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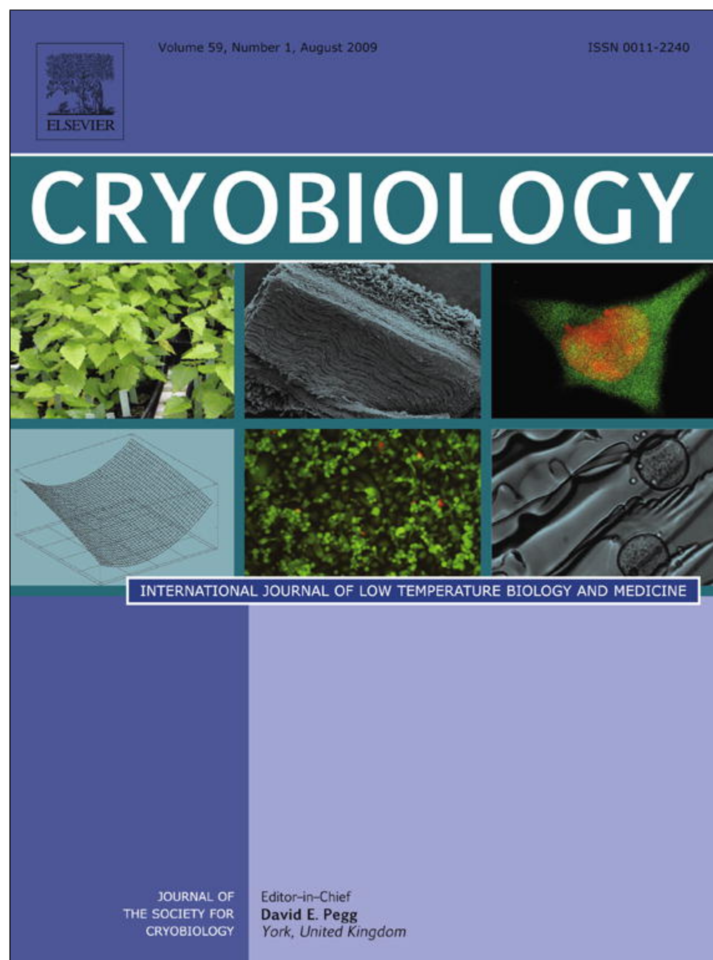


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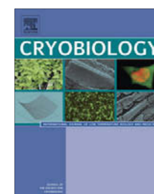

# Establishment and characterization of a fibroblast line from Simmental cattle ☆

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ABSTRACT

A fibroblast line (named SCF36) from ear marginal tissue of Simmental cattle was established successfully by direct culture of explants and cell cryopreservation techniques. Biological analysis showed that the population doubling time of the thawed cells was 42.8 h. The average viability of the cells was 96.8% before freezing and 91.5% after thawing. Measurements of lactic dehydrogenase and malic dehydrogenase isoenzymes showed no cross-contamination of this cell line with other species. Karyotyping showed that the frequency of cells with chromosome number $2n = 60$ was more than 90%. Tests for bacteria, fungi, viruses and mycoplasmas were negative. The efficiencies of expression of enhanced green, yellow and red fluorescent protein genes (pEGFP-$N_3$, pEYFP-$N_1$ and pDsRed1-$N_1$) were between 11.3% and 28.8% after transfection; fluorescence was well distributed in the cytoplasm and nucleus except for some cryptomeric vesicles. This Simmental cattle fibroblast line not only contains the germline of this important cattle breed, which is preserved at the cellular level, but valuable material has also been provided for genomic, postgenomic and somatic cloning research. Moreover, the establishment of these methods may provide both technical and theoretical support for preserving the genetic resources of other livestock and poultry at the cellular level.

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## Introduction

Biodiversity is facing unprecedented challenges, and one of the causes is that high-yielding breeds of domestic livestock are being spread actively throughout the world. Vulnerable animals are threatened by the introduction of foreign species and by industrial pollution. Unless these genomic resources are conserved in some form before they are lost, we will not only lose the genes peculiar to rare breeds but will also find it impossible to explore the cytological and molecular biological mechanisms that are required to reproduce these breeds by somatic cell cloning. The conservation of endangered species and breeds is therefore an urgent requirement. Currently, many strategies are used to conserve the genetic resources of domestic animals. Generative cells, somatic cells, stem cells, zygotes and embryos can all be cryopreserved in cell banks [8]. The establishment of somatic cell banks using low-temperature biological techniques is a new and effective approach to conservation and maintenance of the diversity of livestock and poultry. Not only does this technique preserve precious genetic material, but it also provides an excellent resource for biological research. In addition, modern cloning techniques have made somatic cells an attractive resource for conserving animal genetic materials [31]. Much information has recently been published on the development of fibroblast cell lines from different animals, including the Debao pony [13], Luxi Cattle [12] and the white ear lobe chicken [33].

Simmental cattle originated in the Alps of western Switzerland and have been exported to many countries. They are an important cattle breed and are raised for milk, meat and labor throughout the world. They have many important economic advantages, such as high rates of meat and milk production, high meat quality, a low rate of dystocia, hardiness and disease resistance. This breed therefore enjoys a good international reputation, and it has breeding potential for hybridization with other breeds.

In this experiment, we used a combination of cell viability verification, detection of microorganisms, chromosome analysis, isoenzyme analysis and transfection of fluorescent protein genes to produce an established cell line (SCF36) from Simmental cattle. Our object was to create a cryopreserved genomic resource. This would allow us to transfer advantageous genes from other breeds into the SCF36 cells to obtain a transgenic cell strain. New breeds of cattle could then be produced using somatic cloning techniques. At the same time, we established a fibroblast bank from Simmental cattle that contained 120 fibroblast lines from different individuals (60 male, 60 female, including SCF36). The cells could be used as

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donor cells to revive the breed using somatic cloning techniques if this breed ever becomes endangered. Moreover, with the future development of science and technology, the roles of cell lines will become increasingly prominent and they may be useful in currently unforeseen applications.

## Materials and methods

### *Experimental stock*

Simmental cattle were obtained from the Institute of Animal Science, Chinese Academy of Agricultural Sciences, China. All cattle were treated in accordance with the National Institute of Health (NIH) and United Stated Department Agriculture (USDA) guidelines for the use of animals in research, and all experimental procedures involving cattle were conducted in accordance with the protocols and guidelines for agricultural animal research imposed by the Committee for Ethics of China.

### *Cell cultures*

Ear margin tissues (about 1 $cm^2$ in size) were removed from the Simmental cattle and collected into separate tubes containing DMEM medium (Gibco, USA) with ampicillin (100 U/mL) and streptomycin (100 μg/mL). The samples were rinsed and chopped into 1 $mm^3$ pieces, which were seeded into medium in a tissue culture flask (Corning, USA) containing DMEM (Gibco, USA) +10% fetal bovine serum (Hyclone, USA) in a 37 °C incubator with 5% $CO_2$ in air. Cells were harvested when they reached 80–90% confluence and were divided into prepared culture flasks at ratios of 1:2 or 1:3 [6].

### *Growth curve and estimation of cell viability*

In accordance with the method of Gu et al. [7] and Kong et al. [11], cells at $1.5 \times 10^4\ mL^{-1}$ were seeded into 24-well plates. Data on cell growth and density were monitored and recorded each day until the plateau phase was reached; three wells were counted at each time point. A cell growth curve was then plotted and the population doubling time was calculated from this curve. Cell viability before freezing and after recovery was determined by using the CellTiter-Blue® Cell Viability Assay (Promega, USA). The viabilities of the cells in suspension were evaluated using 0.25% trypan blue (Promega, USA). Both the number of intact cells and the total number of cells in a sample after preservation were counted in a hemocytometer counting chamber, and cell viability was expressed as the percentage of the former relative to the latter. A hemocytometer was also used to enumerate 1000 cells by Butler's dye exclusion method [3]. Counts were done in triplicate.

### *Cryogenic preservation and recovery*

Cells in the logarithmic growth phase were counted with a hemocytometer, and viability was checked by the CellTiter-Blue® Cell Viability Assay (Promega, USA) before freezing. The harvested cells were resuspended in freezing medium containing 40% DMEM, 10% dimethyl sulphoxide (DMSO) (Sigma, USA) and 50% fetal bovine serum to a final concentration of $(3–4) \times 10^6$ viable cells/mL. Single cells were dispensed into in 2 mL aliquots in cryovials (Nalgene Nunc International, Rochester, NY) labeled with the animal name, gender, freezing serial number and date. The cryovials were sealed and kept at 4 °C for 20–30 min to allow the DMSO to equilibrate. Then, cryovials were transferred to a commercially available freezing kit (Nalgene), refrigerated at −80 °C overnight (a process which cools at a rate of 1 °C/min), and subsequently transferred to liquid nitrogen ($LN_2$). [30]. To recover and reseed the cells, the cryovials were rapidly thawed in a water bath at 42 °C. The freezing medium was gradually diluted with cell culture medium. Thawed cells clumps were then transferred to a 15 mL tube and centrifuged at 20 g/min for 5 min. The supernatant was discarded. The pellet was suspended gently in medium and immediately plated onto a culture flask.

### *Microorganism detection in SCF36*

#### *Detection of contamination with bacteria, fungi and yeasts*

The SCF36 cells were cultured in a medium free of antibiotics. Bacterial, fungal and yeast contamination was assessed within 3 days. The detailed procedure used for the contamination test was described by Doyle et al. [4].

#### *Testing for viruses*

Under normal culture conditions, cells were selected randomly for cytopathogenic examination using phase-contrast microscopy, following Hay's hemadsorption protocol [9].

#### *Mycoplasma detection*

Cells were cultured in antibiotic-free medium for at least 1 week, and then fixed and stained with Hoechst 33258 (Sigma, USA) according to the methods of Masover and Becker [14] and Freshney [6] for fluorescent staining of deoxyribonucleic acid (DNA). An ELISA Mycoplasma Detection kit (Roche, Lewes, East Sussex, UK) was used to confirm the results of the DNA fluorescent staining.

### *Chromosome analysis*

Chromosomes were prepared, fixed and stained following standard methods [25]. After Giesma staining, the chromosome numbers per spread were counted for 100 spreads under an oil immersion objective. Relative length to arm ratio and centromeric index and type were counted according to the protocol of Kawarai et al. [22].

### *Isoenzyme analysis*

Isoenzyme patterns of lactic dehydrogenase (LDH) and malic dehydrogenase (MDH) were detected by using a vertical slab non-continuous polyacrylamide gel electrophoresis (PAGE) assay. In brief, the cells were harvested and protein extraction solution (0.9% Triton X-100, 0.06 mmol NaCl:EDTA in mass ratio 1:15) was added after the cell concentration was adjusted to $5 \times 10^7$ cells/mL, then the mixture was centrifuged and the supernatant was stored in aliquots at −80 °C. Liquid sucrose (40%) and the samples were mixed (1:1) and then loaded into the individual lanes of the polyacrylamide gel [24]. Different mobility patterns were indicated by the relative mobility front (RF), which was calculated as the ratio between the distance of migration of the isozyme band and that of the indicator.

### *Expression of fluorescent protein genes in fibroblasts from Simmental cattle*

To obtain the highest efficiency of transfection and low cytotoxicity, transfection conditions were optimized by varying the cell density and the concentrations of plasmid DNAs (Clontech, JAPAN) for three fluorescent proteins (pEGFP-$N_3$, pEYFP-$N_1$ and pDsRed1-$N_1$) and Lipofectamine 2000 (Invitrogen, USA), according to the lipofectamine medium methods of Escriou et al. [5] and Tsuchiya et al. [27]. The cultured cells were observed at 24 h, 48 h, 72 h, 96 h, 1 week, 2 weeks and 1 month after transfection with the three fluorescent protein genes using excitation wavelengths of 405 nm, 488 nm and 543 nm separately. For each experimental group, images were captured from 10 visual fields, and confocal

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microscopy was used to measure the total and positive cell counts in each field to determine the efficiency of transfection.

## Results

### Morphology of fibroblasts from Simmental cattle

Fibroblast-like or epithelial-like cells could be seen migrating from the tissue pieces 5–12 d after explanting (Fig. 1A). When the duration of culture was increased, cells continued to proliferate and were subcultured when they reached 90% confluence. After subculturing, the fibroblasts grew rapidly, gradually outgrowing and excluding other cells such as epithelial cells [32]. After two to three passages, we obtained purified fibroblasts (Fig. 1B). The viabilities of Simmental cattle fibroblasts before freezing and after recovery, as measured by Trypan Blue staining, were 98.6% and 93.7%, respectively. The morphology of the cell was still a typical elongated spindle-shape and the cells were healthy (Fig. 1C and D).

### Growth curve (dynamic state of cells) and cell viability

The growth curve of SCF36 cells had an obvious "S" shape (Fig. 1E) and the population doubling time was 42.8 h. There was a lag time or latency phase of about 48 h after seeding, corresponding to the adaptation to and recovery of the cells from protease damage, then the cells proliferated rapidly and entered an exponential phase. As the cell density increased, proliferation was retarded by contact inhibition; by the sixth day, the cells entered a plateau phase and began to degenerate.

The average viability of the cells was 96.8% before freezing and 91.5% after thawing. These results were not significantly different ($P > 0.05$); therefore the cells were healthy in culture, and freezing had little effect on their viability.

### Microbial analysis

Tests for contamination with bacteria, fungi and yeasts were negative; no microorganisms were observed in the culture media. No viruses were indicated by the cytopathogenic evidence or by the hemadsorption test. Staining with the DNA fluorochrome Hoechst 33258 is the most effective and frequently used method for detecting contamination with mycoplasmas [2]. After staining with Hoechst 33258, fibroblast nuclei appeared as blue ellipses under a fluorescence microscope, showing that the established cell line was mycoplasma negative (Fig. 1F). These results indicated there was no microbial contamination of the SCF36 line.

### Karyogram and chromosome number of SCF36 cells

The chromosome number of Simmental cattle is $2n = 60$, comprising 58 autosomes and two sex chromosomes, XY or XX

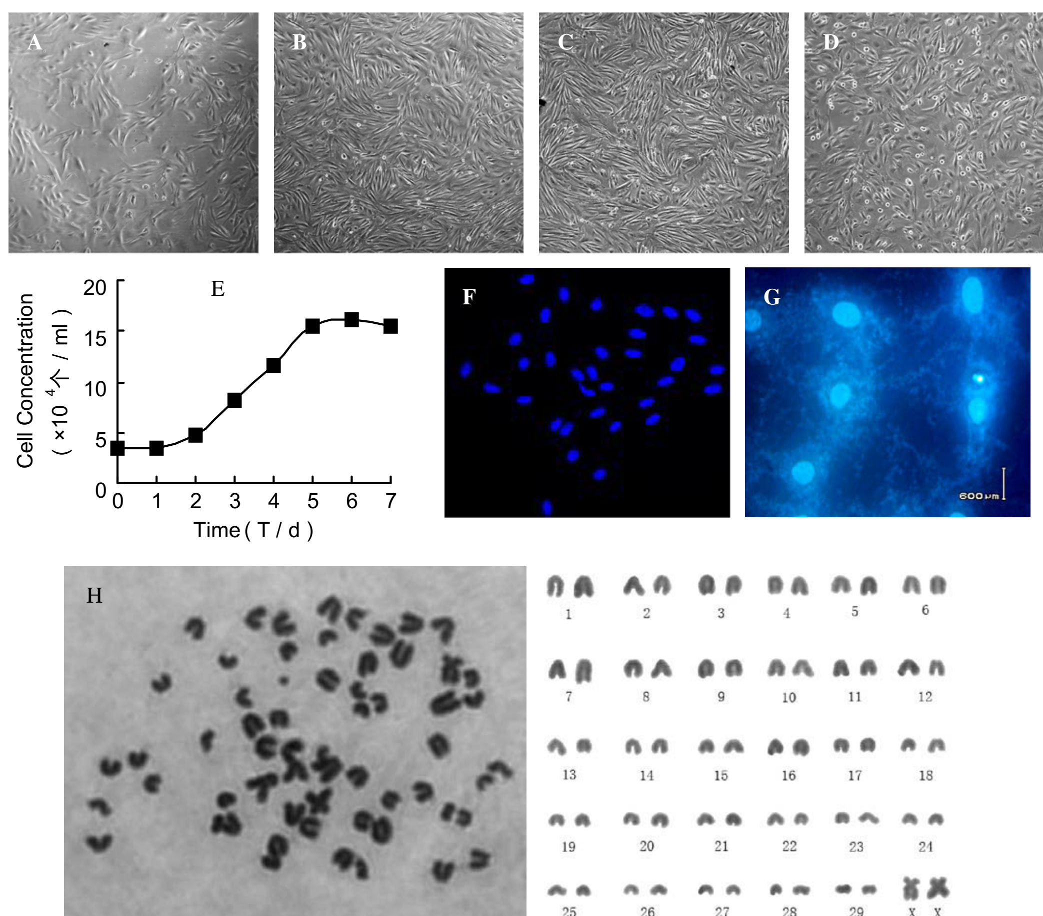


**Fig. 1.** Morphology, growth curve, detection of microbial contamination and karyotype of SCF36 cells. (A) Primary cells (100×), the cells were typical long spindle-shape with growth being slower. (B) Subcultured cells (100×), the cells were subcultured until they reached 90% confluence. After passage, growth accelerated and plateaued after 3–4 days. (C) Cells before cryopreservation (100×) the cells were healthy and in mitotic phase. (D) Cells after recovery (100×), the cells were cultured for 48 h after thawing; (E) Growth curve of SCF36 cells. The curve appeared as a typical "S" shape with cell density on the left axis. Growth curve included latency phase, exponential growth phase and stationary phase. (F). SCF36 mycoplasma negative (200×) (G) Mycoplasma positive control (400×) (H) Chromosome at metaphase (left) and karyotype (right) of SCF36 line (♀) (1000×). The chromosome number of Simmental cattle was $2n = 60$, comprising 58 autosomes and two sex chromosomes, whilst the sex chromosome type was XX (♀).

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(Fig. 1H). All the somatic chromosomes are acrocentric autosomes, and only the two sex chromosomes (X and Y) are metacentric. The chromosome numbers per spread were counted for 100 spreads of the second, fourth and sixth passages, and the frequencies of cells with $2n = 60$ were 98%, 96% and 90%, respectively (Table 1). Aberrations in chromosome number 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.

*Isoenzyme analysis of SCF36 cells*

Isoenzyme polymorphism occurs among species, and even sometimes among breeds, individuals and tissues within a species [18]. Polymorphism analysis of isoenzymes is currently the standard method for the quality control of cell line identification and detection of interspecies contamination. We improved the apparatus and conditions for native polyacrylamide gel electrophoresis, and successfully determined the mobility of the isoenzymes of LDH and MDH.

The LDH bands obtained from Simmental cattle were compared with those from Songhei black pigs, Fatty-tailed sheep, and Angus and Hereford cattle. Five isoenzyme bands (LDH-1, -2, -3, -4, and -5) were observed (Fig. 2A). Patterns of LDH showed clear band differences for different livestock species. Within the same livestock species, there were fewer band differences in the LDH isozymogram. The enzymatic activities were increased in the order LDH-5, LDH-4, LDH-3, LDH-2, LDH-1.

The MDH patterns revealed clear band differences in Songhei black pig, Fatty-tailed sheep, and Angus, Hereford and Simmental cattle fibroblasts (Fig. 2B). Samples from all five domestic animal breeds produced two bands, and there was a single m-MDH band near the cathode and s-MDH bands near the anode. Similar activity was seen from both m-MDH and s-MDH1.

There were significant differences in the isoenzyme patterns of LDH and MDH between the Simmental cattle and other cell lines in our laboratory. These results indicate that the genetic characteristics were stable and there was no cross-contamination of the SCF36 line from different cell lines established in our laboratory at the same time.

*Expression of six fluorescent protein genes in SCF36 cells*

The three fluorescent protein genes pEGFP-$N_3$, pEYFP-$N_1$ and pDsRed1-$N_1$ were all highly expressed with reference to the optimized condition (Fig. 3A–C). Positively expressing cells were observed 12 h after transfection, and the numbers and intensity increased markedly and reached a maximum at 48 h. The green fluorescent proteins (pEGFP-$N_3$) were maximal. The efficiencies of expression of the three fluorescent proteins at 24 h, 48 h and 72 h after transfer were all between 11.3% and 28.8% (Table 2).

Confocal fluorescence microscopy was used to observe the distribution of green, yellow and red fluorescence in the SCF36 cells to determine the subcellular location of the three fluorescent proteins. The results showed 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 showed an intense nuclear signal (Fig. 3D–F). The number of fluorescent cells decreased at 1 week, but a few dispersed positive cells remained after 2 weeks and even after 1 and 2 months post transfection.

The viabilities of cells transfected with pEGFP-$N_3$, pEYFP-$N_1$ and pDsRed1-$N_1$ were 89.5%, 87.6% and 88.6%, respectively; none of these was significantly different from control (91.4%, $P > 0.05$).

## Discussion

We established a fibroblast line (SCF36) from marginal ear tissue of Simmental cattle using an adherent culture method. All the results indicated that the newly established cell line was stable and grows rapidly. We are now able to conserve the genomic resource of Simmental cattle in the long term by freezing fibroblasts in liquid nitrogen and thus achieve the aim of protecting the breed.

**Table 1**
Chromosome number in SCF36 cells.

| Generation | Chromosome number | | | Total cell score | Percentage of 2$n$ (%) |
|---|---|---|---|---|---|
| | Hypodiploid | Diploid | Hyperdiploid | | |
| 2 | 1 | 49 | 0 | 50 | 98 |
| 4 | 1 | 48 | 1 | 50 | 96 |
| 6 | 3 | 45 | 2 | 50 | 90 |

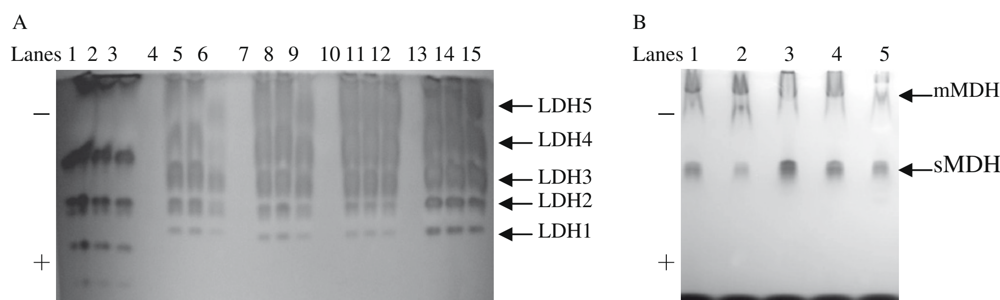


**Fig. 2.** LDH zymotype and MDH zymotype of SCF36 line. These domestic animals had their own unique bands with a different relative mobility. These results showed that there was no cross-contamination between different breeds. (A) SDS–PAGE electrophoresis of LDH, from down to up, there were LDH-1, 2, 3, 4, 5. (Panel A) 1–3 Songhei Black Pig, 4–6 Fatty-tailed Sheep, 7–9 Simmental Cattle, 10–12 Angus, 13–15 Hereford. (B) MDH. from down to up, there were sMDH-1, sMDH-2 and mMDH, whilst s-MDH2 had weaker activity. (Panel B) 1 Angus, 2 Simmental cattle, 3 Hereford, 4 Fatty-tailed sheep, 5 Songhei black pig.

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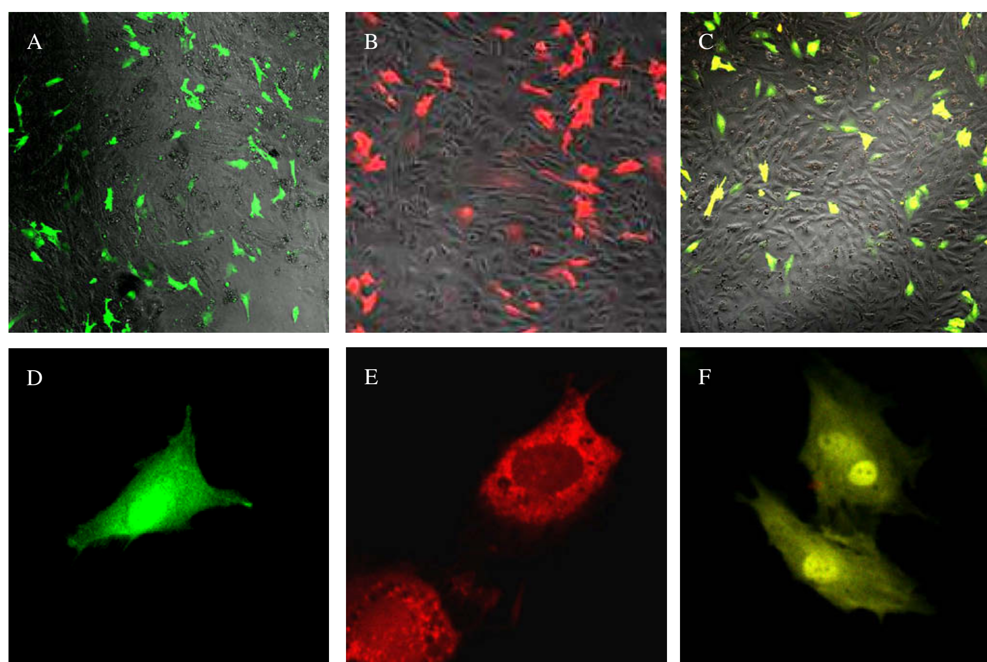


**Fig. 3.** The expression and distribution of pEGFP-$N_3$, pDsRed1-$N_1$ and pEYFP-$N_1$ in SCF36 cell. A, B and C were the transfection results of pEGFP-$N_3$, pDsRed1-$N_1$ and pEYFP-$N_1$ at 48 h after transfection (100×); D, E and F were the subcellular location of pEGFP-$N_3$, pDsRed1-$N_1$ and pEYFP-$N_1$ at 48 h after transfection (400×).

**Table 2**
Efficiency of transfection of three fluorescent proteins.

| Transfection time (h) | pEGFP-N3 (%) | pDsRed1-N1 (%) | pEYFP-N1 (%) |
|---|---|---|---|
| 24 | 11.3 | 13.1 | 12.8 |
| 48 | 24.6 | 28.8 | 23.2 |
| 72 | 22.1 | 24.3 | 20.8 |

In order to ensure full recovery of the cells, they should be frozen within five generations at a concentration greater $3 \times 10^6$ cells/mL, when they show typical fibroblast morphology. The cells may be injured and changed in biological characteristics, especially their hereditary characteristics, after too many passages or digestion with trypsin.

Microbial contamination is the most frequent cause of pollution in cell culture. Air, equipment, serum, tissue samples, etc. can all contaminate the cells. The turbidity of culture media contaminated by bacteria and fungi can be seen with the naked eye. Viruses can be seen under the microscope. However, it is harder to detect contamination with mycoplasmas. Mycoplasmas have no nuclei and can grow and reproduce in currently used media. They are hard to remove and can coexist with cells for a long time. The methods used to detect mycoplasmas include direct culture on solid agar, indirect fluorescence staining of DNA and new DNA-style hybridization. Our microbiological detection results showed that the SCF36 line was purified and free of contamination with mycoplasmas.

Isoenzyme and karyotypic data together can confirm the origin of a cell line and identify possible cross-contamination. The two techniques have been used for many years and are still used today. The practice of combining them has become a standard method for characterizing cell lines [23,17]. Chromosome analysis can relate a cell line to the gender of the animal from which was derived, and also distinguish between normal and malignant cells, because the chromosome number is more stable in normal cells [6]. Given that we aim to conserve the genomic character of Simmental cattle, it is important that the fibroblasts maintain the same diploid character as the cells when in vivo. We improved the freezing procedure and decreased the number of passages to obtain a stable diploid cell line in which about 90% of the cells had $2n = 60$.

According to Ropiquet et al. [20], the chromosome number of Simmental cattle is $2n = 60$, the 58 somatic chromosomes are acrocentric autosomes, and only the two sex chromosomes (X and Y) are metacentric. However, Weber et al. [29] and Liu et al. [12] considered that the 58 somatic chromosomes were acrocentric chromosomes and that the two sex chromosomes (X and Y) were submetacentric. One possible reason for this discrepancy is that long-term natural selection has induced genetic variation. Further study is required to determine the true cause.

LDH and MDH are very important enzymes that participate in the glycolytic pathway and the citric acid cycle, respectively. They are species-specific, and the enzyme contents and activities differ among species, providing a biochemical indicator of species classification by chromatography and electrophoresis. We therefore chose LDH and MDH to determine the species origin of the cells and to measure cross-contamination [16,19]. Biochemical analysis of isoenzyme polymorphism is currently considered the standard method for quality control of cell line identification and interspecies contamination, and is routinely used by the main biological resource centers such as American Type Culture Collection (ATCC) and European Collection of Cell Cultures (ECACC) around the world [2].

LDH is a tetrametric molecule; the H and M subunits are produced by expression of the ldha and ldhb genes, and each tissue has a characteristic and species-specific isoenzyme composition [28]. Moss [15] found between five and eight clear bands in different tissues. Five such bands were found in samples of pectoral muscle. 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. Arai et al. [1] and Ma et al. [13] measured the LDH isoenzyme pattern in equine leucocytes and plasma and in fibroblasts from Debao ponies. In agreement with these studies, five LDH bands, LDH1, LDH2, LDH3, LDH4 and LDH5, were recorded in the samples from Simmental cattle used in the present study.

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MDH is a dimeric enzyme comprising cytosolic (s-MDH) and mitochondrial (m-MDH) subunits. The mobilities of MDH bands are essentially identical among samples from poultry, and the same is true among livestock. However, MDH from livestock migrates more rapidly than that from poultry, and the enzyme content is also greater than in poultry.

Research using fluorescent proteins is mainly focused on tumors, nerves and stem cells [10]. The concentrations of DNA and lipofectin, the DNA incubation time and lipofectin combination, and the presence of serum, can all affect the efficiency of transfection, as shown by research on Vero cells, HeLa cells and various other cell lines [26,21]. In our experiment, the highest efficiency of transfer of the three fluorescent proteins was 28.8% with an optimized plasmid–lipofectin ratio.

We observed that the transfected cells at the stages of reduplication and different phases of cell division, and during growth and reduplication of transfected cells, were not significantly different from the control group. The results showed that the transfected cells had not been affected by fluorescein below a certain range. Moreover, research has indicated that GFP does not affect cell doubling time in tumor and human cell lines, in accordance with our results. The fluoresceins were not distributed homogeneously in all the cell lines; for example GFP was distributed mainly in the nucleus, and DsRed was expressed in a granular substance surrounding the nuclear membrane and formed a red ring profile. Given these exceptions, it was important to determine the distribution of the reporter gene before we analyzed the location of the fusion protein [34].

These results establish a favorable foundation for research on the structure of the genome, functional genomics and transgene work in Simmental cattle.

## Conclusion

The current results indicate that the newly established SCF36 line is stable and grows rapidly. These characteristics suggest that the SCF36 line provides a useful approach for conserving this unique breed and is a basis for further genetic studies on Simmental cattle.

## Acknowledgments

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