Knockout of p16^{INK4a} promotes aggregative growth of dermal papilla cells

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Summary

Objective: Dermal papilla cells (DPCs) are located in the hair follicles and play an important role in hair growth. These cells have the ability to induce hair follicle formation when they display aggregative behavior. DPCs derived from the androgenetic alopecia (AGA) area undergo premature senescence in vitro, associated with p16^{INK4a} expression. The aim of the current study was to investigate the expression of p16^{INK4a} in aggregative and non-aggregative DPCs and the effect of p16^{INK4a} down-regulation in these cells by adenovirus-mediated RNA interference (RNAi).
Method: DPCs were isolated and cultured from healthy human scalp. p16^{INK4a} gene and protein were detected in aggregative and non-aggregative cells. Expression of p16^{INK4a} in DPCs was silenced by infection with rAd5-CDKN1A-1p2shRNA. Cell fate was monitored after infection. The growth of cells was measured by MTT assay. Cell cycle was evaluated by flow cytometry (FCM).
Results: DPCs were isolated by digestion and showed aggregative behavior for six passages. The expression of p16^{INK4a} showed a clear upward trend in non-aggregative cells when compared with aggregative group. p16^{INK4a} expression was silenced by rAd5-CDKN1A-1p2shRNA ($p<0.05$). The p16^{INK4a}-silenced cells grew more rapidly and exhibited a trend towards aggregative growth. There was an increase in the proportion of cells in G1 phase, while those in S phase were reduced after p16^{INK4a} gene silencing ($p<0.05$).
Conclusion: Our results suggest that p16^{INK4a} plays an important role in the premature senescence and aggregative behavior of DPCs. These observations can lead to novel therapeutic strategies for treatment of AGA.

Keywords: hair follicle, transfection, hair/growth and development.

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Introduction

Dermal papilla cells (DPCs) are the main mesenchymal cells located at the bottom of the hair follicle (HF) and compose the dermal papilla. The biological characteristic of these cells is the ability to induce HF formation, both in vivo as well as in vitro. DPCs are a cluster of specialized fibroblasts and occur at the central link in the morphology of the HF and its cyclic growth regulation.[1,2] During the initial stage of HF morphogenesis in the course of embryonic development, the ectodermal epithelial cells proliferate and differentiate downward continuously after being stimulated by dermis signaling molecules to form the hair peg. The hair peg provides feedback to the dermis and induces the formation of dermal concentrate by dermal fibroblast in the dermis, to give rise to the dermal papilla precursor. Some of the aggregative dermal fibroblast cells then differentiate into dermal papilla cells while others turn into dermal sheath cells surrounding the columnar hair follicle epithelial cells.[3-5] In other words, HF formation is directed by an aggregation of dermal mesenchymal cells, which form the origin of DPCs in the embryonic skin. Furthermore, cultured DPCs also have hair-forming activity if they are in an aggregative mode. Aggregative growth is a crucial feature of DPCs to induce hair follicle formation and is characterized by radial growth that occurs prior to fusion of DPCs.[3] DPCs that grow in a radial pattern in an aggregative manner are spindle-shaped with abundant cytoplasm. But the characteristic

of aggregative growth gradually disappears in vitro, eventually ceasing entirely. It was reported that when DPCs of low passage were inoculated into a small incision on the mouse auricle, with high-passage cells and fibroblasts as control, clusters of hair fibers grew on the incision containing low passage DPCs. These hair fibers were thicker and longer than naturally occurring hair on the ear, and were similar to the tentacles from where the DPCs were taken. On the other hand, high-passage DPCs and fibroblasts did not show this phenomenon.[6] These observations confirm that low passages of cultured DPCs with aggregative growth not only have the ability to induce complete regeneration of hair follicles, but also carry the information needed to determine the nature of the hair, as corroborated by subsequent research.[7,8] It should be emphasized that the ability of DPCs to induce HF formation is dependent on their aggregative growth. But the mechanism by which the aggregative behavior disappears is not yet clear.

$p16^{INK4a}$ is a cyclin-dependent kinase (CDK) inhibitor that slows down cell cycle by inhibiting transition from G1 to S phase. Normally, CDK4/6 binds cyclin D to form an active protein complex that phosphorylates retinoblastoma protein (pRB). Once phosphorylated, pRB disassociates from the transcription factor E2F1, thus liberating E2F1 from its cytoplasm bound state, thereby allowing it to enter the nucleus. In the nucleus, E2F1 promotes transcription of target genes that are essential for transition from G1 to S phase.[9,10] Tissue ageing causes $p16^{INK4a}$ concentration to increase dramatically.[11] $p16^{INK4a}$ has also been used as a target to delay certain changes related to ageing in mice.[12] Recent reports have demonstrated that DPCs taken from male androgenetic alopecia (AGA) patients undergo premature senescence in vitro associated with the expression of $p16^{INK4a}$. We hypothesized that non-aggregative growth was a feature of ageing and that aggregative growth characteristics were correlated with $p16^{INK4a}$. We studied $p16^{INK4a}$ expression in DPCs and inhibited $p16^{INK4a}$ expression in DPCs by adenovirus-mediated RNA interference to explore the possible mechanisms of cultured human DPCs losing aggregative growth characteristics.

Method

Ethics statements

The Fourth Hospital of Hebei Medical University institutional review board approved all described studies. The study was conducted according to the Declaration of Helsinki Principles. Informed written consent was obtained from all patients.

Isolation and culture of dermal papilla cells

Specimens were taken from the occipital scalp of six male individuals undergoing surgical excision of benign cutaneous tumors. The patients were not using any hair loss medications when the samples were collected. Dermal papillae were isolated from human scalp hair follicles by digestion with collagenase D and dispase (Sigma, USA) as described earlier.[13] The cells isolated were then cultivated in Dulbecco's modified Eagle's medium (DMEM) containing 15% fetal bovine serum (FBS), 100 IU/mL penicillin, 100 μg/mL streptomycin and 0.4 mM L-glutamine (Sigma, USA) in a 95% humidified atmosphere with 5% CO_2 at 37°C.

RT-PCR

$p16^{INK4a}$ expression at the transcriptional level from different generations of DPCs was tested by RT-PCR. The ddH2O group was regarded as a negative control. Trizol reagent (Suo Bao Lai Biotechnology Co. Ltd, Shanghai) was used to extract total RNA and reverse transcription of the total RNA was carried out using reverse transcriptase and oligo(dT) primers (Tian En Ze technical Co. Ltd, Beijing) according to the manufacturer's instructions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal reference. The primer sequences of RT-PCR were as follows:

- $p16^{INK4a}$ forward primer: 5'-CATCCCCGATTGAAAGAACC-3';
- reverse primer: 5'- AATGGACATTTACGGTAGTGGG-3';
- GAPDH forward primer: 5'-TGAACGGGAAGCTCACTGG-3';
- reverse primer: 5'-GCTTCACCACCTTCTTGATGTC-3'.

PCR was carried out for 40 cycles (95°C for 10 s, 58°C for 20 s and 72°C for 20 s) according to instructions supplied with the Hot Start Fluorescent PCR Core Reagent Kits (Bio Basic) in a real-time fluorescent quantitative PCR cycler (Edinburgh biological technology development Co. Ltd, Shanghai).

Immunohistochemistry

Different passages of cultured DPCs were seeded at a density of 1×10^5 on to flame-sterilized glass coverslips, which were placed in six well plates and cultured in an incubator up to a convergence rate of 30-50%. The cells were then fixed with 4% (v/v) paraformaldehyde for 15 minutes, and treated with 1% (v/v) hydrogen peroxide to block the endogenous peroxidase, followed by incubation in 10x PBS-diluted goat serum at room temperature for 30 minutes. The coverslips were then incubated overnight

with primary antibody diluted in PBS at 4°C. After incubation with primary antibodies, cells were washed thrice with PBS. Secondary and tertiary antibodies (Ding Guo Biotechnology Co. Ltd, Beijing) were used as per the manufacturer's instructions. Immunolocalization was visualized with 3,3'-diaminobenzidine tetrahydrochloride solution (Ding Guo Biotechnology Co. Ltd, Beijing). Cells were counterstained with hematoxylin and mounted with Permount TM Mounting Medium.

Flow cytometry

Flow cytometric analysis was done as described earlier.[14] Confluent DPCs were washed with ice cold PBS and then fixed with 0.5% paraformaldehyde for about 5-10 minutes. Cells were then washed with PBS and blocked with 0.1% BSA. Finally, anti-p16^{INK4a} antibodies were added and incubated overnight at 4°C. Cells were washed twice with PBS, trypsinized and resuspended in 250 µL of fluorescence labeled secondary antibody for analysis.

Transfection

p16^{INK4a} gene sequence was obtained from Genbank (NP-00068.1). RNAi adenovirus targeting p16^{INK4a} (rAd5-CDKN1A-1p2shRNA) and the negative control adenovirus vector (rAd5-HKshRNA-EGFP) were obtained from Wuhan Ximar Biological Co., Ltd. Cells from the 8th generation were seeded in 25 cm^2 cell culture flasks. Cells were divided into three groups, non-transfected cells as blank control group (CON), cells transfected by RNAi targeting p16^{INK4a} via rAd5-CDKN1A-1p2shRNA as gene knockout group (shp16), and cells transfected by rAd5-HKshRNA-EGFP as the negative control group (NC). Diluted adenovirus was added into culture medium according to MOI=100 (concentration of rAd5-CDKN1A-1p2shRNA was 2.4×10^8/mL, concentration of rAd5-HKshRNA--EGFP was 1.2×10^9/mL). Cells were incubated for 4 hours in 37°, 5% CO_2 incubator after transfection, then the medium was replaced by MSCM medium. The cells were finally collected after 48 hours for RNA extraction and detection of protein in order to verify the effects of RNAi.

MTT assay

DPCs of passage 8 were plated in a 96-well plate (1×10^4 per well), and divided into three groups: shp16, NC and CON.Diluted adenovirus was added into culture medium according to MOI=100 (concentration of rAd5-CDKN1A-1p2shRNA was 2.4×10^8/mL, concentration of rAd5-HKshRNA-EGFP was 1.2×10^9/mL). The serum-free medium was replaced by MSCM culture medium after 4 hours. MTT dye dissolved in 0.15 mL of DMSO was added to different groups of wells (5 mg/mL) after 24, 48, 72, 96 and 120 hours. Data were analyzed and growth curves of different groups were plotted.

Immunofluorescence

3×10^5 cells were inoculated in each well of a six-chamber slide and cultured for 24 hours. The samples were prepared as follows: cells were treated with 4% paraformaldehyde for 20 minutes, followed by 0.1% Triton X-100 for 10 minutes at room temperature, then rinsed with PBS twice. The washed cells were treated with 0.2% BSA for 30 minutes at room temperature, followed by treatment with 1,000x diluted p16^{INK4a} antibody. FITC-labeled secondary antibody was then added and incubated at room temperature for 1 hour. The overnight-treated sample was then treated with 5 µg/mL FITC-phalloidin conjugate for 30 minutes at room temperature, washed twice with PBS and stained for 5 minutes with 250x diluted DAPI (Sigma). The samples were protected from light during the procedure.

Statistical analysis

All values are presented as means ± standard deviations of replicate samples. Experiments were repeated a minimum of three times. Differences were assessed using unpaired two-tailed Student's t test and analysis of variance. In all statistical comparisons, $p<0.05$ was defined as significant. SPSS statistics software (Version 15.0) was used for all calculations.

Results

Dermal papillae were intact, spherical (Figure 1A) or ellipsoidal in shape after digestion. Dermal papillae attached quickly within one day and the dermal papilla cells migrated as early as overnight after being plated. On the first day, the rate of attachment was 95%, increasing to 99% by the third day. The initial outgrowth of cells from the dermal papilla explants usually occurred within one day. The outgrowth of cells from DP explants appeared like sunflowers (Figure 1B). The cells appeared spindle-shaped and contained abundant cytoplasm when they formed multilayered parallel arrays. The DPCs proliferated quickly and could be subcultured into passage 2 after 2 weeks of primary culture. At low densities, DPCs were generally spindle-shaped and had a tendency to aggregate and clump into multilayered structures after passage. This aggregative behavior was more pronounced in the first two subcultures and was not observed beyond passage 6. Upon continued passages, the cells became much larger, more flat, polymorphic, proliferated more slowly, and the aggregative behavior gradually disappeared.

We observed that different generations of DPCs consistently expressed $p16^{INK4a}$ mRNA, although the expression was very weak. Compared with the passage 5 group, expression of $p16^{INK4a}$ in the passage 6 group was slightly increased. Remarkably, the expression in the passage 8 group was significantly elevated when compared with the passage 6 ($p<0.01$) and passage 5 ($p<0.01$) groups (Figure 1C). As expected, $p16^{INK4a}$ gene was not detected in the ddH2O group.

Since the results of Western blot analysis of $p16^{INK4a}$ protein were negative, we investigated expression of $p16^{INK4a}$ protein in different generations by FCM and immunocytochemistry. Weak expression of $p16^{INK4a}$ protein was detected in passage 4 and passage 8 groups by immunocytochemistry. On the other hand, $p16^{INK4a}$ was undetectable in the negative control group. We observed a higher expression of $p16^{INK4a}$ protein in the passage 8 group when compared to the passage 4 group. Additionally, expression of $p16^{INK4a}$ protein was also detected by FCM. The FI value of $p16^{INK4a}$ protein was 1.76±0.05 and 2.03±0.06 in passage 4 and passage 8 respectively, which was not significantly different ($p>0.05$).

It was shown by real-time PCR that 72 hours after transfection with rAd5-CDKN1A-1p2shRNA, rAd5-HK-shRNA-EGFP and non-transfected group (shp16, NC and CON group) as described in materials and methods section, the level of $p16^{INK4a}$ mRNA in cells of the shp16 group was significantly lower than the two control groups ($p<0.01$). The relative expression of $p16^{INK4a}$ was 1.00 in the CON group, 0.66 in the NC group and 0.04 in the

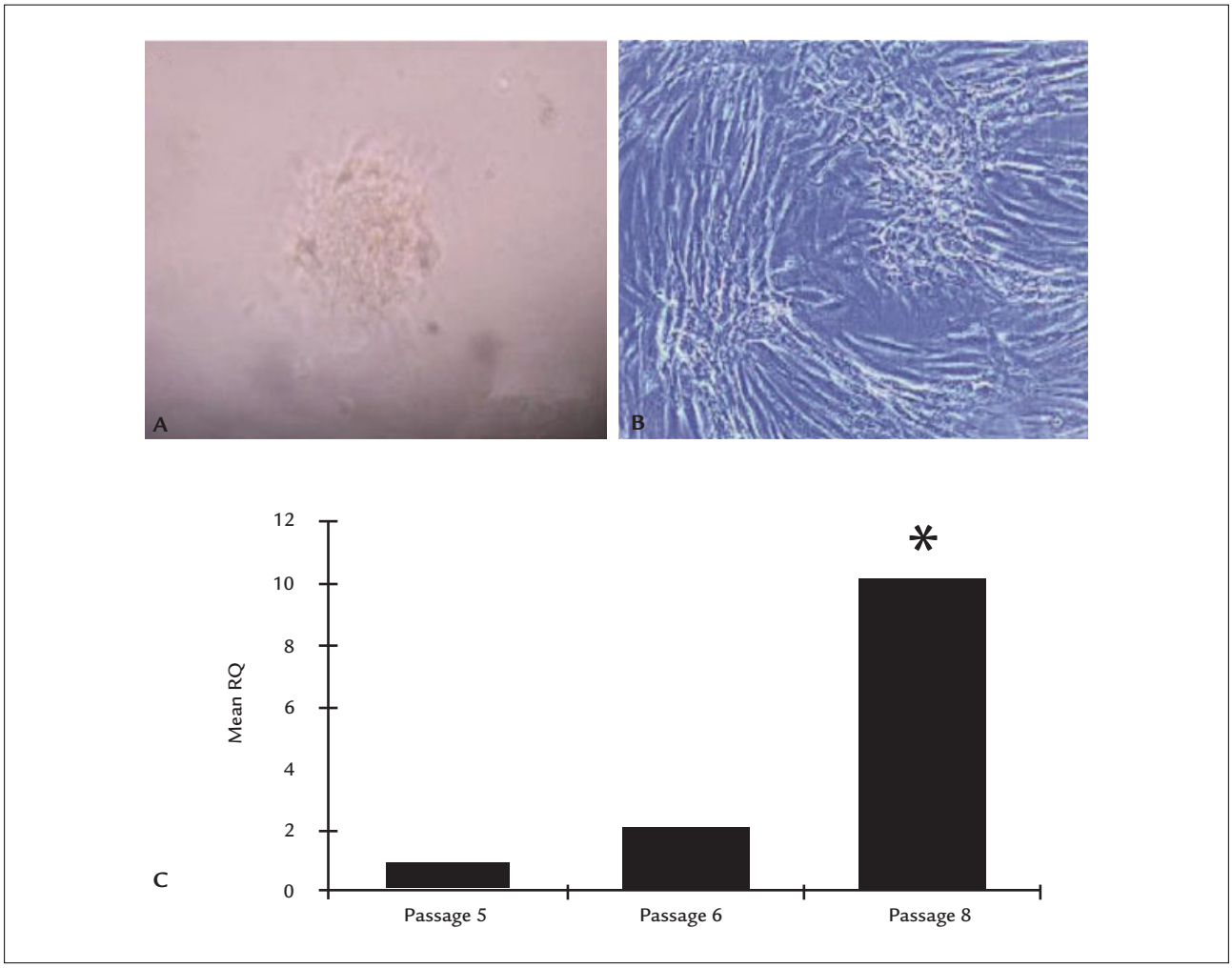

FIGURE 1 A. Dermal papillae were isolated from hair scalp. B. Dermal papilla cells spread out from dermal papillae like a sunflower after adhesion. C. Expression of p16 mRNA in DPCs. The mRNA in the passage 6 group was slightly increased compared with the passage 5 group, and the mRNA in the passage 8 group was significantly elevated compared with passage 6 and passage 5 groups (*$p<0.01$).

shp16 group. After p16 gene knockout, the DPCs morphology tended to fusiform growth, showing a tendency of aggregative growth (Figure 2A and B).

The expression of p16INK4a protein in cells was detected by FCM after transfection. The value of FI in each group is shown in Figure 2C. Expression of p16INK4a protein in the shp16 group was significantly less than the NC ($p<0.05$) and CON ($p<0.05$) groups in the 8th generation. There was no difference in the NC group when compared with the CON group in the 8th generation ($p>0.05$).

The OD 595 nm value of cells from different groups from 1st to 5th day in 96 well plates are shown in Table 1 and growth curves were plotted from these values (Figure 3A). It was evident from these curves that cells grew more rapidly after silencing of the p16INK4a gene, but this effect was more obvious after day 3.

TABLE 1 DPC OD 595 nm value in different groups at different times (±S).

	NC group	CON group	shp16 group
24h	0.196±0.030	0.164±0.039	0.206±0.047
48h	0.266±0.048	0.283±0.063	0.253±0.033
72h	0.325±0.027	0.314±0.064	0.359±0.081
96h	0.369±0.045	0.406±0.075	0.519±0.079
120h	0.465±0.057	0.437±0.070	0.536±0.052

Changes of the cell cycle in different groups were detected by FCM (Figure 3B). The results revealed a statistically significant increase in cells in G1 phase, with a concomitant reduction in cells in S phase after gene silencing.

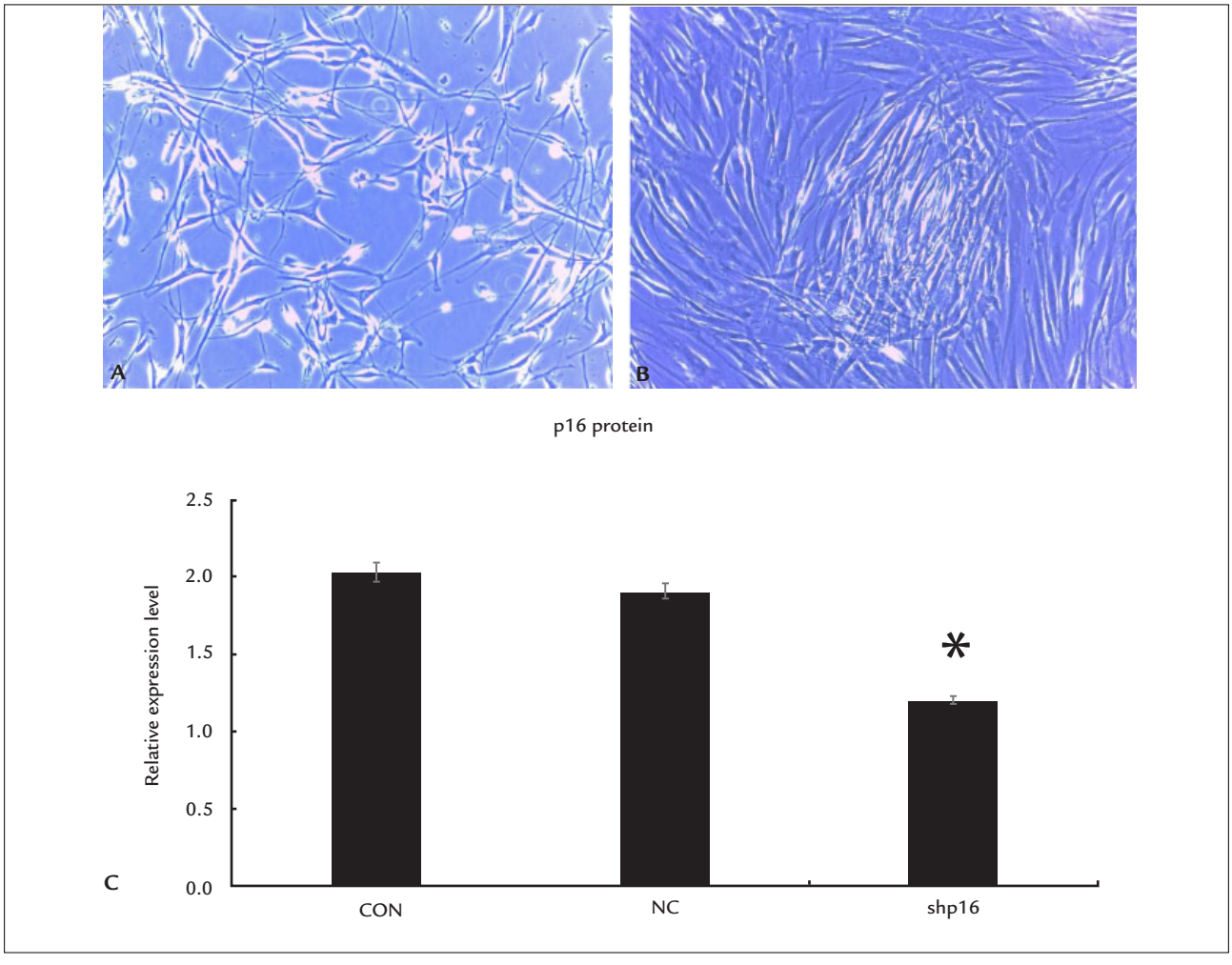

FIGURE 2 A. DPCs of 8 generation without transfection. B. DPCs of 8 generation transfected by adenovirus vector. After p16 gene knockout, the DPCs morphology tended to fusiform growth, promoting a tendency of aggregative growth. C. p16INK4a protein expression detected by FCM in NC, CON and shp16 groups. p16INK4a protein expression in shp16 group was significantly less than that in the NC and CON groups in the 8th generation (*$p<0.05$).

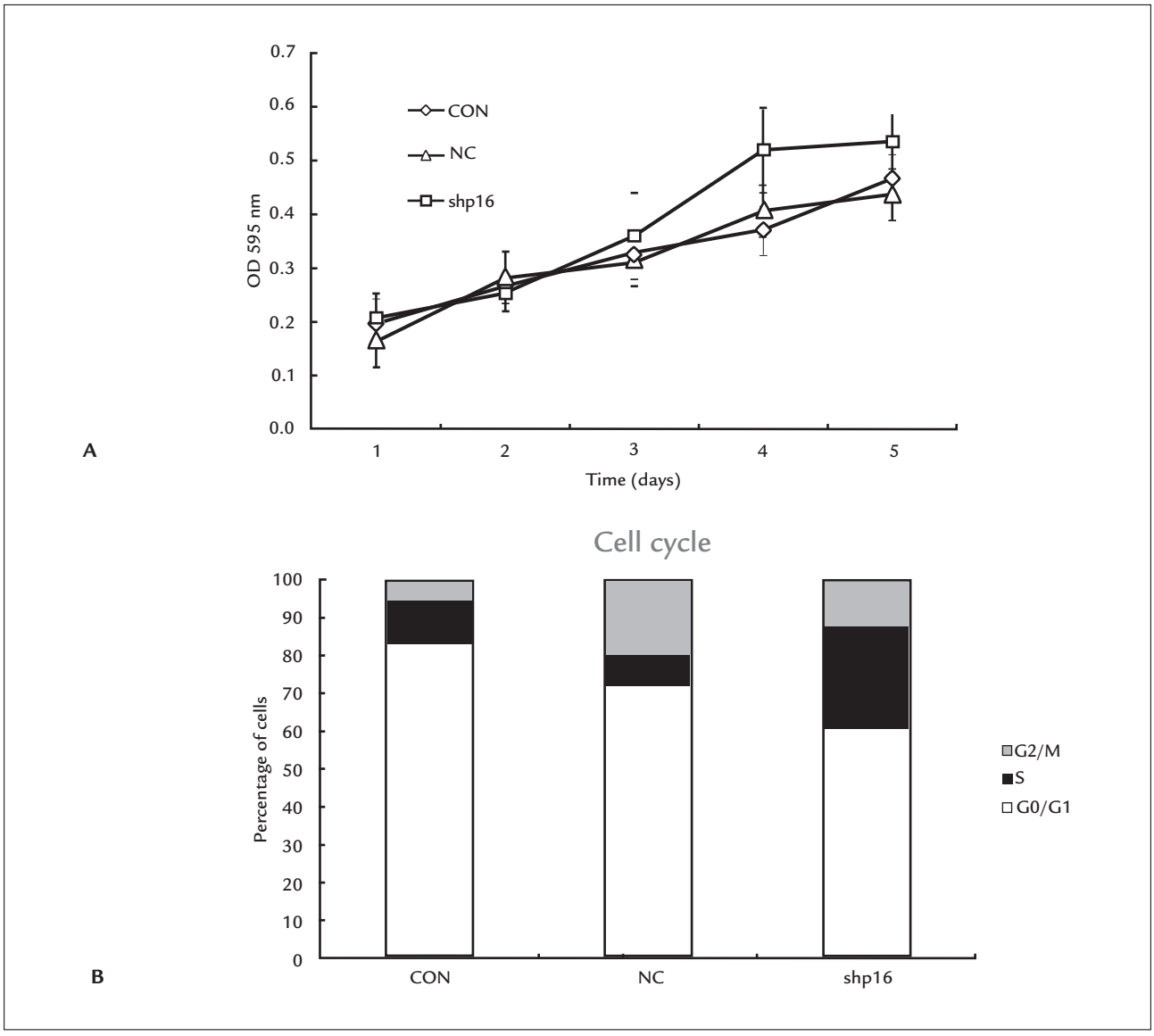

FIGURE 3 A. The growth curves of different groups after transfection. DPCs grew more rapidly after silencing of the $p16^{INK4a}$ gene, and this effect was more obvious after day 3. B. Cell cycle in NC, CON and shp16 groups detected by FCM is shown. Cells in G1 increased, while those in S phase reduced after gene silencing, the difference was statistically significant ($p<0.05$).

Discussion

In this study, we investigated whether $p16^{INK4a}$ has an effect in premature DPC senescence and aggregative behavior. We found that, compared with the aggregative group, the expression of $p16^{INK4a}$ in the non-aggregative group increased as DPCs showed a gradual aging trend. DPCs grew more rapidly and exhibited a trend of aggregative growth when $p16^{INK4a}$ expression was silenced by rAd5-CDKN1A-1p2shRNA. Upon $p16^{INK4a}$ silencing, there was an increase in cells in G1 phase, with a simultaneous reduction in cells in S phase. Our results show that $p16^{INK4a}$ plays an important role in premature senescence and aggregative behavior of DPCs.

Human scalp DPCs were isolated by enzyme digestion in vitro as described in previous studies.[13] We were able to confirm in this study that DPCs were efficiently isolated by collagenase D and dispase. The DPCs showed aggregative growth until passage 6, after which they gradually lost the aggregative growth characteristics in the late period. It is well known that primary DPCs undergo spontaneous senescence during subcultivation. Accordingly, we also observed that late-passage DPCs were more senescent.

Previous studies showed that low-passage DPCs could sustain epidermal cell proliferation[15] and hair growth-promoting capabilities,[16,17] but high-passage DPCs could not. These evidences support the notion that non-aggregative DPCs may have a functional defect. The phenomenon of gradual disappearance of the physiological functions of DPCs when aggregative growth characteristics disappear may be associated with changes in cytokines and cell senescence. A published work suggests that expression of a series of cytokines including FGF7, IGF-1, SCF, VEGF etc. changed after different passages of DPCs.[14,15] Additionally, research by Bahta et al.[18] demonstrated that senescence of balding DPC is associated with increased expression of p16INK4a and pRb but not p53, suggesting that in vitro senescence of balding DPCs is stimulated by environmental stress and not due to replicative senescence. A similar observation has been documented[19] that oxidative stress as a result of passage-induce telomere shortening, and not replicative senescence, was responsible for the commonly observed senescence of dermal fibroblasts.[20]

Work by Yang et al.[21] showed an increase in expression of p16INK4a in balding DPCs from AGA patients with premature senescence, suggesting that androgen/androgen receptor signaling promotes senescence via the p16INK4a pathway in DPCs. We also found weak but gradually increasing expression of p16INK4a in 5, 6, 8 generation DPCs. p16INK4a protein could not be detected by Western blot, possibly owing to its weak expression. Our results suggest that p16INK4a may play an important role during senescence of DPCs and also in the transition from aggregative to non-aggregative growth of DPCs. In order to further clarify the biological characteristics of p16INK4a in DPCs, we constructed a shRNA adenovirus vector targeting p16INK4a and stably transfected DPCs. We found that p16INK4a protein expression decreased significantly after silencing of the p16INK4a gene, along with an increase in cells in G1 phase and an accompanying reduction in cells in S phase, consistent with mechanism of p16INK4a in the cell cycle. There was a tendency for DPCs to show aggregative growth after transfection, suggesting that non-aggregative growth is a form of cellular senescence, which can be influenced by p16INK4a to a certain extent. The expression of β-galactosidase, pRB and p53 remain to be detected in different passages and evaluated for their influence on DPCs in the future work.

In conclusion, we present a potential link between aggregative growth of DPCs and p16INK4a, and to a certain degree reveal the mechanism of aggregative growth of DPCs. We also emphasize the beneficial use of knockdown of the p16INK4a gene, which potentially contributes to the maintenance of aggregative growth and allows more passages of DPCs. Furthermore, this knockdown may provide additional therapies for the treatment of AGA.

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