Modulating hair follicle size with Wnt10b/DKK1 during hair regeneration

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Abstract: Hair follicles have characteristic sizes corresponding to their cycle specific stage. However, how the anagen hair follicle specifies its size remains elusive. Here, we show that in response to prolonged ectopic Wnt10b-mediated β-catenin activation, regenerating anagen hair follicles grow larger in size. In particular, the hair bulb, dermal papilla and hair shaft become

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/exd.12416

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enlarged. While the formation of different hair types (Guard, Awl, Auchene, and Zigzag) is unaffected. Interestingly, we found the effect of exogenous WNT10b was mainly on Zigzag and less on the other kinds of hairs. We observed dramatically enhanced proliferation within the matrix, DP and hair shaft of the enlarged AdWnt10b-treated hair follicles compared with those of normal hair follicles at P98. Furthermore, expression of CD34, a specific hair stem cell marker, was increased in its number to the bulge region after AdWnt10b treatment. Ectopic expression of CD34 throughout the ORS region was also observed. Many CD34 positive hair stem cells were actively proliferating in AdWnt10b-induced hair follicles. Importantly, subsequent co-treatment with the Wnt inhibitor, DKK1, reduced hair follicle enlargement, decreased proliferation and ectopic localization of hair stem cells. Moreover, injection of DKK1 during early anagen significantly reduced the width of prospective hairs. Together, these findings strongly suggest that Wnt10b/DKK1 can modulate hair follicle size during hair regeneration.

**Key words:** Wnt10b – DKK1 –hair follicle size – hair regeneration – hair stem cells

**Introduction**

Hair follicles display different shapes, lengths and thickness during anagen, catagen and telogen phases of the hair cycle (1, 2). Furthermore, in mouse dorsal skin, there are four distinct hair follicle types with different shapes and sizes that successively emerge during hair development. These include Guard (primary hair), Auchene and Awl (secondary hair) and Zigzag (tertiary hair) hairs (3). In general, primary and secondary hair follicles are larger than tertiary hair follicles during anagen (4).
Hair size is strictly controlled by reciprocal epithelial–mesenchymal interactions. Epithelial keratinocytes of the hair matrix supply the hair shaft progenitor cells, while the dermal papilla (DP) is the critical mesenchymal signaling center that regulates epithelial behavior (5). In both human and murine hair follicles, hair size correlates with DP cell numbers (4, 6, 7). A recent study showed that hairs can switch progressively from smaller to larger types during the hair cycle due to increased DP cell numbers (4).

At the molecular level, hair size can be regulated by multiple factors that influence epithelium and/or mesenchyme. Ectopic epidermal expression of the BMP antagonist, Noggin, causes a marked increase in anagen hair follicle size and changes Zigzag hairs to become larger, Awl-like hair follicles (8). Epidermal Eda/Edar is required for primary hair placode formation (9-12), while Sox2+ cell depletion resulted in a loss of primary and secondary hair induction (3). Knocking out Sox2 in the dermal papilla reduced the overall rate of hair growth (13). Sox18 deletion reduced Zigzag hair numbers (14, 15). FGF20 signaling activity also plays a role in primary and secondary hair induction by influencing dermal condensation formation (16). The deletion or up-regulation of each of these molecules not only results in changes of hair size but also hair types. It remains elusive whether or not the hair size can be controlled by molecular mechanisms independent of those regulating the switch of hair types.

Wnts and their DKK antagonists regulate the morphology of ectodermal organs (17-20). Wnt10b is a canonical Wnt member (21) that is expressed during hair induction and hair reconstitution (22, 23). Recent studies have shown that adenovirus mediated Wnt10b expression (AdWnt10b) leads to epithelial and melanocyte cell differentiation and elongated hair shafts (24-26). In our previous studies, we reported that WNT10b over-expression resulted in growth of
vibrissae in vitro and early induction of hair follicles in vivo (27-29). In the present study, we apply multiple injections of Wnt10b-expressing adenoviruses to regenerating hair follicles, with or without the presence of a Wnt antagonist, DKK1. We show that WNT10b treatment leads to enlargements of hair follicles during regeneration through the proliferation and scattering of hair stem cells, which can be partially rescued by DKK1. These findings shed new light on how external macroenvironmental signaling communicates with the hair follicle to specify organ size at the cellular and molecular levels.

Materials and methods

Mice

Animal maintenance and utilization were approved by the Third Military Medical University in China. Female C57BL/6J mice at 8 weeks of age, corresponding to the second telogen phase of the hair cycle (28), were used for the adenovirus injection study. Female C57BL/6J mice at postnatal day98 (P98) were used as controls.

Adenovirus and plasmid.

Adenoviruses including Adwnt10b and AdGFP (control) used in this study were a gift from Dr. T.C. He, University of Chicago, USA. The adenoviruses were propagated in HEK293 cells to a final titer of 1×10^8 according to the published protocol (30). Full length DKK1 CDS sequence was cloned into a pEGFP-N1 vector at Kpn I and Hind III restriction enzyme sites, with the following primers Sense: 5'-CCCAAGCTTATGATGGTTGTGTGCAGCGG-3', Antisense:-5' GGGGTACCTTGTCTCTGGCAGGTGGAGC-3'. pEGFP-N1 plasmid information and expression in skin after injection were presented in our previous studies (31, 32).
Intradermal injection of Adenovirus *in vivo*.

For adenovirus injection, 40ul $1 \times 10^8$ AdWnt10b or AdGFP adenoviruses were injected intradermally once a week for four weeks (Schematic drawing in Fig. 1a and Fig. S1a). The diffusion and expression of adenoviruses were observed by detecting LacZ and GFP in the adenovirus injected area as previously described (28). In the present study, we also observed WNT10b was increased in hair bulb, DP and hair shaft (Fig. S2a). Adenovirus injection experiments were repeated at least 40 times, with a hair regeneration rate of about 85%.

Transfection of naked plasmid *in vivo*.

Naked plasmid was applied to the adenovirus treated area when hair follicles entered anagen (pigmentation appears; Schematic drawing in Fig. 3a and Fig. S4a) or later, during early anagen (P91, schematic drawing in Fig. 4a). 20ul *DKK1* or pEGFP-N1 empty vector plasmid was injected at a concentration of 600 ug/ml (12ug total) to a 12.6 mm$^2$ area in the center of the pigmented region (31, 32). Plasmid injection experiments were repeated five times. Authenticity of the naked plasmid intradermal injection was confirmed by PCR, immunostaining and direct fluorescence as described in our previous studies (31, 32). In the present study, most hair follicles (60.5±6.8%, n=100) in the plasmid injected skin were positive for the encoded GFP and DKK1 one week after *DKK1* treatment (Fig. S4c). The AdWnt10b+*DKK1* plasmid treated skin samples were harvested two weeks after plasmid injection (Fig. 3a). All hair follicles in the collected samples remained in anagen phase as evaluated by TUNEL staining (Fig. S1i and Fig. S4d). Skin samples were harvested one week after receiving a single *DKK1* plasmid injection (Fig. 4a). The size of the central hair bulb and the middle hair shaft width were determined. Hair
shaft length was measured from the epidermis to the tip of the hair bulb. BrdU diluted in PBS (100 mg/kg) was injected to the abdomen 4 hours before euthanasia.

**Histology and immunofluorescence.**

Harvested samples were fixed in 4% paraformaldehyde (PFA) and embedded in paraffin. Sections were cut at 5μm and stained with hematoxylin and eosin (H&E) for 3min. The Adobe Photoshop CS3 ruler tool was used to analyze follicle width. For immunostaining, antigen retrieval was carried out by microwaving the tissues for 10 min in boiled citrate acid plus sodium citrate buffer. Then samples were incubated with primary antibodies against WNT10b (Goat, 1:100, Santa Cruz, CA, USA), β-catenin (Rabbit, Boster, Wuhan, China), Lef1 (Goat, 1:100, Santa Cruz, CA, USA), BrdU (mouse, 1:100, Sigma-Aldrich, St. Louis, MO, USA), Ki67 (mouse, 1:100, Sigma-Aldrich, St. Louis, MO, USA), AE15 (1:2, gift), CD34 (Rabbit, Boster, Wuhan, China), Sox2 (Goat, 1:100, R&D Systems, MN, USA), or β1-integrin (Rabbit, 1:100, Bioss, Beijing, China) overnight at 4°C and with Cy3-labeled fluorescent secondary antibodies (Beyotime, Nantong, China) for 2 hrs at 37°C. Sections were counterstained with DAPI (1:1000, Sigma-Aldrich, St. Louis, MO, USA). Fluorescence was checked by fluorescence microscopy (Nikon, Japan). The relative intensity of β1-integrin protein was measured using Image J.

**Statistic analysis**

40 hair follicles for each group of AdWnt10b, AdGFP, P98, AdWnt10b+DKK1 and AdWnt10b+N1 were analyzed. All experiments were repeated at least 3 times and the determinations were performed in triplicate. Statistical significance was determined using the Student’s t-test (SPSS 13.0, SPSS Inc.; P<0.05). Results are shown as the mean ± SD.

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Results

Prolonged Wnt10b over-expression increased the size of regenerated hair follicles.

To determine whether WNT10b regulates hair follicle size during hair regeneration, we subcutaneously injected AdWnt10b into dorsal skin of the mouse once a week beginning in refractory telogen at P56. The regenerated hairs reached their largest size approximately 4 weeks after the initiation of AdWnt10b treatments (P84; Schematic drawing in Fig. 1a and Fig. S1a), and resembled anagen VI hairs found in P98 control mice. H&E staining (Fig. 1b) showed the width of AdWnt10b-induced hair bulbs and hair shafts were remarkably increased compared to untreated controls (Fig. 1c). In contrast, skin treated with AdGFP remained at telogen phase at P84 (Fig. 1b). The width of medulla cells in the hair fiber was also significantly broadened (Fig. 1e and Fig. S1c-d). The DP of AdWnt10b-treated hair follicles were significantly enlarged compared to those of the P98 normal mouse hair follicles (Fig. 1b-c). AE15 immunostaining showed that the inner root sheath (IRS) of AdWnt10b-infected hair follicles was significantly thicker than those of control hair follicles (Fig. 1d-e). Furthermore, compared to the control follicle, where the outer root sheath (ORS) is composed of one or two cell layers, the ORS of AdWnt10b-infected hair follicles increased to three to five layers (Fig. S1b red line).

Next, we examined whether the larger regenerating follicles originated from primary or secondary hairs. H&E staining showed that there was only one column of medulla cells indicating that these were Zigzag hairs (Fig. S1b). Zigzag hairs have three bends while Guard hairs don’t have any bends (Fig S1i). The ratio of the four different hair types remained similar by AdWnt10b treatment compared to the controls which are in the third anagen cycle (Fig. S1g). So it is unlikely that AdWnt10b induced further conversion of tertiary hair types into the primary hair
In fact, among the four types of regenerated hair follicles, Zigzag hairs were most affected by Wnt10b overexpression (Fig. S1e). About 65% of Zigzag hair follicles had a larger size in mice treated 4-weeks with AdWnt10b compared to controls (Fig. 1f), while the size of other hair types were less affected (Fig. 1f). Unaffected Zigzag hair follicles were comparable in size to normal P98 hair follicles (Fig. S1f). Staining for Sox2, a marker of primary hairs, was not detected in most of the enlarged hair follicles, indicating that the enlarged AdWnt10b treated hairs were not converted to primary hairs (Fig. 1g). Further, the lengths of hair follicles were mostly unchanged compared with those of control mice at P98 (Fig. S1h), and the overall shape of all hair types remained normal after continuous AdWnt10b treatment (Fig. S1i), suggesting that AdWnt10b treatment primarily enlarged the size of Zigzag hairs rather than changing the lengths, shapes, and types of hairs. We hereafter focused on the AdWnt10b treatment-induced changes in the Zigzag hairs.

Aberrant and over-activation of Wnt signaling in AdWnt10b-induced hair follicles.

We next confirmed that 4 weeks of treatment with AdWnt10b induced ectopic Wnt10b signaling. Normally, WNT10b expression was observed in the hair matrix, with very weak expression in the ORS at P98. Prolonged treatment of the skin with ectopic AdWnt10b increased WNT10b expression levels significantly in the hair matrix, hair shaft, and DP (Fig. S2a). Nuclear β-catenin expression within the matrix was also elevated compared to control animals at P98 (Fig. 2a-b and Fig. S2b). Interestingly, β-catenin also accumulated in nuclei of the hair shaft and DP of WNT10b-induced hair follicles compared to controls (Fig. 2a-b). No nuclear β-catenin was detected in the AdGFP-treated hair follicles (Fig. 2a-b). Subsequently, we found that the number of Lef1 positive nuclei was dramatically increased in the matrix and DP of enlarged
AdWnt10b-induced hair follicles, compared to those of P98 control animals (Fig. 2c and Fig. S2c). While in the hair follicle of the AdGFP-treated group, Lef1 was only expressed in the DP region (Fig. S2c).

**AdWnt10b induced hair follicle components show excessive proliferation**

To investigate the mechanisms underlying enlargement of AdWnt10b-induced hair follicles, we examined proliferative activities of hair follicles using BrdU labeling. More BrdU labeled cells were located in the hair shaft (especially in the ORS), matrix and DP of AdWnt10b-treated mice (Fig. 2d-e and Fig. S3c). Ki67 staining confirmed that proliferation was increased in AdWnt10b-treated mice (Fig. S3a-b), while AdGFP-treated hair follicles remained in telogen and contained very few BrdU+ or Ki67+ cells (Fig. S3a-b).

**Increased and aberrant distribution cells with hair stem markers in AdWnt10b induced hair follicles.**

To examine progenitor cells that replenish the hyperproliferative hair follicles, we examined the expression of hair stem cell markers. Six days after the first AdWnt10b injection, expression of β1-integrin, a hair stem cell marker (33), was significantly enhanced in the bulge and the interfollicular epidermis compared to similar regions in the AdGFP treated group and normal telogen hair follicle (P56) (Fig. 2f and Fig. S3d). Moreover, CD34+ cells were also increased in the bulge region of the AdWnt10b-treated hair follicles compared with AdGFP-treated hair follicles (Fig. S3f-g). Interestingly, Ki67 immunostaining showed that the bulge, the DP and especially the second hair germ (HG) all displayed increased cell proliferation (Fig. 3g and Fig. S3e).
Surprisingly, CD34 expression was detected in the hair shaft of regenerated AdWnt10b-treated hair follicles from the bulge to the upper hair bulb, with particularly strong expression observed in the ORS (Fig. 2h). Double staining showed that CD34+/BrdU+ cells were colocalized both in the bulge and ORS of the regenerated AdWnt10b-treated hair follicles, compared to the P98 hair follicles which had very few cells that expressed both markers (Fig. 2i and Fig. S3h-i).

**DKK1 decreased hair follicle sizes by blocking AdWnt10b induced activation of Wnt signaling**

H&E staining showed that the size of most hair bulbs and hair shafts after DKK1 treatment were similar to those of P98 control animals (Fig. 3b). Quantification of hair follicle widths showed a significant decrease in AdWnt10b+DKKI-treated animals compared to AdWnt10b+N1-treated animals (Fig. 3c). Moreover, the DP size of the AdWnt10b+DKKI-treated group decreased compared to the AdWnt10b+N1-treated group and was similar to that of P98 controls (Fig. 3c). However, AdWnt10b+DKKI-treated hair follicles were similar in length to those of P98 control animals (Fig. S4b). Additionally, the proportion of enlarged hair follicles was reduced in the AdWnt10b+DKKI-treated group compared to the AdWnt10b+N1-treated group (Fig. 3d).

We next explored whether DKK1 suppression of Wnt induced β-catenin expression blocked hair follicle enlargement after AdWnt10b treatment. Immunostaining revealed that numbers of cells with β-catenin positive nuclei in the AdWnt10b+N1-treated hair matrix and DP were greater than those found in either the AdWnt10b+DKKI-treated mice or P98 control mice. In addition, β-catenin expression was only associated with the membrane of the hair shaft cells of the AdWnt10b+DKKI-treated hair follicles (Fig. S5a, c). Moreover, Lef1 expression increased in
animals treated with AdWnt10b+N1 but not in AdWnt10b+DKK1 treated or control P98 mice (Fig. S5b, d).

**DKK1 treatment after AdWnt10b induction partially rescued the phenotypes**

It appears that the emaciated hair follicle phenotype observed following treatment with AdWnt10b+DKK1 was due to perturbed proliferation or differentiation. The AdWnt10b+N1 treated group displayed hyperproliferative hair matrix, DP and hair shaft cells (Fig. 3e-f and Fig. S6a), while treatment with AdWnt10b+DKK1 restored proliferation nearly to control levels (Fig. 3e-f and Fig. S6a), which was confirmed by Ki67 immunostaining (Fig. 3g and Fig. S6b). Moreover, proper hair differentiation events (Fig. S7a-b) and hair stem cell localization (Fig. 3h-i) were also partially rescued by DKK1 overexpression.

**Decrease of hair widths after DKK1 treatment**

To further test the influence of DKK1 on hair size regulation, we subcutaneously injected DKK1 at early anagen when the skin became pigmented and examined hair size one week later (Fig. 4a). Most types of hairs displayed decreased widths after DKK1 treatment. Such reduction was particularly clear in secondary hairs and tertiary hairs (Fig. 4b). Unexpectedly, we observed that Awl hairs were significantly shorter after DKK1 treatment; whereas, other hair types retained normal lengths that were comparable to hairs of P98 control animals (Fig. 4b lower panels).

**Discussion**

The precise control of organ size is regulated by complex biological processes involving interactions that coordinate a response to autonomous factors and the extrinsic environment. The
roles of multiple signals including Eda/Edar, BMP, FGF, Sox2/Sox18 in specifying hair type and size have been well characterized (3, 8, 12, 14-16). In the present study, we demonstrate that Wnt10b/DKK1 co-operation could modulate anagen hair follicle sizes, including the hair bulb width, DP sizes as well as the overall thickness of hair shafts. Notably, these events occur without altering hair types.

Injecting up to four weekly doses of highly concentrated AdWnt10b into dorsal skin to continuously activate Wnt signaling significantly increased the size of regenerated hair follicles. It was recently reported that epidermal over-expression of Noggin inhibited BMP signaling and led to increased hair bulb size. This also resulted in the conversion of kinked Zigzag and Auchene hairs into straight Awl-like hairs (8). Another study reported that some Zigzag and Auchene hairs can transform to larger Awl hair types in the following hair cycle (4). In contrast, we show that WNT10b treatment leads to the enlargement of regenerating Zigzag hair follicles but not a switch of hair types.

We also determined how WNT10b exerted its effect on hair follicle enlargement. WNT10b was reported as a very important activator of primary hair follicle development and growth (11), and we also reported WNT10b over-expression resulted in early induction of hair follicles in vivo (26, 28). However, based on the previous and our current studies, we infer that WNT10b has different roles during hair development, regeneration and growth. During development and regeneration, WNT10b functions as an activator to initiate hair induction. After that, WNT10b is strongly expressed in the hair matrix region of anagen VI hair follicles, during the most active growth period of the hair cycle, but was expressed at lower levels in the early anagen, catagen or telogen (26, 28). WNT10b was also shown to promote growth of vibrissae in vitro (29). These
studies indicate that WNT10b might facilitate hair follicle proliferation. Indeed, we demonstrated that the enlarged hair follicles contained greater numbers of nuclear β-catenin, Lef1+, BrdU+ and Ki67+ cells. Further, hair matrix progenitor cells differentiate from hair stem cells during hair regeneration (34, 35). We found aberrant localization of hyperproliferative hair stem cells during WNT10b induction.suggesting that WNT10b might stimulate hair stem cells to proliferate and migrate from the bulge region to replenish hair matrix cells. Taken together, these data suggest that WNT10b may have a proliferative effect on hair matrix and DP cells and that interactive signaling between extra epithelial and mesenchymal cells may later lead to the enlarged hair follicle phenotype. Furthermore, since the hair fiber and IRS are derived from hair keratinocyte precursors (36), differentiation of the increased matrix cell progenitor pool or their progeny may lead to a thickened hair shaft. DKK1 is a specific endogenous Wnt antagonist (37). When hair ORS or DP cells were cultured in nevus sebaceous sebocyte-conditioned media, DKK1 was increased but WNT10b was decreased (38). DKK1 was also reported to promote hair follicle regression (39). Here, by applying DKK1 after AdWnt10b treatment, we found the number of enlarged anagen VI hair follicles was dramatically decreased, suggesting that WNT10b and DKK1 can modulate hair follicle size. Such regulation might act directly at the stem cell levels, since WNT10b treatment leads to stem cell proliferation and scattering, which is suppressed by DKK1 treatment.

How does WNT10b enlarge hair size but not switch the hair type? At the current stage, we do not have a clear molecular explanation. Previous study showed over activation of β-catenin could lead to telogen-anagen transition of hair follicles, with broadened ORS generation (40). However, even though all Wnt-mediated canonical Wnt signaling pathway activation leads to the nuclear accumulation of β-catenin, different Wnt ligands have different functions to regulate hair
follicle activities due to their temporal and spatial expression patterns. For instance, WNT3a functions more on melanocyte differentiation (41), while WNT7b and WNT1 have more influence on regulating hair follicle stem cell homeostasis and hair follicle cycling (42, 43). Although WNT10b is expressed in all the hair types, we might speculate that WNT10b could interact with or regulate those molecules uniquely expressed in Zigzag hair follicles. Second, DP size correlated with hair follicle size. It was reported that epidermal activation of \(\beta\)-catenin results in ectopic hair formation associated with increased fibroblast proliferation (44). It was also shown that inactivation of \(\beta\)-catenin within the developing hair follicle DP leads to reduced proliferation (5). Therefore, it is possible that the WNT10b-induced canonical Wnt/\(\beta\)-catenin pathway could also directly promote proliferation and enlargement of the DP. In addition, WNT10b was reported to promote differentiation of mesenchymal cells toward myofibroblasts (45). However, WNT10b is not expressed in the skin dermal-lineage under normal conditions (26, 28). It may function by mediating epithelial – mesenchymal interactions but not by reprogramming DP cell properties that determines hair types. Furthermore, several lines of evidence suggest that the hair type could be identified by DP markers. Sox2 marks the DPs of the larger primary and secondary hair follicles, while Sox18 marks the DPs of the smaller tertiary Zigzag hair follicles. So we might speculate that different types of DP have different sensitivities to ectopic WNT10b, which led to the larger Zigzag hairs but not those of primary and secondary hair follicles.

Taken together, our data provide compelling evidence that \textit{Wnt10b}/DKK1 can modulate hair follicle sizes by regulating hair matrix, DP and hair stem cell behaviors, including cell proliferation, differentiation and migration (Fig. 4c). Most importantly, our results suggest
potential mechanisms for the control of hair follicle miniaturization which may be utilized during aging or androgenetic alopecia and provide future directions to study the hair follicle response to external insults such as environmental pollution and radiation that target hair stem cells, hair matrix and DP cells.

Acknowledgements

This study was supported by grants 30972645, 11172338 and 11032012 from the National Nature Science Foundation of China and CSTC, Program for New Century Excellent Talents in University (NCET-10-0879), Innovation and Attracting Talents Program for College and University (‘111’ Project) (B06023), China. RW and CMC are supported by US NIH grant AR 42177 and AR 60306. We thank Dr. T.C. He (The University of Chicago) for the generous gifts of Wnt10b and control Adenoviruses. We thank Dr. Chin-Lin Guo (California Institute of Technology), Dr. Eve Kandyba (University of Southern California) and Dr. Lishi Li (The Rockefeller University) for carefully revising the manuscript.

Author contributions

ML, HYG, WQ, XDL performed the experiments. ML, LY, TY and XHL designed the research. ML, CMC and RBW wrote the manuscript.

Conflict of interests

The authors have declared no conflict of interest.
Reference


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**Figures and Legends**

Figure 1. AdWnt10b treatment enlarges hair follicle size without shifting its type. a. Schematic drawing showing the timing of multiple AdWnt10b injections, hair cycle events and checkpoints. b-c. H&E staining and statistical chart showing that the width of HB, DP and HS was significantly increased after continuous AdWnt10b treatment. d-e. AE15 immunostaining and statistical chart revealed the IRS was broadened (Red line), and the hair fiber did not differentiate properly in the AdWnt10b-induced hair follicles (yellow arrow). f. About 65% of Zigzag hair follicles became enlarged, while only few Guard (G), Awl (A), Auchene (Au) hair follicles expanded in size. g. Wnt10b-induced larger Zigzag hair follicles were Sox2 negative while the normal Guard hairs were Sox2 positive. Epi, epidermis; SG, sebaceous gland; HFi, hair fiber; Bu, bulge; HB, hair bulb; HM, hair matrix; DP, dermal papilla; HS, hair shaft. *P < 0.05.

Figure 2. *Wnt10b*-induced hair follicles were accompanied by excessive activation of the Wnt/β-catenin pathway, increased proliferation, and hair stem cell activation and migration. a-b. Immunostaining and statistical chart showed nuclear β-catenin, a key mediator of the Wnt signaling pathway, was dramatically increased in the Matrix, HS and DP in the AdWnt10b-treated group compared within AdGFP and normal P98 hair follicle controls (Red arrow, nuclear β-catenin; pink arrow, cytoplasm β-catenin; white arrow, membrane β-catenin). c. Statistical chart of Lef1, a downstream Wnt signaling target, was also markedly augmented in the Matrix and DP region, but not in the HS. d-e. Immunostaining and statistical chart reveals BrdU-labeled proliferating cells were increased in Wnt10b-induced enlarged hair follicles. f. Statistical graph showing β1-integrin expression was significantly increased in the HG, Bu and...
IFE regions of AdWnt10b-treated hair follicles. g. Ki67+ proliferating cells were located in the bulge, second hair germ and DP region 6d after the first AdWnt10 treatment. h. CD34 was unexpectedly localized to the hair shaft, especially in the ORS region. i. Double staining displayed colocalization of CD34 and BrdU both in the bulge and hair shaft of AdWnt10b-induced hair follicles but not in those of normal control hair follicles. Bu, bulge; HB, hair bulb; DP, dermal papilla; HS, hair shaft; HG, second hair germ; IFE, interfollicular epidermis; EB, epidermal basal layer. *P < 0.05.

Figure 3. Sequential AdWnt10b+DKK1 hair follicle treatment decreased Wnt/β-catenin pathway activation, reduced proliferation in the hair matrix, DP and hair shaft, but maintained the proper localization of hair stem cells. a. Schematic drawing showing the timing of two injections of AdWnt10b followed by DKK1 treatment, hair cycle events and check points. b-c. H&E staining and statistical chart presenting the significantly decreased width of HB, DP and HS after AdWnt10b-DKK1 treatment. The results were similar to those of the P98 normal hair follicles. d. The enlarged hair follicles were significantly reduced from 54.3±4.3 (%) in the AdWnt10b+N1-treated group to 9.8±3.8 (%) in the AdWnt10b+DKK1-treated group. Note, not all hair follicles decreased in size (Red and green arrows in b). e-g. Immunostaining and statistical chart revealed BrdU+ and Ki67+ proliferating cells were decreased in AdWnt10b+DKK1-treated hair follicles compared within the AdWnt10b+N1-treated hair follicles, especially in the hair matrix, DP and hair shaft. The proliferative index was recovered to the P98 normal state. h. Immunostaining for CD34 indicated that after AdWnt10b+DKK1 treatment, hair stem cells were only located in the bulge region of the regenerated hair follicles. i. CD34+ cells were widespread in the hair shaft, especially the ORS region of AdWnt10b+N1.
while not in those of AdWnt10b+DKK1 treated group. N1, control plasmid; Epi, epidermis; SG, sebaceous gland; HB, hair bulb; HM, hair matrix; DP, dermal papilla; HS, hair shaft. *P < 0.05; # no statistical difference.

Figure 4. DKK1 treatment decreased hair width. a. Schematic drawing showing the timing of DKK1 injection, hair follicle status and check points. b. DKK1 treatment narrowed the width of Zigzag, Auchene, and Awl hairs, and shortened the length of Awl hairs. c. Summary diagram showing that hair regeneration could be induced by ectopic WNT10b. Prolonged activation of Wnt signaling in the hair follicle would lead to greater interaction between the hair matrix epithelial cells and the DP mesenchymal cells, producing more proliferation and differentiation and broadening the HB, DP and HS. WNT10b could also promote migration of hair stem cells to sustain matrix proliferation. Interestingly, these processes can be rescued by giving DKK1 to inhibit regenerating hair follicles. Bu, bulge; DP, dermal papilla; HS, hair shaft.

Supplementary information

Figure S1. Prolonged AdWnt10b treatment enlarged hair follicle size without changing the hair length. a. Schematic drawing of working model showing that a subcutaneous injection of AdWnt10b could induce hair regeneration. b. H&E staining shows regenerated hair fibers have only one column of medulla cells, indicating that the Zigzag hair type was not changed in Wnt10b-induced regenerated hair follicles. Increased ORS cell layers were present compared with the normal hair follicles (red line). c-d. WNT10b increased medulla width of regenerated hairs compared with the normal Zigzag hairs. e. WNT10b only increased the width of Zigzag hair fibers. f. The regenerated hair follicles unaffected by AdWnt10b treatment maintained a
normal hair size (Red arrow). g. The ratio of the four different hair types was unchanged after AdWnt10b treatment compared to the P98 controls. h. The length of the WNT10b-induced regenerated hair follicles was not significantly shifted. i. The overview of all hair types were normal compared with AdWnt10b-induced hair fibers and P98 normal hair fibers. j. The absence of TUNEL positive cells in AdWnt10b-induced hair follicles and P98 normal hair follicles revealed collected samples were still in anagen. Data are reported as mean ±SD. *P < 0.01. # no statistical difference.

Figure S2. AdWnt10b treatment increases the activation of the Wnt/β-catenin pathway. a. WNT10b was expressed in the hair matrix and a little bit at the ORS in normal P98 hair follicles. Prolonged AdWnt10b treatment led to increased Wnt10b expression in the hair matrix and ectopic expression in the DP and hair shaft. Wnt10b was not expressed in the AdGFP-treated telogen hair follicles. b. Immunostaining showing overview of β-catenin expression related to Fig. 2a. c. Immunostaining of Lef1, a downstream Wnt signaling target, was also markedly augmented in the Matrix and DP region, but not in the HS. Lef1 was only expressed in the DP region of AdGFP treated hair follicles.

Figure S3. Wnt10b-induced hair follicles were accompanied by hair stem cell activation, proliferation and migration. a-b. Immunostaining and statistical chart showing Ki67+ cells were increased in number in the HM, DP and HS region of AdWnt10b-induced hair follicles. c. Overview of BrdU immunostaining related to Fig. 2d. d. Six days after AdWnt10b treatment, β1-integrin immunoreactivity was significantly increased compared with the AdGFP or the normal P56 control hair follicles. e. Ki67+ proliferating cells were increased in the bulge, second hair germ and DP region 6d after the first AdWnt10 treatment. f-g. Immunostaining and
statistical chart showing that the number of CD34+ bulge stem cells was markedly increased 6d after AdWnt10 treatment, compared with the control group. h-i. Double staining displayed CD34 and BrdU colocalization both in the bulge and hair shaft of AdWnt10b-induced hair follicles but not in those of normal control hair follicles.

Figure S4. Hair regeneration after AdWnt10b+DKK1 sequential treatment. a. Schematic drawing showing our experimental approach to test the role of Wnt10b/β-catenin and DKK1 signaling on hair regeneration. DKK1 was injected subcutaneously in the center of the pigmented region of hair follicles that had regenerated two weeks after AdWnt10b treatment. b. Measurement of follicle length after AdWnt10b+DKK1 co-treatment. AdWnt10b+DKK1 treatment didn’t change the length of the regenerated hair follicles. Follicle lengths were determined at the anagen VI stage in AdWntWnt10b+N1, AdWnt10b+DKK1 and normal P98 groups. c. GFP fluorescence of DKK1-GFP plasmid injected and uninjected skin. Immunostaining showed GFP and DKK1 were increased in the DKK1-GFP plasmid injected skin. d. The absence of TUNEL positive cells in AdWnt10b+N1 and AdWnt10b+DKK1-induced hair follicles revealed the collected samples were still in anagen. *P < 0.01.

Figure S5. Sequential AdWnt10b plus DKK1 treatment decreased Wnt/β-catenin pathway activation. a and c. Immunostaining and statistical chart revealed nuclear β-catenin expression was dramatically reduced in the HM, HS and DP in the AdWnt10b+DKK1-treated hair follicles compared with the AdWnt10b+N1-treated hair follicles. The expression pattern between the AdWnt10b+DKK1-treated group and normal P98 group were most similar to each other (Red arrow, nuclear β-catenin; pink arrow, cytoplasm β-catenin; white arrow, membrane β-catenin).
and d. Immunostaining for Lef1 showed Lef1+ cell numbers were also markedly decreased in the HM, DP region. N1, control plasmid; Epi, epidermis; HB, hair bulb; HM, hair matrix; DP, dermal papilla; HS, hair shaft. *P < 0.05; # no statistical difference.

Figure S6. a. Overview of BrdU immunostaining related to Fig. 3f. b. AdWnt10b+DKK1-treated hair follicles have reduced proliferation in hair matrix, DP and hair shaft. Immunostaining showing Ki67+ cells were prominently diminished in the HM, DP and HS regions of AdWnt10b+DKK1 treated hair follicles. The proliferative index was recovered to the P98 normal state.

Figure S7. Recovered IRS thickness after DKK1 treatment. a-b. AE15 immunostaining for the IRS and statistical chart comparing IRS width. The IRS of AdWnt10b+N1 treated hair follicles (yellow arrow and red line) was broadened. The width was attenuated to P98 level after DKK1 treatment (pink arrow and red line). Some hair follicles that were not rescued show aberrant AE15 expression in the hair fiber medulla cells (Green arrow). *P < 0.05; # no statistical difference.
Figure 1. AdWnt10b treatment enlarges hair follicle size without shifting its type. a. Schematic drawing showing the timing of multiple AdWnt10b injections, hair cycle events and check points. b-c. H&E staining and statistical chart showing that the width of HB, DP and HS was significantly increased after continuous AdWnt10b treatment. d-e. AE15 immunostaining and statistical chart revealed the IRS was broadened (Red line), and the hair fiber did not differentiate properly in the AdWnt10b-induced hair.
follicles (yellow arrow). f. About 65% of Zigzag hair follicles became enlarged, while only few Guard (G), Awl (A), Auchene (Au) hair follicles expanded in size. g. Wnt10b-induced larger Zigzag hair follicles were Sox2 negative while the normal Guard hairs were Sox2 positive. Epi, epidermis; SG, sebaceous gland; HFi, hair fiber; Bu, bulge; HB, hair bulb; HM, hair matrix; DP, dermal papilla; HS, hair shaft. *P < 0.05.
Figure 2. Wnt10b-induced hair follicles were accompanied by excessive activation of the Wnt/β-catenin pathway, increased proliferation, and hair stem cell activation and migration. a-b. Immunostaining and statistical chart showed nuclear β-catenin, a key mediator of the Wnt signaling pathway, was dramatically increased in the Matrix, HS and DP in the AdWnt10b-treated group compared within AdGFP and normal P98 hair follicle controls (Red arrow, nuclear β-catenin; pink arrow, cytoplasm β-catenin; white arrow, membrane β-catenin). c. Statistical chart of Lef1, a downstream Wnt signaling target, was also markedly augmented in the Matrix and DP region, but not in the HS. d-e. Immunostaining and statistical chart reveals BrdU-labeled proliferating cells were increased in Wnt10b-induced enlarged hair follicles. f. Statistical graph showing β1-integrin expression was significantly increased in the HG, Bu and IFE regions of AdWnt10b-treated hair follicles. g. Ki67+ proliferating cells were located in the bulge, second hair germ and DP region 6d after the first AdWnt10 treatment. h. CD34 was unexpectedly localized to the hair shaft, especially in the ORS region. i. Double staining displayed colocalization of CD34 and BrdU both in the bulge and hair shaft of AdWnt10b-induced hair follicles but not in those of normal control hair follicles. Bu, bulge; HB, hair bulb; DP, dermal papilla; HS, hair shaft; HG, second hair germ; IFE, interfollicular epidermis; EB, epidermal basal layer. *P < 0.05.
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Figure 3. Sequential AdWnt10b+DKK1 hair follicle treatment decreased Wnt/β-catenin pathway activation, reduced proliferation in the hair matrix, DP and hair shaft, but maintained the proper localization of hair stem cells. a. Schematic drawing showing the timing of two injections of AdWnt10b followed by DKK1 treatment, hair cycle events and check points. b-c. H&E staining and statistical chart presenting the significantly decreased width of HB, DP and HS after AdWnt10b-DKK1 treatment. The results were similar to those of the P98 normal hair follicles. d. The enlarged hair follicles were significantly reduced from 54.3±4.3 (%) in the AdWnt10b+N1-treated group to 9.8±3.8 (%) in the AdWnt10b+DKK1-treated group. Note, not all hair follicles decreased in size (Red and green arrows in b). e-g. Immunostaining and statistical chart revealed BrdU+ and Ki67+ proliferating cells were decreased in AdWnt10b+DKK1-treated hair follicles compared within the AdWnt10b+N1-treated hair follicles, especially in the hair matrix, DP and hair shaft. The proliferative index was recovered to the P98 normal state. h. Immunostaining for CD34 indicated that after AdWnt10b+DKK1 treatment, hair stem cells were only located in the bulge region of the regenerated hair follicles. i. CD34+ cells were widespread in the hair shaft, especially the ORS region of AdWnt10b+N1 while not in those of AdWnt10b+DKK1 treated group. N1, control plasmid; Epi, epidermis; SG, sebaceous gland; HB, hair bulb; HM, hair matrix; DP, dermal papilla; HS, hair shaft. *P < 0.05; # no statistical difference.
(a) Working model

Refactory telogen

P91

Pigmentation

Check point

(b) Zigzag Guard Auchene Awl

P88 DKK1 P91 DKK1 P98 DKK1 P98 DKK1 P98 DKK1

(c) Uninjected region Injected region Uninjected region Injected region

Uninjected region Injected region

DP Bu DP Bu DP Bu DP Bu

Wnt10b ① Wnt10b ②

Club hair New hair

DP Bu DP Bu

Stem cell emigration

Enlarged follicle

DP cell

Interaction

Matrix cell

Wnt pathway:

Stem cells:

Proliferation:

Differentiation:

Wnt pathway:

Stem cells:

Proliferation:

Differentiation:

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Figure 4. *DKK1* treatment decreased hair width. a. Schematic drawing showing the timing of *DKK1* injection, hair follicle status and check points. b. *DKK1* treatment narrowed the width of Zigzag, Auchene, and Awl hairs, and shortened the length of Awl hairs. c. Summary diagram showing that hair regeneration could be induced by ectopic WNT10b. Prolonged activation of Wnt signaling in the hair follicle would lead to greater interaction between the hair matrix epithelial cells and the DP mesenchymal cells, producing more proliferation and differentiation and broadening the HB, DP and HS. WNT10b could also promote migration of hair stem cells to sustain matrix proliferation. Interestingly, these processes can be rescued by giving DKK1 to inhibit regenerating hair follicles. Bu, bulge; DP, dermal papilla; HS, hair shaft.