Identification of Protein Candidates in Porcine Oocytes during *In Vitro* Maturation

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ABSTRACT

Surface-enhanced laser desorption and ionization time-of-flight mass spectrometry (SELDI-TOF MS) is one of the recently developed proteomic technologies which is based on capturing proteins and peptides by chemically modified surfaces and highly sensitive for the analysis of complex biological samples. In the present study, to gain insights into oocyte maturation and early embryo development, SELDI-TOF-MS was used to find the protein candidates that are specifically or prominently expressed in porcine oocytes at the *in vitro* matured metaphase II (MII) and germinal vesicle (GV) stages. By selected CM10 chip, 16 candidates were found to be up-regulated in GV stage oocytes compared with in MII stage oocytes, their molecular weights were 8,180 (2 candidates), 10,226 (5 candidates), 15,767 (5 candidates) and 16,770 (4 candidates) Da respectively. And the expression of 29 candidates were higher in MII than in GV stage oocytes, their molecular weight were 10,832 (3 candidates), 17,743 (8 candidates), 20,122 (3 candidates), 22,131 (3 candidates), 24,857 (7 candidates) and 33,507 (5 candidates) Da, respectively. The expression of selected 13 candidates (0.2 and 1.0 % error tolerances) were analyzed using real time RT-PCR. The proteins that differentially regulated during oocyte *in vitro* maturation in the pigs may be potential biomarkers of oocyte maturation and quality.

(Key words : Pig oocyte, *In vitro* maturation, SELDI-TOF, Protein candidate)

INTRODUCTION

Oocyte maturation is an important process that prepares the egg for fertilization by spermatozoa. During oogenesis, the mammalian oocyte enters the prophase of the first meiotic division and then progresses to the diplotene stage of prophase I, which is defined as the germinal vesicle (GV) stage. During the following resumption of first meiosis, oocyte chromatin starts to condense and germinal vesicle breakdown (GVBD) is initiated, then the metaphase I spindle is organized and the first polar body is extruded. Immediately thereafter, the oocytes enter meiosis II and are arrested again at the metaphase II (MII) stage.

Differential display (DD)-PCR and microarray studies on the mammalian oocyte or preimplantation embryo are providing researchers with a large quantity of information regarding gene expression and the changing transcriptome. However, transcription profile just one aspect of the complex gene regulatory network that allows cells to respond to intracellular and extracellular signals. It has been estimated that every transcribed gene produces at least three translated proteins, and further variation derives from posttranslational modifications and protein-protein interactions (Gygi *et al.*, 1999). Furthermore, analysis of gene expression does not readily reflect protein abundance (Gygi *et al.*, 1999) or supply information about protein function at the biochemical level as each protein participating in numerous distinct biochemical pathways.

The classic proteomics has not been successfully applied in the field of embryology (Latham *et al.*, 1992; Shi *et al.*, 1994). This approach is straight forward but labor intensive and requires large amounts of starting material. In addition, the technique lacks robustness; proteins with low or high molecular masses or high or low isoelectric points can be underrepresented and difficult to distinguish. Consequently, protein-based research in embryology has concentrated on identifying and localizing individual proteins by Western blot analysis (Wang *et al.*, 2005).

Surface-enhanced laser desorption-ionisation time of flight mass spectrometry (SELDI-TOF MS), an alternative proteomic technology based on capturing proteins and peptides by chemically modified surfaces, is highly sensitive for the analysis of complex biological samples (Siebert *et al.*, 2004; Xiao *et al.*, 2005). Recently, Katz-

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Jaffe *et al.*, (2006) reported candidate proteins in human blastocyst using SELDI-TOF MS, and they have found several protein candidates significantly upregulated in degenerating embryos compared to expanded blastocysts.

Compared to xenopus, little is known about mammalian oocyte maturation. In the present study, we use SELDI-TOF MS to identify molecules that are differentially expressed during porcine oocyte *in vitro* maturation. Furthermore, we used real-time RT-PCR analysis of the oocyte at different stages of maturation to characterize more potential candidate proteins that are differentially expressed during *in vitro* maturation.

MATERIALS AND METHODS

Generation of Embryos

Prepubertal porcine ovaries were collected from a local slaughterhouse and transported to the laboratory at 25℃ in Dulbecco's phosphate-buffered saline (PBS) supplemented with 75 mg/ml penicillin G and 50 mg/ml streptomycin sulfate. Cumulus-oocyte complexes (COCs) were aspirated from follicles 3~6 mm in diameter with an 18-gauge needle into a disposable 10 ml syringe. The COCs were washed three times with Hepes-buffered Tyrodes medium containing 0.1% (w/v) polyvinyl alcohol (Hepes-TL-PVA). For harvesting GV stage oocye, cumulus cells were removed from the COCs by pipetting in the presence of 1 mg/ml hyaluronidase for 2~3 min, and for MII stage oocye, each group of 50 COCs were matured in 500 ml tissue culture medium (TCM)-199 supplemented with 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 0.57 mM cysteine, 10 ng/ml epidermal growth factor (EGF), 10 IU/ml PMSG, 10 IU/ml hCG, 75 mg/ml penicillin G, and 50 mg/ml streptomycin sulfate under paraffin oil at 39℃ for 44 hr. Following maturation, cumulus cells were removed using the same method with GV stage oocyte samples. Harvested GV or MII stage oocytes were snap frozen in liquid nitrogen for 100 or 50 oocytes in each group and stored at −70℃ until mRNA or protein extraction.

Extraction of Protein

Proteins from 100 zona-intact GV oocytes and MII arrested eggs were extracted in Celis lysis buffer (containing 2% (v:v) NP-40, 9.8 M urea, 100 mM dithiothreitol (DTT), 2% ampholines (pH 3.5~10), and protease inhibitors] for 30 min at room temperature as previously described (Wright *et al.*, 2003).

Protein Profiling using SELDI-TOF-MS

To determine the best condition for the discrimination between immature and mature oocyte, various experimental conditions (normal phase ProteinChip Array (NP20 chip) and 3 distilled water (3DW); hydrophobic ProteinChip array (H4 chip) and binding buffer containing 20% acetonitrile/0.1% trifluoroacetic acid; ProteinChip Array for weak cation exchange (CM10 chip) and binding buffer containing 50 mM sodium acetate, pH 4.0; ProteinChip Array for strong anion exchange (SAX10 chip) and binding buffer containing 50 mM Tris-HCl, pH 8.0) were tested. The concentration of all samples (GV oocyte: n=4*100 (4 replicates with 100 oocytes per replicate), MII oocyte: n=4*100) was adjusted to the lowest concentration (0.18 ug/ul by diluting with in water. Each four (n=4) from sample groups were analyzed on four ProteinChip array and resulted in the selection of weak cation exchange ProteinChip Array (CM10 ProteinChip Array) as the most effective ProteinChip Array.

The reminder (GV oocyte: n=10*100 (10 replicates with 100 oocytes per replicate), MII oocyte: n=10*100) was analyzed using CM10 Chip. Each spot on the arrray was incubated with 5 ul binding buffer in a humidity chamber at room temperature for 5 min (twice). 5 ul sample, which was diluted 1:3 with binding buffer, was added to each spot and incubated in a humidity chamber with shaking for 40 min. It was washed with 5 ul binding buffer for 2 min (three times), followed by washing with 5 ul of 3DW (twice). Each air-dried spot was treated with 1 ul of sinapinic acid (SPA) solution saturated in 0.5% trifluoreacetic acid (TFA) and 50% acetonitrile (ACN), and was air-dried for 5 min (twice).

The arrays containing ionized samples were analyzed by ProteinChip Reader (model PBS IIc). The mass spectra of proteins were generated by averaging 100 laser shots with a laser intensity of 190 and a detector sensitivity of 8. Before beginning data collection, the instrument was externally calibrated by All-in 1 protein standard (Ciphergen Biosystems) in accordance with ciphergen protocol. According to the manufacturer, the mass accuracy is within 0.1%.

Data and Statistical Analysis

The data were analyzed with the ProteinChip software 3.1 (Ciphergen Biosystems). The peak intensities were normalized by using the total ion current of m/z between 1,500 and 150,000 Da. To characterize protein peak of potential interest, peaks of similar molecular weight from each sample group were clustered, and then the mean and standard deviation of each sample group were reported, compared, and visualized by Biomarker Wizard.

Real-time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Frozen-thawed GV and MII stage oocytes were used to determine the expression of 14 genes during oocyte maturation. The mRNAs from three sets of 50 oocytes/embryos was extracted by using the Dynabeads mRNA Direct Kit (Dynal Asa, Oslo, Norway) according to the manufacturer's instructions and converted into cDNA by reverse transcription of the RNA by using the Oligo $(dT)_{12\sim18}$ primer and the Superscript reverse transcriptase enzyme (Invitrogen Co., Grand Island, NY). Real time RT-PCR was then performed using the 14 primer sets shown in Table 1. The threshold cycle (Ct) value represents the cycle number at which sample fluorescence is statistically higher than background. The reactions were conducted according to the protocol of the DyNAmo SYBR Green qPCR kit that contains modified Tbr DNA polymerase, SYBR Green, optimized PCR buffer, 5 mM $MgCl_2$, and a dNTP mix that includes d-UTP (Finnzyme, Espoo, Finland). The PCR protocol included a denaturation program (95℃ for 10 min), an amplification and quantification program repeated 40 times (95℃ for 15 sec, 55/60℃ for 15 sec, 72℃ for 15 sec with a single fluorescence measurement), a melting curve program (65~95℃, with a heating rate of 0.2℃ per second and continuous fluorescence measurement), and finally a cooling step (4℃). Fluorescence data were acquired after the extension step during PCR reactions that contained SYBR Green. The PCR products were then analyzed by generating a melting curve. Since the melting curve of a product is sequence-specific, it can be used to distinguish between non-specific and specific PCR products. To use the mathematical model, it was necessary to determine the crossing points (CP) for each transcript. The crossing point is defined as the point at which the fluorescence rises appreciably above the background fluorescence. The relative quantification of gene expression was analyzed by the 2-ddCt method (Livak & Schmittgen, 2001). In all experiments, histone H2a (*H2a*) mRNA served as an internal standard (Cui *et al.*, 2006, 2007a). The sizes of the real time RT-PCR products were confirmed by gel electrophoresis on a standard ethidium bromide-stained 2% agarose gel and visualization upon exposure to ultraviolet light.

Table 1. Primer sequences and conditions used for real time RT-PCR

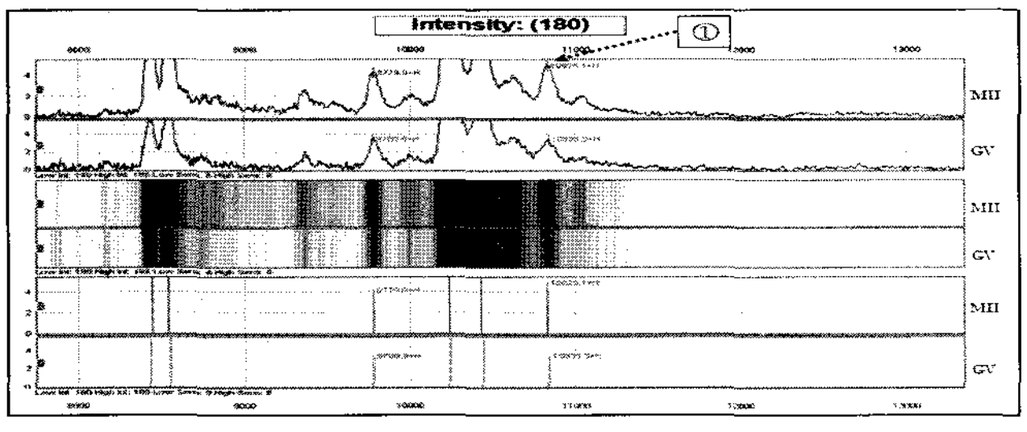
Genes	Primer sequence	Annealing temperature	Base pairs (bp)
Atpk	F: tgctggaggtcaaattaggg R: gcgccatcttcagtggtatt	60℃	242
Rs23	F: aacagcccaaattctgccatc R: atgacctttgcgaccaaatc	55℃	152
Rl32	F: gcccaagatcgtcaaaaaga R: taaccaatgttgggcatcaa	55℃	153
Myl6	F: tggtaaatgctgatggcaaa R: gagatccggcatgttcttgt	60℃	250
Ap4a	F: cctgagagcatgtggtttga R: gcatctatgcctgcttcctc	60℃	195
Pp14a	F: ctggacgtggagaagtggat R: agttctctgtggcgttcgtg	60℃	172
Rs15	F: ctgaaccgaggtctgaggag R: tctccacctggttgaaggtc	55℃	187
Nu4lm	F: tgcccttgcagggttactta R: tcggtaccgtatgtgttgga	55℃	222
Ppia	F: agcactggggagaaaggatt R: aaaactgggaaccgtttgtg	60℃	220
Ppac	F: ccggcagattaccaaagaact R: gtcggagtcgttgccataat	60℃	190
Csk2b	F: atgtccgaaaggccgatg R: atcgcccagatgttggaa	60℃	101
Atp6	F: ctattcccaacacccaaacg R: tgggtgtgaatgagtgtggt	55℃	196
Ucjl1	F: gattaacccggagatgctga R: tgccaatggtctgcttcata	55℃	242
H2a	F: acaacaagaagacccgcatc R: cttggccttgtggtgactct	60℃	167

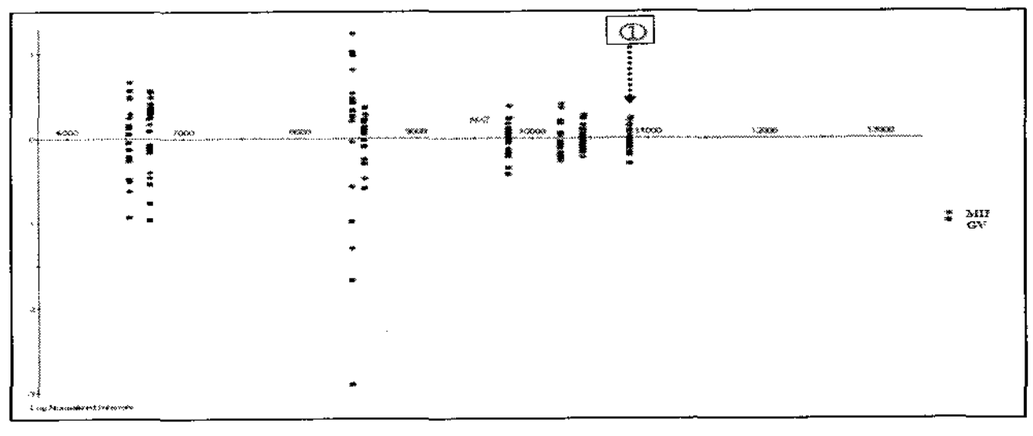
RESULTS

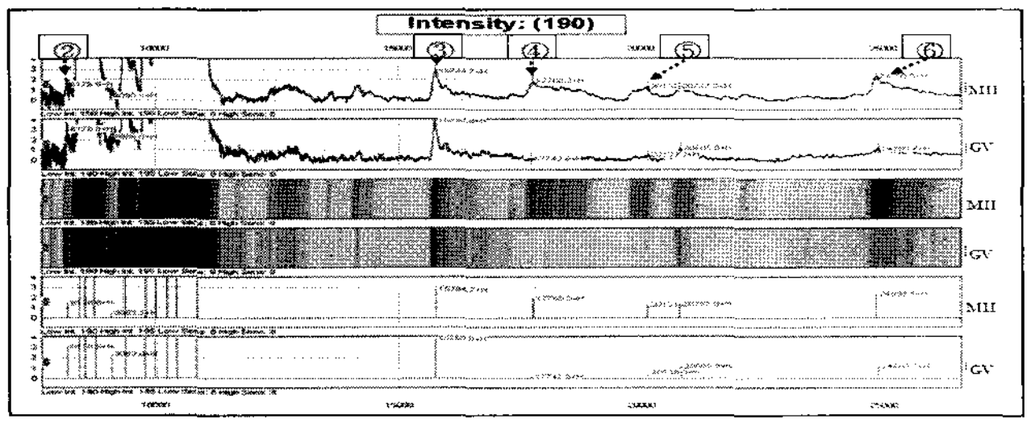
SELDI-TOP-MS Results from GV and MII Stage Oocytes

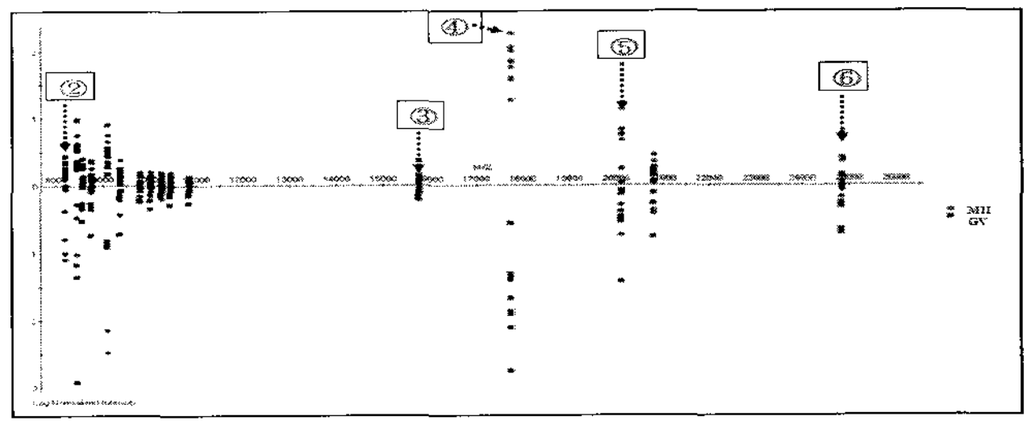
To identify proteins that are specifically or predominantly expressed in MII oocytes, we compared the protein expression profile of porcine GV and MII stage oocytes. The total proteins from both types of oocytes (n=1,400 for both) were extracted and subjected to SELDI-TOF-MS analysis as described above. Fig. 1 shows a gel view of the data enhanced around the 7,000~40,000 Da range for two different stages of oocytes. Scatter plot and box plot of normalized peak intensity for highly expressed proteins in MII or in GV stage oocyte were displayed in Fig. 2 and 3 respectively.

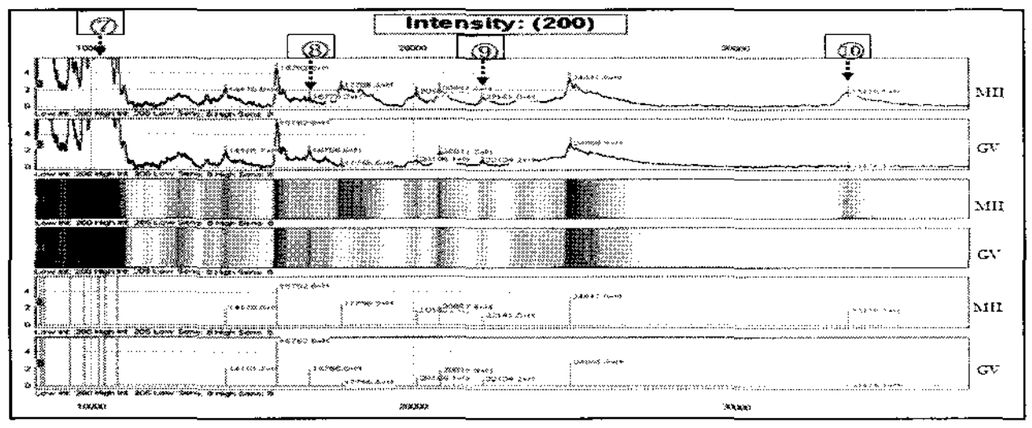
After normalization of the data to total ion current, statistical analysis was performed to determine the significant difference in proteins expression related to oocyte mature situation (immature or mature) on this protein chip type. Comparison of GV stage with MII stage oocytes revealed that 29 protein candidates were significantly highly expressed in MII stage oocyte, at approximately 10,831.79 (①), 17,743.78 (④), 20,122.1 (⑤), 24,857.48 (⑥), 22,131.43 (⑨) and 33,507.95 (⑩) Da, and 16 protein candidates were highly expressed in GV stage oocytes, at approximately 8,175.574 (②), 15,767.13 (③), 10,226.24 (⑦) and 16,770.37 (⑧) Da (Table 2, Fig. 1~3).











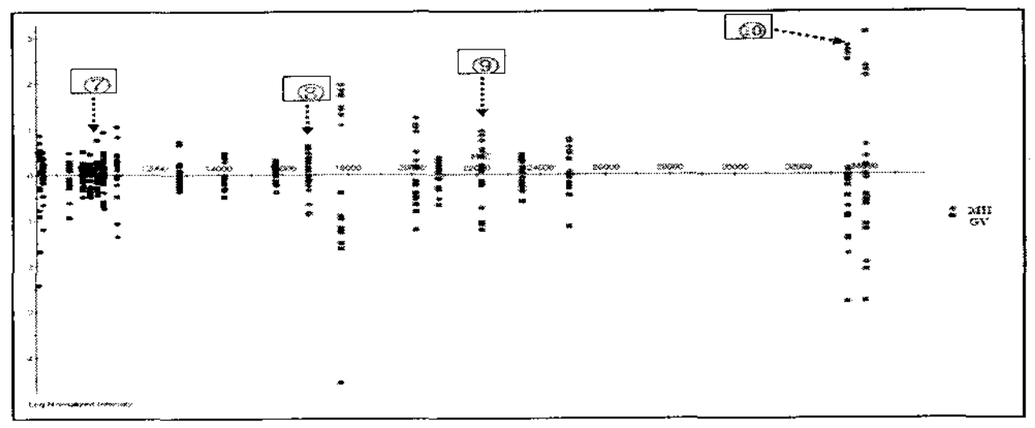


Fig. 1. Gel view of the data at the intensity 180, 190 and 200 between germinal vesicle (GV) and metaphase II (MII) stage oocyte.

Fig. 2. Scatter plot of normalized peak intensity for between germinal vesicle (GV) and metaphase II (MII) stage oocyte.

When GV stage oocytes were compared by statistical analysis with MII stage oocytes, a significant number of differences were observed among the negatively charged proteins. The fold change in expression is illustrated in Fig. 4 for the 45 proteins/biomarkers that were differentially ($p<0.05$) expressed between the two type of oocytes. Negatively charged proteins showing significantly differential expression related to immature and mature oocytes. Fig. 4 (A) or 4 (B) shows protein candidates that up-regulated in GV (Fig. 4 (A)) or MII (Fig. 4 (B)) stage oocytes.

TagIdent tool in the Swiss-prot database (www.expasy.org) was used to search candidate identifications (IDs) for these differentially expressed proteins, using m/z and an approximate isoelectric point range (Table 3 & 4). Molecular weight, error tolerance, specific molecular weight, symbol, name of candidate protein and pI value are listed.

Confirmation of Differentially Expressed Protein Candidates by Real-time RT-PCR

Until now, not so many porcine sequences have been published at the database, moreover, a few specific protein may detect in the porcine, even though they have tested in human or mouse. Therefore, we used real time RT-PCR to confirm mRNA level of the differential expression of protein candidates in GV and MII oocytes. We first analyzed genes that the SELDI-TOF-MS analysis revealed were more highly expressed in GV oocytes, 7 (*Atpk* (3.0±0.4), *Rs23* (4.6±0.6), *Rl32* (10.0 ±1.8), *Myl6* (9.1±1.2), *Ap4a* (3.2±0.2) *Pp14a* (2.1±0.6) and *Rs15* (10.0±1.5)) of these genes were found by real time RT-PCR analysis to be up-regulated in GV compared with their expression in MII stage oocytes (Fig. 5 (A)).

We also subjected protein candidates revealed by the SELDI-TOF-MS analysis to be upregulated in the MII stage compared to the GV stage to verification by real time RT-PCR. These genes were *Nu4lm* (2.3±0.3), *Ppia* (1.7±0.1), *Ppac* (1.6±0.2), *Csk2b* (2.3±0.4), *Atp6* (2.7±0.3) and *Ucp3* (1.5±0.2, Fig. 5 (B)).

DISCUSSION

Until now, the analysis of the message, i.e., mRNA or cDNA, has represented the strategy for rapid, sensitive, and high-throughput study of gene expression in various biological systems. Previously, we have reported transcriptomes of porcine (Cui *et al.*, 2005), bovine

Table 2. Condition of candidates from Q10 chip

No.	Stage	Signal/Noise (S/N)	Height from base line	M. W. (Da)	*P*-Value	Stage
Intensity: 180						
①	MII	4.489	10.718	10,831.79	0.002519	MII
	GV	3.880	7.5233			
Intensity: 190						
②	MII	2.289	3.805	8,179.574	0.008981	GV
	GV	3.150	6.066			
③	MII	2.822	9.176	15,767.13	0.033763	GV
	GV	3.210	10.761			
④	MII	1.422	5.507	17,743.78	0.000239	MII
	GV	0.160	0.554			
⑤	MII	0.933	4.484	20,122.10	0.011369	MII
	GV	0.460	2.559			
⑥	MII	1.644	12.556	24,857.48	0.017892	MII
	GV	1.190	9.045			
Intensity: 200						
⑦	MII	44.244	67.393	10,226.24	0.033763	GV
	GV	53.770	74.546			
⑧	MII	1.456	4.406	16,770.37	0.014306	GV
	GV	2.080	6.382			
⑨	MII	1.356	6.325	22,131.43	0.033763	MII
	GV	0.890	4.074			
⑩	MII	0.667	6.540	33,507.95	0.041227	MII
	GV	0.260	2.830			

Table 3. Candidates for highly expressed proteins in GV stage oocyte

Molecular weight	Error tolerance	MW	Symbol	Candidate protein	pI
8,179.574 Da	1%	8,136.85	GLUC	Glucagon	6.78
		8,241.27	IACS	Sperm-associated acrosin inhibitor	5.19
10,226.24 Da	1%	10,185.73	WFDCP2	WAP four-disulfide core domain protein 2	4.39
		10,195.60	UPTI	Uterine plasmin/trypsin inhibitor	8.87
		10,162.86	CASP1	Caspase-1	9.14
		10,259.43	MGP	Matrix Gla-protein	9.20
		10,189.36	ATPK	ATP synthase f chain, mitochondrial	9.85
15,767.13 Da	0.2%	15,760.54	SOD	Superoxide dismutase	6.04
		15,771.04	TMM10	Transmembrane protein 10	6.84
	1%	15,910.94	PP14B	Protein phosphatase 1 regulatory subunit 14 B	4.75
		15,685.37	RS23	40S ribosomal protein S23	10.50
		15,728.61	RL32	60S ribosomal protein L32	11.32
16,770.37 Da	1%	16,798.86	MYL6	Myosin light polypeptide 6	4.56
		16,704.15	AP4A	Bis (5'-nucleosyl)-tetraphosphatase	5.74
		16,671.92	PP14A	Protein phosphatase 1 regulatory subunit 14A	9.91
		16,908.91	RS15	40S ribosomal protein S15	10.39

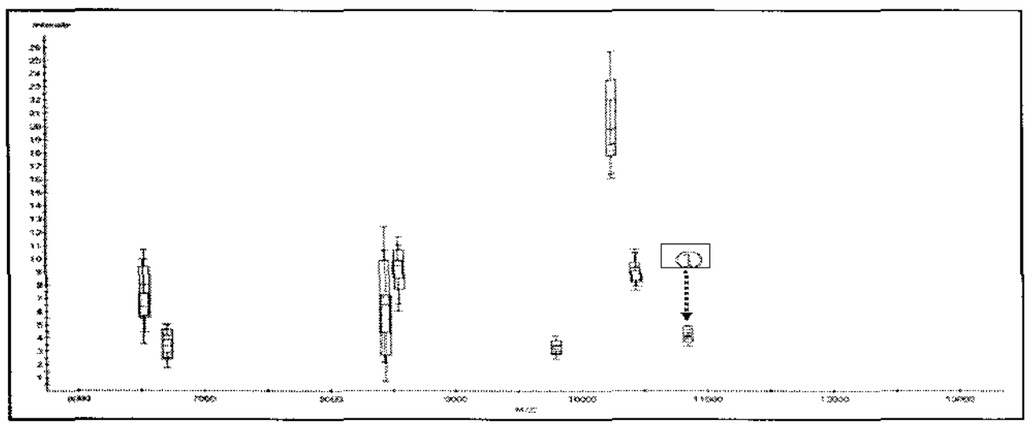
Table 4. Candidates for highly expressed proteins in MII stage oocyte

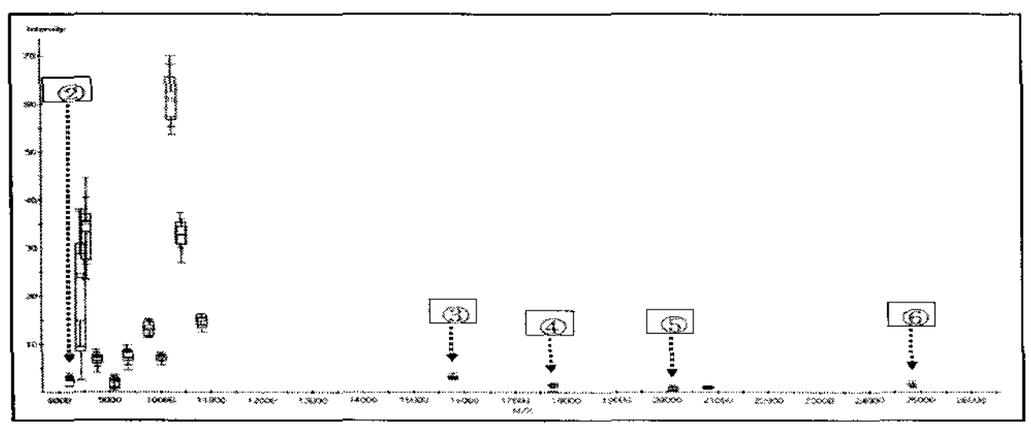
Molecular weight	Error tolerance	MW	Symbol	Candidate protein	pI
10,831.79 Da	0.2%	10,850.27	ICTL	Cathelin	5.11
		10,824.30	NU4LM	NADH-ubiquinone oxidoreductase chain 4L	5.27
	1%	10,803.43	IDH3B	Isocitrate dehydrogenase [NAD] subunit beta, mitochondrial	4.29
17,743.78 Da	0.2%	17,721.16	ENAM	Enamelin precursor	4.90
		17,738.16	PPIA	Peptidyl-prolyl cis-trans isomerase A	8.37
		17,718.68	RS18	40S ribosomal protein S18	10.99
	1%	17,870.56	MMP1	Interstitial collagenase precursor	4.75
		17,707.50	OBP	Odorant-binding protein	4.18
		17,863.51	LACB	Beta-lactoglobulin-1A/1C precursor	4.60
		17,904.28	PPAC	Low molecular weight phosphotyrosine protein phosphatase	7.00
		17,575.29	IL1B	Interleukin-1 beta precursor	8.41
20,122.1 Da	1%	20,014.69	CD3E	T-cell surface glycoprotein CD3 epsilon chain precursor	5.62
		20,073.60	GGT1	Gamma-glutamyltranspeptidase 1 precursor	6.47
		20,081.09	RL11	60S ribosomal protein L11	9.60
22,131.43 Da	1%	22,193.66	IL12A	Interleukin-12 alpha chain precursor	6.12
		22,279.39	KCY	UMP-CMP kinase	6.02
		22,058.87	H1T	Histone H1t	11.58
24,857.48 Da	0.2%	24,859.29	UCHL1	Ubiquitin carboxyl-terminal hydrolase isozyme L1	5.22
		24,942.42	CSK2B	Casein kinase II subunit beta	5.33
	1%	24,785.61	HMGB1	High mobility group protein B1	5.71
		25,085.18	MYOG	Myogenin	5.55
		25,018.42	FA9	Coagulation factor IX	6.65
		25,069.74	CD9	CD9 antigen	7.14
		25,039.26	ATP6	ATP synthase a chain	9.99
33,507.95Da	1%	33,689.82	CDK4	Cell division protein kinase 4	6.76
		33,282.98	ACTHR	Adrenocorticotropic hormone receptor	8.43
		33,226.47	CNN1	Calponin-1	8.92
		33,259.38	UCP2	Mitochondrial uncoupling protein 2	9.86
		33,772.25	UCP3	Mitochondrial uncoupling protein 3	9.51

(Hwang *et al.*, 2005) and mouse (Cui *et al.*, 2007b) oocyte before and after maturation. While this approach will remain important, mRNA molecules are intermediated on the pathway to the ultimate gene products, proteins that are responsible for cellular behavior and plasiticity. Furthermore, it is necessary to consider that the correlation between mRNA and protein levels is usually very low and mRNA level alone does not enough provide information about the presence of different protein isoforms or posttranslational modification of proteins.

In the present study, time-of-flight mass spectrometry was successfully used to analyze the proteome of porcine oocytes. This study has shown that mature (MII) oocyte have differential protein profile compared to the immature (GV) oocyte. Preliminary identification through database searching highlighted 16 and 29 interesting potential candidates which are down and up-regulated in MII stage oocyte respectively. Thirteen candidates gene expression confirmed by real time RT-PCR analysis.

The potential candidate of NADH-ubiquinone oxidoreductase was identified as a MII stage specific protein. NADH-ubiquinone oxidoreductase is the most intricate membrane-bound enzyme of the mitochondrial respiratory chain. In animals, subunit 4L is coded by mitoch-





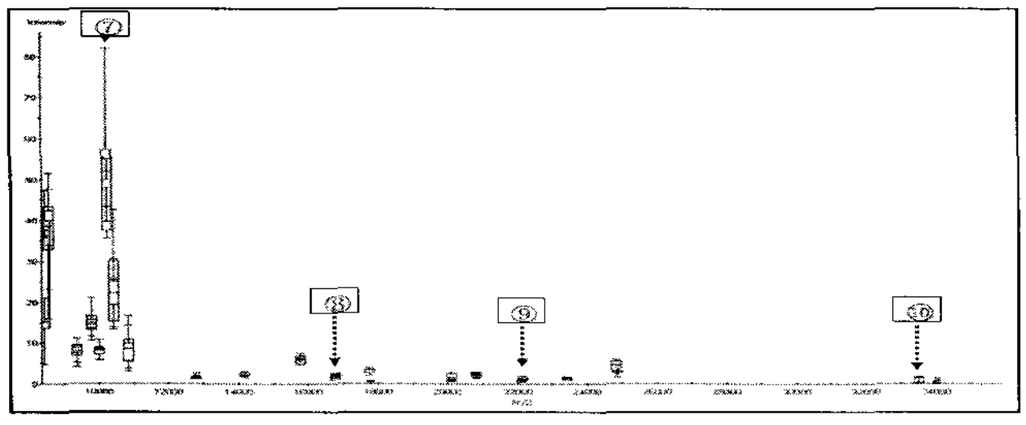
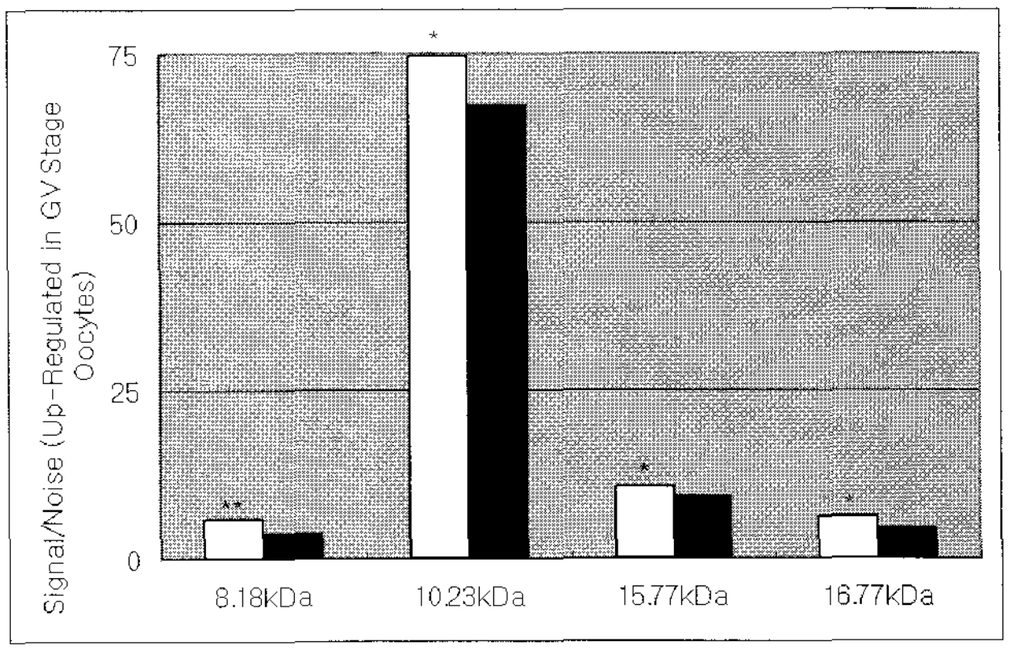
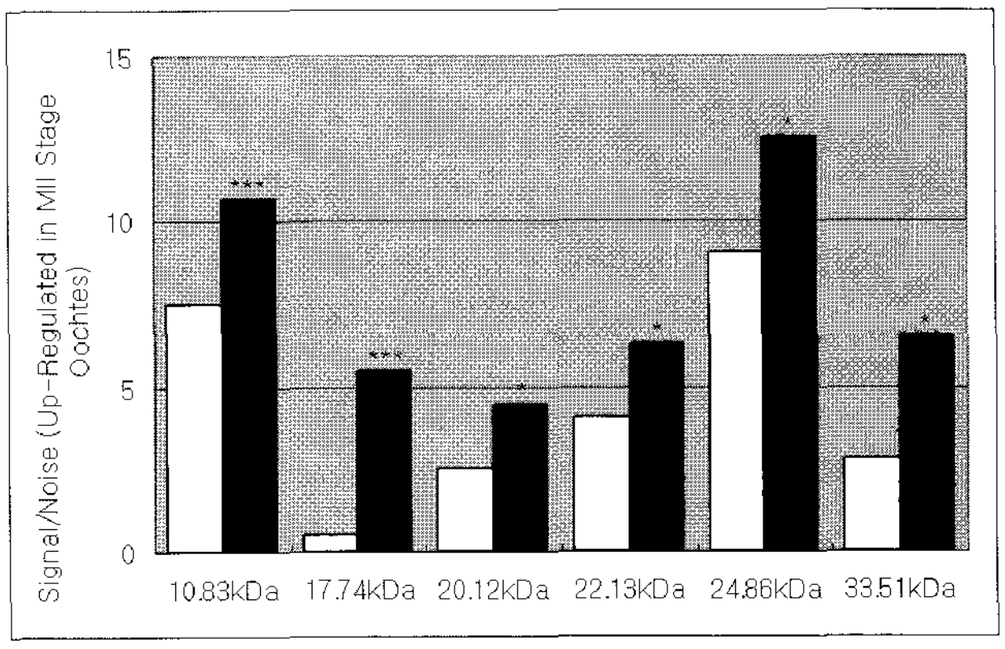


Fig. 3. Box plot of normalized peak intensity for between germinal vesicle (GV) and metaphase II (MII) stage oocyte.



(A)



(B)

Fig. 4. Negatively charged proteins showing significantly differential expression related to oocyte stages. (A), Protein candidates up-regulated in GV stage oocytes. (B), Protein candidates up-regulated in MII stage oocytes. Open bars, GV stage oocytes; solid bars, MII stage oocytes. Bars with different letters differ statistically. *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$.

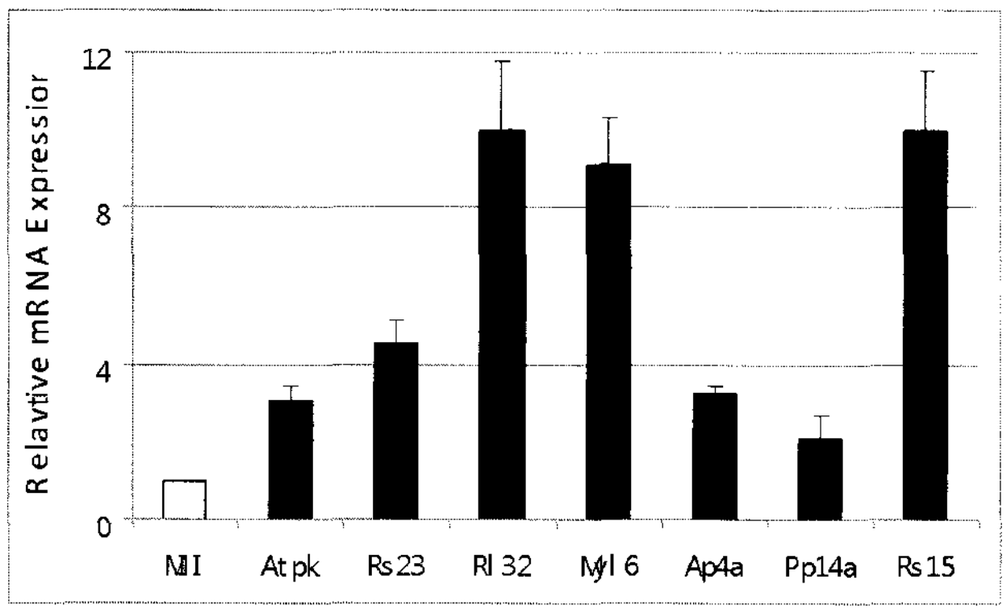
ondrial gene (Cardol *et al.*, 2006) and its expression is regulated by a senescence mechanism (Kodama *et al.*, 1995).

Peptidyl-prolyl cis/trans isomerase A (PPIA) is a potential candidate at the size of 17738 Da which is highly expressed in MII stage oocyte. PPIA is a member of the immunophilin class of proteins that all possess peptidyl-prolyl cis/trans isomerase activity and, therefore, are believed to be involved in protein folding and/or intracellular protein transport. PPIA shows stable mRNA expression across the different stage of preimplantation embryo development in mouse (Mamo *et al.*, 2007).

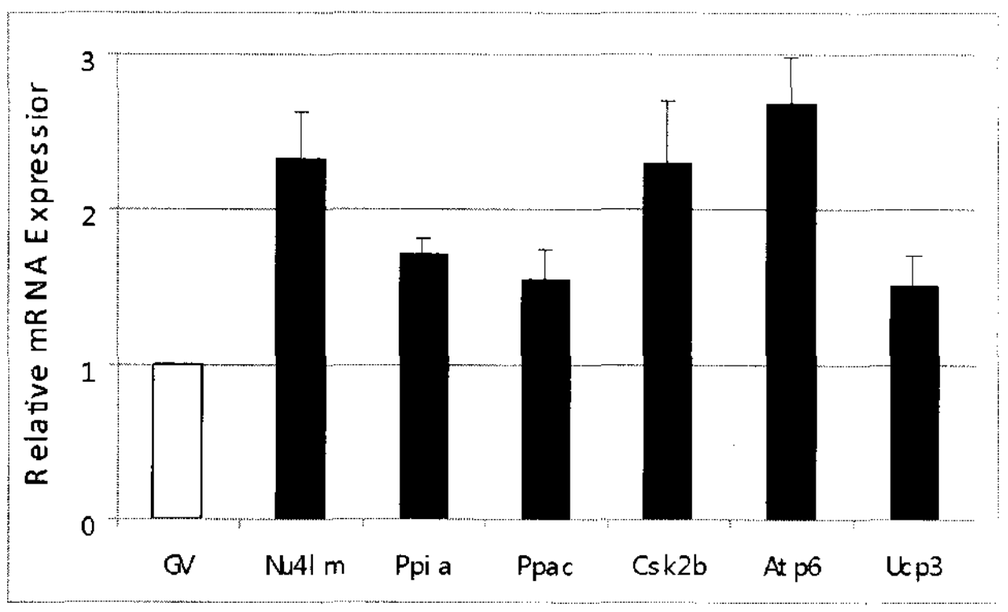
A potential candidate for the 24942 Da (upregulated in MII stage oocyte) is a casein kinase 2 (CK2) subunit beta. CK2 is present in the nucleolus, the site of ribosome biogenesis (Pfaff and Anderer, 1988; Gerber *et al.*, 2000), and copurifies with mammalian Pol I (Rose *et al.*, 1981; Duceman *et al.*, 1981; Belenguer *et al.*, 1989). Interestingly, increased level and activity of CK2 correlate with cell growth and proliferation (Litchfield 2003; Meggio and Pinna 2003), therefore an upregulation of CK2b in mature oocyte might contribute to the early cleavage of preimplantation embryos.

We found that mitochondrial uncoupling protein (UCP) 2 and 3 are abundant in mature oocytes. UCP 3 is a member of the uncoupling protein family (Ricquier and Bouillaud 2000), can uncouple respiratory chain from oxidative phosphorylation (Jaburek *et al.*, 1999). Over-expression of UCP3 in several cell lines and tissue systems reduces mitochondrial membrane potential, whereas mitochondria from UCP3 gene-null mice exhibit high mitochondrial membrane potential (Gong *et al.*, 1997, 2000; Vidal-Puig *et al.*, 2000). However little is known about their biological function during oocyte maturation and early mammalian development.

Interestingly, our previous study (Cui *et al.*, 2005) reported that ATPase 6 was was highly expressed in porcine MII compared to GV stage oocyte and real-time RT-PCR analysis also confirmed that ATPase 6 significantly upregulated in both porcine and mouse MII than GV stage oocyte (Cui *et al.*, 2005). In this study, ATPase 6 (MW, 25039 Da) is one of candidate for upregulated in mature oocyte. Because, ATPase 6 expression is essential for the production of ATP (Oza-



(A)



(B)

Fig. 5. Real time RT-PCR analysis of candidate proteins that are differentially expressed in GV or in MII compared with MII or GV stage oocyte in porcine. Porcine *H2a* mRNA expression was used as an internal standard. (A), Fold differences of mRNA from the same numbers of MII (□) and GV(■) and stages after normalization to the internal standard (porcine *H2a*). Messenger RNA expression of MII (□) was arbitrarily set to one fold. (B), Fold differences of mRNA from the same numbers of GV (□) and MII (■) stages after normalization to the internal standard (porcine *H2a*). Messenger RNA expression of GV (□) was arbitrarily set to one fold.

wa and Nishiyama 1997), so it may has specific functions during oocyte maturation such as in energy production.

In addition, MII stage oocytes exhibit a overall low expression of protein profile than GV stage oocytes. We found that many of the protein candidate highly expressed in GV stage are ribosomal proteins. It is known that the translation of ribosomal protein may be associated with a characteristic maturation of the both nuclear and cytoplasm in porcine oocyte. Although it is not clear what role ribosomal protein plays in specific protein translation, the expression of various types of ribosomal protein in oocyte maturation suggests that they may play critical roles in specific protein synthesis for oocyte maturation and for further fertilization and early preimplantation embryo development.

Onging research is now focused on protein identification by a variety of methods for purification and peptide sequencing. The selective surfaces of the protein chips will assist in protein purification by providing information on the biochemical properties of proteins. The definitive identification of differentially expressed proteins will assist in determining their cellular function during porcine oocyte *in vitro* maturation.

Identification of differentially expressed proteins of mammalian oocytes may lead to an improved understanding of porcine oocyte physiology and of its roles in fertilization and preimplantation embryonic development, and the critical events occurring immediately before implantation.

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