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MicroRNAs in HIV-Associated Nephropathy (HIVAN)

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Abstract

MicroRNAs (miRNAs) play a critical role in multiple biological and metabolic processes. Recent studies suggested that miRNAs are critical in the maintenance of glomerular homeostasis in both physiological and pathological states. However, the role of miRNAs in the pathogenesis of HIV-associated nephropathy (HIVAN) has not been studied. In the present study, we have used a microarray-based approach in combination with real-time PCR to profile the miRNA expression patterns in HIV-1 transgenic mice (Tg26). Our results showed that 13 miRNAs, which belong to 11 miRNA families, were down regulated in HIVAN when compared with control mice. These miRNAs were classified into 20 functional categories. In *in vitro* studies, we examined the expression of specific miRNAs in HIV-1 transduced human podocytes. Our results showed that HIV-1 down regulated miRNAs expression, specifically of miR-200 and miR-33. These studies suggest that miRNAs contributed to the development of the proliferative phenotype of HIVAN. Further functional analysis of these miRNAs in HIVAN animal model will not only enhance understanding of the pathogenesis but would also lead to the development of therapeutic strategies for HIVAN patients.

Keywords

microRNAs; HIV-associated Nephropathy; HIV Transgenic mice; podocytes; epithelial mesenchymal transition

MicroRNAs (miRNAs) are a family of small, 20- to 22- nucleotide (nt)- long noncoding RNAs that have been implicated in the regulation of multiple biological processes (Baskerville et al., 2005; Landgraf et al., 2007). These endogenously produced transcripts play important roles in gene regulation *via* translational repression, inducing mRNA degradation, or transcriptional inhibition (Ding et al., 2009). MiRNAs transcribed initially originated as long primary miRNAs (pri-miRNAs) that are then processed in the nucleus by the enzyme Drosha, yielding precursor miRNAs (pre-miRNAs) which exhibit a characteristic stem-loop (hairpin) structure (Winter et al., 2009). These are exported into the cytosol and further cleaved into functional small-interfering RNAs (mature miRNAs) by the RNase Dicer. One strand of the mature miRNA enters the RNA-induced silencing complex (RISC) and binds to the 3'-untranslated region of the target mRNA (Krol et al., 2010).

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Conflicts of interest

"The authors declare that they have no conflict of interest."

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MiRNAs play an important role in multiple biological and metabolic processes, including developmental timing, signal transduction, and cell maintenance and differentiation (Saal et al., 2009; Liang et al., 2007). Recent studies support and confirm that miRNAs are critical in the maintenance of glomerular homeostasis and the progression of renal disease (Baskerville et al., 2005; Chandrasekaran et al., 2012). Documenting miRNA expression in normal states as well as in different diseased states of kidney provides tissue-specific/enriched miRNAs, which could help in pinpointing the functional role of specific miRNAs in the pathological processes (Kasinath et al., 2011; Sarah et al., 2008; Liu et al., 2004; Bhatt et al., 2011; Kato et al., 2009). Based on miRNA microarray, profiles of miRNAs expression have been reported in several kidney diseases, such as polycystic kidney disease (Pandey et al., 2008), diabetic nephropathy (Lorenzen et al., 2011), ischemia reperfusion injury (Godwin et al., 2010), renal cancer (Gottardo et al., 2007), hypertensive nephrosclerosis (Wang et al., 2010) and kidney fibrosis (Chung et al., 2010; Wang et al., 2011). However, to our knowledge the profile of miRNAs expression and the functional roles of specific miRNAs in HIV-associated nephropathy (HIVAN) have not been investigated till now.

HIVAN is the third most common cause of end-stage disease in the HIV-1-seropositive black American males, which is characterized by collapsing focal segmental glomerulosclerosis and microcystic dilatation of tubules (Atta et al., 2010; Marras et al., 2002). However, the exact mechanisms which contribute to the pathogenesis of HIVAN are not clear. Studies conducted in our laboratory demonstrated that epithelial-mesenchymal transition (EMT) in renal epithelial cells could contribute to the development of proliferative phenotype in HIV-1 transgenic mice (Tg26), the most commonly used mouse model of HIVAN (Kopp et al., 1992; Yadav et al., 2010; Yadav et al., 2012). In addition, mammalian target of rapamycin (mTOR) pathway was activated in HIVAN mice (Kumar et al., 2010; Rehman et al., 2012; Nicoletti et al., 2011). Rapamycin, an inhibitor of mTOR pathway, attenuated the progression of renal injury in HIVAN mice (Kumar et al., 2010; Bonegio et al., 2005). To date, the role of miRNA in the regulation of these processes has not been reported.

Recent data revealed that miR-200 inhibits EMT in cancer cells by targeting Zeb1 and Zeb2, the negative transcription factors of E-cadherin (Gregory et al., 2008; Park et al., 2008). Another study demonstrated that miR-200 could prevent TGF- β induced EMT of renal tubular cells (Kato et al., 2011), thus suggesting that miR-200 might be involved in the progression of HIVAN. Furthermore, the potential role of miRNA in mTOR regulation has previously been reported in human cancer cells (Oneyama et al., 2011; Fornari et al., 2010). Inhibition of mTOR function or introduction of miR-99a showed suppressed tumor growth in certain types of human cancer cells (Oneyama et al., 2011; Nagaraja et al., 2010). Recent report showed that miR-199a regulates mTOR and inhibits tumor cells growth (Fornari et al., 2010). In cardiac cells, miR-199a is a master regulator of a hypoxia-triggered pathway and can be exploited for preconditioning cells against hypoxic damage (Rane et al., 2009). However, the miRNA expression profile and their potential role in the pathological processes of HIVAN need to be further investigated.

In the current study, we addressed a number of important questions: Does HIV-1 modulate miRNA expression in kidney cells? Does the change of miRNA expression contribute to the development of HIVAN? And does miRNA play a role in regulation of the EMT and mTOR pathway in kidney cells? We used a microarray-based approach to profile the miRNA expression patterns in HIV-1 transgenic mice. The miRNAs microarray based expression was further validated by quantitative PCR in HIV-1 transduced human podocytes. Our results showed that several miRNAs were downregulated in HIVAN mice. The expression patterns of miRNAs were similar in HIV-1 transduced human podocytes *in vitro*. These studies suggested that these miRNAs could mediate the pathological processes of HIVAN.

Materials and Methods

Animals

The Ethics Review Committee for Animal Experimentation of Feinstein Institute for Medical Research-North Shore LIJ Health System approved the experimental protocol. HIV transgenic mice (Tg26), which has the proviral transgene, pNL4-3:d1443, encoding all the HIV-1 genes except *gag* and *pol*, were kindly gifted by Prof. Paul E. Klotman (Baylor Medical College, Houston, TX) and were maintained in a laminar-flow facility (Animal Facility of Feinstein Institute). Breeding pairs of FVB/N mice were obtained from Jackson Laboratories (Bar Harbor, ME). Genotyping for Tg26 was performed by DNA amplification of HIV-1 gene using the primers: HIV forward 5'-ACATGAGCAGTCAGTTCTGCCGCAGAC-3' and HIV- reverse 5'-GTGTGGACGCGTAGTCTCAGGAAC-3'.

Renal histological assessment

Kidneys from Tg26 and FVB/N mice were harvested, longitudinally bisected and immersed in 10% formalin for 24 hours and then transferred into 100% ethanol. Tissues were embedded in paraffin and 5µm sections were stained with H&E and PAS. Slides were coded and examined under light microscopy for glomerular injury. Seventy glomeruli per section were examined for scoring; only glomeruli which were sectioned at or close to the hilum were considered. Glomerular lesions were classified as segmental glomerular sclerosis (SGS), global glomerular sclerosis (GGS), and collapsing glomerular sclerosis (CGS).

RNA extraction microRNA microarray

Total RNA was extracted using Trizol (Invitrogen), according to the manufacturer's instructions. Expression of 300 miRNAs (Sanger Version 16.0) was analyzed by microRNA Microarray Service, LC Sciences LLC, Houston, TX. Background was subtracted with regression-based mapping on 5–25% of lowest intensity data points excluding blank spots. Transcripts with undetectable signals lower than 3x background, incorporating spot analysis parameters, were not included. Later, we excluded transcripts with <500 arbitrary signals as these are not identified by quantitative real-time reverse-transcription polymerase chain reactions (qRT-PCR). The ratio of differentially expressed miRNA in Tg26 and FVB/N mice by microRNA array analysis was transformed into log2 scale.

Cells and cell culture

Human podocytes (HPC) were kindly gifted from Dr. Moin A. Saleem (Children's Renal Unit and Academic Renal Unit, University of Bristol, South Mead Hospital, Bristol, UK). The cells were conditionally immortalized by introducing temperature-sensitive SV40-T antigen and additionally transfected with a human telomerase construct. These cells proliferate at a permissive temperature of 33°C and enter growth arrest at a nonpermissive temperature of 37°C. The growth medium contains RPMI 1640 supplemented with 10% fetal bovine serum, 1× penicillin-streptomycin, 1 mM L-glutamine, 1× insulin, transferrin, and selenium (ITS) (Invitrogen) to promote expression of T antigen.

Production of Pseudotyped Retroviral Supernatant

The replication defective virus, the same proviral construct used to generate Tg26 transgenic mice, was prepared as previously described (Husain et al., 2009). Briefly, the HIV-1 proviral pNL4-3 clone was reconstructed by substituting *gag/pol* genes with GFP reporter gene to produce a pNL4-3:ΔG/P-GFP construct. The HIV-1 *gag/pol* and VSV.G envelope genes were provided in *trans* using pCMV R8.91 and pMD.G plasmids, respectively. The viral supernatants were produced by co-transfection of the plasmids into 293T cells with

Transfection Reagent (Effectene; Qiagen Inc.). The negative control virus produced from pHR-CMV-IRES2-GFP-ΔB construct containing HIV-1 long term repeats and GFP reporter gene. The viral supernatant was concentrated by ultracentrifugation at $50,000 \times g$ at 4°C for 3 hr. The virus concentration was titrated by infecting 293T cells with 10-fold serial dilution and FACS analysis for GFP was performed.

Podocyte transduction

HPC were plated in 100 mm plates at a density of 2×10^6 cells with growth medium at a permissive temperature. The cells were infected with pseudotyped retroviral supernatant at MOI 1 of GFP expressing units by tilting at every 20 min interval, and replaced with fresh medium after 2 hr. The control vector was used as the negative control. The cells were harvested after 72 hr of infection, and total RNA was extracted for qPCR analysis.

Real-time PCR

MiRNA expression in renal tissues or human podocytes was performed by qRT-PCR using ABI Prism 7900HT sequence detection system. Briefly, cDNA was synthesized using NCode™ EXPRESS SYBRR® GreenER™ miRNA qRT-PCR Kit (Invitrogen, Life Technologies Corporation, CA) according to the manufacturer's instructions. SYBR Green assays were performed using forward primers from sense strands of specific mature miRNAs and universal reverse primer (Invitrogen). U6 small nuclear (sn) RNA was used as endogenous control to normalize the respective miRNA cycle thresholds (Ct) values. The relative expression level of miRNA was calculated using modified $2^{-\Delta\Delta C_t}$ method (Yuan et al., 2006).

Results

HIV-1 transgenic mice (Tg26) displayed HIVAN phenotype

Kidney sections of Tg26 mice displayed progressive glomerular lesions, e.g. at 4wks of age the average of 4% of glomeruli showed SGS, 13% of glomeruli showed CGS, and no glomeruli with GGS were identified. At 8 wks of age the average of 12% glomeruli showed SGS, 21% of glomeruli showed GGS, and 36% of glomeruli showed CGS (Fig. 1).

Differential miRNAs expression in Tg26 mice

A global miRNA expression profile (total number of 1096 miRNAs analyzed) in Tg26 and FVB/N was developed by microarray. MiRNAs expression levels in Tg26 differed considerably from control FVB/N mice. After excluding miRNAs either expressed at extremely low levels (<500) or statistically not significant ($p > 0.05$), we got 21 miRNAs which belong to 14 different families differentially expressed in Tg26 mice kidneys compared with FVB/N mice kidneys ($p < 0.05$). Of these miRNAs, the following miRNAs were downregulated: miR-497, -140*, -194, -29a, -378*, -145, -30a, -22, -27b, -30c, -378, -143, -181a; whereas miR-1895, -669p*, -669f-3p, -669a-3p, -466f-3p, -466h-3p, -466q, -466i-3p were upregulated (Fig. 2A). Among them, miR-497, -140*, -194, -29a, -378*, -145 were statistically significant at a level of $P < 0.01$.

By comparing the level of miRNAs expression, miR-497, -140*, -194, -29a, -378* and miR-145 were considerably decreased (>1.0 fold); whereas the level of increased miRNAs which belong to 3 miRNAs families, including miR-466, -699 families and miR-1895, were less than 1.0 fold (Fig. 2B). To our knowledge, this is the first time that the expressions of miR-466, -699 families and miR-1895 have been reported in kidneys. Using a computational approach, we identified that these miRNAs are tissue-specific and are not conserved in human tissues.

We confirmed the changes in the expression patterns of some of these miRNAs in kidney tissues using quantitative real-time PCR (qPCR). We also examined and analyzed the expression of the some other miRNAs by qPCR which did not show statistically significant changes in microarray but could potentially play an important role in kidney diseases. Consistently with the microarray analysis, qPCR demonstrated that expression of miR-699*, 466h, -466i, -466f were upregulated; whereas the expression of miR-140, -145, -16, -467, -378, -30c, -331, -497, -30a were diminished in 8 wks old Tg26 mice. The changes ranged from 1.5 to 10.0 folds (Fig. 3).

Microarray analysis of genes involved in HIVAN

Our microarray profiling revealed changes in miRNA expression patterns, indicating that miRNAs play a role in the regulation of development of HIVAN. In order to understand the functional meanings of the differentially expressed miRNAs, we carried out the biological pathway analysis of the predicted target genes of these miRNAs.

We mapped the differentially regulated genes from miRNA target prediction and functional annotations using TargetScan 6.2 (<http://www.targetscan.org>) and miRDB (<http://mirdb.org>). Only commonly predicted targets for different miRNAs were taken for further analysis. Altogether, from both TargetScan and miRDB, a number of 600 genes were identified as miRNA targets. The biological functions of these genes were further categorized into 20 functional groups (Fig. 4), based on the PANTHER classification system (version 7.2) analyses (<http://www.pantherdb.org/panther>).

To identify miRNA-target interaction, we mapped the possible targets of these miRNAs and the molecular pathways they are involved in. We demonstrated 56 genes which could be identified as miRNA targets. These are associated with 36 pathways (Table 1). Of these, major signal transduction pathways were previously described as being involved in various aspects of kidney diseases, such as the PI3 signaling pathway, Wnt pathway, TGF β pathway, cadherin signaling pathway, P53, and the JAK/STAT signaling pathway (Chung et al., 2010; Wang et al., 2011; Kato et al., 2011; Oneyama et al., 2011; Jin et al., 2012; Bhatt et al., 2010; Mori et al., 2009). Interestingly, we found that numbers of miRNAs and their target genes, e.g. Mknk2, Dusp2, Mapk1, Egr1, Arrb1 (Table 1), were involved in oxidative stress response, as well as Angiotensin II-stimulated signaling pathway, which our group has recently demonstrated to be critical in the mechanism of development of HIVAN (Ren et al., 2012; Kumar et al., 2010).

miRNA expression in human podocytes

To examine whether HIV-1 induced the expression changes of miRNA, we transduced HPC with a recombinant retrovirus encoding HIV-1 genes. We selected miRNAs which have been earlier shown to be expressed in kidneys, and analyzed them with real-time PCR. Our results demonstrated that miR-9, -204, -124, -7 and miR-139 were upregulated in HIV transduced HPC cells; whereas, miR-200b, -200c, -33, -211, -375 and miR-22, were downregulated (Fig. 5). Among them, miR-200b, -200c and miR-33 were dramatically decreased (folds>15), indicating these miRNAs could play a major role in HIV-1 induced changes in podocyte phenotype and contribute to the pathologic processes of HIVAN.

Discussion

The present study highlights two important aspects. Firstly, we have established a differential profile of miRNA expression in HIV-1 transgenic mice (Tg26), which had been well characterized and the most commonly used mouse model of HIVAN (Kopp et al., 1992; Kumar et al., 2010; Rehman et al., 2012; Nicoletti et al., 2011; Bonegio et al., 2005).

Secondly, we have identified the differential expression of miRNAs in HIV-1 transduced human podocytes. These studies provide a fundamental baseline in understanding the role of miRNAs expression in human HIVAN.

Previous studies have suggested that several miRNAs were enriched in human kidney, e.g., miR-192, -194, -204, -215, and miR-216 (Sun et al., 2004). MiRNA misexpression has been implicated in the pathogenesis of various kidney diseases and cancer, e.g., polycystic kidney disease, diabetic nephropathy, immunoglobulin A nephropathy, lupus nephritis (Pandey et al., 2008; Lorenzen et al., 2011; Dai et al., 2009). However, miRNAs involved in HIVAN have not been investigated so far. Our microarray analysis revealed that 13 miRNAs, which belong to 11 miRNA families, were down regulated in the kidneys of HIVAN mice. These miRNAs were associated with 20 functional categories (Table 1 and Fig. 4). Comparison of these differentially regulated miRNAs and their potentially targeted genes, revealed that these genes were involved in critical biological processes, such as Wnt signaling (Jin et al., 2012), mTOR signaling (Oneyama et al., 2011), angiotensin II-stimulated signaling (Ren et al., 2012), cadherin signaling (Kato et al., 2011), MAPK (Mitogen-Activated Protein Kinase) signaling (Mori et al., 2009), and TGF- β pathway (Chung et al., 2011). This suggests that miRNAs could play a crucial role in the development of HIVAN. Further functional roles of the specific miRNAs in HIVAN are being pursued further.

Recent studies have implicated that miRNAs are species specific. Landgraf et al profiled miRNAs in mouse, rat, and human tissue, where it was found that only approximately 70 distinct miRNAs were present in each library (Landgraf et al., 2007). Our studies indicated that 8 miRNAs, which belong to 3 miRNAs families, were upregulated in kidneys of HIVAN mice. Interestingly, these miRNAs have not been reported in human kidneys, and were not found to be conserved in human tissues using computational analyses.

Our previous studies have implicated several pathways that are involved in the pathogenesis of HIVAN, e.g., epithelial mesenchymal transition (EMT) was demonstrated to contribute to the proliferative phenotype of HIVAN (Yadav et al., 2010; Yadav et al., 2012). Tg26 mice showed enhanced renal tissue expression of ZEB2 and diminished expression of E-cadherin. Recent studies indicated that specific miRNAs, including miR-200 family (miR-200a-c, miR-141 and miR-429), mediated EMT in cancer cells as well as kidney cells by targeting Zeb1 and Zeb2 (Gregory et al., 2008; Park et al., 2008; Jin et al., 2012; Xiong et al., 2012), thus suggesting that miR-200 might be involved in the progression of HIVAN. In the present study, we evaluated miR-200 expression in HIVAN mice. Microarray analysis showed that miR-200a-c ($\log_2$ of Tg26/Ctr = -0.30, -0.67, and -0.10 respectively) and miR-429 ($\log_2$ of Tg26/Ctr = -0.84) expression was decreased in Tg26 mice when compared to its control. Recent reports suggest that microRNA was differentially expressed between the renal cortex and medulla (Tian et al., 2008), but miR-200 was even more abundantly expressed in the medulla compared with the renal cortex (Kaucsár et al., 2010). Since podocytes were the most abundantly studied in the course of HIVAN, and are only a small fraction of cells in the renal cortex, this would explain that miR-200a-c expression did not show any significant differences in the whole kidney microarray analysis.

We further asked whether miR-200 was mediated in HIV-1 induced podocytes cellular phenotype changes in HIVAN. According to the previous studies, podocytes exhibit proliferative phenotype and loss of differentiation markers in collapsing focal segmental glomerulosclerosis (FSGS) of HIVAN (Husain et al., 2009). Recent studies demonstrated that miRNAs are essential for podocyte structure and function (Harvey et al., 2008; Shi et al., 2008; Ho et al., 2008). In this study, we transduced human podocytes with HIV-1 and evaluated miR-200 expression by real-time PCR. Our results indicated that miR-200 expression was decreased in HIV-1 transduced podocytes, suggesting that miR-200 could

mediate HIV-1 induced cellular phenotype transformation in podocytes and contribute to the pathogenesis of HIVAN. Further *in vivo* studies would be required to explore the functional significance of miR-200 family in the development of proliferative phenotype of HIVAN. This study is currently under investigation.

Activation of mammalian target of rapamycin (mTOR) is another mechanism in the development and progression of HIVAN (Kumar et al., 2010; Rehman et al., 2012; Nicoletti et al., 2011; Bonegio et al., 2005). However, the role of miRNA in mTOR regulation in HIVAN is still not quite clear. Recent efforts have been made to understand the potential role of miRNAs in mTOR regulation and the substantial data have been accumulated in human cancer cells. These studies demonstrated that miR-99, miR-100, and miR-199 could suppress tumor growth by targeting mTOR (Fornari et al., 2010; Nagaraja et al., 2010). Although our microarray analysis in whole kidney tissues did not show significantly decreased expression of these miRNAs, it would be worthwhile to further investigate the functional role of the miRNAs in the regulation of mTOR pathway in HIV-1 induced podocytes.

Recent reports suggest that renin-angiotensin system play an important role in the progression of HIVAN (Ren et al., 2012; Kumar et al., 2010). Angiotensin II induce nephrin dephosphorylation and podocyte injury (Ren et al., 2012). The subtype of angiotensin receptors, including AT₁R and AT₂R, were demonstrated to be decreased and were associated with the advancement of renal lesions in renal tissues of Tg26 mice (Kumar et al., 2010; Salhan et al., 2012). However, the role of miRNAs in the regulation of angiotensin II and HIV-1 induced podocytes injury is still not completely understood. During evaluation of the biological functions of miRNAs in HIVAN, we determined that some of these miRNAs might be involved in angiotensin II-stimulated signaling, e.g., miR-22, miR-378 (Table 1). The functional role of these miRNAs and the others in rennin-angiotensin system and HIVAN need to be investigated further.

In summary, we have established the miRNA expression profile in HIV-1 transgenic mice (Tg26) and involvement of these miRNAs in HIV-1 transduced human podocytes. Our results suggest that miRNAs play a critical role in the pathogenesis of HIVAN. Further functional investigation of these miRNAs in HIVAN animal model will not only enhance understanding of the pathogenesis but would also lead to new treatments for patients with HIVAN.

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Fig. 1. HIV-1 transgenic mice (Tg26) displayed the HIVAN phenotype. (A) Normal kidney section. (B) Kidney section of Tg26 mouse showing HIVAN. One glomerulus showing collapsed glomerular tufts with proliferation and hypertrophy of glomerular epithelial cells (black arrow); other glomerulus showing peripheral foci of segmental sclerosis (white arrow); dilated tubules filled with proteinaceous cast (black arrowheads) Original magnification $\times 200$. (C) Cumulative data showing percentage of normal glomeruli, glomeruli with mesangial expansion (ME), and sclerosed glomeruli (SGS, segmental glomerulosclerosis; GGS, global glomerulosclerosis; CGS, collapsing glomerulosclerosis) in FVB/N mice, 4 wks old Tg26 mice, and 8 wks old Tg26 mice.

Fig. 2.

Differential expression of miRNAs in kidneys of Tg26 and control FVB/N mice. (A) Heatmap depicts triplicate microarray hybridizations, revealing a subset of miRNAs that are differentially expressed in 8 wks Tg26 mice compared with control FVB/N. The color scale shown at the bottom: red denotes expression >0 and green denotes an expression <0 . (B) The relative expression of miRNAs level is present in log2 transformation. Total 8 miRNAs were upregulated (right) and 13 miRNAs were downregulated (left) in Tg26 mice ($P < 0.05$).

Fig. 3.

Validation of miRNAs expression by real-time quantitative PCR. MiR-466h-3p, miR-466i-3p, miR-466f-3p and miR-669* were upregulated and miR-140, miR-145, miR-16, miR-467, miR-378, miR-30c, miR-331, miR-497 and miR-30a were downregulated in 8 wks of HIVAN mice. U6 snRNA was used as endogenous control. The relative expression level of specific miRNA was calculated using modified $2^{-\Delta\Delta C_t}$ method.

Fig. 4. Overrepresented the functional categories of the differential expressed miRNAs in HIVAN. The pie-chart shows 20 functional categories and each pie represents a functional category with an overrepresentation of regulatory pathways of miRNA targets.

Fig. 5.

MiRNAs expression in HIV-1 induced human podocytes. Human podocytes were infected with HIV-1 virus for 72 hours. Real-time PCR analysis showed the expression of miR-9, miR-204, miR-124, miR-7 and miR-139 were upregulated; whereas miR-200b, miR-200c, miR-33, miR-211, miR-375, and miR-22 were decreased. U6 snRNA was used as endogenous control. The relative expression level of specific miRNA was calculated using modified $2^{-\Delta\Delta C_t}$ method.

Table 1

miRNAs and their targets

miRNA	Target Genes	Pathways	Log folds
miR-1895	Lig3, Pcdcd4, Fli1, Uba6, Yes1, Rasa2	Cell cycle, Induction apoptosis, PDGF pathway, Tyrosine kinase pathway, Cytokine-mediated signaling pathway, Ubiquitin proteasome pathway, B cell activation, Cadherin pathway, FGF pathway, EGF receptor pathway	0.78
miR-699p-3p	Nxph1, Atad2, Hspa4l, Ppap2b, Acta2	Cell communication, Ubiquitin proteasome pathway, Response to stress, Wnt pathway, Cadherin pathway, Cell cycle	0.65
miR-669a-3p	Ptpre, Cdc14a, Ank1, Egr1, Rab6, Ankrd45	Cell cycle, cell communication, Angiotensin II-stimulated signaling through G proteins and beta-arrestin, Response response to stress	0.61
miR-669f-3p	Ppm1H, Pcdh11Y, PcdhX, Pcdh8, Tubgcp5, Raph1, Kif20B, VEGFA, Nfkb1A, Stard8, Srgap3	Cell communication, Wnt signaling pathway, Cadherin signaling pathway, Cell cycle, Response to stimulus, Angiogenesis, VEGF pathway, Toll receptor pathway, Apoptosis pathway, PDGF pathway	0.56
miR-446f-3p	Ss18l1, Ube2h, Stat5b, Pcsk2, Arid1b, Foxg1	Apoptosis, Ubiquitin proteasome pathway, Angiogenesis, EGF receptor pathway, Negative regulation apoptosis, Ras Pathway, PDGF pathway, JAK/STAT pathway, Cell communication, Wnt pathway, Insulin/IGF pathway, TGF-beta pathway	0.69
miR-466h-3p	Adra1bRab11b	Apoptosis, Cell communication, Heterotrimeric G-protein signaling pathway, PDGF pathway	0.64
miR-466q			0.56
miR-466i-3p	Gli3, Srgap2, Csrnp3, Lmx1a, Nrf1, Strbp, Csnk1e, Csnk1d	Hedgehog signaling pathway, PDGF signaling pathway, Apoptosis, Cell cycle, Cell communication, Response to stress, Wnt pathway	0.46
miR-497	Atp7a, Zbtb34Prkar2a, Akt3Tlk1Traf3	Heterotrimeric G-protein signaling pathway, Ras Pathway, Hypoxia response via HIF activation, FAS pathway, p53 pathway feedback loops 2, Cell cycle, cytokine-mediated signaling pathway	-3.0
miR-140-3p	Fgf9, Pdgrfra, Ppp2r3a, Nlk, Wnt9a, Foxp2, Bcl2l1	FGF pathway, Angiogenesis, Wnt pathway, Cadherin pathway, Insulin/IGF pathway, TGF-beta pathway, Apoptosis pathway	-1.79
miR-194	Fzd6, Hbegf Slk Cxcl1 Fgfr3	Cadherin pathway, Wnt pathway, EGF, FGF receptor pathway, Integrin pathway, Phosphoserine phosphatase, TGF-beta pathway	-1.32
miR-29a	Col4a5, Col5a3, Col9a1, Atad2b, Dusp2, Pcdha4	Ubiquitin proteasome pathway, Integrin pathway, Oxidative stress response, MAP Kinase Phosphatases, Wnt pathway, Cadherin pathway	-1.17
miR-145	Epb4.1l5, Fli1, Uba6, Add3, Yes1, Rasa2, Nras	Dopamine receptor mediated signaling pathway, PDGF pathway, Ubiquitin proteasome pathway, Adenine and hypoxanthine salvage pathway, B cell activation, Cadherin pathway, PDGF pathway, FGF pathway, PI3 kinase pathway, VEGF pathway, Ras Pathway	-1.16
miR-30a	Cyp24a1, Mkrn3, Celsr3, Klhl20, Foxg1	Vitamin D metabolism, EGF, FGF receptor pathway, p53 pathway, PI3 kinase pathway, Cadherin pathway, Apoptosis pathway, Insulin/IGF pathway, TGF-beta pathway	-0.95
miR-30c			-0.54
miR-22	Arrb1, Esr1, Clic4, Prkar2a, Styx	Wnt pathway, Angiotensin II-stimulated signaling, cytokine pathway, p53 pathway, Dopamine receptor mediated signaling pathway, Beta1 adrenergic receptor pathway, Oxidative stress response	-0.87
miR-27b	Adora2b, Acvr1c, Fbxw7, En2, Fzd3, Cdh11, Mknk2	Heterotrimeric G-protein signaling pathway, Wnt pathway, TGF-beta pathway, Notch pathway, Angiogenesis, Cadherin pathway, Oxidative stress response, p38 MAPK pathway	-0.82
miR-378	Efna5, Arf2, Dcbld2, Frk, Mapk1	Integrin pathway, Insulin/IGF pathway, TGF-beta pathway, Interleukin pathway, PI3 kinase pathway,	-0.51

miRNA	Target Genes	Pathways	Log folds
miR-378*	Etv6, Abl2, Tpm3, Ube2e3, Igfbp5 Zfp97, Gpr22, Prtg	Apoptosis pathway, B cell activation, Angiotensin II-stimulated signaling, Ras Pathway	-1.16
miR-143		Cell communication, Cellular defense response, Intergrin pathway, Ubiquitin proteasome pathway, Cellular component organization	-0.47
miR-181a		Cell communication, Cell cycle, Induction of apoptosis, Cell adhesion	-0.42