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Original Article

Characterization of immortalized human podocytes infected with lentivirus as an *in vitro* model of viral infection-associated podocytopathy

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Abstract: A large number of studies have shown the association of kidney disease with viral infections in the body. Viral infections cause kidney injury in two manners, the systemic inflammation (cytokine storm) and the direct infection of kidney cells. Concerning direct viral infection of podocytes, the mechanism underlying virus-induced podocyte injury remains largely unknown and requires effective experimental models to facilitate its study. Here, we performed molecular characterization of immortalized human podocyte cell line (HPC) infected with lentivirus by RNA-seq. Bioinformatics analysis revealed a strong innate immune response in the cells, including interferon production and signaling. Meanwhile, activations of ferroptosis pathway and TNF-alpha signaling were also found, consistent with an impaired viability of the cells. Lentiviral infection also upregulated expression of APOL1 as observed in patients with HIV associated nephropathy (HIVAN) and diabetic nephropathy (DN). Interestingly, when the lentiviral infected cells were treated with Adriamycin (ADR), the ADR-associated signaling pathways were not interfered and remained activated as that in the cells treated with ADR only, suggesting that the virus and ADR have distinct mechanisms in damaging podocytes. Thus, the lentivirus-infected HPC cells represent a useful *in vitro* model of viral infection-associated podocytopathy.

Keywords: Lentiviral infection, podocyte, innate immune response, APOL1, adriamycin

Introduction

Viral infections of the body are known to be associated with various kidney diseases. The most recent example was the patients infected with COVID-19, who manifested with acute kidney injury (AKI) and collapsing focal segmental glomerulosclerosis (cFSGS) [1-4]. Infections of Hepatitis B and C viruses are also associated with chronic kidney disease (CKD) and are risk factors for CKD [5-7]. Human immunodeficiency virus (HIV) is well known for its effect of kidney disease induction [8, 9]. Furthermore, Epstein-Barr virus, cytomegalovirus, Dengue, Hantavirus, Parvovirus, hepatitis A virus, and hepatitis E virus are all associated with various forms of glomerular diseases, including rapidly progressive glomerulonephritis (RPGN), immune complex glomerulonephritis (ICGN), mesangial proliferative glomerulonephritis (MsPGN), diffuse proliferative glomerulonephritis (DPGN), thrombotic microangiopathy (TMA), collapsing FSGS, membranous glomerulopathy (MN), Henoch Shoenlein purpura (HSP), and membranoproliferative GN (MPGN) [10].

The viruses infecting the body can damage kidney in two major ways. One is to cause systemic inflammation and cytokine storm that act on kidney cells to cause damage; and the other one is to directly infect kidney cells and induce injury. Studies have shown that kidney diseases could occur in the absence of viruses in kidney cells, suggesting that systemic inflammation and the resulting cytokine storm upon viral infection may underlie kidney injury [11]. In contrast, in HIV-associated nephropanty (HIVAN) it has been clearly shown that the virus is present in tubular cells and podocytes of the patients [12-14] and that HIV expressed proteins can directly induce kidney cell damage [15, 16]; for example, the HIV virus was shown to be capable

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of damaging actin cytoskeletons in some cell types [17]. Besides HIVAN, more recent studies showed a direct infection of COVID-19 in kidney tissues to cause injury [18, 19].

Infections of viruses, including HIV, COVID-19, cytomegalovirus, Epstein-Barr-Virus, and parvovirus, are associated with the development of cFSGS in patients. cFSGS is characterized by proteinuria, glomerular condensation and capillary obliteration, podocyte hypertrophy, as well as tubulointerstitial injury. Concerning pathogenesis, cFSGS is linked not only with systemic inflammation but also with direct HIV infection of podocytes; in addition, its development involves apolipoprotein L1 (APOL1) gene. APOL1 is known to have risk variants that confer increased susceptibility to kidney diseases [20]. For example, the cFSGS susceptibility to COVID-19 and HIV infections has been associated with APOL1 risk alleles as shown by the observation that there was an increased prevalence of cFSGS associated with the infection of COVID-19 [21] and HIV [22] for individuals carrying the risk alleles. APOL1 is lowly expressed in human podocytes such that the APOL1 risk alleles do not have any effect normally. However, when APOL1 is upregulated upon infection of HIV [23] and COVID-19 [24, 25], the risk variants would exert their toxic effects on podocytes. Therefore, the risk variation and expression upregulation are two prerequisites for APOL1 to promote virus-induced podocyte injury.

In the present study, we characterized the immortalized human podocyte cell line (HPC) infected with lentivirus and found a strong activation of the innate immunity in the cells. In addition, APOL1 was found to be upregulated in the infected cells. We suggest that this is an appropriate *in vitro* model of viral infection of podocytes, hopefully facilitating the study of virus-associated podocytopathy.

Materials and methods

Cell line, lentivirus, and reagents

The immortalized human podocyte cell line (HPC) was a gift from Dr. Saleem M's laboratory (University of Bristol, UK). Lentivirus stock was made from HEK293 cells after transfection with vector plasmid, pHBLV-CMV-MCS-3FLAG-EF1-ZsGreem-T2A-PURO, together with packaging plasmids, psPAX2 and pMD2.G that encode HIV gag, pol, and VSV-G (Hanbio Inc., Shanghai).

Lentiviral infection of human podocytes in culture, adriamycin treatment, and total RNA preparation

HPC cells were cultured with RPMI1640 medium supplemented with 10% FBS, 1% Pen/Strep and 1% ITS (insulin-transferrin-selenium) at 33°C in 5% CO_2 incubator for growth, followed by trypsinization, replating and culture at 37°C for 10 days to obtain differentiated cells. To 8.4×10^4 differentiated podocytes of in a well of 12-well plate, 2.52 μl of 10^8 TU/ml lentiviruses, which were mixed with polybrene (GM-040901A, Genomeditech, Shanghai) for a concentration of 5 μg/ml, were added for a MOI of 3. Twenty-four hours later, medium was changed with fresh one, and the cells were cultured for 3 days, followed by harvest or treatment with 10 μg/ml ADR for 6 hours. To harvest the cells for RNA, the cells were homogenized with Trizol and the total RNA was extracted [26]. The amount and integrity of RNA samples were assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

Library preparation for transcriptome sequencing

mRNA was purified from total RNA by using poly-T oligo-attached magnetic beads. A standard method and procedure for library preparation for transcriptome sequencing were followed as described [27].

Sequencing data analyses

The methods and procedures for the quality control of the sequencing, reads mapping to the reference genome, and quantification of gene expression level were performed as described [27].

Bioinformatics analyses

Differential expression analysis, enrichment analysis of differentially expressed genes, and gene set enrichment analysis were performed as described [27].

Statistics

Data are presented as the mean ± SD. Differences between 2 groups were analyzed

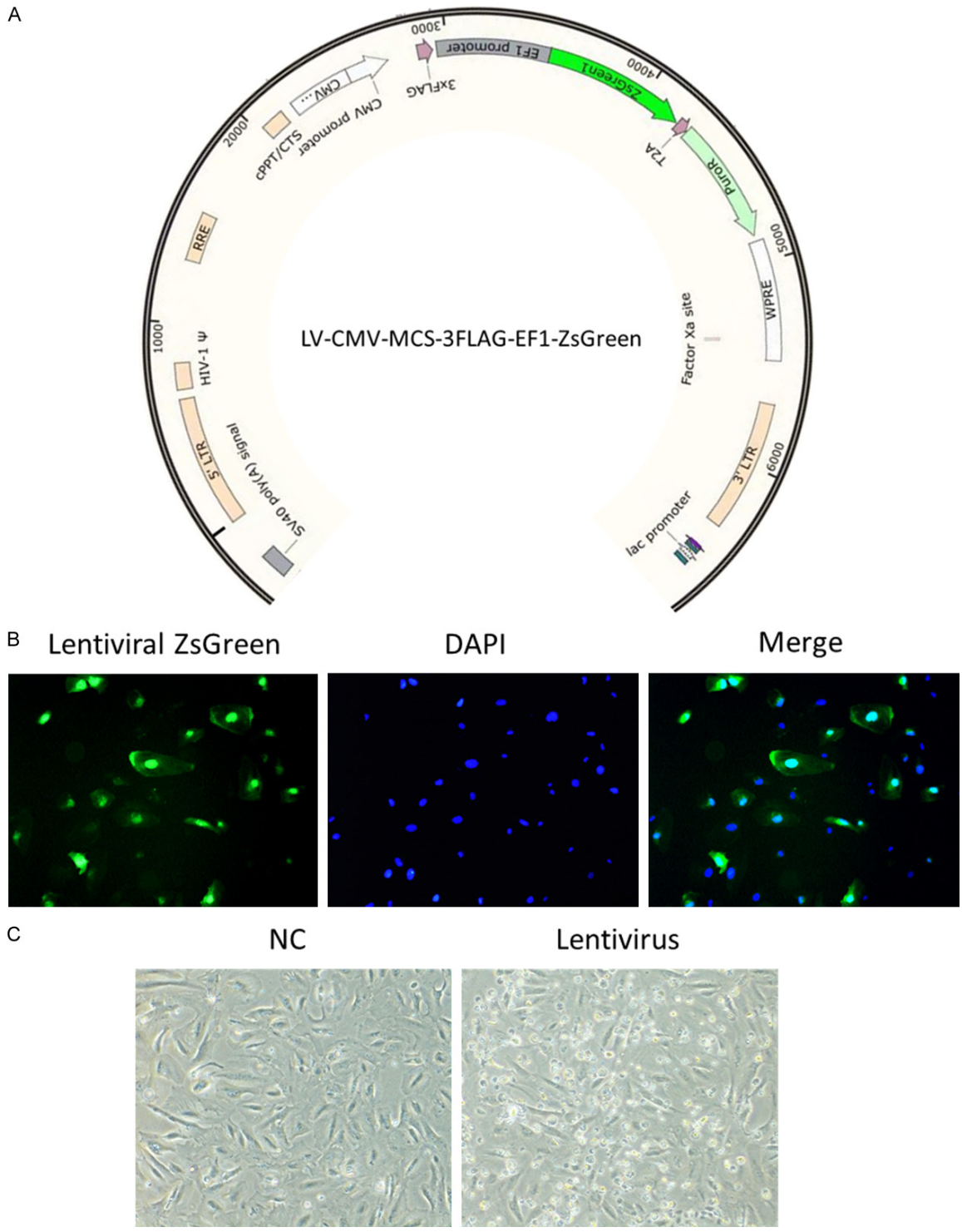

Figure 1. Infection of lentivirus in HPCs induced cell death. A. The map of the lentivirus showing the main elements and genes on the virus genome. B. On the day 3 after virus addition, the green fluorescence of the virus-expressed ZsGreen was observed in most of the cells, suggesting a high infection efficiency. C. The representative result of lentivirus-induced death of HPC cells on day 5 after viral infection.

using a 2-tailed Student's t test and incorporated into GraphPad Prism 5 software (GraphPad Software). $P < 0.05$ was considered statistically significant.

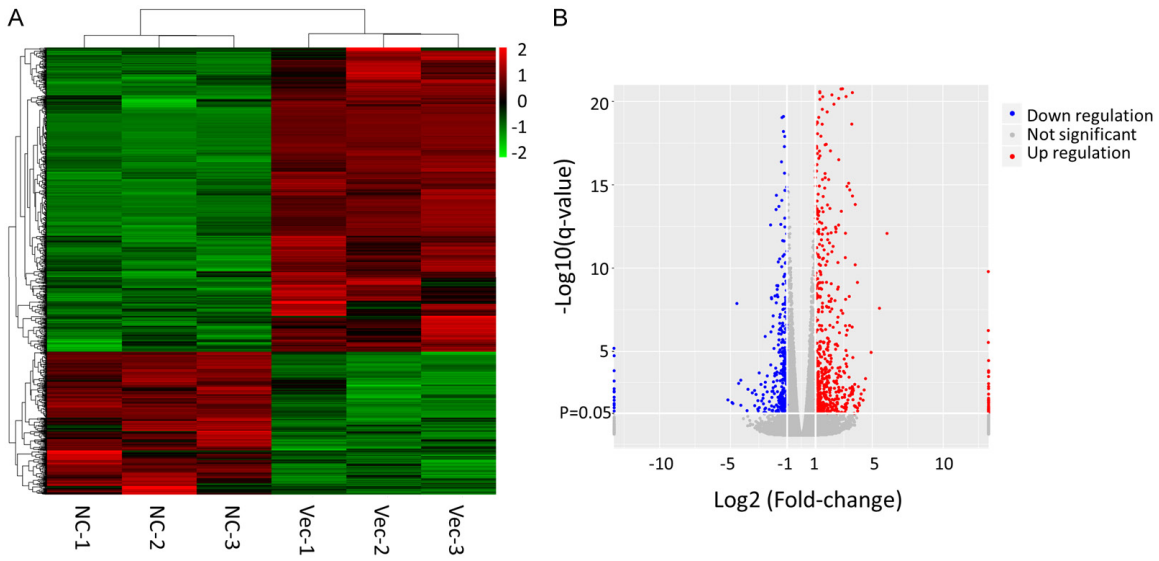

Figure 2. Analysis of differentially expressed genes between control and retroviral infected HPC cells by EdgeR. (A, B) Presentation of differentially expressed genes by heatmap (A) and Volcano (B).

Results

Lentiviral infection of human podocyte in culture resulted in cell death

We used the lentivirus to infect the HPC cells in culture. The lentivirus had ZsGreen expression cassette, allowing detection of successful infection and expression of the virus. We found the green fluorescence of the ZsGreen three days after the infection (**Figure 1A**, **1B**). At the day 5 after infection, marked cell death was observation (**Figure 1C**), demonstrating that lentiviral infection is harmful to the cells.

RNA-sequencing showed lentivirus triggered strong innate immune response in HPC cells

To understand the mechanism underlying lentivirus-induced podocyte injury, we performed RNA-sequencing on the cells infected with lentivirus using the uninfected cells as the control. Differential gene expressions between the two groups of cells were revealed and shown as the heatmap and volcano from the EdgeR analyses (**Figure 2A**, **2B**).

Next, the differentially expressed genes were subject to bioinformatics analyses. In the GO classifications, the terms of viral process and immune system process were found (**Figure 3A**). Similarly, immune system, infectious diseases (viral/parasitic/bacterial), and immune diseases were in the KEGG classification (**Figure 3B**). The association of the differential expressed genes in HPC cells infected by lentivirus reflected an immune response in the cells.

In the GO enrichment analysis, a large number of terms pertinent to both innate and adaptive immune responses were found among the top 30 terms (**Figure 4A**), including MDA-5 signaling pathway, cellular response to exogenous dsRNA, TLR7 signaling, JAK2 activation, CXCR chemokine receptor signaling, etc.

Among the top 30 terms of KEGG enrichment, terms related to innate immune response included IL-17 signaling, cytosolic DNA-sensing, RIG-I-like receptor signaling, NLRP signaling, complement cascades, cytokine-cytokine receptor interaction, hepatitis C, and TLR signaling (**Figure 4B**). Besides, the activations of TNF signaling and ferroptosis were consistent with the observed death of the cells as shown in **Figure 1B**.

We performed GSEA analysis of the differentially expressed genes and showed the results for a couple of terms with significant enrichment (**Figure 5**).

Lentivirus and adriamycin pathways co-existed in the HPC and did not interfere each other

Adriamycin (ADR) is a routine podocyte injury model, which induces podocyte injury through

Figure 3. Bioinformatics analyses of the differentially expressed genes between control and retroviral infected HPC cells. A. GO classifications of the genes; B. KEGG classifications of the genes.

DNA damage, oxidative stress, and cell cycle reentry and mitotic catastrophe. We analyzed the differentially expressed genes between normal control cells and the cells treated with ADR, and found the activations of Wnt, Hippo, TGF-beta, and cytokine-cytokine receptor interaction (**Figure 6A**). We found that these pathways characteristic of ADR remained in the cells infected with lentivirus as shown by comparison with cells only infected with lentivirus (**Figure 6B**). PCA analysis of the groups of cells showed distinct gene regulation effects of lentivirus and ADR (**Figure 6C**). Likewise, when we compared the cells treated with ADR and those treated with both ADR and lentivirus, we still observed marked innate immune response in the latter (**Figure 7**), as in the cells infected with lentivirus compared with normal control cells (**Figure 4**).

APOL1 is upregulated in lentiviral infected HPC

Infection with HIV is believed to be the risk factor for APOL1-associated kidney disease [22] because HIV lentivirus induced innate immune response, resulting in upregulation of APOL1 [23]. To determine whether the lentiviral infection of HPC cells is capable of inducing APOL1 upregulation, we compared the mRNA level of APOL1 in the cells of normal control and those infected with lentivirus. The result showed that the APOL1 mRNA level was 3 folds of that in the control cells (**Figure 8**), indicating that the lentivirus did induce upregulation of APOL1 in the present HPC model.

Discussion

Viruses are known to infect podocytes and cause podocytopathies. However, the pathomechanism is largely elusive. Effective models are desirable to facilitate the study in the direction. In the present study, we show that lentiviral infection of HPC may represent an ideal *in vitro* model for virus-associated podocytopathies.

We performed RNA-seq on the cells infected with lentivirus using the uninfected cells as the control. Differential gene expressions were subject to bioinformatics analyses. In the GO and KEGG classifications, the terms of viral process, immune system process, infectious diseases and immune diseases. In the GO enrichment analysis, many terms related to innate

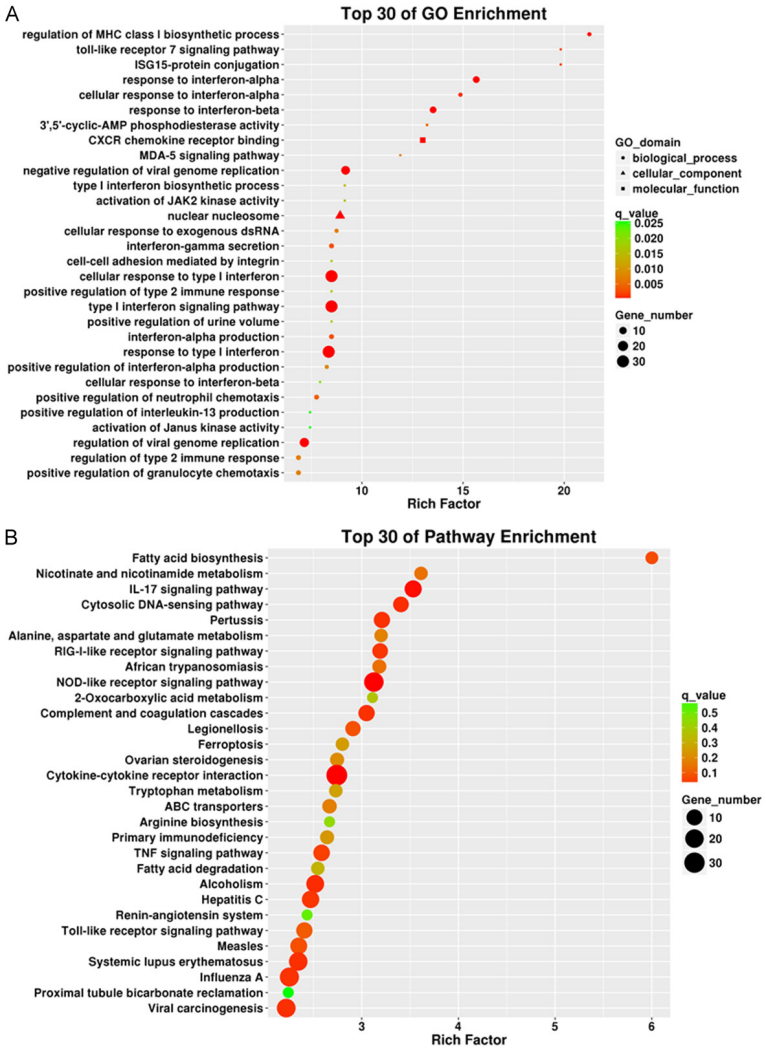

Figure 4. Bioinformatics analyses of the differentially expressed genes between control and retroviral infected HPC cells. A. GO analysis of the genes. B. KEGG pathway analysis of the genes.

and adaptive immune responses were found. The "MDA-5 signaling pathway" suggests MDA-5 was acting as an intracellular sensor of viral RNA and triggered innate immune response. This term, together with the terms "cellular response to exogenous dsRNA" and "TLR7 signaling" (whose ligand is ssRNA), reflected the RNA nature of the lentiviral genome. The terms concerning interferon production and responses to interferons, as well as those of "JAK2 activation" and "CXCR chemokine receptor signaling" indicated innate immune responses in the cells. In fact, lentiviral vector is known to cause innate immune response in immune cells [28, 29]. It has also been shown that TLR7 activation is capable of inducing expression of type I interferons in dendritic cells [30], and Type 1 INFs can activate the JAK-STAT pathway to turn on the transcription of INF-regulated genes (IRG), which inhibit viral entry, translation and replication [31]. These results indicate that, like immune cells, podocytes have the capability of innate immune response upon lentiviral infection. Furthermore, the term "regulation of MHC-I biosynthetic process" suggests that podocytes can have not only innate immune response but also adaptive immune response, consistent with the established notion that podocytes have many properties of immune cells [32-34]. In the analysis of KEGG enrichment, terms related to innate immune response were also found, and the activations of TNF signaling and ferroptosis were consistent with the observed death of the cells as well as the fact that Type 1 INFs are capable of inducing podocyte death [35].

As having shown in above analyses, lentivirus infection caused HPC death likely through innate immune response in the cells. The innate immune response resulted in production of IFNs and other cytokines which act on podocytes in the autocrine manner to induce ferroptosis or other forms of cell death. It is known that podocytopathies can be induced by simultaneous actions of multiple factors [36-38]. It would be interesting to know whether lentiviral innate immune pathways would be affected by other factors, e.g., ADR. The results indicated that lentivirus and ADR affect HPC cells with different molecular mechanisms, and the mechanisms do not interfere each other. However, whether the two mechanisms would converge later to lead to cell death warrants further investigation. This may be the first time showing the relationship of two different stimuli in podocyte injury.

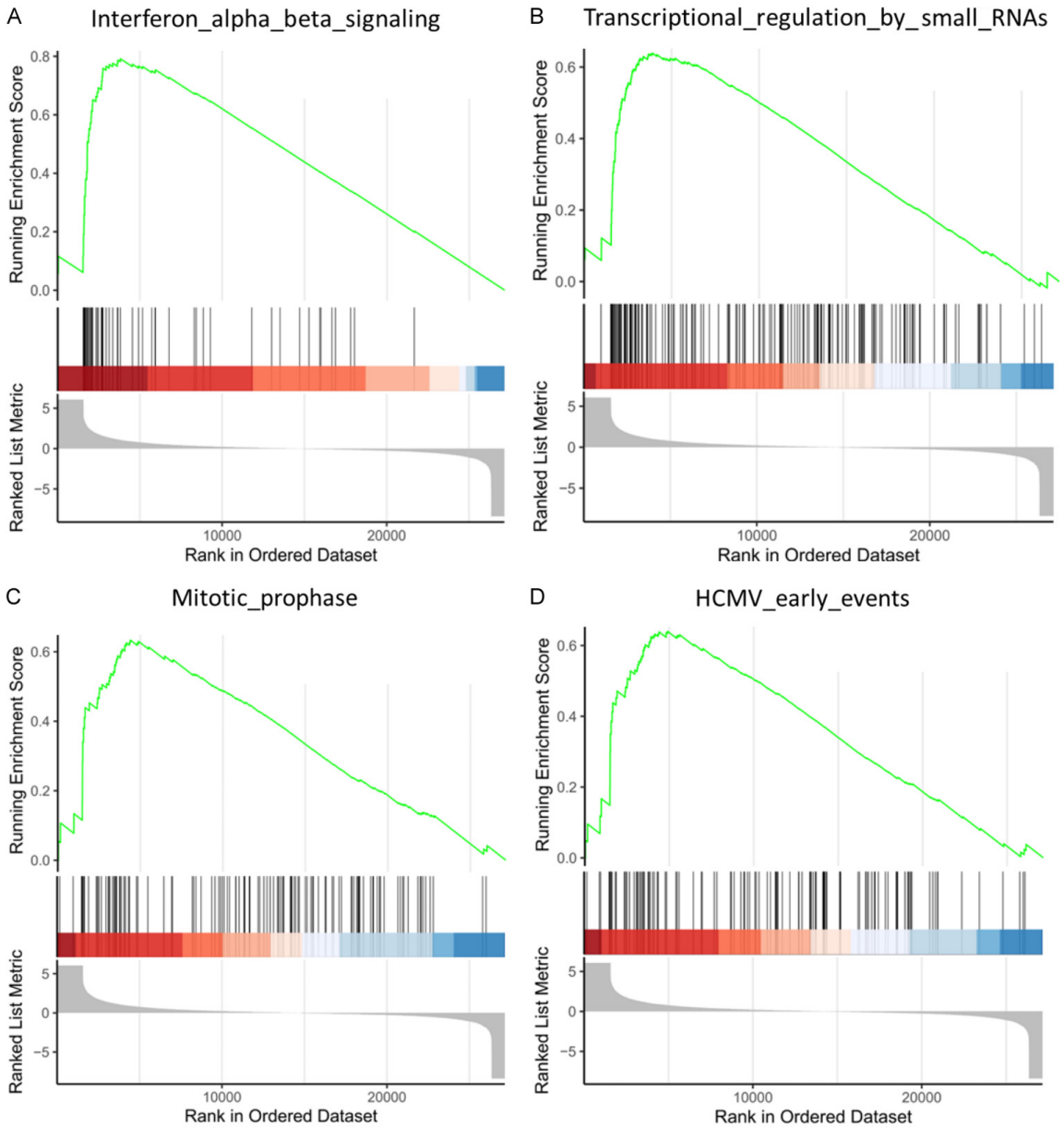

Figure 5. Representative results of GSEA analyses of the differentially expressed genes between control and retrovirus-infected HPC cells. A. Interferon_alpha_beta signaling. B. Transcriptional regulation by small RNAs. C. Mitotic prophase. D. HCMV early events.

Another finding in the lentiviral infected HPC was APOL1 upregulation. Studies have already shown that innate immune pathways are involved in upregulation of APOL1 in podocytes, e.g., type 1 INFs (INF-alpha and -beta) [39-41]. It was found that INF-alpha, INF-beta and INF-gamma are all able to activate APOL1 expression in podocytes [41]. This is why patients carrying APOL1 risk variants could develop cFSGS upon the treatment with INFs [42, 43], and in APOL1 G1 transgenic mice INF-gamma was capable of inducing proteinuria [44]. cGAS-STING signaling can upregulate APOL1 via IFN induction [45]. Systemic immune response with the resulting cytokine storm can upregulate APOL1 and could directly influence podocyte function if the APOL1 is in the form of risk variants [46].

It has been shown that expression of nef and vpr protein of HIV induces podocyte dedifferentiation and injury thereby contributing to the

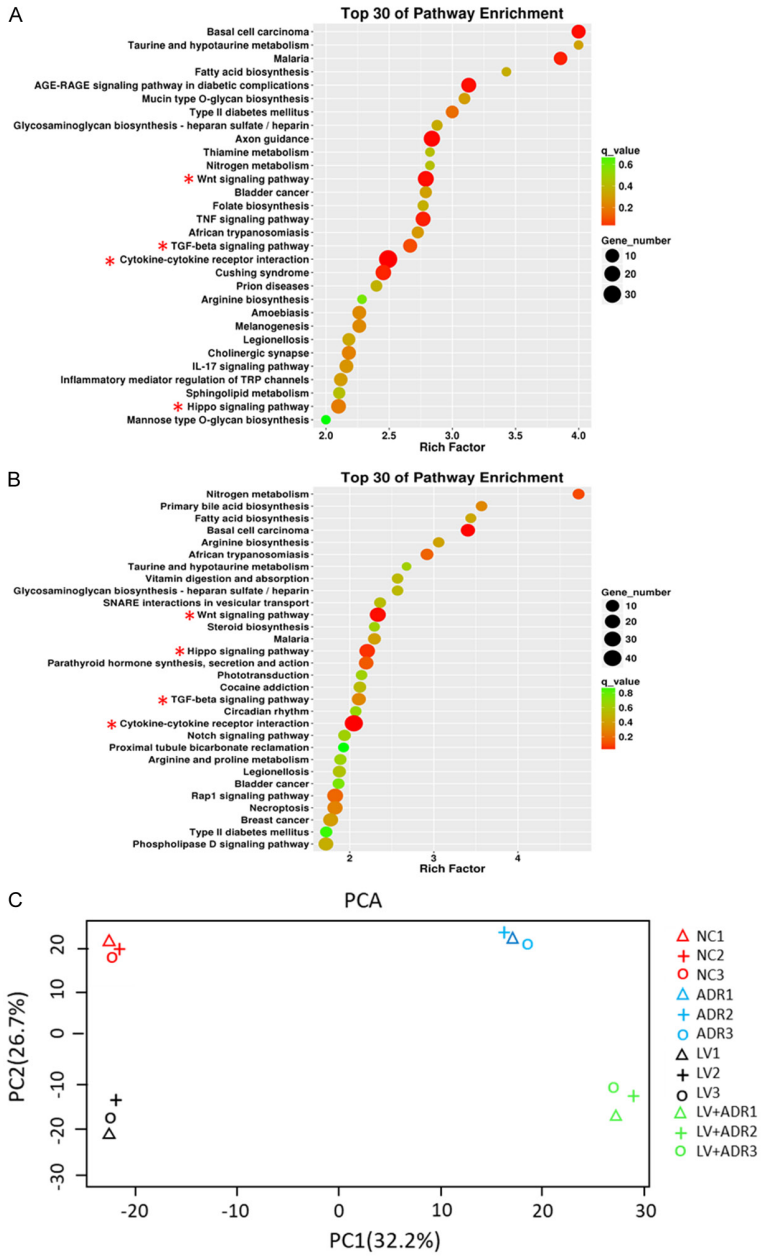

Figure 6. Lentiviral infection and ADR regulate distinct sets of genes and signaling pathways in HPC cells. (A, B) KEGG pathway analyses of the differentially expressed genes between control and ADR-treated HPC cells (A) and between cells infected with lentivirus and that infected with lentivirus and treated with ADR (B). The injurious pathways activated by ADR, as indicated by red *, remained active in the setting of lentiviral infection. (C) PCA analysis of the groups of cells further demonstrate the distinct gene regulation effects of lentivirus and ADR.

development of HIVAN [16, 47, 48]. However, as a vector, the lentivirus used in the present study lacked genes encoding HIV proteins, including nef and vpr. Therefore, the changes of the podocytes after infection of lentivirus did not involve nef and vpr. From this perspective, lentiviral infection of HPC cells does not fully model HIVAN. However, it does induce APOL1 expression which is known to interact with nef to induce podocyte injury [49]. Similarly, although it does not fully model other podocytopathies associated with virus infection, some aspects of the diseases are reflected in this model, including innate immune response and induction of APOL1.

In conclusion, the prominent innate immune response, APOL1 upregulation, and cell death upon lentiviral infection together suggest that lentivirally infected HPC cells may serve as a good *in vitro* model of virus-associated podocytopathy that may facilitate mechanistic studies by conveniently addressing 1) how APOL1 upregulation is induced; 2) how the HPC cells are injured by the virus; 3) whether the infected podocytes secrete factors that may affect adjacent cells of other types in the glomerulus; and 4) how to prevent virus-induced podocyte injury.

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Disclosure of conflict of interest

None.

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Figure 7. Activations of lentiviral pathways were not affected in the cells in the presence of ADR. (A, B) GO enrichment (A) and KEGG pathway enrichment (B) analyses of the differentially expressed genes between cells treated with ADR and those treated with ADR and lentivirus.

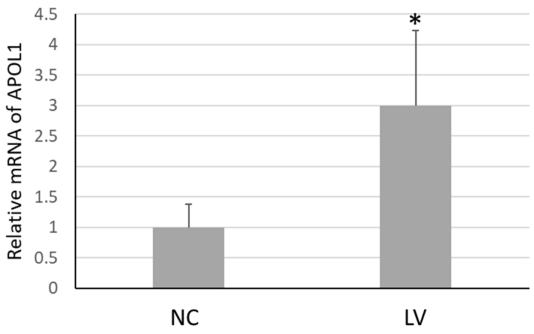

Figure 8. APOL1 was upregulated in HPC cells infected with lentivirus. The mean FPKMs of APOL1 in the control and lentiviral infected cells are compared. NC, normal control. LV, lentivirus. The data represent the mean ± stdev of three samples in each group. T-test was used to calculated the statistical significance. *P=0.027.

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