

Immortalization of human embryonic fibroblasts by overexpression of c-myc and simian virus 40 large T antigen

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Abbreviations: cdk, cyclin-dependent kinase; HEF, human embryonic fibroblast; hTERT, human telomerase reverse transcriptase; PD, population doubling; Rb, Retinoblastoma; SV40 large T antigen, simian virus 40 large T antigen

Abstract

SV40 large T antigen, a viral oncoprotein, is known to immortalize human diploid fibroblast by soaking up cellular RB and p53, but its frequency is extremely low. Additional genetic alteration is necessary for single-step immortalization. We attempted to find out what this alteration is by overexpressing cellular signal mediator genes; c-myc and cyclin D frequently amplified in many cancer cells. Overexpression of cyclin D did not affect the immortalization, but, overexpression of c-myc along with T antigen could immortalize normal human diploid fibroblast. Several cellular markers tested during immortalization process showed that p21, a cyclin-dependent kinase inhibitor and a marker of cellular senescence, disappeared in the life span-extended cells by T antigen and in the immortalized cells by c-myc. p21 was, however, elevated in the senescent cells and in the cells of crisis. Interestingly, p16 was upregulated whenever T antigen is overexpressed. Telomerase activity was also activated only in the immortalized cells. These results suggest that overexpression of c-myc contributes to immortalization of human diploid fibroblast by activating telomerase activity and suppressing p21 activity.

Keywords: immortalization, human embryonic fibroblasts, SV40 large T antigen, telomerase

Introduction

Normal diploid cells in culture undergo a finite numbers

of divisions before they reach a terminally non-proliferating state known as the replicative senescence (Hayflick and Moorhead, 1961). This phenomenon has been reported for various animal cells including human cells (Hayflick and Moorhead, 1961; Rheinwald and Green, 1975; Rohme, 1981; Tassin *et al.*, 1979; Thornton *et al.*, 1983; Medrano *et al.*, 1994). Maximum population doubling numbers to get for animal embryonic fibroblast before reaching the replicative senescence is reported to be proportional to the maximum life span of donor animal (Martin *et al.*, 1970; Schneider and Mitsui, 1976; Rhome, 1981). While human embryonic fibroblasts (HEFs) undergo 50-70 population doublings before reaching the replicative senescence (Hayflick and Moorhead, 1961), mouse embryonic fibroblasts (MEFs) can have only 8-10 population doublings (Rhome, 1981). Replicative senescence has been extensively studied as an *in vitro* model of aging even though its relatedness to *in vivo* aging is not established yet. Numerous reports on change in gene expression and in the activity of gene products during replicative senescence have been released (Dulic *et al.*, 1993; Dimri and Campisi, 1994; Smith and Pereira-Smith, 1996). Cyclin dependent kinase inhibitors (CDKIs), p21 and p16, are increased as human fibroblast reaches the late passage (Noda *et al.*, 1994; Rogan *et al.*, 1995; Tahara *et al.*, 1995; Alcorta *et al.*, 1996; Wong and Riabowol, 1996; Brown *et al.*, 1997). Activation of these two CDKI genes are directly associated with the replicative senescence-related growth arrest (Rogan *et al.*, 1995; Tahara *et al.*, 1995; Alcorta *et al.*, 1996; Wong and Riabowol, 1996; Brown *et al.*, 1997; Stein *et al.*, 1999).

Telomere shortening theory is now well accepted for explanation of replicative senescence (Harley *et al.*, 1990; Campisi, 1997). Telomere shortening as a mitotic clock eventually causes chromosome instability, leading to the activation of the DNA damage response pathway followed by p53 dependent cell cycle arrest and senescence (Vaziri and Benchimol, 1996). When telomerase was artificially expressed to maintain telomere end, normal human fibroblasts have been reported to overcome the "crisis" and eventually to be immortalized, even though its frequency has not been mentioned (Bodnar *et al.*, 1998). However, telomere shortening theory has a limited application. It has not been applied to the most mammalian cells or all human cell types, although it has been stringently applied to some human cell types including human fibroblast (Dickson *et al.*, 2000; Kiyono *et al.*, 1998). Expression of hTERT, the major protein subunit of telomerase, is regulated mainly at the trans-

criptional level (Greider, 1999). Several transcription factors regulating the hTERT transcription including viral oncoprotein E1A have been identified (Greider, 1999; Kim *et al.*, 2001). Among them, c-Myc is the major activator of hTERT transcription (Wang *et al.*, 1998).

DNA tumor viruses like the SV40 virus and the papilloma virus can immortalize normal human cells at very low frequencies. A transforming viral protein of the SV40 virus large T antigen has been well characterized for its immortalization ability (Chang *et al.*, 1986; Mayne *et al.*, 1986). T antigen soaks up the cellular negative growth regulators, pRB, p53, and some related factors to enable cells to grow continuously. Expression of T antigen extends the replication potential of human diploid cells by about 20 population doublings and results in crisis, a non-proliferation state where cell growth and death are balanced (Ide *et al.*, 1984; Wright *et al.*, 1989). Only one out of 10^7 cells escapes the crisis to be immortalized. Therefore, additional genetic alteration is necessary for single-step immortalization. We attempted to find out what this alteration is by overexpression of cellular genes. c-myc and cyclin D were the first candidates since these two genes are frequently amplified in many cancer cells.

Here we report that overexpression of c-myc along with T antigen could immortalize normal human diploid fibroblast maintaining homeostasis of cell growth. Overexpression of c-myc seems to contribute to immortalization of human diploid fibroblast by activating telomerase activity and suppressing p21 activity.

Materials and Methods

Cell culture and Plasmids

A primary human embryonic fibroblast strain was established from an abortus at Hallym University Hospital. Primary normal human embryonic fibroblast cells were grown in DMEM, supplemented with 10% FBS (Biowhittaker, USA) and antibiotics under air atmosphere containing 5% CO₂ at 37°C. The primary culture was designated as population doubling (PD) number zero. The fibroblast cells were split into new dishes at 1:4 dilutions every 3-6 days and the medium was replaced every 3-4 days. One passage was equivalent to two PD for 1:4 split. Plating efficiency was measured to calibrate the correct population doublings but was neglected, since it was near 100%. pSV2-neo is a control vector containing only neo, and pSV2-myc-neo contains neo and wild type c-myc. pSV3-neo contains neo and SV40 large T antigen and pSV3-myc-neo has wild type c-myc and SV40 large T antigen.

Stable transfection and evaluation of immortalization

Human fibroblasts at 70% confluent were transfected

with 3 mg of DNA using lipofectamine (Gibco BRL) for 5 hours according to the manufacturer's instructions. After washing cells, cells were incubated in DMEM-10% FBS for 2 days. And then cells were cultivated in DMEM-10% FBS supplemented with 0.75 mg/ml G418. 5 colonies per each plasmid vector were picked and cultivated continuously in DMEM-10% FBS supplemented with 0.75 mg/ml G418 to test the immortalization.

Western blot analysis

Cells were harvested, washed in PBS, and lysed with extraction buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, pH 8.0, 1% Nondiet P-40) containing a mixture of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 5 mg/ml of aprotinin, 5 mg/ml of leupeptin). After centrifugation, the protein concentration of the supernatants was determined using BCA protein assay reagent (Pierce, USA). The proteins were loaded on a 12% or 15% SDS-PAGE gel and separated. The resulting gels were transferred to an Immobilon-p (polyvinylidene difluoride) filter, and the filters were blocked in 5% nonfat powdered milk in TBST (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20). The blots were probed with either a human p21-specific monoclonal antibody (15091A, Pharmigen, Inc.), anti-p16 monoclonal antibody (Pierce), anti-c-myc (Pharmingen), and anti-SV40 large T antigen (Santa Cruz). The bands were visualized with the ECL kit according to manufacturer's instruction (Amersham Pharmacia, USA) and relative band intensities were determined by scanning densitometry (Molecular Dynamics Inc.).

TRAP assay

Telomerase activity was analyzed by using the PCR-based telomeric repeat amplification protocol (TRAP) using TRAPeze Telomerase Detection Kit (Intergen) according to the manufacturer's instructions.

Results and Discussion

Growth and replicative senescence of a human embryonic fibroblast cell line

A human embryonic fibroblast cell line that was established from an abortus was obtained and tested for the replicative senescence. Population doubling was measured as a serial cultivation with split ratio 1:4. The growth of the embryonic fibroblast slowed down around 64 PD and ceased at 72 PD (Figure 1). This PD number at growth arrest was little higher than the numbers in previous reports that ranged about 50-70 PD (Hayflick and Moorhead, 1961). Morphological change at senescent fibroblast cells through microscopic observation was similar to the result observed in other known human fibroblast lines like IMR90, IMR91, MRC5, and

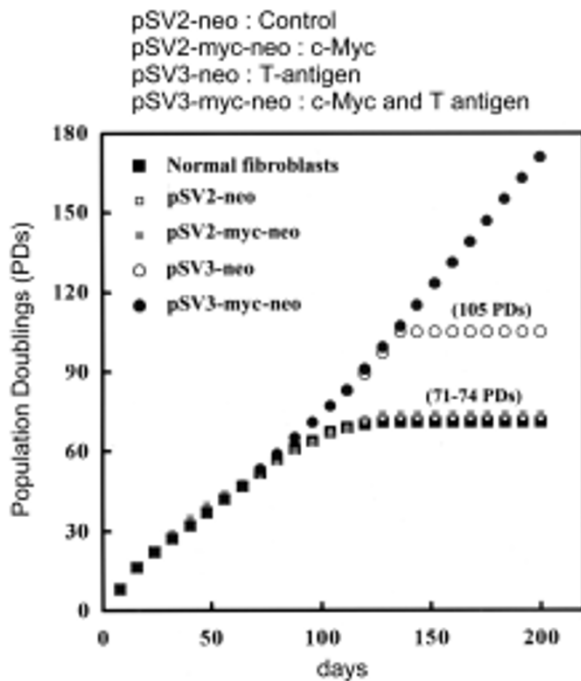


Figure 1. Growth curve of normal, SV40 large T antigen-transfected, c-Myc-transfected, and SV40 large T antigen/c-Myc-transfected human fibroblasts. Primary normal human embryonic fibroblasts were stably transfected with pSV2-neo, pSV2-myc-neo, pSV3-neo, and pSV3-myc-neo vectors. Five clones for each transfection were selected in medium containing G418, and growth for those clones were examined by serial cultivation. Upper panel shows the genes that each vector expresses.

WI38. Cells were elongated and flattened in the senescent cells (Blumenthal *et al.*, 1993; Chen and Ames, 1994). Acid β -galactosidase activity that is called senescence-associated- β Gal and is known to be activated only in both *in vivo* and *in vitro* aged cells was tested. Only senescent cells of this fibroblast strain were stained with blue color at perinuclear area as reported previously (Dimri *et al.*, 1995). Karyotype analysis of G banded-metaphase chromosomes showed the presence of normal 46 chromosomes, indicating that this line is a normal human cell (data not shown).

Immortalization of human fibroblasts with c-myc and SV40 large T antigen

Since overexpression of SV40 large T antigen is not sufficient to immortalize human diploid fibroblast, additional genetic alteration is necessary for single-step immortalization. We attempted to find out what this alteration is by overexpression of cellular genes. c-Myc and cyclin D were the first candidates since these two genes are frequently amplified in many cancer cells. Four vectors were used to study immortalization of human embryonic fibroblast. pSV2-myc-neo expresses wild type c-myc, and pSV3-neo expresses SV40 large T antigen. pSV3-myc-neo expresses both c-myc and SV40 large T

antigen. pSV2-neo expresses only neo and was used as a control vector. Normal human embryonic fibroblasts were transfected with these vectors, and clones were selected with medium containing G418. In each stable transfection, five clones were selected to confirm the clonal variation and analyzed for life span (Figure 1) and immortalization frequency. The clones transfected with either pSV2-neo or pSV2-myc-neo showed the similar life span with normal human fibroblast. The clones transfected with pSV3-neo showed the extended life span (95-105 PDs) compared to that (71-74 PDs) of normal human fibroblasts as expected, but eventually reached the crisis that is equilibrium between cell death and growth. Extension of life span by 20-30 PDs is known to be due to soaking up cellular RB, p53 and related proteins by SV40 large T antigen. Inactivation of p53 and Rb pathway by SV40 large T antigen is enough to overcome replicative senescence but not sufficient to immortalize. The clones overexpressing cyclin D and T antigen had same fate as the clones with pSV3-neo, suggesting that overexpression of cyclin D has no advantage for immortalization (Data not shown). All five colonies transfected with pSV3-myc-neo kept dividing up to 120 PDs without having a growth arrest or a crisis. The result indicates that overexpression of T antigen and c-myc is sufficient to immortalize human diploid fibroblast cells in culture.

Expression of the p21 and p16 protein

To confirm whether T antigen and c-myc are overexpressed in the clones transfected as expected, protein levels of these were analyzed by Western blot analysis. T antigen was expressed highly in the clones transfected with vectors containing T antigen as expected (Figure 2D). The c-myc protein was expressed in all clones including untransfected normal fibroblast and was expressed much highly in the clones transfected with vectors containing c-myc as expected (Figure 2C). To address the molecular mechanism of immortalization, expressions and activities of markers like p21, p16, and telomerase were examined. p21 and p16 are increased as human fibroblast reaches the late passage (Noda *et al.*, 1994; Rogan *et al.*, 1995; Tahara *et al.*, 1995; Alcorta *et al.*, 1996; Wong and Riabowol, 1996; Brown *et al.*, 1997), and activation of these two CDKI genes are directly associated with the replicative senescence-related growth arrest (Rogan *et al.*, 1995; Tahara *et al.*, 1995; Alcorta *et al.*, 1996; Wong and Riabowol, 1996; Brown *et al.*, 1997; Stein *et al.*, 1999). Thus, the protein levels of p21 and p16 were analyzed in all the steps of immortalization process for all clones. Expression of the p21 and p16 proteins much increased in older cells compared to that in younger cells (Figure 2A and 2B) as expected. In the clones transfected with pSV2-neo and pSV2-myc-neo, expression of p21 and

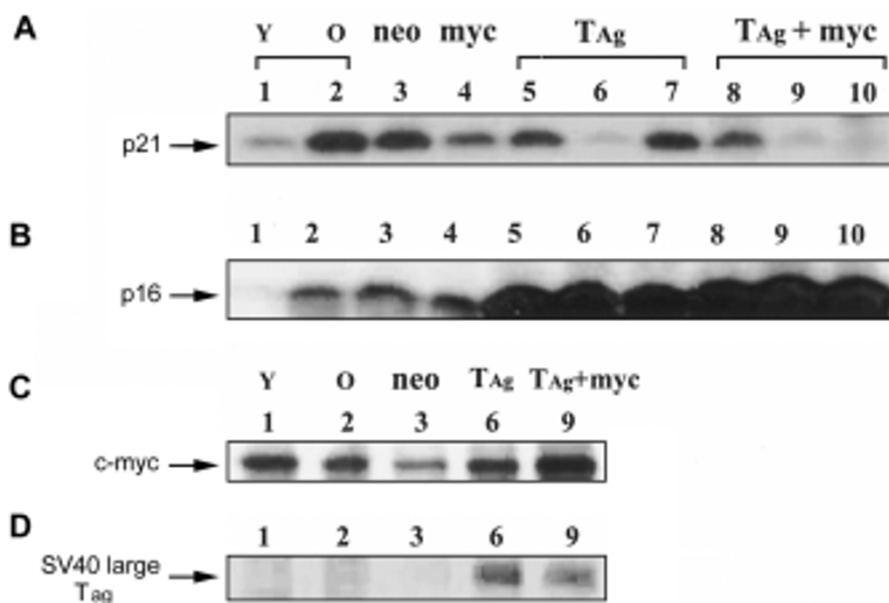


Figure 2. Expression of the p21 and p16 proteins. Expression of the p21 and p16 protein at various growth periods for the clones expressing c-myc or/and T antigen was measured by Western blot analysis. Cells were harvested at 70 to 80% confluence and extracted as mentioned in Material and Methods. (A) Western blot analysis of p21 protein; lane 1, young HEFs (12 PDs); lane 2, old HEFs (72 PDs); lane 3, the clone transfected with pSV2-neo (71 PDs, senescent); lane 4, pSV2-myc-neo-clone (71 PDs, senescent); lane 5, 6, and 7; the clone transfected with pSV3-neo (60 PDs (growing), 84 PDs (extended growth), and 92 PDs (in crisis), respectively); lane 8, 9, and 10; the clone transfected with pSV3-myc-neo (64 PDs (growing), 84 PDs (extended growth), and 110 PDs (immortalized), respectively). (B) Western blot analysis of the p16 protein; Explanation for lanes is the same as mentioned above. (C) Western blot analysis of the c-myc protein; Explanation for lanes is the same as mentioned above. (D) Western blot analysis of the SV40 large T antigen; Explanation for lanes is the same as mentioned above. Abbreviations represent as follows: Y, young; O, old; Tag, T antigen

p16 was elevated as normal fibroblast (Figure 2A and 2B). In the clones transfected with pSV3-neo, expression of the p21 protein increased in the number of PDs occurring replicative senescence, but decreased during the extended life span, and again increased during crisis (Figure 2A). Since T antigen soaks up cellular p53, the p21 level should be low due to no elevation of p21 by p53 whenever T antigen is highly expressed. However, the result showed it was not true. The level of p21 was still high during crisis. Elevation of the p21 protein during crisis implicates a possible role of p21 in crisis although its exact role in crisis and the mechanism for its elevation during crisis are not clear currently. Interestingly, expression of the p16 protein was elevated highly whenever T antigen is expressed (Figure 2B). As previously reported (Hara *et al.*, 1996), our results indicate that T antigen may directly or indirectly upregulate the expression of p16, although its exact mechanism has not been elucidated. In the clones transfected with pSV3-myc-neo, expression of the p21 protein gradually decreased and was undetectable in the immortalized cells (Figure 2A). The result indicates that p21 is tightly regulated by the same pattern as growth is regulated during immortalization process but p16 was upregulated whenever T antigen is expressed.

Analysis of telomerase activity

Since recent reports suggested that the activation of hTERT was required for immortalization of human diploid fibroblast, telomerase activity was examined to test whether it is related to immortalization by c-myc and T antigen. Unlike the clones expressing only SV40 large T antigen, the clones expressing both c-myc and SV40 large T antigen kept growing up to 170 PDs without having crisis (Figure 1). The activity of telomerase was analyzed during this immortalization process by using the TRAPeze telomerase detection kit. The results showed that only immortalized clones with pSV3-myc-neo showed the activity of telomerase (Figure 3), indicating that overexpression of c-myc may re-activate hTERT. Transcriptional activation of hTERT by c-myc has been reported, although it has not been reported that human diploid fibroblast was immortalized by overexpression of c-myc and T antigen. Overexpression of c-myc may also mediate the down-regulation of p21 to overcome crisis for immortalization although the mechanism has not been established.

Overexpression of c-myc with T antigen overcomes the "crisis" while overexpression of T antigen alone reaches it. The result suggests that crisis represents the situation including the lack of telomerase activity, telomere shortening, no G1 arrest due to the lack of pRB, and upregulation of p21, even though the mechanism for upregulation of p21 during crisis is unknown. Telo-

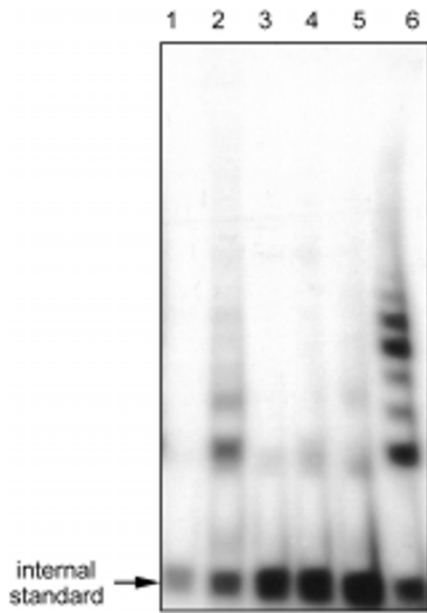


Figure 3. Analysis of telomerase activity. Telomerase activity was analyzed as mentioned in Material and Methods; lane 1, heat-inactivated negative control; lane 2, positive cell control; lane 3, normal HEFs (12 PDs, young); lane 4, the clone transfected with pSV2-myc-neo (71 PD, senescent); lane 5, the clone transfected with pSV3-neo (84 PDs, extended growth); lane 6, the clone transfected with pSV3-myc-neo (110 PDs, immortalized).

mere shortening may induce the expression of p21 by a p53-independent mechanism. This induced-p21 may hold DNA replication by binding PCNA, a replication initiation factor as previously reported. Growth arrest at crisis may be because of blocking of DNA replication by p21 even though no functional pRB is available in the nucleus. Overexpression of c-myc solves the problem of telomere shortening by inducing hTERT. This in turn results in down-regulation of p21 and continuous doubling without growth arrest. This appears to be a likely scenario for immortalization of human diploid fibroblast by overexpression of c-myc and T antigen although the details are not elucidated. We expect that further molecular characterization for detailed process will provide better understanding of this immortalization

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