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Development of an Ussuri catfish *Pseudobagrus ussuriensis* skin cell line displaying differential cytopathic effects to three aquatic animal viruses

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ABSTRACT

An Ussuri catfish *Pseudobagrus ussuriensis* skin (UCS) cell line was developed and subcultured for more than 60 passages. UCS cells consisted of mostly epithelial-like cells and multiplied well in TC199 medium supplemented with 10% fetal bovine serum at 25 °C. Chromosome analysis revealed that most UCS cells had a normal diploid karyotype with 2n = 52. UCS cells showed differential cytopathic effects (CPEs) after inoculation of spring viremia of carp virus (SVCV, a negative-strand RNA virus), grass carp reovirus (GCRV, a multi-segmented double-stranded RNA virus) and *Rana grylio* virus (RGV, a large double-stranded DNA virus), and were indicative of high sensitivities to these three aquatic animal viruses by a virus titration study. The CPE caused by SVCV appeared as rounded and granular cells, grape-like clusters and small lytic plaques. Characteristic CPE containing plaque-like syncytia was induced by GCRV. RGV-infected cells produced typical CPE characterized by cells shrinkage and aggregation, formation of clear plaques and cell sheet detachment. Furthermore, significant fluorescent signals were observed after UCS cells were transfected with green fluorescent protein reporter plasmids, and the development of CPE induced by a recombinant RGV, ΔTK-RGV, in UCS cells was illustrated using a combination of light and fluorescence microscopy. The data from this study suggested that UCS cell line can potentially serve as a useful tool for the comparison study of different aquatic animal viruses and the isolation of some newly emerging viruses in Ussuri catfish farming.

1. Introduction

Ussuri catfish *Pseudobagrus ussuriensis*, a kind of East Asian bagrid catfish, is the recent addition to aquaculture in China because of its great economic value and better productive performance. However, aquaculture is plagued with infectious viral disease problems resulting from its intensification and commercialization, and viral diseases badly affecting many highly valued fish species have been widely reported (Bondad-Reantaso et al., 2005; Qin et al., 2006; Walker and Winton, 2010; Gui and Zhu, 2012; Lei et al., 2012b; Zhang and Gui, 2012).

Members of the family *Rhabdoviridae* are negative-strand RNA viruses and may cause serious diseases in wild and farmed fish (Goodwin, 2002; Zhang et al., 2007; Tao et al., 2008; Ou et al., 2013). Spring viremia of carp virus (SVCV) is an OIE-listed rhabdovirus, causing high mortality in common carp (*Cyprinus carpio*) (Brémont, 2005; Chen et al., 2006). SVCV has also been isolated and identified from other cyprind species and Wels catfish (*Siluris glanis*) (Teng et al., 2007). Aquareoviruses contain eleven-segmented dsRNA genomes and have been isolated from a wide variety of aquatic animals posing a great threat to the aquaculture industry (Ke et al., 2011). As a common fish pathogen involved in hemorrhagic disease, Grass carp reovirus (GCRV) not only infects grass carp (*Ctenopharyngodon idellus*), but also is capable of infecting black carp (*Mylopharyngodon piceus*), topmouth gudeon (*Pseudorasbora parva*) and rare minnow (*Gobiocypris rarus*), causing significant economic losses in freshwater fish aquaculture in China and East Asia (Qiu et al., 2001; Zhang et al., 2003; Pei et al., 2014). Iridoviruses are nucleo-cytoplasmic large DNA viruses and have been recognized as serious pathogens of economically important cold-blooded vertebrates (Zhang et al., 2004; Lei et al., 2012c). *Rana grylio* virus (RGV), an iridovirus isolated in China in 1995, resulted in a high mortality rate in frogs (Zhang et al., 2001). Recent reports suggest that an outbreak of disease occurred amongst farmed Chinese

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giant salamanders (*Andrias davidianus*) is a result of iridovirus infection. Analysis of the complete genome sequence of the iridovirus revealed high similarity to RGV (Chen et al., 2013). Recombinant virus could be used to study viral gene functions and serve as delivery vehicles for biological control of some diseases (Chinchar et al., 2011; He et al., 2013, 2014). A recombinant RGV, ΔTK-RGV, was constructed (He et al., 2012). The virus can express enhanced green fluorescence protein after infection and replication in cells, and the infection can be directly observed under a fluorescence microscope.

Development of diverse and sensitive fish cell lines is of priority interest for isolating and identifying fish viruses (Imajoh et al., 2007; Rougée et al., 2007; Zhou et al., 2007; Dong et al., 2011). Till now, more than 280 fish cell lines have been established, most of which are derived from model fishes or traditional cultured species, such as zebrafish (*Brachydanio rerio*), rainbow trout (*Oncorhynchus mykiss*), grass carp and groupers (Lakra et al., 2011). However, cell lines originating from new species in aquaculture were rare. Since the new species are economically important, the aquaculture industry should benefit from the establishment of those species cell lines by allowing possible pathogens to be detected and studied. As a newly exploited species, there was no cell line from Ussuri catfish. In addition, cell lines derived from catfish were also rare. Fish skin, as the primary interface between the body and the water environment, may participate in a series of physiological reactions and provide an important barrier against microbial pathogens (Zhao et al., 2008; Tsutsui et al., 2009; Salinas et al., 2011). The cell line derived from skin of Ussuri catfish would be helpful for studying the pathology and physiology of this species.

Here, we will try to create an Ussuri catfish skin (UCS) cell line and then analyze its susceptibilities to different aquatic animal viruses (such as SVCV, GCRV and RGV) for developing a cell-pathogen model of Ussuri catfish. In addition, the cytopathic effects (CPEs) of these viruses will be compared in UCS cells, and the development of CPE induced by a recombinant RGV (ΔTK-RGV) will be illustrated using a combination of light and fluorescence microscopy.

2. Materials and methods

2.1. Primary cell culture and subculture

A healthy Ussuri catfish, weighing about 100 g, was obtained from Fisheries Scientific Research Institute in Huai'an, Jiangsu Province of China and used for primary cell culture. After the fish was killed and wiped with 75% (v/v) ethanol, the skin was separated from the muscle and immersed in TC199 medium containing high concentrations of antibiotics (10^3 U ml^{-1} of penicillin and 1000 μg ml^{-1} streptomycin) for 1.5 h. The skin tissues were washed three times with sterile phosphate-buffered saline (PBS), minced thoroughly with scissors, and then transferred to 25 cm^2 cell culture flasks containing 4 ml of TC199 growth medium containing 20% fetal bovine serum (FBS) and low concentrations of antibiotics (100 U ml^{-1} of penicillin and 100 μg ml^{-1} streptomycin). The primary cells were maintained at 25 °C. One half of the growth medium was changed every 4 days until the cells reached 80% confluence.

The confluent cells were sub-cultured at a ratio of 1:2. After a complete monolayer had formed, the old medium was removed and confluent cells were digested with 0.25% trypsin solution from the flask surface and transferred into two fresh flasks. The first 7 passages of the cell line was subcultured in TC199 medium with 20% FBS, and then the concentration of FBS was reduced to 10% later. UCS cells at passages of 7, 30, 45 and 60 were cryopreserved at −80 °C and recovered to evaluate the growth behavior of cells after an interval of 6 months according to methods previously described (Lei et al., 2012a). Briefly, there flasks of cultured cells were harvested and suspended in 5 ml TC199 medium containing 20% FBS and 10% dimethyl sulphoxide (DMSO), and then the cell suspensions were transferred to 2-ml cryo-vials and kept in a foamy box and stored at −80 °C directly. For recovery, the frozen cells were placed in the water with the temperature of 37 °C until the cells were dissolved and then transferred into a 25 cm^2 cell culture flask with 5 ml fresh TC199 medium quickly. After 12 h of culture at 25 °C, the medium was changed with fresh TC199 medium again.

2.2. Cell growth and chromosome analysis

The effects of incubation temperature and FBS on UCS cells growth were studied at the 45th passage level. The UCS cells were seeded into 24-well plates containing 10% FBS and 20 mmol l^{-1} HEPES (*N*-2-hydroxyethylpiperazine-*N*-ethane-sulphonicacid) at an initial density of 1.0×10^4 cells well^{-1} and incubated at 15, 20, 25 and 30 °C, respectively. Every day, triplicate wells of cells at each temperature were trypsinized and counted microscopically using a hemocytometer. The experiment lasted 4 days. In a similar way, the effect of FBS on cells growth was evaluated. 1.0×10^4 cells were seeded into a 24-cell plate containing 20 mmol l^{-1} HEPES and 10% FBS or FBS-free medium and cultured at 25 °C. Four days later, the cells were harvested and calculated as described above.

Chromosome preparations were obtained from UCS cells at passage 35 using a method previously described with some modifications (Lei et al., 2012a). Briefly, the cells were seeded into 25-cm^2 culture flasks and incubated at 25 °C for 10 h. Colchicine was added to the cells to the final concentration of 2 μg ml^{-1} and incubated for 10 h in culture flasks. The cells were trypsinized and harvested by centrifugation (500 g, 10 min). The single cells were suspended in 3 ml hypotonic solution of 0.075 M KCl for 30 min and then premixed with one-tenth volume of cold Carnoy's fixative (methanol:acetic acid = 3:1) for 5 min at room temperature. After centrifugation, the pellet was gently suspended in 5 ml fresh Carnoy's fixative and fixed at 4 °C overnight. The next day, the cells were precipitated and suspended in 0.5 ml fixative. Then, the suspension was dropped on cold glass slides, air dried and stained with 5% Giemsa solution. Hundred photographed cells at metaphase were counted, and chromosome karyotype was analyzed.

2.3. Virus challenge assay

Three aquatic animal viruses (SVCV, GCRV and RGV) from different families were used to test viral susceptibility of the UCS cell line. SVCV, GCRV and RGV were maintained in our laboratory (Chen et al., 2006; Zhang et al., 2001, 2003). Cell cultures and virus propagation were performed as described previously (Lei et al., 2012a). UCS cells cultured in a 6-well plate with eighty percent confluent were infected with each virus at a multiplicity of infection (MOI) of approximately 0.05. CPE caused by these viruses were observed daily under an inverted light microscope. After complete CPE, the supernatant was collected respectively and used for re-infection and virus titration study. For virus titration, the UCS cells were seeded into a 96-well plate and incubated at 25 °C until monolayer was achieved, and the following virus challenge tests were performed as described by Zhang et al. (1999). Briefly, tenfold serially dilutions of virus were inoculated into cells and the CPE were observed every day. The cells were stained with crystal violet solution on day 7 after infection and the titers were calculated by the 50% tissue culture infective dose ($TCID_{50}$) method. In addition, growth curves of SVCV, GCRV and RGV in

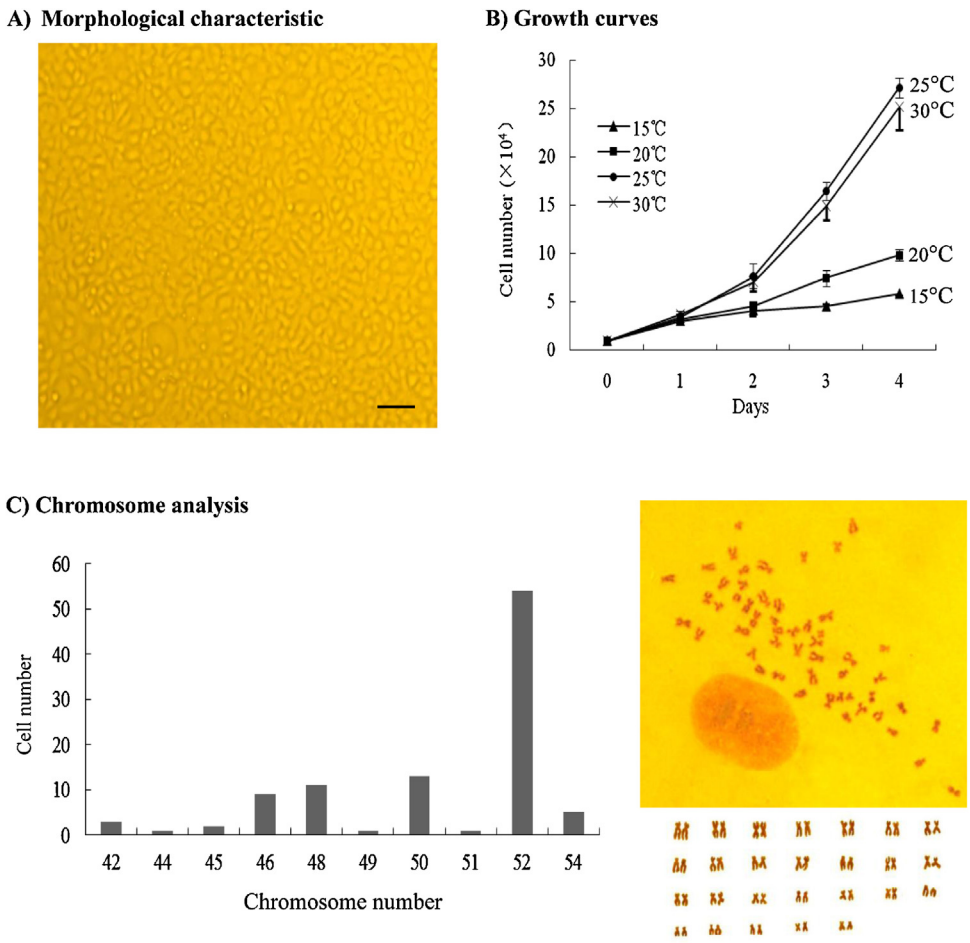

Fig. 1. Characteristics of the UCS cell line. (A) The monolayer of the UCS cells consisted predominantly of epithelial-like cells. Scale bar = 100 μm. (B) Growth curves of UCS cells at different temperatures. (C) Distribution of chromosomes in the UCS cells (left panel) and the metaphase and diploid karyotype (right panels) of UCS cells at passage 35.

UCS cells were performed. UCS cells were grown in 24-well plate and infected with SVCV, GCRV or RGV at MOI of 0.05, after 1 h absorption, the residual virus was removed and then cells were incubated with fresh TC199 medium. Cell cultures were harvested at 12-h intervals from 0 to 72 h after virus inoculation and subjected to three freeze-thaw cycles, and then viral titers of the freeze-thawed samples were determined as mentioned above. The experiments were repeated three times and the averages were presented.

2.4. Cell transfection with pEGFP express vector and infection with recombinant virus

UCS cells were seeded on glass coverslips in a 6-well plate at a density of 2×10^5 cells well^{-1}. Eighty percent confluent monolayers were transfected with 1 μg pEGFP express vector using lipofectamine™ 2000 (Invitrogen) according to the manufacturer's protocol. After 36 h incubation, the cells were fixed and stained with Hoechst 33342 as described previously (Ke et al., 2011). The green fluorescence signals and Hoechst-stained nuclei were observed under a Leica fluorescence microscope, and the percentage of GFP positive cells (transfection efficiency) was confirmed by quantifying 3 microscopic fields.

A recombinant RGV (ΔTK-RGV) containing EGFP gene was constructed in our laboratory (He et al., 2012). UCS cells infection with ΔTK-RGV was performed as described above. The development of CPE induced by ΔTK-RGV was observed using a combination of light and fluorescence microscopy.

3. Results

3.1. Characterization of the UCS cell line

After 24–36 h of explants preparation for the skin tissue, the UCS cells began to migrate from the skin fragments, and primary monolayer was obtained by several stimulations with trypsin. Morphologically, UCS cells consisted of both epithelial-like cells and fibroblastic-like cells at the early passages, and mostly of epithelial-like cells after 7 passages (Fig. 1A). The cell line has successfully been subcultured more than 60 passages. Cells cryopreserved at −80 °C recovered well and grew to confluence within 4 days. Average survival rate was estimated up to 70% of the initial cell population during freezing and thawing.

UCS cells were able to grow at incubation temperatures between 15 and 30 °C with different growth rates (Fig. 1B). The highest growth rate was obtained at 25 °C. No significant growth was observed at 15 °C. However, after monolayer was achieved at 25 °C, the cells could be maintained in sufficiently good condition at 15 °C for as long as 2 months. Growth of UCS cells was highly dependent on the FBS concentration in the culture medium. During 4 days, cells could not grow well in FBS-free medium, but had a very good proliferation in TC199 medium supplement with 10% FBS (data not shown).

Chromosome spreads made from UCS cells revealed the diploid number ranged from 42 to 54, and the modal number was 52, which occupy 54% in the 100 metaphase cells counted at passage 35 (Fig. 1C). The metaphase with a normal diploid number

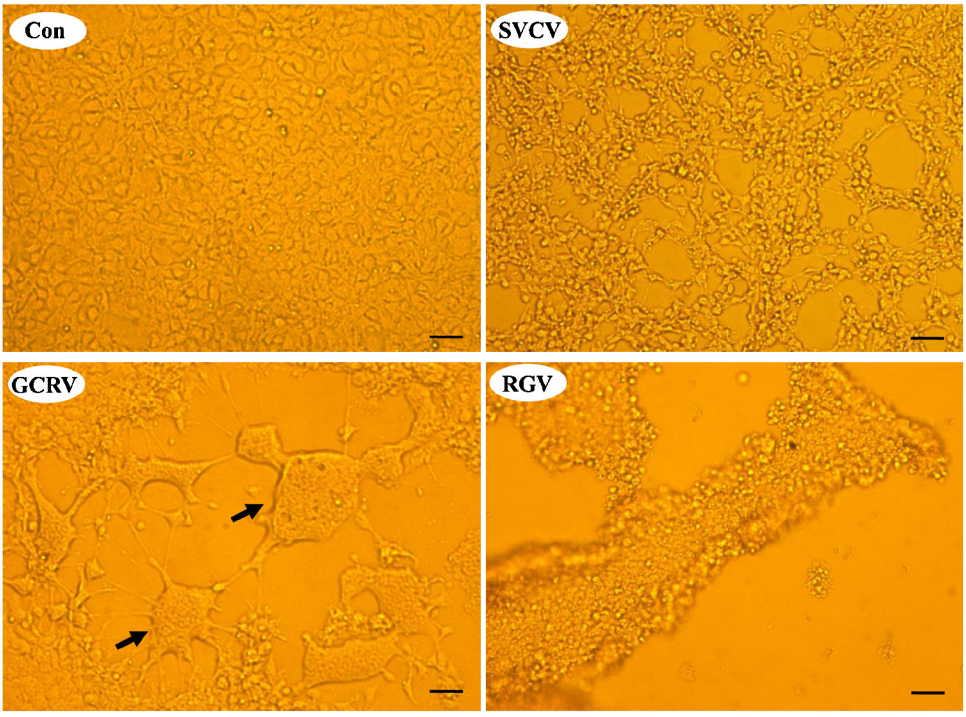

Fig. 2. Differential CPEs of UCS cells infected with three aquatic animal viruses and confluent uninfected UCS cells (Con). The CPE caused by SVCV appeared as rounded and granular cells, grape-like clusters and small lytic plaques at 2 days post-infection (dpi, SVCV). Characteristic CPE containing plaque-like syncytia (arrows) was induced by GCRV at 3 dpi (GCRV). RGV-infected cells detached from the bottom of the flask at 3 dpi (RGV). Scale bar = 100 μm.

displayed the normal karyotype, consisting of twelve pairs of metacentrics (m), nine pairs of submetacentrics (sm) and five pairs of subtelocentrics (st): 2n = 24m + 18sm + 10st, which was identical with the standard karyotype of Ussuri catfish (Xue and Yin, 2008).

3.2. Differential CPEs and viral titers

SVCV, GCRV and RGV were used to test viral susceptibility of the UCS cell line. Differential CPEs were observed in UCS cells within 3 days post-infection (dpi) with these viruses, and no CPE was observed in the mock-infected cells (Fig. 2). Compared with normal cells, the CPE caused by SVCV appeared as rounded and granular cells, grape-like clusters and small lytic plaques at 2 dpi. Following infection with GCRV, characteristic CPE containing plaque-like syncytia was observed within 3 dpi. RGV-infected cells produced typical CPE characterized by cells shrinkage and aggregation, formation of clear and large plaques and cell sheet detachment within 3 dpi. There were still many adherent cells in the RGV-infected cell monolayer after 3 days. However, the SVCV-infected monolayer completely disintegrated at this time point. In addition, we also measured the viral titers in the course of infection. Fig. 3 shows the growth curves of SVCV, GCRV and RGV, indicating that all viruses grew productively in UCS cells. The viral titers of SVCV and RGV rose quickly before 24 h post-infection (hpi) and then remained basically stable, yielding the highest titers of $10^{8.1}$ and $10^{7.3}$ $TCID_{50}$ ml^{-1} at 48 and 60 hpi, respectively. GCRV titer continued to increase throughout the course of infection, and reached a high titer of $10^{8.2}$ $TCID_{50}$ ml^{-1} at 72 hpi.

3.3. DNA transfection and recombinant virus infection

When UCS cells were transfected with pEGFP express vector, clear, multiple and strong green fluorescent signals could be observed in both the cytoplasm and nucleolus after 36 h post transfection (Fig. 4A), and the efficiency of transfection was estimated to be 10%.

ΔTK-RGV has the similar infection characteristics to RGV, and the infected cells can emit green fluorescence (Fig. 4B). Initially, the infected UCS cells exhibited localized of morphological changes such as cells shrinkage and aggregation after 36 h, and weak green fluorescence signals were detected in these pathological cells. As CPE progressed, the aggregation cells were disintegrated and clear and large plaques with green fluorescence were visible at 48 h post-infection. The CPE progressed rapidly and caused the cell sheet detached from the bottom of the flask, and some disintegrated, but some adherent cells with strong green fluorescence in the cell sheet edges still could be observed after 72 h.

4. Discussion

In this study, an Ussuri catfish skin cell line was established by explant method. The UCS cell line has been subcultured for more than 60 passages since March 2011. Some fish cell lines have been reported that mixed population of cells was present in primary monolayer during early cell cultures and then the cells would exhibit a single morphology after subsequent subcultures (Tong et al., 1998; Ishaq Ahmed et al., 2008). Similarly, UCS cells obtained epithelial morphology, which may due to the predominance of epithelial cells over fibroblast cells in cell cultures. The growth temperature range for UCS cells was 15–30 °C with the optimum growth at 25 °C. The cells grew better in medium with FBS than in free-FBS medium demonstrating that FBS was essential for the proliferation of UCS cells. In addition, UCS cells could be maintained in sufficiently good condition at 15 °C for a long time and be able to recover quickly from cryopreservation. A cell line with those cultural characteristics could be advantageously applied in some experiments when the requirements of cell conditions are

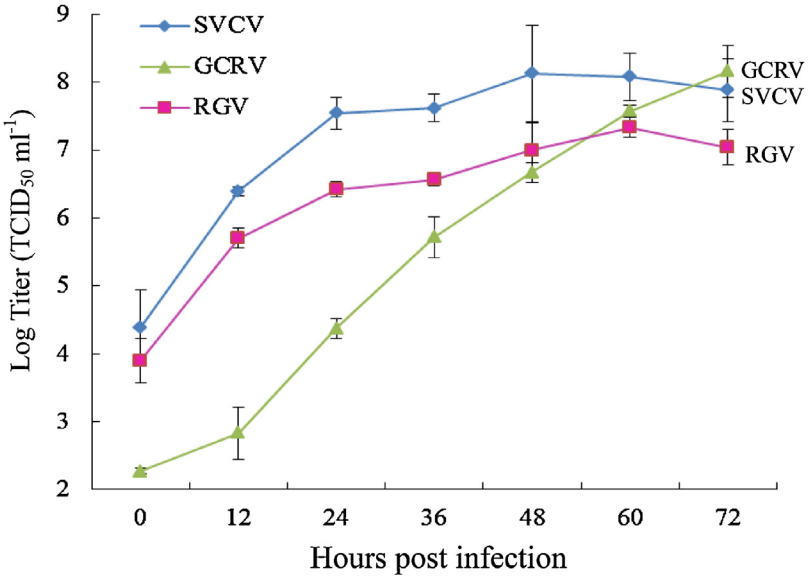

Fig. 3. Growth curves of SVCV, GCRV and RGV in UCS cells. Cells were infected with SVCV, GCRV or RGV at MOI of 0.05 and then harvested at the indicated time points. The replications of viruses were determined by measuring their $TCID_{50}$. The values shown represent averages and standard deviations of three independent experiments.

strict and used for virus isolation from both warm and cold water fish. Karyotype analysis revealed that 54% of the cells possessed a diploid chromosome number of 2n = 52, which was identical with the modal number of Ussuri catfish reported previously (Xue and Yin, 2008). Fish cell lines derived from same or different species always consist of diverse number of chromosome, ranging from 32 to 264, but a diploid chromosome number of 52 is rarely reported in those cell lines (Lakra et al., 2011). To our knowledge, UCS cell line is the first cell line originating from Ussuri catfish which can secrete large amounts of mucus from its skin when stimulated by external condition such as mechanical, drought and cold stress. This skin cell line would be suitable as a good *in vitro* model for conservation genetics and studying the mucus-related gene expressions in Ussuri catfish skin.

Sensitive fish cell lines are the basis for isolation, identification and characterization of fish viruses. Application of the UCS cell line for virus detection was evaluated by its ability to support the infection of SVCV, GVRV and RGV, aquatic animal viruses from three different families. The results revealed that UCS cells were very susceptible to those viruses and displayed differential CPEs. The virus susceptibilities of UCS cells to SVCV and RGV were similar to that of grass carp ovary (GCO) cells, and CPE induced by RGV was like that previously reported for this virus in GCO cell cultures and Singapore grouper iridovirus in grouper spleen cell cultures (Lei et al., 2012a; Qin et al., 2006). SVCV produced the special CPE such as rounded cells, grape-like clusters and small lytic plaques in UCS cells, similar to those of infectious hematopoietic necrosis virus and hirame rhabdovirus, other two fish rhabdoviruses, in flounder spleen cells and *Scophthalmus maximus* rhabdovirus (SMRV) in carp leukocyte cells (Kang et al., 2003; Du et al., 2004). Du et al. (2004) also reported that the major mechanism of cell death occurring during SMRV infection was associated with the induction of apoptosis. Similar CPEs imply that common pathogenic mechanisms and virus–host cell interactions may have evolved in these rhabdoviruses, and apoptosis may be involved in the special CPEs caused by these viruses. GCRV-infected UCS cells showed typical CPE in the form of plaque-like syncytia, which was like the CPE induced by *Scophthalmus maximus* reovirus, another aquareovirus, in Grass carp fins cells (Ke et al., 2011). Up to now, many fusion associated small transmembrane proteins that could induce cell–cell fusion were identified in aquareoviruses, which may be responsible for the typical CPE induced by those viruses (DeWitte-Orr and Bols, 2007; Ke et al., 2011). After inoculation with equivalent amounts of SVCV, RGV and GCRV and removing the unabsorbed viruses, the titer of virus in GCRV-infected cell cultures at 0 hpi was significantly lower than that in SVCV-infected or RGV-infected cell cultures, implying the different abilities of these viruses to absorb UCS cells and the diverse mechanisms of viral adhesion and entry. These results indicated that UCS cell line could be helpful for isolating some newly emerging viruses in Ussuri catfish farming, and that it is possible to preliminarily identify the family of pathogenic virus based on the type of CPE. Furthermore, the differential CPEs of UCS cells to these three viruses suggested that the UCS cell line could potentially serve as a useful tool to compare and elucidate the characteristics and infection mechanisms of aquatic animal viruses from different families.

In the transfection experiments, UCS cells expressed the reporter gene efficiently and produced strong signals, indicating that the UCS cell line could serve as an *in vitro* system for transgenic and genetic manipulation studies such as gene targeting and various constructs. The recombinant RGV (ΔTK-RGV) and RGV have similar infection characteristics, and the infection of ΔTK-RGV could be confirmed through direct observation under a fluorescence microscope. Combination of light and fluorescence microscopy, the development of CPE induced by ΔTK-RGV was clearly illustrated. This research suggested that the UCS cell line was suitable for expression of the exogenous gene in recombinant RGV, and could also help to study the RGV gene function and understand the interaction between RGV and host cells.

To date Ussuri catfish skin cell line has been biologically and phenotypically characterized. Virus susceptibility analysis showed that the cell line was highly susceptible to three aquatic animal viruses from different families, and displayed significant differential CPEs, which suggested that the cell line could be used to study different virus–cell interactions and isolate the possible viral pathogens in Ussuri catfish farming. Transfection using pEGFP vector DNA and infection with recombinant virus indicated that the cell line would also facilitate the identification and analysis of unknown genes of aquatic animal viruses.

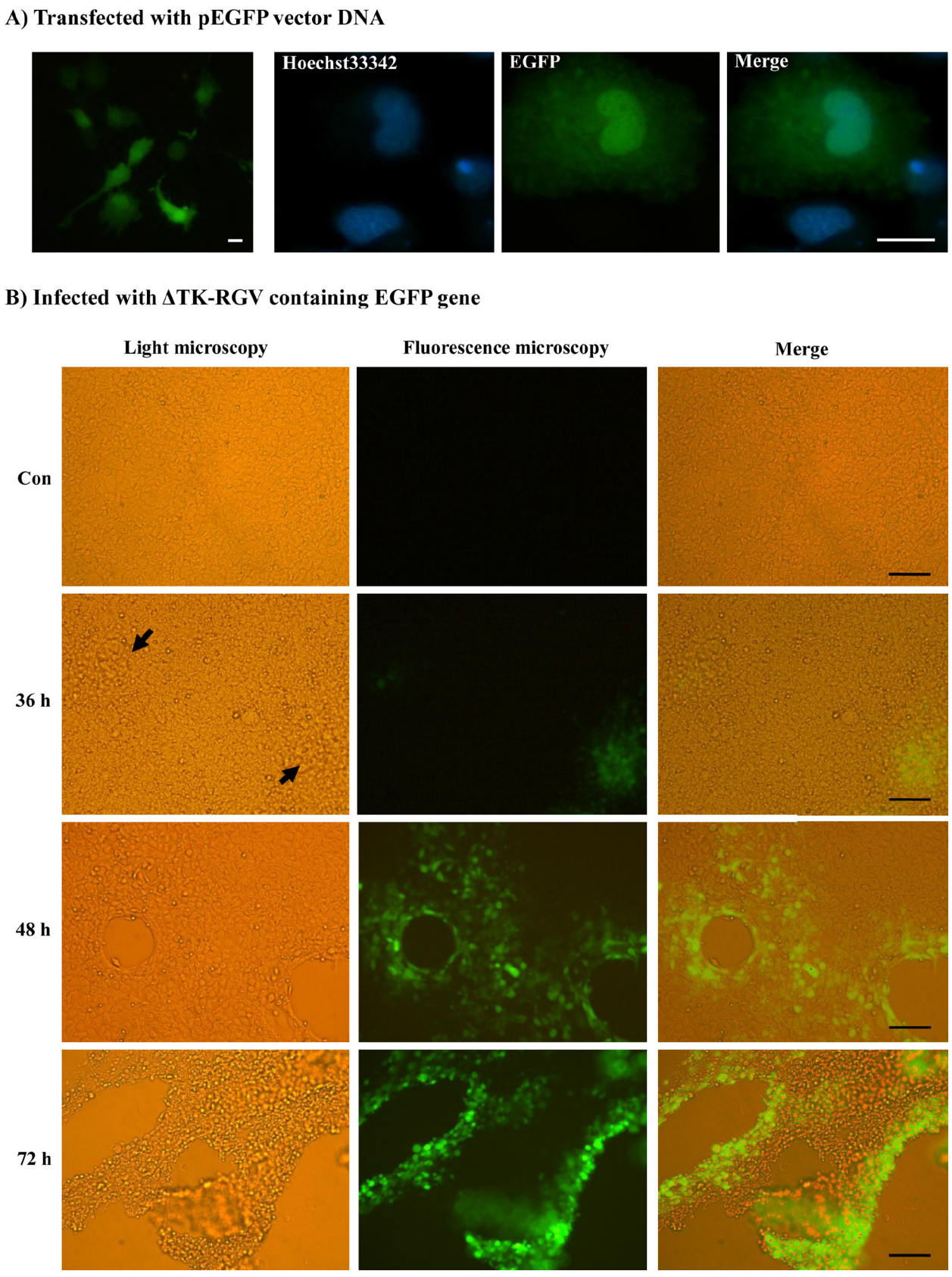

Fig. 4. UCS cells transfection with pEGFP vector DNA and infected with recombinant virus ΔTK-RGV. (A) Clear, multiple and strong green fluorescent signals could be observed in both the cytoplasm and nucleolus of UCS cells after transfection with pEGFP vector DNA. Scale bar = 25 μm. (B) The development of CPE induced by ΔTK-RGV in UCS cells. Initially, the infected cells exhibited localized of morphological changes such as cells shrinkage and aggregation (arrows) after 36 h, and weak green fluorescence signals were detected in these pathological cells. As CPE development progressed, clear and large plaques with green fluorescence were visible at 48 h post-infection. The cell sheet eventually detached from the bottom of the flask, and some disintegrated, but some adherent cells with strong green fluorescence in the cell sheet edges still could be observed after 72 h. The cells with green fluorescence further confirmed the infection of ΔTK-RGV. Scale bar = 200 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Acknowledgments

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