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STUDY ON GENETIC CHARACTERISTICS OF IN VITRO CULTURE AND INTRACELLULAR TRANSDUCTION OF EXOGENOUS FLURESCENT PROTEINS IN FIBROBLAST CELL LINE OF ANGORA GOAT

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ABSTRACT

Establishment of fibroblast cell lines of endangered goat breeds and research on the gene or protein functions based on the cells made a significant contribution to the conservation and utilization of genetic resources. In this study, a fibroblast cell line of Angora goat (AGF31) from 31 samples, frozen in 162 cryogenically-preserved vials with (2–3) $\times 10^6$ cells each, was successfully established by using explants culture and cryopreservation techniques. The cells cultured *in vitro* showed typical morphology of fibroblasts, with a population doubling time of 34.3h; The average viability of the cells was 97.6 % before freezing and 95.9 % after thawing; Chromosome karyotyping and G-banding analysis showed that >90.8% cells were diploid (2n=60) prior to the 4th generation; Neither microbial contamination nor cross-contamination was detected by isoenzyme analyses; To determine cell permeability, intracellular path and stability of exogenous proteins during the transduction, six fluorescent protein genes were transferred into fibroblasts by lipofectamine-mediated method. The transfection efficiency of six fluorescent protein genes fluctuated between 13.4% and 60.8%. ECFP and DsRed were mostly shown in cytoplasmic in dots around the nucleus, and EYFP and EGFP had a slightly stronger expression in the nucleus than in the cytoplasm. Angora goat fibroblasts still kept stable genetic properties and normal biological characteristics *in vitro*, and every index met the standard quality control of American Type Culture Collection.

Key words: Angora goat, Fibroblast cell line, Characterization, Cryopreservation.

INTRODUCTION

Angora goat is an ancient and excellent goat breed in Turkey that originated in the district of Angora in Asia Minor. The most valuable characteristic of the Angora as compared to other goats is the value of the mohair that is clipped, and the mohair is very similar to wool in chemical composition but differs from wool in that it is has a much smoother surface with less fiber diameter. In this new century, with rapid and ongoing industrialization, biodiversity is facing unprecedented challenges worldwide and vulnerable animals are threatened by the introduction of foreign species and massive destruction of natural habitats.

It is crucial mission to protect the genetic resources of endangered animals, and establishing somatic cell lines of special livestock has been proposed as a practical approach for this purpose (Scherf, 2000). The establishment and biological analysis of cell lines of Angora goat could facilitate the conservation and utilization of genetic resources in endangered goats, such as cell lines of Mongolian sheep and Liaoning cashmere goat established in our laboratory (Liu *et al*., 2011; Hu *et al*., 2013). It was therefore providing a very applicable target cells for further gene function and regulation mechanism studies. Our project of this study is to seek a valuable approach to cryopreserve this protected genomic resource in the long term in the form of fibroblasts for the purposes of reviving endangered breed by cloning and supplying a convenient and valuable experimental material for genomic research.

MATERIALS AND METHODS

Cell cultures, Cryogenic preservation and Recovery: Ear margin tissue samples (about 1 cm^2 in size) were sampled from 31 individuals of Angora goat (17 male and 14 female) and chopped into 1mm^3 pieces, which were seeded on the surface of a tissue culture flask in a 37°C incubator with 5% CO_2. The fibroblast was subcultured and harvested following the protocol previously described (Yachida *et al*., 2011; Freshney, 2000). Then cell suspension ($1–3\times10^6$ viable cells/mL) was dispensed into 2mL cryogenic vials labeled with the animal name, gender, freezing serial number, and date. After programmed freezing by a CyroMed freezer (Thermo Scientific), these vials were transferred to liquid nitrogen for long-term storage (Gómez-Fernández *et al*., 2012). To recover and reseed the cells, the frozen vials were taken out from liquid nitrogen and quickly thawed in 42°C water bath, and then the cells were transferred into a flask with complete DMEM medium and cultured at 37°C with

5% CO_2.

Detection of epithelial cells and other cells: For testing mixture of other cell types, the cells were harvested at 85% confluence. Total RNA was extracted using Trizol methods and reverse transcribed to cDNA. The PCR primer pairs of Cytokeratin 19 (CK19) and Osteopontin (OPN) were designed for amplifying the special markers of epithelial cells and osteocytes (CK19 primer F: 5'-GAAGAACCACGAGGAGGAAA-3', R: 5'-CCAGCGACCTCCTTGTTCAG-3'; OPN primer F: 5'-CTGAGCAAACAGACGATC 3' R: 5'-TTAGATCGGCGGAACTTC-3'; GAPDH primer F: 5'-GAAGGTCGGAGTGAACGGAT 3' R: 5'-TCGCTCCTGGAAGATGGTGAT-3').

Growth curve and Estimation of cell viability by Trypan Blue dye: The cells with a concentration of 1.5×10^4cells per milliliter 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 (Fan *et al*., 2009). 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).

Cells apoptosis and cell cycle analysis: Apoptotic cells were evaluated using an Annexin V/FITC kit (Beyotime, Jiangsu, China). Cells were stained according to the manufacturer's protocol and analyzed by flow cytometry (Cytomics FC 500, Beckman Coulter, USA). AGP31 fibroblasts before cryoconservation and after recovery were treated with 70% ethanol overnight and then stained by Propidium Iodide (PI). At last, the determination of cells in G0/G1, S and G2 phase was carried out by Flow Cytometry.

Detection of microorganism of AGF31 cell line: Cells were cultured in medium without antibiotics and tested for contamination with bacteria, fungi and yeasts by using the procedure described by Doyle *et al*. (1990). Routine examination for cytopathogenic effects using phase-contrast microscopy was performed following Hay's haemadsorption protocol (Hay, 1992). *Mycoplasma detection:* Cells were stained with Hoechst 33258 DNA fluorescent staining and Mycoplasma Detection kit (Roche) to detect the four most common *mycoplasma* species (*M. arginini, M. hyorhinis, A. laidlawii, M. orale*).

Chromosome analysis and Isoenzymes 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, centromeric index and type were measured and calculated according to the protocol of Kawarai *et al*. (2006).

Isoenzyme patterns of lactic dehydrogenase (LDH) and malic dehydrogenase (MDH) were detected by using the vertical slab non-continuous polyacrylamide gel electrophoresis (PAGE) assay according to the protocol of Liu *et al*. (2011). Mobility was measured as the ratio of distance migrated by the band to the distance migrated by the indicator dye.

Expression of fluorescent protein genes in Angora goat fibroblastic cell: Using the lipofectamin-mediated method, fluorescent protein vectors pEGFP-N3, pEGFP-C1, pECFP-N1, pECFP-mito, pDsRed1-N1, and pEYFP-N1 plasmid DNA (BD Bioscieces Clontech) were transferred into fibroblasts by Lipofectamine™ 2000 (Invitrogen Corp., Carlsbad, CA, USA) (Tsuchiya *et al*., 2002). The transfection results were observed after being transfected for 24, 48, and 72 h, respectively under excitation wavelength of 405nm, 488nm and 543nm separately using confocal microscopy (Nikon TE-2000-E, Tokyo, Japan). Transfection efficiency was calculated by choosing 10 visual fields and counting the ratio of positive cells in total cells.

RESULTS

Morphology of fibroblasts and Detection of mixed cells: Fibroblast-like or epithelial-like cells could be seen migrating from the tissue pieces 5–10 d after explanting (Fig. 1A). Cells proliferated along the flask wall and were subcultured when confluence reached 80-90%. Fibroblasts mingled with epithelial cells initially (Fig. 1B). For their rapid growth, fibroblasts gradually replaced the epithelial cells in subcultures (Fig. 1C). After 2-4 passages, we obtained the pure fibroblasts (Fig. 1D). In addition, they were viable and grew well after they were thawed (Fig. 1 E, F). The viabilities before cryo-preservation and after recovery of fibroblasts detected by Trypan Blue staining were 97.6%±0.41% and 95.9%±0.23%, respectively.

The epithelial cells and other cells were detected in the fibroblast line using RT-PCR. PCR result indicated that there were both epithelial cells and fibroblasts during the primary and subsequent several passages of the explanted tissues. With the growth of cell passage numbers, the other cells, such as epithelial cells, osteocytes, disappeared in the cell cultures. The result of PCR showed that the mixed cells were replaced after passage 3 (Fig. 2A, B).

Growth curve, Microbial analysis, Karyogram, Cell apoptosis and Cell cycle of Angora goat: The growth curve of the AGF cell line assumed as typical "S" shape (Fig. 3A) and the PDT was about 34.3h. No microorganisms of bacteria, fungi and yeasts were observed in the culture media. No viruses were indicated by the cytopathogenic evidence or by the haemadsorption

test. After staining with Hoechst 33258, fibroblast nuclei appeared as blue ellipses, showing that the established cell line was mycoplasma negative (Fig. 3B). The mycoplasma detection kit (Roche) was used and confirmed a negative outcome (Fig. 2C).

The chromosome number of Angora goat was 2n=60, in which 29 pairs autosomes were all telocentric chromosomes. The X chromosome was the second longest telocentric chromosome, while Y chromosome was the shortest and only metacentric chromosome. The karyotype composition of the Angora goat is 29 (T), XY (T, M) (Table 1; Fig. 3C). The chromosome numbers per spread were counted for 100 spreads of the first, second and fourth passages, and the frequencies with 2n=60 were 93.2%, 91.4% and 90.8%, respectively. Aberrations in chromosome numbers tended to increase with increasing numbers of passages, indicating that *in vitro* culture affected the heritage of cells slightly.

The results showed in Fig. 3D, apoptotic rate after recovery (1.5%) as slightly higher than before cryopreservation (0.5%). Under normal conditions, the percentage of G0/G1 cells was 64.20% before cryopreservation and 64.70% after recovery, respectively (Fig. 6E), there was no clear difference. Therefore, the Angora goat fibroblasts we cultured in vitro can be used as donor cells, which do not need to be treated with normal serum starvation during nuclear transfer.

Isoenzymes analysis of Angora goat cell line: We improved the apparatus and conditions for polyacrylamide gel electrophoresis to determine the mobility of the isoenzymes of LDH and MDH. The LDH bands obtained from Angora goat were compared with those from other species or breeds. Enzymatic activities were in order of LDH-3, LDH-4, LDH-5, LDH-2, LDH-1, where LDH-3, LDH-4 and LDH-5 were dominant while LDH-1 and LDH-2 were scarcely observable (Fig. 4A).

All eight domestic animals had two bands (s-MDH, m-MDH), and there was a cellular solute form s-MDH band near the anode and a mitochondrial form m-MDH band near the cathode (Fig. 4B). Relative enzyme mobility of domestic animals had their own unique bands. These results indicated that each breed had its characteristic bands and there was no cross-contamination of AGF31 cell line from different cell lines at the same time.

Expression results of six fluorescent protein genes in Angora goat fibroblastic cell: The transfection efficiencies of the cyan fluorescent proteins were significantly lower than those of the green, yellow and red fluorescent proteins ($P < 0.01$, Fig. 5), and the green fluorescent proteins were maximal. The expression efficiencies of the six fluorescent proteins at 24-72 h were between 13.4 and 60.8%. We obtained two positive cell strains that expressed EGFP and EYFP stably by screening G418 resistance and monoclonic culture for 1 month (Fig. 6 E, F).

Table1 Chromosome's Parameters of Angora goat（♂）

Chromosome number	Relative length(%)	Centromere morphology	Chromosome number	Relative length(%)	Centromere morphology
1	5.24±0.72	T	17	2.96±0.17	T
2	4.89±0.04	T	18	2.90±0.12	T
3	4.76±0.62	T	19	2.79±0.18	T
4	4.54±0.12	T	20	2.75±0.14	T
5	4.35±0.15	T	21	2.54±0.12	T
6	4.18±0.26	T	22	2.50±0.11	T
7	3.89±0.16	T	23	2.44±0.14	T
8	3.81±0.08	T	24	2.36±0.28	T
9	3.76±0.13	T	25	2.32±0.39	T
10	3.73±0.01	T	26	2.23±0.45	T
11	3.71±0.12	T	27	2.13±0.41	T
12	3.40±0.26	T	28	1.98±0.34	T
13	3.24±0.14	T	29	1.93±0.12	T
14	3.38±0.19	T	X	5.23±0.06	T
15	3.15±0.02	T	Y	1.40±0.31	M
16	3.05±0.04	T			

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

The fluorescence could be observed throughout the cytoplasm and nuclei of control cells except in the cryptomere vesicle. ECFP and DsRed were mostly shown in cytoplasmic (with a punctuate pattern) in dots (Fig. 6A,

arrow a; Fig. 6B, arrow d) around the nucleus (Fig. 6A, arrow b; Fig. 6B, arrow e; Fig. 6 H,I), and uniformly in other parts (Fig. 6A , arrow c; Fig. 6B, arrow f) and showed the weakest fluorescence intensity. The EYFP and EGFP had a slightly stronger expression in the nucleus (Fig. 6C, arrow g; Fig. 6D, arrow i) than in the cytoplasm, but without expression in some vacuoles (Fig. 6C, arrow h), a ribbon-like texture with a large number of non-expressed vacuoles and empty strip zones (Fig. 6D, arrow j) appeared.

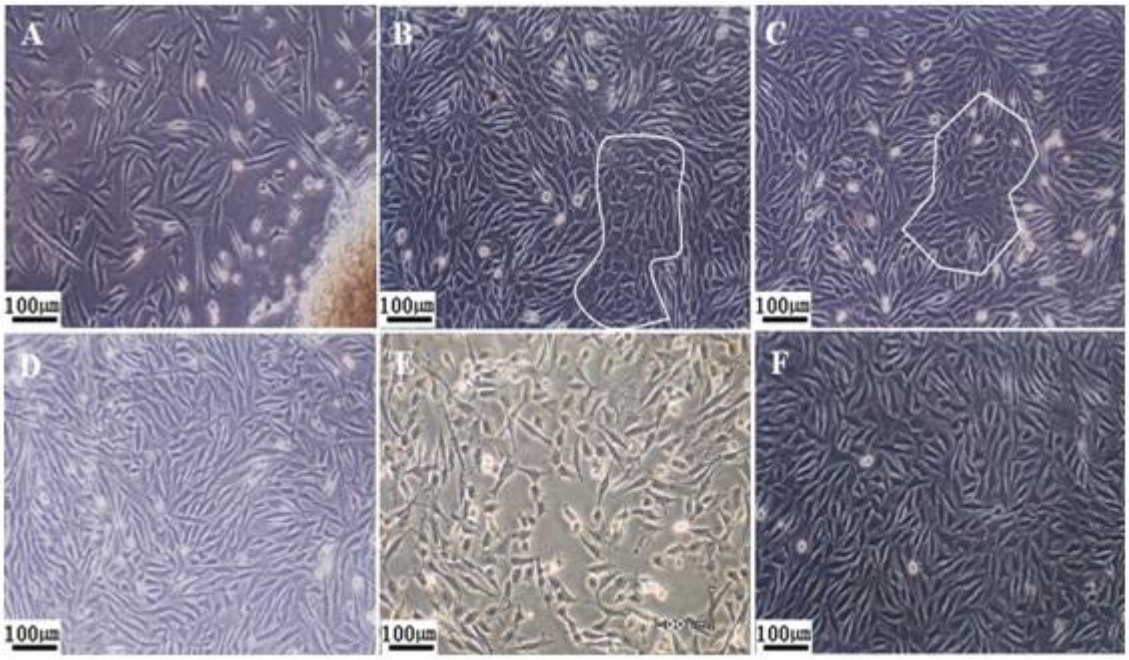

Fig. 1. Culture and Morphology of Angora goat fibroblast cell (100×)
A: Primary Cells of Angora Goat, Fibroblasts and epithelial cells migrated from the tissue; B: Both epithelial cells and fibroblast cells co-grow in the primary cells; C. The cells were subcultured until they reached 90% confluence; Passage 2 fibroblast cells co-grow with other cells; D: After passage, growth accelerated and plateaued after 3–4 days; Cells before cryopreservation, the cells were healthy and in mitotic phase; E: Fibroblasts after thawing and cultured at 12 h; F: Fibroblasts after thawing and cultured at 48 h.

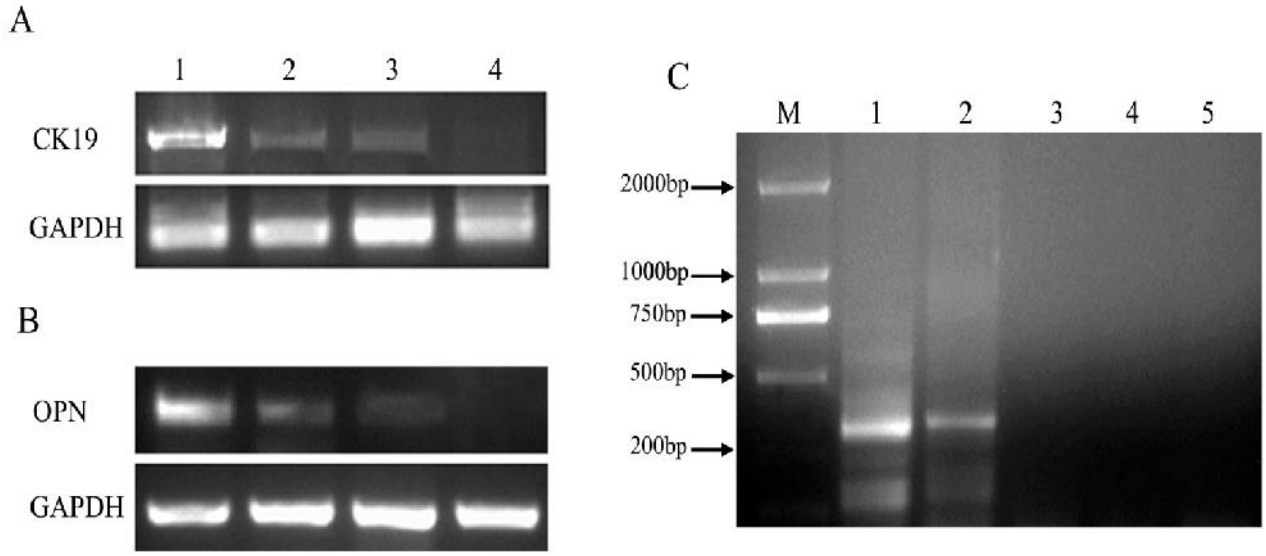

Fig. 2. Detection of epithelial cells and other cells and Mycoplasma detection.
A,B: Cytokeratin 19 (CK19) and Osteopontin (OPN), the special markers of epithelial cells and osteocytes, were detected using RT-PCR;1. Primary cells; 2. Passage 1 cells; 3. Passage 2 cells; 4. Passage 3 cells; C: AGF cell line were cultured and detected by EZ-PCR mycoplasma test kits. M: DL2000; 1, 2. positive control; 3. negative control; 4 and 5. test sample.

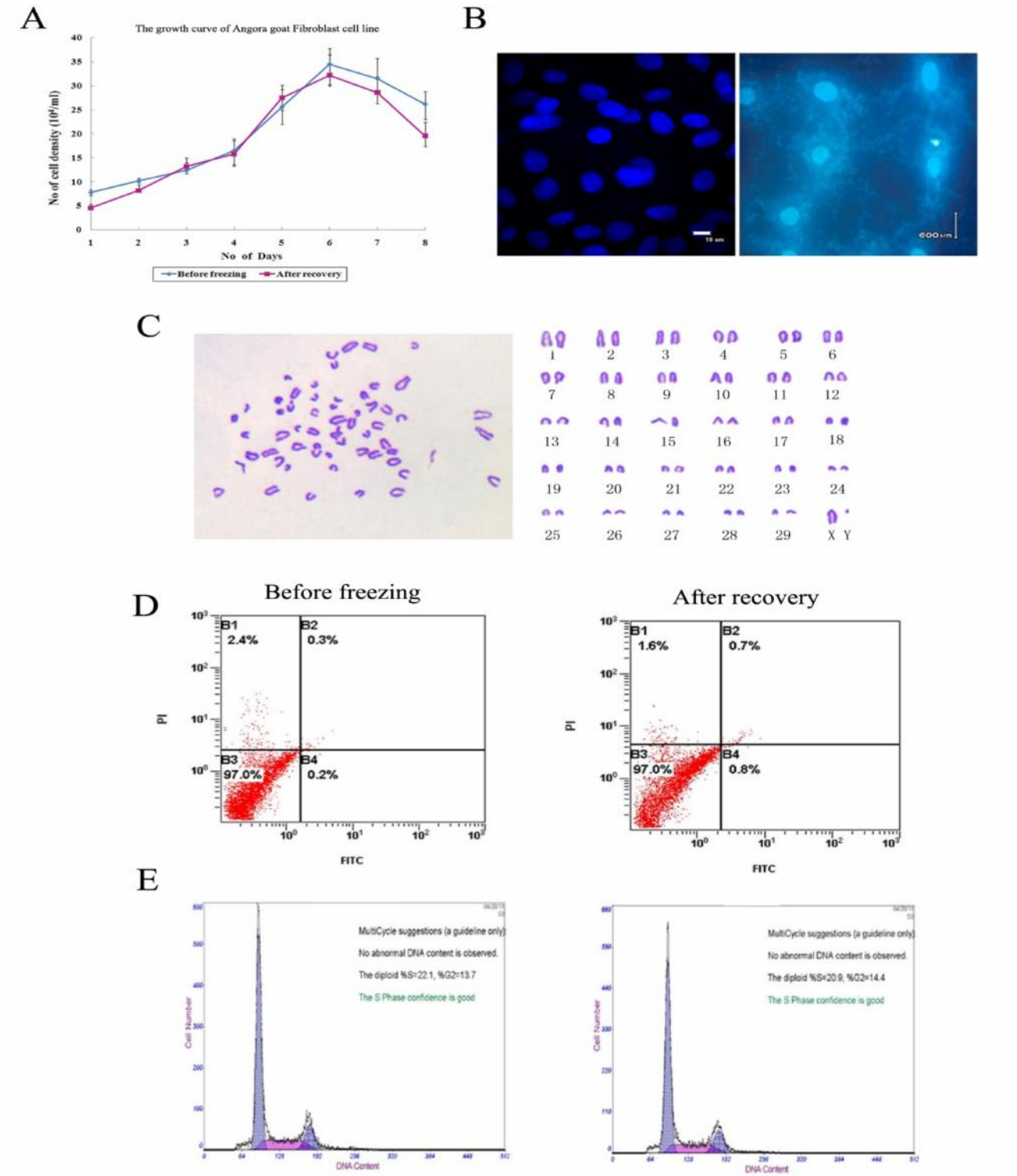

Fig. 3. Detection of Growth curve, microbial contamination, karyotype, cell apoptosis and cell cycle of AGF31 cells.
A: Growth curve of AGF31 cells. The curve appeared as a typical "S" shape with cell density on the left axis. Growth curve included latency phase, exponential growth phase and stationary phase. B: Mycoplasma contamination for the Angora goat fibroblasts Stained with Hoechst 33258 and Positive control of Mycoplasma contamination. C: Chromosome at metaphase (left) and karyotype (right) of the AGF31 cell line XY (♂). D: Apoptotic rates of AGF31 fibroblasts before cryopreservation and after recovery using AnnexinV-FITC/PI by flow cytometry. E: Cell cycle detection of AGF31 fibroblasts before cryopreservation and after recovery by flow cytometry.

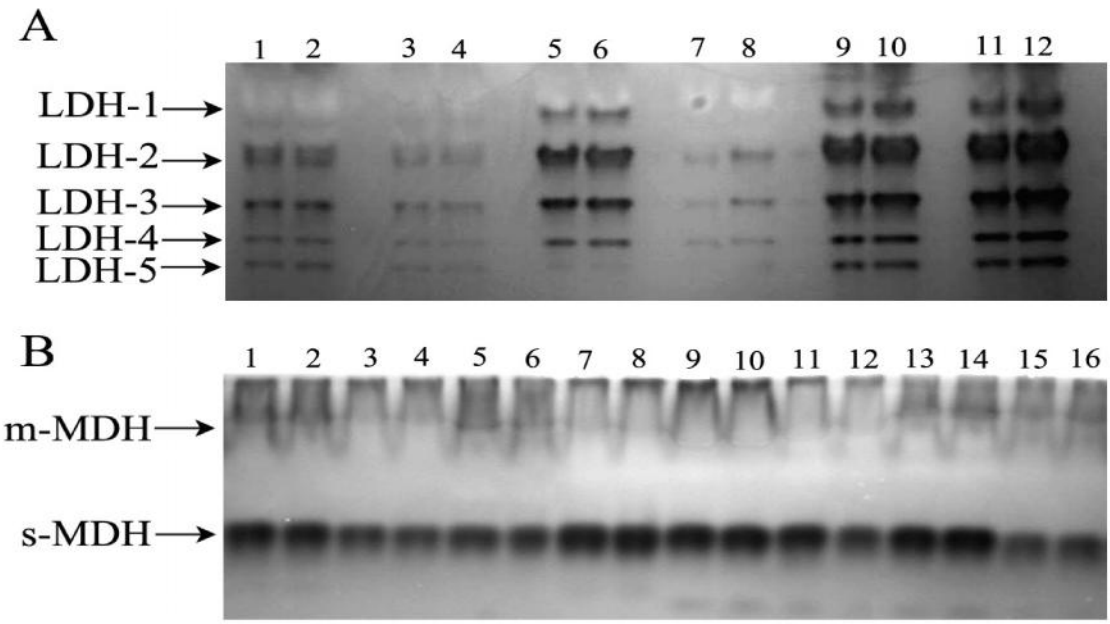

Fig. 4. LDH zymotype and MDH zymotype of AGF31 cell line.
A: SDS–PAGE electrophoresis of LDH, 1,2 Wuzhishan miniature pig, 3,4 Large white pig, 5,6 Luxi cattle, 7,8 Piemontese Bovine, 9,10 Angora goat, 11,12 Mongolian sheep; B: SDS–PAGE electrophoresis of MDH, 1,2 Suffolk sheep, 3,4 Texel sheep, 5,6 Poll Dorset sheep, 7,8 Mongolian sheep, 9,10 Fat-tailed Sheep, 11,12 Mongolian sheep,13,14 Jining black goat, 15,16 Angora goat.

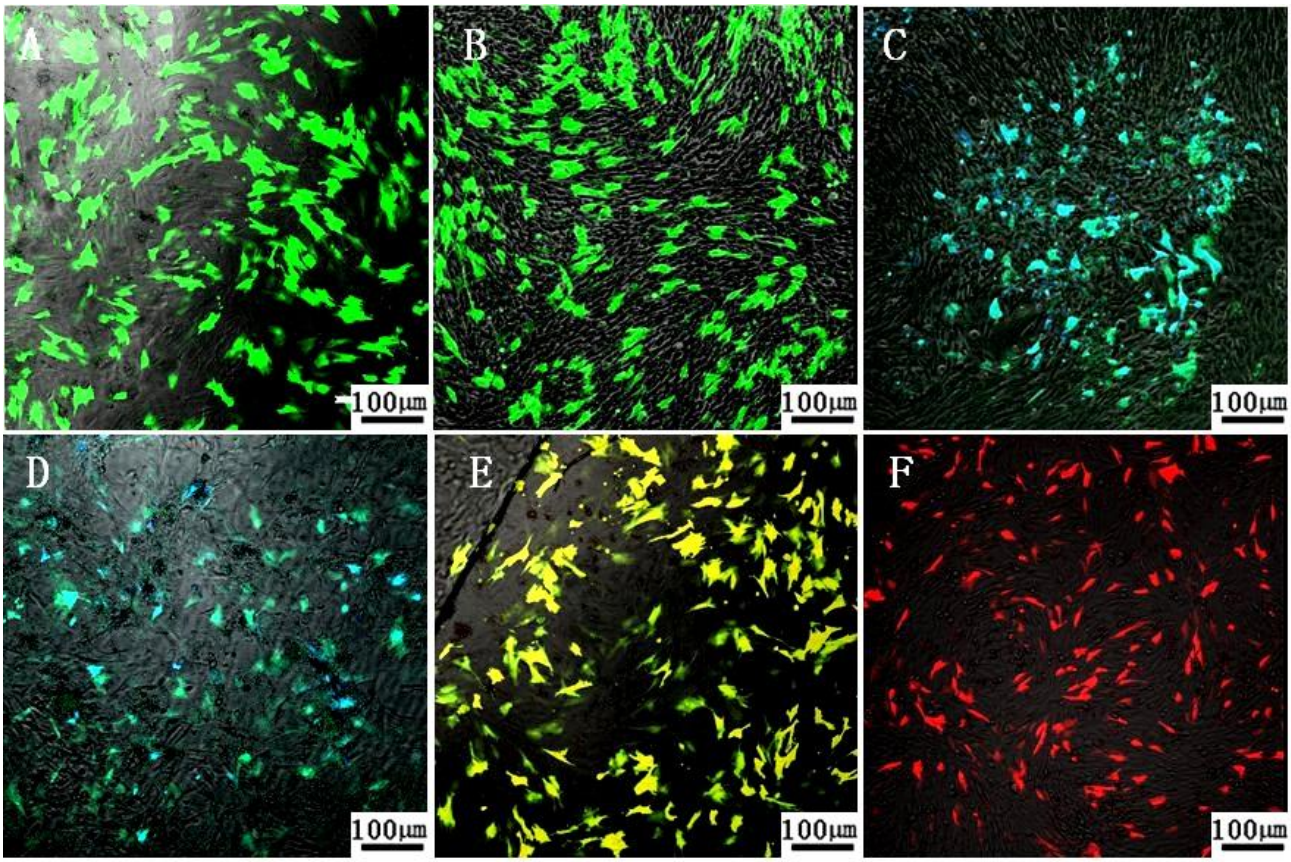

Fig. 5. Comparative Fig.s of six fluorescent proteins at 24h after transfection using Nikon TE-2000-E inverted microscope with excitation wavelengths of 433-588nm to determine the transfection efficiency (100×).
A, B, C, D, E and F were the transfection results of pEGFP-C1, pEGFP-N3, pECFP-C1, pECFP-mito, pEYFP-N1, pDsRed1-N1, respectively.

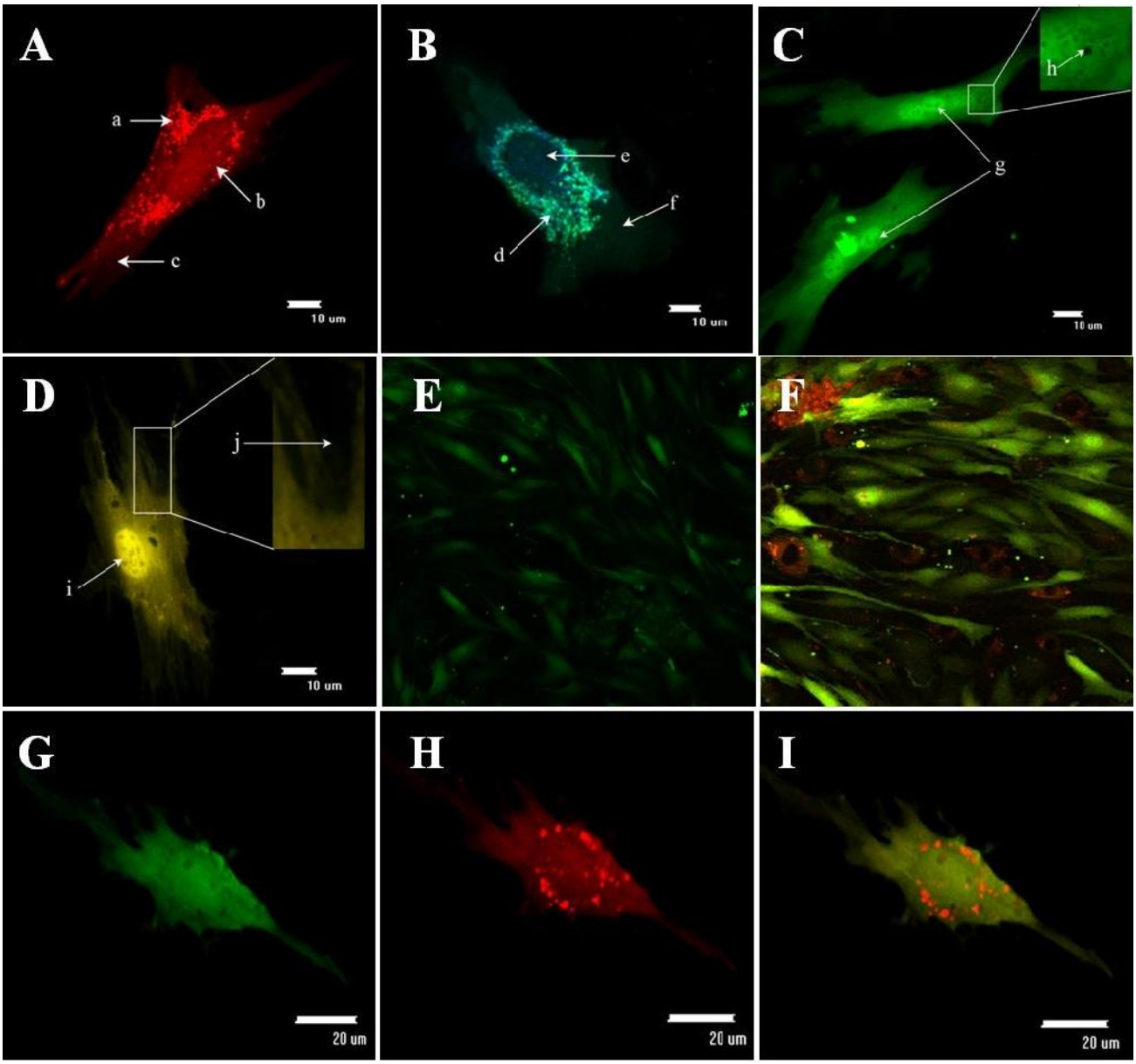

Fig. 6. Expression and distribution of pDsRed1-N1, pECFP-N1, pEGFP-N3, and pEYFP-N1 in Angora goat fibroblast cell (×400).

A, B, C, D: the distribution of the four fluorescent proteins in cells at 48 h after transfection. E, F: two positive cell strains that expressed EGFP and EYFP stably by screening G418 resistance and monoclonic culture. G, H are transfectected by pEGFP-N3 and pDsRed-N1 at the same time excited by 488nm and 543nm separately; I images produced by merging two signals together (G, H).

DISCUSSION

Establishment of Angora goat fibroblast cell line: Tissue adherent culturing method and enzyme digestion are two frequently used methods for primary cell culture. In comparison, the tissue adherent culturing method is simple and feasible, avoiding the injury of enzyme digestion (Yang *et al*., 2009). Morphological results indicated that both epithelial and fibroblast cells were present during the primary and early passages. Different tolerances to trypsin were evident as the fibroblasts fell off first when treated with trypsin and were adherent again quickly after passage, whilst most epithelial cells need the support of growing matrices like collagen or other extracellular matrix components (Hu *et al*., 2009; Anand *et al*., 2012). For this reason, purified fibroblasts could be obtained after 3-4 passages (Zhou *et al*., 2004). The average viability after thawing was above 90%, indicating that freezing had little influence on the viability of the fibroblasts. Hence, it is likely that the Angora goat genome can be preserved by freezing fibroblasts in liquid nitrogen for long-term storage.

Somatic cells, such as diploid fibroblasts, can be used for nuclear donors, and they can be subjected to genetic manipulation *in vitro*. All cells preserved a species should undergo a minimum number of passages (<4), because too many passages and excess trypsin digestion could seriously affect hereditary characteristics of such cells.

Mycoplasma detection, Karyotype analysis and Isoenzymes analysis: Microorganism contamination is a frequent problem in cell culture. The culture media contaminated by bacteria, eumycetes and mycetes can be observed by naked eye and phase contrast microscopy. Using indirect DNA fluorescence staining by Hoechst 33258 is a convenient and effective protocol for detecting mycoplasma contamination, which is commonly used by some cell culture collection institutions such as the ATCC.

Chromosome number and karyotype are reliable indices for identifying the taxonomic and sexual origin of a cell line, and also distinguish between normal and malignant cells (Freshney, 2000). The result showed that the proportion of cells with a 2n =60 was above 90%. A small percentage of cells displayed abnormal chromosomes, presumably as a result of chromosome loss or overlap during preparation or chromosomal damage during culture and passage *in vitro*.

Isoenzyme and karyotypic data together can effectively confirm the origin of a cell line and identify possible interspecies cross-contamination, has become a classical and standard method routinely used by the main **B**iological Resource Centers (ATCC, ECACC, DSMZ and Riken) (Nims *et al*., 1998). Liu *et al*., 2011 and Zhou *et al*., 2004 analyzed the LDH isoenzyme pattern in sheep and plasma of Debao horse fibroblasts. Their results showed that the plasma and Debao pony fibroblast LDH isoenzyme patterns were dominant for LDH-1, LDH-2, and LDH-3; LDH-3, LDH-4 and LDH-5 were dominant in the leukocytes. The present study on the LDH isoenzyme patterns of Angora goat fibroblasts showed that LDH-3 and LDH-4 were dominant. MDH is a dimeric enzyme comprising cytosolic (s-MDH) and mitochondrial (m-MDH) subunits. The present results showed that the MDH of the Angora goat also have s-MDH and m-MDH forms and again, the movement rate of s-MDH was more out of the two. This is in accordance with the MDH activity of ear tissue, indicating that cells cultured *in vitro* have similar MDH activity to original tissues.

Expression of fluorescent protein genes: Enhanced fluorescent proteins with stable structures, high expression levels and not dependent on the germ-line have been used as marker genes to observe the contribution and function of target proteins in cells and organisms (Baird *et al*., 2000). We considered that the expression efficiency of fluorescent protein genes could also be used as a criterion to evaluate the quality of cell lines and cell strains cultured *in vitro*. Under optimal conditions, the expression efficiencies of the six fluorescent proteins at 24-72 h were between 13.4 and 60.8%. Fluorescent proteins expression had no significant effects (P>0.05) on cell morphology, cell proliferation, cell apoptosis rate under certain range to control group (Rong *et al*., 2006). Moreover, fluorescent protein-positive cell strains observed here will provide donor cells for transgenic animal cloning.

In conclusion, we used a primary explants technique and cryogenic preservation technology to establish the AGF31 cell line and proceeded to Biology and Genetics detection according to ATCC quality control procedures, and improved some techniques and methods, for example increased expression of fluorescent proteins. The AGF31 line provides a useful approach for conserving this unique breed of the world and will be an effective experimental material supply for further genetic studies on the Angora goat as well.

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