Germplasm rescue of post-mortem critically endangered Yangtze Sturgeon by cell cryopreservation

Juanjuan Liu (05juanjuan@163.com)
China Three Gorges Corporation https://orcid.org/0000-0001-9638-7075
Binzhong Wang
China Three Gorges Corporation
Yacheng Hu
China Three Gorges Corporation
Hongtao Huang
China Three Gorges Corporation
Xun Zhao
China Three Gorges Corporation
Hejun Du
China Three Gorges Corporation

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Abstract

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 urgently to rescue the genetic resources of every wild Yangtze sturgeon. Here, we isolated and preserved viable cells from the post-mortem critically endangered Yangtze Sturgeon for the first time.

Attempt of rescuing and preserving cell resources were carried out from 8 tissues, brain, kidney, heart, fin, liver, skin, spleen and muscle of over-35-years-old female wild Yangtze sturgeon between 11 and 14 hours after death at 19.8℃ in outdoor concrete pond, and only muscle tissue cells could be successfully sub-cultured and preserved. Furthermore, the cultured cells were assessed by population doubling time, immunofluorescence analysis, microorganism detection, karyotyping and origin identification, and no abnormality was observed in the morphology, structure, growth and ploidy. These results suggest that viable cells could also be successfully isolated and cryopreserved from the wild Yangtze sturgeon in a short time after death, and muscle tissues had more potential to separate living cells than other tissues.

This report is not only of great significance to the germplasm rescue of critically endangered Yangtze sturgeon, but also provides some scientific reference for the germplasm preservation of other endangered fish.

Introduction

Since the 17th century, the explosion of human populations and the intense intervention of human activities have led to the accelerated extinction of many species. In contrast, large animals and freshwater animals are more vulnerable to extinction (Primack & Ji 2000; Turvey et al. 2010). The rescue protection of large endangered species has gradually become the focus of attention. Entering the 21st century, all three large sturgeons in the Yangtze River, the Chinese paddlefish (*Psephuyrus gladius*), Chinese sturgeon (*Acipenser sinensis*) and Yangtze sturgeons (*Acipenser dabryanus*), are critically endangered: the Chinese paddlefish has been declared functionally extinct (Zhang et al. 2020), the reproduction in the wild of Chinese sturgeon has been interrupted in 2013, 2015 and 2017–2020, and unfortunately the reproduction in the wild of Yangtze sturgeon in the wild stopped at the end of last century (IUCN 2010). So far, there are less than 20 wild individuals in captivity, and most of them lose their chance to reproduce as they age or die. At the same time, the wild Yangtze sturgeon resources in the Yangtze River also decreased sharply. Therefore, 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 (Morrell et al. 2017), especially to the cell resources from

threatened or endangered species (Cao et al. 2021; Liu et al. 2019). In contrast to gametes and embryos, somatic cells are more resistant to cryopreservation stress as well as more easily to be collected from valuable species of inaccessible or even dead (Thélie et al. 2019; Wang et al. 2020). In addition, recent progress in modern biotechnologies such as somatic cell nuclear transfer (Matoba et al. 2018) and induced pluripotent stem cells (iPSCs) (Yu et al. 2007; Peng et al. 2019) have also demonstrated their potential implications in the maintenance and rescue of threatened or extinct species. Successful cloning of animals including sheep (Wilmut et al. 1997), mice (Wakayama et al. 1998), pig (Betthauser et al. 2000; Fan et al. 2013) and monkey (Liu et al. 2018) has been reported in several studies. The technique has early been reported to be successful in fish cloning since the 1960s (Tung et al. 1963, Chen et al. 1986; Wakamatsu et al. 2001). In particular, there are also successful cases of cloning cells using postmortem somatic cells (Hoshino et al. 2009; Loi et al. 2001), which provides confidence for the rescue preservation of genetic resources of endangered animals, especially for those found in a short period after death. Therefore, somatic cell cryopreservation can be farsightedly considered as one feasible means to preserve and restore genetic resources (Frankham et al. 2002).

Although many efforts to conserve the postmortem tissue cells are ongoing, mainly in humans and mammals (Wang et al. 2020; Palmer et al. 2001), there are still no reports about the successful preservation cells from dead fish.

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 time. Through the culture of 8 tissues, the viable muscle cells were finally obtained and cryopreserved. This technique has important practical value for further exploration of the rescue conservation of large endangered fish genetic resources.

Materials And Methods

Tissue Collection

The Yangtze sturgeon sampled was raised in outdoor concrete pond of the Chinese Sturgeon Research Institute, China Three Gorges Corporation. It is an over-35-years-old wild female captured from the Yangtze River around 1990. Eight tissues including brain, kidney, heart, fin, liver, skin, spleen and muscle were excised from it dead after 11–14 hours at a pond temperature of 19.8℃ (Fig. 1) and transported to the laboratory within 10 minutes. All tissues were quickly disinfected with 75% alcohol and rinsed several times with 1× phosphate-buffered saline (PBS) containing 1% Antibiotic-Antimycotic solution (Gibco), and temporarily stored in sterile petri dishes for in vitro culture.

Primary cultures, secondary cultures and cryopreservation

All tissue explants were cultured in vitro as described earlier (Liu et al. 2020), respectively. After disinfected and washed several times, the explants were chopped into about 1 mm^3 size pieces and adhered onto 25cm^2 tissue culture flasks (Corning). The small pieces cultured in complete medium

consist of minimum essential medium (MEM, Gibco), 20% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin solution. The flasks were incubated at 25°C in carbon dioxide incubator with 5% CO2 (Thermo Fisher Scientific). In our experience, the culture flasks should not be removed so that sample cells can attach 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 media was changed every 3 day. The 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 every 3 generations with freezing medium (70% MEM, 20% FBS, and 10% dimethyl sulfoxide) in liquid nitrogen freezer and thawing quickly at 37°C water bath. After centrifugation, thawed cells were suspended in complete medium described above and continued to cultivate.

Growth curve of cells

The cells at 12th passage were trypsinized and inoculated in 24-well plates with a density of 50,000 cells per well at 25°C culture temperature. Subsequently, cells maintained in MEM were randomly collected from three wells per day and the average cell density was calculated by a hemocytometer. The test lasted for 9 days. The growth curve was plotted according to the average number of cells counted each time. The population doubling time (DT) of cells was calculated as follows:

$$DT\ (h) = t \times Lg2 / (LgNt - lgNo)$$

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

Immunofluorescence staining

Grow 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, then permeabilized with 0.3% Triton X-100 for 15min. After that, cells incubated with blocking solution (1% bovine serum albumin) for 50 min followed by incubation overnight with the 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 Olympus fluorescence microscope (Japan).

Testing for microbial contaminants

The detection of bacteria and fungi in cultured cells was performed routinely according to the quality control requirements (Langdon et al. 2004). Cell cultures were incubated in test medium (Table 1) for 21 days at 25°C or 37°C under aerobic conditions. Contamination was indicated if colonies appeared on solid media or if any of the liquid media become turbid.

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
Table.2 cell culture results of six kinds of postmortem tissues				

Mycoplasma contamination of cell cultures has been carried out by Hoechst 33258 fluorescent staining (Sigma) and PPLO agar (Becton, Dickinson) plate method (Cobo et al. 2007). The test results were obtained using an inverted microscope and small fluorescent particles showed the mycoplasma contamination.

Chromosome analysis

The 9th passage muscle cells grown to 80–90% confluency cells were used for chromosome analysis. Cells cultured in MEM medium with 20% fetal bovine serum (FBS) at 25°C incubator and treated with colchicine solution (Sigma) at a final concentration of 20 μL mL^{-1} for overnight. Then, cells were trypsinized, centrifuged and gently resuspended in 0.75M KCl for 30–40 min at 30°C water bath. 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, resuspension of muscle cells was dropped onto a pre-cooled 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).

Species Authentication of Muscle cell lines

The mitochondrial cytochrome oxidase subunit I (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'-CTACCAGGATTCGGCATGAT-3' (COI F1) and 5'-GGAAGTGTTGGGGGAAGAAT-3' (COI R1); 5'-GCTTGGTCCTGGCCTTACTA-3' (12S F1) and 5'-GTGCACCTTCCGGTACACTT-3' (12S R1). The PCR reaction system employed was described by Li (Li et al., 2015). Amplified DNA was analyzed by agar gel electrophoresis. The obtained target bands were purified by the purification and recovery kit (Omega, USA), and then sequenced by GENEWIZ Inc., (Suzhou, China).

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 pEGFP-N3 expression vector (Clontech) and the lipofectamine 2000 were diluted by MEM medium supplement with no antibiotics or serum respectively. A total volume of 100 μl this

mixture were processed at room temperature for 20 min, then added into the 12-well plates. After 6 h of culture at 25℃, the medium was replaced by MEM containing 10% FBS. The fluorescence expression signals of 24 h transfection were observed under fluorescence microscope (Olympus Corporation, Japan).

Results

Primary cell culture of post-mortem wild Yangtze sturgeon

Primary cell culture was performed on brain, kidney, heart, fin, liver, skin, spleen and muscle of post-mortem wild Yangtze sturgeon, respectively. Table 2 shows the results of in vitro culture of different tissues. Only spleen and muscle cells could be observed to migrate from their tissues after the explants adhered to dish surface (Fig. 2a-b). Further research showed that only muscle cells could be stably subcultured, which was named WYSM. Fibroblasts WYSM gradually proliferated and then formed a cell monolayer within 15d. Subsequently, primary cells were subcultured at a split ratio of 1:2 or 1:3 according to the growth of the cells. The culture medium used for subcultures was MEM medium contained with 10% FBS and free of antibiotics after the cells exhibited stable growth. The WYSM cells morphologically consisted of spindle-like, fusiform-like and a very small number of epithelioid cells in the first few generations. Thereafter, purified fibroblast-like cells could be observed and WYSM cells were subcultured for more than 25 passages (Fig. 2c-d).

The WYSM cell lines were resuspended in freezing medium and kept for 30min at 4℃,then cell vials were placed in -80℃ overnight and finally transferred to liquid nitrogen every three passages. The revived cells exhibited more than 90% viability and became confluent monolayer within 3–5 days. No significant changes in morphology or in growth were observed after cryopreserved for 150 days.

Growth curve of WYSM cells

As shown in Fig. 3, 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 passage and adaptation of cells to a new environment. After that the cell proliferated at a higher rate and showed an exponential growth during 3–7 d of culture. The population doubling time (DT) of this cell was calculated to be 52.98 h.

Immunofluorescence staining assay

The WYSM cells were strongly positive for fibronectin and specifically labeled by desmin, whereas these cells did not show any staining for pancytokeratin or vimentin (Fig. 4). PBS was used instead of primary antibodies for negative control and all were negative for the proteins tested. These results confirmed WYSM cells a fibroblastic nature and their myogenic origin.

Microbial analysis

The prevention and avoidance of microbial contamination is one of the keys to the success of cell culture, especially for some important and rare cell lines (Drexler et al. 2002; Jean et al. 2017). It's essential to monitor contamination in cell cultures. Here, four kinds of growth medium for the detection of bacteria and fungi in cultured WYSM cells did not become turbid, thus confirming they were not contaminated by bacteria and fungi. Additionally, test results of both methods for broth-agar test and indirect fluorescent staining test were presented negative for mycoplasma infection (Fig. 5). Therefore, the cell lines were confirmed to be free of microbial contamination and could be used for subsequent research.

Karyotype analysis of WYSM cells

A hundred well spread metaphases of WYSM cells at passage 9 were counted (Fig. 6a). Karyotype analysis results indicated that the chromosomes ranged in number from 240 to 267, and the chromosome modal number is 264. The occurrence frequency of 264 chromosomes was 39% (Fig. 6b), which is in accordance with the reported chromosome number of Yangtze sturgeon (Liu et al. 2020).

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 (Fig. 7), which were consistent with the expected results. The sequence alignment analysis of the two fragments showed 100% similarity to the published sequences in the GenBank (AY510085.1), which proved that WYSM cells in our study originated from *A. dabryanus.*

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 WYSM cell line was examined at 24 h post-transfection (Fig. 8). By counting all the cells and green fluorescent cells, the transfection efficiency achieved 15%, indicating that the WYSM cell line had a highly conversion efficiency for exogenous genes.

Discussion

In this research, we successfully cultured and cryopreserved the muscle cells of post-mortem critically endangered Yangtze Sturgeon.

Eight kinds of tissues from wild Yangtze Sturgeon after death have been attempted for primary culture. The results revealed that the rate of apoptosis varies among different tissues after death for the Yangtze sturgeon. Compared with other tissues, only muscle tissue shows better viability and viable cells can migrated from wild Yangtze Sturgeon after death at room temperature. Moreover, muscle cells could be subcultured and cryopreserved. On the contrary, tissues such as the brain, heart, and kidneys seem to be deactivated soon after death and unable to migrate out of cells, or even if cells could be observed

migrating, as in spleen tissue, they could not been subcultured and cryopreserved. These results suggested that muscle, located between skin and visceral tissue, more likely to avoid suffering the effect from environmental oxidation and autolysis of internal organs, thus have the greater potential to maintain viability.

WYSM cells showed a stable growth and have not shown significantly morphological changes up to passage 25. The passage number was restricted to 25th since biological characteristics of cells preserved as germplasm resources may be adversely affected by more passages and repeated digestion of trypsin (Yang et al. 2018; Kumar et al. 2019).

Unlike previously reported cell lines such as fins, kidneys and gonads (Pao et al. 2019; Suryakodi et al. 2021; Xu et al. 2022), the cultured cells were not heterogeneous consisted of a mixture of polymorphic cell, but instead exhibited mostly of fibroblast morphology during the initial passages with only a few epithelial cells. Similar phenomenon was described in the muscle cells derived from turbot and humpback grouper (Gao et al. 2019; Wang et al. 2020). A homogeneous population of fibroblasts cells was observed after the 8th generation. Furthermore, the immunofluorescence staining results supported the opinion that the WYSM cells were fibroblastic in nature due to its 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 (Vo et al. 2019). Myosatellite cell is a kind of muscle derived stem cells that is able to self-renew and have the potential to differentiate, however, it makes up less and less of a fish's body weight as it ages (Koumans et al. 1991; Wang et al. 2012). What is noteworthy, although WYSM cells derived from the aged wild Yangtze sturgeon (over 35 years old), we found that desmin as a myogenic cell protein marker was expressed in WYSM cells at a highly level in the cytoplasmic compartment.

Cryopreservation of cell lines plays an important role in preserving the genetic resources of threatened species (Cetinkaya et al. 2011). In our study, the obtained muscle cells showed more than 90% viability after post-thaw and demonstrated a relatively moderate population doubling times (52.98h) compared to other muscle cell lines, derived from Giant Panda (33.8h) (Yu et al. 2015) and triploid olive flounder (69.88h) (Peng et al. 2016), respectively. These results indicated that the WYSM cells have been 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 testing results suggested that the 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 the preservation of cell line resources.

Furthermore, karyotype analysis revealed that the number of chromosomes 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 of the WYSM cells obtained from the of 12 ~ 14 hour postmortem wild Yangtze sturgeon were aneuploidy. The loss of chromosomes in sturgeon cell lines

may be an adaptation to the environment in vitro culture, and cells preserved as germplasm resources should be avoid excessive subculture as much as possible.

Mitochondrial gene such as 12S rRNA, 18S rRNA and COI genes (Wei et al. 2018; Ahmed et al. 2009) have been commonly used to identify various established cells origin, in addition to chromosome analysis. Here, mitochondrial gene 12S rRNA and COI gene were amplified and sequenced. Not surprisingly, the data obtained from sequencing and BLAST showed that the WYSM cell line was completely originated from Yangtze sturgeon and not cross-contaminated by other cell lines.

It has always been difficult to transfect foreign plasmids with fish cell lines (Schiøtz et al. 2011). In this study, 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.

Conclusions

Preservation of genetic diversity is a major objective in conservation programs, as genetic diversity represents evolutionary potential. In this study, the cell resources of wild *Acipenser dabryanus* were successfully rescued, which provided precious resources for enhancing genetic diversity as well as technical reference and guidance for rescuing and preserving other rare and endangered species.

Declarations

Declarations

Conflict of interest

The authors declare no competing interest.

Ethics approval

The study was carried out in accordance with 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.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

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Author contributions

JJL carried out the laboratory experiments and wrote the paper. BZW and YCH provide technical assistance. XZ and HTH helped to collect sample tissue. JJL and HJD designed the research and conducted review.

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Figures

Figure 1

Morphology and characteristics of wild Yangtze sturgeon after 11-14 hours death

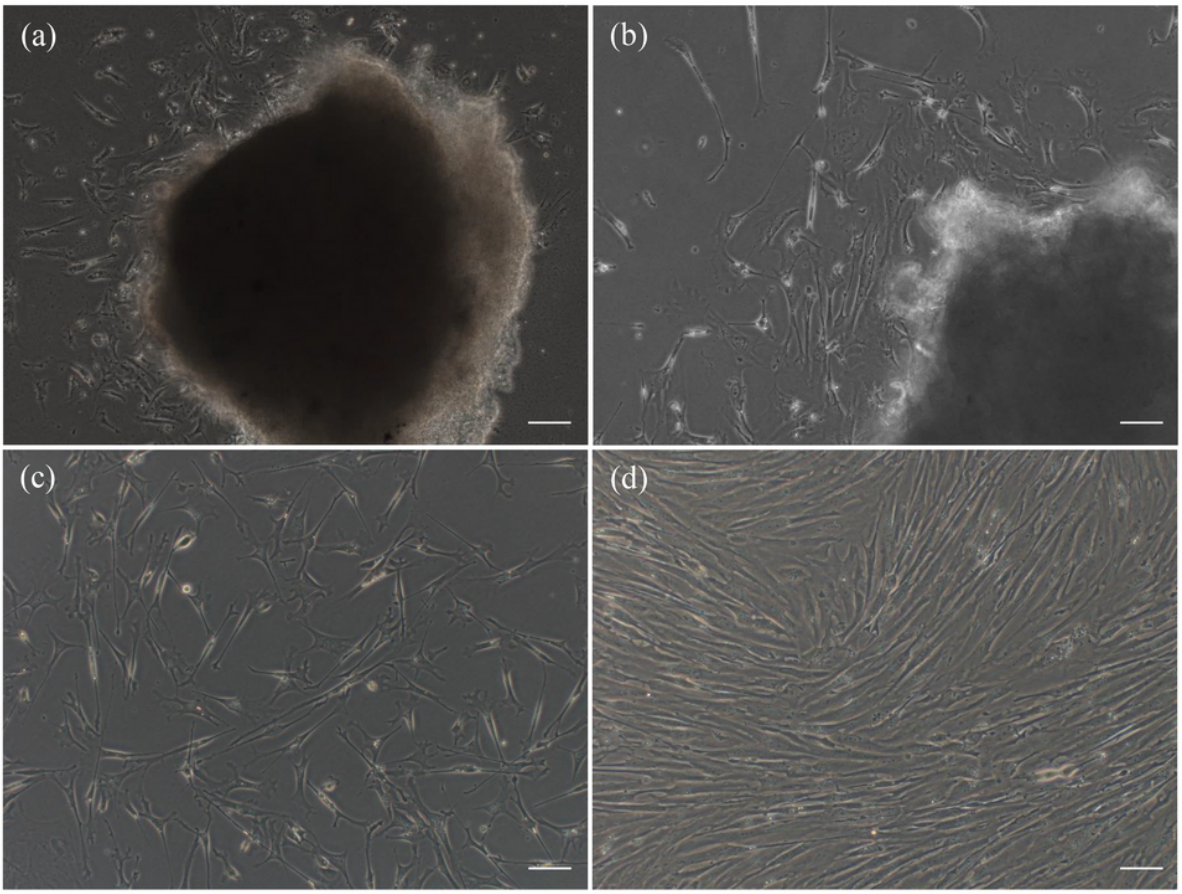


Figure 2

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

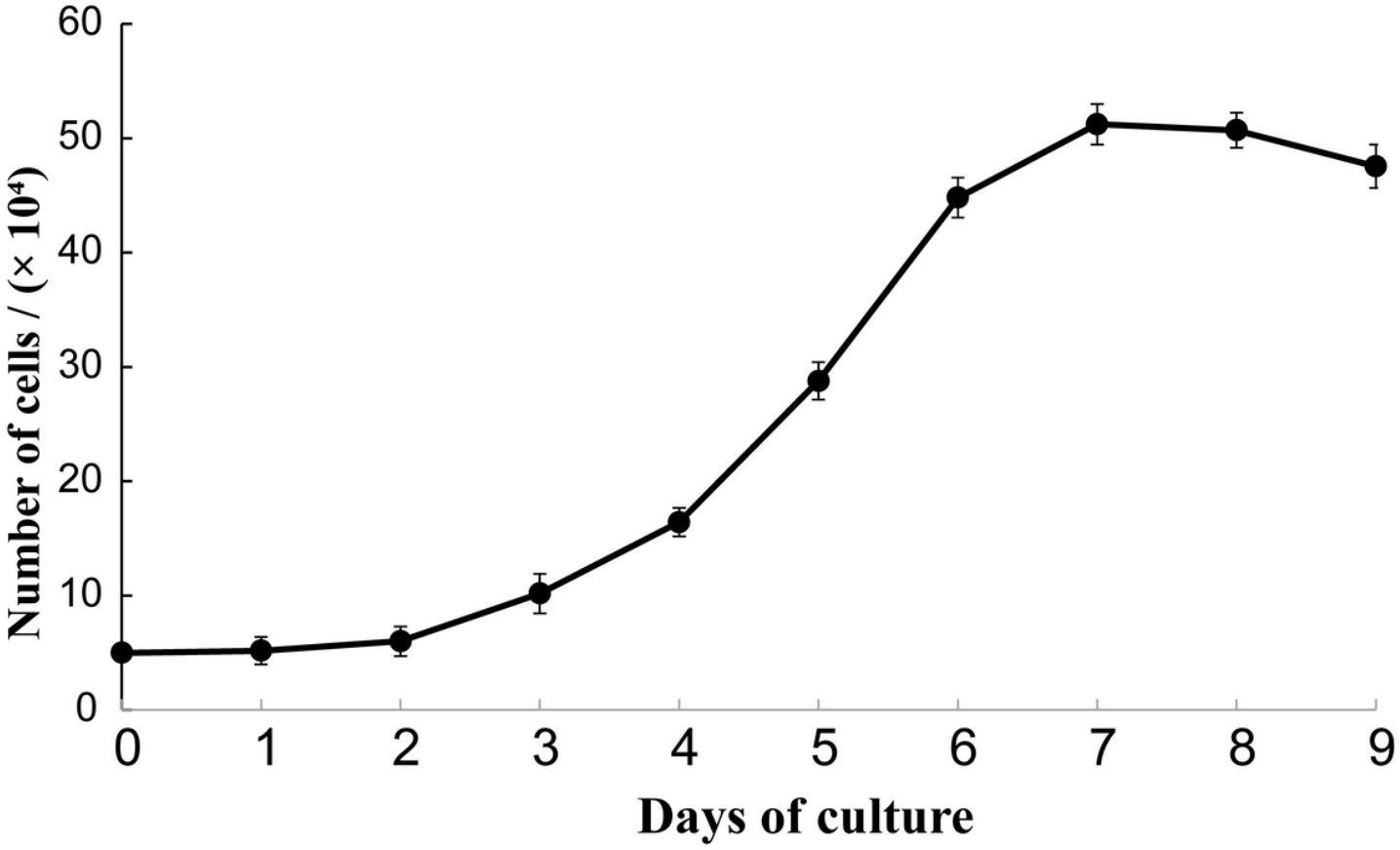


Figure 3

Growth curve of WYSM cells at the 12th passage. The initial cell density was 5×10^4 mL^{-1}

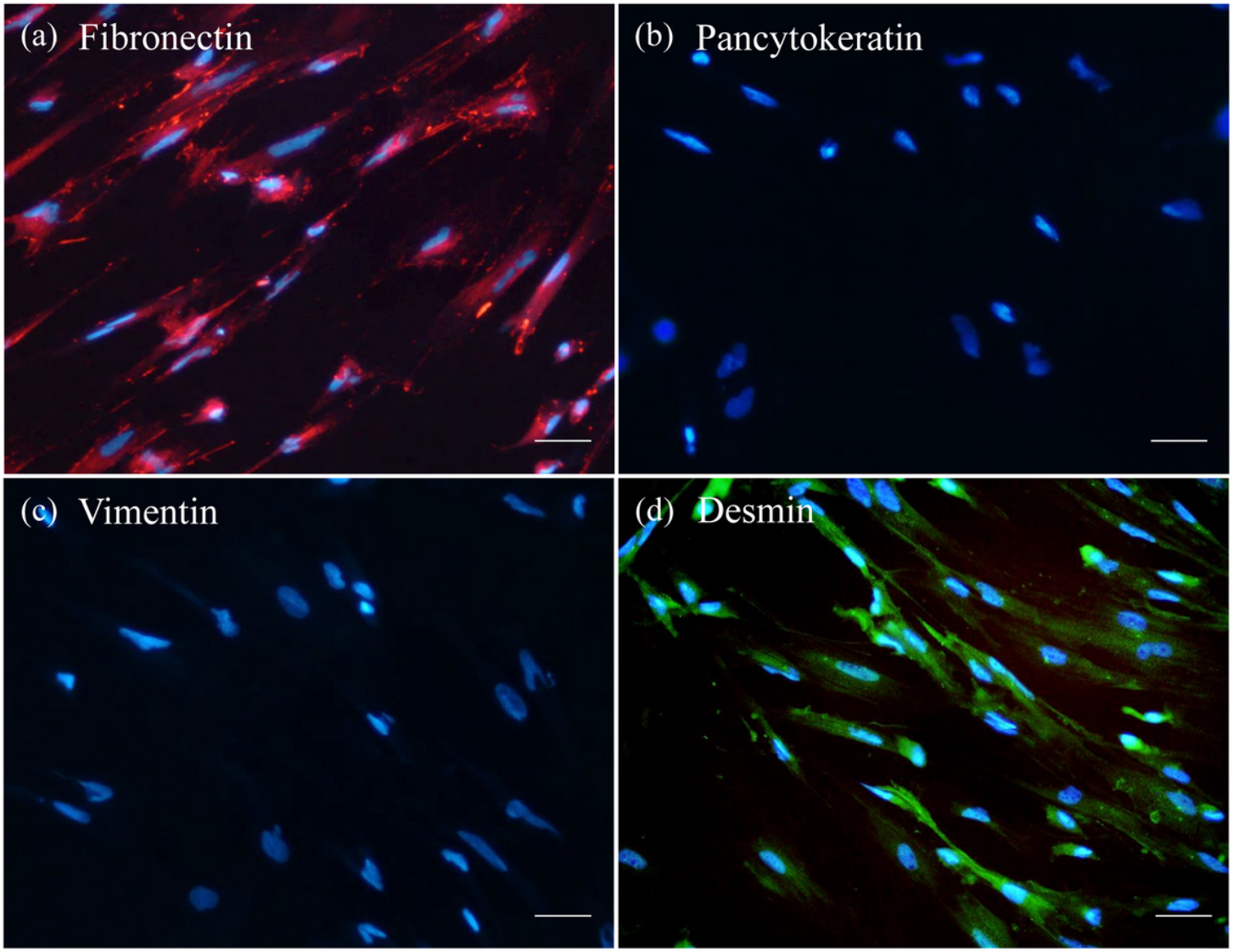


Figure 4

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

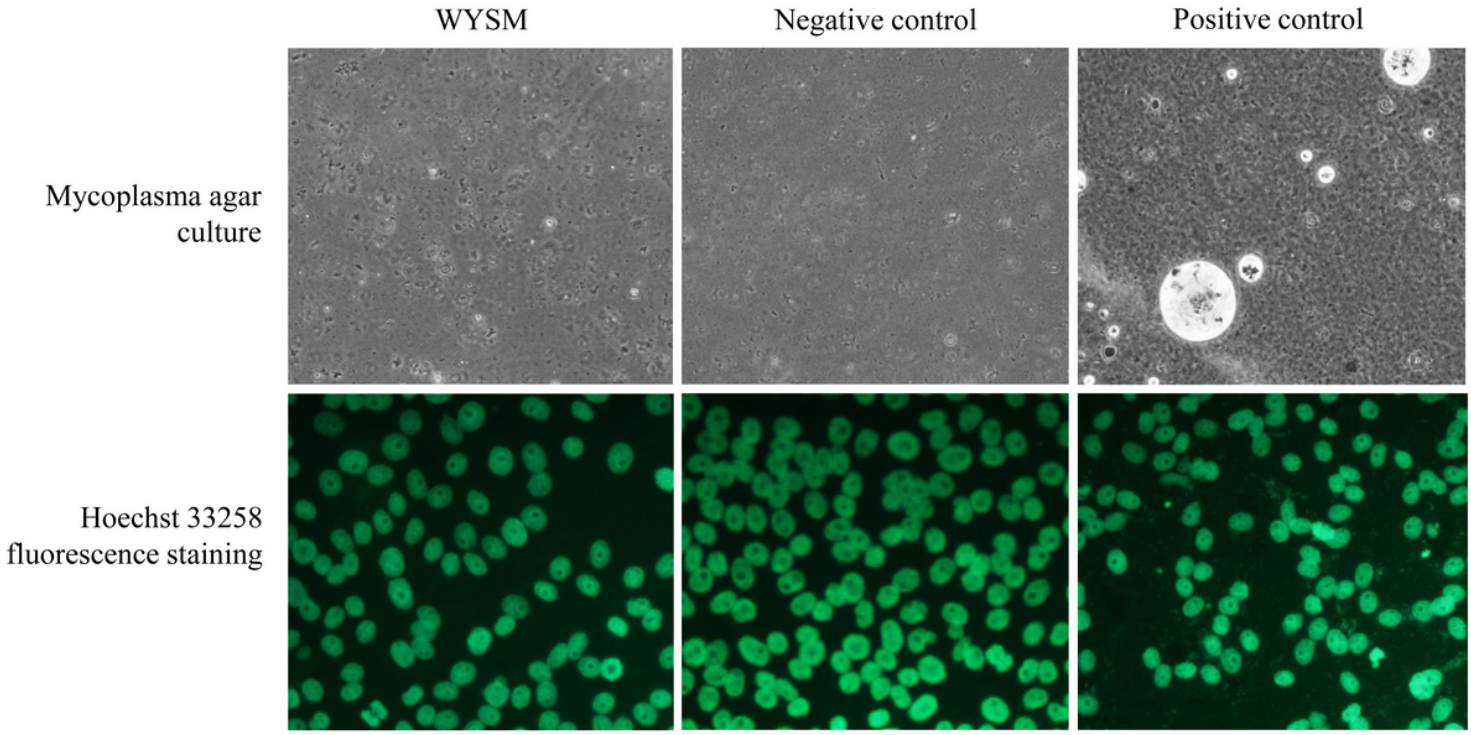


Figure 5

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

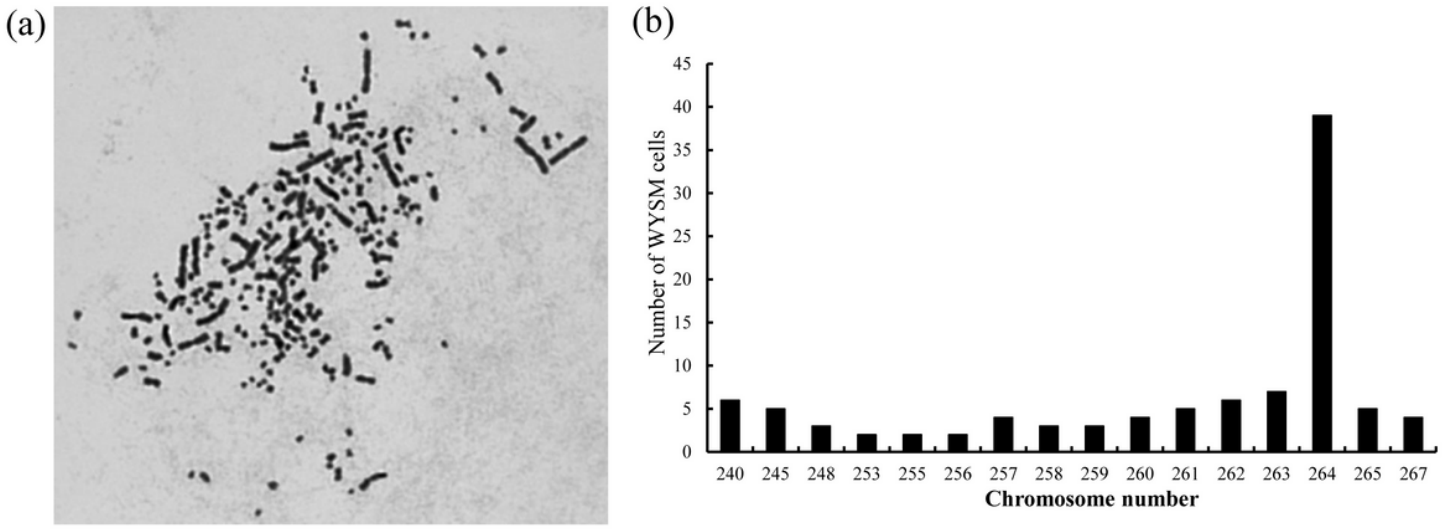
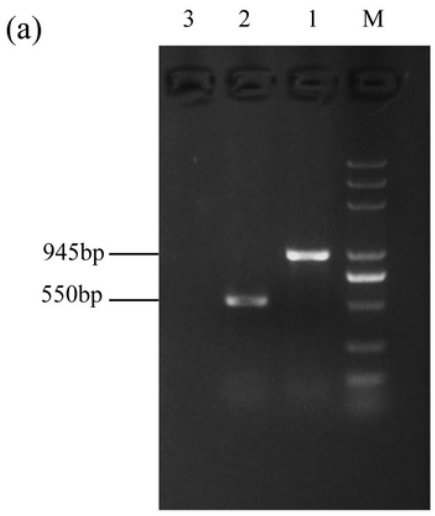


Figure 6

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



(b) 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

Figure 7

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

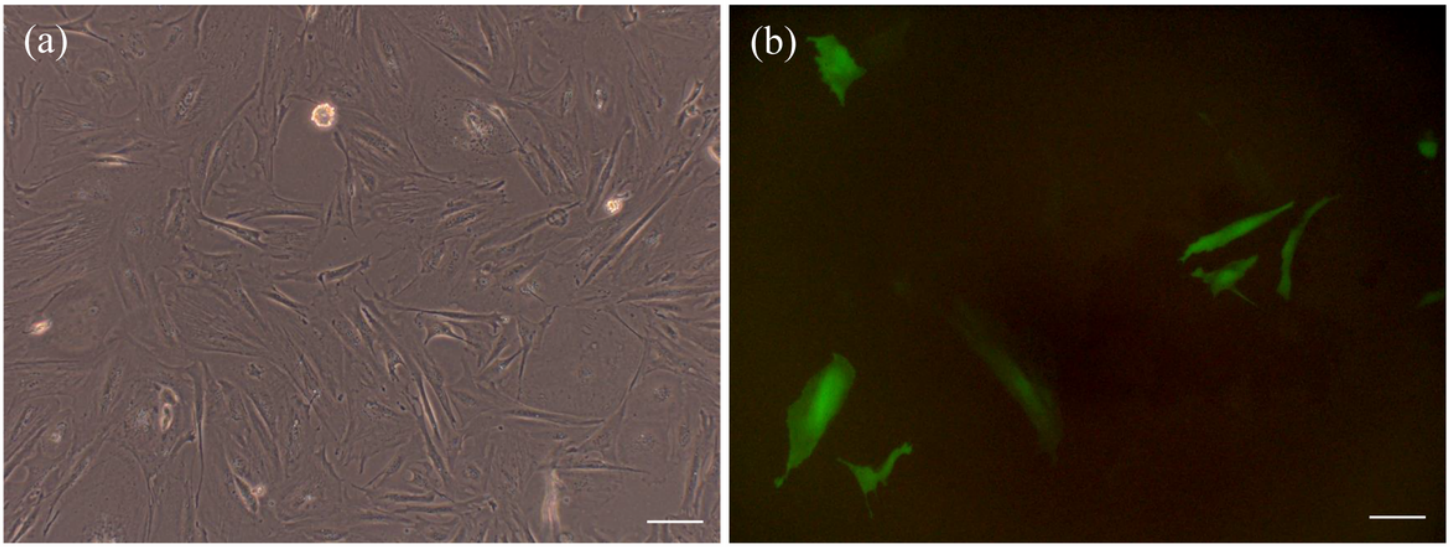


Figure 8

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