

Establishment and Biological characterization of a Large Yorkshire Pig fibroblast line

[Geniş yapılı Yorkshire domuz ırkına ait fibroblast hattının kurulması ve biyolojik karakterizasyonu]*

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ABSTRACT

Aim: This study aimed to establish the fibroblast cell bank of the Large Yorkshire pig in the form of somatic cells, and to study its biological characteristics, to explore long-term conservation and sustainable use of this genetic resource.

Material and methods: Ear marginal tissue of the Large Yorkshire pig was used to establish a fibroblast cell line by direct culture of explants and cryopreservation techniques, and the quality of the cell line was assessed.

Results: Biological analyses showed that the cells were morphologically homogenous fibroblasts, and the estimated population doubling time (PDT) was 72 hours. The growth curve appeared as a typical "S" shape as the cell growth passed through a latent phase, a logarithmic phase and a plateau phase. Tests for bacteria, fungi, viruses and *Mycoplasma* were negative. Analysis of lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) isoenzymes ruled out cross-contamination between cell lines. Karyotyping and G-banding indicated a total chromosome number of $2n = 36$; the proportion of diploid cells in the population was 91–96%. The transfection efficiencies of pEGFP-N3, pEYFP-N1 and pDsRed1-N1 were between 5.14 and 12.13%. Bioindicators of the cell bank met all the standards of the American Type Culture Collection (ATCC).

Conclusion: This study not only shows the effective preservation of the Large Yorkshire pig germplasm at the cellular level, but also provides a valuable experimental resource for fields including cell biology, medicine, genomics, post-genomics, genetic engineering and embryo engineering.

Key Words: Large Yorkshire Pig, fFibroblast line; biological characterization

Conflict of Interest: We declare that there is no conflict of interest.

ÖZET

Amaç: Çalışmada geniş yapılı Yorkshire domuzlarından somatik hücre formunda fibroblast hücre bankasının kurulması, biyolojik karakterizasyonu, uzun dönem saklanması ve genetik kaynak olarak sürdürülebilirliği araştırılmıştır.

Gereç ve Yöntem: Fibroblast hücre hattının kurulmasında kulak kenar dokusu doğrudan kültürü yapılarak kullanılmış, dondurularak saklanma yöntemleri ve hücre hattının kalitesi değerlendirilmiştir.

Bulgular: Biyolojik analizler hücrelerin morfolojik olarak homojen fibroblastlar olduğunu göstermektedir. Populasyonun tahmini ikiye katlanma süresi (PDT) 72 saat olarak bulunmuştur. Büyüme eğrisi latent faz, logaritmik faz ve plato fazını kapsayan "S" şeklindedir. Bakteri, mantar, virus ve *Myoplasma* testleri negatiftir. Laktat dehidrogenaz (LDH) ve malat dehidrogenaz (MDH) analizleri hücre hatları arasındaki kontaminasyon olasılığını ortadan kaldırmaktadır. Karyotipleme G-bantlama, toplam kromozom sayısının $2n = 36$ olduğunu, populasyondaki diploid hücre oranının % 91-96 olduğunu göstermektedir. pEGFP-N3, pEYFP-N1 ve pDsRed1-N1'nin transfeksiyon verimleri % 5.14 ile % 12.13 arasında değişmektedir. Hücre bankasının biyobelirteçleri Amerikan Tipi Kültür Koleksiyonu (ATCC)'nin bütün standartlarını karşılamaktadır.

Sonuç: Çalışma sadece geniş yapılı Yorkshire domuzlarının üreme hücrelerinin hücresel düzeyde etkili bir biçimde saklanması göstermesi bakımından değil, aynı zamanda hücre biyolojisi, tıp, genomics, post-genomics, genetic mühendislik ve embriyo mühendisliği gibi alanlar için deneysel kaynak sağlaması bakımından önemlidir.

Anahtar Kelimeler: Geniş yapılı Yorkshire domuzu, fibroblast hattı, biyolojik karakterizasyon

Çıkar Çatışması: Çıkar çatışması bulunmamaktadır.

Introduction

Animal Genetic Resources are vital resources that constitute an important component of biological and genetic diversity. Livestock and poultry genetic resource conservation is an important area of research, as it is essential for the sustainable development of animal husbandry and biodiversity. The United States Department of Agriculture has emphasized the protection of animal genetic resources, launching projects for the effective use and preservation of national animal genetic resources at the beginning of this century [1]. Major culture collections around the world include the American Type Culture Collection (ATCC), the European Collection of Animal Cell Culture (ECACC) and the German Collection of Microorganisms and Cell Culture (DSMZ) [2]. Animal genetic resource conservation in China has currently fallen behind the United States and Europe. However, because of strong support from the government, it is developing rapidly, with the establishment of specialized conservation farms and a number of gene banks containing national genetic resources, for living animal reserves, semen and embryo preservation, and tissue preservation.

Currently there are several ways to conserve animal genetic resources, including living animal reserves, semen preservation, embryo preservation, and genomic library and cDNA library preservation. With the development of somatic cell cloning technology, animal somatic cells are becoming of interest as a supplement for the preservation of animal genetic resources [3]. Therefore, building a cell bank of important livestock and poultry species can preserve and make use of their genetic resources effectively and permanently at the cellular level. This can also provide valuable experimental material for life science research on the cell biology, genomics, post-genomics and embryo engineering of livestock and poultry species [4]. Currently the cell bank of breeds such as the white ear lobe chicken, Luxi cattle, Simmental cattle, Landrace pigs, and the Qingyuan partridge chicken [5-9] have been established, but the available cell bank of the Large Yorkshire pig has not yet been established in China.

The Large Yorkshire pig, also known as the Large White, originated in the English county of Yorkshire and is one of the world-famous lean meat breeds. Its appearance is characterized by a large physique, high weight by stature, erect ears, straight nose, slightly bowed back, long limbs, and white hair all over the body, and in livestock production this breed shows fast growth velocity, high feed use, good farrowing performance and a high carcass lean meat percentage. The Large Yorkshire pig was introduced to China in the early twentieth century and shows good adaptability after many years of domestication. In China, the Large Yorkshire pig is mainly used as the male parent in a two-way cross, a three-way cross or a multiple cross, with a local sow as

the female parent. The hybrid progenies show obvious hybrid vigor in terms of daily weight gain and feed use, as well as in reproductive ability, giving good economic value [10]. In this study we establish the fibroblast cell bank of the Large Yorkshire pig in the form of somatic cells, and study its biological characteristics, to explore long-term conservation and sustainable use of this genetic resource. This study has laid the foundation for genetic resource preservation of other breeds.

Materials and Methods

Cell culture

Ear marginal tissue samples were taken from 30 Large Yorkshire pigs (10 males and 20 females) that were provided by the Pig Breeding Farm of The Chinese Academy of Agricultural Sciences, Beijing, PR China. The samples were collected into separate tubes which contained DMEM (Gibco, Carlsbad, CA, USA) supplemented with ampicillin (100 U/ml) and streptomycin (100 µg/ml) (Solarbio, China, Beijing). The samples were then rinsed and chopped into 1-mm³ pieces, which were seeded onto the surface of a tissue culture flask containing 90% DMEM and 10% fetal bovine serum (FBS) in a incubator at 37°C with 5% CO₂ [11-13]. When the cells reached 80–90% confluency, they were harvested and divided into prepared culture flasks at 1:2 or 1:3 ratios [11].

Cryopreservation and recovery

The cells were taken in the logarithmic growth phase and counted with a hemocytometer, and their viability was checked using the CellTiter-Blue Cell Viability Assay (Promega, Madison, WI, USA) before freezing. The harvested cells were resuspended in freezing medium containing 40% DMEM, 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) and 50% FBS, to a final concentration between 3×10⁶ and 4×10⁶ viable cells/ml. Single cells were dispensed into sterile plastic cryovials labeled with species, gender, freezing serial number and date. The sealed cryovials were initially kept at 4°C for 20–30 min to allow the DMSO to equilibrate. Then, the cryovials were transferred to a commercially available freezing kit (Nalgene, USA), stored at –80°C overnight (a process in which the temperature decreased at a rate of 1°C/min), and subsequently transferred to liquid nitrogen [12]. To recover the cells, the cryovials were removed from liquid nitrogen and immediately thawed in a 42°C water bath. They were then subsequently transferred with a straw to a culture flask containing 90% DMEM and 10% FBS. The suspension was mixed gently with a pipette to distribute the cells, and then cultured at 37°C with 5% CO₂. The medium was refreshed after 24 h.

Growth dynamics and viabilities

Cells were seeded into 24-well plates at 1.5×10⁴ cells/ml

and cultured for 7 d, following Gu and Kong's method [13-14]. Cell growth and density data were monitored daily by counting three wells every 24 h, until the cells reached the plateau phase. A cell growth curve was then plotted, and the population doubling time (PDT) was calculated from the curve. Cell viabilities, both before freezing and after recovery, were determined using a hemocytometer to enumerate 1000 cells by Butler's dye exclusion method [15].

Microorganism contamination detection

Detection of bacteria and fungi

Cells were cultured in a medium free of antibiotics. Bacteria and fungi contamination was assessed within three days. The detailed procedure used for the contamination test was that described by Doyle [16].

Detection of Mycoplasma

Cells were stained with Hoechst 33258 (ATCC), according to the methods of Freshney and Masover & Becker [11,17], for fluorescent staining of deoxyribonucleic acid (DNA). An ELISA Mycoplasma Detection kit (Roche, Lewes, UK) was used to confirm the results of the DNA fluorescence staining.

Virus Detection

Under normal culture conditions, the cells were selected randomly for cytopathogenic examination using phase-contrast microscopy following Hay's hemadsorption protocol [18].

Karyotype analysis

Chromosomes were prepared, fixed and stained following standard methods [19]. After Giemsa staining, the chromosome numbers per spread were counted by oil immersion objective. Relative length, arm ratio, centromeric index and type were counted according to published protocols [20-21].

Isoenzyme analysis

Isoenzyme patterns of lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) were identified by non-continuous native polyacrylamide (Sigma-Aldrich, USA) gel electrophoresis (PAGE). The stacking gels were at an acrylamide concentration of 5% w/v, and the separating gel was at an acrylamide concentration of 13% w/v. The cells were harvested, and protein extraction solution (0.9% Triton X-100, 0.06 mmol NaCl:EDTA in a mass ratio of 1:15; pH 7.0) was added to cells at a density of 5×10^7 cells/ml. The suspension was centrifuged and stored in aliquots at -70°C . Equal volumes of native gel sample loading buffer (Solarbio) were added to the samples. The gel buffer was prepared which using Tris-citric acid buffer at two different buffer: 0.078 mol/L Tris-citric acid (stacking gels; pH 6.8), and 0.017 mol/L Tris-citric acid (separating gel; pH 8.9). The PAGE apparatus was assembled and run, the electrophoretic

buffer was changed to Tris-glycin (pH 8.7).

The buffered staining mixture was 0.1 M Tris-HCl (pH 8.0) and 100 mM DL-lactate lithium salt (for LDH staining), or 50 mM L-malic acid (for MDH staining). Gel were stained for LDH and MDH by incubation in the dark in 100 ml of the appropriate buffered staining mixture containing 10 mg nicotinamide adenine dinucleotide, 10 mg 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide and 5 mg phenazine methosulfate. The gel was soaked in an enzymatic activity staining solution to locate isoenzyme subtypes by producing a water-insoluble blue color.

Expression of three fluorescent proteins in Large Yorkshire pig fibroblasts

Following the Lipofectamine medium methods of Escriou and Tsuchiya [22-23], the cultured cells were transfected with the plasmid DNAs (Clontech) for three fluorescent proteins (pEGFP-N3, pEYFP-N1 and pDsRed1-N1), and with Lipofectamine 2000 (Invitrogen, USA), at an optimal concentration of 2 μg of plasmid DNA and 6 μl of Lipofectamine 2000. Six hours later, the medium was refreshed. The transfected cells were observed at 24 h, 48 h, 72 h, 96 h, 1 week, 2 weeks and 1 month after transfection for expression of the pEGFP-N3, pEYFP-N1 and pDsRed1-N1 genes using excitation wavelengths of 405, 488 and 543 nm, respectively. For each experimental group, images were captured from ten visual fields, and confocal microscopy was used to measure the total and positive cell counts in each field to determine the transfection efficiencies

Results

Morphology of cultured cells

Fibroblast-like or epithelial-like cells could be seen migrating from the tissue pieces 5–12 days after explanting (Figure 1A). When the cultured cells reached 90% confluence they were subcultured (Figure 1B). After subculturing, the fibroblasts grew rapidly, and gradually outgrew and excluded other cells such as epithelial cells, this result was similar to Zhou's reported [24]. After 3–4 passages, we obtained purified fibroblasts (Figures 1C-D). The viabilities of Large Yorkshire pig fibroblasts before freezing and after recovery, measured by Trypan Blue staining, were 98.3 and 92.5%, respectively.

Growth dynamics

The growth curve of ear marginal tissue fibroblasts of the Large Yorkshire pig had an obvious "S" shape (Figure 2A) and the PDT was about 48 h. There was a lag time or latency phase of about 48 h after seeding, corresponding to the adaptation and recovery of the cells from protease damage, after which the cells proliferated rapidly and entered exponential phase. As the cell density increased, cell proliferation was inhibited by contact inhibition. After the sixth day, the cells no longer proliferated and

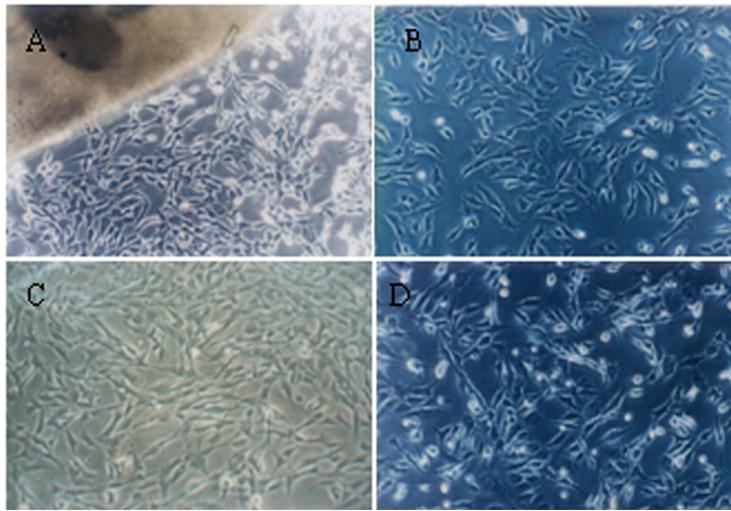


Figure 1. Culture of Large Yorkshire pig fibroblasts. (A) Primary cells of Large Yorkshire ear marginal explants (100×); (B) Subcultured fibroblasts (100×); (C) Fibroblast cells before freezing (100×); (D) Fibroblast cells 24 h after recovery (100×)

entered into plateau phase. They subsequently began to degenerate and entered into a decline phase.

Microbial detection

All the results of *Streptococcus*, fungal and yeast contamination assays were negative; no microorganisms were observed in the culture media by high power microscopy. No viruses were indicated by cytopathogenic evidence or by the hemadsorption test. After staining with Hoechst 33258, the most effective and frequently used method for detecting *Mycoplasma* contamination [19], fibroblast nuclei appeared as blue ellipses, showing that the established cell line was *Mycoplasma* negative (Figure 2B). All these results showed that the cultured cells were not subject to microbial contamination.

Karyotype analysis

The chromosome number of Large Yorkshire pig fibroblasts was $2n = 36$, comprising 34 autosomes and two sex chromosomes, XY or XX (Figure 2D). The parameters, including relative length and centromere morphology are shown in 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 $2n = 36$ were 96, 94 and 91%, respectively.

Isoenzyme analysis of Large Yorkshire pig fibroblasts

Isoenzyme polymorphism occurs among species, and sometimes even among breeds, individuals and tissues within a species [25]. Polymorphism analysis

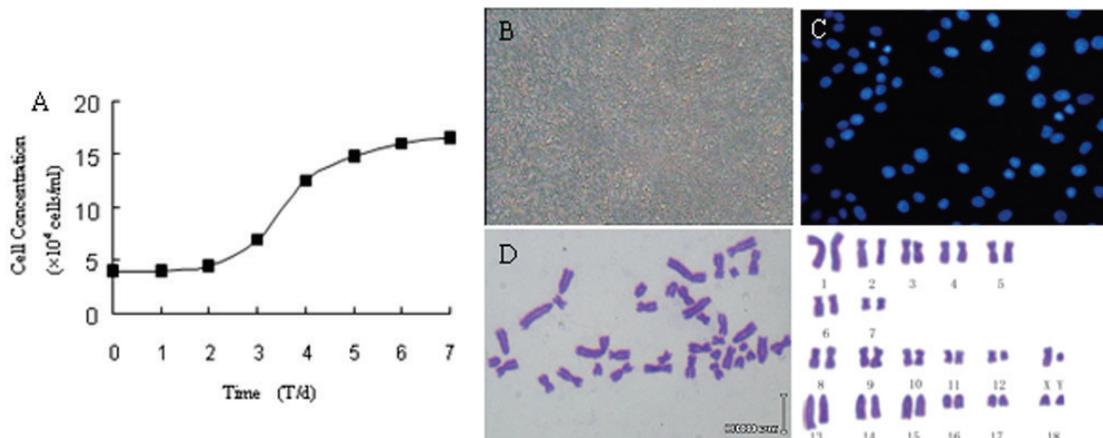


Figure 2. Growth curve, detection of microbial contamination and karyotype of Large Yorkshire pig fibroblasts. (A) Growth curve of Large Yorkshire pig fibroblasts, with cell density on the vertical axis. The curve appeared as a typical “S” shape, including latency phase, exponential growth phase and plateau phase; (B) Negative result of *Streptococcus* detection for Large Yorkshire pig fibroblasts (100×); (C) Negative result of *Mycoplasma* detection for Large Yorkshire pig fibroblasts (200×); (D) Chromosomes at metaphase (left) and karyotype (right) of Large Yorkshire pig fibroblast line (1000×). The chromosome number of Large Yorkshire pig fibroblasts was $2n = 36$, comprising 34 autosomes and two sex chromosomes, whilst the sex chromosome type was XY (♂).

Table 1. Chromosome parameters of the Large Yorkshire

Chromosome number	Relative length (%)	Centromere morphology (%)	Chromosome number	Relative length (%)	Centromere morphology (%)
1	10.32±0.21	SM	11	4.09±0.33	M
2	7.70±0.18	SM	12	1.93±0.25	M
3	6.22±0.24	SM	13	8.29±0.51	T
4	6.03±0.15	SM	14	6.63±0.22	T
5	5.43±0.29	SM	15	6.08±0.11	T
6	5.00±0.36	ST	16	3.56±0.21	T
7	2.86±0.51	ST	17	3.18±0.22	T
8	5.75±0.42	M	18	2.50±0.26	T
9	5.17±0.36	M	X	5.05±0.18	M
10	4.23±0.47	M	Y	2.28±0.15	M

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.

of isoenzymes is currently the standard method for the quality control of cell line identification and detection of interspecies contamination. The protocol for native polyacrylamide gel electrophoresis was optimized, and the mobility of the isoenzymes of LDH and MDH were successfully determined. Five isoenzyme bands (LDH-1, -2, -3, -4 and -5) were observed (Figure 3A), and the LDH patterns from the Large Yorkshire pig were compared with those from Zhiwei goat and Mongolian horse. The results suggested that the LDH banding patterns are species-specific, and there was no cross-contamination among them. According to the enzymatic activity, the order of LDH from Large Yorkshire pig was LDH3, LDH4, LDH2, LDH1 and LDH5, whilst the LDH order from Zhiwei goat is LDH2, LDH1, LDH4, LDH3 and LDH5. The LDH1 band of Mongolian horse was barely observable. The MDH banding patterns from the Large Yorkshire pig were compared to Hu sheep, Gan Jia sheep, Mongolian horse, Xinihe horse. Two MDH isoenzyme bands (s-MDH and m-MDH) were observed in the pattern of Luxi cattle (Figure 3B). There were significant differences in the isoenzyme patterns of LDH and MDH between the above of species and other cell lines in our laboratory.

Expression of fluorescent proteins in the Large Yorkshire pig fibroblasts

The three fluorescent proteins pEGFP-N3, pEYFP-N1 and pDsRed1-N1 were all highly expressed at the optimized conditions (Figures 4A–C). Positive cells were first observed 12 h after transfection, and the number

and intensity of positive cells increased markedly to a maximum value at 48 h. The highest expression was seen for pEGFP-N3. The expression efficiencies of the three fluorescent proteins at 24, 48 and 72 h after transfection are shown in Table 2.

Confocal fluorescence microscopy was used to observe the distribution of the three fluorescent molecules in Large Yorkshire pig fibroblasts to determine the subcellular localization of the three fluorescent proteins. Fluorescence could be observed throughout the cytoplasm and nuclei of control cells, except in the cytoplasmic vesicles. DsRed was mostly shown in the cytoplasm surrounding the nuclear membrane, and formed a red ring profile, whereas EGFP and EYFP showed an intense nuclear signal (Figures 4D–F). The number of fluorescent cells decreased at one week after transfection, but some scattered positive cells remained at two weeks. The transfection efficiencies are shown at the last line of the Table 2.

Discussion

In this study, Large Yorkshire pig ear marginal tissues were used as experimental materials following an adherent culture method, and a fibroblast cell line was successfully established that meets all the criteria of the ATCC. The results show that the newly established line is stable and proliferates rapidly. The genetic resource of Large Yorkshire pigs can be conserved for a long time by freezing fibroblasts in liquid nitrogen, thus achieving the protection of the breed.

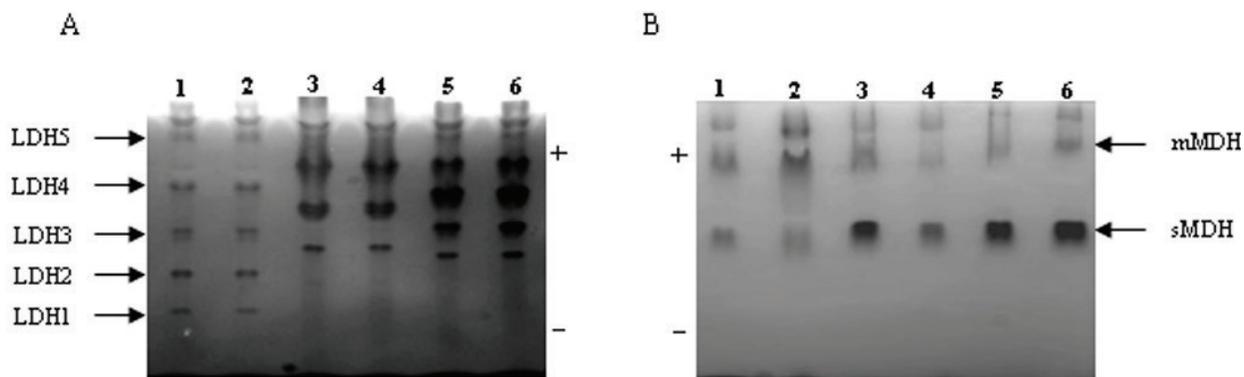


Figure 3. Lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) zymotypes for different breeds. Different subtypes of LDHs and MDHs were distinguished according to differential relative molecular weight and relative mobilities. Different subtypes of LDHs and MDHs were distinguished according to differential relative molecular weight and relative mobilities. Panel A, 1 and 2, Zhiwei goat; 3 and 4, Mongolian horse; 5 and 6, Large Yorkshire. Panel B, 1, Large Yorkshire; 2, Landrace; 3, Hu sheep; 4, Gan Jia sheep; 5, Mongolian horse; 6, Xinihe horse. (non-continuous native polyacrylamide gel, stacking gels = 5%, separating gel = 13%, staining by development process).

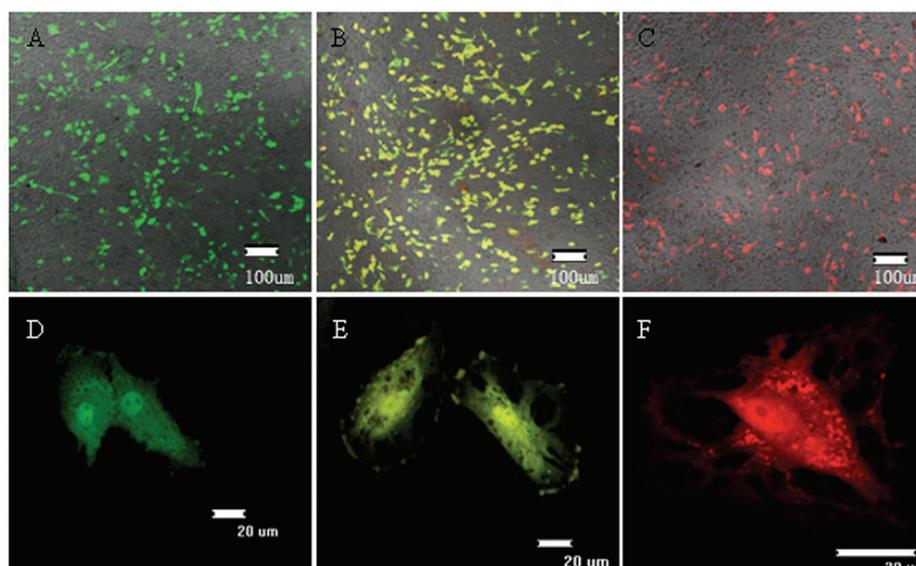


Figure 4. Expression and distribution of pEGFP-N3, pEYFP-N1 and pDsRed1-N1 in Large Yorkshire pig fibroblasts. A, B and C show the transfection results of pEGFP-N3, pEYFP-N1 and pDsRed1-N1, respectively, at 48 h after transfection; D, E and F show the subcellular location of pEGFP-N3, pEYFP-N1 and pDsRed1-N1, respectively, at 48 h after transfection.

Table 2. Transfection efficiencies of three fluorescent proteins

Time/h	Three fluorescent plasmids		
	pEGFP-N3 (%)	pEYFP-N1 (%)	pDsRed1-N1 (%)
24	8.11±0.35	9.25±0.27	5.41±0.18
48	10.36±0.16	12.13±0.72	8.43±0.44
72	9.45±0.51	10.23±0.94	6.58±0.39
336	20.71±0.31	21.52±0.44	12.01±0.11

Microbial contamination is one of the most common phenomena in cell culture resulting in experimental failure. Potential sources of contamination include air, equipment, serum, and tissue samples [26]. Whilst contamination by bacteria, eumycetes, and mycetes can be detected by the naked eye because of the turbidity of the culture media, it is harder to detect *Mycoplasma* contamination. *Mycoplasma* have no nuclei and can grow and reproduce in cell culture media. They are hard to eliminate and can coexist with cultured cells for long periods of time. The methods 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 a ready-to-use technique, it is commonly used by some cell culture collections such as the ATCC. Our microbiological detection results showed that the Large Yorkshire pig fibroblast bank was purified and free of *Mycoplasma* contamination. The *Streptococcus* contamination-detection results showed that the Large Yorkshire pig fibroblast bank was also free of *Streptococcus*.

Karyotype analysis is commonly used for detecting abnormal cells in a population because the chromosome number, shape and structure of normal cells remain very stable. Aberrations in chromosome numbers showed a tendency to increase with passage number, indicating that *in vitro* culture can have a slight effect on the stability of the cells, but suggesting that the cell line was reproducibly diploid. In addition, there were no chromosomal abnormalities, further indicating that the cell lines were stably diploid.

LDH and MDH are pivotal enzymes involved in the glycolytic pathway and citric acid cycle, respectively. They are species-specific and evolutionarily conserved, but the subtypes and activities differ among species, providing a biochemical indicator of species classification by chromatography and electrophoresis. LDH and MDH were therefore selected to determine the species origin of the cells and to detect cross-contamination [27-28]. Biochemical analysis of isoenzyme polymorphism is currently considered the standard method for quality control of cell line identification and interspecies contamination, as demonstrated when Arai *et al* measured the LDH isoenzyme patterns in horse leucocytes and plasma, and in Debaio pony fibroblasts [29]. MDH is a dimeric enzyme composed of cytosolic (s-MDH) and mitochondrial (m-MDH) subunits. The mobilities of MDH bands among poultry are essentially identical, and the same is true among those from livestock. However, MDH from livestock migrates more rapidly than that from poultry, and livestock contains a higher concentration of MDH.

Research using fluorescent proteins mainly focuses on tumor, nerve and stem cells [30]. It has been shown in Vero cells, HeLa cells and other cell lines that transfection efficiencies can be affected by DNA

concentration, Lipofectamine concentration, incubation time, reagent combinations, and the presence of serum [30]. This study has shown the expression of fluorescent proteins in Large Yorkshire pig fibroblasts with no obvious effect on the growth and proliferation of the transfected cells. This indicates that fluorescent protein genes can be safely used as a marker of foreign genes in transgenic animals.

In summary, the Large Yorkshire pig fibroblast bank has been successfully established, containing biologically normal and genetically stable fibroblasts. All aspects of the bank have met the standards for cell line quality supervision of major international culture collections and centers. Results from exogenous gene expression support the vitality of the cells, and their applications in transgenic therapy and genetics. The precious genetic resource of the Large Yorkshire pig has been effectively preserved at a cellular level. This bank can serve as a resource providing biological materials for further experimental work in fields including genetics, biomedical sciences, cell and molecular biology, and immunology.

Acknowledgements

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