAs were used as controls, since they

![Figure 6. HSV-1 infection causes dramatic loss of mature miRNAs.](image)

MEKs were infected with HSV-1 for twelve hours (MOI=1). Total RNA was harvested via Trizol® extraction, and converted to cDNA by reverse transcription. cDNA libraries were used in q-RT-PCR reactions to detect mature miRNAs and control small RNAs with the Qiagen miScript system. Ribosomal, small nucleolar, and small nuclear RNAs were used as controls, as they had been previously shown to be unchanged during HSV-1 infection. Data shown is relative quantity (ΔCq), without normalization to housekeeping genes. Within each primer set, relative quantity was normalized to the noninfected sample. Error bars represent standard error of the mean, from technical duplicates. Data is representative to n=2 independent experiments.
RESULTS

are unaffected by HSV-1 infection. HSV-1 infection resulted in dramatic loss of mature miRNA expression. miR-200b, miR-203, and miR-205 were reduced to 0.74%, 8.44%, and 0.51% of noninfected levels, respectively (Figure 6).

**HSV-1 does not disrupt expression of ago2.** In order to uncover a potential mechanism for HSV-1 suppression of mature miRNA expression and activity, I probed infected cell lysates for expression of various miRNA pathway components. Our laboratory has previously observed that loss of argonaute family members can result in mild loss of mature miRNAs. This observation, combined with the finding that HSV-1 titers are enhanced in the absence of ago2, potentially implicates ago2 as a part of a host restriction that the virus must overcome. To determine if HSV-1 suppresses ago2 expression, I analyzed protein lysates of infected cells by western blot. MEKs were infected with wild-type or UV-inactivated HSV-1 for 6 or 12 hours, at which total protein lysate was collected. Protein concentration was determined and standardized by BCA assay before SDS-PAGE analysis. Lysates were probed for ago2, along with β-tubulin, which is unchanged during HSV-1 infection. No change in ago2 expression occurred during wild-type or UV-inactivated infection at any time point, suggesting that HSV-1 blocks miRNA expression and function independently of ago2 and RISC (Figure 7).
In 2009, Wu and colleagues observed an interaction between HSV-1 and the RNA silencing pathway in mammalian cell culture. This was a particularly significant finding, as it is one of the first demonstrations of a potential antiviral RNAi mechanism in mammals. Previously, studies of antiviral RNAi activity had been restricted to plant and insect infection by RNA viruses. The 2009 study by Wu et al. broke both of these barriers, in that their analysis was conducted in a mammalian system in the context of a double-stranded DNA virus infection.

They demonstrated that HSV-1 is capable of RSS activity, through the observation that HSV-1 infection blocked shRNA-mediated silencing of a GFP reporter. Additionally, they showed that siRNA knockdown of ago2 enhanced viral replication in HEK 293T cells (Wu et al. 2009). Despite their novel finding, there are several critical caveats to their study. Wu et al. carried out experiments in an exogenous, non-physiologically relevant system, and failed to suggest a mechanism. I chose to explore these findings further, by investigating HSV-1 interaction with the endogenous RNA silencing pathway in keratinocytes, the virus’ natural host during lytic infection. Using a mouse knockout model in vitro, I validated the finding that ago2 loss-of-function in the producer cell enhances viral titers (Figure 3A). This result lead me to the hypothesis that ago2 is acting as a host restriction factor, or through an inhibitory mechanism, to limit HSV-1 replication in keratinocytes. Since ago2 is a critical component of the mammalian endogenous RNAi pathway, it is likely that ago2 restricts the virus through the activity of small RNAs. I focused my efforts on the miRNA pathway, since miRNAs are the most abundant small interfering RNAs in mammals. Additionally, it is unlikely that ago2 restricts HSV-1 replication in a manner similar to that suggested by van Rij et al., and Wang et al. (2006). Both groups observed synergistic action between dicer-2 and Ago-2 that produced perfectly complementary siRNAs from viral RNA genomes in drosophila. However, since HSV-1 is a dsDNA virus and does not go though a dsRNA intermediate stage that could be recognized by dicer, it is more likely
that mammalian ago2 is acting in concert with endogenous small RNAs. My finding that HSV-1 infection disrupts endogenous miRNA silencing supports this hypothesis (Figure 5b). It is interesting to note that HSV-1 infection suppressed siRNA and miRNA-type targeting. miR-203 and miR-205 reporters were constructed to contain target sites perfectly complementary to the full-length miRNA. This is typical of siRNA-mediated silencing, and triggers cleavage of the luciferase mRNA. mRNA cleavage is catalyzed by ago2 slicer activity, and requires an enzymatic domain that is only active in ago2. The miR-200b reporter, however, contains mismatches within the target sites, and is representative of miRNA-mediated suppression. mRNA cleavage should not occur in this instance, and translational repression is independent of ago2 catalytic activity. While my observation of HSV-1 suppression of RNA silencing corroborates that of Wu et al., I provide additional evidence that this phenomenon occurs in keratinocytes, and can modulate the endogenous miRNA pathway.

There are several possible mechanisms for HSV-1 suppression of RNA silencing. After observing that HSV-1 disrupts endogenous miRNA-mediated silencing of luciferase reporters, I considered that the virus could either disrupt the activity of RISC, or prevent expression of mature miRNAs. My observation that mature miRNAs are significantly downregulated during HSV-1 infection suggests the latter (Figure 6). I first considered the virus-encoded RNase vhs, that is responsible for the characteristic host cell shutoff observed during infection. Vhs is encoded by the viral U1-41 gene. Vhs is transcribed in the γ (late) set of gene products, but is packaged into the virion tegument allowing it to act early in the viral life cycle. Vhs possesses RNase activity similar to RNase A, and acts to degrade host mRNAs. Vhs is capable of suppressing highly abundant mRNAs, including actin and GAPDH (Strelow et al. 1995). Vhs-mediated RNA degradation is thought to be restricted to mRNAs, as no small nuclear RNA degradation has been observed, and it is theorized that vhs recognizes the 5’ cap of mRNAs.
However, it is entirely plausible that vhs can degrade miRNAs, either as intermediates in the biogenesis pathway or in their mature form. Vhs-mediated degradation could be responsible for the observed loss of mature miRNA during infection, and subsequent blockade of miRNA silencing. However, Wu et al. showed that a vhs-null virus was equally capable of RSS activity, suggesting that vhs does not function in that mechanism. Although, loss of miRNA expression could be distinct from HSV-1 RSS activity, and a vhs-null virus should be used to determine if vhs is involved in miRNA degradation. Loss of mature miRNA could also result from events other than RNA degradation. miRNAs and shRNA-derived siRNAs are produced through a well-characterized biogenesis pathway. Mouse knockout models have demonstrated that in the absence of miRNA processing enzymes, miRNA expression is lost and severe defects in development and tumorigenesis occur. By disrupting or degrading an essential component of the miRNA biogenesis pathway, HSV-1 could efficiently prevent expression of endogenous miRNAs. HSV-1 encodes a RING finger E3 ubiquitin ligase, ICP0, which is capable of catalyzing poly-ubiquitination of cellular targets (Boutell et al. 2013). It is possible that HSV-1 represses, perhaps through degradation by ICP0, an essential miRNA biogenesis component. While I did not observe loss of Ago2 expression during infection, other components of the miRNA biogenesis pathway remain to be tested (Figure 7).

RNAi has been demonstrated as an essential antiviral mechanism for RNA viruses in both plants and insects. Despite lacking analogs of the powerful mammalian adaptive immune systems, plants and insects are able to effectively combat viral infections. Much of the pioneering work in the anti-viral RNAi field has been carried out in drosophila. Dicer-2, in collaboration with Ago-2, cleaves dsRNA viral genomes into siRNAs, which are incorporated into RISC and guide targeted, efficient degradation of viral genetic material. Since these small RNAs are perfectly complementary to the viral genome, they direct RISC to cleave viral RNAs, which is
characteristic of siRNA-type silencing. Endogenous small RNAs have not yet been implicated in the antiviral response. Both double and single-stranded RNA viruses can fall victim to RNAi-mediated restriction, as many ssRNA viruses must undergo a dsRNA replication intermediate. To date, observations of antiviral RNAi activity have mostly taken place in plants or insects infected with RNA viruses. However, recent investigations of viral gene product function suggest that anti-viral RNAi mechanisms are somewhat conserved in mammals. Many viruses have been found to encode gene products that have RSS functions. For example, HIV-1 transcriptional activator protein (tat), is a potent suppressor of RNA silencing (Qian et al. 2009). Inhibition of RNAi machinery, by a multitude of methods, enhances HIV-1 transcription and replication (Bennasser et al. 2005; Qian et al. 2009). Ebola virus also encodes a protein with RSS function, VP35 (Haasnoot et al. 2007). VP35 can rescue tat-deficient HIV-1 viruses, suggesting RSS function is conserved and critical among mammalian RNA viruses. The broad presence of mammalian viral RSS proteins, combined with the observation that inhibition of RNAi machinery enhances viral replication, strongly suggests that RNAi plays an antiviral role in mammals. Wu et al. significantly broadened the understanding of mammalian anti-viral RNAi by demonstrating that HSV-1, a dsDNA virus, also possesses RSS activity (Wu et al. 2006). I expanded upon their findings further by demonstrating that HSV-1 suppresses expression and function of endogenous small RNAs, which has yet to be demonstrated in any viral system. The culmination of evidence implicating the RNAi pathway in mammalian antiviral defense has opened tremendous avenues for further research, particularly into the involvement of endogenous small RNAs.
My study corroborates and expands upon the findings of Wu and colleagues, demonstrating critical HSV-1 interactions with the RNA silencing pathway. Through ago2 loss-of-function studies, I have implicated the host RNAi pathway as a restriction factor for HSV-1. I also provide the first evidence of viral suppression of endogenous miRNAs, at both the levels of function and expression. While a mechanism remains unknown, examination of miRNA pathway components and miRNA precursors will provide clues regarding HSV-1 RSS activity and purpose.

ACKNOWLEDGEMENTS

**Thesis Advisor:** Dr. Rui Yi

**Defense Committee:** Dr. Rui Yi, Dr. Jennifer Martin, Dr. Debra Goldberg

**Assistance with Experimental Design and Data Analysis:** Kent Riemondy

**Funding:** UROP


Clark, Rachael; Chong, Benjamin; Mirchandani, Nina; Brinster, Nooshin K; Yamanaka, Kei-ichi; Dowgiert, Rebecca K; Kupper, T. S. vast majority of CLA+ T cells are resident in normal skin. *Journal of immunology* (2006). at <http://www.ncbi.nlm.nih.gov/pubmed/16849438>


