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Full Length Research Paper

Establishment and biological characteristics of Piedmontese cattle fibroblast line

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A fibroblast line was successfully established from ear marginal tissues of Piedmontese cattle by direct culturing of explants. Biological analysis showed that the population doubling time (PDT) for reviving cells was approximately 24 h. The average viability of the cells was 96.6% before freezing and 92.7% after thawing. Isoenzyme polymorphorism of lactic dehydrogenase (LDH) and malic dehydrogenase (MDH) ruled out cross-contamination among cell lines. Karyotyping showed that the proportion of cells with chromosome number 2n = 60 was above 92%. Tests for bacteria, fungi, viruses and mycoplasmas were negative. The transfection efficiencies of pEGFP-N3, pEYFP-N1 and pDsRed1-N1 were between 7.1 and 30.8%; fluorescences were homogenously distributed throughout cytoplasm and nucleus except in some cryptomeric vesicles. Every index of the Piedmontese cattle cell line met the quality control standards of the American Type Culture Collection (ATCC).

Key words: Piedmontese cattle, fibroblast line, biological characterization.

INTRODUCTION

With high-yield breeds being widely spread in the current world, the genomic diversity of livestock and poultry, on which the survival and sustainable development of human society rest, is being eroded annually. In addition, vulnerable animals are being threatened by industrial pollution. Unless these genomic resources are conserved in some forms before lost, not only the genes peculiar to rare breeds would extinguish forever, but will also be impossible to explore the underlying cell and molecular mechanisms related or to reproduce these breeds by somatic cell cloning. The conservation of endangered species and breeds is therefore of an urgent requirement. Currently, many techniques are applied to conserve the genetic resources of domestic animals. Germ cells, somatic cells, stem cells, zygotes and embryos can all be cryopreserved in cell banks (Guan et al., 2007). Not only do cell banks preserve precious genetic materials, but they also provide excellent resources for biological researches. In addition, modern cloning techniques have made somatic cells an attractive point for conserving animal genetic resources.

Piedmontese cattle, originated in Piedmont region of northern Italy, has been distributed in 14 provinces and cities since firstly introduced into China in 1986 by Institute of Animal Science, Chinese Academy of Agricultural Sciences. This breed possesses good adaptability and docile temperament, and is one of the world's best ultimate hybridization male. Piedmontese cattle is also recognized as one of the breeds with the largest proportion of high-grade site meat for its huge muscular apparatus area and muscular rear. There are other merits including low fat and cholesterol, high amino acid and so on.

The object was to cryopreserve the precious genomic resource, to establish a Piedmontese cattle fibroblast line and to study their biological characteristics, which could serve as an original material for somatic cell cloning, a convenient and helpful resource for genomics. Moreover, with the development of modern science and technology, the roles of limited cell lines will become increasingly

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Abbreviations: PDT, Population doubling time; **LDH,** lactic dehydrogenase; **MDH,** malic dehydrogenase; **ATCC,** American Type Culture Collection.

indispensible and there will be currently unforeseen applications.

MATERIALS AND METHODS

Experimental stock

Piedmontese cattle were obtained from the Institute of Animal Science, Chinese Academy of Agricultural Sciences, China. All cattle were treated in accordance with the National Institute of Health (NIH) and United Stated Department Agriculture (USDA) guidelines for the use of animals in researches, and all experimental procedures involving cattle were conducted in accordance with the protocols and guidelines for agricultural animal research imposed by the Committee for Ethics of China.

Cell culture

Ear marginal tissues (about 1 cm^2) were taken from 10 Piedmontese cattle (four males and six females) and collected into separate tubes containing Dulbecco's Modified Eagle Media (DMEM) medium (Gibco, USA) supplemented with ampicillin (100 U/ml) and streptomycin (100 µg/ml). The samples were rinsed and chopped into 1 mm^3 pieces, which were subsequently seeded into the surface of a tissue culture flask containing DMEM medium and 10% fetal bovine serum (FBS) (Hyclone, USA) in a 37°C incubator with 5% carbon (IV) oxide (CO_2) in air (Guan et al., 2005; Zhou et al., 2004). Cells were harvested when they reached 80 to 90% confluence and were separated into prepared culture flasks at the ratio of 1:2 or 1:3 (Freshney 2000).

Cryopreservation and recovery

Cells in logarithmic phase were enumerated with a hemocytometer, and viabilities were calculated by Trypan Blue exclusion test before freezing. The harvested cells were resuspended in freezing medium containing 40% DMEM medium, 10% dimethyl sulphoxide (DMSO) (Sigma, USA) and 50% FBS to a final concentration of (3 to 5) × 10^6 viable cells per milliliter. The suspension were aliquoted into sterile plastic cryovials labeled with species, breed, gender, cryopreservation serial number and the date, which were then sealed and placed into boxes filled with proper amounts of isopropyl alcohol. These boxes were placed in a -80°C refrigerator overnight and then the cells were transferred into a liquid nitrogen storage system (Werners et al., 2004). To recover and reseed the cells, the cryovials were removed from liquid nitrogen and thawed quickly in a 42°C water bath, and then the cells were transferred into a flask containing complete DMEM. The cells were cultured in a humidified atmosphere at 37°C with 5% CO_2 and the medium was renewed 24 h later.

Growth dynamics and trypan blue exclusion test

Following the method of Gu et al. (2006) and Kong et al. (2007), cells at the concentration of 1.5 × 10^4 ml^{-1} were seeded into 24-well microplates. Data on cell growth and density were calculated and recorded each day until the plateau phase; three wells were counted each time. The cell growth curve was then plotted and the PDT was calculated from this curve. Cell viabilities before cryopreservation and after recovery were determined using the Cell Titer-Blue Cell Viability Assay (Promega, USA). The viabilities of the cells in suspension were evaluated using 0.25% trypan blue (Promega, USA) solution. Both the number of intact cells and the total number of cells in a sample after cryopreservation were counted in a hemocytometer counting chamber, and viability was formulated as the percentage of the former to the latter. Counts were repeated thrice.

Microbial analysis

Detection of bacteria, fungi and yeasts was as described by Doyle et al. (1990). Test for viruses: Hay's hemadsorption protocol was used routinely to examine the samples for cytopathogenesis using phase-contrast microscopy (Hay et al., 1992). Mycoplasma detection, the cells were stained with Hoechst 33258 according to the deoxyribonucleic acid (DNA) fluorescent dyeing protocol (Guan et al., 2005). An enzyme-linked immunosorbent assay (ELISA) mycoplasma detection kit (Roche Diagnostics Corp., Indianapolis, Indiana) that could detect the four of the most common mycoplasma species was used to further confirm the detection results.

Chromosome analysis

Chromosomes were prepared, fixed and stained following standard methods (Suemori et al., 2006). After Giesma staining, the chromosome numbers per spread were counted for 100 spreads under an oil immersion objective. Relative length, arm ratio and centromeric index and type were calculated according to the protocol of Kawarai et al. (2006).

Isoenzyme analysis

Isoenzyme patterns of LDH and MDH were detected using a vertical slab non-continuous polyacrylamide gel electrophoresis (PAGE) assay. In brief, the cells were harvested and protein extraction solution (0.9% Triton X-100, 0.06 mmol NaCl: ethylene diamine tetra-acetic acid (EDTA) in mass ratio 1:15) was added after the cell concentration was adjusted to 5×10^7 cells per milliliter. Then the mixture was centrifuged, and the supernatant was stored in aliquots at -80°C. Sucrose solution (40%) and the samples were blended (1:1(v/v)) and then loaded into the individual lanes of polyacrylamide gel (Simpson, 2003). Different mobility patterns were reflected by the relative mobility front (RF), which was calculated as the ratio of the migration distance of an isozyme band to that of the indicator.

Expression of fluorescent genes in Piedmontese cattle fibroblasts

To obtain the highest transfection efficiency and a low cytotoxicity, transfection conditions were optimized by modifying the cell density and the concentrations of plasmid DNAs (BD Biosciences Clontech, Japan) of three fluorescent plasmids (pEGFP-N3, pEYFP-N1 and pDsRed1-N1) and Lipofectamine 2000 (Invitrogen, USA), according to the lipofectamine medium methods of Escriou et al. (2001) and Tsuchiya et al. (2002). The cells were observed 24, 48, 72 and 96 h, one and two weeks, and one month after transfection for three kinds of fluorescent proteins using excitation wavelengths of 488, 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 counts in each field to determine the transfection efficiency. The effect of the exogenous genes on the cells was measured in terms of cell motilities and apoptosis rates using trypan blue exclusion test and 4',6-diamidino-2-phenylindole (DAPI) staining.

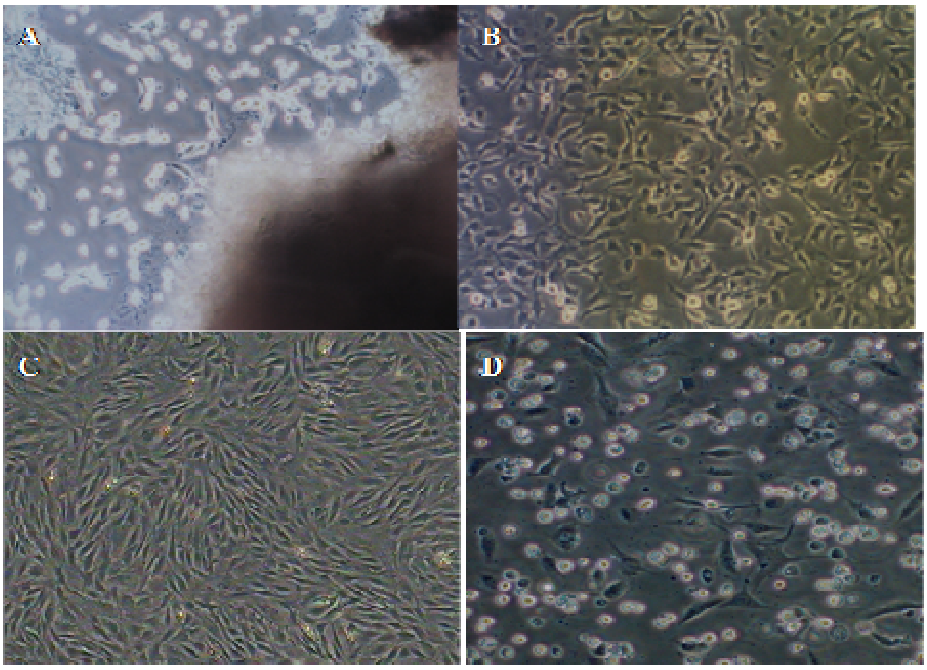

Figure 1. The culture of Piedmontese cattle fibroblasts (10 × 3.3). (A) Primary cells of migrating from ear marginal explants; (B) sub-cultured fibroblasts;(C) cells before freezing; (D) fibroblasts at 12 h after recovery.

RESULTS

Morphology of Piedmontese cattle fibroblasts

Fibroblast-like or epithelial-like cells could be seen migrating from the tissue pieces five to 12 days after explanting (Figure 1A). The cells were sub-cultured when 90% confluent. Afterwards, the fibroblasts grew rapidly, gradually outgrew and excluded other cells such as the epithelial ones (Li et al., 2008). Purified fibroblasts were obtained two to three passages later (Figure 1B), and the morphologically elongated spindle shapes suggested that the cells were still healthy (Figures 1C and 1D).

Growth dynamics and viabilities

The growth curve of Piedmontese cattle ear marginal fibroblasts had an obvious “S” shape (Figure 2A) and the PDT was proximately 24 h. There was a lag time or latency phase of about 24 h after seeding, corresponding to the adaptation and recovery of the cells from trypsinization, and then the cells proliferated rapidly and entered the exponential phase. As the cell density increased, proliferation was retarded by contact inhibition, and then the cells entered a plateau phase and began to degenerate since the sixth day.

The viabilities of Piedmontese cattle fibroblasts before freezing and after recovery, as detected by Trypan Blue exclusion tests, were 96.6 and 92.7%, respectively, the difference between which were non-significant ($P > 0.05$). Therefore, the cryopreservation had little effects on the viability.

Microbial analysis

The culture media showed no increase in turbidity or other changes in the negative control and Piedmontese cattle fibroblasts, which ruled out the contamination from bacteria, fungi or yeasts.

No viruses were detected by cytopathogenic evidence or hemadsorption test. Staining with the DNA fluorochrome Hoechst 33258 (Sigma), one of the most effective and frequently used methods for mycoplasma contamination detection (Barile and Rottem, 1993), was adopted in this research, and the fibroblast nuclei appeared as blue ellipses, suggesting that the newly established cell line was mycoplasma negative (Figure 2B).

The karyogram and chromosome number of Piedmontese cattle fibroblasts

The chromosome number of Piedmontese cattle was 2n = 60, comprising 58 autosomes and two sex chromosomes, XY or XX (Figure 2C). All somatic chromosomes were acrocentric autosomes, and only the two sex chromosomes (X and Y) were submetacentric (Table 1). The chromosome numbers per spread were counted from 100

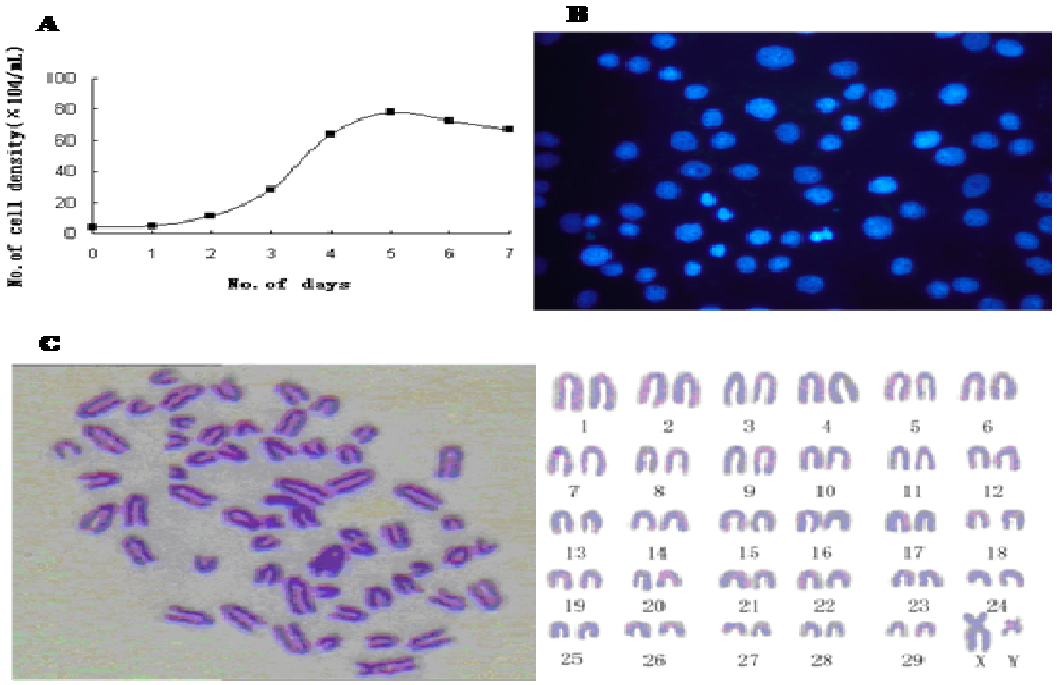

Figure 2. The characterization of Piedmontese cattle ear marginal fibroblast line. Panel A, the growth curve before cryopreservation, the cell number was counted using a hemocytometer. Each value represents the mean of three independent experiments; panel B, mycoplasma contamination for the Piedmontese cattle fibroblasts stained with Hoechst 33258; C, chromosomes at metaphase (left) and karyotype (right) of the Piedmontese cattle (♂), 1000×.

Table 1. Chromosome's parameters of Piedmontese cattle.

Chromosome number	Relative length (%)	Centromere morphology	Chromosome number	Relative length (%)	Centromere morphology
1	5.25 ± 0.21	T	16	3.14 ± 0.29	T
2	4.88 ± 0.18	T	17	2.98 ± 0.11	T
3	4.62 ± 0.32	T	18	2.88 ± 0.17	T
4	4.43 ± 0.39	T	19	2.82 ± 0.04	T
5	4.14 ± 0.14	T	20	2.64 ± 0.29	T
6	3.97 ± 0.38	T	21	2.64 ± 0.22	T
7	3.96 ± 0.34	T	22	2.64 ± 0.14	T
8	3.94 ± 0.51	T	23	2.56 ± 0.24	T
9	3.77 ± 0.06	T	24	2.56 ± 0.17	T
10	3.38 ± 0.23	T	25	2.43 ± 0.21	T
11	3.27 ± 0.09	T	26	2.30 ± 0.48	T
12	3.25 ± 0.17	T	27	2.19 ± 0.13	T
13	3.25 ± 0.29	T	28	2.19 ± 0.33	T
14	3.19 ± 0.19	T	29	2.11 ± 0.31	T
15	3.19 ± 0.31	T	X	5.44 ± 0.10	SM

M1.0 –1.6, metacetricchromosome (M); SM1.7 – 2.9, submetacentricchromosome (SM); ST 3.0 – 6.0, subtelocentric chromosome (ST); T≥7.0, telocentric chromosome.

spreads of the first, fourth and sixth passages, and the proportions of cells with 2n = 60 were 94.8, 92.2 and 91.5% respectively. Aberrations in chromosome numbers tended to increase as passage numbers increased, indicating that *in vitro* culture affected the heritage of cells slightly, yet supporting that the cell line was reproducibly diploid.

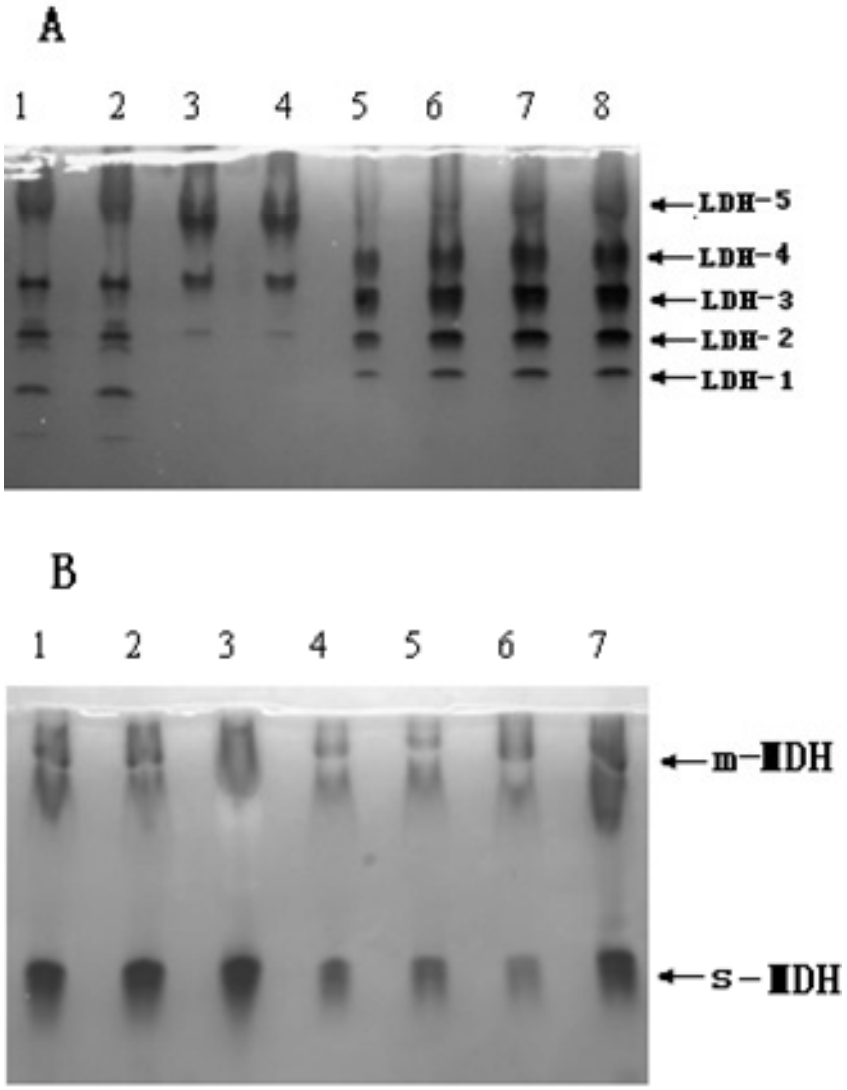

Figure 3. Lactate dehydrogenase(LDH) and malate dehydrogenase (MDH) zymotype for different species. Panel A: 1, 2, Landraze; 3, 4, Bengal tiger; 5, 6, Piedmontese cattle; 7, 8, Zhiwei goat; Panel B: 1, 2,Tan sheep; 3, Piedmontese cattle; 4, 5,Luxi cattle; 6, Hereford cattle; 7, Mongolian horse.

Isoenzyme analysis of Piedmontese cattle cell line

The LDH bands obtained from Piedmontese cattle were compared with those from other species or breeds, and five isoenzyme bands (LDH-1, -2, -3, -4, -5) were observed (Figure 3A). The enzymatic activities were in the order LDH-3, LDH-2, LDH-4, LDH-1, LDH-5; LDH-1, LDH-2 from anode to cathode. LDH-2, LDH-3 and LDH-4 were dominant, while LDH5 was scarcely observable. These domestic animals were characterized by their distinct bands with different relative mobilities. For the same livestock species of different breeds, there were fewer band differences on the LDH isozymogram.

Two MDH isoenzyme bands cytosolic type (s-MDH) and a mitochondrial type (m-MDH) were observed in the pattern of Piedmontese cattle fibroblast line (Figure 3B). All five domestic animals exhibited two bands, and there were significant differences in the isoenzyme patterns of LDH and MDH between the Piedmontese cattle fibroblast line and others established by our laboratory. These results disprove the cross contamination from interspecies and intraspecies.

Expression of three fluorescent protein genes in Piedmontese cattle fibroblasts

The expression of pEGFP-N_3, pEYFP-N_1 and pDsRed1-N_1 on the optimized condition at 24, 48 and 72 h were recorded using laser confocal microscopy upon a specific

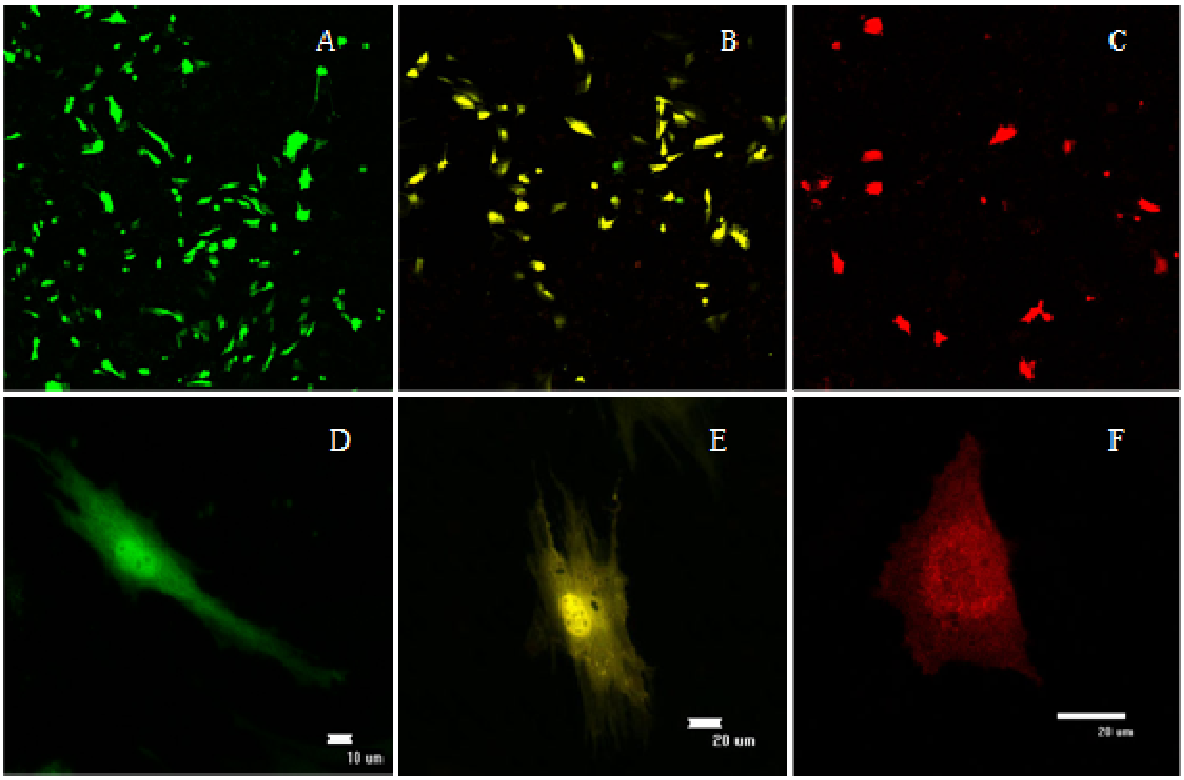

Figure 4. The expression and distribution of pEGFP-N3, pEYFP-N1 and pDsRed1-N1in Piedmontese cattle fibroblasts. A, B and C were the transfection results of pEGFP-N3, pEYFP-N1and pDsRed1-N1at 48 h after transfection (100×); D, E and F were the subcellular location of pEGFP-N3, pEYFP-N1and pDsRed1-N1 at 48 h after transfection (400×).

Table 2. Efficiency of transfection of three fluorescent proteins.

Transfection time (h)	pEGFP-N3 (%)	pEYFP-N1 (%)	pDsRed1-N1 (%)
24	7.1	12.2	11.4
48	22.6	30.8	21.2
72	20.1	22.9	20.4

exiting light (Figure 4). Positive cells were observed 12 h after transfection, and the numbers and intensities increased markedly and reached their maximum at 48 h. The intensity of pEGFP-N3 was the most of the three. The expression ratios of the three kinds of fluorescent proteins at 24, 48 and 72 h after transfection were all between 7.1 and 30.8% (Table 2). The numbers of fluorescent cells decreased at one week, but a few scattered ones remained positive after two weeks and even one to two months (data not shown).

DISCUSSION

Establishment of Piedmontese cattle fibroblast line

The marginal ear tissue fibroblast line from Piedmontese cattle was established using adherent culture method. All the results indicate that the newly established cell line was stable and proliferated rapidly, and the quality of this cell line conformed to the criteria of the ATCC. Thus, the genomic resource of Piedmontese cattle could be conserved through a long-term cryopreservation. After being thawed, the viabilities decreased to some extent, possibly owing to the injury that occurred during the process of freezing and recovery. Due to the fact that serial passage and trypsinization might seriously affect the biological characteristics of the cells, in particular the hereditary ones, all cells should undergo a minimum number of passages. To ensure the motility rate of the cells recovered at late stages, the cells were frozen within five generations at a concentration above 3×10^6 ml^{-1}. The procedures used in this study conformed to the protocols of the ATCC technical bulletin for primary culture, subculture and

cryopreservation.

Microbial detection

Microbial contamination is one of the most frequent phenomena in cell culture. Air, equipment, serum, tissue sample, and handling errors can all be the sources. The turbidity of culture media contaminated by bacteria, eumycetes and mycetes, can be observed even with the naked eye. Viral infections could be identified under the microscope through hemadsorption experiment, but it is hard to detect mycoplasmas, which have no nuclei and can grow and reproduce in currently used media. They are hard to remove and could coexist with cultures for long periods. The method of mycoplasma detection included direct solid agar culture, indirect fluorescence staining of DNA and new DNA-style hybridization. Due to the fact that fluorescent staining of mycoplasma DNA is simple and quick, it is commonly utilized by some cell culture collection institutions. Our microbiological detection results show that the Piedmontese cattle fibroblast bank was purified and free of micoplasma contamination.

Karyotype analysis

Species are characterized by their chromosome numbers, morphology and structure, which remain very stable in the normal cells. Therefore, karyotype analysis constitutes a major method for distinguishing normal cells from the mutants. Karyotype can effectively confirm the origin of a cell line and identify possible cross-contamination. The technique has been used for many years and is still being used today, practice of which has become a classical and standard method for characterizing cell lines (Shepel et al., 1994; Nims et al., 1998).

For the best preservation of Piedmontese cattle's genetic traits, the fibroblasts must remain diploid just as those in vivo. The freezing procedure was ameliorated and the passage number was reduced to obtain a stable diploid cell line in which about 92% of the cells had 2n = 60 chromosomes. Chromosome analysis can relate a cell line to the gender of the animal, and also distinguish between normal and malignant cells, since the chromosome number is more stable in normal ones.

Isoenzyme analysis

Isoenzyme polymorphism is commonly used to identify cell lines and their origins. ATCC consider the biochemical analysis of isoenzyme polymorphism to be the standard method for detecting interspecies contamination (Drexler et al., 1999). LDH and MDH are key enzymes in glycolytic pathway and citric acid cycle respectively. They are species-specific and conservative, but the contents and activities differ among species, providing a biochemical indicator of species classification by chromatography and electrophoresis. Therefore, they were chosen to determine the species' origin of the lines and to detect cross-contamination (Nelson-Rees and Daniels, 1981; Parodi et al., 2002).

In this study isoenzyme zymograms for LDH and MDH were obtained through the improved starch gel electrophoresis method of the ATCC. LDH is a tetrametric molecule; the H and M subunits are produced by the expression of the ldha and ldhb genes, and each tissue has a characteristic and species-specific isoenzyme composition (Washizu et al., 2002). Five LDH bands were found for Piedmontese cattle fibroblasts, namely LDH1, LDH2, LDH3, LDH4 and LDH5. MDH in livestock has a cytosolic type (s-MDH) and a mitochondrial type (m-MDH). The mobilities of MDH bands among poultry are essentially identical, and the same is true among those from livestock, but MDH from livestock migrated more rapidly than that from poultry, and the enzyme content was also higher than that in poultry.

Expression of fluorescent genes

Researches using fluorescent proteins were mainly focused on tumors, nerves and stem cells (Jung et al., 2001). The concentrations of DNA and lipofectin, the DNA incubation time and lipofectin combination, and the presence of serum, can all affect the efficiency of transfection, as shown by the researches on Vero cells, HeLa cells and various other cell lines (Tseng et al., 1999; Rui et al., 2006). In this study, the highest transfection efficiency of the three kinds of fluorescent genes was 30.8% with an optimized plasmid-lipofectin ratio. It was observed that the transfected cells at all the stages of reduplication and cell division and growth were not significantly different from the control group. The numbers of fluorescent cells decreased at one week, but a few scattered positive cells remained after two weeks and even one to two months.

The fibroblasts could be used as a tool for investigating the functions of exogenous genes. This new resource will be important for future identification of breed specific genetic markers and for nuclear transfer and transgenic cloning investigations.

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