

Establishment and biological characterization of fibroblast cell line from the Langshan chicken

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Abstract

Objective: We needed to establish an embryonic fibroblast cell line from the Langshan chicken (LSCEF61) to preserve their important genetic resources at the cellular level.

Material and methods: The cell line was established from 9-day-old embryos by direct explant culture and cryopreservation techniques. Cell morphology, dynamic proliferation and any contamination present were tested, and the karyotype and levels of isoenzymes of lactic dehydrogenase and malic dehydrogenase were analysed. Four types of fluorescent protein exogenous genes for pEGFP-C₁, pEGFP-N₃, pEYFP-N₁ and pDsRed1-N₁ were transfected into the cells.

Results: Results showed that the cells were healthy and were of spindle shaped structure, without change in morphology. Cell growth curves were of typical S-shape. Assays for microbial contamination were negative. The LSCEF61 line showed no cross-contamination when assessed by isoenzyme analysis. Chromosome number ($2n$) = 78 on more than 90% of occasions. The four types of fluorescent protein extro-genes appeared to be expressed effectively with high transfection efficiency between 15.6% and 38.6%.

Conclusion: The cell line met each of the quality control standards required for the American Type Culture Collection. It had not only preserved the genetic resources of the important Langshan chicken at the cellular level, but also provided valuable material for genomic, post-genomic and somatic cell cloning research and other applications.

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Introduction

Diversity of genetic resources in livestock and poultry forms an important part of overall biological diversity, as well as being the basis for survival and sustainable development of humans. Thus, preservation of genetic resources of endangered species is of significant scientific importance. Preservation of individual species, semen samples, embryos, genomic libraries and cDNA libraries are all practical methods. In addition to these methods, modern somatic cell cloning techniques have made somatic cells an attractive resource in the conservation of animal genetic materials (1).

The Langshan chicken, a well-known member of the world's poultry breeds, is of an ancient Chinese genotype. The breed originated in Jiangsu Ru Dong County in Langshan territory and it was listed as one of the 78 nationally protected domestic animals by the Chinese government in 2004. The breed is used for both meat and egg production, and is celebrated for its large shape, black plumage and egg productivity during winter. The Langshan chicken has also been bred in other countries and has been hybridized with local breeds to produce new breeds such as the Australian black chicken. In the present study, tissues from embryos of Langshan chickens were used to culture fibroblasts and thus a fibroblast cell line was developed. We identified the cell line and described its characteristics to allow for future preservation of the line at the cellular level.

Materials and methods

Primary culture and subculture

Nine-day-old chick embryos were rinsed and chopped into samples measuring 1 mm³ in size. The surface of a tissue culture flask was seeded with these tissue pieces and MEM medium containing 10% foetal bovine serum was added. Flasks were inverted and incubated at 37 °C for 2 h in an incubator with 5% CO₂ until the tissue pieces

spontaneously adhered to the flask surface, after which the flasks were turned.

Medium was changed when needed and cultures were observed daily for occurrence of substantial outgrowth of cells from the tissue pieces; cells were then harvested at 80–90% confluence. The cell sheet was rinsed twice in phosphate-buffered saline to remove all traces of trypsin inhibitors in serum supplied to the media and 0.05% trypsin solution was added. Flasks were inverted and incubated for 3 min at 37 °C before being turned over. Subsequently, flasks were shaken gently to detach cells from their walls and medium was added to the cell suspension to terminate trypsinization. Cells were split into new culture flasks at 1:2 ratio and incubated at 37 °C with 5% CO₂.

Cryogenic preservation and recovery

Prior to freezing, cultures were maintained in an actively growing state (log phase or exponential growth) to ensure optimum health and good recovery. Culture medium was changed 24 h prior to harvesting and harvested cells were treated in the same manner as described for subcultures. Cells were counted by haemocytometer and their viability was checked by using the trypan blue exclusion technique. They were then centrifuged at 168g for 8 min to form a pellet, supernatant was removed and the cell pellet resuspended in freezing medium (10% DMSO + 50% foetal bovine serum + 40% MEM) to reach a final cell concentration of $3\text{--}5 \times 10^6$ viable cells/ml. One-millilitre samples of cell suspension were transferred into sterile plastic cryogenic vials labelled with animal name, gender, age, passage number and the date. The sealed vials were then placed into boxes filled with an appropriate amount of isopropyl alcohol, frozen overnight at –80 °C and the cells were transferred into a liquid nitrogen storage system [2].

Frozen tubes were taken from the liquid nitrogen and plunged into a 42 °C water bath for quick thawing. Cells were subsequently transferred to a flask with complete medium, and were cultured at 37 °C with 5% CO₂. Medium was changed 24 h later [3].

Estimation of cell viability

Assays on cell viability before freezing and after recovering were performed using the trypan blue exclusion method. Cells were seeded into six-well plates and 1000 viable cells were counted [4].

Microorganism detection

The Doyle *et al.* [5] and Freshney method [3] was followed for detection of potential bacterial, fungal and yeast

contamination. DNA fluorescence staining was performed using Hoechst 33258 (Sigma, St. Louis, MO, USA) to identify any contamination by mycoplasma. Using the ELISA Mycoplasma Detection Kit (Roche Diagnostics Corp, Indianapolis, IN, USA) the four most common Mycoplasma species (*M. arginini*, *M. hyorhinis*, *A. laidlawii* and *M. orale*) was used to confirm results of the DNA staining.

Growth curves

Following Gu's *et al.* [6] method, 24-well plates were seeded with cells at a concentration of 4×10^4 cells/ml and cultured for 7 days. Cell proliferation and concentration were recorded from three wells per day until the plateau phase was reached. This was repeated 10 times and the set of numbers was averaged. The cell population growth curve was plotted and the population doubling time (PDT) was calculated, based on this curve.

Chromosome analysis

Cells were harvested at 80–90% confluence. Microscope slide preparation and chromosome staining were performed according to the method of Hirofumi *et al.* [7]. Fifty to 100 spreads were sampled for counting chromosome numbers per initial spread. Three important parameters for the analysis of chromosomes are relative length, arm ratio and centromere index. Counts were determined according to the protocol of Kawarai *et al.* [8].

Isoenzyme analysis

Isoenzyme patterns of lactic dehydrogenase (LDH) and malic dehydrogenase (MDH) were detected using vertical slab non-continuous polyacrylamide gel electrophoresis assay. In brief, cells were harvested and protein extraction solution (0.9% Triton X-100, 0.06 mmol NaCl:EDTA in mass ratio 1:15) was added after cell concentration was adjusted to 5×10^7 cells/ml. This was then centrifuged and supernatants were stored in aliquots at –80 °C. Forty per cent sucrose liquid and samples (1:1) were mixed and then loaded on individual lanes of polyacrylamide gel [9]. Different mobility patterns were indicated by relative mobility front (RF), which was calculated as the ratio between distance of migration of the isozyme band and that of the bromophenol blue.

Detection of fluorescent proteins in Langshan chicken cells

Using the method of Tsuchiya *et al.* (2002), the same quantity of fluorescent protein vectors pEGFP-N₃,

pEGFP-C₁, pDsRed-N₁ and pEYFP-N₁ were transfected into the LSCEF61 cells with Lipofectamine™ 2000 transfection reagent (Invitrogen Corp, Carlsbad, CA, USA). Plasmid DNA (µg) to Lipofectamine 2000 (µl) ratio was 1:3. After 8 h, cells were removed from non-serum medium and transferred to medium containing serum. Cell morphology was observed, and the cells were stained with trypan blue once more to estimate cell viability. Cells were observed at 24, 48 and 72 h respectively, after being transfected, to estimate transfection efficiency. Cell morphology was observed by confocal microscopy (Nikon TE-2000-E, Tokyo, Japan), and comparative analysis of expression was made, depending on intensity of staining of the different fluorescent proteins in the cell nucleus and cytoplasm. For each experimental group, images were captured from 10 visual fields, and confocal microscopy was used to measure total and positive cell counts in each field, to determine efficiency of transfection per set of numbers. Multiple comparisons of test data were made to judge differences between the groups.

Results

Morphology of LSCEF61 cells

Two hours after attachment of embryo tissues, several types of epithelial-like and fibroblast-like cells were removed from the tissues. As time progressed, fibroblasts emerged from the vicinity of tissue pieces in large numbers, multiplied rapidly and assumed a typical long, spindle-shape (Fig. 1a). Primary cell growth was slow and medium was changed for them only when it turned yellow. Cells were subcultured when they reached 90% confluence. After passaging, cell population growth accelerated and within 2–3 days, cells covered the entire base of the flask (Fig. 1b). LSCEF61 cells were expanded over 14 passages.

Average cell viability was 96% before freezing and 90.5% after thawing. Results indicate that the cells grew

well, that culture conditions were appropriate and that survival rates were high and not greatly affected by freezing (Fig. 1c,d). To ensure motility rate of the cells recovered at later stages, the cells were frozen in four generations when their frozen concentration exceeded 3×10^6 /ml.

Microbial analysis

Assays for microbial contamination from bacteria, fungi and mycoplasma were negative (Fig. 2). These results indicated that there was no microbial contamination of the LSCEF61 culture.

Growth curves

Growth curves of LSCEF61 after recovery displayed a typical S shape (Fig. 3a). A lag of around 24 h was apparent after the cells were seeded, that was accepted for recovery after possible damage by the protease. After the lag, cells proliferated rapidly and entered the exponential growth phase until they reached a stationary phase after about 6 days. From day 6, growth plateaued and cells began to degenerate. The PDT calculated from the curve data, was 49.5 h.

Chromosome analysis

Number of diploid chromosomes. As chromosomes are small and difficult to differentiate, we sampled 50 cells and only calculated the number of hypodiploid, diploid and hyperdiploid ones. Results are shown in Table 1, from which it is evident that the majority of chromosomes were diploid but over time, frequency of hyperdiploid chromosomes increased. By the 4th generation, the diploid proportion reached 86%, as a result of which it is recommended that only cells in early generations after culturing are frozen and stored.

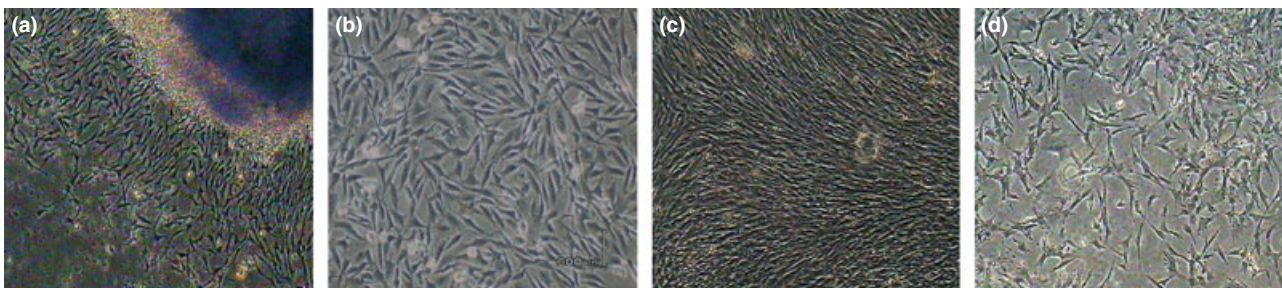


Figure 1. Morphology of LSCEF61 cells. (a) Primary cells ($\times 100$), the cells were typical long spindle-shape with growth being slower. (b) Subcultured cells ($\times 100$), the cells were subcultured after they reached 90% confluence. After passage, growth accelerated and plateaued after 3–4 days. (c) Cells before cryopreservation ($\times 100$), the cells were healthy and in mitotic phase. (d) Cells after recovery ($\times 100$), the cells were cultured for 48 h after thawing.

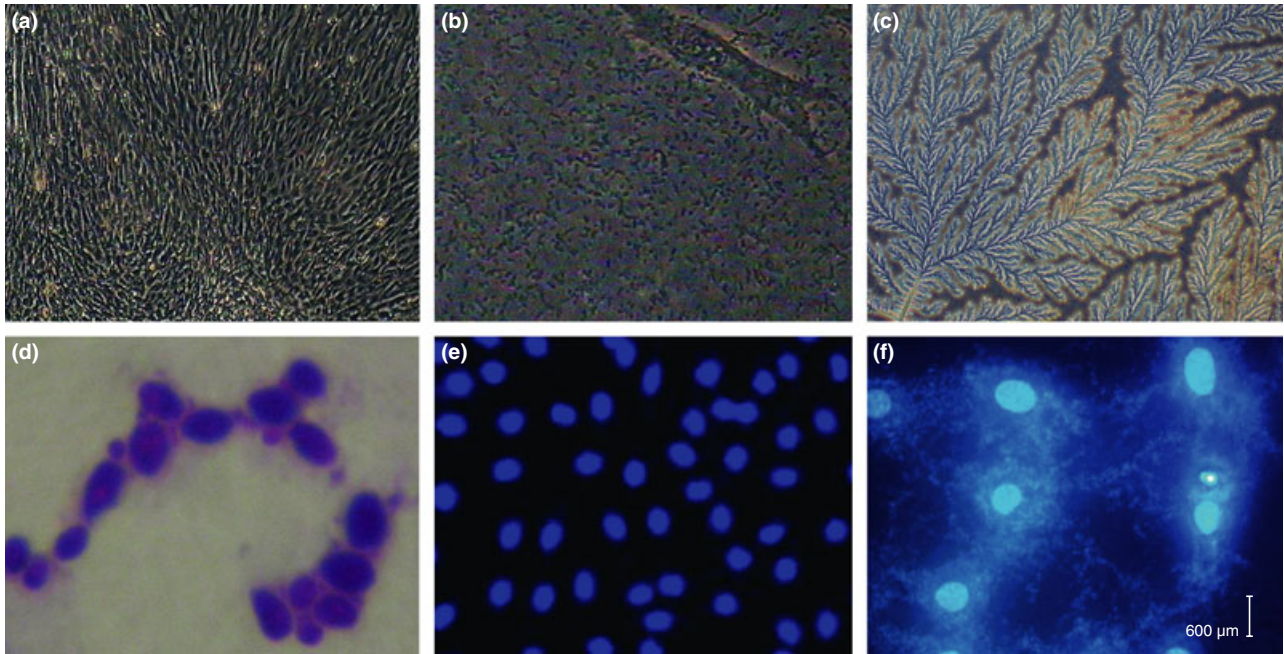


Figure 2. Detection of microbial contamination of LSCEF61 cells. Assays for microbial contamination from bacteria, fungi and mycoplasma were negative. (a) LSCEF61 cells (40×). (b) Bacteria positive control (200×). (c) Mold positive control (100×). (d) Candida positive control (200×). (e) LSCEF61 cells mycoplasma negative (200×). (f) Mycoplasma positive control (400×).

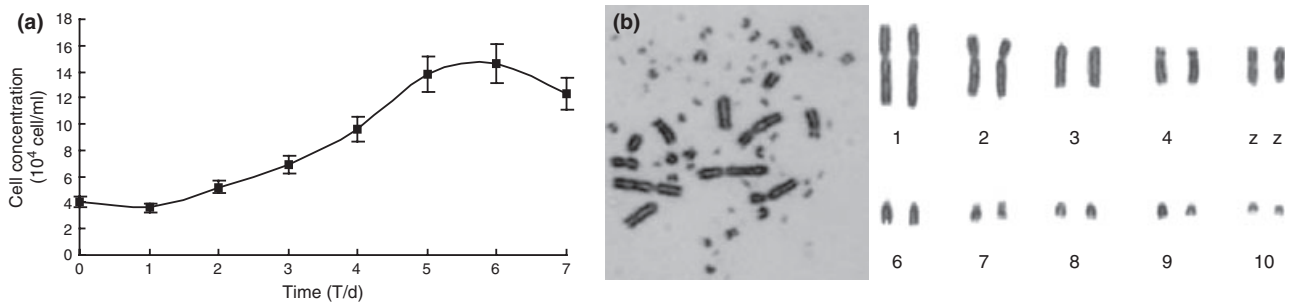


Figure 3. Growth curve and karyotype of LSCEF61 cells. (a) Growth curve of LSCEF61 cells. The curve appeared as a typical ‘S’ shape with cell density on the left axis. Growth curve included latency phase, exponential growth phase and stationary phase. The Population Doubling Time calculated from the curve data was about 49.5 h. (b) Chromosome at metaphase (left) and karyotype (right) of LSCEF61 line (♂) (×1000). Langshan chicken 2n chromosomes number is 78.

Table 1. Chromosome number in LSCEF61 line

Generation	Chromosome number			Total cell score	Percentage of 2n (%)
	Hypodiploid	Diploid	Hyperdiploid		
2	1	46	3	50	92
3	0	45	5	50	90
4	2	43	5	50	86

Fifty spreads were sampled for counting chromosome numbers of hypodiploid, diploid and hyperdiploid chromosomes.

Chromosome morphology

Number of chromosomes per LSCEF61 cell was $2n = 78$, consisting of 10 pairs of macrochromosomes and 29 pairs of microchromosomes, while the sex chromosome type was ZZ (♂). Chromosomes 1 and 7 were classified as type M, 2 and 4 were SM, 3, 6, 8, 9 and 10 were classed as T and the sex chromosome was ZZ. Z chromosomes were classified as type M. In size, the Z chromosome was as large as chromosome 5. These results conform to those from studies on other chicken breeds [11], but there are

discrepancies with reports regarding chromosome 7 [12]. These discrepancies were related to ease of Robertsonian translocation, principally with micro-chromosomes of bird species. However, the exact cause is yet to be fully elucidated. Relative length, centromere index and kinetochore type are shown in Fig. 3b.

Isoenzyme analysis

Pattern of distribution of isoenzyme polymorphisms may be characteristic of a species or tissue [13]. Polymorphism analysis of isoenzymes is currently the standard method used during quality control of cell line identification and interspecies contamination. Isoenzyme patterns of LDH and MDH from Langshan chicken cells were obtained and compared with four other breeds. Five bands were apparent representing LDH1, LDH2, LDH3, LDH4 and LDH5 from anode to cathode (Fig 4a). LDH activity of the Chinese game chicken, Wenchang chicken and Langshan chicken breeds was similar but that of the Beijing duck, Jingning chicken and Langshan chicken were significantly different. Similarity in LDH activity between breeds is indicative of a similar ability to utilize lactic acid in different environments and implies close genetic relationship. Simultaneous shading of an area represents strength or weakness of phenotype enzymatic activity. Progression from lowest to highest LDH activity was as follows: LDH1, LDH4, LDH2, LDH3 and LDH5. Although flow rate of the isodynamic enzyme bands of different breeds appeared to have little obvious difference, smaller differences were apparent. Results illustrate certain differences between breeds and indicate that no cross-contamination from different cell lines established in the laboratory at the same time occurred.

Patterns of MDH are shown in Fig. 4b. One band of m-MDH was located near the anode and two bands of s-MDH were found near the cathode. m-MDH and s-MDH1 had similar activity levels, while activity of s-MDH2 was weaker. Each chicken breed showed characteristic banding, and each band had a different relative mobility. These results confirmed that there was no cross-contamination between different breeds.

Comparison of expression of four fluorescent protein transgenes in LSCEF61 cells

Expression of pEGFP-N₃, pEGFP-C₁, pDsRed1-N₁ and pEYFP-N₁ at 24, 48 and 72 h was recorded by laser confocal microscopy with specific excitation wavelength of light. Results indicated that all four fluorescent proteins were expressed in most positive cells, and the strongest fluorescence intensity and highest transfection efficiency of exogenous genes appeared 48 h after transfection. Expression efficiencies of the four fluorescent proteins 24, 48 and 72 h after transfer were between 15.6% and 38.6% (Table 2). All positive cells appeared shrunken, were shed and disintegrated 24 h after transfection. Number of positive cells increased by 48 h and there were many non-fluorescent vacuoles in cytoplasm in most of positive cells at 48 and 72 h after transfection (Fig. 5). Number of cells expressing fluorescent proteins decreased and fluorescence intensity gradually faded, disappearing by 7 days after transfection, although some cells still expressed fluorescent proteins after 4–5 weeks indicating that the exogenous genes in the fibroblasts can be replicated, transcribed, translated and modified after correct translation. Viabilities of cells transfected with pEGFP-N₃, pEGFP-C₁, pEYFP-N₁ and pDsRed1-N₁ were 88.5%, 87.2%,

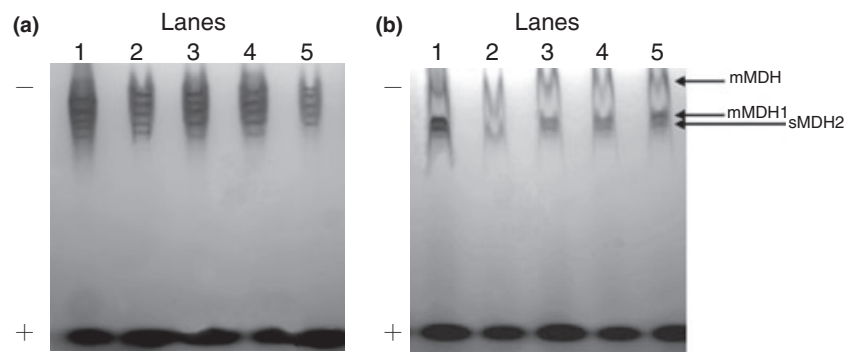


Figure 4. LDH zymotype and MDH zymotype of LSCEF61 line. These birds had their own unique bands with a different relative mobility. These results showed that there was no cross-contamination between different breeds. (a) SDS-PAGE electrophoresis of LDH (left panel). (b) MDH (right panel). The species in left panel were lane 1 = Beijing duck, lane 2 = Chinese game chicken, lane 3 = Langshan chicken, lane 4 = Wenchang chicken and lane 5 = Jingning chicken respectively. The species in right panel were lane 1 = Beijing duck, lane 2 = Jingning chicken, lane 3 = Chinese Game Chicken, lane 4 = Wenchang chicken and lane 5 = Langshan Chicken respectively. On the left panel, from down to up, there were LDH-1, 2, 3, 4, 5. On the right panel, from up to down, there were mMDH, sMDH-1 and sMDH-2, whilst s-MDH2 had weaker activity.

Table 2. The transfection efficiency of four fluorescent proteins

Transfection time (h)	pEGFP-N ₃ (%)	pEGFP-C ₁ (%)	pDsRed1-N ₁ (%)	pEYFP-N ₁ (%)
24	26.4	33.6	15.6	28.2
48	31.8	38.6	18.4	32.3
72	29.5	35.4	16.7	27.8

Cells were examined 24, 48 and 72 h after transfection using a Nikon TE-2000-E inverted microscope with excitation wavelengths of 433–588 nm to determine the transfection efficiency. The number of whole cells and positive cells in 10 visual fields of every well was counted to calculate the transfection efficiency.

89.8% and 86.3% respectively; none of these was significantly different from controls (94.2%, $P > 0.05$). Results showed that fluorescent protein expression had no obvious effect on population growth and proliferation of the transfected cells.

Discussion

Morphological analysis indicated that both epithelial and fibroblast cells were present during primary and early passages. Because of their different levels of tolerance to trypsin, fibroblasts detached from flask walls when treated with trypsin but reattached quickly after passaging, while most epithelial cells were unable to adhere, or only did so in an unstable manner and detached again when vibrated [14]. For this reason, purified fibroblasts could be obtained after 2–3 passages. Average viability after thawing was above 90% indicating that freezing had little influence on viability of the cells. Thus, it seems possible to conserve genomic resources of the Langshan chicken breed by long-term freezing of their fibroblasts in liquid nitrogen.

Chromosomes of poultry breeds have been defined as possessing eight pairs of macrochromosomes and 30 pairs of microchromosomes with the sex chromosomes being Z and W. Range in frequency of diploid chromosomes varies notably between breeds with most of them having from

78 to 82. Macrochromosomes of *Gallus domesticus* average 7.8 ± 0.9 in number but vary between 6 and 9, while there are 31.9 ± 2.5 microchromosomes with a range from 24 to 35. The present study sampled 100 cells and found 10 pairs of macrochromosomes and 29 pairs of microchromosomes with 92% of cells being diploid. Most chromosomes of the Langshan chicken are therefore microchromosomes, and may be lost easily during section preparation and disruption of purity of the chromatin. Thus, it becomes difficult to determine quantity and morphology of the chromosomes whose number in the diploid state may vary from 78. It is important to determine time at which colchicines should be added and length of time over which they are active.

Isoenzymes show polymorphisms between diverse species, races, individuals and tissues, and intercellular pollution can be detected by isoenzyme analysis when 10% of cells are polluted [15]. Kewen and Fengying [16] found between 5 and 8 clear bands five of which were found in samples of pectoral muscle. LDH isoenzymes have also been studied in samples of cardiac muscle, liver and blood of Chinese jungle fowl, and for all three tissues, five bands were observed. In agreement with this, five LDH bands, LDH1, LDH2, LDH3, LDH4 and LDH5, were recorded in the Langshan chicken samples of the present study.

The MDH of poultry is present in both cell solute form (s-MDH) and mitochondrial form (m-MDH), and movement rate of the former is faster than that of the latter. Of s-MDH bands of 1- to 16-day-old embryos, which appeared after 3 days and increased thereafter, all showed deviation towards the positive electrode. The present results showed that MDH of Langshan chicken breed also has s-MDH and m-MDH forms and again, movement rate of s-MDH is the higher of the two. This is in accordance with MDH isoenzyme activity of embryos at early and middle stages of development (1–16 days) and indicates that MDH enzyme activity of cells *in vitro* was similar to that of the original tissues. In the present study, the LDH

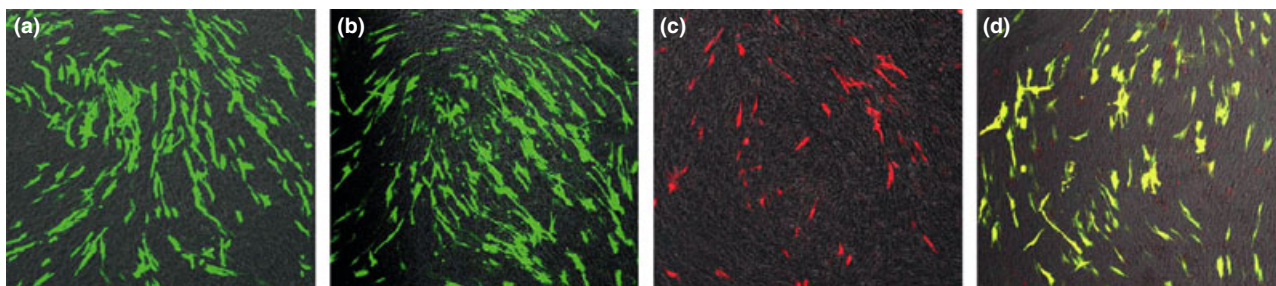


Figure 5. The transfection efficiency of TSF19 cells with pEGFP-N₃, pEGFP-C₁, pDsRed1-N₁, and pEYFP-N₁ using Lipofectamine 2000 ($\times 100$). Comparative figures of four fluorescent protein transfection. a–d shows the results 48 h after transfection of genes for pEGFP-N₃, pEGFP-C₁, pDsRed1-N₁ and pEYFP-N₁.

and MDH isoenzyme bands from Langshan chicken cells were clear, suggesting that cell genetic characteristics were stable and that there was no cross-contamination from one breed to another in the laboratory.

Conclusion

The current results indicate that the newly established LSCEF61 cell line is stable and possesses normal biological characteristics. These characteristics suggest that LSCEF61 line provides a useful approach for conserving this unique breed of chicken in China and would be an effective experimental resource for further genetics studies.

Acknowledgements

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