Derivation and characterization of cell cultures from the skin of the Indo-Pacific humpback dolphin *Sousa chinensis*

Wei Jin · Kuntong Jia · Lili Yang · Jialin Chen · Yuping Wu · Meisheng Yi

Abstract The marine mammalian Indo-Pacific humpback dolphin, once widely lived in waters of the Indian to western Pacific oceans, has become an endangered species. The individual number of this dolphin has significantly declined in recent decades, which raises the concern of extinction. Direct concentration on laboratorial conservation of the genetic and cell resources should be paid to this marine species. Here, we report the successful derivation of cell lines form the skin of Indo-Pacific humpback dolphin. The cell cultures displayed the characteristics of fibroblast in morphology and grew rapidly at early passages, but showed obvious growth arrest at higher passages. The karyotype of the cells consisted of 42 autosomes and sex chromosomes X and Y. The immortalized cell lines obtained by forced expression of the SV40 large T-antigen were capable of proliferation at high rate in long-term culture. Immortalization and long-term culture did not cause cytogenetically observable abnormality in the karyotype. The cell type of the primary cultures and immortalized cell lines were further characterized as fibroblasts by the specific expression of vimentin. Gene transfer experiments showed that exogenetic genes could be efficiently delivered into the cells by both plasmid transfection and lentivirus infection. The cells derived from the skin of the Indo-Pacific humpback dolphin may serve as a useful in vitro system for studies on the effects of environmental pollutants and pathogens in habitats on the dolphin animals. More importantly, because of their high proliferation rate and susceptibility to lentivirus, these cells are potential ideal materials for generation of induced pluripotent stem cells.

Keywords Indo-Pacific humpback dolphin · Skin · Cell culture · Characterization

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Introduction

The Indo-Pacific humpback dolphin (*Sousa chinensis*), a marine mammalian species also named the Chinese white dolphin because of its unique pink skin color of adult, once widely lived in the coastal and inshore waters of the Indian to western Pacific oceans has become endangered. The largest population left inhabits in the Pearl River Estuary of China, one of the world's busiest and most polluted ports. It has been listed in the Convention on International Trade in Endangered Species of Wild Fauna and Flora and as a "Grade 1 National Key Protected Species" in China due to its endangered status. Recent surveys indicated that the individual number of this dolphin in the RPE had significantly declined to approximately 1,500 individuals during recent decades (Jefferson 2000; Huang et al. 2012). Facing the increasing threats, such as overfishing, water pollution, and heavy marine traffic, it is believed that the population size is still declining at a continuous rate of 2.46% per annum, and the decline rate may further accelerate in the future, which raises the high risk of extinction (Huang et al. 2012). Alongside the actions that have been implemented to provide protection to their habitats by governments, researches on ecology and other fields have been conducted for planning of more actions (Karczmarski et al. 2000; Lin et al. 2010; Frere et al. 2011; Li et al. 2012). Besides, the direct concentration on the laboratorial conservation of the germ resources should also be paid to this marine mammal species seriously threatened with human-caused extinction, the event that had happened in the Yangtze River dolphin

(*Lipotes vexillifer*), a fresh water mammal species which had been recently declared as "likely extinct" (Dalton 2006; Turvey et al. 2007). Living cell banking might be the first step towards the laboratorial conservation of its genetic resource.

In vitro culture of cells of marine mammal species has proven to be difficult due to the nature of the cetacean cells and limitation of samples from protected species because of the rare opportunities of sampling live animals for tissues suitable to cell culture. Only a few successes in cell culture in marine mammals have previously been reported. The cell lines established from kidney, lung, and skin tissues of marine cetaceans have been reported to be suitable to genomic analysis and in vitro toxicological evaluations of dolphin cells (Carvan et al. 1994, 1995; Yu et al. 2005; Li Chen et al. 2009).

In this study, we generated the cell cultures of the Indo-Pacific humpback dolphin from the skin of a male animal. The features of the derived fibroblast cells were characterized by morphological observation, gene transfer experiments, immunologic methods as well as cytogenetical analysis.

Materials and methods

Skin tissue collection. The skin tissue sample of the Indo-Pacific humpback dolphin was collected using a noninvasive method from a male adult individual which was rescued for rehabilitation from a recent animal live-stranding event in a shallow river near Foshan city of China. The sampling was under the permission of the Pearl River Estuary Chinese White Dolphin National Nature Reserve and was conducted by veterinarians with professional training. The sample site on back was sterilized with surgical cotton containing 70% alcohol, and the skin tissue fragments were sheared off aseptically by scraping with a blade. The wound (approximately 0.2 cm^2) was treated immediately with haemostatic and anti-inflammatory ointment, and close medical observation had been implemented. The skin tissue was placed into Dulbecco's modified Eagle's medium (DMEM) including penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (5 μg/ml) and transported to laboratory on ice within 2 h.

Culture medium. The medium WDM1 formulated for dolphin skin cell cultures consists of DMEM with high glucose and Ham's F12 (Invitrogen, Carlsbad, CA) at 1:1 ratio, HEPES (15 mM, Sigma, St. Louis, MO), NaCl (10 mM), L-Glutamine (2 mM), MEM Nonessential amino acid (1×, Invitrogen), MEM Amino Acids (3×, Invitrogen), MEM-Vitamin (1×, Invitrogen), hEGF(20 ng/ml, PeproTech, Rocky Hill, CT), Bovine Pituitary Extract (35 μg/ml, Invitrogen),

buffalo Insulin (5 ng/ml, Sigma), transferrin (5 ng/ml, Sigma), penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (5 μg/ml), and fetal bovine serum (15%, Invitrogen). The pH value of the medium was adjusted to 7.4 using saturated NaHCO$_3$ solution.

Primary culture and subculture. For primary culture, the tissue fragments were rinsed with 70% ethanol and washed five times with 20 ml of 1× Hank's balanced salt solution containing 200 U/ml of penicillin, 100 μg/ml of streptomycin, and 10 μg/ml of amphotericin B. The sterile skin sample was transferred into a drop of DMEM in a 10-cm Petri dish. After removing the adipose tissue, the skin sample was cut into l-mm^3 pieces using sterile tweezers, scissors, and scalpel blades. The minced tissue pieces in DMEM were then pipetted into 25-cm^2 tissue culture flasks containing 1 ml of full culture medium WDM1. The flasks were gently inverted, and tissue pieces attaching to the bottom of the flasks were uniformly distributed with a tweezers. After incubated at 37°C for 12 h to allow tissue attachment, additional 4 ml of fresh WDM1 was added, and the flasks were then gently turned over and kept at 37°C for culture in a humidified atmosphere containing 5% CO$_2$. The cultures were monitored daily by an inverted phase microscope, and half of the medium was changed every 3 d.

After a cell monolayer formed, the cells in the flasks were rinsed with phosphate-buffered saline (PBS, pH 7.2) and detached by trypsin-EDTA (Invitrogen) digestion. The suspended cells in fresh WDM1 medium were split into new culture flasks at a ratio of 1: 2 or 1: 3 depending on the cell number. Subsequent subcultures were done at 1:3 split when the cells was at 90–100% confluence. Primary cell cultures and subcultures were monitored and photographed using an inverted phase microscope. Part of cell cultures from each passage was frozen as stocks as previously descripted (Yi et al. 2010).

Plasmids and gene transfer. Transfection was performed by using the Lipofectamine 2000 reagent (Invitrogen) according to the instruction of the manufacturer. We used three plasmids to transfect the skin cell cultures of the Indo-Pacific humpback dolphin. The plasmid pSV40LT-Puro encodes the Simian vacuolating virus 40 (SV40) large T-antigen and puromycin resistance genes. The plasmids pCGFP and pCDsRed (Fig. 6A) express cytoplasmic green fluorescent protein (GFP) and red fluorescent protein (RFP), respectively. To obtain stable cell clones expressing the SV40 large T-antigen, 2 d after transfection, cells were exposed to 1 μg/ml of puromycin (Sigma) in complete medium. After another 2 d later, the dead cells were removed, and the surviving cells were reseeded at 10^5 cells/10-cm dish in complete medium containing 1 μg/ml of puromycin for clonal growth. The puromycin-resistant

cell colonies were picked for expansion and subsequently examined for the SV40 large T-antigen expression by Western blot and immunostaining.

For lentivirus infection, the lentivirus expressing and packaging plasmids pSicoR (Fig. 6*A*), pMLg/pRRE, pRSV-REV, and pMD2.G were used to cotransfect HEK 293T cells, the virus particles were collected and condensed by ultracentrifugation, and then were applied to ScSF cells. The GFP expression was visualized under an invert fluorescent microscope. To evaluate the efficiency of infection, after 72 h of virus infection, cells from three wells of six-well plate were washed with PBS and trypsinized into single cells. The number of GFP positive cells and the total cell number were determined using a hemocytometer under an inverted fluorescent microscope.

Growth curve. Plasmid pSV40LT-Puro stably transfected clone ScSFT-1 cells and nontransfected culture ScSF cells were analyzed for continuous growth as described (Yi et al. 2010). Briefly, single cells were evenly seeded into 10 wells of 12-well plates at 2×10^4 cells per well to grow for 1 to 10 d with daily medium change in half. Cells were harvested at 24-h interval and brought up to a final volume of 1 ml for hemocytometric determination of cell number. The dead cells were excluded from counting by trypan blue staining. The analysis at each time point was triplicated, and the mean cell counts were used to construct the growth curve.

Western blot. The cells were lysed for 30 min on ice in lysis buffer (10 mM Tris–HCl pH 7.5, 0.4 M NaCl, 1% NP-40, 0.4% Triton X-100, 0.2% sodium deoxycholate, 1 mM EDTA, protease inhibitors). The cell lysates were then centrifuged at $12,000 \times g$ for 10 min. The supernatants were boiled in 5× SDS loading buffer, and then separated via sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 12% Tris–HCl gel. The gel-separated proteins were transferred onto a nitrocellulose membrane. The membrane was blocked in 5% nonfat milk in TBS with Tween-20 (TBST; 20 mM Tris–HCl pH 7.6, 150 mM NaCl, 0.05% Tween-20) at room temperature (RT) for 1 h, and then was incubated overnight with primary antibody at 4°C with gentle shaking. Following three 10-min washes in TBST, the membrane was incubated with secondary antibody (alkaline phosphatase-conjugated anti-mouse IgG antibody, Sigma) for 1 h at RT with gentle shaking. After final washes, BCIP/NBT reagents were used for color development.

Immunofluorescent staining. Cells were seeded in 24-well plates to allow overnight attachment. The following day, the cells were washed with PBS, fixed with prechilled methanol at RT for 10 min, and permeabilized with 1% Triton X-100 in PBS for 10 min. The cells were rinsed three times with PBS again and incubated in a blocking solution (5% BSA in PBS) for 30 min at RT. After incubation with primary antibodies, which were diluted in blocking buffer at 1:200, for 1 h at RT or overnight at 4°C, and then with secondary antibody anti-mouse Alexa Fluor 488 (Invitrogen; 1:300 dilution) for 1 h at RT, the cells were washed three times in PBS, subjected to nuclear counterstaining with Hoechst 33342 (1 μg/ml) for 10 min, and embedded in an antifade reagent (20 mM Tris–HCl, 0.5% *N*-propyl gallate, 90% glycerol, pH 8.0) for microscopy (Ono et al. 2001).

Karyotype analysis. Chromosome plates from cell cultures were prepared for karyotyping essentially as previously described (Yi et al. 2010). For each chromosome preparation, three cultures in 35-mm dishes were used. Briefly, cells at 70–90% confluence were incubated for 2–4 h with colchicine (Sigma) at a final concentration of 1 μg/ml, and then trypsinized into single cells. The cells were collected by centrifugation for 5 min at $800 \times g$, and hypotonically treated with 1 ml of 40 mM KCl for 30 min at RT. Following fixation for three times in freshly mixed methanol/glacial acetic acid (3:1) for 30 min, cells was centrifuged, and the cell pellet was resuspended in 0.1–0.5 ml of fixative depending on cell density and dropped onto cold wet slides. The slides were air-dried at RT.

For chromosome G-banding, the slides were aged for 7–10 d at RT. After kept in an oven at 65°C for 2–3 h, the slides were incubated in 0.05% trypsin-EDTA in PBS for 2–5 min at 37°C, rinsed with PBS, stained for 15 min with 10% Giemsa in a phosphate buffer (pH 6.8), and allowed to dry in air. The banded chromosome preparations were visualized and photographed under a light microscope. For preparation of chromosome plates from blood, phytohaemagglutinin-stimulated culture of blood lymphocytes was used to produce metaphase cells as previous description (Bangs and Donlon 2005).

Results

Primary culture and subculture. From the fresh skin tissue sample of the Indo-Pacific humpback dolphin, three independent primary cell cultures (ScSF cells) were obtained. Under the culture condition established in this experiment, at day 10, some single cells were observed at the edges of the tissue pieces attaching on the bottom of culture flasks. These cells were fibroblastic in morphology with spindle-like or polygonal shape viewed under a phase-contrast microscope (Fig. 1*A*). At day 13 of culture, a monolayer of cells around the tissue pieces formed, and afterward, the cells expanded towards outside (Fig. 1*B*) until the cells from all tissue pieces in the culture flask contacted to form a confluent monolayer. At day 15, first subculture was performed at 1:3 split. The subcultures in early passages

Figure 1. Phase contrast photomicrographs of cell cultures derived from the skin of the Indo-pacific humpback dolphin. (*A*) Primary culture at day 10, showing the skin piece attaching to the flask and the growing cells surrounding the sample; (*B*) Primary explant culture at day 13, showing the growing cells and the cell monolayer; (*C*) Cells at day 3 after first subculture from the cell monolayer shown in (*B*); (*D*) Cell morphology of the subcultures. Cells are fibroblastic in morphology with a length of ~100 μm. *Asterisks* in (*A*) and (*B*) indicate the skin piece, *arrows* in (*D*) indicate the mitotic cells. *Scale bars*, 100 μm.
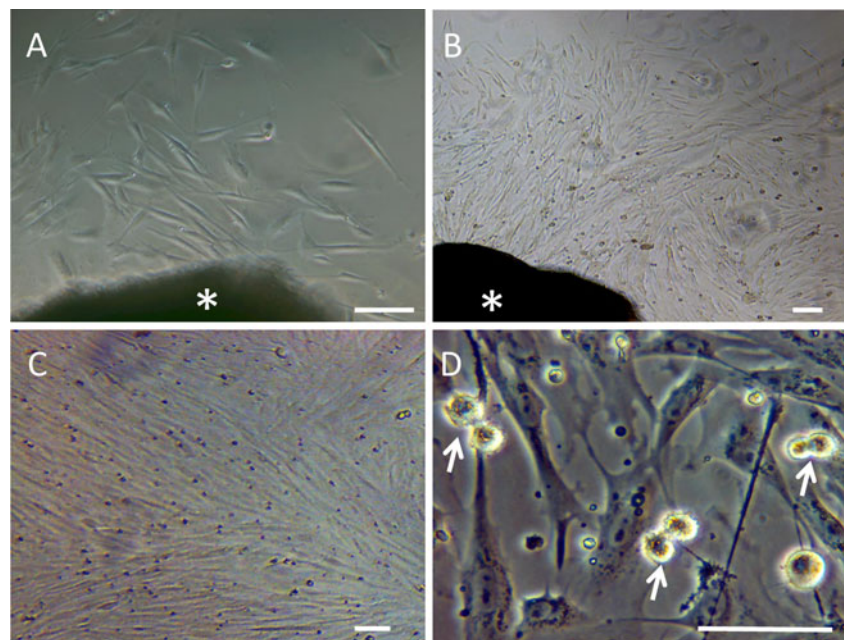
grew rapidly and reached 90–100% confluence in approximately 3–5 d (Fig. 1*C*). Some cells undergoing mitosis could be observed in the subcultures (Fig. 1*D*), which indicated proliferation of the cells. Most subcultured ScSF cells still remained the fibroblast cell morphology, and had clear nuclei with prominent nucleoli (Fig. 1*D*). However, approximately after passage 10, the cells gradually lost their ability of rapid proliferation, and cell growth was totally arrested at passage 17.

Cell immortalization. To obtain cells capable of continuous proliferation, ScSF cells at passage 5 were transfected with plasmid pSV40LT-Puro encoding the SV40 large T-antigen and puromycin-resistance gene under the EF1α promoter (Fig. 2*A*). After 48 h of transfection, puromycin was applied in the medium for selection. The reseeded puromycin-resistance cells in low density exhibited rapid growth, and cell colonies formed within 20–30 d in the presence of puromycin (1 μg/ml) at an efficiency of approximately 300 colonies per 10,000 reseeded cells. Four healthy colonies were picked under a stereomicroscope for cell expansion, from which four cell lines ScSFT-1 to ScSFT-4 were obtained. The expression of exogenous SV40 large T-antigen in the cell lines was verified by Western blot analysis. Using the antibody against SV40 large T-antigen, a band (94 KD) corresponding to SV40 large T-antigen was detected in the cell lysates from both ScSFT-1 and T-2 cells, whereas the signal was undetectable in that of nontranfected ScSF cells (Fig. 2*C*). This expression was also confirmed by fluorescent immunostaining. The SV40 large T-antigen-positive signals were detected in the nuclei of all immortalized ScSFT-1 cells (Fig. 2*D, E*), but no signal was detectable in nontranfected ScSF cells (Fig. 2*F, G*).

Viewed under a microscope, the cultured skin cells exhibited the same morphology of fibroblast before and after immortalization. However, compared to the nontransfected ScSF cells, the SV40 large T-antigen immortalized ScSFT-1 cells displayed a marked increase in growth rate. Growth curve analysis showed that the nontransfected ScSF cells at passage 9 reached the growth plateau at day 6 with cell doubling time 60.15 h, and the immortalized ScSFT-1 cells reached the growth plateau, which was much higher than that of ScSF cells, at day 8 with cell doubling time 41.53 h (Fig. 3). This indicated that the finite lifespan of these primary skin cell cultures had been overcome by immortalization mediated by the oncogene SV40 large T-antigen.

Karyotyping. The karyotype of the Indo-Pacific humpback dolphin has not been previously reported. The chromosomes preparations of the skin cultures ScSF at passages 5, 8 12, 15 and the immortalized ScSFT-1 cells at passages 8, 12, 20, and 28 were prepared. Although we failed to obtain enough metaphases from the ScSF at passages 12 and 15, the results of chromosome counts of 200 metaphase plates at each other passage revealed that the chromosome number of the cultured skin cells before and after immortalization ranged from 30 to 50 with a modal peak at 44. The chromosome number distribution pattern of ScSF cells at passage 5 was shown in Fig. 4*A*, and a standard metaphase plate with 44 chromosomes was shown in Fig. 4*B*. For karyotyping, the chromosome lengths and centromere positions of three to six metaphases with 44 chromosomes from each preparation were measured, and chromosome pairing was conducted according to chromosome lengths, arm ratios, and G-banding patterns. As shown in Fig. 4*C*, The chromosome

Figure 2. The stably transfected cells ScSFT expressing SV40 large T-antigen and puromycin. (A) Schematic diagram of the plasmid used to transfect the ScSF cells; (B) Colonal growth of the ScSFT cells, showing a colony of ScSFT cell formed after plasmid transfection, puromycin selection, and low-density growth; (C) Western blot of transfected ScSFT cells. Puromycin-resistant cell colonies ScSFT-1 and ScSFT-2 (lanes 1 and 2) displayed SV40 large T-antigen (94 KD) positive, whereas no signal was detected in nontransfected ScSF control (lane 3); (D–G) Immunostaining of ScSFT cells, showing SV40 large T-antigen expression in nuclei of the ScSFT-1 cells (E green color), and undetectable expression in nontransfected ScSF cells (G). (D, F) showed the Hochest 33342 nuclear staining (blue). M marker; Scale bar, 100 μm.
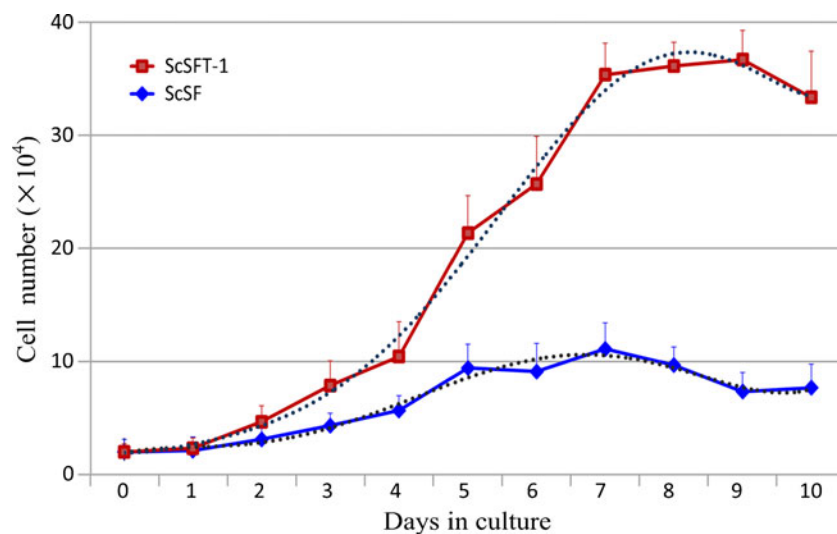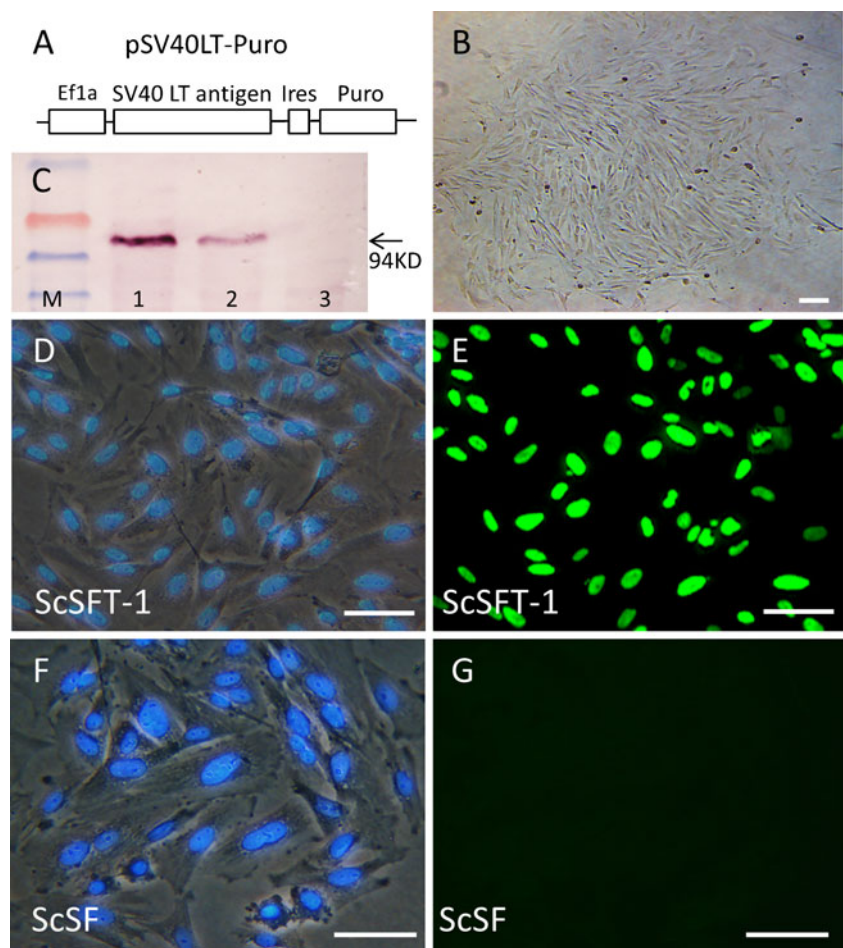

Figure 3. Growth curve of the Indo-Pacific humpback dolphin skin cell culture ScSF and the SV40 large T-antigen transformed cell ScSFT-1. Cells were harvested, and cell numbers were calculated at different time points of culture. The experiments at each time point were triplicated. ScSFT-1 cells (passage 14), which reached the growth plateau at day 8 with cell doubling time 41.53 h, displayed a remarkable increase in growth rate compared to the nontransfected ScSF cells (passage 9), which reached growth plateau at day 6 with cell doubling time 60.15 h. The cell doubling time (DT) was calculated during the exponential growth phase of the cells according to the growth curves and −the formula $DT = t \times [\lg 2 (\lg Nt - \lg No)]$ was applied. No, cell number of first count; Nt, cell number after t hours of culture from the first time of cell counting.

Figure 4. Chromosomal analysis of ScSF cells. (*A*) Chromosome number distribution pattern of ScSF cells at passage 5, showing the modal peak chromosome number 44 of ScSF cell. (*B*) A standard metaphase plate with 44 chromosomes; (*C*) Diploid karyotype of the metaphase plate in (*A*), showing the sex chromosomes and 21 homologous chromosome pairs arranged according to their sizes and G-band patterns. Based on the arm ratios, the chromosome pairs were classified into five groups including sm group (pairs 1 to 8), st group (pairs 9 and 10), m group (pairs 11 to 16), t group (pairs 17 to 21), and sex chromosome group (X and Y). *Scale bar*, 10 μm.
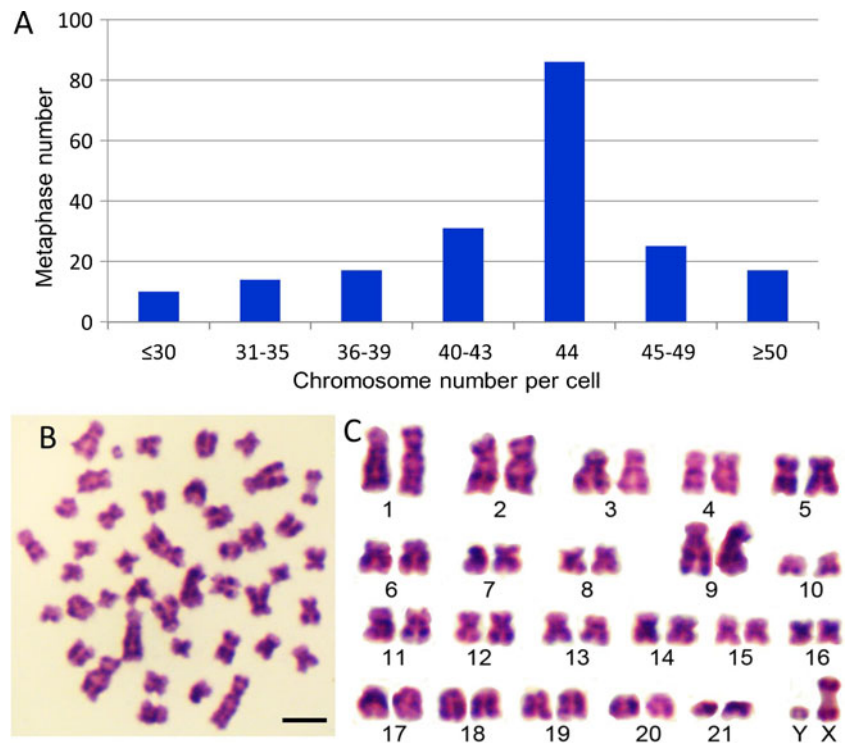
pairs were classified into five groups, submetacentric chromosome group (sm, pairs 1 to 8), subtelocentric chromosome group (st, pairs 9 and 10), metacentric chromosome group (m, pairs 11 to 16), and telocentric chromosome group (t, pairs 17 to 21). The telocentric Y chromosome and metacentric X chromosome were classified separately to sex chromosome group. Therefore, the karyotype of the Indo-Pacific humpback dolphin is 2n=13m+16sm+4st+11t (male, XY) according to this sampled male individual, which is consistent with the result obtained from the peripheral blood sample of the same individual (data not shown).

Cell type characterization. Previous reports indicated that skin tissues of dolphin produced two cell types, fibroblast or epithelial cells (Yu et al. 2005; Wang et al. 2011). In this culture system, the cultures derived from the skin of the Indo-Pacific humpback dolphin were morphologically fibroblastic-like. In order to clearly determine the cell type of the cultures, ScSF and ScSFT cells were subjected to fluorescent immunostaining and Western blot using commercially available human antibodies against vimentin and cytokeratins which are specifically expressed in fibroblasts and epithelial cells, respectively (Yu et al. 2005; Kueper et al. 2008). Western blot analysis revealed that the cell lysates of both ScSF and ScSFT (lines T-1 and T-2) cells strongly reacted with the antibody against vimentin, whereas had a negative reactivity with the antibody against cytokeratin AE1/AE3. This observation was also confirmed by fluorescent immunostaining experiment. Figure 5*C* showed the ScSF cells were immunoreactive to the antibody against vimentin, but negatively reacted with cytokeratin AE1/AE3 monoclonal antibody (Fig. 5*D*). Thus, the majority, if not all, of the cultures derived from the skin of the Indo-Pacific humpback dolphin are fibroblasts.

Efficiency of gene transfer. Plasmid transfection and lentivirus infection were used to deliver vectors containing sequences encoding GFP or RFP into ScSF cells. The transfection with plasmids pCGFP and pCDsRed with CMV promoter both resulted in GFP or RFP expression at an efficiency of approximately 30% estimated by the number of GFP and RFP expressing cells (Fig. 6*B, C*). Lentivirus infection experiment indicated that the ScSF cells exhibited high sensitivity to lentivirus containing a functional EF1α promoter. After 72 h of infection, more than 90% of the cells were GFP positive (approximately 3.33×10^6 GFP-positive cells out of 3.67×10^6 cells; Fig. 6*D*).

Discussion

In this study, we generated cell cultures from the skin of the Indo-Pacific humpback dolphin. The cells were subsequently immortalized and characterized. To our knowledge, this is the first successful cell culture of this marine mammal species.
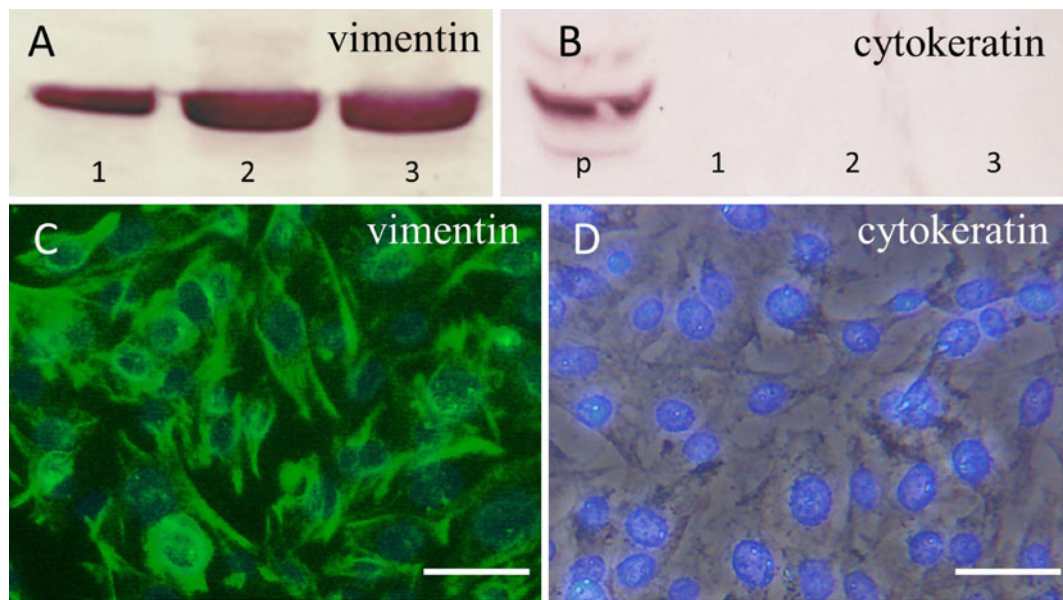
Figure 5. Expression of cell type-specific proteins in cell cultures. (*A, B*) Expression of vimentin and cytokeratin detected by Western blot, showing the positive expression of vimentin in both ScSF and ScSFT cells (*A*), but no detectable expression of cytokeratin in neither ScSF nor ScSFT cells (*B*). *lane 1*, ScSF cells; *lanes 2* and *3*, ScSFT-1 and ScSFT-2 cells respectively; *p*, positive control of cytokeratin expression; (*C*) Cytoplasmic expression of vimentin in ScSF cells detected by fluorecent immunostaining of using antihuman vimentin antibody; (*D*) Negative immunostaining of ScSF cells reacted with antihuman cytokeratin AE1/AE3 monoclonal antibody. *M*, protein marker; *Scale bar*, 100 μm.

The ScSF cultures and the immortalized ScSFT cell lines obtained were characterized as fibroblast cells by morphological observation and detection of cell type-specific protein expression. The cell type was different from that of cell lines previously established from the skin of the Atlantic bottlenose dolphin (*Tursiops truncatus*) (Yu et al. 2005), but consistent with that of the lines derived from the skin of a fresh water cetacean species, the Yangzi finless porpoise (*Neophocaena phocaenoides asiaeorientalis*) (Wang et al. 2011). As predicated, the skin cultures ScSF grew rapidly during in the early subculture passages, and the growth rate slowed down in higher passages due to possible cell senescence, which could be overcome by forced expression of large T-antigen encoded by the early region of SV40, leading to the production of cell lines capable of continuous proliferation (Hahn et al. 2002).

We analyzed the karyotype of the skin cultures. The chromosome plates were firstly prepared from passage 5 to

Figure 6. Transient transfection of ScSF cells with plasmid and infection with lentivirus. (*A*) Schematic diagrams of plasmid pCDsRed, pCGFP (*upper*) and pSicoR (*lower*) enconding RFP and GFP repetively; (*B, C*) ScSF cells transiently transfected with pCDsRed (*B*) and pCGFP (*C*), showing the GFP and RFP expression; (*D*) ScSF cells infected by lentivious particles, showing high efficiency of GFP expression. *Scale bar*, 100 μm.
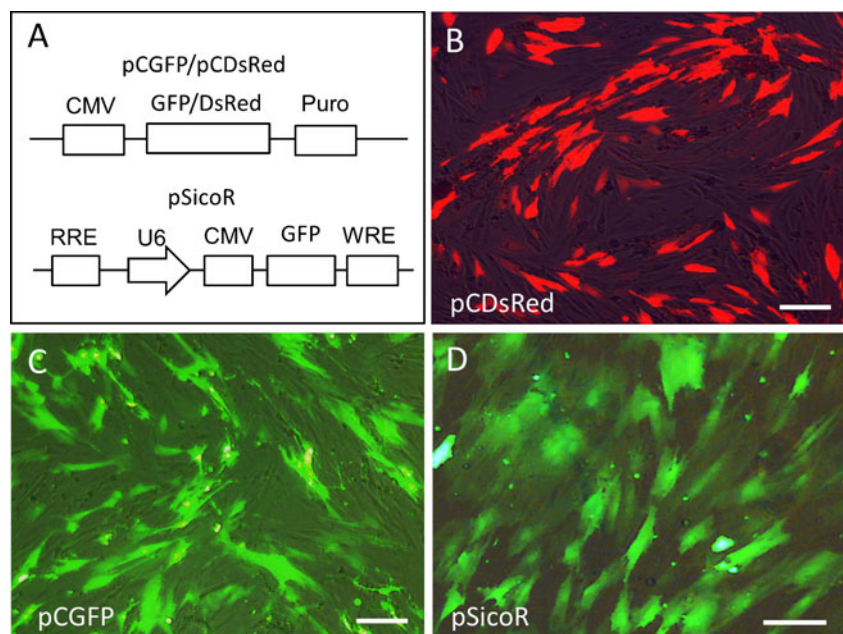
avoid possible aneuploidy events induced by long-term culture maintenance. Previous reports indicated that the normal diploid somatic cells of cetacean species contain 44 or 42 chromosomes (Heinzelmann et al. 2009). Here, we found that the diploid chromosome number of the Indo-Pacific humback dolphin was 44 comprising 13 metacentric, 16 submetacentric, 4 subtelocentric, and 11 telocentric chromosomes including sex chromosomes X and Y in male based on the analysis to this individual. This karyotype pattern was confirmed by the karyotyping analysis to the short-term cultured peripheral blood sample of the same individual. Yu et al. (2005) reported that the karyotype of skin cell cultures derived from the Atlantic bottlenose dolphin was slightly altered after cells were stably transfected with the plasmid encoding SV40 small t- and large T-antigens. To inspect the possible alteration of karyotype of ScSF and ScSFT cells, we collected the metaphases from ScSF cells at higher passages 12 and 15 before the growth was totally arrested, and from immortalized ScSFT cells at passages 20 and 28. However, by comparing the karyotype patterns of all analyzed cell cultures, no alteration had been found, although it was possible that small abnormalities of chromosomes escaped from observation at cytogenetic level. This indicated neither stable expression of SV40 large T-antigens nor long-term culture had led to cytogenetically detectable alteration of the karyotype.

Many programs and plans have been made by governments and other departments to protect this engendered marine mammal species. However, the fact that individual number, especially the number of young individual, is rapidly decreasing caused by increasing threats still raises the concern of human-caused extinction of this species, the event that had recently happened to the Yangzi River dolphin (Turvey et al. 2007). Therefore, it is actually necessary in conducting more researches on cell biology and genetics in order to develop assisted artificial reproduction technologies in this species such as animal cloning and semi-cloning which succeeded recently in fish and mouse (Yi et al. 2009; Yang et al. 2012). Induced pluripotent stem cells (iPSCs) have been generated from somatic cells of many species by direct molecular reprogramming. The capability of iPSCs in production of various cell types and even whole animals not only provides the possibility to apply iPSCs to preserve genetic material of endangered species but also offers hope of rescuing species on the verge of extinction (Ben-Nun et al. 2011). For the Indo-Pacific humpback dolphin, because of its protected status, the only opportunity to obtain stem cells is to generate iPSC from somatic cells, and skin tissue might be the only live sample resource of cell suitable for in vitro culture. The ScSF cells grew rapidly for several passages with a stable karyotype. This has opened a window for introduction of reprogram factors into these cultured fibroblast cells by approaches of either plasmid

transfection or virus infection which have been widely used to generate iPSC. It is essential to estimate the efficiency of gene transfer and exogenous gene expression of newly developed somatic cell cultures before iPSC generation. Our results from plasmid transfection experiment showed that plasmid could be transferred into cells with high efficiency and the CMV promoter normally functioned in the ScSF cells. Compared to plasmid transfection, lentivirus infection led to a gene transfer efficiency of more than 90%. This implies that lentivirus might be the best tool in production of iPS cells from the ScSF cells.

As an endangered marine mammal species, the living animals are not allowed for experiments which would cause damages and diseases. The established cell lines in this study also provide an alternative system for researches on toxicology and pathology. Compared to other marine mammals, the Indo-Pacific humpback dolphins prefer to inhabit in shallow areas to the shore where various toxic pollutants and pathogens are enriched. The cell lines serve as an accurate in vitro system for investigating the responses of dolphin skin to the various environmental pollutants and pathogens. Understanding of the impact of environmental stressors on the Indo-Pacific humpback dolphin will be helpful in monitoring of habitats and choosing sites suitable to off-site conservation while necessary.

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