PHOSPHOPROTEOMICS AND GLYCOPROTEOMICS OF ENDOMETRIAL SECRETION IN ASSISTED REPRODUCTIVE TECHNOLOGY CYCLES WITH IMPLANTATION AND NON-IMPLANTATION

BY

MISS JINDARAT JENKIENGKRI

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN MEDICAL SCIENCE

FACULTY OF MEDICINE

THAMMASAT UNIVERSITY

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ENTITLED

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was approved as partial fulfillment of the requirements for
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Thesis Title	PHOSPHOPROTEOMICS AND GLYCOPROTEOMICS OF ENDOMETRIAL SECRETION IN ASSISTED REPRODUCTIVE TECHNOLOGY CYCLES WITH IMPLANTATION AND NON-IMPLANTATION
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ABSTRACT

Embryo implantation represents a crucial step to success in the assisted reproductive technology treatment (ART) of infertility. The endometrial receptivity assessment during the window of implantation (WOI) has been an important challenge. The analysis of endometrial receptivity using endometrial secretion has been of particular interest as they represent a non-invasive approach and the number of mediators contained may involve in the preparation for embryo to implant and by modulation of endometrial receptivity.

This study aims firstly, to investigate the endometrial secretome based on the phosphoproteomics and glycoproteomics study, and secondly, to identify possible potential phosphoprotein and glycoprotein biomarkers to represent for the phases of human endometrium and receptivity during the WOI of stimulated ART treatment cycle.

The cross-sectional study performed in infertile couples underwent assisted reproductive technology treatment at Thammasat Fertility Center, Thailand, during Dec 2015 – Dec 2016. This study included total of 60 infertile women of which 30 were

patients with implantation and another 30 were patients with non-implantation. Endometrial secretions were also collected from embryo transfer catheter tip at the oocyte retrieval day (day 0/ D0) of 15 implantation plus 15 non-implantation patients designated as group I (baseline or control) and of 30 implantation patients and 30 non-implantation patients at embryo transfer day (day 5/ D5) designated as group II and group III (study groups) respectively. The sample from each group was analysed for phosphoproteins and glycoproteins.

To study phosphoproteins and glycoproteins in endometrial secretion, immobilized metal affinity chromatography (IMAC) and lectin concanavalin A (ConA) were used to enrich phosphorylated proteins and N-glycosylated proteins respectively. After that the proteins were digested into short peptide by trypsin, and analysed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) approaches. Bioinformatic tools were used to compare phosphoproteins and glycoproteins found unique between the groups, searching for gene ontology and category classifications.

In order to validate the predicted differential phosphoproteins and glycoproteins expression of endometrium receptivity biomarkers based on LC-MS/MS data, the western blot analysis using the commercial antibodies against total protein was performed in sample collected at D5 comparing between implantation and non-implantation groups. The results of these western blot analysis were then compared to the corresponding phosphopeptide and glycopeptide obtained from mass spectrometry results.

From all of the endometrial secretion samples in this study, LC-MS/MS analysis resulted in the identification of total 267 phosphoproteins and 110 glycoproteins. In order to identify phosphoproteins and glycoproteins those represent for the phase of endometrium, this study compared the differential phosphoproteins and glycoproteins expression between samples collected at oocyte retrieval day (D0 as pre-receptive phase) and samples collected at embryo transfer day (D5 as receptive phase or window period of implantation). It was found in this study that 267 phosphoproteins and 105 glycoproteins expressed at D0, whereas 220 phosphoproteins and 110 glycoproteins expressed at D5. Moreover, 47 phosphoproteins were found unique at D0 and 5 glycoproteins were found unique at D5.

In order to identify phosphoprotein and glycoprotein those represent for the endometrium receptivity, this study also compared the differential phosphoproteins and glycoproteins expressions at D5 between implantation and non-implantation groups. It was found that 4 phosphoproteins and 1 glycoprotein were found unique in the implantation group, whereas 36 phosphoproteins and 2 glycoproteins were found unique in the non-implantation group.

Regarding validation of the LC-MS/MS results, western blot analysis was performed. This study analysed the total protein expressions in two selected phosphoproteins and one selected glycoprotein: ATP synthase subunit gamma, mitochondrial (ATPG), katanin p60 ATPase containing subunit A1 (KATNA1), and thioredoxin domain-containing protein 2 (thioredoxin2), respectively. The results of western blot of phosphoprotein and glycoprotein validation showed that there were total proteins expressed in samples of implantation and non-implantation groups. These confirmed the results of protein expression by LC-MS/MS, however, there was not quite similar in pattern of expression in which LC-MS/MS showed of the identified proteins were uniquely expressed. This study also analysed the relative quantity of total protein expression. For phosphoprotein validation, there was no significantly different in protein abundances between implantation and non-implantation groups. Whereas, for glycoprotein validation, there was significantly different in protein abundances between implantation and non-implantation groups with similar trend to those of LC-MS/MS result. The results obtained both by western blot analysis in correlation with LC-MS/MS, it is proposed that there was the same parent proteins found in both implantation and non-implantation groups as measured by western blot analysis while apparent post-translational modification forms especially phosphorylated and glycosylated were differentially found in implantation and non-implantation groups as measured by LC-MS/MS.

Based on the findings, it was postulated that 267 phosphoproteins and 105 glycoproteins expressed at D0 may represent pre-receptive phase of endometrium, while 220 phosphoproteins and 110 glycoproteins expressed at D5 may represent receptive phase of endometrium. Moreover, in this study 47 phosphoproteins were found unique at D0 and 5 glycoproteins were found unique at D5. This finding may help especially to improve more sensitivity and specificity values of the ability to

discriminate between pre-receptive and receptive phases of endometrium. Based on their sequential temporal expression with respect to the implantation window, it was considered that the number of phosphoproteins and glycoproteins identified may regulate the functions of human endometrium in preparation for the implantation process during early and mid-luteal phase of stimulated endometrium.

It was also postulated that 4 phosphoproteins and 1 glycoprotein found unique in implantation group may represent the activity for promotion of the endometrium receptivity and may be regarded as potential biomarkers for positive regulation of endometrium receptivity. Whereas 36 phosphoproteins and 2 glycoproteins found unique in non-implantation group may represent barrier activity of the endometrium receptivity and might be regarded as potential biomarkers for negative regulation of endometrium receptivity. All of these functional proteins might reflect important physiological activities and supposedly characteristics of endometrium receptivity.

In conclusion, these preliminary data apply the comprehensive phosphoproteomics and glycoproteomics study of endometrial secretion to evaluate of significance protein profiles. This study showed that a panel of proteins, rather than a single one can improve sensitivity and specificity values of the ability to discriminate between pre-receptive and receptive phases. Also, these receptivity biomarkers may help to increase sensitivity and specificity determining predictive value as definite indicator for endometrium receptivity. Finally, based on the findings in this study, it is suggested that study of biomarkers in endometrium related to implantation process may not be pointed to the difference observed by total proteomics study, but should be considered with more unique insights. Therefore, from results of this study we proposed that regulation of endometrium with implantation might exert at post-translational levels.

The limitation of this study was the endometrial secretion collected during WOI using non-invasive clinical routine procedure elicited the sample containing very low abundance of proteins. Therefore, it is quite difficult for analysis since generally more protein concentration is required and then more demanding processes are needed to perform analysis, especially analysis of phosphoproteins and glycoproteins included

in this study. Furthermore, antiphospho- and antiglyco-specific antibodies for western blot analysis are not always available.

Further study is needed in the larger population or by way of working with other reproductive samples. Other confirmatory methods will be needed to validate these findings. Tests in the other biological fluids would be more easily and relatively non-invasive to collect sample i.e. plasma or urine that are preferred in clinical applications. The continuous dynamic analysis of identified biomarkers starting from pre-receptive to receptive period (time series) is certainly required for further study. To correlate the unique phosphoproteins and glycoproteins found in this study with other approaches e.g. histological, ultrasound, and pinopods for assessing the endometrium receptivity are of interest for future research, and certainly, other PTMs study, such as ubiquitination, acetylation, etc. are also interested.

Keywords: Phosphoproteomics, Glycoproteomics, Endometrial secretion, Window period of implantation, Assisted reproductive technology

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Miss Jindarat Jenkiengkri

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LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
%	Percentage
/	Per
°C	Degree Celsius
A	Alpha
B	Beta
Γ	Gamma
µ	Micron
µg	Microgram
µl	Microliter
µm	Micromolarity
µmol	Micromole
2D-PAGE	Two dimensional-polyacrylamide gel electrophoresis
Abs	Absorbance
ADP	Adenosine diphosphate
APS	Ammonium persulfate
ARF6	ADP-Ribosylation Factors 6
ART	Assisted reproductive technology
ASB	Ankyrin repeat and SOCS (suppressor of cytokine signaling) box
ASB5	Ankyrin repeat and SOCS box protein 5
ATP	Adenosine triphosphate
ATPG	ATP synthase subunit gamma
BMI	Body mass index
BSA	Bovine serum albumin
Ca2+	Calcium ion
$CaCl_2$	Calcium chloride
cAMP	Cyclic adenosine monophosphate

CAN	Acetonitrile
cGMP	Cyclic guanosine monophosphate
CICR	Calcium-induced calcium release
Cm	Centimeter
COH	Controlled ovarian hyperstimulation
COX	Cytochrome C oxidase
CRC	Calcium release channels
CTC	Copper-tartrate-carbonate
Cu^{2+}	Copper ion
$CuSO_4.7H_2O$	Copper sulfate heptahydrate
$CuSO_4.H_2O_2$	Copper (II) sulphate
D0	Day 0 (Day at oocyte retrieval)
D5	Day 5 (Day at embryo transfer)
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
e.g.	Exempli gratia, for example
ER	Endoplasmic reticulum
ESC	Endometrial stromal cell
ET	Embryo transfer
et al.	Et alii, and others
etc.	Et cetera, and other things
FA	Formic acid
FSH	Follicle-stimulating hormone
G	Gram
G	Gravity centrifugal force
GnRH	Gonadotropin-releasing hormone
H	Hour
HCG	Human chorionic gonadotropin
hCG	Human coding gene
HVA	high-voltage activated
i.e.	Id est, that is

IAA	Iodoacetamide
ICAM-1	Intercellular adhesion molecules
Ig	Immunoglobulin
IMAC	Immobilized metal affinity chromatography
IVF	In vitro fertilization
KATNA 1	Katanin p60 ATPase-containing subunit A1
Kb	Kilobase
KCl	Potassium chloride
kDa	Kilodalton
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
LH	Luteinizing hormone
M	Molar
mA	Milliamps
Min	Minute
ml	Milliliter
mM	Millimolar
mRNA	Messenger ribonucleic acid
MW	Molecular weight
Na_2CO_3	Sodium carbonate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
Ng	Nanogram
Nm	Nanometer
Nmol	Nanomole
NO	nitric oxide
OD	Optical density
OXPHOS	Oxidative phosphorylation
p53/TP53	tumor protein 53
PBS	Phosphate buffer saline
PBS-T	Tween-20 in phosphate buffer saline

PCR	Polymerase chain reaction
pH	Potential of hydrogen ion
PR	progesterone receptor
PRKG1	cGMP-dependent protein kinase 1
PTM	Post-translational modification
rFSH	Recombinant Follicle-stimulating hormone
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPM	Round per minute
rRNA	Ribosomal ribonucleic acid
RyR	Ryanodine receptor
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SOCS	Suppressor of cytokine signaling
SR	Sarcoplasmic reticulum
TCA	Tri-chloroacetic acid
TEMED	N,N,N′,N′-tetramethylethylenediamine
TF	transcription factor
TPBS	Tris-Phosphate buffer saline
TRX	Thioredoxin
U	Unit
UV	Ultraviolet
v/v	Volume by volume
VCAM-1	Vascular cell adhesion molecule 1
VDCC	Voltage-dependent calcium channel
w/v	Weight by volume
w/w	Weight by weight
WOI	Window of Implantation

CHAPTER 1
INTRODUCTION

1.1 Background and rationale

Infertility is a worldwide problem. It affects approximately 8-12% of couples at reproductive age (1, 2). Assisted reproductive technology (ART) was introduced to be used as the treatment of infertility several decades ago. Although there have been many advances in ART techniques, but the success rate is now still limited at 30-40% (The 2014 National Summary; The Centers for Disease Control and Prevention/ CDC, Division of Reproductive Health). Failure of the embryo to implant is now considered to be the major limiting step in ART treatment (3-5).

As it is well known, a successful implantation process is a chronological relationship which depends on three important factors: the competent embryo at the blastocyst stage, the endometrium at the receptive status, and the synchronized interaction between both (6-8). It can occur temporally in the only specific limited time called window of implantation (WOI). In human, this period lasts about 4 days during days 20-24 in the mid-secretory phase of endometrium in natural ovulatory cycle (9-12).

In order to achieve successful implantation in ART treatment, assessment of the highest potential embryo and endometrial receptivity status during the WOI are the very important challenges. While in the field of embryo assessment, there is more advance, but in the field of endometrium assessment, there is less. The problems in assessing endometrium are those of many complex interactions between biomarkers and timing of implantation which is important involving synchronous activities, and the aggressive examination may disrupt the nature of the process.

The analysis of endometrial receptivity using endometrial secretion has been of particular interest as it represents a non-invasive approach and the number of mediators contained may involve in the preparation for embryo and modulation of endometrial receptivity. It may contain source of potential biomarkers for diagnosis of endometrial receptivity.

The endometrial secretion can be collected from the tip of embryo transfer catheter immediately after the routine embryo transfer is performed during the specific time of WOI. This way of sampling would represent a non-invasive approach to be used clinically. It has advantage in that it is already a part of clinical practice and can be carried out without disrupting implantation process and also easy to perform.

Proteomics analysis of post-translational modifications (PTMs) is one of the challenging aspects of proteomics study (13-15). This approach points to characterize a selected group of proteins which can provide important functional information. PTMs are the process of covalent addition or removal of modifying groups, which can increase the functional diversity of the entire proteins. PTMs is necessary for stability of protein, turnover of protein, activity regulation, protein localization, protein signaling, and protein interaction with other molecules. Therefore, these PTMs are the real functional molecules. Two of the most common and widely studied PTMs are protein phosphorylation and glycosylation (16-18) due to their vital role for protein function associated with biological and clinical relevance. In addition, various methods for protein phosphorylation and glycosylation enrichment integrated with proteomics tool have been developed providing the great opportunities to capture them which are comprised only a small fraction of the total protein in cell. And currently, there are bio-informatics and databases involvement used as a tool for the identification of proteins which are essential to analyse the huge amount of data.

This study applied the phosphoprotein and glycoprotein enrichment technique coupled with liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis technologies together with the bio-informatics in order to investigate the endometrial secretion for identified phosphoprotein and glycoprotein in association and unique for specific endometrium receptivity biomarkers in stimulated ART treatment cycle.

1.2 Research question

In stimulated ART treatment cycle, what are the phosphoprotein and glycoprotein profiles uniquely expressed in endometrial secretion during window period of implantation?

1.3 Objectives

This study aims firstly, to investigate the endometrial secretion based on the phosphoproteomics and glycoproteomics study, and secondly, to identify possible potential phosphoprotein and glycoprotein biomarkers to represent for the phase of human endometrial and the receptivity during the window period of implantation (WOI) in stimulated ART treatment cycle.

1.4 Keywords

Phosphoproteomics, Glycoproteomics, Endometrial secretion, Window period of implantation, Assisted reproductive technology

CHAPTER 2
REVIEW OF LITERATURE

2.1 Embryo implantation

Implantation is the complex biological process. It depends on coordination through signaling pathways and series of cellular and molecular interactions between the embryo and endometrium to complete the success. A number of growth factors, cytokines, adhesion molecules, and signaling cascades had been mediated to regulate during the implantation process.

The implantation process comprises of continuous three steps, followed by apposition, attachment, and finally invasion (19, 20) as shown in Figure 2.1.

Apposition is the first step when embryo comes closely and assumes the correct orientation for attachment with consequence in the adjacent contact of the trophoblast of embryo to the uterine luminal epithelium. There are many changes to support the contact between embryo and endometrium including decrease in uterine fluid volume, accomplished by chemokines secreted by the embryo and the endometrium (21, 22).

Attachment (23) is the stage in which the trophectoderm incorporate into the endometrium and attachment interaction occurs. In this stage, the outer layer of the trophoblast cells of embryo proliferate and secrete proteolytic enzymes in order to make initial contact with the luminal epithelium (LE) of endometrium, while endometrium also expresses many extracellular matrix components in association with the adhesion of the blastocyst.

The final stage is **invasion** (24) in which the trophoblasts of embryo penetrate locally through the endometrium and fusion of trophoblasts with uterine occurs, and the blastocyst eventually implants and pregnancy are established.

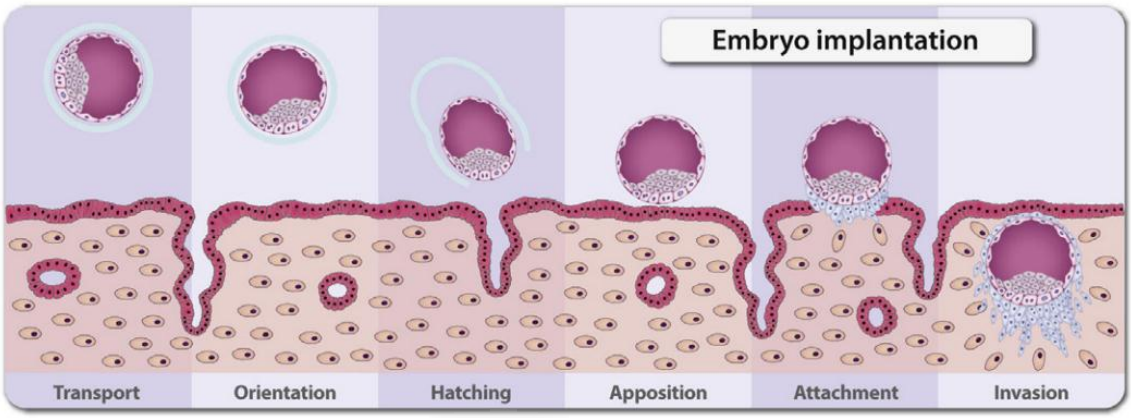


Figure 2.1 Schematic diagram demonstrating implantation steps. [from Weimar CH et al, 2013 (25)]

2.2 Window of implantation

A successful implantation process depends on three essential factors i.e. the viability of embryo at the blastocyst stage, the endometrial receptivity, and a synchronized interaction between embryo and endometrium. The blastocyst must come to attach with the endometrium during the specific and limit period called the “window of implantation (WOI)". This period of WOI temporally last for only about 4 days in the mid-secretory phase during day 20 to 24 of a regular natural menstrual cycle (9, 10, 26-28).

In humans, the endometrium is classified histologically and functionally into proliferative and secretory phases during the regular menstrual cycle. It is clear that estrogen and progesterone modulates endometrium functions and control the phase of endometrium in the reproductive system (29-36). The endometrium phase of response to implantation during the secretory phase is classified into pre-receptive, receptive and non-receptive (refractory) phases. The receptive status of endometrium is during the mid-secretory phase which limited only 4 days spanning from day 7–10 after ovulation. While during both of the pre-receptive phase and refractory phase, the endometrium is not receptive and cannot establish implantation. (as shown in Figure 2.2)

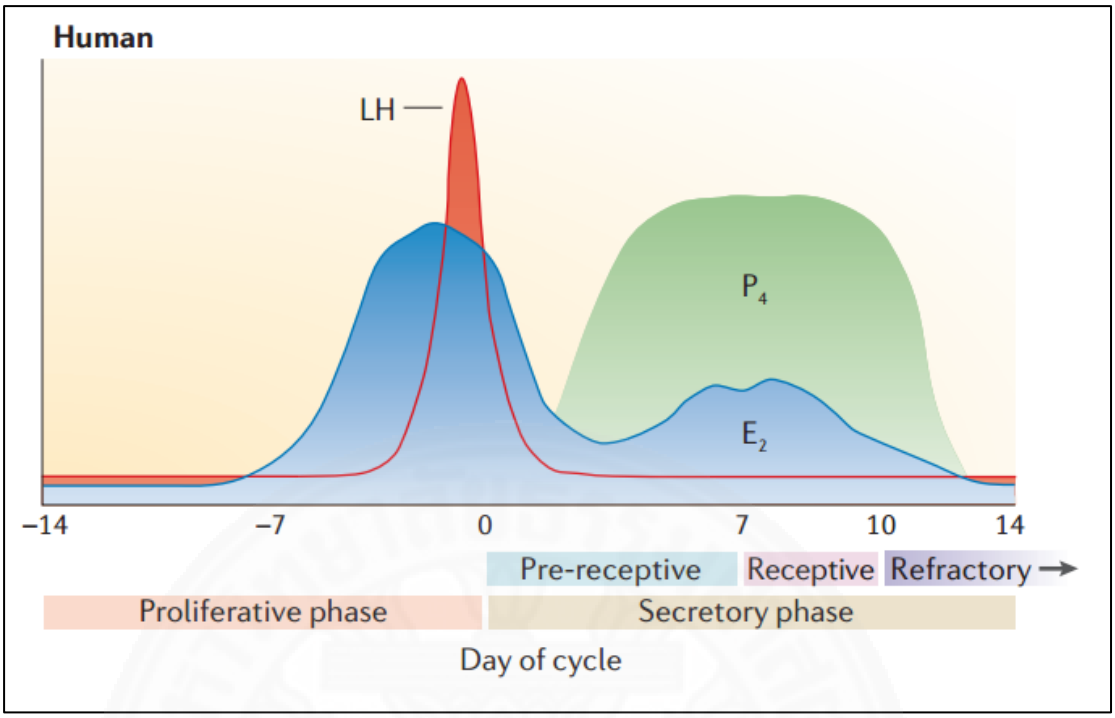


Figure 2.2 The human endometrium becomes receptive status during in the window of implantation at day 7-10 after LH surge. [from Wang H, Dey SK., 2006 (37)]

The implantation window is started by change and prepare of endometrium both structurally and in the biochemical of its secretions. In this period, the cells of the uterus are proliferating and differentiating by the influence of estrogen and progesterone, to become receptive to the incoming blastocyst. The uterine environment is prepared to support embryo growth, and implantation (38). The transition into a receptive endometrium state consists of cellular and molecular changes and the modulated expression of immunity, cytokines, growth factors, and various mediators for implanted embryo (39).

In order to enable the embryo implantation, endometrium has to be changed into decidua which is complex series of events called "**decidualization**". Decidualization is the endometrial remodeling process for preparation of embryo implantation. This process consists of many changes in endometrium including increases thickness, becomes more vascularized, and its glands produce more their secretions (40-43).

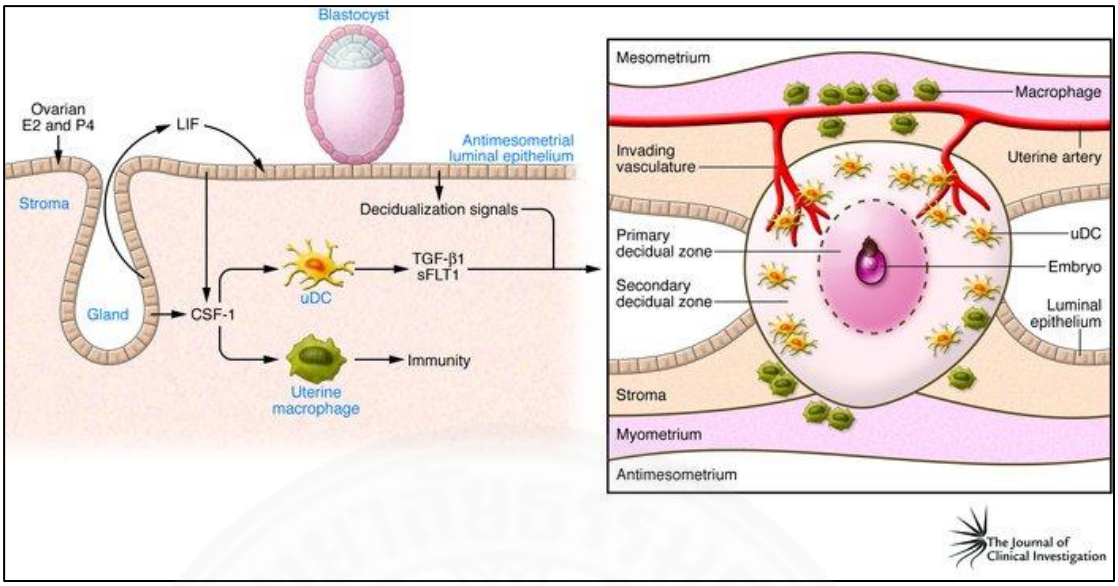


Figure 2.3 The decidualization; the complex series of events in endometrium preparation for blastocyst implantation. [from Pollard JW, 2008 (44)]

2.3 Approaches to assessing endometrial maturation and receptivity

Several analytical methods are utilized to evaluate and identify endometrium function. The standard evaluation of endometrial dating according to Noyes et al. (45) has been widely used. They use the major morphologic criteria assessing by histological tool to monitor and in dating each stage changes throughout the cycle of endometrium. However, this tool has limitation in the difficulty of interpretation and the significant intra and inter-observer variation. Moreover, it requires endometrial tissue obtained from invasive biopsy which can disrupt the process of implantation.

One of the investigations of endometrial receptivity is the attention to pinopod as a receptivity marker of the WOI in the human endometrium (46-49). In a natural cycle, normally the apical surfaces of the epithelial cells lining the uterine cavity are bulging and covered with microvilli. During WOI period, the apical surface develops into large and smooth membrane look like balloon shape called “pinopod”. Pinopod is present at the time of implantation phase between day 19 (5 days after peak of luteinizing hormone: LH+5) and day 21 (7 days after peak of luteinizing hormone: LH+7) and persist for 24 to 48 hours (as shown in Figure 2.1). The pinopod is named due to their pinocytosis function (50). It was revealed that in the rodents, pinopod

develops by the dependent of progesterone and coincides with the implantation window therefore pinopod is a good morphological marker of endometrial receptivity (46, 51). However, in human it is still controversial. The function of pinopod still has not been fully established. The limitation to clinically apply is the using of endometrial tissue biopsy that disruption the process of implantation.

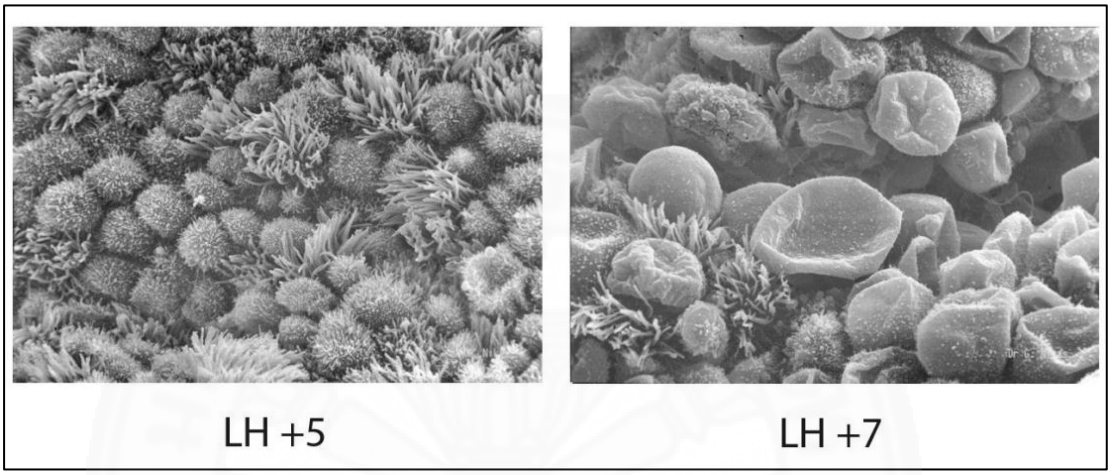


Figure 2.4 Scanning electron microscopy showing pinopod of endometrial epithelium in a natural cycle. [from Nikas G et al. (49)]

One of the techniques to diagnose the endometrium is to perform transvaginal ultrasonography (52-57). Endometrial appearances i.e. endometrial pattern and endometrial thickness have been described as prognostic factors with the chance to pregnant. It has been suggested that triple layer of endometrium appearance with the thick endometrium is associated with higher IVF success rates. This diagnostic tool can be used clinically since it is easy to perform, takes a short time, and non-invasive. However, the data information is still unable to assess the endometrium receptivity clearly.

Subsequent research has pointed to discover the biochemical markers to assess endometrial receptivity. Many complex molecular mediators have been identified, however, none of these molecules has been relied as an endometrial biomarker in clinical practice.

Developments in molecular biology techniques, the “OMICS” era, have enabled the investigation of the molecular components of human endometrium

involving in the process of implantation. Human endometrial transcriptomic analyses have identified existence of differential gene expression patterns regulated across the menstrual cycle in women undergoing ART treatment (58-60). Recent data showed that the transcriptomic approach has been used as a genomic tool to predict endometrial dating in endometrial biopsy that is called endometrial receptivity array (ERA) (61-65). ERA which is consisted of 238 gene signature that differentially expressed to classify the endometrium as 'receptive' or 'non-receptive'. It was shown to be more superior than the histological dating of the endometrium. A sensitivity and specificity of ERA for endometrium dating are 0.99758 and 0.8857 respectively. However, substantial clinical trial is still in progress and the less invasive method in the conception cycles is needed.

2.4 Effects of controlled ovarian hyperstimulation on endometrial protein profiles during the window of implantation

For the endometrium of women undergo controlled ovarian hyperstimulation (COH) which is one of the procedure of the assisted reproductive technology (ART) treatment, the window of implantation is quite different from natural menstrual cycle. COH is the hormonal treatments used to stimulate and induce multiple oocyte production. One of the most important aspects in COH is to suppress and prevent the premature ovulation. In combination with gonadotropins for ovarian stimulation, gonadotropin-releasing hormone (GnRH) analogs either agonists or antagonists, are often used to prevent premature luteinizing hormone (LH) surge. Anyhow, this GnRH analog considerably disturbs the endometrium resulted in menstrual phase not similar to the normal cyclicity. Premature and advances in secretory phase were seen in the post-ovulatory of ART cycles resulting in functional and dys-synchronous changes in the endometrium leading to non-appropriate receptivity and detrimentally effects on pregnancy outcomes (66-75).

A stimulated IVF cycle which is influenced by supraphysiological hormonal levels during control ovarian hyperstimulation (COH) has a significant impact to receptivity of endometrium. Many studies demonstrated that in high responders to ovarian stimulation patients who are performed frozen embryos transferred in a subsequent natural cycle had increased in IVF success rates (76-78).

These findings emphasize that altered hormonal environment in stimulated cycles is adversely impact to success rate.

In many consistent previous studies, the endometrium in IVF cycles compared with natural cycle controls have shown advance shift of secretory changes in the postovulatory and early luteal phase. The advancement in endometrial maturation on the day of oocyte retrieval in stimulated IVF cycles either with GnRH agonist or GnRH antagonist were observed. COH caused also structural and functional changes compared with natural cycles which impact negatively to implantation rates of ART

Alterations and differences in the stimulated endometrium had been revealed in many studies including at a morphology level from endometrial tissues as assessed by using Noyes' criteria for endometrial dating (67, 79). Furthermore, looking into the ultrastructure, pinopods which are organelles presented on the endometrial surface during the implantation phase, following controlled ovarian hyperstimulation (COH) and ovulation induction, pinopod is formed earlier than in the natural state (on days 17 or 18), resulting in asynchrony between endometrium and embryo. It is thus possible that COH and early pinopods formation may have a role in advanced shift of the window of receptivity (80, 81).

Alterations in the stimulated endometrium had been also revealed at a molecular level including gene expression (79, 82, 83), and protein expression (84). Several gene- and protein expression profiles of pre-receptive and receptive endometrium in natural (85-87) and ovarian stimulation cycles (82, 83, 88) have been investigated in the last decade. These findings showed that there is differential in gene and protein expressions comparing between natural cycle and stimulated cycle. These data provide strong evidence that endometrial receptivity is disturbed and lack of synchronized in ART stimulated cycles.

2.5 Endometrial secretion

Endometrial secretions contain a lot of soluble mediators which offer a way to study the complex molecular component of the endometrium. In recent years, less invasive techniques have become more interesting to study endometrial receptivity.

Endometrial secretion could be collected by flushing or lavage (89-92). This technique could isolate proteins which loosely bound to the surface. It is performed

by placing and positioning the catheter into the uterine lumen. The catheter connected to 10 mL syringe was filled with 2-4 mL of sterile normal saline. The saline was slowly infused into the uterine cavity, aspirated and the procedure was repeated a few times (90, 93, 94). However, the limitation of this method is on varying volumes of fluid recovered, which may affect protein contents consequently in other studies using this technique and might also had the varied and inconsistent results.

The other technique to obtain endometrium secretion is the aspiration of endometrial secretion. It could be performed prior to embryo transfer during the window of implantation without disturbance of endometrium and affecting on pregnancy rates (94-97). It could perform by using an embryo transfer catheter introduces passing through transcervically into the uterine cavity. The secretion was gently and gradually suctioned with simultaneously rotating of the catheter. Then, the routine embryo transfer procedure was carried out. However, this technique was still not applied as clinical routine procedure in the ART treatment.

Boomsma C M et al, 2009 (97) utilized the endometrial secretion obtained from endometrial aspiration of patients attending for IVF/ ICSI treatment. They described and offered a non-invasive technique that allows the study of endometrial receptivity and the implantation. The data was analysed using a multiplex immunoassay technique. The 17 selected cytokines panel were included. This study confirmed that the endometrial secretion can be used as material to be analysed for all of these cytokines.

Boomsma C M et al, 2009 (98) also further studied the endometrial secretion analysis to identify a cytokine profile for predicting of pregnancy in IVF. Analysis using a multiplex immunoassay detecting 17 soluble mediators. Data analysis revealed significant associations between MCP-1 ($P = 0.005$) and IP-10 ($P = 0.037$) levels and implantation (negative and positive association, respectively). And in clinical pregnancy, there were significant associations between IL-1b ($P = 0.047$) and TNF-a ($P = 0.023$) levels (negative and positive association, respectively). This study concluded that the value for IL-1b and TNF-a was useful to predict pregnancy in addition to that of embryo quality.

2.6 Proteomics analysis of endometrial secretion

The proteins in the endometrial secretion may be different from those in plasma. They have the temporal and spatial patterns of protein expression influenced by circulating steroid hormone concentrations.

Many studies described the application of proteomics analysis of endometrial secretions. Jessica G. Scotchie et al, 2009 (90) identified proteomics differentially secreted in pre-receptive (4 days after peak of luteinizing hormone: LH+4) and receptive phases (9 days after peak of luteinizing hormone: LH+9). Endometrial lavage was performed in 11 participants and analysed by different gel electrophoresis (DIGE) followed by mass spectrometry. This study had identified a total of 152 unique parent proteins of which 82 were differentially expressed. Most proteins in LH+9 with increased expression have the function in host defense, while proteins with decreased expression had many other functions. They found a total of 14 proteins had changed suggesting these proteins may be involved in altered post-translational modification.

Also in the study by Hannan NJ et al, 2010 (92), who did assessment of the human endometrial secretion and identify protein content differences. This study analysed endometrium lavages collected from fertile and infertile women during the mid-secretory (MS) phase, and from fertile women during the mid-proliferative (MP) phase. Analysis using 2D-differential in gel electrophoresis (2D-DiGE), revealed that when the MP was compared to the MS phase, there were 7 spots of proteins that were significantly decreased. And when fertile was compared to infertile women, there were 18 spots showing differential expression. Mass spectrometry was used to identify proteins. Antithrombin III and alpha-2-macroglobulin were further validated for the production using immunostaining of endometrial biopsies taken immediately following uterine lavage. Their staining pattern localized in endometrium tissue suggested the roles during embryo implantation.

2.7 Proteomics and post-translational modifications (PTMs) of proteins

In the "-OMICs" era, Omics technologies have a broad range of applications in many research in order to know a holistic view of the molecules that

make up a cell in the organisms. Together with the bio-informatic involvement in the process is essential to analyse the huge amount of data.

This technology includes genomics (the study of DNA sequence), transcriptomics (the study of messenger RNAs identification), proteomics (the study of protein identification), and metabolomics (the study of metabolite products).

Both of genomics and transcriptomics are more progress due to advance in tools such as polymerase chain reaction (PCR) method for amplifying signal and microarray technology. But it is important to highlight some limitations i.e. gene or mRNA expression sometime not directly correlated with the actual protein levels due to post-transcriptional process, and rate of protein synthesis and turnover.

Proteomics and metabolomics analysis, which is using mass spectrometry method to analyse, are more functional relevance. Metabolomics have an advantage over the other omics approaches since it is relevant and close to specific phenotype. Moreover, metabolite is the end products of cellular regulatory processes, therefore, changing in the metabolite can be regarded as the amplified response of biological systems to genetic or environmental changes. Anyhow, metabolomics study is still limited due to lack of known database to identify the metabolite (99).

A proteomics approach is now more preferable since proteins are the direct bio-functional molecules in the organisms. Furthermore, proteomics has more known database available to identify than those of the metabolomics study. Proteomics is the study of the expression, localization, functions, post-translational modifications and interactions of proteins at a specific condition and at a specific time. Since the proteins are most likely to be ubiquitously affected in disease and disease response, proteome is thought to hold special promise for biomarker discovery (100, 101).

The proteome is a complexity of structural and regulatory networks that requires continuous modification to meet the dynamic requires of the cell. The vastly complex of human proteome is far beyond the level of human genome. It is estimated that the human genome contains 3.2 billion bases (102) and comprised between 20,000 and 25,000 genes (103) which can encode to the estimated total number of proteins of over 1 million (104). These demonstrated that single gene can encode multiple proteins and make the protein more complexities. Numerous factors contribute to the generation of complex proteomes such as alternative splicing, the subcellular location of proteins,

and the attachment of various modifications to proteins which is called “post-translational modifications (PTMs) of protein’ (as shown in Figure 2.5).

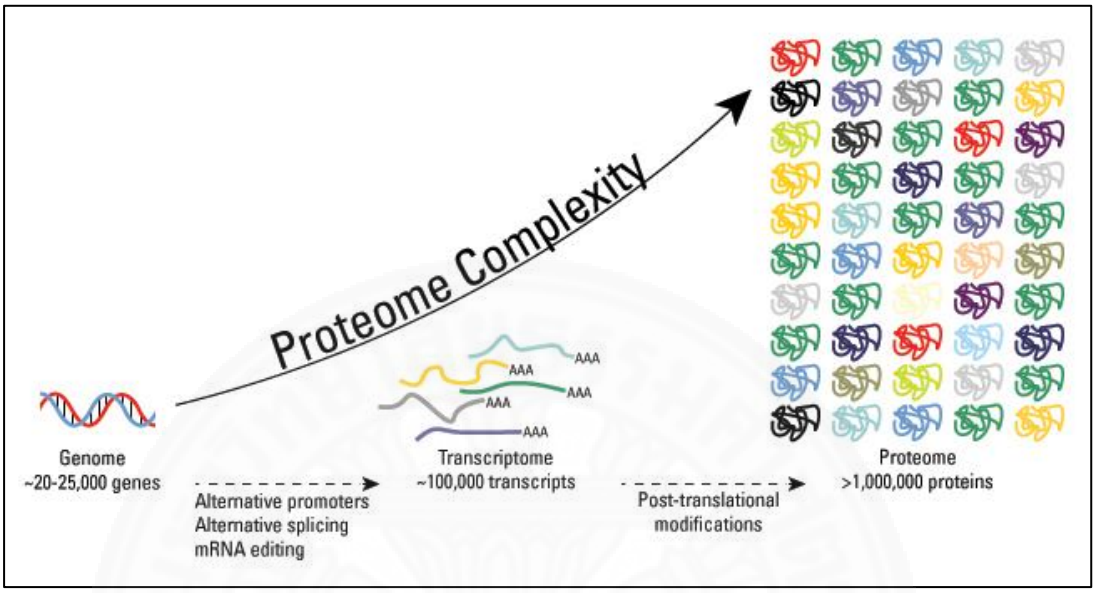


Figure 2.5 The complexity of proteome. [from https://www.thermofisher.com]

Post-translational modifications (PTMs) of protein are the process of covalent addition or remove of modifying groups, which can increase the functional diversity of the entire proteins. PTMs are necessary for stability of protein, turnover of protein, activity regulation, protein localization, protein signaling, and interaction with other molecules. Therefore, these PTMs are the real functional molecules which determine and influence of proteins.

As it is known, there are more than 300 different types of PTMs, including phosphorylation, glycosylation, ubiquitination, methylation, acetylation, etc. (105, 106). Identifying and understanding these PTMs are now become interested in the field of proteomics study both in normal and disease-associated. The characterization of PTMs is particularly important and may provide knowledge insight into the cellular process functions.

Consequently, the analysis of proteins post-translational modifications is still challenging aspects of proteomics with current methodologies. Technically, the main challenges in studying PTMs is they are present at low abundance within cells and high abundance structural proteins may mask the more functional proteins.

Therefore, the development of specific enrichment and purification methods are to be prerequisite process. The developments in the application of enrichment technique, advanced mass spectrometric techniques, and bioinformatics tools optimized for interpretation of huge amount of data have enabled PTMs analysis. The combination of optimization steps results in a high-throughput, high-coverage and simplified workflow thus move beyond the stage where it can successfully address the complex molecular and biological association.

2.8 Phosphoproteomics

Protein phosphorylation is one of an important mechanism task of post-translational modification of proteins (PTMs) for regulating protein functions and activities (18, 107, 108). Phosphorylation is the addition of a phosphate (PO4) group occurring mainly on serine, tyrosine and threonine residues. It is a key reversible modification and controlled by the action of protein kinases and phosphatases enzymes. These kinases and phosphatases constitute about 2% of the human genome (109-111).

It is estimated at least 30% of all cellular proteins in a eukaryotic were phosphorylated and >100,000 potential phosphorylation sites exist, often at multiple sites (112, 113). Each phosphorylation event might have a different effect on protein functions. Protein phosphorylation (kinases) and dephosphorylation (phosphatase) are also a key regulation of many metabolic processes such as signal transduction, transcriptional regulation, cell division, cell differentiation, cell cycle control, and apoptosis. Therefore, understanding the regulation of protein activity by phosphorylation is vital to study many cellular pathways.

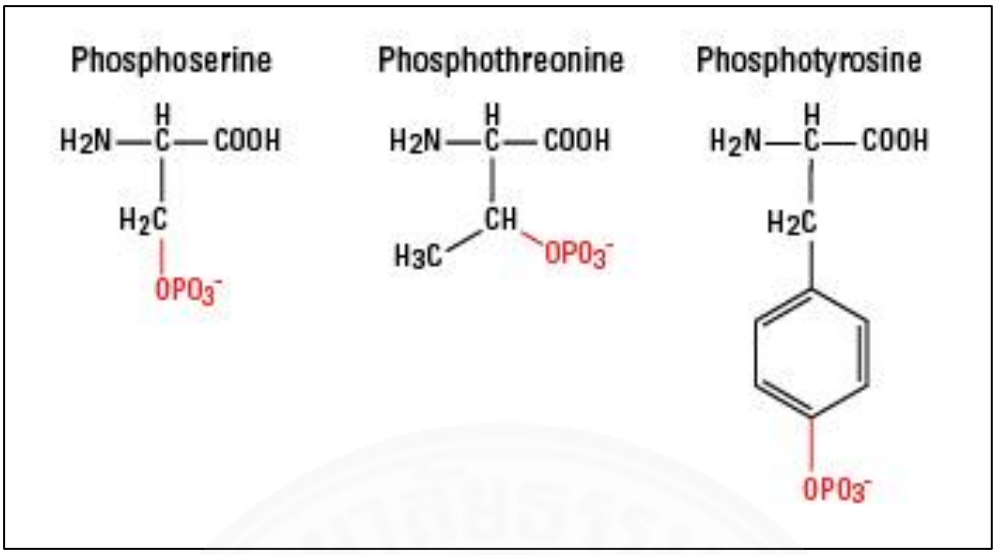


Figure 2.6 Addition of phosphate group on serine, threonine or tyrosine residues. (from https://www.thermofisher.com)

Analysis of the whole compartment of phosphorylated proteins in a cell which called phosphoproteome is an attractive study. In generally, there is only a relatively small percentage of proteins are phosphorylated in any given cell, thus, enrichment prior to proteomic analysis is required. The enrichment for phosphoproteins prior to downstream analysis by using methods such as mass spectrometry enabling to increase the sensitivity of these analyses and the ability of detecting rare and novel phosphoproteins.

Among the different enrichment strategies, immobilized-metal affinity chromatography5 (IMAC) techniques using microcolumns have become popular for phosphopeptide enrichment and enable to identify many of phosphorylation sites in sample (114-117). The principle depends on affinity chromatographic capture methods in which negatively charged of phosphate groups bind to positively charged metal ions immobilized to a support phase (as shown in Figure 2.7). This strategy allows more comprehensive characterization of the phosphoproteome in sample. Therefore, with the recent developments in proteomics, integrated enrichment strategy, and known database available, phosphoproteomics are now known as a subfield of proteomics study with high biological and clinical relevance.

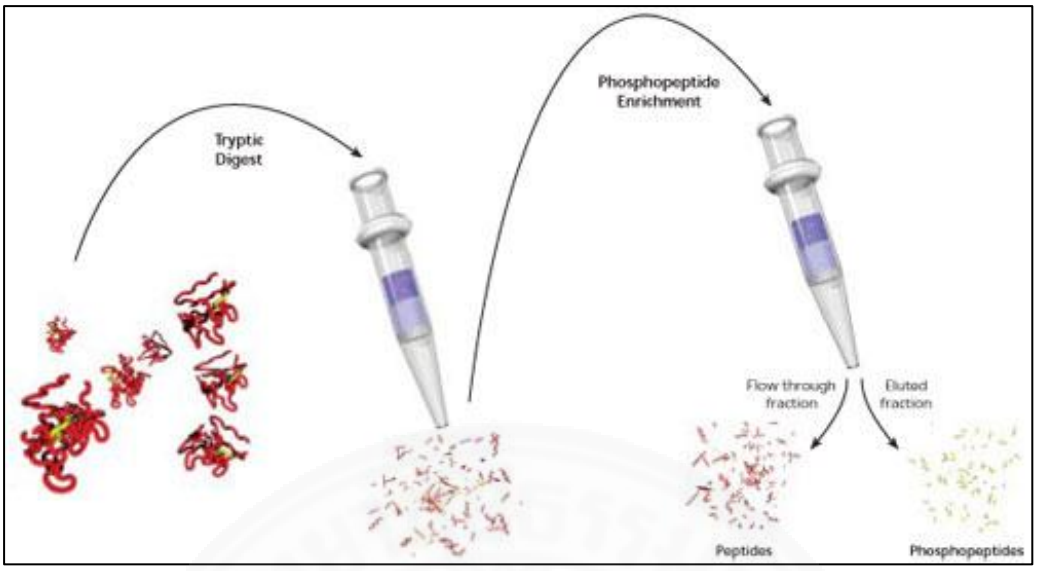


Figure 2.7 Phosphoprotein enrichment. (from http://www.sigmaaldrich.com)

2.9 Glycoproteomics

Protein glycosylation is a common and important form of post-translational modifications of proteins. Glycoprotein is formed by the addition of a sugar moieties or carbohydrate to protein. It has been reported that approximately more than 50% of all proteins typically expressed in a cell are likely to undergo this modification (118).

Glycosylation is a critical role of the biosynthetic-secretory pathway in the endoplasmic reticulum (ER) and Golgi apparatus. In the ER, its role is in quality control of proteins including folding of newly synthesized proteins, degradation of misfolded proteins, and sorting and transport of proteins between organelles (119, 120). The other role of glycosylation is to participate in protein secretion for stability, and also play as ligands for receptors on the cell surface (121). Alterations of glycosylation in many diseases are used for diagnostic and prognostic purposes such as in cancer (122, 123). Recent research also used the comprehensive glycoproteomics in exploration of a glycoprotein-related biomarker (124, 125).

The major class and broadly studied forms are N-linked and O-linked glycosylation (126). N-linked glycosylation is the attachment of the amide group to asparagine residues. It has been associated with secretion. O-linked glycosylation is the link of hydroxyl group to serine or threonine residues. It can be associated with membranes, cytosol, and nucleus (127-129).

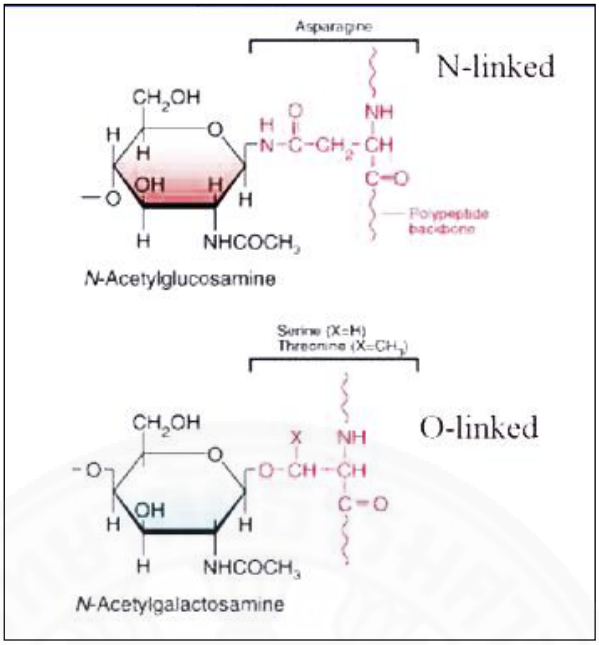


Figure 2.8 N-linked and O-linked glycosylation. (from http://www.nbs.csudh.edu)

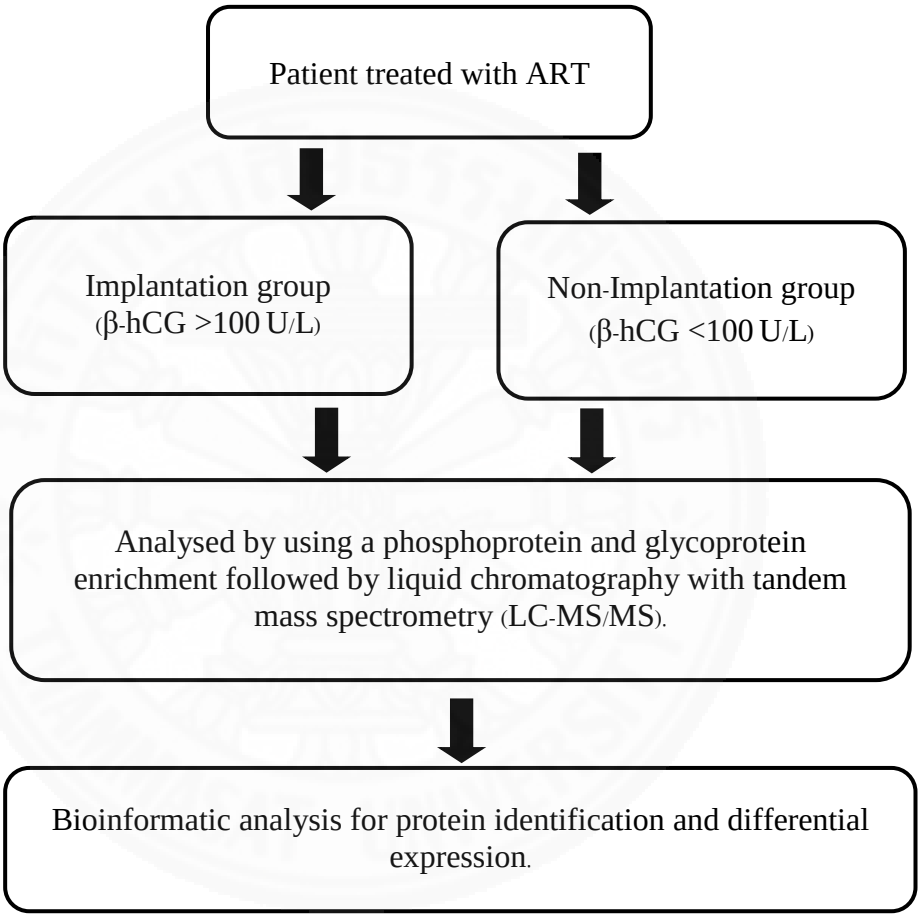
Recent advance in mass spectrometry technology has emerged in glycoproteomics, including the development of glycoprotein enrichment strategy enabling the high-throughput identification of glycosylated proteins have accelerated the analysis. In general, there are complexity and heterogeneity of glycans. Therefore, glycosylation analysis requires the improved depth-of-coverage and sensitivity. The methods specifically targeting glycoproteins are necessary to facilitate their isolation from sample prior to their identification by mass spectrometry-based analysis.

The enrichment glycosylated proteins using lectin-based isolation/ purification strategy targeting glycoprotein fraction have yielded techniques to elucidate the glycoproteome (130-133). Lectin is glycan-binding protein that has strong affinity for specific selected sugar moieties using to separate non-bound glycans and bound glycans and enrich glycoprotein fractions. Advance glycoproteomics is now sufficient to initiate efforts to capture the molecular complexity and make opportunities to increase the understanding of the functional roles of glycoproteins in human health and diseases.

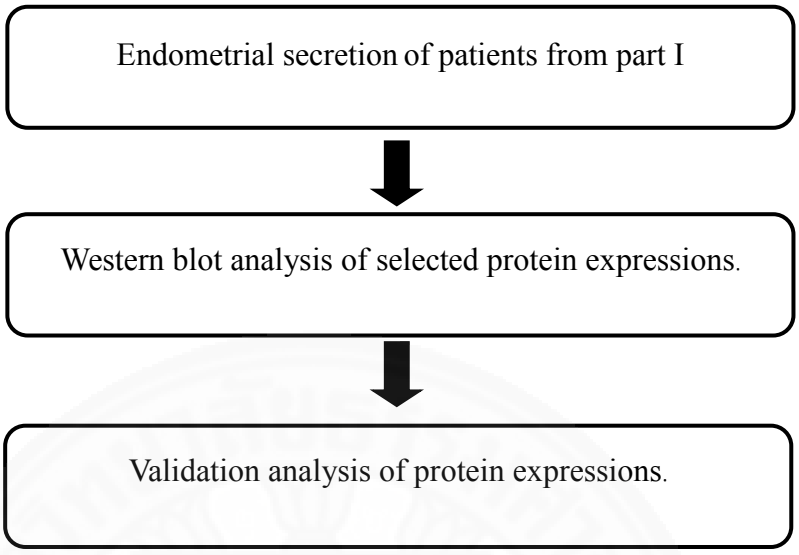
CHAPTER 3
RESEARCH METHODOLOGY

3.1 Experimental design

3.1.1 Part I: phosphoprotein and glycoprotein profiles identification



3.1.2 Part II: validation of the results



3.2 Study on phosphoproteins and glycoproteins differentially expressed in implantation and non-implantation cycles

3.2.1 Volunteers

Infertility couple volunteers treated with assisted reproductive technology at Thammasat Fertility Center, Thailand, during Dec 2015 – Dec 2016 were asked for consent voluntarily to participate in the study.

3.2.2 Inclusion criteria of the volunteers

- aged 25-40 years
- no underlying medical history of diseases
- no uterine anomaly
- no endometrial pathology
- no cervical pathology
- no ovarian dysfunctions
- no tubal factors
- no endometriosis
- negative hepatitis B antigen, VDRL, anti-HIV screenings

- have at least one embryo developing to blastocyst stage at day 5 after oocyte retrieval
- good quality morphological blastocysts defined as 3AA, 3AB, 3BA, 3BB, 4AA, 4AB,4BA and 4BB according to Gardner and Schoolcraft criteria, 1999 (134)

Note: Embryo transfer was performed by only one clinician to control interpersonal variations.

3.2.3 Exclusion criteria of the volunteers

- difficult embryo transfer
- history of difficult embryo transfer
- blood contamination (by visual signs) of the endometrial secretions

3.2.4 Sample size

The sample size in each group was calculated at effect size = 0.75, $\alpha = 0.05$ and $\beta = 0.20$;

$$n_{each} = \frac{2*(Z_{1-\alpha} + Z_{1-\beta})^2 * \sigma^2}{(\mu_1 - \mu_2)^2}$$

n_{each}	=	sample size in each group
α	=	type 1 error = 0.05; $Z_\alpha = 1.96$
β	=	type 2 error = 0.20; $Z_\beta = 0.84$
Power	=	$(1-\beta) = 0.80$
σ^2	=	Variance
$\mu 1 - \mu 2$	=	0.75σ
Effect size	=	0.75

therefore;

$$n_{each} = 2*(1.96+0.84)^2 * \sigma^2 / (0.75\sigma)^2$$
$$= 27.87$$

Sample size is calculated to have 30 persons per group.

3.2.5 Ethical considerations

The cross sectional study was performed in infertile couples in the assisted reproductive technology treatment cycles at Thammasat Fertility Center with the approval of Thammasat University Research Ethic Committee No.1. The number of approved protocol was MTU-EC-OB-1-149/58.

3.2.6 Sample collection

This study included total of 60 infertile couples of which 30 were implantation patients and 30 were non-implantation patients. Endometrial secretions were collected from embryo transfer catheter tip at the oocyte retrieval day (day 0) of 15 implantation plus 15 non-implantation patients designated as group I (baseline or control) and of 30 implantation patients and 30 non-implantation patients at embryo transfer day (day 5) designated as group II and group III (study groups) respectively. The sample from each group was analysed for phosphoproteins and glycoproteins.

3.2.7 Assisted reproductive technologies

The female infertile partners were treated with controlled ovarian hyperstimulation protocol using recombinant Follicle stimulating hormone/ rFSH (Gonal-F®, Merck Serono, Italy), starting on cycle day 2 or 3 (The daily dose of rFSH is individualized and adjusted according to ovarian response). When the leading follicle reaches 14 mm in diameter, 0.25 mg of Gonadotropin releasing hormone (GnRH) antagonist (Cetrotide®, Merck serono, Italy) is daily administered additionally until at least 2 follicles with 18 mm in diameter are obtained. The ovulation induction was triggered with 10,000 IU of recombinant Human chorionic gonadotropin/ rHCG (Ovidrel® Merck Serono, Italy). The oocyte retrieval was performed 36 hours after ovulation induction under transvaginal ultrasound guidance. After oocyte retrieval, the "mock" embryo transfer was carried out immediately. The mock transfer is a trial run of the actual embryo transfer. In this procedure, the clinician passed an empty catheter into the uterine cavity. It allowed the clinician to determine the best position to place the embryo in uterus and to ensure that there are no unexpected or other problems that may make transfer difficult. Immediately after mock embryo transfer was performed, the endometrial secretion at the tip of catheter was then collected. The tip of catheter

was cut off, and then secretion fluid that attached at the tip of the catheter was removed and centrifuged for 10 minutes at 500X g immediately after obtaining the samples. Then the pellets were discarded and suspension were frozen in an eppendorf tube (labeled as D0) and stored at -80°C until assessment.

Thereafter, at 4 hours after retrieval, the oocytes were inseminated with prepared sperm by density gradient centrifugation (Sil-Select Plus®, Fertipro, Belgium) by intracytoplasmic sperm injection (ICSI) technique. After insemination, the embryo was cultured with Global culture media® (Life-Global, USA) under paraffin oil (Life-Global, USA) in the tri-gas (89% nitrogen, 5% oxygen and 6% carbon-dioxide) incubator for 5 days. The routine embryo transfer procedure was carried out thereafter. A maximum of two good quality blastocysts on day 5 is selected to be transferred using Guardia® Access Embryo Transfer Catheter (Cook Medical, USA). The patients who did not have the embryos reaching the blastocyst stage with good morphology and had the difficult transfer or bleeding during transfer were excluded from the study. In the process of embryo transfer, in order to prevent contamination by cervical mucous, clinician inserted the outer sheath catheter by passing through the cervix into the depth of uterine cavity in advance and then introduced the inner catheter loaded with embryo(s) protrusion of placement within the endometrium. And during the catheter removal process, the inner catheter was then withdrawn through the outer sheath, which was prevented the contact with the cervix. Immediately after embryo transfer, the tip of the inner catheter was cut off, and the same procedure was used at D5 as described for the D0 sample.

The luteal support was performed by administration of vaginal progesterone gel 90 mg once daily (Crinone®, Merck Serono, Italy) starting on the oocyte retrieval day and continued until the day of pregnancy test.

3.2.8 Sample preparation

Immediately after mock transfer (D0) and embryo transfer (D5), the tip of the inner catheter was cut off approximately 2 cm and put in 1.0 ml phosphate buffer saline (PBS). After centrifuged for 10 minutes at 500 G, the pellets were discarded and the supernatant was collected in 1.5 ml eppendorf tube and stored at -80°C until assessment.

Samples were normalized for protein concentration using Lowry Protein Assay (135) with BSA as the standard. Equal of protein concentration was taken from each sample. In each group, endometrial secretions from 30 patients were pooled together by equal quantity of total protein to make an appropriate quantity for perform an assay.

3.2.9 Protein quantitation

The "Lowry Assay" (135) was used to determine the protein concentration of each endometrial secretion sample prepared in a microplate, then pre-treated with 50 µl of reagent A that contains copper ion in alkali solution, incubated for 30 minutes in room temperature. Thereafter, 250 µl of reagent B containing folin-ciocalteu reagent was added to each sample, mixed, and incubated for 30 minutes at room temperature. The end product of reaction was a blue color. The absorbance at 750 nm was measured with microplate reader (Rayto, Rayto Life and Analytical Sciences Co., Ltd., Shenzhen, China). Each sample was performed in triplicate.

In order to determine concentrations of protein in the sample, known concentrations of bovine serum albumin (BSA) was prepared simultaneously and used as a standard curve of absorbance. The standard curve was done on each plate.

3.2.10 Phosphoproteins sample preparation

Samples were normalized for protein concentration using Lowry Protein Assay with BSA as the standard. Phosphoproteins were collected by Pierce™ Phosphoprotein Enrichment Kit (Thermo Fisher Scientific Pierce Biotechnology, United States). Negatively charged phosphate groups bound to positively charged metal ions were immobilized to a chromatographic support. (136, 137).

Total of 40 µl of IMAC resin was loaded into a column placed into a 1.5 ml eppendorf tube and pre-washed twice with Lysis/Binding/Wash buffer. Two hundred micrograms of protein in sample was added to the column and inverted several times to mix. Column was incubated for 30 minutes at room temperature with mixing by rocking platform, and then incubated at 4^{o}C overnight.

To remove the unbound proteins, the sample mixture was centrifuged at 1000 G for 1 minute and washed three times with 250 µl of Lysis/Binding/Wash buffer.

The bound phosphoproteins were eluted from the IMAC beads using 100 µl of elution buffer and incubated for 5 minutes at room temperature with mixing by rocking platform. The elution step was repeated for four times.

3.2.11 Glycoproteins sample preparation

The enrichment of N-linked glycan was performed using affinity chromatography on immobilized lectin columns concanavalin A (ConA) affinity kit which recognizes mannose residues to enrich N-glycosylated proteins.

Samples were normalized for protein concentration using Lowry Protein Assay with BSA as the standard. The enrichment of N-linked glycan was performed using affinity chromatography on immobilized lectin columns concanavalin A (ConA) affinity kit (Thermo Fisher Scientific Pierce Biotechnology, United States). Forty microliters of ConA Lectin Resin were loaded into a 1.5 ml eppendorf tube and pre-washed three times with PBS. Two hundred microliters of sample in 5X Binding/Wash buffer 50 µl was added to the tube, and the sample was mixed. Column was incubated for 10 minutes at room temperature with mixing by rocking platform and then incubated at 4^{o}C overnight. To remove the unbound proteins, the sample mixture was centrifuged at 1000 g for 1 minute and washed three times with 80 µl of 5X Binding/Wash buffer. The bound glycoproteins were eluted with 80 µl of elution buffer, incubated for 5 minutes at room temperature with rocking platform. The eluted fractions were collected by centrifugation.

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3.2.12 Sample desalted

The eluted fractions were desalted to clean up the proteins using Zeba™ Spin Desalting Columns, 7K MWCO, 0.5 mL (Thermo Fisher Scientific Pierce Biotechnology, United States). The micro-column was conditioned with 1 ml deionized water for 3 times. Protein was concentrated using Nanosep® Centrifugal Devices (Pall Corporation, Life sciences, USA).

3.2.13 Sample digested

The digestion in solution was performed. The protein was reduced disulfide bonds of cysteine with 5 mmol/l dithiothreitol (DTT) (Sigma, St. Louis, MO, USA) and

incubated at 56°C for 60 min, alkylated free cysteines by incubation with 15 mmol/l iodoacetamide (IAA) (Sigma, St. Louis, MO, USA) in darkness for 60 min at ambient temperature. Protein lysates were digested with trypsin (Promega Corp., Madison, WI, USA) (substrate: enzyme = 1:20) at 37°C overnight.

In order to quantify relative concentrations, sample mixture containing known concentrations of bovine serum albumin (BSA) were prepared simultaneously.

3.2.14 Sample dried

Sample was dried with speed vacuum centrifugal dryer. (Savant AES 1010 Automatic Environmental Speedvac System® , Thermo Fisher Scientific Ltd, MA, USA). The samples were then re-suspended with a 1% (vol/vol) formic acid aqueous solution to a concentration of 100 ng/µl.

3.2.15 Liquid chromatography with tandem mass spectrometry

Phosphopeptide digests and glycopeptide digests were analysed using SYNAPT^{TM} HDMS mass spectrometer (Waters Corp., Manchester, UK) integrated with high performance liquid chromatography using NanoAcquity system (Waters Corp., Milford, MA) which was directly interfaced with a linear trap quadrupole (LTQ)-SYNAPT^{TM} HDMS mass spectrometer (Waters Corp., Manchester, UK). The injections of each sample were resolved on symmetry C_{18} 5 µm, 180-µm × 20-mm Trap column and a BEH130 C_{18} 1.7 µm., 100-µm ×100-mm analytical reversed phase column (Waters Corp., Milford, MA). Following precolumn and analytical column equilibration, each sample was performed for linear elution with 600 nl/min, using 2 eluents; mobile phase A (0.1% formic acid in LC-MS water), mobile phase B (0.1% formic acid in Acetonitrile (ACN). [Glu^1] fibrinopeptide B (Waters Corp., Manchester, UK) was used as an external lock mass for calibration. The time-of-flight (TOF) analyzer of the mass spectrometer was externally calibrated with [Glu^1] fibrinopeptide B. The quadrupole mass analyzer was adjusted to AutoMS mode with scan ranged of mass per change ratio from 300 to 1,800.

The injection of each sample was repeated for three times.

3.2.16 Western blot analysis

The total protein concentration of the sample mixer was measured using Lowry Protein Assay. The sample was prepared to a concentration of 20 µg for loading per lane. Polyacrylamide SDS-gel for protein separation was prepared using TGX FastCast™ acrylamide Solutions (Bio-Rad Laboratories, Inc., California, USA). Then added 10% ammonium persulfate (APS) used as an initiator catalyst for the polymerization of acrylamide and bisacrylamide gels. The polymerization reaction was then adjunct catalyzed by TEMED (N,N,N′,N′-tetramethylethylenediamine). A tracking dye (bromophenol blue) was added to make the dilution concentration of dye from 5X to 1X. The sample mixture was denatured by heating for 5 minutes and centrifuged for 1 minute at 10,000X G. The gel is then connected to the power supply and allowed to run. After separation, proteins were transferred from the gel onto the nitrocellulose membrane using Trans-Blot® Turbo™ Transfer System (Bio-Rad Laboratories, Inc., California, USA). Phosphoprotein Blocker (Blok^{TM}-PO) (EMD Millipore Corporation, MA, USA) was used to block nonspecific binding and then incubated at 4°C overnight with primary antibodies and with the dilution according to manufacturer. Antibody to histone which is housekeeping protein was performed simultaneously to use as a loading control for normalize protein abundance in all the experiments.

Membranes were washed in Tween-20 in PBS (PBS-T) buffer 3-5 times and then incubated with secondary antibody for 60 min at room temperature. Luminata™ Crescendo Western HRP Substrate (Millipore Corporation, Billerica, MA, USA) was used as substrates for chemiluminescence detection. Immune complexes were detected and imaged using ChemiDoc™ XRS+ system (Bio-Rad Laboratories, Inc., California, USA).

For western blot quantitation, we used histone to quantify the loading sample in each lane and then to normalize band intensity with the corresponding loading control. We used the ratio relative to histone for evaluating single protein level variation and compared the relative abundance of one over the other.

3.3 Bio-informatics for data analysis

3.3.1 DeCyder™ MS Differential Analysis Software

All MS raw files were processed with DeCyder™ MS Differential Analysis Software (DeCyder MS) (Version 2.0, Thermo Scientific, USA), for visualization, detection, comparison, and label-free relative quantitation of LC-MS/MS data. The PepDetect module of DeCyder MS was used to analyse the signal intensity maps, detect and quantitate the peptide ions, and charge states assigned. The PepMatch module was then used to match of peptides from different signal intensity maps. The relative quantitation is obtained by normalization with known concentration of bovine serum albumin (BSA).

3.3.2 Mascot algorithm 2.2.1 software

Mascot algorithm 2.2.1 software (Matrix Science, London, UK) was used to search for MS/MS spectra against a homo sapiens database from NCBI (National Center for Biotechnology Information, Bethesda US).

The enzyme specificity was set to trypsin with allowing up to 3 missed cleavages. The precursor mass tolerance was set to 5 parts-per-million (ppm) for the first search (used for nonlinear mass recalibration), fragment mass deviation was set to 0.6 Da. Cysteine carbamidomethylation was selected as fixed modification. In phosphopeptide analysis, methionine oxidation, and phosphorylation on serine, threonine, and tyrosine were selected as variable modifications. In N-glycopeptide analysis, methionine oxidation, and deamidated were selected as variable modifications.

3.3.3 Venn diagram

Venn diagram (http://bioinfo.genotoul.fr/jvenn/) was used as the integrative tool for comparing lists of differentially expressed proteins between study groups.

3.3.4 Universal Protein Resource database

The Universal Protein Resource Knowledge Base (UniProtKB) was used as the central resource on protein sequences and functional annotation.

3.3.5 The Protein ANalysis THrough Evolutionary Relationships Classification System

The PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System was used to explore the comprehensive function information about genes and also facilitate analysis of large numbers of genes.

3.4 Expected benefits and applications

The identification of reliable biomarkers for endometrium receptivity offers great promise to:

- Provide information for the decision to transfer or to freeze all embryos as individualized patient-tailored management in infertility patients.
- Improve efficiency of the ART outcomes.
- Enhance cost-effectiveness of treatment which also reducing patient's emotional and financial stress.
- Empower effectiveness of single ET and pre-implantation genetic screening (PGS) protocols.
- Provide new insights into reproductive molecular interaction targeting especially on the embryo-endometrial interactions.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Patient characteristics

Endometrial secretions collected from women underwent assisted reproductive technology treatment were subjected to analysis, of which 30 endometrial secretions collected from women who achieved implantation and the other 30 endometrial secretions selected from women who did not achieve implantation. Among non-implantation group, 30 samples were randomly selected by 1 out of every 2 samples from the total 61 samples.

The baseline characteristics of infertile female partners were shown in Table 4.1. T-test analysis comparing clinical parameters showed no statistically different in age, BMI, total recombinant gonadotropin dose, number of oocytes per retrieval, number of mature oocytes per retrieval, number of fertilized oocytes, number of blastocysts on day 5, and number of good quality blastocysts transferred (all *P*-value > 0.05).

Table 4.1 The characteristics of infertile female partners in the study groups.

Parameters	**Implantation (*n* = 30)**	**Non-Implantation (*n* = 30)**	***P*-value [a]**
Age (years)	33.87 ± 2.79 (29-39)	34.10 ± 3.43 (27-40)	0.773
BMI	21.42 ± 1.18 (19.40-24.30)	21.80 ± 1.25 (19.70-23.80)	0.236
Total recombinant gonadotropin dose (IU)	2,080 ± 421.48 (1,350-2,700)	2,027.5 ± 496.51 (1,200-2,700)	0.660
Number of oocytes per retrieval	8.70 ± 2.32 (5-14)	8.47 ± 3.03 (4-16)	0.739
Number of mature oocytes per retrieval	7.33 ± 1.83 (3-11)	6.87 ± 2.94 (2-13)	0.464
Number of fertilized oocytes	5.70 ± 1.51 (3-8)	5.3 ± 2.27 (2-10)	0.426
Number of blastocysts on day 5	3.20 ± 1.24 (1-5)	3.06 ± 1.81 (1-7)	0.741
Number of good quality blastocysts[b] transferred	1.67 ± 0.48 (1-2)	1.63 ± 0.49 (1-2)	0.795

All values are mean+SD with range shown in brackets.

a. Mean values of data were compared by t-test.

b. Embryo with good quality blastocyst was defined as 3AA, 3AB, 3BA, 3BB, 4AA, 4AB, 4BA and 4BB according to Gardner and Schoolcraft criteria, 1999 (134).

4.2 Endometrial secretion profile

4.2.1 The protein contents in endometrial secretion

All endometrial samples contained sufficient material for analysed. Data are presented in Table 4.2.

Table 4.2 The protein contents of endometrial secretions in the sample groups.

Groups	D0 sample	D5 sample	
	Implantation + Non-implantation	Implantation	Non-implantation
Patients (n)	15 + 15 = 30	30	30
Protein concentration range (µg/µl)	0.116 – 2.909	0.052 – 2.693	0.041 – 2.779
Median (µg/µl)	0.608	0.488	0.507
25^{th}–75^{th} percentiles (µg/µl)	0.332 – 0.885	0.205 – 0.767	0.190 – 0.779

4.2.2 LC/MS-MS for phosphoproteins and glycoproteins identification

(a) Phosphoprotein identification

LC-MS/MS analysis of IMAC-enriched phosphopeptides resulted in the identification of 267 phosphoproteins from the endometrial secretion collected at D0 and 220 phosphoproteins collected at D5, of which 184 phosphoproteins from the endometrial secretion of implantation group and 216 phosphoproteins from non-implantation group were identified.

The identified phosphoproteins were classified according to Gene Ontology using The PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System. The classification based on cellular components reveal that the majority of the proteins are known to be cell part (38.40%), organelle (30.80%), macromolecular complex (15.30%), membrane (9.30%), extracellular matrix (3.10%), and extracellular region (3.10%) as shown in Figure 4.1A.

The proteins were also classified based on biological processes into those involved in cellular process (27.40%), metabolic process (22.00%), localization (10.70%), multicellular organismal process (9.30%), cellular component organization or biogenesis (8.00%), developmental process (7.30%), response to stimulus (6.00%), biological regulation (4.00%), immune system process (2.70%), biological adhesion (1.30%), and reproduction (1.30%) as shown in Figure 4.1B.

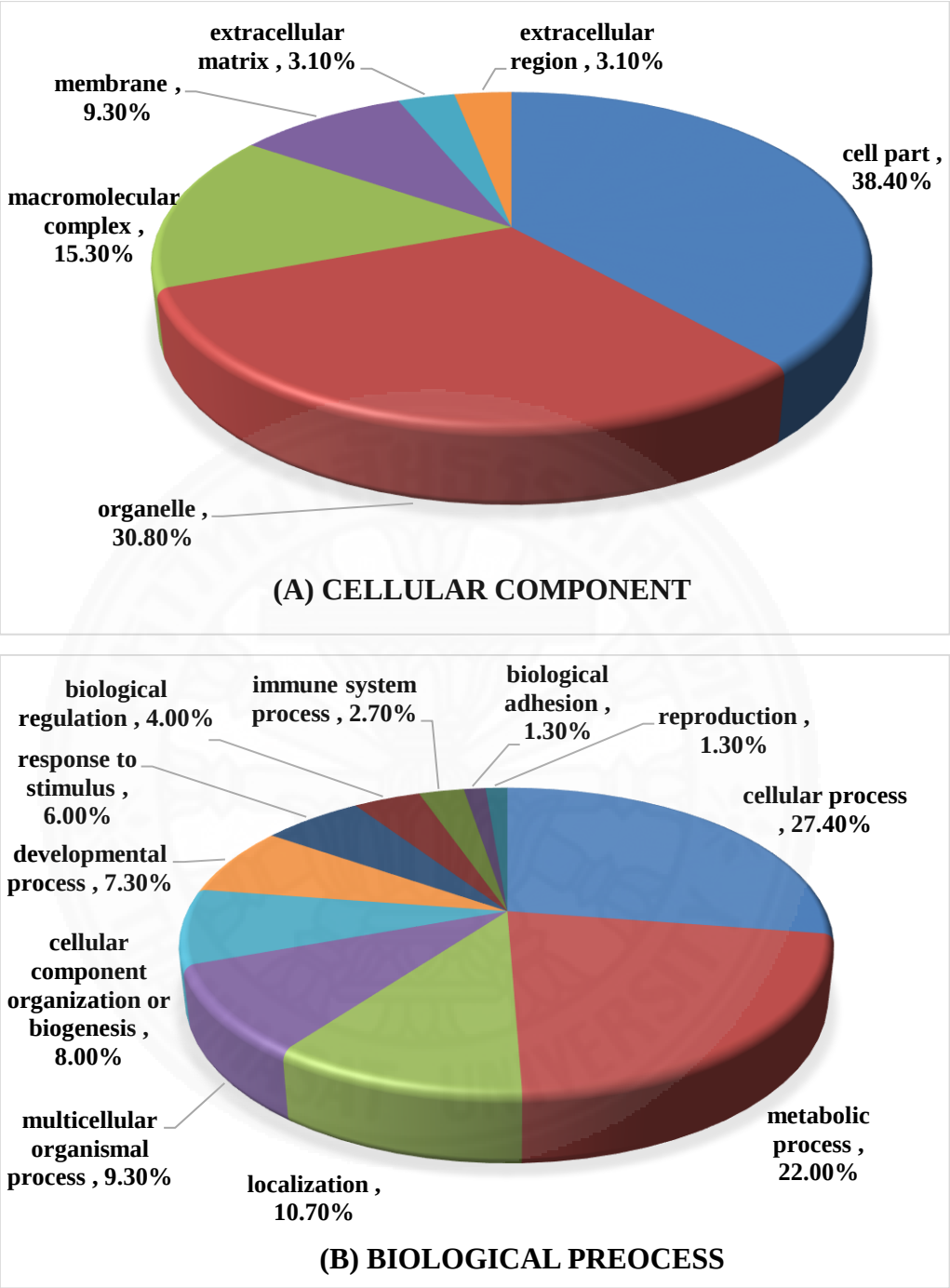

Figure 4.1 Distribution of the phosphoproteins based on Gene Ontology analysis including (A) cellular components and (B) biological processes.

(b) Glycoprotein identification

LC-MS/MS analysis of N-glycosylation peptides resulted in the identification of 105 glycoproteins from the endometrium secretions collected at D0 and 110 glycoproteins collected at D5, of which 108 glycoproteins from the endometrium secretions of implantation group and 109 glycoproteins from non-implantation group were identified

The identified glycoproteins were classified according to Gene Ontology using The PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System. The classification based on cellular components revealed that the majority of the glycoproteins are shown to be cell part (47.10%), organelle (35.30%), macromolecular complex (11.80%), membrane (2.90%), and extracellular region (2.90%) as shown in Figure 4.2A.

The proteins were also classified based on biological processes involved in cellular process (26.40%), metabolic process (22.40%), localization (8.80%), response to stimulus (8.80%), cellular component organization or biogenesis (8.00%), immune system process (8.00%), biological regulation (5.60%), developmental process (4.00%), multicellular organismal process (4.00%), biological adhesion (3.20%), and locomotion (0.80%) as shown in Figure 4.2B.

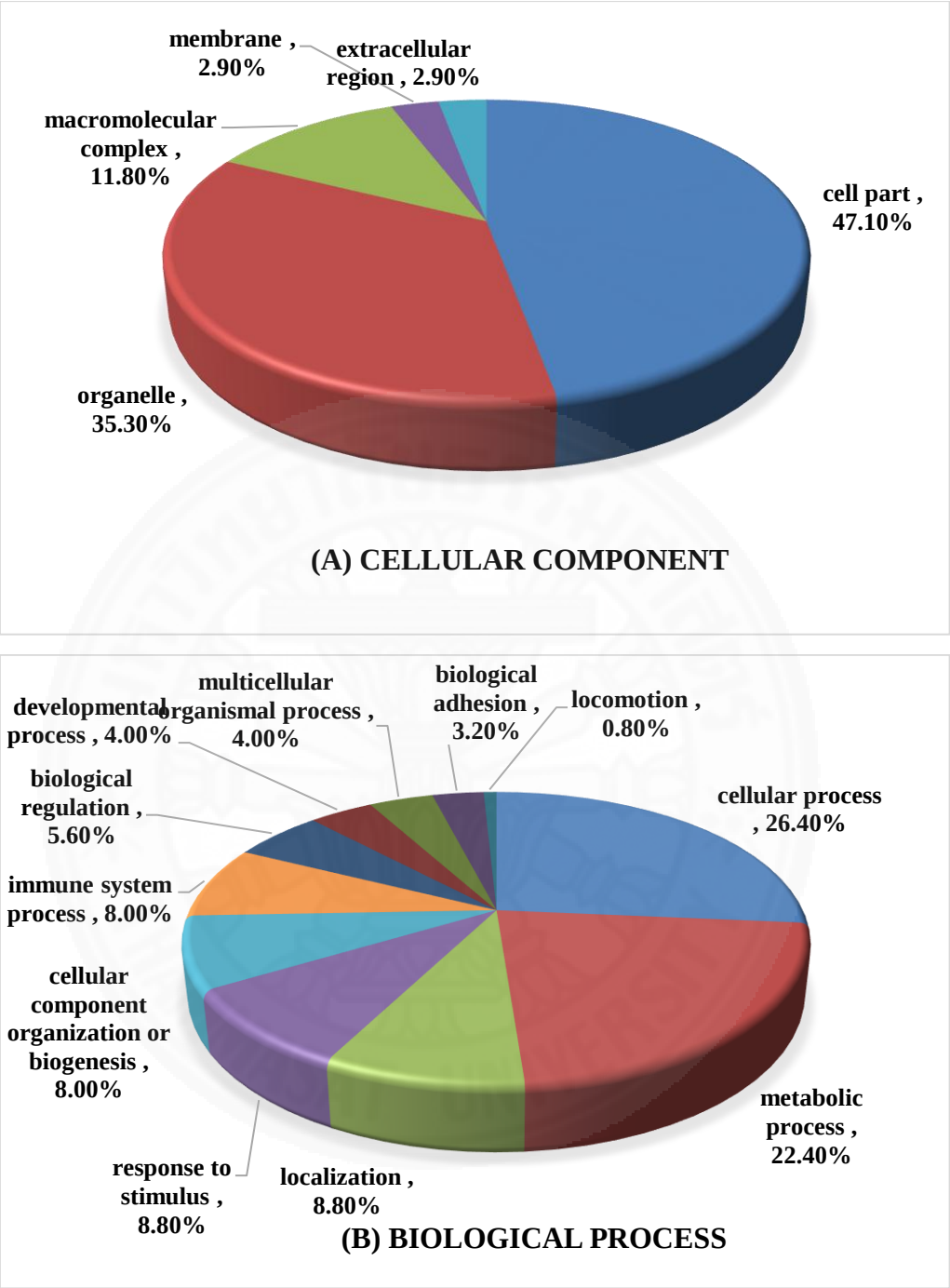

Figure 4.2 Distribution of the glycoproteins based on Gene Ontology analysis, including (A) cellular components and (B) biological processes.

4.3 Analysis of differential phosphoprotein and glycoprotein expressions

4.3.1 Comparison of differentially expressed proteins between endometrial secretion collected at D0 and at D5

(a) Comparison of differentially expressed phosphoproteins between endometrial secretion collected D0 and D5

From the pooled endometrium secretion samples obtained from women treated with assisted reproductive technology, this study had analysed the differences in the phosphoprotein and glycoprotein contents with respect to pre-receptive phase (day at oocyte retrieval /D0) and receptive phase or window period of implantation (day at embryo transfer /D5) of endometrium. It was found in this study that total of 267 phosphoproteins were expressed at D0, of which 47 phosphoproteins were uniquely found and total of 220 phosphoproteins were expressed at D5, with no phosphoproteins uniquely found (as shown in Figure 4.3).

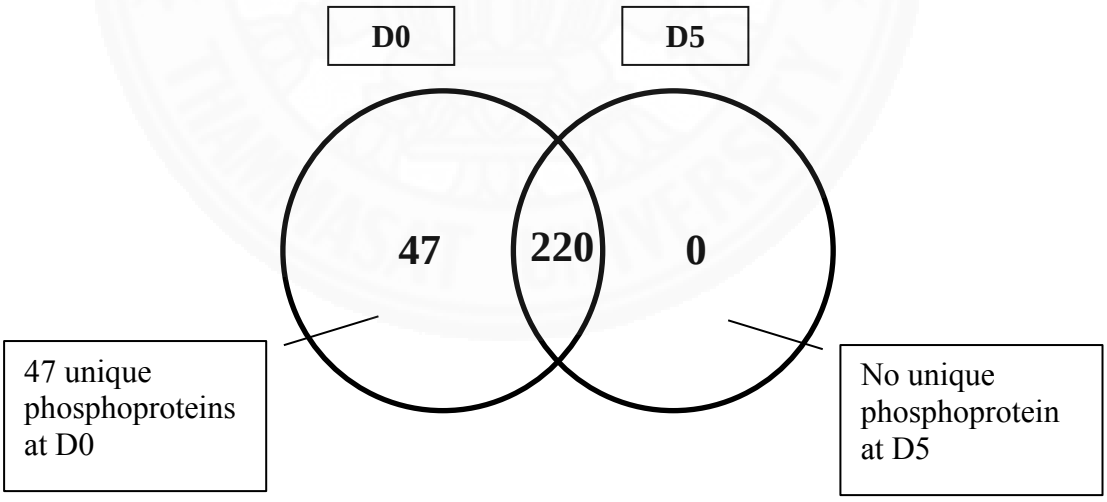

Figure 4.3 Venn diagram demonstrates the overlapped phosphoproteins identified from endometrial secretions at D0 and D5 groups.

In order to provide an overview of the differential phosphoproteins expression between pre-receptive phase (D0) and receptive phase or window period of implantation (D5) of endometrium, this study had classified D0 and D5 samples according to Gene Ontology using The PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System, in which 267 phosphoproteins at D0 and 220 phosphoproteins at D5 were identified. Based on cellular components, the results revealed that both of phosphoproteins in two groups had minor different of percent changed. The details are listed in Table 4.3 and as shown in Figure 4.4

Table 4.3 The identified phosphoproteins at D0 and D5 are classified according to cellular components.

Cellular components	267 Phosphoproteins at D0	220 Phosphoproteins at D5	% Changed from D0 to D5
Cell part	38.40%	37.00%	-0.04%
Organelle	30.80%	31.50%	0.02%
Macromolecular complex	15.30%	14.80%	-0.03%
Membrane	9.30%	9.30%	0.00%
Extracellular matrix	3.10%	3.70%	0.19%
Extracellular region	3.10%	3.70%	0.19%

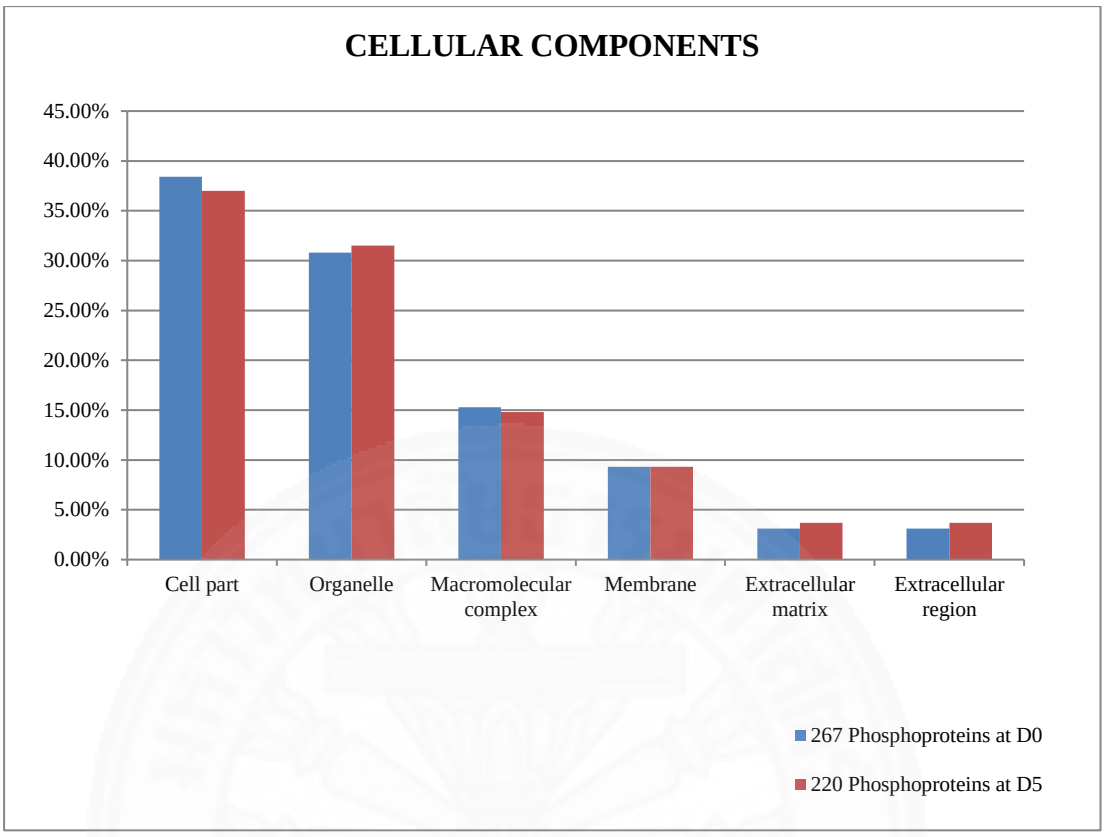

Figure 4.4 The identified phosphoproteins at D0 and D5 are classified according to cellular components.

Overview of 267 phosphoproteins at D0 with 220 phosphoproteins at D5 based on biological processes. The details are listed in Table 4.4 and as shown in Figure 4.5.

Table 4.4 The identified phosphoproteins at D0 and D5 are classified according to biological processes.

Biological processes	267 Phosphoproteins at D0	220 Phosphoproteins at D5	% Changed from D0 to D5
Cellular process	27.40%	26.40%	-3.65%
Metabolic process	22.00%	21.60%	-1.82%
Localization	10.70%	10.40%	-2.80%
Multicellular organismal process	9.30%	9.60%	3.23%
Cellular component organization or biogenesis	8.00%	8.80%	10.00%
Developmental process	7.30%	7.20%	-1.37%
Response to stimulus	6.00%	5.60%	-6.67%
Biological regulation	4.00%	4.00%	0.00%
Immune system process	2.70%	3.20%	18.52%
Reproduction	1.30%	1.60%	23.08%
Biological adhesion	1.30%	1.60%	23.08%

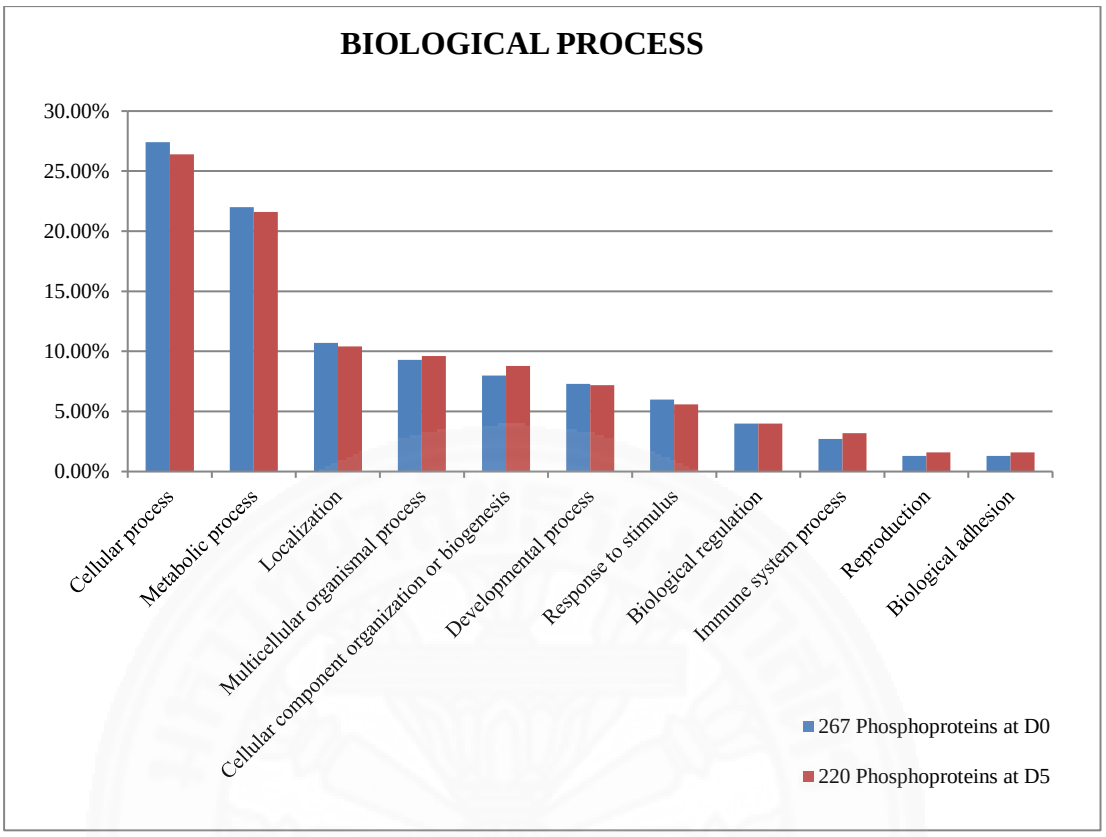

Figure 4.5 The identified phosphoproteins at D0 and D5 are classified according to biological processes.

Analysis the amounts of phosphoproteins differentially expressed from D0 to D5, phosphoproteins which had fold-change >2 were selected (138). It was found in this study that 219 phosphoproteins were differentially expressed, of which 78 phosphoproteins were up-regulated and 141 phosphoproteins were down-regulated from D0 to D5.

Overview of 78 up-regulated phosphoproteins and 141 down-regulated phosphoproteins from D0 to D5 based on cellular components, the details are listed in Table 4.5.

Table 4.5 The identified up-regulated and down-regulated phosphoproteins from D0 to D5 are classified according to cellular components.

Cellular components	78 Phosphoproteins were up-regulated	141 Phosphoproteins were down-regulated
Cell part	36.40%	41.20%
Organelle	24.20%	29.40%
Macromolecular complex	21.20%	14.70%
Extracellular region	9.10%	2.90%
Membrane	6.10%	8.80%
Extracellular matrix	3.00%	3.00%

Overview of 78 up-regulated phosphoproteins and 141 down-regulated phosphoproteins from D0 to D5 based on biological processes, the details are listed in Table 4.6.

Table 4.6 The identified up-regulated and down-regulated phosphoproteins from D0 to D5 are classified according to biological processes.

Biological processes	78 Phosphoproteins were up-regulated	141 Phosphoproteins were down-regulated
Cellular process	32.30%	27.90%
Metabolic process	24.60%	24.10%
Biological regulation	7.70%	1.20%
Immune system process	7.70%	1.20%
Cellular component organization or biogenesis	6.20%	9.60%
Localization	6.20%	9.60%
Developmental process	4.60%	8.40%
Response to stimulus	4.60%	7.20%
Multicellular organismal process	4.60%	7.20%
Biological adhesion	1.50%	1.20%
Reproduction	-	2.40%

Interestingly, it was found in this study that 47 phosphoproteins found unique at D0 with no phosphoproteins found unique at D5. In order to provide an overview of these 47 unique phosphoproteins, The PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System was used. Based on cellular components, they are known to be cell part (45.40%), organelle (27.30%), macromolecular complex (18.20%), and membrane (9.10%).

This study also classified of 47 unique phosphoproteins based on biological processes, it revealed their activities involved in cellular process (32.00%), metabolic process (24.00%), localization (12.00%), developmental process (8.00%), multicellular organismal process (8.00%), response to stimulus (8.00%), biological regulation (4.00%), and cellular component organization or biogenesis (4.00%).

Bioinformatics tools were used to search for phosphoproteins identification. The details of 47 unique phosphoproteins at D0 are listed in Table 4.7.

Table 4.7 Forty-seven phosphoproteins found unique in endometrial secretion at D0.

Accession No.	Uniprotkb ID	Peptide sequence	Protein description
gi|145611434	B2C6G3	QGAKEKQ LLK	abhydrolase domain-containing protein 8
gi|58531795	Q8IZF6	KVSDTPPIV ITKSXK	adhesion G-protein coupled receptor G4
gi|115511036	Q96L96	VEQFPDAS GSLK	alpha-protein kinase 3
gi|444738525	L8EAG2	ATPAAAPV TVTR	alternative protein LAMA5
gi|353252824	-	HNRYYFDS WGQGTLV TVS	anti-tetanus toxoid immunoglobulin heavy chain variable region
gi|1710030	P29374	RKILGQSSP EK	AT-rich interactive domain-containing protein 4A isoform X2

Accession No.	Uniprotkb ID	Peptide sequence	Protein description
gi|18676526	Q5H9F3	KGSQAGAEGQPSTVKR	BCL-6 corepressor-like protein 1 isoform X5
gi|10432807	Q9NXG0	KAAELSVKEYK	Centlein
gi|54633315	Q5QJE6	KDSKIVPGNEK	deoxynucleotidyltransferase terminal-interacting protein 2
gi|23397546	A0A0S2Z662	SALTILQSLSQPEAVSMK	DNA helicase MCM9 isoform 2
gi|13654237	P78527	IPALDLLIK	DNA-dependent protein kinase catalytic subunit isoform 1
gi|307776317	Q96EP1	GAMVRR	E3 ubiquitin-protein ligase CHFR
gi|119623450	-	STPWLLMSR	hCG2045715
gi|387765984	P42858	MATLEKLMKAFESLKSFX	Huntingtin
gi|24474080	-	ELVLTQPPSASGTPGQR	immunoglobulin lambda light chain variable region
gi|78101060	Q14145	XTSGRSGVGVAVT	kelch-like ECH-associated protein 1
gi|119605087	Q9H0B3	MTLQGRADLSGNQGNAAGR	KIAA1683
gi|7661878	Q15058	KTADMPLTPNPVGR	kinesin-like protein KIF14
gi|444299651	Q8NG31	TNLEHTTGQLTTMNRQIAVK	kinetochore scaffold 1

Accession No.	Uniprotkb ID	Peptide sequence	Protein description
gi|578824395	-	GIKSSIGRLFGK	liprin-alpha-2 isoform X16
gi|386642465	Q13126	GTVTTFKVSWSK	methylthioadenosine phosphorylase
gi|116805332	O60682	RPRVAGGGGAGGSAGGGK	musculin
gi|48145973	Q6IB76	LGIKVGESTPDK	NDUFV2
gi|974994783	O15118	APLTDK	niemann-Pick C1 protein isoform X4
gi|76879773	Q8NI08	QNAETATAVATR	nuclear receptor coactivator 7
gi|380036026	A0A087WXV5	SWPSAHSVSNILGIR	paired box protein Pax-1 isoform 2
gi|62087514	Q59GZ2	RMFVFKITTTK	PLEK protein variant
gi|159162550	P11940	AKESKVGPASSVK	polyadenylate-binding protein 1
gi|530378014	-	RHVGVISVEGK	PREDICTED: kelch-like protein 8 isoform X3
gi|530404745	-	CHPGSQSPSQSVVT	PREDICTED: synaptotagmin-16 isoform X2
gi|4406696	Q7Z5A7	AGAAASMAPSPR	protein FAM19A5
gi|578826332	-	LTGLMQSS	regulator of G-protein signaling 6 isoform X13
gi|530398983	B0AZV0	LWQTVVGKTYGLWK	serine-threonine kinase receptor-associated protein isoform X2

Accession No.	Uniprotkb ID	Peptide sequence	Protein description
gi|1477982	-	ETGGDAEAFFGQG	TCR V beta 2-J beta 1.1
gi|409973767	Q15750	PVSVPYXSAQSTS	TGF-beta-activated kinase 1 and MAP3K7-binding protein 1
gi|13236587	Q9BTV4	AANYSSTSTR	transmembrane protein 43
gi|157829353	F4MHK7	SXRCSNTSTLAAR	ubiquitously transcribed tetratricopeptide repeat protein Y-linked transcript variant 96
gi|119612811	Q6ZUT6	KKNQALLRR	uncharacterized protein C15orf52
gi|47678501	Q6IC83	AQLMQYLSLPK	uncharacterized protein C22orf42
gi|34534881	Q9Y6X6	TTKSPRILKHK	unconventional myosin-XVI
gi|40976394	-	KALNXACKSHGHEG	unnamed protein product
gi|530406470	Q6ZTF9	SHMIKKLYK	unnamed protein product
gi|40981144	-	LSLQQLSGK	unnamed protein product
gi|40045182	-	QMGRSSEGR	unnamed protein product
gi|40042354	-	AXMVSMISLR	unnamed protein product
gi|40039202	-	WSYLSRXPSC	unnamed protein product
gi|578811679	-	QENHSSLVSLGGEIQTKSR	zinc finger protein with KRAB and SCAN domains 4 isoform X8

(b) Comparison of differentially expressed glycoproteins between endometrial secretion collected at D0 and D5

This study had analysed the differences in the glycoprotein contents with respect to pre-receptive phase (day at oocyte retrieval /D0) and receptive phase or window period of implantation (day at embryo transfer /D5) endometrium. It was found in this study that total of 105 glycoproteins expressed at D0 and 110 glycoproteins expressed at D5, of which, 5 of them were found unique at D5 with no glycoproteins found unique at D0 (as shown in Figure 4.6).

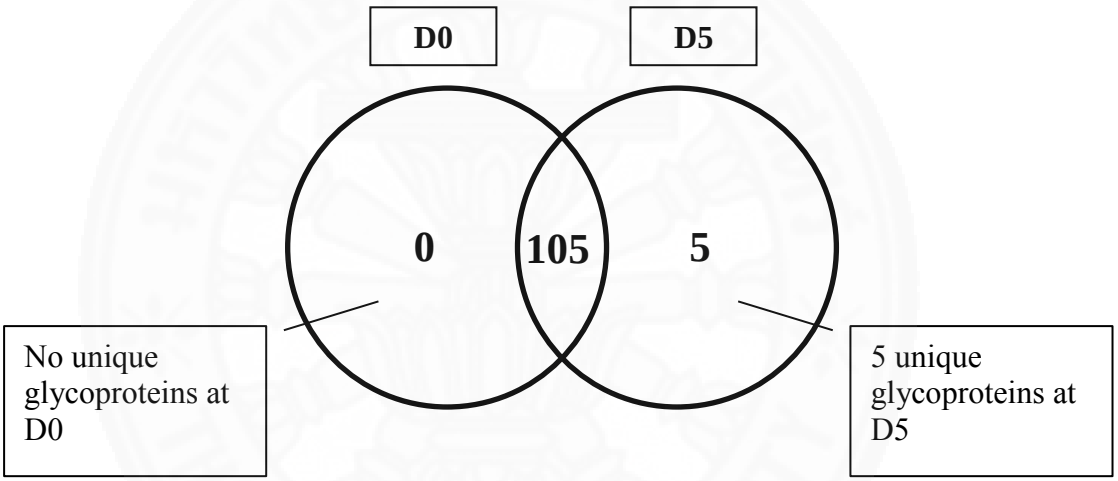

Figure 4.6 Venn diagram demonstrates the overlapped glycoproteins identified from endometrial secretions at D0 and D5 groups.

In order to provide an overview of the differential glycoproteins expression between pre-receptive phase (D0) and receptive phase or window period of implantation (D5) of endometrium, this study had classified D0 and D5 samples according to Gene Ontology using The PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System, in which 105 glycoproteins at D0 and 110 glycoproteins at D5 were identified. Based on cellular components, the results revealed are listed in Table 4.8 and as shown in Figure 4.7.

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Table 4.8 The identified glycoproteins at D0 and D5 are classified according to cellular components.

Cellular components	**105 Glycoproteins at D0**	**110 Glycoproteins at D5**	**% Changed from D0 to D5**
Cell part	46.80%	47.10%	0.01%
Organelle	33.30%	35.30%	0.06%
Macromolecular complex	13.30%	11.80%	-0.11%
Extracellular region	3.30%	2.90%	-0.12%
Membrane	3.30%	2.90%	-0.12%

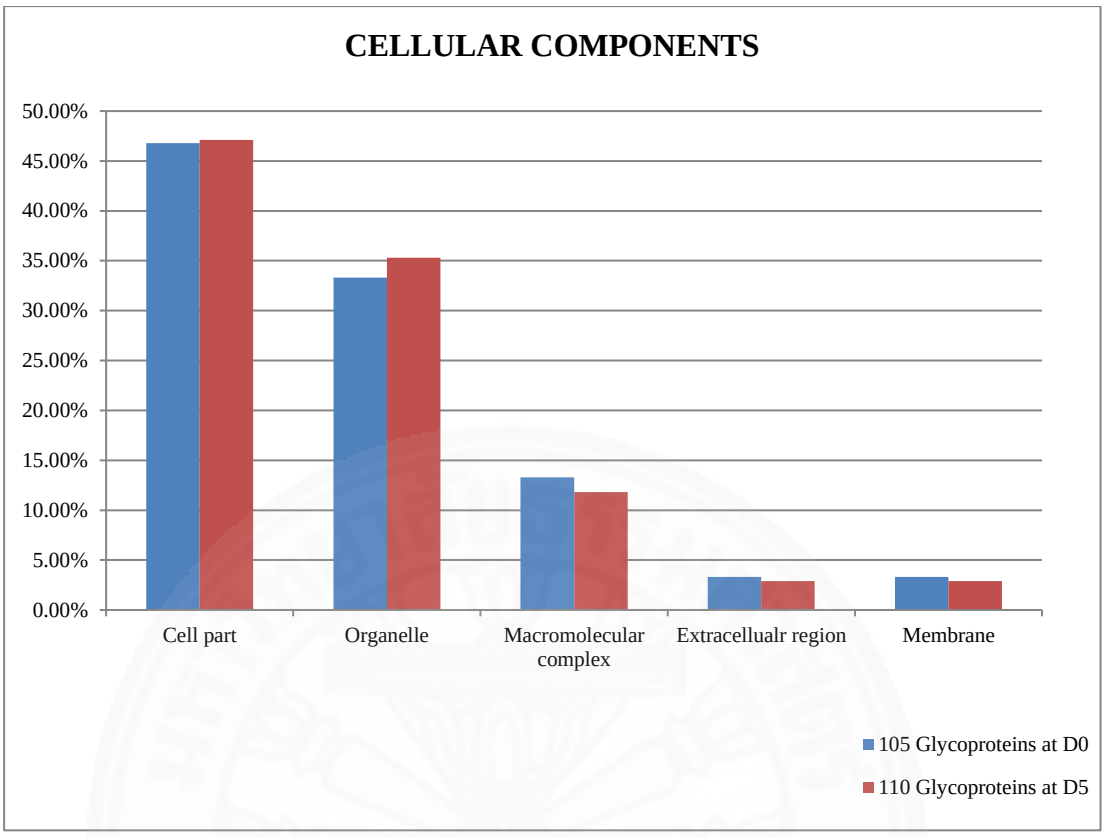


Figure 4. 7 The identified glycoproteins at D0 and D5 are classified according to cellular components.

Overview of 105 glycoproteins at D0 with 110 glycoproteins at D5 based on biological processes, the results revealed that both of glycoproteins in two groups had minor different change. The details are listed in Table 4.9 and as shown in Figure 4.8.

Table 4.9 The identified glycoproteins at D0 and D5 are classified according to biological processes.

Biological processes	105 Glycoproteins at D0	110 Glycoproteins at D5	% Changed from D0 to D5
Cellular process	25.60%	26.40%	0.03
Metabolic process	22.00%	22.40%	0.02
Localization	9.30%	8.80%	-0.05
Response to stimulus	9.30%	8.80%	-0.05
Immune system process	8.50%	8.00%	-0.06
Cellular component organization or biogenesis	6.80%	8.00%	0.18
Biological regulation	5.90%	5.60%	-0.05
Developmental process	4.20%	4.00%	-0.05
Multicellular organismal process	4.20%	4.00%	-0.05
Biological adhesion	3.40%	3.20%	-0.06
Locomotion	0.80%	0.80%	0.00

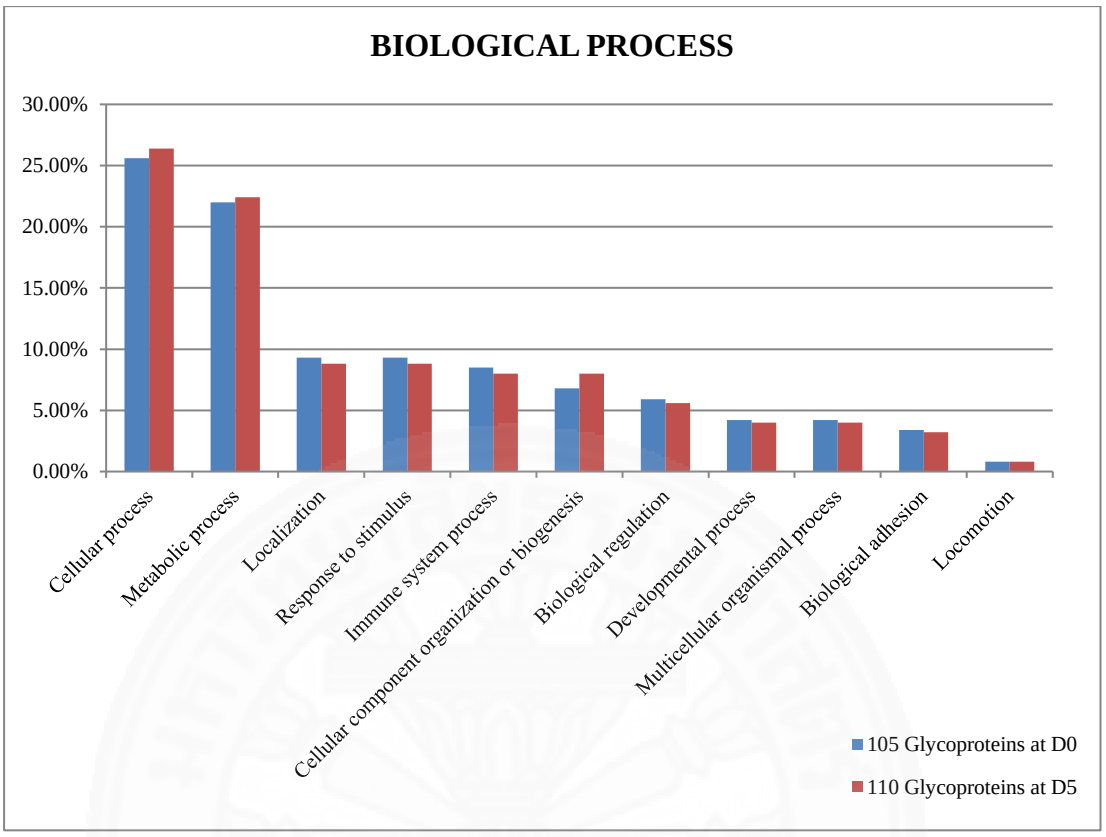


Figure 4.8 The identified glycoproteins at D0 and D5 are classified according to biological processes.

Analysis the amounts of glycoproteins differentially expressed from D0 to D5, which glycoproteins had fold-change >2 were selected (138). It was found in this study that 58 glycoproteins were differentially expressed, of which 34 glycoproteins were up-regulated and 24 glycoproteins were down-regulated from D0 to D5.

Overview of 34 up-regulated phosphoproteins and 24 down-regulated glycoproteins from D0 to D5 based on cellular components, the details are listed in Table 4.10.

Table 4.10 The identified up-regulated and down-regulated glycoproteins from D0 to D5 are classified according to cellular components.

Cellular components	34 Glycoproteins were up-regulated	24 Glycoproteins were down-regulated
Cell part	40.00%	42.90%
Organelle	33.30%	42.90%
Macromolecular complex	20.00%	14.20%
Membrane	6.70%	-

Overview of 34 up-regulated glycoproteins and 24 down-regulated glycoproteins from D0 to D5 based on biological processes, the details are listed in Table 4.11.

Table 4.11 The identified up-regulated and down-regulated glycoproteins from D0 to D5 are classified according to biological processes.

Biological processes	34 Glycoproteins were up-regulated	24 Glycoproteins were down-regulated
Cellular process	28.90%	25.00%
Metabolic process	17.80%	20.00%
Cellular component organization or biogenesis	11.10%	5.00%
Localization	8.90%	-
Response to stimulus	8.90%	15.00%
Multicellular organismal process	6.70%	5.00%
Immune system process	6.70%	10.00%
Biological adhesion	4.40%	5.00%
Biological regulation	4.40%	5.00%
Developmental process	2.20%	10.00%

Interestingly, it was found in this study that 5 glycoproteins were found unique at D5 with no glycoproteins were found unique at D0. In order to provide an overview of these 5 unique glycoproteins, this study used The PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System. Based on cellular components, these proteins are known to be cell part (50.00%), and organelle (50.00%).

Based on biological processes, the results revealed that 5 unique glycoproteins activities involved in cellular process (42.80%), cellular component organization or biogenesis (28.60%), and metabolic process (28.60%).

Bioinformatics tools were used to search for glycoprotein identification. The details of 5 unique glycoproteins at D5 are listed in Table 4.12.

Table 4.12 Five glycoproteins found unique in endometrial secretion at D5.

Accession No.	Uniprotkb ID	Peptide sequence	Protein description
gi|5453543	Q04828	ALEATK	aldo-keto reductase family 1 member C1
gi|578826735	O94986	EGAEK	centrosomal protein of 152 kDa
gi|14249190	Q14781	GSLGDGK	chromobox protein homolog 2
gi|23396771	P82970	KGEDGK	high mobility group nucleosome-binding domain-containing protein 5
gi|30268178	Q70EL2	NASVGGK	ubiquitin carboxyl-terminal hydrolase 45

4.3.2 Comparison of differentially expressed proteins in endometrial secretions collected at D5 between implantation and non-implantation groups

(a) Comparison of phosphoprotein expressions at D5 between implantation and non-implantation groups

For the phosphoproteins analysis, this study identified 220 phosphoproteins at D5. The analysis of the differences in the phosphoprotein contents with respect to receptivity and non-receptivity of endometrium identified total of 40 phosphoproteins differentially expressed, of which 4 phosphoproteins were found unique in implantation group and 36 phosphoproteins were found unique in non-implantation group (as shown in Figure 4.9).

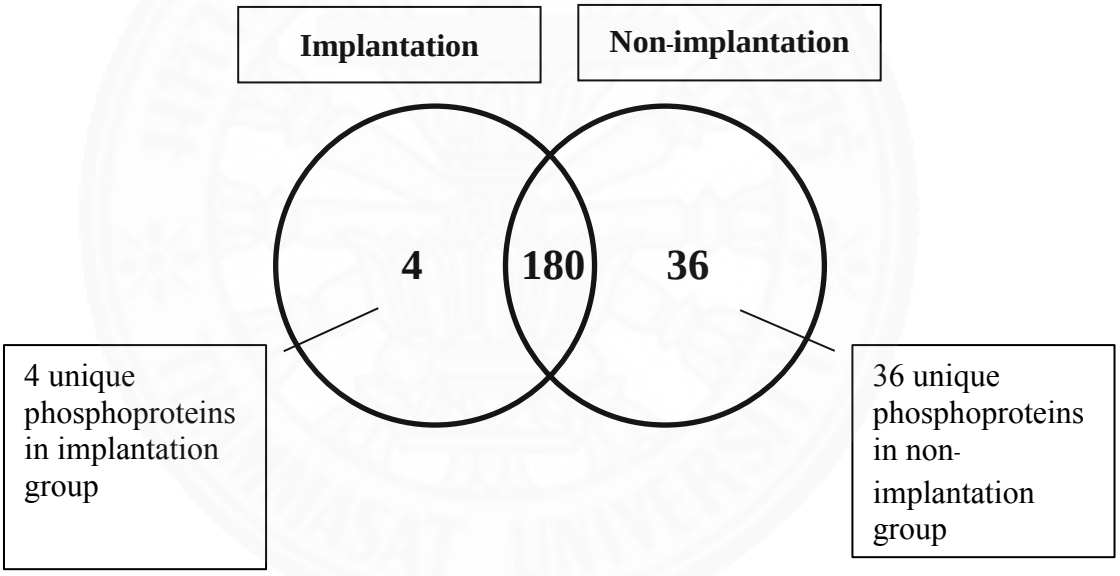


Figure 4.9 Venn diagram demonstrates the overlapped phosphoproteins identified from endometrial secretions at D5 of implantation and non-implantation cycle.

Bioinformatics tools were used to identify phosphoproteins differentially expressed between implantation and non-implantation groups. The details are listed in Table 4.13 and Table 4.14.

Table 4.13 Four phosphoproteins found unique in implantation group.

Accession No.	Uniprotkb ID	Peptide sequence	Protein description
gi|50345988	P36542	SEVATLTAAGK	ATP synthase subunit gamma, mitochondrial isoform L (liver) precursor
gi|119631287	D3DPI2	VSGNTSQR	hCG1641229, isoform CRA_a
gi|62177112	Q9BW62	MQDGASDGEMPK	katanin p60 ATPase-containing subunit A-like 1 isoform X2
gi|51094548	A4D2F6	NNSTILSHAKMSR	similar to Splicing factor, arginine/serine-rich, 46kD

Table 4.14 Thirty-six phosphoproteins found unique in non-implantation group.

Accession No.	Uniprotkb ID	Peptide sequence	Protein description
gi|3088340	P60866	KGPVRMPTKTLR	40S ribosomal protein S20
gi|6808314	Q96Q27	GKGERSPSLSSTFPQGRR	ankyrin repeat and SOCS box protein 5 isoform X2
gi|119612815	-	KGTTIITATNH	chromosome 15 open reading frame 23, isoform CRA_a
gi|2827693	Q6IBW4	RNVELFIATSQK	condensin-2 complex subunit H2 isoform X1
gi|119576538	-	RGRTDDADAQMTWTHR	hCG2045069
gi|379318422	P10412	QSIHLTQHLR	histone H1.4
gi|47027976	Q6PL43	SSGPGCLPAAPLSGTLK	hypothetical protein
gi|519673618	-	GTTVSVSS	immunoglobulin A heavy chain variable region, partial
gi|70798135	P01764	KSYVDSMEGR	immunoglobulin heavy chain variable region
gi|27894373	Q13901	NASKVANKGK	nuclear nucleic acid-binding protein C1D
gi|5453908	Q00169	QKDPVKGMTADD	phosphatidylinositol transfer protein alpha isoform
gi|578820863	P62330	QAEESMVASMR	PREDICTED: ADP-ribosylation factor GTPase-activating protein 6 isoform X4
gi|578814520	Q96JN2	MSLESYGK	PREDICTED: coiled-coil domain-containing protein 136 isoform X9

Accession No.	Uniprotkb ID	Peptide sequence	Protein description
gi|578827434	Q8N5R6	STSEEKNNQSSK	PREDICTED: coiled-coil domain-containing protein 33 isoform X3
gi|578809883	O15018	DSQVPVTSSVVPEAKASR	PREDICTED: PDZ domain-containing protein 2 isoform X9
gi|578802818	Q9BXF6	EKYSTSVVEK	PREDICTED: rab11 family-interacting protein 5 isoform X4
gi|578811844	Q92766	QEITEGELK	PREDICTED: ras-responsive element-binding protein 1 isoform X11
gi|530398781	P48065	KMAVTIPVITSAFK	PREDICTED: sodium- and chloride-dependent betaine transporter isoform X4
gi|530406332	Q13596	MGSMK	PREDICTED: sorting nexin-1 isoform X1
gi|34527855	Q9P2P6	QIDQSSSDQTR	PREDICTED: stAR-related lipid transfer protein 9 isoform X1
gi|578805044	Q96K49	TFRIAK	PREDICTED: transmembrane protein 87B isoform X2
gi|578815847	Q7Z7G8	SEDLGTVQEK	PREDICTED: vacuolar protein sorting-associated protein 13B isoform X4
gi|578832877	A6NN14	AFSQFSTLKK	PREDICTED: zinc finger protein 729 isoform X1
gi|530410547	O75880	KGEIAASIATHMRPYR	Protein SCO1 homolog, mitochondrial isoform X1
gi|1526978	Q92736	HVTTGK	ryanodine receptor 2 isoform X9
gi|2654717	-	CASSPFSSR	T cell receptor beta chain, partial

Accession No.	Uniprotkb ID	Peptide sequence	Protein description
gi|28591	-	KVPEVSTPTLVEVSR	unnamed protein product
gi|10433312	-	MSADIAVEMNCIK	unnamed protein product
gi|40039690	-	EDNSXYIMR	unnamed protein product
gi|40976344	-	TAMPRAGGT	unnamed protein product
gi|7020294	Q9NZM3	MSNTKLLK	unnamed protein product
gi|315434271	P19320	KANMKGSYSLVEAQK	vascular cell adhesion protein 1 isoform c precursor
gi|530365359	B1ALM3	QYFMSIFNR	voltage-dependent L-type calcium channel subunit alpha-1S isoform X1
gi|578834848	P52742	KARXSAGAAKR	zinc finger protein 135 isoform X3
gi|400153449	Q7Z340	SYLGSTSMR	zinc finger protein 551 isoform 2
gi|115344353	Q08EK0	LCEGEAQK	ZNF766 protein

(b) Comparison of glycoprotein expressions at D5 between implantation and non-implantation groups

For the glycoproteins analysis, this study identified 110 glycoproteins at D5. The analysis of the differences in the glycoprotein contents with respect to receptivity and non-receptivity of endometrium identified total of 3 glycoproteins differentially expressed, of which 1 glycoprotein was found unique in implantation group and 2 glycoproteins were found unique in non-implantation group (as shown in Figure 4.10).

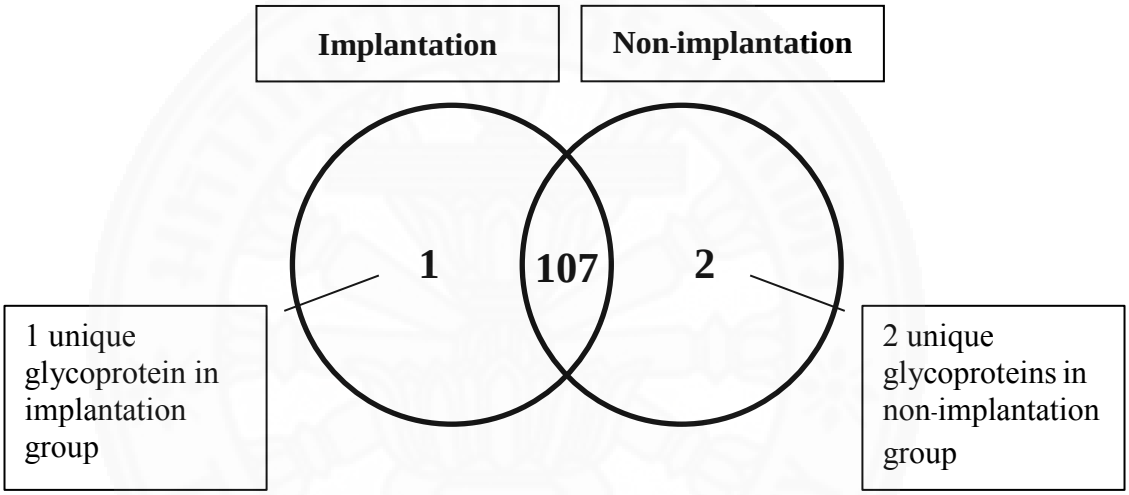


Figure 4.10 Venn diagram demonstrates the overlapped glycoproteins identified from endometrial secretion at D5 of implantation and non-implantation groups.

Bioinformatics tools were used to identify glycoproteins differentially expressed between implantation and non-implantation groups. The details are listed in Table 4.15 and Table 4.16.

Table 4.15 One glycoprotein found unique in implantation group.

Accession No.	Uniprotkb ID	Peptide sequence	Protein description
gi|10835242	Q13976	AYEDAEAK	cGMP-dependent protein kinase 1 isoform 2

Table 4.16 Two glycoproteins found unique in non-implantation group.

Accession No.	Uniprotkb ID	Peptide sequence	Protein description
gi|119599937	-	FQHFRRPR	hCG2038570
gi|148727319	Q86VQ3	KEEKVDELCGALK	thioredoxin domain-containing protein 2 isoform 2

4.4 Western blot analysis

To verify the predicted differential phosphoproteins and glycoproteins expression of endometrium receptivity biomarkers based on LC-MS/MS data, the western blot analysis using the commercial antibodies against total protein was performed in sample collected at D5 compared between implantation and non-implantation group. This study analysed the total protein expressions in two selected phosphoproteins which are ATP synthase subunit gamma, mitochondrial (ATPG) and katanin p60 ATPase containing subunit A1 (KATNA1), and in one selected glycoprotein which is thioredoxin domain-containing protein 2 **(**thioredoxin2) respectively. The results of these western blot analysis were then compared to the corresponding phosphopeptide and glycopeptide mass spectrometry results.

(a) Phosphoprotein validation

The differential expressions and abundances of two candidate phosphoproteins were verified by commercially available antibodies against total protein to ATP synthase subunit gamma, mitochondrial (ATPG) and katanin p60 ATPase containing subunit A1 (KATNA1). From the analysis of the enriched phosphopeptides using LC-MS/MS, this study identified phosphorylated ATPG and KATNA1 uniquely expressed in implantation group.

The results of western blot showed that there was the total protein of ATPG and KATNA1 expressions in samples both of implantation and non-implantation groups (as shown in Figure 4.11A). These results confirmed the predicted protein expressions from LC-MS/MS data.

In order to quantify the total protein expression in each sample, band intensity normalized with histone in western blot analysis was performed. Image Lab software (Bio-Rad Laboratories Inc., California, USA) was used for image acquisition and intensity analysis of each band. The analysis was done in triplicate. In the analysis of ATPG, the mean ± standard deviations (SD) of relative intensity in implantation and non-implantation group were 5.5374 ± 0.84 and 6.0324 ± 1.05 respectively. In the analysis of KATNA1, the mean ± SD of relative intensity in implantation and non-implantation groups were 0.7038 ± 0.09 and 0.7467 ± 0.10 respectively. The detailed are listed in Table 4.17. With respect to SD, the presence of overlapping error bars used

indicating no significantly different in relative quantity of total ATPG and KATAN1 expression between implantation and non-implantation group. (as shown in Figure 4.11B).

Table 4.17 Relative intensity of ATPG and KATNA1.

	Relative Intensity	
	Implantation	**Non-implantation**
ATPG	5.5374 ± 0.84	6.0324 ± 1.05
KATNA1	0.7038 ± 0.09	0.7467 ± 0.10

The data was presented as mean ± standard deviation (SD), n = 3.

ATPG was represented for ATP synthase subunit gamma, mitochondrial.

KATNA1 was represented for katanin p60 ATPase containing subunit A1.

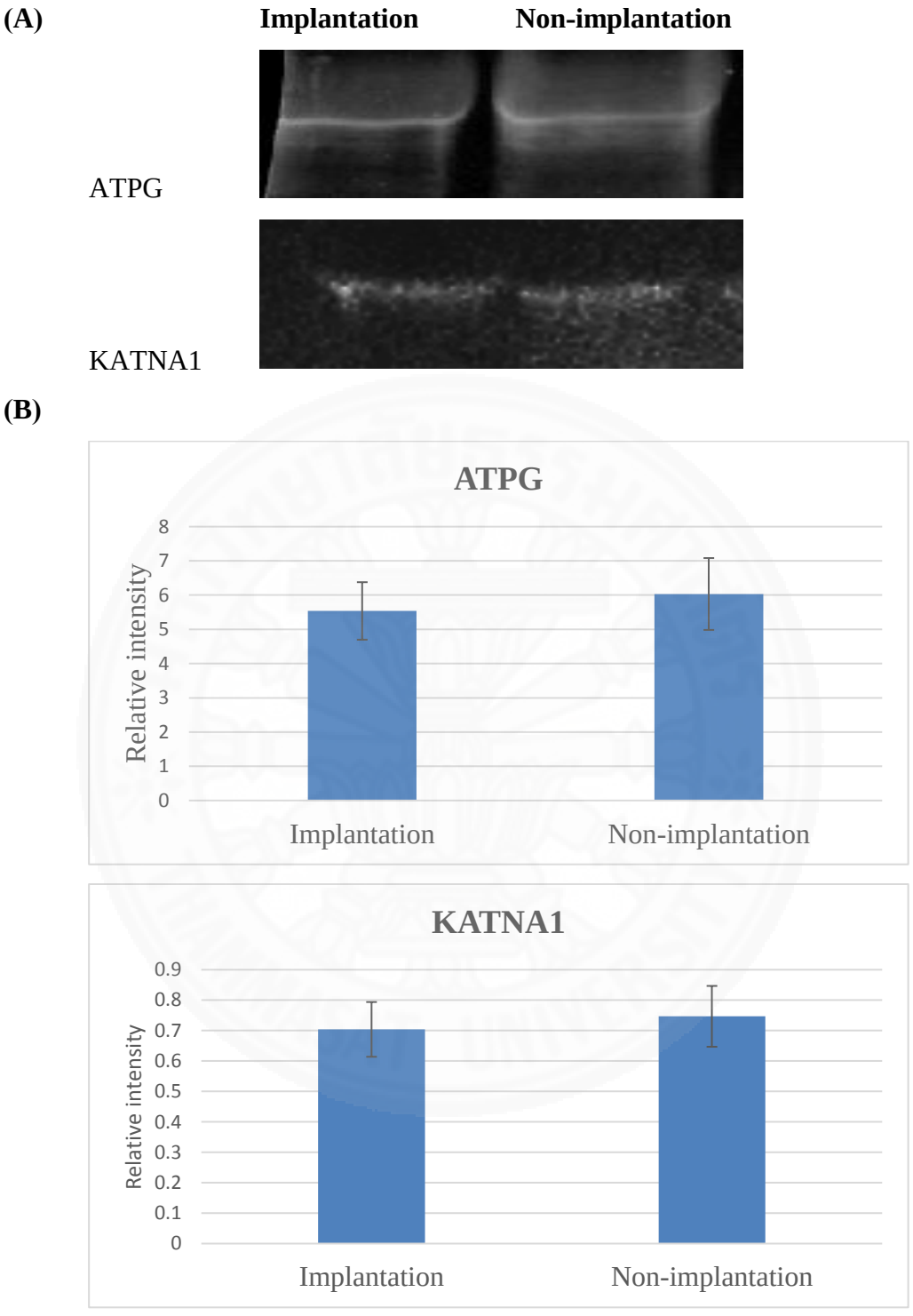


Figure 4.11 Western blot analysis for ATPG and KATNA 1; (A) The illustration of ATPG and KATNA1 expression, (B) Relative intensities of ATPG and KATNA 1 (normalized with histone in western blot analysis) compared between implantation and non-implantation groups, Error bar represent the standard deviation.

(b) Glycoprotein validation

The differential expression and abundance of one candidate glycoprotein was verified by commercially available antibody against total protein to thioredoxin domain-containing protein 2 **(**thioredoxin2). From the analysis of the enriched glycopeptides using LC-MS/MS, this study identified glycosylated of thioredoxin2 uniquely expressed in non-implantation group.

The result of western blot showed that there was the total protein of thioredoxin2 expression in samples of both implantation and non-implantation groups (as shown in Figure 4.12A). These results confirmed of predicted protein expressions from LC-MS/MS data.

In order to quantify the total protein expression in each sample, band intensity normalized with histone in western blot analysis was performed. Image Lab software (Bio-Rad Laboratories Inc., California, USA) was used for image acquisition and intensity analysis. The analysis was done in triplicate. In the analysis of thioredoxin2, the mean ± standard deviation (SD) of relative intensity in implantation and non-implantation group were 8.2486 ± 0.74 and 12.6916 ± 1.08 respectively. The details are listed in Table 4.18. With respect to SD, the presence of non-overlapping error bars used indicating significantly different in relative quantity of total thioredoxin2 expression between implantation and non-implantation group. (as shown in Figure 4.12B).

Due to the differential abundances of thioredoxin2 between implantation and non-implantation groups observed by western blot analysis, it might be estimated that there was positive correlation with glycopeptide mass spectrometry and western blot measured protein abundance for the selected glycoprotein.

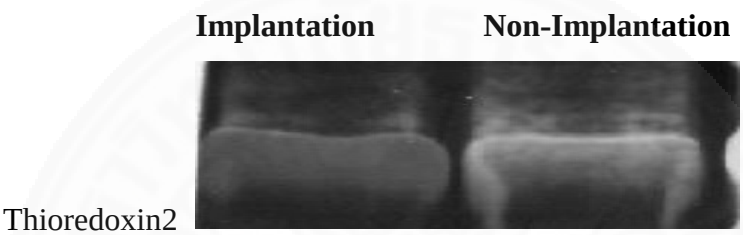
Table 4.18 Relative intensity of Thioredoxin2

	Relative Intensity	
	Implantation	**Non-implantation**
Thioredoxin2	8.2486 ± 0.74	12.6916 ± 1.08

The data was presented as mean ± standard deviation (SD), n = 3.

Thioredoxin2 represented for thioredoxin domain-containing protein 2.

(A)



(B)

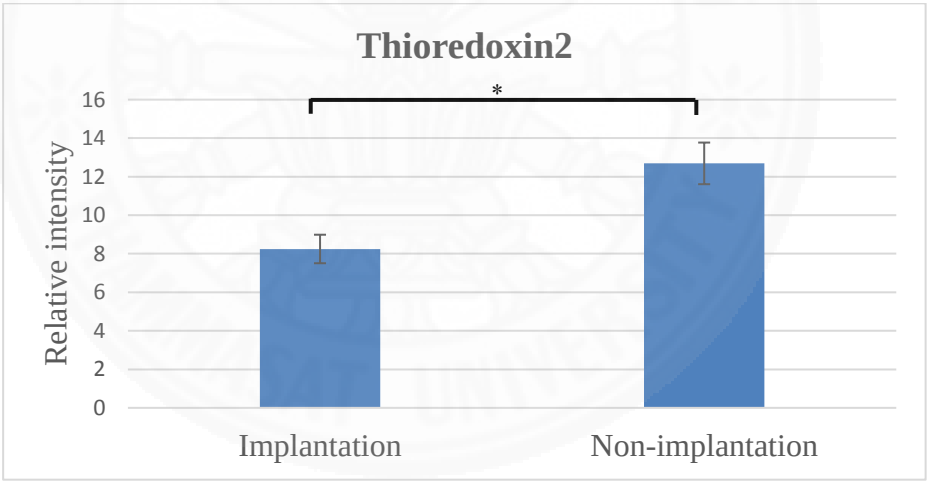


Figure 4.12 Western blot analysis for thioredoxin2; (A) The illustration of thioredoxin2 expression, (B) Relative intensities of thioredoxin2. (normalized with histone in western blot analysis) compared between implantation and non-implantation groups. Error bar represent the standard deviation.

* represents for comparison with significantly different variances.

4.5 Discussion

4.5.1 Protein contents in endometrial secretion

In recent years, many approaches to study the endometrium functions and receptivity pointed to analysis of the contents of endometrial secretions especially during the window of implantation. Since it had been shown to represent for a less invasive means of studying than tissue biopsy. Furthermore, the endometrial secretion offers an alternate sample choice of far less complexity than the tissues (91).

The endometrium secretion collected from tip of embryo transfer catheter which is used in this study for sample collection, is the advantage. It is normally used in the clinical **routine** procedure of embryo transfer process. The limitation of this study is due to the concentrations of total protein in endometrial secretion samples of all studied groups containing very low abundance of proteins, having range between **0.041 - 2.909 µg/µl (median 0.539, 25–75 percentiles 0.259–0.829 µg/µl)**. Therefore, it is quite difficult for analysis since generally more protein concentration is required and then more demanding processes is needed to perform analysis, especially analysis of phosphoproteins and glycoproteins included in this study.

Regarding to analysis of the very small abundance of proteins, the samples from the same group were pooled prior to analysis, in order to provide the amount of proteins enough to perform an assay. Sample pooling strategy is also considered in proteomics analysis (139-142). It has advantage that can be used to minimize the variance of technical in experiment performance when many individuals are analysed. In addition, the protein expression in a pool represents for the majority of proteins which matches the average expression of the individuals included in the pool. Therefore, the significant differences and similarities between study groups might be observed easily and thus giving increase statistical power to detect the differences between study groups. However, it is considered when samples are pooled that all of proteins in individual samples does not always detectable (143).

In context of technique used to collect endometrial secretion and protein contents obtained, technique used in this study was compared with those of collected by endometrium lavage or flushing (89-92). In the study of Scotchie JG et al, 2009 (90) who collected endometrial secretion with a sonohysterography catheter passing into the

uterine cavity and inflated 1.5 ml of the air, then flushed with 4 ml of normal saline. The flushing was then removed into sterile containers and this process was performed twice. The mean volume of fluid recovered after injection was 4.3 ml (range 1.0-8.0 ml) and the mean protein quantitation per sample was **4.92 µg/µl** (range 2.70-7.14 µg/µl). Although the proteins presented in uterine flushing had more amount than those of technique used in this study, it differed and varied in fluid recovered during the lavage procedure. Therefore, the other studies using this technique might also have the varied and inconsistent results. Moreover, this technique is not the clinical routine procedure and might have negative impact to pregnancy rate if done in treatment cycle.

Technique used in this study was also compared with endometrial secretion collected by aspiration technique, which could be performed during window of implantation without detrimental effect on pregnancy rates (95-97). In the study of Boomsma CM et al, 2009 (97), they used a 2-ml syringe connected to an embryo transfer catheter and passed through transcervically into the uterine cavity. The secretion was gently and gradually suctioned with simultaneously rotating of the catheter. Then, the routine embryo transfer procedure was carried out. For this technique, the protein content in the diluted samples varied from **0.04 to 3.15 µg/µl** (median 0.535, 25–75 percentiles 0.280–0.943 µg/µl). Regarding to the protein contents in endometrial secretion, technique used in this study was comparable to that of aspiration technique of Boomsma CM et al, 2009 (97) except that no process of suction with simultaneously rotating the catheter. Therefore, it was not traumatic to endometrium. Furthermore, this aspiration technique was still not yet the routine performed in ART treatment.

4.5.2 Phosphoproteins and glycoproteins represent for pre-receptive and receptive phases or window period of implantation (WOI)

The synchronized maturation and differentiation from the pre-receptive endometrium to receptive endometrium or window period of implantation (WOI) is crucial for embryo implantation. During this period, the endometrium undergoes structural and functional changes influenced by the ovarian steroids which prepare it to be receptive to embryo. Furthermore, the endometrium in stimulated ART cycles is influenced by supraphysiological hormonal levels during controlled ovarian

hyperstimulation (COH) which had been revealed that having a significant alterations and differences from natural menstrual cycle.

In the traditional method used to dating the endometrium, histological assessment according to Noyes et al criteria, 1950 (144) is used as gold standard technique. This technique defined the proliferative and secretory phase of endometrium based on structural and morphological features of the different compartments detected in biopsy endometrium tissue. Since it is an invasive technique which many studies try to avoid by using less invasive techniques that suitable for clinical application. In this context, secretomics obtained by technique used in this study with no disturbance to endometrial function is of interest as possibility to assess endometrial maturation and receptivity.

This study identified phosphoproteins and glycoproteins expression in endometrial secretion at pre-receptive (oocyte retrieval day/D0) and receptive phases or window period of implantation (embryo transfer day/D5) to characterize the proteins associated in the transition from pre-receptive phase to receptive phase of stimulated endometrium. It was found in this study that 267 phosphoproteins and 105 glycoproteins expressed at D0 may represent for **pre-receptive phase** of endometrium, while 220 phosphoproteins and 110 glycoproteins expressed at D5 may represent for **receptive phase or window period of implantation (WOI)** of endometrium.

Regarding to biological processes involved and the cellular components at D0 in comparison with those of D5, it was found only minor different in percent changed from D0 to D5 except phosphoproteins involved in immune system process, reproduction, and biological adhesion which were found more increase (18.52%, 23.08%, and 23.08% respectively).

The minor different percent changed from D0 to D5 might be due to the most of identified proteins are commonly found at both D0 and D5. It may implicate that in the process of endometrium development, numerous proteins involved in preparing the endometrium are produced from early secretory phase or from the end of proliferative phase and most of these actual functional proteins still remain to be determined.

This study also analysed the amounts of proteins differentially expressed from D0 to D5, with >2 fold-changed. It was found in this study that 219

phosphoproteins and 58 glycoproteins were differentially expressed, of which 78 phosphoproteins and 34 glycoproteins were up-regulated, while 141 phosphoproteins and 24 glycoproteins were down-regulated from D0 to D5. This study suggested that the process by which a cell increases or decrease the quantity of protein expression may involves in mechanism of endometrial preparation for implantation process during the early and mid-secretory phases.

Interestingly, it was found in this study that 47 phosphoproteins uniquely found at D0 with no phosphoproteins found unique at D5, and 5 glycoproteins uniquely found at D5 with no glycoproteins found unique at D0. These findings might be representing as **biomarkers for endometrium phase of D0 and D5 in stimulated cycles respectively.** These functional molecules might help to improve sensitivity and specificity values in increasing the ability to discriminate between pre-receptive phase and receptive phase of endometrium. However, the further study it needed.

The 47 unique phosphoproteins have related functions to transcription regulator (Polyadenylate-binding protein 1, paired box protein Pax-1 isoform 2, Nuclear receptor coactivator 7, BCL-6 corepressor-like protein 1 isoform X5, musculin, ubiquitously transcribed tetratricopeptide repeat protein Y-linked transcript variant 96), cytoskeleton (Unconventional myosin-XVI, kinesin-like protein KIF14, Huntingtin, PLEK protein variant, katanin p60 ATPase-containing subunit A-like 1 isoform X2), signal transduction (regulator of G-protein signaling 6 isoform X13, Adhesion G-protein coupled receptor G4), cell division (Centlein, Kinetochore scaffold 1), cell-matrix adhesion (liprin-alpha-2 isoform X16), defensive (anti-tetanus toxoid immunoglobulin heavy chain variable region, immunoglobulin lambda light chain variable region), degradation (E3 ubiquitin-protein ligase CHFR degradation, kelch-like protein 8 isoform X3, Kelch-like ECH-associated protein 1), and receptor (TGF-beta-activated kinase 1 and MAP3K7-binding protein 1).

The 5 unique glycoproteins have related functions to transcription regulator (Chromobox protein homolog 2, High mobility group nucleosome-binding domain-containing protein 5), degradation (Ubiquitin carboxyl-terminal hydrolase 45), and cell division (Centrosomal protein of 152 kDa).

4.5.3 Phosphoproteins and glycoproteins represent for endometrium receptivity

This present data identified the endometrial secretion in women underwent ART treatment cycle and compared phosphoproteins and glycoproteins expressions between implantation and non-implantation groups. This study revealed 4 phosphoproteins and 1 glycoprotein found unique in implantation group that might represent of the endometrium receptivity and might be useful as potential biomarkers for positive endometrium receptivity. Whereas 36 phosphoproteins and 2 glycoproteins found unique in non-implantation group might represent the endometrium non-receptivity and be useful as potential biomarkers for negative endometrium receptivity.

4.5.3.1 Phosphoproteins and glycoproteins represent for promotion of the endometrium receptivity

The 4 phosphoproteins and 1 glycoprotein, which were uniquely expressed in the implantation group were ATP synthase subunit gamma, mitochondrial isoform L (liver) precursor (ATPG), hCG1641229, isoform CRA_a, katanin p60 ATPase-containing subunit A-like 1 isoform X2 (KATNA1), and similar to Splicing factor, arginine/serine-rich, 46kD and cGMP-dependent protein kinase 1 isoform 2, respectively.

One of the five differentially expressed proteins is **phosphorylated ATP synthase subunit gamma, mitochondrial isoform L (liver) precursor (ATPG)**. This protein is a subunit of mitochondrial membrane ATP synthase and is known to be involved with energy metabolism (145-147). The biological process of ATPG associates in ATP biosynthetic process, mitochondrial ATP synthesis, and oxidative phosphorylation. (148-150)

The involvement of ATPG in endometrium function was revealed by Murli Manohar, et al, 2014 (151). They studied the protein expression in human endometrial biopsies which were collected during receptive phase (7 days after the LH peak occurred: LH+7). Comparing of protein expression between fertile and unexplained infertile women, they identified 12 proteins displaying significant expression changes. One of them was **ATP synthase subunit delta** which was down-regulated protein in unexplained infertile compared to fertile women.

Phosphorylated **Katanin p60 ATPase-containing subunit A1** (KATNA1) which was the one found unique in implantation group, is a catalytic subunit of Katanin protein complex, which has function as severs microtubules in an ATP-dependent manner (152-157). Its activity is important for architecture and function of the dynamic re-organization of microtubule, microtubule severing, and mitotic nuclear division. Biological process is associated in cell division and differentiation, intracellular transport, and cell migration (158-162).

Phosphorylated **Similar to Splicing factor, arginine/serine-rich** which was the one that was found unique in implantation group, is the member of the SR protein family, involved in the regulation of pre-mRNA splicing (163, 164). Its function is associated in constitutive and alternative splicing of pre-mRNA which is an important mechanism required for regulating gene expression and thus cause the generating of protein diversity and protein expression**.** The other functional role is on various post-splicing activities, including mRNA nuclear export, nonsense-mediated decay, and mRNA translation (165, 166). Pre-mRNA splicing of SR proteins dynamic activity requires the phosphorylation and dephosphorylation processes (167-169).

The involvement of splicing factor in endometrium was revealed by Nie GY et al, 2000 (170) who studied of alternatively spliced mRNAs for SC35 (**splicing factor SC35**) in uterine expression. They concluded that in the mouse uterus during early pregnancy, there were overall expression level of SC35 mRNA much higher in implantation sites than in inter-implantation sites and also in the pseudopregnant uterus. They also implicated that in the presence of embryo-derived factors, SC35 mRNA was also up-regulated at implantation sites. These all results demonstrated that splicing factor is essential for regulating and plays a critical role during implantation process and establishment of pregnancy in the mouse uterus.

A report by Lopez-Mejia IC et al, 2013 (171), which studied the regulation of epithelial cell invasion during physiological processes of embryo implantation. They found that tissue-specific in district site were different in invasion properties, and the higher expression rate of the fibronectin EDA+ splicing isoform in endometrium resulted from higher expression levels of the **serine/arginine-rich splicing factor 1 (SRSF1)** in fibroblasts from this tissue. These data showed that the cellular

concentrations of the splicing factor SRSF1 can give unique properties to tissues and enable host cell invasion.

Cyclic guanosine monophosphate (cGMP)-dependent protein kinase 1 (PKG1) in glycosylated form found unique in implantation group in this study, is serine/threonine protein kinase. It is a key mediator of the nitric oxide (NO)-cGMP signaling pathway. Protein kinases have important functions in various cellular signal transduction pathways through their substrate phosphorylation. Proteins that are phosphorylated on serines and threonines by PKG1 have implicated in modulating cellular calcium and important functions in signal transduction pathways. The biological role is associated in regulation of platelet activation and adhesion, smooth muscle contraction, cardiac function, gene expression, feedback of the NO-signaling pathway (172-175).

PKG1 has the role associated in progesterone hormone, which modulates endometrium functions and control the phase of endometrium in the secretory phase of the menstrual cycle (29-36). The effects of progesterone were mediated by second messengers i.e. cAMP, **cGMP** through intracellular progesterone receptors (PRs) and transmit signals to the nuclear PRs or other transcription factors (TFs) resulting in classical mechanism (35, 176-179).

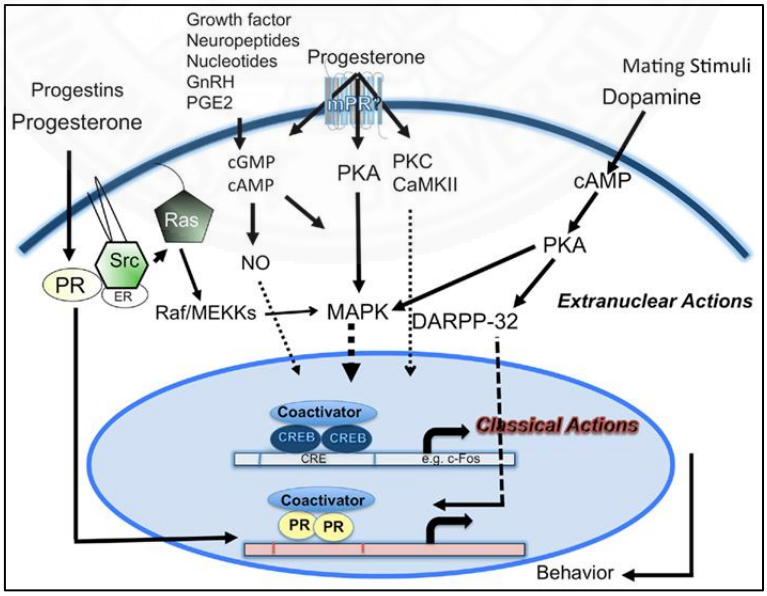


Figure 4.13 The regulatory action of progesterone is involving the mediated signal transduction pathways including cGMP-PKG signaling pathway. [from Mani SK and Oyola MG, 2012 (176)]

The involvement of PKG in endometrium was revealed by Cornwell TL et al, 2001(180) who studied the regulation of PKG in human uterine tissues and its role in the control of uterine and vascular smooth muscle contractility during the menstrual cycle. They found that PKG was localized within various sites of uterine compartments including endometrium. In endometrial stromal cell, PKG was found increasing in secretory phase. They suggested that hormonal system is one of the complex regulations of PKG expression within reproductive tract.

Moreover, in group of proteins identified unique in implantation group, this study selected to investigate the expression of ATPG and KATNA1 in endometrial secretion collected at D5 of implantation and non-implantation groups with immunoblotting. Western blot for total protein expression showed expression of ATPG and KATNA1 in both groups. These results confirmed the protein expression as the predicted results from LC-MS/MS.

4.5.3.2 Phosphoproteins and glycoproteins that represent for barrier of the endometrium receptivity

The 36 phosphoproteins and 2 glycoproteins, which were uniquely expressed in the non-implantation group, were classified into 6 groups according to their similarity of function.

(1) oxidoreductase activity protein

It was found in this study that some of identified proteins have function associated in stress response and oxidoreductase activity i.e. **Protein SCO1 homolog, mitochondrial isoform X1** and **Thioredoxin domain-containing protein 2.** These two proteins had function as cellular redox antioxidants which are essential in maintaining balance levels of reactive oxygen species (ROS) in the cell. As it is well known, ROS and antioxidant enzyme also has an important role in the regulation of endometrial function (135-138).

Protein SCO1 homolog, mitochondrial isoform X1 is essential for the assembly of the catalytic core of cytochrome c oxidase (COX) and has a role in cellular copper homeostasis, mitochondrial redox signaling or insertion of copper into the active site of COX (181-184).

Thioredoxin domain-containing protein 2 (TRX2) is a cellular redox-active disulfide/dithiol protein. It acts as coactivator of thioredoxin reductase, which is enzymatic antioxidant called a TRX system (185-190)**.** TRX2 is localized to the mitochondria (also known as mitochondrial thioredoxin) and regulates the mitochondrial redox environment, essential for the control of mitochondrial reactive oxygen species homeostasis, apoptosis regulation during accumulation of oxidants and cell viability. (191-195)

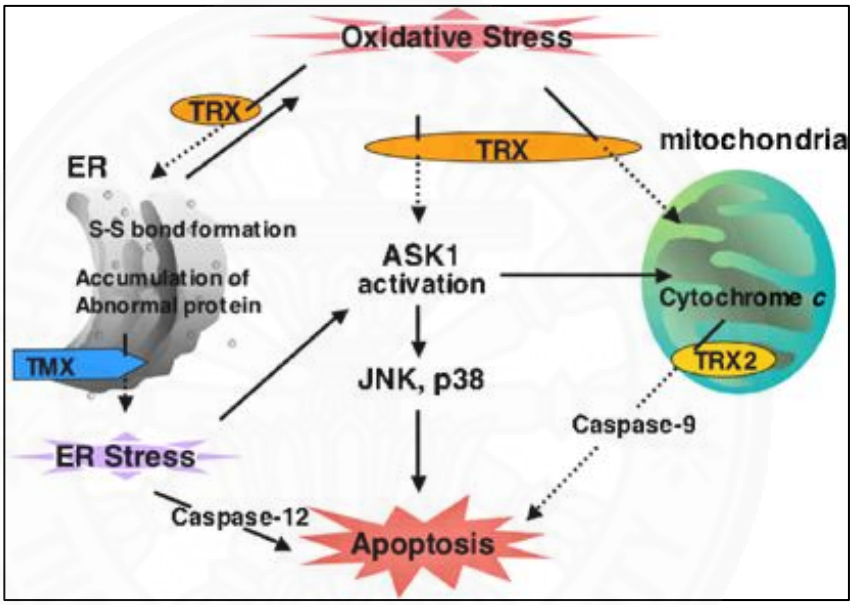


Figure 4.14 Thioredoxin2 (TRX2) localized to the mitochondria and regulates the mitochondrial redox, protecting cytochrome c release, and apoptosis. [from Masutani H et.al., 2005 (196)]

The association of human thioredoxin (hTRX) to the endometrium had been studied by Tetsuo Maruyama et al 1997 (197). They used immunohistochemically analysis in which hTRX was detected in the glands of the late proliferative endometrium. Subsequently, in the early secretory phase, hTRX was expressed strongly and became expressed moderately during the mid to late secretory phases. This result suggested that hTRX is induced in the glands of the endometrium under the influence of estrogen in the proliferative phase and might be accelerated under the cooperation of estrogen and progesterone in the early secretory phase.

(2) Degradation / apoptosis protein

This study identified **Ankyrin repeat and SOCS (suppressor of cytokine signaling) box protein 5 isoform X2 (Asb5)** and **Nuclear nucleic acid-binding protein C1D** which are proteins involved in apoptosis process. Apoptosis can be initiated via various stimuli such as oxidative stress, or DNA damage.

Ankyrin repeat and SOCS (suppressor of cytokine signaling) box protein 5 isoform X2 (Asb5) was involved in mediation of the ubiquitination and subsequent targeting them for proteasomal degradation (198-201).

Nuclear nucleic acid-binding protein C1D is the prototypical member of The C1D family of proteins. Its product induces apoptotic cell death when exceeding a critical level. It can induce apoptosis in a tumor protein 53 (p53/TP53) dependent manner. Biological process is involved in apoptotic process (202-208).

(3) Cell division regulation protein

It was found in this study that some of identified proteins involved in modulation of chromatin compaction for cell division i.e. **Condensin-2 complex subunit H2 isoform X1** and **Histone H1.4.**

Condensin-2 complex subunit H2 isoform X1 plays a role in chromosome assembly/segregation and exert compaction forces on chromatin in preparation for mitosis and meiosis. Regulatory subunit of the condensin-2 complex seems to provide chromosomes condensation and epigenetic regulation of gene transcription and in establishing mitotic chromosome architecture (209-212).

Histone H1.4 is the prototype of the linker histone, which is a protein family forming a critical component of eukaryotic chromatin (213-217). The H1 variants differ in a number of parameters, such as their chromatin binding affinity, evolutionary stability, and euchromatin/heterochromatin distribution, which might be a result of their post-translational modifications such as acetylation and phosphorylation (218-222).

(4) Calcium regulation protein

This study identified protein associated in calcium regulation, those were **Ryanodine receptor 2 (RyR2)** and **Voltage-dependent L-type calcium channel subunit alpha-1S isoform X1 (L-type VDCCs).**

Ryanodine receptor 2 (RyR2) are one type of intracellular calcium release channels (CRC) in the endoplasmic (ER) and sarcoplasmic reticula (SR). Ryanodine receptors mediate the release of calcium ions which is an essential step in muscle contraction and required for cellular calcium ion homeostasis (223, 224). The regulation of RyRs activity required phosphorylation /dephosphorylation processes (225).

Voltage-dependent L-type calcium channel subunit alpha-1S isoform X1 (L-type VDCCs) are a group of voltage-gated ion channels found in the membrane of excitable cells with a permeability to the calcium ion (226, 227). VDCC mediating of the mechanisms for calcium influx into the cells and involving in calcium-dependent processes i.e. muscle contraction, cell division, and gene expression (227, 228).

The role of RYR and VDCC involved in the implantation process had been revealed by Kusama K et al, 2015 (229). This study investigated the role of intracellular calcium mediated through cyclic AMP (cAMP) on decidualization of human endometrial stromal cells (ESCs) and gland maturation events. They found that inhibitors of Ca^{2+} influx through L-type voltage-dependent Ca^{2+} channel (VDCC), nifedipine and verapamil, enhanced the decidual gene expression. They also examined the effect of dantrolene, a blocker of the ryanodine receptor (RyR) that inhibit the release of Ca^{2+} from ER into cytoplasm, the result also showed enhanced decidual markers expression in ESCs.

(5) Defense / immunity protein

This study identified protein involved in immune responses i.e. **Immunoglobulin A heavy chain variable region, Immunoglobulin heavy chain variable region, T cell receptor beta chain, partial.** This study also found **Vascular cell adhesion protein 1 (**known as vascular cell adhesion molecule 1 / VCAM-1) which is functions as a cell adhesion molecule related in leukocyte migration during inflammatory diseases, including cytokines produced in the tissue, and reactive oxygen species (230-233)**.**

(6) Membrane trafficking and /or vesicle transport protein

It was found in this study that some of identified proteins have function as membrane trafficking and /or vesicle transport i.e. **ADP-ribosylation factor GTPase-activating protein 6** (ARF6) which is broadly known for their role in vesicular trafficking and are major regulators of vesicle biogenesis in intracellular traffic. The biological process is associated in ER-Golgi transport, protein transport, transportation. (234-240), **Rab11 family-interacting protein 5 isoform X4** which is involved in regulating endosomal trafficking and various aspects of membrane trafficking (241, 242), **Sorting nexin-1** which is involved in membrane trafficking and protein sorting (243-245), **Vacuolar protein sorting-associated protein 13** (VPS13) which may be involved in protein sorting in post golgi membrane traffic.

Moreover, in group of proteins identified unique in non-implantation group, this study selected one glycoprotein, Thioredoxin domain-containing protein 2 (thioredoxin2) to be investigated for the expression in endometrial secretion collected at D5 of implantation and non-implantation groups with immunoblotting. The results confirmed the total protein expression.

4.5.4 Validation of the LC-MS/MS results

Western blot analysis using the commercial antibodies against total proteins in sample collected at D5 compared between implantation and non-implantation group was performed in order to validate predicted protein expressions from LC-MS/MS data.

The results of western blot on both phosphoprotein and glycoprotein validation showed that there were total proteins expressed in samples of both implantation and non-implantation groups. These confirmed the results of protein expression by LC-MS/MS, however, there was not quite similar in pattern of expression in which LC-MS/MS showed of the identified proteins that uniquely expressed.

This study also analysed relative quantity of total protein expression to validate phosphoprotein. It was found, there was no significantly different in protein abundances between implantation and non-implantation groups. Based on the findings from this study, it is proposed that for the phosphoprotein validation, no correlation

between phosphopeptide mass spectrometry and western blot measured protein abundance for the selected phosphoproteins was observed.

For glycoprotein validation, there was significantly different in protein abundances between implantation and non-implantation groups by western blot analysis with similar trend to those of LC-MS/MS result. It might be estimated that there was positive correlation between glycopeptide mass spectrometry and western blot measured protein abundance for the selected glycoprotein.

In this study, antibodies used to detect the selected proteins cannot differentiate between phospho- or glyco-forms since specific antibodies are not available. Therefore, the results obtained from western blot analysis was detected at levels of total protein expression.

The results obtained both by western blot analysis in correlation with LC-MS/MS, it is proposed that there were the same parent proteins found in both implantation and non-implantation groups as measured by western blot analysis while apparent post-translational modification forms especially phosphorylated and glycosylated were differentially found in implantation and non-implantation groups as measured by LC-MS/MS.

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

These preliminary data apply the comprehensive phosphoproteomics and glycoproteomics study of endometrial secretion to evaluate of significance protein profiles. The investigations of human endometrial secretion, especially directed towards the functional relevance of proteins, may identify novel markers of endometrial receptivity and also obtain significant insights into the mechanisms of implantation during window period of implantation (WOI).

The analysis of endometrial receptivity using endometrial secretion has been of particular interest as they represent a non-invasive approach and the number of mediators contained may involve in the preparation for embryo and modulation of endometrial receptivity. Endometrial secretion collected during the time of embryo transfer in this study offers advantages over other techniques since it is already a part of ART clinical practice that can be carried out without affecting implantation rates. This non-invasive tool also can be used clinically.

This study suggested the number of phosphoproteins and glycoproteins identified at pre-receptive phase (D0) and receptive phase or window period of implantation (D5) of endometrium based on their sequential temporal expression with respect to the period of implantation window. The functional proteins identified are related to of those functions required in human endometrium preparation for implantation process during the early and mid-secretory phases of stimulated cycle. This study postulated that these functional proteins could be potential regulators in each phase of endometrium.

This study identified number of differentially phosphoproteins and glycoproteins expressed at D5 compared between implantation and non-implantation. It is suggested these phosphoproteins and glycoproteins might reflect important difference in the physiological functions between receptivity and non-receptivity of endometrium in stimulated cycle. Dynamic regulation of these molecules may also be considered to play an important role in endometrium receptivity during the events of implantation process.

The phosphoproteins and glycoproteins uniquely found in implantation group might represent activity for promotion of the endometrium receptivity and might be regarded as potential biomarkers for positive endometrium receptivity. These functional proteins might reflect important physiological status of endometrium with receptivity. It is also hypothesized that the other acquired characteristics of endometrium receptivity concerning with identified proteins are the followings; it was found in this study that protein regulating ATP production for active energy produced supporting endometrium activity, cytoskeleton protein with functions in endometrial remodeling, signaling association protein which conduct transduction of progesterone signal, and transcription regulation protein for active transcription functions.

Whereas phosphoproteins and glycoproteins uniquely found in non-implantation group might represent barrier activities of the endometrium receptivity and might be regarded as potential biomarkers for negative endometrium receptivity. These functional proteins might reflect important physiological functions of endometrium with non-receptivity. It is also hypothesized that the other acquired characteristics of endometrium with non-receptivity concerning with identified proteins are the followings; protein relating oxidoreductase activity for balancing intracellular oxidative stress and ROS in the endometrium, degradation or apoptosis regulation proteins for activation of apoptosis process, protein involved in modulation of chromatin compaction for cell division and active proliferation, calcium regulation protein which conduct muscle contraction, defensive /immunity protein with defense function for endometrium, and membrane trafficking protein for active vesicle transports.

Regarding the LC-MS/MS results in relation to western blot analysis which implicated that it is not the difference in total protein express, but the difference in the actual phosphorylation and glycosylation are subjected to differential regulation which is needed to pinpoint. It is suggested that study of biomarkers in endometrium related to implantation process may not be the difference observed by total proteomics study, but the direct and unique functional proteomics should be considered to identify novel markers and also provide more significant insights into the mechanisms of implantation during WOI.

This study showed that a panel of proteins, rather than a single biomarker, improved sensitivity and specificity values, of the ability to discriminate between pre-receptive and receptive phase or window period of implantation (WOI) of endometrium and also in implantation and non-implantation. A subset of the identified phosphoproteins and glycoproteins may have important roles on implantation process as a predictive indicator of the endometrium receptivity for better clinical outcome in ART patients. Although this study had only identified a limited number of proteins, but these proteins may have important function in the initiation and maintenance of the window of receptivity.

The findings and significant propose after discussion;

1. Protein found in pre-receptive and receptive phases (window period of implantation; WOI) of endometrium

- Pre-receptive phase: 267 phosphoproteins and 105 glycoproteins were found at D0 of which 47 phosphoproteins were uniquely found.
- Receptive phase or WOI: 220 phosphoproteins and 110 glycoproteins were found at D5 of which 5 glycoproteins were uniquely found.

2. Biomarkers for receptivity

- Promotion for implantation: 4 phosphoproteins and 1 glycoprotein were uniquely found in implantation.
- Barrier for implantation: 36 phosphoproteins and 2 glycoproteins were uniquely found in non-implantation

3. This study proposed that regulation of endometrium during implantation is exerted at post-translational levels.

The limitation of this study was the endometrial secretion collected during WOI, using non-invasive clinical routine procedure elicited the sample containing very low abundance of proteins. Therefore, it is quite difficult for analysis since generally more protein concentration is required and then more demanding processes are needed to perform analysis, especially analysis of phosphoproteins and glycoproteins included in this study. Furthermore, antiphospho- and antiglyco-specific antibodies for western blot analysis are not always available.

Further study is needed in the larger population or by way of working with other reproductive samples. Other confirmatory methods will be needed to validate these findings. Tests in the other biological fluids would be more easily and relatively non-invasive to collect sample i.e. plasma or urine preferred in clinical applications are also required. The dynamic continuous analysis starting from pre-receptive to receptive period (time series) is certainly required for further study. To correlate the unique phosphoproteins and glycoproteins found in this study with other approaches in assessing the endometrium receptivity e.g. histological, ultrasound, pinopods are of interest for future research, and certainly, other PTMs study, such as ubiquitination, acetylation, etc. are also interested.

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APPENDICES

APPENDIX A
LIST OF MATERIALS AND REAGENTS

Assisted reproductive technology treatment

Gonal-F®, Cetrotide®, and Ovidrel® were purchased from Merck (Serono, Italy). The routine embryo transfer procedure was carried out with Guardia™ Access Embryo Transfer Catheter purchased from Cook Medical (Cook Medical, USA).

Protein Quantitative: Lowry assay

Bovine serum albumin (BSA), CTC, Folin-Ciocalteu phenol reagent, Na_2CO_3, NaOH, SDS were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Phosphoprotein Enrichment

Phosphoproteins were collected using Pierce™ Phosphoprotein Enrichment Kit purchased from Thermo Fisher Scientific Pierce Biotechnology, USA.

Glycoprotein Enrichment

The enrichment of N-linked glycan was performed using affinity chromatography on immobilized lectin columns concanavalin A (ConA) affinity kit purchased from Thermo Fisher Scientific Pierce Biotechnology, USA.

Sample desalted

The eluted fractions were desalted using Zeba™ Spin Desalting Columns, 7K MWCO, 0.5 mL purchased from Thermo Fisher Scientific Pierce Biotechnology, United States.

Protein concentrated

Nanosep® Centrifugal Devices purchased from Pall Corporation, Life sciences, USA was used for protein concentrated.

Sample digested

Dithiothreitol (DTT) and iodoacetamide (IAA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trypsin was purchased from Promega Corp. (Madison, WI, USA)

Immunoblot

Polyacrylamide SDS-gel for protein separation was prepared using TGX FastCast™ acrylamide Solutions purchased from Bio-Rad Laboratories, Inc. (California, USA). TEMED was purchased from USB Corporation (Cleveland, OH USA). Ammonium Persulfate (APS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phosphoprotein Blocker (Blok^{TM}-PO) was purchased from EMD Millipore Corporation. (MA, USA). PageRuler™ Prestained Protein Ladder (Product #26616) was purchased from Thermo Scientific (USA).

APPENDIX B
REAGENTS PREPARATION

Lowry protein assay reagent

Reagent A

Copper-tartrate-carbonate (CTC)	5	ml
20% Sodium carbonate ($Na_2CO_{3)}$	5	ml
0.8N Sodium hydroxide (NaOH)	10	ml
5% Sodium dodecyl sulfate (SDS)	20	ml
Total volume	40	ml

Reagent B

Folin-Ciocalteu phenol reagent	1	ml
Sterile water	5	ml
Total volume	6	ml

Stock reagent

1. Stock solution CTC 100 ml
   - Copper Sulfate Heptahydrate ($CuSO_4.7H_2O$) 0.2 g
   - Tatalic acid 0.4 g
   - Dissolve in sterile water, adjust volume to 100 ml
2. 0.8N Sodium hydroxide (NaOH) 50 ml
   - NaOH 1.6 g
   - Dissolve in sterile water, adjust volume to 50 ml
3. 5% Sodium dodecyl sulfate (SDS) 50 ml
   - SDS 2.5 g
   - Dissolve in sterile water, adjust volume to 50 ml
4. 20% Sodium carbonate ($Na_2CO_{3)}$ 50 ml
   - Na_2CO_3 10 g
   - Dissolve in sterile water, adjust volume to 50 ml

0.1 M Phosphate buffer solution (1x PBS), pH 7.4

Sodium chloride (NaCl)	8	g
Potassium chloride (KCl)	0.2	g
Disodium hydrogen phosphate (Na_2HPO_4)	1.44	g
Potassium dihydrogen phosphate (KH_2PO_4)	0.24	g
Adjust pH to 7.4 with Hydrogen chloride (HCl)		
Dissolve in sterile water, adjust volume to 1,000 ml		

0.1% (v/v) Tween-20 in PBS (PBS-T)

Tween-20	1	ml
1x PBS	10	ml

Antibody dilution in 1:1,000 in PBS-T

Antibody	1	ul
Dilute with PBS-T	1,000	ml

10X SDS-PAGE electrophoresis buffer

Tris-base 25 mM	30	g
Glycine 192 mM	144	g
SDS 0.1 %	10	g
Adjust pH to 8.8		
Adjust volume to 1,000 ml		

20 mM Ammonium bicarbonate (FW 79.06)

Ammonium bicarbonate	79.6	mg
Dissolve in sterile milli Q water		
Adjust volume to 50 ml		

10 mM Ammonium bicarbonate

20 mM Ammonium bicarbonate	25	ml
Sterile milli Q water	25	ml

10 mM Dithiothreitol / 10 mM Ammonium bicarbonate (Fresh prepare)

Dithiothreitol (FW 154.25)	7.7125	mg
Dissolve in 10 mM Ammonium bicarbonate		
Adjust volume to 5 ml		

100 mM Iodoacetamide / 10 mM Ammonium bicarbonate (Fresh prepare)

Iodoacetamide (FW 184)	92.0	mg
Dissolve in 10 mM Ammonium bicarbonate		
Adjust volume to 5 ml		

50% acetronitrite / 10 mM Ammonium bicarbonate

100% acetronitrite	5	ml
20 mM Ammonium bicarbonate	5	ml

10 ng Trypsin in 50% acetronitrite / 10 mM Ammonium bicarbonate

50% acetronitrite / 10 mM Ammonium bicarbonate	2	ml
Add to 20 ng Trypsin		

0.1% (v/v) Formic acid in acetonitrile

100% formic acid (FA)	1	ml
Dilute to a final volume of 1000 mL with acetonitrile		

APPENDIX C

LIST OF 267 PHOSPHOPROTEINS IN ENDOMETRIAL SECRETION

List of 267 phosphoproteins in endometrial secretion and fold changed from D0 to D5.

Accession No.	Uniprotkb ID	Peptide sequence	Protein description	Fold changed
gi|27227743	P02751	TRAGGXGAGRK PPK	fibronectin isoform X21	13.43
gi|323430534	-	INPNSGGTNYTQ K	immunoglobulin variable region	12.58
gi|578828656	P07911	INFACSYPLDMK	PREDICTED: uromodulin isoform X5	11.08
gi|33188463	P82933	SAQAGAIRLAM AK	28S ribosomal protein S9, mitochondrial	10.28
gi|358439847	P49959	NPDSTKNTK	MRE11 meiotic recombination 11 isoform CRA_b	10.27
gi|37182159	Q9Y4P3	YLATCADDR	transducin beta-like protein 2 isoform X1	9.35
gi|3738222	Q14201	YGEKNNAFIVAS FENK	protein BTG3 isoform X1	9.04
gi|119574302	-	GVIDLFTADSLK	chromosome 16 open reading frame 45, isoform CRA_b	8.97
gi|119620215	-	SWAPLLTHCVPN	hCG1988952	8.71
gi|554793148	O95292	CVFEL	Vesicle-associated membrane protein-associated protein B/C	8.29
gi|27574164	P60568	MATSXSTK	interleukin 2 (N-terminal) [human, Peptide Recombinant Partial, 23 aa]	8.02
gi|119584155	-	MALLPAGSPILK	hCG1814615	7.77
gi|7717245	Q9NSJ0	GIPVRK	Fibroblast growth factor	7.72
gi|578832772	Q9Y5B0	QPSMSETMPLYT LCK	PREDICTED: RNA polymerase II subunit A C-terminal domain phosphatase isoform X2	7.71

Accession No.	Uniprotkb ID	Peptide sequence	Protein description	Fold changed
gi|7020294	Q9NZM3	MSNTKLLK	unnamed protein product	7.45
gi|225903424	Q93075	SASRSGGPSSPKRLK	putative deoxyribonuclease TATDN2	7.07
gi|62087868	Q59GG5	QFPPTVSLPLK	galactose-3-O-sulfotransferase 4 variant	6.56
gi|7328135	Q9UPS8	EEFDK	ankyrin repeat domain-containing protein 26 isoform X9	6.12
gi|82407601	P01850	YFGPGTRLTVT	T-cell receptor beta chain	5.92
gi|578835748	Q5JPB2	ALEPGR	Zinc finger protein 831 isoform X5	5.87
gi|530426557	-	ATKEVATR	PREDICTED: RNA-binding motif protein, Y chromosome, family 1 member F/J isoform X2	5.72
gi|119612746	-	GNMKGF	hCG37408, isoform CRA_a	5.72
gi|22027482	Q99742	MKSTLTKRGLHVK	PREDICTED: neuronal PAS domain-containing protein 1 isoform X1	5.59
gi|54607141	Q5THJ4	LLAESLPR	vacuolar protein sorting-associated protein 13D isoform 2	5.48
gi|33945247	P08700	MAPMTQTTSLK	interleukin-3	5.45
gi|578804834	Q8WZ42	LSDTSTLIGDAVELR	PREDICTED: titin isoform X6	5.38
gi|432134244	Q5JUK3	LSRKAPK	PREDICTED: potassium channel subfamily T member 1 isoform X2	5.32
gi|384081587	Q6ZRS4	MPTTASCKTISLLK	coiled-coil domain-containing protein 129 isoform 3	5.27
gi|119615992	Q9P1M5	MQKSSSTR	hCG1979952	5.19
gi|395759238	Q04206	TYETFXSIMKKS	Transcription factor p65	4.98

Accession No.	Uniprotkb ID	Peptide sequence	Protein description	Fold changed
gi|119594950	-	QLLASSDPSLSVSQR	hCG2016942, isoform CRA_f	4.89
gi|151101337	Q96MW1	LEALGVDR	coiled-coil domain-containing protein 43 isoform 1	4.81
gi|119573402	D3DVA5	QTFSFLSGMTGK	rho/rac guanine nucleotide exchange factor (GEF) 2, isoform CRA_a	4.75
gi|224549005	Q96I25	QSTVLAPVIDLK	splicing factor 45	4.70
gi|40042588	-	SVVARF	unnamed protein product	4.65
gi|221316632	P53618	RNAFMMLIHADQDR	coatomer subunit beta	4.63
gi|28207899	Q86TV6	HSPMM	tetratricopeptide repeat domain 7B, isoform CRA_f	4.57
gi|530370661	Q9P242	SASTSGVPPPSVTPLR	neuronal tyrosine-phosphorylated phosphoinositide-3-kinase adapter 2 isoform X1	4.50
gi|27900256	-	KIXKS	unnamed protein product, partial	4.41
gi|119604061	-	GLSEGEGSSVSLQR	hypoxia-inducible protein 2, isoform CRA_b	4.19
gi|103471995	Q8NEF9	EQAPKTR	serum response factor-binding protein 1	4.10
gi|578820677	Q9NPQ8	EHEAMKLVTMFDK	PREDICTED: synembryn-A isoform X2	3.94
gi|22208850	Q96CN9	QRDRALAVLTEK	GRIP and coiled-coil domain-containing protein 1	3.83
gi|21264361	O00187	RTCSEQSL	mannan-binding lectin serine protease 2 isoform 2 precursor	3.65
gi|33187645	Q7Z4S9	NGTADAASK	PREDICTED: SH2 domain-containing protein 6 isoform X1	3.64
gi|40045874	-	YTIGLGQAKMGF	unnamed protein product	3.63
gi|449061960	D6RJB6	NPAQAPK	ubiquitin carboxyl-terminal hydrolase 17-like protein 20	3.63

Accession No.	Uniprotkb ID	Peptide sequence	Protein description	Fold changed
gi|194390424	P26641	ARFASTFFLSPQIYAHK	Elongation factor 1-gamma	3.63
gi|119829187	Q9UMD9	LLKGSRSASVSPTR	collagen alpha-1(XVII) chain	3.62
gi|32187309	Q7Z5Z8	VDPGLSPSR	NPC-A-7	3.58
gi|1658310	Q93033	SLESSLQVSLMSR	immunoglobulin superfamily member 2 isoform 2 precursor	3.52
gi|312283732	Q9H633	AGPVKDR	ribonuclease P protein subunit p21 isoform 3	3.50
gi|119628678	A0A024RDN1	KDNRVK	intraflagellar transport 88 homolog (Chlamydomonas), isoform CRA_d	3.44
gi|226729541	A6NK59	GKEDALSHLTK	PREDICTED: ankyrin repeat and SOCS box protein 14 isoform X3	3.21
gi|34529488	Q6AWC8	AVLTPFLTK	Putative uncharacterized protein LOC100129027	3.18
gi|578816581	Q5VZ89	AGLIFR	PREDICTED: DENN domain-containing protein 4C isoform X2	3.14
gi|27894373	Q13901	NASKVANKGK	nuclear nucleic acid-binding protein C1D	3.10
gi|127139033	P16435	DGKEVKASDRLTMK	NADPH--cytochrome P450 reductase	3.06
gi|194394141	O60287	GPRGRK	nucleolar pre-ribosomal-associated protein 1	2.90
gi|119576260	A0A024R034	NRAHLLR	hCG1990740, isoform CRA_b	2.89
gi|530382158	A0A024QZT4	METLSNASGTFAIR	serpin B9 isoform X1	2.87
gi|119612495	-	KKVTEEPALK	hCG1645224	2.82
gi|578832545	O94885	TVQMMFKEAKFK	PREDICTED: serpin B8 isoform X1	2.77
gi|21757449	Q5XXA6	TAMAGVKLTDKVK	PREDICTED: anoctamin-1 isoform X12	2.73

Accession No.	Uniprotkb ID	Peptide sequence	Protein description	Fold changed
gi|119623995	A0A024RCX2	KLRLHGTSRLASK	proline-rich transmembrane protein 1, isoform CRA_a	2.61
gi|530370275	P28330	LDSATACMAK	PREDICTED: long-chain specific acyl-CoA dehydrogenase, mitochondrial isoform X1	2.55
gi|25140577	Q8IXB1	SGMAAVK	DnaJ homolog subfamily C member 10	2.53
gi|119626347	Q6UWZ7	MSSPETDEEIEKMK	BRCA1-A complex subunit Abraxas isoform X3	2.45
gi|10436783	O94885	AGGSVESLR	PREDICTED: SAM and SH3 domain-containing protein 1 isoform X3	2.43
gi|119629662	-	EGAATLTRKAASGSR	hCG2040419	2.41
gi|40789025	-	QTLGRSTSLTEK	KIAA1029 protein	2.41
gi|558479645	V5LKZ7	FDSDAASPKREPR	MHC class I antigen, partial	2.35
gi|41152097	Q02040	LSGFSDILK	A-kinase anchor protein 17A isoform 1	2.32
gi|333609271	Q96MY7	KKSQAMSKSVTLR	protein FAM161B	2.30
gi|2462488	O43847	WAQFFIHPLMIR	PREDICTED: nardilysin isoform X1	2.21
gi|4156155	O75140	TWRSSATGDEKFADR	DEP domain-containing protein 5 isoform X10	2.20
gi|530419242	Q9ULJ3	EAGPSK	PREDICTED: zinc finger and BTB domain-containing protein 21 isoform X3	2.19
gi|183248	P10071	EAQSHSSTTTEKK	transcriptional activator GLI3 isoform X2	2.16
gi|257467482	Q9UKV8	QCTEVHLK	protein argonaute-2 isoform 2	1.95
gi|40981272	-	LLPLASAITSQVDK	unnamed protein product	1.80
gi|578805044	Q96K49	TFRIAK	PREDICTED: transmembrane protein 87B isoform X2	1.75

Accession No.	Uniprotkb ID	Peptide sequence	Protein description	Fold changed
gi|21595246	P28749	LQDVVSERANH	PREDICTED: retinoblastoma-like protein 1 isoform X1	1.73
gi|578807466	Q9Y2J4	ASSKR	PREDICTED: angiomotin-like protein 2 isoform X4	1.68
gi|10438571	Q9H6I0	AGASVR	cDNA: FLJ22257 fis, clone HRC02873	1.62
gi|40041174	-	TSAVSNTK	unnamed protein product	1.61
gi|974994791	O75164	MTLISPLMLK	PREDICTED: lysine-specific demethylase 4A isoform X3	1.48
gi|45709401	Q9Y2H2	GSYPAAPRGAGS GGLDR	Phosphatidylinositide phosphatase SAC2	1.42
gi|578814520	Q96JN2	MSLESYGK	PREDICTED: coiled-coil domain-containing protein 136 isoform X9	1.39
gi|10438270	Q63HN8	RSLETNGEINLPR	E3 ubiquitin-protein ligase RNF213	0.90
gi|32187323	Q7Z5Z2	SSTQKK	SHUJUN-2	0.79
gi|119571032	-	MMQLEIIDQVV MSYR	hCG1980421, isoform CRA_a	0.70
gi|151301215	O95785	GLSSHARAHLR	PREDICTED: protein Wiz isoform X10	0.57
gi|578836079	Q8IWU5	SIRSVAIEVDGR	extracellular sulfatase Sulf-2	0.53
gi|34304354	Q96KR7	TSSVER	phosphatase and actin regulator 3 isoform 2	0.52
gi|133777217	-	AAMDSR	Chromosome 19 open reading frame 34	0.40
gi|190394	Q01212	SDASHGSSGSRS ASRQTR	profilaggrin, partial	0.39
gi|58530840	P15924	LQSLTENLTK	desmoplakin isoform I	0.38
gi|578837679	-	SLPPLASGGNGR KSR	PREDICTED: uncharacterized protein LOC102724378	0.20
gi|530420998	Q8IZT9	EHVCR	PREDICTED: protein FAM9C isoform X4	0.20

Accession No.	Uniprotkb ID	Peptide sequence	Protein description	Fold changed
gi|35493853	Q9HC10	MTDTQDGPSESSQIMR	otoferlin isoform c	0.16
gi|306873	P17096	KQPPVSPGTALVGSQK	high mobility group protein HMG-I/HMG-Y isoform X3	0.06
gi|578800790	Q8NB59	GTAKSKDEK	PREDICTED: synaptotagmin-14 isoform X3	0.06
gi|444299651	Q8NG31	TNLEHTTGQLTTMNRQIAVK	Kinetochore scaffold 1	-16.67
gi|62177112	Q9BW62	MQDGASDGEMPK	katanin p60 ATPase-containing subunit A-like 1 isoform X2	-16.59
gi|1477982	-	ETGGDAEAFFGQG	TCR V beta 2-J beta 1.1	-16.28
gi|4406696	Q7Z5A7	AGAAASMAPSPR	Protein FAM19A5	-15.64
gi|159162550	P11940	AKESKVGPASSVK	Polyadenylate-binding protein 1	-15.43
gi|34534881	Q9Y6X6	TTKSPRILKHK	Unconventional myosin-XVI	-15.42
gi|7661878	Q15058	KTADMPLTPNPVGR	kinesin-like protein KIF14	-15.01
gi|13654237	P78527	IPALDLLIK	DNA-dependent protein kinase catalytic subunit isoform 1	-14.91
gi|353252824	-	HNRYYFDSWGQGTLVTVS	anti-tetanus toxoid immunoglobulin heavy chain variable region	-14.85
gi|23397546	A0A0S2Z662	SALTILQSLSQPEAVSMK	DNA helicase MCM9 isoform 2	-14.64
gi|379318422	P10412	QSIHLTQHLR	Histone H1.4	-14.61
gi|444738525	L8EAG2	ATPAAAPVTVTR	alternative protein LAMA5	-14.59
gi|58531795	Q8IZF6	KVSDTPPIVITKSXK	Adhesion G-protein coupled receptor G4	-14.47
gi|530404745	-	CHPGSQSPSQSVVT	PREDICTED: synaptotagmin-16 isoform X2	-14.36
gi|387765984	P42858	MATLEKLMKAFESLKSFX	Huntingtin	-14.34

Accession No.	Uniprotkb ID	Peptide sequence	Protein description	Fold changed
gi|40976394	-	KALNXACKSHG HEG	unnamed protein product	-14.23
gi|578824395	-	GIKSSIGRLFGK	liprin-alpha-2 isoform X16	-14.16
gi|13236587	Q9BTV4	AANYSSTSTR	transmembrane protein 43	-14.12
gi|47678501	Q6IC83	AQLMQYLSLPK	Uncharacterized protein C22orf42	-14.08
gi|578812787	P35372	DHPSTANTVDR	PREDICTED: mu-type opioid receptor isoform X1	-14.02
gi|62087514	Q59GZ2	RMFVFKITTTK	PLEK protein variant	-13.98
gi|530398983	B0AZV0	LWQTVVGKTYG LWK	serine-threonine kinase receptor-associated protein isoform X2	-13.96
gi|48145973	Q6IB76	LGIKVGESTPDK	NDUFV2	-13.93
gi|530406470	Q6ZTF9	SHMIKKLYK	unnamed protein product	-13.82
gi|386642465	Q13126	GTVTTFKVSWS K	methylthioadenosine phosphorylase	-13.79
gi|40981144	-	LSLQQLSGK	unnamed protein product	-13.77
gi|3328006	-	LTVXGQPKAAPS VS	immunoglobulin light chain variable region	-13.51
gi|115511036	Q96L96	VEQFPDASGSLK	alpha-protein kinase 3	-13.36
gi|1710030	P29374	RKILGQSSPEK	AT-rich interactive domain-containing protein 4A isoform X2	-13.35
gi|119631287	D3DPI2	VSGNTSQR	hCG1641229, isoform CRA_a	-13.31
gi|307776317	Q96EP1	GAMVRR	E3 ubiquitin-protein ligase CHFR	-13.28
gi|10432807	Q9NXG0	KAAELSVKEYK	Centlein	-13.26
gi|530406332	Q13596	MGSMK	PREDICTED: sorting nexin-1 isoform X1	-13.17

Accession No.	Uniprotkb ID	Peptide sequence	Protein description	Fold changed
gi|530365359	B1ALM3	QYFMSIFNR	voltage-dependent L-type calcium channel subunit alpha-1S isoform X1	-13.17
gi|530378014	-	RHVGVISVEGK	PREDICTED: kelch-like protein 8 isoform X3	-13.11
gi|380036026	A0A087WXV5	SWPSAHSVSNILGIR	paired box protein Pax-1 isoform 2	-13.11
gi|76879773	Q8NI08	QNAETATAVATR	Nuclear receptor coactivator 7	-13.07
gi|70798135	P01764	KSYVDSMEGR	immunoglobulin heavy chain variable region	-12.62
gi|974994783	O15118	APLTDK	Niemann-Pick C1 protein isoform X4	-12.60
gi|50345988	P36542	SEVATLTAAGK	ATP synthase subunit gamma, mitochondrial isoform L (liver) precursor	-12.57
gi|2827693	Q6IBW4	RNVELFIATSQK	condensin-2 complex subunit H2 isoform X1	-12.56
gi|119612811	Q6ZUT6	KKNQALLRR	Uncharacterized protein C15orf52	-12.52
gi|78101060	Q14145	XTSGRSGVGVAVT	Kelch-like ECH-associated protein 1	-12.48
gi|40045182	-	QMGRSSEGR	unnamed protein product	-12.48
gi|18676526	Q5H9F3	KGSQAGAEGQPSTVKR	BCL-6 corepressor-like protein 1 isoform X5	-12.43
gi|119623450	-	STPWLLMSR	hCG2045715	-12.41
gi|145611434	B2C6G3	QGAKEKQLLK	abhydrolase domain-containing protein 8	-12.37
gi|409973767	Q15750	PVSVPYXSAQSTS	TGF-beta-activated kinase 1 and MAP3K7-binding protein 1	-12.34
gi|157829353	F4MHK7	SXRCSNTSTLAAR	ubiquitously transcribed tetratricopeptide repeat protein Y-linked transcript variant 96	-12.34
gi|40042354	-	AXMVSMISLR	unnamed protein product	-12.34
gi|28591	-	KVPEVSTPTLVEVSR	unnamed protein product	-12.30

Accession No.	Uniprotkb ID	Peptide sequence	Protein description	Fold changed
gi|32307132	Q53FP3	GVARFYRSRK	NFS1 nitrogen fixation 1 isoform a variant	-12.17
gi|157676101	A8E194	HSIPLTINKEGEKLR	bcr-abl1 fusion protein	-12.15
gi|54633315	Q5QJE6	KDSKIVPGNEK	deoxynucleotidyltransferase terminal-interacting protein 2	-12.02
gi|40976002	-	TRLQSPQGFXK	unnamed protein product	-11.92
gi|578815847	Q7Z7G8	SEDLGTVQEK	PREDICTED: vacuolar protein sorting-associated protein 13B isoform X4	-11.77
gi|530406899	A1L4K1	KVVQAEMANESR	PREDICTED: fibronectin type III and SPRY domain-containing protein 2 isoform X6	-11.60
gi|119612815	-	KGTTIITATNH	chromosome 15 open reading frame 23, isoform CRA_a	-11.56
gi|71981383	Q26261	ASSQLVVSIFK	Netrin receptor unc-5	-11.46
gi|40039202	-	WSYLSRXPSC	unnamed protein product	-11.38
gi|3088340	P60866	KGPVRMPTKTLR	40S ribosomal protein S20	-11.36
gi|119605818	-	EELTP	interleukin 32, isoform CRA_d, partial	-11.21
gi|119576538	-	RGRTDDADAQMTWTHR	hCG2045069	-11.01
gi|578809883	O15018	DSQVPVTSSVVPEAKASR	PREDICTED: PDZ domain-containing protein 2 isoform X9	-10.98
gi|578804836	Q8WZ42	KMASSSGEGK	PREDICTED: titin isoform X7	-10.94
gi|578832877	A6NN14	AFSQFSTLKK	PREDICTED: zinc finger protein 729 isoform X1	-10.84
gi|578811679	-	QENHSSLVSLGGEIQTKSR	zinc finger protein with KRAB and SCAN domains 4 isoform X8	-10.60
gi|4758412	A0A1L7NY50	SGGLSVEVCGPALSQQWK	polypeptide N-acetylgalactosaminyltransferase 2 precursor	-10.43

Accession No.	Uniprotkb ID	Peptide sequence	Protein description	Fold changed
gi|578802818	Q9BXF6	EKYSTSVVEK	PREDICTED: rab11 family-interacting protein 5 isoform X4	-10.40
gi|578834848	P52742	KARXSAGAAKR	zinc finger protein 135 isoform X3	-10.21
gi|2341018	Q15465	ASSGSGPPSGGALGPR	Sonic hedgehog gene	-10.14
gi|530410547	O75880	KGEIAASIATHMRPYR	Protein SCO1 homolog, mitochondrial isoform X1	-10.04
gi|119605087	Q9H0B3	MTLQGRADLSGNQGNAAGR	KIAA1683	-10.03
gi|116805332	O60682	RPRVAGGGGAGGSAGGGGK	musculin	-10.01
gi|519673618	-	GTTVSVSS	immunoglobulin A heavy chain variable region, partial	-9.60
gi|51094548	A4D2F6	NNSTILSHAKMSR	similar to Splicing factor, arginine/serine-rich, 46kD	-9.57
gi|400153449	Q7Z340	SYLGSTSMR	zinc finger protein 551 isoform 2	-9.36
gi|530381903	-	GVLSPADKTNVK	unnamed protein product	-9.30
gi|11137057	P01834	SAMSASVGDRVTITCR	immunoglobulin kappa chain	-9.26
gi|55963361	Q96NU0	QSSVGAAQGTGVR	contactin associated protein-like 3B	-8.99
gi|285026441	Q96DE0	GLSHPLPGEILSR	U8 snoRNA-decapping enzyme isoform 1	-8.95
gi|24474080	-	ELVLTQPPSASGTPGQR	immunoglobulin lambda light chain variable region	-8.50
gi|62088884	Q59F09	LLSAVDILGEK	Werner syndrome protein variant	-8.29
gi|85544427	O43615	ILDIDNVDLAXGK	Mitochondrial import inner membrane translocase subunit TIM44	-8.09
gi|530438309	-	ENLVQR	PREDICTED: ankyrin repeat domain-containing protein 34A-like	-8.08

Accession No.	Uniprotkb ID	Peptide sequence	Protein description	Fold changed
gi|193785148	-	RARSAPTAVAAATASDMDK	unnamed protein product	-8.05
gi|130980075	A8K8P1	AGVILNR	centromere protein J	-7.97
gi|10433312	-	MSADIAVEMNCIK	unnamed protein product	-7.95
gi|444738643	L8ECD7	AAVPGGR	alternative protein TCHH	-7.80
gi|119623240	-	GEVVVVVQHTSR	chromosome X open reading frame 34, isoform CRA_d	-7.46
gi|578838225	Q9ULL8	TSGQEATESAK	PREDICTED: protein Shroom4 isoform X2	-7.37
gi|47027976	Q6PL43	SSGPGCLPAAPLSGTLK	hypothetical protein	-7.33
gi|2654717	-	CASSPFSSR	T cell receptor beta chain, partial	-7.33
gi|578827434	Q8N5R6	STSEEKNNQSSK	PREDICTED: coiled-coil domain-containing protein 33 isoform X3	-7.33
gi|546231677	Q96Q89	NSSVKK	kinesin-like protein KIF20B isoform 1	-7.21
gi|578820863	P62330	QAEESMVASMR	PREDICTED: ADP-ribosylation factor GTPase-activating protein 6 isoform X4	-6.94
gi|578827491	Q9Y4G6	MTKGITMATAK	PREDICTED: talin-2 isoform X15	-6.73
gi|530389407	Q96DN5	QSTDLGNKESGK	PREDICTED: TBC1 domain family member 31 isoform X3	-6.58
gi|260064072	Q04743	FQGKSMVSEPK	homeobox protein EMX2 isoform 2	-6.23
gi|37930915	-	ASSSPTTRAPTATMALHR	unknown	-6.17
gi|578819622	L8J3J7	RNSVDSDLKSTR	PREDICTED: protein FAM178A isoform X2	-6.06
gi|15141739	-	LVYLPRSR	unnamed protein product	-6.05
gi|987255	Q04656	AIVAVSPGLYR	Copper-transporting ATPase 1	-6.02

Accession No.	Uniprotkb ID	Peptide sequence	Protein description	Fold changed
gi|119629296	-	KVLGVPSAGQRK	hCG2020493	-5.93
gi|578800054	Q96AE4	VPDGMVGFIIGR	PREDICTED: far upstream element-binding protein 1 isoform X3	-5.55
gi|530381236	Q9H013	KVINTPEILR	disintegrin and metalloproteinase domain-containing protein 19	-5.40
gi|578806615	-	AKVTSSF	PREDICTED: uncharacterized protein C3orf20 isoform X4	-5.39
gi|297206791	Q5CZC0	MAKSTKIISSIVSRR	fibrous sheath-interacting protein 2	-5.29
gi|34527855	Q9P2P6	QIDQSSSDQTR	PREDICTED: stAR-related lipid transfer protein 9 isoform X1	-5.25
gi|40039690	-	EDNSXYIMR	unnamed protein product	-5.24
gi|40976344	-	TAMPRAGGT	unnamed protein product	-5.10
gi|315434271	P19320	KANMKGSYSLVEAQK	vascular cell adhesion protein 1 isoform c precursor	-5.05
gi|578842365	P48634	AGSSGSSSGGGGGGPGGR	PREDICTED: protein PRRC2A isoform X2	-4.97
gi|507043259	Q6P2Q9	QAPVH	Pre-mRNA-processing-splicing factor 8	-4.96
gi|2245352	P81133	STKATSTTMAGDGR	Single-minded homolog 1	-4.82
gi|1526978	Q92736	HVTTGK	ryanodine receptor 2 isoform X9	-4.77
gi|62087970	Q59GB4	ANITSLG	dihydropyrimidinase-like 2 variant	-4.73
gi|530376698	O94823	KSSQTPKLSGR	PREDICTED: probable phospholipid-transporting ATPase VD isoform X3	-4.72
gi|119627837	Q8NCS4	KVVRPTR	Transmembrane protein 35B	-4.71
gi|578832879	O14796	TGEEACSDLFSQVSR	PREDICTED: SH2 domain-containing protein 3A isoform X10	-4.59

Accession No.	Uniprotkb ID	Peptide sequence	Protein description	Fold changed
gi|530414965	Q8NFU1	LYRRTLMR	Bestrophin-2 isoform X1	-4.50
gi|114794264	P30101	FVXQEEFSRDGK	Protein disulfide-isomerase A3	-4.41
gi|119583787		STEPSHSR	unnamed protein product	-4.14
gi|578805879	Q8N335	AAAPLK	PREDICTED: glycerol-3-phosphate dehydrogenase 1-like protein isoform X3	-4.11
gi|18027768	Q8WYW0	TRRPGRLVSSCIK	Putative uncharacterized protein pp13842	-4.01
gi|50513360	O75146	EXAATSAAIEDAVR	Huntingtin-interacting protein 1-related protein	-3.85
gi|578811307	Q9H7N4	AGDGGPGR	PREDICTED: splicing factor, arginine/serine-rich 19-like	-3.79
gi|544709756	Q4KMX1	MPGFHLITQLK	LRRTM4 protein	-3.74
gi|119628898	A0A024RDQ1	SPCSSCR	furry homolog (Drosophila), isoform CRA_c	-3.35
gi|578811844	Q92766	QEITEGELK	PREDICTED: ras-responsive element-binding protein 1 isoform X11	-3.00
gi|147712797	A5JTV3	GEEMNSAVFR	mutS homolog 2 protein	-2.82
gi|530398781	P48065	KMAVTIPVITSAFK	PREDICTED: sodium- and chloride-dependent betaine transporter isoform X4	-2.82
gi|578826332		LTGLMQSS	regulator of G-protein signaling 6 isoform X13	-2.66
gi|530416869		HSGLYACSVR	PREDICTED: pregnancy-specific beta-1-glycoprotein 4 isoform X5	-2.64
gi|578809988	P13671	TSNPYR	PREDICTED: complement component C6 isoform X4	-2.60
gi|578806774	Q9H981	KTAISLFEGK	PREDICTED: actin-related protein 8 isoform X2	-2.57
gi|119587297	-	MSSTLNRCSGK	hCG1810906, isoform CRA_b	-2.57

Accession No.	Uniprotkb ID	Peptide sequence	Protein description	Fold changed
gi|578804412	P23760	TTLAGAVPR	PREDICTED: paired box protein Pax-3 isoform X1	-2.40
gi|578840387	O75478	QGGLRLAQARALIKIDVNK	PREDICTED: transcriptional adapter 2-alpha isoform X5	-2.29
gi|6808314	Q96Q27	GKGERSPSLSSTFPQGRR	ankyrin repeat and SOCS box protein 5 isoform X2	-2.16
gi|530403277	Q9UKV3	KISVVSTK	PREDICTED: apoptotic chromatin condensation inducer in the nucleus isoform X1	-2.01
gi|39777586	Q6P158	RISAISVAERVAK	putative ATP-dependent RNA helicase DHX57	-1.78
gi|119571711	-	EGSIGK	hCG1646049, isoform CRA_b	-1.75
gi|530425322	Q969V3	TVQRLLVKAKTQ	PREDICTED: nicalin isoform X1	-1.69
gi|28799780	-	NGPGPTK	unnamed protein product	-1.67
gi|38195091	Q86SR1	SSLNCK	polypeptide N-acetylgalactosaminyltransferase 10	-1.65
gi|578809984	O75962	SADAGSQK	PREDICTED: triple functional domain protein isoform X2	-1.45
gi|5453908	Q00169	QKDPVKGMTADD	phosphatidylinositol transfer protein alpha isoform	-1.39
gi|119619044	Q5QGS0	KKINSGSQGATK	KIAA2022 protein	-1.32
gi|34527290	-	ASVEGR	unnamed protein product	-1.30
gi|190610018	Q8N9U0	LPSDGK	tandem C2 domains nuclear protein isoform 1	-1.25
gi|5454028	P10301	MSSGAASGTGR	ras-related protein R-Ras precursor	-1.21
gi|40043700	-	MAXPGVSVR	unnamed protein product	-0.98
gi|40041404	-	MSSTSPXLQK	unnamed protein product	-0.80
gi|15419021	P01602	KGGSSRSSS	5-kappa immunoglobulin light chain variable region	-0.76

Accession No.	Uniprotkb ID	Peptide sequence	Protein description	Fold changed
gi|7661742	Q9UNZ5	APAKKKGAAAATSSK	leydig cell tumor 10 kDa protein homolog	-0.75
gi|332356381	-	KVPQVSTPTLVEVSR	unnamed protein product	-0.74
gi|13161129	Q9BZ98	MPIPVAAYVSCLSELSACLSR	Putative transcript Y 1 protein	-0.73
gi|282721018	Q9UPP2	KGLSRQMIGEFLGNSK	IQ motif and SEC7 domain-containing protein 3 isoform 1	-0.71
gi| 2495717	Q12766	KPTGADLLTPGSRAPELKGR	PREDICTED: HMG domain-containing protein 3 isoform X3	-0.68
gi|400153758	Q7Z4H7	LSETSRMETFSPAVGNR	HAUS augmin-like complex subunit 6 isoform 2	-0.67
gi|119618706	-	WAGGGAVCSTSR	hCG2016674	-0.46
gi|115344353	Q08EK0	LCEGEAQK	ZNF766 protein	-0.41
gi|119568650	-	QETADSLDNKK	hCG34808, isoform CRA_b	-0.18
gi|4557509	Q14093	AAEIGK	cylicin-2	-0.17

APPENDIX D
LIST OF 110 GLYCOPROTEINS IN ENDOMETRIAL SECRETION

List of 110 glycoproteins in endometrial secretion and fold changed from D0 to D5.

Accession No.	Uniprotkb ID	Peptide sequence	Protein description	Fold changed
gi|5453543	Q04828	ALEATK	aldo-keto reductase family 1 member C1	18.07
gi|30268178	Q70EL2	NASVGGK	Ubiquitin carboxyl-terminal hydrolase 45	18.07
gi|14249190	Q14781	GSLGDGK	Chromobox protein homolog 2	15.17
gi|23396771	P82970	KGEDGK	High mobility group nucleosome-binding domain-containing protein 5	15.17
gi|578826735	O94986	EGAEK	Centrosomal protein of 152 kDa	13.21
gi|112363655	Q8N608	IGKPEIK	Inactive dipeptidyl peptidase 10	12.01
gi|74731224	Q96BD5	TLPLVLK	PHD finger protein 21A	12.01
gi|530393066	Q6P4A7	IAGTK	Sideroflexin-4	11.85
gi|116283869	Q05BJ6	AGEEQAKFENQLKTK	CEP290 protein	11.27
gi|40040732	-	HVXLVR	unnamed protein product	10.59
gi|1052244769	P39900	GIQSLYG	Macrophage metalloelastase	10.59
gi|23943912	Q99570	LCVFFGR	phosphoinositide 3-kinase regulatory subunit 4	9.17
gi|57015374	P49750	FSAPPSR	YLP motif-containing protein 1	6.95
gi|56675643	-	NGGIX	unnamed protein product	5.95
gi|152013050	Q9UPT6	QLSPNGGQEDTRMK	C-Jun-amino-terminal kinase-interacting protein 3	5.31

Accession No.	Uniprotkb ID	Peptide sequence	Protein description	Fold changed
gi|157836012	P46952	YYVGDTXDVLFEK	Human 3-hydroxyanthranilate 3,4-dioxygenase	5.31
gi|205277354	Q7Z407	VNYKEIEK	CUB and sushi domain-containing protein 3 isoform 3	5.21
gi|51094654	A4D255	VFQQKLEK	hypothetical protein MGC26484	5.21
gi|21619816	Q8N532	LMFPF	TUBA1C protein	5.14
gi|32528301	P54760	IVARENGGASHPLLDQR	Ephrin type-B receptor 4	5.11
gi|767918496	-	MLSMDYVVAFQILIR	PREDICTED: PTB domain-containing engulfment adapter protein 1 isoform X4	5.11
gi|384872704	Q8WZ42	KIESTSSLR	Titin	4.66
gi|92091575	Q2TAC2	SQQDIERYK	Coiled-coil domain-containing protein 57 isoform X4	4.06
gi|3462896	O75628	MTLNTEQEAK	Ras-like GTP-binding protein REM	4.06
gi|327200636	Q9NVV4	RFSEXQNER	Human Mitochondrial Poly(A) Polymerase (Papd1)	3.78
gi|124376920	A2RUF6	RNFKLLDTR	DENN/MADD domain containing 2A	3.78
gi|119617471	Q86YJ5	CHILDLIK	E3 ubiquitin-protein ligase MARCH9	3.71
gi|4754909	Q9UQL6	QHEVQLQK	Histone deacetylase 5	3.71
gi|296080706	Q5JY77	AMTKEEAK	G-protein coupled receptor-associated sorting protein 1	3.34
gi|10835242	Q13976	AYEDAEAK	cGMP-dependent protein kinase 1 isoform 2	3.17
gi|22770673	A3R0T8	APKSPAKAK	histone H1	3.17
gi|557878647	P61960	MIRAFPTTTPR	ubiquitin-fold modifier 1 isoform 3	2.95
gi|145611434	B2C6G3	QGAKEKQLLK	abhydrolase domain-containing protein 8	2.64

Accession No.	Uniprotkb ID	Peptide sequence	Protein description	Fold changed
gi|578831203	-	QQEQKQLLK	PREDICTED: trinucleotide repeat-containing gene 6C protein isoform X7	2.41
gi|915392	P49327	ASGRTPEAVQK	fatty acid synthase	0.92
gi|373251213	Q9BU02	AQGLIEVERK	thiamine-triphosphatase isoform 3	0.92
gi|323431585	-	AIQXLAAV	immunoglobulin variable region, partial	0.85
gi|71051535	Q05D60	GMMGDLDPGEY MSMDFTNR	Deuterosome assembly protein 1	0.79
gi|167887738	Q14139	LQVAWRDAQQS SSPAADNLR	ubiquitin conjugation factor E4 A	0.79
gi|110349740	Q9H063	SHEFSR	repressor of RNA polymerase III transcription MAF1 homolog	0.42
gi|16552596	-	STSIVGAK	unnamed protein product	0.42
gi|18845003	P52823	AFVKESLK	stanniocalcin 1	0.40
gi|119592218	-	MEAMEKLK	protein (peptidylprolyl cis/trans isomerase) NIMA-interacting, 4 (parvulin)	0.39
gi|296439467	Q96MD2	LQTWGQIFEKQR	UPF0536 protein C12orf66	0.26
gi|29292348	Q9H0V9	HLTIMMDIDGK	VIP36-like protein	0.03
gi|520261838	Q6ZNB6	VTVPCGR	NF-X1-type zinc finger protein NFXL1	0.00
gi|119591573	-	MRAAPSR	unnamed protein product	0.00
gi|119608295	-	QRLSAVALQISE NVVR	hCG2021757	-18.55
gi|119571632	-	SSEPVQADEPQS PRVR	hCG2040319, partial	-18.55
gi|148727319	Q86VQ3	KEEKVDELCGA LK	thioredoxin domain-containing protein 2 isoform 2	-14.93
gi|119567993	-	QLDMM	unnamed protein product	-7.25

Accession No.	Uniprotkb ID	Peptide sequence	Protein description	Fold changed
gi|28971817	Q8TDM6	AANEEMEALRQIK	disks large homolog 5	-5.98
gi|10190742	Q9HBJ7	MPLFMSKSPTHVK	ubiquitin carboxyl-terminal hydrolase 29	-5.98
gi|119599937	-	FQHFRRPR	hCG2038570	-5.95
gi|50369183	Q9H7P9	AETRASANAPR	Pleckstrin homology domain-containing family G member 2	-5.95
gi|119623343	D3DX33	EMAGAGT	hCG1999854, isoform CRA_a	-4.82
gi|62702352	A0A024R419	MDTNR	KIAA0971, isoform CRA_b	-4.82
gi|194318456	B5A942	GDLTIANLGTSEGRFMQVSAF	soluble MET variant 14	-4.14
gi|353254276	P01764	DNAKSSLYLQMNGLR	immunoglobulin heavy chain variable region	-3.82
gi|732549710	-	DNATNSLFLQMNSLR	immunoglobulin heavy chain variable region, partial	-3.82
gi|2879784	P98177	DKGDSNSSAGWKNSIR	AFX1	-3.22
gi|62913995	Q86UT6	DLLLHDQCQITTLR	NLR family member X1	-3.22
gi|66346698	P54802	RPSLQMNTSIWYNR	Alpha-N-acetylglucosaminidase isoform X2	-2.98
gi|29387351	Q9Y2X7	VPGQPR	GIT1 protein	-2.93
gi|5080758	A0A024R0P7	KAFTLLTK	BC331191_1	-2.47
gi|21749758	-	ASGFGKKKT	unnamed protein product	-2.47
gi|12652961	Q9Y2R9	YVRELKKTQLIK	Mitochondrial ribosomal protein S7	-2.42
gi|317373380	Q8NG48	KNLFPYNPTALLK	Protein Lines homolog 1	-2.42
gi|85397653	Q2M3V2	TAGGR	Ankyrin repeat domain-containing protein SOWAHA	-2.16

Accession No.	Uniprotkb ID	Peptide sequence	Protein description	Fold changed
gi|355332859	P11215	DMMXEGGPPGA EPQ	Integrin Alpha-M Beta-2 Cytoplasmic Tails	-2.07
gi|379317829	H9E7Y3	ADANTAAIQAIL YXR	NADH dehydrogenase subunit 5 (mitochondrion)	-2.01
gi|460417522	P18827	TKQEEFXA	Syndecan1 Peptide	-1.97
gi|194473657	Q8WZ79	FLHFAK	deoxyribonuclease-2-beta isoform 1 precursor	-1.97
gi|5514674	Q9UBC0	MSALRLA	Hepatocyte nuclear factor 6	-1.97
gi|46249807	Q6NUK0	SALSLMLPMGIG DR	PRDM4 protein	-1.91
gi|62089356	Q59EC6	CLSGTANRRERR	ret finger protein isoform beta variant, partial	-1.90
gi|38707981	Q6ZSI9	GQSYEAIR	calpain-12	-1.75
gi|223633999	Q8WUY9	FIIHNVYSVSK	DEP domain-containing protein 1B isoform 1	-1.70
gi|332356380	F6KPG5	YLYEIAR	albumin	-1.69
gi|197304907	P16333	AAANSS	Cytoplasmic protein NCK1	-1.68
gi|46249998	Q8IXI2	GGPRRPKRLAG GAVGWVLLVR	Mitochondrial Rho GTPase 1	-1.61
gi|767995752	-	HLTIKCPPSPR	PREDICTED: formin-like protein 1 isoform X7	-1.57
gi|62739186	P08603	KDQYKVGEVLK	Complement factor H	-1.57
gi|61680421	Q9NZL4	AXQQQVQKLK	Hspbp1 Core Domain	-1.56
gi|149192855	Q5JSZ5	SSDTLAMDMR	protein PRRC2B	-1.56
gi|226958663	Q5VU65	EGAAFVSSR	nuclear pore membrane glycoprotein 210-like isoform 1 precursor	-1.50
gi|29888	P05109	KAMKKATK	Protein S100-A8	-1.50

Accession No.	Uniprotkb ID	Peptide sequence	Protein description	Fold changed
gi|119603310	A0A024R6T8	ELKEYRNNLK	NEFA-interacting nuclear protein NIP30, isoform CRA_a	-1.43
gi|172045901	Q6F5E8	ARTLRHLGLAGCKLPPDALR	Capping protein, Arp2/3 and myosin-I linker protein 2	-1.40
gi|254588216	-	CDPTDISTGHLK	immunoglobulin heavy chain variable region, partial	-1.40
gi|1765864	Q99487	FQTMESVNLMK	platelet-activating factor acetylhydrolase 2	-1.40
gi|21707816	Q8N4G2	KLTGFVKSHMKSR	ADP-ribosylation factor-like 14	-1.39
gi|116242497	Q8IUC8	NMTLGT	Polypeptide N-acetylgalactosaminyltransferase 13	-1.35
gi|40788866	Q6P0Q8	HRDLALVPDELLK	Microtubule-associated serine/threonine-protein kinase 2	-1.22
gi|194319979	Q9BY84	MIGTQIVTER	The Human Dual Specificity Phosphatase 16	-1.19
gi|29544726	Q9P104	DGEAIYQK	Docking protein 5	-1.18
gi|20380982	Q8N165	SMNGRMKQLIK	Serine/threonine-protein kinase PDIK1L	-1.01
gi|119581065	-	MENAEINNIIK	topoisomerase (DNA) II alpha 170kDa, isoform CRA_a, partial	-0.99
gi|58761506	Q9BT25	MSEGGR	HAUS augmin-like complex subunit 8 isoform b	-0.59
gi|221043086	B7Z6D4	AAPEHRSGITAFMK	cDNA FLJ50665, highly similar to Zinc finger protein 406	-0.49
gi|119622471	Q9BRJ9	LGSGQRQSASEREK	mesoderm posterior protein 1	-0.49
gi|37181905	Q9H4B8	XWSEEELQGVLR	Dipeptidase 3	-0.40
gi|54696644	P22692	STSGGKMKVNEAPR	insulin-like growth factor binding protein 4	-0.40
gi|456929		HLYPGEVCPGMDIRNNLT	insulin receptor alpha subunit (246) [human, placenta, Peptide Partial, 18 aa]	-0.37

Accession No.	Uniprotkb ID	Peptide sequence	Protein description	Fold changed
gi|62088458	Q59FM1	DLHILPPPLIPTPPPDDPR	sorting nexin 15 isoform B variant	-0.37
gi|239740376	P51168	EGATMHVKKYLLK	Amiloride-sensitive sodium channel subunit beta isoform X1	-0.36
gi|158258385	-	NQEFEINKDGIPK	unnamed protein product	-0.36
gi|193787585	-	DKMFLIGKLIK	unnamed protein product	-0.29
gi|442564354	L8E853	HIVTFDGQNFK	von Willebrand factor	-0.29
gi|83267877	Q99666	KAVPGNLAK	RANBP2-like and GRIP domain-containing protein 5/6 isoform 1	-0.06

BIOGRAPHY

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