

## Establishment and Characterization of a Fibroblast Line from Duroc

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**Abstract.** A duroc fibroblast cell line has been established and the genetic resources have been long-term stored. We obtained the cell line by primary culture, subculture and cryopreservation, cells' viability were assessed, microbial or other organisms contamination were excluded, isoenzyme analysis and chromosome analysis were detected, three exogenous genes were transfected into the cells, results showed the freezing has little influence to the cell viability, the purity and the hereditary stability of the cell line were in good condition, which could provide abundant genetic materials for other researches in biological field.

**Keywords:** Duroc, fibroblasts, cell bank, genetic resources.

### INTRODUCTION

The diversity of species makes an important role for human's sustainable development, preserving the genetic resources of endangered animals, no matter the wild or the livestock, has a great significance. Many institutions around the world are working on animal genetic resource conservation, such as the American Type Culture Collection (ATCC) and the European Collection of Animal Cell Culture (ECACC). (Hartung *et al.*, 2002). For the pursuit of high yield on products from livestock, the number of species those who has a low yield are being rapid decreased or facing vanishing (Bai *et al.*, 2011), on the other hand, the maturity of hybrid species technology are ruining the diversity as well, the pure species have become more precious. Therefore, it could not be ignored to protect the genetic resources from those livestock. Up to now, technologies on semen cryopreservation, Embryo cryopreservation, cDNA library and genome library are used to preserve the individuals' genetic resources (Liu *et al.*, 2010), but those methods have their shortcomings such as having incomplete genetic information or ethical issues. With the progress of somatic cell clone technology, constructing fibroblast banks has proposed a practical approach to preserve precious genetic

resources, which can also provide abundant genetic materials for transgenic engineering, cell transplantation, induced pluripotent stem(iPS) cell and other biological researches. Since 2003, our laboratory has successfully preserved 96 livestock and avian, including 11 wild animals, more species are collecting through our technology platform.

Duroc is native to America, and it was propagated by red duroc from New York, red duroc from New Jersey and Berkshire swine from Connecticut in the 1860s, it is one of the most famous lean pig breeds in the world. Durocs are widely fed in China for their fast growth, roughage feeding and good taste, while the pure duroc is inefficient in reproductive performance, so that it often conducted as male parent to the hybridization. There would be less purebred existed of this kind of livestock if their genetic resources were not in good storage, which would be a great loss for animal husbandry, even for the whole biosphere. Here we demonstrated the construction of fibroblast bank from duroc. We established the cell line and characterize it with a series of technologies, the genetic resources of duroc would be long-term preserved on cellular level, and this study has laid the foundation for genetic resources preservation for other breeds.

### MATERIALS AND METHODS

#### *Experimental animal*

All animal procedures were approved by the Institutional Animal Care and Use Committee of

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Chinese Academy of Agricultural Sciences, ear marginal tissues from adult duroc pigs were provided by the Animal Husbandry Experimental Base Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing. Duroc were treated in accordance with NIH and USDA guidelines, the use of animals in research and all experimental procedures involving duroc were conducted in accordance with the guidelines for agricultural animal research codified by the Committee for Ethics of Beijing, China.

#### *Cell cultures*

Sample tissues were washed by phosphate-buffered saline (PBS) containing 1% penicillin-streptomycin solution for three times, the hair with impurities and the cuticle with blood were removed, then tissues left were washed by PBS for three times, the samples were sliced into 1 cm<sup>3</sup> pieces and seeded on culture flasks, using tissue explants adherent method for primary culture (Ahmad *et al.*, 2012) culture medium DMEM (Gibco, New York, USA) containing 10 % fetal bovine serum (Biocrom, Berlin, Germany) were added. Flasks were placed in a 37°C incubator with 5% CO<sub>2</sub>. Cells were harvested when they reached 80-90 % confluence and were divided into new flasks at 1:2 ratio for subculture (Li *et al.*, 2009d).

#### *Cryopreservation and recovery*

Cells in the logarithmic phase were harvested to preserve with freezing medium which were composed of 10 % DMSO (Sigma, Santa Clara, USA), 40 % fetal bovine serum and 50 % DMEM at a concentration of 4×10<sup>6</sup>/mL. Cell suspension was distributed into the 2 mL freezing tubes labeled with the animal name, gender, passage and the date. The tubes were conduct by programmed freezing, which was included these steps below: 4° for 30 min, -80° overnight (by a freezing kit, Nalgene Nunc International, Rochester, NY, which has a cool rate of 1°C/min.) and transferred to liquid nitrogen (LN) at last. To recover the cells, tubes were rapidly thawed in a water bath at 42°C, then centrifuged at 20 g/min, the pellet were resuspended with fresh culture medium and reseeded onto the culture flasks at the previous freezing concentration (Li *et al.*, 2009c).

#### *Cell viability and growth curve*

Cell viability before freezing and after recovery were estimated by the Trypan blue assay, cells were seeded onto a six-plate and 1000 viable cells were counted. Cells at a concentration of 2.5×10<sup>4</sup>/mL were seeded onto a 24-plate and cultured for a week, the growth rate and the density were recorded from 3 wells per day, then the cell growth curve were plotted (Kong *et al.*, 2007).

#### *Microorganism detection*

Bacterial and fungal contamination: duroc fibroblasts were cultured in antibiotics free medium, any contamination was assessed within 3 days following the method Liu *et al.* (2008).

#### *Viruses contamination*

Cells were observed by phase-contrast microscopy for cytopathogenic examination, operation in details was similar to Li *et al.* (2009b).

#### *Mycoplasma contamination*

Following the method of Liu (Liu *et al.*, 2011), after culturing with antibiotic-free medium for a week, cells were fixed and stained with Hoechst 33258 (Sigma, Santa Clara, USA) for fluorescent staining of deoxyribonucleic acid (DNA), any contamination by mycoplasma could be observed under a fluorescence microscope.

#### *Isoenzyme analysis*

Isoenzyme patterns of lactic dehydrogenase (LDH) and malicdehydrogenase (MDH) were detected. The cells were harvested at a concentration of 5 × 10<sup>7</sup> cells/mL for protein extraction solution (0.9 % Triton X-100, 0.06 mmol NaCl:EDTA in mass ratio 1:15) then the mixture was centrifuged and the supernatant was stored at -80°C. After that liquid sucrose (40%) and the samples were mixed (1:1) and loaded into the individual lanes of the polyacrylamide gel. Different mobility patterns were indicated, which were calculated as the ratio between the distance of migration of the Isoenzyme band and that of the indicator (Bai *et al.*, 2010).

#### *Chromosome analysis*

Duroc fibroblasts in the logarithmic phase were incubated with culture medium containing 0.1

mg/mL colcemid (Sigma, Santa Clara, USA) for 4 h at 37°C, then harvested, centrifuged and resuspended in 0.075 M KCl at 37°C for 30 min, the cells were centrifuged again and fixed with 3:1 methanol:acetic acid at 37°C for three times, the cell suspension was dropped to slides and stained with Giemsa (Sigma, Santa Clara, USA) (Na *et al.*, 2010b). The chromosomes were counted, the karyotype was prepared.

#### Estimate of exogenous gene transfection

Three fluorescent proteins (pDsRed1-N1, pEGFP-N3 and pEYFP-N1) were transfected into duroc fibroblasts through plasmid DNA (BD Biosciences Clontech product, Japan) and Lipofectamine 2000 (Invitrogen, Carlsbad, USA). The cultured cells were observed at 24, 48, 72, 96 h, 1 week, 2 weeks, and 1 month after transfection. The three fluorescent protein genes were observed respectively under excitation wavelength of 543 nm, 488 nm, and 488 nm and 543 nm under a confocal microscope. In each group 10 visual fields were captured as pictures to assess the expression and the distribution (Wu *et al.*, 2008).

#### Statistical analysis

Statistical analysis was conducted with SPSS 13.0 software. There was statistically significant when  $P < 0.05$ .

## RESULTS

#### Morphological observation of duroc fibroblasts

Fibroblasts were migrating from the tissues after 4 days, cells reached 90 % confluence after a week and then subcultured, purified fibroblasts were obtained after 3 passages and cells growth exhibited a long spindle-like morphology, the edges of cells were distinct and their cytoplasm were homogeneous (Fig. 1).

#### Growth curve and cell viability

We drawn a "S" shape curve depending on the cell's growth status, a momentary decline phase may for cell adaption in 24 h, a obvious rise phase for rapid proliferation from day 2 to day 5, a plateau phase at day 6 to 7 and then a senescence phase were demonstrated. The population of doubling time

was nearly 26 h (Fig. 2). At the same time, we detected the cell viability by a Trypan blue assay, the viability of duroc fibroblasts before freezing and after recovery were 99.2% and 98.7%, respectively.

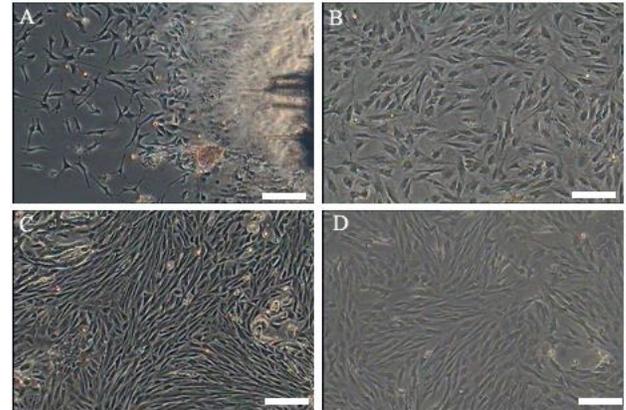


Fig. 1. Morphology of duroc fibroblasts. A Primary cells ( $\times 100$ ), the cells were migrating from attached tissues. B subcultured cells ( $\times 100$ ), the cells were subcultured when they reached 90% confluence. C Cells before cryopreservation ( $\times 100$ ), the cells were in rapid growth and healthy. D Cells after recovery ( $\times 100$ ), the cells showed little effects by freezing after thawing. (Scale bar = 50  $\mu\text{m}$ ).

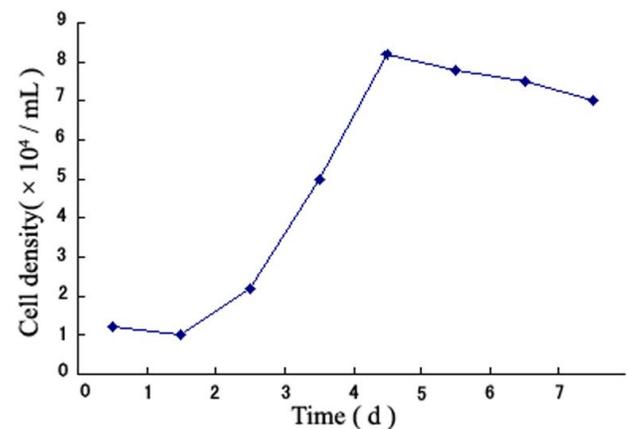


Fig. 2. Growth curve of duroc fibroblasts. The "S" shape curve showed the latency phase, exponential growth phase and stationary phase. The population doubling time was approximately 26h.

#### Microorganisms

All the assays for microorganisms detection showed there were no bacterial or fungal, viruses

and mycoplasma contamination in the culture system.

#### *Isoenzyme analysis*

The isoenzymes of LDH and MDH were detected from duroc fibroblasts, we also detected a same species called Wuzhishan miniature pig. Patterns of LDH showed five clear bands in the order below: LDH 5, LDH 4, LDH 3, LDH 2, LDH 1 (near the anode), there were fewer band differences for the same livestock species. Patterns of MDH showed two bands, m-MDH band near the cathode and s-MDH band near the anode. These results indicated that the genetic characteristics of duroc fibroblasts were stable and the cell line was pure without other cell lines (Fig. 3).

#### *Chromosome analysis*

The duroc fibroblasts were diploid ( $2n=38$ ), containing 12 pairs of macrochromosomes and 6 pairs of microchromosomes, besides that the sex chromosome type is XX. The parameters, including relative length and centromere morphology are given in Table I. This cell line exhibited normal chromosome number and structure. These results conclude that the cell line is reproducibly diploid (Fig. 4).

#### *Expression of exogenous genes*

In this study, three fluorescent proteins were used as marker genes to assess the expression, it showed the high expression levels and species-independent transfect efficiency (Table II). The fluorescence signal of pDsRed1-N1, pEGFP-N3 and pEYFP-N1 were strong at 24h after transfection and the high expression could last for a week, which indicated the exogenous genes were replicated, transcribed, translated and modified in good state, and also rarely effected the growth and proliferation of the duroc fibroblasts (Fig. 5).

## DISCUSSION

The somatic cell bank constructed in our lab is not complicated in technology, but it has a profound significance to preserve the genetic resources in such a large-scale, researchers could utilize those genetic materials at anytime. The

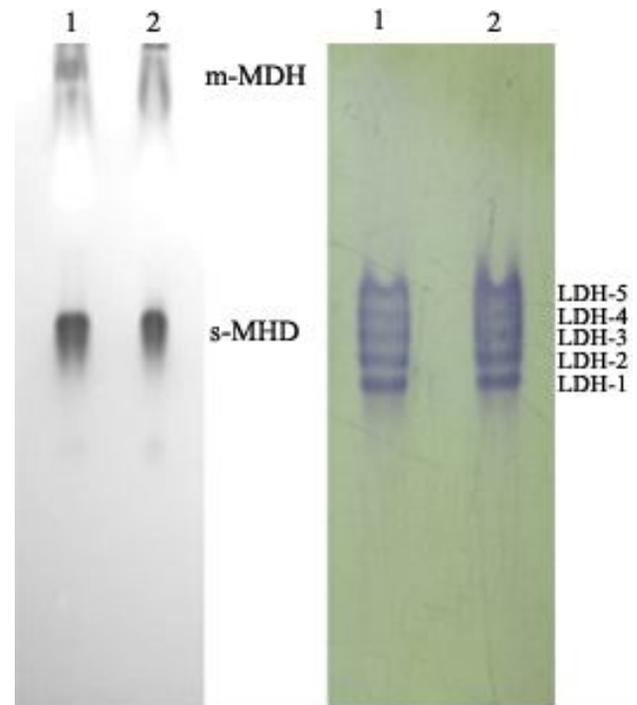


Fig. 3. Isoenzyme patterns of LDH and MDH. Lane 1 was species of Wuzhishan miniature pig and lane 2 was duroc. Electrophoretograms were similar from the same species, both of them showed the clear bands which illustrated there was no other species mixed into the cell line.

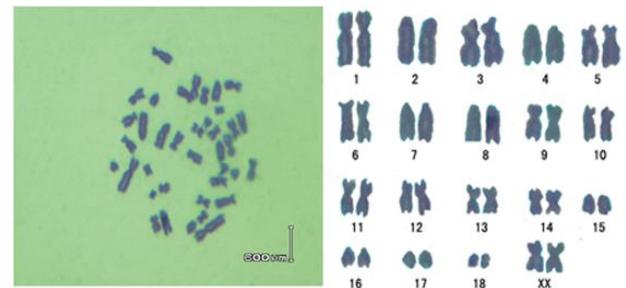


Fig. 4. Chromosomes and karyotype of duroc fibroblasts. The chromosome number of the diploid is  $2n = 38$ , including 12 pairs of macrochromosomes and 6 pairs of microchromosomes, the sex chromosome type is XX.

construction of the superior bank possesses several prerequisites, firstly, creating a mature technology platform, including a flowsheet of sequential cell culture and a system of cryopreservation with a high

**Table I.-** Chromosome parameters of the Duroc.

Chromosome number	Relative length (%)	Centromere morphology (%)	Chromosome number	Relative length (%)	Centromere morphology (%)
1	10.31±0.22	SM	11	4.08±0.35	M
2	7.69±0.19	SM	12	1.93±0.26	M
3	6.21±0.25	SM	13	8.29±0.52	T
4	6.04±0.16	SM	14	6.62±0.23	T
5	5.42±0.30	SM	15	6.07±0.12	T
6	5.01±0.37	ST	16	3.57±0.22	T
7	2.86±0.52	ST	17	3.19±0.23	T
8	5.78±0.41	M	18	2.51±0.27	T
9	5.18±0.38	M	X	5.06±0.19	M
10	4.22±0.48	M	X	5.10±0.14	M

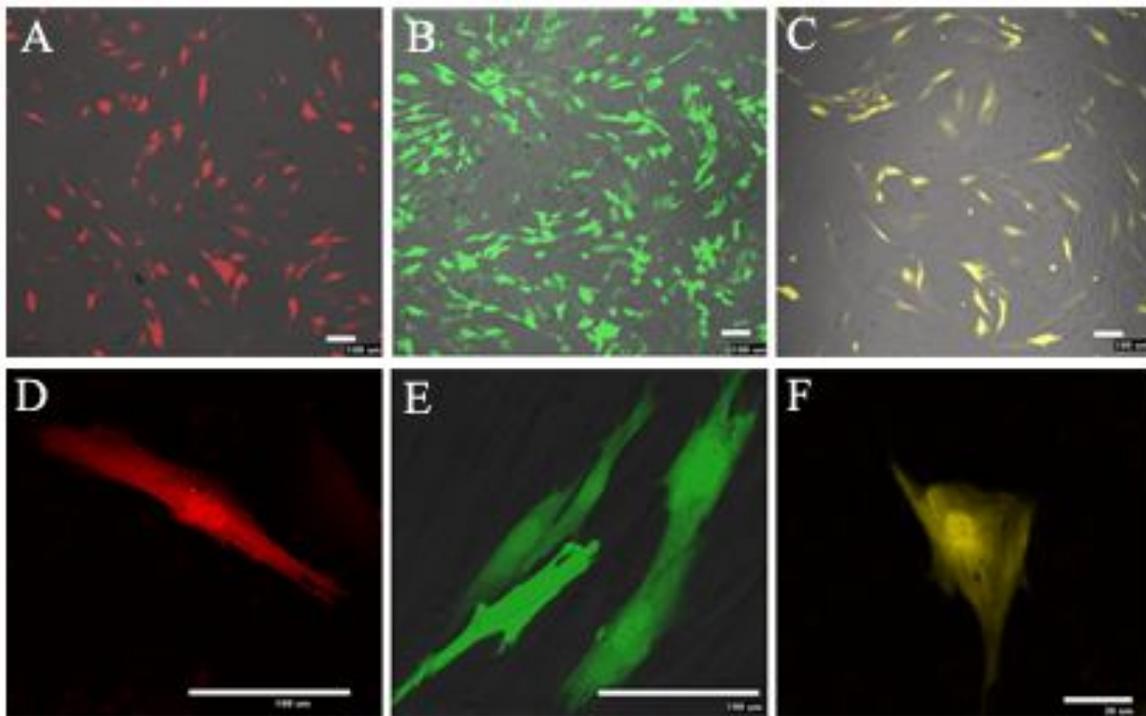


Fig. 5. The expression and distribution of fluorescent proteins in duroc fibroblasts. A, B and C showed the expression and distribution of pEGFP-N3, pDsRed1-N1 and pEYFP-N1 at 48 h after transfection, D, E and F were the close-up pictures. (Scale bar = 50  $\mu$ m)

**Table II.-** The transfect efficiency of three fluorescent proteins in Duroc.

Time/h	pDsRed1-N1 (%)	pEGFP-N3 (%)	pEYFP-N1 (%)
24	29.3	28.7	22.1
48	36.1	31.0	27.5
72	31.8	29.6	25.3

recovery rate. Secondly, ascertaining the source of strains from species, verification of animals should be done before drawing materials, for example, we distinguished the Siberian tigers' fibroblasts (Wu *et al.*, 2008; Guan *et al.*, 2010b) from the zoo or from the wild by investigations. Thirdly, ensuring a pure and a clean cell line, a pure fibroblast cell line could be generally obtained after 2-3 passages for the

difference of attachment time compared with epithelial cell (Guan *et al.*, 2010a). Meantime, excluding cell lines from other species is very important, isoenzymes analysis is currently the standard method for cell line identification and detection of interspecies contamination (Na *et al.*, 2010a). The bacterial, fungal, viruses and mycoplasma are harmful to the cell line, the standards of microorganism detection helps segregated cells from those contamination (Kim *et al.*, 2005).

After the considers above, the essential question is how to make sure the inherited traits from the fibroblasts are stable, the healthy experimental animal should be chose, the viability of the isolated cells should be assessed before freezing and after recovery, the chromosome analysis could illustrate the cells are normal or malignant and show if the chromosome structure is stable (Li *et al.*, 2009a), besides, the high transfection rate of exogenous genes also indicate the fibroblasts are in good growth state and they hold stable hereditary features (Hu *et al.*, 2013).

The construction of fibroblast cell line from duroc enlarged our somatic cell bank, their genetic resources have been long-term stored, and the technology platform give a supply to other field of cell and molecular biology and we are glad to share the freezing materials globally with other researchers who are in need.

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#### Declaration of interest

The authors report no conflicts of interest. The authors along are responsible for the content and writing of the paper.

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