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Singapore grouper iridovirus-encoded semaphorin homologue (SGIV-sema) contributes to viral replication, cytoskeleton reorganization and inhibition of cellular immune responses

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Semaphorins are a large, phylogenetically conserved family of proteins that are involved in a wide range of biological processes including axonal steering, organogenesis, neoplastic transformation, as well as immune responses. In this study, a novel semaphorin homologue gene belonging to the Singapore grouper iridovirus (SGIV), ORF155R (termed *SGIV-sema*), was cloned and characterized. The coding region of *SGIV-sema* is 1728 bp in length, encoding a predicted protein with 575 aa. SGIV-sema contains a ~370 aa N-terminal Sema domain, a conserved plexin-semaphorin-integrin (PSI) domain, and an immunoglobulin (Ig)-like domain near the C terminus. SGIV-sema is an early gene product during viral infection and predominantly distributed in the cytoplasm with a speckled and clubbed pattern of appearance. Functionally, SGIV-sema could promote viral replication during SGIV infection *in vitro*, with no effect on the proliferation of host cells. Intriguingly, ectopically expressed SGIV-sema could alter the cytoskeletal structure of fish cells, characterized by a circumferential ring of microtubules near the nucleus and a disrupted microfilament organization. Furthermore, SGIV-sema was able to attenuate the cellular immune response, as demonstrated by decreased expression of inflammation/immune-related genes such as IL-8, IL-15, TNF-α and mediator of IRF3 activation (MITA), in SGIV-sema-expressing cells before and after SGIV infection. Ultimately, our study identified a novel, functional SGIV gene that could regulate cytoskeletal structure, immune responses and facilitate viral replication.

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INTRODUCTION

During the long co-evolutionary relationship with their host, viruses have developed myriad strategies to manipulate host cellular and immune environments (Alcami & Koszinowski, 2000; Mahalingam *et al.*, 2002). A variety of viruses, especially the large DNA viruses, can encode homologues of host genes, indicating that viruses have the ability to acquire and modify genes from their host in order to benefit themselves during virus–cell interactions (Alcami & Koszinowski, 2000; Bugert & Darai, 2000; Finlay & McFadden, 2006). A detailed insight into the function of viral homologues of host genes may help us deconstruct the fundamental properties of viral pathogenesis and provide directions in developing novel antiviral strategies (Tortorella *et al.*, 2000).

Semaphorins are a large phylogenetically conserved family of secreted, membrane-associated and glycosylphosphatidylinisotol (GPI)-linked proteins that execute functions in multiple systems (Goshima *et al.*, 2002; Kikuchi *et al.*, 1999; Kolodkin *et al.*, 1993; Yazdani & Terman, 2006). To date, more than 20 distinct semaphorins have been identified, and they are widely distributed from worms to mammals, as well as viruses (Goshima *et al.*, 2002; Kikuchi *et al.*, 1999; Nakamura *et al.*, 2000). All semaphorins possess a conserved extracellular Sema domain (~500 aa) in their N terminus. A class-specific C terminus that contains additional sequence motifs like the plexin-semaphorin-integrin (PSI) domain and the immunoglobulin (Ig)-like domain

The GenBank/EMBL/DDBJ accession number for the SGIV-sema sequence of SGIV is YP_164250

One supplementary table and one supplementary figure are available with the online version of this paper.

allows semaphorins to be classified into eight distinct subfamilies (Qu *et al.*, 2002; Sultana *et al.*, 2012; Tamagnone & Comoglio, 2004). Class 1 and 2 semaphorins exist in invertebrates, classes 3–7 express in vertebrates, and class V is specific to viruses (Nakamura *et al.*, 2000; Qu *et al.*, 2002).

Semaphorins were initially identified as axon-guidance cues in the developing central nervous system (Pasterkamp & Kolodkin, 2003). Extensive work revealed that semaphorin-mediated guidance was exerted through regulating cytoskeleton reorganization (Kalil & Dent, 2004; Tran *et al.*, 2007). For example, semaphorin 3A (Sema3A)-induced collapse of the dorsal root ganglion accompanied by a net loss of F-actin and microtubule rearrangement (Fan *et al.*, 1993). Meanwhile, a similar phenomenon was also found in semaphorin 4D (Sema4D), which was reported to be capable of inducing F-actin and β-catenin rearrangement in human umbilical vein endothelial cells (Conrotto *et al.*, 2005). Although semaphorins were initially defined as neuronal repellents, it is now clear that they are widely expressed outside the nervous system and trigger a diverse spectrum of signalling pathways, including modulation of immune response, cell survival, cell migration, apoptosis and viral diseases (Tamagnone & Comoglio, 2004; Zhou *et al.*, 2008). For example, semaphorin 7A (Sema7A), the only GPI-linked member in the family, was reported to facilitate West Nile virus (WNV) pathogenesis in mice through positive regulation of TGF-β1/Smad6 signalling (Sultana *et al.*, 2012).

Intriguingly, recent studies have demonstrated that many viruses are able to encode semaphorin homologues. *Vaccinia virus* (VV), a member of the *Poxviridae* family, was found to encode a semaphorin homologue protein, A39R/SEMAVA (Kolodkin *et al.*, 1993). VV A39R has been reported to promote the production of proinflammatory cytokines in human monocytes, induce actin rearrangement, and inhibit integrin-mediated adhesion of mouse dendritic cells, and is thus involved in the virus-associated immune modulation (Comeau *et al.*, 1998; Walzer *et al.*, 2005). Viral semaphorin homologues were also identified in herpesviruses, like the alcelaphine herpesvirus-1 (AHV-1)-encoded SEMAVB (Ensser & Fleckenstein, 1995). To date, however, no functional semaphorin homologue has been identified in other DNA virus families. Iridoviruses have gained increasing attention because of the high mortality rate and serious systemic diseases they cause in aquatic species. Among them, Singapore grouper iridovirus (SGIV) is a recently identified member belonging to genus *Ranavirus* and family *Iridoviridae* (Qin *et al.*, 2001, 2003), and has caused significant economic losses to grouper aquaculture in China and Southeast Asian countries. The SGIV genome sequence has been determined and *in silico* genomic analysis of SGIV suggests that several viral genes may be acquired from the host and are involved in virus–host interactions (Song *et al.*, 2004).

In this study, we identified a semaphorin homologue encoded by SGIV ORF155R and named it *SGIV-sema*. SGIV-sema shares a conserved Sema domain, a PSI domain, and an Ig-like domain with other reported semaphorins in different species, from fish to mammals, as well as viruses. We found that SGIV-sema was an early gene product during viral infection and predominantly distributed in cytoplasm. Furthermore, SGIV-sema could facilitate viral replication and regulate cytoskeleton rearrangement and immune responses, suggesting that SGIV-sema could modulate the cellular environment to support the infectious process during SGIV infection.

RESULTS

Cloning and characterization of *SGIV-sema* gene

The previously annotated SGIV genome indicates that SGIV ORF155R (GenBank no. YP_164250) may encode a viral semaphorin homologue. Using designed cloning primers, we obtained the full-length coding region (i.e. 1728 bp) of SGIV ORF155R, which encoded a 575 aa peptide with a predicted molecular mass of 64.6 kDa. The predicted amino acid sequence of SGIV ORF155R shared 96 % similarity with its orthologue in grouper iridovirus (GIV, AAV91114), 30 % with its orthologue in canarypox virus (CNPV, NP_955088), and lesser identity with semaphorin homologues found in fowlpox virus (FPV, NP_039010) and AHV-1 (NP_065506). In addition, SGIV ORF155R showed >25 % identity to the semaphorin homologues from *Oryzias latipes* (XP_004067517), *Gallus gallus* (NP_001186678), *Mus musculus* (EDL25928) and *Homo sapiens* (CAJ55400). *In silico* analysis demonstrated that SGIV ORF155R contained a ~370 aa N-terminal Sema domain, a conserved PSI domain and an Ig-like domain near the C terminus, and shared many conserved residues, including 12 conserved cysteine residues, like other reported semaphorins (Fig. S1, available in the online Supplementary Material). Given the context of these conserved domains, we designated SGIV ORF155R as the *SGIV-sema* gene.

Expression pattern of SGIV-sema in SGIV-infected grouper spleen (GS) cells

To determine the expression profile of SGIV-sema during *in vitro* viral infection, we examined its transcriptional and translational kinetics at consecutive time points after SGIV infection. As shown in Fig. 1(a, b), SGIV-sema mRNA could be detected as early as 4 h after SGIV inoculation, and the protein band could be clearly observed at 8 h post-infection (p.i.), with increasing enrichment accompanied by the viral infection process, suggesting it is transcribed and expressed at an early stage of SGIV infection.

To further determine the expression pattern of SGIV-sema, we performed cycloheximide (CHX) and cytosine arabinoside (AraC) inhibition assays on the SGIV-infected GS cells. A previously characterized SGIV gene that is transcribed at an early stage after viral infection (Gong *et al.*, 2010) – *SGIV dUTPase* – was chosen as an indicative control. As shown in Fig. 1c, after viral infection of the

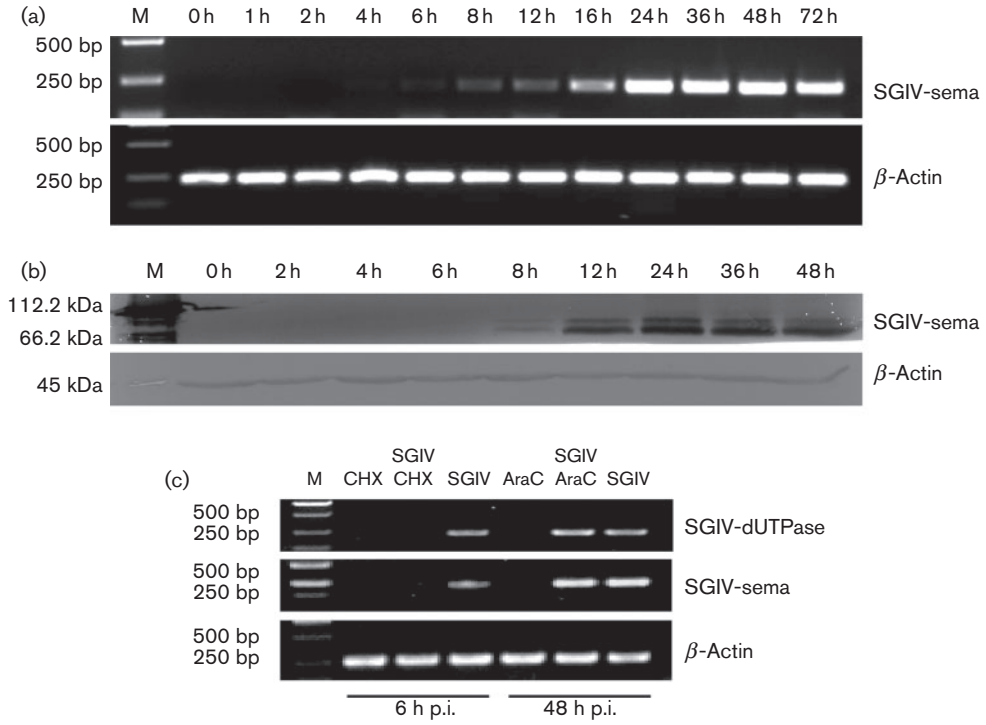

Fig. 1. Temporal expression pattern of SGIV-sema during viral infection. (a) Reverse transcription (RT)-PCR analysis of SGIV-sema expression after SGIV infection in GS cells. (b) Western blot analysis of SGIV-sema expression in SGIV-infected GS cells. (c) RT-PCR detection of SGIV-sema transcripts under drug treatments. Total RNA was isolated from CHX-treated and/or SGIV-infected GS cells at 6 h p.i. and from AraC-treated and/or SGIV-infected GS cells at 48 h p.i. β-Actin was detected under the same conditions as an internal control.

drug-treated cells, the transcription of *SGIV-sema* was totally inhibited in the presence of CHX, but not in the presence of AraC. The expression pattern of SGIV-sema is similar to that of SGIV dUTPase, which can be detected only in the AraC-treated cells, suggesting that SGIV-sema expresses early during viral infection.

Subcellular localization of SGIV-sema

The intracellular distribution of SGIV-sema was assessed by detecting the localization of SGIV-sema–GFP fusion proteins in GS and fathead minnow (FHM) cells. The green fluorescence in the pEGFP–SGIV-sema-transfected cells was diffuse in the cytoplasm and showed a speckled pattern (Fig. 2a, b; lower row). As a control, the empty pEGFP-N3 vector expressed GFP was distributed in whole cells and mainly in the nucleus of fish cells (Fig. 2a, b; upper row), which was consistent with our previous findings (Yan *et al.*, 2013). These results suggest that SGIV-sema is exclusively localized to the cytoplasm, where it executes various functions.

Overexpressed SGIV-sema enhanced SGIV replication during *in vitro* infection

To evaluate the effect of SGIV-sema on SGIV replication, we generated a GS cell line that stably expressed full-length SGIV-sema (GS/pcDNA–SGIV-sema). As a control, we established a stable line that was transfected with empty vector pcDNA3.1 (+) (Fig. 3a). Then, stable GS/pcDNA–sema and GS/pcDNA3.1 cells were infected with SGIV individually, and the viral replication kinetic curves were examined and compared. As shown in Fig. 3b, the replication kinetics of SGIV in GS/pcDNA–SGIV-sema was quite different from that in GS/pcDNA3.1. Specifically, the viral titres in the two stable lines were quite similar before 12 h p.i. After 12 h p.i., the viral titres in GS/pcDNA–SGIV-sema began to increase more quickly than in GS/pcDNA3.1. During the period from 24 to 48 h p.i., the viral titres yielded from SGIV-sema-expressing cells were almost 10-fold higher than those in the control group.

To verify the above results, the transcription kinetics of three indicative SGIV genes, including the immediate-early (IE) gene *ICP18* (Xia *et al.*, 2009), early (E) gene *dUTPase* (Gong *et al.*, 2010) and late (L) gene that encodes the major capsid protein (*MCP*), were measured by real-time PCR. As shown in Fig. 3c, the transcript levels of all these genes were much higher in GS/pcDNA–SGIV-sema cells than in GS/pcDNA3.1 at 24, 48 and 72 h p.i., individually. Taken together, these data suggest that overexpressed SGIV-sema could benefit SGIV replication during viral infection *in vitro*.

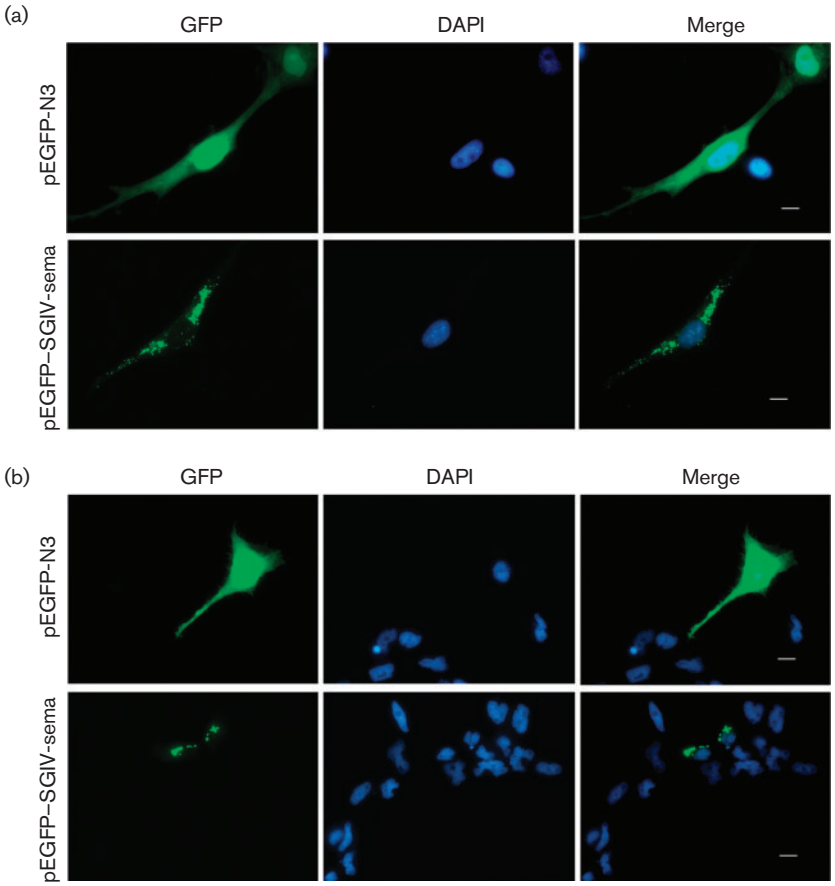

Fig. 2. Intracellular distribution of SGIV-sema. Subcellular localization of SGIV-sema in GS (a) and FHM cells (b) observed by fluorescence microscopy. GS or FHM cells were transfected with pEGFP-N3 (a, b; upper row) or pEGFP–SGIV-sema (a, b; lower row) for 36 h, respectively. Green fluorescence signal in GFP channel indicates the expression of SGIV-sema. DAPI staining of nuclei is shown in blue. Bars, 10 μm.

SGIV-sema did not affect cell proliferation

Given our finding that SGIV-sema was able to promote SGIV replication during *in vitro* infection, we next asked if this was due to its effect on cell proliferation. To address this, the stable cell lines GS/pcDNA–SGIV-sema and GS/pcDNA3.1 were subcultured at the same condition, and the proliferation rates of these stable lines were compared. We found SGIV-sema-expressing cells were not morphologically distinguishable from the control cells (Fig. 4a). Furthermore, both cells displayed typical curves in a time-dependent manner, and no obvious alteration of growth was observed between SGIV-sema-expressing cells and the control cell line (Fig. 4b). Similar results were also obtained in stable FHM cell lines (data not shown). These data indicate SGIV-sema has no impact on fish cell proliferation, implying that its contribution to viral replication may be achieved via other approaches.

SGIV-sema altered the cell cytoskeleton

Considering the special intracellular distribution of SGIV-sema in fish cells, we next tested whether SGIV-sema was related to the cytoskeleton. To conduct these studies, overexpression and immunofluorescence studies were performed in GS cells. As shown in Fig. 5a, cells transfected with empty vector (pEGFP-N3) or pEGFP–SGIV-IGF displayed well-organized, intact microtubule structure throughout the entire cytoplasm (Fig. 5a, upper and lower rows). Intriguingly, in SGIV-sema-expressing cells, the emanative arrangement of microtubules was disrupted and replaced by bright tubulin rings forming at the perinuclear region; only exiguous microtubule protofilaments could be observed (Fig. 5a, middle row). Next, we examined the effect of SGIV-sema on microfilament morphology. We found reduced microfilament organization in SGIV-sema-expressing cells, manifested by almost complete disappearance of F-actin stress fibres from the cytoplasm compared with the control cells (Fig. 5b). These data suggest SGIV-sema could affect the cytoskeletal structure in host cells, which may in turn regulate the process of viral infection.

SGIV-sema regulated the expression of fish immune-related genes

In mammals, emerging evidence suggests that semaphorin is involved in immune modulation (Takamatsu *et al.*, 2010). Next, we sought to determine if SGIV-sema could regulate the immune response of fish cells. As shown in Fig. 6, in the absence of SGIV infection, SGIV-sema evoked a slight reduction in IL-8, IL-15, mediator of IRF3 activation (MITA) and TNF-α, and a relatively large decrease in the production of MHC-II, compared to control cells. As expected, SGIV infection was able to elicit transcriptional

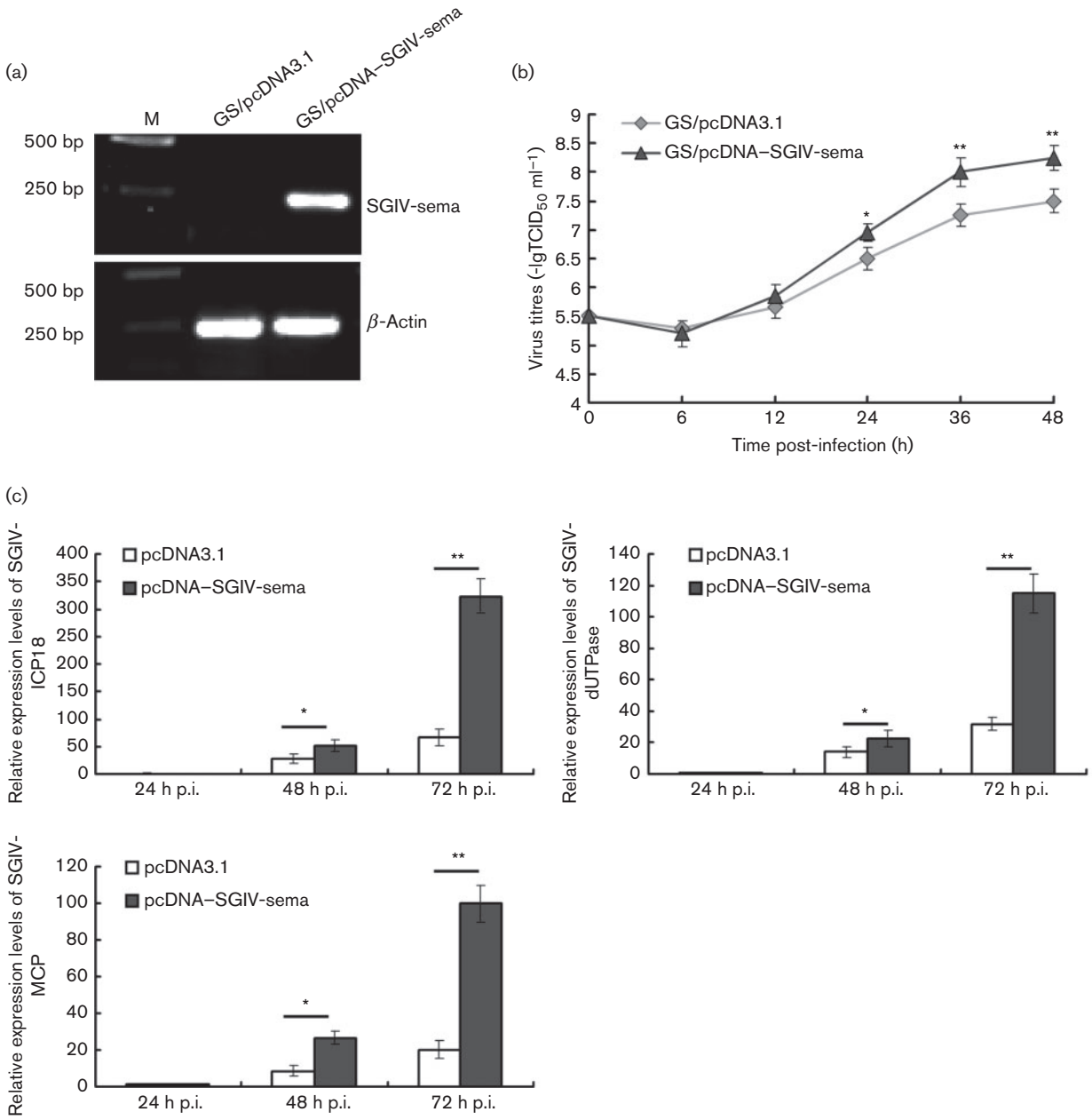

Fig. 3. SGIV-sema enhances viral replication. (a) Validation of SGIV-sema expression in GS/pcDNA–SGIV-sema stable cell line by RT-PCR. M, DNA marker; GS/pcDNA3.1, control stable line; GS/pcDNA–SGIV-sema, stable line with SGIV-sema overexression. (b) Viruses were collected from the lysates of two stable GS cell lines infected by SGIV at different time points (0, 6, 12, 24, 36 and 48 h p.i.), and the viral titres were determined using the $TCID_{50}$ method. $n=3$; means ± SD; $^{**}P<0.01$ compared with the control group. A second experiment provided similar results. (c) Relative mRNA levels of SGIV ICP18, dUTPase and MCP in two stable GS lines after SGIV infection were assessed by real-time PCR, using β-actin as reference gene. The data indicate fold change in gene expression levels in the control cell line at 24 h p.i. $n=3$; mean ± SD; $^{**}P<0.01$ compared with the control group. The experiments were performed three times with similar results.

activation of the given genes. Furthermore, SGIV-sema overexpression resulted in a dramatic reduction of these genes during viral infection, especially for IL-8, MHC-II and TNF-α, which were downregulated by 39 %, 75 % and 55 %, respectively. Of note, we did not find apparent differences in the expression of the hepcidin 1 gene, which encodes an antimicrobial peptide (Zhou *et al.*, 2011), between SGIV-sema expressing and control cells. These data suggest that SGIV-sema negatively tunes cellular immune response through selectively downregulating the

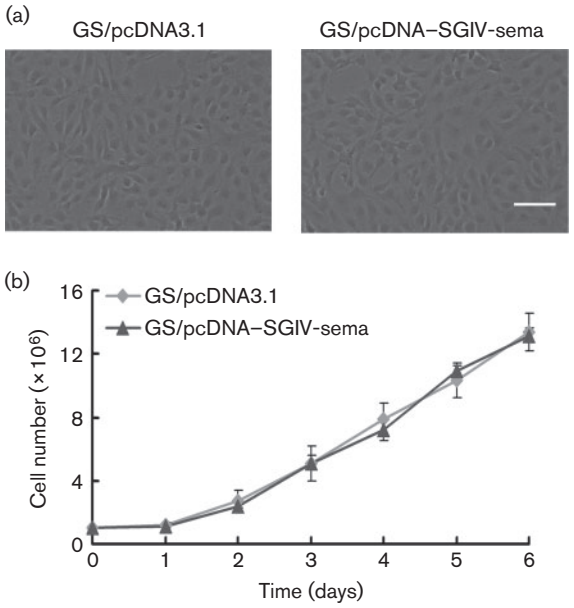

Fig. 4. Effect of SGIV-sema overexpression on cell proliferation. (a) The morphologies of GS cells transfected with pcDNA3.1 or pcDNA–SGIV-sema were observed by phase-contrast microscopy. Bar, 100 μm. (b) Growth curves of GS/pcDNA3.1 and GS/pcDNA–SGIV-sema cell line cultures.

expression of immune-related genes, to facilitate productive viral infection.

DISCUSSION

Semaphorins were initially identified two decades ago as molecular cues for axonal guidance during neural development (Spriggs, 1999). Later, outbreak studies demonstrated that semaphorins are a large and diverse family of proteins that are conserved both structurally and functionally from invertebrates to mammals (Qu *et al.*, 2002; Tamagnone & Comoglio, 2004). To date, >20 distinct semaphorins have been identified from divergent animal phyla, and they are well known to be implicated in many processes, including cell migration guidance, immune response regulation, angiogenesis and cancer progression (Sultana *et al.*, 2012; Yazdani & Terman, 2006). During the ongoing co-evolution of virus and host, some viruses, especially DNA viruses, could encode homologues of cellular proteins to support infectious processes (Holzerlandt *et al.*, 2002). Previous studies have shown that poxviruses such as VV, FPV and CNPV and herpesviruses such as AHV can encode functional semaphorin homologues during infection (Gardner *et al.*, 2001; Ensser & Fleckenstein, 1995). However, as far as we know, no functional semaphorin homologue has been identified from iridoviruses. In the present study, we identified a novel viral semaphorin homologue encoded by SGIV, a recently identified iridovirus belonging to the genus *Ranavirus* of the family *Iridoviridae*.

The semaphorin family is characterized by a Sema domain at the N terminus, a PSI domain, and at times, an Ig-like domain near their C terminus (Sultana *et al.*, 2012; Xu *et al.*, 2000). Based on the previous genomic annotation of SGIV (Song *et al.*, 2004; Teng *et al.*, 2008), we found that SGIV ORF155R encoded a putative protein containing a typical Sema domain, a PSI domain, as well as an Ig-like domain. All these domains share high similarity with that of known semaphorins, including semaphorins from virus, fish, chicken, mouse and human. This suggests that the product of SGIV ORF155R may perform similar functions with vertebrate semaphorins, and prompts the speculation that SGIV ORF155R may be a viral homologue of the semaphorin family. Thus, we designated this gene *SGIV-sema*. Notably, we did not find any *SGIV-sema* homologue gene from other reported iridoviruses, except for another GIV isolated from the yellow grouper *Epinephelus awoara* (Tsai *et al.*, 2005), indicating that *SGIV-sema* is a unique fish iridovirus gene.

According to the temporal kinetic expression profile, the genes of iridoviruses can be categorized into three classes: IE genes, E or delayed-early genes, and L genes (D'Costa *et al.*, 2001). By assessing the expression profiles of SGIV genes during *in vitro* viral infection, we found that SGIV-sema transcripts can be detected as early as 4 h after viral inoculation, and the translational products can be clearly detected at 8 h p.i. Drug inhibitor assays showed only the inhibition of protein synthesis by CHX treatment could block SGIV-sema expression. These data suggest that *SGIV-sema* is an early gene and might produce functional molecules during the early stages after viral infection. Intriguingly, *SGIV-sema* distributes predominantly in the cytoplasm and exhibits a punctate and diffuse cytoplasmic pattern. Our finding is different from that observed in vertebrate semaphorins, which are generally expressed as secreted, transmembrane, or GPI-linked proteins, suggesting that *SGIV-sema* may play special roles distinct from its host homologous gene.

It has been widely proven that viral homologues of cellular genes could ensure virus replication and propagation during infection, like viral IL-10s in herpesviruses and poxviruses, viral Bcl-2s in herpesviruses and adenovirus, as well as orthopoxviruses encoded homologue of interferon-γ receptor (Alcami & Koszinowski, 2000; Polster *et al.*, 2004; Slobedman *et al.*, 2009). Previous studies showed that a WNV-encoded Sema7A homologue contributed to viral pathogenesis through regulation of TGF-β1/Smad6 signalling (Sultana *et al.*, 2012). We next asked if SGIV-sema expression was essential or beneficial to viral infection. To address this question, we generated a stable grouper cell line that constitutively expressed SGIV-sema. We examined and compared the SGIV replication kinetic curves between SGIV-sema-expressing cells and control cells during the time course of infection. We found that the viral titres in SGIV-sema-expressing cells increased more quickly from 12 h p.i. Also, from 24 to 48 h p.i., the viral titres from SGIV-sema-expressing cells were almost 10-fold higher

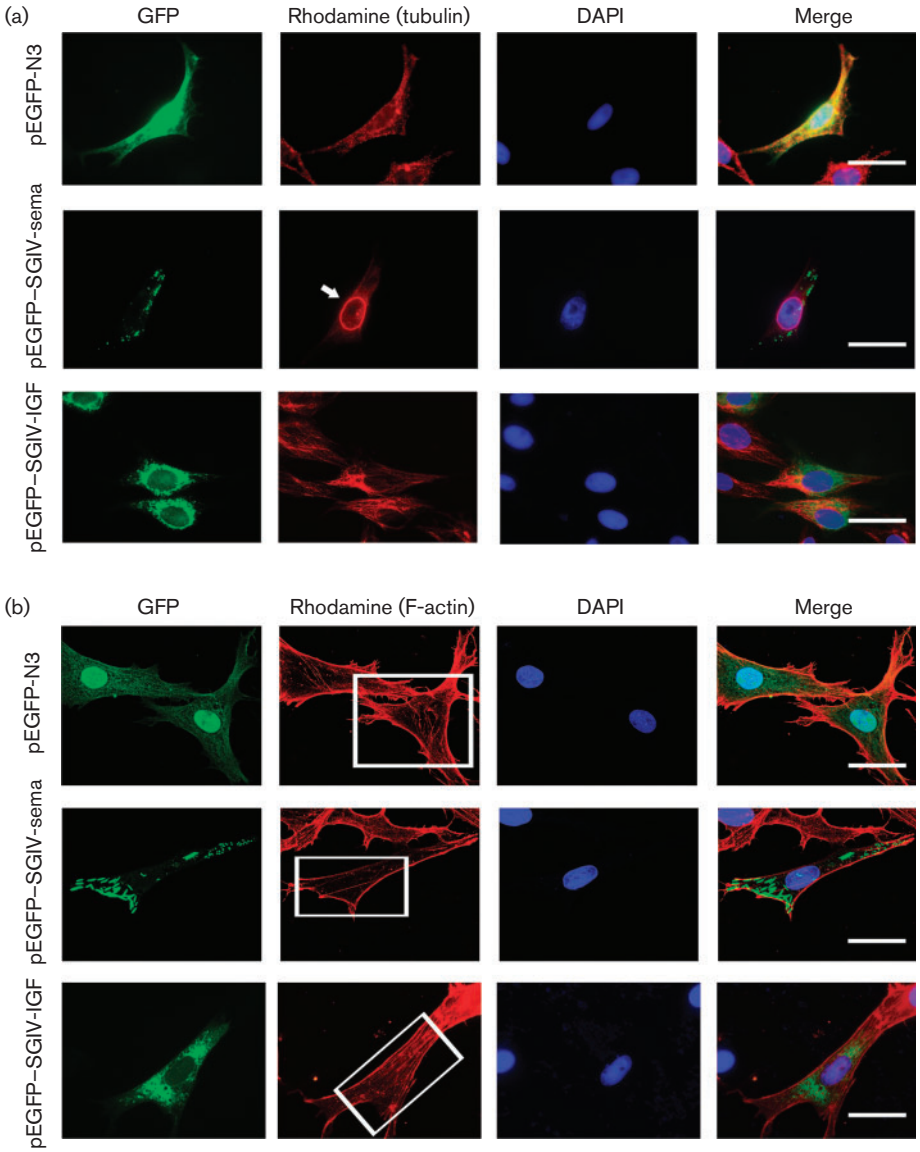

Fig. 5. SGIV-sema causes cytoskeleton alteration. GS cells transfected with pEGFP-N3 (a, b; upper row), pEGFP–SGIV-IGF (a, b; lower row) or pEGFP–SGIV-sema (a, b, middle row) were stained for α-tubulin (a, red) or F-actin (b, red). Nuclei were stained by DAPI (a, b, blue). White arrow denotes the ring structure formed by microtubule reorganization (a, middle row). The rectangles indicate the cytosolic microfilaments (b). Bars, 10 μm.

than those from the control group. Meanwhile, the transcript levels of SGIV functional genes such as *SGIV ICP18*, *dUTPase* and *MCP* were higher in samples of the SGIV-sema-expressing line. These data suggest that SGIV-sema expression plays a beneficial role during SGIV infection.

Accumulating evidence has demonstrated that viral genes may facilitate viral infection through the manipulation of cell proliferation to achieve a favourable cellular environment for viral replication (Spriggs, 1996). Our recent study illustrated that the SGIV-encoded IGF homologue contributed to increased viral yield, principally by promoting host cell proliferation (Yan *et al.*, 2013). To ascertain the underlying mechanism of SGIV-sema-promoted viral replication during *in vitro* infection, we first investigated if SGIV-sema had any impact on cell proliferation. The growth curve analysis based on two stable grouper cell lines showed that GS cells with SGIV-sema overexpression were indistinguishable from the control group, with respect to both cell morphology and proliferation rate. These observations demonstrate that SGIV-sema does not affect the regulation of cell proliferation, indicating that SGIV-sema enhances viral replication through another approach.

Initially defined by their roles in axon guidance, semaphorins were found later to also regulate morphogenetic

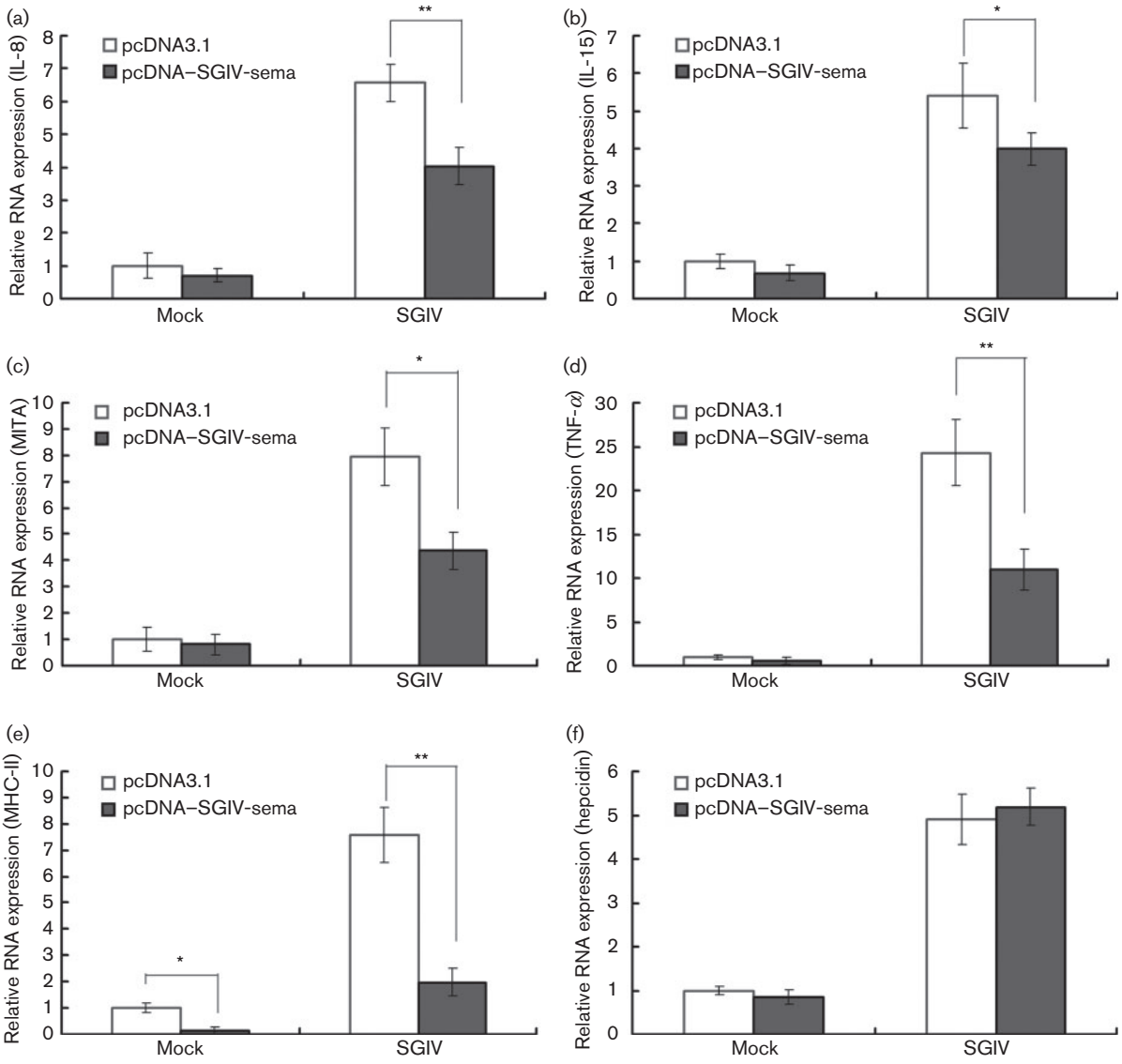

Fig. 6. SGIV-sema downregulates the expression of immune-related genes. GS cells were transiently transfected with SGIV-sema-expressing vectors or control vectors. Then cells were either left uninfected or infected with SGIV for 6 h. Relative mRNA levels of IL-8 (a), IL-15 (b), MITA (c), TNF-α (d), MHC-II (e) and hepcidin 1 (f) were assessed by real-time PCR, using β-actin as a reference gene. The data are normalized to the gene expression levels in cells transfected with empty vector controls and left uninfected. $n=3$; mean ± SD; $^{**}P<0.01$ compared with the control group. The experiments were performed three times with similar results.

events in nonneuronal cells through altering cytoskeletal dynamics (Chisholm, 2008). Considering the special cellular distribution of SGIV-sema, we questioned the possibility of a relationship between SGIV-sema and the cytoskeleton. A number of viruses have been reported to utilize or subvert microfilaments during infection, mainly through regulating the F-actin polymerization dynamics (conformational G-to-F transition) (Taylor *et al.*, 2011). In our study, we found a decreased level of F-actin stress fibres in SGIV-sema-expressing cells (Fig. 5b), along with a stable total β-actin levels (data not shown), suggesting the effect of SGIV-sema on F-actin reduction may be caused by promoting transition from polymeric F-actin to monomeric G-actin, and was, at the least, unlikely through downregulating the expression levels of β-actin. Moreover, SGIV-sema disrupted the emanative network of microtubules and promoted the assembly of bright rings in the perinuclear region. This is consistent with recent work in our laboratory showing that SGIV infection of host cells could change the microtubule structure, which rearranged into a ring-like structure around the nucleus (Huang *et al.*, 2009). These findings allow speculation that SGIV-induced

cytoskeleton alteration may partially depend on the expression of SGIV-sema during viral replication. Furthermore, increasing evidence shows that the cytoskeleton plays critical roles in the replication of many viruses (Jia *et al.*, 2013; Luo *et al.*, 2009). For example, during the infection of respiratory syncytial virus (RSV), microtubules and actin act cooperatively to facilitate efficient viral replication. During this process, microtubules are mainly responsible for the early stage of infection, while actin is in control of viral release (Kallewaard *et al.*, 2005). Besides, frog virus 3 can reorganize microtubules and intermediate filaments at its initial stage of infection (Murti & Goorha, 1983). Thus, it is tempting to speculate that alteration of cytoskeleton by SGIV-sema might favour particular steps of virus infection, and in turn, lead to enhanced viral replication.

Although the roles of semaphorins have been described mainly in the nervous system, their relevance to the immune system is also evident in many studies (Kumanogoh & Kikutani, 2003b). For example, Sema7A (also termed CD108 or Sema-K1), the counterpart of AHVsema, can induce not only the production of proinflammatory cytokines like TNF-α and IL-6, but also monocyte chemotaxis through interaction with VESPR/plexin-C1 (Kumanogoh & Kikutani, 2003a). Furthermore, several virus-encoded semaphorin homologues exhibit the ability to modulate immune responses through binding to their cellular receptors, thus activating intracellular signalling (Comeau *et al.*, 1998). Our previous studies showed SGIV infection could elicit altered expression of numerous genes in the host cells (Huang *et al.*, 2011; Xu *et al.*, 2010). Whether SGIV-sema is involved in the SGIV-induced immune response is another interesting question to be answered. In the present study, we found that SGIV-sema could dramatically reduce the production of immune-related factors, including, but definitely not limited to, IL-8, IL-15, MITA, TNF-α and MHC-II, indicating that SGIV-sema may serve as an important negative regulator of fish immune response to facilitate productive viral infection. Notably, this effect was distinct from the effect observed in VV-encoded A39R/SEMAVA, whose expression contributed to the virally induced acute inflammatory responses (Comeau *et al.*, 1998). The distinct roles of SGIV-sema and VV A39R in the modulation of immune responses suggest that viral semaphorin homologues may perform diverse functions in a viral species-dependent manner, unlike vertebrate semaphorins whose functions are more conserved over the course of evolution. Furthermore, it is important to state that the relationship between SGIV infection and the host immune system is rather complicated, and further studies are needed to uncover this complex network.

Taken together, we identified a novel semaphorin homologue gene, *SGIV-sema*, encoded by SGIV ORF155R. SGIV-sema was an early-expressed transcript during viral replication and predominantly distributed in the cytoplasm. SGIV-sema was beneficial to viral replication during SGIV infection in host cells. SGIV-sema had no effect on cell proliferation; however, SGIV-sema expression could alter cytoskeletal organization, and negatively regulate immune response. Our present study provides additional evidence-based information on SGIV functional genes and provides further insights into SGIV infection and pathogenesis.

METHODS

Cells and virus. Grouper spleen and FHM cells were cultured as previously described (Cui *et al.*, 2011b; Gravell & Malsberger, 1965). SGIV (strain A3/12/98) was originally isolated from a diseased brown-spotted grouper (*Epinephelus tauvina*), and the propagation of SGIV was conducted as described previously (Qin *et al.*, 2003).

Cloning, plasmid construction and computer-assisted analysis. SGIV ORF155R gene (*SGIV-sema*) was cloned from SGIV genomic DNA, by using three different pairs of primers: pcDNA-sema-F/pcDNA-sema-R, pEGFP-sema-F/pEGFP-sema-R and pET-sema-F/pET-sema-R (Table S1). The target PCR products were purified and subcloned into eukaryotic expression vectors pcDNA3.1 (+) and pEGFP-N3, and prokaryotic expression vector pET-32a (+). The resulting constructs were designated pcDNA–SGIV-sema, pEGFP–SGIV-sema and pET–SGIV-sema, respectively.

The similarity of SGIV-sema with other semaphorins was analysed using the BLASTP search program from NCBI (http://www.ncbi.nlm.nih.gov/blast). The conserved domains were predicted using the SMART program (http://smart.embl-heidelberg.de/). Multiple alignments of reported semaphorins protein sequences were performed with CLUSTAL_X 1.83 (http://www.ebi.ac.uk/clustalW/) and edited using the GeneDoc 2.6 programs.

Expression, purification and preparation of the antiserum. pET-SGIV-sema was transformed into *Escherichia coli* Rosseta gami BL21(DE3) and the fusion protein was expressed by exposure to isopropyl 1-thio-β-D-galactopyranoside (0.8 mM) at 37 °C for 6 h. Subsequently, the recombinant protein was purified under denatured conditions by using a HisBind purification kit (Novagen). The polyclonal antiserum against recombinant SGIV-sema was generated by immunizing BALB/c mice after anaesthetization and validated by Western blotting as described in a previous study (Cui *et al.*, 2011a). The animal protocol was approved by the Institutional Review Board of South China Sea Institute of Oceanology, Chinese Academy of Sciences.

Temporal transcription analysis and drug inhibition assay. To determine the temporal transcription of *SGIV-sema* after viral infection, GS cells were either infected or mock infected with SGIV at an m.o.i. of ~0.1 for the indicated time. Total RNA was extracted using TRIzol reagent (Invitrogen) and digested with RNase-free DNase I (TaKaRa). The first-strand cDNA was synthesized with ReverTra Ace qPCR RT kit (TOYOBO), and RT-PCR was performed using primers RT-sema-F/RT-sema-R (Table S1). Simultaneously, to examine the protein expression pattern, Western blot was performed on the protein extracts from samples described above. The primary antibody was anti-SGIV-sema antiserum (host mouse, 1 : 3000), and the secondary antibody was HRP-conjugated goat anti-mouse IgG (1 : 5000) (Pierce). β-Actin was used as a reference gene.

To examine the temporal kinetic class of the *SGIV-sema* gene during viral infection *in vitro*, CHX and AraC (Sigma-Aldrich) were used for *de novo* protein synthesis inhibition and DNA synthesis inhibition, respectively. Briefly, GS monolayer cells were either pretreated with 50 μg CHX ml^{-1} or 100 μg AraC ml^{-1} for 1 h prior to and throughout the SGIV infection. Mock-treated cells were used as the control group. Total RNA was extracted and subjected to RT-PCR as described above. The *SGIV dUTPase* gene, a previously characterized

early gene during SGIV infection (Gong *et al.*, 2010), was used as an indicative control for definition of the kinetic class of *SGIV-sema*.

Intracellular localization analysis. GS or FHM cells were plated on coverslips and cultured in 24-well plates. Cells were transiently transfected with 800 ng of pEGFP–SGIV-sema or pEGFP-N3 empty vectors using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's instructions. About 36 h after transfection, the cells were fixed with 4 % (w/v) paraformaldehyde for 30 min, rinsed with PBS, and permeabilized with 0.2 % (w/v) Triton X-100 for 15 min. Finally, cells were stained with 1 μg 6-diamidino-2-phenylindole ml^{-1} (DAPI; Sigma-Aldrich) and observed by fluorescence microscopy (Leica).

Generation of stable cell lines. To obtain cells that stably expressed SGIV-sema, GS or FHM cells were transfected with pcDNA–sema or empty vector and selected with 800 μg geneticin ml^{-1} (G418) (Gibco) for 4 weeks. The stable line that expressed SGIV-sema was confirmed by RT-PCR. The stable GS lines were termed GS/pcDNA–SGIV-sema and GS/pcDNA3.1, and the stable lines of FHM were termed FHM/pcDNA–SGIV-sema and FHM/pcDNA3.1, respectively.

Viral replication kinetics assay. To investigate the effect of SGIV-sema on SGIV infection *in vitro*, viral replication kinetics were evaluated based on SGIV replication in GS/pcDNA3.1 and GS/pcDNA–SGIV-sema cells, respectively. Briefly, the stable lines were separately seeded in 24-well plates and infected with SGIV at an m.o.i. of ~0.1. The virus-infected cell lysates were harvested at the indicated time points (0, 6, 12, 24, 36 and 48 h p.i.), and viral titres were determined using a 50 % tissue culture infectious dose ($TCID_{50}$) assay (Reed & Muench, 1938). Cytopathic effect (CPE) was observed daily under a light microscope (Leica).

The expression profiles of SGIV ICP18, dUTPase and MCP in GS/pcDNA3.1 and GS/pcDNA–SGIV-sema cells after SGIV infection were examined by real-time PCR using β-actin as a reference gene. RT-qPCR was performed on Roche LightCycler 480 Real-time PCR system (Roche) using the 2 × SYBR Green Real-time PCR Mix (TOYOBO). Primer sequences are listed in Table S1.

Cell proliferation assay. To detect the impact of SGIV-sema on GS cell proliferation, GS/pcDNA3.1 and GS/pcDNA–SGIV-sema cells were seeded in 12-well plates (3×10^5 cells per well). Cell numbers from each line were counted daily for 6 days by using a haemocytometer under the microscope.

Indirect immunofluorescence assay. To detect the impact of SGIV-sema on cytoskeleton structure in fish cells, indirect immunofluorescence examination was performed as previously described (Cui *et al.*, 2011b). Briefly, GS cells grown on coverslips in 24-well plates were transfected with 800 ng of pEGFP–SGIV-sema using Lipofectamine 2000. Meanwhile, empty pEGFP-N3 and pEGFP–SGIV-IGF vectors (Yan *et al.*, 2013) were also separately transfected to serve as experimental controls. About 48 h after transfection, cells were fixed with 4 % paraformaldehyde and permeabilized with 0.2 % Triton X-100 for 15 min. After blocking with 2 % BSA, slides were incubated with rabbit anti-α-tubulin or F-actin (1 : 150) antibodies (Sigma) for 2 h, and then with rhodamine-conjugated goat anti-rabbit antibodies (Pierce) for another 2 h. Subsequently, cell nuclei were stained with 1 μg DAPI ml^{-1} and observed by fluorescence microscopy (Leica) for microtubules and laser scanning confocal microscopy (Leica TCS-SP2) for microfilaments.

Immune-related gene detection by real-time PCR. Real-time PCR was employed to investigate the effect of SGIV-sema on the expression of immune-related genes. Generally, GS cells were transfected with pcDNA3.1 or pcDNA–SGIV-sema, followed by either mock-infection or infection with SGIV (m.o.i. ~ 0.1) for 6 h. Total RNA was extracted and real-time PCR was performed using primers for IL-8, IL-15, MHC-II, MITA and TNF-α genes (Table S1). β-Actin was used as a reference gene, and hepcidin 1 was used as a negative control.

Statistical analysis. The two-tailed Student's *t*-test was used for two-group analyses. Results were expressed as means ± SD, and a *P* value <0.05 was considered statistically significant.

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