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Immortalization of human umbilical vein endothelial cells with telomerase reverse transcriptase and simian virus 40 large T antigen*

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Abstract: Objective: To establish normally conditionally-immortalized human umbilical vein endothelial cells (HUVECs) by ectopic expression of the human telomerase catalytic enzyme (hTERT) and simian virus 40 large T (SV40 LT) antigen. Methods: Primary HUVECs were transfected with recombinant retrovirus containing *hTERT* or *SV40 LT* respectively. Subsequently drug resistant cell clones were screened and expanded for further studies. Endothelial cell biomarkers were confirmed by examination. Results: The morphological phenotype of the transfected cells was similar to the non-transfected cells. Von Willebrand factor, hTERT and SV40 LT could be detected in transfected HUVECs. Moreover, higher telomerase activity in transfected cells was maintained for over 50 population doublings compared with only low level of endogenous telomerase transiently at early population doublings in primary HUVECs. When exposed to TNF-α (tumor necrosis factor-α), the expression of E-selectin in transfected cells was significantly up-regulated, but no alteration of *endothelial lipase* was found. Conclusion: Ectopic coexpression of hTERT and SV40 LT can effectively immortalize HUVECs without tumorigenicity in vitro. Immortalized HUVECs may be an ideal target of further molecular function studies.

Key words: Endothelial cell, Telomerase activity, Immortalization
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INTRODUCTION

Human umbilical vein endothelial cells (HUVECs) are instrumental for defining the biology of endothelial cells. However, primary human cells in culture have a finite proliferative lifespan before they undergo permanent growth arrest, known as replicative senescence. When replicative senescence is bypassed by transformation with viral oncogene such as simian virus 40 large T antigen (SV40 LT), cells can gain additional lifespan. Nonetheless the post-senescent human cell populations expression SV40 LT is still restricted by another barrier represented by entrance into crisis, during which there is a slowing of cellular growth and widespread apoptosis (Macera-Bloch *et al*., 2002). In a number of cell types, crisis can be circumvented by ectopic expression of the catalytic subunit of the telomerase reverse transcriptase enzyme (hTERT) (Lundberg *et al*., 2002; Davies *et al*., 2003; Kudo *et al*., 2002). The hTERT appears to be essential for immortalization, as populations of cells lacking hTERT and expressing only SV40 will still enter into crisis and are not immortalized (Davies *et al*., 2003). Though in some cell types, induction of telomerase activity alone was sufficient for immortalization (Bodnar *et al*., 1998).

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Two previous studies of *hTERT* transfected human endothelial cells had reported conflicting results (Kumazaki *et al*., 2004; O'Hare *et al*., 2001). Meanwhile, immortalization of bone marrow derived endothelial cells with hTERT plus SV40 LT was much more efficient than hTERT alone and non-tumorigenicity was found (MacKenzie *et al*., 2002). And coexpression of hTERT and SV40 LT had been used to immortalize a number of cell types successfully (Davies *et al*., 2003; Kudo *et al*., 2002; Darimont *et al*., 2002). In the present work, we successfully established an immortalized human umbilical vein endothelial cell line preserving normal HUVEC's characteristics by primary HUVECs transfected with *hTERT* plus *SV40 LT*.

MATERIALS AND METHODS

Materails

Primary human umbilicus vein endothelial cells were purchased from Digestech (China); Matrigel was purchased from BD (USA); Retro PT67 packing cell, pLPCX and pLXSN retroviral vectors, G418 and puromycin were purchased from Clontech (USA); von Willebrand factor ELISA kit was from Maxim (China); E-selectin ELISA kit was from Diaclone (USA); Telomerase PCR ELISA kit was purchased from Roche (Germany); PCR primers were synthesized by Sangon Biologic Engineering & Service. pCIneo-*hTERT* was kindly provided by Professor Robert A. Weinberg (USA); Temperature sensitive *SV40 LT* was kindly provided by Professor Kamada (Japan).

Construct retroviral vectors containing *hTERT* or *SV40 LT* and produce retrovirus

hTERT was subcloned from pCIneo-*hTERT* to the *Eco*RI/*Not*I site of pLPCX, and *SV40 LT* was subcloned from pMFG*SV40 tsLT* to the *Eco*RI/*BamH*I site of pLXSN (Fig.1). PT67 retroviral packing cells cultured in DMEM (Dulbecco's modified eagle medium) medium with 10% fetal calf serum were transfected with pLXSN-*SV40 LT* and pLPCX-*hTERT* by standardized calcium phosphate method respectively. Forty-eight hours after transfection, the cells were screened with 500 μg/ml G418 and 4 μg/ml puromycin for 14 d. Drug resistant cells were selected and subcultured. The supernatant was harvested and filtered through a 0.45 μm filter, preserved at –70 °C.

Immortalization of HUVECs

For immortalization of HUVECs, primary HUVECs cultured in RPMI 1640 medium supplemented with 20% fetal calf serum were transfected with the recombinant retrovirus mentioned above for 48 h. Subsequently the transfected HUVECs were selected with 500 μg/ml G418 and 4 μg/ml puromycin at 72 h after transfection for 14 d. Drug resistant cells were selected and expanded for further studies.

Western blot analysis for hTERT and SV40 LT

For hTERT and SV40 LT analysis, cells were lysed with lysis buffer (20 mmol/L PBS, pH 7.4, containing 1% NP40, 0.5% sodium deoxycholate,

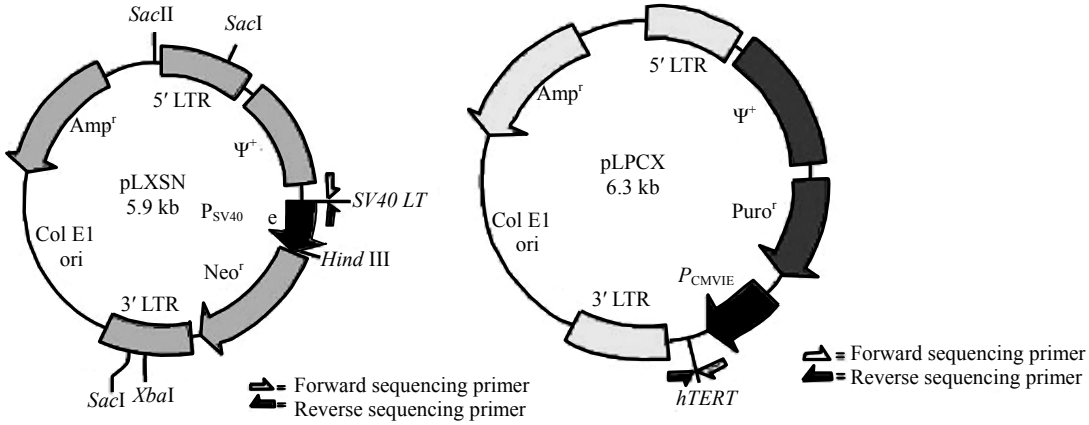

Fig.1 The construction of pLXSN-*SV40 LT* and pLPCX-*hTERT* recombined plasmids
SV40 LT and *hTERT* was subcloned from pMFG*SV40 tsLT* to *Eco*RI/*BamH*I site of pLXSN or pCIneo-*hTERT* to *Eco*RI/*Not*I site of pLPCX respectively

0.1% SDS, and 0.01% protease inhibitor cocktail). The cell debris was removed by centrifugation at 13000 rpm for 30 min and the supernatant was used as sample, electrophoresed on a polyacrylamide gel. Then the proteins were tranfered to a PVDF membrane and the membrane was blocked in skim milk for 2 h. A biotinalyted anti-hTERT/SV40 LT antibody was used to examine protein expression. And the bound antibody was detected using streptavidin-peroxidase conjugate and ECL (enhance luminar) detection system.

PCR-ELISA for the activity of telomerase

Telomerase activity was assayed via Telomerase PCR ELISA kit. The procedure was strictly performed per instruction of manufacturer. Briefly, resuspended cell in lysis buffer was pelleted, and incubated on ice for 30 min, the lysate was centrifuged at 13 000 rpm for 20 min. The supernatant was used in the telomeric repeat amplification protocol. The elongation products were amplified by RT-PCR, and detected by a digoxigenin-labelled telomeric repeat specific ELISA.

Detection of von Willebrand factor

HUVECs were grown on the surface of plastic slide, fixed with 100% ethanol and subjected to immunocytochemistry using a rabbit antibody against von Willebrand factor. Biotinylated sheep anti-rabbit antibody was used as the second antibody. Streptavidin-peroxidase conjugate was used for the indicated substrate. Finally, the reaction was developed with diaminobenzidine substrate. The reaction was terminated with current water. And the slide was restained with haematoxylin for microscopic examination.

Detection of E-selectin and *endothelial lipase*

For E-selectin analysis, HUVECs were cultured to 70%~80% confluence, replaced with fresh medium supplemented with 0 or 50 ng/ml TNF-α for 6 h. The supernatant was used for determination of E-selectin as per instruction provided with the kit. All data were statistically analyzed with student *T*-test.

For *endothelial lipase* analysis, total RNA of HUVECs was converted into cDNA with oligo dT_{15} by M-MuLV reverse transcriptases. RT-PCR was performed with primers as follows: Forward primer 5′-AGAACCTGTGGAAGGAGTTTCG-3′ and reverse primer 5′-CGGATGCGCCTGATATTCA-3′. The reaction conditions for endothelial lipase was 1× (94 °C, 2 min), 30× (94 °C, 15 s; 48 °C, 30 s; 72 °C, 30 s), and 1× (72 °C, 10 min). PCR products were run in 1% agarose gel and the image was analyzed by Kodak Digital Science ID software.

RESULTS

Morphology and biologic characteristics of the transfected cells

The transfected cells grew as confluent monolayer with the cobblestone morphology of normal HUVECs. The cells were homogenous, closely apposed, large, flat, and polygonal, have a characteristically ovoid nucleus with one or two nucleoli. Under inverted microscope, the cytoplasm of the transfected HUVECs appeared reddish after immunocytochemical staining, which confirmed the expression of von Willebrand factor (Fig.2). Under scanning electron microscope, the cells were polygonal, round or fusiform, with many apophysis at cell surface (Fig.3).

The transfected cells of various passages grew steadily with average doubling time of 80 h to 100 h. Non-transfected HUVECs proliferated for only 5 population doublings and then senesced. Under the same culture conditions, the doubling time of the transfected cells was rather stable. The cells had been cultured for more than 50 population doublings up to now.

Analysis for SV40 LT and hTERT and telomerase activity

The expression of SV40 LT and hTERT (Fig.4)

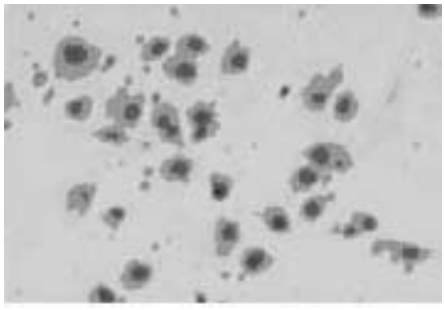

Fig.2 Positive staining of von Willebrand factor in immortalized endothelial cells (×100)

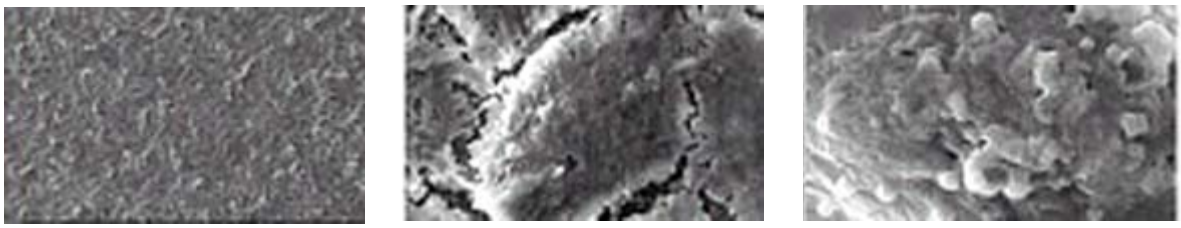

Fig.3 The morphology of immortalized endothelial cells under scanning electron microscope (a) The cells were polygonal, round or fusiform (magnification of ×200); (b) (magnification of ×5000) and (c) (magnification of ×6000) There were many apophyses on their surface

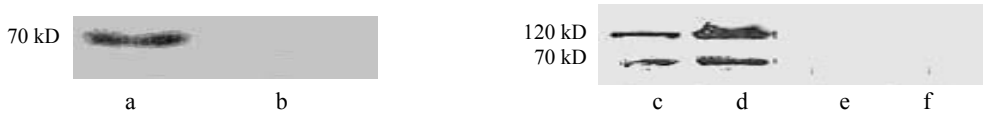

Fig.4 Detection of SV40 LT and hTERT with Western blot assay
a, c and d were from immortalized endothelial cells, and b, e and f were from normal endothelial cells. The expression of SV40 LT and hTERT could be only detected in immortalized endothelial cells

determined by Western blot indicated successful transfection of the two exogenous genes in the transfected cells. The activity of telomerase determined by PCR-ELISA was 0.36 at 12 population doublings and 0.38 at 50 population doublings in transfected cells; compared with 1.12 at first population doubling, 0.06 at third population doublings in non-transfected cells.

Assay of cell function

The expression of *endothelial lipase* and E-selectin in transfected cells as confirmed by RT-PCR or ELISA kit was similar to those in non-transfected HUVECs (Fig.5). When transfected cells were exposed to TNF-α, the expression of E-selectin in the transfected cells was up-regulated significantly (Fig.6) as in non-transfected HUVECs (P<0.01), while the expression of endothelial lipase was not up-regulated significantly (P>0.05).

DISCUSSION

Endothelial cells are single layer cells lining the inner surface of all blood vessels. These cells constitute a major metabolic organ that is critically involved in the generation and the regulation of multiple physiological and pathological processes such as coagulation, hemostasis, inflammation, atherosclero-

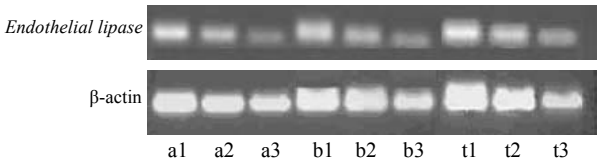

Fig.5 The expression of *endothelial lipase* in immortalized endothelial cells
The expression of *endothelial lipase* was similar with normal HUVECs. After the intervention with TNF-α, the expression of *endothelial lipase* was not up-regulated significantly. a1~3 were transfected cells; b1~3 were normal cells; t1~3 were transfected cells with the intervention of TNF-α

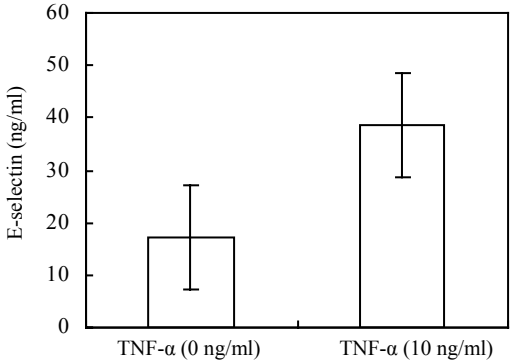

Fig.6 The expression of E-selectin in transfected cells exposed to TNF-α
When the transfected cells were exposed to TNF-α, the expression of E-selectin in them was up-regulated significantly (38.60±1.83 vs 17.19±0.77, P<0.01, student T-test)

sis, angiogenesis and cancerous metastasis dissemination (Bruneel *et al*., 2003). Studies on human endothelial cells are relatively few because of their limited proliferative capability and functional variation of the primary cells. Therefore, normal primary endothelial cells are not ideal targets for long-term studies because they undergo some de-differentiation in culture and have an inherently short replicative life span before senescence. A model endothelial cell line should overcome some of the difficulties encountered in primary endothelial cell culture and provide a consistent model system to study endothelial function. Telomeres are repetitive DNA sequences at the ends of chromosomes synthesized by telomerase (Greenwood and Lansdorp, 2003). The absence of telomerase activity results in progressive shortening of telomeres with each subsequent cell division. When telomeres become critically short so that it cannot maintain the chromatosome stabilization, normal cells will undergo an irreversible growth arrest, referred to senescence (Proctor and Kirkwood, 2003).

A major mechanism regulating telomerase activity in human cells is transcriptional control of hTERT that acts as a reverse transcriptase. Therefore ectopic expression of hTERT can enhance the activity of telomerase, elongate the life span of various cells, and even result in immortalization. Since an *hTERT* vector was used to extend the life span of normal human RPE cells (Bodnar *et al*., 1998), reviving of telomerase activity in primary cells had been proposed as a promising method for extending the life span of many cell types. In some recent studies, the telomeres length in *hTERT* transfected cells did not appear to be longer than normal control cells, sometimes even shorter than the control, which meant that the telomeres length may not be a determinant of the timing of the onset of senescence (Lundberg *et al*., 2002; MacKenzie *et al*., 2002; Terasaki *et al*., 2004). Human telomerase may have a telomere-stabilizing or "capping" function that permits cells to proliferate by protecting telomeres even when they remain shortened. This function of telomerase may be different from its effects on net telomere length. Therefore, the activity of telomerase instead of net telomere length was found in the present study to confirm the transfection of *hTERT*. Other studies indicated that hTERT expression concomitant with inactivation of *pRb* or *p16* could improve the efficiency of immortalization (Noble *et al*., 2004; Veitonmaki *et al*., 2003). SV40 LT can inhibit the function of at least three growth inhibitors (*pRb*, *p53* and *SEN6*), lengthen the life span of fibroblasts and even immortalize the cells (Jha *et al*., 1998). So we transfected these two genes to normal HUVECs simultaneously, expected to establish an immortalized endothelial cell line(s).

In the present study, primary HUVECs showed limited growth potential, entered into a senescence-like state after only 5 population doublings, although transfected HUVECs had been cultured for more than 50 population doublings without any evidence of senescence and the double time was stable under the same conditions as those in non-transfected normal endothelial cells. The result suggested that due to the transfection of *hTERT* and *SV40 LT*, the life span of HUVECs could be extended dramatically. Baseline expression of von Willebrand factor is regarded as key marker distinguishing endothelial cells from other cell types both in vivo and in vitro. In the present study, transfected cells expressed von Willebrand factor, had the morphology of endothelial cells. All of the above suggested that the tranfected cells could not only be cultured in vitro continuously, with no evidence of alteration in growth patterns being detected. In addition, it had been shown that coexpression of hTERT and SV40 LT was not sufficient for tumorigensis in soft-agarose clone test in vitro. E-selectin is a member of the selectin family of endothelial cell adhesion molecules. Expression of E-selectin is both tissue specific and stimulus specific (Burnham *et al*., 2004; Charreau *et al*., 2000), as it is expressed only in endothelial cells in response to induction by cytokines such as interleukin-1β, TNF-α and lipopolysaccharides, which makes E-selectin another key marker distinguishing endothelial cells from other cell types. Endothelial lipase that is also tissue specific is a new member of the TG lipase family (Jaye *et al*., 1999), has triglyceride lipase and phospholipase activities. Therefore, endothelial specific E-selectin and endothelial lipase were used to confirm the cell function. The 50 population doublings transfected cells we cultured expressed E-selectin and endothelial lipase. When the cells were exposed to TNF-α, the expression of E-selectin was up-regulated significantly as in normal HUVECs (Che *et al*., 2002). But the expression of endothelial lipase was not up-regulated significantly in another

study (Jin *et al*., 2003), little is known about this contradictory finding up to now.

In summary, we have generated immortalized HUVECs by ectopic expression of hTERT and SV40 LT without tumorigenic conversion. These cells will be valuable for gaining further understanding of the fundamental mechanisms of endothelial bioactivity and endothelial-related diseases.

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