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ORIGINAL ARTICLE

Vascular endothelial growth factor broadens lentivector distribution in the heart after neonatal injection

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Summary For some applications, the success of gene therapy depends on the efficiency of gene transfer into target organs, however, delivery to many tissues is limited. Efforts have been made to improve the efficiency of gene transfer into target organs such as the brain by using mannitol or vascular endothelial growth factor (VEGF) prior to gene delivery, since these treatments have been reported to increase vascular permeability in experimental animals. Here, we investigated the effect of VEGF pretreatment of neonatal mice on the ability of injected lentivirus (LV) – engineering expression of firefly luciferase (luc) – to enhance the transduction of various organs, including the brain and heart. LV/luc was delivered to VEGF-treated neonatal mice via the temporal vein. Whole-body bioluminescence imaging (WBLI) of luciferase expression showed that VEGF pretreatment does not diminish transgene expression over time since it remained steady for up to 12 weeks. *Ex vivo* imaging of the organs and assessments of organ luciferase activity showed that VEGF pretreatment resulted in significantly increased luciferase expression not only in the heart, but also in the brain, lung, and kidney. This study shows that VEGF may have therapeutic importance to enhance the efficiency of viral gene delivery to the heart, as well as to other target organs.

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Introduction

Some applications of gene therapy depend on high gene transfer efficiencies into target tissues and long-term expression of therapeutic transgenes. Recombinant lentiviral vectors (LVs) have been shown to be effective in transducing non- and/or slowly dividing cells *in vitro* and *in vivo*, including cardiac myocytes [1–4]. Our group has also shown that direct injection of recombinant LVs into neonatal mice led to sustained systemic expression of transgenes with wide distribution in many organs, including the heart [5]. Thus, gene therapy using direct injection of recombinant LVs seems to have therapeutic potential for treatment of cardiac disorders.

As an example in this context, Fabry disease is the second-most prevalent lysosomal storage disorder (LSD). Fabry disease is an X-linked recessive disorder caused by a single gene defect for a lysosomal hydrolase: α-galactosidase A (α-gal A, EC 3.2.1.22) [6]. In Fabry disease, a reduction in α-gal A activity results in the accumulation of galactosylsphingolipid moieties, especially globotriaosylceramide, which leads to cardiac [7] and kidney [8] dysfunction. 'Cardiac variant' Fabry disease has also been elucidated wherein manifestations are only limited specifically to the heart [9]. Enzyme replacement therapy using recombinant enzyme has been available for Fabry disease and can lead to improvement of some manifestations [10–12]; however, this therapy requires biweekly recombinant enzyme infusions throughout life and the benefit of this treatment may possibly be affected by immune responses that have been observed against the enzyme [10,11]. Most problematic, however, is that enzyme replacement therapy is started after the damage to most organs may be irreversible. Therefore, earlier intervention may be more effective than delayed treatment. We have been developing oncoretroviral- [13–15] and lentiviral-mediated gene therapy [16,17] for Fabry disease including delivery to neonatal animals [5].

Regulation of vascular permeability is crucial for organ function, since this barrier prevents transfer of macromolecules, such as serum proteins, from blood to tissues. However, disruption of the regulatory mechanism is often observed in many disease conditions: acute inflammation, pathologies associated with angiogenesis such as tumors, wounds, ischemic diseases, and chronic inflammatory diseases.

Vascular endothelial growth factor (VEGF) plays a role in vasculogenesis, angiogenesis, and even lymphangiogenesis [18,19]. Administration of VEGF into neonatal animals enables increased transport of therapeutic proteins into the brain by increasing the transient and reversible vascular hyperpermeability of the blood–brain barrier (BBB) [20]. It appears that VEGF enhances the activity of an organelle called the vesicular–vacuolar organelle that is found intermittently throughout the endothelial cells (ECs) lining small blood vessels [21]. These organelles are clusters of vesicles and vacuoles that are interconnected with each other and the plasma membrane of the ECs by means of fenestrae that open and close to allow/prevent the flow of macromolecules through the vesicles and into the tissue [22]. This characteristic has been exploited by administering VEGF prior to gene therapy to increase gene transfer efficiency. Studies by Young et al. using an authentic mouse model of an LSD showed increased ingress of administered protein, bone marrow cells, or recombinant viruses into the central nervous system, along with greater therapeutic levels of enzyme and increased life span in animals treated with VEGF compared to animals preconditioned using irradiation or those not conditioned [23]. In studies where LV was directly injected, examination of the brain showed LV-transduced cells present in all areas of the brain and transduced cells included neurons, and glial and endothelial cells [23]. Neonatal gene transfer offers the advantage of administering therapeutic vector before permanent organ and neurological damage has occurred. It also offers the potential to tolerize patients to the therapeutic protein expressed from the vector since the immune system of neonates is still relatively immature [24–26]. Thus, since neonatal gene transfer combined with VEGF treatment to increase distribution has the potential to treat systemic manifestations early in life, it is a promising therapeutic option for Fabry disease and other LSDs.

In the present study, a recombinant LV engineering expression of luciferase in transduced cells (LV/luc) was used to track transgene expression. LV/luc was injected into the temporal vein of neonatal mice, with or without addition of VEGF, and transgene expression was monitored over 12 weeks. *Ex vivo* imaging of the organs showed that VEGF pretreatment increased expression of luciferase in the brain, lung, kidney, and heart compared to organs from the mice that received LV alone. These studies are the first to demonstrate that VEGF treatment prior to viral delivery has a broad systemic effect in improving transduction levels and provide encouraging evidence that this regimen can improve therapeutic outcomes for a variety of disorders, including Fabry disease.

Materials and methods

LV production and determination of titer

The lentiviral vector pHR'cppt-EF-luciferase has previously been described [5]. VSV-g-pseudotyped LV/luc was produced by transient co-transfection of 293T cells with LV/luc and the accessory plasmids pMD.G and pCMVΔR8.91, using the polyethyleneimine-transfection procedure [27–29]. Cell culture medium was changed 16 h post-transfection. Viral supernatants were harvested after 48 h and concentrated by ultracentrifugation at 50,000 × *g* for 2 h. The concentrated virus was suspended in sterile phosphate buffered saline (PBS) and stored at −80 °C until use. The level of p24 antigen in the LV/luc virus preparation was determined using an HIV-1 p24 ELISA kit (PerkinElmer Canada Inc., Vaudreuil-Dorian, QC, Canada) and was found to be ~3100 ng p24/ml.

Animal procedures

The animal experimentation procedures described herein were performed under protocols approved by the University Health Network (UHN) Animal Care Committee. Balb/c mice were maintained at the animal facility of the UHN. Two hours prior to virus injection, 1.7 ng of recombinant mouse $VEGF_{164}$ (R&D Systems, Minneapolis, MN, USA) was administered to 1–3-day-old neonatal mice through the superficial temporal vein in a volume of 100 μl. Control mice received 100 μl of PBS. Concentrated LV (300 ng p24 in 100 μl PBS) was then injected via the superficial temporal vein.

In vivo and *ex vivo* bioluminescent imaging

In vivo bioluminescent imaging was performed at the Advanced Optical Microscopy Facility at the UHN with an IVIS Imaging System (Xenogen, Alameda, CA, USA), which comprised a cooled charged coupled device (CCD) camera mounted in a light-tight camera box. Images and measurements of bioluminescent signals were acquired and analyzed using Living Image software (Xenogen). For whole-body luminescence imaging, mice were anesthetized, administered D-luciferin (Molecular Imaging Products, Ann Arbor, MI, USA) at 100 mg/kg in PBS by intraperitoneal (i.p.) injection, and then imaged 10 min later. For *ex vivo* organ imaging, 2 min after receiving D-luciferin, mice were killed and organs were collected and washed with PBS. Images were then immediately acquired (5 min exposure time). Following imaging, the organs were cut in half. One half of each organ was immersed in optimal cutting temperature (OCT) compound (Pelco International, Redding, CA, USA). The other half was transferred into a microcentrifuge tube, frozen on dry ice, and then stored at −80 °C until use.

Immunohistochemistry

Following *ex vivo* imaging, samples of each organ were cryopreserved in OCT compound and stored at −80 °C. The specimens were cryosectioned to a 5 μm thickness. The sections were mounted on glass slides, air dried for 1 h at room temperature, washed with PBS, and then post-fixed in 4% buffered formalin in 0.1 M sodium phosphate buffer, pH 7.4. Slides were washed with PBS and then incubated in PBS containing 0.1% (v/v) Triton X-100 for 15 min, the samples were treated with 5% (v/v) normal donkey serum in PBS for 30 min. The sections were sequentially reacted with primary antibody solution (1:100 dilution in PBS) at 4 °C overnight, followed by incubation in PBS-containing secondary antibody (1:500 dilution in PBS) labeled with either Alexa488 or Alexa546 for 3 h at room temperature. Antibodies used in this study were as follows: goat anti-luciferase antibody (Chemicon International Inc., Temecula, CA, USA), rat monoclonal anti-mouse CD31 antibody (BD Pharmingen, San Diego, CA, USA), rabbit anti-GATA4 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), rabbit anti-doublecortin (Abcam, Cambridge, MA, USA), rabbit anti-glial fibrillary acidic protein (GFAP) (Lab Vision, Fremont, CA, USA), Alexa488-labeled donkey anti-rabbit or anti-rat IgG antibody (Molecular Probes, Inc., Eugene, OR, USA), and Alexa546-labeled donkey anti-goat IgG antibody (Molecular Probes). Fluorescence signals were analyzed using a confocal laser-scanning microscope LSM-5 and LSM System version 3.98 (Carl Zeiss, Oberkochen, Germany) at the Common Instrument Center of Institute of Development, Aging, and Cancer, Tohoku University.

Measurement of organ luciferase activity

Organs were minced and homogenized using a microfuge pestle in 1× Cell Culture Lysis Reagent (Promega Corp., Madison, WI, USA). Lysates were then spun at 12,000 × *g* for 5 min at 4 °C. The supernatants were transferred to a microcentrifuge tube and luciferase activity was measured using the Luciferase Assay System from Promega, as per manufacturer's instructions. Protein concentrations were measured using the Bio-Rad DC

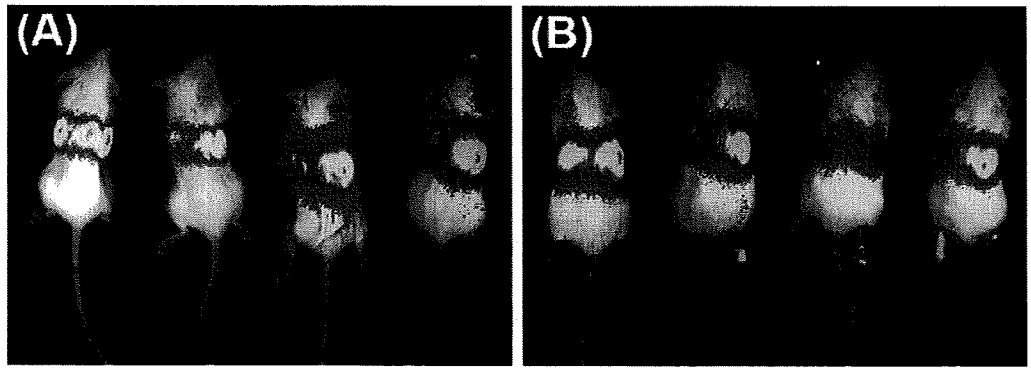

Figure 1 Whole-body luminescence imaging of mice showing long-term luciferase expression. One- to three-day-old neonatal mice were injected with LV/luc in the absence (A) or the presence (B) of VEGF. VEGF was administered 2 h prior to the virus injection via the superficial temporal vein. At 12 weeks post-virus delivery, mice were imaged using a cooled CCD camera following i.p.-administration of D-luciferin from the dorsal side. Shown are images of four representative mice from each group.

Protein Assay (Bio-Rad Laboratories, Mississauga, ON, Canada) as per manufacturer's instructions.

Statistical analysis

Data are presented as the mean ± standard error of mean (SEM). Statistical analyses were performed using Instat version 3 software for Macintosh (GraphPad, San Diego, CA, USA). Statistical analysis was done using the Mann–Whitney test with the level of significance set at $P < 0.05$.

Results

Whole-body bioluminescence imaging

To determine the effect of VEGF administration on the transduction pattern of LV on neonates *in vivo*, 1–3-day-old Balb/c mice were treated with VEGF 2 h prior to injection of LV/luc, whereas control mice received the virus without VEGF pretreatment. No adverse effects from VEGF pretreatment were observed throughout the life of the animals. Transgene expression in these immunocompetent mice was detected by whole-body bioluminescence imaging (WBLI) following administration of the D-luciferin substrate. It was found that the luminescent signals generated by the luciferase activity was detected from the mice in both groups throughout the course of the experiment, beginning at 4 weeks (data not shown) and persisting at similar levels up to 12 weeks (Fig. 1). This pattern is similar to that observed in our previous studies [5]. These results indicate that VEGF administration is well tolerated and does not grossly affect expression of the transgene as measured by WBLI.

VEGF pretreatment increases luciferase expression in the heart and other organs

Following WBLI at week 12, mice were killed and the organs were examined *ex vivo* by measuring the luminescent signal intensity (Fig. 2A). The signal intensity of the brain obtained from the mice receiving VEGF prior to injection of LV was greater than that from VEGF (−) mice (Fig. 2B). It was also found that the lung, heart, and kidney from VEGF (+) mice showed increased signal intensity compared to organs from VEGF (−) mice, while no apparent increases in signal were observed in the liver and spleen (Fig. 2B). It should be noted that this might be due to the high level of the luciferase expression in the liver and spleen that may have saturated the captured signal. Indeed, these organs appear to be the most readily penetrated and transduced by LV as seen in our previous studies [5].

Heart tissue demonstrates the greatest increase in luciferase activity with VEGF pretreatment

To determine the luciferase activity quantitatively in each organ, specimens of each organ were homogenized and the luciferase enzymatic activity in each sample was measured directly. As expected, direct luciferase activity of each of the organs derived from LV/luc-treated mice was found to be 10–1000-fold higher compared to values from untreated mice (Fig. 3). VEGF pre-

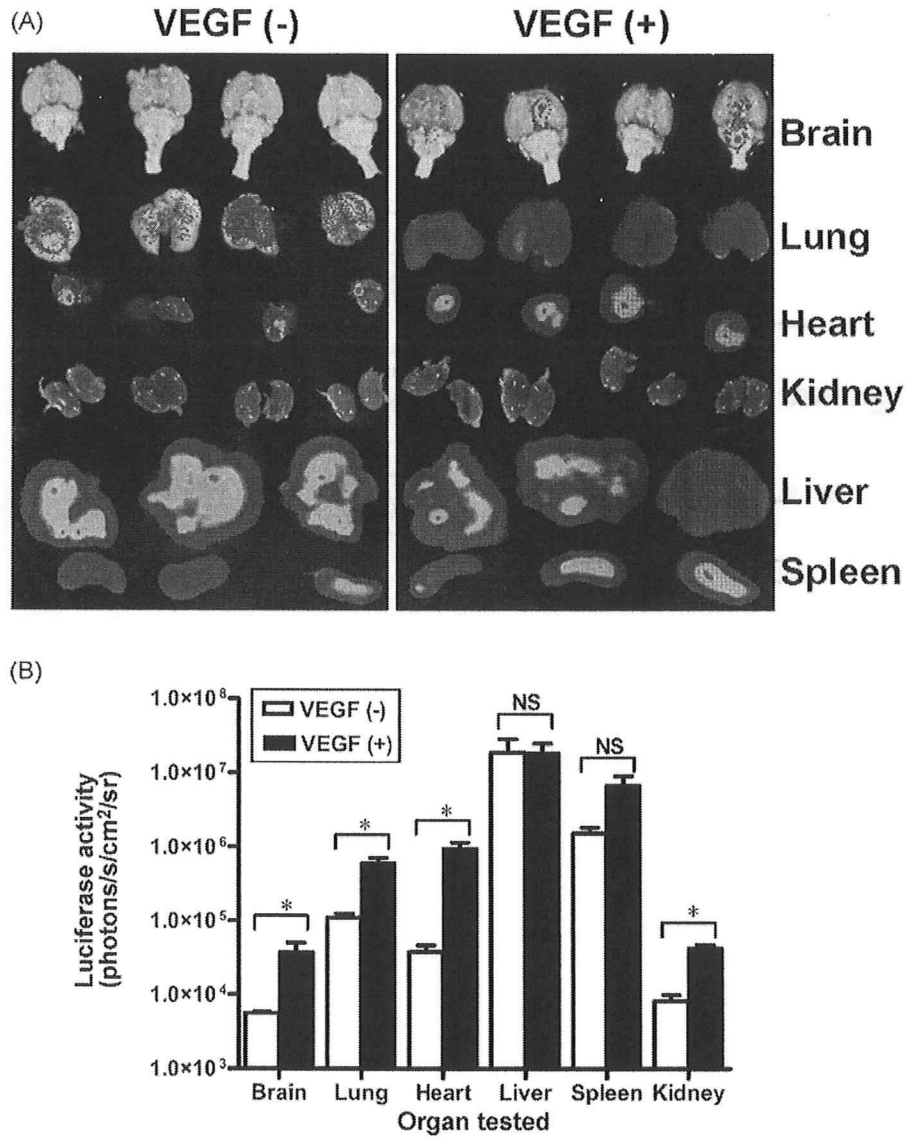

Figure 2 *Ex vivo* imaging of the luciferase expression in the organs at 12 weeks post-virus injection. Following whole-body luminescence imaging, mice were killed and the organs were taken out and imaged. (A) Shown are images of organs from representative mice from each group. (B) The bioluminescent signal from each organ was measured using the Living Image software. Values shown are means ± SD. ($n = 8$ per group, $^*P < 0.05$).

treatment demonstrated a tendency to increase the luciferase activity of each organ from 1.3- to 6.6-fold compared to that without VEGF pre-treatment. Importantly, the activity of the heart was 6.6-fold higher in VEGF-treated mice compared with that of VEGF-untreated mice ($P < 0.05$). Increased luciferase activity in the other organs of VEGF-treated mice could not be demonstrated by this assay. These findings are of particular significance for diseases that have multiple organ involvement and especially important for diseases with cardiac involvement such as Fabry disease and some other metabolic disorders [30–32].

Immunostaining of tissue sections to identify the transduced cells by LV/luc *in situ*

Since the organ luciferase activity of the VEGF-treated mice showed a dramatic increase compared to that observed from the untreated mice, we next examined tissue sections by immunostaining to identify the specific type of cell that was producing reporter enzyme. Tissue sections from the hearts of transduced animals were immunostained using an anti-luciferase antibody combined with either an anti-CD31 antibody as a vascular EC marker (Fig. 4A) or an anti-GATA4 antibody as a myocardial

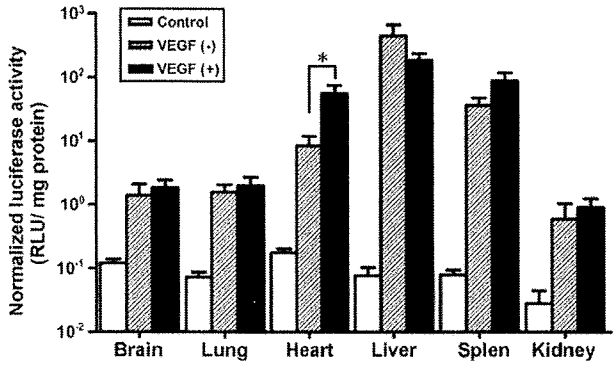

Figure 3 Comparison of the organ luciferase activity. Organs were homogenized and the luciferase activity was measured using a luminometer. Values shown are means ± SEM ($n = 8$ for VEGF (−), $n = 7$ for VEGF (+), and $n = 4$ for non-treated mice; $^*P < 0.05$).

cell marker (Fig. 4B). It was found that both the CD31-positive cells, namely, vascular ECs and the GATA4-positive cells, namely, myocardial cells showed the existence of immunoreactivity against the anti-luciferase antibody in LV/luc-treated mice, whereas only background levels of the fluorescence signal from immunoreactive luciferase was seen in untreated mice. These results suggest that both the vascular ECs and the myocardial cells themselves were transduced by LV/luc injection into neonatal animals.

Tissue sections made from brains of infected animals were also immunostained to identify the type of cells transduced by LV/luc using either an anti-doublecortin antibody as a neuronal cell marker or an anti-GFAP antibody as a glial cell marker. Immunoreactive luciferase in LV/luc mice was detected in neuronal cells, including Purkinje cells of the cerebellum as shown by staining with an anti-doublecortin antibody (Fig. 4C), while no immunoreactive luciferase could be detected in glial cells in LV/luc mice (Fig. 4D). In the liver, both the parenchymal and the vascular ECs showed the presence of immunoreactive luciferase (data not shown) in LV/luc-treated mice. These results indicate that the systemically injected LV/luc transduces not only the ECs but also the perivascular cells in the brain.

Discussion

Angiogenesis is preceded and/or accompanied by enhanced microvascular permeability. Regulation of vascular permeability during angiogenesis is critical to normal and/or patho-physiological functions and the families of proteins that appear to be most important in regulating vascular permeability are the various members of the VEGF families [33]. For example, Young et al. found that administration of VEGF prior to injection of LV into neonates disrupted the neonatal BBB and resulted in increased numbers of cells transduced in the brain [23]. While examination of other organs in that study showed that VEGF had no overt effects on organ development and did not cause tumor development, there was no examination of the effect on transgene expression specifically in the organs themselves nor were the LV-transduced cells identified *in situ* [23]. In the present study, we have used a similar delivery approach using luciferase as a marking transgene to determine the effects of VEGF administration on the transduction of the major internal organs. We found that the luciferase activity achieved in the hearts of mice receiving both LV/luc and VEGF was significantly higher compared to those that did not receive VEGF (Fig. 3). Cellular localization analysis of the heart indicated that both the vascular ECs and the myocardial cells were transduced (Fig. 4A and B).

Transgene expression in LV/luc-administered mice was monitored monthly for 3 months by WBLI and it was found that the expression remained steady in both VEGF (+) and VEGF (−) mice (Fig. 1). Quantification of LV/luc expression by *ex vivo* imaging showed significant increases of luciferase activity in the heart, lung, and kidney of the VEGF-treated mice compared with those of the VEGF-untreated mice (Fig. 2B). The spleen and the liver did not show significant increases in transgene expression mediated by the VEGF treatment but reporter gene expression in these organs was high in all mice treated with LV/luc. Indeed, we may have reached the saturation point of the assay, and as such, differences between groups would be harder to detect. This finding is not surprising since the liver is often the most highly transduced organ when virus is delivered directly into the bloodstream [5,23,34].

The organ luciferase activity assays showed a significant increase in enzyme activity in the hearts of VEGF (+) mice while no significant changes could be detected in other organs (Fig. 3). Since gross sections were made for this assay, it is possible here that the enzyme was diluted in the background of non-transduced tissue and that this had an impact on the activity calculations in contrast to what was observed in the *ex vivo* imaging data. These differences in the results obtained from the two methods of analysis are likely caused by a number of factors including the architecture and vascularization of the organ. Generation of luminescent signals by luciferase requires molecular oxygen and sup-

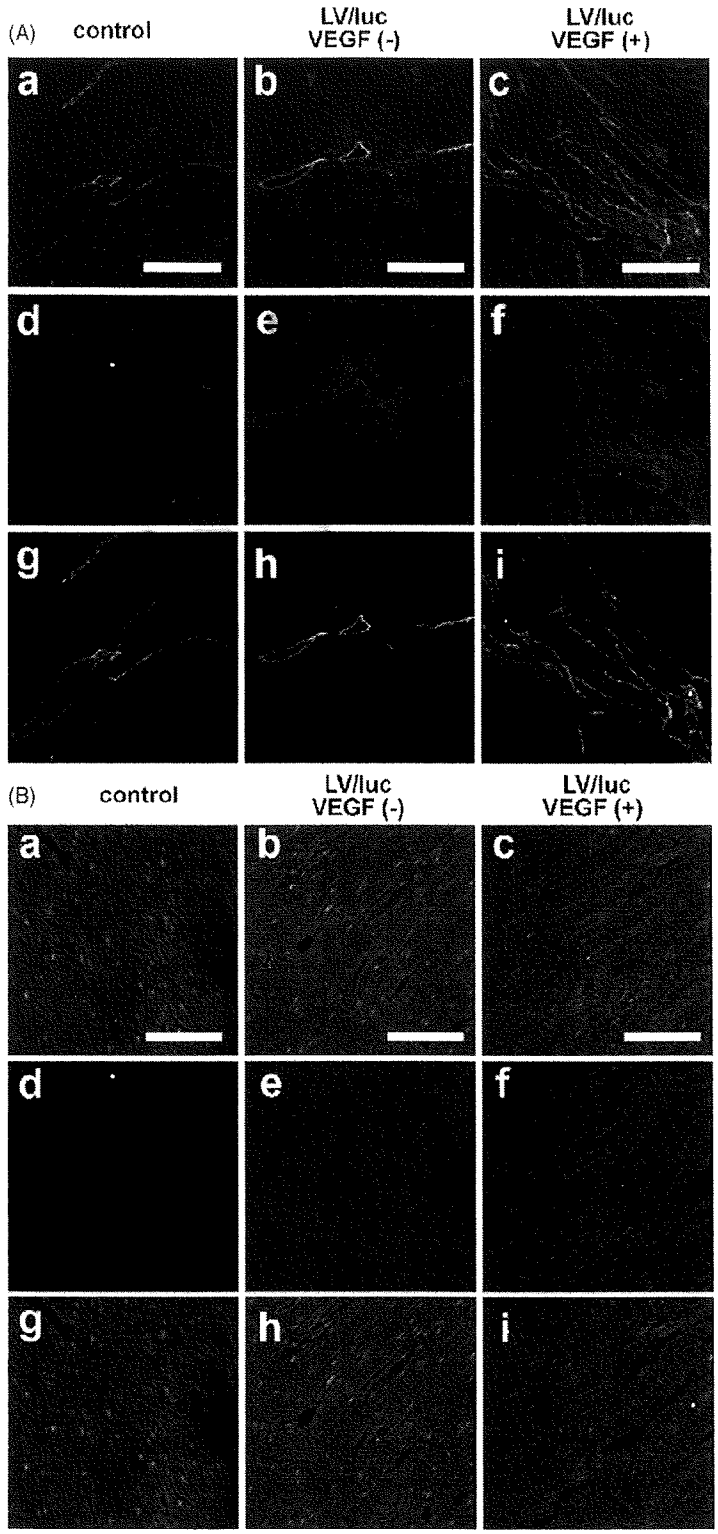

Figure 4 Identification of the cell types that were transduced by LV/luc in the organs. Tissue sections were dually immunostained with an anti-luciferase antibody (shown in red in each panel) and the specific marker of each tissue (shown in green in each panel). Sections of the heart were counter-immunostained using either an antibody to (A) vascular endothelial cell marker, CD31 or (B) myocardial cell marker, GATA4, respectively. Sections of the brains were also counter-immunostained using either an antibody to (C) doublecortin or (D) GFAP, respectively. Images are representative of sections from multiple mice in each group. In (C) and (D), the panels show only merged images. Bar: 100 μm.

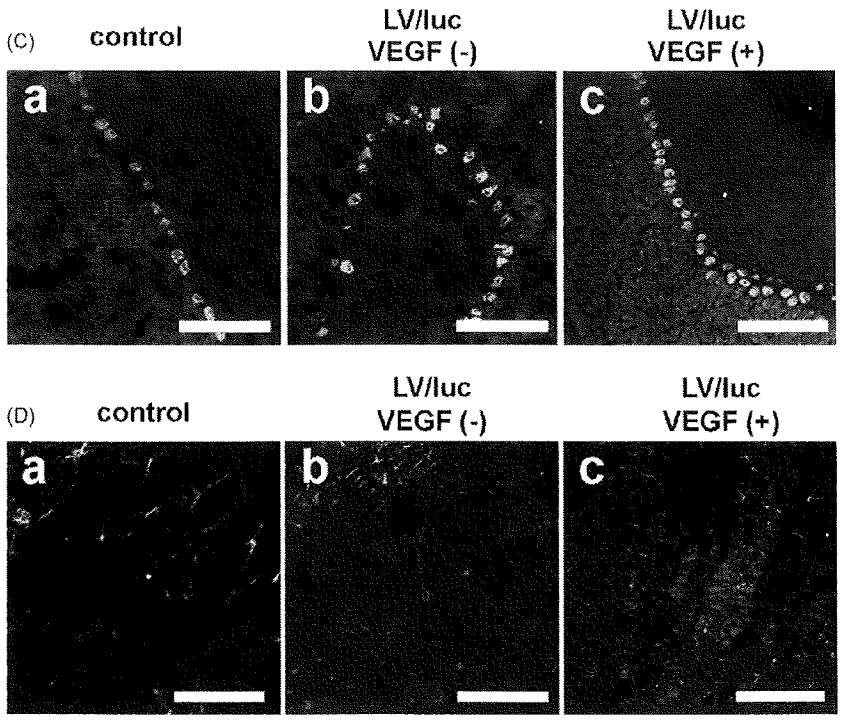

Figure 4 (*Continued*).

ply of the oxygen in each organ highly depends on the vasculature of the tissue. Therefore, the intensity and accuracy with which the luminescent signal could be measured varied among the organs. For instance, it was surprising that little difference in organ luciferase activity was seen in the lungs of VEGF (+) mice compared to that of VEGF (−) mice since the macroscopic observation obtained from the *ex vivo* imaging showed a larger area of luminescence in the VEGF (+) mice than in the VEGF (−) mice. However, the areas of the lungs that were transduced by LV/luc in VEGF (−) mice have a higher intensity signal than the areas transduced in the VEGF (+) mice. These observations may be due to the highly vascularized nature of the lung that allowed a more diffuse distribution of the virus after administration of VEGF, whereas in the VEGF (−) mice the virus appears to have remained concentrated in a smaller area. In the kidney, for example, only small concentrated areas exhibiting luminescence were observed in the VEGF (+) mice whereas no significant difference in organ luciferase activity between both groups was observed. Since random sections were used for the assay, the actual areas transduced by LV/luc may have been inadvertently excluded from the analyses.

Despite the differences in sensitivity of the two methods of transgene detection used in this study, it is clear that pre-administration of VEGF increases the efficiency of treatment with recombinant LVs. The advantages of our experimental approach are: (1) increased persistence in key organs such as the brain and heart; (2) more broad distribution; and (3) early intervention. This protocol has potential therapeutic benefit for the treatment of diseases such as mucopolysaccharidosis type I [35], polyglutamine disorders [36], and other LSDs. Preliminary results from our immunohistochemical analyses show that neuronal cells of the brain and myocardial cells in the heart are transduced by LV/luc and provide evidence that the VEGF pretreatment enhances the efficiency of gene transfer. Future studies will include the staining of tissues from other organs to determine the cell types transduced and the pattern of transduction in all organs. To determine the effect of VEGF on virus delivery to the liver and spleen, similar studies should be performed using reduced amount of virus to allow for the detection of differences between groups in these organs. In addition, testing in a relevant disease model such as Fabry mice will facilitate the evaluation of phenotypical correction and immunological responses to the therapeutic enzyme. It also remains to be determined whether vascular permeability in humans at an analogous stage of development of the mouse is increased by the administration of VEGF and would as such, be affected in the same way by VEGF treatment and viral administration.

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