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Research Article

Germplasm Rescue of Postmortem Critically Endangered Yangtze Sturgeon (*Acipenser dabryanus*) by Cell Preservation

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With the growing sophistication of cloning technology, rescuing cell resources is of great significance for the protection of endangered animals. The Yangtze sturgeon (*Acipenser dabryanus*), one of the three Acipenseriformes species in the Yangtze River, is critically endangered. Natural reproduction of the Yangtze sturgeon has not been detected since 2000. Less than 20 wild individuals are kept in husbandry, and all are too old to breed. Therefore, it is urgent to rescue the genetic resources of every wild Yangtze sturgeon. Here, we isolated and preserved viable cells from critically endangered postmortem Yangtze sturgeon for the first time. Attempts to rescue and preserve cell resources were carried out from 8 tissues, brain, kidney, gonad, fin, liver, skin, spleen, and muscle of an over 35-year-old female wild Yangtze sturgeon between 11 and 14 hours after death at 19.8°C in an outdoor concrete pond, and only muscle tissue cells could be successfully subcultured and preserved. Furthermore, the cultured cells showed high post-thaw cell viability and normal growth with a population doubling time of 52.98 h. Moreover, they were fibronectin- and desmin-positive, characterizing them as fibroblastic confirmed muscle cells with fibroblastic properties and myogenic origin. Tests for microbial contamination of the cell lines were negative. Chromosome analysis demonstrated that muscle cells possess a modal polyploid chromosome number of 264. The mitochondrial sequence data of COI genes and 12S rRNA confirmed that the developed cell line originated from *A. dabryanus*. Furthermore, transfection results indicate that muscle cells could be used for gene manipulation and functional studies. These results suggest that viable muscle cells could also be successfully isolated and cryopreserved from the wild Yangtze sturgeon in a short time after death. This report is not only of great significance for the germplasm rescue of critically endangered Yangtze sturgeon but also provides some scientific reference for the germplasm preservation of other endangered fish.

1. Introduction

Numerous species have experienced accelerated extinction since the 17th century as a result of the explosion of human populations and the intense intervention of human activity. Large creatures and freshwater animals, however, are more susceptible to extinction [1, 2]. Large endangered animals have increasingly grown to be the center of attention due to their need for rescue and conservation. Entering the 21st century, all three large sturgeons in the Yangtze River, the Chinese paddlefish (*Psephuyrus gladius*), Chinese sturgeon (*Acipenser sinensis*), and Yangtze sturgeon (*A. dabryanus*), are critically endangered. The Chinese paddlefish has been declared functionally extinct [3]. The reproduction in the wild Chinese sturgeon was interrupted in 2013, 2015, and 2017–2020. As for the Yangtze sturgeon, unfortunately, it has been unable to naturally reproduce since 2000 [4]. So far, there are less than 20 wild individuals in captivity, and they are too old to reproduce. At the same time, the wild Yangtze sturgeon resources in the Yangtze River also decreased sharply. Thus, how to save or preserve the germplasm resources of these species is a very urgent task at present.

Currently, cryopreservation of gametes, embryos, cells, or tissues in germplasm banks provides unique tools to

preserve valuable genetic material [5], especially the cell resources from threatened or endangered species [6, 7]. In contrast to gametes and embryos, somatic cells are more resistant to cryopreservation stress and more easily collected from rare or even dead species [8, 9]. In addition, recent advances in modern biotechnologies such as somatic nuclear transfer [10] and induced pluripotent stem cells (iPSCs) [11, 12], have also demonstrated their potential implications in the conservation and rescue of threatened or extinct species. Successful cloning of animals including sheep [13], mice [14], pigs [15, 16], and monkeys [17] has been reported in several studies. The study of nuclear transfer in fish was initiated by Tung in the 1960s [18]. Since the 1970s, nuclear transfer in fish has been widely reported, mainly in cyprinid fish such as zebrafish and medaka [19–21]. In particular, there have been successful cases of cloning cells using postmortem somatic cells. Loi et al. successfully cloned a healthy *Ovis orientalis musimon* (a wild endangered animal) from postmortem granular cells of O*vis orientalis musimon,* and Hoshino successfully obtained four live cloned calves from dead bull testicular cells [22, 23]. All these studies provide confidence for the conservation of endangered animal genetic resources, especially those that die suddenly in extreme situations. Therefore, somatic cell cryopreservation can be farsightedly considered a feasible means to preserve and restore genetic resources. However, little research has been carried out on cell lines of fish tissues after death.

In this study, a rescue cell resource preservation attempt was carried out for a wild Yangtze sturgeon that had been dead for a short period. Through the culture of 8 various tissues, a fibroblast cell line from the postmortem muscle of wild Yangtze sturgeon (*A. dabryanus*) was established, which not only preserved the rare germplasm resources of wild Yangtze sturgeon but also provided a useful approach for further exploring the conservation of genetic resources of large endangered fish.

2. Materials and Methods

2.1. Ethics Statement. The study was carried out by the guidelines and regulations of the National Institute of Health Guide for the Care and Use of Laboratory Animals and was approved by the Institutional Review Board on Bioethics and Biosafety of the Chinese Sturgeon Research Institute.

2.2. Tissue Collection. The Yangtze sturgeon samples were reared in the outdoor concrete pond of the Chinese Sturgeon Research Institute, China Three Gorges Corporation. It is a wild female over 35 years old that was rescued from the Yangtze River around 1990. Eight tissues including the brain, kidney, gonad, fin, liver, skin, spleen, and muscle were excised from it dead after 11–14 hours at a pond temperature of 19.8°C and transported to the laboratory within 10 minutes. All tissues were immediately sterilized with 75% alcohol, repeatedly rinsed with 1 × phosphate-buffered saline (PBS) containing 1% Antibiotic-Antimycotic solution (Gibco, USA), and then placed momentarily in sterile Petri dishes for *in vitro* culture.

2.3. Primary Cultures, Secondary Cultures, and Cryopreservation. According to the book [24], MEM is one of the most commonly used media for most cells. Furthermore, MEM was also shown to be suitable for the culture of Yangtze sturgeon fin cells in our previous study [25]. Therefore, we cultured all tissues in an MEM medium, respectively. After being disinfected and washed several times, the explants were chopped into approximately 1 mm^3 pieces and adhered to 25 cm^2 tissue culture flasks (Corning, USA). Small pieces were cultured in a complete medium consisting of a minimum essential medium (MEM, Gibco, USA), 20% fetal bovine serum (FBS, Gibco, USA), and 1% penicillin-streptomycin solution. The flasks were incubated at 25°C in a carbon dioxide incubator with 5% CO_2 (Thermo Fisher Scientific, USA). In our experience, the culture flasks should not be removed so that sample cells can adhere firmly to the flask during the first two days of culture. Next, the flasks were observed under an inverted fluorescence microscope, and half of the culture medium was changed every 3 days. Culture cells were dispersed by standard trypsinization methods and transferred into new flasks. Subsequent routine culturing was performed with 10% FBS instead of 20% and subcultured weekly after trypsinization by diluting the suspensions from 1 : 2 to 1 : 4. Cells were frozen with a freezing medium (70% MEM, 20% FBS, and 10% dimethyl sulfoxide) in a liquid nitrogen freezer and thawed rapidly at 37°C in a water bath. After centrifugation, thawed cells were suspended in the complete medium described above and continued to cultivate.

2.4. Growth Curve of Cells. Cells at the 12th passage were trypsinized and inoculated in 24-well plates with a density of 50,000 cells per well at 25°C culture temperature. Following that, cells cultured in MEM were randomly collected from three wells per day, and the average cell density was calculated by a hemocytometer. This procedure lasted for 9 days. The growth curve was plotted based on the average number of cells counted each time. The population doubling time (DT) of cells was calculated as follows:

$$\mathrm{DT}\,(h) = t \times \frac{\mathrm{Lg2}}{(\mathrm{LgNt} - \mathrm{lgNo})}, \quad (1)$$

where DT is the culture time of the logarithmic growth, t is the incubation time, and No and Nt are the number of cells at the beginning and the end of the logarithmic growth phase, respectively.

2.5. Immunofluorescence Staining. Growth cultured cells were seeded in 12-well plates at 25°C. When the cells grew to 90%, they were processed with the conventional immunostaining method. Cells fixed with 4% paraformaldehyde were rinsed twice in PBS buffer and permeabilized with 0.3% Triton X-100 for 15 min. Thereafter, cells were incubated with a blocking solution (1% bovine serum albumin) for 50 min followed by overnight incubation with primary antibodies at 4°C: anti-fibronectin (Abcam, ab2413, United Kingdom; 1 : 100), anti-vimentin (CST, 5741, USA; 1 : 100), anti-pancytokeratin (Abcam, ab961, United Kingdom; 1 : 100), and anti-desmin (CST, 5332,

USA; 1 : 100). Next, Alexa Fluor anti-rabbit IgG 555 or anti-mouse IgG 488 (CST, USA; 1 : 500) were used as secondary antibodies for immunostaining. Finally, the stained cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and monitored under an Olympus fluorescence microscope (Olympus, Japan).

2.6. Testing for Microbial Contaminants. The detection of bacteria and fungi in cultured cells was routinely performed according to quality control requirements [26]. Cell cultures were incubated in the test medium (Table 1) for 21 days at 25°C or 37°C under aerobic conditions. Contamination indicated whether colonies appeared on solid media or whether any liquid media became turbid.

Mycoplasma contamination of cell cultures was carried out by Hoechst 33258 fluorescent staining (Sigma-Aldrich, USA) and PPLO agar (Becton Dickinson, USA) plate method [27]. Test results were obtained using an inverted microscope, and small fluorescent particles showed the mycoplasma contamination.

2.7. Chromosome Analysis. The 9th passage muscle cells grown to 80–90% confluency cells were used for chromosome analysis. Cells were cultured in an MEM medium with 20% fetal bovine serum (FBS) at a 25°C incubator and treated with colchicine solution (Sigma-Aldrich, USA) at a final concentration of 20 μL mL^{-1} overnight. Then, the cells were then gently resuspended in 0.75 M KCl for 30–40 min at 30°C in a water bath after being trypsinized, centrifuged, and resuspended. Then wild Yangtze sturgeon muscle cells were fixed with a 3 : 1 fixed solution of methanol and acetic acid three times for 15 min each time. Finally, muscle cell resuspension was dropped onto a precooled glass slide and stained with 10% Giemsa solution (Solarbio, China) for 10 min. The glass slides were calculated and 100 chromosome metaphase images were obtained under a light microscope (Leica, Germany).

2.8. Species Authentication of Muscle Cell Lines. The mitochondrial cytochrome oxidase subunit (COI) and 12S rRNA gene amplification experiments were used to confirm the species derivation of the cell line. Total DNA was isolated from the 16th generation cell lines. The primers for amplifying the target gene were used: 5′-CTACCAGGATTC GGCATGAT-3′ (COI F1) and 5′-GGAAGTGTTGGGGGA AGAAT-3′ (COI R1); 5′-GCTTGGTCCTGGCCTTACTA-3′ (12S F1) and 5′-GTGCACCTTCCGGTACACTT-3′ (12S R1). The PCR reaction system employed was described by Li et al. [28]. Amplified DNA was analyzed by agar gel electrophoresis. The target bands obtained were purified by the purification and recovery kit (Omega, USA) and sequenced by GENEWIZ Inc. (Suzhou, China).

2.9. Cell Transfection. Muscle cells from passage 18 were evenly inoculated into 12-well plates at a density of 2.0×10^5 cells per well. Two micrograms of the pEGFP-N3 expression vector (Clontech) and the lipofectamine 2000 were diluted by an MEM medium supplemented with no antibiotics or serum, respectively. This mixture was treated at room temperature for 20 min in a total volume of 100 μl before being applied to the 12-well plates. After 6 h culture at 25°C, the medium was replaced by MEM containing 10% FBS. The fluorescence expression signals of 24 h transfection were observed under a fluorescence microscope (Olympus Corporation, Japan).

3. Results

3.1. Primary Cell Culture of Postmortem Wild Yangtze Sturgeon. Primary culture results from all tissues showed that only spleen and muscle cells could be observed migrating from their tissues after the explants adhered to the dish surface (Figures 1(a) and 1(b)). Further investigation revealed that only muscle cells could be stably subcultured, which was named WYSM. WYSM gradually proliferated and then formed a cell monolayer within 15 days. Following that, primary cells were subcultured at a split ratio of 1 : 2 or 1 : 3, depending on the cell growth. The MEM medium with 10% FBS and no antibiotics was utilized as the culture medium for the subcultures after cells exhibited stable growth. In the initial generations, the WYSM cells morphologically consisted of spindle-like, fusiform-like, and a very limited number of epithelioid cells. Thereafter, purified fibroblast-like cells could be observed, and WYSM cells were subcultured for more than 25 passages (Figures 1(c) and 1(d)).

The WYSM cell lines were resuspended in a freezing medium and maintained at 4°C for 30 minutes before being transported to −80°C overnight, then transferred to liquid nitrogen every three passages. The revived cells exhibited more than 90% viability and became confluent monolayers within 3–5 days. No discernible morphological or growth alterations were observed after 150 days of cryopreservation.

3.2. Growth Curve of WYSM Cells. As shown in Figure 2, the growth curve of WYSM cells had an obvious "S-shape." The seeded cells entered a latency phase of about 2 days after seeding, which was caused by protease damage, mechanical blowing effect during the passage, and cell adaptation to a new environment. After that, the cell proliferated at a higher rate and showed exponential growth during 3–7 days of culture. The population doubling time (DT) of this cell was calculated to be 52.98 h.

3.3. Immunofluorescence Staining Assay. The WYSM cells were strongly positive for fibronectin and specifically labeled by desmin, while these cells showed no staining for pan-cytokeratin or vimentin (Figure 3). PBS was used instead of primary antibodies for the negative control, and all were negative for the proteins tested. These results confirmed the fibroblastic nature of WYSM cells and their myogenic origin.

3.4. Microbial Analysis. Here, four kinds of growth medium for the detection of bacteria and fungi in cultured WYSM

Table 1: Test protocol and results for detection of bacterial and fungal contamination.

Test medium	Temperature (°C)	Gas phase	Observation time (d)	Results
Tryptic soy broth	25 and 37	Aerobic	21	Negative
Thioglycollate medium	25 and 37	Aerobic	21	Negative
Martin-modified medium	25 and 37	Aerobic	21	Negative
Sabouraud dextrose broth	25 and 37	Aerobic	21	Negative

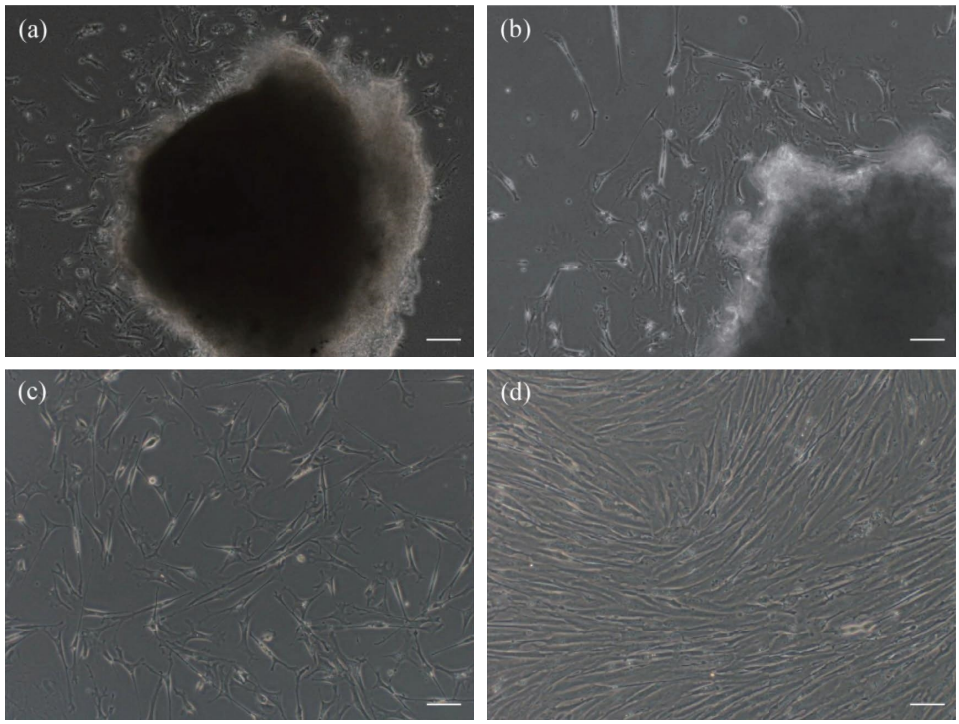


Figure 1: Primary cell culture of wild Yangtze sturgeon tissues. (a) Migrated cells from spleen at 9 d post seeding; (b) migrated cells from muscle at 4 d post seeding; (c) morphological characteristics of muscle cells at 5th passage. (d) Morphological characteristics of muscle cells at 25th passage. Scale bars represent 100 μm.

Figure 2: Growth curves show mean ± SD of cells in triplicate wells. The initial cell density was 5×10^4 mL^{-1}.

cells did not become turbid, confirming that they were not contaminated by bacteria and fungi. Additionally, test results for the broth-agar test and indirect fluorescent staining test were negative for mycoplasma infection (Figure 4). Therefore, the cell lines were confirmed to be free of microbial contamination and could be used for future research.

3.5. Karyotype Analysis of WYSM Cells. A hundred well-spreading metaphases of WYSM cells at passage 9 were counted (Figure 5(a)). Karyotype analysis showed that chromosomes ranged in number from 240 to 267, and the chromosome modal number was 264. The occurrence frequency of 264 chromosomes was 39% (Figure 5(b)).

3.6. Species Authentication of WYSM Cell Lines. The 12S rRNA and COI gene-specific primers of WYSM cells were used for PCR amplification. A 945 bp fragment of 12S rRNA and a 550 bp fragment of COI were obtained (Figure 6), which were consistent with the expected results. The sequence alignment analysis of the two fragments showed 100% similarity to the published sequences in GenBank (AY510085.1), which demonstrated that the WYSM cells in our study originated from *A. dabryanus*.

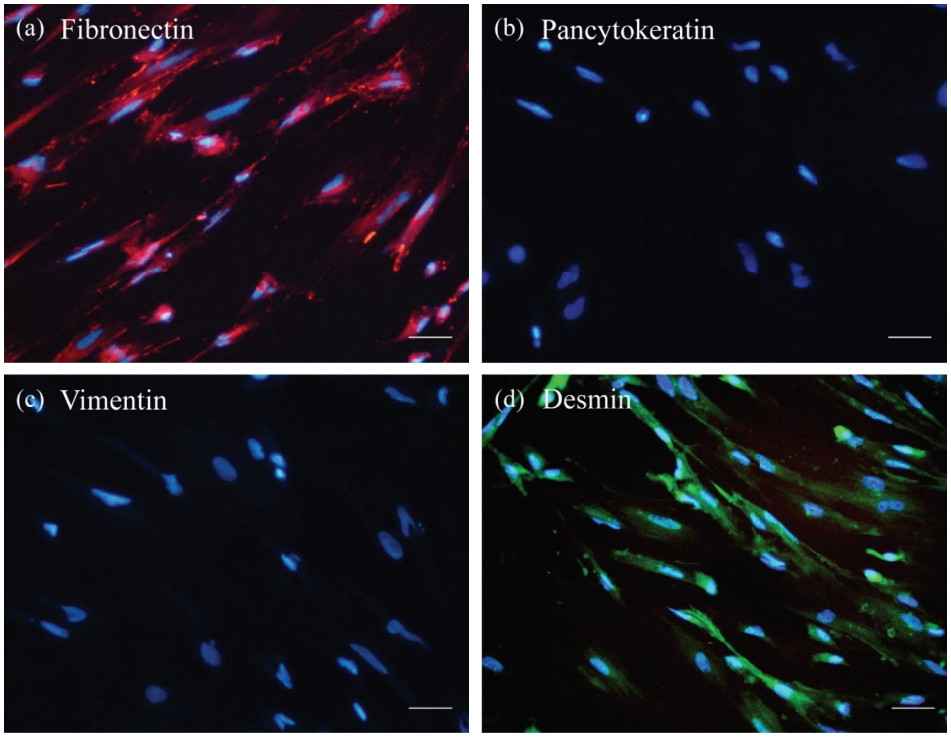


Figure 3: Immunofluorescence staining of WYSM cells. (a) Fibronectin (red) and nucleus marker DAPI (blue); (b) and (c) showed negative staining for pancytokeratin and vimentin, respectively. (d) Desmin (green) and nucleus marker DAPI (blue). Scale bars represent 50 μm.

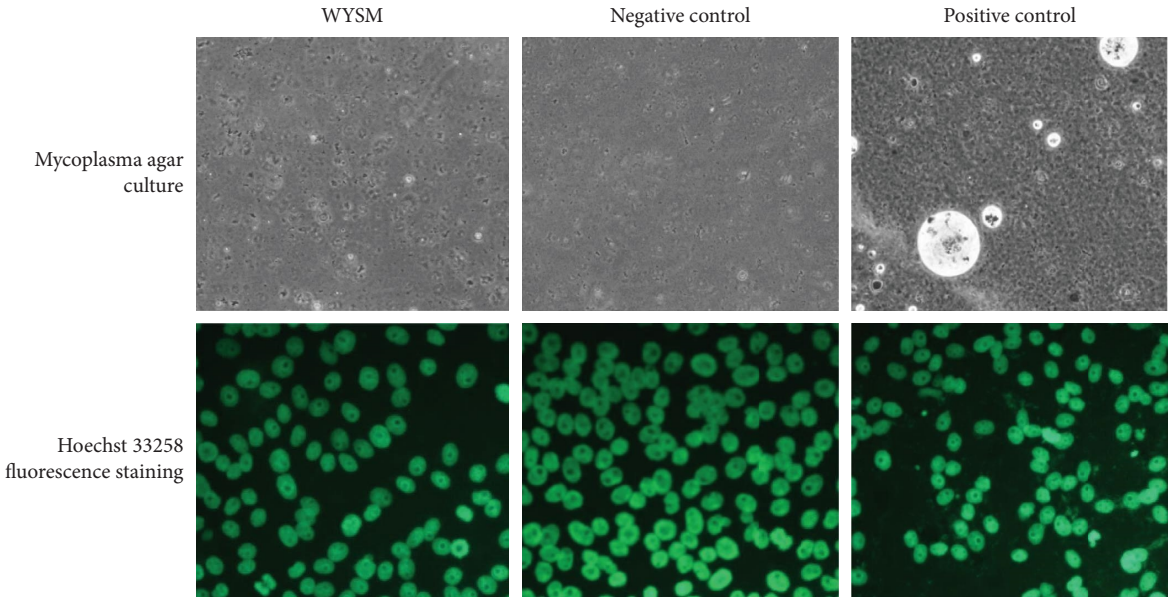
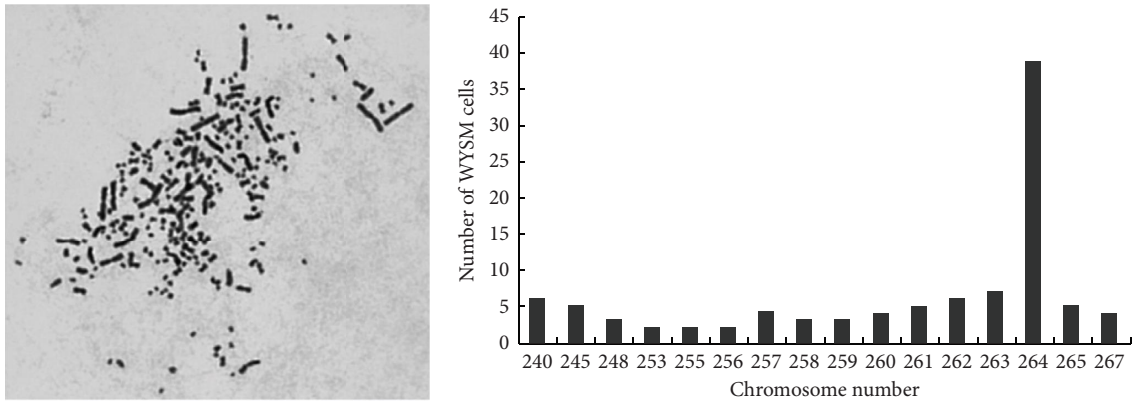


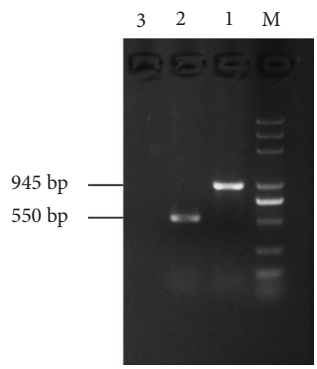
Figure 4: Detection of mycoplasma contamination in cell lines. Mycoplasma colonies seen on positive control agar plates have a fried-egg appearance. The punctate or reticular extracellular fluorescent particles indicated the presence of mycoplasma contamination.

3.7. Cell Transfection. The pEGFP-N3 green fluorescent protein plasmid was successfully transfected into WYSM cells by lipofectamine 2000. The green fluorescent expression of GFP in the WYSM cell line was examined at 24 h post-transfection (Figure 7). By counting all the cells and green fluorescent cells, the transfection efficiency achieved 15%, indicating that the WYSM cell line had a high conversion efficiency for exogenous genes.



(a) (b)

FIGURE 5: Karyotype analysis of WYSM cells. (a) Cellular chromosome of WYSM cells at passage 9 arrested in metaphase. (b) Frequency distribution of chromosomes in 100 spreads. Scale bars represent 10 μm.



12S ribosomal RNA gene 945bp

GCTTGGTCCTGGCCTTACTATCAATTTTAACCCAATTTACACATGCAAGT
CTCCGCACCCCTGTGAGAATGCCCTTAATCCCCCGCCACATAGGGGAAAA
GGAGCAGGTATCAGGCACGCACCCGCAGCCCAAGACGCCTTGCTAAGCCA
CACCCCCAAGGGAACTCAGCAGTGATAAACATTGAGCCATGAGCGCAAGC
TCGACTCAGCCAGAGTTAAGAGGGCCGGTAAAACTCGTGCCAGCCACCGC
GGTTATACGAGAGGCCCCAACTGATAGTCCACGGCGTAAAGCGTGATTAA
AGGACACCTACTACACTAGAGCCAAAAGCCTCCTAAGCCGTCATACGCAC
CTGAAGGCCAGAAGCCCAACCACGAAGGTAGCTCTACCTAACAAGGACCC
CTTGAACCCACGACAACTGAGACACAAACTGGGATTAGATACCCCACTAT
GCTTAGTCATAAACTTTGGTAATAAATTACATATATTACCCGCCAGGGTA
CTACGAGCGCTAGCTTAAAACCCAAAGGACTTGGCGGTGCCCCAGACCCA
CCTAGAGGAGCCTGTTCTAGAACCGATAATCCCCGTTAAACCTCACCACT
TCTTGTCATTACCGCCTATATACCGCCGTCGTCAGCTTACCCTGTGAAAG
ACTAATAGTAAGCAAAAATGGCACACCCAAAAACGTCAGGTCGAGGTGTA
GCGAATGAAGTGGAAAGAAATGGGCTACATTTTCTGACACAGAAAATACA
CGAATAACACTGTGAAACCAGTGATTGAAGGTGGATTTAGCAGTAAAAAG
AAAATAGAAAATTCTTTTGAAGCCGGCTATGGGGCGCGCACACACCGCCC
GTCACTCTCCTCAAAGGAACACCCCAAGTACATAAGTCTACAACCCAAAC
AAGAGGAGGCAAGTCGTAACATGGTAAGTGTACCGGAAGGTGCAC

Cytochrome oxidise subunit I (COI) gene 550bp

CTACCAGGATTCGGCATGATCTCCCACATTGTAGCCTACTATGCCGGCAA
AAAAGAACCTTTTGGCTATATAGGAATAGTGTGGGCCATAATGGCTATTG
GACTACTAGGCTTTATCGTGTGAGCTCATCACATATTTACAGTTGGAATG
GACGTAGACACACGGGCCTATTTTACCTCCGCCACAATAATTATCGCCAT
CCCCACAGGCGTCAAAGTCTTTAGCTGATTAGCCACCCTTCACGGCGGTT
CAATTAAATGAGACACCCCTCTACTATGAGCCCTAGGCTTTATCTTCCTA
TTCACAGTGGGAGGCTTAACGGGAATTGTCTTAGCCAACTCGTCCCTAGA
TATTGTACTTCACGACACCTATTATGTCGTAGCACATTTTCATTATGTAC
TATCAATGGGGGCTGTATTCGCCATTATGGGGGCCTTCGTCCACTGATTC
CCGCTCTTCACAGGTTATACACTACATGGCACCTGATCCAAAATCCACTT
TGCCGTAATATTTGTAGGCGTCAACTTAACATTCTTCCCCCAACACTTCC

(a) (b)

FIGURE 6: Source analysis of WYSM cells in *A. dabryanus* (a) PCR amplification products of 12S rRNA and COI genes, respectively. M, marker: DM2000; lane 1, WYSM 12S rRNA; lane 2, WYSM COI; lane 3, without template (negative control) (b) amplified sequences of 12S rRNA and COI genes of WYSM cells.

4. Discussion

In this work, we successfully cultured and cryopreserved the muscle cells of postmortem critically endangered Yangtze sturgeon.

Eight kinds of tissues from the wild Yangtze sturgeon after death have been attempted for primary culture. The results revealed that the decay degree of fish tissues was significantly different after death. Compared with other tissues, muscle tissue shows better viability, and viable cells can be migrated from the wild Yangtze sturgeon after death at room temperature. Moreover, muscle cells could be subcultured and cryopreserved. In this study, we did not observe the cells migration from the brain, gonad, kidney, and other tissues, or even if cells can be observed migrating, as in spleen tissue, they could not be subcultured and cryopreserved. Based on our experience with cultured animal cells, this may be related to the culture medium used for

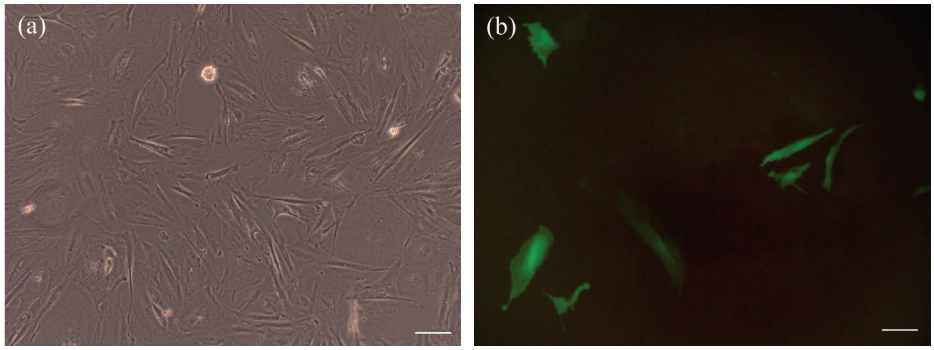


Figure 7: Green fluorescence in 18th passage WYSM cells stably transfected by pEGFP-N3. (a) Light field view of WYSM cells 24 h after transfection. (b) Green fluorescence signal in WYSM cells 24 h after transfection. Scale bars represent 100 μm.

the experiment, but it is possible that this is not the main contributing element. The freshness, oxidation, and microbial contamination of the living tissue may have a greater impact on the smooth migration and passage of tissue cells. Further research may be necessary to determine why muscle tissue in this study is easier to migrate and preserve than other tissues.

WYSM cells showed stable growth and showed no morphological changes up to passage 25. The passage number was restricted to 25 because the biological characteristics of cells preserved as germplasm resources may be adversely affected by more passages and repeated digestion of trypsin [29, 30].

Unlike previously reported cell lines such as fins, kidneys, and gonads [31–33], the cultured cells were not heterogeneous and consisted of a mixture of the polymorphic cells but instead exhibited mostly fibroblast morphology during the initial passages with only a few epithelial cells. A similar phenomenon was described in muscle cells derived from turbot and humpback grouper [34, 35]. A homogeneous population of fibroblast cells was observed after the 8th generation. Furthermore, the immunofluorescence staining results supported the view that the WYSM cells were fibroblastic due to their strong immunoreaction to fibroblastic marker (fibronectin) and a negative reaction to epithelial marker (pancytokeratin), which was different from the positive expression of mesenchymally derived cell marker (vimentin) in some other fibroblasts reported [36]. Myosatellite cells are a type of muscle-derived stem cells that can self-renew and have the potential to differentiate. However, they make up less of a fish's body weight as it ages [37, 38]. Notably, although WYSM cells were derived from the aged wild Yangtze sturgeon (over 35 years old), desmin as a marker of myogenic cell protein was expressed in WYSM cells at a high level in the cytoplasmic compartment.

Cryopreservation of cell lines plays an important role in conserving the genetic resources of threatened species [39]. Compared to DNA extraction, viable cell lines *in vitro* can maintain an organism's genetic information more completely. The preservation of species at the cellular level offers the possibility of the resurrection of endangered and extinct species in the future, and the preservation of cells may reduce material consumption by freezing.

In our study, muscle cells obtained showed more than 90% viability after thawing and demonstrated relatively moderate population doubling times (52.98 h) compared to other muscle cell lines derived from Giant Panda (33.8 h) [40] and triploid olive flounder (69.88 h) [41], respectively. These results indicated that WYSM cells were successfully cryopreserved and revived. In addition, to ensure the quality of cell cryopreservation, microbial contamination of muscle cells was detected using standard test methods. Expectedly, the contamination test results suggested that WYSM cell lines were free of bacteria, fungi, and mycoplasma contamination. Therefore, the cells were suitable for conservation as germplasm resources. This is a prerequisite for maintaining cell line resources.

In addition, karyotype analysis revealed that chromosome numbers increased and decreased in some WYSM cells. Although the chromosome mode number of muscle cells was 264, and 39% metaphase cells exhibited normal chromosomes, most WYSM cells obtained from the wild Yangtze sturgeon 12–14 h postmortem were aneuploidy. The loss of chromosomes in sturgeon cell lines may be an adaptation to the environment *in vitro* culture [42], and cells preserved as germplasm resources should avoid excessive subculture as much as possible.

Mitochondrial genes such as 12S rRNA, 18S rRNA, and COI genes [43, 44] have been commonly used to identify various established cell origin in addition to chromosome analysis. Here, the mitochondrial gene 12S rRNA and the COI gene were amplified and sequenced. Not surprisingly, sequencing and BLAST data showed that the WYSM cell line completely originated from the Yangtze sturgeon and was not cross-contaminated by other cell lines.

Fish cell lines have always been challenging to transfect with foreign plasmids [45]. In this study, the exogenous pEGFP-N3 plasmid was transfected into WYSM cells by lipofectamine 2000, and the foreign GFP gene could be expressed in WYSM cells with 15% transfection efficiency, suggesting that WYSM cell lines could be used as an *in vitro* study model for functional gene analysis and gene targeting.

5. Conclusions

Preservation of genetic diversity is a major objective in conservation programs, as genetic diversity represents evolutionary potential. In this study, the wild *A. dabryanus*

cell resources were successfully rescued, providing precious resources for boosting genetic diversity as well as technical reference and guidance for rescuing and preserving other rare and endangered species.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Disclosure

This manuscript was submitted as a preprint in the link "https://assets.researchsquare.com/files/rs-1344669/v1/cb9ec221-3794-46bf-91a5-92812b89bb79.pdf?c=1657316560".

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Juanjuan Liu carried out the laboratory experiments and wrote the paper. Juanjuan Liu and Hejun Du designed the research and conducted the review. Binzhong Wang and Yacheng Hu provided technical assistance. Xun Zhao and Hongtao Huang helped to collect sample tissues.

Acknowledgments

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