Establishment of a New Fish Cell Line from the Brain of 1 Humpback Grouper (Chromileptes Altivelis), and its Application in Toxicology and Bacterial Susceptibility

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Establishment of a new fish cell line from the brain of humpback grouper (*Chromileptes altivelis*), and its application in toxicology and bacterial susceptibility

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Abstract

Cromileptes altivelis, humpback grouper, belongs to the family Epinephelidae and is one popular farmed fish species because of its high economic value and ornamental value. However, more and more diseases were outbreak with the increasing of *C. altivelis* breeding, resulting in severe economic losses. Today, a new brain cell line of *C. altivelis* (CAB) was established and has been subcultured for more than 40 passages until now. Our results showed that the morphology of CAB cells is epithelial-like and suitable for growth at 26 °C in L15 medium supplemented with 15% fetal bovine serum (FBS). The results of 18S rRNA gene sequencing confirmed that CAB cell line was derived from *C. altivelis*. Moreover, CAB cells were apparently diploid number of chromosomes (2n = 48) by chromosome analysis. In addition, CAB cells could transfect pEGFP-N3 plasmid with high transfection efficiency, indicating that CAB cell line has the potential to investigate the function of exogenous genes *in vitro*. Furthermore, the bacterial susceptibility results suggested that CAB cells were susceptive to *Vibrio harveyi* and *Edwardsiella tarda*. And, heavy metals (Hg, Cd, and Cu) were toxic to the CAB cells and the toxic effect was dose-dependent. In summary, the CAB cell line could be a powerful tool *in vitro* to study functional genes and has the potential application in bacterial susceptibility and toxicology.

Keywords: *Chromileptes altivelis* · Brain cell line · Transfection efficiency · Toxic effect · Bacterial susceptibility

Introduction

Cromileptes altivelis (belongs to Serranidae, Epinephelinae, *Cromileptes*) is mainly distributed in subtropical waters in the South Sea of China (Zhang et al., 2020). As the breeding density of *C. altivelis* increases, diseases are occurring with increasing frequency (Sun et al., 2019). While at the same time, slow growth and low survival rate in the wild greatly limited the amount of *C. altivelis*, which leads to the listing of *C. altivelis* as an endangered species (Afero et al., 2010; Suprayudi et al., 2016; Wei et al., 2018). Fish cell lines is an important material and model for germplasm preservation, gene function analysis and cell engineering breeding, immunology, endocrinology, and functional genomics (Laing et al., 2011; Skrzypski et al., 2016; Zhou et al., 2019). Consequently, establishment of cell lines derived from the endangered *C. altivelis* is of great significance.

Previous studies have shown that diseases caused by bacteria have brought serious damages to aquaculture (Li et al., 2016; Gong et al., 2018). Excitedly, fish cell lines are the convenient tool in monitoring the adverse effect of bacteria (Rachlin et al., 1968). For example, brain cell line of *Trachinotus ovatus* (TOGB) was highly sensitive to virus (grouper nervous necrosis virus (GNNV) and singapore grouper iridovirus (SGIV)) and bacteria (*Vibrio anguillarum* and *V. alginolyticus*) (Li et al., 2016). As far as we know, *V. harveyi* and *Edwardsiella tarda* are two kinds of usual pathogenic bacteria which widely infected many marine and freshwater fish species (Takano et al., 2010; Liu et al., 2016). With the aforementioned issue, there is an urgent need to construct cell lines from *C. altivelis* as an effective tool for monitoring the adverse effect of *V. harveyi* and *E. tarda*.

Besides bacteria, heavy metals are common environmental pollutants, which present extremely cytotoxic to living body and bring a health threat to people (Sapkota et al., 2008; Gong et al., 2018; Renieri et al., 2019). Fish cell cultures also can be applied to detect the environmental pollutant

chemicals as a sensitive biological monitoring system (Segner et al., 2004; Tan et al., 2008; Zhou et al., 2017). Previous study suggested that ovary cell line of *Ictalurus punctatus* and kidney cell line of *Ctenopharyngodon idella* were sensitive to cadmium (Cd) and copper (Cu), respectively, and could indicate the acute cytotoxicity of heavy metals in the aquatic environment (Tan et al., 2008). However, different fish cell lines demonstrated varying degrees of sensitivity to different heavy metals. Thus, establishment of various fish cell lines is essential for detecting the sensitive to heavy metals.

To address this, a new brain cell line from *C. altivelis* (CAB) was established and characterized. Moreover, the application possibility in foreign gene expression of CAB cells was assessed. Besides, the sensitivities of CAB cells to *V. harveyi*, *E. tarda*, and three heavy metals (Cu, Hg, and Cd) have been evaluated. Our study will enrich the resources of fish cell lines, and provides important experimental materials for future research on the prevention and control technology of fish diseases.

Materials and methods

Isolation and cultured of primary brain cells

C. altivelis (approximately 10 cm in length) were obtained from a seafood market in Haikou (Hainan, China). Prior to primary cell culture, fish were soaked in 1% iodophor for 15 minutes. Then the skin of the fish was wiped with 75% ethanol to be sterilized. Brain was taken out aseptically and washed three times with Leibovitz's L-15 medium (Gibco) containing 4% antibiotics (400 μg/ml streptomycin, 400 IU/ml penicillin). After that, it had been digested for 30 min with 1 ml 0.25% trypsin solution (Gibco). Next, the digested mixture was percolated using a 100 mesh screen, and the filtrate was centrifuged at 180 g for 10 min to collect the precipitate. The precipitate was then cultured in 3 ml L-15 medium that containing 20% fetal bovine serum (FBS, Gibco), 20 ng/ml basic fibroblast growth

95 factor (bFGF, Peprotech), 40 ng/ml insulin-like growth factor-I (IGF-I, Peprotech), and 4 % antibiotics
96 at 26 °C in 25 cm^2 cell culture flasks. In daily administration, medium was refreshed every three days
97 until the cells reached at a high confluence (>85%).
98
99 **Subculture, cryopreservation and recovery of cell**
100 The primary cells that formed an intact monolayer were detached from the flasks surface with
101 0.25% trypsin solution for subculture with the rate of 1:2. After more than 20 times of subculture, the
102 content of FBS in L-15 medium was steadily changed from 20% to 15% FBS, and no longer required
103 the bFGF and IGF-I.
104 For long-term storage, CAB cells after initial 5 passages were digested with 0.25% trypsin-EDTA
105 solution. Then the compound was centrifuged at 1000 g for 5 min and resuspended in L-15 medium for
106 eliminating EDTA. After centrifugation 2 times, cells were resuspended in cell freezing medium that
107 contains 40% FBS and 10% dimethyl sulfoxide in L-15 medium. The cells in cryogenic vials were
108 stored at -80 °C overnight and removed into the liquid nitrogen next day. Resuscitation test was
109 performed as follows: CAB cells that stored in liquid nitrogen were taken out quickly and put it into a
110 37 °C water bath immediately for 80 s. Afterwards, cells were added into 25 cm^2 flasks with 4ml L-15
111 medium to culture. Finally, trypan blue test was conducted to analyze the cell viability as described by
112 our previous study (Wang et al., 2020).
113
114 **18S rRNA gene analysis**
115 The genomic DNA of CAB cells at passage 10 was isolated by Tissue DNA kit (Omega Bio-tek,
116 USA). The 18S rRNA was amplified with the following primers: Forward,

5’-CCAGTAGTCATATGCTTGTCT-3’; Reverse, 5’-ACCTTGTTACGACTTTTACTTCC-3’. Afterwards, the obtained PCR product was determined by agarose gel and sequenced.

Optimal growth conditions

The effect of different mediums (L15, DMEM, MEM, M199), culture temperatures (22 °C, 24°C, 26 °C and 28 °C) and concentrations of FBS (5%, 10%, 15%, and 20%) on CAB cells growth were evaluated. The CAB cells at passage 7 were cultured in 12-well plates (2.0×10^4 cells/well). After culturing 1, 3, 5, and 7 days, cells were counted by hemacytometer. Experiments were conducted for three times.

Chromosome analysis

Cells at passage 7 were used for chromosome analysis. Briefly, cells were cultured until a complete monolayer appeared. Then, cells were treated with 0.6 μg/ml of colchicine (Sigma-Aldrich) for 6 h. After that, cells were digested using trypsin and collected by centrifugation at 800g for 10 min. 8 ml of KCl solution (0.075 M) were supplemented to resuspend cells at 26 °C for 30 min. Afterwards, the suspended mixture were incubated with 2 ml of precooled Carnoy’s fixative (methanol : acetic acid = 3 : 1) at room temperature for 5 min. Then, after centrifugation for 10 min at 600 g, cells were collected and fixed twice using 3 ml of Carnoy’s fixative for 30 min. The fixed cells were stained with Giemsa (Solarbio, China) and then counted and photographed the cells chromosome at metaphase by microscope.

Cell cycle analysis

As previously mentioned, the cell cycle of CAB cells at passage 20 was analyzed using flow cytometry (Wang et al., 2020). Firstly, CAB cells were seeded and digested using trypsin after cultured for 24 h and 48 h, respectively. Then, cells were collected and washed with pre-cooled PBS. Following that, 70% pre-cooled ethanol was added into the collected cells and stored at 4 °C overnight. Then, cells were centrifuged at 1000g for 5 min at 4 °C and the sediments were resuspended with pre-cooled PBS. After centrifugation again, sediments were incubated in PI staining fluid (Beyotime, China) for 30 min at 37 °C. The fluorescence signal was detected by a Guava easyCyte (Luminex Corporation, USA). The data were analyzed by Modfit LT (Verity Software House, USA).

Cell transfection

To evaluate the transfection efficiency and detect whether foreign genes can express successfully in CAB cell lines, 2 μg of the pEGFP-N3 plasmid expressed green fluorescent protein (GFP) was transfected into CAB cells at passage 12 using FuGene® 6 Transfection Reagent (Promega, USA) according to the specification. 72 h after transfection, the cells expressed GFP were counted through an inverted fluorescence microscope (Leica, Germany) and the transfection efficiency was expressed as the following formula: Transfection efficiency (%) = (numbers of GFP-positive cells independent light fields / numbers of total cells in 20 independent light fields) × 100.

Bacterial susceptibility assays

To evaluate the susceptibility of *V. harveyi* and *E. tarda* on CAB cells, the CAB cells were cultured in a 6-well plate at 26 °C until a complete monolayer appeared. *V. harveyi* and *E. tarda* with the concentration of 1×10^5 CFU/well were supplemented into the 6-well plate and co-incubated with

cells at 26 °C, respectively. As a control, same volume of PBS was added into the well. After incubation for 1 h and 3 h, the susceptibility of *V. harveyi* on CAB cells was observed using inverted fluorescence microscope (Leica, Germany). Meanwhile, the susceptibility of *E. tarda* on CAB cells was evaluated after infection for 6 h and 9 h.

Cytotoxic effect of heavy metals

To assess the toxic effects of heavy metals on CAB cells, CAB cells were cultured until a complete monolayer appeared in a 96-well plate. Three kinds of heavy metals (copper chloride ($CuCl_2$), mercury chloride ($HgCl_2$), and cadmium chloride ($CdCl_2$)) were diluted by medium to 0.0001, 0.001, 0.01, 0.1, 1, and 10 mM, respectively. Then, heavy metals above were supplemented into the 96-well plate and co-incubated with cells at 26 °C, respectively. As a control, same volume of medium was supplemented into the cells. The viability of cells was evaluated by CCK-8 kit (Bosharp, USA).

Statistical analysis

Data in this study were calculated by SPSS 17.0 software (IBMSPSS. Inc., USA) and presented in the way of means ± SD. The significant part was performed through One-way ANOVA analysis. The statistically significant was marked when P value <0.05.

Results

Primary cell culture and subculture

As shown in Fig. 1, the formed confluent monolayer of the primary cells was occurred at 16 days of incubation. After that, cells were subcultured at a proportion of 1:2 or 1:3 every 4 days. Up to now, the CAB cells have been subcultured more than 40 passages. When cryopreserved cells were revived

for 24 h, a part of cells adhered successfully (Fig. 2a), and the cell morphology was the same as before cryopreservation (Fig. 2b).

Molecular characterization of CAB cells line

PCR identification of CAB cell 18S rRNA gene showed that there was a single band at 1800 bp approximately, which is the expected length (Fig. 3a). The sequencing results were aligned on the NCBI website and results showed that the sequence was 100% homologous to the *C. altivelis* 18S rRNA gene sequence (NCBI Accession No. MZ298593), suggesting that CAB cell line was indeed originated from *C. altivelis*.

Optimum culture condition

The effect of various mediums, culture temperatures and concentrations of FBS on CAB cells were evaluated. The results showed that CAB cells grow the fastest in L15, while in the MEM or DMEM medium, CAB cells couldn't proliferate normally (Fig. 4a). When CAB cells were cultured in L15 medium containing different FBS concentrations, we found that the growth rates of CAB cells in 15% and 20% FBS were significantly increased than that in 10% and 5% FBS, while there was no significant difference between 15% and 20% FBS (Fig. 4b). Considering the costs, 15% was the optimum concentration of FBS for CAB cells cultured. The study of the optimum temperature for CAB cells cultured exhibited that CAB cells could grow at 22 °C, 24 °C, 26 °C, and 28 °C and its optimum temperature was 26 °C (Fig. 4c).

Cell chromosome analysis

100 metaphase plates of CAB cells were randomly select to observe and analyze the chromosome. The results showed that the chromosomes number ranged from 26 to 64 and more than 50% of the cells have 48 chromosomes (2N =48) (Fig. 5).

Cell cycle analysis

The DNA contents of CAB cell lines analyzed by flow cytometric after cultured for 24 h and 48 h were shown in Fig. 6. The results showed that both of two visible peaks (the G0-G1 period and G2-M period) were occurred in cells cultured for 24 h and 48 h, respectively. Moreover, cells at G0-G1 phase (82.90%) and G2-M phase (4.82%) after cultured for 48 h were more than that cultured for 24 h (69.13% and 3.34%), respectively (Fig. 6c). By contrast, the percent of cells at S period after cultured for 24 h (27.54%) were higher than that cultured for 48 h (12.28%) (Fig. 6c).

Transfection efficiency

As shown in Fig. 7, GFP were successfully expressed in CAB cells after transfected with pEGFP-N3 plasmid for 72 h. According to the formula of transfection efficiency, the cell transfection efficiency was approximately 25% (Fig. 7).

Bacteria sensitivity assays for CAB cells

After treated with *V. harveyi* for 1 h, the CAB cells showed shrinking obviously. More apoptotic rounding cells were observed after 3 h of infection with *V. harveyi* (Fig. 8 b and d). In addition, when incubated with *E. tarda* for 6 h, CAB cells became round and most cells were apoptosis with typical characteristics after treated for 9 h (Fig. 8 f and h). During the experiment, the cell morphology of the control group was normal.

Heavy metal cytotoxicity assays for CAB cells

A dose-dependent manner was found in the heavy metal cytotoxicity assays for CAB cells. That is, the higher the concentration of all the three kinds of heavy metal (Cd, Cu, and Hg), the lower the viability of CAB cells (Fig. 9). The results indicated that Cd, Cu, and Hg were toxic to CAB cells. When the concentration of Hg and Cu was decreased to 0.001 mM, the viability of CAB cells assumed a remarkable reduction. Meanwhile, the concentration of Hg was increased to 10 mM, the livability of CAB cells was reduced to approximately 27%. By contract, the cytotoxicity of Cd for CAB cells was detectable at the concentration of 0.01mM. As a result, the calculated IC50 of Hg and Cd was 0.393 mM and 0.445 mM, respectively, while the IC50 of Cu was 1.073 mM.

Discussion

Humpback grouper (*Cromileptes altivelis*) is an important economic species. Nevertheless, its market development was bothered by the slow growth and frequent diseases (Sun et al., 2019). The development of fish cell lines provides new experimental materials and ideas for the research of diseases (Gravell et al., 1965; Li et al., 2017; Wei et al., 2018). Today, this study established a *C. altivelis* brain cell line and evaluated its applications in toxicity studies of the pathogenic infection of bacteria and the impact of environmental heavy metals on fish.

The cell lines, named CAB, were verified to be derived from *C. altivelis*. Its morphologies included fibroblast type and epithelial type in primary cell, and became fibroblast type steadily as the cell passage increased. This phenomenon could also be observed in the brain cell lines from *Epinephelus coioides*, *Epinephelus akaara*, *T. ovatus* and *Sparus aurata* (Wen et al., 2008; Li et al., 2016; María et al., 2020). The suitable growing conditions of CAB cell lines were in L-15 medium

252 containing 15% FBS at 26 °C. Correspondingly, Zhang et al (2014) reported that *C. altivelis* could
253 grow well when the water temperature was between 24 and 32 °C (Wang et al., 2013; Zhang et al.,
254 2014). Ou and Xie (2007) reported that the modal number of diploid chromosome in *C. altivelis*
255 was 48. Correspondingly, the modal chromosome number of CAB cell line is 48. Continuous
256 culturable cell lines are quite imperative in cytogenetic analysis (Wang et al., 2010; Yu et al., 2016).
257 In our study, the analysis of cell cycle by flow cytometric showed that the CAB cells cultured for 48 h
258 possessed a lower percentage of cells in the S phase compared to 24 h, indicating that the CAB cells
259 comprised a diploid cell population that had a firm ability for continuous culture. Similar results were
260 found in the heart cell lines from *Epinephelus lanceolatus* (ELGH) and the snout cell lines from *T.*
261 *ovatus* (GPS) (Guo et al., 2015; Yu et al., 2016). Up to now, CAB cells had been subcultured for more
262 than 40 passages. Given all this, the brain cell line from *C. altivelis* that could be passaged stably had
263 been established successfully by us.

264 High transfection efficiency is an essential index when evaluated whether cell lines can be applied
265 in foreign gene expression (Qin et al., 2006; Zheng et al., 2012; Zhang et al., 2014; Yu et al., 2016). In
266 this study, the transfection efficiency of CAB cells transfected with the pEGFP-N3 plasmid was nearly
267 25%, which was similar with the kidney cell line from *Cynoglossus semilaevis* (Lou et al., 2020), and
268 higher than the *Lateolabrax japonicus* pluripotent embryonic cell line (Chen et al., 2003),
269 demonstrating that the CAB cell line can be used in the study of foreign gene expression.

270 Previous studies indicated that fish cell lines have the clear advantages to predict the acute fish
271 toxicity (Fent., 2001; Huang et al., 2011). More than that, they are effective tools to explore the
272 cytotoxicity of pathogenic bacteria (Qin et al., 2006; Huang et al., 2011; Yu et al., 2016; Wei et al.,
273 2018). For example, *Streptococcus iniae* and *V. alginolyticus* showed strong cytotoxic to the heart

cell line of *E. lanceolatus* (Huang et al., 2011). As we know, *V. harveyi* and *E. tarda* can infect a variety of aquaculture species and cause serious financial losses, especially *V. harveyi* (Li et al., 2011; Liu et al., 2016; Tu et al., 2017). In this study, *V. harveyi* and *E. tarda* showed severe cytotoxicity to CAB cells. Moreover, the cytotoxicity of *V. harveyi* to CAB cells was stronger than that of *E. tarda*. Consistent with those of results *in vitro*, *V. harveyi* could cause acute pathogenicity to *C. altivelis in vivo* as previously described (Sun et al., 2019). Thus, CAB cell line has the potential utility in detecting cytotoxicity of the bacterial pathogens.

To date, heavy metal pollution has become one of the main environmental problems and has caused great harm to environment, aquatic animals, and even human health (Gong et al., 2018). Numerous studies have shown that fish cell lines are effective tools to monitor heavy metal pollution for early warning of their toxicity in the aquatic environment (Gülden et al., 2005; Liu et al., 2017). Previous studies showed that each fish cell line has a great different sensitivity in response to toxicants. It is necessary to detect the toxicity of different heavy metal to different cell lines, so as to establish a sensitive and suitable *in vitro* cell model for the study of early warning of heavy metal toxicity. In this study, the toxicity of three heavy metals (Cd, Hg and Cu) on CAB cells was detected and showed a dose-dependent manner. Results indicated that CAB cells were more sensitive to Hg than Cd and Cu. The similar results were also found in fibroblast SAF-1 cell line from *S. aurata L.* and muscle cell line from *C. altivelis* (Morcillo et al., 2016; Wang et al., 2020). The IC50 showed that the cytotoxicity of Hg (0.393mM) was more remarkable than that of Cd (0.445 mM) and Cu (1.073 mM) in CAB cells. Similarly, in mid-kindey cell line from *T. ovatus*, Hg showed higher toxicity than that of Cd and Cu (Zhou et al., 2017). All these data consistently revealed that CAB cell line could be used for monitoring heavy metal pollution.

Conclusions

In conclusion, our study established a *C. altivelis* brain cell line. The CAB cell line has good application potential in studying foreign gene expression. Furthermore, CAB cell line was sensitive to *V. harveyi* and *E. tarda*, as well as three heavy metals (Hg, Cd, and Cu), indicating that CAB cell line has the potential application in exploring the effect of pathogenic bacteria and heavy metals on host.

Author Declarations

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Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval Animal care was performed conformity with NIH guidelines (NIH Pub. No. 85e23, revised 1996) and was approved by Animal Care and Use Committee of the Hainan University.

Consent to participate Participate consent has been received from all authors.

Consent for publication Permission has been obtained from all authors for this article.

Data availability The study has the data and material.

Authors' contributions Yixuan Liu: Formal analysis, Writing- Original draft preparation; Caoying Wei: Formal analysis, Data Curation, Software, Visualization; Zhiru Liu: Project administration; Zhenjie Cao: Conceptualization, Methodology, Writing- Reviewing and Editing; Yun Sun: Supervision, Writing- Reviewing and Editing; Yongcan Zhou: Data Curation; Shifeng Wang: Validation; Weiliang Guo: Editing.

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Figure Legends

Fig. 1 Morphology of *Cromileptes altivelis* brain (CAB) cells at passage 1.

Fig. 2 CAB cells at 24 h after cell recovery at passage 8 (a) and confluent morphology of CAB cells at 72 h (b)

Fig. 3 PCR amplification of 18S rRNA gene of CAB cells (a) and the partial sequencing results of PCR product (b)

Fig. 4 The optimum growth conditions for CAB cells. (a) Effect of different FBS concentrations on the growth of CAB cells. ■, 20%; ▲, 10%; ●, 10%; ×, 5%. (b) Effect of different cell cultured medium on the growth of CAB cells. ■, L15; ▲, DMEM; ●, MEM; ×, M199. (c) Effect of temperature on the growth of CAB cell. ■, 28 °C; ▲, 26 °C; ●, 24 °C; ×, 22 °C.

Fig. 5 Chromosome frequency distribution of CAB cells at passage 7 (a) and chromosome morphology of CAB cells (b)

Fig. 6 Analysis of DNA content of CAB cells at 24h (a) and 48 h (b). A: G0–G1 phase [(a) 69.13%; (b) 82.90%]; B: G2–M phase [(a) 3.34%; (b) 4.82%]; C: S phase [(a) 27.54%; (b) 12.28%]. (c) Quantification was expressed as the percentage of CAB cells in different phases of the cell cycle; ■, 24 h; □, 48 h.

Fig. 7 Expression of enhanced green fluorescent protein (EGFP) in CAB cells at passage 12. (a) EGFP fluorescence in pEGFP-N3 transfected CAB cells. (b) Nucleus morphology of CAB cells stained by 6-diamidino-2-phenylindole (DAPI). (c) The merged images of EGFP and DAPI image.

Fig. 8 The susceptibility of CAB cells to bacteria. a–d, Cytopathic effect of CAB cells infected with PBS and *Vibrio harveyi* after 1h and 3 h post-infection. e–h, Cytopathic effect of CAB cells infected with PBS and *Edwardsiella tarda* after 6 h and 9 h post-infection. (Scale bar=100μm)

Fig. 9 Cytotoxicity analysis of three heavy metals (Cd, Hg and Cu) to CAB cells. Values are presented as the mean ± SD of three independent experiments. P value < 0.05 was considered statistically significant (*P < 0.05; **P < 0.01)

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491 **Figures**

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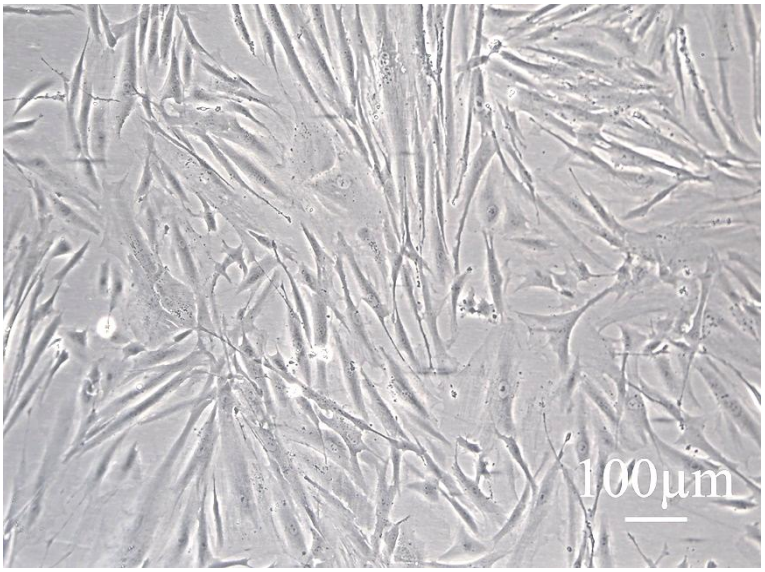
493 **Figure 1**



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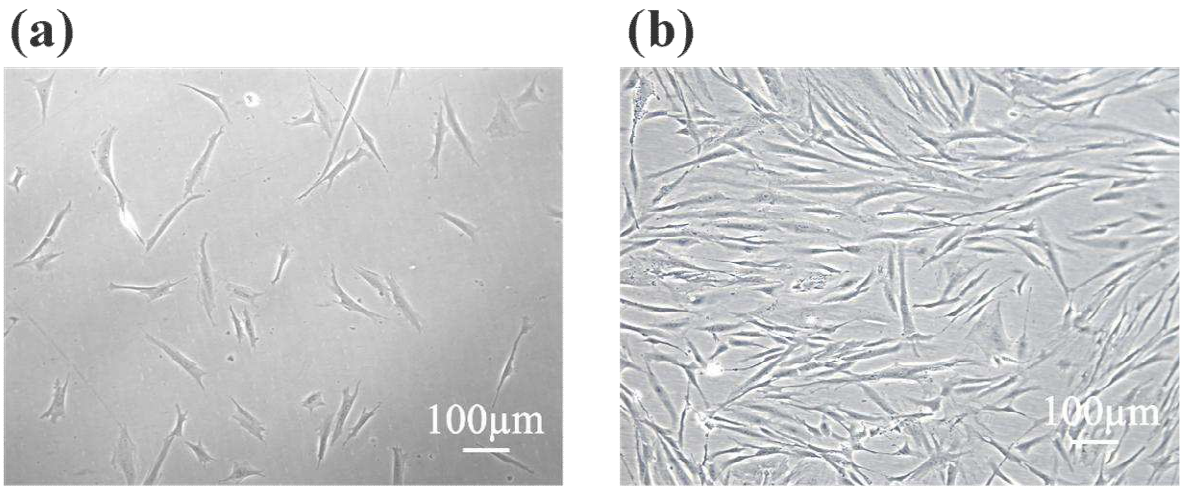
497 **Figure 2**



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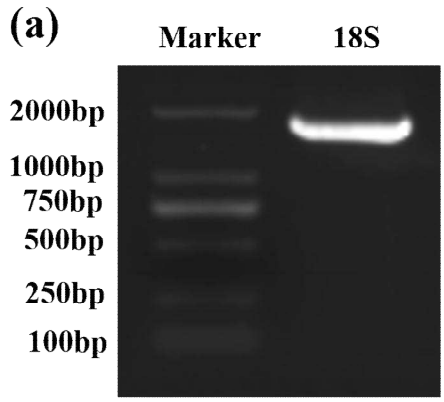

(b)

TACAGGACTCTTTCGAGGCCCTGTAATTGGAATGAGT
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ATAGCGTATCTTAAAGTTGCTGCAGTTAAAAAGCTCGT
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GCCCCCTCGATGCTCTTAGCTGAGTGTCCCGCGGGGT
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TGGAATAGGACTCCGGTTCTATTTTGTGGGTTTTCTCT
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CGGCGCAAG

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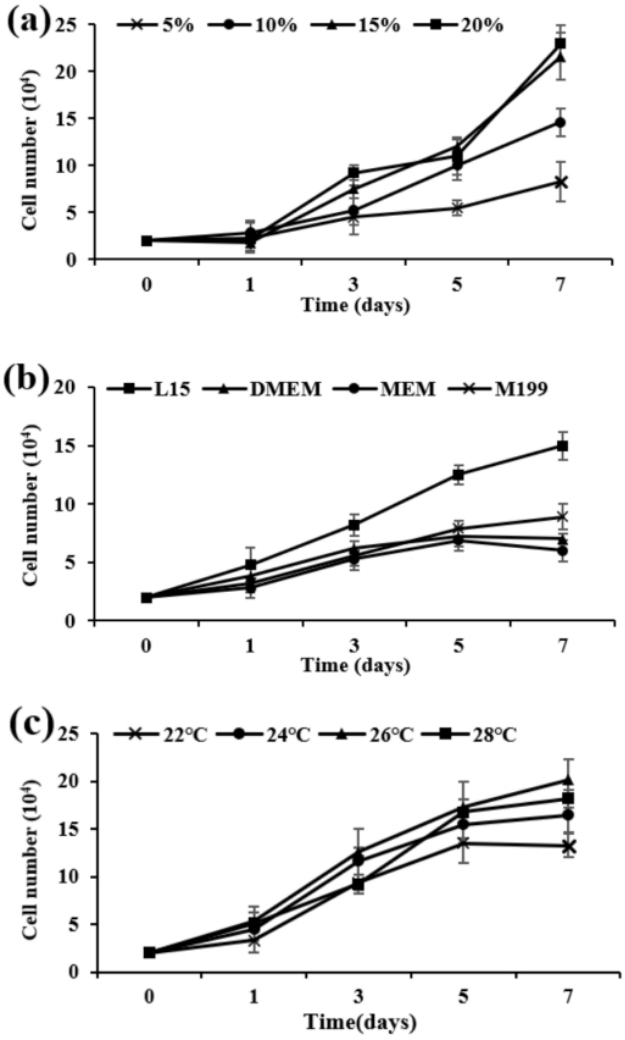

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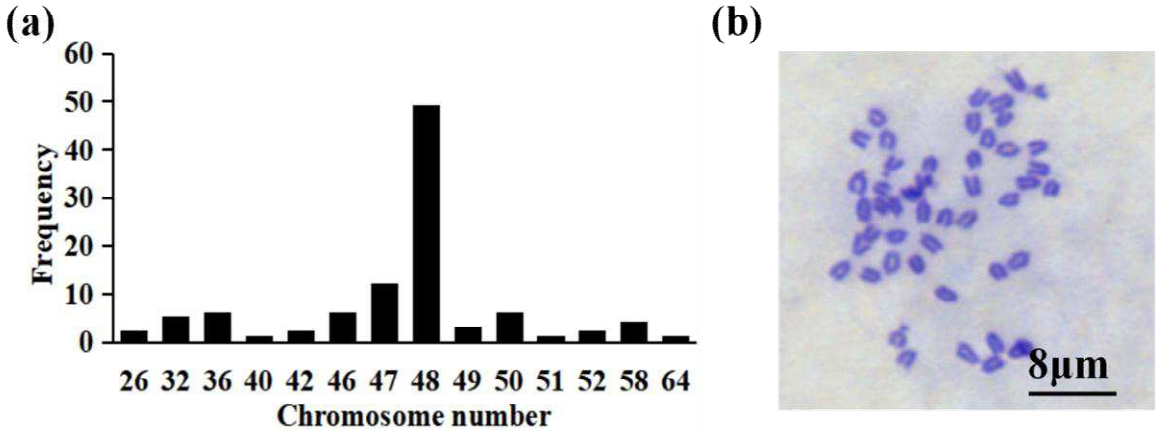
511 **Figure 5**



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514 **Figure 6**

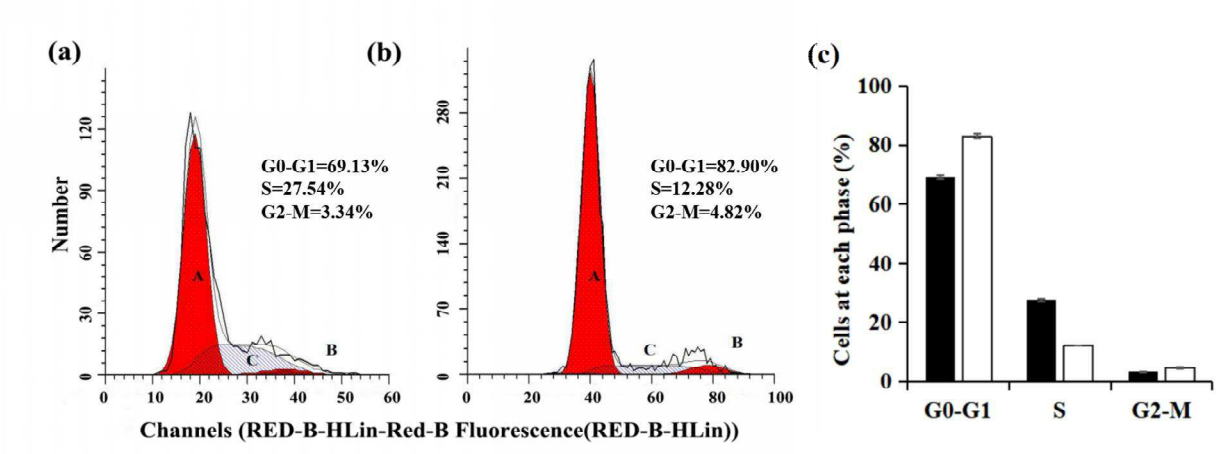

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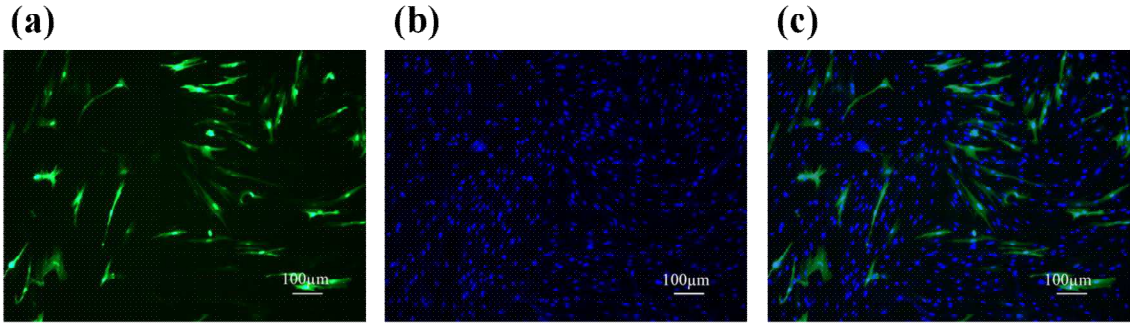
Figure 7



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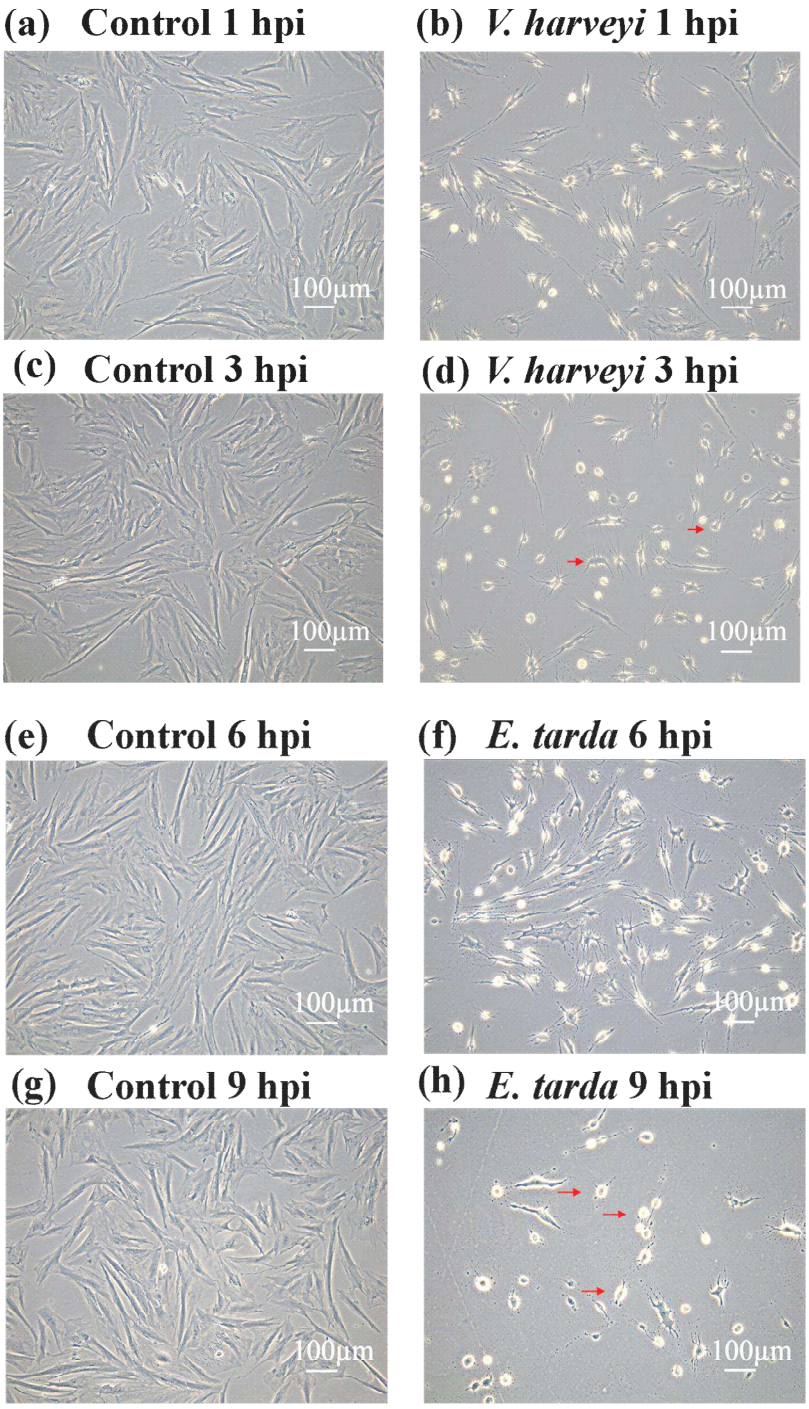

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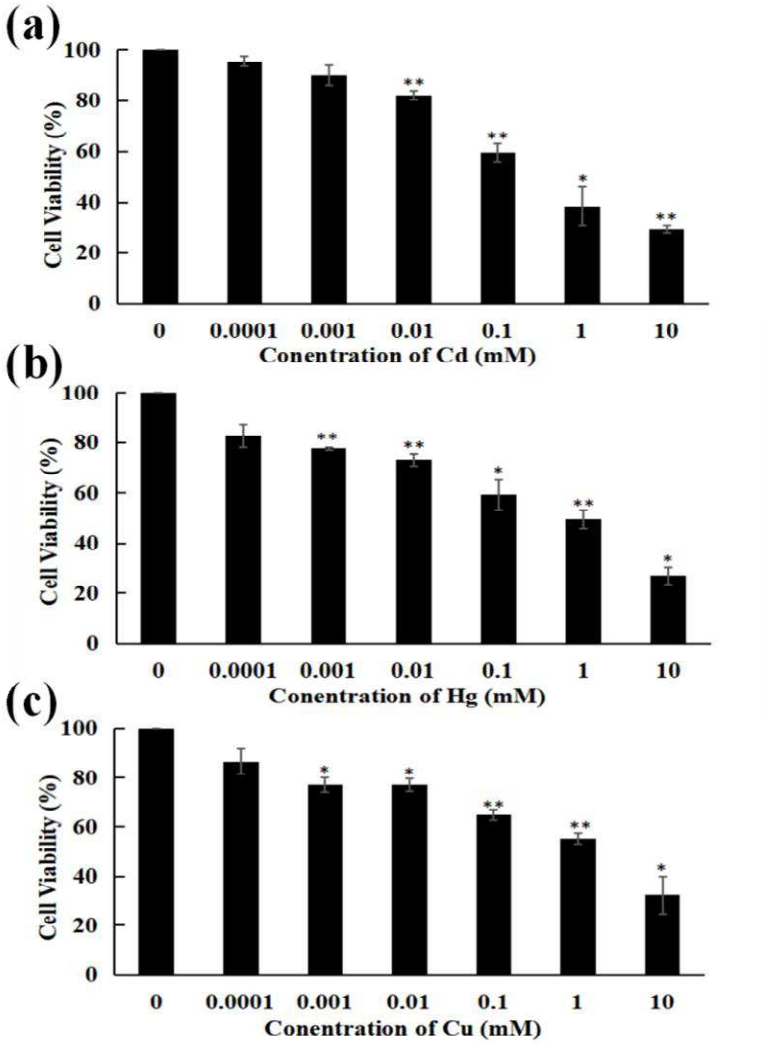
549 **Figure 9**



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