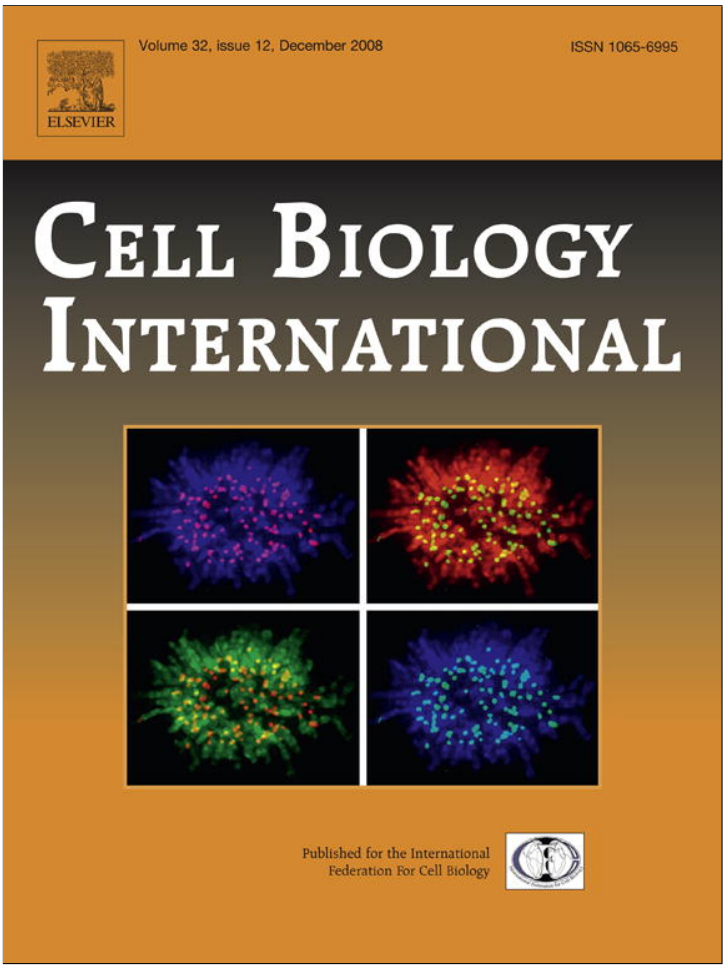

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Establishment and characteristics of white ear lobe chicken embryo fibroblast line and expression of six fluorescent proteins in the cells

Hongmei Wu, Weijun Guan, Han Li, Yuehui Ma*

Institute of Animal Science, Chinese Academy of Agricultural Science, Beijing 100094, PR China

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Abstract

A white ear lobe chicken embryo (WELCE) fibroblast cell bank, containing 322 tubes of frozen cells, was successfully established from primary explants of 57 embryo samples. The cells were morphologically consistent with fibroblasts, and the growth curve was sigmoidal with a population doubling time (PDT) of 48 h. Karyotyping and G-banding indicated a total chromosome number of $2n = 78$; the rate of diploidy in the cell bank was 97.62%. The cells were also free from bacterial, fungal, viral and mycoplasma contamination. Analysis of lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) isoenzymes ruled out cross-contamination between cells. In order to study exogenous gene expression, six fluorescent proteins were transfected into the WELCE cells. The transfection efficiency of these genes was between 10.1 and 41.9%. The corresponding fluorescence was distributed throughout the cytoplasm and nucleus 24 h after transfection. The results indicate that the quality of the cell line meet the quality requirements of the ATCC (American Type Culture Collection).

Keywords: White ear lobe chicken embryo; Fibroblast bank; Biological characteristics

1. Introduction

Biodiversity is facing unprecedented challenges worldwide and vulnerable animals are threatened by the introduction of foreign species, massive destruction of their natural habitats and industrial pollution, as occurs in China. Although China hosts a great variety of indigenous species, information regarding their molecular biology is very limited. In order to preserve the wealth of biodiversity in China, and to uncover the complex underlying interactions between organisms and environment, there is a very urgent need to commence rigorous conservation of endangered species (Weijun, 2002). Preservation of individual animals, semen, embryos, genomic libraries and cDNA libraries are all practical options. In addition, modern cloning techniques have made somatic cells an attractive resource for conserving animal genetic materials (Changxin, 1999). Establishing fibroblast banks from endangered species has been proposed as a practical approach for this purpose; not only does it preserve precious genetic material, but also it provides an excellent resource for biological research.

White ear lobe chickens are found in Guangfeng, Yushan and Shangrao counties in Jiangxi province of China. They are so called because of their yellow feathers and white ears. The breed is one of the rare poultry breeds in China that are precocious egg producers and has been recognized as a good nutritional source since the Song dynasty (approximately 1300 A.C.) (Guifang and Kuanwei, 2003). It was listed among the 138 nationally protected domestic animals by the Chinese government in 2006. In this study, a fibroblast bank from 57 white ear lobe chicken embryos (WELCE) was established successfully using primary explants. In addition, the feasibility of introducing foreign fluorescent proteins into the WELCE cells was demonstrated.

2. Materials and methods

2.1. Primary cell culture, freeze preservation and recovery

Eight-day-old embryos were isolated from white ear lobe chicken eggs and washed three times with phosphate buffered

* Corresponding author. Tel: +86 10 62813463; fax: +86 10 62895351.
E-mail address: yuehui.ma@263.net (Y. Ma).

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saline (PBS). A 1 cm^3 sample was excised, attached to a flask and cultured at 37 °C in a humidified atmosphere of air containing 5% CO_2 for 4–5 h. Modified Eagle's medium (MEM) (Gibco) containing 10% fetal calf serum (Hyclone) was added with the flask inverted and cultured overnight. After three passages, the cultured cells were frozen in a buffer containing 40% MEM, 50% fetal calf serum and 10% DMSO (Sigma). Logarithmic phase cells at a concentration of 4×10^6/ml were incubated in freezing-resistant tubes in this buffer at 4 °C for 20–30 min to allow time for the DMSO to penetrate, put into a cell freezing system with programmable controlled rate, and finally transferred to liquid nitrogen for long-term storage (Jenkins, 1999). Tubes taken from the liquid nitrogen were allowed to thaw in a 42 °C water bath, then transferred to flasks with MEM (Gibco) containing 10% fetal calf serum (Hyclone) and cultured at 37 °C under a 5% CO_2 atmosphere. The medium was renewed after 24 h.

2.2. Cell viability

Cell survival rates before freezing and after recovery were determined using trypan blue. The cells were seeded in 6-well plates at 10^4/well, and counted with a hemocytometer (Qi et al., 2007).

2.3. Cell growth curve

The growth properties of WELCE cells in vitro were assessed by their population doubling time (PDT). Cells were harvested and seeded in 24-well plate at 1.5×10^4/well, cultured for 7 days and then counted every 24 h. Average values were used to draw a growth curve and calculate the population doubling time (Sun et al., 2006).

2.4. Microorganism detection

Bacterial, fungal and yeast contamination: WELCE cells were cultured in a medium free of antibiotics. Bacterial, fungal and yeast contamination was assessed within 3 days. The detailed procedure used for the contamination test was described by Doyle et al. (1990).

Test for viruses: under normal culture conditions, the cells were selected randomly for cytopathogenic examination using phase-contrast microscopy following Hay's hemadsorption protocol (Hay, 1992).

Mycoplasma contamination: mycoplasma was detected by an ELISA mycoplasma detection kit (Roche) and EZ-PCR mycoplasma test kits (Kibbutz Beit Haemek, Israel). The ELISA mycoplasma detection kit identifies the 4 most common mycoplasma species (*Mycoplasma arginini, Mycoplasma hyorhinis, Acholeplasma laidlawii and Mycoplasma orale); the EZ-PCR mycoplasma test kit uses PCR technology to detect mycoplasma contamination in cell cultures. The primer set is specific to the highly conserved 16S rRNA coding region in the mycoplasma genome and allows various mycoplasma species as well as Acholeplasma and Spiroplasma species to be detected with high sensitivity and specificity; simply, bands of the amplified DNA fragments are identified after gel electrophoresis*.

2.5. Karyotype and banding analysis

The cell chromosomes were prepared using a slight modification of the method of Sun et al. (2006). WELCE cells were grown in the presence of 0.1 μg/ml colcemid (Sigma) for 4 h at 37 °C, then harvested, centrifuged and resuspended in 0.075 M KCl prewarmed to 37 °C. After incubation for 30 min at 37 °C, the cells were pelleted again, fixed with 3:1 methanol:acetic acid at 4 °C and washed three times with the fixative. Finally, the cell suspension was dropped on to chilled glass slides and stained with Giemsa (Amresco). The chromosomes were counted, and the modal WELCE cell karyotype was prepared by the method of Ford et al. (1980) with slight modifications. Ten photographs of metaphase chromosomes were selected. The long and short arms of 10 pairs of macrochromosomes were measured. The parameters of relative length, centromere index and kinetochore type were calculated according to Levan et al. (1964). The slides (3–10 days old) were incubated for 2 h at 70 °C, and GTG-banding (G-bands obtained with trypsin and Giemsa) was achieved by treating for 40 s with fresh 0.01% trypsin, rinsing twice with PBS and staining with Giemsa (pH = 6.8) for 10 min. Chromosomal designation followed the International System guidelines for standardized banded karyotypes of the domestic fowl (*Gallus domesticus*) (Ladjali-Mohammedi et al., 1999).

2.6. Isoenzyme analysis

Isoenzyme patterns of lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) were identified by vertical slab non-continuous polyacrylamide gel electrophoresis (PAGE). The WELCE cells were harvested and protein extraction solution (0.9% Triton X-100, 0.06 mmol NaCl:EDTA in mass ratio 1:15) was added until the cell density reached 5×10^7/ml, then the suspension was centrifuged and stored in aliquots at −70 °C. Equal volumes of 40% sucrose and 2.5 μl loading buffer were added to the sample (Zhongxiao and Shuzheng, 1999). Different mobility patterns were indicated by the relative mobility front (RF), which was calculated as the ratio between the distances of migration of the isozyme band and the bromochlorphenol blue.

2.7. Expression of six fluorescent proteins in WELCE cells

To assess the influence of transfection with foreign genes and to lay the foundations for further research on gene transfer, 2×10^4 WELCE cells were seeded in each well of a 24-well plate and transfected with the plasmid DNAs (Clontech) for six fluorescent proteins (pEGFP-C1, pEGFP-N3, pECFP-N1, pECFP-mito, pEYFP-N1 and pDsRed1-N1) and with Lipofectamine 2000 (Invitrogen), according to the lipofectamine media method of Escriou et al. (2001). The medium was renewed 6 h after transfection, and cells were

examined 24, 48 and 72 h, 1 and 2 weeks after transfection using a Nikon TE-2000-E inverted microscope with excitation wavelengths of 433–588 nm to determine the transfection efficiency and the morphology of the positive transfected cells. For each experiment group, images were captured from 10 visual fields, and confocal fluorescence 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 by the cell motility using trypan blue.

3. Results

3.1. WELCE cell morphology

WELCE cells originating from tissue explant pieces grew rapidly, migrated from the tissues one day after the start of the culture and spread out 2–3 days later. Fibroblasts were initially mingled with epithelial cells (Fig. 1A), but the fibroblasts grew rapidly and replaced the epithelial cells gradually after 2–3 passages, and spread on the culture surface 2–4 days after subculturing (Fig. 1B). The cells had fibrous characteristics with turgor vitalis cytoplasm, and during growth they were morphologically fibroblast-like with radiating, flame-like or whirlpool migrating shapes.

3.2. WELCE cell viability

The average viability of the cells was ~96% before freezing and ~92% after thawing. These results were not significantly different ($P > 0.05$), so the cells were healthy in culture and freezing had little effect on their viability.

3.3. WELCE growth curve

The growth curve appeared sigmoidal (Fig. 2). After a latent period, an exponential growth period and a stationary phase were observed. The exponential growth began 2 days after seeding and gave way to the stationary phase. The population doubling time was estimated at 48 h.

3.4. Microorganisms

All the bacterial, fungal and yeast contamination assay results were negative; no microorganisms were observed in the culture media. No viruses were indicated either by cytopathogenic examination or by the hemadsorption test. Mycoplasma testing by both the ELISA detection kit and the EZ-PCR test kits was negative. Agarose gel electrophoresis revealed an ~270 bp fragment in positive samples (Fig. 3). Mycoplasma contamination is a serious and widespread problem in cell culture. Detection by the direct culture procedure is time-consuming and some mycoplasma species are difficult to cultivate. Amplification of the gene sequence with PCR using this primer set enhances not only the sensitivity but also the specificity of detection.

3.5. Karyotype and banding analysis of WELCE cells

The WELCE cells were diploid ($2n = 78$), containing 10 pairs of macrochromosomes and 29 pairs of microchromosomes. The sex chromosome type is ZZ (♂)/ZW (♀). The parameters of relative length, centromere index and kinetochore type are shown in Table 1. This cell line was subjected to G-banding analysis and showed a highly rearranged karyotype (Fig. 4), which exhibited normal chromosome number and structure. The chromosomes were counted at first and third passage and the results showed that 97.6% of the cells were diploid. These results support the conclusion that the cell line is reproducibly diploid.

3.6. Isoenzyme analysis

Lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) isoenzyme patterns were obtained from WELCE cells and compared with those of Langshan chicken embryo

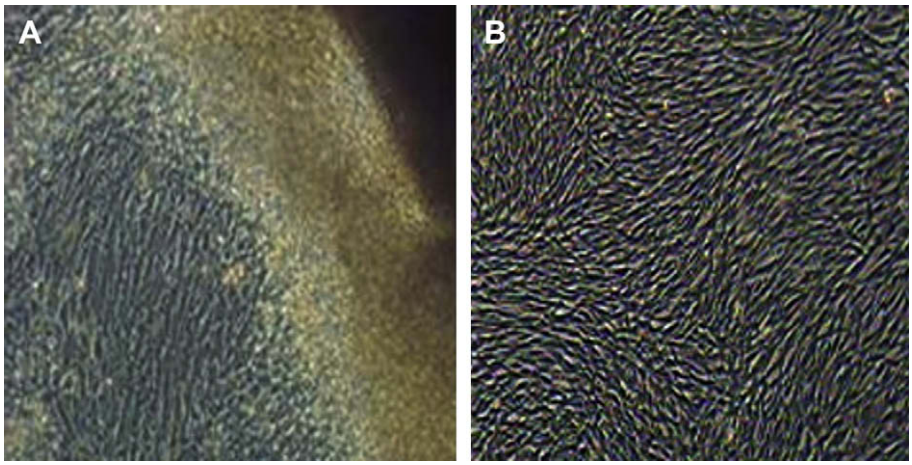

Fig. 1. Morphology of WELCE cells. (A) Primary WELCE cells (×100). Fibroblasts and epithelial cells migrated from the tissue. (B) Fibroblastic cells after two passages (×100). Fibroblasts replaced the epithelial cells in subcultures and spread on the culture surface, forming the fibroblast-like shape.

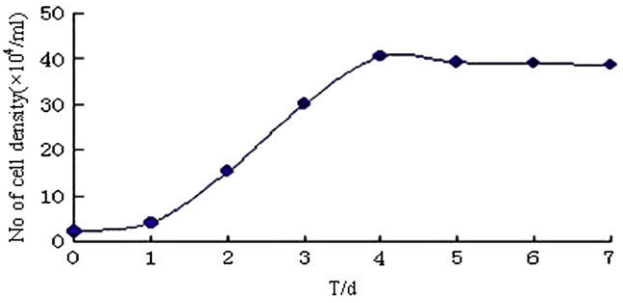

Fig. 2. The growth curve of WELCE cells. Cells were harvested and seeded in 24-well plate at 1.5×10^4/well and cultured for 7 days and then counted every 24 h. Average value represents the mean ± SD of three independent experiments.

fibroblasts. The LDH patterns are shown in Fig. 5. Five bands were observed, corresponding to LDH1, LDH2, LDH3, LDH4 and LDH5 in order from anode to cathode. These bands were very clear and the RF of LDH is given in Table 2. The LDH activities differ in the two lines: LDH2, LDH3, LDH4 and LDH5 predominated while LDH1 was scarcely apparent in white ear lobe chickens. The MDH patterns are shown in Fig. 6: 1 band (m-MDH) near the anode and 1 band (s-MDH) near the cathode. The RF of MDH is shown in Table 3. The different chicken breeds have distinctive band patterns and each band has a different relative mobility. The results show that there was no cross-contamination between the breeds.

3.7. Expression of fluorescent proteins in WELCE cells

Expression of pEGFP-C1, pEGFP-N3, pEYFP-N1, pDsRed1-N1, pECFP-N1 and pECFP-mito in the WELCE cells was observed 24, 48, and 72 h and 1 and 2 weeks after transfection. The results indicated that all six fluorescent proteins were expressed in most positive cells, and the strongest fluorescence intensity and the highest transfection efficiency of the exogenous genes appeared 24 h after transfection (Fig. 7). The transfection efficiencies of the six fluorescent proteins 24, 48 and 72 h after transfer were between 10.1% and 41.9% (Table 4). The highest transfection efficiency

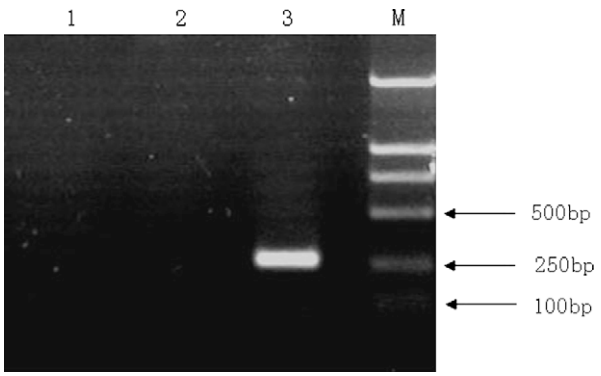

Fig. 3. Mycoplasma detection with EZ-PCR mycoplasma test kits for WELCE cells. WELCE cells were cultured and detected by EZ-PCR mycoplasma test kits. 1, Test sample; 2, negative control; 3, positive control. Mycoplasma testing result was negative.

Table 1
Chromosome's parameters of White ear lobe chicken (♀)

Chromosome	Relative length (%)	Centromere index (%)	Position of centromer
1	23.88 ± 0.170	37.31 ± 0.240	SM
2	20.54 ± 0.164	29.03 ± 0.156	SM
3	11.63 ± 0.096		T
4	10.68 ± 0.088	29.75 ± 0.249	SM
5	5.46 ± 0.036		T
6	3.47 ± 0.022		T
7	3.01 ± 0.020		T
8	4.91 ± 0.033	39.33 ± 3.250	M
Z	13.49 ± 0.099	48.06 ± 0.288	M
W	4.72 ± 0.062	44.44 ± 0.311	M

Ten photographs of metaphase chromosomes were selected of individual, and the long and short chromosome arms in nine pairs of macrochromosomes were measured. The parameters of relative length and centromere index were calculated.

reached 41.9% at 24 h after transfection, which was slightly higher than that of 32.6% in the study of Lei Guo et al. (2007). Confocal fluorescence microscopy was used to observe the distribution of green, yellow and red fluorescence in the WELCE cells to determine the subcellular location of six fluorescent proteins. The results showed that the fluorescence could be observed throughout the cytoplasm and nuclei of control cells except in the cryptomere vesicle. DsRed was mostly shown in cytoplasmic (with a punctuate pattern), whereas EGFP and EYFP showed an intense nuclear signal (Fig. 8). The number of cells expressing fluorescent proteins decreased and the fluorescence intensity gradually faded, disappearing 7 days after transfection, though some cells still expressed fluorescent proteins after 4–5 weeks. The viabilities of cells transfected with pEGFP-C1, pEGFP-N3, pECFP-N1, pECFP-mito, pEYFP-N1 and pDsRed1-N1 were 89.5%, 88.2%, 87.6%, 86.5%, 89.3% and 87.6%, respectively; none of these was significantly different from controls (91.4%, $P > 0.05$).

4. Discussion

4.1. WELCE cell morphology

A WELCE cell bank was successfully established from 57 embryo samples by adherent culture. The biological

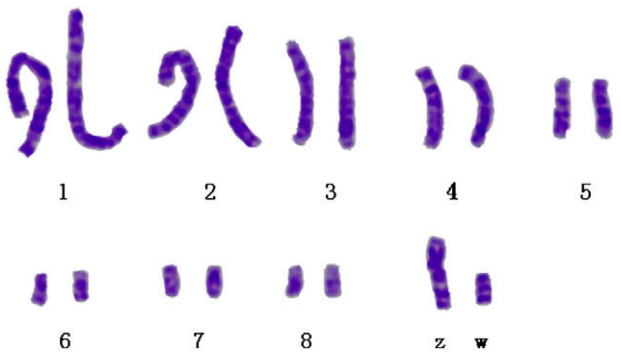

Fig. 4. G-banding karyotyping of cells (♀). G-bands were obtained with trypsin and Giemsa, and exhibited normal chromosome number and structure.

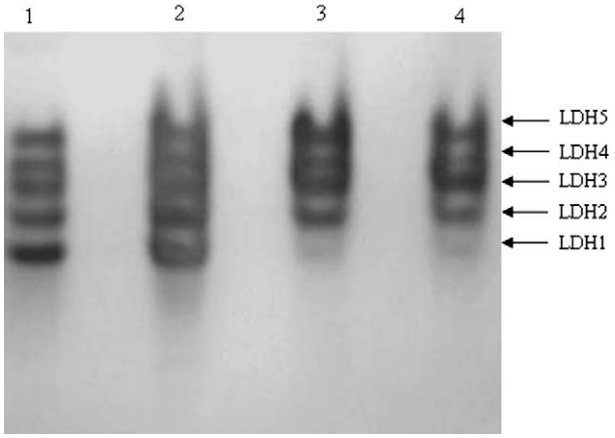

Fig. 5. Isoenzyme patterns of lactate dehydrogenase (LDH) in the WELCE and Langshan chicken fibroblast lines. The WELCE cells were harvested and protein extraction solution was added until the cell density reached to 55×10^7/ml. Equal volume of 40% sucrose and 2.5 ml loading buffer were added to the sample and mixed to do PAGE. 1, 2: White ear lobe chickens; 3, 4: Langshan chicken.

characteristics, especially the genetic characteristics, of the cells may be changed by in vitro culture conditions after many passages, so a minimal number of passages are recommended to conserve them. The WELCE cells were frozen within five passages.

The morphological results indicated that the primary WELCE cells are usually mixed with epithelial cells, which may grow either in groups or scattered. Because epithelial cells and fibroblasts have different tolerances to trypsin, we pay special attention to the digestion time so as to collect the cells needed at the appropriate time. Digestion was terminated with medium containing serum when careful phase-contrast microscopy showed shrinkage of the cytoplasm and increased refraction in most of the fibroblasts. When the wall of the bottle was gently blown with a pipette, the fibroblasts were shed from the wall while the epithelial cells remained attached. Thus, fibroblast suspensions were obtained and inoculated. By these means, a purified fibroblast culture can be obtained after 2–3 passages.

4.2. Chromosome analysis

Cells possess a characteristic chromosome number, shape and structure, which remain very stable in the normal cells. Therefore, karyotype analysis is a major method for distinguishing normal cells from variants. G-banded karyotypes

Table 2
Relative migration distances of LDH

Species	LDH1 (%)	LDH2 (%)	LDH3 (%)	LDH4 (%)	LDH5 (%)
White ear lobe chickens	42.65	38.97	35.29	33.09	30.15
Langshan chicken	43.38	39.71	36.74	33.82	30.88

Different mobility patterns were indicated by the relative mobility front (RF), which was calculated as the ratio between the distances of migration of the isozyme band and the bromochlorphenol blue.

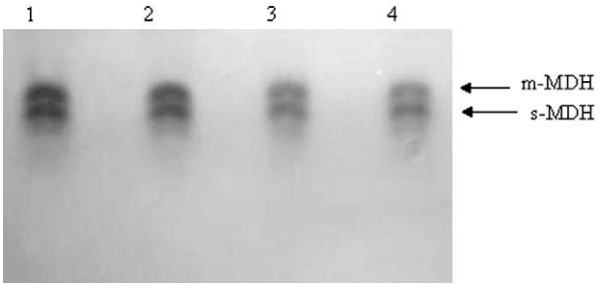

Fig. 6. Isoenzyme patterns of malate dehydrogenase (MDH) in the WELCE and Langshan chicken fibroblast lines. The WELCE cells were harvested and protein extraction solution was added until the cell density reached to 55×10^7/ml. Equal volume of 40% sucrose and 2.5 ml loading buffer were added to the sample and mixed to do PAGE. 1, 2: White ear lobe chickens; 3, 4: Langshan chicken.

were analyzed and no chromosomal aberrations were found; the WELCE cells were all normal diploids. The International Poultry karyotype criterion defines poultry chromatin as comprising eight pairs of macrochromosomes and 30 pairs of microchromosomes with sex chromosomes Z and W, (Ladjali-Mohammedi et al., 1999). Chromosomal diploid numbers range widely in poultry: most species are 78 and 82, and the number of macrochromosomes for *Gallus domestica* is 7.8 ± 0.9, with a range of 6–9, while the microchromosome numbers are 31.9 ± 2.5, with a range of 24–35. The present study conducted on 100 cells shows that the white ear lobe chicken cells are diploid, with 10 pairs of macrochromosomes and 29 pairs of microchromosomes; 97.6% of the cells examined were diploid. Chromosome analysis can relate a cell line to the gender of the animal from which was derived and the result can be compared with that of *Gallus gallus* (Zeng and He, 1986). The diploid chromosome number is $2n = 78$ in both the white ear lobe chicken and *G. gallus* and the chromosomal morphologies are very similar. The G-banding method was used and showed that the centric regions Nos.1 and 2 of the white ear lobe chicken are light banded, whereas the short arm centric region No.1 and the complete centric region No.2 of *G. gallus* are dark banded (Zeng and He, 1986). The G-banding patterns of other breeds are not distinctly different, giving further evidence that *G. domestica* originated from *G. gallus*.

4.3. Isoenzyme analysis

Isoenzyme polymorphism is commonly used to identify cells and their origins (Nims et al., 1998). ATCC consider the

Table 3
Relative migration distance of MDH

Species	m-MDH (%)	s-MDH1 (%)
White ear lobe chickens	20.33	23.58
Langshan chicken	19.20	22.40

Different mobility patterns were indicated by the relative mobility front (RF), which was calculated as the ratio between the distances of migration of the isozyme band and the bromochlorphenol blue.

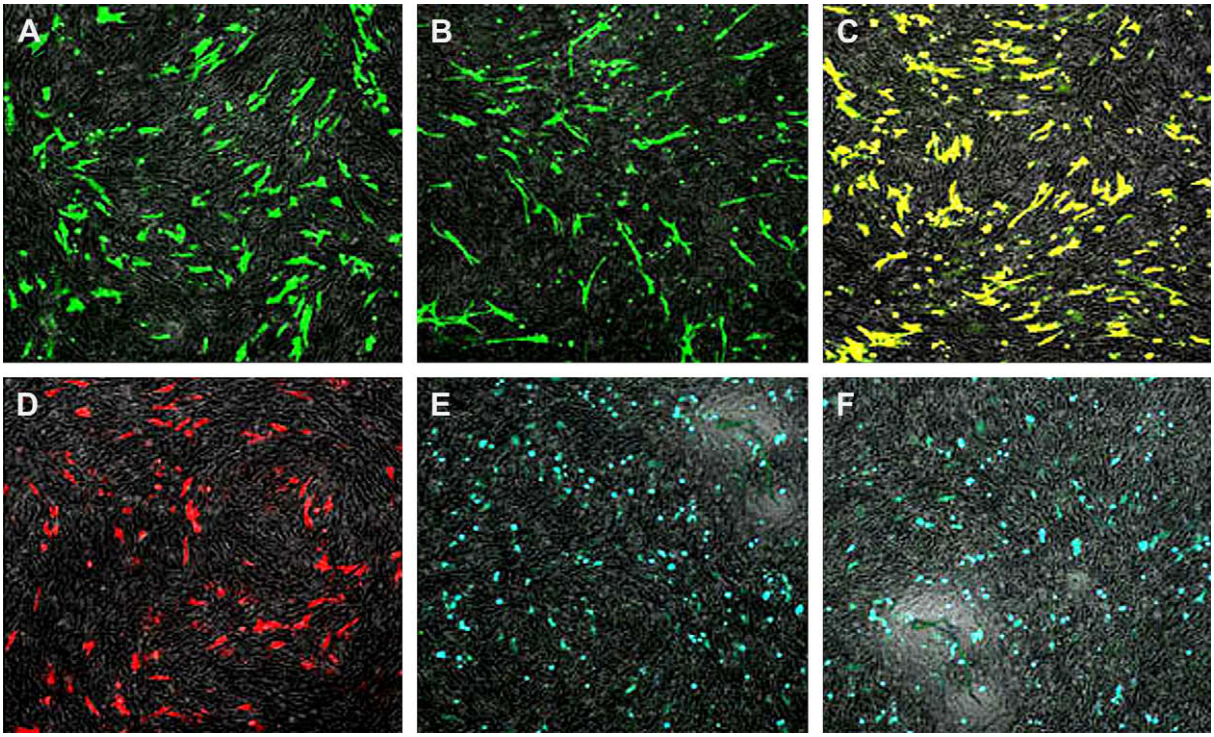

Fig. 7. Comparative figures of six fluorescent proteins at 24 h after transfection using a Nikon TE-2000-E inverted microscope with excitation wavelengths of 433–588 nm to determine the transfection efficiency (×10). A, B, C, D, E and F were the transfection results of pEGFP-C1, pEGFP-N3, pEYFP-N1, pDsRed1-N1, pECFP-N1 and pECFP-mito, respectively.

biochemical analysis of isoenzyme polymorphism to be the regular method for detecting intercellular contamination (Drexler et al., 1999). In the present study we obtained isoenzyme zymograms for LDH and MDH and improved the ATCC starch gel electrophoresis method. Kewen and Fengying (1997) analyzed LDH isoenzymes from eight poultry tissues and obtained 5–8 clear bands. There were five bands in the pectoral muscle. LDH isoenzymes were analyzed in cardiac muscle, liver and blood from Chinese Junglefowl, and all three tissues had five bands (Rusun and Qing, 1997). In accordance with these results, five LDH bands were found white ear lobe chicken: LDH1, LDH2, LDH3, LDH4 and LDH5. MDH in poultry has a cellular solute type (s-MDH) and a mitochondrion type (m-MDH); the shift rate of the former is faster than the latter. The s-MDH bands of 16-day embryos all deviated to the positive electrode; they appeared on day 3 and subsequently increased (Jiangang and Huai Qiu, 1995). The present results show that white ear lobe Chicken has both s-MDH and m-MDH, and the shift rate of the former is faster than the latter, consistent with the MDH isoenzymes during early and middle development (1–16 days) of chicken embryos (Jiangang and Huai Qiu, 1995). Thus the MDH activity in vitro is similar to that in the original tissues. In the present study, the isoenzyme bands of LDH and MDH in WELCE cells were clear, indicating that the genetic characteristics were stable and there was no contamination with other cells.

4.4. Expression of exogenous genes

Six fluorescent proteins with stable structures, high expression levels and species-independent efficiency (Baird et al., 2000) have been used as marker genes to observe the expression, contribution and function of target proteins in live cells and organisms (Heim et al., 1995; Genyang et al., 2003). In this study, positive cells were most abundant and the

Table 4
Transfection efficiencies of six fluorescent proteins in white ear lobe chicken

Time/h	Transfection efficiency of six fluorescent proteins					
	pEGFP-N3 (%)	pEGFP-C1 (%)	pEYFP-N1 (%)	pDsRed1-N1 (%)	pECFP-N1 (%)	pECFP-mito (%)
24	31.8	37.7	41.9	28.3	36.5	26.4
48	28.5	30.6	32.2	19.4	20.3	17.8
72	20.2	24.8	27.7	11.2	13.5	10.1

Cells were examined 24, 48 and 72 h after transfection using a Nikon TE-2000-E inverted microscope with excitation wavelengths of 433–588 nm to determine the transfection efficiency. The number of whole cells and positive cells in 10 visual fields of every well was counted to calculate the transfection efficiency.

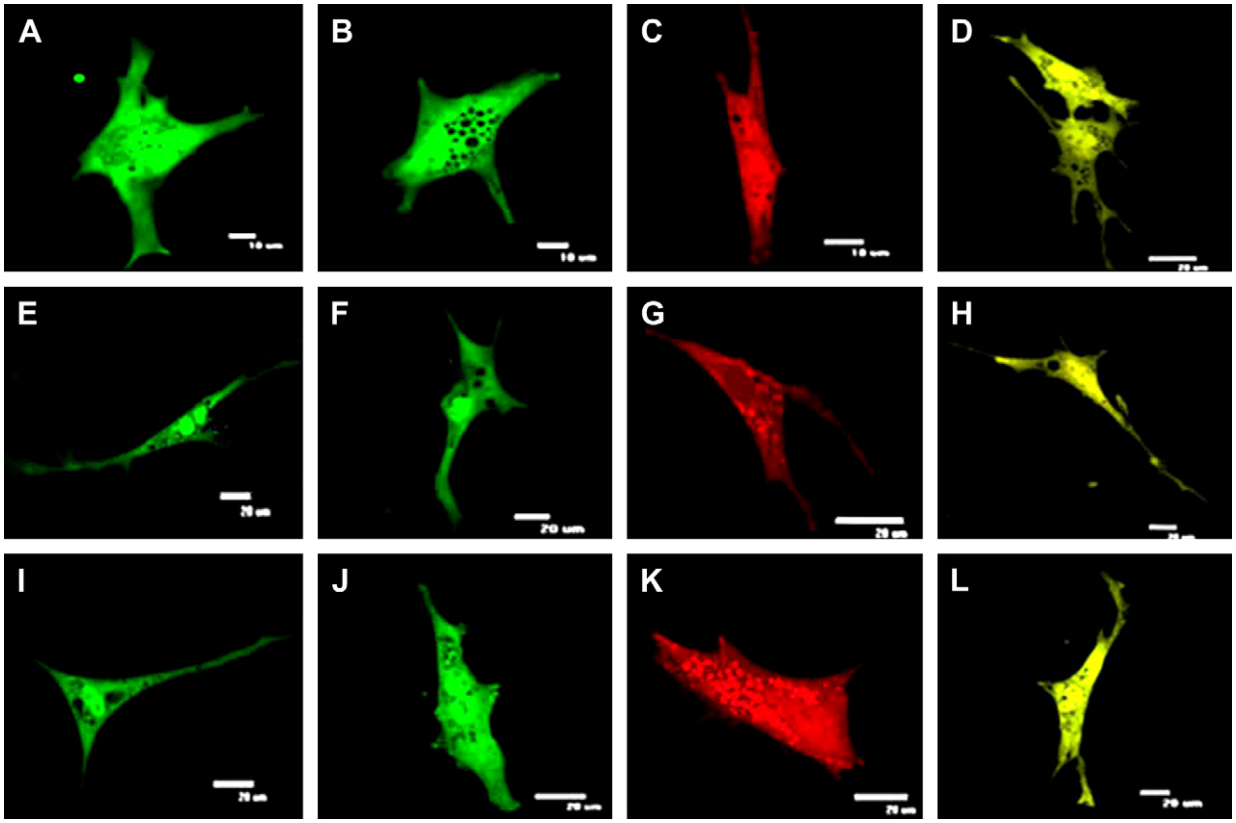

Fig. 8. The expression and distribution of pEGFP-C1, pEGFP-N3, pDsRed1-N1 and pEYFP-N1 in WELCE cell (×40). A, B, C and D were the transfection results of pEGFP-C1, pEGFP-N3, pDsRed1-N1 and pEYFP-N1 at 24 h after transfection; E, F, G and H were the transfection results of pEGFP-C1, pEGFP-N3, pDsRed1-N1 and pEYFP-N1 at 48 h after transfection; I, J, K and L were the transfection results of pEGFP-C1, pEGFP-N3, pDsRed1-N1 and pEYFP-N1 at 72 h after transfection.

fluorescence signal was strong, with the highest transfection efficiency at 24 h after transfection. While the transfection efficiency decreased, strong expression levels were observed after a week, indicating that the exogenous genes in the fibroblasts can be replicated, transcribed, translated and modified after correct translation. The transfected cells were not significantly less viable than the control cells. The results show that fluorescent protein expression had no obvious effect on the growth and proliferation of the transfected cells, which is consistent with the previous findings (Huang and Li, 2001).

Overall, the WELCE cell line established all had characteristics required by the identification criteria of ATCC. The expression of exogenous genes showed that the cells were transfected. The present work not only preserved the germplasm resources of the important white ear lobe chicken at the cell level, but also provided valuable material for genome, post-genome and somatic cell cloning research. The technical platform now established will support the conservation of endangered species and their genetic materials at the cellular level.

Acknowledgements

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