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Regulation of hair follicle development by exosomes derived from dermal papilla cells

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ABSTRACT

Background: Dermal papilla cells (DPCs) play a critical role in the regulation of hair follicle (HF) growth, formation, and cycling. DPCs are thought to regulate HF growth through a paracrine mechanism, in which exosomes may play a critical role.

Methods: DPC-Exos were cutaneously injected into HFs at different HF cycle stages and the effects were evaluated by histological and immunohistochemical analyses. The effects of DPC-Exos on proliferation, migration, and cell cycle status of outer root sheath cells (ORSCs) were evaluated. After treatment of DPC-Exos, changes in mRNA and protein levels of β-catenin and Sonic hedgehog (Shh) in ORSCs were detected.

Results: DPC-Exos were approximately 105 nm in diameter and expressed tumor susceptibility gene 101, cluster of differentiation (CD)9, and CD63. Injection of DPC-Exos accelerated the onset of HF anagen and delayed catagen in mice. Immunohistochemical analyses revealed that β-catenin and Shh levels were upregulated in the skin. In vitro, DPC-Exo treatment enhanced ORSC proliferation and migration, and stimulated the expression of β-catenin and Shh.

Conclusion: DPC-Exos contribute to the regulation of HF growth and development, and provide a potential avenue for the treatment of hair loss.

1. Introduction

In androgenetic alopecia, the hair follicles (HF) exhibits shorter anagen and longer telogen phases, resulting in baldness. Hair loss is detrimental to self-esteem and quality of life. However, currently available treatments are ineffective. Drugs lead to improvement only over the short term, whereas HF transplantation is costly and there is a shortage of donors [1]. There is therefore a need for more effective and novel treatment methods.

Hair growth and HF development involve interactions between epithelial and mesenchymal cells [2]. This interaction is important not only for differentiation during embryogenesis, but also for the regulation of hair cell proliferation and migration in adults [3]. Dermal papilla cells (DPCs) and outer root sheath cells (ORSCs) derived from the mesenchyme and epithelium, respectively, are key regulators of HF development and growth [4,5]. As a type of specialized mesenchymal cell, DPCs not only promote follicular epithelium function and regulate HF growth, formation, and cycling, but also play a role in the pathogenesis of hair loss [6]. Previous studies have reported that DPCs regulate ORSC proliferation and migration [7], and modulate ORSC differentiation [8] as well as HF-specific structure and function [6].

Recent evidence has suggested that DPCs exert their regulatory function of HF growth mainly through a paracrine mechanism [9]. DPCs release various factors including epidermal growth factor, transforming growth factor-β, and keratinocyte growth factor to stimulate follicular epithelium proliferation and differentiation and modulate mesenchymal-epithelial interactions [10].

Exosomes are small vesicles that are an important component of paracrine signaling. They are secreted by various cells and contain proteins, mRNAs, and micro (mi)RNAs and enable cell-cell communication [11]. Exosomes derived from mesenchymal stem cells (MSC-Exos) have been shown to stimulate dermal fibroblast proliferation and migration and modulate scar formation [12]. Exosomes released by keratinocytes affect melanocyte pigmentation [13], and those derived from human amniotic epithelial cells promote wound healing [14]. Based on these observations, we speculated that exosomes secreted by DPCs have a regulatory role

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in HF growth and development, which has not been previously investigated.

In this study, we investigated the function of DPC-Exos in HF cycle regulation and hair cell growth. We found that the DPC-Exos induced anagen while delaying catagen, producing longer hair shafts and larger bulges in mice. Meanwhile, DPC-Exos enhanced the proliferation and migration of ORSCs in vitro. We also demonstrated that these effects were mediated by β-catenin and Sonic hedgehog (Shh) signaling. These data highlight a novel role for DPC-Exos in the regulation of HF growth and development and provide a potential avenue for the treatment of hair loss.

2. Materials and methods

2.1. Isolation and culture of normal DPCs and ORSCs from human scalp

Healthy human scalp specimens (n = 15; from 10 females and five males, age range: 18–50 years) were obtained with informed consent from subjects without systemic diseases who were undergoing cosmetic surgery. ORSCs and DPCs were isolated from scalp HFs by a two-step enzyme digestion method [15]. The ORSC suspension was washed and centrifuged twice at 800 × *g* for 5 min and then resuspended in complete keratinocyte serum-free medium (SFM) [16]. DPCs were resuspended in Dulbecco's modified Eagle's medium/F-12 (1:1) containing 10% FBS. ORSCs and DPCs were transferred to 25-ml flasks and cultured at 37 °C in a humidified atmosphere of 5% CO_2. The study protocol was approved by the Zhejiang University School of Medicine Second Affiliated Hospital Institutional Review Board.

2.2. Isolation and identification of DPC-Exos

DPC-Exos were isolated and purified according to an established protocol [17]. Briefly, DPCs were washed twice with PBS and the culture medium was replaced with Minimal Essential Medium Eagle Alpha Modification (1 ×) (Hyclone, Logan, UT, USA) when the cells reached 70%–80% confluence. They were then cultured for an additional 48 h at 37 °C at 5% CO_2. The supernatant was centrifuged at 300 × *g* for 10 min and then at 1500 × *g* for 10 min, before passage through a 0.22-μm filter (Steritop; Millipore, Billerica, MA, USA). The conditioned medium was transferred to an Ultra-clear tube (Millipore) and centrifuged at 4000 × *g* until the volume of ultrafiltration liquid remaining in the upper compartment was about 200 μL. The ultrafiltration liquid was washed twice with PBS and the ultrafiltration was repeated to 200 μL. For exosome purification, the liquid was ultracentrifuged at 100,000 × g for 2 h. The pelleted exosomes were resuspended in 15 mL PBS and centrifuged at 4000 × g in Ultra-clear tubes until the final volume in the upper compartment was reduced to approximately 200 μL. Exosomes were stored at −80 °C until use.

DPC-Exo concentration and size distribution were determined by tunable resistive pulse sensing on a qNano instrument (Izon Science, Christchurch, New Zealand). Exosome morphology was observed by transmission electron microscopy. Antibodies against cluster of differentiation (CD)9, CD63, and tumor susceptibility gene (TSG)101 (all from Abcam, Cambridge, MA, USA) were used to detect cell surface expression of these proteins by western blotting.

2.3. Reverse transcription (RT)-PCR

ORSCs were grown to 70%–80% confluence, and then treated with DPC-Exos or left untreated. Total RNA was extracted using TRIzol reagent (Ambion, New York, NY, USA) and stored at −80 °C. RT was performed using a high-capacity RNA to cDNA kit (Takara Bio, Otsu, Japan). RT-PCR was carried out with SYBR Green I Master Mix (Takara Bio) and the following forward and reverse primers (synthesized by Sangon Biotech, Shanghai, China): β-catenin, 5′-CGGTCGGTTGATGAGACTACT-3′ and 5′-CAGGGCTCTGTCAAGATCACC-3'; Shh, 5′-CTCGCTGCTGGTATGCTCG-3′ and 5′-ATCGCTCGGAGTTTCTGGAGA-3'; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) 5′-TGAAGGTCGGAGTCAACGG-3′ and 5′-TGGAAGATGGTGATGGGAT-3'. PCR was performed for 40 cycles, and the $2^{-\Delta\Delta Ct}$ method was used to compare the relative mRNA expression levels of target genes to that of GAPDH.

2.4. Western blot analysis

Total cellular protein was extracted with lysis buffer (Beyotime Institute of Biotechnology, Beijing, China) and 40 μg were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis on a 10% polyacrylamide gel. The proteins were transferred to a polyvinylidene difluoride membrane (Millipore), followed by overnight incubation at 4 °C with the following primary antibodies: rabbit polyclonal anti-β-catenin (1:250; Cell Signaling Technology, Danvers, MA, USA), and rabbit polyclonal anti-Shh (1:250; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The blots were incubated for 2 h with horseradish peroxidase-conjugated anti-rabbit IgG (1:5000; Jackson Laboratories, West Grove, PA, USA). GAPDH served as the loading control and was detected with rabbit monoclonal anti-GAPDH antibody (1:1000; Cell Signaling Technology).

2.5. Cell proliferation assay

ORSC proliferation was evaluated with the CellTiter 96 aqueous non-radioactive cell proliferation assay (Promega, Madison, WI, USA). Cells were seeded in 96-well plates at a density of 1.5×10^4 and treated with SFM containing DPC-Exos for 24 h. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) solution was added to each well for 30 min at 37 °C before measuring absorbance at 490 nm on an ELX808 microplate reader (BioTek, Winooski, VT, USA).

2.6. Cell cycle analysis by flow cytometry

ORSCs with or without DPC-Exo treatment for 24 h were digested to obtain a single-cell suspension with 80% precooled ethanol in PBS, and stained with propidium iodide (PI)-RNase using a kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol.

2.7. Transwell migration assay

500 μl of defined SFM containing DPC-Exos were added to the lower chamber of the transwell insert; a 200-μl volume of cell suspension (1×10^6/ml) was added to the upper chamber. Cells on the upper surface of the membrane were carefully wiped off, whereas those on the undersurface of the membrane were fixed with 4% paraformaldehyde for 15 min and then stained with 2% Crystal Violet for 5 min. The number of migrated cells in five random fields was counted.

2.8. Exosome injection into mice

Experiments involving animals were approved by the ethics committee of The Second Affiliated Hospital, Zhejiang University School of Medicine. Female C57 B/L 6 mice (6–8 weeks old) were randomized into PBS and DPC-Exo treatment groups (n = 6 each). DPC-Exos (1.0×10^{10}/ml) were dissolved in sterile PBS and injected

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