Neural progenitor cell-derived nanovesicles promote hair follicle growth via miR-100

Lei Cao
the Affiliated Wuxi No. 2 People's Hospital of Nanjing Medical University

Tian Tian
Nanjing Medical University

Yuanbo Huang
The Affiliated Wuxi People's Hospital of Nanjing Medical University

Shiqin Tao
The Affiliated Wuxi No.2 People's Hospital of Nanjing Medical University

Xiaohong Zhu
The Affiliated Wuxi No.2 People's Hospital of Nanjing Medical University

Mifang Yang
The Affiliated Stomatological Hospital of Nanjing Medical University

Jing Gu
The Affiliated Wuxi No.2 People's Hospital of Nanjing Medical University

Guangdong Feng
The Affiliated Wuxi No.2 People's Hospital of Nanjing Medical University

Yinni Ma
The Affiliated Wuxi No.2 People's Hospital of Nanjing Medical University

Rushan Xia
the Affiliated Wuxi No. 2 People's Hospital of Nanjing Medical University

Wenrong Xu
The Affiliated Wuxi People's Hospital of Nanjing Medical University

Lei Wang
(✉ nydwanglei@njmu.edu.cn)
Nanjing Medical University Affiliated Wuxi People's Hospital

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Abstract

Background: Accumulating evidence shows that mesenchymal stem cell-derived extracellular vesicles (EVs) hold great promise to promote hair growth. However, large-scale production of EVs is still a challenge. Recently, exosome-mimetic nanovesicles (NV) prepared by extruding cells have emerged as an alternative strategy for clinical-scale production. Here, ReNcell VM (ReN) cells, a neural progenitor cell line was serially extruded to produce NV.

Results: The ReN cell-derived NV (ReN-NV) were found to promote Dermal papilla cell (DPC) proliferation. In addition, on a mouse model of depilation-induced hair regeneration, ReN-NV were injected subcutaneously, resulting in an acceleration of hair follicle (HF) cycling transition at the site. The underlying mechanism was indicated to be the activation of Wnt/β-catenin signaling pathway. Furthermore, miR-100 was revealed to be abundant in ReN-NV and significantly up-regulated in DPCs receiving ReN-NV treatment. miR-100 inhibition verified its important role in ReN-NV-induced β-catenin signaling activation.

Conclusion: These results provide an alternative agent to EVs and suggest a strategy for hair growth therapy.

Background

Hair loss, characterized by shorter anagen and longer telogen phases of hair follicles (HFs), is a common medical problem which may cause both cosmetic and psychological problems. Currently, there are several therapeutic approaches, such as oral and topical medication, hair transplantation, and so on. All of these treatments have limitations. Drugs provide only short-term improvement, while surgical means are limited by a number of donor follicles\textsuperscript{[1]}. Therefore, there is a high demand to explore new approaches for alopecia.

HF, which contains both epithelial and mesenchymal compartment, is a complicated organ which undergoes cycles of growth (anagen), regression (catagen), quiescence (telogen) and regeneration\textsuperscript{[2]}. Epithelial–mesenchymal interactions play a critical role during HF development. Dermal papilla cells (DPCs), one population of mesenchymal cells in HF, is necessary for the morphogenesis and growth of the HFs. It is also thought to be a reservoir of multipotent stem cells\textsuperscript{[3]}. Dermal papilla (DP) size is thought to be well-correlated with hair cycle\textsuperscript{[4]}.

During the anagen phase, the cell number of DP increases and DPCs act as a signaling center to guide the surrounding epithelial cells to proliferate and migrate\textsuperscript{[5]}. DPCs release various cytokines and growth factors, which are involved in the regulation of HF cycle and hair growth. It is suggested that DPCs exert their regulatory function of HF growth mainly through paracrine and autocrine\textsuperscript{[6]}. Several signaling proteins, such as Wnt/β-catenin and Akt are up-regulated in DPCs after minoxidil treatment and led to the proliferation of DPCs\textsuperscript{[5]}.
Extracellular vesicles (EVs), composed of exosomes and microvesicles, are membranous nanovesicles (30-1000 nm in diameter) released by most cell types into the extracellular space. They can deliver internal prolifer proteins, mRNAs and microRNAs into target cells and are well tolerated by the body \[^7\]. Stem cell-derived EVs are believed to inherit the growth-promoting properties of stem cells. As previous reports, mesenchymal stem cell (MSC)-derived EVs could promote wound healing by inducing proliferation and migration of fibroblast, as well as angiogenesis \[^8\]. According to Rajendran et al, MSC-derived EVs (MSC-EVs) have a potential to activate DP cells, prolonged survival, induce growth factor activation \textit{in vitro}, and promotes hair growth \textit{in vivo} \[^11\]. It is reported that oxidized-sodium-alginate (OSA)-encapsulated EVs could increase the level of Wnt3a and β-catenin, which might be used to treat alopecia \[^9\]. In addition, it has been shown that exosomes from bone marrow cells (BMC) prevent alopecia areata (AA) progression and sufficed for partial hair regrowth \[^10\]. These studies show that EVs may play an important role in hair growth cycle by regulating certain signaling pathway. Compared with stem cells, EVs are more stable and reservable, have no risk of aneuploidy, a lower possibility of immune rejection following in vivo allogeneic administration, and may provide alternative therapy for various diseases \[^11\]. However, stem cells release relatively low quantities of EVs and purication of EVs is cumbersome, which results in a relatively low yield \[^12\]. Also, the instability and low long-term retention of EVs have hindered the development of EV-based treatments.

Revealed by a previous literature, exosome-mimetic nanovesicles (NV) prepared by extruding cells can deliver therapeutic effectors \[^13\]. The NV prepared by extruding stem cells produce 250-fold yield compared with that of exosomes \[^14\]. Thus, NV can be an alternative strategy for clinical-scale production. Furthermore, ReNcell VM (ReN) cells, a neural progenitor cell line derived from the ventral mesencephalon region of the human fetal brain, remain stable proliferation and differentiation capacity for more than 45 passages \[^15\]. Considering ReNcells partially maintain the characteristics of stem cells \[^16\], we hypothesize that ReNcell-derived NV (ReN-NV) may hold potential for promoting hair growth.

In this study, ReN-NV were prepared by extruding ReNcells through serial porous membranes. On DPCs the effects of ReN-NV were observed \textit{in vitro}. Using a mouse model of depilation-induced hair regeneration, ReN-NV were subcutaneously administered and the structural changes in HFs were analyzed. Furthermore, the possible underlying mechanism were investigated by Western blotting, immunofluorescence staining, and RNA sequencing. This study provides the potential for developing ReN-NV as a novel strategy for promoting hair growth.

**Results**

1. **Preparation and characterization of ReN-NV**

Human neural progenitor ReNcells were cultured and cell spheres formed (Supplemental Fig. 1). The cells were harvested and homogenated. After nucleus removal, the production was serially extruded through membrane filters with pore size of 1, 0.4 and 0.2 μm to obtain ReN-NV (Fig. 1a). NTA showed that the size
of ReN-NV was < 200 nm mainly with peaks at 129 and 170 nm (Fig. 1b). TEM revealed spherical shapes
in ReN-NV (Fig. 1c). In addition, to evaluate cellular uptake of ReN-NV, we labeled the membrane of ReN
cells by expressing palm-tomato and then prepared ReN-NV. Fluorescence microscopy showed
individual tdTomato-labeled ReN-NV (Fig. 1d).

2. ReN-NV treatment increases DPC proliferation by β-catenin signaling pathway

Dermal papillae were isolated by improved two-step enzyme method (Supplemental Fig. 2). To
investigate the biological effects of ReN-NV, primary human DPCs were cultured and incubated with ReN-
NV. After 3, 6, 12 or 24h of incubation, DPCs were washed, fixed and immunostained with α-SMA (a
marker for DPCs). Fluorescence images revealed the uptake of ReN-NV by DPCs in a time-dependent
manner (Fig. 2a, b). Furthermore, the proliferation of DPCs after NV treatment was evaluated by CCK-8
assay. The proliferation of DPCs incubated with ReN-NVs at the concentration of 60μg/mL was greater
than other groups (P< 0.001 versus PBS group at 72 h). The treatment of 30μg/ml ReN-NV also produced
significant higher cell proliferation than that of PBS at 72 h (P< 0.05) (Fig. 2c). These results indicate that
ReN-NVs exert a promotional effect on DPC proliferation in a dose-dependent manner.

Next, to assess the effect of ReN-NVs on the hair-inductive activity of DPCs, we treated DPCs with ReN-
NVs, 293-NVs and PBS, and evaluated the expression of proteins associated with anagen induction, such
as β-catenin, C-myc and CyclinD1. It was showed that the nucleus expression of β-catenin in ReN-NV
group was significant higher than that in control group. While no significant change was seen between
293-NV group and control group (Fig. 3a, b). Furthermore, the expressions of C-myc and Cyclin D1, both
of which were crucial downstream signaling effectors of β-catenin, were measured. Treatment with ReN-
NV showed a substantial increase in C-myc and Cyclin D1 levels. However, no change was seen upon
treatment with 293-NV when compared to PBS (Fig. 3c, d). Above results indicate that β-catenin signaling
pathway is involved in promoting DPC proliferation activity induced by ReN-NV treatment.

3. ReN-NV treatment accelerates HF growth

To study the role for ReN-NV in promoting hair growth in vivo, ReN-NV, 293-NV or PBS were injected
subcutaneously on a mouse model of depilation-induced hair regeneration. Immunofluorescence staining
for Ki67, a proliferation marker, was performed 6 days after the daily injection. For quantitative analyses,
the number of Ki67-positive cells was counted in hair matrix keratinocytes in the hair bulb. Among the
three groups, ReN-NV treatment group exhibited the highest number of Ki67-positive cells (Fig. 4a, b). In
addition, The morphology of ReN-NV-treated HFs was characteristic of anagen phase, with thicker skin,
larger hair bulbs and more bulbs in the subcutis (Fig. 5a). A histological analysis of H&E-stained tissue
sections suggested that the skin in HFs treated with ReN-NVs was the thickest (325.4±14.6μm),
compared to 293-NV (280.5±15.2μm) and PBS groups (266.6±12.4μm) (P<0.05) (Fig. 5b). The hair bulb
diameter of ReN-NV group(55.67±5.8) was larger than 293-NV(41.67±5.8) and PBS group(35.67±2.3)
(P<0.05) (Fig. 5c). HFs in ReN-NV-treated sites were mostly in IV-VI of anagen. It was also demonstrated
that 74.3% HFs treated with ReN-NV were at anagen V stage, while 40% or 48.3% HFs in PBS or 293
group, respectively (Fig. 5d). Furthermore, the activation of β-catenin signaling pathway is essential for
telogen/anagen conversion. Entry into anagen phase was induced in mice treated with ReN-NVs, as evidenced by positive immunofluorescence staining for β-catenin (Fig. 5e). The level of β-catenin was higher in ReN-NV group compared with that in both 293-NV and PBS group. Together, these results indicate that ReN-NVs have anagen-promoting activity of the HF cycle via the regulation of β-catenin signaling pathway.

4. ReN-NV significantly increases miR-100 level in DPCs

miRNAs are primary bio-regulatory molecules packaged in MSC-EVs. Given the similar size to MSC-EVs, we hypothesize that miRNAs play an important role in ReN-NV-induced HF growth. The miRNA profiles in ReN-NV and 293-NV were detected by RNA sequencing. Using TPM_average > 1000, fold change > 1.5 and p < 0.05 as the threshold cutoff, 32 miRNAs were revealed to be significantly up- or down-regulated in ReN-NV compared with 293-NV (Fig. 6a). Subsequently, we selected three miRNAs, miR-100, let-7i and let-7b for further analysis, the expression of which showed most significant differences between ReN-NV and 293-NV (Fig. 6b). The up-regulation of these miRNAs was confirmed by RT-PCR (fold change = 168.6, 49.1 and 17.5, respectively) (Fig. 6c). Furthermore, we evaluated the amount of the miRNAs in DPCs after incubation with PBS, 293-NV or ReN-NV. Compared with PBS treatment, ReN-NV treatment resulted in remarkable increases in all the three miRNA levels in DPCs, while 293-NV produced no significant change (Fig. 6d). Interestingly, the highest fold change is observed for miR-100 with an 6.0-fold change compared with 1.4 for let-7i and 1.3 for let-7b. These results show that miR-100 is significantly abundant in ReN-NV and delivered to DPCs following the uptake of ReN-NV. It is hypothesized that miR-100 might be involved in ReN-NV-induced HF growth.

5. ReN-NV-derived miR-100 promotes β-catenin signaling pathway

According to previous reports, miR-100 repress multiple β-catenin negative regulators and increase β-catenin signaling. To elucidate the biological function of miR-100 delivered by ReN-NV, synthetic anti-miR-100 was transfected 12 h before the co-incubation of DPCs and ReN-NV, and then Western blottings were performed to access β-catenin expression in nucleus. Intervention with anti-miR-100 suppressed the ReN-NV-induced up-regulation of β-catenin level (Fig. 7a, b). Consistent with the trend of β-catenin, the expressions of C-myc and CyclinD1 were increased by ReN-NV and this effect was impaired when anti-miR-100 was applying (Fig. 7c,d). Next, to confirm the role of miR-100 in β-catenin up-regulation induced by ReN-NV in vivo, cholesterol-conjugated anti-miR-100 was loaded into ReN-NV and the modified ReN-NV were injected subcutaneously on a mouse model of depilation-induced hair regeneration. After daily injection for 6 consecutive days, immunofluorescence staining for β-catenin was carried out. Increased fluorescent signals in the hair bulbs were observed after ReN-NV injection compared with that under PBS treatment. However, the increase was suppressed when anti-miR-100 was loaded (Fig. 7e). Taken together, these results indicate that ReN-NV-derived miR-100 enhances the nucleus expression of β-catenin, thereby increasing C-myc and CyclinD1 levels, resulting in the acceleration of HF growth.

Discussion
In this study, we aimed to obtain evidences for direct roles of ReN-NV in hair growth. DPCs, derived from mesenchymal components, are capable of releasing growth factors that direct epithelial cells to proliferate. Thus, DPCs are considered to be a key signaling center responsible for the induction and maintenance of follicular development, regulation of the hair cycle, as well as the regeneration of HFs\textsuperscript{[17]}. First of all, we have successfully fabricated ReN-NV from ReN cells using a serial extrusion through membrane filters with diminishing pore sizes (1, 0.4, 0.2 μm), and the production was identified to be in accordance with the previous reports\textsuperscript{[13]}. Then, the biological function of ReN-NV on DPCs was investigated and the underlying mechanisms were explored. Using fluorescently labeled ReN-NV, we confirmed that their uptake by DPCs in a time-dependent manner when incubate them with cells. In addition, we found that ReN-NV promoted the proliferation of DPCs as determined by CCK-8 analysis. As well known, cell cycle control is important for cell proliferation. β-catenin, a regulator of many gene such as CyclinD1 and C-myc, plays an important role in cell cycle progression. Also, β-catenin accumulates in the cytoplasm and then translocates into the nucleus, leading to increased expressions of downstream molecules. Here, ReN-NV were revealed to have the capacity to promote DPC proliferation. The mechanism may be due to the up-regulation of β-catenin signaling pathway.

To study the effect of ReN-NV \textit{in vivo}, C57BL/6 mouse is an ideal model animal to observe hair cycle, since the dorsal hair in these mice has a time-synchronized growth cycle. During telogen phase, the depilated skin of C57BL/6 mice is pink, while it darkens at initiation of anagen\textsuperscript{[18]}. The HFs in the anagen phase exhibited typical features, such as a larger hair bulb, thicker skin, more bulbs in the subcutis, as well as larger amounts of melanin\textsuperscript{[19]}. Herein, we investigated the effect of ReN-NV on hair re-growth on a mouse model of depilation-induced hair regeneration. As shown in our study, ReN-NV was injected subcutaneously once a day. After injection for 5 consecutive days, sections from dorsal skin of mice revealed that ReN-NV group had the greatest number of Ki67-positive cells, which expressed mostly in hair bulb, compared to 293-NV and control groups. The expression level of Ki67 in hair matrix area is an important marker to assess the proliferation of hair follicle cells\textsuperscript{[20]}. These data indicated that ReN-NV might enhance hair follicle cells proliferation and promote telogen-to-anagen transition of hair follicles. Furthermore, a histological analysis of H&E-stained tissue sections demonstrated that the thickest skin, largest bulb diameter and highest percentage of anagen VI HFs could be found in HFs treated with ReN-NVs, exhibiting that ReN-NVs significantly induced earlier anagen entry and accelerated hair follicle growth compared with control group.

miRNA profiling analysis revealed that miR-100 was much more abundant in ReN-NV than 293-NV. It was striking that miR-100 in DPCs was up-regulated as high as 6 fold after incubation with ReN-NV. In previous studies, miR-100 plays a critical role in tissue regeneration. A. Aguirre et al. identified miR-99/100 and let-7a/c were critical regulators of cardiomyocyte regeneration, which improve heart function recovery after injury. It is also demonstrated that miR-100 promotes MSC osteogenesis\textsuperscript{[21]}. Recently, miR-100 activate Wnt signaling pathway by repressing multiple Wnt negative regulators, along with increased nuclear β-catenin expression\textsuperscript{[22]}. It is well known that Wnt/β-catenin signaling pathway is crucial for the regeneration of hair cycle, abnormity of which may cause hair loss of different types. In addition, the
maintenance of the anagen phase is dependent on β-catenin\textsuperscript{[20]}. However, there is few study focus on the interaction between miR-100 and hair follicle cells. Based on the above mentioned studies, we speculated that miR-100 may play an important role in ReN-NV promoting proliferation of DPCs through Wnt/β-catenin signaling pathway. To further confirm the role of miR-100, anti-miR-100 was applied together with ReN-NV \textit{in vitro} and \textit{in vivo}. miR-100 knock down significantly suppressed ReN-NV-induced increases of β-catenin, Cyclin D1 and C-myc. Taken together, miR-100 in ReN-NV promoted the proliferation of dermal papilla cells by up-regulation of Cyclin D1 and C-myc via the activation of β-catenin pathway.

**Conclusions**

In summary, NV were prepared from stable ReN cell line by serial extrusion and found to promote DPC proliferation and HF growth. The underlying mechanism was indicated to be the activation of Wnt/β-catenin signaling pathway mediated by ReN-NV-derived miR-100. This study provides the potential for developing ReN-NV as a novel strategy for promoting hair growth. In the future, it could be beneficial for large-scale production of ReN-NV for further studies and alopecia therapies. In addition, other drug delivery system, such as microneedle patch, could be used to improve treatment compliance of ReN-NV.

**Materials And Methods**

1. **Isolation and identification of ReN-NV**

ReN cells were purchased from Millipore (USA). The cells were cultured in DMEM/F12 (Life Technologies, USA) supplemented with 2% B27 (Life Technologies), 20 μg/mL EGF (Abm, Canada), 10 μg/mL bFGF (Abm) and 2 μg/mL heparin and incubated with 5% CO\textsubscript{2} at 37 °C. ReN cell spheres were dissociated with Accutase (Life Technologies). The cells were centrifuged at 3,000 \( \times \) g for 10 min to remove the nucleus after homogenate by ultrasonication. The supernatants were extruded serially through 1 μm, 400 nm and 200 nm polycarbonate porous membranes (Whatman, UK) using a mini extruder (Avanti Polar Lipids, USA). Subsequently, the extruded ReN-NV were purified in a layer between 10% and 50% (v/v) iodoxanol by ultracentrifugation (L-80XP, Bechman Coulter, USA) at 100,000 \( \times \) g for 2 h. The ReN-NV pellet was resuspended in PBS after a wash step by another ultracentrifugation with PBS.

ReN-NV were observed by a Tecnai G2 transmission electron microscope (FEI, USA). The total protein concentration was determined by BCA protein assay (Pierce, USA). The size distribution and number were analyzed by NTA using a ZetaView system (Particle Metrix, Germany). As a control, HEK293 cell-derived NV (293-NV) were prepared and identified following an identical procedure.

2. **Isolation and culture of human DPCs**

The study using clinical samples was approved by the Review Board of Nanjing Medical University (2018143). Healthy human scalp specimens were obtained with informed consent from subjects undergoing cosmetic surgery. Improved two-step enzyme method was used as described previously to isolate the dermal papillae\textsuperscript{[23]}. The human dermal papillae were cultured in DMEM (Life Technologies)
containing 10% heat-inactivated fetal bovine serum (Life Technologies) and 1% Pen Strep, then incubated at 37°C in an atmosphere of 95% air and 5% CO₂.

3. The cellular uptake of ReN-NV

To label ReN-NV with tdTomato, ReNcells were stably transduced with packaged lentivirus vectors to express tdTomato fused to palmitoylation signal (palm-tdTomato) which labels cell membrane[24]. The tdTomato-labeled ReN-NV were prepared by extruding the cells, and observed using a Ti-E fluorescence microscope with a 100× objective (Nikon, Japan). DPCs were incubated with 30 μg/mL tdTomato-labeled ReN-NV at 37 °C. At given time points, the cells were washed by PBS and immunostained with anti-α-SMA (Santa Cruz, USA) and Alexa 488-conjugated secondary antibodies (Life Technologies). After staining by DAPI (Sigma, USA), the cells were imaged by fluorescence microscope with a 20×objective (Nikon). Images were processed and analyzed by ImageJ software (NIH, USA). All settings of imaging and processing were kept constant, and the relative fluorescence intensities were calculated.

4. Cell proliferation assay

Cell proliferation rates were measured by using a Cell Counting Kit-8 (CCK-8) (Dojindo, Japan). DPCs (5×10⁴ cells/well) were seeded in a 96-well plate and incubated with PBS, ReN-NV (30 or 60μg/mL) or 293-NV (60μg/mL). Cell proliferation of each group was calculated at the time point of 24, 48 and 72 h, respectively. CCK-8 reagent (10 μL) was added to each well and incubated for 2 h. Then, O.D. 450 nm value in each well was verified by a microplate reader. The experiment was repeated three times.

5. Antisense miRNAs

The antisense nucleotide of miR-100 (anti-miR-100) and negative control (anti-NC) were synthesized and modified with 2’Ome by GenePharma (China). The sequences were as follows: 5’- CACAAGUUCGGAUCUGGGU-3’ for anti-miR-100, 5’-CAGUACUUUGUGUAGUACAA-3’ for anti-NC. DPCs were transfected with 100 nM antisense miRNAs in 6-well plates with lipo2000 (Life Technologies) according to manufacturer’s instructions, followed by ReN-NV treatment 12 h later. To applying antisense miRNAs in vivo, anti-miR-100 and anti-NC were conjugated with cholesterol on the 3’terminus (GenePharma), and then loaded into ReN-NV following our previous study[25]. Briefly, 100 nM cholesterol-conjugated anti-miR-100 or anti-NC were incubated with 100 μg ReN-NV in 200 μL of PBS at 37 °C for 1 h. The antisense miRNAs inserted into ReN-NV membrane through a hydrophobic interaction. After washing with PBS at 140,000g for 90 min, the modified ReN-NV were resuspended and stored at -80 °C prior to use.

6. Western blotting analysis

Cells or tissues were lysed or homogenized in RIPA buffer with protease inhibitors (Pierce). After centrifugation at 12,000 rpm for 30 min at 4°C, the supernatants were loaded in 10% SDS-PAGE gel for electrophoresis (Bio-Rad, USA). Then the samples were transferred onto PVDF membrane (Millipore) and
incubated with antibodies as follows: anti-β-catennin, anti-C-myc, and anti-Cyclin D1 were from Abcam (UK). After incubation with HRP-conjugated secondary antibodies, the signals were visualized using ECL Substrates (Bio-Rad). Histone H3 or β-actin served as the control group.

7. Immunofluorescence staining

For immunofluorescence assay, the mouse skin was isolated and cryosectioned in 5-μm thickness. The sections were treated with 0.3% Triton X-100 for 30 min and 3% BSA for 2 h, and immunostained with anti-β-catennin (Abcam, UK) or anti-Ki67 (Cell Signaling Technology, USA) at room temperature (RT) for 2 h. After washing 5 times by PBST (PBS containing 0.1% Triton X-100), the samples were incubated with Alexa 488 or Alexa 594-conjugated secondary antibodies (Life Technologies) for 1 h at RT. After washing 5 times by PBST, staining by DAPI, and mounting with ProLong Diamond Antifade Mountant (Life Technologies), the tissue slides were imaged by an FV-1200 confocal microscope (Olympus, Japan). Images were processed and analyzed by ImageJ software (NIH). All settings of imaging and processing were kept constant.

8. Animals

Six-week-old female C57BL/6 mice were purchased from the Animal Core Facility of Nanjing Medical University (Nanjing, China). All animal experiments were carried out in compliance with institutional guidelines and were approved by the Animal Care and Use Committee of Nanjing Medical University. Mice were randomly divided into control (PBS, n=5), 293-NV (n=5) and ReN-NV (n=5) groups. One-hundred micro-liter PBS, 293-NV (500 μg/mL), or ReN-NV (500 μg/mL) were injected once daily into the dorsal skin of C57BL/6 mice from depilation day (p.d.) 0. The mice sacrificed at p.d. 6. The dorsal skin samples were harvested for further analysis as previously described.

9. Quantitative histomorphometry

To investigate the progression of HFs in the hair cycle, we performed H&E staining of skin biopsy sections. The dorsal skin tissue fixed in 4% paraformaldehyde was embedded in paraffin and then cut into sections at a thickness of 5μm, followed by staining with hematoxylin and eosin. In histological analysis, measurement of skin thickness, bulb diameter and anagen HF percentage is a well-known method to classify the hair cycle stages. Skin thickness was measured as the distance from the epidermis to the subcutaneous fat. Bulb diameter was measured at the level of the largest diameter of the hair bulbs with clearly visible DP. In our study, HF growth was evaluated according to three parameters: skin thickness, diameter of hair bulbs, anagen HF percentage. At least 50 HFs per sample were analyzed.

10. miRNA sequencing and analysis

For miRNA sequencing, RNA was isolated by Trizol (Life Technologies) extraction from ReN-NV and 293-NV. Qubit 2.0 and Agilent 2100 bioanalyzer were used to quantify the samples. The cDNA libraries were produced using a NEB Next Ultra small RNA Sample Library Prep Kit for Illumina according to the
manufacturer’s instructions. Subsequently, the library preparations were sequenced on an Illumina Hiseq 2500 platform and paired-end reads were generated. Using Bowtie software, the clean reads were compared with Silva database, GtRNAdb database, Rfam database and Repbase database sequence alignment to filter ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA) and other ncRNA and repeats. The remaining reads were used to detect known miRNA and new miRNA predicted by comparing with known miRNAs from miRBase. The miRNA levels were calculated and normalized to transcripts per million (TPM). A heatmap analysis of miRNA expression levels was created based on the TPM values of miRNAs in ReN-NV and 293-NV (using $\text{TPM}_{\text{average}} > 1000$, 1.5-fold change and $p < 0.05$ as the threshold cutoff).

11. RT-PCR for miRNA evaluation

Total RNA was extracted from ReN-NV, 293-NV and cells using Trizol reagent. For NV RNA extraction, 100 μg NV were used with 10 femto-moles cel-miR-39 as an external reference and a RNeasy MinElute Spin Column (Qiagen, Germany) was employed. To detect miRNAs, miRcute Plus miRNA First-Strand cDNA Kit, qPCR Kit and primers (TIANGEN, China) were used following the manufacturer’s instructions. The results were normalized to cel-miR-39 or U6 snRNA levels. Relative expression was calculated by the comparative $2^{-\Delta\Delta Ct}$ method. All experiments were performed at least three times independently.

12. Statistical analysis

All data were analyzed statistically using Graph Pad Prim software (USA). Results were expressed as mean ± SEM after at least three independent tests. The significances among groups were analyzed using the Student’s $t$-test or One-way ANOVA. $P$ value < 0.05 was regarded as statistical significant.

List Of Abbreviations

EV: extracellular vesicle; NV: nanovesicle; DPC: dermal papilla cell; HF: hair follicle; DP: dermal papilla cell; miR-100: microRNA-100; MSC: mesenchymal stem cell; OSA: oxidized-sodium-alginate

Declarations

Ethics approval and consent to participate

The study using clinical samples was approved by the Review Board of Nanjing Medical University (2018143). All animal experiments were carried out in compliance with institutional guidelines and were approved by the Animal Care and Use Committee of Nanjing Medical University/IACUC-1910003.

Consent for publication

All authors agree to be published.

Availability of data and materials
All data generated or analyzed during this study are included in this published article.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors’ contributions**

Lei Cao, Yuanbo Huang, Rushan Xia: conception and design of the experiment, as well as manuscript writing; Shiqin Tao, Xiaohong Zhu: collection an assembly of data and data interpretation; Mifang Yang: edit the manuscript, support and discussions; Jing Gu, Yinni Ma, Guangdong Feng: perform the experiment; Wenrong Xu: technical and financial support; Tian Tian, Lei Wang: conception and design, financial support and the preparation. All authors read and approved the final manuscript.

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**Figures**
Figure 1

a) ReN cells are processed through a series of steps: nucleus removal, serial extrusion, density gradient centrifugation, and isolation & wash to produce nanovesicles.

b) Concentration of particles/mL vs. size (nm) graph showing 129 and 170 particles/mL.

c) Electron microscopy image of nanovesicles.

d) Fluorescence image showing nanovesicle uptake.

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Hoechst/α-SMA/ReN-NV

3h 6h 12h 24h

ReN-NV uptake by DPCs

b) Percentage internalization vs. time (hr) graph.

CCK8 assay on DPCs

c) Graph showing relative absorbance over time (hr) for different conditions.
Figure 4
Figure 5
Figure 6
Figure 7

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.
• DPC.jpg
• ReNcell.jpg