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Identification of Developmental Related Genes in the Lab Animal

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실험동물 발생 유전자의 확인

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Abstract The present study was conducted 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 mouse oocytes at the *in vitro* matured metaphase II (MII) and germinal vesicle (GV) stages. By using selected CM10 chip, found 16 candidates which were up-regulated in GV stage oocytes compared with in MII stage oocytes, molecular weight are 8180 (2 candidates), 10226 (5 candidates), 15767 (5 candidates) and 16770 (4 candidates) Da respectively. And 29 candidates were higher in MII than in GV stage oocytes, molecular weight are 10832 (3 candidates), 17744(8 candidates), 20122 (3 candidates), 22131 (3 candidates), 24857 (7 candidates) and 33507 (5 candidates) Da, respectively. All (45) candidate (0.2 and 1.0 % error tolerances) were performed real time RT-PCR analysis and further selected 13 more potential candidates.

요 약 본 연구에서는 쥐의 미성숙 난자의 체외 성숙과정에서 매우 특이적으로 발현되는 후보 단백질 변화를 동정하려는 목적으로 체외 성숙 과정에서 GV 단계의 미성숙 난자와 MII 단계의 난자를 실험 시료로 사용하였다. 그리고 단백질 Chip은 선행 실험에서 가장 효과적인 CM10을 사용하여 SELDI -TOF MS 분석 장치를 이용하여 후보단백질을 동정하였다. MII 단계의 미성숙 난자와 비교하여 GV 단계의 미성숙 난자에서 16개의 후보단백질이 높게 발현되었으며, 이때 발현된 후보 단백질 각각의 분자량은 8180(후보단백질 2개), 10226 (5개), 15767(5개), 16770(4개) 달톤(dalton)이였다. 또한 29개의 후보단백질은 MII 단계의 미성숙 난자에서 높게 발현되었고 이들의 분자량은 각각 10832(3개)17743(8개)20122(3개)22131(3개) 24857(7개) 33507(5개) 달톤 이였다. 한편 전체 후보 단백질 45개의 분석을 Real time RT-PCR에서 수행하여 13개의 잠재적인 후보단백질을 확인 동정하였다.

Key Words : Mouse oocyte, in vitro maturation, SELDI-TOF, protein candidate

1. 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 the resumption of first meiosis, chromatin starts to condense, germinal vesicle breakdown (GVBD) is initiated, the metaphase I spindle is organized and the first polar body is extruded. Immediately thereafter, the oocytes enter meiosis II and are then arrested again at the metaphase II (MII) stage.

The classic proteomics approach involves two-dimensional polyacrylamide gel electro- phoresis, which has limited success in the field of embryology [1]. This approach is straightforward but labor intensive and

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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 under represented and difficult to distinguish. Consequently, protein-based research in embryology has concentrated on identifying and localizing individual proteins by western blot analysis [2].

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[3]. Recently, Katz-Jaffe et al. [4] reported candidate proteins in human blastocyst using SELDI-TOF MS, they have found several protein candidates significantly upregulated in degenerating embryos compared to expanded blastocysts.

Given how little is known about mammalian oocyte maturation compared to oocyte maturation in xenopus. In the present study, we take a proteomic (SELDI-TOF MS) approach to identify molecules that are differentially expressed during mouse 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.

2. Materials and methods

2.1 Generation of embryos

To obtain GV stage oocyte, 5-week-old B6C3 F1 female mice (C57BL/6 female x C3H/He male) were superovulated by 5 IU pregnant mare serum gonadotropin (PMSG, Sigma, St. Louis, MO), GV stage oocytes were collected 45 h after the PMSG injection from ovary by slicing. The GV oocyte collection medium was M2 (Sigma) supplemented with 300 M dibutyryl cyclic adenosine monophosphate (dbcAMP, Sigma) to inhibit germinal vesicle breakdown (GVBD) during oocyte collection. GV stage oocytes were then cultured in M16 (Sigma) medium supplemented with 0.4% BSA at 37ºC in a humidified atmosphere of 5% CO2 and 95% air to examine rates of maturation (MII). 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.

2.2 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 [5].

2.3 Protein profiling using SELDI-TOF-MS

To determine the best condition for the discrimination between immature and mature oocyte, each four (n=4) from sample groups were analyzed on four ProteinChip (NP20, H4, CM10 and SAX10) 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, MII oocyte: n=10*100) was analyzed using CM10 Chip.

2.4 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 weightfrom each sample group were clustered, and then the mean and standard deviation of each sample group was reported, compared, and visualized by Biomarker Wizard.

2.5 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 the oocyte maturation. The mRNAs from three sets of 50 GV or MII stage oocytes were 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)12-18 primer and the Superscript reverse transcriptase enzyme (Invitrogen Co., Grand Island, NY).

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

Genes	Primer sequence	AT (℃)	BP	Genes	Primer sequence	AT (℃)	BP
Atpk	F: tgctggaggtcaaattaggg R: gcgccatcttcagtggtatt	60	242	Nu4lm	F: tgcccttgcagggttactta R: tcggtaccgtatgtgttgga	55	222
Rs23	F: aacagcccaaattctgccatc R: atgacctttgcgaccaaatc	55	152	Ppia	F: agcactggggagaaag R: aaaactgggaaccgtttgt	60	220
Rl32	F: gcccaagatcgtcaaaaaga R: taaccaatgttgggcatcaa	55	153	Ppac	F: ccggcagattaccaaag R: gtcggagtcgttgccataa	60	190
Myl6	F: tggtaaatgctgatggcaaa R: gagatccggcatgttcttgt	60	250	Csk2b	F: atgtccgaaaggccgatg R: atcgcccagatgttggaa	60	101
Ap4a	F: cctgagagcatgtggtttga R: gcatctatgcctgcttcctc	60	195	Atp6	F: ctattcccaacacccaaa R: tgggtgtgaatgagtgtggt	55	196
Pp14a	F: ctggacgtggagaagtggat R: agttctctgtggcgttcgtg	60	172	Uchl1	F: gattaacccggagatg R: tgccaatggtctgcttc	55	242
Rs15	F: ctgaaccgaggtctgaggag R: tctccacctggttgaaggtc	55	187	H2a	F: acaacaagaagacccgc R: cttggccttgtggtgactct	60	167

Real time RT-PCR was then performed using the 14 primer sets shown in (Table 1).

3. Results

3.1 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 mouse GV and MII stage oocytes. To do this, the proteins of both types of oocytes (n=1,400 for both) were extracted and subjected to SELDI-TOF-MS analysis as described above.

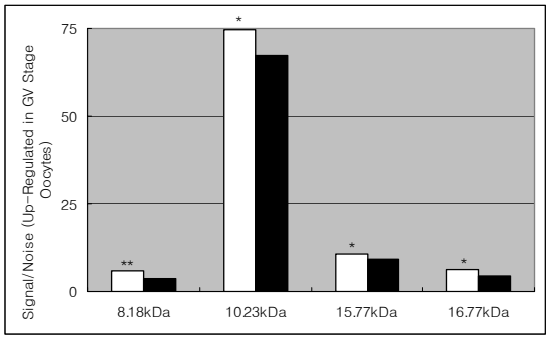
After normalization of the data to total ion current, statistical analysis was performed to determine any significant difference in protein expression related to mature situation (immature or mature) on this protein chip type. Comparison of GV stage with MII stage oocytes revealed 29 protein candidates were significantly highly expressed in MII stage oocyte, at approximately 10831.79, 17743.78, 20122.1, 24857.48, 22131.43 and 33507.95 Da, and 16 protein candidates were highly expressed in GV stage oocytes, at approximately 8175.57, 15767.13, 10226.24 and 16770.37 Da.

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. 1 for the 45 proteins/ biomarkers that were differentially ($P<0.05$) expressed between the oocytes. TagIdent tool in the Swiss-prot database was used to search for candidate identifications(IDs) for these differentially expressed proteins, using m/z and an approximate isoelectric point range (Table 2 & 3).

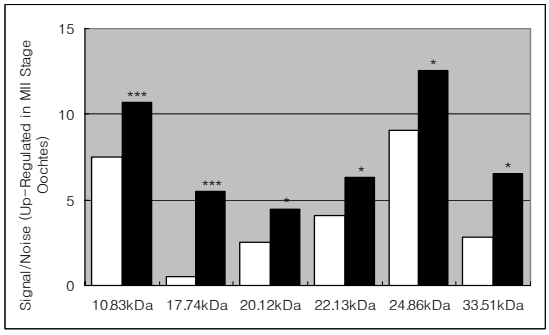
3.2 Confirmation of differentially expressed protein candidates by real-time RT-PCR

We used real time RT-PCR to confirm mRNA expression of the differential expression of protein candidatesin GV and MII oocytes. We first analyzed genes that the SELDI-TOF-MS analysis revealed were more highly expressed in GV oocytes, 7 (Atpk, Rs23, Rl32, Myl6, Ap4a, Pp14a and Rs15) 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.(2a).

We also subjected protein candidates revealed by the SELDI-TOF-MSanalysis to be upregulated in the MII stage compared to the GV stage to verification by real time RT-PCR. These genes were Nu4lm, Ppia, Ppac, Csk2b, Atp6 and Uchl1 Fig. (2b).



(a)



(b)

[Fig. 1] 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

4. Discussion

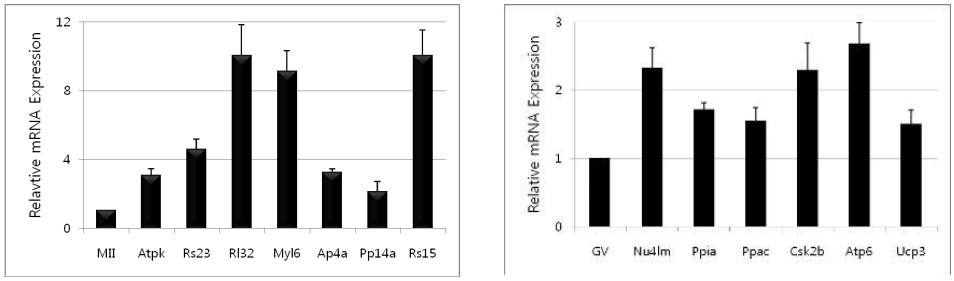
Until now, the analysis of the message, i.e., mRNA or cDNA, has represented the strategy for rapid, sensitive, and high-throughput study of gene expression in various biological systems.

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 provide information about the presence of different protein isoforms or post translational modification of proteins.

In the present study, time-of-flight mass spectrometry was used to successfully analyze the proteome of mouse oocyte. 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 down and upregulated in MII stage oocyte respectively. All (45) candidate (0.2 and 1.0 % error tolerances) were performed real time RT-PCR analysis and further selected 13 more potential candidates.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 respir -atory chain.

[Table 2] Candidates for highly expressed proteins in GV stage oocyte

Molecular Weight	Error tolerance	MW	Symbol	Candidate protein	pI
8179.574 Da	1%	8136.85	GLUC	Glucagon	6.78
		8241.27	IACS	Sperm-associated acrosin inhibitor	5.19
10226.24 Da	1%	10185.73		WAP four-disulfide core domain protein 2	4.39
		10195.60	UPTI	Uterine plasmin/trypsin inhibitor	8.87
		10162.86	CASP1	Caspase-1	9.14
		10259.43	MGP	Matrix Gla-protein	9.20
		10189.36	ATPK	ATP synthase f chain, mitochondrial	9.85
15767.13 Da	0.2%	15760.54		Superoxide dismutase	6.04
		15771.04	TMM10	Transmembrane protein 10	6.84
	1%	15910.94	PP14B	Protein phosphatase 1 regulatory subunit 14B	4.75
		15685.37	RS23	40S ribosomal protein S23	10.50
		15728.61	RL32	60S ribosomal protein L32	11.32
16770.37Da	1%	16798.86	MYL6	Myosin light polypeptide 6	4.56
		16704.15	AP4A	Bis (5'-nucleosyl)-tetraphosphatase	5.74
		16671.92	PP14A	Protein phosphatase 1 regulatory subunit 14A	9.91
		16908.91	RS15	40S ribosomal protein S15	10.39



(a) (b)

[Fig. 2] Real time RT-PCR analysis of candidate proteins that are differentially expressed in GV(a) or in MII(b) compared with MII(a) or GV(b) stage oocyte in mouse. Mouse H2a mRNA expression was used as an internal standard.

Peptidyl-prolyl cis/trans isomerase A (PPIA) is a potential candidate for the 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

[Table 3] Candidates for highly expressed proteins in MII stage oocyte

Molecular weight	Error tolerance	MW	Symbol	Candidate protein	pI
10831.79 Da	0.2%	10850.27	ICTL	Cathelin	5.11
		10824.30	NU4LM	NADH-ubiquinone oxidoreductase chain 4L	5.27
	1%	10803.43	IDH3B	Isocitrate dehydrogenase [NAD] subunit beta, mitochondrial	4.29
17743.78 Da	0.2%	17721.16	ENAM	Enamelin precursor	4.90
		17738.16	PPIA	Peptidyl-prolyl cis-trans isomerase A	8.37
		17718.68	RS18	40S ribosomal protein S18	10.99
	1%	17870.56	MMP1	Interstitial collagenase precursor	4.75
		17707.50	OBP	Odorant-binding protein	4.18
		17863.51	LACB	Beta-lactoglobulin-1A/1C precursor	4.60
		17904.28	PPAC	Low molecular weight phosphotyrosine protein phosphatase	7.00
		17575.29	IL1B	Interleukin-1 beta precursor	8.41
20122.1 Da	1%	20014.69	CD3E	T-cell surface glycoprotein CD3 epsilon chain precursor	5.62
		20073.60	GGT1	Gamma-glutamyltranspeptidase 1 precursor	6.47
		20081.09	RL11	60S ribosomal protein L11	9.60
22131.43 Da	1%	22193.66	IL12A	Interleukin-12 alpha chain precursor	6.12
		22279.39	KCY	UMP-CMP kinase	6.02
		22058.87	H1T	Histone H1t	11.58
24857.48 Da	0.2%	24859.29	UCHIL1	Ubiquitin carboxyl-terminal hydrolase isozyme L1	5.22
	1%	24942.42	CSK2B	Casein kinase II subunit beta	5.33
		24785.61	HMGB1	High mobility group protein B1	5.71
		25085.18	MYOG	Myogenin.	5.55
		25018.42	FA9	Coagulation factor IX	6.65
		25069.74	CD9	CD9 antigen	7.14
		25039.26	ATP6	ATP synthase a chain	9.99
33507.95Da	1%	33689.82	CDK4	Cell division protein kinase 4	6.76
		33282.98	ACTHR	Adrenocorticotropic hormone receptor	8.43
		33226.47	CNN1	Calponin-1	8.92
		33259.38	UCP2	Mitochondrial uncoupling protein 2	9.86
		33772.25	UCP3	Mitochondrial uncoupling protein 3	9.51

embryo development in mouse [6].

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 and copurifies with mammalian Pol I [7]. Interestingly, increasedlevel and activity of CK2 correlate with cell growth and proliferation [8], 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 can uncouple respiratory chain from oxidative phosphorylation [9].

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 [10].

In addition, MII stage oocytes exhibit a 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 thetranslation of ribosomal protein may be associated with a characteristic maturation of the both nuclearand cytoplasm in mouse oocyte. Although it is not clear what role ribosomal protein plays in specific protein translation, the expression of various types of ribosomal protein in oocye maturation suggests that they may play critical roles in specific protein synthesis for oocyte maturation andfor further fertilization and early preimplantation embryo development.

Onging research is now focused on protein identification involving 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 cellular function during mouse oocyte in vitro maturation.

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

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<Research Interests>
Animal Reproductive Physiology