PRESENCE OF NONCODING RNA AND EXOSOMAL BIOGENESIS IN HIV INFECTION

by

Angela Schwab A Thesis Submitted to the Graduate Faculty of George Mason University in Partial Fulfillment of The Requirements for the Degree of Master of Science Systems Biology

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A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at George Mason University

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DEDICATION

This thesis is dedicated to my supportive family: Mom, Dad, George & Kara, Tim, and all my extended family (too many to mention by name).

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LIST OF ABBREVIATIONS

Human Immunodeficiency Virus	HIV
Hepatitis A Virus	HAV
Antiretroviral Therapy	ART
Multivesicular Body	MVB
Endosomal Sorting Complex Required for Transport	ESCRT
Tumor Susceptibility Gene 101	TSG101
Short interfering RNA	siRNA
Hepatocyte Growth Factor Regulated Tyrosine Kinase Substrate	HRS
Vascular Protein Sorting 4	VPS4
Reverse Transcriptase Polymerase Chain Reaction	rt-PCR
Quantitative Polymerase Chain Reaction	qPCR
Western Blot	
Phorbal 12-mystrate 13-acetate	PMA

ABSTRACT

PRESENCE OF NONCODING RNA AND EXOSOMAL BIOGENESIS IN HIV INFECTION

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HIV-1 infection can be treated with antiretroviral drugs, but an efficient system in quantifying latent HIV infection is necessary in a clinical setting. This study shows how nanoparticles can be used to capture exosomes that contain HIV-1 RNA transcripts in patient serum, CVL, and CSF. Multiple viral RNA transcripts of different lengths were found in exosomes from infected cells, including a novel transcript termed "TAR-*gag*". Exosomes from HIV-infected cells cause recipient cells to become more susceptible to future infection. This study also shows how siRNA can be used to knock down key proteins involved in the formation of exosomes to decrease the amount of viral RNA transcripts released in these exosomes.

INTRODUCTION

HIV

HIV-1 is a retrovirus that infects human immune cells (Schwab et al., 2015). The HIV-1 envelope (env) protein binds to a CD4 receptor and CCR5 co-receptor on a T cell (Archin et al., 2014). The membranes of the cell and virus can then fuse, bringing the nucleocapsid into the cytosol (Archin et al., 2014). Once inside the cell, the virus can reverse transcribe to double-stranded DNA, move into the nucleus, and integrate into the host genome (Archin et al., 2014). When the T cells are activated, the provirus can be transcribed; however the provirus in resting T cells typically does not transcribe new viral particles (Archin et al., 2014). HIV-1 can also transcribe miRNAs, the most common being TAR RNA (Narayanan et al., 2013). Tat is one HIV protein that regulates HIV transcription by binding to TAR, allowing a positive transcription elongation complex (P-TEFb) to also bind and catalyze HIV genome transcription (Jaworski et al., 2014a). The Nef protein can cause cell survival of apoptosis in cells (Jaworski et al., 2014a). HIV-1 is predominantly found in CD4+ T cells, but can also be found in other cell types (Archin et al., 2014). Myeloid cells and macrophages have shown to harbor HIV latency (Archin et al., 2014; Bernard et al., 2014). Asterocytes and microglia in the brain have also shown the potential for latent infection during ART treatment (Archin et al., 2014).

Antiretroviral Therapy (ART)

People who are infected with HIV can receive antiretroviral therapy (ART) to decrease the amount of viral load in their bodies (Lorenzo-Redondo et al., 2016). These people become latently infected with the virus with viral reservoirs throughout their body without actively producing the virus (Pasternak et al., 2008). These ART drugs increase the life expectancy of HIV-positive patients, but are very expensive and cannot completely cure the patients. ART is not a cure for the virus, because the virus integrates into the host DNA. CD4+ T cells contain provirus that can replicate when ART treatment is suspended (Archin et al., 2014). Lorenzo-Redondo et al. believe true latency can never be attained even while on ART since they discovered virus being produced in lymphoid tissue (Lorenzo-Redondo et al., 2016). They believe it is important to understand how HIV acts differently in different locations in the body (Lorenzo-Redondo et al., 2016), such as in the brain, that may be harder to reach with ART.

One of the ART drugs is a non-nucleoside reverse transcriptase inhibitor (NNRTI) that is used as first line of defense to control HIV infection (Newman et al., 2014). These prevent the viral RNA from reverse transcribing into provirus (Archin et al., 2014). Another ART is a protease inhibitor (PI) that is a second line of defense booster drug (Newman et al., 2014). ART drugs can perform a number of protective tasks in infected cells including inhibiting viral fusion with the cell membrane and inhibiting CCR5 expression on the cell surface (Archin et al., 2014).

Patients must be monitored often to assess if the ART drugs are preventing HIV from actively producing virus (Newman et al., 2014; Rottinghaus et al., 2014). Used most

often to detect viral load are dried blood spots (DBS) (Newman et al., 2014; Rottinghaus et al., 2014). DBS are easy to obtain from a finger prick, but the actual testing of the viral load is expensive (Newman et al., 2014). Newman et al suggest to pool the blood samples and to only use polymerase chain reaction (PCR) and sequencing on the samples that test positive (Newman et al., 2014). Using this method, they were able to predict with 90% accuracy which patients were failing first-line ART (Newman et al., 2014). It is important to sequence the samples to better understand the reason the patients are failing treatment (Newman et al., 2014).

Detection of viral RNA using PCR

Typically, reverse transcriptase PCR (rt-PCR) and real time qualitative PCR (RT-qPCR) are used to detect and quantify the amount of virus in a cell or outside a cell (Jaworski et al., 2014a; Pasternak et al., 2008), however the primers used are not always specific enough to give an accurate account for the amount of virus in the sample (Archin et al., 2014; Shan et al., 2013). Shan et all suggested using an Oligo-dT primer in rt-PCR to make cDNA of the full viral mRNA with a poly(a) tail (Shan et al., 2013). A set of primers to amplify a downstream region, such as the highly conserved transactivation response (TAR) element, could then be used to quantify the cDNA (Shan et al., 2013). Shan et al also showed how this method could be used to assess viral load in patient plasma (Shan et al., 2013). Pasternak et al believe the use of semi nested real-time PCR is a more sensitive method of detecting low levels of HIV RNA in PBMCs (Pasternak et al., 2008). They first amplified a region of gag DNA before using TaqMan to quantify the

amount of gag DNA present (Pasternak et al., 2008). This method allowed detection of previously undetectable levels of DNA (Pasternak et al., 2008).

Exosomes

Exosomes are small vesicles, 30-120 nm in length, released from all cells in the body and found in various bodily fluids such as semen and urine (Akers et al., 2013; Jaworski et al., 2014b; Narayanan et al., 2013; Schorey and Bhatnagar, 2008; Teow et al., 2016; Ung et al., 2014), and can be transported through the bloodstream and the lymphatic system (Nour and Modis, 2014). They are formed by inward folding of the endosomal membrane to form multivesicular bodies (MVBs), a process carried out by the endosomal sorting complex required for transport (ESCRT) pathway (Jaworski et al., 2014b; Nour and Modis, 2014; Schorey and Bhatnagar, 2008; Valiathan and Resh, 2008). Firstly, the protein HRS binds ESCRT-1, including TSG101 (Valiathan and Resh, 2008). ESCRT II and ESCRT III are then brought in to assist in the sorting of cargo, until Vps4 disassembles the entire complex, causing the final fission of the membrane (Schorey and Bhatnagar, 2008; Valiathan and Resh, 2008). VPS4 disrupts ESCRT III by interacting with CHMP2 and CHMP4 (Adell et al., 2014). Ubiquinated cargo is typically sorted into MVBs using the ESCRT pathway, but proteins that are not ubiquinated can be sorted into the vesicles with a transferrin receptor (Schorey and Bhatnagar, 2008). These MVBs can be sent to the lysosome for degradation, or released from the cell through the cell membrane as exosomes (Nour and Modis, 2014; Schorey and Bhatnagar, 2008; Teow et al., 2016; Valiathan and Resh, 2008). This budding of the membrane to form MVBs is a

similar process to how new virions are formed (Meng et al., 2015; Schorey and Bhatnagar, 2008; Valiathan and Resh, 2008). TSG101 (Goff et al., 2003), CHMP2 (Votteler and Sundquist, 2013), CHMP3 (Muzioł et al., 2006), CHMP4, and VPS4 (Votteler and Sundquist, 2013) are known to be involved in viral budding. TSG101 assists in viral release, whereas CHMP3 can be manipulated to inhibit viral release (Zamborlini et al., 2006). ESCRT II, however may only be indirectly involved in the budding of viruses by simply activating ESCRT III (Ghoujal et al., 2012).



Figure 1: ESCRT pathway. The ESCRT pathway consists of 5 multi-protein complexes. ESCRT-0 includes STAM 1&2 and HRS. ESCRT-I includes VPS37 A, B, C, and D; MVB12 A&B; Tsg101; and VPS28. ESCRT-II includes EAP30; EAP20/VPS25; EAP45/VPS36; and GLUE. ESCRT-III includes CHMP7; CHMP4 A, B, and C; CHMP6; CHMP1 A&B; CHMP2 A&B; CHMP3; and DUB. ESCRT-IV includes VPS4 A&B; CHMP5; and LIP5. The proteins highlighted in red are those targeted by my siRNA. The blue star represents ubiquitin. The purple arrow represents transmembrane cargo. Arrows represent where the proteins are known to interact.

The contents of exosomes depend on the originating cell. It was first thought that exosomes simply carry waste material out of cells, but now exosomes are known to carry functional proteins, mRNA, miRNA, and lipids (Columba Cabezas and Federico, 2013; Jaworski et al., 2014b; Ung et al., 2014). Exosomes are important in cell-to-cell communication and can carry antigens when released from tumor cells to cause an antitumor reaction in recipient cells (Schorey and Bhatnagar, 2008; Ung et al., 2014). Virally infected cells can also incorporate viral proteins and nucleic acid into exosomes (Jaworski et al., 2014b; Pegtel et al., 2010). This has been demonstrated with HIV (Jaworski et al., 2014b; Narayanan et al., 2013; Schorey and Bhatnagar, 2008), HCV (Jaworski et al., 2014b; Nour and Modis, 2014), EBV (Jaworski et al., 2014b; Narayanan et al., 2013; Pegtel et al., 2010; Schorey and Bhatnagar, 2008), HAV (Jaworski et al., 2014b; Nour and Modis, 2014) and several other viruses. These exosomes are noninfectious, but can spread the proteins and nucleic acids to neighboring cells, making them more susceptible to future infection (Laganà et al., 2013; Nour and Modis, 2014; Sampey et al., 2015).

Exosomes can be detected and isolated using certain biomarkers, such as the proteins Alix, TSG101, CD63, CD81, and CD9 (Jaworski et al., 2014b; Schorey and Bhatnagar, 2008). Most exosomes contain Rab and annexin proteins that are involved in membrane fusion (Schorey and Bhatnagar, 2008). There are some miRNAs from cells that have been identified within exosomes that can cause phenotypic changes in the recipient cells (Laganà et al., 2013; Narayanan et al., 2013). There are currently several databases set up online to predict the possible effects of exosomal RNAs on recipient cells (Laganà et al., 2013).

HIV and exosomes

HIV RNA has been found in exosomes in various forms, such as mRNA and miRNA (Bernard et al., 2014; Narayanan et al., 2013; Teow et al., 2016). TAR RNA has been found in exosomes isolated from cell culture supernatant and also from primary patients who are latently infected (Narayanan et al., 2013). Studies have shown how HIV-infected U1 cells release TAR and other miRNAs in exosomes after activation with PMA (Bernard et al., 2014). This current study also shows an increase of TAR in exosomes after HIV-infected cells are activated with PMA. These miRNA-containing exosomes are endocytosed by neighbor cells and can cause RNA interference in these recipient cells, as well as activation of cytokine release (Bernard et al., 2014). HIV proteins, such as Nef (Jaworski et al., 2014b; Teow et al., 2016) and Gag (Narayanan et al., 2013; Teow et al., 2016), have also been found in exosomes from HIV-infected cells. When uninfected cells are exposed to exosomes from HIV-1 infected cells, they become more susceptible to future HIV-1 infection (Narayanan et al., 2013). Exosomes from uninfected cells can potentially protect the host from infection. Host protein APOBEC3g can travel between cells via exosomes and prevent HIV transcription in recipient cells (Teow et al., 2016). Exosomes isolated from breast milk and semen of uninfected individuals seem to provide protection against HIV infection (Teow et al., 2016). So exosomes can useful as well as harmful during infection.

Nanoparticles

Previous studies have shown how hydrogel nanoparticles can be used to trap biomarkers (Luchini et al., 2008) and even virions (Jaworski et al., 2014a). Hydrogels are made of a N-isopropylacrylamide-acrylic acid (NIPAm-AAc) core and a NIPAm shell; the core bait and shell can bind different materials (Longo et al., 2009). This type of hydrogel works well in solution since it has a high water content and is easy to make (Luchini et al., 2008). Luchini et al incubated the hydrogels with lysozymes for different time periods to determine how quickly they can take up proteins in solution (Luchini et al., 2008). They discovered most of the proteins were taken up by the hydroges after just one minute, and by 60 minutes all of the protein was incorporated into the hydrogels (Luchini et al., 2008). Jaworski et al used various hydrogels with different cores to capture different types of material (Jaworski et al., 2014b). NT080 targets exosomes and viral particles; NT082 targets exosomes and Tat viral protein; NT086 targets the HIV-1 virion as well as viral proteins (Jaworski et al., 2014b). They obtained the nanoparticles from Ceres Science and incubated them with viral proteins Tat, Nef, and p24 and assessed their ability to capture the proteins by WB (Jaworski et al., 2014b). They even demonstrated that the nanoparticles can be used to capture material in primary serum samples (Jaworski et al., 2014b).

siRNA

Genes can be silenced in many ways: on the actual DNA strand called epigenetics, or in post-translational modifications by destroying the mRNA called RNA interference

(Martínez et al., 2015). One type of RNA interference can be performed by doublestranded microRNAs, also called short interfering RNAs (siRNA) (Martínez et al., 2015). The siRNAs tend to be about 19-24 base pairs long (Martínez et al., 2015). They originate from non-coding regions of the genome, are transcribed and sent to the cytosol, then are processed by DICER and integrated into an RNA-induced silencing complex (RISC) (Martínez et al., 2015). Only one strand of the siRNA is used by RISC, called the antisense strand (Martínez et al., 2015). The antisense strand is used to find complementary mRNA, which is then degraded or repressed (Martínez et al., 2015). The siRNAs can be artificially synthesized and inserted into cells to cause silencing of genes (Martínez et al., 2015). This can usually be done using lipid nanoparticles, since the siRNAs themselves will not enter the cells on their own (Martínez et al., 2015). These lipid nanoparticles tend to be cationic to envelope the nucleic material, which is negatively charged, and can easily be endocytosed by cells (Jensen et al., 2014). Yan et al developed polymer nanoparticles that encapsulate siRNA for more efficient delivery into cells, protecting the siRNA from nucleases (Yan et al., 2012). Research has shown cationic liposomes used for DNA delivery into cells can cause cytotoxicity at high concentrations (Lv et al., 2006; Masotti et al., 2009).

siRNA can be used to knockdown any gene. Previous studies show that specifically knocking down EAP20 in ESCRT-II will not inhibit viral formation and release (Langelier et al., 2006), but the knockdown of CHMP6 did inhibit viral formation and release (Meng et al., 2015). It is hypothesized CHMP6 knockdown inhibited the virus because CHMP6 is needed to recruit CHMP4, which is more directly involved in viral

release (Meng et al., 2015). While these studies focused on viral release, they did not consider how the knockdown of these proteins affected exosomal release. This current study uses siRNA to knockdown the same proteins to disrupt exosomal formation instead of viral formation.

MATERIALS AND METHODS

Nanoparticle capture of exosomes in cell culture:

First, take cell supernatant, 5 ml, and centrifuge at 25,000x for 5 minutes to remove cells. Filter supernatant through 0.22 μ m filter into new tube. Add 15 μ l of NT80 and 15 μ l of NT82 to between 500 and 1500 μ l of filtrate. Place on rotator at 4°C overnight. Centrifuge at 14,000 rpm for 10 minutes, remove supernatant. Wash NT pellet with 1 ml PBS once, and resuspend final NT pellet in 50 μ l PBS. Keep on ice for a few hours, at 4°C overnight, or at -80°C for longer.

RNA isolation:

First, add 3 volumes of Trizol to sample, mix well, and incubate at room temperature for 15-30 minutes. At this point your virus-infected samples will no longer be infectious. Next, add 1/5 volume of Chloroform, vortex for 30 seconds at least, and incubate at room temperature for 3 minutes. Centrifuge at 12,000 rpm for 5 minutes. Replace top layer to a new Eppendorf microtube. If desired for future DNA or protein isolation, save lower layers at 4°C. Add 1 µl Glycogen to the top layer, mix well by inverting, incubate at room temperature for 2 minutes. Add equal volume of isopropanol, mix by inverting several times, incubate at 4°C or on ice for 10 minutes. Centrifuge at 12,000 rpm at 4°C for 10 minutes. If no pellet is seen at this point, add 1 µul Glycogen again and centrifuge again. Discard supernatants carefully. Wash RNA pellets with 600 µl 75% ethanol, mix by inverting several times, and centrifuge at 12,000 rpm at 4°C for 5 minutes. Remove as much of supernatant as possible and dry under a vacuum for 15 minutes. Resuspend pellet in 15-20 µl of nuclease free, or PCR-grade, water. Keep at -20°C after measuring the concentration at λ =260/280.

RT Reaction:

Two master mixes should be made. The first contains 3 µl PCR-grade water, 1 µl of dNTPs, and 1 µl of reverse primer per sample. The second master mix contains 1.25 µl PCR-grade water, 4 µl 5x GoScript Buffer (Promega), 2 µl MgCl2 (for final concentration of 2.5 mM), 0.25 µl RNasin (final 20 U), and 0.5 µl GoScript Rt (Promega) per sample. To begin, 5 µl of the first mix is placed in each well. Then 7 µl of RNA (typically 12-850 ng) is added to the appropriate wells. This mixture is heated to 70°C for 5 minutes, then immediately placed on ice for 5 minutes. Next, 8 µl of the second master mix is added to each well. This final mixture is incubated at 25°C for 5 minutes, then heated to 42°C for 50 minutes, then to 70°C for 15 minutes. The final product is kept at - 20°C overnight, or at -80°C for longer.

RT-qPCR:

Standard concentrations of 8E5 DNA are used as positive controls as well as a size standard. Dilutions were made to be 1×10^4 , 2×10^3 , 4×10^2 , 8×10^1 , and 16×10^0 copies of DNA per 2 µl. For one sample, 7.84 µl PCR-grade water, 10 µl iQ SuperMix 2x (BioRad), 0.06 µl forward primer (100 µM), 0.06 µl of reverse primer (100 µM), and 0.04 µl of probe (100 µM) are used. After the reaction mix is made, 2 µl of DNA is added to make the total volume of each well ~20 µl. Standards are duplicated, and samples are triplicated. A list of primers and probes used can be found in **Table 1**.

Primer/Probe name	Primer/Probe Sequence	Location in HIV-1 genome
1: TARfII-F	5'- GGT CTC TCT GGT TAG ACC AGA TCT G -3'	1-25
2: TAR+59R*	5'- CAA CAG ACG GGC ACA CAC TAC -3'	99-119
3: LTR+341-R*	5'- ACC CAT CTC TCT CCT TCT AGC C -3'	320-341
4: GAG1-F	5'- TCA GCC CAG AAG TAA TAC CCA TGT -3'	835-849
5: GAG2-R	5'- CAC TGT GTT TAG CAT GGT GTT T -3'	886-909
6: Gag 1625R*	5'- GCT GGT AGG GCT ATA CAT TCT TAC -3'	1155-1178
7: Vpr-R208 [*]	5'- TAA ACG GCA GTT GTT GCA GA -3'	5293-5312
8: Env-2187R*	5'-TGG GAT AAG GGT CTG AAA CG-3'	7917-7936
9: 5T25 [*]	5'- TTT TTT TTT TTT TTT TTT TTT TTG AAG -3'	9178+
EAP20-F	5'-TTC CTG GGC TAC TAC GAT GG-3'	
EAP20-R	5'-ATG CTG GAC TGT TTG TGC AG-3'	
EAP45-F	5'-TAC AGC GAC CAG CAG CTA TG-3'	
EAP45-R	5'-CGA GAG TGG TCG GAG TCA AT-3'	
CD63-F	5' CCC TTG GAA TTG CTT TTG TCG 3'	
CD63-R	5' CGT AGC CAC TTC TGA TAC TCT TC-3'	
VPS4A-F	5'-GAG AGC AAG GGC AGT GAT AGT GA-3'	
VPS4A-R	5'-CGG CAC CCA TCA GCT GTT-3'	
CHMP6-F	5'-GGA GGC CAT GGT TCA GAG TA-3'	
CHMP6-R	5'-CCA GGA GCT CGT CTA TTT GC-3'	
TSG101-F	5'-GTT GGG GCT TAT TCA GGT CA-3'	
TSG101-R	5'-ACT GGG ATT GGG AGG GTA TC-3'	

Table 1: Primers used in PCR. All primers marked with * were used in rt reaction.



DNA isolation of cell pellets:

Follow the protocol for Wizard [®] Genomic DNA Purification Kit from Promega. First, resuspend the cells in 50 μ l PBS. Then add 200 μ l nuclei lysis solution and mix very well. Add 2 μ l RNase A to remove RNA contamination. Incubate at 37°C for 10 minutes. Let samples cool to room temperature, then add 70 μ l protein precipitation solution and mix very well. Place on ice for 5 minutes, then spin the samples at 13,000 rpm for 4 minutes. Replace the supernatant into new tubes; store the protein pellet at -20°C if needed for further analysis. Add 1 μ l glycogen to the supernatant then equal volume of isopropanol. Invert tube 2-3 times to mix. Place on ice for 5 minutes at 4°C. Discard the supernatant, then add 800 μ l of 70% ethanol and invert to mix. Spin the samples at 13,000 rpm for 5 minutes at 4°C. Discard the supernatant for 20 minutes. Resuspend the pellets in 20 μ l nuclease-free water and store at -20°C for a week, or -80°C for longer periods of time.

siRNA Transfection:

Follow Lipofectamin ® RNAiMAX Reagent Protocol 2013 from Life Technologies. First, seed ~ $2x10^5$ adherent cells in 24-well plate. On the following day, dilute Lipofectamine ® RNAiMAX Reagent in Opti-MEM ® Medium by adding 25 µl Opti-MEM ® Medium to 1.5 µl Lipofectamine ® RNAiMAX Reagent in new Eppendorf. Next dilute siRNA in Opti-MEM ® Medium by adding 25 µl Opti-MEM ® Medium to 1.5 µl siRNA (10 µM). The siRNA sequences can be found in **Table 2**. Next add the

diluted siRNA to the diluted Lipofectamine \circledast RNAiMAX Reagent by adding 25 µl of diluted siRNA to 25 µl of diluted Lipofectamine \circledast RNAiMAX Reagent in a new Eppendorf. Incubate at room temperature for five minutes. Then remove cell media and add 50 µl siRNA-lipid complex to cells. Also add ~250 µl fresh exosome free media to cells. Remove siRNA-lipid complex from cells on the following day, and replace with PMA (for monocytes such as U1 cells) and with 1 ml cell media. Let cells grow ~3 days, then harvest cell supernatant and pellet for analysis.

siRNA Name	siRNA Sequence (5'-3')
siTSG101	CUCAAUGCCUUGAAACGAA[dT][dT]
	UUCGUUUCAAGGCAUUGAG[dT][dT]
siVPS4A	CCAAGGAGGCCCUCAAAGA[dT][dT]
	UCUUUGAGGGCCUCCUUGG[dT][dT]
siRAB5A	GUCCUAUGCAGAUGACAAU[dT][dT]
	AUUGUCAUCUGCAUAGGAC[dT][dT]
siRAB27A	CAUUAGACCUACGAAUAAA[dT][dT]
	UUUAUUCGUAGGUCUAAUG[dT][dT]
siVPS36	GACAUUGAGCUAAGAGAUA[dT][dT]
	UAUCUCUUAGCUCAAUGUC[dT][dT]
siVPS25	GACCUGUCUCCCUUUACUU[dT][dT]
	AAGUAAAGGGAGACAGGUC[dT][dT]
siCHMP6IP	GCAAUCACUCAGGAACAAA[dT][dT]
	UUUGUUCCUGAGUGAUUGC[dT][dT]
siPCD6IP	GCAGUAAUAUGUCUGCUCA[dT][dT]
	UGAGCAGACAUAUUACUGC[dT][dT]
siCD63	GGCUAUGUGUUUAGAGAUA[dT][dT]
	UAUCUCUAAACACAUAGCC[dT][dT]

Table 2: siRNA sequences

Bradford Assay:

Wash cell pellet with PBS. Add 35 μ l of cell lysis buffer to the pellet. Place on ice for 20 minutes, vortexing every 5 minutes. Centrifuge at 4°C for 10 minutes at 10,000 rpm. Replace the supernatant to new Eppendorf tube. Use BSA as a standard with 0. μ g/ μ l, 1 μ g/ μ l, 3 μ g/ μ l, and 5 μ g/ μ l concentrations. Add 1 μ l sample to 159 μ l diH₂O, in duplicate. Then add 40 μ l of the Bradford Buffer and mix well, careful to not create many bubbles. Measure absorbance of the wells. Compare the absorbance of the samples to the absorbance of the standards to determine the protein concentration.

Western Blot:

Add laemmli buffer to samples with a 1:1 ratio. Heat to 95°C for 2 minutes, mix the sample, and heat another 2 minutes. Prepare 4-20% Tris-glycine gel, and add the running buffer. Load 10-15 μ l sample onto gel, using standard molecular weight. Run the gel at 100 V for 15 minutes, then at 200 V for 45 minutes until dye reaches the end of the gel. Prepare the transfer tank with the transfer buffer. Place membrane in methanol for 5 minutes before assembling the transfer. Assemble the transfer with two filter papers, then the gel, then the membrane, then two filter papers again all on the negative side. Run at 50 mA overnight, then increase to 150 mA for one hour. Place membrane in blocking solution: 0.25 g dehydrated milk in 5 ml PBS/T20. Rock at 4°C for 30 minutes. Remove milk, and add the antibody solution: 1-25 μ l Ab in 5 ml PBS/T20. Rock at 4°C overnight. Remove antibody and wash the membrane with PBS/T20 for 10 minutes. Add the

secondary antibody: 1-3 μ l Ab in 5 ml PBS/T20. Rock at 4°C for 2 hours. Remove secondary antibody and wash the membrane in PBS/T20 for 10 minutes twice. Wash then with PBS for 10 minutes once. Add 600 μ l developing solution to the membrane, and image the florescence. After imaging, wash the membrane with PBS 5 minutes 3 times and store at 4°C in PBS. If the membrane needs to be examined for a second protein, it can be stripped with a stripping buffer for 5 minutes, washed with PBS 10 minutes 3 times, then blocked for 30 minutes like usual before adding the other antibody solution.

RESULTS

Nanoparticles can capture exosomes in primary samples

Patients who are latently infected with HIV-1 must be routinely checked for detectable levels of HIV in their blood. Typically this means a finger prick and an expensive dried blood spot test (Newman et al., 2014). Other methods use PCR to quantify the amount of viral RNA being produced in the patient, instead of how many copies of the virus are being released from the cells (Pasternak et al., 2008; Shan et al., 2013). Since I know exosomes can carry viral RNA between cells (Sampey et al., 2015), I asked if these exosomes can be used to assess the latency of infection in patients. Previous studies have already shown how nanoparticles can be used to capture exosomes and virions in HIV-infected samples and primary serum (Jaworski et al., 2014b). I decided to further pursue this idea of using nanoparticles to quantify viral load in patient samples. Therefore, I obtained CVL, Serum, and CSF from various infected patients. It is important to note that all serum, CVL, and PBMC samples acquired from the Women's Interagency HIV Study (WIHS) were obtained from patients with viral load below detectable levels. Patient demographics from WIHS is shown in **Table 3**.

Table 3: Patient Demographics.

WIHS Patient Number	Group	Race	Average Age
30403086	ART Responder	Hispanic	35
30409062	ART Responder	African American	45
30409733	Elite Controller	African American	29
30306305	ART Responder	White	34
30306608	ART Responder	White	33
30409149	ART Responder	White	45
30100361	ART Responder	African American	54

I incubated the samples with nanoparticles previously shown to selectively trap exosomes (Jaworski et al., 2014b). After incubation overnight at 4°C, total RNA was isolated from the trapped exosomes for RT reaction and RT-qPCR. Results in **Figure 2A** show TAR and genomic RNA can be quantified from one ART responder patient. There was less RNA found in the CVL than in the serum. Also, there was about a 2-fold difference between TAR and genomic RNA. **Figure 2B** shows no TAR found in the CVL of the ART responder patient, and a high amount of TAR RNA from the Elite controller CVL. In the Elite controller CVL, there was a 2-log difference between TAR and genomic RNA. could be found in two out of ten CSF samples from HIV-infected patients. Collectively, these data show that nanoparticles can be used to concentrate HIV RNA in serum, CVL, and CSF.





HIV-1 RNA in NT080/082-captured material from ART responder #30409086

HIV-1 RNA in NT080/NT082-captured material from CVL sample from HIV-1 infected patients (WIHS)





Figure 2: Nanoparticle capture of exosomes in primary samples. Serum, CVL, and CSF were obtained. Samples were incubated with NT080/082 overnight at 4°C. Total RNA was isolated then subjected to RT reaction with TAR+59R and 5T25 primers. RT-qPCR analysis was performed with TAR primers on the TAR RT and with *gag* primers on the 5T25 RT. Patients used were #30409086 (**A**), 30409062 (**B**), and 30409733 (**B**), obtained from WIHS, and ten CSF samples from HIV infected patients (**C**), obtained from NIH. Standard deviation was found from 3 independent measurements.

Nanoparticles are capturing exosomes, not virions

To further investigate the use of nanotraps particles in capturing material in patient samples, I then used a different nanoparticle known to selectively capture HIV virions (Jaworski et al., 2014b). Patient CVL was obtained from 4 ART responder patients, and serum was obtained from 5 ART responder patients. The samples were incubated with NT080/082 or with NT086 overnight at 4°C. Total RNA was isolated from the captured exosomes for RT-qPCR analysis. Results in **Figure 3A** show TAR and genomic RNA was found in 3 of the 4 NT080/082-captured exosomes from patient CVL. Using NT086, which should capture virus instead of exosome (Jaworski et al., 2014b), TAR and genomic RNA was found in larger quantities in all 4 of the patient CVL (Figure 3B), suggesting the CVL may contain virus. On average, there was more TAR found in the CVL than genomic RNA. Results in Figure 3C show more TAR than genomic RNA found in exosomes from patient serum, about a 2-fold difference in 3 of the patients. Using NT086, which should capture virus instead of exosome (Jaworski et al., 2014b), TAR and genomic RNA was found in equal or less quantities in 4 of the 5 patient serum (Figure 3D), suggesting these samples most likely did not contain virus. It seems the CVL samples have much lower amount of HIV RNA than serum samples. The serum data is more trustworthy, with smaller standard deviation. Overall, it seems the ART patients are not producing virus and the NT080/082 nanoparticles capture most if not all of the extracellular viral RNA.



5x10

4x10³

3x10

2x10

1x10 0

30306305 30306608 30409062 30100361 30409086

HIV-1 RNA in NT086-captured material from serum of ART responders

200

150

100

50

0

30306305

30306608

30409062

HIV-1 RNA in NT086-captured material from CVL of ART responders





Short and Long RNA Transcripts in Cells and Exosomes from Patients on cART These RNA transcripts are found in extracellular material, and must also be found within cells. It is important to know the percent to which these RNAs are released from the cell to understand the extent to which these patients are still producing viral RNA. Therefore, I obtained patient PBMCs and serum from WIHS. These patients were under various cART treatments (Figure 4B). The serum was incubated with NT080/082 overnight at 4°C. Total RNA was isolated from the exosomes and subjected to RT with primers specific for TAR and the 3' end. RNA and DNA were isolated from the PBMCs, and the RNA was subjected to RT in the same manner. TAR RNA, genomic RNA, and pro-viral DNA were quantified by RT-qPCR with the DNA and RNA from the PBMCs and serum in Figure 4A. In each patient, more TAR RNA was found than genomic RNA. Intracellular TAR RNA and genomic RNA were found in 2 of the 4 patients. Exosomal TAR RNA and genomic RNA were found in 3 of the 4 patients. RT-qPCR was also performed on the PBMC DNA with GAPDH primers and compared to the amount of HIV provirus found. Data in Figure 4 show the patient with a smaller percentage of proviral DNA also had less TAR RNA and genomic RNA inside and outside of the cell. These data suggest a direct relationship between HIV-1 proviral DNA, transcription of TAR, and packaging of TAR into exosomes.



Figure 4: TAR and genomic RNA in patient PBMC and serum. PBMC and Serum from 4 patients on various cART treatments (B) were obtained. The serum was incubated with NT080/082 overnight at 4°C. Total RNA was isolated from the nanoparticles and subjected to RT with primers specific for TAR and the 3' end. RNA and DNA were isolated from the PBMCs. The RNA was subjected to RT with primers specific for TAR and the 3' end. A) RT-qPCR was performed on the RNA and DNA to quantify provirus and GAPDH inside the cells and on TAR and genomic RNA inside and outside the cells. D) The percentage of total DNA that consisted of GAPDH and provirus were also found.

Different Lengths of RNA within Exosomes from Infected Cells

I have established that short and long RNA transcripts can be packaged into exosomes and released from the infected cells in vivo. However, TAR RNA and genomic RNA do not fully represent all the RNA transcripts made by HIV-infected cells that could potentially be packaged into exosomes. Therefore, I decided to quantify multiple HIV RNA transcripts that could be packaged into exosomes for a more complete understanding of exosomal content. Here, I isolated exosomes from cell supernatant with NT080/082 and used numerous reverse primers to find different lengths of HIV-1 RNA in the exosomes. Specifically, U1, OM10, J1.1, and ACH2 cells were grown for 3 days before the supernatant was harvested. The supernatant from each cell line was then incubated with NT080/082 overnight at 4°C. Total RNA was isolated from the captured exosomes and subjected to RT with primers specific for the 3' end, env, pol, gag, LTR, and TAR. RT-qPCR was performed on the cDNA with TAR primers (gag with the 3' end). Data in Figure 5 indicate mostly TAR can be found in exosomes from infected cells. In U1 supernatant, 4.5x10⁴ copies of TAR were quantified; in OM10 exosomes, 5.5×10^6 copies; in J1.1, 2×10^5 copies; and in ACH2, 5.5×10^4 copies of TAR were found. The amount of RNA quantified decreased with the LTR reverse primer to 2.5×10^4 in U1 exosomes, $2x10^6$ in OM10 exosomes, $1x10^5$ in J1.1 exoxomes, and $1x10^4$ in ACH2 exosomes, a 2- or 3-fold difference in each cell line. A novel long transcript I termed "TAR-gag" was found in significant quantities in U1 exosomes $(1.5 \times 10^4 \text{ copies})$, in OM10 exosomes $(2x10^6 \text{ copies})$, and in J1.1 exosomes $(1x10^5)$. TAR-gag was not found in large quantity in the ACH2 cells. The amount of RNA quantified from pol, env, and

the 3' end were 10% of the TAR levels in U1, OM10, and J1.1 exosomes. Overall, the results indicate TAR and TAR-*gag* are selectively packaged into exosomes, while longer RNA transcripts are not.



Figure 5: Different lengths of HIV-1 RNA transcripts in exosomes. 4 cell lines (U1, OM10, J1.1, and ACH2) were grown for 3 days before the supernatant was harvested. The supernatant was incubated with NT080/082 overnight at 4°C. Total RNA was isolated and subjected to RT with primers specific for TAR, U5 of LTR, *gag, pol, env*, and the 3' end. RT-qPCR was performed with primers specific for TAR for all transcripts except the 3', with which *gag* primers were used.

TAR-gag RNA Does Not Cause Translation within Cells

Since TAR-gag RNA is a novel RNA transcript, and it was previously unknown whether it was poly-adenylated and translatable, I decided to investigate whether it could be translated into p24 and/or p55 in the recipient cells. Jurkat and U937 cells were plated and treated with either ACH2 or U1 supernatant or exosomes concentrated by NT080/NT082. The cell lysates were run on two SDS-PAGE gels, along with cell supernatant from ACH2 and U1 culture as well as cell supernatant concentrated using NT080/NT082 from ACH2 and U1 culture (to ensure the cell lysates were not contaminated by virus of free p24 (Narayanan et al., 2013)). Results from Figure 6A indicate that p24 and p55 can be found in concentrated ACH2 supernatant while concentrated U1 supernatant only contains p24. However, when these supernatants were added to Jurkat cells, no p24 or p55 was observed within the recipient cells. Figure 6B demonstrates similar results with p24 and p55 again appearing in ACH2 supernatant but only p24 appearing in U1 supernatant. When these supernatants were added to U937 cells, no p24 or p55 was observed within the cells. Collectively, these data show that the novel RNA TAR-gag does not cause translation of p24 or p55.



Figure 6: Cells treated with exosomes containing TAR-gag RNA. ACH2 and U1 cells were pretreated for five days with a cART cocktail consisting of equal parts of indinavir, LAM, TDF, and FTC at 10 μ M. 1 mL of cell supernatant was collected and treated incubated with NT080/NT082 overnight at 4 °C to isolate exosomes. 0.750 mL of fresh Jurkat cells and 1 mL of fresh U937 cells were plated and treated with either 1 mL of cell supernatant from ACH2 or U1 cell culture or concentrated ACH2 or U1 exosomes collected from the NT080/NT082 particles. The cells were then incubated for 72 hours before being harvested and lysed. The resulting Jurkat (A) and U937 (B) cell lysates were run on a Western blot for p24 and p55.

siRNA Knockdown of Key ESCRT Proteins in monocytes/macrophages

Previous studies showed how RNA transcripts are packaged into exosomes and cause recipient uninfected cells to become more susceptible to future infection (Sampey et al., 2015). In order to minimize this adverse effect, I sought to decrease the amount of exosomes released from infected cells. My rationale was that previous studies have used siRNA to knock down ESCRT proteins that are involved in viral budding (Goff et al., 2003), but they failed to take into account how exosomal release was affected. In order to confirm that siRNA can indeed knock down protein translation, I transfected monocytes with siRNA specific for 5 ESCRT proteins of interest and CD63 as a control. After 4 days, I treated the cells with PMA so they would transform into macrophages. Then after 3 days, I harvested the cells and cell supernatant for analysis. The cells were then lysed for WB and RT-qPCR. Figure 7B shows TSG101 and CHMP6 mRNA levels remained relatively constant, whereas EAP20, CD63, EAP45, and VPS4A mRNA levels decreased to 35%, 30%, 27%, and 30%, respectively, of the negative control cells. The WB analysis of the proteins showed a decrease in TSG101 and CHMP6, an increase in EAP20, and no change in CD63, EAP45, or VPS4. Together, the data indicate that siRNA effectively decreased the mRNA or the protein level in these cells.



Figure 7: siRNA knockdown of ESCRT in U1. A) U1 cells were seeded then transfected with siRNA or the reagent Lipofectamine [®] with no siRNA the next day. After 4 days, the supernatant was harvested and replaced with fresh media with PMA. After 3 days the cells and supernatant were harvested. The cells were lysed and RNA was isolated from one set of duplicates, while the other set of duplicates was used for WB. The RNA was subjected to RT with oligo-dT primers. RT-qPCR was performed with primers specific for the 6 targets of the siRNA. The percent of the control (Lipo+, PMA-) was found. Student's T-test was performed between the Lipo+, PMA+ cells and the Lipo+,PMA- and siRNA-treated cells. WB analysis was done on with antibodies specific for the 6 targets of the siRNA and on actin as a loading control.

siRNA knockdown of ESCRT Effect on Exosome Release in U1 Cells

Having established that siRNA was able to penetrate U1 cells and decrease protein translation, I then focused on the cell supernatant to examine exosomal release. Specifically, I expected siRNA-treated cells to excrete less exosomes. In order to test this, the cell supernatant was harvested from monocytes 4 days after siRNA transfection. The supernatant was then incubated with NT080/082, which has previously been shown to trap exosomes in solutions of small volume (Jaworski et al., 2014b). The exosomes collected by the nanoparticles were then lysed for WB and RT-qPCR analysis. Figure **8A,B** show the amount of HIV RNA quantified was around 8×10^4 for TAR but about 3.5×10^4 for TAR-gag, a 2-fold decrease. This is similar to the levels of RNA seen in Figure 5. There was a statistically significant decrease in TAR and TAR-gag RNA in all the siRNA-treated cells except siTSG101. The most dramatic decrease was seen in siCD63-treated cells, which served as our positive control. The genomic RNA level of 5×10^4 in the control only decreased in the siEAP20- and siCD63-treated cells (Figure 8C). The WB analysis in Figure 8D shows a decrease in alix, CD63, and actin levels in siEAP20-treated cells, suggesting these cells had the most vivid decrease in exosomal release. The siCHMP6-treated cells showed little to no protein levels, but there were also no protein bands on the Tris-glycine gel (data not shown), suggesting this sample had been compromised. Collectively, EAP20 knockdown decreased exosome release including the three lengths of HIV RNA, and TSG101 knockdown did not change the level of exosome release or HIV RNA.



Figure 8: siRNA knockdown of exosomal HIV-1 RNA transcripts in U1. The supernatant harvested 4 days after siRNA transtection (Figure 5A) was incubated with NT080/082 overnight at 4°C. Total RNA was then isolated and subjected to RT with primers specific for TAR, *gag*, and the 3' end. RT-qPCR was performed with TAR primers in A and B, but with *gag* primers in C. Student's T-test was performed between the negative control and each siRNA-treated sample. Percentage of the negative control (Lipo+) were also found. D) The duplicate supernatant was used for WB of exosomal markers alix and CD63 and also actin as a loading control.

PMA Activation Increases Exosomal Release in siRNA U1 Cells

PMA causes monocytes to differentiate into macrophages (called PMA activation), which also triggers latent HIV to actively begin transcription (Sen et al., 2015). Since macrophages are physiologically distinct from monocytes, I hypothesized that U1 cells after PMA activation would act differently than they would without PMA. Therefore, I examined exosomal release of U1 cells after PMA activation in the same manner in which I examined the supernatant before activation. Figure 9A shows the TAR RNA levels increase from 8×10^4 before PMA activation (Figure 8A) to 4×10^6 after PMA activation. TAR-gag RNA levels increase from 3.5×10^4 before PMA (Figure 8B) to 1×10^{6} after PMA (Figure 9B). Again, there is about a 2-fold difference between TAR and TAR-gag RNA. The genomic RNA increases from 5×10^4 before PMA activation (Figure 8C) to 1×10^7 after activation (Figure 9C). TAR RNA in siTSG101 and siCHMP6-treated cells increased significantly from the negative control cells in all three lengths of HIV RNA. Again, the largest decrease in viral RNA was found in the siCD63treated cells, which served as a positive control. The WB analysis shows an increase in CD63 and actin from non-PMA-treated cells to PMA-treated cells, as expected (Figure 9D). The CD63 level of all siRNA-treated cells is similar to the non-PMA-treated cells, indicating that exosomal release was reduced in the siRNA-treated cells. Altogether, the effect of the siRNA before PMA activation changed dramatically after the cells were activated.



Figure 9: Effect of PMA activation on exosomal HIV-1 RNA transcripts. The supernatant harvested 3 days after PMA activation (Figure 5A) was incubated with NT080/082 overnight at 4°C. Total RNA was then isolated and subjected to RT with primers specific for TAR, *gag*, and the 3' end. RT-qPCR was performed with TAR primers (A&B) and *gag* primers (C). Student's T-test was found between the negative control (Lipo+, PMA+) and each of the siRNA-treated samples. Percentage of the negative control was also found. D) WB analysis of the duplicate samples was also performed with exosomal markers Alix and CD63 and also actin as a loading control.

siRNA Knockdown ESCRT in CHME5/HIV Cells

To gain a better understanding of the effects of siRNA knockdown of ESCRT, I turned my focus to another cell line: CHME5/HIV. These cells are fetal microglia (Lucas et al., 2010; Zhang et al., 2015). The CHME5/HIV cells were transfected with the same siRNA as used for U1 cells, and after 4 days the cells and cell supernatant were harvested for analysis. **Figure 10** shows a statistically significant decrease in mRNA from the Lipofectamin®-treated control cells to the siEAP20 and siCD63-treated cells. These data indicate Lipofectamin® caused a decrease in mRNA transcription but a slight increase in protein translation in the cells. The WB shows an increase in CHMP6, CD63, and VPS4 after transfection with Lipofectamin® but then a decrease with the siRNA, suggesting the siRNA can counter the effect of Lipofectamin®. Together, the mRNA or protein levels decreased with siRNA treatment.



Figure 7: siRNA knockdown of ESCRT in CHME5/HIV. A) CHME5/HIV cells were seeded then transfected with siRNA the next day. After one day, the supernatant was removed and replaced with exosome-free DMEM. After 3 days the cells and supernatant were harvested. The cells were lysed and RNA was isolated and subjected to RT with Oligo-dT primers. RT-qPCR was performed with primers specific for the 6 targets of the siRNA. B) percentage of the negative control (Lipo-) were found. Student's T-test was performed between the Lipo+ cells and the Lipo- and siRNA-treated cells. WB analysis was also performed with the duplicate cells with antibodies specific for the 6 targets of the siRNA and actin as a loading control.

siRNA knockdown of ESCRT Effect on Exosome Release in CHME5/HIV Cells After establishing that siRNA was able to knockdown the mRNA and protein levels in CHME5/HIV cells, I then looked outside the cells to examine exosomal release. I did not use PMA since this different cell line does not require differentiation and activation, but I examined the cell supernatant in the same manner as with the U1 cells. Figure 11A and **Figure 11B** show the level of TAR RNA and TAR-gag RNA decreases significantly from the untreated control with siRNA specific for ESCRT II and ESCRT IV. Specifically, TAR RNA decreases from 3000 copies in the negative control exosomes to 1000 copies in the cells treated with siRNA specific to EAP45, EAP20, and VPS4A. TAR-gag decreased from 750 copies in the negative control to 200 copies in the cells treated with siRNA specific for EAP45 and EAP20 and to 100 copies in the exosomes from cells treated with siVPS4A. TAR and TAR-gag RNA levels significantly increased with siCHPM6. Specifically, TAR RNA increased to 7000 and TAR-gag increased to 1250 copies in the siCHMP6-treated cells. Genomic RNA was also analyzed, but the levels quantified were negligible and insignificant (Figure 11C), suggesting no full length RNA was being packaged into exosomes. It is important to note that gag RNA is present in these cells, but no gag protein could be made (Dull et al., 1998). The WB analysis in Figure 11D shows an increase in alix with siEAP45 and siEAP20. CD63 and actin increased when Lipofectamin® was added. Actin decreased with siTSG101 and ESCRT II specific siRNA but increased with siVPS4A and siCHMP6. Collectively, the ESCRT II knockdown consistently decreases exosomal release in both U1 and CHME5/HIV cells.



Figure 81: siRNA knockdown of exosomal HIV-1 RNA transcripts in CHME5/HIV. The cell supernatant harvested 4 days after transfection (Figure 7A) was incubated with NT080/082 overnight at 4°C. Total RNA was isolated and subjected to RT with primers specific for TAR, *gag*, and the 3' end. RT-qPCR was then performed with primers for TAR (A&B) and *gag* (C). Student's T-test was found between the negative control (Lipo-) and Lipo+ and each of the siRNA-treated samples. Percentage of the negative control was also found. WB analysis was performed on the duplicate supernatants with antibodies for exosomal markers alix and CD63 and also actin as a loading control.

DISCUSSION

Nanoparticles can indeed be used in a clinical setting to capture exosomes, which can then be used to quantify TAR being released from infected cells. Nanoparticles are easy to use, and using PCR analysis only takes a day or two to get results. **Figures 2-4** show this method being used to analyze different kinds of primary bodily fluids. The patient samples used were taken while the patients were currently on ART treatment. **Figure 3** shows that the vesicles captured by the nanoparticles are most likely exosomes and not virions. TAR RNA can be quantified to track latent infection. Serum showed higher numbers of HIV RNA than CVL, most likely because there are more latently infected cells in the bloodstream. Comparing the level of TAR and genomic RNA in the exosomes to the levels inside PBMC cells and to the level of provirus found, my data indicate that cells with a higher level of provirus will make more TAR and genomic RNA in the cell that can be packaged into exosomes. This makes logical sense; the more HIV-1 DNA, the more RNA will be made.

Since TAR RNA and genomic RNA are not representative of all HIV RNA transcripts, I used various reverse primers in an RT reaction to quantify the amount of each transcript. I expected to see an even downward slope, but instead I saw in 3 of the 4

cell lines the level of RNA quantified drops off beyond *gag* (**Figure 5**). Perhaps the polymerase was interrupted while transcribing the provirus, but since the amount of TAR-*gag* is so much greater than the amount of longer lengths of RNA this suggests that TAR-*gag* may be made for a purpose. This RNA is not translated in recipient uninfected cells and therefore is likely not poly-adenylated (**Figure 6**). Currently, the function and length of this TAR-*gag* transcript is unknown. Current research is focusing on exactly where this transcript ends within the *gag* sequence.in J1.1 exosomes.

Since exosomes containing TAR can cause negative effects on recipient cells, it is important clinically to decrease the amount of exosomes released from infected cells. Previous studies have used siRNA to knockdown ESCRT proteins to decrease the amount of virus released from infected cells (Langelier et al., 2006; Meng et al., 2015). Figure **7B-G** show the level of mRNA and protein of the knockdown proteins. It seems the siRNA did not destroy the mRNA of TSG101 or CHMP6 (Figure 7B,D), however the WB shows lower levels of TSG101 protein, and slightly lower levels of CHMP6 protein. The mRNA of the other genes does decrease significantly, but the protein levels remain the same or even increase, in the case of VPS25/EAP20, (Figure 7C,E-G). Typically, the level of mRNA does not coincide with the level of protein in a cell. The siRNA may have initially decreased the level of TSG101 and CHMP6 mRNA, but the cells transcribed more to replace what was lost in the timeframe of this experiment. Even though the mRNA decreased in the other genes, this does not mean the protein levels will also decrease, as the proteins themselves are not destroyed. Again, perhaps the time frame of this experiment should be altered to better understand the extent of siRNA transfection

reaction in the cells. The increase of TSG101 and CHMP6 mRNA with a decrease in protein suggest the cell was over-compensating for the loss of protein after siRNA treatment. The increase in EAP20 protein and a decrease in mRNA after siRNA treatment suggest a higher half-life of this protein.

Given the fact that mRNA levels of TSG101 and CHMP6 were not decreased in Figure 7B&D, one can explain how the levels of TAR RNA were also not decreased in Figure 8. However, this does not explain the increase in exosomal TAR RNA. Since TSG101 is involved in the viral release pathway (Goff et al., 2003; Langelier et al., 2006; Meng et al., 2015; Prescher et al., 2015; Votteler and Sundquist, 2013), a knockdown of TSG101 will decrease the amount of virions released from the cells but not necessarily decrease HIV RNA transcription. The increase in TAR in siTSG101-treated cells suggests these exosomes contain more concentrated TAR RNA.

When the cells were activated with PMA, I observed 100- to 1000-fold increase of the HIV RNA transcripts (**Figure 9**). When the cells differentiate into macrophages, they begin to actively produce the virus. I expected the percentage of TAR and TAR-gag to be similar before and after PMA activation, even though the actual quantification increases. However, TAR-gag levels were not knocked down in any of the siRNA-treated samples after PMA activation. This suggests TAR and TAR-gag are being packaged into exosomes by different pathways before and after PMA activation. The WB of exosomal markers shows there are more exosomes being released from the PMA activated cells, as expected, and less exosomal release in the siRNA-treated cells. Future experiments can focus on how PMA activation affects the ESCRT pathway. Since the levels of RNA do

not decrease as significantly after PMA activation, it would be interesting to see if PMA activation increases translation of the ESCRT proteins, and thus replacing the proteins lost after siRNA treatment.

It seems lipofectamine caused a decrease in mRNA transcription but a slight increase in protein translation in the cells (**Figure 10**). The siRNA can counter this effect of lipofectamine. Again, it was unclear if the siRNA was actually knocking down all the genes and protein. The primers for the PCR and the antibodies for the WB were difficult to use, and often the results were repeated multiple times before the levels found were believable. In the future, membrane proteins should be isolated before WB analysis of the ESCRT pathway to eliminate cytosol protein interference and potentially eliminate nonspecific binding of the antibodies. Also, most specific primers should be used for PCR analysis of the mRNA.

The WB analysis of the CHME5/HIV exosomes showed an increase of Alix in the siVPS4A and siCHMP6 cells (**Figure 11D**). Knocking down CHMP6 may cause an increase in exosomal release, or perhaps the siRNA for this gene was nonfunctional. However, the HIV-1 TAR and TAR-*gag* transcripts of the siVPS4A exosomes were decreased to 22% and 11%, respectively. The increase in Alix suggests an increase in exosomal release, but the decrease in RNA transcripts suggests less HIV-1 RNA is being packaged into the increase amount of exosomes. VPS4 is also involved in the HIV release pathway (Langelier et al., 2006), so disrupting this protein also disrupts viral release. TAR is not depleted completely to 0% because the virus can use non-ESCRT pathways to facilitate release (Meng et al., 2015). The virus may have been captured by

the nanoparticles along with exosomes since the nanoparticles are not 100% efficient and specific for exosomes alone. ESCRT II caused the most dramatic decrease in exosomal release, whereas siVPS4A and siCHMP6 may have increase exosomal production.

The TAR, TAR-*gag*, and genomic RNA levels decreased in ESCRT II knockdown in both the U1 and the CHME5/HIV cells. This study represents the use of siRNA in only two cell lines. In the future, the siRNA should be tested on multiple cell types and even with mice *in vivo*. Scrambled RNA should also be used for another control in future experiments. Since CHMP6 is only indirectly related to viral release (Langelier et al., 2006; Meng et al., 2015), future studies should also focus on discovering why the HIV RNA transcript levels were so high for these knockdown cells.

CONCLUSION

In conclusion, nanoparticles are an efficient method of capturing extracellular material. In a clinical setting, nanoparticles can assist in the monitoring of infection in latently-infected patients in various bodily fluids including serum, CVL, and CSF. Exosomes contain various lengths of HIV RNA transcripts, but mainly contain TAR and TAR-*gag* transcripts. The TAR-*gag* transcript is not translatable in recipient cells and is therefore considered a long non-coding RNA transcript. SiRNA can be used to knock down key proteins in the ESCRT pathway to prevent release of exosomes containing the HIV RNA transcripts. PMA activation increases release of HIV RNA transcripts in exosomes by 2 logs. TSG101 knockdown did not reduce exosome release and even increased RNA transcript packaging into exosomes in PMA activated cells. ESCRT II knockdown decreased exosomal release and seems to be the most important step in the ESCRT pathway for packaging of RNA and release of exosomes.

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