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Establishment and Biological Characteristics of Qing Kedan Chicken Embryonic Fibroblast Line for Genetic Resource Conservation

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Abstract: A fibroblast cell line of Qing Kedan chicken was successfully isolated, purified and cryopreseved through direct culturing of explants, serial passage and cryogenic techniques. Assays for biological characteristics were conducted and the results suggested that: the cells cultured with a Population Doubling Time (PDT) of about 20 h, constituted a typical fibroblast cell line in morphology; tests for microbial contamination regarding bacteria, fungi, viruses and mycoplasmas were exclusively negative; Lactate Dehydrogenase (LDH) and Malate Dehydrogenase (MDH) polymorphism analysis disproved the existence of cross-contamination from other cell lines; diploid cells making up 85-93% of the population verified the hereditary stability of the fibroblast line. pEGFP-N3, pEYFP-N1 and pDsRed1-N1 were transfected into the Qing Kedan chicken embryonic fibroblasts with transfection efficiencies between 12.6 and 39.7%. To locate their cellular distribution, transfected cells were observed at 24, 48 and 72 h with confocal microscopy and the fluorescences could be observed throughout the cytoplasm and nuclei of positive cells except in cryptomere vesicles. This research preserved the precious germplasm resource of Qing Kedan chicken at cell level and hence served as an enlightening reference for those of other poultry in the world.

Key words: Qing Kedan chicken, embryonic fibroblast line, genetic resource conservation, somatic cell cryopreservation, China

INTRODUCTION

Genetic resources an indispensible basis for sustainable development and the maintenance of ecological balance are responsible for the security of the whole society, the extirpation of which would by any means, lead to severe problems currently and tomorrow and extensively undermine the prosperity of the modern world. Extinction of any species would mercilessly and eternally, forfeit human beings opportunities to investigate the unknown biological and molecular mechanisms concerning them and worse still the desire and attempt would never be fulfilled to revive them through somatic cell cloning. Faced with a serious situation of diminishing biodiversity, it is high time to launch a campaign to conserve endangered species currently, the pragmatic measures of which involve the preservation of individual animals, embryos, semen, genomic libraries, cDNA libraries, etc. (Guan *et al*., 2005). Other than above mentioned techniques, rapid-developing cloning technique would also enormously benefit the preservation of animal resources (Wu, 1999).

Qing Kedan chicken, originating in the provinces of Hubei, Jiangxi, etc. which literally means cyan-shell egg layers has been domesticated distinctively in China for centuries. According to the serum assay by the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, one of the most influential authorities on biological sciences in China, Qing Kedan chicken pertains to an infrequent genotype-specific group with tremendous intrinsic medicinal value and is categorized into four major subbreeds, namely, yellow feathered, black feathered, white feathered and iridescent feathered. It is generally benign-tempered, gregarious, disease resistant, harsh environment adaptive, easily fed up with a routine similar to that of local chicken and feeds mainly on corn and cereal, especially fond of grass, greengrocery, leaves, carrot, etc. (http://www.greeneggs.cn/layers.html).

The aim was to establish a chicken embryo fibroblast cell line from the Qing Kedan breed and study its growth characteristics, karyotype, isoenzyme polymorphism and expression of exogenous fluorescent genes.

MATERIALS AND METHODS

Primary cell culture, cryopreservation and recovery: About 8 days old embryos, isolated from Qing Kedan chicken eggs (Jinagsu Institute of Poultry Science, Jiangsu, China) were rinsed three times with Phosphate

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Buffered Saline (PBS, pH 7.4), chopped into 1.0 mm^3 pieces and then seeded on the bottom of a tissue cultivation flask containing MEM (Gibco, USA)+10% (v/v) fetal bovine serum (Hyclone, USA) in incubator at 37°C with 5% CO_2 (Zhou *et al*., 2004). Primary cells were subcultured into more flasks when 80-90% confluent in the ratio of 1:2 or 1:3 (Freshney, 2000).

Cell viability: Viabilities before cryopreservation and after recovery were calculated through Trypan Blue (Promega, USA) exclusion test as described by Butler (1999). The cells were seeded in 6 well microplates, 1000 of which were counted under a microscope for calculation (Qi *et al*., 2007).

Cryopreservation and reseeding: Media were refreshed 24 h prior to cryopreservation to guarantee sufficient nutrition. Monoplast suspension was prepared by harvesting cells with 0.1% (w/v) trypsin. After centrifuged at 168×g, room temperature for 8 min, the supernatant was discarded and the pellet was resuspended at a concentration of 3.0×10^7 cells per milliliter in a solution containing 10% (v/v) Dimethyl Sulfoxide (DMSO) (Sigma, USA), 50% (v/v) FBS (Hyclone, USA) and 40% (v/v) MEM (Gibco, USA) and then subpackaged into sterile cryovials labeled with the species, gender, cryopreservation serial number and the date. The vials, sealed and kept at 4°C for 20-30 min to equilibrate the DMSO were transferred subsequently into liquid nitrogen after programmed cryopreservation for long term storage (Ren *et al*., 2002). Before reseeding, the cells were removed from liquid nitrogen and thawed at 42°C until a small amount of ice left in cryovials. Afterwards, the cell suspension was transferred into earlier aliquoted complete MEM medium and centrifuged at 168×g, room temperature for 8 min to eliminate the DMSO and the pellet was resuspended in fresh medium. Finally, the suspension was seeded into sterile flasks and cultured at 37°C with 5% CO_2. Media were renewed 24 h later (Freshney, 1992).

Cell growth curve: The proliferation properties of Qing Kedan chicken embryonic fibroblast line *in vitro* were best epitomized by its Population Doubling Time (PDT). The cells, harvested and seeded in a 24 well microplate at a concentration of 1.5×10^4 per well were cultivated upto7 days and counted at intervals of 24 h. Mean values were adopted to plot a growth curve and to calculate the PDT correspondingly (Sun *et al*., 2006).

Microorganism detection test for bacteria, fungi and yeast: Cells were cultivated in an antibiotic free medium and checked out for contamination 3 days later according to the protocol described by Doyle *et al*. (1990).

Test for viruses: Under normal culture conditions, cells were selected randomly for cytopathogenic examination with phase-contrast microscopy according to Hay (1992) hemadsorption protocol.

Mycoplasma detection: According to the protocol from ATCC, cells were cultured in an antibiotic free medium for at least 1 week and then fixed and stained with Hoechst 33258 with the protocols for fluorescent staining of DNA as described by Masover and Becker (1998). The results were further confirmed with an ELISA mycoplasma detection kit (Roche, Lewes, East Sussex, UK) which was designed to identify the four major types of mycoplasmas, namely *M. arginini*, *M. hyorhinis*, *A. laidlawii* and *M. orale*.

Karyotype and banding analysis: Cells at exponential phase were cultured in media with 0.1 μg mL^{-1} colcemid (Gibco/BRL) at 37°C for 4 h harvested centrifuged at 168×g, room temperature for 8 min and with the supernatant removed, resuspended in 0.075 mol L^{-1} KCl prewarmed to 37°C. After incubation at 37°C for 30 min, the cells were pelleted again, fixed with methanol and acetic acid mixture in the ratio of 3:1 (v/v) at 4°C and washed 3 times with the fixative. Microslide preparation and chromosome staining were performed as described by Suemori *et al*. (2006). Representative chromosome sets were photographed and analyzed. The percentage of the diploid was calculated according to results from 100 spreads. Several parameters were calculated as follows:

$$\text{Arm ratio} = \frac{\text{Long arm length (q)}}{\text{Short arm length (p)}}$$

$$\text{Centromere index} = \frac{\text{Short arm length}}{\text{Chromosome length}}$$

$$\text{Relative length} = \frac{\text{Single chromosome length}}{\text{Total autosome length} + \text{X-chromosome length}}$$

Isoenzyme analysis: Isoenzyme patterns of Lactate Dehydrogenase (LDH) and Malate Dehydrogenase (MDH) were obtained through vertical slab non-continuous Polyacrylamide Gel Electrophoresis (PAGE) assay. Protein extraction solution (0.9% Triton X-100, 0.06 mmol L^{-1} NaCl, NaCl: EDTA in mass ratio 1:15) was added into harvested cells at a concentration of 5.0×10^7 ML^{-1} and the supernatant was stored in aliquots at -80°C after the mixture centrifuged at 1200×g, 4°C for 2 min. About 40% (w/v) sucrose solution and the samples

were blended in the ratio of 1:1 (v/v) and then loaded in specific lanes of the polyacrylamide gel (He and Zhang, 1999). Different mobility patterns, reflected by the relative mobility front (RF) were obtained by calculating the ratio of the migration distances of the isoenzyme bands to that of bromophenol blue.

Expression of three kinds of fluorescent proteins in Qing Kedan chicken embryonic fibroblast line: To achieve the highest transfection efficiency with trivial cytotoxicity, optimal conditions were screened through variation of cell densities and plasmid DNA (Clontech, Japan) and Lipofectamine 2000 (Invitrogen, USA) concentrations according to the lipofectamine medium protocols as described by Escriou *et al*. (2001) and Tsuchiya *et al*. (2002), the cells observed at 24, 48 and 72 h after transfection were detected for the expression of three kinds of fluorescent proteins with excitation wavelengths of 488 (pEGFP-N3), 488 and 543 (pEYFP-N1) and 543 nm (pDsRed1-N1), respectively. For each experimental group, images were captured from 10 visual fields. Total and positive cells in each field were counted under a confocal microscope (Nikon TE-2000-E, Japan) to calculate transfection efficiencies. The cytotoxicities of the exogenous genes on the cells were measured in the term of viabilities through Trypan Blue exclusion test.

RESULTS

Cell morphology: Qing Kedan chicken embryonic fibroblasts, migrating from the tissue pieces 2-3 days after explantation, exhibited typically fibrous and fusiform morphologies with centrally located oval-shaped nuclei (Fig. 1a). These cells were subcultured till 80-90% confluent. Due to their stronger proliferation potential, fibroblasts would after serial passage, gradually and eventually, overwhelm their epithelial counterparts adulterated and show characteristically multipolar or bipolar appearances (Fig. 1b).

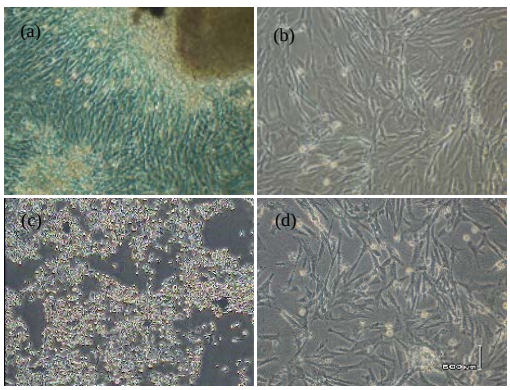

Fig. 1: The culture of Qing Kedan chicken embryonic fibroblasts; a) Primary cells of embryonic explants; b) subcultured fibroblasts; c) dissociate cells treated with trypsin; d) fibroblasts 24 h after recovery

Cell viability: Cells were harvested and cryopreserved after 3-4 passages. The viabilities, formulated as mean±SD were 97.8±0.9% and 95.4±0.8% before (Fig. 1b) and after (Fig. 1d) cryopreservation, respectively the difference between which was not significant. The results suggested that the cells were viable under these culture conditions and were seldom injured during cryopreservation.

Growth curve analysis: The growth curve of Qing Kedan chicken embryonic fibroblast line exhibited a typical S shape (Fig. 2a) with a Population Doubling Time (PDT) of approximately 20 h. The lag time or latency phase, corresponding to the adaptation and recovery period of the cells against trypsin damage was negligible after subculture. Afterwards, they proliferated rapidly and entered exponential phase. As the cell density increased, proliferation receded as a result of contact inhibition. Cell population was maximal at the 4th day followed by a plateau phase since the 5th day.

Microorganism detection: Tests for contamination with bacteria, fungi and yeasts were negative no microorganisms were observed in the culture media. The results of the hemadsorption assay ruled out the possibilities of viruses presence. According, to the observation under a confocal microscope after staining with Hoechst 33258, potentially the most effective and frequently used method for detecting mycoplasma contamination (Barile and Rottem, 1993), it was indicated that the newly established cell line with its cells nuclei appearing as clear blue ellipses was mycoplasma negative (Fig. 2b). The positive control in the results of Li *et al*. (2009) could serve as a reference for the validity of the negative result obtained in this research.

Karyotype analysis: A typical genome of Qing Kedan chicken, 2n = 78 was composed of 10 and 29 pairs of microchromosomes whose sex chromosome type is ZZ (♂) (Fig. 2c) or ZW (♀). The parameters of the chromosomes are shown in Table 1. The average chromosome number was counted from 100 spreads from the first, seecond and fourth passages with frequencies of diploid cells 93, 91 and 85%, respectively. Aberrations in chromosome numbers had a tendency to be magnified as serial passage stepped further indicating that culture *in vitro* would ineluctably disturb the hereditary stability of the cells to some extent yet it was also suggested that the cell line was reproducibly diploid.

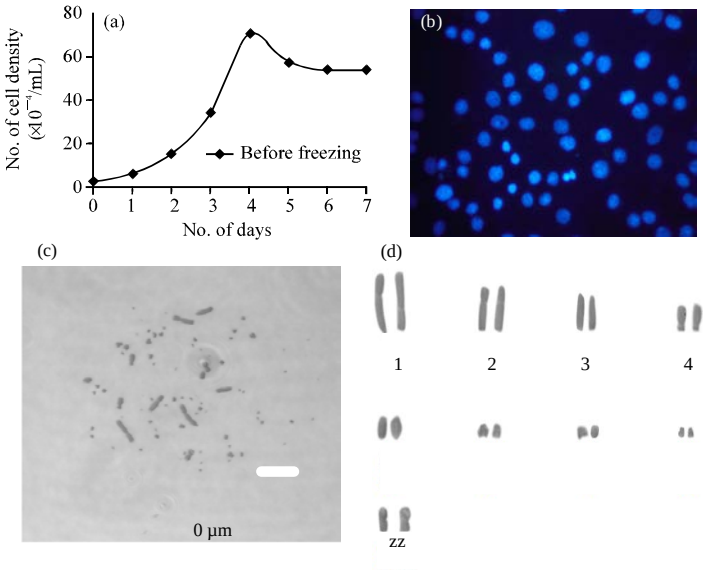

Fig. 2: The characterization of Qing Kedan chicken embryonic fibroblast line. a) The growth curve of Qing Kedan chicken embryonic fibroblasts before cryopreservation, cells were enumerated with a hemocytometer. Each value represents the mean of three independent experiments; b) Mycoplasma contamination detection for Qing Kedan chicken embryonic fibroblasts stained with Hoechst 33258; c) Chromosomes at metaphase (left) and karyotype (right) of Qing Kedan chicken (♂)

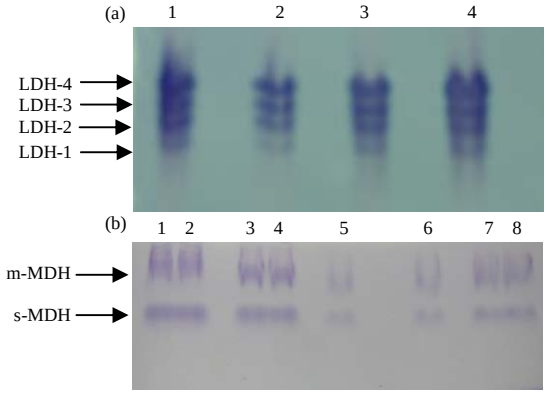

Fig. 3: Lactate Dehydrogenase (LDH) and Malate Dehydrogenase (MDH) zymotype for different subspecies. a) 1: You Xima chicken; 2: Chinese Game chicken; 3: Qing Kedan chicken; 4: Dagu chicken. b) 1 and 2: Silkie Bantam; 3 and 4: Qing Kedan chicken; 5 and 6: You Xima chicken; 7 and 8: Dagu chicken

Isoenzyme analysis: Lactate Dehydrogenase (LDH) and Malate Dehydrogenase (MDH) isoenzyme patterns obtained from Qing Kedan chicken embryonic fibroblasts were compared with those of other chicken. It was observed that four LDH isoenzyme bands, namely LDH4, LDH3, LDH2 and LDH1 (Fig. 3a) and two MDH isoenzyme bands, namely m-MDH and s-MDH (Fig. 3b)

Table 1: Chromosome parameters of male Qing Kedan chicken

Chromosome No.	Relative length (%)	Centromere Arm ratio	Morphology
1	22.79	1.49	M
2	18.29	1.73	SM
3	15.38	+∞	T
4	10.60	2.65	SM
5	7.27	+∞	T
6	5.66	2.05	SM
7	5.22	+∞	T
8	4.93	1.83	SM
Z	9.97	1.06	M
Z	9.95	1.04	M

M: Metacentric chromosome; SM: Submetacentric chromosome; ST: Subtelocentric chromosome; T: Telocentric chromosome

were arranged in order from cathode to anode and their RFs for Qing Kedan chicken are 33.33, 37.97, 41.67, 45.37, 60.64 and 72.91%, respectively. The results ruled out the possibilities of cross-contamination among species and subspecies.

Expression of exogenous fluorescent proteins: The expression of pEGFP-N3, pEYFP-N1 and pDsRed1-N1 in Qing Kedan chicken embryonic fibroblast line was observed at 24, 48 and 72 h after transfection, respectively. Positive cells whose quantity and fluorescent intensity gradually waned at 48 or 72 h arrived at their summits 24 h after transfection (Table 2). Confocal fluorescent microscopy was adopted to observe green, yellow and red fluorescent proteins in Qing Kedan chicken fibroblasts to locate their subcellular distribution

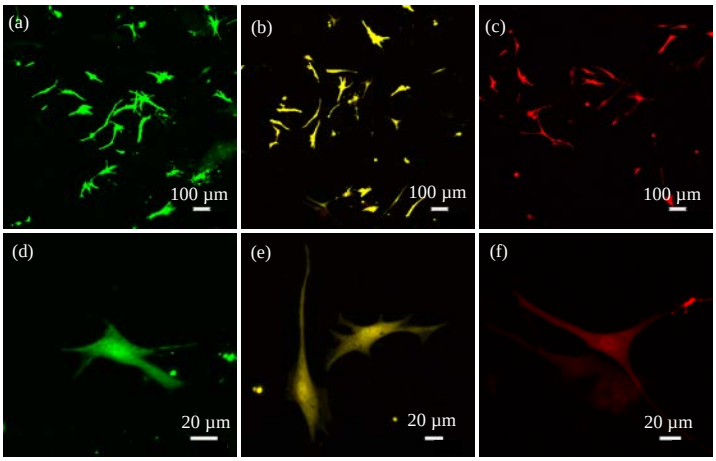

Fig. 4: Comparative figures of pEGFP-N3, pDsRed1-N1 and pEYFP-N1 expression in Qing Kedan chicken embryonic fibroblasts; a and d) pEGFP-N3, 48 h; b and e) pEYFP-N1, 48 h; c and f) pDsRed1-N1, 48 h

Table 2: Transfection efficiencies of three kinds of plasmids in Qing Kedan chicken embryonic fibroblasts

Transfection time (h)	pEGFP-N3 (%)	pEYFP-N1 (%)	pDsRed1-N1 (%)
24	33.1	39.7	29.5
48	27.9	30.9	18.3
72	21.4	28.1	12.6

and the fluorescences could be observed throughout the cytoplasm and nuclei of positive cells except in cryptomere vesicles (Fig. 4). The viabilities of the cells transfected with pEGFP-N3, pEYFP-N1 and pDsRed1-N1 were 92.1, 92.7 and 91.9%, respectively none of which was significantly different from that of the control group (95.1%).

DISCUSSION

Establishment of Qing Kedan chicken embryonic fibroblast line: A Qing Kedan chicken embryonic fibroblast cell line was successfully isolated, purified and cryopreserved through adherent culture. However, the biological characteristics, especially those determining hereditary traits might be undermined as a result of trypsinization *in vitro* so a minimal number of passages to be more specific within 5 in this research were recommended to reduce the injury to the lowest level.

Morphology, one of the most essential standards for culture evaluation is usually observed with light or electron microscopy. In the study the cells possessed fibrous characteristics with turgor vitalis cytoplasm and exhibited typically fibroblast-like morphology with radiating, flame-like or whirlpool-like migrating shapes during growth. It could be observed that cells migrated from the tissue pieces 2-3 days after explanting in accordance with a recent research concerning white ear lobe chicken embryonic fibroblast line (Wu *et al*., 2008).

Typically, epithelial cells and fibroblasts would emerge simultaneously around tissue explants at the beginning of culture *in vitro*. However, the innate propensities for easy adherence and trypsinization of fibroblasts (Ren *et al*., 2002) to the contrary of their epithelial counterparts would enable them, gradually and eventually predominate in the culture population by means of which a pure fibroblast would be obtained after 2-3 passages (Li *et al*., 2003; Zhou *et al*., 2005).

Microorganism detection: It is not uncommon for culture contaminated by one or several kinds of microbes, the results of which would typically be growth cessation and mass detachment. If these signs occurs there would subsequently follow a decimation of the population in nearly all situations. Among all the possible microbial contaminants, bacteria, fungi, yeasts and mycoplasmas, the symptoms of which are easy to detect, might top the list, yet there exist a few types of contamination which are covert and indistinguishable and so are the cases for virus infection until the later stage. The negative results of these assays disproved the existence of the contaminants and verified the health of the cell line.

Karyotype analysis: The genetic stability of cell line, a basis for genetic resource conservation, prescribes that all fibroblasts should remain diploid uniformly as cells *in vivo*. As cells cultured *in vitro* have a tendency to mutate and the mutants would still possess a proliferative potential just as the normal ones, the loss of genetic stability would gradually and eventually debase the importance of somatic cells cryopreservation. Therefore, karyotype analysis a classic technique for distinguishing normal cells from mutants should serve as a routine for

culture evaluation. However, due to the limit of the research technique, it was not an effortless task to match the microchromosome pairs which would ineluctably construct an enormous obstacle for enumeration and description and the data could be misleading if there existed some cases of chromosome drift or loss.

Chromosome numbers for the diploid varies a lot in poultry yet those of most species are 78 or 82. The numbers of macrochoromosomes for *Gallus gallus domestica* is 7.8±0.9, ranging from 6-9 while those of micro chromosomes are 31.9±2.5, from 24-35. An earlier research once conducted a comparison among 48 species including 6 subspecies from 12 orders of Aves and it was posited that a mode for the top three biggest pairs of choromosomes, submetacentric No. 1 and 2 and a subtelocentric or telocentric No. 3 were fairly common or alike in most birds (Takagi and Sasaki, 1974).

In this research, the number of passages was minimized to obtain a stable diploid cell line, 85-93% of whose population possessed 2n = 78 chromosomes in accordance with the results concerning Silkie Bantam by Li *et al*. (2009) and white ear lobe chicken embryonic fibroblast line by Wu *et al*. (2008). Despite of the existence of hypodiploid, hyperdiploid cells and some polyploid ones which had a tendency to increase as serial passage stepped further, they constituted a minor proportion of the whole.

Isoenzyme analysis: Isoenzyme polymorphism, commonly used to identify cultures ancestries (Nims *et al*., 1998) was adopted by ATCC as routine for detecting interspecies contamination (Drexler *et al*., 1999). LDH and MDH, crucial enzymes in the glycolytic pathway and citric acid cycle, respectively species-specific and relatively conservative during evolution and the contents and activities of which vary among species and subspecies could serve as biochemical indicators for species classification by chromatography and electrophoresis. LDH a tetrametric molecule, composed of two kinds of subunits, H and M produced by the expression of *ldha* and *ldhb* genes is a tissue-specific and species-specific isoenzyme complex (Washizu *et al*., 2002). The five isoenzymes of LDH, namely LDH1, LDH2, LDH3, LDH4 and LDH5 are in accordance with different composition of subunits, H4, H3M, H2M2, HM3 and M4, respectively. For human beings, the contents are the most for LDH1 and LDH2 in heart, kidney and erythrocytes, LDH3 in lung, spleen, pancreas, thyroid, adrenal and lymph nodes, LDH4 and LDH5 in skeletal muscles and liver. MDH is a dimeric enzyme including cytosolic MDH (s-MDH) and mitochondrial MDH (m-MDH) types. The former is composed of subunits A and B and having three forms, namely A2, AB and B2 while the later by C and D with three other ones C2, CD and D2. The mobilities of MDH from poultry are fairly uniform so are those of livestock yet MDH from poultry migrates slower and are less in content compared with that from livestock.

Among the five types of LDH, LDH1, LDH2, LDH3 and LDH4 are obvious while LDH5 is rarely observable. The LDH pattern of Qing Kedan chicken embryonic fibroblast line as shown in Fig. 3a, possessed four bands distinct from those of other chicken and the MDH profile exhibited two. Parallel analysis concerning the polymorphism of the two kinds of enzymes, exactly illustrated in earlier research among interspecies and intraspecies, best supported the validity of the technique researchers adopted (Nims *et al*., 1998; O'Brien *et al*., 1977). Therefore, it is reasonable to use these assays as standards for identification of genetic relationships, hereditary stability and the purity of a cell line. The results suggested that the isoenzyme patterns of LDH and MDH in Qing Kedan chicken embryonic fibroblasts were distinct from other chicken and hence disproved the existence of cross-contamination with other cell lines.

Expression of exogenous genes: The three fluorescent proteins due to their numerous merits such as structure stability, high expression levels and species-independent efficiencies (Baird *et al*., 2000) have been widely and frequently used as markers to investigate the expression, distribution and functions of target proteins in live cells and organisms (Cheng *et al*., 2003; Heim *et al*., 1995). Current researches utilizing fluorescent proteins are mostly in fields concerning tumor cells, nerve cells and stem cells (Jung *et al*., 2001). Plasmid DNA and lipofectine concentration, incubation time as well as the existence of serum, all have tremendous influences on their transfection efficiencies according to earlier investigations regarding Vero cells, Hela cells, etc. (Rui *et al*., 2006; Tseng *et al*., 1999).

About 24 h after transfection, the three types of fluorescences could be observed in both cytoplasm and nuclei except cryptomere vesicles. At 48 and 72 h, the three fluorescences waned and some cells became irregular in morphology yet the fluorescent genes were still expressed at a relatively high level which potently validated the expressibility of exogenous genes in the fibroblasts. The transfected cells exhibited no obvious difference compared with the control group in viabilities and morphology during all the stages of cell cycle. Hence, it was suggested that the expression of the three fluorescent proteins in accordance with earlier researches, had trivial negative effects on the cell line (Huang and Li, 2001).

CONCLUSION

The Qing Kedan chicken embryonic fibroblast line was qualified for all the criteria from ATCC whose establishment conserved the genetic resources of Qing Kedan chicken in the form of somatic cells and amounted to great convenience to researches in realms concerning cell biology, biomedical sciences, genomics, somatic cell cloning, embryonic engineering and pharmacology.

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NOMENCLATURE

ATCC = American Type Culture Collection
MEM = Modified Eagle Media
DMSO = Dimethyl Sulfoxide
ELISA = Enzyme-Linked Immunosorbent Assay
FBS = Fetal Bovine Serum
LDH = Lactate Dehydrogenase
MDH = Malate Dehydrogenase
PAGE = Polyacrylamide Gel Electrophoresis
PBS = Phosphate Buffered Saline
PDT = Population Doubling Time
RF = Relative mobility Front

REFERENCES

Baird, G.S., D.A. Zacharias and R.Y. Tsien, 2000. Biochemistry, mutagenesis and oligomerization of DsRed, a red fluorescent protein from coral. Proc. Natl. Acad. Sci. USA., 97: 11984-11989.

Barile, M.E. and S. Rottem, 1993. Mycoplasmas in cell culture. In: Rapid Diagnosis of Mycoplasmas, Kahane, L. (Ed.). Plenum Press, New York, NY, USA., pp: 155-193.

Butler, M., 1999. Cell Counting and Viability Measurements. In: Animal Cell Biotechnology Methods and Protocols, Nigel, J. (Ed.). Humana Press Inc., New Jersey, USA., pp: 131-144.

Cheng, G.Y., X.M. Cheng and X.Y. Bai, 2003. The gene construction and location in tubular epithelial cells of fused by green fluorescence protein and human kidney gene NaDC3. J. Cell. Biol., 25: 170-173.

Doyle, A., R. Hay and B.E. Kirsop, 1990. Animal Cells, Living Resources for Biotechnology. Cambridge University Press, Cambridge, MA., USA., ISBN-13: 9780521352239, Pages: 191.

Drexler, H.G., W.G. Dirks and R.A.F. MacLeod, 1999. False human hematopoietic cell lines: Cross-contaminations and misinterpretations. Leukemia, 13: 1601-1607.

Escriou, V., M. Carriere and F. Bussone, 2001. Critical assessment of the nuclear import of plasmid during cationic lipid-mediated gene transfer. J. Gene. Med., 3: 179-187.

Freshney, R., 2000. Culture of Animal Cells: A Manual of Basic Technique. 4th Edn., John Wiley and Sons Inc., New York.

Freshney, R.I., 1992. Animal Cell Culture: A Practical Approach. 2nd Edn., Oxford University Press, Oxford, UK.

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

Hay, R.J., 1992. Cell Line Preservation and Characterization. In: Animal Cell Culture: A Practical Approach, Freshney, R.I. (Ed.). Oxford University Press, Oxford, UK., pp: 95-148.

He, Z.X. and S.Z. Zhang, 1999. Electrophoresis. Science Press, Beijing, China.

Heim, R., A.B. Cubitt and R.Y. Tsien, 1995. Improved green fluorescence. Nature, 373: 663-664.

Huang, Q. and C.Y. Li, 2001. Research of green fluorescent protein cosmid as marker gene in a living cell. Chin. J. Oncol., 21: 264-266.

Jung, S., C. Ackerley, S. Ivanchuk, S. Mondal, L.E. Becker and J.T. Rutka, 2001. Tracking the invasiveness of human astrocytoma cells by using green fluorescent protein in an organotypical brain slice model. J. Neurosurg., 94: 80-89.

Li, G.F., Y. Li, Z.S. Zhao and D.Q. Li, 2003. The fibroblast culture of sheep ear *in vitro*. China Herbivores, 23: 5-7.

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

Masover, G.K. and F.A. Becker, 1998. Detection of Mycoplasmas in Cell Cultures by Cultural Methods. In: Methods in Molecular Biology, Miles, R.J. and R.A.J. Nicholas (Eds.). Humana Press Inc., Totawa, NJ., USA., pp: 207-215.

Nims, R.W., A.P. Shoemaker and M.A. Bauternschub, 1998. Sensitivity of isoenzyme analysis for the detection of interspecies cell line crosscontamination. *In vitro* Cell. Dev. Biol. Anim., 34: 35-39.

O'Brien, S.J., G. Kleiner, R. Olson and J.E. Shannon, 1977. Enzyme polymorphisms as genetic signatures in human cell cultures. Science, 195: 1345-1348.

Qi, Y., Y. Tu, D. Yang, Q. Chen and J. Xiao *et al*., 2007. Cyclin a but not cyclin D1 is essential for c-myc-modulated cell-cycle progression. Cell. Physiol., 210: 63-71.

Ren, F.L., Y. Li and Y. Zhang, 2002. *In vitro* cultivation and freezing of bovine skin fibroblast cells. Scalper Mag., 28: 8-10.

Rui, R., Y. Qiu, Y. Hu and B. Fan, 2006. Establishment of porcine transgenic embryonic germ cell lines expressing enhanced green fluorescent protein. Theriogenology, 65: 713-720.

Suemori, H., K. Yasuchika, K. Hasegawa, T. Fujioka, N. Tsuneyoshi and N. Nakatsuji, 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: 926-932.

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

Takagi, N. and M. Sasaki, 1974. A phylogenetic study of bird karyotypes. Chromosoma, 46: 91-120.

Tseng, W.C., F.R. Haselton and T.D. Giorgio, 1999. Mitosis enhances transgene expression of plasmid delivered by cationic liposomes. Biochim. Biophys. Acta (BBA): Gene Struct. Expression, 1445: 53-64.

Tsuchiya, R., F. Yoshiki, Y. Kudo and M. Morita, 2002. Cell type-selective expression of green fluorescent protein and the calcium indicating protein, yellow chameleon, in rat cortical primary cultures. Brain Res., 956: 221-229.

Washizu, T., M. Nakamura, N. Izawa, E. Suzuki and S. Tsuruno *et al*., 2002. The activity ratio of the cytosolic MDH/LDH and the isoenzyme pattern of LDH in the peripheral leukocytes of dogs, cats and rabbits. Vet. Res. Commun., 26: 341-346.

Wu, C.X., 1999. The theory and technology of animal genetic resources preservation. J. Yunnan Univ. China, 21: 7-10.

Wu, H., W. Guan, H. Li and Y. Ma, 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: 1478-1485.

Zhou, X.M., Y.H. Ma, W.J. Guan and D.M. Zhao, 2004. Establishment and identification of Debao pony ear marginal tissue fibroblast cell line. Asian-Australian. J. Anim. Sci., 17: 1338-1343.

Zhou, X.M., Y.H. Ma, W.J. Guan, J. Wen and H. Li, 2005. Establishment and characteristics of a Beijing fatty chicken embryo fibroblast cell line. Anim. Vet. Sci., 3: 209-215.