Dermal exosomes containing miR-218-5p promote hair regeneration by regulating β-catenin signaling

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The progression in the hair follicle cycle from the telogen to the anagen phase is the key to regulating hair regrowth. Dermal papilla (DP) cells support hair growth and regulate the hair cycle. However, they gradually lose key inductive properties upon culture. DP cells can partially restore their capacity to promote hair regrowth after being subjected to spheroid culture. In this study, results revealed that DP spheroids are effective at inducing the progression of the hair follicle cycle from telogen to anagen compared with just DP cell or minoxidil treatment. Because of the importance of paracrine signaling in this process, secretome and exosomes were isolated from DP cell culture, and their therapeutic efficacies were investigated. We demonstrated that miR-218-5p was notably up-regulated in DP spheroid–derived exosomes. Western blot and immunofluorescence imaging were used to demonstrate that DP spheroid–derived exosomes up-regulated β-catenin, promoting the development of hair follicles.

INTRODUCTION

People affected by moderate hair loss turn to topical treatments like minoxidil (antihypertensive potassium channel opener) (1) and finasteride (dihydrotestosterone-suppressing 5α-reductase inhibitor) (2), the only Food and Drug Administration–approved treatments for inducing hair regrowth. Both are designed not for hair loss treatment but serendipity. Researchers kept studying the mechanism of hair induction hair regrowth. Both are designed not for hair loss treatment but serendipity. Researchers kept studying the mechanism of hair induction

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The interactions between the epithelial and mesenchymal cells are vital in regulating the cycle of hair growth (12). As the main mesenchymal component of the follicular unit, DP cells induce the transition from telogen to anagen and the formation of new follicles. Thus, regulating DP cells is critical for increasing cell division and follicle growth rate. Two-dimensionally (2D) cultured DP cells have demonstrated no therapeutic effect on hair follicle growth (13). It has been reported that 3D spheroid cultures result in a partial restoration of the inductive capabilities of DP cells, which may enable them to induce de novo hair follicles in human skin (13). DP cells have to aggregate into hair follicle areas to be effective (14). Thus, spheroid culture therapy should be an effective way to regain the capacity for hair regeneration in vitro. However, a comprehensive understanding of the molecular mechanisms that underlie this regenerative process is required. DP cells induce the progression of the hair cycle and regeneration, which requires cross-talk with the surrounding environment (15). Exosomes have been extensively investigated because of their role in cell-cell communication and potential in treating diseases (16–18). The differentially expressed secretome or exosomes from DP spheroids and DP cells could be the key to regulating hair follicle cycles. Recently, DP cell–derived secretome and extracellular vesicles (8, 19, 20) have demonstrated their effect in promoting hair growth, and their mechanism is under immense investigation. Compared with mesenchymal stem cell–derived exosomes (8, 21), DP cell–derived exosomes were demonstrated to be efficient transforming growth factor–β activators and proven to be important in promoting proliferation of human hair follicle DP cells and hair growth (22). 3D culture has been proven to be a way to enrich certain proteins and microRNAs (miRNAs) in secretome (23). Young and co-workers (24) demonstrated that exosomes derived from 3D DP cells (3D DP-XOs) promoted the proliferation of DP cells and outer root sheath cells and increased the expression of growth factors in DP cells. However, the importance of differentially expressed secretome and miRNAs from 2D DP cells and 3D DP cells and possible mechanisms of 3D DP cells or 3D DP-XOs in promoting hair regeneration have not been demonstrated. In this study, we first verified the hair restoration capacity of DP spheroids in C57BL/6 mice. Then, the therapeutic efficacy of DP secretome was compared with that of the DP spheroids. Different factors and exosomes were expressed when DP cells aggregate. Considering DP cells’ aggregative behavior and DP spheroids’ ability to induce new hair follicle formation in vivo, we hypothesized that the differentially expressed factors and miRNAs would be previously undefined therapeutics for hair regrowth.

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RESULTS

DP spheroids enhance the expressions of β-catenin and CD133 in vitro and transplantation engraftment in vivo

C57BL/6 mice are widely used in hair physiology studies because, after their backs are depilated, all hair follicles in the area enter the pause phase (catagen) when mice reach 7 weeks old (25, 26). DP cells were isolated from the whiskers of C57BL/6 mice (27) and cultured. Passages 3 to 6 were used in this study. Figure 1A shows that DP cells grew out from the hair follicle bulb and exhibited a spindle-like shape when they formed swirly parallel arrays centered on the bulb. After passage, DP cells displayed flattened, polygonal morphology (Fig. 1B). DP spheroids were formed by passaging DP cells into ultralow attachment flasks. The spheroids were 150 to 300 μm in diameter (Fig. 1C and fig. S1) and expressed strong immunofluorescent signals of CD133 (green) and β-catenin (red; Fig. 1D). The expression of CD133 and β-catenin were lower in 2D cultured DP cells (fig. S2). In spheroids, β-catenin expression was enhanced due to the increased cell-cell contact (13). CD133-positive DP cells exhibited hair-inducing capacity in vivo (28). This was further confirmed via flow cytometry. Enhanced signal intensity can be seen in the gated spheroid cells (fig. S3).

To compare cell survival rates after transplantation, DP cells and DP spheroids stained with DiD (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt) were injected into the depilated backs of C57BL/6 mice. Scaffolds play an important role in 3D cell culture and engineering (29). Hair keratins were used as cell culture scaffolds (8, 30), as they are autologous, degradable, and biocompatible. The viability of DP cells was preserved during encapsulation and maintained in the porous microarchitecture of keratin hydrogels. Networks of keratin hydrogels and spheroids self-assembled inside hydrogels are shown in Fig. 1 (E and F), respectively. 2D cells or 3D spheroids were collected and distributed in phosphate-buffered saline (PBS) or keratin hydrogel (10^5 cells/20 μl) for subcutaneous administration. To improve the cell survival rate after transplantation, we compared DP cells/PBS, DP cells/keratin, DP spheroids/PBS, and DP spheroids/keratin capacity.
(Fig. 1G). IVIS (in vivo imaging system) imaging revealed that spheroids demonstrated an enhanced retention and survival rate after injection (Fig. 1, H and I). Spheroids allow higher transplantation rate and functional benefit (31, 32). Keratin further supported cell attachment and proliferation (8). DP spheroids/keratin hydrogel maintained high cell viability after engraftment onto the dorsal skin of mice.

**DP spheroids enhance the expression of β-catenin, CD133, and Ki67 in hair follicles in vivo**

DP cells and DP spheroids were injected into one side of each mouse after depilation (10 one-time injections, evenly distributed on the treated side, 10⁵ cells in 20 μl per injection), or 5% minoxidil was topically administered on the treated side daily (Fig. 2A). Ten days later, dorsal skin samples were taken from both the injected sites (left) and nontreated sites (right). β-Catenin, CD133, and Ki67 were stained (Fig. 2, B to D) to compare the hair follicle–inducing mechanism of DP cells, DP spheroids, and 5% minoxidil. Corresponding quantification results are shown in Fig. 2 (E to G).

Minoxidil exerts a vasodilator effect on hair follicles, leading directly to the proliferation of follicle cells (33). On the treated side, 5% minoxidil promoted the expression of Ki67 to 37.2% on day 10, but not β-catenin (2.3%) nor CD133 (7.2%), which suggests that the proliferation and growth of whole hair follicle cells were promoted nonspecifically. In contrast, the injection of 2D DP cells enhanced the expression of β-catenin (19.1%) and CD133 (9.8%) on the treated side, and β-catenin (19.9%) and CD133 (6.8%) on the untreated side. Moreover, the injection of 3D DP spheroids enhanced the expression of β-catenin (32.6%) and CD133 (19.1%) on the treated side, and β-catenin (27.8%) and CD133 (13.7%) on the untreated side. Both showed cell migration on the untreated site and enhanced expressions of β-catenin, CD133, and Ki67 on the untreated side compared with minoxidil. In particular, DP spheroids showed much stronger β-catenin and CD133 staining compared with the DP cell
group. Minoxidil, however, did not alter β-catenin or CD133 expression on either side. The Ki67 signals on the treated side were 37.2, 37.4, and 39.5% for minoxidil, 2D DP cells, and 3D DP cells, respectively, with no significant difference; however, the Ki67 signals on the nontreated side were significantly different at 7.1, 12.6, and 17.5%, respectively. Only the minoxidil group showed a significant difference between the treated side and untreated side. The mechanism of minoxidil is not on regulating DP cells directly but is believed to promote angiogenesis, while the beneficial effects on the untreated sides of DP cell (in PBS or in keratin) groups were likely due to the paracrine effects of injected DP cells. The injection of keratin hydrogel could also promote endogenous DP recruitment by providing a bioactive matrix. Cell migration and viability likely contribute to this remote promotion. This study demonstrated that implanted cells not only influence the hair follicles at the injected site but also regulate the hair follicles in an untreated area. DP spheroid engraftment was more effective in regulating hair follicle growth. In addition, because the migration of cells in the skin was quite limited (Fig. S4), paracrine effects could also be an important mechanism in this process.

**DP spheroid treatment accelerates hair regrowth in C57BL/6 mice**

Minoxidil, the gold standard treatment for hair loss, was used as the positive control. Cultured DP cells were reported to gradually lose their hair-inductive capacity gradually (4). We have already showed the limited therapeutic effect of 2D-cultured DP cell administration in Fig. 2. Thus, the hair follicle induction capacity of 3D spheroids was studied here.

We photographed the state of hair regrowth in depilated mice on days 0, 10, 15, and 20 after treatment. The hair growth area was analyzed via morphological observation (Fig. 3, A and B). On day 10, black pigmentation started to show in the minoxidil- and cell-treated groups. All mice in the DP spheroids/keratin group developed much darker skin than the other groups. On day 15, the treated side of the minoxidil group showed 35% fur coverage, compared with just 10% for the untreated side. The DP spheroids/PBS group had an average of 40% fur coverage, and the fur coverage of the DP spheroids/keratin group was approximately 90%. The engraftment and survival rates of cells in PBS were uneven, and so were the resulting therapeutic effects. In contrast, keratin enhanced the overall spheroid engraftment and cell survival rate.

Masson trichrome and hematoxylin and eosin (H&E) staining demonstrated that the DP spheroids/keratin treatment group resulted in larger hair follicles and more collagen distribution compared with the other groups (Fig. S5, A and B). The transition of the resting follicles into the anagen phase led to increased follicle size (34). In contrast, the control group had smaller follicles and a thinner collagen layer. The injection of DP spheroids did not induce inflammation in the skin tissue because the DP cells were autologous. In addition, the keratin hydrogels did not evoke an acute immune response in mice.

**DP spheroids accelerate the onset of hair follicle anagen phase by up-regulating β-catenin**

To elucidate the molecular mechanism, we evaluated the protein levels of β-catenin, extracellular signal–regulated protein kinases 1 and 2 (Erk1/2), phospho-ERK1/2 (p-Erk1/2), the secreted frizzled-related protein 2 (SFRP2), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) by Western blot (Fig. 4, A and B) in the depilated dorsal skin 20 days after treatment. The results showed that the expression of β-catenin and p-Erk1/2 in the DP spheroid groups was up-regulated, and that of SFRP2 was down-regulated in comparison to the control and minoxidil groups. The WNT/β-catenin pathway has a critical role in regulating the hair cycle by promoting hair growth (35). β-Catenin is a key regulator of hair follicle growth, and it is reported to be the primary initiator of the anagen phase (36). SFRP2 has been reported to be involved in several cellular activities and biological processes via the down-regulation of β-catenin in nuclear translocation. These results indicate that DP spheroids may activate hair follicle development by up-regulating β-catenin signaling in the WNT pathway. We also performed immunofluorescence staining of SFRP2 and β-catenin (Fig. 4, C to E) to further confirm the down-regulation of SFRP2 and up-regulation of β-catenin in the spheroids/keratin group. Thus, 3D spheroid culture could be an effective strategy to improve DP therapeutic potency before it is administered as a hair loss treatment.

**Comparison of protein and miRNA profiles between DP cells and DP spheroids**

DP spheroids affected not only the local injection site but also the regulation of the hair follicle cycle in a nontreated area due to paracrine signaling. DP cells stimulate follicular development and modulate mesenchymal-epithelial interactions by releasing various growth factors and exosomes (24, 37) (Fig. 5A). Exosomes and secretome from DP cells and DP spheroids were isolated and detected. Compared with the other treatment groups, spheroid secretome resulted in significantly higher expression levels of basic fibroblast growth factor (bFGF) and tissue inhibitor of matrix metalloproteinase 2 (TIMP2; Fig. S6). Transmission electron microscopy (FEI Talos F200X) images showed the morphology of exosomes (Fig. 5B). The average size of DP cell–derived exosomes (DP-XOs) was around 180 nm, and the average size of DP spheroid–derived exosomes (DP spheroid–XOs) was around 130 nm (Fig. 4C). Figure S7 indicates the identifying exosomal makers: Alix, CD9, and CD81. To compare miRNA content in the exosomes, we performed miRNA arrays and found that DP spheroid–XOs expressed mir-218-5p at significantly higher levels than DP-XOs (Fig. 5D and fig. S8).

As shown in Fig. 5E, bFGF can be accepted by cell membrane-bound FGFR and up-regulate the expression of p-Erk1/2, which up-regulates the expression of β-catenin (38). TIMP2 was known to inhibit the expression of matrix metalloproteinase 2 (MMP2) (39), which might hinder the migration and proliferation of DP cells (40). However, the mechanism behind MMP2-mediated regulation of hair follicles is still not clear and needs to be further studied. Growth factors are involved in the promotion of hair growth cycles, and the bFGF mechanism has been demonstrated before (41, 42). bFGF and TIMP2 levels were approximately doubled in spheroid secreteome compared with 2D cell secretome. In addition, mir-218-5p in 3D DP-XOs was up-regulated 25-fold in comparison with 2D DP-XOs. Thus, determining how exosomes regulate this process and the importance of mir-218-5p in mediating the hair cycle was the main focus of this study.

**DP spheroid–derived exosome treatment for hair regrowth**

Cell-based therapy is a promising step forward in the pursuit of achieving hair follicle neogenesis. However, harnessing of cellular components to be therapeutic catalysts would make for an even more convenient therapeutic product, obviating the cell as the therapeutic
carrier. Both exosomes and secretome might contribute to the hair growth process, while differentially expressed miR-218-5p in exosomes might contribute to the higher efficacy of 3D spheroids in promoting hair growth compared with 2D DP cells. Also, according to literature, miR-218-5p directly targets SFRP2, and thus, it up-regulates the WNT/β-catenin pathway (43). Therefore, miR-218-5p up-regulated exosomes were the main research object in this study.

The recruitment of DP cells to the hair follicle is crucial to hair regrowth (44). We demonstrated that spheroid-derived exosomes could effectively promote DP migration compared with exosomes from 2D cells (fig. S9), and enhanced cell mobility could also be important to their aggregate and migration behavior in vivo (45).

Then, in vivo studies were performed on C57BL/6 mice. Exosomes were administrated to one side of the dorsal skin (Fig. 2A) and compared with a 5% minoxidil topical treatment. In morphological observation (Fig. 6A) and corresponding quantification (Fig. 6B), the therapeutic efficacy of 2D DP-XOs was not significantly different from that of minoxidil, while 3D DP spheroid–XOs accelerated hair growth to an extent that was comparable to cell treatment. To explore the changes of SFRP2 and β-catenin expression in the skin after treatment, we harvested dorsal skin samples from different groups on day 15. Immunostaining confirmed that β-catenin expression was increased in the groups treated with DP spheroid–XOs when compared with those treated with 2D DP-XOs or minoxidil. DP

**Fig. 3. Dorsal hair growth experiment on C57BL/6 mice.** (A) Observation of hair coverage. Mice were divided into five groups (n = 5) and treated on their left halves. Mice were imaged on days 0, 10, 15, and 20, respectively. (B) Quantification of hair coverage on days 10, 15, and 20. Both the left (treated) and right (untreated) sites were recorded. n = 5; n.s., no significant difference; *P < 0.05, **P < 0.01, and ***P < 0.001. Photo credit: S.H., North Carolina State University.
spheroid–XOs can regulate hair growth cycles by down-regulating SFRP2 and up-regulating β-catenin (Fig. 6, C and D). Because miR-218-5p directly targets SFRP2, miR-218-5p mimics and inhibitors were used to further validate the importance of miR-218-5p enrichment.

**miR-218-5p plays a crucial role in exosome-mediated hair regrowth**

One of the main challenges in miRNA treatment is delivery. The in vitro model does not accurately reflect whether miR-218-5p plays an important role in regulating hair follicle growth when considering the diverse types of cells in the follicles (46). Here, an mmu-miR-218-5p mimic was mixed with polyethyleneimine (PEI) solution (in vivo-jetPEI; Polyplus Transfection, Illkirch, France) according to the manufacturer’s instructions. The PEI/negative control, PEI/mimics, or PEI/inhibitors polyplex solution (10 μl per site) was carefully administered, subcutaneously, into the dorsal skin of depilated mice (Fig. 6E). By targeting SFRP2, miR-218-5p mimics promoted hair follicle development, while miR-218-5p inhibitors inhibited the onset of the anagen phase in the hair follicle cycle. We can see notable hair regrowth effects with the treatment of miR-218-5p mimic, as compared with the control group and the miR-218-5p inhibitors. However, these effects (50 to 90% hair coverage) were less potent than those from exosome treatment (95 to 100% hair coverage) on day 20. We reason that this is because exosomes contain a variety of miRNAs and proteins, and miR-218-5p is not the only one (although a very important one) that was able to contribute to promoting hair growth.

We used a different strategy to promote hair growth. As illustrated in Fig. 6G, both miR-218-5p–loaded exosomes and miR-218-5p mimics encapsulated by in vivo PEI promoted the transcript of miR-218-5p. The genetic structure of miR-218-5p showed that it would target...
SFRP2 directly. To demonstrate the regulatory effect that miR-218-5p and WNT signaling have on the hair growth cycle, we examined transcriptional mediators, SFRP2, and β-catenin by Western blot. Skin samples (day 15) showed that miR-218-5p mimics robustly increased endogenous β-catenin expression, while treatment with miR-218-5p inhibitors showed decreased β-catenin expression (Fig. S10).

Together, these data support the theory that the main therapeutic pathway involves the stimulation of WNT signaling by miR-218-5p–overexpressed exosomes through a down-regulation of SFRP2, a WNT signaling inhibitor, and up-regulation of β-catenin.

**DISCUSSION**

Given the temporary efficacy of finasteride and minoxidil, and the limited number of treatments available, the need to discover new therapies for preventing hair loss and enhancing hair regrowth is urgent (47). Alternative solutions are challenging, especially bioproducts.

Replenishing DP cells with in vitro–cultured DP cells is a reasonable approach for driving the telogen-to-anagen transition in the hair follicle cycle. However, DP cells will lose their hair-inducing capacity over time when they are cultured on a flat, plastic surface. Horne and co-workers (48) observed that cultured DP cells (over six passages) could not induce any hair follicles to transition to anagen after implantation in situ. DP cells have to agglomerate in the hair follicle bulb to promote folliculogenesis (49, 50). Osada et al. have shown that DP cells regained their hair follicle inductive capacity after they were cultured in suspension as spheroids (14). In our study, we verified a higher expression of CD133 and β-catenin in DP spheroids compared with DP cells, which means that DP spheroids could exhibit better hair growth inductive capacity in vivo.

Secretome from DP spheroids is also different from that of dissociated cells. Researchers have shown that dermal spheres are morphologically akin to DP cells in the anagen phase with expression profiles different from 2D cells but greatly similar to intact DP cells.

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Fig. 5. Secretome from 2D DP cells and 3D DP spheroids. (A) Schematic illustrating how DP spheroids promote the hair cycle transition from catagen to telogen via the migration and secretion of factors and exosomes. In anagen, an abundant source of growing DP cells is inside the follicle bulge. DP cells drop during catagen. The replenishment of DP cells promotes the onset of anagen. (B to E) Characterization of 2D DP-XOs and 3D DP-XOs. (B) TEM images. Exosomes are indicated by yellow arrows. Scale bars, 500 μm. (C) Size distribution by NanoSight. (D) Top 10 up-regulated and 10 down-regulated miRNAs of 3D DP-XOs compared with 2D DP-XOs. (n = 3 biological replicate and n = 3 technical replicates for each biological replicate). (E) Schematic illustrating FGF2- and TIMP2-driven hair follicle regulation and miR-218-5p–induced promotion of hair follicle development.
Evidence of how the transplanted hair follicles affect the environment of the bald area and paracrine effects involved were not sufficient. Our studies demonstrated that DP cells exerted their regulatory capacity on hair cycles mainly through a paracrine mechanism. The expression of β-catenin was up-regulated not only in the injection sites but also in nontreated sites. DP cells release various growth factors and vesicles to regulate follicular biology, and it was established that cultivation in spheroids most efficiently preserves the initial phenotype of these cells in vitro. Rather than introducing more DP cells to accumulate into hair follicles, exosomes were injected to regulate hair follicle cycles. Spheroid-derived exosomes, with a higher level of miR-218-5p compared with 2D DP-XOs, also enhanced the expression of β-catenin and down-regulated SFRP2, which positively regulated hair follicle growth and maintained the anagen phase of the hair cycle.

C57BL/6 mice are useful models for screening agents that promote hair growth because their skin produces pigment only during the anagen phase (52). Human hair loss has hormonal, environmental, and genetic causes. Because of its complexity, cell or secretome therapy may be more advantageous in treating hair loss compared to introducing more DP cells.
with minoxidil, since the primary therapeutic mechanism of minoxidil is the increase in cutaneous blood flow to the treated site. Increased blood flow causes hyperpolarization of cell membranes, allowing more nutrients to reach the follicles and cells. However, if DP cells remain dormant, little hair regrowth is likely to occur. In this study, we demonstrated that spheroids drive the hair cycle from telogen to anagen in healthy mice. Disease-related models or hormone-related hair loss models are needed to fully demonstrate the advantages of exosome therapy in comparison to minoxidil. Another limitation of this study lies in the interaction of exosomes with follicle cells. We focused on animal studies rather than cell studies because we were not sure which types of follicle cells were being regulated. More cell studies are needed to explore mechanisms in the future.

Collectively, we demonstrated that miR-218-5p–overexpressed exosomes accelerated the onset of anagen, and spheroid culture provided a potential avenue for cell therapy. miR-218-5p regulated hair follicle development by down-regulating WNT signaling inhibitor SFRP2, thus promoting β-catenin, creating a positive feedback loop. Our study may offer the following advantages compared with current hair regeneration practice. For people not suitable for invasive surgical procedures, they can benefit from the administration of the factors and exosomes by a minimally invasive approach, e.g., needle-free injection, microneedle patches, and sprays. Compared with the commercially available minoxidil, exosomes represent a natural product with fewer side effects. Overall, studies outlined here may represent new therapeutic strategies for hair treatment.

**MATERIALS AND METHODS**

**Isolation of DP cells from C57BL/6 mice**

The vibrissa pads were cut off from a euthanized C57BL/6 mouse (27) and then rinsed in PBS three times. Hair follicles were dissected and incubated with 0.25% dispase (STEMCELL Technologies) for 20 min in the incubator. Then, a horizontal cut was made above the DP. The hair follicle bulbs were then transferred into a rat-tail collagen I (Sigma-Aldrich)–coated dish. We used Eagle’s minimal essential medium (MEM; Gibco), 10% fetal bovine serum (FBS; Corning), 1% penicillin-streptomycin, and bFGF (10 ng/ml; Fisher Scientific) as DP medium until DP cells grew out from bulbs about 3 days later. Then, hair follicle bulbs were washed and placed in fresh medium. DP cells reached confluence after 1 week.

**2D cell and 3D spheroid culture**

For 2D cultures, rat-tail collagen I–coated flasks were used for passage. Passages 3 to 6 DP cells were used for this study. For 3D culture, DP cells were seeded in an ultralow attachment 96-well plate (Corning, 5 × 10^4 per well) to count the number spheroids. Spheroids were formed 2 days after seeding. Days 5 to 7 spheroids were used in the animal study.

DP medium [MEM, 10% FBS, 1% penicillin-streptomycin, and bFGF (10 ng/ml)] was used for cell culture. To collect secretome or exosomes, the DP medium was changed to basic MEM medium (no FBS) once cells reached 80% confluence. Then, conditioned medium was collected after 3 days.

**Secretome and exosome isolation and analysis**

Secretome was concentrated via Amicon Ultra-15 centrifugal filter units (3-kDa cutoff) and washed with PBS once. Exosomes were concentrated via Ultra-15 centrifugal filter units (100-kDa cutoff) and washed with PBS once.

The mouse Angiogenesis Array (RayBio) and the miRNA PCR (polymerase chain reaction) Array Mouse miFinder (Qiagen) were used according to the manufacturer’s instructions.

**Preparation of keratin hydrogels**

Keratin hydrogel was prepared according to a previous publication, with modifications (6). Briefly, human hair (local barber) was washed with water and then cut into small pieces. Then, hair fragments were treated with 2.5% (w/v) peracetic acid overnight before being washed thoroughly with running water. Hair fragments were immersed in 150 mM tris base for 2 hours to extract proteins. Then, a clear solution was obtained after fibers were removed via a 40-μm sieve. This solution was purified by dialysis against DI water (with 3-kDa molecular cutoff dialysis membrane; Fisher Scientific) and freeze dried (lyophilized). The resulting lyophilized solid was further ground into a fine powder. Fifteen percent keratin hydrogels (w/v) were prepared by reconstituting the fine powder with PBS. Spheroids were dispersed in keratin solution and left in a 37°C incubator overnight while the keratin formed a hydrogel.

**Scanning electron microscopy**

The morphology of the hydrogel was visualized via scanning electron microscopy (SEM). Samples were fixed with 2% glutaraldehyde and then dehydrated in gradient ethanol, successively, for 10 min each. Then, they were then dried in hexamethydisilazane (Sigma-Aldrich). Gels were sputter coated with gold before imaging (JEOL 6010LA SEM, JEOL Ltd., Japan).

**Animals and in vivo studies**

Seven-week-old male C57BL/6 mice were purchased from the Jackson laboratory and allowed to adapt to their new environment for 1 week. Hair was removed by using depilatory cream to observe the pink skin (10). Then, animals were randomly divided into groups (n = 5) to study hair regrowth. Minoxidil was topically applied daily as a positive control. All other treatments were administered subcutaneously. All work with mice was in accordance with the Institutional Animal Care and Use Committee at North Carolina State University.

Cell treatment dose: 1.0 × 10^6 cells in 200 μl PBS were subcutaneously injected into 10 spots (20 μl per site) on the left side of the dorsal skin.

Exosome treatment dose: 2.0 × 10^5 exosomes in 200 μl of PBS were subcutaneously injected into 10 spots (20 μl per site) on the left side of the dorsal skin.

Negative control, miR-218-5p mimics, and inhibitors (Sigma-Aldrich) were administered according to the protocol provided by in vivo-jetPEI (Polyplus-transfection). Briefly, mice received 10 injections per depilated dorsal skin. miRNA mimic (400 ng) or inhibitor was dissolved in 90 μl of 5% glucose (w/v) solution, and then 10 μl of in vivo-jetPEI solution was added. The solution was immediately vortex mixed and incubated for 15 min at room temperature before injection. Control groups were injected with mimic control.

**Skin tissue histology**

Mice were euthanized, and the whole dorsal skin was removed. Skin tissue was fixed in 4% paraformaldehyde and then cut into 5-μm-thick sections by Cryostat. H&E and Masson trichrome were stained according to protocols. For immunofluorescence histochemistry,
the primary antibodies used were mouse anti-β-catenin (Abcam, ab6301), rabbit anti-CD133 (ab19898), rabbit anti-Ki67 (ab16667), and rabbit anti-SFRP2 (ab137560). The secondary antibodies used were goat anti-mouse immunoglobulin G (IgG; Alexa Fluor 488; ab150113), goat anti-rabbit IgG (Alexa Fluor 488; ab150077), and goat anti-mouse IgM (Alexa Fluor 555; ab150114).

**Western blot**

Samples were loaded and compared with a standard ladder (Precision Plus Protein Standards, Bio-Rad). Primary antibodies were as follows: mouse anti-β-catenin (Abcam, ab6301), rabbit SFRP2 (ab137560), β-catenin (Abcam, ab6301), rabbit SFRP2 (ab137560), rabbit anti-CD133 (ab19898), rabbit anti-Ki67 (ab16667), β-catenin (Abcam, ab6301), rabbit anti-CD133 (ab19898), rabbit anti-Ki67 (ab16667), and goat anti-rabbit IgG (Alexa Fluor 555; ab150114).

**Statistical analysis**

All quantitative experiments were conducted in triplicate unless otherwise indicated. Data were shown as means ± SEM and analyzed by two-tailed, unpaired Student’s t-test for comparison between the two groups. Data comparisons between more than two groups were done by using one-way analysis of variance (ANOVA) followed by the post hoc Bonferroni test. Data between grouped data were analyzed by using two-way ANOVA. Differences with a P value less than 0.05 were considered statistically significant.

**SUPPLEMENTARY MATERIALS**

Supplemental material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/30/eaba1685/DC1

**REFERENCES AND NOTES**


(2010).


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Dermal exosomes containing miR-218-5p promote hair regeneration by regulating β-catenin signaling

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