Global Science Research Journals

ISSN: 2505-001X Vol. 4(9), pp. 261-268, September, 2016.

http://www.globalscienceresearchjournals.org/

Global Journal of Biological and Biomedical Research

Full Length Research Paper

An Assessment of fibroblast line from embryos of Xiaoshan chicken

Jiang Yat-sen Zemin

Department of Pathology, Chinese PLA General Hospital, Beijing, 100853, PR China.

Accepted 23 August, 2016

A fibroblast line from embryos of Xiaoshan chicken was established successfully by direct culture of explants and cryopreservation techniques. The cells were morphologically consistent with fibroblasts, and the population doubling time (PDT) was about 46.72 h. According to karyotyping and G-banding, the diplontic cells with 78 chromosomes accounted for 98.58±1.27% of the total cells. The cells were tested for microbial contamination and they were free of infections from bacteria, fungi, viruses and mycoplasmas. There were no cross-contamination from other cell lines as revealed by isoenzyme polymorphism analysis of lactate dehydrogenase (LDH) and malate dehydrogenase (MDH). Three fluorescent protein genes were transfected into the Xiaoshan chicken embryonic fibroblasts and the transfection efficiencies of these genes were between 12.72 and 35.89%. All the tests showed that the quality of the cell line conformed to the quality criteria of the American type culture collection (ATCC). This work had not only preserved the precious genetic resources of Xiaoshan chicken, but also explored a new protocol to preserve the endangered animal breeds.

Key words: Xiaoshan chicken, fibroblasts, genetic conservation, biological characteristics.

INTRODUCTION

Animal genetic resources are an important element of biodiversity. Genetic diversity of China livestock and poultry has being seriously shrinking, and some high-quality species are on the edge of imminent extinction as the exacerbation of environmental pollution and the development of modern animal husbandry. While some government departments, organizations and experts appeal for conservation and management of the livestock and poultry genetic resources, but there is still a massive loss committed. If these genetic resources have not been preserved in any forms before their extinction, not only the genetic resources will lost evermore, but also it becomes impossible to investigate the unknown cell and molecular mechanisms in regard to the extinct livestock and poultry to regenerate them through somatic cell cloning. Therefore, it is of urgent need to employ practical measures to conserve endangered species (Guan et al., 2002). At present, preservation in terms of individual animals, semen, embryos, genomic libraries and cDNA libraries are all available methods. In addition, modern cloning techniques have made somatic cells an attractive resource for conserving animal genetic materials (Wu, 1999). Thus, establishment of animal cell line can not only save the genetic resources on cell level, but also provide a precious experiment material for cell biology, genomics, post-genomics and embryonic engineering.

Xiaoshan chicken, also known as Yue chicken, originated in Jiangsu Province of China. It is characterized with big fatty body, high fecundity and tender flesh. As one of the local chicken breeds in China, Xiaoshan chicken has enjoyed a high reputation ever since the Yue State during the Sping and Autumn Period. Xiaoshan chicken was listed in 138 national protected species by Chinese government in 2006. This research successfully constructed a qualified fibroblast line through primary explantation and programmed cryopreservation.

*Corresponding author: E-mail: jiang.zemin28@hotmail.com

The genetic resources of this valuable local breed have thus been permanently preserved in the form of somatic cells, and this technique system will provide technical and theoretical support for conservation of other animal genetic resources at cell level.

MATERIALS AND METHODS

The 8-day old embryos of Xiaoshan chicken in this research were provided by chicken breeding farm of Chinese Academy of Agricultural Sciences, Beijing, China.

MEM (Gibco, USA), special grade fetal bovine serum (Biochrom, German), DMSO (Sigma, USA), Hoechst 33258 (Invitrogen, USA), Lipofectamine 2000 (Invitrogen, USA), polyacrylamide gel (Sigma, USA).

Isolation and culture of Xiaoshan chicken embryonic fibroblasts

The Xiaoshan chicken eggs incubated for 8 days were sterilized using alcohol swabs, and then the embryos were isolated and washed three times with phosphate buffered saline (PBS). The embryos were cropped into pieces of 1 mm^3 in size and seeded onto the surface of a tissue culture flask, and cultured inverted at 37°C in a humidified atmosphere, 5% CO_2 for 3 to 4 h. Modified Eagle's medium (MEM) containing 10% fetal bovine serum (FBS) was added into the flask. The medium was refreshed after 2 to 3 days. The cells were harvested at 80 to 90% confluence using 0.25% trypsin (m/v) solution and were separated into culture flasks at the ratio of 1:2 or 1:3 (Guan et al., 2005; Zhou et al., 2004).

Cryopreservation and recovery

The cells in logarithmic growth phase were harvested and counted with a hemocytometer. The viability before freezing was checked by the CellTiter-Blue® Cell Viability Assay (Promega, USA). The harvested cells were resuspended in freezing medium containing 40% DMEM, 10% dimethyl sulphoxide (DMSO) and 50% FBS to a final concentration of (3 to 4) $\times 10^6$ viable cells/ml. The cell suspension was dispensed into sterile plastic cryovials labeled with species, gender, freezing serial number and the date. First, the sealed cryovials were kept at 4°C for 20 to 30 min to allow the DMSO to equilibrate. Then, cryovials were transferred to a commercially available freezing kit (Nalgene, USA), and refrigerated at -80°C overnight (a process in which temperature decreased at a rate of 1°C min). Whereafter, the cryovials were transferred to liquid nitrogen (LN2) for long term storage (Werners et al., 2004).

When recovering the cells, the cryovials were taken out from liquid nitrogen, and rapidly thawed in 42°C water bath, which were subsequently transferred to culture flask containing 90% MEM and 10% FBS with a straw. The suspension was pipeted gently to make it well-distributed, and then cultured at 37°C with 5% CO_2. The medium was refreshed after 24 h.

Estimation of cell viability by trypan blue exclusion test

Viabilities before freezing and after recovery were determined using trypan blue exclusion test. The number of non-viable cells was determined by counting 1000 cells (Qi et al., 2007).

Growth dynamics

Cells were plated onto 24-well plates at the density of approximately 1.5×10^4 cells per well. The cells were cultured for 7 days and counted every day (3 wells each time). The mean cell counts at each time point were then used to plot a growth curve, based on which the PDT was calculated (Qi et al., 2007; Hirofumi et al., 2006).

Microbial detection

Detection of bacteria and fungi

The cells were cultured in complete MEM media free of antibiotics and observed for the presence of bacteria and fungi at 3 days after subculture according to the method described by Doyle et al., 1990).

Detection of mycoplasmas

According to protocol of the ATCC, the cells were cultured in antibiotic-free medium for at least 1 week, and then fixed and stained with Hoechst 33258 (Simpson, 2003).

Detection of viruses

Hay's hemadsorption protocol was used to examine the samples for cytopathogenesis using phase contrast microscopy (Hay et al., 1992; Wu et al., 2008).

Karyotyping and chromosome analysis

Chromosome spreads were prepared, fixed and stained following standard methods (Costa et al., 2005). Cells were harvested when 80 to 90% confluent, and subjected to hypotonic treatment and fixed, then the chromosome numbers were counted from 100 spreads under an oil immersion objective upon Giemsa staining. Relative length, centromeric index and kinetochore type were calculated according to the protocol described by Sun et al. (2006).

Isoenzyme polymorphisms

Isoenzyme patterns of lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) were identified by polyacrylamide gel electrophoresis (PAGE). The protein extraction solution (0.9% Triton X-100, 0.06 mmol NaCl: EDTA in mass ratio 1:15) was added to the harvested cells at the density of 5×10^7/ml. Then the suspension was centrifuged and stored in aliquots at -70°C. Equal volumes of 40% sucrose and 2.5 ml loading buffer were added to the sample. The PAGE apparatus was used with the electrophoretic buffer and was changed into Tris-glycin (pH 8.7), the gel buffer was prepared into discontinuous system using two kinds of Tris-citric acid buffer at different concentrations: 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 the band migrating from the point of origin. Different mobility patterns were reflected by the relative mobility front (RF), which was calculated as the ratio of the distance of the isozyme migration to that of bromophenol blue.

Expression of fluorescent protein genes in Xiaoshan chicken fibroblasts

According to the methods described by Wu et al. (2008) three plasmids carried fluorescent protein genes (pDsRed1-N1, pEGFP-N3 and pEYFP-N1) (BD Bioscieces Clontech product, USA) were

transfected into the Xiaoshan chicken fibroblasts using Lipofectamine 2000. The tranfected cells were observed at 24, 48, 72, 96, 1 week, 2 weeks and 1 month after transfection. Expression of the three fluorescent protein genes was observed under a confocal microscope (Nikon TE-2000-E, Japan) with excitation wavelengths of 543, 488, 488 and 543 nm respectively. In each group 10 visual fields were captured to take pictures and to calculate the transfection efficiencies which was formulated as the ratio of positive cell numbers to the total cell numbers (Wu et al., 2008). At 48 h after transfection, DAPI staining was adopted to locate the nuclei. By trypan blue and DAPI staining, the viability and apoptosis rate of the transfected cells were detected to analyze the influence of exogenous gene transfection on the cells.

RESULTS

Morphological observation of Xiaoshan chicken fibroblasts

Cells sprouted out from small tissue pieces at 1 day after being plated on the bottom of tissue culture flasks, and then it continued to proliferate and were subcultured when 80 to 90% confluent within about 3 days. The cells displayed typical fibrous and fusiform morphology with centrally located oval-shaped nuclei. However, there existed some ones that were morphologically epithelial cells. The fibroblasts grew rapidly and replaced the epithelial cells gradually after 2 to 3 passages, and then a relatively purified fibroblast line was obtained. The viabilities of Xiaoshan fibroblasts before freezing and after recovery evaluated through trypan blue exclusion tests were 98.36±1.41 and 91.17±0.92% respectively (p<0.5) (Figure 1A, B and C).

Growth dynamics

The growth curve of Xiaoshan chicken fibroblast before cryopreservation and after recovery displayed typical “S” shape (Figure 2). Lag phase of approximately 24 h was observed after the cells were plated, which was corresponding to the recovery period against trypsin damage. Afterwards, they proliferated rapidly and entered the exponential growth phase until the stationary phase after about 5 days. From day 5, growth stagnated and the population began to degenerate gradually. The PDT calculated from the curve was about 46.72 h.

Microorganism detection

The medium was clear all the time and no abnormalities could be observed under the microscope. The results indicated that the Xiaoshan chicken fibroblasts were free of bacterial contamination. Mycoplasma detection suggested that the fibroblasts were free of mycoplasmas. Would there be abundant punctiform and filiform blue fluorescence in the nucleoli, it could be concluded that the cells were contaminated by mycoplasmas (Figure 1D) just as the positive result of Li et al. (2009). As is shown by the cytopathogenic evidence and the hemadsorption test, tests for virus contamination were all negative as well.

Karyogram and chromosome number

The chromosome number of diploid Xiaoshan chicken is 78, including 9 pairs of macrochromosomes and 30 pairs of minichromosomes. The sex chromosome type is ZZ(♂)/ ZW(♀) (Figure 3). The parameters including relative length, centromere index and kinetochore type were shown in Table 1. In this experiment 100 representative spreads at metaphase of passage 3 to 5 were observed under the microscope to count the chromosome numbers, and the mean proportion of diploid cells was 98.58±1.27%.

Isoenzyme polymorophisms

The profiles of isoenzyme polymorphisms might be characteristic in a specific species or tissue (MacLeod et al., 1999). Polymorphism analysis of isoenzymes is currently a standard method for the quality control to identify cell lines and to prevent interspecies contamination. Isoenzyme patterns of LDH and MDH were obtained using vertical slab non-continuous PAGE, stained by Coomassie brilliant blue and compared with those from other species (Figure 4). The LDH RFs were in the order of LDH5, LDH4, LDH3, LDH2, LDH1 (Figure 4A); while the two bands of MDH were Mitochondrial MDH (m-MDH) and cytosolic MDH(s-MDH) (Figure 4B). The three chicken breeds had their own characteristic bands, each band with a different migration rate (Tables 2 and 3). The results indicated that there was no cross-contamination from other cell lines.

Expression of exogenous genes in Xiaoshan chicken fibroblasts

The three fluorescent genes, EGFP, EYFP, DsRed1 were all highly expressed at 24 h according to the optimized conditions. The transfected cells increased gradually within 48 h (Figures 5 and 6), and the transfection efficiencies were listed in Table 4. The number of transfected cells decreased gradually as time passed by. However, fluorescence still could be observed in a few cells at 2 weeks after transfection.

DISCUSSION

Xiaoshan chicken fibroblast line was successfully established from 62 embryo samples by adherent culture, and was cryopreserved in 184 cryovials within 5 passages

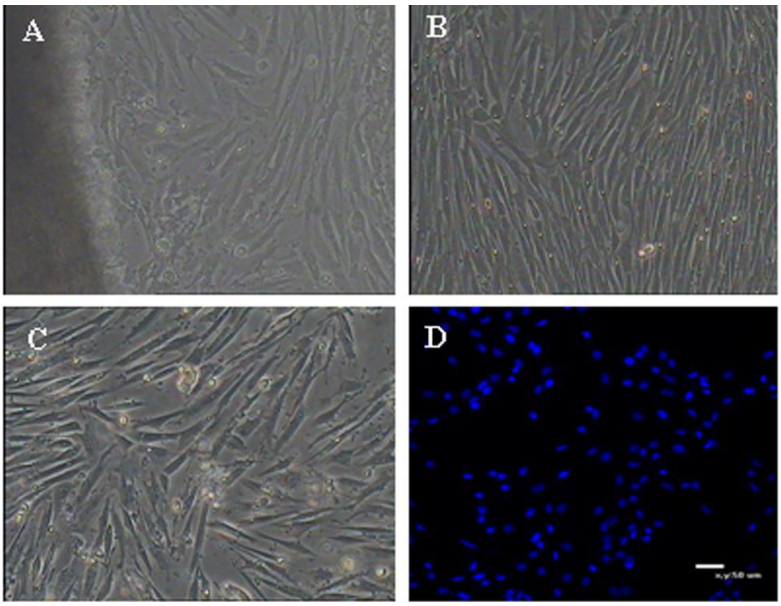

Figure 1. Morphology of Xiaoshan chicken fibroblasts A. Primary cells grew out from the embryo explants; B. Cells before cryopreservation; C. Cells at 24 h after recovery from cryo-storage. D. Xiaoshan chicken fibroblasts stained with Hoechst 33258. Bar = 100 um.

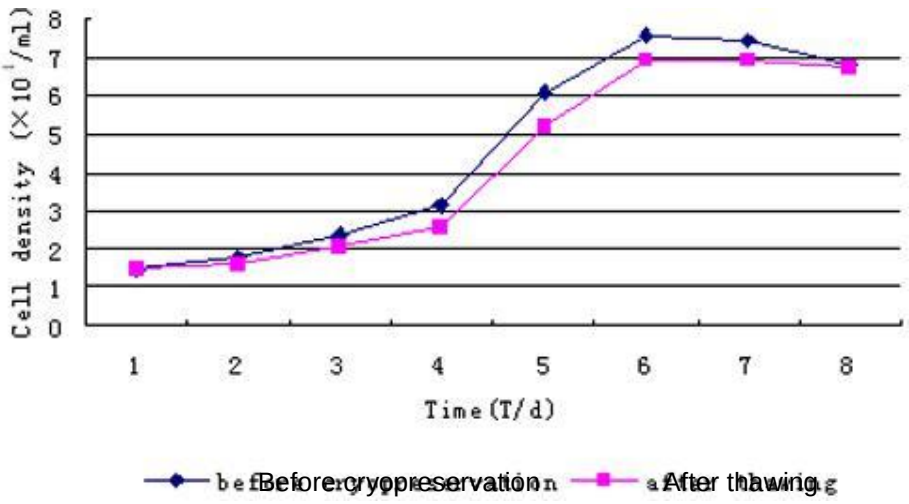

Figure 2. The growth curve of Xiaoshan chicken fibroblasts. The growth curve was typical "S" shape. Lag of around 48 h was observed after cells were seeded. Then, cells proliferated and entered the logarithmic phase. The PDT was approximately 47 h. From the fiveth day, cells entered the plateau phase.

at a density of about 3.0×10^6 cells/ml. The biological characteristics, especially the genetics related ones, may be altered by *in vitro* culture after many passages, so a minimal number of passages are recommended to protect them from degeneration.

Morphological observation indicated that there were both epithelial cells and fibroblasts in the primary cultures of the explanted tissues. Due to their different levels of tolerance to trypsinization, the fibroblasts detached from the flasks earlier when treated with trypsin and adhered again quickly after passage, whilst most epithelial cells were difficult to adhere, or only did so in an unstable manner and fell off when vibrated (Xue et al., 2001). For this reason, a purified fibroblast line could be obtained after 2 to 3 passages.

Isoenzyme polymorphisms and karyotyping together can effectively confirm the origin of a cell line and identify possible cross-contamination. The genetic stability of cell

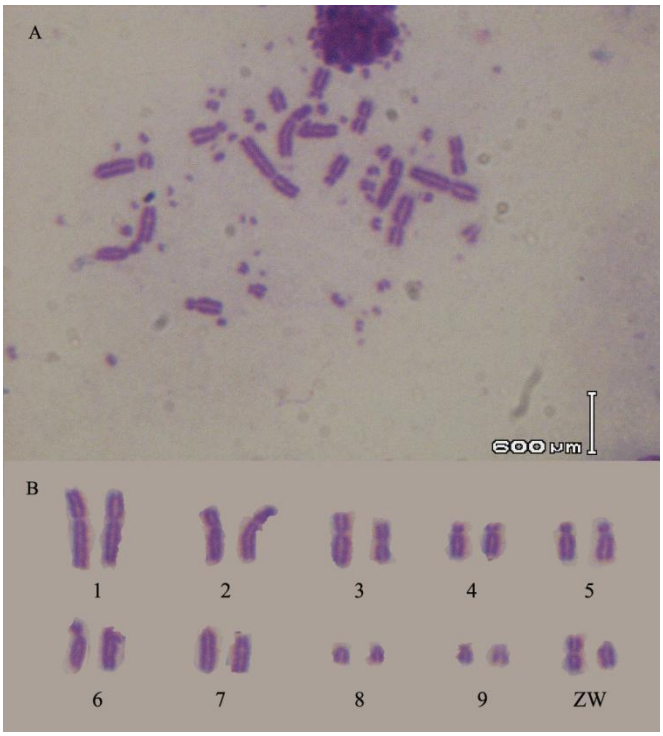

Figure 3. Chromosomes at metaphase (A) and karyotype (B) of the Xiaoshan chicken fibroblasts (♂).

Table 1. Chromosome parameters of Xiaoshan chicken (♂).

Choromosome No.	Relative length (%)	Centromere index (%)	Kinetochore type
1	23.25±0.12	39.43	SM
2	21.18±0.31	37.32	SM
3	19.75±0.35	31.27	M
4	15.78±0.58	25.98	SM
5	11.25±0.95	19.32	SM
6	11.17±0.19	14.62	SM
7	6.82±0.64	0	T
8	4.17±0.32	0	T
9	3.92±0.55	0	T
Z	13.28±0.53	33.71	M
W	6.91±0.31	0	T

Note: SM, Submetacentric chromosomes; T, telocentric chromosomes; M, metacentric chromosomes.

line is critical to preserve the genetic resources, the fibroblasts must maintain the same diploidy as cells *in vivo*. International standards of poultry karyotype were as 8 pairs of large chromosomes with sex chromosomes Z and W, and 30 pairs of microchromosomes (Ladjali-Mohammedi et al., 1999). Avian diploid chromosome number are greatly diversified, the majority of which ranged from 78 to 82. Chicken macrochromosome number is 7.8±0.9, fluctuating from 6 to 9, and the microchromosome number is 31.9±2.5, the range fluctuating from 24 to 35. In this study, results showed that the diploid chromosome number of Xiaoshan chicken was 78±0.9 in 100 cells, with 10 pairs of macrochromosomes and 29 pairs of microchromosomes. The proportion of 2n = 78 cells were 98% as detected in 100 cells. Most Xiaoshan chicken chromosomes were very small ones, which were easily lost in the preparation process and by the interference of dye, rendering the difficulty to count chromosome number and to observe the morphology. Therefore, time point and duration of

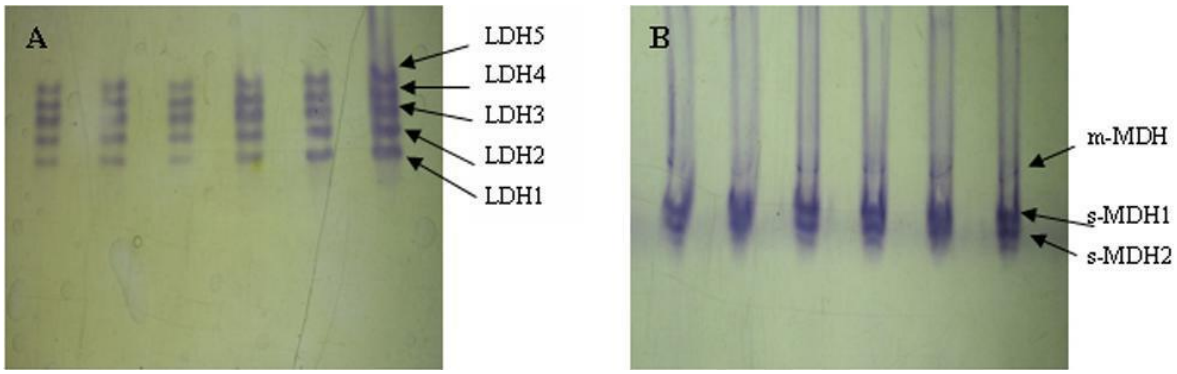

Figure 4. Isoenzyme patterns of LDH and MDH from three cell lines. 1 2 Xianju chicken; 3 4 Xiaoshan chicken; 5 6 Langshan chicken.

Table 2. Relative migration fronts of LDH.

Breeds	LDH1 (%)	LDH2 (%)	LDH3 (%)	LDH4 (%)	LDH5 (%)
Xianju chicken	38.52	33.82	30.14	25.67	19.18
Xiaoshan chicken	37.18	31.89	28.97	23.76	17.13
Langshan chicken	36.71	33.18	29.16	24.29	18.53

Table 3. Relative migration fonts of MDHs.

Breeds	m-MDH (%)	s-MDH1 (%)	s-MDH2 (%)
Xiaoshan chicken	9.13	14.85	23.87
Xianju chicken	9.58	15.13	21.53
Langshan chicken	8.96	14.57	22.86

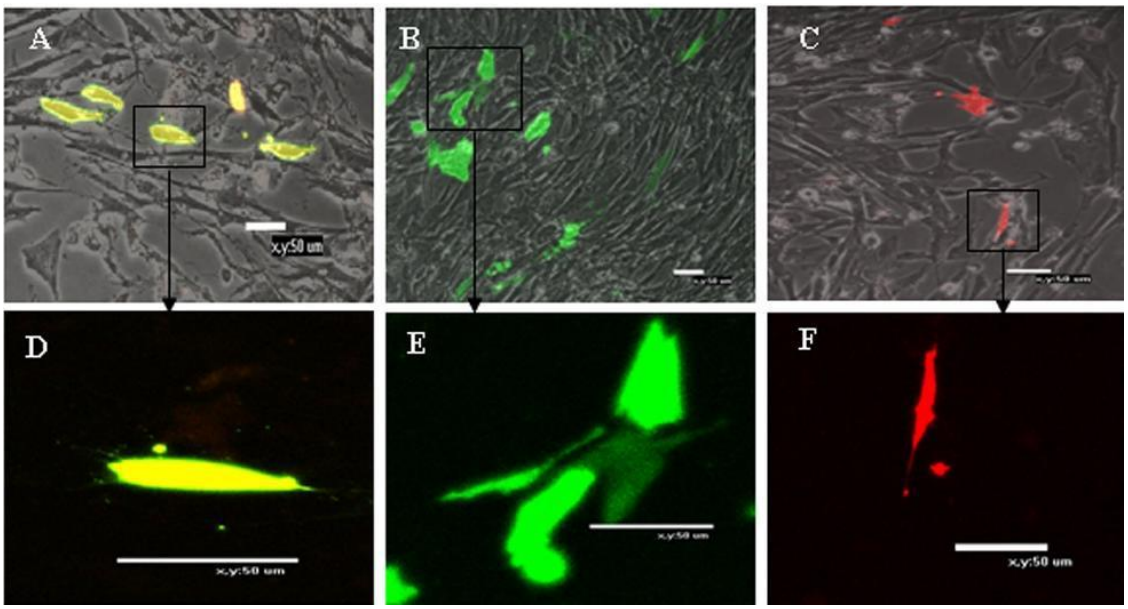

Figure 5. Expression of three fluorescent protein genes at 48 h after transfection. The photos were taken using a laser scanning confocal microscope (Nikon TE-2000-E, Japan) with the excitation wavelengths of 488 and 543 nm. a and d, b and e, c and f were the transfection results of pDsRed1-N1, pEGFP-N3 and pEYFP-N1 respectively. Bar = 50 um.

Table 4. Transfection efficiencies of three fluorescent protein genes.

Time (h)	Three fluorescent plasmids		
	pEGFP-N3 (%)	pEYFP-N1 (%)	pDsRed1-N1 (%)
24	14.18±0.31	16.26±0.72	14.74±0.32
48	21.41±0.55	20.43±0.36	20.16±0.57
72	18.63±0.27	19.21±0.31	18.78±0.19

colchicine administration should be precisely controlled in the experiments. The cells were incubated with 0.1 μg/ml colchine for 4 h when 70 to 80% confluent. Low-osmotic treatment is another major influential factor of karyotyping which aims to cell swelling and surface loosening of chromosome. So the low-osmotic duration must be tightly controlled within 40 min.

Isoenzyme polymorphisms exist in different species, different races, different individuals and even different tissues of the same species. Therefore, biochemical analysis of isozyme polymorphisms constitutes a standard method for the detection of cell line cross contamination in the current important biological resource centers throughout the world such as the American type culture collection (ATCC), European collection of animal cell cultures (ECACC) and Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) (Drexler et al., 1999). It could be distinguished if the proportion of other cells would be more than 10% (Nims et al., 1998). Isoenzyme polymorphism was commonly selected to confirm the species origin of the cells, distinguish between normal and tumor cells, and act as biochemical index of animal classification. In almost all vertebrate tissues examined, the isoenzyme LDH has been shown to consist of five distinct bands. Using isoelectric focusing at a pH range of 3.0 to 9.0, a good and clear separation of all five LDH bands in chicken organs was achieved by Heinova et al. (1999). Moreover, m-MDH and s-MDH were both obtained during early and middle development (1 to 16 days) of chicken embryos and newly hatched chicks. In this study we detected the isoenzyme patterns of LDH and MDH, and improved the ATCC starch gel electrophoresis method. The LDH and MDH isoenzyme bands of Xiaoshan chicken fibroblasts were clear and distinct with the similar isoenzyme activities to that of the original tissues.

The three enhanced fluorescent protein genes in the present study are characterized with stable structure, effective and germ-line independent expression. They are characterized with brighter fluorescences, more efficient transcription and expression than lacZ, CAT and other common fluorescent markers in animal cells (Heim et al., 1995). High transfection efficiencies were obtained at 48 h after transfection. When the transfection efficiencies decreased, intensified fluorescences could still be observed even after 2 weeks, indicating that the exogenous genes could be replicated, transcribed, translated and subsequently modified in the fibroblasts. The results provided solid theoretical and technical basis for structural genomics, functional genomics and transgenic researches concerning the Xiaoshan chicken in the future.

In summary, the Xiaoshan chicken fibroblast line was successfully established, containing biologically normal and genetically stable fibroblasts, all aspects met the cell line quality control standards of major international culture collection centers. Exogenous gene expression results supported their strong vitality and rationalized their applications in transgenic therapies and genetics. The precious genetic resource of Xiaoshan chicken were well preserved at cell level and will be used in the future as biological materials for genetics, biomedical sciences, cell and molecular biology, immunology and so on.

ACKNOWLEDGEMENTS

This research was supported by the Ministry of Agriculture of China for Transgenic Research Program (2011ZX08009-003-006, 2011ZX08012-002-06) and the central level, scientific research institutes for R & D special fund business (2011cj-9).

REFERENCES

Costa UM, Reischak D, da Silva J, Ravazzolo AP (2005). Establishment and partial characterization of an ovine synovial membrane cell line obtained by transformation with Simian Virus 40 T antigen. J. Virol. Meth., 128(2): 72-78.

Doyle A, Hay R, Kirsop BE (1990). Animal cells: living resources for biotechnology, Cambridge University Press, Cambridge, U K. pp: 81-100.

Drexler HG, Dirks WG, MacLeod RA (1999). False human hematopoietic cell lines: cross-contaminations and misinterpretations. Leukemia, 13(10): 1601-1607.

Guan W, Ma Y, Zhou X, Liu G, Liu X (2005). The establishment of fibroblast cell line and its biological characteristic research in Taihang black goat. Rev. China Agric. Sci. Technol., 7(6): 25-33.

Hay RI (1992). Cell line preservation and characterization. In: Freshney, R.I. (Ed.), Animal Cell Culture: A Practical Approach, 2nd ed. Oxford University Press, Oxford. pp: 104-135.

Heim R, Cubitt AB, Tsien RY (1995). Improved green fluorescence. Nature, 6516(373): 663-664.

Heinova D, Rosival I, Avidar Y, Bogin E (1999). Lactate dehydrogenase isoenzyme distribution and patterns in chicken organs. Res. Vet. Sci., 67(3): 309-312.

Hirofumi S, Kentaro Y, Kouichi H, Tsuyoshi F, Norihiro T, Norio N (2006). Efficient establishment of human embryonic stem cell lines and long-term maintenance with stable karyotype by enzymatic bulk passage. Biochem. Biophys. Res. Commun., 345(3): 926-932.

Ladjali-Mohammedi K, Bitgood JJ, Tixier-Boichard M, Ponce de Leon

FA (1999). International System for Standardized Avian Karyotypes (ISSAK): Standardized banded karyotypes of the domestic fowl (*Gallus domesticus*). Cytogenet Cell Genet., 86(3): 271-276.

Li L, Yue H, Ma J, Guan W, Ma Y (2009). Establishment and characterization of a fibroblast line from Simmental cattle. Cryobiology, 59(1): 63-68.

MacLeod RA, Dirks WG, Matsuo Y, Kaufmann M, Milch H, Drexler HG (1999). Widespread intraspecies cross-contamination of human tumor cell lines arising at source. In. J. Cancer., 83(4): 555-563.

Nims RW, Shoemaker AP, Bauternschub MA, Rec LJ, Harbell JW (1998). Sensitivity of isoenzyme analysis for the detection of interspecies cell line cross-contamination. *In Vitro*. Cell. Dev. Biol. Anim., 34(1): 35-39.

Qi Y, Tu Y, Yang D, Chen Q, Xiao J, Chen Y, Fu J, Xiao X, Zhou Z (2007). Cyclin A but not cyclin D1 is essential in c-myc-modulated cell cycle progression. J. Cell. Physiol., 210(1): 63-71.

Simpson RJ (2003). Proteins and Proteomics: A Laboratory Manual, Science Press, Beijing.

Sun Y, Lin C, Chou Y (2006). Establishment and characterization of a spontaneously immortalized porcine mammary epithelial cell line. Cell Biol. Int., 30(12): 970-976.

Weijun Guan (2002). The construction and identification of the cell bank of species of domestic animal on the brink of extinct. Rev. China Agric. Sci. Technol., 6(2): 66-67.

Werners AH, Bull S, Fink-Gremmels J, Bryant CE (2004). Generation and characterization of an equine macrophage cell line (e-CAS cells) derived from equine bone marrow cells. Vet. Immunol. Immunopathol., 97(3): 65-76.

Wu CX (1999). The theory and technology of the conservation of animal genetic resources-the species foundation of animal agricultural continuing development in 21 century. J. Yunnan Univ., 21(3): 7-10.

Wu HM, Guan WJ, Li H, Ma YH (2008). Establishment and characteristics of white ear lobe chicken embryo fibroblast line and expression of six fluorescent proteins in the cells. Cell Biol Int., 32(12): 1478-1485.

Xue QS (2001). The principle and technique of *in vitro* culture. Science Press. Beijing, pp. 432-444.

Zhou XM, Ma YH, Guan WJ, Zhao DM (2004). Establishment and identification of Debao pony ear marginal tissue fibroblast cell line. Asian-Austr. J. Anim. Sci., 17(5): 1338-1343.