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Attempt at Conserving the Genetic Resources of Tan Sheep by Conserving its Fibroblast Line

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ABSTRACT

Tan sheep is one of the most distinctive local sheep breeds in China, while investigations concerning its cell biology and molecular genetics are still scarce. To preserve the valuable genetic resources of Tan sheep, we established a Tan sheep fibroblast line and identified its biological characteristics, including cell morphology, growth curve, karyotype, isoenzyme polymorphism and expression of exogenous fluorescent genes. The results showed that the viability of the fibroblasts before freezing was 96.75±3.24 and 93.42±2.87% after thawing. The growth curve of Tan sheep ear marginal fibroblasts displayed an obvious "S" shape and there was no microbial contamination and cross-contamination; all somatic chromosomes were acrocentric autosomes and only the two sex chromosomes were submetacentric; the transfection efficiencies of the three fluorescent protein genes were 20.6-36.2%. It could be concluded that the this study has not only provided the biological characteristics of Tan sheep at cellular level but also made a valuable contribution to the preservation of the genetic resources of the Tan sheep.

Key words: Tan sheep, fibroblast line, primary explant technique, biological characteristics, genetic conservation

INTRODUCTION

Biodiversity is facing unprecedented challenges worldwide and local breeds are threatened by the introduction of foreign species, massive destruction of their natural habitats and industrial pollution. The genetic significance of local breeds as a reservoir of genetic variations and major genes has been realized (Horst, 1989) and variation needs to be known and how it can be conserved and exploited effectively (Karp *et al.*, 1998). Traditionally, *in vitro* conservation involves materials like semen, embryos and oocytes, however, a number of reasons why these techniques cannot be used for endangered breeds globally. Instead, storage of somatic cells constitutes an optimal choice, each somatic cell contains the full genetic code of the whole animal which can be readily collected.

Tan sheep is one of the most distinctive local sheep breeds in China and was listed among the 138 nationally protected domestic animal breeds by the Chinese government in (2006) (http://www.agri.gov.cn/blgg/t20060609_626418.htm). To date, efforts have been focused on analyzing various quantitative traits of the Tan sheep. While investigations concerning its cell biology and molecular genetics are still scarce. In this study, Tan sheep fibroblasts from the ear marginal tissue of adult sheep (20 males and 23 females) were isolated and cultured and their biological characteristics were subsequently analyzed.

MATERIALS AND METHODS

Preparation of fibroblasts: During all the experiments, all procedures and protocols were in compliance with the Statement on Animal Care and Usage in Research and Teaching. The ear marginal tissue samples of Tan sheep were chopped into 1 mm^3 pieces using ophthalmic scissors. Then the tissue pieces were plated on the bottom of culture flasks with DMEM containing 10% Fetal Bovine Serum (FBS) and cultured at 37°C, 5% CO_2 and saturated humidity until near confluence.

When they reached 85% confluence, the cells were subcultured with 0.25% trypsin (m/v) at a ratio of approximate 1:3. Cells were cultured in fresh medium 24 h prior to freezing to make sure the nutrition was sufficiently absorbed. For freezing, cells were trypsinized and suspended in freezing media (DMEM containing 30% FBS and 10% DMSO) at the concentration of 1.5×10^6 cells mL^{-1}. The suspension was aliquoted in cryovials and stored at -70°C for 24 h before being transferred to liquid nitrogen. For recovery the cryovials were thawed in 37°C water bath and the cells were then transferred to culture flasks with medium containing 10% FBS.

Viability and growth kinetics: Cell viability before freezing and after recovery was determined using Butler's dye exclusion method (Butler, 1999). Growth curve of the cells was plotted following the method of Gu *et al.* (2006).

Tests for microbial contamination: The procedure used for detection of bacteria, fungi and yeast was as described in Doyle *et al.* (1990). The cells were stained with Hoechst 33258 according to the DNA fluorescent staining protocol to detect mycoplasmas which is also recommended by American type culture collection.

Chromosomal analysis: Karyotypes were prepared following the protocol described in the Reading Conference report (Ford *et al.*, 1980). Diploid percentage was determined by analyzing 100 spreads.

Isoenzyme polymorphism: The electrophoretic mobilities of Lactate Dehydrogenase (LDH) and Malate Dehydrogenase (MDH) were determined using the polyacrylamide gel electrophoresis protocol described by Macy (1979) of American type culture collection.

Expression of fluorescent genes in Tan sheep fibroblasts: The fluorescent protein genes pEGFP-C1, pEGFP-N3, pEYFP-N1 and pDsRed1-N1 were transfected into cells with Lipofectamine 2000 (Invitrogen Corp., Carlsbad, California) in serum-free medium. The cells were observed at 24, 48 and 72 h after transfection using laser confocal microscope (Nikon TE-2000-E, Japan).

RESULTS

In primary culture, fibroblast-like or epithelial-like cells could be seen migrating from the tissue pieces 5-12 days after explanting (Fig. 1a). Fibroblasts grew rapidly and gradually replaced the epithelial cells in the entire population. Cells were well spread on the flask bottom, forming characteristically multipolar or bipolar shapes (Fig. 1b). The viability (formulated as mean±SD) was 96.75±3.24% before freezing (Fig. 1c) and 93.42±2.87% after thawing (Fig. 1d), the difference between which is non-statistically significant ($p>0.05$) and assumed an obvious "S" shape (Fig. 2). The population doubling time was approximately 24 h.

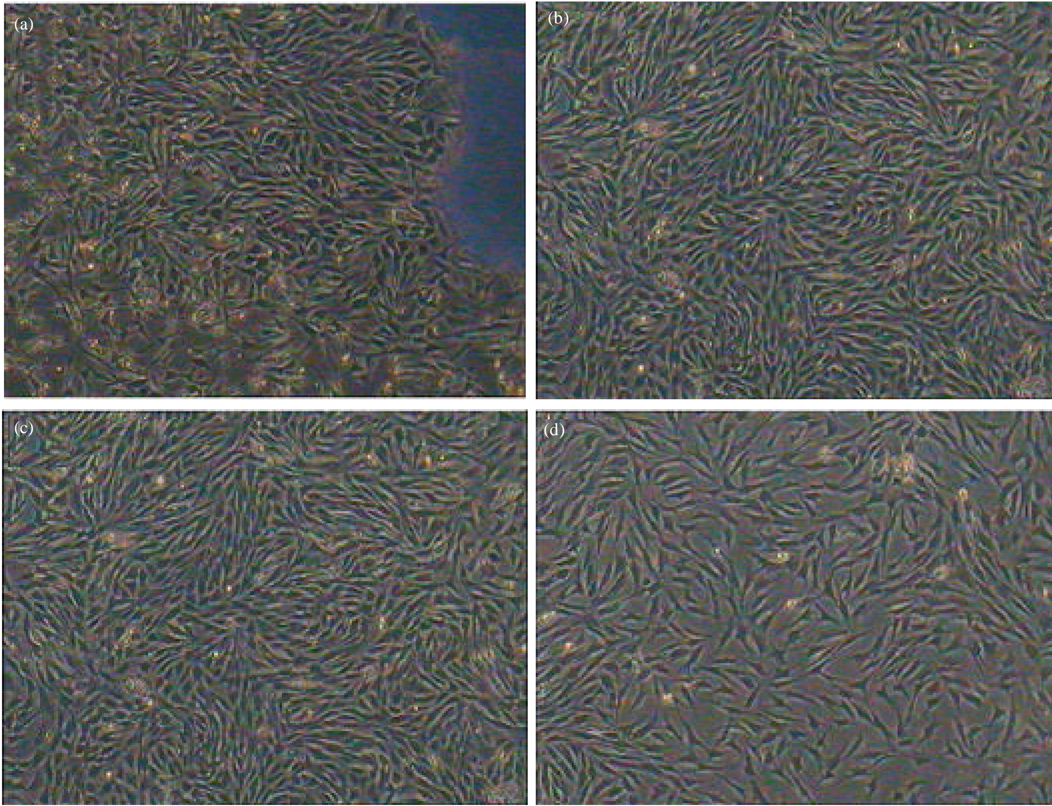

Fig. 1(a-d): Morphology of Tan sheep fibroblasts cultured *in vitro*, (a) 6 days after explanting, (b) Near confluence (c) Before cryopreservation and (d) 24 h after recovery

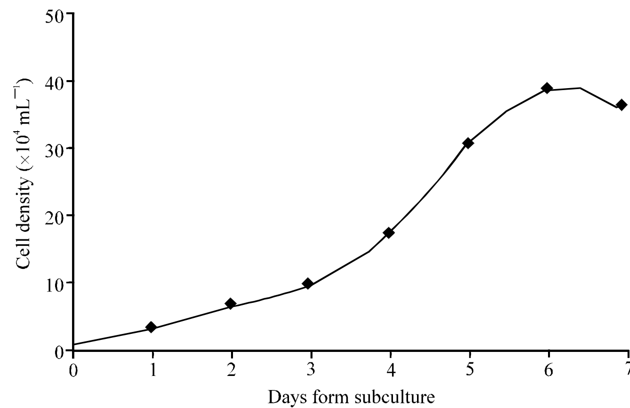

Fig. 2: Growth curve of Tan sheep fibroblasts

A pure cell culture can easily become contaminated with microorganism. Upon analysis, the culture medium tested did not become turbid or display other visible abnormalities whereas the positive control was visibly turbid with precipitation. No viruses were indicated by the cytopathogenic evidence or by the hemadsorption test. The nuclei appeared as clear blue ellipses

stained with Hoechst 33258, disproving the existence of mycoplasmas. The results indicated there was no microbial contamination in the Tan sheep cell line.

Detailed information for karyotype preparation is available in the Reading Conference report (Ford *et al.*, 1980). In Tan sheep the diploid chromosome number is 54, consisting of 52 autosomes and two sex chromosomes, XY or XX. The chromosomal properties were shown in Table 1, the chromosome numbers per spread were counted for 100 spreads of the 1-3 passages and the diploid frequency were 94.5-97.8%. Aberrations in chromosome numbers tended to increase with increasing numbers of passages, indicating that *in vitro* culture affected the heritage of cells slightly but supporting the inference that the cell line was reproducibly diploid. These results showed that all autosomes were acrocentric and the two sex chromosomes (XY) were submetacentric (Fig. 3).

The LDH bands of Tan sheep were compared with those of Songliao Black pig and five bands (LDH-1,-2,-3,-4,-5) were observed in all samples (Fig. 4a). The MDH bands were compared with those of Suffolk sheep and two bands (s-MDH, m-MDH) were observed (Fig. 4b). The isoenzyme patterns of LDH and MDH of Tan sheep fibroblasts were clearly distinguishable from those of other cell lines in our laboratory.

Table 1: Chromosome parameters of Tan sheep fibroblasts (male)

Chromosome Number	Relative length (%)	Centromere index (%)	Kinetochore type	Chromosome Number	Relative length (%)	Centromere index (%)	Kinetochore type
1	8.45±0.025	45.09	M	15	2.89±0.025	0	T
2	8.94±0.023	44.88	M	16	2.84±0.031	0	T
3	7.19±0.021	47.29	M	17	2.79±0.036	0	T
4	3.89±0.015	0	T	18	2.76±0.025	0	T
5	3.81±0.022	0	T	19	2.72±0.024	0	T
6	3.80±0.019	0	T	20	2.72±0.017	0	T
7	3.50±0.024	0	T	21	2.71±0.014	0	T
8	3.43±0.029	0	T	22	2.64±0.036	0	T
9	3.41±0.027	0	T	23	2.52±0.032	0	T
10	3.25±0.032	0	T	24	2.49±0.019	0	T
11	3.19±0.026	0	T	25	2.46±0.027	0	T
12	3.15±0.031	0	T	26	2.07±0.018	0	T
13	2.99±0.023	0	T	X	3.86±0.021	18.90	ST
14	2.93±0.033	0	T	Y	2.58±0.025	52.48	M

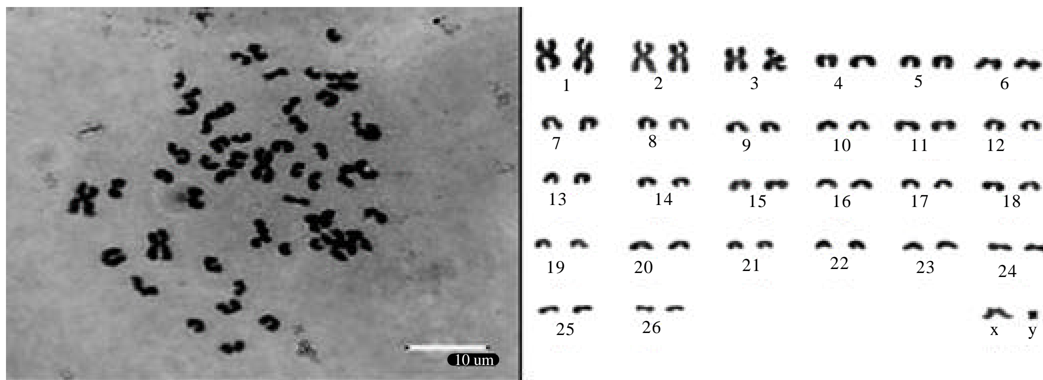

Fig. 3: Karyotype of Tan sheep fibroblasts (male)

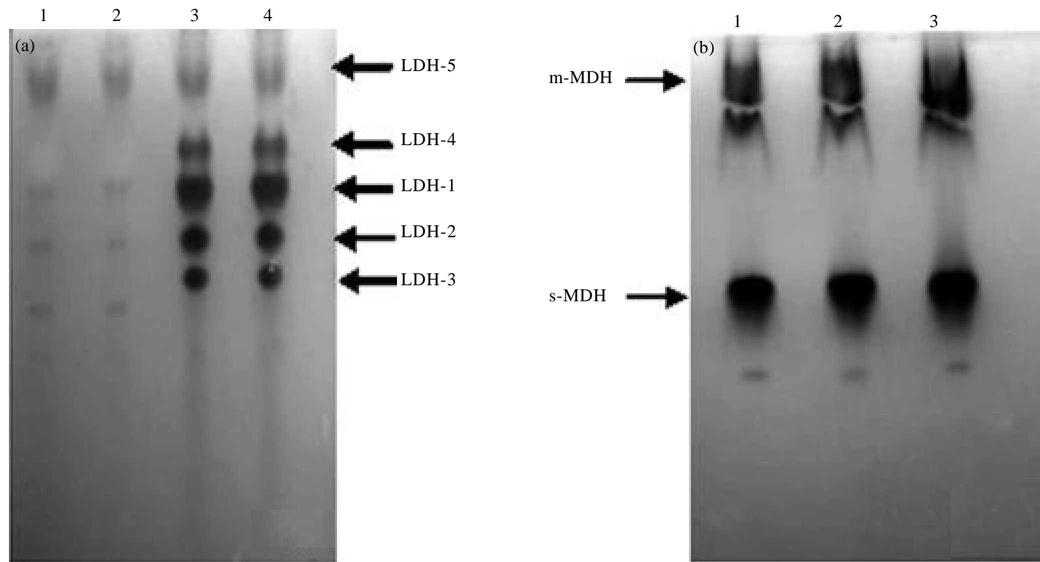

Fig. 4(a-b): Isoenzyme patterns of (a) LDH Lanes1 and 2: Songliao Black pig; Lane 3 and 4: Tan sheep (b) MDH, Lanes 1 and 2: Tan sheep; Lane 3: Suffolk sheep

Using the method of Tsuchiya *et al.* (2002), the fluorescent protein genes pEGFP-N3, pDsRed1-N1 and pEYFP-N1 were transfected into cells with Lipofectamine 2000 (Invitrogen Corp., Carlsbad, California) in serum-free medium. The cells were observed at 24, 48 and 72 h after transfection using laser confocal microscope (Nikon TE-2000-E, Japan). The results indicated that the transfection efficiencies of the three fluorescent protein genes were between 20.6 and 36.2%. And for all the three genes the strongest fluorescence intensities and the highest transfection efficiencies appeared at 48 h after transfection (Fig. 5).

DISCUSSION

Traditionally *in vitro* conservation involves materials like semen, embryos and perhaps oocytes. Placement in liquid nitrogen allows indefinite storage, while recovery is relatively straightforward. There are, however, a number of reasons why these techniques cannot be used for endangered breeds globally. Deep freezing of semen and embryos can only be performed on numerable species and furthermore requires species specific techniques (Groeneveld *et al.*, 2006). It requires substantial infrastructure which is not generally available in all countries, not to mention considerable costs, an issue also pointed out by Woolliams and Wilmut (1999). Instead, storage of somatic cells constitutes an optimal choice as illustrated by Corley-Smith and Brandhorst (1999). The principle rests on the fact that each somatic cell contains the full genetic code of the whole animal which can be readily collected, for instance, from ear notching, making the sampling process cheap and fast and thus also applicable in countries with poor facilities. In addition somatic cells can be collected from every animal easily, for instance, from ear margin, making the collection of samples cheap and fast.

Morphology, one of the most important qualitative criteria of epidermal tissue reconstitution, is commonly evaluated with light and electron microscopy. In this study the cells exhibited fibrous appearances with turgor vitalis cytoplasm and during growth they showed typically fibroblast-like morphology with radiating, flame-like or whirlpool-like migrating patterns.

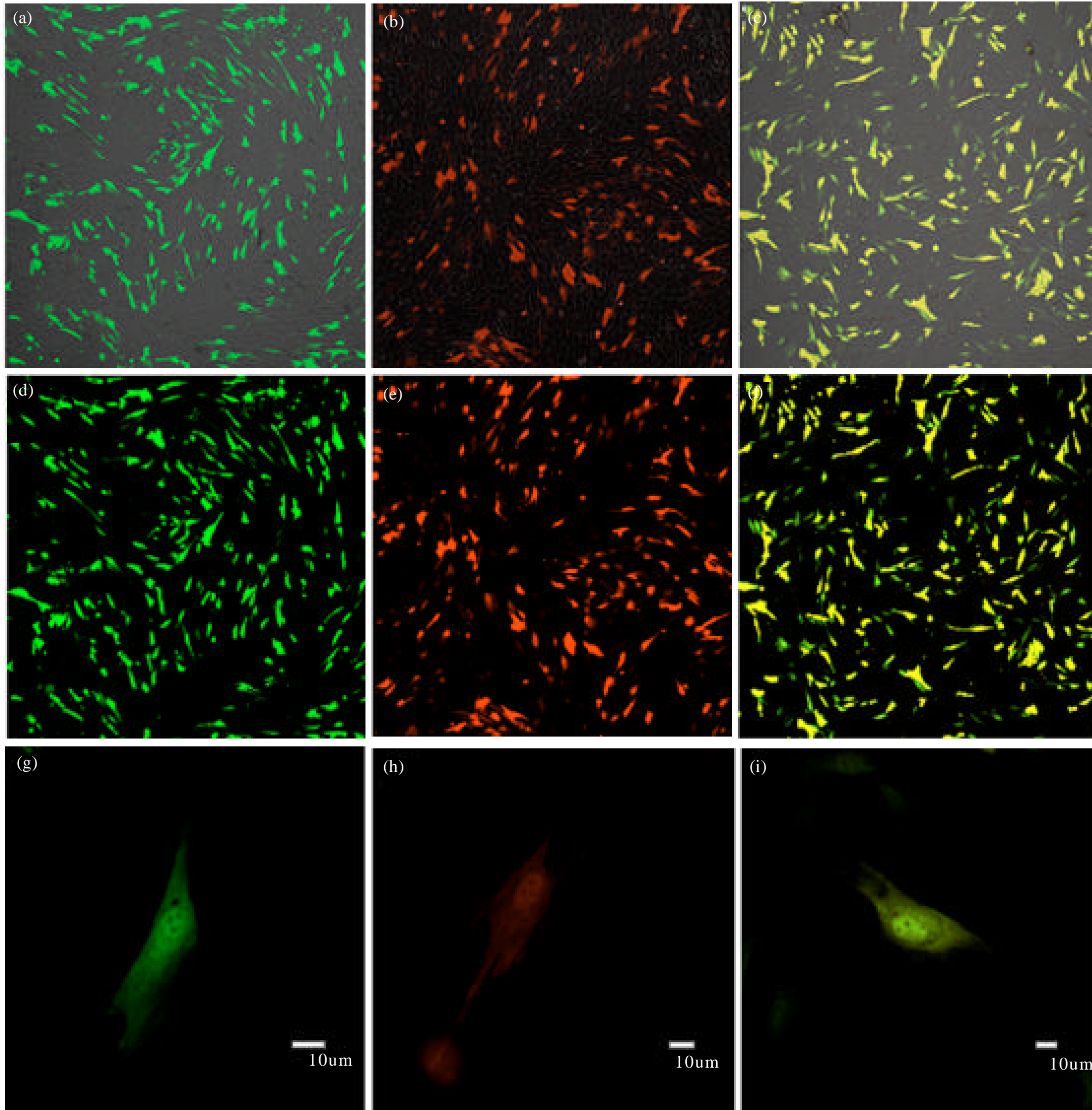

Fig. 5(a-i): Tan sheep fibroblasts cultured for 48 h after transfected by pEGFP-N3 (a, d, g), pDsRed-N1 (b, e, h) and pEYFP-N1 (c, f, i), (a), (b), (c) Merged pictures of phase contrast and fluorescence; (d, e, f, g, h), I pictures in fluorescence; a, b, c, d, e, f (100×); G, H, I (400×)

The genetic stability of cell line is critical to preserve the genetic resources, prescribing that the fibroblasts must maintain the same diploid character as those *in vivo*. As most mutants cultured *in vitro* still possessed the division capability, after successive cell divisions a significant differentiation would appear in the cell lines it which cannot be subsequently used in breed conservation. Chromosome analysis can identify the gender of the animal from which a cell line was derived and also distinguish between normal and malignant cells, due to the instability of chromosome number in the neoplastic ones (Freshney, 2000). Chromosome number per spread was counted from 100 spreads of cells from passages 1, 2 and 3. The normal number of chromosomes

(2n = 54) was observed in 96.56±2.89% of the cells, further validating the stability of these cells. Enzyme polymorphism, evidenced by the existence of isoenzymes, occurs among species and races, as well as tissues within an organism (O'Brien *et al.*, 1977). Isoenzymes can be separated chromatographically or electrophoretically, displaying characteristic distribution patterns. Biochemical analysis of isoenzyme polymorphism is currently considered as a standard method for quality control of cell line and is routinely used by leading biological resource centers (Parodi *et al.*, 2002).

Apart from the need to determine the tissue of origin of a culture, it is also important to avoid cross-contamination from other cell lines. Isoenzyme analysis is an extremely reliable and straightforward technique which provides a rapid and reliable identification of species of origin. While the species of origin of a cell line can usually be determined with only two isoenzyme tests (lactate dehydrogenase and glucose-6-phosphate dehydrogenase), specific identification of a cell line would require a larger battery of tests (Halton *et al.*, 1983). This procedure retained the advantage of rapid testing while also provided a useful level of specificity for identification purposes. As demonstrated above, biochemical analysis of isoenzyme polymorphism is currently considered to be a standard method for cell line quality control and identification and detection of interspecies contamination and is routinely used by the leading biological resource centers around the world. In this study, the isoenzyme bands of LDH and MDH of Tan sheep fibroblasts were clear, indicating that the cultures were free of contamination by other cell types.

The researches about fluorescent protein are mainly focused on tumor cell, nerve cell and stem cell (Jung *et al.*, 2001). We could see that the transferring cells at the state of reduplication and different dividing phase and the growth and reduplication of transfected cell were no transparent difference with control group. The result showed that the transfected cells were not been effected by fluorescein under certain range.

CONCLUSION

In this study, the quality of the Tan sheep fibroblast cell line was evaluated in the aspects of morphology, viability, microorganism detection, chromosome analysis, isoenzyme analysis and exogenous gene transfection. The viability of the fibroblasts before freezing was 96.75±3.24 and 93.42±2.87% after thawing. The growth curve of Tan sheep ear marginal fibroblasts displayed an obvious “S” shape and there was no microbial contamination and cross-contamination; all somatic chromosomes were acrocentric autosomes and only the two sex chromosomes were submetacentric; the transfection efficiencies of the three fluorescent protein genes were 20.6-36.2%. It could be concluded that the this study has not only provided the biological characteristics of Tan sheep at cellular level but also made a valuable contribution to the preservation of the genetic resources of the Tan sheep.

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