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REVIEW

The role of Wnt signaling members in the uterus and embryo during pre-implantation and implantation

Filiz Tepekoy · Gokhan Akkoyunlu · Ramazan Demir

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Abstract Wnt family members are best known for their roles in cell fate determination, differentiation, proliferation and apoptosis during embryonic development. Wnt signaling becomes effective during these cellular processes through the proper interaction between its ligands, receptors, effectors and inhibitors. Here we review Wnt signaling in terms of embryonic development to the blastocyst stage implantation with emphasis on endometrial changes that are critical for receptivity in the uterus. The relationship between Wnt signaling and implantation clearly reveals that, Wnt family members are critical for both early embryonic development and changing of the endometrium before implantation. Specific Wnt signaling pathway members are demonstrated to be critical for endometrial events such as decidualization and endometrial gland formation in addition to cyclic changes in the endometrium controlled by reproductive hormones. In conclusion, specific roles of Wnt members and associated factors for both uterine function and embryonic development should be further investigated with respect to the efficiency of human ARTs.

Keywords Wnt · Uterus · Embryo · Implantation

Introduction

The Wnt family consists of secreted, cysteine-rich glycoproteins which are in the forefront with their roles in embryogenesis [1] . During embryonic development Wnts affect a number of cell types in terms of cell fate determination, differentiation, proliferation and apoptosis [2–5]. After the discovery of mouse proto-oncogene Int-1 in 1982 [6], it was shown that Int-1 and the drosophila segment polarity gene Wingless had a common origin, leading to conceive the term Wnt (combination of Wg (Wingless) and Int) [7].

The roles of Wnt signaling in embryonic development include embryonic axis formation, axon guidance and remodeling in the central nervous system, proper formation of organs such as lung and kidneys [8]. The physiological roles of Wnt signaling in adult tissues are notable, since abnormal activation of Wnt signaling in adult tissues is associated with a number of human diseases including osteoporosis [4], a variety of types of cancer and other degenerative disorders [9].

Wnt signaling pathways include Wnt ligands, G-protein coupled transmembrane frizzled receptors (FZD) and low density lipoprotein receptor-related protein (LRP) co-receptors. Up to now, 19 Wnt ligands (Wnt1, Wnt2, Wnt2b/13, Wnt3, Wnt3a, Wnt4, Wnt5a Wnt5b, Wnt6, Wnt7a, Wnt7b, Wnt8a, Wnt8b, Wnt9a, Wnt9b, Wnt10a, Wnt10b, Wnt11, Wnt16), 10 FZDs (FZD1, FZD2, FZD3, FZD4, FZD5, FZD6, FZD7, FZD8, FZD9, FZD10) and 2 LRPs (LRP-5 and −6) have been identified in mammals [10].

Wnt signaling is transduced via three different pathways following Wnt ligand binding to their FZD or LRP receptors: canonical (Wnt/β-catenin), Wnt/Ca^{2+} and planar cell polarity pathways [11–13]. Canonical Wnt signaling is sustained by β-catenin in the cytoplasm [14]. Besides being the key member of Wnt signaling, β-catenin is also responsible for cell adhesion interacting with type II cadherins near the cell surface [15]. In Wnt signaling, β-catenin protein is localized in

Capsule Specific roles of Wnt ligands, receptors, effectors and inhibitors and their interaction with the factors associated with implantation in terms of both embryonic development and uterine function are reviewed.

F. Tepekoy · G. Akkoyunlu (✉) · R. Demir
Department of Histology and Embryology, Medical Faculty, Akdeniz University, Antalya 07070, Turkey
e-mail: gokhan_akkoyunlu@hotmail.com

the cytoplasm in association with a destruction complex consisting of Axin, adenomatous polyposis coli (APC), glycogen synthase kinase 3B (GSK-3B) and casein kinase Iα (CK1α), where it becomes activated or degraded depending on the signaling's being on or off [9]. When β-catenin is bound to Axin and APC proteins, it is phosphorylated by GSK-3B and CK1α resulting with its degradation by ubiquitin/proteasome pathway [9, 16] (Fig. 1a). Upon interaction of FZD with a Wnt ligand (on state), it cooperates with a member of the LRP family [17]. The arising signal is transduced to the destruction complex via Disheveled (Dsh) to avoid β-catenin phosphorylation and degradation [18]. Thus, β-catenin is able to translocate to the nucleus in an unphosphorylated form (Fig. 1b).

In the nucleus, β-catenin acts as a transcriptional co-regulator of Wnt target genes by displacing the transcription repressor Groucho protein family (TLE) and allowing members of the T cell factor/Lymphoid enhancer factor (TCF/LEF) transcription factor family to regulate Wnt target gene transcription [11, 19].

Wnt signaling can be regulated by two main classes of antagonist one of which is secreted FZD related proteins (sFRP) that show structural similarity to extracellular domains of the FZD family and in this manner prevent Wnt ligands binding to their FZD receptors and transducing their signal [20] . The other class of antagonists is Dickkopf (Dkk) family proteins that lead to removal of LRP- 5/6 co-receptors via Kremen-mediated endocytosis [21–23].

Here we reviewed key literature on Wnt signaling through reproductive tract and considered what has been learned about implantation process.

Wnt signaling in uterine receptivity

The endometrium is a dynamic tissue, the architecture of which is changed during embryonic development, estrous cycle and pregnancy. The architecture of endometrium is changed under the control of steroid hormones, cytokines and morphogenes that orchestrate appropriate positioning of the various specialized cell types within the tissue. Among the group of morphogenes, Wnts are considered as important factors playing a role in development and functioning of the endometrium [24].

Ablation of noncanonical Wnt ligands has revealed the importance of these ligands in development of female reproductive system. Among Wnt ligand family members

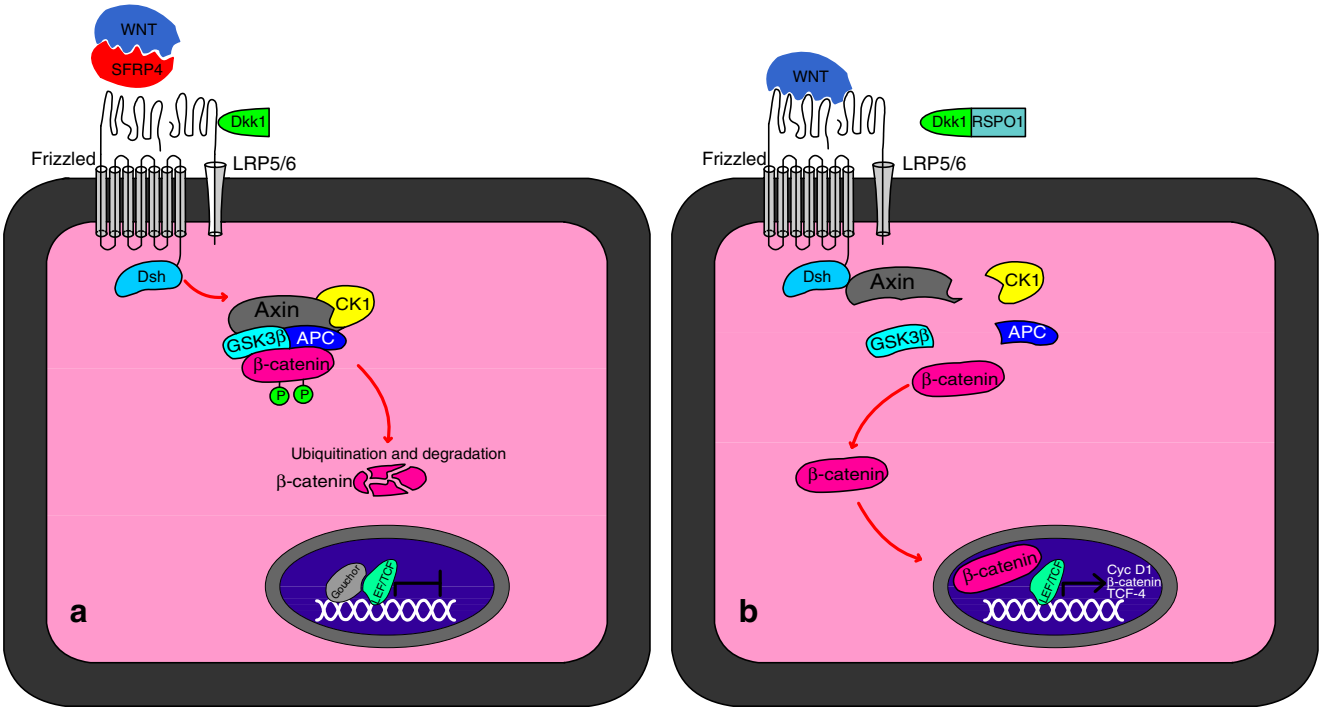

Fig. 1 Schematic illustration of potential canonical WNT signaling pathway- Resting state (**a**): SFRP4 binds free WNT molecules in the extracellular space and DKK binds the LRP co-receptor. GSK3b phosphorylates CTNNB1 and causes ubiquitination and proteosomal degradation. In the nucleus transcription repressor Groucho protein family (TLE) supresses members of the TCF/LEF transcription factor family and inhibits Wnt target gene transcription. Activated state (**b**): A WNT ligand binds a FZD receptor/LRP co-receptor complex. As a result CTNNB1 breaks from the APC/AXIN/GSK3b destruction complex in an unphosphorylated state. RSPO1 binds DKK to repress inhibition. CTNNB1 translocates to the nucleus in an unphosphorylated form. It binds LEF/TCF transcription factor to regulate target gene expression. Abbreviations: *APC* adenomatous polyposis coli, *CTNNB1* b-catenin, *DKK* dickkopf, *Dsh* Disheveled, *FZD* frizzled, *GSK3b* glycogen synthase kinase 3b, *LRP* low density lipoprotein receptor-related protein, *RSPO1* r-spondin 1, *SFRP4* secreted frizzled-related protein 4

canonical Wnt7a ligand and noncanonical Wnt4 and Wnt5a ligands have been identified in the female reproductive tract [25]. These genes are associated with proper prenatal and postnatal development of the Mullerian duct, since null mutation of these genes or alteration of their expression by endocrine disruptors have an impact on Mullerian duct [26]. Wnt4-null mice die at birth as a result of numerous defects besides failing to form Mullerian ducts [27].

Several Wnt ligands and their frizzled receptors are spatially and temporally expressed in the uterus during the menstrual cycle in women and downregulated in patients with endometrial cancer [28]. Uterus receptivity prior to implantation and initiation of implantation process are also regulated by Wnt genes [24]. Wnt ligands Wnt1, Wnt3, Wnt3a, Wnt7a which activate canonical (Wnt/β-catenin signaling) and Wnt2,Wnt4,Wnt5a,Wnt5b,Wnt6,Wnt7b,Wnt11 which activate non-canonical signaling pathway [10] have been proven to play important roles in peri-implantation events in mice, human [29, 30] and sheep [31] (Table 1).

A number of transcription factors are also associated with Wnt pathway. The nuclear localization of forkhead box protein O1 (FOXO1), a transcription factor and a promoter of apoptosis is controlled by Wnt4 in adult mouse uterus. When Wnt4 was conditionally ablated, FOXO1 was determined to be expressed in the nucleus, suggesting that FOXO1 actively promoted the transcription of pro-apoptotic genes [32]. Inhibition of transcription factors, Krüppel Like Factors (KLF) with co-addition of si*KLF9* + *siKLF1,* resulted in significant reductions in both prolactin (*PRL)* and *Wnt4* transcript levels, and numerically lower transcript levels for bone morphogenetic protein 2 (BMP2) in peri-implantation mouse uterus. Knockdown of *KLF9* alone was associated with the reduction of *Wnt4*, while *KLF13* knockdown alone caused a significant reduction in *BMP2* but not *PRL* and *Wnt4* transcript levels [33]. Identification of relation between Wnt signaling and various transcription factors associated with uterine receptivity might reveal hormonal regulation of Wnt signaling in uterus.

Wnt signaling is affected by hormonal regulation during menstrual cycle and pregnancy, besides being associated with structural and functional changes in the endometrium during pre-implantation and implantation processes.

Hormonal regulation during menstrual cycle and pregnancy

During the menstrual cycle, balance of the signaling pathways in the endometrium changes according to the differential exposure to steroid hormones. Wnt signaling members also show differential expression at different stages of menstrual cycle.

Differentiating Wnt gene expression profiles at different stages of estrus cycle and pregnancy were identified in different species [34, 35]. During porcine estrus cycle and pregnancy,

Table 1 Mouse and human Wnt proteins and their known roles in reproductive tract

Mouse and human Wnt proteins	Localization	References
Wnt1	mouse inner cell mass	[67]
Wnt2	human endometrial epithelial/stromal cells	[30]
Wnt2B (13)	mouse gastrulation	[67]
Wnt3	mouse gastrulation	[67]
Wnt3A	mouse blastocyst	[67]
Wnt4	mullerian duct formation, decidualizing human endometrial stroma and is associated with BMP-2 within these cells, development of endometrial glands in mice	[27] [35] [49]
Wnt5A	expressed in the uterine mesenchyme, mediates progesterone functions in human decidualization	[38] [39]
Wnt5B	mouse blastocyst	[67]
Wnt6	post-implantation uterine development of proliferating stromal cells of mice	[37]
Wnt7A	mouse endometrial luminal epithelium, endometrial gland morphogenesis in mice	[38] [48]
Wnt7B	mouse uterus stromal adhesion molecules	[51]
Wnt8A	mouse gastrulation	[67]
Wnt8B	mouse neurulation	[67]
Wnt9A (14)	mouse mural trophoblast and inner cell mass cells surrounding the blastocoele	[67]
Wnt9B (15)	mouse urogenital system development	[26]
Wnt10A	human first trimester villous cytotrophoblasts	[30]
Wnt10B (12)	human first trimester villous cytotrophoblasts	[30]
Wnt11	mouse uterus epithelial adhesion molecules	[51]
Wnt16	human placenta, mouse uterus during implantation	[41] [42]

gene expression levels of Wnt5a showed variation during pregnancy and corresponding day of estrus cycle and Wnt7a was differentially expressed at different stages of estrus cycle and early pregnancy, though Wnt4 and β-catenin gene expressions were maintained at the constant level in the endometrium of cyclic and pregnant pigs [34]. However content of estrogen (E2) in the uterine luminal flushings as well as blood serum level of progesterone (P4) in cyclic gilts of pigs showed no statistically significant correlations with Wnt4, Wnt5a, Wnt7a, β-catenin and Cdh1 gene expression. Moreover, treatment of endometrial tissue explants with different concentrations of E2 or P4 added separately or simultaneously had no effect on Wnt5a, Wnt7a, β-catenin and Cdh1 gene expression [34]. Diethylstilbestrol (DES) exposure suppressed the expression of Wnt4, Wnt5a, Wnt7a, Wnt11, Wnt16, and FZD10 in the neonatal mouse uterus [24]. In equine endometrium, Wnt2, Wnt5a, Wnt9b, Wnt10b, Wnt11 and Wnt16 were upregulated and Wnt4, Wnt7a and sFRP1 were downregulated at high P4 levels. Besides, Wnt5a, Wnt2b, Wnt11, Wnt16 and Dkk1 were increased while Wnt2, Wnt5b, Wnt7a and sFRP1 expressions were downregulated during early pregnancy [35].

LRP-6 and FZD6 expressions are also present in human endometrium and determined to be equally expressed in proliferative and secretory phase endometrium [28]. Wnt receptor related proteins were also found to be associated with endometrial functions, since mRNA levels of sFRP4 were found to be lower during the secretory phase of the menstrual cycle in repeated implantation failure (RIF) endometrium [36].

β-catenin was expressed both in glandular and stromal cells, and was highly expressed during both the proliferative and secretory phases of endometrial cycle. Besides β-catenin, other intracellular modulators of the Wnt signalling, GSK-3 and Dishevelled homolog-1 (Dvl-1) were also found to be expressed in proliferative and secretory phase human endometrium. [28].

Wnt signaling inhibitor Dkk1 was shown to have roles in endometrial function and its expression was affected by cyclic changes of the uterus. In a recent study it was determined that, Dkk1 mRNA expression was significantly elevated in the mid-secretory phase of the menstrual cycle in human endometrium [37]. Dkk1 expression was also found to be increased in response to decidualization in vitro [28].

The effect of varied level of hormones on the expression of Wnt signaling members during different stages of menstrual cycle and pregnancy suggests an interaction between these hormones and Wnt pathway. It must be identified whether Wnt signaling is affected by hormones directly or through other mechanisms changing under the control of hormonal regulation.

Uterus in Pre-implantation period: endometrial gland and luminal epithelium

Luminal epithelium (LE) forms endometrial glands through invading the mesenchyme during embryonic development and this process results in an extensive network of epithelial glands throughout the stroma [38]. In adult females, during the secretory phase of menstrual cycle, further development of these endometrial glands occur. Evidence that uterine glands are required to support pregnancy was provided [39].

Recent studies provide that Wnt signaling control endometrial gland formation and ablation of specific Wnt signaling components results in infertility due to implantation failure [40, 41]. Canonical Wnt ligand Wnt7a was identified as one of the epithelial Wnt genes, since it was expressed only in endometrial LE [42]. It was found that, postnatal ablation of Wnt7a after birth in the mouse uterus caused infertility depending on disruption of endometrial gland morphogenesis. Although uterus histology was not affected by an experimental model of Wnt7a conditional null allele, mutant uteri lacked endometrial glands and as a result, implantation did not occur in the uteri of mutant mice [40]. Wnt4 was also shown to be associated with development of endometrial glands in mice [41]. In Wnt4 ablated mouse models, the amount of endometrial glands were reduced compared to control mice [32], hypertrophy and pseudostratification of the LE occured which may account for the difficulty of embryo invasion [41]. Wnt4 was associated with a number of transcription factors such as FOXO1 and KLF9 in mouse uterus [32]. Wnt16 was shown to be highly abundant in the entire stroma, but not in luminal and glandular epithelium during uterine morphogenesis [24].

Among Wnt receptors, only FZD6 and FZD10 expressions were identified in developing mouse uterus [24]. Wnt receptor expressions were found to be affected by P4. FZD10 expression was suppressed at postnatal days (PNDs) 10 and 20 in mice exposed to P4 during PNDs 3–9. FZD6 was inhibited in the epithelium on PND20 but not affected on PND10 [43].

Wnt genes were also found to be associated with cell adhesion molecules, since in a study, ablation of cadherin1 (Cdh1) was related to decrease in epithelial Wnt genes (Wnt7a and Wnt11) and Wnt receptors (FZD6 and FZD10) whereas stromal Wnt4 and epithelial Wnt7b were shown to be elevated in the uteri of Cdh1 ablated mice [44].

Besides Wnt ligands, the leading intracellular effector of Wnt signaling pathway β-catenin is also associated with Cdh1 providing its maintenance and contributing to its proper functioning [45]. β-catenin binds to the cytoplasmic domain of Cdh1 [46]. Knocking out β-catenin [47] or its downstream target gene, the transcription factor Lef1 [48], perturbed gland formation in neonatal uteri. β-catenin and Cdh1 genes in the human endometrium were shown to be unaffected by steroid hormones and independent of the phase of the estrous cycle by other studies [28, 49]. On the other hand, β-catenin showed higher mRNA amount in the ovine epithelial cells on days 16, 18 and 20 [31, 50]. In the murine uterus, balance in the expression of β-catenin was necessary for proper endometrial gland formation. In a study, conditional activation of β-catenin caused an increased amount of enlarged glands in

the uterus, whereas upon ablation of β-catenin, the endometrium contained no glands [47]. β-catenin protein was present predominantly in the uterine epithelia and was not different between control and mutant uteri with Wnt7a depletion during postnatal development of mice [40].

Wnt signaling is necessary for endometrial gland formation and suggested to have roles in adhesion of LE cells. Investigation of Wnt signaling inhibitors is also necessary for making suggestions on the balance between different Wnt ligands during these processes.

Uterus in implantation period: decidualization

Decidualization is the differentiation process of the endometrial stromal cells into decidual cells. This process is primarily induced by P4. Decidualization is initiated at the time of blastocyst attachment to the uterine epithelium on day 4.5 of pregnancy in mice. Following the attachment reaction, the stromal cells surrounding the implanting blastocyst proliferate and differentiate to form the decidual bed. During pregnancy, decidual cells produce hormones and cytokines that are critical for embryo development, secrete factors that control trophoblast invasion during pregnancy [51]. The roles of Wnt signaling pathway in decidualization are identified both in mouse and human.

Among 19 Wnt ligands identified in mammals, Wnt4 is one of the most studied Wnt ligand in terms of its localization and function in the endometrium. Wnt4 is among several genes that are altered within 24 h of BMP2 addition in the mouse uterine stromal cells. Also, during in vitro decidualization of human endometrial stromal tissue, Wnt4 gene was induced markedly in stromal cells in response to the hormone mixture (progesterone, estrogen and 8-bromo-cAMP) and Wnt4 mRNA was also expressed during human stromal decidualization and its expression profile was related with BMP2. Thus, the expressions of BMP2 and Wnt4, which were induced during stromal decidualization in the mouse, were conserved in the human stromal cells undergoing decidualization [52]. Studies reveal that Wnt4 is not the only Wnt ligand that is associated with BMP2, since BMP2 was shown to be responsible for the upregulation of Wnt6 of murines [53]. Wnt6 was shown to be expressed in proliferating stromal cells of mice during post-implantation uterine development. Moreover, genetic depletion of Wnt6 was proven to impair normal stromal cell proliferation with a prolonged cell-cycle length, while its deficiency exhibited no apparent influence on decidual polyploidization [54]. Wnt5a is another noncanonical Wnt ligand expressed in the uterine mesenchyme [42]. During human endometrial decidualization, Wnt5a was involved in mediating the P4 functions [55]. Human Wnt16 was detected in FOXO1- dependent endometrial decidualizing cells [56] in addition to its presence in the placenta [57]. Wnt16 expression was also present in the adult mouse uterus during implantation [58].

In a study analyzing the array results from the decidua from women with ectopic pregnancies with little or no decidualization, it was found that 658 genes were differentially expressed depending on the degree of endometrial decidualization. Wnt/ß-catenin signaling was indicated to be one of the top canonical pathways associated with these genes. Decidualization was found to be associated with up-regulation of Dkk1 and down-regulation of sFRP4 and sFRP1 genes [59]. Both in vitro studies with cell lines and also with human endometrial primary cells reveal that, secreted protein prokineticin1 (PROK1) increased Dkk1 mRNA expression and this increase was provided via the calcineurin-nuclear factor of activated T-cells (NFAT) pathway. Though PROK1-mediated Dkk1 expression via the NFAT pathway had an inhibitory effect on prokineticin receptor 1 (PROKR1) Ishikawa cell proliferation, PROK1 and Dkk1 expressions were shown to influence decidualization in human primary endometrial stromal cells [37].

In β-catenin knock out or knock down mouse models, the decidual response of the uterus and proper gland formation decreased, providing that β-catenin is also a necessary factor for decidualization and fertility [47].

These investigations show that there are ligands, receptors, effectors and inhibitors of Wnt signaling affecting decidualization via interaction with different signaling molecules including hormones and growth factors. Interestingly, both Wnt ligands and Wnt inhibitors have inducing roles in decidualization. Thus, it can be suggested that specific Wnt ligands stimulate decidualization while other ligands have inhibitory effects on decidual events and inhibition of those ligands with Wnt inhibitors is necessary for induction of decidualization. If the specific effect of Dkk1 in decidual cells is identified, it can be revealed that there is a balance between Wnt ligands during decidualization.

Wnt signaling in embryo during Pre-implantation and implantation processes

Wnt family members are known to be localized in the embryo at early stages of the development before implantation. It's demonstrated that, both Wnt ligands and receptors show specific expression patterns in terms of their localizations in the developing mouse embryo and the developmental stage of the embryo. Wnt signaling pathway members are associated with events such as competency, hatching, attachment and trophoblast cell

proliferation and differentiation for achievement of a successful implantation process.

Wnt signaling in Pre-implantation embryo development

Wnt family members show expression at early stages of embryonic development. It has been reported that Wnt3a and Wnt4 were expressed at the 8-cell stage in the mouse [60]. At the peri-gastrula stages FZD5 was expressed in the distal visceral endoderm (dVE) and later in the anterior visceral endoderm (aVE), whereas FZD10 expression was present in the delaminating epiblast of the primitive streak. In addition to these, it was revealed that, FZD7 was expressed both in the extraembryonic region of the pregastrula and in the epiblast of 6.0 to 7.0 days post coitum (dpc) embryos, becoming more anteriorly restricted at 7.5 dpc [8]. Wnt pathway was also related with factors that regulate germ layer differentiation in the developing embryo, as it was indicated that the Wnt/β-catenin signalling pathway regulated SRY HMG-box 17 transcription factor (Sox17) expression for visceral endoderm pattering and definitive endoderm formation in mouse [61].

At the start of gastrulation FZD10 expression was shown in the epiblast compartment of the primitive streak of mouse embryo, thus a possible role in the epithelial– mesenchymal transition in the primitive streak was suggested. Continued expression of FZD10 in the entire primitive streak, from the allantois to the node, at 7.5 dpc, proved its role of transducing mesoderm-inducing Wnt signals. FZD10 was known to be a putative receptor for Wnt2b, Wnt3, Wnt7b, and Wnt8a, expressed before gastrulation and later Wnt3a, Wnt5a, Wnt5b, or Wnt11, which were all expressed in the posterior region of the gastrula [62–64, 8]. In the mouse gastrula FZD8 was expressed in the dVE, starting at 5.5 dpc [65]. After movement of the dVE to the future anterior region of the embryo at 5.75 dpc, FZD8 expression also shifted in this more proximal direction. At later stages, FZD8 was expressed both in the aVE and in the anterior primitive streak, marking the anterior mesendoderm, and was excluded from the node [66]. Wnt ligands also have roles in generation of the germ layers, since the null mutation of Wnt3 demonstrated the importance of canonical Wnt signaling in mesoderm formation in mouse embryos [8].

Wnt7b was also shown to be another potential FZD ligand at this stage of development and to be expressed throughout the blastocyst [67]. However, Wnt7b expression was restricted to the extraembryonic region of the forming egg cylinder at implantation, 4.75 dpc [8] Among Wnt ligands expressed in the mouse blastocyst (e.g., Wnts 4, 5b, 7b, 10b, 1, 5a, 7a, 11, and 13), a number of them are newly detectable in the blastocysts (Wnt 1, 5a, 7a, 11, and 13). These Wnt ligands specific for blastocyst during embryonic development were evaluated in terms of their upregulation in the blastocyst in vitro. The results of these evaluation reveal that though several of the Wnt genes (7a, 7b, 10b, and 13) were upregulated during in vitro development of the blastocysts, Wnt5a and Wnt11 were expressed at relatively low levels. These results suggest that upregulated expressions of certain Wnt genes in blastocysts require exposure to the uterine factors during the morula-to-blastocyst transition. Wnt11 expression was found to be associated with the onset of the E2 surge by in vivo studies held in different time courses of embryonic development. However, in vitro studies which included E2 treatment to blastocyst were failed to demonstrate an upregulation in Wnt11 expression in blastocysts suggesting that this upregulating activity of E2 might require the uterine environment [68].

Among Wnt downstream effectors β-catenin and GSK-3 kinases are the most studied ones in the blastocyst in terms of their possible functions and interactions with other signaling cascades. Even several Wnts and FZDs were detectable in the pre-implanting embryo, canonical Wnt signaling was suggested to be dispensable for blastocyst formation. Gene knock-out studies showed that mutant mouse embryos lacking β-catenin developed into blastocysts [69]. Another Wnt effector GSK-3 has been demonstrated to be a key regulator of cellular fate and a participant in the differentiation events during embryonic development through its participation in the Wnt signal transduction pathway [70].

In bovine embryos, expression of GSK-3A and GSK-3B isoforms can be seen from the two-cell stage to the blastocyst stage and phosphorylation of both isoforms increases as development progresses, thus GSK-3 is suggested to be critical for embryonic development. [71]. Chemical inhibition of GSK-3 by using different chemicals affected both embryonic development and β-catenin phosphorylation in bovine embryos. β-catenin phosphorylation was decreased, but embryonic development was increased after treatment with GSK-3 inhibitor CT9921. However, treatment with another inhibitor of GSK-3, lithium chloride (LiCl), decreased the proportion of zygotes reaching the blastocyst stage. β-catenin phosphorylation was also shown to be associated with phosphatidylinositol-3 kinase (PI3K) pathway, as inhibition of PI3K resulted in an increase in phosphorylation of β-catenin. [71]. In addition to GSK-3, phosphorylation of β-catenin is regulated by different kinases: GSK-3 which phosphorylates at Threonine and Serine residues Thr41, Ser33, and Ser37 [72]; CK1 which phosphorylates at Ser45, priming β -catenin for subsequent phosphorylation by GSK-3 [73, 74]; protein kinase B (AKT) and protein kinase A (PKA) which phosphorylate at Ser552 and Ser675 [75–77].

Upregulation of several factors specific for pre-implantation process such as heparin-binding epidermal growth factor-like growth factor (HB-EGF), epiregulin, and beta-cellulin were suggested to be associated with the signals

emerging from blastocyst, since these factors were upregulated only in the region of the uterine epithelium adjacent to the blastocyst [78, 79].

Specific expression patterns of Wnt signaling members at different stages of embryonic development, suggests specific roles for Wnt signaling during this process. Wnt signaling in developing embryo is considered to be associated with uterine environment possibly through different signaling mechanisms in the receptive uterus. The uterine mechanisms affecting Wnt signaling in developing embryo must be well characterized in order to better understand the relation between uterus and embryo in terms of Wnt signaling members.

Wnt signalling in embryo during implantation

Competency, hatching and attachment

Blastocyst competency for implantation is related with maternal β-catenin, as female mice with maternal β-catenin depletion produced reduced number of pups when crossbred with wild-type males in comparison to those of wild-type to wild-type mating although oocytes with conditional deletion of β-catenin develop into blastocysts. Reciprocal embryo transfer experiments also revealed that silencing of Wnt-β-catenin pathway negatively affected blastocysts' competency to implant [80].

It was demonstrated that, the Wnt/ β-catenin signaling pathway was active in expanded blastocyst but was inhibited in hatched blastocysts. Wnt/ β-catenin inhibitor Dkk1-treated blastocysts showed increased ability to hatch on Day 7 and Day 8 of in vitro culture. Conversely, treatment of Day 6 blastocysts with Wnt/β-catenin activator LiCl in culture increased the accumulation of β-catenin but reduced the ability of blastocysts to hatch on Day 7 and Day 8 of in vitro culture. Thus, it was found that, hatching ability of pig blastocyst was inversely correlated with the activation of Wnt/ β -catenin signaling pathway [81]. Wnt inhibitor Dkk1 has different effects on different embryonic cells and their functions related to implantation. Through inhibiting Wnt signaling, Dkk1 can have both inhibitory and also activating effect on embryonic cell function related to embryo hatching, attachment and implantation [82].

Using spheroids–endometrial cells co-culture assay, it was revealed that the environmental toxicant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) dose-dependently (0.1–10 nM) suppressed BeWo and Jeg-3 trophoblastic spheroids attachment onto the endometrial epithelial RL95-2 and Ishikawa cells. Besides this, treatment of the BeWo or RL95-2 cells with TCDD at 1 and 10 nM for 24 h significantly suppressed epithelial cadherin (E-cadherin) and β-catenin expression proving a relation between Wnt/ β-catenin signaling and blastocyst's attachment to endometrium. Interestingly, it was demonstrated that, addition of Wnt3a conditioned medium or LiCl (40 μM) restored TCDD-induced reduction of β -catenin and E-cadherin expression, though it had no effect on GSK-3B expression in BeWo and RL95-2 cells [82].

Trophoblast cell proliferation, migration and differentiation

In blastocyst trophoectoderm cells, Wnt antagonist Dkk1 was downregulated, while canonical ligand Wnt3a was induced at higher levels, leading to intracellular accumulation of dephospho β-catenin [80]. It was found that activation of Wnt/β-catenin pathway by LiCl, inhibited trophoectoderm cell proliferation whereas inhibition of Wnt/β-catenin pathway by Dkk1 induced trophoectoderm cell proliferation [81].

In vitro studies both with cell lines (SGHPL-5 cells) corresponding to trophoblast cells and with explant cultures of first trimester villous found out that Wnt3a was associated with proliferation and migration of human cytotrophoblasts which have critical functions for invasion of these cells into endometrium [83]. In vitro studies in which Wnt3a treatment was applied, resulted in the induction of β-catenin/TCF target genes cyclinD1, TCF-4 and β-catenin. Even the reduction of these factors (e.g. β-catenin) was shown to be compensated by Wnt3a addition [83]. Dkk1 decreased the migration of SGHPL-5 cells in the absence of compensating Wnt ligand (Wnt3a) [37]. Wnt3a-induced migration in primary first-trimester villous explant cultures was also decreased in the presence of either Dkk1 or the PI3K inhibitor [83]. Dkk1 was found to induce pig trophoectoderm cell proliferation [81], whereas supplementation of Dkk1 significantly decreased bromodeoxyuridine (BrdU) labeling of human cytotrophoblasts [83].

Since, there is a possibility that a noncanonical Wnt receptor/ pathway may affect AKT or GSK-3, Dkk1 failed to inhibit Wnt-induced phosphorylation of these kinases in trophoblastic SGHPL-5 cells [83]. Modulation of Wnt/β-catenin signaling by LiCl or Dkk1 also altered the expression of Caudal type homeobox 2 (CDX2). CDX2 expression in the LiCl-treated blastocysts was reduced and showed an irregular pattern compared with those of control and Dkk1-treated blastocysts. Since CDX2 was related to trophectoderm differentiation [84], it was suggested that Wnt/β-catenin signaling may involve in regulating trophectoderm fate during the development of pig blastocyst [81].

Studies with human embryonic stem cell lines revealed that expression of pGSK-3B and the Wnt antagonist, Dkk1 were enhanced after the cells' gaining syncytiotrophoblast characteristics and also that inhibition of fibroblast growth factor (FGF) directed the cells to syncytiotrophoblast [85].

In Wnt3a-stimulated SGHPL-5 cells and first-trimester villous explant cultures, migration of the cells was decreased after treatment with Wnt inhibitor Dkk1 or PI3K chemical inhibitor LY294002, suggesting that both Wnt and PI3K signaling pathways contribute to Wnt-dependent trophoblast

motility. Thus, it was suggested that, migration in primary explant cultures was induced by Wnt3a in the presence of either Dkk1 or the PI3K inhibitor [86]. Incubation with recombinant Wnt3a increased phosphorylation of AKT at Ser473 as well as Wnt-dependent phosphorylation of GSK-3B at Ser9 was noticed. As, Dkk1 failed to inhibit Wnt-induced phosphorylation of AKT or GSK-3B, it was suggested that a noncanonical Wnt receptor/ pathway may affect the particular kinases [86].

Matrix metalloproteinase (MMP) secretion was activated by Wnt3a through canonical or PI3K/AKT pathway. Wnt3a also stimulated pro-MMP-2 accumulation and this accumulation was inhibited by Dkk1 or LY294002 in extravillous trophoblast. Since Wnt3a was not effective in alteration of MMP-2 mRNA levels, it was suggested that Wnt3a has post-transcriptional effects on elevation of MMP-2 concentration [86].

Wnt signaling has both inductive and inhibitory effects on blastocyst development during pre-implantation process. Blastocyst competency, attachment, cytotrophoblast proliferation, trophoblast cell migration is found to be activated by Wnt signaling ligands and effectors while blastocyst hatching, trophoectoderm cell proliferation, generation of syncytiotrophoblast layer require inhibition of Wnt signaling. During all these processes, signals that regulate Wnt family members to be activated or inhibited remain to be further investigated.

Conclusion

During pre-implantation and implantation processes, signaling molecules affecting both developing embryo and uterus undergoing structural and functional changes play effective roles for completion of these processes successfully.

Wnt signaling pathway is included in critical processes for endometrial changes related to implantation such as decidualization and endometrial gland formation. Wnt signaling is also suggested to be associated with cyclic changes of endometrium including its ligands, receptors and inhibitors.

Specific Wnt ligands and receptors are localized in the developing embryo from very early stages of the development. During blastocyst formation, specific Wnt ligands and effectors are detected to be upregulated. Besides being included in embryo development, Wnt signaling is also suggested to be critical for the processes that prepare embryos for implantation, such as embryo competency, hatching, attachment and trophoblast cell proliferation, differentiation and migration for a proper invasion process.

According to our observations, functional studies proving the roles of Wnt signaling in implantation are limited to specific Wnt ligands. These studies must be broadened to include more Wnt ligands and receptors. Through functional studies, the relation between specific ligands, receptors, effectors and inhibitors must be clearly identified.

Another critical point, Wnt family members are observed to be associated with reproductive hormones. Thus, wider range of studies including growth factors and other mediators related to these hormones must be done in order to investigate these factors in terms of their association with Wnt signaling pathway in terms of implantation process.

Emerging roles of Wnt signaling pathway during implantation will be clarified by investigating the relation between critical factors for implantation with Wnt ligands, receptors, effectors and inhibitors.

Implantation failure is considered as the major barrier in human fertility. The main reasons of the implantation failure can be originated from uterine factors as it is pointed out in the major number of papers, as well as from embryonic factors. Since the implantation and peri-implantation processes involve a crosstalk between the embryo and uterus, both factors must be considered to avoid implantation failure. If the roles of Wnt signaling members are better understood during these processes, implantation barrier can be overcome with the manipulation of this pathway especially through IVF technologies.

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Conflict of interest The authors declare that they have no conflict of interest.

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