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Establishment and Biological Characteristic Research of Altay Sheep Fibroblast Cell Bank

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Abstract: A fibroblast bank of Altay sheep consisting of 182 tubes of frozen cells was successfully abtained from primary explants of 57 individual animals through attachment cultivation and cryopreservation biotechniques and each vial contains 1.3×10^7 cells, respectively. The results of quality assays and biological characteristic researches showed that the cells cultured with a Population Doubling Time (PDT) of about 24 h, constituted a typical fibroblast in morphology; assays for microbial contamination regarding bacteria, fungi, viruses and mycoplasmas are all negative; analysis of Lactate Dehydrogenase (LDH) and Malate Dehydrogenase (MDH) isoenzymes ruled out the possibilities of cross-contamination between species; the ratio of diploid cells is 98.6%. In order to study the expressibility of exogenous genes, six kinds of fluorescent protein-coding plasmids were transfected into the Altay sheep fibroblast with transfection efficiencies between 22.3 and 46.7%. This research could not only preserve the genetic resources of Altay sheep but also provide both technical and theoretical basis for preserving those of other mammals and poultry at cell level.

Key words: Altay sheep, fibroblast line, biological characterization, genetic resources conservation, somatic cells cryopreservation

INTRODUCTION

Genetic resources, a material basis of human subsistance and sustainable development, is closely related to the security of global economics, culture and environment. Haven't genetics resources been preserved by any means before its totally extinguishment, not only would this treasure be lost forever but also the attempt and desire to make researches on the unknown biological and molecular mechanisms of cells from various livestock and poultry before their extinction would never be fulfilled, not to mention to make them resurgent through somatic cell cloning. Accordingly, it is high time to launch a campaign to conserve endangered species (Guan *et al*., 2005) in the current world, the practical steps of which may include the preservation of individual animals, semen, embryos, genomic libraries and cDNA libraries, etc. Besides above mentioned methods, the development of modern cloning technique facilitates the conservation of animal genetic materials in a tremendous extent (Wu, 1999).

Altay sheep, oringinally named as Fuhai big-tail sheep, domesticated by Kazakh herders for hundreds of years is an immemorial breed in China and was firstly documented in The New Encyclopedia of Tang Dynasty as early as >1000 years ago. Due to its numerous merits such as cold temperature resistance, flesh and fat fecundity and so forth, Altay sheep constitute a precious animal germplasm resource distinctively in China and enjoy a high reputation both home and abroad (http://cif.mofcom.gov.cn/cif/html/shi_html/ppsp/2009/2/1233485461958.html).

MATERIALS AND METHODS

Primary cell culture, freeze preservation and recovery: Ear margin tissues (about 1 cm^2 in size) were abtained from 8 Altay sheep (1 male and 7 females) and placed separately into DMEM containing tubes supplemented with ampicillin (100 U mL^{-1}) and streptomycin (100 μg mL^{-1}). These samples were brought back to the lab immediately after its isolation, rinsed and chopped into 1 mm^3 pieces and then seeded on the bottom of a tissue cultivation flask containing DMEM+10% fetal bovine serum in a 37°C incubator with 5% CO_2 (Freshney, 2000; Guan *et al*., 2005; Zhou *et al*., 2004). Primary cells were passaged into more flasks when 80-90% confluent at a ratio of 1:2 or 1:3 (Freshney, 2000).

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Cell viability: Survival rates before cryopreservation and after recovery were calculated through Trypan Blue Exclusion test. The cells, seeded in 6 well plates at a concentration of 10^4 per well were counted under a microscope with a hemocytometer (Qi *et al*., 2007).

Cryopreservation and reseeding: Cells were cultured in fresh medium 24 h before freezing to guarantee sufficient nutrition. Monoplast cell suspension was prepared by harvesting cells with 0.25% trypsin. After the cell suspension centrifuged at 1000 rpm for 8 min, discard the supernatant was abandoned and resuspend the pellet at a concentration of 4×10^6 cells mL^{-1} in 10% Dimethyl Sulfoxide (DMSO), 30% FBS and 60% DMEM and make it subpackaged into sterile cryogenic vials labeled with the species name, gender, freezing serial number and the date. The vials, sealed and kept at 4°C for 20-30 min to equilibrate the DMSO were then transfered in liquid nitrogen after programmed freezing for long term storage (Ren *et al*., 2002). For reseeding, remove the cryopreserved cells from liquid nitrogen and thaw at 42°C until a small amount of ice left in the cryovial. Transfer the cell suspension to previously aliquoted complete DMEM medium and centrifuged at 1000 rpm for 10 min to remove the DMSO. Resuspend the pellet in fresh medium and seed the suspension into sterile flasks and make the cells cultured at 37°C with 5% CO_2. Medium should be renewed 24 h later (Freshney, 1992).

Cell growth curve: The proliferation properties of Altay sheep cells *in vitro* were best epitomized by their Population Doubling Time (PDT). Cells were harvested and seeded in a 24 well plate at a concentration of 1.5×10^4 per well, cultivated for a span of 7 days and counted at an interval of 24 h. Average values were adopted to plot a growth curve and calculate the PDT (Sun *et al*., 2006) accurately.

Microorganism detection

Test for bacteria, fungi and yeasts: Cultivate the cells in a medium free of antibiotics and check it out after 3 days. The method described by Doyle *et al*. (1990) could be referred to for more detailed procedure.

Test for viruses: Under normal culture conditions, the cells were selected randomly for cytopathogenic examination with phase-contrast microscopy according to Hay's hemadsorption protocol (Hay, 1992).

Mycoplasma detection: According to the protocol from the ATCC, cells were cultured in medium free of antibiotics for at least 1 week and then fixed and stained with Hoechst 33258 and DAPI with the methods of Masover and Becker (1998) and Freshney (2000) for fluorescent staining of DNA. The results were confirmed by ELISA with the ELISA mycoplasma detection kit (Roche, Lewes, East Sussex, UK) which is capable of identifying the four major types of mycoplasmas, namely *M. arginini*, *M. hyorhinis*, *A. laidlawii* and *M. orale*.

Karyotype and banding analysis: Metaphase spreads were prepared with cells at the exponential phase of growth cultivated with 0.1 μg mL^{-1} colcemid (Gibco/BRL). The cells, treated with a hypotonic KCl/HEPES/EDTA solution were harvested following standard cytogenetic procedures. Slides of fixed cells were trypsin-Giemsa banded to identify individual metaphase chromosomes. Representative chromosome sets were photographed and analyzed. Microslide preparation and chromosome staining were performed according to the description by Suemori *et al*. (2006). The percent of diploid was counted from 100 spreads. Several parameters were calculated according to these equations:

Arm ratio = Long arm length (q) vs. Short arm length (p)

Centromere exponent = Short arm length vs. Chromosomal length

Relative length = Single chromosomal length vs. (Total autosome lengths+X-chromosome)

Isoenzyme analysis: Isoenzyme patterns of Lactic Dehydrogenase (LDH) and Malic Dehydrogenase (MDH) were detected by vertical slab non-continuous Polyacrylamide Gel Electrophoresis (PAGE) assay. Protein extraction solution (0.9% Triton X-100, 0.06 mmol NaCl/EDTA in mass ratio 1:15) was added into harvested cells with a concentration of 5×10^7 mL^{-1} and after the mixture centrifuged, the supernatant was stored in aliquots at -80°C. The 40% sucrose solution and the samples (1:1) were blended and then loaded in seperate lanes of the polyacrylamide gel (He and Zhang, 1999). Different mobility patterns were indicated by the Relative mobility Front (RF) which was calculated as the ratio of the migration distance of the isozyme band to that of bromophenol blue.

Expression of six kinds of fluorescent proteins in Altay sheep cells: To achieve the highest transfection with trivial cytotoxicity, optimal conditions were screened through various cell denstities and concentrations of plasmid DNA (BD Biosciences Clontech) and Lipofectamine 2000 (Invitrogen) according to the Lipofectamine Medium Methods of Escriou *et al*. (2001)

and Tsuchiya *et al.* (2002). The cells treated were observed 24, 48, 72 and 96 h, 1 and 2 weeks and 1 month after transfection to detect the expression of six fluorescent protein with excitation wavelengths of 405, 488 and 543 nm, respectively. For each experimental group, images were captured from 10 visual fields and confocal microscopy was used to count the total and positive cell numbers in each field to calculate the transfection efficiencies. The effects of the exogenous genes on the cells were measured in terms of motility and apoptosis rates through Trypan Blue and DAPI staining.

RESULTS

Cell morphology: Fibroblasts, migrating from the tissue pieces 7-14 days after explantation, exhibited typically fibrous and fusiform morphologies with centrally located oval-shaped nuclei (Fig. 1a). These cells were subcultured till 80-90% confluent. Due to their more potent proliferation potential, fibroblasts would, after serial passages, gradually overwhelm epithelial cells adulterated and show characteristically multipolar or bipolar appearances (Fig. 1b).

Cell viability: Cells were harvested and cryopreserved after 3-4 passages. The viabilities, formulated as mean±SD were 96.54±3.22% (Fig. 1c) and 92.36±2.77%, respectively before and after cryopreservation (Fig. 1d), the difference between which is not significant ($p>0.05$). The results suggested that the cells were viable under these culture conditions and were seldom damaged during cryopreservation.

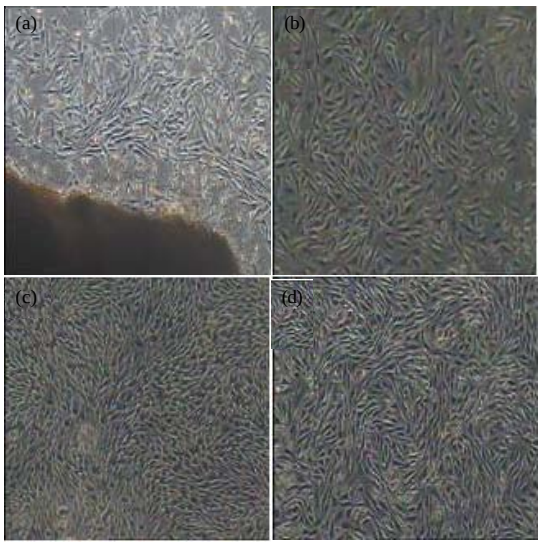

Fig. 1: The culture of Altay sheep fibroblasts (10×3.3). a) Primary cells of ear marginal explants from Altay sheep; b) subcultured fibroblasts of Altay sheep; c) fibroblasts before cryopreservation; d) fibroblasts 12 h after recovery

Growth curve analysis: The growth curve of Altay sheep ear marginal fibroblasts exhibited a typical S shape (Fig. 2) with a Population Doubling Time (PDT) approximately 48 h. There was a lag time or latency phase of about 24 h after seeding, corresponding to the adaptation and recovery of the cells against protease damage after which they proliferated rapidly and entered exponential phase. As the cell density increased, proliferation was reduced by contact inhibition and there come the plateau phase since the 8th day.

Microbial analysis: Tests for contamination with bacteria, fungi and yeasts were negative; no microorganisms were observed in the culture media. The results of the hemadsorption assay ruled out the possibilities of viruses' presence. Observing under a fluorescent microscope after staining with Hoechst 33258, potentially the most effective and frequently used method for detecting mycoplasma contamination (Barile and Rottem, 1993), it was indicated that the established cell line with its fibroblast nuclei appear as blue ellipses was mycoplasma negative (Fig. 2b).

Karyotype analysis: A typical genome of Altay sheep, 2n = 54 was composed of 52 autosomes and 2 sex chromosomes, XY or XX (Fig. 2c). The chromosomes whose parameters were listed in Table 1 were except sex chromosomes subtelocentric (X) or metacentric (Y), chromosome 1, 2 and 3 metacentric, exclusively telocentric. The average chromosome number was counted from 100 spreads of the first, second and fourth passages with frequencies of diploid cells 92.2, 91.6 and 90.7%, respectively. Aberrations in chromosome numbers had a tendency to be magnified as serial passages stepped further indicating that culture *in vitro* would ineluctably disturb the hereditary stability of cells in some degree. However, it also suggested that the cell line was reproducibly diploid.

Isoenzyme analysis: Lactate Dehydrogenase (LDH) and Malate Dehydrogenase (MDH) isoenzyme patterns obtained from Altay sheep fibroblast cells were compared with those of other species and breeds. It was observed that five LDH isoenzyme bands, namely LDH3, LDH2, LDH1, LDH4 and LDH5 (Fig. 3a) and two MDH isoenzyme bands, nameyly m-MDH and s-MDH (Fig. 3b) were arranged in order from anode to cathode, respectively. The results ruled out the possibilities of cross-contamination among species and subspecies.

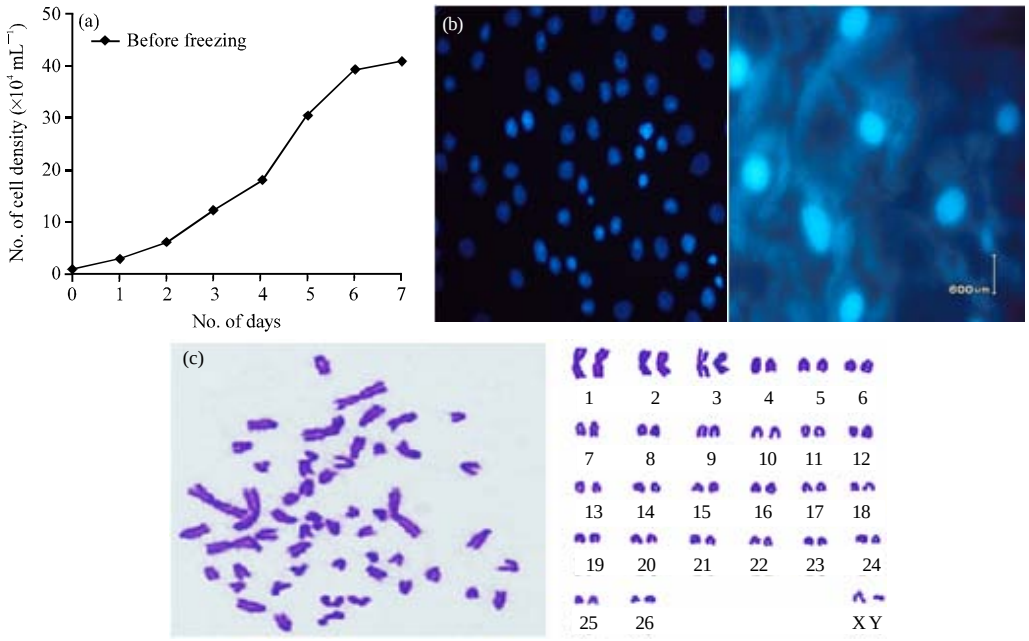

Fig. 2: The characterization of fibroblast cell line of Altay sheep ear marginal fibroblast cell line. a) The growth curve of Altay sheep fibroblast before cryopreservation, cells were enumerated with a hemocytometer. Each value represents the mean±SD of three independent experiments; b) mycoplasma contamination detection for the Altay sheep fibroblasts stained with Hoechst 33258 and its positive control; c) chromosome at metaphase (left) and karyotype (right) of the Altay sheep (♀), 1000x

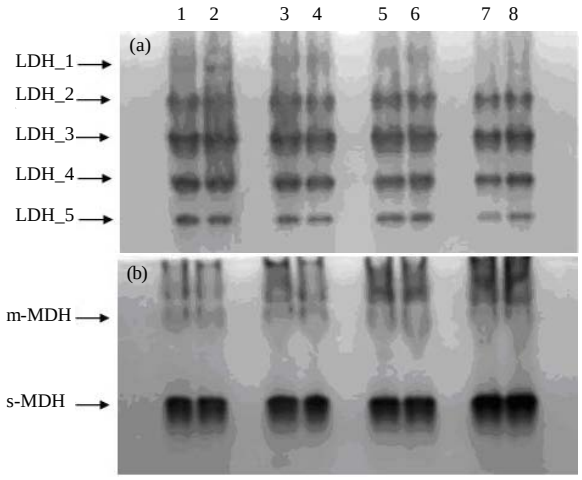

Fig. 3: Lactate Dehydrogenase (LDH) and Malate Dehydrogenase (MDH) zymotype for different species and subspecies; a) 1: Zhiwei goat; 2: Jining black goat; 3: Taihang black goat; 4: Mongolian sheep; 5 and 6: Luxi cattle; 7: Simmental bovine; 8: Angus bovine; 9: Piemontese bovine; 10 and 11: large white pig; b) 1 and 2: Aaijiao chicken; 3 and 4: Baier chicken; 5 and 6: Big Yorksire; 7 and 8: Landrace

Table 1: Chromosome parameters of Altay sheep (male)

Chromosome number	Relative length (%)	Centromere index (%)	Centromere morphology
1	8.41±0.024	45.12	M
2	8.95±0.022	44.78	M
3	7.17±0.024	47.41	M
4	3.86±0.017	0	T
5	3.85±0.018	0	T
6	3.77±0.022	0	T
7	3.52±0.025	0	T
8	3.42±0.027	0	T
9	3.45±0.023	0	T
10	3.22±0.035	0	T
11	3.16±0.023	0	T
12	3.13±0.037	0	T
13	2.95±0.021	0	T
14	2.94±0.035	0	T
15	2.87±0.024	0	T
16	2.83±0.032	0	T
17	2.77±0.034	0	T
18	2.75±0.023	0	T
19	2.76±0.024	0	T
20	2.72±0.017	0	T
21	2.71±0.014	0	T
22	2.64±0.033	0	T
23	2.52±0.032	0	T
24	2.47±0.021	0	T
25	2.46±0.027	0	T
26	2.05±0.019	0	T
X	3.88±0.023	18.90	ST
Y	2.56±0.023	52.48	M

M: Metacentric chromosome; SM: Submetacentric chromosome; ST: Subtelocentric chromosome; T: Telocentric chromosome

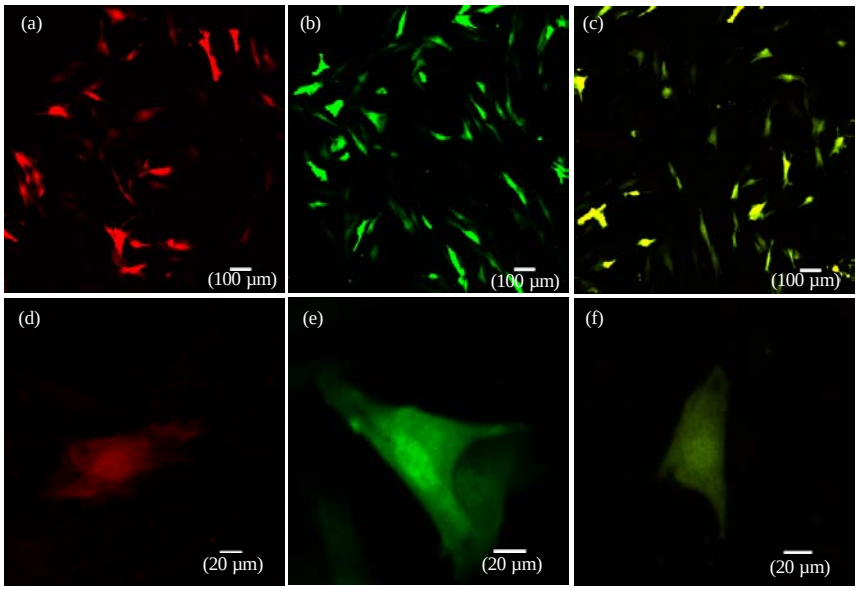

Fig. 4: Comparative figures of pDsRed1-N1, pEGFP-N3 and pEYFP-N1 expression in Altay sheep fibroblasts (10x); a) pDsRed1-N1, 48 h; b) pEGFP-N3, 72 h; c) pEYFP-N1, 72 h

Table 2: Transfection efficiencies of six fluorescent genes in the Altay sheep fibroblast cells

Transfectin time (h)	Transfection rate of six fluorescent protein genes (%)		
	pEGFP-N3	pEYFP-N1	pDsRed1-N1
24	28.9	23.5	16.1
48	42.8	36.1	29.6
72	51.7	42.9	26.9

Exogenous expression of fluorescent proteins: The expression of pEGFP-C1, pEGFP-N3, pEYFP-N1, pDsRed1-N1, pECFP-N1 and pECFP-mito in the Altay sheep fibroblast cells was observed 24, 48 and 72 h, 1 and 2 weeks after transfection. Positively cells, whose quantity and fluorescent intensity waxed as time passed by and arrived at their summits at 48 or 72 h, firstly emerged 12 h after transfection (Table 2). Confocal fluorescent microscopy was adopted to observe the distribution of green, yellow, cyan and red fluorescent proteins in the Altay sheep fibroblast cells to locate the subcellular distribution of six fluorescent proteins and the fluorescences could be observed throughout the cytoplasm and nuclei of positive cells except in cryptomere vesicles (Fig. 4). The expression of fluorescent proteins waned at 1 week, however, there existed a few positive cells scattered after 2 weeks, 1 month and even 2 months. The viabilities of cells transfected with pEGFP-C1, pEGFP-N3, pECFP-N1, pECFP-mito, pEYFP-N1 and pDsRed1-N1 were 88.2, 87.5, 86.3, 87.5, 88.3 and 89.6% respectively, none of which was significantly different from that of the control group (90.3%, $p>0.05$).

DISCUSSION

Establishment of Altay sheep fibroblast line: An Altay sheep fibroblast cell bank was successfully established from 182 samples through adherent culture. However, the biological characteristics, especially the genetic characteristics of the cells might be disturbed *in vitro* conditions after serial passages, so a minimal number of passages, in other words, within 10 in this research were recommended to prevent them from being deeply hurt.

Morphology, one of the most important qualifications in epidermal tissue reconstitution, is usually observed with light and electron microscopy. In the study, the cells had fibrous characteristics with turgor vitalis cytoplasm and during growth they exhibited typically fibroblast-like morphology with radiating, flame-like or whirlpool migrating shapes. Liu *et al*. (2008) research on the Luxi cattle fibroblasts, it could be observed that fibroblast-like or epithelial-like cells migrated from the tissue pieces 5-12 days after explanting with phase-contrast microscopy. Generally speaking, epithelial cells and fibroblasts would emerged simultaneously around tissue explants at the beginning of culture *in vitro*. However, the innate propensities for easy adherence and trypsinization of fibroblasts (Ren *et al*., 2002), to the contrary of their epithelial counterparts would enable them, gradually and eventually, predominate in the culture population by means of which a pure fibroblast would be obtained after 2-3 passages (Zhou *et al*., 2005; Li *et al*., 2003).

Karyotype analysis: The genetic stability of cell line, a basis for genetic resources conservation, prescribes that all the fibroblasts should remain diploid uniformly as cells *in vivo*. As cells cultured *in vitro* has a tendency to mutate and the mutants would still possess a proliferative potential just as the normal ones, the loss of genetic stability would gradually and eventually, debase the importance of somatic cells cryopreservation. Therefore, karyotype analysis, a major method for distinguishing normal cells from mutants, constitute an indispensible criterion for culture evaluation. Researchers ameliorated the cryopreservation procedure and minimize the number of passages to obtain a stable diploid cell line, 98.6% of whose population possess 2n = 60 chromosomes corresponding to the reports of Liu *et al*. (1993) and Zhan *et al*. (1994). Despite of the existences of hypodiploid and hyperdiploid cells and some polyploid cell which had a tendency to increase as serial passages stepped further, they constituted a minor proportion of the whole population.

Isoenzyme analysis: Isoenzyme polymorphism, commonly used to identify cultures' ancestries (Nims *et al*., 1998) was adopted by the ATCC as routine for detecting interspecies contamination (Drexler *et al*., 1999). LDH and MDH, crucial enzymes in the glycolytic pathway and citric acid cycle, respectively, species-specific and relatively conservative during evolution, the contents and activities of which differ among species and subspecies could serve as biochemical indicators for species classification by chromatography and electrophoresis. LDH, a tetrametric molecule; composed of two kinds of subunits, H and M produced by expression of the ldha and ldhb genes, is a tissue-specific and species-specific isoenzyme complex (Washizu *et al*., 2002). MDH is a dimeric enzyme including cytosolic MDH (s-MDH) and mitochondrial MDH (m-MDH). The results that the isoenzyme patterns of LDH and MDH in Altay sheep fibroblast cells were clear authenticate their genetic stability and ruled out the possibilities of cross contamination among interspecies and intraspecies.

Expression of exogenous genes: Six fluorescent proteins, due to their numerous merits such as stable structures, high expression levels and species-independent efficiencies (Baird *et al*., 2000) have been widely and frequently, used as markers to investigate the expression, distribution and function of target proteins in live cells and organisms (Heim *et al*., 1995; Cheng *et al*., 2003). The 24 and 48 h after transfection, the 6 types of fluorescences could be observed in both cytoplasm and nucleus except cryptomere vesicles. At 72 h, *EGFP* and *EYFP* gene, nearly unchanged were still expressed in cytoplasm and nucleus, yet some cells became irregular in morphology. In spite of the decreased transfection efficiencies, high expression levels sustained even after 1 week, potently validating the expressibility of exogenous genes in the fibroblasts. The viabilities of the transfected cells were not significantly lower than the those of the control ones. The results indicated that the expression of fluorescent proteins constituted no obvious cytotoxicity to the transfected cells, corresponding to previous findings (Huang and Li, 2001).

CONCLUSION

In sum, the Altay sheep fibroblast cell line mentioned above corresponded to all the criteria for identification of ATCC whose establishment contributed to the conservation of the genetic resources of Altay sheep and served as precious biomaterial for future investigations in realms such as cell biology, biomedicine, genomics, post-genomics as well as genetic and embryonic engineering.

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