Wnt6 Is Essential for Stromal Cell Proliferation During Decidualization in Mice

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ABSTRACT

Postimplantation uterine development involves extensive stromal cell proliferation and decidual transformation with polyploidization, which is essential for normal pregnancy establishment. However, it remains largely unknown how stromal cell proliferation versus decidual polyploidization is differentially regulated during decidualization. Utilizing Wnt6-mutant mice, we show here that Wnt6 deficiency impairs stromal cell proliferation without much adverse effects on decidual polyploidization. Applying a primary stromal cell culture model, we further reveal that loss of Wnt6 prolongs the cell cycle length via downregulating cyclin B1 expression, thus attenuating stromal cell proliferation. Our study provides the first genetic evidence that Wnt6 is critical for normal stromal cell proliferation in mice, highlighting the concept that there are differential machineries governing the process of stromal cell proliferation versus decidual transformation during early pregnancy. This finding has high clinical relevance because Wnt signaling is known to be important for human implantation and endometrial function.

decidualization, proliferation, stromal cell, Wnt6

INTRODUCTION

Successful implantation requires reciprocal interactions between the implantation-competent blastocyst and the receptive uterus [1, 2]. In mice, the onset of blastocyst attachment with the uterine luminal epithelium occurs in the evening of Day 4 of pregnancy (Day 1 = vaginal plug) [3]. With the initiation of attachment, endometrial stromal cells surrounding implanting blastocysts undergo decidual transformation (i.e., decidualization). The progression of decidualization, from the antimesometrial pole on later Day 5 to the mesometrial pole on Day 7 and thereafter, orients the shape of implantation chambers in an antimesometrial-mesometrial direction, accommodating the growing embryo. In both mice and humans, stromal-decidual transformation eventually embeds the embryo into the antimesometrial endometrial bed. Because decidualization involves dynamic tissue remodeling, Wnt proteins, as secreted signaling molecules linked to morphogenic events [4, 5], are likely to participate in this process.

In fact, recent studies have provided evidence showing that Wnt signaling is a potentially important player during implantation and decidualization [6, 7]. For example, the canonical Wnt-β-catenin pathway is dynamically activated in the uterine compartments in close association with embryo implantation and decidualization [8–11]. We have also recently demonstrated that silencing of Wnt-β-catenin signaling in mice does not adversely affect the uterine preparation for receptivity but, remarkably, blocks blastocyst competency to implantation [12]. Moreover, uterine conditional deletion of β-catenin or constitutive stabilizing β-catenin derails the normal implantation and decidualization [13], while uterine selective depletion of Wnt4 also results in implantation and decidualization failure in mice [14]. In humans, Dickkopf-1 (DKK-1), a secreted inhibitor of canonical Wnt signaling, is upregulated by progesterone during endometrial stromal cell decidualization [15, 16], whereas an aberrant expression of DKK-1 is associated with impaired implantation in women [17, 18]. Moreover, WNT5A is involved in mediating the progesterone functions during the progression of human endometrial decidualization [19]. These findings demonstrated that Wnt-β-catenin signaling is a functional player during embryo implantation and decidualization in both mice and humans.

However, despite all abovementioned findings from gene expression and mutation studies, it remains largely unknown how dysfunctional Wnt activity impairs the initiation and progression of decidualization with respect to the underlying mechanism. Particularly, it is unclear how Wnt signaling differentially regulates stromal cell proliferation versus stromal-decidual transformation with polyploidization. Employing multiple approaches, we herein demonstrate that Wnt6 is spatiotemporally expressed in proliferating stromal cells during postimplantation uterine development. Moreover, genetic deletion of Wnt6 impairs normal stromal cell proliferation with a prolonged cell-cycle length, while its deficiency exhibits no apparent influence on decidual polyploidization. This finding provides evidences that there are different machineries governing the process of stromal cell proliferation versus decidual transformation during decidualization.

MATERIALS AND METHODS

Generation of a Wnt6-Deficient Mouse Line

To clone the mouse Wnt6 locus, a 129/Sv genomic phage library was screened using the mouse cDNA as a probe. Four phage clones were purified, and a genomic contig of 22 kbp was established by restriction mapping. A...
16kbp fragment of one phage was subcloned and modified to generate a targeting vector with a 9.5-kbp 5’ homology region and 1.6-kbp 3’ homology region flanking a floxed PGK-neobpA cassette (pMC1-neo-polA) to replace a 3.0-kbp fragment of the Wnt6 locus harboring exon 3 and 4 of the gene. The targeting vector was linearized and introduced into cells of the E14 embryonic stem (ES) cell line [20]. Three hundred and forty-two G418-resistant colonies were screened by Southern blot analysis, and seven correctly targeted ES cell lines were identified. Wnt6<sup>flu<sup>ES cells (129/Ola) were microinjected into Naval Medical Research Institute (NMRI) albino mouse blastocysts to generate chimeras. Males with high degree of chimerism were mated to NMRI females for germine transmission. F1 heterozygous males were crossed to NMRI females, and heterozygous offspring were intercrossed. Mice and embryos were genotyped by Southern blot analysis or by PCR. Wnt6 null mice backcrossed to CD1 background were housed in the animal care facility at the Institute of Zoology, Chinese Academy of Sciences, according to the institutional guidelines for the care and use of laboratory animals.

Implantation and Decidualization

Virgin female mice were mated with fertile or vasectomized males of the same strain to induce pregnancy or pseudopregnancy, respectively. Implantation sites on Day 4 at 0000 h and Days 5 and 6 of pregnancy were visualized by intravenous injections of Chicago Blue dye [21]. To induce artificial decidualization, one uterine horn of pseudopregnant mice was infused with sesame oil (25 μl) on Day 4. Mice were euthanized 4 days after the oil infusion. The weight of infused and noninfused (control) uterine horns were recorded, and fold increases in uterine weights served as an index of decidualization [22]. To analyze cell proliferation, mice received intraperitoneal injection of 0.3 mg/10 g body weight bromodeoxyuridine (BrdU) solution 20 h before being euthanized.

Uterine Stromal Cell Culture

Uterine stromal cells were isolated and cultured as previously described with some modifications [23, 24]. Three to four pseudopregnant Day 4 mouse uterine horns were cut into small pieces (2–3 mm). Tissue pieces were first digested in 3 ml fresh medium (HBSS +antibiotic; Gibco) containing 6 mg/ml dispase (Gibco) and 25 mg/ml pancreatic (Sigma), and then incubated in fresh medium (3 ml) containing 0.5 mg/ml collagenase (Sigma) at 37°C for 30 min. The digested cells were passed through a 70-μm filter to obtain the stromal cells. Cells were plated at 60-mm dishes and 96-wells plates, containing phenol red-free Dulbecco modified Eagle medium (DMEM) and Ham F-12 nutrient mixture (1:1) (Gibco) with 10% charcoal-stripped fetal bovine serum (CS-FBS) and antibiotic. After 1 h, the medium was replaced with fresh medium (DMEM/F-12, 1:1) without CS-FBS for serum starvation experiments or with fresh medium (DMEM/F-12, 1:1) with 10% FBS for the proliferation assay. After starvation, the cells were cultured in proliferation medium (DMEM/F12, 1:1, with 1% CS-FBS). Cells were collected for flow cytometry or protein extraction. Proliferation assays were performed using MTS reagent (Promega) according to the manufacturer’s directions. Stromal cells were seeded on 96-well plates (4 × 10<sup>4</sup> cells/well), and cell numbers were analyzed 0, 24, 48, 72, and 96 h postaddition of MTS. The experiments were repeated three times. Immunostaining of cytokeratin (1:100; DakoCytomation) and vimentin (1:50; Santa Cruz Biotechnology, Inc.) revealed that primary stromal cells isolated exhibited nearly 97% purity.

Quantitative Real-Time PCR

A total of 1–3 μg RNA was used to synthesize cDNA. The expression levels of different genes were validated by real-time reverse transcription-polymerase chain reaction (RT-PCR) TaqMan analysis using the ABI 7500 sequence detector system according to manufacturer’s instructions (Applied Biosystems). All the primers for real-time PCR are listed in Table S1 (all the Supplemental Data are available online at www.biolreprod.org). All the real-time PCR experiments were repeated at least three times.

In Situ Hybridization

In situ hybridization was performed as previously described [3]. Briefly, frozen sections (10 μm) were mounted onto poly-L-lysine-coated slides and fixed in 4% paraformaldehyde solution in PBS at 4°C. After prehybridization, sections were hybridized at 45°C for 4 h in 50% formamide buffer containing <sup>35</sup>S-labeled sense or antisense cRNA probes. After hybridization, sections were incubated with RNase A (20 μg/ml) at 37°C for 20 min, and RNase A-resistant hybrids were detected by autoradiography using liquid emulsion. Sections hybridized with the sense probes served as negative controls.

Flow Cytometry

Flow cytometric analysis was performed as previously described [24, 25]. Cultured stromal cells or Day 7 decidual cells were digested and harvested. After centrifugation (500 g, 4°C, 5 min), the cell pellet was suspended in 0.25 ml PBS; 1 ml of –20°C cold 80% ethanol was added dropwise under constant, gentle vortexing. Samples were incubated for 30 min on ice and subsequently overnight at –20°C before subjected to staining. Cell suspensions were removed from –20°C storage and allowed to adapt to room temperature (RT) for about 10 min. After centrifugation (500 × g, RT, 5 min), pellets were resuspended in 1 ml PBS (RT) and pelleted again (500 × g, RT, 5 min). Cell sediments were suspended in an appropriate volume (0.5 ml) of staining solution (PBS containing 30 mg/ml propidium iodide (PI) and 0.3 mg/ml DNase-free RNase A) that saturated all the DNA with PI. Samples were incubated overnight at 4°C or for 30 min at 37°C in the dark. They were then returned to RT and subjected to flow cytometry. The experiments were repeated three times.
Immunohistochemistry

Implantation sites were fixed in 10% neutral buffer formalin and then embedded in paraffin. Tissue sections (5 μm) were deparaffinized, rehydrated, and incubated with rabbit anti-phospho-histone H3 (pH3) (1:200; Cell Signaling) and rat anti-BrdU (1:200; Abcam) antibodies. After staining, sections were counterstained with hematoxylin. For detecting the alkaline phosphatase activity in implantation sites, frozen tissue sections (10 μm) were used. The slides were fixed in cold acetone for 15 min and rinsed three times in PBS. The BCIP/NBT (5-bromo-4-chloro-3-indolylphosphate toluidinium and nitro blue tetrazolium) kit was used for staining according to the manufacturer’s protocol (Zhongshan Golden Bridge Biotechnology Co., Ltd.). The sections were then incubated in buffered BCIP/NBT Tris-HCl solution for 5 min at RT. The deep blue color indicated the activity of alkaline phosphatase.

Western Blot Analysis

Protein extraction and Western blot analysis were performed as described previously [26]. Cultured stromal cells were lysed in 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, and a protease inhibitor cocktail (Roche). The samples were kept on ice for 30 min and then centrifuged at 13,000 rpm for 30 min at 4°C. The concentration of protein was detected with a bicinchoninic acid protein assay kit according to the manufacturer’s instructions (Beyotime Institute of Biotechnology). Proteins were subjected to 10% polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene fluoride membrane (Millipore). After the transfer, membranes were blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for 2 h at RT, and incubated overnight with primary antibodies in TBST. Antibodies to cyclin B1 (1:1000; Abcam) and β-actin (1:3000, AC-74; Sigma) were used. After incubation with the primary
antibodies, the membranes were washed in TBST three times and then incubated with their specific secondary antibodies (1:3000; Zhongshan Golden Bridge Biotechnology Co., Ltd.) in 5% silk milk for 2 h at RT. Bands were visualized using Thermo Supersignal West Pico chemiluminescent substrate according to the manufacturer’s instructions. The relative band intensity was acquired by using Quantity One software.

**Statistical Analysis**

Statistical analysis was performed with the SPSS11.5 program. Comparison of means was performed using the independent-samples Student t-test. The data are shown as means ± SEM.
RESULTS

Wnt6 Is Spatiotemporally Expressed in Proliferating Stromal Cells During Postimplantation Uterine Development

Previous reports have demonstrated that many Wnt genes, such as Wnt4, Wnt5a, Wnt7a, Wnt7b, Wnt11, and Wnt16 were expressed in an implantation-specific manner in mouse uteri [8, 9, 11], but the spatiotemporal expression of Wnt6 during embryo implantation and decidualization remained unknown. Using quantitative RT-PCR and in situ hybridization analyses, we observed that Wnt6 was dynamically expressed in the uterus during postimplantation development on Days 5–8 of pregnancy (Fig. 1, A and B). For example, while Wnt6 was expressed at low levels in the uterus on Days 1–4, its expression exhibited a dramatic upregulation in stromal cells surrounding the implanting blastocyst on Day 5 (Fig. 1B). Moreover, following the initiation of decidualization, Wnt6 transcript was intensely detected in proliferating stromal cells in the secondary decidual zone on Day 6 (Fig. 1B). When the pregnancy proceeds to Day 8, Wnt6 expression shifted from the antimesometrial stroma to the mesometrial region (Fig. 1B), which correlated well with the shift of stromal cell proliferation activity during the same periods as labeled by BrdU staining (Supplemental Fig. S1). This spatiotemporal expression pattern of Wnt6 on Days 5–8 of pregnancy motivated us to surmise that Wnt6 could be a critical regulator of stromal cell proliferation during decidualization. We, thus, generated a Wnt6 null mutant mouse line and subsequently explored the consequence of Wnt6 deficiency on embryo implantation and decidualization.

FIG. 5. Connexin43 and Bmp2 are expressed normally in WT and Wnt6 null uteri during decidualization. A) In situ hybridization of Connexin43 and Bmp2 mRNAs in the WT and Wnt6<sup>−/−</sup> implantation sites on Days 7–8 of pregnancy (bar = 1000 μm). M, mesometrial pole; AM, antimesometrial pole; sdz, secondary decidual zone; em, embryo. B) Quantitative RT-PCR analysis reveals a comparable expression level of Connexin43 and Bmp2 mRNAs in WT and Wnt6<sup>−/−</sup> implantation sites on Days 6–8 of pregnancy. Messenger RNA quantities are normalized against GAPDH. Experiments were repeated three times. Data are shown as means ± SEM.

Wnt6 IS ESSENTIAL FOR DECIDUALIZATION
Decidualization Is Defective in Wnt6−/− Mice

To assess the physiological relevance of Wnt6 in implantation and decidualization, we first examined pregnancy outcome in Wnt6+/− females crossed with the same background strain of wild-type (WT) males. As illustrated in Figure 2A, mice lacking Wnt6 showed compromised term pregnancy with substantially reduced litter size compared with WT littermates, suggesting that Wnt6 signaling is crucial for normal pregnancy. In addition, no parturition defects were observed in the Wnt6 mutant female (data not shown). Because Wnt6 is extensively expressed in stromal cells surrounding the implanting blastocyst (Fig. 1B), we then analyzed the window of embryo implantation in Wnt6+/− females. As shown in Figure 2B, normal initial embryo-uterine attachment reaction was observed in Wnt6+/− mice when examined on the morning of Day 5. Moreover, the morphology of embryo-uterine attachment was normal in Wnt6+/− females (data not shown). This finding was further confirmed by observations of comparable expression of implantation marker genes Cox2, Hbegf, and Bmp2 (bone morphogenetic protein 2) in Wnt6+/− and WT attachment sites (Fig. 2C), suggesting that Wnt6 is dispensable for the initiation of embryo implantation. However, we noted that Wnt6 deficiency greatly hampered the process of decidualization. A significant decrease of weight and size of implantation sites were observed in mice lacking Wnt6 on Days 7–8 of pregnancy (Fig. 2, D and E).

Employing the oil-induced deciduoma model [22], we also observed a differential expression pattern of Wnt6 in the decidualizing stromal cells in a similar fashion as that of normal pregnancy (Fig. 3A). Moreover, while WT mouse uteri exhibited a robust decidual response 4 days after intraluminal oil injection on Day 4 of pseudopregnancy, the mutant uteri showed a remarkably reduced decidual response: among the 12 Wnt6+/− mice that we detected, six had a lower degree of decidualization and the other six lacked the decidual response (Fig. 3, B and C). These results reinforced the notion that Wnt6 is critical for normal uterine decidualization. In addition, this impaired decidualization was an intrinsic uterine defect as evidenced by a lack of an effect of Wnt6 deficiency on the expression of progesterone synthesis key enzymes, 3β-hydroxysteroid dehydrogenase (3β-HSD) and cytochrome P450 cholesterol side-chain cleavage enzyme (P450Scc), in the Wnt6+/− corpus luteum (Supplemental Fig. S2).

Wnt6 Is Not Essential for Stromal-Decidual Transformation and Polyploidization

Stromal cell differentiation into polyplody decidual cell is one of the hallmark events during postimplantation uterine development [27]. To characterize the decidual defects in Wnt6+/− females, we first performed alkaline phosphatase activity staining, a sensitive marker for differentiated decidual cells. As shown in Figure 4A, WT and Wnt6−/− decidual tissues exhibited similar alkaline phosphatase expression pattern and activity, regardless of the reduced size of the decidual zone in the absence of Wnt6. We then analyzed the status of decidual polyploidy, an indicator of terminal
differentiation of decidual cells. We also observed comparable ratio levels of polyploid decidual cells in WT and Wnt6−/− mice by flow cytometry analysis (Fig. 4B). This is consistent with normal expression of Connexin43 and Bmp2, which are known to be essential for normal decidualization [28, 29] in Wnt6−/− females on Days 7–8 of pregnancy (Fig. 5, A and B). These observations suggested that Wnt6 is dispensable for stromal-decidual transformation and polyploidization.

Wnt6 Deficiency Prolongs the Cell Cycle Length, Thus Hampering Uterine Stromal Cell Proliferation During Decidualization

The shift of Wnt6 expression from the antimesometrial pole to the mesometrial region (Fig. 1B) and the apparent normal decidual polyploidization even in the absence of Wnt6 (Fig. 4B) led us to further explore whether Wnt6 deficiency impairs stromal proliferation during decidualization. We performed BrdU incorporation and pH3 staining experiments to analyze the cell cycle status during stromal proliferation. As shown in Figure 6, A and B, Wnt6−/− uteri exhibited an increased portion of BrdU-positive cells that were in the S phase of the cell cycle and a decreased number of pH3-staining cells in the M phase of cell cycle at the mesometrial region on Days 7–8 of pregnancy. This result suggested that loss of Wnt6 attenuates the proliferation activity of stromal cells during decidualization, perhaps by altering cell cycle progression. Indeed, through successive BrdU injection, we further analyzed the cell cycle length as previously described [30, 31] and observed that loss of Wnt6 prolonged the cell cycle length by about 6 h (16.6 h in WT vs. 22.5 h in null) when analyzed on Day 7 (Fig. 6C). We subsequently examined the expression of key cell cycle regulators cyclins (Ccn) to reveal the underlying cause of this obvious deferral of cell cycle progression resulting from Wnt6 deficiency. As shown in Figure 6D, while the expressions of Ccnb1, Ccn1, Ccn3, and Ccne1 are comparable in WT and Wnt6−/− females, we noted that Ccnb1, a key G2/M checkpoint factor, was significantly downregulated. This aberrant expres-
sion may contribute to the accumulation of BrdU-positive cells in the S phase and a slow progression of cell cycle from G2 through M phase, depicting reduced number of pH3-positive cells in Wnt6−/− decidua.

Loss of Wnt6 Attenuates Stromal Cell Proliferation via Decreasing Cyclin B1 Expression in Culture

To clarify the cell cycle progression defects during stromal cell proliferation in the absence of Wnt6, we used WT and Wnt6−/− deficient primary stromal cell culture models. As shown in Figure 7A, the loss of Wnt6 attenuated stromal cell proliferation activity in culture. Pretreatment by serum starvation would induce stromal cells arrested in the G0/G1 stage of cell cycle, while synchronized cells can return to an active cell cycle after supplementation with serum. Accessing the speed of the cell cycle progression, we observed that Wnt6−/− stromal cells exhibited a slow transition of cell cycle from the G0/G1 stage into the S phase and later into the G2/M phase 12–24 h after the termination of serum starvation (Fig. 7B). As illustrated in Figure 7C, a significantly reduced ratio of Wnt6−/− stromal cells was observed in the S phase as well as G2/M stage of cell cycle in comparison with WT cells. This defective cell cycle progression was temporally associated with a postponed and largely reduced expression of cyclin B1 in mutant cells (Fig. 7D), which is similar to that observed in vivo (Fig. 6D). These results provide an additional line of evidence that Wnt6 is essential for normal stromal cell proliferation.

DISCUSSION

An appropriate endometrial decidualization is essential for normal pregnancy establishment. The process of decidualization involves extensive stromal cell proliferation and stromal-decidual transformation with polyploidization. Despite increasing knowledge of the wide range of signaling molecules involved in this process [1, 2], it has remained largely unknown whether stromal cell proliferation and decidual transformation are regulated independently during decidualization. In the present study, we provide genetic evidence showing that Wnt6 is essential for postimplantation decidualization; its deficiency leads to impaired stromal cell proliferation correlating with abnormal cell cycle gene expression.

Previous studies have revealed that Wnt signaling plays an important role in cell proliferation in various developmental and pathophysiological events [32–34]. For example, ectodermal Wnt6 could promote the proliferation of limb cells through increasing the expression of Pax-3 and Paraxis in chicken limb myogenesis [35]. It has also been demonstrated that activation of the canonical Wnt pathway facilitates cell proliferation via promoting the cell cycle G1-phase progression through its target gene cyclin D1 [36, 37]. However, cyclin D1 expression can barely be detected in uteri during decidualization [38]. In fact, cyclin D3 is the major D-type cyclin expressed in the postimplantation uterus in mice [38]. Previous studies have demonstrated that silencing of cyclin D3 can abrogate heparin-binding EGF (HB-EGF)-promoted stromal cell polyploidy [24, 27], whereas overexpression of cyclin D3 restores normal polyploidization in mice lacking death effector domain-containing protein [25]. In this respect, we observed a comparable expression of cyclin D3 in Wnt6−/− mice, which exhibit apparently normal decidual transformation, further supporting the notion that cyclin D3 is essential for normal decidual polyploidization.

By contrast, cyclin B1, a potential target of Wnt pathway in transformed tumor cells [39] is remarkably downregulated in the absence of Wnt6, resulting in a prolonged cell cycle length and attenuated stromal cell proliferation. In humans, cyclin B1 is highly expressed in the proliferative versus the secretory endometrium [40]. Moreover, high expression levels of cyclin B1 can also be observed in ectopic endometrial cells associated with abnormal cell cycle regulation of endometriosis [41], which supports our hypothesis that decreased cyclin B1 expression in Wnt6−/− mice is responsible for the abnormal stromal cell proliferation. Because it has been shown that cyclin B1-specific antibody are frequently found in healthy humans who have no history of cancer [42], our findings raise a cautionary note that this anti-cyclin B1 immunity may hamper the normal endometrial decidual response during menstrual cycle and early pregnancy in women.

In our search for the underlying mechanism of impaired stromal cell proliferation, we noted that an upregulated expression of Wnt4 could partially compensate for the loss of Wnt6 during decidualization (Wang et al., unpublished results). In fact, similar genetic compensation among the same family of signaling molecules has been observed in previous studies [43, 44]. For example, upregulation of cyclooxygenase-1 could rescue female infertility in the cyclooxygenase-2 CD1 background knockout mouse [44]. In addition, the maternal HB-EGF deficiency also could be rescued by amphiregulin to sustain pregnancy success [43]. Future investigations are warranted to identify the potential mechanisms governing Wnt4 compensation in the absence of Wnt6 during decidualization.

In summary, this study provides genetic evidences that Wnt6 plays an important role in stromal cell proliferation during decidualization. This work may be of clinical relevance because there is evidence that Wnt signaling is also dynamically involved in endometrial function in humans [15–19].

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