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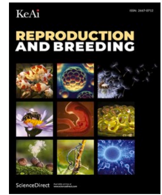

The infection of murine gammaherpesvirus-68 delays the early embryonic development of zebrafish

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ABSTRACT

Zebrafish is a model animal for aquaculture as well as bio-medicine. Murine gamma-herpesvirus 68 (MHV68) is widely regarded as a model for the study of human gamma herpesviruses such as Kaposi's sarcoma-associated herpesvirus (KSHV). In this study, the infection of MHV68 on zebrafish was investigated through incubating fertilized zebrafish eggs with MHV68. The development of zebrafish delayed or stopped at early stage separately when the zebrafish eggs were incubated with MHV68 at different dose for 2 h. The lytic gene of MHV68, including ORF65, RTA and ORF57, were detected in the genome of larvae hatched from the infected eggs. The transcription of the latent gene LANA but not of these lytic genes was detected by RT-PCR. The mRNA level of growth factor TGF-β1, but not of FGF3, was obviously decreased in the larvae from the MHV68 treated eggs. The mRNA levels of cytokines, such as IFNφ3, ISG15 and TNFα, varied post MHV68 infection; however, the transcription of the cytosolic DNA sensors, including DDX41, DHX9 and cGAS, did not change significantly. Moreover, the data generated in zebrafish cell line demonstrated that MHV68 could infect ZF4 cells. All the data support the conclusion that MHV68 could infect zebrafish embryo in the egg envelope and go into latent infection, which will delay the development of the embryos through some unknown mechanism.

1. Introduction

For decades the study of human diseases has relied heavily on the mouse model, but several aspects of murine biology limit its routine use. Zebrafish displays several favorable advantages as an effective model organism, which complements nicely with the application spectrum of mouse [1]. This animal has emerged as a good model organism for human disease and drug screening in recent years besides its application in aquaculture, which has been successfully employed in elucidating the mechanism of embryogenesis [2]. There are many chemicals, artificial or natural, have developmental toxicity in zebrafish embryos like Buckminsterfullerene aggregates (nC60), fullerol, methylmercury (MeHg), carbon nanotubes and chlorinated phosphate esters (CPEs) [3].

In zebrafish, a series of stages for development of the embryo have been generally described and defined. As well, 315 genes essential for early zebrafish development have been identified [4]. Among those molecules, the transforming growth factor-β (TGF-β) superfamily and the fibroblast growth factors (FGFs) have been investigated extensively and clearly. TGF-β belongs to a family of regulatory cytokines that have pleiotropic functions in a broad range of cell lineages involved in numerous physiological and pathological processes such as embryogenesis, carcinogenesis, and the immune response [5]. TGF-β superfamily signaling pathways emerged with the evolution of multicellular animals, suggesting that these pathways contribute to the increased diversity and complexity required for the development of zebrafish. Fibroblast growth factors (FGFs) have been implicated in diverse cellular processes including apoptosis, cell survival, chemotaxis, cell adhesion, migration, differentiation, and proliferation [6].

Herpesviruses represent a group of double-stranded DNA viruses distributed widely within the vertebrates. The herpesviridae family contains eight viruses that infect humans and consists of three subfamilies, namely alpha-herpesvirinae, beta-herpesvirinae and gamma-herpesvirinae [7]. Gamma-herpesviruses are of primary interest due to the two human viruses, Kaposi's sarcoma-associated herpesvirus (KSHV; also referred to as HHV-8) and Epstein-Barr virus (EBV), which are associated with several human malignancies, including B-cell lymphomas, nasopharyngeal carcinoma, and Kaposi's sarcoma [8]. However, the investigations are greatly hampered by the lack of permissive

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cell lines and animal models for both KSHV and EBV. Murine gamma-herpesvirus 68 (γHV68 or MHV68) is closely related to KSHV and EBV. MHV68 infection in laboratory mice leads to a robust acute infection in the lung and a long-term latent infection in the spleen, which makes it a suitable model to study KSHV and EBV [9].

Although zebrafish has been used for the study of human and mammalian viruses in several reports, such as herpes simplex virus type-1 (HSV-1), zebrafishes were infected with viruses through injection in most studies [10]. To our knowledge, no reports showed that mammalian virus could infect zebrafish by directly contact inoculation in water. To explore the possibility of interspecies transmission of herpes virus between mammals and fish, the infection of zebrafish with MHV68 was conducted in this study, in which the fertilized zebrafish eggs were incubated with MHV68. Our data demonstrated that the early development of zebrafish embryos was dampened or inhibited by MHV68 infection with different dosage. The PCR and RT-PCR data identified the integration of MHV68 into zebrafish embryos and the latent infection of MHV68, which implied the interspecies transmission of MHV68 between mammals and zebrafish.

2. Materials and methods

2.1. Ethics statement

Animal experimenters were licensed after attending a training course on laboratory animals held by the Institute of Experimental Animals, Hunan Province, China. Fish work was performed in strict accordance with the recommendations in the Guidelines for the Care and Use of Laboratory Animals of the National Advisory Committee for Laboratory Animal Research in China and was approved by the Animal Care Committee of Hunan Normal University.

2.2. Cells and virus

NIH 3T3 cells were kept in the lab and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. ZF4 cell line was a kind gift from Dr. Pin Nie (Institute of Hydrobiology, CAS) and cultured in DMEM/F-12 (1:1) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. NIH 3T3 cells were cultured at 37 °C with 5% CO_2; ZF4 cells were cultured at 28 °C with 5% CO_2. MHV68 was a kind gift from Dr. Pinghui Feng (University of Southern California) and propagated in NIH3T3 cells as previously described [11].

2.3. Fish maintenance and embryo production

Wild-type AB line zebrafish were obtained from China Zebrafish Resource Center/CZRC (Wuhan, China). Zebrafish maintenance, breeding and staging were performed as previously described [12]. Zebrafish embryos were acquired by natural spawning and cultured at 28 °C.

2.4. Virus titration

Virus titer of MHV68 was determined by plaque assay on NIH3T3 cells as previously described [11]. Briefly, the 10-fold serially diluted virus supernatant were added onto NIH3T3 cells and incubated for 2 h at 37 °C. The supernatant was removed after incubation and DMEM containing 2% FBS and 0.75% methylcellulose (Sigma) was added. Plaques were counted at day 4 post-infection.

2.5. Virus infection

Fertilized zebrafish eggs were collected into 24-well plate (10 embryos/well) with 500 μl aerated-water, MHV68 or whole-cell lysates of NIH3T3 cells was added at the one-cell stage separately. The embryos were washed three times after incubation in the virus for 2 h and subsequently cultured in aerated-water at 28 °C for observation.

2.6. Quantitative real-time PCR

Total RNAs were extracted from fertilized zebrafish eggs and ZF4 cells using Trizol Reagent (TaKaRa, Japan) following the manufacturer's protocol. Then 1 μg of extracted RNA was reverse-transcribed into cDNA through Revert Aid TM First Strand cDNA synthesis Kit (Thermo, USA), which was used for subsequent quantitative real-time PCR (q-PCR). q-PCR was performed to quantify the zebrafish cytokine mRNA expression in vivo and in ZF4 cells. The primers used for amplifying zebrafish cytokines were listed in Table 1 and the qPCR program was: 1 cycle of 95 °C/10min, 40 cycles of 95 °C/15s, 60 °C/1min, followed by dissociation curve analysis (60°C–95 °C) to verify the amplification of a single product. The threshold cycle (CT) value was determined by using the manual setting on the 7500 Real-Time PCR System and exported into a Microsoft Excel Sheet for subsequent data analyses where the relative expression ratios of target gene in treated group versus those in control group were calculated by $2^{-\triangle\triangle CT}$ method.

3. Results

3.1. The early embryonic development of zebrafish was impaired by MHV68 infection

The fertilized eggs of zebrafish were co-cultured with MHV68 for 2 h at one-cell stage to see if this mammalian virus could infect the embryos of zebrafish coated with egg envelope. The embryos pre-cultured with MHV68 at low dose (1000 PFU/embryo) showed no difference in every developmental stage comparing with those embryos pre-cultured with control NIH3T3 whole cell lysate (WCL), which hatched maturely into larva at 45–59 h post fertilization (hpf) (Fig. 1A). However, the embryos pre-cultured with MHV68 at the dose of 5000 PFU/embryo showed delayed embryonic development, which finally hatched maturely into larva at 59–72 hpf (Fig. 1B).

Table 1
Primers used in the study.

Gene	GenBank accession	Primer sequences(5′-3′)
RTA	NP_044887	GAACTTGCTCTTCGGCGTCT ATGCCTCAACTTCTCTGGAT
ORF57	NP_044895	ACAACAAAGTATGAGCCTGC GATACCGCCTTTTCAGACAC
ORF65	NP_044903	GGTCTGGAATAACCCTAAG GGACAGTATTGGCAAAGACC
TGFβ1	NM_182873	GAACTCGCTTTGTCTCCA ACTTATCCGTGCTCTGCT
FGF	NM_131291	GTGGCAATCAAGGGACTGTT GCCGTGATGCATAAGTGTTG
IFNφ1	NM_207640	GAGCACATGAACTCGGTGAA TGCGTATCTTGCCACACATT
IFNφ3	NM_001111083	TTCTGCTTTGTGCAGGTTTG GGTATAGAAACGCGGTCGTC
ISG15	NM_001204169	TCATAACTCGGTGACGATGCAG TGATCCGCTGACCGTTTTCG
TNFα	AY427649	GCGCTTTTCTGAATCCTACG TGCCCAGTCTGTCTCCTTCT
DDX41	KR559928	GTGATGCGTCTGCGGGGTA GAGGTGCTGATGTTGGTCC
DHX9	NM_001201444	CAGAGCCCTTCATCAGCGA CCACCCGAACTTCATCCCA
cGAS	XM_680019	GAGACCAGGCTGTGTTTCCC TTACTCCGTCCCCGTTTTAG
MAVS	FN178460	ATTCATCACTGCTCTGCGGAAG GTTGTAACGGTTGCTGTGGCT
GAPDH	NM_001115114	GTGTCAGGACGAACAGAGGC GGGTGGAGTCGTACTGGAAC

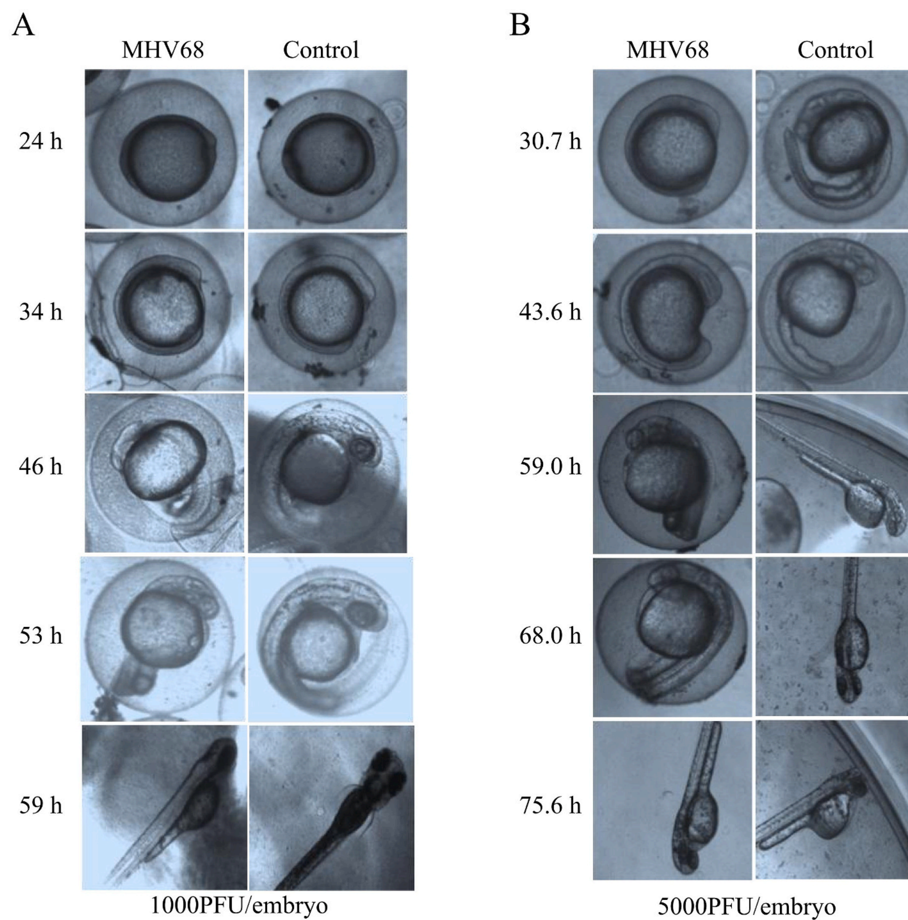


Fig. 1. The development of zebrafish embryos infected with MHV68 at low dose. The fertilized eggs of zebrafish were co-cultured with MHV68 at different doses (5000 PFU/embryo or 1000 PFU/ embryo) or whole cell lysates of NIH3T3 (as control) at the one-cell stage for 2 h independently; then the fertilized eggs were cultured at 28 °C after three times of wash. The embryonic development of zebrafish was observed under stereomicroscope and photographed at indicated time point post fertilization. A. The zebrafish embryos was treated with MHV68 by the dose of 1000 PFU/embryo. B. The zebrafish embryos was treated with MHV68 by the dose of 5000 PFU/embryo.

When the MHV68 amount was increased to the dose of 25000 PFU/ embryo, the embryos pre-cultured with MHV68 presented even obviously damped development (Fig. 2). The impacts of MHV68 on the embryonic development of zebrafish were quantified by the scores of each developmental stage, which reflected the average developmental speed of each group (Fig. 3). It is interesting that the overall embryonic developmental process of zebrafish group treated with NIH3T3 whole cell lysate was faster than that of zebrafish group without any treatment, which might be explained that some components of NIH3T3 whole cell lysate favored the early embryonic development of zebrafish.

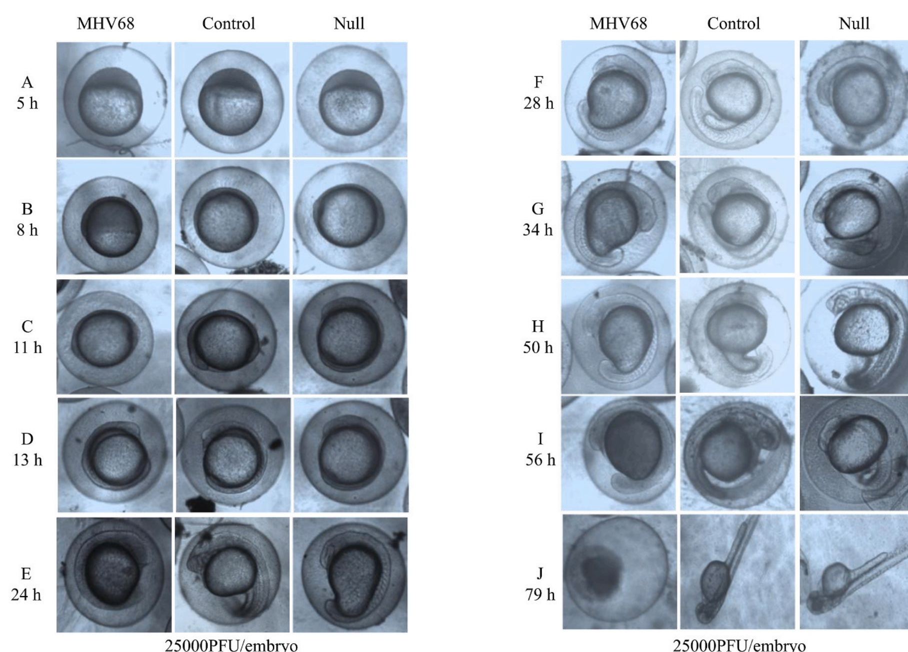
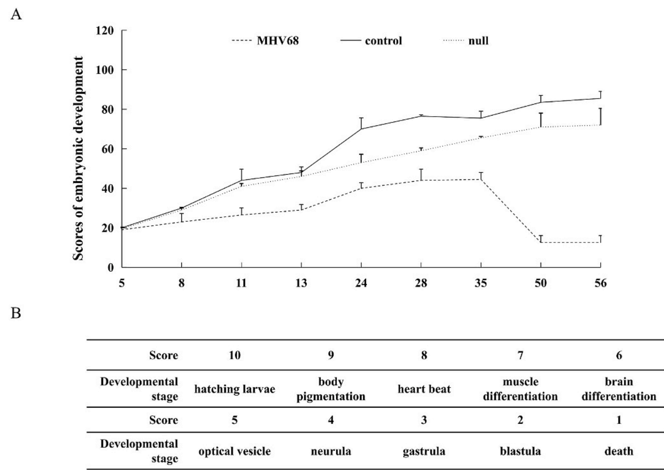


Fig. 2. The zebrafish embryos was treated with MHV68 by high dose.
The fertilized eggs of zebrafish were treated with MHV68 (25000 PFU/embryo) or whole cell lysates of NIH3T3 (as control). Embryos were treated with nothing termed Null. These embryos were observed under stereomicroscope and photographed in the order of time (Ã J). A: blastocyst; B & C: neurula; D: vesicle; E & F: brain differentiation; G: muscle differentiation; H: heartbeat; I: body pigmentation; J: larva. (Reference to Control).



Score	10	9	8	7	6
Developmental stage	hatching larvae	body pigmentation	heart beat	muscle differentiation	brain differentiation
Score	5	4	3	2	1
Developmental stage	optical vesicle	neurula	gastrula	blastula	death

Fig. 3. The scoring system of zebrafish embryonic development with/without MHV68 treatment.
Zebrafish embryos of the three groups (in Fig. 2) were determined to which stage they survive at a time point and each stage was given a specific score. Finally, the total scores was calculated at every time point. A. The scores of zebrafish embryonic development of each group. X-axis: time point (hours post fertilization, hpf). Y-axis: total scores. The map stands for one of the three independent assays. B. The given score for each embryonic developmental stage.

All the MHV68 treated embryos tended to be away from normal development process after they struggled to the muscle differentiation stage and most of them began to die at 59 hpf (Figs. 2 and 3). No embryo pre-cultured with MHV68 at dose of 25000 PFU/embryo could hatch maturely into larva. All these data suggested that, this mammalian virus could infect fertilized zebrafish eggs with envelope and impact the embryonic development of zebrafish.

3.2. MHV68 genes were detected in the genome of zebrafish larvae hatched from MHV68 infected embryos

The genomic DNA was extracted from the zebrafish larvae and used to detect the integration of MHV68, which were hatched from the fertilized eggs co-cultured with MHV68 for 2 h as mentioned above (Fig. 1B). The primers were designed specifically to amplify the lytic genes of MHV68 separately, which included ORF65, RTA and ORF57 (Table 1). The specific bands of ORF65, RTA and ORF57 were all detected in the genomic DNA from the larva hatched from MHV68 treated fertilized eggs but not in the control DNA, which were identified by DNA sequencing (Fig. 4B, arrow indicated). However, the mRNA transcriptions of these lytic genes were not detected by RT-PCR, which suggested that there was no lytic replication of MHV68 in viral treated zebrafish embryos. To investigate mechanism that MHV68 integrated into the genome of zebrafish, the RT-PCR of LANA was recruited, whose expression was crucial for the latent infection of MHV68. The specific band for LANA was detected in the lane of MHV68 infected embryos but not control embryos, which was further identified by DNA sequencing (Fig. 4C). The PCR and RT-PCR data demonstrated that MHV68 could infect the embryo of zebrafish, which were covered with egg envelope.

3.3. The mRNA expression of growth factors in zebrafish embryo changed in response to MHV68 infection

To investigate the mechanism behind the impaired embryonic development by MHV68, mRNA transcriptions of two growth factors, TGFβ1 and FGF3, at different embryonic developmental stages were determined by q-PCR. Embryos treated with MHV68 (as mentioned in Fig. 1) were collected for total RNA isolation at 5, 12, 24, 36, 48 and 56 h post infection (hpi) and used for q-PCR separately as well as embryos treated with NIH3T3 WCL. The mRNA transcription level of TGFβ1 was consistently higher in control group than MHV68 group (Fig. 5A). But there were no obvious difference between the FGF mRNA transcription levels from the two groups.

3.4. The mRNA expression of several cytokines in zebrafish embryo changed in response to MHV68 infection

To investigate the innate immune response of the embryos treated with MHV68, mRNA expression profiles of several antiviral cytokine during the early embryonic developmental stage were examined by q-PCR, which included IFNφ1, IFNφ3, ISG15, TNFα. In general, the mRNA

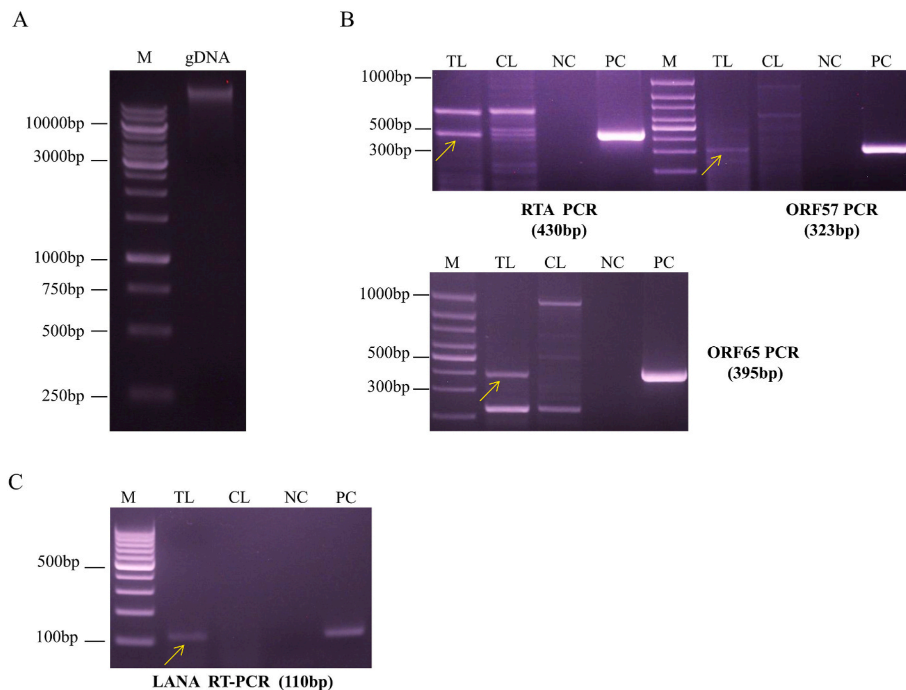


Fig. 4. MHV68 genes were detected in zebrafish larvae treated with MHV68.
The zebrafish larvae from the eggs treated with MHV68 (5000 PFU/embryo, Fig. 1A) were collected after hatching. The genomic DNA of zebrafish larvae was isolated and the integration of exogenous MHV68 DNA was determined by PCR. A. The genomic DNA of zebrafish larvae. B. The PCR amplification of ORF65, RTA and ORF57. C. The RT-PCR of LALA. M: 100bp DNA ladder; gDNA: the genomic DNA of zebrafish larvae; TL: larvae of test group; CL: larvae of control group; NC: negative control; PC: positive control. Yellow arrows stand for the indicated MHV68 genes accordingly.

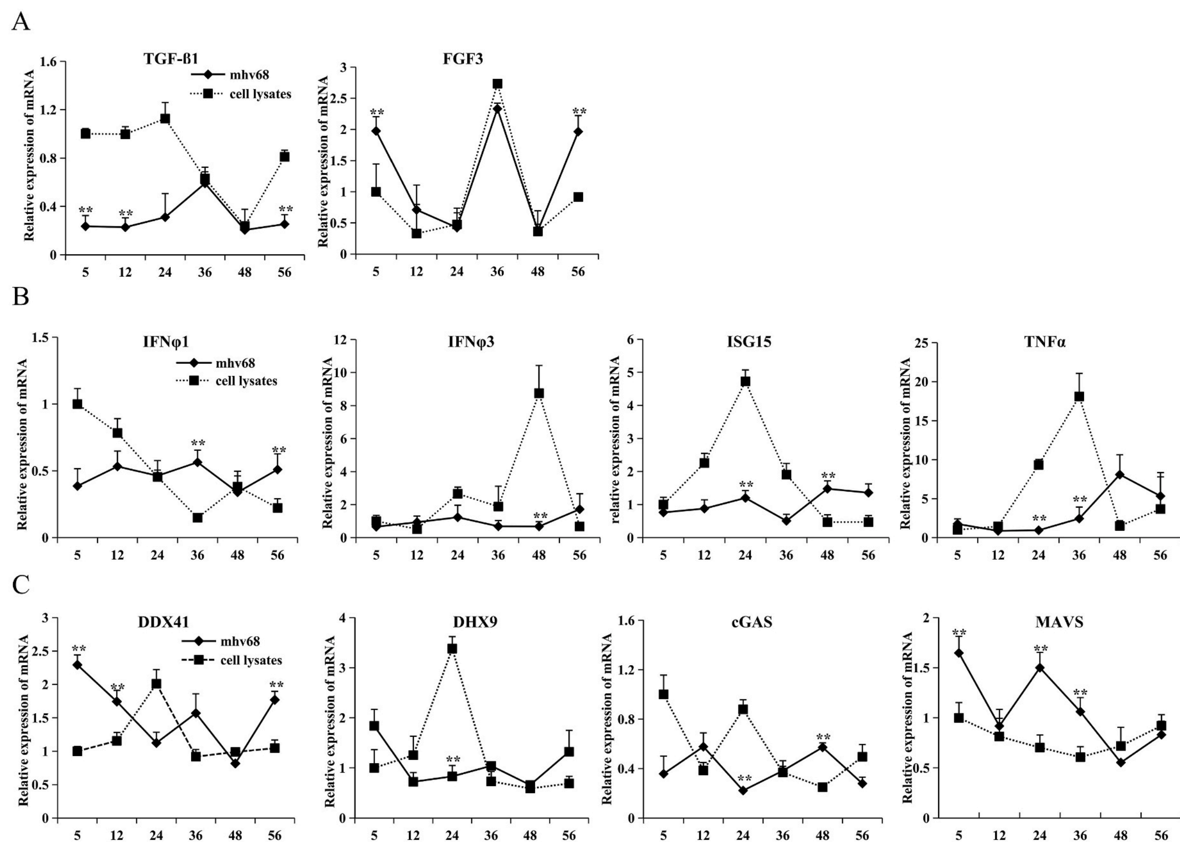


Fig. 5. The mRNA expression of growth factors (TGFβ1, FGF3), cytokines (IFNφ1, IFNφ3, ISG15, TNFα) and sensors (DDX41, DHX9, cGAS, MAVS) during embryonic development.

The fertilized eggs of zebrafish were treated with MHV68 (5000 PFU/embryo) as MHV68 group, whole cell lysates of NIH3T3 (cell lysates) as control group or without treatment as mock (data not shown) at the one-cell stage for 2 h separately, then the fertilized eggs were cultured in fresh water at 28 °C after three times of wash. The embryos were collected at indicated time points post fertilization and applied to RNA isolation. The indicated mRNA transcription was determined by Q-PCR. A. The mRNA expression of TGFβ1 and FGF3 in zebrafish embryo. B. The mRNA expression of IFNφ1, IFNφ3, ISG15 and TNFα in zebrafish embryo. C. The mRNA expression of DDX41, DHX9, cGAS and MAVS in zebrafish embryo. Data are representative of three independent experiments and are presented as mean ± SD (n = 3). *P < 0.05, **P < 0.01.

expression of IFNφ1 in the MHV68 group was not affected compared with the control group (Fig. 5B). However, the mRNA expression of IFNφ3, ISG15, TNFα were obviously damped at the specific development stage. About the mRNA expression of IFNφ3, there is a precipitate down-regulation at 48 hpi in the MHV68 group (0.7 fold) than control group (8.7 fold) (Fig. 5B). About the ISG15, it was also an obviously increasing at 24 hpi in the control group (4.7 fold) (Fig. 5B), but the expected up-regulation was damped in the MHV68 group (1.2 fold). About the TNFα, there is a sharply increasing at 36 hpi in the control group (18.1 fold), while the expected up-regulation was decreased to 2.4 fold in the MHV68 group (Fig. 5B). These results clearly showed that the latent infection of MHV68 could restrain the expression of several antiviral cytokine during the early embryonic developmental stage in zebrafish.

3.5. The mRNA expression of host cytosolic sensors in zebrafish embryo changed in response to MHV68 infection

To investigate whether the MHV68 could been recognized by cytosolic DNA sensors in zebrafish embryo and trigger the innate immune response after MHV68 infection. The mRNA expression of host cytosolic sensors, such as DDX41, DHX9, cyclic GMP-AMP synthase (cGAS) were tested by qPCR. The data showed that the mRNA expression of the three sensors were not triggered to a high level in the MHV68 group (Fig. 5C). Besides, the mRNA expression of the key antiviral adaptor protein MAVS, which responds specifically to RNA virus infection in the RIG-I/MAVS/IFN signaling in zebrafish [13], was also examined. The results showed that the mRNA expression of MAVS in MHV68 group seemed a little higher in compared with control group in the MHV68 infection (Fig. 5C). The reason for this remains to be explored.

3.6. The mRNA expression of growth factors, cytokines and sensors in ZF4 cells changed in response to MHV68 infection

To see what happen in vitro model, ZF4 cells were infected with MHV68 and the genes about growth factors, cytokines and sensors were determined by q-PCR at different time points. The data showed that the mRNA transcription level of TGFβ1 and FGF3 were consistently lower in MHV68 group compared with control group, especially at 36 hpi (Fig. 6A), which indicated that MHV68 infection dampened expression of the two important development related genes in vitro. The really interesting here is that mRNA transcription level in IFNφ1, IFNφ3, ISG15 but not TNFα, compared to unapparent induction in embryo, is consistently higher in ZF4 cells control group (Fig. 6B). At last, the mRNA expression of the three DNA sensors and MAVS were not triggered to a high level in ZF4 cells infected with MHV68, which is similar to the data from zebrafish embryos in vivo (Fig. 6C).

4. Discussion

Zebrafish has been successfully employed as model organism for many years, especially in elucidating the embryogenesis, evolution and development of the immune system. A variety of factors that can affect

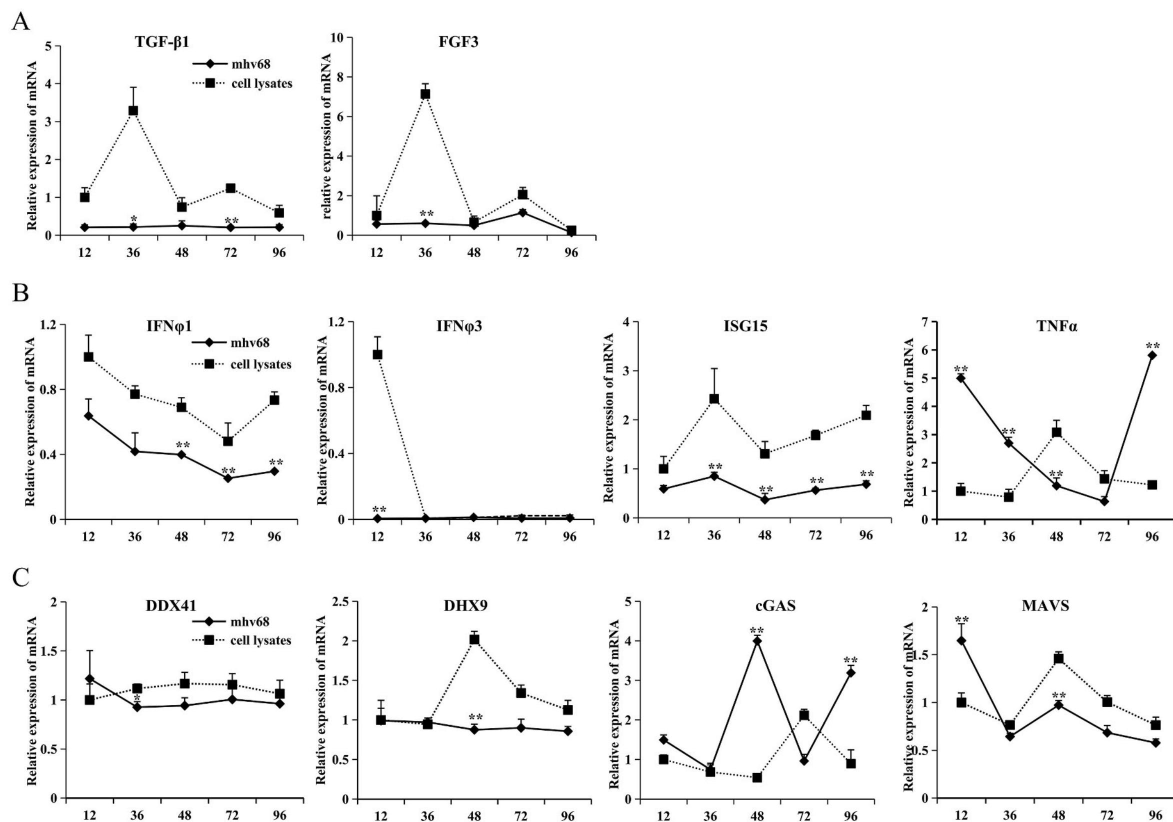


Fig. 6. The mRNA expression of growth factors (TGFβ1, FGF3), cytokines (IFNφ1, IFNφ3, ISG15, TNFα) and sensors (DDX41, DHX9, cGAS, MAVS) in ZF4 cells at the indicated time point.
The ZF4 cells in 6-well plate (2 × 106 cells/well) were treated with MHV68 (MOI = 0.02) as MHV68 group or whole cell lysates of NIH3T3 (cell lysates) as control group. The cells were collected at indicated time points post infection and applied to RNA isolation. The indicated mRNA expression was determined by Q-PCR. A. The mRNA expression of TGFβ1 and FGF3 in the ZF4 cells. B. The mRNA expression of IFNφ1, IFNφ3, ISG15 and TNFα in the ZF4 cells. C. The mRNA expression of DDX41, DHX9, cGAS and MAVS in the ZF4 cells. Data are representative of three independent experiments and are presented as mean ± SD (n = 3). *P < 0.05, **P < 0.01.

the embryonic development of zebrafish has been explored including natural substances and synthetic substances. For example, microcystin and cyanobacterial can affect on embryo-larval development [14]. Endosulfan I and endosulfan sulfate disrupt zebrafish embryonic development [15]. Many of these mechanisms have also been found, including some related genes and proteins. Micro-RNA expression in zebrafish embryonic development has been studied too [16]. Given the versatility of zebrafish for embryogenesis, we were interested in the feasibility of establishing a model to dissect the connection between virus and early embryonic development.

The viruses that infect mammals are normally adapted to propagate at 37 °C and fail to replicate at 28 °C, a temperature optimal for zebrafish growth. However, many mammalian viruses have been reported that can successfully infect zebrafish. Chikungunya virus (CHIKV) could replicate in zebrafish and disseminates to various organs after microinjected into the zebrafish embryo, which demonstrates that zebrafish could be used as a valuable model to dynamically visualize replication, pathogenesis and host responses to human virus [17]. In this paper, zebrafish larvae inoculated with MHV68 demonstrated that zebrafish could be used as a new mode for herpesvirus.

Herpesviruses are important pathogens in vertebrate, which are wide spread among mammals, birds and fish, and most are thought to have evolved in the same host species over long periods. The precise specialized interaction with host has resulted in a high level of host specificity and even the evolution of distinct species within the same host. In general, alpha-herpesviruses are viewed as having the broadest host range with respect to species (excepting VZV) and cell type, beta-herpesviruses can infect a variety of cell types but are usually restricted to infections of their natural hosts, and gamma-herpesviruses are restricted with respect to host and cell type [18]. Although natural infections with most herpesviruses are restricted to a single species, some of these viruses can infect other species experimentally or accidently. Therefore, it was no surprise we detected MHV68 DNA in zebrafish embryos treated with the virus.

After the virus DNA detection, we tried to detect the MHV68 mRNA in virus-treated zebrafish. However, the negative results were obtained. The gamma-herpesviruses establish a delicate balance between life-long latency in the host and immune control of the infection. When the nature host, mice, is treated with MHV68, intranasal infection of mice with MHV68 causes an acute respiratory infection that is rapidly resolved, followed by the establishment of latency. Levels of latent virus in the spleen peak around 14 days after infection, drop quickly, and remain stable for life [19]. We speculate that MHV68 also established a state of latent infection in zebrafish and might be short of conditions for its replication cycle, and the temperature is likely to be one of reason.

Zebrafish is susceptible to infection by Gram-positive and Gram-negative bacteria, mycobacteria, protozoa and viruses [20]. The zebrafish innate immune response involves phagocytic cells such as macrophages and neutrophils, cytokines and their signaling molecules and adaptive humoral and cellular immunity [21]. For pathogens such as DNA and RNA viruses are sensed by different sensor/receptors. Cytosolic DNA viruses, like MHV68, are sensed by a set of sensors, including cGAS, DHX9 and DDX41. They ultimately activate the IRF3 and/or NF-κB- responsive genes, including type I interferons (IFNs) and pro-inflammatory cytokines. But we failed to detect high-level expression of IFNs and cytokines as expected in zebrafish embryos infected

with MHV68. Maybe the three sensors may not be the exactly cytosolic sensors of MHV68 infection in the zebrafish.

In this study, we see that early embryonic development of zebrafish were damped by MHV68 infection and MHV68 genes, ORF65, RTA, ORF57, were detected in larvae by PCR, and the latent infection gene, LALA were detected in larvae by RT-PCR. From the results it can be inferred that the delayed embryonic development is caused by MHV68. But clearly the exact mechanism needs further exploration and study.

Author contributions

H. Feng, J. Yan and M. Chi conceived the project. J. Yan and J. Xiao designed and optimized the experiment system. M. Chi and J. Yan provided zebrafish maintenance and embryo production. J. Xiao and M. Chi performed MHV68 production. J. Yan and M. Chi performed the PCR analysis. J. Yan and M. Chi conducted morphological examination of zebrafish egg. J. Yan and M. Chi performed the data analysis. J. Yan, M. Liu and H. Feng prepared the original manuscript. H. Feng revised the manuscript. All authors reviewed and approved the submitted manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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