Efficient immortalization of cells derived from critically endangered Tsushima leopard cat (*Prionailurus bengalensis euptilurus*) with expression of mutant *CDK4*, *Cyclin D1*, and telomerase reverse transcriptase

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Received: 22 June 2018 / Accepted: 28 August 2018 / Published online: 17 September 2018
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Abstract Tsushima leopard cat is the subspecies of Amur cats, and it is classified as the most highest class of critically endangered animals. Although the protection activity is highly recognized, the number of animals is decreasing due to the human activity and invasion of domestic cats and infectious disease. In this study, we succeeded primary culture of normal fibroblasts derived from the Tsushima leopard cat (*Prionailurus bengalensis euptilurus*). Furthermore, we introduced the human derived mutant Cyclin

Dependent Kinase 4, *Cyclin D1*, and telomere reverse transcriptase. We showed that the expression of these three genes efficiently immortalized cells derived from Tsushima leopard cat. Furthermore, we showed that the chromosome pattern of the established cells is identical with the original one. These data indicate that our method of immortalization is useful to establish cell lines from critically endangered cats, which potentially contributes to the re-generation of

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critically endangered animals from cultured cell with reproductive technique, such as somatic cloning.

Keywords Endangered animals · Immortalization · Cellular senescence · Tsushima leopard cat

Introduction

The Tsushima leopard cat is a subspecies of the Amur wild cats, which is one of the original species of Tsushima island in Japan (Masuda and Yoshida 1995). The origin of Tsushima leopard cat is supposed to come from the main land of China based on sequencing analysis of mitochondrial DNA (Izawa 1991; Park 2011). Due to the hunting and traffic kill of the human activities, the number of individuals of Tsushima leopard cat is decreasing, year and year. The number of Tsushima leopard cat is estimated around 100, which is classified as the highest position as the critically endangered animals (Izawa 1991). From this crisis condition of Tsushima leopard cat, Ministry of environment of Japan started the breeding under the human controlled condition. Although the large efforts and costs have been paid to the breeding program, the successful case is very limited. These situations indicate that the Tsushima leopard cat is one of the most critically endangered animals in Asia.

In the 1970's, the preservation of biological specimens of all endangered animal species, such as somatic cells, germ line cell, and DNA was started as "Frozen Zoo Project" in the San Diego Zoo (Associated-Press). The number of endangered animals is increasing year and year, and protection activity cannot cover the all of them. Against this situation, we started the preservation of cultured cells from critically endangered species in Japan in 2011 (Fukuda et al. 2012, 2014, 2018). Although the research group is so small when compared with that in United States and United Kingdom, we are intensively working on the cell preservation of endemic species of Japan. Although the Asian area is classified as "hot spot" of the animal diversity and involves large number of endemic species, these cellular preservation projects are pretty minor and only supported by the personal efforts of the scientists.

The primary cells cannot continue cell proliferation infinitely. In brief, after a certain number of cell

divisions and cell proliferation reaches to a halt, which is called as cellular senescence (Hayflick and Moorhead 1961). Even before cellular senescence, primary cells stop proliferation due to cellular stress and lack of appropriate growth factors (Mavrogonatou et al. 2018). Cellular senescence and stress can be characterized with the accumulation of senescence associated protein, p16 (Ohtani et al. 2010). The accumulation of p16 protein is known to suppress the kinase complex, which drives the cell cycle, Cyclin Dependent kinase 4 (CDK4) and Cyclin D. As result of the suppression of kinase activity of CDK4-Cyclin D complex, the Retinoblastoma (RB) protein keeps binding with transcriptional factor, E2F (Shiomi et al. 2011). Our research group previously reported that the expression of mutant *CDK4* (R24C mutant), *Cyclin D1*, and telomerase reverse transcriptase (*TERT*) allows us to bypass the cellular senescence, and induce the infinite cell proliferation of human cells (Shiomi et al. 2011). Furthermore, the expression of mutant *CDK4*, *Cyclin D1*, and *TERT* keeps original cellular differentiation ability from myoblasts to mature cardiac myocyte, with intact chromosome pattern. In addition to this, we previously reported that cellular immortalization can be achieved in various animals, including bovine and swine (Donai et al. 2014), monkey (Kuroda et al. 2015a), midget buffalo (Fukuda et al. 2016), prairie vole (Katayama et al. 2016, 2017), and sea turtle (Fukuda et al. 2018) with the expression of human derived mutant *CDK4*, *Cyclin D1*, and *TERT*. The application of this immortalization method with the combination of mutant CDK4, Cyclin D to multiple species is based on the high conservation of amino acid sequence during the evolution of animals. In fact, amino acid sequence of CDK4 of midget buffalo is around 95% identical with that of human (Fukuda et al. 2016). However, interestingly, we also showed that the expression of mutant *CDK4*, *Cyclin D1*, and *TERT* can extend the cell division of African savannah elephant derived cell, but does not induce cellular immortalization (Fukuda et al. 2016). The finding in elephant suggested that cellular immortalization method with mutant *CDK4*, *Cyclin D1*, and *TERT* is not universal in all animals. We have no data if the Tsushima leopard cat derived cells can be immortalized with mutant *CDK4*, *Cyclin D1*, and *TERT*. In this study, we obtained the primary cell from the Tsushima leopard cat, and expressed human derived *CDK4*, *Cyclin D1* and *TERT* in the cells by

lentivirus-mediated gene transfer. This study will contribute to the question on how basic mechanism of cell cycle regulation has been conserved in the process of the animal evolution.

Materials and methods

Primary cell culture of Tsushima leopard cat

The skin tissue was obtained from the Tsushima leopard cat, which was killed by a traffic accident. From the skin tissues, we successfully obtained the primary cells derived from Tsushima leopard cat. From the morphology of the cells, the obtained primary cells were supposed to be derived from the fibroblasts. We carried out the primary culture of the cells in DMEM (cat. no. 044-29765, Wako Pure Chemical Industries, Tokyo, Japan) medium containing 10% fetal bovine serum (cat. no. FB-1365/500, Wako Pure Chemical Industries) and 1% antibiotic–antimycotic mixed stock solution (cat. no. A5955-100ML, SIGMA, Tokyo, Japan) under 5% CO₂ and 37 °C.

The introduction of mutant CDK4, Cyclin D, TERT through lentiviral vectors

The preparations of lentiviral vectors expressing mutant CDK4, Cyclin D, and TERT were performed using the protocol, which is described in our previous work (Donai et al. 2013). The efficiency of gene introduction was estimated by the transduction of lentiviral vector which expresses enhanced green fluorescence protein (EGFP). We named the cell transduced with the R24C mutant *CDK4*, *Cyclin D1*, and *TERT* as K4DT cells, from the last characters of the introduced genes. We also generated K4D cells, which were transduced only with R24C mutant *CDK4* and *Cyclin D1*. The titre of the TERT lentiviral vector sometimes was lower when it was compared with that of the mutant CDK4 and Cyclin D (Fukuda et al. 2016). Cells expressing both mutant CDK4 and Cyclin D genes could be enriched due to their growth advantage against non-transduced cells. However, cells expressing TERT could be enriched only after telomere shortening. From this situation, we additionally transduced the cells with a TERT expressing retroviral vector with the hygromycin selection

marker. Thus, we named the hygromycin B resistant cells K4DT + T cells, which are expected to uniformly express TERT.

Cell cycle analysis

For the cell cycle analysis, Muse cell cycle assay kit (Merck Millipore, Darmstadt, Germany) and Muse cell analyzer (Merck Millipore) were used. The detailed method for the analysis was based on the protocol provided by the manufacture. Statistical significance was evaluated with non-parametric Steel method with six samples for each experimental group.

Genomic PCR reaction

Genomic DNAs of cultured cells were obtained by the commercially available extraction kit using NeucleoSpin Tissue (cat. no. 740952, Takara Bio, Shiga, Japan). Amplification reaction was carried out using KOD-FX Neo (cat. no. KFX-201, TOYOBO, Osaka, Japan) based on the protocol provided from the manufacture. Sequence of the primers are listed in below. For the detection of *Cyclin D1* cassette, forward and reverse primers were used, TF806 (5'-GGCACCAAAATCAA CGGGACTTT-3') and TF807 (5' -TTCCTCGCA-GACCTCCAGCA-3'). For the detection of R24C mutant *CDK4* cassette, forward and reverse primers were used, TF806 (5'-GGCACCAAAATCAACGGGACTTT-3') and TF808 (5'- ACGAACTGTGCTGATGG-GAAGGC-3'). For the detection of TERT cassette, forward and reverse primers were used, TF961 (5'-CTGCTCCTGCGTTTGGTGGATGATT-3') and TF962 (5'-GTCCTGAGTGACCCCAGGAGTGGCA-3'). For internal control of genomic amplification, we designed the forward and reverse primers, TF965 (5'-AGCCCCCTCAGCAAGTCTAGCTCCT-3') and TF966 (5'-CCGGCAGCTCCAGCCCCGAAA) for the genomic region of cat derived Tuberous Sclerosis Type II gene (*TSC2*). Since *TSC2* gene is known as unique sequence in human, mouse and rat genomes and pseudogenes do not exist in their genome, we used *TSC2* as internal control. PCR products were detected in 2% agarose gel with ethidium bromide staining and gel imaging system (STAGE-S3, Amzsystem, Osaka, Japan).

Western blotting

The cells were solubilized in a solution containing 50 mM Tris–HCl, pH 7.4, 0.15 M NaCl, 1% Triton X-100, 2.5 mg/ml, sodium deoxycholate (Cat. no. 194-08311, Wako Pure Chemical Industries) and a protease inhibitor cocktail (1/200 dilution, #25955-11, Nacalai Tesque, Kyoto, Japan). The detailed procedure for western blot was described in our previous manuscripts (Fukuda et al. 1999, 2000, 2008). Primary antibodies used in this study are listed in below. Antibody against Cyclin D (1:5000, code no. 553, MBL, Nagoya, Japan), CDK4 (1:2500, code no. K0065-3, MBL) and α -tubulin (1:1000, cat. no. sc-32293, Santa Cruz Biotechnology, Dallas, TX, USA) were used. Secondary antibodies used in this study are listed in below. Sheep anti-mouse IgG linked horseradish peroxidase (HRP) (1:2000, code no. NA931 V, GE Healthcare, Little Chalfont, UK) and a donkey anti-rabbit IgG linked HRP (1:2000, code no. NA934 V, GE Healthcare). The results of the western blotting were detected with a Pierce Western Blotting and ECL substrate (cat. no. NCI3109, Thermo Scientific, Waltham, MA, USA) with an Image Quant LAS-4000 mini (GE Healthcare).

Stretch PCR assay

Enzymatic activity of the telomere elongation was detected with commercially distributed kit ‘TeloChaser’ (code no. TLK-101, TOYOBO, Osaka, Japan). The detection procedure was performed by using the protocol provided by the manufacture with 1.0×10^5 cells. We used 1.5×10^4 cell of Hela cell as positive control.

Population doublings

Population doublings (PD) were measured to infer the cell proliferation rate with sequential passages. PD value represents the number of cell divisions, obtained from the following formula; $PD = \log_2 (a/b)$ where “a” is the number of cells counted at each passage and “b” is the number of cells seeded at the start of each passage (Qin et al. 2014). To obtain the value of “a”, cells were seeded at a concentration of 5×10^4 cells/well (the value of “b”) in a 35 mm dish. When the cells in one of the wells reached confluence, all cells on the plate were treated with Accutase (Code,

A11105-01, Life Technologies, Waltham, MA, USA) and the number of cells in each well (the value of “a”) was counted at the same time (Qin et al. 2014). Results are shown as the mean with standard deviation, from the data obtained from triplicated experiments.

Senescence associated beta-galactodisase (SA-beta-Gal) staining and Karyotype analysis

To detect the cellular senescence, SA-beta-Gal staining was used. The staining was carried out at passage 2, with Senescence Detection Kit (cat. No. K320-250, Bio Vision, Milpitas, CA, USA). The karyotype analysis was carried out in 50 mitotic phase cells. We detected the number of chromosome, and we also performed karyotype analysis with G banding pattern.

Results

The establishment of primary cells

The Tsushima leopard cat (*Prionailurus bengalensis euptilurus*) is genetically close to the wild cats which exist in the main land of China, such as Leopard cat (*Prionailurus bengalensis*). Appearance of Tsushima leopard cat is very close to that of leopard cats in main land (Fig. 1a), which is good agreement with the characterization as sub-species of *Prionailurus bengalensis*. The primary cells were cultured in the Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% fetal calf serum. The growth of the primary cells was very slow when compared with the primary cells derived from mouse or human, suggesting that there is room for the optimization of cultured condition, such as basal medium and addition of the growth factors. The cell morphology of primary cells let us consider that the majority of them were fibroblasts (Fig. 1b).

Transduction of mutant CDK4, Cyclin D1 and TERT by lentivirus- and retrovirus-mediated gene transfer

We first selected VSV-G pseudotyped lentivirus vectors to deliver the transgenes into the Tsushima leopard cat derived cells, since they have been reported to be applicable to various types of species

Fig. 1 Appearance and cell morphology, gene introduction into Tsushima leopard cat derived cells. **a** Appearance of Tsushima leopard cat. **b** The cell morphology of the Tsushima leopard cat derived cells. **c** Fluorescence in cells transduced with the

recombinant lentivirus expressing enhanced green fluorescence protein (EGFP). CS-CMV-EGFP (upper panel); control (lower panel)

(Miyoshi 2004) and successfully applied to various animal cells in our hand (Katayama et al. 2017). We first monitored the transduction efficiency of lentiviral vector which expresses the Enhanced Green Fluorescence Protein (EGFP). As shown in Fig. 1c, we observed the intense expression of EGFP protein in Tsushima leopard cat derived cells, indicating that transduction with lentiviral vector is an effective method for the gene delivery into the wild cat derived cells. Although the transduction efficiency was not so high, around 6 percent of the cells expressing EGFP were easily detectable under the microscope (Fig. 1c).

Then, we exposed the cells to a mixture of lentiviral vectors, expressing mutant CDK4 and Cyclin D and TERT or those without TERT. We named the resultant cells K4DT cells and K4D cells for CDK4, Cyclin D1, TERT, respectively, from the characters of the transgenes. To ensure that all cells express TERT, we additionally introduced *TERT* gene with retroviral vectors with hygromycin B-resistant gene. We selected the recombinant cells with 100 $\mu\text{g/ml}$ of hygromycin B and named the hygromycin B-resistant cells as K4DT + T cells.

The cellular morphology and characterization of K4DT + T cells derived from Tsushima leopard cat

We established K4D cells (lentivirally transduced with mutant CDK4 and Cyclin D), K4DT cells (lentivirally transduced with mutant CDK4, Cyclin D, and TERT) and K4DT + T cells (K4DT cells retrovirally transduced with TERT). As shown in Fig. 2, we detected the amplification products which are specific to *Cyclin D1*, *CDK4*, *TERT* expression cassette (Fig. 2a). The products were detected with

expected combination, indicating that the expression cassettes were integrated into the cells. Furthermore, we detected the expression of mutant CDK4 and Cyclin D at the protein level with western blot analysis. As shown in Fig. 2b, we detected the signals of mutant CDK4 and Cyclin D at the expected molecular weight with the expected combination. Then, we measured telomerase activity by the stretch PCR assay. As shown in Fig. 2c, we observed the enzyme activity of K4DT + T cells derived from Tsushima leopard cat, although we also observed a slight increase of the enzymatic activity in K4D cell.

Fig. 2 The detection of genomic cassette by genomic PCR and the protein expression by western blotting, the telomerase activity by Stretch PCR. **a** The detection of genomic cassette. Lane1; parental cells, Lane2; K4D, Lane3; K4DT + T. The amplification products from the expression cassette of Cyclin D (Upper left), CDK4 (Lower left), TERT (Upper right), and internal control (Lower right, TSC2, Tsushima leopard cat derived Tuberous Sclerosis Type II gene). **b** The detection of protein expression. Lane1; K4DT + T, Lane2; K4D, Lane3; parental cells. **c** The detection of the telomerase activity by stretch PCR

From these data, we concluded that our established K4DT + T cells gained in enzymatic activity for telomere elongation.

Extended life span of K4D and K4DT + T cells

We evaluated if the K4D and K4DT + T cells have growth advantage over the parental cells. At the beginning, a specific number (5×10^4 cells/35 mm diameter dish) of parental cells, K4D cells and K4DT + T cells was seeded. K4D and K4DT + T cells showed constant proliferation whereas the parental cells almost stopped proliferation soon after the plating at the passage 2 (Day 9, Fig. 3a). At this time point, the parental cells already showed the positive staining of SA-beta gal, indicating that these cells underwent pre-mature senescence (Fig. 3b), which was considered to be caused by culture stress. In contrast to the parental cells, K4D and K4DT + T cells did not show any positive staining of SA-beta gal staining (Fig. 3b). K4D and K4DT + T cells showed negative staining for SA-beta gal even at passage 20 (data not shown). From these data, we concluded that expression of human R24C mutant CDK4 and Cyclin D dramatically extended the life span of cells derived from Tsushima leopard cat.

Cell cycle analysis of the established cells

We next analyzed the cell cycle profile of the established cells. The expression of viral oncogenes such as HPV16 E6/E7 frequently causes a tetraploid genome, which is one of the characteristics of cancer cells (Incassati et al. 2006). Since the primary cells stopped proliferation, we carried out the cell cycle analysis at passage 2. Both K4D cells and K4DT + T cells showed similar cell cycle profiles as parental cells with small fraction of S phase cells (Fig. 4a–c). We also evaluated six samples for each group and evaluated the potential effect of cell cycle stage (Table 1). As the statistical analysis, we did not detect any significant difference between cell lines, indicating that only small proportion of cells was expressing both mutant CDK4 and Cyclin D1 at this passage or human CDK4 and Cyclin D1 could not affect the cell cycle of the Tsushima leopard cat derived cells, as far as within passage 2. We also carried out the cell cycle analysis at passage 7, as shown in Fig. 4d, e, indicating that the duration of the cell cycle was dramatically

reduced in both K4D cells and K4DT + T cells, since we observed decreased ratio of G0/G1, and increased ratio of S and G2/M (wild type cell can not proliferate until passage 7, therefore the data have been compared between wild type cells at passage 2 and K4D and K4DT + T cells at passage 7). The results suggest that the cells expressing both mutant *CDK4* and *Cyclin D1* were enriched during the serial passages of the cells. We also observed that the ratio of the debris was significantly lower in K4D and K4DT + T cell. Although we can not explain the exact reason for the decreased debris, the K4D and K4DT + T cells showed smaller cell size and an increase in cell proliferation.

Karyotype analysis

We evaluated the karyotype of K4DT + T cell (Day 89, passage 12). For getting the chromosome number, we evaluated 50 mitotic cells and we observed that all cells have $2n = 38$, indicating that the K4DT + T cells are keeping the original number of chromosomes (Table 2). Chromosome number of Tsushima leopard cat is known to be identical to another critically endangered wild cat, Iriomote wild cat (*Prionailurus bengalensis iriomotensis*) (Wurster-Hill et al. 1987; Masuda and Yoshida 1995). Interestingly, our established K4DT + T Tsushima leopard cat derived cells showed the intact chromosome pattern of the original cells (Fig. 5). From these results together with telomerase activation in the cells detected by stretch PCR, we concluded that K4DT + T cells are considered to be a virtually immortalized cell line maintaining normal chromosomal number without chromosomal instability.

Discussion

In this study, we established primary cells and recombinant cells from Tsushima leopard cat (*Prionailurus bengalensis euptilurus*). We previously reported that expression of mutant *CDK4* and *Cyclin D1*, and *TERT* enables us to efficiently establish immortalized cell lines from various species (Shiomi et al. 2011; Fukuda et al. 2012, 2016; Donai et al. 2014; Kuroda et al. 2015a, b; Katayama et al. 2016). The immortalization method for Tsushima leopard cat is identical with that of midget buffalo (Lowland anoa)

Fig. 3 Growth curve and SA-beta-Gal staining of the Tsushima leopard cat derived cells and the immortalized cells. **a** Analysis of the cell proliferation. The result shows the each accumulated population doubling of parental, K4D and K4DT + T cells

through sequential passaging. **b** Detection of senescent cells of parental, K4D and K4DT + T cells at passage 2 by SA-beta-Gal staining. Upper panels, staining features with lower magnification. Lower panels, staining features with higher magnification

Fig. 4 Cell cycle analysis of parental, K4D and K4DT + T cells at different passages. **a** Parental cells, **b** K4D cells, **c** K4DT + T cells at passage 2. **d** K4D cell, **E**. K4DT + T cells at passage 7. The representative data of each experimental group were listed. The mean and standard deviation of the ratio of each cell cycle stage from six samples are shown in Table 1

Table 1 Cell cycle analysis of wild type, K4D, and K4DT + T Tsushima wildcat derived cell

	Cell cycle phase			
	G0/G1	S	G2/M	Debris
WT (P2)	75.1 ± 0.7	5.7 ± 0.8	15.2 ± 0.57	80.3 ± 1.5
K4D (P2)	73.2 ± 1.3	4.2 ± 0.2	15.1 ± 0.5	53.8 ± 2.8
K4DT + T (P2)	72.1 ± 1.3	5.65 ± 0.5	15.1 ± 0.3	60.0 ± 0.7
K4D (P7)	52.6 ± 1.4**	7.5 ± 0.4	18.1 ± 0.38*	20.6 ± 0.6**
K4DT + T (P7)	52.2 ± 0.8**	8.2 ± 0.1*	17.7 ± 0.4**	23.3 ± 0.5**

**Statistical significance at 1% and *significance at 5% level between WT (P2) and K4D (P7), WT and K4DT + T (P7). The significance was evaluated by steel method

derived cells (Fukuda et al. 2016). As previously shown in the buffalo study, cDNA size of TERT is relatively longer, and selection with antibiotics was effective to detect the telomere extension activity. In our unpublished data of midget buffalo derived cells, simple mixture and infection of CDK4, Cyclin D, and

TERT expressing lentiviral vector was not enough to detect telomerase activity, which is possibly explained by the relatively low titre of TERT lentivirus (unpublished data), whereas additional introduction of TERT gene into the K4DT cells, followed by antibiotic selection was enough to detect the enzymatic activity

Table 2 Karyotype analysis of K4DT + T Tsushima leopard cat derived cell

	Chromosome number			Diploid/total
	37	38	39	
K4DT + T	0	50	0	50/50

of telomerase. Additional TERT introduction followed by selection with antibiotics was also useful to establish the immortalized Tsushima leopard cat derived cell.

The amino acid sequence of CDK4 and Cyclin D is pretty well conserved during the animal evolution (Fukuda et al. 2018). Based on the conservation of the amino acid sequence, the introduction of human derived CDK4 and Cyclin D enables us to overcome the negative feedback system, which is based on the senescence protein p16. We compared the amino acid sequence of CDK4 and Cyclin D between cat and human. In CDK4, we observed 96% homology with amino acid sequence. In Cyclin D, the homology was 95% between human and cat. This amino acid conservation enabled us to establish an immortalized cell line from Tsushima leopard cat with the introduction of human derived genes.

In the sequential passage experiment, we observed that the primary cells showed positive staining in the passage 2 (Day 9). Although the cells were positive for the senescence associated beta-gal staining, the growth speed of wild cat derived cells was very slow when compared with that of mouse embryonic fibroblasts or human fibroblasts. This situation indicates that positive staining of primary cells in SA-beta gal staining is due to the pre-mature senescence of the

cells. When the cultured cells are exposed to various types of cellular stresses, the cells get into the senescence phenotype, which is defined as pre-mature senescence (Mavrogonatos et al. 2018). The cell culture condition of the primary cells is probably not fully optimized to the cells from the Tsushima leopard cat.

We observed that our established K4DT + T cell was keeping the original chromosome pattern of the cells. As far as we tested 50 mitotic cells, the result was perfect data, $2n = 38$ at passage 12. The original chromosome pattern might be important characteristic to use this cell as in vitro model for infectious disease. For example, one of the threats for the extinction of Tsushima leopard cat is the infection with FIV (feline immunodeficiency virus) (Hayama et al. 2010). In case of critically endangered animals, the infection experiment to investigate the molecular mechanisms is close to be impossible. Our established cell line might help how FIV virus will be transmitted among the domestic cat to wild cat.

Furthermore, the intact chromosome condition might be possible to use as the material for the regeneration of individuals with developmental technology, such as somatic cloning. The conservation of the wild condition is the best resolution, but the number of Tsushima leopard cats already reached around 100, which is close to the wild extinction. For the somatic cloning, K4DT + T cell would be more suitable than K4D cell, since these cells can proliferate infinitely. The infinite cell growth would enable us to distribute cells to scientists world wide. Although this species does not reach to the extinction yet, the conservation of biological specimen including cultured cell, and germ cell has scientific significance to keep the diversity of the animals.

Fig. 5 Karyotype analysis of Tsushima leopard cat derived K4DT + T cells.

a G-banding metaphase spread of K4DT + T cell.
b Aligned chromosome of Tsushima leopard cat derived K4DT + T cell. Sex chromosomes are indicated as X and Y

Acknowledgement We thank Dr. Hiroyuki Miyoshi (RIKEN, BioResource Center) for providing lentiviral constructs. This work was supported by the Grant from JSPS, KAKENHI, Grant Number 17H03624.

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