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Attenuated retinoic acid signaling is among the early responses in mouse uterus approaching embryo attachment

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Abstract

The uterus is transiently receptive for embryo implantation. It remains to be understood why the uterus does not reject a semi-allogeneic embryo (to the biological mother) or an allogeneic embryo (to a surrogate) for implantation. To gain insights, we examined uterine early response genes approaching embryo attachment on day 3 post coitum (D3) at 22 hours when blue dye reaction, an indication of embryo attachment, had not manifested in mice. C57BL/6 pseudo-pregnant (control) and pregnant mouse uteri were collected on D3 at 22 hours for microarray analysis. The self-assembling-manifold (*SAM*) algorithm identified 21,858 unique probesets. Principal component analysis indicated a clear separation between the pseudo-pregnant and pregnant groups. There were 106 upregulated and five downregulated protein-coding genes in the pregnant uterus with fold change (fc) >1.5 and *q* value <5%. Gene ontology (GO) analysis of the 106 upregulated genes revealed 38 significant GO biological process (GOBP) terms (P <0.05), and 32 (84%) of them were associated with immune responses, with a dominant natural killer (NK) cell activation signature. Among the top eight upregulated protein-coding genes, *Cyp26a1* inactivates retinoic acid (RA) while *Lrat* promotes vitamin A storage, both of which are expected to attenuate RA bioavailability; *Atp6v0d2* and *Gjb2* play roles in ion transport and transmembrane transport; *Gzmb*, *Gzmc*, and *Il2rb* are involved in immune responses; and *Tdo2* is important for kynurenine pathway. Most of these genes or their related pathways have functions in immune regulations. RA signaling has been implicated in immune tolerance and immune homeostasis, and uterine NK cells have been implicated in immunotolerance at the maternal-fetal interface in the placenta. The mechanisms of immune responses approaching embryo attachment remain to be elucidated. The coordinated effects of the early response genes may hold the keys to the question of why the uterus does not reject an implanting embryo.

Keywords: Embryo attachment, Pseudo-pregnant uterus, Pregnant uterus, Natural killer cell activation signature, Retinoic acid bioavailability, Transmembrane transport and ion transport

Embryo attachment to the uterine luminal epithelium (LE) is an initial process of embryo implantation, which is essential for mammalian reproduction. Because of increased vascular permeability at the site of embryo attachment, embryo attachment in rodents can be visualized as faint blue bands along the uterine horns following intravenous injection of blue dye, a phenomenon called "blue dye reaction." It becomes detectable around midnight of day 4 post coitum (D4 at 0 hour, equivalent to D3 at 24 hours) in mice, depending on the strains and individual variations within the same strain. In C57BL/6 mice, faint blue bands along the uterine horns start to appear on D3 at 22–24 hours[1]. We defined the "approaching embryo attachment" time window for C57BL/6 mice on D3 at 22 hours based on two criteria: 1) lack of faint blue bands to indicate that the uterus has not fully progressed to embryo attachment that could be visualized by the blue dye reaction, and 2) presence of healthy-looking blastocysts to confirm on-time pregnancy.

An embryo is semi-allogeneic to the biological mother and it is allogeneic to a surrogate. It remains to be understood why the uterus does not reject an embryo and becomes transiently receptive for embryo implantation. We hypothesized that early uterine response genes approaching embryo attachment might offer valuable insights and tested the hypothesis in mice. Young C57BL/6 female mice were randomly assigned to pseudo-pregnant group (control, mated with vasectomized males) and pregnant group (mated with stud males). They were checked every morning for a vaginal plug to indicate mating activity on the previous night. The mating night was defined as day 0 post coitum (D0). On D3 at 22 hours, mice were intravenously injected with Evans blue dye and dissected 5 minutes later for detecting blue dye reaction to indicate embryo attachment[1,2].

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The uterine horns were flushed with 1× phosphate buffer saline and the uterine flushing was examined for the presence and developmental stage of embryos. The uterine horns from the pseudo-pregnant mice had no blue dye reaction and no embryos. In the pregnant group, only the mice with uterine horns that had no faint blue bands but healthy-looking blastocysts flushed were included. Uterine tissues were processed for RNA isolation and microarray profiling (n = 4 mice/group). The differentially expressed genes between the pseudo-pregnant uteri and pregnant uteri represented uterine early response genes approaching embryo attachment.

We retained 21,858 probesets with unique annotation from a total of 35,556 probesets in the microarray dataset (gene expression omnibus [GEO] number: GSE247638). Principal component analysis visually demonstrated a distinct separation between the pseudo-pregnant group and pregnant group (Fig. 1A). Using the self-assembling-manifold (*SAM*) algorithm[3] and employing criteria of fold change (fc) >1.5 and q value <5%, we identified 106 upregulated and 10 downregulated probesets in the pregnant group (Fig. 1B), with the latter containing only five protein-coding genes (Table S1, http://links.lww.com/RDM/A41). A more lenient threshold of fc >1.2 and q value <10% resulted in 858 upregulated and 334 downregulated probes in the pregnant group (Table S1, http://links.lww.com/RDM/A41). These early response genes predominantly express in the endometrium, which encompasses the uterine luminal epithelium (LE), as well as the glandular epithelium (GE), stromal cells, immune cells, and endothelial cells within the stromal compartment.

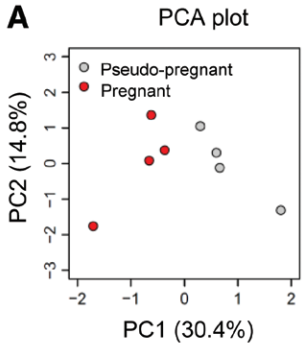
The uterine epithelium undergoes dynamic changes during embryo implantation[4]. LE is the normal site for embryo attachment. Among the most upregulated and downregulated protein-coding genes (Fig. 1C), *Atp6v0d2* (ATPase H^+ transporting V0 subunit D2)[5,6], *Cyp26a1* (Cytochrome P450 family 26 subfamily A member 1)[7], *Gjb2* (gap junction protein beta 2)[8], *Npl* (N-acetylneuraminate pyruvate lyase)[6,9], and *Fxyd4* (FXYD domain containing ion transport regulator 4)[6] are specifically or mainly (*Cyp26a1*) expressed in the LE. The upregulation or downregulation of these genes in the pregnant uteri (compared to the pseudo-pregnant uteri) on D3 at 22 hours (Fig. 1Da) were consistent with that in the post-embryo attachment D4.5 LE (compared to the pre-embryo attachment D3.5 LE) by microarray (Fig. 1Db) (GEO number: GSE44451)[6] and RNA-seq[10]. Certain genes exhibited wide individual variations, such as *Atp6v0d2* (Fig. 1Dc), likely attributable to individual variations in embryo attachment timing[1]. GE-specific genes, such as *Prss28* and *Prss29*[11], were among the early response genes (Fig. 1C). *Lif*, a GE-specific gene, and its receptor *Lifr*, an LE-specific gene, are critical for embryo implantation[12]. Interestingly, *Lifr*, but not *Lif*, was an early response gene (Table S1, http://links.lww.com/RDM/A41).

The stromal layer beneath the embryo attachment site undergoes sequential changes in the early hours of embryo attachment, including increased blood vessel permeability (visualized by the blue dye reaction), local edema, appearance of alkaline phosphatase (ALPL), and histological decidualization[13]. The manifestation of decidualization marker genes, such as *Abp1*, occurs a few hours after the blue dye reaction, around D4 at 4 hours in C57BL/6 mice[1]. While *Alpl* was upregulated in the pregnant uteri on D3 at 22 hours, *Abp1* did not show significant changes (Table S1, http://links.lww.com/RDM/A41). *Tdo2* (tryptophan 2,3-dioxygenase), a stroma-specific gene that is upregulated in the decidualized endometrium[14], was among the most upregulated genes in the pregnant uteri (Fig. 1C). TDO2 catalyzes the metabolism of tryptophan into kynurenine, and the kynurenine pathway is implicated in establishing and maintaining immune-privileged sites[15]. Despite normal fertility in the TDO2-deficient mice[14], any potential contribution of TDO2 to immune regulation at the embryo attachment site cannot be entirely ruled out. Embryo implantation coincides with an increased number of immune cells in the stroma[16], and immune-related genes, such as granzymes (*Gzmb*, *Gzmc*, *Gzmd*), were among the most upregulated genes in the pregnant uteri (Fig. 1C).

The gene ontology (GO) analysis unveiled a predominant upregulation of immune-related GO biological process (GOBP) terms in the pregnant uteri. Among the 38 significant GOBP terms (P <0.05) associated with the 106 upregulated probesets (fc > 1.5, q value < 5%) (Fig. 1E, Table S2, http://links.lww.com/RDM/A42), 32 (84%) were linked to immune responses, specifically, enhanced immune activation and inflammation in the pregnant uterus. Prominent among them were cytotoxicity genes such as cytotoxic mediators (granzymes GZMB, GZMC, GZMD, and natural killer cell granule protein 7 [NKG7]), NK cell surface markers (killer cell lectin-like receptors [KLRs]), chemokines and chemokine receptors involved in immune cell recruitment (CCL6, XCL1, CCR2, CCR3), as well as innate immune cell recognition and signaling (TLR13, EOMES, AIM2, STAT4). Collectively, the data are most consistent with an NK cell activation signature, which continues during the embryo implantation process as NK cells are highly enriched in the decidual compartment on D7.5[17]. Additionally, *Lilrb3* and *Lilrb4* (leukocyte immunoglobulin-like receptor subfamily B members) exhibited upregulation (Table S1, http://links.lww.com/RDM/A41). The known function of LILRB4 in negatively regulating NK inflammation suggests that the uterine upregulation of *Lilrb4* may serve to suppress inflammation upon embryo attachment.

Other significant GOBP terms (P <0.05) included response to retinoic acid, cell surface receptor signaling pathway, protein maturation, regulation of cell migration, osteoclast differentiation, and positive regulation of gene expression, etc. (Fig. 1E, Table S2, http://links.lww.com/RDM/A42). Of note were GOBP terms (P >0.05) "transmembrane transport" and "ion transport" that included some of the most upregulated genes (eg, ATP6V0D2, GJB2, TRPV6, and TRPM6) (Fig. 1C). ATP6V0D2 is a subunit in V-ATPase for H^+ transport and GJB2 is a gap junction protein. We demonstrated that they were early response genes in the LE upon embryo attachment, and inhibitors of V-ATPase or gap junctions impaired uterine transformation for embryo implantation[5,8]. The upregulation of *Atp6v0d2* is coincident with uterine epithelial acidification, most likely lysosomal acidification, upon embryo attachment[5]. *Gjb2* is the dominant gap junction protein gene in the LE upon embryo implantation[6]. Its initial upregulation, specifically in the LE of embryo attachment site, signifies enhanced intercellular communications in the LE to prepare for embryo implantation[8].

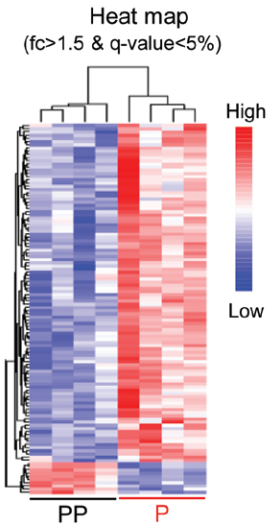
The GOBP term "response to retinoic acid (RA)" includes CYP26A1, GJB2, TFRC, LRAT (Table S2, http://links.lww.com/RDM/A42). Three of the genes, *Cyp26a1*, *Gjb2*, and *Lrat*, are among the top eight most upregulated genes (q < 5%, Fig. 1C, and Table S1, http://links.lww.com/RDM/A41). RA, a physiologically active metabolite of vitamin A (retinol), plays a vital role in reproduction[18]. RA bioavailability is regulated by multiple factors[19], such as the storage of vitamin A as retinyl esters by lecithin-retinol acyltransferase (LRAT/*Lrat*), RA synthesis by retinol dehydrogenases (RDH/*Rdh*), and retinaldehyde dehydrogenases (RALDH), and RA inactivation by CYP26A1/*Cyp26a1*, as well as binding proteins[19] and retinol carrier transthyretin[20],

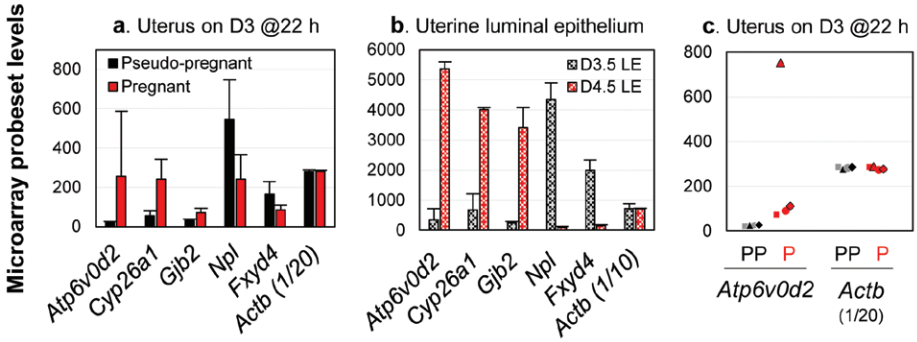


C

Top 30 upregulated and all downregulated protein-coding genes with fc>1.5 & q-value <10%

q-value	Up (preg/pseudo-preg = 6.4~1.9)	Down (preg/pseudo-preg = 0.38~0.66)
<5%	***Atp6v0d2***, *Tdo2*, ***Cyp26a1***, *Prss28*, *Prss29*, *Gzmb*, *Guca2b*, ***Gjb2***, *Gzmc*, *Il2rb*, *Lrat*, *Ms4a14*, *Gzmd*, *Ncr1*, *Bmp2*, *Trpv6*, *Clec4a3*, *Gp49a*, *Trpm6*, *Rrm2*, *Klri2*, *Cwh43*, *Klrd1*, *Pyhin1*, *E2f8*, *Nkg7*, *Cd53*, *Stat4*, *Tnc*, *Klrb1f*	*Taf1d*, ***Npl***, *Slfn3*, *Gpr128*, ***Fxyd4***, *Slc15a2*, *Myd88*, *Slc24a4*, *Galm*, *Inha*, *Mab21l3*, *Rdh9*, *Cbr2*, *Pfkfb3*, *Cdc23*, *Meig1*, *Vaultrc5*, *Thrsp*, *Wfdc6a*, *Tmem38b*, *Rnu5g*, *Olfr767*, *Ube2ql1*, *Msx1*, *Atp1b1*, *Ces1d*
5-10%		





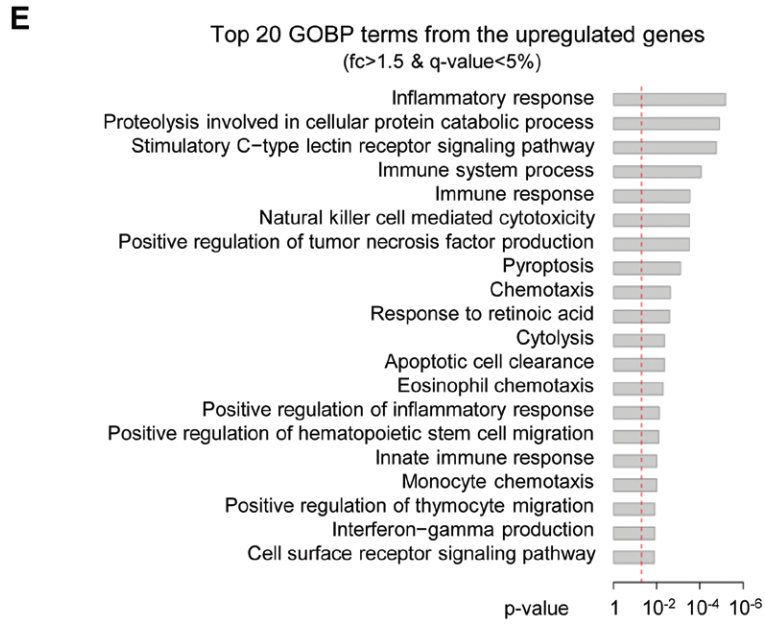


Fig. 1. Microarray detection of uterine early response genes prior to embryo attachment in mice. (A) PCA of all eight samples. Separation of the pregnant (red dots) and pseudo-pregnant (gray dots) mainly by PC1 (30.4%). (B) Heat map of the differential expressed probesets with fc >1.5 and *q* value <5%. PP, uteri from pseudo-pregnant mice, which were mated with vasectomized mice; P, uteri from pre-embryo attachment pregnant mice, which were verified by the presence of blastocysts but absence of blue dye reaction; fc >1.5: P/PP>1.5 for upregulated probesets and P/PP<0.6667 (1/1.5) for downregulated probesets in the pregnant uterus. (C) The top 30 upregulated and all downregulated protein-coding genes with fc >1.5 and *q* value <10%, arranged by fc values from high to low for upregulated genes and low to high for downregulated genes. Blue, *q* value <5%; black, *q* value = 5%–10%; bold, LE-specific genes or LE-dominant gene (*Cyp26a1*) shown in (D). The fc values using *SAM* algorithm in (C) may differ from those using RMA linear values shown in (D). (D) Comparisons of expression levels of selected LE-specific/dominant genes in two microarray datasets, pseudo-pregnant and pregnant mouse uteri on D3 at 22 h (n = 4/group, GEO number: GSE247638) (a) and pregnant uterine LE on D3.5 (D3 at 11 h, pre-embryo attachment) and D4.5 (D4 at 11 h, post-embryo attachment) (n = 3/group, GEO number: GSE44451) (b). Error bar, standard deviation. (c) Individual probeset levels of *Atp6v0d2* and loading control *Actb* in the uterus on D3 at 22 h. (E) Top 20 GOBP terms (P <0.05) from upregulated genes in the pregnant uteri with fc >1.5 and *q* value <5%. Fc: fold change; GEO: gene expression omnibus; GOBP: gene ontology biological process; LE: luminal epithelium; PCA: principal component analysis; *SAM*: self-assembling-manifold; RMA: Robust Multi-array Average.

etc. RA signaling involves RA bioavailability and RA binding to retinoic acid receptors (RAR) and retinoid X receptors. Uterine RAR-mediated RA signaling proves crucial for embryo implantation[21]. The upregulation of *Lrat*, which promotes vitamin A storage, and *Cyp26a1* (Fig. 1C, Table S1, http://links.lww.com/RDM/A41), which inactivates RA, as well as the downregulation of *Rdh9* ($q < 10\%$, Table S1, http://links.lww.com/RDM/A41), which promotes RA synthesis, would suggest attenuated uterine RA bioavailability and a fine balance of uterine RA signaling approaching embryo attachment. RA signaling has been implicated in immune tolerance and immune homeostasis[22,23], its specific role in immune tolerance during embryo attachment warrants further investigation.

Among the four upregulated genes classified in the GOBP term "response to retinoic acid (RA)" (Table S2, http://links.lww.com/RDM/A42), we validated the initial upregulation of GJB2/*Gjb2* in the LE at the embryo attachment site[8]. A notable limitation of this current study was the lack of confirmation for the differential expression of *Cyp26a1* (4.38x, $q < 5\%$), *Lrat* (2.13x, $q < 5\%$), and *Rdh9* (0.59x, $q < 10\%$), which are involved in regulating RA bioavailability, in the D3 at 22 hours pseudo-pregnant and pregnant uterine tissues. This limitation can be partially mitigated by information in the literature. The upregulation of *Cyp26a1* in the uterine epithelium upon embryo implantation has been well-documented[7,24]. Our microarray analysis (GEO number: GSE44451) of D3.5 LE (pre-embryo attachment) and D4.5 LE (post-embryo attachment) showed upregulation of *Cyp26a1* (5.98x, $P < 0.01$) and *Lrat* (3.27x, $P < 0.05$) and downregulation of *Rdh9* (0.31x, $P < 0.0001$) in the D4.5 LE[6]. Similarly, one study reported upregulation of *Cyp26a1* and *Lrat* in the LE (collected *via* microdissection) of pregnant C57BL/6 mice on pre-embryo attachment D3 PM (unspecified afternoon hours) compared to D3 AM (unspecified morning hours) using RNA-seq, and this upregulation continued further on post-embryo attachment D4 AM[10]. This study also showed downregulation of *Rdh9* from D3 to D4 AM, while there was no obvious difference between D3 AM and D3 PM[10]. These temporal regulation patterns indicate that *Cyp26a1* and *Lrat* are upregulated before embryo attachment and may respond earlier than *Rdh9* to the approaching embryo attachment.

Since the uterine functions during early pregnancy are under the control of estrogen (E_2)-estrogen receptor α (ERα) signaling and progesterone (P4)-progesterone receptor (PR) signaling, we searched the binding sites for all the genes in Fig. 1C in the publicly available ChIP-seq database, CistromeDB. Approximately half of these genes exhibited distinctive binding peaks for both ERα and PR in ovariectomized mouse uteri based on the genome browser peaks, suggesting coordinated regulation by ERα and PR (Table S3, http://links.lww.com/RDM/A43)[25,26]. Since the ChIP-seq data were derived from ovariectomized mouse uteri treated with E2 for 1 hour[25] or P4 for 24 hours[26], these experimental conditions may not capture all the binding sites of ERα and PR in their target genes at the time of embryo attachment.

Given that this study employed whole uterine tissues for microarray analysis, it is anticipated that certain cell type-specific changes may be obscured in the whole uterus; in addition, only 61.5% of the probesets in our microarray were unique probesets and microarray did not cover all the transcripts. Despite these limitations, this well-controlled study still provides important information on the uterine early response genes approaching embryo attachment. The obtained data suggest that immune responses with an NK cell activation signature, attenuated RA signaling, and coordinated transmembrane transport and ion transport may hold keys to the question why the uterus does not reject an implanting embryo.

Supplemental materials

Supplementary information is linked to the online version of the paper on the *Reproductive and Developmental Medicine* website.

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Author contributions

X.Y. conceived the project; H.D., S.X., and X.Y. designed the experiments; H.D. performed majority of the animal studies; S.X. participated in data collection; T.Z. did bioinformatics analysis of the microarray data; T.E.M. extracted ERα and PR binding sites from CistromeDB; W.T.W. and X.Y. analyzed the immune aspect of the data; X.Y. and T.Z. drafted the manuscript; all authors participated in manuscript revisions.

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Conflict of interest

All authors declare no conflict of interest.

Data availability statement

Data available on request.

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