Post-Transcriptional Regulation of Astrocyte-Tissue Inhibitor of Metalloproteinases-1 (TIMP-1) in HIV-Associated Neurocognitive Disorders

By:

Mayuri V. Thete

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HIV-1 leads to several central nervous system (CNS) problems termed as HIV-associated neurocognitive disorders (HAND). Tissue inhibitor of metallopoteinases-1 (TIMP-1) / matrix metalloproteinase (MMP) imbalance has been observed in HAND and several other neuroinflammatory conditions. Astrocytes are major contributors to brain TIMPs, and they regulate TIMP / MMP balance [1, 2]. Differential regulation of TIMP-1 in acute versus chronic neuroinflammation is relevant to HAND neuropathogenesis and long-term neurodegeneration[3]. However, the underlying mechanisms are still being uncovered. Our previous work has shown the neuroprotective role of TIMP-1 *via* MMP-dependent and independent manners [4, 5]. In this study, we investigated regulation of astrocyte TIMP-1 in HAND. First, microarray analyses were performed to analyze micro RNA (miRNA) changes in IL-1β activated astrocytes. For further studies, TIMP-1 3' untranslated region (TIMP-1 3'UTR) cloned downstream of firefly luciferase and miRNA overexpression constructs were used to investigate miRNA-mediated TIMP-1 3'UTR post-transcriptional regulation. Firefly luciferase activity and endogenous astrocyte TIMP-1 levels were measured in parallel experiments. A total of 12 miRNAs were significantly increased and four were significantly decreased; seven of those were further confirmed by real-time PCR (RT^2-PCR). The most increased were miR 155, miR 146b and miR 29b, whereas, the most downregulated one was miR 518e. Overexpression of miR 155, miR 146b, miR 29b altered both firefly luciferase activity *via*

and endogenous astrocyte TIMP-1 levels in IL-1β and/or HIV-1 activated astrocytes. Comparable responses were obtained in luciferase activity changes and *de novo* TIMP-1 protein levels corroborating post-transcriptional regulation of astrocyte-TIMP-1. Thus, our data suggests that astrocyte-TIMP-1 is regulated post-transcriptionally by miRNAs during HAND. Given the emerging role of miRNAs in several neuroinflammatory and neurodegenerative diseases, our data uncover a novel mechanism of TIMP-1 regulation in astrocytes that may have significant impact on future studies on MMP/TIMP balance in HIV-associated neuroinflammation.

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List of Publications and Abstracts

List of Publications (In Preparation)

1. Thete M, Ghorpade A. Astrocyte-Tissue Inhibitor of Metalloproteinases-1 (TIMP-1) is regulated post-transcriptionally in HIV-Associated Neurocognitive Disorders by miR 155 and 146b. (In preparation for PLOS one)

Abstracts and Poster Presentations

1. **Thete M**, Ghorpade A. Post-transcriptional Regulation of Astrocyte-Tissue Inhibitor of Metalloproteinases-1 (TIMP-1) In HIV-1-Associated Neurocognitive Disorders. Research Appreciation Day, University of North Texas Health Science Center. Published online: 03/2015.

2. Thete M, Ghorpade A. Post-transcriptional Regulation of Astrocyte-Tissue Inhibitor of Metalloproteinases-1 (TIMP-1) In HIV-1-Associated Neurocognitive Disorders. Society on Neuroimmune Pharmacology.

3. Thete M, Ghorpade A. Post-transcriptional Regulation of Astrocyte-Tissue Inhibitor of Metalloproteinases-1 (TIMP-1) with IL-1β/HIV-1 activated miRNAs. Texas Center For Health Disparities Conference, University of North Texas Health Science Center. Published online: 05/2015. (Received second place poster award)

Table Of Contents

Page

Table

II. Materials And Methods

List of Illustrations

Figure

Table

III. Results

List Of Illustrations

Figure

Table

IV. Overall Discussion

List Of Illustrations

Figure

Abbreviations

AIDS, Acquired immunodeficiency syndrome; **ANOVA,** Analysis of variance; **ART**, Anti-retroviral therapy; **BBB,** Blood brain barrier; **BLAST**; Basic local alignment search tool **CNS,** Central nervous system; **CSF**, Cerebral spinal fluid; **DAPI,** 4',6-diamidino-2-phenylindole; **DMSO**: Dimethyl sulfoxide; **DNA**: Deoxyribonucleic acid. **ECM,** Extracellular matrix; **GFAP,** Glial fibrillary acid protein; HAD, HIV-associated demented, **HAND,** HIV-associated neurocognitive disorders; **HIV-1,** Human immunodeficiency virus-1; **IL,** Interleukin; LPS, Lipopolysaccharide; **miRNA**: micro-RNA; **MMP,** Matrix metalloproteinases; **mRNA**: messenger ribonucleic acid; **MTT,** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **NF-κB,** Nuclear factor-kappaB; **PBS,** Phosphate-buffered saline; **PTC**, Papillary thyroid cancer **TIMP**, Tissue inhibitor of metalloproteinases; **UTR**, Untranslated region.

Chapter 1

Introduction

Introduction

List of Illustrations

Figure

Table

Introduction

1.1 Human Immunodeficiency Virus (HIV)-1

The societal burden of the human immunodeficiency virus or HIV-1 infections, a retrovirus that leads to acquired immunodeficiency syndrome (AIDS) has prevailed over the past several decades. HIV-1 infection leads to suppression of immune system allowing several opportunistic infections to thrive in the body. HIV-1 enters the brain *via* infected monocytes. In the thirty years since the discovery of HIV, an estimated 75 million people have become infected and almost half have died. In 2010, around 33 million HIV infections were reported. In 2013, 1.8 million new HIV infections, 29.2 million prevalent HIV cases and 1.3 million deaths were reported. Of those with HIV+, 40-70% experience central nervous system (CNS) problems, together called HIV-associated neurocognitive disorders (HAND). The most severe form is known as HIV-associated dementia (HAD) [6-8]. HAND is characterized by motor, cognitive, as well as, behavioral abnormalities [9].

1.2 HIV-Associated Neurocognitive Disorders (HAND)

Since effective antiretroviral therapy (ART), the pattern of neurological complications associated with HIV-1 CNS infection is changing. Patients are living longer, have lower viral loads and low level of chronic CNS inflammation. HIV travels in the body and can infect cells by binding of the viral gp120 to the CD4 receptor on the surface of the cell. Primarily, cells expressing these receptors are T-lymphocytes and macrophages, which differentiate from blood monocytes. Following interaction between CD4 and gp120, with the help of gp41, fusion occurs between the cellular membrane and the viral envelope. Enzymes that are packaged in the virus, such as reverse transcriptase (RT) and integrase, which are required immediately after internalizing infection, are discharged in

the host cell cytoplasm after uncoating of the capsid. Double-stranded DNA is then transcribed from the single-stranded RNA of the virus leading to the generation of the provirus after nuclear transport and integration. Further transcription allows viral RNA synthesis, which is exported outside the nucleus with the use of transcription machinery of the host cell, ultimately leading to generation of viral proteins and assembly of virions. Infected monocytes (and maybe CD4 T-lymphocytes) act as viral reservoirs/vehicles, and transport the virus across the blood brain barrier (BBB). As per one well-accepted model, the blood brain barrier is damaged due to the migration of infected monocytes with the disruption of adhesion molecules. As monocytes enter the CNS, they differentiate into macrophages. This causes release of cytokines and chemokines, which lead to further blood brain barrier impairment [10]. Other brain cells like astrocytes are infected in non-productive manner (**Figure 1.2**). *In vivo* studies identified that, during infection, HIV-1 proteins were present in the neuronal tissues [7-9].

In the case of HIV, dysfunction of the caudate and connected prefrontal regions of the brain was also reported. This dysfunction leads to cognitive impairment [11]. In the post-ART era, three different levels of cognitive impairment are recognized and forms three different levels of neurocognitive changes: ANI, asymptomatic neurocognitive impairment; MND, mild neurocognitive disorder and the most severe form, HAD, HIV-associated dementia. All together, these three forms, collectively known as HIV-associated neurocognitive disorders (HAND, as shown in **Figure 1.1)**.

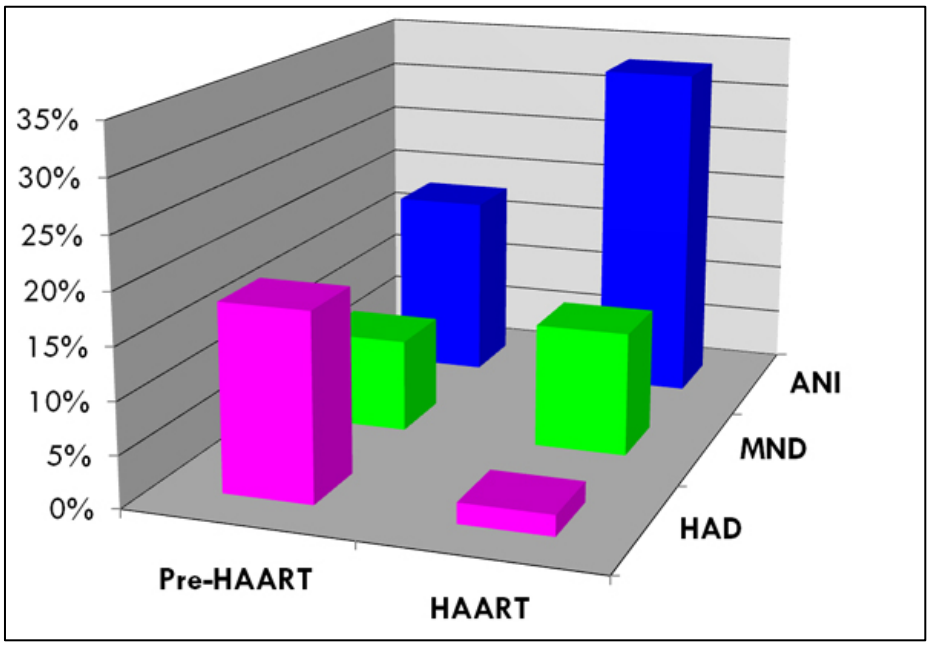

Figure 1.1: HAND in the pre- and post-ART eras**.** HAND subtypes and their incidences in the post highly active antiretroviral therapy (HAART) era, now commonly referred to as anti-retroviral therapy (ART).[12]

(Link:http://www.apa.org/pi/aids/resources/exchange/2013/01/pharmacologic-interventions.aspx)

1.3 Role of astrocytes in CNS

Astrocytes are the most abundant cells in the brain and are intricately involved in CNS homeostasis. Astrocytes facilitate neuronal development and repair and are involved in remodeling the extracellular matrix (ECM). Astrocytes participate both at regulating the neurovascular junctions at the blood brain barrier and are critical components of the tripartite synapse in brain parenchyma. As major producers of tissue inhibitor of metalloproteinases (TIMP) in the brain, they regulate the delicate matrix metalloproteinases (MMP) / TIMP balance, thus regulating ECM remodeling [4, 8, 13].

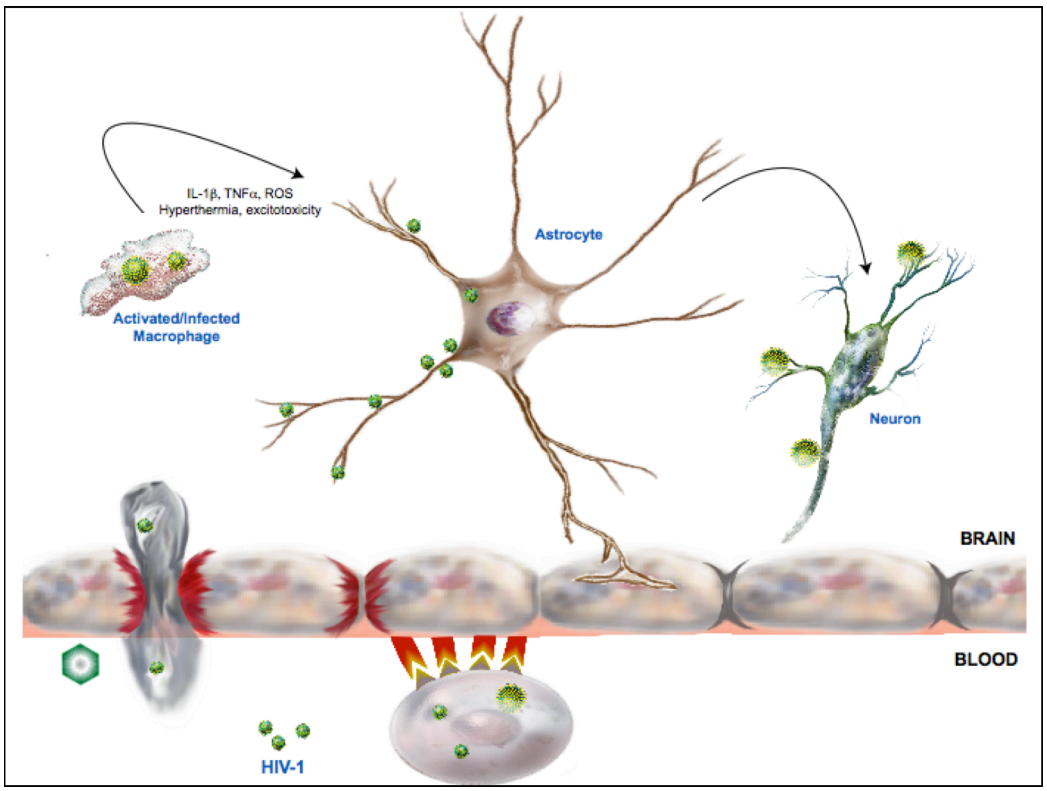

Figure 1.2: Proposed mechanisms of HIV entering the CNS leading to HAND. HIV enters the brain with the help of infected monocytes thus crossing the blood brain barrier. The astrocytes further activated via the infected microglia and contribute to neuropathogenesis.

1.4 Astrocytes and the MMP/TIMP Balance

Astrocytes are vital cells of the CNS and their inflammatory responses play a significant role in HAND neuropathogenesis. Matrix metalloproteinases participate in the degradation and turnover of ECM and are important for several physiological processes. Hence, regulation of ECM turnover is important. A class of inhibitors, TIMP-1 regulates MMP activity in the brain extracellular milieu. Thus, TIMPs are regulators of MMPs [1, 2]. It is important to maintain this TIMP-1/MMP balance for CNS homeostasis. This balance is necessary to be maintained for several physiological processes like cell growth, migration, death *etc*. However, in various neuroinflammatory conditions including HAND, TIMP-1/MMP imbalance has been observed [5]. There are four types of TIMPs, TIMP-1, TIMP-2, TIMP-3 and TIMP-4. TIMPs have a smaller C and larger N-terminal domains, bound by disulfide bonds. Inhibition of MMP activity takes place due to the non-covalent binding at the zinc-binding site between the N-terminal and the MMP substrate molecules [1, 14].

There is differential regulation of TIMP-1 with acute *vs* chronic neuroinflammation. Cytokines are known to control MMP/TIMP-1 expression [15]. In the case of acute activation of brain cells by HAND-relevant stimuli, increase in TIMP-1 levels was observed, which may reflect innate repair mechanisms. However, in case of chronic activation, decreased TIMP-1 levels were reported. In chronic condition, TIMP-1 downregulation leads to loss of neuroprotection both *via* loss of MMP activity regulation and loss of direct neuroprotection. TIMP-1 levels were shown to be downregulated in HAD patients as compared to their age-matched controls [3, 16]. Underlying

mechanisms of this downregulation are not clearly defined. While a few reports documented mechanisms that may participate in this duality, notably, extracellular regulation *via* anti-inflammatory mediators, such as TGF-β and regulation of TIMP-1 mRNA stability, we have yet to uncover every detail involved in this process. It is crucial to dissect the complete picture as it can lead to development of effective therapeutic strategies [13].

TIMPs	Function/Roles
TIMP-1	Made up of 184 amino acids, glycoprotein, soluble, stimulated by interleukin (IL-1β), IL-6, phorbol esters, *etc* [1].
TIMP-2	Made up of 194 amino acids, unglycosylated, insoluble, its expression is constitutive [1, 14].
TIMP-3	Insoluble form, unglycosylated, separate from the transformed cells [14].
TIMP-4	Recently identified, overexpression is reported in human heart [14].

Table 1.1: Description of TIMP family: TIMP-1, TIMP-2, TIMP-3 and TIMP-4

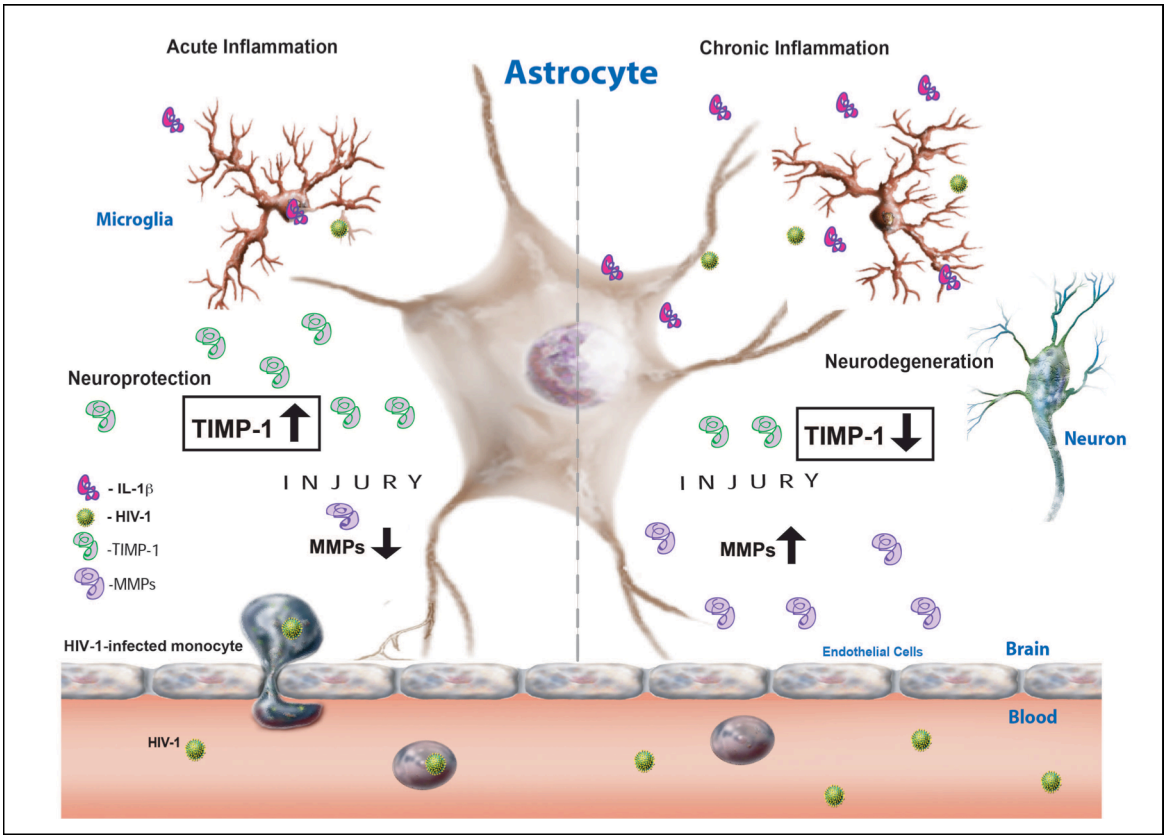

Figure 1.3: Regulation of TIMP-1 levels in acute *vs* chronic condition.

With acute inflammation TIMP-1 levels are elevated which reflects attempted repair response and plays a role in neuroprotection, both *via* regulation of MMP activity and direct neuroprotection. However in case of chronic activation these neuroprotective functions mediated by TIMP-1 are lost with declining levels. (Adapted from Gardner *et al*, 2006)

1.5 Role of miRNAs in post-transcriptional regulation

MicroRNAs (miRNA) are approximately 22 nucleotides long, small, non-coding RNA molecules [17]. In 2004, it was first discovered that miRNAs regulate immune response. miRNAs either repress mRNA translation or reduce mRNA stability [18]. They function in regulation of gene expression and silencing. Gene expression in any given cell can be controlled at any one of seven critical steps (**Figure 1.4**, adapted from Principles of Biochemistry, Lehninger, 2010). Post-transcriptional regulation of gene expression is mediated in part by the RNA interference pathway (**Figure 1.5**). Approximately 550 miRNAs regulate mRNA translation *via* the RNA interference pathway [19]. miRNAs are first transcribed as long RNA transcript called primary miRNA, further cleaved by drosha into shorter precursor miRNA. Pre-miRNAs are exported from the nucleus *via* exportin 5 and are cleaved in the cytoplasm by dicer and RNAase 3 enzymes and then loaded on the RISC complex. This leads to mRNA blocking (miRNA) or mRNA cleavage. Both ultimately lead to translational repression. Thus, the miRNA recognition elements within the 3' untranslated region (3'UTR) of target genes can either repress or block mRNA translation by either imperfect or perfect binding. We investigated how miRNAs regulated the 3'UTR region of TIMP-1 *via* the RNA interference pathway (**Figure 1.6**).

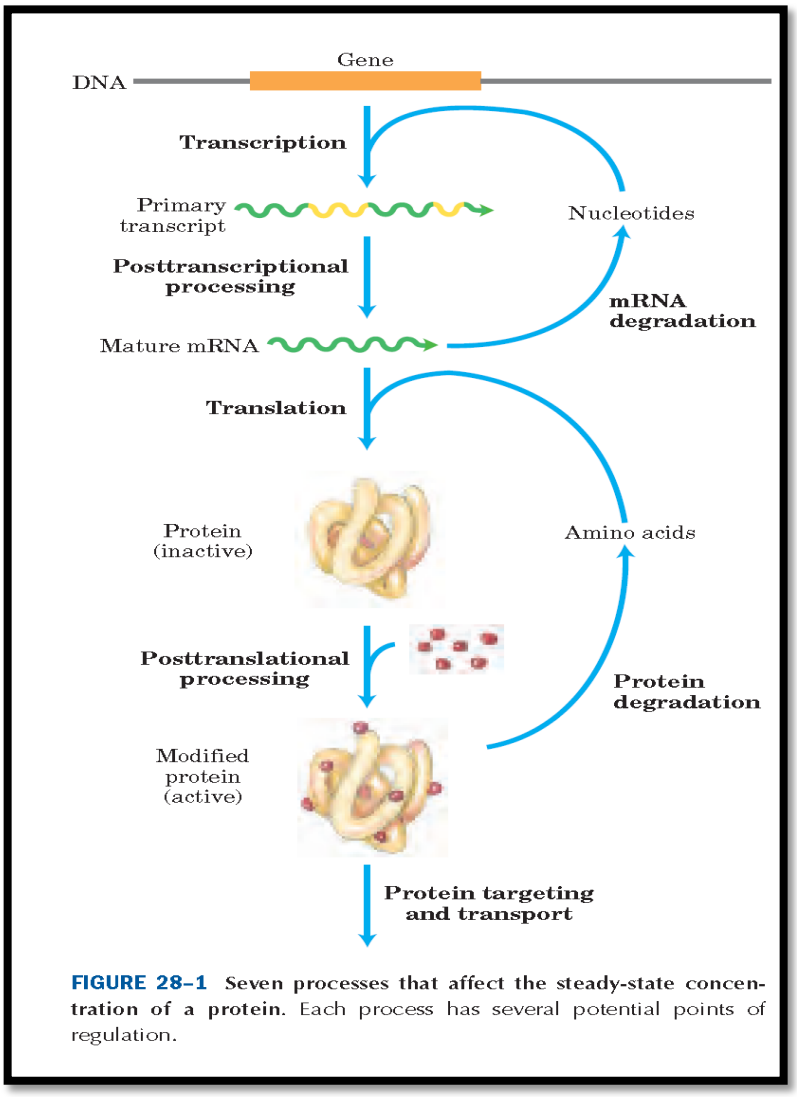

FIGURE 28–1 Seven processes that affect the steady-state concentration of a protein. Each process has several potential points of regulation.

Figure 1.4 Seven key regulatory steps in protein formation. miRNAs participate in the RNA interference pathway *via* post-transcriptional processing. (adapted from Principles of Biochemistry, Lehninger, 2010)

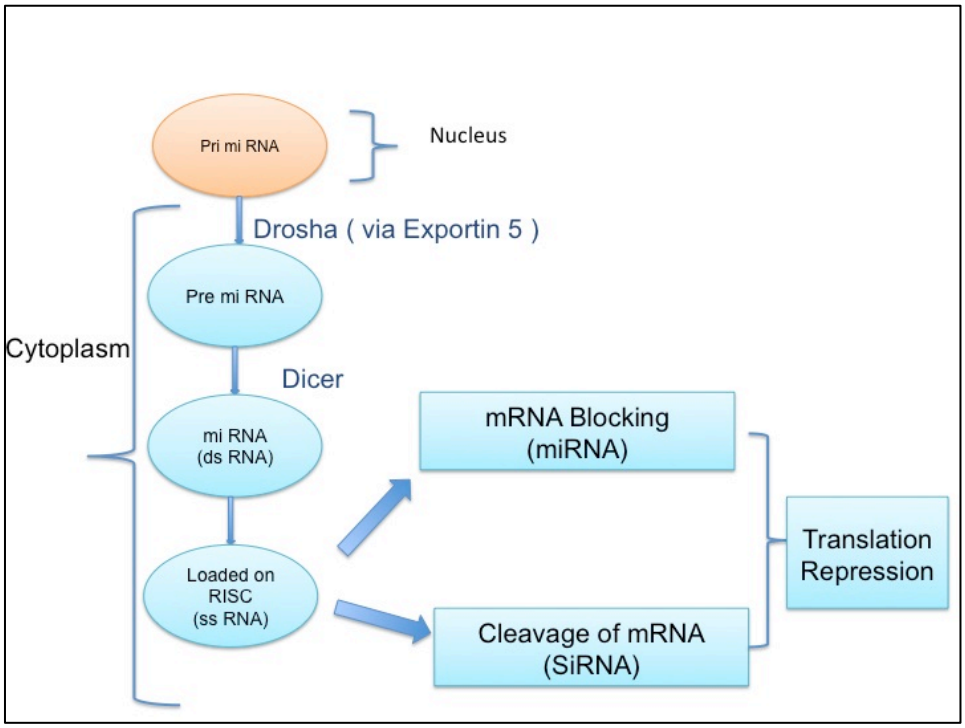

Figure 1.5 RNA Interference Pathway

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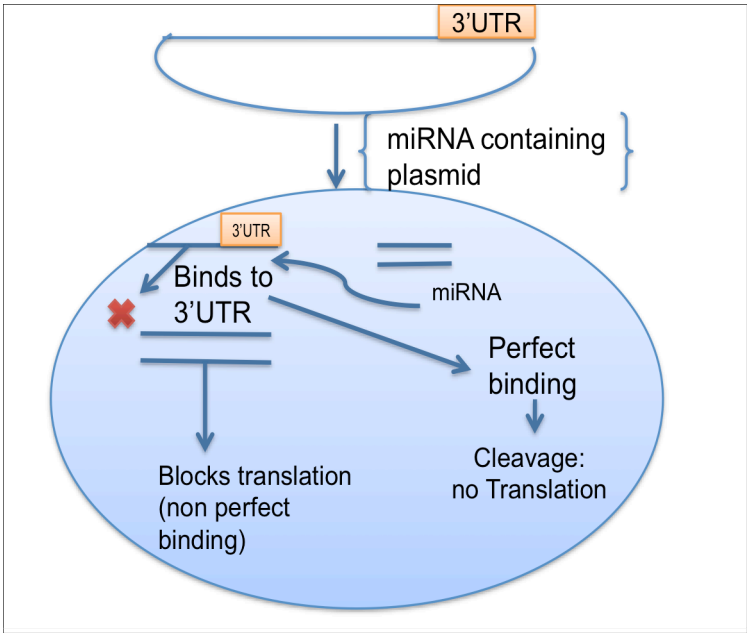


Figure 1.6 Regulation of TIMP-1 3'UTR *via* miRNAs

1.6 Objectives Of The Present Study

Our long-term goal is to investigate mechanisms involved in astrocyte-TIMP-1 regulation. **In this study, our objective was to delineate the role of miRNAs in post-transcriptional regulation.** As discussed earlier, miRNAs participate in the RNA interference pathway *via* post-transcriptional processing. miRNA by binding to the 3'UTR of target genes can stop or reduce translation by imperfect binding [18]. Our overarching hypothesis is that "**astrocyte-TIMP-1 expression in neuroinflammation is controlled, in part, *via* miRNA-dependent post-transcriptional regulation**." To address this hypothesis we propose the following specific aims:

Aim 1: To elucidate which specific miRNAs likely play a role in regulating TIMP-1 3'UTR in the context to HAND. Astrocyte miRNA changes were analyzed by microarray analysis. Upregulated, downregulated and unchanged miRNA targets were confirmed using RT^2-PCR in multiple astrocyte donors. To identify which miRNAs among the altered targets are most likely to regulate TIMP-1, BLAST searches using the TIMP-1 3'UTR sequences for matched putative binding sites were performed. Expression levels of 16 miRNAs were significantly changed, twelve were increased and four were decreased compared to untreated controls. These miRNAs were validated using RT^2-PCR. miRNAs with consistent trends in multiple donors were used for further analyses.

Aim 2: To evaluate if TIMP-1 3'UTR-regulated reporter gene expression is subject to post-transciptional regulation *via* specific miRNAs. *First*, we used reporter assays in such that the luciferase reporter is controlled by TIMP-1 3'UTR. HAND-relevant stimuli were used to activate astrocytes and mimic disease scenario. Overexpression constructs for altered miRNAs were employed in conjunction with

reporter assays. Luciferase activity will be measured by comparing it to appropriate controls. To confirm if the change in reporter gene activity is reflected at the protein level for TIMP-1, endogenous TIMP-1 levels were measured in primary human astrocytes with HAND-relevant stimuli in both acute and chronic conditions. Astrocytes were treated with HIV-1_{JRFL} to ensure the mechanism is similar to that during diseased condition. Specificity of responses were assessed using both upregulated and downregulated targets. Unchanged miRNAs were identified for nonspecific changes.

Aim 3: To confirm the role of miRNAs in astrocyte neuroinflammatory responses. To confirm that the changes obtained in TIMP-1 levels are due to specific miRNAs, inhibitors for miRNA were used in the setting of HAND-relevant stimuli and effects on TIMP-1 protein were evaluated. In parallel experiments, responses of astrocytes to IL-1β, a known inducer of the reactive phenotype of the astrocytes were evaluated using immunocytochemistry. These studies confirmed the post-trancriptional regulation of TIMP-1 expression *via* miRNAs.

Expected Outcomes, Potential Pitfalls and Alternatives: Primary human fetal astrocytes will be used for all experiments as cellular models closest to human disease. While it is not certain that all fetal astrocytes will behave exactly as adult astrocytes, to address this, first, multiple astrocyte donors across a range of gestational ages will be used. Consistent trends obtained in these cells reflect a trend that is likely preserved over time and age. In future studies, primary human adult brain tissues from patients will be used to obtain final *in vivo* confirmation of the results obtained *in vitro*. We expect to uncover altered patterns of miRNA levels in astrocytes activated with inflammatory stimuli and HIV-1 viral particles. There is a possibility that the miRNA that are upregulated in microarray might not show consistent trends of expression in RT^2-PCR. In that case, we

can use miRNA prediction software to validate whether that miRNA should be used for further testing. The luciferase activity in the same donor might not show same trend in case of endogenous TIMP-1 levels. The donor samples can be tested for CCL2/CXCL8 response in order to study if donor has appropriate cytokine response. Donor variations could be one of the reasons for such difference in results. Trying the experiment with a different donor might give consistent results by alleviating donor-specific problems. In knockdown studies, the results obtained should be ideally reversed as compared to the overexpression studies. This might not necessarily replicate. In such case the inhibitor specificity should validated. Reactive responses of astrocytes may change with the treatments and transfection. To avoid any toxicity (if present) to the cells, lower concentrations of plasmids or reagents might be tested and optimized in order to reduce the toxicity.

Significance and Overall Impact: Taken together, in this study, we expected to identify miRNAs that are altered in astrocytes in relationship to inflammation and HIV-1, confirm it in multiple biological replicates of human astrocytes, demonstrate post-transcriptional control using reporter gene assays that correspond to changes in *de novo* TIMP-1 protein levels in astrocytes. Lastly, miRNA inhibitors were employed to confirm the specificity of these outcomes. In summary, this study will provide important insight into the miRNA-based regulation of astrocyte-TIMP-1 at the post-transcriptional level. We anticipate that these results will not only prove important for miRNA inhihibitor based therapeutic interventions for HAND, but will have significant impact on the field of glial biology and help deciphering neuroinflammatory responses of glia neurodegeneration

Chapter 2

Materials And Methods

Materials And Methods

List of Illustrations

Figure

Table

2.2 Materials And Methods

2.1 Materials

A. Plasmid constructs:

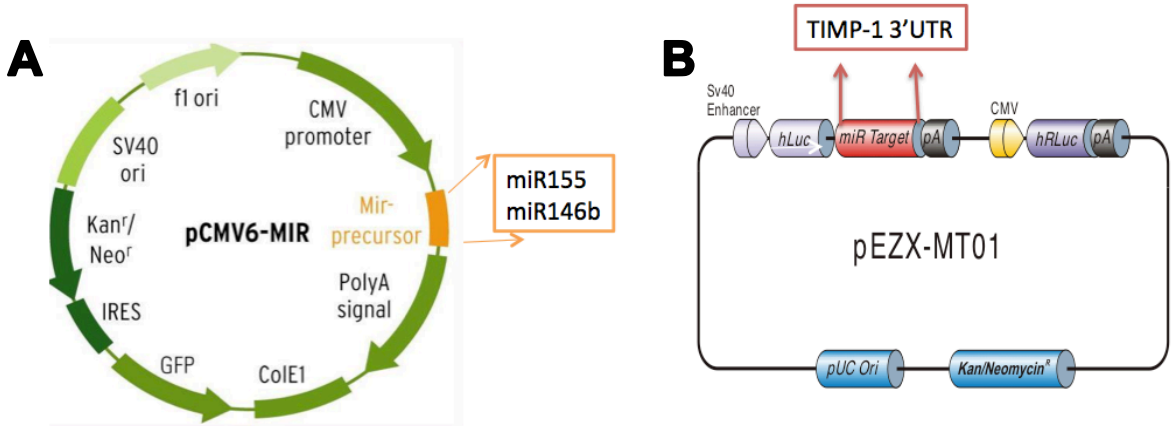


Figure 2.1 (**A**) The plasmid backbone pCMV (Origene) with CMV promoter-driven miR 155 and 146b, 29b, 518e, 23a, 1226 and 708 cloned for overexpression was used for proposed studies. Insertion location for miR 155 and 146b, 29b, 518e, 23a, 1226 and 708 is illustrated as examples (**B**) TIMP-1 3'UTR vector (Genecopoeia): SV40-driven firefly luciferase expression is regulated by TIMP-1 3'UTR. CMV-driven, *Renilla* luciferase activity is intended to serve as the internal control.

B. Viral Stocks:

HIV-1_{JRFL} was used for the activation of astrocytes. This strain is derived from the frontal lobe of the brain. This viral strain was propagated in monocyte-derived macrophages. Peripheral blood monocytes were isolated from HIV and Hepatitis B negative donors with the help of local Red Cross facilities. Isolated monocytes were allowed to differentiate culture for 7 days and then infected with HIV. Cell

supernatant samples were collected over a period of three to four weeks and virus preparations were quantified by analysis of reverse transcriptase activity and HIV-1 p24 levels by commercially available ELISA tests.

C. Plasmids Used:

miR 155, miR 146b, miR 29b, miR 518e and miR 708 overexpression plasmids purchased from Origene were used in these studies. Control 3'UTR and TIMP-1 3'UTR obtained from Genocopeia. Plasmid concentration for transfections was 0.5 µg per 1.6 million cells in each reaction.

D. Astrocyte Media:

Astrocytes culture media (ASM) was used for culturing the cells as well for plating. ASM consisted of DMEM-/F12 media (Life technologies) supplemented with fetal bovine serum (FBS) (10%), 1% penicillin-streptomycin-neomycin (PSN) and 1% Fungizone. Media was stored at 4°C. Prior to every experiment media was warmed in a water bath maintained at 37°C.

2.2 Methods

A. Isolation and Cultivation of Primary Human Astrocytes: Human fetal brain tissues were used to isolate astrocytes under the guidelines of University of Washinghton, UNTHSC and the National Institute of Health (NIH). First, tissues were homogenized and dissociated followed by three washes in HBSS and finally suspended in ASM as described above. Primary cultures were plated in 150 cm^2 flasks and passaged once a week with Trypsin-EDTA at least two times prior to using for experimentation. For individual experiments, cells were plated in either

48 well or 96 well plates or flasks as per the requirement of the experimental design.

B. Transfection: Human astrocytes in suspension were re-suspended in the media and thoroughly mixed in order to avoid clumps and obtain a single cell suspension. These cells once uniformly mixed were then divided into tubes as per the number of transfection. For each transfection 1.6 million cells were used. For one transfection /1.6 million cells, 20 μl (16.4 μl nucleofector + 3.4 μl supplement) of nucleofection reagent (amaxa's nucleofection kit) and 0.5 μg plasmid DNA was used. In case of co-transfection with two plasmids both plasmids were used a concentration of 0.5 μg per 1.6 million cells. After addition of the nucleofection reagents and the plasmid DNA, the cell suspension was mixed. 20 μl was added onto each well of the shuttle plate (96 well plate provided by amaxa). The program code was followed as per the manufacturer's instructions (program: CLL, 133, Neuron basic, standard). The shuttle plate was loaded in the Amaxa nucleofector machine and with help of the program intended for astrocytes for electroporation. Around 100 μl of media was added on each well and was kept for 10 min to allow the cells to recover from the shock of transfection. Later, from each well the transfected cells were transferred into a new tube and resuspended in the media. These cells were then plated on a 48 well plate (0.15 million cells/well).

C. Astrocyte Activation. Following transfection cells were allowed to recover for 24 h. The media from each well was aspirated. And astrocytes were treated with IL-1β (20 ng/ml) and/or HIV-1_{JRFL} (10 ng/ml), resuspended in media and 400 μl of the mixed reagents was used for each well. Astrocytes were treated for 24 h.

D. Metabolic Activity Determination: MTT (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide was added to the media in the ratio of (1:2). Cell supernatant samples were collected for ELISA in a 96-well plate (200 μl/well) and were stored at -20°C. The remaining media was aspirated and MTT and ASM solution (1:2) was added 300 μl/well. These 48 well plates were then incubated for approximately 30-45 min. After that, the MTT solution was aspirated and 250 μl of Dimethyl sulfoxide (DMSO) was added in each well in order to dissolve the formazan crystals. Plates were placed on the shaker at room temperature for 10 min. This solution was transferred on the clear assay plates and readings were taken using the microplate reader at 490 nm. Ratios to mock controls (just transfected cell with transfection reagents and not with any of the plasmids) were calculated for normalization of enzyme linked immune sorbent assay (ELISA) measurements.

E. Microarray: Primary human astrocytes were cultured in 75 cm^2 flasks for 24 h and treated with IL-1β for 8 h. Total RNA was isolated using Qiagen miRNA isolation kit from astrocytes as per manufacturer's instructions (Qiagen).

F. Luciferase activity: Astrocyte media was first aspirated and the cells were lysed using 65ul/well of lysis buffer (1X, luc-pair Firefly/*Renilla* luciferase activity kit according to manufacturer's directions Genecopia). The plate was placed on the shaker for 10-15 min. Next, lysates (20 μl/well) were transferred on an opaque (white bottom 96 well plate) plate, suitable for luminometer (GloMax multi+ luminometer, Promega). Firefly solution (firefly substrate + firefly reagent) and Renilla solution 2 (renilla substrate + renilla reagent) is prepared (vortexed) and

incubated for around 10 mins at 37 degrees. Then, 100ul/well of Firefly solution one was added to all wells using a multichannel pipette. Gently the solution is mixed with the lysate and plate is tapped. The plate was then incubated at 37 degrees for 5 mins and reading was taken on the luminometer (integration 2 sec, program: bright glow). Next, Renilla solution two was added 100/well and mixed gently using pipette. After 5 min incubation Renilla luciferase activity was measured. These readings could be then viewed using the Promega's software (view results).

G: TIMP-1 Quantification*:* TIMP-1 levels were measured using TIMP-ELISA kit (R&D Systems, Minneapolis, MN). The supernatants from transfected astrocytes (treated/untreated) were employed. Standards were made following serial dilution as per the protocol (starting with 200 μl of standard and 200 μl diluent), blank was also incorporated on the standard curve. The samples were then diluted as per requirement (so that, they fit in the standard curve). Diluent of 100ul/well was added in all the columns. Standards were loaded in duplicates and samples were loaded in triplicates/quadruplets on the columns. The plate was then kept to vortex on the shaker for 2 hr. Further, the plate was washed thrice using wash buffer and conjugate (200ul/well) was added. Then the plate was allowed to vortex for 1 hour, washing step was repeated and substrate [substrate A + substrate B, ratio (1:1)] was added 200ul/well. Washing step was repeated after the incubation time and 50ul/well of stop solution was added to stop the reaction. The readings were then taken within 15 min on the microplate reader.

H: miRNA Isolation and Real-time Polymerase Chain Reaction (RT^2-PCR):

T-25 flasks were used and around 4 milion cells per flask were plated. After recovery and treatment, these flasks were treated with trypsin (1ml per flask) and incubated for 5 min. After that, 4-5ml of media was added to each flask and mixed well using pipette. The cells along with the media were transferred in to a new tube and were centrifuged at 1500 rpm for 10 min. Supernatant obtained was discarded carefully and the pellet was resuspended in 700ul trizol reagent (Qiagen) The trizol extracts could be stored at -80 degrees. RNA was isolated from these trizol extracts using Qiagen's miRNA easy kit (Catalog no. 217004). And the concentration of the RNA was measured using nanodrop. Followed by conversion to cDNA as depicted in **Figure 2.2.** StepOne plus was used for RT^2-PCR assays and applied Biosystems miRNA expression kits were used. In the setting, 2 hours (20ul) reaction was run with the Taqman reagents. And the default set up cycle was followed for 40 cycles. Atleast triplicates of each of the samples were used for analyses. The normalizing control used was SnU6 (Small nuclear ribonucleic acid), which is around 150 nucleotides long. RT^2-PCR reaction was allowed to run for two hours. The data obtained was exported and analysis was done of each target in comparison to the control SnU6.

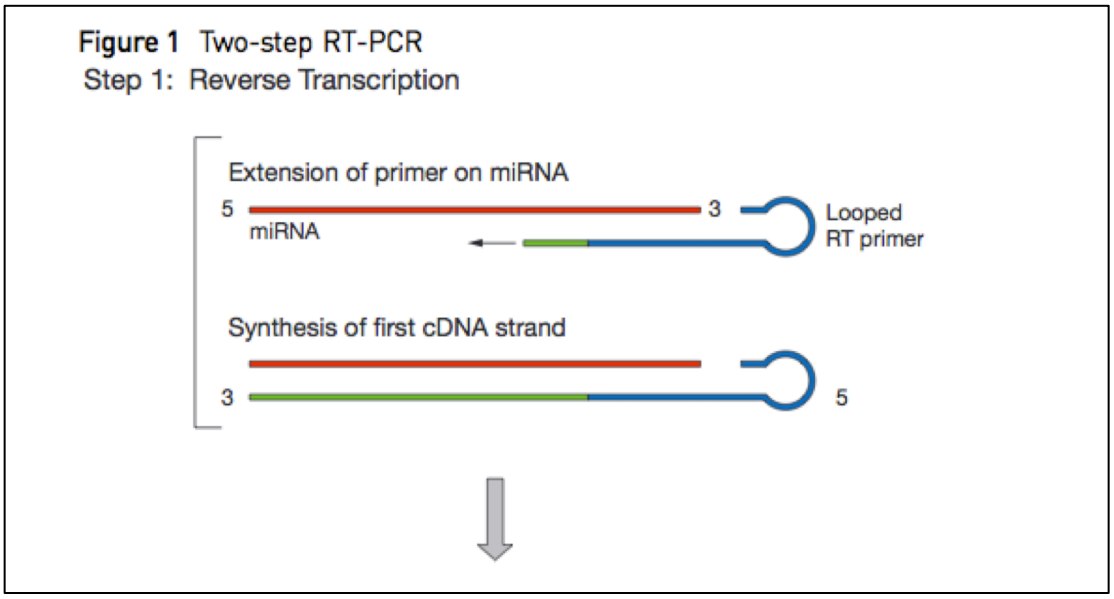
I: miRNA inhibitors: MiRidian miRNA inhibitors (Dharmacon miR 155/146b/29b/518e) were used at 100 nM/ 1.6 million cells and 20ul transfection reagent, specific to each miRNA. Along with these, a negative control inhibitor was used. Inhibitors were transfected into astrocytes using the Amaxa nucleofection kit (Lonza). Transfected astrocytes were stimulated using HAND-

relevant stimuli and after 24h, their endogenous TIMP-1 levels were measured.

J: Immunocytochemistry: After 24 h IL-1β stimulation, transfected astrocytes were fixed using 4% paraformaldehyde following their blocking for half an hour. Later cells were stained using with 250ul/well of primary antibody (GFAP rabbit/ chicken) in the ratio of (1:700) with the blocking buffer. And were allowed to sit at room temperature for about 2 hr. The plate was then washed thrice using 1X PBS. Later, 250ul/well of secondary antibody (Alexa -594, red) in the ratio of (1:500) was added on the cell. Again the plate was then washed thrice using 1X PBS. Lastly, cells were treated with around 200-250ul DAPI (blue), a nuclear label in the ratio of (1:1000). Imaging was carried out using 20X magnification.

K: Statistical analysis: One way one-way analysis of variance ANNOVA was used along with Tukey's post hoc test with help of a software, Graphpad Prism 6.0 (GraphPad Software, La Jolla, CA). Multiple comparisons could be made using this test and the significance was adjusted to $p<0.05$.

A



B

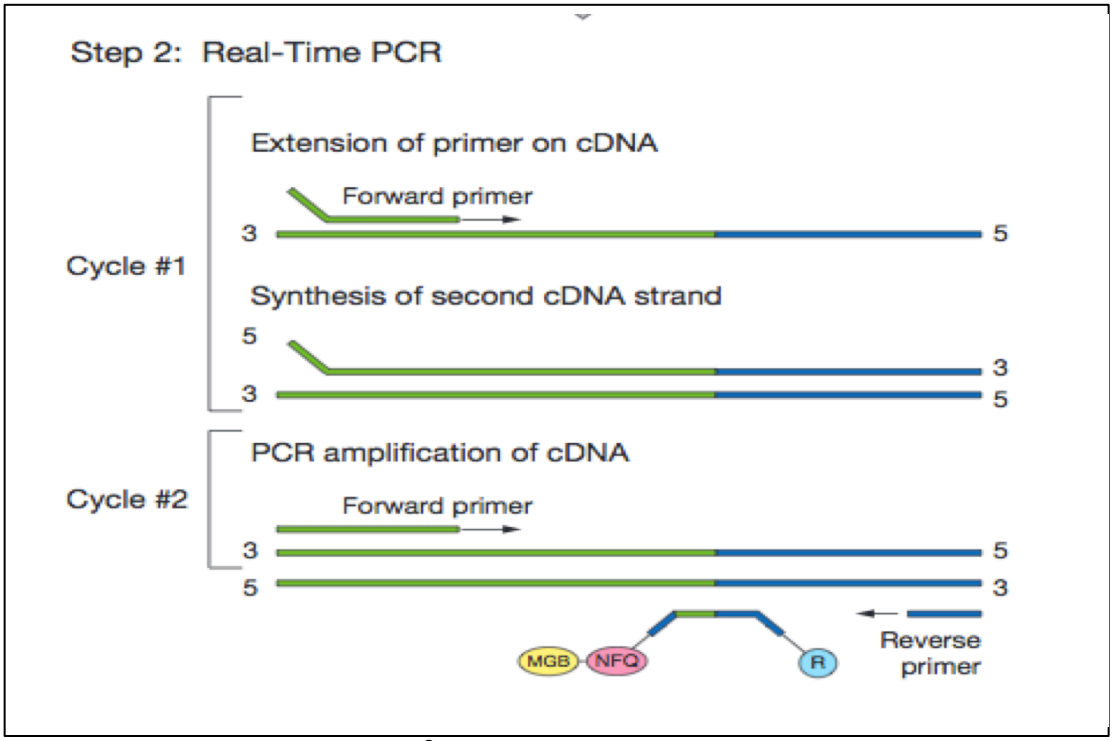


Figure 2.2 Two step RT^2-PCR of miRNAs.(Dharmacon, product sheet)

2.3 Experimental Design: In brief, miRNAs were screened using microarray analysis after treating with IL-1β. Further miRNA expression was confirmed by RT^2-PCR. Luciferase activity and endogenous TIMP-1 levels were measured using HAND relevant stimuli or HIV-1_{JRFL} to study the effect of miRNA on astrocyte TIMP-1 regulation. Also the morphological phenotypes of transfected and activated astrocytes were evaluated using immunocytochemistry. These experiments were conducted in multiple primary human astrocyte donors, in multiple replicates per treatment condition. An overview of the experimental design is depicted below.

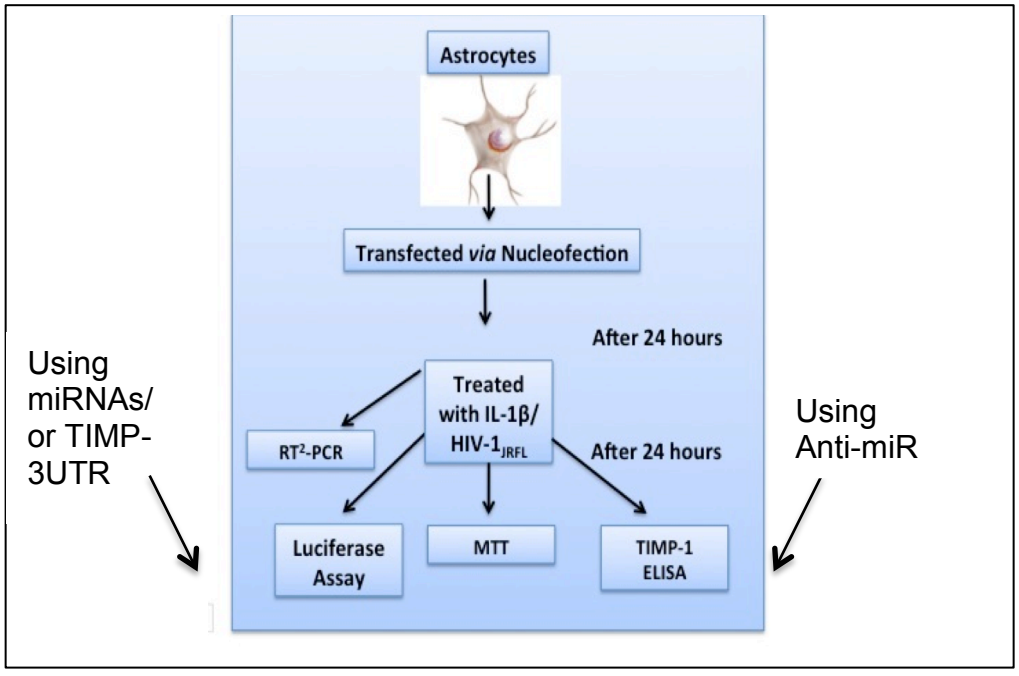


Figure 2.3 Experimental Design: Briefly, the steps depicting how these experiments were conducted following treatment with HAND-relevant stimuli.

Chapter 3

Results

Results

List Of Illustrations

Figure

Table

Figures

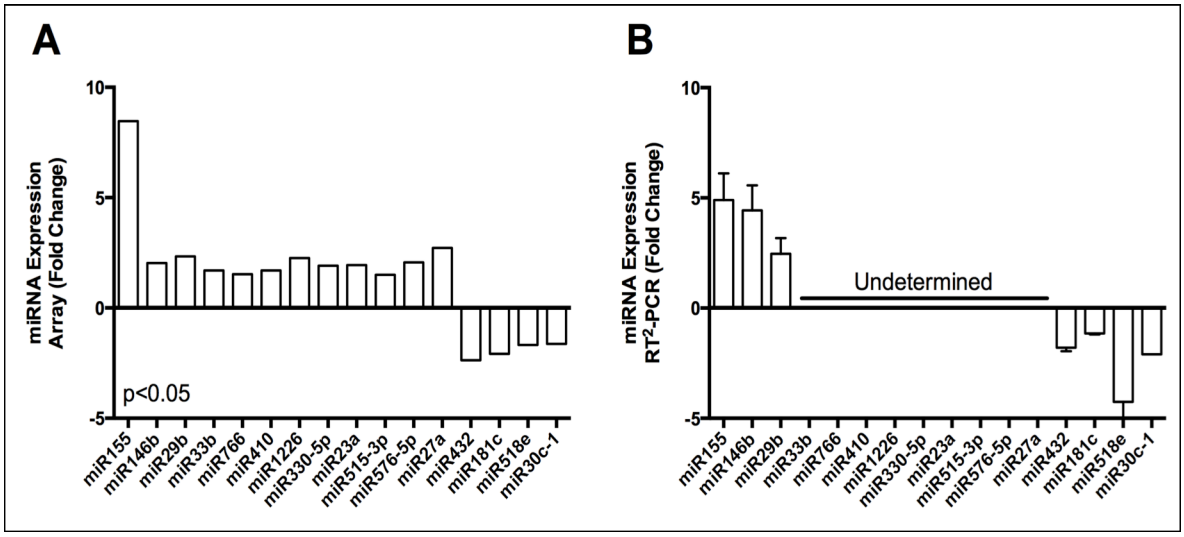
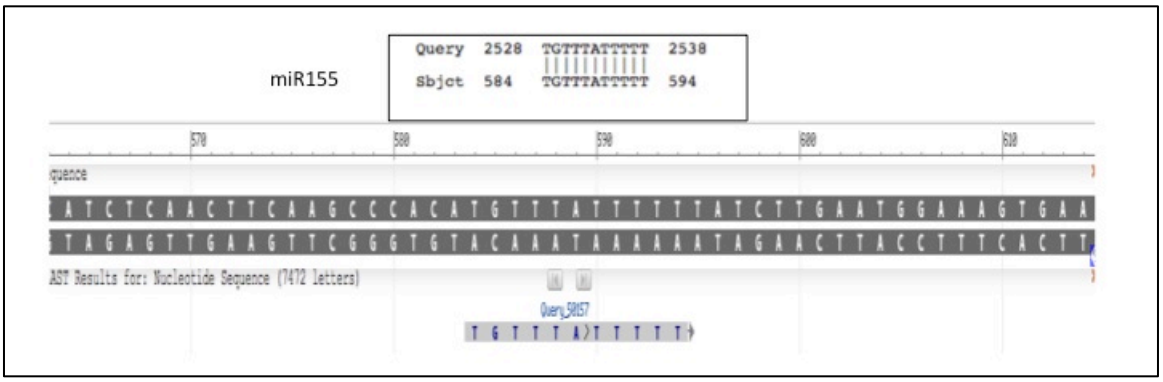


Figure 3.1: IL-1β activation alters human astrocyte miRNA profiles. *Microarray was performed to evaluate miRNAs changed in response to IL-1β activation in primary human astrocytes, 8 h post-treatment. Microarray analyses showed increases in 12 miRNAs, while decreases in the levels of 4 miRNAs* ***(A).*** *Three biological replicate astrocyte donors were used for microarray analyses and p values in panel A reflect changes confirmed in all three. Upregulated and downregulated miRNAs were confirmed by RT^2-PCR analyses (**B**), ($p<0.05$).*

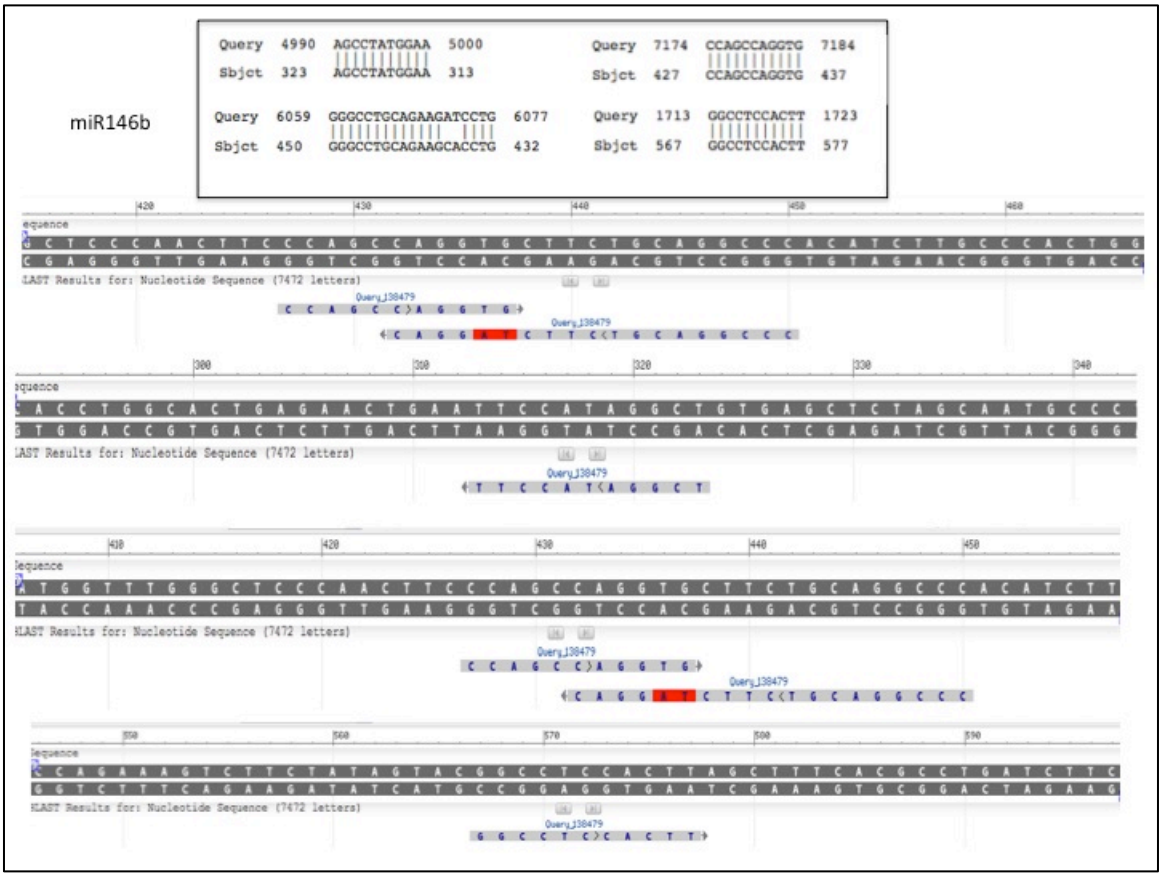
	miRNA	Functions
1	***155***	Crucial role in varied physiological and pathological processes like hematopoietic lineage differentiation, immunity, inflammation, viral infections, cancer, cardiovascular diseases and Down's syndrome [20].
2	**146b**	The predictive status of HIV disease progression and response therapy could be hypothesized in PBMCs and plasma with differential regulation of miR146b. Diagnostic marker in some types of cancer [21].
3	***29b***	May play a role in negating the phenotype of HER-2 positive breast cancer. Helps in providing therapeutic implications for developing cancer chemo-resistance [22].
4	**33b**	Potential role in tumor suppression. Inhibits stemness and metastasis in breast cancer cells. Therapeutic for several metabolic diseases [23, 24].
5	**766**	Could modulate one or more cancer types depending on the source of tissue. [25]
6	**410**	Capability to stimulate neonatal cardiomyocyte proliferation. Role in lung cancer progression [26].
7	**1226**	Act as a tumor suppressor by inducing cell death. Increases the number of reactive oxygen species. Induction of loss of mitochondrial trans-membrane potential and reduces the cell survival [27].
8	**330-5p**	May play a role in adenocarcinoma. Tumor suppression in case of pancreatic cancer cells. [28]
9	***23a***	Specifically interacts with genes crucial for bone development. Expression in squamous carcinoma cells, have strong predictive potential [29].
10	**515-3p**	Levels of miRNA in the maternal plasma can be correlated to the placental weight. Act as a prognostic marker in prostate cancer [30].
11	**576-5p**	Associated with survival in correlation to tumor biology. May serve as a biomarker for glioma detection with grater sensitivity and specificity [31].
12	**27a**	Might be involved in regulating CYP3A4 gene regulation. May help in regulating resistance in case of adenocarcinoma [32].
13	**432**	Serves as a potential biomarker for certain infectious diseases in cattle. Expression of this miRNA could lead to cessation of cell cycle. Thus, could be used as a therapeutic approach for Huntington's disease [33].
14	**181-c**	Regulatory role in differentiation of human embryonic stem cells. Related to tumor relapse in osteocarcinoma [34].
15	***518e***	Act as a non-invasive diagnostic biomarker in case of preeclampsia. [35]
16	**30c-1**	Serves as a biomarker for lung adenocarcinoma. Might be involved in the recovery in case of stroke [36].

Table 3.1: Summary of miRNAs altered in IL-1β treated astrocytes.

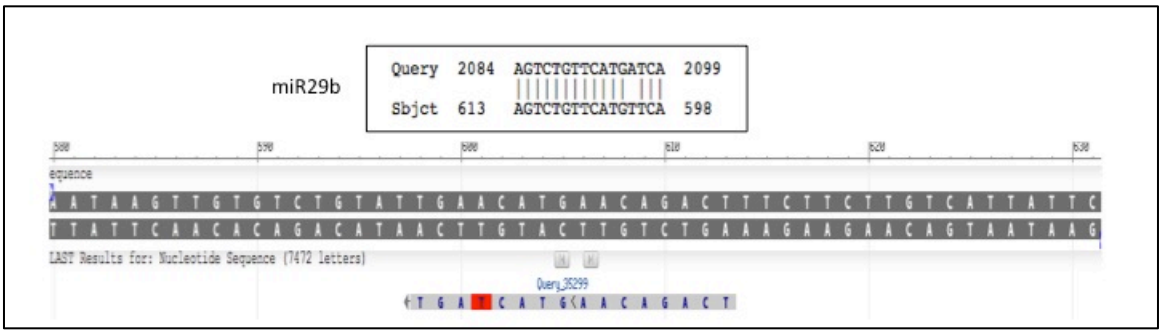
A


miR155
Query 2528 TGTTTATTTTT 2538
Sbjct 584 TGTTTATTTTT 594

B


miR146b
Query 4990 AGCCTATGGAA 5000
Sbjct 323 AGCCTATGGAA 313
Query 7174 CCAGCCAGGTG 7184
Sbjct 427 CCAGCCAGGTG 437
Query 6059 GGGCCTGCAGAAGATCCTG 6077
Sbjct 450 GGGCCTGCAGAAGCACCTG 432
Query 1713 GGCCTCCACTT 1723
Sbjct 567 GGCCTCCACTT 577

C



D

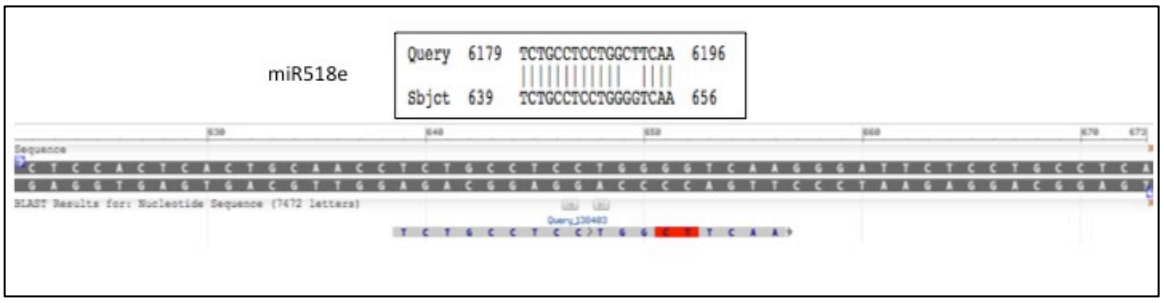


Figure 3.2: TIMP-1 3'UTR putative miRNA binding sites analysis*. BLAST search tool identified putative binding sites on the TIMP-1 3'UTR for selected miRNAs that were increased or decreased, in response to IL-1β stimulation. Putative miRNA binding sites to the TIMP-1 3'UTR sequences were identified. This analysis showed that miR 155 has one (**A**), miR 146b has four (**B**), miR 29b has one (**C**) and miR 518e has one binding site (**D**) in the TIMP-1 3'UTR.*

(In BLAST, Query: indicating the TIMP-1 3'UTR sequence and Subject: indicating miRNA sequence)

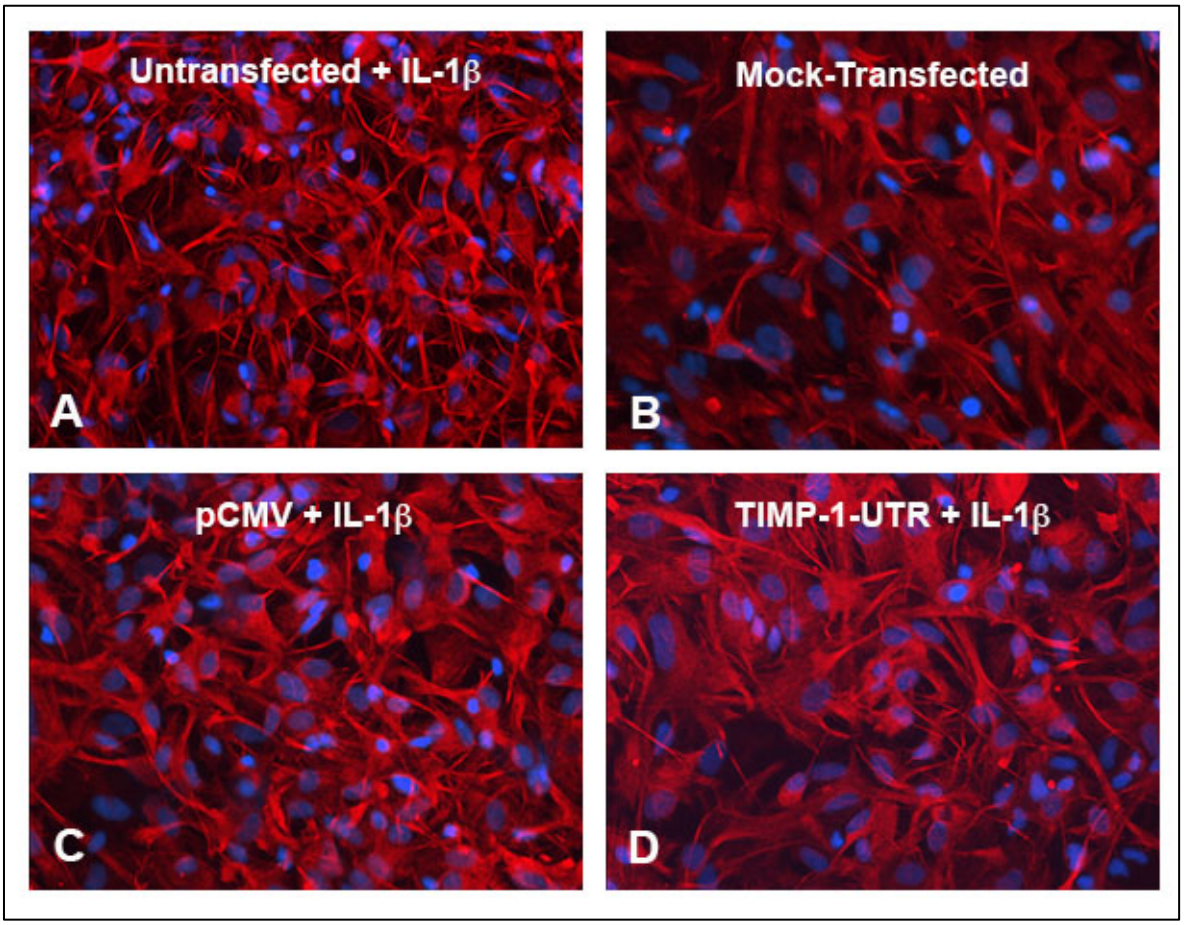


Figure 3.3: ***Comparable reactive responses of transfected astrocytes to IL-1β***. *Astrocytes were transfected with TIMP-1 3'UTR +/- IL-1β or the miRNA vector backbone (pCMV) and treated with or without IL-1β for 24 h. Control and Mock-transfected astrocytes were maintained in parallel. Astrocytes were immunostained for glial fibrillary acidic protein (GFAP, red) as the astrocyte marker and counterstained with DAPI (blue), to identify nuclei. Representative panels are shown for untransfected control astrocytes with IL-1β activation used for comparisons (**A**), mock-transfected control (**B**), pCMV + IL-1β (**C**) and TIMP-1-UTR+ IL-1β (**D**). Three independent astrocyte donors were immunostained post-transfection.*

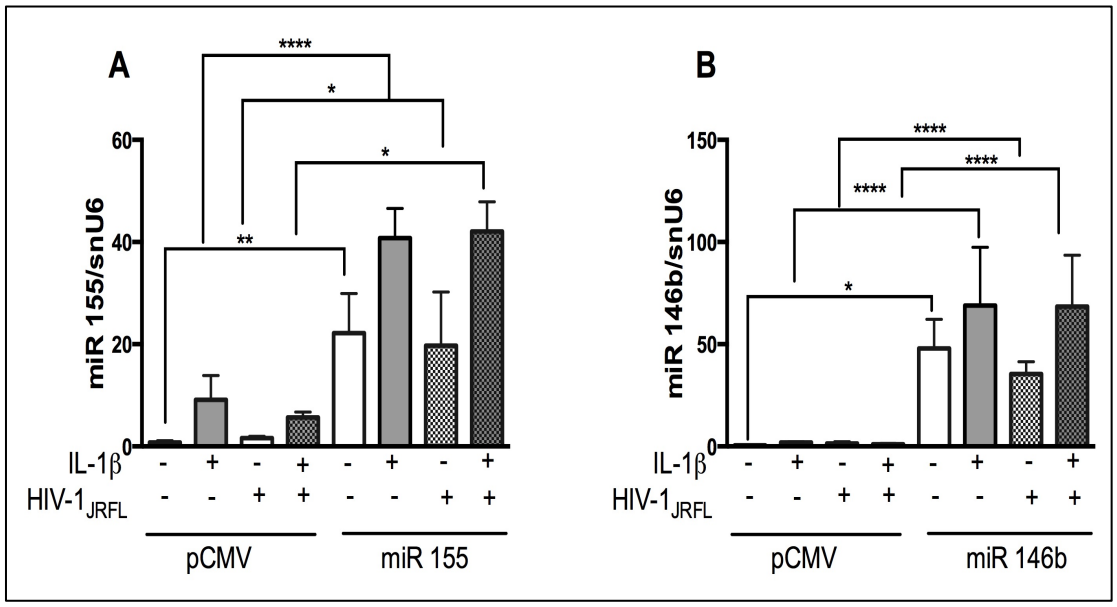


Figure 3.4*: Successful overexpression of miRNAs in human astrocytes with or without IL-1β and HIV-1$_{JRFL}$ activation**. *Human astrocytes were transfected with the empty pCMV, miR 155 and miR 146b expressing vectors followed by IL-1β/ HIV-1$_{JRFL}$ / IL-1β+ HIV-1$_{JRFL}$ treatment. At 24 h, miR 155 (**A**) and miR 146b (**B**) levels were measured with SnU6 as the internal normalizing control target. (*p<0.05, **p<0.01,*p<0.0001)*

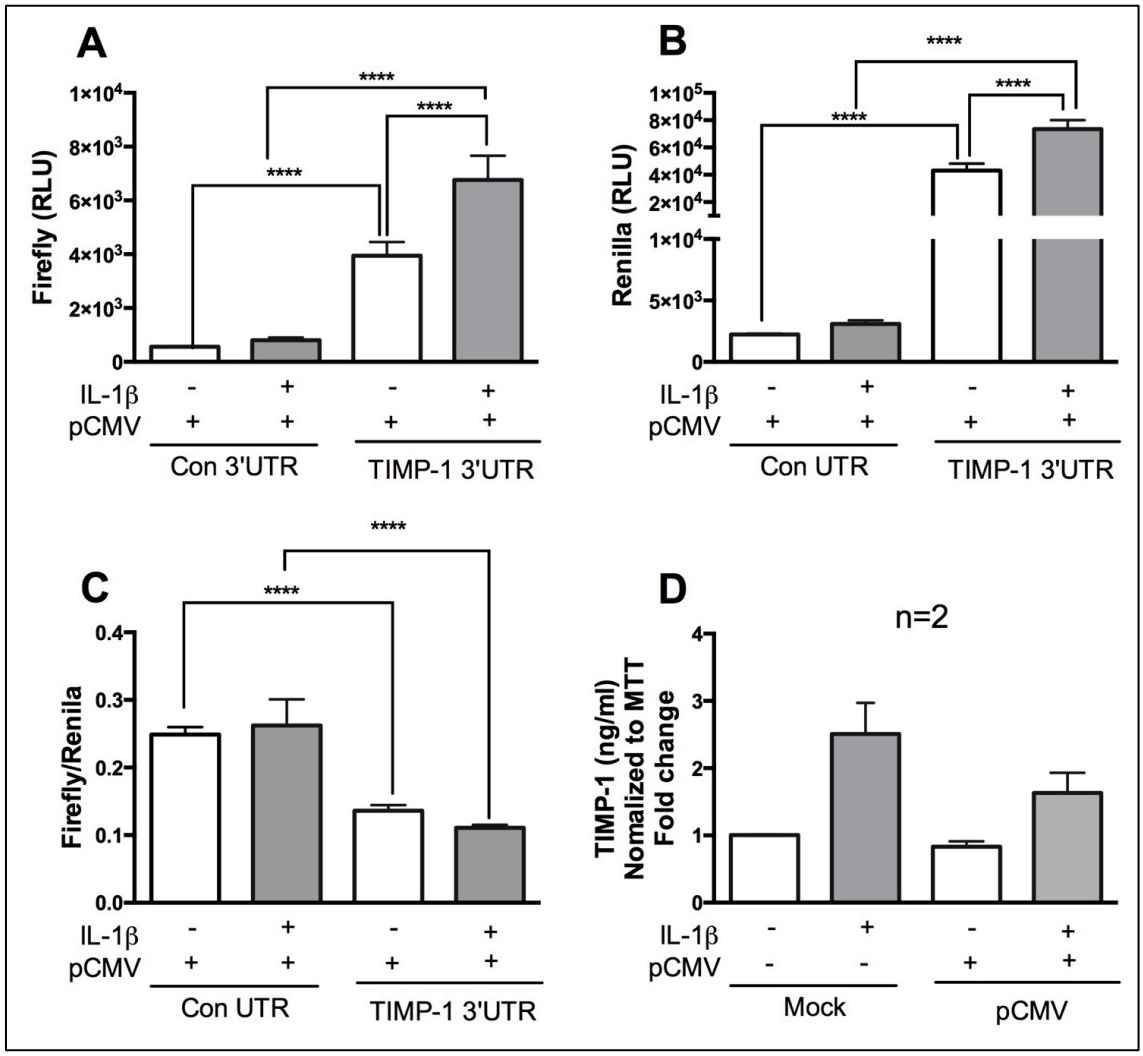


Figure 3.5*: Baseline luciferase activity and TIMP-1 levels in transfected astrocytes**. *Baseline activity of Con 3'UTR or TIMP-1 3'UTR with the co-transfection of empty miRNA vector, pCMV, was measured 24 h post IL-1β treatment. Firefly luciferase activity (**A**), Renilla luciferase activity (**B**) and firefly / Renilla ratio (**C**) are shown. Culture sups TIMP-1 levels were measured in pCMV (**D**). (*p<0.0001) (**A**), (**B**) and (**C**) show representative data. Similar trend has been observed in 3 donors (n=3). (**D**) depicts fold changes in two biological replicates (n=2).*

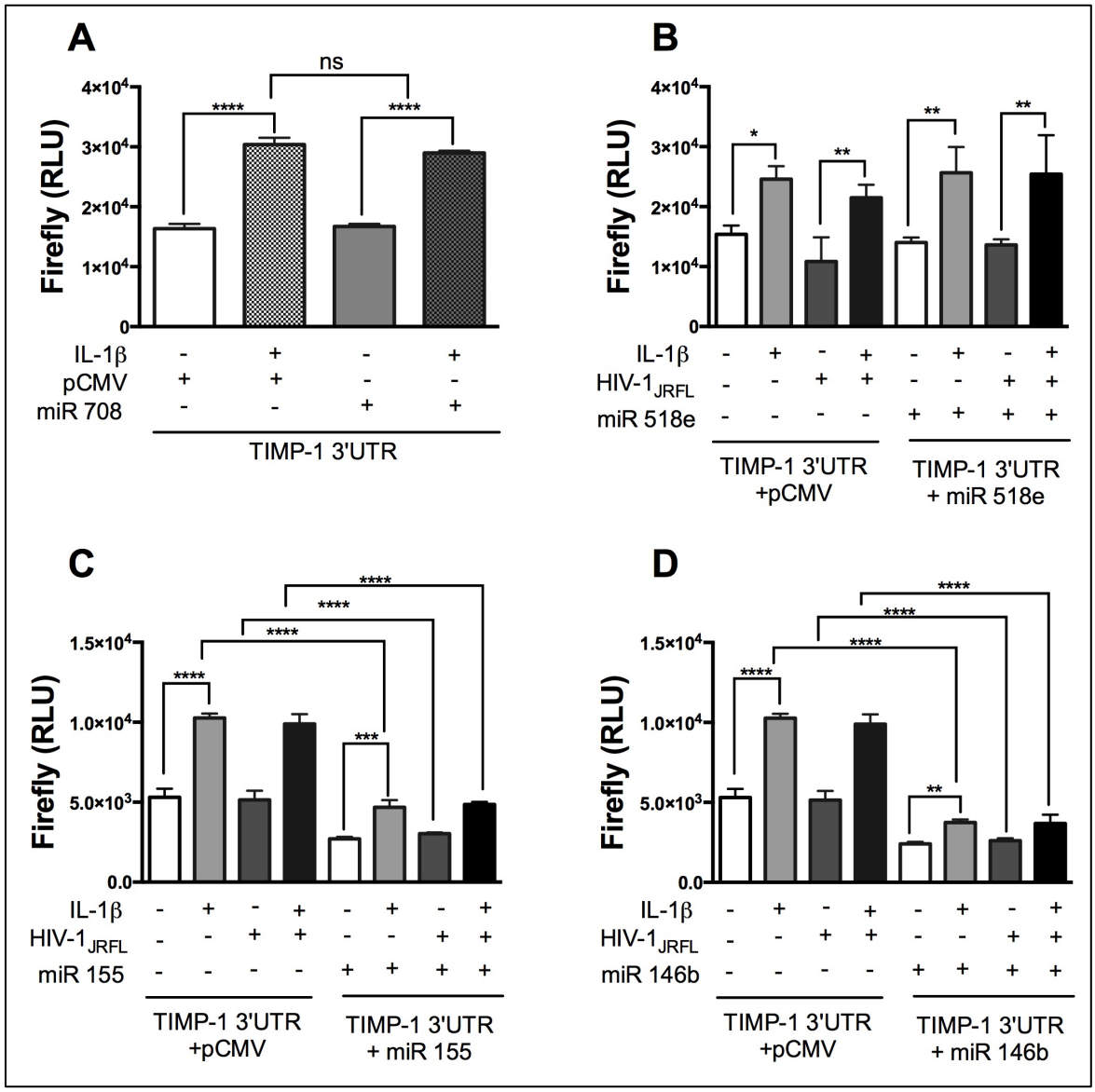


Figure 3.6**: **Astrocyte miRNAs 155 and 146b regulate firefly luciferase expression through TIMP-1 3'UTR activity**. Astrocytes were co-transfected with either control TIMP-1 3'UTR or TIMP-1 3'UTR along with control miRNA (pCMV) and miRNAs. Firefly luciferase activity was measured with the transfection of miR 708 (**A**), miR 518e (**B**), miR 155 (**C**) and miR 146b (**D**) (*p<0.05, **p<0.01, ***p<0.001,*p,0.0001). Comparable trends were observed in two independent astrocyte donors (n=2).*

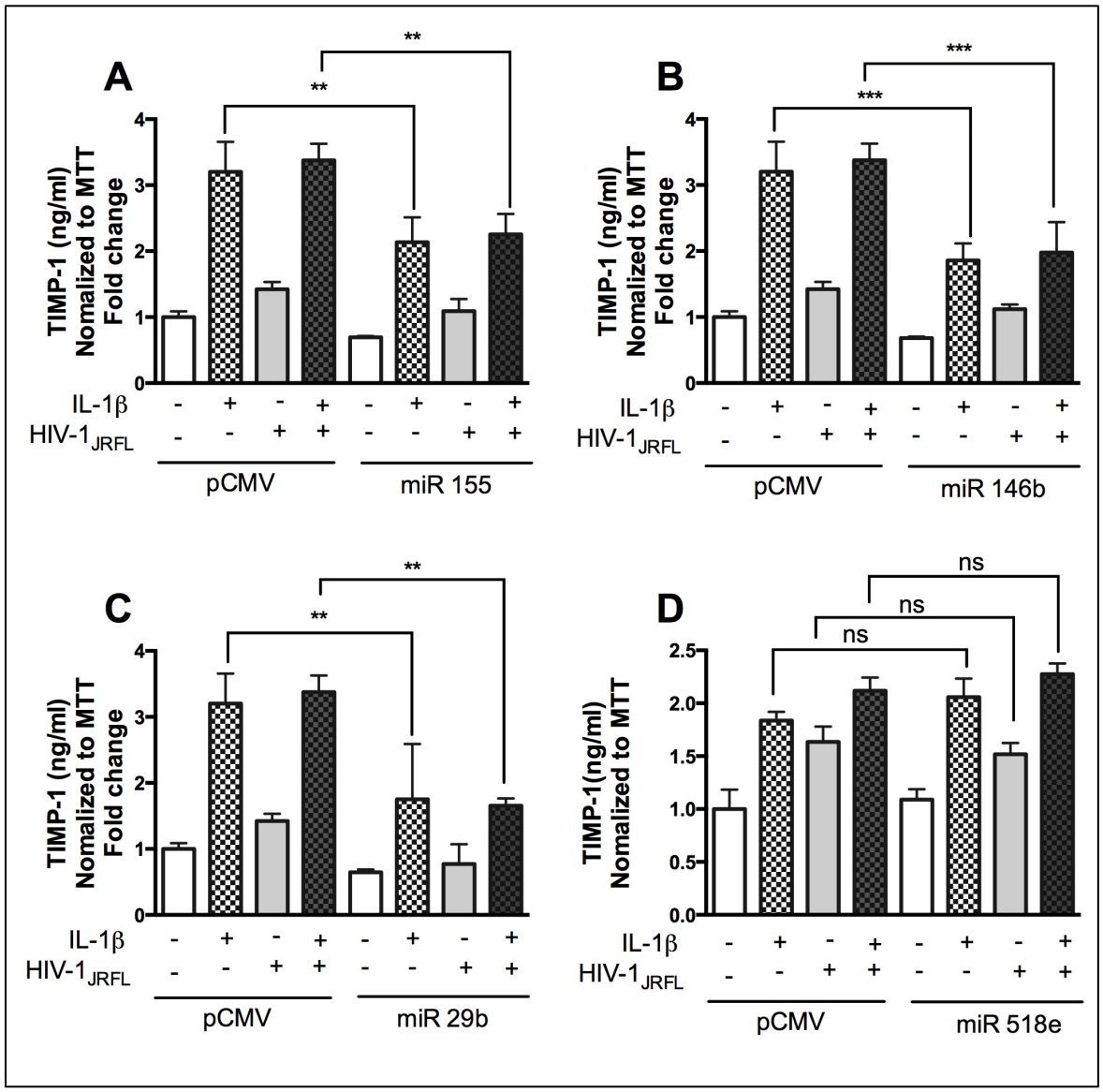


Figure 3.7*: *miRNA overexpression regulates astrocyte-TIMP-1 in context of HAND. *Secreted TIMP-1 levels in astrocytes transfected with miR 155 (**A**), miR 146b (**B**), miR 29b (**C**) and miR 518e (**D**) were evaluated with MTT normalization. TIMP-1 levels were measured 24 h post activation with IL-1β, HIV-1$_{JRFL}$, alone and in combination. (**p<0.01, ***p<0.001), Comparable trends were observed in two donors (n=2).*

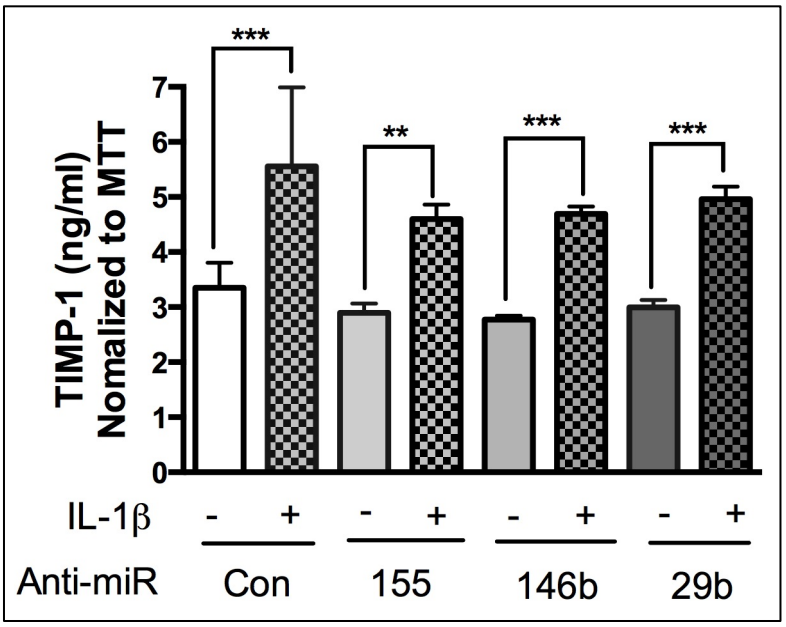


Figure 3.8: Inhibition of specific target miRNAs reverses miRNA-mediated TIMP-1 post-transcriptional regulation. *miRNAs were inhibited by transfecting astrocytes with 100 nM of inhibitor, miRidian inhibitors (Dharmacon). Anti-miR Con is essentially non-targeting oligonucleotide, control for miRNA inhibitors. Inhibition of the miRNAs (miR 155, 146b and 29b) used for overexpression studies of astrocytes, nullified the effect of IL-1β-induced miRNA upregulation as TIMP-1 levels were not altered compared to Anti-miR Con. (**p<0.01, ***p<0.001)*

Experimental Design:

To investigate the role of miRNAs in astrocyte-TIMP-1 downregulation.

Rationale: TIMP-1/MMP imbalance is observed in various physiological disorders including HAND [37]. Our laboratory discovered the neuroprotective role of TIMP-1 [38], thus making it crucial to study the regulation of TIMP-1. miRNAs are known to bind to the 3'UTR of target and regulate the expression [39]. Furthermore, miR 146b has been shown to be involved regulating the 3'UTR of the TNF receptor factor 6 and IL-1 receptor associated kinase 1. Hence, the role of miR 146b was proposed as a negative regulator [40]. Taken together data show IL-1β/HIV-1_{JRFL} treated primary human astrocytes showed higher expression with miR 146b and miR 155 as compared to the baseline expression, which was mimicked by transfection with miRNA overexpression vectors (**Fig. 3.4**). Additionally, upon transfection with a TIMP-1 3'UTR reporter plasmid, a decrease in the luciferase activity was also seen with miRNA overexpression (**Fig 3.6**). Since the 3'UTR was linked to the luciferase construct and co-transfected with various miRNAs, changes in the luciferase activity can be directly correlated to the regulation of TIMP-1 3'UTR *via* miRNAs. To better understandon the role of miRNAs in astrocyte-TIMP-1 regulation, endogenous TIMP-1 levels were also measured with miRNA-overexpression in the context of HAND-relevant astrocyte stimulation (**Fig 3.7**). Decrease in endogenous TIMP-1 levels was observed as compared to the empty vector control (pCMV). This study elucidates role of miRNAs in post-transcriptional regulation of TIMP-1. Multiple miRNA, as confirmed by microarray and RT^2-PCR, were tested for possible roles in astrocyte TIMP-1 regulation, along

with comparison miRNAs, which were unchanged or downregulated in IL-1β activated astrocytes.

3.1 IL-1β alters miRNA expression profiles of primary human astrocytes that target TIMP-1 3'UTR

In order to evaluate whether astrocyte TIMP-1 is post-transcriptionally regulated, first, microarray analyses were performed to analyze changes in miRNA profiles in the presence of IL-1β, a known inducer of astrocyte-TIMP-1. Summarized data showed miRNAs were either upregulated or downregulated with IL-1β stimulated in primary human astrocytes (**Fig. 3.1**). Significantly altered miRNAs expression changes were confirmed using RT^2-PCR. miR 155, miR 146b and miR 29b were the most consistently increased, while miR 518e was the most significantly down regulated. The miR 708 remained unchanged in the IL-1β activated astrocytes. Thus, we selected these miRNAs for further experiments. miR 155 increased almost eight-fold, miR 146b increased almost two-fold, miR 29b increased almost three-fold and miR 518e decreased around three-fold with HAND-relevant stimuli in microarray (**Fig. 3.1A**) *($p<0.05$)*. In RT^2-PCR, around a five-fold, increase in both miR 155 and miR 146b, a three-fold increase in miR 29b and around four-fold decrease with miR 518e in the expresssion was observed (**Fig. 3.1B**). Hence, these miRNAs were selected to study the role of miRNAs in the post-transcriptional regulation of TIMP-1 3'UTR.

In order to analyze which of these miRNAs upregulated in IL-1β activation, would regulate TIMP-1 3'UTR, Basic Local Alignment tool (BLAST) analysis was

performed to evaluate wheather the miRNAs elevated with IL-1β have putative binding sites for TIMP-1 3'UTR. One putative binding site for miR 155 **(Fig. 3.2A**), four binding sites for miR 146b **(Fig. 3.2B)** and one binding site for miR 29b **(Fig. 3.2C)**, and miR 518e **(Fig. 3.2D)** on TIMP-1 3'UTR were identified. Thus, BLAST confirmed that IL-1β induces a panel of miRNAs that are likely to be involved in regulating TIMP-1 3'UTR and were further used in this study to investigate post-transcriptional regulation of astrocyte-TIMP-1.

3.2 Successful overexpression of TIMP-1 3'UTR targeting miRNAs in human astrocytes

The approach of overexpression of selected miRNAs in astrocytes was employed to investigate their functional outcomes in context of TIMP-1 regulation. Before conducting any experiments for reporter gene expression assays, or *de novo* regulation of astrocyte TIMP-1, we first checked if overexpression constructs alter inflammatory responses of astrocytes. Astrocytes respond to IL-1β by exhibiting a typical reactive phenotype such as activated cell bodies and long processes. Astrocytes were transfected with either TIMP-1 3'UTR +/- IL-1β or pCMV (miR backbone) +/- IL-1β and their phenotype was compared to activated, untransfected, as well as, transfected control cells. IL-1β stimulated untransfected astrocytes showed reactive phenotype with contracted cell bodies and long processes (**Fig. 3.3A**). Clearly, cells responded to IL-1β as expected. However in case of mock, which is the transfected control, astrocytes were flat with larger cell body and fewer processes. This was expected as the process of transfection

provides a toxic shock to cells (**Fig. 3.3B**). Furthermore, with the transfection of pCMV or TIMP-1 3'UTR, IL-1β activation didn't completely resemble non-transfected IL-1β activated cells, astrocyte like phenotype of larger cells recovered and the cells appear activated by IL-1β. Hence, GFAP/DAPI staining showed that the astrocytes are responding to the IL-1β and showed activated phenotype with transfections. TIMP-1 3'UTR and pCMV transfected astrocytes (**Fig. 3.3C/D**) were immunostained in parallel and showed no significant dampening of reactive phenotypes in response to IL-1β.

Astrocytes transfected with miR 155 and miR 146b overexpressing plasmids were treated with IL-1β/ HIV-1_{JRFL} / IL-1β+ HIV-1_{JRFL} for 24 h and TIMP-1 levels were analyzed using RT^2-PCR. Around 40-50 fold increase with the presence of miR 155 (**Fig 3.4A**) and miR 146b (**Fig 3.4B**) respectively was observed*, (****p<0.0001).* Further, the fold change was higher with the induction by HAND relevant stimuli 24 h post-treatment (IL-1β/ HIV-1_{JRFL} / IL-1β+ HIV-1_{JRFL}) *(****p<0.0001)*. Overexpression of the plasmid miR 146b, miR 155 was confirmed in transfected astrocytes using RT^2-PCR PCR.

3.3 Effects of HIV-1 and IL-1β activation on TIMP-1 3'UTR-regulated Firefly luciferase activity in miRNA-overexpressing astrocytes

After the successful confirmation of the overexpression of, these miRNAs (miR 155, miR 146b) furthermore, understanding about the regulation of TIMP-1 3'UTR *via* luciferase reporter assay system could be obtained. In this reporter construct, TIMP-1 3'UTR is linked to the firefly luciferase. Hence, if the miRNA is

regulating the TIMP-1 3'UTR, the changes would be reflected in the firefly luciferase activity. *Renilla*, is incorporated in the plasmid as an internal normalizing control and hence its activity is expected to remain unchanged across all the conditions unless the promoter is sensitive to the stimuli to which cells are exposed and/or it is regulated in an indirect manner. *Renilla* is driven by CMV promoter, which is known to be a much stronger promoter in comparison to SV-40 that is driving the Firefly activity. Also IL-1β appears to regulate the CMV promoter resulting in significant changes in the *Renilla* activity across all the conditions in conjunction with firefly luciferase activity changes. While this appears counterintuitive, as *Renilla* is in the same plasmid molecule, all cells that receive the Firefly luciferase reporter will automatically receive the *Renilla* as well. In particular, it is noteworthy fact that while they are regulated with two distinct promoters only the firefly luciferase activity is subject to the regulation via TIMP-1 3'UTR whereas the *Renilla* is independent of it. As the firefly luciferase activity is through TIMP-1 3'UTR, we focused on firefly activity as a direct measure to evaluate TIMP-1 3'UTR regulated reporter activity. Firefly luciferase activity was determined 24 h post-IL-1β treatment, in co-transfected astrocytes with TIMP-1 3'UTR and pCMV. Significantly higher firefly luciferase activity was seen with IL-1β stimulation in mock as well as TIMP-1 3'UTR + pCMV. In comparison to control 3'UTR+pCMV -/+IL-1β, TIMP-1 3'UTR+pCMV -/+IL-1β showed significantly high firefly luciferase activity (**Fig 3.5A**)*, (****p<0.0001)*. Equivalent increase in the *Renilla* luciferase activity was seen (**Fig 3.5B)***, (***p<0.001)***. However, significant decrease in the firefly/ *Renilla* ratio was observed in TIMP-1

3'UTR+pCMV-/+ IL-1β in comparison to mock-/+IL-1β (**Fig 3.5C),** *(***p<0.001)*. Therefore, the astrocytes as expected were induced with IL-1β. As explained above, only the firefly luciferase activity is subject to control through the TIMP-1 3'UTR and the CMV promoter driving the *Renilla* luciferase is sensitive to the stimuli. Taken together, the *Renilla* in this system does not serve as a proper internal control. Hence, in further experiments data analysis of the firefly luciferase activity of the astrocytes will be the primary measure of TIMP-1 3'UTR function.

miR 708 could potentially serve as a good target for the comparison since, miR 708 was unchanged in the microarray and does not have any binding sites for TIMP-1 3'UTR. This study would enlighten us whether the the changes in miRNAs are specific to our target (TIMP-1 3'UTR) and not nonspecifically observed across all miRNAs. In comparison to TIMP-1 3'UTR +pCMV -/+IL-1β (control for this experiment) miR 708 -/+IL-1β did not show any significant difference in the firefly luciferase activity. However, statistically significant increase with IL-1β stimulation was seen, concluding that the astrocytes are responding to stimuli **(Fig 3.6A,,** ****p<0.001)*. To introduce disease relevance, HIV-1_{JRFL}, another HAND-relevant stimuli derived from frontal lobe of the brain, was used activate astrocytes. Significant reduction in the firefly luciferase activity was seen with the overexpression of miR 155 **(Fig 3.6B,** ****p<0.001)* and miR 146b **(Fig 3.6C)(** ****p<0.001)*, 24 h post treatment (IL-1β / IL-1β+ HIV-1_{JRFL}). However, no change in the firefly luciferase activity was seen with the overexpression of miR 518e (IL-1β/ IL-1β+ HIV-1_{JRFL}) **(Fig 3.6D,** ****p<0.001).* Thus, overexpression of miR 146b and miR 155, consistently decreased the TIMP-1 3'UTR luciferase

activity. miR 518e downregulated in the array **(Fig 3.5A)** did not significantly change its firefly activity, therefore acting as a control to identify the specificty of other miRNAs.

3.4 Endogenous TIMP-1 levels in HIV-1 and IL-1β-activated astrocytes reflect miRNA-specific post-transcriptional regulation

In parallel experiments along with assessing the luciferase activity, endogenous TIMP-1 levels were measured. IL-1β/ IL-1β +HIV-1_{JRFL} treatment with elevated miRNAs significantly decreased TIMP-1 levels, specifically, the overexpression of miR 155 **(Fig 3.7A,** ****p<0.001)*, miR 146b **(Fig 3.7B,** ****p<0.001)*, and miR 29b **(Fig 3.7C,** ****p<0.001)* as compared to pCMV-transfected astrocytes. However, TIMP-1 levels remained unchanged with the overexpression of miR 518e **(Fig 3.7D***)*. Changes in firefly luciferase activity were also reflected at the protein level. The fold change in luciferase activity was reflected in the endogenous TIMP-1 levels. Thus, these data show that TIMP-1 3'UTR may be regulated by miR 155 miR 146b and miR 29b at post-transcriptional level. The overexpression of miR 518e, that was downregulated in the microarray, did not affect TIMP-1 3'UTR-driven luciferase activity, further confirmed that these changes are induced specifically due to miRNA activity.

Lastly, we inhibited miR 155, miR 146b and miR 29b using specific antisense oligonucleotide along with a non-specific negative control (or Anti-miR-Con). Endogenous TIMP-1 levels were measured 24 h post treatment. The inhibitory effects of miRNAs induced by IL-1β activation of astrocytes were

nullified and no difference in the TIMP-1 levels was observed as compared to IL-1β-activated miR-Con **(Fig 3.8)**. This, further confirms that miR 155, miR 146b and miR 29b regulate TIMP-1 3'UTR at post-transcriptional level.

Chapter 4

Discussion

Discussion

List Of Illustration

Figure

Discussion and future plans

HIV-1 can lead to several CNS impairments together termed as HAND [41]. In acute versus chronic neuroinflammation, differential regulation of TIMP-1 is relevant to HAND neuropathogenesis. However, the underlying mechanisms are still being uncovered [13]. In case of acute inflammation the TIMP-1 levels increase, which is known to have a neuroprotective role [5]. However, in chronic condition, TIMP-1 levels are downregulated leading to the loss of neuroprotection and increased MMP activity. Thus, it is important to study the potential mechanisms involved in TIMP-1 downregulation. There are multiple proposed mechanisms for TIMP-1 downregulation, including the production of anti-inflammatory cytokines, such as, through the signaling of transforming growth factor (TGF-β) [3]. TGF-β has a novel extracellular loop in relation to TIMP-1 regulation. In acute or chronic condition, stimulation in combination with TGF-β and IL-1β showed the same response as observed with IL-1β induction in acute or chronic condition [4]. TIMP-1 is also regulated at the level of mRNA stability, in such that, IL-1β activation increases TIMP-1 mRNA stability almost two-fold in acute activation, which is then lost and brought back to baseline levels in chronic neuroinflammation [3].

These mechanisms; however, do not represent the complete picture of the complex circuitry in TIMP-1 regulation. In our study, we propose, miRNA-mediated TIMP-1 downregulation. This study investigated the post-transcriptional regulation of astrocyte TIMP-1 with IL-1β/HIV-1_{JRFL} activated miRNAs. miRNAs are known to regulate at the post-transcriptional level *via* the RNA interference

pathway. In human HIV+ brain, miRNAs play a role in astrocyte survival by regulation of caspase-6 [42]. Overall, miRNA profiles are altered in neuroinflammation and neurodegeneration and emerging as critical players in disease processes. miRNAs confirmed to be altered in astrocytes treated with IL-1β, were investigated for probable TIMP-1 3'UTR binding with BLAST. miR 155, miR 146b, miR 29b and miR 23a satisfied both the confirmatory analyses. A pro-inflammatory role for miR 155 has been identified in astrocytes [42]. IL-1-like receptor kinase and TNF receptor-associated factor 6 protein levels were shown to be reduced due to the cytokine signaling *via* miR 146b. miR 146b has been reported to play a role in innate immunity by controlling toll-like receptors and cytokine signaling using the negative feedback loop. miR 146b and miR 155 have been known to be elevated in response to TNF-α, LPS and IL-1β in a monocyte derived cell line [43]. The downregulated miR 518e in our array data, as well as, the unchanged miR 708 served as controls for validating miR155 and 146b-specific effects. miR 155 plays a role in various physiological and pathological processes involved in lineage differentiation, immunity, inflammation, viral infections, cancer, cardiovascular diseases and Down syndrome [20]. The predictive status of HIV disease progression and response therapy could be hypothesized with differential regulation of miR146b in PBMCs and plasma. miR 146b also serves as a diagnostic marker in some types of cancer PTC [21]. Previously, miR 29b was known to play a role in negating the phenotype of HER-2 positive breast cancer and aids in in providing therapeutic implications for developing cancer chemoresistance [22]. miR 23a specifically interacts with

genes crucial for bone development. Downregulation of miR 708 contributes to development of hepatocarcinoma cells and prevents mobility [44].

Reactive astrogliosis is identified by astrocytes hypertrophy, as well as, by recruitment astrocytes at the site of injury, reactive astrogliosis is known to be the phenotypic characteristic of astrocytes [45]. Thus, reactive phenotype as expected was observed with IL-1β stimulation and confirmation of their overexpression was attained with either miR backbone or TIMP-1 3'UTR. Using luciferase reporter assay it was confirmed that the miR 155, miR 146b and miR 29b decreased the TIMP-1 3'UTR-controlled luciferase activity in astrocytes activated with IL-1β/HIV-1_{JRFL}. Downregulation of miR 29b has been observed in various diseases like Huntington's, Alzheimer's and schizophrenia [46-48]. Treatment with HIV-1_{JRFL} was employed in order to introduce disease relevance to this study. Thus, in parallel experiments along with changes in luciferase activity, endogenous TIMP-1 levels were also assayed. Downregulation in the luciferase activity was reflected in the endogenous TIMP-1 levels.

Our findings confirm that with the corresponding decrease in the luciferase activity significant decrease in the endogenous TIMP-1 levels was observed. These data show that miRNAs can regulate TIMP-1 *via* the 3'UTR at the post-transcriptional level. Furthermore, confirmation was achieved using miR 708, which was unchanged in the array and does not have any binding sites on TIMP-1 3'UTR. As expected miR 708 did not show any significant change in the TIMP-1 3'UTR activity as well the endogenous TIMP-1 levels as compared to the control. It can be suggested from this that the downregulation is specific to our target TIMP-1.

Furthermore, miR 518e, found to be downregulated in the array, upon IL-1β activation, did not alter TIMP-1 luciferase activity or endogenous TIMP-1 levels. It is more evident that TIMP-1 downregulation we observed with miR 155, miR 146b and miR 29b in response to IL-1β. To get a proof of concept on this finding, knock down studies were performed targeting these miRNAs. The results obtained with overexpression model of the miRNAs were nullified with the inhibition of these miRNAs. Suggesting the endogenous changes in TIMP-1 are due these specific miRNAs (miR 155, miR 146b and miR 29b). Eventually, using these findings, we can propose that TIMP-1 downregulation in case of neuroinflammation or neurodegeneration might be regulated *via* miRNAs (miR 155, miR 146b) at the post-transcriptional level. This potential mechanism provides a better understanding on the TIMP-1 down regulation during neuroinflammation in context of HAND. However, there might be several other mechanisms underlying which need to be explored. Better insights on how each of these miRNA regulate IL-1β response is important. Although we know that the TIMP-1 is downregulated endogenously, but exactly how, or through what series of signaling cascades it downregulate, is unclear so far. Hence, we made an attempt in this study to shed light towards better understanding the dual role of TIMP-1 in the setting of IL-1β/HIV-1 . miR 155, miR 146b and miR 29b might serve to have a therapeutic potential in context of HAND. TIMP-1 level downregulation might be counteracted upon with the help of these Anti-miR. Combined effect of these miRNAs could be evaluated to identify which approach is more dominating over the other. Also, it will give an idea of which miRNAs can or cannot regulate in synergy. Further,

additional approaches such as inhibiting these miRNAs in the HIV+ patient samples would be executed. This will give us better understanding about the mechanism of TIMP-1 downregulation in case of chronic inflammation.

Similar experiments (luciferase reporter assays and TIMP-1 ELISA) with the miR 23a will be conducted. Role of miR 23a in the post-transcriptional regulation of TIMP-1 will be identified. TIMP-1 promoter regulation would be studied by mutating CCAAT sites. CCAAT mutation analysis will be performed using single or combination mutations, to identify those CCAAT sites, which are crucial for the regulation TIMP-1 promoter activity. All together, regulation of TIMP-1 at transcriptional/post-transcriptional levels will be identified in context of HAND.

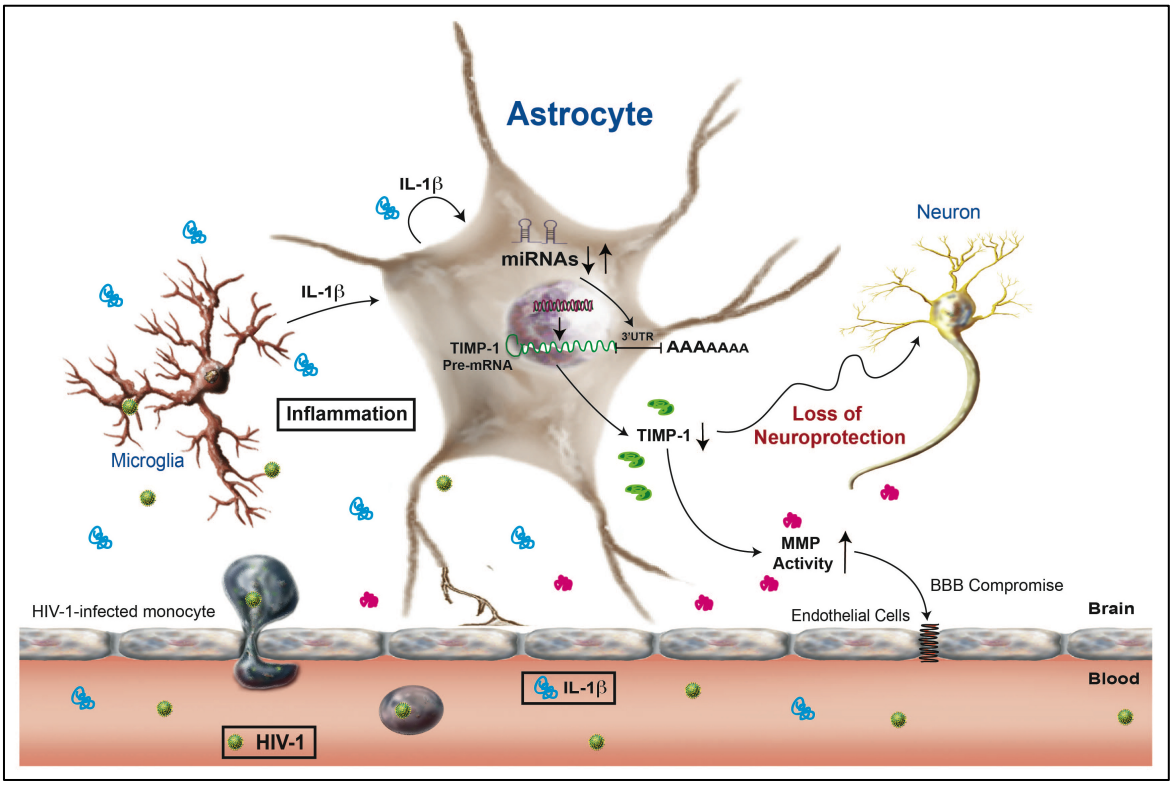


Figure 4.1: Regulation of TIMP-1 *via* miRNAs in HAND. HIV-1 crosses the blood brain barrier *via* infected monocytes, further infecting microglia, by capturing the host cell machinery. IL-1β produced by infected/activated microglia induces brain inflammation. With sustained neuroinflammation TIMP-1 downregulation is known to occur, leads to loss of neuroprotection both *via* loss of MMP inhibition and loss of direct neuroprotection. We propose that the miRNA post-transcriptionally regulate the 3'UTR of the TIMP-1.

Chapter 5

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