ELSEVIER

Cell Biology International 31 (2007) 1257–1264

Cell Biology International

www.elsevier.com/locate/cellbi

Replicative senescence, telomere shortening and cell proliferation rate in Gaddi goat's skin fibroblast cell line

Neelam Gupta [a], Ritu Taneja [a], Alok Pandey [a], Manishi Mukesh [a], Hari Singh [b], S.C. Gupta [a,*]

[a] *Transgenic Research Laboratory, DNA Fingerprinting Unit, National Bureau of Animal Genetic Resources, Karnal 132001, Haryana, India*
[b] *Department of Biochemistry, Kurukshetra University, Kurukshetra, Haryana, India*

Received 3 February 2007; revised 3 May 2007; accepted 12 May 2007

Abstract

We assessed aging in continuous donor skin fibroblast cell line GGM5 up to the 25th passage by *in vitro* replicative senescence, telomere dynamics and chromosomal abnormalities. Cell proliferation rate increased from 0.84 ± 0.26 (primary cells) to 1.20 ± 0.17 (13–15 passage group) per day and reduced to 0.65 ± 0.14 in 22–25 passages. Cell proliferation rate was reduced by 45.7% after 87.62 CPDs. Cell viability reduced from 100% to 97.4% up to the 25th passages. Frequency of β gal^+ cells increased in successive passages and days in culture. The correlation coefficient between frequency of β gal^+ cells and growth rate was −0.50 to −0.61. Loss of mean TRF length was 13.8 nucleotides (passage 15) to 95.4 nucleotides per cell division in later passages. All cells showed Robertsonian translocation in 22–25 passaged cells. The SCNT pre-implantation embryos production was highest (22.5%) in donor cells used from 10–15 passages as compared to early (≤5) and late (22–25 passages). Our findings supports that cell proliferation rates, β gal staining, mean TRF loss and karyological profile are useful marker for evaluation of competent nuclear donor.

Keywords: Aging; Senescence; Robertsonian translocation; Cell proliferation; Goat; Telomere

1. Introduction

Primary cells that are grown in culture do not proliferate indefinitely and after a period of rapid proliferation, their division rate slows down, ultimately ceasing altogether (Hayflick, 1965). Such cells become unresponsive to mitogenic stimuli, but can remain viable for extended periods of time because of senescence (Goldstein, 1990; Dimri et al., 1995). The shift is accompanied by changes in nuclear structure, gene expression, protein processing, and metabolism (Witkowski, 1985). *In vivo* senescence serves as a mechanism that limits the proliferative capacity of normal cells, avoiding incorporation of cellular changes, degradation, and chromosomal deformities because of further cell divisions (Millis et al., 1989). *In vitro*, senescent cells show selected cell specific changes in structure, remain metabolically active, resisting apoptotic death for long period of time and can be detected by β-galactosidase staining (Dimri et al., 1995).

Telomeres play an important role in genomic stability (Hande et al., 1999). Telomeres shorten during each cell division by 50–100 bp owing to the end replication problem of DNA polymerase, which is because of suppression of telomerase activity in somatic cells (Velicescu et al., 2003). Thus, the life span of primary human cells is limited in terms of number of cell divisions depending on the telomere length of cells in starting cultures (Allsopp et al., 1992). When telomeres reach a critical shortening in length, senescence is induced, resulting into permanent proliferation arrest and typical changes in cell morphology (Allsopp et al., 1992; Schwartz et al., 2001). Telomeres are essential for complete replication and telomere shortening leads to genomic instability (Greider and

* Corresponding author. Tel.: +91 184 228 3891/91 989 622 6228; fax: +91 184 226 7654.
E-mail address: guptasc@yahoo.com (S.C. Gupta).

1065-6995/$ - see front matter © 2007 International Federation for Cell Biology. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.cellbi.2007.05.004

Blackburn, 1996; Velicescu et al., 2003). Intact telomeres enable cells to distinguish chromosome ends from double strand breaks (DSB) in the genome, while uncapped chromosomal ends are at great risk for degradation, recombination or fusion by cellular DNA repair system (Zneimer et al., 2000; Velicescu et al., 2003). Chromosome degradation causes loss of genetic information, and if unchecked can leads to cell death (Kim et al., 2002). Senescence and telomere shortening and resulting chromosomal aberrations are ultimately reflected in reduced cell proliferation rate and cell viability (Gupta et al., 2003). *In vitro* embryo production, particularly by SCNT, is associated with compromised pre- and post-implantation development (Mastromonaco et al., 2006). In order to improve SCNT success, careful attention is required to the establishment of viable, normal cell lines since good quality embryos depend upon the chromosomal integrity of the donor cell (Slimane Bureau et al., 2003).

Our study attempted to evaluate the process of *in vitro* aging in terms of cell proliferation rates, degree of senescence, telomere size and chromosomal stability of goat skin fibroblast cell line during multiple passaging, for developing competent donor cells for SCNT experiment of goats.

2. Materials and methods

2.1. Cell line

The skin tissues (1 cm × 1 cm) of ear pinna of adult Gaddi goats (2 years of age) were collected by biopsy from its main breeding tract in Himachal Pradesh. The skin samples were brought aseptically to the laboratory in complete media (DMEM + Ham's F12 supplemented with 15% FBS -Hyclone, SH30071.03), L-glutamine and antibiotics (Sigma) at 4 °C. Primary cultures were harvested on 15th day after attaining 70–80% confluency by usual Trypsin-EDTA method.

2.2. Cell proliferation rate

Cells were serially passaged by re-seeding at $3.2 \times 10^4/\text{cm}^2$ in culture flasks (Nunc) and harvesting at 70–80% confluency from passage 1 to passage 25. In each harvesting, the live and dead cell counts were made by the dye exclusion method, using a Neubar's hemocytometer. From the data on cell count at the time of seeding, harvesting and duration of culture for each passage, the cell proliferation rate (r) per day was calculated according to the formula of Cristofalo and Phillips (1989).

$$\text{cell proliferation rate } (r) = \frac{(\log N_H - \log N_I)}{T_2 - T_1}$$

Where,

N_H = no. of cells harvested;
N_I = no. of cells initially seeded;
T_1 = time at seeding (h);
T_2 = time till harvesting (h).

2.3. Mean population doubling time (MPDT)

The mean population doubling time (MPDT), expressed as the time taken to double the existing cell population, was calculated using the formula reported by Cristofalo and Phillips (1989) and Davis (1994).

$$\text{MPDT} = 24/r$$

Where r is the cell proliferation rate.

2.4. Cumulative population doublings (CPD)

The cumulative population doubling for each cell line was calculated by following formula (Cristofalo and Phillips, 1989)

$$\text{CPD} = \sum_{i=1}^{m} T_2 - T_1/r$$

Where

$T_2 - T_1$ = duration in passage; and
r = cell proliferation rate.

The data generated on cell proliferation rate, mean population doubling time, cumulative population doublings and cell viability during 25 passages was grouped into passage intervals of 1–3, 4–6, 7–9, 10–12, 13–15, 16–18, 19–21 and 22–25.

2.5. Growth curve analysis

Growth curve analysis of skin fibroblasts was carried out at every 5, 15 and 25th passage. The cells harvested at 4, 14 and 24th passages were reseeded at 40,000 cells/25 cm^2 in 16 flasks. Two flasks were harvested at an interval of 24 h consecutively for 8 days. The rounded cells were counted with the help of hemocytometer from one flask separately. The growth curve was plotted for cell counts in 1–8 days.

2.6. In vitro cell senescence

The *in vitro* cell senescence was studied during 5, 15 and 25th passage using the method described by Dimri et al. (1995). In this experiment, the cells in monolayer in other flask of growth curve study was washed twice with full DPBS (Hyclone) and were fixed in 3% formaldehyde (Glaxo, India) for 5 min at room temperature. The cells were stained with 1 mg/ml X-gal (Roche Diagnostics) substrate staining solution. The cultures were incubated at 37 °C for 18–20 h, and washed with 2 ml of full DPBS. The cells in monolayer were scored under inverted microscope for the β-gal$^+$ blue-stain. A minimum of 400 cells per treatment were scored and percentage of β-gal$^+$ cells was calculated.

2.7. DNA isolation

The genomic DNA was isolated from primary skin fibroblast cells (zero passage), mid stage (15 passages) and late stage (25 passages). The method described by Laird (1991) was suitably modified for isolation of genomic DNA from monolayer-cultured cells. At 50–70% confluency, cell lysis buffer (100 mM Tris–HCl pH 8.5, 0.5 M EDTA, 20% SDS, 5 M NaCl and 20 mg/ml Proteinase K) was added to the monolayer for one hours at 37 °C with agitation. One volume of isopropanol as added to the cell lysate, and samples were stirred until precipitation was complete. DNA was recovered by lifting the aggregate precipitate from the solution using a disposable yellow tip. Quality and quantity of DNA were checked by gel electrophoresis using 0.8% agarose (Sigma).

2.8. Telomere length assay

The effect of replicative aging of cells in culture in multiple passaging was studied by telomere length assay at 0, 15 and 25th passages for all cell lines to determine telomeric DNA loss because of cell divisions in multiple passaging. Telomere length assay was performed using Telo TAGGG Telomere Length Assay Kit (Roche Diagnostics, Cat. No. 229136). To determine the terminal restriction fragment (TRF) length 1.5 μg of DNA was digested with HinfIII