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Short communication

Arm-specific telomere dynamics of each individual chromosome in induced pluripotent stem cells revealed by quantitative fluorescence *in situ* hybridization

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ABSTRACT

We have reported that telomere fluorescence units (TFUs) of established induced pluripotent stem cells (iPSCs) derived from human amnion (hAM933) and fetal lung fibroblasts (MRC-5) were significantly longer than those of the parental cells, and that the telomere extension rates varied quite significantly among clones without chromosomal instability, although the telomeres of other iPSCs derived from MRC-5 became shorter as the number of passages increased along with chromosomal abnormalities from an early stage. In the present study we attempted to clarify telomere dynamics in each individual chromosomal arm of parental cells and their derived clonal human iPSCs at different numbers of passages using quantitative fluorescence in situ hybridization (Q-FISH). Although no specific arm of any particular chromosome appeared to be consistently shorter or longer than most of the other chromosomes in any of the cell strains, telomere elongation in each chromosome of an iPSC appeared to be random and stochastic. However, in terms of the whole genome of any specific cell, the telomeres showed overall elongation associated with iPSC generation. We have thus demonstrated the specific telomere dynamics of each individual chromosomal arm in iPSCs derived from parental cells, and in the parental cells themselves, using Q-FISH.

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1. Introduction

Normal human cells exhibit a limited capacity for proliferation in culture (Hayflick, 1965). This phenomenon is considered to be attributable to reduction of telomere length as an indicator of the number of divisions a cell has undergone. Telomeres protect chromosomes against degeneration, reconstruction, fusion and loss, as well as contributing to pairing of homologous chromosomes (Blackburn, 1991). Telomeres are repetitive G-rich DNA sequences found at the ends of linear eukaryotic chromosomes and appear to play a key role in preventing genomic instability (Blackburn, 2001). The end-to-end chromosome fusions observed in some tumors could play a role in genetic instability associated with tumorigenesis, and may be the result of telomere loss (Blackburn, 1991). The human telomere is a simple repeating sequence of six bases, TTAGGG, located at the ends of chromosomes (Moyzis et al., 1988). Telomere lengths of cultured fibroblasts (Takubo et al., 2010) and human tissues (Aida et al., 2008) show marked heterogeneity among individual telomeres, and in terms of mean or median values in individual cells. Heterogeneity of telomere length and telomerase expression has also been reported previously among induced pluripotent stem cells (iPSCs) and cultured fibroblasts (Wang et al., 2012). In the present study we attempted to clarify telomere dynamics in each individual chromosomal arm of both parental cells and their derived clonal human iPSCs after different numbers of passages using quantitative fluorescence in situ hybridization (Q-FISH).

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Q-FISH and image analyses were performed as described previously. The Q-FISH method we have developed and employed is highly accurate and reproducible, having already been used in a number of published studies (Izumiyama-Shimomura et al., 2014; Nakamura et al., 2014; Poon and Lansdorp, 2001a, 2001b; Takubo et al., 2010; Terai et al., 2013).

Previously we have measured individual telomere lengths of chromosomal arms in human fibroblast strains using Q-FISH to clarify the morphologic signs of chromosomal instability (Takubo et al., 2010). We have demonstrated a linear correlation between telomere fluorescence units (TFUs) estimated by Q-FISH and telomere length measured by Southern blotting (Takubo et al., 2010). We have reported that TFUs of iPSCs derived from human amnion (hAM933) and fetal lung fibroblasts (MRC-5) were significantly longer than those of the parental cells, and that the telomere extension rates varied quite significantly among clones without chromosomal instability, although the telomeres of other iPSCs derived from MRC-5 became shorter as the number of passages increased, along with chromosomal abnormalities from an early stage (Terai et al., 2013). Here, using Q-FISH, we investigated the specific telomere dynamics of each individual chromosomal arm in iPSCs cloned from parental cells, and also the parental cells from which they were derived, after different numbers of passages in culture.

2. Materials and methods

2.1. Ethics statement

Human amnion cells were collected by scraping tissue from surgical specimens, with signed informed consent from the donors concerned, and under ethical approval from the Institutional Review Board of the National Institute for Child Health and Development, Japan. The surgical specimens were irreversibly de-identified. All experiments involving the handling of human cells and tissues were performed in line with the tenets of the Declaration of Helsinki.

2.2. Human cell culture

Human amniotic membrane (hAM)-derived cells were independently established in our laboratory (Cui et al., 2007, 2011; Fukawatase et al., 2014; Makino et al., 2009; Nagata et al., 2009; Nishino et al., 2010, 2011). hAM-derived cells and MRC-5 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and penicillin–streptomycin. Human iPSCs were generated in our laboratory using the procedures described by Yamanaka and colleagues (Cui et al., 2007, 2011; Fukawatase et al., 2014; Makino et al., 2009; Nagata et al., 2009; Nishino et al., 2010, 2011; Takahashi and Yamanaka, 2006; Takahashi et al., 2007). The iPSCs were established from hAM-derived cells and MRC-5, and designated hAM933 iPSCs and MRC-5 iPSCs, respectively. Briefly, to produce VSV-G (vesicular stomatitis virus G glycoprotein) retroviruses, 293FT cells (Invitrogen) were plated at 2×10^6 cells per 10-cm culture dish in DMEM supplemented with 10% FBS, and incubated overnight. On the following day, the cells were co-transfected with pMXs-OCT4, SOX2, KLF4 or c-MYC, pCL-GagPol, and pHCMV-VSV-G vectors using the TransIT-293 reagent (Mirus Bio LLC, Madison, WI, USA). The virus-containing supernatants were collected 48 h after incubation. The supernatants were filtered through a 0.45-mm pore size filter, centrifuged, and then resuspended in DMEM supplemented with 4 mg/ml polybrene (Nakarai Tesque, Kyoto, Japan). The parental cells were seeded at 1×10^5 cells per well in 6-well Plates 24 h before infection. A 1:1:1:1 mixture of OCT4, SOX2, KLF4 and c-MYC viruses was then added to the parental cells (Cui et al., 2007, 2011; Fukawatase et al., 2014; Makino et al., 2009; Nagata et al., 2009; Nishino et al., 2010, 2011; Takahashi and Yamanaka, 2006; Takahashi et al., 2007). In a separate experiment, infection with the retrovirus carrying the EGFP gene was performed to estimate the infection efficiency. One half of the medium was changed every day, and colonies were picked up at around day 28. They were maintained on irradiated mouse embryonic fibroblasts (MEFs) in iPSellon medium (Cardio Incorporated, Kobe, Japan) supplemented with 10 ng/ml recombinant human basic fibroblast growth factor (bFGF, Wako Pure Chemical Industries, Ltd., Osaka, Japan). We used iPSCs that had been chosen randomly from stable lines in our laboratory.

We measured telomere lengths of each individual chromosomal arm in the parental cells (hAM933 and MRC-5), two iPSC lines (hAM933 iPSCs-2, hAM933 iPSCs-3) derived from different colonies originating from hAM933, and two iPSC lines (MRC-5 iPSCs-16 and MRC-5 iPSCs-40) cloned from the same iPSCs (MRC-5 iPSCs) at different numbers of passages (MRC-5 iPSCs-16; passages 22 and 59; MRC-5 iPSCs-40; passages 21 and 62).

We checked cell contamination by short tandem repeat (STR) genotyping of the parental cells (hAM933 and MRC-5), two iPSC lines (hAM933 iPSCs-2, hAM933 iPSCs-3) derived from different colonies originating from hAM933, and two iPSC lines (MRC-5 iPSCs-16 and MRC-5 iPSCs-40) cloned from the same iPSCs (MRC-5 iPSCs) after different numbers of passages (data not shown) (Cui et al., 2007, 2011; Fukawatase et al., 2014; Makino et al., 2009; Nagata et al., 2009; Nishino et al., 2010, 2011; Takahashi and Yamanaka, 2006; Takahashi et al., 2007). Genomic DNA was isolated from samples of the cultured cells using DNeasy columns (Qiagen). This DNA was used as a template for STR analysis employing the PowerPlex 16 System (Promega) and ABI PRISM instrumentation. Numbers shown denote the bp lengths of the 15 autosomal fragments. The analysis was carried out at BEX Co., Ltd.

2.3. Measurement of telomere lengths of individual arms in metaphase spreads using Q-FISH and image analysis

For karyotype analysis and quantitative analysis of telomeres, metaphase chromosomes were fixed and then hybridized using the peptide nucleic acid-FISH preparation method described previously (Poon and Lansdorp, 2001a; 2001b).

A Cy3-labeled $(CCCTAA)_3$ peptide nucleic acid probe (telo C) (Fasmac, Atsugi, Catalog No. F1002, Japan) was used to label the telomeres, and a FITC-labeled CTTCGTTGGAAACGGGGT peptide nucleic acid probe (CENP1; a non-specific centromere probe; Fasmac, custom made, Japan) was used for labeling the centromere. The chromosome preparations were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Molecular Probes, Eugene, OR, USA).

Analysis of fluorescence images was performed as described previously (Takubo et al., 2010). Digital images were recorded with a CCD camera, AxioCam MRm (Zeiss, Oberkochen, Germany), mounted on an Axio Imager M1 (Zeiss) epifluorescence microscope equipped with a triple band-pass filter for Cy3/FITC/DAPI (61010 Chroma Technology, Corp., Rockingham, VT, USA) and a 63× oil objective lens (Zeiss EC Plan-NEOFLUAR 63×/1.25 ∞/0.17). Microscope control and image acquisition were performed with the ISIS system (MetaSystems, GmbH, Altlussheim, Germany).

A calibration system was used to ensure reliable quantitative estimation of telomere length in the various samples. To correct for daily variations in lamp intensity and alignment, images of fluorescent beads (orange beads, size 0.2 μm, Molecular Probes Inc.) were imaged just prior to acquisition of images from the samples. The fluorescence intensities of the beads and the telomeres

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