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Establishment and Characterization of a Fibroblast Line from Inner Mongolia Cashmere Goats

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Abstract: A fibroblast line (named SCF36) from 33 Inner Mongolia Cashmere goats ear marginal tissues was established successfully by means of using primary explant technique and cell cryoconservation technology. This fibroblast line contains 336 cryovials with 8×10^6 cells, respectively. Biological characteristics of the fibroblast line showed that the cells cultured *in vitro* were all morphologically typical fibroblast and the cell Population Doubling Time (PDT) was approximately 2 days. The cell average viability before freezing was 97.37±2.25 and 91.43±.22% after thawing. Chromosome analysis showed that >90% of the whole population were diploid. Isozyme analysis of Lactic Dehydrogenase (LDH) and Malic Dehydrogenase (MDH) showed that the SCF36 cells had no cross-contamination with other species and the genetic characteristics of the cell line were stable *in vitro*. Tests for cell line contamination with bacteria, fungi and mycoplasmas were also negative. The transfection efficiency of three fluorescent proteins were relatively high, indicating that the exogenous genes could be effectively expressed in the cells. The cell line met all criteria from the American Type Culture Collection (ATCC). Not only has the germline of this important sheep breed been preserved at the cell level but also valuable material had been provided for genome, postgenome and somatic cloning research and so on.

Key words: Inner Mongolia Cashmere goats, fibroblast, cell line, biological characteristics, marginal tissues

INTRODUCTION

With the improvement of people's living standard and the development of science and technology, biodiversity is facing unprecedented challenges. The diversity of livestock and poultry genetic resources is an important part of biodiversity and is the basis for human society to keep living and achieve sustainable development. While some of the livestock species are facing extinction crisis, vulnerable animals are threatened by the introduction of foreign species and by industrial pollution. While some government departments, organizations and experts have appealed for conservation and management of livestock and poultry genetic resources, there has still been a massive loss (Woelders *et al*., 2006). If these genomic resources are not conserved in some form, researchers will not only lose the genes of rare breeds but will also find it seriously hard to study the cytological and molecular biological mechanisms that are required to reproduce these breeds by somatic cell cloning. Therefore, genetic resource preservation of livestock and poultry is extremely urgent. At present, many methods are used to conserve the genetic resources of domestic animals. Generative cells, somatic cells, stem cells, zygotes and embryos can all be cryopreserved in cell banks and rapid thawing can not only keep the same cell's damage but also can modify collagen synthesis and differentiation of fibroblasts (Guan *et al*., 2007). Low-temperature biological techniques is a effective approach to conservation and maintenance of the diversity of livestock and poultry. In addition, modern cloning techniques have made somatic cells an attractive resource for conserving animal genetic materials. Therefore, the establishment of somatic cell banks is more necessary (Wiebe and May, 1990). Spontaneously Immortalized Porcine Mammary Epithelial Cell line (SI-PMEC) from the mammary gland of a lactating sow had been established (Sun *et al*., 2006). Much information has recently been published on the development of fibroblast cell lines from different animals including the Debao pony (Zhou *et al*., 2004), Luxi cattle (Liu *et al*., 2008) and white ear lobe chicken (Wu *et al*., 2008).

Inner Mongolia White Cashmere goats originated in the Western region of Inner Mongolia, living in the typical continental plateau climate. Cashmere goats come in shades of black, gray, brown and white. In Mongolia, nomads usually maintain mixed herds. Goats outnumber people by four to one and roam almost as wildlife-usually keeping within a few kilometers of their owner's ger. Researchers saw no one looking after the herd pictured. Goats grow cashmere as an undercoat which provide

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nomads with their main source of cash income. But a goat's usefulness does not stop there. Mongolians milk goats throughout the summer producing various dairy products. Excess milk is churned and dried into a kind of nomads' candy for use in the winter. In October, the bucks straddle the does which then carry pregnancies through the winter. In winter Mongolian goats see their only shelter of the year-usually a 3-sided barn. A small luxury when pregnant in extreme subzero temperatures. The kids drop in April and the goats get a month's maternity before dehairing begins in May and June.

In order to seek for a more effective way to save down the genetic resources of Inner Mongolia White Cashmere goats. In this study, researchers used a combination of cell viability verification, detection of microorganisms, chromosome analysis, isoenzyme analysis and transfection of fluorescent protein genes to establish a fibroblast cell line from the Inner Mongolia White Cashmere goats ear marginal and also identified its biological characteristics. The aim is to supply a convenient and effective resource for genomic research at the cellular level. Furthermore, preservation and maintenance of domestic animal resources by means of the cryoconservation technology provided valuable materials for genomic, postgenomic, somatic clone and other research fields of life science. To some extent, the roles of cell lines will become increasingly prominent and they may be useful in currently unforeseen applications and the keep of characterization and quality control (Stacey and Masters, 2008).

MATERIALS AND METHODS

Cell culture: Ear marginal tissue samples (about 1 cm^2) were taken from 32 Inner Mongolia Cashmere goats and collected into separate tubes containing DMEM supplemented with ampicillin (100 U mL^{-1}) and streptomycin (100 μg mL^{-1}). The samples were washed eight times with Phosphate Buffered Saline (PBS) and the tissue samples were placed in 60 mm Petri dishes and cut into small pieces (1 mm^3) using ophthalmic scissors. The tissue blocks were seeded into medium in a tissue culture fask (Corning, USA) containing DMEM (Gibco, USA) +10% fetal bovine serum (Hyclone, USA) in a 37°C incubator with 5% CO_2. Cells were harvested by means of 0.25% trypsin+0.02% Ethylenediamine Tetraacetic Acid (EDTA) when they reached 80-90% confluence and split into new flasks at the ratio 1:2 or 1:3 (Freshney, 2000).

Cryogenic preservation and recovery: The fibroblasts in logarithmic growth phase were enumerated with a hemocytometer and cell viability was checked by Trypan Blue staining before freezing. When the cells reach a final cell density of 80-90%. The cultured cells were harvested by centrifuging at 1200 rpm for 8 min. The harvested cell pellet was re-suspended in a freezing medium containing 10% Dimethylsulfoxide (DMSO), 50% FBS and 40% DMEM to reach a final cell density of 3-5×10^6 viable cells/mL. Cell suspensions were splitted into sterile cryovials labeled with breed, gender, passage number and the date. The cryovials were sealed and kept at 4°C for 20-30 min to allow the DMSO to equilibrate and then put into the programmed cryopreservation system for 12 h and finally transferred to liquid nitrogen for long-term storage. When recovered, the frozen vials were taken out from the liquid nitrogen and thawed in 42°C water bath kettle and then transferred into a flask with complete medium of DMEM containing 10% FBS. The cells were cultured at 37°C with 5% CO_2. The medium was renewed after 24 h (Doyle *et al*., 1990).

Viability assay: Cell viability was determined by the Trypan blue staining exclusion test before freezing and after recovery. Cells were digested and seeded in 6 well plates and 1000 cells were stained and checked for cell viability rate.

Growth curve: By the traditional method, cells at final cell density of 4×10^4 mL^{-1} were seeded into 24 well plates and cultured for 7 days. Cell density data were monitored and recorded each day until the plateau phase. Three wells were counted reach time and the mean was deemed to a point of growth curve. The cell growth curve was then plotted and the Population Doubling Time (PDT) was calculated on the basis of this curve (Kim *et al*., 2005; Zhou *et al*., 2005).

Microorganism detection: Following the methods of the Doyle *et al*. (1990) method, the passaged and thawed cells were cultured in antibiotic-free medium containing DMEM and 10% fetal bovine serum for at least 1 week and were fixed and stained with Hoechst 33258, respectively for mycoplasma contamination detecting. The 4 most common mycoplasma species (*Mycoplasma arginini*, *Mycoplasma hyorhinis*, *Acholeplasma laidlawii* and *Mycoplasma orale*) could be detected using an ELISA mycoplasma detection kit by confirming the results of the DNA fluorescent staining (Masover and Becker, 1998).

Chromosome analysis: Cells at the exponential growth phase were treated with 0.1 μg mL^{-1} colcemid for 6 h at 37°C and were treated with a hypotonic KCl/HEPES/EDTA solution and then harvested and fixed. After Giesma staining, the chromosome numbers were

counted from 100 spreads under an oil immersion objective (Suemori *et al*., 2006). The parameters of relative length, centromere index and arm ratio index were calculated according to the protocol of Kawarai *et al*. (2006).

Isoenzyme analysis: The isoenzyme analysis of Lactate Dehydrogenase (LDH) and Malate Dehydrogenase (MDH) from Inner Mongolia Cashmere goats ear marginal tissue fibroblasts were performed by a vertical slab non-continuous Polyacrylamide Gel Electrophoresis (PAGE) assay, the cells were harvested using the normal method and protein extraction solution (0.9% Triton X-100, 0.06 mmol NaCl:EDTA in mass ratio 1:15) was added when the cell concentration was reached to 5×10^7 cells/mL, then the mixture was centrifuged and the supernatant was stored at -80°C. The mixture of liquid sucrose (40%) and the samples were loaded into the individual lanes of the polyacrylamide gel. The electrophoretic mobilities of LDH and MDH were determined the relative mobility front (RF) (Simpson, 2003).

Expression of fluorescent proteins in Inner Mongolia Cashmere goats fibroblasts: The transfection conditions were optimized by varying cell density and plasmid DNA and Lipofectamine 2000 (Invitrogen) concentrations. Cells in the logarithmic growth phase were transfered using the fluorescent protein vectors pEGFP-N3, pECFP-N1, pDsRed1-N1 and pEYFP-N1 in the serum-free medium using Lipofectamine 2000 transfection reagent (Invitrogen Corp., Carlsbad, California). The plasmid DNA (micrograms) to Lipofectamine 2000 (μL) ratio was 1:3. After 5 h, the supernatant was removed and substituted with medium containing serum and then the cells were observed 24, 48 and 72 h after transfection under a confocal microscope to estimate the transfection efficiency. The relative fluorescence intensity of the 6 different fluorescent proteins was evaluated and the positive cells were selected througth G418 resistance marker.

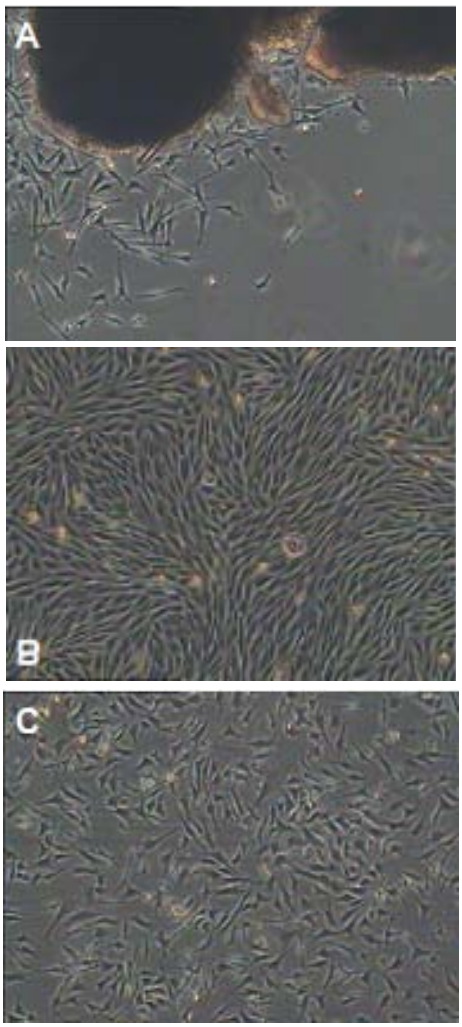

Fig. 1: Morphology of Chinese Game chicken embryo fibroblasts cultured *in vitro*; A) primary cells (40x), the cells were typical long spindle-shape with growth being slower; B) subcultured cells (40x), the cells were subcultured until they reached 90% confluence. After passage, growth accelerated and plateaued after 3-4 days; C) cells after recovery (40x), the cells were cultured for 48 h after thawing

RESULTS

Cell morphology and viability: After explanting, fibroblast-like and epithelial-like cells could be seen in 5-12 days (Fig. 1A). With the extension of incubation time, the cells increased gradually, the cells were pasaged when the cells convergence degree reached 80-90% in the whole culture bottle (Fig. 1B). The pasaged cells growed rapidly and the proportion of fibroblast-like cells also increased gradually. After two to three passages, epithelial-like cells were disappeared and fibroblasts were purified almost completedly (Fig. 1C). As measured by Trypan Blue staining, the viabilities of Inner Mongolia Cashmere goats fibroblasts before freezing and after recovery were 97.6 and 94.5%, respectively. The cell activity had no significant difference before cyropreservation and after recovery. The morphology of the cell was still a typical elongated spindle-shape. Above all, the culture conditions were appropriate and cells were not significantly affected by cryopreservation.

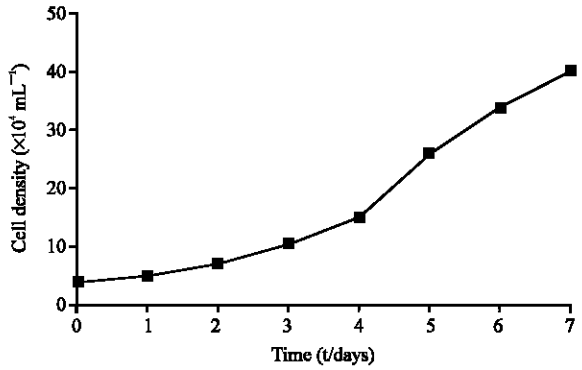

Fig. 2: The growth curve of Inner Mongolia Cashmere goats fibroblast line. The growth curve was an typical "S" shape. A lag phase of around 36 h was observed after cells were seeded. Then, cells proliferated and entered the logarithmic phase until they reached the stationary phase after about 6-7 days

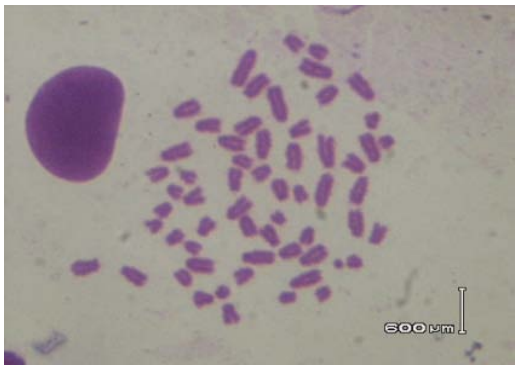

Fig. 3: Karyotype of Inner Mongolia Cashmere goat fibroblasts

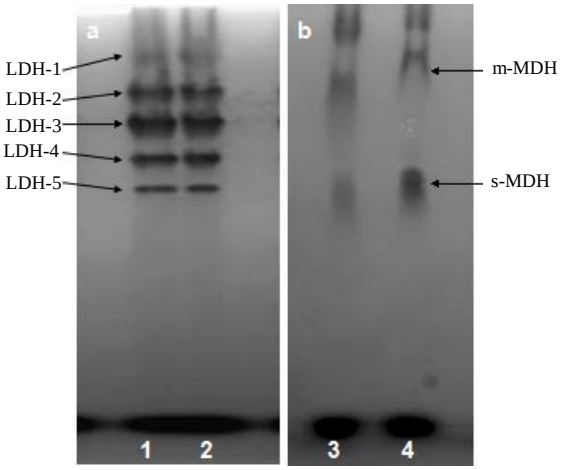

Fig. 4: LDH zymotype and MDH zymotype of Inner Mongolia Cashmere goat and Ujumqin sheep fibroblasts line. 1, 3) Ujumqin sheep; 2, 4) Inner Mongolia Cashmere goat. These results showed that there was no cross-contamination between different breeds; a) SDS-PAGE electrophoresis of LDH; b) MDH

Growth dynamics: The growth curve of ear marginal tissue fibroblasts from Inner Mongolia Cashmere goats before cyropreservation and after recovery had an typical "S" shape (Fig. 2). There was a lag time or latency phase of about 24 h after seeding which needed a recovery period after trypsin damage and cryopreservation effect then the cells proliferated rapidly and entered exponential phase. Along with the cell density increased, proliferation was restrained as a result of contact inhibition and the cells entered the plateau phase after about 6-7 days and began to degenerate. The PDT was about 72 h calculated from the curve.

Microbial analysis: Tests for contamination with bacteria, fungi and yeasts were negative; no microorganisms were observed in the culture media all the time under the microscope. The results indicated that the Inner Mongolia Cashmere goats fibroblasts were free of bacterial contamination. By testing, the cells have no mycoplasma contamination. If there was abundant punctiform and filiform blue fluorescence in the nucleoli, it could be concluded that the cells were contaminated by mycoplasmas. As is shown by the cytopathogenic evidence and the hemadsorption test, tests for virus contamination were negative.

Chromosome and karyotype analysis: The chromosome number of Inner Mongolia Cashmere goat is 2n = 60 including 29 pairs of euchromosomes and two sex chromosomes, XY or XX (Fig. 3). The X chromosome is the longest acrocentric chromosome and the Y chromosome is the shortest submetacentric chromosome.

All somatic chromosomes were acrocentric autosomes. The chromosome numbers per spread were counted for 100 spreads of the first, second and fourth passages and the ratio of cells with 2n = 60 were 93.5, 92.6 and 91.3%, respectively. Abnormality in chromosome numbers was inclined to increase with increasing numbers of passages which indicated that *in vitro* culture affected the hereditary property of cells slightly but supporting the evidence that the cell line was reproducibly diploid.

Isoenzyme analysis of Inner Mongolia Cashmere goat cell line: The LDH and MDH bands obtained from Chinese Game chicken embryo fibroblasts were compared with those from other species (Fig. 4). The isoenzyme

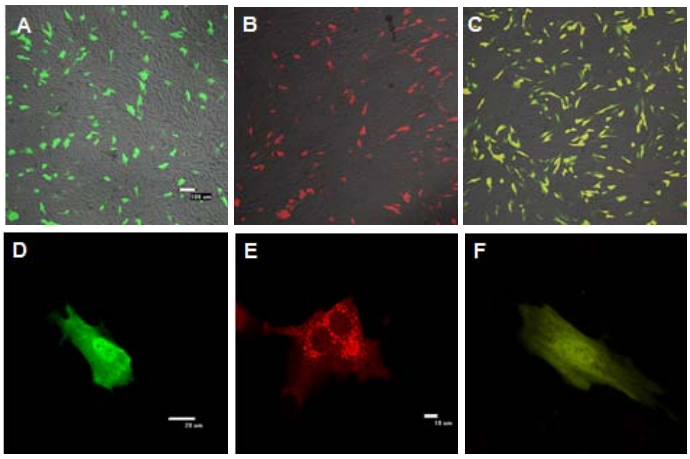

Fig. 5: The expression and distribution of pEGFP-N1, pDsRed1-N1 and pEYFP-N1 in SCF 36 cell; A-C) the transfection results of pEGFP-N1, pDsRed1-N1 and pEYFP-N1 at 48 h after transfection (100x); D-F) the subcellular location of pEGFP-N1, pDsRed1-N1 and pEYFP-N1 at 48 h after transfection (400x)

result showed 5 LDH isoenzyme bands and two MDH isoenzyme bands. The 5 bands were apparent representing LDH1, LDH2, LDH3, LDH4 and LDH5 from anode to cathode, two MDH isoenzyme bands were m-MDH and s-MDH and one band of cytosolic MDH was located near the anode and another bands of mitochondrial MDH was found near the cathode. The results indicated that the genetic characteristics of Inner Mongolia Cashmere goat fibroblasts were stable *in vitro* and there was no cross-contamination with cells from other species.

Expression of fluorescent protein genes in fibroblasts from Inner Mongolia Cashmere goat: The 3 fluorescent protein genes *pEGFP-N1*, *pEYFP-N1* and *pDsRed1-N1* were transferred into the Inner Mongolia Cashmere goats fibroblasts with reference to the optimized condition when the cell density reached 75-80% (Fig. 5A-C). The 3 fluorescent genes expressing were observed at 24, 48 and 72 h. The 2 weeks and 1 month after transfection using laser confocal microscopy. Expression of the three gene in fibroblasts were positive and the transfection efficiencies at 24, 48 and 72 h after transfection were between 9.0 and 32.6%. Positively, expressing cell numbers and intensity increased markedly and reached a maximum at 48 or 72 h. The yellow fluorescent proteins (pEGFP-N1) were maximal. The subcellular location of the three fluorescent proteins were observed using confocal fluorescence microscopy. The results indicated that the fluorescence could be observed throughout the cytoplasm and nuclei of control cells except in the cryptomere vesicle. DsRed was mostly shown in the cytoplasm surrounding the nuclear membrane and formed a red ring profile whereas EGFP and EYFP displayed an intense nuclear signal (Fig. 5D-F). The number of fluorescent cells decreased at 1 week but a few dispersed positive cells remained post transfection after 2 weeks and even after 1 month. The viabilities of transfected cells with pEGFP-N1, pEYFP-N1 and pDsRed1-N1 were 88.6, 85.7 and 88.3%, respectively. Among them, no one was significantly different from that of the control group (91.3%, $p>0.05$).

DISCUSSION

A fibroblast line from marginal ear tissue of Inner Mongolia Cashmere goat was established using an Adherent Culture Method. All the results showed that the newly established cell line was stable and growed rapidly. Primary cell appeared epithelial cells and fibroblasts together. As described in the earlier studies, fibroblasts adhere more easily to flasks and can be trypsinized more easily whereas it is not easy to adhere for epithelial cells (Ren *et al*., 2002). Owing to these differences, fibroblasts will quickly outgrow their epithelial counterparts. In this manner, when the cells were treated over 2-3 passages, researchers could obtain a pure fibroblast line (Li *et al*., 2003). Researchers are now able to preserve the genomic resource of Inner Mongolia Cashmere goat in a long term by freezing fibroblasts in liquid nitrogen and thus achieve the purpose of protecting the breed. To ensure full recovery of the fibroblasts, the cells within five passages were frozen when the cells reached a density of 3×10^6 cells/mL. Because of too many passages or digestion with trypsin, the cells may be injured and changed in biological characteristics, especially their hereditary characteristics (Liu *et al*., 2008).

Microbial contamination is a frequent and pivotal pollution phenomenon in cell culture. Due to the growth and reproduction of bacteria, the culture liquid becomes turbid and yellow in color and abundant bacteria can be seen under the inverted microscope. When emerged bacterial contamination and without treatment for a long time, it will constitute a threat to the cells and the cells will thaw rapidly in the morphology. Tests for microbial contamination by bacteria, fungus, viruses and mycoplasmas showed that the Inner Mongolia Cashmere goat fibroblast cell line were pure and purified and free of microorganism contamination.

The hereditary stability of cell line is critical to preserve the genetic resources. Various factors *in vivo* can cause cell mutations with the mutational cells growing and passaged, it will finally results in the variation of the cell line and then the cell line loses the significance of breed conservation. Karyotype analysis has indicated the induction of many chromosome changes, leading to the heterogeneity of chromosome number (Costa *et al*., 2005) and the loss of their functional reactivity (Dzhambazov *et al*., 2003).

Hence, karyotype analysis is a major method to distinguish normal cells and mutational cells. After testing the mean frequency of diploid cells was 93.65±3.37% which indicated the Inner Mongolia Cashmere goat fibroblasts cultured *in vitro* were stable diploid, in accordance with the reports of Zhan *et al*. (1994). With increasing number of passages, chromosome rearrangements may appeared. Almost every passage has a frequency of diploid cells of above 90% which further validating the genetic stability of these cells. Therefore, it could be inferred that there not appeared chromosomal structure variation in the Inner Mongolia Cashmere goat fibroblasts.

LDH and MDH are very important enzymes that participate in the glycolytic pathway and the citric acid cycle, separately. They are species-specific (Washizu *et al*., 2002; Arai *et al*., 2003) and could provide a biochemical indicator of species classification by chromatography and electrophoresis which also could reveal species or tissue characteristic distribution patterns. Biochemical analysis of isoenzyme polymorphism is currently considered to be the standard method for cell line identification and detection of interspecies contamination (Wu, 1999) and is routinely used by the leading biological resource centers around the world (Parodi *et al*., 2002). Researchers have modified the apparatus and conditions for polyacrylamide gel electrophoresis and successfully identified the mobility of MDH and LDH from the Inner Mongolia Cashmere goat fibroblasts cultured *in vitro*.

The enzyme LDH generally consist of five distinct isoenzymes in almost all vertebrate tissues (Hall *et al*., 1990). A clear separation of all five LDH isoenzymes from heart, liver, lung and muscle in Small Tail Han sheep and German Merino was achieved by Li *et al*. (2003). In this study, the isoenzyme bands of LDH and MDH in the Inner Mongolia Cashmere goat fibroblasts and the bands were clear and also the isoenzyme activities *in vitro* was similar to that in the original tissues. The results indicated that the genetic characteristics of the Inner Mongolia Cashmere goat fibroblasts were stable. It was thus demonstrated that the Inner Mongolia Cashmere goat genetic resources could be conserved by freezing their fibroblasts in liquid nitrogen for a long time.

Fluorescent protein research mainly focus on tumors, nerves and stem cells, the study found that the GFP tag enables tracking of highly migratory and invasive ostrocytoma cells under direct vision (Jung *et al*., 2001). The consistence of DNA and lipofectin, the lipofectin combination and DNA incubation time and the presence of serum can all affect the efficiency of transfection as shown by research on Vero cells, HeLa cells and various other cell lines (Tseng *et al*., 1999; Rui *et al*., 2006). In the study, the highest efficiency of transfer of the three fluorescent proteins was 29.3% with an optimized plasmid-lipofectin ratio.

Researchers observed the transfected cells at reduplication and different dividing phases transfection had no significant effects ($p>0.05$) on cell morphology, growth and proliferation, apoptosis rate or reduplication status compared with untransfected control under optimal conditions. As the result indicted, the transfected cells had not been affected by fluorescein below a certain range.

The 3 fluoresceins were not distributed homogeneously in all the cell lines. For instance, GFP was distributed mainly in the nucleus and DsRed was expressed in a granular substance surrounding the nuclear membrane and formed a red ring profile. The Inner Mongolia Cashmere goat fibroblasts can be widely used as means for investigating the functions of exogenous genes. This new genic resource will be important for future identification for breed-specific genetic markers and for nuclear transplantation and transgenic cloning investigations.

CONCLUSION

The newly line is established successfully and its property is stable and grows rapidly, suggesting that it may be useful in the conservation of this unique breed. It also provides a new method for genetic research in the future on the Inner Mongolia Cashmere goat.

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