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Identification of stathmin 1 during peri-implantation period in mouse endometrium by a proteomics-based analysis

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ABSTRACT

In this work we aimed to identify the differentially expressed proteins and their potential roles during peri-implantation period through proteomics-based approach. Adult healthy female mice were mated naturally with fertile males to produce pregnancy. The models of pseudopregnancy, delayed implantation, and artificial decidualization were established. The protein profile between pre-implantation (D1) and implantation (D5) period was compared by two-dimensional electrophoresis (2-DE) and identified by mass spectrometry (MS). 2-DE yielded comparative images presenting over 500 protein spots in D1 and D5 mouse endometrium. 15 proteins were identified, of which stathmin 1, Apo-A1, hnRNP H3, transgelin 2 and arginase 1 were validated by western blotting. Stathmin 1 expression did not change in pseudopregnancy, but activation of implantation, or induction of decidualization increased it dramatically. Under non-pregnant status, progesterone alone or in combination with17β-estradiol increased it dramatically. Our results clarified the protein profile in mouse endometrium during implantation. The specific expression profile of stathmin 1 suggested that it should be involved in implantation and serve as a potential regulator of this process. These findings may contribute to the better understanding of the molecules events during embryo implantation, and subsequently improve the ability to treat infertility.
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1. Introduction

Embryo implantation is established and maintained by a network of complex molecules that are involved in physiological changes of the endometrium, including hormones, cytokines, growth factors, and their interactions [1]. It can only occur over a restricted period after ovulation called “implantation window” lasting about 24 h in mouse spanning early on day 4 to middle of the day 5 of pregnancy. Comparing to the commonly used one-by-one research strategy, high throughput techniques theoretically have the advantages in discovering the molecular mechanism in a global extent.

As well as the significant alterations in transcriptional and post-transcriptional levels, for the events relating to translation, post-translational modification, and subcellular localization, proteomics techniques should be more informative because these events are difficult to detect either by studying nucleotide sequence variation or by measuring the quantity of RNA or miRNA. In previous studies, we identified some proteins in endometrial carcinoma by two-dimensional electrophoresis (2-DE)-based proteomic strategies, some of which might serve as potential genetherapy target or prognosis biomarker of endometrial carcinoma [2,3]. Considering the fact that the embryo implantation process shares similar biological characteristics with cancer development and progression [4,5], we presume a significant change of the protein profile during peri-implantation period. Therefore, herein we clarified the proteins profile in embryo implantation process, using 2-DE and mass spectrometry (MS)-based approaches. In addition, a series of animal models were established for strict validation of the unbiased candidates. As a result, we successfully identified 15 differentially expressed proteins, and the specific expression profile of stathmin 1 was further validated in a series of animal models, including pseudopregnancy, delayed implantation, artificial decidualization and ovariectomization. As clinicians, we think these findings can contribute to the better understanding of the molecules events during embryo implantation, and subsequently improve the ability to treat infertility, to prevent early pregnancy loss and to increase success rate of IVF.

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2. Materials and methods

2.1. Preparation for animal specimens

The Institutional Animal Care and Treatment Committee of Sichuan University reviewed and approved all animal experiments herein (04/21/2013, shown in supplemental file). The adult healthy female C57BL6/J mice (6–8 weeks of age, nonfertile and 18–20 g each) were mated naturally with fertile males to produce pregnancy. The morning of finding a vaginal plug was designated as day 1 (D1) of pregnancy. Implantation was assumed to be taking place on the day 5 (D5), which was confirmed by the visualization of implantation site by intravenous injections (0.1 ml/mouse) of 1% Chicago Blue B dye solution. Anesthetized pregnant mice were euthanized at 9:00 on D5 and the fresh endometrial tissue samples were immediately frozen in liquid nitrogen for subsequent experiments.

Pseudopregnancy was induced by caging adult females with vasectomized males and the finding of a vaginal plug was designated as D1 of pseudopregnancy. To induce artificial decidualization, on D5 of pseudopregnancy when the uterus was optimally sensitized for artificial deciduogenic stimuli, 25 μl olive oil was infused into the lumen of one uterine horn. The contralateral uterine horn, which was not infused, served as a control. To induce delayed implantation, the pregnant mice on D4 were ovariectomized and injected with progesterone (1 mg/mouse) intraperitoneally to maintain delayed implantation on D5 and 6. Then the mice were injected intraperitoneally with progesterone (1 mg/mouse) combined with 17β-estradiol (0.1 μg/mouse) on D7 to terminate delayed implantation.

To test the effects of steroid hormones under physiological condition, the mice were ovariectomized 2 weeks before steroid hormones injection. The ovariectomized mice were injected intraperitoneally with 17β-estradiol (0.1 μg/mouse/day) and/or progesterone (1 mg/mouse/day) for 3 days. 24 hrs later the anesthetized mice were euthanized and the uterine tissues were isolated and frozen in liquid nitrogen. All steroid hormones were dissolved in olive oil and the ovariectomized mice injected with olive oil served as controls.

2.2. 2-Dimensional gel electrophoresis

Mice uterine tissues were ground into powder in liquid nitrogen and lysed in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 65 mM DTT, 0.2% carrier ampholyte, 1% (v/v) cocktail). After centrifugation at 13,000 rpm for 30 min followed by vortex, sonication and incubation for 2 h, the supernatant was precipitated with cold acetone/trichloroacetic acid. The precipitation was redissolved in rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 65 mM DTT, 0.2% carrier ampholyte), after centrifugation at 2000 rpm for 5 min, and the protein concentration was determined by Brad-ford concentration assay (Bio-Rad, Hercules, CA). Rehydration buffer containing 500 μg protein was load passively onto an Ready Strip™ IPG strip (17 cm, pH 3–10 non-linear, Bio-Rad, Hercules, CA) for 16 h. Then the first dimensional isoelectric focusing (IEF) was performed on a PROTEAN IEF CELL system (Bio-Rad, Hercules, CA) until a total of 100,000 V-hr was reached. The focused strips were then equilibrated for reduction and alkylation in equilibration buffer (75 mM Tris pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, traces of bromophenol blue) containing 1% (w/v) DTT for 15 min and subsequently in equilibration buffer containing 4% (w/v) iodoacetamide for another 15 min. Then the equilibrated strips were transferred onto 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using a PROTEAN II xi Cell system (Bio-Rad, Hercules, CA) under a constant current of 30 mA/gel at 15 °C until the tracking dye migrating to the bottom of the gel. The protein spots in gels were visualized by Coomassie Brilliant Blue R-250 staining. Image acquisition and analysis was performed using PDQuest 7.1 software (Bio-Rad, Hercules, CA). Spot intensity was quantified by calculation of spot volume after normalization of the image by taking the ratio of intensity of one spot to the total spots and expressed as a fractional intensity. Those spots with 2.5-fold or more changes in intensity and frequencies higher than 40% were selected as differentially expressed spots for identification.

2.3. In-gel digestion

Gel spots were excised and digested using Trypsin Gold according to the manufacturer's instructions (Promega, Madison, WI). The spots were dehydrated twice in 100% ACN for 5 min after destained twice with 0.1 ml destaining solution (50% 50 mM NH_4HCO_3, 50% ACN) for 30 min at room temperature. Then spots were incubated with 10 μl of 12.5 μg/ml Trypsin Gold at 4 °C for 15 min and then covered with 15 μl of digestion buffer (40 mM NH_4HCO_3, 10% ACN) at 37 °C overnight. Saving the liquid, peptides were extracted twice with 80 μl of 50% ACN, 5% TFA by ultrasonication for 15 min. Then all extracts were collected and dried in a SpeedVac at room temperature. Peptides were resolved in 5 μl of 50% ACN, 0.1% TFA.

2.4. Mass spectrometry (MS) analysis and database searching

MALDI-Q-TOF MS (matrix-assisted laser desorption/ionization quadrupole time-of-flight tandem mass spectrometry) was performed on a 4800 MAIDI TOF/TOF™ mass spectrometer (Applied Biosystems, Foster, CA). Peptide mass maps were acquired in positive ion reflector mode, and monoisotopic peak masses were automatically determined within the mass range 800–3500 Da with a signal/noise ratio minimum set to 5 and a local noise window width of 200 *m/z*. After MS acquisition, 10 ions of maximum intensity were selected for MS/MS analysis. The MS together with MS/MS spectra were processed with the search algorithm GPS Explorer 3.6 (Applied Biosystems, Foster, CA) and MASCOT 2.1 (Matrix Science, London, UK) software against the NCBInr protein sequence database. The MS/MS data were retrieved against the *Mus musculus* subset of the sequences with the following parameter settings: Trypsin cleavage, one missed cleavage allowed, cysteine carbamidomethylation set as fixed modification, methionine oxidation allowed as variable modification, peptide mass tolerance set to 50 ppm, MS and MS/MS tolerance set to ±0.15 and ±0.25 Da, and minimum ion score confidence interval for MS/MS data set to 95%. Only those individual MS/MS spectrum with statistically significant ion scores exceeding the threshold (based on MS/MS data) were considered acceptable.

2.5. Western blotting

Tissue specimens were ground into powder in liquid nitrogen and lysed in RIPA lysis buffer (50 mM Tris–HCl (pH 7.4), 0.25% SDS, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM PMSF). Lysates were subjected to 10% SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes. After 1 h blocking with 5% dried skimmed milk in TBS-Tween 20, the membranes were incubated with primary antibodies overnight at 4 °C. The blots were labeled with peroxidase-conjugated secondary antibodies and visualized by electrochemiluminescent detection. The specific antibodies used were as follows: anti-mouse stathmin, anti-mouse apoA-I, anti-mouse hnRNP E1, anti-mouse arginase-I, and anti-mouse transgelin-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The

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