Establishment and Identification of a Debao Pony Ear Marginal Tissue Fibroblast Cell Line*

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ABSTRACT : The Debao pony ear marginal tissue fibroblast cell line (NDPEM 2/2) was successfully established using either primary explant technique or collagenase technique. The characterizations of the cell line were identified as following: the cells were adherent and of density limitation; population doubling time (PDT) of cells made with the two techniques were 35.9 h and 48 h, respectively; chromosome analysis showed that the frequency of cell chromosome number to be 2n=64 was 91.3%-92.8%. Confirmed by isoenzyme analysis, this cell line had no cross- contamination. Tests for microbial contamination from bacteria, fungi, virus or mycoplasma were negative. This newly established cell line meets all the standard quality controls of ATCC. It will provide a precious genetic resource for the conservation of the Debao pony breed, as well as effective experimental material for genetic studies on Debao ponies. ***(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 10 : 1338-1343)***

Key Words : Debao Pony, Ear Marginal Tissue, Fibroblast Cell Line, Primary Explant Technique, Collagenase Technique

INTRODUCTION

The Debao pony breed in China was newly found in 1981. This breed inhabits only around Debao town in Guangxi Autonomous Region. The Debao pony in China is a natural breed arising from long-term genetic evolution and is among the 78 nationally protected domestic animal breeds (Editing Committee of Report on Domestic Animal Genetic Resources in China, 2004). In 1981, total number of individuals of this breed was 2000. Adult Debao ponies grow no taller than 106 cm, making this breed very promising for entertainment in the future (Editing Section of Horse and Ass Breeds in China, 1986).

In spite of many measures that were taken to preserve Debao ponies by national and local governments, the population of this breed has continuously decreased. Many purebred ponies were transported to other places and crossbred with other breeds. Protection of this breed from extinction is imperative.

At present, there are many strategies to conserve the genetic resources of domestic animals, of which live animal preservation is the main one and will be so for a long time (Wu, 1999). Nevertheless, establishment of cell banks using low temperature biological techniques is another effective approach (Shi, 1989). Generative cells, somatic cells, as well as zygotes and embryos can be cryopreserved at cell banks. However, most cell banks emphasizing conservation and utilization of animal resources, especially animal genetic resources, cryopreserve mainly generative cells and embryos (Ho et al., 1997; Oishi, 1997; Simon, 1999). Our project, in contrast, aims to cryopreserve somatic cells of nationally protected animals and to establish a cell bank for these animals for the purposes of reviving endangered breeds by cloning techniques and supplying a convenient and effective resource for genomic research on these animals. In this experiment, a fibroblast cell line of the Debao pony was established, and characterizations of the cell line were identified according to ATCC quality control methods.

MATERIALS AND METHODS

Biopsy of the ear marginal tissue of the Debao pony

Ear tissue samples (about 1 cm^2 in size) were isolated from 5 Debao ponies (3 male and 2 female), collected in five separate labeled tubes containing PBS (antibiotics added) and brought to the laboratory immediately.

Primary culture

The tissue samples were chopped finely and rinsed. Half of the pieces of each sample were seeded onto the surface of a tissue culture flask. DMEM medium with 10% fetal bovine serum was added to the flask and the flask was placed inverted in a 37°C incubator with 5% CO_2, for 18-24 h until the tissue pieces adhered spontaneously to the surface, and the flask was then turned over. The medium was changed when needed and the cultures were observed daily for a substantial outgrowth of cells from tissue pieces (Freshney, 2000).

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The other half of each sample was transferred to a 75 cm^2 flask, drained of PBS, and re-suspended in 4.5 ml of growth medium with serum. Half a milliliter of crude collagenase (2,000 units/ml) was added to the media to give a final concentration of 200 units/ml. Cultures were incubated at 37°C for 4-48 h without agitation, checked for effective disaggregation by gently moving the flask, and left up to 5 d or more if disaggregation was slow (sometimes replenished with fresh medium and collagenase). The cells were collected by centrifuging at 150 g for 5 min when most cells have disaggregated and soft tissue was observed. The cells were then washed once with PBS and combined in fresh medium, seeded in a 75 cm^2 flask at 5×10^5-1×10^6 cells/ml, and incubated at 37°C, 5% CO_2 (E, 1995).

Subculture

Cells were harvested when they reached 80-90% confluence. The cell sheet was rinsed twice with 0.9% NaCl to remove all traces of trypsin inhibitors in the serum supplied to the media, then 2.5 ml 0.25% trypsin solution was added. The flasks were incubated inverted in a 37°C incubator for 3 min, and were then turned over. The progress of the enzyme treatment after 30 seconds was checked under phase-contrast microscope. Flasks were gently shaken to shed cells from the flask walls when cells rounded up and detached. Growth medium was then added to the cell suspensions to stop the trypsinization. Cells were split into prepared culture flasks and incubated at 37°C, 5% CO_2 (Freshney, 2000).

Cryogenic preservation

Cells in logarithmic growth phase were supplied with fresh media 24 h before the freezing procedure to ensure optimal health and good recovery. Cells were then harvested using the same protocol as described for the subculture. The concentration of the cells in suspension was enumerated using a hemocytometer, with the viability of the cells being checked by trypan blue staining at the same time. Cells were pelleted by centrifuging at 150 rpm for 8min., the supernatant removed, and then re-suspended in cell freezing media (10% DMSO+90% fetal bovine serum) to give a final cell concentration of $1\sim3\times10^6$ viable cells/ml. Cells were then allocated 1 ml each into sterile plastic cryogenic vials labeled with animal name, gender, cell line name, passage number, and the date. The vials were sealed and placed into boxes filled with the proper amount of isopropyl alcohol. The boxes were placed in a -80°C freezer overnight, and then cells were transferred to liquid nitrogen storage system quickly and efficiently (Hay, 1978; Freshney, 2000).

Estimation of cell viability by dye exclusion

Cell viability before freezing and after thawing was determined using a hemocytometer by Butler's dye exclusion method (Butler, 1999).

Growth curve

Culture at concentration of $2\sim4\times10^4$ cells/ml was seeded into 24 well plates. Cell concentration was monitored each day by counting cells in three of the wells until they reached the plateau phase. Cell growth curve was then plotted and the population doubling time (PDT) was determined based on the curve (E, 1994).

Chromosome analysis

Chromosome fixation and spreading was performed following standard methods (Harnden, 1974). The chromosome numbers per spread for 50-100 spreads were counted. Karyotypes were prepared following the protocol provided by the Reading Conference report (Ford et al., 1980).

Isoenzyme analysis

The electrophoretic mobility of lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) were determined using the protocol contributed by Marvin L. Macy, ATCC, with following modifications: the vertical slab polyacrylamide gel electrophoresis apparatus was used and the electrophoretic buffer was changed into Tric-glycin (pH=8.7) and gel buffer was prepared into discontinuous system using two kinds of buffers: 0.078 mol/L Tris-citric acid (pH=8.9) and 0.017 mol/L Tris-citric acid (pH=6.8) (Yang and Wu, 1999). Electrophoretic mobility was defined by number and intensity of enzyme bands, as well as the distance of band migration from the point of origin for each sample (Freshney, 2000).

Screening for contamination

Test for bacteria, fungi and yeasts contamination : Monolayer cell cultures were cultured on two types of test media. The detailed procedure used for bacteria, fungi and yeasts contamination test was described by Doyle et al. (1990).

Test for the presence of viruses: Routine examination for cytopathogenic effects using phase-contrast microscopy was performed following Hay's protocol (Hay, 1992).

Haemadsorption test was also performed in order to detect the presence of certain viruses that do not produce a CPE in cultured cells (Hay, 1992).

Mycoplasma detection

Cells were cultured in media free of antibiotics for at least one week, then fixed and stained with Hoechst 33258 according to Freshney's method (2000).

The ELISA Mycoplasma Detection kit (Roche, Lewes, East Sussex, UK) was used to confirm the results of above

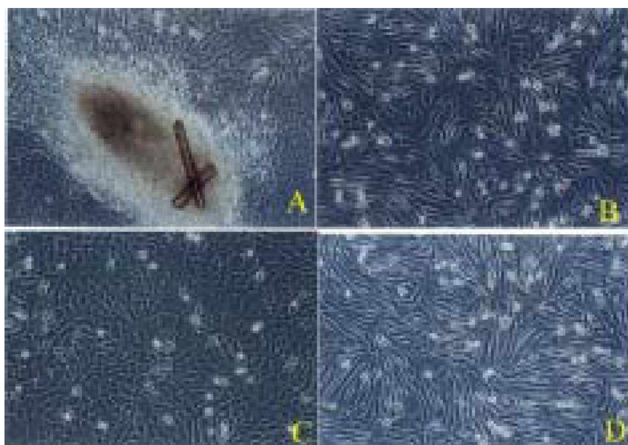

Figure 1. Cells cultured from Debao pony ear marginal tissue using primary explants technique and collagenase technique. A: primary cells prepared by primary explants technique ×100, a few of fibroblastic or epithelial cells migrating from explanted tissue, B: fibroblast cells after primary cells prepared by primary explants technique subcultured two passages ×100, fibroblast cells replaced the epithelial cells in subcultures and were well spread on the culture surface, forming characteristic multipolar or bipolar shapes, C: primary cells prepared by collagenase technique ×100, cells dispersed well after dissociation in crude collagenase. They grew rapidly and appeared fibroblast-like or epithelial-like, D: fibroblast cells after primary cells prepared by collagenase technique subcultured two passages ×100, fibroblast cells replaced the epithelial cells in subcultures and were well spread on the culture surface, forming characteristic multipolar or bipolar shapes.

DNA staining for mycoplasma. The kit was able to detect the four most common Mycoplasma species (A: *M. arginini*, B: *M.hyorhinis*, C: *A. laidlawii, and* D: *M.orale*).

RESULTS

Morphology of NDPEM 2/2

Cells produced from direct tissue explant pieces technique grew slowly. Only a few fibroblastic or epithelial cells were observed migrating from tissue half a month after planting (Figure 1A). Cells then continued to proliferate, and were subcultured when they reached 90% confluence. Fibroblasts grew rapidly, and replaced the epithelial cells gradually in subcultures. Cells were well spread on the culture surface, forming characteristic multipolar or bipolar shapes (Figure 1B).

Cells prepared by collagenase disaggregation grew rapidly. A few were observed spreading on the culture surface 2 or 3 days after seeding. Cells appeared fibroblast-like or epithelial-like, and reached almost 90% confluence at ten days after seeding (Figure 1C). Fibroblasts outgrew other cells in the common medium and excluded other cells gradually after several passages of subculturing (Figure 1D).

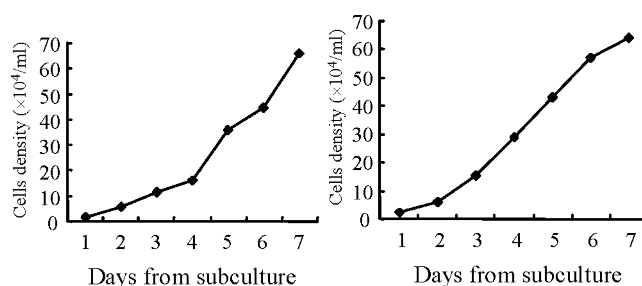

Figure 2. The growth curve of Debao pony ear marginal tissue fibroblast cell. A: Cells prepared by primary explant technique grew a little slowly before the exponential (logarithmic) phase, the population doubling time was 48 h, B: Cells prepared by disaggregation technique using crude collagenase grew rapidly, the population doubling time was 35.9 h.

Viability of NDPEM 2/2 before freezing and after thawing

Cells were frozen after subculturing 3~4 passages. The average viability was 95.6% before freezing. Some ampoules were selected and the cells were recovered. The average viability of cells was 92.2% after thawing. The results revealed that cells were healthy in culture conditions, and that freezing had little influence on the viability of the cells.

Growth curve

Figure 2 shows the growth curve of Debao pony ear marginal tissue fibroblast cells. The growth curve appeared to be of "S"shape. Population doubling times for cells prepared by primary explant and collagenase were 48 h and 35.9 h respectively. The results showed that, the cells prepared by disaggregation technique, to certain extent, grew more rapidly than those prepared by primary explant technique.

Chromosome analysis of NDPEM 2/2

According to Rothfels and co-workers, the correct horse chromosome number was 2n=64. Horses had 13 pairs of metacentric/submetacentric and 18 pairs of acrocentric autosomes. The X chromosome was the second largest submetacentric, while the Y chromosome was one of the smallest acrocentrics (Bowling et al., 1997). Our results were consistent with their conclusion. The chromosome numbers per spread for 100 spreads of first passage and second passage were counted, and the results showed that the frequencies of cells to be 2n=64 were between 93.1% and 92.8%, supporting that the cell line was a steady diploid one. Two pictures of prepared karyotypes are shown in Figure 3.

Isoenzyme analysis

Enzyme protein polymorphism (isoenzymes) occurs among species and, sometimes, among races, individual,

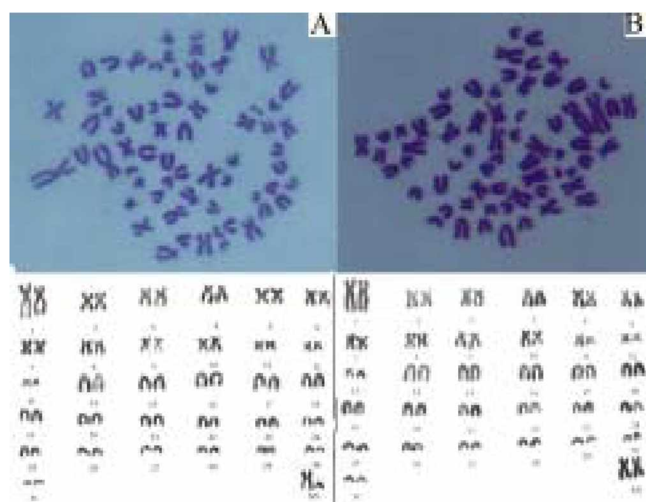

Figure 3. Karyotype of Debao pony. 2n=64, there were 13 pairs of metacentric/submetacentric, 18 pairs of acrocentric autosomes, and a pair of sex chromosomes. A: Conventional Giemsa-stained karyotype of Debao pony stallion. B: Conventional Giemsa-stained karyotype of Debao pony mare.

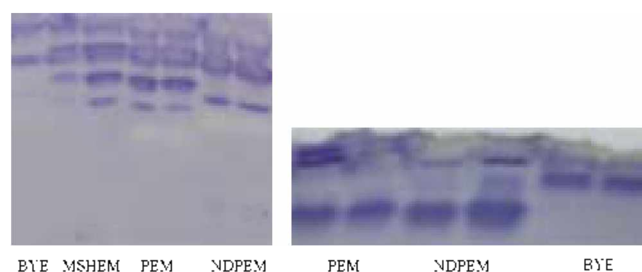

Figure 4. The banding patterns of the isozyme of LDH (left picture) and MDH (right picture) for the Debao pony, Picdmont, Mongolian sheep ear marginal tissue fibroblast cell lines and Beijing yellow embryo fibroblast cell lines, Five isoenzymes bands of LDH (LDH-1, -2, -3, -4, -5) and two isoenzymes bands of MDH were observed in the NDPE. LDH-1, -2 and –3 were dominant, LDH-4 and -5 were scarcely observed. There were significant differences for their isoenzyme patterns of these two enzymes between the NDPE and other cell line in our laboratory. DPEM2/2: Debao pony ear marginal tissue fibroblast cell line; BYE2/2: Beijing yellow embryo fibroblast cell line; MSHEM2/2: Mongolian sheep ear marginal tissue fibroblast cell line; PEM2/2: Picdmont ear marginal tissue fibroblast cell line.

and tissues within a species (O'Brien et al., 1977). Those isoenzymes may be separated chromatographically or electrophoretically, and the distribution patterns may be characteristic of species or tissue. Biochemical analysis of isoenzyme polymorphism is currently considered to be the standard method in quality control of cell line identification and interspecies contamination, and is routinely used by the main Biological Resource Centers around the world (i.e., ATCC, ECACC, DSMZ and Riken) (Parodi et al., 2002). However, this test requires specific equipment and expensive reagents, and is difficult to operate. We have improved the apparatus and electrophoretic conditions with reference to polyacrylamide gel electrophoresis (Yang and Wu, 1999), and successfully determined the mobility of the two isoenzymes (MD and LDH). The results obtained in terms of number and intensity of enzyme bands from the point of origin for Debao pony ear marginal tissue fibroblast cells (NDPEM 2/2) were compared with that of Picdmont ear marginal tissue fibroblast cells (PEM 2/2), Mongolian sheep ear marginal tissue fibroblast cells (MSHEM 2/2) and Beijing Yellow embryo fibroblast cells (BYE 2/2). The banding patterns of the isoenzyme of LDH and MDH for those cell lines were shown in Figure 4.

Screening for contamination

Test for bacteria, fungi and yeast contamination : The culture didn't become turbid or display other changes in negative controls or in media collected from cell cultures while there was obvious growth of microorganisms in the positive controls.

Test for the presence of viruses : No presence of viruses was indicated either by routine cytopathogenic effect examination, or by additional haemadsorption test.

Mycoplasma detection : A number of methods have been developed to detect mycoplasma contaminations with various efficiencies (Masover and Becker, 1998; Barile and Rottem, 1993; Uphoff and Drexler, 2002). Among them the direct demonstration of mycoplasmas by deoxyribonucleic acid (DNA) fluorenchrome staining (e.g., by Hoechst 33258) seems to be the most frequently applied method. This method is also recommended by ATCC to detect mycoplasmas. However, in our study, we found the results of tests using this technique are often not easy to interpret. The staining pattern observed may be confused with the cell debris (Garner et al., 2000). Interpretation of DNA staining patterns requires a greater level of expertise than other tests. To make sure of an accurate detection of mycoplasma, we confirmed the results obtained from DNA staining with ELISA using the ELISA Mycoplasma Detection kit. This kit contains polyclonal antibodies against the four most common Mycoplasma species (A: *M. arginini*, B: *M. hyorhinis*, C: *A. laidlawii* and D: *M. orale*). The results of the two tests showed that the established cell line was negative for mycoplasma (cells stained with Hoechst 33258 were shown as Figure 5).

DISCUSSION

Animal diversity is a valuable treasure for agricultural development particularly and socio-economic development generally. Man depends on these bioresources for his continued existence and sustainable development. However, with economic development, many local breeds are endangered or facing extinction because their performance.

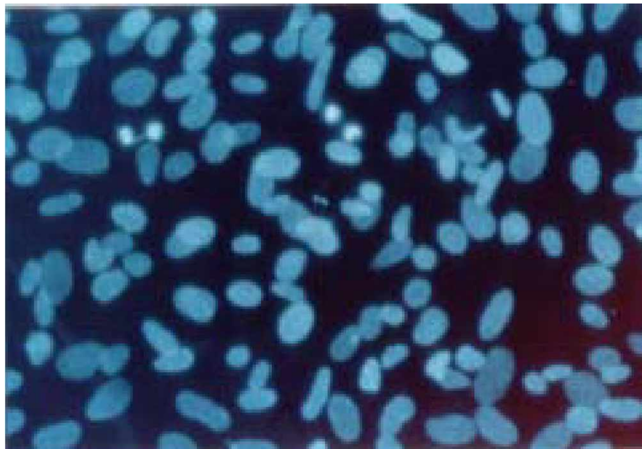

Figure 5. Debao pony ear marginal tissue fibroblasts stained with Hoechst 33258. Mycoplasma uninfected cells were seen as fluorescing nuclei against a negative background, whereas cultures infected with mycoplasma contain both fluorescing nuclei and extranuclear mycoplasmal DNA.

food conservation efficiency or lean meat percent are much lower than that of imported breeds. Today, many measures are taken to conserve animal genetic resources in many countries. With the report from Ian Wilmut of the cloning of Dolly, the application of cloning technology to conservation efforts for endangered species seems appropriate (Ryder and Benirschke, 1997). Moreover, rapid development and wide application of nuclear transfer using somatic cells broadens the choice of making transgenic domestic animals (Lee and Piedrahita, 2003). In this experiment, a fibroblast cell line for the Debao pony was established, after consideration of its future function for the conservation of this breed and some fine genes.

Procedures we used in this study conformed to the protocols of ATCC technique bulletin for primary culture, subculture and freezing. Moreover, we characterized the established cell line according to ATCC quality control procedures. We found that, firstly, the morphology of the primary culture cells, prepared either by direct explant or by collagenase disaggregation, appeared to be a mixture of fibroblasts and epithelial cells. However, after several passages of subculturing, fibroblastic cells outgrew the epithelial cells and excluded the epithelial cells gradually. The reason may be that the two types of cells have different tolerance to trypsin in the subculture. Fibroblast cells are usually more easily disaggregated and detached from the surface of a flask. In addition, fibroblast cells adhere and attach faster than epithelial cells, which barely attach or attach unstably by themselves, and require certain growth substrates such as collagen to sustain them (Xiao, 2001; Ren et al., 2002). Thus, the fibroblast cells could have outgrown the epithelial cells through enzymic digestion and repetitive attachments.

Secondly, the growth curves indicated that the growth speed of the cells obtained by primary explant technique is a little slower compared with that of the cells obtained by disaggregation technique in crude collagenase, and PDT were 35.9 h and 48 h for the two cell preparations, respectively. The technique of primary explant is particularly suitable for small amounts of tissue, such as skin biopsies. It has a rather lower risk of losing cells compared with enzymatic disaggregation. Its disadvantages lie in the poor adhesiveness of some tissues and the selection of cells in the outgrowth (Freshney, 2000). Moreover, on some horse, pig and sheep ear tissue samples in our experiments, no cells migrated out even after three months of culturing. We suggest, based on our observations, that the technique of primary explant in combination with enzymatic disaggregation is a very applicable and effective technique for thick tissue samples such as ear marginal tissue, also for samples obtained from precious sources.

Isoenzyme and karyotypic data together can confirm the origin of a cell line and identify possible cross-contaminations. The two techniques had been applied for many years and are still used today. The practice of combining these two techniques together has become the most classical and standard one in characterizing cell lines (Committee on Standardized Genetic Nomenclature for Mice, 1972; Nelson et al., 1981; Shepel et al., 1994; Nims et al., 1998). Several researchers' were able to show, using the above-mentioned combined method that many commonly used cell lines were cross-contaminated with HeLa (Nelson-Rees and Flandermeyer, 1977; Lavappa, 1978). Chromosome analysis even 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 (Freshney, 2000). In this experiment, we used the combined method to test the established cell line, and the results showed that there was no cross-contamination, and the cell line was derived from the Debao pony. Moreover, we have improved the protocol of electrophoresis by trying a local apparatus and different buffers, thus making this technique feasible under our conditions.

CONCLUSION

The current results indicate that the newly established cell line is stable and grows rather rapidly, and the identification for this cell line conforms to the requirement of quality control of ATCC. These characteristics suggest the Debao pony ear marginal tissue fibroblast cell line provides a useful approach for conserving this unique breed in China and would be an effective experimental material supply for further genetic studies on the Debao pony as well.

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