Molecular Reproduction & Development 77:51–58 (2010)

Aberrant Protein Expression Is Associated With Decreased Developmental Potential in Porcine Cumulus–Oocyte Complexes

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SUMMARY

Oocyte developmental competence is progressively obtained during pubertal development in females. Poor developmental potential in oocytes derived from prepubertal females suggests that essential processes required for oocyte development have not been fulfilled. The objective of this experiment was to analyze the protein profiles of porcine cumulus–oocyte complexes (COC) derived from cyclic and prepubertal females to identify alterations in protein abundance that correlate with developmental potential. COC complexes, aspirated from prepubertal and cyclic ovaries, were pooled into three replicates of 400 COCs each per treatment in ~100 μl SOF-HEPES medium. Protein samples were extracted and analyzed by two-dimensional differential in gel electrophoresis (2D-DIGE). Over 1,600 proteins were resolved on each of the three replicate gels. Sixteen protein spots were identified by mass spectrometry, representing 14 unique, differentially expressed proteins (volume ratio greater than 1.3). Glutathione-*S*-transferase and pyruvate kinase 3 were more abundant in COCs derived from cyclic females, whereas soluble epoxide hydrolase and transferrin were more abundant in prepubertal derived COCs. Abundance of several glycolytic enzymes (enolase 1, pyruvate kinase 3, and phosphoglycerate kinase) was increased in COCs derived from cyclic females, suggesting glucose metabolism is decreased in prepubertal derived COCs. We conclude that the abundance of proteins involved in metabolism and oxidative stress regulation is significantly altered in prepubertal derived COCs and may play a role in the mechanisms resulting in developmental competence.

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Mol. Reprod. Dev. 77: 51–58, 2010. © *2009 Wiley-Liss, Inc.*

Received 14 May 2009; Accepted 31 July 2009

Published online 2 September 2009 in Wiley InterScience (www.interscience.wiley.com).
DOI 10.1002/mrd.21102

INTRODUCTION

The quality of an oocyte is difficult to predict due to the numerous processes that must be completed during oocyte growth and development. During the growth phase of the oocyte, essential transcripts are accumulated and stored for utilization during early embryo development before the embryonic genome is activated (Trounson et al., 2001; Marchal et al., 2002; Calvert et al., 2003). Therefore, the oocyte has to establish the correct transcriptome and proteome during early oocyte growth to support good developmental competence. Previously, our laboratory has shown that transcript abundance is altered in porcine oocytes derived from cycling and prepubertal females, possibly resulting in altered developmental potential in prepubertal-derived oocytes (Paczkowski et al., 2007). Oocytes obtained from cyclic females have twice the developmental potential of prepubertal-derived oocytes, and transcript abundance of genes involved in metabolism, DNA metabolism, and oxidative stress were altered in prepubertal-derived oocytes

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(Paczkowski et al., 2007). Although the identity of the proteome is directed by the transcriptome, there is often poor-to-no correlation observed between mRNA and protein levels (Gygi et al., 1999). Therefore, analyzing the transcriptome alone does not provide all the necessary information on the cellular mechanisms at work within a given cell.

Global analysis of mammalian proteomes identifies changes in tissues and cells induced by disease, drug therapy, and changes in environment or physiological development. Few investigators have attempted to characterize the oocyte proteome, although a few studies have been completed in bovine (Levesque and Sirard, 1994), porcine (Ellederova et al., 2004), and murine oocytes (Calvert et al., 2003) using one-dimensional gel electrophoresis (1DE) or two-dimensional gel electrophoresis (2DE) techniques. However, proteomic techniques, such as two-dimensional differential in gel electrophoresis (2D-DIGE), require large amounts of starting material for accurate analysis. Practical and ethical limitations in working with oocyte material, due to the difficulty in collecting large number of oocytes, especially donated human oocytes, are difficult to overcome. Previous reports have utilized a large number of oocytes for 2DE techniques, ranging from 200 and 600 porcine oocytes for analytical and micropreparative gels, respectively (Ellederova et al., 2004) to 2,850 murine oocytes (Calvert et al., 2003). In our laboratory, we reported differences in the oocyte proteome extracted from 1,500 porcine oocytes (Paczkowski et al., 2005).

Protein synthesis in mammalian oocytes is required not only for the resumption of meiosis (Kastrop et al., 1990; Wu et al., 1996; Tomek et al., 2002) but also for the attainment of cytoplasmic maturation and is influenced by maternal age. Prepubertal-derived ovine oocytes incorporate less methionine during in vitro maturation (Ledda et al., 2001; Kochhar et al., 2002), indicating decreased protein synthesis compared to adult-derived oocytes. Altered protein profiles are associated with decreased developmental competence in prepubertal-derived bovine (Levesque and Sirard, 1994; Gandolfi et al., 1998) and porcine oocytes (Paczkowski et al., 2005). Prepubertal-derived bovine oocytes display a similar protein profile to defective adult-derived oocytes and have reduced embryonic development, suggesting that poor development, and therefore poor oocyte competence, may be associated with an altered protein profile (Levesque and Sirard, 1994). In our laboratory, we previously observed differential expression of 292 proteins extracted from 1,500 porcine oocytes derived from cyclic and prepubertal females using 2DE. However, only seven differentially expressed proteins were identified with high confidence by mass spectrometry. These proteins included peroxiredoxin 2, protein disulfide isomerase, and phospholipase C, which are all elevated in prepubertal-derived oocytes, suggesting an altered ability to respond to increased oxidative and cellular stress due to the in vitro environment during oocyte maturation (Paczkowski et al., 2005).

Cumulus cells play a dynamic role in oocyte development and are an essential component of the follicular microenvironment that influences the health of the follicle and oocyte developmental potential. Follicular cells synthesize and secrete hormones and proteins that influence oocyte development; however, oocytes direct their own development via secreted factors that interact with surrounding cumulus cells to regulate essential processes required by the oocyte (Gilchrist et al., 2008). Altered gene and protein expression in cumulus cells may influence oocyte development and could potentially be a noninvasive method to predict oocyte quality. Lysosomal cysteine proteinases, associated with pro-apoptotic pathways, are increased in cumulus cells surrounding prepubertal- and developmentally incompetent adult-derived oocytes compared to competent adult-derived oocytes (Bettegowda et al., 2008). Abundance of constitutive and neosynthesized proteins is also increased in bovine cumulus cells from adult females compared to prepubertal-derived cumulus cells (Gandolfi et al., 1998; Khatir et al., 1998).

To our knowledge, only one previous study has attempted to characterize the proteome of approximately 2,000 intact murine COCs by 2DE. Over 250 proteins in mature COCs were identified, including proteins involved in glycolysis, oxidative and heat stress regulation, and gene/protein expression, which are essential for oocyte development (Meng et al., 2007). However, to date, reports have not been published comparing the proteomes of developmentally competent versus incompetent COCs to determine if the molecular networking between oocytes and cumulus cells are altered. Several reports have indicated that prepubertal females have reduced developmental potential compared to adult females (Marchal et al., 2001; Grupen et al., 2003; Sherrer et al., 2004; Kauffold et al., 2005; Leoni et al., 2007) and represent an excellent model for analyzing the proteome of developmentally incompetent COCs. The objective of the current experiment was to identify differentially expressed proteins in porcine COCs derived from prepubertal (incompetent COCs) and cyclic females (competent COCs). We hypothesize that prepubertal-derived COCs will exhibit aberrant expression of essential, regulatory proteins and increased expression of detrimental proteins.

RESULTS

To identify alterations in the protein expression profile of COCs derived from cyclic and prepubertal females that are associated with decreased developmental potential, proteins were separated using 2D-DIGE (Applied Biomics, Hayward, CA). Average protein content was not significantly different between COCs derived from cyclic and prepubertal females (Table 1); therefore, differences in protein abundance observed in the 2D-DIGE analysis reflect actual differences in sample protein abundance rather than significant differences in cell numbers. Gel-to-gel variation between treatments was observed in the current experiment. The total number of proteins resolved on the three replicate gels and the number of proteins with increased or decreased abundance in COCs derived from cyclic compared to prepubertal females are shown in Table 2. Differential protein expression was determined for protein spots with a volume ratio greater than 1.3 on at least two out of the three gels. The number of spots resolved on the gels ranged from 1,600 to 1,800 proteins (gel 1: 1,733 proteins; gel 2:

TABLE 1. Average Protein Content of Pooled COCs (n = 400) and Average Protein Content of Individual COCs Derived From Cycling and Prepubertal Females Determined Using the Bio-Rad Protein Assay

Age	Average protein content, μg (400 COCs; mean ± SEM)	Average protein content, μg (per COC)
Prepubertal	238.33 ± 12.58	0.596
Cycling	258.33 ± 22.05	0.646

Average protein content was not significantly different between COCs derived from cycling and prepubertal females.

1,800 proteins; gel 3: 1,603 proteins), and the number of proteins with increased or decreased abundance in COCs derived from cyclic females ranged from 10 to 22 proteins (0.578–1.372%) and 4 to 21 proteins (0.23–1.248%), respectively.

The 2D-DIGE gels of cyclic and prepubertal-derived COC proteins are shown in Figure 1. Increased protein abundance in COCs derived from cyclic females resulted in a red spot, increased abundance in prepubertal-derived COCs resulted in a green spot, and equal protein abundance between the two protein sources resulted in a yellow spot. Sixteen protein spots, representing 14 unique proteins, were differentially expressed, determined by average volume ratio, and identified with high confidence by mass spectrometry. Volume ratios greater than 1.3 represented a 30% change in protein abundance. Protein spot number and average volume ratio, representing the fold change in protein abundance in COCs derived from cyclic compared to prepubertal females, are depicted in Tables 3 and 4. Eleven proteins displayed increased abundance in COCs derived from cyclic females (Table 3), and four proteins displayed increased abundance in prepubertal-derived COCs (Table 4). Two proteins (stress-induced phosphoprotein 1 and enolase 1) were each represented by two distinct spots on the 2D-DIGE gels and could suggest post-translational modification. Expression of stress-induced phosphoprotein 1 (Fig. 1) displayed both increased and decreased abundance in COCs derived from cyclic compared to prepubertal females (spots 4 and 5; Tables 3 and 4). Stress-induced phosphoprotein 1, spot 4, displayed increased abundance in COCs derived from cyclic compared to prepubertal females, indicated by an orange-red protein spot color. Stress-induced phosphoprotein 1, protein spot 5, displayed decreased abundance in COCs derived from cyclic females, indicated by a green spot color. This suggests differential expression of a post-translational modification due to differences in developmental competence.

DISCUSSION

Essential transcripts and proteins required for early development are accumulated and stored during the growth phase of the oocyte (Trounson et al., 2001; Marchal et al., 2002; Calvert et al., 2003). Oocytes with decreased developmental potential may not have obtained the proper complement of transcripts and proteins during this critical period (Levesque and Sirard, 1994). Identifying perturbations in mRNA or protein abundance could lead to methods to enhance oocyte developmental potential, thereby improving the overall efficiency of assisted reproductive technologies.

In the current experiment, we utilized 2D-DIGE to identify changes in the proteome of porcine COCs that are correlated to differences in developmental potential. The use of COCs provides an opportunity to observe differences in protein abundance in GV oocytes and cumulus cells without the interference of oocyte maturation conditions. Furthermore, proteome analysis of COCs enables the investigation of molecular networking between the oocyte and surrounding cumulus cells which is essential for oocyte developmental competence. Prepubertal-derived COCs are less developmentally competent, indicated by reduced percentages of oocytes developing to the blastocyst stage after in vitro fertilization and culture compared to COCs derived from cyclic females (Marchal et al., 2001; Grupen et al., 2003; Sherrer et al., 2004; Kauffold et al., 2005; Leoni et al., 2007). Previous studies in our laboratory have confirmed that prepubertal-derived porcine oocytes matured in vitro have a similar ability to undergo nuclear maturation compared to oocytes derived from cycling females; however, prepubertal-derived oocytes display a decreased ability to undergo embryonic development compared to oocytes derived from cycling females (Paczkowski et al., unpublished data).

Protein samples, derived from cyclic and prepubertal-derived COCs, were separated and analyzed on the same acrylamide gel using fluorescent labeling (2D-DIGE) by Applied Biomics. 2D-DIGE reduces gel-to-gel variation between treatment groups, compared to 2DE, thus increasing the ability to identify differentially expressed proteins (Marouga et al., 2005). However, some of the limitations of standard 2DE methods apply, including gel-to-gel variation between biological replicates as multiple gels are required for experimental design, and decreased ability to visualize and identify lower abundant proteins (Marouga et al., 2005). High resolution was observed in the current experiment on three gel replicates, with over 1,600 proteins spots resolved on each of the gels. However, replicates were subjected to variation between gels, indicated by the broad range of total proteins resolved (1,600–1,800), and proteins displaying increased or decreased abundance in COCs derived from

TABLE 2. Number of Protein Spots Resolved on Three 2D-DIGE Gels and the Number of Proteins With Increased or Decreased Abundance in COCs Derived From Cycling Versus Prepubertal Females

	Total number of proteins resolved	Number of proteins with increased abundance	Number of proteins with decreased abundance
Replicate 1	1,733	10 (0.578%)	4 (0.23%)
Replicate 2	1,800	15 (0.834%)	21 (1.167%)
Replicate 3	1,603	22 (1.372%)	20 (1.248%)

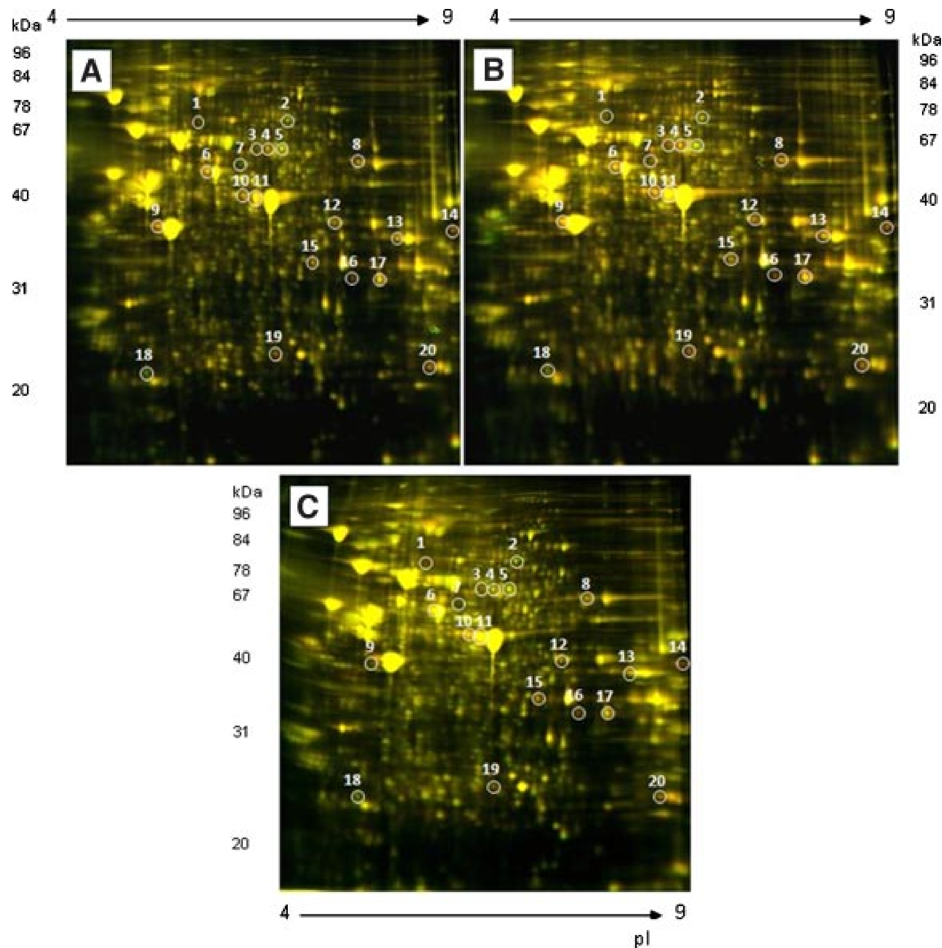

Figure 1. Protein spots resolved on three 2D-DIGE replicates selected for identification by mass spectrometry: (**A**) replicate 1, (**B**) replicate 2, and (**C**) replicate 3. Protein samples from COCs derived from cyclic females were labeled with Cy5 and samples from prepubertal-derived COCs were labeled with Cy3. Protein samples were mixed and an equal concentration of proteins from each sample was loaded onto the 2D-DIGE for analysis. Increased protein abundance in COCs derived from cyclic females resulted in a red protein spot, increased abundance in prepubertal-derived COCs resulted in a green protein spot, and equal protein abundance between the two protein sources resulted in a yellow protein spot.

cyclic and prepubertal females (range from 10 to 22 proteins and 4 to 21, respectively). Due to the limitations in gel electrophoresis techniques to separate and visualize low abundance proteins, the differentially expressed proteins identified in the present experiment probably represent abundant proteins in COCs and likely do not accurately represent global changes in the proteome of COCs.

Five proteins with increased abundance in COCs derived from cyclic females are involved in glucose metabolism (fructose 1,6-bisphosphate aldolase, phosphoglycerate kinase 1, enolase 1, lactate dehydrogenase A chain, and pyruvate kinase 3). The interaction between cumulus cells and oocytes play an integral role in the regulation of glycolysis. Oocyte-secreted factors promote glycolysis in the cumulus cells (Sugiura et al., 2005, 2007); however, only fully grown oocytes are capable of supporting higher glycolytic activity. Glucose metabolism and expression of glycolytic enzymes in cumulus cells and preantral follicles are increased in fully grown, competent oocytes compared to oocytes that are still growing (Sugiura et al., 2005). Elevated levels of glycolysis in bovine (Krisher and Bavister, 1999) and feline oocytes (Spindler et al., 2000) are associated with increased developmental potential to the blastocyst stage. The increased abundance of enzymes involved in glycolysis and gluconeogenesis in COCs derived from cyclic females-suggests less than optimal glucose metabolism in prepubertal-derived COCs, resulting in poor oocyte developmental potential in prepubertal females.

Plasminogen activator (PA) inhibitor 1 (PAI-1) and nexin-1 (PN-1) are protease inhibitors that regulate the activity of proteases involved in degradation of the ovarian extracellular matrix near ovulation (Kouba et al., 2000; Zhang et al.,

TABLE 3. Protein Spots Identified by Mass Spectrometry With Increased Abundance in COCs Derived From Cycling Compared to Prepubertal Females

Spot[a]	Protein identification	Ratio ± SEM[b]	No. of peptides[c]	Sequence coverage (%)	Expected MW	Expected pI	Organism[d]	Accession number
3	Zona pellucida 1	1.69 ± 0.050	5	10	79,631.9	8.03	Pig	AAA31144
20	Glutathione-*S*-transferase	1.587 ± 0.084	7	37	25,375.5	8.87	Pig	NP_999015
4	Stress-induced phosphoprotein 1	1.567 ± 0.341	17	31	62,528.4	6.40	Mouse	NP_058017
14	Nexin-1	1.563 ± 0.033	16	45	44,056.2	9.48	Pig	NP_999452
14	Plasminogen activator inhibitor type 1, member 2	1.563 ± 0.033	10	29	43,974.2	9.35	Human	NP_006207
11	Enolase 1	1.47 ± 0.046	11	26	47,139.2	7.01	Rat	AAH63174
8	Pyruvate kinase 3	1.44 ± 0.035	18	38	57,808.0	7.18	Mouse	NP_035229
16	L-lactate dehydrogenase A chain	1.427 ± 0.043	15	47	36,595.3	8.18	Pig	P00339
9	Beta actin	1.417 ± 0.012	14	53	40,194.1	5.55	Human	AAH12854
13	Fructose 1,6 bisphosphate aldolase	1.353 ± 0.023	14	56	39,264.3	8.39	Human	4ALD
12	Phosphoglycerate kinase 1	1.3 ± 0.03	16	51	44,530.1	8.02	Pig	AAT77773

[a]Location of protein spot on gel.
[b]Volume ratio of proteins extracted from COCs derived from cycling versus prepubertal females.
[c]Number of peptides matching the identified sequence.
[d]Species corresponding to identified peptide sequences.

TABLE 4. Protein Spots Identified by Mass Spectrometry With Decreased Abundance in COCs Derived From Cycling Compared to Prepubertal Females

Spot[a]	Protein identification	Volume ratio ± SEM[b]	No. of peptides[c]	Sequence coverage (%)	Expected MW	Expected pI	Organism[d]	Accession number
7	Soluble epoxide hydrolase	−1.84 ± 0.289	20	44	62,726.9	6.06	Pig	NP_001001641
5	Stress-induced phosphoprotein 1	−1.563 ± 0.104	16	30	62,542.4	6.4	Mouse	NP_058017
2	Transferrin	−1.39 ± 0.043	23	46	76,901.4	6.73	Pig	CAA30943
18	Apolipoprotein A1	−1.38 ± 0.057	18	53	30,306.7	5.48	Pig	P18648

[a]Location of protein spot on gel.
[b]Volume ratio of proteins extracted from cycling versus prepubertal females.
[c]Number of peptides matching the identified sequence.
[d]Species corresponding to identified peptide sequences.

2007). *PN-1* is increased in oocytes and in granulosa, thecal, and stromal cells isolated from early antral to preovulatory stage follicles (Zhang et al., 2007). Expression of *PA*, *PAI-1*, and *PN-1* increases 6 hr post-hCG stimulation; however, levels of *PAI-1* and *PN-1* decrease prior to ovulation while *PA* levels remain elevated, suggesting that controlled expression of these enzymes direct the timing of ovulation and prevent early proteolysis of follicular walls (Peng et al., 1993; Cao et al., 2006). Increased abundance of PN-1 and PAI-1 in COCs derived from cyclic females may represent mature follicle growth and enhanced protection against early proteolysis and ovulation compared to prepubertal-derived COCs. Alternatively, follicular fluid concentration of PN-1 is higher in nonatretic follicles compared to atretic follicles (Cao et al., 2006); therefore, lower levels of PAI-1 and PN-1 in prepubertal females may indicate that a higher proportion of COCs were isolated from atretic follicles.

Heat stress (Matsuzuka et al., 2004; Camargo et al., 2007) and oxidative stress (Takahashi et al., 2000; Balasubramanian et al., 2007) hinder embryonic development, indicating that regulation of cellular stress is an essential factor in oocyte developmental competence. Heat shock protein 70 (HSP70) is an indicator of cellular stress, as levels are elevated during heat shock in oocytes; this is associated with decreased developmental potential in bovine oocytes (Camargo et al., 2007). Stress-induced phosphoprotein 1 (STI1) mediates the protein maturation and folding activity of HSP70 and HSP90 (Song and Masison, 2005). Abundance of *STI1* increases in cells after heat shock (Nicolet and Craig, 1989; Lassle et al., 1997), although total protein levels are not affected (Lassle et al., 1997). STI1 migrates as a single band during electrophoresis but undergoes modifications after heat treatment, indicated by multiple bands on a Western blot, suggesting that STI1 undergoes stress-induced phosphorylation (Lassle et al., 1997). In the current experiment we resolved two distinct protein spots identified as STI1, suggesting a post-translational modification event. Interestingly, protein spot number 4 was increased in COCs derived from cyclic females and spot number 5 was increased in prepubertal-derived COCs. The data suggest that post-translational modification of STI1 is altered in COCs derived from cyclic versus prepubertal females, and phosphorylation of STI1 may indicate cellular stress in prepubertal-derived porcine oocytes.

Oxidative stress is induced by the accumulation of reactive oxygen species (ROS), such as superoxide anions, hydrogen peroxide, and hydroxyl free radicals (Tarin et al., 1998; Gupta et al., 2006). Decreased oocyte compe-

tence (Tarin et al., 1998; Tatemoto et al., 2000) and embryonic development (Takahashi et al., 2000), and increased apoptosis in oocytes (Tatemoto et al., 2000; Balasubramanian et al., 2007), are associated with oxidative stress. Reduced concentrations of glutathione-*S*-transferase (GST), involved in the maintenance of ROS levels, is a marker of oxidative stress (Neefjes et al., 1999; Hemachand and Shaha, 2003; Tarin et al., 2004; Yousef et al., 2007). In the current experiment, reduced abundance of GST was observed in prepubertal-derived COCs, suggesting prepubertal-derived COCs are unable to respond appropriately to cellular stress.

Transcript abundance of beta-actin (*ACTB*), microfilaments in the ovarian cytoskeleton, decreases after oocyte growth in preovulatory oocytes (Bachvarova et al., 1989). Contradictory studies of ACTB expression in relation to differences in developmental potential have been reported. Abundance of *ACTB* was not significantly different in ovine oocytes derived from prepubertal and adult females (Leoni et al., 2007). However, our 2D-DIGE analysis demonstrated elevated protein abundance of ACTB in COCs derived from cyclic compared to prepubertal females. In accordance with the current experiment, abundance of *ACTB* was decreased in oocytes matured in vitro compared to oocytes matured in vivo (Kim et al., 2004), supporting the hypothesis that reduced ACTB abundance is associated with developmentally incompetent oocytes.

Apoliproprotein A1 (APOA1) is a protein component of high-density lipoproteins (HDL), a cholesterol transporter. APOA1 protein was identified in metaphase II porcine oocytes but not in germinal vesicle stage oocytes (Novak et al., 2004). APOA1 may be synthesized and secreted by the cumulus cells and transported into the oocyte (Hermann et al., 1998; Novak et al., 2004). Plasma concentrations are elevated during the preovulatory stage (Azogui et al., 1992; Tonolo et al., 1995; Brizzi et al., 2003). Lower concentrations of APOA1 and HDL in follicular fluid are associated with increased fertilization and developmental potential of oocytes (Ng et al., 1993; Monisova et al., 2007). Therefore, decreased abundance of APOA1 in COCs derived from cyclic females suggests increased developmental potential compared to prepubertal-derived COCs.

In conclusion, we have identified differential expression of 14 unique proteins correlated with altered developmental potential in COCs derived from cyclic and prepubertal females. Specifically, proteins involved in glycolysis and the regulation of cellular stress and ovulation are increased in COCs derived from cyclic females. As metabolism is a critical component in oocyte quality, aberrant protein abundance of glycolytic enzymes likely result in poor developmental potential of oocytes from prepubertal females. Analysis of cumulus cells for the presence or abundance of glycolytic enzymes could be an efficient, noninvasive method to predict oocyte quality, as growing oocytes are not capable of supporting high glycolytic activity. Proteins involved in cholesterol and iron transport are increased in prepubertal-derived COCs and could suggest the oocytes are still actively growing and are less developmentally competent. Further analysis of transcripts and proteins differentially expressed between good and poor quality oocytes will elucidate mechanisms required for the establishment of good developmental potential. The discovery of biomarkers that are associated with oocyte developmental potential could then lead to the development of noninvasive methods to accurately predict the quality of an oocyte, thereby increasing the percentage of oocytes developing to the blastocyst stage and improving embryo quality.

MATERIALS AND METHODS

For in vitro maturation, fertilization, and culture, all reagents were purchased from Sigma–Aldrich (St Louis, MO) unless otherwise stated. To allow for adequate equilibration of the media to the in vitro culture environment, maturation medium was prepared at least 4 hr prior to the addition of oocytes, and fertilization and culture media were prepared at least 12 hr prior to use.

COC Collection and 2D-DIGE

Porcine ovaries from cross-bred cycling and prepubertal (noncycling) females were obtained from two different abattoirs (Momence Packing Co., Momence IL and Indiana Packers Corp., Delphi, IN, respectively), transported to the laboratory at 30–34°C, and washed with warm saline (0.9% NaCl). Cumulus–oocyte complexes (COCs) were collected by vacuum aspiration of 2–6 mm follicles using an 18 G needle. COCs were washed in protein-free HEPES-buffered synthetic oviductal fluid medium supplemented with 0.1% polyvinylalcohol (PVA; SOF-HEPES), and selected for two or more layers of cumulus cells. COCs were pooled in groups of 400, with three replicates per treatment, and frozen in SOF-HEPES at −80°C. Prior to shipment, samples were thawed, centrifuged for 1 min at 1,000 rpm, the supernatant removed, and the samples refrozen.

Samples were shipped to Applied Biomics on dry ice for 2D-DIGE. Briefly, the cellular pellets were lysed in 30 mM Tris–HCl, pH 8.8, containing 7 M urea, 2 M thiourea, and 4% CHAPS, and sonicated at 4°C. Samples were shaken at room temperature for 30 min followed by centrifugation at 14,000 rpm for an additional 30 min. The supernatants were removed and protein quantification was determined using the Bio-Rad Protein Assay (Hercules, CA).

Protein samples from COCs derived from cyclic females were labeled with Cy5 and samples from prepubertal-derived COCs were labeled with Cy3. Internal controls for each gel were created from a 1:1 mixture of protein from cyclic and prepubertal-derived COCs and labeled with Cy2. Diluted CyDye (1.5 dilution with DMF from 1 nmol/μl stock) was added to each sample, vortexed, and incubated on ice for 30 min in the dark. Lysine (10 mM) was added and the samples were vortexed and incubated for an additional 15 min. Protein samples extracted from the cyclic and prepubertal-derived oocytes and the internal control were mixed and 2× 2D sample buffer (8 M urea, 4% CHAPS, 20 mg/ml DTT, 2% pharmalytes, and trace amount of bromophenol blue), 100 μl destreak solution, and rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mg/ml DTT, 1% pharmalytes, and trace amount of bromophenol blue) were added. An equal concentration of proteins from each sample was loaded onto 13 cm, pH 3–10, linear IPG strips under 1 ml of mineral oil, and isoelectric focusing (IEF) was performed using the IPGphore II (GE Healthcare, Piscataway, NJ) for 12 hr at 20°C at 50 uA/strip, followed by 500 V for 1,000 Vhr, 1,000 V for 2,000 Vhr, and 8,000 V for 22,000 Vhr.

IPG strips were incubated in equilibration buffer 1 (50 mM Tris–HCl, pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS, trace amount of bromophenol blue, and 100 mg/ml DTT) for 15 min with slow shaking. The strips were rinsed with equilibration buffer 2 (50 mM Tris–HCl, pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS, trace amount of bromophenol blue, and 450 mg/ml

iodoacetamide) for 10 min with slow shaking. Strips were rinsed in SDS–gel running buffer and transferred into gradient SDS–gels (9–12% SDS–gel prepared using low florescent glass plates). The gels were sealed with 0.5% (w/v) agarose solution and all three gels were run simultaneously with the Hoefer SE600 (Hoefer, Inc., San Francisco, CA) at 15°C.

Gels were scanned using Typhoon TRIO following the company's protocols and analyzed by Image Quant software (version 5.0). Protein spot normalization and in-gel and cross-gel analyses were performed using DeCyder software (version 6.0) and the ratio change of protein abundance obtained. Sixteen differentially expressed protein spots with a volume ratio greater than 1.3 on at least two out of the three gels were selected and excised using an Ettan Spot Picker. Selected spots were subjected to in-gel trypsin digestion (Promega, Madison, WI), peptide extraction, desalting, and MALDI–TOF/TOF (Applied Biosystems, Piscataway, NJ). Proteins were identified by peptide mass fingerprinting using Mascot (Matrix Science, Inc., Boston, MA) and compared to a mammalian database. Mascot search criteria included one missed cleavage, no fixed modifications, two variable modifications (oxidation and carbamidomethyl), precursor tolerance of 100 ppm, peptide charge of +1, and fragment tolerance of 0.3 Da. Proteins that were identified with greater than a 95% confidence interval and with at least three peptides matching to the predicted sequence were included in the analysis.

ACKNOWLEDGMENTS

This research was supported by the Purdue Research Foundation.

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