Chapter 3

Results

Section 1
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Rotavirus Induced miR-142-5p Elicits Proviral Milieu by Targeting Non-Canonical TGFβ Signalling and Apoptosis in Cells

### 4.1 Introduction

MicroRNAs (miRNAs), 19-22nt long small non coding RNA, constitute a new class of negative regulators which repress gene expression by pairing with their target messenger RNAs (mRNAs) (Chen *et al.*, 2004). miRNAs are encoded from the human genome and regulate thousands of target mRNAs illustrating important regulatory role of miRNAs in cellular differentiation, proliferation, development and apoptosis pathways (Slaby *et al.*, 2009; Small *et al.*, 2011). A single miRNA can target multiple sequences with imperfect complementarities (Guo *et al.*, 2010; O'Neill *et al.*, 2011). It is therefore not surprising that miRNA deficiencies or excesses have been implicated in various cancer (Liston *et al.*, 2010; O'Connell *et al.*, 2010; Pallante *et al.*, 2010), asthma (Tan *et al.*, 2007; Polikepahad *et al.*, 2010), cardiovascular (Contu *et al.*, 2010), neurological (Haramati *et al.*, 2010; Miller *et al.*, 2010), psychiatric (Maes *et al.*, 2009), skin diseases (Sonkoly *et al.*, 2009; Buckner *et al.*, 2010) and microbial infections (Li *et al.*, 2010; Zhang *et al.*, 2010).

Increasing number of studies now focus on direct regulation of mammalian viruses by host miRNAs. Human liver-specific miR-122 has been shown to enhance hepatitis C virus (HCV) replication (Jopling *et al.*, 2005), while other human miRNAs, including miR-199a-3p, miR-210, and miR-125a-5p, are found to repress hepatitis B virus (HBV) replication (Liu *et al.*, 2011). Recent studies revealed that different herpes viruses [Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpes virus (KSHV)] are targeted by different cellular miRNAs, including the miR-17/92 and miR-106b/25 clusters (Kang *et al.*, 2011; Riley *et al.*, 2012; Skalsky *et al.*, 2012) and coxsackievirus is targeted by miR-342-5p (Wang *et al.*, 2012). Other human miRNAs such as miR-323, miR-491, and miR-654 target influenza virus; miR-27 and miR-93 target vesicular stomatitis virus (VSV); and miR-28, miR29a, miR-125b, miR-150, miR-223, and miR-382 target the human immunodeficiency virus (HIV-1) (Houzet *et al.*, 2011). Most surprisingly, in a survey of more than 25,000 individual sequences of HCV, HIV-1, human papillomavirus (HPV) and HBV, it was found that there is a strong conservation and preservation of cellular miRNA-targeted sites within these viruses (Russo *et al.*, 2011). Based on all these reported
studies it can be concluded that the consortium of host miRNAs delivers a first layer of bioactive encounter against invasion of any foreign pathogen and fine-tunes the cell's overall antiviral arsenal.

Rotavirus (RV), a member of Reoviridae family, is the most important etiologic agent of severe nonbacterial diarrhea in children worldwide (Estes et al., 2007). RV has six structural proteins (VP1-VP4, VP6, VP7) forming icosahedral structure and produces six nonstructural proteins (NSP1 to NSP6) upon entry in the host. NSPs mainly control the cellular machinery, virus replication and assembly (Gonzalez et al., 1991; Mattion et al., 1991; Poncet et al., 1997; Afrikanova et al., 1998; González et al., 1998; Fabbretti et al., 1999; Michel et al., 2000; Vende et al., 2002; Taraporewala et al., 2006; Harb et al., 2008; Bagchi et al., 2010; Bhowmick et al., 2012). Inspite of extensive research on RV replication and propagation, till date, there is no reported study on miRNA regulation during RV life cycle.

In this study, we have analyzed cellular miRNA expression profile during RV infection in HT29 colorectal cancer (CRC) cells. This analysis identified miR-142-5p as a proviral miRNA which helps RV propagation by blocking host mediated early apoptosis. Furthermore RV NSP5 was identified to modulate the expression of hsa-miR-142-5p and confer viral propagation by blocking non-canonical Transforming Growth Factor Beta (TGFβ) signalling and the resulting apoptosis. The TGFβ non-canonical pathways are linked to epithelial to mesenchymal transition (EMT) which leads to cancer progression in microsatellite stable (MSS) CRC cells having an unimpaired TGFβ receptor 2 (TGFβR2) (Levy et al., 2005; Pino et al., 2010). Through this study the role of RV NSP5 in regulating EMT in TGFβ stimulated HT29 cells was also investigated.

4.2 Results

4.2.1 Identification of Differentially Expressed miRNAs in Rotavirus Infected HT29 Cells

Differentially regulated host miRNAs of HT29 cells, infected with simian RV-SA11 strain, were first analyzed using miRNA microarray. Microarray was performed using miRNA expression profiling platform (Thermo Scientific Dharmacon), comprised of probes to capture human, mouse and rat mature miRNAs in the Sanger database. Total RNA was isolated from confluent monolayers of HT29 cells either infected with SA11 strain [2 Multiplicity of infection (M.O.I.)] or mock infected for a specific time using miRNA isolation kit and subsequently processed for
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microarray analysis. The data (Fig 4.1A) was analyzed at the P*<0.05 significance level. Amongst differentially expressed miRNAs, five were up-regulated (red in heat map) and eleven were down-regulated (green in heat map) in the RV-SA11 infected cells. The cluster analysis revealed complete separation of RV-SA11 infected and the mock infected groups based on expression profiles of the differentially expressed miRNAs as shown in Fig 4.1B.

To validate reliability of miRNA microarray data, four miRNAs were selected for real time PCR (RT-PCR) analysis depending on their degree of differential expression as well as target sites. Increased expression of miR 142-5p was detected in RV-SA11 (simian) and KU (human) infected cells relative to the mock infected cells (Fig 4.2 A, B). Results were in agreement with the microarray data, which showed an induction of miR 142-5p at 8 hour post infection (hpi). However, RT-PCR analysis showed a low level of induction of miR-142-5p at 1hpi, which was not reflected in the microarray data. Reduced expression of miR-215, miR-194 and miR-192 in RV (SA11 and KU) infected HT29 cells relative to the mock infected cells were also reconfirmed by RT-PCR (Fig 4.2 A, B). Similar results were obtained in 293T cells, infected with the RV strains (Fig 4.2 C). RV infection in HT29 and 293T cells was confirmed by analyzing expression of nsp3 mRNA (Fig 4.1C). Throughout this study expression of NSP3 transcript and protein was assessed to confirm RV infection as NSP3 plays a significant role in modulating the host translational machinery and at the same time enhancing translation of rotaviral mRNAs (Piron et al., 1998). Overall, results were in agreement with the microarray data and confirmed regulation of miRNAs during RV infection to be strain independent (Fig 4.2).
**Fig 4.1:** (A) Heat map for differentially expressed miRNAs in RV infected cell [Time point A (1 hpi) and B (8 hpi)] and control cells. Red indicates an induction and green indicates a reduction of expression in infected cells compared to control cells, with color intensity correlating with level of change. 16 miRNA signals were found to be differentially expressed among the factor levels A, B and Control at the $P^*<0.05$ significance level. (B) Agglomerative hierarchical clustering using cosine correlation distance metric revealing distinctly clustered control samples from the A and B time points, indicating that factors relating to the sample labels are the predominant source of variation. (C) HT29 and 293T cells were infected with SA11 and KU strains (2 M.O.I.). Total RNA was isolated at specified time points and abundance of viral nsp3 mRNA was determined by real time analysis. Fold change in transcript was calculated by normalizing relative gene expression to gapdh using formula $2^{-\Delta\Delta cT}$. Result shown is obtained as mean of three independent experiments ($P < 0.05$).
4.2.2 Rotavirus Induced miR-142-5p Positively Regulates RV Infection

To experimentally define the role of differentially regulated miRNAs during SA11 infection, miR-142-5p was chosen for further investigation. HT29 cells were either mock transfected or transfected with the miR-142-5p specific hairpin inhibitor at 70-80% confluency. Transfection efficiency of miRNA inhibitor was analyzed by quantitating the expression of the respective miRNA (Fig 4.3A). 24 h post transfection, cells were infected with the RV strains (SA11 or KU) (2 M.O.I.). Total RNA and whole cell lysates were prepared for assessing RV gene transcript and
protein expression (Fig 4.4 A, B) by q-PCR and immunoblot analysis respectively. Remarkably, in the loss of function experiment, anti-miR-142-5p (hairpin inhibitor) transfection resulted in a statistically significant reduction of both RV NSP3 mRNA (Fig 4.4A) and protein (Fig 4.4B) levels in HT29 cells compared to the mock transfected and RV (SA11 and KU) infected condition. Maximum effect of the inhibitor was obtained at 100nM concentration (Fig 4.3 B). End point (24 hpi) viral titers by plaque assay were also measured in hsa-miR-142-5p inhibitor transfected cells, infected with either SA11 or KU strains. As shown in Fig 4.4 C hsa-miR-142-5p inhibitor significantly reduced RV titers compared to the mock transfected virus infected cells. Overall, these results indicate the pro-rotaviral potential of miR 142-5p for RV strains of animal as well as human origin.

**Fig 4.3:** (A) HT29 cells were transfected with miR-142-5p inhibitor and infected with RV strain. RNA was isolated at 24 hpi followed by real time analysis. Results revealed decrease in miR-142-5p expression due to presence of inhibitor. Fold change in transcript was calculated by normalizing relative gene expression to rnu6b using formula $2^{-\Delta\Delta C_t}$. Results are obtained as mean of three independent experiments (P < 0.05). (B)
HT29 Cells were transfected with hsa-miR-142-5p inhibitor. 24 h after transfection infected with RV strain at 2 M.O.I. Total RNA was isolated and nsp3 mRNA levels were analyzed over mock infected control cell by real time PCR. Fold change in transcript was calculated by normalizing relative gene expression to gapdh using formula $2^{-\Delta\Delta C_T}$. Result revealed significant reduction of nsp3 transcript at 100nM miR-142-5p inhibitor. Result shown is obtained as mean of three independent experiments (P < 0.05).

Figure 4.4: Role of hsa-miR-142-5p Inhibitor in RV Infection. (A) (B) HT29 cells, transfected with 100nM miR-142-5p inhibitor, infected with SA11 and KU RV (2 M.O.I.) for 0-9 hpi. (A) RNA was isolated at specified time points and expression of nsp3 and vp6 transcripts were analyzed by real time PCR compared to mock infected control cells. Fold change in transcript was calculated by normalizing relative gene expression to gapdh using formula $2^{-\Delta\Delta C_T}$. (B) Total cellular extracts were prepared and separated by SDS-PAGE to assess expression of viral protein NSP3 by immunoblotting. Results revealed reduced NSP3 expression in presence the inhibitor. Membrane was reprobed with GAPDH antibody as internal control. (C) HT29 cells transfected with hsa-miR-142-5p inhibitor were infected with SA11 and KU strains (2 M.O.I.). Virus yield was determined by plaque assay method at 24 hpi. Results shown (A, B and C) are obtained as mean of three independent experiments (P < 0.05).
4.2.3 hsa-miR-142-5p Regulates TGF Beta Signalling

Downstream effect of upregulated miR-142-5p was studied further for understanding the mechanism of promotion of RV by miR-142-5p. KEGG pathway suggested regulation of the entire TGFβ signalling by hsa-miR-142-5p because multiple proteins of this signalling are targeted by miR-142-5p. Thus TGFβ signalling in the context of RV infection and elevated miR-142-5p expression was analyzed. Among different target molecules, TGFβR2 and SMAD3 expression were assessed in whole cell lysates and total RNA of SA11 infected HT29 cells by immunoblotting and RT-PCR, respectively. Results revealed no significant change (1.2-1.6 fold) in the transcript levels of tgfβr2 and smad3 in RV-SA11 infected HT29 cells (Fig 4.5 A left panel). However, expression of both the proteins was downregulated (Fig 4.5 A right panel) in virus infected cells compared to the mock infected cells. Similar results were obtained in KU infected HT29 cells (data not shown). Consistent with the previous report, (Barreto et al., 2010), tgfβ mRNA level was found to be induced by more than twofold in RV-SA11 infected cells compared to mock infected cells (Fig 4.5 B). Furthermore, immunoblotting revealed reduction of TGFβR2 and SMAD3 protein in HT29 cells transfected with different concentrations of miR-142-5p mimic (10nM-100nM), with maximum effect at 100nM (Fig 4.5 C right panel). However, the ectopic expression of miR-142-5p mimic had no effect on tgfβr2 and smad3 transcripts (Fig 4.5 C left panel).

TGFβR2 and SMAD3 were further confirmed as the direct targets of miR-142-5p by luciferase assay. Using the TargetScan database, two miR-142-5p binding sites were found within the 3’Untranslated Region (UTR) of TGFβR2 and SMAD3. Target sites for miR 142-5p within the 3’UTR region of these proteins are pictorially represented in Fig 4.5 D. Among the target sites, one is conserved while another one is variable among different species. To determine if miR-142-5p targets the 3’UTRs of TGFβR2 and SMAD3, different luciferase reporter plasmids (pMIR-REPORT) were constructed containing the wild type and mutated miR-142-5p binding sites. pMIR-UTR-TGFβR2 and pMIR-UTR-SMAD3 constructs have wild type miR-142-5p target sites whereas pMIR-UTR-ΔSMAD3 contain mutated sequence and pMIR-UTR-SC have a scrambled sequence, considered as a negative control. (Fig 4.6). Cotransfection of pMIR-UTR-TGFβR2 and pMIR-UTR-SMAD3 with mature miR-142-5p mimic resulted in a significant reduction of the luciferase expression (Fig 4.5 E) in 293T cells. Indeed when pMIR-UTR-ΔSMAD3 and pMIR-UTR-SC were cotransfected with mature miR-142-5p mimic there was an increased luciferase
signal (close to the control luciferase expression) confirming that TGFβR2 and SMAD3 are direct targets of miR-142-5p. Renilla luciferase was used as an internal control.

**Figure 4.5: miR-142-5p Regulates TGFβ Signalling Pathway.** (A) HT29 cells were infected with RV-SA11 at an M.O.I. of 2. Total RNA was isolated (left panel) and whole cell lysates (right panel) were prepared at specified time points followed by real time analysis and immunoblotting respectively. Results reveal change at translational level of TGFβR2 and SMAD3 whereas transcripts for both genes did not change. (B) Total RNA, isolated from RV-SA11 infected HT29 cells, show time dependent increase in tgfβ mRNA. (C) HT29 cells were transfected with increasing concentrations of miR-142-5p mimic followed by real time analysis (left panel) and western blotting (right panel). TGFβR2 and SMAD3 protein expression reduced in presence of the mimic in dose dependent manner. (D) The schematic of tgfbr2 and smad3 mRNA shows two potential binding sites for miR-142-5p in its 3'UTR. One binding site is highly conserved among different species while another one is poorly conserved. (E) Targeting of tgfbr2 and smad3...
smad3 3’UTR results in translational suppression. 293T cells were transfected with the pMIR-REPORT luciferase construct containing the miR-142-5p binding site in 3’UTR, mutant binding site and scrambles sequences, treated with the miR-142-5p mimic followed by luciferase analysis. (A) (B) (C) and (E) Fold change in transcript was calculated by normalizing relative gene expression to gapdh using formula $2^{-\Delta\Delta c_T}$ and in immunoblotting membrane was reprobed with GAPDH to ensure equal loading. Results shown are obtained as mean of three independent experiments (P < 0.05).

![Graphical Representation of pMIR-REPORT luciferase constructs](image)

**Fig 4.6:** Graphical Representation of pMIR-REPORT luciferase constructs. Sequence for wild type binding sites and mutated binding sites are are depicted within green box.

### 4.2.4 Overexpression of hsa-miR-142-5p and Impaired TGFβ Signalling was Regulated by RV Encoded NSP5

To identify the viral protein(s) responsible for regulating miR-142-5p, HT29 cells were transfected with NSP1-NSP6 followed by assessing expression of miR-142-5p by RT-PCR. Results revealed 10±2 fold upregulation of miR-142-5p expression in NSP5 transfected cells (Fig 4.7A). Over expression of NSPs in transfected cells was verified by immunoblotting with specific antibodies (Fig 4.8 A). Furthermore transfection of NSP5 in 293T cells resulted in reduction of TGFβR2 and SMAD3 protein (Fig 4.7B), whereas no change was obtained at transcriptional level (Fig 4.8 B). For further studies, NSP5 expressing HT29 cell line (NSP5-HT29) was established by selecting the cells with Blasticidin (10μg/ml). Over expression of miR-142-5p in stable cell (NSP5-HT29) was confirmed by RT-PCR (Fig 4.7C). TGFβR2 and SMAD3 expression was partially restored in NSP5-HT29 cells transfected with hsa-miR-142-5p inhibitor (Fig 4.7D).
Figure 4.7: RV NSP5 Upregulates miR-142-5p and Subsequently Downregulates TGFβR2 and SMAD3.
(A) HT29 cells transfected with different RV NSPs followed by real time analysis after 24 h of transfection. Results reveal induction of miR-142-5p in cells transfected with NSP5. Fold change in transcript was calculated by normalizing relative gene expression to rnu6b using formula 2-ΔΔcT. Results shown are mean of three independent experiments (P < 0.05). (B) HT29 cells were transfected with CAG-NSP5 followed by immunoblotting after 24 h post transfection. NSP5 expression is correlated with decreased TGFβR2 and SMAD3 protein. Membranes were reprobed with GAPDH as internal control. (C) NSP5 expression in stable NSP5 HT29 cells was confirmed by immunoblot and elevated level of miR-142-5p was checked by real time analysis. (D) NSP5 over expressing HT29 cell was transfected with miR-142-5p inhibitor at increasing concentration. Whole cell lysates were prepared followed by immunoblotting analysis which indicates restoration of TGFβR2 and SMAD3 in presence of the inhibitor. GAPDH was used as an internal loading control. Results shown in (C upper panel) is obtained as mean of three independent experiments (P < 0.05).
Fig 4.8: (A) HT29 cells were transfected with different RV NSP-CAG vectors. Whole cell lysates were prepared and probed with specific NSP antibody to confirm expression of NSP-CAG plasmids. All membranes were reprobed with GAPDH as internal control. (B) HT29 cells were transfected with increasing concentrations of CAG-NSP5 followed by real time analysis after 24h of transfection. Results revealed no change in transcript level of tgfβr2 and smad3. Fold change in transcript was calculated by normalizing relative gene expression to gapdh using formula 2^{-ΔΔCT}. Results are obtained as mean of three independent experiments (P ≤ 0.05).

4.2.5 NSP5 Blocks TGFβ Non-Canonical Signalling Mediated Early Apoptosis to Ensure Viral Replication

Besides canonical i.e. SMAD dependent pathways, TGFβ can induce the apoptosis through TRAF6-TAK1-JNK/p38 pathway activation (Zhang 2009). Transcriptional upregulation of TGFβ (Fig 4.9B) in RV infected cells was observed. In addition TGFβ secretion from RV infected cells has also been reported earlier (Barreto et al., 2010). To ensure own propagation, RV would require to nullify TGFβ mediated cellular apoptosis. To assess this, HT29 cells were infected with RV-SA11 and whole cell lysates were prepared from 0-12 hpi. Immunoblot analysis revealed significantly reduced phosphorylation of p-38 MAPK, JNK and ERK1/2 after 3hpi (Fig 4.9A right panel). Although during early time point of RV infection significant phosphorylation of p-38 MAPK, JNK and ERK1/2 was observed upto 2 hpi followed by decrease from 3 hpi (Fig 4.9A left panel). Same cellular extracts were reprobed with both NSP3 and NSP5 antibody. As mentioned earlier, NSP3 expression was considered as establishment of RV infection but it was not related with phosphorylation of the above mentioned proteins. However, reduced phosphorylation of p-38 MAPK, JNK and ERK1/2 were correlated with expression of NSP5 at 3
In a parallel experiment, HT29 and NSP5-HT29 cells were incubated with TGFβ1 ligand for 24 h and whole lysates were prepared. Immunoblotting analysis revealed enhanced phosphorylation of p38 MAPK, JNK and ERK1/2 proteins in HT29 cells after TGFβ1 incubation. In NSP5-HT29 cells, TGFβ1 treatment failed to enhance their phosphorylation (Fig 4.9B). On the contrary, HT29 cells, transfected with hsa-miR-142-5p mimic, exhibited reduced phosphorylation of p-38 MAPK, JNK and ERK1/2 following TGFβ1 treatment (Fig 4.9B). However, in HT29-NSP5 cells, transfected with miR-142-5p inhibitor and treated with TGFβ1, enhanced phosphorylation of these proteins was observed (Fig 4.9B). Consistent with the activation of p-38 MAPK, JNK and ERK1/2, a concomitant reduction in c-JUN phosphorylation was also observed in RV-SA11 infected HT29 cells (data not shown). Correspondingly, HT29 cells incubated with TGFβ1 ligand exhibited induced phosphorylation of c-JUN, but in NSP5-HT29 cells TGFβ1 failed to induce phosphorylation of c-JUN (data not shown). Overall, the results indicated that NSP5 regulates expression of hsa-miR-142-5p which further effectively inhibits TGFβ signalling.

TGFβ can induce the apoptosis via phosphorylated p-38 MAPK, JNK and ERK1/2 i.e. non-canonical pathways. Apoptosis was measured in this study by measuring DNA fragmentation using Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay according to the manufacturer’s protocol. Results revealed a significant increase in TUNEL-positive cells in TGFβ1 treated HT29 cells (46±5%) compared to TGFβ1 treated NSP5-HT29 cells (10±3%) (Fig 4.10A). In comparison, hsa-miR-142-5p mimic transfected HT29 cells showed significantly reduced cell death (18±4%) and hsa-miR-142-5p inhibitor transfected NSP5-HT29 cells show increased cell death (30 ±4%) upon induction with TGFβ1 (Fig 4.10A). Furthermore, immunoblot analysis of the whole cell lysates of TGFβ1 incubated HT29 cells revealed cleavage of caspase 3 and 9 which was not observed in TGFβ1 incubated NSP5-HT29 cells (Fig 4.10B). In addition, absence of caspase 9 and 3 cleavage in hsa-miR-142-5p mimic transfected HT29 cells in presence of TGFβ1 (Fig 4.10B) confirmed anti apoptotic role of hsa-miR-142-5p.
Figure 4.9: NSP5 Downregulates TGFβ Non-canonical Pathways by Upregulated hsa-miR-142-5p.

(A) HT29 cells were infected with RV-SA11 at an M.O.I. of 2. Whole cell lysates were prepared at specified time point and immunoblotting performed with specified antibodies. Membranes were reprobed with GAPDH to confirm equal loading. (B) HT29 and NSP5 expressing HT29 cells were transfected with hsa-miR-142-5p mimic and hsa-miR-142-5p inhibitor respectively followed by incubation with TGFβ1 ligand. Whole cell lysates were prepared followed by analysis of phosphorylation of JNK, p-38 MAPK, ERK. NSP5 overexpression resulted in modulation of TGFβ signaling. Membranes were reprobed with GAPDH as internal control.
4.2.6 TGFβ Induces EMT Transition in HT29 Cells Whereas Stable Expression of NSP5 Blocks EMT in HT29 Cell

Previous studies have related TGFβ signalling with the regulation of EMT in HT29 cells (Ellenrieder et al., 2001; Chen et al., 2012). To further demonstrate whether ectopically expressed RV NSP5 can inhibit TGFβ1 induced EMT in HT29 cells, both HT29 and NSP5-HT29 cells were incubated with TGFβ1 ligand. After 48 h of incubation, bright field microscopy at 20X revealed a dramatic change of the cellular phenotype of HT29 cells. Cells became dissociated with reduced cell-cell contacts and acquired a more spindle phenotype (Fig 4.11A). These phenotypic characteristics were consistent with cells undergoing the EMT (Ellenrieder et al. 2001; Christiansen et al., 2006). The morphological changes induced by TGFβ1 were not observed in
NSP5-HT29 cells (Fig 4.11A). HT29 cells after TGFβ1 treatment showed enhanced N-Cadherin and reduced E-Cadherin expression whereas transfection with hsa-miR-142-5p mimic showed opposite results (Fig 4.11B). Similarly NSP5-HT29 cells transfected with miR-142-5p inhibitor and treated with TGFβ1 also revealed increased N-Cadherin and decreased E-Cadherin expression (Fig 4.11B). Furthermore, immunostaining TGFβ1 treated HT29 cells with E-Cadherin antibody followed by confocal microscopy revealed reduced expression of E-Cadherin and transformation to a spindle shape (Fig 4.12). But ectopic expression of RV-NSP5 or miR-142-5p keeps the cells in epithelial state, indicated by circular shape and increased E-Cadherin expression even after treatment with TGFβ1 (Fig 4.12). Overall, the results suggested that HT29 cells shift towards mesenchymal status from epithelial phenotype when treated with TGFβ1 ligand but presence of NSP5 or miR-142-5p inhibits the EMT.

Considering that the EMT is characterized by enhanced cellular motility and invasion (Kalluri et al., 2009; Duldulao et al., 2012), migratory capacity and invasive potential of HT29 and NSP5-HT29 cells after TGFβ1 treatment was analyzed using wound healing assay. The assay further confirmed a significant inhibitory effect of NSP5 overexpression on TGFβ1 induced migration in HT29 cells two days and four days after incision of the wound (Fig 4.13). In addition to wound healing assay, soft agar assay which confirms anchorage independent growth in semisolid medium was done. HT29 cells produce bigger colonies compared to NSP5-HT29 cells on soft agarose within 2 weeks after TGFβ1 induction (Fig 11C). Overall, it can be hypothesized that NSP5 over expression through induction of hsa-miR-142-5p not only prevents TGFβ induced apoptosis during RV replication but it can also prevent TGFβ1 induced EMT in cells.
Figure 4.11: Acquisition of NSP5 resistant morphologic changes consistent with EMT and anchorage-independent growth in HT29 cells following TGFβ1 treatment. (A) HT29 and NSP5 expressing HT29 cells were treated with TGFβ1 ligand (5ng/1000μl). After 48 h of incubation HT29 and NSP5-HT29 cells were assessed for morphologic changes consistent with EMT by bright field microscopy. Spindle-shaped cells with loss of polarity (green arrows), increased intercellular separation (red arrows), and pseudopodia (white arrows) were noted in the HT29 cells but not in NSP5 expressing cells. Figure shown is representative of three experiments. (B) HT29 and NSP-HT29 cells transfected with hsa-miR-142-5p mimic and hsa-miR-142-5p inhibitor respectively followed by incubation with TGFβ1 ligand. Whole cell lysates were prepared and western blotting performed with N-Cadherin and E-Cadherin antibody. Membranes were reprobed with GAPDH confirming equal loading. (C) HT29 cells and NSP5 expressing HT29 cells were plated and treated with TGFβ1 for 2 weeks followed by staining with Crystal Violet. Pictures of colonies grown on soft agarose were shown which reveal large colonies in HT29 compared to NSP5 expressing HT29. All experiments were replicated in triplicate.
Fig 4.12: HT29 and NSP5-HT29 cells were treated with TGFβ ligand (5ng/1000μl). After 48 h of incubation cells were fixed with paraformaldehyde and stained with E-Cadherin antibody followed by rhodamine-labeled (red) rabbit secondary antibody. The cells were visualized with confocal microscope at 100X magnification. Microscopic images clearly revealed spindle shape of HT29 cells (indicated by white arrow) and reduced expression of E-Cadherin after TGFβ treatment. Indeed NSP5-HT29 cells and miR-142-5p overexpressed HT29 cells retain their circular shape (indicated by yellow arrow) and normal expression of E-Cadherin after TGFβ incubation.
Fig 4.13: Scratch wound healing assay. HT29 cells and NSP5 expressing HT29 cells were seeded 35mm dishes, transfected with hsa-miR-142-5p mimic and inhibitor. 24 h transfection cells were scratched at 70-80% confluency followed by incubation with TGFβ1 up to 4 days. Migration distance was measured at 2 days and 4 days after TGFβ1 treatment. NSP5 expressing cells significantly reduced rate of migration in HT29 cells incubated with TGFβ1.

4.3 DISCUSSION

Host miRNA expression is significantly influenced by viral infection, which can be attributed to both host antiviral defense and viral factors altering the cellular environment (Jopling et al., 2005; Houzet et al., 2011; Kang et al., 2011; Liu et al., 2011; Russo et al., 2011; Riley et al., 2012; Skalsky et al., 2012; Wang et al., 2012). In course of this study we have investigated the impact of RV infection on cellular miRNAs and their subsequent downstream signalling. Micro array analysis has identified significant number of differentially expressed cellular miRNAs during RV-SA11 infection in vitro (Fig 4.1A). A subset of upregulated and downregulated miRNAs were further validated in human (KU) and simian (SA11) strains using the specific primers (Fig 4.2A and B). Among differentially expressed miRNAs, hsa-miR-142-5p was identified to have...
proviral function. Transfection of hsa-miR-142-5p inhibitor in HT29 cell effectively reduced RV replication (both SA11 and KU) as evidenced by reduced mRNA, protein and viral titer in the presence of the inhibitor (Fig 4.3 and Fig 4.4).

Since hsa-miR-142-5p showed proviral function, its regulation and function was studied in detail. RV-NSP5 was found to modulate the expression of miR-142-5p, which is a previously unknown property of NSP5 (Fig 4). NSP5 is an O-glycosylated phosphoprotein that self-assembles into dimers and has nonspecific RNA-binding activity (Gonzalez et al., 1991; Vende et al., 2002). NSP5 interacts with NSP2 (Poncet et al., 1997; Afrikanova et al., 1998), resulting in up-regulation of its hyperphosphorylation, and formation of viroplasm-like structures (Afrikanova et al., 1998; Fabbretti et al., 1999). It is also known to interact with the virus polymerase VP1 and the non-structural 11 kDa protein NSP6 (Matthion et al., 1991; González et al., 1998). The present study has explored the potential of NSP5 in upregulation of hsa-miR-142-5p for successful viral propagation. NSP5 overexpression was also found to induce expression of Serum Response Factor (SRF) protein which is considered as one of the important transcriptional factors regulating the expression of hsa-miR-142-5p (Data not shown). Based on this result it can be hypothesized that NSP5 may regulate the expression of miR-142-5p through SRF (Marsico et al., 2013).

KEGG pathway analysis tool identified different components of TGFβ signaling as direct targets of hsa-miR-142-5p; concurrently our results also showed downregulation of two key components of TGFβ signalling (TGFβR2 and SMAD3) during both RV infection and NSP5 overexpression (Fig 4.5, 4.7). Luciferase reporter assays further confirmed the presence of target sequence of the miRNA within the 3’ UTR region of both the proteins (Fig 4.5E). Mutations within the target sequences enhanced the luciferase intensity as a consequence of non binding of the candidate miRNA to the target sequence (Fig 4.5E). HT29, categorized as a MSS colon carcinoma cell (Polyak et al., 2009), has nonsense mutation in SMAD4 (Pino et al., 2010). This abrogates the canonical pathways of TGFβ although the non-canonical pathways such as phosphorylation of JNK, p-38MAPK, ERK1/2 etc, are activated following TGFβ treatment. RV infection leading to TGFβ activation and secretion has been reported earlier (Barreto et al., 2010) and has also been confirmed during this study (Fig 4.5B). TGFβ in turn induces phosphorylation and activation of JNK, p-38 MAPK, ERK1/2, all of which were phosphorylated immediately after RV infection (0 hpi-3 hpi) (Fig 4.9A). However phosphorylation of these proteins was reduced
with the progression of infection (> 3 hpi) which is probably related with the expression of viral proteins and induced miR-142-5p (Fig 4.9A). Furthermore, enhanced phosphorylation of JNK, p-38 MAPK, ERK1/2 was observed in HT29 cells but not in NSP5-HT29 cells following treatment with TGFB1 ligand (Fig 4.9C). Concurrently, transfection of hsa-miR-142-5p mimic in HT29 cells and hsa-miR-142-5p inhibitor in NSP5-HT29 cells reversed the results (Fig 4.9C) This confirms the role of miR-142-5p in regulating non-canonical pathways of TGFB. Phosphorylation of JNK and p-38 MAPK but not ERK at 8 hpi in RV-SA11 infected (10 M.O.I.) HT29 cell (Holloway et al., 2006) has been reported earlier. However, during this study, time course analysis revealed the increased phosphorylation of JNK and p-38 MAPK at 0.5-2 hpi, with significant reduction at later time points (3-10 hpi) (Fig 4.9A). Reduced phosphorylation of c-JUN was also observed during RV infection which is in agreement with the reduced phosphorylation of p-38 MAPK, JNK and ERK1/2.

TGFB1 treated HT29 cells revealed increased cell death and cleavage of caspase 9 and 3 whereas in presence of hsa-miR-142-5p mimic, cleavage of caspases and cell death were significantly reduced (Fig 4.10). Concurrently in TGFB1 treated NSP5-HT29 cells, cleavage of caspases (9 and 3) and TUNEL-positive cells were significantly low compared to HT29 cells (Fig 4.10) but ectopic expression of hsa-miR-142-5p inhibitor resulted in increased cleavage of caspases. Based on these results it is postulated that apoptotic effect of TGFB1 on HT29 cells can be neutralized either by overexpression of NSP5 or using hsa-miR-142-5p mimic.

In addition to modulation of apoptosis, TGFB also induces EMT in HT29 cells (Halder et al., 2005; Pino et al., 2010). EMT is initiated as a result of TGFB induced phosphorylation and activation of ERK1/2, JNK and p38 MAPK in HT29 cell. Since previous results confirmed the downregulation of TGFB non-canonical signaling in cells overexpressing NSP5 through upregulation of miR-142-5p, EMT was analyzed in NSP5-HT29 cells. As expected, TGFB induced EMT was significantly impaired in NSP5-HT29 cells (Fig 4.11, Fig 4.12). Supportingly, fast cellular migration and large colony in soft agarose assay were observed in HT29 cells upon TGFB1 induction but not in NSP5-HT29 cells (Fig 4.11, Fig 4.13). EMT is considered as an important factor of cancer progression. It has been associated with features of the advanced stage of the disease including metastasis, resistance to chemotherapy and generation of cancer cells with stem cell-like characteristics (Polyak et al., 2009). In micro satellite stable cells lacking SMAD4, TGFB induced growth inhibition is impaired, but pro-oncogenic functions remain
active. Inhibition of TGFβ induced non-canonical signalling of hsa-miR-142-5p can be exploited as a treatment modality of microsatellite stable colon cancers.

On the whole, the results unravel differential expression of miRNAs during RV infection in the host cells. Role of RV encoded NSP5, in absence of any other viral protein or viral infection, in regulating the induction of hsa-miR-142-5p has been established. This study also highlights the mechanism by which RV exploits the cellular miRNA for its own survival by targeting TGFβ mediated early apoptosis.

**Figure 4.14: Graphical Representation of Signalling.** RV infection leads to upregulation of TGFβ which may lead to early apoptosis preventing virus infection. RV counteracts this by upregulation of hsa-miR-142-5p. RV NSP5 upregulate this miRNA which target several candidates of TGFβ signaling. As HT29 is a microsatellite stable cell, canonical pathways are not activated (indicated by red cross in the figure). By blocking non-canonical pathways (JNK, p-38MAPK, ERK1/2 mediated) hsa-miR-142-5p block apoptosis and epithelial to mesenchymal transition. Dotted lines indicate findings of this study.