

## Establishment and characterization of a fibroblast-like cell line from *Anabarilius grahami* (Cypriniformes: Cyprinidae)

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**Abstract:** Though Yunnan province contains some 562 known species of fish, no cell lines from any of these have been made available to date. To protect germplasm resources and provide an effective tool in solving problems at cellular level of *Anabarilius grahami*, a fish endemic to Fuxian Lake, Yunnan, China, we established and characterized the major features of a continuous cell line (AGF II) from the caudal fin tissue of *A. grahami*. This AGF II cell line consists of fibroblast-like cells and has been subcultured more than 60 times over the course of a year. The cell line was maintained in DMEM/F12 supplemented with 10% FBS, with a cellular doubling time of 51.1 h. We continued with more experiments to optimize the culture and storage conditions, and found a variety of interesting results: cells could grow at temperature between 24 °C and 28 °C, with the optimal temperature of 28 °C. Likewise, the growth rate of *A. grahami* fin cells increased when the FBS proportion increased from 5% to 20%, with the optimal growth at the concentrations of 20% FBS; cells were able to grow in L-15 and DMEM/F12 with optimal growth at L-15; DMSO is a better cryoprotectant than Glycerol, EG and MeOH for AGFII cells with optimal concentration of 5% DMSO. Chromosome analysis also showed that the distribution of chromosome number varies from 38 to 52, with a modal peak at 48 chromosomes, accounting for 39.8% of all cells. Using the same primer pairs specific to mtDNA, the AGF II cell sequences obtained by PCR were identical to those from muscle tissues of *A. grahami*. Both chromosome analysis and PCR amplification confirmed the AGF II cells were from *A. grahami*, also indicating that that current long-term artificial propagation of *A. grahami* has been successful. Finally, we noted that when cells were transfected with pEYFP-N1 and pECFP-N1 plasmid, bright fluorescent signals were observed, suggesting that this cell line may be suitable for use in transfection and future gene expression studies.

**Keywords:** Characterization; Fibroblast-like cell line; *Anabarilius grahami*; Cryopreservation

*In vitro* cultures of animal cells have provided a powerful tool in studying different a variety of subjects, including immunology (Bols et al, 2001), environmental toxicology (Bahich & Borenfreund, 1991), endocrinology (Bols & Lee, 1991), virology (Wolf, 1988), biotechnology and aquaculture (Bols & Lee, 1991), and resources protection (Zhou et al, 2008; Chen & Qin, 2011). Since the first reported fish cell line in 1962 (Wolf & Quimby), over 280 lines have been established, most of derived from freshwater and anadromous fish species (Wolf & Mann, 1980; Fryer & Lannan, 1994; Lakra et al, 2011). To date, more than 50 cell lines have been established in China including over 20 species (Chen & Qin, 2011). Among these fish, most are economically important species, such as *Anguilla japonica* (Ku et al, 2010), *Chrysophrys major* (Chen et al, 2003), *Lateolabrax*

*japonicus* (Ye et al, 2006) and *Epinephelus akaara* (Huang et al, 2009); nevertheless, only a few are rare fish, such as *Acipenser sinensis* (Zhou et al, 2008) and *Gobiocypris rarus* (Tan et al, 2009). In Yunnan province alone, home to china's largest biodiversity hotspot, there are 562 known species of fish (Yang & Li, 2010), and yet no cell lines are available. Clearly, more attention should be paid to the rare and endemic fish cell cultures in future.

Received: 03 September 2012; Accepted: 30 October 2012

Foundation items: This work was supported by the Global Environment Foundation/The World Bank Project (GEF-MSP grant No. TF051795); the Yunnan Development and Reform Commission

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One such native species endemic to Fuxian Lake, Yunnan, *Anabarilius grahami* (Regan) belongs to Cypriniformes, Cyprinidae and has been assessed as a vulnerable species on the China species red lists (Wang & Xie, 2004) and evaluated as a threatened fish due its declining numbers (Liu *et al.*, 2009). Fuxian Lake, the second deepest lake in China (Ley *et al.*, 1963; Yang, 1984), has a great wealth of endemic fish, some 4.7% of all Yunnan fish species (Yang & Chen, 1995). However, because of exotic species invasion and over-exploitation, the production of *A. grahami* has sharply declined in recent years (Yang & Chen, 1995; Li *et al.*, 2003a; Xiong *et al.*, 2006). To date, a few reports on its Karyotype (Zan & Song, 1980), ecology (Yang, 1994; Li *et al.*, 2003a), diet composition (Qin *et al.*, 2007), artificial propagation (Li *et al.*, 2003b,c), disease control (Li *et al.*, 2003d), embryonic development (Ma *et al.*, 2008) and genetic diversity (Yang *et al.*, 2008; Liu *et al.*, 2011) have been made available. Unfortunately, little research has been carried out on *A. grahami*'s cell culture of. To further protect this valuable species and aid in its recovery, Li *et al.* (2003b) succeeded in launching artificial cultivation and the release of some fingerlings into Fuxian Lake annually since 2007. Liu *et al.* (2009) also noted that an investigation of the restocked population is necessary. Aquaculture by artificial cultivation has inherent limitations, e.g. germplasm degradation, reduction of genetic diversity, and a weakened ability to defend against disease and environmental stress, (Chen, 2002; Su *et al.*, 2012). A cell culture of *A. grahami* could provide an effective tool in studying these problems on cellular level.

## MATERIALS AND METHODS

### Primary cell culture

A healthy juvenile of *A. grahami* (10 cm total length) was obtained from the tenth generation of artificial cultivation from the Endangered Fish Conservation Center (EFCC), Kunming Institute of Zoology, Chinese Academy of Sciences. The specimen was disinfected with potassium permanganate. Caudal fin tissue was clipped and transferred to flasks using aseptic techniques and then washed three times with Hanks' balanced salt solution (HBSS) supplemented with antibiotics (penicillin, 400 IU/ml; streptomycin, 400 ug/ml; fungizone, 10 ug/ml). The washed tissue was minced into about 1 mm<sup>2</sup> in 10 cm diameter Petri dishes, and transferred into T-25 flasks (Corning) containing 3 mL of growth medium (DMEM/F12, Gibco) supplemented with 20% fetal bovine serum (FBS, Gibco), 100 IU/ml of penicillin, and 100 ug/ml of streptomycin. All flasks were incubated at 28 °C. After 3 days, 3 mL of growth medium was added into the uncontaminated flasks. Monolayers of primary cells were obtained after 30-40

days (Clem *et al.*, 1961; Chen & Qin, 2011; Freshney, 2010).

### Sub-culture and maintenance

Each flask was examined to ensure it was free of microbial contamination and normal morphology of the cells. The spent medium was removed to a new flask and then combined with the same volume of fresh medium, making a conditioned medium. The cells were washed with HBSS and covered with 1 mL of 0.25% trypsin-EDTA solution (Invitrogen). Each culture was examined under an inverted microscope (Olympus, CKX41) to ensure that cells had separated from the flask surface. The trypsin solution was decanted and the cells were dislodged by gently tapping the flask. A volume of 10 mL fresh or conditioned medium was added to the flask and shaken to obtain a cell suspension. Finally, 5 mL of the suspension was transferred to a new flask and incubated at 28 °C. The cells were confluent within 3-4 days (Zhou *et al.*, 2008; Chen & Qin, 2011).

### Growth of cells

Cells were placed at an initial density of  $4.8 \times 10^4$ /ml into a T-25 flask with DMEM/F12+10% FBS at 28 °C. Cells from the flasks were collected and counted daily for 9 days using a haemocytometer; this process was repeated three times (Freshney, 2010).

### Effect of medium on cell growth

The effect of medium on cell growth was evaluated in 4-well. Cells were respectively cultured in DMEM/F12, DMEM, M199 and L-15 with 10% FBS at 28 °C. Cells of duplicate wells in each medium were trypsinized and counted with a haemocytometer daily for 5 days (Huang *et al.*, 2009; Ou *et al.*, 2010).

### Effect of temperature and FBS concentrations on cell growth

Cells were seeded into 4-well containing L-15 medium with 10% FBS and incubated at 20 °C, 24 °C, 28 °C and 32 °C, successively. Duplicate wells were used for each temperature. In the same manner, L-15 medium containing 5%, 10%, 15%, 20% and 25% FBS were assessed for their effect on cell growth at 28 °C. In the three tests, the average number of cells was calculated daily for 4 days using a haemocytometer (Ku *et al.*, 2010; Lakra *et al.*, 2011).

### Effect of cryoprotectant on cell cryopreservation

The procedure of cell cryopreservation as follows: cells were propagated for 3-4 days in T-75 flasks until confluent monolayers had formed and cell suspension from each flask was collected into a 50 mL centrifuge tube and then centrifuged at 200 r/min for 8 min. The pellets were suspended in FBS containing optimal

cryoprotectant to obtain a concentration of  $5\text{--}6\times 10^6$  cells/ml. Cell suspensions were then transferred to 2-mL cryovials and kept sequentially at  $-20\text{ }^{\circ}\text{C}$  for 4 h,  $-80\text{ }^{\circ}\text{C}$  for 16 h and finally stored in liquid nitrogen  $-196\text{ }^{\circ}\text{C}$  (Freshney, 2010; Chen & Qin, 2011). The frozen cells were recovered in a water bath at  $37\text{ }^{\circ}\text{C}$ , transferred to L-15 medium (10% FBS) in a T-25 flask and incubated at  $28\text{ }^{\circ}\text{C}$ . The medium was decanted from the flask 24 h after incubation and replaced with 5 mL of fresh L-15 medium. The flask was incubated at  $28\text{ }^{\circ}\text{C}$  until the cells reached confluence.

We progressively examined the effects of cryoprotectants, DMSO, Glycerol, ethylene glycol (EG) and Methanol (MeOH) on cell cryopreservation, allowing us to then examine suitable concentrations of better cryoprotectant at 5%, 7.5%, 10%, 12.5%, and 15%. The quality of the cells recovered after thawing was assessed according to two criteria: (1) viability of cells, assessed by Trypan blue exclusion assay; and (2) adhesion ability of the thawed cells, estimated by counting the cells and then seeding them in 4 wells with L-15+10% FBS, and dissociated with 0.25% Trypsin-EDTA after 24 h at  $28\text{ }^{\circ}\text{C}$ . The cell adhesion percentage was the ratio of the total numbers of viable cells recovered after adhesion to the number of viable cells after thawing (Mauger, 2006).

#### Cell origin identification

Total DNA was isolated from fresh *A. grahami* muscle tissue and the AGF II cell line using the DNeasy Tissue Kit (BioTeke). Primers PDL1: 5'-ACCCCTGGC TCCCAAAGC-3' and PDH1: 5'-ATCTTAGCATCTTCA GTG-3' were used to amplify the 925 bp of D-loop gene using PCR (Liu et al., 2002). The PCR conditions were modified as follows: 50  $\mu\text{L}$  mixture of PCR containing 5  $\mu\text{L}$  of  $10\times$  buffer, 1  $\mu\text{L}$  of 10  $\mu\text{M}$  PDL1 and PDH1 primers, 3  $\mu\text{L}$  of 10 mM dNTP, 0.25  $\mu\text{L}$  Supernew Taq, 1  $\mu\text{L}$  DNA and 36.75  $\mu\text{L}$  ddH<sub>2</sub>O. PCR was ran at  $94\text{ }^{\circ}\text{C}$  denaturation for 3 min followed by 35 cycles of  $94\text{ }^{\circ}\text{C}$  for 45 s,  $52\text{ }^{\circ}\text{C}$  for 45 s and  $72\text{ }^{\circ}\text{C}$  for 90 s, and a final extension of 10 min at  $72\text{ }^{\circ}\text{C}$ . The purified fragments were then sequenced by Sangon Biotech (Shanghai) (Yang et al, 2008; Liu et al, 2011).

#### Chromosome preparation

Chromosome spreads were prepared from AGF II cells at passages 32. About  $10^6$  cells were seeded into a T-75 flask and grown in L-15 medium supplemented with 10% FBS. After 1 day incubation at  $28\text{ }^{\circ}\text{C}$ , 0.2  $\mu\text{g}/\text{mL}$  of colcemid (Sigma) was added to the medium for 2 h to arrest the cells in metaphase. The majority of cells (70%) were dislodged from the flask by gentle tapping and then harvested by centrifugation (200 g, 8 min). Cell pellets were suspended in a hypotonic solution consisting of 0.4% KCl for 15 min, and thereafter fixed in a 3:1

mixture of methanol:acetic acid. Chromosomes spread on a glass slide were then prepared according to the conventional drop-splash technique, followed by staining with 5% Giemsa for 8 min (Zan & Song, 1980; Freshney, 2010) and then the number of chromosomes was counted under a Leica DMR microscope (Leica Mikroskopie und Systeme GmbH).

#### Cell transfection

Cells were seeded in 24-well plates and transfected with the plasmid DNA of two fluorescent proteins (pEYFP-N1 and pECFP-N1) by Lipofectamine 2000 (Invitrogen) at a ratio of 1:2. The medium was renewed 6 h after transfection, and cells were observed under a Leica inverted fluorescence microscope 24 h after transfection. The transfection efficiency was determined by counting the number of fluorescent protein-positive cells as well as the total cell number in 20 independent optical fields at a magnification of  $\times 400$ , after 60h post-transfection (Ouyang et al, 2010).

## RESULTS

#### Primary cell culture and maintenance

After 5-6 days, cells migrated out from the edge of the caudal fin tissues and grew well, forming a monolayer within 40 days at  $28\text{ }^{\circ}\text{C}$ . The initial cells consisted of both epithelioid-like and fibroblast-like cells (Figure 1A). After 6 subcultures, the fibroblast-like cells, named AGF II (AG, *Anabarilius grahami*; F, Fin), became predominant (Figure 1B). To date of this study, the cell line has been subcultured more than 60 passages.

#### Growth of cells

After subculture, AGF II cells progress through a characteristic growth pattern: a lag phase (1-3 day), exponential phase (3-7 day) and stationary phase (7-9 day) (Figure 2). The AGF II cells' population doubling time during exponential growth was determined to be 51.1 h, at a seeding density of  $4.8\times 10^4$  cells/mL with DMEM/F12+10% FBS at  $28\text{ }^{\circ}\text{C}$ .

#### Effect of medium on cell growth

The effects of different medium on cell growth are presented in Figure 3. The cells adhered the first day with L-15, DMEM/F12 and DMEM were more than with M1640. Afterward, cells were able to grow in L-15 and DMEM/F12, with optimal growth at L-15. No obvious growth was observed at DMEM and M1640.

#### Effect of temperature and FBS concentration on cell growth

Our observations showed that AGF II Cells grew at  $20\text{ }^{\circ}\text{C}$ ,  $24\text{ }^{\circ}\text{C}$  and  $32\text{ }^{\circ}\text{C}$  with optimal growth at  $28\text{ }^{\circ}\text{C}$  (Figure 4A). The cells grew well between  $24\text{ }^{\circ}\text{C}$  and

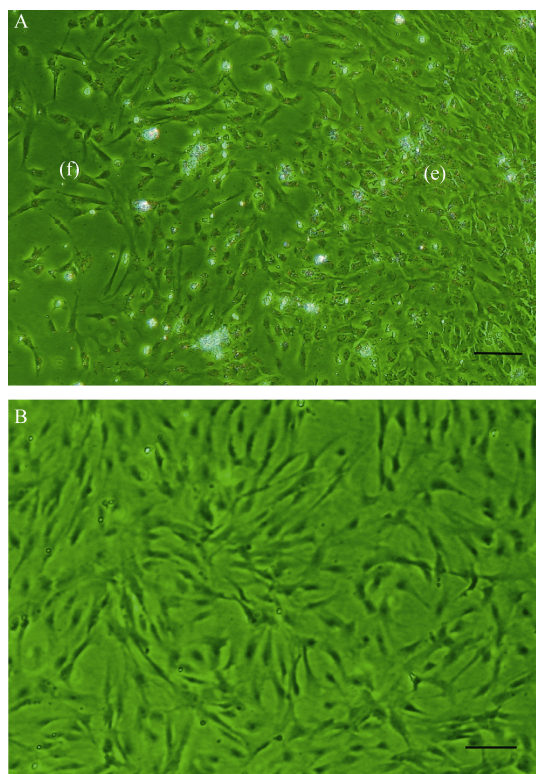


Figure 1 Photomicrograph of the *Anabailius grahami* cell line A: Epithelioid-like (e) and fibroblast-like (f) cells from fin explants were present after 40 days culture at 28 °C; B: Monolayer of AGFII cells at passage 13. Bar=100 μm.

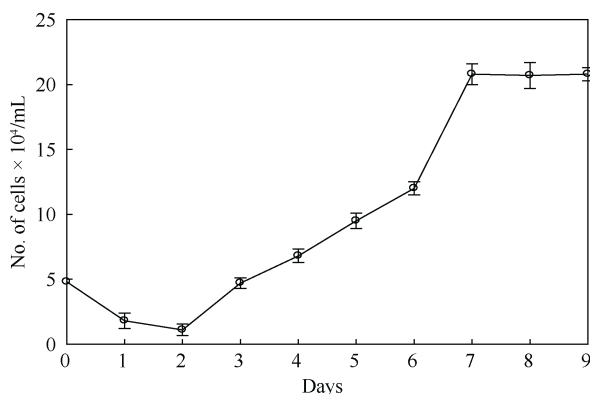


Figure 2 Growth curve of AGF II cells with DMEM/F12+10% FBS at 28 °C

28 °C, but when the culture temperature declined to 20 °C, no obvious growth was observed, and the cells staid at the lag phase. When the temperature was raised to 32 °C, aside from no growth being observed, the number of viable cells declined.

Growth of AGF II cells at varying levels of FBS concentration is shown in Figure 4B. Our results show that serum was required for the cell to survive and grow, and that when concentration of FBS varied between 5% and 20%, the higher the concentration of FBS, there was a faster cell growth rate, but there was no faster growth

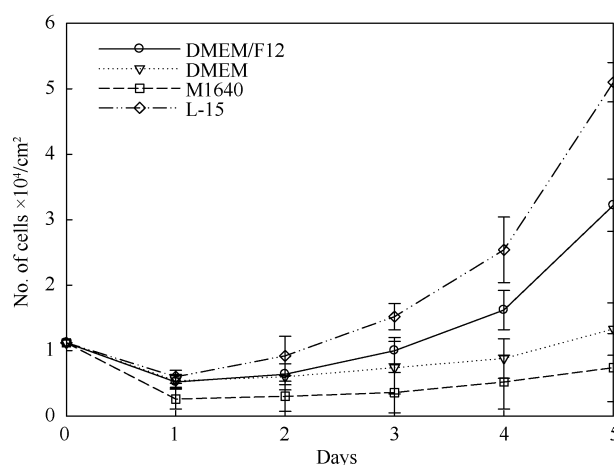


Figure 3 Effect of four different media on AGF II cell growth

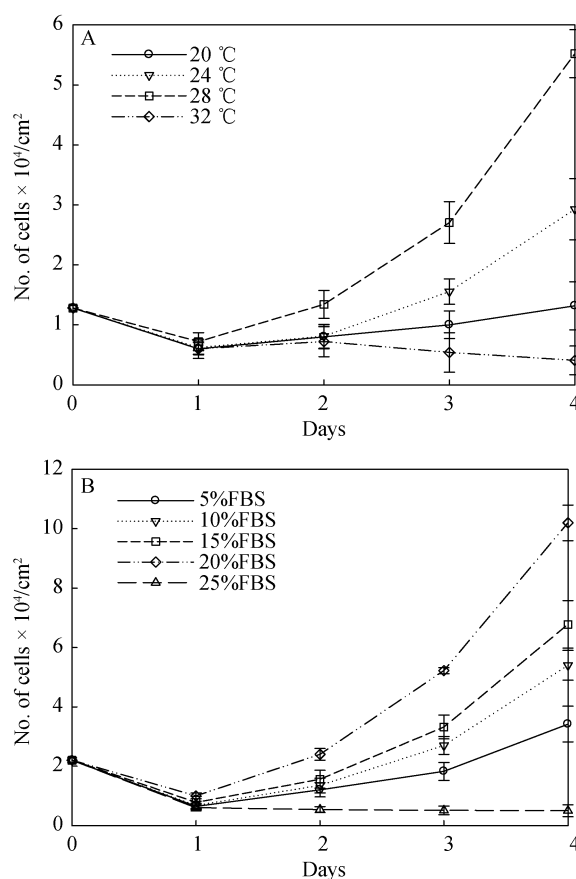


Figure 4 Effect of (A) temperature and (B) FBS concentration on AGF II cell growth

rate observed when the concentration of FBS was increased to 25%.

#### Effect of cryoprotectant on cell cryopreservation

A given cryoprotectant and concentration of cryoprotectant can affect the quality of thawed cells. Our data showed that the viability and ability of cells to recover after cryopreservation were significant lower

than that of the fresh control (Figure 5A). DMSO treatment gave better re-viability percentage ( $80.36 \pm 5.3\%$ ) and re-adhesion percentage ( $44.24 \pm 7.5\%$ ) than others ( $P < 0.01$ ). The re-viability percentage between Glycerol ( $61.29 \pm 4.5\%$ ) and EG ( $60.29 \pm 5.1\%$ ) treatment was not significant ( $P > 0.05$ ), nor was the re-adhesion percentage. MeOH treatment gained worst re-viability and re-adhesion percentages, ( $28.3 \pm 4.5\%$ ) and ( $2.42 \pm 1.0\%$ ), respectively. For both the percentages of viability and adhesion of cryopreserved cells, we noted a discernible relationships of  $\text{DMSO} > \text{Glycerol}$  and  $\text{EG} > \text{MeOH}$ . We likewise found that DMSO is the best cryoprotectant for AGFII cells.

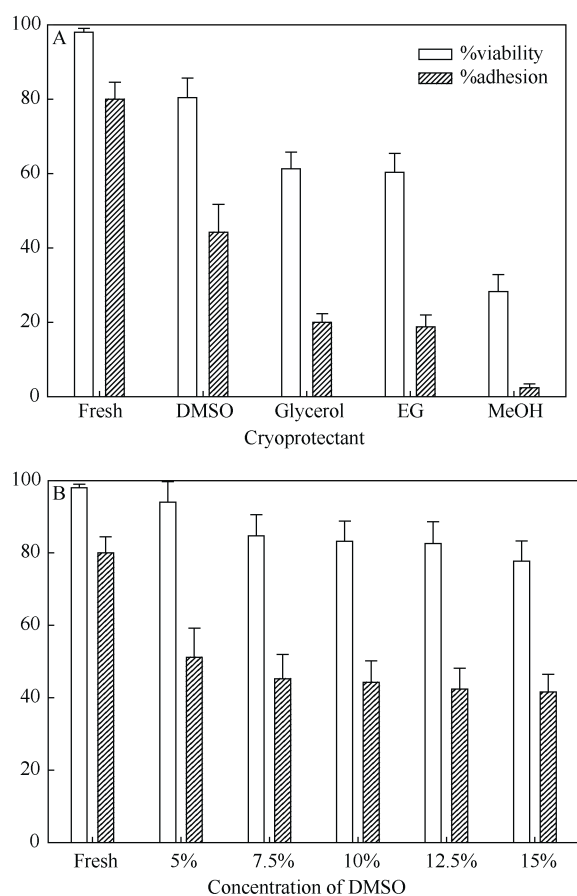


Figure 5 Effect of different cryoprotectant (A) and concentration of DMSO (B) on viability and adhesion percentage of recovered AGF II cells at passage 21 after cryopreservation

In addition to the above-mentioned results, all graded concentrations of DMSO treatment led to more cell losses than those observed with the fresh control ( $P < 0.05$ ). Among the 5 treatments, 5% proved to be the optimal concentration, whose re-viability and re-adhesion percentage were respectively ( $94.03 \pm 5.7\%$ ) and ( $51.2 \pm 8.0\%$ ). As concentrations of DMSO increase, the percentage of viability and adhesion decreased. More

than 70% of the thawed cells were viable after cryopreservation for a month, and more than 40% could be recovered after seeding with the 4 other treatments (Figure 5B).

### Cell origin identification

PCR products of the D-loop gene showed a band near 1 000 bp by agarose gel electrophoresis (Figure 6). Sequencing of this gene obtained a 925 bp length, and then comparative analysis showed these sequences from fresh *A. grahami* muscle tissue and from AGF II at passage 3, 10 and 20 were identical. These data confirmed that the origin of the established AGF II cell lines were from *A. grahami*.

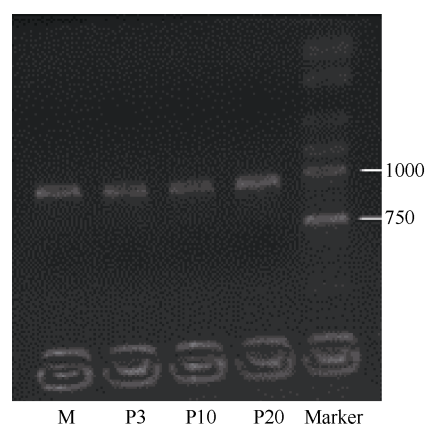


Figure 6 Photograph of agarose gel electrophoresis from PCR products

Total DNA from line M was from fresh muscle of *A. grahami*, and line P3, P10 and P20 were representing the AGF II cell line at passages 3, 10 and 20, respectively.

### Chromosome analysis

Chromosome morphology of AGF II is presented in Figure 7. The result of chromosome counts of 108 metaphase plates revealed that the chromosome number of AGF II cells were widely distributed between 38 and 52, with a modal peak at 48 chromosomes, and 39.8% of all cells contained 48 chromosomes. Aneuploidy was observed in the AGF II cell line, though they were small in proportion, such as 14.8%, 12.1% and 10.2% of all cells contained 50, 49, 47 chromosomes, respectively. Diploid karyotype formula of *A. grahami* fin cell line was 14 m (metacentric chromosome)+20 sm (sub-metacentric chromosome)+14 st (subtelocentric chromosome).

### Cell transfection

After electroporation, we observed yellow and cyan fluorescent proteins in the cell line after 24 h transfection (Figure 8). The transfection efficiency was 35% for pEYFP-N1 and 10% for pECFP-N1, indicating the suitability of this cell line for transfection and gene expression studies.

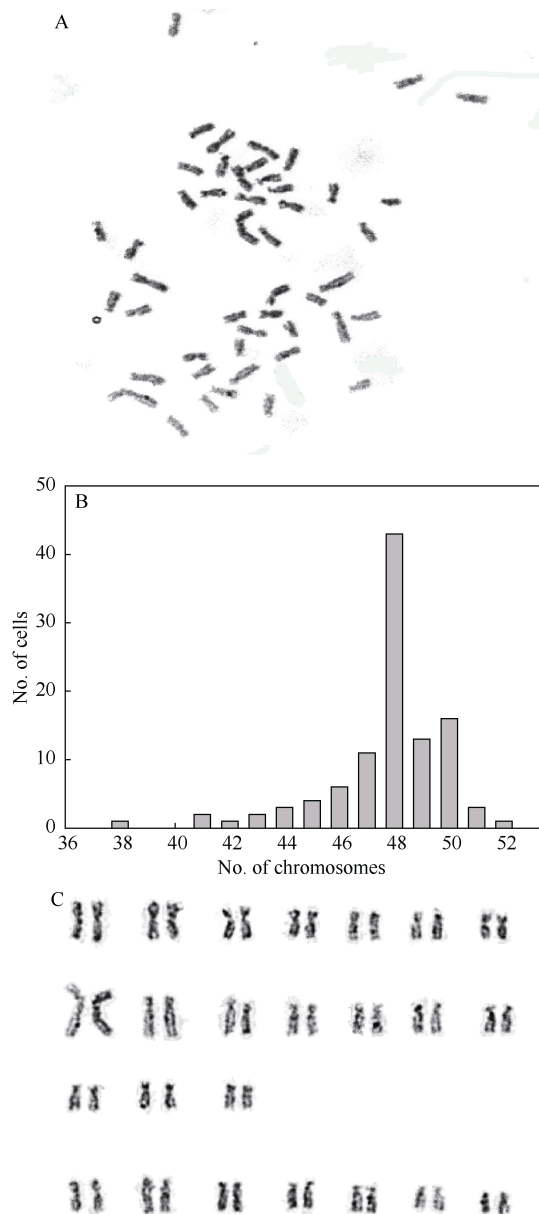


Figure 7 Chromosome distribution (A) metaphase (B) and diploid karyotype (C) of *A. grahami* fin cells at passage 32. 108 metaphases were counted

## DISCUSSION

The AGF II is the first cell line of *Anabarilius grahami* and is also the first cell line developed from Fuxian Lake (Yang, 1994). The morphology of initial cells is the same as other fin cell lines (Zhou et al, 2008; Ku et al, 2010). Different cell lines require various culture and cryopreservation conditions (Chen & Qin, 2011; Lakra et al, 2011), suggesting that it is necessary to optimize conditions of each cell line and significant to identify cell origin to ensure the AGF II comes from *A. grahami*, as well as cell application.

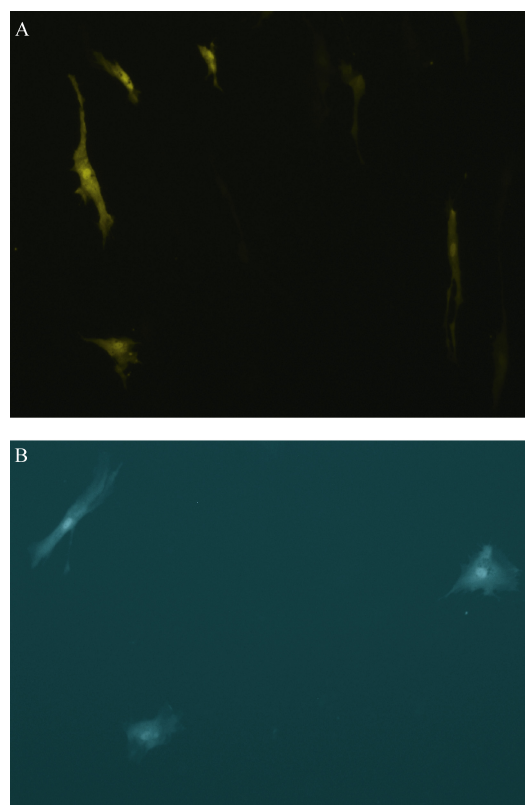


Figure 8 Fluorescent micrographs of yellow fluorescent protein (YFP, A) and cyan fluorescent protein (CFP, B) expression in transfected AGFII cells using pEYFP-N1 and pECFP-N1, respectively ( $\times 400$ )

## Culture conditions

Though different media are used to culture different cell lines, DMEM/F12 comes close to being an all purpose culture medium for the cells of mammals, birds, reptiles, amphibians as well as fish (Wolf & Quimby, 1969), and L-15 does not require CO<sub>2</sub> buffering, so they have also successfully used in fish cell lines (Leibovitz 1963).

The optimal incubation temperature of AGF II cells (24 °C-28 °C) is higher than optimal growth temperature of adult fish (23 °C-26 °C) and fertilized eggs (20 °C) of *A. grahami* (Li et al, 2003a; Ma et al, 2008). Long-term aquarium environment may contribute to these differences to a certain extent, because the temperature of *A. grahami* could adapt to 28 °C-29 °C in artificial breeding.

FBS seems to be the most popular choice of supplements in the tissue culture media as it is easy to obtain in large volumes as well as for to the presence of both known and unknown growth factors (Lakra et al, 2011). In fact, when using a FBS free medium, the AGF II cells did not adhere to the substratum flask, suggesting that FBS may play a key role in promoting cell attachment and proliferation, which is consistent with the case of the *Acipenser sinensis* fin cell lines (Zhou et al, 2008). However, FBS concentrations are not usually



much higher than 20%, as there is evidence that high serum concentrations may inhibit cell growth (Freshney, 2010; Chen & Qin, 2011).

### Cell cryopreservation

Freezing lower vertebrate cells is done using the same procedure as for homeotherm cells, with optimal storage in liquid nitrogen (Wolf & Mann, 1980). However, less attention has been paid to the effects of different cryoprotectant and concentration on cell cryopreservation. DMSO is the most common cryoprotectant for cultured cells (Mauger et al, 2006; Freshney, 2010), yet Glycerol, EG and MeOH are mostly used in cryopreservation of germ cells but not somatic cells (Cabrita et al, 2010). All cryoprotectants have proved to have some negative effects toward fish cells, since the percentage of viability and adhesion of thawed cells is lower than fresh cells, which may be relative to the mild toxicity of cryoprotectant toward fresh cells, apart from the effect of lower temperature (Borenfreund, 1989; Muchlisin, 2005; Mauger et al, 2006; Cabrita et al, 2010). DMSO has been demonstrated to be better than other cryoprotectant for *A. grahami* cell cryopreservation. Similarly, concentration of DMSO did not influence cell cryopreservation, because all different DMSO concentration could obtain relative higher viability and adhesion than Glycerol, EG and MeOH.

### Cell identification

Identifying the cell origin after establishment of the cell line has been finished is essential. Chromosome analysis is the most commonly used method (Freshney, 2010), and because the mitochondrial DNA control region (D-loop) is highly variable and can be used for phylogenetic analysis (Yang et al, 2008); accordingly they were used to verify whether the AGF II was actually derived from *A. grahami*. Both the PCR amplification and chromosome analysis confirmed that the AGF II cells were from *A. grahami*. The D-loop is an important non-coding region of mitochondrial DNA that possesses a high rate of evolutionary change (Yang et al, 2008). All sequences of D-loop gene from *A. grahami* brought about a discovery of difference between this *A. grahami* and the wider *A. grahami* species was not obvious. That relatively high genetic diversity of from the EFCC may contribute in this matter (Yang et al, 2008).

A modal chromosome number ( $2n=48$ ) of AGF II cell line by Karyotype analysis was consistent with Zan & Song's (1980) results. The percentages of cells

containing modal chromosomes to total cells varied from 20% to 70%, depending on different species and cell lines (Chen & Qin, 2011). The percentage in AGF II is 39.8%, which is imperfect, but because the materials' origin was not explicitly described in most studies—i.e. the use of a wild or domestic population and of which generation—so comparing our findings with their results is not significant or clearly useful. Conversely, AGF II is from the tenth generation of *A. grahami* through artificial propagation, suggesting that to some degree, long-term artificial propagation of *A. grahami* has been successful and may further benefit by frequently adding wild *A. grahami* species from different locations (Yang et al, 2008). Both aneuploidy and heteroploidy were widespread in fish cell lines (Wolf & Quimby, 1969; Kang et al, 2003; Ye et al, 2006; Cheng et al, 2010), which may be connected with the growth status of cell lines (Wolf & Quimby, 1969).

### Cell application

As other cell lines, AGF II is also capable of accepting exogenous genes (Zhou et al, 2008; Ou et al, 2010; Cheng et al, 2010), so we also assessed the application of the *A. grahami* cell line for exogenous gene manipulation by examining the ability of AGF II cells to express the YFP and CFP gene. Cases where different cell lines lead to different transfection efficiency (Zhou et al, 2008; Ku et al, 2010) and the ability of the same cell to express different gene is also distinct (Hameed et al, 2006) may potentially be the reason that transfection efficiency of pECFP-N1 is lower than that of pEYFP-N1.

In summary, we establish a freshwater fish cell line, AGF II, from the caudal fin of *A. grahami*, and subcultured more than 60 passages. This cell line could provide an important tool for the further study of exogenous gene manipulation among freshwater fish. We also expect that the results of this study will attract more researchers to pay attention to cell culture of native fishes, and protect fish germplasm resource of Yunnan province.

**Acknowledgements:** We thank Wenhui NIE, Jinhuan WANG, Weiting SU and Yi HU (Cell Bank of the Kunming Institute of Zoology, CAS) for their help and support in experiment, and also Wansheng JIANG (State Key Laboratory of Genetic Resources and Evolution of the Kunming Institute of Zoology, CAS) for their assistance in improving the manuscript.

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