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Non-invasive Bioluminescence Imaging of Myoblast-Mediated Hypoxia-Inducible Factor-1 Alpha Gene Transfer

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Abstract

Purpose—We tested a novel imaging strategy, in which both the survival of transplanted myoblasts and their therapeutic transgene expression, a recombinant hypoxia-inducible factor-1 α (HIF-1 α -VP2), can be monitored using firefly luciferase (fluc) and *Renilla* luciferase (hrl) bioluminescence reporter genes, respectively.

Procedures—The plasmid pUbi-hrl-pUbi-HIF-1 α -VP2, which expresses both hrl and HIF-1 α -VP2 using two ubiquitin promoters, was characterized *in vitro*. C2c12 myoblasts stably expressing fluc and transiently transfected with pUbi-hrl-pUbi-HIF-1 α -VP2 were injected into the mouse hindlimb. Both hrl and fluc expression were monitored using bioluminescence imaging (BLI).

Results—Strong correlations existed between the expression of hRL and each of HIF-1 α -VP2, VEGF, and PlGF ($r^2 > 0.83$, $r^2 > 0.82$, and $r^2 > 0.97$, respectively). *In vivo*, both transplanted cells and HIF-1 α -VP2 transgene expression were successfully imaged using BLI.

Conclusions—An objective evaluation of myoblast-mediated gene transfer in living mice can be performed by monitoring both the survival and the transgene expression of transplanted myoblasts using the techniques developed herein.

Keywords

Cell-mediated gene transfer; Bioluminescence imaging; Hypoxia-inducible factor-1 α ; Molecular imaging

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Conflict of interest. None.

Introduction

Cell- and gene-based therapies have been proposed as promising treatments for ischemic heart disease based on their proven efficacy in pre-clinical animal studies [1–6]. Recent clinical trials using either form of therapy, however, have not shown comparable success for unclear reasons [7]. The lackluster results associated with gene therapy have been attributed to either suboptimal delivery/expression of therapeutic vectors or poor patient responsiveness. The mis-delivery of cells and their poor survival *in vivo* have also been thought to be responsible for the negative if not confounding trial results. In an attempt to improve the efficacy of either type of treatment, various groups have explored combining cell and gene therapies into a single treatment modality in which cells are genetically modified to overexpress therapeutic genes, *e.g.*, vascular endothelial growth factor (VEGF), protein kinase B (AKT/PKB), with the intent to enhance both angiogenesis and the long-term cell viability [8–11]. Such a combined therapy has been shown to be more effective than either gene or stem cell therapy alone and may represent a new paradigm for therapeutic angiogenesis [11]. To help facilitate its evaluation and translation into the clinics, we and others have developed novel molecular imaging techniques for non-invasive monitoring of cell survival and transgene expression in living subjects [12–14].

Our laboratory has previously demonstrated the feasibility of non-invasively imaging reporter gene expression in small animals using bioluminescence imaging (BLI) and positron emission tomography (PET) [15]. Both techniques rely on using a reporter gene, which when expressed can lead to protein products (reporter proteins) capable of interacting with systemically delivered substrates (reporter probe). With BLI, the bioluminescence reporters [*e.g.*, firefly luciferase (fluc) and *Renilla luciferase* (hrl)] can oxidize their substrates in a biochemical reaction to produce visible light as signal. In contrast, the PET reporters [*e.g.*, mutant herpes simplex virus type 1 thymidine kinase (HSV1-sr39tk)] can trap intracellularly their radiolabeled substrates (PET reporter probe), which produce high-energy gamma rays via positron emission. By quantifying the amount of visible light emitted with a cooled charge-coupled camera in the case of BLI or the amount of coincident gamma rays with a PET scanner with the latter approach, the magnitude and location of reporter gene expression can be accurately determined. By linking the expression of a reporter gene to that of a therapeutic gene in a vector construct, we can indirectly monitor therapeutic gene expression by imaging only the reporter gene expression [16]. Labeling donor cells with a BLI or PET reporter gene therefore allows direct imaging of cell survival following transplantation [17]. In the context of combined gene and cell therapy, it may therefore be possible to image cell survival directly using one reporter gene/reporter probe system and the expression of their transgene indirectly via another reporter gene/reporter probe system.

The goal of this study was to test the hypothesis that it is feasible and experimentally practical to non-invasively image transplanted cells and their transgene expression using two different bioluminescence reporter gene/reporter probe systems, namely fluc/D-luciferin and hrl/coelenterazine. C2c12 mouse skeletal myoblasts (C2c12) were genetically modified to stably express a multimodality reporter gene (mrfp-fluc-sr39tk) encoding a fusion protein of

monomeric red fluorescent protein (mRFP), FL, and HSV1-sr39tk under the control of an ubiquitin (Ubi) promoter [18]. Our group has shown that embryonic stem cells can develop into teratomata, and that such growth can be controlled using suicide gene therapy with HSV1-tk [19]. These genetically modified cells (C2c12-3F) were then transfected with a plasmid vector carrying two ubiquitin (Ubi) promoters, one driving the expression of hrl and the other mediating the constitutive expression of a recombinant hypoxia-inducible factor-1 alpha fused to two repeats of the HSV viral protein (VP) 16 (HIF-1 α -VP2) under both hypoxic and normoxic conditions.

Using BLI and the constructs we built, we aimed to (1) assess cell viability using FL expression as a surrogate and (2) indirectly monitor HIF-1 α -VP2 transgene expression by tracking hRL expression. The cell culture and animal study results obtained should help validate a powerful imaging strategy for objectively assessing both combined gene and cell therapy, while raising hope for performing similar evaluation in the clinics using PET reporter gene/reporter probe systems as we have previously validated [20].

Methods

Construction of Dual Promoter Plasmid Vectors

A fragment of human HIF-1 α (amino acids 1–390) was isolated from a commercially available full-length HIF-1 α plasmid (ATCC John Hopkins collection) via polymerase chain reaction (PCR) and cloned into a pcDNA3.1 expression vector using NheI and BamHI, upstream of two repeats of the transactivation domain of herpes simplex virus VP16 previously cloned in between BamHI and XhoI. The PGL3-Ubi-hrl plasmid was digested with BglII/BamHI to release the Ubi-hrl-SV40polyA fragment, which was then ligated into a puromycin resistance gene containing pcDNA3.1(+) vector digested with BglII/BglII. The fragment consisting of Ubi-HIF-1 α -VP2 and BGH polyA was amplified via PCR and ligated into the previous construct pcDNA3.1-Ubi-hrl-SV40polyA using MluI/KpnI. The final experimental construct (pUbi-hrl-pUbi-HIF-1 α -VP2) containing two ubiquitin promoters, with the upstream one driving hrl expression and the downstream one driving HIF-1 α -VP2, was confirmed by sequencing (Sequetech, Mountain View, CA).

Transient Transfection of 293T and C2c12 Cell Lines

293T human embryonic kidney cells (ATCC, Manassas, VA) were grown in minimal essential medium (MEM, Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 10 U/ml penicillin, and 10 μ g/ml streptomycin at 37°C and 5% CO₂. C2c12 mouse skeletal myoblasts (ATCC) were grown in DMEM high glucose (Gibco, Carlsbad, CA) supplemented with 10% FBS, 10U/ml penicillin, and 10 μ g/ml streptomycin under the same temperature and gas conditions. C2c12 and 293T cells plated at 1 and 3 $\times$ 10⁶/10 cm dish, respectively, were transiently transfected 24 h later with increasing doses (0, 2.5, 5, and 10 μ g) of the experimental dual promoter plasmid (pUbi-hrl-pUbi-HIF-1 α -VP2), a fixed dose (20 ng) of pcDNA3.1-CMV-fluc and balanced with pcDNA3.1(+) using Lipofectamine 2000 (20 μ l/10 μ g DNA; Invitrogen, Carlsbad, CA). Twenty-four hours post-transfection, cells were harvested, analyzed for luciferase expression, and the supernatants were assayed

for downstream VEGF and PlGF levels using enzyme-linked immunosorbent assay (ELISA) according to manufacturer's protocol (R&D Biosystems, Minneapolis, MN).

In Vitro Assays for Firefly Luciferase and Renilla Luciferase Activities

The *in vitro* firefly and *Renilla* luciferase assays were performed as previously described [21]. Briefly, cell pellet samples were lysed for 15 min on ice in 1× passive lysis buffer (Promega, Madison, WI), followed by centrifugation at 13,000 rpm and 4°C for 5 min. Supernatants mixed with coelenterazine (0.5 µg/sample; Nanolight Technology, Pinetop, AZ) and LARII substrate (100 µl; Promega, Madison, WI), respectively, were measured for hRL and FL activities using a luminometer (Turner Biosystems, Sunnyvale, CA). Supernatants were mixed with Bradford protein assay reagent (1 ml, Bio-Rad, Hercules, CA) and measured for protein content using a spectrophotometer (Beckman Coulter, Brea, CA). hRL activities were normalized by both protein concentration and transfection efficiency (FL enzyme activity) and expressed as unitless index (*i.e.*, (hRL relative light unit (hRL RLU) per microgram of protein)/(FL RLU/per microgram of protein)).

Western Blotting for HIF-1α-VP2 Expression

293T and C2c12 cells transfected with the experimental plasmid construct were harvested 24 h later. Cell pellets were washed with ice-cold PBS, lysed in urea-containing buffer (6 M urea, 75 mM Tris-HCl pH 7.4, and B-mercaptoethanol) on ice for 15 min, and centrifuged at 13,000 rpm and 4°C for 15 min to remove cell debris. To detect both hRL and HIF-1α-VP2 proteins, supernatants were loaded into and resolved using a 4–12% NU-PAGE gradient gel (Invitrogen, Carlsbad, CA), and subsequently transferred onto a nitrocellulose membrane. The membrane was blocked with 5% non-fat dry milk in Tris-buffered saline with 0.01% Tween (TBST) for 1 h, incubated separately with mouse monoclonal antibodies against either VP16 (1:200, Santa Cruz, Santa Cruz, CA), RL (1:5,000, Chemicon, Billerica, MA), or α-tubulin overnight at 4°C, washed three times with TBST prior to probing with a secondary goat anti-mouse antibody (1:3,000, Cell Signaling, Danvers, MA) for 1 h at room temperature. Washing of the membrane thrice with TBST was done before development with an enhanced chemiluminescence kit (Pierce, Rockford, IL). The intensity corresponding to different target proteins was determined using Image J software and normalized to the intensity of α-tubulin, which served as a loading control.

VEGF and PlGF Quantitation with Enzyme-Linked ImmunoSorbent Assay

C2c12 cells were transfected with experimental vectors pUbi-hrl-pUbi-HIF-1α-VP2 and CMV-hrl-CMV-HIF-1α-VP2 and harvested 24 h later. Supernatants were collected, centrifuged at 13,000 rpm for 10 min to remove debris, and subsequently assessed for VEGF and PlGF protein levels using VEGF and PlGF ELISA assay kits (R&D Systems, Minneapolis, MN) according to the manufacturer's protocols. Serial dilutions of recombinant human VEGF and PlGF served as standards.

Generation of C2c12-3F Cell Line

C2c12 cells were transduced with a lentiviral vector containing an ubiquitin promoter driving the expression of a multimodality reporter gene fusing monomeric red fluorescent

protein (mrfp), fluc, and a mutant herpes simplex virus type 1 thymidine kinase (HSV1-sr39tk) PET reporter gene [mrfp-fluc-sr39tk] (C2c12-3F) as previously described [18]. To ensure that transient transfection of C2c12-3F with the experimental vector does not affect FL expression, C2c12-3F cells were plated in a 12-well plate (2.5×10^5 cells/well) for 24 h and co-transfected with increasing amounts of the experimental vector (0.5 and 1 μ g plasmid). Twenty-four hours later, cells were washed three times with phosphate-buffered saline and assayed for RL using a cooled charge-coupled device (CCD) after administration of coelenterazine at a concentration of 2.5 μ M. After disappearance of the RL-mediated signal, cells were washed with PBS and D-luciferin (1 mg/ml) was added to evaluate FL expression. To determine if the amount of light generated correlated with cell number, various amounts of C2c12-3F cells (2.5×10^4 , 5×10^4 , 1×10^5 , and 1.5×10^5) were plated in a 12-well plate for 24 h. Twenty-four hours later, cells were assayed for FL expression using a cooled CCD after administration of D-luciferin (1 mg/ml). Image analysis was performed using Living Image software by placing a fixed size region of interest over single wells and measuring the total flux in photons per second per square centimeter per steradian (p/s/cm²/sr). All experiments were performed in triplicate and data are expressed as mean $\pm$ SD.

Bioluminescence Imaging of Living Animals

C2c12-3F cells (1×10^6) transiently transfected with the experimental vector, pUbi-hrl-pUbi-HIF-1 α -VP2, and control C2c12-3F (1×10^6) were injected into the right and left thigh muscles, respectively, of Swiss-Webster mice ($n=4$). Bioluminescence imaging of injected cells was done immediately and 24 h after cell injection using a cooled CCD as previously described (IVIS 100, Xenogen) [22]. On the day of the scan, mice were placed in an imaging chamber under continuous gas anesthesia (2% isoflurane). Two 2-min scans were acquired before and after intravenous administration of the substrate, coelenterazine (10 μ g), with the first scan serving as the baseline. Thirty minutes after the *Renilla* luciferase scan, mice were injected with D-luciferin (substrate for FL, 375 mg/kg body weight) intraperitoneally and received consecutive scans until the peak signal was obtained. The FL-mediated signal obtained 24 h after injection of cells was expressed as percentage of the initial FL signal.

To prove the feasibility of using reporter genes to monitor cell viability, in a different set of mice ($n=5$) various amounts of C2c12-3F cells transiently transfected with the experimental vector pUbi-hrl-pUbi-Hif-1 α -VP2 (5×10^4 , 1×10^5 , 2×10^5 , 4×10^5 , 8×10^5 , and 1.5×10^6 cells) were injected in the right and left legs and optical imaging was performed immediately after cell administration. For image analysis, region-of-interest (ROI) analysis was performed using Living Image 2.50 software (Xenogen Inc., Alameda, CA) by quantifying the signal intensity within the ROI as p/s/cm²/sr.

Statistics

Data are expressed as mean $\pm$ SD. Correlations were assessed by least square linear regression to obtain the Pearson's correlation coefficient. The significance of correlation is calculated by the *t* test against the null hypothesis. *P* values <0.05 are considered statistically significant.

Results

Construction of Plasmid Vectors

The HIF-1 α -VP2 fusion gene was constructed by truncating HIF-1 α at amino acid 390 and fusing it to two repeats of the transactivation domain of herpes simplex virus VP16 downstream (Fig. 1a). The dual promoter plasmid vector constructed in the present study is shown in Fig. 1b and is based on a vector system containing two identical promoters. This vector contains both hrl and HIF-1 α -VP2 at separate locations with each gene placed downstream of a constitutive ubiquitin promoter.

In Vitro Reporter Enzyme Assays Confirm Robust Expression of Hrl and HIF-1 α -VP2 in Transfected Cells

To confirm proper vector construction, show cell line independent expression, and assess the feasibility of indirectly monitoring HIF-1 α -VP2 expression through hRL expression, 293T cells and C2c12 cells were transfected with increasing doses of the experimental plasmid pUbi-hrl-pUbi-HIF-1 α -VP2. The pUbi-hrl-pUbi-Hif-1 α -VP2 vector was used for the entire study because it yielded the best expression of both transgenes, compared to all other dual promoter constructs made. Twenty-four hours after transfection, we assessed hRL reporter activity and HIF-1 α -VP2 expression using *in vitro* luciferase assay and western blotting. The hRL activity was found to increase linearly with plasmid dose up to 10 μ g ($r^2=0.95$; $P<0.05$, Fig. 2).

Similarly, Western blot also showed increasing amount of both HIF-1 α -VP2 and hRL proteins with increasing plasmid dose up to 10 μ g, confirming successful transfection of the experimental vector in both 293T (Fig. 3a) and C2c12 (Fig. 3b) cells. Furthermore, the expression of HIF-1 α -VP2 protein quantified by densitometry and normalized for α -tubulin correlated linearly with *in vitro* hRL activity (Fig. 3c).

In Vitro ELISA Detected Downstream Angiogenic Effect of HIF-1 α -VP2

To study whether the recombinant hybrid HIF-1 α -VP2 protein can effectively induce downstream angiogenic gene expression, we performed ELISA to quantify the amount of VEGF and PlGF in the supernatant of cells transfected with increasing doses of the experimental vector pUbi-hrl-pUbi-Hif-1 α -VP2. The VEGF and PlGF levels in the supernatant were found to correlate linearly with the *in vitro* hRL activity ($r^2=0.82$ and $r^2=0.97$ respectively, Fig. 4). At the highest dose of 10 μ g, VEGF and PlGF levels were three- and fourfold, respectively, higher than the baseline levels, further confirming the functionality of the HIF-1 α -VP2 fusion protein and the dose-dependent upregulation of the downstream angiogenic genes.

In Vitro Experiments Show a Correlated Expression Between Cell Number and Detected Light Using Luciferase Reporter Genes

To assess the effect of transient transfection of C2c12-3F cells with the experimental vector on FL expression, C2c12-3F cells were plated in a 12-well plate (2.5×10^5 cells/well) for 24 h and co-transfected with increasing amounts of the experimental vector (0.5 μ g and 1 μ g plasmid). Twenty-four hours later, FL expression was assayed in a cooled CCD camera. As

shown in Fig. 5a, there is no significant difference in FL-mediated light emission after transient transfection with the dual promoter construct.

To determine whether measured optical signal corresponded to the number of viable cells, various numbers of cells were plated in a 12-well plate and assayed for FL expression in a CCD camera. The number of cells strongly correlated with FL-mediated light production ($r^2=0.97$, Fig. 5b), supporting the concept that light emission correlates well with viable cell number.

BLI Revealed the Feasibility of Concomitantly Imaging Transplanted Cells and HIF-1 α -VP2 Transgene Expression in the Living Subject

To assess the feasibility of non-invasively imaging transplanted cells and their HIF-1 α -VP2 transgene expression, we injected C2c12-3F and C2c12-3F cells that transiently express our experimental vector into the left and right thighs of mice ($n=4$), respectively. Immediately and 24 h after cell delivery, mice were imaged for both FL and hRL expression. As shown in Fig. 6a for a representative mouse, the FL-mediated signal was initially robust (maximum $5-10 \times 10^4$ p/s/cm²/sr) in both legs immediately after cell implantation, whereas hRL signal was detected only in the right leg (maximum 4×10^5 p/s/cm²/sr). After 24 h, the FL signal dropped drastically to 3.4×10^4 p/s/cm²/sr and 7.8×10^3 p/s/cm²/sr in the right and left leg, respectively. On average the FL signal decreased to $42 \pm 37\%$ compared to baseline (Fig. 6b), while the hRL expression became undetectable (data not shown). These data showed that cell viability and transgene expression can be evaluated concomitantly using a reporter gene strategy.

To confirm that cell number correlates with measured detected light *in vivo*, we injected different amounts of C2c12-3F cells transiently transfected with the experimental vector in the right (R) and left (L) legs ($n=5$) and optical imaging was performed immediately after cell administration. We found a highly significant correlation ($r^2=0.92$) between injected number of cells and FL-mediated light production (Fig. 7a). In the same group of animals, a good correlation between FL expression and hRL signal was observed ($r^2=0.76$, $P<0.05$; Fig. 7b).

Discussion

The success of combined cell and gene therapy will likely depend, at least in part on the survival of engrafted cells and the long-term expression of the therapeutic transgene. Non-invasive, repetitive monitoring of transplanted cells and their therapeutic transgene expression has been difficult to accomplish because probes to directly image therapeutic transgene expression are not readily available. However, simultaneous imaging of both transplanted cell survival and their therapeutic transgene is crucial for a better understanding of the effectiveness of combined cell and gene therapy, and has been shown for the first time in this study to be feasible. In this study, we were able to image both injected cells and indirectly HIF-1 α -VP2 expression using a dual promoter construct. Direct imaging of HIF-1 α -VP2 was not possible because no imaging probe exists for HIF-1 α -VP2. The development of such a probe would also be challenging due to the nuclear localization of HIF-1 α -VP2.

The indirect imaging approach is a valuable alternative. Its utility, however, requires a robust coupling between the expression of the reporter (hrl) and the therapeutic (HIF-1 α -VP2) genes. We were able to satisfy this requirement by demonstrating a strong correlation between the hRL activity and the HIF-1 α -VP2 expression, or its downstream angio-genic (VEGF and PlGF) protein levels in cell culture. These data underscore the feasibility of using this construct for indirect monitoring of HIF-1 α -VP2 expression in living animals.

One of the greatest strengths of non-invasive molecular imaging is its potential to repeatedly interrogate cell survival and therapeutic gene expression. Previously, cell survival has been routinely evaluated by traditional histology and quantitative PCR both with inherent limitations to monitor cell survival within the same living subject [23, 24]. Our imaging results demonstrate a large drop in FL signal (on average only 42% of the baseline signal) while the hRL signal was undetectable 24 h after cell administration. The significant FL signal after 24 h supports the concept that the drastic drop in FL signal is reflective of cell death rather than technical issues or delivery of dead cells. If all cells were dead or dying during delivery, one would only expect background levels after 24 h because of the very short half-life of FL (~2 h) and the lack of adenosine triphosphate necessary to mediate light generation. This decrease in cell survival is consistent with several other studies, which have observed a significant acute donor cell death up to 90% within the first 24 h of cell delivery, regardless of the cell type (except undifferentiated embryonic stem cells) [19]. Cell immunogenicity, ischemia, and the cellular micro-environment may all account for this acute donor cell death. The disappearance of hRL signal can be attributed to the drastic cell death early after cell delivery, as implied by the corresponding ~60% drop in FL signal. In addition to the acute donor cell death, transient transfection of the vector construct may also account for the disappearance of hRL signal 24 h after transplantation. While we used a lipofect-amine-based approach, we did not observe any acute toxicity or major cell death after transfection. It is worth mentioning that light detection was lower in the right leg (C2c12-3F cells transiently transfected with the experimental plasmid) compared to the left leg (C2c12-3F). Because transient transfection does not affect cell viability (Fig. 5a), factors like variations in injection technique (location and depth of cell injection) and animal positioning in the CCD camera can account for the differences in light detection and should be considered when performing longitudinal studies. We choose a transient expression strategy in this study, while other studies that report beneficial effects have been performed with physical or viral delivery methods, which can lead to better transfection efficiency and more long-term transgene expression [25–28]. Although it is not entirely clear whether long-term transgene expression would be most ideal, considering that continuous transcriptional activation of angiogenic genes may lead to unwanted side effects such as vascular malformation [29]. For cases in which therapeutic efficacy has been shown to hinge on long-term transgene expression, either stable transfection or viral transduction can be performed. Because we used a transient expression strategy in this study, we did not collect data on cell survival and long-term therapeutic gene expression, and in turn we did not perform histological analysis. In future studies where long-term viability and biology are evaluated, the use of more traditional tools such as histology will be important to elucidate the mechanisms that could contribute to the changes in cell survival and biology of injected cells.

The rationale for using HIF-1 α as the therapeutic gene stems from earlier studies that administration of one single angiogenic factor (*e.g.*, VEGF) may not be sufficient to achieve therapeutic efficacy. In addition, stand alone VEGF treatment for therapeutic neovascularization can have deleterious side effects such as enhanced vascular permeability, edema, and inflammation [29, 30]. Thus, increasing VEGF while avoiding its side effects is preferred from a therapeutic perspective, and this can be achieved by delivering a master switch gene such as HIF-1 α , which controls the expression of various downstream angiogenic growth factors. HIF-1 α is a key player in oxygen homeostasis and is an upstream gatekeeper of key angiogenic growth factors necessary for adequate neovascularization [31, 32]. HIF-1, a heterodimer composed of an inducible HIF-1 α and a constitutively expressed Hif-1 β present in the nucleus, is controlled by oxygen levels [33]. HIF-1 α is predominantly upregulated in a hypoxic environment and activates downstream angiogenic factors by binding to their hypoxia responsive elements [34]. In contrast, during normoxia, HIF-1 α gets degraded or its transcriptional activation is blocked. From a therapeutic viewpoint, an oxygen tension-independent activation of HIF-1 α is preferred, and this can be achieved by selectively deleting the region between amino acids 390–826 which contains both the transactivation domain and the oxygen-dependent region (ODD) [35, 36]. Deleting the latter and replacing it with the VP16 transactivation domain of HSV results in a constitutively active and oxygen-independent HIF-1 α -VP16 [26]. HSV-VP16 is a potent transactivator, and we have found in our preliminary studies that two VP16 domains back-to-back further increases transactivation (data not shown). Therefore, in the current study we used the hybrid HIF-1 α -VP2 to further increase upregulation of downstream genes involved in angiogenesis.

The therapeutic efficacy of this synthetic fusion gene (HIF-1 α -VP16) has been validated in pre-clinical studies for either cardiac or peripheral ischemia, and has recently been shown to be promising in a phase I clinical trial [25–27, 37, 38]. However, other published data have also shown Hif-1 α -VP16 to be only as potent as VEGF alone, even though it upregulates additional angiogenic genes [25]. Previous evidence for the efficacy of HIF-1 α -VP16 was based mainly on surrogate endpoints such as increased capillary density and improved organ function, because HIF-1 α expression could not be directly monitored over time [28]. Our indirect imaging approach, which robustly couples the expression of HIF-1 α and hrl, allows monitoring of the temporal kinetics of therapeutic transgene expression. This strategy offers a generalizable tool in which the therapeutic gene of interest can be serially monitored during combined cell and gene therapy, so that its expression can be closely correlated with other endpoints to more objectively evaluate therapeutic efficacy. Further refinement of this strategy should help facilitate the continual optimization and evaluation of HIF-1 α -VP16 therapy in pre-clinical studies.

Bioluminescence imaging has the advantage of being high throughput and low cost, but is limited clinically by the limited imaging depth. To overcome this problem, the optical reporter genes used in this study can be replaced with PET reporter genes such as HSV1-sr39tk and dopamine type 2 receptor [20]. The concepts presented herein for simultaneous cell and transgene imaging would remain exactly the same, in which one PET reporter gene can be used to monitor cell survival and the other can be used to indirectly image therapeutic

gene expression. This modification should help facilitate the optimization of cell-mediated HIF-1 α -VP16 gene transfer in large animals and open the door for monitoring of combined cell and gene therapy in clinical trials.

Conclusion

We demonstrated in this study that BLI can be used to simultaneously image injected cells and their expression of a therapeutic gene using two different optical reporter gene systems. This strategy should help facilitate the evaluation and optimization of combined cell and gene therapies in living subjects by allowing for an objective evaluation of both cell survival and the kinetics of transgene expression. Further translation of this strategy can be accomplished by replacing the optical reporter genes with the PET reporter genes we have previously validated. Our future studies are aimed at evaluating the potential beneficial effects of genetically modified cells on both cell survival after transplantation, as well as functional improvement following ischemia.

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Fig. 1.

Plasmid Vector Construction. **(a)** Construction of an oxygen insensitive form of HIF-1α. HIF-1α (amino acid 1–390) was fused to two repeats of the Herpes Simplex Virus protein VP16. Abbreviations: wtHIF-1α, wild type HIF-1α gene; dHIF-1α, deletion of the oxygen dependent domain; ODD, oxygen dependent domain; TAD N, transactivation domain N-terminus; TAD C, transactivation domain C-terminus; P567T and P658Q, mutations to replace two proline residues by a threonine and glutamine at position 567 and 658, respectively **(b)** Schematic of dual promoter plasmid vector construct. A dual promoter construct was cloned with one promoter driving the expression of the reporter gene and the second promoter driving the expression of the therapeutic gene (Hif-1α-VP2). Abbreviations: pUbi, ubiquitin promoter.

Fig. 2.

In vitro Enzymatic Activity of hRL. Cells were transiently transfected with increasing amount of plasmid pUbi-hrl-pUbi-Hif-1α-VP2 (0, 2.5, 5, and 10 μg). Twenty-four hours later, hRL enzymatic activity was measured and correlated linearly with the amount of plasmid transfected ($r^2 > 0.95$).

Fig. 3. Correlation Between the Amount of Plasmid Transfected and the Protein Levels of hRL and HIF-1α-VP2 using Immunoblot Analysis. **(a)** 293T cells and **(b)** C2c12 cells were transiently transfected with increasing doses of the dual promoter construct and blotted for hRL and HIF-1α-VP2 proteins, whose bands are shown for the plasmid dose indicated. **(c)** Correlation between the enzymatic hRL activity and the amount of HIF-1α-VP2 protein assessed by immunoblot analysis normalized to α-tubulin ($r^2 = 0.82$).

Fig. 4.

Downstream Angiogenic Effect of HIF-1 α -VP2. Cells were transiently transfected with increasing amounts of the plasmid pUbi-hrl-pUbi-Hif-1 α -VP2 (0, 2.5, 5, and 10 μ g) and assessed for VEGF and PIGF levels 24 hrs later using ELISA. The VEGF and PIGF levels are expressed as fold-induction over the baseline levels for PIGF and VEGF and plotted against the corresponding hRL activity at each plasmid dose ($r^2 = 0.82$ and $r^2 = 0.97$ respectively).

Fig. 5.

In Vitro Evaluation of FL-mediated Light Emission. **(a)** C2c12-3F cells were transiently transfected with increasing amounts of the plasmid pUbi-hrl-pUbi-Hif-1 α -VP2 (0, 0.5 and 1 μ g) and assayed for FL expression in a CCD camera. There was no significant difference in FL-mediated light production between parental C2c12-3F cells and C2c12-3F cells transfected with the plasmid. **(b)** Correlation between different numbers of plated cells and light emission assessed with a CCD camera. All experiments were done in triplicate and data are expressed as means $\pm$ SD.

Fig. 6. Non-invasive Bioluminescence Imaging of Transplanted Cells and Their Therapeutic Transgene Expression. Mice (n=4) were injected intramuscularly with C2c12-3F (1×10⁶) and C2c12-3F cells (1×10⁶) transiently transfected with pUbi-hrl-Ubi-HIF-1α-VP2 (C2c12-3F + hrl) into the left and right thighs, respectively. Bioluminescence imaging of FL and hRL activities were performed immediately post-implantation. (a) A representative bioluminescence image is shown demonstrating only hRL activity in the right thigh. (b) Values of FL-mediated light emission for each individual animal immediately and 24 hours after cell injection. Data are expressed as p/s/cm²/sr. Abbreviations: L, left leg and R, right leg.

Fig. 7.

Correlated Luciferase Reporter Gene Expression in Vivo. Mice (n=5) were injected with different numbers of C2c12-3F cells transiently transfected with the dual promoter plasmid (pUbi-hrl-pUbi-Hif-1 α -VP2). **(a)** Correlation between various numbers of injected cells and light production using the firefly luciferase reporter gene system. **(b)** Correlation between FL- and hRL-mediated light emission immediately after cell administration.