

## Establishment and biological research of the Jining Grey goat fibroblast line

Chunyu BAI<sup>1,2,\*</sup>, Dianjun WANG<sup>3,\*</sup>, Xiaohua SU<sup>1</sup>, Minghai ZHANG<sup>2</sup>, Weijun GUAN<sup>1</sup>, Yuehui MA<sup>1,\*\*</sup>

<sup>1</sup>Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing, 100193 – P.R. CHINA

<sup>2</sup>College of Wildlife Resources, Northeast Forestry University, Harbin, 150040 – P.R. CHINA

<sup>3</sup>Department of Pathology, Chinese People's Liberation Army General Hospital, Beijing, 100853 – P.R. CHINA

Received: 13.06.2011 • Accepted: 11.11.2011

**Abstract:** Using Jining Grey goat ear marginal tissues as experimental materials, we succeeded in establishing a fibroblast cell bank, including 32 samples, by means of a primary explant and cryopreservation technique. The results from the biological analysis suggested that the population doubling time of the cell line was approximately 48 h. The diploid accounted for 98.35%, the isozyme analysis of the lactic dehydrogenase and malic dehydrogenase disproved cross contamination, and the results of the bacterium, fungi, virus, and mycoplasma detection tests were all negative. The transfection rates of 3 fluorescent protein genes were high, which supported the opinion that exogenous genes could be effectively expressed in the cells. This research has not only preserved the precious germplasm resources of the Jining Grey goat at the somatic cell level, but it has also provided valuable materials for the study of genomics, postgenomics, and somatic cloning.

**Key words:** Jining Grey goat, fibroblast cell bank, cryogenic preservation, genetic conservation

### Introduction

Animal genetic resources are an important element of biodiversity. With the development of animal husbandry in China, livestock has become commercialized and people prefer animal species with high economic value. As a result, local breeds with lower economic value are suffering from group decrease and population shrinkage, and, worse of all, impending extinction. China has a total of 596 local livestock breeds, among which 17 have become extinct, 336 are being threatened at different levels, and only 243 are relatively safe (1). Effective measures should be taken to protect

endangered species, as such a situation will lead to the extinction of animal genetic resources, which signifies an eternal loss of the objects for studies on cellular and molecular biology. Thus, genetic resource conservation for endangered, precious, native livestock breeds is imperative and should not be ignored. At present, the preservation of individual animals, semen, embryos, genomic libraries, and cDNA libraries are all practical options. Modern cloning techniques have made somatic cells an attractive resource for conserving animal genetic materials (2). With further development of animal cloning technology, the establishment of a somatic cell bank is extremely important for the

\* Authors who made an equivalent contribution to this paper.

\*\* E-mail: yuehui\_ma@hotmail.com

recovery of endangered species. In brief, ear marginal tissues or embryonic tissues are isolated for adherent culture, *in vitro*, to establish the fibroblast cell bank. Recently, somatic cell banks have been established for the endangered breeds of Simmental cattle (3), Ujumqin sheep (4), Texel sheep (5), Jining Black Grey goat (6), etc.

The Jining Grey goat (JGG) inhabits the Heze and Jining areas of Shandong Province, China. It includes 2 subspecies, the Jining White Grey goat and the Jining Black Grey goat. The Jining White Grey goat, which is also known as the JGG, is superior to the Jining Black Grey goat (JBG) in breeding performance and the economic value of their fur. Moreover, it is world-famous for the attractive good quality of its kid-pelt, a traditional commodity in international markets (7,8). To preserve this valuable genetic resource, in this study, a fibroblast bank of the JGG was established, and its biological characteristics were identified. This study has preserved the genetic resources at the somatic cell level. The cell bank will provide valuable experimental materials for genomics, postgenomics, somatic cloning, and other fields of life sciences in the future.

## Materials and methods

### Materials

The 32 JGG ear marginal tissue samples were provided by a goat breeding farm in Shandong Province, China. Except where otherwise indicated, all of the chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

### Isolation and culture of ear marginal fibroblasts from the JGG

Ear marginal tissue samples (about 1 cm<sup>2</sup>) were acquired from 32 JGGs (15 males and 17 females) and placed into separate tubes containing Dulbecco's minimum eagle's medium (DMEM) supplemented with 100 U/mL ampicillin and 100 U/mL streptomycin. The samples were washed 5 times with phosphate buffered saline and chopped into 1 mm<sup>3</sup> pieces, which were seeded on the bottom surfaces of tissue culture flasks containing 90% DMEM and 10% fetal bovine serum (FBS), and cultured at 37 °C in an incubator with 5% CO<sub>2</sub> (9,10) and saturated humidity.

When reaching 80%–90% confluence, the cells were harvested using 0.25% trypsin and separated into prepared culture flasks at ratios of 1:2 or 1:3 (10,11).

### Cryogenic preservation and recovery

Cells at the logarithmic growth phase were collected and counted with a hemocytometer, and then resuspended in frozen solution (10% dimethyl sulfoxide + 40% FBS + 50% DMEM), at a density of  $4 \times 10^6$  viable cells per milliliter. The cell suspension was aliquoted into sterile plastic cryovials that were labeled with the breed name, sex, freezing serial number, and the date. The vials were sealed and kept at 4 °C for 20–30 min to equilibrate the DMSO, put into a programmed freezing system, and finally transferred to liquid nitrogen for long-term storage (12).

The cryovials were removed from the liquid nitrogen and quickly thawed in a 42 °C water bath. When the ice clump was almost thawed, the cells were transferred into a flask with 90% DMEM and 10% FBS, gently blown into uniform single cell suspension, and cultured at 37 °C, 5% CO<sub>2</sub>. The medium was changed after 24 h.

### Estimation of cell viability by trypan blue exclusion test

Viabilities before freezing and after recovery were determined using the trypan blue exclusion test. The number of nonviable cells was determined in a visual field of 1000 cells (13).

### Cell growth curve

The cells were seeded in 24-well plates at a density of approximately  $1.5 \times 10^4$  cells per well, cultured for 7 days, and counted every day (3 wells each time). The mean cell numbers at each time point were then plotted to time, and the population doubling time (PDT) was determined based on this curve (14–16).

### Chromosome analysis

The chromosomes were prepared, fixed, and stained following standard methods (17). The cells were harvested when they reached 50%–70% confluence. After hypotonic treatment, fixation, and Giemsa staining, the chromosome numbers were counted from 100 spreads under an oil immersion objective. Relative length, centromeric index and type were calculated according to the protocols of Kawarai et al. (18).

### Isoenzyme analysis

The isoenzyme patterns of lactic dehydrogenase (LDH) and malic dehydrogenase (MDH) were detected using the vertical slab noncontinuous polyacrylamide gel electrophoresis (PAGE) assay. According to the method described by Simpson (19), the cells were harvested, and the protein extraction solution (0.9% Triton X-100 and 0.06 mM NaCl:EDTA at a mass ratio of 1:15) was added after the cell concentration was adjusted to  $5 \times 10^7$  cells/mL. The mixture was then centrifuged and the supernatant was stored in aliquots at  $-80^\circ\text{C}$ . Liquid sucrose (40%) and the samples were mixed (1:1) and loaded into the individual lanes of the polyacrylamide gel (20).

### Microbial analysis

#### Detection of bacteria and fungi

The cells were cultured in DMEM containing 10% FBS, which was free of antibiotics, to test for the presence of bacteria and fungi at 3 days after subculture, according to the method of Doyle et al. (21).

#### Detection of mycoplasmas

According to the protocols of the American Type Culture Collection (ATCC), the cells were cultured in an antibiotic-free medium for at least 1 week, and then fixed and stained with Hoechst 33258 (22).

#### Detection of viruses

Hay's hemadsorption protocol was used routinely to exclude the cells from cytopathogenesis, using phase-contrast microscopy (23).

#### Expression of fluorescent protein genes in the JGG fibroblasts

According to the methods of Wu et al., the 3 fluorescent protein genes, which are carried by 3 kinds of plasmids, pDsRed1-N1, pEGFP-N3, and pEYFP-N1, were transfected into the fibroblasts through a combination of plasmid DNA (BD Biosciences Clontech product) and Lipofectamine 2000 (Invitrogen). The cells were observed at 24, 48, 72, and 96 h, 1 and 2 weeks, and 1 month after transfection, using a laser scanning confocal microscope (Nikon TE-2000-E, Japan). Expression of the 3 fluorescent protein genes was observed under excitation wavelengths of 405, 488, and 543

nm, separately. In each experimental group, 10 visual fields were picked to take pictures and calculate the transfection efficiency (24).

## Results

### Morphological observation of the fibroblasts from the JGG

At about 5–7 days after the tissue explants adhered to the flasks, fibroblast cells were observed sprouting from the margins of these tissue pieces (Figure 1a). The cells showed typical fusiform morphology with centrally located oval nuclei. The cells covered the bottom of the flasks within 3–5 days and formed a monolayer. The fibroblasts grew rapidly and gradually replaced the epithelial cells in the subculture. The cells had fibroblastic characteristics with turgor vitalis cytoplasm, and, during growth, they were morphologically fibroblast-like with radiating, flame-like or whirlpool-like migrating patterns (Figure 1b).

### Cell viabilities before cryopreservation and after recovery

The method of the trypan blue exclusion test was adopted to calculate cell viabilities before cryopreservation and after recovery. The viabilities of the culture were  $98.24 \pm 0.35\%$  (Figure 1c) before freezing and  $96.83 \pm 0.54\%$  after thawing (Figure 1d). These results showed that the cells were healthy with good viability under these culture conditions, and freezing did cell viability little harm.

### Growth dynamics

The growth curve of the JGG ear marginal tissue fibroblasts exhibited a typical "S" shape (Figure 2) and the PDT was about 48 h. The latent phase was about 2 days, followed by an exponential phase of 4 days, which gave way to the stationary phase afterwards.

### Karyogram and chromosome number

The chromosome number of the JGG was  $2n = 60$ , comprising 58 autosomes and 2 sex chromosomes, XY or XX (Figure 3). The relative length and centromere type are shown in Table 1. The chromosome numbers per spread were counted for 100 spreads of the first and the third passages, and the results showed that  $98.35 \pm 0.21\%$  and  $97.62 \pm 0.53\%$  of the cells were diploid, supporting the conclusion that the cell line is reproducibly diploid.

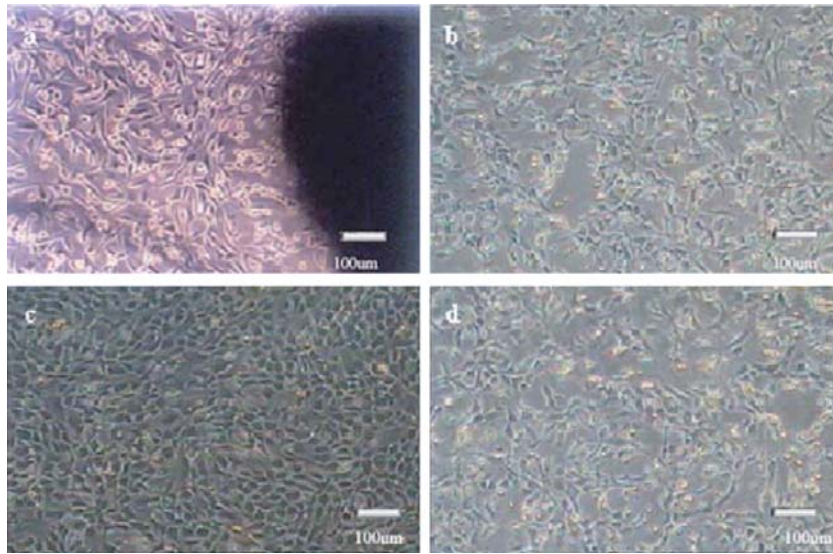


Figure 1. Morphology of the JGG fibroblast cells: a) primary cells grew out from the ear marginal explants, b) subcultured cells, c) cells before cryopreservation, and d) cells at 24 h after recovery from cryostorage. Bar = 100  $\mu\text{m}$ .

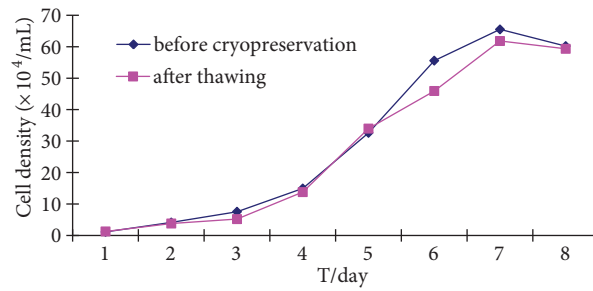


Figure 2. The growth curve of the JGG fibroblasts. The growth curve was a typical “S” shape. A lag of around 48 h was observed after cells were seeded. The cells then proliferated and entered the logarithmic phase. The PDT was approximately 48 h. From the sixth day, cells entered the plateau phase.

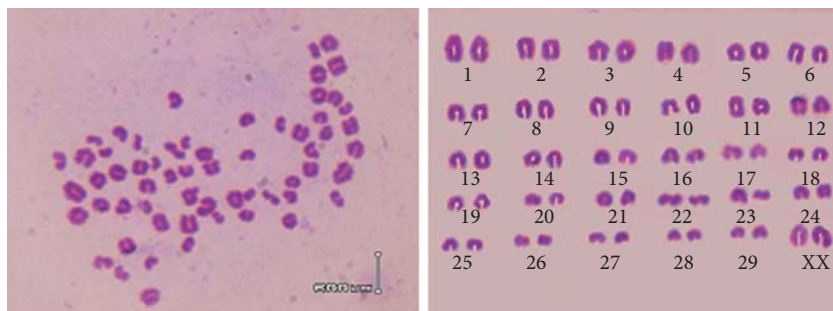


Figure 3. Chromosomes at metaphase (left) and karyotype (right) of the JGG fibroblasts ( $\delta$ ). The karyotype of the JGG fibroblasts consisted of 30 pairs of chromosomes. The sex chromosome type was XX( $\text{♀}$ )/XY( $\text{♂}$ ).

Table 1. Chromosome parameters of the JGG fibroblasts (♂).

Chromosome number	Relative length (%)	Centromere type	Chromosome number	Relative length (%)	Centromere type
1	4.89 ± 0.67	T	17	2.96 ± 0.137	T
2	4.79 ± 0.23	T	18	2.90 ± 0.22	T
3	4.45 ± 0.56	T	19	2.81 ± 0.16	T
4	4.43 ± 0.20	T	20	2.72 ± 0.14	T
5	4.37 ± 0.34	T	21	2.68 ± 0.11	T
6	4.16 ± 0.24	T	22	2.50 ± 0.14	T
7	4.05 ± 0.12	T	23	2.39 ± 0.16	T
8	4.00 ± 0.08	T	24	2.32 ± 0.24	T
9	3.86 ± 0.10	T	25	2.15 ± 0.36	T
10	3.69 ± 0.03	T	26	2.10 ± 0.41	T
11	3.58 ± 0.12	T	27	2.01 ± 0.41	T
12	3.55 ± 0.26	T	28	1.84 ± 0.34	T
13	3.48 ± 0.14	T	29	1.72 ± 0.11	T
14	3.44 ± 0.19	T	X	4.65 ± 0.07	T
15	3.16 ± 0.02	T	Y	1.36 ± 0.1	M

### Isoenzyme analysis

In this study, the isoenzyme patterns of LDH and MDH were obtained using vertical slab noncontinuous PAGE, stained by Coomassie brilliant blue, and compared with those from other species, which were cultured at the same time (Figures 4 and

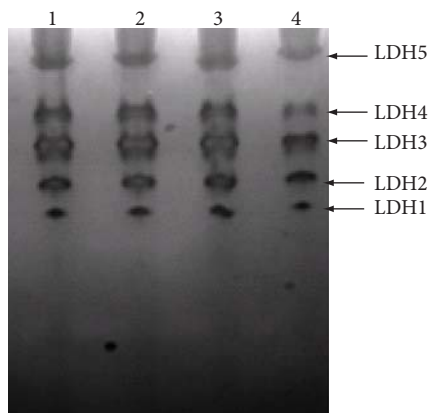


Figure 4. Isoenzyme patterns of LDH from 4 cell lines: lane 1, Suffolk sheep; lane 2, Texel sheep; lane 3, Poll Dorset sheep; and lane 4, Jining Grey goat.

5). The relative mobility fronts of LDH were in the order of LDH-5, LDH-4, LDH-3, LDH-2, and LDH-1, from cathode to anode (Figure 4), while the 2 bands of MDH were m-MDH and s-MDH (Figure 5). The results indicated that there was no cross-contamination from other cell lines.

### Microbial analysis

#### Detection of bacteria and fungi

The medium was clear at all times and no abnormal changes could be observed under the microscope. The results indicated that our JGG ear marginal fibroblasts were free of bacteria and fungi contamination. In the positive control group, fungi or bacteria did not affect cells very much during the initial stage of contamination, but the culture medium became turbid as they propagated (Figure 6a). The accumulation of acor metabolin made the culture medium flavo-green. The colony or hypha appearing silky or tree-shaped could be easily observed under an inverted microscope, as well as by the naked eye (Figure 6b).

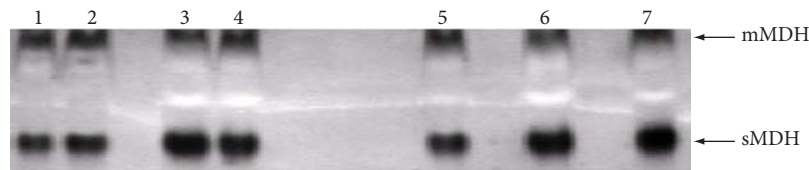


Figure 5. Isoenzyme patterns of MDH from 7 cell lines: lane 1, Suffolk sheep; lane 2, Texel sheep; lane 3, Poll Dorset sheep; lane 4, Angora goat; lane 5, Jining Grey goat; lane 6, Taihang Black goat; and lane 7, Jining Black Grey goat.



Figure 6. Test for the presence of microbial organisms: a) fibroblasts contaminated by fungi, b) fibroblasts contaminated by bacteria, and c) fibroblasts stained with Hoechst 33258 for mycoplasma contamination, negative, bar = 100 µm.

### Detection of mycoplasmas

After being stained with Hoechst 33258, the cells showed smooth surfaces, round nuclei with blue fluorescence, and no filamentous blue fluorescence around the nuclei when observed under a fluorescence microscope. If there was abundant punctiform and filiform blue fluorescence in the cell nucleoli, it could indicate that the cells were contaminated by mycoplasmas (Figure 6c). The result suggested that the fibroblasts were free of mycoplasmas.

### Detection of viruses

Tests for virus contamination were negative. No cytopathogenic evidence was detected by the hemadsorption test.

### Expression of exogenous genes in the fibroblasts of the JGG

The 3 fluorescent protein genes, DsRed1, EGFP, and EYFP, were all highly expressed with the highest transfection efficiencies at 48 h (Figure 7). The transfection efficiencies are listed in Table 2. The number of transfected cells decreased gradually as time passed. However, fluorescence could still be observed in a few cells at 2 weeks after transfection. The transfection result provided solid evidence for

the future application of JGG fibroblasts in structural genomics, functional genomics, and transgene research.

### Discussion

The ear marginal fibroblast line from the JGG was established using the adherent culture method. During the early formation stage of the cell line, epithelial cells and fibroblasts grew simultaneously. As opposed to the epithelial cells, fibroblasts could be trypsinized more readily and adhered more easily (12). Because of these differences, the fibroblasts would quickly outgrow their epithelial counterparts. In this manner, a pure fibroblast culture was obtained after 2–3 passages (25). The JGG fibroblasts were cryopreserved in 52 vials, within 5 passages, at a density of about  $3 \times 10^6$  cells/mL. The viabilities were  $98.24 \pm 0.35\%$  before freezing and  $96.83 \pm 0.54\%$  after thawing, indicating that cryopreservation and recovery had little impact on the cells. The growth curve of the JGG fibroblasts showed a typical “S” shape, consisting of the latent phase, exponential phase, and plateau phase. The cells after passage had a latent phase of about 48 h, which was a result of trypsinization. Thereafter, the cells experienced

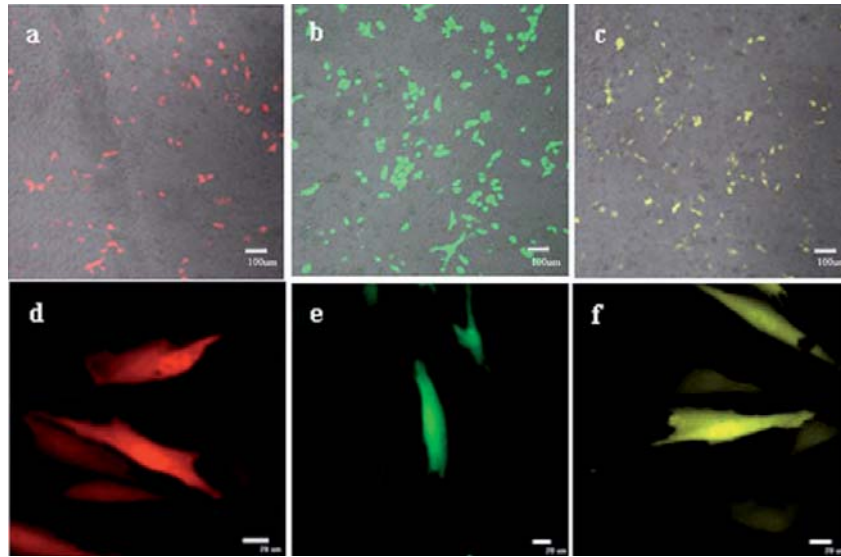


Figure 7. Expression of 3 fluorescent protein genes at 48 h after transfection: a and d, b and e, and c and f are the transfection results of pDsRed1-N1, pEGFP-N3, and pEYFP-N1, respectively, and a, b, and c bar = 100  $\mu$ m, d, e, and f bar = 20  $\mu$ m. The photos were taken using a laser scanning confocal microscope, at excitation wavelengths of 408, 488, and 543 nm.

Table 2. Transfection efficiencies of 3 fluorescent protein genes in the JGG fibroblasts.

Time (h)	3 fluorescent protein genes (%)		
	EGFP	EYFP	DsRed1
24	27.51 $\pm$ 0.21	22.14 $\pm$ 0.36	15.22 $\pm$ 0.59
48	37.33 $\pm$ 0.52	28.67 $\pm$ 0.61	30.95 $\pm$ 0.42
72	35.26 $\pm$ 0.26	26.92 $\pm$ 0.51	28.46 $\pm$ 0.37

exponential proliferation, followed by a plateau phase. Finally, cell growth ceased as a result of contact inhibition.

As the diploid frequency was 98.3% and 97.6%, we could conclude that the JGG fibroblast culture was stably diploid. Although some hypodiploid, hyperdiploid, and polyploid cells did emerge in the culture as the passages increased, the incidence was still very rare in our study (below 2%). Hence, there was seldom a chromosome number variation in the JGG fibroblasts.

Microbial contamination is the most frequent pollution in cell culture. Air, equipment, serum, tissue sample, etc. are all sources of cell culture

contamination. The turbidity of culture media contaminated by bacteria and fungi can be distinguished with the naked eye and these microorganisms could be removed using a bacteria filter. Virus infection was ruled out by detecting the cytopathogenic effects in the culture. However, it is hard to exclude mycoplasma contamination. Mycoplasmas have no nuclei and can grow and reproduce simultaneously with the cultured cells. They are hard to remove and could coexist with the cells for a long time. The methods for mycoplasma detection include direct solid agar culture, indirect fluorescence staining of the DNA, and new DNA probe hybridization. Because fluorescent staining of mycoplasma DNA is ready-to-use, it is commonly

used by major cell culture collection institutions, such as the ATCC. Our microbiological detection results showed that the JGG fibroblast bank was healthy and free of mycoplasma contamination.

Isoenzyme polymorphism, evidenced by the existence of distinctive patterns, occurs among species and sometimes among subspecies, as well as among tissues from the same species (26). Isoenzymes can be separated chromatographically or electrophoretically, revealing species or tissue characteristic distribution patterns. Biochemical analysis of isoenzyme polymorphism is currently considered to be a criterion for cell line identification and the detection of interspecies contamination, and is routinely used by leading culture centers around the world. In this study, the clear bands and specific isoenzyme patterns confirmed that the genetic characteristics of the JGG fibroblasts were stable and there was no contamination with cells from other cell lines.

In the transfection assay, the fluorescent signals were strong, with the highest transfection efficiency at 48 h. The transfection efficiencies decreased with time, while high expression levels were observed after 1 week, indicating that the exogenous genes in the fibroblasts could be replicated, transcribed, translated, and subsequently modified.

In addition, there were no differences in the cell morphology, cell viability, growth kinetics, karyotype

analysis, and exogenous expression of the fluorescent protein genes of the JGG cells and the JBG cells of Li et al. (6). However, the isoenzyme analysis result showed that there were differences in the shapes and mobility of the LDH and MDH. It is suggested that JGGs and JBGs are 2 distinct subspecies, with some differences in gene sequence, which need further microarray analysis to understand.

The biological characteristics of the JGG cells, especially the genetic characteristics, may change when cultured in vitro, as a result of serial passage. Thus, as few passages as possible are recommended to protect the genetic properties of the fibroblasts.

It is potently supported that our cell bank makes a great contribution to the preservation of the genetic resources of the precious native species JGG and provides useful biomaterials for future studies in cell biology, medicine, genomics, post-genomics, and both genetic and embryonic engineering.

### Acknowledgments

This study was supported by the “863” National Major Research Program (2006AA10Z198, 2007AA10Z170), the National Key Technology Research and Development Program (2006BAD13B08), the National Scientific Foundation of China (30671539), and the Ministry of Agriculture of China for Transgenic Research Program (2008ZX08009-003).

### References

1. Ma, Y., Fen, W.: The evaluation of livestock and poultry breed resources. *Ecol. Dom. Anim.*, 2002; 3: 1–5.
2. Wu, C.: The theory and technology of the conservation of animal genetic resources—the specy foundation of animal agricultural continuing development in 21 century. *J. Yunnan Univers.*, 1999; 21: 7–10.
3. Li, L.F., Yue, H., Ma, J., Guan, W.J., Ma, Y.H.: Establishment and characterization of a fibroblast line from Simmental cattle. *Cryobiology*, 2009; 59: 63–68.
4. Na, R.S., Zhao, Q.J., Jin da, P., Su, X.H., Chen, X.W., Guan, W.J., Ma, Y.H.: Establishment and biological characteristics of Ujumqin sheep fibroblast line. *Cytotechnology*, 2010, 62: 43–52.
5. Li, L.F., Guan, W.J., Li, H., Zhou, X.Z., Bai, X.J., Ma, Y.H.: Establishment and characterization of a fibroblast cell line derived from Texel sheep. *Biochem. Cell. Biol.*, 2009; 87: 1–8.
6. Li, X.C., Yue, H., Li, C.Y., He, X.H., Zhao, Q.J., Ma, Y.H., Guan, W.J., Ma, J.Z.: Establishment and characterization of a fibroblast cell line derived from Jining Black Grey goat for genetic conservation. *Small Ruminant Res.*, 2009; 87: 17–26.
7. Yao, F.: Chinese Caprine resource for fur skin. *Modern Livestock and Poultry Breeding Industry*. 2004; 6: 44–45.
8. Jin, J.: Rarest Jining Gray goat. *Jilin J. Anim. Husb. Vet. Med.*, 1997; 5: 22.
9. Freshney, R.I.: *Culture of Animal Cells: A Manual of Basic Technique*, 4th ed. Wiley-Liss, New York. 2000; 49–175.
10. Guan, W.J., Ma, Y.H., Zhou, X.Y., Liu, G.L., Liu X.D.: The establishment of fibroblast cell line and its biological characteristic research in Taihang Black goat. *Rev. China Agric. Sci. Technol.*, 2005; 7: 25–33.



11. Zhou, X.M., Ma, Y.H., Guan, W.J., Zhao, D.M.: Establishment and identification of Debao pony ear marginal tissue fibroblast cell line. *Asian-Aust. J. Anim. Sci.*, 2004; 17: 1338–1343.
12. Ren, F., Li, Y., Zhang, Y.: In vitro cultivation and freezing of bovine skin fibroblast cells. *J. Yellow Cattle Sci.*, 2002; 28: 8–10.
13. Freshney, R.I.: *Culture of Animal Cells: A Manual of Basic Technique*. 3rd ed. John Wiley & Sons, New York. 1994.
14. Qi, Y., Tu, Y., Yang, D., Chen, Q., Xiao, J., Chen, Y., Fu, J., Xiao, X., Zhou, Z.: Cyclin A but not cyclin D1 is essential in c-myc-modulated cell-cycle progression. *J. Cell. Physiol.*, 2007; 210: 63–71.
15. Suemori, H., Yasuchika, K., Hasegawa, K., Fujioka, T., Tsuneyoshi, N., Nakatsuji, N.: Efficient establishment of human embryonic stem cell lines and long-term maintenance with stable karyotype by enzymatic bulk passage. *Biochem. Biophys. Res. Commun.*, 2006; 345: 926–932.
16. Weingartl, H.M., Sabara, M., Pasick, J., van Moorlehem, E., Babiuk, L.: Continuous porcine cell lines developed from alveolar macrophages Partial characterization and virus susceptibility. *J. Virol Methods.*, 2002; 104: 203–216.
17. Costa, U.M., Reischak, D., da Silva, J., Ravazzolo, A.P.: Establishment and partial characterization of an ovine synovial membrane cell line obtained by transformation with Simian Virus 40 T antigen. *J. Virol Methods.*, 2005; 128: 72–78.
18. Sun, Y.L., Lin, C.S., Chou, Y.C.: Establishment and characterization of a spontaneously immortalized porcine mammary epithelial cell line. *Cell. Biol. Int.*, 2006; 30: 970–976.
19. Kawai, S., Hashizaki, K., Kitao, S., Nagano, S., Madarame, H., Neo, S., Ishikawa, T., Furuichi, M., Hisasue, M., Tsuchiya, R., Tsujimoto, H., Yamada, T.: Establishment and characterization of primary canine hepatocellular carcinoma cell lines producing alpha-fetoprotein. *Vet. Immunol. Immunopathol.*, 2006; 113: 30–36.
20. Simpson, R.: *Proteins and Proteomics: A Laboratory Manual*. Science Press, Beijing. 2003.
21. Doyle, A., Hay, R., Kirsop B.: *Animal Cells: Living Resources for Biotechnology*. Cambridge University Press, Cambridge, U.K. 1990; 81–100.
22. Masover, G., Becker, F.; Detection of mycoplasmas in cell cultures by cultural methods. In: Miles, R.J., Nicholas, R.A.J., Eds. *Methods in Molecular Biology*. Vol. 104: *Mycoplasma Protocols*. Humana Press Inc., Totawa, New Jersey. 1998; 207–215.
23. Hay, R.I.: Cell line preservation and characterization. In: Freshney, R.I., Ed. *Animal Cell Culture: A Practical Approach*, 2nd ed. Oxford University Press, Oxford. 1992; 104–135.
24. Wu, H., Guan, W., Li, H., Ma, Y.: Establishment and characteristics of white ear lobe chicken embryo fibroblast line and expression of six fluorescent proteins in the cells. *Cell. Biol. Int.*, 2008; 32: 1478–1485.
25. Zhou, X.M., Ma, Y.H., Guan, W.J., Wen, J., Li, H.: Establishment and characteristics of a Beijing fatty chicken embryo fibroblast cell line. *Chinese J. Anim. Vet. Sci.*, 2005; 36: 209–215.
26. Men, Z.M., Liu, X., Ma, H.M., Han, J.L.: Karyotype analysis of Lanzhou fat-tailed sheep. *J. Gansu. Agric. Univers.*, 2002; 37: 158–160.