Medaka haploid embryonic stem cells are susceptible to Singapore grouper iridovirus as well as to other viruses of aquaculture fish species

Yongming Yuan,1 Xiaohong Huang,2 Lei Zhang,1 Yi Zhu,1 Youhua Huang,2 Qiwei Qin2 and Yunhan Hong1

Correspondence
Yunhan Hong
dbsyhy@nus.edu.sg
Qiwei Qin
qinqw@scsio.ac.cn

1Department of Biological Sciences, National University of Singapore, Singapore
2Key Laboratory of Marine Bio-resources Sustainable Utilization, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou, PR China

Viral infection is a challenge in high-density aquaculture, as it leads to various diseases and causes massive or even complete loss. The identification and disruption of host factors that viruses utilize for infection offer a novel approach to generate viral-resistant seed stocks for cost-efficient and sustainable aquaculture. Genetic screening in haploid cell cultures represents an ideal tool for host factor identification. We have recently generated haploid embryonic stem (ES) cells in the laboratory fish medaka. Here, we report that HX1, one of the three established medaka haploid ES cell lines, was susceptible to the viruses tested and is thus suitable for genetic screening to identify host factors. HX1 cells displayed a cytopathic effect and massive death upon inoculation with three highly infectious and notifiable fish viruses, namely Singapore grouper iridovirus (SGIV), spring viremia of carp virus (SVCV) and red-spotted grouper nervous necrosis virus (RGNNV). Reverse transcription-PCR and Western blot analyses revealed the expression of virus genes. SGIV infection in HX1 cells elicited a host immune response and apoptosis. Viral replication kinetics were determined from a virus growth curve, and electron microscopy revealed propagation, assembly and release of infectious SGIV particles in HX1 cells. Our results demonstrate that medaka haploid ES cells are susceptible to SGIV, as well as to SVCV and RGNNV, offering a unique opportunity for the identification of host factors by genetic screening.

INTRODUCTION

Food demand is increasing steadily worldwide, and innovative technologies are urgently needed to meet future food demands. With an annual growth of 6.9% from 1970 to 2007, aquaculture has been the fastest-growing animal food-producing sector (FAO, 2008), now representing a major global industry with total production exceeding 50 million tons and estimated values of almost US$80 billion (FAO, 2009). A major challenge in aquaculture is viral infection, which brings about massive death in larval production (Walker & Winton, 2010). Viral infection becomes more serious in high-density aquaculture, because it often causes massive or even complete loss. Little is known about treatments for fish viral diseases. The development of virus-controlling biotechnologies holds enormous potential for cost-efficient and sustainable aquaculture to meet the increasing demands for fish production.

The identification of host factors that viruses used for infection is essential to elucidate the mechanisms by which these viruses cause diseases. Likewise, an understanding of how viruses depend on host factors to complete the infectious cycle may accelerate the development of antiviral strategies. Most intriguingly, targeted disruption of genes encoding host factors essential for viral infection would represent a revolutionarily novel biotechnology to create virus-resistant cells and even virus-resistant whole animals. However, host factors for viral infection have remained largely undetermined. One of the exceptions in aquatic organism is the laminin receptor, which acts as a binding protein for Taura syndrome virus infection in the whiteleg shrimp and whose knockdown leads to organism lethality (Stockwell, 2002).

Genetic screening is a powerful tool to identify genes essential for diverse cellular processes through the production of genome-wide mutations with discernible phenotypes. The best example is the classical work pioneered by Muller (1927), which induced numerous mutations and led to the unbiased elucidation of genes crucial for biological processes in Drosophila. The elusiveness of the generation and recovery of biallelic mutations in diploid cells is a major barrier to the application of this approach.

One supplementary figure, one table and two data figures are available with the online version of this paper.
in vertebrate organisms. Haploid cells offer a powerful system for genetic analyses of molecular events because any recessive mutations of essential genes will show a clear phenotype due to the absence of a second gene copy. Carette et al. (2009) initially demonstrated the power of haploid genetic screening in KBM7, a near-haploid human chronic myeloid leukaemia cell line (Kotecki et al., 1999). By generating a library of KBM7 mutant cells using a gene-trap retrovirus and screening for mutant cells resistant to a range of bacterial toxins and cytotoxic viruses, they identified host genes required for cell death by the toxins or viruses (Carette et al., 2009, 2011b). Specifically, human cytidine monophosphate N-acetylneuraminic acid synthetase (CMAS) and solute carrier family 35 (UDP-galactose transporter), member A2 (SLC35A2) were thus identified as host factors essential for influenza infection (Carette et al., 2009), and the cholesterol transporter Niemann–Pick C1 as essential for Ebola virus entry (Carette et al., 2011b). This near-haploid cell line has also been used to assign human genes to phenotypes by global gene disruption (Carette et al., 2011a).

We recently generated three genuine haploid embryonic stem (ES) cell lines HX1–HX3 from the laboratory fish medaka (Oryzias latipes) and demonstrated the applicability of these haploid ES cells for whole animal production by semi-cloning (Yi et al., 2009). This was followed by similar success in mouse haploid ES cell derivation and semi-cloning (Leeb & Wutz, 2011; Li et al., 2012; Yang et al., 2012). The availability of medaka haploid ES cells offers a unique opportunity for haploid genetic screening in fish.

So far, more than 25 virus species have been reported in diverse fish species of importance in aquaculture. Nine are listed by the Office of International Epizootic (OIE) as highly infectious and notifiable viruses (http://www.oie.int). These include Singapore grouper iridovirus (SGIV), spring viremia of carp virus (SVCV) and viral nervous necrosis virus (VNNV). SGIV is a highly infectious pathogen that causes massive death in wild and farmed groupers and many other marine teleosts (Qin et al., 2003), and its genome is a circular dsDNA of 140 131 bp and predicts 162 ORFs (Song et al., 2004). We reported recently that SGIV induces paraptosis-like cell death in its natural host species such as groupers but apoptosis in cells of non-host species (Huang et al., 2011a). SVCV has an 11 kb RNA genome and causes massive death by haemorrhaging and necrosis of internal organs in many fresh water species of the families Cyprinidae, Siluridae, Salmonidae, Esocidae, Centrarchidae and Poeciliidae in many countries. VNNV is non-enveloped, has a 4.9 kb RNA genome and causes a highly destructive disease in larvae of more than 30 marine fish species from 14 families of the order Perciformes. A total of five isolates have been reported for VNNV, which originate from the striped jack, barfin flounder, tiger puffer, red-spotted grouper [red-spotted grouper nervous necrosis virus (RGNNV)] and orange spotted grouper (Epinephelus coioides). Susceptibility to RGNNV has been reported for medaka fry (Furusawa et al., 2006) and differentiated somatic cell lines (Adachi et al., 2010).

As a prelude for genetic screening for host factors that pathogens use for infection in aquaculture-important fish species, this study aimed to test the viral susceptibility of medaka haploid ES cells. We showed that medaka haploid ES cells are susceptible to SGIV, as well as to SVCV and RGNNV. By gene expression profiles and light microscopy, we demonstrated that SGIV infection in HX1 cells was able to elicit an immune response and apoptosis. Most importantly, we revealed by growth curve analysis and electron microscopy that HX1 cells are susceptible to SGIV and produce infectious particles following virus replication. Our data establish medaka haploid ES cells as a unique system for the identification of host factors by genetic screening.

RESULTS

Viral susceptibility screening using a cytopathic effect (CPE) assay

To screen the candidate viruses with potential infectivity towards medaka stem cells, we tested nine viruses that are classified as highly infectious and notifiable viruses by the OIE. Our results indicated that three of the nine listed viruses were able to cause a distinct CPE in the medaka haploid ES cell line (Table 1). In SGIV-infected HX1 cells, typical CPE was observed at 3 days post-inoculation (p.i.) and became more evident at 4 days p.i., when control cells retained their normal shape and grew into a full monolayer, whereas the majority of SGIV-infected cells lost attachment to the substrata and started to collapse or lyse (Fig. 1a, a’). A similar CPE was found in the medaka diploid ES cell line MES1 (Fig. 1b, b’). In SGIV-infected cells, the CPE became more evident at 4 days p.i., when control cells retained their normal shape and grew into a full monolayer, whereas the majority of SGIV-infected cells lost attachment to the substrata and started to collapse or lyse (Fig. 1a, a’). A similar CPE was found in the medaka diploid ES cell line MES1 (Fig. 1b, b’). In the diploid spermatogonial cell line SG3 (Fig. 1c, c’). CPE was also observed in HX1 cells infected with SVCV (Fig. S1a, available in JGV Online) and RGNNV (Fig. S1b).

Molecular analyses of viral RNA and protein expression

Based on the above CPE screening, the potential susceptibility of medaka ES cells to SGIV, SVCV and RGNNV was verified further by reverse transcription-PCR (RT-PCR) and Western blotting. RT-PCR analysis revealed that HX1 cells at 4 days p.i. expressed viral structural genes from SGIV, SVCV and RGNNV (Fig. 2a). In SGIV-infected medaka cells of the HX1, MES1 and SG3 lines, Western blotting revealed the correlative expression of viral protein ORF018 (NCBI Protein no. YP_164113.1) and ORF158 (NCBI Protein no. YP_164253.1) (Fig. 2b). The former encodes a highly conserved structural protein identified in mature virions (Wang et al., 2008) and the latter encodes an SGIV-specific histone H3-binding protein (Tran et al., 2011). A time-course detection of viral gene expression level in HX1 was performed with...
semi-quantitative RT-PCR. The transcription of immediate-early gene orf086 (Xia et al., 2009) could be detected as early as 2 h p.i., whereas the RNA of early gene orf158 and late gene orf018 was not detectable until 4 and 8 h p.i., respectively (Fig. 2c). The above results demonstrated SGIV entry and gene expression in medaka stem cells including haploid HX1 cells.

**Host response at the molecular level**

It has been shown that SGIV induces apoptosis in cells of non-host species such as fathead minnow but paraptosis-like cell death in cells of natural host species such as grouper spleen cells (Huang et al., 2011a). We wanted to examine the host-cell response at the molecular level by determining the time-course expression of a selected set of cellular genes (Fig. 2c). Five medaka genes homologous to those involved in the immune response were identified by sequence comparison, which included interferon (ifn), mx, stat1 and viperin (vpr) (Fig. 2c). Interestingly, induction of RNA expression was found for all of these immune-responsive genes as early as 2 h p.i. but was undetectable in the mock-inoculated control (not shown). The four immune-responsive genes fell into three groups according to their induced expression patterns. The first group included ifn and mx, which displayed a steadily increasing or persistent high level of expression throughout the period examined. The second was represented by stat1, whose RNA level peaked at 2 h p.i. and decreased subsequently. The third was vpr, which was upregulated at 2 h p.i. and persisted at a comparably low level. The expression level of retinoblastoma (rb) was stable both in mock and virus-infected cell. Concurrently, cell death-associated genes, namely p53 and p21, and more importantly, caspase 3a (cas3a), an apoptosis-specific gene, exhibited obvious upregulation at 4 h p.i. compared with the mock-infected control cells (Fig. 2c), strongly suggesting that SGIV infection induces the apoptosis pathway in HX1 cells. These data established that SGIV infection elicits molecular processes characteristic of a cellular immune response and apoptosis in medaka haploid ES cells.

**Host response at the cellular level**

We continued our examination into the cellular phenotype change after SGIV infection. A hallmark of apoptotic cells is DNA fragmentation, which at the early stage will form a series of differently sized chromosomal DNA fragments. Such fragments will form a series of ladders that differ in

---

**Table 1. Virus CPE screening in medaka haploid ES cells**

<table>
<thead>
<tr>
<th>Class</th>
<th>Family</th>
<th>Virus name (abbreviation)</th>
<th>CPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA virus</td>
<td><em>Iridoviridae</em></td>
<td>Infectious spleen and kidney necrosis iridovirus (ISKV)</td>
<td>No</td>
</tr>
<tr>
<td>DNA virus</td>
<td><em>Herpesviridae</em></td>
<td>Koi herpes virus (KHV)</td>
<td>No</td>
</tr>
<tr>
<td>RNA virus</td>
<td><em>Rhabdoviridae</em></td>
<td>Infectious haematopoietic necrosis virus (IHNV)</td>
<td>No</td>
</tr>
<tr>
<td>RNA virus</td>
<td><em>Birnaviridae</em></td>
<td>Infectious pancreatic necrosis virus (IPNV)</td>
<td>No</td>
</tr>
<tr>
<td>RNA virus</td>
<td><em>Nodaviridae</em></td>
<td>Red-spotted grouper nervous necrosis virus (RGNNV)</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*In the OIE list of highly infectious and notifiable viruses, 25 fish viruses have been reported. They belong to seven families: *Iridoviridae* (ten), *Herpesviridae* (three), *Nodaviridae* (four), *Rhabdoviridae* (three), *Birnaviridae* (one), *Orthomyxoviridae* (one) and *Reoviridae* (three).
size by ~200 bp upon agarose gel electrophoresis. We clearly observed such a ladder in SGIV-infected HX1 cells at 48 h p.i. (Fig. 3a). Concurrently, we also detected nuclear fragmentation, as evidenced by the appearance of apoptotic bodies in SGIV-infected HX1 cells at 48 h p.i. (Fig. 3b). HX1 cells were further analysed by staining with FITC–Annexin V, which served as sensitive probe to detect the externalization of phospholipid phosphatidylserine in the early stage of apoptosis, together with Hoechst 33342 and propidium iodide (PI). Hoechst 33342 stains the nuclei of both live and dead cells, whereas PI stains the nuclei of dead cells only. Early apoptotic cells are positive for annexin V staining but negative for PI staining. Late apoptotic cells are positive for both annexin V and PI staining. SGIV-induced apoptosis in HX1 cells was seen at the early stage until 36 h p.i. and at the late stage until 72 h p.i. (Fig. 3c). These results demonstrated that SGIV infection induces apoptosis in medaka haploid ES cells.

**Infectivity of SGIV produced in medaka haploid ES cells**

SGIV derived from HX1 and grouper spleen (GS) cells was inoculated back into both cell lines (Fig. 4). HX1-derived SGIV was comparable to the GS-derived counterpart in producing CPE in both HX1 (Fig. 4b, c) and GS cells (Fig. 4e, f). Furthermore, the resulting viral growth curve indicated that SGIV from either HX1 or GS cells retained its infectivity and could replicate in the host. In cultures of HX1 or GS cells, viruses from the two sources gave similar growth curves and titres. However, the titre of virus in HX1 cells increased more slowly than in GS cells and was about tenfold lower than that in GS cells at 72 days p.i. (Fig. 4 g, h). The P value from Student’s t-test (n=3) indicated there was no significant difference between the two curves in the same host. Therefore, SGIV produced in medaka haploid ES cells resembles SGIV produced in its natural host cells in infectivity.

**Electron microscopy of SGIV propagation and release**

Electron microscopy of ultrathin sections revealed the presence of numerous virus particles in the cytoplasm of SGIV-infected HX1 cells (Fig. 5a, b). There were also fully matured particles in the budding cytoplasm (Fig. 5a), suggesting that they were in the process of release from the cells. Virions were distributed throughout the cytoplasm. At a higher magnification, three major stages of virus assembly were clearly visible in the cytoplasm (Fig. 5b). At stage I, four- and five-sided intermediates of SGIV assembly were seen together with elongated tubular-like materials, suggesting continuity of the assembly intermediate with cellular membrane compartments. At stage II, empty hexagonal capsids were apparent. At stage III, the assembly process was complete, leading to the production of mature virus particles. Similar stages have been described for African swine fever virus (Rouiller et al., 1998). These results suggest that medaka haploid ES cells allows SGIV to complete the infectious cycle.

**DISCUSSION**

In this study, we have demonstrated for the first time that stem cell lines from a small freshwater fish like medaka are susceptible to infection by SGIV and other viruses. Specifically, we have shown in more detail that SGIV, a large DNA virus, can efficiently infect even medaka haploid ES cells. Here, four independent lines of evidence clearly point to the viral susceptibility of medaka stem cells...
including the haploid ES cell line HX1. First, CPE occurred for all three medaka stem cell lines tested, including the haploid HX1 line, and was similar to that observed in groupers, the natural host, following SGIV infection.

Secondly, SGIV gene transcription and protein synthesis took place in all three stem cell lines. Thirdly, electron microscopy revealed SGIV propagation, maturation and budding in HX1 cells. Finally, and most convincingly, we showed that HX1-derived SGIV had infectivity not only in GS cells as the natural host but also in medaka haploid ES cells. These results clearly demonstrate medaka haploid ES cells as the first potential system for genetic screening to identify host factors essential for infection by these virus species of importance in aquaculture.

Medaka live in freshwater habitats and are a member of the order Beloniformes. Carp are freshwater species of the order Cypriniformes. Groupers are marine species of the order Perciformes. The separation between Cypriniformes and Beloniformes plus Perciformes has been estimated to be approximately 300 million years ago (Yamanoue et al., 2006). Our finding that the medaka haploid ES cell line HX1 is susceptible to infection by SVCV, SGIV and RGNNV, whose natural host species belong to Cypriniformes and Perciformes, underscores its potential use in genetic screening for host factors crucial for viral infection in many cyprinids and perches of importance in aquaculture. In this regard, the sequenced medaka genome (Kasahara et al., 2007) will contribute greatly to the identification of host factors and to investigation of the host-cell response to viral infection.

Recently, we revealed that SGIV induces paraptosis in its natural host species but apoptosis in non-host cells (Huang et al., 2011a). This finding is in agreement with our observation in this study that haploid ES cells of the HX1 line from medaka as a non-host species are capable of apoptosis. SGIV-induced apoptosis was supported by four independent lines of evidence. First, apoptosis-associated genes, in particular caspase 3a, were induced by SGIV infection. Secondly, chromosomal fragmentation generated DNA ladders visible on gels. Thirdly, nuclear fragmentation led to the formation of apoptotic bodies. Lastly, cells positive for annexin V, a marker of apoptosis, were induced and increased in number during SGIV infection. In addition, we revealed that SGIV also elicited an immune
response in medaka haploid ES cells, as evidenced by the apparent induction and upregulation of immune-responsive genes such as \textit{ifn} and \textit{stat1}. It has been reported that fish virus-induced interferon exerts an antiviral function through the Stat1 pathway (Yu et al., 2010). These results suggest that medaka haploid ES cells resemble cells of the natural host species at molecular and cellular levels in response to SGIV infection, and thus provide a system for understanding the mechanisms underlying viral infection and antiviral strategies.

**METHODS**

**Cell culture.** The medaka cell lines were maintained at 28 °C in medium ESM4 as described previously (Hong & Schartl, 2006; Yi et al., 2010). These included the diploid ES cell line MES1 (Hong et al., 1996), the diploid spermatogonial cell line SG3 (Hong et al., 2004) and HX1, one of the three haploid ES cell lines (Yi et al., 2009). The grouper embryonic cell line GP (Qin et al., 2003) and grouper spleen cell line GS (Huang et al., 2011a) were maintained at 25 °C in Eagle’s minimal essential medium containing 10% FBS as described elsewhere.

**Virus preparation and challenge.** Susceptibility to the nine virus species list in Table 1 was tested independently in three laboratories. A first test was performed as a custom service by the Agri-Food and Veterinary Authority of Singapore (http://www.ava.gov.sg/), which identified SGIV and SVCV as infectious agents in medaka haploid ES cells based on apparent CPE. We further examined these two virus species and RGNNV. SGIV (strain A3/12/98) originally isolated from diseased brown-spotted grouper (Epinephelus tauvina) was propagated in GP cells as described previously (Qin et al., 2003). RGNNV and SVCV were propagated in GB cells (Huang et al., 2011b).
and CO cells (Teng et al., 2007). For instance, SGIV was inoculated onto confluent GP cells at an m.o.i. of ~0.1. Following the appearance of an apparent CPE, cells containing SGIV were harvested and centrifuged at 3000 g for 10 min at 4 °C, and the cell debris together with partial supernatant were collected and stored at -80 °C until use. Medaka cell lines were similarly challenged with different viruses with partial supernatant were collected and stored at 70 % confluency for further analysis.

**Virus replication kinetics assay.** To determine whether medaka haploid ES cells were able to propagate infectious virus comparable to that produced in the natural host cells, SGIV particles derived from the infected HX1 and GS cultures were inoculated back into the two cell types to test the viral growth curve. In detail, cells of HX1 and GS (1 x 10^6 cells per well) were seeded into six-well plates and cultured for 24 h. SGIV collected from HX1 and GS cells was inoculated back onto seeded cells at an m.o.i. of 0.1. The infected cells together with supernatant were harvested at the indicated time points and stored at -80 °C for titre assays. Virus titres were determined with serially diluted GS cells to determine the TCID_{50} after 72 h of incubation.

**RT-PCR analysis.** RNA isolation from cell culture and RT-PCR analyses were performed as described previously (Hong et al., 2004; Yi et al., 2009). The GenBank accession numbers of target genes are listed in Table S1, and the sequences of the genes *stat1* and *p21* were identified using amino acid sequence alignment (Data S1 and S2). RT-PCR primers were designed according to the relative sequence (Table S1). PCR was performed in a 20 μl volume containing 10 ng cDNA for 30 (for β-actin as a loading control) or 35 cycles (other genes) of 95 °C for 30 s, 60 °C for 20 s and 72 °C for 1 min. The primers used are listed in Table S1. PCR product (4 μl) was loaded and analysed by 2 % agarose gel electrophoresis.

**Genomic DNA isolation.** HX1 cells were harvested by trypsinization and lysed in lysis buffer [10 mM Tris/HCl (pH 8.0), 1 mM EDTA, 1 % SDS, 100 μg proteinase K ml^{-1}], and the lysate was incubated at 50 °C for 3 h. After phenol/chloroform extraction, the DNA was precipitated by adding 2 vols ethanol at -20 °C overnight and then centrifuged at 12000 g for 15 min. The DNA pellets were resuspended in distilled water with RNase A (50 μg ml^{-1}) and then analysed by 1.5 % agarose gel electrophoresis.

**Western blot analysis.** Western blot analysis was performed essentially as described previously (Xu et al., 2005) using antibodies against the cellular β-actin (clone AC-74; Sigma) and SGIV proteins ORF018 (Wang et al., 2008) and ORF158 (Tran et al., 2011).

**Cell staining.** HX1 cells grown in six-well plates at 60 % confluency were mock infected or infected with SGIV. Live cells were stained with MitoTracker Red (Invitrogen) plus Hoechst 33342 (1 μg ml^{-1}) or with an FITC : Annexin V Apoptosis Detection kit II (BD Pharmingen) plus nuclear dyes PI (1 μg ml^{-1}) and Hoechst 33342 (1 μg ml^{-1}). After staining, the cells were analysed by fluorescence microscopy.

**Light microscopy.** Light microscopy analyses were carried out as described previously (Hong et al., 2010; Yang et al., 2012; Yi et al., 2009).

**Electron microscopy.** SGIV-infected HX1 cells were subjected to electron microscopy as described previously (Huang et al., 2011a). Briefly, cells at 36 h p.i. were fixed in 2.5 % glutaraldehyde overnight, washed with PBS and post-fixed in 1 % osmium tetroxide for 1 h. After dehydration in an increasing ethanol series, the cells were embedded in Epon resin and ultrathin sections were cut at 200 nm thickness with a UC6/PC6 ultramicrotome (Leica). Sections were double stained with uranyl acetate and lead citrate. Samples on grids were examined and documented at 120 kV on a JEM-1400 electron microscopy (JEOL).

**ACKNOWLEDGEMENTS**

We thank J. Deng for fish breeding and C. M. Foong for laboratory management. This work was supported by the National Research Foundation Singapore (NRF-CRP7-2010-03) and the National Basic Research Program of China (973 Program: 2012CB114402).

**REFERENCES**


