Urotensin II promotes the proliferation and secretion of vascular endothelial growth factor in rat dermal papilla cells by activating the Wnt-β-catenin signaling pathway

Congjuan Liao,[1] Zhen Huang,[2] Liuting Chen,[2] Xiaorong Fan,[1] Jun Peng,[1] Xiaoqing Tan,[1] Jianshe Yang,[3] Xusheng Zhang[1]

[1]People's Hospital of Longgang District & The Second Hospital of the Chinese University of Hong Kong, Shenzhen; [2]Shenzhen Hospital of Beijing University of Chinese Medicine, Shenzhen; [3]Shanghai Tenth People's Hospital, Tongji University School of Medicine, Shanghai, China

ABSTRACT

Introduction. Urotensin II (U II) is a kind of active peptide with a variety of biological effects, such as promoting cell proliferation and endocrine effects. The aim of this study is to investigate the effect of urotensin II on the proliferation and secretion of vascular endothelial growth factor (VEGF) in cultured rat dermal papilla cells (DPCs), and to explore its molecular mechanism. Materials and Methods. We used the DPCs isolated from the thoracic aortas of Wistar-Kyoto rats to run the CCK8 and ELISA assay, RC-PCR and Western blotting techniques to identify the effect of Urotensin II on the proliferation and secretion of VEGF in DPCs, data were analyzed by one-way ANOVA or t-test. Results. U II can increase the mRNA expression of proliferation markers Ki67 and PCNA. In addition, the Wnt/β-catenin pathway was activated by U II, but Wnt inhibitor DKK1 reversed the effect of U II. Conclusions. U II promoted the proliferation and secretion of VEGF in rat DPCs through activation of the Wnt-β-catenin signaling pathway.

Correspondence: Jianshe Yang, Shanghai Tenth People's Hospital, Tongji University School of Medicine, Shanghai, 200072, China.
Tel.: +86.2166302721.
E-mail: yangjs@impcas.ac.cn

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Introduction

Alopecia is a common hair disease. Although alopecia is not a threat to people's health, some patients may have low self-esteem and thus affect social interaction. Therefore, more and more attention has been paid to the molecular mechanisms related to hair growth and development. Dermal papilla cells (DPCs) are a group of dermal-derived cells located in the base of hair follicle, which constitute the hair papilla together with the extracellular matrix, blood vessels, and nerves. DPCs play an important role in hair growth, development, and regeneration.[1] DPCs, as a source of nutrients and growth factors, support the proliferation and growth of keratinocytes, promote the induction of new hair follicles and maintain hair growth.[2] The loss of hair follicle function induced by DPCs senescence is the main mechanism of androgenetic alopecia. The biological effects of DPCs are accomplished by a variety of cell growth factors and a series of signaling pathways, among which vascular endothelial growth factor (VEGF) is an important autocrine growth factor of DPCs. It is believed that VEGF may regulate the periodic circulation of hair follicles and promote hair

growth by inducing the proliferation and migration of DPCs or inducing the formation of blood vessels around hair follicles.[3,4]

The Wnt signaling pathway regulates various cell functions, such as the direction of cell differentiation, proliferation, migration, polarity distribution, and gene expression, by activating cell-surface receptor-mediated signal transduction pathways.[5] Studies have shown that activation of the Wnt/β-catenin signaling pathway plays an important role in hair follicle regeneration.[6] Kishimoto *et al*. demonstrated that freshly isolated mouse DPCs could induce epithelial cells to form new hair follicles, but the cells would gradually lose their ability to induce hair follicles during cultured *in vitro*. However, the induction ability of mouse DPCs cultured *in vitro* was maintained by the classical Wnt signal stimulation, indicating that the Wnt signal plays an important role in the functional maintenance of DPCs.[7]

Urotensin II (U II) is a somatostatin-like cyclic peptide, which is the strongest vasoconstricting peptide found *in vivo* so far.[8] Recent studies have found that U II is an endogenous mitogen, which can promote a variety of cell division *in vitro*, including vascular smooth muscle cells,[9] glomerular mesangial cells,[10] and liver oval cells.[11] At the same time, U II also has endocrine effects, which can affect the secretion of plasma prolactin, thyrotropin, and other bioactive factors.[12,13]

However, the biological effect of U II on DPCs has not been reported. Our study investigated the effect of U II on proliferation and VEGF secretion of DPCs cultured *in vitro*. Furthermore, the role of the Wnt/β-catenin signaling pathway in U II modulated activities was also investigated.

Materials and Methods

Material

Fetal bovine serum (Gibco, USA), high glucose DMEM (Gibco, USA), penicillin, streptomycin (Gibco, USA), Cell Counting Kit-8 (Beyotime, China), Mouse VEGF ELISA Kit (Beyotime, China), Antibody against β-actin (Cell signaling, USA), antibodies against β-catenin (Cell signaling, USA), TRIzol® reagent (Invitrogen, USA) SYBR® Green Premix Pro Taq HS qPCR Kit (Accurate Biotechnology, China), Evo M-MLV RT Kit with gDNA Clean for qPCR (Accurate Biotechnology, China), Primers for RT-PCR were synthesized in BGI Genomics.

Cell culture

DPCs were isolated from the thoracic aortas of 4- to 6-week-old male Wistar-Kyoto rats weighing 120-180 g and cultured by the tissue adherence method.[1] Cells were cultured in DMEM supplemented with 10% fetal bovine serum and maintained in a humidified atmosphere of 5% CO2 at 37°C. AFs at 80-90% confluence were passaged with 0.25% trypsin-EDTA. Cells from passages three to six were used.

CCK8 assay

The cells were digested and stained with trypan blue for viable cell counting. Cells were seeded in 96-well plates at a density of 3000 cells per well and incubated for 24 hours. After the cells adhered to the wall, 100μl DMEM medium containing different concentrations of U II was added to each well. The concentrations of U II were 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10} mol /L, respectively. Medium without U II was added to the control Wells. Three duplicate Wells were set for each concentration. After incubation for 48 h, the original culture medium of each well was removed, and a fresh medium containing 10% CCK8 was added. Then incubated for another 1h, the OD value of the band at 450nm was measured on a microplate reader. The cell proliferation rate was calculated using the following formula: proliferation rate = (OD value of experimental Wells containing U II-OD value of blank control Wells)/(OD value of control Wells without U II-OD value of blank control Wells).

ELISA test

Cells were seeded in 24-well plates at a density of 15000 cells per well. After the cells adhered to the wall, 500μl DMEM medium containing different concentrations of U II was added to each well. The concentrations of U II were 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} mol/L, respectively. Medium without U II was added to the control Wells. Three duplicate Wells were set for each concentration. After incubating for 72 h, the original culture medium was replaced by a serum-free medium followed by incubating for another 24 hours. The supernatant was then collected separately. The concentration of VEGF in the culture supernatant was detected by the VEGF ELISA kit.

RT-PCR

Cells were seeded in 6-well plates at a density of 1×10^5 cells per well. After the cells adhered to the wall, 2ml DMEM medium containing 10^{-8} mol /L U II was added. Medium without U II was added to the control Wells. After incubation for 48h, the cells were harvested and total RNA was extracted with TRIzol® reagent. The mRNA expression levels were determined by an SYBR green-based real-time PCR assay using the protocol as follows: Pre-denaturation at 95°C for 30 sec, and a total of 35 cycles of denaturation at 95°C for 5 sec, annealing at 58°C for 30 sec, and extension at 72°C for 50 sec, followed by a final extension at 72°C for 10 min. The experiments were performed thrice and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used

as an internal control. The relative mRNA expression levels of each gene were determined. The primers used for PCR are shown in Table 1.

Western blotting

Cells were seeded in 6-well plates at a density of 1×10^5 cells per well. After incubation for 24h, cells were treated with U II and/or DKK1 for 48h. Then the cells were harvested with lysis buffer and collected the lysates. The protein samples were separated on 10% SDS-PAGE gels, transferred to polyvinylidene fluoride membranes (Millipore, MA, US), and blocked with 5% nonfat milk/TBST for 1 h at room temperature. The membranes were subsequently incubated overnight at 4°C with primary antibodies. The membranes were washed with TBST three times and incubated with secondary antibodies for 1 h at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence (Millipore, MA, US). The density of the bands was quantified with the Quantity One image analysis software (Bio-Rad). The optical density of each band was determined and subsequently normalized to that of GAPDH.

Data analysis

All data are expressed as mean±SD. The mean differences between the groups were analyzed by one-way ANOVA or t-test using SPSS 22.0 statistical software. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Urotensin II promotes dermal papilla cells proliferation *in vitro*

In order to verify the effect of U II on the proliferation of rat DPCs, we cultured the cells with a medium containing different concentration of U II, and detected the proliferation of DPCs using CCK8 after 48h. As shown in Figure 1A, 10^{-6}, 10^{-7} and 10^{-8} mol /L U II could significantly promote the proliferation of DPCs (com-

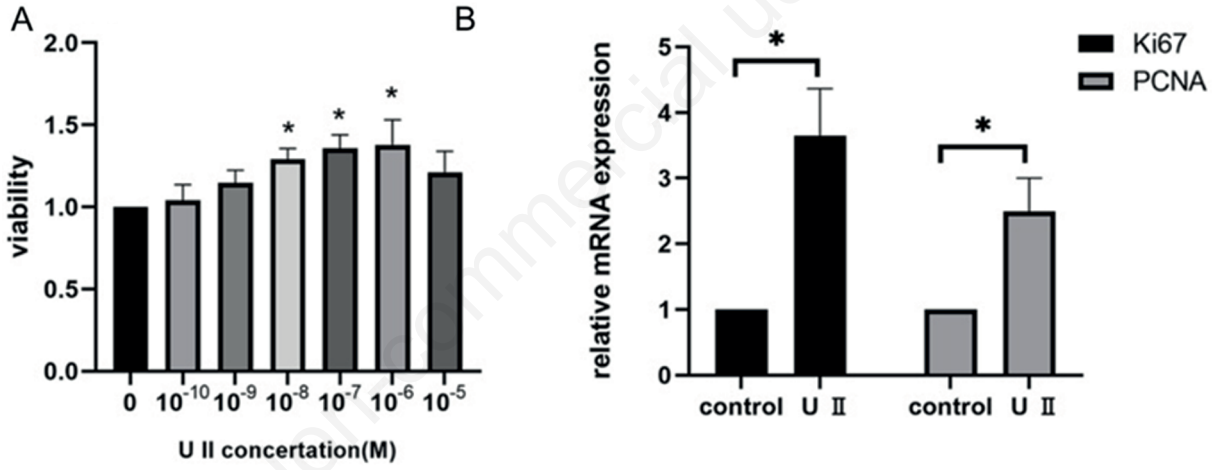

Figure 1. Urotensin II promotes the proliferation of dermal papilla cells. A) Dermal papilla cells were treated with different concentrations of Urotensin II. The effect of proliferation was measured by CCK8 (mean±SD, n=3). *P<0.05 *vs.* control group; B) Dermal papilla cells were treated with 10^{-8} mM Urotensin II for 48h. The mRNA expression level of proliferation-related genes was measured by RT-PCR assay (mean±SD, n=3). *P<0.05 *vs.* control group.

Table 1. Primer sequence list.

Gene		Primer
Ki67	Forward	5'- CTTTATGGCTGCTGGGTGCT -3'
	Reverse	5'- GAGGTT-GAAGCCGGACACAC -3'
PCNA	Forward	5'-CAATTTCTAGCAACGCCTAAGAT-3'
	Reverse	5'-AAGAGGAAGCTGTGTGTCCATAGAG-3'
VEGF	Forward	5'-ATA GCA GAT GTG AAT GCA GAC CA-3'
	Reverse	5'-TCA CAG TGA ACG CTC CA CAG GA-3'
GAPDH	Forward	5'-GGC TGC CCA GAA CAT CAT-3'
	Reverse	5'-CGG ACA CAT TGG GGG TAG-3'

PCNA, proliferating cell nuclear antigen; VEGF, vascular endothelial growth factor; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

pared with the control group, P<0.05). RT-PCR results showed that cultured with 10^{-8} mol /L U II significantly increased the mRNA expression of Ki67 and PCNA, which are the markers of proliferation (Figure 1B).

Urotensin II promoted vascular endothelial growth factor secretion in rat dermal papilla cells *in vitro*

VEGF is one of the bioactive factors secreted by DPCs. In order to explore the effect of U II on the capability of secreting VEGF by DPCs, the concentration of VEGF in the supernatant of the DPCs culture medium was detected by ELISA kit. As shown in Figure 2, 10^{-6}, 10^{-7} and 10^{-8} mol /L U II increased the concentration of VEGF in the supernatant. When treated with 10^{-8} mol/L U II for 48h, VEGF mRNA expression in DPCs was significantly higher than that in the control group. These results indicate that U II can promote the secretion of VEGF by DPCs.

Urotensin II promoted the proliferation and secreting of vascular endothelial growth factor by rat dermal papilla cells via the Wnt/β-catenin signaling pathway

The Wnt/β-catenin pathway is one of the key factors in regulating the proliferation and induction capability of DPCs.[14] In order to investigate whether U II promotes the proliferation and secreting of VEGF in DPCs via the Wnt/β-catenin pathway, we treated cells with DKK1, an inhibitor of the Wnt/β-catenin signaling pathway. The results of WB showed that U II up-regulated the protein expression of β-catenin, while simultaneous treatment with DKK-1 and U II inhibited the up-regulated of β-catenin protein expression (Figure 3A). CCK8 results showed that DKK-1 can inhibit the proliferation of DPCs induced by U II (Figure 3B). PCR

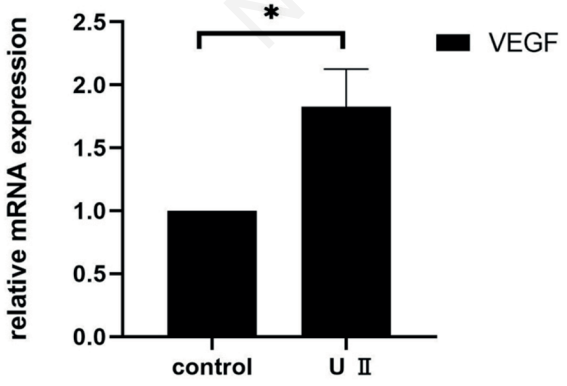

Figure 2. Urotensin II promotes vascular endothelial growth factor mRNA expression in dermal papilla cells. Dermal papilla cells were treated with 10^{-8} M Urotensin II for 48h. The mRNA expression level of vascular endothelial growth factor was measured by RT-PCR assay (mean±SD, n=3). *P<0.05 *vs.* control group.

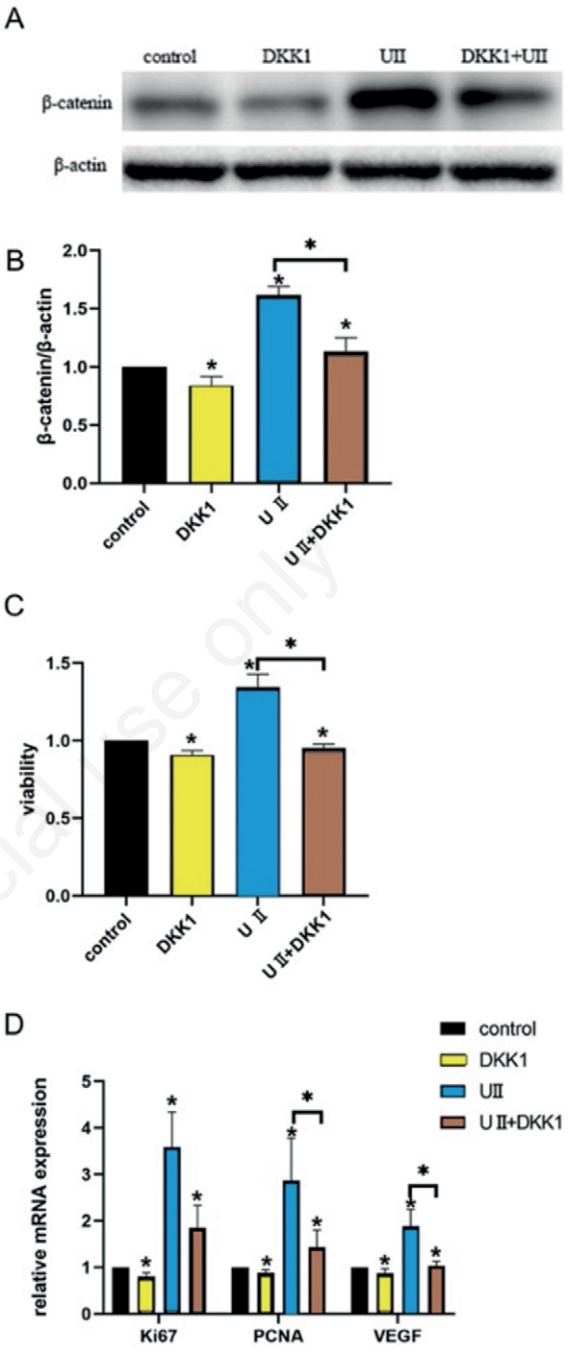

Figure 3. Urotensin II activates the wnt-β-catenin pathway, whereas DKK1 reverses the effect of Urotensin II. A) Western blot analysis was used to measure the effect of DKK1 on β-catenin protein expression in Urotensin II-treated dermal papilla cells; B) Quantitative analysis of β-catenin protein expression in Urotensin II-treated dermal papilla cells with or without exposure to DKK1(mean±SD, n=3). *P<0.05 *vs.* control group; C) The proliferation of U II-treated dermal papilla cells with or without exposure to DKK1 was measured by CKK8 (mean±SD, n=3). *P<0.05 *vs.* control group; D) The mRNA expression level of Ki67, PCNA, and vascular endothelial growth factor in Urotensin II-treated dermal papilla cells with or without exposure to DKK1 were measured by RT-PCR assay (mean±SD, n=3). *P<0.05 *vs.* control group.

results showed that U II treatment up-regulated the mRNA expression of Ki67, PCNA, and VEGF, while DKK1 reversed the effects of U II (Figure 3C).

Discussion

Studies have found that the number of DPCs is closely associated with baldness. The reduction of DPCs may lead to the atrophy of hair follicles while stimulating the proliferation of dermal papilla cells can promote hair growth.[15]

U II can promote the proliferation of vascular smooth muscle cells, glomerular mesangial cells and liver oval cells.[9-11] In order to confirm whether U II has a proliferative effect on DPCs, we performed a CCK-8 assay. Our results showed that 10^{-6}, 10^{-7} and 10^{-8} mol /L U II could significantly promote the proliferation of DPCs. Therefore, U II may be involved in the regulation of the hair follicle cycle by increasing the number of DPCs in the hair follicle. 10^{-5} mol /L U II also had a proliferative effect on DPCs, but the effect was not as obvious as 10^{-6} mol /L U II, possibly because the excessive concentration of U II would activate some negative feedback mechanisms.

Ki-67, which was used as a marker of proliferation, was not expressed during the cell quiescent phase, but expressed in other stages of the cell cycle.[16] The concentration of PCNA varies periodically throughout the cell cycle and peaks during the DNA replication phase. After the completion of replication, its concentration decreased rapidly. Therefore, the expression of PCNA can also be used as a marker of cell proliferation.[17] Here, we demonstrated that 10^{-8} mol /L U II significantly increased the mRNA expressions of Ki67 and PCNA, which further indicated that U II may promote the proliferation of DPCs by up-regulating the mRNA expression of PCNA and Ki67.

It has been reported that rabbit macrophages and human vascular endothelial cells were induced to secrete VEGF by U II,[18,19] suggesting that U II is likely to be an important factor in promoting VEGF secretion from DPCs. To confirm the effect of U II on the VEGF secretion from DPCs, the concentration of VEGF in the supernatant of DPCs cultured medium was detected by ELISA. Our results showed that treatment with 10^{-6}, 10^{-7}, and 10^{-8} mol /L U II significantly promoted VEGF secretion by DPCs. RT-PCR results showed that the mRNA expression of VEGF in the U II-treated group was significantly increased compared with the control group. In hair follicles, VEGF, which is secreted by DPCs, can activate the hair cycle and promote hair growth.[16] Our data showed the significance of U II stimulating VEGF secretion of DPCs, which will lead us to consider the potential of promoting hair growth by U II.

The wnt/β-catenin signaling pathway plays an important role in maintaining and regulating hair follicle proliferation and growth cycle.[20-22] Classical Wnt/β-catenin signaling is a key factor to initiate hair follicle development, and ectopic expression of Wnt repressor DKK1 or lack of β-catenin protein expression inhibits hair follicle formation.[23,24] Overexpression of β-catenin in skin epithelial cells increased hair follicle formation, whereas overexpression of DKK1 has an inhibitory effect on hair follicle formation.[25,26] To confirm whether U II promotes the proliferation and VEGF secretion of dermal papilla cells via the Wnt/β-catenin pathway, we treated cells with DKK1, a Wnt/β-catenin pathway inhibitor. Subsequently, the hallmark of Wnt signaling, β-catenin, was detected by WB. The results showed that U II up-regulated the expression of the β-catenin protein, which indicated the activation of the Wnt/β-catenin pathway. However, the DKK1 intervention reversed the U II effect. In addition, RT-PCR was used to detect the mRNA expression of Ki67, PCNA, and VEGF after DKK1 treatment. The results showed that Ki67 and PCNA mRNA expression was significantly decreased, and VEGF mRNA expression was also down-regulated after DKK-1 treatment. These results further demonstrated that U II promoted the proliferation and VEGF secretion of DPCs via the Wnt/β-catenin pathway.

Conclusions

In summary, our results suggest that U II promotes the proliferation of DPCs and increases the VEGF secretion by DPCs via activating the Wnt/β-catenin pathway. Our study provides new evidence to reveal the biological effects of U II.

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