

Establishment and characterization of fibroblast cell line of the Songliao Black pig

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Liu, X., Li, L.-F., Li, X., Ma, Y. and Guan, W. 2009. **Establishment and characterization of fibroblast cell line of the Songliao Black pig.** *Can. J. Anim. Sci.* **89**: 463–466. The Songliao Black pig was the first lean-meat maternal breed bred in China. To preserve genetic diversity of this breed, we designed a procedure to use ear marginal tissue of one male pig to establish a fibroblast cell line (designated SBPF18), which was subsequently characterized according to the population doubling time, karyotype analysis, isoenzyme assay, DNA fingerprint and sex determination, meanwhile microbial contamination assays were conducted to demonstrate the cell line was contamination-free. This method is suitable for establishing the fibroblast cell line from small amounts of tissue, and could further facilitate genomic studies and genetic modification.

Key words: Songliao Black pig, fibroblast cell line, primary explants technique, cryogenic preservation

Liu, X., Li, L.-F., Li, X., Ma, Y. et Guan, W. 2009. **Établissement et caractérisation d'une lignée de fibroblastes du porc noir Songliao.** *Can. J. Anim. Sci.* **89**: 463–466. Le porc noir Songliao est la première race maternelle à viande maigre hybridée en Chine. Pour préserver la diversité génétique de la race, les auteurs ont mis au point une méthode de laquelle un peu de tissu marginal de l'oreille d'un mâle a servi à établir une lignée de fibroblastes (appelée SBPF18), qu'on a ensuite caractérisée d'après le temps de doublement de la population, l'analyse du caryotype, le dosage des isoenzymes, l'identification du code génétique et la détermination du sexe. Les auteurs ont également procédé à des essais de contamination bactérienne pour prouver que la lignée n'était pas polluée. La procédure se prête très bien à l'établissement d'une lignée de fibroblastes à partir d'une petite quantité de tissu et pourrait faciliter les études en génomique ainsi que la manipulation génétique.

Mots clés: Porc noir Songliao, lignée de fibroblastes, technique des explants primaires, conservation par cryogénie

The Songliao Black pig was the first lean-meat maternal swine bred successfully in northern China, in 1988. As well as its high reproducibility and meat quality, the breed also has strong adaptability and resistance. For example, anti-freeze test showed the Songliao Black could endure -28°C for up to 15 min (Zhang 2007). Therefore, the Songliao Black is very suitable for raising in northeast China, and is usually crossbred with other breeds, even with wild boars for considerable heterosis (Jin et al. 2008). Because the Songliao Black is of economic importance for Chinese agriculture and the meat industry, it has been ranked as a nationally protected swine breed. Until now, most studies have focused on analyzing various quantitative traits of the Songliao Black (Zhang et al. 2007); however, investigations concerning cell biology and genetic background are still scarce. The purpose of our study was to establish and characterize a porcine fibroblast cell line (designated SBPF18) of the Songliao Black pig, which

will not only contribute to precious genetic resource preservation, but provide excellent cellular resource for further genomic and genetic research.

During all the experiments, all procedures and protocols were in compliance with the Statement on Animal Care and Usage in Research and Teaching (Animal Care Committee of the Institute of Animal Science 2004). Ear margin tissue samples (each about 1 cm^2 in size) were collected from one 8-mo-old Songliao Black pig (δ) and placed into sterile tubes containing DMEM medium supplemented with ampicillin (100 U mL^{-1}) and streptomycin ($100\text{ }\mu\text{g mL}^{-1}$). Samples were chopped finely with crossed scalpels into about 1-mm^3 pieces, which were transferred to a 50-mL sterile centrifuge tube and washed with DMEM. The pieces were seeded on the surface of a tissue culture flask, and DMEM medium

Abbreviations: LDH, lactate dehydrogenase; MDH, malate dehydrogenase

with 10% FBS was added. Incubated flasks were inverted in a 37°C incubator with 5% CO₂ for 3–6 h until tissue pieces adhered to the flask surface; the medium was changed weekly, and a substantial outgrowth of fibroblastic-like or epithelial-like cells was observed. After two 25-min incubations in warm trypsin (0.27%), fibroblasts were separated from epithelial-like cells. The fibroblast was subcultured and harvested following the protocol previously described (Freshney 2005). Before cryogenic storage, 10% DMSO was selected as cryoprotective agent and a CyroMed freezer (Thermo Scientific) was used for controlled-rate cell freezing overnight. Finally, cells were transferred to a liquid nitrogen freezer for long-term cryogenic storage. We randomly selected three vials and thawed them by gently agitating them in a water bath at 37°C. The contents of each vial were then transferred to one 25-cm² tissue culture flask, and incubated at 37°C. The Butler's dye exclusion method (Butler 1999) was used to detect cell viability after recovery. Meanwhile, several methods, including Hoechst 33258 staining, were used to demonstrate the absence of microbial- and cross-contamination. We also studied the karyotype of this cell line following the method of Rooney and Czepulkowski (1986). In total, 50 to 100 spreads were sampled for counting chromosome analysis. To further characterize isoenzyme activities, a discontinuous SDS-PAGE electrophoresis system was used to determine motility of lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) with optimizations on electrophoretic buffer (containing Tris-glycin pH 8.7) and two kinds of gel buffers (containing 0.078 mol L⁻¹ Tris-citric acid pH 8.9 and 0.017 mol L⁻¹ Tris-citric acid pH 6.8, respectively) (He and Zhang 1999). To identify the DNA fingerprint, one modified multiplex PCR system was used for amplifying 10 porcine microsatellite loci (SW72, SW2185, SW983, SWR2516, SW2093, SWR136, SW2, SW395, SW349, SW435) (Guo et al. 2004); primer sequences of all loci are accessible at GenBank (<http://www.ncbi.nlm.nih.gov>). At the same time, a pair of primers (SSRYU:TGAACGCTTTCATTGTGTGGTC and SSRYL: CCTGTAGCC TCTGTGCCTCCT) were designed and synthesized to amplify a 161-bp fragment from the swine SRY locus to conduct sex determination.

The average viability of thawing cells was up to 93.7% ($\pm 3.1\%$ SD) (Fig. 1A). Isoenzyme patterns for LDH and MDH of the Songliao Black were obtained and compared with those of the Tan sheep, whose cell lines were established at the same time (Fig. 1B). These domestic animals showed species-specific bands with different relative mobilities. These results also indicate that there was no cross-contamination among different cell lines established in our laboratory. The chromosome number of the Songliao Black pig is $2n = 38$ and frequencies of cells that were $2n = 38$ was 93.5–94.8%. The karyotype of the male was $2n = 38$, XY (Fig. 1C, right panel). Five pairs of chromosomes of the Songliao

Black were submetacentric (SM), two pairs were subtelocentric (ST), five pairs were metacentric (M) and six pairs were telocentric autosomes (T), and X and Y chromosomes were all metacentric (Fig. 1C, right panel). The population doubling time, calculated based on the growth curve (Fig. 1D), averaged 72 h, while genotype of the SBPF18 cell line were SW72(123/135), SW2185(188/198), SW983(222/230), SWR2516(227/251), SW2093(183/191), SWR136(302/302), SW2(205/205), SW395(167/175), SW349(313/321), and SW435(191/191) (Fig. 1E). The existence of a clear 161-bp DNA band amplified from the SPY locus on agarose gel (2%) after 40 min electrophoresis further confirmed that the SBPF18 originated from a male Songliao Black pig (data not shown).

In this study, we used the combined methods of cell viability, microorganism detection, chromosome analysis, isoenzyme analysis, DNA fingerprint and sex determination to detect the established cell line (named SBPF18) of the Songliao Black pigs. Among all the methods used in this study, the warm trypsinization acted as one of the most crucial steps to determine cell line quality. It is well known that prolonged exposure of the tissue to trypsin at 37°C may damage cells, so minimizing the exposure could preserve maximum viability. A simple method of minimizing damage to the cells is to soak the tissue in trypsin at 4°C for 6–18 h to allow penetration of the enzyme with little tryptic activity (Freshney 2005). However, it did not work well for fibroblasts of the Songliao Black pig, although the underlying mechanism is still unclear. Based on preliminary experiments, we found that harvesting cells after 25-min incubations in warm trypsin (0.27%) optimized viable cell yield. Based on this procedure, 10 other fibroblast cell lines of the Songliao Black (five males and five females) have been established successfully (data not shown). Given the fibroblast cell line of the Songliao Black as a case study, our method is particularly suitable for small amounts of tissue, such as skin biopsies. We believe that the procedure reported here will also greatly facilitate establishment and characterization of fibroblast cell lines for other valuable domestic animals.

Presently, farm animals supply an estimated 30% of all food and agricultural needs. However, big farms and modern agriculture, both in China and around the world, often depend on only a few breeds of livestock. Though at least 4000 breeds of farm animals have been developed, hundreds of breeds have disappeared. Since 1993, the United Nations Food and Agriculture Organization (FAO) and the United Nations Environment Programme (UNEP) have published the World Watch List for Domestic Animal Diversity to document the state of global livestock genetic diversity. Monitoring, describing, and characterizing existing breeds constitute a vital part of conservation, allowing us to understand each species' status, as well as its unique qualities and potential. According to the FAO, which publishes a list

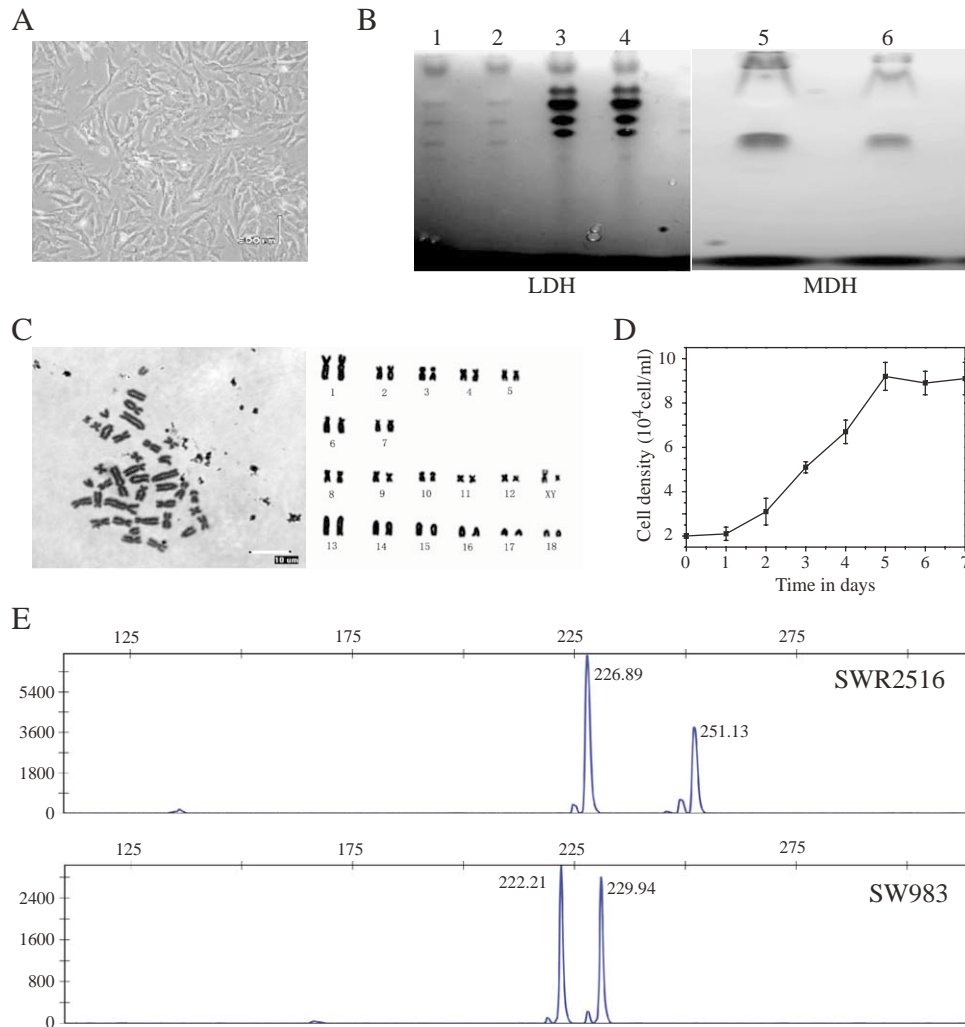


Fig. 1. Construction and characterization of fibroblast cell line of the Songliao Black pig. (A) Quantitating cell recovery after freezing. After thawing, fibroblasts were cultured for 12 h, ($\times 100$); (B) SDS-PAGE electrophoresis of lactate dehydrogenase (LDH) (left panel) and malate dehydrogenase (MDH) (right panel) of the Songliao Black pig (lanes 1, 2, 5) and the Tan sheep (lanes 3, 4, 6). On the left panel, from top to bottom, there were LDH-1, 2, 3, 4, 5. Fibroblasts of both species were cultured in our laboratory at the same time. Differences between two isoenzymic electrophoretic patterns of the Songliao Black pig and the Tan sheep demonstrated no cross-contamination; (C) chromosome at metaphase (left) and karyotype (right) of the Songliao Black pig (♂) by the standard trypsin-Giemsa technique ($\times 1000$); (D) growth curve of revival cells. Growth curves of cells following subculture are shown with cell density on the left axis. The data shown are means \pm standard deviation and are representative of three independent experiments for those randomly selected three vials; (E) genotype assays of swine microsatellite loci SWR2516 and SW983 for the SBPF18 cell line.

of endangered farm animals, more than 800 breeds are still in danger of disappearing (Scherf 2000). People are beginning to become aware of how serious the situation is, and experts warn that we are losing genetic diversity in China. Recently, the Chinese Ministry of Science and Technology and the Ministry of Agriculture initiated efforts to collect genetic resources from various farm animals breeds and protect them. The Songliao Black pig, as the first lean-meat swine bred in China, was put on the list to be protected. This study presents the

special genetic diversity of the Songliao Black breed, such as characteristic patterns of karyotypes, DNA fingerprint and population doubling time. These characteristics suggest that a marginal ear tissue-derived fibroblast cell line would not only preserve a vital genetic resource of the Songliao Black pig for sustainable use, but further serve as a convenient platform to provide valuable cellular material for genomics and proteomics studies and somatic cell cloning in the future (Lee and Piedrahita 2003).

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