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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 $4 \times 10^6$/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 $1.5 \times 10^4$/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 $5 \times 10^7$/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, $2 \times 10^4$ 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