Uterine secretome and its modulation in rat (*Rattus norvegicus*)

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Abstract

The present study identifies uterine fluid (UF) proteins that display differential abundance during the embryo-permissive phase in nonconception and conception cycles in rats. UF samples were collected from nonpregnant rats in the proestrous ($n=17$) and metestrous ($n=18$) phases and also from pregnant ($n=17$) and pseudopregnant ($n=17$) rats on day 4 post coitus. UF protein profile in the metestrous phase was compared with that in the proestrous phase. Similarly, UF protein profile of the pregnant rats was compared with that of the pseudopregnant rats. Two-dimensional PAGE, followed by densitometric analysis of the paired protein spots, revealed differential abundance of 44 proteins in the metestrous phase, compared with that in the proestrous phase. Of these, 29 proteins were identified by matrix-assisted laser desorption/ionization time-of-flight or liquid chromatography–tandem mass spectrometry. Functional groups such as proteases, protease inhibitors, and oxidoreductases were enriched in differentially abundant proteins. Total protease activity in UF was found to be significantly ($P<0.05$; t -test) higher in the proestrous phase, compared with that in the metestrous phase. Furthermore, 41 UF proteins were found to be differentially abundant in pregnant rats, compared with pseudopregnant rats. Of these, 11 proteins could be identified. Immunoblotting analysis confirmed significantly higher ($P<0.05$; t -test) abundance of β -actin, Rho-specific guanine nucleotide dissociation inhibitor alpha (Rho-GDI α), and peroxiredoxin-2 and -6 in the metestrous phase, compared with that in the proestrous phase. Compared with pseudopregnant rats, pregnant rats had significantly higher ($P<0.05$; t -test) levels of UF β -actin and Rho-GDI α . Furthermore, these proteins could be detected in the culture supernatants of endometrial epithelial cell lines, thereby providing an evidence of their secretion from endometrial epithelial cells. Data obtained from the study expand our knowledge on the uterine milieu that favours embryo implantation.

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Introduction

Reproductive tract fluids provide an optimal microenvironment for movement of spermatozoa and ova. These fluids also provide a conducive medium for fertilization, mobility, and development of pre-implantation embryos (Aguilar & Reyley 2005). It is believed that the identification of the constituents of reproductive tract fluids can provide significant insights into mechanisms underlying various reproductive events. Attempts have been made to screen follicular fluid, cervical-vaginal fluid, amniotic fluid, uterine fluid (UF), etc., for the presence of specific proteins, presumed to be of relevance in reproductive functions (Jozwik *et al.* 1999, Inagaki *et al.* 2003, Bedaiwy *et al.* 2007, Heng *et al.* 2008, Keeler *et al.* 2009). However, these studies focused only on the particular proteins of interest. Recent advances in proteomic technologies have spurred several efforts toward the identification of the total protein repertoire of human reproductive tract fluids (Tsangaris *et al.* 2006, Casado-Vela *et al.* 2009, Zegels *et al.* 2009, Jarkovska *et al.* 2010). As a result,

now there exists substantial information about the proteins, which collectively contribute toward creating extracellular microenvironments in vicinity of various reproductive tissues.

Investigations to assess the significance of UF in female reproduction date back to 1970s. Proteins, carbohydrates, and other metabolites secreted by the endometrial cells and components of serum transudates are known to be the major constituents of the UF. It is postulated that these components serve as regulators of endometrial cell volume and nutrition, and also as the mediators of intracellular and extracellular communication (Hafez 1977). UF also functions as a transport and support medium for spermatozoa and embryos (Salleh *et al.* 2005). It provides an immunosuppressive environment for implantation, as well as nutrition to a growing embryo (Halperin *et al.* 1998). It was previously believed that the embryo derives its nutrition from uterine luminal fluid only until it establishes contact with the maternal vasculature. However, it has become apparent now that uterine glands and their secretions continue playing an active role in terms of nutrition till the first trimester of

pregnancy in humans (Burton *et al.* 2002). Levels of leukemia inhibitory factor, mucin-1, glycodelin, vascular endothelial growth factor, and proprotein convertase 5/6 in the UF were found to be suboptimal in women with infertility (Mackenna *et al.* 1993, Aplin *et al.* 1996, Mikolajczyk *et al.* 2006, Hannan *et al.* 2011, Heng *et al.* 2011). These studies showed the utility of UF analysis in gauging endometrial function.

In recent years, few proteomics-based investigations have been undertaken to investigate the UF protein complement of humans (Parmar *et al.* 2008, Scotchie *et al.* 2009, Hannan *et al.* 2010) and some animal species such as pigs and ewes (Lee *et al.* 1998, Koch *et al.* 2010). These studies revealed cycle- or pregnancy-dependent modulation in the UF protein profile. However, to our knowledge, there are no reports on the global protein profile of rat UF. Considering the utility of rodent models in assessing the functional relevance of human genes/proteins associated with endometrial receptivity, we considered it worthwhile to identify the proteins that define the luminal milieu of uterine cavity in rats (*Rattus norvegicus*). Protein profiles of the proestrous and metestrous phase UF samples were compared. These investigations were expected to aid in the identification of secreted proteins that may contribute to endometrial preparation toward receptivity. UF proteomes of pregnant and pseudopregnant rats were also compared with an aim to identify those proteins that modulate the uterine microenvironment during embryo attachment.

Materials and methods

The study was approved by the Institute's Animal Ethics Committee. Rats (*R. norvegicus*) were housed in individual cages in a temperature- and humidity-controlled room with a 14 h light:10 h darkness cycle between 23 and 25 °C. Vaginal smears of sexually mature, healthy female rats (200–250 g body weight) were checked daily for three consecutive estrous cycles and only those animals with regular cyclicity were selected.

Nonconception cycle

Nonpregnant animals were allotted to two groups – proestrous ($n=17$) and metestrous ($n=18$) based on the vaginal smear examination. The animals were killed by CO₂ asphyxiation and their uterine horns were quickly excised for retrieval of the UF. For the proestrous group, samples collected from 11 animals were used for 2D proteomics studies (three pools with each containing a pooled sample of three to four animals), three animals for immunoblot analysis, and three animals for protease assays. For the metestrous group, UF samples of 12 animals were investigated for 2D proteomics, three animals for immunoblot analysis, and three animals for protease assays.

Conception cycle

The presence of a sperm plug in the vaginal smears of female rats caged with male rats confirmed mating and was considered as day 1 post coitus or day 1 of pregnancy. Pseudopregnancy was induced by caging adult female rats with vasectomized males. Continuation in the diestrous phase for three consecutive days was an indicator of pseudopregnancy. Pregnant ($n=17$) and pseudopregnant ($n=17$) animals were killed on day 4 post coitus. Uterine horns were excised from pregnant and pseudopregnant rats. For both the groups, UF samples from 11 animals were used for 2D proteomics studies, three for immunoblot analysis, and three for protease assays. For immunoblot and protease assays, individual samples were used.

Collection of UF and uterine tissues

Both the uterine horns were ligated at their tips and also at their conjoint portion. Associated fat and connective tissues were removed from the uterine horns. Each horn was flushed at one end with 0.5 ml saline containing 1 × protease inhibitor cocktail (Amersham Biosciences) and fluid was collected from the other end of the horn. Total volume retrieved from both the horns was around 1.05 ml. For protease assays, fluid samples were collected in saline without protease inhibitors. After collection, samples were immediately centrifuged at 135 *g* for 30 min at 4 °C to remove cells or debris, and the supernatants were stored at –20 °C for proteomic analysis. Protein amount (100–120 µg) recovered from the UF of one animal was insufficient for 2D proteomic analysis; hence, samples were pooled from three or four animals presenting the same condition (proestrous or metestrous phases/pregnancy or pseudopregnancy). Samples were pooled only after confirmation by the histological analysis of the uterine sections of each animal. For histological and immunohistochemical analysis, uterine horns were collected in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄·H₂O, and 2 mM KH₂PO₄) and fixed in 10% buffered formalin for 24 h, washed in 70% vol/vol ethanol, passed through ascending grades of ethanol followed by xylol (xylene:ethanol, 1:1), and xylene treatment. Tissues were then embedded in paraffin wax (Merck Limited). Serial sections of 5 µm were cut and stained with hematoxylin/eosin for histological analysis.

Protein extraction

UF proteins were concentrated by acetone precipitation. Protein pellets were solubilized in rehydration buffer containing 9 M urea, 10 mM dithiothreitol (DTT), and 0.5% 3-((3-chlamidopropyl) dimethylammonio)-1-propanesulfonate. Protein concentration was determined using Bradford's assay (Bradford 1976). Samples to be

compared were subjected to isoelectric focusing and SDS-PAGE in the same run. Immobililine pH gradient strips (Amersham Pharmacia Biotech) of 11 cm length (in the linear range of pH 4–7) were actively rehydrated with 200 µg protein in rehydration buffer containing 20 µl DeStreak solution (Amersham Pharmacia), 4 µl ampholine (Amersham Pharmacia) at 50 V for 16 h, followed by isoelectric focusing at 100 V for 1 h, 250 V for 1 h, 500 V for 1 h, 6000 V for 2 h, and 8000 V for 4 h in a IEF cell (Bio-Rad) at 20 °C. Strips with focused proteins were incubated in equilibration buffer I (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, and 65 mM DTT) for reduction and in equilibration buffer II (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, and 250 mM iodoacetamide) for alkylation. Proteins were resolved in the second dimension by 12% SDS-PAGE and 2D gels were stained using a mass-spectrometry-compatible silver staining method (Shevchenko *et al.* 1996). Stained gels were scanned using ImageScanner III (GE Healthcare Biosciences, Pittsburgh, PA, USA).

Densitometric analysis of 2D gels

For each group, to be compared with its counterpart (proestrous with metestrous; pregnant with pseudopregnant), three representative 2D gels were prepared using the respective samples, each pooled from three or four animals. Each gel representing the proestrous group was compared with all the three gels representing the metestrous group, thus generating nine data sets. A similar strategy was adopted for the comparative analysis of 2D gels representing the pregnant and pseudopregnant groups. Densitometric analysis of gels was carried out using IMP software, version 6 (GE Healthcare Biosciences) to identify differentially abundant proteins. To normalize the total protein load across the gels, total gel spot volume was calculated. Each of the paired spots was analyzed for its volume. Paired spots, which showed twofold or more change in their volume (when compared between proestrous and metestrous gels or between pregnant and pseudopregnant gels), were considered to be differentially abundant.

Excision and identification of spots

Differentially abundant spots were manually excised from 2D gels and trypsin digested according to the protocol published previously (Shevchenko *et al.* 1996). In brief, excised gel plugs were washed four times with Milli Q water by centrifugation at 9391 g for 10 min each. Destaining of the gel plugs was carried out in freshly prepared Farmer's reagent (30 mM potassium ferricyanide and 100 mM sodium thiosulfate) for 20 min at room temperature with shaking until they became colorless. After washing twice with water, gel plugs were incubated with 25 mM ammonium bicarbonate (ABC) for 15 min at RT. This was followed by dehydration in 50% acetonitrile (ACN) and rehydration

in 25 mM ABC for 5 min each. Gel plugs were dehydrated first in 50% ACN and then in 100% ACN. Proteins were reduced to peptides by incubating the dried gel plugs in 4 ng/µl sequencing grade trypsin (Sigma-Aldrich) in 20–25 µl reaction volume for 16 h at 37 °C. Peptides were eluted from gel matrix by mincing the gel plugs. Peptide extraction was done, first in 50% ACN with 0.1% TFA, then in 50% ACN with 0.5% TFA, and finally in 50% ACN with 1% TFA. Supernatants containing tryptic peptides were collected, lyophilized, and stored at –20 °C until MALDI-TOF/TOF or LCMS/MS analysis.

Digested peptides, suspended in 2 µl 50% ACN containing 1.0% TFA, were spotted onto a MALDI-TOF/TOF plate, and mass spectra of trypsin-digested gel spots were generated on a Biflex III MALDI-TOF (Bruker Daltonik, GmbH, Bremen, Germany) or Proteome Analyzer 4700 mass spectrometer (Applied Biosystems). MS/MS identification was carried out using Mascot (Matrix Science, London, UK) to search Swiss Prot or MSDB database. Search parameters used were Rodentia (Rodents) as taxonomy, Carbamidomethyl (C) as fixed modification, and Oxidation (M) as variable modification, peptide tolerance at 100 p.p.m., and maximum 1 missed cleavage by trypsin. The criteria used for a positive identification was significant Mowse score. Mowse score ≥ 64 was considered significant ($P < 0.05$). BioTools (Bruker Daltonik) linked to Matrix Science and those spots that showed statistically significant Mowse score were confirmed by MS/MS for the most abundant peaks from MS.

Differentially abundant proteins were annotated by their GO terms such as biological process, molecular function, and cellular location using Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources 6.7 (<http://david.abcc.ncifcrf.gov/home.jsp>).

Total protease activity in UF

Total protease activity was estimated in rat UF using QuantiCleave protease assay kit (Thermo Scientific, Rockford, IL, USA) as per the manufacturer's instructions. Trypsin, a standard protease, was serially diluted fivefold in the range of 100–0.03 µg/ml. Succinylated casein (2.0 mg/ml) was used as a substrate. UF samples were diluted to 1:10 to ensure that the absorbance of the blank (without the substrate) remained below 0.500. To the wells containing succinylated casein or only assay buffer, 50 µl diluted samples or trypsin standards were added. 2,4,6-Trinitrobenzene sulfonic acid was added to each well after incubation for 20 min at 37 °C. The change in absorbance at 450 nm (ΔA_{450}), calculated by subtracting the A_{450} of the blank (sample or standards without substrate) from that of the corresponding casein well, was indicator of the proteolytic activity. A standard curve (ΔA_{450} against trypsin standard

concentration) was plotted to assess total protease activity of UF samples. Total protease activity was expressed in $\mu\text{g}/\text{mg}$ of UF protein.

Collection of culture supernatants from human endometrial epithelial cell lines

Two human endometrial epithelial cell lines RL95-2 (CRL-1671) and HEC-1A (HTB-112) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). RL95-2 cell line was maintained in a 1:1 mixture of DMEM (Sigma-Aldrich) and nutrient mixture F-12 containing 15 mM HEPES, L-glutamine, 1.2 g/l sodium bicarbonate, and 5.0 mg/l insulin. HEC-1A was grown in McCoy's 5A modified medium (Sigma-Aldrich) with 10 mM HEPES, L-glutamine, and 2.2 g/l sodium bicarbonate. To collect the supernatants, cells were grown in media without sera for 24 h. Cell viability was checked by trypan blue exclusion method. Supernatants collected from the cultures having more than 95% live cells were centrifuged at 135 g to get rid of cellular contamination. Protein pellets obtained by acetone precipitation of culture supernatants were suspended in 100 μl rehydration buffer and stored at -20°C until used.

Western blotting

Protein samples (20 μg) extracted from UF or culture supernatants were resolved on 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Merck Millipore, Billerica, MA, USA). After blocking, blots were incubated overnight with respective primary antibody (against β -actin/Rho-GDI α /peroxiredoxins) at 4°C . β -Actin antibody (MAB raised in mouse) was procured from Sigma-Aldrich; rabbit MABs to peroxiredoxins (-2 and -6) from Epitomics (Burlingame, CA, USA); and rabbit polyclonal antibody to Rho-GDI α was obtained from Invitrogen (Life Technologies). Antibodies used were cross-reactive to human as well as rat/mouse antigens. After washing with PBST (0.05% Tween 20 in PBS), blots were incubated for 1 h with respective secondary antibody (goat anti-rabbit or goat anti-mouse) conjugated to HRP (Dako, Glostrup, Denmark) at room temperature. Immunoreactive bands on the blots were detected using enhanced chemiluminescence (ECL Plus, Amersham Biosciences).

After chemiluminescent detection, blots were stained with Coomassie blue. Intensities of all the bands for each protein sample were determined and summed up using Image Quant (IQTL) software (GE Healthcare Biosciences) to obtain the total intensity, as an indicator of the total protein load. Intensity of the immunoreactive band for the protein of interest on the luminogram was also measured using IQTL software. Ratios of the immunoreactive band intensity to the total protein

intensity were compared to calculate the fold change in the abundance of the protein of interest between different conditions.

Immunohistochemical localization

Uterine sections of 5 μm thickness were deparaffinized and rehydrated using descending grades of methanol. Endogenous peroxidase activity was quenched by incubating the uterine sections in 0.3% H_2O_2 for 30 min. This was followed by microwave treatment of the sections for 10 min in antigen unmasking solution (Vector Laboratories, Burlingame, CA, USA). Sections were blocked with 2% horse serum (for the sections to be incubated with mouse MABs to β -actin) or with goat serum (for the sections to be incubated with polyclonal antibodies against peroxiredoxins or Rho-GDI α) in PBS for 1 h at RT. Sections were then incubated with the respective primary antibodies for 16 h at 4°C . The negative control sections were incubated with rabbit or mouse IgGs in place of primary antibodies. This was followed by incubations of the sections with biotinylated secondary antibodies raised in either goat or horse (Vector Laboratories) for 1 h at 37°C . This was followed by incubation in avidin-biotin-HRP complex for 30 min followed by the addition of 1 mg/ml diaminobenzidine (Sigma-Aldrich) prepared in 0.001% H_2O_2 in PBS. Sections were then counterstained with hematoxylin and gradually dehydrated, cleared in xylene, and mounted.

The staining intensities for immunoreactive antigens were determined using the image analysis software – Aperio Image scope version v11.2.0.780 (Aperio, Vista, CA, USA). At least ten areas from each section were randomly selected for measuring the integrated optical density (IOD).

Figure 1 Representative 2D gels of uterine fluid proteins collected in the proestrous and metestrous phases of the estrous cycle. Total protein (200 μg) sample was resolved first in the pH range of 4–7 and then in second dimension on 12% SDS-PAGE. Circled spots represent those protein spots that were found to be in higher abundance compared with their counterparts in the gel representative of the other phase. These protein spots were identified by MALDI-TOF/TOF or LCMS/MS.

Statistical analysis

Nonparametric unpaired Student's *t*-test was used to determine the significance of difference in the relative levels of immunoreactive antigens on immunoblots and also to evaluate the difference in the intensities of immunolocalized antigens in endometrial tissue sections. This test was also used to determine the difference in total protease activity between proestrous and metestrous groups. The level of statistical significance was selected as $P < 0.05$.

Results

UF proteins displaying differential abundance during the metestrous phase in a nonconception cycle

Image Master Platinum (IMP) software-assisted analysis of the silver-stained two-dimensional (2D) gels of rat UF

collected in the proestrous and metestrous phases of the estrous cycle, revealed the presence of at least 341 and 336 polypeptides respectively (Fig. 1). Two hundred and twenty-six spots were found paired between proestrous and metestrous gels. Comparison of spot volume of paired spots led to the identification of 44 protein spots as differentially abundant by at least twofold. Twenty-three proteins were found to be more abundant in metestrous phase while 21 proteins more abundant in proestrous phase fluid, when compared with each other. Differentially abundant protein spots were excised from the gels and trypsin digested. Twenty-nine proteins were identified using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF/TOF) or liquid chromatography–tandem mass spectrometry (LCMS/MS) with a significant Mowse score (Table 1). Reports of MALDI-TOF/TOF or LCMS/MS analysis are shown as supplementary data.

Table 1 Characteristics of uterine fluid proteins displaying more abundance in the metestrous phase compared with proestrous phase. Proteins were identified by MALDI-TOF/TOF or LCMS/MS. Full-length amino acid sequences of all the proteins were analyzed for the presence of classical and nonclassical signals required for the extracellular secretion using Signal P and Secretome P tools respectively.

Spot Id	Protein identity	Uniprot access key	MOWSE score	Experimental MW (kDa)	Experimental pI	Known function	Presence of classical signals for extracellular secretion	Presence of nonclassical signals for extracellular secretion	Presence in human uterine fluid
M1	Alpha-1-antitrypsin	A1AT_RAT	90	50 ^a	4.5 ^a	Inhibitor of serine proteases	Yes	Yes	Yes ^{b,c}
M2	Contrapsin-like protease Inhibitor 1	SPA3K_RAT	167	46	4.7 ^a	Inhibitor of serine proteases	Yes	Yes	–
M3	Actin, cytoplasmic 1	ACTB_RAT	641	42	5.2	Cellular cytoskeleton	No	No	Yes ^c
M4	14-3-3 Zeta isoform	1433_RAT	140	27	4.7	Regulation of signaling pathway	No	No	Yes ^c
M6	Proteasome subunit alpha type-5	PSA5_RAT	236	26	4.6	Responsible for the chymotrypsin-like activity of the proteasome	No	No	–
M8	Rho GDP-dissociation inhibitor 1	GDIR1_RAT	124	26 ^a	5.1	GDP/GTP exchange reaction of the Rho proteins	No	No	Yes ^c
M9	Peroxiredoxin-6	PRDX6_RAT	304	25	5.2 ^a	Regulation of phospholipid turnover and protection against oxidative injury	No	No	Yes ^c
M10	Peroxiredoxin-6	PRDX6_RAT	187	25	5.6	Same as above	No	No	Yes ^c
M11	Phosphatidylethanolamine-binding protein 1	PEBP1_RAT	56	22 ^a	5.4	Serine protease inhibitor	No	No	Yes ^c
M12	Contraception-associated protein 1	PARK7_RAT	114	22 ^a	6.3	Sensor for oxidative stress	No	No	–
M14	Peroxiredoxin-2	PRDX2_RAT	133	22	5.4	Elimination of peroxides	No	No	Yes ^c
M16	Metastasis-associated protein MTA-1	MTA1_RAT	62	24 ^a	5.0 ^a	Modifies histone proteins	No	No	–
M17	Rat copper–zinc containing superoxide dismutase	SODC_RAT	192	16	5.3 ^a	Antioxidant oxidoreductase	No	Yes	–
M18	Transthyretin chain A rat	TTHY_RAT	230	15	5.7	Transports thyroid hormone, vitamin A	No	Yes	Yes ^{b,c}
M19	Transthyretin chain A rat	TTHY_RAT	196	15	6.0 ^a	Same as above	No	Yes	Yes ^{b,c}
M20	Coactosin-like protein	COTL1_RAT	100	15	5.4	F-actin binding protein	No	Yes	–

^aDeviation from the theoretical molecular weight (MW) or pI, predicted from the full-length amino acid sequence of the respective protein. These deviations may be attributed to posttranslational modifications. ^bHannan *et al.* (2010). ^cScotchie *et al.* (2009).

Figure 2 Enrichment of differentially abundant proteins in the Gene Ontology (GO) groups classified based on their molecular function (A), biological process (B), and cellular component (C). Only those groups that had statistically significant P values (<0.05 ; t -test) are shown.

Full-length amino acid sequences of 29 differentially abundant proteins (retrieved from UniProtKB at <http://www.uniprot.org/>) were screened for the presence of classical and nonclassical secretory signals using online tools SignalP 4.0 (www.cbs.dtu.dk/services/SignalP/) and SecretomeP 2.0 (www.cbs.dtu.dk/services/SecretomeP/) respectively. Of these, alpha 1 antitrypsin and contrapsin-like protease inhibitor 1 had classical signal sequences for the extracellular secretion whereas copper-zinc containing superoxide dismutase, transthyretin, coactosin-like protein, 40S ribosomal protein SA, protease activator complex subunit 1, ferritin, and cofilin 2 were found to have nonclassical signals for the extracellular secretion (Table 1).

Differentially abundant proteins such as alpha-1-antitrypsin (A1AT), β -actin, and peroxiredoxin-2 and -6 were found to be associated with biological processes such as response to organic or inorganic substances, cellular homeostasis, response to wounding, and response to oxidative stress, as determined using the DAVID database (Fig. 2A). Molecular functions of the differentially abundant proteins such as phosphatidylethanolamine binding protein 1, copper-zinc containing superoxide dismutase, 14-3-3 zeta protein, metastasis-associated protein-1, and peroxiredoxins were enzyme binding, antioxidant activity, and peptidase and endopeptidase activity (Fig. 2B). When categorized based on the cellular component, the majority of differentially abundant proteins were cytosolic or extracellular (Fig. 2C).

UF proteins displaying differential abundance during the embryo attachment stage in a conception cycle

Analysis of the silver-stained 2D gels of UFs collected from pregnant and pseudopregnant rats on day 4 post coitus (the day of embryo attachment) revealed the presence of at least 364 and 347 polypeptides respectively (Fig. 3). Two hundred and thirty-five spots were

Figure 3 Representative 2D gels of uterine fluid proteins collected from pregnant and pseudopregnant rats on the day 4 post coitus. Proteins (200 μ g) were resolved in the pH range of 4–7 and then in the second dimension by 12% SDS-PAGE. Circled spots represent those protein spots that were more abundant in the pregnant rats compared with pseudopregnant rats. These protein spots were identified by MALDI-TOF/TOF or LCMS/MS.

found to be paired in the gels representing the UF protein profile of pregnant and pseudopregnant rats. Spot volume comparison of the paired spots led to the identification of 41 protein spots as differentially abundant by at least twofold. Circled spots (Fig. 3) indicate the protein present in higher abundance compared with its counterpart in the other phase. Twenty-one proteins were found to be more abundant in pregnant rats and 20 proteins more abundant in pseudopregnant rats. Excision from the gel and trypsin digestion followed by MALDI-TOF/TOF or LCMS/MS analysis identified 11 proteins with a significant Mowse score (Table 2). Among these, only serum albumin was found to have classical and nonclassical signals for the extracellular secretion. Reports of MALDI-TOF/TOF or LCMS/MS analysis are shown as supplementary data.

Total protease activity in UF

Total protease activity in the UF was significantly higher ($P < 0.05$) in the proestrous phase, compared with that in the metestrous phase (Fig. 4). Although protease activity was significantly ($P < 0.05$) higher in pregnant animals compared with nonpregnant rats, this did not differ significantly between pregnant and pseudopregnant rats.

Detection of β -actin, Rho-GDI α , and peroxiredoxin-2 and -6 in endometrial epithelial culture supernatants

Immunoblotting of the protein extracts of serum-deprived culture supernatants of two endometrial epithelial cell lines, RL95-2 and HEC1-A, revealed the presence of β -actin, Rho-specific guanine nucleotide dissociation inhibitor alpha (Rho-GDI α), and peroxiredoxins (Fig. 5). These results suggested a possibility of these proteins being secreted by the endometrial epithelium in the UF.

Validation of 2D data

Immunoblotting experiments using specific antibodies were carried out to validate the 2D data. UF β -actin was found to be 1.70-fold ($P = 0.0093$) and Rho-GDI α to be 2.73-fold ($P = 0.0314$) more abundant in the metestrous phase, compared with that in the proestrous phase (Fig. 6). Peroxiredoxin-2 and peroxiredoxin-6 were also found to be 3.58- ($P = 0.006$) and 2.07-fold ($P = 0.0095$) respectively more abundant in the metestrous phase (Fig. 6). Thus, immunoblotting results were in concordance with 2D data.

Pregnant and pseudopregnant animals were compared for the abundance of these proteins in the UF.

Table 2 Characteristics of uterine fluid proteins displaying more abundance in the proestrous phase compared with metestrous phase. Proteins were identified by MALDI-TOF/TOF or LCMS/MS. Full-length amino acid sequences of all proteins were analyzed for the presence of classical and nonclassical signals required for extracellular secretion using Signal P and Secretome P tools respectively.

Spot Id	Protein identity	Uniprot access key	MOWSE score	Experimental MW (kDa)	Experimental pI	Known function	Presence of classical signals for extracellular secretion	Presence of nonclassical signals for extracellular secretion	Presence in human uterine fluid
P1	Contrapsin-like protease inhibitor 1	SPA3K_RAT	360	46	5.3 ^a	Inhibitor of serine proteases	Yes	Yes	–
P2	Anti-trypsin precursor	A1AT_RAT	356	46	4.9 ^a	Inhibitor of serine proteases	Yes	Yes	–
P3	Creatine kinase, B chain	KCRB_RAT	436	43	5.3	Energy transduction	No	No	Yes ^{b,c}
P4	Transaldolase	TALDO_RAT	166	38	6.6	Pentose metabolism	No	No	–
P5	Aldose reductase	ALDR_RAT	239	36	6.8 ^d	Catalysis of NADPH-dependent reduction	No	No	–
P6	40 S ribosomal protein SA	RSSA_RAT	233	38	4.8	Signaling pathways	No	Yes	–
P8	Tropomyosin alpha-4 chain	TPM4_RAT	338	32 ^d	4.8	Regulation of smooth muscle contraction	No	No	Yes ^c
P9	Annexin V (Lipocortin V)	ANXA5_RAT	514	36	4.9	Positive regulation of apoptosis	No	No	Yes ^c
P12	Protease activator complex subunit 1	PSME1_RAT	269	29	5.77 ^d	Degradation of incorrectly folded proteins	No	Yes	–
P14	Proteasome subunit alpha type 1	PSA1_RAT	96	30	6.6 ^d	Same as above	No	No	–
P15	High-mobility group protein B1	HMG1_RAT	190	25	6.6 ^d	Histone binding protein	No	No	–
P20	Ferritin heavy chain	FRIH_RAT	74	21	5.8	Ferroxidase activity	No	Yes	–
P21	Cofilin-2	COF2_MUS	73	18	6.6 ^d	Actin reorganization	No	Yes	–

^aA different isoform of the same protein reported in Table 1. ^bHannan *et al.* (2010). ^cScotchie *et al.* (2009). ^dDeviation from the theoretical molecular weight (MW) or pI, predicted from the full-length amino acid sequence of the respective protein. These deviations could be attributed to posttranslational modifications.

Figure 4 Estimation of total protease activity in the uterine fluid (UF) samples collected from the nonpregnant rats in their proestrous and metestrous phases (A) and also in the samples collected from the pregnant and pseudopregnant rats on day 4 post coitus (B). Total protease activity was expressed as µg/mg protein recovered from the UF. * $P < 0.05$, where P is the level of significance of the difference (by t -test).

β -Actin was 3.01-fold ($P = 0.0002$) and Rho-GDI α was 1.64-fold ($P = 0.02$) more abundant in the pregnant rats, compared with the pseudopregnant rats (Fig. 7).

Peroxiredoxin-6 was 1.4-fold more abundant in the UF of pregnant rats, compared with that in the pseudopregnant rats (Fig. 7). Whereas, no significant change was found in the abundance of peroxiredoxin-2 in the UF of pregnant and pseudopregnant rats.

Detection of β -actin, Rho-GDI α , and peroxiredoxin-2 and -6 in the endometrial tissues

β -Actin, Rho-GDI α , and peroxiredoxin-2 and -6 were localized in the glands as well as in the stroma of rat endometrium. Their localization was found to be more intense in the glandular epithelium compared with that in the stroma (Fig. 8A). Semiquantitative analysis of the intensities of immunoreactive antigens in the endometrial tissues demonstrated significantly ($P < 0.05$) higher expression of β -actin, Rho-GDI α , and peroxiredoxins in the metestrous phase (Fig. 8A, a, b, c, and d) compared with that in the proestrous phase (Fig. 8A, e, f, g, and h and Supplementary Figure S1, see section on supplementary data given at the end of this article). This was similar to the

pattern detected in the UF samples, collected in proestrous and metestrous phases. These proteins could also be localized in the luminal epithelial compartment of endometrium. Intensities of the immunoreactive proteins appeared higher in the metestrous phase, than in the proestrous phase endometrium (Fig. 8D). This was suggestive of the possibility that the endometrial epithelium could be one of the sources from which these proteins are secreted into the uterine lumen.

Pregnant animals also demonstrated significantly higher intensities of endometrial β -actin, Rho-GDI α , and peroxiredoxins compared with pseudopregnant animals (Fig. 9 and Supplementary Figure S2).

Discussion

The uterine lining (i.e. endometrium) acquires competency for embryo attachment during the receptive phase in a menstrual cycle. It also assumes a secretory phenotype (Denker 1994). Uterine secretions contribute toward creating an optimal milieu for embryonic growth, development, and attachment. The majority of evidence for this notion originated from studies in animal models. Restriction of embryos to the oviduct, by ligation of the tubo-uterine junction, led to a halt in their development at the early blastocyst stage (Kirby 1962). Embryos remained unattached or became degenerated in the uterine cavity of pregnant ewes, whose uteri lacked endometrial epithelial glands and their secretions (Gray *et al.* 2002). In humans also, infertility in some cases was found to be associated with altered levels of proteins such as leukemia inhibitory factor, proprotein convertase, and mucin-1 in the UF (Aplin *et al.* 1996, Mikolajczyk *et al.* 2006, Heng *et al.* 2011). Thus, investigations of the UF in the receptive phase assume significance as these may lead to the identification of the biomarkers of endometrial function. In addition,

Figure 5 Detection of β -actin, peroxiredoxins, and Rho-GDI α in the culture supernatants of human endometrial epithelial cell lines, RL95-2 and HEC1-A. Protein samples precipitated from the culture supernatants of cell lines were immunoprobed with the respective antibodies.

Figure 6 Immunodetection to compare the levels of β -actin, Rho-GDI α , and peroxiredoxin-2 and -6 in the metestrous and proestrous uterine fluid (UF). M1, M2, and M3 and P1, P2, and P3 represent the protein extracts of UF samples collected from three individual animals in metestrous (M) or proestrous phase (P) respectively. UF proteins were resolved by SDS-PAGE and blotted onto a nylon membrane. Blots were immunoprobed with respective antibodies and immunoreactive antigens were detected by ECL (A). The density of the band corresponding to the respective immunoreactive antigen on the luminogram was measured using Image Quant software (IQTL). After immunoprobing, the blots were stained with Coomassie blue to determine the total protein load in each lane (B). Total protein intensity of each sample was measured by summation of all the protein bands using IQTL. The ratio of the intensity of the immunoreactive band for the protein of interest to total protein intensity was calculated for each sample (C). Mean ratios ($\pm$ s.d.) were calculated for each group. Significance of the difference between mean ratios for proestrous and metestrous groups was calculated by Student's *t*-test. * $P < 0.05$, ** $P < 0.01$, where *P* is the level of significance of the difference.

proteomic analysis of the UF, collected before or during embryo attachment, may provide significant insights into the uterine milieu that favours embryo implantation.

The present study demonstrated differential abundance of 44 UF proteins in the metestrous or receptive phase, compared with the proestrous phase. This indirectly suggested hormone-dependent modulation in the UF protein repertoire during a nonconception cycle. When categorized according to the 'molecular

function', the majority of differentially abundant proteins (e.g. A1AT, contrapsin-like protease inhibitor, proteasome subunit alpha 5, and phosphatidylethanolamine binding protein-1) were enriched in the groups typified by serine or threonine-type endopeptidase activity and peptidase inhibitor activity. Previous reports have also demonstrated the presence of proteases in the UF of rats, mice, and rabbits (Joshi *et al.* 1970, Beier 1974, Hoversland & Weitlauf 1982). Our 2D data

Figure 7 Immunodetection to compare the levels of β -actin, Rho-GDI α , and peroxiredoxins in the uterine fluid (UF) samples collected from pregnant (Pg) and pseudopregnant (Pp) rats on day 4 post coitus. Pg1, Pg2, and Pg3 and Pp1, Pp2, and Pp3 represent the protein extracts of UF samples collected from three individual pregnant (Pg) and pseudopregnant (Pp) animals. UF proteins were resolved by SDS-PAGE and blotted onto nylon membrane. Blots were immunoprobed with respective antibodies and the immunoreactive antigens were detected by ECL (A). The density of the band corresponding to the respective immunoreactive antigen on the luminogram was measured using Image Quant (IQTL) software. After immunoprobing, the blots were stained with Coomassie blue to determine the total protein load in each lane (B). Total protein intensity for each sample was measured by summation of all the bands using IQTL. The ratio of the intensity of the immunoreactive band for the protein of interest to total protein intensity was calculated for each sample (C). Mean ratios ($\pm$ s.d.) were calculated for each group. Significance of the difference in the mean ratios between pregnant and pseudopregnant groups was calculated by Student's *t*-test. * $P < 0.05$, *** $P < 0.001$, where *P* is the level of significance of the difference.

Figure 8 (A) Immunohistochemical localization of β -actin (a and e), Rho-GDI α (b and f), peroxiredoxin-2 (c and g), and peroxiredoxin-6 (d and h) in the metestrous (a, b, c, and d) and proestrous (e, f, g, and h) phase endometrial tissues. Panels i and j represent the sections where primary antibodies were replaced with rabbit and mouse IgGs respectively. Magnification 40 $\times$. Panels B and C display results of the semiquantitative analysis of the intensities of immunoreactive antigens in glandular epithelial or stromal compartments respectively. * P value < 0.05, ** P value < 0.001, *** P value < 0.0001 (t -test). Panel D shows immunolocalization of β -actin (a and e), Rho-GDI α (b and f), peroxiredoxin-2 (c and g), and peroxiredoxin-6 (d and h) in the luminal epithelial compartments of metestrous (a, b, c, and d) and proestrous (e, f, g, and h) phase endometrial tissues. Panels i and j represent negative control sections where primary antibodies were replaced with rabbit and mouse IgGs respectively. Magnification 100 $\times$.

demonstrated hormone-dependent modulation in the abundance of proteases as well as protease inhibitors in the UF. This was further supported by detection of significantly higher total protease activity in the proestrous phase, compared with that in the metestrous phase. Regulation of the UF protease activity probably ensures the survival of embryos in the uterine cavity as well as their invasion into the endometrium.

Interestingly different isoforms of A1AT precursor and contrapsin-associated protein showed differential abundance in the proestrous and metestrous phases of a nonconception cycle. In humans also, different

isoforms of A1AT were overrepresented in different phases of the menstrual cycle (Parmar *et al.* 2008, Scotchie *et al.* 2009). This raises the possibility that in different hormonal milieu, different isoforms of a protein are expressed.

For validation of 2D data, we focused on four proteins – β -actin, Rho-GDI α , and peroxiredoxin-2 and -6, annotated as cytosolic proteins by Gene Ontology (GO) analysis on the basis of cellular component. It was intriguing to detect the presence of cytosolic proteins in the UF. Although others have also reported the presence of cytosolic proteins such as tubulin and

Figure 9 (A) Immunohistochemical localization of β -actin (a and e), Rho-GDI α (b and f), peroxiredoxin-2 (c and g), and peroxiredoxin-6 (d and h) in the pregnant (a, b, c, and d) and pseudopregnant (e, f, g, and h) rat endometrial tissues. Panels i and j represent the sections where primary antibodies were replaced with rabbit and mouse IgGs respectively. Magnification 40 $\times$. Panels B and C display results of the semiquantitative analysis of the intensities of immunoreactive antigens in glandular epithelial and stromal compartments respectively. ***P* value < 0.001, ****P* value < 0.0001 (*t*-test).

cofilin in the endometrial secretome (Scotchie *et al.* 2009), these are not considered as conventional secretory proteins. When analyzed *in silico*, these proteins were found to have no signals (classical and/or nonclassical) for extracellular secretion. The presence of these proteins in the UF can be attributed to two possibilities. One possibility was that these proteins may find their way into the UF because of the contamination with endometrial cells and their subsequent rupture during sample collection. We ruled out this possibility by immediately centrifuging the samples after collection to get rid of a cellular contamination. The other possibility was that these proteins in the UF indeed represent a physiological event and are released into the uterine cavity through extracellular secretion or cellular apoptosis. It has been reported that the endometrial epithelium undergoes apoptosis during the receptive phase in rodents (Parr *et al.* 1987). There is another possibility that some proteins (including those which lack classical secretory signals) are released into the UF by an apocrine mode, which involves release of secretory vesicles, along with some cytoplasmic contents into the lumen. Indeed, scanning electron microscopy-based evidence exists which indicates that endometrial secretion occurs via an apocrine mode (Hafez *et al.* 1975, 1977). Detection of these proteins in the culture supernatants of human endometrial epithelial

cell lines strengthens the possibility of their *in vivo* secretion by the endometrial epithelium. It will be interesting to investigate whether β -actin, Rho-GDI α , and other UF proteins execute their conventional functions (for example, β -actin-mediated cytoskeleton arrangement) or they assume novel functions in an extracellular milieu.

Levels of specific proteins in the UF may vary either because of differential secretion into the luminal cavity or due to differential synthesis by the endometrium. We observed relatively higher expression of endometrial β -actin, Rho-GDI α , and peroxiredoxins in the metestrous phase, compared with that in the proestrous phase. Pregnant rats also demonstrated higher expression of endometrial β -actin, Rho-GDI α , and peroxiredoxins compared with pseudopregnant rats. The expression patterns of these proteins in the endometrial tissues were similar to those found in the UF, when compared between pregnancy and pseudopregnancy or between proestrous and metestrous phases. Thus, an increased abundance of these proteins in the UF may be attributed to an increased synthesis in the endometrial epithelium, during the receptive phase in a nonconception cycle and also during pregnancy. These proteins were found to be localized in the luminal epithelium of endometrium, thereby strengthening the possibility of their secretion from the endometrium into the uterine cavity.

Table 3 Characteristics of proteins displaying differential abundance in the uterine fluid of pregnant and pseudopregnant animals. Proteins were identified by MALDI-TOF/TOF or LCMS/MS. Full-length amino acid sequences of all proteins were analyzed for the presence of classical and nonclassical signals for extracellular secretion.

Spot Id	Protein identity	Uniprot access key	MOWSE score	Experimental MW (kDa)	Experimental pI	Higher abundance	Known function	Presence of classical signals for extra-cellular secretion	Presence of non-classical signals for extra-cellular secretion	Presence in human uterine fluid
SB22	Serum albumin	ALBU_RAT	322	46 ^a	5.8 ^a	Pregnant	Regulation of the colloidal osmotic pressure of blood and transporter protein	Yes	Yes	–
SB26	Aldose reductase	ALDR_RAT	179	36	6.8 ^a	Pregnant	Converts glucose into fructose	No	No	–
SB23	Actin, cytoplasmic 1	ACTB_RAT	359	40 ^a	5.2	Pregnant	Cytoskeleton protein	No	No	Yes ^{b,c}
SB24	Actin, cytoplasmic 1	ACTB_RAT	87	38 ^a	5.6 ^a	Pregnant	Same as above	No	No	Yes ^c
SB27	Proteasome subunit alpha, type 1	PSA1_RAT	84	30	6.6 ^a	Pregnant	ATP-dependent proteolytic activity	No	No	–
SB31	14-3-3 sigma protein	1433S_MOUSE	108	30	4.7	Pregnant	Signaling pathways	No	No	Yes ^c
SB32	Rho GDP-dissociation inhibitor 1	GDIR1_RAT	110	26 ^a	5.1	Pregnant	GDP/GTP exchange reaction of the Rho proteins	No	No	Yes ^c
SB34	Peroxiredoxin-6	PRDX6_RAT	184	25	5.6	Pregnant	Regulation of phospholipid turnover and protection against oxidative injury	No	No	Yes ^c
SB36	Actin	ACTB_RAT	103	20 ^a	4.3 ^a	Pregnant	Cytoskeleton protein	No	No	Yes ^c
SB40	Peptidylpolycistrans isomerase	PPIA_RAT	70	17	5.8 ^a	Pregnant	Folding of proteins	No	No	–
SB1	Alpha enolase	ENOA_RAT	102	58 ^a	5.6 ^a	Pseudopregnant	Receptor and activator of plasminogen and stimulates immunoglobulin production	No	No	–

^aDeviation from the theoretical molecular weight (MW) or pI, predicted from the full-length amino acid sequence of the respective protein. These deviations may arise due to posttranslational modifications. ^bHannan *et al.* (2010). ^cScotchie *et al.* (2009).

Interestingly, in humans also, expression of endometrial β -actin has been reported to be upregulated in the receptive phase (Chen *et al.* 2009). In the same study, expression of endometrial Rho-GDI α was also found to be higher in the receptive phase. Rho-GDI are negative regulators of Rho family GTPases, which control various processes, including cell adhesion, migration, proliferation, and actin-dependent cytoskeletal morphogenesis (Hall & Nobes 2000). Our investigation of the rat UF revealed the same pattern, i.e. higher levels of Rho-GDI α in the receptive phase compared with the proestrous

phase. Thus, the pattern of cycle-dependent abundance, at least for some proteins, in UF was found to be akin to that in endometrium. This reinforces the utility of UF analysis in assessing the endometrial function.

Peroxiredoxins in the UF were found to be more abundant in the metestrous phase, compared with the proestrous phase, in nonpregnant rats. Peroxiredoxins are known to play an important role in protection against oxidative injury (Immenschuh & Baumgart-Vogt 2005). It is well established that embryo metabolism results in reactive oxygen species, excess of which can

induce development block and retardation of embryonic growth (Guerin *et al.* 2001). Increased levels of peroxiredoxins in the UF probably protect the embryo from oxidative injury.

We scanned available proteomic data on the human UF to investigate whether rodents and humans share any similarity in terms of their UF protein repertoire and hormonal modulation. Eleven proteins, including β -actin and A1AT precursor, have been reported in the human UF also (Table 3). As observed in rats, β -actin levels in the human UF were higher and those of tropomyosin α 4 chain, lower in the receptive phase, compared with the pre-receptive phase. Thus, to some extent, receptivity-associated events appear to be conserved in rats and humans.

Uterine luminal fluid samples of sheep, cattle, and pigs have been previously investigated to define adaptation in the uterine microenvironment in response to the presence of the conceptus (Bazer *et al.* 2012). In the present study, gestational UF, collected at the time of embryo attachment, was investigated for its protein complement. Results indicated a differential abundance of 41 proteins in the UF of pregnant animals, compared with pseudopregnant animals. These proteins could have been secreted by either the embryo or the endometrium in response to embryonic signals or by both.

2D as well as immunoblotting data demonstrated higher levels of β -actin in the UF of pregnant animals, compared with pseudopregnant animals. The presence of β -actin has also been reported in the sheep UF during initial embryo attachment (Lee *et al.* 1998). In the present study, different forms of β -actin were detected in the 2D gels representing the proteome of gestational UF. It is not clear whether different forms of β -actin originate because of a possible proteolytic action in the UF or whether these are secreted as such from the embryo or endometrium during the embryo attachment stage. In 1D immunoblots, truncated actin fragments (of 38 and 20 kDa, as observed in the 2D gels) were not detected. This could be attributed to immunoprobings of 1D blots with a MAB directed against an N-terminal epitope, probably absent in the truncated fragments of β -actin.

To sum up, the study investigated proteomic alterations in the rat UF during the period when there is a maximum likelihood of embryos getting implanted in a nonconception cycle. Several proteins, not reported earlier for their presence in the rat UF, have been identified. Some of the proteins showed the similar pattern of abundance in the receptive phase, to that reported in humans. The study also led to the identification of proteins that are differentially secreted in the gestational UF, during the embryo attachment stage. It will be of interest to investigate whether modulation in the levels of specific protein influences embryonic development and/or implantation and whether any of these can be used as an indicator of the functional normalcy of embryo and/or endometrium.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-12-0461>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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