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ORIGINAL ARTICLE

Coxsackievirus B3 used as a gene therapy vector to express functional FGF2

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Current gene therapies are predominantly based on a handful of viral vectors. The limited choice of delivery vectors has been one of the stumbling blocks to the advancement of gene therapy. Therefore, the development of novel recombinant vectors should facilitate the application of gene therapies. In this study, we examined coxsackievirus B3 (CVB3) as a novel recombinant vector for the delivery and expression of a foreign gene *in vitro* and *in vivo*. A recombinant CVB3 complementary DNA was constructed by inserting a gene encoding human fibroblast growth factor 2 (FGF2). The recombinant virus (CVB3–*FGF2*) efficiently expressed FGF2 in HeLa cells and human cardiomyocytes *in vitro* and in mouse hindlimbs *in vivo*. The injection of the recombinant virus into mice with ischemic hindlimbs protected the hindlimbs from ischemic necrosis. CVB3–*FGF2* injection significantly improved the blood flow in the ischemic limbs for over 3 weeks compared with that in the phosphate-buffered saline- or CVB3-injected controls, suggesting that FGF2 expressed from CVB3–*FGF2* is functional and therapeutically effective. The virulence of CVB3 was also drastically attenuated in the recombinant virus. Thus, CVB3 can be modified to express a functional foreign protein, supporting its use as a novel viral vector for gene therapy.

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Keywords: coxsackievirus B3; viral vector; human fibroblast growth factor 2

INTRODUCTION

Gene therapy is a method of treating disease by introducing a functional gene into cells that have an abnormal genome, including the presence of a pathological gene or the absence of a normal functional gene. A number of diseases, including hereditary diseases and some cancers, are caused by an abnormal genome, so gene therapy is an attractive treatment modality.[1,2] The first clinical gene therapy was administered in 1990, with great anticipation and hope that incurable diseases could be treated.[3] Since then, gene therapy has been investigated for the treatment of a number of diseases,[4-6] although several adverse events have occurred during its application.[7,8] Because most of these events are caused by insertional mutagenesis when the viral vector integrates into a host chromosome, the development of novel nonintegrating vectors has become an important goal.

For successful gene therapy, a gene delivery method must be selected that is suitable for the specific purpose of each treatment. Gene-delivery techniques can be divided into two categories, viral and nonviral. During viral gene delivery, the gene to be delivered is carried into abnormal cells and tissues by a virus. The viruses predominantly used for gene delivery include retroviruses, lentiviruses, adenoviruses and adeno-associated viruses.[9] In contrast, nonviral gene delivery can be achieved with physical methods, such as microinjection, gene gun, electroporation, impalefection, hydrostatic pressure, continuous infusion and sonication, or chemical methods such as lipofection.[10] Although nonviral delivery may have some advantages, the efficacy of this system is too low for clinical use. Therefore, most clinical applications of gene therapy have been based on viral delivery systems.[11]

Coxsackievirus B3 (CVB3) is a nonenveloped RNA virus with a 7400 bp, single-stranded positive-sense RNA genome and belongs to the genus *Enterovirus* of the family *Picornaviridae*.[12] The genome of CVB3 encodes a single open reading frame, which produces four structural viral proteins (VP1–4) and seven nonstructural viral proteins ($2A^{pro}$, 2B, 2C, 3A, 3B, $3C^{pro}$ and $3D^{pol}$). This viral genome is also flanked by a 5′-NTR (5′ nontranslated region) and a polyadenylated 3′-NTR.[13] CVB3 enters its target cells through the coxsackievirus and adenovirus receptor (the main receptor) and the membrane protein decay accelerating factor (a coreceptor).[14,15] Recently, several studies have reported that CVB3 can be used as a vector to express foreign genes, such as hepatitis C virus E2 and some cytokines.[16-18] However, no recombinant CVB3 vector has yet been used in a disease model. Therefore, whether CVB3 can be used in clinical gene therapy must be confirmed.

We constructed a recombinant CVB3 expressing a gene encoding human fibroblast growth factor 2 (FGF2) as the model protein, and tested whether the FGF2 expressed from the recombinant virus was biologically functional in a model of ischemic disease. We confirmed that FGF2 was expressed by CVB3–*FGF2* in cell cultures and in mouse hindlimbs. The injection of CVB3–*FGF2* improved the blood flow in ischemic hindlimbs and protected them from ischemic necrosis. In contrast to wild-type CVB3, which caused inflammation in the heart and the subsequent death of the injected mice, the recombinant virus induced no inflammation in the animals. Taken together, these results suggest that CVB3 can be used as an effective viral delivery vector for gene therapy.

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RESULTS

Construction of recombinant coxsackievirus expressing FGF2 (CVB3–*FGF2*)

Construction and characterization of recombinant CVB3. To determine whether CVB3 can be used as a viral vector for gene therapy, we constructed a recombinant CVB3 encoding FGF2, designated CVB3–*FGF2*, using an infectious complementary DNA (cDNA) of CVB3 to express the model protein in this study, as described in Materials and methods (Figure 1a). The infectious recombinant virus was isolated from the supernatant of COS-7 cells transfected with the viral cDNA containing the *FGF2* gene. To check the one-step growth rate of CVB3–*FGF2*, we infected HeLa cells with CVB3 as a control or with the recombinant virus (multiplicity of infectivity of 10) and collected the supernatants at the indicated times. CVB3–*FGF2* showed a growth rate almost identical to that of CVB3 (Figure 1b).

FGF2 expressed by CVB3-*FGF2 in vitro*

FGF2 expression in vitro. To confirm that FGF2 was actually expressed from CVB3–*FGF2 in vitro*, HeLa cells and human cardiomyocytes (hCMs) were infected with CVB3 or CVB3–*FGF2*. In western blotting and immunofluorescence assay analyses, FGF2 protein was only detected in the CVB3–*FGF2*-infected HeLa cells and hCMs (Figures 2a and b). We used an enzyme-linked immunosorbent assay (ELISA) to confirm that FGF2 was released from the recombinant-virus-infected hCMs into the cell supernatant. The assay showed that FGF2 was approximately 10-fold higher in the supernatant of hCMs infected with CVB3–*FGF2* than in that of CVB3-infected hCMs (Figure 2c). Together, these results show that CVB3–*FGF2* expressed FGF2 *in vitro* and that the foreign protein expressed by CVB3 was effectively released into the extracellular environment from the recombinant-virus-infected cells.

Attenuation of CVB3–*FGF2* virulence in mice

Safety of CVB3–FGF2. If CVB3–*FGF2* is to be used as an agent for gene therapy, it must produce no adverse effects *in vivo*. To check this, BALB/c mice were injected with CVB3 or CVB3–*FGF2* (1×10^6 plaque-forming units per mouse) *via* an intraperitoneal route, and the survival rates of these mice were determined. Whereas all the mice injected with CVB3 died within 7 days post infection (pi), the mice injected with the recombinant virus showed no mortality (Supplementary Figure 1a). This phenomenon was consistent with the histological data for BALB/c mice administered viruses intramuscularly insofar as a severe inflammatory pattern was observed at 7 days pi in the hearts and pancreases of mice injected with CVB3, whereas the CVB3–*FGF2*-injected mice showed no cardiac and only slight pancreatic symptoms (Supplementary Figure 1c). Mice were also injected intramuscularly with 1×10^6 plaque-forming unit of CVB3 or CVB3–*FGF2* to check the viral growth rates in the hearts and pancreases of the injected mice. The viral titers detected at 3 days pi in the hearts and pancreases of the mice injected with the recombinant virus were about 1–2-fold lower on a log scale than those detected in the hearts and pancreases of CVB3-injected mice (Supplementary Figure 1b). Although the *in vivo* growth results (Supplementary Figure 1b) are not consistent with the *in vitro* growth results (Figure 1b), these data show that the virulence of CVB3–*FGF2* was somewhat attenuated, at least *in vivo*. However, if we want a safer viral vector, another strongly attenuated viral backbone is required from which to construct the recombinant virus.

Expression of FGF2 from recombinant virus in mouse hindlimbs

FGF2 expression in vivo. The CVB3–*FGF2* vector constructed in this study was considered applicable to a mouse ischemic disease model. Therefore, we first tested whether CVB3–*FGF2* effectively expressed FGF2 in mouse hindlimbs. As shown in Figure 3a, the

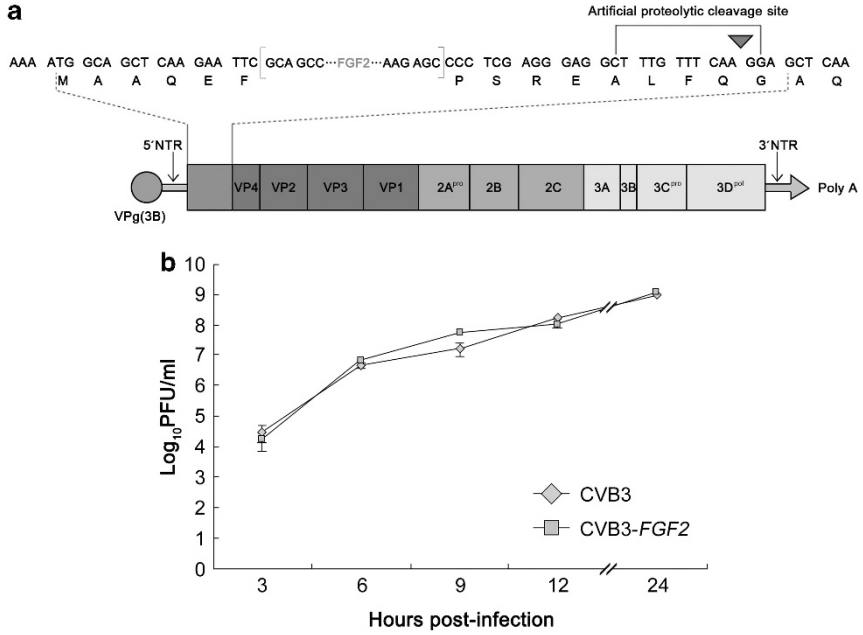

Figure 1. Construction of recombinant coxsackievirus expressing FGF2. (**a**) The red box on the left of viral protein 4 (*VP4*) represents *FGF2*. The additional sequences located 5′ to *FGF2* indicate the translation start codon (ATG) and the restriction site (GGATCC) for *EcoR*I. The sequences 3′ to *FGF2* represent the restriction site (CTCGAG) for *Xho*I and an artificial proteolytic cleavage site (encoding ALFQG) for the coxsackieviral protease $3C^{pro}$. The inverted triangle indicates the site cut by $3C^{pro}$. (**b**) The growth rate of CVB3–*FGF2*. HeLa cells were infected with CVB3 or CVB3–*FGF2* at a multiplicity of infectivity of 10. After infection for 1 h, the supernatants of the infected cells were collected at the indicated times and analyzed for the one-step growth of the viruses using a plaque assay (described in detail in the Materials and Methods). The data presented are the means ± s.d. of triplicate determinations. The color reproduction of this figure is available at the *Gene Therapy* online.

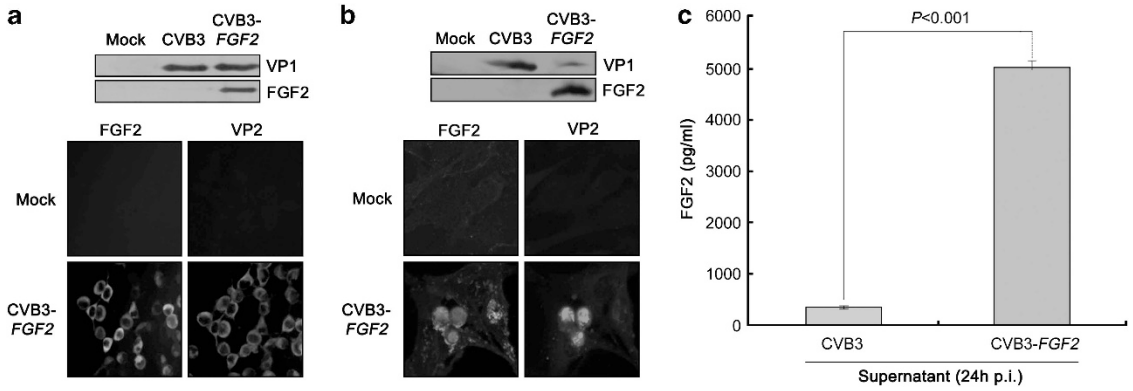

Figure 2. The expression of FGF2 from CVB3-*FGF2 in vitro*. (**a**, **b**) FGF2 expressed from CVB3-*FGF2* was detected in recombinant-virus-infected HeLa cells (multiplicity of infectivity = 10 for 8 h; (**a**) and hCMs (multiplicity of infectivity = 200 for 24 h; (**b**) using western blotting and immunofluorescence assay. On an immunoblot, α-VP1 and α-FGF antibodies were used to detect the capsid protein VP1 of CVB3 and FGF2, respectively. The α-VP2 antibody used in the immunofluorescence assay was directed against the capsid protein VP2 of CVB3.[19,20] The images obtained with immunofluorescence assay are magnified × 200. 'Mock' indicates no infected cells. (**c**) FGF2 expressed from CVB3-*FGF2* was detected in the supernatant of hCMs infected with CVB3-*FGF2* (multiplicity of infectivity = 200). After infection for 24 h, the supernatants of hCMs infected with CVB3 or CVB3-*FGF2* were harvested and the amount of FGF2 was measured with an ELISA kit for human FGF.

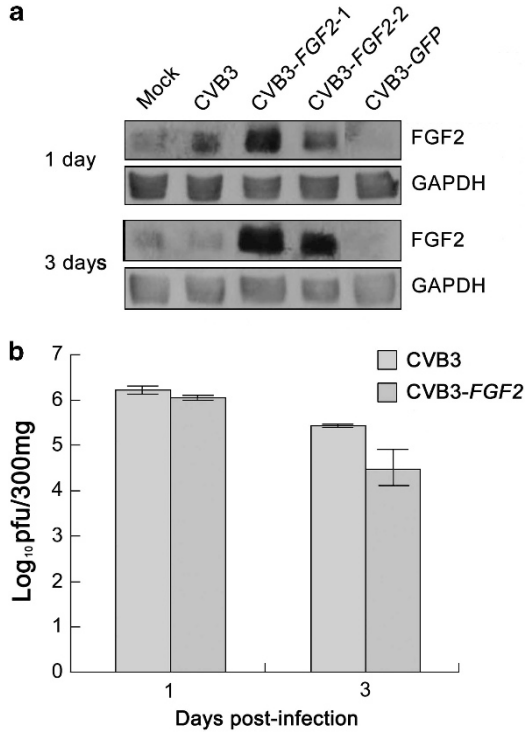

Figure 3. Expression of FGF2 in BALB/c mouse hindlimbs after CVB3-*FGF2* injection. (**a**) Expression of FGF2 in mouse hindlimbs. CVB3, CVB3-*GFP* or CVB3-*FGF2* (1×10^6 plaque-forming unit per mouse, respectively) were injected into each mouse hindlimb ($n = 3$ / group) via an intramuscular route and FGF2 was detected by western blotting with anti-FGF2 antibody on days 1 and 3 pi. (**b**) Viral titers in BALB/c mouse hindlimbs. The mouse hindlimbs ($n = 3$ / group) were injected with CVB3 or CVB3-*FGF2* (1×10^6 plaque-forming unit per mouse, respectively) via an intramuscular route. CVB3-*FGF2* replication in the mouse hindlimbs was slightly lower than the replication of CVB3 on days 1 and 3 pi (described in detail in the Materials and Methods).

expression levels of FGF2 detected in mouse hindlimbs injected with CVB3-*FGF2* were significantly higher than those detected in the same region in mice injected with phosphate-buffered saline (PBS, mock), CVB3, or CVB3-*GFP*, on both days 1 and 3 pi. The viral titer of CVB3-*FGF2* detected in the mouse hindlimbs was a little lower than that of CVB3 (Figure 3b). In summary, CVB3-*FGF2* can replicate and express FGF2 in mouse hindlimbs *in vivo*.

CVB3-*FGF2* protects against ischemic necrosis and improves blood flow in a model of ischemic vascular disease

Effects of CVB3-FGF2 in a disease model. We tested whether FGF2 expressed from CVB3-*FGF2* is functional and therapeutically effective in a disease model *in vivo*. It has been reported that FGF2 protein stimulates therapeutic arteriogenesis in ischemic mouse models.[21-23] Therefore, we generated ischemic hindlimbs by unilateral femoral artery ligation, as previously described,[24,25] and then injected them with CVB3-*FGF2*, CVB3-*GFP*, CVB3 or PBS. CVB3-*FGF2* significantly protected the hindlimbs from ischemic necrosis compared with PBS, CVB3 and CVB3-*GFP* in BALB/c mice ($P < 0.05$; Figure 4), whereas there were no differences in limb salvage among the PBS, CVB3 and CVB3-*GFP* groups, which all showed significant limb necrosis (Figure 4). This therapeutic effect of CVB3-*FGF2* was also observed in C57BL/6J mice. Limb salvage was observed in 70% and 62.5% of mice in the PBS- and CVB3-injected groups, respectively, whereas all hindlimbs (100%) were salvaged in the CVB3-*FGF2*-injected group (Figures 5a and b) in C57BL/6J mice at 21 days pi, confirming that CVB3-*FGF2* effectively prevents ischemic necrosis. Consistent with these limb salvage data, laser Doppler perfusion imaging showed that CVB3-*FGF2* injection significantly increased the blood flow in the ischemic limbs for over 3 weeks compared with that in the CVB3- and PBS-injected groups ($P < 0.01$ vs CVB3 at weeks 2 and 3; $P < 0.05$ vs PBS at 3 weeks; Figures 5c and d), confirming that FGF2 expressed from CVB3-*FGF2* is functional and therapeutically effective *in vivo*.

DISCUSSION

The use of CVB3 as a viral gene delivery vector has been reported previously. Some cytokines, such as murine interleukin 4 and interferon γ, have been expressed from recombinant CVB3.[16] Lim *et al.*[26] also expressed green fluorescent protein (GFP) in mouse hearts from a recombinant CVB3 variant for at least 8 weeks. The *Renilla* reporter protein was also transiently and extensively expressed from an attenuated recombinant CVB3 in mouse organs.[27] However, because reporter proteins were used in these studies, we do not know whether a foreign gene delivered by

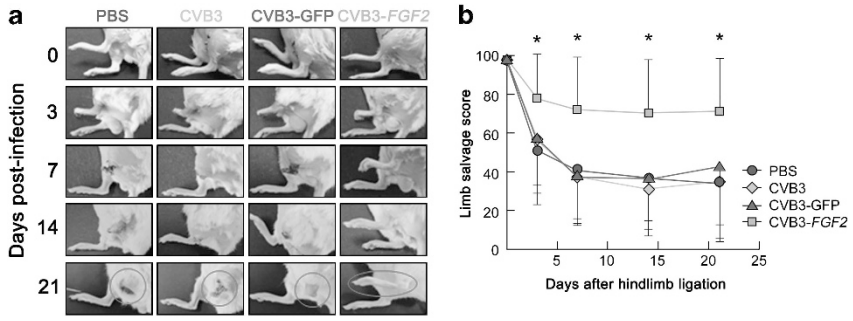

Figure 4. Therapeutic effect of CVB3-*FGF2* in BALB/c mice. Hindlimb ischemia was induced by femoral artery ligation and the removal of distal arterial branches in BALB/c mice. CVB3-*FGF2*, CVB3-*GFP*, CVB3 or PBS was injected into the ischemic limbs and limb salvage was determined on days 3, 7, 14 and 21 after injection. (**a**) Representative photographs of hindlimbs. The hindlimbs in which ischemia was generated are marked with red circles. (**b**) Limb salvage scores. The CVB3-*FGF2* group showed significantly higher limb salvage than the other groups (*$P<0.05$). $n=7$-22 (PBS, $n=11$-22; CVB3, $n=7$-19; CVB3-*GFP*, $n=17$-19; CVB3-*FGF2*, $n=18$-20). The color reproduction of this figure is available at the *Gene Therapy* online.

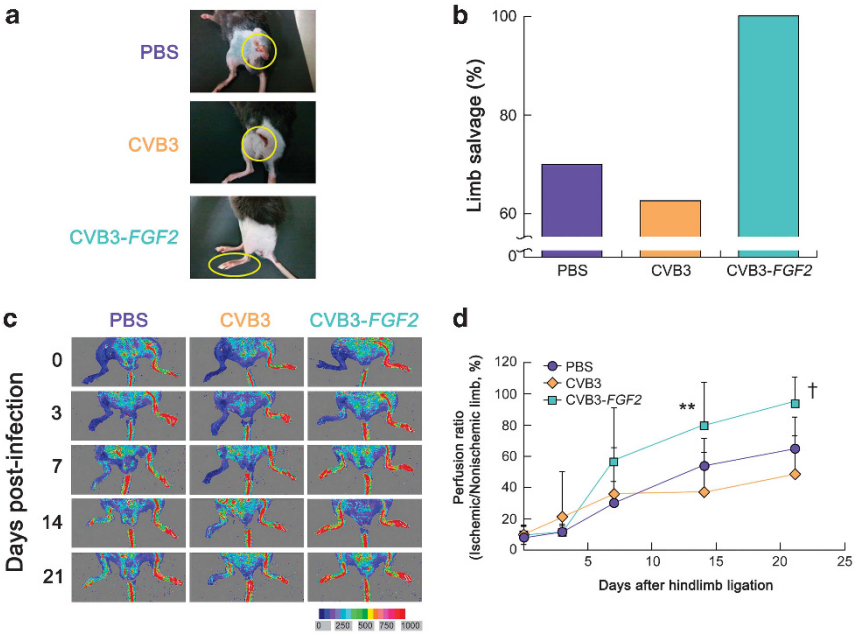

Figure 5. Protection from ischemic necrosis by CVB3-*FGF2* in C57BL/6J mice. Hindlimb ischemia was induced by femoral artery ligation and the removal of distal arterial branches in C57BL/6J mice. CVB3-*FGF2*, CVB3 or PBS was injected into the ischemic limbs and limb salvage was determined 3 weeks after injection. (**a**) Representative photographs of hindlimbs. The hindlimbs in which ischemia was generated are marked with yellow circles. (**b**) The limb salvage rate shows an improvement in hindlimb ischemia produced by CVB3-*FGF2* ($n=5$-10). Hindlimb ischemia was generated, and CVB3-*FGF2*, CVB3 or PBS was injected, as described in the legend to Figure 4, and the blood flow in the hindlimbs was measured using a laser Doppler perfusion imager. (**c**) Representative laser Doppler perfusion images. (**d**) Measured blood flow in the ischemic limbs, normalized to that in the contralateral nonischemic limbs. Injection of CVB3-*FGF2* significantly improved hindlimb ischemia compared with the effect of the CVB3 or PBS control (**$P<0.01$ vs CVB3, $^{\dagger}P<0.05$ vs PBS, $n=5$-11).

recombinant CVB3 actually produces a therapeutic effect on the organs of a mouse model of disease.

The purpose of this study was to test whether CVB3 can be used as a gene delivery vector, producing a therapeutic effect. To test this, we constructed a recombinant CVB3 expressing FGF2 as the model protein, and showed that the recombinant virus can be used as an efficient vector for therapeutic purposes *in vivo* (Figures 4 and 5). However, although CVB3 was used to make the recombinant virus, it will be necessary to use attenuated strains of CVB3, including YYFF,[28] in future research into the application of attenuated CVB3 as a safe viral vector for gene therapy.

In this study, we found that CVB3-*FGF2* did not significantly increase the density of capillaries in the mouse hindlimbs (Supplementary Figure 2), suggesting that the main mechanism by which CVB3-*FGF2* injection ameliorated hindlimb ischemia was FGF2-induced therapeutic arteriogenesis, not angiogenesis. It has been shown that although CVB3-*FGF2* effectively protects mice from ischemic necrosis, the expression of FGF2 in mouse hindlimbs is transient, and FGF2 is not detected 14 days pi (data not shown). However, several previous studies have demonstrated that the transient expression of FGF2 is enough to trigger arteriogenesis.[21-23] Furthermore, because arteriogenesis involves complex host responses, including the proliferation of vascular cells and the remodeling of the vascular structure, a significant increase in perfusion might only become apparent days or weeks after FGF2 expression. Therefore, when FGF2 was transiently expressed from CVB3-*FGF2* in mouse hindlimbs, it was functional and effective, as indicated by an improvement in blood flow and protection from ischemic necrosis in the hindlimbs injected with the recombinant CVB3 (Figures 4 and 5).

Although there are obstacles to be overcome in the use of CVB3 as a gene delivery vector, such as the pathogenic risks of live virus and the effect of preexisting neutralizing antibodies directed against CVB3 on delivery efficiency,[16,17,29] CVB3 has several merits as a gene delivery vector for gene therapy. First, CVB3 does not generate a DNA intermediate that could insert into a host chromosome during replication.[12,26] Host chromosomal integration usually causes insertional mutagenesis in the host cell, which can lead to the formation of tumors, and is one of the main reasons that gene therapy is not widely used in human patients. Second, CVB3 can effectively replicate in nondividing cells, including myocytes, as lentiviruses do.[30] Third, recombinant CVB3 can more effectively express a foreign gene than recombinant adenovirus. According to Kim *et al.*,[27] recombinant CVB3 can express the *Renilla* reporter gene in mouse organs more efficiently than can recombinant adenovirus.

In summary, we have demonstrated that CVB3 can effectively express a functional foreign gene and that FGF2 expressed from recombinant CVB3 has biological activity, improving hindlimb ischemia. All mice injected with CVB3–*FGF2* survived, whereas all mice injected with CVB3 showed both cardiac and pancreatic inflammation and died within 7 days of infection, suggesting that the virulence of CVB3 was abolished in the recombinant CVB3–*FGF2*. Taken together, our data suggest that recombinant CVB3 is a good candidate gene therapy vector.

MATERIALS AND METHODS

Cells and viruses

The cervical HeLa–UVM cancer cell line (here denoted 'HeLa cells'), COS-7 cells and human cardiac myocytes (hCMs) were maintained in Dulbecco's modified Eagle's medium (Gibco–BRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and grown at 37 °C in a humidified 5% CO_2–air mixture.[31] The wild-type CVB3, an H3 Woodruff variant strain (donated by Prof. E Jeon, Samsung Medical Center, Seoul, Korea),[32] was grown and titered in HeLa cells. In this paper, 'CVB3' means wild-type CVB3. The cDNA of CVB3 was used as the backbone for the construction of the recombinant virus CVB3–*FGF2*.

Construction of a recombinant infectious viral cDNA encoding FGF2

To generate a recombinant CVB3 genome that expresses FGF2, we used an infectious cDNA of CVB3 in pBlueScript, as reported previously,[28] and the cDNA of *FGF2*. The CVB3 genome was modified to include an in-frame synthetic polylinker containing *Eco*RI and *Xho*I sites, into which the foreign gene could be inserted. The *FGF2* sequence was amplified by PCR from *FGF2* cDNA, which was donated by the Korea National Institute of Health. The primer sequences for *FGF2* were 5′-GCGTCGAATTCGCAGCCGGGA GCATCAC-3′ (sense) and 5′-GGTTCCTCGAGGGGCTCTTAGCAGACATTGG-3′ (antisense). We used a previously reported strategy with which GFP and *Renilla* luciferase were expressed from *Coxsackievirus*.[26,27] The sequence encoding FGF2 was inserted immediately upstream from the gene encoding the viral VP4 capsid protein. Flanking *FGF2* on the 3′ side was an artificial cleavage site for viral protease $3C^{pro}$.[26] The first six amino acids (MAAQEF) of the CVB3 VP4 capsid protein were positioned at the amino terminus of FGF2. During viral replication, viral $3C^{pro}$ cleaved the monocistronic fusion polyprotein at the artificial cleavage site (ALFQG), releasing FGF2 from the CVB3 protein and allowing normal viral protein packaging (Figure 1a).

Production of progeny virus

To produce progeny virus, the cDNA (2 μg) of each virus was transfected into COS-7 cells in six-well plates using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), as directed by the supplier. Three days after transfection, the cultures were frozen and thawed three times, and then centrifugally cleared of cell debris. The cleared supernatants were used to culture HeLa cells to produce each replication-complete viral stock (passage 2). The progeny viruses were stored as aliquots at −85 °C. The viral titers were routinely determined by plaque assay in HeLa cells, as described below.

Plaque assays and one-step growth curves

The levels of infectious virus were determined by plaque assay[33] in individual organ homogenates, which were prepared with an HT10 homogenizer (IKA-Korea, Anyang, Korea). To analyze the one-step growth of the viruses, HeLa cells in 24-well plates were infected with CVB3 or CVB3–*FGF2* at an multiplicity of infectivity of 10. After 1 h at 37 °C, the unattached virus was removed by washing the cells twice with prewarmed PBS (pH 7.4), and 1 ml of prewarmed Dulbecco's modified Eagle's medium containing 10% FBS was added. The supernatants were harvested at the indicated times. After the samples were serially diluted, a plaque assay was performed in HeLa cell monolayers.

Animal testing

Four-week-old inbred male mice (strain BALB/c or C57BL/6J) were administered 1×10^6 plaque-forming unit of CVB3 or CVB3–*FGF2* by intraperitoneal injection or injection directly into the mouse hindlimbs. After injection, the BALB/c mice were used to analyze the survival rate of the mice injected with each virus, and the BALB/c mouse organs were harvested to check the viral titers, the expression levels of FGF2, and inflammation. The mouse model of hindlimb ischemia was constructed in both BALB/c and C57BL/6J mice. The numbers of mice and the infection routes used in each animal experiment are described in detail in the legend to each figure. All procedures were reviewed and approved by the Animal Care and Use Committee of the Catholic University of Korea.

Histology and image analysis

The mouse organs were fixed in 10% buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin (to test for inflammation). The stained sections were subjected to image analysis with a Leica microscope (Leica, Solms, Germany) in transmitted-light mode, and the images were captured with a SPOT CCD microscope digital camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA).

Western blot analysis

Cells infected with CVB3 or CVB3–*FGF2* were lysed in M-PER buffer (Pierce, Rockford, IL, USA) that included 1 mM PMSF, as reported previously.[28] Anti-VP1 monoclonal antibody (α-VP1; NCL-Entero, Novocastra Laboratories Ltd, Newcastle, UK), anti-VP2 peptide antibody (α-VP2)[19,20] or anti-FGF2 antibody (α-FGF2; Abcam, Cambridge, UK) was used as the primary antibody for immunoblotting to detect viral protein VP1 of CVB3 or FGF2 protein, respectively.

Immunofluorescence analysis

HeLa cells or hCMs grown on glass coverslips (Corning, Corning, NY, USA) in six-well plates were used to detect the viral protein or FGF2 protein expression after infection with CVB3 or CVB3–*FGF2*. All the procedures used to detect immunofluorescence were performed as previously described.[34,35] To detect viral protein VP2 and FGF2 expressed in the virus-infected cells, the fixed cells were doubly immunolabeled with α-VP1 and α-FGF2 (both diluted 1:100) for 2 h at room temperature. After washing, the cells were incubated for 1 h at room temperature with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (diluted 1:100) and Texas-Red-conjugated goat anti-rabbit IgG (diluted 1:100). After washing, the slides were mounted and examined for immunofluorescence using a Leica microscope (× 200 magnification).

ELISA to detect FGF2

An ELISA was performed on the supernatants (diluted 1:5) obtained at the indicated times from hCMs infected with CVB3–*FGF2*. FGF2 was detected with an ELISA kit (R&D Systems, Minneapolis, MN, USA), according to the

manufacturer's instructions. Absorbance was measured at 450 nm with an ELISA reader (Multiskan EX, Thermo Scientific, Waltham, MA, USA).

Generation of hindlimb ischemia and injection of CVB3-*FGF2* into the hindlimbs

Hindlimb ischemia was generated as previously described.[36] Briefly, the femoral artery was ligated and all arterial branches were removed from C57BL/6J mice aged 8-10 weeks. CVB3-*FGF2* or CVB3 (1×10^6 plaque-forming unit per mouse) in 100 μL of PBS or 100 μL of PBS, used as the negative control, was injected intramuscularly into the ischemic hindlimb on day 0 and day 1 after the generation of hindlimb ischemia. Ischemic necrosis was determined from the limb salvage score = ((14−limb necrosis score)/14) × 100. The limb necrosis score was calculated as previously described, according to the following rating system: 0 = none; 1 = 1-3 tips; 2 = 4-5 tips; 3 = 1-3 toes; 4 = 4-5 toes; 5 = 1/3 foot; 6 = 2/3 foot; 8 = whole foot; 10 = 1/3 leg; 12 = 2/3 leg; and 14 = whole leg.

Blood flow measurement in ischemic hindlimbs

The blood flow in the hindlimbs was determined using a laser Doppler perfusion imager (Moor Instruments, Axminster, UK), as previously described.[37] The mean values for perfusion were obtained from stored digital color-coded images. The blood flow levels in the ischemic (left) limbs were normalized to those in the contralateral nonischemic (right) limbs to avoid data fluctuations caused by variations in ambient light and temperature.

Measurement of capillary density

Three weeks after the injection of CVB3-*FGF2* into the ischemic hindlimbs, fluorescein-isothiocyanate-conjugated dextran (average molecular weight = 2 000 000 Da; Sigma-Aldrich, St Louis, MO, USA) was intravenously injected through the jugular vein under anesthesia to visualize the blood vessels. The mice were euthanized 15 min after the dextran injection and their calf muscles were removed, fixed with 4% paraformaldehyde at 4 °C overnight, and frozen sectioned. The specimens were stained with DAPI and observed under a fluorescence microscope (Olympus IX71). The capillary density was calculated from at least four randomly selected fields per mouse.

Statistical analysis

All data are expressed as means ± s.d. Unless otherwise stated, the differences between the values were analyzed with one-way ANOVA and *t*-test. Survival was analyzed using the Kaplan-Meier method. A value of $P < 0.05$ was considered statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Gene Therapy website (http://www.nature.com/gt)