Establishment and Biological Characterization of Fibroblast Cell Line From the Wenchang Chicken

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Abstract: The fibroblast cell line from the Wenchang chicken was successfully established by the attachment culture method and the cryopreservation biotechniques. Cell morphology, dynamic proliferation and any contamination present were tested, the karyotype, levels of isoenzymes of lactic dehydrogenase and malic dehydrogenase were analysed. Results showed that 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; Isoenzyme analysis confirmed that there was no cross-contamitation in the culture. The three types of fluorescent protein extro-genes appeared to be expressed effectively with high transfection efficiency between 15.6% and 38.6%. The results indicate that the quality of the cell line meet the quality requirements of the ATCC (American Type Culture Collection).
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Keywords: Wenchang chicken; fibroblast; biological characteristics

1. Introduction

Diversity of genetic resources in livestock and poultry forms an important part of overall biological diversity, as well as being the basis for survival and sustainable development of humans. Thus, Livestock and poultry breed resources were an indispensible part of overall biological breed resources and formed a basis for human survival and sustainable development of the society. Preservation of individual species, semen samples, embryos, genomic libraries and cDNA libraries are all practical methods. In addition to these methods, modern somatic cell cloning techniques have made somatic cells an attractive resource in the conservation of animal genetic materials (Changxin, 1999).

The Wenchang chicken, is a well-known member of the world's poultry breeds, It originated from Wenchang territory of Hainan province and was listed as one of the nationally protected domestic animals by the Chinese government in 2006. The breed is used for both meat and egg production, and is celebrated for its Fresh Delicate Succulent and strong fragrance. In the present study, tissues from embryos of Wenchang chickens were collected for fibroblasts culture and thus a fibroblast cell line was developed. The cell line were identified and its characteristics were described to allow for future preservation at the cellular level.

2. Material and Methods

Isolation, primary culture and subculture

The Wenchang chicken eggs incubated for 7 days were cleaned using alcohol cotton for disinfection, and then the embryos were isolated and washed three times with phosphate buffered saline (PBS). After cleaning, the embryos were placed in 60mm Petri dishes and cut into small pieces (1mm3) using ophthalmic scissors. Then sample was excised, attached to a flask and cultured at 37℃in a humidified atmosphere of air containing 5%CO2 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.

The medium was changed after 2-3d. The cells were then harvested at 80-90% confluence. The cell sheet was rinsed twice in phosphate-buffered saline and 0.05% trypsin solution was added. Flasks were inverted and incubated for 3 min at 37℃ before being turned over. Subsequently, flasks were shaken gently to detach cells from their walls and medium was added to the cell suspension to terminate trypsinization. Cells were split into new culture flasks at 1:2 ratio and incubated at 37℃with 5%CO2.

Cryogenic preservation and recovery

After three passages, the cultured cells were frozen in a buffer containing 40% MEM, 50% fetal bovine serum and 10% DMSO. One-millilitre samples of cell suspension were transferred into sterile plastic cryogenic vials labelled with animal name, gender, age, passage number and the date. The sealed vials were then placed into boxes filled with an appropriate amount of isopropyl alcohol, frozen overnight at -80℃ and the cells were transferred into a liquid nitrogen storage system （Werners et al., 2004. Tubes taken from the liquid nitrogen were allowed to thaw in a 42℃ water bath, then transferred to flasks with MEM (Gibco) containing 10% fetal calf serum (Hyclone) and cultured at 37℃ under a 5% CO2 atmosphere. The medium was renewed after 24 h.

Estimation of 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).

Cell growth kinetics

Cells were seeded in 24-well plates at a density of approximately 1.5×105 cells/well and cultured for 7d and then counted every 24 h (3 wells each time). Average values were used to draw a growth curve and calculate the population doubling time (Sun et al., 2006).

Microorganism detection

The Doyle et al. (Doyle et al., 1990)and Freshney(Freshney,1999) method was followed for detection of potential bacterial, fungal and yeast contamination. DNA fluorescence staining was performed using Hoechst 33258 (Sigma, St. Louis, MO, USA) to identify any contamination by mycoplasma. Using the ELISA Mycoplasma Detection Kit (Roche Diagnostics Corp, Indianapolis, IN, USA) the four most common Mycoplasma species (M. arginini, M. hyorhinis, A. laid-lawii and M. orale) was used to confirm results of the DNA staining.

Chromosome analysis

The cells were harvested at 80%–90% confluence. Chromosomes were prepared, fixed, and stained following the standard methods (Suemori et al. 2006). Chromosome numbers were counted for 50–100 individual chromosome spreads. This method focuses on three important parameters: relative chromosome length, arm ratio, and centromere index.

Detection of fluorescent proteins in Wenchang chicken fibroblast cells

Using the method of Tsuchiya et al. (2002), the same quantity of fluorescent protein vectors pEGFP-N3, pDsRed-N1 and pEYFP-N1 were transfected into the Wenchang chicken fibroblast cells with Lipofectamine TM 2000 transfection reagent (Invitrogen Corp, Carlsbad, CA, USA). The cultured cells were observed at 24 h, 48 h, 72 h, 96 h, 1 week, 2 weeks and 1 month after transfection. 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

Morphology of Wenchang chicken fibroblast cells

Two hours after attachment of embryo tissues, several types of epithelial-like and fibroblast-like cells were removed from the tissues. 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 showed typical fibroblast-like morphology as radiating, flame-like or whirlpool migrating shapes (Fig.1B).

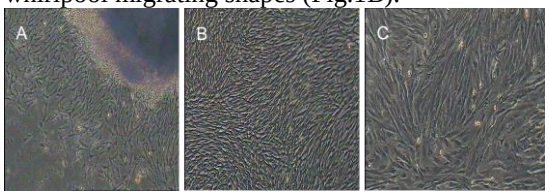

Figure 1. Morphology of the Wenchang chicken fibroblast cells. (A) Primary the Wenchang chicken fibroblast cells (×40). Fibroblasts and epithelial cells migrated from the tissue. (B) Cells before cryopreservation(×40), the cells were healthy and in mitotic phase.(C) Cells after recovery (×40), the cells were cultured for 48 h after thawing.

Growth dynamics

The growth curve appeared sigmoidal, and the population doubling time (PDT) was approximately 48 h. A lag of around 24 h was apparent after the cells were seeded, that was accepted for recovery after possible damage by the protease. After the lag, cells proliferated rapidly and entered the exponential growth phase until they reached a stationary phase after about 6 days.

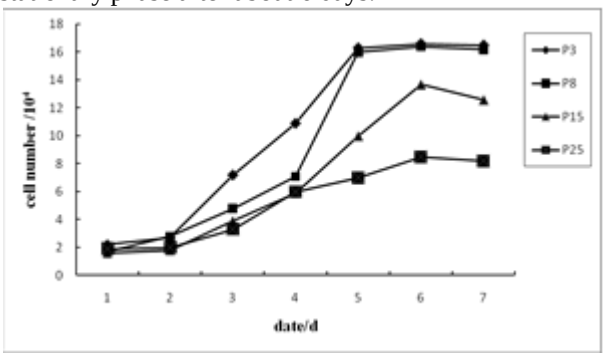

Figure 2. Growth curve of the Wenchang chicken fibroblast cells.

Microorganism detection

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. In our experiment we could see smooth fluorescent nucleoli, and the background was clear under fluorescence microscope. These suggested that the fibroblalsts were free of mycoplasmas. These results indicated that there was no microbial contamination of the fibroblast cell line from the Wenchang chicken culture

Chromosome analysis

The Wenchang chicken fibroblast cells were diploid (2n = 78), containing 10 pairs of

macrochromosomes and 29 pairs of microchromosomes. The sex chromosome type is ZZ (♂)/ZW (♂). This cell line was subjected to G-banding analysis and showed a highly rearranged karyotype (Fig.3), which exhibited normal chromosome number and structure. In vitro culture conditions affected the chromosomal stability to a limited extent. However, it still supported the conclusion that the cell line is reproducibly diploid.

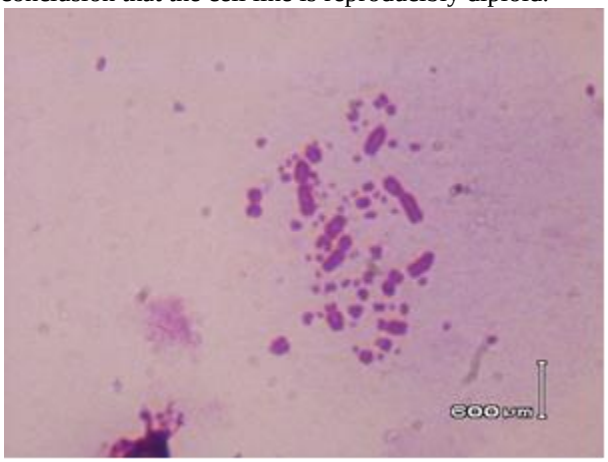

Figure 3. Karyotype of Wenchang chicken embryo fibroblasts

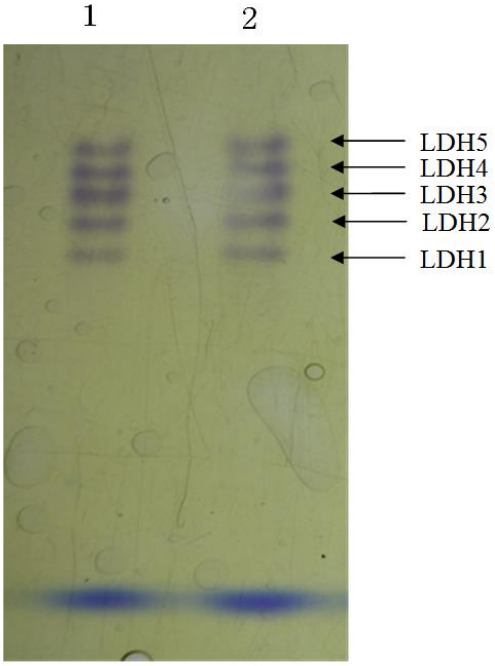

Figure 4. Isoenzyme patterns of LDH in the Wenchang chicken and Qingyuan spotty plumage chicken. Lines 1: Qingyuan spotty plumage chicken; lines 2: Wenchang chicken.

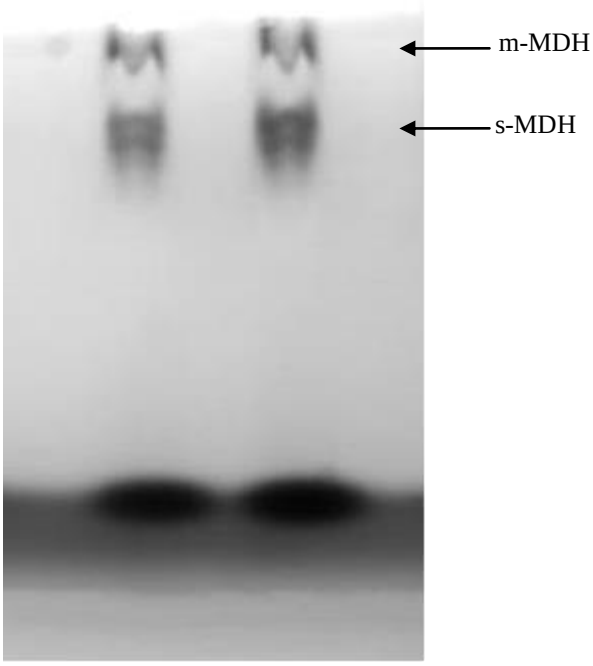

Figure 5. Isoenzyme patterns of MDH in the Wenchang chicken and Qingyuan spotty plumage chicken. Fibroblast lines 1: Qingyuan spotty plumage chicken; lines 2: Wenchang chicken.

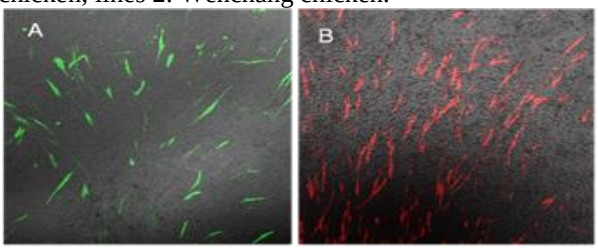

Figure 6. Transfection efficiency of the Wenchang chicken fibroblast cells with pEGFP-N3 and pDsRed1-N1 using Lipofectamine 2000 (× 100). Comparative figures of two fluorescent protein transfection. (A) 48h after transfection of genes for pEGFP-N3; (B) 72h after transfection of genes for pDsRed1-N1.

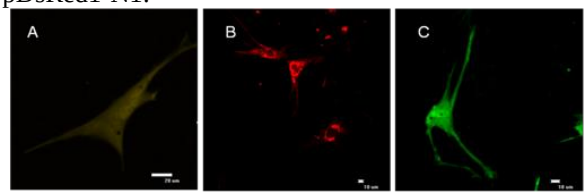

Figure 7. Expression and distribution of pEYFP-N1, pDsRed1-N1 and pEGFP-N3 in the Wenchang chicken fibroblast cell (×40). (A) transfection results of pEYFP-N1 at 24 h after transfection; (B) transfection results of pDsRed1-N1 at 48h after transfection; (C) transfection results of pEGFP-N3 at 72h after transfection.

Isozyme analysis

Pattern of distribution of isoenzyme polymorphisms may be characteristic of a species or tissue (MacLeod et al., 1999). The LDH and MDH bands obtained from Wenchang chicken embryo fibroblasts were compared with those from other species. The LDH patterns are shown in Fig.4. Five bands were observed, corresponding to LDH1, LDH2, LDH3, LDH4 and LDH5 in order from anode to cathode. Two MDH isoenzyme bands were m-MDH and s-MDH (Fig.4). 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.

Expression of fluorescent proteins in Wenchang chicken fibroblast cells

Expression of pEGFP-N3, pDsRed-N1 and pEYFP-N1 in the Wenchang chicken fibroblast cells was observed 24, 48, and 72 h and 1 and 2 weeks after transfection. Results indicated that all three fluorescent proteins were expressed in most positive cells, and the strongest fluorescence intensity and highest transfection efficiency of exogenous genes appeared 48 h after transfection. Confocal fluorescence microscopy was used to observe the distribution of green, yellow and red fluorescence in the Wenchang chicken fibroblast cells to determine the subcellular location of three fluorescent proteins. The results showed that the fluorescence could be observed throughout the cytoplasm and nuclei of control cells except in the cryptomere vesicle. All positive cells appeared shrunken, were shed and disintegrated 24h after transfection. Number of positive cells increased by 48h and there were many non-fluorescent vacuoles in cytoplasm in most of positive cells at 48 and 72 h after transfection (Fig.6). DsRed was mostly shown in cytoplasm, whereas EGFP and EYFP showed intense nuclear signal (Fig.7). 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. Results showed that fluorescent protein expression had no obvious effect on population growth and proliferation of the transfected cells.

4. Discussions

A fibroblast line from embryo samples of the Wenchang chicken were established by using an adherent culture method. Morphological analysis indicated that both epithelial and fibroblast cells were present during primary and early passages. Fibroblasts adhere more easily to flasks and can be trypsinized more readily, whereas epithelial cells do not adhere in a short time and are easily shed using gentle mechanical agitation (Ren,2002).Therefore , fibroblasts will quickly outgrow their epithelial counterparts. The cells were treated with trypsin over 2-3 passages, then a pure fibroblast culture was obtained (Zhou et al., 2005; Li et al., 2003). Average viability after thawing was above 90% indicating that freezing had little influence on viability of the cells. Thus, it seems possible to conserve genomic resources of the Wenchang chicken breed by long-term freezing of their fibroblasts in liquid nitrogen. After freezing and thawing, the cell survival rate decreased to some extent, possibly owing to cell injury that occurred during the process of refrigeration and recovery.

Morphological analysis indicated that both epithelial and fibroblast cells were present during primary and early passages. 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. Fibroblasts detached from flask walls when treated with trypsin but readhered quickly after passaging, while most epithelial cells were unable to adhere, or only did so in an unstable manner and detached again when vibrated(Xue, 2001). For this reason, purified fibroblasts could be obtained after 2–3 passages.

Karyotype analysis is a major method for distinguishing normal cells from variants. 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). Range in frequency of diploid chromosomes varies notably between breeds with most of them having from 78 to 82. Macrochromosomes of Gallus domestics average 7.8 ± 0.9 in number but vary between 6 and 9, while there are 31.9 ± 2.5 microchromosomes with a range from 24 to 35. Most chromosomes of the Wenchang chicken are microchromosomes, and may be lost easily during section preparation and disruption of purity of the chromatin. Thus, it is important to determine time at which colchicines should be added and length of time over which they are active.

Enzyme protein polymorphism, evidenced by the existence of isoenzymes, occurs among species and sometimes among genera, as well as among tissues within an organism (O'Brien et al., 1977). In the present study we have obtained the apparatus and conditions for polyacrylamide gel electrophoresis, and successfully determined the mobility of MDH and LDH which were isolated from the Wenchang chicken embryo fibroblasts cultured in vitro. Kewen and Fengying (1997) analyzed LDH isoenzymes from eight poultry tissues and obtained 5-8 clear bands. In accordance with these results, five LDH bands were found in the Wenchang chicken: LDH1, LDH2, LDH3,

LDH4 and LDH5. MDH in poultry has a cellular solute type (s-MDH) and a mitochondrion type (m-MDH)，and movement rate of the former is faster than that of the latter. These two kinds of MDH isozymes have different electrophoresis mobilities which are obviously divided as two band groups in the common electrophoretogram, that is, the sMDH band group and the mMDH band group.

Fluorescent proteins were rapidly becoming an important reporter molecules for monitoring gene expression and protein localization in vivo, in situ and in real time. DNA concentration, lipofectine concentration, DNA incubation time and lipofectine combination, and the presence of serum can all affect cell transfection efficiency.

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