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Kevin S Jones, *Howard University*



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Zebrafish: Modeling for Herpes Simplex Virus Infections

Thessicar Evadney Antoine,¹ Kevin S. Jones,² Rodney M. Dale,³ Deepak Shukla,¹ and Vaibhav Tiwari^{1,4}

Abstract

For many years, zebrafish have been the prototypical model for studies in developmental biology. In recent years, zebrafish has emerged as a powerful model system to study infectious diseases, including viral infections. Experiments conducted with herpes simplex virus type-1 in adult zebrafish or in embryo models are encouraging as they establish proof of concept with viral-host tropism and possible screening of antiviral compounds. In addition, the presence of human homologs of viral entry receptors in zebrafish such as 3-O sulfated heparan sulfate, nectins, and tumor necrosis factor receptor superfamily member 14-like receptor bring strong rationale for virologists to test their *in vivo* significance in viral entry in a zebrafish model and compare the structure–function basis of virus zebrafish receptor interaction for viral entry. On the other end, a zebrafish model is already being used for studying inflammation and angiogenesis, with or without genetic manipulations, and therefore can be exploited to study viral infection-associated pathologies. The major advantage with zebrafish is low cost, easy breeding and maintenance, rapid lifecycle, and a transparent nature, which allows visualizing dissemination of fluorescently labeled virus infection in real time either at a localized region or the whole body. Further, the availability of multiple transgenic lines that express fluorescently tagged immune cells for *in vivo* imaging of virus infected animals is extremely attractive. In addition, a fully developed immune system and potential for receptor-specific knockouts further advocate the use of zebrafish as a new tool to study viral infections. In this review, we focus on expanding the potential of zebrafish model system in understanding human infectious diseases and future benefits.

Introduction

HERPES SIMPLEX VIRUS (HSV) CAUSES significant health problems from periodical skin and corneal lesions to encephalitis.¹ The current model of HSV-1 entry suggests multiple roles for both heparan sulfate (HS) and a highly modified form of it called, 3-O sulfated HS (3-OS HS).^{2–4} HS mediates the initial viral binding or attachment to the host cells via HSV-1 glycoprotein B (gB)² followed by the fusion of viral envelope with the host cell membrane during viral penetration.^{3,4} During the latter process of fusion it is the 3-O-sulfotransferase (3-OST) enzymatic modification of HS chains that generate 3-OS HS, which can bind gD and initiate the formation of a multi-protein fusion complex involving additional glycoproteins such as gB, gH and gL.⁴ Using primary human cell culture model and a mouse model we have demonstrated the *in vivo* significance of HS and 3-OS HS in HSV-1 infection.^{5–8} Quite interestingly, zebrafish *Danio rerio* widely expresses 3-OSTs, which are required for embryonic development and also for generating HSV receptors.^{9–14} Supporting the idea that zebrafish 3-OSTs can generate the

receptors, we have found evidence that similar to the human 3-OST-3 isoform, zebrafish encoded 3-OST-3 also allows HSV-1 entry.⁹ In addition, zebrafish embryos are susceptible to HSV infection and the zebrafish model is now emerging as a new model system for host-pathogen interactions, largely because the zebrafish larvae are transparent and thus, highly suited for *in vivo* imaging and cost effective high throughput screening. Based on the above attractive promises, zebrafish system provides a unique opportunity to understand many aspects of HSV infection such as host cell tropism, clinical pathologies of the disease and associated inflammation together with immune response to the infection via live *in vivo* imaging. In this review we focus on available tools to understand HSV entry and spread at molecular level and develop zebrafish system for therapeutic intervention.

Expression of Viral Entry Receptors in Zebrafish Embryos

The first step in viral infection involves host cell interaction through cell surface receptors.¹ The current model of herpes

¹Departments of Ophthalmology and Visual Sciences & Microbiology/Immunology, University of Illinois at Chicago, Chicago, Illinois.

²Department of Biology, Howard University, Washington, District of Columbia.

³Department of Biology, Loyola University Chicago, Chicago, Illinois.

⁴Department of Microbiology and Immunology, Midwestern University, Downers Grove, Illinois.

simplex virus (HSV) infection suggests that viral entry and spread are dynamic and multistep processes, which start with initial viral attachment or binding to a cell. It has been proposed that HSV-1 glycoprotein B and C mediate the initial attachment or binding to cell surface through heparan sulfate (HS).² After initial docking, the virus-cell fusion is mediated by 3-O sulfated heparan sulfate (3-OS HS). The latter modification in HS chain is catalyzed by an enzyme called 3-O sulfotransferases (3-OSTs).³ Whereas the unmodified HS aids in viral attachment to a cell, the modified version of HS mediates virus-cell fusion.⁴ In a nutshell, it is clear that heterogeneous HS plays a major role at multiple steps during HSV-1 infection. We have provided clinical significance of 3-OST-3 generated 3-OS HS receptor during HSV-1 entry and spread in primary cultures of human corneal fibroblasts.^{5,6} In addition, phage display screening generated peptides against HS and 3-OS HS significantly impaired HSV infection in both cell culture and *in vivo* mouse corneal model.^{7,8} Recently, our group provided the first evidence of zebrafish encoded 3-OST modified HS receptor mimicking human 3-OST generated HS in allowing HSV-1 entry and spread.⁹⁻¹¹ Our results raised the possibility that zebrafish can provide a reasonable ground to study the role of 3-OST generated receptors in HSV-1 infection by making 3-OST isoform-specific knockouts. Zebrafish embryos can be microinjected with morpholinos at 1-4 cell stage to target the specific 3-OST gene. Since this knockdown can be effective for up to 10 days postfertilization, it can allow long-term imaging of viral infection compared with control knockouts.

Zebrafish embryos are known to express HSV entry receptors, including multiple isoforms of 3-OST, an enzyme that modifies HS (Fig. 1). Using *in situ* hybridization, a study by Cadwallader and Yost demonstrated the expression pattern of various isoforms of HS modifying enzymes such as 2-OSTs,

6-OSTs, and 3-OSTs in zebrafish embryos.¹²⁻¹⁴ The authors further reported seven 3-OST genes that showed homology to mouse and human counterparts of 3-OSTs. Among these two zebrafish genes, 3-OST-3X and 3-OST-3Z were found highly homologous to human 3-OST-3A and 3-OST-3B. The two human genes share 100% identity throughout the catalytic domain.¹⁴ Overall, the members of the 3-OST family in zebrafish shared 63% similarity within the catalytic domain to the corresponding human isoform with the exception of zebrafish 3-OST-5, which showed only 53% similarity to human 3-OST-5.^{10,14} The *in situ* hybridization data further showed extensive 3-OST brain expression localized in the specific brain subdivisions.¹⁴ For instance, 3-OST-2 was expressed in developing the brain, otic vesicle, and olfactory areas during the early developmental stage, while 3-OST-3X was observed in the neural tube and lateral plate mesoderm.¹⁴ Similarly, zebrafish 3-OST-6 was expressed at high levels in the hind-brain with no expression in the spinal cord region.¹⁴ Interestingly, structural and cell adhesion properties of zebrafish HS bearing syndecan proteins were reported to be shared with higher vertebrates.¹⁵ The diverse expression of the 3-OST family members in the zebrafish embryo model system (Table 1) was the main rationale to test the role of zebrafish encoded 3-OST isoforms in mediating HSV-1 infection. Using Chinese hamster ovary (CHO-K1) cells transiently expressing zebrafish encoded 3-OST-2 and 3-OST-3 isoforms resulted in HSV-1 infection.^{9,10} More direct and visual evidence of HSV-1 infection was demonstrated by using green fluorescent protein (GFP)-tagged HSV-1 (K26GFP) virions, which clearly infected CHO-K1 cells expressing zebrafish encoded 3-OST-3. The infection was also confirmed by high resolution electron microscopy.^{9,16} Future studies to characterize the other members of the zebrafish 3-OST family will determine their role in mediating HSV infection of specific cells and tissues. Because

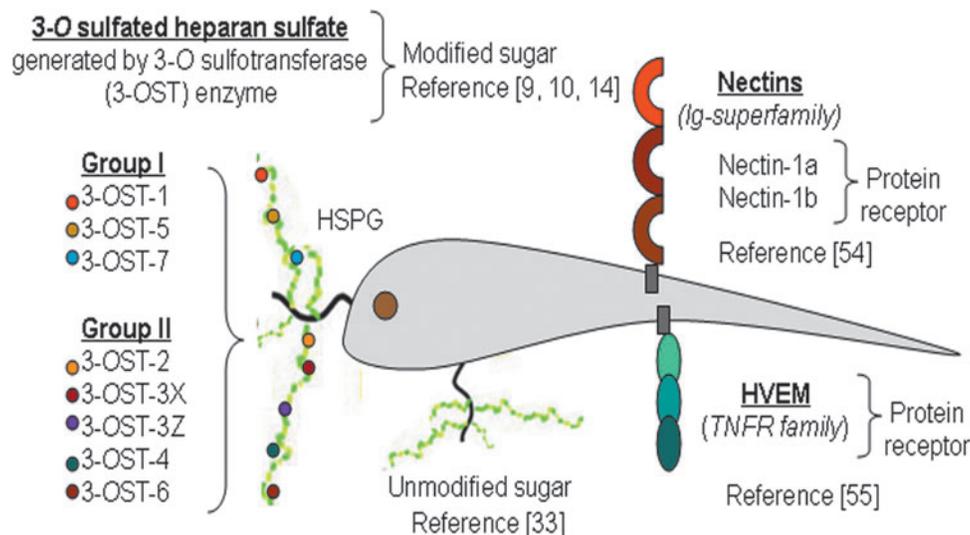


FIG. 1. Expression of Herpes simplex virus (HSV-1) glycoprotein D entry receptors in zebrafish. Cartoon indicates that the presence of human homologs of both protein receptors (nectins and tumor necrosis factor receptor superfamily member 14-like receptor) and heparan sulfate proteoglycans (HSPG) containing 3-O sulfated heparan sulfate (3-OS HS) in zebrafish embryos provide rationale to study *in vivo* significance of receptors. Based on phylogenetic comparison between zebrafish, the mouse and human 3-OST gene family has been divided in to two subgroups. Our published data indicate that zebrafish encoded 3-OST-2 and 3-OST-3 generated HS receptors facilitate HSV-1 entry in a cell culture model. Color images available online at www.liebertpub.com/zeb

TABLE 1. EXPRESSION PROFILE OF HEPARAN SULFATE 3-O-SULFOTRANSFERASE (3-OST) ISOFORMS IN ZEBRAFISH

Isoforms	Tissue distribution	Known function(s)
3-OST-1	CNS, tail bud, eye, heart, gut	Not known
3-OST-2	CNS, otic vesicle	HSV-1 entry
3-OST-3X	CNS, pancreas, kidney, otic vesicle	HSV-1 entry
3-OST-3Z	CNS, heart, gut, pancreas, liver, kidney	HSV-1 entry
3-OST-4	CNS, eye, otic vesicle, olfactory system	HSV-1 entry
3-OST-5	CNS, eye, otic vesicle, olfactory system	Not known
3-OST-6	CNS, eye, kupffer's vesicle, olfactory system	Not known
3-OST-7	CNS, eye, otic vesicle, olfactory system	Not known

HSV-1, herpes simplex virus type-1; CNS, central nervous system.

HSV-1 is a neurotropic virus, it is therefore critical to determine the role of the 3-OS HS receptor in promoting pathology in the brain region of zebrafish embryo during HSV-1 infection. Similarly, 3-OST-specific isoforms, such as 3-OST3Z, 3-OST-4, and 3-OST-6, are also expressed in the heart and eye regions¹⁴ providing a strong rationale to study the wider role of 3-OST during HSV pathogenesis in the zebrafish embryo model. Further, an advantage of using a zebrafish model is to

test peptides generated against specific 3-OST isoforms.^{7,8} The kinetics of inhibition can be determined in short duration of time together with toxicological evaluation, which again is not possible with present murine and rabbit models against HSV infection. The information generated by the zebrafish experiments can identify useful inhibitors to prevent HSV-1-induced pathological damages especially during ocular corneal infection or neuronal damages.

Zebrafish models can also be used to identify new host receptors or homologs. A study by Petrella *et al.* characterized coxsackievirus and adenovirus receptor by using a zebrafish homologue of the coxsackievirus and adenovirus receptor (CAR).¹⁷ The identified homologue of zebrafish CAR was found to be <50% identical in the extracellular domain to that of human CAR, but the expression of zebrafish CAR resulted in infection of the transfected CHO-K1 cells by both adenovirus type 5 and coxsackievirus B3.¹⁷ Interestingly, picornavirus and adenovirus have been isolated from fish,¹⁸ but there is no evidence yet that zebrafish CAR functions naturally as a receptor. However, the presence of viral entry receptors gives an opportunity to study their role in the zebrafish model and also to characterize their structure–function in relation to virus–cell interactions.

Routes of Viral Infections in Zebrafish Model

There are various possible routes of experimental viral infection in adult zebrafish or zebrafish embryos. As indicated in Figure 2, using tissue culture plates or dishes, it is

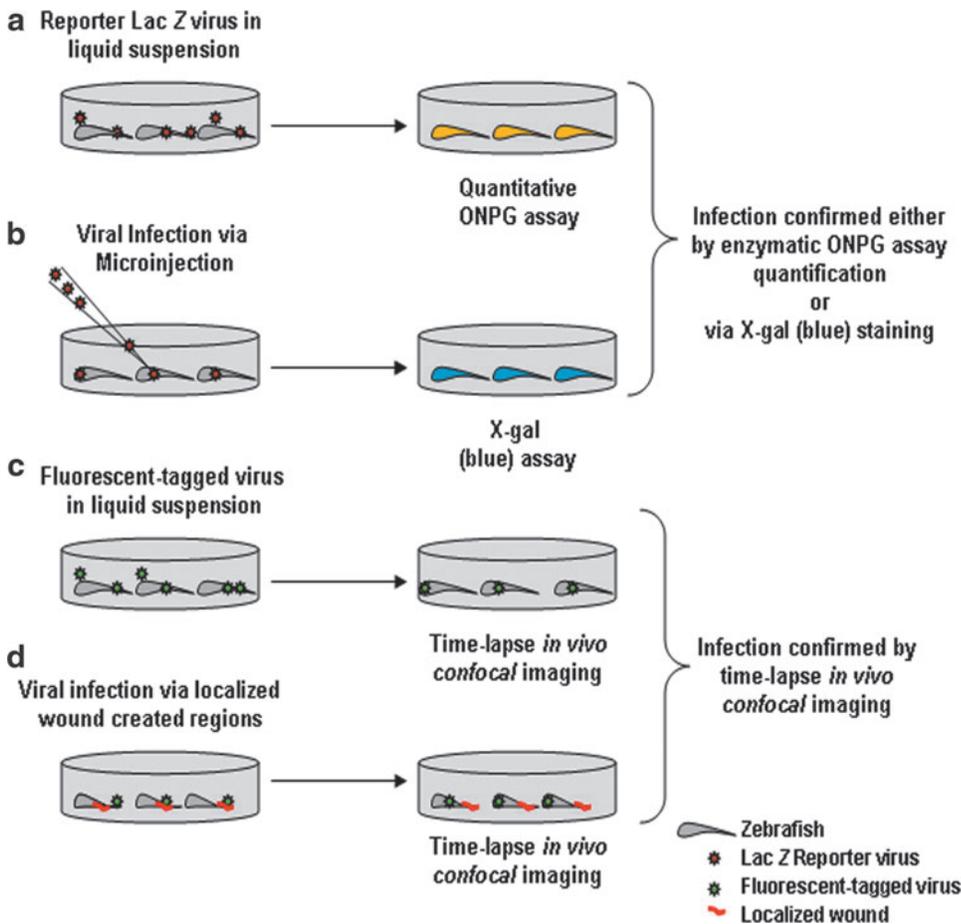


FIG. 2. Cartoon suggesting possible routes of viral infection in zebrafish model. (a) Reporter virus infection in a liquid aqueous medium containing zebrafish embryos in tissue culture plates. (b) Cell- or tissue-specific microinjection of viral inoculums with the reporter virus in zebrafish. (c) Fluorescently tagged virus infection in the zebrafish model in a liquid suspension for *in vivo* imaging. (d) Localized wound or injury created in zebrafish adults followed by placing them in an aqueous medium containing the reporter virus may facilitate opportunistic viral infections. Color images available online at www.liebertpub.com/zeb

possible to infect zebrafish embryos by incubating them in an E3 media containing the *Lac Z* reporter virus system to analyze viral infection. The infection in zebrafish can be determined either through enzymatic reactions, which can be quantified by a plate reader or through the substrate staining method, which gives blue color through X-gal assay to infected embryos in a manner similar to cell culture assays.⁵ As presented in Figure 3, HSV-1 infection in 3-day-old zebrafish embryos demonstrated through both blue X-gal staining (Fig. 3b) and quantitative soluble substrate o-nitrophenyl- β -D-galactopyranoside assay (Fig. 3c). If zebrafish embryos are infected with fluorescently tagged virus particles, the goal will be to track virions in real time using the fluorescent stereomicroscope. The later technique can be useful to study viral tropism, viral spread, and the kinetics of viral load in real time. The other advantage of this technique is to visualize cell- or tissue-specific pathology associated with the viral disease. Fluorescent imaging has allowed studying inflammatory responses at damaged tissue sites as well.^{19,20} Studies

have shown that adult zebrafish can also be infected with virus using intraperitoneal (i.p.) microinjections in anesthetized fish. Using the KOS strain of HSV-1, Burgos *et al.* for the first time demonstrated that i.p. microinjection of HSV-1 resulted in acute infection.²¹ In the infected adult zebrafish, HSV-1 DNA was later detected and quantified by polymerase chain reaction (PCR) and immunohistochemistry for HSV-1 VP16 tegument protein.²¹ Interestingly, wounds or sites created in adult zebrafish through scraping to induce injury may also facilitate viral infection. Since the wounds are localized,²² viral infection to the area of interest can be generated to study viral spread to nearby areas and inflammation to the site of injury. One other interesting possibility is to test if cell-associated virus, for example, varicella zoster virus—a member of α -herpes virus coinubated with intact zebrafish embryos or adult zebrafish can move and this transfer can be tracked and visualized.

Viral Tropism in Zebrafish Model

Understanding viral trafficking from infected areas to the uninfected areas and visualizing their localization in tissue-specific areas may shed light on viral host tropism. For instance, studies done with HSV-1 in the adult zebrafish model indicated that microinjection of virus in the abdominal cavity resulted in viral spread from the ventral area to the dorsal midbody region, and finally to the brain where the highest concentration of the virus was quantified. The later clearly confirms the neurotropism of HSV-1 in the zebrafish adult model.²¹ These results are very exciting as HSV-1 is associated with several neurological diseases. In the same study, when adult zebrafish were treated with the antiviral drug acyclovir, the treatment resulted in significant decrease in viral load in the infected regions, while in contrast, a higher viral load and higher mortality were observed when zebrafish was treated with cyclophosphamide.²¹ In a separate study, Liu *et al.* further demonstrated a unique example of viral tropism in an adult zebrafish model.²³ To learn the mechanism of intrahepatic cholangiocarcinoma (ICC) associated with hepatitis B and hepatitis C viruses (HCV), a conditional expression system with the hepatitis B virus X protein and HCV core protein was generated in the liver of zebrafish, which showed signs of liver fibrosis and development of ICC.²³ Interestingly, the profiles of biomarkers in zebrafish ICC were similar to those of human ICC. In addition, the study further demonstrated a loss of ICC formation in the presence of doxycycline treatment.²³ Ding *et al.*, generated a zebrafish larvae model expressing NS5B that amplified the HCV replicon showing expression of HCV core RNA and protein.²⁴ When ribavirin and oxymatrine, two known anti-HCV drugs were used, the amplification of HCV replicon was inhibited suggesting the use of zebrafish larvae model for screening and evaluation of anti-HCV drugs.²⁴ In a separate study, it was shown that the HCV core protein with thioacetamide could induce HCV in the zebrafish model in a shorter period of time compared with a mouse model.²⁵ A recent study by Zhao *et al.* demonstrated that the HCV internal ribosome entry site (IRES) in the zebrafish larvae model mimics mammalian hepatic steatosis and fibrosis at gene levels and, therefore, recommends the feasibility of the zebrafish embryo model for screening anti-HCV drugs targeting HCV-IRES-mediated gene expression.²⁶

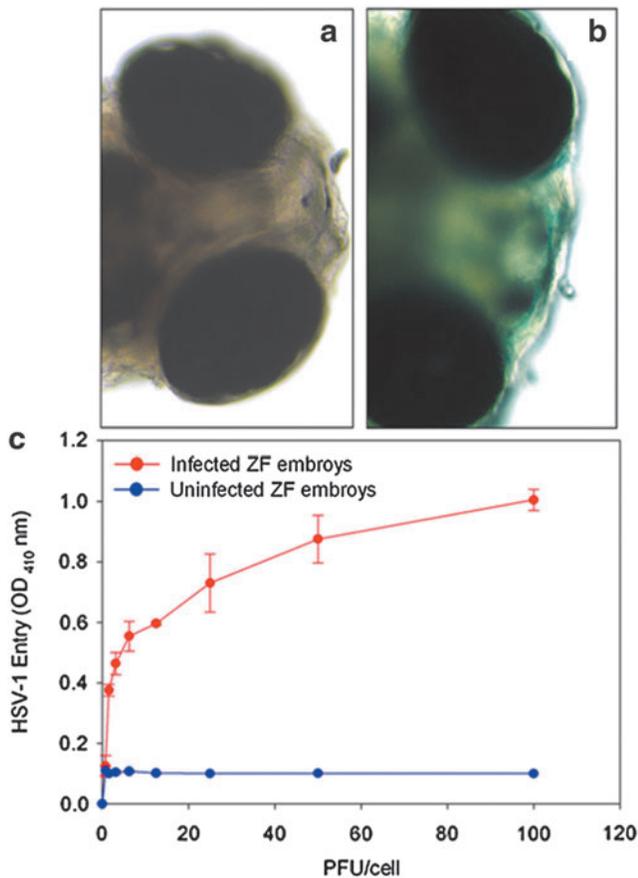


FIG. 3. HSV-1 infection in zebrafish embryo model. The wild-type zebrafish embryo wild India Klein strain kept in a 96-well plate format either mock infected with $1 \times$ phosphate buffer saline (a) or infected (b) with *Lac Z* expressing reporter HSV-1 gL86 at 10^6 plaque forming units for 24 h. X-gal staining in b demonstrated blue regions in HSV-1 infected zebrafish embryo. (c) Quantitative o-nitrophenyl- β -D-galactopyranoside (ONPG) assay further generated dose-response curves for HSV-1 infected zebrafish embryos in a 96-well format. Color images available online at www.liebertpub.com/zeb

Viral Infection in Zebrafish and Inflammation

Studying molecular and cellular basis of virus induced tissue damage through inflammation is another potential area of understanding human viral diseases in the zebrafish model. To date, most of the studies are relied on *in vitro* assays or fixed tissue samples. The optical transparency and the availability of transgenic fluorescent reporters (Table 2), for instance, BACmpx::GFP or lysC::DsRED2 transgenic zebrafish larvae can be useful for studying leukocyte migration specifically to damaged lateral line neuromasts after HSV-1 infection. Since zebrafish has a short breeding cycle, a large number of fish at a lower cost can be obtained to perform high-throughput screening of small molecules for antiviral or anti-inflammatory activity *in vivo*. Zebrafish embryos and adult forms are already emerging as a powerful tool to study inflammation due to remarkable similarity of its immune mechanism with the human immune system.²⁷⁻²⁹ The major components of the immune recognition system and defense signaling pathways are conserved between fish and mammals. In this regard, it has been shown that 48 h postfertilization, zebrafish embryos develop innate immunity with the activation of macrophages and neutrophils, while adaptive immunity is functional after 4 days postfertilization.²⁸ In addition, variants of Toll-like receptors have been identified in zebrafish. Similar homologues of the complement system and mammalian cytokines such as the interferon gene have also been identified.^{30,31} Therefore, zebrafish embryos or adult fish infected with different virus or viral strains may provide important information regarding critical components of host defense and associated pathology with the human disease. In addition, identification of cytokine-specific signaling during viral infection in the zebrafish model will allow screening of anti-inflammatory drugs that modify or block specific pathways.

It is worth mentioning that linear glycosaminoglycans (GAGs) such as HS actively participate in activation of chemokines and cytokines and therefore do play a major role in the inflammatory process.³² Interestingly, the expression levels of HS vary depending on the age of zebrafish.³³ Using liquid chromatography and mass spectrometry, it has been demonstrated that HS disaccharides are present in the zebrafish embryos, larvae, and adult fish.³³ Structural analysis of HS showed a different compositional profile during development. The compositional diversity was highest during early development, decreased at 1 dpt (day post fertilization), then increased at later time points.³³ Therefore, it will be interesting to investigate the HSV infectivity and associated

inflammation in different stages of zebrafish development and zebrafish HS and 3-OS HS knockouts.

Zebrafish Model: Overall Advantages to Study Viral Infections

The usage of the zebrafish model is expanding rapidly to study microbial infection, including viral pathogenesis.^{30,31,34,35} Although only few human viruses^{21,23-26,36-39} have been reported to infect zebrafish, the potential to take advantage of the zebrafish model is growing. There are several advantages of utilizing zebrafish to use microbial infections such as its small size, rapid breeding cycle, low maintenance cost offers large-scale infection analysis that can be very useful in defining infection requirements and confirming them in previously defined mammalian models^{40,41} (Table 3). Female zebrafish can be mated weekly and produce 100-200 progeny per clutch. Embryos develop externally in eggs 0.7 mm in diameter, larvae are only 2-4 mm long, while adults grow to a maximum of 4 cm making them easy to analyze and care for any stage of life. As mentioned previously, another advantage with the model is optical transparency, which lasts ~3 weeks at the larval stage.⁴² A double zebrafish mutant, *mitfa*^{w2/w}, *roy*^{a9/a9}, referred to as Casper lacks all iridophore- and melanophore-based pigment, has been developed where infection can be easily visualized in adult stages.⁴³ This unique property gives researchers an opportunity to inoculate zebrafish embryos with fluorescently labeled viruses to analyze progression of infection from the route of inoculation to other regions of the embryos in real time in a noninvasive fashion.⁴⁴ The information regarding virus dissemination to various tissues and organs can be translational as they can be used in the understanding and the treatment of human infections. The limitation with the existing murine and rabbit models is our inability to track viral particles (entry and spread) in whole animals, observe in real-time the pathological changes in cells and tissues, and relatively long breeding cycles. In addition, they are expensive to maintain. The optical clarity in zebrafish embryos together with the expression of viral entry receptors (Fig. 1),¹¹ and a well-developed immune system²⁸⁻³⁰ as well as evolutionarily conserved host defense strategies^{27,35} make zebrafish an excellent model to study multiple events of viral pathogenesis.

An additional strength of utilizing zebrafish in viral disease modeling is their putative usage for generating kinetics of a

TABLE 2. LIST OF SUGGESTED TRANSGENIC ZEBRAFISH, WHICH WILL BE INTERESTING TO STUDY IN CONTEXT WITH VIRAL INFECTIONS

<i>Transgenic reporter zebrafish</i>	<i>References</i>
G2R Cre reporter: Gene function	56
BA Cmps:: GFP: Inflammation	57
LysC:: DsRED2: Inflammation	57
MPO: GFP: Inflammation	58
Casper (roy): <i>In vivo</i> imaging	44
FGF reporter: Chemical screening	59
Red REGCO: Retinotectal system	60
VEGEF-2 RCFP: Angiogenesis	61
HuC-GFP: GFP-tagged neurons	62

TABLE 3. ATTRIBUTES OF ZEBRAFISH IN COMPARISON TO CURRENTLY USED MODEL FOR HSV

<i>Advantages of Zebrafish model for HSV-1</i>	<i>Zebrafish</i>	<i>Mouse</i>	<i>Rabbit</i>
Annual husbandry costs	Low	High	High
Time-lapse imaging	Yes	No	No
Large-scale high throughput	Yes	No	No
Duration of infection	Fast	Slow	Slow
Live immune response	Yes	No	No
Breeding cycle	Fast	Slow	Slow
Whole organism screening	Yes	No	No
Cost of gene knockout TALEN/CRISPR	Low	High	High
Neurotropism	Yes	Yes	Yes
Acyclovir screening	Yes	Yes	Yes

viral infection at different stages of development. The correlation of GAG expression and viral attachment is an important aspect of an age-based viral infection approach. The differential expression of GAGs at different stages of the zebrafish lifecycle provides a basis of studying viral infections in zebrafish as they develop from embryos to adults.⁴⁵

The most practical aspect of the zebrafish model is to generate clinically relevant information and design improvement in the disease therapy. Clearly, zebrafish also provides an inexpensive whole-animal vertebrate model for screening a library of small molecules, cell- or tissue receptor-specific compounds against viral pathogens or anti-angiogenic or anti-inflammatory compounds.^{24,46-49} One study has already employed this model to confirm the antiviral effect of acyclovir using HSV-1 infected zebrafish.²¹ Ideally, zebrafish in a 96-well format can be treated with antibodies⁵⁰ or compounds targeting specific cells/tissues or modified antiviral peptides with an objective to have higher efficacy. For instance, peptide generated against human 3-OS HS^{7,8} can be tested against the zebrafish 3-OS HS model since virus is able to infect zebrafish. Likewise, elucidating the antiviral activity of new drugs or peptides can be done and activation or blockage of specific pathways may be identified. Oncolytic adenoviruses (Ad) are great tools created with the objective for gene therapy.⁵¹ Interestingly, tumor regression in the ventral region has been documented in the zebrafish model when the oncolytic Ad virus was used.⁴¹ In one study, Davis

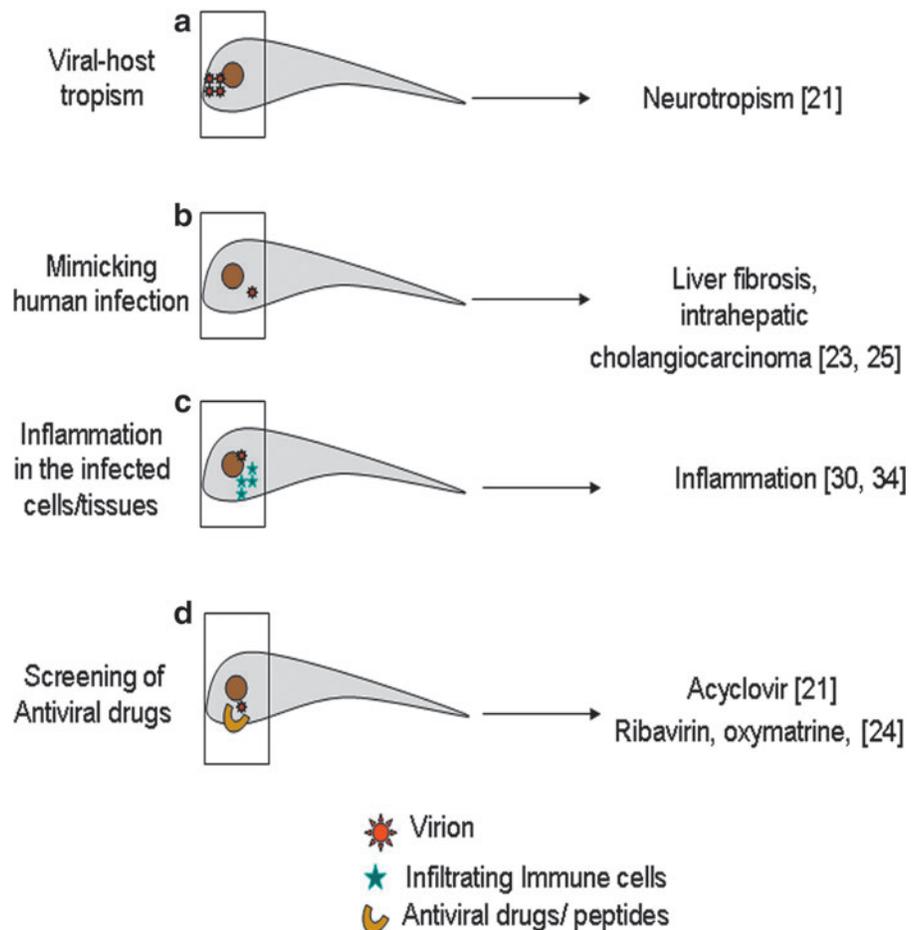
et al., suggest the use of a zebrafish model to determine the proper dosages of protein kinase C modulator required for the HIV-1 reservoir eradication.⁵²

In a similar fashion, if we consider recent advancements made in the field of high resolution fluorescent confocal imaging and molecular cloning, it is possible to label specific molecules in both zebrafish and viral particles to study their interactions at molecular and cellular levels. In addition, it is also possible to generate two fluorescently tagged viruses to study coinfection and super infection models in zebrafish embryos. Similarly, the transparent model together with imaging may also prove useful for antiviral drug administration and drug trafficking and toxicological studies.⁵³ Taken together, the zebrafish model has generated multiple interests to study viral infections and how they cause human diseases.

Conclusions

The investigations using human viruses to infect zebrafish to study viral infection has just started. One bigger question that is often asked is whether zebrafish embryos or adults can provide or mimic a model system for human viral infections. As indicated in Figure 4, there are multiple ways where the zebrafish model has provided clues to understand human viral infections. One published report on productive HSV-1 infection in zebrafish establishes the feasibility of developing this model system to study viral infections.²¹ Similarly, it is very encouraging to

FIG. 4. Significance of investigating human viral infection in zebrafish model (a–d). Understanding viral tropism for specific host cells and tissues (a), cell- and tissue-specific disease pathology mimicking human infections (b), inflammatory response to the damage sites (c), and screening of antiviral drugs (d) in zebrafish is likely to generate clinical interest. Color images available online at www.liebertpub.com/zeb



note that HSV-1 entry receptor generating enzymes 3-OST-2 and 3-OST-3 are expressed in zebrafish embryos and their products facilitate viral entry.^{9,11} However, whether 3-OST generated HS receptors are the natural receptors for HSV-1 entry in the zebrafish model remains to be tested. Some recent studies with human viruses also address that cell- and tissue-specific pathologies can be visualized with the zebrafish model.^{23,38–41} The future goal is to complement what we have learned in the mouse model and use genetic and embryological benefits of the zebrafish system to address human viral infections. There are multiple strengths in the zebrafish model, which can bring virologists, immunologists, and cell biologists together. For instance, availability of transparent zebrafish allows visualizing viral infection together with inflammatory response through labeled immune cells at the damaged tissue sites using live animal imaging. The above information will provide direct visual evidence of viral dissemination kinetics and ability of some viruses to replicate in host-specific cells and tissues. For instance, HSV-1 dependence on neurons and ocular cells or hepatitis tropism for hepatocytes can be visually confirmed. In addition, visual evidence generated with virulent clinical strains will be very useful to understand disease pathology. Zebrafish also provides an attractive model system for exploring new antiviral agents against resistant viruses or viruses, where other model systems have not been developed. Similarly, studies utilize knockdown and knockout technology of targeted genes to determine their effect on viral infection. Screening of mutagenized zebrafish against different viruses may also provide valuable information on viral disease at the molecular level. One challenge that relates to lower temperature, as zebrafish is maintained at 28°C while most of human viruses require 37°C for optimum productive infections, needs to be kept in mind.

Currently published studies have used a variety of approaches to study viral infection in the zebrafish model. For instance, Burgos *et al.* used i.p. inoculation of viral culture supernatants to anesthetize adult zebrafish, PCR for the detection of viral DNA load in different body regions after sacrificing and dissecting the animals, and histopathological immunodetection of HSV-1 glycoproteins for studying the effects of antiviral drug during immunosuppression.²¹ As indicated in Figure 3, we infected zebrafish embryos by keeping them in a liquid suspension in a 96-well format containing reporter HSV-1 virus. A similar study used fluorescence-labeled HCV subreplicon injected at 1–8 cell-stage zebrafish larvae to study viral tropism in the liver using fluorescent microscopy.²⁴ The same study also used conventional reverse transcriptase-PCR and western blot analysis to confirm the effect of antiviral drugs in zebrafish larvae.²⁴ We found that creating localized wound or injury to anesthetized adult zebrafish kept in an aqueous medium containing a virus supernatant provides an opportunity to facilitate viral infection, which can be visualized or quantified using reporter viruses (Fig. 2d). In terms of receptor expression, the differences in HS compositional profile during zebrafish development have been shown already.³³ The differences also point to the possibility that the levels of HSV infectivity during zebrafish development from early stages of embryos to adult form may vary and a lot of it can be monitored using time-lapse confocal imaging. Since our group has already identified and characterized HS recognizing anti-HS and anti-3-OS HS peptides,^{7,8} it will be interesting to test the effects of the peptides in blocking HSV infection and associ-

ated inflammation in the zebrafish model. Similarly, testing HSV infectivity in receptor-specific knockouts will also be very useful to understand their *in vivo* significance. Taken together, these types of experiments tap our strength with molecular tools such as live-cell imaging, molecular cloning, and generating mutations; it is quite realistic to say that in the future, we can design a better zebrafish model to study viral infection to better understand human infections and develop therapeutics to combat them.

Acknowledgments

We sincerely thank the Western University of Health Sciences, Pomona, CA facility for zebrafish embryo imaging (IACUC protocol # R10/IACUC/026). This work was supported by the National Institutes of Health R15 (1R15AI088429-01A1) to V.T.

Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Vaibhav Tiwari, PhD

Department of Microbiology and Immunology

Midwestern University

555 31st Street

Downers Grove, IL 60515

E-mail: vtiwar@midwestern.edu