Biochemical and Biophysical Research Communications 461 (2015) 211–216

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

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ARTICLE INFO

Article history:
Received 9 February 2015
Available online 9 April 2015

Keywords:
Embryo implantation
Proteomics
Two-dimensional electrophoresis
Mass spectrometry
Stathmin 1

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.

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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http://dx.doi.org/10.1016/j.bbrc.2015.02.171
0006-291X/

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

equivalent loadings were confirmed using anti-mouse GAPDH antibody. Experiments were independently performed in triplicate.

3. Results

3.1. Comparison of the protein expression profiles of mouse endometrium between pre-implantation and implantation period

To obtain a comprehensive comparison of the protein expression profile of mouse endometrium between pre-implantation and implantation period, a comparative 2-DE analysis was conducted with 5 pairs of mouse endometrium on D1 and D5. Image analysis was performed using PDQuest 7.1 software and yielded well resolved and reproducible protein profiles for both D1 and D5 endometrium (Fig. 1). Coomassie staining showed a matching rate of 94.5% and an average of 512 ± 45 and 565 ± 58 spots in D1 and D5 specimen, respectively. Differentially expressed spot was defined as spot with 2.5-fold or more change in intensity and frequency higher than 40% (recurrence more than 2 times in the 5 pairs). According to the criteria, 16 up-regulated and 2 down-regulated spots were selected and subjected to MS/MS identification, and 15 were unambiguously identified by MS/MS and searched against the NCBInr protein database (Table 1). To eliminate the redundancy of proteins with multiple isoforms, only one isoform belonging to the species *M. musculus* with the highest MASCOT score was selected for those proteins, and the MS/MS data shown in Table 1 pertained to that isoform of the protein accordingly. Of these 15 proteins, the majorities (14 of 15 proteins) were up-regulated in D5 in comparison with D1, and arginase 1 was the only one down-regulated protein in D5. Subcellular analysis showed that these proteins were located in cytoplasm (6/15), nucleus (1/15), and secreted (3/15). Database searching got a result of "unnamed protein product" in 3 spots, even their protein score C.I were all perfect in MS/MS identification. These proteins were reported to be involved in regulation of microtubule system, mRNA transport and protein synthesis, metabolism, and etc. Due to only 15 proteins were identified, the pathway and GO (Gene Ontology) analyses from the UniProt and GO databases were not conducted.

3.2. Validation of differentially expressed proteins by western blotting

5 of the 15 proteins, including stathmin 1 (oncoprotein 18), apolipoprotein A-1 (Apo-A1), heterogeneous nuclear ribonucleoprotein H3 (hnRNP H3), transgelin 2 and arginase 1, were selected for further examination of their dynamics during the peri-implantation period. Selection of these proteins was comprehensively based on the facts as follows: 1) significant and ubiquitous expression difference between D1 and D 5 endometrium; 2) perfect reliability of MS identification with high protein score and C.I.; 3) active biological function with potential involvement in embryo implantation. To strengthen the result reliability, the samples for western blotting were not the same samples for 2-DE analysis. As shown in Fig. 2, expression of stathmin 1 was detected from D1 to D8, but up-regulated apparently and specially in implantation period (D5). Apo-A1 had a similar profile to stathmin 1, which was detected in peri-implantation period (D1 to D8), but remarkably up-regulated in implantation period (D5). Expression of hnRNP H3 was slight on D1 to D3 and almost undetectable in D4; but up-regulated in implantation and post-implantation periods (D5 to

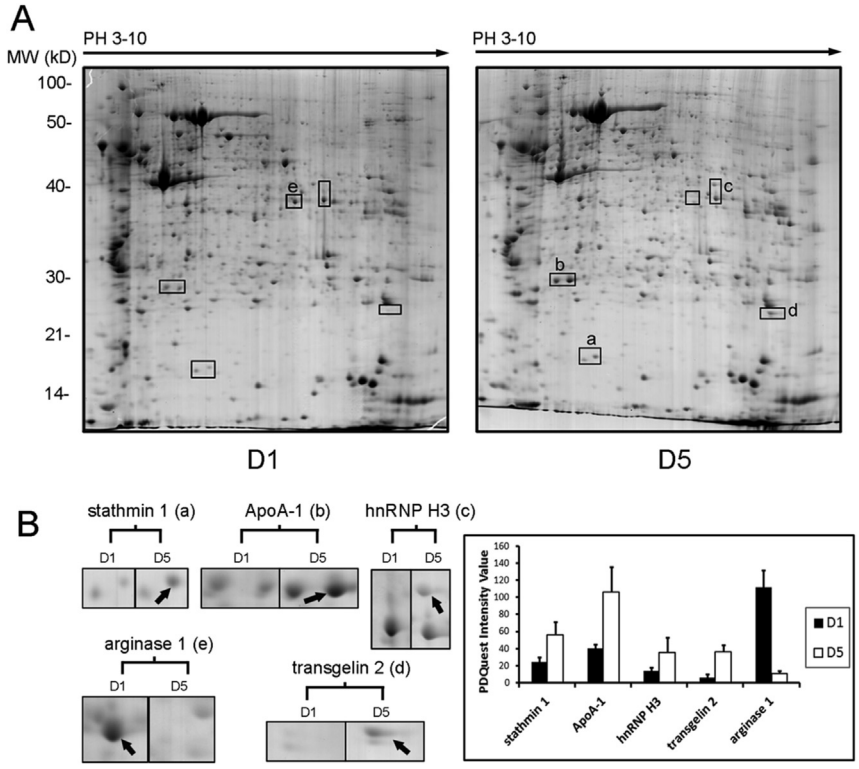

Fig. 1. Comparison of the protein profile of mouse endometrium between D1 and D5. An average of 512 ± 45 and 565 ± 58 spots were visualized in D1 and D5 gel image, respectively, with a matching rate of 94.5% (A). Five representative spots with differential expression were marked as a, b, c, d, and e. (B) Cropped 2-DE gel images of the five representative spots. Stathmin 1, ApoA-1, hnRNP H3, and transgelin 2 were significantly up-regulated in implantation period (D5) ($P < 0.05$), and arginase 1 was significantly down-regulated in D5 ($P < 0.05$). Spot volume was quantified from the intensity of spot using PDQuest 7.1 software, and values were recorded as mean ± S.D., and representative of five independent experiments.

Table 1
Data of 15 proteins identified by MALDI-TOF-MS/MS.

	Protein name	Accession no.	Protein score	Score C. I. %	Protein MW	Protein PI	Peptide count	Subcellular location	Function
Up-regulated									
1	Stathmin 1	gi|14625464	437	100	17205.9	5.95	13	Cytoplasm	Regulation of the microtubule filament system
2	Apolipoprotein A-I	gi|160333304	193	100	30596.6	5.51	11	Secreted	Cholesterol metabolism
3	Albumin	gi|26986064	300	100	23609.3	5.48	11	Secreted	Regulation of the colloidal osmotic pressure
4	Unnamed protein product	gi|74196535	360	100	35721.9	6.38	12	N/A	N/A
5	Heterogeneous nuclear ribonucleoprotein H3	gi|14141159	87	98.307	35216.4	6.86	5	Nucleus	mRNA metabolism and transport
6	Transgelin 2	gi|30519911	236	100	22381.2	8.39	15	Cytoplasm	Not determined
7	mCG4061	gi|148669988	166	100	25,801	6.85	15	N/A	N/A
8	Glutathione S-transferase mu 7	gi|113679874	345	100	25692.9	6.34	19	Cytoplasm	Conjugation of reduced glutathione to hydrophobic electrophiles
9	Eukaryotic translation initiation factor 4H	gi|15808988	118	99.999	27324.4	6.67	9	Cytoplasm	Stimulate the initiation of protein synthesis
10	Poly(rC)-binding protein 1	gi|149036630	199	100	35489.9	7.03	12	Cytoplasm	mRNA metabolism and transport
11	Unnamed protein product N/A	gi|74183933	82	94.264	42074.3	6.64	6	N/A	N/A
12	mCG8461, isoform CRA_c	gi|148684042	290	100	29,988	5.57	11	N/A	N/A
13	Albumin	gi|26986064	365	100	23609.3	5.48	12	secreted	Regulation of the colloidal osmotic pressure
14	Unnamed protein product	gi|26341396	408	100	64960.7	5.49	14	N/A	N/A
Down-regulated									
15	Arginase 1	gi|7106255	454	100	34786.2	6.51	15	Cytoplasm	Regulation of urea metabolism

D8), with the maximum in D5. Arginase 1 expression was detected in pre-implantation period (D1 to D4), followed by remarkably decrease in implantation and post-implantation periods (D5 to D8). For transgelin 2 expression, no apparent change was observed during the peri-implantation period from D1 to D8.

3.3. Expression profile of stathmin 1 in a series of animal models

To further determined the potential association with the presence and status of embryo, endometrial decidualization, and steroid hormone status, we examined the stathmin 1 expression in a series of established animal models, including pseudopregnancy, delayed implantation, artificial decidualization, and non-pregnant ovariectomization (Fig. 3). Western blotting showed that in pseudopregnancy model there was no apparent alteration of stathmin 1 expression on D1 to 8 of pseudopregnancy. In delayed implantation model there was no apparent alteration of stathmin 1 expression during the delayed implantation period from D1 to 7, but it increased dramatically 24 h after activation of implantation by 17β-estradiol treatment on D7. In artificial decidualization model, there was no apparent alteration of stathmin 1 expression on D1 to 5 of pseudopregnancy, but after decidualization induction on D5 by olive oil infusion, it increased gradually on D6 to 8 and achieved a maximum on D8. In ovariectomization model stathmin 1 expression decreased in non-pregnant ovariectomized mouse endometrium, in comparison with normal mouse endometrium. However, treatment with progesterone alone or in combination with 17β-estradiol increased its expression dramatically. The 17β-estradiol treatment alone had similar effect, but less dramatic than progesterone.

To discover whether the change of stathmin 1 expression during implantation period was a tissue-specific manner, the expression of stathmin 1 in heart, liver, kidney, lung, and brain, was also examined. Western blotting showed that there was no apparent

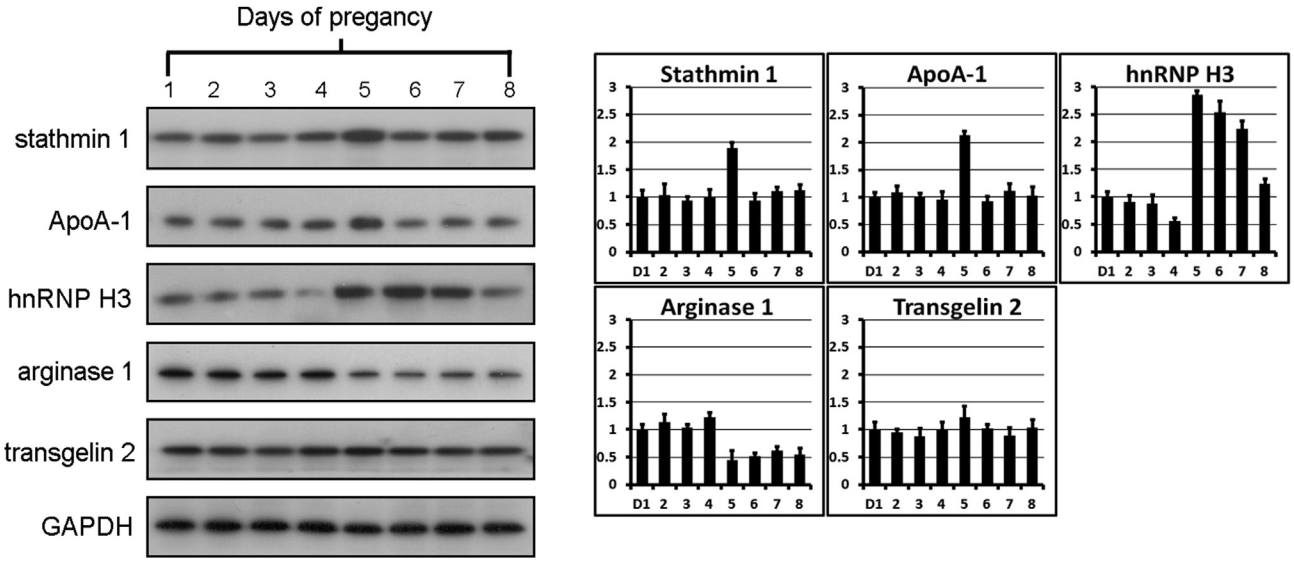

Fig. 2. Expressions of stathmin 1, ApoA-1, hnRNP H3, arginase 1, and transgelin 2 during peri-implantation period from D1 to D8 by western blotting. The intensity of band normalized to GAPDH, serving as a loading control, was quantified using Quantity One 4.6 software, and the value on D1 was subjectively set at 1. The value was recorded as mean ± S.D., and representative of three independent experiments.

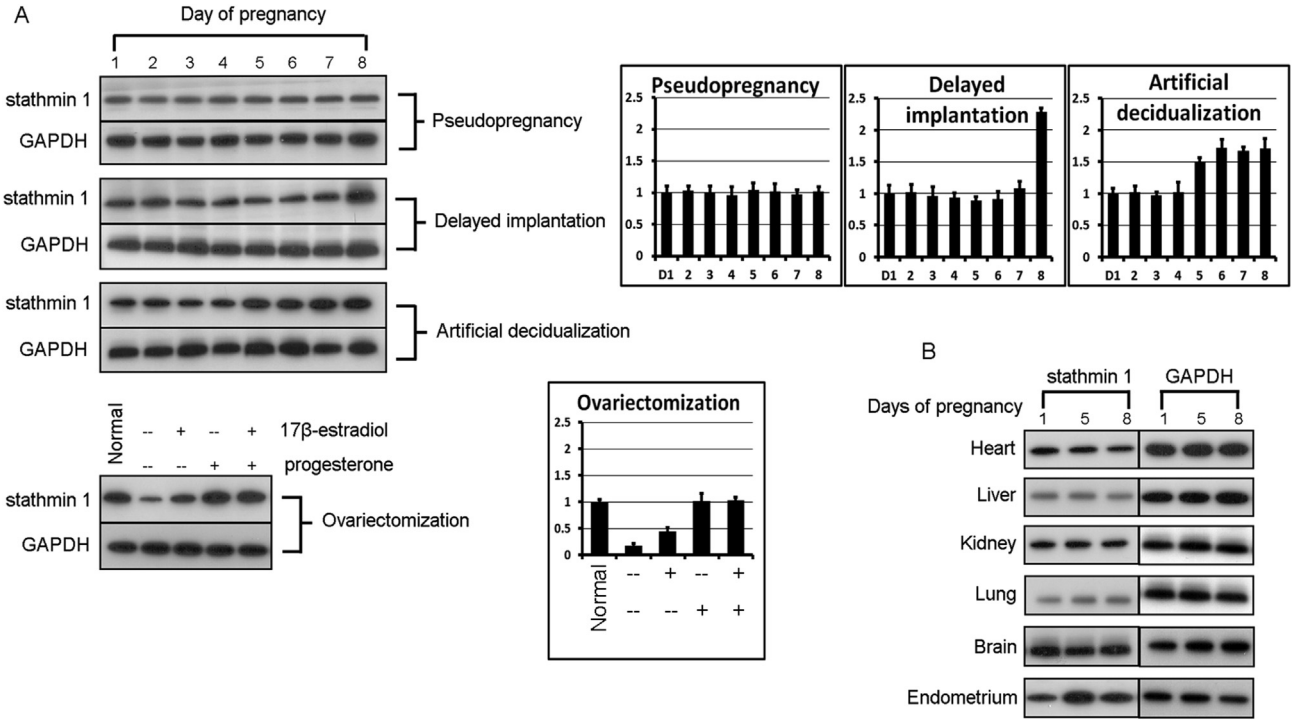

Fig. 3. (A) Expression of stathmin 1 during peri-implantation period in the models of pseudopregnancy, delayed implantation, artificial decidualization, and ovariectomization; (B) Expression of stathmin 1 in various tissues during peri-implantation period. GAPDH served as control to equal loading and the bands were representative of three independent experiments. The intensity of band normalized to GAPDH, serving as a loading control, was quantified using Quantity One 4.6 software, and the value on D1 or in normal (ovariectomization) was subjectively set at 1. The value was recorded as mean ± S.D., and representative of three independent experiments.

alteration of stathmin 1 expression in the above tissue specimens during implantation period (Fig. 3).

4. Discussion

In this study, we found 18 spots between pre-implantation and implantation endometrium, and 15 spots were successfully identified by MS/MS. Even protein expression profiles with perfect stability and repeatability were obtained, but the limited number of differentially expressed spots seemed not proportionate to the complexity of molecular crosstalk in embryo implantation. We think it was partially due to the strict selection criteria relating to not less than 2.5-fold change and 40% recurrent frequency. In addition, we used a general method in protein extraction procedure for soluble and hydrophilic proteins, which had limited effect on extracting hydrophobic proteins locating in membrane and nucleus. So most of the 10 identified proteins with subcellular localization information were located in cytoplasm (6/10) or secreted (3/10). We think that improvement of the separation of the protein extracts into less complex mixtures would lead to more extensive representation of the exhibited proteins and yield spectra with more information.

The dynamic expressions from D1 to D8 of stathmin 1, Apo-A1, hnRNP H3, and arginase 1 were confirmed by western blotting. Only transgelin 2 failed to be found apparent alteration. Based on the expression profile in implantation period and literature review, stathmin 1 was further selected as a potential regulator of embryo implantation for validation with a series of established models. Stathmin 1, also known as oncoprotein 18, is a highly conserved 17 kDa phosphoprotein that has 4 serine residues at its N-terminus [6], acting as a regulator of microtubule filament system by preventing assembly and promoting disassembly of microtubule during cell-cycle progression [7,8]. It also promotes tubulin catastrophes by binding to microtubule plus ends, and stimulates the exposed β-subunit of the terminal tubulin dimmers to hydrolyze GTP [8]. The stathmin 1 phosphorylation by various kinases suppresses its microtubule destabilizing activity [9]. While the majority of researches on stathmin 1 have focused on the roles in controlling microtubule dynamics, the participation of cytoskeleton system in implantation process suggests the potential interaction between stathmin 1 and implantation. During the implantation or pregnancy endometrial morphology is dynamically regulated by steroid hormones, and cytoskeleton system participates in the cyclic architectural modifications of endometrium. The control of cytoplasmic actin is also crucial for regulating endometrium rearrangement during pregnancy. Pampfer et al. first reported that the up-regulation of stathmin 1 mRNA expression in the mouse endometrium on days 5–7 of pregnancy and in expanded and hatched blastocysts [10]. Later, Tamura et al. found the higher expression of stathmin 1 in the implantation sites than inter-implantation sites on days 6–7 of pregnancy [11]. It was localized in the glandular and luminal epithelium, stromal cells and vascular endothelium before implantation of embryo into the uterus. In addition, expression profiles of stathmin 1 family genes, including SCG10, SCLIP, and RB3, were also characterized in murine uterus during the peri-implantation period [12]. Consistently, our results also showed an apparent up-regulation of stathmin 1 in mouse endometrium on D5, strengthening the implantation-specific expression manner of stathmin 1. In addition, we also examined its expression in various tissue specimens, and failed to find apparent changes during peri-implantation period in other tissues. The temporal and spatial characters of stathmin 1 expression powerfully suggested its potential association with implantation.

To further determine whether stathmin 1 expression was associated with the presence and status of blastocysts, and

endometrial decidualization, we measured the influence of pseudopregnancy and delayed implantation on stathmin 1 expression. Our results showed that there was no apparent alteration of stathmin 1 expression on D1 to 8 in pseudopregnancy model, which was non-pregnant and only exposed to progesterone. However, when the artificial decidualization was induced by intraluminal infusion of olive oil, stathmin 1 expression increased remarkably in the pseudopregnant mouse 24 h later. These results showed that the up-regulation of stathmin 1 expression was dependent on decidualization, but not affected by progesterone. It was possible that the physiological changes in endometrium that regulated stathmin 1 expression were also involved in decidualization process. Tamura et al. found that stathmin 1 was expressed specifically in the glandular epithelium and stromal cells of human endometrium. Knockdown of stathmin 1 expression in stromal cells before exposure to decidualizing agents remarkably suppressed decidualization, strongly suggesting that stathmin 1 should be involved in the preparation or initiation of decidualization [13,14]. Delayed implantation is a developmental arrest at the blastocyst stage which is suitable for discovering the molecular interaction between embryo and endometrium. We found that the stathmin 1 expression did not increase on D5-7 in normal pregnant mouse when the implantation was delayed by ovariectomization and injection with progesterone, but was up-regulated dramatically 24 h after activation of implantation by 17β-estradiol treatment. These findings suggested that not only the presence but the status of blastocysts should be important to the stathmin 1 expression. Stathmin 1 expression was only up-regulated by activated blastocysts, but not affected by dormant blastocysts.

Progesterone contributed to preparing the uterus for implantation and development of a fertilized blastocyst, and estrogen was a critical determinant that specified the duration of uterine "implantation window" [1]. Therefore, we established a non-pregnant ovariectomization model to clarify the potential effect of steroid hormones on stathmin 1 under physiological status. We found that stathmin 1 expression decreased in non-pregnant ovariectomized mouse. However, treatment with progesterone alone or in combination with 17β-estradiol increased its expression dramatically. The expression profiles of stathmin 1 were comparative between the pregnant mouse and the non-pregnant ovariectomized and steroid-treated mouse, suggesting that the effects of steroid hormones, especially progesterone, on stathmin 1 might be, at least partially, associated with the up-regulation of stathmin 1 during implantation period. However, in another study, no apparent influence of steroid hormones on stathmin 1 was observed in ovariectomized rat [13]. Therefore, so far, we cannot determine whether the up-regulation of stathmin 1 is an up-stream cause or a down-stream effect of steroid hormone change in implantation, and the exact relation between them requires to be elucidated.

In summary, we presented a profile of differentially expressed proteins during peri-implantation period using proteomic approaches. Stathmin 1, hnRNP H3, ApoA-1, arginase 1, and transgelin 2 were confirmed by western blotting. The specific expression profile of stathmin 1 was further examined in a series of established models, including pseudopregnancy, delayed implantation, artificial decidualization, and non-pregnant ovariectomization, which suggested that expression of stathmin 1 should be dependent on activated blastocysts and decidualization, but not affected by dormant blastocysts. Future studies will focus on the spatiotemporal expression characteristics and the diverse functions of these proteins during implantation, which will provide insight into the detailed mechanisms of blastocyst-endometrium crosstalk. In addition, effects of dysregulation of these proteins on pregnant outcomes and the association with pregnancy-related diseases will also be worthy of being studied.

Conflict of interest

There is no conflict of interest.

Acknowledgments

This work was supported by the Natural Science Foundation of China (Grant number: 81170592), Special Fund from National Excellent Doctoral Dissertation (Grant number: 201079), and Program for New Century Excellent Talents in University (Grant number: NCET-10-0594). We thank Prof. Canhua Huang, State Key Laboratory of Biotherapy, West China University Hospital, Sichuan University, for the kind and valuable instruction and assistant in proteomics techniques. We thank Bioms Sci-tech Co., Ltd. (Beijing, China) for the excellent MS services.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.171.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.02.171.

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