

Research article

## HIV-1 expression induces cyclin D<sub>1</sub> expression and pRb phosphorylation in infected podocytes: cell-cycle mechanisms contributing to the proliferative phenotype in HIV-associated nephropathy

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### Abstract

**Background:** The aberrant cell-cycle progression of HIV-1-infected kidney cells plays a major role in the pathogenesis of HIV-associated nephropathy, however the mechanisms whereby HIV-1 induces infected glomerular podocytes or infected tubular epithelium to exit quiescence are largely unknown. Here, we ask whether the expression of HIV-1 genes in infected podocytes induces cyclin D<sub>1</sub> and phospho-pRb (Ser780) expression, hallmarks of cyclin D<sub>1</sub>-mediated G<sub>1</sub> → S phase progression.

**Results:** We assessed cyclin D<sub>1</sub> and phospho-pRb (Ser780) expression in two well-characterized models of HIV-associated nephropathy pathogenesis: HIV-1 infection of cultured podocytes and HIV-1 transgenic mice (Tg26). Compared to controls, cultured podocytes expressing HIV-1 genes, and podocytes and tubular epithelium from hyperplastic nephrons in Tg26 kidneys, had increased levels of phospho-pRb (Ser780), a target of active cyclin D<sub>1</sub>/cyclin-dependent kinase-4/6 known to promote G<sub>1</sub> → S phase progression. HIV-1-infected podocytes showed markedly elevated cyclin D<sub>1</sub> mRNA and cyclin D<sub>1</sub> protein, the latter of which did not down-regulate during cell-cell contact or differentiation, suggesting post-transcriptional stabilization of cyclin D<sub>1</sub> protein levels by HIV-1. The selective suppression of HIV-1 transcription by the cyclin-dependent kinase inhibitor, flavopiridol, abrogated cyclin D<sub>1</sub> expression, underlying the requirement for HIV-1 encoded products to induce cyclin D<sub>1</sub>. Indeed, HIV-1 virus deleted of *nef* failed to induce cyclin D<sub>1</sub> mRNA to the level of other single gene mutant viruses.

**Conclusions:** HIV-1 expression induces cyclin D<sub>1</sub> and phospho-pRb (Ser780) expression in infected podocytes, suggesting that HIV-1 activates cyclin D<sub>1</sub>-dependent cell-cycle mechanisms to promote proliferation of infected renal epithelium.

## Background

Proliferation and dedifferentiation of HIV-1 infected renal epithelium is singularly unique compared to the effects of HIV-1 infection in other non-lymphoid tissues. Indeed, unlike the atrophy and cell-death of infected central nervous system [1], cardiac [2], or gastro-intestinal [3] parenchyma, aberrant cell-cycle progression of infected renal epithelium is a defining feature of HIV-associated nephropathy (HIVAN) [4,5]. Yet, the mechanisms are largely unknown whereby HIV-1 subverts the cell-cycle controls that normally maintain renal epithelial in G<sub>0</sub>. Nonetheless, several observations from *in vitro* and *in vivo* models of HIVAN pathogenesis suggest that HIV-1 expression itself may be a key pathogenic factor. Infection of cultured podocytes with replication incompetent HIV-1 demonstrated that HIV-1 gene expression leads to the proliferation and dedifferentiation of the infected podocytes [6–8], recapitulating the phenotypes observed in HIVAN specimens [9,10]. Similarly, renal expression of replication incompetent HIV-1 proviral transgenes in mice and rats produces a proliferative renal lesion that is indistinguishable both histopathologically and clinically from human HIVAN [11–14]. These observations raise the possibility that HIV-1 gene products may interact with mitogenic pathways in infected renal epithelium to cause aberrant cell-cycle engagement.

In comparison to the two general alternative mechanisms utilized by known transforming viruses to promote cell-cycle progression, namely, by activating or bypassing endogenous D-type cyclins (herein, referred to as "cyclin D"), it has not been established whether HIV-1 gene products trigger either cyclin D-dependent or cyclin D-independent proliferation in non-lymphoid tissues [15]. In infected renal epithelium, HIV-1 could disrupt the inhibitory binding of pRb to E2F independent of endogenous cyclin D, analogous to transforming viral mechanisms that bypass and down-regulate endogenous cyclin D, such as SV-40 T antigen binding to pRb [16], HHV-8 *v*-cyclin binding to and activating cyclin-dependent kinase-6 (CDK) [17], and activation of cyclin E by EBV-induced *c-myc* [18]. Alternatively, HIV-1 could activate cyclin D-dependent proliferation, as exemplified by RSV *v*-src-mediated signaling [19]. In most tissues, the selective activation of these cell-cycle pathways is readily discernable. Indeed, if cells depend on cyclin D-mediated G<sub>1</sub> → S transition, passage from G<sub>0</sub> through the restriction point in G<sub>1</sub> is dependent on the *de novo* expression of cyclin D transcript, accumulation of nuclear cyclin D protein, and cyclin D/CDK-4/6 phosphorylation of pRb [20]. Then, although nuclear-localized cyclin D decreases in S phase in individual cells [21], total cyclin D in asynchronously proliferating cell populations remain elevated compared to quiescent cells [20]. In renal epithelial and mesenchymal cells, cyclin D<sub>1</sub> is the dominant D-type cyclin activated

during cell-cycle progression, both during nephrogenesis [22] and in non-viral-mediated kidney cell proliferation [23].

Here, we ask whether infected podocytes expressing HIV-1 genes show the hallmarks of cyclin D<sub>1</sub>-dependent proliferation, specifically the up-regulation of cyclin D<sub>1</sub> transcript and protein expression and the subsequent phosphorylation of pRb on serine 780, a target site of active cyclin D<sub>1</sub>/CDK-4/6 that is not shared with cyclin E/CDK-2 complexes [24]. Utilizing two well-characterized models of HIVAN pathogenesis, HIV-1 transgenic mice [11–13] and HIV-1 infection of cultured podocytes [6–8] we find not only these hallmarks of cyclin D<sub>1</sub>-dependent proliferation, but also dysregulation of cyclin D<sub>1</sub> expression that normally occurs with cell-cell contact and differentiation [25].

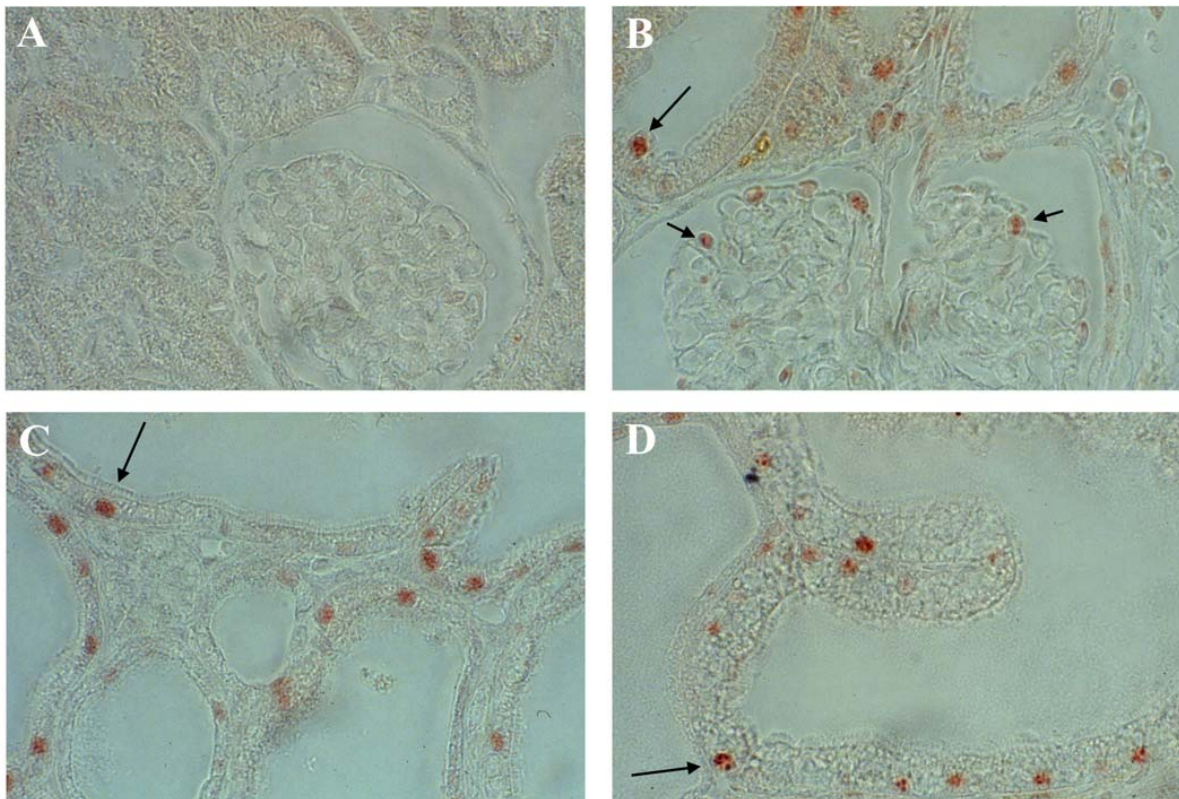
## Results

### Phospho-pRb (Ser780) expression *in vivo*

Cyclin D<sub>1</sub> is characterized as the major D-type cyclin controlling G<sub>1</sub>-phase progression in kidney cells, both during normal and abnormal proliferation [22,23]. To determine whether cyclin D<sub>1</sub> may be active in promoting G<sub>1</sub> → S progression in kidneys expressing HIV-1 genes, we utilized a well-characterized HIV-1 transgenic mouse model (Tg26) of HIVAN [11–13]. Secondary to renal expression of the HIV-1 NL4-3  $\Delta$ *gag-pol* proviral transgene, adolescent Tg26 transgenics develop a progressive, proliferative renal disease marked by podocyte and tubular epithelial hyperplasia that is indistinguishable both clinically and histopathologically from human HIVAN. In order to determine if cyclin D<sub>1</sub> plays a role in this hyperplasia, kidneys from three proteinuric 40-day old transgenic siblings and three non-transgenic littermates were stained by immunohistochemistry with antibody to phospho-pRb (Ser780), a site of cyclin D<sub>1</sub>/CDK-4/6 phosphorylation not targeted by cyclin E/CDK-2 complexes that disrupts pRb/E2F binding for G<sub>1</sub> → S progression [24]. Figure 1 depicts the intense, heterogeneous nuclear staining of glomerular podocytes and tubular epithelium from hyperplastic nephrons in the diseased transgenics versus the absent staining of nephrons in the non-transgenics consistent with the very low mitotic index in normal kidneys of adult animals [26]. This suggests that cyclin D<sub>1</sub> is promoting cell-cycle progression during the proliferation of epithelium in diseased kidneys.

### Cyclin D<sub>1</sub> and phospho-pRb (Ser780) expression *in vitro*

HIV-1 expression in cultured podocytes recapitulates the phenotypic abnormalities induced by HIV-1 expression in infected podocytes *in vivo*, specifically proliferation, loss of contact-mediated growth control, and dedifferentiation [6–8]. To determine if these phenotypes were marked by enhanced cyclin D<sub>1</sub> and phospho-pRb (Ser780) expres-



**Figure 1**

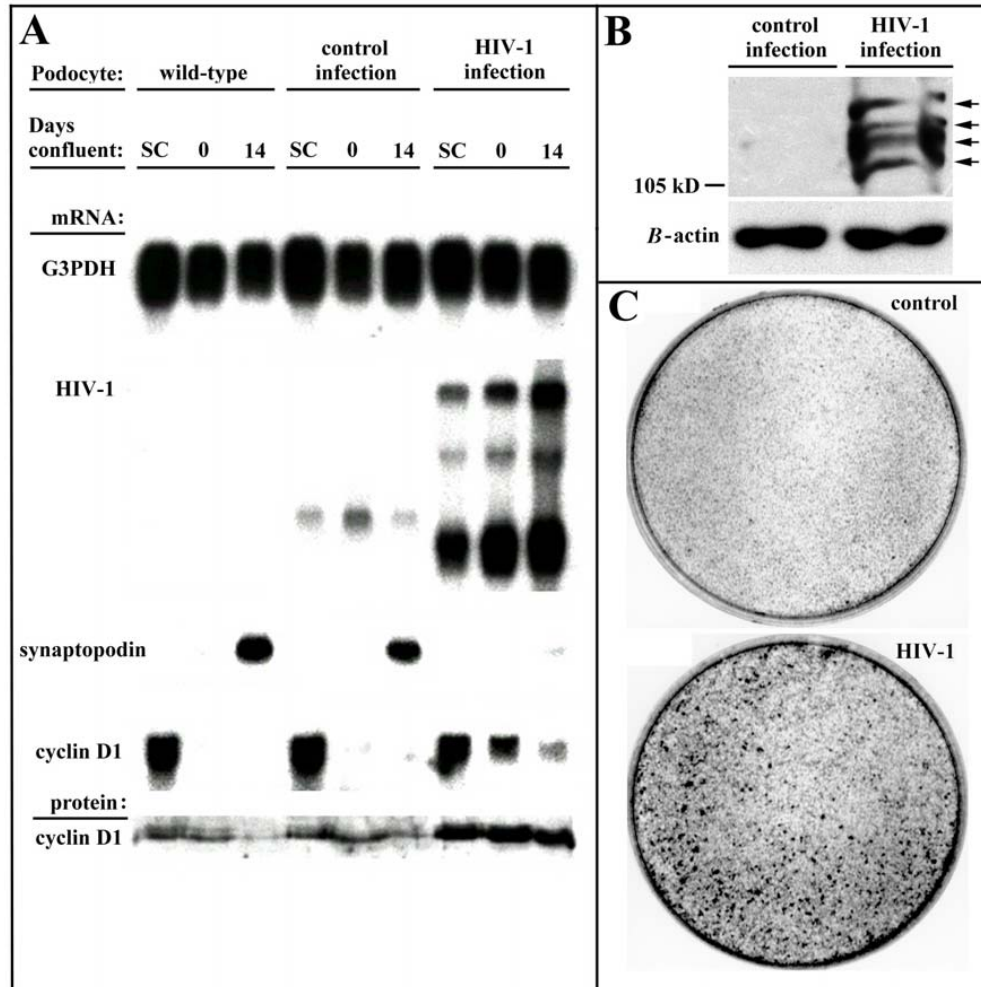
**In situ immunodetection of phosphorylated serine 780 on pRb in Tg26 kidneys.** Phospho-pRb (Ser780) is not detected in the nuclei of epithelium of normal kidneys (A), but is readily detected in podocytes (short arrows) and tubular epithelial cells (long arrows) in hyperplastic nephrons of diseased transgenic kidneys at cortical (B), juxta-medullary (C), and medullary (D) depths. The prominent staining in many, but not all nuclei, is characteristic of asynchronously proliferating cells. (magnification = 40 $\times$ )

sion, we utilized the podocyte cell-culture protocol developed by Rieser et al. [27,28] to study both sub-confluent podocyte growth and podocyte growth during the formation of mature cell-cell contacts at confluence. In this protocol, normal podocytes are growth-arrested 4–5 days after initial confluence and are fully differentiated with mature cell-cell contacts approximately 10 days later. Figure 2A shows that cyclin D<sub>1</sub> message levels are nearly equivalent between mock ("wild-type")-, control virus-, and HIV-1-infected subconfluent podocytes, although only the HIV-1-infected subconfluent podocytes had elevated levels of cyclin D<sub>1</sub> protein. In contrast, cell-cell contact in wild-type and control-infected, but not HIV-1-infected podocytes, caused a severe down-regulation of cyclin D<sub>1</sub> transcript and protein concomitant with the up-regulation of synaptopodin, a podocyte differentiation marker [7,28], by the end of the 14-day differentiation period. Although HIV-1 infected podocytes showed some decrease in cyclin D<sub>1</sub> message by the end of the differentiation period, cyclin D<sub>1</sub> protein levels remained elevated, suggesting activation by HIV-1 of mechanisms that stabi-

lize cyclin D<sub>1</sub> protein levels. Moreover, HIV-1-infected podocytes, but not control-infected podocytes, had readily detectable phospho-pRb (Ser780) at the end of the differentiation period, suggesting that the stably up-regulated cyclin D<sub>1</sub> protein remained active in promoting cell-cycle progression (Figure 2B). The ongoing proliferation of HIV-1-infected podocytes following cell-cell contact led to the formation of multiple foci (Figure 2C) with intense, heterogenous nuclear cyclin D<sub>1</sub> immunofluorescence consistent with ongoing asynchronous proliferation in this population of cells (Figure 3). These data indicate that HIV-1 expression in infected podocytes induces cyclin D<sub>1</sub> and phospho-pRb (Ser780) expression, hallmarks of cyclin D<sub>1</sub>-mediated G<sub>1</sub> → S progression. The data, however, do not prove that cyclin D<sub>1</sub> expression alone is sufficient to cause cell-cycle progression.

#### **Requirement of HIV-1 expression for induction of cyclin D<sub>1</sub>**

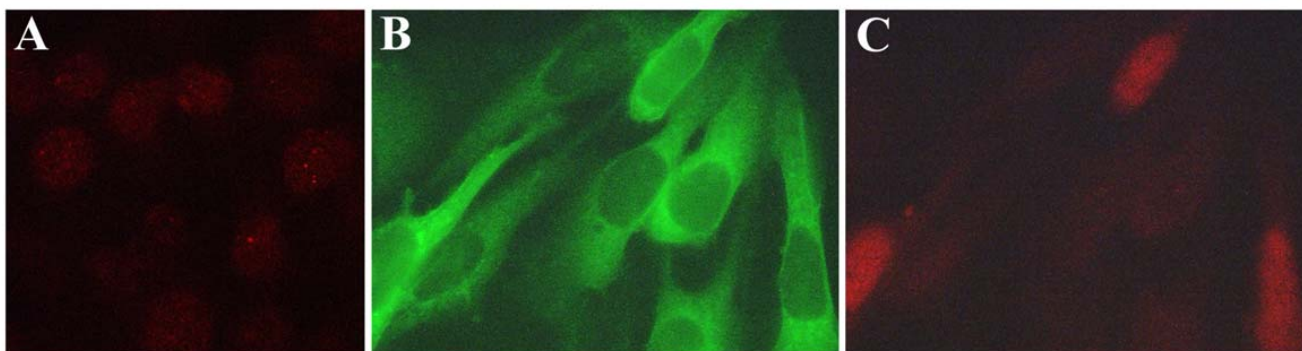
We sought to further confirm that HIV-1 expression, and not some idiosyncratic effect of infection, was directly inducing cyclin D<sub>1</sub> expression in infected podocytes. First,



**Figure 2**

**Expression of cyclin D<sub>1</sub> and phospho-pRb (Ser780) in HIV-1-infected podocytes.** (A) Northern and western blot detection of cyclin D<sub>1</sub> expression in wild-type, control-infected, and HIV-1-infected podocytes during subconfluent (SC) growth at 33°C and during 14 days of differentiation of confluent podocytes at 37°C shows that neither the cyclin D<sub>1</sub> transcript nor the elevated cyclin D<sub>1</sub> protein in HIV-1-infected podocytes (full-length, singly-spliced, and multiply spliced HIV-1 transcripts) are significantly down-regulated by podocyte cell-cell contact or by podocyte differentiation (synaptopodin transcript) when compared to wild-type or control-infected (one transcript from LTR sequences) podocytes. (B) Immunodetection of phospho-pRb (Ser780) on several phosphorylated forms (arrows) of pRb in the asynchronously proliferating, HIV-1-infected podocytes that is absent in the quiescent, control-infected podocytes after 14 days of differentiation at 37°C. (C) Abundant foci formation in confluent HIV-1-infected podocytes but not in confluent control-infected podocytes due to the ongoing proliferation of HIV-1-infected podocytes after cell-cell contact. Blots are representative of two independent experiments.





**Figure 3**  
**Heterogenous nuclear localization of cyclin D<sub>1</sub> in HIV-1-infected podocytes.** Immunofluorescent detection of nuclear cyclin D<sub>1</sub> in quiescent, wild-type podocytes after 14 days of differentiation at 37°C shows light speckled staining in all nuclei (A). This differs markedly from the intense nuclear staining of cyclin D<sub>1</sub> in some, but not all nuclei, of the asynchronously proliferating, HIV-1-infected podocytes (GFP positive podocytes) (B and C). (magnification = 100×)

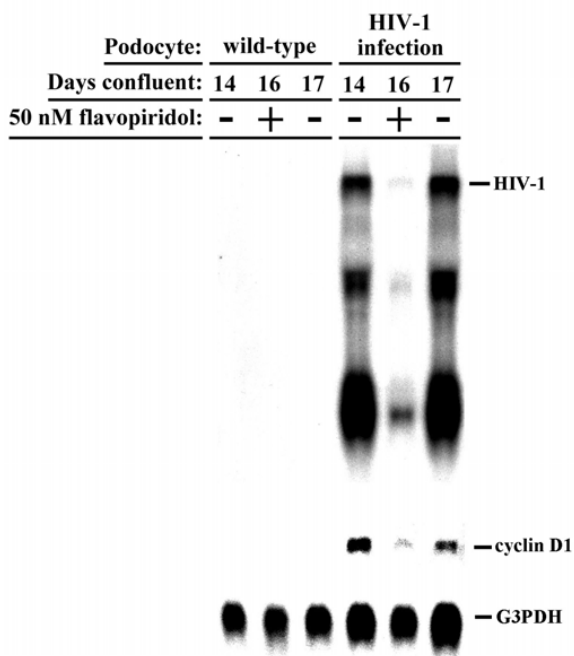
we utilized the small molecule cyclin-dependent kinase inhibitor, flavopiridol, which at low concentrations (e.g., less than 100 nM) can selectively suppress HIV-1 transcript elongation without altering cellular transcripts in cell types of various lineages, including podocytes [7,29,30]. Cyclin D<sub>1</sub> transcript levels were followed before, during, and after treatment of confluent wild-type or HIV-1 infected podocytes with 50 nM flavopiridol, a dose that suppresses HIV-1 transcription by 70% [7]. Analysis of HIV-1, cyclin D<sub>1</sub>, and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) transcript levels in podocytes treated with flavopiridol for 2 days, followed by drug washout for 1 day, showed that cyclin D<sub>1</sub> expression paralleled HIV-1 expression in infected podocytes before, during and after suppression of HIV-1 genes (Figure 4). Secondly, we utilized single gene mutants of HIV-1 [8] to determine which HIV-1 gene(s) may be involved with up-regulating cyclin D<sub>1</sub> expression. Analysis of cyclin D<sub>1</sub> transcript levels in podocytes infected with HIV-1 singly mutated with premature stop codons in the genes encoding the various structural and regulatory proteins of HIV-1 (except tat, which is present in every virus) showed that nef-deficient virus did not significantly up-regulate cyclin D<sub>1</sub> expression when compared to the other mutant viruses (Figure 5). Taken together, these data indicate that induction of cyclin D<sub>1</sub> expression requires HIV-1 gene expression and that HIV-1 nef is important for this induction.

### Discussion

The mechanisms whereby HIV-1 subverts cell-cycle controls in infected renal epithelium have been unclear. Here, we demonstrate that podocytes expressing HIV-1 genes display the hallmarks of cyclin D<sub>1</sub>-dependent cell-cycle progression, specifically, that cyclin D<sub>1</sub> transcript and protein expression are markedly up-regulated, and that levels of phospho-pRb (Ser780), a target of cyclin D<sub>1</sub>/CDK-4/6

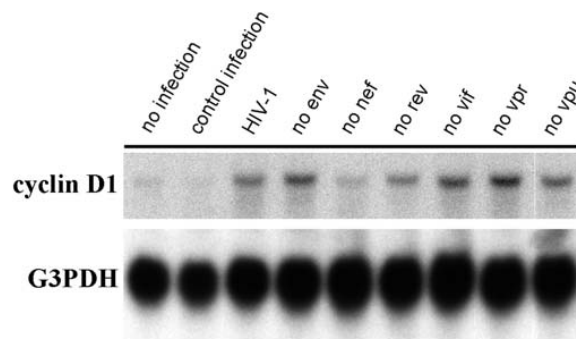
but not cyclin E/CDK-2 complexes [24], are induced *in vitro* and *in vivo*. In addition, preliminary micro-array gene profiling of HIV-1-infected tubular epithelium showed that cyclin D<sub>1</sub> expression is up-regulated several fold when compared to uninfected tubular epithelium (Michael Ross, personal communication). This suggests that the HIV-1-induced proliferation of infected renal epithelium may be caused by a cyclin D<sub>1</sub>-dependent mechanism, unlike other viruses that bypass a requirement for G<sub>1</sub>-phase cyclins to trigger aberrant cell-cycle progression. Moreover, normal cellular controls on cell-cycle progression appears to be dysregulated by HIV-1 expression because cell-cell contact and differentiation, events that normally lead to the down-regulation of cyclin D<sub>1</sub> expression [25], did not significantly alter cyclin D<sub>1</sub> protein levels in HIV-1 infected podocytes. This suggests a novel stabilization of cyclin D<sub>1</sub> protein in podocytes by HIV-1. We are exploring whether this occurs from enhanced translation of cyclin D<sub>1</sub> message and/or decreased degradation of cyclin D<sub>1</sub> protein and whether other D-type cyclins may be involved in phosphorylating pRb in HIVAN.

Prior studies in HIV-1 transgenic animals support the model that HIV-1 gene products interact with endogenous mitogenic pathways in infected renal epithelium. [11–14]. Kidney transplants between normal and transgenic Tg26 siblings suggested that specific HIV-1 proteins within the kidney, and not circulating factors, transform infected renal epithelium [13]. Indeed, HIV-1 transgenic mice generated with an HIV-1 proviral construct mutated in the Nef "PXXP" SH3-binding motif for Src-family kinases failed to develop nephropathy [31], and a recent *in vitro* analysis of podocytes infected with single gene mutants of HIV-1 further suggest that Nef plays a central role in causing the proliferative phenotype in HIVAN [8]. Utilizing these same single gene mutant viruses, we showed



**Figure 4**  
**Parallel expression of HIV-1 and cyclin D<sub>1</sub>.** Northern blot detection of HIV-1, cyclin D<sub>1</sub>, and G3PDH transcripts before (day 14), during (two days with drug, day 16), and after (one day after drug washout, day 17) treatment of wild-type and HIV-1-infected podocytes with 50 nM flavopiridol, a small molecule CDK inhibitor of HIV-1 transcription, showing that cyclin D<sub>1</sub> expression parallels HIV-1 expression. Blot is representative of two independent experiments.

here that Nef appears to play a role in the up-regulation of cyclin D<sub>1</sub> by HIV-1 *in vitro*. Importantly, these single gene mutants do not exclude the possibility that other HIV-1 gene(s) may cooperate with *nef* to induce cyclin D<sub>1</sub>, particularly *tat*, which is present in every virus. Cumulatively, however, these observations do suggest a mechanism whereby HIV-1 expression in renal epithelium may induce cyclin D<sub>1</sub>: activation of Src-family kinases by Nef would be expected to up-regulate cyclin D<sub>1</sub> gene expression and promote the synthesis and stabilization of cyclin D<sub>1</sub> protein via Src-Ras-MAPK and Src-PI3K-mTOR signal transduction pathways, respectively [32-34]. Interestingly, our unpublished *in vitro* observations and published data showing marked expression of basic fibroblast growth factor in the interstitium of HIVAN kidneys [35] suggest that soluble mitogens may be important cofactors with HIV-1 expression to induce cyclin D<sub>1</sub>. Thus, interrupting HIV-1 gene expression should down-regulate cyclin D<sub>1</sub> and correct the phenotypic abnormalities in infected podocytes. In support of this notion, we show



**Figure 5**  
**Mapping of HIV-1 genes that induce cyclin D<sub>1</sub> expression.** Northern blot detection of cyclin D<sub>1</sub> and G3PDH transcripts from wild-type, control-infected, and HIV-1-infected podocytes infected with single gene mutants of HIV-1 (*tat* present in every virus). Similar to wild-type and control-infected podocytes, podocytes infected with *nef*-deficient HIV-1 do not significantly up-regulated cyclin D<sub>1</sub> expression compared to podocytes infected with other single gene mutant viruses.

here that cyclin D<sub>1</sub> expression parallels HIV-1 expression before, during, and after suppression of HIV-1 transcription with flavopiridol, and recently, we showed that longer periods of treatment with flavopiridol ameliorate the phenotypic abnormalities of infected podocytes [7].

**Conclusions**

The following results of this study suggest that HIV-1 expression leads to cyclin D<sub>1</sub>-mediated G<sub>1</sub> → S progression in infected podocytes: cyclin D<sub>1</sub> transcript and protein levels are markedly up-regulated, phospho-Rb (Ser780) levels are increased, cyclin D<sub>1</sub> protein and phospho-Rb (Ser780) levels do not decrease with podocyte cell-cell contact and differentiation, and the up-regulation of cyclin D<sub>1</sub> requires the expression of HIV-1 genes, particularly HIV-1 *nef*. In sum, these results suggest HIV-induced cell-cycle mechanisms may contribute to aberrant epithelial proliferation in HIVAN.

**Methods**

**Podocyte cell culture and infection**

Murine podocytes immortalized with interferon-inducible temperature-sensitive SV-40 T antigen (*ts*-SV-40 T) were isolated and infected with replication incompetent GFP-expressing HIV-1 virus, replication incompetent GFP-expressing single-gene mutants of HIV-1, or replication incompetent GFP-expressing control lentivirus containing GFP but no HIV-1 genes as previously described [7,8]. In all cases, 70% to 80% of podocytes showed GFP expression 4 days post infection (data not shown). Podocytes were used before passage twenty and maintained in

RPMI 1640 (Life Technologies) supplemented with 10% fetal calf serum, 1 × PenStrep (Life Technologies), and 20 U/ml recombinant interferon-gamma (Life Technologies) at 33°C. Unless otherwise indicated, all studies were conducted at cell confluency and 14 days after inactivation of *ts-SV-40 T* at the non-permissive temperature (37°C) when wild-type podocytes are fully differentiated [27,28].

### **Immunohistochemistry**

Kidneys from three 40 day-old Tg26 heterozygous transgenic siblings demonstrating 2+ proteinuria (Chemstrip 2GP, Roche) and three of their non-transgenics littermates were collected in accordance with Animal Use and Care Committee guidelines, fixed (10% buffered formalin), then paraffin-embedded (Department of Pathology, Mount Sinai School of Medicine). Four micron full coronal kidney sections from each animal were adhered to lysine-coated slides and used for immunodetection of phospho-Rb (Ser780) as follows: sections were deparaffinized and hydrated by placing in xylene for 8 minutes, followed by 3 minutes each of 100%, 96%, 80%, 70%, 50%, 30% ethanol, ending with 10 minutes in water; sections then underwent antigen retrieval (Glyca Reagent, Biogenex) followed by immunodetection (Super Sensitive Detection Kit, Biogenex) of phospho-Rb (Ser780) after a 12 hour incubation with a 1:1000 dilution of rabbit anti-phospho-Rb (Ser780) antibody (Cell Signaling) or control rabbit anti-sera (Biogenex) at 4°C.

### **Immunofluorescence**

Following the 14 day podocyte differentiation protocol on coverslips to establish mature podocytes, wild-type and HIV-1 infected podocytes underwent immunofluorescent detection of cyclin D<sub>1</sub> as follows: coverslips were washed three times with 1 × PBS, fixed in 60% acetone/3.7% formaldehyde at -20°C for 20 minutes, then washed twice with 1 × PBS; the coverslips were then incubated with a 1:200 dilution of rabbit anti-cyclin D<sub>1</sub> antibody (Santa Cruz Biotechnology) or control rabbit sera (Biogenex) for 2 hours at room temperature followed by three washes with 1 × PBS; the coverslips were then incubated with a 1:250 dilution of rhodamine-labeled donkey-anti-rabbit anti-sera (Chemicon) for 45 minutes to detect the primary antibody; following three final washes with 1 × PBS, the coverslips were mounted on glass slides using ProLong Antifade (Molecular Probes) for fluorescent detection of GFP and cyclin D<sub>1</sub> on an Olympus IX70 microscope at 100 × magnification.

### **Podocyte cell-cell contact**

Total RNA and protein were extracted from wild-type, control-infected, and HIV-1 infected podocytes during the Reiser, et al. protocol to study subconfluent podocyte growth and podocyte growth during establishment of mature cell-cell contacts between differentiated podocytes

[27,28]. Briefly, RNA and protein were extracted from parallel plates of podocytes using TRIZOL Reagent or RIPA buffer, respectively, under three conditions: 1) during sub-confluent growth at 33°C; 2) immediately upon reaching confluency (day zero); and 3) on day 14 after switching confluent cultures to 37°C. At the end of the differentiation protocol (day 14), additional plates were stained to detect foci formation as follows: cells were washed with 1 × PBS, fixed in methanol for 1 minute, stained with bromophenol blue for 1 minute, then washed again with 1 × PBS. In experiments utilizing the single gene mutant of HIV-1, total RNA was extracted from infected podocytes 14 days after switching confluent cultures to 37°C. All experiments were performed twice.

### **Flavopiridol treatment**

After the 14-day podocyte differentiation protocol described above, total RNA was extracted from wild-type and HIV-1-infected podocytes before treatment with flavopiridol (Developmental Therapeutics Program, National Cancer Institute) (day 14), two days after suppression of HIV-1 transcription with media containing 50 nM flavopiridol (day 16), and one day after drug washout using media without flavopiridol (day 17). The experiment was performed twice.

### **Cyclin D<sub>1</sub> transcript and protein**

RNA and protein samples were analyzed as follows: Podocyte RNA (10 µg) was analyzed by northern blot using probes for HIV-1 *nef-LTR* [7], mouse cyclin D<sub>1</sub> (cDNA generated by RT-PCR from mouse podocytes and verified by sequence analysis), synaptopodin [7], and G3PDH. Following 7.5% SDS/PAGE, podocyte protein (20 µg) was analyzed by western blot for cyclin D<sub>1</sub> (ECL detection of a 1:1000 dilution of rabbit anti-cyclin D<sub>1</sub> antibody, Santa Cruz BioTechnology). Protein (250 µg) from the control-infected or HIV-1-infected podocytes on day 14 of the differentiation protocol was also analyzed by western blot for total pRb (ECL detection of a 1:1000 dilution of rabbit anti-Rb, Santa Cruz Biotechnology) and phospho-Rb (Ser780) (ECL detection of a 1:500 dilution of rabbit anti-phospho-Rb (Ser780) antibody binding, Cell Signaling). Protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories), and blotting of equivalent amounts of protein was confirmed by transient staining of membranes with Ponceau S solution (0.5% Ponceau S/1% acetic acid) and by western blot for β-actin (Chemicon). Signal intensity was calculated using the UN-SCAN-IT gel v4.3 (Silk Scientific).

### **Authors' contributions**

P.J.N. conceived and designed the study; performed the immunohistochemistry, immunofluorescence, and western blotting; and drafted the manuscript. M.S. cultured the podocytes and performed the northern blotting. M.H.

designed and prepared the single gene mutants of HIV-1. I.H.G. mentored the authors and participated in the study's design and coordination.

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