The role of HIV-1 microRNAs in viral latency

Per Johnsson
**Title (English)**

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**Abstract**

The Human Immunodefi ciency Virus type 1 (HIV-1) is the causing agent of Acquired Immune Deficiency Syndrome (AIDS). HIV-1 is capable to latently infect cells, thus causing long-term infection and depletion of the human immune system. A possible role for RNA interference in regulation of viral latency was studied. The function of a previously described HIV-1 encoded microRNA, miR-N367, and several computationally predicted microRNAs were studied. Data suggest miR-N367 to be operative in transcriptional modulation of HIV-1 LTR activity. Moreover, one computationally predicted miRNA, H4miRNA, appears expressed during infection and functioning in the regulation of the host cell gene BAF170. These data suggest the use of microRNAs to be operative during HIV-1 infection and be part of the regulatory complexity of HIV-1 latency.

**Keywords**

HIV-1, RNA interference, microRNA, Transcriptional gene silencing

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The role of HIV-1 microRNAs in viral latency

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Sammanfattning


Ett decennium sedan revolutionerades den molekylära biologin i och med Andrew Fires och Craig Mellos upptäckt av RNA interferens (RNAi). RNAi är små RNA molekyler som reglerar uttryck av cellulära proteiner. Upp till 30% av de cellulära proteinerna har rapporterats att vara under reglering av RNAi. Många virus, så som herpes- och adenovirus, har även observerats att utnyttja denna cellulära mekanism till sin fördel.

Syftet med detta projekt var att studera det samband som råder mellan HIV virusets reglering av latens och RNAi. Resultaten visar att en funktionell RNA molekyl via RNAi kan reglera det virala uttrycket redan på transkriptionell nivå. Dessutom föreslår vi en mekanism där det virala uttrycket regleras via att en obalans av RNAi som induceras under HIV infektion.

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## Table of Contents

Abbreviations ..............................................................................................................................................3

1. Introduction ..................................................................................................................................................4

2. Specific aim of the project....................................................................................................................................6

3. Background ..................................................................................................................................................7
   3.1 HIV-1 genome and infection .....................................................................................................................7
   3.2 Latency and viral expression ...................................................................................................................8
   3.3 RNA interference .....................................................................................................................................9
      3.3.1 Post-Transcriptional Gene Silencing (PTGS) ..................................................................................11
      3.3.2 Transcriptional Gene Silencing (TGS) ...........................................................................................12
   3.4 Epigenetics ...........................................................................................................................................13
   3.5 microRNAs and virus ............................................................................................................................14
      3.5.1 MicroRNA and HIV-1 .....................................................................................................................14

4. Materials and methods ....................................................................................................................................16
   4.1 Cell culture .............................................................................................................................................16
   4.1.1 HEK293T-G cells .................................................................................................................................16
   4.1.2 Jurkat cells and Jurkat TREx cells ......................................................................................................16
   4.1.3 1G5 and TZM-b1 cells .......................................................................................................................17
   4.1.4 ACH-2 cells .......................................................................................................................................17
   4.2 Selection of stable cell line .....................................................................................................................17
   4.3 Electroporation of ACH-2 cells ..............................................................................................................17
   4.4 RNA isolation and qRT-PCR analysis .....................................................................................................17
      4.4.1 Primers used for qRT-PCR .............................................................................................................18
   4.5 HIV-1 infection .......................................................................................................................................18
   4.6 Detection of other HIV-1 expressed miRNAs ......................................................................................19
   4.7 ChIP analysis .........................................................................................................................................19

5. Results .........................................................................................................................................................20
   5.1 vmiR-N367 ............................................................................................................................................22
   5.2 Detection of predicted HIV-1 expressed miRNAs .................................................................................23
      5.2.1 Target prediction of H4miRNA .......................................................................................................26
   5.3 Regulation of BAF170 ............................................................................................................................26

6. Discussion .....................................................................................................................................................30
   6.1 HIV the last decades ...............................................................................................................................30
   6.2 HIV-1 and miRNAs ..................................................................................................................................30
      6.2.1 vmiR-N367 .......................................................................................................................................30
      6.2.2 H4miRNA .......................................................................................................................................31
      6.2.3 miRNA specificity ............................................................................................................................31
      6.2.4 A potential role for miRNAs in viral expression and treatment .....................................................32

7. Prospective ....................................................................................................................................................33

8. Acknowledgements ........................................................................................................................................34
9. References ..............................................................................................................35
Abbreviations

Ago    Argonaute
AIDS   Acquired Immune Deficiency Syndrome
DNMT  DNA methyltransferase
dsRNA  double stranded RNA
EBV    Epstein-Barr virus
Efa    Efavirenz
HAART  Highly Active Antiretroviral Therapy
HIV    Human Immunodeficiency Virus
LTR    Long Terminal Repeat
miRNA microRNA
mRNA   Messenger RNA
Nev    Nevirapine
Nt     Nucleotide
Pasha  DGCR8
PTGS   Post Transcriptional Gene Silencing
qRT-PCR Quantitative Real-Time PCR
RISC   RNA-induced silencing complex
RNAi   RNA interference
RNApol II RNA polymerase II
SIV    Simian Immunodeficiency Virus
TAR    Transactivation-responseTat responsive element
Tet    Tetracycline
TGS    Transcriptional Gene Silencing
TRBP   TAR RNA binding protein
vmiRNA Viral specific microRNA
vRT    Viral Reverse Transcriptase
WHO    World Health Organization
1. Introduction

Human immunodeficiency virus (HIV) and its association with Acquired Immune Deficiency Syndrome (AIDS) were first described in the late 1970s/early 1980s. AIDS, the syndrome of HIV infection that causes immune depletion, was observed in the late 1970s while HIV was defined a few years later in the early 1980s by Francoise Barré-Sinoussi [2] and Jay A. Levy [22]. Since then, the number of people infected with HIV has increased to approximately 35 million people worldwide and AIDS remains a leading cause of death. Almost 7000 people continue to be infected each day and approximately 2.1 million people die from AIDS each year. The most afflicted region remains sub-Saharan Africa, which accounts for more than 75% of people with HIV. However, it is important to note that increased research and development in the field of HIV prevention is showing results. In 2001, 3.2 million people were newly infected with HIV worldwide. Today, this number has been reduced to 2.5 million people. On the other hand there has been an increase in Eastern Europe during the last years, where the number of HIV infected persons increased by nearly 150 percent from 2001 to 2007. The total number of people living with HIV is still increasing. This is probably due to more effective drug treatments, which extend the time before outbreak of AIDS and the cause of death [58].

Upon initial infection, the virus is poorly cleared by the immune response and stays in a latent phase in resting CD4⁺ cells. The virus remains hidden in the host genome and eventually re-emerges, causing the CD4⁺ cell count to decrease and leading to AIDS. Since most of the conventional therapeutic drugs are designed to target actively replicating virus, the drugs are ineffective in clearing the virus from latently infected cells. By nature, HIV evolves very fast and multi drug resistant HIV strains can evolve in infected individuals on multiple therapies. While new drugs continue to be needed, alternate ways to protect from infection or clear virus from infected people have been proposed. Protection from infection by vaccination has long been a major focus, but trials so far have not shown any promising results. Recently Merck™ terminated a vaccine trial when results surprisingly indicated more infected individuals among the vaccinated individuals than the control group [46]. Other strategies to defeat HIV-1 infection have also been investigated. Depletion of virus via activation of latently infected cells has been proposed [21]. Recent findings also indicate that microRNAs might be involved in
establishment and maintenance of viral latency [4, 32, 33], thus previously unknown pathways, and possible new targets for drug development, may be involved in HIV latency. While activation of latency infected cells and its association to cellular factor NFκB has been extensive evaluated, a mechanism explaining early establishment and maintenance of viral latency still remains to be formulated. This process will be essential to understand in order to defeat HIV infection.
2. Specific aim of the project

Recent findings have proposed that HIV-1 expresses viral microRNAs (vmiRNAs) upon infection. The aim of this study was to investigate the effect of the previously observed vmiR-N367 in HIV-1 infected cells [4, 32]. vmiR-N367 is encoded within the viral Nef gene and expression of vmiR-N367 has been proposed to regulate viral latency via modulation of the viral promoter. This study intended to study the effects of the proposed vmiR-N367. Overexpression and knockdown of vmiR-N367 was conducted and viral transcriptional activity and chromatin changes to the viral promoter were studied.

To date, most work on HIV-1 encoded microRNAs remains vmiR-N367 [4, 32, 33] and miR-TAR [17]. However, it remains unknown whether there are other HIV-1 expressed microRNAs and what their respective function may be with regards to viral and/or cellular regulation. To investigate this, the entire HIV-1 HXB2 genome was screened for putative HIV-1 expressed miRNAs [42]. Screening of computationally predicted virally expressed miRNAs was performed in HIV-1 infected cells using quantitative real time PCR (qRT-PCR). Targets for the predicted viral miRNAs were also predicted and expression of possible mRNA targets was further evaluated using qRT-PCR.
3. Background

3.1 HIV-1 genome and infection

Human immunodeficiency virus-1 (HIV-1) is a retrovirus belonging to the lentivirus subfamily. Like other lentiviruses, HIV-1 integrates its viral genome into the genome of the host cell. Viral reverse transcriptase (vRT) is carried in infectious particles and during infection of the host cell the vRT transcribes viral RNA to DNA. The DNA is then integrated in host genome through the action of the viral integrase protein. HIV-1 is composed of two positive, single-stranded RNA molecules encoding nine genes (Fig. 1). Three of the genes, *gag*, *pol* and *env*, encode proteins necessary for the virus particle while the six other genes, *tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu* encode regulatory proteins. Each end of viral RNA contains a sequence called long terminal repeat (LTR) and once viral DNA is integrated in genome, the 5′-LTR functions as the promoter and regulates viral transcription and expression.

HIV-1 primarily infects vital cells in the human immune system such as CD4+ T-helper cells. Eventually, this will cause decreased levels of CD4+ cells leading to AIDS, and those infected die from opportunistic infections due to failure of the immune system. Without treatment, most HIV-1 infected people die from AIDS within 10-15 years [19].

**Fig 1.** (A) Structure of HIV-1 genome. 9 genes are encoded within the genome that is flanked by LTR-regions. (B) 5′-LTR consists of U3, R and U5 region. The viral promoter containing the TATA-box is found within U3 while TAR is found within R region. Several transcription factor binding sites are also found in the 5′-LTR. Picture modified from [55].
The primary cellular receptor for HIV-1 is CD4. Upon infection, HIV-1 first binds to the CD4 receptor followed by binding to a co-receptor, CCR5. The CCR5 co-receptor is crucial for cellular entry but is also dispensable for human physiology. Some people are homozygous for a deletion of CCR5 and show reduced risk for HIV-1 infection. The CCR5 co-receptor is therefore an interesting target for therapeutic approaches [5].

A major challenge for effective drug development to treat HIV-1 is overcoming the fact that HIV-1 has evolved several ways to avoid the human immune system. The reverse transcriptase of HIV-1 that turns the viral genome into DNA lacks proof reading mechanisms. The genetic variability of HIV-1 is therefore very high, making it hard for human immune system to produce specific antibodies and completely defeat infection. In addition, HIV-1 can enter a state of latency. During latency phase, the infected cells express no viral specific factors that can be recognized by the human immune system. The virus can therefore hide in the infected cells and avoid the immune system.

HIV-1 infected individuals today are treated with a combination of antiretroviral drugs called highly active antiretroviral therapy (HAART). HAART consists of at least three anti-retroviral drugs, normally two different nucleoside analogues targeting vRT, and also an inhibitor of the viral protease. By hitting several viral factors simultaneously, the risk of developing multidrug resistant mutants is reduced. However, the treatment cannot affect HIV-1 in latent infected cells and therefore HAART is not a way to completely cure infected individuals. Furthermore, there is still a risk of multidrug resistant HIV-1 strains to evolve in people treated with HAART. How HIV-1 is establishing and maintaining viral latency is still widely unknown and there is currently no drug treatment able to hit HIV-1 in latent infected cells. Latently infected cells therefore represent a major barrier for curing HIV-1 infection and it is crucial to understand the molecular mechanisms involved.

### 3.2 Latency and viral expression

The 5’-LTR of HIV-1 consists of several important sites for virus-host cell interactions. The LTR consists of three sequence elements, U3, R and U5. The basal promoter including the TATA-box and several important binding sites for transcriptional factors are found within U3. In addition to virus-host cell interactions, regulation of chromatin
structure adds an additional level of complexity to the regulation of HIV-1 expression. Regardless of site of integration, 5’-LTR is always packed by two nucleosomes named Nuc-(0) and Nuc-(+1). Nuc-(+1) is located near the viral transcriptional start site. Several cellular binding factors bind upstream of Nuc-(+1) and induce remodeling of chromatin structures [55].

In addition to virus-host cell interactions and chromatin regulation, the 5’-LTR consists of a functional RNA region called transactivation-response element (TAR-element). The TAR-element binds the cis-acting viral-encoded Tat protein and has a pivotal role in activation of viral transcription. Tat induces phosphorylation of RNApol II, resulting in transcription of full-length transcripts. Without Tat only a few number of full-length transcripts are processed and mainly short transcripts are released. T-cell activation is required in order to get increased quantities of Tat. This is achieved via T-cell receptor activation of resting CD4+ T-cells by antigen and/or cytokines such as Interleukine-2 or Tumor necrosis factor-alpha. Activation of resting CD4+ T-cells will also increase levels of nuclear localized NF-AT and NF-κB. NF-AT and NF-κB interact with sites within the 5’-LTR, inducing transcriptional activation of the virus [55]. The activation of HIV-1 latency infected cells is a well-studied process. However, a mechanism explaining the establishment of viral latency when the HIV-1 genome is integrated into the host cell still remains to be formulated. A possible role for RNAi pathway has been proposed to be part of this regulation [33].

3.3 RNA interference

RNA interference (RNAi) is a mechanism for regulation of gene expression. Ever since described by Fire et al in 1998 [9], the number of known cellular mechanisms and cellular genes under the control of RNAi has increased. The effector sequences of the RNAi pathway are 19-25 nucleotides (nt) long non-coding RNA molecules and up to a third of the human genes are estimated to be regulated by these sequences [23]. The effector sequences are either miRNAs or siRNAs and are processed in a similar fashion. miRNAs are encoded within the cellular genome, transcribed, and further processed to
functional molecules (Fig 2A). siRNAs can be introduced experimentally via transfections or result from transposons and virally expressed dsRNAs (Fig 2B).

**Fig 2.** The RNAi pathway. (2A) miRNAs are transcribed from the cell’s genome, processed by Drosha in the nucleus before translocation to cytoplasm via Exportin 5. miRNAs are further processed by Dicer and loaded on to RISC in a similar way as siRNAs. (2B) siRNAs are the result of transposons, viruses or endogenous genes expressing dsRNA or when dsRNA is introduced experimentally. dsRNAs are processed by Dicer to 19-22 long siRNAs. siRNAs are then loaded on to RISC causing post-transcriptional gene silencing. Picture modified from www.ambion.com.

Apoptosis, cellular differentiation and developmental timing are processes reported to be under regulation of RNAi. It has also been argued that the RNAi pathway is part of the innate immune system and used to protect against transposable elements and RNA viruses [43, 48]. Some viruses use the cellular RNAi pathway in order to express viral specific effector molecules necessary for establishment of viral infection [37, 50]. The number of mechanisms involving RNAi continues to grow and RNAi has in many aspects changed the view on the cell and its regulatory mechanisms.
RNAi consists of two distinct pathways, post-transcriptional gene silencing (PTGS) and transcriptional gene silencing (TGS). PTGS was described by Fire et al [9] and acts on mRNA in the cytoplasm. Morris et al [30] described TGS in human cells in 2004. TGS acts at the transcriptional level in the nucleus. Even though PTGS and TGS act at different cellular levels, the processing of functional miRNAs is very similar.

### 3.3.1 Post-Transcriptional Gene Silencing (PTGS)

PTGS was first described in *Caenorhabditis elegans* [9]. It is defined by homology-dependent target degradation or translational inhibition. Screens for miRNA targets suggest that a large part of human genes are under regulation of miRNAs. It has been predicted that between 10% [14] and 30% [23] of the human genes might be regulated by miRNAs. A precursor called pri-miRNA is first transcribed by RNApolymerase II (RNAPol II) in the nucleus. This is a dsRNA-like hairpin structure with imperfect pairing. The pri-miRNA is then further processed in the nucleus by the RNase type III endonuclease Drosha and Pasha (also called DGCR8). Drosha cleaves pri-miRNA at a point where a mismatch occurs and a 70 nt long hairpin structure called the pre-miRNA is formed [20]. The pre-miRNA hairpin structure is formed and exported to the cytoplasm by Exportin 5 [28]. In the cytoplasm, the pre-miRNA structure is further processed. The loop structure is processed to double stranded RNA (dsRNA) by a second RNase type III endonuclease called Dicer [7, 16]. Dicer cuts off the loop structure and a mature miRNA with a 2 nt 3’-overhang is formed. This mature miRNA is loaded into the RNA-induced silencing complex (RISC) in a process that is still poorly understood. One of the miRNA strands called “the passenger strand” is degraded. The other strand, called the “guiding strand”, guides RISC to the mRNA target. The best-characterized component of RISC is the Argonaute (Ago) protein, which is the mediator of RNA silencing [36, 52]. There are four different Ago proteins (Ago 1-4) in mammals but only Ago2 functions to induce siRNA target degradation [26, 38]. Depending on sequence complimentarily, the mRNA target is either translationally repressed, or degraded via endonucleolytic cleavage. The mRNA target is degraded if there is perfect sequence complementarity to the miRNA, while translational repression occurs when there is imperfect pairing.
3.3.2 Transcriptional Gene Silencing (TGS)

TGS was first observed in tobacco plants by Matzke et al [29]. Tobacco plants showed reduced transgene expression and analysis indicated methylation of the suppressed gene. Most of the studies have since been performed in the model organism *Schizosaccharomyces pombe* and the phenomenon was not reported in human cells until 2004 [30]. In contrast to PTGS, TGS acts at transcriptional level in the cellular nucleus and effects promoter activity.

Many of the molecular mechanisms concerning how siRNAs are guided to promoter regions in genomic DNA to mediate TGS in human cells remain unknown. First, siRNAs have to gain access to nuclear DNA, and second, TGS is mediated in the nucleus. Two models for how siRNAs gets access to the nucleus have been proposed, one where the siRNA-protein complex is actively transported into the nucleus, and one where siRNAs gain access to nuclear DNA during cell division when no nuclear membrane is present [31]. In addition, two different models have also been proposed to explain how siRNAs mediate TGS via chromatin changes (Fig 3). siRNAs might mediate TGS via direct interaction with the DNA promoter in a RNA-DNA dependent way (Fig 3A), or via a RNA-RNA intermediate where the siRNAs recognizes a longer RNA that overlaps with the promoter, followed by targeting of the promoter DNA sequence (Fig 3B) [45]. Recently, data supporting the RNA-RNA model has been reported [11]. It was shown that RNA polymerase II transcribes a low copy-number promoter transcript, which the siRNA anti-sense strand can interact with. Blocking of these promoter associated transcripts resulted in loss of TGS, thus supporting the RNA-RNA intermediate model.

![Fig 3.](image)

(3A) RNA-RNA intermediate model for initiation of TGS. siRNAs interact with a longer RNA molecule which overlaps with promoter DNA. An RNA-RNA intermediate then interact with promoter DNA.
DNA causing TGS. (3B) siRNAs interact in a direct way with promoter causing TGS. Picture modified from [31].

TGS seems to be mediated via epigenetic changes where methylation of histone 3 lysine 9 di-methylation (H3K9me2) and histone lysine 27 tri-methylation (H3K27me3) occurs [12, 56]. Methylation of DNA has in some cases also been observed but might not be a conserved mechanism. To date, components shown to be involved in RNA-mediated TGS are Ago-1, Ago-2, RNApol II and DNA methyltransferase 3a (DNMT3a). Ago-1 and Ago-2 appear to be functional before methylation of histones is observed, while DNMT3a seem important for a more heritable, long-term form of epigenetic gene silencing, possibly involving DNA-methylation [12].

3.4 Epigenetics
Epigenetics is the study of meiotically and mitotically heritable changes in gene expression. Three distinct mechanisms are known to modify epigenetic marks; RNA-associated silencing, DNA methylation, and histone modifications [8]. DNA methylation can directly prevent binding of transcription factors to DNA and thus create silenced methylated regions [51]. DNA methyltransferases are known to regulate methylation of DNA. DNA methyltransferase 3b (DNMT3b) has been reported to mediate de novo methylation while DNMT3a augments existing methylation [15]. Cells where DNMT is inhibited lose TGS activity [56].

It is also clear that chromatin structure has a pivotal role in transcriptional activity. Chromatin is composed of nucleosomes, which consist of an octamer of histone proteins [27]. Heterochromatin is a condensed form of chromatin and is related to low transcriptional activity. It can be formed via deacetylation and methylation of specific lysine residues on histone tails. Deacetylation by histone deacetyltransferase (HDAC) creates a more condensed and inactive chromatin structure while acetylation by histone acetyltransferase (HAT) creates relaxation of chromatin and gene activation. In addition, methylation of histones is known to regulate transcriptional activity. Methylation of histone 3 lysine 9 (H3K9) and histone 3 lysine 27 (H3K27) are both known to create a silent state of DNA. H3K9me2 and H3K27me3 are observed and upregulated upon RNA-
mediated TGS [56]. Altogether, this proposes a model where TGS is dependent on both DNA methylation and histone methylation. Whether DNA methylation and methylation of histones are simultaneous events or not remains to be determined.

3.5 microRNAs and virus

The RNAi pathway has long been known to be part of the antiviral defense mechanism in plants [43]. Several pieces of evidence now also support the theory that the RNAi pathway is part of the immune response in mammalian cells [43]. However, some viruses have developed mechanisms to avoid antiviral activity of RNAi and use the RNAi pathway to propagate. Some viruses interfere with the RNAi pathway, while others encode viral specific miRNAs (vmiRNAs) [44]. Influenza virus [24] and adenovirus [1] have been reported to express suppressors of the RNAi pathway. Viruses expressing vmiRNAs during infection have in several cases also been observed. Simian Virus 40 encodes a vmiRNA in order to escape cytotoxic T-cells [50]. Epstein-Barr virus [37], HIV-1 [32, 55] and most recently Herpes Simplex virus-1 [54] have also been proposed to express vmiRNAs with the potential to regulate viral latency.

The ever-increasing number of miRNAs being associated with viral infection indicates that they play an important functional role during viral infection. This makes the interaction between viruses and host cells even more complex, while also enlightening a new spectrum of targets for drug development.

3.5.1 MicroRNA and HIV-1

Screening for miRNAs in HIV-1 infected cells show that large numbers of cellular miRNAs are downregulated during HIV-1 infection [59]. However, a few have also been observed to be upregulated [53]. This suggests a mechanism through which HIV-1 acts upon the RNAi pathway. Two proteins have been proposed to interfere with the RNAi pathway [10]. These are the viral-encoded Tat protein and the TAR RNA-binding protein (TRBP). TRBP is a cellular protein first identified through its binding interaction with the TAR region in the HIV-1 genome. TRBP has now also been shown to bind to Dicer [10]. TRBP binds to the TAR HIV-1 region when viral transcription is activated. This might
therefore decrease the amount of TRBP for Dicer and consequently interfere with the RNAi pathway. Tat has also been observed to interfere with the cellular RNAi pathway by binding to Dicer [3]. Tat is a multifunctional protein and is also a viral encoded transactivator essential for viral 5’-LTR activation [41].

HIV-1 has not only been reported to interfere with the cellular RNAi pathway; some groups have also proposed that HIV-1 expresses vmiRNAs [4, 17, 32]. Predictions indicate that HIV-1 encodes several vmiRNAs, which have the potential to regulate a broad spectrum of cellular genes. Of special interest for viral expression and regulation of latency is the HIV-1 TAR element [17] and viral miRNA-N367 (vmiR-N367) [4, 32]. HIV-1 TAR element has been reported to be a substrate for Dicer-processing, hence resulting in functional miRNAs with potential to regulate viral expression [17]. vmiR-N367 was first described in 2004 [32] and proposed to regulate Nef expression. Later, vmiR-N367 was also proposed to be a regulator of HIV-1 transcription [33]. Since vmiR-N367 is transcribed from the viral 3’-LTR, it also has the potential to bind to 5’-LTR and modulate viral expression via TGS in a manner beneficial for viral persistence [55]. Interestingly, the vmiR-N367 site frequently appears modified in HIV-1 infected long-term non-progressors [40]. However, other groups have had difficulties to reproduce the detection of viral encoded miRNAs and the data suggesting that HIV-1 processes HIV-1 TAR element and/or expresses vmiR-N367 are disputed [25]. Lately, Lin et al [25] presented data where miRNAs from HIV-1 infected cells were cloned and sequenced. Lin et al [25] did not succeed to detect any viral encoded miRNAs in their studies.
4. Materials and methods

4.1 Cell culture

HEK293T-G and TZM-b1 cells were cultured in Dulbecco's Modified Eagle Medium (BioWhittaker, MD, USA) supplemented with 10 % heat inactivated fetal bovine serum (Invitrogen, Carlsbad CA) and 50 IU/mL Penicillin/50 µg/mL Streptomycin mix (Gibco, BRL, UK) at 37 °C and 5 % CO. Jurkat cells, Jurkat T-REx, 1G5, and ACH-2 cells were maintained in Roswell Park Memorial Institute medium (BioWhittaker, MD, USA) supplemented with 10 % heat inactivated fetal bovine serum (Invitrogen, Carlsbad CA) and 50 IU/mL Penicillin/50 µg/mL Streptomycin mix (Gibco, BRL, UK) at 37 °C and 5 % CO. Jurkat T-REx cells were also maintained in 5 µg/ml of blasticin. All cells were split every second to third day.

4.1.1 HEK293T-G cells

HEK293T-G is a Human Embryonic Kidney cells line expressing SV40 T antigen. This maintains a high copy number of plasmids containing a SV40 promoter and allows them to be expressed episomally. HEK293T-G cells also stably express the chimeric G-protein subunit that makes cells better adhere to the growing surface.

4.1.2 Jurkat cells and Jurkat TReX cells

Jurkat cells are an immortalized line of T-lymphocyte cells. They are CD4⁺ and therefore a cell line often used for HIV-1 infection. Jurkat T-REx cells express a tetracycline (tet) repressor protein (pcDNA6/TR Invitrogen). The tet-repressor protein is used for tet-inducible miRNA expression systems. A plasmid expressing a miRNA of interest is introduced into Jurkat T-REx cells. Transcription of miRNA is under regulation of the tet-repressor protein and upon addition of tet, tet will bind to the tet-repressor protein and transcription of the miRNA starts.
4.1.3 1G5 and TZM-b1 cells

1G5 cells are an extension of the Jurkat cell line and the TZM-b1 cells are an extension of the HeLa cell line. A viral 5’-LTR has been fused to Luciferase and introduced into the cell lines. Luciferase expression can therefore be used as measurement of viral replication and transcription.

4.1.4 ACH-2 cells

ACH-2 cells are a T-lymphoblast cell line. The cell line is infected with HIV-1, which contains a defective TAT element. This result in a HIV-1 infected cell line that constitutively produces low levels of HIV until stimulated. The cells are therefore often used as a model cell line to study viral latency [6].

4.2 Selection of stable cell line

Block-IT (Invitrogen) was used to establish a stable tet-inducible cell line. Jurkat T-REx cells were transfected using Nucleofector Kit V (Amaxa biosystems) according to manufacturer’s protocol. Cells were put under zeocin drug selection 24h hours post transfection at a concentration of 400 μg/ml. Medium was changed every second to third day and selection was continued for 10-14 days before cells were expanded. Expression of the miRNA was induced at a final concentration of 1μg/ml of tetracycline.

4.3 Electroporation of ACH-2 cells

The respective ODNs were transfected (BioRad Electroporator, 100nM) into ACH-2 cells (1x10^7) in triplicate and viral RNA assessed by qRT-PCR at 24 and 48 hours post-transfection.

4.4 RNA isolation and qRT-PCR analysis

Unless stated otherwise, total cellular RNA was isolated according to manufacturer’s instructions using the QIAamp Viral RNA Mini kit and the RNeasy Mini kit, respectively, automated by the Qiacube (Qiagen, Valencia, CA). All RNA samples
subject to qRT-PCR were prepared according to the following procedure; Isolated RNA in nuclease free water was DNase treated using RQ1 RNase-Free DNase™ (Promega BioSciences, San Luis Obispo, CA) according to manufacturer’s instructions for 30 minutes at 37 °C. Following treatment, samples were standardized (equal amounts of RNA used for reverse transcription reaction) and subject to reverse transcription PCR using the iScript™ cDNA Synthesis Kit (BioRad, Hercules, CA) according to instructions. Controls not subject to reverse transcription did not receive the RT enzyme. All qRT-PCR was carried out using an Eppendorf Mastercycler ep Realplex. qRTPCR was carried out using SYBR® GreenER™ qPCR SuperMix (Invitrogen, Carlsbad, CA).

Thermal cycling parameters started with 2 minutes at 50 °C, 8 minutes at 95 °C, followed by 40 cycles of 95 °C for 15 seconds, 55 °C for 15 seconds and 68 °C for 15 seconds. Specificity of the PCR products was verified by melting curve analysis.

### 4.4.1 Primers used for qRT-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luciferase Forward</td>
<td>5´ CCTGGAACAAATTGCTTTTAC 3´</td>
</tr>
<tr>
<td>Luciferase Reverse</td>
<td>5´ GTTTTCATAGCTTCTGCCAAC 3´</td>
</tr>
<tr>
<td>GAPDH Forward</td>
<td>5´ AGGGGTCATTGATGGCAACAATATCCA 3´</td>
</tr>
<tr>
<td>GAPDH Reverse:</td>
<td>5´ TTTACCAGAGTTAAAAGCAGCCCTGGTG 3´</td>
</tr>
<tr>
<td>HIV-1 LTR (ChIP) Forward</td>
<td>5´CACACAAGGCTACTTCCCTGA 3´</td>
</tr>
<tr>
<td>HIV-1 LTR (ChIP) Reverse</td>
<td>5´GGCCATGTGATGAATGCTA 3´</td>
</tr>
<tr>
<td>H4AS</td>
<td>5´ AGGGAAGATCTGGCCTTCTCTA 3´</td>
</tr>
<tr>
<td>Baf170 Forward</td>
<td>5´CCACCAACAAGTCCCTGTCT 3´</td>
</tr>
<tr>
<td>Baf170 Reverse</td>
<td>5´GGAAACATTTTGATCGGCAGT 3´</td>
</tr>
</tbody>
</table>

### 4.5 HIV-1 infection

100*10^{-12} g p24 of HIV-1b (MDR isolate 10076-4) was used for all HIV-1 infections unless stated otherwise.
4.6 Detection of other HIV-1 expressed miRNAs

qRT-PCR miRNA screen (QuantiMir™) was performed on small RNAs isolated from HIV-1 infected cultures (MirVana™ small RNA isolation kit). 1G5 cells (2x10⁶) were infected with 10 ng of HIV-1b virus (170 ng/ml HIV-1 MDR, isolate 10076-4) and cellular miRNAs isolated at days 1 and 6 post-infection. The miRNAs were detected using qRT-PCR from standardized input of small RNAs using first strand cDNA synthesis from SBI System Biosciences and predicted primer sets based on those putative miRNAs determined previously (Table 1).

4.7 ChIP analysis

A ChIP analysis was performed on the HIV-1 LTR in TZM-bl cells transfected with various U6 expressing miRNA cassettes. TZM-bl cells (2.0 x 10⁵ / well) were transfected in triplicate with pTatDSRed (0.2 µg) and either pmiR-GFP, pmiR-TAR, or pmiR-N367 (0.8 µg)). The respective samples were pooled together 48 hours later and assessed by ChIP for H3K4me2 (07-030, Upstate) or H3K27me3 (07-449, Upstate). The sample lanes are Input (In), no antibody pulldown (No), H3K4me2 pulldown (K4), and H3K27me3 pulldown (K27) (Fig 7B).
5. Results

If HIV-1 is dependent on miRNA regulation in order to establish and maintain infection, Dicer should be essential and play a vital role during infection. Therefore, if Dicer expression is decreased, a significant change in viral expression should be observed. To investigate this, Jurkat T-REx cells were transfected with a tet inducible Dicer knockdown plasmid followed by infection with HIV-1. Dicer and viral RNA expression was measured by qRT-PCR 48 hours post HIV-1 infection (Fig 4A). When Dicer expression was decreased (Fig 4A), viral RNA expression was also decreased (Fig 4B). The reduced level of viral RNA upon Dicer knockdown indicates that Dicer activity has a functional role in viral infection. To further confirm these data, a Jurkat T-REx cell line with a stably expressed tet-inducible Dicer knockdown plasmid was established. Dicer knockdown was verified 48 hours post tet-induction using qRT-PCR. After this time, dDicer expression was indeed significantly decreased (Fig 5A). The cell line was then

Fig 4. (4A) Dicer expression is down-regulated 48 hours post HIV-1 infection/post tet-induced Dicer knock-down. (4B) When Dicer expression is down-regulated, so is also viral RNA.
infected by HIV-1 while Dicer knockdown was induced. RNA was purified 48 hours post infection and viral RNA expression measured using qRT-PCR. Again, viral RNA expression decreased when Dicer was knocked down simultaneously (Fig 5B). Together, the results indicate that processing by Dicer, and functional miRNAs, are important to establish HIV-1 infection.

To study the functional role for Dicer during latent phase, Dicer was knocked down in ACH-2 cells (Fig 6A). ACH-2 cells are infected with an HIV-1 strain defective for the TAT element, thus constitutively produces low levels of HIV-1 [6]. The cells are therefore often used to study viral latency. In contrast to previous data, HIV-1 expression in ACH-2 cells increased 3 days post Dicer knock-down (Fig 6B). The data therefore suggest a multifunctional role for Dicer, where Dicer processing first has a functional role to establish HIV-1 infection, and later works to maintain viral latency.
5.1 vmiR-N367

Several groups have reported that HIV-1 expresses vmiRNA-N367 (Fig 7A) [4, 32, 33]. vmiR-N367 is encoded within the viral Nef gene. Since Nef overlaps with the 3’-LTR, miRNAs encoded within this region could also potentially bind to the 5’-LTR. vmiR-N367 may therefore have the potential to regulate viral expression through TGS, via binding to the 5’-LTR. In order to investigate if previously detected vmiR-N367 has the capacity to regulate HIV-1 expression, phosphothioate oligodeoxynucleotides (ODNs) with the potential to mimic and increase the effect of vmiR-N367 (miR-N367sense) were transfected into ACH-2 cells. Also, phosphothioate ODNs antisense to vmiR-N367 (miR-N367antisense), which bind and inhibit function of vmiR-N367, were transfected into ACH-2 cells. ODNs bind to target RNA and inhibit RNA expression via activation of the RNase H pathway that cleaves the targeted RNA [60]. The effects of the ODNs in ACH-2 cells were studied 1 and 2 days post transfection. While no significant change was seen 24 hours post transfection, cells transfected with ODN vmiR-N367sense show decreased viral expression 48 hours post infection, indicating that viral expression is indeed affected by vmiR-N367 (Fig 7C). Furthermore, cells transfected with ODN vmiR-N367antisense showed increased viral expression 48 hours post infection, reinforcing the transcriptional inhibition effect of vmiR-N367 (Fig 7C).

To deduce whether the observed change of viral expression was a consequence of epigenetic regulation, a plasmid expressing predicted vmiR-N367 was constructed. TZM-bl cells were transfected with different miRNA-expressing cassettes and a ChIP analysis of the HIV-1 LTR was performed 48 hours post transfection, to assess H3K4me2 and H3K27me3. When vmiR-N367 was overexpressed, H3K27me3 is observed (Fig 7B). The data therefore suggest that vmiR-N367 has the potential to regulate HIV-1 expression via a miRNA dependent pathway where epigenetic changes occur.
Fig 7. (A) Predicted secondary structure of HIV-1 expressed miRNA with the Dicer cleavage site pointed out [32, 33]. (B) ChIP pulldown to assess H3K4me2 from transfected TZM-bl cells over expressing different miRNA cassettes. H3K27me3 is enriched when miR-N367 is transfected. No enrichment is observed upon transfection with control miRNAs miR-GFP and miR-TAR. (C) Effects of miR-N367 sense and antisense ODNs relative to control day 1 and day 2 post transfection. When cells transfected with miR-N367sense, viral RNA is decreased at 48 hours post transfection. An increase of viral RNA is observed at the same time point when cells transfected with miR-N367antisense.

5.2 Detection of predicted HIV-1 expressed miRNAs

To date it is unknown whether HIV-1 is expressing other vmiRNAs except vmiR-N367 and TAR RNA. One possible way to discover undefined miRNAs is via computational based approaches. Viral encoded miRNAs were predicted [42] to further investigate a possible role for miRNAs during HIV-1 infection and latency phase. Nine different vmiRNAs and one negative control (H1-H10) were predicted to be putatively expressed and primers were constructed for detection of vmiRNAs in HIV-1 infected 1G5 cells (Table 1). Cells were infected with 10 ng of HIV-1 (170 ng/ml p24 HIV-1 MDR isolate 10076-4) and cellular RNA was isolated (MirVana, Ambion) at day 1 and day 6 post-infection. vmiRNAs were detected using QuantiMir RT-PCR (SBI System Biosciences) from standardized input of small RNAs. miRNA-16 was used as positive control and negative control consisted of H1-10 pooled without antisense primers added. qRT-PCR
Table 1. Predicted vmiRNA (R22 based screening, Isidore) and position in the viral genome.

<table>
<thead>
<tr>
<th>HIV-1 miRNA Name</th>
<th>Predicted mature miRNA sequence</th>
<th>Target site in HIV-1, gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1miRNA</td>
<td>TACTTGAAGAACTGCTGAG</td>
<td>8954-8972, Nef, 5' 3' LTRs</td>
</tr>
<tr>
<td>H2miRNA</td>
<td>CCTGTACTGGGTCTCTG</td>
<td>9117-9135, 5' and 3' LTRs</td>
</tr>
<tr>
<td>H3miRNA</td>
<td>TCGACGGCAAATCCAGGCTT</td>
<td>232-250, 5' and 3' LTRs</td>
</tr>
<tr>
<td>H4miRNA</td>
<td>AGGGAAGATCTGGCCCTTCGTA</td>
<td>1634-1654, Pol</td>
</tr>
<tr>
<td>H5miRNA</td>
<td>GCCGAGGGAAATTTCCTCATAGG</td>
<td>5934-5955, Env</td>
</tr>
<tr>
<td>H6miRNA (Neg Control)</td>
<td>CTCAGGTACCTTTAAAGCAGA</td>
<td>8565-8585, Nef</td>
</tr>
<tr>
<td>H7miRNA</td>
<td>GGAACGATCTGGCGAGCCCTG</td>
<td>8034-8055, Env Rev</td>
</tr>
<tr>
<td>H8miRNA</td>
<td>TGATTTGAAGGAGATTGAGG</td>
<td>8090-8110, Env Rev</td>
</tr>
<tr>
<td>H9miRNA</td>
<td>TACTTGAAGAATCTGCTGAG</td>
<td>8954-8972, Nef, 5' 3' LTR</td>
</tr>
<tr>
<td>H10miRNA</td>
<td>TTTGCGCTGTACTGGGTCTCTCG</td>
<td>9113-9135, 5' and 3' LTR</td>
</tr>
</tbody>
</table>

indicated that amplification of target sequences had occurred in several cases. Melting curves were further analyzed and H4miRNA, H5miRNA, H7miRNA as well as H8miRNA all looked promising with single peaks at 70-75°C (Fig 8). qRT-PCR amplified samples were also loaded on 8% acryl amide gel in order to verify size of amplification (Fig 9). A band of 69 base pairs is expected from amplified miRNAs. This is seen for positive control miRNA-16 (Fig 9) and also for H4miRNA and H8miRNA.

![Fig 8. Melting curves. Screening of predicted viral encoded miRNAs was performed in 1G5 cells. RNA was analyzed using qRT-PCR and melting curves were studied.](image-url)
Fig 9. (A) Screening of 10 predicted viral encoded miRNAs in HIV-1 infected 1G5 cells day. qRT-PCR products from day 1 (A) and day 6 (B) post infection were loaded on 8% acryl amide gel in order to verify size of amplification. H2, H4, H5, H7 and H8 are all of expected size at day 1 and/or day 6 post infection.

However, when compared to uninfected cultures, H4miRNA (Fig 10 A) appeared to be expressed in both infected and uninfected cells (Fig 10 B). No significant change of H4miRNA expression is seen upon HIV-1 infection (Fig 10 B). These data therefore suggest that H4miRNA contains sequence homology to HIV-1 and is expressed within the context of the cell. However, the actual target for H4miRNA remains unknown.

Fig 10. (A) Predicted structure of H4miRNA. (B) HIV-1 infected and uninfected cells were screened for H4miRNA expression. H4miRNA was expressed at detectable level in both infected and uninfected cells and no significant change of expression was observed upon HIV-1 infection.
5.2.1 Target prediction of H4miRNA
H4miRNA were screened against the entire human genome (R22 based screening methodology) to predict putative targets for H4miRNA (Appendices 1). Of several targets, those showing the greatest homology and corresponding to a gene previously shown to be involved in the HIV-1 lifecycle were selected for further analysis. Finally, BAF170 was selected for further studies. BAF170 is part of the SWI/SNF complex [62], a complex reported to regulate chromatin structure and also observed to bind to the 5′-LTR of the viral genome [13]. BAF170 and the SWI/SNF complex are therefore of special interest regarding viral transcription and regulation of latency via chromatin changes. BAF170 was therefore chosen for further expression analysis in HIV-1 infected Jurkat cells.

5.3 Regulation of BAF170
In order to establish whether BAF170 is regulated during HIV-1 infection, HEK293T-G cells were transfected with pro-viral DNA plasmids for two different human HIV-1 strains (HX10 and NL4-3) and Simian Immunodeficiency Virus (SIV). Empty plasmid pUC was transfected as a control. RNA was purified 48 hours post transfection and BAF170 expression analyzed using qRT-PCR. BAF170 was upregulated in cells transfected with pSIV, pHX10 and pNL4-3 (Fig 11) thus suggesting a conserved mechanism for BAF170 regulation during viral infection.

![Fig 11. BAF170 expression in 293T-G cells 48 hours post transfection with pro-viral plasmids. BAF170 is upregulated in cells transfected with SIV, HX10 and NL4.](image-url)

![Fig 12. BAF170 expression in 293T-G cells 48 hours post transfection with pro-viral plasmids. BAF170 is upregulated in cells transfected with SIV, HX10 and NL4.](image-url)
To further study if changes in BAF170 expression are a consequence of HIV-1 infection, a system where HIV-1 infection was blocked by anti-retroviral drugs was developed. 1G5 cells were treated with two different anti-retroviral drugs named Efavirenz (Efa) and Nevirapine (Nev). Efa and Nev are approved non-nucleoside analogue reverse transcriptase inhibitors and are used as therapeutic drugs. Cells were treated with drugs 24 hours post infection and drug treatment was repeated daily according to table 2.

RNA was purified 48 hours post infection and BAF170 expression assessed by qRT-PCR. BAF170 expression was upregulated in cells without drug treatment while no upregulation was observed in cells treated with anti-retroviral drugs (Fig 12). Since upregulation of BAF170 was lost in anti-retroviral drug treated cells, this suggests that BAF170 upregulation is indeed a consequence of viral infection. However, it was also observed that BAF170 is decreased in cells treated with drugs as compared to the control, indicating off-target effects. This can probably be avoided if the concentration of drugs is optimized for 1G5 cells.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells</td>
<td>Cells no Virus</td>
</tr>
<tr>
<td>HIV</td>
<td>No Drug + virus</td>
</tr>
<tr>
<td>Efa + Nev</td>
<td>2 drugs (Efa 25nM, Nev 150nM)</td>
</tr>
<tr>
<td>Efa + Nev + HIV</td>
<td>2 drugs (Efa 25nM, Nev 150nM) + virus</td>
</tr>
</tbody>
</table>

Table 2. 4 different treatments of 1G5 cells. Drug treatment of cells started 24 hours pre HIV-1 infection. Drug treatment was repeated daily and RNA purified 48 hours post infection.

![Fig 12](image_url). 1G5 cells were treated with anti-retroviral drugs Efavirenz and Nevirapine (Table 2). BAF170 expression was assessed 48 hours post infection. BAF170 was upregulated in HIV-1 infected cells while upregulation was lost upon drug treatment of infected cells compared to...
Upregulation of BAF170 indicates that changes in chromatin structure occur in the cell. Since it has already been described that chromatin structure has a pivotal role for viral life cycle and latency [55], BAF170 regulation may be of interest in order to understand this process. To study in more detail how BAF170 is regulated during early infection, a 72 hour time-study of infected 1G5 cells was set up in triplicate. RNA was purified 24, 48 and 72 hours post infection. BAF170 and Luciferase expression were analyzed using qRT-PCR (Fig 13). 1G5 cells carrying luciferase under the control of an HIV-1 5’-LTR promoter, thus higher viral expression activates transcription of the 5’-LTR and results in more expression of luciferase. Analysis of BAF170 and luciferase over a 72 hour period showed that both are upregulated at the last time point. Only minor changes in expression were seen earlier during infection.

**Fig 13.** 1G5 cells were infected by HIV-1. Luciferase and BAF170 expression were analysed 24 hours, 48 hours, and 72 hours post infection. Only minor changes were observed 24 hours and 48 hours post infection. 72 hours post infection both Luciferase and BAF170 expression had increased.
Taken together, these data suggest a complex interplay between HIV-1 and BAF170. The level of H4miRNA remains the same in HIV-1 infected cells (Fig 10). Since the HIV-1 genome contains sequence homology to H4miRNA and it is possible that H4miRNA is targeting HIV-1 during infection, thus causing an imbalance of H4miRNA (Fig 14). This imbalance might subsequently result in less H4miRNA targeting of BAF170 via the RNAi pathway causing upregulation of BAF170 (Fig 14C). This imbalance could provide greater access of BAF170 to the Swi/Snf complex and possibly increased Swi/Snf mediated gene expression of integrated virus which functions to modulate transcription of integrated HIV virus.

**Fig 14.** (A) Uninfected cell. BAF170 is under regulation of H4miRNA. (B) Cell is infected by HIV-1. (C) The total amount of H4miRNA remains the same upon infection but H4miRNA targeting is partially relocated and now also binding to the viral genome, thus causing an increase of BAF170 expression.
6. Discussion

6.1 HIV the last decades
Almost 30 years after it was identified, HIV continues to be a crucial global issue. Drugs have been developed and life expectancy for HIV-infected individuals has greatly increased. However, there is still no way to completely obliterate the virus from the human body, even though viral levels are often below detectable levels during treatment. Due to the nature of HIV, whereby mutations often occur, infected individuals always face the risk of developing multidrug resistant HIV strains.

Several vaccination trials have been set up, so far without any success. Most recently in 2007, Merck™ decided to terminate a vaccine trial when it was discovered that more HIV infections had occurred in vaccinated subjects compared to placebo treated individuals [46]. This was highly unexpected and all planned HIV vaccine trials were postponed. This clearly illustrates the need of more basic research and one must also face the fact that a vaccine may not be developed for HIV. It is still too early to give up faith of finding a vaccine, but other methods to treat HIV should continue to be examined and researched.

6.2 HIV-1 and miRNAs

6.2.1 vmiR-N367
In order to defeat HIV and eliminate infection from infected individuals, it is important to understand how viral latency is established and maintained. A mechanism for this remains to be formulated and will be essential to completely understand the regulatory mechanisms of HIV. A possible role for miRNAs and the RNAi pathway has been proposed [17, 33]. Viral-expressed miRNAs, specifically vmiR-N367 have been proposed and detected by several groups [4, 32], while others have failed to detect the same [25]. In this report we did not intend to study whether vmiR-N367 is expressed or not. The intention was rather to study the effect of vmiR-N367 and other potential vmiRNAs during infection. However, noteworthy in the experiments conducted is that different methods have been used for miRNA identification, including different protocols
and procedures for RNA isolation. Moreover, it is possible that the observed vmiRNAs are expressed during specific time points during infection, and that may also be the reason why some groups have been successful in identifying vmiRNAs, while others have failed to do so.

Data generated in this thesis-work indicated that vmiR-N367 has the potential to regulate viral expression (Fig. 7C) and also has the potential to induce methylation of the viral 5’-LTR (Fig 7B, miR-N367, K27). Taken together, a mechanism for HIV where latency and viral expression is regulated via TGS and small non-coding RNAs is proposed. Whether this occurs in nature or not still remains to be elucidated but the modulation of viral expression by vmiR-N367 might have therapeutic interest.

**6.2.2 H4miRNA**

Nine vmiRNAs were predicted [42] and HIV-1 infected cells were screened for expression of these predicted vmiRNAs. Out of the nine predicted vmiRNAs, H4miRNA turned out to be the most promising candidate. However, upon closer examination, this small RNA appeared to be cellular expressed and in part operative in BAF170 gene expression. Since BAF170 is involved in the previously described SWI/SNF complex that induces chromatin modification, this protein was of special interest concerning viral latency. Data in this report shows upregulation of BAF170 during early infection (Fig 11, Fig 12, Fig 13). This upregulation is also seen in several strains of HIV-1 and also for SIV (Fig. 11), but is lost when infected cells are treated with HIV-1 inhibitors (Fig 12). Taken together, these data suggest a mechanism where H4miRNA shifts from targeting BAF170 to target HIV-1 during infection (Fig 14), thus causing an increase of BAF170 expression. Even though this observation has to be further evaluated, the results in this report indicate a far more complex regulatory landscape that is operative in both cellular and viral gene regulation, which previously has been distinctly overlooked.

**6.2.3 miRNA specificity**

It is known that miRNAs often have several targets and hundreds to thousands of possible targets for a single miRNA are often proposed. Differentiating between a direct effect and an off target effect is difficult and extensive work is often required in order to verify
targets for a specific miRNA. Changing the target site a few bases upstream or downstream might induce unexpected off-target effects [57]. In fact, not all 19-21 bases have to be complementary with the target sequence. It has been showed that position 2-9, so called the “seed sequence”, is often conserved while the other nucleotides are less important. The specificity might therefore be solely within these 8-9 bases. Off targets effects must therefore always be considered. Whether the effects observed in this study were a direct effect or an off target effect have to be evaluated more extensively.

6.2.4 A potential role for miRNAs in viral expression and treatment

Can miRNAs be a new way to target HIV in infected cells? This certainly must be investigated and it is thus important to understand how HIV regulates miRNAs in infected cells. Data presented in this report strongly support a multifunctional role for Dicer and subsequently for miRNA processing. HIV-1 infection is first dependent on functional Dicer activity during early infection where decreased viral expression was observed when Dicer was knocked down (Fig 4, Fig 5). Interestingly, the opposite was observed when Dicer was knocked down in latently infected ACH-2 cells, suggesting a functional role for Dicer to maintain viral latency (Fig 6).

It is also known that the TAR RNA binding protein (TRBP) binds to Dicer as well as to the 5’-LTR of the HIV-1 genome [10]. This could affect the overall miRNA expression in infected cells. Whether vmiR-N367 is expressed by HIV-1 or not, the observation that vmiR-N367 can direct histone modifications and subsequently regulate gene expression suggests that vmiR-N367 might be of interest for drug development. Long term silencing of genes via TGS has been showed [61] and this indicates the potential of treatment for a specific time frame, thus keeping the virus in a silent mode. In addition to being a potential treatment of HIV, RNA-induced TGS also has the potential to treat other human diseases, such as cancer diseases, that often are caused by deregulation of oncogenes and/or anti-tumor genes [31].
7. Prospective

Expression of vmiR-N367 should be further evaluated. Specifically, analysis of miRNA-N367 expression should be performed in HIV-1 infected cells for different HIV-1 strains and at different time points. Reports today show different and conflicting data [3, 4, 17, 25, 32]. Some groups have succeeded in amplifying vmiR-N367 while other groups have failed to detect the same. Notable is that different methods for RNA isolation have been used in conducted experiments so far [17, 25, 32]. This is something that can affect the outcome of the miRNA analysis and has to be further evaluated. Furthermore, using expression constructs for vmiR-N367 to study how overexpression or downregulation of vmiRNA-N367 affects viral infection is of great interest and is also something to consider for future experiments.

In this report, no direct data of H4miRNA binding to BAF170 and/or HIV-1 is presented. To verify targets of miRNAs are difficult and there is no consensus method of achieving this. Overexpression of H4miRNA via transfection of siRNA and then assessing BAF170 and HIV-1 expression should be attempted, however off targets effects must always be considered. To further evaluate targets for H4miRNA, the target sequence can be put in to 3’-UTR of luciferase. Luciferase expression can then be studied when H4miRNA is overexpressed or during HIV-1 infection. Recently, Orom et al [34] presented a method where biotinylated synthetic miRNAs are used for identification of miRNA targets. This should also be considered when H4miRNA targets are identified.
8. Acknowledgements

I would like to give Dr Kevin V Morris my greatest thanks for accepting me to join his group at TSRI and personally supervising me during this time. I highly appreciate the support I have received and for always having time for questions and scientific discussions. During this time I have been given invaluable experience and knowledge on TGS, microRNAs and HIV. Thank you also for making my time in San Diego even more pleasant by learning my how to “duck dive” when surfing.

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Thanks also to Stefan Schwartz for reviewing this report.
9. References


H4miRNA predicted cellular targets. The sequence was screened against the entire human genome using (R22 based screening methodology). A few putative targets presented out of the several candidate target genes.