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Canonical Wnts, specifically Wnt-10b, show ability to maintain dermal papilla cells

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ABSTRACT

Although Wnts are expressed in hair follicles (HFs) and considered to be crucial for maintaining dermal papilla (DP) cells, the functional differences among them remain largely unknown. In the present study, we investigated the effects of Wnts (Wnt-3a, 5a, 10b, 11) on the proliferation of mouse-derived primary DP cells *in vitro* as well as their trichogenesis-promoting ability using an *in vivo* skin reconstitution protocol. Wnt-10b promoted cell proliferation and trichogenesis, while Wnt-3a showed those abilities to a limited extent, and Wnt-5a and 11 had no effects. Furthermore, we investigated the effects of these Wnts on cultured DP cells obtained from versican-GFP transgenic mice and found that Wnt-10b had a potent ability to sustain their GFP-positivity. These results suggest that canonical Wnts, specifically Wnt-10b, play important roles in the maintenance of DP cells and trichogenesis.

1. Introduction

Dermal papilla (DP) cells are highly associated with the development of hair follicles (HFs) and regulation of the hair cycle [1–3]. However, it is also well known that primary DP cells isolated from HFs lose their ability for HF induction and do not promote hair growth after culturing in standard medium (e.g., DMEM containing 10% FBS) [4–6]. Wnts are considered to be involved with inductive signaling that gives HF induction and hair growth promotion abilities to DP cells, and their expressions (e.g., Wnt-3a, 5a, 10b, 11) have been reported in HFs of postnatal skin [7]. They are divided into two groups according to their signal transduction pathways [8]; canonical Wnt signaling, in which β-catenin stabilization occurs, and non-canonical Wnt signaling, in which Ca^{2+} flux (non-canonical Wnt/Ca pathway) or activation of the Jun N-terminal (non-canonical planar cell polarity, PCP pathway). Wnt-3a, 5a, and 11 are known to mediate the canonical, non-canonical Wnt/Ca, and non-canonical PCP pathways, respectively.

We recently reported that Wnt-10b mediates canonical signaling, while it also promotes the proliferation of primary DP cells, and maintains their ability for HF induction and hair growth [9]. Furthermore, serially passaged DP cells in cultures containing Wnt-10b sustained those abilities. However, the effects of biological functions of other Wnts on DP cells, including Wnt-3a, 5a, and 11, are not fully reported in the context of comparison with Wnt-10b.

In the present study, we investigated the effects of Wnt-3a, 5a, 11, and 10b on DP cells that were primarily cultured or passaged. Only Wnt-10b evidently promoted proliferation of cultured DP cells, and successfully maintained their abilities for HF induction and hair growth, while only limited and insufficient maintenance were observed with Wnt-3a, 5a and 11 showed no effects. Furthermore, we evaluated the effects of Wnts on DP cells derived from versican-GFP transgenic mice, as it is known that the effects of GFP expression, driven by the versican promoter, on DP cells is positively correlated to their HF-inducing ability in this mouse model. After the first DP cell culture for 10 days, more than 60% of the cells maintained strong GFP-positivity in cultures with Wnt-10b, whereas only 22.8% of the cells were weakly GFP positive in cultures with Wnt-3a. No or few GFP-positive cells remained in the culture with medium alone, or that with Wnt-5a or 11. Taken together, our results suggest that Wnt-10b has a potent ability to sustain the HF-inducing ability of DP cells, while they also indicate that canonical Wnts, specifically Wnt-10b, play an important role in the maintenance of DPs and trichogenesis.

2. Materials and methods

2.1. Reagents

Wnt-3a and 5a recombinant proteins were purchased from R&D Systems Inc. (Minneapolis, MN, USA)

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2.2. Animals

Inbred 4-week-old C3H/HeN and 8-week-old Balb/c nude (*nu/nu*) mice were purchased from Japan SLC (Hamamatsu, Japan), and versican-GFP transgenic mice were provided from Shiseido Research Institute. DP cells were obtained from adult C3H/HeN mice, while primary skin epithelial cells and dermal fibroblasts came from postnatal day 2 (P2) C3H/HeN mice [10]. Balb/c nude mice were used for hair reconstitution experiments. All animal procedures were conducted in accordance with our institutional guidelines as well as those of the National Institutes of Health.

2.3. Primary cultures of DPs after isolation from vibrissae

DPs were isolated from vibrissae of 4-week-old C3H/HeN mice using a microdissection method, as previously reported [6,9]. Briefly, the mystacial pad was cut open and the skin inverted, then follicles were removed with micro-scissors. Collagen capsules surrounding the vibrissae follicles were removed to expose the follicle base and DP samples were dissected out using fine forceps. Each sample was placed in a 6 cm dish and quietly cultured for 4 days in DMEM containing 10% FBS. DP cells appeared only around the DP samples and showed rapid outgrowth during the following 2 days. By day 10, cells had spread throughout the dishes and a high level of alkaline phosphatase (ALP) activity was expressed in nearly all of the cultured DP cells. DP outgrowths were harvested with 0.25% trypsin–EDTA after 10 days and used for culturing of DP cells after washing with saline.

2.4. Preparation of Wnt-10b and Wnt-11

We previously established Wnt-10b- and Wnt-11-secreting COS cell lines (Wnt-10b-COS and Wnt-11-COS, respectively) by introducing the Wnt-10b cDNA gene, and reported that those culture supernatants contained bioactive Wnt-10b and 11 proteins, respectively [11,12]. In the present study, Wnt-10b-COS and Wnt-11-COS cells were separately seeded into 10 cm dishes, then cultured in DMEM containing 10% FBS. After 48 h, the supernatants were collected and used as culture media containing Wnt-10b or 11 for DP cells after filtration with a 0.22 μm filter membrane.

2.5. Cultures of DP cells

An outline of the DP cell culture protocol is shown in Fig. 1A. One thousand harvested primary DP cells from DP cultures were seeded into 6 cm culture dishes, then cultured in DMEM containing 10% FBS alone or with Wnts. Wnt-3a and 5a were added to the culture medium at a final concentration of 100 ng/ml. Supernatants from the cultures of Wnt-10b-COS and Wnt-11-COS cells, containing Wnt-10b and 11, respectively, were collected and used as culture media, with the medium changed every 4 days. DP cells were harvested on day 10 with 0.25% trypsin–EDTA and used for RT-PCR experiments, TOPFLASH assays, or transplantation. DP cells cultured in Wnt-10b-COS or Wnt-11-COS supernatant were passaged every 10 days until the end of the 5th culture.

2.6. Alkaline phosphatase (ALP) activity

ALP activity was observed using an Alkaline Phosphatase (AP) Live Stain kit (Invitrogen), according to the manufacturer's protocol. Briefly, the cells were washed twice in fresh medium, then an appropriate amount of 1 × AP Live Stain solution was directly applied to the cell culture, after which incubation was performed at 37°C for 30 min. The cells were then rinsed well with fresh medium 3–4 times and observed under a fluorescent microscope using a standard FITC filter.

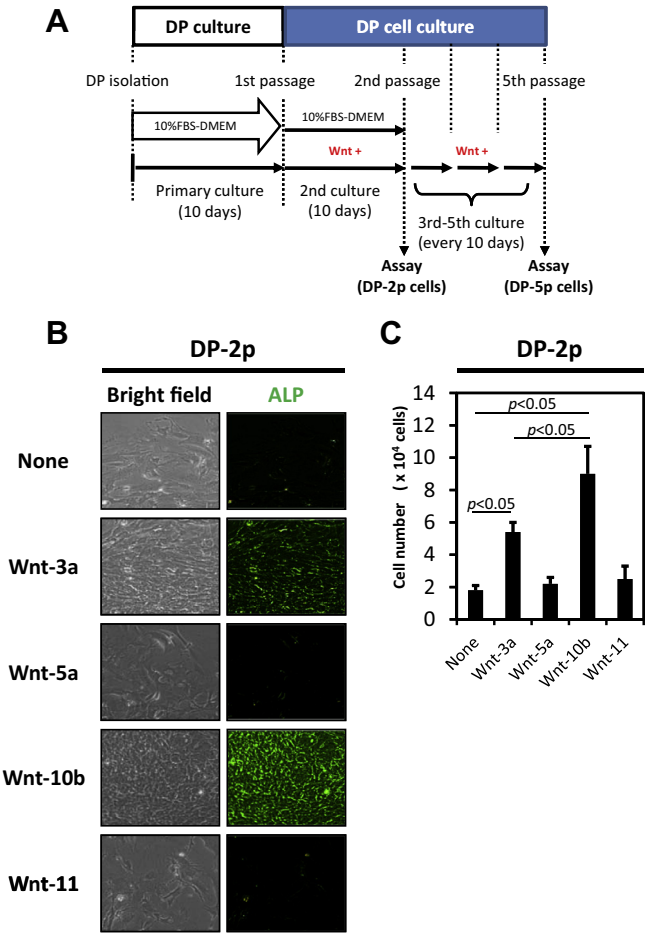

Fig. 1. Wnt-10b and 3a promote proliferation of primary DP cells. (A) Experimental design and procedures. DP cultures: DP specimens were isolated from vibrissae of C3H/HeN mice using a microdissection method, then placed in 6 cm dishes and quietly cultured in DMEM containing 10% FBS. DP outgrowths were harvested with 0.25% trypsin–EDTA on day 10 and used for DP cell cultures. DP cell cultures: Primary DP cells (1×10^3) harvested from DP cultures were seeded into 6 cm culture dishes and cultured in DMEM medium containing 10% FBS alone, or with Wnts. DP cells harvested on day 10 were used as DP-2p cells. DP cells cultured with Wnts were passaged every 10 days until the end of the 5th culture, then harvested and used as DP-5p cells. (B) Morphology and ALP activity of DP cells after the second culture. (C) Numbers of DP cells after the second culture.

2.7. Proliferation assay

DP cells were plated at a density of 50 cells per well in flat-bottom 96-well plates, and cultured with or without Wnts. After 10 days, cell numbers were determined using the trypan blue exclusion method.

2.8. TOPFLASH assay

Involvement of the canonical Wnt signaling pathway was examined using reporter assays. 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 [13]. DP cells were transfected with the reporter plasmids. The culture medium was removed 4 h later, and replaced with new medium with or without Wnts. After 48 h of incubation, the cells were lysed and luciferase activity was quantified using a luciferase reporter assay kit (Clontech, Worcester, MA, USA), as recommended by the man-

ufacturer, and normalized using the level of β-gal as the internal control.

2.9. RT-PCR

Total RNA was purified using Trizol (Invitrogen, Carlsbad, CA, USA) following the protocol of the manufacturer. One microgram of DNase-treated total RNA was used for first-strand cDNA. This reaction was performed using a random primer (Invitrogen) and M-MLV reverse transcriptase (Promega, Madison, WI, USA). 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 at 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.

2.10. Quantitative real-time polymerase chain reaction (qPCR) analysis

Total RNA (1 μg) was extracted from cultured cells using TRIzol reagent (Invitrogen). Reverse transcription and qPCR were performed with a SYBR PrimeScript RT-PCR kit II (TaKaRa), according to the manufacturer's instructions using primers purchased from TaKaRa Bio Inc. (forward: ATGGGATTGAAGACACTCAGGACAC, reverse: TGCATCACACTGCTCAAATCCA). The amount of versican PCR product was calculated relative to the internal control β-actin, and then compared between the experimental and control groups using the ΔΔCT method.

2.11. Hair reconstitution assay

A hair reconstitution experiment was performed as previously reported [9,10,14]. Briefly, epidermal keratinocytes were prepared from P2 C3H/HeN mice, then mixture solutions containing epidermal keratinocytes (2.5×10^7/ml) and DP cells (2.5×10^7/ml) passaged with DMEM medium alone or media containing Wnts were used for transplantation into Balb/c nude mice ($n = 3$). Silicon transplantation chambers, kindly supplied by Dr. J. Kishimoto (Shiseido Research Institute), were implanted into dorsal sites of the Balb/c nude mice where the skin had been removed, then 200 μl of mixture was injected into each of the chambers. One week after transplantation, the roof of the chamber was cut off to facilitate drying of the applied site and the chamber was removed 2 weeks after transplantation.

2.12. Statistical analysis

Data are expressed as the mean ± SD of five independent experiments. Statistical significance was tested using Tukey's test. Results were considered significant at $p < 0.05$.

3. Results

3.1. Wnt-10b and Wnt-3a promoted proliferation of primary DP cells

DP cells were harvested from primary DP cultures on day 10, and 1×10^3 cells were cultured for 10 days in 6 cm culture dishes in DMEM containing 10% FBS alone or with the Wnts (Fig. 1A). We examined the morphology of DP cells cultured with each Wnt as well as their ALP activity (Fig. 1B). DP cells in cultures containing Wnt-10b presented cuboid or polygonal shapes, were tightly assembled, and maintained a high level of ALP activity, while those in cultures with Wnt-3a showed similar shapes, though had a distinctly lower level of ALP activity. DP cells cultured in medium alone or those with Wnt-5a or 11 were sparsely distributed with various shapes, including some with a thin spindle-like morphology as commonly seen with fibroblasts. DP cell proliferation was examined by counting after 10 days of culture. Wnt-10b and 3a promoted cell proliferation, with an approximately 5-fold increase in the presence of Wnt-10b and a 3-fold increase with Wnt-3a, as compared to medium alone (Fig. 1C). No significant proliferation was observed in cultures of DP cells with Wnt-5a or 11.

3.2. Wnt-responsiveness and trichogenesis-promoting ability of DP cells treated with Wnts

Primary DP cells were harvested from DP cultures, then cultured with Wnts for 10 days. After the second culture, cells (DP-2p) were examined for retained canonical Wnt responsiveness, DP-marker expression such as Sox-2 and versican, and trichogenesis-promoting ability. DP-2p cells from cultures with Wnt-10b (DP-2p/Wnt-10b) or Wnt-3a (DP-2p/Wnt-3a) had a similar level of pTOPFLASH reporter activities (Fig. 2A). However, no reporter activity was found in DP cells cultured with medium alone, Wnt-5a, or 11. Lef-1, a representative downstream gene in canonical Wnt signal cascades, was clearly expressed in DP-2p/Wnt-10b cells, while it was considerably reduced in DP-2p/Wnt-3a cells (Fig. 2B). Furthermore, Sox-2 was distinctly expressed in DP-2p/Wnt-10b cells, though only faintly in DP-2p/Wnt-3a cells. The expression of versican was similar to that of Lef-1 and clearly observed clearly in DP-2p/Wnt-10b cells, while it was considerably reduced in DP-2p/Wnt-3a cells (Fig. 2B, C).

Since versican expression is known to be correlated with the HF induction ability of DP cells [15], we also examined the trichogenesis-promoting ability of DP-2p cells *in vivo* using skin reconstitution assays (Fig. 2D). DP-2p cells were obtained from the dorsal skin of P2 C3H/HeN mice, then co-transplanted with epithelial cells into silicon chambers set on dorsal sites of Balb/c nude mice. Abundant hair growth was observed in the reconstituted skin with DP-2p/Wnt-10b cells, whereas scant hair growth was seen in the skin with DP-2p/Wnt-3a cells applied. Furthermore, no hair growth was seen in skin that received DP-2p cells from cultures with medium alone (Fig. 2D, None), Wnt-5a, or 11. These results suggest that Wnt-10b gives DP cells an ability to maintain HF induction and

Table 1
Gene-specific primers used in the present study.

Genes	Primer sequences	Product size (bp)	Genebank accession no.	Temp (°C)
Versican	Forward: 5′- gacgactgtcttggtgg Reverse: 5′- atatccaaacaagcctg	285	NM_001081249	58
Lef-1	Forward: 5′- actgtcaggcgacacttcc Reverse: 5′- tgcacgttgggaaggagc	541	NM_010703	58
Sox-2	Forward: 5′- acatgatggagacggagctg Reverse: 5′- aatctccgcagcgaaacgac	554	NM_011443.3	58
Gapdh	Forward: 5′-accacagtccatgccatcac Reverse: 5′-tccaccaccctgttgctgta	452	NM_008084	58

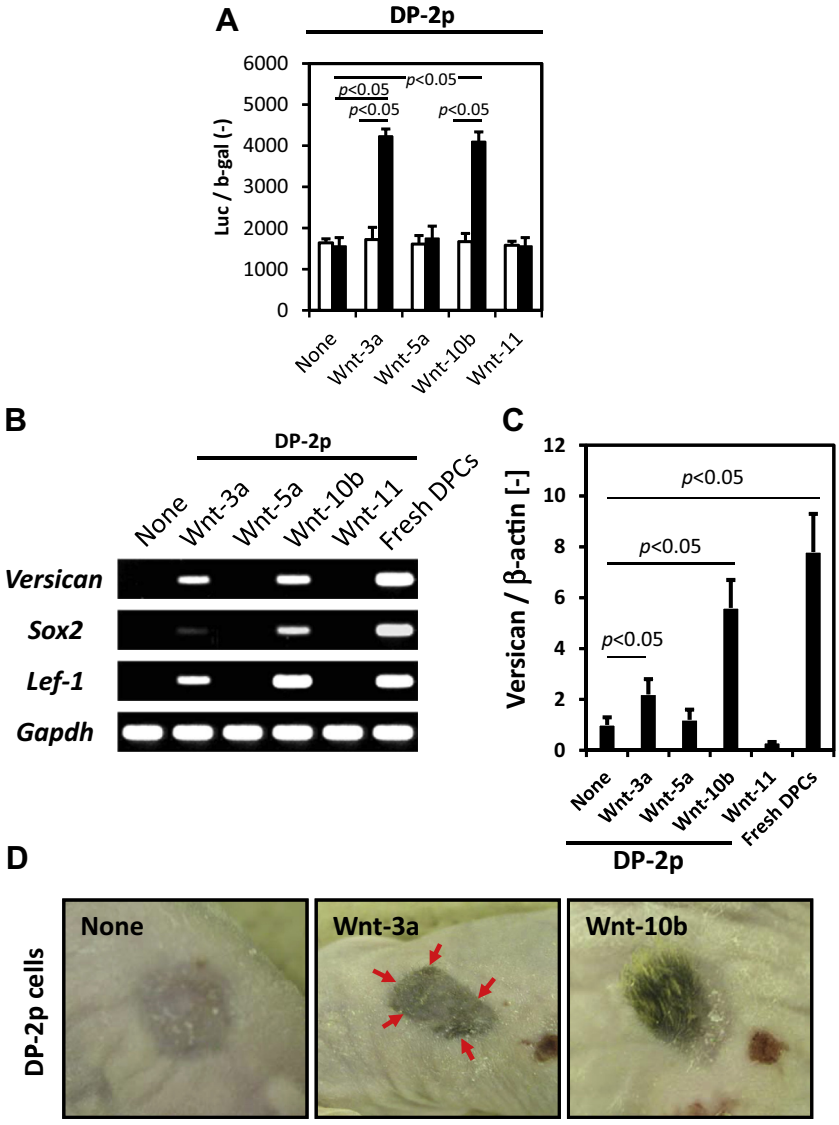

Fig. 2. Wnt responsiveness and trichogenesis promoting ability of DP cells treated with Wnts. (A) TOPFLASH assay findings. DP cells from the second culture with Wnt-3a (DP-2p/Wnt-3a cells) and with Wnt-10b (DP-2p/Wnt-10b cells) had similar levels of reporter activities. (B, C) RT-PCR analysis of expressions of versican, Sox-2, and Lef-1 in DP cells from the second culture, and quantification of versican expression. (D) HF induction ability of DP-2p cells cultured in medium alone (None), and of DP-2p/Wnt-3a (Wnt-3a) and DP-2p/Wnt-10b (Wnt-10b) *in vivo*, as shown in skin reconstitution assay findings.

hair growth, while Wnt-3a has the same characteristic, though to a limited extent.

Although Wnt-3a was used at a final concentration of 100 ng/ml in the present study, we also investigated the effects of much higher Wnt-3a concentrations, such as 250 and 500 ng/ml, on DP cells. However, DP-2p cells from cultures with Wnt-3a at such high concentrations showed limited HF induction and hair growth *in vivo* in the skin reconstitution, which also coincided with a decreased expression of versican, as compared to that of DP-2p/Wnt-10b cells.

3.3. Only Wnt-10b showed ability to maintain DP cells after serial passages

Although we previously reported sustained HF induction ability of serially passaged DP cells in the presence of Wnt-10b [9], it is not known whether other Wnts including Wnt-3a has the same ability to maintain DP cells in culture after passaging. We compared DP cells which were cultured for five passages in the presence of Wnt-10b (DP-5p/Wnt-10b cells) to those cultured with Wnt-3a (DP-5p/Wnt-3a cells) (Fig. 3). The DP-5p/Wnt-10b cells showed excellent proliferation, which was seemingly unchanged or quite similar to that of DP-2p/Wnt-10b cells, including keeping their original cuboid or polygonal shape (Fig. 3A, B). On the other hand, DP-5p/Wnt-3a cells showed reduced proliferation after five passages. As for the expressions of DP markers, DP-5p/Wnt-10b cells maintained the expression of versican and Sox-2 at a similar level to that of DP-2p/Wnt-10b cells (Fig. 3C, D), whereas those DP markers in DP-5p/Wnt-3a cells were down-regulated to a negligible level or lost. In addition, the expression of Lef-1 was decreased in DP-5p/Wnt-3a cells and no trichogenesis-promoting ability was observed in DP-5p/Wnt-3a cells (Fig. 3E, Supplementary Table S1).

3.4. Cultures of HFs and DP cells from versican-GFP transgenic mice

To further investigate the effects of Wnt-3a and 10b, we used DP cells obtained from versican-GFP transgenic mice (Fig. 4A). It

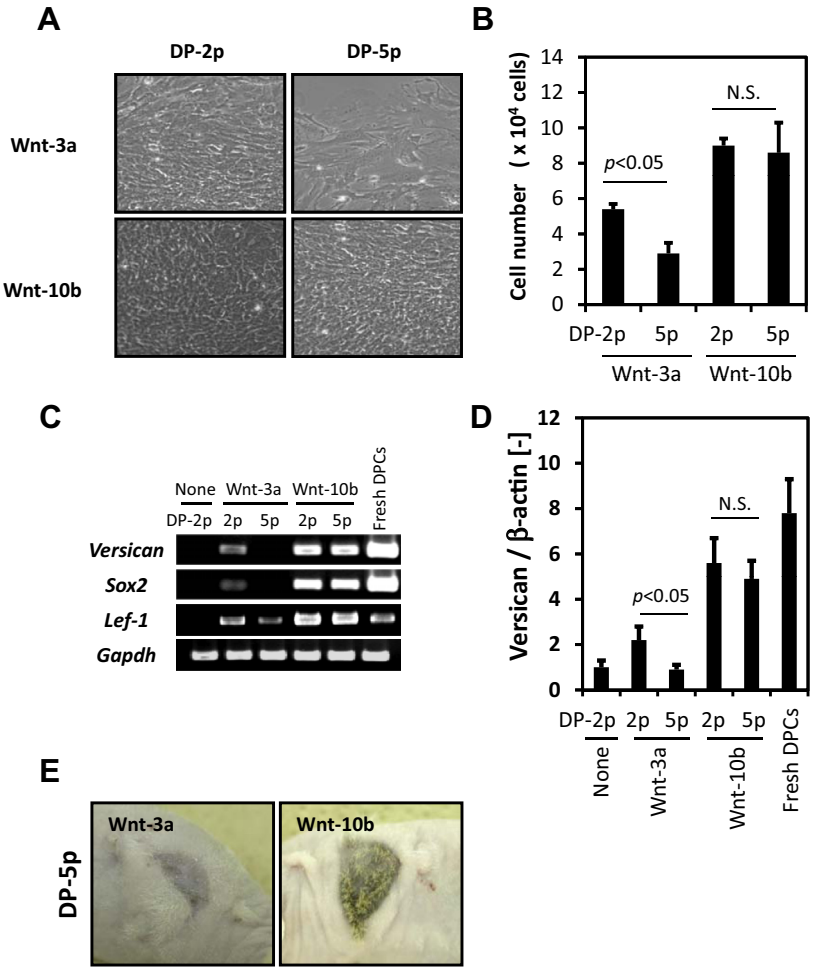

Fig. 3. Wnt-10b alone showed ability to maintain DP cells after serial passages. (A) Morphology of DP cells after the second and fifth cultures. (B) Numbers of DP cells after the second and fifth cultures. (C, D) RT-PCR analysis of expressions of versican, Sox-2, and Lef-1 in DP cells from the second and fifth cultures, and quantification of versican expression. (E) HF induction abilities of DP-5p/Wnt-3a (Wnt-3a) and DP-5p/Wnt-10b (Wnt-10b) cells *in vivo*, as shown in skin reconstitution assay findings.

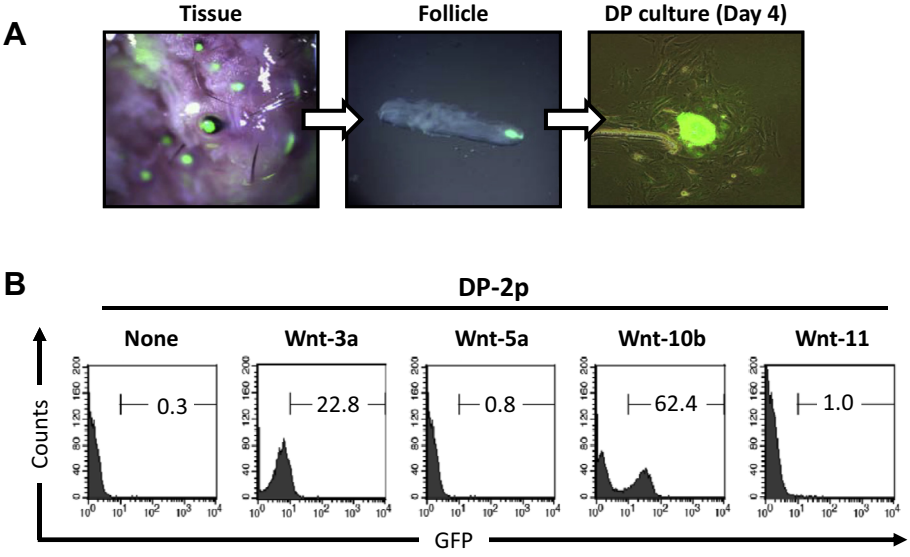

Fig. 4. Cultures of HF specimens and DP cells obtained from versican-GFP transgenic mice. (A) HF samples from versican-GFP transgenic mice were isolated and subjected to primary cultures. Representative images show the appearance of clean-shaven skin (left), an isolated follicle (middle), and a culture dish on day 3 (right). (B) Flow cytometry analysis of DP-2p cells cultured with Wnts. Following primary cultures, FACS-sorted GFP-positive DP cells were then cultured with Wnts for 10 days. Subsequently, DP cells after the second culture (DP-2p cells) were analyzed for GFP expression. Wnt-10b- and Wnt-3a-treated DC-2p cells contained 62.4% and 22.8%, respectively, GFP positive cells, whereas there were few GFP positive cells in DC-2p cells cultured in medium alone, or when treated with Wnt-5a or 11.

is known that the expression of versican coincides with hair induction ability *in vivo* [14]. We cultured their DP cells with Wnts for 10 days and analyzed their GFP-positivity. More than 60% of the cells in cultures with Wnt-10b firmly maintained their GFP-positivity [mean fluorescence intensity (MFI) for entire population 42.8 ± 9.8], whereas 22.8% of the cells were GFP-positive in cultures with Wnt-3a (MFI 11.1 ± 3.2) (Fig. 4B). No or few GFP positive cells remained in cultures with medium alone, Wnt-5a, or 11. Based on these findings, we concluded that Wnt-10b is the most potent of the tested Wnts for maintaining the HF-inducing ability of DP cells.

4. Discussion

HFs are composed of epithelial and DP cells [16], and it has been speculated that they interact in a reciprocal manner. Wnts are expressed in HFs throughout life from embryo to adult, and considered to be critical for their development and maturation [7]. In mature anagen follicles, Wnt-3a is expressed in differentiating inner root sheath (IRS) cells, while Wnt-5a has been found in dermal papilla and root sheath cells, Wnt-10b in IRS cells within the matrix, and Wnt-11 in outer root and dermal sheath cells, implying that they are deeply involved in follicle morphogenesis. However, their roles remain largely unknown.

In a previous study, we showed that Wnt-10b mediates canonical signaling [11,17]. Thereafter, we investigated the effects of Wnts (Wnt-3a, 5a, 10b, 11) on epithelial cell differentiation and hair growth, and found that only Wnt-10b demonstrated evident promotion of epithelial cell differentiation and hair shaft growth, in contrast to Wnt-3a, 5a, and 11 [12]. Subsequently, in the present study we examined the effects of Wnt-10b on DP cells and noted that Wnt-10b promoted their proliferation, and also maintained their ability to induce the growth of HFs and hair [9]. Furthermore, these biological activities were sustained in serially passaged DP cells in cultures containing Wnt-10b. Apart from the elucidated biological actions of Wnt-10b on epithelial cells and DP cells, the effects of other Wnts such as Wnt-3a, 5a, and 11 on DP cells were not evaluated.

In the present study, we investigated the effects of Wnt-3a, 5a, 11, and 10b on the proliferation of cultured mouse DP cells *in vitro*, as well as their trichogenesis-promoting ability *in vivo* using skin reconstitution assays. We found that Wnt-10b prominently promoted proliferation and maintained their trichogenesis-promoting ability. Wnt-3a had the same biological effects, though to a limited extent, while no promoting effect was seen with Wnt-5a or 11. The concentration of Wnt-3a used in the present study (100 ng/ml) was considered to be sufficient for its maximum biological action, based on results of TOPFLASH assays that revealed the same level of activity at much higher concentrations (250 and 500 ng/ml). In addition, limited hair production was also seen when DP cells were cultured with Wnt-3a at those higher doses and then used in the *in vivo* reconstitution assays. However, there were documents showing that Wnt-3a-treated DP cells induced HF induction or hair growth via Wnt/β-catenin signaling [15,18], thus leading to full regeneration of skin hair, using the same *in vivo* skin reconstitution assay. Although the reasons for the differences from our results are unclear, we think the method of DP cell stimulation may have an influence. In our experiments, we simply added Wnt-3a to cultures of DP cells, while DP cells were cultured with feeder cells transfected with a Wnt-3a-producing viral vector [15] or directly transfected with the Wnt-3a-producing vector [18].

To further investigate the effects of Wnt-3a and 10b, we utilized DP cells obtained from versican-GFP transgenic mice [14]. In these transgenic mice, versican expression appears in the condensed mesenchyme in the skin of embryos and is also observed in the dermal papilla during the anagen phase after birth. It is also known that its expression coincides with the hair induction ability of DP cells *in vivo*. According to a previous study, the relative number of GFP fluorescent cells from these mice decreases significantly in culture, reaching approximately less than 20% of the initial number of GFP positive cells remaining after the first passage (4 days) [14]. However, in the present study, more than 60% of the DP cells were GFP positive with a high MFI value after 10 days in cultures with Wnt-10b, while 22.8% were positive with a relatively low MFI value in cultures with Wnt-3a. We consider that these findings support our results from the DP cell culture experiments. Thus, we concluded that Wnt-10b has a potent ability to sustain the expression of versican in DP cells and maintain their HF-induction ability.

In HFs, the DP is located at the bottom of the follicle, where it is surrounded by undifferentiated epidermal matrix cells (known as Wnt-10b producers) and IRS cells (known as Wnt-3a and 10b producers) [7]. It is very reasonable to consider that DP cells are profoundly affected by Wnts produced by neighboring cells. Our results suggest that canonical Wnts, specifically Wnt-10b, have an excellent ability to maintain the HF-inducing activity of DP cells.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.07.108.

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