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Assessment of hypoxia and TNF-α response by a vector with HRE and NF-kappaB response elements

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1. ABSTRACT

Hypoxia and inflammatory cytokine activation (H&I) are common processes in many acute and chronic diseases. Thus, a single vector that responds to both hypoxia and inflammatory cytokines, such as TNF-α, is useful for assesing the severity of such diseases. Adaptation to hypoxia is regulated primarily by hypoxia inducible transcription factor (HIFα) nuclear proteins that engage genes containing a hypoxia response element (HRE). Inflammation activates a multitude of cytokines, including TNF-α, that invariably modulate activation of the nuclear factor kappa B (NF-kB) transcription factor. We constructed a vector that encompassed both a hypoxia response element (HRE), and a NF-kappaB responsive element. We show that this vector was functionally responsive to both hypoxia and TNF-α, *in vitro* and *in vivo*. Thus, this vector might be suitable for the detection and assessment of hypoxia or TNF-α.

2. INTRODUCTION

Hypoxia and inflammatory cytokine activation (H&I) are common processes in many acute and chronic diseases. Ischemic-reperfusion injury, due to blood vessel occlusion is a well-studied form of H&I. Following occlusion of the vessel, anterograde blood flow ceases and there is a shift from aerobic to anaerobic tissue metabolism. Upon reperfusion, a sudden influx of oxygen perturbs the adaptive metabolism, generating substantial toxic free radical stress, which may, in turn, lead to cell death. This is followed by an obligate inflammatory phase to clear necrotic tissues before adaptive remodelling and neovascularisation re-establishes normal tissue oxygen tension (1). H&I is also prevalent and clinically significant in chronic inflammatory diseases such as arthritis, atherosclerosis, heart failure, cancer, rhabdomyolysis, inflammatory bowel disease and autoimmune disorders. In such instances, inflammation alters the cellular micromilieu, and with the perpetual presence of

inflammatory cells, imposes greater metabolic demands to cause vasculopathy, further reducing tissue perfusion and increasing hypoxia (2).

Adaptation to hypoxia is regulated primarily by hypoxia inducible transcription factor (HIF) nuclear proteins that activate genes encompassing a hypoxia response element (HRE). Canonical oxygen sensing by HIFs occurs through perpetual synthesis and degradation by oxygen-dependent hydroxylation, rendering HIFs susceptible to ubiquitylation and proteosomal degradation. As oxygen tension drops, the absence of hydroxylation stabilizes HIFs which translocate into the nucleus. In hypoxia, HIFα binds HRE-responsive gene promoter-enhancer elements and regulate homeostasis by induction of erythropoietin, vascular endothelial growth factor, glucose transporter or other molecules, essential to adaptation to hypoxia (3).

Inflammation is activated by leukocyte accumulation and release of a multitude of cytokines and other mediators of inflammatory responses. Inflammatory cytokines, such as TNF-α invariably modulate receptor-mediated inhibitor-kappa-kinase (IKK) phosphorylation of inhibitor-of-kappa-B (IkB), allowing dissociation and suppression by IkB of nuclear factor kappa B (NF-kB). Dissociation of IkB enables translocation of NF-kappaB into the nucleus, where it transcriptionally regulates the cellular response to inflammation. NF-kappaB has also been implicated as an oxygen-sensitive transcription factor (4). A growing body of evidence suggests that both the HIFα and NF-kappaB transcription factors establish mechanistic cross-talk (5), interdependent of specific gene expression (6-9). Further, HIFα can be stabilized by inflammatory actuators, such as lipopolysaccharides, tumor necrosis factors or interleukins (2). Given the concomitant prevalence of H&I in various acute and chronic diseases, it is logical to jointly utilize both HRE and NF-kB-mediated mechanisms within a single vector to detect and assess the severity of hypoxic and TNF-α mediated responses.

A strategy for reducing viral vector associated risks *in vivo* is to perform cell mediated vector transduction *ex vivo* prior to transplantantation *in vivo*. EPCs have been extensively studied as delivery vehicles for gene therapy due to their role in promoting therapeutic neovascularization in addition to their amenability to viral transduction. EPCs transduced with HRE and NF-kB-regulated viral vectors provide a means of identifying where these cells are incorporated and responsive to either hypoxia or TNF-α. These vectors can be used broadly to study hypoxia or TNF-α stimuli, physiologically or in clinically relevant vector design to regulate gene expression. Our results show that combined promoter regulation with HRE and NF-kB-responsive elements react proportionately to either hypoxia or TNF-α cytokine activation and provide a novel mechanism for the detection of these stimuli *in vitro* and *in vivo*.

3. MATERIALS AND METHODS

3.1. Cell culture

Human cord blood mononuclear cells were purchased from Lonza (Cambrex, Massachusetts) and seeded onto 100 mm^2 fibronectin-coated culture dishes (BD Biosciences). Cells were supplemented with endothelial growth medium (EGM-2) (Lonza), 10% FBS and 100 units/ml penicillin, 100 µg/ml streptomycin and cultured at 37°C, 5% CO_2. Endothelial progenitor colonies were isolated and characterized as previously described (10,11). Following 72 hours of mononuclear cell culture in fibronectin coated dishes with endothelial growth supplemented media (EGM-2 media, Lonza), cultures were then washed extensively to remove any remaining non-adherent mononuclear cells from the adherent endothelial progenitor colonies. The progenitor colonies were subsequently cultured in EGM-2 therafter. After ~15 days, endothelial colony-forming cells produce a visible outgrowth of endothelial progenitor cells (EPC) with classical endothelial cobblestone morphology. The outgrowths were detached using HyQtase solution (Thermo-Fisher Scientific) and expanded in EGM-2 media for no more than 5 passages on fibronectin-coated dishes prior to experimentation, so as to maintain the progenitor phenotype.

HEK293FT (Invitrogen) were maintained in DMEM supplemented with 10% FBS for viral packaging. When necessary, before validation in a primary human EPC, we performed vector optimization in the highly stable and transduction permissive HT1080 cell line in accordance with the provider's directions, HT1080 cells (MEM 10% FBS media; American Type Culture Collection). This ensured cost effectiveness, availability and reduced risk of influencing the cell responses (such as by genetic drift due to multiple cell passages associated with a primary cell), while performing vector editing and served as a check to ensure vector design and responsiveness are not cell dependent.

3.2. Construction of lentiviral vectors

A 3' polylinker (5'CACCGTTTAAACGAA TTCGCTAGCCTGCAGGTCGACTCCGGAGTTA-ACCGTACGGATATC) was cloned into a pLenti6/V5-D-TOPO vector to generate pLenti-linker according to the manufacturer's protocol (Invitrogen). A 620-bp *Eco*RI-*Nco*I fragment containing the internal ribosome entry site (IRES) from plasmid pIRES2-AcGFP1 (Clontech) was ligated into a 4.3.-kb *Eco*RI-*Xba*I fragment of pIRES (Clontech) along with a 1.7.-kb *Nco*I-*Xba*I fragment containing the firefly luciferase (Luc) gene from plasmid pGL3 (Promega); this yielded plasmid pIRES-Luc. The gene coding for the *Aequorea coerulescens* green fluorescent protein (AcGFP1) was amplified from pIRES2-AcGFP1 (Clontech) by PCR using *Eco*RI-containing (underlined) and *Sal*I-containing (underlined) primers (Table 1). The PCR product was digested with *Eco*RI-*Sal*I

Table 1. Primer sequences used in vector design

Target	Oligonucleotide Sequence (5'-3')
AcGFP1 (*Eco*RI-containing)	ATACCGGAATTCCAACCATGGTGAGCAAGGGC
AcGFP1 (*Sal*I-containing)	CTGTCCCCGCGGTCACTTGTACAGCTCATC
CMV promoter region (upstream; *Cla*I-containing)	ATAGCCATCGATAAGCTTGGGGTTTAGTGAACCGTCAGATC
CMV promoter region (behind; *Cla*I-containing)	ATAGCCATCGATAAGCTTGGGACGCAAATGGGCGGTAGG
CMV minimal promoter (*Spe*I-containing)	CCACACTGGACTAGTTCTAGAGTCGGTG

and ligated into *Eco*RI-*Sal*I-digested pIRES-Luc to generate pGFP-IRES-Luc (GIL). The 2.9.-kb *Eco*RI-*Sal*I-fragment containing the GFP-IRES-Luc cassette was ligated with *Eco*RI-*Xho*I-digested vector pLenti-linker to generate pLenti-GFP-IRES-Luc (pLenti-GIL).

To excise the CMV sequence as pLenti-ΔCMV-GIL or minimally present the otherwise constitutively active CMV construct as pLenti-mCMV-GIL, the area upstream of CMV and the area behind CMV, or the area of the minimal CMV, were amplified by PCR using *Cla*I-containing (underlined) primers or a *Spe*I-containing (underlined) primer (Table 1). The two PCR products were digested with *Cla*I-*Spe*I and respectively ligated into *Cla*I-*Spe*I-digested pLenti-GFP-IRES-Luc to generate pLenti-ΔCMV-GIL and pLenti-mini-CMV-GIL. Eight copies of the hypoxia response element (HRE) consensus sequence (GCCCTACGTGCTGTCTCACACAG-CCTGTCTGACC TCTCGACCCTACCGGGCCTGAGGCCACAAGCTC) of the human erythropoietin (EPO) gene enhancer were respectively inserted into pLenti-ΔCMV-GIL, upstream of GFP and Luc gene to generate pLenti-HRE-GIL. Four copies of the NF-kappaB binding sequences (NF-kB) of the human vascular cell adhesion molecule (VCAM) promoter (CTGGGTTTCCCCTTGAAGGGATTTCCCTC) were respectively inserted into: 1) pLenti-ΔCMV-GIL 5' of the GFP gene, 2) pLenti-HRE-GIL 3' region of the HRE sequences and 5' region of the GFP gene, or 3) pLenti-HRE-mCMV-GIL 3' region of the HRE sequences and 5' region of the mimimal CMV promoter to generate: 1) pLenti-NF-kB-GIL, 2) pLenti-HRE-NF-kB-GIL and 3) pLenti-HRE-NF-kB-mCMV-GIL.

3.3. Lentivirus production

Lentiviral particles were generated using ViraPower™ Lentiviral Expression System (Invitrogen) according to the manufacturer's directions. Lentiviral expression constructs were cotransfected with ViraPower™ packaging mix: pLP1, pLP2, and pLP/VSVG using Lipofectamine2000 (Invitrogen) into the HEK293FT producer cell line. Supernatants were collected 48 hours after transfection, centrifuged and filtered through a 0.4.5-µm filter. Viral titer was determined by blasticidin selection (10 µg/ml) on HT1080 cells 48 hours after transduction with 10-fold serial dilutions of the lentiviral supernatant. After 10 days of selection, the cells were stained with crystal violet and colonies were counted.

3.4. Lentiviral transduction

The titrated lentivirus was diluted into fresh medium to obtain a suitable Multiplicity of Infection (MOI). EPCs cultured in EGM-2 media (or HT1080 cells during vector optimization studies) were cultured in MEM and supplemented with 10% FBS were transduced with lentiviruses at MOI of 1 or 5 for 16 hours at 37°C, 5% CO_2 in a humidified atmosphere. Following transduction, the viral media was removed and replaced with fresh, complete culture medium. The transduced cells were further cultured for 4 days for *in vitro* or *in vivo* analysis.

3.5. *In vitro* hypoxia and TNF-α treatment

Lentiviral transduced EPCs were passaged into multi-well plates and distributed into four experimental groups: 1) normoxic conditions (5% CO_2), 2) inflammatory cytokine (hTNF-α, (10 µg/ml), 3) hypoxia (5% CO_2 and 1% O_2), and 4) H&I (hTNF-α, (10 µg/ml), 5% CO_2 and 1% O_2) for 16 h prior to analysis.

3.6. *In vivo* murine model of hypoxia and inflammatory cytokine activation

Immunodeficient male $Rag2^{-/-}/Il2rg^{-/-}$ mice (T, B, and NK cell deplete; aged 6-10 weeks) received human EPCs transduced with lentiviral vectors. The experimental protocol was approved by the Queen's University Animal Care Committee in accordance with national and international guidelines for animal care and the use of laboratory animals. Mice were only handled within laminar-flow hoods. Lentiviral-transduced EPCs were collected and suspended in 300 µl of sterile saline (total cell number ~1 × 10^6) and slowly administered via tail vein injection. Mice were allowed to incorporate human EPCs for two-weeks post-injection and no adverse events were observed before separating animals into four groups: 1) normoxic, 2) inflammatory cytokine (10 µg/kg, hTNF-α), 3) hypoxic (10% O_2), and 4) H&I (10 µg/kg, hTNF-α, 10% O_2) for 16 h. Animals were immediately sacrificed in location and tissues from liver, spleen, heart, kidney, lung and brain were collected and analyzed for GFP and luciferase expression.

3.7. Analysis of GFP and luciferase expression

Cells and tissues were analyzed for GFP by FACS (FC500 Beckman Coulter) or microscopy (Leica DM-IRB), whereas luciferase was quantified

following cell/tissue lytic analysis using the Bright-Glo™ Luciferase Assay System (Promega) in a Lumat LB 9507 Luminometer (Berthold). The protein concentrations of the lysates were measured for normalization via BCA Protein Assay Kit (Thermo Fisher Scientific).

3.8. Statistical analysis

Data are expressed as mean ± SD. Statistical analyses were performed using Prism 4 software (GraphPad Software Inc.). Statistical significance between data sets was assessed using one-way ANOVA and Tukey's multiple comparison test ($P<0.0.5$ was considered significant).

4. RESULTS AND DISCUSSION

4.1. Combined promoter vector generation

An emerging requirement in vector development is to overcome simple monogenic delivery (the delivery of a single gene by a single vector) by using bicistronic vectors (delivery of two genes by a single vector) in order to better provide additional gene products and greater efficacy (i.e. gene adjuvants-genes that augment or synergize the efficacy of each other). This efficacy was recently demonstrated using dual protein kinase-B (Akt) and heme-oxygenase-1 (HO-1) expression in human EPCs for cell-mediated gene therapy (11), where combined Akt/HO-1 gene delivery into EPCs was more effective than either gene alone in promoting EPC-mediated recovery following infarction. However, this required adding two viral vectors, doubling the viral particle exposure to EPCs prior to transplantation. Further, the exogenous genes expressed in that model were constitutive and not regulated by stress. Additionally, constitutive viral transgene expression increases the risk of transactivation of neighbouring genome sequences (12). To jointly detect inflammatory and hypoxic responses, we created a single functional vector for high fidelity visualization and quantification of GFP and Luc activity using the promoters of NF-kappaB and HIF1α. To construct this promoter, the pLenti6/v5-D-TOPO lentiviral vector was first modified by inserting nine restriction enzyme sites into the multiple cloning site (MCS) of the vector and generated the pLenti-linker vector (Figure 1A). A GFP-IRES-Luc cassette was then subcloned into pLenti-linker vector, under the ubiquitous and constitutive regulation of the cytomegalovirus (CMV) promoter to generate bicistronic combinatorial lentiviral vector, pLenti-GIL (GFP-IRES-Luc; Figure 1A). To reduce the risk of side effects to viral exposure, cells are first transduced *ex vivo*.When the human EPCs were stably transduced with pLenti-GIL, GFP was expressed in over 90% of cells at 5 multiplicities of infection (MOI) (Figure 1 B). When compared to control cells that were either non-transduced or transduced with a Lac-operon at equal titer, luciferase activity was only observed in the pLenti-GIL EPCs (Figure 1C). When the bicistronic orientation was reversed by placing Luc upstream and the GFP downstream of the internal ribosomal entry site (IRES), the vector showed a similar transduction efficiency, however, intensity of GFP was lower (data not shown). This is consistent with prior reports that showed that the expression diminishes when Luc or GFP are placed downstream of the IRES promoter (13). When the CMV was excised from the expression vector, there was no measurable Luc activity (Figure 1D). When a minimal CMV enhancer was inserted into the vector, there was minimal promoter leakage or IRES drive, even when human fibrosarcoma cells were transduced and subjected to permissible and optimal condition (Figure 1E-F).

4.2. Construction of expression vectors responsive to hypoxia and inflammatory cytokine stimulation

To develop HRE and NF-kB-responsive vector, we first developed pLenti-ΔCMV-GIL (deleted CMV) and pLenti-miniCMV-GIL (minimal CMV) by removing or partially deleting the internal CMV promoter. The cells transduced with pLenti-ΔCMV-GIL and pLenti-miniCMV-GIL did not express GFP and Luc (Figure 1D-F). We subsequently generated three vectors, pLenti-NFkB-GIL, pLenti-HRE-GIL and pLenti-HRE-NFkB-GIL by respectively inserting NF-kB-responsive elements (four copies), HRE (eight copies) or both into pLenti-ΔCMV-GIL (Figure 2A). The number of repeat sequences and orientation of each sequence was empirically changed for optimizing the responses to hypoxia or TNF-α.

Previous reports demonstrated cross talk between HREs and NF-kappaB promoter activity (14-15). However, we failed to see such a cross talk by the vectors that we developed (Figure 2B) since hypoxia did not increase Luc activity in the pLenti-NF-kB and neither did TNF-α increase Luc activity in the pLenti-HRE alone. The responsiveness of the initial vector to hypoxia was lower than that expected for use in assessment of hypoxia and TNF-α mediated responses. To overcome this problem and to enhance transactivation without loss of promoter specificity, we reinserted a minimal CMV promoter into the vector, a strategy previously shown to amplify transcriptional sensitive activity (16). This significantly improved the promoter response to hypoxia or TNF-α (Figure 2C). Importantly, as previously reported, the minimal CMV eliminated the significant differences between responses to hypoxia and TNF-α, synergisticially amplifying the combined promoter effect of HRE and NF-kappaB response elements (Figure 2C, purple bar & D).

4.3. *In vitro* response of vectors to hypoxia and inflammatory cytokine stimulation

We tested the responsiveness of the pLenti-NFkB-GIL, pLenti-HRE-GIL and pLenti-HRE-NFkB-GIL in response to normoxia, hypoxia and TNF- that drives the expression of NF-kB promoter.

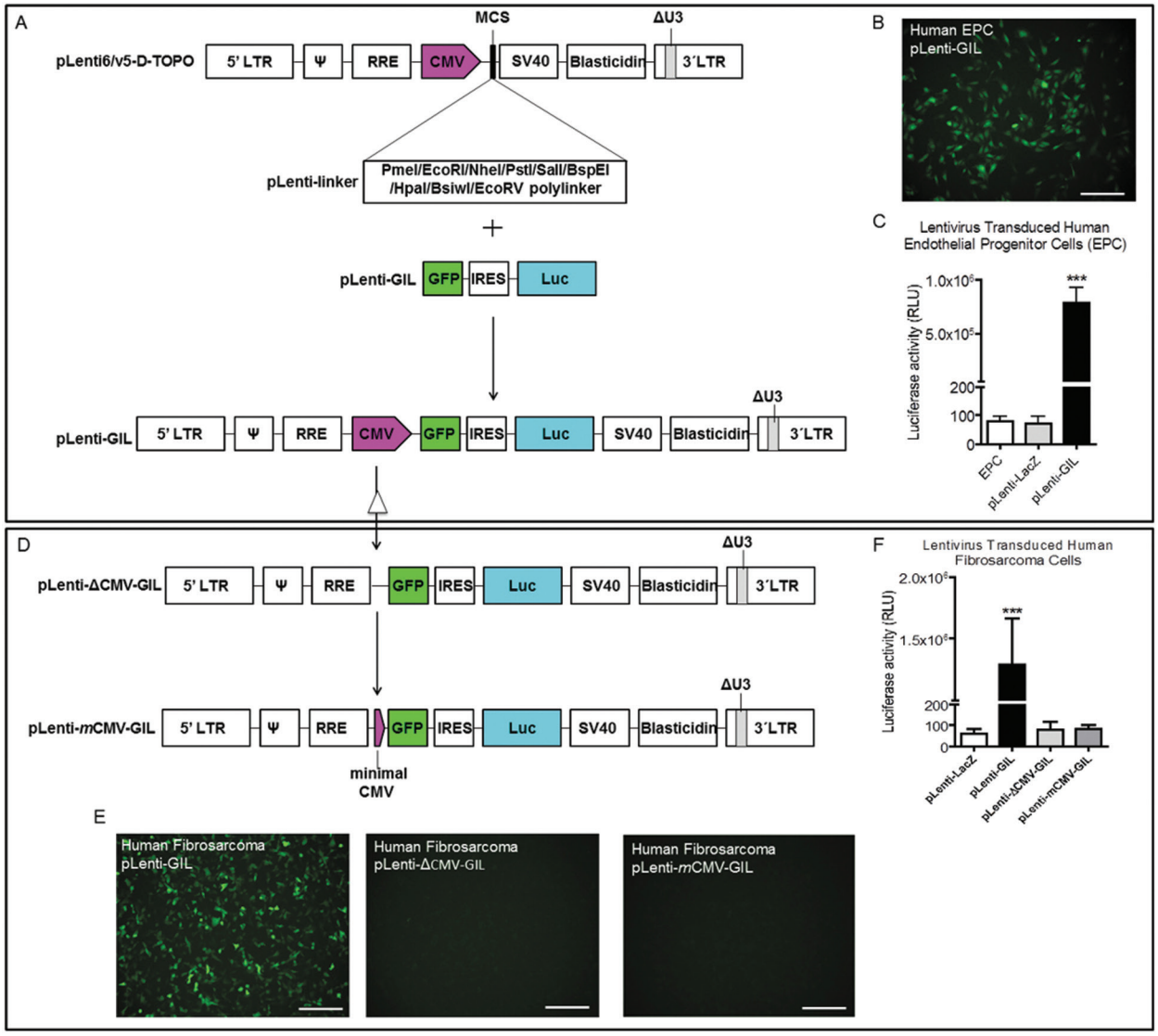

Figure 1. Construction and validation of lentiviral backbones for gene expression. A) Nine restriction enzyme sites were introduced into multiple cloning sites (MCS) of pLenti6/v5-D-TOPO self-inactivating vector. Gene cassettes of GFP, IRES and Luc were subsequently cloned into the vector to generate a bicistronic lentiviral vector of pLenti-GIL. B) EPCs were transduced with pLenti-GIL (MOI: 1). Both pLenti-LacZ and non-virus transduced cells were used as control. Expression of GFP was observed at day 4 after transduction and C) luciferase activity was quantified in parallel. Data shown are representative of two independent experiments (N=3); ***P<0.0.01 vs. non-viral transduced EPC control; RLU=relative light unit; MOI=Multiplicity of Infection. Scale Bar = 100 μM. D) The CMV promoter was deleted from pLenti-GIL to generate pLenti-ΔCMV-GIL. In pLenti-miniCMV-GIL, the majority of the CMV promoter was deleted and the minimal CMV promoter was retained. E) CMV-modified lentiviral vector function was confirmed in HT1080 cells and GFP expression was determined in parallel with F) luciferase activity. Data shown are representative of two independent experiments (N=3); ***P<0.0.01 versus transduced with pLenti-LacZ control; RLU=relative light unit; Scale Bar = 100 μm.

4.3.1. *In vitro* response to hypoxia

Hypoxia induced up to three-fold increase in luciferase activity in pLenti-HRE-GIL and pLenti-HRE-NFkB-GIL transduced EPCs as compared to transduced EPCs that were exposed to normoxia (Figure 2B, blue).

4.3.2. *In vitro* response to TNF-α

EPCs transduced with the same vectors showed twelve-fold increase in luciferase activity when exposed to normoxia and treated with TNF-α, as compared to transduced EPCs that were exposed to normoxia alone (Figure 2B, red).

4.3.3. *In vitro* response to hypoxia and TNF-α

EPCs that were transduced with pLenti-HRE-NFkB-GIL showed up to fifteen-fold higher luciferase activity when exposed to hypoxia and treated with TNF-α as compared with control EPCs exposed to normoxia alone (Figure 2B, purple).

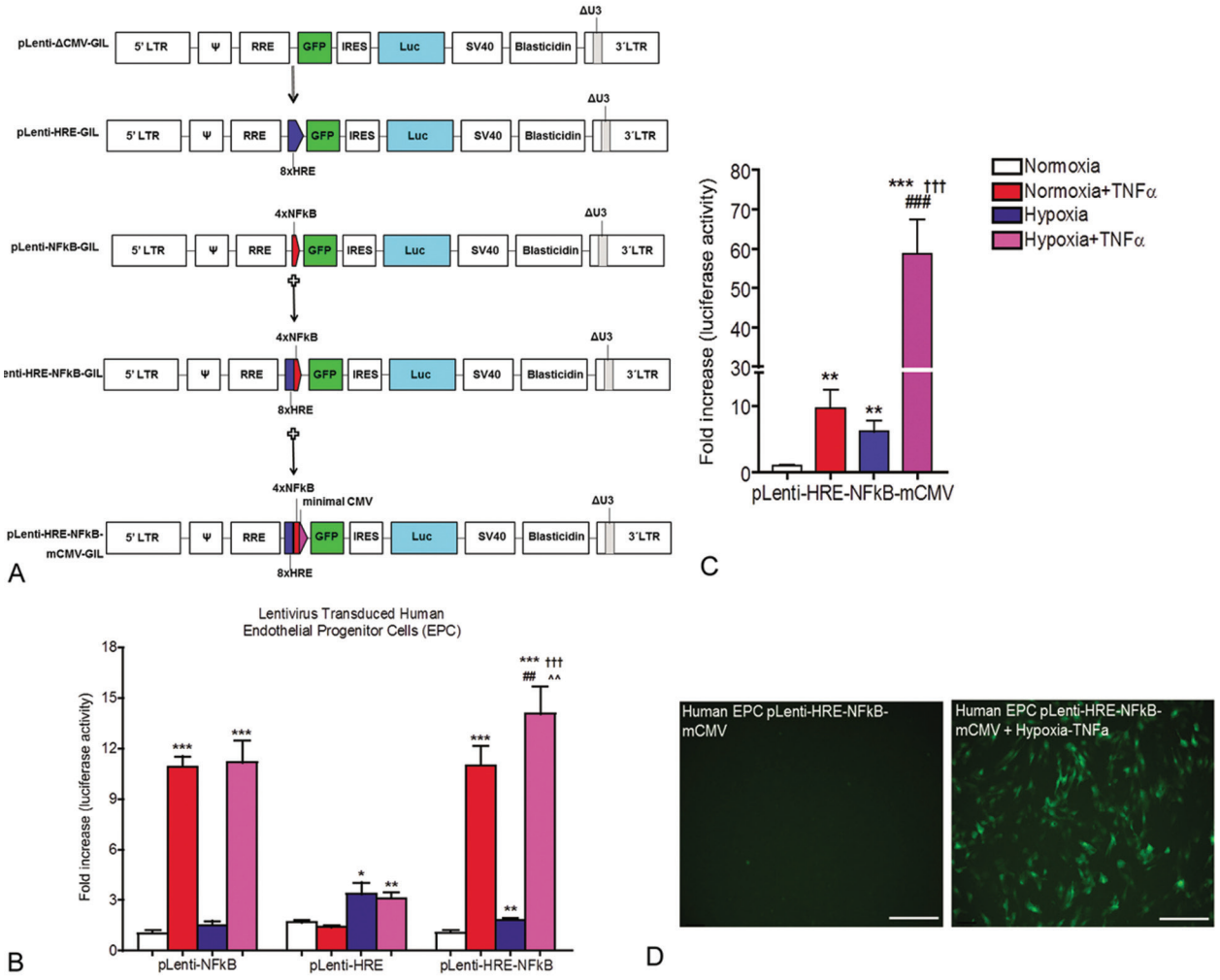

Figure 2. Schematic diagram of lentiviral vectors. A) Lentiviral vectors were respectively incorporated with eight copies of hypoxia responsive element (HRE), four copies of nuclear factor-kappa B (NF-κB)-responsive element, HRE with NF-κB, and HRE with NF-κB and minimal CMV. B) Induction of luciferase activity in EPCs transduced with the regulated lentiviruses. EPCs were transduced with pLenti-NFkB-GIL, pLenti-HRE-GIL, and pLenti-HRE-NFkB-GIL in a 12-well plate at 37°C, 5% CO2 in a humidified atmosphere. After four days, the EPCs were exposed to normoxia, normoxia with TNF-α (10μg/ml), hypoxia (1%O_2), and hypoxia with TNF-α for 16 hours. Cells were collected to detect luciferase expression over 16 hours. Data shown are representative of two independent experiments; *, p < 0.0.5; **, p < 0.0.1; ***, p < 0.0.01. vs normoxia-only control.

These findings show that pLenti-HRE-NFkB-GIL is responsive to both hypoxia, and TNF-α alone or both. For this reason, the vector, can be used for the assessment of all conditions that lead to hypoxia and inflammatory cytokine activiton. Hypoxia and inflammatory cytokine activation coincide in a multitude of disease processes. Recent studies have established that regulation of HRE and NF-kappaB elements may be cooperative. pLenti-HRE-NFkB-GIL vector can be employed *in vitro* for the assessment of hypoxia and TNF-α (Figure 2).

4.3.4. *In vivo* response to hypoxia and TNF-α

To examine the expression system *in vivo*, human EPCs were transduced with the pLenti-8HRE-4NFkB-mini CMV-GIL-regulated lentivirus (Figure 3A). To avoid rejection, these cells were injected via tail vein into $Rag2^{-/-}$/$Il2rg^{-/-}$ immune deficient mice. Two weeks later, luciferase activity was measured in various tissues of animals that were exposed to hypoxia for 16 hours and treated with and without TNF-α (16 hours). In heavily vascularized tissues such as lung, liver and spleen, luciferase activity was ~2-5-fold higher in response to hypoxia or TNF-α alone (17). However, when hypoxia was combined with treatment with TNF-α there was > thirty-fold increase in luciferase activity in these tissues (Figure 3B). This could be the result of differential EPC distribution after intravenous infusion as previosly reported, which can vary according to cell size or mechanisms of EPC adhesion (18-20). Alternatively, these organs may be more or less susceptible to H&I due to their perfusion (21). A limitation to our study is that

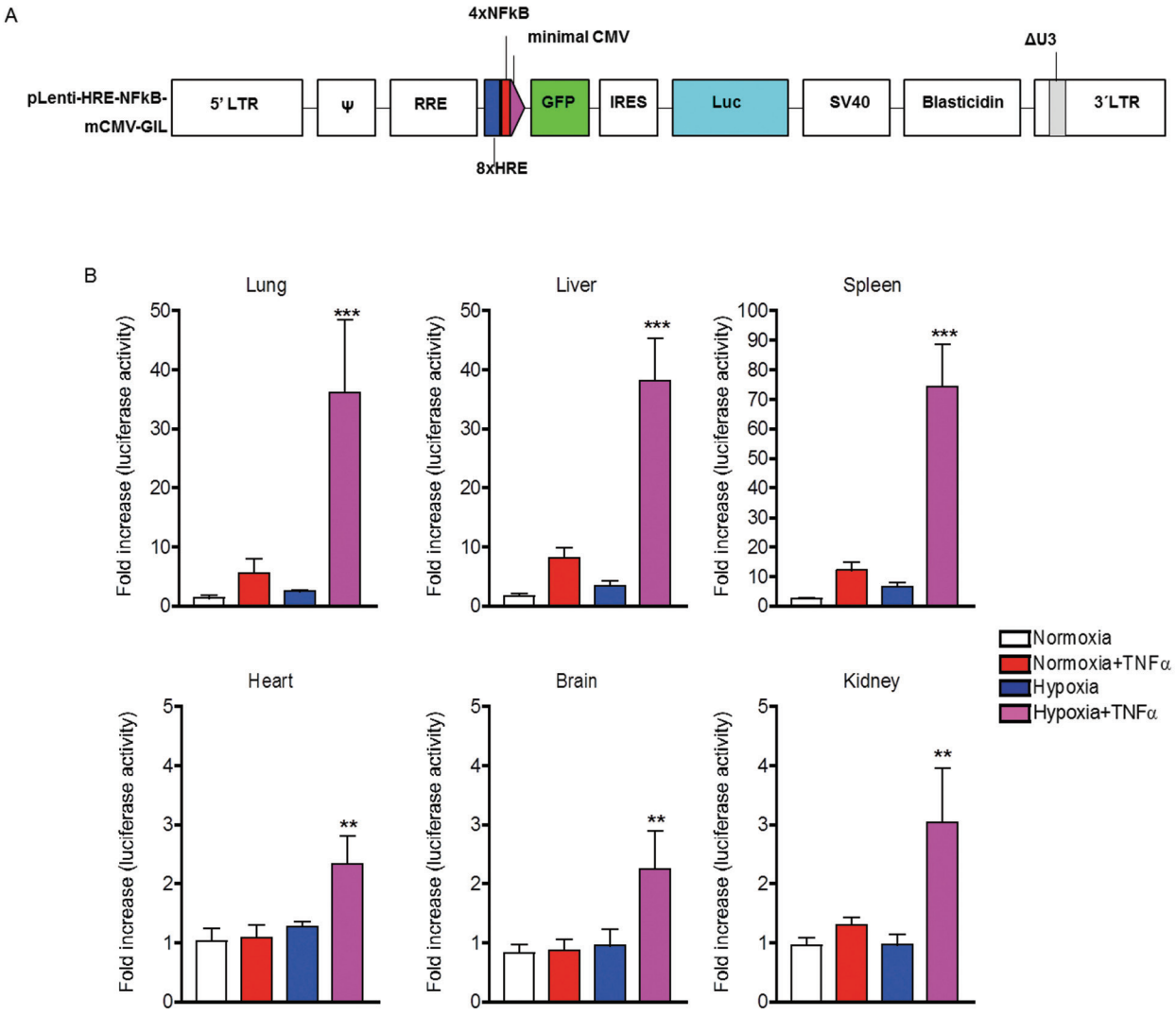

Figure 3. Luc activity in mice transduced with pLenti-8HRE-4NFkB-mini CMV-GIL-regulated lentivirus. A) Transduction of human EPCs with the vector. B) Mice were injected with lentivirus-transduced EPCs by tail vein injection. Luc activity in kidney, liver, heart, brain, spleen and lung was analyzed in mice under normoxia, normoxia with TNF-α (10 μg/kg), hypoxia (10% O_2), and hypoxia with TNF-α. Data shown are representative of two independent experiments with similar results; *, $p < 0.0.5$; **, $p < 0.0.1$; ***, $p < 0.0.01$ versus normoxia-only control.

these organs were homogenized for luciferase analysis; future studies should examine EPC distribution and the colocalization of nuclear translocation in NF-kB and HIF-transcription factors by immunohistochemistry. In constrast in the heart, brain and kidney, there was only a 2-3 fold increase in luciferase activity when these tissues were subjected to both hypoxia and TNF-alpha (Figure 3B). Based on such evidence, the vector may be used to assess hypoxic and TNF-α cytokine-associated effects *in vitro* and *in vivo*.

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Abbreviations: AAV9: adeno-associated-virus 9, CMV: cytomegalovirus, DMEM: Dulbecco's Modified Eagle Medium, EBM-2: endothelial basal medium, EGM-2 endothelial growth medium, EPC: endothelial progenitor cells, FACS: fluorescence activated cell sorting, FBS: fetal bovine serum, GFP: green fluorescent protein, H&I: hypoxia and inflammatory cytokine activation, HIF: hypoxia inducible factor, HRE: hypoxia response element, IKK: inhibitor-kappa kinase, IkB: inhibitor-of-kappa B, IRES: intermediate ribosomal entry site, Luc: firefly luciferase, MCS: multiple cloning site, MOI: multiplicity of infection, NF-kB: nuclear factor-kappa B, PCR: polymerase chain reaction, TNF-α: tumor necrosis factor alpha, VCAM: vascular cell adhesion molecule

Key Words: Hypoxia, Inflammation, HRE, NF-kappa B, Vector design

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