Journal of Dermatological Science 83 (2016) 45–51

Contents lists available at ScienceDirect

Journal of Dermatological Science

journal homepage: www.jdsjournal.com

Wnt/β-catenin signaling pathway activates melanocyte stem cells *in vitro* and *in vivo*

Haiying Guo[a], Yizhan Xing[a], Yingxin Liu[a], Yan Luo[a], Fang Deng[a], Tian Yang[a], Ke Yang[b,*], Yuhong Li[a,*]

[a] Department of Cell Biology, Third Military Medical University, Chongqing 400038, China
[b] Chongqing Stem Cell Therapy Engineering Technical Center, Children's Hospital of Chongqing Medical University, Chongqing 400014, China

ARTICLE INFO

Article history:
Received 26 February 2016
Received in revised form 7 April 2016
Accepted 15 April 2016

Keywords:
Wnt signaling pathway
Wnt3a
Wnt10b
Melanocyte stem cells
Melanogenesis

ABSTRACT

Background: Melanocyte stem cells (McSCs) are the origin of melanocytes that are periodically refreshed in skin and hair follicle. Previously, we reported that Wnt3a could promote melanogenesis, but the mechanism of McSCs activation remains unclear.
Objective: We aimed to illustrate the roles of Wnt/ß-catenin signaling pathway during McSC activation.
Methods: Adenovirus-mediated overexpression of Wnt3a and Wnt10b were used. *In vitro* experiments were performed on the immortalized melanocyte progenitor cell line iMC23, wheres *in vivo* experiments were performed in Dct-LacZ mice. Immunofluorescence and western blot were used to determine the protein expression.
Results: Wnt3a promotes the differentiation and melanogenesis of iMC23, by activating Wnt/β-catenin signaling pathway. Wnt3a induces hair follicle regeneration and McSC activation. Detailed analysis indicats that Wnt3a activated Wnt/β-catenin signaling pathway, thus promoting the differentiation of McSCs during this process. Wnt10b, another canonical Wnt signaling ligand, induces hair follicle regeneration and McSC activation as well.
Conclusion: Wnt/β-catenin signaling pathway activates McSCs both *in vitro* and *in vivo*.

1. Introduction

Melanocytes synthesize melanin within the melanosome and thus protect individuals from harmful UV rays. Melanocyte dysfunction leads to diseases such as melanoma and pathological pigmentation [1,2]. Melanocytes are terminally differentiated cells, but they undergo periodic refreshment, along with the periodical regeneration of hair follicles. Hair follicle is an accessory mini-organ of skin. Hair cycle consists of anagen, catagen and telogen phases [3]. During the hair follicle telogen phase, McSCs (melanocyte stem cells) in the hair follicle bulge are resting. At anagen, new melanocytes are derived from the differentiation of McSCs, which are reported to reside in the bulge area and hair germ area of hair follicles [4,5]. McSC niche deficiencies potentially result in pigmentary disorders, thus leading to hair graying or vitiligo [6,7].

Many signaling pathways are involved in the regulation of melanogenesis and McSCs activation, such as Wnt (wingless-type mouse mammary tumor virus integration site), Notch, and TGFβ (transforming growth factor β) [8–12]. Wnt signaling pathway plays an important role in development, regeneration and tumorigenesis [13]. Wnt family consists of at least 19 types of secreted proteins. These proteins act in an autocrine or paracrine fashion. Wnt signaling pathway is typically divided into canonical Wnt signaling pathway and non-canonical Wnt signaling pathway. When canonical Wnt ligand binds with its receptor Frizzled and coreceptor LRP5/6, ubiquitin degradation of plasma β-catenin is abrogated. After sufficient accumulation, β-catenin translocates intothe nucleus, and binds to LEF/TCF, thereby activating the expression of target genes. Thus, the canonical Wnt signaling pathway is also called Wnt/β-catenin pathway. Among the canonical Wnt signaling ligands, Wnt7a, Wnt7b and Wnt10b induce hair follicle regeneration [14–16]. Both *in vitro* and *in vivo* studies reported that deletion of Wnt1 and Wnt3a changed the fate of neural crest cells, thus leading to the loss of the function of melanogenesis [17,18]. Wnt7a induces the translocation of β-catenin in McSCs [10]. Wnt3a promotes the melanogenesis of melanocytes [19,20], whereas Wnt10b promotes differentiation of

* Corresponding authors.
E-mail addresses: 78210114@163.com (K. Yang), liyuhongtmmu@hotmail.com (Y. Li).

http://dx.doi.org/10.1016/j.jdermsci.2016.04.005
0923-1811/

mouse hair follicle melanocytes [21]. However, the role of canonical Wnt signaling pathway in the activation and quiescence of McSCs has not been systematically elucidated, partly due to a lack of *in vitro* culture for McSCs. Previously, we established an immortalized melanocyte progenitor cell line [22], characteristics similar to McSCs. We also established an adenovirus-mediated gene expression model [16], which could manipulate genes locally in skin. Using use of these models, we systematically researched the role of canonical Wnt signaling pathway in McSC differentiation, both *in vitro* and *in vivo*.

2. Methods

2.1. Animals and skin samples

Dct-LacZ transgenic mice were kindly provided by Jackson [23]. The generated colony was backcrossed with C57BL/6J. Mice were housed in community cages at the animal facility of Third Military Medical University. All of the animal-related procedures were in strict accordance with the approved institutional animal care and maintenance protocols. All experimental protocols were approved by the Research Committee of Third Military Medical University.

2.2. Reverse transcription polymerase chain reaction

Total cellular RNA was isolated with TRIzol reagent (Invitrogen, USA) according to manufacturer's protocol. Single-stranded cDNA was synthesized with a ReverTra Ace reverse transcription kit (Toyobo, Japan). PCR reactions for Frizzled 1 to Frizzled 10, LRP5 and LRP6 were performed as previously described [19]. The experiment was repeated for three times.

2.3. Adenovirus amplification

Adenoviruses including Wnt1, AdWnt3a, AdWnt10b and AdGFP (control) were gifts from Dr. Tong-Chuan He, University of Chicago, USA. The adenoviruses were propagated in HEK293 cells as previously described [24]. After purification on cesium chloride gradients, adenoviruses were dialyzed in storage buffer. Then their titers were determined and diluted with storage buffer to the ultimate titers of 10^8PFU (plaque-forming unit)/mL.

2.4. Intradermal administration of adenoviruses

A total of 50 μL AdWnt3a or AdGFP vehicle control were administered intradermally into the dorsal skin of female Dct-LacZ mice or C57BL/6J mice (8-week-old). For the histochemical analyses, dorsal skins were harvested for X-gal staining at 4 and 14 days post administration. Three mice were used for each time point in each group.

2.5. Histological analysis and immunostaining

For X-gal staining, dorsal skin was fixed in 4% paraformaldehyde at 4 °C for 1 h, stained with X-gal staining solution (Beyotime, China) at room temperature for 24 h, and post-fixed overnight with 4% paraformaldehyde at 4 °C. The pH of X-gal staining solution is approximately 7.0. The skin was embedded in paraffin and sectioned into 6-μm sections. Sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin.

Immunostaining was performed on 5-μm paraffin-embedded sections from dorsal skin. Sections were deparaffined, rehydrated, and boiled in citrate buffer solution. After blocking in 10% goat serum in PBS, sections were incubated with the following primary antibodies: rabbit anti-GFP (1:100, Beyotime, China), rabbit anti-Wnt3a (1:100, Abcam, USA), goat anti-TRP2 (1:200, Abcam, UK; or 1:100, Santa Cruz, USA), rabbit anti-TRP1 (1:100; Santa Cruz, USA), and mouse anti-β-catenin (1:100; Beyotime, China). Alexa Fluor 488(Invitrogen, USA) or CY3 (Beyotime, China) labeled secondary antibodies were used.

2.6. Cell culture and adenovirus infection

The immortalized cell line of mouse melanocyte progenitor, iMC23, was cultured in DMEM medium (Hyclone, USA) containing 10% FBS (Gibco, USA) [22]. For adenovirus infection, iMC23 cells were plated onto 6-well or 12-well plates at a density of 1×10^4 cells/cm^2 in the growth medium for 12 h. Then, the cells were grown in medium supplemented with adenoviruses. Phase-contrast and bright-field microscopy were used to identify the differentiation of iMC23 cells by the presence of typical dendritic cell morphology and melanin granules. The experiments were repeated three times.

For the conditioned medium, JB6CI 30-7b cells were plated in 6-well plates at a density of 1×10^4 cells/cm^2, and AdWnt3a or AdGFP (control) was added into the growth medium. Twenty-four hours later, the growth medium was changed into normal DMEM without any adenovirus. Another 48 h later, the supernatant was collected and used as conditioned medium (CM). iMC23 cells were cultured in 12-well plates and treated with CMs. The CMs were changed every day. After 3 days, tyrosinase activity assays were performed.

2.7. Reporter assay

IMC23 cells were seeded into 6-well plates and transfected with 2 μg of Tcf4/Lef1 reporter plasmid DNA per well. Thirty-six hours after infection, the cells were lysed, and the lysates were harvested for use in a luciferase assay with Promega's Luciferase Assay Kit (Promega, USA). Each assay condition was performed three times.

2.8. Tyrosinase activity assay

IMC23 cells cultured in 6-well plates were infected with AdWnt1, AdWnt10b, AdWnt3a or AdGFP for 72 h, trypsinized and counted. Briefly, 1×10^5 cells were treated with 200 μL 1% TritonX-100/PBS at −80 °C for 30 min and thawed at 37 °C. Then, the extracts were clarified by centrifugation. Then, 50 μL of the supernatant was transferred into 96-well plates, and 10 μL of 2 mg/mL L-Dopa (Sigma, USA) was added. After incubation for 2 h at 37 °C, absorbance was measured at 490 nm. The experiments were performed at least three times.

2.9. Western blot analysis

Cell lysates were separated by 10% SDS-PAGE and transferred to PVDF membranes. The membranes were incubated with rabbit anti-Wnt3a antibody (1:1000, Abcam, USA), rabbit anti-TRP1 antibody (1:1000, Santa Cruz, USA), rabbit anti-tyrosinase antibody (1:1000, Bioworld, USA), rabbit anti-β-catenin antibody (1:1000, Bioworld, USA) or rabbit anti-β-catenin (Phospho-Ser675) antibody (Cell Signal, USA) at 4° C overnight. Blots were then incubated with HRP-conjugated secondary antibody (Invitrogen, USA) for 1 h. Bands were visualized on the membranes using an ECL western blotting detection system.

2.10. Statistical analysis

Significant differences were evaluated using a *t*-test, and $p < 0.01$ was considered statistically significant.