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Discovery of putative oocyte quality markers by comparative ExacTag proteomics

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Abstract

Purpose—Identification of the biomarkers of oocyte quality, and developmental and reprogramming potential is of importance to assisted reproductive technology in humans and animals.

Experimental design—PerkinElmer ExacTag™ Kit was used to label differentially proteins in pig oocyte extracts (oocyte proteome) and pig oocyte-conditioned *in vitro* maturation media (oocyte secretome) obtained with high- and low-quality oocytes.

Results—We identified 16 major proteins in the oocyte proteome that were expressed differentially in high- versus low-quality oocytes. More abundant proteins in the high-quality oocyte proteome included kelch-like ECH-associated protein 1 (an adaptor for ubiquitin-ligase CUL3), nuclear export factor CRM1 and ataxia-telangiectasia mutated protein kinase. Dystrophin (DMD) was more abundant in low-quality oocytes. In the secretome, we identified 110 proteins, including DMD and cystic fibrosis transmembrane conductance regulator, two proteins implicated in muscular dystrophy and cystic fibrosis, respectively. Monoubiquitin was identified in the low-quality-oocyte secretome.

Conclusions and clinical implications—A direct, quantitative proteomic analysis of small oocyte protein samples can identify potential markers of oocyte quality without the need for a large amount of total protein. This approach will be applied to discovery of non-invasive biomarkers of oocyte quality in assisted human reproduction and in large animal embryo transfer programs.

Keywords

ATM; Cystic fibrosis; Dystrophin; Infertility; Oocyte

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1 Introduction

The ability to efficiently and reproducibly create embryos *in vitro* that are competent to produce normal offspring is a goal that would facilitate a variety of technological advances in assisted human and animal reproduction, such as *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI), IVF with gender sorted spermatozoa, creation of transgenic embryos, somatic cell nuclear transfer in animals, stem cell technology, cryopreservation and *in vitro* development of embryos followed by non-surgical embryo transfer. Unfortunately, it is often difficult to assess the developmental and reprogramming potential of oocytes or embryos without inflicting irreparable damage to them. Various morphological, biochemical, genomic and proteomic approaches to discover and then characterize novel markers that would permit non-invasive assessments of oocyte and embryo quality have been pursued with modest success.

Pig oocytes, obtainable in much larger quantities than rodent or non-human primate oocytes, are an excellent biomedical model as they mimic some of the temporal events of oocyte maturation and early development observed in humans. For example, pig is the only known animal species besides the rat that makes the major transition from maternal control of development to zygotic control of development during the four-cell stage as in the human [1]. It is relatively easy to create model batches of good- and poor-quality pig oocytes by using an *in vitro* maturation (IVM) system with and without gonadotropins. Before the preovulatory gonadotropin surge, mammalian oocytes are arrested at the dictyate stage of meiosis I. The gonadotropin discharge induces a cascade of events including chromatin condensation, germinal vesicle breakdown, re-initiation of the oocyte cell cycle, expansion of the oocyte-surrounding cumulus mass, first polar body extrusion and second meiotic arrest at metaphase-II [2]. Pincus and Enzmann [3] first observed that mammalian oocytes released from large ovarian follicles spontaneously resume meiosis and many of them are able to reach metaphase of second meiosis when cultured *in vitro*. However, several decades of research have been necessary to optimize maturation media by including gonadotropins, to reach the maximum maturation rate and post-fertilization development achievable *in vitro*. It is now well accepted that the presence of follicle stimulating hormone (FSH) and luteinizing hormone (LH) in the IVM medium is beneficial to IVM, fertilization and post-fertilization development of bovine [4], ovine [5] and murine [6] oocytes. In pig oocyte maturation, FSH in particular stimulates normal oocyte maturation to metaphase-II [7]. Similarly, gonadotropins stimulate IVM of human oocytes [8]. Altered systemic levels of FSH and LH are associated with human reproductive pathologies such as the polycystic ovary syndrome, endometriosis and ovarian hyperstimulation syndrome, and this hormonal imbalance affects oocyte growth and maturation [9]. It is also worth noting that ovarian stimulation with high doses of gonadotropins can result in poor oocyte quality and depletion of the ovarian reserve [10,11]. Indeed, even in livestock, forcing the ovulation of follicles that are not physiologically “ready” gives rise to low-quality oocytes/ embryos – or an insufficient uterine environment – that results in late embryonic loss [12]. Altogether, there is clear evidence that altering the maturation media by varying the level of gonadotropins and altering the base medium will result in differing degrees of subsequent oocyte quality and development of the embryos. This difference in oocyte quality is not due to immaturity of oocytes matured without gonadotropins, which still reach metaphase II at a rate of approximately 55% [13]. It is thus relatively straightforward to generate a physiologically and pathologically relevant animal model of high- and low-quality oocytes by maintaining or withholding gonadotropins from IVM-culture medium. In the present study, such a model has been combined with quantitative proteomic analysis to identify novel biomarkers of oocyte quality. The paucity of reliable oocyte biomarkers hinders the identification of the underlying causes of low oocyte/embryo quality in human and large animal reproductive disorders.

To date, most oocyte quality biomarker studies have been performed by conventional SDS-PAGE analysis and proteomic identification of excised protein bands or spots. These methods have a limited quantitative resolution and require of high total protein content. Perkin-Elmer ExacTag™ Kit, as used in the present study, enables direct, simultaneous quantification of proteins in up to ten samples with extremely low protein content using isobaric mass tags with any tandem mass spectrometer. As described by the manufacturer, Perkin Elmer, ExacTag™ kit is used for labeling proteins through direct conjugation to the thiol group of the cysteine residues or indirectly to Lys-residues through an adapter linker that converts the ϵ -amino group of lysine into a thiol group. Relative abundance of labeled proteins is determined from the strength of the signal for each label in a LC-MS/MS by using free ExacTag™ analysis software. We used this innovative technique to demonstrate that withholding FSH and LH from oocyte maturation medium significantly alters the oocyte proteome. This allowed us to identify a number of proteins differentially expressed in high- and low-quality pig oocytes, some of which could be exploited as non-invasive biomarkers of oocyte quality in assisted human production and in large animal embryo transfer programs.

2 Materials and methods

2.1 Oocyte production

Porcine ovaries were collected at a local slaughterhouse from animals that were not subjected to hormonal stimulation. Ovaries were transported to the laboratory in warm 0.9% saline supplemented with penicillin and streptomycin. Oocytes were collected by aspiration with an 18-gauge needle attached to a 10-mL sterile syringe. Groups of 100 oocytes with high developmental capacity (“high quality oocytes”) were generated by IVM of prophase I oocytes for 44 h in a serum-free TCM-199 medium [14] supplemented with FSH and LH (Gibco BRL; supplemented with 0.1% PVA w/v, 3.05 mM D-glucose, 0.91 mM sodium pyruvate and 10 μ g/mL gentamicin. The following media components were added fresh before use: 0.57 mM cysteine, 0.5 μ g/mL LH, 0.5 μ g/mL FSH, 10 ng/mL epidermal growth factor (EGF)). Oocytes with reduced developmental potential (“low-quality oocytes”) were generated by using the same media lacking gonadotropins. Both high- and low-quality oocytes were matured cumulus-enclosed and the cumulus cells were removed at the end of IVM. We observed high rates of first polar body (PB1) extrusion (>70%) in all experiments, while this rate was only somewhat reduced by gonadotropin withholding (~50–60% PB1). A typical experiment with high starting quality of GV-stage oocytes had the following maturation and development parameters: maturation rate of 75.5% of MII oocytes with gonadotropins ($n=538$) and 59.0% without gonadotropins ($n=114$); blastocyst formation rate of 38.9% in ova fertilized after maturation with gonadotropins ($n=228$) and 25.4% in ova matured without gonadotropins ($n=68$). After maturation, the oocytes and the “spent” culture media were stored at -80°C until ExacTag™ quantitative proteomic analysis was performed. For oocyte storage, zonae were carefully removed by pronase treatment and the zona-free oocytes were deposited in batches of 100 in a minimal volume of protein-free TLH medium in 0.6 μ L Eppendorf tubes. Oocytes in tubes were gently spun for 5 s in a benchtop microcentrifuge at 6000 rpm and the remaining medium was carefully removed by aspiration from tube under a stereomicroscope. Cellular debris was removed from the culture media by centrifugation for 10 min at $2000 \times g$ prior to storage in a final volume of ~500 μ L/tube.

2.2 ExacTag™ quantitative proteomic analysis

Quantitative proteomic analysis was performed by using the ExacTag™ (Perkin Elmer) labeling kit according to the manufacturer’s directions. This kit is designed for high sensitivity, which makes it optimal for work with low concentration protein samples. In

control experiments using different concentrations of bovine serum albumin with ExacTag kit, we were able to detect protein at concentrations as low as 0.001 pmol. The preparation of oocytes was dissolved in 1 mL of 2-D gel loading buffer (2D-Xtract; G Biosciences) made from dried powder and used immediately. The solution also contained 10 mM DTT and a protease cocktail (Protearrest). The solution was incubated at room temperature for 30 min prior to precipitation with six volumes of chilled acetone. The solution was then incubated at -20°C for 4 h. The precipitate was collected by centrifugation at $10\,000 \times g$ for 10 min. The pellet was not allowed to dry and was resuspended in 100 μL of extraction buffer (from the Calbiochem ProteoExtract kit). The solubilized protein was labeled by using the ExacTag labeling kit (Perkin Elmer) according to the manufacturer's recommendations. A volume of 67.2 μL of sample was added to 8.4 μL of dissolution buffer and 8.4 μL of denaturant buffer as formulated in the kit. To this mixture, 1 μL of a 100-mM DTT solution was added and incubated at 95°C for 10 min. The sample was allowed to cool before adding the specific labeling reagents provided in the kit (HG-460 and HG-472), which reacted with the reduced cysteines to attach the mass tag. The reaction was allowed to incubate for 3 h at RT. After incubation, 1 μL of 100 mM DTT was added to quench the reaction and 50 μL was removed from each tube and was mixed together and precipitated with six volumes of cold acetone. The pellet was re-dissolved into extraction buffer (Calbiochem; ProteoExtract) and the proteins were digested with trypsin using the Calbiochem ProteoExtract trypsin digest kit according to the manufacturer's protocol with two modifications: the trypsin in the kit was replaced with Promega's proteomics grade trypsin and the incubation time was extended to 4 h. Proteomic analysis of tagged oocyte proteins was performed as described below for oocyte secretome analysis. The same analysis was conducted twice, each time with batches of 100 ova *per* group (high- and low-quality oocytes).

2.3 NanoSpray LC-MS/MS of oocyte-conditioned media

Culture media for the secretome analysis were recovered immediately after oocyte maturation ended, and cleared of cellular debris by centrifugation for 5 min at 3000 rpm in an IEC Micromax centrifuge. Media were kept frozen until use, and digested with trypsin immediately before proteomic analysis, as described above. The digested peptides were analyzed by using LC-MS/MS using an Agilent 1100 chromatography system coupled to an LTQ mass spectrometer. Ten microliters of peptides were injected using an auto-sampler. The peptides were first trapped onto a C-18 peptide CapTrap (Michrom) at high flow rate (0.5 mL/min) for 10 min. Then, the flow was switched using the 8-port valve on the LTQ to allow the trapped peptides to elute onto a 0.050×100 mm C-18 capillary column (Agilent) using a split flow rate of ~ 400 nL/min. The peptides were eluted using a 40–90% gradient of ACN over 100 min. The MS/MS spectra were collected using Xcalibur 2.2 software. Identifications were made by using BioWorks 3.2 software. The threshold for DTA file formation was set at 200 with a peptide tolerance of 0.5 Da. The resulting BioWorks search was filtered for Xcorr scores of 1.5, 2.0 and 2.5 for $m = 1, 2$ and 3, respectively. The resulting matches were used to compile a FASTA file for quantification using the ExacTag software 3.0 according to the manufacturer's recommendations.

2.4 Proteomic data analysis and comparisons

Protein IDs and quantitative data generated by ExacTagTM labeling were compared between high- and low-quality oocyte groups by using ExacTagTM software version 3.0, which incorporates analytical tools and an integrated Mascot Protein Search. The most significant hits at $p < 0.05$ were evaluated between treatments. Each experiment was performed in duplicate. Such analyses were performed separately for the oocyte proteome (*i.e.* the cytosolic protein extracts of high/low-quality oocytes) and for the oocyte secretome (*i.e.* medium recovered from high/low-quality oocyte culture). The presence of some of the

identified high/ low oocyte marker proteins were confirmed by Western blotting and immunocytochemistry. To address the paucity of sequence data in the porcine genome/ proteome, we applied *de novo* sequencing using Peaks (BioInformatics Solutions BSI) software in conjunction with other search engines. The software allows the user to combine searches with homology searches performed using SPIDER.

2.5 Antibodies for Western blotting and immunocytochemistry

Anti-CFTR (cystic fibrosis transmembrane conductance regulator) mouse IgM (Abcam cat #ab2784) was raised against a synthetic peptide corresponding to amino acid residues 103–117 within the extracellular loop of human CFTR [15], and recognizes a predominant band of ~25 kDa in oocyte extracts. The same antibody has been shown to recognize the full length CFTR (~170 kDa) in mouse and human sperm extracts [16]. It is thus possible that the protein detected in porcine ova is a fragment of the extra-cellular loop of partially degraded CFTR protein. Anti-dystrophin rabbit IgG (Abcam #ab15277) was raised against a synthetic peptide corresponding to amino acids 3661–3677 of human DMD protein (~430 kDa) and recognizes a band migrating in the appropriate range (*i.e.* on top of 10% gel, above the highest-migrating molecular marker) in oocyte extracts resolved on 10% gels. The anti-ATM (ataxia-telan-giectasia mutated) mouse IgG (cat. #2C1; GeneTex, San Antonio, TX, USA) was raised against truncated recombinant protein corresponding to amino acids 2577–3056 of human ATM and shown to be specific to ATM alone by Western blotting and immunoprecipitation [17,18]. In the present analysis, this antibody recognized two specific bands of ~60 kDa and ~150 kDa in oocyte extracts resolved on a 10% gel, that probably resulted from the fragmentation of the nascent ~370 kDa ATM protein under the conditions described below for the extraction of oocyte proteins. At present, we cannot exclude a posttranslational cleavage or alternative splicing being responsible for these fragments. However, given the extensive characterization of this antibody in previous studies, it is not likely that the observed bands were a result of non-specific binding to a protein other than ATM. A prominent ATM fragment of ~150 kDa has been reported to result from caspase-mediated cleavage of full length ATM protein [19,20]. Interestingly, the proteolytic fragments of ATM retain their ability to bind to DNA [20]. Anti-FBN1 mouse IgG (Abcam #ab3090) was raised against amino acids 451–909 of human fibrillin-1 (FBN1) and its specificity is well established. FBN1 is a glycoprotein of ~320–350 kDa that contains multiple EGF-like repeats and cysteine-rich TB repeats. The antibody is highly specific to FBN1 as it recognizes a unique domain not shared with fibrillin-2 and other related proteins. It is well established that FBN1 is posttranslationally processed by enzymatic cleavage of both the C-terminal domain and the leader sequence [21]. This processing is the likely reason why the above antibody recognized a band of ~55 kDa in oocyte extracts in our experiments. This band is consistent with FBL1 intermediate of ~55 kDa described by Kielty *et al.* [22]. After proteolytic cleavage, only the 55 kDa FBN1 but not the nascent 350 kDa protein, is observed [22]. Rabbit anti- kelch-like ECH-associated protein 1 (KEAP1) antiserum was raised against full-length recombinant KEAP1 protein [23]. This serum recognizes the anticipated KEAP1 band of ~65 kDa as well as several bands of higher mass, which are thought to be inducible KEAP1 forms with retarded mobility on SDS-PAGE [23]. Alternatively, some of the KEAP1 bands observed in oocyte extracts could be attributed to KEAP1 ubiquitination [24]. Anti-ubiquitin mouse IgG MK-12-3 (MBL International, Wooburn, MA, USA) was raised against purified bovine erythrocyte ubiquitin. Anti-ubiquitin mouse IgM FK-1 (Biomol, Plymouth Meeting, PA, USA) was raised against a crude preparation of ubiquitinated lysozyme. Anti- β -actin rabbit IgG for loading control was purchased from Abcam (ab8227).

2.6 Western blotting

For oocyte-conditioned media samples, media were recovered from culture dishes conditioned by equal numbers of oocytes for individual treatments. Media were kept frozen until analysis, and diluted and boiled in Laemmli loading buffer containing 50 mM Tris (pH 6.8), 150 mM NaCl, 20% glycerol, 2% SDS, 5% β -mercaptoethanol, 1 mM PMSF and 0.01% bromophenol blue. Protein concentration was normalized by using Bradford assay. For oocyte protein analysis, zonae were removed by pronase and oocytes were deep frozen in aliquots of 100/tube. Samples were boiled with Laemmli loading buffer as described above. Gel electrophoresis was performed in 10% Tris-Glycine gels (Cambrex Bio Science, Rockland, ME, USA) by loading 10 μ M protein *per* lane and separated proteins were transferred to PVDF membranes (Millipore, Bedford, MA, USA) using an Owl wet transfer system (Fisher Scientific, Houston, TX, USA) at a constant 50 V for 4 h. The membranes were sequentially incubated with 10% non-fat milk (1 h), the primary antibody (1/1000 to 1/2000 dilution, overnight), HRP-conjugated species-specific secondary IgG (1/10 000 dilution, 1 h) and chemiluminescent substrate (SuperSignal, Pierce, Rockford IL, USA). Membranes were exposed on Kodak film and developed by using an automated developer. Relative density of major bands was measured by using a Kodak Densitometry System. Band density data were entered in MS-Excel tables and compared between treatments by ANOVA using SAS version 8.2. β -Actin (ACTB) antibody was used for loading control and normalization of densitometric data.

2.7 Immunocytochemistry

Zonae from oocytes were removed by pronase and the oocytes were fixed in 2% formaldehyde [25]. Zona-free ova were permeabilized with 0.1% Triton X-100 and incubated sequentially with the primary antibody followed by a mixture of fluorescently conjugated, secondary species specific IgG and 5 μ g/mL DAPI, a blue fluorescent DNA stain (Molecular Probes). Ova were washed and mounted on coverslips in VectaShield anti-fade medium. Slides were examined by using a Nikon Eclipse E800 microscope equipped with infinity-corrected 40 $\times$, 60 $\times$, and 100 $\times$ lenses and a high-resolution CCD camera (Cool Snap CCD), operated by MetaMorph 4.6 software. For each treatment and antibody, the experiments were repeated twice with 20–30 ova *per* treatment group. The first antibody was replaced by a non-immune serum of an appropriate species for negative controls. Preimplantation embryos shown in Supporting Information Figs. 1 and 2 were generated using conventional IVF and embryo culture methods described previously [26].

3 Results

This study was undertaken with the hope of identifying potential non-invasive, quantifiable biomarkers to be found within the proteome or secretome of the predicted high- or low-quality porcine oocytes. Proteome is defined for the purpose of this study as the content of cytosolic proteins assessed in an equal number of high- and low-quality oocytes by quantitative ExacTagTM proteomics. Secretome is defined as a quantifiable amount of proteins secreted in the culture medium by the oocyte–cumulus complexes during oocyte maturation from GV stage (prophase I) to metaphase II.

We generated a quantitative proteome of predicted “high” and “low” quality porcine ova. High-quality oocytes were obtained by maturation in a serum-free TCM-199 medium supplemented with FSH and LH. Low-quality oocytes were matured in the same maturation medium, but without gonadotropins. Extracts of high/low-quality oocyte batches then were compared by using the ExacTagTM technology. A total of 503 significant protein IDs were recorded in the combined proteome of high- and low-quality oocytes (Supporting Information Table). Sixteen predominant proteins (Table 1) were identified by ExacTagTM

that differed in quantity between oocytes of high and low predicted developmental competence (Fig. 1). The proteome analysis was performed on two independently generated groups of high- and low-quality oocytes. Nine out of these 16 proteins showed the same tendencies between high- and low-quality ova in each replicate, meaning that if the protein in question was higher in high-quality ova in the first replicate, it was also higher in high-quality ova in the second replicate. Table 1 demonstrates that the differences in relative abundance of individual proteins between high- and low-quality ova were significantly more pronounced in the first trial than in the second trial, which can be attributed to day-to-day differences in oocyte quality, influenced by timing of ovary harvest and weather conditions.

To examine if any of the proteins identified in Table 1 could be secreted by the maturing oocytes and/or by the surrounding cumulus cells, we harvested the oocyte–cumulus complex-conditioned medium and subjected it to Nanospray LC-MS/MS analysis. We found a total of 110 statistically significant protein IDs in the IVM medium conditioned with oocyte–cumulus complexes, but not in the control medium unexposed to maturing oocytes (Table 2). Eight out of 16 proteins identified as differentially expressed in the oocyte proteome were also abundant in the IVM secretome (Fig. 1). The most abundant secretome proteins were major proteins related to cellular physiology and pathology. Among them, dystrophin (DMD) and CFTR protein were readily detectable in oocytes by immunofluorescence (Figs. 2A–D), along with several proteins present in both oocyte secretome and proteome. The CFTR protein showed a striking association with the oolemma. While this pattern of CFTR distribution could suggest localization in the perivitelline space and possible secretion (consistent with CFTR presence in the secretome samples), it is most likely a reflection of CFTR insertion in the oolemma. The oocyte content of CFTR protein measured by densitometry against ACTB was as much as 41% higher in high-quality oocyte extracts than in low-quality oocyte extracts (Fig. 2E). Based on immunohistochemistry, CFTR was diffusely distributed in the ooplasm of oocytes in preantral and small antral follicles, and accumulation at the oolemma became obvious only in large antral follicles. A high content of CFTR was also detected in mural and cumulus granulosa cells (Fig. 3), suggesting that the cumulus cells in particular could also contribute CFTR to the IVM secretome. Interestingly, granulosa cells and oocytes of atretic follicles showed lower intensity of labeling with anti-CFTR antibody than healthy follicles (Fig. 3). Dystrophin was detected in the ooplasm, sometimes in a polarized manner reflecting the polarized distribution of cytoplasmic inclusions/organelles (Figs. 2C and C'). Very low levels of dystrophin labeling were detected in granulosa cells on ovarian tissue sections, which in some cases showed dystrophin accumulation in the oocyte cortex (Fig. 3). Most likely, dystrophin in the IVM secretome was contributed by the oocyte rather than by cumulus cells.

Three of the abundant proteins identified in the oocyte secretome and proteome (compare Table 2 with Supporting Information Table), including fibrillin (FBN1), KEAP1 and ATM protein kinase were further examined by semi-quantitative, densitometry-based Western blotting (Fig. 4). Relative Western blot band density of ATM, KEAP1 and FBN1 was compared with band density of β -actin in high/low-quality oocyte extracts. Consistent with the ExacTag data, KEAP1 protein showed an increase of 42.2% in high-quality ova (KEAP1-ACTB ratio of 0.0064 in low-quality ova *versus* 0.0091 in high-quality ova) and ATM protein also showed some, albeit a modest increase in good oocytes. Importantly the FBN1 protein, found in the secretome, had a ratio to ACTB of 0.125 in low-quality oocytes as opposed to FBN1/ACTB ratio of 0.187 (*i.e.* an increase of 49.6%) in high-quality oocytes (Fig. 4). We next examined the localization of these proteins in porcine oocytes by immunofluorescence. While FBN1 and KEAP1 showed diffuse ooplasmic distribution (Supporting Information Figs. 1 and 2), ATM was concentrated in a large granular pattern extending from high concentration in the oocyte cortex (Fig. 5). No such pattern was

observed in cumulus cells. Nuclear export factor CRM1, previously studied in porcine and murine oocytes and zygotes [27], was also among the highly abundant proteins in the high-quality oocytes (Fig. 1).

To our knowledge, the only protein identified so far as a non-invasive biomarker of embryos with high developmental potential that is found in both the mouse and human embryo secretome is ubiquitin. Ubiquitin was found in the culture media conditioned by high-quality human or mouse embryos but not in the media conditioned by poor embryos [28]. In our studies, we detected unconjugated 8.5 kDa monoubiquitin predominantly in the oocyte maturation media conditioned by the low-quality oocytes, while high mass bands, representing the polyubiquitinated proteins were detected predominantly in the media conditioned by high-quality oocytes (Fig. 6).

4 Discussion

Two of the most significant, potential biomarkers of oocyte quality identified here, CFTR and DMD are highly important disease-related proteins. The CFTR protein is a transmembrane chloride channel belonging to ABC family of transporter proteins [29]. Recessive autosomal mutation of CFTR causes the pulmonary disease cystic fibrosis (CF) [30]. CF is associated with male infertility due to congenital absence of the vas deferens. This may have an effect on sperm fertilizing ability, as shown in CFTR mutant mice [16]. Female CF patients are frequently infertile, with increased maternal morbidity and mortality [31]. It is not clear, however, whether this female infertility is caused by abnormal oocyte structure and function. We have not found reports of CFTR expression in mammalian oocytes, though overexpression of CFTR *via* cRNA/mRNA injection into *Xenopus* eggs is a popular model in CF research [32]. In the present study, CFTR content was increased in the high-quality oocyte proteome in addition to being present in the oocyte-cumulus cell secretome. It is possible that CFTR channels, present on the sperm equatorial segment [16] and on the oolemma (this study) participate in sperm-oolemma interactions during fertilization, or support the functionality and structural organization of sperm plasmalemma and oolemma. The present study provides grounds for further investigation into the possible role of CFTR transmembrane channel regulator in fertilization, both on sperm and oocyte side, and in embryo development.

Dystrophin (DMD) content was increased in the low-quality oocyte proteome. Also, it appeared that the oocytes within the atretic follicles (Figs. 3F–F'') had a stronger DMD labeling than oocytes in healthy follicles. Inversely, only weak labeling of CFTR was associated with granulosa cells and oocytes in atretic follicles. Deletions and point mutations of the *Dmd* gene are associated with Duchenne muscular dystrophy and with a group of related skeletal and cardiac muscle diseases. Dystrophin is the protein component of dystrophin-glycoprotein complex necessary to maintain the connection between the extracellular matrix and the muscle plasma membrane, the sarcolemma [33]. Reduced ovarian reserve and poor response to ovarian hyperstimulation have been reported in women suffering from myotonic dystrophy 1, a type of muscular dystrophy caused by mutation of dystrophin related *Mdy1*, a myotonin kinase gene [34]. However, there is no indication of infertility in patients with Duchenne muscular dystrophy. Dystroglycan, a transmembrane protein component of the dystrophin-associated glycoprotein, is important for oogenesis in *Drosophila* [35], but we have not found reports of dystrophin or dystroglycan in mammalian oocytes.

Among 16 oocyte proteins identified by ExacTag, ATM, DMD, ryanodine receptor (RyR) and five other proteins were also identified in the oocyte/IVM secretome. This suggests that oocyte-synthesized proteins are secreted in IVM medium during oocyte maturation. It is

possible that the extent of secretion is commensurate with the abundance of these proteins in the ooplasm. Alternatively, cytoplasmic fragmentation in poor-quality ova could cause massive release of some of these proteins even if they lack a signal sequence for secretion. Not to be overlooked is the contribution of cumulus cells to the examined IVM secretome.

Among the proteins that showed the most obvious difference between high- and low-quality oocyte proteomes was nuclear export factor CRM1 (XPO1/exportin 1). The CRM1 protein, which was as much as two times more abundant in high-quality ova, is a central receptor in nuclear export pathway involved in the assembly of 40S and 60S ribosomal subunits [36]. Of particular interest to gamete and embryo biology is the implied involvement of CRM1 in the shuttling of DNA methyltransferase 1 between nuclei and cytoplasm of mouse zygotes and two-cell embryos [37]. Data generated in mouse suggest that by controlling the movement of DNA methyltransferase 1, CRM1 may have an effect on the maintenance of DNA methylation patterns in the early embryo [37]. A specific inhibitor of CRM1, LBM1 blocks embryo development at the activation of the embryonic genome (two-cell stage in mouse, four-cell in pig) [27]. It is thus possible, and worthy of additional inquiry, that the oocyte levels of CRM1 have bearing on developmental and reprogramming potential of oocytes in the mouse and in other species, including pigs and humans.

Transcriptome analyses revealed that the altered expression of genes in the ubiquitin-proteasome pathway (UPP) is associated with poor developmental competence of non-human primate oocytes and embryos [38]. Our previous proteomic and biochemical studies of porcine oocytes and zygotes revealed at least three physiologically relevant, highly abundant proteins with turnover controlled by UPP that may be important for developmental competence of mammalian oocytes and embryos. These include the cell protecting, drug-resistance related factor, the major vault protein [39], ubiquitin-C-terminal hydrolase UCHL1 (PGP9.5; [40]), and mitochondrial transcription factor-1 (TFAM) [41]). In addition to the identification of ubiquitin in porcine oocyte secretome, at least two of the 16 ExacTag-identified oocyte-proteome proteins, ATM and KEAP1 are directly involved in the regulation of the UPP. The *Atm* gene, which is mutated in humans suffering from a sterility-associated neurological disorder called ataxia-telangiectasia (A-T), encodes the ATM-protein, a kinase involved in DNA repair [42] and in the regulation of UPP [43]. In our analysis, ATM protein was upregulated in high-quality oocytes. Localization of ATM shows accumulation in large clusters and numerous granule-like structures in the oocyte cortex, distinct from the cortical granules that undergo exocytosis following fertilization. The KEAP1 protein is a substrate adaptor protein for the E3-type ubiquitin-ligase CUL3, that marks other proteins for proteasomal degradation [44]. Similar to ATM, KEAP1 is upregulated in high-quality oocytes examined by ExacTagTM.

Other notable oocyte proteins with a connection to UPP have been identified in our ExacTagTM trial: RyR, upregulated in the high-quality oocytes, is involved in calcium signaling during fertilization and has been shown to be translated only during the final stages of bovine oocyte maturation [45]. The number of RyR receptors is thought to be regulated by UPP in skeletal muscle [46]. The IP3- receptor, which is also involved in oocyte calcium response during fertilization, is regulated by UPP in mouse ova [47]. Extensive literature exists on the role of the EGF-receptor in ovarian function and its localization in oocyte and granulosa cells has been documented (*e.g.* [48]). The downregulation of EGF-receptor is mediated by UPP [49]. Nuclear export factor CRM1 (XPO1), discussed above, is known to directly interact with HECT type E3 ubiquitin ligase SMURF1 during TGF- β -signaling [50], and is essential for swine embryo development beyond the four-cell stage [27]. The mRNA for β 1-adrenergic receptor, which was more abundant in low-quality oocytes, is present in mature *Xenopus* oocytes and down regulated after fertilization [51]. This receptor protein is down regulated by UPP in response to

prolonged stimulation with agonists of the G protein-coupled receptors [52]. Albumin is commonly used as a substrate for ubiquitination and proteasomal degradation assays. Connective tissue growth factor is expressed in rat granulosa cells, with highest expression in cumulus/ corona cells immediately surrounding the oocyte [53]. It was less abundant in normal oocytes. Dystrophin, less abundant in high-quality ova, is a substrate of UPP during physiological and pathological muscle fiber degradation [54]. As discussed above, the dystrophin-associated dystroglycan complex affects oocyte polarity in *Drosophila* [35]. Cortactin-binding protein, more abundant in high-quality ova, interacts with actin-binding protein cortactin, present in *Xenopus* oocytes [55]. Fatty acid-synthase expression has not been reported in mammalian oocytes, but is known to interact with ubiquitin-specific protease 2a and other components of UPP [56]. Mannose-6-phosphate/IGF II receptor, IS10 transposase, glutaminase and hydroxyvitamin D3-hydroxylase has not been reported in the oocyte or in connection with UPP.

Some of the above-described putative biomarkers of oocyte quality were found to migrate at less than predicted mass in SDS-PAGE. As elaborated in Section 2.5, the protein fragments found for ATM and FBN1 have been shown conclusively to originate from posttranslational processing or degradation of the full length nascent proteins [19–22,57]. Consequently, it is not likely that the Western blotting patterns of these proteins in oocyte extracts are due to non-specific antibody–protein reactions. More likely, these proteins are partially degraded in the oocyte in preparation for a widespread proteolysis of maternally stored proteins that occurs after oocyte activation by the fertilizing spermatozoon. We have shown such a pattern of post-fertilization proteolysis for at least two abundant oocyte proteins that are turned over by the UPP, including the major vault protein of the vault particle [39] and ubiquitin-C-terminal hydrolase UCHL1 [58]. The targeted, widespread degradation of maternally stored ooplasmic proteins during oocyte maturation or shortly after fertilization has been documented in detail in invertebrates [59,60]. Our results suggest that in addition to maternal mRNA degradation in the zygote, similar “wiping of the slate” also occurs at the protein level in mammals.

In conclusion, the isobaric tag-analysis proved to be a powerful tool for the discovery of potential oocyte quality markers. Further efforts will be focused on the optimization of ExacTag™ analysis of culture media conditioned by oocytes and embryos of varied quality.

Clinical Relevance

The ability to efficiently and reproducibly create embryos *in vitro* that are competent to produce normal offspring is a goal that would facilitate a variety of technological advances in assisted human and animal reproduction, such as assisted fertilization and embryo cryopreservation in humans, and creation of transgenic embryos, somatic cell nuclear transfer and non-surgical embryo transfer in animals. Unfortunately, it is often difficult to assess the developmental and reprogramming potential of oocytes or embryos without inflicting irreparable damage to them. Various approaches to discover novel non-invasive markers of oocyte and embryo quality have been pursued with modest success. In the present study, we have identified 16 proteins expressed differentially in high-*versus* low-quality porcine oocytes, using the Perkin Elmer, ExacTag™ kit for differential labeling of proteins in quantitative tandem mass spectrometry. We also found that poor-quality oocytes secreted several proteins implicated in human disease, such as dystrophin and cystic fibrosis transmembrane conductance regulator. Altogether, our data show how a direct, quantitative proteomic analysis of small oocyte protein samples can identify potential markers of oocyte quality without the need for SDS-PAGE of large amount of total oocyte protein. Some of the identified proteins could be exploited as non-invasive

biomarkers of oocyte quality in assisted human production and in large animal embryo transfer programs.

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Abbreviations

ATM	ataxia-telangiectasia mutated
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
EGF	epidermal growth factor
FBN1	fibrillin-1
FSH	follicle stimulating hormone
ICSI	intracytoplasmic sperm injection
IVF	<i>in vitro</i> fertilization
IVM	<i>in vitro</i> maturation
KEAP1	kelch-like ECH-associated protein 1
LH	luteinizing hormone
RyR	ryanodine receptor
UPP	ubiquitin-proteasome pathway

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Figure 1.

Sixteen proteins identified by ExacTagTM as differentially expressed in high- versus low-quality porcine oocytes. Complete annotations are shown in Table 1. Proteins that were also identified in oocyte secretome are annotated in gray. Numeric values (no units) represent the relative abundance of the identified proteins in 100 high/low-quality oocytes based on ExacTagTM analysis. Data from one replicate are shown.

Figure 2. Identification of CFTR and DMD in porcine metaphase II oocytes by immunocytochemistry (A–D) and Western blotting (E). (A, B) CFTR localizes distinctly to the oolemma. (C) Diffuse cytoplasmic labeling of DMD. (D) Negative control with a non-immune rabbit serum and anti-rabbit IgG. Corresponding perfocal differential interference contrast (DIC) images are shown in panels A'–D'. (E) Western blotting of CFTR in bad (lanes 1 and 2) and good (lanes 3 and 4) oocytes. Membrane was reprobed with ACTB antibody and subjected to densitometric analysis. CFTR/ACTB ratio shows an increase of 6–41% in good oocytes, depending on which good/bad oocyte lanes are paired for comparison.

Figure 3.

Immunolocalization of CFTR (red; left column) and DMD (green; center column) in porcine ovarian tissue sections. (A–A'') Small preantral follicles display similar intensity of diffuse labeling of CFTR and DMD in oocytes and granulosa cells. (B–B'') Large preantral follicle shows signs of CFTR and DMD translocation toward oocyte cortex. (C–C'') Early stage antral follicle; note the increased DMD labeling in oocyte, compared to surrounding granulosa cells. (D–D'') Oocyte surrounded by healthy cumulus cells inside a large antral follicle. Note the distinct localization of CFTR in the oolemma. (E–E'') Detail of oolemma labeling (arrowheads) in a similar oocyte inside a large antral follicle. (F–F'') Adjacent healthy (left) and atretic (right) follicles show inverse patterns of CFTR and DMD labeling, with CFTR labeling intense in healthy follicle's granulosa cells and oocyte (left arrowhead), and weak in the atretic follicle with apparently apoptotic granulosa cell nuclei, wherein the oocyte (right arrowhead) and granulosa cells are both intensely labeled. (G–G'') Negative control with a mixture of non-immune mouse and rabbit sera followed by fluorescently conjugated anti-mouse IgM and anti-rabbit IgG. Inset shows a Western blot of oocyte extract with anti-DMD antibody.

Figure 4.

Western blotting and quantitative densitometry of ATM, KEAP1 and fibrillin (FBL). Protein membranes with lanes with equivalent loads of high-quality oocyte (H) and low-quality oocyte (L) protein extracts were probed with antibodies against ATM, KEAP1 and FBL, then stripped and re-probed with antibody against actin B (ACTB), a housekeeping protein standard for densitometry and protein loading. All three proteins were detected in both oocyte proteome and secretome. ATM and KEAP1, but not FBL, were among 16 proteins identified as differentially expressed by ExacTag. Both ATM and KEAP1 proteins were more abundant in high-quality oocyte extracts.

Figure 5. Detection and localization of ATM in porcine metaphase II oocytes. (A, B) Immunofluorescence of ATM (red), superimposed onto parfocal DIC images, reveals clusters of ATM-positive granules in cortical ooplasm. (C) Western blot of ATM in extracts of 170 ova (lane 1) and zona pellucida protein from 120 ova (lane 2, negative control).

Figure 6.

Detection of ubiquitin in media conditioned by high- and low-quality oocytes using antibodies MK-12-3 (A) and FK-1 (B; reprobing of a membrane shown in (A)). Ubiquitin is an 8.5 kDa, 76-aminoacid protein, a mass corresponding to the band detected in good oocyte medium (arrowhead). Lane pairs 1 and 2 and 3 and 4 are from two separate replicates of oocyte maturation. Lanes 1 and 3 = maturation medium exposed to high-quality oocytes; lanes 2 and 4 = maturation medium exposed to low-quality oocytes.

Table 1
Protein IDs of 16 oocyte proteins expressed differentially in the high and low quality oocyte proteomes

Protein identifier	Name	No. of peptides	Mean	Low Q #1	Low Q #2	High Q #1	High Q #2	Score
1 gi 19919838 gb AAM08402.1 AF490841_1	Glutaminase [<i>Sus scrofa</i>]	2	374.92281	0.1871	0.2436	0.3334	0.2359	26.1
2 gi 24528344 emb CAD56043.1	Hypothetical protein [<i>Sus scrofa</i>]	3	308.94739	0.2247	0.2495	0.3088	0.2169	28.08
3 gi 2796173 gb AAB97525.1	β -1 adrenergic receptor [<i>Sus scrofa</i>]	3	319.57778	0.3887	0.205	0.2157	0.1906	28.09
4 gi 6581131 gb AAF18474.1 AF210153_1	Nuclear export factor CRM1 [<i>Sus scrofa</i>]	2	386.84	0.1688	0.2384	0.3428	0.2501	28.9
5 gi 47522816 ref NP_999160.1	25-Hydroxyvitamin D3 1 α -hydroxylase [<i>Sus scrofa</i>]	2	299.25456	0.3333	0.2136	0.249	0.2041	36.09
6 gi 51870493 emb CAG15151.1	Kelch-like ECH-associated protein 1 [<i>Sus scrofa</i>]	3	385.47212	0.2146	0.2512	0.312	0.2221	38.07
7 gi 5738092 gb AAD50250.1 AF162223_9	IS10-right transposase [<i>Shigella flexneri</i>]	2	297.42333	0.2206	0.2578	0.3026	0.219	42.11
8 gi 52353332 ref NP_001005208.1	Albumin [<i>Sus scrofa</i>]	2	270.30917	0.203	0.2498	0.2884	0.2587	52.09
9 gi 2317892 gb AAC48756.1	Connective tissue growth factor [<i>Sus scrofa</i>]	2	267.655	0.326	0.2122	0.2683	0.1936	66.07
10 gi 164648 gb AAA31119.1	Ryanodine receptor [<i>Sus scrofa</i>]	6	357.45188	0.194	0.2316	0.3293	0.2452	68.08
11 gi 58416122 emb CAI26302.1	Dystrophin [<i>Sus scrofa</i>]	2	337.19333	0.3258	0.2096	0.2558	0.2088	80.08
12 gi 38322755 gb AAR16306.1	Cortactin-binding protein 2 [<i>Sus scrofa</i>]	3	602.28125	0.2124	0.2404	0.3043	0.2428	104.1
13 gi 61676044 gb AAX51683.1	Fatty acid synthase [<i>Sus scrofa</i>]	3	320.32816	0.1802	0.228	0.3413	0.2506	144.09
14 gi 47522840 ref NP_999172.1	Epidermal growth factor receptor [<i>Sus scrofa</i>]	2	260.23227	0.1933	0.2276	0.3302	0.2489	186.08
15 gi 16506695 gb AAL23907.1 AF339885_1	Mannose-6-phosphate/insulin-like growth factor II receptor [<i>Sus scrofa</i>]	2	641.405	0.3325	0.2027	0.2339	0.231	194.09
16 gi 46810446 gb AAT01608.1	Ataxia-telangiectasia mutated protein [<i>Sus scrofa</i>]	5	291.1325	0.2027	0.2427	0.3202	0.2345	214.08

Table 2

Protein IDs of 111 most abundant proteins found in the secretome of porcine oocyte–cumulus complexes

Accession	Mass	Score (%)	Coverage (%)	Query matched	Name	UC score
1 gi 47523672	40 246	66	37	22	2'-5' Oligoadenylate synthetase [<i>Sus scrofa</i>]	66
2 gi 47524464	40 727	66	25	18	2'-5' Oligoadenylate synthetase 1 [<i>Sus scrofa</i>] [MASS =40726]	66
3 gi 47523686	97 157	67	13	18	Androgen receptor AR [<i>Sus scrofa</i>]	67
4 gi 46810446	350 436	83	18	72	Ataxia-relangiectasia mutated protein [<i>Sus scrofa</i>] [MASS =350434]	83
5 gi 55741486	223 109	66	27	64	β -Myosin heavy chain [<i>Sus scrofa</i>]	66
6 gi 47523000	53 787	63	4	6	Calcitonin receptor-like receptor [<i>Sus scrofa</i>]	63
7 gi 17226307	53 656	63	4	6	Calcitonin receptor-like receptor [<i>Sus scrofa</i>] [MASS =53656]	63
8 gi 1937	565 209	66	10	57	Calcium release channel [<i>Sus scrofa</i>] [MASS =565197]	66
9 gi 47522612	126 658	66	19	25	Catalytic subunit of G- $\beta\gamma$ -activated [<i>Sus scrofa</i>]	66
10 gi 47522672	84 790	65	18	13	CD18 leukocyte adhesion molecule [<i>Sus scrofa</i>]	65
11 gi 58530634	54 784	67	23	21	Cell division cycle 20 [<i>Sus scrofa</i>] [MASS =54783]	67
12 gi 47523438	79 125	65	23	18	cGMP-gated cation channel [<i>Sus scrofa</i>]	65
13 gi 52694696	56 846	61	16	13	Cholesterol 7 α -hydroxylase [<i>Sus scrofa</i>]	61
14 gi 61097883	101 234	71	27	28	Class 3 phosphoinositide-3-kinase [<i>Sus scrofa</i>]	71
15 gi 47523048	256 078	86	17	70	Coagulation factor V [<i>Sus scrofa</i>]	86
16 gi 47523630	93 062	66	16	26	Complement component C7 [<i>Sus scrofa</i>]	66
17 gi 47522844	186 806	66	17	37	Complement component C3 [<i>Sus scrofa</i>]	66
18 gi 48675959	188 615	66	16	29	Complement component C5 [<i>Sus scrofa</i>]	65
19 gi 38322755	164 982	66	22	44	Cortactin-binding protein 2 [<i>Sus scrofa</i>] [MASS =164982]	66
20 gi 46452255	168 187	85	18	33	Cystic fibrosis transmembrane conductance regulator [<i>Sus scrofa</i>] [MASS =168187]	85
21 gi 38322754	168 203	85	18	33	Cystic fibrosis transmembrane conductance regulator, ATP-binding cassette (sub-family C, member 7) [<i>Sus scrofa</i>] [MASS =168203]	85
22 gi 47523114	36 454	66	29	20	Cytosolic malate dehydrogenase [<i>Sus scrofa</i>]	66
23 gi 47523212	53 354	75	16	8	Decay-accelerating factor CD55 [<i>Sus scrofa</i>]	75
24 gi 47523520	96 118	66	19	23	Delayed rectifier potassium channel Kv2.1 [<i>Sus scrofa</i>]	66
25 gi 47522904	111 424	65	25	34	Dihydropyrimidine dehydrogenase [<i>Sus scrofa</i>]	65
26 gi 47523582	88 242	71	8	9	Dipeptidyl peptidase IV [<i>Sus scrofa</i>]	71
27 gi 66363554	182 388	67	22	48	DNA methyltransferase 1 [<i>Sus scrofa</i>] [MASS =182388]	67
28 gi 47523002	177 860	66	15	35	Dual oxidase 1 [<i>Sus scrofa</i>]	66

Accession	Mass	Score (%)	Coverage (%)	Query matched	Name	UC score
29 gi 58416122	424 860	74	19	90	Dystrophin [<i>Sus scrofa</i>]	74
30 gi 35384836	32 884	62	27	14	Electron transfer flavoprotein α -subunit precursor [<i>Sus scrofa</i>] [MASS =32884]	62
31 gi 47522810	34 940	65	13	5	Estrogen sulfotransferase [<i>Sus scrofa</i>]	65
32 gi 48976131	312 699	66	12	49	Fibrillin 1 [<i>Sus scrofa</i>]	66
33 gi 47523826	78 173	67	13	14	Follicle-stimulating hormone receptor [<i>Sus scrofa</i>]	67
34 gi 47523806	33 667	66	21	9	Four and a half LIM domains 1 protein, isoform C [<i>Sus scrofa</i>]	66
35 gi 11125367	38 009	66	31	18	Four and a half LIM domains 1 protein, isoform C [<i>Sus scrofa</i>] [MASS =38009]	66
36 gi 40353111	4402	66	37	16	Fructose-bisphosphate aldolase A [<i>Sus scrofa</i>] [MASS =4402]	66
37 gi 41688293	161 725	66	16	39	FYVE and coiled-coil domain containing 1 [<i>Sus scrofa</i>] [MASS =161725]	66
38 gi 41688313	86 556	65	16	17	FYVE and coiled-coil domain containing 1 [<i>Sus scrofa</i>] [MASS =86555]	65
39 gi 47522754	83 107	66	24	26	Gastrin-binding protein [<i>Sus scrofa</i>]	66
40 gi 47522694	66 895	66	18	15	Glutamic acid decarboxylase [<i>Sus scrofa</i>]	66
41 gi 52631983	37 497	73	24	10	Glutaminyl-tRNA synthetase [<i>Sus scrofa</i>] [MASS =37497]	73
42 gi 47523200	23 922	65	30	8	Glycolipid transfer protein [<i>Sus scrofa</i>]	65
43 gi 3297938	13 195	62	24	7	Goosecoid protein [<i>Sus scrofa</i>] [MASS =13195]	62
44 gi 47522950	83 973	66	45	68	Heart calpastatin [<i>Sus scrofa</i>]	66
45 gi 47523018	123 220	66	16	39	Heat-stable enterotoxin receptor [<i>Sus scrofa</i>]	66
46 gi 55742658	344 800	66	11	51	Huntingtin [<i>Sus scrofa</i>]	66
47 gi 24528344	49 300	66	18	8	Hypothetical protein [<i>Sus scrofa</i>] [MASS =49300]	66
48 gi 47523430	154 956	66	16	30	IGF-1 receptor [<i>Sus scrofa</i>]	66
49 gi 51317288	129 799	66	15	21	Insulin receptor substrate-1 [<i>Sus scrofa</i>] [MASS =129798]	66
50 gi 47522764	88 266	66	20	23	Integrin α -1 subunit [<i>Sus scrofa</i>]	66
51 gi 5738092	46 028	66	16	7	IS10-right transposase [<i>Shigella flexneri</i>]	66
52 gi 41529164	118 288	62	12	13	KIAA1885 protein [<i>Sus scrofa</i>]	62
53 gi 41529172	67 962	68	13	11	KIAA1949 protein [<i>Sus scrofa</i>] [MASS =67962]	68
54 gi 57117921	27 219	73	34	18	Kruppel-like factor 9 [<i>Sus scrofa</i>]	73
55 gi 47522788	35 852	69	18	10	L-36 lactose binding protein [<i>Sus scrofa</i>]	69
56 gi 47523782	75 660	66	14	18	Lactoferrin [<i>Sus scrofa</i>]	51
57 gi 32130549	77 523	66	17	18	Lactoferrin [<i>Sus scrofa</i>] [MASS =77522]	30
58 gi 17467354	77 682	66	16	19	Lactoferrin [<i>Sus scrofa</i>] [MASS =77681]	51
59 gi 41688298	77 612	66	18	20	Lactotransferrin [<i>Sus scrofa</i>] [MASS =77611]	51

Accession	Mass	Score (%)	Coverage (%)	Query matched	Name	UC score
60 gi 56711370	40 660	69	42	16	LIM and cysteine-rich domains protein 1 [<i>Sus scrofa</i>]	68
61 gi 4235639	86 580	65	10	7	Low density lipoprotein receptor [<i>Sus scrofa</i>] [MASS =86579]	65
62 gi 3201962	88 723	66	11	15	Low density lipoprotein receptor [<i>Sus scrofa</i>] [MASS =88723]	66
63 gi 3153895	88 776	66	11	15	Low density lipoprotein receptor [<i>Sus scrofa</i>] [MASS =88776]	66
64 gi 47523452	72 745	65	13	13	Low-affinity Na-dependent glucose cotransporter [<i>Sus scrofa</i>]	65
65 gi 38569737	1 161 011	66	20	37	Mannose receptor C1 [<i>Sus scrofa</i>] [MASS =161125]	66
66 gi 16506695	252 550	84	12	48	Mannose-6-phosphate/insulin-like growth factor II receptor [<i>Sus scrofa</i>] [MASS =252551]	84
67 gi 3928021	103 780	66	15	16	Mast/stem cell growth factor receptor [<i>Sus scrofa</i>] [MASS =103780]	65
68 gi 3928015	108 288	66	15	16	Mast/stem cell growth factor receptor [<i>Sus scrofa</i>] [MASS =108287]	65
69 gi 3928019	108 288	66	15	16	Mast/stem cell growth factor receptor [<i>Sus scrofa</i>] [MASS =108287]	65
70 gi 3928017	108 316	66	15	16	Mast/stem cell growth factor receptor [<i>Sus scrofa</i>] [MASS =108315]	65
71 gi 47575836	5969	66	77	10	Metallothionein [<i>Sus scrofa</i>]	1
72 gi 1827460	4 705	65	46	7	Metallothionein isoform [<i>Sus scrofa</i>] [MASS =4705]	65
73 gi 1827471	4728	65	85	6	Metallothionein isoform [<i>Sus scrofa</i>] [MASS =4728]	16
74 gi 1827456	6022	66	77	10	Metallothionein isoform [<i>Sus scrofa</i>] [MASS =6022]	1
75 gi 18202363	5959	66	77	10	Metallothionein-IC (MT-IC)	1
76 gi 18202369	5969	65	74	7	Metallothionein-IE (MT-IE)	29
77 gi 18202365	5955	65	57	9	Metallothionein-IF (MT-IF)	65
78 gi 47523864	83 337	75	22	22	Methylmalonyl-CoA mutase [<i>Sus scrofa</i>]	75
79 gi 47523888	32 910	66	46	25	Mitochondrial solute carrier family 25 member 6 [<i>Sus scrofa</i>]	66
80 gi 57527982	68 550	65	27	23	Moesin B [<i>Sus scrofa</i>]	65
81 gi 33439992	100 652	65	17	27	Myocardin [<i>Sus scrofa</i>]	65
82 gi 55741490	223 148	77	26	70	Myosin heavy chain 2a [<i>Sus scrofa</i>]	20
83 gi 5360748	223 234	75	26	67	Myosin heavy chain 2b [<i>Sus scrofa</i>] [MASS =223235]	32
84 gi 5360750	223 171	77	25	68	Myosin heavy chain 2x [<i>Sus scrofa</i>] [MASS =223172]	2
85 gi 12060489	223 296	66	27	65	Myosin heavy chain slow isoform [<i>Sus scrofa</i>] [MASS =223297]	66
86 gi 47523638	44 084	69	35	17	Nexin-1 [<i>Sus scrofa</i>]	69
87 gi 41529174	217 915	74	11	29	NFBD1 [<i>Sus scrofa</i>] [MASS =217916]	74
88 gi 47523738	44 839	66	35	30	pD1A1 chaperone [<i>Sus scrofa</i>]	66
89 gi 47523762	134 710	65	28	36	Plasma membrane Ca ²⁺ pump (PMCA1b) [<i>Sus scrofa</i>]	65
90 gi 47523406	83 155	66	18	18	Poly-Ig receptor [<i>Sus scrofa</i>]	66

Accession	Mass	Score (%)	Coverage (%)	Query matched	Name	UC score
91 gi 62461592	183 904	84	18	53	Procollagen α 1(V) [<i>Sus scrofa</i>]	84
92 gi 62086218	145 245	74	27	51	Procollagen α 2(V) [<i>Sus scrofa</i>] [MASS =145244]	74
93 gi 62086220	172 158	82	20	54	Procollagen α 3(V) [<i>Sus scrofa</i>] [MASS =172158]	82
94 gi 164648	565 165	66	10	54	Ryanodine receptor [<i>Sus scrofa</i>]	66
95 gi 164646	565 112	66	9	54	Ryanodine receptor [<i>Sus scrofa</i>] [MASS =565100]	66
96 gi 48675951	62 767	65	18	18	Soluble epoxide hydrolase [<i>Sus scrofa</i>]	65
97 gi 5069468	25 421	65	23	6	Sp1 transcription factor [<i>Sus scrofa</i>] [MASS =25421]	65
98 gi 60116610	140 058	66	12	18	Sterol response element binding protein cleavage-activating protein [<i>Sus scrofa</i>] [MASS =140057]	66
99 gi 2581864	1 184 175	66	1	18	Submaxillary apomucin [<i>Sus scrofa domestica</i>]	66
100 gi 51234755	15 084	65	21	7	T-cell receptor α [<i>Sus scrofa</i>] [MASS =15084]	65
101 gi 51234709	15 881	65	20	7	T-cell receptor α [<i>Sus scrofa</i>] [MASS =15881]	65
102 gi 47523544	191 399	66	13	24	Tenascin C [<i>Sus scrofa</i>]	66
103 gi 47523454	118 708	68	7	15	Toll-like receptor 8 [<i>Sus scrofa</i>]	68
104 gi 47523094	174 306	69	27	68	Topoisomerase II [<i>Sus scrofa</i>]	69
105 gi 47522924	63 668	72	14	14	T-plasminogen activator [<i>Sus scrofa</i>]	72
106 gi 833800	1 076 840	77	17	19	Transferrin [<i>Sus scrofa</i>] [MASS =76952]	77
107 gi 47522864	144 858	70	31	51	Unconventional myosin [<i>Sus scrofa</i>]	70
108 gi 47522618	116 876	65	27	45	Vinculin [<i>Sus scrofa</i>]	65
109 gi 12230718	272 394	71	12	33	von Willebrand factor precursor (vWF)	71
110 gi 1066466	270 363	66	12	39	Zonadhesin	66