CryoLetters **31** (2), 130-138 (2010)

ESTABLISHMENT AND CRYOPRESERVATION OF A FIBROBLAST CELL LINE DERIVED FROM BENGAL TIGER (PANTHERA TIGRIS TIGRIS)

W.J. Guan[1*], C.Q. Liu[1, 2*], C.Y. Li[3], D. Liu [4], W.X. Zhang[1], Y.H. Ma[1**]

[1]Institute of Beijing Animal Science and veterinary, CAAS, Beijing, 100094, China
[2]Bioscience Department, Bengbu Medical College, Bengbu, 233000, China
[3]College of Wildlife Resources, Northeast Forestry University, Harbin, 150040, China
[4]The Northeast Tiger Wooden Land of Heilongjiang; Harbin 150028; China
[*]These authors contributed equally to this work.
[**] Corresponding author: e-mail: wjguan86@iascaas.net.cn; Yuehui.ma@263.net

Abstract

The Bengal tiger ear marginal tissue fibroblasts cell line (BTF22), containing 157 tubes of frozen cells, was successfully established by using primary explants technique and cell cryoconservation technology. Biological analysis showed that the population doubling time (PDT) for revival cells was approximately 28 h. Measurement of LDH and MDH isoenzymes showed no cross-contamination among the cells. Karyotyping showed that the frequency of cells with chromosome number 2n=38 was 90.6–92.2%. Tests for bacteria, fungi, viruses and mycoplasma were negative. Plasmids encoding the fluorescent proteins pEGFP-N3, pEGFP-C1, pECFP-N1, pECFP-mito, pDsRed1-N1, and pEYFP-N1 were transfected into cells to study exogenous gene expression in the cells. The plasmid transfection efficiency was between 4.4% and 31.9%. Every index of the BTF22 cell line meets all the standard quality controls of American type Culture Collection (ATCC). Not only has the germline of this important Bengal tiger species been preserved at the cell level, but also valuable material had been provided for genome, postgenome and somacloning research. Moreover, the establishment of this technical platform would provide both technical and theoretical support for storing the genetic resources of other animals and poultry at the cell level.

Keywords: Bengal tiger; Fibroblast Cell line; Establishment; Characterization; Cryopreservation

INTRODUCTION

Tiger (*Panthera tigris Linnaeus*, 1758) is a special species only found in Asia and considered as a symbol of beauty, power, and bravery. There are four generally accepted tiger subspecies in China, Siberian tigers (*P. t. altaica*), Indochinese tigers (*P. t. corbetti*), South China tigers (*P. t. amoyensis*), and Indian or Bengal tigers (*P. t. tigris*). Today an estimated fewer than 4580 Bengal tigers survive in Bangladesh, Nepal, eastern India, Bhutan, Burma and southwest China, and less than 30 now exist in China (14, 27).

The tiger is warranted the highest level of protection by the Convention on International Trade in Endangered Species of Wild Fauna & Flora (CITES). In 1989, the Chinese government brought Bengal tiger into category I of National Protected Animal breed. In order to preserve the wealth of biodiversity in China, and to uncover the complex underlying interactions between organisms and environment, there is a very urgent need to commence rigorous conservation of endangered species. Preservation of individual animals, semen, embryos, genomic libraries and cDNA libraries are all practical options. In addition, modern cloning techniques have made somatic cells an attractive resource for conserving animal genetic materials (22, 31).

Up to now, there is little research information about Bengal tiger and no report about its skin fibroblast cell incubation. In our report, we used the combined method of cell viability, microorganism detection, chromosome analysis, isoenzyme analysis, and fluorescent protein genes transfection to detect the established Bengal tiger cell line (named BTF22 line) overall. Our project is to cryopreserve this international protected genomic resource for the purposes of reviving endangered breed by cloning and supplying a convenient and effective resource for genomic research. Moreover, with the development of science and technology, the roles of limited cell lines will become increasingly prominent, and there will be currently unforeseen applications.

MATERIALS AND METHODS

Cell cultures

Ear tissue samples (about 1 cm^2 in size) were sampled from 22 individuals of Bengal tiger (12 male and 10 female) and collected into separate tubes containing Dulbecco's modified Eagle's medium (DMEM) medium with ampicillin (100U/ml) and streptomycin (100μg/ml). Primary cell culture and subculturing for details of the procedure see Zhou et al. (32) and Liu et al. (16).

Growth curve and Estimation of cell viability by Trypan Blue dye

In accordance with the method of Gu et al. (9), cells at 1.5×10^4 ml^{-1} were seeded into 24-well plates. Cell growth and density data were monitored and recorded on a daily basis for density until they reached the plateau phase. Cell growth curves were plotted, and the population doubling time (PDT) was calculated based on the growth curve. Assays of cell viability before freezing or after recovery were carried out by using the Trypan Blue vital stain method, and 1,000 cells were counted for viability (23).

Cryogenic preservation and recovery

Cells in logarithmic growth phase were enumerated with a hemocytometer. Harvested cells were resuspended in freezing medium (10% DMSO+ 90% fetal bovine serum) to a final density of $(3–4)\times10^6$ viable cells/ml. Single cells were dispensed into sterile plastic cryogenic vials labeled with animal name, gender, freezing serial number and the date. The vials were sealed and kept at 4°C for 20–30 min to allow the DMSO to equilibrate, then after programmed freezing they were transferred to storage in liquid nitrogen storage system quickly and efficiently.

To recover and reseed the cells, the frozen tubes were removed from liquid nitrogen storage system and quickly thawed in 42°C water bath, and then the cells were transferred into a flask with complete medium and cultured at 37°C with 5% CO_2 (8).

The measurement of microorganism of BTF22 cell line

Tests for contamination with bacteria, fungi and yeasts: for details of the procedure used, see Doyle et al. (6).

Test for viruses: Hay's hemadsorption protocol was used routinely to examine the samples for cytopathogenesis using phase-contrast microscopy (10).

Mycoplasma detection*:* The cells were stained with Hoechst 33258 according to the DNA fluorescent staining protocol to detect whether they were contaminated by mycoplasma (19).The enzyme-linked immunosorbent assay Mycoplasma Detection kit (Roche Diagnostics Corp) was used to detect the four most common mycoplasma species (*M.arginini, M.hyorhinis, A.laidlawii, M.orale*).

Chromosome analysis

Chromosomes were prepared, fixed and stained following standard methods (11). After Giesma staining, the chromosome numbers per spread were counted for 100 spreads under an oil immersion objective. Relative length to arm ratio and centromeric index and type were counted according to the protocol of Sun et al. (26) and Kawarai et al. (13).

Isoenzyme analysis

The electrophoretic mobilities of lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) were determined using the polyacrylamide gel electrophoresis protocol contributed by Wu et al. (31). Mobility was measured as the ratio of distance migrated by the band to the distance migrated by the indicator dye.

Expression of fluorescent protein genes in Bengal tiger fibroblastic cell

To obtain the highest transfection efficiency and low cytotoxicity, transfection conditions were optimized by varying cell density and concentrations of fluorescent protein vectors pEGFP-N3, pEGFP-C1, pECFP-N1, pECFP-mito, pDsRed1-N1, and pEYFP-N1 plasmid DNA (BD Bioscieces Clontech) and Lipofectamine™ 2000 (Invitrogen Corp., Carlsbad, CA), according to lipofectamine media methods of Escriou et al. (7) and Tsuchiya et al. (29). To estimate the transfection efficiency, the cells were observed after being transfected for 24, 48, and 72 h, respectively under excitation wavelength of 405nm, 488nm and 543nm separately using confocal microscopy (Nikon TE-2000-E, Japan). The effect of the exogenous genes on the cells was measured by the cell motility using trypan blue.

RESULTS

Morphology of fibroblasts from Bengal tiger

We used a primary explants technique and cell cryogenic preservation technology to establish the BTF22 cell line and proceeded to Biology and Genetics detection. Fibroblast-like or epithelial-like cells could be seen migrating from the tissue pieces 5-12 d after explanting (Fig. 1A). Cells continued to proliferate and were subcultured when they reached 90% confluence (Fig.1B). After subculture, the fibroblastic cells grew rapidly, gradually outgrowing and excluding other cells such as epithelial cells. After 2-3 passages, we obtained the purified fibroblasts (Fig.1C, D).The motilities of Bengal tiger fibroblasts before freezing and recovery were 97.6% and 95.7%, respectively.

Growth curve (cell dynamic state observation)

The growth curve of the BTF cell line appeared as typical "S" shape (Fig. 1E) and the PDT was 28 h. There was a lag time or latency phase of about 28 h after seeding, corresponding to the adaptation and recovery of the cells from protease damage, then the cells proliferated rapidly and entered exponential phase. As the cell density increased, proliferation was retarded by contact inhibition; by the sixth day, the cells entered the plateau phase and began to degenerate.

Table 1 Chromosome's Parameters of Bengal tiger

Chromosome number	Relative length(%)	Centromere morphology	Chromosome number	Relative length(%)	Centromere morphology
1	10.23±0.97	SM	11	4.08±0.72	M
2	9.38±1.02	M	12	3.42±0.76	SM
3	8.48±0.94	ST	13	3.41±0.64	M
4	6.68±0.86	M	14	3.22±0.59	T
5	6.41±0.80	SM	15	3.08±0.57	T
6	5.87±0.85	ST	16	2.89±0.15	SM
7	5.71±0.72	M	17	2.44±0.23	SM
8	5.31±0.77	SM	18	2.25±0.64	M
9	5.32±0.69	SM	X	5.34±0.72	M
10	4.47±0.71	SM			

Note: M 1.0 ~ 1.6 Metacetricchromosome (M) ; SM 1.7 ~ 2.9 Submetacentricchromosome (SM) ; ST 3.0 ~ 6.0 Subtelocentric chromosome (ST) ; T ≥7.0 Telocentric chromosome;

Microbial analysis

Tests for contamination with bacteria, fungi and yeasts were negative; no microorganisms were observed in the culture media. No viruses were indicated by the cytopathogenic evidence or by the hemadsorption test. Staining with the DNA fluorochrome Hoechst 33258

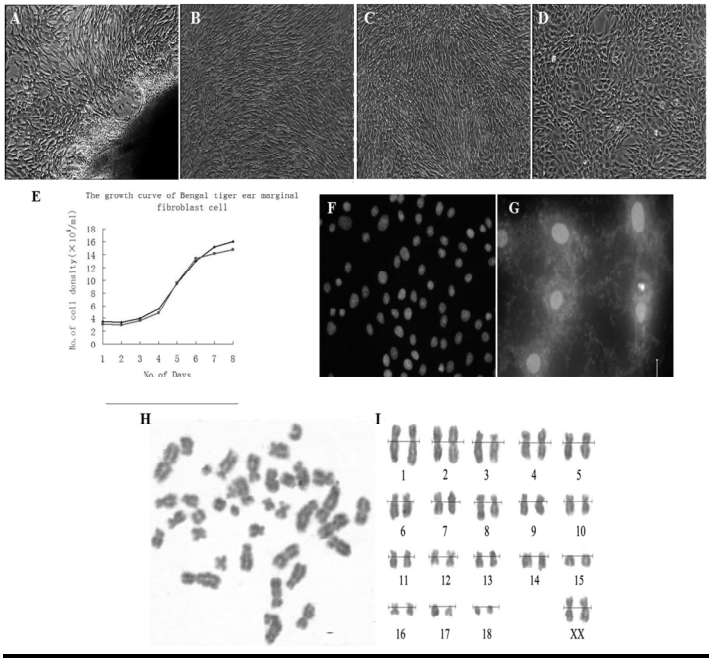

Figure 1. Morphology, growth curve, detection of microbial contamination and karyotype of BTF22 cells. (**A**) Primary cells (100×), the cells were typical long spindle-shape with growth being slower. (**B**) Subcultured cells (100×), the cells were subcultured until they reached 90% confluence. (**C**) Cells before cryopreservation (100×) the cells were healthy and in mitotic phase. (**D**) Cells after recovery (100×). (**E**) Growth curve of BTS22cells. The curve appeared as a typical "S" shape with cell density on the left axis. Growth curve included latency phase, exponential growth phase and stationary phase. (**F**). BTS22 mycoplasma negative (200×) (**G**) Mycoplasma positive control (400×) (H) Chromosome at metaphase (left) and karyotype (right) of BTS22 line (♀) (1000×).

is the most effective and frequently used method for detecting mycoplasma contamination (4). Under a fluorescence microscope after staining with Hoechst 33258, fibroblast nuclei appeared as blue ellipses, showing that the established cell line was mycoplasma negative (Fig. 1F).

Karyogram and the chromosome number of Bengal tiger

The chromosome number of Bengal tiger was 2n=38, comprising 36 autosomes and two sex chromosomes, XY or XX (Fig. 1G, H). According to the position of the centromere and satellite, we divided the autosomes into five groups; Group A: 2, 4, 7, 11 and 13 are metacentric chromosomes (M); Group B: 1, 5, 8, 9, 10, 12, 16 and 17 are submetacentric chromosomes (SM); Group C: 3 and 6 are subtelocentric chromosome (ST); Group D: 14 and 15 are telocentric chromosome (T); Group E: 18 is metacentric chromosomes, satellite. The

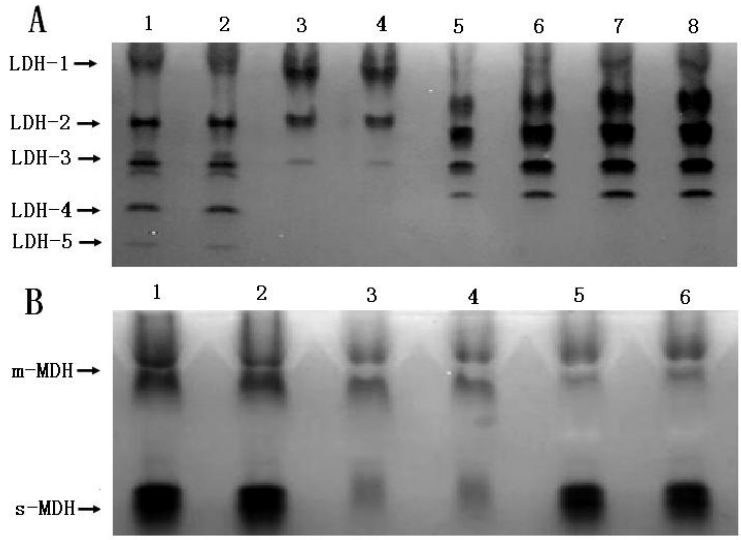

Figure 2. LDH zymotype and MDH zymotype of BTF22 cell line. These animals had their own unique bands with a different relative mobility. These results showed that there was no cross-contamination between different species.
(A) SDS–PAGE electrophoresis of LDH, from up to down, there were LDH-1, 2, 3, 4, 5. Panel A: 1,2 Zhiwei goat; 3,4 Simmental bovine; 5,6 Bengal tiger; 7,8 Changbai pig **(B)** MDH from up to down, there were mMDH and sMDH. Panel B: 1,2 Zhiwei goat; 3,4 Bengal tiger; 5,6 Changbai pig

heterosomes (X, X) were the metacentric chromosomes (Table 1). The karyotype composition of the Bengal tiger is 12 (M) + 16 (SM) + 4 (ST) + 4 (T), XY (M, M), which was similar to that reported by Levan et al (15) and Zhang et al (1993). The chromosome numbers per spread were counted for 100 spreads of the first, second and fourth passages, and the frequencies of cells with 2n=38 were 92.2%, 91.4% and 90.6%, respectively.

Isoenzymes analysis of Bengal tiger cell line

Polymorphism analysis of isoenzymes is currently the standard method for the quality control of cell line identification and detection of interspecies contamination (5, 18). We improved the apparatus and conditions, and successfully determined the mobility of the isoenzymes of LDH and MDH. The LDH bands obtained from Bengal tiger were compared with those from other species, and five isoenzyme bands (LDH-1, -2, -3, -4, -5) were observed (Fig. 2A). Eenzymatic activities were in order LDH-3, LDH-4, LDH-2, LDH-5, LDH-1; LDH-2, LDH-3 and LDH-4 were dominant, while LDH-1 and LDH-5 were scarcely observable.

Two MDH isoenzymes bands (s-MDH, m-MDH) were observed in the Bengal tiger, and there was a single m-MDH band near the cathode and a single s-MDH band near the anode (Fig. 2B). There were significant differences in the isoenzyme patterns of LDH and MDH between the Bengal tiger and other cell lines. These results indicated that there was no cross-contamination of BTF22 cell line from different cell lines established in our laboratory at the same time.

Expression results of 6 fluorescent protein genes in Bengal tiger fibroblastic cell

Positively expressing cells were observed 12 h after transfection and the numbers and intensity increased markedly and reached a maximum at 48 h or 72 h. The transfer efficiencies of the cyan fluorescent proteins (pECFP-C1, pECFP-mito Fig. 3A, B) were significantly lower than those of the green, yellow and red fluorescent proteins ($p < 0.01$), and the green fluorescent proteins (pEGFP-C1 and pEGFP-N3, Fig. 3C, D) were maximal. The expression efficiencies of the six fluorescent proteins were between 4.4 and 31.9. The numbers of fluorescent cells decreased at 1 week, but a few dispersed positive cells remained after 2 weeks and even after 1 and 2 months. By screening G418 (Geneticin) resistance selection and monoclonic culture for 1 month, we obtained three positive cell strains that expressed EGFP, EYFP, and DsRed stably.

Confocal fluorescence microscopy was used to observe the distribution of green, yellow and red fluorescence in the Bengal tiger cells to determine the subcellular location of six fluorescent proteins. DsRed was mostly shown in cytoplasmic (with a punctuate pattern Fig. 3I), whereas EGFP and EYFP showed an intense nuclear signal (Fig. 3G, H). The viabilities of cells transfected with pEGFP-C1, pEGFP-N3, pECFP-N1, pECFP-mito, pEYFP-N1 and pDsRed1-N1 were 90.1 %, 88.5%, 82.5%, 81.4%, 89.2% and 85.4%, respectively; none of these was significantly different from controls (90.8%, $P > 0.05$).

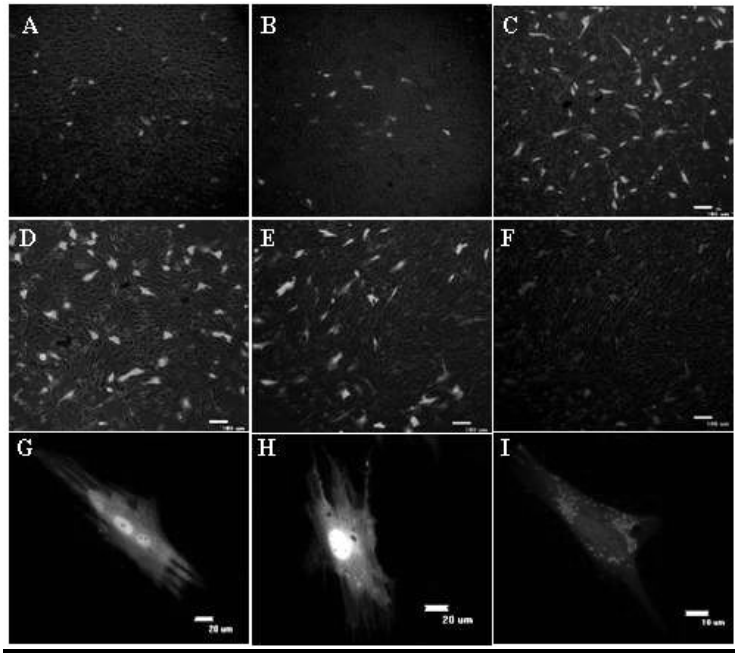

Figure 3. Expression and distribution of pEGFP-C1, pEGFP-N3, pECFP-C1, pECFP-mito, pEYFP-N1 and pDsRed1-N1 in BTF22 Cell. Comparative figures of six fluorescent proteins at 24h after transfection using TE-2000-E inverted microscope with excitation wavelengths of 433-588nm to determine the transfection efficiency (100×). A, B, C, D, E and F were the transfection results of pECFP-C1, pECFP-mito, pEGFP-C1, pEGFP-N3, pEYFP-N1and pDsRed1-N1, respectively; G, H and I were the transfection results of pEGFP-N3, pEYFP-N1 and pDsRed1-N1 at 48 h after transfection(×400).

DISCUSSION

Establishment and Cryopreservation of Bengal tiger group fibroblast cell line

Because too many passages and excess trypsin digestion could adversely affect the biological characteristics of such cells, especially hereditary characteristics, all cells used to preserve a species should undergo a minimum number of passages. If we use these abnormal cells as nuclear donors, the risk of abnormal cloned embryos will increase, whereas the success ratio of cloning will reduce. Hence, the incubation conditions of nuclear donor cells, and whether these cells have normal characteristics and stable chromosome, are critical elements of cloning success ratio.

Different tolerances to trypsin were evident as the fibroblasts fell off first when treated with trypsin and were adherent again quickly after passage, while most epithelial cells were not adherent or stably adherent and fell off only when treated with mechanical agitation. For this reason, purified fibroblasts could be obtained after 2-3 passages (32). The procedures used in this study conformed to the protocols of the ATCC technical bulletin for primary culture, subculture and freezing (2). The average viability after thawing was above 90%, and freezing had little influence on the viability of the fibroblasts. Hence, it is likely that the Bengal tiger genome can be preserved by freezing fibroblasts in liquid nitrogen for long-term storage.

Mycoplasma detection

Microbial contamination is the most frequent pollution phenomenon in cell culture. Air, equipment, serum, tissue sample, etc. could all contaminate the cells. The turbidity of culture media contaminated by bacteria, eumycetes and mycetes, can be seen with the naked eye. Viruses can be seen under the microscope. But it is harder to detect mycoplasma contamination. Mycoplasmas have no nuclei and can grow and reproduce in currently used media. They are hard to remove and could coexist with cells for a long time. Because fluorescent staining of mycoplasma DNA is simple and quick, it is commonly used by some cell culture collection institutions. Our microbiological detection results showed that the BTF22 cell line was purified and free of micoplasma contamination.

Karyotype analysis

Isoenzyme and karyotypic data together can effectively confirm the origin of a cell line and identify possible cross-contamination. The practice of combining them has become a classical and standard method for characterizing cell lines (20, 25). Because we want to conserve the genomic character of Bengal tiger, the fibroblasts must maintain the same diploid character as the cells in vivo. We improved the freezing procedure and decreased the number of passages to obtain a stable diploid cell line in which about 90% of the cells had 2n =38. Chromosome analysis can relate a cell line to the gender of the animal from which was derived, and also distinguish between normal and malignant cells, since the chromosome number is more stable in normal cells.

Isoenzymes analysis

Biochemical analysis of isoenzyme polymorphism is currently considered the standard method for quality control of cell line identification and interspecies contamination, and is routinely used by the main biological Resource Centers around the world, such as ATCC, ECACC(European Collection of Cell Cultures), DSMZ(German Resource Centre for Biological Material) and Riken(Japan Collection of Microorganisms)(21). LDH and MDH are very important enzymes participating in the glycolytic pathway and citric acid cycle,

respectively. We therefore chose LDH and MDH to determine the species origin of the cells and to measure cross-contamination.

LDH is a tetrametric molecule; the H and M subunits are produced by expression of the ldha and ldhb genes, and each tissue has a characteristic and species-specific isoenzyme composition (30). Arai et al. (1) and Ma et al. (17) analyzed the LDH isoenzyme pattern in the leukocytes and plasma of horse and Debao pony fibroblasts. Their results showed that the plasma and Debao pony fibroblast LDH isoenzyme patterns were dominant for LDH-1, LDH-2, and LDH-3; LDH-3 and LDH-4 were dominant in the leukocytes. The present study on the LDH isoenzyme patterns of Bengal tiger fibroblasts showed that LDH-2, LDH-3, and LDH-4 were dominant. MDH is a dimeric enzyme comprising cytosolic (s-MDH) and mitochondrial (m-MDH) subunits. The mobilities of MDH bands among poultry are essentially identical, and the same is true among mammals. But MDH from mammals migrates more rapidly than that from poultry, and the enzyme content is also greater than in poultry.

Expression of fluorescent protein genes

Research using fluorescent proteins is mainly focused on tumor cell, nerve cell and stem cell (12). We considered that the expression efficiency of exogenous genes could also be used as an efficient method to identify and evaluate the quality of cell lines cultured *in vitro.* Six fluorescent proteins with stable structures, high expression levels and species-independent efficiency have been used as marker genes to observe the expression, contribution and function of target proteins in live cells and organisms (3). DNA concentration, lipofectamine concentration, the DNA incubation time and lipofectamine combination, and the presence of serum, can all affect transfection efficiency, which is identical in research on Vero cells, HeLa cells and some other cell lines (24, 28). The results show that fluorescent protein expression had no obvious effect on the growth and proliferation of the transfected cells.

Overall, the newly established BTF22 cell line had all characteristics required by the cell line identification criteria of ATCC. The expression of exogenous genes showed that the cells were transfected well. The BTF22 line provides a useful approach for conserving this important international species in China and will be an effective experimental material supply for further genetic studies on the Bengal tiger as well.

Acknowledgements: This research was supported by the Ministry of Agriculture of China for transgenic research (2008ZX08009-003), the“863”National Major Research Program (2007AA10Z170), National Key Technology R&D Program (2006BAD13B08).

REFERENCES

1. Arai T, Inoue A, and Uematsu Y (2003) *Res Vet Sci* **75**, 15-19.
2. ATCC (2007) *Technical Bulletin* **8**, 1-4.
3. Baird GS, Zacharias DA, and Tsien RY (2000) *Proc Natl Acad Sci* **7**, 1984-1989
4. Barile ME, and Rottem SM (1993) Mycoplasmas in cell culture. In: *Kahane L., Adoni A. Eds*. Rapid diagnosis of mycoplasmas. Plenum Press, New York, pp155-193
5. Bowling AT, Breen M, Chowdhary BP, Hirota K, Lear T, Millon LV, Ponce de Leon FA, Raudsepp T, and Stranzinger G (1996). *Chromosome Res* **5**, 433–443.
6. Doyle A, Hay R, and Kirsop BE (1990) Animal cells, living resources for biotechnology. Cambridge University Press, Cambridge UK, pp81-100
7. Escriou V, Carriere M, and Bussone F (2001) *J Gene Med* **3**,179-187
8. Freshney RI (2000) Culture of animal cells: *A manual of basic technique* (4th eds). New York Wiley-Liss, New York, pp149-175
9. Gu YP, Li HZ, and Mik J (2006) *Exp Cell Res* **312**, 841-843.

10. Hay RI (1992) Cell line preservation and characterization. In: *Animal cell culture: a practical approach* (2nd eds). Oxford University Press, Oxford, 104-135
11. Hirofumi S, Kentaro Y, Kouichi H, Tsuyoshi F, Norihiro T, and Norio N (2006) *Biochem Biophys Res Commun* **345**,926-932
12. Jung S, Ackerley C, and Ivanchuk S (2001) *Neurosurg* **94**, 80-89
13. Kawarai S, Hashizaki K, and Kitao S (2006) *Vet Immunol Immunopathol* **113**, 30-36.
14. Kun W, Zhihe Z, Wenping Z, Xiao X, Xu L, Guangxin H, Fujun S, Liang Z, Rong H, and Bisong Y (2008) *Conserv Genet* **9**: 225-228.
15. Levan A, Fredga K, and Sandbergm AA. (1964) *Hereditas* **52**: 201-220.
16. Liu CQ, Guo Y, Guan WJ, Ma YH, and Tang XX (2008) *Tissue Cell* **40**, 417-424.
17. Ma YH, Zhou XM, Guan WJ, and Zhao DM (2004) *Asian-Aust J Anim Sci* **17**, 1338-1343;
18. MacLeod RA, Dirks WG, and Matsuo Y (1999) *Int J Cancer* **83**, 555-563.
19. Masover GK, and Becker FA (1998) Detection of mycoplasmas in cell cultures by cultural methods. In: Miles RJ, Nicholas RAJ, et al, (Eds). Methods in molecular biology. vol. 104, Mycoplasma protocols. Totawa NJ: Humana Press Inc. pp207-215.
20. Nims RW, Shoemaker AP, and Bauternschub MA (1998) *In Vitro Cell Dev Biol-Animal* **34**, 35-39.
21. Parodi B, Aresu O, and Bini D (2002) *BioTechniques* **32**, 432-440.
22. Pip E, and Franck JPC (2008) *Can J Zool* **86**, 10-16.
23. Qi Y, Tu Y, Yang D, Chen Q, Xiao J, Chen Y, Fu J, Xiao X, and Zhou Z (2007) *J Cell Physiol* **210**, 63-71.
24. Rong R, Yan Q, Hu YL, and Fan BQ (2006) *Theriogenology* **65**, 713-720.
25. Shepel, LA, Morrissey LW, and Hsu LC (1994) *Genomics* **19**, 75-85.
26. Sun YL, Lin CS, and Chou YC (2006) *Cell Biol Int* **30**, 970-976.
27. Taro S, Junco N, Vladimir VA, Alexander B, Seigo Hi, and Dale RM (2006) *Conserv Genet* **7**, 799-802
28. Tseng WC, Haselton FR, and Giorgio TD (1999) *Biochim Biophys Acta* **5**, 53-64.
29. Tsuchiya R, Yoshiki F, KudoY, and Morita M (2002) *Brain Research* **956**, 221-229.
30. Washizu T, Nakamura M, and Izawa N (2002) *Vet Res Commun* **26**, 341-346.
31. Wu H, Guan W, Li H, and Ma Y (2008) *Cell Biol Int* **32**, 1478-1485.
32. Zhou XM, Ma YH, Guan WJ, and Zhao DM (2004) *Asian-Aust J Anim Sci* **17**, 1338-1343.

Accepted for publication 18/2/2010