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Application of Proteomics to Identify Fertility Markers in Angus Bull Sperm

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ABSTRACT

The goal of the study was to ascertain sperm proteins as fertility markers in Angus bull sperm using proteomics and validating the markers through comparative sperm biology between Angus and Holstein bulls for which there are reliable fertility data available. We aimed to determine proteins differentially expressed in sperm from Angus bulls with different fertility phenotypes. Two-dimensional differential gel electrophoresis with mass-spectrometry, functional gene clusters, canonical pathways and protein networks, using integrated discovery bioinformatics software and ingenuity pathway analysis were used to identify and analyze sperm proteome. We identified 80 proteins that were differentially expressed in sperm of our experimental population. Using computational biology approaches we demonstrated involvement of structural proteins such as outer dense fiber of sperm tails 2 and enzymes including kinases, and phosphatases having functions in essential pathways in glycolysis/gluconeogenesis and free scavenging. The results are significant because analyzed proteins in Angus sperm are determinants of fertility, gene-environment interactions, as well as potential biomarkers for animal breeding.

1. Introduction

In addition to providing a half of the genome, sperm also transfer essential transcripts and proteins to the oocyte. Any defects in sperm proteins derived from either genome or epigenome vitally affect male fertility and early embryonic development (Dada *et al.* 2012). The prevalence of male infertility among the American infertile couples was found to be 17% in 2010 according to the Society for Assisted Reproductive Technology data (SART). Male infertility is also a concern in cattle reproduction as well because thousands of cows can be inseminated using cryopreserved sperm from a single bull. Since male fertility has such a paramount influence on genetic improvement of the herd, much greater considerations of male selection, their management, and replacement are needed (Rahman *et al.* 2017). Using high quality sperm and proper fertilization techniques are critical for maintaining the conception rates with artificial insemination (AI) in the field (Kwon *et al.* 2015b).

Sperm proteins can be classified by their physiological functions or their cellular locations. Locations of several sperm proteins were demonstrated to be acrosomal, mitochondrial, nuclear matrix, cytoskeletal (i.e., tubulins and actins) and membrane proteins (i.e., aquaporins). Most of the sperm proteins were structural proteins such as ODF2 and tubulin which are located in the flagellum play important roles in sperm physiology (Hoyer-Fender *et al.* 1998; Donkor *et al.* 2004). On the other hand, some other sperm proteins such as kinases and superoxide dismutase (SOD) are synthesized in the cytosol and have enzymatic functions. Although the significance of sperm in cattle reproduction has been obvious, sperm proteins and molecular mechanisms of uncompensable infertility in Angus breed are vastly undefined. Because the bulls were similar in their genotypes, epigenetics (such as posttranslational modifications of proteins that

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influence gene expression without any changes in DNA through environmental factors such as nutrition, management, and climate) may play important roles in male fertility. Therefore, the purpose of this study was to uncover sperm proteome of Angus bulls to identify possible protein markers affecting male fertility. To accomplish our goal, we used a quantitative proteomics methods 2D-DIGE and matrix assisted laser desorption/ionization time-of-flight mass spectrometry (Choi *et al.* 2008) and bioinformatics. This pioneering and comprehensive proteomics study of Angus bull sperm is significant because the results are hypothesis generators and potential fertility markers to determine and measure semen quality and bull fertility.

2. Materials and Methods

2.1. Experimental Design

The frozen semen samples and the fertility data from four Angus bulls with different fertility index and satisfactory semen quality were obtained from Alta Genetics, Inc. (Watertown, WI, USA). Cryopreserved semen samples were washed three times to remove the cryoprotectants, and then the proteins were extracted for 2D-DIGE analysis. In addition, MALDI-TOF/TOF analysis was used to identify proteins. Further, bioinformatics and pathway analyses were carried out to identify the protein networks and pathways. All chemicals were purchased from Sigma-Aldrich Chemicals, St. Louis, MO, USA except those stated.

2.2. Determination of Bull Fertility and Isolation of Sperm

Fertility of the bulls was tested through artificial insemination (AI) of 1,265 cows on seven farms. Frozen semen samples from four bulls were distributed to seven herds, and cows were bred in standing heat. The pregnancy diagnoses were performed by rectal palpation on day 40 post insemination. The breeding numbers and the conception rates of the four bulls are presented (Figure 1). Sperm were isolated from four Angus bulls with different fertility using percoll gradient according to Feugang *et al.* (2009). Sperm pellets were washed with PBS (Gibco, Grand Island, NY, USA) three times, centrifuged at 500 g and aliquoted as 10×10^6 spermatozoa per tube. The pellets were kept at -80°C prior to shipping, and shipped on dry ice to Appliedbiomics (Hayward, CA, USA) for 2D-DIGE and mass spectrometry.

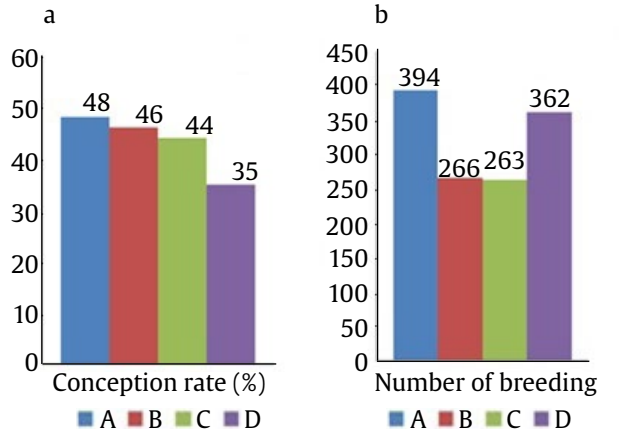

Figure 1. Fertility ranking of the bulls whose sperm were used in 2D-DIGE analysis. (a) four bulls are shown from A to D based on the number of breeding, (b) conception rates obtained from the field following AI for the same bulls are also displayed. Note that the fertility of these bulls gradually decreases from bull A to bull D, so Bull A and Bull B were considered as higher fertility than Bull C and Bull D

2.3. Isolation of Proteins from Sperm and CyDye Labeling

Sperm pellets were resuspended in 120 µl of 2-D cell lysis buffer (30 mM Tris-HCl, pH 8.8, 7 M urea, 2 M thiourea, and 4% CHAPS) supplemented with protease inhibitor cocktail (Roche San Francisco, CA, USA) followed by two seconds of sonication (VerTis, Gardiner, NY, USA). Samples were then incubated on a rotator at room temperature (RT) for 30 minutes and centrifuged at 13,000 g at 4°C for 30 minutes. The supernatant was collected, and the protein concentration was measured using Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Proteins were labeled for each sample. Thirty micrograms of proteins were mixed with 0.7-0.9 µl of diluted CyDye (1:5 diluted with DMF from 1 nmol/µl stock) and kept in the dark on ice (~4°C) for 30 minutes. The four samples (total concentration 20 µg/gel) were mixed together to create the internal standard. Samples from each group were labeled with Cy2, Cy3, and Cy5, respectively. The labeling reaction was stopped by adding 0.7-0.9 µl of 10 mM Lysine to each sample and incubating in the dark on ice for an additional 15 min. The labeled samples were then mixed together, and 130 µl destreak solution (GE Healthcare, Piscataway Township, NJ, USA) and 100 µl of Rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mg/ml DTT, 1% Pharmalyte, and trace amount of bromophenol blue) were added to the labeling mix for a total volume of 260 µl. The samples were put on a rotator for 15 min.

and centrifuged at 16,060 g for another 15 min. The labeled samples were then loaded onto a strip holder and immersed with a 13 cm IPG strip. Two 2D-DIGE gels were replicated for each experiment, for Bull C *vs*. Bull A and similarly for Bull D *vs*. Bull B. Since the protein expression patterns from two high and two low fertility bulls were compared in the same gel, only two gels were used.

2.4. Isoelectric Focusing (IEF) and Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), Image Scan and Data Analysis

Once the labeled samples were loaded, IEF (pH3-10 linear) was run according to the protocol provided by Amersham BioSciences (GE Healthcare, Piscataway Township, NJ, USA). The IPG strips were then incubated in the freshly made equilibration buffer-1 (50 mM Tris-HCl, pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS, trace amount of bromophenol blue, and 10 mg/ml Dithiothreitol; DTT) with gentle shaking for 15 min. Then the strips were rinsed in the fresh equilibration buffer-2 (50 mM Tris-HCl, pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS, trace amount of bromophenol blue, and 45 mg/ml DTT) with gentle shaking for 10 min. Next, the IPG strips were rinsed in the SDS-gel running buffer prior to transferring into 12% SDS-gels. The SDS-gels were run at 15°C until the dye ran out of the gels. Gel images were scanned immediately following the SDS-PAGE using typhoon TRIO (Amersham BioSciences, GE Healthcare, Piscataway Township, NJ, USA). The scanned images were then analyzed by Image Quant software (version 6.0, Amersham BioSciences, GE Healthcare, Piscataway Township, NJ, USA), followed by in-gel analysis using DeCyder software version 6.0 (Amersham BioSciences, GE Healthcare, Piscataway Township, NJ, USA). The fold changes of the protein expression levels were obtained from in-gel DeCyder analysis.

2.5. Identification of Differentially Expressed Proteins Using Mass Spectrometry

Protein spots of interest were excised from preparative gels (~600 µg of protein) by using an Ettan spot picker (Amersham Biosciences, GE Healthcare, Piscataway Township, NJ, USA) and digested with trypsin (Promega, Madison, WI USA). The trypsin-digested peptides were extracted out and de-salted using C-18 ziptip (Millipore, Billerica, MA, USA). Then, the desalted peptides were used for MALDITOF protein identification (MALDI-TOF/TOF mass spectrophotometer, ABI-4700 from Applied Biosystems, Inc, Foster City, CA, USA). Using the Mascot search engine (Matrix Science, Boston, MA, USA), protein databases of national center for biotechnology (NCBI)/SwissProt (http://www.ncbi.nlm.nih.gov/) and (www.uniprot.org) were searched for >95% matches of high-quality mass spectra. For the differentially expressed protein data, a ratio of protein expressions from relatively lower fertility bulls over relatively higher fertility bulls was calculated as C/A and D/B. Subsequently, an average of these two ratios was taken for each protein using the equation [(C/A + D/B)/2] to find the mean, for more accuracy. Eighty proteins were identified to be different in spermatozoa from the four bulls based on their fertility index.

2.6. Bioinformatics and Pathway Analysis of the Differentially Expressed Proteins

Functional gene annotation clustering of the differentially expressed proteins was performed using DAVID bioinformatics database (http://david.abcc.ncifcrf.gov) to reveal their molecular functions in biological processes. The pathway analysis was completed using ingenuity pathway, IPA (http://www.ingenuity.com) to determine a functional interactome between the differentially expressed proteins and bull fertility. The GenInfo Identifier (GI) accession numbers of 80 proteins were imported into the IPA software prior to data analysis. Then, the unmapped proteins were determined and manually converted into their human counterparts using ENSEMBL database (http://www.ensembl.org) with their identity (%). Afterwards, the pathway analysis was performed using IPA with the proteins that were mapped automatically and manually. The proteins that would be further analyzed using bioinformatics tolls were selected according to IPA interactome results.

2.7. Sperm Proteins as Markers Across Breeds

Through harnessing the power of comparative biology, we performed a comprehensive literature

search on proteins of bovine sperm to uncover potential fertility markers that can be used in selection of breeding bulls. The proteins were included in the following groups according to their molecular physiology: Chromatin proteins, seminal plasma proteins, acrosome proteins, ATP synthesis proteins, capacitation proteins, cytoskeletal proteins, and other proteins.

3. Results

3.1. Protein Analysis by 2D-DIGE

We detected approximately 2,000 protein spots in each of the 2D gels developed using sperm from four bulls with varying fertility per gel, Bull C/Bull A and Bull D/Bull B, respectively (Figure 2a and b). Among all the data obtained from the two 2D gels, 80 of the differentially expressed protein spots were detected. Overall about 4% of all protein spots were differentially expressed between relative high (A and B) and low fertility bulls (C and D), and the expression levels of these proteins ranged from -4.65 to 8.2.

3.2. Differentially Expressed Proteins in Sperm from Bulls with Varying Fertility

We found total of 80 proteins that were differentially expressed in the sperm from the four bulls having varying fertility (Table 1). These expressed proteins exhibited various levels of fold differences among the bulls. While most of these proteins corresponded to known proteins, others were similar to known proteins, or to predicted proteins or hypothetical proteins.

3.3. Bioinformatics and Pathway Analysis of the Differentially Expressed Proteins

According to the IPA output, 47 canonical pathways and networks were identified (Table 2 and 3). Twelve of these canonical pathways were

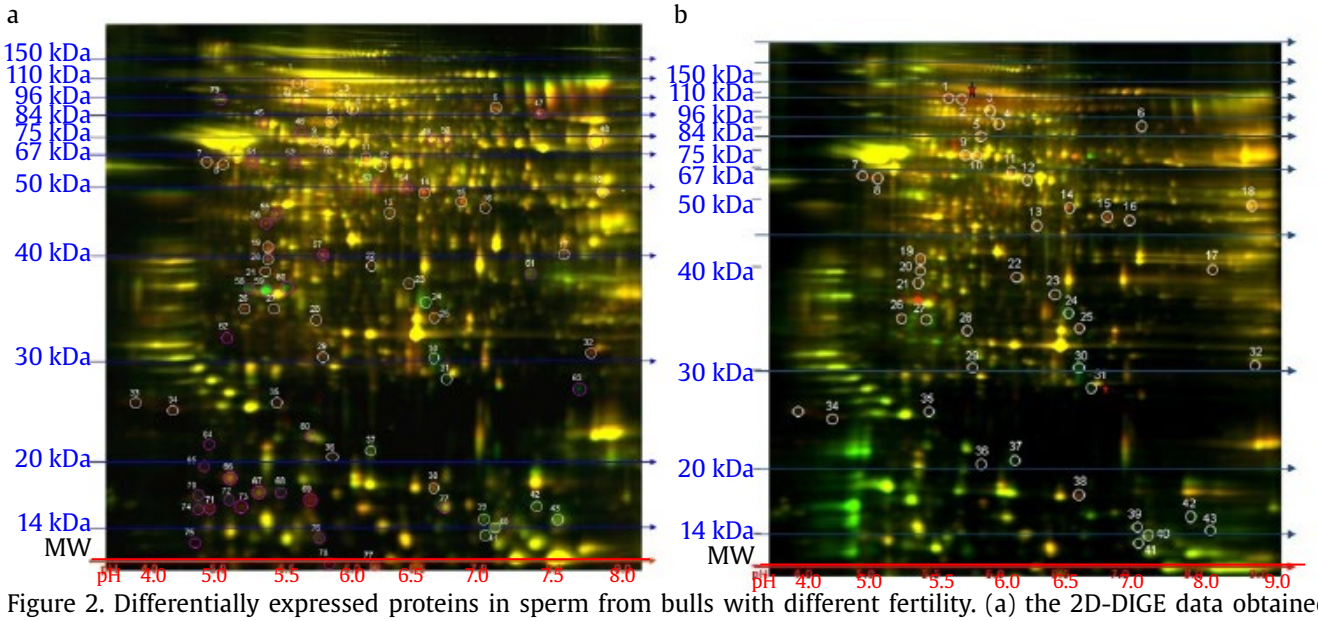

Figure 2. Differentially expressed proteins in sperm from bulls with different fertility. (a) the 2D-DIGE data obtained by using spermatozoa from (Bull C *vs.* Bull A). (b) 2D-DIGE data obtained by using spermatozoa from (Bull D *vs.* Bull B). The 2D-DIGE gel pictures analyzed by Image Quant and DeCyder software are displayed. The same amounts of protein extracts from spermatozoa were labeled separately and subjected to a 13 cm IPG strip (pH 3-10) and then transferred to SDS-PAGE. Differentially expressed proteins are circled; dotted circles indicate the proteins sequenced for identification. Molecular weights of markers are listed on the left, while the pH ranges are indicated at the bottom. Spots 2 and 31 are Outer Dense Fiber of Sperm Tails 2 (ODF2) and manganese-dependent superoxide dismutase (MnSOD), (Red asterisk), respectively

Table 1. Sperm proteins detected using 2D-DIGE analysis and LC-MSMS. Differentially expressed proteins in the spermatozoa from the four bulls with different fertility were represented. This table is composed of the spot numbers, gi accession numbers, and molecular weights of these 80 proteins

Top ranked protein name (species)	Ratio bull C/bull A	Ratio bull D/bull B	Average ratio	Accession no.	Protein MW	Protein PI	Pep. count	Protein score	Protein score C. I. %	Total ion score	Total ion C. I. %
PREDICTED: similar to testis specific 10 isoform 1 [Bos taurus]	1.71	8.95	5.33	gi|194671321	81188	5.65	13	164	100	63	99.8
Outer dense fiber of sperm tails 2 [Bos taurus]	1.64	14.8	8.21	gi|84000345	75450.6	7.52	13	235	100	166	100
PREDICTED: similar to EF-hand domain (C-terminal) containing 1 isoform 1 [Bos taurus]	1.11	1.37	1.24	gi|76650703	73984.5	5.78	26	473	100	272	100
Outer dense fiber of sperm tails 2 [Bos taurus]	1.46	1.56	1.51	gi|84000345	75450.6	7.52	27	554	100	397	100
Outer dense fiber of sperm tails 2 [Bos taurus]	1.62	1.49	1.55	gi|84000345	75450.6	7.52	29	697	100	514	100
Chain E, leech-derived tryptase inhibitorTRYPSIN COMPLEX	2.03	2.07	2.05	gi|3318722	23457.4	8.26	2	73	96.6	56	98.9
Tubulin, beta 2C [homo sapiens]	1.89	1.86	1.87	gi|23958133	49808	4.83	20	543	100	324	100
Tubulin, beta, 2 [homo sapiens]	2.09	1.93	2.01	gi|5174735	49799	4.79	22	585	100	330	100
PREDICTED: similar to outer dense fiber of sperm tails 2 isoform 10 [canis familiaris]	1.18	1.42	1.3	gi|73967892	52075.9	5.81	26	536	100	348	100
PREDICTED: similar to outer dense fiber of sperm tails 2 isoform 10 [canis familiaris]	1.3	1.62	1.46	gi|73967892	52075.9	5.81	23	604	100	434	100
Tubulin, alpha 1a [mus musculus]	2.79	1.51	2.15	gi|6678465	49927.6	4.97	15	688	100	520	100
Enolase 1 [Bos taurus]	1.03	-1.3	-0.35	gi|87196501	47296.4	6.37	21	666	100	458	100
Hypothetical protein LOC511761 [bos taurus]	1.69	1.49	1.59	gi|115495817	30193.3	6.21	15	427	100	279	100
PREDICTED: similar to tubulin alpha-3 chain (alpha-tubulin 3) [canis familiaris]	3.2	1.83	2.51	gi|73996007	49913.6	4.97	15	577	100	418	100
Tubulin, alpha 1a [mus musculus]	1.97	2.53	2.25	gi|6678465	49927.6	4.97	14	442	100	314	100

Table 1. Continued

Top ranked protein name (species)	Ratio bull C/bull A	Ratio bull D/bull B	Average ratio	Accession no.	Protein MW	Protein PI	Pep. count	Protein score	Protein score C. I. %	Total ion score	Total ion C. I. %
short-chain acyl-CoA dehydrogenase [bos taurus]	1.68	1.96	1.82	gi|77735757	44523.9	8.82	15	352	100	220	100
Lactate dehydrogenase a-like 6B [bos taurus]	2.25	1.41	1.83	gi|78369344	41565.8	8.91	17	646	100	483	100
Phosphoglycerate kinase 2 [bos taurus]	1.64	1.68	1.66	gi|174840786	44729.3	8.51	19	482	100	332	100
mCG20287 [mus musculus]	2.63	1.98	2.3	gi|148676266	39342.1	5.14	13	435	100	261	100
mCG20287 [Mus musculus]	2.71	2.3	2.5	gi|148676266	39342.1	5.14	11	376	100	231	100
TUBB2C protein [homo sapiens]	3.8	1.9	2.85	gi|14124960	25858.4	4.95	13	299	100	147	100
hCG1992406, isoform CRA_b [homo sapiens]	3.29	2.29	2.79	gi|119576011	42187	5.03	8	265	100	194	100
Chain D, cytochrome Bc1 complex from bovine	1.64	1.48	1.56	gi|4139395	27269.5	6.49	13	301	100	179	100
Hypothetical protein LOC510569 [bos taurus]	-2.93	-2.41	-2.67	gi|156120505	17804.6	5.94	4	77	98.5	39	45.3
Hypothetical protein TP0959 [treponema pallidum	2.03	1.82	1.92	gi|15639943	13894	8.89	7	74	64.7		
PREDICTED: similar to ENSANGP00 000002667 [bos taurus]	3.01	1.77	2.39	gi|194674718	19864.8	5.62	5	310	100	253	100
mCG20287 [mus musculus]	2.35	1.52	1.93	gi|148676266	39342.1	5.14	14	426	100	310	100
Unnamed protein product [mus musculus]	2.56	2.45	2.5	gi|26355849	32237.9	5.56	9	323	100	223	100
Phosphatidyletha nolamine-binding protein 4 [bos taurus]	3.15	1.32	2.23	gi|77735827	25129.6	5.87	4	60	35.7	23	0
hypothetical protein LOC510569 [bos taurus]	-3.19	-2.92	-3.05	gi|156120505	17804.6	5.94	6	309	100	234	100
Manganous superoxide dismutase; MnSOD [bos taurus]	-2.91	-6.4	-4.65	gi|7555818	24574.6	8.7	6	313	100	254	100
PREDICTED: hypothetical protein LOC736 248 isoform 2 [pan troglodytes]	4.37	4.23	4.3	gi|114585016	43100.4	5.07	10	460	100	342	100
TUBB2C protein [homo sapiens]	2.13	1.86	1.99	gi|14124960	25858.4	4.95	13	473	100	303	100

Table 1. Continued

Top ranked protein name (species)	Ratio bull C/bull A	Ratio bull D/bull B	Average ratio	Accession no.	Protein MW	Protein PI	Pep. count	Protein score	Protein score C. I. %	Total ion score	Total ion C. I. %
TUBB2C protein [homo sapiens]	2.2	2.29	2.24	gi|14124960	25858.4	4.95	10	258	100	140	100
PREDICTED: similar to ENSANG P00000002667 [bos taurus]	2.08	1.35	1.71	gi|194674718	19864.8	5.62	6	276	100	212	100
	2.18	1.52	1.85	gi|194674718	19864.8	5.62	6	286	100	222	100
PREDICTED: similar to ENSANG P00000002667 [bos taurus]	-1.91	-1.97	-1.94	gi|148744160	18156.9	6.71	12	282	100	142	100
ACP1 protein [bos taurus] heat shock protein, alpha-crystallin-related, B9 [bos taurus]	1.94	2.15	2.04	gi|94966950	16773.2	8.22	8	476	100	355	100
PREDICTED: similar to Acrosomal protein SP-10 precursor (acrosomal vesicle protein-1) isoform 3 [Ca	-1.67	-1.32	-1.49	gi|73954519	26598.9	5.08	6	101	100	53	99.2
acrosomal vesicle protein 1 [bos taurus]	1.24	-1.63	-0.19	gi|115495399	28934.4	4.53	7	113	100	55	99.6
Alpha enolase [bos taurus]	-1.4	-1.37	-1.38	gi|4927286	47247.3	6.44	8	154	100	105	100
Chain B, refined 1.8 angstroms aesolution crystal structure Of porcine epsilon-trypsin	-2.07	-2.09	-2.08	gi|999627	8813.5	6.67	2	119	100	96	100
Chain B, refined 1.8 angstroms resolution crystal structure Of porcine epsilon-trypsin	-2.14	-2.88	-2.51	gi|999627	8813.5	6.67	2	104	100	79	100
Outer dense fiber of sperm tails 2 [bos taurus]	1.07	1.46	1.26	gi|84000345	75450.6	7.52	20	373	100	282	100
PREDICTED: heat shock 60kDa protein 1 (chaperonin) [bos taurus]	1.13	1.06	1.09	gi|119888228	74984.7	9.05	23	1,060	100	865	100
plasma glutamate carboxypeptidase precursor [bos taurus]	1.07	2.41	1.74	gi|115495837	51646.3	5.55	11	453	100	389	100
Chain A, the refined three-dimensional structure of cat muscle (M1) pyruvate kinase, at a resolutio	1.6	2.37	1.98	gi|157833510	57877.9	7.23	19	178	100	64	99.9

Table 1. Continued

Top ranked protein name (species)	Ratio bull C/bull A	Ratio bull D/bull B	Average ratio	Accession no.	Protein MW	Protein PI	Pep. count	Protein score	Protein score C. I. %	Total ion score	Total ion C. I. %
Glyceraldehyde-3-phosphate dehydrogenase, spermatogenic [bos taurus]	1.1	1.89	1.49	gi|110626121	43260.3	8.32	19	596	100	422	100
Tektin 3 [bos taurus]	1.04	2.73	1.88	gi|149773556	56645.5	6.42	28	661	100	414	100
Tektin 3 [bos taurus]	1.04	-4.64	-1.8	gi|149773556	56645.5	6.42	26	571	100	363	100
Tubulin, alpha 1a [mus musculus]	2.5	2.51	2.5	gi|6678465	49927.6	4.97	14	452	100	317	100
Chain A, cytochrome Bc1 complex from bovine	1.29	2.46	1.87	gi|4139392	49181.3	5.46	25	845	100	588	100
5'-nucleotidase, cytosolic IB [bos taurus]	1.42	1.35	1.38	gi|84370143	63970.7	8.8	20	416	100	261	100
actin-like 7A [bos taurus]	1.88	-1.25	0.31	gi|84370183	48984.7	6.29	21	610	100	427	100
PREDICTED: similar to actin-related protein T1 (ARP-T1) [bos taurus]	1.56	1.91	1.73	gi|61878077	42103.3	5.39	14	306	100	202	100
actin-related protein T2 [bos taurus]	2.35	-1.11	0.62	gi|84000199	41886.4	5.48	20	470	100	281	100
WBP2 N-terminal like [bos taurus]	2.22	-1.64	0.29	gi|126723634	31945.6	5.61	6	288	100	248	100
PREDICTED: similar to sp32 [bos taurus]	-7.14	3.07	-2.03	gi|194666681	61196.7	5.11	9	362	100	301	100
PREDICTED: similar to sp32 [bos taurus]	-14.1	7.21	-3.44	gi|194666681	61196.7	5.11	9	422	100	354	100
PREDICTED: similar to sp32 [bos taurus]	-4.52	2.73	-0.89	gi|194666681	61196.7	5.11	11	509	100	454	100
PREDICTED: similar to voltage-dependent anion channel 2 [equus caballus]	-2.33	-1.07	-1.7	gi|149689995	31524.5	7.46	6	96	100	63	99.9
Tyrosine 3-monoox ygenase/ tryptophan 5-monooxy genase activation protein, zeta polypeptide [homo sapi	3.7	1.3	2.5	gi|68085578	27695.8	4.73	8	130	100	40	65.6
3-oxoacid CoA transferase 2 [bos taurus]	-56.7	1.1	-27.8	gi|148223655	55973.1	7.15	3	76	98.4	45	75.5
PREDICTED: similar to leucine rich repeat containing 37A [bos taurus]	1.27	-6.7	-2.71	gi|194676234	280818.2	4.87	5	102	100	91	100

Table 1. Continued

Top ranked protein name (species)	Ratio bull C/bull A	Ratio bull D/bull B	Average ratio	Accession no.	Protein MW	Protein PI	Pep. count	Protein score	Protein score C. I. %	Total ion score	Total ion C. I. %
Chain A, 12-Bromodo decanoic acid binds Inside the Calyx of bovine beta-lactoglobulin	1.85	-4.32	-1.23	gi|6980895	18355.4	4.76	12	612	100	463	100
Seminal vesicle secretory protein 109 [bos taurus]	1.55	-3.42	-0.93	gi|47564036	15470.2	4.91	8	557	100	457	100
Chain A, bull seminal plasma Pdc-109 fibronectin type Ii module	1.34	-2.87	-0.76	gi|20663779	12787.8	5.08	9	610	100	516	100
Phosphodi esterase 6D, cGMP-specific, rod, delta [bos taurus]	-1.6	-1.95	-1.77	gi|27806061	17378.8	5.57	4	223	100	197	100
Sperm acrosome associated 3 [bos taurus]	3.37	-1.1	1.13	gi|157279923	18086.8	5.87	2	127	100	107	100
PREDICTED: similar to Thioredoxin domain-containing protein 5 precursor (thioredoxin-l ike protein p	-1.23	-7.94	-4.58	gi|194223000	39011.2	5.56	6	50	0		
seminal vesicle secretion 8 [bos taurus]	3.03	-2.32	0.35	gi|28849949	16129.7	4.9	6	175	100	117	100
PREDICTED: similar to sp32 [bos taurus]	-3.18	-1.1	-2.14	gi|194666681	61196.7	5.11	2	142	100	131	100
PREDICTED: similar to sp32 [bos taurus]	2.63	-1.49	0.57	gi|194666681	61196.7	5.11	4	95	100	81	100
PREDICTED: similar to thioredoxin domain-containing protein 5 precursor (thioredoxin-like protein p	-1.32	-5.99	-3.65	gi|194223000	39011.2	5.56	6	50	0		
Chain A, crystal structure of apo-bovine alpha-lactalbumin	1.27	-5.52	-2.12	gi|12084466	14176.8	4.8	7	218	100	139	100

Table 1. Continued

Top ranked protein name (species)	Ratio bull C/bull A	Ratio bull D/bull B	Average ratio	Accession no.	Protein MW	Protein PI	Pep. count	Protein score	Protein score C. I. %	Total ion score	Total ion C. I. %
Sperm acrosome associated 5 [bos taurus]	-1.26	-2.45	-1.85	gi|94966873	17568.2	5.55	4	127	100	91	100
PREDICTED: similar to sp32 [bos taurus]	-1.48	-2.51	-1.99	gi|194666681	61196.7	5.11	6	563	100	535	100
Protease, serine, 2 [rattus norvegicus]	1.63	1.27	1.45	gi|6981420	25942.7	4.71	1	68	87.2	61	99.6
Thioredoxin domain containing 3 (spermatozoa) [bos taurus]	-1.34	-1.49	-1.42	gi|114053199	46889.1	4.94	17	269	100	134	100
Acrosomal vesicle protein 1 [bos taurus]	1.27	-2.08	-1.41	gi|115495399	28934.4	4.53	6	100	100	60	99.9

Table 2. Ingenuity Canonical Pathways using IPA. The IPA results of canonical pathways were obtained using the 2D-DIGE data and sorted by the significance level of the canonical pathways. A total of 47 canonical pathways with their p-value and related proteins were represented here

Ingenuity canonical pathways	log (p-value)	Ratio	Molecules
Glycolysis/gluconeogenesis	4.96E00	4.55E-02	PGK2,ENO1,GAPDHS,LDHAL6B
14-3-3-mediated Signaling	3.05E00	2.46E-02	TUBB2C,YWHAZ,TUBA3C/TUBA3D
Propanoate metabolism	2.43E00	3.51E-02	LDHAL6B,ACADS
Purine metabolism	2.05E00	1.11E-02	NT5C1B,HSPD1,PDE6D
Mitochondrial dysfunction	1.72E00	1.47E-02	SOD2,CYC1
Aldosterone signaling in epithelial cells	1.59E00	1.23E-02	HSPB9,HSPD1
Germ cell-sertoli sell junction signaling	1.58E00	1.24E-02	TUBB2C,TUBA3C/TUBA3D
Phenylalanine, tyrosine and tryptophan biosynthesis	1.57E00	5.88E-02	ENO1
Gap junction signaling	1.56E00	1.19E-02	TUBB2C,TUBA3C/TUBA3D
Riboflavin metabolism	1.5E00	5E-02	ACP1 (includes EG:11431)
Sertoli cell-sertoli cell junction signaling	1.48E00	1.05E-02	TUBB2C,TUBA3C/TUBA3D
Breast cancer regulation by stathmin1	1.42E00	9.95E-03	TUBB2C,TUBA3C/TUBA3D
Protein ubiquitination pathway	1.19E00	7.41E-03	HSPB9,HSPD1
Galactose metabolism	1.18E00	2.33E-02	LALBA
Cell cycle: G2/M DNA damage checkpoint regulation	1.17E00	2.04E-02	YWHAZ
Retinol metabolism	1.17E00	2.27E-02	NT5C1B
β-alanine metabolism	1.14E00	2.13E-02	ACADS
Phototransduction pathway	1.09E00	1.79E-02	PDE6D
Cysteine metabolism	1.09E00	1.89E-02	LDHAL6B
Protein kinase a signaling	1.06E00	6.27E-03	YWHAZ,PDE6D
Myc mediated apoptosis signaling	1.05E00	1.64E-02	YWHAZ
Butanoate metabolism	1.04E00	1.67E-02	ACADS
ERK5 signaling	1.02E00	1.59E-02	YWHAZ
Pyruvate metabolism	9.99E-01	1.52E-02	LDHAL6B
Valine, leucine, and isoleucine degradation	9.99E-01	1.52E-02	ACADS
PDGF signaling	9.69E-01	1.37E-02	ACP1 (includes EG:11431)
Aminosugars metabolism	9.69E-01	1.41E-02	PDE6D
TR/RXR activation	8.96E-01	1.12E-02	ENO1
Axonal guidance signaling	8.9E-01	4.71E-03	TUBB2C,TUBA3C/TUBA3D

Table 2. Continued

Ingenuity canonical pathways	log (p-value)	Ratio	Molecules
IGF-1 signaling	8.34E-01	9.62E-03	YWHAZ
Nicotinate and nicotinamide metabolism	8.26E-01	9.9E-03	NT5C1B
Type I diabetes mellitus Signaling	7.95E-01	8.55E-03	HSPD1
PI3K/AKT signaling	7.61E-01	7.52E-03	YWHAZ
p70S6K signaling	7.47E-01	7.87E-03	YWHAZ
Fatty acid metabolism	7.47E-01	8.06E-03	ACADS
Cardiac β-adrenergic signaling	7.2E-01	7.19E-03	PDE6D
Relaxin signaling	7.14E-01	6.9E-03	PDE6D
Pyrimidine metabolism	6.94E-01	7.09E-03	NT5C1B
Oxidative phosphorylation	6.86E-01	6.94E-03	CYC1
Acute phase response signaling	6.29E-01	5.81E-03	SOD2
RAR activation	6.2E-01	5.65E-03	NT5C1B
Ephrin receptor signaling	6.15E-01	5.1E-03	ACP1 (includes EG:11431)
NRF2-mediated oxidative stress response	6E-01	5.26E-03	SOD2
ERK/MAPK signaling	5.92E-01	5.05E-03	YWHAZ
cAMP-mediated signaling	5.38E-01	4.65E-03	PDE6D
Role of macrophages, fibroblasts and endothelial cells in rheumatoid arthritis	4.11E-01	3.04E-03	PRSS1/PRSS3
G-Protein coupled receptor signaling	2.48E-01	1.92E-03	PDE6D

Table 3. Networks created by IPA analysis. The data obtained from the 2D-DIGE analysis were analyzed using IPA software and sorted by the significance level of the networks. The interactions of the proteomics were generated by overlaying the first and the second networks

Top functions	Score	Focus molecules	Molecules in network
Free radical scavenging, cancer, hematological disease	33	13	AARS,ACADS,ACP1 (includes EG:11431),ACPP,Akt,Alpha tubulin,CD3, CENPJ, CYC1,Cytochrome c,ENO1,Enolase, ERK1/2,GZMK, HSP, HSPB9, HSPD1,Jnk,LALBA, mannitol, MLXIP,ODF1,PACS2,PEBP4,PI3K (complex), PRSS1/PRSS3, SIRT3,SOD2,TAOK2,TPD52 ,TUBA3C/TUBA3D, TUBB1, TUBB2C,Tubulin, YWHAZ
Cellular assembly and organization, cellular development, embryonic development	9	3	ACRV1,ACTL7A,Odf2,YBX2
Carbohydrate metabolism, small molecule biochemistry, antigen presentation	3	1	A2M,SPACA3
Cell-to-cell signaling and interaction, reproductive system development, and function, cellular development	3	1	Gstp1 (includes others),WBP2NL
Embryonic development, endocrine system development and function, organ development	3	1	HNF1A,PGCP,TGFB1 (includes EG:21803)

Table 3. Continued

Top functions	Score	Focus molecules	Molecules in network
Cell cycle, cellular compromise, cellular growth and proliferation	3	1	ACRBP,NUMA1,progesterone
RNA damage and repair, gene expression, RNA post-transcriptional modification	3	1	HNRNPD,L-lactate dehydrogenase,LDHAL6B
Drug metabolism, lipid metabolism, small molecule biochemistry	3	1	5'-nucleotidase,NT5C1B,RDH
Genetic disorder, neurological disease, cell morphology	2	1	CACNA1E,E2F4,E2f,EFHC1,TEX11,Voltage Gated Calcium Channel
Cell death, liver necrosis/cell death, cellular movement	2	1	ATP,GAPDHS,glyceraldehyde-3-phosphate dehydrogenase (phosphorylating),HSPA2,MAPK3,MITF,NTHL1,SH3BP4
Neurological disease, genetic disorder, organismal injury and abnormalities	2	1	APP,Crem,DNMT3A,DNMT3B,FGF2,MAPT,PAPOLB,PBX4,PDPK1,PGK2,Pgk,PSEN1,SP3,UBC
Cell-to-cell signaling and interaction, cellular assembly and organization, tissue development	2	1	3',5'-cyclic-GMP phosphodiesterase,3',5'-cyclic-nucleotide phosphodiesterase, ARL1,ARL2, ARL3,ARL15, C9orf25, Ca2+,CDC42,CETN3,GRK1,GRK7,HRAS,KRAS,NRAS,Pde,PDE6 d),PDE6D,PTGIR, RAB13, RAB18, RAD23A, RAP1A,RAP2B, RHEB,RHOA,RHOB, RND1,RPGR

statistically significant by enriching above the threshold as shown (Figure 3a). Based on the DAVID software, glycolysis/gluconeogenesis is the first cluster with 3.17 of enrichment score and enolase 1 was the first significantly expressed protein in this pathway (Figure 3b) the same pathway was also confirmed by IPA as significant ($p<0.0001$) in the canonical pathway (Figure 3a).

Additionally, free radical scavenging and cellular assembly or organization, cellular development, and embryonic development networks were identified to be first and second networks, respectively (Table 3). The first and second networks had a ratio containing focused molecules over score; 13/33 and 3/9, respectively. Based on the IPA results, most of the proteins in our sperm proteomics data were found to be enzymes with important functions. Their locations and functions were listed in Table 4. The final interactome was created by overlaying the two networks mentioned earlier (Figure 4) by selecting differentially expressed two proteins: outer dense fiber of sperm tails 2 (ODF-2) from the first network and manganese superoxide dismutase (SOD) from the second network. According to 2D-DIGE results, MnSOD was 4.65 times more abundant in spermatozoa from relatively higher

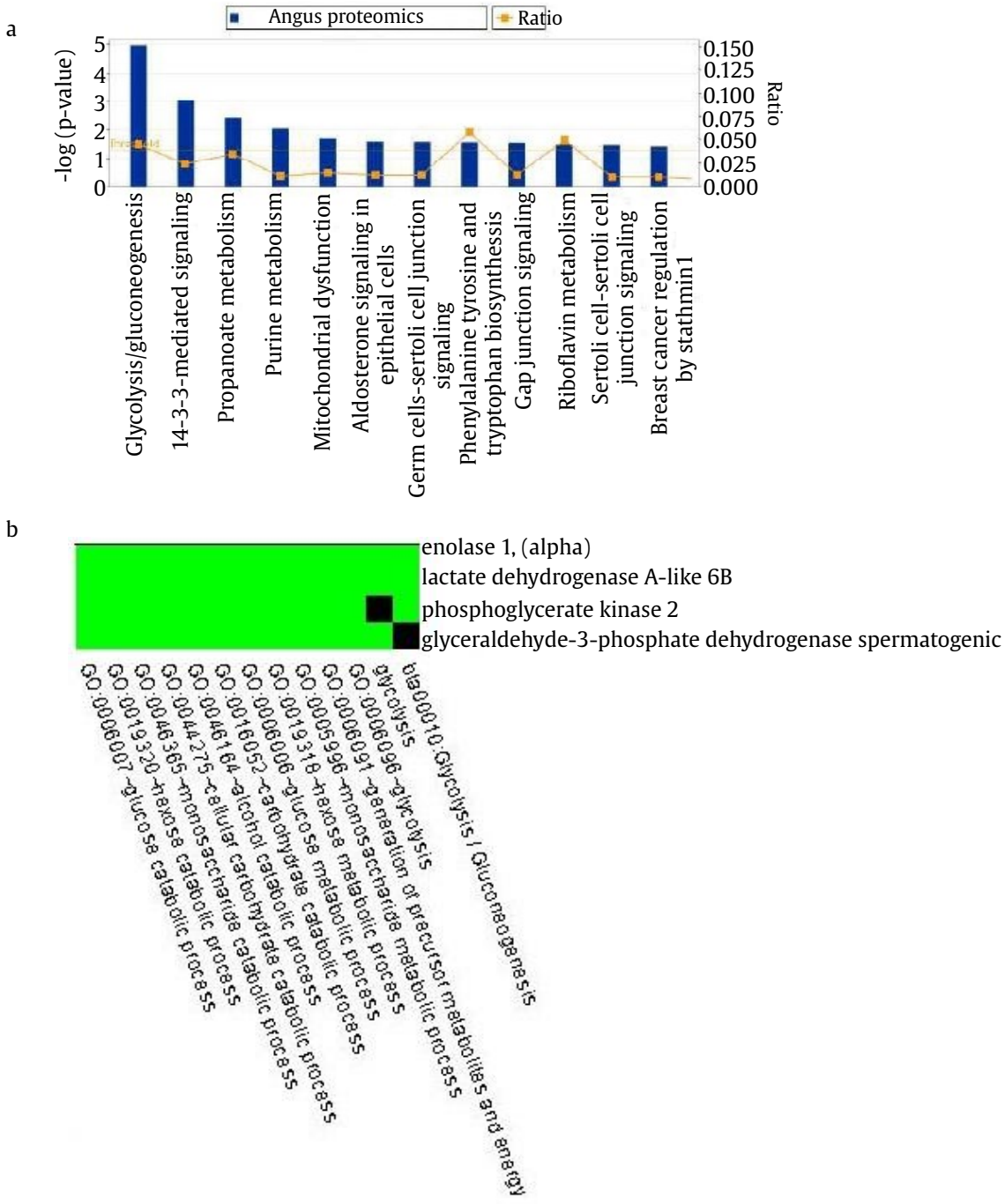

Figure 3. IPA canonical pathways and proteins of cluster 1 by DAVID. (a) this figure shows 12 canonical pathways generated using IPA, which were statistically significant by enriching above the threshold (out of 47). The bars in the graph represent the total molecules involved in these pathways while the ratio (yellow line) shows the proteins given for each pathway in the data, (b) the glycolysis is shown to be the first cluster of functional gene annotation according to DAVID bioinformatics tool. In this figure, the horizontal axis demonstrates the functions of the related proteins that are represented in the vertical axis

Table 4. Protein cellular locations and physiology. The locations and functions of the detected sperm proteins detected were generated using IPA analyses

Id	Symbol	Entrez gene name	Location	Type(s)
77735757	ACADS	acyl-CoA dehydrogenase, C-2 to C-3 short chain	Cytoplasm	enzyme
148744160	ACP1 (includes EG:11431)	acid phosphatase 1, soluble	Cytoplasm	phosphatase
194666681	ACRBP	acrosin binding protein	Extracellular Space	other
115495399	ACRV1	acrosomal vesicle protein 1	Cytoplasm	other
84370183	ACTL7A	actin-like 7A	Nucleus	other
61878077	ACTRT1	actin-related protein T1	Cytoplasm	other
84000199	ACTRT2	actin-related protein T2	unknown	other
115495817	C15orf26	chromosome 15 open reading frame 26	unknown	other
76650703	EFHC1	EF-hand domain (C-terminal) containing 1	Cytoplasm	other
4927286	ENO1	enolase 1, (alpha)	Cytoplasm	transcription regulator
110626121	GAPDHS	glyceraldehyde-3-phosphate dehydrogenase, spermatogenic	Cytoplasm	enzyme
94966950	HSPB9	heat shock protein, alpha-crystallin-related, B9	Cytoplasm	other
156120505	IZUMO4	IZUMO family member 4	unknown	other
78369344	LDHAL6B	lactate dehydrogenase A-like 6B	Cytoplasm	enzyme
84370143	NT5C1B	5'-nucleotidase, cytosolic IB	Cytoplasm	phosphatase
84000345	Odf2	outer dense fiber of sperm tails 2	Cytoplasm	other
27806061	PDE6D	phosphodiesterase 6D, cGMP-specific, rod, delta	Cytoplasm	enzyme
77735827	PEBP4	phosphatidylethanolamine-binding protein 4	Cytoplasm	other
115495837	PGCP	plasma glutamate carboxypeptidase	Extracellular Space	peptidase
174840786	PGK2	phosphoglycerate kinase 2	Cytoplasm	kinase
6981420	PRSS1/PRSS3	protease, serine, 1 (trypsin 1)	Extracellular Space	peptidase
7555818	SOD2	superoxide dismutase 2, mitochondrial	Cytoplasm	enzyme
157279923	SPACA3	sperm acrosome associated 3	Cytoplasm	enzyme
149773556	TEKT3	tektin 3	Cytoplasm	other
6678465	TUBA3C/TUBA3D	tubulin, alpha 3c	Cytoplasm	other
14124960	TUBB2C	tubulin, beta 2C	Cytoplasm	other
126723634	WBP2NL	WBP2 N-terminal like	Cytoplasm	other
68085578	YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	Cytoplasm	enzyme

fertility bulls (Bulls A and B) compared to that from their lower fertility counterparts (Bulls C and D) in the experimental population. On the other hand, ODF2 protein was up regulated in spermatozoa from low fertility animals (Bulls C and D) although their expression levels varied in the individual bulls. The average of protein expression ratios obtained from relatively lower fertility bulls (Bulls C and D) over high fertility bulls (Bulls A and B) (Table 1).

3.4. Sperm Proteins as Fertility Markers and Likely Protein-Protein Interaction

In search for potential fertility markers, we have determined sperm proteins that have been identified in this study and through the literature search. The fertility makers are mainly chromatin/nuclear proteins, seminal plasma proteins, proteins in acrosome, proteins which regulate ATP synthesis, capacitation related proteins, and sperm cytoskeletal proteins (Table 5).

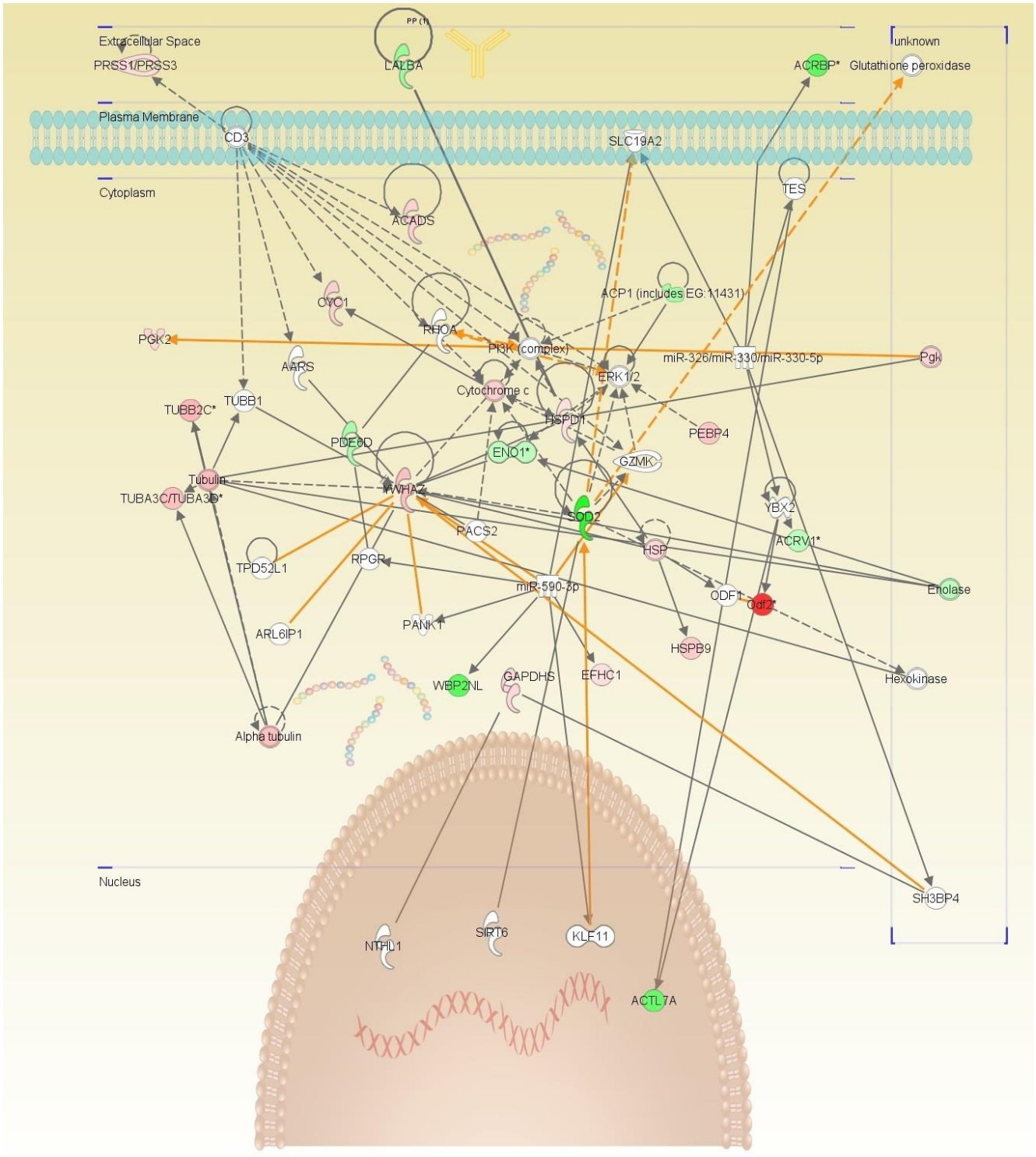

Figure 4. The merged interactome by IPA Networks 1 and 2. The final interactome was created by overlaying of these two networks free radical scavenging and cellular assembly or organization, cellular development, and embryonic development networks. The proteins and their interactions between others including as well as their locations in the cell are also included in the figure. The red and green colors of each protein represent up-regulated and down-regulated proteins in spermatozoa from lower fertility bulls (Bull C and D), respectively

Table 5. Comparison of sperm protein markers reported among different bovine species belonging to different categories of sperm physiology pathways in current literature

Category of protein	Protein name	Function	Holstein and other bovine bull sperm	Angus bull sperm
Sperm chromatin proteins	Protamines (PRM)	Substitute for histones in the sperm chromatin spermatid to sperm development phase of spermatogenesis. They compact sperm DNA into a highly condensed, stable and inactive complex	Holstein (Fortes *et al.* 2014; Dogan *et al.* 2015)	No reports
	HIST1H2BA/ TH2B	Testis specific histone variant specifically required to direct the transformation of dissociating nucleosomes to protamine in male germ cells	Holstein (Kutchy *et al.* 2017)	No reports
	H3K27ac	Functions as histone acetyltransferase and regulates transcription via chromatin remodeling. Acetylates all four core histones in nucleosomes	Holstein (Kutchy *et al.* 2018)	No reports
	H3K27me3	Catalytic subunit of the PRC2/EED-EZH2 complex, which methylates 'Lys-9' (H3K9me) and 'Lys- 27' (H3K27me) of histone H3, leading to transcriptional repression of the affected target gene	Holstein (Kutchy *et al.* 2018)	No reports
Seminal plasma proteins	Osteopontin	Binds tightly to hydroxyapatite and appears to form an integral part of the mineralized matrix. Probably important to cell-matrix interaction	Holstein (Cancel *et al.* 1997; Cancel *et al.* 1999)	No reports
Acrosomal proteins	Acrosomal tyrosine-phosphorylated proteins	Role in acrosomal formation of spermatids during spermiogenesis	Japanese Black cattle (Harayama *et al.* 2010)	No reports
	IZUMO1	Fusion of sperm to egg plasma membrane	Japanese Black cattle (Fukuda *et al.* 2016)	No reports
ATP synthesis proteins	Adenylate kinase 1 (AK1)	Catalyzes the reversible transfer of the terminal phosphate group between ATP and AMP. Plays an important role in cellular energy homeostasis and in adenine nucleotide metabolism	Holstein (D'Amours *et al.* 2012)	No reports
	Enolase 1 (ENO1)	Multifunctional enzyme, plays part in glycolysis, plays a part in various processes such as growth control, hypoxia tolerance and allergic responses. May also function in the intravascular and pericellular fibrinolytic system due to its ability to serve as a receptor and activator of plasminogen on the cell surface of several cell-types such as leukocytes and neurons. Stimulates immunoglobulin production	Hanwoo (Park *et al.* 2012)	No reports
	ATP synthase H+ transporting mitochondrial F1 complex β subunit (ATP5B)	ATP synthesis and hydrolysis of proton transport	Holstein (Peddinti *et al.* 2008)	No reports
Capacitation proteins	Na+/K+-ATPase	Its role is to create the electrochemical gradient of sodium and potassium, providing the energy for active transport of various nutrients	Holstein (Thundathil *et al.* 2006)	No reports

Table 5. Continued

Category of protein	Protein name	Function	Holstein and other bovine bull sperm	Angus bull sperm
Cytoskeletal proteins	Outer dense protein fiber 2 (ODF2)	A major component of sperm tail outer dense fibers (ODF). ODFs are filamentous structures located on the outside of the axoneme in the midpiece and principal piece of the mammalian sperm tail and may help to maintain the passive elastic structures and elastic recoil of the sperm tail. May have a modulating influence on sperm motility. Functions as a general scaffold protein that is specifically localized at the distal/subdistal appendages of mother centrioles	Holstein (Petersen *et al.* 1999; Wang *et al.* 2014)	Current study
Other proteins	Super oxide dismutase (SOD)	Destroys radicals which are normally produced within the cells and which are toxic to biological systems	Holstein (Bansal and Bilaspuri 2008)	Current study

4. Discussion

Molecular and cellular attributes of sperm are important for fertilization, egg activation and embryonic development. Some bulls that produce high numbers of spermatozoa with normal morphology exhibit low fertility after hundreds of AI (DeJarnette and Marshall 2005). Despite the importance of male fertility in reproduction for both basic and applied science, there is no sufficient method to determine sperm quality other than conventional semen analysis. Indeed, traditional approaches to estimate male fertility such as evaluating of sperm morphology and motility might not be accurate all the time (Bartoov *et al.* 1993). Molecular mechanisms of how male fertility can be fully determined by evaluating the sperm quality still remain a mystery. The objective of this study was to identify differentially expressed proteins in spermatozoa from bulls with different fertility taking advantage of both wet lab and computational biology and bioinformatics approaches.

While proteomics is an important high-throughput method providing a panoramic view of proteomes in the cell, bioinformatics is a powerful approach to predict and discover the functions and interactions of the given proteins. Sperm proteome profiling has been reported in human (Martinez-Heredia *et al.* 2006; Li *et al.* 2007), in murine (Cao *et al.* 2006; Baker *et al.* 2008a, 2008b), in porcine (van Gestel *et al.* 2007) and in bovine (Lalancette *et al.* 2006; Peddinti *et al.* 2008; D'Amours *et al.* 2010) and results generated by these researchers provided important insights about identities of diverse proteins. Previously, expressions of nine proteins, including two isoforms of epididymal sperm-binding protein E12 and proteasome subunit α type-6, were shown to be differentially expressed among high and low fertility Holstein bulls. Recently, using sperm from low *vs*. High fertility bulls, three proteins; enolase (ENO1), voltage dependent anion channel 2 (VDAC2), and ubiquinol-cytochrome-c reductase complex core protein 2 (UQCRC2) were detected to be the fertility markers in bulls (Park *et al.* 2012; Park *et al.* 2013; Kwon *et al.* 2015a). Our group previously showed that the expression of certain proteins in spermatozoa from high-fertility Holstein bulls was implicated in energy metabolism, cell communication, spermatogenesis, and cell motility (Peddinti *et al.* 2008). However, according to our literature search, sperm proteomics profiling of Angus bulls has been elusive.

In our study reported here, there were more than 80 sperm protein spots that notably differed among the bulls with varying fertility. By comparing the protein spot sequences to the databases, we identified most of these proteins. Our 2D-DIGE results demonstrated that ODF2 was up-regulated in spermatozoa from relatively low fertile bulls (Bulls C and D). ODF2 is a cytoskeletal structural protein in spermatozoa and it is abundantly present in flagella. Thus, it is associated with sperm morphology and motility, and that its absence or decreased expression leads to abnormal morphology and infertility (Petersen *et al.* 1999; Wang *et al.* 2014; Yoon *et al.* 2016). The structure of ODF2 protein is an α-helical and is similar to leucine zipper motif (Brohmann *et al.* 1997). Three or four testis-specific transcripts

of *odf2* gene have been detected in rat and bull spermatozoa (Brohmann *et al.* 1997; Schalles *et al.* 1998) and mutations in *odf2* gene are known to result certain tail abnormalities in spermatozoa (Tarnasky *et al.* 2010). Previously, ODF2 and Cenexin were shown to be the alternative splice variants of exon 3b of *odf2* in mouse testis (Huber *et al.* 2008). Our results from the 2D-DIGE experiments demonstrated the importance of ODF-2 in sperm motility and ultimately bull fertility and the results are supported by (Cao *et al.* 2006). Protein variations can be induced by additional posttranslational modifications such as phosphorylation, cleavage, and glycosylation (Flickinger *et al.* 2001). Indeed, our results showed that many of the differentially expressed protein spots corresponded to ODF2, suggesting the possible posttranslational modifications (PTM) occurring in this protein. For example, it was revealed that a tyrosine phosphorylation in sperm ODF2 took place during capacitation (Mariappa *et al.* 2010).

The other protein of significant function was SOD based on our 2D-DIGE and bioinformatics results. The SOD is an important antioxidant that dismutase O_2- into H_2O_2, improves cell survival by reducing the level of ROS. It is plausible that this increased expression of SOD reflects a defensive response to protect the spermatozoa against oxidative stress (Mruk *et al.* 2002; Fujii *et al.* 2003; Cui *et al.* 2008; Yoon *et al.* 2016). The SODs are scavenger antioxidants catalyzing the neutralization reaction of superoxide radicals into H_2O_2 and oxygen in the cell. Because of the cytoplasmic reduction and environmental changes, spermatozoa become vulnerable to oxidative stress in the course of spermatogenesis (Agarwal and Prabakaran 2005). On the other hand, SOD has also a protective effect against oxidation, enhancing sperm motility (Lindemann *et al.* 1988; Kobayashi *et al.* 1991). Likewise, there was an increase in sperm motility and viability and a decrease in the LPO levels when bull spermatozoa were subjected to Mn^{2+} treatment in presence of oxidative stress (Bansal and Bilaspuri 2008).

Another study showed that the expression of MnSOD in bovine blastocysts increased when the culture media was supplemented with fetal calf serum (FCS), which could improve cryotolerance of these blastocysts (Rizos *et al.* 2003). In addition, it was suggested that SOD activity in bovine spermatozoa might be a metabolic indicator of membrane integrity. Since the same study revealed a correlation between malondialdehyde production and SOD activity, measuring this enzyme in spermatozoa might predict oxidative stress-induced damage (Beconi *et al.* 1991). In contrast, a study concluded that male infertility was not related to the SOD activities in both human spermatozoa and seminal plasma where the semen was obtained from men with normozoospermia and oligoasthenozoospermia (Hsieh *et al.* 2002). However, fertility scores of human patients were not as reliable as those obtained from livestock animals. In another study, the MnSOD activity in human spermatozoa was detected to be negligible. However, compared to human blood plasma, the abundance of total SOD activity in the seminal plasma was 20 times higher. According to the same study, it was concluded that the minimal activity of SOD enzymes in spermatozoa might be the reason of its protection against internal and external superoxide radicals (Peeker *et al.* 1997).

We established here that MnSOD was up-regulated in spermatozoa from relative higher fertile bulls (Bulls A and B) compared to their low fertile counterparts based on the 2D-DIGE results. Therefore, these differentially expressed proteins could potentially play key roles in spermatozoa and may be involved in male fertility and could be used to predict superior sires (Kwon *et al.* 2015a, 2015c). We concluded that the abundance of SOD in spermatozoa differs among the bulls with different fertility in a given population. This might be an indicator of excessive oxidative stress caused by cryopreservation or centrifugation in spermatozoa, affecting sperm motility and ultimately male fertility.

Protein based molecular markers provide reliable information about the elite sire as well as its progeny. However, comparing Angus cattle across other cattle breeds especially Holstein for recognizing the protein molecular markers for sire selection, we find no report for Angus. Different categories of sperm associated proteins are chromatin/nuclear proteins, seminal plasma proteins, proteins in acrosome, proteins which regulate ATP synthesis, capacitation related proteins, and sperm cytoskeletal proteins. We have report about the sperm chromatin related proteins as potential markers for selection (Fortes *et al.* 2014; Dogan *et al.* 2015; Kutchy *et al.* 2017; Kutchy *et al.* 2018) out of all these reported proteins no reports are available for Angus cattle one of the well reputed cattle breed in the USA. Therefore, we realize urgent need of selection of Angus bulls based on protein as markers of selection and hence

dire need to report the potential protein markers in Angus sperm and related proteins.

In conclusion evaluating semen quality and predicting bull fertility are vital for precision livestock agriculture. With the increasing uses of artificial insemination bull effects on herd is becoming more prevalent. Low heritability of the fertility traits implies that much of the differences in bull fertility are related to environment, management, nutrition and epigenetics. As such, sperm functional genomes such as proteomes reflect sperm fertility, and the differentially expressed proteins in high fertility *vs*. Low fertility can be harnessed as potent fertility markers in sperm evaluation and marker assisted selection. There is a need for reliable phenotypic data in order to identify such fertility markers. Compared to the dairy cattle, there is a disparity of phenotypic data in beef cattle. To remedy this, beef producers should collect phenotypic data and keep records including the pedigree information. Through comparative biology, sperm fertility proteins identified in dairy bulls can be studied to determine to what extent the protein markers can be used for beef bulls. Sperm protein markers can be combined with other sperm parameters and used as complementary tests in genomic selection. Comprehensive studies aimed at sperm functional genome and epigenome in larger sample sizes during the entire year for multiple years are expected to further fundamental science and technology of bull fertility.

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