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BRIEF COMMUNICATION

Prolonged gene expression in mouse lung endothelial cells following transfection with Epstein–Barr virus-based episomal plasmid

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The development of a strategy to deliver a gene to pulmonary endothelium will be useful for gene function study and for pulmonary gene therapy. Cationic lipidic vectors are efficient in gene transfer to pulmonary endothelium via the vascular route; however, gene expression is transient and lasts for only a few days. In this study, we show that pulmonary gene transfer via cationic lipidic vectors can be significantly improved using an Epstein–Barr virus (EBV)-based expression plasmid. Systemic administration of cationic liposomes followed by the EBV-based plasmid led to gene expression in the lung that lasted for more than 3 weeks. Prolonged and high levels of gene expression can also be obtained in primary mouse lung endothelial cells (MLEC) following lipofection with an EBV-based plasmid. These results suggest the utility of this gene transfer protocol in studying the expression of cloned genes in lung endothelial cells and in pulmonary gene therapy.
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Somatic gene transfer to the pulmonary endothelium may be a useful strategy for modifying the phenotype of endothelium and/or vascular smooth muscle in disorders such as primary pulmonary hypertension, ARDS, or pulmonary metastatic disease. It may also provide a useful research tool to study the function of cloned genes in pulmonary endothelial cells *in vitro* and *in vivo*. Among the viral vectors employed for such potential use,[1,2] adenoviral vectors have proved to be limited with respect to efficiency in part because of difficulty in assuring significant residence time in the lung and/or paucity of receptors for adenovirus on endothelium. Also, adenoviral vectors are only moderately effective in infecting primary mouse lung endothelial cells *in vitro*. First-generation cationic lipidic vectors also produce modest degrees of gene transfer to lung after i.v. administration.[3,4] Modification of cationic lipid composition and DNA:lipid ratios led to considerably higher gene transfer efficiency in the lung with the endothelial cells being the major cell type transfected.[5–10] However, gene expression in the lung via this gene transfer protocol is transient and lasts for only a few days. In the current study, we investigate whether *in vitro* and *in vivo* gene transfer to mouse lung endothelial cells (MLEC) can be significantly improved using an EBV-based expression plasmid.

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EBV plasmid is a replicating episomal vector that has been developed to overcome the problem of rapid elimination of intracellularly delivered plasmid DNA in nonviral vector-mediated gene transfer. The viral elements required for episomal replication and nuclear retention are the *cis*-acting replication origin (oriP) of the EBV gene and the EBV nuclear antigen-1 (EBNA-1), which interacts with the oriP region.[11–13] Plasmids containing the oriP and EBNA-1 sequences are maintained as low-copy number DNA episomes in the cell nucleus and replicate once per cell cycle in primate cells. Most studies suggest that long-term replication of EBV-derived plasmids occurs only in primate cells but not in rodent cells.[11,14] Nonetheless, a more persistent gene expression was found in a number of rodent tissues following nonviral method-mediated delivery of EBV plasmid compared to non-EBV plasmid possibly because of the nuclear retention function and the enhancer activity of transgene expression induced by EBNA-1.[15–18] We hypothesize that lipofection of MLEC can also be significantly improved both *in vitro* and *in vivo* using the EBV-based plasmid.

Figure 1 shows the maps of three different plasmids used in this study. Plasmids pGEG.GL3 and pG.GL3 contain the firefly luciferase gene under the control of a CAG promoter. pGEG.GL3 also contains an EBV EBNA-1 gene driven by a CAG promoter. In addition, pGEG.GL3 contains the EBV oriP. Plasmid pNGVL3-Luc contains the firefly luciferase gene under the control of a cytomegalovirus (CMV) promoter. In the initial experiment, we compared the level of gene expression in the lung with

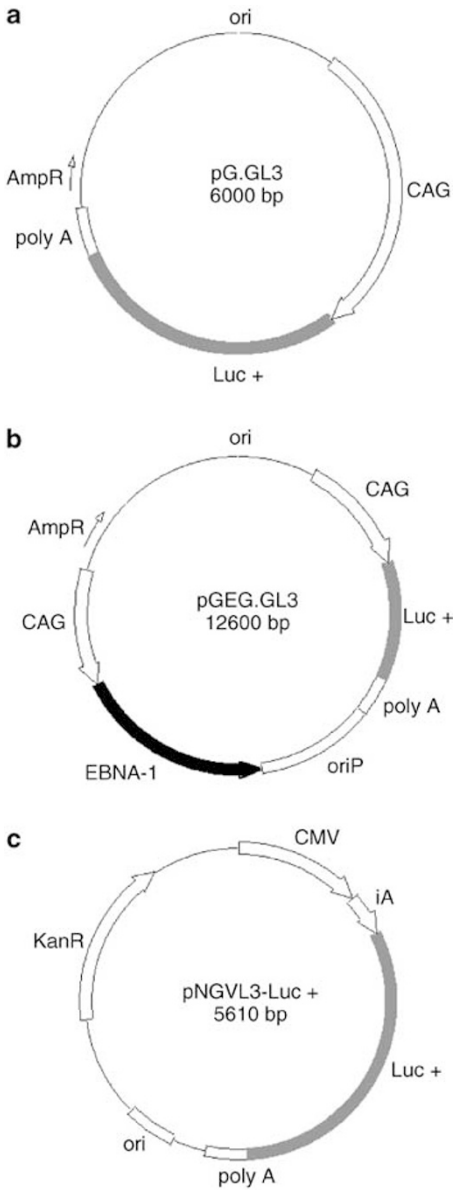

Figure 1 *Plasmid structure: (a) pG.GL3, (b) pGEG.GL3, (c) pNGVL3-Luc. CAG: the chicken β-actin promoter-cytomegalovirus (CMV) enhancer; EBNA-1: Epstein–Barr nuclear antigen-1; oriP: EBV latent origin of replication; Luc: luciferase.*

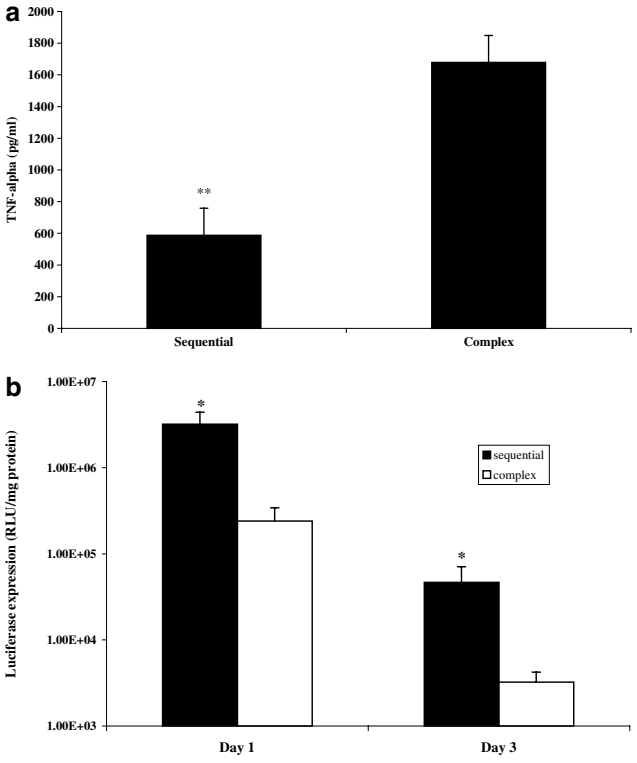

Figure 2 *TNF-α cytokine response (a) and luciferase gene expression in mouse lungs (b) following i.v. injection of cationic liposome/DNA complexes or cationic liposomes followed by plasmid DNA. DOTAP:cholesterol liposome/pGEG.GL3 complexes were prepared at a +/− charge ratio of 6/1 as described[19] and injected into female CD-1 mice (Charles River Laboratories, Wilmington, MA, USA; eight mice in each group) via tail vein at a dose of 35 μg DNA per mouse. In a different group, mice received tail vein injection of DOTAP:cholesterol liposomes (1.3 mmol lipid/mouse) followed by pGEG.GL3 (35 μg/mouse). At 2 h following injection, mice were bled from the retro-orbital sinuses under anesthesia. The blood was allowed to stay at 4°C for 4 h and then centrifuged at 14 000 g for 10 min at 4°C. Serum levels of TNF-α were determined with the specific cytokine immunoassay kit (R&D Systems, Minneapolis, MN, USA). In a separate experiment, groups of eight mice received i.v. injection of DOTAP: cholesterol liposome/pGEG.GL3 complexes or sequential injection of DOTAP:cholesterol liposome followed by pGEG.GL3 as described above. At days 1 and 3 following injection, mice were killed and lungs were removed and homogenized in 1 ml of ice-cold lysis buffer (0.05% Triton X-100, 2 mM EDTA, and 0.1 M Tris, pH 7.8) with a tissue tearer for 20 s at high speed. The homogenates were then centrifuged at 14 000 g for 10 min at 4°C. Of the supernatant 10 μl was analyzed with the luciferase assay system (Promega, Madison, WI, USA) using an automated LB953 luminometer (Berthod, Bad Wildbad, Germany). The protein content of the supernatant was measured with the Bio-Rad protein assay system (Bio-Rad, Hercules, CA, USA). Luciferase activity was expressed as relative lights units (RLU) per milligram of tissue protein. Data were expressed as means ± s.d. and analyzed by the two-tailed unpaired Student's t-test using the PRISM software program (GraphPad Software, San Diego, CA, USA). *P < 0.05; **P < 0.01 (versus complex injection).*

pGEG.GL3 using two different gene transfer protocols, that is, complex injection and sequential injection. With a non-EBV plasmid, we have shown recently that sequential injection of cationic liposomes and plasmid is more efficient in pulmonary gene transfer than injection of cationic liposome/DNA complexes.[19] Furthermore, sequential injection is associated with a significantly reduced proinflammatory cytokine response.[19] A similar result was observed in this study with an EBV-based plasmid. As shown in Figure 2a, serum levels of TNF-α in sequential injection group were only 30% of those in complex injection group. Furthermore, sequential injection of DOTAP:cholesterol liposomes and pGEG.GL3 led to a significantly higher level of gene expression in the lung than complex injection (Figure 2b). We and others have shown that proinflammatory cytokines can significantly inhibit transgene expression in either viral or nonviral vector-mediated gene transfer.[20–23] The improved pulmonary gene transfer via sequential injection protocol is probably because of a decreased inhibitory effect of cytokines on transgene expression. Thus, sequential injection protocol was used in subsequent studies.

Figure 3 shows the luciferase expression in the lungs over time following sequential injection of DOTAP:cholesterol liposomes (+/− charge ratio of 6:1) followed by

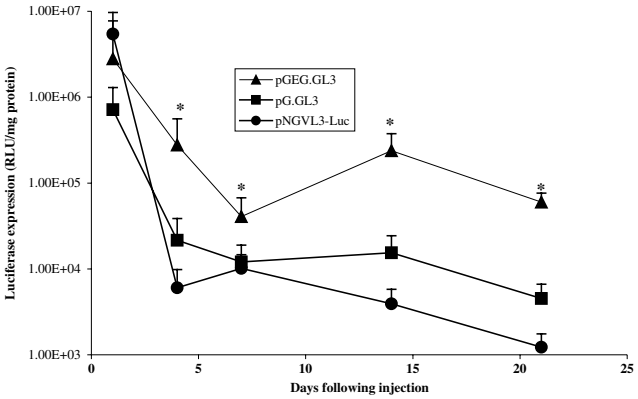

Figure 3 *Prolonged gene expression in mouse lungs following sequential injection of cationic liposomes and an EBV-based plasmid. Groups of eight mice received tail vein injection of DOTAP:cholesterol liposomes (1.3 mmol lipid/mouse) followed by pGEG.GL3, pG.GL3, or pNGVL3-Luc (35 μg/mouse). At different time points, mice were killed and the level of gene expression in the lung was assayed as described in the legend to Figure 2. The level of luciferase activity in lungs of mock-transfeced mice is less than 1000 RLU/mg protein. *P<0.05 (versus pG.GL3).*

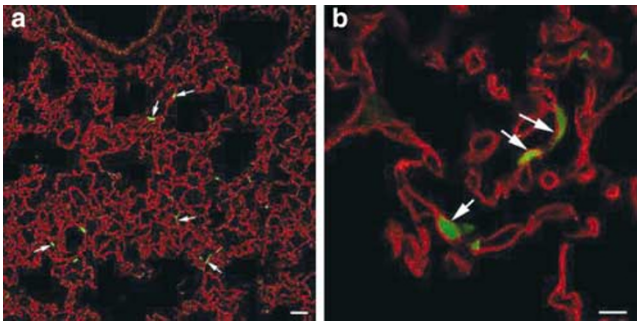

Figure 4 *Immunofluorescence detection of transgene-expressing cells. Mice received tail vein injection of DOTAP:cholesterol liposomes followed by pGEG.EGFP. After 1 day, mice were killed and lungs were perfused intravascularly with PBS followed by 2% paraformaldehyde in PBS, and inflated with this fixative to near total lung capacity. The lungs were rinsed with cold PBS and immersed in 30% sucrose in PBS at 4°C overnight. The lungs were then quickly frozen in OCT with dry ice. Lung cryo-sections (5 μm) were then cut. Following three washes in PBS containing 0.5% bovine serum albumin and 0.15% glycine (PBG buffer), sections were incubated in a 1:100 dilution of rat anti-mouse PECAM (PharMingen, San Diego, USA) for 1 h at RT, washed with PBG three times, and labeled with Cy3-labeled goat anti-rat IgG (Jackson ImmunoResearch Laboratories) for 1 h at RT. The sections were mounted in Gelvatol (Monsanto, St Louis) and the images were collected using a Leica TCS NT confocal microscope at 1024 × 1024 pixel resolution.*

pGEG.GL3, pG.GL3, or pNGVL3-Luc (35 μg/mouse). Peak expression of luciferase occurred on day 1 for all plasmids. However, of the three plasmids, gene expression in pNGVL3-Luc group declined most rapidly with time and was not different from background by 21 days. Gene expression in the pGEG.GL3 group declined during the first week following sequential injection, but then there was a rebound in gene expression over the subsequent 2 weeks. A similar phenomenon was observed in a study with complex injection.[24] The rapid decline in gene expression in the first few days following injection is probably largely because of the inhibitory effect of cytokines. Despite a decreased cytokine response in sequential injection (Figure 2a), the level of TNF-α in the serum might be sufficient to cause significant inhibition on transgene expression. Nevertheless, the levels of gene expression in pGEG.GL3 group were significantly higher than those in pG.GL3 or pNGVL3-Luc group at all time points examined. Of note is that even after 3 weeks, there is a detectable level of gene expression in lungs of mice injected with pGEG.GL3.

Having characterized pulmonary gene transfer with pGEG.GL3, the sequential injection protocol was further evaluated using pGEG.EGFP as a reporter gene. No green cells were observed in the lungs of mice treated with a control plasmid (pGEG.GL3) (data not shown). In contrast, there was localized gene expression throughout the distal lung of mice that received pGEG.EGFP (Figure 4a). The cell type of transfected cells was further analyzed by anti-platelet endothelial cell adhesion molecule-1 (PECAM-1) immunofluorescence staining of the lung sections. Constitutive expression of PECAM-1 is a fundamental characteristic of endothelial cells.[25] As shown in Figure 4a and b, EGFP signal was substantially colocalized with PECAM labeling, confirming that endothelial cells were the major cell type transfected.

We then examined whether primary MLEC can also be efficiently transfected via EBV-based plasmid. Primary MLEC were prepared by modification of an immunobead protocol.[26] Briefly, mouse lungs were finely minced and digested in collagenase (Type I, 100 μg/ml). Cell suspensions were incubated with a monoclonal antibody (rat anti-mouse) to PECAM-1 (BD Pharmingen, San Diego, CA, USA) for 30 min at 4°C. PECAM-1 has been used extensively as a reliable marker to isolate endothelial cells.[26] The cells were washed twice with buffer to remove unbound antibody, and resuspended in binding buffer containing the appropriate number of washed magnetic beads coated with sheep anti-rat IgG (Dynal, Oslo, Norway). Attached cells were washed four to five times in cell culture medium, and then were digested with trypsin/EDTA to detach the beads. Bead-free cells were centrifuged and resuspended for culture. At approximately passage 2, cells were incubated with fluorescent-labeled di-acetylated LDL (diI-LDL), which is taken up only by endothelial cells and macrophages, and sorted to homogeneity by FACS. The enriched PECAM and diI-LDL population were subcultured in DMEM/F-12, 20% FBS, 6% plasma-derived human serum, 2 mM glutamine, and 30 μg/ml endothelial-derived growth factor. Figure 5 shows gene expression in MLEC following transfection with pGEG.GL3, pG.GL3, or pNGVL3-Luc. More persistent and significantly higher level gene expression was achieved with pGEG.GL3. It should be noted that transfection of MLEC in this study was performed using a nonoptimized reagent (DOTAP:cholesterol liposomes). Gene expression in MLEC should be further improved via optimization of transfection condition. This will greatly facilitate the use of this gene transfer protocol as a research tool to study the functions of cloned genes in MLEC *in vitro*. EBV plasmid coupled with cationic liposome and HVJ-liposome has also been shown to efficiently transfect human primary cells *in vitro*.[27]

One of the potential concerns over the use of EBV plasmid is the oncogenicity of EBNA-1 protein. Transgenic mice harboring the EBNA-1 gene driven by B lymphocyte-specific enhancer developed B-cell lymphoma.[28] However, several studies have shown that EBNA-1

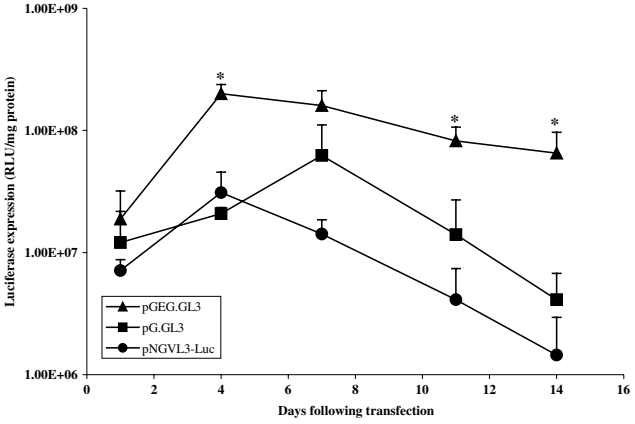

Figure 5 *Prolonged gene expression in primary lung endothelial cells following lipofection with an EBV-based plasmid. Primary MLEC were prepared as described in the text. Cells of density 1 × 10⁵ cells/well in a 48-well plate were transfected with 1.5 μg of plasmid (pGEG.GL3, pG.GL3, or pNGVL3-Luc) complexed to DOTAP:cholesterol liposomes at a +/− charge ratio of 2:1 in serum-free medium. At 4 h later, the transfection medium was removed and replaced with complete medium. At days 1, 4, 7, and 11 following transfection, the cells were lysed using ice-cold lysis buffer (0.05% Triton X-100, 2 mM EDTA, and 0.1 M Tris, pH 7.8), the homogenate centrifuged at 14 000 g for 10 min at 4°C and the supernatant analyzed for luciferase expression. *P < 0.05 (versus pG.GL3).*

itself is insufficient for B lymphocyte transformation *in vitro* in the absence of the latent viral properties EBNA-2,[29] EBNA-3A,[30] EBNA-3C,[30] and LMP-1.[31] Furthermore, 293 cells stably transfected with EBNA-1 did not grow in soft-agar plates and were sensitive to serum depletion.[32] It is unlikely that the current gene expression system will induce tumor in the host. Further studies are required to address the safety of long-term gene therapy using EBV plasmid.

In summary, we have shown that systemic administration of cationic liposomes followed by an EBV plasmid led to a prolonged gene expression in the lung, which lasted for more than 3 weeks. An efficient transfection of primary MLEC can also be achieved using EBV plasmid. These results suggest the utility of this gene transfer protocol in studying the expression of cloned genes in lung endothelial cells and in pulmonary gene therapy.

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