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Transcript analysis identifies differential uterine gene expression profile beyond the normal implantation window in mice

Qiliang Xin [a, 1], Meiling Li [a, 1], Haibin Wang [b, c], Sheng Cui [a], Guoliang Xia [a, **], Shuangbo Kong [b, c, *]

[a] State Key Laboratory of Agro-biotechnology, College of Biological Sciences, China Agricultural University, Beijing 100193, PR China
[b] Reproductive Medical Center, The First Affiliated Hospital of Xiamen University, Medical College Xiamen University, Xiamen, Fujian 361102, PR China
[c] Fujian Provincial Key Laboratory of Reproductive Health Research, Medical College Xiamen University, Xiamen, Fujian 361102, PR China

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ABSTRACT

Uterine receptivity is defined as a state when the uterine milieu is favorable for blastocyst implantation and it can only last for a limited time period. In this study, by utilizing the embryo transfer model, it was observed that a portion of blastocysts could initiate implantation even when transferred beyond the timing of normal uterine receptivity, while their mid-gestational embryo development exhibited severe retardation, suggesting that the uterine status beyond the normal implantation window is unconducive for normal implantation. We further performed microarray analysis to explore the molecular basis that distinguishes the normal and defective uterine receptivity. A total of 229 genes was found to be differentially expressed, and a large amount of them were epithelium-expressing genes and responsive to progesterone signaling.

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1. Introduction

Embryo implantation is one of the crucial steps in mammalian embryo development. In placental mammals, uterine sensitivity to implantation is classified into pre-receptive, receptive and non-receptive (refractory) phases [1–3]. In normal pregnancy, when implantation-competent blastocysts are ready to initiate implantation, the uterus differentiates into an altered state called uterine receptivity, which sustained for a limited time period. In mice, the normal uterine receptive phase is on day 4 of pregnancy or pseudo-pregnancy (day 1 = day of vaginal plug). At this stage, the uterine environment is conducive to blastocyst growth, attachment and subsequent events of implantation. In contrast, the uterine environment is unfavorable to blastocyst survival in the refractory phase after day 5 of pregnancy [1,4]. Embryo implantation is a dynamic developmental event that involves a series of physical and physiological interactions between the blastocyst trophectoderm and various endometrial cell-types, including both the uterine epithelial and stromal compartment. It has been observed that the ultrastructure of luminal epithelium (LE), such as LE cell surface components, lateral adherent junctions and gap junction channels, need remolding during the establishment of uterine receptivity. The morphological transformations are likely associated with the differential gene expression in the LE, which would affect the trophectoderm-uterine epithelium crosstalk that fosters the normal blastocyst implantation. In response to the implanting embryo, the surrounding uterine stroma undergoes extensive cellular proliferation and transformation, a process known as decidualization, to accommodate embryonic growth and invasion. Decidualization can also occur in response to artificial stimulus by oil injection or endometrium scratch in the receptive uterus to form deciduoma.

In mice, progesterone (P4), coordinating with estrogen (E2), is obligatory for uterine preparation for a receptive state [1,5–7]. In mice, there are two main P4 receptor isoforms as PRA and PRB, arising from alternative promoter usage in the same gene *Pgr* [8]. The P4 mainly functions through its nuclear receptor PRA to coordinate the molecular networks for the establishment of uterine

Abbreviations: LE, Luminal epithelium; PSP, Pseudo-pregnancy; P4, Progesterone; E2, Estrogen; IS, Implantation site; Cyp26a1, Cytochrome P450 26A1; PR, Progesterone receptor.

* Corresponding author. Fujian Provincial Key Laboratory of Reproductive Health Research, Medical College Xiamen University, Xiamen, Fujian 361102, PR China.
** Corresponding author.
E-mail addresses: glxiachina@sohu.com (G. Xia), Shuangbo_kong@163.com (S. Kong).

[1] Authors contributed equally to this work.

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receptivity in mice. Numerous defects in female mice lacking the *Pgr* gene that encodes PR have been demonstrated, including failure in ovulation, mammary gland development, and embryo implantation along with uterine hyperplasia and inflammation, reflecting the critical role of P4/PR pathway in female reproduction [9]. Furthermore, the molecular and genetic evidence has also indicated that locally produced signaling molecules in the uterus, including cytokines, homeobox transcription factors, coordinate with these hormone receptors and serve as autocrine, paracrine and juxtacrine factors to specify the uterine receptivity [8].

In this study, by using the embryo transfer strategy, we showed that a portion of blastocysts can initiate implantation even when transferred on day 5 beyond the normal timing of uterine receptivity, while the mid-gestational development of these embryos exhibited severe retardation, suggesting that the uterine status on day 5 of pseudopregnancy (PSP) is unconducive for normal implantation. So we inferred that critical physiological changes occur in the uteri between days 4 and 5 of PSP. In order to explore the underlying molecular differences between these two different uterine states, we applied the microarray approach to analyze the uterine gene expression profile on day 4 *versus* day 5 of PSP. Using this approach, here we show that, 229 out of about 24, 000 genes examined were significantly differentially expressed between days 4 and 5 PSP uteri. Further analyses revealed that a large proportion of (46.7%) differentially expressed genes were uterine epithelium-expressing genes and responsive to P4 signaling, which may compromise the epithelial cell differentiation program and thus the normal implantation.

2. Materials and methods

2.1. Mice

Adult 8 weeks CD-1 male and female mice used in this study were purchased from the Vital River Laboratory Animal Technology Co. Ltd. All animal experiments were performed in accordance with the guidelines and regulations of the Institutional Animal Care and Use Committee of China Agricultural University. Adult female mice were mated with intact or vasectomized males of the same strain to induce pregnancy or PSP, respectively. The morning of seeing a vaginal plug was defined as day 1 of pregnancy or PSP.

2.2. Embryo transfer and examination of pregnancy outcome

Day 4 blastocysts from the normal pregnant female donors were transferred to the uteri of recipients in the morning (10:00 h) on days 4–6 of PSP. Recipient mice were sacrificed to examine the implantation status at 10:00 h on indicated days. The number of implantation site (IS) was recorded by tail vein injection (0.1 ml/mouse) of Chicago Blue dye (Sigma-Aldrich, C8679) solution (1% in saline) at 48 h after embryo transfer. Mice were sacrificed 5 min later and ISs were demarcated as discrete blue bands along the uterine horns since the vascular permeability is increased at the implantation sites [10,11]. In the mice without implantation sites, the uterine horns were flushed with saline to recover the unimplanted blastocysts. Mice without implantation sites but no recovery of blastocysts were excluded from the experiments. Recipients were examined for subsequent developmental events on day 12 or observed for delivery of pups at term. For each time point, at least 5 mice were included for the analysis.

2.3. Experimentally induced decidualization in pseudo-pregnant mice

To induce artificial decidualization, 25 μl of sesame oil (Sigma-S3547) was infused intraluminally in one uterine horn on days 4–6 of PSP. The contra-lateral horn served as control. At least 4 mice were examined in each time point for this experiment. Mice were sacrificed 96 h later. Uterine weights of the infused and non-infused (control) horns were recorded. Fold increases in uterine weights were used as an index of decidualization [12].

2.4. RNA extraction, amplification, labeling and hybridization

For days 4 and 5 PSP mouse uteri ($n = 4$ mice per group), the samples were pooled and total RNA were extracted from the mouse uteri using Trizol reagent (Invitrogen-15596018, Carlsbad, CA) according to manufacturer's instructions. The RNA was cleaned up with RNeasy Kit (Cat #74104, Qiagen, Hilden, Germany) and the quantities and qualities were determined by spectrophotometer and 1% formaldehyde denaturing gel electrophoresis.

Affymetrix GeneChip mouse Genome Array, which contains more than 45,000 probe sets representing approximately 24,000 of the best characterized mouse genes, was used in microarray analysis. Hybridization, data capture, and analysis were performed by Capital Bio Corporation (Beijing, China), a service provider authorized by Affymetrix Inc. (Santa Clara, CA).

2.5. Microarray data analysis

All the data were viewed pre-normalized by tree clustering to remove outlying microarray data, leaving three arrays (three replicates) per time point.

The hybridization data were analyzed using Affymetrix GeneChip Command Console Software (AGCC), which uses statistical criteria to generate a ‘present’ or ‘absent’ call for genes represented by each probe set on the array. Afterwards, genes with ‘absent’ scores were filtered out and the remaining genes were analyzed. Microarray data were normalized using the Robust Multiarray Average (RMA) method. The affy suite of the bio-conductor package (http://www.bioconductor.org) was used to calculate expression values, which refer to the quantile normalization of Robust Multiarray Average method (each performed at the individual probe level). Significance Analysis of Microarrays (SAM) was used to identify genes that are differentially expressed [13].

2.6. Validation of microarray data by Real-time PCR

Differentially expressed genes of select subsets in days 4 and 5 of PSP uteri were validated by Real-time PCR as described [10]. Total RNA was extracted from days 4 and 5 PSP uteri using Trizol reagent (Invitrogen-15596018, Carlsbad, CA) according to the manufacturer's protocol. A total of 3 μg RNA was used to synthesize cDNA with the oligo dT primers. Quantitative Real-time PCR was performed with SYBR Green dye method (Takara, DRR820A) on an ABI PRISM 7500 system. All expression values were normalized against Gapdh. The primer sequences for Real-time PCR were listed in Supplementary Table 1. All Real-time PCR experiments were repeated at least 3 times.

2.7. Statistical analysis

Statistical analysis was performed with the SPSS 11.5 program (SPSS Inc., Chicago, IL, USA) for embryo transfers and validation experiment data. Comparison of means was presented by making use of the independent-samples Student *t*-test. The data are shown as means ± SD., P values of statistical significance are represented as $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

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