

Article

Expression of Wilm's Tumor Gene (*WT1*) in Endometrium with Potential Link to Gestational Vascular Transformation

Peilin Zhang 

Pathology, New York Presbyterian - Brooklyn Methodist Hospital, 506 6th St., Brooklyn, NY 11215, USA; Pez9008@NYP.org

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Abstract: Background: Wilm's tumor 1 gene (*WT1*) is a transcription factor with versatile cellular functions in embryonic development, the maintenance of adult tissue functions, and regeneration. *WT1* is known to be regulated by progesterone and it is abundantly expressed in endometrium, but its function is unclear. Design: in this observational and descriptive study, *WT1* expression was detected by immunohistochemical staining in endometrium of various physiological and pathological conditions. Result: *WT1* was detected in endometrial stromal cells and vascular smooth muscle cells, in both proliferative and secretory phases of menstrual cycles. *WT1* appeared increased in vascular smooth muscle cells in spiral artery in early pregnancy and it was also detected in regenerative endothelial cells and smooth muscle cells in decidual vasculopathy at term. *WT1* expression appeared decreased in endometrial stromal cells in adenomyosis (endometriosis). Conclusion: *WT1* potentially links the hormonal effects on endometrial decidualization and may play a role in gestational vascular transformation during pregnancy and restoration after pregnancy.

Keywords: *WT1* gene; endometrium; decidual vasculopathy; spiral artery remodeling; regeneration; steroid hormones

1. Introduction

Endometrial decidualization is characterized by the morphological transformation of epithelium, endometrial stromal tissue and terminal segments of the spiral artery, through the concerted action of steroid hormones (estrogen and progesterone), secreted by the ovary and subsequently corpus luteum after ovulation [1,2]. Embryonic implantation occurs in a narrow window of time after fertilization and the fertilized embryo attaches and invades into endometrial tissue, through complex interactions between the developing trophoblast and the decidualized endometrium [2]. Extravillous trophoblasts derived from the early trophoblast invade the decidualized endometrial stromal tissue and the spiral artery, leading to trophoblast-dependent spiral artery remodeling, by forming the endovascular trophoblastic “plugs” with concurrent replacement of smooth muscle wall and endothelium [3]. Uterine natural killer cells (NK) play critical roles in trophoblast-dependent spiral artery remodeling and the formation of endovascular trophoblastic “plugs” is associated with phenotypic switch of the endovascular trophoblasts to express CD56, a defining molecular marker for NK cell lineage [4–8]. Meanwhile, endometrial spiral artery is undergoing trophoblast-independent remodeling morphologically characterized by arterial wall hypertrophy/hyperplasia (mural hypertrophy/hyperplasia) and endothelial vacuolization, and this morphological transformation occurs at a distance from the extravillous trophoblasts, without direct trophoblastic interaction [9]. The molecular mechanism of trophoblast-independent remodeling of spiral artery (mural hypertrophy/hyperplasia) is likely through endocrine (hormonal) actions, but the precise hormonal action on the smooth muscle cells of the vascular wall is poorly understood. During endometrial

decidualization and subsequent embryonic/blastocyst implantation, estrogen, progesterone and human chorionic gonadotrophin (hCG) play critical roles in decidual, trophoblastic and fetal development, and the maintenance of pregnancy in the first trimester. However, the role of these steroid and peptide hormones on spiral artery remodeling is yet to be defined [2].

Wilm's tumor 1 gene (*WT1*) is a tumor suppressor gene discovered in patients of Wilm's tumor, a high grade pediatric malignancy with versatile molecular functions [10–15]. *WT1* is a transcription factor regulating a large number of target gene expressions, mediating a variety of cellular functions in organ development, homeostasis and diseases [16,17]. Mutations of *WT1* gene were shown to be associated with Denys–Drash syndrome and Frasier syndrome, and deletion of the short arm of the chromosome 11 containing *WT1* gene is associated with WAGR syndrome (Wilm's tumor, aniridia, genitourinary anomalies, and mental retardation) [15,18,19]. *WT1* is interacting with many different cellular partner proteins important for various cellular activities [10,15]. Normal *WT1* gene function is critical for the development of urogenital system, mesothelial cell function and vascular endothelial cell functions [15]. Clinically, *WT1* gene is also important for adult kidney function and cardiovascular functions [15,20]. In pathology practice, immunohistochemical staining for *WT1* serves as a marker for ovarian carcinoma, mesothelial cell and endothelial cell lineages [21,22]. There is abundant *WT1* gene expression in the endometrium by RNA microarray studies (human protein atlas <https://www.proteinatlas.org/>), but the function of *WT1* in endometrium is yet to be defined, especially when the endometrium undergoes significant changes during the menstrual cycle and pregnancy. As initial steps, expression of *WT1* protein in endometrium by immunohistochemical staining was attempted to locate the *WT1* protein expression during menstrual cycles and pregnancy. These results will provide the global view of *WT1* gene expression in endometrium during the changes of menstrual cycle and pregnancy, with particular emphasis on maternal vascular remodeling during pregnancy and vascular restoration after pregnancy.

2. Material and Methods

This study is exempt from Institutional Review Board (IRB) approval according to section 46.101(b) of 45CFR 46, which states that research involving the study of existing pathological and diagnostic specimens in such a manner that subjects cannot be identified is exempt from the Department of Health and Human Services Protection of Human Research Subjects. Endometrial and placental tissues submitted for pathology examination for a variety of clinical indications were included in the study using the routine hematoxylin and eosin (H+E) stain. Proliferative endometrium, secretory endometrium, decidua and implantation sites were obtained from the submitted pathology specimens during clinical practice. Second trimester placentas were obtained from inevitable abortion specimens due to chorioamnionitis. Paraffin-embedded tissues from routine surgical pathology specimens and routine H+E stained pathology slides were examined by light microscopy using the Amsterdam criteria for placental examination [23]. Immunohistochemical staining for *WT1* was used to highlight the specific cell types of the muscular vessels, endometrium and myometrium, fetal villous stem villous arteries and umbilical cord vessels. CD34 was also used to highlight the endothelium of the endometrial or decidual vessels in comparison. Immunohistochemical staining procedures were performed in the routine clinical pathologic application on paraffin-embedded tissues, using the Leica Biosystems Bond III automated immunostaining system, following the manufacturer's instructions. No special procedures were employed for the study. *WT1*, CD34 and CD56 monoclonal antibodies were purchased for clinical applications from Dako Agilent, under the catalogue number M3561, M7165 and M730401-2. Monoclonal antibody against smooth muscle myosin heavy chain (SMYOHc) was from Cell Marque (Sigma-Aldrich, St. Louis, MO, USA, under the catalogue number 298M-16.

In total, 3 cases of proliferative endometrium, 3 cases of secretory endometrium, 2 cases of therapeutic abortion specimens at 10 weeks' gestation, 2 case of inevitable abortion at 17 weeks, 8 cases of term placentas and umbilical cords at 39–40 weeks, with and without decidual vasculopathy, were examined by light microscopy and H+E stain, as well as immunostaining for *WT1*, CD34, CD56

and SMYOHC. The immunostaining results were presented as qualitative measures of *WT1* gene expression, and the methods were not calibrated for the direct quantitative measurement of *WT1* protein expression.

3. Results

In small muscular arteries or arterioles of the lower extremities and the ovaries, *WT1* was detected by immunohistochemical staining, within the endothelial cells only. The staining signals appeared to be both cytoplasmic and nuclear. The smooth muscle layers of the vascular walls showed no or minimal immunostaining signals (Figure 1). The adventitia layer of the artery was evident on H+E staining slides, but no *WT1* signals were present within the adventitia. In proliferative endometrium, *WT1* expression was present in the nuclei of stromal cells and the endothelial cells, as well as the smooth muscle cells of the spiral artery (Figure 2, top panel). The endometrial glands, epithelium and lymphocytes showed no staining signals. In myometrium, the endothelial cells of the artery showed *WT1* staining signals, as well as the entire myometrium, but no smooth muscle cells of muscular artery were staining for *WT1* (Figure 2 bottom panel). The adventitia layer was not evident in the endometrial arteries, and no convincing staining signals for *WT1* were noted for the adventitial layers of myometrial arteries. In contrast to those of the proliferative endometrium, the endometrial stromal cells in adenomyosis (endometriosis) showed no nuclear staining signals for *WT1* (Figure 3, bottom panel). In secretory endometrium, *WT1* expression was identified in the stromal cells, endothelial cells and smooth muscle cells of the spiral artery walls (Figure 4, top panel), similar to those of proliferative endometrium (Figure 2). There appeared to be stronger staining signals for *WT1* in smooth muscle layers of spiral artery, but the immunohistochemical staining method was not calibrated for the quantitative measurement of *WT1* expression. *WT1* was seen in the endothelial cells of the myometrial segment of the spiral artery, and no significant *WT1* signals were present in the smooth muscle layers or adventitial layers (Figure 4, bottom panel).

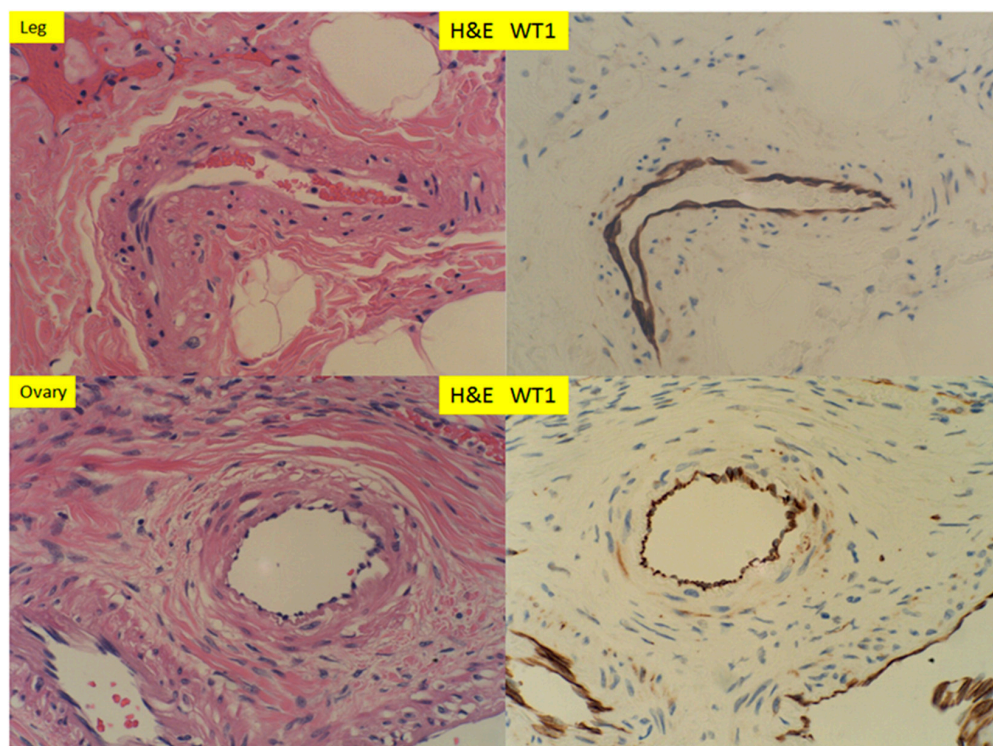


Figure 1. Hematoxylin and eosin (H+E) staining and immunostaining for Wilm’s tumor 1 gene (*WT1*) expression of muscular arteries in the soft tissue of the lower leg, after traumatic amputation and the ovary tissue of peri-menopausal women (400× magnification).

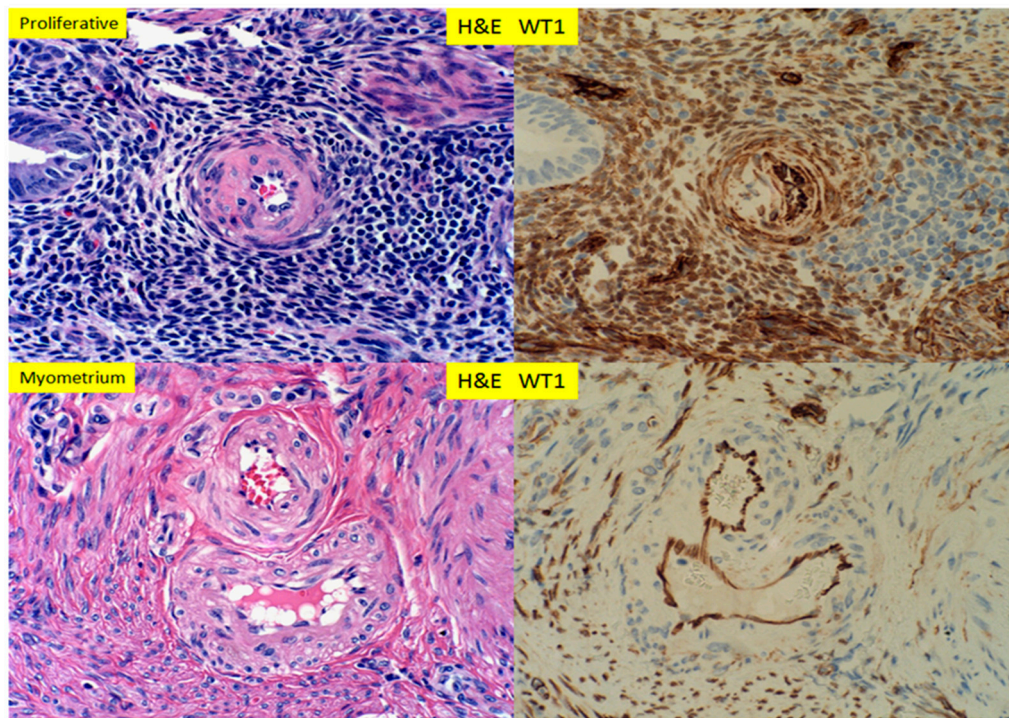


Figure 2. H+E staining and immunostaining for *WT1* expression of proliferative endometrium, with glandular, stromal cells and spiral artery and myometrium with spiral artery (400× magnification).

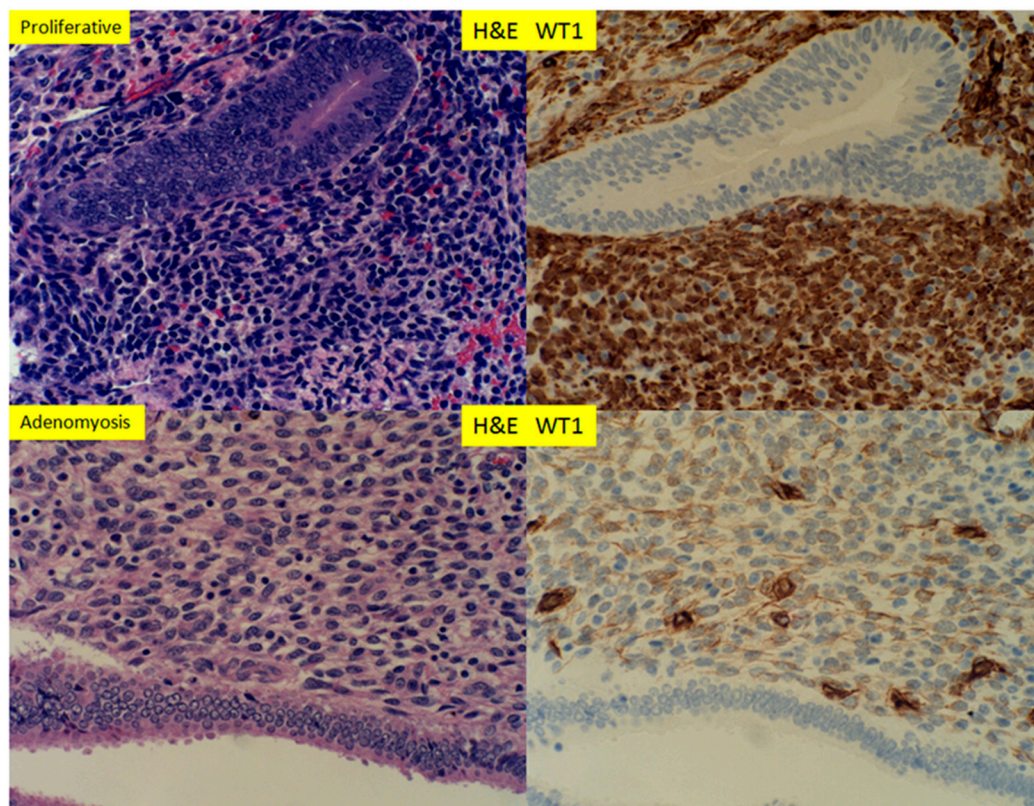


Figure 3. H+E staining and immunostaining for *WT1* expression of proliferative endometrium and adenomyosis in hysterectomy specimen. Noted a significant reduction of *WT1* signals within the stromal cells (400× magnification).

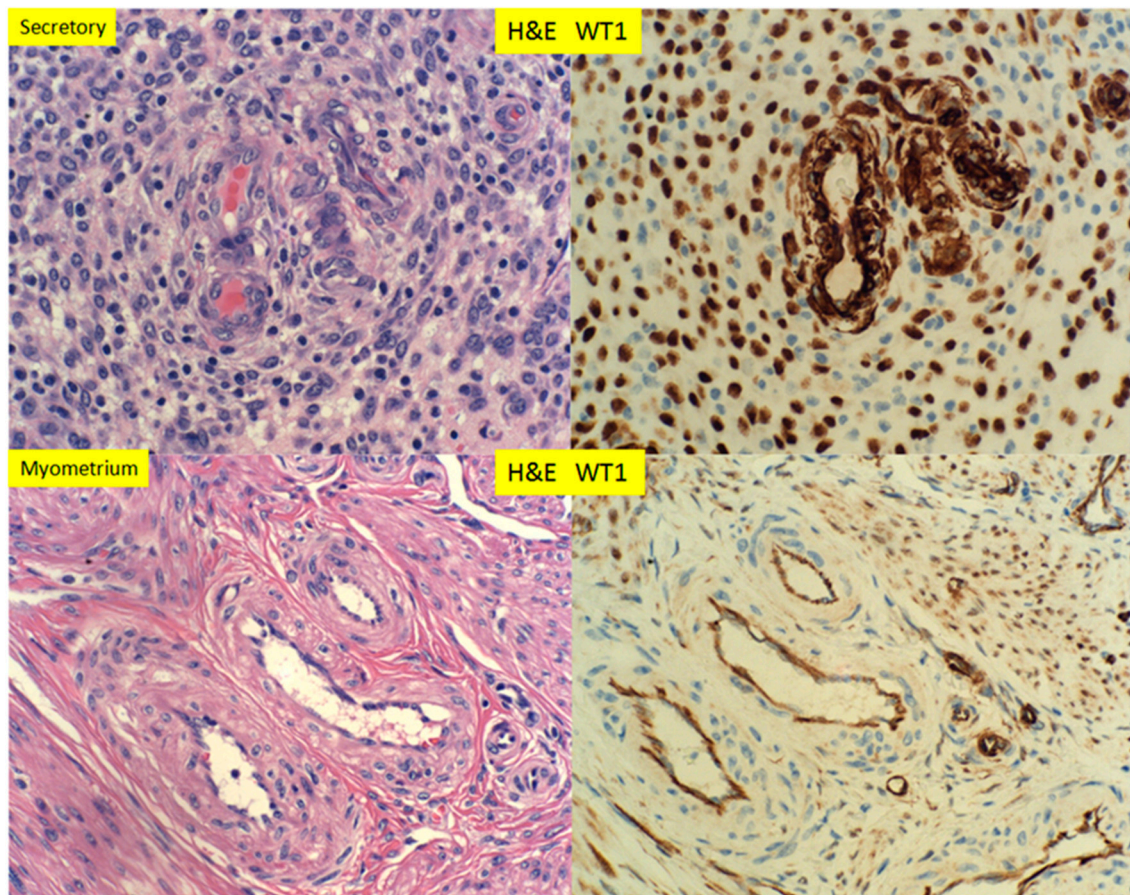


Figure 4. H+E staining and immunostaining for *WT1* expression of secretory endometrium and myometrium (400× magnification).

In early pregnancy, at 10 weeks, when the spiral arteries were undergoing remodeling in both the trophoblasts dependent and independent manners, the decidual cells expressed *WT1* strongly within the nuclei (Figure 5). There were strong nuclear signals for *WT1* in smooth muscle walls of the spiral artery, which showed mural hypertrophy/hyperplasia (Figure 5, top panel). There appeared to be cytoplasmic *WT1* signals within the endothelial cells in the arterial lumen. In contrast, the spiral arteries with trophoblastic “plugs” and the fibrinoid replacement of muscular walls were negative for *WT1* expression (Figure 5, bottom panel). At 17 weeks’ gestation (Figure 6), the expression patterns of *WT1* in decidua, spiral artery with hypertrophy/hyperplasia and those with trophoblastic plugs were similar to those at 10 weeks’ gestation (Figure 5). There was no difference in *WT1* expression pattern and intensity between those of 10 weeks’ and 17 weeks’ gestation. No *WT1* staining signals were noted in the adventitia layers of vessels, at either 10 weeks’ or 17 weeks’ gestation.

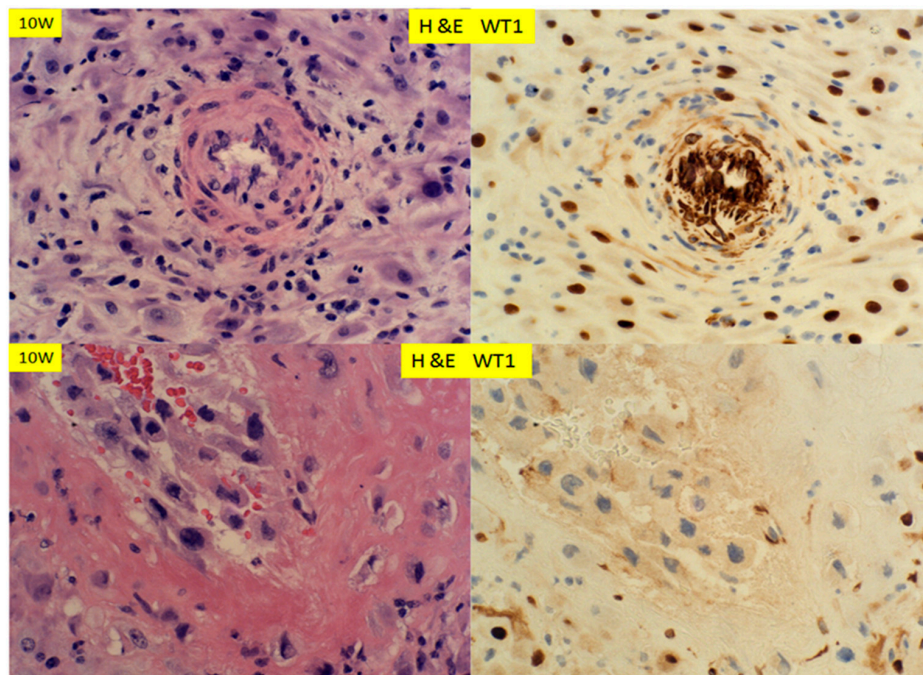


Figure 5. H+E staining and immunostaining for *WT1* expression of decidua at 10 weeks' gestation of therapeutic abortion. Spiral artery with mural hypertrophy/hyperplasia (top panel) and endovascular trophoblastic plug (bottom panel) (400× magnification).

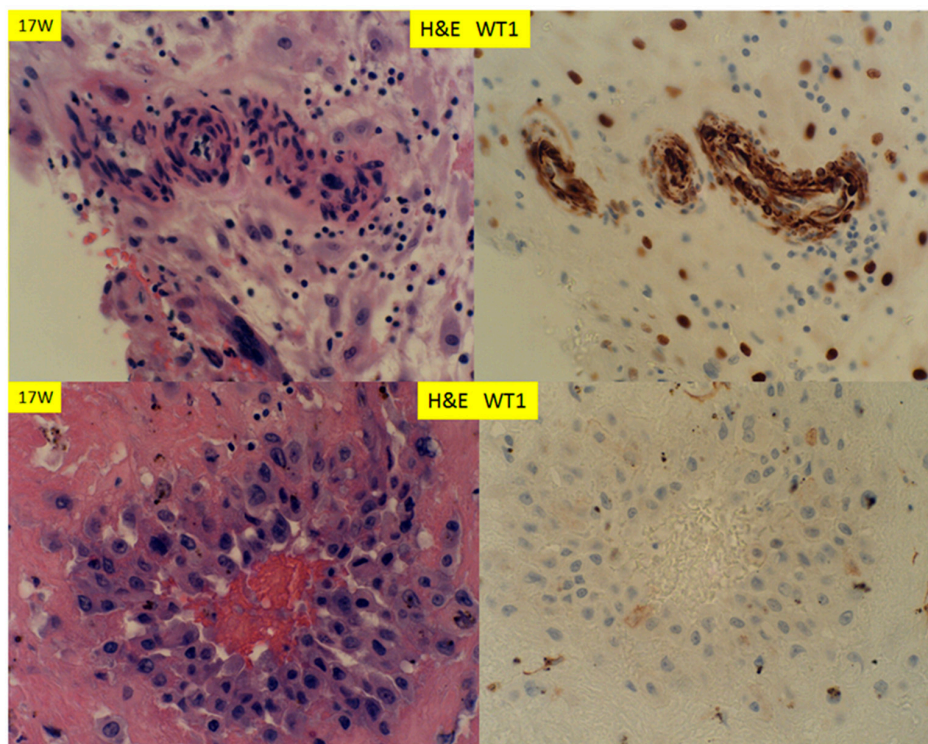


Figure 6. H+E staining and immunostaining for *WT1* expression of decidua at 17 weeks gestations, with inevitable abortion and chorioamnionitis. Spiral artery with mural hypertrophy/hyperplasia (top panel) and endovascular trophoblastic plug (bottom panel) (400× magnification).

There are two types of decidual vasculopathy; mural arterial hypertrophy/hyperplasia and classic type, including atherosclerosis and fibrinoid medial necrosis. Mural hypertrophy/hyperplasia in the

membrane roll (decidua vera) showed *WT1* expression in endothelial cells and smooth muscle cells (Figure 7 top panel), and it appeared that the expression in the smooth muscle cells was somewhat decreased in comparison to those in early pregnancy (Figure 5; Figure 6). There was no *WT1* expression in classic decidual vasculopathy, including atherosclerosis and fibrinoid medial necrosis. However, *WT1* was detected in the regenerating endothelial cells and smooth muscle cells of the arterial walls (Figure 7, bottom panel). The fibrinoid medial necrosis was partially present in the same vessel, with regenerating endothelium and smooth muscle wall, and the fibrinoid medial necrosis was negative for *WT1* expression (Figure 7, bottom panel). To illustrate this point of regeneration further, the endovascular trophoblasts can be identified by immunostaining for CD56, and the regenerating smooth muscle cells by smooth muscle myosin heavy chain (SMYOHC), as well as *WT1* expression (Figure 8).

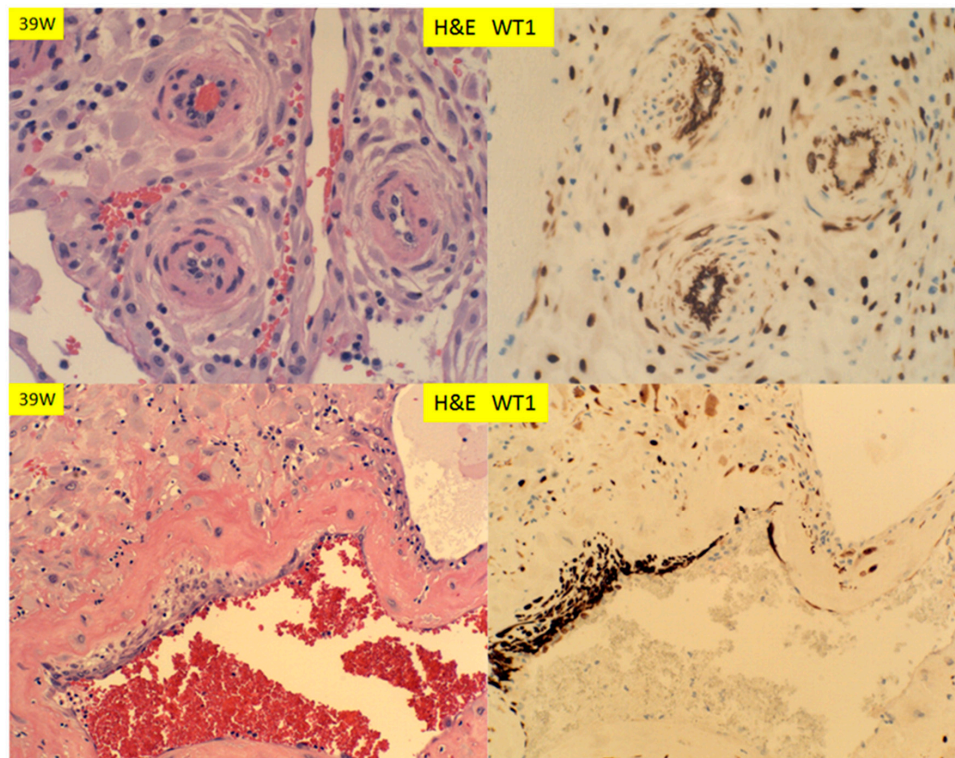


Figure 7. H+E staining and immunostaining for *WT1* expression of decidual vessels in the membrane roll (vera) and decidua basalis at 39 weeks (decidual vasculopathy). Spiral artery at decidua vera (top panel) and partially restoring spiral artery after vasculopathy (bottom panel) (400× magnification).

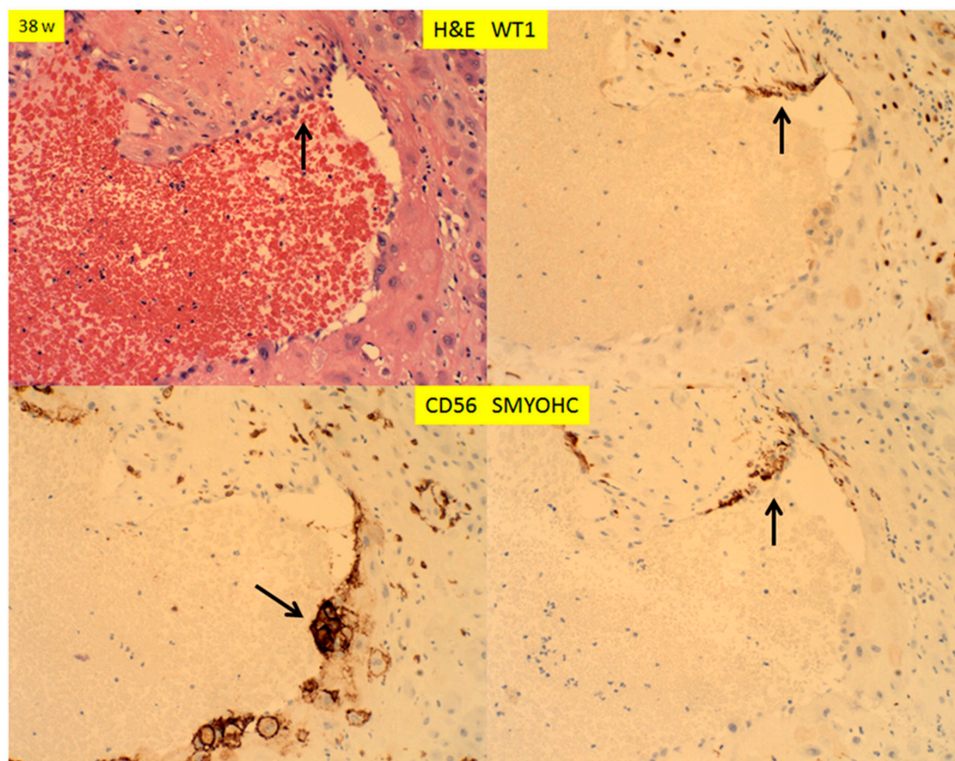


Figure 8. H+E staining and immunostaining for *WT1*, CD56 and smooth muscle myosin heavy chain (SMYOHc) expression of decidual vessels in the membrane roll (vera) and decidua basalis at term (decidual vasculopathy), at 38 weeks. Arrows indicate the focus of regenerating endothelium and smooth muscle cells with the remaining endovascular trophoblasts (200× magnification).

In tubal pregnancy, *WT1* expression was noted to be abundant in the tubal epithelium, smooth muscle wall and endothelium of the vessels, as well as small capillary endothelial cells (Figure 9). This is in contrast to the endometrium, where *WT1* was only expressed in the endometrial stromal cells, not in the endometrial glandular epithelium.

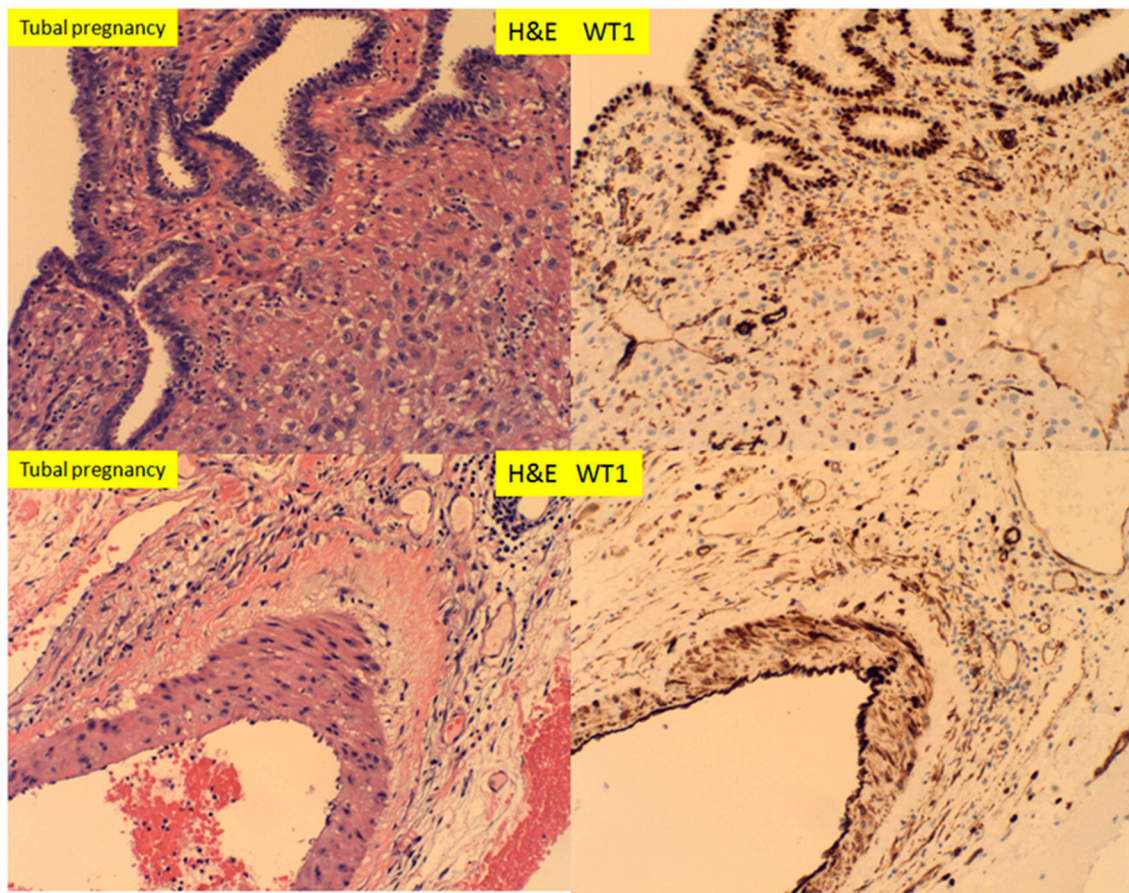


Figure 9. H+E staining and immunostaining for *WT1* expressions of tubal pregnancy, at 11 weeks after salpingectomy. Noted the tubal epithelium and tubal vessels with *WT1* expression and no *WT1* expression in the extravillous trophoblasts (400× magnification).

In placental stem villi at term, the muscular artery showed intense *WT1* staining signals, but in the luminal endothelial cells, there appeared to be no staining (Figure 10, top panel). This *WT1* staining pattern in stem villous artery was in direct contrast to CD34 staining signals, in which only the luminal endothelial cells were positive (Figure 10, top and bottom panels). The *WT1* expression patterns in stem villous artery were similar to those in the umbilical artery and veins in umbilical cord at term (Figure 11). There were no adventitia layers of these vessels, as these vessels are anatomically different from those in the systemic circulation [24].

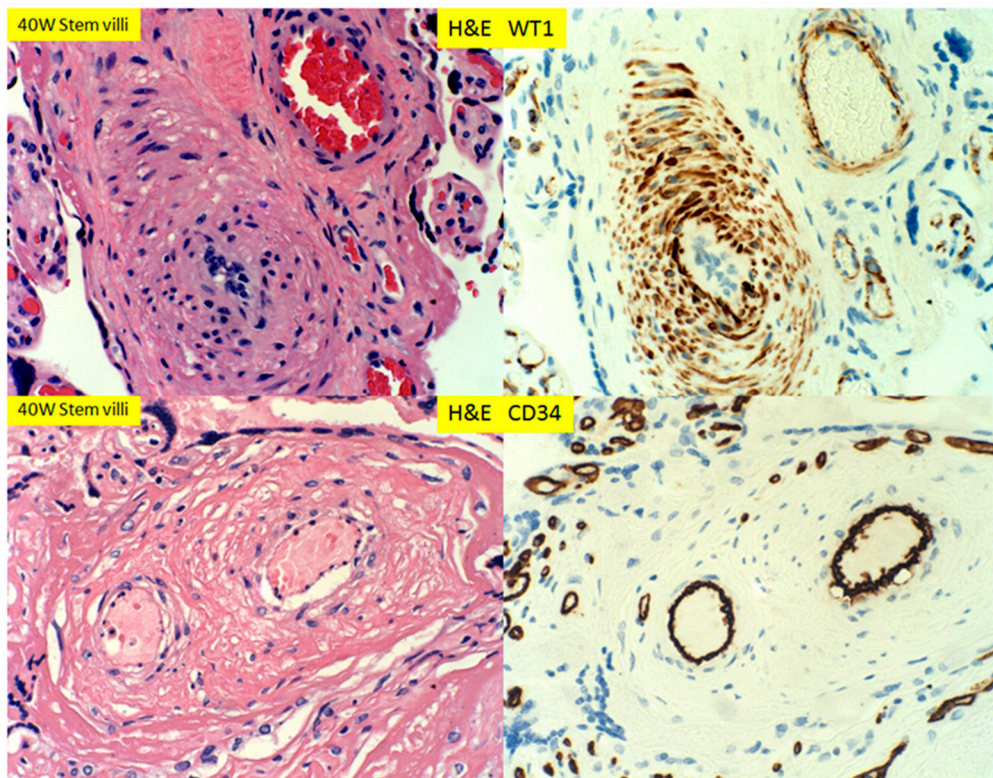


Figure 10. H+E staining and immunostaining for WT1 and CD34 expression of muscular arteries of stem villi of 39 week placentas. WT1 was noted in smooth muscle wall but not in endothelium, whereas CD34 was only seen in endothelium (400× magnification).

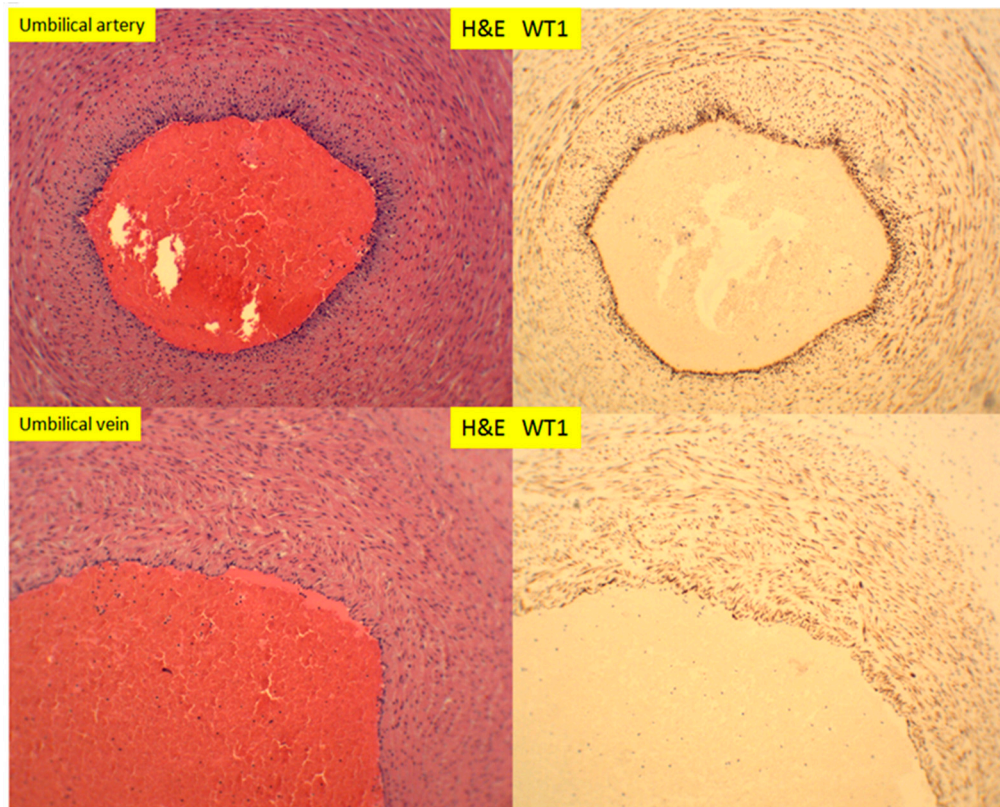


Figure 11. H+E staining and immunostaining for WT1 expression of umbilical artery and vein of 39-week placenta (100× magnification).

4. Discussion

WT1 gene expression has been studied extensively in a variety of tissues and organs and many aspects of *WT1* gene functions are known (Figure 12). *WT1* is important for organogenesis of urogenital system, endothelial cell system and mesothelial cell system during embryogenesis, and its versatile cellular function is likely to be conferred through not only regulating multiple target gene expression, but also receiving signals from multiple factors in growth and development [10,15]. Our interest in *WT1* gene expression is centered on the role of *WT1* gene in decidual vasculopathy of pregnancy, in relation to preeclampsia and other pregnancy related complications. The *WT1* function in endometrium appeared at least in part to be through regulation by the steroid hormones (estrogen and progesterone) and insulin-like growth factors (IGF), which are known to be critical for decidualization, implantation and fetal development [25–27]. *WT1* has been shown to be up-regulated by progesterone in in vitro models, and it can also be self-regulated [25,28]. Our current *WT1* expression data in endometrium is consistent with the known function of progesterone in both menstrual cycles and pregnancy. However, the basal expression of *WT1* in the stromal cells of proliferative endometrium and the epithelium of the fallopian tubes appears to be unrelated to the progesterone effect, and there is no estrogen or progesterone receptor expression in the tubal epithelium (data not shown). The potential link of *WT1* expression in stromal cells to the estrogen function could be through amphiregulin, which was shown to be transcriptionally activated by *WT1* [29], although the amphiregulin and estrogen effects was demonstrated in mammary tissue [30]. In the current study, the *WT1* expression is abundant in the stromal cells of the secretory endometrium and the decidua in first and second trimesters, as well as the smooth muscle wall of the spiral arteries. The spiral artery transformation in secretory phase of endometrium and remodeling in early pregnancy correlates with the expression of *WT1* in the smooth muscle wall, as well as in the stromal cells (decidua cells). There is no *WT1* expression in villous trophoblasts or extravillous trophoblasts, including the endovascular trophoblasts. There appears no *WT1* expression in uterine NK cells or lymphocytes (data not shown), although *WT1* functions are known to be important for myeloid cells and myeloid leukemia [31].

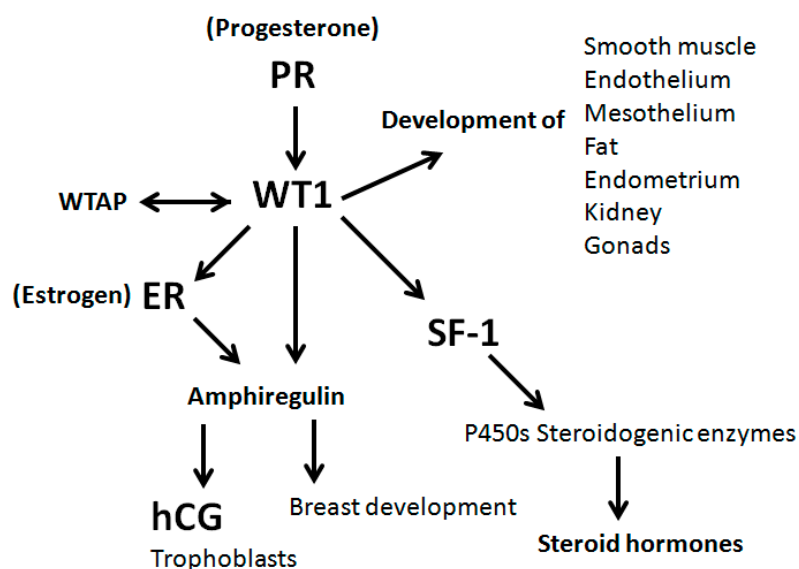


Figure 12. Schematic representation of *WT1* gene expression, in relation to other steroid and peptide hormones and receptors in regulation of organ development, homeostasis and disease development.

Spiral artery remodeling in early pregnancy is critical for normal pregnancy, and two separate molecular mechanisms are proposed: trophoblasts-dependent or trophoblasts-independent [3,32]. Trophoblasts-dependent remodeling is associated with the direct invasion of extravillous trophoblasts into the spiral artery, with the replacement of the smooth muscle wall and endovascular trophoblasts

plugs in the lumen [7]. *WT1* gene function is not known to play any role in the process of trophoblastic invasion into the vessels, as there is no *WT1* expression in trophoblasts or NK cells. It is unclear if these remodeled arteries contain the adventitia layer, but no *WT1* expression was observed in the entire transformed arterial wall. However, the smooth muscle hypertrophy/hyperplasia of trophoblasts-independent spiral artery remodeling appears to correlate with *WT1* gene expression in the smooth muscle cells in early pregnancy. Both of the mechanisms result in the narrowing of the vascular lumen, thus limiting the maternal blood flow into the intervillous spaces and embryo, leading to the hypoxic environment which was shown to be critical for fetal development [33]. Furthermore, in the third trimester, after the trophoblasts-dependent smooth muscle remodeling, the spiral artery restores the endothelial layers and the smooth muscle layers within decidua and superficial (inner) segments of myometrium, and the restoration of endothelium and smooth muscle cells appears to be associated with increased *WT1* expression (Figures 7 and 8). The presence of endovascular trophoblasts, fibrinoid medial necrosis of spiral artery wall, regenerating endothelial cells and smooth muscle cells within the same vessel, captured the dynamic changes and interactions among these cells at the specific time of gestation. *WT1* and *WT1* associated protein (WTAP) have been shown to regulate vascular smooth muscle proliferation [34,35]. The restoration of the spiral artery also appears associated with the regression (involution, de-plugging) of endovascular trophoblasts.

The adventitia layer of muscular arteries is equivalent to the serosal layer of visceral organs, and the serosal surface is covered by mesothelium which expresses *WT1* [36]. In mice model, *WT1* gene deletion resulted in fetal death, due to the abnormal development of vasculature [10,20,36]. In young adult mice, *WT1* deletion can lead to multiple organ failures including renal failure, diminished splenic erythropoiesis, bone and fat development as well as abnormal circulating IGF1 and cytokines levels [10,20]. The serosal mesothelial cells served as progenitor cells, not only to the visceral fat tissue, but also to the smooth muscle cells of the vessels such as coronary arteries, and *WT1* is critical for maintenance of mesothelial progenitor cells [36–39]. Based on the current data, the endothelial cells appear important to restoration of smooth muscle wall of the spiral artery after trophoblasts-dependent remodeling, and no adventitia layer or serosal mesothelial layers were observed for the spiral artery of endometrium, placental stem villous arteries or umbilical vessels. *WT1* functions in spiral artery remodeling during pregnancy, and restoration after pregnancy requires more in vitro and animal model studies, and this process is likely to be important for further risk of cardiovascular disease after pregnancy.

It is interesting to note that *WT1* expression was reduced in adenomyosis (endometriosis). It is known that defective decidualization of the endometrium is related to endometriosis and IGF family members appear important for decidualization [40–42]. *WT1* regulates the expression of multiple IGFs, IGF binding proteins and IGF receptors, and the *WT1* function appears more complex in physiological condition during menstrual cycles and pregnancy [20,43]. It is also interesting to note that *WT1* expression is abundant in the tubal epithelium but not in tubal stromal cells, and the fallopian tubes do not undergo decidualization during pregnancy. The fact that tubal pregnancy occurs occasionally raises interesting questions whether decidualization of endometrium is absolutely required for embryonic implantation, and how embryonic implantation occurs in tubal pregnancy in the absence of decidualization. Finally, *WT1* is a gene with multiple functions and the upstream factors influencing the *WT1* gene function/expression in the regeneration of endothelial cells and smooth muscle cells as well as in endometriosis will likely yield important information, not only about the decidual vasculopathy and preeclampsia, but also the pathogenesis of endometriosis. Searching for these important upstream factors of *WT1* gene expression requires establishment of in vitro cellular and animal models, as well as quantitative biochemical studies.

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Conflicts of Interest: The author declares no conflict of interest.

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