Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical and Biophysical Research Communications 342 (2006) 28–35
BBRC
www.elsevier.com/locate/ybbrc

Wnt-10b promotes differentiation of skin epithelial cells in vitro

Yukiteru Ouji *, Masahide Yoshikawa, Akira Shiroi, Shigeaki Ishizaka

Program in Tissue Engineering and Department of Parasitology, Nara Medical University, Kashihara, Nara, Japan

Received 16 January 2006
Available online 2 February 2006

Abstract

To evaluate the role of Wnt-10b in epithelial differentiation, we investigated the effects of Wnt-10b on adult mouse-derived primary skin epithelial cells (MPSEC). Recombinant Wnt-10b protein (rWnt-10b) was prepared using a gene engineering technique and MPSEC were cultured in its presence, which resulted in morphological changes from cuboidal to spindle-shaped and inhibited their proliferation. Further, involvement of the canonical Wnt signal pathway was also observed. MPSEC treated with rWnt-10b showed characteristics of the hair shaft and inner root sheath of the hair follicle, in results of Ayoub Shklar staining and immunocytochemistry. Further, the cells expressed mRNA for differentiated epithelial cells, including *keratin 1*, *keratin 2*, *loricrin*, *mHa5*, and *mHb5*, in association with a decreased expression of the basal cell marker *keratin 5*. These results suggest that Wnt-10b promotes the differentiation of MPSEC.

Keywords: Wnt-10b; Skin epithelial cell; β-Catenin; Differentiation

Wnt proteins are secreted, lipid-modified glycoproteins that activate cell-surface receptor-mediated signal transduction pathways to regulate a variety of cellular activities, including cell fate determination, proliferation, migration, polarity, and gene expression [1–3]. Thes proteins are heavily involved in morphogenesis during embryogenesis, as well as in the regeneration of various adult tissues including skin and hair follicles [4–6]. The binding of Wnt proteins to their receptors, frizzled (FZ) proteins, and low-density lipoprotein receptor-related protein (LRP) family members, results in activation of a conserved ''canonical'' signaling pathway [2]. Cytoplasmic β-catenin is known to translocate to the nucleus and forms active transcription complexes with members of the T cell factor/lymphoid enhancer factor (TCF/LEF) family [7,8]. By controlling the complex formation with TCF/LEF transcription factors, Wnt proteins play fundamental roles in various cells and tissues.

Mouse Wnt-10b was initially isolated from lymphoid tissue and suggested to be involved in lymphoid development [9–11]. Thereafter, it was shown to play various roles in a wide range of biological actions, such as adipogenesis [12], bone formation [13,14], tumor development [15], and axis determination [16]. However, few studies have been conducted concerning the role of Wnt-10b with the differentiation of skin epithelial cells and development of hair follicles. In an extensive study of gene expression by the Wnt family in skin tissue, Wnt-10b was found to be expressed in developing hair follicles, with the earliest and most marked localization in placodes [17], which suggested Wnt-10b as a candidate to promote the differentiation of skin epithelial cells and development of hair follicles.

To demonstrate the in vitro effects of Wnt-10b on the differentiation of skin epithelial cells, we isolated mouse-derived primary skin epithelial cells (MPSEC) from adult mouse skin tissue and cultured them in the presence of recombinant Wnt-10b (rWnt-10b). Since Wnt-10b protein is not commercially available, we prepared rWnt-10b using a gene engineering technique. The presence of rWnt-10b in the culture caused morphological changes in cultured MPSEC, inhibited their proliferation, and led to activation of the canonical Wnt signal pathway. MPSEC treated with rWnt-10b showed characteristics of the hair shaft and inner

* Corresponding author. Fax: +81 744 29 8847.
E-mail address: oujix@naramed-u.ac.jp (Y. Ouji).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.01.104

root sheath of the hair follicle, as seen in the results of Ayoub Shklar (AS) staining, which was used to detect epithelial cells undergoing differentiation and immunocytochemistry. Further, they expressed mRNA for differentiated epithelial cells, including *keratin 1*, *keratin 2*, *loricrin*, *mHa5*, and *mHb5*, in association with decreased expression of the basal cell marker *keratin 5*.

Our results suggest that Wnt-10b promotes the differentiation of MPSEC. Considering that the expression of Wnt-10b in hair follicles is very limited to the earliest placodes [17], Wnt-10b may play an important role with the differentiation of epithelial cells in the hair follicle, especially in initiating their differentiation.

Materials and methods

Mice. Inbred female C3H/HeN mice were purchased from Japan SLC (Hamamatsu, Japan) and used at the age of 7–10 weeks. They were housed in group cages at the animal facilities of Nara Medical University.

Cell preparation and culture conditions. Adult MPSEC were isolated from dorsal skin areas, as reported previously [18]. Briefly, the isolated skin samples were washed several times with phosphate-buffered saline (PBS) and incubated overnight at 4 °C in 0.25% trypsin. Detachment of the dermis from the epidermis was performed gently using forceps, after which cells were scrubbed from the epidermis-facing surface of the dermis using the belly of the forceps and used as MPSEC. The MPSEC thus obtained were suspended in Epilife™ serum-free culture medium (KURABO, Osaka, Japan) and plated in 96-well plates coated with collagen type I (Nippon Ham, Tsukuba, Japan), and kept in a humidified atmosphere containing 5% CO_2 at 37 °C. The culture medium was changed every 2 days. In addition, mouse pre-adipocyte 3T3-L1 and COS-7 cells were purchased from the Japanese Collection of Research Bioresources (Tsukuba, Japan) and cultured in DMEM containing 10% FBS.

Construction of Wnt-10b expression vector and production of stable transfectants. Mouse Wnt-10b cDNA, a kind gift from Dr. T.F. Lane (Harvard Medical School), was subcloned into pCAGIRESzeocinpA (kindly provided by Dr. H. Niwa, Center for Developmental Biology, Kobe, Japan) to create the plasmid pCAGIRESzeocinpA-wnt10b, which contained the Zeocin resistance gene driven by the CAG promoter. COS-7 was transfected with the plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and stable transfectants were selected in medium containing 400 μg/ml of Zeocin (Invitrogen), then analyzed by immunoblotting and immunohistochemistry. Cell lysates and supernatants concentrated using Vivapore 10/20 (Sartorius AG, Goettingen, Germany) were prepared as samples, which were then run on 10% SDS–PAGE and blotted onto a PVDF membrane. The blots were blocked with 5% non-fat dried milk in PBS containing 0.1% Tween 20 (TPBS) and then incubated with goat anti-mouse Wnt-10b (C-19) polyclonal antibody (diluted 1:200; Santa Cruz Biotech. CA) overnight at 4 °C, followed by horseradish peroxidase-conjugated anti-goat IgG (diluted 1:2000, Zymed Lab., CA) for 2 h. After washing, the blots were detected by the addition of True Blue™ (KPL, Gaithersburg, MD) as a substrate solution. The clone that produced Wnt-10b protein most abundantly was selected and named Wnt-COS, and then the supernatants of Wnt-COS cells cultured in Epilife serum-free medium were used as rWnt-10b for the differentiation of MPSEC.

For immunocytology, cells were plated and cultured on Lab-Tek Chamber Slides (Nunc, Rochester, NY), then fixed with 4% paraformaldehyde at 4 °C for 30 min and permeabilized in methanol at −20 °C for 5 min. The chamber slides were then exposed to the primary anti-Wnt-10b antibody, followed by a secondary antibody (Alexa 488 rabbit anti-goat IgG, diluted 1:200, Molecular Probes, Oregon). Fluorescence was examined under a Zeiss Axiovert 200 microscope.

Adipogenetic differentiation. 3T3-L1 cells (2.5×10^5 cells/ml) were seeded in a 24-well plate. On day 8, lipid inducers (5 μg/ml insulin, 1 μM dexamethasone, and 0.25 mM isobutylmethylxanthine) were added to the culture. After 2 days, the cell culture medium was removed and replaced with medium with or without rWnt-10b. On day 6, fat accumulation was visualized by staining the lipids with Oil Red O and the amount of stain incorporated into the cells was determined quantitatively by measuring optical density (OD) at 540 nm after dissolving with 2-propanol.

Demonstration of canonical Wnt signaling pathway. Involvement of the canonical Wnt signaling pathway was examined by reporter assays and immunocytochemistry. Two reporter plasmids, pTOPFLASH, carrying the TCF-binding consensus sequence followed by the luciferase gene, and pFOPFLASH, carrying the dominant-negative TCF-binding sequence instead of the wild-type sequence in pTOPFLASH, were kindly supplied by Dr. B. Vogelstein [19]. MPSEC were transfected with the reporter plasmids, and 4 h later the cell culture medium was removed and replaced with medium with or without rWnt-10b. After 48 h of incubation in the medium, the cells were lysed and luciferase activity was quantified using a luciferase reporter assay kit (Clontech, Worcester, MA), as recommended by the manufacturer, and normalized using β-gal levels as an internal control.

Translocation of β-catenin was immunocytochemically detected in MPSEC cultured in Wnt-COS supernatant for 48 h. MPSEC were fixed with cold acetic acid–methanol and blocked with HISTOMOUSE™ blocking solution (Zymed Lab.). The cells were treated with rabbit anti-β-catenin antibody (diluted 1:200, Santa Cruz Biotech.) at room temperature for 1 h, then washed three times with TPBS and treated with the secondary antibody Alexa 488 (anti- rabbit IgG, diluted 1:200, Molecular Probes).

RT-PCR. Total RNA was purified using Trizol (Invitrogen) following the protocol of the manufacturer. One microgram of DNase-treated total RNA was used for the first-strand cDNA. This reaction was performed using a random primer (Invitrogen) and M-MLV reverse transcriptase (Promega, Madison, WI). For PCR analysis, 0.5 μg of cDNA was used as a template and amplification was performed using the primer sequences shown in Table 1. The general PCR conditions were 25–30 cycles of 94 °C for 2 min, 94 °C for 30 s, 52–62 °C for 30 s, and 72 °C for 1 min. The PCR products were run on 1.5% agarose gels.

Determination of differentiated MPSEC by Ayoub Shklar (AS) staining and immunohistochemistry. As an assessment of epidermal differentiation, we employed Ayoub Shklar (AS) staining. This method has been reported to be a quick and reliable histological means to determine the progression of epidermal differentiation and hair follicle formation [20]. In the present study, MPSEC were cultured with or without medium containing rWnt-10b in a 24-well plate for 5 days. The plate was rinsed with PBS and

Table 1
List of gene-specific primers

Genes	Forward primer sequence	Reverse primer sequence	Size of product (bp)	GenBank Accession No.
Keratin 5	tgggacaggaagagaggtgatc	accaaaaccaaatccactgccg	497	AF306785
Keratin 1	gagattttcaggaggaggcttc	ttcatcctcgtacttggtcctg	700	NM_008473
Keratin 2	ggttgcatctctagctccat	gtactcttggttctgtctctcc	915	NM_010665
Loricrin	tcaccagaaaaagcagccca	tggcactgatactgttggga	813	M34398
mHa5	gaggttggtttggagaaggcat	cataccagtcttcagcatccct	611	AF020790
mHb5	atcaccaccgtctctgtcaatg	cgtaagtaggcacagtccacat	431	AF021836
Gapdh	accacagtccatgccatcac	tccaccaccctgttgctgta	452	NM_008084

fixed with 10% formalin for 30 min at room temperature, then rinsed again with PBS and stained with an acid fuchsin solution for 3 min. The acid fuchsin solution was removed and incubation continued with an Aniline Blue-Orange G solution for 45 min, after which the solution was removed and the cells were washed several times with 95% ethanol. The stained cells in 95% ethanol were then photographed.

For immunostaining with AE13 and AE15 (kindly provided by Dr. T.T. Sun), which are known to recognize the hair shaft and IRS, respectively, in mouse hair follicle tissue [21,22], MPSEC were fixed with cold acetic acid–methanol and blocked with HISTOMOUSE™ blocking solution (Zymed Lab.). The cells were then treated with the mouse monoclonal antibodies at room temperature for 1 h, after which they were washed three times with TPBS and treated with the secondary antibody Alexa 594 (anti-mouse IgG, diluted 1:200, Molecular Probes).

Cell proliferation assay. MPSEC were plated at a density of 50 cells per well in flat-bottom 96-well plates and cultured with or without rWnt-10b. After 5 days, the culture plates were used for cell proliferation assays with a CyQUANT® Cell Proliferation Assay Kit (Molecular Probes).

Statistical analysis. Data are expressed as means ± SD of five independent experiments. Statistical significance was tested using Student's *t* test.

Results

Establishment of bioactive Wnt-10b-producing cells

Wnt-10b protein is not commercially available at the present time. In order to obtain Wnt-10b protein, we first constructed a plasmid carrying the Wnt-10b cDNA gene and introduced it into COS-7 cells. Among 12 clones selected by Zeocin, those producing rWnt-10b were screened by a Western blotting assay. The clone that produced rWnt-10b protein most abundantly was selected, named Wnt-COS, and used for the following experiments. As shown in Fig. 1A, rWnt-10b protein was immunocytochemically

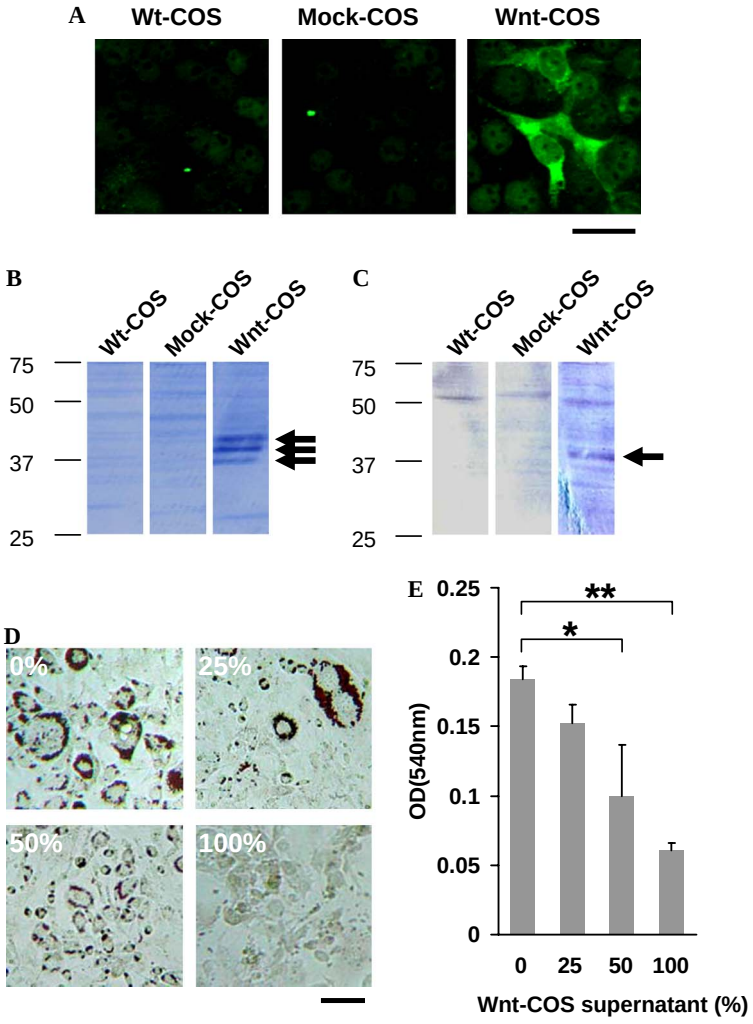


Fig. 1. Establishment of bioactive Wnt-10b-producing cells. (A) Immunofluorescent analysis. Wnt-10b-immunopositivity was found in the cytoplasm of the Wnt-COS clones. Scale bar = 10 μm. (B,C) Western blotting analyses of cell lysates (B) and concentrated supernatants (C). Wt-COS, non-transfected; Mock-COS, transfected with vector alone; Wnt-COS, transfected with Wnt-10b expression vector. Wnt-10b was detected as three bands in the total cellular extracts, while one band was detected in the concentrated supernatant from Wnt-COS cells. (D,E) Adipogenesis in 3T3-L1 cells. Lipid accumulation was visualized by Oil Red O staining. The lipid inducers insulin (5 μg/ml), dexamethasone (1 μM), and isobutylmethylxanthine (0.25 mM) induced lipid accumulation in 3T3-L1 cells, while the addition of Wnt-COS supernatant inhibited lipid accumulation in a dose-dependent manner. Scale bar = 20 μm (D). Oil Red O, incorporated intracellularly, was dissolved by 2-propanol and determined quantitatively at 540 nm. *$p < 0.005$; **$p < 0.0001$ (E).

demonstrated in Wnt-COS cells (Wnt-COS), but not in the parental COS cells (Wt-COS) or in COS cells transfected with the plasmid vector alone (Mock-COS). Strong intracellular immuno-positive signals were found in the cytoplasm of Wnt-COS cells. In accordance with our immunocytological results, rWnt-10b was also detected in total cellular extracts in 3 bands by Western blotting only from Wnt-COS cells (Fig. 1B). Further, the supernatant from the culture of Wnt-COS cells contained secreted rWnt-10b, which was demonstrated as a single band (Fig. 1C). We considered that the size differences between the Wnt-10b proteins from the cellular extract and supernatant were due to different levels of glycosylation.

Next, we examined whether the rWnt-10b produced by Wnt-COS cells was functionally active using a preadipocyte cell line (3T3-L1). It is well known that Wnt-10b inhibits the differentiation of preadipocytes into lipid-carrying adipocytes [12,13,23,24]. 3T3-L1 cells were treated with adipocyte inducers for 2 days, then subjected to a 4-day culture in the absence or presence of Wnt-COS supernatant samples at various ratios (Fig. 1D). A distinct accumulation of intracellular lipid droplets, as shown by Oil Red O staining, was found in the 3T3-L1 cells cultured without Wnt-COS supernatant (Fig. 1D, 0%). However, the addition of Wnt-COS supernatant inhibited the accumulation of lipid droplets in 3T3-L1 cells in a dose-dependent manner. This dose-dependent inhibitory effect by Wnt-COS supernatant on lipid accumulation was quantitatively confirmed by measuring the absorbance of the eluted lipids (Fig. 1E). Supernatants from the cultures of Wt-COS and Mock-COS cells showed no inhibitory effects on lipid accumulation.

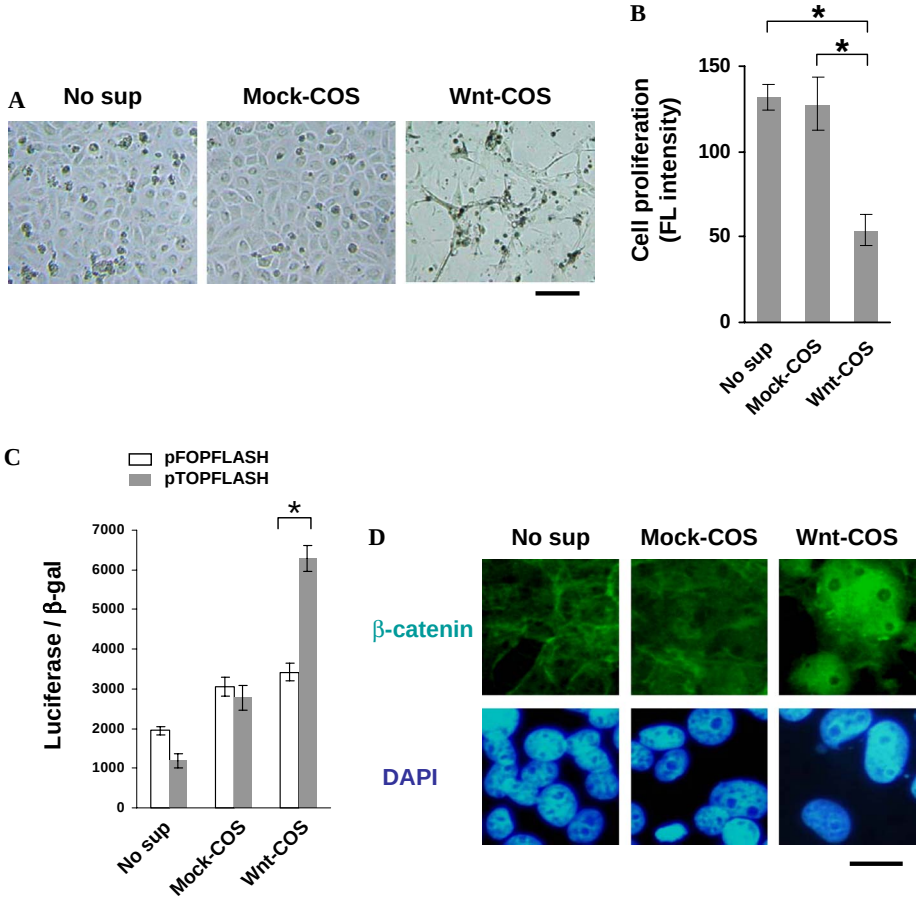


Fig. 2. Effects of rWnt-10b on MPSEC and involvement of canonical Wnt signaling pathway. (A) MPSEC were cultured in supernatants from Mock-COS cells (Mock-COS), Wnt-COS cells (Wnt-COS), or normal culture medium (No sup). Those in medium with Mock-COS supernatant or without supernatant presented cuboid or polygonal shapes and were tightly assembled. In medium with Wnt-COS supernatant, MPSEC were distributed diffusely and most were spindle-shaped. Scale bar = 20 μm. (B) The proliferation of MPSEC was assessed using a proliferation kit. Cell growth in Wnt-COS supernatant was significantly suppressed, as compared to culturing with Mock-COS supernatant or in normal culture medium. *$p < 0.005$. (C) Involvement of the canonical Wnt signaling pathway in MPSEC was assessed using TCF-reporter plasmids. MPSEC were transiently transfected with either pTOPFLASH or pFOPFLASH, and then cultured in Mock-COS culture supernatant (Mock-COS), Wnt-COS culture supernatant (Wnt-COS), or normal culture medium (No sup). *$p < 0.001$. (D) β-Catenin localization was determined immunocytochemically. Cells were immunostained with anti-β-catenin antibody (green, upper). Nuclei were stained with DAPI (blue, lower). The photographs shown were taken at the same position. Immunopositivity against β-catenin was faintly observed throughout the entire cytoplasm in MPSEC cultured with Mock-COS supernatant (Mock-COS) or in normal culture medium (No sup), while it was strongly detected in the nuclei of MPSEC cultured with Wnt-COS supernatant (Wnt-COS). Scale bar = 10 μm.

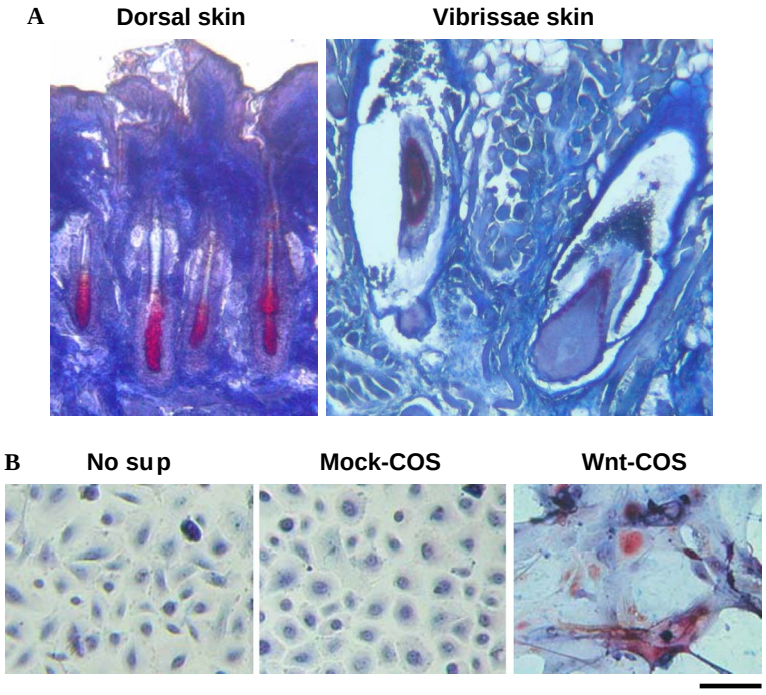
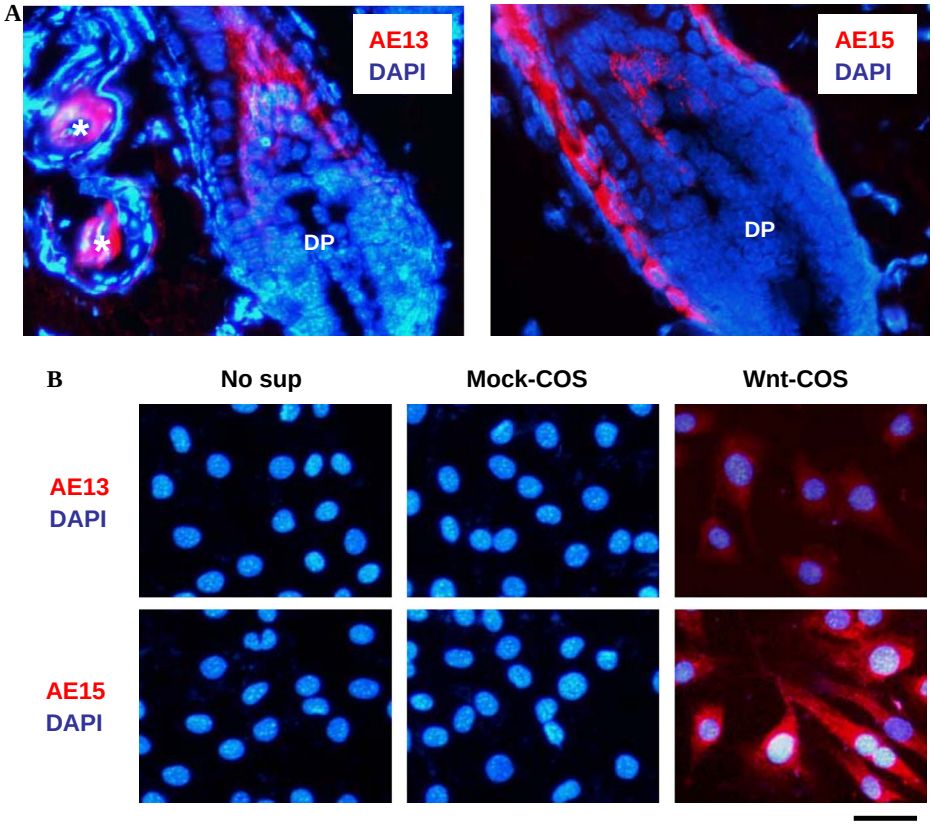


Fig. 3. Ayoub Shklar (AS) staining of mouse follicle tissue and MPSEC. (A) AS staining was performed using mouse dorsal skin and vibrissae tissue samples. The hair shaft and inner root sheath (IRS) were stained reddish brown. (B) AS staining was performed in MPSEC cultured in supernatant from Mock-COS cells (Mock-COS), supernatant from Wnt-COS cells (Wnt-COS), or normal culture medium (No sup). Those cultured in Wnt-COS supernatant were stained reddish brown, while those in Mock-COS supernatant or without supernatant remained unstained. Scale bar = 20 μm.



Effects of rWnt-10b on the proliferation of MPSEC

MPSEC, plated at a density of 50 cells per well in flat-bottom 96-well plates, were cultured for 5 days in the presence or absence of COS culture supernatants (Figs. 2A and B). MPSEC cultured in Mock-COS supernatant (Mock-COS) or in medium alone (No sup) presented distinctly cuboid or polygonal shapes and were assembled tightly (Fig. 2A). However, MPSEC cultured in Wnt-COS supernatant (Wnt-COS) presented a quite different morphology, as they became spindle-shaped and were distributed diffusely. The number of MPSEC cultured in Wnt-COS supernatant after 5 days was low as compared to the number of cells cultured in Mock-COS supernatant or medium alone. Further, suppression of cell proliferation by Wnt-COS supernatant was also demonstrated by a cell proliferation assay (Fig. 2B).

Involvement of canonical Wnt signaling pathway

Next, we examined whether the canonical Wnt signaling pathway was activated in MPSEC after exposure to Wnt-COS supernatant. For this purpose, 2 reporter plasmids, pTOPFLASH and pFOPFLASH [19], were transiently transfected into MPSEC. As shown in Fig. 2C, a high level of pTOPFLASH activity was induced by the Wnt-COS-supernatant, whereas pFOPFLASH activity was not changed, suggesting that Wnt-10b in the Wnt-COS supernatant induced transcriptional activity from the TCF-responsive promoter through the β-catenin/TCF complex in MPSEC. Activation of the canonical pathway was further confirmed by investigating the intracellular localization of β-catenin (Fig. 2D). The translocation of β-catenin into nuclei was observed in MPSEC cultured in Wnt-COS supernatant, whereas β-catenin existed mostly in the cytoplasm in MPSEC cultured in Mock-COS supernatant or medium alone.

Differentiation of MPSEC promoted by rWnt-10b

We considered that the changes in cell shape and cell number caused by Wnt-COS supernatant were associated with the differentiation of MPSEC. To evaluate the differentiation of MPSEC caused by rWnt-10b, AS staining was applied to the cultured MPSEC, as this method has been reported to be a quick and reliable method for determining the progression of epidermal differentiation and hair follicle formation [20]. According to those reports, differentiated epidermal cells are stained red and undifferentiated epidermal cells are stained blue. Before employing this staining method for MPSEC, we utilized it with mouse dorsal skin and vibrissae tissue samples (Fig. 3A), and found that the hair shaft and inner root sheath (IRS) were stained reddish brown. MPSEC cultured in Mock-COS supernatant (Mock-COS) or medium alone (No sup) were stained blue, whereas those cultured in Wnt-COS supernatant became reddish brown (Fig. 3B), suggesting that rWnt-10b induced the differentiation of MPSEC.

We performed an immunocytological study of cultured MPSEC as well as vibrissae tissue using the monoclonal antibodies AE13 and AE15, which are known to recognize the hair shaft and IRS in mouse hair follicle tissue, respectively [21,22], as we confirmed (Fig. 4A). MPSEC cultured in Wnt-COS supernatant were stained weakly with AE13 and strongly with AE15 (Fig. 4B). However, no staining was observed in MPSEC cultured in Mock-COS supernatant or medium alone. These results indicate that the MPSEC had differentiated into hair shaft- and IRS-like cells.

Gene expression analysis by RT-PCR

To confirm the differentiation of MPSEC by rWnt-10b from the aspect of gene expression, we performed an RT-PCR analysis. *keratin 5* was selected as a basal cell marker, while *keratin 1* (spinous cells), *keratin 2* (granular cells), *loricrin* (granular cells), and *mHa5* and *mHb5* (cortex) were used as differentiation markers of epithelial cells. MPSEC cultured in Mock-COS supernatant or medium alone distinctly expressed *keratin 5*. However, *keratin 5* expression nearly disappeared in MPSEC cultured in Wnt-COS supernatant (Fig. 5), while the markers of differentiated epithelial cells (*keratin 1*, *keratin 2*, *loricrin*, *mHa5*, and *mHb5*), all of which were absent in MPSEC cultured in Mock-COS supernatant or medium alone, were detected in the cells cultured in Wnt-COS supernatant.

Discussion

Information regarding Wnt proteins is rapidly accumulating, however, little is known about the biological roles of Wnt-10b in skin. It was recently reported that the expression of Wnt-10b was upregulated in placodes at the onset of follicle morphogenesis and in postnatal hair follicles beginning a new cycle of hair growth [17], suggesting that the protein may have an important role in the initiation of hair follicle development. In the present study, we prepared recombinant Wnt-10b (rWnt-10b) using a gene transfer

Fig. 4. Immunocytological study of mouse follicle tissue and MPSEC. (A) An immunocytological study of mouse vibrissae tissue was performed using the monoclonal antibodies AE13 and AE15, which recognized the hair shaft and IRS, respectively, in mouse hair follicle tissue. DP, dermal papilla; asterisks, hair shafts in cross-section. (B) An immunocytological study of MPSEC cultured in supernatant from Mock-COS cells (Mock-COS), supernatant from Wnt-COS cells (Wnt-COS), or normal culture medium (No sup) was performed. MPSEC cultured in Wnt-COS supernatant were stained with the marker for hair follicle-specific antibodies (AE13 and AE15), while those cultured in Mock-COS supernatant or without supernatant remained unstained. Scale bar = 20 μm.

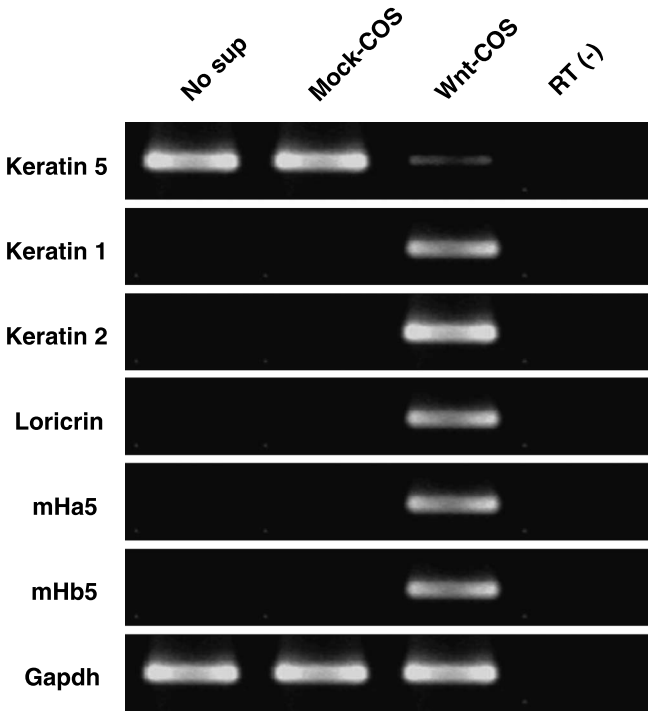


Fig. 5. Gene expression analysis by RT-PCR The expression of hair follicle differentiation-related markers in MPSEC was examined by RT-PCR. *Keratin 5* mRNA expression was decreased in MPSEC cultured in Wnt-COS supernatant, while markers of differentiated epithelial cells (*keratin 1*, *keratin 2*, *loricrin*, *mHa5*, and *mHb5*) were detected. RT (−): mRNA samples not reverse transcribed.

technique to evaluate the role of Wnt-10b with epithelial differentiation, as analyses of the biological function of Wnt-10b have not been vigorously conducted in vitro, partly due to the lack of commercially available Wnt-10b protein. We successfully obtained COS cell lines secreting bioactive Wnt-10b and used the culture supernatant in our experiments.

It is well known that hair follicles contain 2 types of cells, dermal papilla cells, and epithelial cells of the root sheath and hair shaft [25]. The former are derived from the mesenchyme, while the latter come from the surface epithelium, with the hair follicle formed through reciprocal inductive interactions between them [26]. We utilized mouse-derived primary skin epithelial cells (MPSEC) as a substitute for undifferentiated skin epithelial cells, as they have been used as a model of keratinocyte differentiation, and were indeed immunopositive for the basal cell marker keratin 5 (data not shown) and also expressed *keratin 5* mRNA in the present experiments. Therefore, we concluded that these cells retain an ability to differentiate into in hair follicle epithelial cells.

We cultured MPSEC in the presence of rWnt-10b and examined the effects on their growth and differentiation. The cuboidal cell shape of undifferentiated MPSEC was gradually changed to spindle-shaped after exposure to rWnt-10b and cell proliferation was suppressed in a dose-dependent manner. Further, MPSEC treated with rWnt-10b were colored reddish brown following AS staining, and became immunopositive for AE13 and AE15, which suggested differentiation. Red coloring following AS staining is accepted as one of the indicators of differentiated skin epithelial cells [20], and AE13- and AE15-immunopositivity results have also been reported in hair shaft and IRS tissues, respectively [21,22]. In the present experiments as well, the hair shaft and IRS were stained reddish brown by AS, and were immunopositive for AE13 and AE15, respectively. These results suggest that rWnt-10b promoted MPSEC to differentiate into hair shaft and IRS. Further, gene expression analysis revealed that rWnt-10b-treated MPSEC expressed the mRNA of *keratin 1*, *keratin 2*, *loricrin*, *mHa5*, and *mHb5* in association with a decrease in *keratin 5* expression, suggesting keratinocyte differentiation as well as differentiation into hair shaft tissues. Taken together, our results demonstrate that rWnt-10b promoted the differentiation of MPSEC.

We observed the involvement of the Wnt-canonical pathway in MPSEC treated with rWnt-10b, using a reporter assay with pTOPFLASH and an immunocytochemical demonstration of translocation of β-catenin into the nuclei. Although the mechanisms by which rWnt-10b promoted the differentiation of MPSEC remains unknown, we speculated that the Wnt-canonical pathway activated by rWnt-10b and the following modification of transcription via TCF/LEF complex are important for initiating differentiation of MPSEC.

Acknowledgments

We thank Dr. L. Lane (UCLA, CA) for the Wnt-10b cDNA, Dr. H. Niwa (RIKEN, Center for Developmental Biology, Kobe, Japan) for the pCAGIRESzeocinpA vector, Dr. T.T. Sun (New York University School of Medicine, NY) for the AE13 and AE15 antibodies, Dr. Sasaki (Nippon Ham, Tsukuba, Japan) for the collagen, and Dr. B. Vogelstein (Howard Hughes Medical Institute) for the pTOPFLASH and pFOPFLASH plasmids. This research was funded by a Grant-in-Aid for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (KAKENHI).

References

[1] R.T. Moon, B. Bowerman, M. Boutros, N. Perrimon, The promise and perils of Wnt signaling through beta-catenin, Science 296 (2002) 1644–1646.
[2] M. Peifer, P. Polakis, Wnt signaling in oncogenesis and embryogenesis—a look outside the nucleus, Science 287 (2000) 1606–1609.
[3] P. Polakis, Wnt signaling and cancer, Genes Dev. 14 (2000) 1837–1851.
[4] M. Bienz, H. Clevers, Linking colorectal cancer to Wnt signaling, Cell 103 (2000) 311–320.
[5] L. Alonso, E. Fuchs, Stem cells in the skin: waste not, Wnt not, Genes Dev. 17 (2003) 1189–1200.
[6] F.J. Staal, H. Clevers, Tcf/Lef transcription factors during T-cell development: unique and overlapping functions, Hematol. J. 1 (2000) 3–6.
[7] A. Bejsovec, Wnt signaling: an embarrassment of receptors, Curr. Biol. 10 (2000) R919–R922.

[8] A. Wodarz, R. Nusse, Mechanisms of Wnt signaling in development, Annu. Rev. Cell Dev. Biol. 14 (1998) 59–88.
[9] G. Hardiman, S. Albright, J. Tsunoda, T. McClanahan, F. Lee, The mouse Wnt-10B gene isolated from helper T cells is widely expressed and a possible oncogene in BR6 mouse mammary tumorigenesis, Gene 172 (1996) 199–205.
[10] T. Reya, M. O'Riordan, R. Okamura, E. Devaney, K. Willert, R. Nusse, R. Grosschedl, Wnt signaling regulates B lymphocyte proliferation through a LEF-1 dependent mechanism, Immunity 13 (2000) 15–24.
[11] M. van de Wetering, W. de Lau, H. Clevers, WNT signaling and lymphocyte development, Cell 109 (2002) S13–S19.
[12] S.E. Ross, N. Hemati, K.A. Longo, C.N. Bennett, P.C. Lucas, R.L. Erickson, O.A. MacDougald, Inhibition of adipogenesis by Wnt signaling, Science 289 (2000) 950–953.
[13] C.N. Bennett, C.L. Hodge, O.A. MacDougald, J. Schwartz, Role of Wnt10b and C/EBPalpha in spontaneous adipogenesis of 243 cells, Biochem. Biophys. Res. Commun. 302 (2003) 12–16.
[14] C.N. Bennett, K.A. Longo, W.S. Wright, L.J. Suva, T.F. Lane, K.D. Hankenson, O.A. MacDougald, Regulation of osteoblastogenesis and bone mass by Wnt10b, Proc. Natl. Acad. Sci. USA 102 (2005) 3324–3329.
[15] K.R. Brennan, A.M. Brown, Wnt proteins in mammary development and cancer, J. Mamm. Gland Biol. Neoplasia 9 (2004) 119–131.
[16] T. Ishikawa, Y. Tamai, A.M. Zorn, H. Yoshida, M.F. Seldin, S. Nishikawa, M.M. Taketo, Mouse Wnt receptor gene Fzd5 is essential for yolk sac and placental angiogenesis, Development 128 (2001) 25–33.
[17] S. Reddy, T. Andl, A. Bagasra, M.M. Lu, D.J. Epstein, E.E. Morrisey, S.E. Millar, Characterization of Wnt gene expression in developing and postnatal hair follicles and identification of Wnt5a as a target of Sonic hedgehog in hair follicle morphogenesis, Mech. Dev. 107 (2001) 69–82.
[18] J.J. Reiners, T. Rupp, Conversion of xanthine dehydrogenase to xanthine oxidase occurs during keratinocyte differentiation: modulation by12-O-tetradecanoylphorbol-13-acetate, J. Invest. Dermatol. 93 (1989) 132–135.
[19] V. Korinek, N. Barker, P.J. Morin, D. van Wichen, R. de Weger, K.W. Kinzler, B. Vogelstein, H. Clevers, Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC−/− colon carcinoma, Science 275 (1997) 1784–1787.
[20] T.C. Troy, K. Turksen, ES cell differentiation into the hair follicle lineage in vitro, in: Methods in Molecular Biology, Embryonic Stem Cells Methods and Protocols, Humana Press, Totowa, NJ, 2002, pp. 255–260.
[21] D. Dhouailly, C. Xu, M. Manabe, A. Schermer, T.T. Sun, Expression of hair-related keratins in a soft epithelium: subpopulations of human and mouse dorsal tongue keratinocytes express keratin markers for hair-, skin- and esophageal-types of differentiation, Exp. Cell Res. 181 (1989) 141–158.
[22] M.H. Lynch, W.M. O'Guin, C. Hardy, L. Mak, T.T. Sun, Acidic and basic hair/nail (hard) keratins: their colocalization in upper cortical and cuticle cells of the human hair follicle and their relationship to "soft" keratins, J. Cell Biol. 103 (1986) 2593–2606.
[23] C.N. Bennett, S.E. Ross, K.A. Longo, L. Bajnok, N. Hemati, K.W. Johnson, S.D. Harrison, O.A. MacDougald, Regulation of Wnt signaling during adipogenesis, J. Biol. Chem. 277 (2002) 30998–31004.
[24] K.A. Longo, W.S. Wright, S. Kang, I. Gerin, S.H. Chiang, P.C. Lucas, M.R. Opp, O.A. MacDougald, Wnt10b inhibits development of white and brown adipose tissues, J. Biol. Chem. 279 (2004) 35503–35509.
[25] L.C. Sperling, Hair anatomy for the clinician, J. Am. Acad. Dermatol. 25 (1991) 1–17.
[26] R.F. Oliver, C.A. Jahoda, Dermal-epidermal interactions, Clin. Dermatol. 6 (1988) 74–82.