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Establishment and Biological Characteristics of Hereford Cattle Fibroblast Bank

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Abstract: A fibroblast line from kindey tissue of Hereford cattle was established successfully by direct culture of explants and biology cryopreservation techniques. The cell line contained 101 tubes of frozen cells from 34 primary kidney samples. Biological analysis showed that the cells were morphologically consistent with fibroblasts and the growth curve was sigmoidal with a Population Doubling Time (PDT) of 35 h.The average viability of the cells was 95.8% before freezing and 93.4% after thawing. Cross-contamination among cell lines was excluded by isoenzyme analysis of Lactate Dehydrogenase (LDH) and Malate Dehydrogenase (MDH). The frequency of cells having the diploid chromosome number (60) was 97.5%. Detection of bacteria, fungi, viruses and mycoplasmas was verified negative. At 24, 48 and 72 h after transfection, the expression efficiency of fluorescent protein genes (*pEGFP-N3*, *pEYFP-N1* and *pDsRed1-N1*) were between 18.6~32%; The fluorescence could be observed well-distributed in cytoplasm and nucleus in addition to some cryptomere vesicles at 12 h after transfection.

Key word: Hereford cattle, biological characteristic, fibroblast, livestock, poultry, China

INTRODUCTION

The genetic diversity of livestock and poultry is an important part of biodiversity however, it is facing unprecedented challenges worldwide. One of the crucial causation is that vulnerable animals are threatened by industrial pollution and the introduction of high-yielding foreign species which are being spread actively all over the world. If there is no proper measures taken to preserve the resources before they are extinct, these previous characteristic genes will be lost and the related molecular biological mechnisms required to reproduce those breeds by somatic cell cloning also cannot be explored thus it's urgent to commence conservation of endangered species and breeds (Guan *et al*., 2005).

Currently the genetic resources of domestic animals are preserved by many strategies for instance generative cells, somatic cells, stem cells, embryos and zygotes can all be cryopreserved in cell banks (Guan *et al*., 2007). Nevertheless, establishment of somatic cell banks using low-temperature biological techniques is a new effective approach to conserving and maintaining the diversity of domestic animals. Not only does this technique conserve valuable genetic materials but it also supplies an eximious resource for biological research. Otherwise, development of cloning techniques has made somatic cells an captivating resource for preserving animal genetic materials.

Hereford cattle originated in Hereford County of western England. It is the world's most acient middle and small early maturing beef cattle breed and disperses in many countries worldwide. The breed is divided into two types: the horned and the hornless. Hereford cattle has the typical beef cattle body-chunky neck, plump muscular cylindrical soma, broad back, generous hip, muscularity, short and thick limbs and that the soma looks like rectangular shape from side-looking. Moreover, its clothing hair all over the body is red except that of the head, neck down, belly, lower limbs and the tip of tail is white, its skin is orange and the horn is wax yellow or white. While the outstanding property of Hereford cattle is its good adaptability. Under the conditions of plateau pastures in the dry cold Winter (-48~~50°C) or the very hot Summer (38~40°C), it can be grazed and live a normal breeding life, showing good adaptability and production performance.

In this research, the object was to seek an effective approach to conserving important germplasm resource of domestic animals. This established fibroblast line of Hereford cattle was a valued and high-performance materials for genomic and transgenic research. Advantageous genes from other breeds could be transferred into this cell line to study gene function. Using this cell line, the breed could be revived by somatic cloning techniques if this breed ever becomes endangered. Moreover with the development of modern somatic cell cloning techniques, limited cell lines will have increasingly prominent roles in research and may be useful in currently unforeseen applications.

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MATERIALS AND METHODS

Cell cultues: The kidney tissue samples were washed with PBS for three times and chopped into 1 mm^3 pieces which were planted in a culture flask (Corning, USA) and cultured at 37°C in a humidified atmosphere of air containing 5% CO_2 (Freshney, 2000; Gu *et al*., 2006). About 3~4 h later when the tissue pieces adhered to the flask surface, DMEM (Gibco, USA) containing 10% fetal bovine serum (Hyclone, USA) was added with the flask inverted. Cells were gathered when they attained 80-90% confluence and were divided into prepared tissue culture flasks at 1:2 or 1:3 ratios (Freshney, 2000).

Cryogenic preservation and recovery: Cells in logarithmic growth phase were reckoned with a hemocytometer and viability was examined by pigmentation of Trypan Blue before freezing. Harvested cells were frozen in a freezing solution containing 40% DMEM, 10% Dimethyl Sulphoxide (DMSO) (Sigma, USA) and 50% fetal bovine serum with the cell density adjusted to (3~6) $\times 10^6$ viable cells/mL. Single cell suspension was dispensed into sterile plastic cryogenic vials labeled with animal name, gender, freezing serial number and the date. The vials were hermetical and kept at 4°C for 20-30 min to allow time for the DMSO to equilibrate then they were transferred to liquid nitrogen for long-term effective storage (Werners *et al*., 2004).

To recover and reseed the cells, the cryogenic vials were rapidly thawed in a 42°C water bath. Then thawed cells were transferred to a 15 mL centrifuge tube and centrifuged at 20 g min^{-1} for 5 min. The supernatant was discarded and the sedimentum was resuspended gently using complete DMEM and immediately transferred to a tissue culture flask to culture at 37°C under a 5% CO_2 atmosphere.

Cell growth curve: Cells at 1.5×10^4 mL^{-1} were seeded into 24 well plates, counted every 24 h until the plateau phase was reached. The growth property of cells *in vitro* was estimated by their PDT. Average cell densities at each time point were used to plot a growth curve and calculate the PDT (Sun *et al*., 2006).

Cell viability: Cell viability before freezing and after recovery were detected using trypan blue. The cells were seeded in 6 well plates at 104/well and counted using a hemocytometer (Qi *et al*., 2007).

Microorganism detection: The Doyle and Freshney Method was followed to test bacteria, fungi and yeasts contamination (Doyle *et al*., 1991). The cultured cells were stained with Hoechst 33258 according to the method of DNA fluorescent staining protocol to detect mycoplasma contamination (Guan *et al*., 2005). An ELISA mycoplasma detection kit (Roche) was used to verify the results of DNA staining for mycoplasma. The four most common mycoplasma species (*Mycoplasma arginini*, *Mycoplasma hyorhinis*, *Acholeplasma laidlawii* and *Mycoplasma orale*) could be identified by this kit.

Chromosome analysis: Chromosomes were prepared, fixed and stained complying with standard methods (Suemori *et al*., 2006). After Giesma staining, the chromosome numbers of 100 spreads were counted under an oil immersion objective. Relative length arm ratio index and centromeric index were calculated following the protocol of Sun *et al*. (2006) and Kawarai *et al*. (2006).

Isoenzyme analysis: Isoenzyme patterns of LDH and MDH were detected by using the vertical slab non-continuous Polyacrylamide Gel electrophoresis (PAGE). In brief, protein extraction solution (0.9% Triton X-100, 0.06 mmol NaCl/EDTA in mass ratio 1:15) was added to the harvested cells the density of which was adjusted to 5×10^7 mL^{-1} then the mixture was centrifuged and the supernatant was stored at -80°C. After that equal volumes of 40% sucrose were added to the sample and then the prepared sample could be loaded to do the electrophoresis (He and Zhang, 1999). Mobility was measured as the ratio of the migration distance of the isoenzyme band to that of the indicator dye.

Expression of fluorescent protein gene in Hereford cattle fibroblasts: Following the method of Tsuchiya *et al*. (2002), the three fluorescent protein vectors pEGFP-N3, pDsRed1-N1 and pEYFP-N1 were transfected into cells using Lipofectamine 2000 (Invitrogen). The ratio of plasmid DNA (μg) to Lipofectamine 2000 (μL) was 1:3. The admixture of plasmid DNA and Lipofectamine 2000 was adequately diluted by nonseum medium gently before transfection. After transfection 8 h, the nonserum medium was removed and replaced by medium containing 10% serum. The cells were observed and appraised at 12, 24, 48 and 72 h employing confocal microscope (Nikon TE-2000-E, Japan) to confirm the transfection efficiency and the morphology of the positive transfected cells. For each experiment group, images were captured from 10 visual fields and confocal fluorescence microscopy was used to measure the total and positive cell counts in each field to determine the transfection efficiency. The effect of the exogenous genes on the cells was measured in terms of cell viability and apoptosis using Trypan Blue and DAPI.

RESULTS AND DISCUSSION

Cell morphology and viability: About 6 days after explanting, cells could be observed migrating from the borderline of tissue pieces (Fig. 1a). The cellular morphology was elongated spindle-shape which was typical fibroblast cell morphous. With the duration of culture, cells continued to proliferate and bespread antapical half of culture flask gradually. Then cells were subcultured when they reached 90% confluence. Epithelial cells were also found in mixture with fibroblasts, however, after the third subculturing, we obtained purified fibroblasts because the fibroblasts grew rapidly and excluded other cells such as epithelial cells gradually during every subculturing (Fig. 1b) (Li *et al*., 2008).

The average viability of the cells was 95.8% before freezing and 93.4% after recovery. Statistics analysis showed that these results were not significantly different ($p>0.05$), the morphology of the cells was still typical elongated spindle-shape and the cells were healthy in vitro culture and freezing had little harmful effect on cell viability (Fig. 1c and d).

Growth curve (dynamic state of cells): The growth curve of fibroblasts from Hereford cattle appeared sigmoidal (Fig. 2a). There was a lag time or latency phase of about 72 h after seeding in this period, the cells were adapting and repairing from protease damage then the cells proliferated rapidly and entered exponential growth phase. With the cell density increasing, proliferation was retarded by contact inhibition, after 6 days, the cells entered the stationary phase and began to degenerate. Analysis of the curve data shown that the PDT was approximately 35 h.

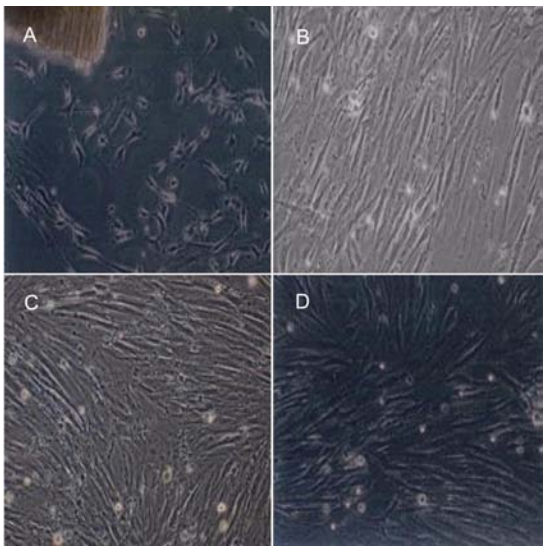

Fig. 1: A) Cells migration from the borderline of tissue pieces; B) Epithelial cells in mixture with fibroblasts after third subculturing and C, D) the results of average viability of the cells before and after freezing

Microbial analysis: Bacteria, fungi and yeasts were negative with contamination testing; no microorganisms were detected in the culture media. No viruses were observed by the cytopathogenic evidence and the hemadsorption test. After staining with Hoechst 33258, fluorescence microscopy indicated that the fibroblast nuclei appeared as blue ellipse which shown that the established cell line was mycoplasma negative (Fig. 2b). These results were confirmed by DNA staining with an ELISA mycoplasma detection kit (Roche Diagnostics Corp) and the results of the two tests confirmed that this cell line was negative for mycoplasma.

Karyogram and chromosome number of Hereford cattle: The chromosome number of Hereford cattle was 2n = 60, containing 58 euchromosomes and two sex-chromosomes,

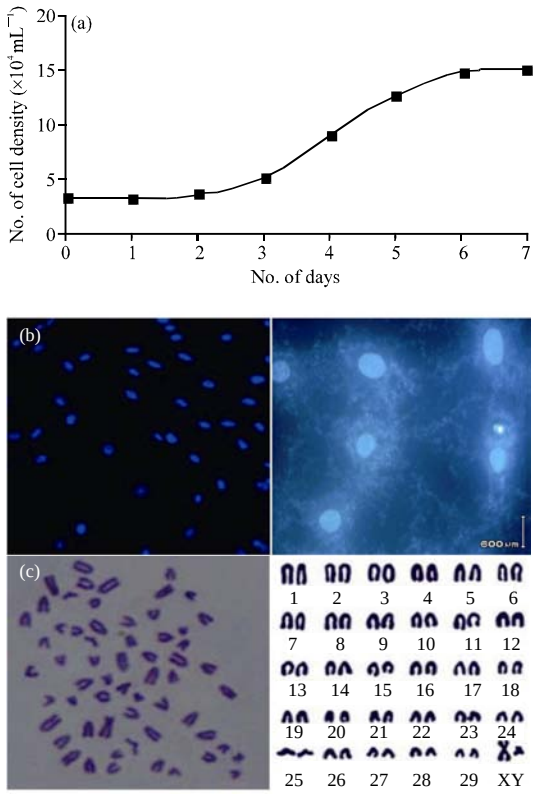

Fig. 2: a) Grouth curve (dynamic state of cells); b) Microbial analysis and c) Karyogran and chromosome number of Hereford cattlet

whose type was XY or XX (Fig. 2c). Besides the two sex-chromosomes being submetacentric, all euchromosomes were acrocentric. The detailed parameters of relative length, arm ratio index, centromere index and centromere type are shown in Table 1. In this experiment, the chromosome of abnormal morphous was not found. The chromosome numbers per spread were counted for 100 spreads of the 1st, 2nd and 3rd passages, the results showed that 97.5% of the cells were diploid. The frequency of hypodiploid and multiploid were always under 2.5%. These results suggested that the cell line was reproducibly diploid and with was great genetic stabilization.

Isoenzyme analysis: Isoenzyme polymorphism exists among species even sometimes among breeds individuals and tissues in the same species. Currently, the isoenzyme polymorphism analysis is the standard method for the quality control of cell line identification and detection of interspecies contamination. The apparatus and conditions of native polyacrylamide gel electrophoresis was improved by the laboratory using this method, the mobility of the isoenzyme was determined successfully.

The LDH and MDH bands obtained from Hereford cattle were compared with those of Songhei pig, Fatty-tailed sheep, Simmental cattle and Angus cattle. The LDH patterns are shown in Fig. 3a. About 5 isoenzyme bands were observed clearly, corresponding to LDH-1, LDH-2, LDH-3, LDH-4 and LDH-5 in order from anode to cathode. The enzymatic activities were increased in the order LDH-5, LDH-4, LDH-3, LDH-2, LDH-1. Patterns of LDH bands showed the character which presented differences among different livestock species and fewer differences in the same livestock species.

Table 1: Chromosome's parameters of hereford cattle

Chromosome number	Relative length (%)	Centro-mere type	Chromosome number	Relative length (%)	Centro-mere type
1	4.26±0.043	T	17	2.25±0.023	T
2	4.32±0.051	T	18	1.93±0.023	T
3	2.47±0.028	T	19	2.46±0.025	T
4	2.93±0.026	T	20	3.24±0.036	T
5	2.71±0.033	T	21	4.13±0.042	T
6	2.78±0.029	T	22	4.39±0.043	T
7	3.01±0.043	T	23	4.92±0.047	T
8	2.27±0.023	T	24	3.94±0.033	T
9	3.58±0.035	T	25	4.25±0.043	T
10	2.57±0.027	T	26	4.10±0.045	T
11	3.24±0.033	T	27	3.61±0.034	T
12	3.31±0.039	T	28	3.78±0.036	T
13	3.24±0.042	T	29	3.15±0.032	T
14	2.84±0.028	T	X	2.80±0.024	M
15	3.58±0.033	T	Y	1.20±0.009	SM
16	2.74±0.022	T	-	-	-

M1.0-1.6, Metacetricchromosome (M) SM1.7-2.9, Submetacentri-cchromosome (SM); ST 3.0-6.0, Subtelocentric chromosome (ST) T≥7.0, Telocentric chromosome

In the MDH patterns, two bands (m-MDH and s-MDH) were examined from all five domestic animal breeds, containing Songhei pig, Fatty-Tailed sheep, Simmental cattle, Angus cattle and Hereford cattle fibroblasts (Fig. 3b). The differences of bands were observed among those breeds and similar activities were seen from both m-MDH and s-MDH bands.

There were significant differences in the isoenzyme patterns of LDH and MDH between the Hereford cattle and other cell lines in the laboratory. All these results proved that the genetic characteristics of fibroblast cell line from Hereford cattle were stable and there was no cross-contamination of the line from other cell lines established in the laboratory.

Expression of three fluorescent protein genes in Hereford fibroblasts: Expression of pEGFP-N3, pEYFP-N1 and pDsRed1-N1 was observed at 12, 24, 48 and 72 h and 1 and 2 weeks after transfection. The three fluorescent protein genes were all highly expressed with reference the optimized conditions (Fig. 4). Positively expressing cells appeared at 12 h after transfection and the strongest fluorescence intensity and the highest transfection efficiency of the exogenous genes were observed at 48 h.

The Green Fluorescent Proteins (pEGFP-N3) were highest, especially. The expressing efficiencies were between 18.6 and 32.0% for the three fluorescent proteins at 24, 48 and 72 h (Table 2).

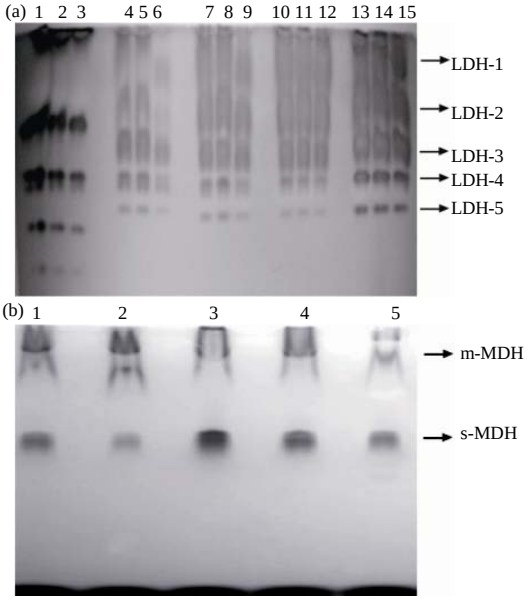

Fig. 3: a) The LDH pattrens; b) Two bands (m-MDH and s-MDH) examined from all five domestic animal breeds containing Songhei pig, Fatty-Tailed sheep, Simmental cattle, Angus cattle and Hereford cattle fibroblasts

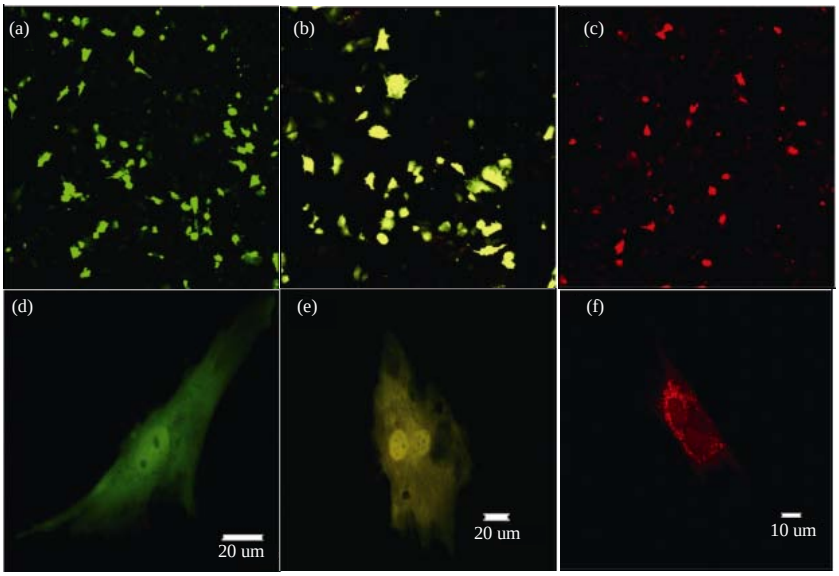

Fig. 4: a-f) The three fluorescent protein genes were all highly expressed with reference the optimized

Table 2: Efficiency of transfection of three fluorescent proteins

Transfection time (h)	pEGFP-N3 (%)	pEYFP-N1 (%)	pDsRed1-N1 (%)
24	18.6	19.2	18.9
48	25.4	32.0	27.1
72	22.3	24.8	23.2

The distribution of green, yellow and red fluorescence was observed using confocal microscope to determine the subcellular location of the three fluorescent proteins. The observation results indicated that the fluorescence extended all over the cytoplasm and nuclei of cells, except in the cryptomere vesicle. EGFP and EYFP displayed intense nuclear signals but DsRed mostly appeared in the cytoplasm surrounding the nuclear membrane and formed a accumulative ring circumscription.

The number of positive cells began to decrease at 1 week however, a few dispersed fluorescent cells remained after 2 weeks and even after 1 and 2 months. The motility rate of cells transfected of pEGFP-N3, pEYFP-N1 and pDsRed1-N1 were 91.5, 89.8.6 and 90.7%, respectively; none of these was significantly different from the control group (93.5%, $p>0.05$).

Establishment of Hereford cattle fibroblast line: The kindey tissue fibroblast line of Hereford cattle was successfully established using an adherent culture method. All the results indicated that the newly established cell line was stable and grew rapidly. *In vitro* culture conditions, the biological characteristics, especially the hereditary characteristics of the cells may be changed after many passages or trypsin digestion so, the slightest times of passages are recommended to conserve them. Thereore, the cells of this cell line were frozen within three passages and the cellular density was at a greater 3×10^6 mL^{-1} in order to ensure the cell motility rate for succedent recovery.

Morphological observations indicated that there were both fibroblast and epithelial cells appeared during the primary culture and early passages. Nevertheless, epithelial cells and fibroblasts have different tolerances to trypsin. When they were treated with trypsin, the detached speed of epithelial cells was slower than fibroblasts and when they were subcultured, the adhering velocity of fibroblasts was swifter than epithelial cells which were removed easily by mechanical agitation. After 2 or 3 passages, purified fibroblasts were obtained using these procedures.

Microbial contamination detection: In cell culture, microbial contamination is the most frequent pollution phenomenon. Many factors can induce the inquination in cells, for example air, equipment, serum, tissue sample, handling errors, etc.

The contamination caused by bacteria, eumycetes and mycetes can be seen with the naked eye for the turbidity of culture media. Viral contamination can be found under the inverted microscope for the pathological changes. Nevertheless, only the mycoplasma is harder to detect. Because the characteration of mycoplasma was no nucleus. Moreover, they can grow and reproduce in media even coexist with cells for a long time, accordingly, once mycoplasmas contaminated the cells, they were hard to

remove. The detecting methods of mycoplasma include direct solid agar culture and indirect fluorochrome stain of DNA and new DNA-probe hybridization. Furthermore, the pragmatic and rapid method is fluorochrome stain of mycoplasma which is commonly applied by cell culture collection institutions.

Karyotype analysis: Karyotype analysis data is an effective method to confirm the origin of a cell line. The technique has been applied for many years and is still used today. In practice, the technique has become a classical and standard method for characterizing cell lines (Shepel *et al*., 1994; Nims *et al*., 1998).

Except confirming the origin of a cell line, chromosome datas can also distinguish normal and variant cells because the chromosome number is more stable in normal cells (Freshney, 2000). In this research, the chromosome number of the Hereford cattle was 2n = 60 including 58 euchromosomes and two sex chromosomes and the ratio of diploid cells was 97.5%. The research objective was to preserve the genomic characteristic of Hereford cattle, requiring the fibroblasts maintaining the diploid character as the cells *in vivo*. The karyotype analysis showed that the established cell line was stable and satisfactory.

Isoenzyme analysis: One of the major problems in cell culturing was the misidentification or cross-contamination of cell lines. Isoenzyme polymorphism is a important standard in identifying interspecies cross-contamination and cellular origins (Li *et al*., 2008). Moreover, biochemical analysis of isoenzyme polymorphism is currently considered the standard method for quality control of cell line identification and interspecies cross-contamination and is routinely used around the world by the main biological resource centers such as European Collection of Cell Cultures (ECACC), American Type Culture Collection (ATCC) and American Type Culture Collection (ATCC) (Barile and Rottem, 1993).

LDH and MDH are species-specific and constant but the enzyme content and activity are different among species, the biochemical indicator of classifying species can be showed efficiently by chromatograms and electrophoresis (Parodi *et al*., 2002).

LDH was a tetrameric molecule, composing of the two subunits H and M which correspond to the *ldha* and *ldhb* gene products (Drexler *et al*., 1999). Prior research discovered 5-8 clear bands in different tissues. In samples of pectoral muscle, five clear bands were found (Moss, 1979). About 5 bands of the LDH isoenzymes have also been found in samples of cardiac muscle, liver and blood from Chinese junglefowl. In addition, LDH isoenzyme pattern was measured in horse leucocytes and plasma and Debao pony fibroblasts (Arai *et al*., 2003; Zhou *et al*., 2004).

MDH was a dimer and composed of cytosolic MDH (s-MDH) and mitochondrial MDH (m-MDH). MDH electrophoresis mobility among poultry and livestock is essentially identical however, MDH from livestock migrates faster compared with poultry and the enzyme content is also greater than in poultry.

Expression of fluorescent protein genes: The fluorescent proteins have been used as labeled genes to observe the expression and function of target proteins in live cells and organisms because they have stable structures, high expression levels and species-independent efficiency (Baird *et al*., 2000; Cheng *et al*., 2003). Some factors can affect transfection efficiency including DNA concentration, lipofectine concentration, the DNA incubation time, lipofectine combination and the presence of serum and so on and these factors have been confirmed by research on Vero cells, HeLa cells and various other cell lines (Escriou *et al*., 2001; Rui *et al*., 2006). In this experiment, the highest transfection efficiency are 32%, emerging at 48 h after transfection. After a week, strong expression levels of fluorescent protein were observed when the transfection efficiency degraded, revealing that the ectogenous genes in the fibroblasts can be replicated, transcribed, translated and modified. Comparing with the control cells during growth and reduplication, transfected cells were not significantly different. The results indicated that fluorescent protein expression had no obvious effect on the growth and proliferation of the transfected cells which was consistent with previous research (Huang and Li, 2001).

CONCLUSION

This study shows that all indexes of the Hereford cattle cell line met all the quality control standards of the American Type Culture Collection (ATCC). This cell line have not only preserved the genetic resources of the Hereford cattle at the cellular level but also provided valuable materials for more wider fields including genomics, postgenomics, somatic cloning and otherwise. Furthermore this study may provide both technical and theoretical supports for preserving genetic resources of other livestock and poultry at the cellular level.

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