Decreased expression of WNT2 in villi of unexplained recurrent spontaneous abortion patients may cause trophoblast cell dysfunction via downregulated Wnt/β-catenin signaling pathway

Ning Li¹, Shuhong Li², Yanwei Wang¹, Jiahui Wang¹, Kai Wang³, Xin Liu¹, Yan Li⁴ and Juan Liu¹*

¹ Central Laboratory, the Affiliated Yantai Yuhuangding Hospital of Qingdao University, Yantai, Shandong, China
² Department of Obstetrics and Gynecology, the Affiliated Yantai Yuhuangding Hospital of Qingdao University, Yantai, Shandong, China
³ School of Agriculture, Ludong University, Yantai, Shandong, China
⁴ College of Life Science, Yantai University, Yantai, Shandong, China

Abstract

WNT2 has been reported to be important for placental development, especially for the proper vascularization of the placenta. However, its precise role in first-trimester trophoblast cells is still unknown. WNT2 expression in the villous tissues of unexplained recurrent spontaneous abortion (URSA) patients was compared with that of healthy women by Western blot. The function of WNT2 in HTR-8/SVneo trophoblast cells was evaluated by altering the cellular WNT2 level through overexpression and shRNA knockdown. The molecular mechanism of the effect of WNT2 on trophoblast cells was investigated. The association of WNT2 with the Wnt/β-catenin signaling pathway was studied through Western blot and immunofluorescence. Results showed that WNT2 protein expression was significantly decreased in villi of the URSA group compared with the control group. In vitro studies showed that WNT2 could promote human trophoblast cell proliferation and migration through activating the Wnt/β-catenin signaling pathway. Moreover, upon the knockdown of WNT2, trophoblast cell proliferation and migration were significantly suppressed. In conclusion, our study indicated that WNT2 plays an important role in trophoblast function. WNT2 insufficiency might cause impaired trophoblast cell proliferation and migration via downregulation of Wnt/β-catenin signaling pathway.

Keywords: HTR-8/SVneo; migration; proliferation; unexplained recurrent spontaneous abortion; Wnt/β-catenin signaling pathway

Introduction

Recurrent spontaneous abortion (RSA) refers to two or more consecutive miscarriages before 20 weeks gestation with the same partner (Zong et al., 2016). It is estimated that RSA affects approximately 1–5% women at the reproductive age around the world (Zong et al., 2016). The etiology of RSA is extremely complex (Rai and Regan, 2006). Although many etiological factors have been considered as causes of RSA, including infection, chromosomal abnormality, anatomic deformation, endocrine, metabolic, and autoimmune diseases, approximately 50% of cases have an unknown cause and are referred to as unexplained recurrent spontaneous abortion (URSA) (Li et al., 2002). It is well known that trophoblast cells are essential to both placental and fetal development (Pollheimer et al., 2006). Defects in trophoblast cell function have been associated with pregnancy-related complications such as URSA, intrauterine growth retardation, and pre-eclampsia (Ferreira et al., 2011; Pennington et al., 2012; Zong et al., 2016). Recent evidence suggested that Wingless (Wnt) signaling is critical for physiological processes of human trophoblasts and might be involved in the pathogenesis of URSA (Knofler and Pollheimer, 2013; Li et al., 2015).

The Wnt signaling pathway is a highly conserved signal transduction pathway and plays important roles in cell
proliferation, differentiation, apoptosis, and migration (Peifer and Polakis, 2000). Wnt signals transduce through at least three distinct pathways, commonly referred to as the canonical Wnt/β-catenin, the Wnt/Ca\(^{2+}\) and the planar cell polarity pathway (Clevers, 2006). The canonical Wnt/β-catenin pathway has been extensively studied and has intricate functions in reproduction (Sonderregger et al., 2010b). Signaling through this pathway is initiated by the binding of a typical Wnt ligand to a Frizzled (Fzd) receptor and lipoprotein receptor-related protein (LRP) coreceptor, which ultimately leads to the accumulation and translocation of β-catenin to the nucleus followed by the activation of target genes which promote cell cycle progression, differentiation, and migration such as CyclinD1, c-Myc, c-Jun, and matrix metalloproteinases (MMPs) (Gordon and Nusse, 2006). So far, 14 of 19 Wnt ligands and 8 of 10 Fzd receptors have been detected in placenta, suggesting that Wnt signaling may control trophoblast function (Sonderregger et al., 2007). It has been reported that recombinant Wnt ligand Wnt-3A can enhance migration and invasion of trophoblast cells and first-trimester cytotrophoblasts (CTBs) via activating the canonical Wnt/β-catenin pathway (Sonderregger et al., 2010a). Furthermore, recent studies of our group revealed that expression of β-catenin was significantly decreased in villi and deciduas of URSA patients compared to that of healthy women, indicating that downregulated Wnt/β-catenin signaling in URSA might be associated with the inhibited trophoblast proliferation (Li et al., 2015).

Secreted glycoprotein WNT2, one of the canonical Wnt ligands (Jung et al., 2015), has been reported to play an important role in placental development (Ferreira et al., 2011; Zhang et al., 2013). Previous studies in mice showed that Wnt2 gene is most highly expressed during early placental development, and homozygous mutation of the Wnt2 gene in mice resulted in a number of placentation defects such as oedema, decreased numbers of capillaries and fibrinoid deposition (Monkley et al., 1996). In human placenta, WNT2 can be detected at all stages of placenta development (Sonderregger et al., 2007). Moreover, the decreased expression of WNT2 in human third trimester placenta might be associated with the pathogenesis of severe preeclampsia. Recent studies suggested that reduced WNT2 expression in placenta caused by high WNT2 promoter methylation (WNT2PrMe) was associated with low birthweight percentile in the neonate (Ferreira et al., 2011). However, to date, the expression level of WNT2 in normal pregnant women and URSA patients has not been elucidated. The precise role of WNT2 in first-trimester trophoblast cells remains unknown.

In the present study, the expression of WNT2 in first-trimester villi of normal pregnant women and URSA patients was examined. The function of WNT2 in first-trimester trophoblast cells was also studied in vitro. We demonstrated that the expression of WNT2 is lower in villi of URSA patients than in normal pregnant women, and that WNT2 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

All the participants were recruited at the Department of Obstetrics and Gynecology, the Affiliated Yantai Yuhuangding Hospital of Qingdao University. A total of 30 outpatients who had experienced at least two consecutive first-trimester miscarriages of unexplained etiology were enrolled in this study. The karyotypes of both the patient and their male partners were normal. Patients were excluded if they had any infectious, metabolic, anatomic, endocrine, or autoimmune diseases. Moreover, conceptuses with chromosomal abnormalities were also excluded. In this study, all the URSA patients underwent surgical abortion at hospital after the failed pregnancy was confirmed by ultrasound. The villous tissues of URSA patients were obtained after surgical abortion. Another group of 30 randomly selected healthy women undergoing a legal termination of apparently normal early pregnancies at the same facility were recruited as controls, with inclusion criteria described as before (Li et al., 2015). In all 30 normal cases, fetal heart activity had been identified within 2 weeks before sample collection. There was no significant difference in age or gestation between the URSA and control groups (Table 1). All procedures in this study were approved by the ethics review board of Yantai Yuhuangding Hospital. Moreover, informed consent was acquired from all participants. Freshly obtained villous

### Table 1: Characteristics of URSA and normal control women (mean ± SE and range of values).

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Age (years)</th>
<th>No. of miscarriages</th>
<th>Gestational age (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>URSA</td>
<td>30</td>
<td>30 ± 0.52 (24–35)</td>
<td>2.45 ± 0.09 (2–4)</td>
<td>8.55 ± 0.18 (7–12)</td>
</tr>
<tr>
<td>Normal</td>
<td>30</td>
<td>29.5 ± 0.50 (25–36)</td>
<td>0</td>
<td>8.20 ± 0.11 (7–9)</td>
</tr>
</tbody>
</table>

URSA versus normal controls: Age, \(P = 0.512\) (Student’s t-test). Gestational age, \(P = 0.62\) (Mann–Whitney U-test). URSA, unexplained recurrent spontaneous abortion; SE, standard error.

---

Decreased WNT2 causes trophoblast dysfunction

N. Li et al.

Tissues were immediately frozen and stored in liquid nitrogen for mRNA and protein extraction.

Western blot

Total proteins were extracted and Western blot analysis was performed as previously described (Li et al., 2015) using the following primary antibodies: anti-WNT2 (ab109222, rabbit, Abcam Inc, Cambridge, Massachusetts), anti-β-catenin (9587, rabbit, Cell Signaling Technology, Danvers, MA, USA), anti-active-β-catenin (anti-ABC, 05-665, mouse, Merck Millipore, Billerica, MA), anti-β-actin (sc-81178, mouse, Santa Cruz Biotechnology, Dallas, TX, USA) and anti-Lamin B (13435, rabbit, Cell Signaling Technology). The secondary antibodies used were HRP-conjugated goat anti-rabbit IgG or HRP-conjugated goat anti-mouse IgG (ZB-2301, ZB-2305, 1:5000, ZhongShan Goldenbridge Biotechnology, Beijing, China). Normal rabbit or mouse IgG (ZDR-5003 and ZDR-5006, ZhongShan Goldenbridge Biotechnology, Beijing, China) were used as negative control. Densitometric analysis was performed by analyzing the gray level intensity of target bands derived from scanned films, processed using ImageJ.

Immunohistochemistry and quantification

Immunohistochemistry was performed as described previously (Li et al., 2015). Dilutions of primary and secondary antibodies were anti-WNT2 1:50 and horseradish peroxidase-conjugated goat anti-rabbit IgG 1:5000. The rabbit IgG was used as negative control to demonstrate the staining specificity. Sections were examined with bright-field microscopy (DM LB2, Leica, Nussloch, Germany).

The sections were blind evaluated by two independent pathologists. The immunohistochemical staining of WNT2 was graded on a semiquantitative scale. Briefly, staining intensities were documented according to the following categories: 0, no staining/no color; 1, weak staining/pale brown color; 2, distinct staining/dark brown color; 3, strong staining/brownish-black color.

Cell culture and lentivirus transduction

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% CO2 humidified air according to standard procedures.

The knockdown of WNT2 gene in the HTR-8/SVneo cells was performed using a short hairpin RNA (shRNA) lentiviral vector (Abmgood Inc., Nanjing, China) according to the manufacturer’s protocol. Briefly, cells were seeded at a density of 5 × 10^5 cells per well in 6-well plates. When the cells reached 70% confluency, the cells were infected with a WNT2-specific shRNA virus that included shWNT2-1 and shWNT2-2 or a non-specific control shRNA. Stably transduced cells were selected with puromycin and successful knockdown of WNT2 was confirmed by Western blot. The RNA interference sequences against WNT2 were as follows: WNT2-si1: 5’-GCAAAGGAAAGGAAAGGAA-3’; WNT2-si2 5’-GATCCAAAGGAAAGGAA-3’. Scrambled nucleotides (5’-CGCTAATTCGACTCGGATA-3’) were used as negative control.

WNT2-overexpressing HTR-8/SVneo cells were generated by infecting with the recombinant lentiviruses carrying the WNT2 coding sequence (Abmgoodchina Inc., Nanjing, China). The lentivirus transduction was carried out as described above. The HTR-8/SVneo cells infected with the recombinant lentiviruses carrying the empty vector were defined as negative control cells. Stably infected cells were selected with puromycin and successful overexpression of WNT2 was confirmed by Western blot.

RNA isolation and quantitative PCR

RNA isolation and quantitative PCR (qPCR) analysis was performed as previously described (Li et al., 2015). The relative expression levels of downstream targets of Wnt/β-catenin in HTR-8/SVneo cells were calculated using the 2^-ΔΔCt method (Livak and Schmittgen, 2001). All experiments were performed in triplicate. The primer sequences are presented in Table 2. The average level of MMP-2, MMP-9, c-Myc, and CyclinD1 mRNA expression in negative control cells was set at 1.0.

Cell proliferation assay

The effect of WNT2 protein on proliferation of trophoblast cells was measured using 3-(4,5-dimethylthiazol-2-yl)-2,
5-diphenyl-tetrazolium bromide (MTT) colorimetric assay. Cells were seeded in 96-well plates at a density of $3 \times 10^5$ per well and cultured in 180 μL DMEM/F12 with 10% FBS until 60% confluency. Subsequently, the normal culture medium was replaced with DMEM/F12 containing 3% FBS. On each day, MTT (0.5 mg/mL final concentration) 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

The migration ability of trophoblast cells was evaluated through wound healing assay. Cells were seeded into the 6-well plates at a density of $5 \times 10^5$ 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. 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. (2012). Briefly, collected HTR-8/SVneo cells were resuspended in hypotonic buffer (42 mM KCl, 10 mM Hepes pH 7.4, 5 mM MgCl₂, 10 μg/mL each aprotinin and leupeptin). Nuclei homogenates were removed by centrifugation at 14000g for 10 min at 4°C.

Immunofluorescence

HTR-8/SVneo cells were cultured on microscope cover glasses (Fisherbrand, USA) until 60% confluency. Then the cells were subsequently cultured in medium with 200 ng/mL recombinant WNT2 (rWNT2, Abnova, Taiwan) or 30 μM LiCl (Sigma, USA) which was used as a positive control (Zhou et al., 2012), respectively. Untreated HTR-8/SVneo cells were used as negative control for treatment. After 30 h incubation, the cells were fixed in 4% paraformaldehyde for 15 min at RT 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 antibodies (9587, rabbit, Cell Signaling Technology, Danvers, MA, USA) at 1:50 dilution for 1 h at 4°C overnight. After washes in PBS, cells were incubated with FITC-labeled goat anti-rabbit secondary antibodies (ZSGB-BIO, Bejing, China) at 1:200 dilution at room temperature. Negative controls for staining were processed in the same manner except primary antibodies were replaced by normal rabbit IgG. 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. Significant differences were analyzed using Student’s t-test and Mann–Whitney U-test.
(for parametric data) or Mann-Whitney U-test (for nonparametric data). Mean ± 2SE of the reference value was considered as the cut-off value for classification as lower or higher than the reference value. Frequencies between the two groups were compared using Fisher’s exact test. Differences were considered to be statistically significant when \( P < 0.05 \).

Results

Expression of WNT2 and active \( \beta \)-catenin in villous tissues of normal and URSA group

The expression levels of WNT2 and active \( \beta \)-catenin in villous tissues from 30 normal pregnant women and 30 URSA patients were detected by Western blot (Figure 1A). Quantification analysis showed that WNT2 protein expression was decreased in villous tissues of URSA patients compared to normal pregnant women (Mann–Whitney U-test, \( P < 0.001 \)) (Figure 1B). In 70% of the URSA women the WNT2 expression levels were at least 2SE lower than those from normal pregnant women in villous tissues (Mann–Whitney U-test, \( P < 0.001 \)) (Figure 1B). Moreover, the expression of active \( \beta \)-catenin was significantly decreased in villous tissues of URSA patients compared to normal pregnant women (Fisher’s exact test, \( P < 0.001 \)) (Figure 1B). The proportion of samples in villous tissues of URSA women with active \( \beta \)-catenin levels at least 2SE lower than those in normal pregnant women was 86.67% (Fisher’s exact test, \( P < 0.001 \)) (Figure 1B).

![Figure 2](image)  
**Figure 2** Representative immunostaining of WNT2 in villi of normal and URSA groups. WNT2 was mainly localized in villous cytotrophoblast and syncytium. The immunostaining of WNT2 were reduced in villi of URSA group (C and D) compared to the normal group (A and B). E and F, negative controls with rabbit IgG instead of primary antibody. S, syncytium; vCTB, villous cytotrophoblast. (A,C,E) Scale bar 100 \( \mu \)m. (B,D,F) Scale bar 50 \( \mu \)m.
Localization of WNT2 protein in the villi of normal and URSA group

Immunohistochemical staining showed that WNT2 could be detected in villous cytotrophoblast and syncytium (Figure 2). Moreover, WNT2 showed a stronger staining intensity in the control group (Figures 2A and 2B) than in the URSA group (Figures 2C and 2D). Table 3 summarized the results which were categorized by the intensity of immunostaining. The staining intensity of WNT2 in villi tissues of the URSA group was weaker than the normal controls (Fisher’s exact test, $P < 0.001$).

Knockdown of WNT2 suppressed trophoblast cell proliferation and migration

We evaluated the effect of knocking down of endogenous WNT2 expression by shRNA in HTR-8/SVneo human trophoblast cells (Figures 3A to 3C). Western blot analysis showed that the relative intensity of WNT2 expression to $\beta$-actin was significantly lower in cells transfected with shWNT2-1 or shWNT2-2 compared to control shRNA (shCtrl) (Figure 3A). Proliferation (Figure 3B) and migration (Figure 3C) assays demonstrated that shWNT2-2 significantly suppressed cell proliferation and migration compared to shCtrl (both $P < 0.05$). These results suggest that WNT2 plays a crucial role in trophoblast cell function and that its reduction leads to impaired cell proliferation and migration.
villous trophoblasts cells. The effect of WNT2 knockdown was verified by Western blot (Figure 3A). WNT2 expression was significantly decreased in HTR-8/SVneo cells transfected with both knockdown vectors (shWNT2-1 and shWNT2-2) compared to the negative control stable cell line (shCtrl). Knockdown of WNT2 resulted in significant inhibition of HTR-8/SVneo cell proliferation and migration compared with the control group (Figures 3B and 3C).

**WNT2 overexpression promoted trophoblast cell proliferation and migration in vitro**

To determine the role of WNT2 in trophoblast cell proliferation and migration, we stably transduced WNT2 overexpression plasmids into HTR-8/SVneo. The overexpression of WNT2 in HTR-8/SVneo cells was confirmed by Western blot (Figure 4A). Moreover, the secreted WNT2 protein could also be detected in culture medium of WNT2-overexpression cells but not in that of negative control cells (Figure 4A). In accordance with the results from WNT2 knockdown in trophoblast cells, both proliferation and migration in HTR-8/SVneo cells displayed significant increase compared to negative control cells (Figures 4B and 4C). Furthermore, the expression levels of MMP-2, MMP-9, c-Myc, CyclinD1 were upregulated in WNT2-overexpressing HTR-8/SVneo cells (Figure 4D).

**WNT2 promotes cell proliferation and migration via nuclear translocation of β-catenin**

To elucidate the underlying molecular mechanism by which WNT2 affects trophoblast proliferation and migration, the Wnt/β-catenin signalling pathway in HTR-8/SVneo cells was evaluated. Western blot revealed that rWNT2 led to...
elevated levels of both β-catenin and active β-catenin in HTR-8/SVneo cells (Figure 5A, top). Moreover, subcellular fractionation and immunofluorescence was performed after cells were cultured with rWNT2 or LiCl for 30 h to determine whether the accumulated β-catenin proteins in cytoplasm enter the nucleus. Subcellular fractionation showed that the levels of β-catenin and active β-catenin were both increased in the nucleus of rWNT2 treated cells.

**Figure 5** WNT2 promoted 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. The relative intensity of β-catenin and active β-catenin expression was shown in the right panel. The results are expressed as mean ± SE of three independent experiments (Student’s t-test; * P < 0.05). (B) Representative immunofluorescence images showing the translocation process of β-catenin from the membrane to nucleus in rWNT2 and LiCl treated cells. 30 μM LiCl treated cells were used as positive control. Antibody against β-catenin was labelled with FITC and nuclei were labelled with DAPI. Scale bars, 20 μm.
compared to those of untreated cells (Figure 5A, bottom). Immunofluorescence staining showed that besides the localization on the membrane, β-catenin could also be detected in the nucleus of the rWNT2 treated cells, demonstrating its nuclear translocation (Figure 5B). As a positive control, LiCl was found to markedly activate β-catenin expression and nuclear translocation (Figures 5A and 5B). In contrast, nuclear translocation of β-catenin could hardly be observed in the untreated cells (Figure 5B).

Discussion

Accumulating data indicated that abnormal expression of Wnt/β-catenin signaling might be associated with pathogenesis of URSA (Bao et al., 2013; Li et al., 2015). In the present study, we found that besides the decreased expression of active β-catenin, WNT2 was also significantly reduced in villi of URSA compared to that of healthy women. Immunohistochemical staining showed that WNT2 was predominantly expressed in the CTBs and the staining intensity in URSA is lower than in the control group, suggesting that WNT2 may play an important role in trophoblast function. Trophoblast cells are the most important cells in early pregnancy and are crucial to both placental and fetal development (Zong et al., 2016). Numerous autocrine factors present at the fetal-maternal interface could regulate trophoblast proliferation, differentiation, and migration (Knofler, 2010). Deranged placenta secretions could influence trophoblast function, and adversely affect pregnancy outcome, increasing maternal and fetal morbidity and mortality (Bischof et al., 2000; Zong et al., 2016). It had been reported that decreased expression of WNT2 in the villous syncytiotrophoblast and the extravillous trophoblast (EVT) of preeclampsia (PE) might be associated with the dysfunction of the trophoblast which can contribute to the pathogenesis of PE (Zhang et al., 2013). In the present study, in vitro studies also showed that HTR-8/SVneo cell proliferation and migration were significantly suppressed after knocking down WNT2 expression by shRNA. Taken together, all these results indicate that dysregulated expression of WNT2 may cause URSA through suppression of trophoblast cell proliferation and migration.

WNT2 is an important member of the Wnt family (Smolich et al., 1993). It has been reported that the expression of WNT2 is always associated with anchorage-independent cell survival, metastasis, and tumor invasion (Huang et al., 2015; Jung et al., 2015). In addition, the expression of WNT2 is implicated in activating/stabilizing β-catenin, similar to other canonical (β-catenin-mediated) Wnt ligands (Jung et al., 2015). In order to elucidate the underlying molecular mechanism by which WNT2 regulates the function of trophoblast cells, the Wnt/β-catenin signalling pathway in HTR-8/SVneo cells was characterized. Our results clearly showed that β-catenin which is a frequently used surrogate marker of Wnt pathway activation entered the nucleus when trophoblast cells were treated with rWNT2 (Wang et al., 2013), suggesting that the presence of abundant WNT2 in growth media successfully activated the canonical Wnt signalling pathway. Moreover, the transcription levels of Wnt target genes, MMP-2 and MMP-9, were increased in WNT2-overexpression cells. MMP-2 and MMP-9 have been reported to be the critical proteases involved in trophoblast migration (Bischof et al., 2000; Sonderegger et al., 2010a). Previous studies suggested that induction of the MMP-2 could be one of the critical mechanisms promoting WNT3-dependent trophoblast migration (Sonderegger et al., 2010a). We speculated that WNT2 might promote trophoblast migration through similar mechanism.

In our study, we demonstrated that WNT2 protein expression was decreased in villi of URSA, while the cause of reduced WNT2 gene expression in URSA was not investigated. It has been reported that methylation of promoter CpG sites is usually associated with a decreased expression of the gene (Costello and Vertino, 2002). A recent study showed that high WNT2 promoter methylation (WNT2PrMe) in post-delivery placenta is associated with reduced expression of the WNT2 gene, indicating that high WNT2PrMe could be a risk factor for low birth weight percentile in the neonate (Ferreira et al., 2011). Whether the cause of reduced WNT2 gene expression in URSA is also associated with DNA methylation needs further study.

Conclusion

In conclusion, our study demonstrated that the expression of WNT2 is lower in villi of URSA patients than in normal pregnant women. In vitro studies showed that WNT2 protein could promote the proliferation and migration of first-trimester trophoblast cells via activating the canonical Wnt/β-catenin pathway. Moreover, WNT2 insufficiency might lead to impaired trophoblast cell proliferation and migration. Our findings revealed a mechanism by which trophoblast function may be regulated and that may contribute to the pathogenesis of URSA. One limitation for this study is that the villous tissues from URSA would not be as fresh as those from healthy pregnant women, because the villous tissues would have been in distress due to the failed pregnancy. Further study into promoter methylation of WNT2 in villous tissues of URSA will help us to understand the precise molecular mechanisms of URSA pathogenesis.

Acknowledgments and funding

This work was supported by the National Natural Science Foundation of China (Grant no. 81501313; 81571490; 81670874; 81601291).
81302233), Shandong Provincial Natural Science Foundation, China (Grant no. ZR2015HQ031; ZR2014HQ068; ZR2016HL05) and Yantai Science and Technology Program (Grant no. 2015WS024; 2015WS019; 2016WS002; 2016WS012).

**Declaration of conflicting interests**

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**References**


Received 26 March 2017; accepted 13 June 2017.

Final version published online