Establishment and Biological Characteristic Research of Yuxizhiwei sheep Fibroblast Cell Bank

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Abstract: An ear marginal fibroblast cell bank was established from the Yuxizhiwei sheep which is an excellent Chinese livestock breed using attachment culture and freezing biotechniques. This bank included 30 ear samples and had stocks of 136 cryogenically preserved vials, each containing 1.7×10^7 cells. Establishment and biological characteristic research results of the cell bank showed that the cells revealed typical fibroblast morphology and grew well in vitro, the growth curve consisted of latent phase, logarithmic growth phase and stationary phase, the cell population doubling time (PDT) was 24 hours, and there was no microbe contamination (bacteria, epiphyte, virus or mycoplasma) in the culture. In addition, lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) zymography indicated that this cell bank was free of cross-contamination. We also determined that the diploid rate of the cell bank was 90.2%~91.4% and measured the expression ratios of three fluorescent protein genes which were between 11.3% and 30.2%. The quality identity of this cell bank thus satisfied the standards of the American Type Culture Collection (ATCC). This study has not only opened up new ways to conserve genetic resources of important and endangered animal breeds in the form of somatic cells, but also provided valuable experimental materials for cell biology, medicine, genomics, post-genomics, embryo engineering and so on.
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1. Introduction

China is one of the countries which has the world's most abundant resources of livestock and poultry breeds. For thousands of years, the working people have carefully selected, cultivated many excellent and distinctive local varieties of livestock and poultry. In recent years, with the social and economic development, China imported a large number of foreign varieties with local livestock and poultry species hybridization to improve production, while this also making a serious damage to the local genetic resources of our country. Some species are endangered, some have been extinct, and some reduced in number of individuals in varying degrees. Almost 596 livestock and poultry breeds are prevalent in China, of which 17 have become deracinated and 336 are under different levels of threat (Zhou et al., 2004). Therefore, the conservation of animal genetic resources and species diversity is one of the themes of today's biological researches.

At present, there are several conventional preservation methods, such as in vivo preservation of semen, embryos, genomic library, cDNA library and so on. For a long time live animal preservation is the main method to conserve genetic resources of domestic animals among the many strategies (Wu., 1999). Most cell banks emphasize conservation and uti-lization of animal resources,in particular animal generative cells and embryos (Ho et al., 1997; Simon, 1999; Park et al., 2009). Nevertheless, establishment of somatic cell banks using in vitro culture and low temperature biological techniques is a new effective approach to conserve and maintain the diversity of domestic animals (Shi, 1989). The development of somatic cell cloning techniques is one effective way to preserve animal genetic materials (Hong et al., 2005; Yuna et al., 2008). More and more articles were publicated on the development of fibroblast cell lines from various animals, including the Debao pony (Zhou et al., 2004), Beijing fatty chicken (Zhou et al., 2005), sheep (Chen et al., 2006), Taihu pig (Zhang et al., 2008), Luxi cattle (Liu et al.2008), Texel sheep (Li et al., 2009a) and Silkie Bantam (Li et al., 2009b).

Yuxizhiwei sheep is a Mongolian sheep originated from Central Asia and the Far East, and the breed is formed through a long-term domestication selection by Henan people. It is a good local breed of Henan Province. The breed has plump naked body, tender meat, evenly distributed fat, high production rate and early sexual maturity. Yuxizhiwei sheep is strongly resistant to crude feed, disease and heat, especially it is with strong climbing ability and suitable for mountain grazing. In this study we have established fibroblast cell bank of Yuxizhiwei sheep and studied on its biological characteristics with the aim of long-term preservation of somatic cells of this breed to provide valuable

material for further researches in cellular engineering, molecular biology and embryo engineering.

2. Material and Methods

2.1 Cell cultures

Ear tissue samples (about 1 cm^2 in size) were sampled from 30 individuals of Yuxizhiwei sheep and collected into separate tubes containing Dulbecco's modified Eagle media (DMEM) medium with Ampicillin (100U/ml) and Streptomycin (100μg/ml). Then the tissue samples were rinsed and chopped finely into pieces about 1 mm^3 in size. Afterwards the tissue pieces were seeded on bottom surface of a tissue culture flask and incubated invertedly at 37 ℃, 5% CO_2 and saturated humidity for 2-3 h, until the tissue pieces adhered spontaneously to the surface, the flask was then turned over and added DMEM medium containing 10% fetal bovine serum (FBS) to proceed the primary culture in a 37℃ incubator with 5% CO_2 (Guan et al., 2005). Cells were harvested when they reached 80-90% confluence using trypsinization, and split into prepared culture flasks under the ratio 1:2 or 1:3 (Freshney, 2000).

2.2 Cryogenic preservation and recovery

Cells in logarithmic growth phase were enumerated with a hemocytometer, and checked cell viability by trypan blue staining before freezing. The cells were next centrifuged to form a pellet at 1000 rpm for 8 min, the supernatant was removed. Harvested cells were re-suspended in freezing media (10% Dimethyl sulfoxide (DMSO)+50% FBS+40% DMEM) to reach a final cell density of 3-4×10^6 viable cells/ml. Cells were allocated 1ml each into sterile plastic cryogenic vials labeled with animal name, gender, freezing serial number, and the date. The vials were sealed and placed in 4℃ freezer for 20-30 min in order to let DMSO penetrate into cells adequately and after program freezing transferred to liquid nitrogen storage system quickly and efficiently （Werners et al., 2004; Ren et al.,2002）

To recover and reseed the cells, the frozen tubes were removed from liquid nitrogen and quickly thawed in a 42℃ water bath, and then the cells were transferred into a flask with complete medium. Cells were cultured at 37℃ with 5% CO_2 and the medium was renewed 24 h later.

2.3 Growth curve and Estimation of cell viability by Trypan Blue dye

According to Gu et al (2006) and Kong et al (2007) method, cells at the concentration of 1.5×10^4 cells/ml were seeded into 24 well plate. Monitor and record cell growth and density data per day until cells reached the plateau phase, and for each time point counting was carried out in three wells to get an average. Cell growth curve was then plotted and the Population doubling time (PDT) was calculated based on the curve. Cell viability before freezing and after recovery was determined using a hemocytometer to enumerate 1000 cells by Trypan Blue staining exclusion method (Butler., 1999).

2.4 Chromosome analysis

Chromosome fixation preparation and chromosome staining were performed following standard methods (Suemori et al., 2006). One hundred well-spread metaphases were prepared. The chromosome number per spread was counted under oil immersion objective after Giemsa staining. Relative length, arm ratio and centromeric index and type were calculated according to the protocols of Sun et al (2006) and Kawarai et al (2006).

2.5 Isoenzyme analysis

The electrophoretic mobilities of Lactate dehydrogenase (LDH) and Malate dehydrogenase (MDH) were determined using polyacrylamide gel. Electrophoresis protocol contributed by Marvin L. Macy at American Type Culture Collection (ATCC). Electrophoric mobility was defined by number and intensity of enzyme bands, as well as the distance of band migration from the point of origin for each sample (Freshney, 2000).

2.6 The measurement of microorganism

Detection of bacteria and fungus: The detailed procedure used for bacteria and fungus contamination test was referred to Doyle et al (1990).

Detection of viruses: Routine examination for cytopathogenic effects was performed using phase-contrast microscopy following Hay's haemadsorption protocol (Hay, 1992).

Detection of mycoplasmas: Cells were cultured in media free of antibiotics for at least one week, then fixed and stained with Hoechst 33258 according to DNA fluorescent staining of Freshney (2000). The ELISA Mycoplasma Detection kit (Roche, Lewes, East Sussex, UK) was used to confirm the results of DNA fluorescent staining.

2.7 Expression of three fluorescent protein genes in Yuxizhiwei sheep fibroblastic cells

To obtain the highest transfection efficiency and low cytotoxicity, transfection conditions were optimized by varying cell density as well as plasmid DNA (BD Bioscieces Clontech product) and Lipofectamine 2000 (Invitrogen) concentrations, according to lipofectamine media methods of Escriou et al (2001) and Tsuchiya et al (2002). The cells were observed 24 h, 48 h and 72 h after transfection under

excitation wavelength of 405 nm, 488 nm and 543 nm separately.

3. Results

3.1 Morphology of Yuxizhiwei sheep fibroblast cells

It could be observed fibroblastic-like or epithelial-like cells migrated from tissue pieces 5-12 d after explanted (Figure 1A). With the cultural time extended, cells then continued to proliferate and were subcultured when they reached 80~90% confluence (Figure 1B). After subculture, the fibroblast cells grew rapidly, outgrew and excluded other cells like epithelial cells gradually (Ren et al., 2002). After about 2-3 passages, we could gain the purified fibroblast cells (Figure 1C,D). The motility rate of fibroblast cells of Yuxizhiwei sheep before freezing and after recovery measured by trypan blue staining were 99.0% and 98.7%.

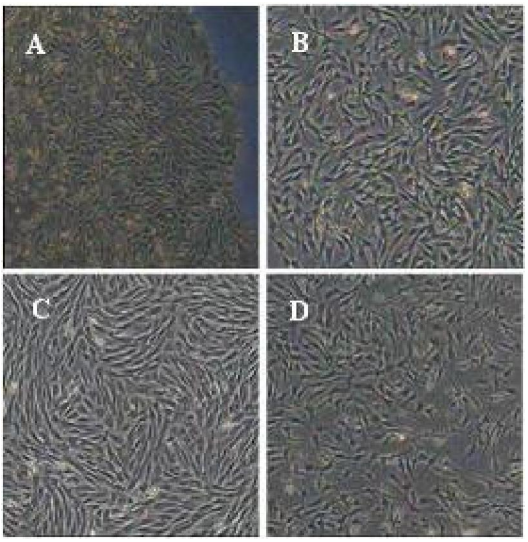

Figure 1. Fibroblast Cells of Yuxizhiwei sheep ear marginal explants. A: Primary Cells; B: Subcultured fibroblast cells; C: Fibroblasts cells before freezing; D: Fibroblasts cells after recovery.

3.2 Growth curve

The growth curve of Yuxizhiwei sheep ear marginal tissue fibroblast cells showed obvious "S" shape (Figure 2) and the PDT of the cells was about 24 h. There was a lag time or laterncy phase about 24 h after cells were seeded, corresponding to the adaptation of cells after recovery and repairment of the trypsin damage, then the cells proliferated rapidly and entered exponential phase. With the cell density accreted, cells growth was influenced by contact inhibition from the sixth day when the cells entered the plateau phase, and cells begun to degenerate.

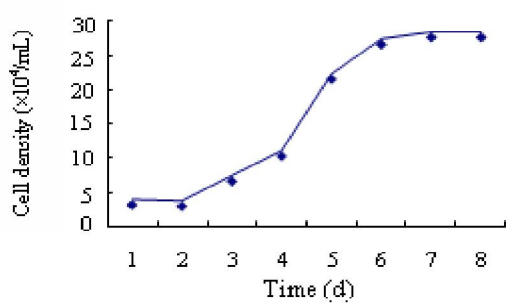

Figure 2. The growth curve of Yuxizhiwei sheep fibroblast cells

3.3 Karyogram and the chromosome number of Yuxizhiwei sheep

The chromosome number of Yuxizhiwei sheep was 2n=54, 52 autosomes and two sex chromosomes XY or XX (Figure 3). Somatic chromosomes No.1-3 were submetacentric chromosomes, No.4-26 and two sex chromosomes (X X) were acrocentric autosomes (Table 1). Chromosome numbers aberration rate showed increasing tendency following the increasing of passages, which indicated culture in vitro affected the heritage of cells slightly, supporting that the cell line was a steady diploid one.

3.4 Isoenzymes analysis of Yuxizhiwei sheep cell line

The distribution patterns of isoenzyme polymorphisms may be characteristic of a species or a tissue. Polymorphism analysis of isoenzymes is currently the standard method for the quality control of cell line identification and detection of interspecies contamination. Isoenzyme patterns for LDH and MDH in Yuxizhiwei sheep fibroblasts were obtained and compared with those from Texel sheep, Mongolian sheep, Angus bovine. Patterns of LDH were shown in figure 4A and revealed clear band differences.

For different livestock species, the same livestock species and different breeds, there are less band differences on the LDH isozymogram. These results show that there was no cross-contamination of the Yuxi Zhiwei sheep fibroblasts from different cell lines established in our laboratory at the same time. The order of LDH activity from low to high is LDH5＜LDH1＜LDH2＜LDH4＜LDH3.

The MDH patterns were shown in figure 4B for Black Grey goat， Black goat, Angora goat, Suffolk sheep, Texel sheep, and Yuxizhiwei sheep ear tissue fibroblasts. All the six domestic animals had two bands, and there was a single m-MDH band near the cathode and a single s-MDH bands near the anode, m-MDH had weaker activity. These results showed that there was no cross-contamination between different breeds.

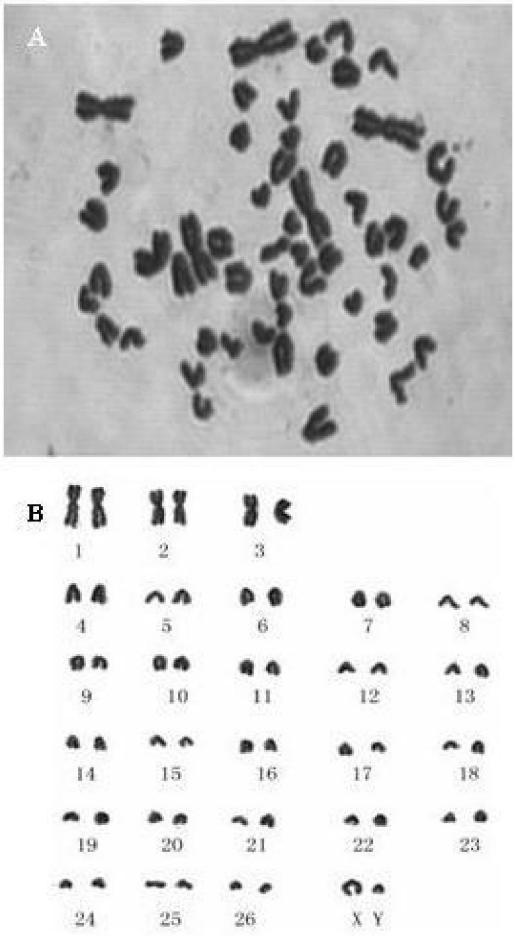

Figure 3. Chromosome metaphase and karyotype of Yuxizhiwei sheep (♂). A: Chromosome metaphase; B: chromosome karyotype

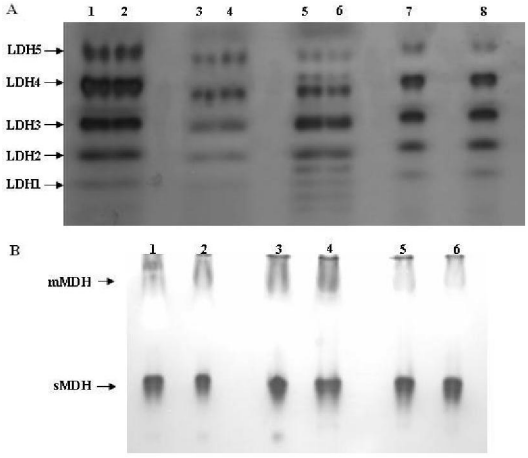

Figure 4. Lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) zymotype for different subspecies. A: 1and 2 Texel sheep, 3and 4 Mongolian sheep, 5and 6 Yuxizhiwei sheep, 7and 8 Angus bovine. B: 1 Black grey Goat, 2 Black Goat, 3 Angora, 4 Suffolksheep, 5 Texel Sheep, 6 Yuxizhiwei sheep.

Table 1. Chromosome characters of Yuxizhiwei sheep (♂)

No.	Relative length（%）	Type
1	10.34±0.12	SM
2	9.03±0.14	SM
3	8.54±0.14	SM
4	5.34±0.12	T
5	412±0.20	T
6	4.25±0.25	T
7	4.21±0.14	T
8	3.42±0.02	T
9	3.23±0.14	T
10	3.32±0.15	T
11	3.34±0.31	T
12	3.17±0.12	T
13	2.95±0.21	T
14	2.71±0.14	T
15	2.78±0.15	T
16	2.68±0.13	T
17	2.51±0.42	T
18	2.49±0.10	T
19	2.43±0.01	T
20	2.33±0.05	T
21	2.14±0.31	T
22	2.13±0.12	T
23	1.95±0.21	T
24	1.71±0.14	T
25	1.78±0.15	T
26	1.68±0.13	T
X	5.01±0.42	T
Y	0.94±0.10	M

Note: M Metacetric chromosome;
SM Submetacentric chromosome;
ST Subtelocentric chromosome;
T Telocentric chromosome.

3.5 Microbial analysis

All results of bacteria, fungi and yeast contamination assays were negative, there were no microorganisms observed in culture media. No presence of viruses was indicated either by the cyto-pathogenic effect examination or by the haemadsorption test. DNA fluorescent staining by Hoechst33258 was most effective and frequently used mothod to detect mycoplasma contamination. Under fluorescent microscope after stained by Hoechst 33258, nuclear of the fibroblast appeared blue elliptic, which showed the established cell line was mycoplasma negative (Figure 5).

3.6 Transfection results of three fluorescent protein genes in Yuxizhiwei sheep fibroblastic cells

Under defined excitation wavelengths (pEGFP-N3, 488 nm; pDsRed1-N1, 543 nm; pEYFP-N1, 495 nm), the expression of pEGFP-N3,

pDsRed1-N1 and pEYFP-N1 was observed at 24 h, 48 h, and 72 h after transfection using laser confocal microscope. The result indicated that all the three fluorescent proteins were expressed in positive cells, and the maximum fluorescence intensity and transfection efficiencies of the exogenous genes appeared at 48 h after transfection. The expression efficiencies of the three fluorescent protein genes at 24 h, 48 h, and 72 h after transfection were all between 11.3% and 30.2% (Table 2).

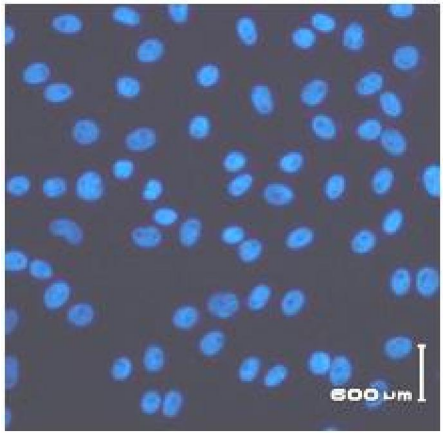

Figure 5. Mycoplasma negative Yuxizhiwei sheep fibroblasts stained by Hoechst 33258

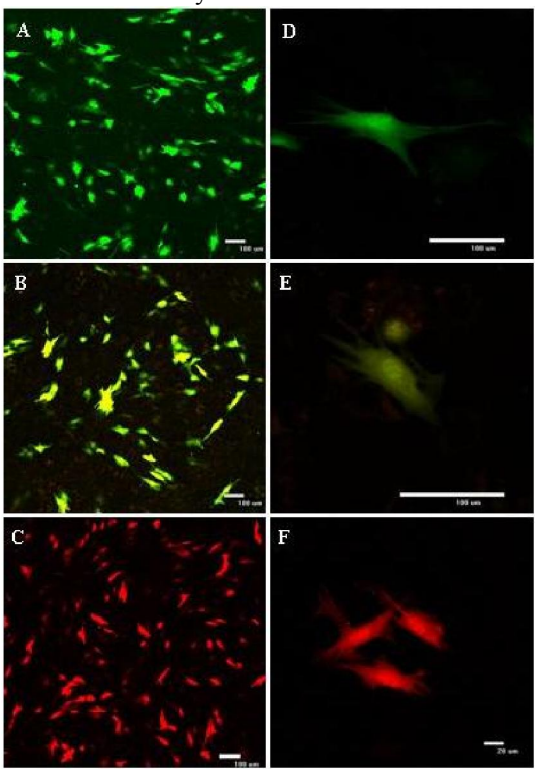

Figure 6. Comparative figures of pEGFP-N3,pEYFP-N1 and pDsRed1-N1 expression in Yuxi Zhiwei sheep fibroblasts. A and D pEGFP-N3, 48 h; B and E pEYFP-N1, 48 h; C and F pDsRed1-N1, 48 h.

At 24h and 48h after transferring, the 3 fluorescences could be observed in cytoplasm and nucleus well-distributed except cryptomere vesicle. At 72h after transferring, pEGFP-N3, pDsRed1-N1 and pEYFP-N1 gene still expressed steadily in cytoplasm and nucleus, which were nearly unchanged, but some cells morphous were irregular and semilism (Figure 6).

Table 2. Transfection efficiency for three fluorescent proteins.

Transfection time (h)	pEGFP-N3 (%)	pEYFP-N1 (%)	pDsRed1-N1 (%)
24	13.6	11.3	17.8
48	30.2	16.8	20.3
72	27.5	15.6	18.8

4. Discussions

4.1 Establishment of Yuxizhiwei sheep fibroblast cell line

We established the Yuxizhiwei sheep ear marginal tissue fibroblast cell line (LXCEM 2/2) using adherent culture method. All measured results indicated that the newly established cell line was stable and grew rather rapidly, and the identification for this cell line conformd to the requirement of quality control of ATCC. So we can conserve the Yuxizhiwei sheep fibroblast cell line by freezing the cells in liquid nitrogen for long-term conservation. In order to ensure the motility rate of cells after recovery, freezing should be keep within 5 generations, cell density should exceed 3×106/ml with cell morphous showing typical fibroblast. Because too many passages and trypsinization affect the biological characters of cells, especially the hereditary characters. Procedures we used in this study conformed to the protocals of ATCC technique 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 we added the transfection of 3 fluorescent protein genes.

4.2 karyotype analysis

In this experiment, the chromosome number of the Yuxizhiwei sheep was found to be 54, with 52 autosomes and 2 sex chromosomes. Because we want to conserve the genomic characteristics of the Yuxizhiwei sheep, the in vitro established fibroblast cell line must maintain diplont characteristics the same as in vivo. The result showed that the proportion of normal diploid cells was above 90%, indicating that the Yuxizhiwei sheep fibroblasts we cultured were stable diploid. Chromosome analysis relates to the gender of the animal from which cells derived. It is also an index to distinguish normal and

malignant cells. In experiment operation it is important to determine the proper time point at which to add colchicine, generally when 70%-90% of the cells are on dividing. It is also important to determine the hypotonic time, which is generally controlled within 25-40 minutes. We achieved the best results when the cells were subjected to colchicine at a final concentration of 0.1μg/ml for 1-2 h.

4.3 Isoenzymes analysis

Isoenzymes show polymorphisms between different species, races, individuals and tissues, and intercellular pollution can be detected by isoenzyme analysis when 10% of cells are polluted. ATCC use isoenzyme polymorphism analysis as a regular method to detect intercellular pollution.

LDH and MDH are very important enzymes participating in the two energy metabolic pathways of glycolytic cycle and citric acid cycle in the bodies of animals, and with different constancy and specificity in different species. LDH is a tetetrametric molecule consisted by the H and M subunits which separately are the ldha and ldhb genes expressed products, and each tissue has a characteristic composition of isoenzymes in a species-dependent manner. In our current experiment, the LDH isoenzyme pattern of Yuxizhiwei sheep fibroblasts showed that LDH-2, LDH-3, and LDH-4 were dominant.

MDH is a dimer enzyme and composed of cytosolic MDH(s-MDH) and mitochondria MDH (m-MDH). The MDH band mobilities among livestock were essential identical, and the same among poultry.

4.4 Mycoplasma detection

A pure cell culture can easily become contaminated with bacteria or fungi. Air, equipment, serum, tissue samples and handling errors can all be sources of such contamination. If contaminated by germ, eumycete and mycetes, the cell media could be turbid and observed by naked eye. Viruses could be observed under microscope. But mycoplasma contamination couldn't be found by naked eye and microscope.

The mycoplasmas have no nucleus and could grow and reproduce in media. It is hardly to be removed and could coexist with cells for long time. Thus it is more difficult to be found than germ, eumycete, mycetes and virus. The methods for mycoplasma detection include direct solid agar culture in microbiology, indirect DNA fluorescent staining and new DNA probe hybridization. Because DNA fluorescent staining is simple and quick, it was commonly used by some cell culture collection institutions like ATCC. Our measurement results of microbiological detections showed that the Yuxi Zhiwei sheep fibroblast cell bank was purified and free from microbial contamination.

4.5 Expression of fluorescent protein genes

The researches about fluorescent protein gene transfection are mainly focused on tumor cells, nerve cells and stem cells (Jung et al., 2001). DNA concentration, lipofectine concentration, the incubate time of the DNA and lipofectine combinant, and serum all can affect the transfection efficiency which is identical with the researches on Vero cell, Hela cell and some other cell lines (Tseng et al., 1999; Rui et al., 2006). In our experiment, the transfection efficiency could reach 30.6% with optimized ratio of plasmid and lipofactamine. The numbers of fluorescent cells reduced at 1 week, however, there remained a few dispersed positive cells after 2 weeks even 1 or 2 months. These fibroblasts can be widely used as tools for investigating the functions of exogenous genes.

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Abbreviations:

ATCC, American Type Culture Collection; DMEM, Dulbecco's modified Eagle media; DMSO, Dimethyl sulfoxide; ELISA, Enzyme-linked immunosorbent assay; FBS, Fetal bovine serum; LDH, Lactate dehydrogenase; MDH, Malate dehydrogenase; PAGE, Polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PDT, Population doubling time; RF, Relative mobility front.

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