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AZT誘発ミトコンドリア機能障害に対する分子治療方法の開発

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厚生労働科学研究費補助金（エイズ対策研究事業）
（総括）研究報告書

AZT 誘発ミトコンドリア機能障害に対する分子治療方法の開発

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研究要旨

ヌクレオシド系抗 HIV 薬であるアジドチミジン(AZT)は、HIV に対する有効な作用を発揮する反面、重篤な心筋ミオパチーを誘発するが、その分子機構は不明な点が多い。昨年度までに、私たちは AZT を効率よくその活性化体に変換する酵素チミジル酸キナーゼ(tmpk)の変異型を用いて、AZT 代謝物の培養心筋細胞に対する効果を検討を行い、AZT 代謝物のうち活性化代謝物 AZT3 リン酸が強いミトコンドリア機能障害を誘発することを見いだした。本年度は、AZT3 リン酸が誘発するミトコンドリア機能障害を防止できる薬剤をスクリーニングし、その結果低濃度のシクロスポリン A が有効であるということが明らかとなった。

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A. 研究目的

抗 HIV 治療法として用いられている HAART による長期的副作用の内、AZT をはじめとする NRTI によるミトコンドリア機能障害による重篤な心筋ミオパチー等が、問題とされているが、その原因(分子機構)は未だ明らかとなっていない。本研究では、AZT 代謝物が誘発するミトコンドリア機能障害の詳細な分子機構の詳細な解析から、ミトコンドリア機能障害を誘発する責任分子(群)すなわち AZT 代謝物が標的とする分子(群)を同定することを目的とする。さらにこの副作用を持たない新規薬物、あるいは、副作用に対する保護作用を示す薬物をスクリーニングすることにより、HAART 治療における AZT 誘発ミトコンドリア機能不全症を防ぐ分子標的薬の開

発を目指す。

B. 研究方法

ラット心筋由来細胞 H9c2 に AZT 代謝に関与するチミジル酸キナーゼ(thymidylate kinase: tmpk) cDNA を導入したものをを用いた。(1) 上記細胞の AZT および類薬感受性を調べるために種々の濃度の薬物存在下で 4 日間培養後、細胞の生存率を MTT 法により定量した。(2) AZT (100 μ M)が誘発するミトコンドリア機能不全を介する細胞死に対する保護作用をもつ可能性のある薬剤として Cyclosporin A (CsA)を 0.01 μ M \sim 1 μ M の終濃度で添加し、一定時間細胞を培養後、細胞内 ATP 量をルシフェラーゼ法により定量した。(3) 細胞内に蓄積する AZT 代謝物に関する HPLC を用いる昨年度の検討において、tmpk cDNA を導入した細胞では、AZT-3 リン酸が蓄積していたが、その前段階である AZT-2 リン酸もまた蓄積していた。AZT を細胞に添加すると両者が細胞内に蓄積し、細胞死を誘導する。このどちらがより強い細胞死

誘導活性を持つかを検討するために、AZT-2リン酸からAZT-3リン酸への変換過程を担うヌクレオシド二リン酸キナーゼ(nucleoside diphosphate kinase: NDK)に対するRNAi効果をもつベクターを導入し、主にAZT-2リン酸が蓄積すると予測される細胞系（以後NDK/RNAi）を作製した。従来のtmpk-cDNA導入細胞系（以後、Tmpk：主にAZT-3リン酸が蓄積）、今回作製したNDK/RNAi、あるいは遺伝子導入していない細胞（親株：主にAZT-1リン酸が蓄積）を用い、200 μ M AZT存在下で一定時間培養を行い、アポトーシス誘導率（アネキシンVを用いて測定）および細胞の生存率（MTT法）を定量した。

（倫理面への配慮）

本研究において、倫理面において配慮が必要とされる研究は行わない。また、本研究においては、安全対策を必要とするレンチウイルスベクターの使用が含まれているが、申請者らはすでにこのウイルスベクター系を使うことに対する十分な安全対策を施した遺伝子組み換え実験計画を東北大学遺伝子組換え実験安全専門委員会に申請し、承認済みである。この試験計画を試行するにあたり、試験に使用した大腸菌、細胞および組換えDNA分子は、オートクレーブ等により不活化して廃棄した。

C. 研究結果

(1) AZTおよび類薬による細胞死誘導効果の比較

AZTおよび類薬としてd4T, ddC, ddI, Lamivudineを種々の濃度で使用した。4日間薬剤の存在する状態で培養後の、細胞の生存率測定を行った。その結果、Tmpk遺伝子導入細胞に対する細胞死誘導効果は、AZTがもっとも強く、d4T, Lamivudine, ddI, ddCの順であった。

(2) AZT誘発細胞死のCsAによる保護効果の検討

今回使用した濃度のCsA自身によるミトコンドリア機能障害による細胞のATP含量の低下効果は、観察されなかった。次に100 μ M AZT存在下で種々の濃度のCsAを共存させ4日間培養を行い、その後細胞のATP含量をルシフェラーゼ法で測定した。その結果、CsAが存在しない状態では、細胞のATP含量がコントロールに比して40%減少したのに対し、0.01 μ MのCsA濃度では、それが25%の減少に止まった。それ以上の用量のCsAでは、細胞のATP含量はCsA 0.01 μ M用いたものよりも若干減少するが、CsAを用いない対照群と比して有意に細胞のATP含量は亢進した。

(3) 3種類のAZT代謝物の細胞死誘導活性の比較

NDKに対するRNAi効果をもつベクターを導入した細胞においては、NDKのタンパク質発現レベルは対照群に比して、80%程度に減少した。次に、親株、Tmpk、およびNDK/RNAi用いて、200 μ M AZT存在下、2日間培養後、細胞の生存率をMTT法で定量した。その結果、親株では共存するAZTにより細胞の生存率がAZTを添加しない対照に比して12%減少し、Tmpkでは、41%、NDK/RNAiでは53%、それぞれ細胞の生存率が低下した。また、アポトーシス誘導率については、親株では共存するAZTにより対照に比して1.9倍、Tmpkでは、2.6倍、NDK/RNAiでは、5.5倍増加する結果となった。

D. 考察

Tmpk-cDNA導入細胞をAZTで処置するとミトコンドリアの機能障害が誘発され、ミトコンドリア内膜の膜電位消失、アポトーシスの進行が起きることは既に確認している。今回の研究では、ミトコンドリア内膜の膜電位形成に重要な役割をもつ mitochondrial permeability transition pore (mPTP)の開口

抑制作用をもつ CsA を用いた。CsA は、mPTP の開口を抑制することで、ミトコンドリア内膜の膜電位消失を抑制し、その結果、AZT 誘発細胞死の抑制を行うことができるのではないかと考えて、研究を行った。CsA は、通常免疫抑制剤として使用されるが、今回用いた CsA の濃度は免疫抑制剤として使用する濃度の 1/1000 程度である。今回検討した CsA 濃度においては、AZT 誘発ミトコンドリア機能障害による細胞の ATP 含量低下を効果的に抑制することができた。

次に AZT が代謝されて生ずる各代謝物（1リン酸体、2リン酸体、3リン酸体）が細胞に与える影響を詳細に検討するために、遺伝子導入していない細胞（親株）、tmpk-cDNA 導入細胞、および tmpk-cDNA 導入細胞にさらに NDK に対する RNAi 効果をもつベクターを導入した細胞を用い、AZT 処理後の細胞の生存率などの検討を行った。AZT 代謝物の細胞毒性としては、従来 AZT-1 リン酸が持つことが知られていた。本研究で Tmpk cDNA を発現する細胞を構築したことで、AZT-3 リン酸を効率よく生ずる系を構築し、昨年度までに AZT-3 リン酸の方が AZT-1 リン酸よりも細胞毒性が強いことを明らかにしてきたが、AZT-2 リン酸については、不明であったので、tmpk-cDNA 導入細胞にさらに NDK に対する RNAi 効果をもつベクターを導入した細胞を作製し用いた。これらの細胞群を用いた検討の結果、細胞内に蓄積する AZT 代謝物の中で AZT-2 リン酸が AZT-3 リン酸よりも細胞毒性が高いということを明らかにした。しかしながら、細胞の中に蓄積している代謝物の HPLC による詳細な分析は現在進行中であり、tmpk-cDNA 導入細胞にさらに NDK に対する RNAi 効果をもつベクターを導入した細胞において AZT の主代謝物として蓄積してい

る物質の同定を行う必要がある。

E. 結論

本研究の検討結果から、AZT 代謝物が誘発するミトコンドリア機能障害は、従来原因物質として考えられていた AZT-1 リン酸ではなく、活性化体もしくは活性化中間体の AZT-3 リン酸あるいは AZT-2 リン酸であることを明らかにした。さらに AZT 誘発ミトコンドリア機能障害を低濃度の CsA が抑制できることを明らかにした。今後、これらの知見を元に NRTI 誘発心筋ミオパチーの詳細な分子機構の検討および、その研究成果に基づく分子標的予防方法についての開発を進めていく必要がある。

F. 健康危険情報

特になし

G. 研究発表

1. 論文発表

Sato T., RamsuBir, S., Higuchi, K., Yanagisawa, T., and Medin, JA. Vascular endothelial growth factor broadens lentivector distribution in the heart after neonatal injection. *J. Cardiol.* 54: 245-254, 2009.

2. 学会発表

国内

Kinoshita, K., Takayanagi, S., Sato, T., Maeda, K., Haraguchi, M., Yanai, K., Fukunaga, K., Yanagisawa, T., and Sukegawa, J. Cell surface expression of histamine H3 receptor is regulated by proteins interacting with the carboxy-terminus of the receptor. 10th MOVD 2009. June 1-3, 2009, Sendai, Japan.

Sato, T., Neschadim, A., Medin, JA. and Yanagisawa, T. Gap junction-mediated bystander killing highlights the utility of the TmpkF105Y/AZT system for cancer suicide gene therapy. 第15回日本遺伝子治療学会年会. July 9-11, 2009, 大阪

佐藤岳哉、助川淳、柳澤輝行 新規遺伝子治療法 tmpk/AZT 系の抗ガン治療法としての有効性の検証 (In vivo Bystander 効果の確認)、第60回日本薬理学会北部会 September 26, 2009, 富山

木下和樹、高柳詩織、前田恵、原口満也、佐藤岳哉、谷内一彦、福永浩司、柳澤輝行、助川淳

ヒスタミン H3 受容体に結合する細胞内タンパク質の機能解析、第60回日本薬理学会北部会 September 26, 2009, 富山

木下和樹、廣田佳那、佐藤岳哉、前田恵、原口満也、福永浩司、柳澤輝行、助川淳

ヒスタミン H3 受容体に結合する細胞内タンパク質の機能解析、第83回日本薬理学会年会、March 16-18, 2010, 大阪

佐藤岳哉、Shobha Ramsbir, 樋口公嗣、Jeffrey Medin、柳澤輝行

新生マウスに対する VEGF 前投与は遺伝子治療効果を改善する新生マウスに対する VEGF 前投与は遺伝子治療効果を改善する、第83回日本薬理学会年会、March 16-18, 2010, 大阪

H. 知的所有権の出願・取得状況(予定を含む)

1. 特許取得
なし。

2. 実用新案登録
なし。
3. その他
なし。

別紙 4

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書 籍 名	出版社名	出版地	出版年	ページ

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Sato T., Ramsbury, S., Higuchi, K., Yanagisawa, T., and Medin, J. A.	Vascular endothelial growth factor broadens lentivector distribution in the heart after neonatal injection.	J. Cardiol.	54	245-254	2009



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

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Immunologic
techniques;
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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 pCMV Δ R8.91, 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 $50,000 \times g$ 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 VEGF₁₆₄ (R&D Systems, Minneapolis, MN, USA) was administered to 1–3-day-old neonatal mice through the superficial temporal vein in a volume of $100 \mu\text{l}$. Control mice received $100 \mu\text{l}$ of PBS. Concentrated LV (300 ng p24 in $100 \mu\text{l}$ 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 $5 \mu\text{m}$ 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 0.1M 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-gial 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 $1 \times$ Cell Culture Lysis Reagent (Promega Corp., Madison, WI, USA). Lysates were then spun at $12,000 \times g$ 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

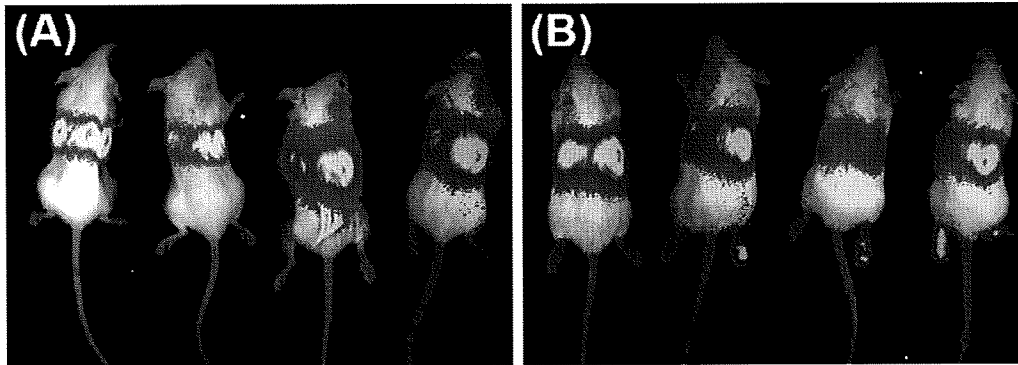


Figure 1 Whole-body luminescence imaging of mice showing long-term luciferase expression. One- to three-day-old neonatal mice were injected with LV/luc in the absence (A) or the presence (B) of VEGF. VEGF was administered 2 h prior to the virus injection via the superficial temporal vein. At 12 weeks 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 \pm 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-

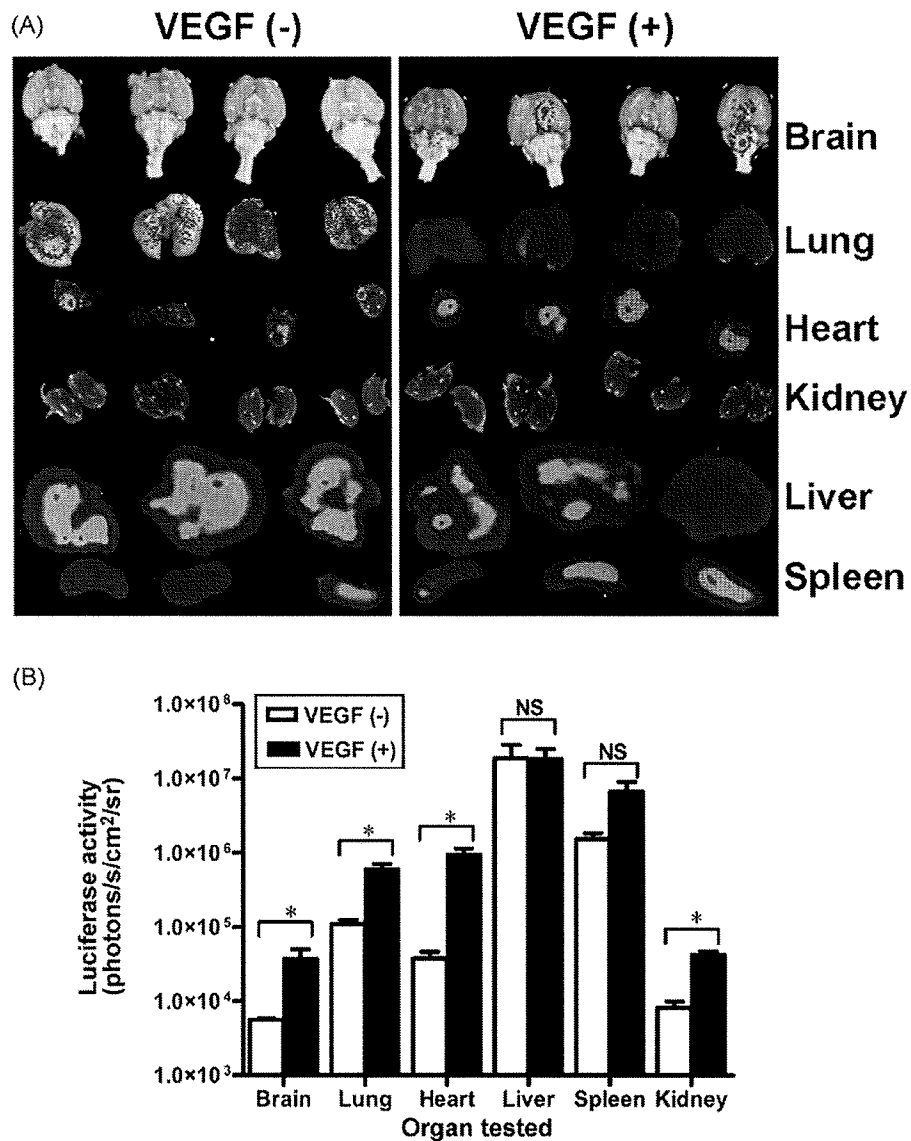


Figure 2 *Ex vivo* imaging of the luciferase expression in the organs at 12 weeks post-virus injection. Following whole-body luminescence imaging, mice were killed and the organs were taken out and imaged. (A) Shown are images of organs from representative mice from each group. (B) The bioluminescent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

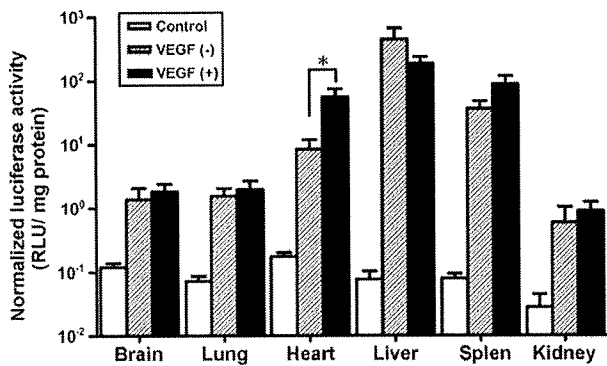


Figure 3 Comparison of the organ luciferase activity. Organs were homogenized and the luciferase activity was measured using a luminometer. Values shown are means \pm SEM ($n=8$ for VEGF (-), $n=7$ for VEGF (+), and $n=4$ for non-treated mice; * $P < 0.05$).

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 permeabil-

ity 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-

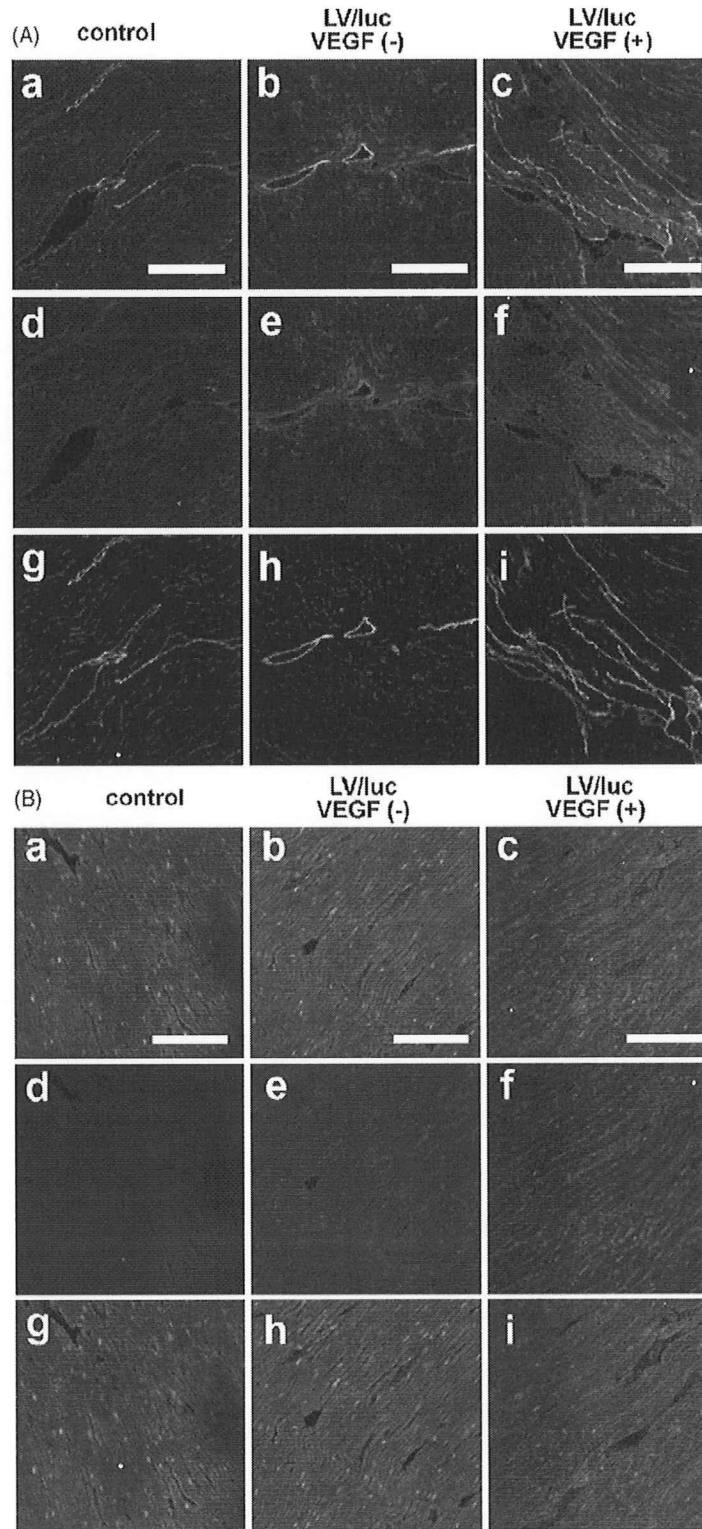


Figure 4 Identification of the cell types that were transduced by LV/luc in the organs. Tissue sections were dually immunostained with an anti-luciferase antibody (shown in red in each panel) and the specific marker of each tissue (shown in green in each panel). Sections of the heart were counter-immunostained 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 μ m.

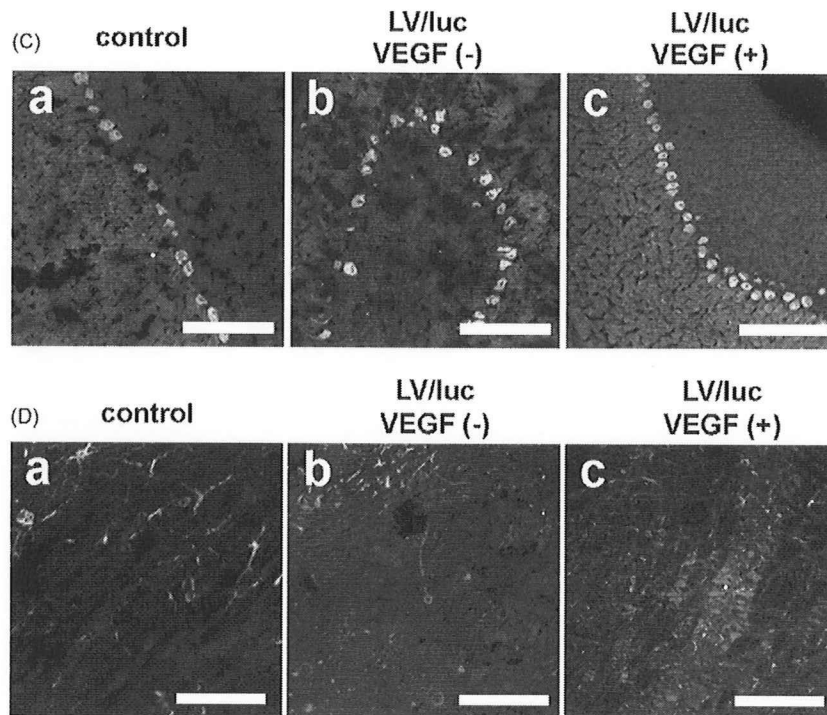


Figure 4 (Continued).

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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References

- [1] Higuchi K, Ayach B, Sato T, Chen M, Devine SP, Rasaiah VI, Dawood F, Yanagisawa T, Tei C, Takenaka T, Liu PP, Medin JA. Direct injection of kit ligand-2 lentivirus improves cardiac repair and rescues mice post-myocardial infarction. *Mol Ther* 2009;17:262–8.
- [2] Boink GJ, Verkerk AO, van Amersfoort SC, Tasseron SJ, van der Rijt R, Bakker D, Linnenbank AC, van der Meulen J, de Bakker JM, Seppen J, Tan HL. Engineering physiologically controlled pacemaker cells with lentiviral HCN4 gene transfer. *J Gene Med* 2008;10:487–97.
- [3] Hurttila H, Koponen JK, Kansanen E, Jyrkkänen HK, Kivelä A, Kylätie R, Ylä-Herttua S, Levonen AL. Oxidative stress-inducible lentiviral vectors for gene therapy. *Gene Ther* 2008;15:1271–9.
- [4] Niwano K, Arai M, Koitabashi N, Watanabe A, Ikeda Y, Miyoshi H, Kurabayashi M. Lentiviral vector-mediated SERCA2 gene transfer protects against heart failure and left ventricular remodeling after myocardial infarction in rats. *Mol Ther* 2008;16:1026–32.
- [5] Yoshimitsu M, Sato T, Tao K, Walia JS, Rasaiah VI, Sleep GT, Murray GJ, Poepl AG, Underwood J, West L, Brady RO, Medin JA. Bioluminescent imaging of a marking transgene and correction of Fabry mice by neonatal injection of recombinant lentiviral vectors. *Proc Natl Acad Sci USA* 2004;101:16909–14.
- [6] Brady RO, Gal AE, Bradley RM, Martensson E, Warshaw AL, Laster L. Enzymatic defect in Fabry's disease. Ceramidetrihexosidase deficiency. *N Engl J Med* 1967;276:1163–7.
- [7] Frustaci A, Chimenti C, Ricci R, Natale L, Russo MA, Pieroni M, Eng CM, Desnick RJ. Improvement in cardiac function in the cardiac variant of Fabry's disease with galactose-infusion therapy. *N Engl J Med* 2001;345:25–32.
- [8] Sheth KJ, Roth DA, Adams MB. Early renal failure in Fabry's disease. *Am J Kidney Dis* 1983;2:651–4.
- [9] Nakao S, Takenaka T, Maeda M, Kodama C, Tanaka A, Tahara M, Yoshida A, Kuriyama M, Hayashibe H, Sakuraba H, Tanaka H. An atypical variant of Fabry's disease in men with left ventricular hypertrophy. *N Engl J Med* 1995;333:288–93.
- [10] Eng CM, Guffon N, Wilcox WR, Germain DP, Lee P, Waldek S, Caplan L, Linthorst GE, Desnick RJ, International Collaborative Fabry Disease Study Group. Safety and efficacy of recombinant human alpha-galactosidase A-replacement therapy in Fabry's disease. *N Engl J Med* 2001;345:9–16.
- [11] Schiffmann R, Kopp JB, Austin 3rd HA, Sabnis S, Moore DF, Weibel T, Balow JE, Brady RO. Enzyme replacement therapy in Fabry disease: a randomized controlled trial. *JAMA* 2001;285:2743–9.
- [12] Weidemann F, Breunig F, Beer M, Sandstede J, Turschner O, Voelker W, Ertl G, Knoll A, Wanner C, Strotmann JM. Improvement of cardiac function during enzyme replacement therapy in patients with Fabry disease: a prospective strain rate imaging study. *Circulation* 2003;108:1299–301.
- [13] Medin JA, Tudor M, Simovitch R, Quirk JM, Jacobson S, Murray GJ, Brady RO. Correction in trans for Fabry disease: expression, secretion and uptake of alpha-galactosidase A in patient-derived cells driven by a high-titer recombinant retroviral vector. *Proc Natl Acad Sci USA* 1996;93:7917–22.
- [14] Qin G, Takenaka T, Telsch K, Kelley L, Howard T, Levade T, Deans R, Howard BH, Malech HL, Brady RO, Medin JA. Preselective gene therapy for Fabry disease. *Proc Natl Acad Sci USA* 2001;98:3428–33.
- [15] Takenaka T, Murray GJ, Qin G, Quirk JM, Ohshima T, Qasba P, Clark K, Kulkarni AB, Brady RO, Medin JA. Long-term enzyme correction and lipid reduction in multiple organs of primary and secondary transplanted Fabry mice receiving transduced bone marrow cells. *Proc Natl Acad Sci USA* 2000;97:7515–20.
- [16] Yoshimitsu M, Higuchi K, Dawood F, Rasaiah VI, Ayach B, Chen M, Liu P, Medin JA. Correction of cardiac abnormalities in fabry mice by direct intraventricular injection of a recombinant lentiviral vector that engineers expression of alpha-galactosidase A. *Circ J* 2006;70:1503–8.
- [17] Yoshimitsu M, Higuchi K, Ramsuvar S, Nonaka T, Rasaiah VI, Siatskas C, Liang SB, Murray GJ, Brady RO, Medin JA. Efficient correction of Fabry mice and patient cells mediated by lentiviral transduction of hematopoietic stem/progenitor cells. *Gene Ther* 2007;14:256–65.
- [18] Bates DO, Lodwick D, Williams B. Vascular endothelial growth factor and microvascular permeability. *Microcirculation* 1999;6:83–96.
- [19] Nagy JA, Vasile E, Feng D, Sundberg C, Brown LF, Detmar MJ, Lawitts JA, Benjamin L, Tan X, Manseau EJ, Dvorak AM, Dvorak HF. Vascular permeability factor/vascular endothelial growth factor induces lymphangiogenesis as well as angiogenesis. *J Exp Med* 2002;196:1497–506.
- [20] Proescholdt MA, Heiss JD, Walbridge S, Mühlhauser J, Capogrossi MC, Oldfield EH, Merrill MJ. Vascular endothelial growth factor (VEGF) modulates vascular permeability and inflammation in rat brain. *J Neuropathol Exp Neurol* 1999;58:613–27.
- [21] Qu H, Nagy JA, Senger DR, Dvorak HF, Dvorak AM. Ultrastructural localization of vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) to the abluminal plasma membrane and vesiculovacuolar organelles of tumor microvascular endothelium. *J Histochem Cytochem* 1995;43:381–9.
- [22] Dvorak HF, Brown LF, Detmar M, Dvorak AM. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am J Pathol* 1995;146:1029–39.
- [23] Young PP, Fantz CR, Sands MS. VEGF disrupts the neonatal blood–brain barrier and increases life span after non-ablative BMT in a murine model of congenital neurodegeneration caused by a lysosomal enzyme deficiency. *Exp Neurol* 2004;188:104–14.
- [24] Sarzotti M. Immunologic tolerance. *Curr Opin Hematol* 1997;4:48–52.
- [25] Ponder KP. Immunology of neonatal gene transfer. *Curr Gene Ther* 2007;7:403–10.
- [26] Waddington SN, Kennea NL, Buckley SM, Gregory LG, Themis M, Coutelle C. Fetal and neonatal gene therapy: benefits and pitfalls. *Gene Ther* 2004;11(Suppl 1):S92–7.
- [27] Baekelandt V, Claeys A, Cherepanov P, De Clercq E, De Strooper B, Nuttin B, Debysse Z. DNA-Dependent protein kinase is not required for efficient lentivirus integration. *J Virol* 2000;74:11278–85.
- [28] Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, Behr JP. A versatile vector for gene and oligonucleotide transfer into cells in culture

- and in vivo: polyethylenimine. *Proc Natl Acad Sci USA* 1995;92:7297–301.
- [29] Sharp TV, Wang HW, Koumi A, Hollyman D, Endo Y, Ye H, Du MQ, Boshoff C. K15 protein of Kaposi's sarcoma-associated herpesvirus is latently expressed and binds to HAX-1, a protein with antiapoptotic function. *J Virol* 2002;76:802–16.
- [30] Linhart A, Lubanda JC, Palecek T, Bultas J, Karetova D, Ledvinova J, Elleder M, Aschermann M. Cardiac manifestations in Fabry disease. *J Inherit Metab Dis* 2001;24(Suppl 2):75–83.
- [31] Shah JS, Hughes DA, Sachdev B, Tome M, Ward D, Lee P, Mehta AB, Elliott PM. Prevalence and clinical significance of cardiac arrhythmia in Anderson-Fabry disease. *Am J Cardiol* 2005;96:842–6.
- [32] Wilcken DE. Overview of inherited metabolic disorders causing cardiovascular disease. *J Inherit Metab Dis* 2003;26:245–57.
- [33] Bates DO, Harper SJ. Regulation of vascular permeability by vascular endothelial growth factors. *Vascul Pharmacol* 2002;39:225–37.
- [34] Carbonaro DA, Jin X, Petersen D, Wang X, Dorey F, Kil KS, Aldrich M, Blackburn MR, Kellems RE, Kohn DB. In vivo transduction by intravenous injection of a lentiviral vector expressing human ADA into neonatal ADA gene knockout mice: a novel form of enzyme replacement therapy for ADA deficiency. *Mol Ther* 2006;13:1110–20.
- [35] Kobayashi H, Carbonaro D, Pepper K, Petersen D, Ge S, Jackson H, Shimada H, Moats R, Kohn DB. Neonatal gene therapy of MPS I mice by intravenous injection of a lentiviral vector. *Mol Ther* 2005;11:776–89.
- [36] Torashima T, Koyama C, Iizuka A, Mitsumura K, Takayama K, Yanagi S, Oue M, Yamaguchi H, Hirai H. Lentivector-mediated rescue from cerebellar ataxia in a mouse model of spinocerebellar ataxia. *EMBO Rep* 2008;9:393–9.

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