

# Establishment and characterization of an immortalized human dermal papilla cell line

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**Establishment of immortalized human dermal papilla cells (DPCs) retaining the characteristics of DPCs would be a great help for hair researchers. We recently established a simian virus 40T (SV40T)-transformed human DP cell line (SV40T-DPC). However, the cell line senesced around passage 25 and ceased proliferation. In this study, we introduced the human telomerase reverse transcriptase (hTERT) gene into SV40T-DPC and established an immortalized human DP cell line. The cell line, SV40T-hTERT-DPC, did not induce tumors when inoculated into nude mice. SV40T-hTERT-DPC maintained morphology of early passage DPCs, expressed markers of DPCs, and retained responses to Wnt/ $\beta$ -catenin and bone morphogenic protein (BMP) signaling pathways known to be required for hair-inducing activity of DPCs. The data strongly suggest that SV40T-hTERT-DPC retains many characteristics of human DPCs *in vivo* without malignant transformation. [BMB reports 2011; 44(8): 512-516]**

## INTRODUCTION

The mammalian hair follicle contains dermal papilla (DP) and dermal sheath cells derived from the mesenchyme (1). It also contains epithelial cells of outer and inner root sheaths, matrix, and hair shaft, which are derived from the epithelium (1). DP, a cluster of specialized fibroblasts, is believed to secrete autocrine and paracrine factors that regulate the growth and activity of the various cells in the follicle, thereby playing a key role in the regulation of hair cycling and growth (2, 3).

Cultured dermal papilla cells (DPCs) are believed to be useful for the study of their role in relation to hair growth and hair regeneration (4-6). However, DPCs cultured *in vitro* show

drastic morphological change and possess a relatively short replicative life span. Establishment of immortalized human DP cell lines with primary characteristics of DPCs would help hair biologists to obtain DPCs without tedious and tricky cultivation from hard-to-obtain human biopsies. Also, immortalized human DP cell lines would supply sufficient number of cells resulting in more reproducible *in vitro* results.

We recently introduced the simian virus 40 large T antigen (SV40T-Ag) into cultured human scalp DPCs, enabling them to proliferate beyond their normal replicative life span and established a SV40T-transformed human DP cell line designated SV40T-DPC. We used this cell line for screening androgen-regulated genes (7). However, SV40T-DPC also senesced and ceased proliferation around passage 25. Since the ectopic expression of human telomerase reverse transcriptase (hTERT) is known to allow transformed cells to escape from a proliferation barrier known as "crisis", leading to immortalization (8), we presently introduced the hTERT gene into SV40T-DPC. Here, we report the establishment and characterization of an immortalized human DPC line with primary characteristics of DPCs.

## RESULTS AND DISCUSSION

### Establishment of an immortalized human DP cell line (SV40T-hTERT-DPC)

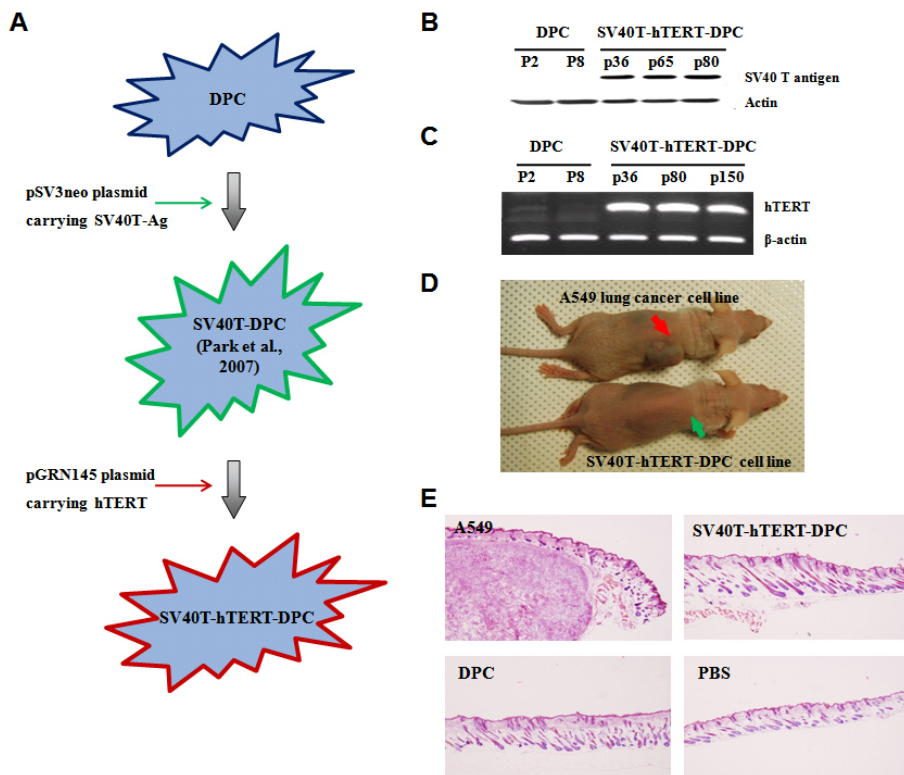
We introduced a hTERT expression plasmid into SV40T-DPC. Hygromycin-resistant cells were selected and named SV40T-hTERT-DPC (Fig. 1A). SV40T-hTERT-DPC proliferated beyond passage 160 without showing senescence. Expression of SV40T antigen and hTERT in SV40T-hTERT-DPC was identified by immunoblot and RT-PCR analyses (Fig. 1B and 1C). Our cell line did not induce tumors when inoculated into nude mice, whereas the A549 human lung cancer cell line used as a positive control developed tumors (Fig. 1D). Consistent with this, no histological alterations were observed in mice where SV40T-hTERT-DPC were inoculated, whereas significant histological changes were induced when A549 cells were inoculated (Fig. 1E). These data demonstrate the successful establishment of an immortalized cell line without malignant transformation.

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<http://dx.doi.org/10.5483/BMBRep.2011.44.8.512>

Received 28 March 2011, Accepted 1 June 2011

**Keywords:** Dermal papilla, Hair follicle, hTERT, Immortalization, SV40T-transformation



**Fig. 1.** Establishment of an immortalized human DP cell line, SV40T-hTERT-DPC. (A) Scheme of the immortalization. (B) Detection of SV40T antigen. Equal amounts of total protein from passage 2 (lane 1), and passage 8 (lane 2) primary DPCs, and SV40T-hTERT-DPC (passage 36, 65, & 80) were used for immunoblot. Actin was used as an internal control. (C) Detection of hTERT mRNA. Equal amounts of total RNA from passage 2 (lane 1), and passage 8 (lane 2) DPCs, and SV40T-hTERT-DPC (passage 36, 80, & 150) were used for RT-PCR analysis. (D) Result of the tumorigenicity assay. Nude mice were injected with either A549 human lung cancer cell line (up) or SV40T-hTERT-DPC (down) and tumor formation was observed for 6 weeks. (E) Histology of back skin inoculated with A549, SV40T-hTERT-DPC, primary DPC (passage 2), and PBS only are shown. Scale bar = 1.0 mm.

#### Expression of DP marker proteins in SV40T-hTERT-DPC

We next examined expression of DPC markers in SV40T-hTERT-DPCs by immunostaining. Antibody against cytokeratin-8 (a keratinocyte marker) demonstrated that primary DPCs and SV40T-hTERT-DPC did not express cytokeratin-8 (Fig. 2, top panel), providing strong evidence that there is no epithelial cell contamination in the established cell line. On the other hand,  $\alpha$ -smooth muscle actin, one of the known markers of cultured DPCs (9), was highly expressed in SV40T-hTERT-DPC (Fig. 2, middle panel). In addition, the use of an antibody against biglycan, a proteoglycan expressed in growing DP *in vivo* (10), showed that SV40T-hTERT-DPC as well as DPCs expressed this protein (Fig. 2, bottom panel). These results demonstrate that SV40T-hTERT-DPC still maintain the characteristics of primary DPCs.

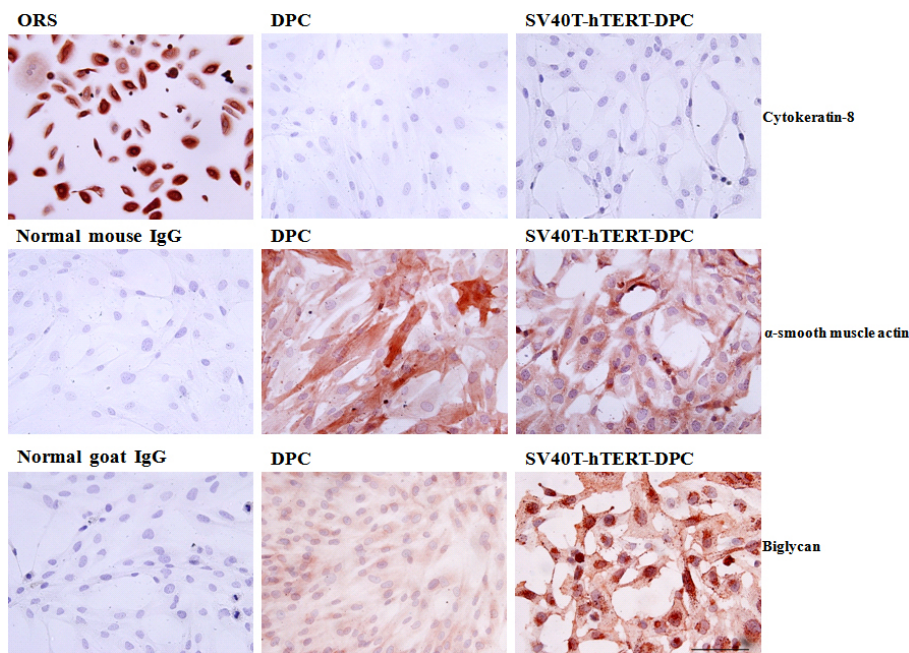
#### Wnt/ $\beta$ -catenin signaling is maintained in SV40T-hTERT-DPC

DP *in vivo* is one of the sites of Wnt/ $\beta$ -catenin signaling and  $\beta$ -catenin activity in the DP regulates morphogenesis and regeneration of hair (11, 12). Also, maintenance of Wnt signaling through the  $\beta$ -catenin pathway is required for hair-inducing activity of DPCs and maintains DPCs capable of in hair growth (13, 14). Recent studies showed that cultured human DPCs respond to Wnt3a (15, 16). We, therefore, examined whether SV40T-hTERT-DPC maintained responsiveness to Wnt/ $\beta$ -catenin signaling. Cells were transiently transfected with the

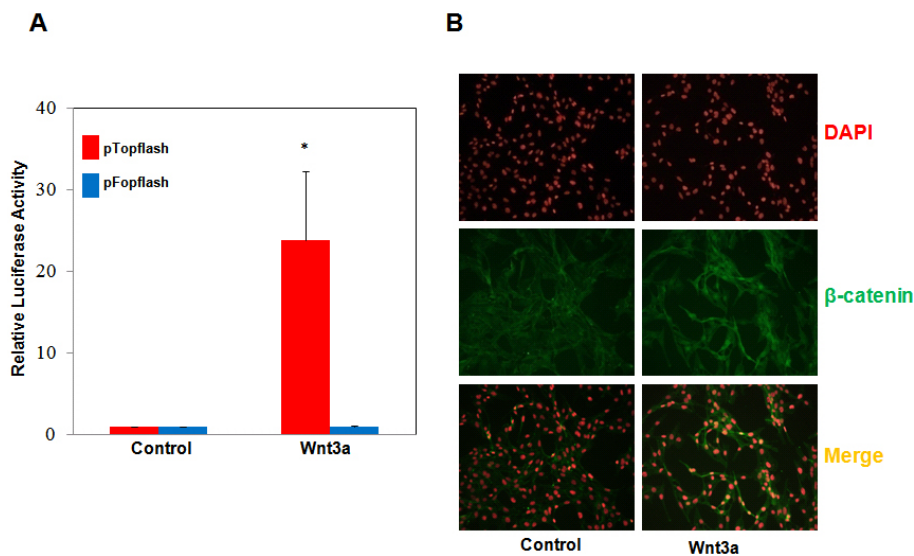
$\beta$ -catenin responsive TCF reporter plasmid (pTopflash) and corresponding control plasmid (pFopflash) to assess the activity of  $\beta$ -catenin signaling in the presence of Wnt3a. Wnt3a significantly stimulated the transcriptional activity of pTopflash but not pFopflash (Fig. 3A). Consistent with this, nuclear translocation of  $\beta$ -catenin was observed in SV40T-hTERT-DPC by Wnt3a treatment (Fig. 3B). These results demonstrate that the Wnt/ $\beta$ -catenin signaling pathway is maintained in SV40T-hTERT-DPC and suggests that this cell line maintains the potential to induce hair follicles if a suitable microenvironment is provided.

#### BMP signaling is maintained in SV40T-hTERT-DPC

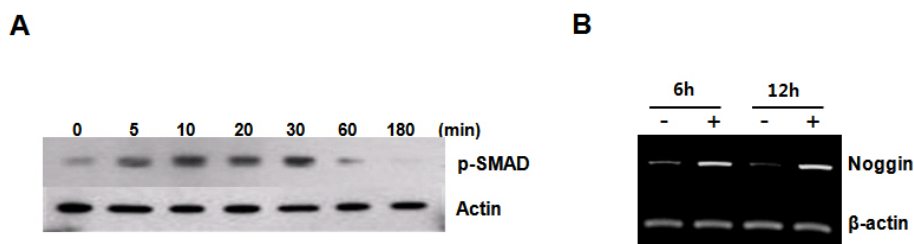
Recently, Rendl *et al.* (17) showed that BMP-treated mouse DPCs have improved hair inductivity. They also showed that ablation of BMP receptor 1a (BMPR1A) in cultured DPCs fails to induce hair follicles. These data demonstrated the connectivity between BMP signaling and hair-inducing activity of DPCs. We, therefore, investigated whether SV40T-hTERT-DPC maintained responsiveness to BMP signaling. Since BMPs induce phosphorylation of Smad1/5/8 to transduce the BMP signaling (18), we examined the level of phospho-Smad1/5/8 (p-SMAD) in the presence of BMP4. The level of p-SMAD was increased in 5 min and maintained up to 30 min when SV40T-hTERT-DPC were treated with BMP4 (Fig. 4A). We also observed that noggin, a transcriptional target gene of BMPs (17,



**Fig. 2.** Immunostaining for cell markers. DPCs (passage 1) and SV40T-hTERT-DPC (passage 70) were incubated with antibodies against cytoke-  
ratin-8 (top panel),  $\alpha$ -smooth muscle actin (middle panel), and biglycan (bottom panel). Outer root sheath follicular  
keratinocytes (ORS; passage 1) were cultured as described previously (22) and used as a positive control for the  
cytoke-  
ratin-8. DPCs (passage 1) were  
also incubated with corresponding  
normal IgG as a negative control.  
Scale bar = 0.1 mm.



**Fig. 3.** Wnt/ $\beta$ -catenin pathway activa-  
tion in SV40T-hTERT-DPCs. (A) Deter-  
mination of luciferase activity. Cells  
were transiently transfected with either  
pTopflash or pFopflash plasmids and  
treated with Wnt3a for 6 h. (\* $P < 0.005$ ) (B) Nuclear accumulation of  
 $\beta$ -catenin by Wnt3a treatment. Cells  
were treated with either 0.1% BSA (Control) or 250 ng/ml Wnt3a for 6 h and immunostained with  $\beta$ -catenin anti-  
body (middle). DAPI nuclear counter-  
staining is shown in red and merged  
images are shown in bottom panel.



**Fig. 4.** BMP signaling is maintained in  
SV40T-hTERT-DPCs. (A) Immunoblot  
of pSMAD. Cells were treated with  
50 ng/ml rhBMP4 for indicated times.  
Actin was used as an internal control.  
(B) Induction of the expression of  
noggin. Cells were treated with 50  
ng/ml rhBMP4 for indicated times and  
analyzed by RT-PCR.  $\beta$ -actin was  
used as an internal control.

19, 20), was increased in the presence of BMP4 as examined by RT-PCR analysis (Fig. 4B). Similar data was also obtained when SV40T-hTERT-DPC was treated with BMP6 (data not shown). These results demonstrate that BMP signaling is indeed maintained in SV40T-hTERT-DPC. We, therefore, expect this cell line to induce hair follicles if suitable factors or micro-environment is provided.

Very recently, Won *et al.* (21) reported the establishment of an immortalized human DP cell line by transducing human DPCs with a combination of viral vectors containing SV40T and c-myc. The cell line maintained the characteristics of primary human DPCs. However, the cell line senesced when cultured over 30 passages. Therefore, strictly speaking, the cell line was transformed but not immortalized. We believe that SV40T-hTERT-DPC is a genuine immortalized human DP cell line.

In summary, we successfully established an immortalized human DP cell line that expresses DPC markers such as  $\alpha$ -smooth muscle actin and biglycan. Also, this cell line maintains responses to Wnt/ $\beta$ -catenin and BMP signaling pathways, which are known to be required for hair-inducing activity of DPCs. Altogether, our data show that SV40T-hTERT-DPC retains many characteristics of human DPCs *in vivo* without malignant transformation. Although further characterization may be needed, we believe this cell line is potentially useful for studies of human hair follicle induction and hair growth.

## MATERIALS AND METHODS

### Establishment of immortalized cell line and primary culture of human DPCs

In our previous study, early passage (passage 2) human DPCs were transfected with SV40T expressing pSV3neo plasmid and the transformed human DP cell line was named SV40T-DPC (9). In this study, SV40T-DPCs (passage 13) were transfected by microporation with pGRN145 plasmid (ATCC #MBA-141) carrying the hygromycin-resistant and hTERT genes. Hygromycin resistant cells were selected. The established cell line was maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Gaithersburg, MD, USA) with 10% fetal bovine serum (FBS). Primary DPCs were cultured as described previously (22). Briefly, DP was isolated from the bulbs of dissected anagen hair follicles, transferred onto plastic dishes coated with bovine type 1 collagen and cultured in DMEM with 20% FBS. After the cell outgrowth become sub-confluent, cells were sub-cultured with split ratio of 1 : 4 and maintained in DMEM with 10% FBS.

### Immunoblotting

Total cell lysates (3  $\mu$ g/lane) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the resolved proteins were transferred to nitrocellulose membranes. The membranes were blocked with 5% milk in phosphate-buffered saline for 1 h, and they were pro-

bed with mouse anti-SV40TAg antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and polyclonal rabbit phospho-SMAD antibody (Cell Signaling Technologies, Beverly, MA, USA). Horseradish peroxidase (HRP)-conjugated streptavidin (Pierce, Rockford, IL, USA) were used as the secondary antibodies at a 1 : 5,000 dilution. The band was visualized using ECL Plus (Amersham, Buckinghamshire, UK). The membranes were also probed with mouse monoclonal antibody against actin (Chemicon, Temecula, CA, USA).

### Immunocytochemistry

DPCs were plated in an eight-chamber slide (Nunc Lab-Tek, Roskilde, Denmark) at a density of 30,000 cells per well and cultured in serum free DMEM for 24 h. Cell were washed with phosphate-buffered saline (PBS) and immersed in cold methanol for 10 min. After blocking with 10% normal goat serum for 1 h at room temperature, sections were incubated with cytokeratin 8 antibody (DAKO, Carpinteria, CA, USA),  $\alpha$ -smooth muscle actin antibody (R&D Systems, Minneapolis, MN, USA) and biglycan antibody (R&D Systems) at 4°C overnight. The slides were then rinsed with PBS, incubated with secondary antibody-HRP conjugate antibody for 30 min, and rinsed again with PBS. The slides were incubated with AEC for 10 min, counterstained with Mayer's hematoxylin and mounted.

### Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated using TRIzol reagent (Gibco-BRL, Grand Island, NY, USA). cDNA was synthesized from 3  $\mu$ g of total RNA using the cDNA synthesis kit containing the Superscript II reverse transcriptase and oligo (dT) primer according to the instructions of the manufacturer's instructions (Gibco-BRL). One microliter of cDNA was amplified with each of the forward and reverse primers. For the detection of hTERT, 30 cycles (1 min at 94°C, 45 s at 58°C, and 45 s at 72°C) of amplification was performed with 5'-CGGAAGAGT-GTCTGGAGCAA-3' and 5'-GGATGAAGCGGAGTCTGGA-3'. For the detection of noggin, 30 cycles (1 min at 94°C, 45 s at 57°C, and 45 s at 72°C) of amplification was performed with 5'-GTGCAAGTGCTCGTGCTAGA-3' and 5'-AAGTCCCCCG-TCTCTTTGT-3'. For the detection of actin, 22 cycles (1 min at 94°C, 45 s at 58°C, and 45 s at 72°C) of amplification was performed with 5'-GGGAAATCGTGCCTGACATT-3' and 5'-GGAGTTGAAGGTAGTTTCGTG-3'. PCR products were separated by electrophoresis on a 1% agarose gel and visualized under UV light.

### Reporter assay

SV40T-hTERT-DPC were transfected with 0.45  $\mu$ g of either pTopflash, carrying the TCF binding consensus sequence followed by the luciferase gene, or pFopflash, carrying the dominant-negative TCF-binding sequence and 10 ng pRenilla as reporter control using microporation. At 24 h post-transfection, the cells were stimulated with rmWnt3a (250 ng/ml) (R&D

Systems) for 6 h. The luciferase assay was performed using the dual luciferase assay reporter kit according to the manufacturer's instructions (Promega, Madison, WI, USA).

### Tumorigenesis assay

BALB/c nude female, 6-week-old mice were purchased from Orient company (Busan, Korea). SV40T-hTERT-DPC and A549 human lung cancer cells were suspended in PBS and three mice each were subcutaneously injected with each cell line with 200  $\mu$ l ( $5 \times 10^6$  cells) at their right oxters. The mice were observed weekly for 4-6 weeks before they were sacrificed.

### Acknowledgements

This work was supported by the Korea Research Foundation (KRF) grant funded by the Korea government (MEST) (2009-0072162). This work was also supported by the Grant of the Korean Ministry of Education, Science and Technology (The Regional Core Research Program/Anti-aging and Well-being Research Center).

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