Developmental and Comparative Immunology 88 (2018) 55–64

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/devcompimm

# Establishment of fin cell lines and their use to study the immune gene expression in cyprinid fishes with different ploidy in rhabdovirus infection

Jun Xiao[1], Yongming Fu[1], Wei Zhou, Lingzhi Peng, Jun Xiao, Shaojun Liu, Hao Feng*

*State Key Laboratory of Developmental Biology of Freshwater Fish, College of Life Science, Hunan Normal University, Changsha, 410081, China*

ARTICLE INFO

*Keywords:*
Innate immunity
Ploidy
Fish cell line
SVCV
Transcriptome

ABSTRACT

Triploid hybrid (3n = 150) of red crucian carp (♀, 2n = 100) and allotetraploid (♂, 4n = 200) display improved disease resistance and stress resistance than their parents. In order to elucidate their innate immune mechanisms, three novel cell lines from the caudal fin of red crucian carp, triploid hybrid and allotetraploid (named 2nFC, 3nFC and 4nFC accordingly) were established and characterized respectively. 2nFC, 3nFC and 4nFC showed fibroblast-like morphology and characteristics. They have been subcultured for more than 100 passages since the initial primary culture. Viral infection experiments showed that 2nFC, 3nFC and 4nFC were susceptible to spring viraemia of carp virus (SVCV) infection. Intriguingly, 3nFC performed the stronger resistance ability against SVCV than 2nFC and 4nFC, which indicated that 2nFC, 3nFC and 4nFC might be used as the suitable *in vitro* models for exploring and analyzing the differences among these three cyprinid fishes in antiviral innate immune mechanisms. Based on this, we analyzed the transcriptome profile of 2nFC, 3nFC and 4nFC in the context of SVCV infection. The KEGG enrichment analysis showed that the differentially expressed genes (DEGs) were primarily enriched to immune-related signaling pathways. However, some signaling pathways against viral infection were activated remarkably in 2nFC and 3nFC but not in 4nFC. Overall, the establishment of 2nFC, 3nFC and 4nFC provided us a suitable platform to elucidate the innate immunity of fishes with different ploidy and clear genetic relationship.

## 1. Introduction

Polyploidization exists in vertebrates and plants, however, it happens more frequently in plants (Mable, 2004, 2013). Polyploidization in vertebrates seldom shows positive effects and more often results in lethal consequences because viable gametes fail to form during meiosis (Liu et al., 2016). Fortunately, allotetraploid (AT; 4n = 200) has been developed by crossing red crucian carp (*Carassius auratus*red var., ♀, 2n = 100) with common carp (*Cyprinus carpio* L., ♂, 2n = 100) and subsequently selective breeding. Both male and female individuals of allotetraploid are fertile and this allotetraploid population has propagated 26 generations (Liu et al., 2001). Triploid hybrid (3n = 150) was developed through the hybridization between the male allotetraploid and the female diploid red crucian carp (Chen et al., 2009; Liu et al., 2004; Shen et al., 2006). Triploid hybrid possesses many merits, such as fast growing and good taste, which make it an economic important species in Chinese fresh water aquacultural industry (Liu, 2010). Especially, triploid hybrid displays much improved disease resistance and stress resistance than its parents (Liu, 2010). As to these three fish species with different ploidy and clear genetic relationship, extensive studies had been conducted to explore their development, reproduction and physiology, however, there were few reports about the immunity of these fish species (Duan et al., 2016; Long et al., 2006; Zhang et al., 2005).

Cell lines are valuable *in vitro* models to study virology, pathology, developmental biology and immunology of both lower and higher vertebrates (Lakra et al., 2011; Zhang et al., 2003). Up to now, a lot of fish cell lines have been established from different fish species, such as zebrafish (*Danio rerio*), rainbow trout (*Oncorhynchus mykiss*), crucian carp (*Carassius auratus*), grass carp (*Ctenopharyngodon idella*), orange spotted grouper (*Epinephelus coioides*), black carp (*Mylopharyngodon piceus*) etc (Driever, 1993; Luque et al., 2014; Qin et al., 2006; Xue et al., 2018; Zhang and Gui, 2004; Zuo et al., 1986). Many studies reported that fish fibroblasts were able to express abundant cytokines and immune-related receptors, which suggested that fibroblasts might play an important role in both the innate and adaptive immunity in fish (Ingerslev et al., 2010; Villena, 2003). Allotetraploid, diploid red crucian carp and their triploid hybrid offspring offer a unique system for

* Corresponding author.
*E-mail address:* fenghao@hunnu.edu.cn (H. Feng).
[1] These authors contribute equally to this paper.

https://doi.org/10.1016/j.dci.2018.07.007
Received 8 February 2018; Received in revised form 7 July 2018; Accepted 7 July 2018
Available online 12 July 2018
0145-305X/ 

the study of the evolution of the innate immunity with several advantages. For example, their known parentage separates them from natural polyploids, and it is easy to trace the fate of progenitor genes (Liu et al., 2016). However, for the studies on the innate immunity, there is a lack of species-specific cell lines of fishes with different ploidy, because they are non-model animals.

In this study, we have established and characterized three cell lines from the primary culture of the caudal fins of diploid red crucian carp, triploid hybrid and allotetraploid separately. These three cell lines were designated as 2nFC, 3nFC and 4nFC accordingly and they showed fibroblast-like morphology. Two marker genes for epidermis (containing epithelial cells) and dermis (containing fibroblasts) were investigated to further determine the cell types of 2nFC, 3nFC and 4nFC. In natural and aquacultural condition, red crucian carp, triploid hybrid and tetraploid fish are facing many pathogenic microbes, such as spring viraemia of carp virus (SVCV) (Yan et al., 2016). Therefore, the susceptibility of 2nFC, 3nFC and 4nFC to SVCV was examined in the current study. At the same time, some antiviral genes like IFNa, PKR, Viperin and Mx1, which induced by fish rhabdoviruses were investigated by qPCR method. Next-generation sequencing (NGS) technologies have provided a new approach for exploring the whole genome and transcriptome information involved in fish immunity in recent years (Morozova and Marra, 2008). By sequencing RNA from infected and uninfected samples, it is possible to identify immune-related genes which are differentially expressed and further lead to a better understanding of molecular mechanisms underlying the host immune response to pathogenic stimuli (Petit et al., 2017). Therefore, we employed RNA-seq technology to investigate the transcriptome of 2nFC, 3nFC and 4nFC in the context of SVCV infection. Overall, the establishment of 2nFC, 3nFC and 4nFC constituted a suitable platform to study the immune gene expression in cyprinid fishes with different ploidy in rhabdovirus infection.

## 2. Materials and methods

### 2.1. Primary cell culture and subculture

Healthy red crucian carp (6 g in weight), triploid hybrid (19.8 g in weight) and allotetraploid (146 g in weight) were collected from the Engineering Research Center of Polyploid Fish Breeding and Reproduction of State Education Ministry in Hunan Normal University. After thoroughly removing the surface mucus on the body of the fish, the caudal fins were clipped, disinfected with 75% ethanol and washed with phosphate-buffered saline (PBS) containing 200 U/ml penicillin and 200 μg/ml streptomycin (HyClone). Thereafter, the fins were minced into pieces (approximately 1 $mm^3$) by using sterile scissors and then placed in fetal bovine serum (FBS; Gibco). Tissue pieces were seeded into 8-$cm^2$ dishes and incubated at 26 °C with 5% CO2 equilibrium. After 1 h, 3 ml DMEM (Gibco) with 30% FBS and 20 ng/ml bFGF (Sigma) was added to the dishes.

Fifty percent of the media was changed every 3–5 days with fresh DMEM supplement with 30% FBS, 20 ng/ml bFGF, 100 U/ml penicillin and 100 μg/ml streptomycin. Subculture was carried out at a split ratio of 1:2 subsequently by trypsinization when primary cell cultures grew to 90–100% of confluence. After 15 passages, the concentration of FBS in the medium was decreased from 30 to 15%, and the bFGF was decreased to 10 ng/ml.

### 2.2. Cell growth and cell transfection

Growth characteristics of 2nFC, 3nFC and 4nFC were evaluated by cell doubling-time assay. Cells at a density of $2.0 \times 10^5$ cell/well were seeded into 6-well plates and incubated at 26 °C. Every other day, cells were trypsinized and collected for hemocytometric determination of cell number. All experiments were undertaken in triplicate.

2nFC, 3nFC and 4nFC were seeded into 6-well plates at a density of $2 \times 10^5$ cell/well separately. After being cultured for 24 h, cells were transfected with pEGFP-N1 by using LipoMax (SUDGEN), Lipofectamine®2000 (Invitrogen) or calcium phosphate according to the manufacturer's instruction respectively (Liu et al., 2017). Green fluorescence signals were detected by fluorescence microscope, and the transfection efficiency was determined by counting green fluorescent protein-positive and total cells from 20 random fields at 48 h post transfection.

### 2.3. Chromosomal analysis and flow cytometry

Chromosomal analyses of the 2nFC, 3nFC and 4nFC were performed at passage 50. Exponentially growing cells in 10-cm dishes were treated with colcemid (0.1 mg/ml) for 3 h. The cells were harvested and followed by hypotonic treatment with 0.075 M KCl at 26 °C for 25–30 min, then fixed in methanol-acetic acid (3:1, v/v) with three changes. Then cells were stained according to a previously described method (Xiao et al., 2014). Chromosome metaphases were observed and photographed with Pixera Pro 600ES (US). For each cell line, 100 good-quality metaphase spreads were analyzed.

Cells in 10-cm dishes were collected and washed three times with PBS. The cells were re-suspended in pre-cooling ethanol and fixed overnight at 4 °C. The fixed cells were washed three times with PBS and re-suspended in PBS at a concentration of $1 \times 10^6$ cells/ml. PI (50 μg/ml) and RNase A (20 μg/ml) were added into the cell suspension to stain the cells in the dark for 30 min. After PI staining, the cells were analyzed with flow cytometer for the DNA content measurement.

### 2.4. Virus production and infection

The strain 741 of SVCV (SVCV-741) was kindly provided by Dr. Yong' an Zhang (Institute of Hydrobiology, CAS). The virus was propagated in EPC cells at 25 °C in the presence of 2% FBS. Virus titer was determined by plaque assay in EPC cells as previously described (Xiao et al., 2017). Briefly, the 10-fold serially diluted virus supernatants were added onto EPC cells and incubated for 2 h at 25 °C. The supernatant was removed after incubation and DMEM containing 2% FBS and 0.75% methyl cellulose (Sigma) was added. Plaques were counted at day-3 post infection.

For viral infection, 2nFC, 3nFC or 4nFC were seeded into 12-well plates at $1 \times 10^5$ cells/well at 26 °C for 24 h. SVCV were added into the culture media at the dose of 0.003, 0.03, 0.3 or 3 MOI, respectively. After 1 h incubation, the media containing viruses was replaced with fresh media after washing twice with DMEM. Anther 24 h later, the supernatants were collected for viral titer measurement using plaque assay as previously described (Wu et al., 2017).

### 2.5. RNA-seq samples collection

2nFC, 3nFC and 4nFC were separately propagated in 10-cm dishes at 26 °C. When the cells were 100% confluent, the cells were infected with SVCV at MOI of $3 \times 10^{-3}$ while the control cells were only exposed to the DMEM growth media. After 1 h incubation, the virus containing media was replaced with fresh DMEM (2% FBS) after washing twice with DMEM. Thereafter, the SVCV infected and non-infected cells were incubated at 26 °C for 36 h and harvested for RNA extraction respectively.

### 2.6. RNA extraction, sequencing, de novo assembly and annotation

Total RNA isolation was performed by following the instruction of the TRIzol reagent product manual (Invitrogen). Then RNA was treated with DNase I to remove any genomic DNA traces. The concentration and quality of RNA was examined through NanoDrop and agarose gel electrophoresis. Double-stranded cDNA was synthesized from mRNA. cDNA library preparation and sequencing reactions were conducted