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Hypoxia and HIF-1 inhibition enhance lentiviral transduction efficiency: a novel strategy for gene delivery optimization

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Abstract

Lentiviral vectors are widely used for stable gene delivery, but their transduction efficiency can be limited by suboptimal experimental conditions. Here, we investigated the role of oxygen concentration and hypoxia-inducible factor 1 (HIF-1) signaling in lentiviral packaging and transduction. We found that packaging lentivirus under hypoxic conditions (10% O_2) significantly increased viral titers and transduction efficiency by approximately 10%. However, hypoxic conditions during viral entry impaired infection efficiency, likely due to HIF-1α-mediated cellular protective mechanisms. Pretreatment of cells with the HIF-1 inhibitor PX-478 reversed this effect, enhancing viral entry and genome integration in a dose-dependent manner. Combining hypoxic virus packaging with PX-478 pretreatment synergistically improved transduction efficiency by 20%. These findings suggest that HIF-1 inhibition and controlled hypoxia significantly enhance lentiviral transduction efficiency, establishing a versatile strategy with broad applicability across viral vector-dependent biomedical applications.

Keywords Lentiviral transduction, Gene delivery optimization, Oxygen tension, Hypoxia, HIF-1 inhibitor, Transduction efficiency

Introduction

Lentivirus is an enveloped, plus-strand RNA virus belonging to the *Orthoretrovirinae* subfamily of *Retroviridae* [1]. It utilizes viral reverse transcriptase and integrase to integrate its genome into the host genome, enabling stable gene delivery even in non-dividing cells [2]. Most lentiviral vectors are derived from human immunodeficiency virus-1 (HIV-1) and have been optimized for biotechnological applications. For instance, the retroviral envelope glycoprotein (Env) is often replaced with the vesicular stomatitis virus G glycoprotein (VSV-G) to enhance virion stability and broaden cell tropism [3]. Additionally, viral accessory proteins (e.g., *env*, *vif*, *vpr*, *vpu*, *nef*) are deleted in second-generation vectors to improve safety by reducing viral propagation [4]. These advancements have established lentiviral vectors as a safe

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and efficient tool for stable transgene delivery [5]. Today, lentiviral vectors are widely used in neuroscience, hematology, developmental biology, and stem cell research [6, 7], as well as in clinical applications such as gene therapy [8] and chimeric antigen receptor (CAR) T cell generation [9, 10].

Recombinant lentivirus is typically produced in human embryonic kidney-derived cells (HEK-293T). These cells are transfected with lentiviral transfer and helper plasmids, after which virions are packaged and purified from the culture medium [11]. Key factors influencing transduction efficiency include transfection methods, vector quality and concentration, chemical facilitators, cell confluency, DNA ratios, incubation time, and temperature [12, 13].

Standard cell culture incubators regulate CO_2 and temperature but rarely control O_2 levels. The oxygen concentration in these incubators, termed "normoxia," is typically 18–21%, significantly higher than the physiological oxygen levels (2–10%, termed "physioxia") found in most human tissues [14–16]. Hyperoxic conditions commonly used in cell culture can disrupt redox homeostasis, elevate reactive oxygen species (ROS) production, and cause DNA damage, leading to oxidative stress [17, 18]. In contrast, physioxic conditions have been shown to enhance the proliferation and differentiation of stem cells [19–21], fibroblasts, and adipocytes [22].

The clinical translation of lentiviral-based therapies faces particular challenges in immune cell engineering. CAR-T cell manufacturing, for instance, requires high-efficiency transduction of activated T lymphocytes - a cell type notoriously resistant to viral entry. Currently, several studies are dedicated to enhancing viral transduction efficiency in T cells, such as using chemical enhancers (e.g., polybrene [23]) or physical methods (e.g., spinoculation [24]). However, these approaches may induce cytotoxic effects and compromise therapeutic efficacy. This highlights the need for novel transduction enhancement strategies while preserving cell viability and maintaining therapeutic potential.

Based on these findings, this study investigated the role of physioxia in lentiviral packaging and transduction, focusing on its effects on exogenous gene integration and protein expression. We also examined whether hyperoxia could reverse the effects of physioxia on viral transduction. By dissecting these phase-specific oxygen effects and employing HIF-1α inhibitors, this study establishes an oxygen-modulated paradigm to optimize lentiviral gene delivery, which holds great potential for improving transduction efficiency in recalcitrant cell targets such as CAR-T cells and hematopoietic stem cells.

Materials and methods

Cells and cell culture

HEK-293T cells were obtained from our institute's cell resource center and cultured in DMEM medium (Servicebio, G4515), while K562 cells were maintained in RPMI-1640 medium (Servicebio, G4530). Both cell lines were supplemented with 10% (v/v) fetal bovine serum (ExCell Bio, FSP500) and penicillin-streptomycin-amphotericin B solution (Beijing Solarbio Science & Technology Co., Ltd, P7630). Cells were incubated at 37 °C in a humidified incubator with 5% CO_2 (Thermo Fisher Scientific, Inc.).

Lentiviral vector and packaging

The second-generation lentiviral packaging system was used to investigate the role of oxygen in lentiviral packaging and transduction [25]. The transfer vector pCDH-EF1 (Addgene, #72266) was modified to include the mNeonGreen (mNG) fluorescent protein upstream of the WPRE element. Lentiviral vectors pseudotyped with vesicular stomatitis virus glycoprotein (VSV-G) and packaging plasmid pPAX2 were purchased from Addgene. Polyethylenimine (PEI 40 K, Maokangbio, CAS 49553-93-7) was used as the transfection reagent (1 mg/mL).

HEK-293T cells were seeded in 10 cm dishes and incubated for 24 h to reach 70–80% confluency. Prior to transfection, the medium was replaced with fresh medium. PEI 40 K was diluted in 150 mM NaCl and incubated at room temperature for 3 min. Packaging, envelope, and transfer plasmids were mixed in a 4:4:5 ratio (w/w) with 150 mM NaCl. The PEI/DNA mixture was prepared at an N/P ratio of 18 [26] and incubated for 20 min before being added to the cells. After 8–12 h, the medium was replaced with fresh medium. Viral supernatants were collected at 24-, 48-, and 72-hours post-transfection, pooled, and stored at 4 °C. The supernatant was centrifuged at 5,000 × g for 10 min to remove cell debris, filtered through a 0.45 μm filter, and ultracentrifuged at 50,000 × g for 2 h at 4 °C. The viral pellet was resuspended in 500 μL PBS and stored at -80 °C.

Viral infection

K562 cells were seeded at 5×10^5 cells/mL in 6-well plates and transduced with lentivirus under different oxygen conditions. After 8 h, the virus-containing medium was replaced with fresh medium. For environment-switching groups, cells were transferred to new oxygen conditions during medium replacement. After 48 h, cells were imaged using a Nikon microscope and analyzed by flow cytometry. Infection efficiency was calculated as the percentage of fluorescent cells relative to the total cell population.

Hypoxic/hyperoxic and PX-478 treatment

Cells were incubated in sealed chambers with controlled oxygen levels: physioxia (10% O_2, 5% CO_2, 85% N_2) or hyperoxia (30% O_2, 5% CO_2, 65% N_2). Oxygen concentrations were continuously monitored using a portable gas detector (ADKS-1, Changzhou Edkors Instrument Co., Ltd), and confirmed stable at the beginning, middle, and end of experiments. K562 cells were pretreated with PX-478 (20 μM) or vehicle for 16 h before hypoxia treatment and transduction.

CCK-8 assay

Cell viability was assessed using the Cell Counting Kit-8 (CCK-8, Dojindo, CK04). Cells were seeded in 96-well plates (1×10^4 cells/well) and treated with PX-478 or vehicle for 16 h. CCK-8 reagent (10 μL) was added to each well, and plates were incubated at 37 °C for 2 h. Absorbance was measured at 450 nm using a microplate reader (Synergy H4, BioTek Instruments).

Flow cytometry analysis

Cells were harvested, washed with PBS, and resuspended in PBS containing 0.5% BSA. Samples were analyzed using a BD LSRFortessa Cell Analyzer, with 10,000 events recorded per sample. Data were analyzed using FlowJo software (v10.8.1).

Quantitative real-time PCR

Genomic DNA was extracted using the Universal Genomic DNA Kit (CWBIO, CW2298M) according to the manufacturer's instructions. Total RNA was extracted using TRIzol reagent (Servicebio, G3013) and reverse transcribed using oligo(dT), dNTPs, RNase inhibitor, and MLV reverse transcriptase. cDNA or gDNA was amplified using SYBR Green qPCR Supermix (US Everbright Inc, S2024) on a CFX Duet Real-Time PCR Detection System (Bio-Rad). Primers for ACTB were: forward, 5′-CACCATTGGCAATGAGCGGTTC-3′; reverse, 5′-AGGTCTTTGCGGATGTCCACGT-3′. Primers for mNG were: forward, 5′-ATGGGCTGGTTCTTCAGGTA-3′; reverse, 5′- CCCAACGACAAGACCATCAT-3′. Primers for HIF1A were: forward, 5′- TATGAGCCAGAAGAACTTTTAGGC-3′; reverse, 5′- CACCTCTTTTGGCAAGCATCCTG-3′. Primers for GLUT1 were: forward, 5′- TTGCAGGCTTCTCCAACTGGAC-3′; reverse, 5′- CAGAACCAGGAGCACAGTGAAG-3′. Data were normalized to ACTB and analyzed using the 2^(-ΔΔCq) method.

Statistical analysis

Data were analyzed using GraphPad Prism (v10). Statistical significance was determined by ordinary one-way ANOVA with multiple comparisons (three or more groups) or unpaired t-tests (two groups). Results are expressed as mean ± standard deviation (SD) from at least three replicates. A p-value < 0.05 was considered statistically significant.

Results

Lower oxygen concentration during viral packaging enhances transduction efficiency

Lentivirus was packaged in HEK-293T cells under normoxic (21% O_2) and hypoxic (10% O_2) conditions. After 3 days of transfection, viral particles were collected, concentrated, and analyzed. Fluorescence microscopy revealed lower mNeonGreen (mNG) expression in 293T cells under hypoxic packaging compared to normoxia (Fig. 1A). However, viruses packaged under hypoxia exhibited significantly higher transduction efficiency in

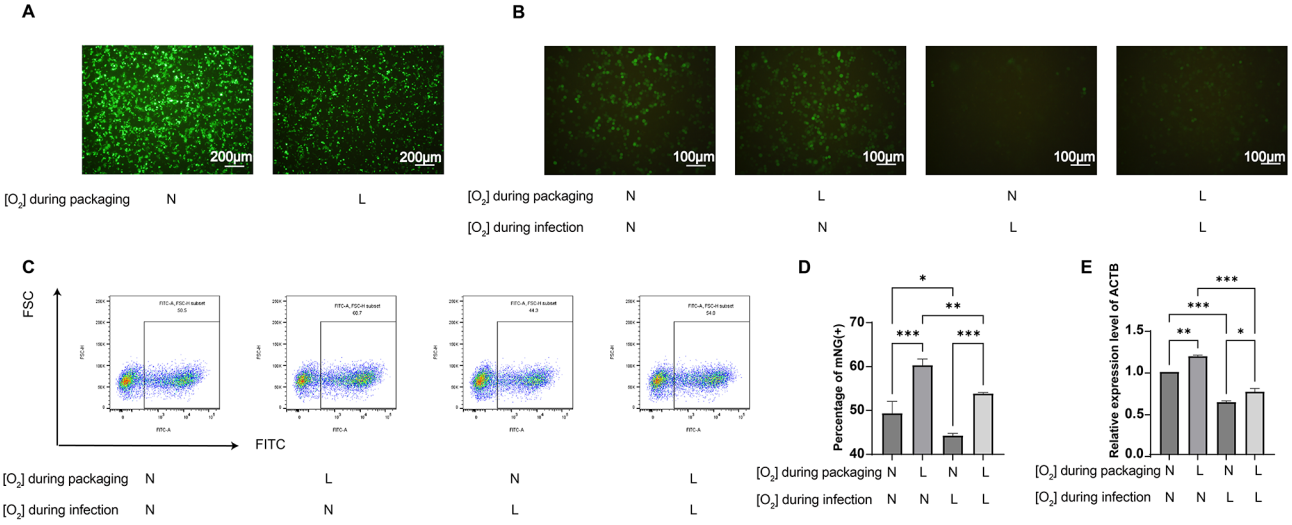

Fig. 1 Effect of oxygen concentration on viral packaging and transduction efficiency. (**A**) Fluorescence images of 293T cells packaged under normoxia (N) or hypoxia (L). (**B**, **C**, **D**) Fluorescence images, flow cytometry plots, and quantification of K562 cells transduced under normoxia (N) or hypoxia (L). (**E**) qPCR analysis of viral genome integration. $n = 3$, $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, data are presented as the mean ± standard deviation

K562 cells, as evidenced by increased fluorescence intensity (Fig. 1B), flow cytometry analysis (Fig. 1C), and quantification of fluorescent cells (Fig. 1D) ($p < 0.001$). qPCR confirmed enhanced viral genome integration in cells transduced with hypoxically packaged viruses (Fig. 1E) ($p < 0.001$). These results demonstrate that hypoxic packaging improves viral transduction efficiency, while hypoxic conditions during transduction reduce infection efficiency.

Hypoxia inhibits viral entry, and hyperoxia fails to reverse this effect

The HIV-1 Gag protein transfers into the target cells in 1–3 h [27], with approximately 80% of Gag transferred by 6 h [28]. To investigate the impact of oxygen on viral entry, we divided the transduction process into two phases: viral entry (0–8 h) and post-entry gene expression (8–48 h). After 8 h co-culture of viral particles and cells, the medium containing viral particles was replaced with fresh medium to remove the free virus, and the cells were subsequently transferred to different oxygen environments. Hypoxia during the entry phase significantly reduced transduction efficiency (Fig. 2A, top row; Fig. 2B, top row; Fig. 2C, first three columns). In contrast, hypoxia during the post-entry phase had minimal effects (Fig. 2A, middle row; Fig. 2B, middle row). Hyperoxia did not reverse the inhibitory effects of hypoxia on viral entry (Fig. 2A, right column; Fig. 2B, right column) and also impaired viral entry (Fig. 2C, last three columns). These findings suggest that hypoxia primarily inhibits viral entry, and hyperoxia exacerbates this effect.

The HIF-1 inhibitor PX-478 reverses hypoxia-induced inhibition of viral entry and enhances genome integration

Hypoxia-inducible factors (HIFs) are heterodimeric transcription factors composed of an oxygen-regulated α subunit (HIF-1α or HIF-2α) and a constitutively expressed β subunit. HIF-1 is a key regulator of cellular responses to hypoxia and is stabilized under low oxygen conditions, where it activates the transcription of genes involved in angiogenesis, metabolism, and survival [29]. PX-478 is a well-characterized HIF-1α inhibitor that reduces HIF-1α mRNA levels and inhibits its translation, without affecting HIF-1β expression, even under hypoxic conditions [30].

To investigate whether hypoxia inhibits viral entry through HIF-1α activation, we pretreated K562 cells with PX-478 prior to viral infection. The optimal concentration of PX-478 (20 μM) was determined using a CCK-8 assay, as higher concentrations (e.g., 40 μM) showed minimal effects on cell viability (Fig. 3A, $p<0.0001$). Importantly, PX-478 treatment effectively suppressed hypoxia-induced upregulation of both HIF1A and its downstream target GLUT1 (a key glucose transporter mediating metabolic adaptation to hypoxia) (Fig. 3B, $p<0.0001$). K562 cells pretreated with PX-478 were then infected with lentivirus under normoxic or hypoxic conditions. Remarkably, PX-478 pretreatment significantly increased the percentage of fluorescent cells, regardless of the oxygen environment (Fig. 3C, D, E, $p<0.0001$). This enhancement exhibited a dose-dependent effect, with a 10% increase in fluorescence-positive cells observed at 20 μM PX-478. Furthermore, qPCR analysis confirmed that PX-478 treatment also enhanced viral genome integration, supporting its role in facilitating viral entry and stable transduction (Fig. 3F, $p = 0.0003$).

Hypoxic virus packaging combined with PX-478 pretreatment synergistically enhances viral infection efficiency

To further optimize lentiviral transduction, we investigated the combined effects of hypoxic virus packaging

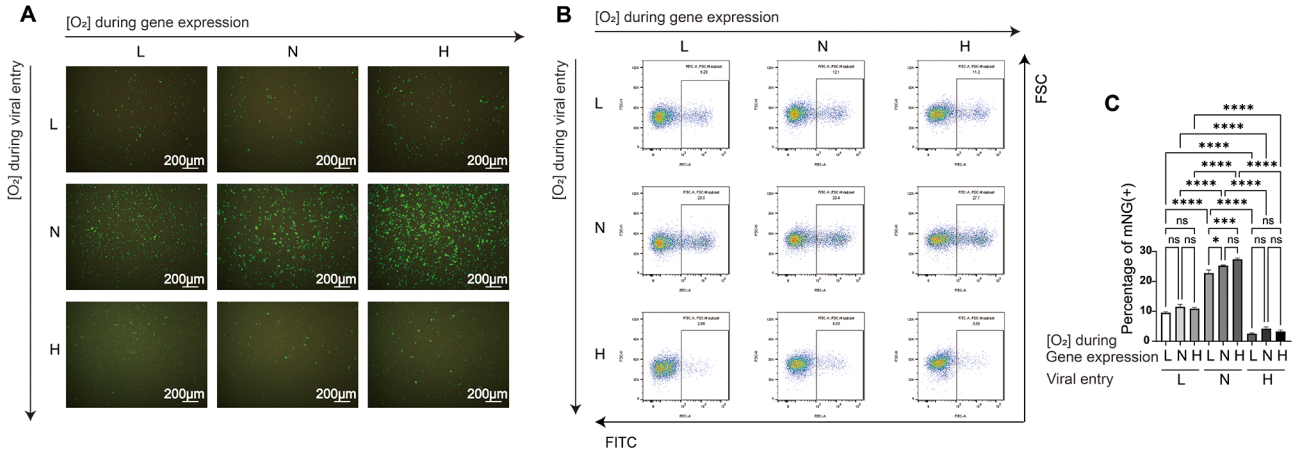

Fig. 2 Impact of hypoxia and hyperoxia on viral infection efficiency. (**A**, **B**, **C**) Fluorescence images, flow cytometry plots, and quantification of infected cells under normoxia (N), hypoxia (L), or hyperoxia (H). $n = 3$, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$, data are presented as the mean ± standard deviation

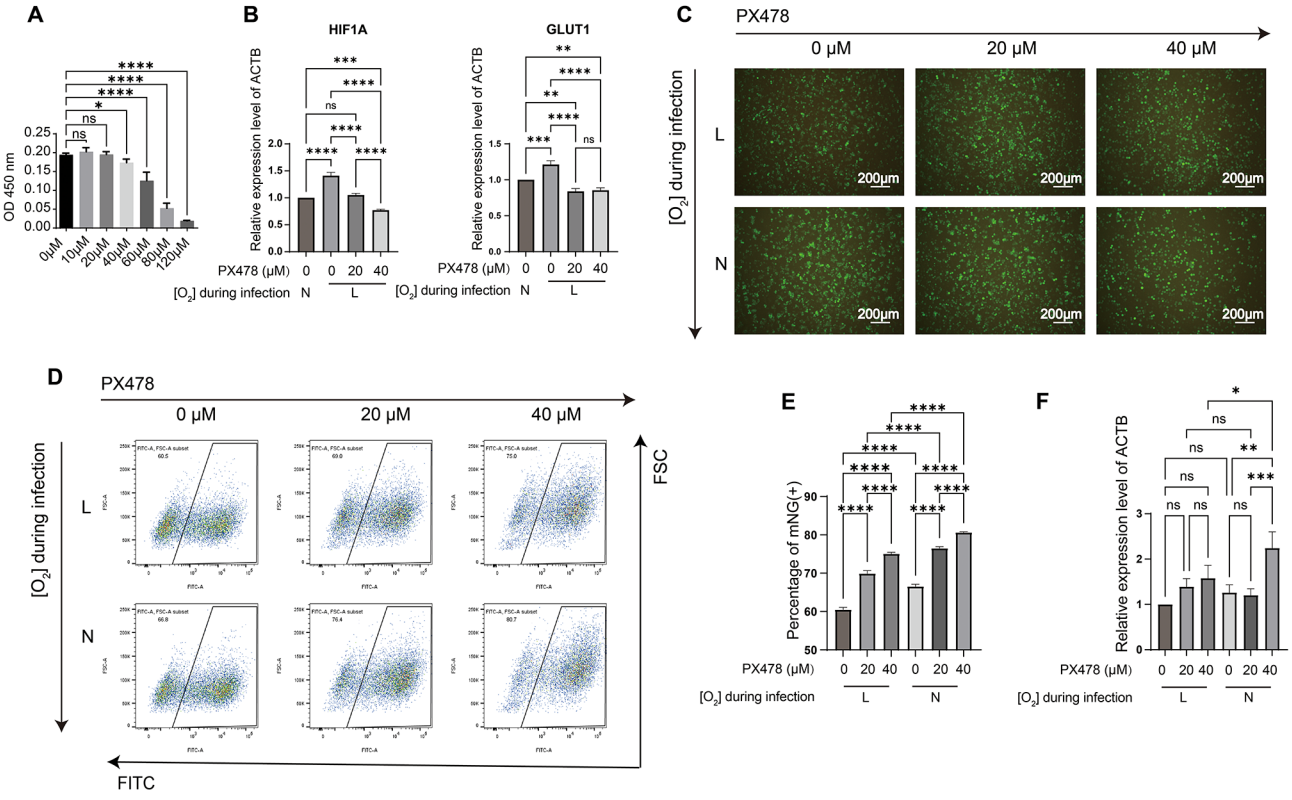

Fig. 3 PX-478 reverses hypoxia-induced inhibition and enhances viral genome integration. (**A**) CCK-8 assay of PX-478-treated K562 cells. $n = 6$. (**B**) mRNA expression levels of HIF1A and GLUT1 with or without PX478 treatment and under hypoxia. $n = 3$. (**C**, **D**, **E**) Fluorescence images, flow cytometry plots, and quantification of infected cells with or without PX-478 pretreatment. $n = 3$. (**F**) qPCR analysis of viral genome integration. $n = 3$. ns: not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$, data are presented as the mean ± standard deviation

and HIF-1 inhibition. Lentivirus was packaged in HEK-293T cells cultured under normoxic or hypoxic conditions. K562 cells were pretreated with PX-478 (20 μM) and then infected with viruses packaged under hypoxia. This combination was compared to infections using normoxically packaged viruses without PX-478 pretreatment.

The combination of hypoxic packaging and PX-478 pretreatment led to a 20% increase in the proportion of fluorescently labeled cells (Fig. 4A, B, C, $p<0.0001$), indicating improved transduction efficiency. Additionally, qPCR analysis revealed a 0.5-fold increase in the rate of viral genome integration (Fig. 4D, $p = 0.0081$), suggesting enhanced stable gene delivery.

Discussion

The lentiviral vector system is a powerful tool for gene delivery, offering stable integration into both dividing and non-dividing cells, broad tissue tropism, and the ability to deliver complex genetic elements without viral protein expression post-transduction [31]. Given its widespread use, significant efforts have been made to optimize lentiviral production and transduction efficiency, including improvements in vector design, culture conditions, and transfection protocols [32–34]. In this study, we explored the role of oxygen concentration in lentiviral packaging and transduction, demonstrating that hypoxic conditions during packaging significantly enhance viral titers and transduction efficiency by approximately 10%. However, hypoxic conditions during viral entry impair infection efficiency, likely due to hypoxia-induced cellular protective mechanisms mediated by HIF-1α.

The lentiviral transduction process involves multiple steps: viral attachment, membrane fusion, reverse transcription, nuclear import, and genome integration. The viral RNA is reverse transcribed and forms a pre integration complex with integrase. After the pre integration complex enters the nucleus, integrase catalyzes its integration into the host genome [35]. Viral entry efficiency is closely associated with the kinetics of viral internalization, with the majority of entry processes typically completed within 8 h [36] Based on this time frame, we roughly divided the viral infection process into two distinct phases: (1) viral entry (0–8 h post-infection, encompassing membrane attachment, fusion, and intracellular trafficking) and (2) post-entry protein expression (8–48 h post-infection, including reverse transcription, nuclear import, and transgene expression). By exposing cells to

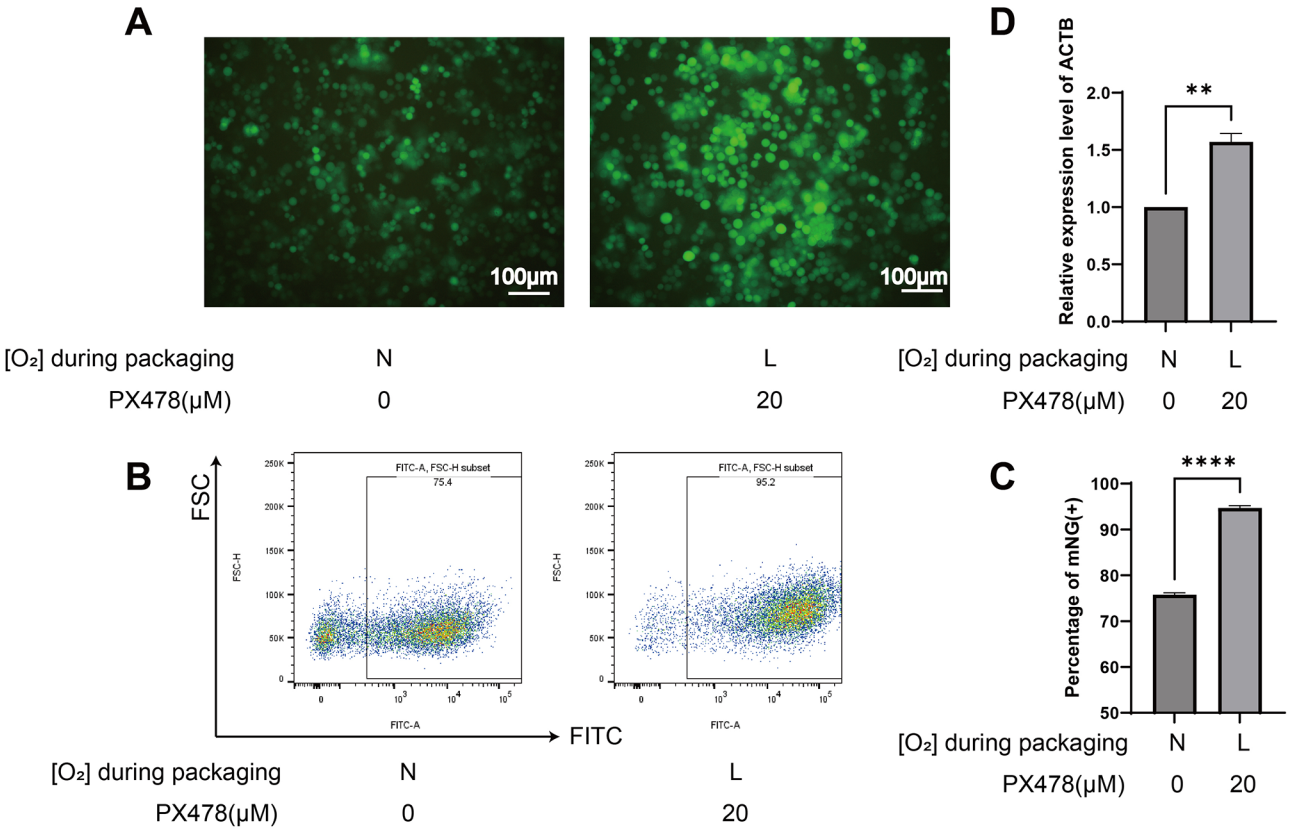

Fig. 4 Synergistic enhancement of transduction efficiency by hypoxic packaging and PX-478 pretreatment. (**A**, **B**, **C**) Fluorescence images, flow cytometry plots, and quantification of infected cells. $n = 3$. (**D**) qPCR analysis of viral genome integration. $n = 3$, $^{**}p < 0.01$; $^{****}p < 0.0001$, data are presented as the mean ± standard deviation

different oxygen environments during these phases, we observed that hypoxic conditions (mimicking physiological oxygen levels) significantly inhibited viral entry. Notably, restoring normoxic or hyperoxic conditions during the post-entry phase failed to reverse this inhibitory effect. This phenomenon may be attributed to hypoxia-triggered cellular protective mechanisms, such as altered endocytic trafficking or activation of stress-responsive pathways (e.g., HIF-1α signaling), which likely impair viral entry efficiency irreversibly [37].

HIF-1α is a key transcription factor activated under hypoxic conditions. Under normoxic conditions, HIF-1α is rapidly degraded by the ubiquitin-proteasome pathway, mediated by prolyl hydroxylase domain (PHD) enzymes and the von Hippel-Lindau (VHL) tumor suppressor protein. However, under hypoxic conditions, the hydroxylation of HIF-1α is inhibited, leading to its stabilization, nuclear translocation, and subsequent binding to hypoxia-responsive elements (HREs) in the promoters of target genes [38, 39]. This transcriptional activation regulates a wide range of biological processes, including metabolism, inflammation, thereby playing a critical role in cellular adaptation to low oxygen environments [40–42]. In this study, we employed the HIF-1 inhibitor PX-478 to pretreat cells prior to viral infection and observed a significant enhancement in viral infection efficiency, which exhibited a dose-dependent relationship with the concentration of PX-478. In addition to PX-478, we further validated the role of HIF-1α in lentiviral transduction using KC7F2 (Fig.S1), a HIF-1α inhibitor that inhibited its protein synthesis at the translation level [43]. KC7F2 pretreatment increased transduction efficiency by approximately 5% under normoxic conditions. While this enhancement was slightly lower than the 10% improvement observed with PX-478, the concordant effects of two inhibitors support the conclusion that HIF-1α inhibition itself, rather than off-target effects, drives the improved viral entry. Importantly, these findings collectively establish HIF-1 inhibitors as a novel class of transduction enhancers, with potential applicability across diverse viral delivery systems. By modulating cellular pathways associated with hypoxia response, HIF-1 inhibitors can potentially increase viral entry in target cells. Furthermore, the combined use of PX-478 pretreatment and viruses packaged under hypoxic conditions synergistically increased the percentage of infection-positive cells by approximately 20%. Interestingly, HIF-1 inhibitors can enhance viral infection even under normoxic conditions, likely due to localized hypoxia in conventional culture systems [44]. These findings suggest that

HIF inhibitors could serve as valuable tools for optimizing viral-based applications, even under normoxic conditions.

The enhancement in transduction efficiency holds particular significance for clinical applications requiring high-efficiency gene delivery. In ex vivo gene therapy settings—such as CAR-T cell engineering [45] and hematopoietic stem cell (HSC) transduction [46]—even modest improvements in transduction rates can substantially impact therapeutic outcomes. Our strategy of combining hypoxia-packaged viruses with HIF inhibitors could potentially enhance transduction while maintaining cell viability better than traditional chemical enhancers that often induce cytotoxicity at effective concentrations. The hypoxia/HIF-targeting biological strategy offers unique advantages: (1) potential synergy when combined with existing methods; and (2) oxygen tension control being readily implementable in closed-system bioreactors for clinical-scale production. Moreover, since HIF-1α inhibitors like PX-478 are already in clinical trials as anticancer agents [47], their repurposing as transduction enhancers may facilitate rapid translation to Good Manufacturing Practice (GMP)-compliant cell therapy production systems.

In this study, we continuously monitored the oxygen concentration in the culture environment to standardize hypoxic and normoxic conditions. However, it is important to note that the measured ambient oxygen levels may not fully represent the actual dissolved oxygen concentrations within the cell culture medium. Future studies employing direct measurement tools (e.g., fluorescence-based oxygen probes or microelectrodes) to quantify dissolved oxygen at the cellular level may provide more precise insights into how oxygen dynamics regulate lentiviral transduction efficiency.

In brief, this approach offers a promising strategy for improving infection efficiency in hard-to-transduce cell lines or optimizing viral-based applications in research and industry. The 20% synergistic improvement achieved through combined hypoxic packaging and PX-478 pretreatment demonstrates the potential of this approach for clinical applications requiring high-efficiency transduction. Further studies are needed to elucidate the underlying mechanisms and validate the broad applicability of HIF inhibitors as viral infection enhancers.

Conclusion

In this study, we demonstrated that hypoxic conditions during lentiviral packaging significantly enhance viral titers and transduction efficiency, while hypoxia during viral entry impairs infection efficiency due to HIF-1α-mediated cellular protective mechanisms. By employing the HIF-1 inhibitor PX-478, we successfully reversed the inhibitory effects of hypoxia on viral entry and achieved a dose-dependent enhancement of transduction efficiency. Furthermore, the combination of hypoxic virus packaging and PX-478 pretreatment synergistically improved transduction efficiency by 20%. Our findings demonstrate that controlled hypoxia and HIF-1 inhibition significantly enhance lentiviral transduction efficiency, providing a versatile platform-optimization strategy for viral vector-based biomedical applications, including therapeutic development, cellular engineering, and molecular delivery systems.

Abbreviations

ANOVA	Analysis of Variance
CAR-T	Chimeric antigen receptor T cells
CCK-8	Cell Counting Kit-8
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal bovine serum
HEK-293T	Human embryonic kidney 293T cells
HIF-1	Hypoxia-inducible factor 1
HIV-1	Human immunodeficiency virus-1
mNG	mNeonGreen
PEI	Polyethylenimine
PCR	Polymerase chain reaction
PHD	Prolyl hydroxylase domain
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
VSV-G	Vesicular stomatitis virus glycoprotein
WPRE	Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12896-025-00969-3.

Supplementary Material 1

Author contributions

Qianyu Huo and Wentian Wang devised the project, handled most of the experiments, performed the numerical calculations, and wrote the initial draft of the manuscript. Jiawen Dai, Xu Yuan, Dandan Yu, and Bingqi Xu participated in performing a portion of the experiments and contributed to data analysis. Ying Chi, Huiyuan Li, and Xiao Lei Pei critically revised the manuscript for important intellectual content, integrated feedback from all co-authors, and helped finalize the manuscript. All authors provided critical feedback and contributed to shaping the research, analysis, and manuscript. Lei Zhang and Guoqing Zhu supervised the project, provided funding support, and offered strategic direction for the research.

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Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

This study did not involve human participants, animals, or any clinical trials. All cell lines used in this study were obtained from commercial sources or institutional cell banks and were handled in accordance with standard laboratory protocols. Ethical approval was not required for this research.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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