Animal Science Journal (2011) **82,** 215–222 doi: 10.1111/j.1740-0929.2010.00824.x

ORIGINAL ARTICLE

Establishment and characterization of a fibroblast cell line derived from Mongolian sheep

Chang-Qing LIU,[1,2]* Yu GUO,[2]* Wei-Jun GUAN[1] and Yue-Hui MA[1]

[1]*Institute of Beijing Animal Science and Veterinary, CAAS, Beijing, and* [2]*Bioscience Department, Bengbu Medical College, Bengbu, China*

ABSTRACT

The Mongolian sheep ear marginal tissue fibroblast cell line (MSF32) from 32 samples was successfully established by using primary explants technique and cell cryoconservation technology. MSF32 cells were adherent, with a population doubling time of 28.2 h. Chromosome analysis showed that >90.2% of cells were diploid (2n = 54) prior to cell passage 4. Isoenzyme analyses of lactate dehydrogenase and malate dehydrogenase showed that the MSF22 cells had no cross-contamination with other species. Tests for cell line contamination with bacteria, fungi, viruses and mycoplasmas were also 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 12.3% and 63.3%. Every index of the MSF32 cell line meets all the standard quality controls of American Type Culture Collection (ATCC). Not only has the genetic resources of the Mongolian sheep been preserved at the cell level, but also valuable materials had been provided for genome, postgenome and somacloning research.

Key words: *characterization, cryopreservation, establishment, fibroblast cell line, Mongolian sheep.*

INTRODUCTION

Biodiversity is facing unprecedented challenges worldwide and vulnerable animals are threatened by the introduction of foreign species, massive destruction of their natural habitats and industrial pollution, as occurs in China. There are 596 known Chinese domestic animal breeds, but 17 have become extinct and 336 breeds are threatened to various degrees (Liu *et al*. 2008). It is particularly crucial to protect the genetic resources of endangered animals and to utilize modern scientific methodologies to accomplish this feat. 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 (Roberto *et al*. 2009). Establishing fibroblast lines from endangered species has been proposed as a practical approach for this purpose; not only does it preserve precious genetic material, but also it provides an excellent resource for biological research (Wu *et al*. 2008).

Mongolian sheep is an ancient sheep breed in China that originated from the Inner Mongolian grasslands. This animal was one of the 78 nationally protected domestic species, which was listed in 2000 by the Chinese government. There are many important genetic traits in the Mongolian sheep, such as meat quality, disease resistance and resistance to cold weather, which have breeding potential by hybridization.

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 cell line (named MSF32 line) overall. Our project seeks a different approach to cryopreserve this nationally protected genomic resource for the purposes of reviving endangered breeds 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 unwonted meaning.

Correspondence: Wei-Jun Guan, Institute of Beijing Animal Science and Veterinary, CAAS, Beijing 100193, China. (Email: wjguan86@iascaas.net.cn)
*These authors contributed equally to this work.
Received 2 October 2009; accepted for publication 15 April 2010.

© 2011 The Authors
Journal compilation © 2011 Japanese Society of Animal Science

MATERIALS AND METHODS

Cell cultures

Ear tissue samples (approximately 1 cm^2 in size) were sampled from 32 individuals of Mongolian sheep (12 male and 20 female) and collected into separate tubes containing Dulbecco's modified Eagle's medium (DMEM) with ampicillin (100 U/mL) and streptomycin (100 μg/mL). The samples were rinsed and chopped into 1 mm^3 pieces, which were seeded on the surface of a tissue culture flask containing DMEM+ 10% fetal bovine serum in a 37°C incubator with 5% CO_2 (Zhou *et al*. 2004; Liu *et al*. 2008). Cells were harvested when they reached 80–90% confluence and were divided into prepared culture flasks at 1:2 or 1:3 ratios (Freshney 2000).

Growth curve and estimation of cell viability by trypan blue dye

The cells with a concentration of 1.5×10^4 cells/mL were seeded into 24-well plates. After culturing for 7 days, three wells 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 (Gu *et al*. 2006). Cell viability before freezing or after recovery was determined using a hemocytometer to enumerate 1000 cells by Trypan Blue vital stain method (Qi *et al*. 2007).

Cryogenic preservation and recovery

Prior to freezing, the culture should be maintained in an actively growing state (log phase or exponential growth) to ensure optimum health and good recovery. Harvested cells were resuspended in freezing medium (10% dimethyl sulfoxide + 50% fetal bovine serum + 40% DMEM) to a final density of $(3–5) \times 10^6$ viable cells/mL. Subsequently, 1 mL of the cell suspension was allocated into one sterile plastic cryogenic vial labeled with the animal name, gender, passage number, freezing serial number and 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 quickly and efficiently transferred to a liquid nitrogen storage system.

To recover and reseed the cells, the frozen tubes were removed from the liquid nitrogen storage system and quickly thawed in a 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 (Freshney 2000).

The measurement of microorganism of MSF32 cell line

Tests for contamination with bacteria, fungi and yeasts: for details of the procedure used, see Doyle *et al*. (1990). *Testing for viruses*: Hay's haemadsorption protocol was used routinely to examine the samples for cytopathogenesis using phase-contrast microscopy (Hay 1992). *Mycoplasma detection:* Cells were stained with Hoechst 33258 according to the DNA fluorescent staining protocol to detect whether they were contaminated by mycoplasma (Masover & Becker 1998). The enzyme-linked immunosorbent assay Mycoplasma Detection kit (Roche Diagnostics Corp., Indianapolis, IN, USA) was used to detect the four most common mycoplasma species (*M. arginini, M. hyorhinis, Acholeplasma laidlawii, M. orale*).

Chromosome analysis

Chromosomes were prepared, fixed and stained following standard methods (Suemori *et al*. 2006). After Giemsa 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*. (2006) and Kawarai *et al*. (2006).

Isoenzymes analysis

Isoenzyme patterns of lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) were detected by using the vertical slab non-continuous polyacrylamide gel electrophoresis (PAGE) assay. In brief, the cells were harvested, and protein extraction solution (0.9% Triton X-100, 0.06 mmol NaCl/EDTA in mass ratio 1:15) was added after the cell concentration was adjusted to 5×10^7 mL cells/mL, then centrifuged, and the supernatant was stored in aliquots at −80°C. Forty percent sucrose liquid and the samples (1:1) were mixed and then loaded in the individual lanes of the polyacrylamide gel (Wu *et al*. 2008). Different mobility patterns were indicated by the relative mobility front (RF), which was calculated as the ratio between the distance of migration of the isozyme band and the bromophenol blue.

Expression of fluorescent protein genes in Mongolian sheep 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 Biosciences Clontech, Palo Alto, CA, US) and Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA), according to lipofectamine media methods of Escriou *et al*. (2001) and Tsuchiya *et al*. (2002). To estimate the transfection efficiency, the cells were observed after being transfected for 24, 48 and 72 h, respectively, under excitation wavelength of 405 nm, 488 nm and 543 nm separately using confocal microscopy (Nikon TE-2000-E, Tokyo, Japan). For each experimental group, images were captured from 10 visual fields, and the confocal microscope was used to measure the total and positive cell counts in each field to determine the transfection efficiency. The effect of the exogenous genes on the cells was measured by the cell motility using trypan blue.

RESULTS

Morphology of fibroblasts from Mongolian sheep

We used a primary explants technique and cell cryogenic preservation technology to establish the MSF32 cell line and proceeded to biology and genetics detection. The culture conditions were optimal, and the cells were healthy. Fibroblast-like or epithelial-like cells could be seen migrating from the tissue pieces 5–12 days after explanting (Fig. 1A). When the time in culture was increased, cells continued to proliferate and were subcultured when they reached 90% confluence (Fig. 1B). After subculturing, the fibroblasts grew rapidly and replaced the epithelial cells gradually

© 2011 The Authors
Journal compilation © 2011 Japanese Society of Animal Science

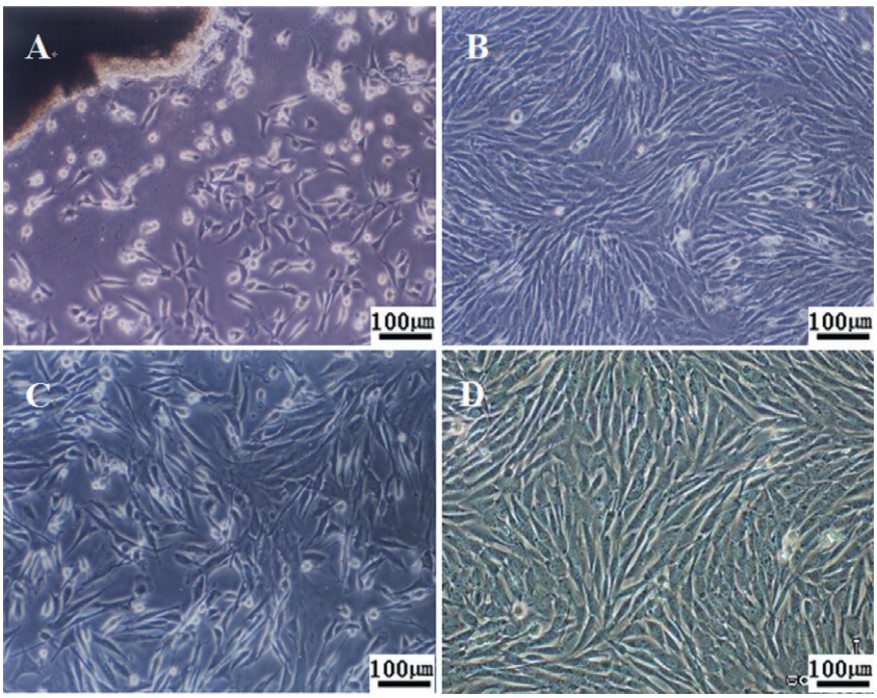

Figure 1 Culture and morphology of Mongolian sheep fibroblast cells. (A) Primary cells of Mongolian sheep, fibroblasts and epithelial cells migrated from the tissue. (B) Subcultured cells of Mongolian sheep, the cells were subcultured until they reached 90% confluence. After passage, growth accelerated and plateaued after 3–4 days. (C) Cells before cryopreservation; the cells were healthy and in mitotic phase. (D) Mongolian sheep cells after recovery; the cells were cultured for 48 h after thawing.

after 2–3 passages (Fig. 1C,D). The motilities of Mongolian sheep fibroblasts before freezing and recovery measured by trypan blue staining were 98.5% and 96.7%.

Growth curve (cell dynamic state observation)

The growth curve of the MSF cell line appeared as a typical 'S' shape (Fig. 2A) and the PDT was approximately 28.2 h. There was a lag time or latency phase of about 24 h after the cells were seeded, corresponding to the adaptation and recovery of the cells from protease damage, then the cells proliferated rapidly and entered the 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.

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 haemadsorption test. Staining with the DNA fluorochrome Hoechst 33258 is the most effective and frequently used method for detecting mycoplasma contamination (Barile & Rottem 1993). 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. 2B).

Karyogram and the chromosome number of Mongolia sheep

According to Levan *et al*. (1964) and Li *et al*. (2007), the chromosome number of sheep was 2n = 54, in which three pairs were metacentric/submetacentric chromosomes and 23 pairs were telocentric autosomal chromosomes. The X chromosome was the longest telocentric chromosome, and the Y chromosome is the shortest metacentric chromosome. Our present results are in accordance with this conclusion. The karyotype composition of the Mongolian sheep is 4 m + 23 t (Table 1), namely, chromosomes 1, 2, 3 and Y are metacentric chromosomes; chromosomes 4 to 26 and X are telocentric chromosomes (Fig. 2C).

The chromosome numbers per spread were counted for 100 spreads of the first, second and fourth passages, and the frequencies of cells with 2n = 54 were 91.4%, 90.6% and 90.2%, respectively. Aberrations in chromosome numbers tended to increase with increasing numbers of passages, indicating that *in vitro* culture affected the heritage of cells slightly, but supporting the inference that the cell line was reproducibly diploid.

© 2011 The Authors
Journal compilation © 2011 Japanese Society of Animal Science

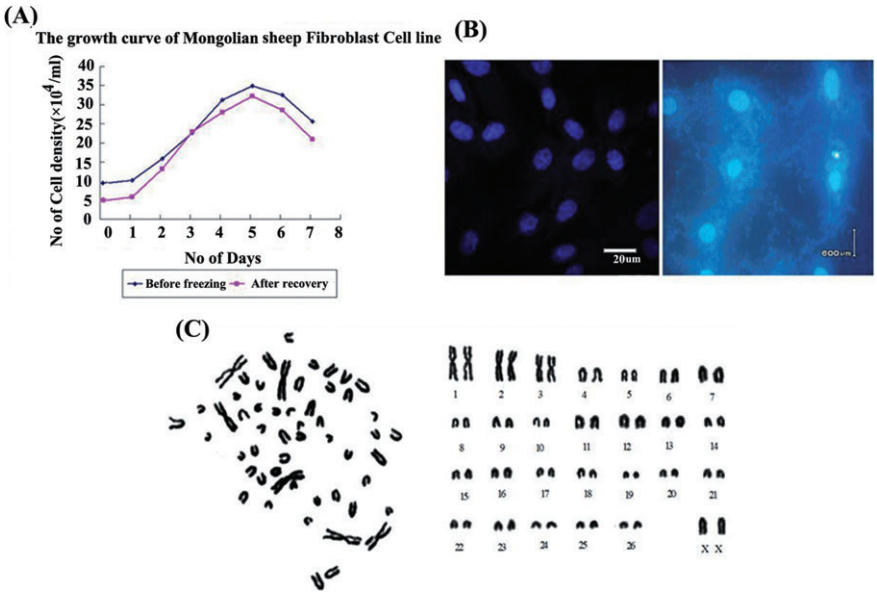

Figure 2 Growth curve, detection of microbial contamination and karyotype of MSF32 cells. (A) Growth curve of MSF32 cells. The curve appeared as a typical 'S' shape with cell density on the left axis. Growth curve included latency phase, exponential growth phase and stationary phase. (B) Mycoplasma contamination for the Mongolian sheep fibroblasts stained with Hoechst33258 and positive control of Mycoplasma contamination stained with Hoechst33258. (C) Chromosome at metaphase (left) and karyotype (right) of the MSF32 cell line. The chromosome number of Mongolian sheep was $2n = 54$, comprising 52 autosomes and two sex chromosomes, while the sex chromosome type was XX (♀).

Table 1 Chromosome's parameters of Mongolian sheep

Chromosome number	Relative length (%)	Centromere morphology	Chromosome number	Relative length (%)	Centromere morphology
1	9.73 ± 0.74	M	15	3.08 ± 0.17	T
2	8.18 ± 0.25	M	16	2.89 ± 0.15	T
3	6.98 ± 0.34	M	17	2.64 ± 0.18	T
4	5.68 ± 0.16	T	18	2.49 ± 0.14	T
5	5.41 ± 0.10	T	19	2.41 ± 0.17	T
6	5.17 ± 0.15	T	20	2.32 ± 0.18	T
7	4.81 ± 0.02	T	21	2.28 ± 0.08	T
8	4.61 ± 0.17	T	22	2.22 ± 0.11	T
9	4.27 ± 0.18	T	23	2.11 ± 0.28	T
10	3.97 ± 0.01	T	24	2.03 ± 0.39	T
11	3.78 ± 0.12	T	25	1.94 ± 0.22	T
12	3.42 ± 0.26	T	26	1.46 ± 0.12	T
13	3.21 ± 0.14	T	X	4.94 ± 0.12	T
14	3.12 ± 0.19	T	X	4.35 ± 0.16	T

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.

Isoenzyme analysis of Mongolian sheep cell line

The distribution patterns of isoenzyme polymorphisms may be a characteristic of a species or a tissue (MacLeod *et al.* 1999). Polymorphism analysis of isoenzymes is currently the standard method for the quality control of cell line identification and detection of interspecies contamination. We improved the apparatus and conditions for native PAGE, and successfully determined the mobility of the isoenzymes of LDH and MDH. The LDH bands obtained from Mongolian sheep were compared with those from other species or breeds, and five isoenzyme bands (LDH-1, -2, -3, -4, -5) were observed (Fig. 3A). Enzymatic activities were in order LDH-3, LDH-4, LDH-5, LDH-2, LDH-1; LDH-3, LDH-4 and LDH-5 were dominant, while LDH-1 and LDH-2 were scarcely observable.

All five domestic animals had two bands (s-MDH, m-MDH), and there was a single m-MDH band near the cathode and a single s-MDH band near the anode (Fig. 3B). Similar activity was seen from both m-MDH

© 2011 The Authors
Journal compilation © 2011 Japanese Society of Animal Science

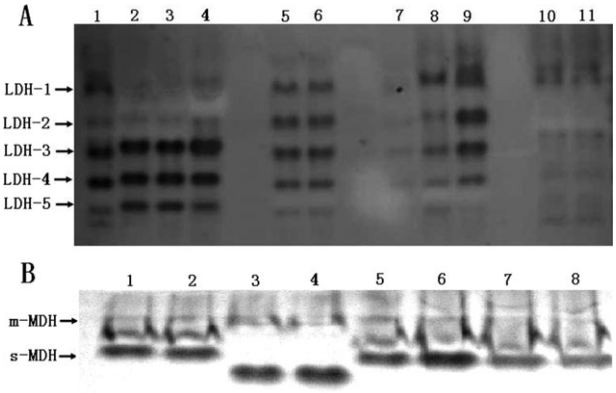

Figure 3 LDH zymotype and MDH zymotype of MSF32 cell line. These domestic animals had their own unique bands with a different relative mobility. These results showed that there was no cross-contamination between different breeds. (A) Sodium dodecyl sulfate – polyacrylamide gel electrophoresis of LDH, from up to down, there were LDH-1, 2, 3, 4, 5. Panel A: 1 Zhiwei goat, 2 Jining black goat, 3 Taihang black goat, 4 Mongolian sheep, 5,6 Luxi cattle, 7 Simmental bovine,8 Angus bovine, 9 Piemontese bovine, 10,11 large white pig. (B) MDH from up to down, there were mMDH and sMDH. Panel B: 1,2 Qingyuan partridge chicken; 3,4 Mongolian sheep; 5,6 Beijing duck; 7,8 Langshan chicken; 9,10 Tibitan chicken.

and s-MDH. These domestic animals had their own unique bands with a different relative mobility. These results indicated that there was no cross-contamination of MSF32 cell line from different cell lines established in our laboratory at the same time.

The expression results of six fluorescent protein genes in Mongolia sheep fibroblastic cells

The six fluorescent protein genes pECFP-C1, pECFP-mito, pEGFP-C1, pEGFP-N3, pEYFP-N1 and pDsRed1-N1 were all highly expressed with reference to the optimized condition for pEGFP-N3 (Fig. 4). 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) 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) were maximal. The expression efficiencies of the six fluorescent proteins at 24 h, 48 h and 72 h after transfer were between 12.3 and 63.3. 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 resistance and monoclonic culture for 1 month, we obtained three positive cell strains that stably expressed EGFP, EYFP and DsRed.

Confocal fluorescence microscopy was used to observe the distribution of green, yellow and red fluorescence in the Mongolian sheep cells to determine the subcellular location of six fluorescent proteins. The results showed that the fluorescence could be observed throughout the cytoplasm and nuclei of control cells except in the cryptomere vesicle. DsRed was mostly shown in cytoplasm (with a punctuate pattern), whereas ECFP, EGFP and EYFP showed an intense nuclear signal (Fig. 5). The viabilities of cells transfected with pEGFP-C1, pEGFP-N3, pECFP-N1, pECFP-mito, pEYFP-N1 and pDsRed1-N1 were 90.2%, 88.7%, 84.5%, 83.1%, 89.2% and 84.6%, respectively; none of these was significantly different from controls (92.2%, $P > 0.05$).

DISCUSSION

Establishment of Mongolian sheep fibroblast cell line

Morphological results indicated that the primary Mongolian sheep cells are usually mixed with epithelial cells. 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 (Zhou *et al*. 2004). 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 Mongolian sheep genome can be preserved by freezing fibroblasts in liquid nitrogen for long-term storage.

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 (<4). The procedures used in this study conformed to the protocols of the American Type Culture Collection (ATCC) technical bulletin for primary culture, subculture and freezing. Moreover, we characterized the established cell line according to ATCC quality control procedures and improved some techniques and methods, for example increased expression of the six fluorescent proteins.

Mycoplasma detection

Microbial contamination is the most frequent pollution phenomenon in cell culture. 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

© 2011 The Authors
Journal compilation © 2011 Japanese Society of Animal Science

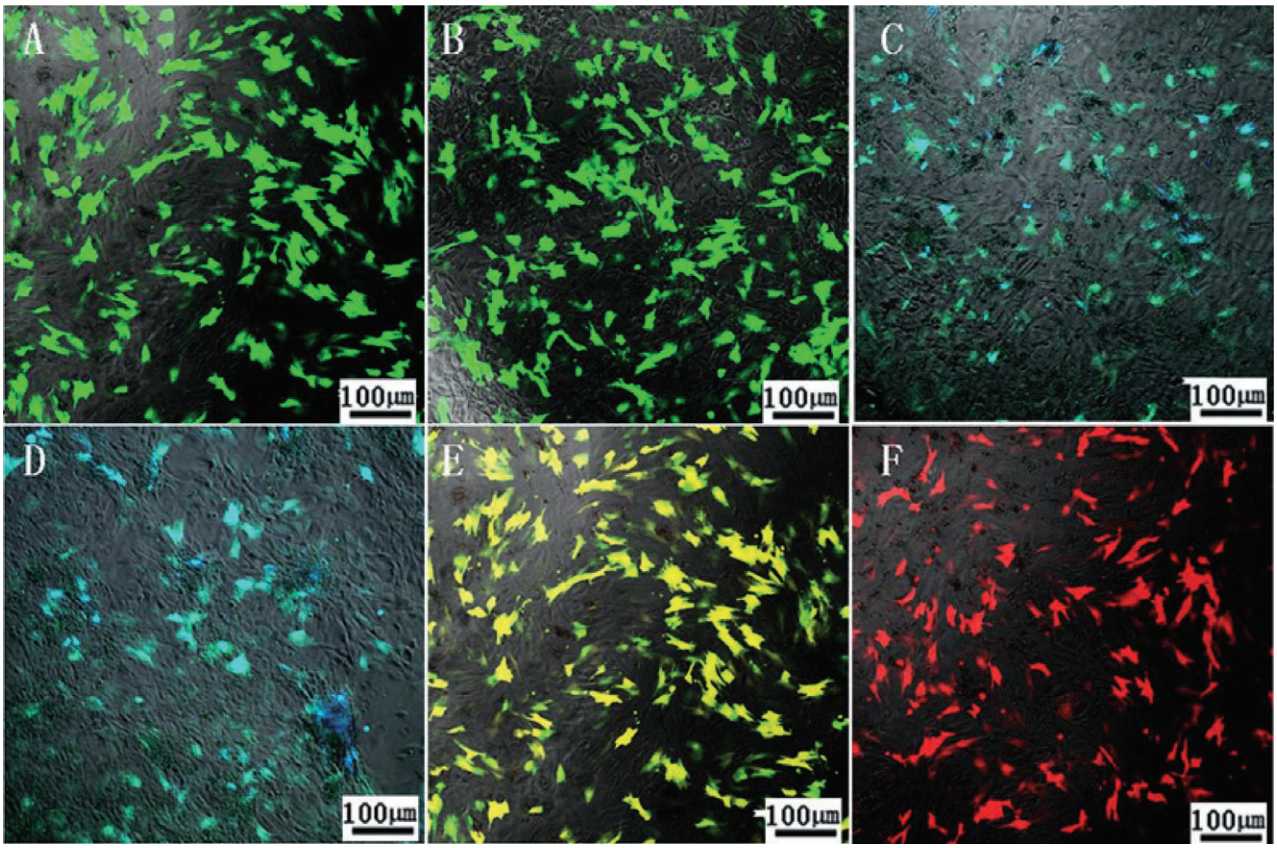

Figure 4 Comparative figures of six fluorescent proteins at 24 h after transfection using TE-2000-E inverted microscope with excitation wavelengths of 433–588 nm to determine the transfection efficiency. A, B, C, D, E and F were the transfection results of pEGFP-C1, pEGFP-N3, pECFP-C1, pECFP-mito, pEYFP-N1, pDsRed1-N1, respectively.

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 such as the ATCC. Our microbiological detection results showed that the MSF32 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 (Shepel *et al*. 1994; Nims *et al*. 1998). Because we want to conserve the genomic character of Mongolian sheep, the fibroblasts must maintain the same diploid character as the cells *in vivo*. The result showed that the proportion of cells with a 2n = 54 was above 90%, MSF32 cell line was a stable diploid cell line. Chromosome analysis can relate a cell line to the gender of the animal from which it was derived, and also distinguish between normal and malignant cells, since the chromosome number is more stable in normal cells (Freshney 2000).

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 (ATCC, ECACC, DSMZ and Riken) (Parodi *et al*. 2002). LDH and MDH are very important enzymes participating in the glycolytic pathway and citric acid cycle, respectively. They are species-specific and constant, but the enzyme contents and activities differ among species, providing a biochemical indicator of species classification by chromatography and electrophoresis. 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 (Washizu *et al*. 2002). Arai *et al*. (2003) and Zhou *et al*. (2004) analyzed the LDH isoenzyme pattern in the leukocytes and plasma of Debao horse and pony fibroblasts. Their results showed that the plasma and Debao pony fibroblast

© 2011 The Authors
Journal compilation © 2011 Japanese Society of Animal Science

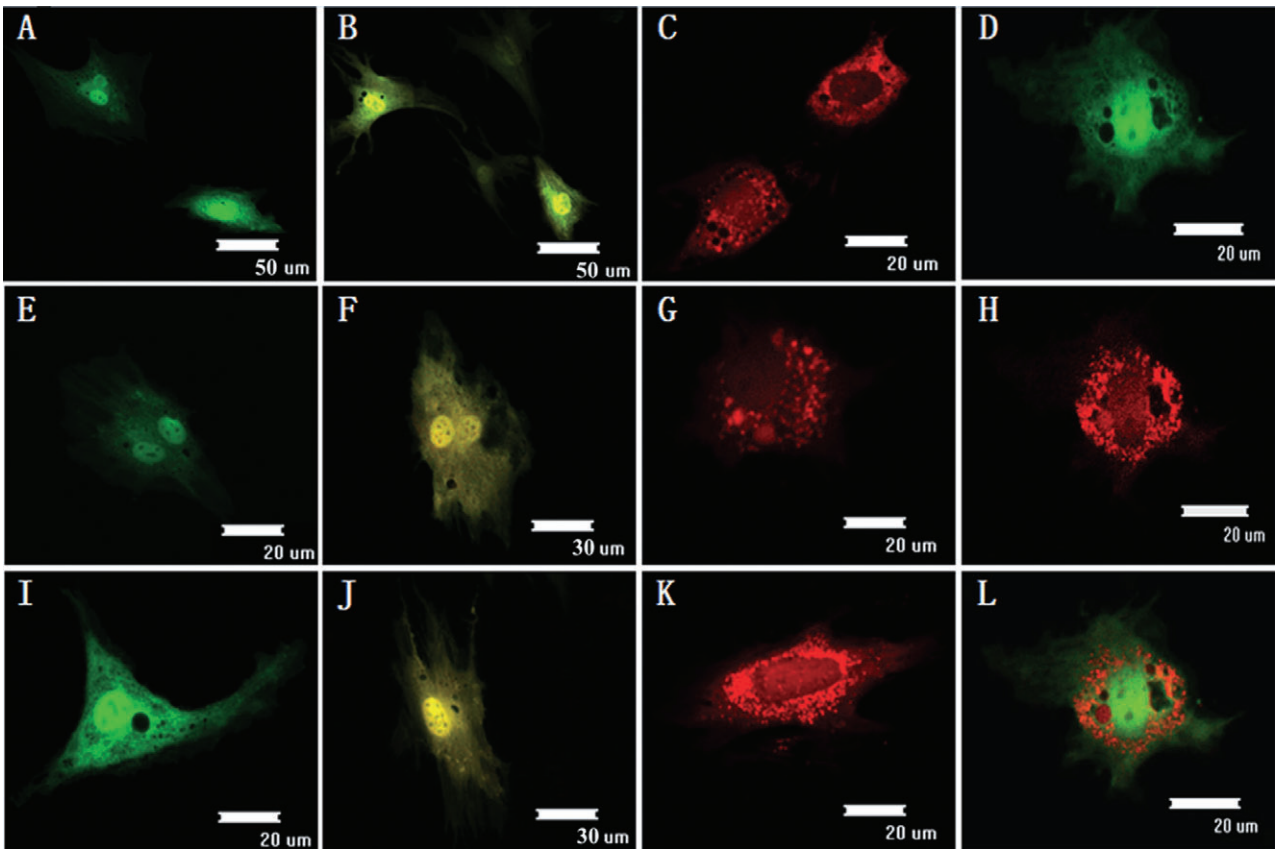

Figure 5 The expression and distribution of pEGFP-N3, pDsRed1-N1 and pEYFP-N1 in Mongolian sheep cell. A, B and C were the transfection results of pEGFP-N3, pDsRed1-N1 and pEYFP-N1 at 24 h after transfection. E, F and G were the transfection results of EGFP-N3, pDsRed1-N1 and pEYFP-N1 at 48 h after transfection. I, J and K were the transfection results of pEGFP-N3, pDsRed1-N1 and pEYFP-N1 at 72 h after transfection. D, H are transfectected by pEGFP-N3 and pDsRed-N1 at the same time excited by 488 nm and 543 nm, separately. I images produced by merging two signals together (D, H).

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 Mongolian sheep fibroblasts showed that LDH-3, LDH-4 and LDH-5 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 livestock. But MDH from livestock migrates more rapidly than that from poultry, and the enzyme content is also greater than in poultry.

Expression of fluorescent protein genes

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 (Baird *et al*. 2000). 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*. In this study, positive cells were most abundant and the fluorescence signal was strong, with the highest transfection efficiency at 48 h after transfection. DNA concentration, lipofectine concentration, the DNA incubation time and lipofectine 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 (Tseng *et al*. 1999; Escriou *et al*. 2001; Rong *et al*. 2006). The transfected cells were not significantly less viable than the control cells. The results show that fluorescent protein expression had no obvious effect on the growth and proliferation of the transfected cells under a certain range, which is consistent with previous findings (Huang & Li 2001). Moreover, it is quite important for researchers to determine the distribution of reporter genes before analyzing the location of fusion protein.

Conclusions

Overall, the newly established MSF32 cell line had all characteristics required by the cell line identification criteria of ATCC. The expression of exogenous genes showed that the cells were well transfected. The

© 2011 The Authors
Journal compilation © 2011 Japanese Society of Animal Science

MSF32 line provides a useful approach for conserving this unique breed in China and will be an effective experimental material supply for further genetic studies on the Mongolian sheep as well. The technical platform now established will support the conservation of endangered species and their genetic materials at the cellular level.

ACKNOWLEDGMENTS

This research was supported by the National High Technology Research and Development Program (863) of China (grant no. 2006AA10Z198, 2007AA10Z170), Anhui Provincial Key Science Foundation for Outstanding Young Talent (grant 2010SQRL123ZD) and National Scientific Foundation of China (grant no. 30671539).

REFERENCES

Arai T, Inoue A, Uematsu Y. 2003. Activities of enzymes in the malate-aspartate shuttle and the isoenzyme pattern of lactate dehydrogenase in plasma and peripheral leukocytes of lactating Holstein cows and riding horses. *Research in Veterinary Science* **75,** 15–19.

Baird GS, Zacharias DA, Tsien RY. 2000. Biochemistry, mutagenesis, and oligomerization of DsRed, a red fluorescent protein from coral. *Proceedings of the National Academy of Sciences of the United States of America* **7,** 1984–1989.

Barile ME, Rottem SM. 1993. Mycoplasmas in cell culture. In: Kahane L, Adoni A (eds), *Rapid Diagnosis of Mycoplasmas*, pp. 155–193. Plenum Press, New York.

Doyle A, Hay R, Kirsop BE (eds). 1990. *Animal Cells, Living Resources for Biotechnology*, pp. 81–100. Cambridge University Press, Cambridge.

Escriou V, Carriere M, Bussone F. 2001. Critical assessment of the nuclear import of plasmid during cationic lipid-mediated gene transfer. *The Journal of Gene Medicine* **3,** 179–187.

Freshney RI. 2000. *Culture of Animal Cells: A Manual of Basic Technique*, 4th edn, pp. 149–175. New York Wiley-Liss, New York.

Gu YP, Li HZ, Mik J. 2006. Phenotypic characterization of telomerase-immortalized primary non-malignant and malignant tumor-derived human prostate epithelial cell lines. *Experimental Cell Research* **312,** 841–843.

Hay RI. 1992. Cell line preservation and characterization. In: Freshney RI (ed.), *Animal Cell Culture: A Practical Approach*, 2nd edn, pp. 104–135. Oxford University Press, Oxford.

Huang Q, Li CY. 2001. Research of green fluorescent protein cosmid as marker gene in a living cell. *Chinese Journal of Clinical Oncology* **21,** 264–266.

Kawarai S, Hashizaki K, Kitao S. 2006. Establishment and characterization of primary canine hepatocellular carcinoma cell lines producing alpha-fetoprotein. *Veterinary Immunology and Immunopathology* **113,** 30–36.

Levan A, Fredga K, Sandbergm AA. 1964. Nomenclature for centromeric position on chromosomes. *Hereditas* **52,** 201–220.

Li X, Wu D, Chen S, Leng X. 2007. Study on the chromosomal aberrations of liangshan semifine-wool sheep and tibetan sheep. *Acta Veterinaria et Zootechnica Sinica* **38,** 400–406.

Liu CQ, Guo Y, Guan WJ, Ma YH, Tang XX. 2008. Establishment and biological characteristic research of luxi cattle fibroblast cell bank. *Tissue and Cell* **40,** 417–424.

MacLeod RA, Dirks WG, Matsuo Y. 1999. Widespread intraspecies cross-contamination of human tumor cell lines arising at source. *International Journal of Cancer* **83,** 555–563.

Masover GK, Becker FA. 1998. Detection of mycoplasmas in cell cultures by cultural methods. In: Miles RJ, Nicholas RAJ (eds), *Methods in Molecular Biology. Mycoplasma Protocols*, Vol. **104**, pp. 207–215. Humana Press Inc, Totawa, NJ.

Nims RW, Shoemaker AP, Bauternschub MA. 1998. Sensitivity of isoenzyme analysis for the detection of interspecies cell line cross-contamination. *In Vitro Cellular & Developmental Biology-Animal* **34,** 35–39.

Parodi B, Aresu O, Bini D. 2002. Species identification and confirmation of human and animal cell lines: a PCR-based method. *BioTechniques* **32,** 432–440.

Qi Y, Tu Y, Yang D, Chen Q, Xiao J, Chen Y, Fu J, Xiao X, Zhou Z. 2007. Cyclin A but not cyclin D1 is essential in c-myc-modulated cell cycle progression. *Journal of Cellular Physiology* **210,** 63–71.

Roberto GC, Ana Sancha MB, Marta SM, Severino GN, Rita de Cássia Ramos do EQ, José Teodorico de AF, Arturo SV. 2009. Physical and chemical characterization of lamb meat from different genotypes submitted to diet with different fibre contents. *Small Ruminant Research* **81,** 29–34.

Rong R, Yan Q, Hu YL, Fan BQ. 2006. Establishment of porcine transgenic embryonic germ cell lines expressing enhanced green fluorescent protein. *Theriogenology* **65,** 713–720.

Shepel LA, Morrissey LW, Hsu LC. 1994. Bivariate flow karyotyping, sortiing, and peak assignment of all rat chromosomes. *Genomics* **19,** 75–85.

Suemori H, Yasuchika K, Hasegawa K, Fujioka T, Tsuneyoshi N, Nakatsuji N. 2006. Efficient establishment of human embryonic stem cell lines and long-term maintenance with stable karyotype by enzymatic bulk passage. *Biochemical and Biophysical Research Communications* **345,** 926–932.

Sun YL, Lin CS, Chou YC. 2006. Establishment and characterization of a spontaneously immortalized porcine mammary epithelial cell line. *Cell Biology International* **30,** 970–976.

Tseng WC, Haselton FR, Giorgio TD. 1999. Mitosis enhances transgene expression of plasmid delivered by cationic liposomes. *Biochimica et Biophysica Acta* **5,** 53–64.

Tsuchiya R, Yoshiki F, Kudo Y, Morita M. 2002. Cell type-selective expression of green fluorescent protein and the calcium indicating protein, yellow cameleon, in rat cortical primary cultures. *Brain Research* **956,** 221–229.

Washizu T, Nakamura M, Izawa N. 2002. The activity ratio of the cytosolic MDH/LDH and the isoenzyme pattern of LDH in peripheral leukocytes of dogs, cats, and rabbits. *Veterinary Research Communications* **26,** 341–346.

Wu H, Guan W, Li H, Ma Y. 2008. Establishment and characteristics of white ear lobe chicken embryofibroblast line and expression of six fluorescent proteins in the cells. *Cell Biology International* **32,** 1478–1485.

Zhou XM, Ma YH, Guan WJ, Zhao DM. 2004. Establishment and identification of Debao pony ear marginal tissue fibroblast cell line. *Asian-Australian Journal of Animal Science* **17,** 1338–1343.

© 2011 The Authors
Journal compilation © 2011 Japanese Society of Animal Science