Establishment and Characterization of a Fibroblast Line from Sinihe Horse

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Abstract: For preserving the genetic resources of Sinihe Horse and exploring a new method to preserve endangered species, a Sinihe Horse fibroblast cell line was established successfully by the attachment culture. The cell line was preserved using the cryopreservation biotechniques. 48 Sinihe horse ear marginal tissue samples was used to form the cell line and has stocks of 156 cryovials, with each containing 4.5×106 cells respectively. Based on the research, Biological characteristics showed the following: During the cells proliferation, the cells were exhibited density-dependent inhibition; analysis of microbial contamination from bacteria, mycoplasma and fungi were negative; the cells population doubling time was 35.7 h; and chromosome number of the cells were 64, and the diploid frequency was higher than 80%. Isoenzyme assays of lactic and malic dehydrogenases showed no cross-contamination of this cell line with other species. Theree fluorescent proteins, pEGFP-N1, pEYFP-N1 and pDsRed1-N1, were transfected into the cells to study exogenous gene expression. The interrelated fluorescence distributed throughout the cytoplasm and nucleus 12h and 48h after transfection.The Sinihe Horse fibroblast cell line preserves the genetic resources of the Sinihe Horse at the cellular level and valuable material had been provided for genome, postgenome and somatic cloning research in this species.
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1. Introduction

Biological diversity mainly manifest in the genetic diversity， it is important to maintain the genetic diversity of livestock and poultry. Utilizing modern scientific methodologies to protect the genetic resources of endangered animals is particularly important. Various methods can be applied to preserve genetic resources, such as preservation of individual animals, embryos, semen, genome, complementary DNA libraries and so on[1]. In addition to the above methods, somatic cell cloning techniques can be used to conserve animal genetic materials[2].

Sinihe Horse is an ancient horse breed in China that originated in Inner Mongolia autonomous region ewenki autonomous banner Shini River. Sinihe Horse grows under the condition of nature extensively and There are many important genetic traits, such as strong body, speed, endurance and strong adaptability.

In order to research a more efficient method to save down the genetic resources of Sinihe Horse. In this study, we used the compositive method of cell viability, microorganism detection, chromosome analysis, isoenzyme analysis, and fluorescent protein genes transfection to detect the established cell line. To test and verify exogenous gene expression in the Sinihe Horse fibroblast cells, fluorescent protein genes were transfected into the cells as reporter genes. The objective of this study is to cryopreserve genomic resource from Sinihe Horse for the purposes of reviving endangered breed by cloning and supplying an effective and convenient resource for genomic research on breed qualities and breed enhancement. Moreover, with the development of modern Biotechnology and Clinical Technologies and Systems, the limited cell lines will play a prominent role on medical science research and may be useful in the unforeseen applications.

2. Material and Methods

2.1 Cell culture

Ear marginal tissue samples were obtianed from 28 Sinihe Horse (12 males and 16 females) and placed into Freezing Tube containing DMEM supplemented with ampicillin (100 U/ml) and streptomycin (100 g/ml). The samples were sent back to the lab within 24h for further analysis. In the shortest possible time, The samples were washed eight times at least with phosphate buffered saline (PBS) and cut into $1mm^3$ pieces, which were immediately seeded on the surface of a tissue culture flask containing high glucose DMEM+10% fetal bovine serum and incubated at 37.5℃ in a 5% CO_2 atmosphere[3]. when the cells reached 80% confluence, The media were refreshed, and next day, they were washed two times and they were passaged with 0.25% trypsin digesting and separated into prepared culture flasks at the ratio of 1:2 or 1:3[4].

2.2 Cryogenic preservation and recovery

Prior to freezing, the cells should be maintained in an actively state (log phase or exponential growth phase) to guarantee optimal health status and good recovery. 24 h before the cryopreserved, the culture medium was changed. The harvested cells were counted using a hemocytometer, and cell viability was checked by Trypan Blue staining. The cells were subsequently centrifuged at 1200rpm/min for 8 min, the supernatant was removed, and then the cell pellet was resuspended in Dimethyl sulfoxide (50% fetal bovine serum + 10% DMSO + 40% DMEM) to ensure a final cell concentration of $3\text{-}5\times10^6$ viable cells per milliliter. Then, 1ml of the cell suspension was put into one sterile freezing tube labeled with the animal name, gender, cell line, passage number, and date. These freezing tube were sealed and placed into frozen storage box filled with the proper amount of isopropyl alcohol. Subsequently, the box were placed in a −80°C freezer overnight, and the freezing tube were keep in reserve in a liquid nitrogen storage system[5]. when the cells were recovered and reseeded, freezing tubes should be taken out from liquid nitrogen and quickly thawed in a 42℃ water bath, Cell suspension in freezing tubes were transferred into sterile centrifuge tubes, and the cells were subsequently centrifuged at 1200rpm/min for 8 min, and then the resuspended cells were transferred into a flask with complete DMEM. The cells were cultured at 37.5℃ in a 5% CO_2 atmosphere and the medium was renewed 24h later[6].

2.3 Viability assay

Test of cell viability before freezing or after recovery were carried out using the trypan blue vital stain method. Though conventional counting, cells were digested and seeded in 6-well plates and 1000 cells were stained and checked for cell viability rate[7].

2.4 Growth curve

According to the traditional method, cells were seeded into 24-well plates at a final cell density of 4×10^4/ml and cultured for 7 days. Cell density were calculated and recorded each day (3 wells each time) until the last day. The mean cell counts at each time point were then used to plot the growth curve, the PDT was calculated in accordance with growth curve[7-9].

2.5 Microorganism detection

2.5.1 Detection of microbes

Cells were cultured in DMEM containing 10% fetal bovine serum without antibiotics and tested for the presence of bacteria and fungus at 3d after subculture according to the method of Doyle[10].

2.5.2 Test for viruses

Hay's hemadsorption protocol was used routinely to examine the samples for cytopathogenesis using phase-contrast microscopy[11].

2.5.3 Test for Mycoplasma

Cells were cultured in antibiotic-free medium for about 1 week, and then fixed and stained with Hoechst 33258 (Sigma, USA) according to the methods of Masover and Becker[12] and Freshney[13] for fluorescent staining of deoxyribonucleic acid. An ELISA Mycoplasma Detection kit (Roche, Lewes, East Sussex, UK) was used to confirm the results of the DNA fluorescent staining.

2.6 Chromosome analysis

Cells at the exponential growth phase were treated with 0.1 μg/mL colcemid for 5h at 37℃and were treated with a hypotonic KCl/HEPES/EDTA solution, and then harvested and fixed. After Giesma staining, the chromosome numbers were counted from 100 spreads under an oil immersion objective[8,14]. The parameters of relative length, centromere index and arm ratio index were calculated according to the protocol of Kawarai [15].

2.7 Isoenzyme analysis

Isoenzyme patterns of lactic dehydrogenase (LDH) and malic dehydrogenase (MDH) from Sinihe Horse ear marginal tissue fibroblasts were detected by using a vertical slab non-continuous polyacrylamide gel electrophoresis (PAGE) assay. Generally, the cells were harvested using the common method and protein extraction solution (0.9% Triton X-100, 0.06 mmol NaCl:EDTA in mass ratio 1:15) was added when the cell density was reached to 5×10^7cells/mL, then the mixture was centrifuged and the supernatant was stored at -80℃ .The mixture of liquid sucrose (40%) and the samples were loaded into the individual lanes of the polyacrylamide gel. The electrophoretic mobilities of LDH and MDH were determined the relative mobility front (RF)[16].

2.8 Expression of fluorescent proteins in Sinihe Horse fibroblasts

To get the low cytotoxicity and highest transfection efficiency, optimized transfection conditions by varying cell density, plasmid DNA and Lipofectamine 2000 concentrations were detected, according to lipofectamine media methods of Escriou[17] and Tsuchiya[18]. The cultured cells were observed 24h, 48h, 72h, after cells in the logarithmic growth phase were transfered using the fluorescent protein vectors pEGFP-N3, pECFP-N1, pDsRed1-N1, and pEYFP-N1 in the serum-free medium under excitation wavelength of 405, 488 and 543 nm separately. Each experiment group picked 10 visual fields to take pictures, and statistics all the cells and the positive cells of each field to count the

transfection efficiency by confocal microscopy. Finally, the positive cells could be selected through G418 resistance marker[19].

3. Results

3.1 Cell morphology and viability

After seeding, fibroblast-like and epithelial-like cells could be seen in 4-12 days(Figure. 1 A). With the extension of incubation time, the cells growth rate increased gradually, the cells were pasaged when the cells convergence degree attained 80%-90% in the whole culture bottle(Figure. 1B).The pasaged cells of culture medium were refreshed and the pasaged cells growed rapidly, and the proportion of fibroblast-like cells also increased gradually. After two to three passages, epithelial-like cells were disappeared, and fibroblasts were purified almost completedly (Figure. 1C). As measured by Trypan Blue staining, the viabilities of Sinihe Horse fibroblasts before freezing and after recovery were 97.6% and 94.5% respectively. The cell activity had no significant difference before cyropreservation and after recovery. The morphology of the cell was still a typical elongated spindle-shape. In brief, the culture conditions were appropriate and cells were not significantly affected by cryopreservation.

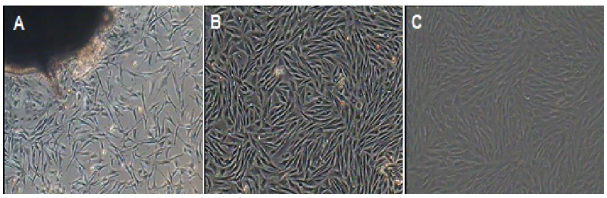

Figure 1. Morphology of Sinihe Horse fibroblasts cultured in vitro. (A) Primary cells (40×), the cells were typical long spindle-shape with growth being slower. (B) Subcultured cells (40×), the cells were subcultured until they reached 90% confluence. After passage, growth accelerated and plateaued after 3–4 days. (C) Cells after recovery (40×), the cells were cultured for 48h after thawing;

3.2 Growth dynamics

The growth curve of ear marginal tissue fibroblasts from Sinihe Horse had an obvious "S" shape (Figure. 2) and the PDT was about 24 h according to the growth curve. We can clearly see that there was a lag time or latency phase of about 24h after seeding, which conformed to the adaptation and recovery of the cells from protease damage, then the cells growed rapidly and entered exponential phase. With the cell density increased, proliferation was slow on account of contact inhibition; when the cell proliferated to the sixth day, the cells entered the plateau phase gradually and began to degenerate.

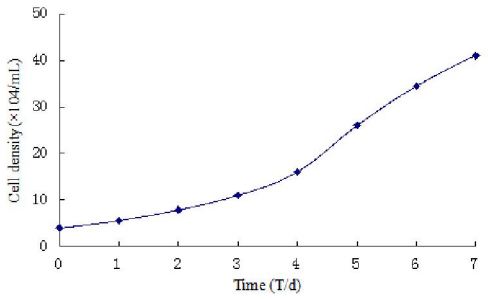

Figure 2. The growth curve of Sinihe Horse fibroblast line. The growth curve was an typical "S" shape. A lag phase of around 36 h was observed after cells were seeded. Then, cells proliferated and entered the logarithmic phase until they reached the stationary phase after about 6–7 days.

3.3 Microbial analysis

Experimental results Indicated that the tests for contamination with bacteria, fungi and yeasts were negative; there are no microorganisms in the culture media, and the growth state of cells was active. No viruses were detected by the cytopathogenic evidence or by the hemadsorption test. The detecting of mycoplasma contamination by staining with the DNA fluorochrome Hoechst 33258, which is the most effective and frequently used method in the mycoplasma contamination test. Fibroblast nuclei appeared as blue ellipses, showing that the established cell line was mycoplasma negative under a fluorescence microscope after staining with Hoechst 33258[20].

3.4 Chromosome and karyotype analysis

The chromosome number of Sinihe Horse is 2n=64, comprising 62 autosomes and two sex chromosomes, XY or XX (Figure 3), 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 analysis result was in accordance with this conclusion. The chromosome numbers per spread were counted for 100 spreads and the frequencies of cells with 2n=60 were 92.5%, and there were no aberrations in chromosome numbers and morphological characteristics. Above all, in vitro culture condition is optimal for the cell growth.

3.5 Isoenzyme analysis of Sinihe Horse cell line

The distribution patterns of isoenzyme polymorphisms are regarded as characteristic of a species or a tissue[21]. And the polymorphism analysis of isoenzymes is currently the standard method for the quality control of cell line identification and

detection of interspecies contamination. The LDH and MDH bands obtained from Sinihe Horse fibroblasts were compared with those from Mongolian horse (Figure.4). The Isoenzyme result showed five LDH isoenzyme bands and two MDH isoenzyme bands. Five bands were apparently representing LDH1, LDH2, LDH3, LDH4 and LDH5 from anode to cathode, two MDH isoenzyme bands were m-MDH and s-MDH, and one band of cytosolic MDH was located near the anode and another bands of mitochondrial MDH was found near the cathode. These results show that there was no cross-contamination between different breeds and the genetic characteristics of Sinihe Horse fibroblasts were stable in vitro.

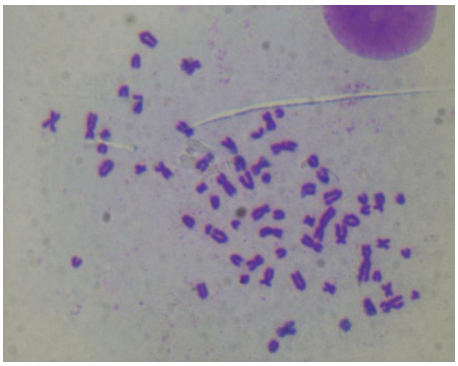

Figure 3. Karyotype of Sinihe Horse fibroblasts.

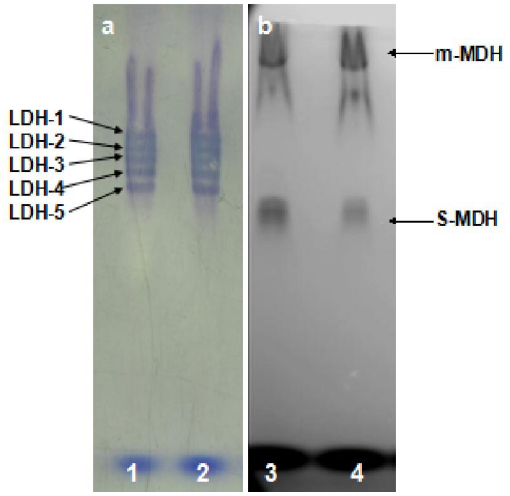

Figure 4. LDH zymotype and MDH zymotype of Sinihe Horse fibroblasts line and Mongolian horse. (1,3) Sinihe Horse; (2,4) Mongolian horse. These domestic animals had their own unique bands with a different relative mobility. These results showed that there was no cross-contamination between different breeds. (a) SDS-PAGE electrophoresis of LDH (left panel). (b) MDH (right panel)

3.6 Expression of fluorescent protein genes in fibroblasts from Sinihe Horse

Three fluorescent protein genes pEGFP-N1, pEYFP-N1 and pDsRed1-N1 were transferred into the Sinihe Horse fibroblasts according to the optimized condition when the cell density reached 75%-80%. 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 (Figure.5). The numbers of fluorescent cells decreased at 1 week, but a few dispersed positive cells remained after 2 weeks and even after 1 and 2 months. The subcellular location of the three fluorescent proteins were observed using confocal fluorescence microscopy. The results indicated that the fluorescence could be observed throughout the cytoplasm and nuclei of control cells, except in the cryptomere vesicle. DsRed was mostly shown in the cytoplasm surrounding the nuclear membrane, and formed a red ring profile, whereas EGFP and EYFP displayed an intense nuclear signal. Though the viabilities of transfected cells with pEGFP-N1, pEYFP-N1 and pDsRed1-N1 were 85.6%, 84.7% and 87.3% respectively. Among them, no one was significantly different from that of the control group (91.3%, $P > 0.05$).

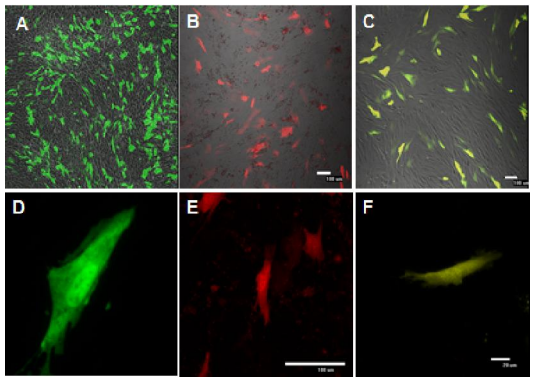

Figure 5. The expression and distribution of pEGFP-N1, pDsRed1-N1 and pEYFP-N1 in Sinihe Horse fibroblasts. A, B and C were the transfection results of pEGFP-N1, pDsRed1-N1 and pEYFP-N1at 48 h after transfection (100×); D, E and F were the subcellular location of pEGFP-N1, pDsRed1-N1 and pEYFP-N1at 48 h after transfection (400×).

4. Discussions

Animal genetic resources are regarded as a part of the biodiversity. There are extremely rich genetic resources However, our native animal genetic resources have not more attention recently, some are already gone extinct and some are faced the danger of extinction. Therefore, animal genetic resources must be considered as the insurance of the future, since

they may have an important potential to ameliorate our social and economic life.

Storage of somatic cells may open an option as pointed out by Corley-Smith and Brandhorst[22]. In our study, the marginal ear tissue fibroblast line from Sinihe Horse had been established successfully using an adherent culture method. All the above results indicate that the newly established cell line is stable and proliferates rather rapidly, and the identification of the cell line accords with the quality control requirement of ATCC. Therefore, genetic resources of Sinihe Horse are conserved by the means of freezing fibroblasts in liquid nitrogen and obtain the aim of protecting the breed. When the cells density reached 3×10^6/ml, the cells should be frozen within five generations, because the cells in low passages can keep enough activity and ensure full recovery of the cells subsequently. Owing to trypsin digestion, the cells may be injured and changed in biological characteristics, especially hereditary characteristics after too many passages[23,24].

The cells can be proliferated healthily only in the optimal growing conditions.therefore, Microbial contamination is the most frequent pollution phenomenon in cell culture. Air, equipment, serum, tissue sample, etc. could all contaminate the cells. If the culture media are contaminated by bacteria, eumycetes and mycetes, we can see the culture media is turbidy clearly with the naked eye. Viruses can be seen under the microscope. But it is hard to detect mycoplasma contamination.

The direct solid agar culture, Indirect fluorescence staining of DNA and new DNA-style hybridization can be used to detect mycoplasma contamination. Because fluorescent staining of mycoplasma DNA is simple and quick, it is regularly used by some cell culture collection institutions such as the ATCC. To avoid the microbial contamination, we perfomed stict detection. Our microbiological detection results showed that the Sinihe Horse fibroblast bank was purified and free of micoplasma contamination.

In order to confirm the origin of a cell line and identify possible cross-contamination of Sinihe Horse fibroblast bank, we carried on isoenzyme and karyotypic test. The two techniques have been used for many years and are still used today. The practice of combining them has become a classical and standard method for characterizing cell lines[25,26].

The hereditary stability of cell line is critical to preserve the genetic resources, the fibroblasts must maintain the same diploid character as the cells in vivo. The freezing procedure was improved so that the cells have a better hereditary stability. Though testing of karyotypic analysis, the mean frequency of diploid cells was 94.7%±3.74%, which indicated the Sinihe Horse fibroblasts cultured in vitro were stable diploid. With increasing number of passages, chromosome rearrangements may appeared[27]. Almost every passage has a frequency of diploid cells of above 90%, which further validating the genetic stability of these cells. Therefore, it could be inferred that there not appeared chromosomal structure variation in the Inner Mongolia cashmere goat fibroblasts.

Isoenzymes harbor polymorphisms across species, races, individual, or diverse tissues, and intercellular cross contamination can be detected by isoenzyme analysis[26]. Enzymatic content and activity are also different among species, which provids a biochemical indicator of species classification by analyzing chromatography and electrophoresis. therefore, we choose MDH and LDH to determine the species origin of the cells and to measure cross-contamination among species[28,29].

The LDH isoenzymes have also been studied in samples of cardiac muscle, liver and blood from Chinese junglefowl, and for all three tissues, five bands were observed. In accordance with all above studies, five LDH bands, LDH1, LDH2, LDH3, LDH4 and LDH5, were recorded in the samples from Sinihe Horse fibroblasts used in the present study. The mobilities of MDH bands are essentially identical among poultry samples. However, MDH from livestock migrates more rapidly than that from poultry, and the enzyme content is also greater than that in poultry. In this study, the isoenzyme bands of LDH and MDH of Sinihe Horse fibroblasts were clear and conformed to the above basic characteristisc, which indicated that the genetic feature were stable and there was no cross-contamination with cells of other species.

Fluorescent protein research mainly focus on tumors, nerves and stem cells[30]. DNA concentration, lipofectine concentration, the DNA incubation time and lipofectine combination, and the presence of serum, can affect transfection efficiency. In our study, the three fluorescent proteins was 31.3% with an optimized plasmid–lipofectin ratio. We could see that the transfected cells at the state of reduplication and different dividing and growth phases exhibited no obvious differences with control groups. The result showed that the transfected cells had not been affected by fluorescin under a certain range. The expressing location of fluorescent protein was different in cells from different species, for example GFP mainly expressed in nucleus in COS cell and DsRed expressed some granula-expression product surrounding nuclear membrane and formed a red ring profile. Considering these features of fluorescent protein transfection, it is very necessary for

researcher to detect the distribution of reporter gene before we analyzed the location of fusion protein.

5. Conclusion

The newly cell line from Sinihe Horse ear marginal tissue is established successfully, and its property is stable and grows rapidly, suggesting that it may be useful in the conservation of this unique breed. It also provides a new method for genetic research in future on Sinihe Horse.

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