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Bovine and porcine fibroblasts can be immortalized with intact karyotype by the expression of mutant cyclin dependent kinase 4, cyclin D, and telomerase

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ABSTRACT

Cattle and pigs comprise the most economically important livestock. Despite their importance, cultured cells from these species, which are useful for physiological analyses, are quite limited in cell banks. One of the reasons for the limited number of cell lines is the difficulty in their establishment. To overcome limitations in cell-line establishment, we attempted to immortalize bovine and porcine fibroblasts by transduction of multiple cell cycle regulators (mutant cyclin dependent kinase 4, cyclin D and telomerase reverse transcriptase). The transduced cells continued to display a stable proliferation rate and did not show cellular senescence. Furthermore, cell cycle assays showed that induction of these exogenous genes enhanced turnover of the cell cycle, especially at the G1-S phase. Furthermore, our established cell lines maintained normal diploid karyotypes at 98–100%. Our study demonstrated that bypassing p16/Rb-mediated cell arrest and activation of telomerase activity enabled efficient establishment of immortalized bovine- and porcine-derived fibroblasts. The high efficiency of establishing cell lines suggests that the networks of cell cycle regulators, especially p16/Rb-associated cell cycle arrest, have been conserved during evolution of humans, cattle, and pigs.

1. Introduction

In 1961, Hayflick described the limitation of cell division in primary cells (known as the Hayflick limit) (Hayflick and Moorhead, 1961). Primary cells cannot continue cell division infinitely. When primary cells reach their Hayflick limit, the cells show a phenotype called cellular senescence (Campisi and d'Adda di Fagagna, 2007). However, cancer-derived cells such as HeLa and 293T cells can proliferate infinitely and do not show cellular senescence (Graham et al., 1977; Scherer et al., 1953). Cellular proliferation beyond the Hayflick limit is defined as immortalization. At the beginning of a primary culture, the Hayflick limit is a major restriction for establishment of cell lines. To overcome this limitation, various types of methods have been studied, and continue to be studied.

In previous studies, the introduction of oncogenic proteins such as simian vacuolating virus 40 (SV40) T antigen or human papillomavirus-derived E6/E7 protein have been reported to be effective for the establishment of cell lines (Tsao et al., 2002). SV40T antigen and E6/E7 are known to bind to negative cell cycle regulators such as p53 and the retinoblastoma gene product (pRb), which are known to act as tumor suppressor proteins in the genome (Shay et al., 1991). Furthermore, strong telomerase activity has been reported to be essential for the immortalization phenotype of

Abbreviations: Rb, retinoblastoma; SV40, Simian vacuolating virus 40; hTERT, human telomerase reverse transcriptase; CDK4, cyclin-dependent kinase 4; BFFs, bovine fetal fibroblasts; PEFs, pig embryonic fibroblasts; CMV, cytomegalovirus; VSV-G, vesicular stomatitis virus G glycoprotein; PDL, population doubling level; SA-β-Gal, senescence-associated β-galactosidase; TERC, telomerase RNA component; Bmi-1, B cell-specific Moloney murine leukemia virus integration site 1.

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cells (Counter et al., 1992). The combination of human telomerase reverse transcriptase (hTERT) and oncogenic protein expression have been reported to efficiently immortalize human primary cells (Tsuruga et al., 2008). However, the expression of these oncogenic proteins can induce genomic instability such as polyploidy and other chromosomal abnormalities (Duensing et al., 2000; Ray et al., 1990). Furthermore, oncogenic proteins may affect the biological characteristics of the original primary cells. To overcome the limitation of oncogenic proteins, Shiomi et al. (2011) conducted cellular immortalization by expression of mutant cyclin-dependent kinase 4 (CDK4), cyclin D, and hTERT. Notably, the expression of these three genes allows efficient establishment of human immortalized myogenic cell lines that maintain their original phenotype.

Cattle and pigs are the most economically important animals among livestock. The economic value of livestock depends on various factors, such as meat quality. For example, the grade of fat marbling in meat has a huge impact on market meat prices, but the underlying molecular mechanisms are not well understood. As an experimental model for fat cell differentiation, 3T3-L1 mouse fibroblasts have been used in previous studies. 3T3-L1 cells can be efficiently induced to adipocytes by treatment with isobutylmethylxanthine, insulin, and dexamethasone (Rubin et al., 1978). Interestingly, primary bovine fibroblasts cannot differentiate into adipocytes in the same manner. This observation suggests that understanding the difference of cellular and genetic backgrounds between 3T3-L1 and bovine fibroblasts is important to elucidate the underlying mechanisms of adipocyte differentiation. To study cellular differentiation in livestock, we need to efficiently establish fibroblasts or pre-adipocytes, because bovine- and porcine-derived cells are quite limited in public cell banks such as the American Type Culture Collection. Furthermore, the cost for sampling to start a primary culture is another limitation in studies of livestock.

These factors lead us to the hypothesis that expression of mutant CDK4, cyclin D1, and hTERT might efficiently establish bovine- and porcine-derived immortalized cells with the intact phenotype of the primary cells. As far as we know, our study is the first report of successful immortalization with intact karyotypes in livestock derived cells. The application of this immortalization protocol might facilitate cellular experiments in livestock and contribute to the welfare of farm animals.

2. Materials and methods

2.1. Preparation of bovine and porcine fibroblasts

Both bovine fetal fibroblasts (BFFs) and pig embryonic fibroblasts (PEFs) were maintained in the laboratory of animal breeding and genetics, Tohoku University Graduate School of Agricultural Science. They were cryopreserved in liquid nitrogen until use. Detailed methods to obtain the primary cultured fibroblasts are described in our previous paper (Fukuda et al., 2012).

2.2. Lentiviral vector construction and establishment of immortalized cell lines

To immortalize BFFs and PEFs, CSII-CMV-hTERT, CSII-CMV-cyclin D1, and CSII-CMV-hCDK4R24C were introduced into each cell line. The preparation and recombination of lentiviral construction have been described previously (Sasaki et al., 2009). The production of recombinant lentiviruses with vesicular stomatitis virus G glycoprotein (VSV-G) are also described in a previous study (Miyoshi, 2004).

2.3. Cell culture

Cell culture medium consisted of Dulbecco's modified Eagle's medium (cat. no. 08459-35, Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (cat. no. 12483-020, Invitrogen, Carlsbad, CA) and 100× antibiotic–antimycotic mixed solution (cat. no. 02892-54, Nacalai Tesque). All cells were maintained at 37 °C with 5% CO_2.

2.4. F-actin staining (phalloidin)

Bovine and porcine cells were seeded at densities of 5×10^4 and 1×10^5 cells in a 35 mm glass based dish (cat. no. 3910-035, AGC TECHNO GLASS, Shizuoka, Japan). Glass surface were coated with Atelocollagen solution (1:100 dilution, cat. no. IPC-50, KOKEN, Tokyo, Japan). After 48 h incubation, F-actin staining was carried out with Alexa Fluor 546 Phalloidin (1:100 dilution, cat. no. A22283, Invitrogen) in accordance with the manufacturer's protocol. Fluorescence micrograph was captured by BZ-8100 (Keyence, Osaka, Japan).

2.5. Population doubling level assay

Bovine and porcine cells were seeded at densities of 5×10^4 and 1×10^5 cells per well in a 6-well plates, respectively. Cells were passaged when one well of the 6-well plate became confluent. The cell number was counted using an automatic cell counter (Countess, Invitrogen), and cell growth was evaluated in triplicate experiments. Passaging was repeated until senescence. The population doubling level (PDL) was obtained from the number of cells using the formula as follows: $PDL = \log_2(a/b)$, where "a" is the number of cells counted in the passage and "b" represents the number of seeded cells (Qin et al., 2012). The results of the PDL assay were plotted as means of triplicate samples.

2.6. Western blotting

To obtain total protein extract, cells were lysed in a solution containing 50 mM Tris–HCl (pH 7.4), 0.15 M NaCl, 1% Triton X-100, 2.5 mg ml^{-1} sodium deoxycholate (cat. no. 194-08311, Wako Pure Chemical Industries, Osaka, Japan), and a protease inhibitor cocktail (1:200 dilution, cat. no. 25955-11, Nacalai Tesque). Cell lysates were separated by SDS-PAGE and then transferred to hydrophobic polyvinylidene fluoride membranes (cat. no. IPVH00010, Merck Millipore Corporation, Billerica, MA). After the membranes were blocked with 3% nonfat dry milk/PBS with 0.05% Tween 20, they were probed with mouse anti-human cyclin D1 (1:4000 dilution, cat. no. 554180, BD Biosciences, Franklin Lakes, NJ), mouse anti-human CDK4 (1:4000 dilution, cat. no. 610147, BD Biosciences), and mouse anti-α-tubulin (1:1000 dilution, cat. no. sc-32293, Santa Cruz Biotechnology, Inc. Dallas, TX) antibodies. The blots were then incubated with horseradish peroxidase-conjugated sheep anti-mouse IgG (1:2000 dilution, cat. no. NA931V, GE Healthcare, Buckinghamshire, UK). Immunoreactive proteins were detected using an ImageQuant LAS-4000 mini (GE Healthcare) with enhanced chemiluminescence (cat. no. NCI3109, Thermo Scientific, MA).

2.7. Telomerase assay

Telomerase activity was measured using a Telochaser (Toyobo, Osaka, Japan) based on the stretch-PCR method. According to the manufacturer's protocol, cell extracts were obtained from 1×10^5 trypsinized cells. After 30 min of telomerase extension at 30 °C, PCR was performed under the following conditions: 2.5 min of denaturation at 95 °C, followed by 26 cycles of 30-s denaturation at 95 °C,