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# Letter to the Editor

## THE CULTURE OF FIBROBLASTS FROM DIAPHRAGM OF GIANT PANDA

Dear Editor:

Giant panda (*Ailuropoda melanoleuca*) is a precious rare animal of the world and is regarded as the Treasure of China. It is classified as an endangered species by IUCN (Wang, 1998). It is estimated that only 1000 giant panda live in the wild, and most of them are distributed in the Sichuan province of China. To protect this endangered species, more studies, besides habitat conservation, are being developed. Since we put forward the research plan of giant panda cloning in 1997, the cell culture and the foundation of cell pools of giant panda have become more important and indispensable (Chen et al., 1999; Liu et al., 1999). Cells or tissues are difficult to obtain because the giant panda is rare and is listed in the First Category of State Key Protected Wildlife List. We only had three chances for cell culture because we could not obtain the tissues and culture the cells within a short time (generally within 0.5 and 15 h, respectively) after the giant panda died. Here we report that we obtained the tissue over 2.5 h after the giant panda died and successfully cultured cells over 30 h after they were placed in sterile saline containing 200 U/ml penicillin and 200 μg/ml streptomycin at 4° C.

The big piece of diaphragm was cut over 2.5 h after the animal keeper discovered the death of the giant panda (female, 22-mo old, Wolong Nature Reserve, China). The diaphragm was rinsed three times with sterile saline containing 200 U/ml penicillin and 200 μg/ml streptomycin, then preserved, and carried to the laboratory in same liquid at 4° C. The diaphragm was rinsed three times with sterile saline containing 200 U/ml penicillin and 200 μg/ml streptomycin again and minced finely with crossed scalpels after 30 h. Small pieces were disaggregated in cold 0.25% trypsin (GIBCO BRL, Life Technologies, Rockville, Maryland) for 10 h (4° C), and then transferred to 37° C for 30 min. The disaggregated tissues and cells were seeded in culture flasks. To this was added Dulbecco modified Eagle medium/nutrient mixture F12 (Ham) at 1:1 ratio (DMEM/F12; GIBCO BRL, Life Technologies) and 20% fetal bovine serum (FBS; GIBCO BRL, Life Technologies). The cultures were incubated at 37° C in a humidified atmosphere of 5% $CO_2$.

The primary spindle-shape fibroblasts, named XXJ, from the diaphragm of giant panda were cultured after 3 d and subcultured normally. Phase-contrast microscopic observation showed the XXJ cells take on normal morphology characteristics (Fig. 1). The growth characteristics of XXJ cells were measured over a period of 8 d in culture. The fourth passage XXJ cells ($3 \times 10^4$) were seeded in Nunclon 24-well dish (Nunc, Roskilde, Denmark) and incubated with 0.5 ml DMEM/F12 supplemented with 15% FBS at 37° C in a humidified atmosphere of 5% $CO_2$. Three wells were trypsinized and counted by hemacytometer counts at the same time everyday, and then the growth curve was drawn (Fig. 2).

Further, the cytoskeleton of XXJ cells were analyzed by immunochemical methods. The fifth passage XXJ cells cultured on 8 × 8-mm cover glass were fixed in 3.7% paraformaldehyde for 20 min at room temperature (RT). They were then washed twice by 0.01 mol/L phosphate-buffered saline (PBS) (pH 7.4) containing 0.01% Triton X-100 (T-PBS). The cover glasses were treated with 0.1% Triton X-100 containing 3 mg/ml bovine serum abumin (BSA) for 30 min at 37° C, then washed with T-PBS three times at RT. The free aldehydes were reduced in 0.01 mol/L PBS (pH 7.4) containing 150 mmol/L glycine and 3 mg/ml BSA for 30 min at 37° C. Microtubules or vimentin were labeled with monoclonal antibody to β-tubulin (1:160, T-4026; Sigma Chemical Co., St. Louis, MO) or vimentin (1:200, V-6630; Sigma) for 0.5 h at 37° C, then washed with T-PBS three times at RT. Then both were incubated with fluorescein isothiocyanate (FITC)–conjugated anti-mouse IgG (1:80, F-4143; Sigma) for 0.5 h at 37° C and washed with T-PBS three times at RT. Microfilaments were labeled with phalloidin–FITC (1:200,

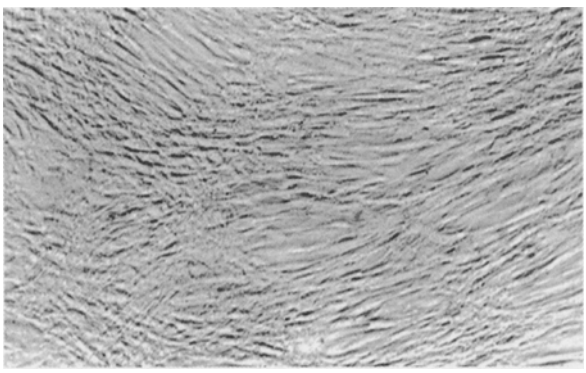

FIG. 1. The phase-constrast micrograph of XXJ cells. Magnification: ×100.

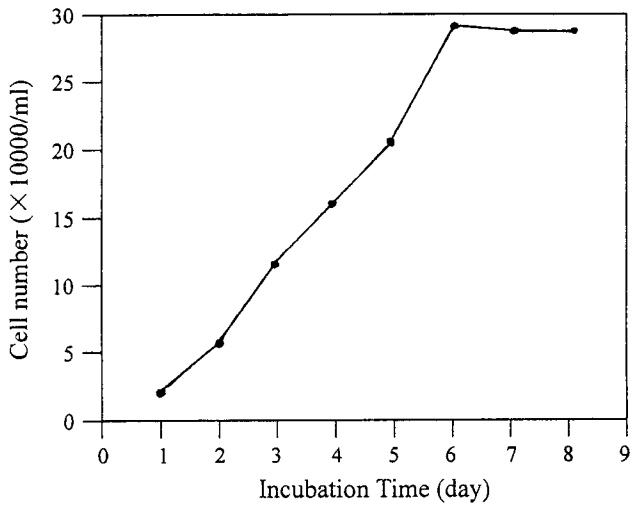


FIG. 2. The growth curve of XXJ cells.

P-5282; Sigma) and washed with T-PBS three times at RT. Deoxyribonucleic acid were labeled with 10 μg/ml propidium iodide for 10 min and then washed with T-PBS for 10 min. Laser confocal microscopic observations (Leica TCS-4D, Heidelberg, Germany) of the cultured cell showed that they have normal cytoskeleton (Fig. 3).

In summary, we have successfully cultured normal fibroblasts from the diaphragm of giant panda, for the first time, after the tissue was placed in sterile saline containing 200 U/ml penicillin and 200 μg/ml streptomycin, at 4° C over 30 h, which is valuable for further development of our giant panda cloning research and endangered wildlife conservation.

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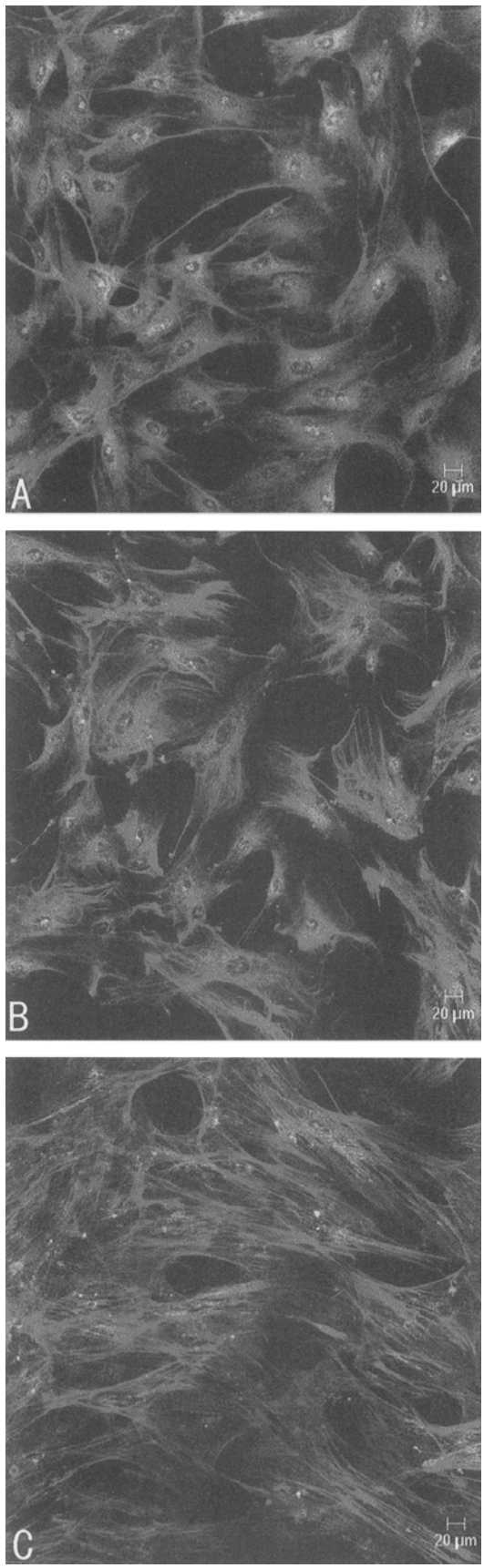


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FIG. 3. Confocal micrograph of XXJ cells by immunochemical analysis. (*A*) Stained microtubules and nuclei, (*B*) stained vimentin and nuclei, and (*C*) stained microfilament and nuclei.