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Establishment and biological characteristics of Luxi cattle fibroblast bank

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Abstract

A fibroblast line from ear marginal tissue of Luxi cattle (LXCEM2/2) was successfully established by direct culturing of explants. Biological analysis showed that the population doubling time (PDT) for reviving cells was approximately 24 h. Measurement of lactic dehydrogenase (LDH) and malic dehydrogenase (MDH) isoenzymes showed no cross-contamination among the cells. Karyotyping showed that the frequency of cells with chromosome number 2n = 60 was 90.7–92.2%. Tests for bacteria, fungi, viruses and mycoplasma were negative. The efficiencies of expression of pEGFP-N3, pEYFP-N1 and pDsRed1-N1 were between 6.3% and 31.6% at 24 h, 48 h and 72 h after transfer; at 24 h, fluorescence was well distributed in the cytoplasm and nucleus except for some cryptomeric vesicles. Every index of the Luxi cattle cell line meets the quality control standards of the American Type Culture Collection (ATCC). Not only has the germline of this important cattle breed been preserved at the cell level, but also valuable material had been provided for genome, postgenome and somacloning research. Moreover, the establishment of this technical platform may provide both technical and theoretical support for storing the genetic resources of other animals and poultry at the cell level.

Keywords: Luxi cattle; Fibroblast bank; Biological characterization

1. Introduction

Because high-yield breeds are being actively spread worldwide, the genomic diversity of livestock and poultry, on which the survival and sustainable development of human society depends, is being eroded annually. There are 596 known Chinese domestic animal breeds, but 17 have become extinct and 336 breeds are threatened to various degrees. Unless these genomic resources are conserved in some form before they are lost, we will not only lose the genes peculiar to the breeds but also find it impossible to explore the cytological and molecular biological mechanisms required to reproduce those breeds by somatic cell cloning. It is therefore very urgent to commence conservation of our nation's threatened breeds (Guan et al., 2005). Generative cells, somatic cells, stem cells, zygotes and embryos can all be cryopreserved in cell banks. However, cell banks emphasizing conservation and utilization of animal resources, especially animal genetic resources, mainly cryopreserve generative cells and embryos.

Luxi cattle are one of the four most important cattle breeds in China. They have many important economic advantages such as high rates of meat production and high meat quality and they enjoy a good international reputation. In 2006, the Chinese government made them one of 138 state-level protected domestic animal breeds. Currently, there are many strategies to conserve the genetic resources of domestic animals, of which live animal preservation is the most important

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and will be so for a long time (Wu, 1999). Nevertheless, establishment of somatic cell banks using low temperature biological techniques is a new effective approach to conserving and maintaining the diversity of domestic animals (Shi, 1989).

Our project seeks a different approach to conserving important domestic animals in imminent danger. Using a characteristic Chinese cattle breed to initiate the establishment of cell lines and the study of their biological characteristics, our aim is to cryopreserve this nationally protected genomic resource for the purposes of reviving endangered breeds by cloning, and supplying a convenient and effective resource for genomic research. Moreover, with the development of science and technology, the roles of limited cell lines will become increasingly prominent and there will be currently unforeseen applications.

2. Materials and methods

2.1. Cell cultures

Ear tissue samples (about 1 cm^2) were taken from 8 Luxi cattle (1 male and 7 females) and collected into separate tubes containing DMEM supplemented with ampicillin (100 U/ml) and streptomycin (100 μg/ml). The samples were rinsed and chopped into 1 mm^3 pieces, which were seeded on the surface of a tissue culture 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). Cells were harvested when they reached 80–90% confluence and were divided into prepared culture flasks at 1:2 or 1:3 ratios (Freshney, 2000).

2.2. Cryogenic preservation and recovery

Cells in logarithmic growth phase were enumerated with a hemocytometer, and viability was checked by Trypan Blue staining before freezing. Harvested cells were resuspended in freezing medium (10% DMSO + 90% fetal bovine serum) to a final density of (3–4) × 10^6 viable cells/ml. Single cells were dispensed into sterile plastic cryogenic vials labeled with animal name, gender, freezing serial number and the date. The vials were sealed and kept at 4 °C for 20–30 min to allow the DMSO to equilibrate, then after programmed freezing they were transferred to storage in liquid nitrogen quickly and efficiently (Werners et al., 2004; Ren et al., 2002).

2.3. Growth curve and estimation of cell viability by Trypan Blue

In accordance with the method of Gu et al. (2006) and Kong et al. (2007), cells at 1.5×10^4 ml^{-1} were seeded into 24-well plates. Cell growth and density data were monitored and recorded each day until the plateau phase was reached; three wells were counted for each time point. A cell growth curve was then plotted and the population doubling time (PDT) was calculated from this curve. Cell viability before freezing and after recovery was determined using a hemocytometer to enumerate 1000 cells by Butler's dye exclusion method (Butler, 1999).

2.4. Measurement of microorganisms in cell line

- *Tests for contamination with bacteria, fungi and yeasts*: for details of the procedure used, see 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, 1992).
- *Mycoplasma detection*: cells were cultured in antibiotic-free medium for at least 1 week, then fixed and stained with Hoechst 33258 (ATCC) according the methods of Masover and Becker (1998) and Freshney (2000) for fluorescent staining of deoxyribonucleic acid (DNA). An ELISA Mycoplasma Detection kit (Roche, Lewes, East Sussex, UK) was used to confirm the results of the DNA fluorescent staining.

2.5. Chromosome analysis

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

2.6. Isoenzyme analysis

The electrophoretic mobilities of lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) were determined using the polyacrylamide gel electrophoresis protocol contributed by Marvin L. Macy of ATCC. Mobility was measured as the ratio of distance migrated by the band to the distance migrated by the indicator dye.

2.7. Expression of fluorescent protein gene in Luxi cattle fibroblasts

To obtain the highest transfection efficiency and low cytotoxicity, transfection conditions were optimized by varying the cell density and the 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 cultured cells were observed 24 h, 48 h, 72 h, 96 h, 1 week, 2 weeks and 1 month after transfection with six fluorescent protein genes using excitation wavelengths of 405 nm, 488 nm and 543 nm separately. For each experimental group, images were captured from 10 visual fields, and confocal microscopy was used to measure 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 motility and apoptosis using Trypan Blue and DAPI staining.

3. Results

3.1. Morphology of Luxi fibroblasts

Fibroblast-like or epithelial-like cells could be seen migrating from the tissue pieces 5–12 d after explanting (Fig. 1A). When the time in culture was increased, cells continued to proliferate and were subcultured when they reached 90% confluence (Fig. 1B). After subculturing, the fibroblasts grew rapidly, gradually outgrowing and excluding other cells such as epithelial cells (Ren et al., 2002). After 2–3 passages, we obtained purified fibroblasts (Fig. 1C, D). The motilities of Luxi fibroblasts before freezing and after recovery as measured by Trypan Blue staining were 99.0% and 98.7%, respectively.

3.2. Growth curve (dynamic state of cells)

The growth curve of ear marginal tissue fibroblasts from Luxi cattle had an obvious "S" shape (Fig. 2A) and the PDT was about 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 protease damage, then the cells proliferated rapidly and entered exponential phase. As the cell density increased, proliferation was retarded by contact inhibition; by the sixth day, the cells entered the plateau phase and began to degenerate.

3.3. Microbial analysis

Tests for contamination with bacteria, fungi and yeasts were negative; no microorganisms were observed in the culture media. No viruses were indicated by the cytopathogenic evidence or by the hemadsorption test. Staining with the DNA fluorochrome Hoechst 33258 is the most effective and frequently used method for detecting mycoplasma contamination (Barile and Rottem, 1993). Under a fluorescence microscope after staining with Hoechst 33258, fibroblast nuclei appeared as blue ellipses, showing that the established cell line was mycoplasma negative (Fig. 2B).

3.4. Karyogram and chromosome number of Luxi cattle

The chromosome number of Luxi cattle was 2n = 60, comprising 58 autosomes and two sex chromosomes, XY or XX (Fig. 2C). All somatic chromosomes were acrocentric autosomes, and only the two sex chromosomes (XY) were submetacentric (Table 1). The chromosome numbers per spread were counted for 100 spreads of the first, second and fourth passages, and the frequencies of cells with 2n = 60 were 92.2%, 91.6% and 90.7%, respectively. Aberrations in chromosome numbers tended to increase with increasing numbers of passages, indicating that in vitro culture affected the heritage of cells slightly, but supporting the inference that the cell line was reproducibly diploid.

3.5. Isoenzyme analysis of Luxi cattle cell line

Isoenzyme polymophism occurs among species, and even sometimes among races, individuals and tissues within a species (O'Brien et al., 1977). We improved the apparatus

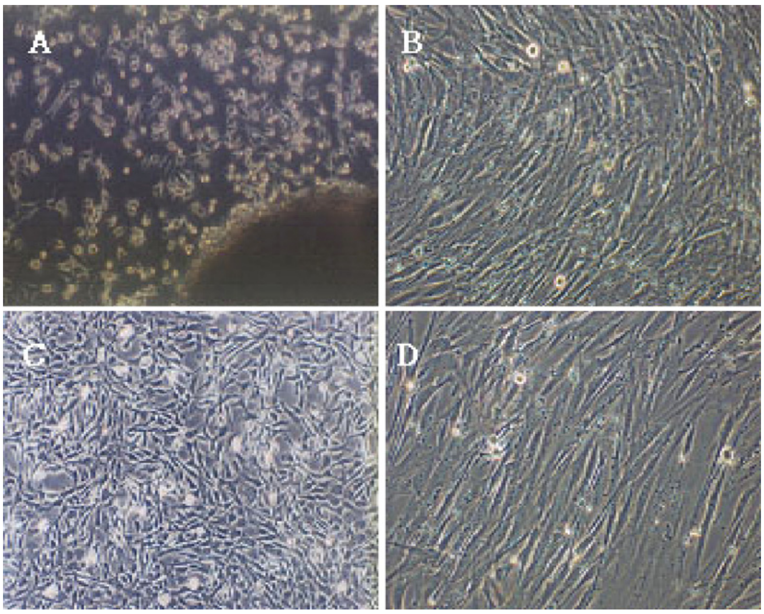

Fig. 1. The culture of Luxi cattle fibroblasts (10 × 3.3). (A) Primary cells of Luxi cattle ear marginal explants; (B) subcultured fibroblast cells of Luxi cattle; (C) fibroblasts cells before freezing; (D) fibroblasts cells after recovery 12 h.

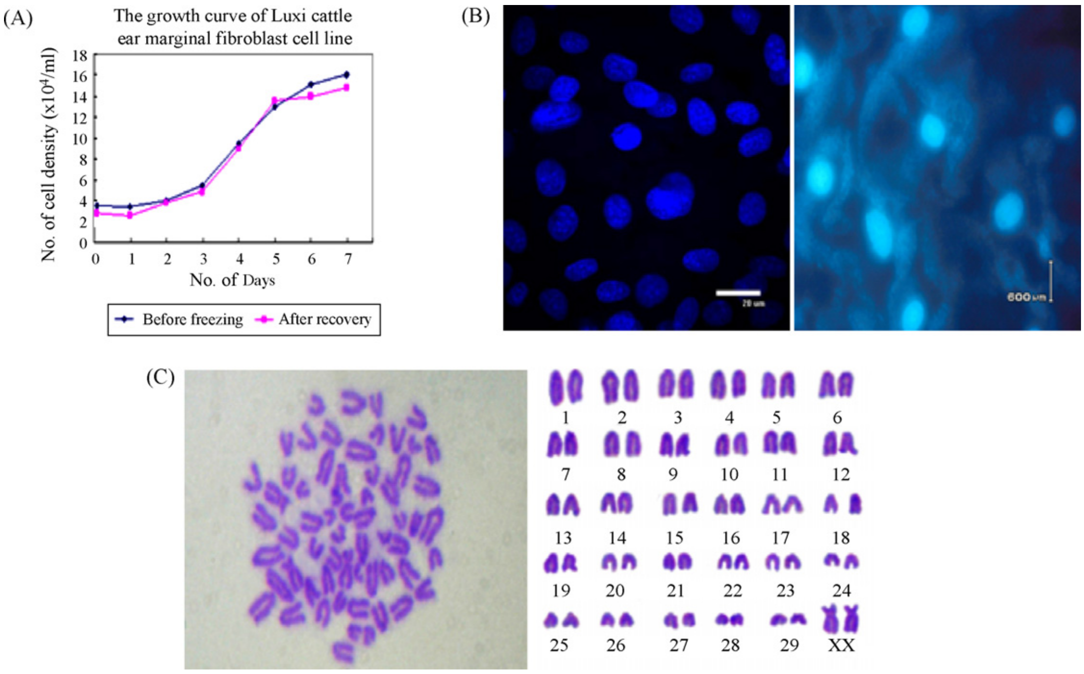

Fig. 2. The characterization of fibroblast cell line of Luxi cattle ear marginal fibroblast cell line. Panel A, the growth curve of Luxi cattle fibroblast cell before freezing and stored in liquid nitrogen for 6 months, the cell number was determined using a hemocytometer. Each value represents the mean–S.D. of three independent experiments; panel B, mycoplasma contamination for the Luxi cattle fibroblasts stained with Hoechst 33258 and positive control of mycoplasma contamination stained with Hoechst 33258; panel C, chromosome at metaphase (left) and karyotype (right) of the Luxi cattle (♀), 1000×.

and conditions for native polyacrylamide gel electrophoresis, and successfully determined the mobility of the isoenzymes of LDH and MDH.

The LDH bands obtained from Luxi cattle were compared with those from other species or breeds, and five isoenzyme bands (LDH-1, -2, -3, -4, -5) were observed (Fig. 3A). The enzymatic activities were in the order LDH-3, LDH-4, LDH-2, LDH-1, LDH-5; LDH-1, LDH-2, LDH-3 and LDH-4 were dominant, while LDH5 was scarcely observable. Two MDH isoenzyme bands (s-MDH, m-MDH) were observed in the Luxi cattle (Fig. 3B). There were significant differences in the isoenzyme patterns of LDH and MDH between the Luxi cattle and other cell lines in our laboratory.

3.6. Expression of six fluorescent protein genes in Luxi cattle fibroblasts

The six fluorescent protein genes pECFP-C1, pECFP-mito, pEGFP-C1, pEGFP-N3, pEYFP-N1 and pDsRed1-N1 were all highly expressed with reference the optimized condition for pEGFP-N3 (Fig. 4). Positively expressing cells were observed 12 h after transfection and the numbers and

Table 1
Chromosome's parameters of Luxi cattle

Chromosome number	Relative length (%)	Centromere morphology	Chromosome number	Relative length (%)	Centromere morphology
1	5.58 ± 0.26	T	16	3.26 ± 0.32	T
2	5.12 ± 0.16	T	17	3.01 ± 0.09	T
3	4.68 ± 0.34	T	18	2.97 ± 0.19	T
4	4.49 ± 0.41	T	19	2.97 ± 0.06	T
5	4.23 ± 012	T	20	2.71 ± 0.31	T
6	4.05 ± 0.45	T	21	2.70 ± 0.24	T
7	3.87 ± 0.38	T	22	2.60 ± 0.12	T
8	3.86 ± 0.57	T	23	2.58 ± 0.27	T
9	3.81 ± 0.04	T	24	2.21 ± 0.19	T
10	3.76 ± 0.22	T	25	2.14 ± 0.22	T
11	3.61 ± 0.11	T	26	2.09 ± 0.53	T
12	3.56 ± 0.19	T	27	2.07 ± 0.10	T
13	3.41 ± 0.33	T	28	1.85 ± 0.35	T
14	3.36 ± 0.20	T	29	1.75 ± 0.32	T
15	3.27 ± 0.41	T	X	4.47 ± 0.11	SM

Note: M 1.0–1.6, metacetricchromosome (M); SM 1.7–2.9, submetacentricchromosome (SM); ST 3.0–6.0, subtelocentric chromosome (ST); T ≥7.0, telocentric chromosome.

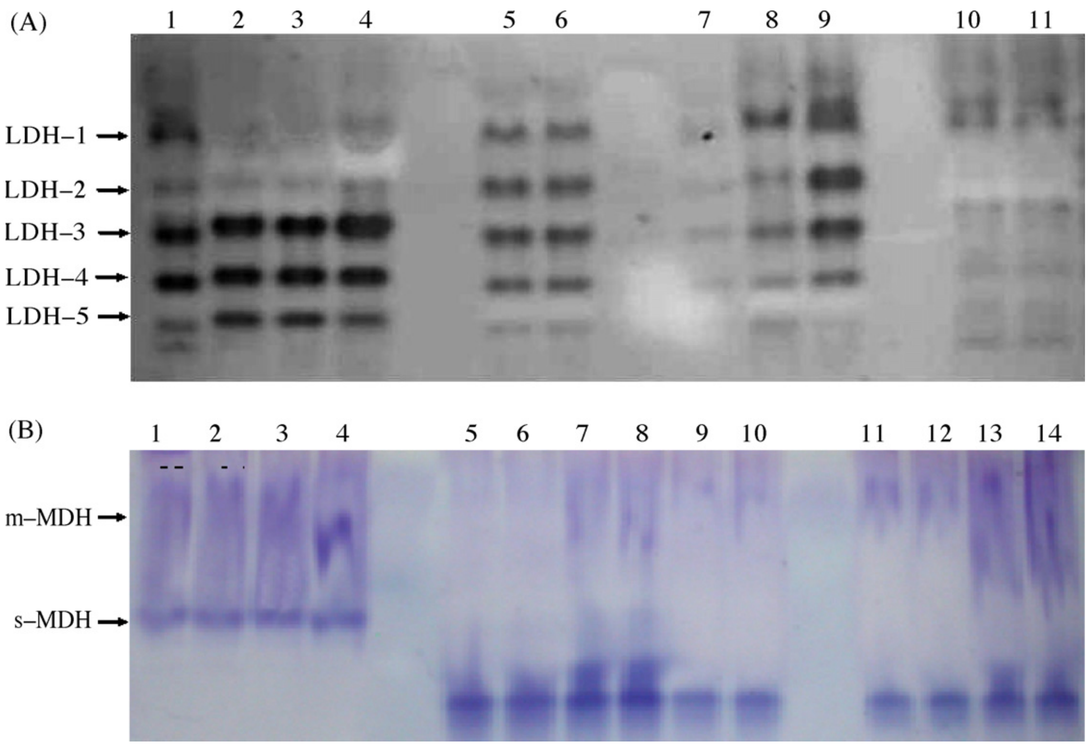

Fig. 3. Lactate dehydrogenase(LDH) and malate dehydrogenase (MDH) zymotype for different species. Panel A: 1, Zhiwei goat; 2, Jining black goat; 3, Taihang black goat; 4, Mongolian sheep; 5 and 6, Luxi cattle; 7, Simmental bovine; 8, Angus bovine; 9, Piemontese bovine; 10 and 11, large white pig. Panel B: 1 and 2, Aaijiao chicken; 3 and 4, Baier chicken; 5 and 6, Big Yorksire; 7 and 8, Landrace, 9 and 10, Songliao black pig; 11 and 12, Piemontese bovine; 13 and 14 Luxi cattle.

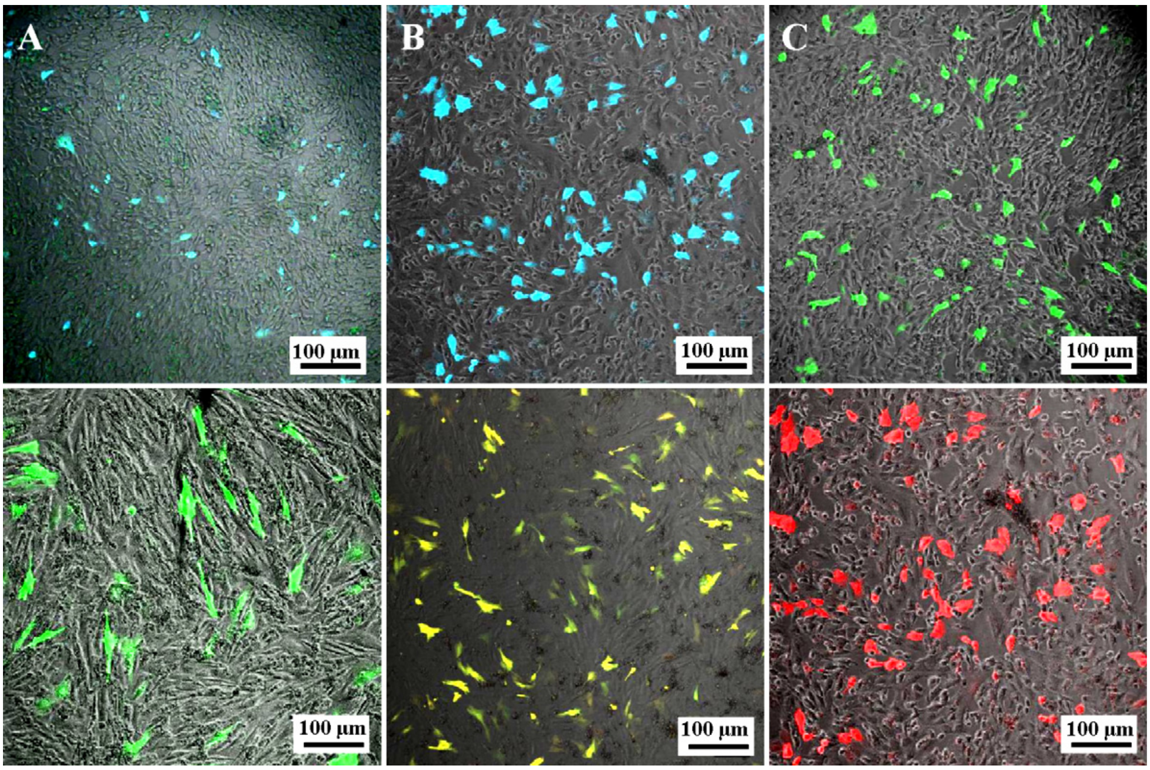

Fig. 4. Comparative figures of pECFP-C1, pECFP-mito, pEGFP-C1, pEGFP-N3, pEYFP-N1, pDsRed1-N1 fluorescent protein expression (10×). (A) pECFP-N1, 48 h; (B) pECFP-MITO, 48 h; (C) pEGFP-C1, 48 h; (D) pEGFP-N3, 48 h; (E) pEYFP-N1, 48 h; (F) pDsRed1-N1, 48 h.

Table 2
Transfection efficiencies of six fluorescence genes in the Luxi fibroblast cells

Transfection time (h)	Six fluorescent protein genes transfection rate (%)					
	pEGFP-C1	pEGFP-N3	pECFP-N1	pECFP-mito	pEYFP-N1	pDsRed1-N1
24	12.3	10.2	6.7	6.3	9.1	8.6
48	27.3	31.6	17.2	14.3	23.2	21.9
72	26.5	30.1	19.8	13.7	25.3	19.9

intensity increased markedly and reached a maximum at 48 h or 72 h. The transfer efficiencies of the cyan fluorescent proteins (pECFP-C1, pECFP-mito) were significantly lower than those of the green, yellow and red fluorescent proteins ($p < 0.01$), and the green fluorescent proteins (pEGFP-C1 and pEGFP-N3) were maximal. The expression efficiencies of the six fluorescent proteins at 24 h, 48 h and 72 h after transfer were between 6.3 and 31.6 (Table 2). The numbers of fluorescent cells decreased at 1 week, but a few dispersed positive cells remained after 2 weeks and even after 1 and 2 months.

3.7. Morphology, motility and apoptosis rate of transfected positive cells

Using confocal microscopy we observed that a few positive cells began to shrink and disaggregate, becoming amotic and even dead. Most of the transfected cells had spindle shapes, but the proliferation rate was obviously slower than for non-transfected cells, in accordance with the findings of Yuan et al. (2004). The cell motility rates were between 95.3% and 97.6%, not significantly different from the contrast cell at 98.5% ($p > 0.05$). Using DAPI, the cell nuclei showed an evenly dispersed blue fluorescence, and there were deeply staining blue granules and fluorescent particles (apoptotic bodies) in a few cells. The apoptosis ratio of the transfected groups was 0.6%, probably representing normal apoptosis since the value for the control group was 0.5% (Fig. 5).

4. Discussion

4.1. Establishment of Luxi cattle fibroblast line

We established the marginal ear tissue fibroblast line from Luxi cattle (LXCEM 2/2) using an adherent culture method. All the results indicate that the newly established cell line is stable and proliferates rather rapidly, and the identification of this cell line conforms to the quality control requirement of ATCC. Thus, we can conserve the genomic resource of Luxi cattle long-term by freezing fibroblasts in liquid nitrogen and achieve the aim of protecting the breed. In order to ensure full recovery of the cells subsequently, they should be frozen within five generations at a density greater 3×10^6 ml^{-1}, when they show typical fibroblast morphology. The cells may be injured and changed in biological characteristics, especially hereditary characteristics, after too many passages or trypsin digestion. The procedures used in this study conformed to the protocols of the ATCC technical bulletin for primary culture, subculture and freezing. Moreover, we characterized the established cell line according to ATCC quality control procedures and improved some techniques and methods, for example increased expression of the six fluorescent proteins.

4.2. Mycoplasma detection

Microbial contamination is the most frequent pollution phenomenon in cell culture. Air, equipment, serum, tissue sample, etc. could all contaminate the cells. The turbidity of culture media contaminated by bacteria, eumycetes and mycetes, can be seen with the naked eye. Viruses can be seen under the microscope. But it is harder to detect mycoplasma contamination.

Mycoplasmas have no nuclei and can grow and reproduce in currently used media. They are hard to remove and could coexist with cells for a long time. The method used to detect mycoplasma include direct solid agar culture, indirect fluorescence staining of DNA and new DNA-style hybridization. Because fluorescent staining of mycoplasma DNA is simple and quick, it is commonly used by some cell culture collection institutions such as the ATCC. Our microbiological detection results showed that the Luxi cattle fibroblast bank was purified and free of micoplasma contamination.

4.3. Karyotype analysis

Isoenzyme and karyotypic data together can effectively confirm the origin of a cell line and identify possible cross-contamination. The two techniques have been used for many years and are still used today. The practice of combining them has become a classical and standard method for characterizing cell lines (Shepel et al., 1994; Nims et al., 1998).

Because we want to conserve the genomic character of Luxi cattle, the fibroblasts must maintain the same diploid character as the cells in vivo. We improved the freezing procedure and decreased the number of passages to obtain a stable diploid cell line in which about 90% of the cells had $2n = 60$. Chromosome analysis can relate a cell line to the gender of the animal from which was derived, and also distinguish between normal and malignant cells, since the chromosome number is more stable in normal cells (Freshney, 2000).

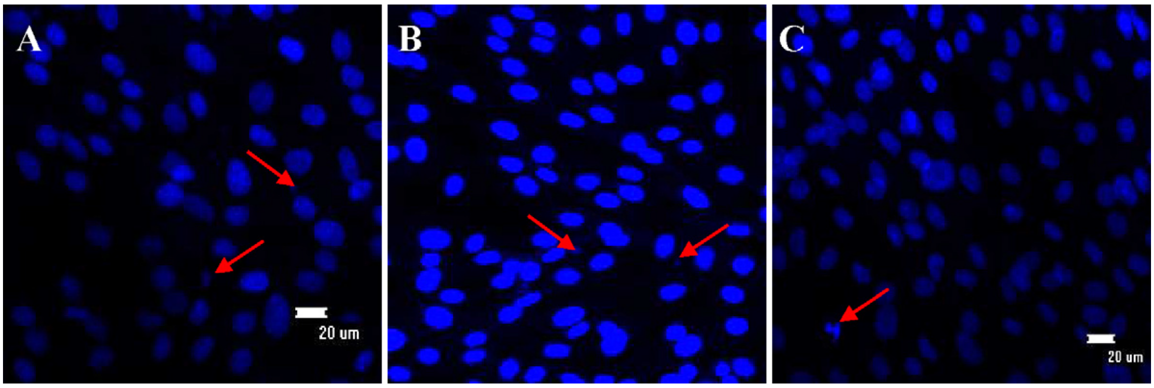

Fig. 5. Apoptosis examine of pEGFP-N3 and pEYFP-N1 transfect fibroblast cell stained by DAPI. (A) and (B) are the apoptosis examine of pECFP-N1 and pEGFP-C1 transfect fibroblast cell stained by DAPI; (C) is control group which is non-transfected.

4.4. Isoenzyme analysis

LDH and MDH are very important enzymes participating in the glycolytic pathway and citric acid cycle, respectively. They are species-specific and constant, but the enzyme contents and activities differ among species, providing a biochemical indicator of species classification by chromatography and electrophoresis. We therefore chose LDH and MDH to determine the species origin of the cells and to measure cross-contamination (Nelson-Rees et al., 1981; Parodi et al., 2002). Biochemical analysis of isoenzyme polymorphism is currently considered the standard method for quality control of cell line identification and interspecies contamination, and is routinely used by the main biological Resource Centers around the world (ATCC, ECACC, DSMZ and Riken) (Parodi et al., 2002).

LDH is a tetrametric molecule; the H and M subunits are produced by expression of the ldha and ldhb genes, and each tissue has a characteristic and species-specific isoenzyme composition (Washizu et al., 2002). Arai et al. (2003) and Zhou et al. (2004) measured the LDH isoenzyme pattern in horse leucocytes and plasma and Debao pony fibroblasts. MDH is a dimeric enzyme comprising cytosolic (s-MDH) and mitochondrial (m-MDH) subunits. The mobilities of MDH bands among poultry are essentially identical, and the same is true among livestock. But MDH from livestock migrates more rapidly than that from poultry, and the enzyme content is also greater than in poultry.

4.5. Expression of fluorescent protein genes

Research using fluorescent proteins is mainly focused on tumor, nerve and stem cells (Jung et al., 2001). DNA concentration, lipofectine concentration, the DNA incubation time and lipofectine combination, and the presence of serum, can all affect transfection efficiency, which is identical in research on Vero cells, HeLa cells and some other cell lines (Tseng et al., 1999; Escriou et al., 2001; Rong et al., 2006). In our experiment, the highest transfer efficiency of the six fluorescent proteins was 31.6% with an optimized plasmid-lipofactamine ratio. The numbers of fluorescent cells decreased at 1 week, but a few dispersed positive cells remained after 2 weeks and even after 1 and 2 months. By screening G418 resistance and monoclonic culture for 1 month, we obtained three positive cell strains that expressed EGFP, EYFP and DsRed stably.

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