Am J Physiol Cell Physiol 317: C390–C397, 2019.
First published May 15, 2019; doi:10.1152/ajpcell.00038.2019.

RESEARCH ARTICLE | *Metabolism, Oxidative Stress and Cell Signaling*

MiR-144 mediates Nrf2 inhibition and alveolar epithelial dysfunction in HIV-1 transgenic rats

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Submitted 1 February 2019; accepted in final form 10 May 2019

Kukoyi AT, Fan X, Staitieh BS, Hybertson BM, Gao B, McCord JM, Guidot DM. MiR-144 mediates Nrf2 inhibition and alveolar epithelial dysfunction in HIV-1 transgenic rats. *Am J Physiol Cell Physiol* 317: C390–C397, 2019. First published May 15, 2019; doi:10.1152/ajpcell.00038.2019.—Chronic HIV infection causes redox stress and increases the risk of acute and chronic lung injury, even when individuals are adherent to antiretroviral therapy. HIV-1 transgene expression in rats inhibits nuclear factor (erythroid-derived 2)-like 2 (Nrf2), which regulates antioxidant defenses and alveolar epithelial cell (AEC) barrier function, but the mechanism is unknown. In this study, we present novel evidence that these pathological effects of HIV are mediated by microRNA-144 (miR-144). HIV-1 transgene expression in vivo increases the expression of miR-144 in the alveolar epithelium, and this can be replicated by direct exposure of naïve primary AECs to either Tat or gp120 ex vivo. Further, treating naïve primary AECs with a miR-144 mimic decreased the expression and activity of Nrf2 and inhibited their barrier formation. In contrast, treatment with a miR-144 antagomir increased the expression and activity of Nrf2 and improved barrier function in primary AECs isolated from HIV-1 transgenic rats. Importantly, either delivering the miR-144 antagomir intratracheally, or directly activating Nrf2 by dietary treatment with PB123, increased Nrf2 expression and barrier formation in HIV-1 transgenic rat AECs. This study provides new experimental evidence that HIV-induced inhibition of Nrf2 and consequent AEC barrier dysfunction are mediated via miR-144, and that these pathophysiological effects can be mitigated in vivo by either directly antagonizing miR-144 or activating Nrf2. Our findings suggest that targeting the inhibition of Nrf2 in individuals living with HIV could enhance their lung health and decrease the lung-specific morbidity and mortality that persists despite antiretroviral therapy.

alveolar epithelium; HIV; miR-144; Nrf2; oxidative stress

INTRODUCTION

Pulmonary complications remain a significant cause of morbidity and mortality in people living with HIV despite antiretroviral therapy (ART). For example, the Veterans Aging Cohort Study showed a 4.5-fold increase in nonopportunistic bacterial infections and an even higher rate of tuberculosis (7). Intuitively, these infectious complications are not surprising given the devastating effects of the virus on various components of the immune system. However, the same study also showed a significant increase in the incidence of noninfectious pulmonary diseases, specifically chronic obstructive pulmonary disease (COPD), pulmonary hypertension, and interstitial lung disease (7).

Although the mechanisms by which HIV affects the lungs are still poorly understood, prior work from our lab suggests a major role for chronic expression of HIV-related viral proteins within the lungs, causing oxidative stress and toxicity to the airway epithelium (14). Specifically, individuals living with HIV on ART may have undetectable virus but, nevertheless, have had genomic integration of HIV into multiple target cells that then produce and release HIV-related proteins that have been shown to have a range of toxicities. Of note, HIV proviruses can integrate into human airway epithelial cells (2), and we identified that alveolar macrophages harbor latent HIV, even in individuals who are well suppressed on ART (5). Our group and others have studied this chronic condition experimentally in HIV-1 transgenic mice and rats in which there is no active viral replication, but in which chronic expression of HIV-related proteins produce an AIDS-like phenotype (1, 12–15, 21). We previously determined that these HIV-related proteins are present within the alveolar space of HIV-1 transgenic rats, as had been previously identified in HIV-infected humans (9). We have also previously shown that these proteins, specifically, the transactivator of transcription (Tat) and glycoprotein 120 (gp120), impair the alveolar epithelial barrier, an important component of the lung's innate immune defense and surface area for gas exchange (14). These HIV-mediated derangements in alveolar epithelial function would explain, at least in part, the predisposition of persons living with HIV (PLWH) to pneumonia, as well as to noninfectious lung diseases, such as emphysema, in which alveolar epithelial injury is a cardinal feature (6, 25).

Previous studies in our laboratory have also identified that the alveolar epithelial barrier defects in the HIV-1 transgenic rat model are mediated by decreased expression and function of epithelial tight junction proteins, such as zonula occludens (ZO-1), occludin, and selected members of the claudin family of proteins (14). These proteins are responsible for limiting the permeability of the epithelial barrier by maintaining cell-to-cell tight junctions that form the scaffolding of the epithelium. Interestingly, we have also identified that this HIV-mediated alveolar epithelial barrier dysfunction is driven, in large part,

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by oxidative stress and depletion of glutathione in the alveolar space, which appears to be triggered by downregulation of the transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2 or NFE2L2) and its downstream effectors, including glutamate-cysteine ligase catalytic subunit (GCLC) and NAD(P)H dehydrogenase (quinone 1) (NQO1) (10). These findings likely contribute to the well-known effects of chronic HIV infection on glutathione homeostasis, including our findings that even otherwise healthy PLWH have profoundly decreased levels of glutathione in their alveolar space (4). Further, we made the somewhat surprising discovery that Nrf2 also regulates the expression of tight junction proteins within the alveolar epithelium, in addition to its regulation of antioxidant defenses (10). However, the mechanism(s) by which HIV-related proteins inhibit Nrf2 and, thereby, dysregulate antioxidant and barrier functions within the alveolar epithelium have not yet been elucidated. In other biological contexts, it has been identified that selected microRNAs (miRNAs) inhibit Nrf2 expression (11, 18), and therefore, we speculated that HIV-related proteins might induce one or more miRNAs that dampen Nrf2 expression and function as a potential mechanism for the impairments we had observed.

miRNAs are a group of 21- to 23 nucleotide-long noncoding RNAs that regulate gene expression by binding to the 3′ UTR of target mRNA transcripts and, thereby, inhibiting their translation. Importantly, HIV has been shown to influence the expression of multiple miRNAs. As just one example, in a model of acute HIV infection, a microarray analysis of miRNA expression showed upregulation of miR-223 and downregulation of miR-21, 26a, and 155, among others (26), and these alterations appear to mediate HIV replication and the apoptosis of infected CD4 cells (26). In parallel, although several miRNAs have been shown to inhibit Nrf2, including miR-21, miR-27a, miR-144, and miR-155, they have not been examined in the context of the effects of HIV on the lung. Therefore, we performed an initial screen for this study and identified that of these four miRNAs, only miR-144 expression was increased in the alveolar epithelial cells of HIV-1 transgenic rats. Interestingly, miR-144 has previously been found to negatively affect alveolar epithelial barrier function outside the setting of HIV (3), in addition to its ability to bind to the 3′-UTR of Nrf2 and impair Nrf2 function (11, 19). Therefore, we focused on miR-144 as a potential mechanism by which HIV-related proteins inhibit Nrf2 expression and function and, thereby, disrupt alveolar epithelial barrier integrity.

MATERIALS AND METHODS

Cell culture. Primary alveolar epithelial cells (AECs) were isolated as previously described (8) from 9- to 12-mo-old HIV-1 transgenic (HIV-1 Tg) rats (this construct was originally developed by Reid et al. (23) and littermate wild-type rats, both of which are in the Fischer 344 background, and cultured in DMEM/F12 (Cellgro, Manassas, VA) with 10% FBS (Atlanta Biologicals, Lawrenceville, GA) and the antibiotic-antimycotic agents penicillin, streptomycin, and ampicillin (Sigma-Aldrich, St. Louis, MO) at 37°C in 5% CO_2 (22). HIV-1 Tg rat and wild-type rat breeders were originally obtained from Harlan Laboratories (Indianapolis, IN), from which we have developed and maintained a colony in our animal facility. AEC monolayers were established on permeable transwell plates for 5–6 days, as previously described (14). All procedures were approved by the Institutional Animal Care and Use Committee at Emory University.

RNA isolation, reverse transcription, and real-time PCR. Total RNA was extracted using the Quick-RNA kit (Zymo Research, Irvine, CA). Reverse transcription and real-time PCR (Bio-Rad, Hercules, CA) were performed with primer pairs (10) and normalized to 9S.

microRNA isolation. The mirVana miRNA isolation kit was used to extract and purify miRNAs (ThermoFisher Scientific, Waltham, MA). cDNA was synthesized from 250 ng miRNA/sample using the miScript II RT kit (Qiagen, Germantown, MD). On the basis of in silico analysis of Nrf2 3′-UTR and previous publications, quantitative PCR (qPCR) was performed for miR21, miR27a-3p, miR155-5p, and miR-144-3p gene expression with primers obtained from Qiagen using QuantiTect SYBR Green PCR kit, normalized to SNORD25, and relative levels were determined by the comparative cycle threshold method, as we have used previously (14).

miR-144 overexpression. Primary AECs from 9- to 12-mo-old wild-type rats were transfected with either miR-144-3p mimic from Qiagen (5′-UACAGUAUAGAUGAUGUACU-3′; 5 nM final concentration) or 5 nM of a miRNA negative control (Qiagen) using

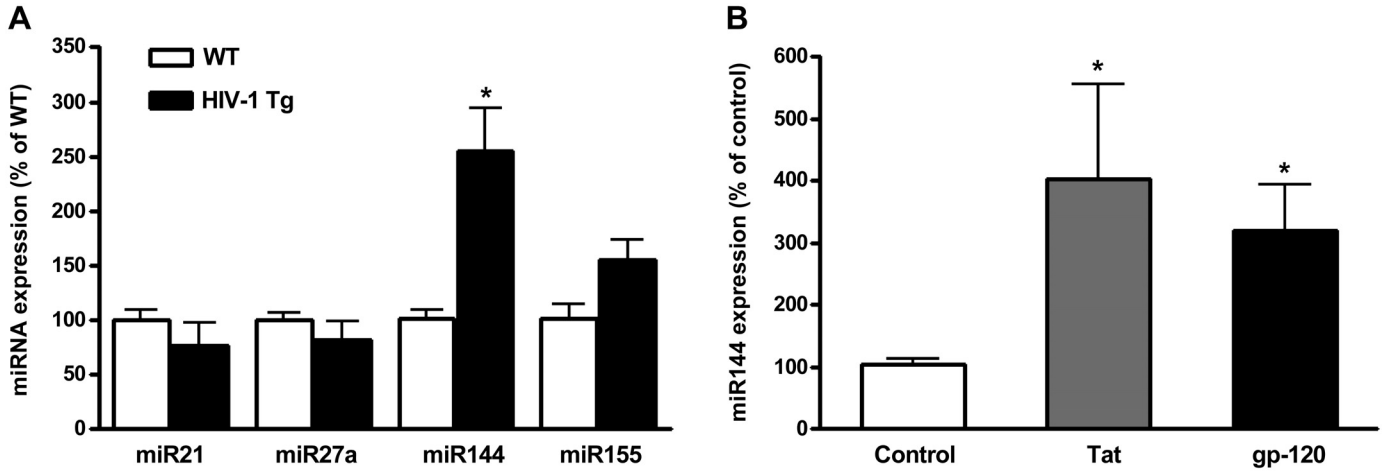

Fig. 1. HIV-1 viral proteins induce miR-144 expression in primary alveolar epithelial cells (AECs). Primary AECs from HIV-1 transgenic (Tg) and wild-type (WT) rats were isolated and the relative expression of miR-21, miR-27a, miR-144, and miR-155 was determined by RT-PCR. *A*: miR-144 expression was significantly increased in the AECs of HIV-1 Tg rats when compared with miR-144 expression in AECs from wild-type rats. *B*: in parallel, treating AECs from wild-type rats with Tat and gp120 for 72 h recapitulated the induction of miR-144 seen in the AECs of HIV-1 transgenic rats. $*P < 0.05$ compared with untreated wild type. Data shown are means ± SE ($n = 3–6$ in *A* and $n = 8–11$ in *B*).

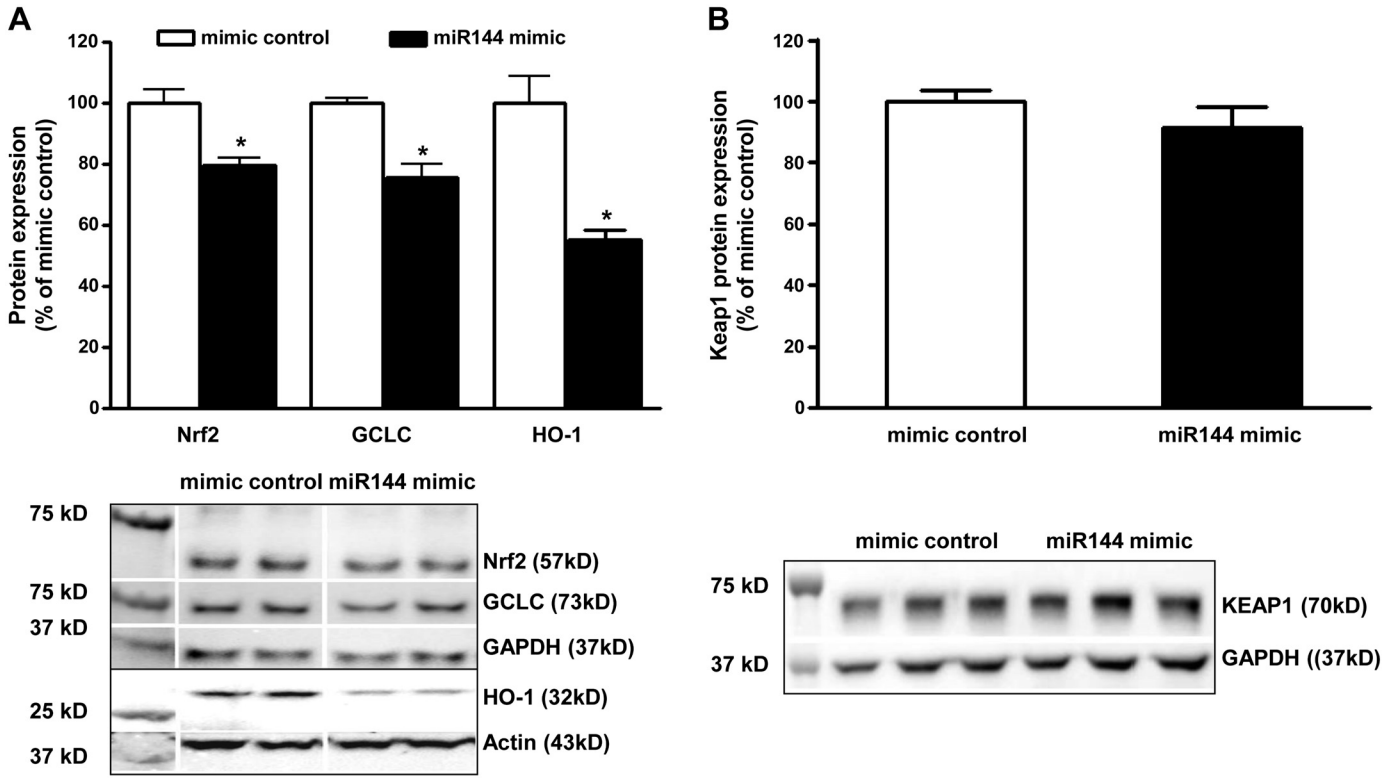

Fig. 2. Overexpression of miR-144 inhibits nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and glutamate-cysteine ligase catalytic subunit (GCLC) protein expression in naïve primary alveolar epithelial cells (AECs) isolated from wild-type rats. AECs isolated from wild-type rats were transfected with either a miR-144 mimic or a negative control mimic. Relative protein expression of Nrf2 and its downstream effectors, GCLC and heme oxygenase 1 (HO-1), were quantified by Western immunoblotting after 72 h posttransfection. *A*: there was a significant reduction of Nrf2, GCLC, and HO-1 protein expression in the cells treated with the miR-144 mimic compared with cells treated with the control mimic. *B*: there was no change in Keap1 protein level when cells were treated with miR144 mimic. $^*P < 0.05$ compared with treatment with the control mimic. Data shown are means ± SE ($n = 6$ in each group). Representative Western blots are shown.

Lipofectamine 3000 (Thermo Fisher Scientific) and assessed at 96 h posttransfection for protein expression, respectively.

miR-144 silencing. Primary AECs from 9- to 12-mo-old HIV-1 Tg rats were isolated and transfected with a miR-144-3p inhibitor from Qiagen (5′-UACAGUAUAGAUGAUGUACU-3′, 50 nM) or an miRNA inhibitor negative control (50 nM) using Lipofectamine 3000 (Thermo Fisher Scientific). After incubation for 96 h, the cells were collected for protein expression.

Western immunoblotting. Total proteins were isolated from primary AECs derived from wild-type or HIV-1 Tg rats using Laemmli sample buffer (Bio-Rad) (10), electrophoresed in 4–20% polyacrylamide gels (NuSep, Germantown, MD), transferred to a PVDF membrane, and incubated with primary antibodies against Nrf2 (SC-722x), heme oxygenase 1 (HO-1; SC-10789), and actin (SC-1616) from Santa Cruz Biotechnology (Heidelberg, Germany), Keap1 (10503-2-AP) from ProteinTech (Rosemont, IL) and glutamate-cysteine ligase catalytic subunit [GCLC (55435) from Abcam, Cambridge, MA] before incubation with secondary antibodies, with GAPDH (G9545 from Sigma-Aldrich, St. Louis, MO) as the loading control, as we have done previously (9). Immunoreactive bands were captured with ChemiDoc XRS system (Bio-Rad).

Epithelial monolayer permeability assay. Barrier function of AEC monolayers was quantified by paracellular flux of FITC-labeled Dextran (Sigma-Aldrich, St. Louis, MO) over 2 h, as previously described (9, 14).

miR-144 inhibitor treatment in vivo. Eight HIV-1 transgenic rats (4 male and 4 female rats) were divided into two treatment groups (2 male and 2 female rats per each group). 50 nM of a miR-144 inhibitor or a negative control (in 100 μl of PBS) was delivered intratracheally into HIV-1 transgenic rats (total of three treatments over a 7-day period) after which AECs were harvested for gene expression and epithelial barrier function analysis.

Dietary treatment with the Nrf2 activator PB123. In selected experiments, three wild-type rats and six HIV-1 transgenic rats were

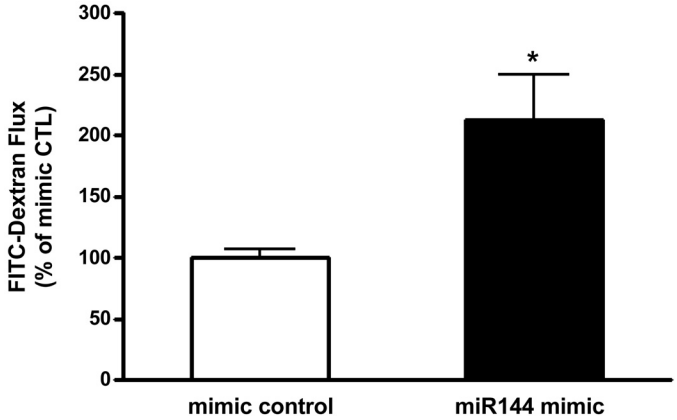

Fig. 3. Overexpression of miR-144 likewise impairs barrier function in naïve primary alveolar epithelial cells (AECs) isolated from wild-type rats. Primary AECs isolated from wild-type rats were transfected with either a miR-144 mimic or a negative control (CTL) mimic after culturing for 24 h. Epithelial barrier function was assessed by measuring relative flux of FITC-labeled dextran across the monolayers after 4 days posttransfection. Primary AECs treated with the miR-144 mimic had a significant increase in FITC-dextran permeability ($^*P < 0.05$), reflecting decreased barrier function. Data shown are means ± SE ($n = 3$ in each group).

fed the Lieber-DeCarli liquid diet (Research Diets, New Brunswick, NJ) ± the Nrf2 activator PB123 (60 mg/l; Pathways Bioscience, Aurora, CO) for 2 wk before harvesting their AECs.

Statistical analyses. One-way ANOVA with Student-Newman-Keuls post hoc tests was performed for multiple comparisons, and Student's *t*-test was used for single comparisons using Prism (GraphPad, San Diego, CA). All data are presented as means ± SE, and significance was accepted at $P < 0.05$.

RESULTS

HIV-1 viral proteins induce miR-144 expression in primary alveolar epithelial cells. To assess for possible changes in microRNA expression in HIV-1 Tg rats, primary AECs from HIV-1 Tg rats and their wild-type littermates were isolated. Levels of miR-21, miR-27a, miR-144, and miR-155 were assessed by microRNA RT-qPCR. As shown in Fig. 1*A*, miR-144 expression was significantly increased ~2.5-fold in AECs from HIV-1 Tg rats. In contrast, miR-21, miR-27a, and miR-155 expression was the same in AECs from HIV-1 Tg and wild-type rats. In parallel, the increased expression of miR-144 in the HIV-1 Tg rats in vivo was reproduced by direct treatment ex vivo of naïve primary rat AECs isolated from wild-type rats with either of the HIV-related proteins Tat (transactivator of transcription) or gp120 (glycoprotein 120), as shown in Fig. 1*B*.

Pathophysiological effects of HIV-1 transgene expression on Nrf2 and alveolar barrier function can be reproduced by overexpression of miR-144 in primary AECs of wild-type rats ex vivo. As previously noted, an in silico analysis revealed a miR-144 binding site on the 3′-UTR of Nrf2 mRNA. To determine the effects of miR-144 on Nrf2 expression and AEC barrier function, wild-type rat primary AECs were transfected with a miR-144 mimic or a control mimic ex vivo, and protein expression of Nrf2 and GCLC, HO-1 (Nrf2 downstream effectors) was then assessed by Western blotting. As shown in Fig. 2*A*, the expression of those proteins was significantly reduced after transfection with the miR-144 mimic, while Keap1 expression did not change (Fig. 2*B*). In parallel, miR-144 overexpression significantly impaired barrier function in monolayers formed from these primary AECs, as reflected by an approximately twofold increase in paracellular permeability (Fig. 3). Those data suggest that overexpression of miR-144 in normal AECs from WT rats could mimic the effect seen in HIV-1 Tg rats.

In parallel, the pathophysiological effects of HIV-1 transgene expression can be reversed by inhibiting miR-144 expression in primary AECs ex vivo. We next determined the effects of silencing miR-144 ex vivo in primary AECs derived from HIV-1 Tg rats using a specific miR-144 inhibitor (antagomir). We first identified that antagonizing miR-144 significantly

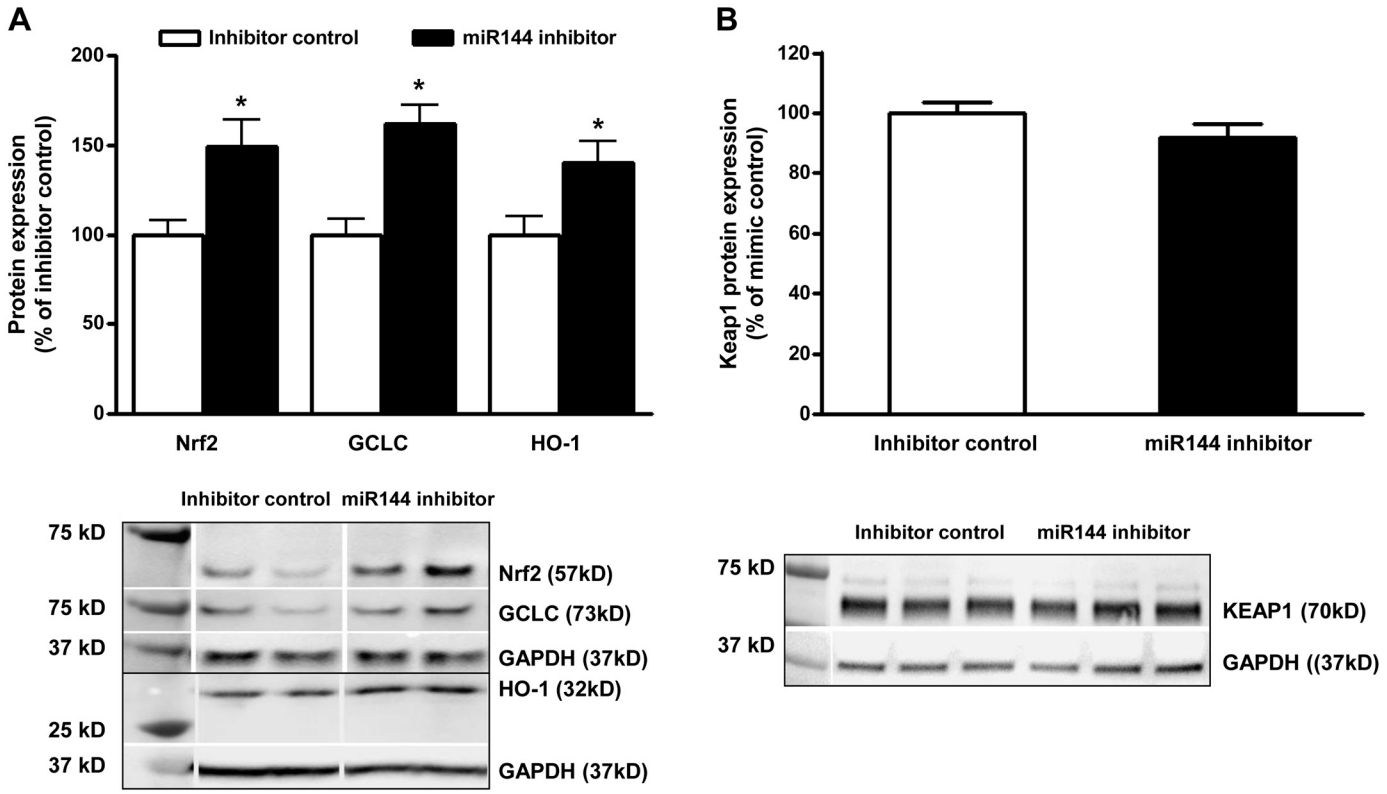

Fig. 4. Inhibition of miR-144 in HIV-1 transgenic (Tg) rat primary alveolar epithelial cells (AECs) ex vivo enhances protein expression of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and its downstream effector glutamate-cysteine ligase catalytic subunit (GCLC) and restores barrier function. Primary AECs isolated from HIV-1 Tg rats were transfected with a miR-144 inhibitor or a negative control inhibitor. Relative protein expression of Nrf2 and its downstream effectors, GCLC and HO-1, was assessed by Western blotting 72 h posttransfection. *A*: primary AECs treated with a miR-144 inhibitor showed a significant increase in the levels of Nrf2, GCLC, and heme oxygenase 1 (HO-1) compared with cells with a control inhibitor. *B*: there was no change in Keap1 protein level when cells were treated with miR144 inhibitor. *$P < 0.05$ compared with protein expression of HIV-1 Tg rat AECs transfected with the control inhibitor. Data shown are means ± SE ($n = 4$ in each group). Representative Western blots are shown.

increased the protein levels of Nrf2, GCLC, and HO-1, indicating an increase in both Nrf2 expression and activity, as reflected by downstream induction of a Nrf2-dependent antioxidant (Fig. 4*A*). The miR144 antagomir did not affect Keap1 protein expression as shown in Fig. 4*B*. In parallel, miR-144 inhibition significantly improved barrier function in monolayers derived from primary AECS of HIV-1 Tg rats (Fig. 5). The data indicate that miR-144 plays an important role in the HIV-1 transgene-induced alveolar epithelial dysfunction.

Effects of miR-144 on Nrf2 and alveolar barrier function can be reversed in vivo by delivering the miR-144 antagomir intratracheally. To extend this study and determine the potential therapeutic benefit of antagonizing miR-144 in vivo, we delivered three intratracheal treatments of either the miR-144 inhibitor (antagomir) or a control inhibitor over the course of 1 wk to HIV-1 Tg rats and then isolated their AECs to assess their expression of Nrf2 and their ability to form tight monolayers in culture. Consistent with the effects of antagonizing miR-144 ex vivo, treatment with the miR-144 antagomir in vivo significantly increased the expression of Nrf2, GCLC, and HO-1 in primary AECs from HIV-1 Tg rats (Fig. 6*A*). In parallel, treatment with the miR-144 antagomir in vivo significantly improved barrier function in monolayers formed by these primary AECs, as evidenced by an ~50% reduction in paracellular permeability (Fig. 6*B*).

Dietary treatment with the Nrf2 activator PB123 in vivo also improved barrier function in the alveolar epithelium of HIV-1 transgenic rats. Although miR-144 antagomir rescued alveolar epithelial barrier function in HIV-1 Tg rats in vivo, we were interested in a practical therapy that would be potentially easier to administer in future clinical studies. To that end, we used the novel Nrf2 activator PB123 to determine whether Nrf2 activation could overcome the inhibitory effects of miR-144 on the Nrf2/ARE axis. Therefore, we treated HIV-1 transgenic rats and their littermate controls PB123 (60 mg/l) in their diets for 2 wk and compared barrier integrity in monolayers formed by their primary AECs. Consistent with the findings that antagonizing miR-144 improved Nrf2 function and barrier formation in AECs from HIV-1 Tg rats, treatment with the Nrf2 activator PB123 in vivo increased gene expression of Nrf2 and its downstream effectors, GCLC and HO-1 (Fig. 7*A*) and restored AEC barrier formation ex vivo, as reflected by paracellular flux of FITC-dextran (Fig. 7*B*).

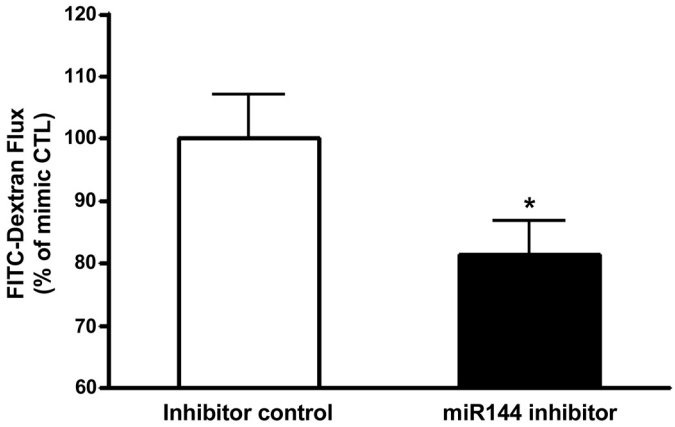

Fig. 5. Inhibition of miR-144 expression restores the epithelial barrier function in the primary alveolar epithelial cells (AECs) derived from HIV-1 transgenic (Tg) rats. Primary AECs isolated from HIV-1 Tg rats were transfected with a miR-144 inhibitor or an inhibitor control (CTL) after culturing for 24 h. Assessment of epithelial barrier function by FITC-labeled dextran permeability 4 days posttransfection showed a significant decrease in permeability across the epithelial monolayer after treatment with the inhibitor. $*P < 0.05$ compared with the HIV-1 Tg rat AECs transfected with the negative control inhibitor. Data shown are means ± SE ($n = 5$ or 6 in each group).

DISCUSSION

We previously determined that HIV-related viral proteins impair epithelial barrier function by inhibiting Nrf2 expression and function (10). In this study, we determined that exposure to HIV-related viral proteins increases expression of miR-144 and that upregulation of this miRNA impairs epithelial barrier function via inhibition of Nrf2 and its downstream effectors. We further identified that HIV-mediated inhibition of Nrf2 and the consequent alveolar epithelial barrier dysfunction can, in turn, be abrogated by either inhibiting miR-144 or activating Nrf2 in the airway in vivo. Taken together, these new experimental findings implicate the previously unrecognized induction of miR-144 as a fundamental mechanism by which chronic expression of HIV-related proteins in the alveolar space inhibits the critical defense pathways regulated by Nrf2 and, thereby, impairs antioxidant responses and epithelial barrier integrity.

We began by determining the levels of selected microRNAs in the HIV-1 transgenic rat model that are known to inhibit Nrf2. We initially targeted miR-21 and miR-155, based on evidence of alteration in these miRNAs by HIV infection in other systems (26). Interestingly, analysis in silico (using the miRNA database at http://mirdb.org) identified that multiple other miRNAs, including miR-27a and miR-144, have binding sites in the 3′-UTR of Nrf2. However, of these four selected miRNAs, only miR-144 expression was significantly increased in the primary AECs from HIV-1 transgenic rats compared with their wild-type littermates. Although we did not screen for all other mRNAs known to potentially target Nrf2, the circumstantial evidence linking miR-144 and the collected findings in our study provide a strong case to implicate miR-144 in HIV-related alveolar epithelial cell dysfunction. Consistent with this, we were able to replicate the same pattern of miR-144 expression in wild-type AECs by directly exposing them to HIV-related proteins ex vivo. Further, overexpressing miR-144 in naïve wild-type AECs recapitulated the effects of HIV-1 transgene expression, whereas antagonizing miR-144 expression mitigated the pathophysiological effects of chronic exposure to HIV-related proteins in HIV-1 transgenic AECs. Taken together, these experimental findings provide novel evidence that HIV-related viral proteins induce miR-144 within the alveolar epithelium which, in turn, mediates the pathophysiological effects of HIV in the lung that we had previously identified.

The striking similarities between the effects of miR-144 overexpression and Nrf2 impairment on epithelial barrier function raised the intriguing possibility that miR-144 was, indeed, exerting its effects by inhibiting Nrf2. Nrf2 is the master transcription factor that regulates antioxidant defenses via activation of multiple genes important in the cellular response to oxidative stress, including the glutamate-cysteine ligase, catalytic subunit (GCLC), NAD(P)H dehydrogenase, and quinone 1 (NQO1) (17). In the alveolar epithelium, we previously

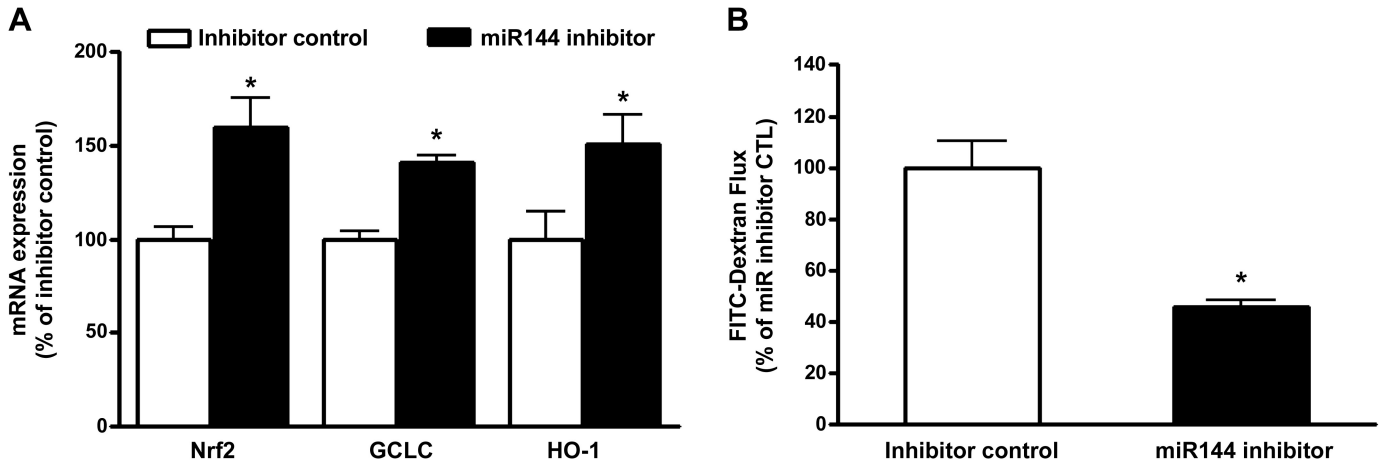

Fig. 6. Intratracheal treatment of HIV-1 transgenic (Tg) rats with the miR-144 inhibitor in vivo increases gene expression of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and its downstream effector glutamate-cysteine ligase catalytic subunit (GCLC) and restores barrier function in the alveolar epithelium. Primary alveolar epithelial cells (AECs) were isolated from HIV-1 Tg rats after intratracheal treatment with either a miR-144 inhibitor (50 nM ×3 times) or negative control inhibitor in vivo for 1 wk. Freshly isolated AECs were extracted and real-time RT-PCR was performed for Nrf2, GCLC, and heme oxygenase 1 (HO-1) expression. *A*: cells derived from HIV-1 Tg rats treated with the miR-144 inhibitor had a significant increase in Nrf2, GCLC, and HO-1 mRNA expression. *B*: in parallel, alveolar epithelial barrier function (as reflected by FITC-labeled dextran permeability across cultured monolayers) was significantly improved after treatment with the inhibitor. $*P < 0.05$ compared with gene expression of cells from rats treated with control inhibitor. Data shown are means ± SE ($n = 4$ in each group).

determined that manipulating Nrf2 expression, either via RNA silencing or vector-mediated overexpression, directly affected epithelial barrier function (10). Specifically, Nrf2 silencing increased epithelial barrier permeability and decreased expression of tight junction proteins, while overexpressing Nrf2 normalized the expression of tight junction proteins and restored barrier function in alveolar epithelial cells isolated from HIV-1 transgenic rats (10). In addition, treating AECs from HIV-1 Tg rats with sulforaphane, a Nrf2 activator, also restored epithelial barrier function (10).

Our findings are, in fact, consistent with multiple other reports showing a direct regulatory effect of miR-144 on Nrf2 (24, 27, 28). As an example, increased miR-144 expression resulted in decreased Nrf2 expression and as a result, impaired antioxidant capacity in a chronic myeloid leukemia cell line (24). To assess whether this regulatory relationship between miR-144 and Nrf2 held true in the HIV-1 transgenic rat model, we determined the effects of overexpressing and silencing miR-144 on the Nrf2-ARE axis. As predicted, overexpression of miR-144 in wild-type AECs downregulated Nrf2 and its downstream effector GCLC, HO-1 (reflecting a reduction in Nrf2 activity), and silencing miR-144 in the HIV-1 Tg rat AECs rescued Nrf2,GCLC, and HO-1 expression.

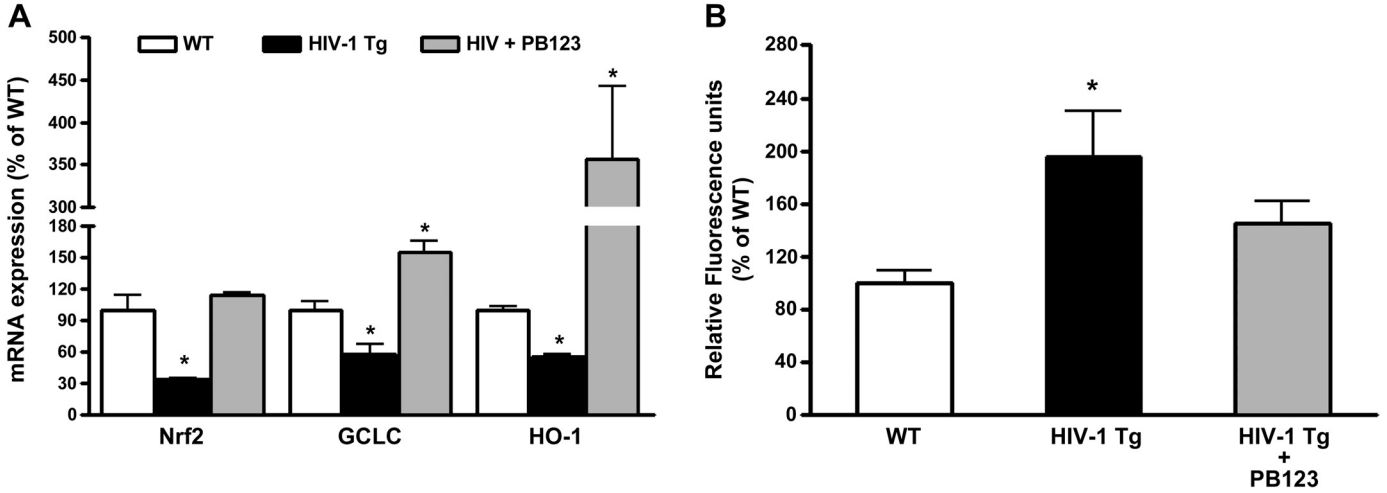

Fig. 7. Treatment of HIV-1 Tg rats with an nuclear factor (erythroid-derived 2)-like 2 (Nrf2) activator, PB123, in vivo restores alveolar epithelial barrier function. Primary alveolar epithelial cells (AECs) were isolated from wild-type rats, HIV-1 transgenic (Tg) rats, and HIV-1 Tg rats given the Nrf2 activator PB123 in their liquid diets for 2 wk. *A*: mRNA expression of Nrf2 and its downstream effectors, glutamate-cysteine ligase catalytic subunit (GCLC) and heme oxygenase 1 (HO-1), was analyzed from isolated AECs by RT-qPCR. Gene expression of all three targets was significantly increased ($*P < 0.05$; $n = 3$). *B*: alveolar epithelial monolayer permeability was determined by FITC-labeled dextran permeability. Monolayers derived from HIV-1 Tg rats had increased paracellular permeability compared with monolayers derived from wild-type (WT) rats. In contrast, prior treatment with PB123 in vivo restored barrier function in AEC monolayers derived from HIV-1 Tg rats, such that their paracellular permeability was the same as AEC monolayers derived from wild-type rats. $*P < 0.05$ compared with FITC-labeled dextran permeability of the AECs derived from wild-type rats. Data shown are means ± SE ($n = 3$).

On the basis of this newly identified mechanistic pathway in which HIV-related proteins induce the expression of miR-144, combined with the known effects of miR-144 on Nrf2 and our earlier studies revealing the role of Nrf2 in regulating alveolar epithelial barrier function, we next turned our attention to determining the relevance of this pathway in vivo. To that end, we treated HIV-1 transgenic rats intratracheally with the miR-144 antagomir to determine whether this therapeutic approach could mitigate the effects of chronic exposure of the alveolar epithelium to HIV-related proteins in vivo. In parallel, to provide additional evidence that the pathophysiological effects of chronic exposure to HIV-related proteins on the alveolar epithelium are, in fact, mediated by decreased activity of Nrf2 in vivo, we also treated HIV-1 transgenic rats with the Nrf2 activator PB123 as a dietary supplement. Consistent with our proposed pathway, we determined that either direct antagonism of miR-144 or activation of Nrf2 in vivo could reverse the pathophysiological effects of chronic HIV-related protein expression on alveolar epithelial barrier function.

Many important questions remain about this pathway, including the mechanism(s) by which HIV-related viral proteins increase miR-144 expression. Our analysis in silico and published data from colleagues (19) indicate that miR-144 can bind directly to the 3′-UTR of Nrf2 mRNA and, thereby, block Nrf2 protein translation. However, it is also possible that miR-144 inhibits Nrf2 expression and signaling more indirectly by limiting expression of one or more targets that influence Nrf2. For example, silencing of Brahma-related gene 1 (Brg1) was found by Yue and colleagues (16) to diminish the effects of miR-144 on Nrf2 expression and activity. Whether or not this gene is mediating a role in our model is as yet undetermined. However, taken together, these data provide compelling evidence that the inhibition of Nrf2 and the consequent alveolar epithelial barrier dysfunction that we previously characterized in the HIV-1 Tg rat model are mediated either directly or indirectly by miR-144. Specifically, we determined that HIV-related viral proteins induce the expression of miR-144, which, in turn, suppresses Nrf2 activity and, thereby, impairs barrier function. In parallel, either the direct antagonism of miR-144 or the activation of Nrf2 in vivo restored barrier formation in alveolar epithelial cells from HIV-1 transgenic rats. Although these experiments do not rule out other potential effects of miR-144 on barrier function, the accumulated data make a strong case for the primacy of Nrf2 impairment in HIV-mediated lung epithelial dysfunction. Given the ongoing work in the area of oncology, demonstrating the therapeutic potential of miRNA antagonism (20), our findings also offer an important window into a new therapeutic pathway by which we might improve the lung health of people living with HIV.

In conclusion, this new study suggests a novel therapeutic approach to enhance lung health in individuals living with HIV, even when they are adherent to antiretroviral therapy, either by antagonizing miR-144 or, perhaps more feasibly, through the activation of Nrf2 with dietary supplements of phytochemicals, such as PB123.

ACKNOWLEDGMENTS

We thank Robert Raynor and S. Todd Mills for invaluable technical expertise and assistance with these experiments.

GRANTS

B. S. Staiteh was supported by National Institute on Alcohol Abuse and Alcoholism Grant K08 AA024512. D. M. Guidot was supported by National Heart, Lung, and Blood Institute Grant R01 HL125042, and A. T. Kukoyi was supported by a minority supplement on the same award.

DISCLOSURES

B. M. Hybertson, B. Gao, and J. M. McCord own the company (Pathways Bioscience) that produces PB123 and provided the data on PB123, as well as the compound itself for the experiments in this study. None of the other authors has any conflicts of interest, financial or otherwise, to disclose.

AUTHOR CONTRIBUTIONS

X.F. and D.M.G. conceived and designed research; A.T.K., X.F., B.M.H., and B.G. performed experiments; A.T.K., X.F., B.M.H., B.G., and J.M.M. analyzed data; A.T.K., X.F., B.S.S., B.M.H., B.G., and J.M.M. interpreted results of experiments; A.T.K., X.F., B.M.H., and B.G. prepared figures; A.T.K. and X.F. drafted manuscript; X.F., B.S.S., and D.M.G. edited and revised manuscript; D.M.G. approved final version of manuscript.

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