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Expression and Regulation of Dickkopf2 During Periimplantation in Mice

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Abstract. Successful implantation depends on active dialogue between the maternal endometrium and the implanting blastocysts that is well controlled by groups of regulators at the molecular level. Dickkopf2 (Dkk2) is a member of Dickkopf family normally acting as an antagonist of canonical Wnt/beta-catenin signaling, which has been proven to participate in tumorigenesis and early embryo development. In order to explore the potential function of Dkk2 in embryo implantation, the present study investigated the uterine expression and regulation profiles of Dkk2 during periimplantation in mice. Using reverse transcription-polymerase chain reaction, immunohistochemistry and Western blotting, we showed that the mRNA and protein levels of Dkk2 began to increase in the glandular epithelium on day 4, continued to increase on day 5 and then decreased from day 6 of pregnancy. Moreover, on days 5–8 of pregnancy, Dkk2 was increasingly expressed in the deciduum of the uterus, especially around the implanting embryos. In addition, upregulation of Dkk2 was also observed in uteri treated with estrogen (estradiol-17 β) as well as in oil-induced artificial decidualization, indicating that the expression of Dkk2 could be induced by both steroid hormone (estrogen) and the process of decidualization. Furthermore, in the postimplantation uterus, the Dkk2 protein showed an inversed expression with active beta-catenin from day 6 onward, supporting the notion that Dkk2 plays an inhibitory role against canonical Wnt signaling in the context of the decidualizing stroma. Collectively, our data suggests that Dkk2 expression is associated with uterine receptivity changes as well as the process of decidualization and that it might play important roles through inhibition of canonical Wnt signaling in the periimplantation uterus.

Key words: Decidualization, Dickkopf2 (Dkk2), Implantation, Mouse, Uterus

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Wnt signaling pathways play important roles in various processes such as fetal development, tumorigenesis and homeostasis. When Wnt ligands bind to two receptors, Frizzled (Fz) proteins and lipoprotein receptor-related proteins 5 and 6 (LRP5/6), classic canonical Wnt signaling is activated. As a result, beta-catenin accumulates in the cytoplasm, then translocates into the nucleus and finally activates the expression of diverse Wnt target genes [1]. The Wnt signaling pathway has been demonstrated to be involved in the female reproductive tract and to play important roles in mediating estrogen-induced uterine responses [2–5]. There is also evidence that some Wnt signaling related genes exhibit spatiotemporal expression in the uterus during mouse periimplantation and that they are tightly regulated by critical implantation-related genes, such as *LIF* and *Hoxa-10* [6]. Furthermore, during mouse preimplantation embryo development and embryo activation, many Wnt signaling related genes show a dynamic expression pattern within the embryo that might determine the competency of blastocysts for implantation [7]. More recently, it has been demonstrated that embryo-derived Wnt7a can trigger an implantation-related response in the uterus, resulting in uterine upregulation of nuclear beta-catenin, a downstream event of canonical Wnt signaling activation [8]. This evidence highlights Wnt signaling as an important participator in the context of

embryo-uterine interaction during early pregnancy.

Wnt activity can be inhibited by two main classes of antagonists. The first group consists of Wnt ligand binding proteins that are structurally similar to the extracellular domains of the Frizzled (Fz) family, which exert their functions by preventing extracellular Wnt ligands from interacting with Frizzled receptors. On the other hand, the second class of antagonists, including the Dickkopf (Dkk) family proteins, do not prevent Wnt from associating with Frizzled but directly interact with LRP5/6 coreceptors to form a ternary structure, resulting in a rapid removal of the receptors via Kremen-mediated endocytosis.

Dkk2 is a member of Dkk family. It has recently been proposed that Dkk2 could normally act as a Wnt inhibitor by binding to Kremen2 (Krm2) and LRP5/6, and it appears to be involved in cell differentiation as well as epithelial-mesenchymal transformation [9–11]. This has raised our interest in determining whether Dkk2 plays an important role in embryo implantation by mediating Wnt signaling, since there have been other Wnt antagonists proven to play critical roles for the process of embryo implantation [12].

Previous work in our lab has confirmed that Dkk1 and its receptor Kremen1 played important roles during the implantation process in the mouse [13], and it has more recently been demonstrated that they could promote trophoblast cell invasion [14]. Due to the structural similarities and possible competitive/cooperative relationships of Dkk2 and Dkk1, investigation of the potential role of Dkk2 in embryo implantation is worthwhile. For this purpose,

we investigated the distribution and regulation pattern of *Dkk2* in murine uteri during periimplantation in the present study.

Materials and Methods

Animals and treatments

Adult mice (weight 22–25 g) of the outbred Kunming white strain were purchased from the Experimental Animal Center (Institute of Zoology, Chinese Academy of Sciences, Beijing, China) and raised at 25 °C with a constant photoperiod (14 h light:10 h dark cycle). The Guidelines for the Care and Use of Animals in Research were followed. Animals were allowed free access to water and food. Virgin female mice were mated with fertile or vasectomized males of the same strain. The presence of a vaginal plug was used to designate day 1 of pregnancy or pseudopregnancy. Pregnancy was confirmed by recovering embryos from the reproductive tracts during days 1–4 and the presence of implantation sites during pregnancy days 5–7. Uteri from days 1–5 of pseudopregnancy were collected for further analysis.

Artificial decidualization was induced by injection of 10 μ l sesame oil intraluminally into one uterine horn on day 4 of pseudopregnancy, whereas the contralateral horn without any infusion served as a control. Uterine tissues were collected on days 5–8 of pregnancy, and the decidualizing reaction was confirmed by increased uterine weight and histological examination.

To induce delayed implantation, pregnant mice were ovariectomized at 0800–0900 h on day 4 of pregnancy and maintained with a daily subcutaneous injection of progesterone (P4, 2 mg/mouse) from days 5–7. These animals were divided into two groups: the activated group and the dormant group. To activate dormant blastocysts and initiate implantation, the activated group mice were injected with estradiol-17 β (E2, 100 ng/mouse) on day 7. Their uteri were collected after 24 h of treatment, and only uteri with implantation sites were used. In the other group, the P4-treated mice were sacrificed for uterus collection on day 7.

To determine the effects of steroid hormones (E2, P4) on the expression of *Dkk2* in the uterus, mature female mice were ovariectomized. After a 2-week rest period, the ovariectomized mice were treated with an injection of estradiol-17 β (E2, 100 ng/mouse), progesterone (P4, 2 mg/mouse) or a combination of the same doses of progesterone and estradiol-17 β (E2+P4), respectively. The mice were sacrificed for uterus collection at 2, 4, 6, 12 and 24 h after the steroid hormone treatments. Estradiol-17 β and progesterone were dissolved in sesame oil and injected SC and the controls received the sesame oil only.

Reverse transcription-polymerase chain reaction

Total RNAs were isolated from uteri with Trizol (Gibco BRL, Grand Island, NY, USA) reagent according to the manufacturer's instructions. A total of 2 μ g RNA was reversed transcribed using Moloney Murine Leukemia Virus Reverse Transcriptase (NEB, New England, USA) and 2 μ g of random hexamer primers (Takara, Ohtsu, Japan). The primers used for amplifying mouse *Dkk2* and *18s* were as follows: the sense and antisense primers for *Dkk2* were 5-ACCCCTGCAGCAGTGATAAG-3 and 5-TGGCTTTGGAAG-AGTAGGTG-3, respectively, and the sense and antisense primers

for *18s* were 5-AATCAGGGTTCGATTCCGGA-3 and 5-CCA-AGATCCAACCTACGAGCT-3, respectively.

Amplifications were carried out with 30 cycles for *Dkk2* and 28 cycles for *18s*. The thermal cycling conditions were 94 °C for 30 sec, 58 °C for 30 sec and 72 °C for 30 sec.

Immunohistochemistry

The procedures followed a protocol described previously [15]. Mouse uteri were cut into small pieces, fixed in Bouin's solution, dehydrated and embedded in paraffin as described previously [16]. Sections (5 μ m) were cut, deparaffinized and then rehydrated. Endogenous peroxidase activity was blocked by incubating the sections in 3% hydrogen peroxide for 10 min. Nonspecific binding was blocked in 10% normal goat serum in PBS for 40 min. The sections were then incubated with the *Dkk2* (R&D, Minneapolis, USA), Kremen2 (ZYMED, South San Francisco, California, USA) or active beta-catenin (Upstate, New York, USA) antibodies, respectively, overnight at 4 °C, and were subsequently incubated with horseradish peroxidase-conjugated secondary antibodies (Zhongshan Biotechnology, Beijing, China) for 1 h at 37 °C. The second antibody was detected with 3, 3'-diaminobenzidine solution (Zhongshan Biotechnology, Beijing, China). For some sections, primary antibodies were replaced with preimmune IgGs as negative controls.

Western blot analysis

The procedures for Western blot analysis followed a protocol described previously [13] with some modifications. Protein samples were boiled in SDS β -mercaptoethanol sample buffer, and 100 μ g samples were loaded onto each lane of 15% polyacrylamide gels. After separation by electrophoresis, proteins in the gels were blotted onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). After blocking with 5% fat-free milk diluted in TBS+0.1% Tween-20 (TBST), the membranes were probed with the *Dkk2* antibody (R&D) at 4 °C overnight. Horseradish peroxidase-conjugated rabbit anti-goat IgG was then used to detect proteins by enhanced chemiluminescence (Zhongshan Biotechnology). Anti-beta-actin (Zhongshan Biotechnology) antibody was used in the same western blot as a loading control.

Results

Expression of *Dkk2* during the periimplantation period

Since the *Dkk2* transcripts have been detected in normal murine uteri [17], we first examined the expression pattern of *Dkk2* mRNA during the mouse periimplantation period by semiquantitative RT-PCR. As shown in Fig. 1A, *Dkk2* was undetectable from days 1 to 3, whereas on day 4 of pregnancy, the level of *Dkk2* mRNA was significantly increased. This high level lasted to day 7 of pregnancy, and the signal then decreased on day 8.

To further examine the localization of *Dkk2* in the pregnant uterus, immunohistochemistry was performed from day 1 to day 8. As shown in Fig. 1B, on day 1, a basal level of *Dkk2* protein was detected localizing in both the luminal and glandular epithelium. On days 2 and 3, it was barely detectable in the uteri (data not shown), which was in agreement with our RT-PCR results. On day

Fig. 1. Dkk2 expression in the uterus during the periimplantation period. A: RT-PCR analysis of *Dkk2* (upper) and *18S* (lower) genes in mouse uteri from days 1 to 8 of pregnancy. B: Immunohistochemistry analysis of Dkk2 in mouse uteri during early pregnancy on day 1 (a), 4 (b and e), 5 (c and f), 6 (d and g), 7 (h) and 8 (i) and in the negative controls (primary antibody replaced by goat IgG; j). Cross-sections were used for all samples and are shown at 40 $\times$ (b, c, d, h and i), 100 $\times$ (a, e, f, g and j), respectively. Le, luminal epithelium; ge, glandular epithelium; em, embryo; de, deciduas.

4 of pregnancy, a highly increased level of Dkk2 was observed, especially localized in the glandular epithelium. From day 5 onward, Dkk2 protein began to increase in the primary decidualization zone, initially around the implanting embryo, and this was followed by a rapid increase on days 6 to 8 in the decidualizing area. On the other hand, while the level of Dkk2 remained high in the glandular epithelia of day 5 uteri, it decreased significantly in the day 6 uteri. In fact, there were only small parts of the glandular epithelia of day 6 uteri that were still expressing Dkk2, and these parts exhibited much weaker staining.

Expression of Dkk2 in pseudopregnant and delayed implantation mouse uteri

The expression pattern of Dkk2 for days 1 to 4 of pseudopregnancy is similar to that for normal pregnancy. Nonetheless, on day 5 of pseudopregnancy with the absence of an implanting embryo, the expression of Dkk2 was significantly decreased in both the luminal and glandular epithelium compared with that of the normal pregnancy (only days 4 and 5 are shown in Fig. 2).

In the delayed implantation model, the uterus remains in the receptive state under the control of P4 while the blastocysts

Fig. 2. Immunohistochemical analysis of Dkk2 protein during pseudopregnancy and delayed implantation. The expression patterns of Dkk2 on days 4 (A) and 5 (B) in pseudopregnant uteri, in the P4-treated delayed uterus on day 7 of pregnancy (C), and at the implantation site after E2 treatment in the delayed uterus (D) are shown at their original magnification, $\times 200$.

undergo dormancy, and implantation does not occur until an estrogen injection rapidly activates the blastocysts. The P4-primed uteri contained a basal level of Dkk2 protein in the luminal epithelium (Fig. 2C). After embryo implantation was initiated by estrogen, Dkk2 protein was strongly detected in luminal epithelial cells along with an increasing density in the subepithelial stroma around the implantation chamber (Fig. 2D), and this was similar to the expression pattern observed on day 5 of pregnancy.

Expression of Dkk2 in mouse uterus of artificial decidualization

To determine whether embryonic signals influence the expression of Dkk2 during decidualization, artificial decidualization was induced in one uterine horn by intrauterine oil infusion on day 4 of pseudopregnancy, while the contralateral horn without treatment served as a control. Dkk2 protein was unobservable in the control uterine horn, while a remarkable signal could be detected in the decidualized cells that was maintained until day 8 of pseudopregnancy with artificial decidualization (Fig. 3), and this was similar to that observed in normal pregnancy from days 5 to 8.

Effects of steroid hormones on Dkk2 expression

Dkk2 was notably induced on day 4 of pregnancy, at which point the uterus is exposed to rising levels of progesterone from corpora lutea with a small surge of estrogen. This expression pattern suggested that induction of Dkk2 might be regulated by steroid hormones. To validate this hypothesis, ovariectomized mice were treated with single injections of E2, P4 or a combination of E2 and P4 (E2+P4), respectively. As shown in Fig. 4, the level of Dkk2 protein was low in the uteri of ovariectomized animals. In the E2-treated group (Fig. 4A), the protein level gradually increased after treatment, with a peak at 6 h post-injection, and this level lasted for 24 h. In the P4-treated group (Fig. 4B), there was no evident

Fig. 3. Immunohistochemical analysis of Dkk2 protein in artificial decidualization uteri. This figure shows the expression patterns of Dkk2 in control uterine horns (A) and in the oil-induced deciduum on days 5 (B), 6 (C), 7 (D) and 8 (E) of pseudopregnancy. F is the negative control. Original magnification: $\times 200$.

Fig. 4. Ovarian steroid hormone regulation of Dkk2 in the uteri of ovariectomized mice. Dkk2 was induced by E2 (A) and E2+P4 (C), but not by P4 (B) alone. This figure shows immunostaining analysis of Dkk2 in the uteri 2 h (b, h and n), 4 h (c, i and o), 6 h (d, j and p), 12 h (e, k and q) and 24 h (f, l and r) after steroid hormone treatment and in the control uterus horns (a, g and m). Original magnification: $\times 200$. Western blotting (on the right side) also showed the same time-dependent expression pattern of Dkk2. β -actin was used as a loading control. Similar results were observed in two independent experiments.

change in the Dkk2 signal, which remained at a low level in the uteri. In the E2+P4 group (Fig. 4C), a similar effect was observed as seen in the E2 group. The western blot results further confirmed the results of immunohistochemistry.

Expression of active beta-catenin in the periimplantation period

Previous studies have shown that Dkk2 function is related to

Fig. 5. Expression of Krm2, Dkk2 and active β -catenin (ABC) during decidualization. Immunostaining showed Krm2 (a, d, g and j), Dkk2 (b, e, h and k) and ABC (c, f, i and l) localization in the mouse uteri on days 6 ($\times 40$, $\times 200$) and 7 ($\times 40$, $\times 200$) of pregnancy. Em, embryo; de, deciduas; s, stroma.

canonical Wnt/ β -catenin signaling by binding to its receptor Kremen2 (Krm2). We first detected the localization of Krm2 during decidualization, but found that Krm2 is not colocalized with Dkk2 as shown in Fig. 5. In addition to investigating the relationship of Dkk2 and dephosphorylated (active) β -catenin (ABC) during implantation, we prepared serial sections of mouse periimplantation uterus and examined the expression of Dkk2 and active β -catenin with immunostaining. As shown in Fig. 5, Dkk2 expression was restricted to around the implanting embryo on days 6 and 7. However, there was no or very weak expression of active β -catenin in the same areas and strong staining in the outer layer of deciduas where there was no Dkk2.

Discussion

In this study, the expression and regulation patterns of Dkk2 in the mouse uterus during periimplantation were examined. The Dkk2 protein was highly detected in both the epithelial cells of the preimplantation uterus and deciduum of the postimplantation uterus, and these expression patterns were induced by estrogen and the process of decidualization separately.

Dkk2, a typical secretory protein, was strongly expressed in the uterine glandular epithelium on days 4 and 5 of pregnancy and then decreased on day 6, and this was associated with uterine receptivity changes during this period. In fact, its expression pattern was somewhat similar to LIF, which has been demonstrated to play critical roles for uterine preparation and embryo implantation [18–20]. Indeed, there is evidence that Wnt signaling and LIF mediate over-

lapping functions in maintenance of the pluripotency of mouse ES cells [21]. Furthermore, some members of the Wnt family, such as *Wnt4* and *sFRP4*, have been reported to aberrantly express in the uterus of *Lif* null mice [6]. However, the potential interactions between *Dkk2* and LIF signaling in the context of embryo implantation are still unknown and await further study.

Another characteristic feature of *Dkk2* expression was that it markedly increased around implanting embryos with the initiation and expansion of decidualization. Decidualization is a biological process in which uterine endometrial stroma cells proliferate and differentiate into large epithelioid decidual cells. In the uteri of pregnant mice, the differentiating stromal cells surrounding the blastocysts initially formed a primary decidual zone (PDZ) on day 5, and the secondary decidual zone (SDZ) began to be formed around the PDZ on day 6. Our results showed that expression of *Dkk2* could be upregulated in the decidualizing stroma by both the embryo and oil, suggesting that there is tight correlation with the process of decidualization. Several genes have been proven to be critical for the process of decidualization, and some of them, such as *BMP2*, share similar expressional features to *Dkk2* [22]. *BMP2* has been demonstrated to be a critical gene for decidual response in mice and humans [23–25]. Uterine specific ablation of *BMP2* gene has resulted in complete infertility due to defective decidualization in mice [25]. Indeed, *Dkk2* has been reported to regulate *BMP2*-induced osteoblast differentiation through downregulation of Wnt/ β -catenin signaling [23]. However, the exact role of *Dkk2* and its hierarchical interaction with *BMP2* and other decidualization related genes requires further detailed exploration.

In the present study, we observed a significant increase of *Dkk2* on day 4 of pregnancy, and this coincided with the preimplantation estrogen surge of E2 from corpora lutea. Using an established hormone treatment protocol, we further demonstrated that *Dkk2* is upregulated by estrogen in the absence of an implanting embryo, with a peak at 6 h after E2 treatment. It has previously been demonstrated that several members of the Wnt signaling pathways are regulated by estrogen in the mouse uterus without the involvement of classical estrogen receptors (ERs), including *Wnt4*, *Wnt5*, *frz2*, *sfrp2* and active β -catenin [26]. Moreover, a report from Suzuki *et al.* showed that exposure to diethylstilbestrol (DES), an analog of E2, significantly induces expression of *Dkk2* in mouse oviducts and uteri [27]. The results of the present study revealed another member of the Wnt signaling pathway that is regulated by E2. In mice, steroid hormone waves orchestrate the changes in the uterine epithelium. During early pregnancy, estrogen stimulates luminal and glandular epithelium proliferation and differentiation, making the uterus receptive to blastocysts so that implantation can be initiated. This effect is achieved through activation of a myriad of downstream target genes, and the exact role of *Dkk2* in this complex system requires further investigation.

There is established evidence demonstrating that Wnt signaling plays critical roles in the process of implantation. For example, targeted gene deletion of *Wnt7a* in mice results in the complete absence of uterine glands and infertility [28], and *Fzd4*^{−/−} mice fail to become pregnant and exhibit impaired corpora lutea formation and function [12]. Under normal conditions, formation of the primary and secondary decidual zones may be driven by Wnt pathway

activity [29]. *Dkk2* normally functions as an antagonist of canonical Wnt signaling by binding to Kremen2 (*Krm2*) to inhibit β -catenin activity, but other data shows that *Dkk2* can activate rather than inhibit the Wnt/ β -catenin signaling pathway in *Xenopus* embryos [30]. In the present investigation, our colocalization experiment of *Dkk2* and active β -catenin further suggested an inhibitory effect for *Dkk2* against active β -catenin in the context of the decidualizing uterus. However, our results showed that *Krm2* did not co-exist with *Dkk2* in this area on days 6 and 7, which indicated that *Dkk2* exerted its function without binding to *Krm2* in this context. This phenomenon has been partially explained by the recent finding that *Krm2* can function in the Wnt pathway in the absence of *Dkks* [30]. Given the results above, it is possible that *Dkk2* acted as an inhibitor of canonical Wnt/ β -catenin in decidualization through a receptor(s) other than *Krm2*. Further detailed studies are needed to uncover the underlying mechanisms.

Our previous study demonstrated that *Dkk1* participated in mouse decidualization [13], and the present study suggests that *Dkk2* might be another member involved in this process. Indeed, there is much evidence suggesting that *Dkks* take part in brain, lens and tooth development in mice, all of which involve the process of epithelial-mesenchymal transformation [1, 17, 31, 32], and are hallmarks of uterine decidualization. In fact, *Dkk2* homozygous mutant mice are viable and fertile, but smaller in size than their wild-type littermates. Mahua *et al.* [10] attributed this phenomenon to a bone density defect. In the present study, we suggested the alternative explanation that the *Dkk2* mutant mouse could have defective implantation and decidualization that could potentially harm further development.

In conclusion, this study established the expression and regulation profiles of *Dkk2* within the context of the mouse periimplantation uterus. Our data suggests that *Dkk2* might play an important role by taking part in Wnt signaling during embryo implantation.

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