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Establishment and characterization of a Chinese Game chicken embryo fibroblast bank

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Abstract
For preserving the genetic resources of the Chinese Game chicken and exploring a new method to preserve endangered animals, a Chinese Game chicken embryo fibroblast cell bank was established by the attachment culture method and the cryopreservation biotechniques. This bank includes 43 embryo samples and has stocks of 178 cryovials, with each containing 4.0×10^6 cells respectively. Quality assays and biological characteristics of the bank established showed that the cells cultured in vitro were all typical fibroblast and the cell population doubling time (PDT) was about 2 days. The cell viability before freezing was 97.37% ± 2.25% and 91.43% ± 3.22% after thawing. Tests for microbial contamination by bacteria, fungus, viruses and mycoplasmas were all negative. The frequency of cells having the diploid number of chromosomes (78) was 94.65% ± 3.29%. Isoenzyme analysis confirmed that there was no cross-contamitation in the culture. The fluorescence could be observed well-distributed in cytoplasm and nucleus except for some cryptomere vesicles at 24 h after transfection, and the expression efficiency of pEGFP-C1, pEGFP-N3, pEYFP-N1, pECFP-N1, pECFP-mito and pDsRed1-N1 were all between 9.5%~34.6% at 24 h, 48 h and 72 h after transfection. This research provided both technical and theoretical support for preserving the genetic resources of other animals and poultry at the cell level.

Keywords
Chinese Game chicken fibroblasts; biological characteristics; genetic conservation

Introduction

The genetic diversity of livestock and poultry is an important part of biodiversity, and is the basis for human society to keep living and to achieve sustainable development.

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If these genetic resources have not been preserved in any way before extinction, not only the genetic resources will lost evermore, but also the research on biological mechanisms of various unknown cells and molecules of the extinct livestock and poultry will not be realized as well as the wish to regenerate the cells and molecules through somatic cell cloning. Therefore, there is a very urgent need to commence conservation of endangered species (Guan, 2002). At present, 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 of endangered species has been proposed as a practical approach for this purpose; not only does it preserve precious genetic materials, it also provides an excellent resource for biological research.

Chinese Game chicken has a history of two thousand years, belonging to the fancy chicken breeds, and is famous at home and abroad for cock being good at fighting. And owing to its muscular breast and legs, Chinese Game chicken has been selected to breed meat chicken. In a word, Chinese Game chicken is a precious poultry germplasm resource in China. Furthermore, it was among the 138 nationally protected domestic animals listed by the Chinese government in 2006 (http://www.agri.gov.cn/BLGG/ t20060609_ 626418.htm).

To preserve this valuable genetic resource, in this study we established a fibroblast bank of Chinese Game chicken and identified its biological characteristics. It had preserved the genetic resources at the cellular level. Moreover, it also provided valuable materials for genomic, postgenomic, somatic clone and other research fields of life science.

Materials and methods

Materials

The 43 Chinese Game chicken embryos required in our experiment were provided by the poultry institute of Jiangsu province of China.

Except where otherwise indicated, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Isolation and culture of Chinese Game chicken embryo fibroblasts

All procedures were in accordance with the National Institutes of Health Animal Care and Use Guidelines. The Chinese Game 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 ($1mm^3$) using ophthalmic scissors. These pieces were washed three times in calcium- and magnesium- free PBS (pH 7.4), and then attached to flasks 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 bovine 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 bovine serum and 10% DMSO. Logarithmic phase cells at a concentration of 4.0×10^6/mL were incubated in freezing-resistant tubes in this buffer at 4°C for 20-30 min to allow for the DMSO to penetrate, then put into a cell freezing system with programmable cooling 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 bovine serum and cultured at 37°C under a 5% CO_2 atmosphere. The medium was renewed after 24 h.

Estimation of cell viability by trypan blue staining

Cell viability was determined using trypan blue staining (Xue, 2001; Weingartl et al., 2002). The number of dead cells was determined in a visual field of 1000 cells (Yitao et al., 2007).

Growth curve analysis

Cells were seeded in 24-well plates at a density of approximately 1.5×10^5 cells/well and cultured for 7 d and then counted every day (3 wells each time). The average cell counts at each time point were then plotted against time and the PDT was determined based on this curve (Costa et al., 2005; Kim et al., 2005, Hongmei et al.,2008).

Detection of microbes

Detection of bacteria and fungus. Cells were cultured in MEM containing 10% fetal bovine serum without antibiotics and tested for the presence of bacteria and fungus at 3d after subculture according to the method of Doyle et al. (1990).

Detection of viruses. The Routine examination for cytopathogenic effect using phase-contrast microscope was performed according to Hay's haemadsorption protocol (Hay, 1992).

Detection of mycoplasmas. According to the American Type Culture Collection protocol, cells were cultured in medium free of antibiotics for at least one week and then fixed and stained with Hoechst 33258 and DAPI dye using Masover (1998) and Freshney's method (2000) for fluorescence staining of DNA. Results of DNA staining were confirmed by ELISA using the ELISA mycoplasma detection kit (Roche, Lewes, East Sussex, UK) This kit could identify the four most common mycoplasma species: *M. arginini, M. hyorhinis, A. laidlawii, and M. orale.*

Chromosome analysis

Metaphase spreads were prepared from cells at the exponential growth phase following treatment with 0.1 μg/mL colcemid (Gibco/BRL). The cells were treated with a

hypotonic KCl/HEPES/EDTA solution and harvested according to standard cytogenetic procedures. The percent of diploid was counted from 100 cells. Representative chromosome sets were photographed and analyzed. Karyotypes were prepared following the protocol described in the Reading Conference report (Ford et al., 1980). The parameters of relative length, centromere index and arm ratio index were calculated according to Levan et al. (1964).

Isozyme analysis

The electrophoretic mobilities of lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) were determined using the polyacrylamide gel electrophoresis according to protocol provided by Marvin (1977). Improvements were made, including the vertical slab polyacrylamide gel electrophoresis apparatus used and the electrophoretic buffer changing into Tric-glycin (pH 8.7). In addition, the gel buffer was prepared into discontinuous system using two kinds of Tris-citric acid buffer with different concentration: 0.078 mol/L (pH 8.9) and 0.017 mol/L (pH 6.8). Electrophoretic mobility was defined by numbers and intensity of enzyme bands, as well as the distance of band migrating from the point of origin (Freshney, 2000). Mobility was expressed as the ratio of the distance the isoenzyme band migrated to that of the indicator dye.

Liposome-mediated transfection of plasmid DNA

To obtain the highest transfection efficiency and low cytotoxicity, transfection conditions need to be optimized by varying cell density as well as pEGFP-C1, pEGFP-N3, pEYFP-N1, pECFP-N1, pECFP-mito and pDsRed1-N1 (BD Biosciences Clontech product) and Lipofectamine 2000 (Invitrogen) concentrations, according to the lipofectamine media methods of Escriou et al. (2001), and Tsuchiya et al. (2002).The cultured cells were observed at 24 h, 48 h, 72 h, 96 h, 1 week, 2 weeks and 1 month after transfection. The six fluorescent protein genes were transfected under excitation wavelength of 405 nm, 488 nm and 543 nm separately. In each experiment group 10 visual fields were picked to take pictures and calculate the transfection efficiency which refered to the ratio of the positive cells to the total cells in a visual field under confocal microscope.

Results

Morphological observation

Chinese Game chicken embryo fibroblasts originating from tissue explant pieces grew rapidly, spread on the wall of the culture flask in one or two days and formed cell monolayer afterwards. Fibroblasts mingled with epithelial cells initially (fig. 1A). For their rapid growth, fibroblasts gradually replaced the epithelial cells in subcultures. The cells had fibrous characteristics with turgor vitalis cytoplasm, and during growth they showed tipical fibroblast-like morphology as radiating, flame-like or whirlpool migrating shapes (fig.1B).

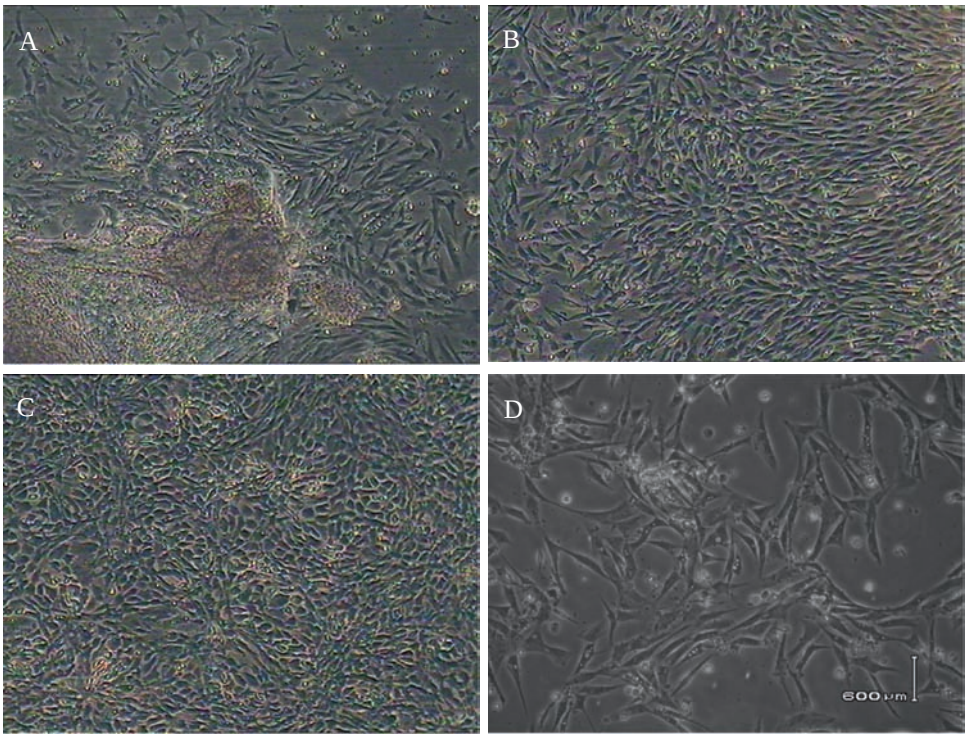

Figure 1. Morphology of Chinese Game chicken embryo fibroblasts cultured in vitro. A: 1-2 day after explanting (100×); B: near confluence (100×); C: Chinese game Chicken embryo fibroblasts before cryopreservation (100×); D: 24 h after recovery from cryostorage (200×). This figure is published in colour online, see http://www.brill.nl/ab.

Cell viability before cryopreservation and after recovery

The method of trypan blue dye exclusion test was adopted to test cell survival rate before cryopreservation and after recovery. The viability (expressed as mean value ± SD) of the cultures before freezing was 97.37% ± 2.25% (fig. 1C) and 91.43% ± 3.22% after thawing (fig. 1D), this difference was not significant ($P > 0.05$). These results showed that the cells were healthy when cultured in vitro and that freezing had done little harm to their viability.

Growth curve analysis

The growth curve of Chinese Game chicken embryo fibroblasts appeared sigmoidal (fig. 2) and the population doubling time (PDT) was approximately 48 h. There was a lag time or latency phase of about 24 h after seeding, corresponding to the adaptation and recovery of the cells from protease damage; after that the cells proliferated rapidly and entered exponential phase. As the cell density increased, proliferation reduced by contact inhibition and the cells began to enter the plateau phase after the fifth day.

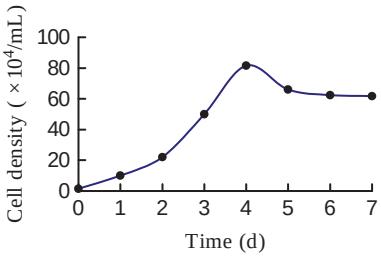

Figure 2. Growth curve of Chinese Game chicken embryo fibroblasts.

Microorganism detection

Detection of bacteria and fungus. The medium was clear all the time and no abnormal changes could be observed under the microscope. The results indicated that our Chinese Game chicken embryo fibroblasts were free of bacterial contamination. In addition, we did not observe any thallus aggregation, indicating that the cells were free of fungus, while there was obvious growth of microorganisms in the positive control group.

Detection of viruses. We performed a hemadsorption test to detect the presence of viruses, which was negative. In addition, no cytopathogenic effects were observed in the cultures.

Detection of mycoplasmas. If there was abundant punctiform and filiform blue fluorescence in the cell nucleoli, it is indicated that the cells were contaminated by mycoplasmas (Barile, 1993). However, 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.

Chromosome analysis

The chromosome number of diploid Chinese Game chicken is 78, including 9 pairs of macrochromosomes and 30 pairs of minichromosomes. The sex chromosome type is ZZ(♂)/ZW(♀). According to the size of chromosomes their features were described as follows:

No. 1, submetacentric chromosome, the largest pair;
No. 2, submetacentric chromosome, the secondly largest pair;
No. 3, submetacentric chromosome;
No. 4, telocentric chromosome, with a size approximately the same as the third pair;
No. 5, metacentric chromosome Z and the other is a submetacentric chromosome W;
No. 6, submetacentric chromosome;
No. 7, submetacentric chromosome;
No. 8, telocentric chromosome;

No. 9, telocentric chromosome;
No. 10-No.39, acrocentric chromosome or telocentric chromosome;

The karyotype and chromosome parameters of Chinese Game chicken embryo fibroblasts were shown in fig. 3 and table 1 respectively. In this experiment 100 well-spread metaphases of 3-5 passage were observed under the microscope to count the chromosome numbers, and the mean frequency of diploid cells was 94.65% ± 3.29% (mean value±SD). Aberrations in chromosome number tended to increase with increasing number of passages (data not shown), showing that 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.

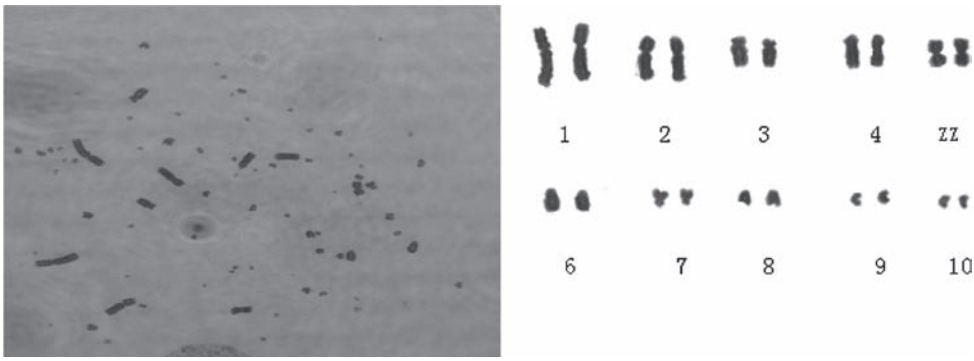

Figure 3. Karyotype of Chinese Game chicken embryo fibroblasts (♀).

Table 1.
Chromosome parameters of Chinese Game chicken embryo fibroblasts (♀).

Chromosome number	Relative length (%)	Arm ratio index	Centromere index (%)	Kinetochore type
1	24.20±0.242	1.78±0.02	35.80±0.261	SM
2	18.12±0.165	1.76±0.023	35.82±0.245	SM
3	13.54±0.132	1.92±0.040	33.00±0.207	SM
4	11.65±1.163	1.60±0.024	34.82±0.224	T
Z	6.26±0.086	1.02±0.012	49.60±0.195	M
W	6.18±0.046	1.18±0.016	48.42±0.224	M
6	5.66±0.044			T
7	5.28±0.05			T
8	4.70±0.048			T
9	4.65±0.052			T

Isozyme analysis

The LDH and MDH bands obtained from Chinese Game chicken embryo fibroblasts were compared with those from other species.The Isoenzyme patterns showed five

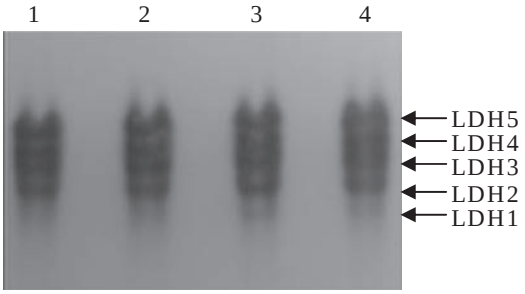

Figure 4. Isoenzyme patterns of LDH in the Chinese Game chicken and Dagu chicken. Lines 1 and 2: Chinese Game chicken; lines 3 and 4: Dagu chicken.

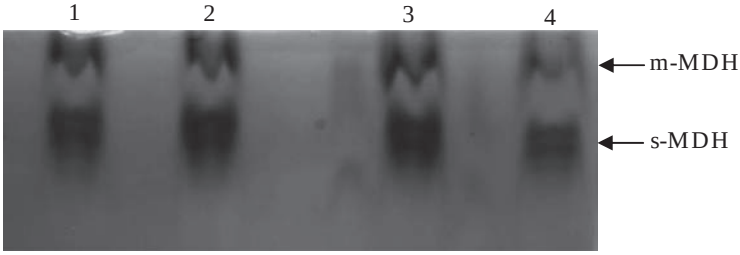

Figure 5. Isoenzyme patterns of MDH in the Chinese Game chicken and Dagu Chicken. Fibroblast lines 1 and 2: Chinese Game chicken; lines 3 and 4: Dagu Chicken.

LDH isoenzyme bands and two MDH isoenzyme bands (figs 4 and 5). The enzymatic mobilities were in the order of LDH-1, LDH-2, LDH-3, LDH-4, LDH-5; two MDH isoenzyme bands were m-MDH and s-MDH (fig. 5). The results indicated that there was no cross-contamination with cells from other species.

Liposome-mediated transfection of plasmid DNA

Expression of pEGFP-C1, pEGFP-N3, pEYFP-N1, pECFP-N1, pECFP-mito and pDsRed1-N1 in the Chinese Game chicken fibroblasts was observed at 24 h, 48 h, 72 h, 1 week and 2 weeks after transfection. In most positive cells all the six fluorescent proteins were expressed, and the transfection efficiencies at 24 h, 48 h and 72 h after transfection were between 9.5% and 34.6% (table 2). In addition, at 48 h after transfection there observed the strongest fluorescence intensity (fig. 6) and the highest transfection efficiency 34.6 % which was slightly higher than that of 32.6% in the study of Lei Guo et al. (2007), Confocal fluorescence microscope was used to observe the distribution of green, yellow, cyan, and red fluorescence in the Chinese Game chicken fibroblasts to determine the subcellular location of the six fluorescent proteins. The results showed that fluorescences could be observed in cytoplasm and nuclei except cryptomere vesicles. DsRed was mostly shown in cytoplasm, whereas EGFP and EYFP showed intense nuclear signal (fig. 7). The number of cells expressed fluorescence

Table 2.
Transfection efficiency of six fluoscent protein genes in Chinese Game chicken. Cells were examined at 24, 48, and 72 h after transfection using a Nikon TE-2000-E inverted microscope with excitation wavelengths of 405 nm, 488 nm and 543 nm. The transfection efficiencies were calculated by counting the numbers of total cells and positive cells in 10 visual fields of every well.

Time (h)	Six fluorescent protein genes					
	pEGFP-C1 (%)	pEGFP-N3 (%)	pEYFP-N1 (%)	pECFP-N1 (%)	pECFP-mito (%)	pDsRed1-N1 (%)
24	29.6	28.7	30.3	26.7	9.5	12.1
48	34.6	33.4	33.1	29.3	11.7	14.8
72	31.2	30.6	32.4	28.1	10.1	12.3

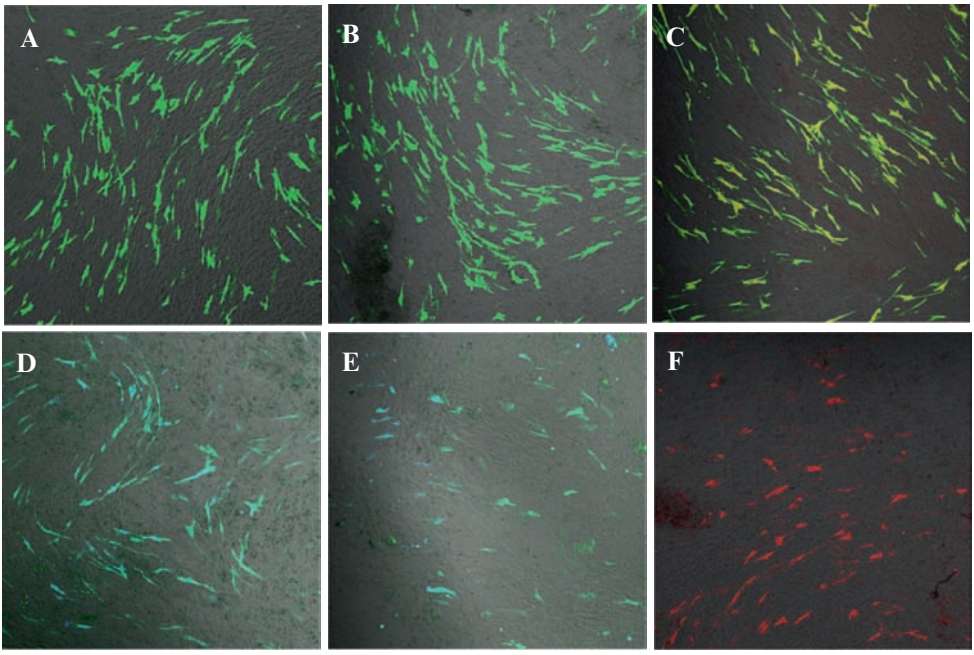

Figure 6. Transfection photos of six fluorescent protein genes. These photos were taken 48 h after transfection, using a Nikon TE-2000-E confocal fluorescence microscope with excitation wavelengths of 405 nm, 488 nm and 543 nm to determine the transfection efficiency (100×). A: pEGFP-C1; B: pEGFP-N3; C: pEYFP-N1; D: pECFP-N1; E: pECFP-mito; F: pDsRed1-N1. This figure is published in colour online, see http://www.brill.nl/ab.

reduced at 1 week; however, there remained a few dispersed positive cells after 2 weeks even 1 month and 2 months. The viabilities of transfected cells with pEGFP-C1, pEGFP-N3, pEYFP-N1, pECFP-N1, pECFP-mito and pDsRed1-N1 were 89.6%, 87.5%, 85.2%, 87.5%, 86.3% and 88.3% respectively. Among them, no one was significantly different from that of the control group (91.2%, $P > 0.05$).

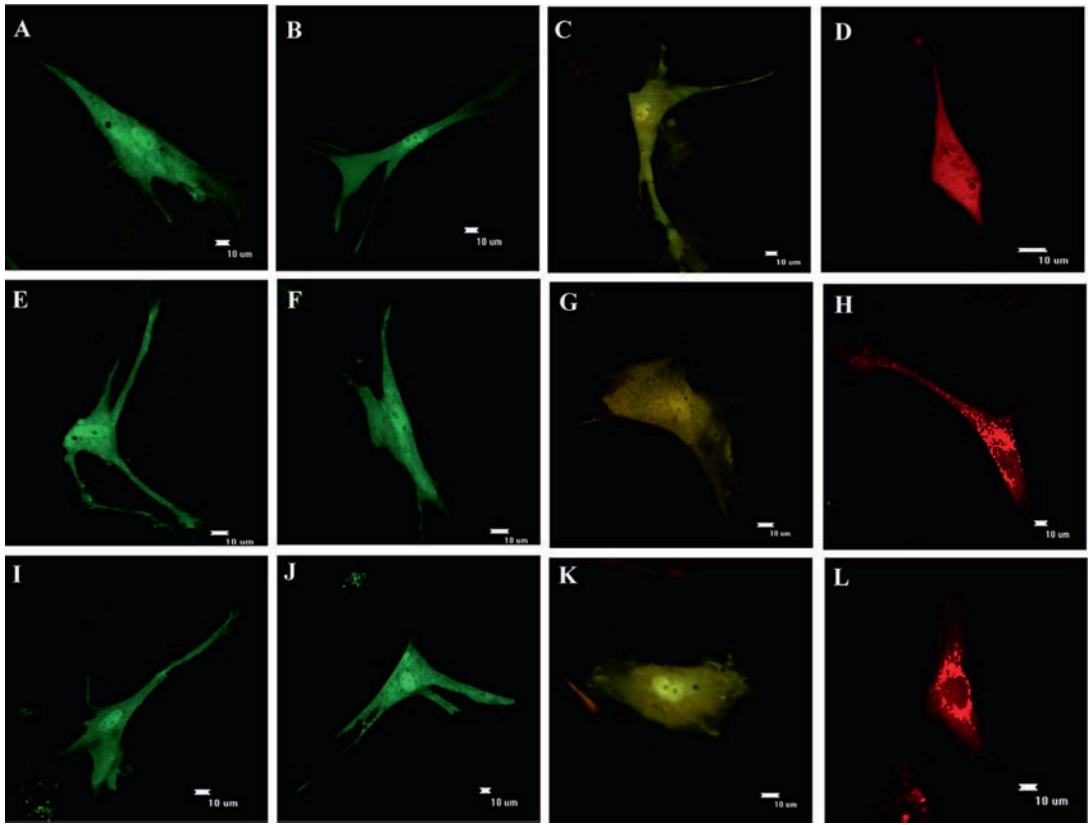

Figure 7. The expression and distribution of pEGFP-C1, pEGFP-N3, pEYFP-N1 and pDsRed1-N1 (respectively, from left to right column) in Chinese Game chicken (400×). A, B, C and D: expression photos 24 h after transfection; E, F, G and H: expression photos 48 h after transfection; I, J, K and L: expression photos 72 h after transfection. This figure is published in colour online, see http://www.brill.nl/ab.

Discussion

At the early stage of the formation of the cell line, epithelial cells and fibroblasts will initially grow together. 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). Because of these differences, fibroblasts will quickly outgrow their epithelial counterparts. In this manner 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).

Due to the growth and reproduction of bacteria, the consumption of nutrient, the culture liquid becomes turbid and yellow in color, and abundant spot-shaped thallus particles can be seen under the inverted microscope. Sometimes cells would be covered by thallus, which constitutes a threat to the cells. Tests for microbial contamination by bacteria, fungus, viruses and mycoplasmas showed that the Chinese Game chicken embryo fibroblast cell bank were pure and without microorganism contamination.

The genetic stability of cell line is critical to preserve the genetic resources; the fibroblasts must maintain the same diploid character as cells in vivo. The cells cultured in vitro easily varied. The varied cells still have dividing capability, which finally results in the variation of the cell line and then the cell line loses the significance of breed conservation. Therefore, karyotype analysis is a major method for distinguishing normal cells from variants. That the mean frequency of diploid cells was 94.65%±3.29% indicated the Chinese Game chicken embryo fibroblasts cultured in vitro were stable diploid, in accordance with the reports of Liu et al. (1993) and Zhan et al. (1994). At continuous subcultivation the cells are unstable, and with increasing number of passages there appear chromosome rearrangements which lead to loss of functional reactivity (Dzhambazov et al., 2003). However, the frequency of diploid cells was above 90%, further validating the genetic stability of these cells. Hence, it could be inferred that chromosomal structure variation was rare in the Chinese Game chicken embryo fibroblasts.

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). Isoenzymes can be separated chromatographically or electrophoretically, revealing species or tissue characteristic distribution patterns. Biochemical analysis of isoenzyme polymorphism is currently considered to be the standard method for cell line identification and detection of interspecies contamination, and is routinely used by the leading biological resource centers around the world (i.e., American Type Culture Collection, European Collection of Cell Cultures) (Parodi et al., 2002.) However, this test requires dedicated equipment and expensive reagents, as well as a high level of technical expertise. We have modified the apparatus and conditions for polyacrylamide gel electrophoresis, and successfully determined the mobility of MDH and LDH which were isolated from Chinese Game chicken embryo fibroblasts cultured in vitro.

LDH is a kind of important enzyme which participates in glucose metabolism. It catalyzes lactate to be oxidized to pyruvate and at the same time NAD^+ is reduced to

NADH, or it catalyzes the reverse reaction of the two substances. It plays an important role in the energy metabolism in mammalian and its generation is double controlled by metabolites and genetic gene. There are two kinds of subunits, namely, subunits A and B, which can form five different tetramers, i.e. LDH1 (B4), LDH2 (A1B3), LDH3 (A2B2), LDH4 (A3B1) and LDH5 (A4). Thus five bands will be shown in electrophoresis. Due to the different genetic regulations and expressions of the A and B subunits during the tissue differentiation, the LDH isozyme shows specificity of species and tissue or organ on the electrophoresis bands. Once formed, they could be inherited stably so that the stability of the species can be guaranteed. Thus, the LDH isozyme is not only an inherited marker to identify the animal species, but also a fine mode for gene expression and regulation analysis during the development of the animal individual.

MDH is one of the important dehydrogenases in the tricarboxylic acid cycle, which can oxidize the malic acid into the oxaloacetic acid. MDH exits abundantly in tissues and cells of animals. It has types which are sMDH (cytoplasm type) encoded by A and B genes and mMDH (mitochondria type) encoded by C and D genes; wherein, the former forms three kinds of molecular forms, namely A2, AB and B2, the latter forms also three kinds of molecular forms, namely C2, CD and D2. 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.

In this study, the isoenzyme bands of LDH and MDH of Chinese Game chicken embryo fibroblasts were clear, indicating that the genetic characteristics were stable and there was no contamination with cells of other species. Five LDH bands and two MDH bands were observed in the Chinese Game chicken embryo fibroblasts, and the same results were obtained with same way in white ear lobe chicken (Hongmei, et al., 2008). Analysis of these genes in this way has shown both interspecies and intraspecies isoenzyme polymorphism previously (O'Brien et al., 1977; Nims et al., 1998). Therefore, these tests can be used to identify the relationship between species, and also heredity distribution, in addition to confirming the origin and purity of explanted cells.

The researches about fluorescent protein are mainly focused on tumor cells, nerve cells and stem cells (Jung et al., 2001). DNA and lipofectine concentration, the incubation time of the DNA and lipofectine combination, and serum, all can affect the transfection efficiency which is identical with researches on the Vero cells, Hela cells and some other cell lines (Tseng et al., 1999; Escriou et al., 2001; Rong et al., 2006). In this study, the transfection efficiency of the six fluorescent protein genes could achieve 34.6% with the optimized ratio of plasmid and lipofectamine. The number of cells expressed fluorescence reduced at 1 week, however, there remained a few dispersed positive cells after 2 weeks even 1 month and 2 months. The number of cells expressing fluorescence reduced at 1 week, however, there scattered some positive ones at 2 weeks even 1 month and 2 months. Through G418 resistance screening and monoclone culture during 1 month, 3 positive cell strains were obtained which can express EGFP, EYFP and DsRed stably.

At 24 h and 48 h after transfection, the six kinds of fluorescences could be observed in cytoplasm and nuclei well-distributed except cryptomere vesicles. At 72 h after transfection, EGFP and EYFP gene still expressed steadily in cytoplasm and nuclei, which were nearly unchanged, yet morphology of some cells became irregular. Besides, at 72 h, cells transfected with DsRed gene mainly expressed some granular expression products which formed a red annular profile surrounding nuclear membranes.

We could see the transfected cells at reduplication and different dividing phases, moreover, the growth and reduplication of the transfected cells were non-significantly different from the control group. The result showed that the transfected cells were not affected by fluorescin under certain range. Some researches supported that GFP did not obviously affect cell doubling time in tumor and human cell lines, this was identical with our results. Besides, fluorescins were not distributed homogeneously in all cell lines, for example GFP mainly distributed in nucleus in COS cell and DsRed expressed some granular expression product surrounding nuclear membrane and formed a red ring profile. Due to these exceptions, it was quite important for researchers to determine the distribution of reporter proteins before analyzing the location of fusion proteins.

A Chinese Game chicken embryo fibroblast cell bank was successfully established from 43 samples by adherent culture. The biological characteristics of the cells, especially the genetic characteristics, may change when cultured in vitro for many passages, so it is recommended to conserve them at early passages. We contend that our cell bank makes a valuable contribution to the preservation of the genetic resources of the Chinese Game chicken and provides useful biomaterials for future studies in cell biology, medicine, genomics, post-genomics, and both genetic and embryonic engineering.

Acknowledgements

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