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Thesis for the Degree of Doctor of Philosophy

Genetically engineered virus-based prophylactic and therapeutic approaches against Spring Viremia of Carp Virus (SVCV) and Viral Hemorrhagic Septicemia Virus (VHSV)

By

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Pukyong National University

February 2024

Genetically engineered virus-based prophylactic and therapeutic approaches against Spring Viremia of Carp Virus (SVCV) and Viral Hemorrhagic Septicemia Virus (VHSV)

유전자 엔지니어링 재조합 바이러스를 기반으로 하는 Spring Viremia of Carp Virus (SVCV) 및 Viral Hemorrhagic Septicemia Virus (VHSV) 예방 및 치료

Advisor: Prof. Ki Hong Kim

By
Mariem BESSAID

A thesis submitted in partial fulfillment for the requirements
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Genetically engineered virus-based prophylactic and therapeutic approaches against Spring Viremia of Carp Virus (SVCV) and Viral Hemorrhagic Septicemia Virus (VHSV)

A dissertation

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Genetically engineered virus-based prophylactic and therapeutic approaches against Spring Viremia of Carp Virus (SVCV) and Viral Hemorrhagic Septicemia Virus (VHSV)

Mariem BESSAID

Abstract

Fish rhabdoviruses are considered as one of most devastating viruses for the worldwide fish rearing field. Up to date, there is still no effective control strategy to be used for treating those viral diseases or to prevent their infection. During our study, we focused on two important rhabdoviruses, spring viremia of carp virus (SVCV) and viral hemorrhagic septicemia virus (VHSV) which are infamous cause of high mortalities in freshwater and marine fish, and we attempted the development of therapeutic and prophylactic approaches using reverse genetics in order to control those two viruses' propagation and to understand more about their genes function and interaction with their host.

In chapter 1, we have studied one of SVCV gene's function, the phosphoprotein, since it is an essential component for virus replication, and it has a role in the suppression of type I interferon of the host cells. For that reason, we have generated recombinant snakehead expressing SVCV P gene by inserting the P gene into SHRV genome between its N and P gene. The *in vitro* viral growth test showed a significantly higher titer of rSHRV-A-Psvcv compared to the control virus rSHRV-A-eGFP. In addition, the result of the luciferase assay that was conducted to verify the effect of SVCV P gene when it is inserted into SHRV genome on type I interferon, showed that comparing to the control virus, rSHRV-A-Psvcv seemed to suppress more the interferon response. After confirming that *in vitro*, the role of SVCV P gene was

investigated *in vivo* through conducting a virulence test by infecting zebrafish with either rSHRV-A-Psvcv or rSHRV-A-eGFP. As a result. high mortality occurred from the fish infected with rSHRV-A-Psvcv compared to the control virus group with a significantly higher viral growth (at 7d at 15°C and at 3d at 28°C). Through this findings, SVCV P gene could reverse the virulence of SHRV *in vivo* and it may have an effect on the replication of the virus by delaying the immune response which can be explained by the late appearance of the immune relevant pathways enriched through RNA-seq analysis.

In the second part of this thesis, the development of a therapeutic tool has been carried out to restrain SVCV infection *in vivo*, using artificial microRNA (AmiRNA) targeting SVCV P gene transcript. synthesized AmiRNA mimics and AmiRNA-expressing vector system were used to determine the downregulation ability of the three candidates of AmiRNAs (AmiR-P1, -P2, and –P3) against SVCV P gene transcript, among which AmiR-P3 was chosen since it showed a higher inhibitory activity. Based on the *in vitro* results, we rescued (SHRVs) expressing the chosen SVCV P gene-targeting AmiRNA (rSHRV-AmiR-P3) or control AmiRNA (rSHRV-AmiR-C) in order to overcome any limitation of AmiRNA mimics or the AmiRNA-expressing vector systems in *in vivo*. After verifying *in vitro* that the expression of AmiR-P3 and AmiR-C was successful through rSHRVs, we evaluated the availability of rSHRV-AmiR-P3 for *in vivo* control of SVCV. For that, zebrafish were infected with either rSHRV-AmiR-C or rSHRV-AmiR-P3 followed by SVCV infection or infected with SVCV followed by either rSHRV-AmiR-C or rSHRV-AmiR-P3 infection. as a result, there was no significant difference in survival rates between groups of fish infected with rSHRV-AmiR-C or rSHRV-AmiR-P3 before SVCV infection, however, the survival rate in the group of fish infected with SVCV

followed by infection with rSHRV-AmiR-P3 was significantly higher than in the group of fish infected with rSHRV-AmiR-C. Based on the present study results, we could verify that rSHRV could be a suitable system for a successful expression of AmiRNA, and rSHRV expressing AmiR targeting SVCV P gene could be used as an alternative to control SVCV infection in fish for therapeutic purpose.

In chapter 3, prophylaxis was developed based on SVCV glycoprotein in order to provide protection of fish against SVCV infection. we have used chimeric rSHRV-Gsvcv as a live attenuated vaccine for the protection of zebrafish against SVCV. At first two experiments were conducted where zebrafish were immunized with two different doses of chimeric rSHRV-Gsvcv (1×10^4 pfu or 1×10^3 pfu) and after SVCV challenge, both doses showed significant high survival rate comparing to the control group which lead to the choice of the lowest dose to be used in the third experiment where a third control group wad added (rSHRV). After immunization, the immunized fish were challenged with SVCV to evaluate the protectivity of rSHRV-Gsvcv. Consequently, chimeric rSHRV-Gsvcv showed a significantly higher survival rate compared to the control groups. Based on this, chimeric rSHRV-Gsvcv can be considered as a successful tool to be used *in vivo* to protect fish against SVCV infection.

In chapter 4, we have focused on VHSV's G protein (glycoprotein) which has always been an interesting element to study its function and role related to the viral replication and general mechanism. The manipulation of glycoprotein by exchanging its signal peptide with a high secreted antimicrobial peptide gene "piscidin" was performed in order to increase recombinant VHSV titer. rVHSV expressing piscidin signal peptide exchanged-glycoprotein

(rVHSV-PspvG) was produced the *in vitro* comparison of its titer showed a lower titer comparing with rVHSV-wild titer. This could be related to the fact that overexpression of the protein can lead to its accumulation into the ER causing ER stress which results in the initiation of the unfolded protein response (UPR). This UPR activates protein kinase R-like ER kinase (PERK) that phosphorylates eukaryotic initiation factor 2α (eIF2 α), attenuating global translation. To investigate this fact, antioxidants (ER stress reliver; BHA and SA), PERK inhibitor (GSK) or ER stress inducer (Tunicamycin) were used to determine the relation between ER stress and PERK pathway on VHSV growth. The result of the viral growth showed that the highest titer of both rVHSV-PspvG and rVHSV-wild reached from the cells treated with PERK inhibitor and the lowest one was shown from the ER stress inducer treated cells. Through this result, VHSV replication seems to be related to the ER stress and influenced by the activation of PERK pathway. The effect of piscidin signal peptide on the virus replication and on the immunogenicity of glycoprotein was determined by conducting virulence test of rVHSV-PspvG and DNA vaccine based on G gene *in vivo*. The virulence test result showed that a late and lower mortality was triggered by rVHSV-PspvG at both injected doses (1×10^5 pfu or 1×10^3 pfu) in comparison with rVHSV-wild injected fish. The DNA vaccine data revealed that PspvG-immunized fish showed significantly lower survival rate compared to the original vG. These findings can interpret that the secretion of the glycoprotein influences the virus replication and the protective ability of glycoprotein *in vivo*.

In the present study, we could prove that recombinant viruses generated through reverse genetics can serve for several purposes such as the study of a heterologous gene such as in our case the usage of rSHRV-A-Psvcv, for the

delivery of therapeutic tools or for prophylactic reasons. In addition, viral replication depends on the ER stress and the pathways related to that and the secretion of the glycoprotein could impact the virus growth and its protection ability.

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General introduction

In virology, reverse genetics refers to the recovery of a live recombinant virus from a copy of its original genome. During the recent decades, reverse genetics has been applied to many viruses including rhabdoviruses which have been the subject of many investigations and studies (Stéphane Biacchesi, 2011; Stobart & Moore, 2014; Ye et al., 2014). The first trial of recovering a recombinant rhabdovirus was attempted by Schnell et. Al (1994) who produced recombinant rabies virus through a full cDNA copy of the viral RNA genome driven by T7 polymerase promoter, with the viral N, P, L proteins. Since then, reverse genetics systems were established for mammalian rhabdoviruses such as vesicular stomatitis virus (VSV; (Lawson et al., 1995)). Moreover, the generation of recombinant piscine rhabdoviruses was applied first for snakehead rhabdovirus (SHRV; (Johnson et al., 2000)), followed by infectious hematopoietic necrosis virus (IHNV; (Stéphane Biacchesi et al., 2000)), then viral hemorrhagic septicemia virus (VHSV; (Ammayappan et al., 2011; M. S. Kim & Kim, 2011)) and hirame rhabdovirus (HIRRV; (Ryu et al., 2022)). This technique allowed researchers to study viral genes functioning and virus mechanism, to develop attenuated live vaccines and to deliver heterologous molecules *in vitro* and *in vivo*, through the production of recombinant viruses (M. S. Kim & Kim, 2012; Kwak et al., 2021; Lee et al., 2021; Vakharia et al., 2019). Recombinant viruses which are produced by reverse genetic technique are considered to be safer than the naturally attenuated viruses. Consequently, studies on the development of vaccine candidates based on the reverse genetically produced viruses are raising during the years in the aquaculture field (Choi et al., 2019; S. Y. Kim et al., 2023).

Piscine rhabdoviruses are a widespread RNA virus group in marine and freshwater fish around the world. They are enveloped, negative-sense single-stranded RNA viruses, and considered as lethal pathogens causing morbidity that leads to significant economic issues in aquaculture and can have an environmental impact on the wild fish communities (Walker & Winton, 2010). In this thesis, we have focused on two critical rhabdoviruses, Spring viremia of carp virus (SVCV) and viral hemorrhagic septicemia virus (VHSV).

Spring viremia of carp virus (SVCV) is an enveloped negative-sense single stranded RNA virus, belonging to *Sprivivirus* genus. The RNA genome consists of about 11 kilo-base pair (kbp) nucleotides encoding 5 structural proteins arranged in the order: Nucleoprotein (N), Phosphoprotein (P), Matrix protein (M), Glycoprotein (G) and RNA-dependent RNA polymerase (L), and they assemble together to give a bullet shape virion (Walker et al., 2021). Isolated first by Fijan et al (1971), SVCV is a highly pathogenic virus to Cyprinid fish mainly common carp (*Cyprinus carpio*) causing high mortality which lead to severe economic loss (Ahne et al., 2002). Many studies have been conducted to explore SVCV mechanism and to establish strategies for the control of its infection in fish. However, up to date, no commercial effective vaccine or therapy is available that can provide protection against SVCV.

In the present study, using reverse genetics, we aimed to understand more about SVCV genes function on the virulence and to develop prophylactic and therapeutic ways to control its infection *in vivo,* by generating different forms of rSHRV. Indeed, SHRV is a *Novirhabdovirus*, was firstly detected in snakehead fish (*Ophicephalus striatus*) in Thailand (Wattanavijam et al., 1986), but there was no notice about SHRV-related disease after that. It has been

revealed on some occasion that SHRV triggers mortality in zebrafish (*Danio rerio*) (Alonso et al., 2004), but several preliminary studies conducted in our laboratory showed that SHRV is able to replicate in zebrafish, but with no occurred mortality. According to that, SHRV presented a possible candidate to be used as a tool for several purposes in our studies.

The phosphoprotein (P protein or gene) is an essential component of the RNP complex which is important for viral protein transcription and for virus replication (Leyrat et al., 2011; Roy, 1981; Wang et al., 2020). It has also a role in the virulence and in the host cell invasion strategy adopted by the virus since it possesses a suppressive effect on the Type I interferon (S. Li et al., 2016; Vakharia et al., 2019; Wang et al., 2020) and also interacts with some of the antiviral molecules of the host cells (Liu et al., 2023). In the present study, we investigated the role of SVCV P gene in the *in vivo* virulence by generating rSHRV harboring SVCV P gene in its genome between N and P gene (rSHRV-A-Psvcv) and comparing its virulence with a control avirulent rSHRV expressing eGFP as a heterologous gene between N and P gene (rSHRV-A-eGFP) (Chapter I).

SVCV P gene is an important element for virus virulence and replication, making it an interesting target to be manipulated in order to control SVCV infection. Over the past few years, RNA interference (RNAi), a posttranscriptional gene silencing process mediated by small RNAs, has been used for developing therapies to control several diseases (Seo et al., 2019; Waring et al., 2018). microRNAs (miRNA), one of the RNAi tools discovered first by Lee et al. (1993), are small non-coding RNAs that regulate several biological functions in many organisms by triggering degradation or inhibition of translation of the target gene mRNA. The study of miRNA effect in fish has

been conducted and proved in response to VHSV (Kwak et al., 2019; Najib et al., 2017). Recently, artificial microRNA (AmiRNA), mimic of endogenous miRNA, considered as an interesting tool since it can be made by the replacement of the central stem region of the endogenous miRNA with the complementary sequence of our target gene and proved to be safer and well tolerated by the host cells more than other small RNAs such as small interfering RNA (siRNA). The effect of AmiRNA was determined lately by Kwak & Kim, (2021) *in vitro* and they showed that AmiRNA could inhibit the replication of VHSV when it is delivered by recombinant virus. However, the application and the delivery of AmiRNA by recombinant virus *in vivo* was not reported before. In this thesis, we have developed a therapeutic tool to control SVCV replication and infection to be used *in vivo* by generating rSHRV expressing AmiRNA targeting SVCV P gene (Chapter II).

Recombinant rhabdoviruses have been proved to be useful tools for the delivery of heterologous genes such as antigens or as live attenuated vaccines through the manipulation of their genetic material. In viruses, glycoprotein, an envelope protein, is one of the most important genes that plays a key role not only in the assembly of the virion but also in virulence. Also, it is the main target of neutralizing antibodies which makes it the target to be used as antigen for the development of vaccines (E. Lorenzen et al., 2000; N Lorenzen & LaPatra, 2005; Niels Lorenzen et al., 2001). Several trials of vaccination based on SVCV G gene have been conducted and showed protectivity against SVCV infection (Embregts et al., 2017; Emmenegger & Kurath, 2008; C. Li et al., 2023; Zhang et al., 2018; Zhao et al., 2022). However, live attenuated viruses-based vaccines seemed to have better effectiveness and more advantageous since they can stimulate their host as the original virus, leading to more durable immunity with

high protectivity by inducing both humoral and cellular immune response (M. S. Kim et al., 2011; M. S. Kim & Kim, 2011; 2012). In Chapter III of this thesis, we modified the SHRV genome by exchanging its G gene with SVCV's and used the chimeric rSHRV-Gsvcv as our prophylactic method in protecting zebrafish against SVCV.

In addition to SVCV, VHSV is a rhabdovirus belonging to Novirhabdovirus genus. Its genome encodes 5 structural proteins arranged as: Nucleoprotein (N), Phosphoprotein (P), Matrix protein (M), Glycoprotein (G) and RNA-dependent RNA polymerase (L), with a non-virion gene (NV) located between G and L genes. VHSV is an infamous pathogen causing viral hemorrhagic septicemia disease in both fresh water and marine fish leading to severe mortalities which made it notifiable by the World Organization of Animal Health (WOAH)(Escobar et al., 2018; Skall et al., 2005; Walker et al., 2022). VHSV has always been an interesting virus, and many studies have been done in order to investigate the role of viral genes in virulence and replication of the virus (Ito et al., 2018; Yusuff et al., 2019) . One of these genes is glycoprotein which is the unique envelope protein that presented the target of several research to explore its function related to VHSV virulence and replication (Abdellaoui et al., 2022; Einer-Jensen et al., 2014; Gaudin et al., 1999; S. Y. Kim et al., 2023) but also used as an antigen for the development of many vaccines' candidates since it is the main target for neutralizing antibodies (M.-J. Kim et al., 2023; S. Y. Kim et al., 2022; Martinez-Lopez et al., 2014; Pereiro et al., 2012; Puente-Marin et al., 2018).

Briefly, the biosynthesis of the glycoprotein starts from the endoplasmic reticulum (ER) where it is located by the signal peptide to be translated into

monomers, then oligomerized to make the final trimeric form that will be packaged in the Golgi apparatus. Afterwards, the trimeric glycoprotein is transported and displayed on the membrane for the virus budding and release (Albertini et al., 2008). According to this fact, increasing the expression of glycoprotein can be a way to elevate virus titer. However, viral infection and protein overexpression can simulate the ER stress which lead in its turn to the activation of the untranslated protein response (UPR). The UPR results in the activation of the three stress sensors one of them is the protein kinase R-like ER kinase (PERK) pathway that phosphorylates the eukaryotic initiation factor 2α (eIF2 α) causing the attenuation of the global translation of the host cells (Hetz & Papa, 2018; Pavio et al., 2003). The purpose of the present study consists of increasing VHSV titer, so we manipulated G gene by exchanging its signal peptide with antimicrobial peptide (piscidin) gene's signal peptide (PspvG). Then using reverse genetics, we generated rVHSV expressing PspvG (rVHSV-PspvG), determined its viral growth *in vitro* comparing to rVHSV-wild and its relationship with the ER stress and PERK pathway. Furthermore, we have investigated the effect of the piscidin signal peptide on G gene immunogenicity *in vivo* through DNA vaccine against VHSV infection (Chapter IV).

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Chapter I: Insertion of spring viremia of carp virus (SVCV)'s P gene into snakehead rhabdovirus (SHRV) genome increased virulence

1. Introduction

Spring viremia of carp virus (SVCV) belongs to the genus Sprivivirus of the family Rhabdoviridae (Walker et al., 2021) and is responsible for the contagious spring viremia of carp disease (SVCD) that has caused significant mortalities in common carp (Cyprinus carpio) and other cyprinid fish species (Ahne et al., 2002). SVCV is a negative single-strand RNA virus with around 11 kb genome encoding five structural genes, nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and RNA-dependent RNA polymerase (L). Among those proteins, the P protein is known to suppress host cells' type I interferon responses (Li et al., 2016; Wang et al., 2020) and facilitate apoptosis through p53 stabilization (Li et al., 2019). However, the contribution of the P protein in the *in vivo* virulence of SVCV has been poorly investigated.

The generation of genetically engineered recombinant viruses using reverse genetic technology has made it possible to analyze each viral gene's function. Previously, we reported the generation of recombinant snakehead rhabdoviruses (SHRVs) for the delivery of CRISPR/Cas9 (Kwak et al., 2021) or for the expression of heterologous viral genes (Lee et al., 2021). The first SHRV detection was reported from snakehead fish (*Ophicephalus striatus*) in Thailand (Wattanavijam et al., 1986), since then the occurrence of SHRV-related disease has not been reported. There have been only a few papers reporting mortalities in zebrafish (*Danio rerio*) by intraperitoneal injection of SHRV (Alonso et al., 2004; Phelan et al., 2005). Many of our preliminary experiments to determine the virulence of SHRV in zebrafish showed that less than 1 x 104 PFU of SHRV did not induce mortality in adult zebrafish, which led us to postulate that it may be possible to use SHRV as a tool to analyze the involvement of heterologous

viral proteins in the *in vivo* virulence by engineering SHRV genome to have heterologous viral genes. In the present study, to know whether the SVCV P protein is involved in the *in vivo* virulence, we generated a recombinant SHRV that had the SVCV P gene between the N and P genes in the genome (rSHRV-A-Psvcv) and compared its virulence in zebrafish with the control rSHRV expressing enhanced green fluorescent protein (eGFP; rSHRV-AeGFP) instead of SVCV P protein.

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2. Materials and Methods

2.1.Cells and viruses

Epithelioma papulosum cyprini (EPC) cells were used for virus propagation and recombinant virus rescue. Cells were grown at the optimal temperature of 28°C in complete Leibovitz's medium (L15, Sigma) supplemented with 10% fetal bovine serum (FBS, Welgene) and penicillin-streptomycin (Welgene). For virus propagation, a 2% FBS-supplemented L15 medium was used. Baby hamster kidney (BHK-21) cells for minigenome assay were grown at 37°C in complete Dulbecco's Modified Eagle Medium (DMEM, Welgene) supplemented with 10% FBS and antibiotics. Stocks of the previously rescued recombinant SHRV expressing eGFP (rSHRV-AeGFP; Kwak et al., 2020) and SVCV-K1 (isolated from wild common carp in Korea 2010 by researcher senior from the Diagnostic Biochemistry Laboratory, Pukyong National University) were made by propagation on EPC cells. When cytopathic effect (CPE) appeared broadly, supernatants were collected, centrifuged, filtered through 0.45 μm syringe filters, aliquoted, then stored at -80 °C until further use.

2.2.Minigenome assay

To verify the interaction between SHRV RNP complex with the SVCV P protein, BHK-21 cells were co-transfected with SHRV minigenome expressing plasmid driven by CMV promoter (2 μg; previously constructed by Lee et al., (2021) and helper vectors of pCMV-SHRV N (0.5 μg), pCMV-SHRV L (0.5 μg), and either pCMV-SHRV P (0.3 μg) or pCMV-SVCV P (0.3 μ) using Fugene HD. Cells were incubated at 37°C overnight, then the temperature was shifted to 28°C. The cells were observed daily for the expression of fluorescence.

2.3.Rescue of SVCV P gene-expressing recombinant SHRV (rSHRV-A-Psvcv)

To construct a vector containing the SVCV P gene between the N and P genes of the SHRV genome, a previously constructed SHRV full genome vector that has the eGFP gene between the N and P genes (pSHRV-AeGFP; Kwak et al., 2020) was used as a backbone. The SVCV P gene ORF was amplified from a full-length cDNA of SVCV using a forward primer containing the *Kpn*I site at its 5' region and a reverse primer containing the *Mlu*I site at the 3' region (Table 1-1) and was cloned into the pGEM-T easy vector (Promega), then designated as pT-Psvcv. After the verification of the P gene sequence, both pSHRV-AeGFP and pT-Psvcv were digested by *Kpn*I and *Mlu*I, then the SVCV P gene ORF was ligated between the N and P genes of pSHRV-AeGFP by replacing eGFP gene ORF, then designated as pSHRV-A-Psvcv.

The recombinant virus (rSHRV-A-Psvcv) was produced using reverse genetic technology. Briefly, pSHRV-A-Psvcv vector (2 μg) was co-transfected into T7 polymerase-expressing EPC cells with previously constructed pCMV-SHRV N (0.5 μg), pCMV-SHRV P (0.3 μg), pCMV-SHRV L (0.2 μg) vectors (Kwak et al., 2020) using Fugene HD (Promega) as a transfection reagent at a ratio of 1 : 3 with fresh L-15 medium and incubated overnight at 28 °C, then the medium was replaced with complete L-15 medium. When broad CPE was observed, the supernatant was collected, centrifuged, filtered through a 0.45 μm pore-sized filter, and then propagated on an EPC monolayer for passages. The virus stock made from the sixth passage was aliquoted and stored at -80 °C. The generation of the SVCV P gene-inserted rSHRV was verified by sequencing and reverse transcription PCR (RT-PCR) using primers in Table 1-1. The virus titer was determined by plaque-forming assay.

2.4.Viral growth

EPC cells grown to 80% confluence were inoculated with either rSHRV-AeGFP or rSHRV-A-Psvcv at MOI 0.001 and incubated at 28 °C. The supernatant was sampled at different time points (6, 12, 24, 48, and 72 h) to determine the viral growth using plaque assay.

2.5.Type I interferon response

To determine the effect of SVCV P gene insertion on type I interferon response, EPC cells harboring a reporter vector for Mx gene expression (pOFMx-reporter; (Kim & Kim, 2012)) were infected with either rSHRV-AeGFP or rSHRV-A-Psvcv at MOI 0.01 and 0.001, and incubated at 28 °C. The supernatant was sampled at 12, 24, and 48 h post-infection. Then the type I interferon response was determined by measuring the secreted luciferase (Metridia luciferase) using Ready to Glow Secreted Luciferase reporter systems Kit (Takara) in a Victor III multilabel plate reader (PerkinElmer).

2.6.*In vivo* virulence

The *in vivo* virulence of rSHRV-A-Psvcv was performed using adult zebrafish (average weight 0.3g) as a model fish. Experiments were conducted under two temperatures, 15°C and 28°C. Fish were divided into three groups of three replicates at each temperature, and each replicate contained 20 fish. Fish in each group were intraperitoneally (i.p.) injected with 1 x 10^4 pfu/20 µl/fish of rSHRV-AeGFP or rSHRV-A-Psvcv or 20 µl of L-15 medium alone (control group). Fish were maintained at 15 °C or at 28 °C until the end of the experiment. The cumulative mortality of two replicates was monitored for 21d.

To enumerate virus replication in fish, one replicate among three replicates in each group at each temperature was randomly chosen, and 3 fish from each group were randomly sampled at 6 h, 12 h, 24 h, 3 d, 7 d, and 14 d post-injection (dpi). Viral copy number was analyzed by quantitative reverse transcription polymerase chain reaction (RT-qPCR) using Light Cycler 480 (Roche). Briefly, RNA was extracted from fish samples using a Hybrid R kit (GeneAll, Korea) and used as the template for the one-step RT-qPCR that was performed using AccuPower Dual HotStart RT-qPCR kit (Bioneer, Korea). The sequences of a TaqMan probe and a primer set designed to bind to the N gene of SHRV are in Table 1-1.

2.7.Total RNA extraction, library construction and sequencing for RNA-seq analysis

Zebrafish (average weight 0.3g) were injected with the same concentration of rSHRV-AeGFP or rSHRV-A-Psvcv as in the *in vivo* virulence test or for negative control (NC) with L-15 media. Spleens of 3 fish from each group were isolated at 12h, 24h and 72h after injected, pooled, and treated with RNAlater (Invitrogen) before storage at -80°C. Total RNA was extracted using Trizol reagent (Invitrogen)in accordance with the manufacturer's instructions. The total RNA purity was analyzed using Nanodrop 8000 spectrophotometer (Thermofisher Scientific) and the quality was evaluated with an Agilent 2100 Bioanalyzer (Agilent Technologies) with an RNA integrity number (RIN) value above 9.0. Then, the library was prepared using Illumina's Truseq stranded mRNA library prep kit, following the manufacturer's instructions. All the libraries paired-end sequencing was conducted by Illumina Novaseq 6000 system (2x100 nucleotide read length).

2.8.Identification of differentially expressed genes (DEGs)

Total Illumina sequence raw reads were filtered to eliminate adapters and poor-quality reads via Cuatadapt (v4.1). Clean reads were mapped to zebrafish refence genome (GCF_000002035.6) by Tophat (v2.0.13) and the aligned results were added to Cuffdiff (v2.2.0) to obtain differentially expressed genes (DEGs). For library normalization and dispersion estimation, geometric and pooled methods were applied. The gene expression level of each gene was measured based on the fragments per kilobase of exon per million mapped reads (FPKM). The DEGs between control and vaccinated groups were identified based on the false discovery rate (FDR) < 0.05 and log2 (fold change) > 1.0 or < -1.0. All DEGs were mapped to terms in Kyoto Encyclopedia of Genes and Genomes (KEGG) database and the data were considered significant at a $p < 0.05$.

2.9.Statistical analysis

Statistical analyses were performed using GraphPad Prism 9.0 (GraphPad Software Inc., USA). To analyze statistical significance in cumulative mortality among groups, Kaplan-Meier survival plots were generated, and the statistical significance was determined by the log-rank test. The data on the viral copy number and type I interferon response were analyzed by Student's *t*-test. Differences with $p<0.05$ were considered statistically significant.

Table 1-1 Primers used in Chapter I

Primer		Sequence 5'→3'
For the construction of pT-Psvcv		
SVCV-P-KpnI-F		GGTACCATGTCTCTACATTCGAAATTGTCAGAAAGTC
SVCV-P-MluI-R		ACGCGTTTACAACCTATATTTTTGATACAACTTATTGTACAATCTCC
For RT-PCR (verification of the insertion of SVCV P gene into the rescued rSHRV)		
Mid SHRV N to mid SVCV P	SHRV-midN-F	GGAGTATTGTACACATGCGCGTT
	SVCV_mid_P-R	CTCTGTACGTATGCCAGGTCGG
Mid SHRV G to SHRV L (the first 900bp)	SHRV_G_TM_F	ACTCTCTGGCCATCTCTCTCGG
	SHRV_6397R	GCTTGCGTGTCTCTGACTTGATTAGC
Backbone vector	T_easy_OC_AmpR_F2	TGCGGCGACCGAGTTGCTCTTGC
	T_easy_OC_R	GGCGTAATCATGGTCATAGCTGT
For real time qPCR		
SHRV-IPC2-Fwd		TGGATTCAGTGTAAA GGAGGTTC
SHRV-IPC2-Rev		CAGTCTCGGGCTTGACTAATG
SHRV-IPC2-Prob-Cy5		ATTCTCCAGCGGCGCCTCAATC

3. Results

3.1. Minigenome assay

BHK cells co-transfected with SHRV minigenome (Fig. 1a) and helper vectors were monitored for 7 days to verify the interaction between SHRV N, L, and SVCV P genes. Cells having the SHRV minigenome together with SHRV N, P, and L genes showed fluorescence (Fig. 1b), but cells supplied with the SVCV P gene instead of the SHRV P gene showed no fluorescence for 7 days.

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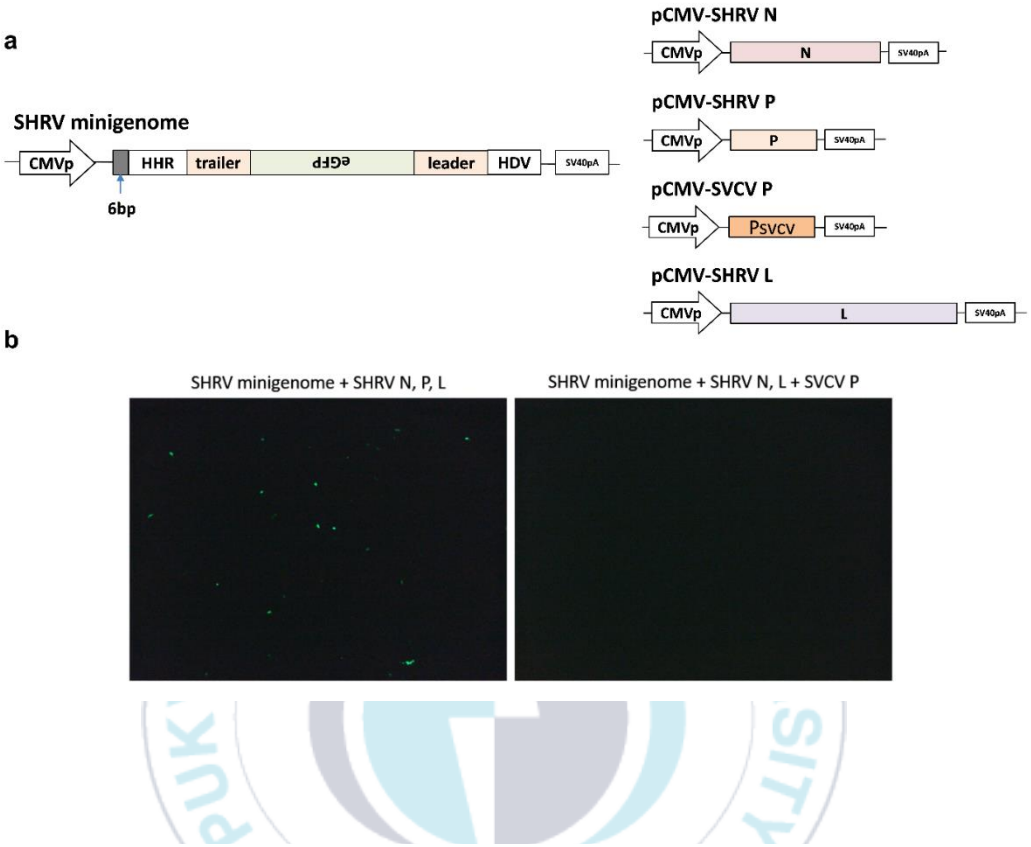

Figure 1. SHRV minigenome assay. a: SHRV minigenome construct and the helper vectors constructs. b: Comparison of SHRV minigenome functioning when the construct was co-transfected with SHRV P helper plasmid or SVCV P helper plasmid.

3.2.Rescue of rSHRV-A-Psvcv and *in vitro* growth

The rSHRV-A-Psvcv was successfully rescued by the co-transfection of EPC cells with pSHRV-A-Psvcv (Figure 2-a) and helper vectors. The generation of rSHRV-A-Psvcv was confirmed by sequencing, RT-PCR (Figure 2-b), and plaque assay (Figure 2-c). The *in vitro* growth of rSHRV-A-Psvcv was compared with that of rSHRV-AeGFP, in which the plaque number of rSHRV-A-Psvcv was significantly higher than that of rSHRV-A-eGFP at 48 and 72 h (Figure 2-d).

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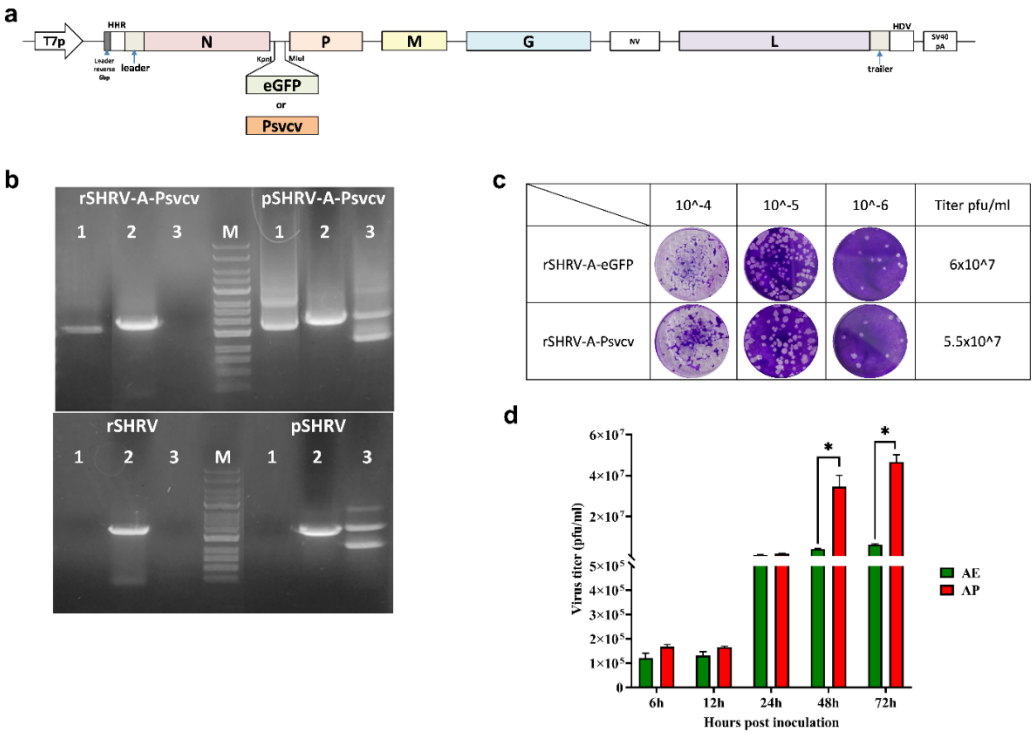

Figure 2. Rescue of rSHRV-A-Psvcv and *in vitro* viral growth. (a) Construct Schematic for the generation of rSHRV-A-Psvcv. (b) Verification of rSHRV-A-Psvcv production by RT-PCR: lane 1 presents the amplified region from the middle of SHRV N gene to SVCV P gene (1643bp), lane 2 is the amplified region between SHRV G gene and SHRV L gene (1823bp) and lane 3 represents the amplified region from the backbone vector (1097bp), M is DNA size marker. (c) the result of plaque assay determining the viral stock titer and verifying the production of rSHRV-A-Psvcv. (d) viral growth comparison of rSHRV-A-Psvcv and rSHRV-A-eGFP by plaque assay. The asterisk refers to the significant difference at $p < 0.05$.

3.3.*In vitro* antiviral response against rSHRV-A-Psvcv

To examine the effect of SVCV P gene insertion into SHRV genome on type I interferon response, the expression level was analyzed by luciferase assay following virus infection. Cells infected with rSHRV-A-Psvcv showed significantly lower luciferase activity than cells infected with rSHRV-AeGFP at both MOIs of 0.01 and 0.001 (Figure 3-a, b).

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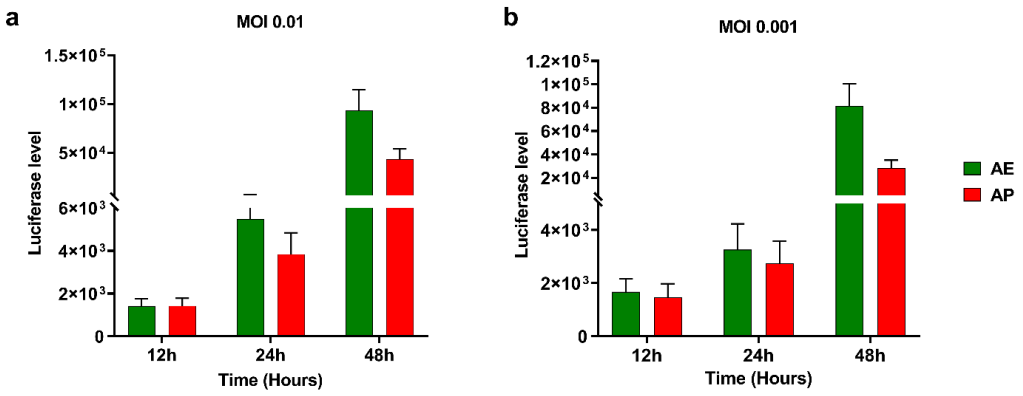

Figure 3. *In vitro* effect of SVCV P gee inserted into SHRV genome on Type I interferon response. (a) and (b) shows the luciferase activity from cells inoculated with rSHRV-AeGFP or rSHRV-A-Psvcv at MOIs of 0.01 and 0.001, respectively.

3.4.Cumulative mortality and viral replication in zebrafish

To verify the *in vivo* virulence of rSHRV-A-Psvcv, mortality of zebrafish was monitored for 21 days following the infection of rSHRV-A-Psvcv and rSHRV-AeGFP at 15°C and 28°C. No mortality was observed in the groups of fish infected with rSHRV-A-Psvcv and rSHRV-AeGFP at 28 °C. Mortality was noticed only from the group of fish infected with rSHRV-A-Psvcv at 15°C (cumulative mortality 60% in replicate 1 and 40% in replicate 2), while no death was observed from the group of fish infected with rSHRV-AeGFP at 15 °C (Figure 4-a).

The *in vivo* viral growth analysis using RT-qPCR showed that the peak titers of rSHRV-A-Psvcv (at 7 d at 15 °C and at 3 d at 28 °C) were significantly higher than those of rSHRV-AeGFP (Figure 4-b).

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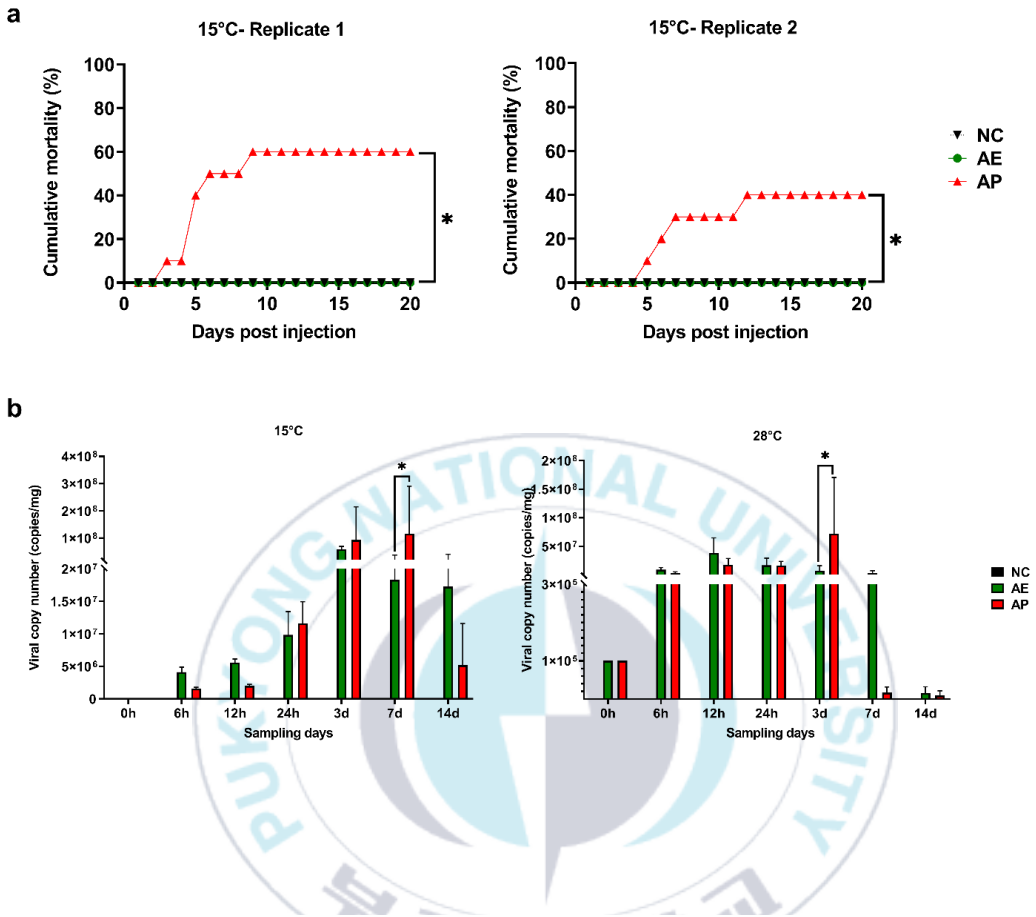

Figure 4. *In vivo* virulence test of rSHRV-A-Psvcv. (a) cumulative mortality of zebrafish infected with rSHRV-A-eGFP or rSHRV-A-Psvcv at 15°C. (b) Comparison of the *in vivo* viral growth of rSHRV-A-eGFP and rSHRV-A-Psvcv at 15°C and 28°C. The asterisks show the significant difference at $p<0.05$.

3.5.Transcriptome and analysis of DEGs

Nine cDNA libraries from spleen of the control group (NC), rSHRV-A-eGFP (AE) and rSHRV-A-Psvcv (AP) were made and analyzed by Illumina sequencing. On average, a total read of 416,639,683 raw reads were obtained. The quality score (Q30) was 94.6%. The reads were mapped to the reference genome of zebrafish *Danio rario*, representing 82.52%-87.71% of mapped rate (Table 1-2).

To identify the DEGs between the control group and rSHRV-A-eGFP (AE) or rSHRV-A-Psvcv (AP) infected group at each time point, pairwise comparison was carried out: NC vs AE 12h, NC vs AE 24h, NC vs AE 72h, NC vs AP 12h, NC vs AP 24h, NC vs AP 72h. a total of 1308 DEGs were identified from all the groups applying to the criteria for the FDR < 0.05 and the absolute value of fold change > 2. According to the time, the up regulate DEGs For NC vs AE and NC vs AP increased. At 12h, 93 up regulated and 54 down regulated were identified for NC vs AE and 20 up regulated and 333 down regulated from NC vs AP. At 24h, 175 up regulated and 107 down regulated were identified for NC vs AE and 155 up regulated and 29 down regulated from NC vs AP. At 72h, 146 up regulated and 12 down regulated were identified for NC vs AE and 155 up regulated and 65 down regulated from NC vs AP (Figure 5).

The KEGG pathway analysis was performed to explore the pathway related to each virus infection. At 12h, for both groups (NC vs AE and NC vs AP), the enriched pathways were not related to viral infection (Table 1-3). “Motor proteins”, “ECM-receptor interaction” and “calcium signaling pathway” were enriched in AE group and “Cell cycle”, “steroid hormone biosynthesis” and “motor proteins” in AP group. At 24h, antiviral response related pathways

were activated in the AE group naming “Toll-like receptor signaling pathway”, “NOD-like receptor signaling pathway”, “RIG-I-like receptor signaling pathway” and “Cytokine-cytokine receptor interaction” then at 72h only “Cytokine-cytokine receptor interaction” was enriched in AE. Meanwhile, from AP group, at 24h “motor proteins” and ‘cell cycle” were enriched, then at 72h, “Cytokine-cytokine receptor interaction”, “Toll-like receptor signaling pathway” and “RIG-I-like receptor signaling pathway” were enriched (Table 1-3).

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Table 1-2. Summary of RNA-seq (transcriptome) analysis

Experimental Group	Raw Reads	Q30 (%)	Mapping Rate
NC 12h	38,035,375	94.53	86.58
AE 12h	47,554,204	94.86	86.44
AP 12h	37,886,226	94.82	84.24
NC 24h	49,887,246	94.35	87.71
AE 24h	47,647,760	94.76	84.96
AP 24h	44,727,530	94.39	87.54
NC 72h	46,388,872	94.69	86.83
AE 72h	53,067,432	94.57	83.80
AP 72h	51,445,040	94.76	82.52

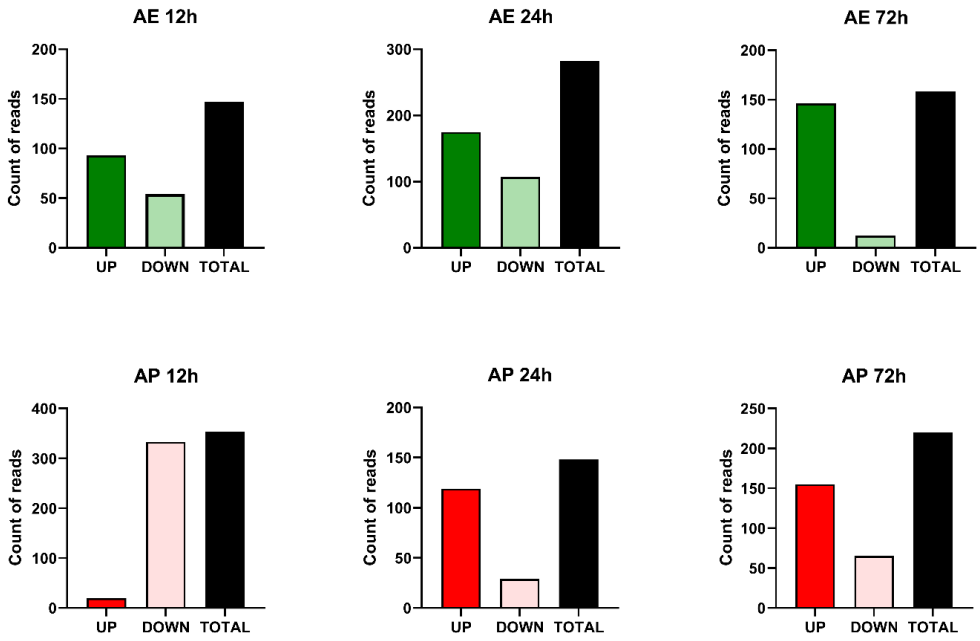

Figure 5. Number of Differentially of up regulated and down regulated reads for rSHRV-A-eGFP (AE) and rSHRV-A-Psvcv (AP) at 12h, 24h and 72h post infection.

Table 1-3. KEGG pathway enrichment result

GROUP	DESCRIPTION	P ADJUST	COUNT
NC-AE 12h	Motor proteins	2.30E-08	42
	ECM-receptor interaction	0.000303944	19
	Calcium signaling pathway	0.0287246	37
NC-AP 12h	Cell cycle	0.003642784	24
	Steroid hormone biosynthesis	0.013978694	13
	Motor proteins	0.096628814	24
NC-AE2 4h	Toll-like receptor signaling pathway	0.000216398	16
	NOD-like receptor signaling pathway	0.004877917	18
	RIG-I-like receptor signaling pathway	0.007447268	10
	Cytokine-cytokine receptor interaction	0.018842247	19
NC-AP 24h	Motor proteins	8.79E-06	34
	Glycolysis / Gluconeogenesis	0.058721322	11
NC-AE 72h	Cytokine-cytokine receptor interaction	7.47E-05	36
	Cell cycle	0.011609215	27
NC-AP 72h	Cytokine-cytokine receptor interaction	0.001616223	20
	Toll-like receptor signaling pathway	0.001616223	13
	RIG-I-like receptor signaling pathway	0.045710597	8

4. Discussion

The P protein of rhabdoviruses is essential for viral replication through interaction with N and L proteins (Ivanov et al., 2011; Riedel et al., 2020). In the present SHRV minigenome analysis, helper vectors composed of SHRV N protein, SHRV L protein, and SVCV P protein could not induce the expression of the minigenome-encoding eGFP, suggesting that SVCV P protein did not participate in the formation of the ribonucleoprotein complex with SHRV N and L proteins. This result led to a hypothesis that a recombinant SHRV that additionally expresses SVCV P protein can be used as a tool to analyze the role of SVCV P protein in the *in vivo* virulence which is independent of the role of P protein as a part of the ribonucleoprotein complex.

In the *in vitro* growth results, rSHRV-A-Psvcv showed significantly higher titers than rSHRV-AeGFP. Furthermore, in the *in vivo* virulence results, no mortality was recorded from the zebrafish group injected with rSHRV-AeGFP. However, mortality was noted from the fish group injected with rSHRV-A-Psvcv accompanying hemorrhage and ophthalmia, the symptoms related to SVCV infection. In spite of no direct participation of SVCV P protein in SHRV replication, the higher replication ability of rSHRV-A-Psvcv and the induction of zebrafish mortality by infection with rSHRV-A-Psvcv suggested that the virulence of SHRV was enhanced by the SVCV P gene and the enhanced virulence might be mediated by the SVCV P protein's characteristics.

It was reported that the phosphorylation of the SVCV P protein by TBK-1 had an impact on the level of phosphorylated IRF3, IRF7, and mediator of IRF3 activation (MITA) (Li et al., 2016). Moreover, (Huang et al., 2022) reported that the SVCV P protein promoted the expression and accumulation of IRF2a (belongs to the IRF1 subfamily and negatively regulates IFN response),

resulting in the reduction of STAT1 activity to disrupt the IFN-mediated signaling. However, these results only indirectly suggested the possible role of the SVCV P protein in viral virulence. In the present study, we could demonstrate the role of the SVCV P protein in viral virulence by a recombinant viral system not by a recombinant protein expression system. In this study, EPC cells infected with rSHRV-A-Psvcv showed significantly lower type I interferon response compared to the cells infected with rSHRV-A-eGFP, suggesting that the SVCV P protein expressed by the recombinant SHRV still possess the inhibitory activity against type I interferon response. Therefore, type I interferon suppressive activity of the SVCV P protein might be one of the causes of the enhanced virulence of rSHRV-A-Psvcv.

The *in vivo* virulence of a virus is a complex phenomenon involving diverse viral and host factors (Rothenburg & Brennan, 2020). In this study, despite 40-60% mortality of zebrafish by rSHRV-A-Psvcv infection at 15 °C, zebrafish kept at 28 °C did not show any mortality by rSHRV-A-Psvcv infection. In the viral growth, the titers of rSHRV-AeGFP and rSHRV-A-Psvcv increased according to time-lapse at 15 °C but did not show a gradually increasing pattern at 28 °C. Although further supplemental experiments are needed, the present results suggest that low temperature-mediated immunosuppression and weakening might increase the sensitivity of zebrafish to viral virulent factors.

Following the analysis of the RNA-seq, the KEGG analysis showed that the immune-relevant pathways (Toll-like receptor signaling pathway (TLRs), NOD-like receptor signaling pathway (NLRs), RIG-I-like receptor signaling pathway (RLRs, Cytokine-cytokine receptor interaction) from the fish infected with rSHRV-A-eGFP appeared at early time of infection (24h) then decreased.

However, for the rSHRV-A-Psvcv, the stimulation of those pathways was triggered lately at 72h post infection. Host immune system tend to eliminate pathogens such as viruses, after recognition by pattern recognition molecules/pathogen recognition receptors (PRRs) which are classified into TLRs, NLRs, RLRs (Loo et al., 2007; Takeuchi & Akira, 2009; Wei et al., 2016). The activation of those pathways usually occurs at an early stage of the infection and their initiation leads to the induction of the Type I interferon response and its related genes such as the IRF3 (Yokota et al., 2010).

The late appearance of those pathways in the fish samples infected with rSHRV-A-Psvcv compared to the one infected with rSHRV-A-eGFP, could be related to the presence of SVCV P which has a role in suppressing the genes that are related to those pathways, so it give time to the virus to replicate and weaken the host system which lead to increase the virulence and the mortality that occurred during the *in vivo* experiment.

The present study showed a way to analyze the role of a viral gene in virulence using a recombinant SHRV system. As the SHRV titer used in this study could not induce mortality in zebrafish, the occurrence of mortality by recombinant SHRVs expressing a heterologous gene can be an indication of whether the heterologous gene is involved in the virus virulence. The initiation of those pathways leads to the induction of several cytokines like type I interferon (IFN).

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Chapter II: Generation of recombinant SHRV expressing artificial microRNA targeting SVCV P gene and *in vivo* therapeutic usage against SVCV infection

1. Introduction

Spring viremia of carp virus (SVCV) belongs to the genus Sprivivirus in the family Rhabdoviridae (Walker et al., 2021) and has a negative-strand RNA genome encoding five structural proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and RNA-dependent RNA polymerase (L). SVCV has caused significant mortalities in common carp (Cyprinus carpio) and other cyprinid fish species (Ahne et al., 2002), and its highly lethal infection has posed a major threat to the aquaculture industry, leading to being listed as a notifiable disease by the World Organization for Animal Health (WOAH). However, therapeutic, and prophylactic strategies adopted for SVCV have been limited to specific types of non-commercialized vaccines or mainly to severe hygienic measures (Ashraf et al., 2016).

Regulation of gene expression through microRNAs (miRNAs), known as RNA interference (RNAi), has been extensively investigated in eukaryotes to uncover the role of genes of interest or to use as a tool for the therapy against cancers (O'Brien et al., 2018; Rupaimoole & Slack, 2017). Primary miRNAs (pri-miRNAs) transcribed in the nucleus are cleaved by the microprocessor complex composed of Drosha/DGCR8 to give the precursor miRNAs (pre-miRNAs) which are exported to the cytoplasm by exportin-5, then processed by the RNase III endonuclease Dicer giving the miRNA duplex consisting of around 22 nucleotides. One strand (guide miRNA) is chosen from the miRNA duplex and assembled with the RNA-induced silencing complex (RISC). The target mRNA having the complementary sequence to guide miRNA is silenced through mRNA cleavage or translation inhibition (Davis & Hata, 2010;

Filipowicz et al., 2008; Gregory et al., 2005; V. N. Kim et al., 2009; Siomi & Siomi, 2010).

RNAi-mediated knockdown of gene expression has been done with synthesized small interfering RNA (siRNA), short- or long-hairpin RNA (shRNA or lhRNA), or artificial miRNA (Kotowska-Zimmer et al., 2021). Artificial miRNA is designed based on an endogenous primary miRNA, but the original stem region sequence is replaced with a specific sequence complementary to the target gene sequence. Compared to shRNA or siRNA, artificial miRNA offers an alternative tool that presents a higher RNAi efficiency with reduced cytotoxicity and a broad usage spectrum when suitable promoters are available (Jopling et al., 2005; Zeng et al., 2005). Artificial miRNAs have been studied for their therapeutic features against diseases such as cancers or viral-related diseases in mammals (Waring et al., 2018; Wen et al., 2022).

In fish rhabdoviruses, *in vitro* inhibitory effect of shRNA targeting the L gene or G gene on VHSV replication was reported (M. S. Kim & Kim, 2011; Ruiz et al., 2009). Recently, the inhibition of SVCV replication using siRNAs targeting SVCV N gene and P gene transcripts (Gotesman et al., 2015), or SVCV L gene transcript (Fouad et al., 2019) in Epithelioma papulosum cyprini (EPC) cells was reported. Furthermore, Fouad et al. (Fouad et al., 2022) reported *in vivo* inhibitory role of siRNAs targeting SVCV M gene and L gene transcripts in common carp. However, more efficient delivery tools are required to use RNAi as an *in vivo* measure for the control of fish viruses.

The successful application of reverse genetic technology to produce recombinant Novirhabdoviruses has extended the use of recombinant viruses

for uncovering viral virulence mechanisms, enhancing vaccine protective ability, and delivery of diverse functional genes or proteins (M. S. Kim & Kim, 2019; Kwak et al., 2021). Recently, Kwak et al. (Kwak et al., 2019) reported the delivery of microRNA-30e using recombinant VHSV. Furthermore, Kwak and Kim (Kwak & Kim, 2021) produced self-replication-limiting VHSV by inserting the viral P gene-targeting artificial microRNA cassette into the viral genome.

In the present study, we generated a new recombinant snakehead rhabdovirus (SHRV) expressing SVCV P gene-targeting artificial microRNA (rSHRV-AmiR-P) and evaluated *in vivo* therapeutic effect of the recombinant SHRV against SVCV in zebrafish (*Danio rerio*).

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2. Materials and Methods

2.1.Cells and viruses

Epithelioma papulosum cyprini (EPC) cells and baby hamster kidney-21 (BHK-21) cells were cultured in Leibovitz's L-15 medium (Gibco) at 28 °C and in Dulbecco's modified Eagle's medium (DMEM, Welgene) with 5% CO_2 at 37 °C, respectively. Each medium was supplemented with 10% fetal bovine serum (FBS, Welgene) and antibiotic-antimycotic solution (Anti-Anti, Gibco). SVCV (K1 strain isolated from common carp in the year 2010 in Korea) and SHRV (ATCC-VR1386) were propagated on EPC cells with 2% FBS and antibiotics. Once cytopathic effect (CPE) broadly appeared, the supernatant was collected by centrifugation and filtered through a 0.45 μm syringe filter, then stored at -80°C. The titration of each virus was determined by plaque assay.

2.2.Suppression of SVCV P gene transcript by artificial microRNA (AmiRNA) mimics

The suppressive effect of AmiRNA mimics on SVCV P gene expression was analyzed by dual luciferase assay. Several sites of AmiRNA targeting the SVCV P gene were predicted by the Block-iT RNAi Web Designer tool (Invitrogen), among which the top three sites were selected and designated as AmiR-P1, AmiR-P2, and AmiR-P3, then the three AmiRNA mimics were synthesized by Bioneer (Korea). As a control mimic (AmiR-C), the AccuTarget miRNA negative control (Bioneer) was used. The region of the SVCV P gene containing the three sites of AmiRNA was amplified by PCR using primers containing *Xba*I (forward) and *Sal*I (reverse) enzyme sites (Table. 2-1). The amplicon was cloned into pGEM-T easy vector (Promega), digested with *Xba*I

and *Sal*I enzymes, then inserted into the downstream of the firefly luciferase gene of the pmiRGLO vector (Promega). The constructed plasmid was designated as pmiRGLO-Psvcv. BHK-21 cells (1 x 10^4 cells/well in a 96-well plate) were transfected with the pmiRGLO-Psvcv (30 ng) along with each of the synthesized AmiRNA mimics targeting SVCV P gene or the control mimic (100 μM) using Fugene HD (Promega) as the transfection reagent. To determine the effect of each mimic on SVCV P gene expression, firefly and *Renilla* luciferase activities were measured at 24 h post-transfection using the Dual-Glo Luciferase Assay kit (Promega) following the manufacturer's instruction with Victor X3 plate reader (PerkinElmer). The experiment was conducted in triplicate, and the mean value of the relative luciferase ratio of the control mimic was normalized to 1. To verify whether the inhibition was dose-dependent or not, BHK-21 cells were co-transfected with the pmiRGLO-Psvcv and AmiR-P3 at 25, 50, or 100 nM, then the activity of luciferases was analyzed according to the above method.

2.3.Construction of vectors expressing AmiR-P1, AmiR-P3, and AmiR-C

To construct artificial microRNA-expressing vectors based on miR-155 scaffold, a previously constructed plasmid (pcDNA3.1-eGFP-amiR-C-VHSV-P; (Kwak & Kim, 2021)) containing two cassettes driven by CMV promoter was used as a backbone vector. The stem-loop region of the microRNA and the VHSV P gene in the previously constructed plasmid (pcDNA3.1-eGFP-amiR-C-VHSV-P) was replaced with the present AmiR-C, AmiR-P1, or AmiR-P3 sequence and the SVCV P gene, respectively, using the overlap cloner kit (Elpis, Korea), then designated as peGFP-AmiR-C-SVCV-P, peGFP-AmiR-P1-SVCV-P, and peGFP-AmiR-P3-SVCV-P. The primers used for the construction are in

Table 1. As a second step, the constructed plasmids (3 μg) were transfected into BHK-21 cells (5 x 10^5 cells/ml) using Fugene HD. To estimate the expression of the SVCV P gene under the effect of each artificial microRNA, cells were harvested at 48 h post-transfection, lysed with RiboEx (GeneAll), and extracted RNA using a Hybrid R kit (GeneAll, Korea). One μg of the extracted RNA was used for cDNA synthesis using RT Master Premix-Oligo dT (Elpis), then semi-quantitative RT-PCR was performed to verify whether the AmiR-P3 expressed through vector system can suppress the expression of SVCV P gene transcript. The primers used for the PCR are in Table 2-1.

2.4.Generation of recombinant SHRVs expressing artificial microRNA

To verify the effect of AmiR-P3 on SVCV *in vivo*, rSHRVs expressing AmiR-C or AmiR-P3 were rescued using reverse genetics. The pri-miRNA sequence of AmiR-C or AmiR-P3 was inserted between the N and P genes of the SHRV genome using restriction enzymes (*Kpn*I and *Mlu*I) and T4 DNA ligase (Elpis). The constructs were designated as prSHRV-AmiR-C and prSHRV-AmiR-P3, respectively. EPC cells were co-transfected with the constructed plasmids and previously constructed helper vectors (Kwak et al., 2020) - CMV-SHRV N, CMV-SHRV P, CMV-SHRV L, and T7 RNA polymerase expressing plasmid (CMV-T7pol) - using Fugene HD. When typical CPE appeared, the supernatant was collected, centrifuged, filtered with a 0.45 μm syringe filter, then passaged on EPC cells. The titration of viral stocks was carried out by plaque assay. The produced viruses were designated as rSHRV-AmiR-C and rSHRV-AmiR-P3, respectively.

To verify the expression of AmiR through the recombinant SHRVs, BHK-21 cells were inoculated with rSHRV-AmiR-C or rSHRV-AmiR-P3 at MOI 0.01. At 72 h post-inoculation, cells were harvested, and small RNA was extracted with a Hybrid-RTM miRNA kit (GeneAll). cDNA synthesis and AmiR quantification were done using HB Multi Assay Kit system II (HeimBiotek, Korea). The primers used in both cDNA synthesis and quantitative PCR (qPCR) for AmiR-C, AmiR-P3, and small RNA U6 (a reference gene) were provided by Heimbiotek. qPCR was performed with LightCycler 480 (LC480) (Roche), and the expression level of both AmiR-C and AmiR-P3 was analyzed via the comparative threshold method ($2^{-\Delta\Delta Ct}$).

2.5. Effect of rSHRV-AmiR-P3 on SVCV infection-mediated mortality in zebrafish

To verify whether AmiR-P3-expressing rSHRV can reduce mortality by SVCV infection, zebrafish (average weight 0.3g) were divided into eight groups: Group 1, negative control group injected with 20 µl of L-15 medium alone; Group 2, positive control group injected with 1 x 10^3 pfu/20 µl of SVCV; Group 3, fish injected with 1 x 10^4 pfu/20 µl of rSHRV-AmiR-C; Group 4, fish injected with 1 x 10^4 pfu/20 µl of rSHRV-AmiR-P3; Group 5, fish injected with 1 x 10^4 pfu/20 µl of rSHRV-AmiR-C, then injected with 1 x 10^3 pfu/20 µl of SVCV one day later; Group 6, fish injected with 1 x 10^4 pfu/20 µl of rSHRV-AmiR-P3, then injected with 1 x 10^3 pfu/20 µl of SVCV one day later; Group 7, fish injected with 1 x 10^3 pfu/20 µl of SVCV, then injected with 1 x 10^4 pfu/20 µl of rSHRV-AmiR-C one day later; Group 8, fish injected with 1 x 10^3 pfu/20 µl of SVCV, then injected with 1 x 10^4 pfu/20 µl of rSHRV-AmiR-P3 one day later. The experiment was performed with duplicate (12 fish/tank) at 14 ± 1 °C, and the

mortality was recorded daily. At the end of the experiment, to enumerate the quantity of SVCV from dead fish, RT-qPCR was conducted with 2 x SYBR Green Premix (Enzynomics, Korea), 2 µl of synthesized cDNA, and 5 pMol of primers (amplifying around 100 nt of SVCV N gene) using LightCycler 480 (Roche).

2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 9 (GraphPad Software, USA). Differences in dual luciferase assay and in semi-quantitative RT-PCR were analyzed using one-way ANOVA followed by Tukey HSD post-hoc test, and the relative quantification of artificial miRNAs was analyzed using Student's *t*-test. Survival kinetic data were analyzed by Kaplan-Meier survival analysis based on the Log-Rank (Mantel-Cox) test. The *p*-values below 0.05 were considered statistically significant.

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Table 2-1 primers used in Chapter II

Primers		Sequence (5'-3')
For the construction of pmiRGLO-Psvcv		
SVCV-P_Luc-XbaI-F		**TCTAGA**GATCCCCGATTACCTCAGAGAGAATAG
SVCV-P_Luc-SalI_R		**GTCGAC**GAAATCAAGCTCTTATAATTTGGGTCTACG
For the construction of peGFP-AmiR-P3-SVCV-P		
Fragment 1	SVCV-P-SV40p-OC-F	CAGGATGGTGAGCAAGGGCGATGTCTCTACATTCGAAATTGTCAGAAAG
	SVCV-P-amiR3_OC_R	GTCAGTCAGTGGCCAAAACTTCGTTATAACGGCCTCGGTACAGCATACAG CCTTC
Fragment 2	SVCV-P-amiR3_OC_F	GTTTTGGCCACTGACTGACTTCGTTATCGGCCTCGGTACAGGACACAAG
	SV40_OC_eGFP_R	CGCCCTTGCTCACCATCCTGTCTCTTGATCAGATC
For the construction of peGFP-AmiR-P1-SVCV-P		
Fragment 1	SVCV-P-SV40p-OC-F	CAGGATGGTGAGCAAGGGCGATGTCTCTACATTCGAAATTGTCAGAAAG
	miRNA-SVCV-P-OC-R	GTCAGTGGCCAAAACGAATAGCAGCATCACAGTGGACAGCATACAGCCTT C
Fragment 2	miRNA-SVCV-P-OC-F	GTTTTGGCCACTGACTGACGAATAGCAATCACAGTGGACAGGACACAAG GCC
	SV40_OC_eGFP_R	CGCCCTTGCTCACCATCCTGTCTCTTGATCAGATC
For the construction of peGFP-AmiR-C-SVCV-P		
Fragment 1	SVCV-P-SV40p-OC-F	CAGGATGGTGAGCAAGGGCGATGTCTCTACATTCGAAATTGTCAGAAAG
	SVCV-P-SV40pA-OC-R	CCGCACCAATCGAATTCCCTTACAACCTATATTTTTGATACAACTTATTGTA CAATC
Fragment 2	SV40_OC_F	GGGAATTCGATTGGTGCGGGACTCTGGGGTTCG
	SV40_OC_eGFP_R	CGCCCTTGCTCACCATCCTGTCTCTTGATCAGATC

3. Results

3.1. Suppression of SVCV P gene transcript by artificial microRNA (amiRNA) mimics

The RNAi effect of the designed artificial microRNAs targeting the SVCV P gene was determined by dual luciferase assay (Figure 6-a) using the corresponding three different AmiR mimics. The luciferase value estimated from the cells transfected with AmiR targeting SVCV P gene mimics was significantly lower than the one from the cells transfected with the control mimic (Figure 6-b). As AmiR-P3 mimic showed the lowest luciferase value among the three mimics, AmiR-P3 was selected and further tested to know the effect of different doses. The two concentrations (25 nM and 50 nM) of AmiR-P3 mimic showed similar luciferase values to each other, but the 100 nM of AmiR-P3 mimic showed a significantly lower value than the two lower concentrations (Figure 6-c).

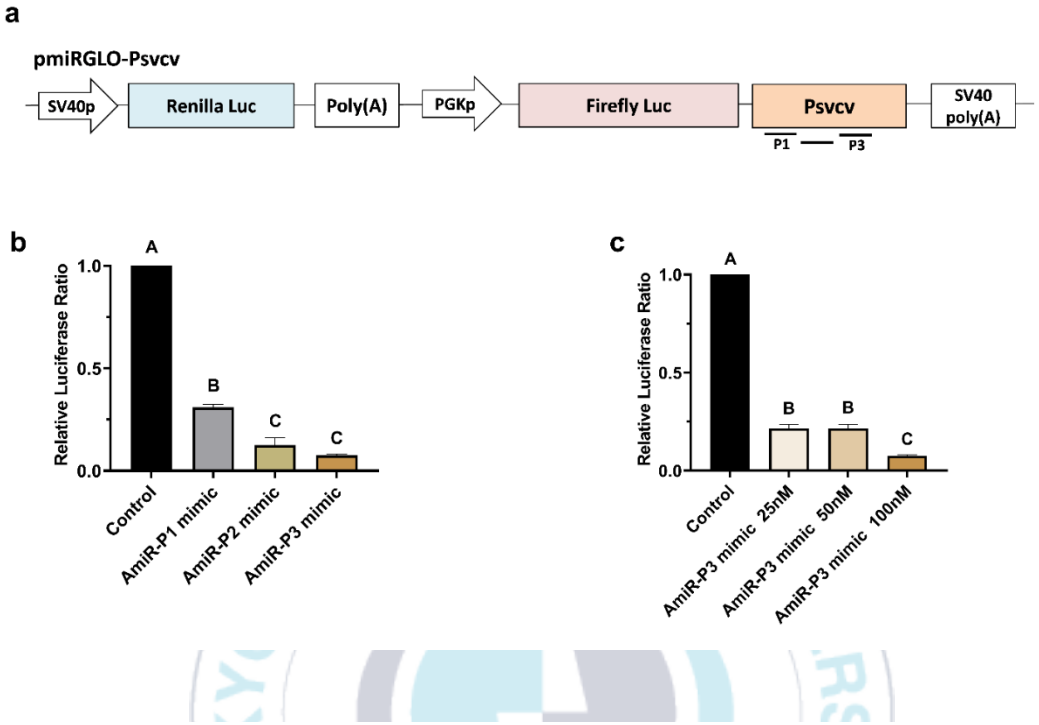

Figure 6. RNAi effect of the designed AmiR targeting SVCV P gene on the target gene expression. (a) Schematic of expression vector used for the dual Luciferase assay. (b) The relative Luciferase ratio measured from BHK-21 cells transfected with the top three selected SVCV P targeting AmiRNA mimics (AmiR-P1, AmiR-P2, AmiR-P3) along with the expression vector harboring SVCV P gene downstream Firefly Luciferase (pmiRGLO-Psvcv). (c) The relative Luciferase ratio measured from BHK-21 cells co-transfected with different concentrations of AmiR-P3 (25nM, 50nM, 100nM) and pmiRGLO-Psvcv. The asterisks indicate statistical significance ($p<0.05$).

3.2.Suppression of SVCV P gene transcript by artificial microRNA-expressing vector system

To verify the present artificial primary microRNA can inhibit the target mRNA in a sequence-specific manner, vectors expressing AmiR-P1 or AmiR-P3 and SVCV P gene (Figure 7-a) were transfected into BHK-21 cells, then the P gene expression was determined by semi-quantitative RT-PCR. The intensity of the amplified PCR bands from the cells transfected with the vector expressing AmiR-P3 or AmiR-P1(peGFP-AmiR-P1-SVCV-P and peGFP-AmiR-P3-SVCV-P) was significantly decreased compared with the one corresponding to the P gene expression from the cells transfected with the vector expressing AmiR-C (peGFP-AmiR-C-SVCV-P). Moreover, the band intensity from the cells transfected with the vector expressing AmiR-P3 was clearly lower than the one from the cells transfected with the vector expressing AmiR-P1 (Figure 7-b, c).

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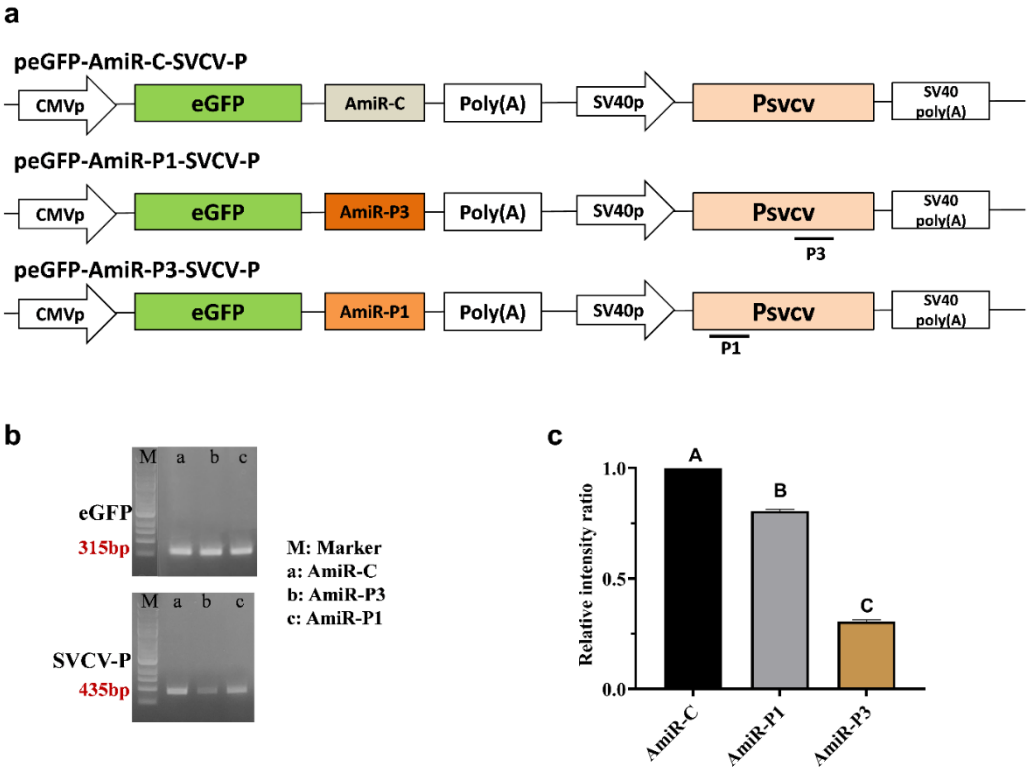

Figure 7. Suppression of SVCV P gene targeting AmiRNA vector. (a) Schematic of AmiRNA expression vectors used for the verification of AmiRNA suppressive effect on SVCV P gene expression. the expression vector contains eGFP and AmiRNA (SVCV P targeting AmiRNA-P3 or scrambled sequence in the stem region for the control microRNA) cassette driven by CMV promoter and SVCV P gene expressing cassette driven by SV40 promoter. (b) Semi-quantitative RT-PCR electrophoresis bands intensity were used for measuring AmiR-P3 mediated SVCV P gene silencing by image analysis. Asterisks indicates statistical significance ($p<0.05$).

3.3.Generation of rSHRVs expressing AmiR-C or AmiR-P3

Recombinant SHRVs expressing AmiR-C and AmiR-P3 (Figure 8-a) were successfully rescued, and titers of stocks were measured by plaque assay. The plaque number of rSHRV-AmiR-C and rSHRV-AmiR-P3 was 4.4 x 10^6 pfu/ml and 4.7 x 10^6 pfu/ml, respectively. The expression of mature AmiR-C and AmiR-P3 was verified by the infection of BHK-21 cells with rSHRV-AmiR-C or rSHRV-AmiR-P3, then the expression of each mature microRNA was measured by qPCR. The results showed that the expression of AmiR-P3 or AmiR-C successfully occurred when cells were infected with each corresponding rSHRV (Figure 8-b).

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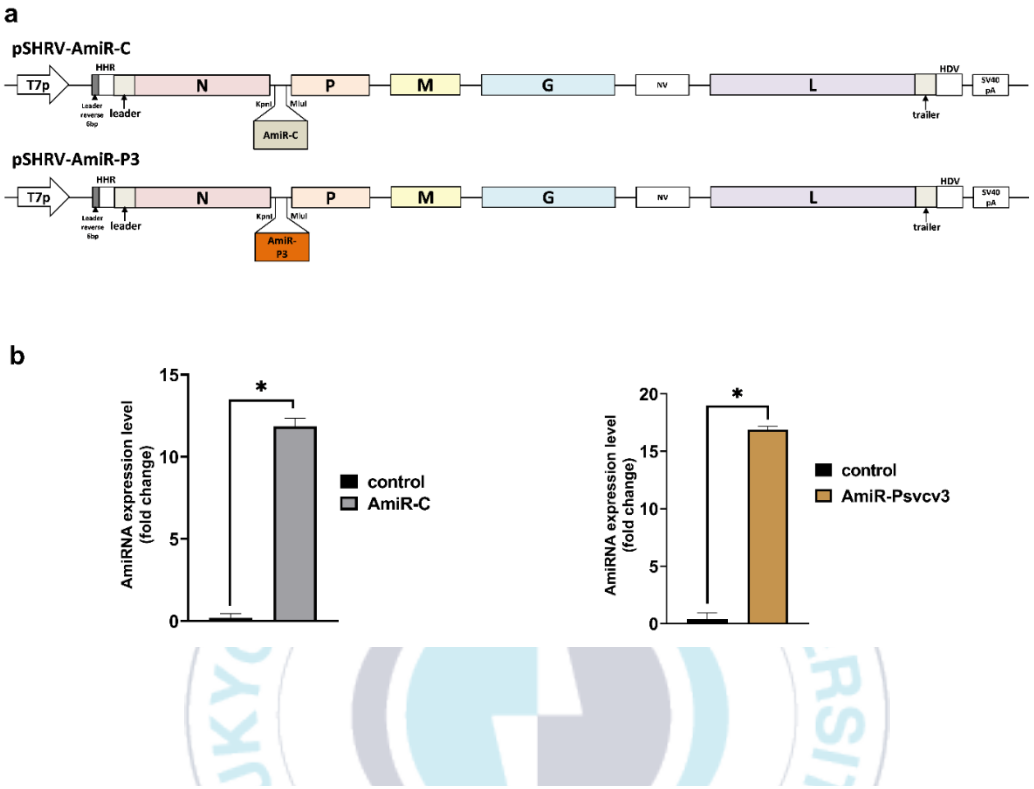

Figure 8. Expression of AmiRNA targeting SVCV P gene through recombinant SHRV (rSHRV). (a) Schematic of SHRV full viral genome expression vector expressing AmiR-C or AmiR-P3 between N and P genes for the virus rescue. (b) Quantification of AmiRNAs expression level by relative qPCR from BHK-21 cells infected with rSHRV-AmiR-C or rSHRV-AmiR-P3. Asterisks indicates statistical significance ($p<0.05$).

3.4.Effect of rSHRV-AmiR-P3 on SVCV infection-mediated mortality in zebrafish

Zebrafish infected with SVCV alone showed 100% mortality in 10 days (Figure 9-a). On the other hand, zebrafish infected with rSHRV-AmiR-C or rSHRV-AmiR-P3 before SVCV infection showed the highest survival rates against SVCV infection. However, there were no statistical significances between groups infected with rSHRV-AmiR-C and rSHRV-AmiR-P3 (Figure 9-a). Although the survival rates of fish infected with rSHRV-AmiR-C or rSHRV-AmiR-P3 after SVCV infection were lower than groups infected with the rSHRVs before SVCV infection, fish infected with rSHRV-AmiR-P3 showed significantly higher survival rates than fish infected with rSHRV-AmiR-C in both duplicates (Figure 9-a). All dead fish in the groups of fish infected with SVCV showed more than 1 x 10^8 copies/mg of body weight (Figure 9-b).

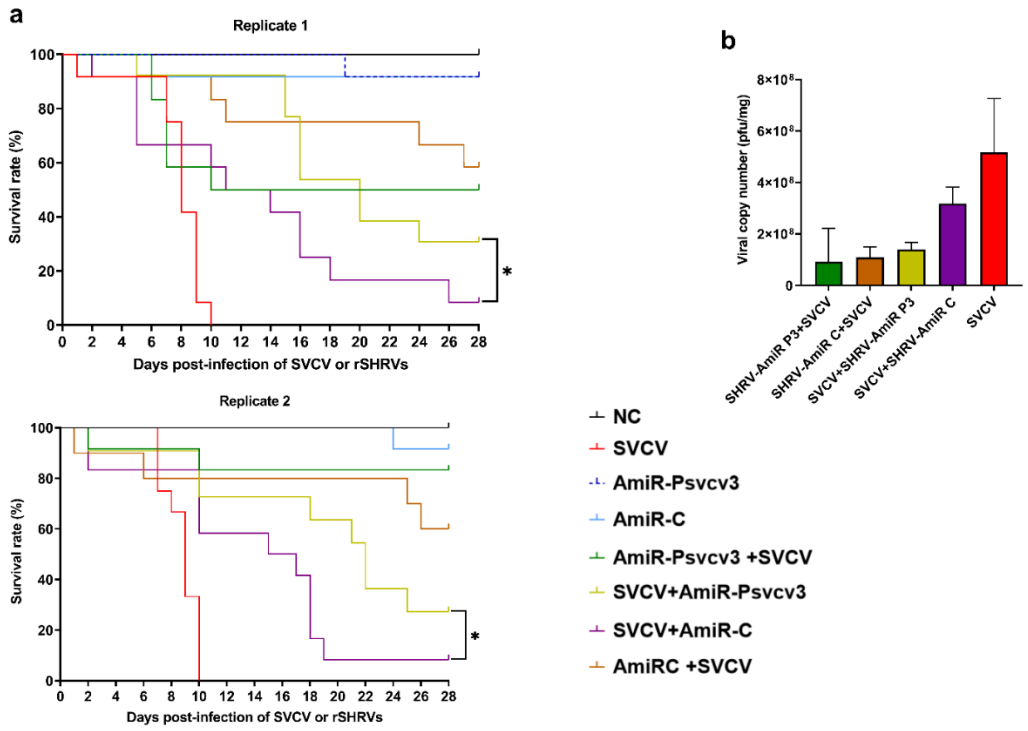

Figure 9. Therapeutic effect of AmiR-P3 delivered by rSHRV *in vivo* against SVCV infection. (a) Relative survival rate (RPS) was analyzed by Kaplan-Meier survival. (b) RT-qPCR quantification of SVCV copy numbers from the dead fish samples. Asterisk refers to $p<0.05$.

4. Discussion

The P protein of SVCV plays an important role in viral replication through the suppression of host cell's immune responses such as type I interferon (Li et al., 2016). Moreover, the P protein is essential for forming the ribonucleoprotein complex with the N and L proteins to transcribe viral genes and generate viral full genomic and antigenomic strands (Ahne et al., 2002). Therefore, the suppression of the P gene expression or P protein function would lead to decreased SVCV replication. Synthesized siRNA-based RNAi has been exploited to suppress SVCV gene transcripts (Fouad et al., 2019; 2022; Gotesman et al., 2015), in which only prophylactic effects (siRNA administration before SVCV infection) were verified. However, EPC cells treated with synthesized siRNA simultaneously or after SVCV infection did not show any inhibitory effect in virus replication (Fouad et al., 2019), indicating the therapeutic use of siRNA against viral diseases requires more enhanced strategies.

In the present study, not only mimics but also artificial primary microRNAs produced by plasmid vectors showed efficient knock-down of target P gene transcript, suggesting that the constructed artificial microRNAs can be used as a tool for the control of SVCV through the P gene-specific RNAi. Furthermore, the dose-dependent effect of mimics in this study suggests that highly efficient artificial microRNA expression systems would be needed for effective RNAi.

Although the prophylactic use of siRNAs can be a way to prevent or lessen viral diseases in fish, the difficulties in the prediction of viral infection time and in the enumeration of effective RNAi duration make RNAi unpractical

to be used in aquaculture farms as a prophylactic tool. The practicality of RNAi in aquaculture farms can be enhanced by the development of effective therapeutic RNAi. In antiviral therapeutic RNAi, the quantity and duration of virus-targeting siRNAs in hosts would be critical factors to induce effective RNAi-mediated antiviral response.

Compared to synthesized siRNAs, viral vectors would be a more efficient way to deliver siRNAs, since viruses can efficiently enter host cells as well as continuously express siRNAs in infected cells, which can make it possible to compete with replicating target viruses. In this study, we explored *in vivo* therapeutic effect of recombinant SHRV expressing SVCV P gene-targeting artificial microRNA against SVCV replication. SHRV belongs to the genus *Novirhabdovirus* and has the NV gene in the genome, characteristic of the genus Novirhabdovirus (J et al., 1992; Kurath et al., 1985). Although SHRV was first isolated from snakehead fish (Ophicephalus striatus) during an epizootic ulcerative syndrome (EUS) outbreak in Thailand (Wattanavijam et al., 1986), the role of SHRV in the EUS was thought to be not significant because a fungal species, Aphanomyces invadans, was identified as the cause of EUS (Iberahim et al., 2018) and snakehead fish artificially infected with SHRV did not show any EUS associated symptoms (Frerichs et al., 1993). There are a few data on the virulence of SHRV in fish. Alonso et al. (Alonso et al., 2004; Phelan et al., 2005) reported that adult zebrafish infected by intraperitoneal injection with 1 x 105 pfu/ml of SHRV induced 66.4% cumulative mortality. Phelan et al. (Phelan et al., 2005) also reported mortalities (more than 40%) of adult zebrafish by intraperitoneal injection of SHRV. In our several preliminary *in vivo* virulence experiments, zebrafish infected with less than 1 x 104 pfu/ml of SHRV did not show any mortality or any abnormal symptoms. Therefore, we used SHRV as

an *in vivo* delivery tool for artificial microRNA targeting the SVCV P gene. The generated recombinant SHRVs (rSHRV-AmiR-C and rSHRV-AmiR-P3) successfully expressed artificial microRNAs (AmiR-C and AmiR-P3, respectively) in BHK-21 cells, suggesting that cytoplasmic RNA viruses could be used as an effective tool for the delivery of microRNAs.

In the present *in vivo* experiment, not only rSHRV-AmiR-P3 but also rSHRV-AmiR-C showed a prophylactic effect against SVCV infection when fish were infected with the rSHRVs before SVCV infection. Novirhabdoviruses including SHRV, usually induce strong type I interferon responses in fish (He et al., 2021), which might be the cause of the present prophylactic effect of pre-infection with rSHRVs as well as no observable RNAi effect by rSHRV-AmiR-P3. The therapeutic effect of rSHRV-AmiR-P3 against SVCV was clearly shown when fish were treated with rSHRVs after SVCV infection, in which fish treated with rSHRV-AmiR-P3 showed significantly higher survival rate than fish treated with rSHRV-AmiR-C.

In conclusion, although more elaborated designed experiments that can evaluate the effect of competition between SHRV and SVCV, the present results proved that artificial microRNA expressing recombinant SHRVs can be used as *in vivo* therapeutic tools against SVCV infection in fish.

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Chapter III: Protection against spring viremia carp virus (SVCV) by immunization with chimeric snakehead rhabdovirus rSHRV-Gsvcv

1. Introduction

Viruses in the Rhabdoviridae family account for lots of morbidity and mortality in vertebrates (Ahne et al., 2002; OIE, 2023; Way et al., 2003). In fish, several rhabdoviruses are designated as notifying diseases by the World Organization for Animal Health (WOAH), such as spring viremia carp virus (SVCV), infectious hematopoietic necrosis virus (IHNV), and viral hemorrhagic septicemia virus (VHSV). Among them, SVCV is distinct from IHNV and VHSV in that it does not have the NV gene in the genome. SVCV belongs to the genus Sprivivirus and has five structural genes encoding nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and RNA-dependent polymerase (L) (Ahne et al., 2002). SVCV has been recognized as the most lethal virus in cyprinid fishes, especially common carp (Ahne et al., 2002) and designated as a disease to slaughter all infected fish and all fish at risk in many countries (Padhi & Verghese, 2008). There have been reports on the prophylactic measures against SVCV (Ashraf et al., 2016), especially, the high effectiveness of DNA vaccines containing an SVCV G protein expression cassette (Embregts et al., 2017; Emmenegger & Kurath, 2008; Kanellos et al., 2006; Zhao et al., 2022), however, no commercial vaccine has yet been developed.

Live vaccines based on attenuated viruses are known to be advantageous compared to inactivated vaccines or subunit vaccines in the induction of protective immune responses due to the possible stimulation of hosts like original viruses ((Kim et al., 2011; Kim & Kim, 2011). Non-virulent or weak-virulent viruses can be used as a delivery tool for antigens of virulent pathogens by generating chimeric viruses expressing heterologous antigens. In mammals,

adenovirus or adeno-associated virus have been used to deliver heterologous antigens (Peruzzi et al., 2009; Syyam et al., 2022).

In our previous study, we generated a chimeric recombinant snakehead rhabdovirus (SHRV) by replacing SHRV G gene ORF in the genome with SVCV G gene ORF (rSHRV-Gsvcv) and analyzed replication characteristics at different temperatures (Lee et al., 2021). SHRV belongs to the genus Novirhabdovirus and was first isolated from snakehead fish (Ophicephalus striatus) in Thailand (Wattanavijarn et al., 1986). Little information is available on the virulence of SHRV in fish, and only (Alonso et al., 2004) and (Phelan et al., 2005) reported mortalities of adult zebrafish (Danio rerio) by intraperitoneal injection of SHRV. In our preliminary experiments, however, we found no mortality in adult zebrafish by challenging with 1 x 104 pfu/fish of either wild-type SHRV or rSHRV-Gsvcv, while mortality was reached 100% by SVCV infection. In the present study, to evaluate the protection potential of the chimeric rSHRV, zebrafish were immunized with rSHRV-Gsvcv and monitored cumulative mortality by SVCV challenge.

2. Materials and Methods

2.1.Cells and viruses

Epithelioma papulosum cyprini (EPC) cells were grown in Leibovitz medium (L-15, Sigma) supplemented with 10% fetal bovine serum (FBS, Welgene) and 1% penicillin-streptomycin (Welgene) at 28°C. rSHRV (constructed by Kwak et al., 2020; based on wild SHRV ATCC-VR1386) and wild SVCV was propagated in EPC cells in L-15 with 2% FBS and antibiotics at 28°C. For the preparation of viral stock, a monolayer of EPC cells was infected with the virus, then the supernatant was collected and centrifuged at 4,000 rpm for 10 min when the extensive cytopathic effect (CPE) was observed. After filtration through a 0.45 μm pore size filter, the supernatant was aliquoted and stored at -80°C until further use.

2.2. Immunization and challenge

2.2.1. Experiment I

Zebrafish (average weight 0.3g) were divided into two groups with 2 replicates (12 fish/tank) and i.m. injected with L-15 alone (control group) or 1×10^4 pfu/20μl/fish of rSHRV-Gsvcv. At 5 weeks post-immunization, fish were challenged with 1×10^3 pfu/20μl /fish of wild-type SVCV. The water temperature was maintained at 15°C throughout the experiment. Mortality was monitored for 15 days.

2.2.2. Experiment II

Zebrafish (average weight 0.3g) were divided into two groups with 2 replicates (15 fish/tank) and i.m. injected with L-15 alone (control group) or 1×10^3 pfu/20μl/fish of rSHRV-Gsvcv. At 4 weeks post-immunization, fish were challenged with 1×10^2 pfu/20μl /fish of wild-type SVCV. The water temperature was maintained at 15 °C throughout the experiment. Mortality was monitored for 20 days.

2.2.3. Experiment III

Zebrafish (average weight 0.3g) were divided into three groups with 2 replicates (10 fish/tank) and I.M. injected with L-15 alone (control group), 1×10^3 pfu/20μl/fish of rSHRV-wild, or 1×10^3 pfu/20μl/fish of rSHRV-Gsvcv. At 4 weeks post-immunization, fish were challenged with 1×103 pfu/20μl /fish of wild-type SVCV. The water temperature was maintained at 15 °C throughout the experiment. Mortality was monitored for 15 days.

2.3.Statistical analysis

Statistical analysis was performed using GraphPad Prism version 9 (GraphPad Software, USA). The challenge result was analyzed by Kaplan-Meier survival analysis based on the Log-Rank (Mantel-Cox) test. The *p*-values below 0.05 were considered statistically significant.

3. Results

3.1. Immunization and protective effect of chimeric rSHRV-Gsvcv

In experiment I, fish immunized with 1 x 10^4 pfu/fish of rSHRV-Gsvcv showed significantly higher survival rates against the SVCV challenge (Figure 10-a). In experiment II, ten times lower titer of rSHRV-Gsvcv (1 x 10^3 pfu/fish) also induced significantly higher survival rates (Figure 10-b). In experiment III, fish immunized with 1 x 10^3 pfu/fish of rSHRV-wild showed 90-100% mortality, which was similar to the mortality of the control group (100%), while fish immunized with 1 x 10^3 pfu/fish of rSHRV-Gsvcv showed 10-20% mortality (Figure 10-c).

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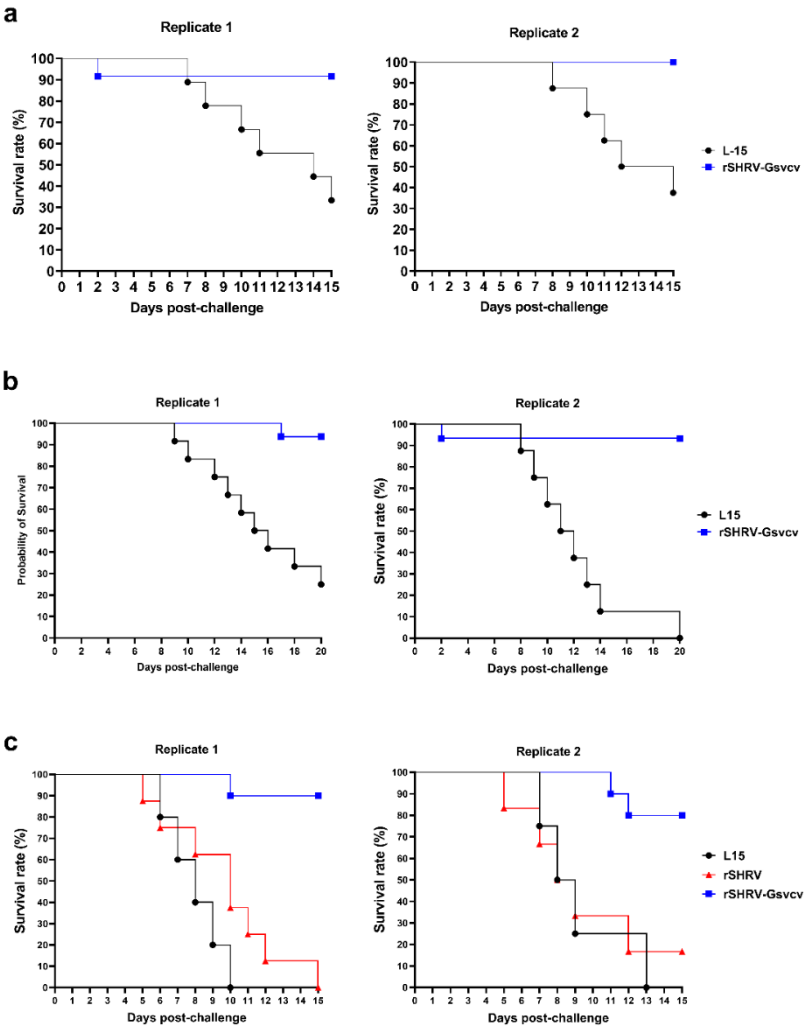

Figure 10. Chimeric rSHRV-Gsvcv protective effect against SVCV infection. (a) and (b) refers to the survival rate obtained from experiment I and experiment II, respectively, showing the usage safety and the protective effect of the chimeric virus depending on the injected dose. (C) represent the survival rate of the chimeric rSHRV-Gsvcv comparing to a control virus rSHRV-wild.

4. Discussion

The glycoprotein of rhabdoviruses plays a critical role in viral infection, which has led to the development of glycoprotein-based vaccines (Takano et al., 2001). In the present study, we used chimeric SHRV expressing SVCV G gene and confirmed its protective ability against SVCV infection.

In the immunization experiments with the chimeric SHRV-Gsvcv, SVCV induced high mortality rate between 75% and 100% in zebrafish injected with only L-15 alone, while rSHRV-Gsvcv did not induce infection-mediated mortality. Although the investigation of the virulence mechanism of SVCV was not the aim of the present study, this result suggests that the SVCV G protein might not be involved in SVCV virulence or SVCV G protein alone cannot induce virulence in zebrafish.

In this study, fish immunized with rSHRV-Gsvcv showed more than 80% survival rates, while fish immunized with rSHRV showed 90-100% mortality, suggesting the SVCV G gene in the chimeric virus was the main factor for the high protection. The present results suggest that SHRV can be used as an antigen-delivery tool in fish through the generation of recombinant chimeric viruses. Although the wide temperature range (15-30 °C) for SHRV replication was known (Johnson et al., 2000), the host species range of SHRV has not been investigated. To extend the availability of chimeric SHRVs, the possible host range should be investigated. Furthermore, in terms of safety, as the chimeric SHRVs still possess replicative ability in host cells, the possibility to induce pathological symptoms in infected fish cannot be excluded. Therefore, safer forms like single-cycle chimeric SHRVs should be considered to enhance the chance to get permission as practical vaccines.

5. Reference

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Chapter IV: Effects of exchanging VHSV G protein's signal peptide with piscidin signal peptide on immunogenicity and virus replication

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1. Introduction

Viral hemorrhagic septicemia virus (VHSV), listed by WOAH, is the causative agent of the viral hemorrhagic septicemia disease which is one of the main causes of morbidity and significant mortality in both wild and cultured fish worldwide (Ahmadivand et al., 2015; Eaton et al., 1991; Faisal & Schulz, 2009; W.-S. Kim et al., 2009; Schlotfeldt & Ahne, 1988). As a member of the Rhabdoviridae family, VHSV is an enveloped Novirhabdovirus, has a negative-stranded RNA genome of about 11 kb encoding six structural genes in the order 3' leader-N-P-M-G-NV-L-trailer 5' (Brown et al., 1979; Eaton et al., 1991; Meyers & Winton, 1995).

Viral genes are essential for virus replication and its virulence (Ammayappan & Vakharia, 2011; Thoulouze et al., 2004). Like other viruses' glycoprotein, VHSV glycoprotein represents the essential viral element not only for the entry of the virus to the host cells, for efficient assembly, and for the virion release, but also for inducing the adaptive immune response (Bernard et al., 1983). For that reason, the glycoprotein has been used as a target antigen for several types of vaccines such as live attenuated viral vaccines (Kim et al., 2015; Nishizawa et al., 2011; Kim et al., 2023).

DNA vaccines based on the G gene have been well-established for many rhabdoviruses, including VHSV. In the report of Einer-Jensen et al. (2009), one injection of mixed DNA vaccine (VHSV G and IHNV G DNA vaccines) induced long-lasting protection against both IHNV and VHSV. Also, each DNA vaccine could provide cross-protection in the early days against other Novirhabdoviruses and specific long-lasting immunity (LaPatra et al., 2001; McLauchlan et al., 2003). On many other occasions, the full G protein-encoding

DNA vaccine was proved to be efficacious in triggering protection to salmonids after virus challenge (Kurath et al., 2007; E. Lorenzen et al., 2000; LORENZEN et al., 1998; N. Lorenzen et al., 2002) and when it is administered through i.m. route, higher protection was conferred comparing to the one provided by killed or subunit vaccines (Kurath, 2005; E. Lorenzen et al., 2000; Purcell et al., 2012). However, the effect of the secreted form of G gene in inducing antiviral response and the production of the neutralizing antibodies is still less explored. Martinez-Lopez et al. (2013) showed that the versatility of the transmembrane-deleted glycoprotein could be enhanced by increasing its expression through the modification of the original signal peptide with pleurocidin gene signal peptide. This secreted glycoprotein form could stimulate the host immune response and confer higher protection than the original form. In the present study, in addition to the full G gene-based DNA construct, we have designed a secreted soluble form of G gene-based DNA vaccine from which we have replaced its N-terminal region (signal peptide) with the one of a high secreted antimicrobial peptide, piscidin, from rockfish.

This strategy seemed to be interesting to be used for increasing the viral titer by increasing the expression of the envelope protein G. However, in case of gene overexpression, the protein translation could be not efficient. It is known that viruses, not excluding rhabdoviruses, trigger the integrated stress response of host cells which involves shutting down protein translation (Harding et al., 1999). One of the important compartments of host cells related to protein expressing at first stages is the endoplasmic reticulum (ER). The entrance of protein to the ER affects its folding capacity. The misfolded or the unfolded proteins would accumulate in the ER triggering its stress. The ER stress is restored through the activation of three sensors among them, protein kinase R-

like ER kinase (PERK) which phosphorylate the eukaryotic initiation factor 2α (eIF2α) leading to the attenuation of the global host cell translational machinery (Arnaud et al., 2010; Donnelly et al., 2013; Shi et al., 1998; Wek et al., 2006). Viral replication is related to PERK pathway since viruses are dependent on the host cell translation system and viral infection can initiate the immune response (Arnaud et al., 2010; Datan et al., 2016; Wang et al., 2019). It was proved that TGEV infection is implicated in the ER stress and stimulated the unfolded protein response (UPR) which activated the PERK/ phosphorylated eIF2α leading to the suppression of TGEV replication (Xue et al., 2018).

In this chapter, we have generated VHSV expressing piscidin signal peptide-exchanged glycoprotein (rVHSV-PspvG). We have evaluated the function of signal peptide exchanged VHSV glycoprotein on viral replication *in vitro* and its relationship with the ER stress and the PERK pathway in comparison with the rVHSV-wild.

2. Materials and methods

2.1.Cells and virus

Epithelioma papulosum cyprini (EPC) cells were grown in Leibovitz medium (L-15, Sigma) supplemented with 10% fetal bovine serum (FBS, Welgene) and 1% penicillin-streptomycin (Welgene) at 28°C. The wild type VHSV (KJ2008) and the recombinant VHSVs were propagated in EPC cells in L-15 with 2% FBS and antibiotics at 28°C. For the preparation of viral stock, a monolayer of EPC cells was infected with the virus, then the supernatant was collected and centrifuged at 4,000 rpm for 10 min when the extensive cytopathic effect (CPE) was observed. After filtration through a 0.45 μm pore size filter, the supernatant was aliquoted and stored at -80°C until further use.

2.2.Secretory effect of different signal peptides

Along with the original VHSV glycoprotein's (vG) signal peptide, we selected hepcidin and piscidin to compare their signal peptide secretory level with the vG signal peptide. To do so, the signal peptide of Metridia luciferase (Met luc) was replaced with vG, hepcidin or piscidin signal peptide by PCR using overlapped primers then amplified with primers set containing NheI and MluI restriction enzyme site. Each amplicon was inserted into PFC vector by ligation using T4 DNA ligase (Promega, USA). The constructs were designated as pvGsp-Metluc, pHepsp-Metluc and pPspMetluc, respectively. The constructs schematic is represented by Figure 12-a. All the primers used for plasmids construction are listed in Table 4-1.

To determine the secretory level of each signal peptide, secreted Luciferase activity was performed. Briefly, the constructed plasmids expressing the original Met luc gene or the exchanged signal peptide-Met luc gene, were transfected into EPC cells using Fugene HD (Promega) according to the manufacturer's instructions. The supernatant was collected at 24h, 48h and 72h post transfection, then the secreted luciferase activity was analyzed using Ready-To-Glow Secreted Luciferase Reporter assay kit (Clontech) according to the manufacturer's instructions by victor X3 plate reader (PerkinElmer) the activity was measured 5times wit 5s interval.

2.3.Generation of rVHSV-PspG and comparison of its replication with rVHSV-wild harboring the original G gene

The original signal peptide of vG was replaced with piscidin signal peptide by PCR using an overlapped forward primer and specific reverse primer, and the amplicon was designated as PspvG. Then, PspvG was amplified by PCR using forward and a reverse primer that contains AgeI and NotI site. PspvG was digested then cloned into pGEM-Teasy vector and designated as PspvG-T. After verifying the construct sequence, the constructed PspvG-T and VHSV (KJ2008full) full genome expressing plasmid constructed by (M. S. Kim & Kim, 2011) (pVHSV-wild) were digested by AgeI and NotI, then PspvG was inserted into pVHSV-wild, in place of the original G gene, by ligation reaction performed with T4DNA ligase (Promega), resulting pVHSV-PspvG (Figure 13-a). The used primers are mentioned in Table 4-1.

rVHSV-PspvG rescue was carried out by co-transfection of the constructed pVHSV-PspvG (1.5 μg) with pCMV-N (500ng), pCMV-P (300ng), pCMV-L (200ng) with pCMV-T7 RNAP (T7 polymerase expressing plasmid),

that were constructed previously (M. S. Kim & Kim, 2011), into EPC cells expressing VHSV G gene (M. S. Kim et al., 2015) at 15ºC. After total CPE was observed, the supernatant was collected, centrifuged at 4000rpm for 10min at RT, filtered through 0.45µm syringe filter, then propagated onto EPC cells for 4 consecutive passages. At the 5th passage, the virus was collected, and the same procedure was done as mentioned above, then virus stock was stored at -80ºCuntil plaque assay was performed to determine the virus titer.

The comparison of rVHSV-wild and rVHSV-PspvG viral titer was performed by plaque assay. Briefly, both viruses were serially diluted from 10^{-4} to 10^{-6}, then inoculated into EPC cells monolayer. After 2h of incubation at 15ºC, the inoculum was discarded, and cells were overlaid with plaque media (0.8% of agarose in fresh L-15) and incubated at 15ºC. After 7days when plaque appeared, cells were fixed with 10% formalin, stained with 10% crystal violet, followed by washing step with distilled water, then the plaque-forming unit (PFU) was counted, and titer of both viruses was determined.

2.4.*In vitro* investigation of the effect of antioxidants, PERK inhibitor, and ER stress stimulator on the viral growth of rVHSV-wild and rVHSV-PspvG

In order to determine the cause of the lower titer of rVHSV-PspG titer compared to rVHSV-wild, several chemicals were used to determine the related phenomenon and pathway that can be a cause of the low titer. EPC cells were grown to 85% confluency in L-15 supplied with 10% FBS and 1% antibiotics. Cells were then treated with either 20µM of Butylated hydroxy anisole (BHA, Sigma-Aldrich), 700µM of Sodium Ascorbate (SA, Sigma-Aldrich), 10 µM of the PERK inhibitor GSK2656157 (GSK, Sigma-Aldrich) or 2µg/ml of the ER

stress inducer Tunicamycin (Tu, Sigma-Aldrich) and non-treated cells were use as control. Cells were incubated at their optimal temperature (28ºC) for 48h. After 48h, the media was discarded and rVHSV-wild or rVHSV-PspvG were inoculated at MOI 0.01 in L-15 supplied with 2%FBS for 2h at 15ºC (temperature was shifted gradually until 15ºC). after incubation the inocula were discarded and replaced with L-15 supplied with 2% FBS and incubated again at 15ºC. To determine the viral growth of both viruses at the different conditions mentioned above, supernatant was sampled at 1d, 3d, 5d, 7d, then titration was performed by plaque assay as was mentioned before.

2.5.Virulence test of rVHSV-PspvG in olive flounder

Fingerlings of olive flounder (average weight 2.56g) obtained from culture facility in Hadong, were randomly divided into 7 groups of 15 fish each and acclimated gradually at 14ºC until injection. Fish were infected either with 50µl of 10^3, 10^5pfu of rVHSV-wild or rVHSV-PspvG. For the control group, fish were injected with 50µl of L-15. The injection was carried out through an intraperitoneal route. The mortality was recorded for 4 weeks, and dead fish were immediately sampled and stored until analysis.

To evaluate the replication of rVHSV-PspvG comparing to rVHSV-wild, total RNA was extracted from spleen and head kidney of dead olive flounder using Hybrid R kit (GeneAll), then 1ug was used in one-step quantitative qPCR using QIAGEN one-step qPCR (Qiagen) following the manufacturer's instructions.

2.6.DNA vaccine based on Piscidin signal peptide-exchanged glycoprotein

2.6.1. Plasmids construction

For the immunization, DNA plasmids expressing different forms of glycoproteins were constructed. The full glycoprotein gene was amplified by PCR using specific primers set, containing NheI and NotI enzyme site, then cloned into T easy vector (pT-Vg). vG was inserted then into NheI-NotI digested PFC vector using T4 DNA ligase (Promega). The construct was designated as pvG. The transmembrane region and the C-terminus region were deleted by PCR using specific primers set to produce the secreted for of glycoprotein. The amplicon was assembled with PFC vector using overlap cloner kit (Elpis). The plasmid was designated as pvG△TMC. PspvG was inserted into PFC vector by restriction enzyme digestion using PspvG-T plasmid used for the generation of rVHSV-PspvG. The construct was designated as pPspvG. Using the latter construct, the transmembrane and the C-tail region were deleted by PCR using primers set mentioned in Table 4-1. The amplicon was assembled with PFC vector using overlap cloner kit (Elpis). The constructed plasmids were named as PspvG△TMC. All the plasmids were verified by sequencing before the immunization test. Figure 16-a shows the constructs used for the DNA vaccine. All the primers used for the plasmids' construction are mentioned in Table 4-1.

2.6.2. Olive flounder immunization and challenge

Olive flounder (*Paralichthys olivaceus*) fingerlings (average weight 1.14g) were obtained from Busan fishery resources research institute

(부산광역시 수산자원연구소). The fish were acclimated in sea water at 20ºC before the injection. For the immunization, fish were randomly divided into 6 groups in duplicates. 5μg of each plasmid (pvG, pvG△TMC, pPsp-vG and pPsp-G△TMC) in 30μl of PBS were intramuscularly (i.m.) injected into fish. as a positive control, 5ug of peGFP plasmid in 30μl of PBS were injected and for negative control group, fish were injected with 30μl of PBS. After 30 days of immunization, fish were intraperitoneally challenged with 10^4pfu of wt rVHSV in 30μl of L-15. The challenge test was performed at 14ºC, and the mortality was recorded for 20days.

2.7.Statistical analysis

Statistical analysis was performed using GraphPad Prism version 9 (GraphPad Software, USA). The secreted luciferase activity, the *in vitro* viral growth, and the virus titer from the dead olive flounder from the virulence test were analyzed by 2way ANOVA followed by Tukey's multiple comparison test. The challenge result was analyzed by Kaplan-Meier survival analysis based on the Log-Rank (Mantel-Cox) test. The *p*-values below 0.05 were considered statistically significant.

Table 4-1 Primers used in Chapter IV

Primers	**Sequence 5'→3'**	
For the construction of Metridia luciferase plasmids		
vG signal peptide in MET Luc plasmid	vGsp1-Metluc-F	TCATCATAAAAAGCACCACATCAAAGAGCACCGAGTTCGAC
	vG-sigpep2-F	TTCTTGGTGATTCTGGTCATCATCATAAAAAGCACC
	vG-sigpep3-NheI-F	GCTAGCATGGAATGGAATACTTTTTTCTTGGTGATTCTGGTCA
	MetLuc-MluI-R	ACGCGTTCACCTGTCGCCGGCCATG
Piscidin signal peptide in MET Luc plasmid	MetLuc-piscidin SP1-F	CTTGTGTTGTCCATGGTTGTTCTCATGGCTAAGAGCACCGAGTTCGAC
	VSP-piscidin-NheI-F2	GCTAGCATGAAGTGTATCGTGATCTTTCTTGTGTTGTCCATGGTTGTTC
	MetLuc-MluI-R	ACGCGTTCACCTGTCGCCGGCCATG
Hepcidin signal peptide in MET Luc plasmid	hep-sp1-MetLuc-F	TCTGCATCCTCCAGTCCGCAGCCAAGAGCACCGAGTTCGAC
	MetLuc-hep-F2	CTGCTGCAGTCATCATCGCATGCGTCTGCATCCTCCAGTCCGCA
	MetLuc-hep-NheI-F3	GCTAGCATGAAGTTCTCACGTGTGGCTCTCGCTGCTGCAGTCATCATCGCAT
	MetLuc-MluI-R	ACGCGTTCACCTGTCGCCGGCCATG

For the generation of rVHSV-PspvG			
For the RT-PCR of PspvG	PspvG-AgeI-OC-F		TACACAACAAGTCACCGGTATGAAGTGTATCGTGAT
	VHS-R2-SacII		CCGCGGTCAGACCATCTGGCTTCTGGAG
For the construction of DNA vaccine plasmids			
pvG	vhsv-G-R-Not1		GCGGCCGCTCAGACCATCTGGCTTCTGGA
	vhsv-G-F-Nhe1		GCTAGCGCCACCATGGAATGGAATACTT
pvG△TMC and pPspvG△TMC	Fragment 1	Neo_Kan_253_OC_F	CTGAAGCGGGAAGGGACTGG
		vGdeltaTm-R	**ATTGAATGACCAATCCGAAG**GAATTAGATTG
	Fragment 2	vGdeltaTmCd-OC-F	**CTTCGGATTGGTCATTCAA**TACGCGTCTGATCATAATCAGCC
		Neo_Kan_253_OC_R	TCCCTTCCCGCTTCAGTGACAAC

3. Results

3.1.Secretory effect of different signal peptides

The secretory effect of each signal peptide was determined through the secreted luciferase value comparing with the original signal peptide of Met luc (Figure 12-b). At 24h post transfection, the secreted luciferase value was higher from the cells transfected with Met luc sp, vG sp and Pisc sp exchanged Met luc expressing plasmid than from the ones transfected with Hep sp, but there was not significant difference between all the groups. However, at 48h and 72h post transfection, the Pisc sp secretory effect showed significant higher luciferase value comparing with vG or Hep sp. consequently, Pisc sp was selected to verify the antigenicity of vG when it is highly expressed and its effect on the virus replication.

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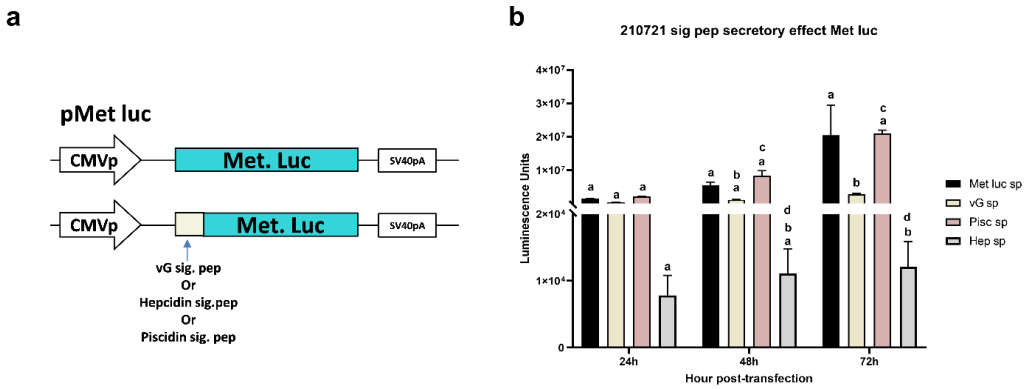

Figure 11. Piscidin signal peptide secretory ability. (a) Constructs schematics used for the luciferase assay. (b) Comparison vG, piscidin and hepcidin signal peptide secretory effect by estimation secreted luciferase activity. The different letters on each bar shows the significant difference and the same letter refers to no significant difference at $p < 0.05$

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3.2.Rescue of rVHSV-PspG and its replication comparing with rVHSV-wild harboring the original G gene

The rescued rVHSV-PspG was titrated by plaque assay and compared with the original vG-harboring rVHSV-wild. The result of plaque assay of the virus stock showed lower titer of rVHSV-PspG compared to rVHSV-wild titer. The plaques size and form of both viruses were not different (Figure 13-b).

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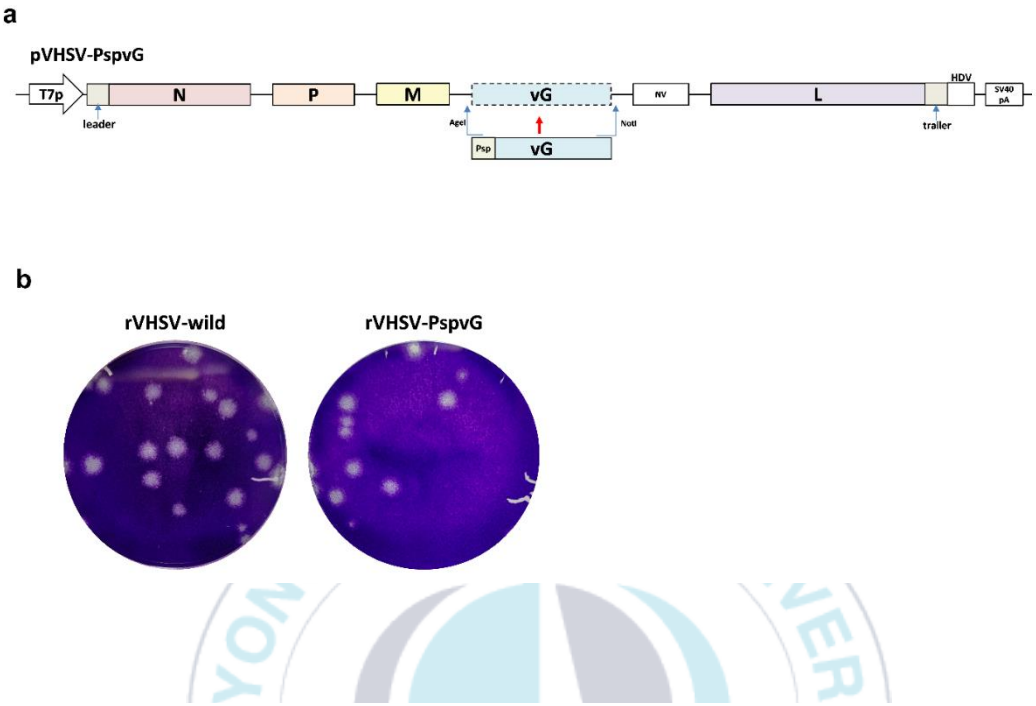

Figure 12. Rescue of rVHSV-PspvG. (a) construct schematic used for the generation of rVHSV-PspvG. (b) plaque assay result of rVHSV-wild and rVHSV-PspvG.

3.3.*In vitro* investigation of the effect of antioxidants, PERK inhibitor, and ER stress stimulator on the viral growth of rVHSV-wild and rVHSV-PspvG

For both rVHSV-wild and rVHSV-PspvG, the result of the *in vitro* viral growth at 1d and 3d showed a non-significant difference in the virus titer from all the treated and the non-treated cells. But at 5d and 7d, the titer of rVHSV-wild from the cells treated with the antioxidant BHA was significantly higher than the ones from the non-treated cells or the cells that were treated with the antioxidant SA or the ER stress inducer (Tu) but was not significantly higher comparing to the titer calculated from the cells treated with PERK inhibitor. At 5d and 7d, the titer of rVHSV-PspvG that was recorded from the cells treated with PERK pathway inhibitor (GSK) was significantly higher than the titer that was recorded from all the other cells. In addition, during the whole days, the titer estimated from the cells treated with the ER stress inducer (Tu) was significantly low. The detailed growth is shown in Figure 14.

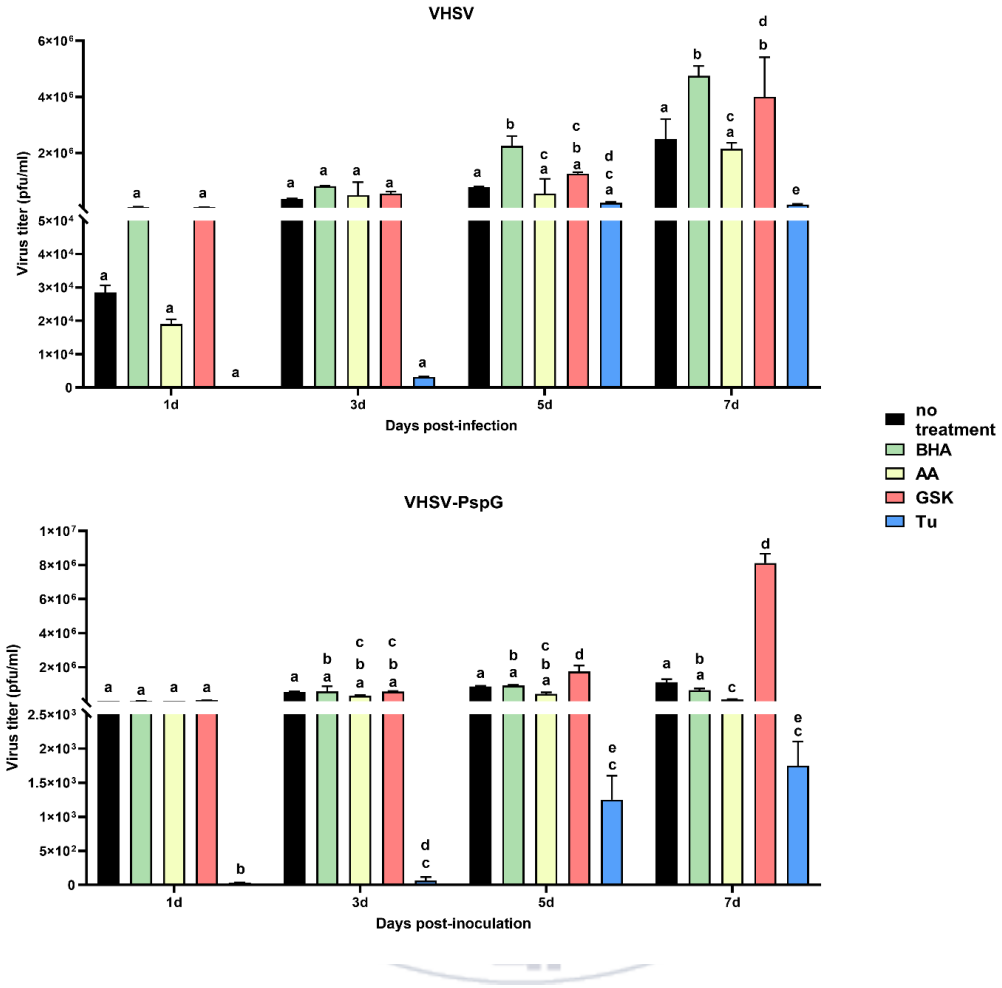

Figure 13. *In vitro* effect of antioxidants, PERK pathway inhibition and ER stress stimulation on rVHSV-wild and rVHSV-PspvG replication.

3.4.Virulence test of rVHSV-PspvG in olive flounder

The *in vivo* virulence test was conducted for 27days. During this period, the NC fish group showed no mortality and were healthy. The mortality started at day 5 post infection from the group injected with rVHSV-wild 10^5pfu to reach the 100% of death at 17dpi followed by the group injected with rVHSV-PspvG 10^5pfu which recorded 81% of mortality at 21dpi until the end of the test. rVHSV-wild 10^3pfu injected fish group showed same mortality rate as the group that was infected with rVHSV-PspvG 10^5pfu. Compared to these 3 groups, the mortality from rVHSV-PspvG 10^3pfu started later at 12dpi and was slow to reach the lowest mortality rate recorded during this experiment of 68% at 19dpi until the end of the experiment (Figure 15-a).

To verify each virus concentration in dead fish, virus copy number was determined from spleen and head kidney extracted RNA by quantitative qPCR. For both viruses, the estimated virus titer from spleen was not significantly different between groups. However, from the head kidney, the lowest titer was shown from both concentrations of rVHSV-PspvG infected fish samples compared to the rVHSV-wild infected fish samples (Figure 15-b).

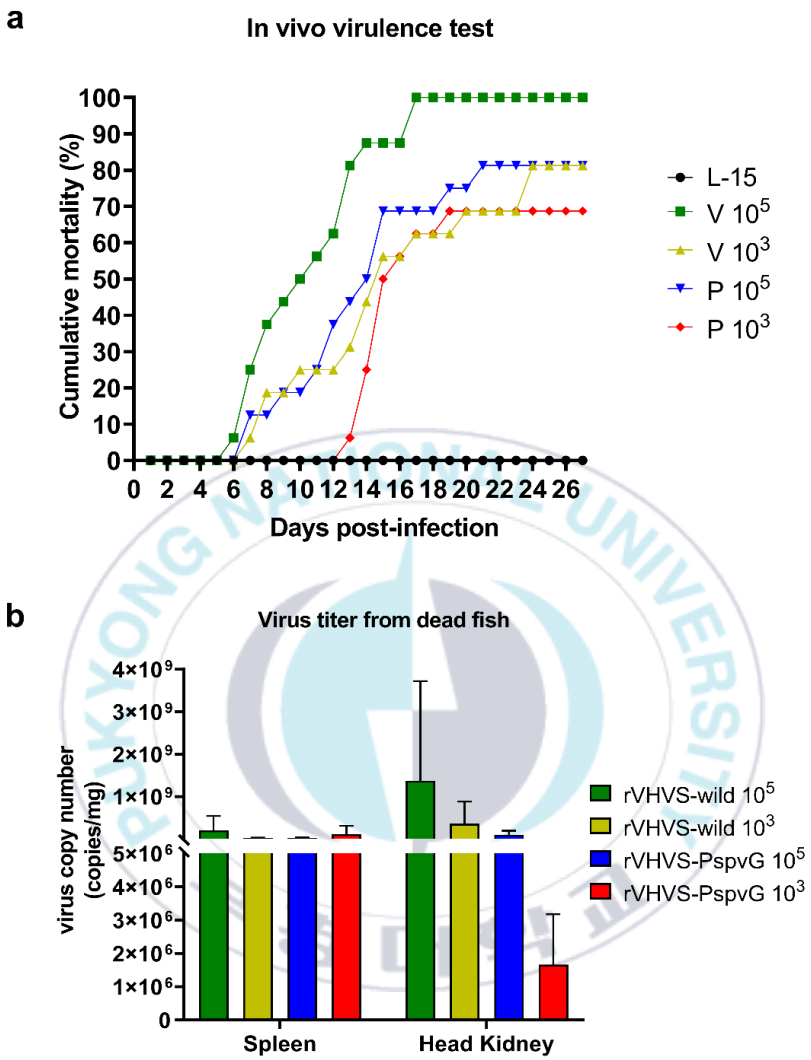

Figure 14. *In vivo* virulence test of rVHSV-PspvG. (a) Cumulative mortality of olive flounder following the infection with rVHSV-wild or rVHSV-PspvG. (b) Comparison of both virus titers from the dead fish (spleen and head kidney) samples.

3.5.DNA vaccine based on Piscidin signal peptide-exchanged glycoprotein

In the challenge test, the immunized olive flounder with full vG showed 100% survival rate in both replicates during the whole test followed by the group of fish immunized by PspvG plasmid showing 62% and 55% of survival rate in replicate 1 and replicate 2, respectively. In replicate 1, the fish immunized with the secreted PspvG△TMC showed a higher survival rate of 50% than the group injected with the original form of the secreted glycoprotein (vG△TMC) which showed 70% mortality (Figure 16-b).

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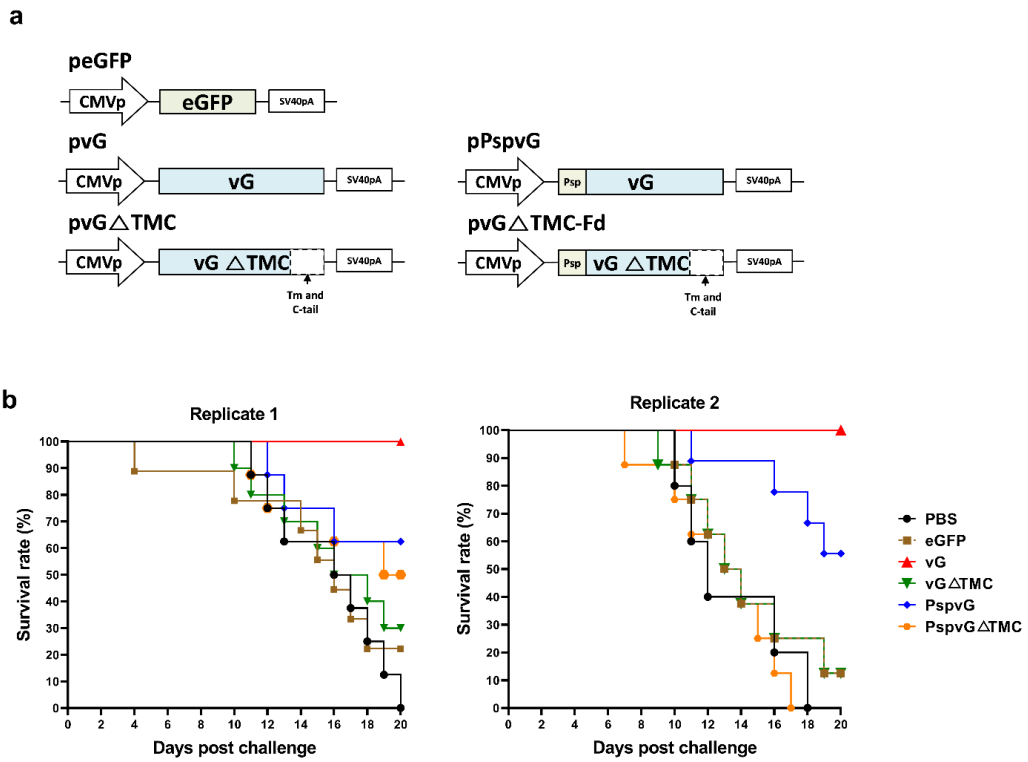

Figure 15. Protective effect of vG-based DNA vaccine in olive flounder against VHSV infection. (a) Schematic representation of the DNA plasmids expressing full G gene (vG), transmembrane (Tm) and C-tail-deleted G gene (secreted form-vG△TMC), Psp-exchanged G gene (PspvG) or Psp-exchanged secreted G gene (PspvG△TMC). (b) Survival rate represents the protectivity of each form of glycoprotein followed by VHSV infection.

4. Discussion

The endoplasmic reticulum (ER) is one of the important cell compartments that serves for the glycoprotein synthesis during the initial stages after translocating the protein by the signal peptide. It is where the envelope protein is folded in an appropriate way before its transport to the cell surface. However, protein misfolding, for example in case of overexpression, the unfolded proteins will be accumulated in the ER which can lead to the oxidative stress of the ER resulting in the shutdown of the host cell translation machinery (Malhotra & Kaufman, 2007; Ron & Walter, 2007). We have generated rVHSV expressing Glycoprotein harboring piscidin signal peptide which was verified to have higher secretory effect than the original signal peptide, in order to increase viral titer. However, the result obtained in this study showed that rVHSV-PspvG have lower titer than rVHSV-wild. Usually, viruses including rhabdoviruses, manipulate host cells via a variety of developed mechanisms to translate viral mRNAs and to inhibit cellular host mRNA translation in order to ensure an efficient viral replication (Breyne & Ohlmann, 2018; Gale et al., 2000). Meanwhile, one of the pathways related to the innate immune response to viral infection or in case of misfolded protein accumulation in the ER, RNA-regulated Kinase (PKR)-like endoplasmic reticulum Kinase (PERK) pathway is activated by the phosphorylation of eukaryotic initiation factor 2α which lead to suppress the global translation (Lv et al., 2015; Wu et al., 2022). So, the low viral could be a response to the ER stress that could have occurred

Glycoprotein of rhabdoviruses is a trimeric envelope protein and considered as one of the viral structural proteins that plays a critical role not only, in the virus assembly and its release by budding of ascent virions from the cytoplasmic

membrane of the host cell, but also in its entry by mediating host membrane fusion. In this study, we have focused on determining the role of glycoprotein when its signal peptide replaced with an antimicrobial peptide, piscidin, on viral replication and on the host cell machinery.

Following the overexpression of glycoprotein. To verify this fact, we have used antioxidants to prevent ER-stress, PERK pathway inhibitor and ER stress inducer. The pre-infection antioxidant (BHA) treatment of cells resulted in a higher virus titer compared to the one collected from the non-treated cells. On the other hand, the PERK pathway inhibition resulted in a higher viral yield for both rVHSV-wild and rVHSV-PspvG compared to the virus titer estimated from the non-treated cells. Moreover, when we triggered the cells ER stress by treatment with the ER stress inducer Tu, virus titer was significantly lower, in addition the cell apoptosis that was observed during the test from the day 3 (Tu treated cell with no virus infection).

According to this *in vitro* test result, the generated rVHSV-PspvG virulence was elucidated *in vivo* using olive flounder. Except for the negative group, mortality has occurred in all the infected groups with both viruses. However, the slowest and the lowest mortality rate was recorded from the fish group infected with rVHSV-PspvG 10^3pfu. In addition, the virus copy number estimated from the head kidney of dead fish from the latter group was the lowest. This result could be explained by the fact that the secretion excess of glycoprotein by piscidin signal peptide, plus to the viral infection might lead to the ER stress which trigger the suppression of the host cell translation machinery.

DNA vaccine based on G gene have been explored enough to show its protectivity against viral infection (Chico et al., 2009). However, in several trial

that were carried out to investigate the potential use of the secreted form of G gene and its ability in inducing the immune response against VHSV infection, the result showed that vG△TMC has very low to no protection against viral infection. The stimulation of the immune response of host cells is easily triggered by the trimeric form of the glycoprotein. The trimerization of the G gene helps the protein to be transported outside of the ER and exposed to the cell receptors to induce the neutralizing antibodies production. In other occasions, modified or immunostimulant-fused truncated G gene seems to confer an innate and adaptive immune response against VHSV (Puente-Marin et al., 2019; 2023).

In the present study, in addition to the full vG form, we investigated the effect of the antimicrobial peptide piscidin signal peptide on the full G and the secreted G form immunogenicity and protectivity against VHSV. The result of the immunization experiment showed that full G gene vG has high protection ability as it was proved before (Millard et al., 2017; Pereiro et al., 2012). However, the full PspvG showed lower protection against VSHV comparing to the original vG, but higher than the two secreted forms of vG (vG△TMC and PspvG△TMC). This could be related to the overexpression of the glycoprotein which could cause the ER stress then unfolded protein accumulation leading to the suppression or shutdown of the translation machinery, then less amount of glycoprotein synthesis. This result also confirmed the previous results of the preliminary studies conducted in our laboratory, that the secreted form could not provide protection against VHSV infection.

In this chapter, we could confirm that virus replication depends on the host protein synthesis system and that VHSV replication is related to the activation or the inhibition of PERK pathway which is an essential pathway for the restoration of the ER state in case of stress caused by pathogenic infection. In addition, we confirmed the protective potential that VHSV G gene has *in vivo* in case of viral infection. However, the mechanism of secreted form of glycoprotein should be more explored and further studies should be performed in order to increase the versatility and the immunogenicity of vG△TMC in order to confer higher protection against VHSV.

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