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Establishment of Cell Lines Derived From the Genus *Macaca* Through Controlled Expression of Cell Cycle Regulators

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ABSTRACT

Nonhuman primates are useful animal models for the study of human diseases. However, the number of established cell lines from nonhuman primates is quite limited compared with the number established from other experimental animals. The establishment of nonhuman primate cell lines would allow drug testing on those cell lines before moving experiments into primates. In this study, we established nonhuman primate primary cell lines by introducing the genes for CDK4R24C, cyclin D1, and hTERT. These cell lines proliferated more rapidly than primary cells and bypassed cellular senescence. Karyotype analysis showed that the chromosome patterns were intact in the immortalized cell lines. Furthermore, we showed that the expression of introduced genes could be precisely controlled through the Tet-Off system with the addition of doxycycline. The present study shows that introduction of the CDK4R24C, cyclin D1, and hTERT genes are effective methods of establishing nonhuman primate cell lines. J. Cell. Biochem. 116: 205–211, 2015. © 2014 Wiley Periodicals, Inc.

KEY WORDS: NONHUMAN PRIMATE MODEL; MACAQUE MONKEY; CDK4R24C; CYCLIN D1; hTERT

The genomic sequence of nonhuman primates such as chimpanzees has high homology (more than 90%) with that of humans [Ebersberger et al., 2002; Fujiyama et al., 2002; Sakaki et al., 2003]. Because of this high homology, animal experiments with *Macaques*, a genus of primates is important data for toxicological and pharmacological evaluation before clinical trials.

Despite the importance of macaque monkeys as experimental animals, the number of established cell lines from these nonhuman primates is quite limited. In the American Type Culture Collection (ATCC), only 12 cell lines have been established from nonhuman primates, whereas more than 1400 cell lines have been derived from mice. The establishment of nonhuman primate cell lines would allow drug testing on those cell lines before moving experiments into primate animal models. For accurate preclinical evaluation of toxicology and pharmacology at a reasonable cost, the establishment of nonhuman primate cell lines is important.

Regarding the difficulty in establishing primate cell lines, the Hayflick limit is the first obstacle. Primary cells cannot proliferate indefinitely because of cellular senescence. To overcome this limitation, several approaches have been used, such as expression of the simian vacuolating virus 40 large T (SV40T) antigen [Ozer et al., 1996; Jha et al., 1998; Fukuda et al., 2012] and human telomerase reverse transcriptase (hTERT) [Bodnar et al., 1998]. SV40T is an oncogenic protein that promotes cell cycle turn over. However, the expression of oncogenic proteins such as SV40T can cause genomic instability and/or chromosome abnormality in the

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host cells. Expression of hTERT was discovered to be essential for infinite cell division. Bodnar et al. (1998) reported that introduction of hTERT was sufficient to immortalize human dermal fibroblasts [Bodnar et al., 1998]. However, Itahana et al. (2003) showed that suppression of p16 tumor suppressor function was required for the immortalization of human embryonic fibroblasts, in addition to expression of hTERT [Itahana et al., 2003]. These results indicate that the essential conditions for cell immortalization differ depending on the cell type and tissue of origin. As an improved method for immortalization, Sasaki et al. (2009) reported that the expression of hTERT, cyclin D, and mutant CDK4 allows efficient immortalization of ovarian epithelial cells [Sasaki et al., 2009]. Furthermore, Shiomi et al. (2011) demonstrated the usefulness of the hTERT, cyclin D, and mutant CDK4 immortalization method in human myogenic cells [Shiomi et al., 2011]. Notably, a high percentage of the established cell lines maintained a normal karyotype pattern [Shiomi et al., 2011]. This evidence suggests that transfection with hTERT, cyclin D1, and mutant CDK4 can induce immortalization regardless of the cell/tissue origin.

In the present study, we establish immortalized macaque cell lines by expressing the genes for hTERT, mutant CDK4, and cyclin D1 in *Macaca fascicularis* and *Macaca fuscata*. The introduced genes were controlled by the tetracycline-regulated gene repression (Tet-OFF) system. The efficient establishment of macaque-derived cell lines will provide an alternative to animal experiments and contribute to animal welfare.

MATERIALS AND METHODS

ISOLATION AND CULTURE OF MACAQUE FIBROBLAST CELLS

Primary Japanese macaque fibroblast cells (NC1 primary cells) and crab-eating macaque fibroblast cells (RC1 primary cells) were isolated from the muscle tissues of a Japanese macaque (*M. fuscata*, 12-year-old male) and crab-eating macaque (*M. fascicularis*, 12-year-old female). The experiments were conducted under the guidelines of the Animal Care Committee of the Sapporo Medical University (approval number: 12–066). To culture primary cells, small muscle tissues were digested with dispase and plated on 100-mm cell culture dishes (BD Biosciences, Franklin Lakes, NJ) coated with atelocollagen (Koken, Tokyo, Japan). The primary cell cultures were maintained in DMEM (Nacalai Tesque, Kyoto, Japan) containing 10% fetal bovine serum (FBS) and 1% antibiotics (Nacalai Tesque).

VIRAL VECTOR CONSTRUCTION AND VIRAL TRANSDUCTION

Construction of the lentiviral vector plasmids CSII-CMV-Tet-Off, CSII-TRE-Tight-cyclin D1, and CSII-TRE-Tight-CDK4R24C was previously described [Shiomi et al., 2011]. In brief, the EF1α promoter in CSII-EF-RfA (a gift from Dr. H. Miyoshi, RIKEN) was replaced with a tetracycline-inducible promoter, TRE-Tight, from pTRE-Tight (Clontech, Mountain View, CA) to generate CSII-TRE-Tight-RfA. Human cyclin D1, human mutant CDK4 (CDK4R24C: an INK4a-resistant form of CDK4), and hTERT were inserted into the entry vector via a BP reaction (Invitrogen, Carlsbad, CA). These segments were then recombined with CSII-TRE-Tight-RfA through an LR reaction (Invitrogen) to generate CSII-TRE-Tight-cyclin D1, CSII-TRE-Tight-CDK4R24C, and CSII-TRE-Tight-hTERT. The rtTA segment from pTet-Off Advanced (Clontech) was amplified by PCR, recombined with the donor vector pDONR221 via a BP reaction (Invitrogen) to generate pENTR221-Tet-Off, and then recombined with a lentiviral vector, CSII-CMV-RfA, through an LR reaction (Invitrogen) to generate CSII-CMV-Tet-Off. Recombinant lentiviruses with vesicular stomatitis virus G glycoprotein were produced as described previously [Miyoshi, 2004]. NC1 primary cells and RC1 primary cells at passage number two were seeded at a density of 5×10^4 cells/well in a 12-well plate and inoculated with CSII-CMV-Tet-Off, CSII-TRE-Tight-cyclin D1, and CSII-TRE-Tight-CDK4R24C lentiviruses at a multiplicity of infection of five for each virus in the presence of 6 μg/mL of polybrene.

POPULATION DOUBLING ASSAY

Primary macaque cells and recombined macaque cells (NC1 Tet-Off cells and RC1 Tet-Off cells) were seeded at 1×10^5 cells/well in a 6-well plate (BD Biosciences). When the cells reached confluency, both primary cells and recombined cells were harvested and the total number of cells in each well was determined using Coulter automated cell counter (Invitrogen). Population doubling (PD) was used as the measure of the cell growth rate. PD was calculated from the formula $PD = \log_2(A/B)$, where A is the number of harvested cells and B is the number of plated cells [Fukuda et al., 2012; Qin et al., 2012]. Experiments were carried out in triplicate, and the average and standard deviation (SD) were calculated.

SA β-GALACTOSIDASE STAINING

To detect cellular senescence, senescence-associated β-galactosidase (SA-β-gal) staining was used (Senescence Detection Kit; BioVision, Milpitas, CA). Experiments were conducted at passage number 10 (NC1 primary cells and NC1 Tet-Off cells) and 5 (RC1 primary cells and RC1 Tet-Off cells). Primary macaque cells and recombined macaque cells were fixed with Fixative Solution for 10 min at room temperature. After the cells were washed twice with PBS, Staining Solution Mix was added and incubated overnight at 37 °C, following the manufacturer's protocol. Pictures of positively stained cells were captured with an inverted microscope (Nikon TS-100) and digital camera. The rate of positive staining was calculated as the mean ± SD from six randomly selected microscopy fields.

KARYOTYPE ANALYSIS OF IMMORTALIZED CELLS

Cells were treated with colcemid at a final concentration of 0.02 μg/mL the day before harvesting. After trypsinization, cells underwent hypotonic solution and fixed in Carnoy's fluid. Fixed cells were dropped onto a glass slide and stained using the G-banding stain method. Twenty metaphase cells were identified by karyotype analysis. The results obtained from this analysis are shown according to the International System for Human Cytogenetic Nomenclature (ISCN).

CONTROLLED IMMORTALIZATION WITH THE TET-OFF GENE EXPRESSION SYSTEM

The tetracycline-regulated gene repression system (Tet-Off system) has been widely used to control gene expression [Gossen and

Bujard, 1992]. In this system, the expression of the transfected genes in the target cells expected to decrease after the addition of doxycycline (DOX) to the cell culture medium. Cells were seeded at a density of 5×10^4 cells/well in a 6-well plate (BD Falcon) and cultured with or without 100 ng/mL DOX in DMEM containing 10% FBS and 1% antibiotics. Subsequent processes and analysis were performed the same way as described in the population doubling assay section.

IMMUNOBLOTTING ANALYSIS

Primary cells and Tet-Off cells cultured with or without DOX were homogenized in lysis buffer (1 M Tris-HCl, pH 7.4, 3 M NaCl, Triton X-100, sodium deoxycholate, protease inhibitor cocktail [Nacalai Tesque]). The detailed method for western blotting was described in our previous manuscript [Fukuda et al., 2008]. A mouse monoclonal antibody against human cyclin D1, a mouse polyclonal antibody against human CDK4 (1:1000; BD Biosciences), and a mouse monoclonal antibody against TetR (1:1000; Clontech) were used as the primary antibodies. The secondary antibody used was sheep anti-mouse IgG conjugated to horse radish peroxidase (1:2000; GE Healthcare, Little Chalfont, Buckinghamshire, UK). Signals on the membranes were detected using an ECL kit (GE Healthcare) and scanned with an ImageQuant LAS-4000 Mini system (GE Healthcare).

TELOMERASE DETECTION ASSAY

In this study, we detected telomerase activity according to the telomeric repeat amplification protocol (TRAP) method using a TRAPeze® Telomerase Detection Kit (Millipore, Billerica, MA). We obtained cell extracts from established cells cultured in 6-well plates (BD Falcon). Cell extract from 293 T cells were used as a positive control. The telomerase extension reaction was performed at 37 °C for 90 min. PCR and polyacrylamide gel electrophoresis (PAGE) were conducted according to the manufacturer's recommendations. To visualize, the gel was stained with GelRed™ (Biotium, Hayward, CA), and the image was captured by using a UV transilluminator at 254 nm.

STATISTICAL ANALYSIS

To test for statistical differences, we used the Mann–Whitney U test, which measures the distribution difference of two independent samples in nonparametric mode. A *P*-value less than 0.05 was considered statistically significant.

RESULTS

IMMORTALIZED MACAQUE CELLS SHOW HIGH PD VALUES COMPARED WITH PRIMARY CELLS

Primary macaque cells were enlarged (Fig. 1A upper left panel and lower left panel) as a morphological feature after primary culture. NC1 Tet-Off cells and RC1 Tet-Off cells expressing CDK4R24C, cyclin D1, and hTERT were smaller and more spindle-shaped than primary cells (Fig. 1A, upper right panel and lower right panel). To evaluate the cell proliferation rate, we measured the population doubling value. Figure 1B shows the population doubling rates of NC1 primary cells (diamonds) and NC1 Tet-Off cells (squares). NC1 Tet-Off cells proliferated more rapidly than NC1 primary cells, with a statistically significant difference between NC1 Tet-Off cells and NC1 primary cells after the first measurement. Increased cellular proliferation was also detected in RC1 Tet-Off cells, when compared with proliferation in RC1 primary cells (Fig. S1). These results suggest that the introduction of CDK4R24C, cyclin D1, and hTERT genes can be used to establish cell lines even from relatively old monkeys (approximately 12 years old).

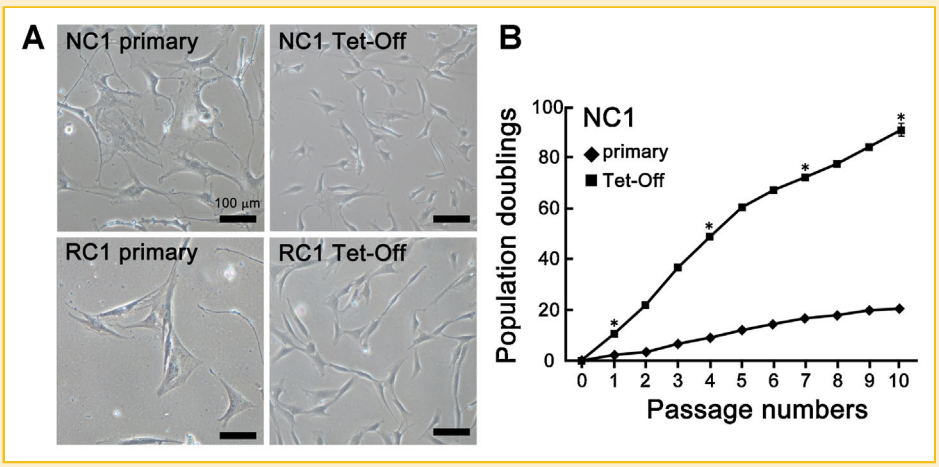


Fig. 1. The cell morphology and population doubling of recombined nonhuman primate-derived cells and the corresponding primary cells. (A) Cellular morphology of primary macaque cells and established cell lines. The size of NC1 primary cells (upper left panel) and RC1 primary cells (lower left panel) was enlarged, indicative of cellular senescence. NC1 Tet-Off cells (upper right panel) and RC1 Tet-Off cells (lower right panel) established by the introduction of CDK4R24C, cyclin D1, and hTERT were smaller, with a more spindle-shaped morphology, when compared with primary cells. Scale bars: 100 μm. (B) The results of the population doubling assays in NC1 primary cells and NC1 Tet-Off cells. The mean values for NC1 primary cells (diamonds) and NC1 Tet-Off cells (squares) were plotted. Triplicate samples were assessed at each measurement point to obtain the standard deviation. *$P < 0.05$.

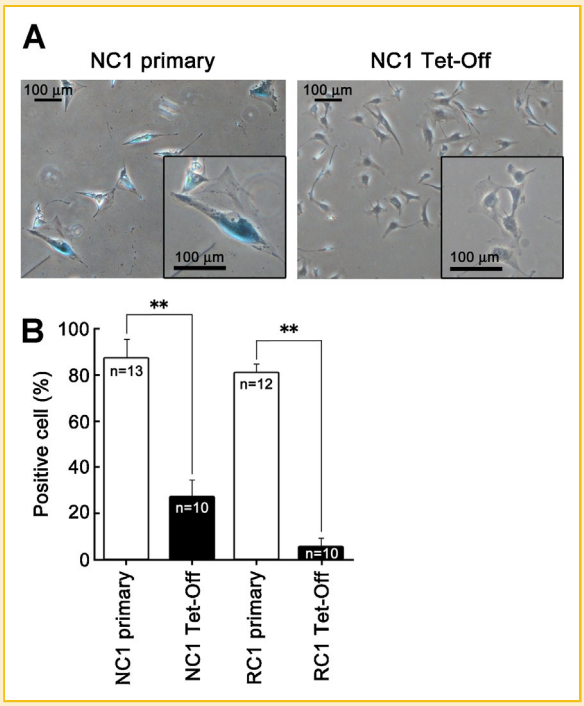


Fig. 2. Detection of cellular senescence with SA-β-gal staining in recombined and primary cells. (A) SA-β-gal staining for the detection of cellular senescence. Results in NC1 primary cells (left panel) and NC1 Tet-Off cells (right panel) are shown. SA-β-gal-positive staining is shown in blue in NC1 primary cells. Scale bars: 100 μm. (B) Percentage of SA-β-gal-positive primary cells and Tet-Off cells. $^{**}P < 0.01$.

DETECTION OF CELLULAR SENESCENCE

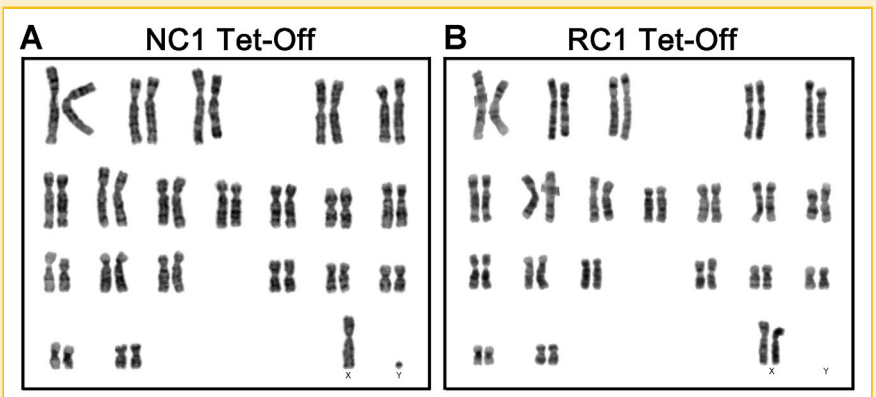
Positive staining for SA-β-gal is characteristic of senescent cells [Dimri et al., 1995]. After the population doubling assay, we detected SA-β-gal activity in each cell line. The majority of NC1 primary cells showed positive staining (Fig. 2A, left panel), but the rate of positive staining in NC1 Tet-Off cells was low (Fig. 2A, right panel). The positive rate was also low in RC1 Tet-Off cells (Fig. S2). To determine the ratio of cells with SA-β-gal positive staining, we counted 10 to 13 microscopy fields. The results showed a statistically significant difference between primary cells and Tet-Off cells (Fig. 2B). These results demonstrated that expression of the CDK4R24C, cyclin D1, and hTERT genes allowed the cells to bypass cellular senescence.

KARYOTYPE ANALYSIS

To determine the karyotype of NC1 Tet-Off cells and RC1 Tet-Off cells, we analyzed the cells using the G-band method. NC1 Tet-Off cells (Fig. 3A) and RC1 Tet-Off cells (Fig. 3B) had normal 42XY diploid and 42XX diploid karyotypes, respectively. Although 100% of NC1 Tet-Off cells had the normal karyotype of *M. fuscata*, 10% of RC1 Tet-Off cells (2/20 cases) had a chromosomal abnormality (fusion of chromosome at 14p12) (Figs. 3C and S3). The results of our study showed that combined expression of cyclin D1, mutant CDK4, and hTERT allowed us to establish cell lines with intact karyotypes.

SUPPRESSION OF IMMORTALIZATION GENES WITH DOXYCYCLINE

We expressed mutant CDK4, cyclin D1, and hTERT using the tetracycline-regulated gene repression (Tet-OFF) system in NC1 Tet-



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Cell line	Diploid/Total (%)	Chromosome number 41	42	43	Others
NC1 Tet-Off	20/20 (100)	ND	20/20	ND	
RC1 Tet-Off	20/20 (100)	ND	20/20	ND	2/20 add(14)(p12)

Fig. 3. Karyotype analysis in established nonhuman primate cells. (A) and (B) Representative results of the karyotype analysis. NC1 Tet-Off cells (A) and RC1 Tet-Off cells (B) showed 2n = 42 normal karyotypes. (C) Ratio of diploid and normal karyotype in NC1 Tet-Off cells and RC1 Tet-Off cells. In RC1 Tet-Off cells, a chromosome fusion abnormality was observed in two cases.

Off cells and RC1 Tet-Off cells. To determine the role of cell cycle drivers in the continuous proliferation of these cells, we examined the expression of CDK4R24C and cyclin D1 in the presence of DOX. Cyclin D1 and CDK4R24C were not detected in primary cells (Fig. 4A, lane 1 and 5), but they were detected in Tet-Off cells treated with vehicle (Fig. 4A, lanes 2 and 6). CDK4 and cyclin D1 expression dramatically decreased at 2 days and 4 days after the administration of 100 ng/mL DOX (Fig. 4A, lanes 3, 4, 8, and 9). After the removal of DOX and the replacement of fresh medium, CDK4 and cyclin D1 protein levels recovered (Fig. 4, lanes 5 and 10). These cell cycle regulators are controled Tet repressor (TetR) in Tet-Off cells. We detected TetR expression in Tet-Off cells (Fig. 4B, lanes 1, 3 to 5, and 7 to 10), but not primary cells (Fig. 4B, lanes 2 and 6).

We also evaluated the effect of DOX on cell proliferation. Cell numbers in the presence of DOX were significantly lower when compared with cell numbers in the presence of vehicle (Fig. 5A). Furthermore, Tet-Off cells treated with DOX exhibited characteristics of cellular senescence, such as a larger cell volume (Fig. 5B). From these results, we concluded that DOX administration suppressed the introduced transgenes, resulting in slower cell proliferation, similar to that observed in the primary cells.

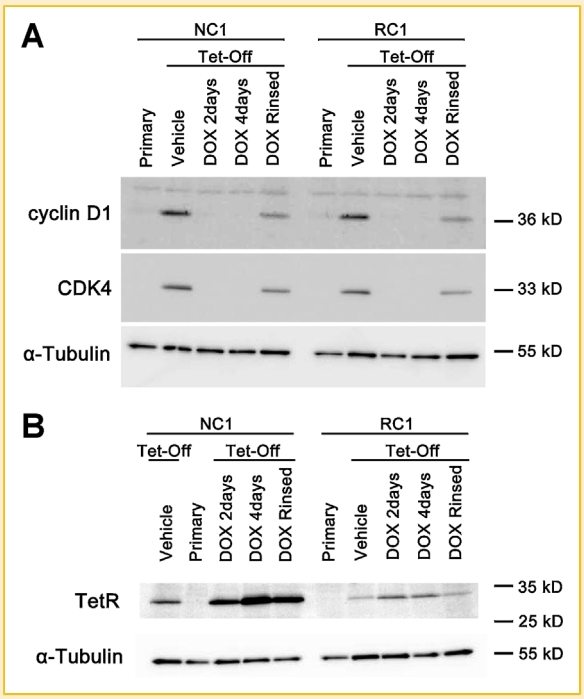

Fig. 4. Immunoblotting analysis for detection of cell cycle regulators. (A) Detection of cyclin D1 and CDK4 under the control of doxycycline (DOX). Lanes 1 and 6, untreated NC1 primary cells and RC1 primary cells, respectively; lanes 2 and 7, NC1 Tet-Off cells and RC1 Tet-Off cells, respectively, treated with vehicle; Lane 3 and 8, NC1 Tet-Off cells and RC1 Tet-Off cells, respectively, treated with DOX for 2 days; lanes 4 and 9, NC1 Tet-Off cells and RC1 Tet-Off cells, respectively, treated with DOX for 4 days; lanes 5 and 10, NC1 Tet-Off cells and RC1 Tet-Off cells, respectively, cultured in fresh medium after treatment with DOX for 4 days. (B) Detection of TetR protein between primary cells and Tet-Off cells. Lanes 2 and 6, NC1 primary cells andRC1 primary cells, respectively; lanes 1 and 3 to 5, NC1 Tet-Off cells respectively; lanes 7 to 10, RC1 Tet-Off cells respectively.

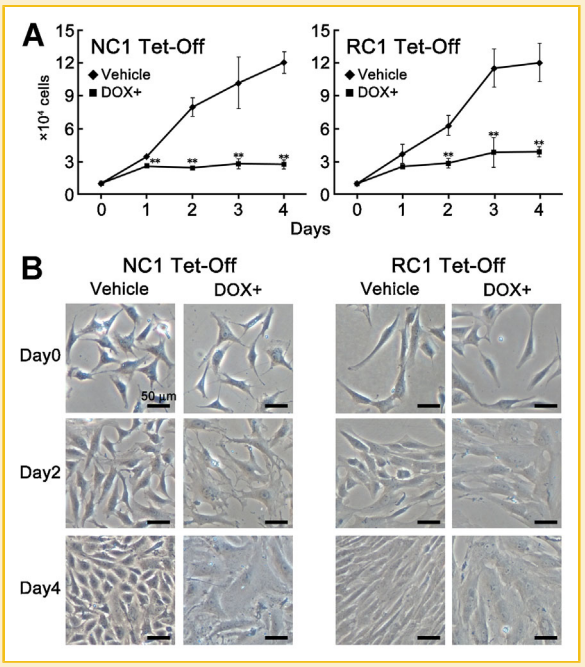

Fig. 5. Cell growth of NC1 Tet-Off cells (left) and RC1 Tet-Off cells (right) in the presence of DOX. (A) The mean cell number with (squares) and without (diamonds) 100 ng/mL DOX was plotted. Triplicate samples were assessed at each time point (1, 2, 3, and 4 days) to obtain the mean and standard deviation. $^{**}P < 0.01$. (B) Cell morphology of NC1 Tet-Off cells (left) and RC1 Tet-Off cells (right) in the presence of DOX. Representative pictures are shown for day 0, day 2, and day 4 with vehicle (left) and DOX (right) treatments. Scale bars: 50 μm.

DETECTION OF TELOMERASE ACTIVITY

hTERT was also expressed under the control of the Tet-Off system, and we assessed telomerase activity with the TRAP assay. In the electrophoresis pattern for 293 T cells, we detected a 6-bp ladder (Fig. 6, lane 2, positive control), which is indicative of telomerase activity. Notably, telomerase activity was also detected in both of the established recombined cell lines (Fig. 6, lanes 3, 4, 5, and 6). Although we observed nonspecific amplification in the negative control (Fig. 6, lane 1, negative control), the electrophoretic pattern was different, indicating the specificity of the detection method. From these results, we concluded that the established cell lines had telomerase activity.

DISCUSSION

In the present study, we established two macaque cell lines by introducing three genes, encoding CDK4R24C, cyclin D1, and hTERT. Both of the macaque cell lines established escaped senescence. In addition, the monkey cells showed increased cell proliferation, whereas primary cultured monkey cells showed decreased cell proliferation after sequential passages.

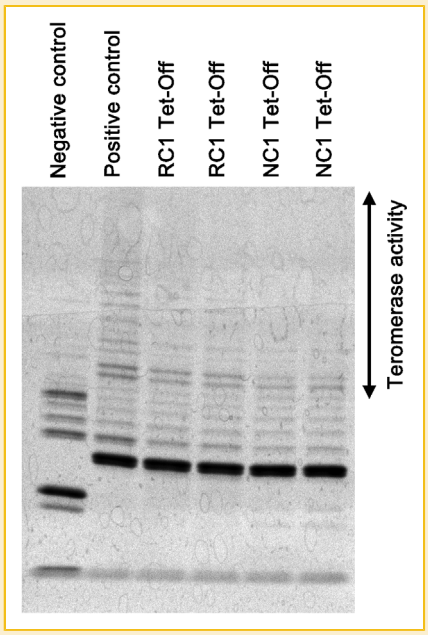

Fig. 6. Detection of telomerase activity with the TRAP assay. Lane 1, CHAPS buffer as a negative control; lane 2, 293T cells as a positive control; lanes 3 and 4, NC1 Tet-Off cells; lanes 5 and 6, RC1 Tet-Off cells. The double-headed arrow indicates the 6-bp ladder resulting from telomerase activity.

Nonhuman primates, such as the Japanese macaque (*M. fuscata*) and crab-eating macaque (*M. fascicularis*), have been used as experimental animals because of their similarity to humans [Daniel et al., 1985; Kanki et al., 1985]. For the safety evaluation of new vaccines and therapeutic drugs for human immunodeficiency virus, nonhuman primate models are frequently used before clinical trials in humans [Letvin, 1990], However, experiments in nonhuman primary models are expensive. Furthermore, from the standpoint of animal welfare, the replacement of animal models with in vitro models is encouraged, in accordance with guidelines for animal experiment practices based on the 3Rs (replacement, reduction, and refinement) [Hobson-West, 2009]. Another point to consider is that nonhuman primate models are not genetically regulated. Consequently, the results of experiments in nonhuman primate models exhibit relatively wide variation, when compared to the results from experiments in small rodents with a uniform genetic background, such as inbred strains or closed colonies. Established cell lines provide experimental material with a uniform genetic background, especially when the cells have been immortalized.

There are several approaches for the establishment of cell lines, including the introduction of hTERT or oncogenic proteins, such as SV40T or E6 and E7 proteins derived from the human papilloma virus. Although expression of SV40T is effective for establishing cell lines [Ozer et al., 1996; Jha et al., 1998], prolonged expression has been reported to cause chromosomal abnormalities [Ray et al., 1990]. In our previous study, we reported that approximately 16% of pig embryonic fibroblast cells established by SV40T expression showed chromosomal aneuploidy at passage 10 [Fukuda et al., 2012]. In the present study, we established cell lines from macaque fibroblast cells, and a relatively high percentage of NC1 Tet-Off cells and RC1 Tet-Off cells maintained a normal karyotype (NC1 Tet-Off cells: 100%, RC1 Tet-Off cells: 90%), when compared with the percentage observed with oncogenic protein expression. A chromosome fusion abnormality was observed in this study, but polyploidy was not detected. Polyploidy is induced by chromosomal instability, which is thought to have a large effect on the malignant transformation of cells [Kahyo et al., 2011]. From these situations, we concluded that combined expression of hTERT, cyclin D1, and mutant CDK4 (CDK4R24C) in nonhuman primate cells maintains the original karyotype of the primary cells more accurately than expression of oncogenic proteins.

Cellular senescence hinders the establishment of cell lines. Although we introduced human-derived genes for cyclin D1, CDK4R24C, and hTERT, we observed increased proliferation in both of the nonhuman primate cell lines. At the amino acid level, the homology between human and monkey proteins is high for CDK4 and cyclin D1: 100% for CDK4 and 99% for cyclin D1 from *M. mulatta*; 99% for CDK4 and 99% for cyclin D1 from *M. fascicularis*. This suggests that the human-derived genes encode proteins that can form complexes with endogenous nonhuman primate molecules and function properly. Given the high level of homology, cellular immortalization might be possible in nonhuman primate cells. We detected telomerase activity in NC1 Tet-Off and RC1 Tet-Off cells (Fig. 6, lanes 3, 4, 5, and 6), indicating that hTERT functioned normally in monkey cells by virtue of its compatibility with endogenous subunits, such as monkey telomere RNA component and dyskerin.

Our study showed that CDK4R24C, cyclin D1, and hTERT expression can be used to immortalize cells from other species, in addition to human cells. The establishment of immortalized cell lines from a wide variety of species will contribute to our understanding of molecular evolution and genetic diversity.

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