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Protective immune responses in rabbits induced by a suicidal DNA vaccine of the VP60 gene of rabbit hemorrhagic disease virus

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ABSTRACT

A suicidal DNA vaccine based on a Semliki Forest virus (SFV) replicon was evaluated for the development of a vaccine against rabbit hemorrhagic disease virus (RHDV). The VP60 gene of RHDV was cloned and inserted into pSCA1, an SFV DNA-based replicon vector. The resultant plasmid, pSCA/VP60, was transfected into BHK-21 cells, and the antigenicity of the expressed protein was confirmed using indirect immunofluorescence and a western blot assay. In addition, immunogenicity was studied in rabbits. Fifteen rabbits were injected intramuscularly twice with pSCA/VP60 at 2-week intervals. They were challenged with an RHDV isolate 2 weeks after the second immunization. In all cases, anti-RHDV antibodies were detected by ELISA. Additionally, the lymphocyte proliferation response was tested by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide method, and neutralizing antibodies were measured by microneutralization tests. Our results showed that RHDV-specific antibodies and an RHDV-specific cell-mediated immune response were strongly induced in rabbits. Furthermore, all of the rabbits were protected against challenge with wild type RHDV. In conclusion, we demonstrated that the suicidal DNA vaccine is a promising vaccine candidate that facilitates the prevention of rabbit hemorrhagic disease caused by RHDV.

1. Introduction

Rabbit hemorrhagic disease (RHD) is a highly contagious and lethal infection that affects both wild and domestic rabbits. Its etiological agent, the rabbit hemorrhagic disease virus (RHDV), is considered the single most economically important disease of rabbits worldwide. The disease was first recognized in China (Liu et al., 1984) but subsequently spread to other areas of Asia, multiple European countries, Mexico, and elsewhere (Nowotny et al., 1997). The etiological agent was identified as a calicivirus, a positive-sense, single-stranded RNA virus that is antigenically related to the European brown hare syndrome virus (Wirblich et al., 1994). The complete genome of RHDV has been elucidated for the German isolate (Meyers et al., 1991) and was shown to comprise a single-stranded positive sense RNA genome of 7437 nucleotides. The genome contains two open reading frames; the first of which is 2344 nucleotides in length (ORF1) and encodes a large polyprotein containing the viral non-structural proteins as well as the viral coat protein at the C-terminus. The coat protein has an apparent molecular weight of 60 kDa. In total, 180 copies of this protein are assembled to produce native virus capsids (Barcena et al., 2000, 2004).

Because the lack of a suitable cell culture system for RHDV has hindered large-scale production of the virus as a source of vaccine antigens, commercially available vaccines are still produced from tissues collected from experimentally infected rabbits. However, this strategy raises serious concerns about biological safety, contaminant residues and animal welfare. In the past 20 years, the capsid gene (VP60) has been successfully expressed in several heterologous systems (Bertagnoli et al., 1996a,b; Castanon et al., 1999; Fernandez-Fernandez et al., 2001; Fischer et al., 1997) and has been shown to induce full protection of rabbits against a lethal challenge with RHDV. However, it should be considered that in veterinary medicine, the simplicity of the procedures and the cost per dose could be a crucial aspect that limits the practical use of a vaccine. As mentioned above, although several recombinant VP60 proteins have been obtained from insect cell lines or in *Pichia pastoris*, the scaling up of these sources for antigen enrichment purposes is both difficult and expensive.

The goal of this study was to develop a new suicidal DNA vaccine, which has been shown to potentially induce high-level humoral and cell-mediated immunity against a variety of antigens and provide protection for animals (Berglund et al., 1998; Deshpande et al., 2002; Klinman, 2003; Sun et al., 2007), and to evaluate its immunogenicity and protective immunity in rabbits.

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2. Methods

2.1. Cell culture and virus propagation

BHK-21 cells were seeded at a density of 10^6 cells/ml in Dulbecco's Modified Eagle Medium (DMEM) and supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 mg/ml streptomycin in an incubator at 37 °C and 5% CO_2. RHDV JX/CHA/97 is a virulent strain of RHDV that was isolated in 1997 from an outbreak of RHDV in Jiaxing, China.

2.2. Construction of suicidal DNA vaccine

Viral RNA was extracted from a viral suspension according to the Trizol R protocol (Invitrogen, USA), and first-strand cDNA synthesized by RT-PCR with M-MLV reverse transcriptase (Promega) was used as a template. The cDNA template was included in a high-fidelity PCR with forward primer VP60F (5′- CGGGATCC*BamHI*GCCACCATG *kozak sequence* GCCACCATGGGCGTGGTGGCCGC -3′) and VP60R (5′- TCCCCCGGG *SmaI* TCAGACATAAGAAAAGCC-3′). The primers were designed according to the sequence of the JX/CHA/97 strain (GenBank accession No. DQ205345). The forward primer contained a Kozak sequence (underlined above) and an initiation codon (ATG) for optimal initiation of translation, and the reverse primer contained a stop codon (TAG) for correct termination. The PCR product was cloned into the dephosphorylated *Sma*I and *BamH*I sites of pSCA1, the SFV DNA-based replicon vector (kindly provided by Dr. Sun, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, China), and the resultant plasmid was named pSCA/VP60. The recombinant plasmid was confirmed by sequence analysis.

2.3. Detection of VP60 expression in BHK-21 cells

PSCA/VP60 was introduced into BHK-21 cells grown in 35 mm wells using Lipofectamine 2000 reagent (Invitrogen). 48 h after transfection, cells were analyzed for expression of RHDV proteins. A monolayer of cells cultured on cover slips was fixed in cold 100% acetone (−20 °C for 30 min). Samples were incubated with rabbit anti-RHDV serum (37 °C for 30 min) in a humid box and then with fluorescein-conjugated goat anti-rabbit serum (Sigma) for 30 min at 37 °C. Fluorescence was observed under a Nikon microscope.

For western blot analysis, the transfected cells were washed twice with PBS (pH 7.4) and lysed in lyses buffer 48 h after transfection. The solubilized proteins were separated by SDS–PAGE and electrophoretically transferred to a nitrocellulose (NC) membrane (Hybond-C Super, Amersham). To eliminate possible non-specific binding, the membranes were blocked with 10% skim milk in PBST (0.5% Tween 20 in PBS) overnight and incubated with mouse anti-VP60 antiserum (1:500) for 1 h at 37 °C. Horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (Beijing zsbio) was used as the secondary antibody. Protein bands were visualized by 3, 3′-diaminobenzidine tetrahydrochloride (DAB) (Sigma–Aldrich) staining.

2.4. Immunization of rabbits and virus challenge

The DNA vaccine was prepared by diluting the purified plasmid DNA preparation to 1 μg/μl in PBS. 2-month-old New Zealand White rabbits, free from anti-RHDV antibodies, were obtained from the Laboratory Animal Centre of Shanghai Veterinary Research Institute, China. The rabbits were randomly assigned to three groups (five rabbits in each group) and housed in separate rooms. Two experimental groups were vaccinated intramuscularly with either pSCA/VP60 (Group 1) or the pSCA1 vector control (Group 2). The third group was injected intramuscularly with PBS, which served as a negative control (Group 3). Groups of five rabbits were inoculated twice at 2-week intervals with 500 μg of the pSCA/VP60 DNA vaccine for primary administration and with 500 μg of the pSCA1/VP60 DNA vaccine for booster immunization. The diluted DNA was injected into the quadriceps muscle of both legs and groin using a syringe. Rabbits inoculated with the same amount of control pSCA1 DNA and PBS were used as controls. Serum was collected at weeks 0, 1 and 2 post-immunization. 2 weeks after the final immunization, all of the rabbits were challenged intramuscularly with 100 LD_{50} of RHDV (Chinese isolate JX/CHA/97). The animals were observed daily, and deaths were monitored until 14 days post infection. 2 weeks after challenge, the surviving animals were bled and euthanized. The livers from all of the experimental animals were frozen at −80 °C immediately after death.

2.5. Antibody responses against RHDV

Serum samples from rabbits were analyzed by an indirect ELISA test using the recombinant VP60 protein of RHDV as the antigen. The VP60 protein was expressed in *Escherichia coli* BL21 (DE3) using the PET 32a expression system (Novagen), and the recombinant product was purified by dialysis. Then, 96-well flat-bottomed plates (Corning Costar) were coated with recombinant VP60 protein in 0.1 M carbonate/bicarbonate buffer (pH 9.6) and incubated overnight at 4 °C. After blocking with 5% BSA in PBS, plates were incubated with duplicate twofold serial dilutions of test sera for 1 h at 37 °C. Rabbit anti-rabbit IgG HRP (KPL) at a 1:2000 dilution was then added for 1 h at 37 °C, followed by the addition of the substrate 2 mM sulfuric acid. Absorbance was determined at 450 nm using a Bio-Rad microtiter plate reader.

2.6. Lymphocyte proliferation assay

Blood was collected from the jugular vein of all experimental rabbits in blood-collecting tubes containing 3.8% sodium citrate. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation in Ficoll-Paque Plus (density 1.077; Amersham Biosciences) for 20 min at 18 °C. The PBMCs were plated in 96-well flat-bottom plates at 100 μl/well (10^5 cells/well). Subsequently, 100 μl/well of medium with or without 20 μg/ml of RHDV virus particles was added and mixed. Concanavalin A (5 μg/ml; Sigma) was used as a positive control. Each sample of PBMCs was plated in triplicate. The proliferative activity was measured according to the method described by a standard protocol. Briefly, after 96 h of incubation, 20 μl of MTS 3-(4,5-dimethylthylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (inner salt; Promega) was added to each well, and the plates were incubated for a further 5 h. At the end of the incubation, the OD of the plate wells was read at 490 nm. The stimulation index (SI) was calculated as the ratio of the average OD value of wells containing antigen-stimulated cells to the average OD value of wells containing only cells with medium.

2.7. Cytokine assays

The supernatant collected in the T lymphocyte proliferation assay was used to examine the levels of the Th1-type cytokine IFN-γ and the Th2-type cytokine IL-4 using a commercially available ELISA kit (R&D Minneapolis, MN, USA) according to the manufacturer's instructions.

2.8. Detection of viral RNA

Total RNA was extracted from the samples (serum and liver from rabbits) with RNeasy (Qiagen, Germany) and was used imme-

diately for cDNA synthesis. cDNA synthesis was performed using SuperScript II reverse transcriptase (RT) (Invitrogen, USA). The primers were designed based on the sequence of the VP60 gene from RHDV strain JX/CHA/97. The forward and reverse primers were 5′-CCAATAAAATCGATTCAGACA-3′ and 5′-TAACAGCCCCAA-CATT CCGAC-3′, respectively. The 743 bp of the VP60 gene were amplified and then visualized and photographed under UV illumination.

2.9. Statistical analysis

To evaluate the protective immune responses among the different groups, the mean values from all groups were compared with Student's *t*-test using SPSS v.12.0 software. *P*-values of <0.05 were considered statistically significant.

3. Results

3.1. Expression of VP60 in BHK-21 cells

To demonstrate expression of the RHDV VP60 proteins, transfected BHK-21 cells were analyzed by IFA. Cells transfected with pSCA/VP60 showed specific green fluorescence, but the negative control, which was transfected with the same amount of pSCA1, and non-transfected cells did not show any fluorescence emission (Fig. 1). The *in vitro* expression of VP60 was also detected by western blot analysis. The results showed that rabbit hyperimmune serum against VP60 protein reacted with the 60 kDa protein in the lysates of DNA construct-transfected cells, which indicates that VP60 could be expressed in BHK-21 cells (Fig. 2).

3.2. Antibody responses against RHDV

To evaluate the immunogenicity of the recombinant replicon plasmid pSCA/VP60, it was injected into rabbits as described in the Section 2. Blood was collected at week 2 after the first vaccination and week 4 after the second vaccination to test for the presence of anti-VP60 antibodies. Total anti-VP60 antibody response was determined by an indirect ELISA. The mean antibody level of the pSCA/VP60-vaccinated group was significantly higher ($0.01 < P < 0.05$) than those of the pSCA1 and PBS control groups (Fig. 3). After the booster immunization, the mean antibody level increased, but this increase was not statistically significant ($P > 0.05$). It is important to note that all of the rabbits from group 1 were seropositive. The induction of specific antibodies was detected by western blot at week 2 only in animals immunized with pSCA/VP60 (data not shown).

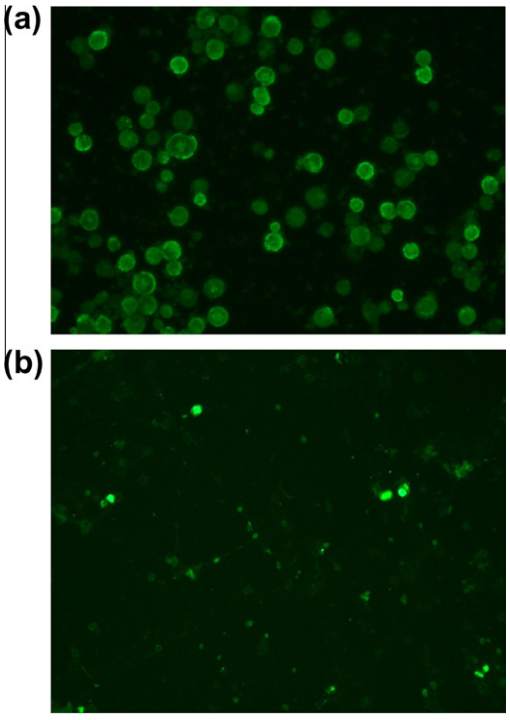

Fig. 1. IFA results of VP60 expression in BHK-21 cells transfected with pSCA1/VP60 (a) pSCA1 (b).

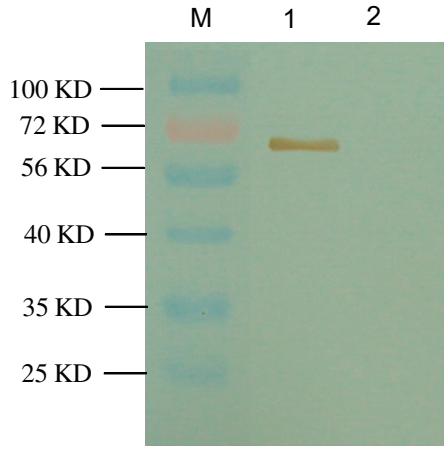

Fig. 2. Western blot analysis of the cell lysates transfected with pSCA/VP60 against anti-VP60 hyperimmune sera. Lane 1, lysates of pSCA/VP60 transfected cells. Lane 2, lysates of pSCA1-transfected cells.

3.3. RHDV-specific T cell proliferation

To investigate cellular immunity responses induced by pSCA/VP60, we analyzed the lymphocyte proliferative responses of all rabbits at 0, 1 and 3 weeks post-immunization. The results indicated that pSCA/VP60 induced obvious and intense lymphocyte proliferative responses (Fig. 4).

3.4. Cytokines

ELISA kits were used to detect the production of IFN-γ and IL-4 in sera at weeks 1, 2, 3 and 4 post-immunization. The results show

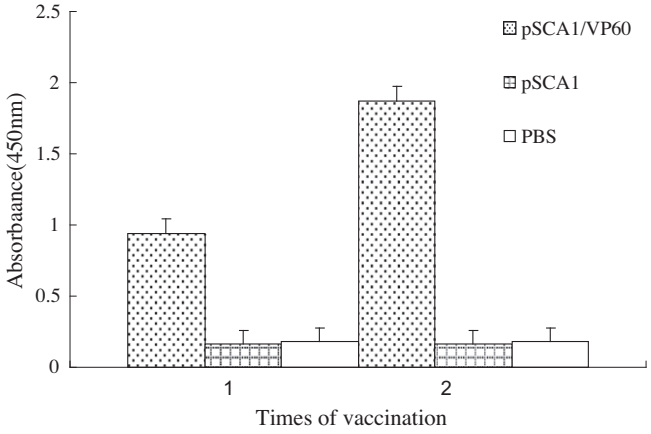

Fig. 3. Anti-VP60 antibody levels in rabbits after immunization. Sera were tested for antibodies at 1:32 dilution. The results were obtained from the mean ELISA absorbance values of three sera from each group.

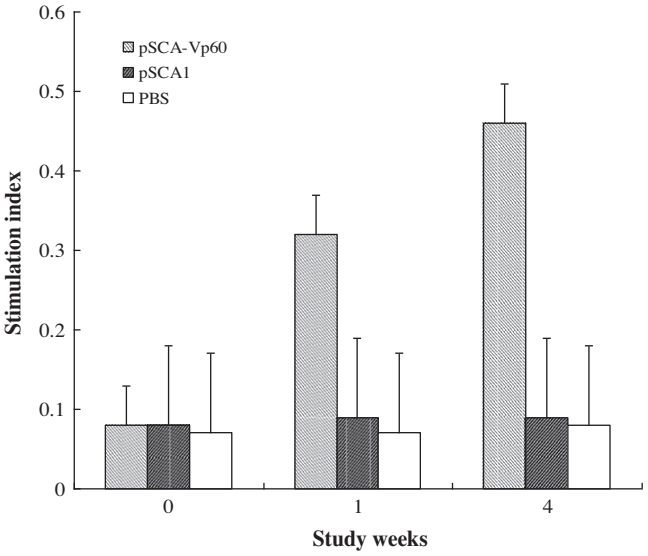

Fig. 4. Specific proliferation of PBMCs in immunized rabbits. The data are presented as the mean absorbance value of each group (SI > 2).

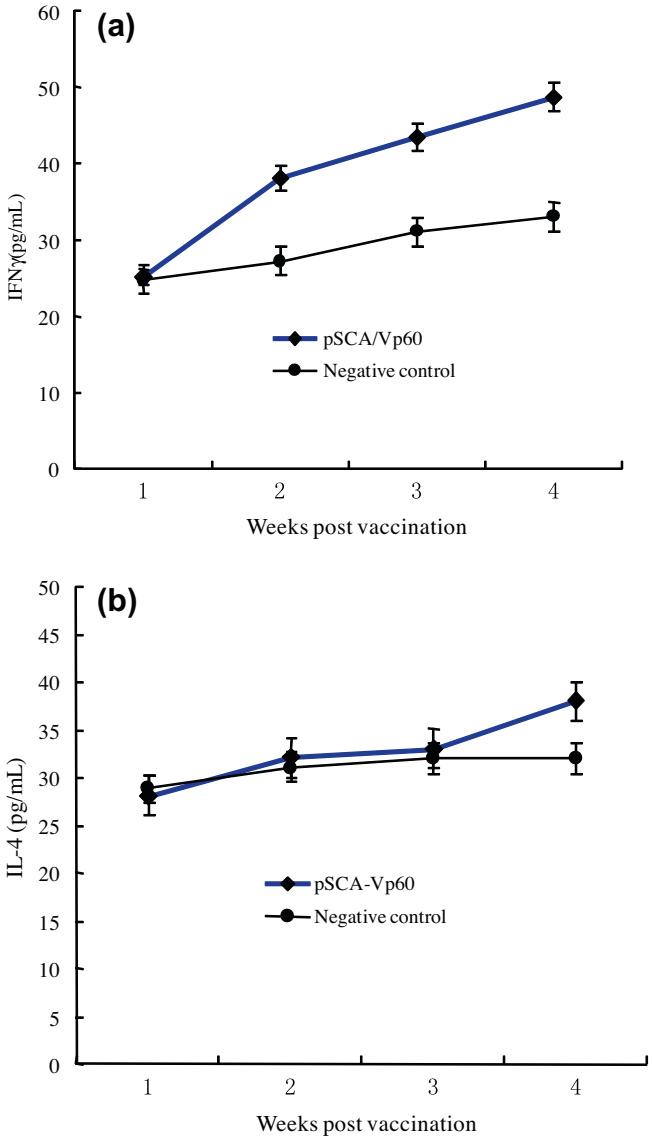

Fig. 5. Both IFN-γ and IL-4 are produced in response to RHDV following vaccination of groups of rabbits with pSCA/Vp60. Sera were analyzed for (a) IFN-γ and (b) IL-4 using ELISA. Statistical significance ($P < 0.05$) was determined by Student's *t*-test. The data are representative of two experiments.

that the mean levels of IFN-γ and IL-4 were significantly higher in the rabbits inoculated with pSCA/VP60 (Fig. 5) compared with those inoculated with PBS.

3.5. Protection against RHDV challenge

To test whether this vaccine could induce a protective effect against a lethal RHDV infection, the rabbits were infected with a wild type RHDV strain, JX/CHA/97, 14 days after the last immunization. All rabbits were housed in an isolation facility and examined for 14 days after the challenge. The results showed that all rabbits from the control group displayed the clinical symptoms and typical lesions of the disease and died within 48–72 h. All vaccinated rabbits survived without any clinical signs of disease. The antibody titers of rabbits vaccined with pSCA/VP60 were ranged from 500 to 1000 in the subsequent 2 weeks. No RHDV could be detected by ELISA in the livers collected from vaccinated animals that were sacrificed 14 days after the challenge. In contrast, all animals in the control groups died between 48 and 72 h after challenge and had anti-VP60 antibody titers below the detection limit of the test. RHDV could be detected in all liver homogenates from these animals (data not shown).

3.6. Detection results of viral RNA

To detect viremia in rabbits after RHDV challenge, the VP60 gene sequence of RHDV was amplified from the livers of the rabbits. As expected, a 743-bp fragment was amplified by RT-PCR in all rabbits from the PBS/pSCA1 group (data not shown), but all rabbits from the pSCA/VP60 group were negative. The data indicate that vaccination with pSCA/VP60 could significantly reduce disease onset and decrease viral replication in rabbits.

4. Discussion

Rabbit hemorrhagic disease (RHD) is a contagious and highly lethal viral disease of rabbits. Therefore, it is important to prevent and control the disease with effective vaccines against its etiological agent, RHDV. Unfortunately, the lack of an efficient *in vitro* propagation system for RHDV has hindered the production of the virus as a source of vaccine antigens (Farnos et al., 2009), and commercially available vaccines are still produced from tissues collected from experimentally infected rabbits. It has been reported that the capsid protein (VP60) of RHDV shows good immunogenicity, and rabbits inoculated with enough recombinant VP60 could be fully protected from wild type RHDV (Frantz et al., 2011). Therefore, the capsid protein gene has been expressed in a number of heterologous hosts (Fernandez et al., 2011; Fischer et al., 1997; Marin et al., 1995; Perez-Filgueira et al., 2007) to obtain a recombinant engineered vaccine against RHDV. However, none of these new types of vaccine against RHDV is available for field use.

DNA vaccines have been shown to induce protective cellular and humoral immune responses and can overcome many problems associated with conventional vaccination (Alarcon et al., 1999; Gerdts et al., 1997). However, in all cases, the protection level is generally low compared with that of attenuated or inactivated vaccines (Oka et al., 2001; Tacket et al., 1999). Recently, suicidal DNA vaccines have emerged as an important strategy for enhancing immunogenicity and improving the biosafety of conventional DNA vaccines (Berglund et al., 1998;Leitner et al., 2003).

In the present study, we constructed a suicidal DNA vaccine against RHDV and assessed its immunogenic properties and its ability to protect against viral challenge. Our results showed that the recombinant plasmid pSCA/VP60 could not only express the VP60 protein faithfully but could also induce stronger specific immune responses in rabbits. Specifically, higher antibody titers were detected in rabbits immunized with pSCA/VP60. The difference in the stimulation index (SI) of the lymphocyte proliferation assay of suicidal DNA vaccine pSCA/VP60 was statistically significant in comparison to control groups. Lethal challenge experiments showed that pSCA/VP60 could provide full protection for rabbits against virulent RHDV challenge. Our results also showed that no RHDV was detected in rabbits vaccinated with pSCA/VP60, which suggests that the suicidal DNA vaccine pSCA/VP60 could decrease RHDV replication *in vivo*, which is very important for eradicating RHD.

In conclusion, an RHDV suicidal DNA vaccine pSCA/VP60 was successfully constructed for the first time. Compared to control groups, the suicidal DNA vaccine pSCA/VP60 was able to elicit a significant humoral and cellular immune response and provide adequate protection for rabbits challenged with a lethal dose of RHDV. Therefore, we conclude that suicidal DNA technology shows promise as a tool for the development of a candidate vaccine for RHDV, although much work still needs to be performed.

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