Comparison of Urinary Proteome Between Recurrent Spontaneous Abortion Model Mice and Normal Pregnant Mice During the Peri-implantation Period

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Abstract

Objective: To preliminarily investigate whether pregnancy implantation failure can be reflected through alterations in the urinary proteome. Methods: Urine samples were collected from recurrent spontaneous abortion (RSA) model mice during the implantation period (E3.5 and E4.5) at the time of abortion, and from normal pregnant mice during the implantation period (E3.5 and E4.5). These samples were analyzed using label-free quantitative proteomics technology with liquid chromatography-tandem mass spectrometry (LC-MS/MS). Differential proteins in the urinary proteome were screened (FC $>$ 1.5 or $<$ 0.67, P $<$ 0.050) for protein function and biological pathway analysis. Results: A total of 4 RSA model mice, none of which produced offspring; 3 normal pregnant mice had normal pregnancies and produced 7, 8, and 9 offspring respectively at E21. At E3.5, 23 differential proteins could be identified; at E4.5, 21 differential proteins could be identified. By retrieving differential proteins and related literature reports through the Uniprot database and Pubmed database, nearly half of the differential proteins were found to be related to implantation. Enrichment analysis of differential proteins through the David database identified numerous biological pathways related to the implantation process. Conclusion: Equivalent to the stage when blood human chorionic gonadotropin levels in pregnant women have not yet changed and existing methods cannot observe and accurately determine pregnancy status, the urinary proteome of RSA model mice already exhibited changes during the implantation period compared with normal pregnant mice, and the known functions of some of these altered proteins were related to implantation.

Full Text

Preamble

Comparison of Urinary Proteome Between Recurrent Spontaneous Abortion Model Mice and Normal Pregnant Mice During Implantation

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Objective: To preliminarily explore whether pregnancy implantation failure can be reflected through changes in the urinary proteome.

Abstract

Objective: To investigate whether pregnancy implantation failure can be detected through alterations in the urinary proteome.

Methods: Urine samples were collected from recurrent spontaneous abortion (RSA) model mice during the implantation period (E3.5 and E4.5) and from normal pregnant mice during the same period (E3.5 and E4.5). Label-free quantitative proteomics analysis was performed using high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS). Differentially expressed proteins were identified (fold change >1.5 or <0.67, P<0.05) and subjected to functional and biological pathway analysis.

Results: Four RSA model mice were established, none of which delivered offspring. Three normal pregnant mice carried to term, delivering 7, 8, and 9 pups respectively at E21. At E3.5, 23 differential proteins were identified, and at E4.5, 21 differential proteins were identified. Database searches of Uniprot and PubMed revealed that nearly half of these differential proteins were associated with implantation. The differential proteins were enriched in numerous biological pathways related to the implantation process through DAVID database analysis.

Conclusions: These findings demonstrate that during the implantation period —when blood human chorionic gonadotropin levels have not yet changed and existing methods cannot reliably detect or confirm pregnancy—the urinary proteome of RSA model mice already shows significant alterations compared to normal pregnant mice. Notably, some of these altered proteins have known functions related to implantation.

Keywords: urinary proteome; recurrent spontaneous abortion mouse model; implantation

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1. Introduction

Implantation is a critical process in pregnancy during which the blastocyst attaches to, invades, and becomes embedded within the maternal endometrium, establishing connections with maternal blood vessels to enable nutrient exchange. Only after successful implantation can the blastocyst continue development by acquiring nutrients from maternal blood; otherwise, pregnancy terminates. During implantation, the embryo develops to the blastocyst stage and enters the uterus in preparation for attachment, while the endometrium must simultaneously differentiate to a receptive state. Through the coordinated actions of immune cells, cytokines, growth factors, chemokines, and adhesion molecules, the blastocyst can successfully implant [1–5]. In humans, implantation efficiency is extremely low, with a success rate not exceeding 30% [6], and current methods cannot monitor the implantation period, let alone effectively determine whether implantation has succeeded or provide interventions to promote it.

Urine, produced by renal filtration of blood to eliminate metabolic waste, is not subject to homeostatic regulatory mechanisms and thus can more sensitively preserve subtle physiological changes [7]. Numerous studies support this concept: in diabetic mouse models, urinary glucose disturbances precede blood glucose changes [8]; in a transgenic mouse model of Alzheimer' s disease, the urinary proteome showed alterations in 29 proteins before amyloid plaque deposition in the brain, with 24 of these proteins previously reported to be associated with Alzheimer' s disease or serve as biomarkers [9]. Furthermore, research has demonstrated that the urinary proteome can reflect embryonic development during normal pregnancy in rats [10]. This study attempts to establish a mouse model of recurrent spontaneous abortion (RSA), collect urine samples during the implantation period for proteomic analysis, and compare them with those from normal pregnant mice to explore whether the implantation process can be monitored through urine.

BALB/c, CBA/J, and DBA/2 are common inbred mouse strains with modest husbandry requirements, strong reproductive capacity, and low breeding costs, providing a stable and abundant domestic laboratory animal source. The CBA/J female × DBA/2 male mating produces a well-established RSA model characterized by recurrent spontaneous abortion with relatively constant abortion rates, while CBA/J females mated with BALB/c males undergo normal pregnancy [10], serving as an appropriate control. Abortion in the mouse RSA model involves recurrent resorption of chromosomally normal embryos and occurs during the implantation period, specifically between embryonic day 3.5 (E3.5) and E4.5. Current evidence suggests that immunological factors are the primary cause of spontaneous abortion in this model [11–13].

2. Materials and Methods

2.1 Experimental Animals

Seven 14-week-old CBA/J female mice, four 14-week-old DBA/2 male mice, and three BALB/c male mice were purchased from Beijing Huafukang Bioscience Co. All mice were housed in a standard environment (temperature (22$±$1)°C, humidity 65–70%). After three days of acclimatization, experiments were initiated. All procedures were reviewed and approved by the Animal Welfare and Ethics Committee of the College of Life Sciences, Beijing Normal University (approval number: CLS-AWEC-B-2022-003).

2.2 Experimental Procedures

2.2.1 RSA Mouse Model Establishment CBA/J females were divided into two groups: RSA group (n=4) and Control group (n=3). RSA group females were co-housed with DBA/2 males at a 1:1 ratio at 16:00. The following morning at 7:00, females were checked for vaginal plugs; those with plugs were considered successfully modeled and designated as embryonic day 0.5 (E0.5). Control group females were co-housed with BALB/c males following the same protocol, with plug-positive females designated as E0.5.

2.2.2 Urine Sample Collection Females were housed individually. Pre-mating urine samples were collected from each female and designated as D0, then stored at -80°C. Post-mating urine samples were collected during the implantation period at E3.5 and E4.5, and similarly stored at -80°C.

2.2.3 Urine Sample Processing Protein Extraction: Frozen urine samples were thawed at 4°C, then centrifuged at 12,000×g for 30 min at 4°C. Five hundred microliters of supernatant were transferred to 2 mL tubes, mixed gently with three volumes of ice-cold ethanol, and precipitated overnight at -20°C. The precipitated mixture was centrifuged at 12,000×g for 30 min at 4°C, the supernatant was discarded, and ethanol was allowed to evaporate. Protein pellets were resuspended in lysis buffer (8 mol/L urea, 2 mol/L thiourea, 25 mmol/L dithiothreitol, 50 mmol/L Tris), centrifuged at 12,000×g for 30 min at 4°C, and the supernatant was transferred to new 1.5 mL tubes. Protein concentration was determined using the Bradford method.

Enzymatic Digestion: One hundred micrograms of urinary protein were diluted with 25 mmol/L NH_4HCO_3 to a final volume of 200 L. Twenty millimolar dithiothreitol (DTT, Sigma) was added, vortexed, and heated at 97°C for 10 min, then cooled to room temperature. Fifty millimolar iodoacetamide (IAA, Sigma) was added, vortexed, and incubated in the dark at room temperature for 40 min. For filtration, 200 L of UA solution (8 mol/L urea, 0.1 mol/L Tris-HCl, pH 8.5) was added to a 10 kDa ultrafiltration tube (Pall, Port Washington, NY, USA) and centrifuged at 14,000×g for 5 min at 18°C; the filtrate was discarded and the wash repeated. The IAA-treated protein sample was added to

the filter and centrifuged at 14,000×g for 30 min at 18°C. The filter was washed twice with 200 L UA solution and twice with 25 mmol/L NH_4HCO_3 solution, centrifuging each time at 14,000×g for 30 min at 18°C. Trypsin (Trypsin Gold, Promega, Fitchburg, WI, USA) was added at a 1:50 enzyme-to-protein ratio and incubated at 37°C for 15 h. After digestion, peptides were collected by centrifugation at 13,000×g for 30 min at 4°C. Peptide mixtures were desalted using HLB solid-phase extraction columns (Waters, Milford, MA), lyophilized, and stored at -20°C.

2.2.4 LC-MS/MS Analysis Lyophilized peptides were dissolved in 0.1% formic acid, quantified using a BCA kit, and diluted to 0.5 g/ L. For each sample, 6 L was pooled and fractionated using a high-pH reversed-phase peptide separation kit (Thermo Fisher Scientific). Ten fractions were collected, lyophilized, and reconstituted in 0.1% formic acid. Both fractions and individual samples were spiked with iRT reagent (Biognosys, Switzerland) at a 10:1 sample-to-iRT ratio to calibrate peptide retention times.

The ten fractions were separated using an EASY-nLC1200 chromatography system (Thermo Fisher Scientific, USA), and peptides were analyzed on an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific, USA) in Data-Dependent Acquisition (DDA) mode to generate ten raw files. These were imported into Proteome Discoverer software (version 2.0, Thermo Scientific) for library generation using Swiss-iRT and Uniprot-mouse databases. Based on the library, a DIA method with 39 variable windows was established for individual sample analysis. Individual samples (1 g peptide each) were separated using the EASY-nLC1200 system and analyzed by DIA mode on the Orbitrap Fusion Lumos Tribrid mass spectrometer using the newly established method to generate raw files.

2.2.5 Label-Free DIA Quantification Individual sample raw files from DIA acquisition were analyzed using Spectronaut Pulsar software (Biognosys AG, Switzerland). Peptide abundance was calculated from the summed peak areas of fragment ions in MS^2 spectra, and protein abundance was derived from the sum of constituent peptide abundances.

2.2.6 Statistical Analysis Each sample was analyzed in three technical replicates, and mean values were used for statistical analysis. Differentially expressed proteins between RSA and Control groups were identified using a two-tailed unpaired t-test with the following criteria: fold change (FC) $ $1.5 or $ $0.67, and $P<0.05$. Identified proteins were functionally annotated using Uniprot (https://www.uniprot.org/) and DAVID database (https://david.ncifcrf.gov/), and relevant literature was retrieved from PubMed (https://pubmed.ncbi.nlm.nih.gov) for functional interpretation.

3. Results

3.1 RSA Mouse Model Establishment

Following model establishment, behavioral observations were conducted throughout gestation. RSA group mice showed normal water intake, whereas Control group mice exhibited significantly increased water consumption. At E13.5, RSA group mice showed no obvious physical changes, while Control group mice showed markedly increased body size. All four RSA group mice failed to deliver offspring, confirming successful model establishment. All three Control group mice underwent normal pregnancy, delivering 7, 8, and 9 pups respectively at E21.

3.2 Differential Protein Analysis

3.2.1 Functional Analysis of Differential Proteins Comparison of urinary proteomes between RSA and Control groups at E3.5 identified 23 differentially expressed proteins (FC$ \$1.5 $ or $ \$ 0.67, P < 0.05$). $Uniprot database search results are represented in Table 1. Literature review via PubMed revealed that 9 of these type plasminogen activator is expressed in trophoblast cells during implantation in mice and humans, participatin receptor for TGF-$ \$ superfamily members, mediating TGF-β signaling pathways that regulate trophoblast invasion and uterine decidualization during implantation in mice and humans [19–21]. Sphingomyelin phosphodiesterase participates in sphingolipid metabolism; during mouse implantation, altered uterine sphingolipid metabolism is accompanied by increased sphingomyelin phosphodiesterase expression at the maternal-embryonic interface [22]. Sphingolipid metabolism regulates uterine decidualization and vascular stability, and its dysregulation is also a cause of human miscarriage [23]. Furin regulates E-cadherin processing-mediated cell adhesion and non-polar inner cell mass differentiation in mouse blastocysts [24, 25]. Altered peptidyl-prolyl cis-trans isomerase activity has been reported to cause miscarriage due to implantation failure [26]. Collagen alpha-1(IV) chain is highly expressed in the mouse endometrium and myometrium before embryo implantation, with decreased expression after implantation [27]; in human females, downregulation of Collagen alpha-1(IV) chain reduces endometrial epithelial adhesive capacity, leading to implantation failure and infertility [28].

Most remaining differential proteins are enzymes involved in protein, glycoprotein, nucleic acid, and lipid metabolism, as well as cell signal transduction, proliferation, motility, and adhesion. Although not previously reported in implantation studies, their functions may participate in the blastocyst invasion process. For example, N-acetylglucosamine-6-sulfatase mediates hydrolysis of heparan sulfate and keratan sulfate; mouse blastocyst trophoblast cells express heparan sulfate proteoglycans, while uterine epithelial cells express several complementary heparan sulfate-binding proteins that support blastocyst attachment to the endometrium [29]. Prostaglandin F2 receptor negative regulator inhibits prostaglandin F2α binding by reducing receptor number, limiting its function,

while prostaglandins play important roles in endometrial decidualization during mouse blastocyst implantation [30]. Notably, Solute carrier family 12 member 3 was absent in Control group urinary proteomes but present in all RSA group mice. This protein is associated with immunological factors and serves as an interleukin-18 receptor, facilitating IL-18-induced production of cytokines including interferon-γ (IFNG), interleukin-6, interleukin-18, and chemokine ligand 2 (CCL2). IFNG plays a critical role in normal mouse pregnancy by initiating endometrial vascular remodeling, angiogenesis at implantation sites, and maintenance of decidual components [31]; IL-18 regulates human embryo implantation, with either overactivation or deficiency causing implantation failure [32]; IL-6 deficiency reduces implantation sites in mouse uterus [33]; and CCL2 is considered predictive of human recurrent miscarriage and recurrent implantation failure [34]. This suggests Solute carrier family 12 member 3 may serve as a urinary protein biomarker for predicting miscarriage.

At E4.5, comparison of urinary proteomes between RSA and Control groups identified 21 differential proteins (FC\$ \$1.5 or \$ $0.67, P <$ $0.05), with Uniprot search results presented in Table 2. Literature review revealed that 11 of these proteins are direct$ $1 is a component of intercellular desmosomal junctions mediating cell adhesion through interactions with plaquep$ $1 redistributes on uterine epithelial cell plasma membranes to prepare for blastocyst invasion [36, 37]. Coxsackievir$ $epithelial migration; it is expressed in mouse blastocyst trophoblast and endometrial epithelial cells during implan$ $endometrial interactions [38]. OX-2 membrane glycoprotein participates in heterotypic cell adhesion; it express$ receptor expressed in trophoblast and endometrial cells, Endoglin maintains endometrial receptivity and regulates trophoblast proliferation and invasion, which is essential for embryo implantation [44, 45]; it also participates in trophoblast invasion of uterine spiral arteries to remodel uterine vasculature and maintain vascular homeostasis [46]. Corticosteroid-binding globulin is the major transporter for glucocorticoids and progesterone in vertebrate blood, a specific protein in the endometrium of women with repeated implantation failure [47]; its synthesis and secretion increase during hamster implantation [48], and continuous progesterone production from the corpus luteum is required to maintain decidualization during mouse implantation [49]. Kallikrein 1 is expressed in rat and human uterus, functioning in blastocplantation endometrium [50, 51], possibly by dilating vessels, inhibiting platelet aggregation, and promoting cell proliferation and trophoblast invasion to establish and maintain placental blood flow [52]. TGF-beta receptor type-1 participates in blastocyst and uterine embryonic development, being crucial for human blastocyst implantation and maintenance of uterine decidualization [53]; knockout of this gene in mice causes implantation defects, disorganized trophoblast cells, reduced uterine natural killer cells, and impaired spiral artery remodeling [54]. Transcobalamin-2 transports vitamin B12 into cells and has been implicated in recurrent implantation failure in Korean women [55].

Although not previously reported in implantation studies, remaining differential proteins may also participate in blastocyst invasion. For example, Adipocyte enhancer-binding protein 1 positively regulates collagen fiber formation, potentially participating in extracellular matrix organization and remodeling. Leuko-

cyte surface antigen CD47 is an adhesion protein mediating cell interactions, participating in cell adhesion, proliferation, and angiogenesis, while also mediating cellular responses to interleukin-1 and IL-6 production, both of which function during blastocyst implantation [20]. Galectin-3-binding protein promotes integrin-mediated cell adhesion and may inhibit tumor cell invasion. Neprilysin participates in placental development and may regulate endometrial IL-1 and endothelin-1 metabolism to maintain optimal conditions for blastocyst implantation [56]. Leucine-rich repeat-containing protein 19 is a pathogen recognition receptor that activates TRAF2/NF- B signaling to induce pro-inflammatory cytokine expression [57]; studies have shown that the TRAF2/NF- B axis regulates mitochondrial dynamics and apoptosis, causing chorionic villous dysfunction that is a key factor in recurrent miscarriage [58].

3.2.2 Biological Pathway Analysis of Differential Proteins DAVID database analysis of E3.5 differential proteins enriched 10 biological processes (Table 3). These pathways primarily involve cell migration; protein, nucleic acid, and carbohydrate metabolism; and angiogenesis, likely related to trophoblast invasion of the uterine decidua and establishment of maternal blood connections during implantation. Notably, the transforming growth factor beta receptor signaling pathway plays an important role in mouse and human blastocyst implantation and placentation [20].

Analysis of E4.5 differential proteins enriched 16 biological processes (Table 4). These pathways primarily involve cell adhesion and motility; protein hydrolysis and processing; and angiogenesis, also likely related to trophoblast invasion and maternal blood connection establishment. Studies have shown that epithelial-mesenchymal transition participates in female implantation, enabling uterine luminal epithelial cells and decidualized stromal cells to acquire mesenchymal cell motility and migratory capacity to accommodate trophoblast invasion [59]. SMAD proteins, as downstream signaling molecules of the TGF-beta superfamily, also participate in mouse and human blastocyst implantation and placentation [20].

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4. Outlook

Recurrent spontaneous abortion (RSA) is defined as three or more consecutive pregnancy losses before 28 weeks with the same partner, though some consider two consecutive losses to confer similar risk [60]. Approximately 2–5% of reproductive-age women are affected by RSA, causing significant psychological trauma and economic burden. Multiple factors contribute to RSA, including maternal age, anatomical and chromosomal abnormalities, genetics, endocrinology, placental disorders, infection, immunity, smoking and alcohol consumption, psychological factors, and exposure to environmental pollutants such as heavy metals and radiation [60–62]. However, the cause remains unknown in

approximately 50% of patients, and these unexplained cases are often associated with immunological factors [63]. The complex etiology and lack of specific clinical manifestations make RSA difficult to diagnose and predict. Even when abnormal findings are identified, most patients cannot receive evidence-based treatment, with empirical and combined multiple therapies remaining the main approach [60]. Identifying highly selective biomarkers for RSA to enable timely and sensitive pregnancy monitoring, exploring new diagnostic methods, and assisting precision treatment are urgent challenges. The ability of urinary proteomics to reflect changes during the implantation period in RSA mice compared to normal pregnant mice suggests that urinary proteomics can monitor implantation development, provide insights into implantation failure mechanisms, and facilitate identification of potential intervention targets.

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Note: Figure translations are in progress. See original paper for figures.