Int J Clin Exp Pathol 2017;10(6):6827-6835
www.ijcep.com /ISSN:1936-2625/IJCEP0052087

Original Article

Decreased secretion of WNT3 by deciduas of URSA patients leads to trophoblast cell dysfunction

Ning Li[1*], Yanwei Wang[1*], Yan Li[2], Juan Liu[1], Xin Liu[1], Wenting Wang[1], Cuicui Yu[3], Jiahui Wang[1], Shuhong Li[4]

[1]Central Laboratory, Departments of [3]Anesthesiology, [4]Obstetrics and Gynecology, The Affiliated Yantai Yuhuangding Hospital of Qingdao University, Yantai, Shandong, China; [2]College of Life Science, Yantai University, Yantai, Shandong, China. [*]Equal contributors.

Received March 3, 2017; Accepted April 26, 2017; Epub June 1, 2017; Published June 15, 2017

Abstract: It is well known that rapid development of placental structures during the first weeks of gestation is critical for embryonic survival and maintenance of pregnancy, in which WNT3 has been reported to play a critical role. However, the precise role WNT3 plays in the first-trimester trophoblast cells remains unknown. In present study, the expression level of WNT3 in the decidual tissues was compared between the unexplained recurrent spontaneous abortion (URSA) patients and healthy control women through Western blot. The function of WNT3 on trophoblast cells was investigated by altering the extracellular WNT3 level *in vitro*. The molecular mechanism of the effect of WNT3 on trophoblast cells was investigated by studying the association of WNT3 with the Wnt/β-catenin signalling pathway through Western blot and immunofluorescence. Results showed that WNT3 protein expression was significantly decreased in deciduas of the URSA group compared with those of the control group, suggesting that WNT3 might regulate trophoblast function mainly through a paracrine way during early pregnancy. *In vitro* studies showed that WNT3 could promote human trophoblast cell proliferation and migration via activation of the Wnt/β-catenin signaling pathway. In conclusion, our study indicated that WNT3 deficiency might lead to impaired trophoblast cells proliferation and migration via the downregulation of Wnt/β-catenin signaling pathway.

Keywords: Wnt/β-catenin signaling pathway, unexplained recurrent spontaneous abortion, WNT3, HTR-8/SVneo

Introduction

Rapid development of placental structures during the first weeks of gestation is critical for embryonic survival and maintenance of pregnancy [1]. The proliferation, migration and invasion of the most important cells in early pregnancy, human trophoblasts, are crucial processes for normal placental and fetal development [2]. Defects in trophoblast cell function are closely associated with adverse pregnancy complications, including recurrent spontaneous abortion (RSA) which was defined as two or more consecutive miscarriages before 20 weeks gestation with the same partner [2, 3]. RSA occurs in 1%-5% women of reproductive age, and has a negative effect on human reproductive health [4]. Except for some verifiable etiological factors, such as infection, endocrine disorder, anatomic deformation, chromosomal abnormality and metabolic and autoimmune diseases, there are still approximately 50% of cases with unknown cause and are referred to as unexplained recurrent spontaneous abortion (URSA) [5]. Previous studies showed that besides a strong intrinsic molecular program mediating the function of trophoblast, endocrine secretions from uterine cells are likely necessary for early stages of trophoblast development [6, 7]. Endometrial secretion disorder could be a potential cause of early stage trophoblast dysfunction [6]. However, the soluble decidual factors controlling growth and differentiation of trophoblast cells during early gestation remain poorly elucidated.

Recently, accumulating evidence suggests that the canonical Wnt pathway involving nuclear recruitment of β-catenin and activation of Wnt dependent transcription factors are critically involved in endometrial function, implantation and placental development [8]. Failures in Wnt signalling are associated with gestational diseases such as URSA [9, 10]. Some study reported that Wnt ligands could play a role in early placental function and differentiation in an

Table 1. Characteristics of URSA and control subjects (mean ± SD and range of values)

	n	Age (years)	No. of miscarriages	Gestational age (weeks)
URSA	50	30.02±3.07 (24-35)	2.54±0.65 (2-4)	8.56±1.13 (7-12)
Control	50	29.72±3.23 (25-36)	0	8.20±0.69 (7-9)

URSA versus control: Age, P = 0.512 (Student's t-test). Gestational age, P = 0.62 (Mann-Whitney U-test). URSA, unexplained recurrent spontaneous abortion; SD, standard deviation.

autocrine manner [11]. However, detection of various Wnt ligands in human endometrial cells suggested that paracrine activation of the Wnt pathway could also regulate trophoblast development and function [12]. Therefore, how decidua-derived Wnt ligands control growth and differentiation of trophoblast cells during early gestation still waits to be explained.

WNT3, an activator of the canonical Wnt/β-catenin pathway, plays a critical role in developmental process [13]. In mice, Wnt3 is expressed prior to gastrulation and its targeted deletion causes an early developmental arrest [14]. In human, homozygous nonsense mutation within the *WNT3* coding region (Q83X) resulted in the loss of all limbs with craniofacial and urogenital defects in affected fetuses [15]. WNT3 may also regulate trophectoderm lineage differentiation in human blastocysts [16]. Moreover, WNT3 showed elevated expression in the proliferative endometrium, suggesting that it might regulate trophoblast development and function through paracrine way [12]. However, the precise role of decidua-secreted WNT3 in first-trimester trophoblast cells is unknown.

In the present study, the expression of WNT3 in deciduas of normal pregnant women and URSA patients was examined. The function of WNT3 in first-trimester trophoblast cells was also studied *in vitro*. We demonstrated that the expression of WNT3 was lower in deciduas of URSA patients than in normal pregnant women, and that WNT3 protein could promote the proliferation and migration of first-trimester trophoblast cells via activating the canonical Wnt/β-catenin pathway.

Materials and methods

Clinical samples

A total of 50 patients underwent spontaneous abortion were enrolled in this study, all of whom were outpatients at the Department of Obstetrics and Gynecology, Yantai Yuhuangding Hospital, Shandong province. The inclusion criteria were: i) had experienced at least two consecutive first-trimester miscarriages of unexplained cause; ii) failed pregnancy was confirmed by ultrasound; iii) diagnosed as URSA after excluding any verifiable causes; iv) the karyotypes of both the patient and their male partners were normal; v) patient's male partners had normal semen quality according to the criteria from the 2010 World Health Organization guidelines [17]. The exclusion criterion was abortion caused by verifiable causes.

Another group of 50 randomly selected healthy women undergoing a legal termination of apparently normal early pregnancies at the same facility were recruited as control, with inclusion criteria described before [10]. All women in control group had at least one living child, with no history of spontaneous abortion, ectopic pregnancy, preterm delivery, or stillbirth. In all 50 normal cases, fetal heart activity had been identified within 2 weeks before sample collection. There was no significant difference in age or gestational weeks between the URSA and control groups (**Table 1**). All procedures were approved by the ethics review board of Yantai Yuhuangding Hospital. Moreover, the informed consent was also obtained from all participants. The study was conducted in accordance with the Declaration of Helsinki guidelines.

RNA isolation and quantitative PCR

Quantitative PCR was performed as described previously [10]. β-actin was used as endogenous reference. The relative expression levels of downstream targets of Wnt/β-catenin in HTR-8/SVneo cells were calculated by the 2-ΔΔCt method [18]. All experiments were performed in triplicate. The primer sequences were as follows:

MMP-2 forward: 5'-GGCACCCATTTACACCTACA-3', reverse: 5'-TCTGAGCGATGCCATCAAATA-3'; c-Myc forward: 5'-CGACTCTGAGGAGGAACAAGA-AGA-3', reverse: 5'-TGCGTAGTTGTGCTGATGTGT-G-3'; CyclinD1 forward: 5'-AGCTGTGCATCTAC-

ACCGACAA-3', reverse: 5'-TGTTTGTTCTCCTCCGCCTCTG-3'; β-actin forward: 5'-ACCATGTACCCTGGCATTG-3', reverse: 5'-GTCATACTCCTGCTTGCTGAT-3'.

Western blot

Total proteins were extracted and Western blot analysis was performed as described previously [10]. using the following primary antibodies: anti-WNT3 (ab32249, rabbit, Abcam Inc, Cambridge, Massachusetts), anti-β-catenin (9587, rabbit, Cell Signaling Technology, Danvers, MA, USA), anti-active-β-catenin (anti-ABC, clone 8E7, 05-665, mouse, Merck Millipore, Billerica, MA), anti-β-actin (sc-81178, mouse, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and anti-Lamin B (13435, rabbit, Cell Signaling Technology).

Cell culture and proliferation assay

HTR-8/SVneo cells were cultured in DMEM/F12 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin (GIBCO) at 37°C with 5% CO_2 humidified air according to standard procedures.

To evaluate the effect of WNT3 protein on proliferation of trophoblast cells, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) colorimetric assay was performed. Cells were seeded in 96-well plates at a density of 3×10^3 per well and cultured in 180 μl DMEM/F12 with 10% FBS until 60% confluency. Subsequently, cells were treatment with 200 ng/ml recombinant WNT3 (H00007473-P01, Abnova, Taiwan) for 1-3 days at 37°C. On each day, 0.5 mg/ml final concentration of MTT was added followed by 2 h incubation. Then the medium was removed and 100 μl per well DMSO (Sigma, Aldrich) was added. The plates were kept in darkroom for 15 min and the optical density (OD) value of each well was measured spectrophotometrically at 490 nm.

Wound healing assay

Wound healing assay was used to evaluate the migration ability of trophoblast cells. Cells were seeded into the 6-well plates at a density of 5×10^5 cells per well and incubated in medium containing 10% FBS. To avoid the effect of serum on cell migration and induce cell synchronization, cells were starved with serum-free medium for 24 h before the assay when they reached 90% confluency. Then a 200 μl pipette tip was used to scratch a straight wound tract through the middle of the cell monolayer. Each well was gently washed with PBS to remove the detached cells. Then the cells were cultured with recombinant WNT3 (rWNT3, H00007473-P01, Abnova, Taiwan). Migration distance was calculated by subtraction of the gap distance at 18 h from that at 0 h (immediately after scratching). Results were obtained from 3 independent experiments with 10 measurement points each.

Subcellular fractionation

Subcellular fractionation was performed according to Wang et al [19]. Briefly, collected HTR-8/SVneo cells were resuspended in hypotonic buffer (42 mM KCl, 10 mM Hepes pH 7.4, 5 mM $MgCl_2$, 10 μg/ml each aprotinin and leupeptin). Nuclei homogenates were removed by centrifugation at 14000×g for 10 min at 4°C.

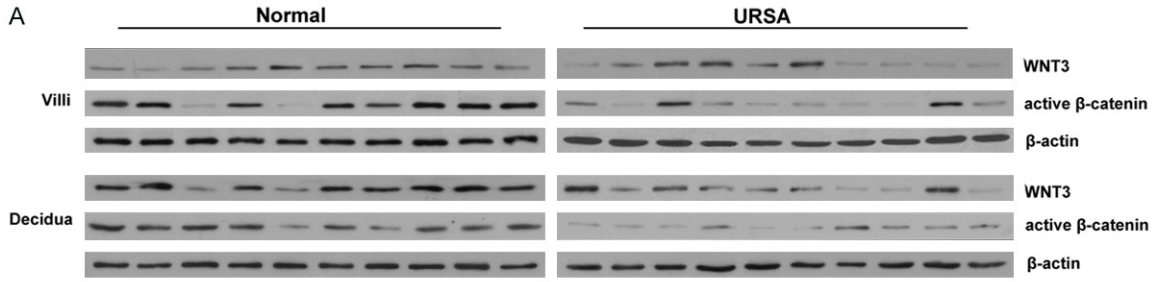
Immunofluorescence

HTR-8/SVneo cells were cultured on microscope cover glasses (Fisherbrand, USA) until 60% confluency. After complete removal of the normal culture medium, cells were subsequently cultured with rWNT3 (H00007473-P01, Abnova, Taiwan). Cells treated with 30 μM lithium chloride (LiCl, Sigma, USA) were used as positive control [20]. After 30 h incubation, the cells were fixed in 4% paraformaldehyde for 15 min at room temperature and permeabilized with 0.1% Triton X-100 for 5 min. Then the fixed cells were incubated with 3% bovine serum albumin (BSA) in PBS solution at room temperature for 60 min, washed three times with PBS, and incubated with anti-β-catenin primary antibody (9587, rabbit, Cell Signaling Technology, Danvers, MA, USA) at 1:50 dilution for 1 hour at 4°C overnight. After washes in PBS, cells were incubated with FITC-labeled goat anti-rabbit secondary antibodies (ZSGB-BIO, Beijing, China) at 1:200 dilution at room temperature for 1 h. Normal rabbit IgG was used as negative control. The nuclei were labeled with DAPI (1:1000) for 10 min. Images were obtained by confocal laser scanning microscopy (LSM-510, Carl Zeiss, Jena, Germany).

Statistical analysis

Statistical analyses were performed using SPSS 18.0. The Student's t-test or the Mann-Whitney U-test was used to compare the statistical significance of differences. Mean ± 2SD of

A



B Relative intensity of WNT3 and active-β-catenin normalized to β-actin

		Normal	URSA
Villi	WNT3	0.33±0.19 (n=50) [2/50]	0.38±0.26 (n=50) [3/50]
	Active-β-catenin	0.76±0.27 (n=50) [3/50]	0.28 ±0.22# 54% [27/50]*
Decidua	WNT3	0.81 ±0.30 (n=50) [2/50]	0.27 ±0.23# 52% [26/50] *
	Active-β-catenin	0.66±0.22 (n=50) [4/50]	0.32±0.19# 38% [19/50] *

Figure 1. Decreased expression of WNT3 and active β-catenin in villi and deciduas of normal pregnant women and URSA patients. Representative protein expression (A) and the quantification (B) of WNT3 and active β-catenin in villi and deciduas by Western blots and densitometry. The proportion of URSA group with low levels of WNT3 and active β-catenin (at least 2SD lower than normal values) is also presented (B). Values are mean ± SD. #Values significantly lower than that of normal group (Mann-Whitney U-test, $P < 0.05$). *Significantly higher proportion of samples differed from normal samples (Fisher's exact test, $P < 0.05$). $n = 50$ for each group.

the reference value were considered as the cut-off value for classification as lower or higher than the reference value. Frequencies between the two groups were compared Fisher's exact test. Differences were considered to be statistically significant when $P < 0.05$.

Results

Decreased expression of WNT3 and active β-catenin in decidual tissues of normal pregnant women and URSA patients

During pregnancy, the uterine decidual tissue and villous tissue affect each other through secretion of regulatory factors. Therefore, the expressions of WNT3 and active β-catenin, which is a downstream component of WNT3 and marker for Wnt signalling activation, in decidual tissues and villous tissues from 50 normal pregnant women and 50 URSA patients were evaluated by Western blot (**Figure 1A**). Quantification analysis showed weak expression of WNT3 in villous tissues from both groups but high expression in decidual tissues from normal pregnant women. For URSA patients there was lower level of WNT3 expression in decidual tissues than normal pregnant women ($P < 0.001$). There were 52% of the URSA women whose WNT3 expression levels were at least 2SD lower than those from normal pregnant women (**Figure 1B**). Moreover, the expression of active β-catenin was significantly decreased in both the decidual and villous tissues of URSA patients compared to normal pregnant women ($P < 0.001$) (**Figure 1B**). The proportion of samples from URSA women with active β-catenin levels at least 2SD lower than those from normal pregnant women was 54% ($P < 0.001$, **Figure 1B**) and 38% in villous tissues and decicual tissues respectively.

Recombinant WNT3 promoted trophoblast cell proliferation and migration in vitro

Cell proliferation assay and wound healing assay showed that the cells treated with recombinant WNT3 (rWNT3) displayed increased cell proliferation and migration compared to untreated cells (**Figure 2A** and **2B**). Furthermore, the mRNA expression level of MMP-2, c-Myc, CyclinD1 were upregulated in the rWNT3 treated cells (**Figure 2C**).

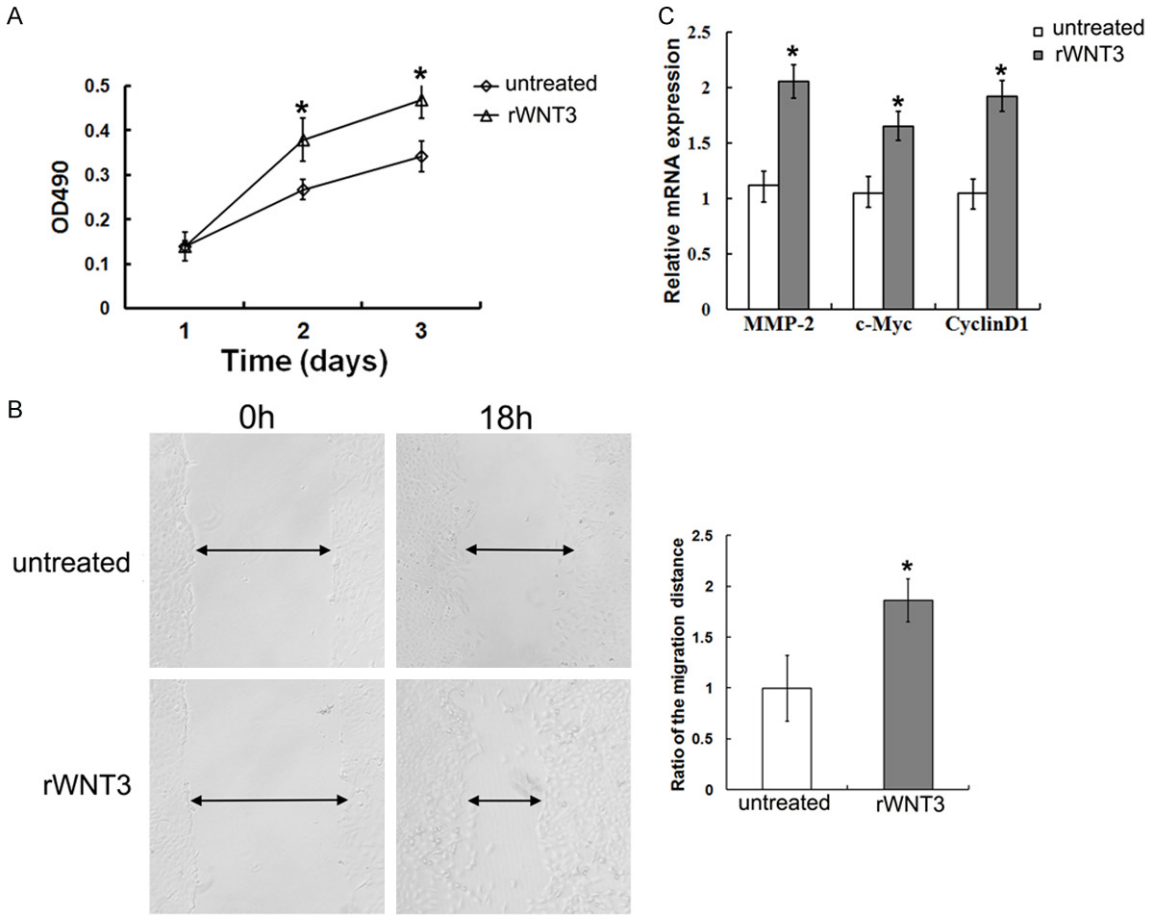


Figure 2. WNT3 promoted trophoblast cell proliferation and migration *in vitro*. Proliferation (A) and migration (B) of HTR-8/SVneo cells treated with 200 ng/ml rWNT3 or negative control medium were evaluated by MTT assay (n = 3, Student's t-test; *P < 0.05) and wound healing assay, respectively. Ratio of the migration distance in wound healing assay was shown in the right panel. Ratio of the migration distance = (Gap distance$_{0\ h}$-Gap distance$_{18\ h}$)$_{rWNT3}$/(Gap distance$_{0\ h}$-Gap distance$_{18\ h}$)$_{untreated}$. Results were obtained from 3 independent experiments with 10 measurement points each (Student's t-test; *P < 0.05). (C) Quantification of MMP-2, c-Myc and CyclinD1 mRNA expression in HTR-8/SVneo cells treated with rWNT3. The expression levels of MMP-2, c-Myc and CyclinD1 in HTR-8/SVneo cells were calculated by the $2^{-\Delta\Delta Ct}$ method (n = 3, Student's t-test; *P < 0.05). The average level of MMP-2, c-Myc and CyclinD1 mRNA expression in negative control cells set as 1.0.

WNT3 promoted cell proliferation and migration via nuclear translocation of β-catenin

To explore the underlying molecular mechanism of the growth-enhancing effect of rWNT3 on trophoblast cell, the Wnt/β-catenin signalling pathway in HTR-8/SVneo cells was characterized. Western blot revealed that rWNT3 resulted in elevated levels of both β-catenin and active β-catenin in HTR-8/SVneo cells (**Figure 3A**, top). In order to determine whether the accumulated β-catenin proteins in cytoplasm might enter the nucleus, subcellular fractionation and immunofluorescence was performed 30 h after cells were cultured either with rWNT3. Subcellular fractionation showed that the level of β-catenin and active β-catenin both increased in the nucleus of the cells treated with rWNT3 (**Figure 3A**, bottom). Immunofluorescence staining showed that besides the localization on the membrane, β-catenin could also be detected in the nucleus of the rWNT3 cultured cells, demonstrating its nuclear translocation (**Figure 3B**). As a positive control, LiCl was found to markedly activate β-catenin expression and nuclear translocation (**Figure 3A** and **3B**). In contrast, nuclear translocation of β-catenin could be hardly observed in the untreated cells (**Figure 3B**).

Discussion

Recent study showed that interference with the Wnt signalling pathway might result in URSA [9,

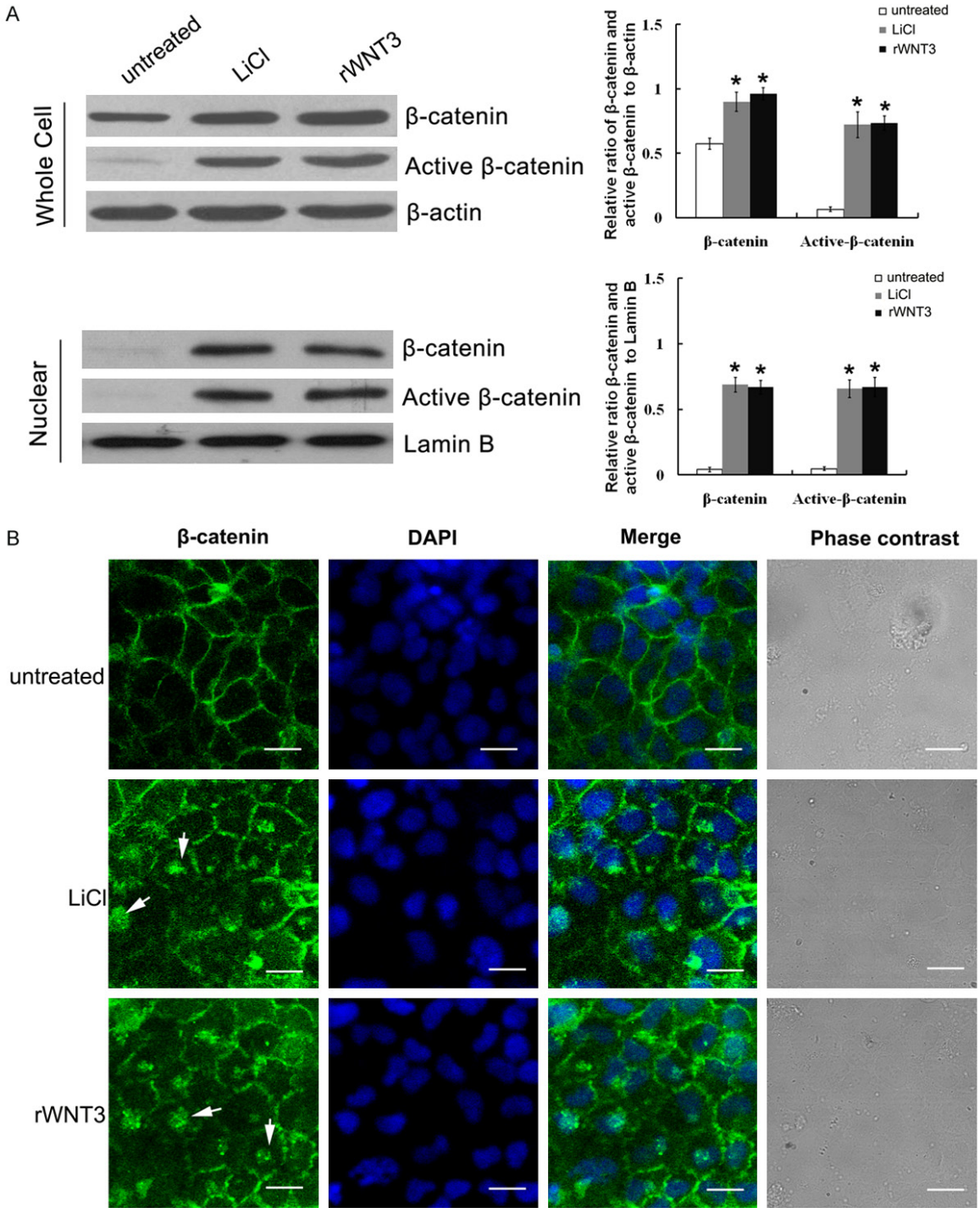


Figure 3. WNT3 promotes cell proliferation and migration via nuclear translocation of β-catenin. A. Western blot analysis of β-catenin and active β-catenin in whole cell (top) and nuclear extract (bottom). β-actin and Lamin B were used as internal control of whole cell and nuclear extract, respectively. Quantification of β-catenin and active β-catenin expression was shown in the right panel. The results are expressed as mean ± SD of three independent experiments (Student's t-test; *$P < 0.05$). B. Representative immunofluorescence images demonstrating the translocation process of β-catenin from the membrane to nucleus in rWNT3 and LiCl treated cells. 30 μM LiCl were used as positive control. Nuclear translocation of β-catenin could be hardly observed in the untreated cells. Antibody against β-catenin was labeled with FITC and nuclei were labeled with DAPI. Scale bars, 20 μm.

10]. However, as the activator of the canonical Wnt/β-catenin pathway, Wnt ligands that control growth and differentiation of trophoblast cells during early gestation are not well understood.

In the present study, we found that WNT3 was weakly expressed in villous tissues and highly expressed in decidual tissues, indicating that WNT3 affected the trophoblasts through decidual secretion. We also found decreased WNT3 expression in URAS patients. Meanwhile, active β-catenin level was observed to reduce in both tissue types in URAS patients comparing to normal pregnant women. Therefore, we speculated that decidua-derived WNT3 insufficiency might lead to impaired trophoblast cells proliferation and migration via the downregulation of Wnt/β-catenin signaling pathway.

WNT3 is a secreted, palmitoylated glycoprotein that can act through autocrine or paracrine modes [12, 16]. Previous studies showed that WNT3 can be detected in the first trimester placenta, whereas the expression level is low suggesting that WNT3 might not regulate the first trimester trophoblast function via autocrine way [21]. It has been appreciated that besides regulation by trophoblastic factors through the autocrine way, trophoblast proliferation, differentiation and migration might also be regulated in a paracrine way by uterine factors [22]. Maternal uterine environment is critical for human placental morphogenesis and early stage trophoblast development [23, 24]. Besides acting as a source of nutrients, endometrium also secretes a variety of growth factors, such as epidermal growth factor EGF) and vascular endothelial growth factor (VEGF), that bind to trophoblast-specific integrins and modulate trophoblast migration, invasion, or both *in vitro* models [22, 25]. Deranged uterine secretions could adversely affect pregnancy outcome, increasing maternal and fetal morbidity and mortality [6]. Therefore, paracrine interactions between the extravillous trophoblast and the maternal decidua are important for successful embryonic implantation [26]. Accumulating evidence showed that a large number of Wnt-related genes have all been identified as being expressed in endometrial samples and in endometrial epithelial/stromal cells, suggesting that these factors could potentially regulate trophoblast development and function in a paracrine manner [1, 12]. It was also found that WNT3 was elevated in the proliferative endometrium [12]. Interestingly, in the present study, WNT3 was significantly decreased in deciduas of URSA. These results raised the possibility that WNT3 might regulate trophoblast function mainly through a paracrine way during early pregnancy.

In present study, the function of WNT3 on HTR-8/SVneo trophoblast cells was validated by altering the extracellular WNT3 level *in vitro*. rWNT3 could promote trophoblast cell proliferation and migration by activating the Wnt/β-catenin signalling pathway which was characterized by β-catenin accumulation in the cytoplasm, translocation into the nucleus and activation of transcription of Wnt target genes including MMP-2, cyclin D1 and c-myc. As a key component of the Wnt signalling pathway, β-catenin is a frequently-used surrogate marker of Wnt pathway activation [27]. Our results clearly showed that β-catenin entered the nucleus after trophoblast cells were treated with rWNT3, suggesting that the presence of abundant WNT3 in growth media successfully activated the canonical Wnt signalling pathway. MMP-2 has been identified as a novel Wnt target in invasive trophoblasts [28]. It was thought to degrade decidual extracellular matrix proteins and thereby facilitate cell invasiveness [28]. The present study showed that secreted WNT3 activated the secretion of MMP-2, suggesting that WNT3 might promote trophoblast motility through elevated MMP-2.

It has been reported that histone methylation plays an important role in diverse biological processes including transcription. Polycomb repressive complex 2 (PRC2)-mediated histone H3 lysine 27 trimethylation (H3K27me3) has been mainly linked to transcription repression [29], and analysis of previous ChIPseq results of H3K27me3-enriched genes revealed that the WNT signaling pathway is highly enriched for H3K27me3 [30]. Moreover, Jiang *et al*. found that the H3K27me3 level of WNT3 gene promoter might affect the expression of WNT3 and endoderm differentiation at early stage [30] Whether reduced WNT3 gene expression in URSA is also associated with histone methylation needs further study.

In conclusion, our study demonstrated that the expression of WNT3 was lower in deciduas of URSA patients than in normal pregnant women, suggesting that WNT3 might regulate trophoblast function mainly through paracrine way

during early pregnancy. *In vitro* studies showed that rWNT3 protein could promote the proliferation and migration of first-trimester trophoblast cells via activation of the canonical Wnt/β-catenin pathway. Our findings add to our knowledge of the regulation of trophoblast function and how Wnt signalling might contribute to the pathogenesis of URSA. Further study into histone methylation of WNT3 in decidual tissues of URSA will help us to understand the precise molecular mechanisms of URSA pathogenesis.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant no. 81501313; 81571490), Shandong Provincial Natural Science Foundation, China (Grant no. ZR2015HQ031; ZR2014HQ068; ZR2016HL-05), Yantai Science and Technology Program (Grant no. 2015WS024; 2015WS019; 2016-WS002; 2016WS012), Yantai Yuhuangding Hospital Youth Scientific Research Foundation (Grant no. 201526).

Disclosure of conflict of interest

None.

Address correspondence to: Shuhong Li, Department of Obstetrics and Gynecology, The Affiliated Yantai Yuhuangding Hospital of Qingdao University, Yantai 264000, Shandong, China. E-mail: yiyu28@163.com; Jiahui Wang, Central Laboratory, The Affiliated Yantai Yuhuangding Hospital of Qingdao University, Yantai 264000, Shandong, China. Tel: +86-535-6691999-81533; Fax: +86-535-626724; E-mail: jiahui.wang@outlook.com

References

[1] Meinhardt G, Saleh L, Otti GR, Haider S, Velicky P, Fiala C, Pollheimer J and Knofler M. Wingless ligand 5a is a critical regulator of placental growth and survival. Sci Rep 2016; 6: 28127.

[2] Zong S, Li C, Luo C, Zhao X, Liu C, Wang K, Jia W, Bai M, Yin M, Bao S, Guo J, Kang J, Duan T and Zhou Q. Dysregulated expression of IDO may cause unexplained recurrent spontaneous abortion through suppression of trophoblast cell proliferation and migration. Sci Rep 2016; 6: 19916.

[3] Kaur R and Gupta K. Endocrine dysfunction and recurrent spontaneous abortion: An overview. Int J Appl Basic Med Res 2016; 6: 79-83.

[4] Vaiman D. Genetic regulation of recurrent spontaneous abortion in humans. Biomed J 2015; 38: 11-24.

[5] Rai R and Regan L. Recurrent miscarriage. Lancet 2006; 368: 601-611.

[6] Burton GJ, Scioscia M and Rademacher TW. Endometrial secretions: creating a stimulatory microenvironment within the human early placenta and implications for the aetiopathogenesis of preeclampsia. J Reprod Immunol 2011; 89: 118-125.

[7] Knofler M and Pollheimer J. IFPA award in placentology lecture: molecular regulation of human trophoblast invasion. Placenta 2012; 33 Suppl: S55-62.

[8] Sonderegger S, Pollheimer J and Knofler M. Wnt signalling in implantation, decidualisation and placental differentiation–review. Placenta 2010; 31: 839-847.

[9] Bao SH, Shuai W, Tong J, Wang L, Chen P and Duan T. Increased Dickkopf-1 expression in patients with unexplained recurrent spontaneous miscarriage. Clin Exp Immunol 2013; 172: 437-443.

[10] Li S, Li N, Zhu P, Wang Y, Tian Y and Wang X. Decreased beta-catenin expression in first-trimester villi and decidua of patients with recurrent spontaneous abortion. J Obstet Gynaecol Res 2015; 41: 904-911.

[11] Knofler M and Pollheimer J. Human placental trophoblast invasion and differentiation: a particular focus on Wnt signaling. Front Genet 2013; 4: 190.

[12] Tulac S, Nayak NR, Kao LC, Van Waes M, Huang J, Lobo S, Germeyer A, Lessey BA, Taylor RN, Suchanek E and Giudice LC. Identification, characterization, and regulation of the canonical Wnt signaling pathway in human endometrium. J Clin Endocrinol Metab 2003; 88: 3860-3866.

[13] Teh C, Sun G, Shen H, Korzh V and Wohland T. Modulating the expression level of secreted Wnt3 influences cerebellum development in zebrafish transgenics. Development 2015; 142: 3721-3733.

[14] Yoon Y, Huang T, Tortelote GG, Wakamiya M, Hadjantonakis AK, Behringer RR and Rivera-Perez JA. Extra-embryonic Wnt3 regulates the establishment of the primitive streak in mice. Dev Biol 2015; 403: 80-88.

[15] Niemann S, Zhao C, Pascu F, Stahl U, Aulepp U, Niswander L, Weber JL and Muller U. Homozygous WNT3 mutation causes tetra-amelia in a large consanguineous family. Am J Hum Genet 2004; 74: 558-563.

[16] Krivega M, Essahib W and Van de Velde H. WNT3 and membrane-associated beta-catenin regulate trophectoderm lineage differentiation in human blastocysts. Mol Hum Reprod 2015; 21: 711-722.

[17] Organization. WH. WHO laboratory manual for the examination and processing of human se-

men. 5th edition. Geneva, Switzerland: World Health Organization; 2010.
[18] Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25: 402-408.
[19] Wang K, Diao LH, Gong Y, Liu X and Li Y. NEMO differentially regulates TCR and TNF-alpha induced NF-kappaB pathways and has an inhibitory role in TCR-induced NF-kappaB activation. Cell Signal 2012; 24: 1556-1564.
[20] Zhou AD, Diao LT, Xu H, Xiao ZD, Li JH, Zhou H and Qu LH. beta-Catenin/LEF1 transactivates the microRNA-371-373 cluster that modulates the Wnt/beta-catenin-signaling pathway. Oncogene 2012; 31: 2968-2978.
[21] Sonderegger S, Husslein H, Leisser C and Knofler M. Complex expression pattern of Wnt ligands and frizzled receptors in human placenta and its trophoblast subtypes. Placenta 2007; 28 Suppl A: S97-102.
[22] Burton GJ, Jauniaux E and Charnock-Jones DS. Human early placental development: potential roles of the endometrial glands. Placenta 2007; 28 Suppl A: S64-69.
[23] Burton GJ, Jauniaux E and Charnock-Jones DS. The influence of the intrauterine environment on human placental development. Int J Dev Biol 2010; 54: 303-312.
[24] Hempstock J, Cindrova-Davies T, Jauniaux E and Burton GJ. Endometrial glands as a source of nutrients, growth factors and cytokines during the first trimester of human pregnancy: a morphological and immunohistochemical study. Reprod Biol Endocrinol 2004; 2: 58.
[25] Amirchaghmaghi E, Rezaei A, Moini A, Roghaei MA, Hafezi M and Aflatoonian R. Gene expression analysis of VEGF and its receptors and assessment of its serum level in unexplained recurrent spontaneous abortion. Cell J 2015; 16: 538-545.
[26] Bischof P, Meisser A and Campana A. Paracrine and autocrine regulators of trophoblast invasion. Placenta 2000; 21 Suppl A: S55-60.
[27] Wang K, Li N, Yeung CH, Li JY, Wang HY and Cooper TG. Oncogenic Wnt/beta-catenin signalling pathways in the cancer-resistant epididymis have implications for cancer research. Mol Hum Reprod 2013; 19: 57-71.
[28] Sonderegger S, Haslinger P, Sabri A, Leisser C, Otten JV, Fiala C and Knofler M. Wingless (Wnt)-3A induces trophoblast migration and matrix metalloproteinase-2 secretion through canonical Wnt signaling and protein kinase B/AKT activation. Endocrinology 2009; 151: 211-220.
[29] Cao R and Zhang Y. The functions of E(Z)/EZH2-mediated methylation of lysine 27 in histone H3. Curr Opin Genet Dev 2004; 14: 155-164.
[30] Jiang W, Wang J and Zhang Y. Histone H3K27me3 demethylases KDM6A and KDM6B modulate definitive endoderm differentiation from human ESCs by regulating WNT signaling pathway. Cell Res 2013; 23: 122-130.