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REPORT

Establishment and characterization of a fibroblast cell line from the Mongolian horse

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Abstract A fibroblast line was successfully established from Mongolian horse ear marginal tissue by using a primary explant technique and cell cryogenic preservation technology. Biological analysis showed the following: The cells were adherent and exhibited density-dependent inhibition of proliferation; assays of microbial contamination from bacteria, fungi, and mycoplasma were negative; the population doubling time of the cells was 33.9 h; and a 2n chromosome number of 64 at a frequency higher than 80%. A lack of cross-contamination of this cell line with other species was confirmed by isoenzyme analysis of lactic and malic dehydrogenases. In order to study exogenous gene expression, four fluorescent proteins, pEGFP-N3, pEGFP-C1, pDsRed1-N1, and pEYFP-N1, were transfected into the cells. The corresponding fluorescence was distributed throughout the cytoplasm and nucleus 12 h after transfection. This cell line not only preserves the genetic resources of the Mongolian horse at the cellular level but also provides valuable materials for genomic, postgenomic, and somacloning research in this species.

Keywords Mongolian horse · Fibroblast line · Establishment · Characterization

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It is important to maintain the genetic diversity of livestock and poultry in order to preserve their biological diversity. It is particularly crucial to protect the genetic resources of endangered animals and to utilize modern scientific methodologies to accomplish this feat. The preservation of individual animals, semen, embryos, genome, and complementary DNA libraries are all practical options in this regard. In addition to these methods, modern somatic cell cloning techniques can be used to conserve animal genetic materials (Wu 1999).

The Mongolian horse (*Equus caballus*) is an ancient horse breed in China that originated from the inner Mongolian grasslands. This animal was one of the 78 nationally protected domestic species, which was listed in 2004 by the Chinese government. There are many important genetic traits in the Mongolian horse, such as endurance, disease resistance, and resistance to cold weather, which have breeding potential by hybridization with other species.

In our report, we used the combined method of cell viability, microorganism detection, chromosome analysis, isoenzyme analysis, and fluorescent protein genes transfection to detect the established cell line (named MHF34 line) overall. Our object is to cryopreserve this genomic resource for the purposes of reviving endangered breed by cloning and supplying a convenient and effective resource for genomic research. Moreover, with the development of science and technology, the roles of limited cell lines will become increasingly prominent, and there will be currently unforeseen applications.

Ear margin tissues (about 1 cm^2 in size) were sampled from Mongolian horse and collected into separate tubes containing Dulbecco's modified Eagle's medium (DMEM) medium with ampicillin (100 U/ml) and streptomycin (100 μg/ml). The samples were immediately brought back

to the laboratory for further analysis. Each ear margin tissue sample was rinsed and then cropped to 1 mm^3 in size. Tissue pieces were seeded on the bottom of a tissue culture flask in a 37°C incubator with 5% CO_2 for 2 h until the tissue pieces spontaneously adhered to the flask surface, and then DMEM containing 10% fetal calf serum was added. Cells were harvested when they reached 80–90% confluence and were divided into prepared culture flasks at 1:2 or 1:3 ratios (Freshney 2000).

Prior to freezing, the culture was maintained in an actively growing state (log phase or exponential growth) to ensure optimal health and good recovery. The culture medium was changed 24 h prior to harvesting. The subculture protocol described above was then used for the harvested cells. The cells were enumerated with a hemocytometer, and cell viability was checked by Trypan Blue staining. The cells were subsequently centrifuged to form a pellet at 168 g for 8 min, the supernatant was removed, and then the cell pellet was re-suspended in media for freezing (10% DMSO+50% fetal bovine serum+40% DMEM) to reach a final cell concentration of $3–5\times10^6$ viable cells per milliliter. Subsequently, 1 ml of the cell suspension was allocated into one sterile plastic cryogenic vial labeled with the animal name, gender, cell line, passage number, and date. These vials were sealed and placed into boxes filled with the proper amount of isopropyl alcohol. Then, these boxes were placed in a −80°C freezer overnight, and the vials were transferred to a liquid nitrogen storage system (Werners et al. 2004).

To recover and reseed the cells, the frozen tubes were removed from liquid nitrogen and quickly thawed in 42°C water bath, and then the cells were transferred into a flask with complete medium and cultured at 37°C with 5% CO_2. The medium was renewed 24 h later (Freshney 1992).

Assays of cell viability before freezing or after recovery were carried out by using the Trypan Blue vital stain method. Cells were seeded in a six-well plate, and 1,000 cells were counted for viability (Yitao et al. 2007).

The cells with a concentration of 2.5×10^4 cells per milliliter were seeded into 24-well plates. After culturing for 7 d, three wells were monitored on a daily basis for density until they reached the plateau phase. Cell growth curves were plotted, and the population doubling time (PDT) was calculated based on the growth curve (Gu et al. 2006).

For details of the procedure used to detect potential bacteria, fungi, and yeast contamination, see Doyle et al. (1990). The cells were stained with Hoechst 33258 according to the DNA fluorescent staining protocol to detect whether they were contaminated by mycoplasma (Guan et al. 2005a,b). The enzyme-linked immunosorbent assay Mycoplasma Detection kit (Roche Diagnostics Corp., Indianapolis, IN) was used to detect the four most common mycoplasma species (*Mycoplasma arginini*, *Mycoplasma hyorhinis*, *Acholeplasma laidlawii*, and *Mycoplasma orale*), and to confirm the results of DNA staining for mycoplasma.

The cells were harvested at 80–90% confluence. Microslide preparation and chromosome staining were performed according to the description by Suemori et al. (2006). Fifty to 100 spreads were sampled for counting chromosome numbers per initial spread. There are three important parameters for the analysis of chromosomes, including relative length, arm ratio, and centromere index. Counts were determined according to the protocol of. Kawarai et al. (2006).

Isoenzyme patterns of lactic dehydrogenase (LDH) and malic dehydrogenase (MDH) were detected by using the vertical slab non-continuous polyacrylamide gel electrophoresis (PAGE) assay. In brief, the 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 the cell concentration was adjusted to 5×10^7 cells per milliliter, then centrifuged, and the supernatant was stored in aliquots at −80°C. Forty percent sucrose liquid and the samples (1:1) were mixed and then loaded in the individual lanes of the polyacrylamide gel (He and Zhang 1999). Different mobility patterns were indicated by the relative mobility front (RF), which was calculated as the ratio between the distance of migration of the isozyme band and the bromophenol blue.

Using the Tsuchiya et al. (2002) method, the fluorescent protein vectors pEGFP-N3, pEGFP-C1, pDsRed-N1, and pEYFP-N1 were transfected into the Mongolian horse cells with Lipofectamine™ 2000 transfection reagent (Invitrogen Corp., Carlsbad, CA). The plasmid DNA (μg) to Lipofectamine 2000 (μl) ratio was 1:3. After 8 h, the cells were removed from non-serum medium and transferred to the medium that contained serum. To estimate the transfection efficiency, the cells were observed after being transfected for 24, 48, and 72 h, respectively. Multiple comparisons were made from the test data to judge the difference between the groups. Cell morphologies were observed under confocal microscope (Nikon TE-2000-E, Japan). For each experimental group, images were captured from ten visual fields, and confocal microscope was used to measure the total and positive cell counts in each field to determine the transfection efficiency.

Ear marginal tissues were sampled from Mongolian horses. We used a primary explant technique and cell cryogenic preservation technology to establish the MHF34 line and proceeded to Biology and Genetics detection. Assays of microbial contamination from bacteria, fungi, and mycoplasma were negative. The culture conditions were optimal, and the cells were healthy (Fig. 1*a–d*). A few epithelioid and fibroblast-like cells grew from different tissues. The morphology of the cell was typical long spindle-shape. The cells were subcultured when they reached 90%

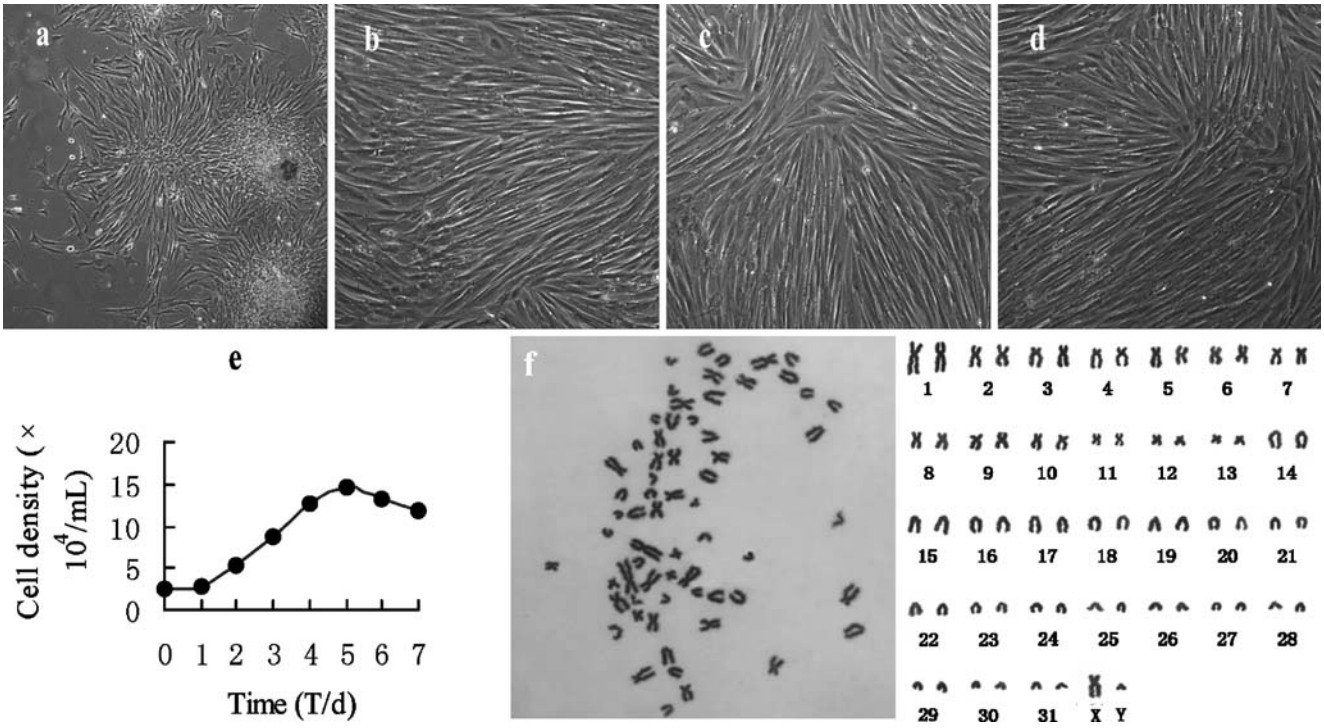

Figure 1. Morphology, growth curve and karyotype of MHF34 line. (*a*) Primary cells (×100), the cells were typical long spindle-shape with growth being slower. (*b*) Subcultured cells (×100), the cells were subcultured until they reached 90% confluence. After passage, growth accelerated and plateaued after 3–4 d. (*c*) Cells before cryopreservation (×100), the cells were healthy and in mitotic phase. (*d*) Cells after recovery (×100), the cells were cultured for 48 h after thawing; (*e*) growth curve of MHF34 line. The curve appeared as a typical "S" shape with cell density on the left axis. Growth curve included latency, exponential growth, and stationary phases. The population doubling time calculated from the curve data was about 33.9 h. (*f*) Chromosome at metaphase (*left*) and karyotype (*right*) of MHF34 line (♂) (×1,000). Mongolian horse 2n chromosomes number is 64, in which 13 pairs are metacentric/submetacentric chromosomes and 18 pairs are telocentric autosomal chromosomes. The X chromosome is the second longest submetacentric chromosome, and the Y chromosome is the shortest telocentric chromosome.

confluence. After passage, growth accelerated. The cells were contact-inhibited if they were not subcultured promptly. As a result, the cell proliferation became slow, not forming stratified clones and occurring apoptosis simultaneously (Table 1).

The growth curve of the MHF34 line appeared as typical "S" shape (Fig. 1e). There was a lag time or latency phase of about 24 h after the cells were seeded, corresponding to the adaptation of primary cells in recovery that were repairing the protease damage. The cells then proliferated rapidly and entered the exponential growth phase. They reached a stationary phase after about 3 d. The PDT calculated from the curve data was about 33.9 h.

Assays of microbial contamination from bacteria, fungi, and mycoplasma were negative. These results indicated that there were no microbial contamination of the MHF34 line.

Because Mongolian horse chromosomes are small and difficult to differentiate, only the hypodiploid, diploid, and hyperdiploid numbers were calculated for 50 cells. Table 2 showed that the majority of the cells were diploid, but the chromosome number changed over time with an increasing frequency of hyperdiploid occurrences. The diploid proportion could in fact drop to 84% by the fourth generation in culture, indicating that in vitro culture affected the heritage of cells slightly but supporting the inference that the cell line was reproducibly diploid.

According to Rothfels and Siminovitch (1958), the horse 2n chromosome number is 64, in which 13 pairs are metacentric/submetacentric chromosomes and 18 pairs are telocentric autosomal chromosomes. The X chromosome is the second longest submetacentric chromosome, and the Y chromosome is the shortest telocentric chromosome. Our present results are

Table 1. The cell density of MHF34 line after 11 d in culture (×10^4 cell per milliliter)

Time (d)	0	1	2	3	4	5	6	7	8	9	10	11
Cell density (×10^4cell/ml)	2.5	2.93	5.43	8.85	12.8	14.7	13.2	11.9	12.1	11.8	12.0	11.6

Table 2. Chromosome number in MHF34 line

Generation	Chromosome number			Total cell score	Percentage of 2n (%)
	Hypodiploid	Diploid	Hyperdiploid		
2	1	46	3	50	92
3	2	44	4	50	88
4	2	42	6	50	84

in accordance with this conclusion. The karyotype composition of the Mongolian horse is 8 m+6 sm+19 t, namely, chromosomes 1, 4, 6, 8, 9, 11, 12, and 13 are metacentric chromosomes; chromosomes 2, 3, 5, 7, 10, and X are submetacentric chromosomes, and chromosomes 14 to 31 and Y are telocentric chromosomes (Fig. 1*f*). However, Chao et al. (1992) reported that horse chromosomes 1, 9, 11, and 12 were submetacentric chromosomes, which may be due to chromosome translocation or distortion. Further analysis is needed to determine a concrete cause for this discrepancy.

The distribution patterns of isoenzyme polymorphisms may be a characteristic of a species or a tissue (Bowling et al. 1997; MacLeod et al. 1999). Polymorphism analysis of isoenzymes is currently the standard method for the quality control of cell line identification and detection of interspecies contamination. Isoenzyme patterns for LDH and MDH in Mongolian horse fibroblasts were obtained and compared with Texel Sheep, White Grey Goat, Songliao Black Pig, and Large Yorkshire. Patterns of LDH showed clear band differences for different livestock species (Fig. 2*a*). For the same livestock species and different breeds, there are less band differences in LDH isozymogram. These domestic animals had their own unique bands with a different relative mobility. These results indicated that there was no cross-contamination of MHF34 line from different cell lines established in our laboratory at the same time. The order of LDH activity from low to high is LDH4<LDH3<LDH5<LDH2<LDH1.

The MDH patterns reveal clear band differences in Texel Sheep, White Grey Goat, Songliao Black Pig, Large Yorkshire, and Mongolian Horse fibroblasts (Fig. 2*b*). All five domestic animals had two bands, and there was a single m-MDH band near the cathode and two s-MDH bands near the anode. Similar activity was seen from both m-MDH and s-MDH1, while s-MDH2 had weaker activity. These domestic animals had their own unique bands with a different relative mobility. These results indicated that there was no cross-contamination between different breeds.

The four fluorescent protein genes, pEGFP-N3, pEGFP-C1, pDsRed-N1, and pEYFP-N1, were all highly expressed with reference to the optimized condition. Positive cells were observed at the 12th hour after transfection. The numbers and intensity increased markedly and reached the summit at 48 or 72 h (without data shown). The numbers of fluorescent cells decreased at 1 wk, but a few dispersed positive cells remained after 2 wk and even after 1 and 2 mo. By screening G418 resistance and monoclonic culture for 1 mo, we obtained three positive cell strains that expressed EGFP, EYFP, and DsRed stably.

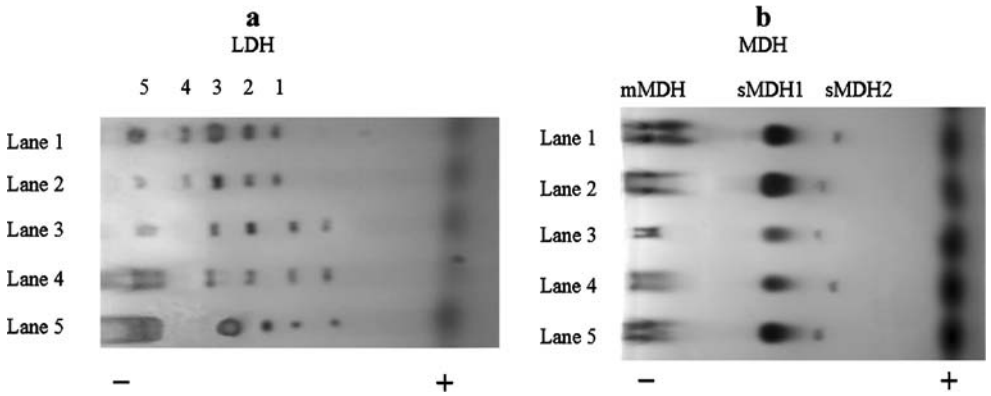

Figure 2. LDH zymotype and MDH zymotype of MHF34 line. These domestic animals had their own unique bands with a different relative mobility. These results showed that there was no cross-contamination between different breeds. (*a*) SDS-PAGE electrophoresis of LDH (*left panel*). (*b*) MDH (*right panel*). The species in the *left* and *right panels* were *lane 1* Texel Sheep, *lane 2* White Grey Goat, *lane 3* Songliao Black pig, *lane 4* Large Yorkshire, and *lane 5* Mongolian horse respectively; lanes *1–4* were control species. On the *left panel*, from *right* to *left*, there were LDH-1, LDH-2, LDH-3, LDH-4, and LDH-5. On the *right panel*, from *right* to *left*, there were m-MDH, s-MDH-1, and s-MDH-2, while s-MDH2 had weaker activity.

Discussion

All of the cultured Mongolian horse fibroblasts were harvested when they reached 80–90% confluence. Because too many passages and excess trypsin digestion could adversely affect the biological characteristics of such cells, especially hereditary characteristics, all cells used to preserve a species should undergo a minimum number of passages.

Our morphological results indicated that there were both epithelial cells and fibroblasts present during the primary and early passages of the explanted tissue. Different tolerances to trypsin were evident as the fibroblasts fell off first when treated with trypsin and were adherent again quickly after passage, while most epithelial cells were not adherent or stably adherent and fell off only when treated with mechanical agitation (Xue 2001; Ren et al. 2002). For this reason, purified fibroblasts could be obtained after two to three passages (Guan et al. 2005a, b; Zhou et al. 2005).

The average viability after thawing was above 93%, and freezing had little influence on the viability of the fibroblasts. Hence, it is likely that the Mongolian horse genome can be preserved by freezing fibroblasts in liquid nitrogen for long-term storage, and recovery and exam activity average 3 yr.

In this report, the chromosome number of the Mongolian horse was 64, including 62 autosomes and two sex chromosomes. There are 62 autosomes in these horse cells based upon our present findings, but some other experts consider these to be acrocentric chromosome because a shallow band on the centromeric summit has been described previously (Chen et al. 1995). Therefore, further karyotype research will be needed in the future. Because we want to conserve the genomic characteristics of the Mongolian horse, the fibroblasts derived from this species must maintain in vivo diplont characteristics. The result showed that the proportion of cells with a 2n of 64 was above 80%, i.e., the MHF34 line was a stable diplont cell line. Chromosome analysis can relate a cell line to the gender of the animal from which it was derived. It can also distinguish between normal and malignant cells, since the chromosome number is more stable in normal cells.

Isoenzymes harbor polymorphisms across species, races, individual, or diverse tissues, and intercellular cross contamination can be detected by isoenzyme analysis when 10% of the cells are affected (Nims et al. 1998). LDH and MDH are detected in specific quantities depending upon the animal species because enzymatic content and activity are also different among species. These biochemical indicators are reliable tools with which to distinguish different species by utilizing chromatograms and electrophoresis. Thus, we utilized LDH and MDH to confirm the origin and detect no cross-contamination of the MHF34 line.

The LDH isoenzyme is a tetramer that is composed of the two subunits A and B. The subunit phenotypes for A and B are LDH-1-5, corresponding to B4, B3A, B2A2, B1A3, and A4, respectively. Because of differences in molecular structure, under certain pH conditions, a protein may acquire a different electrical charge during PAGE, and then a different sized band on the gel will be obtained. LDH-A4 quickly catalyzes pyruvic acid to lactic acid in an anaerobic environment, so it mainly exists in skeletal muscle; LDH-B4 exists in heart in great quantities and promotes the synthesis of pyruvic acid while generating three molecules of carboxylic acid that can serve to produce more energy. The expression of LDH is thus doubly controlled by its gene, and the end metabolite and differences in its expression therefore exist among different organs or visceral organs in the same individual, between juveniles and adults, and of course between species. Each tissue contains a characteristic composition of isoenzymes in a species-dependent manner. Arai et al. (2003) and Ma et al. (2004) analyzed the LDH isoenzyme pattern in the leukocytes and plasma of Debao horse and pony fibroblasts. Their results showed that the plasma and Debao pony fibroblast LDH isoenzyme patterns were dominant for LDH-1, LDH-2, and LDH-3; LDH-3 and LDH-4 were dominant in the leukocytes. The present study on the LDH isoenzyme patterns of Mongolian horse fibroblasts showed that LDH-1, LDH-2, LDH-3, and LDH-4 were dominant.

MDH is a dimer that is composed of cytosolic MDH (s-MDH) and mitochondrial MDH (m-MDH). MDH electrophoresis band mobility among poultry and livestock is essentially identical, but the migration velocity of MDH in livestock is faster compared with poultry, with greater enzyme content also present. The former migrates towards the anode, the latter closer to the cathode. The cell extracts, electrophoresis conditions, and staining times must be controlled to obtain a clear isoenzyme zymogram. In our present analysis, two MDH isoenzyme patterns were detected after staining—the s-MDH isoenzyme pattern was thin and deep and the m-MDH isoenzyme pattern was broad and shallow.

In conclusion, the current results indicate that the newly established MHF34 line is stable and grows rather rapidly. The MHF34 line provides a useful approach for conserving this unique breed in China and will be an effective experimental material supply for further genetic studies on the Mongolian horse as well.

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