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Protein markers in Angus bull spermatozoa for fertility

Xiaojun Wang

PROTEIN MARKERS IN ANGUS BULL SPERMATOZOA FOR FERTILITY

By

Xiaojun Wang

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By

Xiaojun Wang

Approved:

_____

Erdogan Memili
Assistant Professor of Animal and
Dairy Sciences (Major Professor and
Director of Dissertation)

_____

E. David Peebles
Professor of Poultry Science
(Committee Member and Graduate
Coordinator)

_____

Attila Karsi
Assistant Research Professor
CVM, Department of Basic Science
(Committee Member)

_____

Abdullah Kaya
Product Technology Development
Specialist in Alta Genetics
(Committee Member)

_____

Melissa J. Mixon
Asc VP&Ext Professor
Interim Dean of College of Agriculture
and Life Sciences

Name: Xiaojun Wang

Date of Degree: August 7, 2010

Institution: Mississippi State University

Major Field: Genetics

Major Professor: Erdogan Memili

Title of Study: PROTEIN MARKERS IN ANGUS BULL SPERMATOZOA FOR FERTILITY

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Candidate for Degree of Master of Science

In the field of mammalian reproduction, the success rate of fertilization largely relies on spermatozoa fertility potential. Total proteins change to a large degree during gametogenesis to activate gametes. There are some techniques that are most popular for proteomic studies of fertilization, including SDS-PAGE, 2D-DIGE, Western Blotting and Immunocytochemistry, as well as Mass Spectrometry. Proteins and their cofactors play important roles at different stages of gametogenesis, fertilization and embryo development. However, insufficiencies in the construction mode of fertility hinder the techniques in determination the sperm fertility. This study focused on identifying and tracking crucial proteins for fertility based on comparison between high- and low-fertility sperms by means of 2D-DIGE and immunoblotting. We identified 18 proteins that varied significantly, including Outer dense fiber 2 (ODF2) of sperm tails and Manganous superoxide dismutase (MnSOD). Differences in these proteins suggest that posttranslational modification and deoxydation of sperm proteins might be associated with fertility.

Key words: fertility, proteins, ODF2 and MnSOD

DEDICATION

This work is dedicated to my parents, Qingfang Li and Ming Wang for their unconditional support.

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ATP	Adenosine triphosphate
2D-DIGE	Two-dimensional difference gel electrophoresis
ADAM	A Disintegrin And Metalloproteinase
APS	Ammonium persulfate
BSA	Bovine Serum Albumin
DTT	Dithiothreitol
ESI	Electrospray ionization
H_2O_2	Hydrogen peroxide
HRP	horseradish peroxidase
IEF	Isoelectric focusing
IgG	Immunoglobulin G
MnSOD	manganese superoxide dismutase
MS	Mass spectrometry
PBS	Phosphate buffered saline
PVDF	Polyvinylidene fluoride
PT	perinuclear theca
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE)
TBS	Tris Buffered Saline
TCA	Trichloroacetic Acid in Acetone
TEMED	N, N, N', N'-tetramethylethylenediamine
TLRR	leucine-rich repeat protein
TSSK	Testis Specific Serine Kinase

CHAPTER I

PROTEOMIC STUDY OF PROTILITY IN BULL SPERMATOZOA

Introduction

Fertilization is the process of fusion between spermatozoa and oocyte, by which a new individual forms. Fertilization sets the first stage for embryogenesis and beyond in development of full term offspring. The importance of fertilization relies on two functions: 1) transferring genes to the next generation and 2) initiating the reaction in egg cytoplasm to proceed towards further development (Gilbert 2003). Sex of offspring is determined during fertilization resulted by combination of the two haploid genomes which is derived from two parents. Fertility significantly impacts the efficient production of animals as a result of its dominant role in increasing numbers of cattle. Following the formation of a zygote, developmentally regulated events lead to embryogenesis.

Maternal transcripts and proteins mostly support embryos during the early stages of development following fertilization (Flach, Johnson et al. 1982; Bolton, Oades et al. 1984). Fertilization and early embryogenesis both involve several developmentally regulated events that must take place properly for successful gametogenesis and embryogenesis. For instance, capacitation of the sperm, the attachment of sperm to the oocyte, and the number of sperm binding to the zona pellucida all affect the rate of successful fertilization. Similarly, the molecular and cellular health of developing embryos is critical for the formation of blastocysts with high developmental competency.

Therefore, well orchestrated mechanisms ensure the accuracy of the whole pathway in animal development.

Gametogenesis is a process of producing haploid gametes through one mitotic division and two meiotic divisions. Gametogenesis includes development of male and female germ cells, through spermatogenesis and oogenesis, respectively. At the beginning of spermatogenesis, a stem cell, called the spermatogonium, develops to one primary spermatocyte through mitosis. Secondly, the primary spermatocyte which contains duplicate genomes divides into two secondary spermatocytes through Meiosis I, followed by Meiosis II. In all, four haploid spermatids are formed by the deviation of two secondary spermatocytes at the end of the second meiosis. At last, four identical mature sperm cells are constructed by a series of differentiations. The main difference between spermatogenesis and oogenesis is the number of gametes each parental stem cell produces, which are four in spermatogenesis and only one oocyte in oogenesis. That is a result of the first and second meiosis in oogenesis during which only one cell is kept in each division. Healthy gametes are a prerequisite for fertilization and preimplantation embryonic development.

It takes about ten days for spermatozoa to gain the fertilizing capability in the epididymis (Robaire and Hermo 1988) and it is their flagellum that generates the force to reach the oocyte (Mortimer 1997). Fertilization is not only a step of fusion between an oocyte and sperm. Indeed, it has four main stages: 1) recognition and interaction between sperm and egg; 2) entry of sperm into oocyte; 3) diffusion and reformation of both nucleuses and 4) rearrangement of egg cytoplasm (Gilbert 2003). Cleavage occurs after fertilization, but cytoplasmic volume does not change even after the cell has gone through several mitotic divisions. The amount of genetic material increases by many folds during

this period. The first mitotic division in early cleavage was found to determine embryo quality and pregnancy rate (Shoukir, Campana et al. 1997; Sakkas, Shoukir et al. 1998; Lundin, Bergh et al. 2001). The zygote develops to morula during cleavage and starts to differentiate after it reaches the 64-cell stage.

Beef cattle traits have been grouped to indicate profitability, which include reproduction, growth, carcass and commercial beef production (Anderson and Lewis 1990).The Angus first attracted people's attention and demonstrated their breed's value by better crossing the winter and more weight gained during the next spring (American Angus Association). Genetically, Angus cattle have advantage in strong maternal traits, carcass quality and calving ease. Besides, their vigorous growth, convenience traits and versatility help make Angus cattle invaluable to industries (American Angus Association) Furthermore, breed improvement records and reliable genetic selection tools increase the application of Angus cattle. So far, Angus cattle have been the most wide spread cattle in the US.

The impact of spermatozoa not limited to contribute through half of the total genome of the zygote. It is known that metabolic reactions multiply during oogenesis, such as lactate dehydrogenase activation (Mangia and Epstein 1975), and a considerable increase of ATP (Mangia, Erickson et al. 1976). Huge amounts of proteins are synthesized and increased linearly throughout the entire period of oocyte growth (Canipari, Pietrolucci et al. 1979). Although not many nuclear-encoded translations exist in mature spermatozoa, there is still some protein synthesized by mitochondrial-encoded RNA (Premkumar and Bhargava 1972; Hecht and Williams 1978; Alcivar, Hake et al. 1989) for capacitation or for replacing the proteins degraded during swimming or other actions prior to fertilization (Gur and Breitbart 2008).

The chemicals in both cytoplasm and membrane play vital roles in regulating fertilization and embryogenesis. Proteins synthesized during gametogenesis assist fertilization and early embryo development. For example, the integrin superfamily is one group of heterodimeric receptors identified as exracellular matrix ligands on the oocyte surface. Not only a protein phosphatase in oocyte, integrins can also serve as signaling receptor to mediate intracellular Ca^{2+} ions, pH and inositol turnover as well (Schwartz 1993; McNamee, Liley et al. 1996). Multiple molecules in both oocyte and sperm usually play critical roles after fertilization. Mitosis –promoting factor (MPF) regulates meiotic division in oogenesis and continues its role in the biphasic cell cycle of early blastomeres (Schorderet-Slatkine and Drury 1973; Ford 1985; Maller 1985).

In order to discover and understand regulatory elements in fertility, specific proteins related in this process are important clues to begin with. Their interactions with other proteins or gene networks in a systems biology framework will help construct the networks of regulation. The purpose of this review is to provide a narrative on spermatozoa proteins and their roles in fertility. Proteins which determine the success of spermatogenesis are expected to define fertile sperms. These can be used as protein markers to determine abnormal or low fertile sperms. While industries continue to improve Angus breed, translation of basic research findings is expected to help achieve this goal.

It is important to expand our knowledge on fertility and early embryonic development for both advancement in basic science and discovery, and for biotechnology. Puzzles in the process of fertility and early embryo development will be resolved by more comprehensive studies on the protein markers. Further advancement in

this area of research will be enabled through hypothesis driven projects on the functions of the critical spermatozoal proteins. It will become easier to trace the molecular causes of fertility deficiencies after the construction of the whole system. Reasons of infertility will be analyzed more precisely and quickly. Biotechnology and biopharmaceutical industries will benefit from basic knowledge to better understand and improve fertility in mammals.

The significance of identifying key proteins in bull spermatozoa is not limited to applications in cattle. Experiments aimed at studying humans are restricted to other model systems. It is known that a large number of molecules having been identified to be critical in fertilization processes are highly conserved in both the mammalian model system and in humans. In fact, recently completed bovine genome sequence and annotation reviews have shown that cows and humans share more similarities than previously thought both at genomic and physiological levels (Elsik, Tellam et al. 2009).

Approaches for the Study of Sperm Proteomes

SDS-PAGE electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is one of the most popular proteomic approaches in biochemical experiments to separate proteins. SDS gel usually consists of stacking gel and separating gel (or resolving gel). The stacking gel compresses the sample proteins and enables them to run at the same point of initiation. The separating gel sorts the proteins by their charge/ weight. Acrylamide and Bisacrylamide work as cross linkers in the gel, together with Tris buffer. Adding of Ammonium persulfate (APS) and N, N, N', N'-tetramethylethylenediamine

(TEMED) initiate the cross link of Acrylamide and Bisacrylamide. Protein samples are usually denatured in the reducing system and loaded with protein ladder in the wells. The negatively-charged proteins migrate across the gel towards the anode in the electric field. The running system is determined by the concentration and size of the gel and molecular weight of protein samples. Following electrophoresis, Coomassie Brilliant Blue R-250 is widely used to stain and visualize the proteins in the gel.

SDS-PAGE was first published as a method in scientific study in 1970 (Laemmli 1970). The principle of SDS-PAGE relies on the same attachment of SDS in each unit of peptic length, which induces the same charge per mass unit. Proteins are denatured by SDS, which is an anion detergent and rounded with negative charge. Therefore, the charge of protein is relative with its size which enables us to separate proteins according to their molecular weight. The molecular weight of target proteins can be approximated in the gel by the markers whose molecular weights have already been determined. The amount and purity of protein samples can simply be viewed according to the densities and numbers of the bands. SDS-PAGE is hence critical for proteomics as a result of its broad application in protein size modification, protein identification, purity examination and protein quantification. Blotting application also expands its usage. However, unlike native electrophoresis, SDS-PAGE cannot be applied to keep protein's native state because of the detergent. Another limitation is that the proteins with similar molecular weight cannot be separated by SDS-PAGE. Furthermore SDS-PAGE is not a suitable method for low-molecular weight proteins.

Two-dimensional differential in gel electrophoresis (2D- DIGE)

From the same principles of SDS-PAGE, Two-dimensional difference gel electrophoresis (2D-DIGE) was developed and it enables separation of proteins with different Molecular Weight as well as Isoelectric Point. The 2D DIGE technique was not described in the literature until 1975 (O'Farrell 1975). In 1997, 2D DIGE was developed to run more than one sample by prelabeling with fluorescent cyanine dyes (Unlu, Morgan et al. 1997). Trichloroacetic Acid in Acetone Protocol (TCA precipitation) or Bio-Rad protein assay is carried out to extract proteins. For the experiments having more than two samples, the internal control which contains the whole proteins in each sample will benefit much for the quantification accuracy. Linear or nonlinear (Bjellqvist, Sanchez et al. 1993) program is run after the samples are loaded in the IPG strips. The first dimension will separate the proteins according to their PI in an IPG strip. In the second dimension step, IPG strips are loaded in the SDS-PAGE where proteins will be separated again by their charge or molecular weight. To visualize the protein spots, both Coomassie Brilliant Blue and silver staining are used (Merril, Goldman et al. 1981Oakley, Kirsch et al. 1980). However, they are not as good and sensitive as detection methods relying on fluorescent compounds and radiolabeling proteins (Gorg, Weiss et al. 2004). The diluted protein samples and internal controls are prelabeled with the fluorescent cyanine dyes (CyDye, Cy2, Cy3, and Cy5 separately) before being loaded in IPG strips so that three groups of samples can be visualized individually in different wavelengths.

The length of IPG strip varies form 7-24 cm and its PH range is between 2.5 and 12. More than 5,000 proteins can be resolved in the gel and this technique can detect as little as 1 ng of proteins per spot (Gorg, Weiss et al. 2004). This is the only technique to parallel large sets of protein mixtures. The protein expression level, isoforms or

modification can be reflected from the protein mapping. Since the dyes cannot bind to the proteins without lysine, 2D DIGE exhibites different sensitivities towards proteins contain low or high amount of lysine.

Western Blotting (also known as immunoblotting)

Protein signals can be detected through specific antibodies in Western Blotting. The antibodies bind to their target proteins which work as antigens in a membrane and emit signals by the conjugated enzymes. Cells and tissues need to be prepared and resolved in reducing agent. SDS-PAGE electrophoresis with positive control is needed so that the proteins can be separated in the gel and later transferred to a membrane. The positive control works as a marker to confirm the existence of target protein. The transfer process can be monitored by reversible staining or Ponceau S staining of the membrane. In order to prevent non-specific binding and to decrease background, the membrane is incubated in blocking buffer at room temperature for about an hour. Traditional blocking buffer is usually produced by adding blocking agent (0.5% casine, 5% non fat milk and 5% Bovine serum albumin) in TBS (50 mM Tris.HCl, pH 7.4 and 150 mM NaCl). Followed by three rounds of washing, the membrane is then incubated with primary antibody, which binds to the target protein. Following washing, secondary antibodies conjugated with horseradish peroxidase or alkaline phosphatase is added into the membrane. The horseradish peroxidase or alkaline phosphatase enzymes react with substrates and emit light or other detectable signal. Final signals can be detected and visualized by X-ray film and digital images.

Transfer of proteins from the gel to the membrane can be accomplished in several apparatuses. Wet transfer is less likely to increase the background caused by drying of the

membrane and it is recommended that large proteins (>100 kDa) are transferred more effective in wet transfer. Semi-dry transfer decreases the transfer time to an hour or less and currently it is the most widely used transfer. Dry transfer takes only eight minutes or less, but the application is restricted by its transfer efficiency. Nitrocellulose and Polyvinylidene fluoride (PDVF) are the most popular membranes in Western blotting. Fixing or staining of gels will lower the efficiency of transfer, especially for the proteins more than 50 kDa (Perides, Plagens et al. 1986). Therefore, the electrophoresis separation of proteins cannot be examined by staining the gel until the transfer step finishes. The capacity of analyzing and identifying target proteins leads to the popularity of Western Blotting. Protein concentration can be quantified by this technique according to the signal intensity. However, it cannot show the localization of this protein in the cell, which can be accomplished by immunocytochemistry. Although Western Blotting has been widely utilized, its reliability is reduced by its nonspecificity (Larsson 1988). Degradation of target protein causes visualization of multiple bands.

Immunocytochemistry

Immunocytochemistry is a common technique that uses antibody to examine and visualize the presence of a specific protein in cells. Ethanol is dipped on the solid support and the surface is then flamed to be sterilized where cells would attach. Cells are then fixed after rinsing with PBS and being frozen at -20 ℃. The cells are blocked by gentle rocking with blocking buffer followed by incubation with primary antibody. Next, the cells are washed and added with secondary antibody, followed by four rounds of washing. At last, the whole support and cells together are dried and sealed with nail polish. The signal can be detected under fluorescent microscope.

Immunocytochemistry is widely used to determine the existence of a specific group of molecules and localize them in the cells. It is also utilized for the studies of protein processing and interaction. The application of immunocytochemistry is expanded by combination with other techniques, such as Formaldehyde-Induced Fluorescence, Autoradiography and Microinjection. However, since the immune system is adjustable and flexible, immunocytochemistry is questioned for its specificity. Secondly, immunocytochemistry cannot characterize the charge, size or hydrophobicity of the molecules (Larsson 1988). Compared to other immunoassays, it is expensive and difficult to quantify the signals to determine the amounts of proteins expressed.

Mass spectrometry

Mass spectrometry (MS) is a powerful technique to ionize and determine elemental composition regarding mass-to-charge ratio. An ion source, a mass-selective analyzer, and an ion detector are three main modules in an MS instrument. The procedures in this analytical technique generally include five steps. Firstly, a sample is vaporized prior to an ion source. Secondly, the ion source converts gas phase sample molecules into positively charged ions. Then the magnetic field in the instrument accelerates all the ions. The high speed ions are finally separated based on various masses. At last, the detector collects all the data and analyzes them.

Tandem mass spectrometer is the main MS to identify proteins (Boyd 2005). Electrospray ionization (ESI) and matrix assisted laser desorption ionization (MALDI) are two major developed methods of MS, which are able to analyze molecules of very high molecular weight. The importance of Mass spectrometry is not limited to protein identification but is also important for its wide application in isotope dating, atom probe,

protein characterization, etc. Mass spectrometer was even utilized to analyze respired gas in hospital (Riker and Haberman 1976). Mass spectrometry is a high throughput technique and always couples with other techniques, such as SDS-PAGE and 2D DIGE

The combination of MS and liquid chromatography (LC) enhances the separation and can identify more than 1,000 proteins (Washburn, Wolters et al. 2001; Peng, Elias et al. 2003; Liu, Sadygov et al. 2004). However, MALDI has its limitation on protein quantification due to its nonlinear signal intensity and ESI-MS/MS has a high requirement of high purity protein samples and has a poor tolerance for electrolytes and detergents (Barnidge, Dratz et al. 1999; Ardrey 2003)

Proteomes of Bull Spermatozoa

Huge changes in transcription and translation occur during spermatogenesis (Hecht 1988; Hake, Alcivar et al. 1990). Considering the limited contents in the sperms, proteins that still exist in the spermatozoa should be very important for sperm maturation and fertilization. Jmjd1a, for example, is a regulator in regulating histone modification through demethylation in testis. Jmjd1a deficiency induces many infertile symptoms, so the essential role of Jmjd1a was determined for gene expression and spermatogenesis (Liu, Zhou et al. 2010). In spermatids, KIFC1 (Kinesin family member C 1) motor, which is a member of the Kinesin-14 subfamily, associates with nucleoporin-containing complex and is probably involved in acrosome elongation and manchette motility (Yang and Sperry 2003; Yang, Jefferson et al. 2006). TLRR is a leucine-rich repeat protein which interacts with KIFC1 (Wang and Sperry 2008). Associating with the manchette, TLRR binds with testis specific isoform of protein phosphatase-1 (PP1) and was suggested to take part in cytoskeleton modulation (Wang and Sperry 2008).

A number of enzymes mediating the process of spermatogenesis have been discovered. The Testis Specific Serine Kinases (TSSKs) are a group of kinases in germ cells or in testis, which include TSSK1, TSSK2, TSSK3 and others (Bielke, Blaschke et al. 1994; Kueng, Nikolova et al. 1997; Zuercher, Rohrbach et al. 2000). Since Specific Serine Kinases are only synthesized postmeiotically in spermatogenesis, they were proposed to act during sperm maturation, capacitation and fertilization. Sosnik et al (Sosnik, Miranda et al. 2009) demonstrated Tssk6's essential role in maintaining sperm structural integrity and suggested its function to regulate actin dynamics. Trypsin is another enzyme that is active during spermatogenesis. In addition to being secreted into the intestine to digest proteins, trypsin also functions in the initiation of meiosis, spermiogenesis, and fertilization (Miura, Ohta et al. 2009). Miura and his colleague also implied the involvement of trypsin in the entry site of the meiosis cycle.

Fertilization

Proteins that localize on the sperm membrane usually play critical roles on fertility through their functions in cell-cell interaction. One of the sperm membrane proteins, SP22 was found to be highly correlated with fertility and may be active in both zona penetration by sperm to and the fusion of sperm and oocyte membranes (Klinefelter, Welch et al. 2002). This protein can also be expressed in the ovary and reaches its peak in CL during pregnancy (Benoit, LaVoie et al. 2007).

Existing in ciliated cells, Sperm Flagellar Protein 2 (SPEF2, also called KPL2) was first detected in 1999 and was suggested to transmit signal by its ATP/GTP binding site and proline-rich domain (Ostrowski, Andrews et al. 1999). Spermatogenesis needs the support from somatic Sertoli cells during the whole process (Kimmins, Kotaja et al.

2004; Kimmins and Sassone-Corsi 2005). Short-tail sperm defect were found to be caused by abnormal expressed SPEF2 (Sironen, Thomsen et al. 2006). After SPEF2 was detected in Sertoli cells and intercellularly between spermatozoa, it was implied to regulate the organization of sperm tails by interacting with intra-flagellar transport protein IFT20 (Sironen, Hansen et al. 2010). Integrin family also plays an important role in fertility, especially sperm and egg interaction. The fact that integrin beta 1 facilitated the rate of fertility confirmed the importance of integrin beta1 to assist the binding between sperm and egg plasma membranes (Baessler, Lee et al. 2009). Another family is ADAMs, which is widely spread with a disintegrin and metalloprotease domain, and joins in the process of fertility, embryogenesis, etc (Edwards, Handsley et al. 2008). Fertilin β (ADAM2) is thought to be an important protein in sperm-egg binding by interacting with integrin through its disintegrin domain (Evans 2002). Recently, Testase 1(ADAM 24), a sperm membrane protein, was shown to partially control the number of sperms that interact with occytes (Zhu, Gupta et al. 2009).

Egg activation

In addition to half of the genome, sperm also injects thier cytoplasm into oocyte, in which many proteins from sperm will regulate egg activation. The condensed cytoplasm forms the structure named perinuclear theca (PT), which includes the subacrosomal layer (SL) and the postacrosomal sheath (PAS) (Oko and Morales 1994; Oko, Aul et al. 2001; Robaire 2001). Experiments have shed lights into the trace molecules performing oocyte activation from sperm head, which was able to activate oocyte alone while sperm tail could not (Kimura, Yanagimachi et al. 1998). PAWP is a sperm-specific WWI domain-binding protein only detected in PT. The role of PAWP was

appreciated from the observation that it was critical in meiotic resumption and pronuclear development during egg activation by a PPXY motif (Wu, Sutovsky et al. 2007). In the same year, Wu et al (2009) also found PAWP was dependent on manchette microtubules during spermiogenesis and PAS was the only structure mediating egg activation (Wu, Sutovsky et al. 2007).

Ca^{2+} increase is the most remarkable event during egg activation. Its active role has been proven by the experiment results showing that egg activation was occurred following Ca^{2+} injection in the absence of sperm (Swann and Ozil 1994; Schultz and Kopf 1995). The proteins that induce the calcium oscillation in egg cytosol should be responsible for its activation (Swann, Larman et al. 2004). PLC ζ protein, which is exclusively expressed in spermatids (Saunders, Larman et al. 2002) is a novel form of phospholipase C family. It has been proved to cause Ca^{2+} oscillation by injecting pure cRNA into oocytes (Swann, Larman et al. 2004).

Early embryonic development

The ciliary structure of sperm tails is mainly separated into fibrous sheaths and outer dense fiber proteins. As a fibrous sheath protein, TSGA10 is expressed in embryos and increases during embryo development, which provides evidence to suggest its involvement in ciliary cell to cell division (Behnam, Modarressi et al. 2006). There are 11 isotypes in the Protein Kinase C (PKC) family and many of them participate in spermatogenesis, fertilization and embryogenesis. In early embryo, PKC isotypes overcome changes in both concentration and location. High concentrations of PKC α, γ and ζ are detected in the nuclei in two-cell stage, although none of PKC α and γ appears around pronuclei in the zygote (Baluch and Capco 2008) (Dehghani and Hahnel 2005).

However, these isotypes can only be detected in a narrow location after the two-cell stage, except PKC ζ (Capco 2001). PKC ζ is highly enriched until the end of the eight-cell stage, where it decreases before the blastocyst stage (Pauken and Capco 1999; Pauken and Capco 2000). It is concluded that PKC family plays a role by phosphorylating special substrates in embryogenesis with their cofactors (Kalive, Faust et al. 2010).

Besides proteins that mediate the process of embryonic development, there are some proteins aimed to protect the embryo from radiations. As early as 1996, Norimura and his colleagues already found the activation and increasing of p53 which was induced by irradiation. More data have shown the existence of at least three mechanisms regulated by p53 and by p21 (Toyoshima 2009).

Conclusions

Successful fertilization is the beginning of a new life, where halves of the genomes of two parents are combined. A series of developmentally regulated events must be accomplished before fertilization occurs, which are called gametogenesis (spermatogenesis and oogenesis). A stem cell is infertile until it undergoes Mitosis, Meiosis I and Meiosis II. Spermatogenesis and oogenesis are quite similar, but the main difference is the number of gametes that each stem cell produces (four in spermatogenesis and one oocyte in oogenesis). Cleavage occurs after fertilization, which is followed by early embryogenesis. Genetically, Angus cattle are widely raised due to strong maternal traits, carcass quality and calving ease. Industries are also benefit from their vigorous growth, convenience traits and versatility.

Translation slows down in nuclease of mature spermatozoa, but mitochondrial-encoded translations still take place. These translated proteins are synthesized only when they are essential for capacitation and fertilization. Both cytoplasm and membranes have proteins that regulate and assist fertilization and embryogenesis. Therefore, these proteins can give us clues as to the processes related with fertility regulation. They can be applied as protein markers to determine abnormal or low fertile sperms. They have the potential to improve the Angus breed in industry and enlarge basic research findings. A better understanding and fertility improvement, through the use of precise and rapid analysis, will not only benefit in basic science but will also benefit mammal biotechnology and biopharmaceutical industry.

Many approaches have been applied on proteomic studies of bull fertility. The principle that the charge of the protein is positive in relation to its size enables us to separate proteins according to their molecular weight. SDS-PAGE has become a basic technique with wide application when it is integrated with other techniques. Finally, we are able to determine protein size, modification, identification, purity, and amount. 2D-DIGE accelerates the development of proteomic by direct and global two dimension views of several hundreds of proteins from the sample (Chevalier, 2010). Western Blotting and Immunocytochemistry are mainly used to detect the amount and location of target proteins. Many mass spectrometry techniques are dedicated to provide powerful analysis of the elemental composition of proteins.

Protein variety changes during the formation of mature gametes as well as their DNAs and RNAs. Proteins that remain in spermatozoa are considered to be critical for maturation, fertilization, etc. The observation that one specific protein deficiency induces many fertility symptoms confirms the importance of this protein for fertility. Many

protein families might function as mediators or maintain structural integrity of spermatozoa. Proteins and other molecules play their roles at different stages of gametogenesis. Membrane proteins usually serve in cell-cell interaction during fertilization and may be expressed in spermatozoa, oocyte, or both. It is believed that proteins transmit signals in the pathways including many protein families. Integrin and ADAM families widely exist in gametes and have been shown to control sperm-egg binding. Egg activation requires the participation of proteins in spermatozoa, especially cytoplasmic proteins injected into the occytes. They are critical in meiotic resumption and pronuclear development during egg activation, such as PAWP and PLC ζ. Cell division cannot occur successively without the proteins in spermatozoa. Proteins can either function in a short period or during most of the process. The Protein Kinase C (PKC) family is one of those proteins that play a role during spermatogenesis, fertilization and embryogenesis. There are also some proteins aimed to protect the embryo from radiations, etc.

Future Prospects

Although hundreds of proteins have been considered to be involved in fertilization, only a few protein pathways have been analyzed completely. The specific targets these proteins work on are not clear. Other elements, such as Ca^{2+} and K^{+}, also participate with proteins. But little is known about the mechanism how all of these factors are mediated and function together. Despite considerable amounts of research thus far, we are still at the beginning of understanding the proteomics of gametes. The next challenge is to identify the biological networks by which these proteins regulate the beginning of life.

Key chains of pathways might be the first step where scientists can have a breakthrough, because it is easier to find the significant events that occur in the complete network of biochemical pathways. In fact, endeavors have been made and many pathway databases have been created. However, they are far from completion. In the long term, the focus should be on the improvement of these databases. Advanced techniques will no doubt accelerate the development of network construction.

The application of this knowledge will definitely bring benefits to the animal agriculture industry. In research focused on the use of protein markers to measure fertilization potential, attention is drawn to the essential proteins that widely spread in spermatozoa that are closely linked with fertility. In this time of research, should be gamete samples quickly examined so that the higher fertile bulls would be selected. Obviously, the labor and cost of collecting fertility data and data processing can be decreased. It will become possible to increase fertility based on reasonable regulation and reconstruction of these mechanisms. These research findings will finally lead to financial profit once they are implemented.

CHAPTER II

PROTEIN MARKERS IN ANGUS BULL SPERMATOZOA FOR FERTILITY

Introduction

Male fertility is one of the most important factors affecting mammalian reproduction. It is genetic; hence protein variations in cattle cause differences in the fertility and efficiency of production. Semen is categorized as compensable or uncompensable by fertility traits (Evenson 1999; Saacke, Dalton et al. 2000; Watson 2000; Dejarnette 2005), and uncompensable fertility is independent of sperm dosage among males. Despite the importance of sperm in cattle reproduction, however, molecular mechanisms of uncompensable infertility and relative proteins are not quite clear yet.

Scientists began treatment of sterility before 1902, and spermatolysis was the first research object during the molecular research of male fertility (Martin, Carnett et al. 1902). In the earliest days, immunologic studies was one of the most important methods used to study the factors of sterility (Kiddy, Stone et al. 1959). Scientists once considered immunologic male infertility to be the main reason; however, the later measurement of seminal leukocytes in routine semen analysis shows its shortage in prognostic value (Tomlinson, Barratt et al. 1993). With the development of molecular biology, it was found that protein and DNA contents in sperm from an infertile human male had deficiencies in protamine that vary over time (Bench, Corzett et al. 1998).

Preliminary data show that there are protein markers for sub-optimal bull fertility, and many sperm proteins related to male infertility have been identified. Compared to sperms from low-fertility bulls, sperms from high-fertility bulls have a higher expression of proteins involved in energy metabolism, cell communication, spermatogenesis, and cell motility (Peddinti, Nanduri et al. 2008). The critical role of carbohydrates and glycoproteins has been assumed during sperm-oviduct adhesion (Lefebvre, Lo et al. 1997; Suarez SS 1998; Revah, Gadella et al. 2000; Talevi and Gualtieri 2001; Sostaric, van de Lest et al. 2005; Ignotz, Cho et al. 2007) , sperm-oocyte interactions (Tulsiani, Yoshida-Komiya et al. 1997; Gougoulidis, Trounson et al. 1999; Amari, Yonezawa et al. 2001; Tanghe, Van Soom et al. 2002; Thys, Nauwynck et al. 2009), and embryo implantation (Whyte and Allen 1985; Biermann, Gabius et al. 1997). Hepatocyte growth factor (HGF), a pleiotropic cytokine, was reported to influence mitogenesis, motility, differentiation, and functions on the maintenance of sperm motility (Janescha A. 2008). In the asthenozoospermic samples, scientists have found 17 protein spots at different amounts (Martinez-Heredia, de Mateo et al. 2008). These include cytoskeletal Beta actin, Annexin-A5, Cytochrome C oxidase-6B, Histone H2A, Prolactin-inducible protein and precursor, and so on.

It has been known that molecular defects in the sperm might cause sub-par fertility. However, the area regarding the mode of bovine fertility is still a puzzle (Talevi and Gualtieri 2001). The molecular mechanisms of uncompensatory infertility are still unknown, and the gap in the knowledge is due to the fact that molecular mechanisms of uncompensatory infertility remain unclear (Dejarnette 2005). Furthermore, scientists only discovered and demonstrated a small portion of actual genes and proteins crucial to regulate bull fertility [small ratio of actual genes and proteins regulating bull fertility

have been demonstrated to be crucial]. There is no set of systematically identified protein markers that indicate fertility differences among bulls. It has been discovered that molecular defects are the cause of uncompensatory traits, and a number of spermatozoa proteins such as fertilin and integrins have been proposed to play critical roles in fertilization. However, underlying mechanisms are not defined (Fusi, Tamburini et al. 1996; Pate, White et al. 2008). Research results have already proven that change in reproductive performance can generate return on investment three times more than in product performance (Hansen 2009).

Therefore, we used our established methods in quantitative proteomics 2D-DIGE and systems biology modeling to solve these problems. We also applied canonical protein interaction pathways and networks coupled with functional assays. We have so far identified differentially expressed proteins as molecular markers associated with fertility. In addition, we also identified biological functions of these proteins in the context of protein interaction networks and putative molecular markers associated with high-fertility phenotype. These molecular biomarkers can be used to predict bull fertility, thereby improving animal production efficiency and genetics.

Materials and Methods

Determination of bull fertility

The frozen semen samples and the fertility data from 4 Angus bulls with satisfactory semen quality were generously donated by Alta Genetics, Inc. (Watertown, WI).

Fertility of the bulls was tested through artificial insemination of 1,265 cows on seven farms. Frozen semen samples from 4 bulls were distributed to seven herds, and

cows bred in standing heat. Then, the pregnancy diagnoses were performed by veterinary palpation, following 40 days of post insemination. Based on the conception rates, 2 bulls were considered high fertility and 2 bulls were considered low fertility

Table 2.1 Fertility ranking of the bulls used in proteomics analysis

Bull Code	Conception Rate (%)	Number of Breeding
A	48	394
B	46	266
C	44	263
D	35	362

Isolation of motile sperms

Viable sperm were isolated from 4 Angus bulls with varying fertility using percoll gradient according to Feugang et al. (Feugang, Kaya et al. 2009). Briefly, motile spermatozoa were purified through 45% and 90% (v/v) percoll gradient from frozen spermatozoa. Cell pellets containing motile sperm were washed with PBS three times and stored as 1×10^7 sperms/tube at -80 ℃ before shipping in dry ice to Appliedbiomics (Appliedbiomics) for 2D-DIGE.

Isolation of spermatozoa proteins

Proteins of spermatozoa were isolated by scientists at Appliedbiomics (Appliedbiomics) using their established methods. Briefly, sperm pellets were resuspended in 120 µl of 2-D cell lysis buffer (30 mM Tris-HCl, pH 8.8, containing 7 M

urea, 2 M thiourea and 4% CHAPS, Protease Inhibitor Cocktail from Roche Molecular Biochemicals) followed by sonication for 2 seconds for three times (speed setting 4) using VirSonic ultrasonic Cell Disrupter, (VerTis, Gardiner, NY) on ice to lyse the cell pellets. Samples were incubated on a rotator at room temperature for 30 minutes and spun down at 16,000 g at 4 ℃ for 30 minutes. The supernatant was collected, and the protein concentration was measured using Bio-Rad protein assay (Bio-Rad Laboratories).

CyDye labeling

Proteins were labeled by scientists at Appliedbiomics (Appliedbiomics) using their established methods. Briefly, for each sample, 20-24 μg of protein was 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 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 minutes. The labeled samples were then mixed together, and 130 μl destreak solution (GE Healthcare) and 100 μl of Rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mg/ml DTT, 1% pharmalytes, 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 minutes and spun down at 16,060 g for another 15 minutes. The labeled samples were then loaded onto a strip holder and immersed with a 13-cm IPG strip.

IEF and SDS-PAGE

Proteins were labeled by scientists at Appliedbiomics (Appliedbiomics) using their established methods. Once the labeled samples were loaded, IEF (pH3-10 linear)

was run according to the protocol provided by Amersham BioSciences. 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 DTT) with gentle shaking for 15 minutes. 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 minutes. 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^{o}C until the dye front run out of the gels.

Image scan and data analysis

Image and data analyses were performed by scientists at Appliedbiomics (Appliedbiomics) using their established methods. Briefly, gel images were scanned immediately following the SDS-PAGE using Typhoon TRIO (Amersham BioSciences). The scanned images were then analyzed by Image Quant software (version 6.0, Amersham BioSciences), followed by in-gel analysis using DeCyder software version 6.0 (Amersham BioSciences). The fold changes of the protein expression levels were obtained from in-gel DeCyder analysis.

Isolation and electrophoresis of sperm proteins for immunoblotting

Thirty million sperm cells were lysed in 40 µl RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, Thermo Scientific, Protease Inhibitor Cocktail from Roche Molecular Biochemicals) by sonication (Ultrasonic Cell Disruptor, Microson) of the samples followed by incubation on a rotator at room temperature for one hour. The cell lysates were then centrifuged at 16.000 g at

4^{o}C for 15 minutes. Protein concentration was determined using a 2D Quant kit (GE healthcare). The protein samples were denatured with the same volume of laemmli buffer, and 20 µg total proteins were size separated using 10% SDS-PAGE.

Immunoblotting

Proteins were transferred to Polyvinylidene Fluoride (PVDF, Millipore, NJ) membrane through semi-dry transfer (20V, 60 min by Owl HEP). Non-specific sites on the membranes were blocked using 2% BSA in case-in blocking buffer (Bio-Rad, CA) at room temperature for 90 minutes. The membranes were then blotted with the primary antibodies against outer dense fiber of sperm tails 2 (ODF2) and manganous superoxide dismutase (SOD), (1: 250, 0.8 µg goat polyclonal IgGs in 10 ml TBS), (Santa Cruz Biotechnology, CA). Next, the membranes were washed with washing buffer (0.1% (v/v) Tween-20, 10 mM Tris, and pH 7.4, 0.9% NaCl) three times.

The membranes were then incubated with donkey anti-goat IgG, HRP-conjugated secondary antibodies (1:100,000 dilution in TBS, Santa Cruz) at room temperature for one hour. Following three washes, the signals were detected using the Enhanced Chemiluminescence Western Blotting Kit (Millipore) according to the manufacturer's instructions. Anti-actin antibodies (sc-1615, Santa Cruz Biotechnology) were used as loading control for each membrane.

Results

Protein analysis by 2D-DIGE

We detected around 2,000 protein spots in each of the 2D gels (Figure 2.1). Among the 4 bulls, there were 77 detectable spots of differentially expressed proteins. While most of the proteins had similar expression levels, some protein spots were differentially expressed in sperm from bulls with varying fertility. The expression levels ranged from -4.65 to 8.21.

Differentially expressed proteins in sperm from bulls with varying fertility

We cut out 30 of the differentially expressed protein spots. Of these 30 spots, we identified 18 proteins that were differentially expressed in spermatozoa from bulls with varying fertility (Table 2.2).

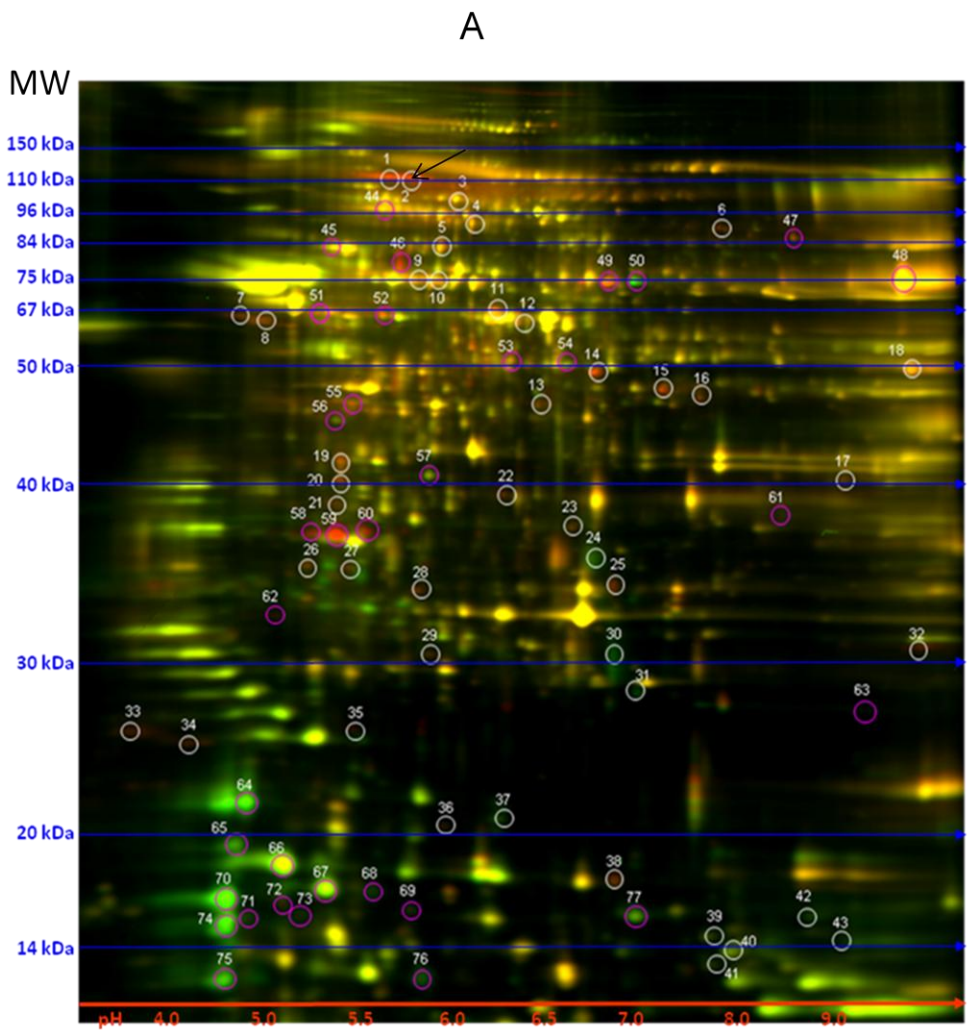


Figure 2.1 (A&B) 2D-DIGE gel image of two groups of spermatozoa (A&C, B&D) analyzed by Image Quant and DeCyder software (A) gel image of group A&C; (B) gel image of group B&D.

The same amount 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 Manganous Superoxide Dismutase (MnSOD), (Arrows), respectively.

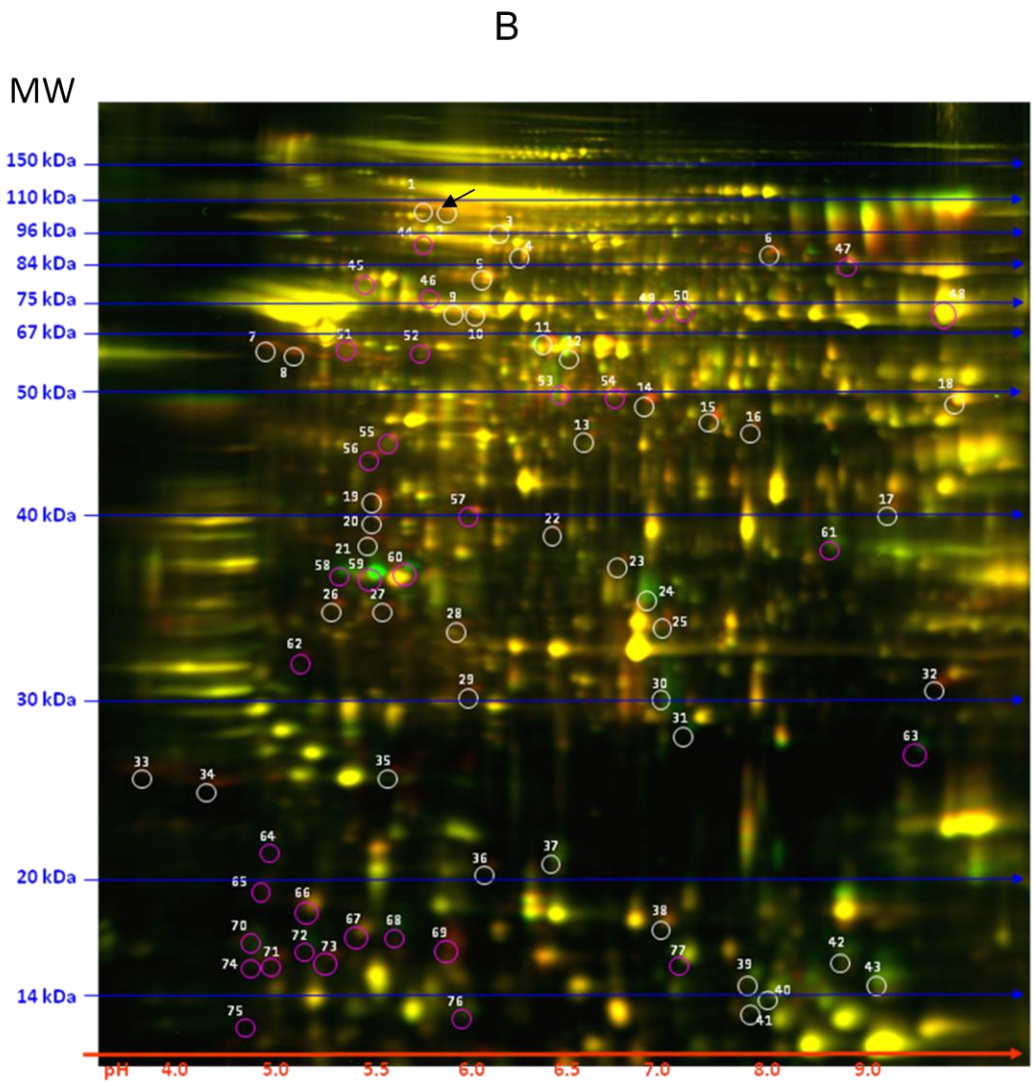


Figure 2.1 (continued)

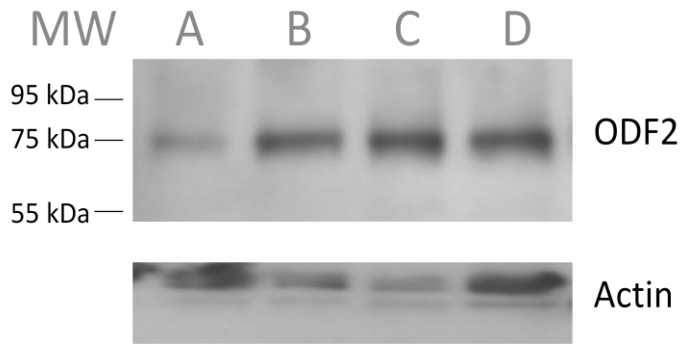
Table 2.2 Sperm proteins identified from 4 bulls with varying fertility by 2D-DIGE analysis

Entrez gene name	Location	Functions	Ratio low/high	Accession no.	Protein mw	Pi
Acrosin binding protein	Extracellular space	Unknown	-2.14	Gi|194666681	61,197	5.11
Chromosome 19 open reading frame 36	Unknown	Unknown	-3.05	Gi|156120505	17,805	5.94
Heat shock protein, alpha-crystallin-related, B9	Unknown	Protein binding	2.04	Gi|94966950	16,773	8.22
Outer dense fiber of sperm tails 2	Cytoplasm	Structural molecule activity; protein binding	8.21	Gi|84000345	75,451	7.52
Superoxide dismutase 2, mitochondrial	Cytoplasm	Superoxide dismutase activity; protein binding; oxidoreductase activity; oxygen binding; manganese ion binding; metal ion binding	-4.65	Gi|7555818	24,574.60	8.70
Tubulin, alpha 3e	Unknown	Nucleotide binding; gtpase activity; structural molecule activity; gtp binding	2.87	Gi|6678465	49,927.60	4.97
Tubulin, beta 2C	Cytoplasm	Nucleotide binding; gtpase activity; structural molecule activity; GTP binding; MHC class I protein binding; unfolded protein binding	2.65	Gi|14124960	25,858.40	4.95
Chain E, Leech-Derived Tryptase inhibitortrypsin COMPLEX	Unknown	Unknown	2.05	Gi|3318722	23,457.40	8.26
Mcg20287 [Mus musculus]	Unknown	Unknown	2.3	Gi|148676266	39,342.10	5.14
Hcg1992406, isoform CRA_b [Homo sapiens]	Unknown	Unknown	2.79	Gi|119576011	42,187.00	5.03
PREDICTED: similar to ENSANGP00000002667 [Bos taurus]	Unknown	Unknown	2.39	Gi|194674718	19,864.80	5.62
Chain b, refined 1.8 angstroms resolution crystal structure of porcine epsilon-trypsin	unknown	unknown	-2.08	Gi|999627	8,813.50	6.67
PREDICTED: similar to leucine rich repeat containing 37A [Bos taurus]	Unknown	Unknown	-2.71	Gi|194676234	280,818.20	4.87

Most of these proteins were expressed at higher levels in the high-fertility sperm. The amount of Outer dense fiber of sperm tails 2 (ODF2) was detected 8.21 times more in low-fertility spermatozoa. The protein (gi|194671321), which was detected five times more abundant in highly fertile spermatozoa, is similar to testis-specific 10 isoform 1. Tubulin proteins and heat shock proteins were also much higher in highly fertile spermatozoa. Meanwhile, five proteins were found to express preliminary abundance in low-fertility spermatozoa. Manganous superoxide dismutase (MnSOD) was 4.65 times higher in high-fertility sperms, and hypothetical protein LOC510569 was 3.05 times higher.

Spermatozoal ODF2 and MnSOD proteins detectable using immunoblotting

To confirm the expression of ODF2 and MnSOD in bull spermatozoa, we performed immunoblotting experiments. Our results showed that both of these proteins were detectable in spermatozoa of the 4 bulls studied (Figures 2.2 and 2.3). The expression levels of ODF2 were almost negatively correlated with the conception rate, but there is no sufficient evidence to show any tendency between SOD and conception rate. However, the SOD levels of bull A and bull D were higher than B and C, which means SOD levels and breeding levels, were positively related.



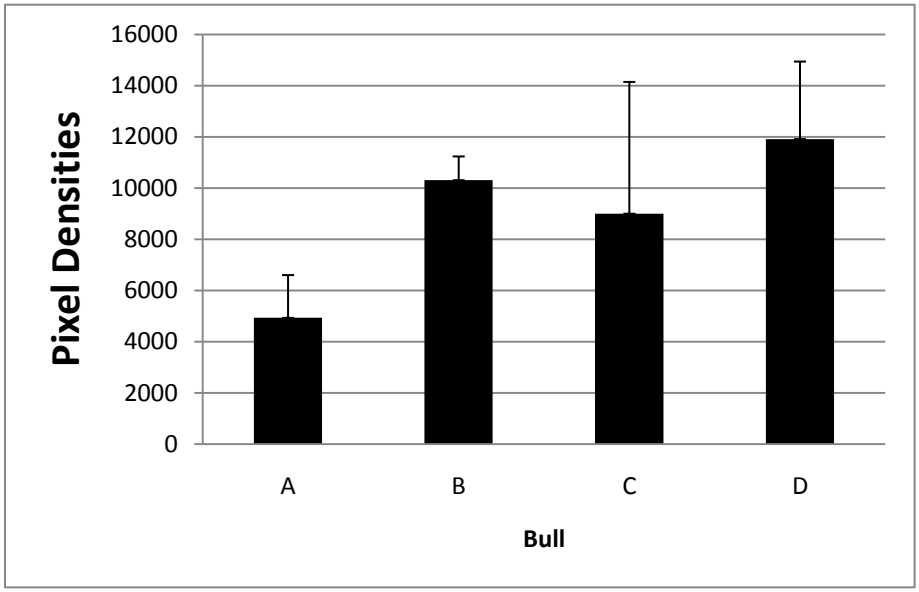
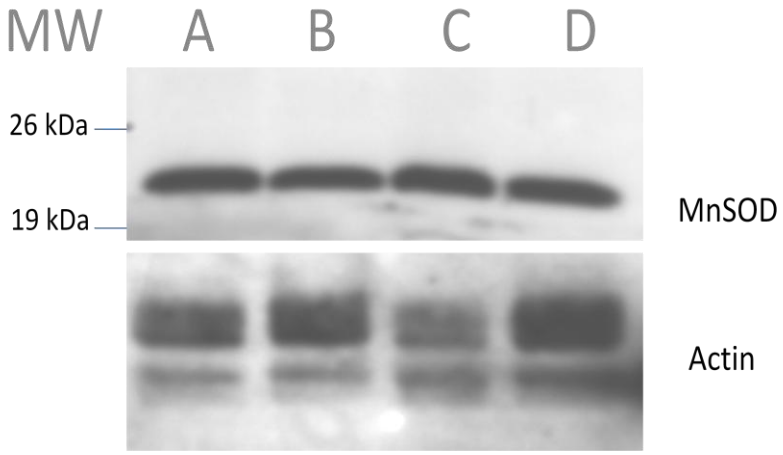


Figure 2.2 ODF2 Expression levels by Western Blot

Expression of ODF2 was detected using western blotting in various fertility sperms from 4 bulls. Proteins were extracted by sonication for five times at 5 watts, and 30 µg of total proteins were loaded in each well. Actin was used as a loading control.



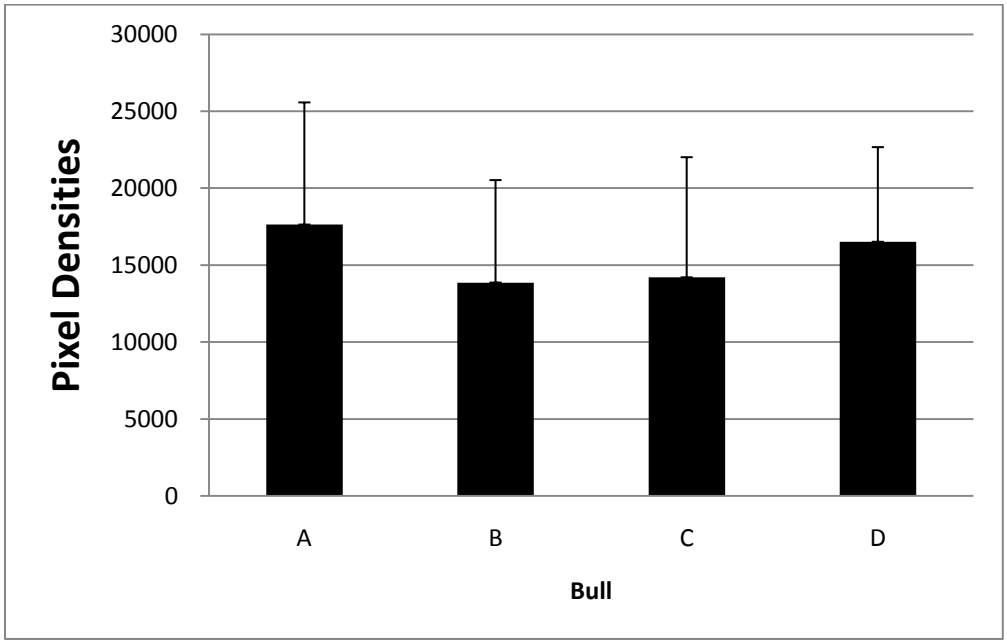


Figure 2.3 MnSOD Expression levels by Western Blot

Expression of MnSOD was detected using western blotting in various fertility sperms from 4 bulls. Proteins were extracted by sonication for five times at 5 watts, and 30 µg of total proteins were loaded in each well. Actin was used as a loading control.

Discussion

The ability of spermatozoa to fertilize the oocyte and activate oocytes, as well as support embryonic development, is an important determinant of bull fertility. Development of male gametes involves a series of developmentally regulated events during spermatogenesis. Capacitation is also known to determine fertility potential of sperm (Austin 1951; Chang 1951). Some bulls that produce high numbers of sperm with normal sperm morphology exhibit low fertility after hundreds of artificial inseminations (AIs), (DeJarnette and Marshall 2005). Molecular mechanisms of this important biological phenomenon remain a mystery. Despite its importance for both basic and applied science, there is no conventional method to determine semen quality. In addition, traditional approaches to estimate fertility potential, particularly by morphology and motility, have not been fully accurate (Bartoov, Eltes et al. 1993). The objective of this study was to identify proteins differentially expressed in spermatozoa of bulls with high and low fertility.

Our comprehensive proteomics approach to identify proteins differentially expressed in spermatozoa of bulls with varying fertility produced fruitful outcomes. We have shown that there were more than 70 protein spots that differed notably between high- and low-fertility bull sperm. By comparing the protein spot sequences to the databases, we recognized 11 of these proteins and confirmed their expression of two proteins, ODF2 and MnSOD, using immunoblotting. Analysis results from DeCyder software show the predominantly abundant ODF2 in the high-fertility sperms and MnSOD in low-fertility sperms.

As a major component of sperm tail protein, ODF2 was first detected by Brohmann in 1997(Brohmann, Pinnecke et al. 1997; Hoyer-Fender, Petersen et al. 1998).

ODF2 is located in centrosomes and basal bodies, and it is widely expressed in cerebellum and muscle tissue (Schweizer and Hoyer-Fender 2009). By blasting sequence of ODF2 [Bos Taurus], we found high levels of similarities between cow ODF2 protein and those of mice and humans, 98% and 97%, respectively. The structure of ODF2 protein is an α-helical, which is similar to leucine zipper motif (Brohmann, Pinnecke et al. 1997). The translation of ODF2 was proposed to be modified when its three or four testis-specific transcripts were detected in rat and bull sperms (Brohmann, Pinnecke et al. 1997; Schalles, Shao et al. 1998).

MnSOD is synthesized in the cytosol and posttranslationally modified for transport to the mitochondrion (Wispe, Clark et al. 1989; Shimoda-Matsubayashi, Matsumine et al. 1996). It is a tetrameric enzyme that catalyzes the dismutation of two superoxide radicals into H2O2 and oxygen (Knox, Cowey et al. 1981). The observation that MnSOD mutant was inhibited at 37 ℃ demonstrated the vital role of MnSOD in the protection against high temperature (Narasipura, Chaturvedi et al. 2005). Recently, SODs, as they function in *A. fumigatus,* were revealed to neutralize their own fungal oxidants intracellularly. (Lambou, Lamarre et al. 2010).

The proteins abundant in high-fertility sperms should be positive for fertility, and their dysfunction might reduce the rate of fertility. Protein variations can be induced by additional posttranslational modifications such as phosphorylation, cleavage, and glycosylation (Flickinger, Rao et al. 2001). It has been proven that protein tyrosine phosphorylation (PYP) changes occurred in ODF2 during sperm capacitation (Mariappa, Aladakatti et al. 2010). According to the property of ODF2, it probably ensures the integrity of sperm structure. Without enough ODF2 to sustain the morphology of the sperm tail, the spermatozoa are less likely to be active and motile for successful

fertilization. MnSOD protects the sperm from oxygen. The higher amounts of MnSOD can be explained by the intracellular excessive damage in the sperm. Our results suggest that posttranslational modification and deoxydation of sperm proteins are involved in bull fertility.

Our results also reflect the different performances of sp32 protein, which is a 32-kDa protein formed by posttranslational modification of a 61-kDa precursor (Baba, Niida et al. 1994). Baba et al. also found that this protein binds with both amino- and carboxyl-terminal sequences of proacrosin in sperm and might be involved in the package of the acrosin zymogen. According to its function between capacitation and tyrosine phosphorylation, sp32 was believed to be involved with the changes in capacitation (Bravo, Aparicio et al. 2005). The 2D-DIGE result showed that sp32 was expressed more than two times higher in low-fertility sperm cells. This confirmed the hypothesis by Bravo that sp32 can work as a marker to indicate the capacitation changes. Therefore, the success of capacitation and hence fertility potential can be linked to sp32. Although sp32 was detected before 1988 (Williams, Hawgood et al. 1988), exact molecular mechanisms of action for sp32 are not known in detail. Our result provides the groundwork to develop the pathway in which sp32 participates and affects fertility potential.

The main limitation of this study is the limited sample size due to limited fertility data available for Angus bulls. The range of proteins was restricted to these 4 bulls even though there might still have been some proteins existing in other bulls that were associated with low fertility. Frozen and thawed steps might induce the protein oscillation and affect the performance of proteins. Although the technique of 2D-DIGE has been optimized, not all the proteins can be soluble in the lysis buffer. Insoluble proteins and other conjugated proteins might be lost.

The proteins identified between high- and low-fertility sperms can be either the upstream reasons that result in low fertility or the outcomes of other pathways causing low fertility. They provide clues to trace the deep-seated pathways. Furthermore, the whole mode of low fertility is not comprehensive until combined with extracellular proteins. Proteins of semen plasma were reported to influence sperm-egg binding and fertility (Killian, Chapman et al. 1993). It was also demonstrated that fertility data in dairy bulls were relative with proteins that exist in cauda epididymidis and accessory sex glands (Moura, Koc et al. 2006; Moura, Chapman et al. 2007).

The proteins identified here enrich our knowledge on molecular causes of low fertility, and they can be considered as biomarkers while judging the fertility of sperm. The protein markers identified would facilitate the method of fertility evaluation and reduce the cost associated with low fertility. Studies on additional sperm proteins and mechanisms by which they regulate bull fertility are expected to generate a whole systematic model of protein markers.

Conclusion

Crucial proteins for fertility identifying and tracking are based on comparisons between high- and low-fertility sperms by means of 2D-DIGE and immunoblotting. We identified 18 proteins that varied significantly, including Outer dense fiber of sperm tails 2 (ODF2) and Manganous superoxide dismutase (MnSOD). Differences in these proteins between high and low fertility bulls suggest that posttranslational modification and deoxydation of sperm proteins might be associated with fertility. The proteins identified here enrich our knowledge on molecular causes of low fertility, and they can be considered as biomarkers while judging the fertility of sperms. The protein markers

identified would facilitate the method of fertility evaluation and reduce the cost associated with low fertility. Studies on additional sperm proteins and mechanisms by which they regulate bull fertility are expected to generate a whole systematic model of protein markers.

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APPENDIX A

DATA ON 2D DIGE ANALYSIS

Table A.1 Spot ratios in High/ Low fertile spermatozoa

	Gel-1	Gel-2			Gel-1	Gel-2	
Assigned spot#	**Ratio: 3 / 1**	**Ratio: 4 / 2**	**Average**	**Assigned spot#**	**Ratio: 3 / 1**	**Ratio: 4 / 2**	**Average**
1	1.71	8.95	5.33	41	-1.4	-1.37	-1.38
2	1.64	14.78	8.21	42	-2.07	-2.09	-2.08
3	1.11	1.37	1.24	43	-2.14	-2.88	-2.51
4	1.46	1.56	1.51	44	1.07	1.46	1.26
5	1.62	1.49	1.55	45	1.13	1.06	1.09
6	2.03	2.07	2.05	46	1.07	2.41	1.74
7	1.89	1.86	1.87	47	1.6	2.37	1.98
8	2.09	1.93	2.01	48	1.1	1.89	1.49
9	1.18	1.42	1.3	49	1.04	2.73	1.88
10	1.3	1.62	1.46	50	1.04	-4.64	-1.8
11	2.79	1.51	2.15	51	2.5	2.51	2.5
12	1.03	-1.3	-0.35	52	1.29	2.46	1.87
13	1.69	1.49	1.59	53	1.42	1.35	1.38
14	3.2	1.83	2.51	54	1.88	-1.25	0.31
15	1.97	2.53	2.25	55	1.56	1.91	1.73
16	1.68	1.96	1.82	56	2.35	-1.11	0.62
17	2.25	1.41	1.83	57	2.22	-1.64	0.29
18	1.64	1.68	1.66	58	-7.14	3.07	-2.03
19	2.63	1.98	2.3	59	-14.09	7.21	-3.44
20	2.71	2.3	2.5	60	-4.52	2.73	-0.89
21	3.8	1.9	2.85	61	-2.33	-1.07	-1.7
22	3.29	2.29	2.79	62	3.7	1.3	2.5
23	1.64	1.48	1.56	63	-56.74	1.1	-27.82
24	-2.93	-2.41	-2.67	64	1.27	-6.7	-2.71
25	2.03	1.82	1.92	65	1.85	-4.32	-1.23
26	3.01	1.77	2.39	66	1.55	-3.42	-0.93
27	2.35	1.52	1.93	67	1.34	-2.87	-0.76
28	2.56	2.45	2.5	68	-1.6	-1.95	-1.77
29	3.15	1.32	2.23	69	3.37	-1.1	1.13
30	-3.19	-2.92	-3.05	70	-1.23	-7.94	-4.58
31	-2.91	-6.4	-4.65	71	3.03	-2.32	0.35
32	4.37	4.23	4.3	72	-3.18	-1.1	-2.14
33	2.13	1.86	1.99	73	2.63	-1.49	0.57
34	2.2	2.29	2.24	74	-1.32	-5.99	-3.65
35	2.08	1.35	1.71	75	1.27	-5.52	-2.12
36	2.18	1.52	1.85	76	-1.26	-2.45	-1.85
37	-1.91	-1.97	-1.94	77	-1.48	-2.51	-1.99
38	1.94	2.15	2.04	78	1.63	1.27	1.45
39	-1.67	-1.32	-1.49	79	-1.34	-1.49	-1.42
40	1.24	-1.63	-0.19	80	1.27	-2.08	-1.41