Establishment of Big Bone chicken fibroblast cell bank and study of its biological characteristics

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ABSTRACT. A fibroblast bank of Big Bone chicken was established using tissue adherent culture method. This cell bank included 29 embryo samples, had stocks of 147 cryogenically-preserved vials each containing $2\sim3\times10^6$ cells, and met all the cell line quality control standards established by the American Type Culture Collection (ATCC). The cells cultured *in vitro* showed the typical morphology of fibroblasts. The growth curve assumed the "S" shape, and the cell population doubling time (PDT) was about 30h. Cell viability was 98.1% before cryopreservation and 96.6% after recovery. All tests for microbial contamination were negative. The isoenzyme pattern was of species specificity. The frequency of diploid cells was 91%. The transfection efficiency of three fluorescent protein genes fluctuated between 10.6% and 26.5%. These results showed that the cells cultured *in vitro* grew well and had stable genetic properties. This research thus does not only preserve the poultry genetic resources of Big Bone chicken at the cell level, but also opens new ways for preserving important genetic resources of endangered animals in the form of somatic cells.

KEY WORDS: Big Bone chicken, fibroblast cell bank, biological characteristics

INTRODUCTION

China has a total of 596 livestock and poultry species, among which 17 species have gone extinct, 336 species are subject to threats of different degrees and only 243 species are considered to be relatively safe (MA & FENG, 2002). If we do not take effective protection measures immediately, these endangered domestic animals will become extinct in the next 20 years (SCHERF, 2000). Therefore, it is imperative to launch the protection work of genetic resources of endangered livestock and poultry in China and elsewhere in the world.

At present, preservation of individual animals, semen, embryos, genomic libraries and cDNA libraries are all practical methods for preserving animal genetic resources. However, due to certain restrictions these methods still have some drawbacks. With the development and maturity of somatic cell cloning, animal somatic cells receive more attention as a supplement to the preservation of animal genetic resources (LIU et al., 2008; WU et al., 2008; LI et al., 2009a). Establishing somatic cell banks of special livestock and poultry species could not only preserve the genetic resources at the cell level effectively and permanently, but also provide valuable experimental material for research in the life sciences of these species, such as cell biology, genomics, post-genomics and embryo engineering.

The Big Bone chicken, which is also named Zhuanghe chicken, is mainly distributed throughout the Zhuanghe County in the Liaoning Province (XU & CHEN, 2003). This breed was listed among the 138 nationally-protected domestic animals by the Chinese government in 2006. It has a burly physique, broad chest, a wide, long back, sturdy legs and a plump abdomen. Its outstanding advantage is its big eggs, which have thick solid shells and low breakage rates. Moreover, it has good meat with well-distributed subcutaneous fat and fresh flavour. In a word, the Big Bone chicken is an excellent native breed raised for meat and egg.

The purpose of this study is to preserve the genetic resources of Big Bone chicken breed in the long term in the form of fibroblasts and to provide technical and theoretical references for the cellular preservation of other poultry breeds. In this study, we established a fibroblast bank of this chicken breed and checked the quality of cells during cultivation and cryo-preservation.

MATERIALS AND METHODS

Materials

The embryos of Big Bone chicken were provided by the Poultry Institute of Jiangsu Province.

Primary culture

Eight days old embryos of Big Bone chicken were rinsed 3 times with phosphate buffered saline (PBS) and the brain, eyes, limbs and viscera were removed. Then, the tissue samples were rinsed again three times with PBS and cropped into pieces of about $1mm^3$ in size. These small pieces were uniformly seeded on the bottom wall of culture flasks. The flasks were inverted and incubated at 37°C, 5% CO_2 and at saturated humidity for about 4 ~ 5h to let the tissue pieces adhere to the flasks. When tissue samples adhered firmly, the flasks were turned over and complete minimum essential medium (MEM) containing

10% foetal bovine serum (FBS) was added to produce the primary culture.

Subculture

When cell confluence (measurement for cell density in the culture) reached 80% ~ 90%, flasks were sub-cultured. The cells were rinsed three times with PBS after the culture medium had been removed, then 0.10% trypsin (m/v) was added and cells were incubated at 37°C for 5min with the flask turned over. When we observed under phase contrast microscopy that a large number of cells were retracted into a round shape and intercellular space had enlarged, the flask was shaken gently to detach the cells from the bottom wall. Complete medium was added to stop the trypsinization when most cells detached. The cells were split into new flasks at a ratio of 1:2 or 1:3 and continually incubated.

Cryogenic preservation and recovery

The culture medium was changed 24h prior to freezing. Cells were collected conventionally when the cell confluence reached 80% ~ 90%, and then centrifuged at 1000rpm for 8min. Afterwards, the supernatant was discarded and the appropriate amount of freezing medium (10% dimethyl sulfoxide (DMSO) + 50% FBS +40% MEM) was added, adjusting the cell density to 4×10^6 cells/mL. Then the single cell suspension was dispensed into 1mL aliquots in 2mL cryovials. The cryovials were placed sequentially at 4°C for 1h, at -20°C for 1h, at -70°C for 12h, and then transferred to liquid nitrogen for long-term preservation.

The vials were taken out from the liquid nitrogen and quickly thawed in a water bath of 42°C. When the ice clump had thawed to bean size, the cells were transferred into a flask with complete medium and gently pipetted into uniform suspension and cultured at 37°C and 5% CO_2. The medium was refreshed 24h later (FRESHNEY, 2000).

Microorganism detection

Cells were cultured in medium without antibiotics and tested for the presence of bacteria and fungus three days after subculturing. Following the method of DOYLE et al. (1990), cells were seeded on tryptone and wort medium, respectively, to detect possible bacteria or fungus contamination of the cells with cells deliberately infected with bacteria and fungus as positive controls.

In accordance with the DNA fluorescent staining method of ATCC, cells were stained with Hoechst 33258 and observed under the fluorescence microscope (FRESHNEY, 2006). For confirming the accuracy of the results, the mycoplasma detection kit (Roche) was used, which can identify the four most common *mycoplasma* species: *M. arginini, M. hyorhinis, A. laidlawii,* and *M. orale*.

Routine examinations for virus injuries such as plaque or barren spot were performed under the phase contrast microscope in the daily culture. In addition, some cells were randomly selected for hemadsorption tests (checking *in-vitro* for hemagglutinating viruses).

Cell viability

The trypan blue dye exclusion test was used to determine cell viability before cryo-preservation and after recovery (XUE, 2001). One thousand cells were counted to calculate cell viability.

Growth curve

Cells were seeded in 24-well plates at a density of approximately 1.5×10^5 cells/mL and cultured for 10 days. The cell density was counted every 24h, each time for three wells. The average cell density at each observation point was plotted against time and the cell population doubling time (PDT) was determined from this curve.

Chromosome analysis

According to the method of SUN et al. (2006b), chromosomal preparations were carried out using cell monolayers that reached 80% ~ 90% confluence and were in the exponential growth phase. After Giemsa staining, 100 well-spread metaphases were observed under the oil immersion lens. The relative length, arm ratio index, and centromere index were measured and calculated according to CONFERENCE (1960) and the standard of LEVAN et al. (1964) to determine the centromere type.

Isoenzyme analysis

In the process of cell culture, several breeds are cultured at the same time using the same apparatus and reagent, making cross-contamination a possibility (NIMS et al., 1998). Because isoenzymes vary within species and even within individuals or tissues of the same species, NIMS et al. (1998) suggested that even if only 10% of cells are contaminated, this could still be detected by isoenzyme analysis. Currently, analysis of isoenzyme polymorphisms has been adopted as a standard method for detection of interspecies cross-contamination by many world-famous culture collection centres such as ATCC, ECACC and DSMZ (DREXLER et al., 1999). In animals, lactic dehydrogenase (LDH) and malic dehydrogenase (MDH) are important enzymes of the metabolic pathways glycolysis and tricarboxylic acid cycle. The electrophoretic mobilities of these two isoenzymes, LDH and MDH, were determined using polyacrylamide gel electrophoresis (FRESHNEY, 2000) with some modifications. Isoenzyme patterns of Big Bone chicken were obtained and compared with other breeds simultaneously cultured in the laboratory. The technical details of the procedure were as follows:

Sample preparation

Cells were harvested and rinsed three times with PBS, then the protein extraction solution was added, adjusting the cell density to 5×10^7 cells/mL. After centrifugation at 4°C and 1000rpm for 2min, the supernatant was aliquoted and stored at –70°C.

Electrophoresis

Every sample well was loaded with 20 ~ 50μL loading solution, which was prepared by mixing the sample with 40% sucrose solution (1:1) and 2.5μL bromophenol blue

solution. Then the gel electrophoresis took place at 4°C and 120V. When the bromophenol blue migrated into the gel below the spacer, the voltage was changed to 220V. When the bromophenol blue had migrated to the bottom (0.5 ~ 1cm), the electrophoresis was finished.

Staining

The spacer gel was cut off and the gel rinsed twice with distilled water. After that, the gel was placed at 37°C for 2h to stain in the dark inside an incubator. Afterwards photographs were taken (HE & ZHANG, 1999).

Transfection of fluorescent protein genes

Using the lipofectamine-mediated method with some modifications (TSUCHIYA et al., 2002), three fluorescent protein genes including DsRed1, EGFP and EYFP were transfected into the in-vitro fibroblasts of Big Bone chicken with 2μg plasmid DNA (pDsRed1-N1, pEGFP-C1, pEYFP-N1, respectively) and 6μL lipofectamine (Lipofectamine 2000). Transfection was conducted when cell confluence reached 60% ~ 70%.

Six to eight hours after transfection, the transfection solution was discarded and complete MEM containing 10% serum was added. The transfection results were observed at 24h, 48h, 72h, 1 week, and 2 weeks after transfection, under a laser scanning confocal microscope. The transfection efficiency was calculated by choosing 10 visual fields in every well and counting the total cells and the positive cells in every visual field. The distribution of fluorescent proteins in positive cells was determined by detecting the fluorescent intensity under the high power lens of the laser scanning confocal microscope.

RESULTS

Cell morphology

In primary culture, the cells grew out of the edge of tissue blocks 1 day after seeding, and soon after the cells rapidly multiplied outwards around tissue blocks in a radiated manner. After 2 to 3 days, cells spread along the flask wall and the confluence reached 80% ~ 90%. In primary culture, fibroblasts mingled with epithelial cells. With sequential passages, epithelial cells were excluded from the culture, and we obtained pure fibroblasts. Primary cells had typical fibroblast morphology, showing the shape of a shuttle or irregular triangle with plump cytoplasm and a clear nucleolus (Fig. 1A). After subculture, cells grew well and showed the flame-like or whirlpool-like shapes (Fig. 1B).

Microorganism detection

Detection of bacteria and fungus

In the actual test group and negative control, the medium was clear, showing no turbidity or other abnormal changes. In contrast, apparent turbidity and precipitation was observed in the positive control group.

Detection of mycoplasmas

After being stained with Hoechst 33258, the cells showed smooth surfaces, round or oval nuclei with blue fluorescence, and no filamentous blue fluorescence around nuclei when observed under fluorescence microscope (Fig. 2). This result indicates that the *in vitro* cultured fibroblasts were free of *mycoplasma*. To reconfirm this result, the mycoplasma detection kit (Roche) was used, also with a negative outcome.

Detection of viruses

Virus injuries were not found in cells under the phase contrast microscope, and also the result of hemadsorption test was negative.

Cell viability

The viability detected by trypan blue staining was 98.1% before cryo-preservation and 96.6% after recovery, with a non-significant difference ($P>0.05$). Cells after recovery grew well and adhered to the flask wall in 30 ~ 60min, and had spread along the bottom wall by 48h later. Growth velocity and morphology of cells before cryo-preservation and after recovery showed no significant difference.

Growth curve

The growth curve of Big Bone chicken assumed an “S” shape. Thus, cells experienced the latent phase, the exponential growth phase, the stationary phase and the decline phase, and the PDT was about 30h (Fig. 3). In addition, the growth curve reflected the dynamic growth state of cells. Cells were at the latent phase within 0 ~ 2d and reached the exponential growth phase afterwards. Cell density was at its maximum at day 4. From the 5^{th} day onwards, cells entered the stationary phase and began to degenerate and die after day 7.

Karyotype analysis

This study analyzed 100 well-spread metaphases of the P1 ~ P3 fibroblasts of Big Bone chicken. From the karyotype and chromosome parameters, no abnormality in chromosome structures was detected (Fig. 4, Table1). The frequency of diploid cells was 91%. Generally, if cells with a certain chromosome number account for more than 75% of the total, this chromosome number can therefore be regarded as the number of diploids. Therefore, it could be confirmed that the cultured cells were stable diploid and the chromosome number of diploid was 78, in agreement with studies on other chicken breeds.

Isoenzyme analysis

In the LDH isoenzymogram, four bands representing LDH1, LDH2, LDH3 and LDH4 were distinct from the anode to the cathode. The LDH activity of the four breeds was similar, LDH2, LDH3 and LDH4 displaying heavy shading and LDH1 light shading (Fig. 5). In the MDH isoenzymogram, there was a cellular solute form (s-MDH) near the anode and a mitochondrial form (m-MDH) near the cathode (Fig. 6). Relative enzyme mobility of the four breeds was similar although small differ-

ences were visible. These results indicate that each breed had its characteristic bands and that there was no cross-contamination between different breeds.

Transfection analysis of three fluorescent protein genes

The three fluorescent genes pDsRed1-N1, pEGFP-C1 and pEYFP-N1 are widely used in living cells and organisms as marker genes to dynamically observe the expression, distribution and function of target proteins (HEIM et al., 1995; CHENG et al., 2003). Here, the expression of these genes in the Big Bone chicken fibroblasts was observed at 24h, 48h, 72h, 1 week, and 2 weeks after transfection under the laser scanning confocal microscope. The results showed that the highest number of cells with fluorescent signals, the strongest fluorescence intensity and the highest transfection efficiency appeared 48h after transfection (Fig. 7, Table 2).

Twenty four hours after transfection, the exogenous genes had begun to become expressed in some cells and all three experimental groups showed obvious fluorescent signal. Besides, a few positive cells became shrunken, deciduous and disintegrated. Forty eight hours after transfection, the number of positive cells increased in all three groups, many bright dots could be seen in the visual field, and the transfection efficiency reached its maximum at this time. Seventy two hours after transfection, there was no further increase in positive cells, and in most of the positive cells fluorescent proteins were not expressed in the vacuoles. From the 7th day onwards, the number of positive cells decreased and fluorescence intensity weakened gradually. However, two weeks later, there were still a small number of cells expressing fluorescence.

The red fluorescence was distributed in dots around the nucleus (Fig. 7D, arrow a; b) and uniformly in other parts (Fig. 7D, arrow c) and showed the weakest fluorescence intensity. Intensity of the green fluorescence was the strongest, with a stronger expression in the nucleus (Fig. 7E, arrow d) and some vacuoles, but without expression in the cytoplasm (Fig. 7E, arrow e). The yellow fluorescence had a slightly stronger expression in the nucleus (Fig. 7F, arrow f) than in the cytoplasm, where a ribbon-like texture with a large number of non-expressed vacuoles and empty strip zones (Fig. 7F, arrow g) appeared.

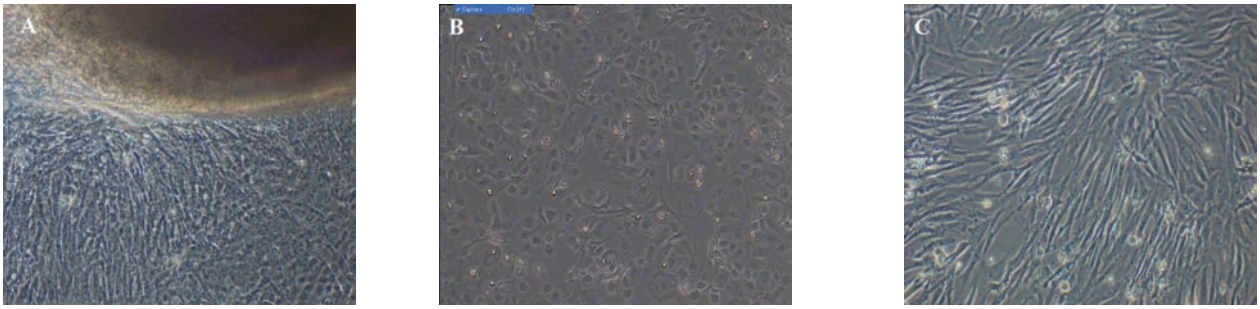

Fig. 1. – Primary and subcultured cells of Big Bone chicken (100×). A: Primary Cells grew out from the edge of tissue blocks at 1d after seeding. B: Primary cells: fibroblasts mingled with epithelial cells. C: Pure subcultured fibroblasts.

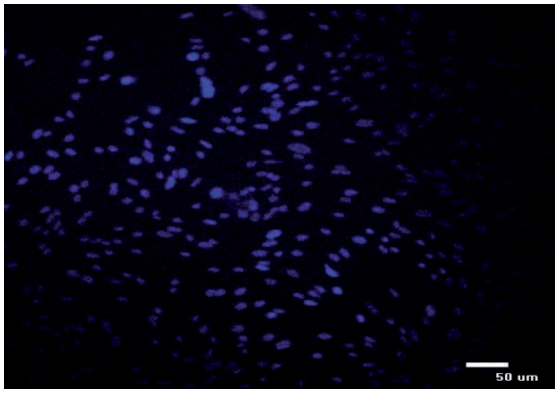

Fig. 2. – Detection of mycoplasmas by Hoechst33258 staining. This figure shows *mycoplasma* negative fibroblasts of Big Bone chicken.

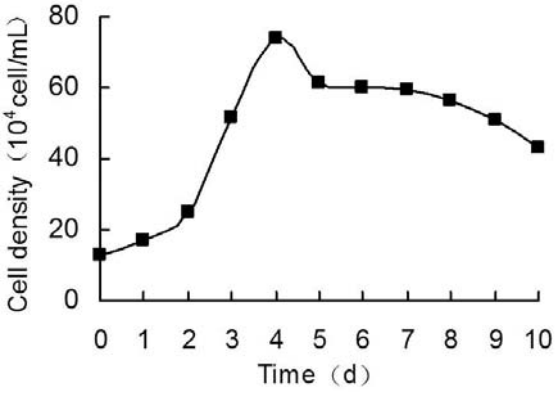

Fig. 3. – Growth curve of Big Bone chicken fibroblasts.

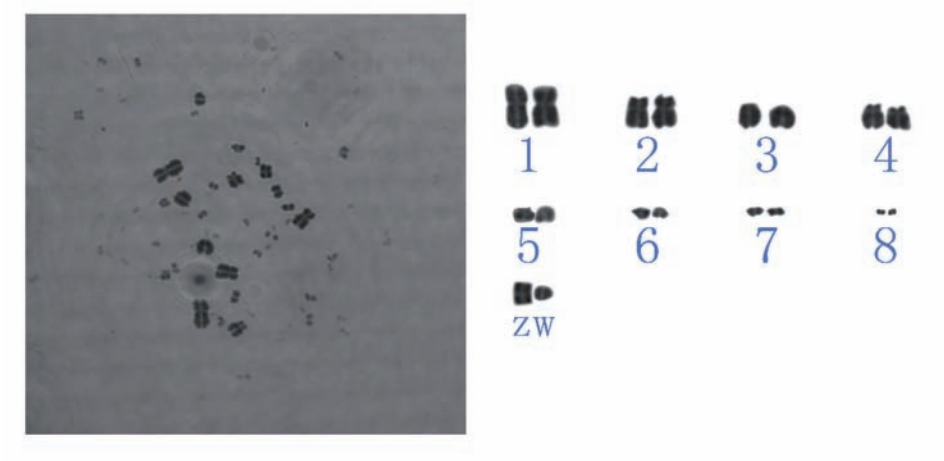

Fig. 4. – Chromosome karyotype of Big Bone chicken ZW (♀)

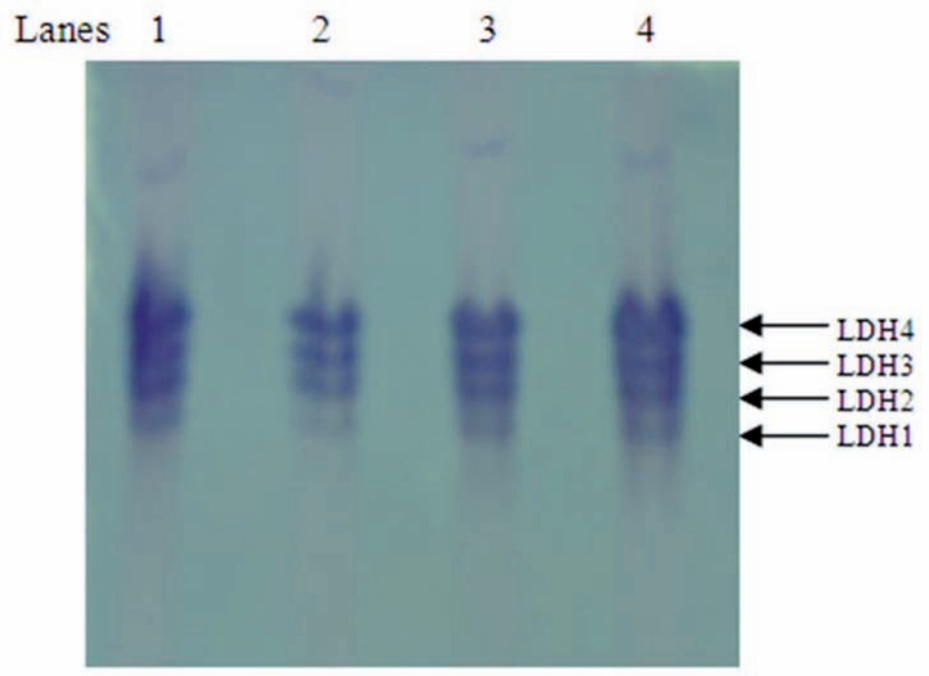

Fig. 5. – LDH isoenzyme typing of four chicken breeds. Lane 1: Youxima chicken. Lane 2: Chinese Game chicken. Lane 3: Qingkedan chicken. Lane 4: Big Bone chicken.

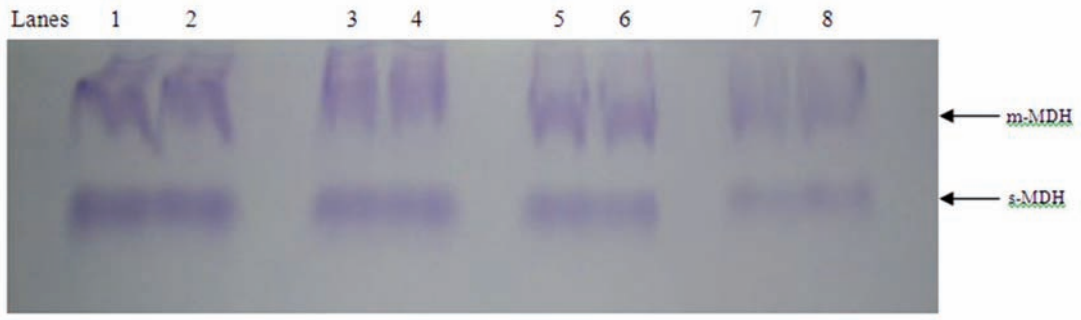

Fig. 6. – MDH isoenzyme typing of four chicken breeds. Lanes 1 & 2: Youxima chicken. Lanes 3 & 4: Chinese Game chicken. Lanes 5 & 6: Qingkedan chicken. Lanes 7 & 8: Big Bone chicken.

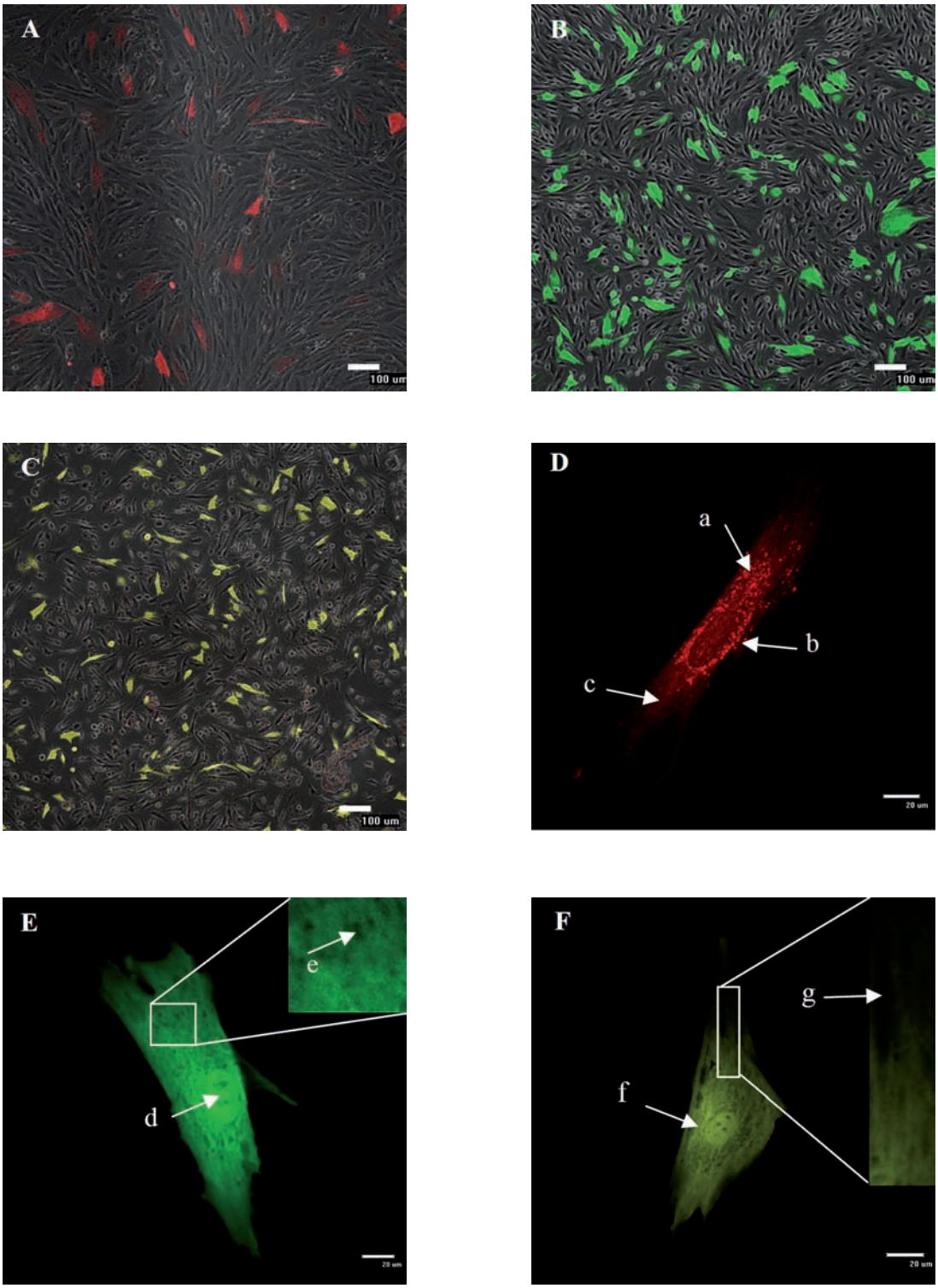

Fig. 7. – The expression of DsRed1, EGFP and EYFP in fibroblasts of Big Bone chicken. A & D: The transfection results of DsRed at 48h (100×, 400×). B & E: The transfection results of EGFP at 48h (100×, 400×). C & F: The transfection results of EYFP at 48h (100×, 400×). DsRed1, EGFP and EYFP are the red, green, and yellow fluorescent protein genes being located in plasmid DNA of pDsRed1-N1, pEGFP-C1, pEYFP-N1, respectively. D, E, F: The distribution of the three fluorescent proteins in cells. Arrows a and b show dot-like, red fluorescence while arrow c indicates the uniform fluorescent signal. Arrow d points towards the stronger green fluorescence in the nucleus and arrow e shows the vacuoles without fluorescence. Arrow f indicates the stronger yellow fluorescence in the nucleus and arrow g the empty strip zones without fluorescence.

TABLE 1

Chromosome parameters of Big Bone chicken (♀)

Number	Relative length (%)	Arm ratio	Centromere index (%)	Centromere type
1	21.73	1.27	44.05	M
2	16.52	1.71	36.90	SM
3	12.52	∞	0	T
4	10.95	1.86	34.97	SM
5	8.70	∞	0	T
6	7.00	∞	0	T
7	6.61	∞	0	T
8	5.39	∞	0	T
Z	10.61	1.03	49.26	M
W	7.04	1.19	45.66	M

$$\text{* relative length} = \frac{\text{a single chromosome length}}{\text{all haploid chromosome length} + \text{chromosome X length}} \times 100\%$$

$$\text{arm ratio index} = \frac{\text{the long arm length}}{\text{the short arm length}}$$

$$\text{centromere index} = \frac{\text{the short arm length}}{\text{the chromosome length}} \times 100\%$$

According to the Levan criteria for classification, the ones with arm ratio index in the range of 1.0 ~ 1.7 and centromere index in the range of 50.0% ~ 37.5% are metacentric chromosomes (M), the ones with arm ratio index in the range of 1.7 ~ 3.0 and centromere index in the range of 37.5% ~ 25.0% are submetacentric chromosomes (SM), the ones with arm ratio index >7.0 and centromere index in the range of 12.5% ~ 0.0% are telocentric chromosomes (T).

TABLE 2

Transfection efficiencies of three fluorescent protein genes in Big Bone chicken fibroblasts

Transfection time (h)	pDsRed1-N1 (%)	pEGFP-C1 (%)	pEYFP-N1 (%)
24	10.6	22.9	19.6
48	17.8	26.5	23.4
72	16.2	25.1	22.6

DISCUSSION

Cell culture

Tissue adherent culturing method and enzyme digestion are two frequently used methods for primary cell culture (FRESHNEY, 2006). The concentration and action time changes, to some extent, in enzyme-injured cells and thus affects normal development of cells severely (LI et al., 2009b; YANG et al., 2009). In comparison, the tissue adherent culturing method is simple and feasible, avoiding the injury of enzyme digestion. Furthermore, cells that are cultured using this method have good homogenicity and strong vitality. We used the tissue adherent culturing method to primary culture fibroblasts of Big Bone chicken, and managed to grow cells well with typical fibroblast morphology. Passage changes the growing environment of cells, and trypsinization causes some injuries (RYAN & MAXWELL, 1986; PARK et al., 2006; CHO et al., 2008). Consequently after high numbers of passages, cells usually vary in their biological characteristics, especially in genetic parameters (SUN et al., 2006a). For preservation, times of passage should be decreased considerably, keeping the number to a maximum of five passages. In addition, for ensuring the cell viability after recovery, the freezing density should be more than 1×10^6 cells/mL (FRESHNEY, 2006).

Because the tolerance of epithelial cells and fibroblasts to trypsin is different when trypsinized, fibroblasts can be detached from the flask wall more readily and adhere more quickly after passage, whereas most epithelial cells need the support of growing matrices like collagen or other extracellular matrix components. Otherwise, they fail to adhere in a short time and are easily shed off when using gentle mechanical agitation. Because of these differences, fibroblasts will quickly outgrow their epithelial counterparts. Consistent with earlier studies, pure fibroblasts of Big Bone Chicken were obtained here after two to three passages, and in order to minimize the injuries caused by trypsinization, passage numbers were limited to five to guarantee the best vitality (GUAN et al., 2005; ZHOU et al., 2005).

Micro-organism detection

Micro-organism contamination is a common problem in cell culture and may originate from air, equipment, operation, serum and tissue samples. Bacteria and fungus grow rapidly, consume nutrients, produce toxins that inhibit cell growth, and kill cells within a short time (FRESHNEY, 2000). After normal culturing for five days, we did not observe, by naked eye and phase contrast

microscopy, any turbidity or other evidence of contamination from bacteria and fungi. Using indirect DNA fluorescence staining, a convenient and reliable protocol, the existence of *mycoplasma* was also ruled out (MASOVER & BECKER, 1998; FRESHNEY, 2000). These results suggest that the Big Bone Chicken fibroblasts are free of infections from microorganisms and suitable for genetic resource preservation.

Cell viability before freezing and after recovery

Generative cells, somatic cells, stem cells, zygotes and embryos can all be cryopreserved in cell banks (GUAN et al., 2007). In the freezing process when the temperature goes down to 0°C, cells suffer from physical and chemical injuries (HAN & BISCHOL, 2004). Adding cryoprotectant during freezing can prevent ice crystals and injuries caused by them. In this study, we prepared the freezing solution as 10% DMSO, 50% FBS, 40% MEM. After conventional harvest of cells, an appropriate amount of freezing solution was added to prepare the cell suspension. Then cells were placed at 4°C for 1h, at -20°C for 1h, at -70°C for 12h in order, and transferred to liquid nitrogen for long-term preservation. For recovery, the freezing tube was taken out from liquid nitrogen and thawed quickly in a 42°C water bath. The key point for cryogenic preservation and recovery of cells was programmed freezing and quick thawing. We found a difference between cell viabilities before cryo-preservation and after recovery, which could probably be related to injuries from freezing and recovery. Nevertheless, the average viability after recovery was 96.6% which indicated that freezing and recovery had little influence on the overall cell viability. Thus, it seems feasible to preserve fibroblast cell banks of Big Bone chicken using such long-term cryo-preservation techniques.

Growth curve

After seeding, there was a latent phase of about 48h, which was probably the adaptive phase during which cells recovered from injuries caused by trypsinization. Afterwards, cells entered the exponential growth phase. Finally, from the 4th day on, after reaching the maximum cell density, cell growth became slow and stopped while some cells were floating. This was probably the stationary phase. The available growing space was gradually occupied, which generated contact and density inhibition, eventually causing the culture to enter the stationary phase and ultimately, the decline phase.

Karyotype analysis

Chromosome number and karyotype are the basis of cytogenetics. They are reliable indices for identifying the taxonomic and sexual origin of a cell line, and also allow assessing whether the cell line is stable or variable. Thus, they represent the characteristics of a certain cell line and are not only used to analyse genetic stability but also to test for cross-contamination. According to the international karyotype standard, poultry contain eight pairs of macrochromosomes, the sex chromosomes Z and W, and 30 pairs of microchromosomes (LADJALI-MOHAMMADI et al., 1999). Because the purpose of our experiment is to conserve the genetic resources of Big Bone chicken, the fibroblasts must maintain their diplont character similar to *in vivo* cells. We improved the freezing technique and decreased the passages necessary to obtain a stable diploid cell line. Karyotypic analysis was conducted on 100 cells, and our results indicate that the Big Bone chicken cells are diploid with nine pairs of macrochromosomes and 30 pairs of microchromosomes; 91% of the cells examined were diploid. A small percentage of cells displayed abnormal chromosomes, presumably as a result of chromosome loss or overlap during preparation or chromosomal damage during culture and passage *in-vitro*. Because most chromosomes are microchromosomes, which are easily missed in the process of slide making, chromosome number and morphology of chicken karyotypes are difficult to determine.

Isoenzyme analysis

We analysed the isoenzyme patterns of LDH and MDH in our study and obtained clear, distinct bands.

ZENG & CHEN (1997) retrieved five to eight clear bands in the electrophoretic analysis of eight kinds of chicken tissues; in the pectoral muscle, five bands were found, among which LDH4 and LDH5 predominated over others. This pattern might coincide with characteristics of these breeds, which are not good at flying. LIU & YU (1997) analysed LDH isoenzymes in the cardiac muscle, in the liver and from blood samples of Chinese *Gallus gallus* using thin layer isoelectric focusing electrophoresis, and found five bands in all three types of tissues. In contrast, we obtained four bands solely from fibroblasts of Big Bone chicken embryos. This could be either explained by breed difference or the heterogenous origins of the fibroblasts. However, the LDH bands of the four breeds were clear and distinct, thus suggesting that the Big Bone Chicken fibroblast cell line we established is not contaminated by cells from other breeds in the same lab.

Similarly to mammals, MDH of birds includes a cellular solute form (s-MDH) and a mitochondrial form (m-MDH), and the migration rate of the former is higher than that of the latter (JOHN & CHARLES, 1987). In our experiment, there were two MDH bands, which is in accordance with the MDH activity of chicken embryos during the first 16 days of development (MA & QIU, 1995), indicating that cells cultured *in-vitro* have similar MDH activity to biological tissues.

Expression of exogenous genes

The three enhanced fluorescent protein genes screened in our experiment have stable structures, high-level expression and are not dependent on the germ-line (HEIM et al., 1995; BAIRD et al., 2000; CARRIE et al., 2003). Concentrations of DNA and lipofectin, the DNA incubation time, lipofectin-plasmid DNA complexes, and the presence of serum all can affect the efficiency of transfection, as shown by research on Vero cells, HeLa cells and various other cell lines (TSENG et al., 1999; RUI et al., 2006; SHU et al., 2007). In our experiment, the transfer efficiency varied from 10.6% to 26.5% with the optimized ratio of plasmid and lipofactamine. After 2 weeks a few dispersed positive cells remained showing fluorescent

signals. This means some that cells gain the ability to express DsRed, EGFP and EYFP stably. After transfection, cells at different dividing phases could be seen, and the growth of transfected cells was not different to the control group, while some transfected cells changed their morphology. This result showed that the transfected cells were to some extent not affected by fluorescent protein. It is very possible that when cells accumulate enough exogenous fluorescent protein, morphologically-changed cells will greatly increase, which leads to the growth changes of cells finally. Furthermore, fluorescent proteins were not homogeneously distributed; for example, EGFP was mainly found in the nucleus and DsRed expressed surrounding the nuclear membrane and forming a red ring profile (see Fig. 7). These differences of fluorescent proteins are typical for molecular tags (labelling) for investigating the functional role of the genes of interest. From these results, it can be concluded that Big Bone chicken fibroblasts possess the potential to be used in transgenic studies.

CONCLUSIONS

In conclusion, our results strongly indicate that the established fibroblast cell line of Big Bone chicken embryos has stable genetic properties and normal biological characteristics. Moreover, we have been able to preserve the unique genetic resource of the Big Bone chicken at the cellular level, and thus provide effective technical and theoretical references and suitable biological material for related studies.

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