

A cell-based system for screening hair growth-promoting agents

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Abstract Androgen-inducible transforming growth factor β (TGF- β 1) derived from dermal papilla cells (DPCs) is a catagen inducer that mediates hair growth suppression in androgenetic alopecia (AGA). In this study, a cell-based assay system was developed to monitor TGF- β 1 promoter activity and then used to evaluate the effects of activated TGF- β 1 promoter in human epidermal keratinocytes (HaCaT). To accomplish this, a pMetLuc-TGF- β 1 promoter plasmid that expresses the luciferase reporter gene in response to TGF- β 1 promoter activity was constructed. Treatment of HaCaT with dihydrotestosterone, which is known to be a primary factor of AGA, resulted in a concentration-dependent increase in TGF- β 1 promoter activity. However, treatment of HaCaT with the TGF- β 1 inhibitor, curcumin, resulted in a concentration-dependant decrease in TGF- β 1 expression. Subsequent use of this assay system to screen TGF- β 1 revealed that HaCaT that were treated with apigenin showed decreased levels of TGF- β 1 expression. In addition, treatment with apigenin also significantly increased the proliferation of both SV40T-DPCs (human

DPCs) and HaCaT cells. Furthermore, apigenin stimulated the elongation of hair follicles in a rat vibrissa hair follicle organ culture. Taken together, these findings suggest that apigenin, which is known to have antioxidant, anti-inflammatory, and anti-tumor properties, stimulates hair growth through downregulation of the TGF- β 1 gene. In addition, these results suggest that this assay system could be used to quantitatively measure TGF- β 1 promoter activity in HaCaT, thereby facilitating the screening of agents promoting hair growth.

Keywords TGF- β 1 · Hair growth · Apigenin · Dermal papilla cell · Hair follicle

Hair growth is a cyclically controlled process [19] that consists of three distinct phases in mammals: anagen (growing phase), catagen (regressing phase), and telogen (resting phase) [4, 11, 16]. Hair follicle regression during catagen reflects a tightly coordinated process that is characterized by apoptosis and terminal differentiation of the proximal epithelial hair bulb, perifollicular proteolysis, and matrix remodeling, as well as termination of follicular melanogenesis [8, 12]. The growth and development of hair follicles are influenced by a variety of growth factors and cytokines, with the most widely described being keratinocyte growth factor, interleukin-1, and transforming growth factor β (TGF- β) [1, 2, 22].

It was recently reported that TGF- β 1 controls murine hair follicle regression (catagen) in vivo [18]. TGF- β 1 transcripts are up-regulated during late anagen and the onset of catagen [21], and TGF- β 1 is required for timely entry into the catagen phase [2]. In addition, TGF- β 1 is involved in the regulation of hair follicle regression, and is capable of inducing premature catagen in vivo via the induction of apoptosis and inhibition of keratinocyte proliferation

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[15, 17, 18, 21]. Furthermore, androgen-inducible TGF- β 1 derived from dermal papilla cells (DPCs) has been shown to mediate the suppression of hair growth and epithelial cell growth in androgenetic alopecia (AGA) [6, 7]. Collectively, TGF- β 1 operates as a catagen inducer and indirectly suppresses hair growth.

Transforming growth factor type β 1 is a member of a large family of multifunctional secreted polypeptides that are potent regulators of cell growth, apoptosis, differentiation, and matrix production [3] that also act as powerful morphogens during embryogenesis [5, 13]. The 5'-flanking sequences of the TGF- β 1 gene contain five distinct regulatory regions including a region with enhancer-like activity (-1,132 to -731), two regions that exert negative regulatory activity (-1,362 to -1,132 and -731 to -453), a region that exerts positive regulatory activity (-453 to -323), and a second promoter region (+1 to +271). In addition, the sequences downstream from the +1 start site are also required for expression of the human TGF- β 1 mRNAs is likely to be independently regulated and transcribed from this second promoter region [10]. Therefore, monitoring the activity downstream of the +1 start site in the TGF- β 1 promoter could potentially provide an appropriate method of screening for agents that promote hair growth.

In this study, a pMetLuc-TGF- β 1 vector system was constructed for use in the screening of agents that promote hair growth. To accomplish this, we initially constructed a pMetLuc-TGF- β 1 promoter vector that expresses the luciferase gene in different levels in response to differing levels of TGF- β 1 promoter activity. We then evaluated this cell-based reporter system to determine if it could be utilized to screen for agents that promote hair growth. During the evaluation of this system, we found that apigenin may be a possible agent that promotes hair growth.

SV40T-DPCs (human DPCs) were kindly provided by Professor Y.K. Sung at Kyungpook National University, Republic of Korea. The SV40T-DPCs were then transfected with pSV3neo plasmid carrying the SV40 T antigen and the neomycin-resistance gene [14]. These DPCs (SV40T-DPC) were then grown in low glucose Dulbecco's Eagle's medium (DMEM; Gibco, MD, USA) supplemented with 10% fetal bovine serum (Gibco, MD, USA), 1% penicillin/streptomycin (Gibco, MD, USA). The cells were then cultured at 37°C in a 95% air/5% CO₂ environment. Additionally, HaCaT (American Type Culture Collection, VA, USA) were incubated in high glucose DMEM (Gibco, MD, USA) supplemented with 10% fetal bovine serum (WelGene, Seoul, Korea), penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C in a 95% air/5% CO₂ environment.

The pMetLuc-TGF- β 1 constructs were generated by PCR amplification using genomic DNA obtained from HaCaT cells as a template in conjunction with the

following primer set: 5'-GGGAAGCTTTGGAAGGATCCTTAGCAGGGG-3' and 5'-GGGTCGACCGCGGAGGGAGGTGGGA-3'. PCR was conducted using KOD XL DNA polymerase (Novagen®, CA, USA) according to the manufacturer's instructions, with the following modifications: samples were subjected to initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 5 s, extension at 72°C for 2 min, and a final extension at 74°C for 10 min. Following digestion with *Hind*III and *Sal*I, the PCR products were extracted from the agarose gel. The purified fragments were then subcloned into the pMetLuc-reporter vector, which carried the luciferase reporter gene without a promoter.

HaCaT cells were transfected with the pMetLuc-TGF- β 1 promoter vector using Superfect transfection reagent according to the manufacturer's instructions (QIAGEN Co., ON, Canada). After 24 h of transfection, various chemicals [curcumin, dihydrotestosterone (DHT), apigenin] were added to the culture media. Curcumin and apigenin were dissolved in dimethylsulfoxide (DMSO; Sigma Chemical Co., MO, USA), while DHT was dissolved in ethanol (Sigma Chemical Co., MO, USA). Following stimulation, 50 µl of the culture media were used to measure the reporter gene expression. The luciferase activity was then assayed using the Ready-To-Glow™ secreted luciferase reporter assay (Clontech, CA, USA). The luminescence was measured using an LB953 luminometer (Berthold, Bad Wildbad, Germany).

After culturing the HaCaT cells on the chemical (curcumin, DHT, apigenin)-treated dishes for 24 h, the conditioned medium was harvested and the concentration of TGF- β 1 was determined using an ELISA kit (Quantikine human TGF- β 1 immunoassay, R&D systems, MN, USA) according to the manufacturer's instructions.

Cell proliferation was evaluated using an MTT assay. Briefly, SV40T-DPCs and HaCaT cells were seeded into a six-well plate at a density of 1×10^5 cells/well and then cultured in serum-free DMEM for 24 h. The cells were then treated with either a vehicle (DMSO diluted 1:1,000 in DMEM) as a control or with various concentrations of apigenin for 3 days. Next, 200 µl of MTT solution (1 mg/ml; USB, OH, USA) was added to each well, after which the plates were incubated for 3 h at 37°C in the dark. The supernatants were then removed and 1 ml DMSO (Duchefa, Haarlem, Netherlands) was added to each well to dissolve the formazan products. The samples were then incubated at room temperature for 30 min, after which the absorbance at 540 nm was measured using an ELISA reader (BioTek Instruments, VT, USA). The results were then expressed as a percentage of the control cells.

All experiments were performed in triplicate and statistical significance was determined using the Student's *t* test. A $P < 0.05$ was considered to be statistically significant.

Fig. 1 Diagram of the pMet-Luc-TGF- β 1 promoter plasmid (a) and representation of the regulatory region in the TGF- β 1 promoter (b)

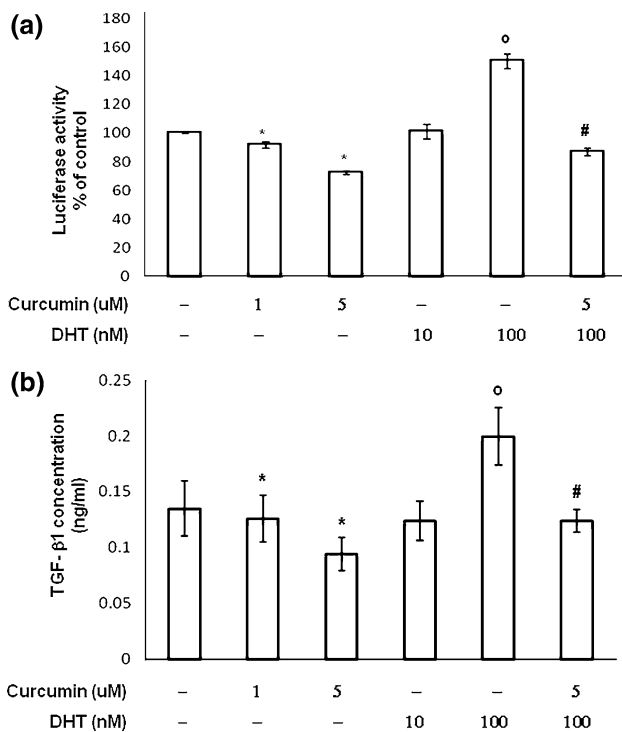
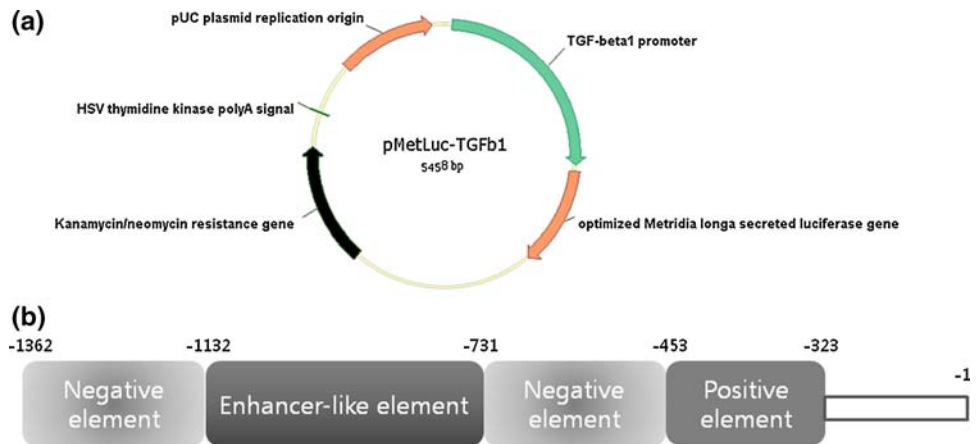


Fig. 2 Transforming growth factor β 1 promoter activity in response to treatment with curcumin and DHT in transfectant HaCaT cells. Transfectant HaCaT cells were treated with the indicated concentrations of curcumin, DHT or curcumin plus DHT for 16 h. Cultured media were then assayed for luciferase activity. Data are expressed as the mean \pm SD. The results were confirmed by four independent experiments, each of which was conducted in triplicate. * P < 0.05 versus controls (decrease of luciferase activity), ° P < 0.05 versus controls (increase of luciferase activity), # P < 0.05 versus DHT

We constructed a pMetLuc-TGF- β 1 promoter plasmid to quantify changes in TGF- β 1 promoter-dependent expression in human keratinocytes (Fig. 1a). The pMetLuc-TGF- β 1 promoter plasmid permits expression of the secreted luciferase reporter gene in a TGF- β 1 promoter-dependent manner and contains the neomycin phosphotransferase gene to confer geneticin resistance. The TGF- β 1 promoter was cloned from genomic DNA isolated from human DPCs

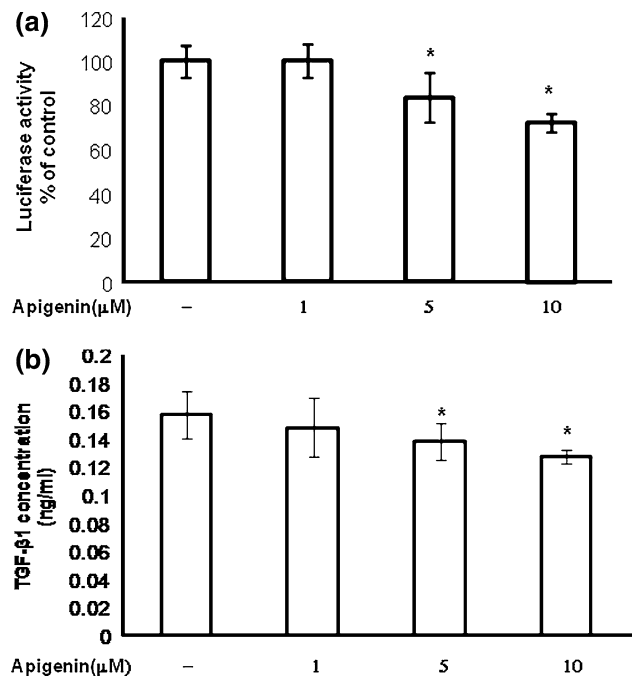


Fig. 3 a Activity of TGF- β 1 promoter in response to apigenin in transfected HaCaT cells. Transfected HaCaT cells were treated with the indicated concentrations of apigenin for 16 h. The cultured media was then assayed for luciferase activity. Data are expressed as the mean \pm SD. The results were confirmed by four independent experiments, each of which was conducted in triplicate. * P < 0.05 versus controls. b Effect of apigenin on TGF- β 1 production in HaCaT cells. HaCaT cells that were treated with apigenin showed increased TGF- β 1 production. The graph shows the results of three different experiments conducted in triplicate. Data are expressed as the mean \pm SD. * P < 0.05 versus controls

using the aforementioned primers. This promoter contains five distinct regulatory regions, including a region with enhancer-like activity, two negative regulatory regions, a positive regulatory region, and a second promoter region (Fig. 1b) [10].

To determine if human epidermal keratinocytes (HaCaT) expressed the luciferase gene in a TGF- β 1 promoter-dependent manner, the reporter enzyme activities were assayed

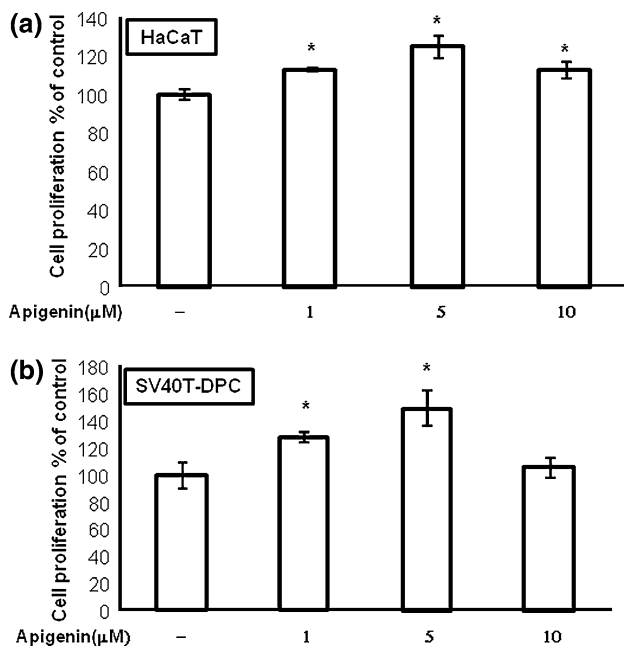


Fig. 4 Effects of apigenin on the proliferation of HaCaT (a) and SV40T-DPC (b) cells. HaCaT cells (a) and SV40T-DPCs (b) were treated with vehicle or the indicated concentrations of apigenin for 3 days. Cell proliferation was then evaluated by MTT assay. The values shown represent the mean \pm SD of triplicate measurements of four separate experiments. Values are shown as percentages of the control. * $P < 0.05$ versus controls. AP apigenin

using a detection method capable of measuring the enzyme activity in transfectant cells. In this study, curcumin was introduced as a negative control because it is known to inhibit TGF- β 1 expression via inhibition of activating protein-1. In addition, DHT was used as a positive control because it up-regulates the expression of TGF- β 1. [6, 9, 10, 20] As shown in Fig. 2, curcumin reduced the reporter luciferase activity in a concentration-dependent manner, whereas DHT increased TGF- β 1 promoter activity. These results suggest that transformed HaCaT cells may potentially be used to screen for agents that affect TGF- β 1 promoter activity. Consistent with these findings, TGF- β 1 production was inhibited by curcumin and induced by DHT (Fig. 2b).

When screening for TGF- β 1 inhibitors using this system, apigenin was identified as a candidate for the promotion of hair growth. As shown in Fig. 3a, treatment of the transfectant HaCaT cells with apigenin attenuated the TGF- β 1 promoter activity when compared with that of the control. These findings were further confirmed by the attenuated expression of TGF- β 1 protein by apigenin (Fig. 3b). Specifically, treatment with 5 and 10 μM apigenin inhibited TGF- β 1 production in a concentration dependent.

It has also been reported that TGF- β 1 inhibits human keratinocyte proliferation in vitro [23]; therefore, we evaluated apigenin to determine if it could inhibit the TGF- β 1-induced effects via downregulation of the

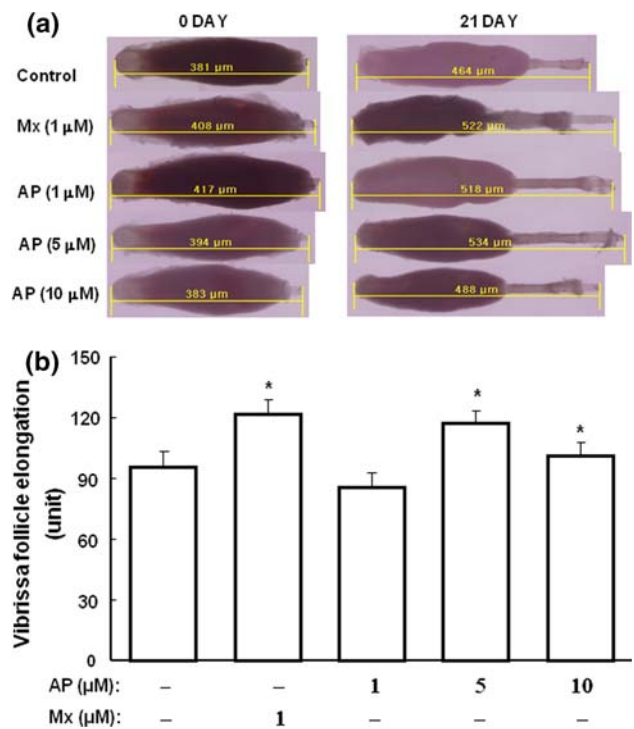


Fig. 5 The effects of apigenin on hair growth in rat vibrissa follicles. a Representative photomicrographs of rat vibrissa follicle organ cultures. Hair follicle organ cultures were performed in triplicate using hair follicles obtained from rat vibrissa. Apigenin was found to significantly increase hair shaft growth, especially at concentrations of 5 and 10 μM . The results were confirmed by three independent experiments. b Quantification of rat vibrissa follicle organ cultures. * $P < 0.05$ versus controls. Mx minoxidil sulfate, AP apigenin

TGF- β 1 gene. To accomplish this, cell proliferation assays were performed using human DPCs (SV40T-DPCs) and HaCaT cells. We found that treatment with apigenin at concentrations of 1 and 5 μM significantly increased the proliferation of HaCaT when compared to treatment with a vehicle-treated control. However, treatment with higher concentrations of apigenin (10 μM) were less effective than treatment with 5 μM apigenin, although the amount of enhanced hair growth induced by 10 μM apigenin was significantly higher than that of the vehicle-treated control (Fig. 4a). In this study, we found that apigenin induces cell proliferation in HaCaT cells. However, it is generally accepted that the properties of HaCaTs are different from those of primary keratinocytes, especially hair matrix keratinocytes. Therefore, we evaluated apigenin to determine if it exerts the same proliferation effect in human DPCs as it does in HaCaT cells. We found that the proliferation of SV40T-DPCs increased significantly in response to treatment with apigenin at concentrations of 1 and 5 μM when compared to treatment with the vehicle-treated control (Fig. 4b).

To determine if apigenin also exerts a direct effect on the growth of whole hair follicle cells, we evaluated the

elongation of hair follicles in rat vibrissa. To accomplish this, we isolated rat vibrissa follicles and then quantified the changes of hair shaft and follicle size in organ cultures *in vitro*. The length of vibrissa follicles that were treated with apigenin at concentrations of 5 and 10 μM increased significantly when compared with vehicle-treated controls (Fig. 5). Specifically, the size changes in the length of vibrissa follicles that were treated with 5 μM apigenin, 10 μM apigenin or vehicle were 117.1 ± 6.6 , 101.0 ± 6.8 , and 95.9 ± 7.6 U, respectively. However, treatment with a higher concentration of apigenin (10 μM) was less effective than treatment with 5 μM apigenin, although the increase in hair growth induced by treatment with 10 μM apigenin was significantly higher than of the increase in growth induced by the vehicle-treated control. The addition of minoxidil sulfate (which was dissolved in DMSO) to vibrissa cultures as a positive control also resulted in a significant increase in the growth of the hair shaft. Specifically, minoxidil sulfate induced a 121.8 ± 6.9 U increase in the growth of the hair shaft. These findings indicate that apigenin promotes the growth of hair shaft.

Taken together, these results suggest that pMetLuc-TGF- β 1 reporter-transfected cells can be used to screen hair growth-promoting agents and to monitor signal transduction pathways that are involved in the hair growth cell cycle. In addition, apigenin, which was selected as a suppressor of TGF- β 1 expression by a cell-based system that utilizes a pMetLuc-TGF- β 1 reporter, was found to induce hair cell growth, suggesting that apigenin might be useful as an adjunctive therapy for the treatment of AGA.

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