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Functional characterization of seven-band grouper immunoglobulin like cell adhesion molecule, Nectin4 as a cellular receptor for nervous necrosis virus

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ABSTRACT

Nectin-4/PVRL4 belonging to the family of immunoglobulin-like cell adhesion molecules was identified as a potential cellular receptor for several animal viruses. Here we show that nervous necrosis virus that causes viral nervous necrosis in teleosts uses the same receptor in its life cycle. Transfection of SSN-1 cell lines with an expression vector encoding Nectin-4 rendered them to be more susceptible to NNV. Immunofluorescence microscopy on Nectin-4 expressing cells revealed that the protein interacted with NNV specifically. A virus binding assay indicated that Nectin-4 was a bonafide receptor that supported virus attachment to the host cell whereas siRNA directed against Nectin-4 blocked NNV infections in grouper primary brain cells. Results of the present study will improve our understanding of the pathogenesis of NNV infection and provide a target for the development of novel antiviral interventions in marine finfish aquaculture.

1. Introduction

Nectins are calcium-independent type-I trans-membrane cell-cell adherens proteins of the immunoglobulin superfamily. They consist of four members (viz., Nectin-1, 2, 3 and 4) and share three Ig like extracellular domains comprising of an N-terminal variable region like (V) domain, two constant region like domains (C2), a transmembrane domain (TM) and a cytoplasmic region [1]. Previous studies have demonstrated that several neurotropic viruses exploit Nectins as cellular receptors to mediate their entry [2–8]. From our preliminary studies, we identified Nectin-4 as a potential molecule to be evaluated in the context of NNV pathogenesis. Nectin-4 is normally localized at the adherens junctions and its expression is tissue specific in higher vertebrates [9]. In fish, brain has the highest expression level of Nectin-4 followed by the gill and kidney [10]. Nectin-4 is an established cellular receptor for measles virus (MeV), canine distemper virus (CDV), and peste de petits ruminant virus (PPRV) [6–8].

Nervous necrosis virus belongs to the genus Betanodavirus. It is the causative agent of viral nervous necrosis (VNN) in teleost fish. VNN is characterized by a neuropathological condition in the central nervous system [11]. The nodaviral genome consists of two single-stranded positive-sense RNA molecules, RNA1 and RNA2 that encode 100 kDa RdRp and 42 kDa capsid protein respectively [11,13]. During RNA replication, a sub-genomic RNA3 encoding B2 protein is produced co-terminal with the 3' terminus of RNA1 [11,12]. VNN can be transmitted both vertically and horizontally. It accounts for high levels of mortality in marine finfish aquaculture [11,14]. NNV affects more than 120 species of around 30 families of farmed and wild fish populations [15].

Although there are extensive studies on receptor involvement in viral pathogenesis in higher vertebrates, there are gaps in the understanding of receptors associated with finfish viral pathogens. It has been reported that, the heat shock cognate protein 70 (HSP 70) and sialic acid can act as cellular receptors in GF-1 and SSN-1 cells for NNV entry [16,17]. Nectins characterized from seven-band grouper demonstrated that they might play an important role as receptors in NNV infection process [10]. In light of these observations, the objective of the present study was to evaluate the functional role of grouper Nectin-4 in NNV pathogenesis.

2. Materials and methods

2.1. Cloning and transfection of plasmids

Expression constructs of grouper Nectin-4 (GenBank accession number: MK522074) based on pcDNA3.1 and pEGFP were generated by restriction cloning with Nectin-4 open reading frame (ORF). Primers used for the cloning are listed in Table .1. ORF was inserted into pcDNA using the HindIII/EcoRI digestion. It was inserted into pEGFP using EcoRI/KpnI digestion. All plasmids were transformed into *Escherichia coli* DH5α and purified endotoxin free using Geneall Exfection LE

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Table 1
Primers and template oligonucelotide sequences for siRNA generation used in this study.

Purpose	Primer	Sequence (5′-3′)
Template oligonucleotide sequences for siRNA generation	Nectin-4S1	5′-TAATACGACTCACTATAGGGAGACAGG**GCAGCGGTACCCCAATATTCA**
	Nectin-4S2	5′- **GCAGCGGTACCCCAATATTCA**CCTGTCTCCCTATAGTGAGTCGTATTA
	Nectin-4AS1	5′- TAATACGACTCACTATAGGGAGACAGG**TGAATATTGGGGTACCGCTGC**
	Nectin-4AS2	5′- **GCAGCGGTACCCCAATATTCA**CCTGTCTCCCTATAGTGAGTCGTATTA
	ScrambleS1	5′-TAATACGACTCACTATAGGGAGACAGG**GCCCTCAACCACTATCCACTA**
	ScrambleS2	5′- **TAGTGGATAGTGGTTGAGGG**CCCTGTCTCCCTATAGTGAGTCGTATTA
	ScrambleAS1	5′-TAATACGACTCACTATAGGGAGACAGG**TAGTGGATAGTGGTTGAGGGC**
	ScrambleAS2	5′- **GCCCTCAACCACTATCCACTA**CCTGTCTCCCTATAGTGAGTCGTATTA
Primers for pcDNA3.1 cloning	Nectin-4/HindIII-F	**GAGAGAAAGCTT**ATGGAG GGT TGT CTC TGT TAT G
	Nectin-4/EcoRI-R	**GAGAGAGAATTC** CAG GGA TCC TTC TTG AAG TGG
Primers for pEGFP cloning	Nectin-4/EcoRI-F	**GAGAGAGAATTC** ATG GAG GGT TGT CTC TGT TAT G
	Nectin-4/KpnI-R	**GAGAGGTACC** CAG GGA TCC TTC TTG AAG TGG
Nectin-4 qPCR	qNectin-4F	CGGACCACAGTTTGTCAACGG
	qNectin-4R	AGGCTTATGACCGGAGGCAC
NNV RNA2 qPCR	qNNV.R2.F	AAATTCAGCCAATGTGCCCC
	qNNV.R2.R	ATTTGGCAACGACTGCACCA

*Bold underlined sequences represent siRNA template and bold sequences denote restriction sites.

megaprep kit (Geneall, S. Korea) according to the manufacturer's instruction.

2.2. Single step growth curve analysis and virus quantification

SSN-1 cells transfected with pcDNA-Nectin-4 or empty plasmid in 24-well plates were infected with NNV at 10^5 $TCID_{50}$ dose in serum-free Leibovitz-15 (L15) media (Gibco, USA). After 1 h of adsorption period, the cells were rinsed once and re-fed with L15 containing 10% fetal bovine serum (FBS) (Atlas, USA). At various times after infection, cells were scraped into 1 mL serum-free L15 and used for virus quantification. For titer determination, cells collected were lysed by three freeze-thaw cycles and virus titer was determined by $TCID_{50}$ method. For copy number determination, RNA was isolated from cells, reverse transcribed and subjected to quantitative PCR based on RNA-2 segment as described previously [18].

2.3. Virus-binding assay

Binding of NNV to SSN-1 cells expressing Nectin-4 was performed as described by Gardner et al. [19] with slight modifications. Briefly, pcDNA-Nectin4 or empty vector transfected adherent target cells were washed in buffer (20 mM Tris·HCl, pH 8.0, 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$ and 0.5% BSA) and incubated with an equal volume of viral supernatant (10^6 $TCID_{50}$) for 15 min, 30 min, 45 min and 60 min at 25 °C with gentle agitation every 15 min. After washing five times with buffer to remove unbound virus, cells were lysed according to the manufacturer's instructions for RNA preparation (MRC, USA). Total cellular RNA was analyzed for NNV RNA-2 content by qPCR.

2.4. Immunofluorescence assay

SSN-1 cells seeded onto 4-well Chamber Slide™ (SRL, S. Korea) were transfected with 0.5 μg pEGFP-Nectin-4 using Lipofectamine 2000 reagent (Invitrogen, USA). At 48 h after transfection, the medium was removed from each well and cells were infected with NNV using a multiplicity of infection (MOI) of 1 in L15 media. After incubation for 24 h, wells were washed with PBS three times. Cells were fixed with 4% paraformaldehyde for 10 min at room temperature and then permeabilized for 15 min with 0.5% Triton X-100 in PBS. Slides were blocked using blocking buffer (5% goat serum v/v in PBST) and then incubated with monoclonal *Anti*-NNV coat protein antibody [20] overnight at 4 °C. Primary antibody was detected with TRITC-conjugated anti-mouse IgG antibody (Sigma, USA) for 1 h at room temperature. Slides were mounted with coverslips using Vectashield mounting reagent (Vector Laboratories, USA) and imaged using an Olympus BX53 fluorescence microscope.

2.5. Primary brain cell culture

Healthy seven-band grouper juveniles with mean body weight of 7.8 g were maintained in the wet lab facility at 20–22 °C and daily fed with a commercial diet. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Chonnam National University (CNU IACUC-YS-2018-3). Starved fish were euthanized using MS222 overdose. Brain tissues were dissected out aseptically and washed in sterile phosphate buffered saline. Tissues were homogenized mechanically by mincing and pipetting in tubes containing DPBS with penicillin (100 U/mL) and streptomycin (100 mg/mL) followed by treatment with 0.025% trypsin-EDTA for 5 min. To this suspension, L15 medium supplemented with 15% FBS was added. The suspension was centrifuged at 3000 rpm for 5 min. Pelleted cells were resuspended by adding complete L15, plated in 25 cm^2 flasks and incubated at 25 °C. These flasks were refreshed with fresh medium after 2 days and every 3 days thereafter. Confluent cells were subcultured at a ratio of 1:2.

2.6. siRNA preparation and transfection

siRNAs were synthesized by *in vitro* transcription (IVT) method as described by Zhu et al. [21] with modifications. Briefly, purified complimentary oligonucleotides with leader sequence (Table 1) were annealed in 20 mM Tris-acetate (pH 7.9), 10 mM magnesium acetate, 50 mM potassium acetate, and 1 mM DTT by incubating at 85 °C for 1 min followed by incubation at 37 °C for 10 min to produce a template. An aliquot of 1 μL of annealed template [Nectin-4-S1 and Nectin-4-S2 to produce Nectin-4 sense strand (Nectin-4S); Nectin-4-AS1 and Nectin-4-AS2 to produce Nectin-4 antisense strand (Nectin-4-AS); Scramble-S1 and Scramble-S2 to produce Scramble sense strand (Scramble-S); Scramble-AS1 and Scramble-AS2 to produce Scramble antisense strand (Scramble-AS)] was used in 20 μL IVT reaction containing 1X Buffer, 100 mM DTT, 20 units of RNase inhibitor, 100 μM each of rNTPs and T7 RNA polymerase (Promega, USA). The mixture was incubated at 37 °C for 2 h, DNase I treated and purified using RNA cleanup column (Invitrogen, USA). Sixty micrograms of complementary RNA strands were annealed at 90 °C for 1 min following incubation at 37 °C for 10 min and further treated with 600 U of RNase H (TaKaRa, Japan) at 30 °C for 1 h to remove the leader sequence. The RNA was precipitated with ethanol and resuspended in nuclease-free water.

For transfection experiments, primary brain cells developed as described earlier were seeded into a 96-well plate at density of 5×10^3 cells per well at 24 h before the experiment. Cells were

transfected with specific or scrambled siRNA (10 nM, 3pmol/well) using Lipofectamine 2000 reagent (0.1 mL/well; Invitrogen). At 6, 12 and 24 h post-transfection the cells were analyzed by qPCR for the expression of Nectin-4 to confirm gene silencing. At 48 h post-transfection, transfected or control cells were infected with 10^5 $TCID_{50}$ dose of virus. At 6, 12 and 24 h post-infection, wells were washed with PBS three times and cells were harvested. Viral gene and protein expression levels in the harvested cells were analyzed using qPCR and western blotting respectively.

2.7. Western blotting

Samples of total cell lysate were prepared with passive lysis buffer (Promega, USA) containing a mixture of protease inhibitors (Sigma, St. Louis, MO, USA), Samples were incubated in protein sample buffer (final concentration: 50 mM Tris, pH 6.8, 1% SDS, 10% glycerol, 1% β-mercaptoethanol, and 0.01% bromophenol blue) for 5 min at 95 °C before running SDS-PAGE at room temperature followed by immunoblotting with detection antibodies. Primary antibodies were identified with horse radish peroxidase (HRP) conjugated secondary antibody (Dako, Denmark) and 3,3′,5,5′- tetramethyl Benzidine (TMB) substrate (Sigma, St. Louis, MO, USA). Primary antibody used was monoclonal *anti*-NNV capsid protein antibody.

2.8. Gene expression analysis

Specific primers were designed using the primer blast server (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) based on *H. septemfasciatus* Nectin-4 sequence. First strand cDNA was synthesized using ReverTra Ace qPCR RT Kit (Toyobo, Japan) according to the manufacturer's protocol (500 ng DNase treated RNA per 10 μL reaction). Quantitative real-time PCR (qPCR) was performed on an Exicycler 96 Real-Time Quantitative Thermal Block (Bioneer, Korea) using AccuPower 2 × GreenStar qPCR Master Mix (Bioneer, Korea). All real-time PCR reaction contained the following: 1.0 μL of each primer (5 μM), 5.0 μL SYBR Green supermix, 2.0 μL RNase/DNase-free water, and 1.0 μL 200 ng/μL cDNA. The PCR reaction mixture was denatured at 95 °C for 30 s and then subjected to 40 cycles of 95 °C for 10 s and 60 °C for 20 s followed by dissociation curve analysis to verify the specificities of amplicon. PCR amplifications were performed in triplicates for each sample. Relative mRNA expression was calculated using the Livak and Schmittgen method [22]. EF-1α was used as internal reference gene.

2.9. Statistical analysis

Data were subjected to one way analysis of variance (ANOVA) and multiple Student t-tests using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). Each experiment was replicated thrice for replicability. $P < 0.05$ was considered statistically significant. Error bars are expressed as mean ± standard deviation.

3. Results

3.1. Viral growth curve analysis in Nectin-4 transfected SSN-1 cells

When transfected SSN-1 cells were experimentally infected with NNV, cytoplasmic vacoulation formed at 24–48 h post infection. In contrast, no visible cytopathic effects were seen in wild-type cells at this time point. Transfected cells displayed extended plaque formation at three days post-infection compared to mock-transfected group (Fig. 1A). Comparison of replication kinetics of NNV in a single-step growth curve demonstrated significantly faster replication in cell lines expressing Nectin-4 compared to untransfected cells ($P < 0.05$) based on RNA2 copy number and viral titer (Fig. 1B and C).

3.2. Interaction analysis of Nectin-4 with NNV

To understand the interaction of Nectin-4 with NNV, virus binding assay was performed. Results showed that the binding of viral particles was more pronounced in Nectin-4 expressing transfected cells compared to the untransfected group. Virus binding was evident from 15 min post-incubation in the transfected group. In the control group, the binding was evident at 45 min post-incubation. The copy number was different compared with the transfected cells at this time point (Fig. 2A). To further examine the interaction of Nectin-4 with NNV, pEGFP- Nectin-4 transfected cells were infected with NNV and then immunostained with NNV capsid protein specific antibodies. Co-localizing signal (yellow) of NNV and Nectin-4 was observed (Fig. 2B).

3.3. In vitro siRNA silencing and viral replication

To elucidate the role of Nectin-4 in NNV infection, we transfected specific siRNA to primary brain cells in order to knock down the Nectin-4 gene expression. Cells transfected with specific or scrambled siRNA displayed similar morphology as control cells, indicating no cytotoxicity to cells. Expression analysis by qPCR showed that transfection with scrambled siRNA did not alter Nectin-4 levels. In contrast, transfection of Nectin-4 specific siRNA resulted in nearly complete knockdown of Nectin-4 in cells at 48 h post transfection (Fig. 3A).

At 48 h post-transfection, cells were infected with NNV and NNV RNA2 expression levels were examined using qPCR, immunoflourescence and western blotting. The viral copy number was reduced significantly at all time points post-infection in the transfected cells whereas there were increasing viral copies in control cells (Fig. 3B). Results of immunoflourescence and western blot also demonstrated the same results, showing increased capsid protein concentration in untransfected cells and absence or faint protein expression in Nectin-4 silenced cells after NNV infection (Fig. 3C and D).

4. Discussion

The initial encounter of virus to its host cell is mediated by viral surface components such as glycoprotein or sites on the viral capsid so that virus can bind to specific receptors on target cell surface. Preference of receptor usage varies with the viruses. Some viruses share a common receptor in their pathogenesis [23,24]. Understanding the interaction between the viruses and their host receptors are necessary to obtain complete insight into their infection biology. In the present study, we evaluated the possible role of a cell surface protein Nectin-4 as a receptor in the pathogenesis of nervous necrosis virus. Nectin family proteins are known as potential receptors for a variety of animal viruses predominantly those known to cause neurodegenerative diseases [2–8]. They were initially identified as the receptors for poliovirus and known as poliovirus receptors (PVR) [25]. Nectin proteins identified include *Nectin-1* or *PVRL1 (CD111), Nectin-2* or *PVRL2 (CD112), Nectin-3* or *PVRL3 (CD113)* and *Nectin-4 or PVRL4* [2,9,26,27].

Nectin-4 or PVRL4 localized in the adherens junctions of the cells regulates several cellular activities including cell movement, and polarization. It has been implicated as the receptor for several viruses [28]. It is important for the formation of adherens junction during the polarization of epithelial cells together with the cadherins [29]. Our previous study [10] has identified Nectin-4 from the seven-band grouper and demonstrated its association with NNV during the infection process. It showed increased transcriptional modulation in brain gill and the kidney [10]. Previous reports have suggested that Nectin-4 is a cellular receptor for several animal viruses including MeV, CDV, and PPRV [6–8].

SSN-1 cells transfected with Nectin-4 expression construct displayed a significant increase in replication kinetics of NNV compared to control cells. Single step viral growth curve and plaque assay demonstrated a faster replication of NNV with an increased titer and NNV RNA2 copy

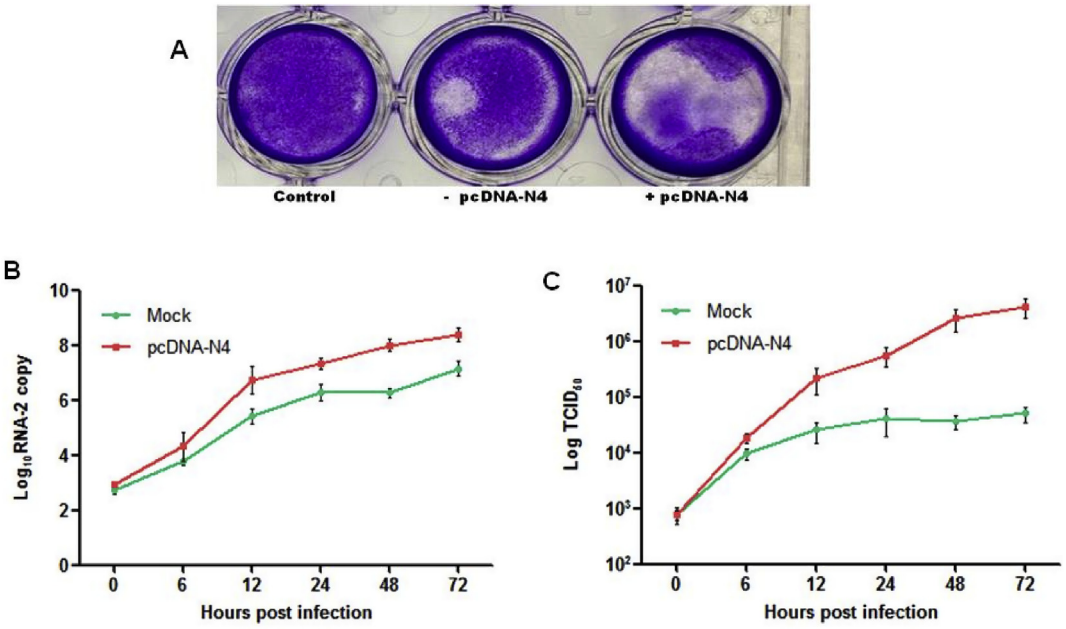

Fig. 1. (A) Plaque formation (3 dpi) on SSN-1 cells expressing Nectin-4 compared with mock (empty vector) transfected cells and control. **(B, C)** Single-step growth curve of NNV in SSN-1 cell lines. Viral titration was performed in SSN-1 cells using with freeze-thawed cell lysate from virus infected wild and transfected cells (**B**). NNV RNA-2 copy number was estimated from total RNA of virus infected wild and transfected cells (**C**). All infections were performed in triplicate. Error bars denote standard deviations from the means.

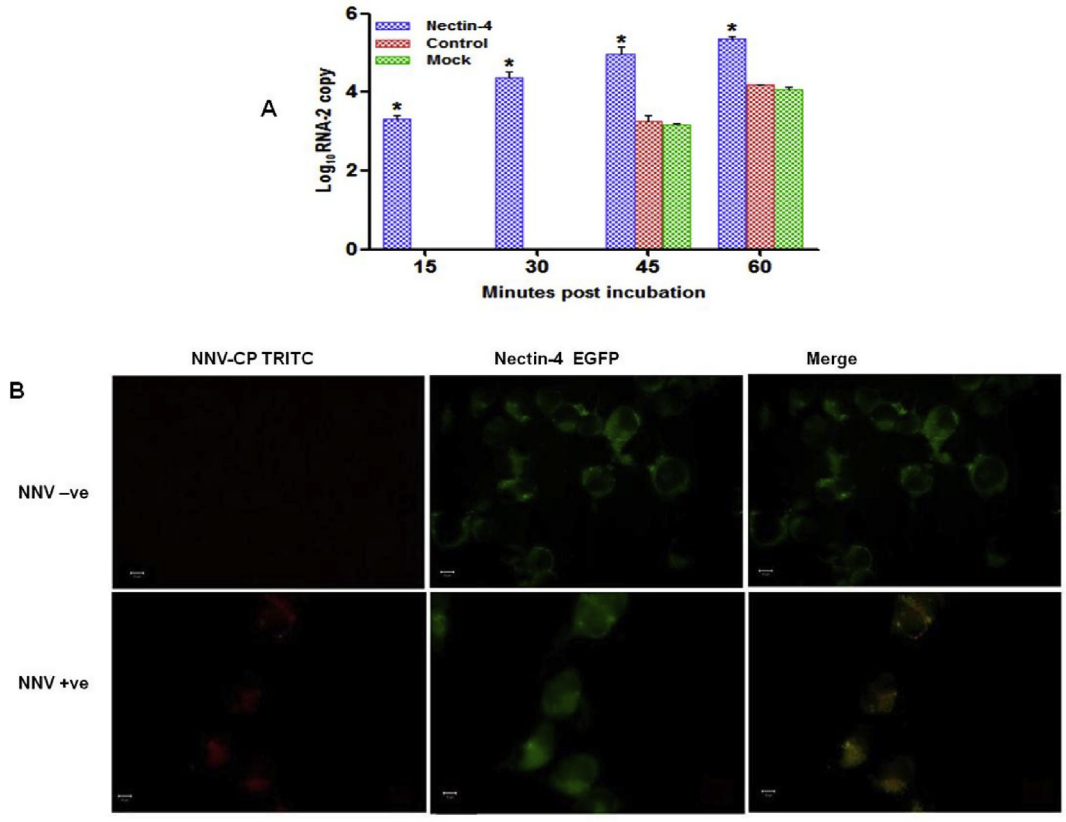

Fig. 2. (A) Virus binding assay on Nectin-4 expressing SSN-1 cells compared with control and mock transfected cells. 10^8 $TCID_{50}$/ml NNV was incubated with Nectin-4 expressing cells for 1 h at 25 °C and virus attachment was subsequently measured using RT-PCR at 15 min intervals. Error bars indicate standard deviations. * indicates significant difference at $P < 0.05$. **(B)** Localization of Nectin-4 and NNV coat protein analyzed by immunofluorescence staining.

number in the growth curve. Similar results were derived in case of canine Nectin-4 expressing Vero cells which displayed syncitia and plaque formation at 2 days post infection (dpi) with CDV [7]. Experimental infection of Nectin-4 lentiviral transduced sheep kidney epithelial (PO) cells with PPRV resulted in syncytia formation at 2dpi and faster replication kinetics [8]. Our results were in concurrence with

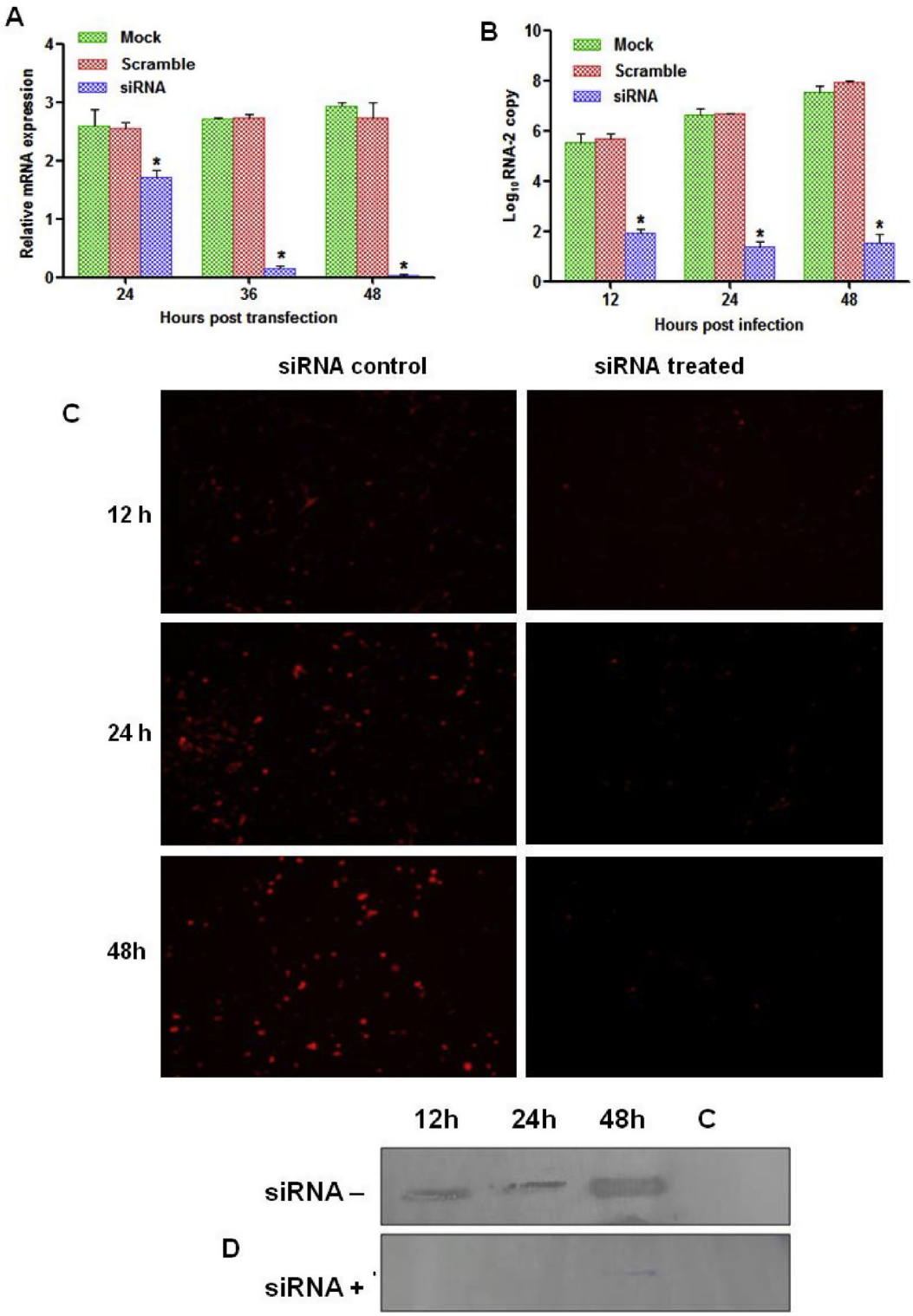

Fig. 3. siRNA downregulation of Nectin-4 gene expression and its effects on NNV replication. Primary brain cells were transfected with Nectin-4-specific siRNAs or scrambled siRNA as a negative control. **(A)** Levels of Nectin-4 mRNA were analyzed by real-time PCR at 24, 36 and 48 h post-transfection. **(B, C)** After siRNA transfection, cells were infected with NNV (10^5 $TCID_{50}$), NNV RNA2 copy number **(B)** and capsid protein expression by Immunoflourescence (C) and western blot **(D)** were examined at 12, 24, and 48 h post-infection. Error bars indicate standard deviations. * indicates significant difference at $P < 0.05$.

these observations showing cytoplasmic vacuolation and plaque formation at 3 dpi in grouper Nectin-4 expressing SSN-1 cells. This suggests the involvement of Nectin-4 in the NNV infection.

Virus binding and immuno-colocalization experiments were performed to elucidate the role of Nectin-4 in NNV entry into cells. The time-dependent virus binding assay was performed using Nectin-4 expressing SSN-1 cells. It demonstrated increased uptake of the virus at 15 min post-infection compared to the control group. A similar approach on CHO cells expressing human Nectin-4 has shown that Nectin-4 is a bonafide receptor used by MeV for the attachment to the host cell [6]. Immunostaining of Nectin-4 with NNV capsid protein showed a positive co-localization, implying their significant interaction within.

This observation was supported by our previous study showing that, Nectin-4 interacted well with NNV-capsid protein on docking simulation [10]. Viruses that use Nectin family proteins as their cellular receptors can interact with their concerned Nectin receptors with high affinity using adhesive interface located at N-terminal loop domains, especially the Ig-V domain [30]. Nectin-4 was predicted to possess three potential N-linked glycosylation motifs in its N-terminal ectodomain which could be glycosylated according to the previously studies of crystal structure of human Nectins [10,31]. Studies by Chi et al. [32] and Costa et al. [33] have stated that NNV capsid protein is heavily glycosylated implying its possible benefit in determining specific receptor motif at the cell surface. Previous studies have reported that NNV can gain entry into the cell by receptor-mediated endocytosis targeting sialic acid residues on SSN1 cell membrane as well as heat shock cognate protein 70 (HSC70) on GF-1 cell as potential receptors [16,17]. These findings indicate that NNV capsid proteins might recognize the distinct glycan motifs of Nectin-4 at host cell surface and contribute to its cellular entry.

To confirm these results, we performed siRNA directed Nectin-4 knockdown in primary grouper brain cells and infected them with NNV. Results demonstrated that Nectin-4 expression was effectively reduced following siRNA transfection. Scrambled siRNA did not inhibit NNV infection. However, nectin-4 siRNA blocked the NNV infection. Similar observations were obtained showing that MeV was blocked in MCF7 and NCI–H358 cells with Nectin-4 silenced and that canine nectin-4 silenced MDCK cells restricted CDV infection suggesting unrestricted Nectin-4 surface expression was essential for viral infection [6,34].

In summary, results of the present study demonstrated that NNV uses Nectin-4 as a receptor for cellular entry. Overexpression of the Nectin-4 enhanced viral replication kinetics whereas its silencing resulted in diminished viral interaction with host cells. Identification of grouper Nectin-4 as a potential receptor highlights the important role of this protein in NNV pathogenesis. It contributes to our understanding of viral nervous necrosis in teleost.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.08.019.

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