JOURNAL OF INTEGRATED OMICS
A METHODOLOGICAL JOURNAL
HTTP://WWW.JIOMICS.COM

ORIGINAL ARTICLE | DOI: 10.5584/jiomics.v3i2.136

Global proteomic profiling of the membrane compartment of bovine testis cell populations

Michelle L. Colgrave[1,2†*], Sally Stockwell[1,2†], Aimee Grace[3,4], Mary McMillan[3,4], Rhonda Davey[3], Sigrid Lehnert[1,2], Sabine Schmoelzl[2,3,4]

[1]CSIRO Animal, Food and Health Sciences, 306 Carmody Rd, St Lucia, QLD 4067; [2]CSIRO Food Futures National Research Flagship, 5 Julius Avenue, North Ryde, NSW 2113, Australia; [3]CSIRO Animal, Food and Health Sciences, F. D. McMaster Laboratory, Armidale, NSW 2530; [4]School of Environmental and Rural Science, University of New England, Armidale, NSW 2351; [†]*These authors have contributed equally to the work.*

Received: 24 April 2013 **Accepted:** 03 June 2013 **Available Online**: 16 July 2013

ABSTRACT

Spermatogonial stem cells hold enormous potential in mammalian reproductive medicine through the preservation of gametes, the restoration of fertility, enhancement of germ-lineage genetic manipulation and the improvement in our understanding of stem cell biology. Here we describe the protein profiles of the membrane compartment of bovine testicular cell isolates which were enriched for germ cells using differential plating. The isolated cells were characterised with antibodies to UCHL1 (previously known as PGP9.5) for type A spermatogonia; DDX4 (previously known as VASA) for germ cells and vimentin for Sertoli cells. Ultracentrifugation techniques were used to specifically isolate cell membranes, with membrane protein identifications significantly increased when compared to whole cell lysates. We utilised the filter-aided sample preparation protocol for improved digestion efficiency of membrane proteins. Using ESI-LC-MS/MS, we compared the proteins present in two cell populations. A total of 1,387 proteins were identified in bovine testis cell isolates, of which 39% were membrane-associated. A total of 64 proteins were differentially expressed in the non-adhered fraction (enriched for undifferentiated germ cells) compared to the adhered fraction, of which 16 were unique to this cell population and the remaining 48 showed a two-fold change (increase when compared to the adhered cell population) as judged by spectral counting. This analysis revealed a number of candidate germ cell markers including the known markers, DDX4 and UCHL1. The proteomic profiles generated in this study support and complement transcription data on gene expression and histological levels, and reinforce the potential of proteomics in identifying and characterising the protein effectors of self-renewal and/or differentiation in stem cells.

Keywords: Spermatogonial stem cells; germ cells; bull testis; cell surface; proteomics; membrane.

Abbreviations

BSA: bovine serum albumin; **DBA**: *Dolichos biflorus* agglutinin; **DMEM/F12**: Dulbecco's Modified Eagle Medium: Nutrient Mixture F12; **FASP**: filter-aided sample preparation; **PBS**: phosphate buffered saline; **PGP9.5/UCHL1**: ubiquitin carboxy-terminal hydrolase L1; **qRT-PCR**: Quantitative Reverse Transcribed Polymerase Chain Reaction; **RT**: room temperature; **SSC**: spermatogonial stem cell; **TBS**: Tris-buffered saline; **VASA/DDX4:** ATP-dependent RNA helicase DDX4.

1. Introduction

The mammalian spermatogonial stem cell (SSC) population is set aside early during embryonic development and differentiation of SSC in adults results in the continual production of sperm in the testis.[1]. Spermatogonial stem cells are a subset of the undifferentiated spermatogonial cell population, and are defined by the ability to colonise testis tissue after transplantation [2]. At present there are no definitive markers that can be used to distinguish SSC from other cell types in the testis of any species, limiting the ability to identify and purify SSC populations [3-5]. A gene expression array -based whole genome approach to identify biomarkers employed a rare condition of defective spermatogenesis to sepa-

***Corresponding author:** Michelle L. Colgrave, CSIRO Animal, Food and Health Sciences, 306 Carmody Rd, St Lucia, QLD 4067 Australia; Tel: +61 (0)7 3214 2697; Fax: +61 (0)7 3214 2900. Email address: michelle.colgrave@csiro.au

rate spermatogonial markers, but the markers identified by this approach could not be confirmed as spermatogonial stem cell markers [5]. Pluripotency markers such as SSEA4 have been found to be expressed in human repopulating SSC [6], but such markers have not been confirmed for bovine SSC.

Spermatogonial stem cells reside on the basal membrane of seminiferous tubules in close association to Sertoli cells. While no specific markers are known so far for SSCs, there are several established markers for spermatogonia and Sertoli cells in different species. Probable ATP-dependent RNA helicase (DDX4; VASA) is a highly conserved molecular marker for the germ cell lineage across species, expressed in primordial germ cells and undifferentiated spermatogonia as well as throughout the germ cell lineage [7, 8]. Established markers for bovine undifferentiated spermatogonia are ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCHL1) [9], and binding of *Dolichos biflorus* agglutinin (DBA), a plant lectin binding to sugar residues on the surface of bovine undifferentiated spermatogonia [10-12]. Sertoli cells, which are the only other cell population present in seminiferous tubules besides germ cells, can be identified by their expression of GATA4 [13] and vimentin [14, 15].

Proteomics provides a feasible route to profiling testis cell populations, complementing the genomics and molecular biology efforts. Improvements in sensitivity, accuracy and speed of mass spectrometric analysis when combined with automated methods for data acquisition and processing have enabled protein profiling experiments in a high throughput manner. Over recent years, proteomic studies have been applied to the study of SSC from rats [16], mice [17-19], humans [20], chickens [21] and dogfish [22]. Proteomics can reveal information about protein expression (presence and quantity) and post-translational modification which facilitates our understanding of protein interactions and function in complex systems. For example, proteomics has played a critical role in understanding complex biological processes, such as that of spermatogenesis [23].

Methods for the isolation and culture of SSC from the testes of mice have been available for decades [24, 25]. Since then, human SSC have been successfully cultured [26]. A major impediment to the development of systems to culture cattle SSC is the lack of specific markers for isolation and comparison of stem cell populations. While genomic and transcriptomic studies in mice and humans have provided candidate markers, no such list or "molecular signature" is available for studies of bovine SSC. The identification of suitable markers is further confounded by the requirement that proteins with utility for sorting of cell populations must be surface-exposed. Plasma membrane proteins are typically of low abundance and/or of low solubility in cell lysates, hampering their identification and necessitating membrane fractionation prior to analysis.

In the current investigation, a global proteomic profiling experiment was applied to the analysis of membrane-enriched fractions from bovine testis cell populations generated by differential plating. The presence or absence of proteins in each population was assessed and the relative abundance was examined. The relative abundance of each candidate germ cell marker was assessed first by protein score and secondly by spectral counting. A short list of candidate germ cell markers was investigated further for differential protein or gene expression by immunohistochemistry and qRT-PCR.

2. Materials and Methods

2.1. *Cell isolation*

All animal experimentation was approved by the Armidale Animal Ethics Committee (Animal Research Authority 11-09). Testes of 15-30 g weight were harvested from prepubertal bull calves at slaughter and transferred to the laboratory in cold phosphate buffered saline (PBS; no calcium or magnesium; Invitrogen, Carlsbad, CA, USA) + penicillin (100 U/mL) and streptomycin (100 µg/mL) (Pen/Strep, Gibco, Grand Island, NY, USA). Single cell isolations were prepared according to Herrid *et al* [12] with some modification. The tunica vaginalis and the epididymis were removed and each testis weighed and washed in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12; Invitrogen) + Pen/Strep. The seminiferous tissue was dissected free, minced finely in fresh DMEM/F12 + Pen/Strep and ground gently in a tea strainer using a 5 mL syringe plunger to remove interstitial cells. The remaining tubules were then washed several times in a 50 mL Falcon tube with the DMEM/F12 solution and digested in collagenase (Type IV; Sigma-Aldrich, St Louis, MO, USA; 2 mg/mL; 20 mL/10 g of tubules) in a shaking 37°C water bath until individual tubules were observed by microscopy. The tubule suspension was washed repeatedly in PBS + Pen/Strep and treated with 0.025 % trypsin/ethylenediaminetetraacetic acid (Gibco) at 37°C for 5-10 min to digest the tubules. During incubation, 1 mL DNase1 (7 mg/mL; Sigma-Aldrich) was added. When dissociation of tubules was observed, the reaction was stopped with 2 mL of foetal bovine serum (Gibco). The solution was filtered through 70 µM cell strainers (BD Biosciences, North Ryde, NSW, Australia) and the resultant cell suspension pelleted by centrifugation. Red blood cells were removed by incubation at 37°C in 5 mL of 0.83% NH_4Cl for 7 min. The NH_4Cl was neutralised by the addition of PBS, the cells pelleted again and resuspended in DMEM/F12 + 10% foetal bovine serum for culture. Cell counts were performed using a haemocytometer and cell viability assessed using Trypan Blue.

2.2. *Cell culture and differential plating*

The isolated testis cells were cultured overnight at a concentration of 2 x 10^6 cells/mL in T175 flasks at 37°C and 5% CO_2. The following day, the supernatant containing non-adhered cells including the undifferentiated germ cells

was collected and the cells washed once with PBS. The non-enriched, adhered fraction was washed once and then gently resuspended in PBS using a cell scraper. Both cell fractions were collected by centrifugation at 800 x *g* for 5 min. Cell viability was again determined using Trypan Blue. Smears of both cell populations were air-dried on glass slides (Superfrost; Menzel Glaser, Braunschweig, Germany) and stored at -80°C until analysed. The remaining cells were used to prepare membrane extracts.

2.3. *Characterisation of cell smears*

Cell smears were equilibrated to room temperature prior to immunocytochemical staining. The cells were fixed in Modified Davidson's Fixative for 2 min, rinsed in Tris-buffered saline (TBS) (2 x 5 min) and blocked in 0.3% hydrogen peroxide for 15 min at RT. After further washes in TBS + 0.1% Tween 20 (TBS/Tween 20: 2 x 5 min) the cells were incubated with the primary antibody in TBST, 0.5% BSA for 30-60 min at RT. Germ cells were characterised using DDX4 (Abcam, Cambridge, UK; 1:2,000) and UCHL1 (Dako Australia, Campellfield, VIC, Australia; 1:700) and Sertoli cells were highlighted with Vimentin (Zymed, USA; 1:100). The cell smears were washed again in TBS/Tween 20 (2 x 5 min) and incubated for 30 min at RT with EnVision+ Dual Link System Peroxidase secondary antibody (Dako). Following further washes, the chromogen diaminobenzidine (Dako) was used to visualise the reaction. Cells were counterstained in haematoxylin, dehydrated through alcohol and xylene and mounted in SHUR/Mount (ProSciTech, Thuringowa, QLD, Australia). Primary antibodies were replaced with buffer for negative controls. Cell counts were performed manually from two fields of view and at least 500 cells.

2.4. *Immunohistochemistry*

Paraffin sections (5 µm) were dehydrated through xylene and graded alcohol dilutions to water. Antigen retrieval was performed by immersing the slides in heated 10 mM Tris-base, 1 mM EDTA, 0.05% Tween 20 and heated in an 800 W microwave on 30% power for 15 minutes and allowed to cool. The slides were rinsed in tap water. Binding of non-specific proteins was blocked by incubating sections in a detergent solution of tris-buffered saline 0.05% Tween 20 (TBST) for 30 minutes. For immunofluorescence, DDX6 (Santa Cruz Biotechnology, Santa Cruz, TX, USA) was used at 2 µg/mL, NAP1L4 (Abcam, UK) at 0.2 µg/mL and TKTL1 (Santa Cruz Biotechnology) at 0.5 µg/mL, GATA-4 (Santa Cruz Biotechnology) at 1 µg/mL, DDX4 (previously known as VASA) (Abcam) at 0.5 µg/mL, Vimentin (Zymed, USA) 1/100, and DBA-biotin (B1035, Vector Laboratories, Burlingame, CA, USA) at 10 µg/mL. Parallel sections were incubated with a mixture of rabbit and mouse immunoglobulin at 2 µg/mL as a control (Sigma-Aldrich). Cocktails of primary antibodies containing DBA-biotin, a mouse and a rabbit primary antibody, or control immunoglobulin, were incubated for 45 minutes at room temperature. Slides were washed in TBST, and then the secondary antibodies, streptavidin-alexafluor 350 (10 µg/mL), (Invitrogen), goat anti-mouse Alex 488 (A11001) 1:250, chicken anti-rabbit Alex 594 (Invitrogen) applied, incubated at room temperature for 30 minutes, washed in TBS, cover slipped in Prolong Gold (Invitrogen) and kept in the dark until photographed using a AxioImager and Axiovision software (Carl Zeiss, Oberkochen, Germany). For the brightfield images, antibody against DDX6 was used at 2 µg/mL, NAP1L4 at 0.2 µg/mL, TKTL1 at 0.5 µg/mL, and staining was visualised using the Expose Mouse and Rabbit Specific HRP/DAB detection kit (Abcam), counterstained with haematoxylin, dehydrated through alcohol and xylene and then mounted with DPX (Merck, Darmstadt, Germany).

2.5. *Membrane Fraction Preparation*

Differentially plated cells were harvested as described above from a single testis of two different animals. Protein samples were kept separate as biological replicates throughout the analysis. All membrane preparation steps were performed at 4°C where possible. The cell isolates were suspended in PBS and mechanically lysed by repetitive passes through a 21 G needle. The solution was further disrupted by probe sonication on ice for 5 min. Cell debris was removed by centrifugation at 1,000 x *g* for 10 min. The supernatant was clarified at 15,000 x *g* for 30 min removing larger membrane particles. Cell membranes were collected by centrifugation at 240,000 x *g* for 1 h. Membrane pellets were washed twice with PBS (20 min; 240,000 x *g*) and stored at -80°C if required.

2.6. *Protein Concentration Determination*

The isolated membrane pellet was resuspended in PBS and sonicated briefly at 4°C in a sonic water bath. Protein concentration was determined from an aliquot of the cell suspension using a commercial bicinchoninic acid assay kit (Pierce, Thermo Fisher Scientific, Scoresby, VIC, Australia).

2.7. *Filter-Aided Sample Preparation (FASP)*

Reduction, alkylation and digestion was performed using the filter-aided sample preparation protocol developed by Wisniewski *et al* [27]. In brief, up to 30 µL of the membrane pellet suspension containing up to 300 µg of protein was mixed with 200 µL of 8 M urea in 0.1 M Tris/HCl (UA solution; pH 8.5) in a Microcon YM10 filter unit (Millipore, Merck Kilsyth, VIC, Australia) and centrifuged at 14,000 x *g* for 15 min. The unit was washed with another 200 µL of UA (14,000 x *g* for 15 min). The flow-through from the collection tube was discarded and 100 µL of iodoacetamide solution (0.05 M in UA) was added. The filter unit was mixed at 600 rpm at 20°C for 1 min then incubated for a further 20 min at 20°C. Excess iodoacetamide was removed by centrifu-

gation at 14,000 x *g* for 10 min and the filter washed three times with 100 µL of UA (14,000 x *g*; 15 min). Ammonium bicarbonate (0.05 M in H_2O; 100 µL) was added to the column and washed through at 14,000 x *g* for 10 min. This step was repeated twice. Digests were performed on the filters with 40 µL of trypsin (Promega, Madison, WI, USA) in ammonium bicarbonate at an enzyme to protein ration of 1:60. The filter units were mixed at 600 rpm for 1 min and incubated overnight at 37°C in a humid chamber. The filter units were transferred to fresh collection tubes, centrifuged at 14,000 x *g* for 10 min to collect the tryptic peptides and washed with a further 40 µL ammonium bicarbonate. The eluate was dried down in a vacuum concentrator and resuspended as required for proteomic analysis.

2.8. *Chromatography*

Tryptic peptides were chromatographically resolved using a Shimadzu Prominence LC20 HPLC system with a C18 Vydac column (75 µm x 15 cm, 300 Å, 5 µm). Protein digests were reconstituted in 0.1% formic acid and 1 µg was injected on-column. A linear gradient at a flowrate of 800 nL/min from 1-40% solvent B over 80 min was utilised where solvent A was 0.1% formic acid and solvent B was 0.1% formic acid in 90% acetonitrile.

2.9. *Mass Spectrometry*

The eluate from the HPLC system was directly coupled to the nanoelectrospray ionisation source of the TripleTOF™ 5600 system (AB/Sciex, Foster City, CA, USA). Data were acquired in information dependent acquisition (IDA) mode. The IDA method consisted of a high resolution TOF-MS survey scan followed by 20 MS/MS in a second with a maximum accumulation time of 50 ms. First stage MS analysis was performed in positive ion mode over the mass range *m/z* 300-2000 with a 0.5 s accumulation time. The ionspray voltage was set to 2600 V, the curtain gas was set to 25, the nebuliser gas to 20 and the heated interface was set to 150°C. Tandem mass spectra were acquired over the mass range *m/z* 100-2000 using rolling collision energy (CE) for optimum peptide fragmentation.

2.10. *Database Searching and False Discovery Rate Analysis*

All data were processed using ProteinPilot v4.0 (AB/Sciex) with integrated false discovery rate analysis [28]. The spectral sets (either individually or combined) were searched against all bovine proteins present in Uniprot database (version 20110718; 70,452 proteins). Search parameters were defined as cysteine alkylation with iodoacetamide, trypsin as the digestion enzyme and no restrictions were placed on taxonomy. Modifications were set to the "generic workup" and "biological" modification sets provided with this software package, which consisted of all modifications listed in Unimod, for example, acetylation, methylation and phosphorylation. The generic workup modifications set contains 59 potential modifications that may occur as a result of sample handling, for example, oxidation, dehydration and deamidation. The identification of proteins was recorded in the Results section if the protein was identified at a 1% global false discovery rate (FDR).

2.11. *Quantitative Reverse Transcribed Polymerase Chain Reaction (qRT-PCR)*

Bovine specific primers were designed with Primer3 (http://frodo.wi.mit.edu) using bovine sequences in the NCBI GenBank databases. The primers used in this study are listed in Supplemental Table 1. Appropriate reference genes, *ATP5G2* (ATP synthase) and *RPS26*, were determined using the sheep GeNorm kit (PrimerDesign, Southampton, UK). Total RNA was extracted from frozen cell samples using an RNeasy Midi kit (Qiagen, Hilden, Germany). cDNA was synthesised using a Superscript III first strand synthesis kit (Invitrogen). qRT-PCR reactions were carried out in triplicate in an iQ5 real time thermal cycler (Bio-Rad, Hercules, CA, USA). Each reaction contained 1× IQ SYBR Green Supermix (Bio-Rad), 0.5 µM of each forward and reverse primer and cDNA transcribed from 10 ng RNA. Interplate controls and negative controls were included in each assay.

2.12. *Statistical analysis*

Cell counts from immunocytochemical characterisation were expressed as percentages of positive cells and analysed using analysis of variance (ANOVA). Differences with p values < 0.05 were considered significant.

C_t values from qRT-PCR were converted into expression data using the Excel add-in Genex (Bio-Rad). Statistical analyses of gene expression data were performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). A ranked Mann Whitney test was applied to determine statistically significant differences between treatment groups. Differences of $p < 0.05$ were considered to be significant. Results are presented as dot plots with means ±SEM shown.

2.13. *Bioinformatics*

Identified proteins were classified according to known gene ontology using the GO analysis toolkit for the agricultural community (AgriGO) [29]. The GO annotation terms were further reduced using REViGO [30] to remove redundant terms and to visualise the data. Enrichment was determined against the bovine genome. The molecular function and biological processes of the identified proteins were obtained from Protein ANalysis THrough Evolutionary Relationships (PANTHER) [31], the Database for Annotation, Visualisation and Integrated Discovery (DAVID) [32, 33] or the European Bioinformatics Institute's web based tool for gene ontology searching (QuickGO) [34]. Where annota-

tions for bovine proteins were unavailable, molecular function and process were determined from sequence homology and classification in other species. Predictions of transmembrane domains, signal peptides and non-classical secretion were derived from the Centre for Biological Sequence Analysis (CBS) prediction servers TMHMM [35], SignalP 4.0 [36] and SecretomeP 2.0 [37] respectively.

3. Results

Differential plating

Testis tissue from two pre-pubertal bull calves was subjected to enzymatic digestion and subsequent cell isolation. At this stage of development, the germ cell population is more likely to contain only gonocytes and undifferentiated spermatogonia [17]. After overnight culture, adhered and non-adhered cell populations were harvested for cell characterisation and membrane fractionation. Cell viability at harvest was greater than 90%. Cell smears were treated with antibodies to the known germ cell markers UCHL1 and DDX4, and the Sertoli cell marker Vimentin. These markers were present in both cell populations, however, cell counts demonstrate significant germ cell enrichment in the non-adhered fraction (UCHL1 = 9%, representing a 5x increase, DDX4 = 6% representing a 7x increase, $p < 0.01$) and slightly higher Sertoli cell numbers in the adhered population (Vimentin = 24% representing a 1.3x increase) (Figure 1).

Global Protein Profiling

Mass spectrometry analysis, followed by database searches identified a total of 1,387 proteins at a 1% false discovery rate (FDR) when all data were combined and searched together (Supplemental Table 2). In the non-adhered populations 1,150 proteins were identified, while in the adhered population 988 proteins were identified (Supplemental Tables 3,4). A total of 767 proteins (55%) were found to be expressed in both sub-populations.

The goal of this study was to help identify proteins that are expressed by undifferentiated spermatogonia, but not present on other cells in bovine testis tissue. To this end, we performed a comparative analysis to identify proteins that were either: (1) present in the non-adhered population, but not in the adhered population; or (2) present in greater abundance in the non-adhered fraction.

The preliminary abundance levels were assessed based on the total protein score, the number of peptides mapping to a given protein and the percentage of sequence coverage (Supplemental Table 2). In practice, proteins that were identified by at least two unique peptides (resulting in a score $\geq$ 4.0) in both non-adhered populations (NA1 and NA2), but not detected in either of the adhered cell populations (A1 and A2) were considered unique. Proteins with at least two unique peptides in both NA1 and NA2 and that were detected in A1 and/or A2 were considered common. Common proteins with a protein score ratio $\geq$ 2 were considered to be present in greater abundance. Proteins passing either of these criteria are listed in Supplemental Table 5 (proteins more abundant in non-adhered) and Supplemental Table 6 (proteins more abundant in adhered) and were subjected to further scrutiny.

Based on the above criteria, a total of 89 proteins were differentially expressed in the non-adhered fraction, of which 21 were only detected in the non-adhered cell population and the remaining 68 were present with protein scores that differed by two-fold. A total of 105 proteins appeared to be differentially expressed in the adhered cell population, with 20 proteins only detected in the adhered population and 85 proteins showing protein scores that differed by two-fold. As the protein score and number of peptides identified are dependent on the protein size and other factors (ionisation efficiency, peptide size), we also examined the relative abundance by spectral counting, that is the total number of MS/MS spectra that identify a given protein was determined for each analysis. Only peptide spectrum matches (PSM) identified with $\geq$ 95% confidence were considered.

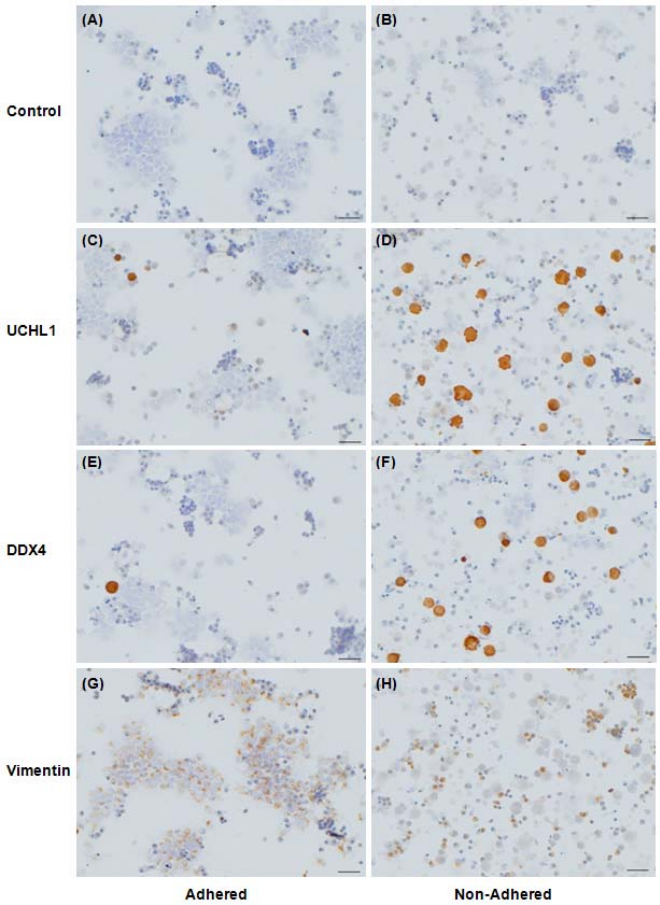

Figure 1. Immunocytochemistry of adhered (A,C,E,G) and non-adhered (B,D,F,H) bovine testis cell isolates. A,B: no primary antibody (control); C,D: UCHL1 antibody (germ cells); E,F: DDX4 antibody (germ cells); G,H: Vimentin antibody (Sertoli cells). The scale bar represents 50 μm.

Only proteins with spectral counts in two replicates greater than 4 were considered. Proteins that showed variation in the spectral count between the biological replicates were excluded (Supplemental Tables 7 and 8).

With the added stringency of spectral counting, a total of 64 proteins passed the criteria for differential expression in the non-adhered fraction (Table 1), of which 16 were only detected in the non-adhered cell population and the remaining 48 were present with protein scores that differed by two-fold. Likewise, a total of 78 proteins were differentially expressed in the adhered cell population, with 19 proteins only detected in the adhered population and 59 proteins showing protein scores that differed by two-fold.

Of the proteins identified in the literature as mammalian germ cell markers, DDX4 and UCHL1 were detected in the non-adhered population, however, these two proteins did not pass the strict criteria applied to be considered differentially expressed. DDX4 was detected only in the non-adhered population, but was identified by only a single peptide in both replicates. Likewise, UCHL1 was detected in both non-adhered populations, but by a single peptide in one replicate. For Sertoli cell markers, vimentin was detected in both, adhered and non-adhered fractions with only sightly higher representation in the adhered cell population (ratio 0.9) (Supplemental Table 8).

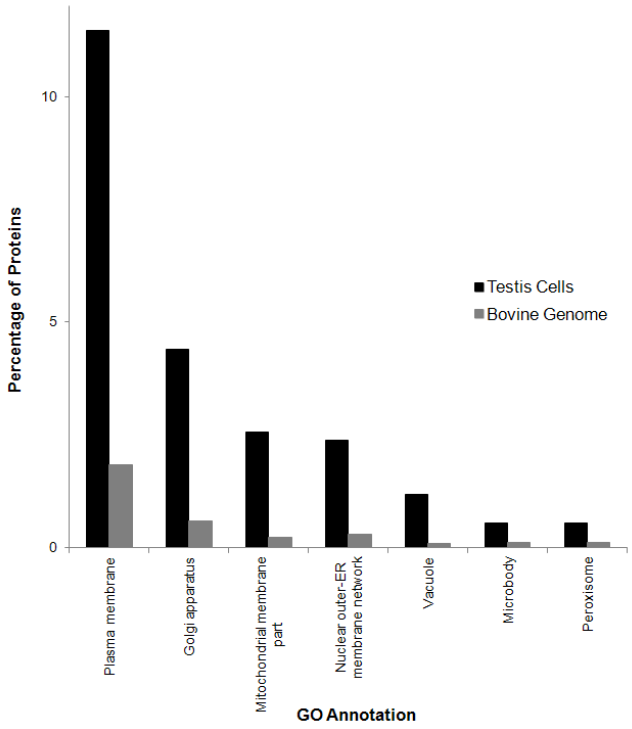

Figure 2. Subcellular localization annotations of membrane proteins in bovine testicular cell isolates. Localisation annotations determined from Gene Ontology using AgriGO (http://bioinfo.cau.edu.cn/agriGO/)

Functional annotation

AgriGO analysis of the cellular component of the total list of 1,387 proteins demonstrated a clear enrichment for membrane associated proteins. Of the total number, 536 (38.65%) were membrane proteins compared to 28.45% present in the bovine genome. Cell organelle localisation shows that the greatest proportion of these membrane proteins are associated with the plasma membrane (11.45%) (Figure 2), however, all organelle membrane proteins are enriched in our samples when compared to genomic levels.

The web-based PANTHER classification system was used to group the differentially expressed adhered and non-adhered proteins according to their molecular function and the biological processes in which they are involved. Figure 3 shows the distribution of the molecular functions in both groups. More proteins in the adhered group are classified as having a binding function (44.8%) when compared to the

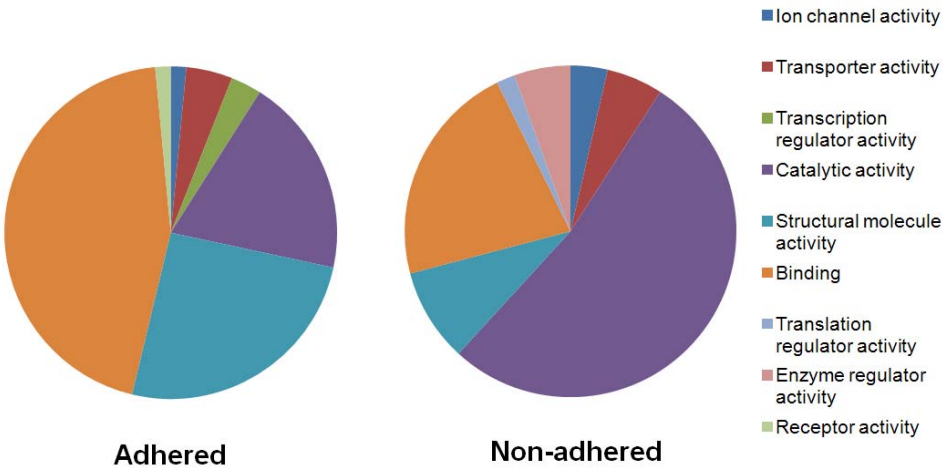

Figure 3. Distribution of molecular function annotations of membrane proteins in adhered and non-adhered subpopulations of bovine testicular cell isolates. Annotations determined from Gene Ontology using PANTHER (http://www.pantherdb.org/)

non-adhered (21.8%). Similarly, more proteins with a structural function (e.g. keratin) were identified in the adhered cells (25.4%) than the non-adhered population (9.1%). The non-adhered fraction contains a higher proportion of proteins associated with catalytic activity (52.7%) than that of the adhered cells (19.4%).

The broader GO annotation terms displayed in Figure 3 encompass a number of "child" terms and these are listed for the non-adhered cells only in Table 1 along with their biological processes. The prediction of transmembrane helices (TM) in the identified proteins are also listed in Table 1 along with the presence of a signal peptide cleavage site (SP) or the involvement of the protein in non-classical secretion (NCS), that is, secretion not triggered by cleavage of a signal peptide. Of the 64 proteins found to be differentially expressed in non-adhered cells, only two, malectin and hypoxia up-regulated protein 1, contain a transmembrane domain, however, both proteins are associated with the ER membrane. The same two proteins also have a signal peptide cleavage site along with 4 others, and 30 candidates demonstrate non-classical secretion.

Gene expression

To identify candidates for gene expression markers for testicular germ cells, a number of candidate molecules were selected from the proteins found exclusively or preferentially in the non-adhered cells (Table 1 and Supplemental Tables 2 and 5). The expression patterns of genes corresponding to these proteins were examined in human testis tissue images at www.proteinatlas.org. Genes which showed an expression pattern similar to early spermatogonia (situated in low numbers on the basement membrane in seminiferous tubules) were included as candidates for investigation of RNA expression by qRT-PCR. Ten candidate genes (*ASB9, ATIC, DDX6, FSCN1, IQGAP1, NAP1L4, PFN1, PHGDH, TKTL1* and *TLN1*) and four established testis cell marker genes (*DDX4, GATA4, UCHL1* and *VIM*) were assessed for transcription levels relative to the reference genes *ATP5G2* (ATP synthase) and *RPS26* in samples from four non-adhered and four adhered cell fractions (Supplemental Table 1). Of the ten investigated candidates, three showed higher ($p < 0.05$) expression levels in non-adhered cells (*DDX6, NAP1L4, TKTL1*) (Figure 4). *ASB9* showed a trend towards higher gene expression in non-adhered cells, but the difference was not statistically significant.

Immunohistochemistry

For three candidate germ cell markers which showed significantly higher levels of RNA expression in non-adhered cells (*DDX6, NAP1L4 and TKTL1)*, protein expression was investigated by antibody staining of peri-pubertal testis tissue sections (Figure 5). None of the three candidate markers showed any co-staining with vimentin, indicating no expression by Sertoli cells. DDX6 did show co-staining with DBA-biotin, consistent with expression by early spermatogonia and also stained cells which did not show binding of DBA-biotin or vimentin-antibody, consistent with expression by more advanced germ cells (Figure 5 B, E-H). Although NAP1L4 showed high background when visualized for brightfield micrography, in fluorescence micrography the same antibody exhibited a tight overlap with DBA-biotin staining, consistent with expression by early spermatogonia (Figure 5 C, I-L). TKTL1 showed staining by cells towards the centre of seminiferous tubules, consistent with expression in advanced spermatogonia (Figure 5 D, M-P).

4. Discussion

In this study, membrane fractionation combined with proteomics analyses was employed to examine enriched undifferentiated spermatogonial cell populations with the aim of uncovering novel surface markers.

The application of proteomics to mammalian spermatogenesis has been recently reviewed [23] Proteome reference maps have been generated for whole testes from pigs (yielding 337 identifications) [38], mice (504 identifications) [39] and humans (1430 identifications) [40]. 2D-gels have been the proteomic tool of choice for studies of germline stem cells in chickens [21], in germline and embryonic SCs in mice [18] and of rat spermatogonia [41] revealing 56, 166 and 102 proteins respectively. More recently, the spermatogonial stem cell niche of dogfish has been examined yielding 16 protein identifications [22]. In this study, we report the comprehensive proteomic profiling of bovine testis cell populations which enabled the identification of 1,387 proteins, of which 1,150 were identified in the population enriched for undifferentiated spermatogonia. Using optimised membrane fractionation and enzymatic digestion protocols, we show an enrichment for membrane proteins (39% compared to 28% present in the total bovine proteome) and we demonstrated a 6-fold enrichment for proteins associated with the plasma membrane (Fig. 3).

In contrast to global proteomic profiling studies employing whole cell lysates, this study focussed specifically on the membrane-fraction of testis cell populations. Methods for proteomic studies of cell membrane preparations include "cell shaving" and FASP technology [27]. Since the testis cell isolation procedure included the use of trypsin, an enzyme-based method for retrieval of cell surface-exposed proteins was not considered a promising approach. Application of the FASP technology allows an increased rate of membrane protein identification as these rather insoluble proteins may be solubilised and treated in the presence of detergents and chaotropes not typically used in gel-free proteomic studies because of their interference in down-stream analyses. The resulting protein preparation was enriched in membrane proteins and can therefore be expected to yield an increased identification rate for low abundance proteins that may be useful as markers of undifferentiated spermatogonia.

The testicular cell isolates were differentially plated to yield

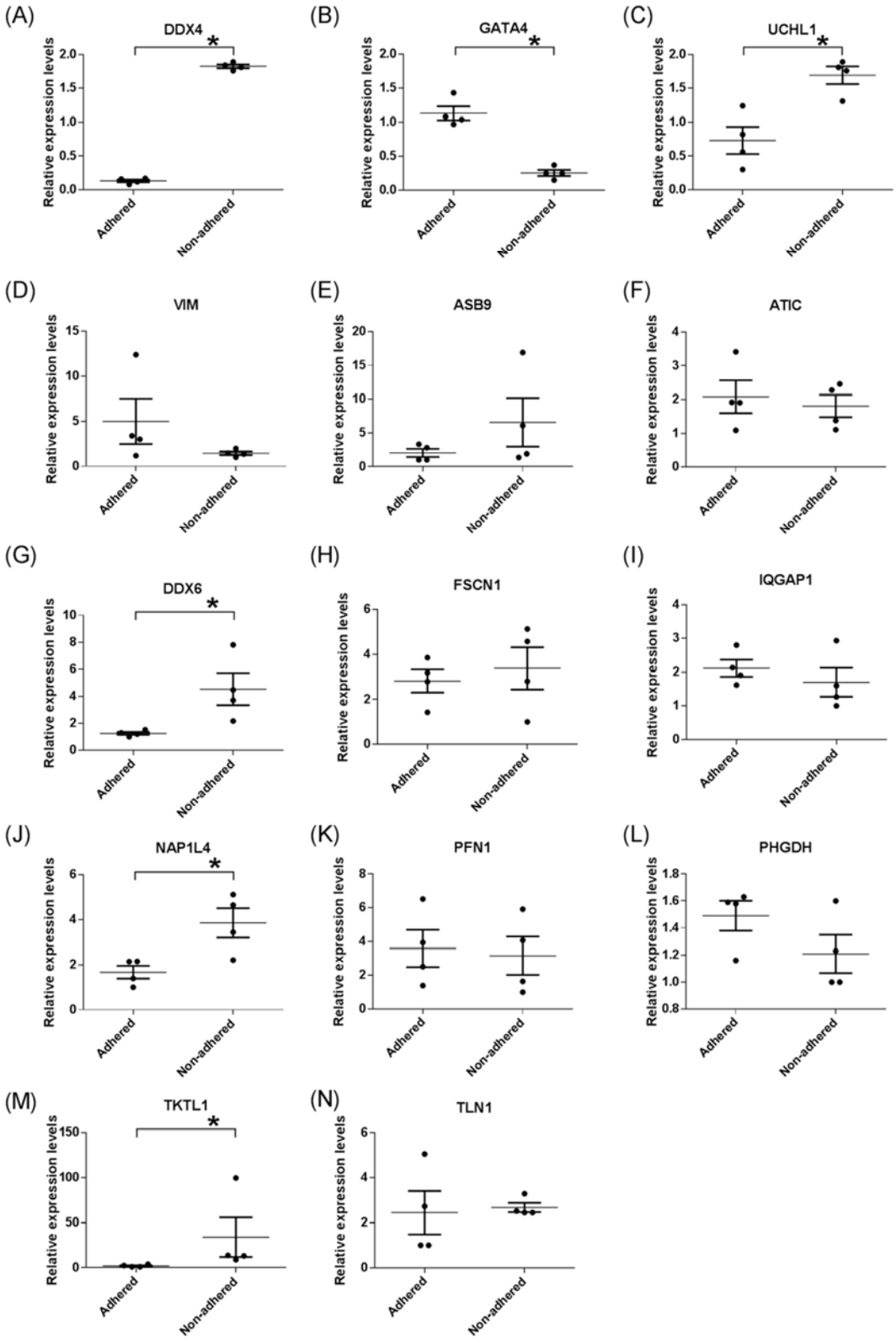

Figure 4. Comparison of candidate gene expression in adhered and non-adhered testis cell fractions. (A-D) Established testis cell markers. (E-N) Candidate markers. Gene expression levels are graphed as a ratio relative to reference gene expression levels (y-axes). Each data point represents the average of three technical replicates. Horizontal bars represent the mean of four biological replicates, with error bars representing the standard error of the mean. * denotes a statistically significant difference between cell fractions in a Mann-Whitney U test ($p < 0.05$).

two cell populations: adhered and non-adhered. The non-adhered population, enriched for undifferentiated spermatogonia, was the target of our investigation as it is well established that the majority of undifferentiated spermatogonia remain in suspension during the initial culture period, whereas their support cells, the Sertoli cells are expected to be present in both populations with a slight enrichment in the adhered population [12]. This was confirmed by immunocytochemical examination of cell smears that showed significant enrichment for the known germ cell markers UCHL1 and DDX4 in the non-adhered population. Label-free relative quantification of the identified proteins following LC-MS/MS analysis was undertaken and the proteins were classed as differentially expressed if they passed multi-

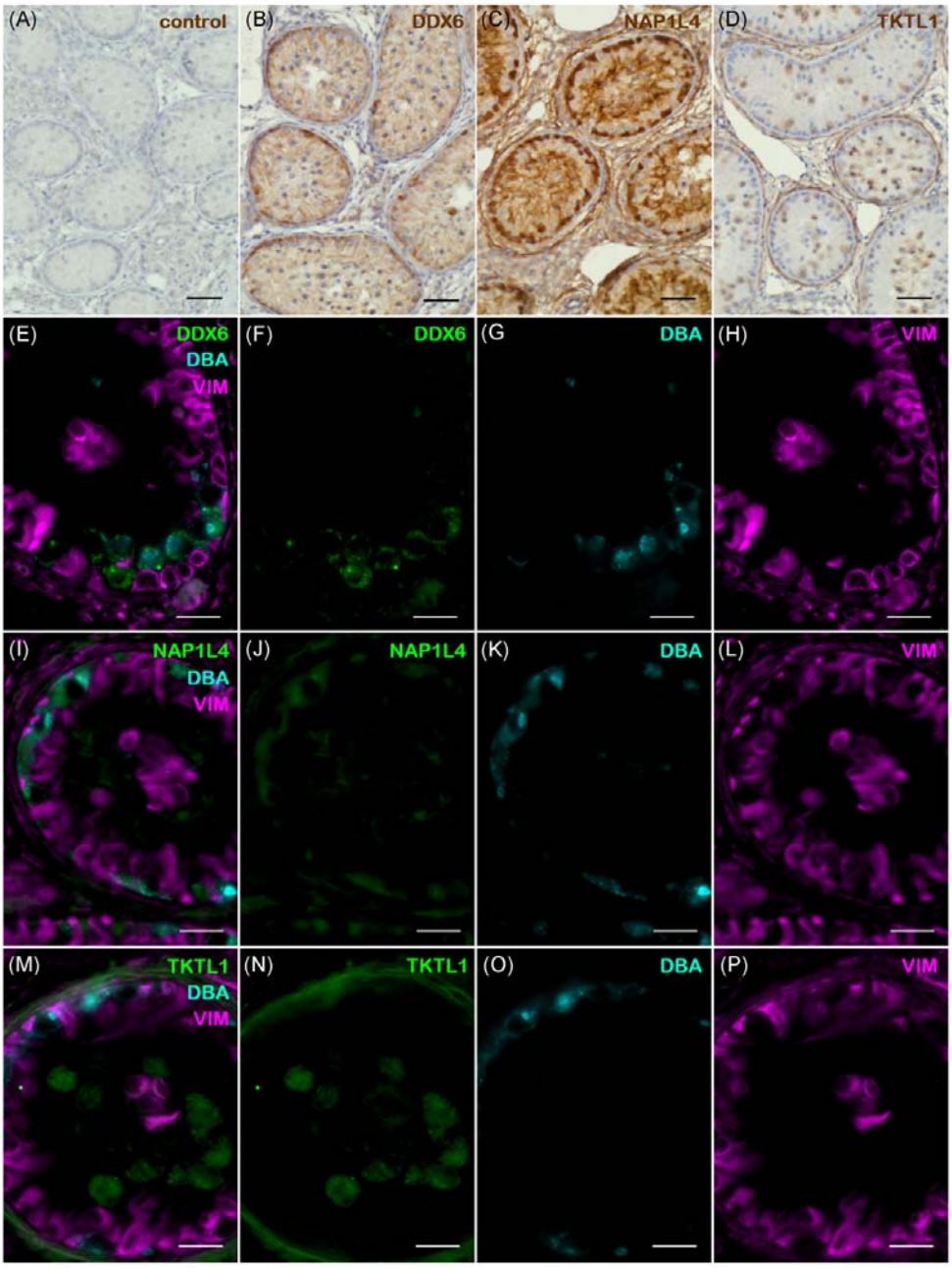

Figure 5. Expression of candidate markers in peripubertal bovine testis tissue. (A-D) Overview brightfield micrographs, scale bars are 50 µm. (E-P) Detailed fluorescence micrographs with (E, I, M) merged images, scale bars are 20 µm. (A) Control, section parallel stained with secondary antibody cocktail. (B, F) Tissue stained with DDX6-antibody. (C, J) Tissue stained with NAP1L4-antibody. (D, N) Tissue stained with TKTL1-antibody. (E-H) Tissue co-stained with DDX6-antibody (E, F), DBA-biotin (E, G) and Vimentin-antibody (E, H); (I-L) tissue co -stained with NAP1L4-antibody (I, J), DBA-biotin (I, K) and Vimentin-antibody (I, L); (M-P) tissue co-stained with TKTL1-antibody (M, N), DBA-biotin (M, O) and Vimentin-antibody (M, P).

ple criteria based on the number of peptides identified, the total protein score and the spectral count. One of the constraints in our experimental system was that the differential plating did not result in a homogenous population, it only served to enrich the fraction for undifferentiated spermatogonia. However, it is well understood that other cell types would be present, for example red blood cells. In fact, both hemoglobin subunit alpha and beta were present in the differentially expressed lists for the non-adhered population. An additional confounding factor lies in the fact that the protein profile would be expected to change as a result of the attachment of the cells to the plates. This was observed, with structural proteins such as the keratins, collagens and spectrin present in higher abundance in the adhered fraction. With these constraints in mind, the protein populations were classified according to their molecular function. As expected, the adhered population was dominated by proteins associated with binding and structural molecule activity. In contrast, the non-adhered population was dominated by proteins associated with catalytic activity and to a lesser extent enzyme and translation regulator activities.

Of the 64 proteins that appear to be more abundant in the non-adhered cell population, 15 show localisation or close association with the plasma membrane (NSF, TGM2, ATIC, PSMC6, MDH2, ACLY, PHGDH, ENO1, IQGAP1, EHD2, ATP6V1A, ATP6V1B1, TLN1, FSCN1 and CAP1) according to the Human Protein Atlas (www.proteinatlas.org) or literature reference. For 6 of those proteins (TGM2, ATIC, MDH2, EDH2, TLN1, and CAP1), no previous reports exist that would indicate a specific role in mammalian testis or germ cells.

Vesicle-fusing ATPase (or *N*-ethylmaleimide-sensitive fusion protein, NSF) is primarily localised to microvesicles and is involved with vesicle-to-plasma membrane fusion during exocytosis. It plays a critical role in the sperm acrosome reaction, a calcium-dependent exocytosis event, which is required for fertilization [42]. In this study, NSF was uniquely detected in the non-adhered cell fraction (5 and 7 peptides in the two biological replicates).

Several of the proteins identified that appear to be more abundant in the non-adhered population share similar functions including cell surface remodelling, cell adhesion and migration. TGM2 was detected with higher protein score and spectral count ratios in the non-adhered fraction. Likewise, IQGAP1, TLN1, EHD2 and FSCN1 were also detected in the non-adhered fraction with higher protein score ratios along with higher spectral count ratios. The protein glutamine gamma-glutamyltransferase 2 (TGM2) has been demonstrated to play a role in conferring stem cell-like properties to mammary epithelial cells [43]. TGM2 additionally plays a role in remodelling extracellular matrices and promotes cell adhesion with the highest concentrations observed at cell-cell and cell-substratum contact points [44]. Although initally thought to be an intracellular enzyme, TGM2 has been demonstrated to bind to the pericellular fibronectin coat [45]. Ras GTPase-activating-like protein IQGAP1 is localised to the plasma membrane and cell junctions and also plays a role in cell surface remodelling (through organisation of the actin cytoskeleton) and cell adhesion. IQGAP1 through its interaction with CDC42 co-localises to the periphery of both Sertoli and germ cells regulating their adhesion [46]. Talin-1 (TLN1) is a plasma membrane protein that shows higher concentrations in areas of cell-cell contact. TLN1 binds to integrin-β and plays a critical role in cell adhesion and morphogenesis [47]. A recent report by Sharma et al [48], demonstrated that talin-1 enhances the survival , migration and differentiation of cardiac stem cells. EH-domain containing 2 protein (EHD2) is a cell membrane protein also involved with membrane reorganisation and membrane trafficking between the plasma membrane and endosomes [49]. A recent genome-wide profiling study found that EHD2 was up-regulated in primordial germ cells and embryonic germ cells compared to embryonic stem cells [50]. Fascin (FSCN1) plays a role in cell motility and migration through its involvement in actin bundling and formation of cell protrusions [51]. Fascin-3 is a testis-specific isoform [52] that has been shown to increase during sperm capacitation [53].

Two of the proteins identified as more abundant in the non-adhered cell population were enzymes initially assumed to be cytosolic in nature. ATP citrate lyase (ACLY) was detected with a higher protein score ratio (5.28) and spectral count ratio (4.75) in the non-adhered fraction, whereas α-enolase was uniquely detected in the non-adhered cell fraction (6 peptides detected in each of the two biological replicates). ACLY is the primary enzyme responsible for the synthesis of acetyl-CoA in many tissues and is primarily localised to the cytoplasm but also the plasma membrane. Lipid synthesis is required not only for membrane production, but also for lipid-based post-translational modification of proteins. Rat testicular germ cells were observed to possess ACLY and post-meiotic increases in the activity of anabolic enzymes were noted [54]. Alpha-enolase (ENO1) is a multifunctional cytoplasmic enzyme involved in glycolysis and cell growth control that is also expressed on the cell surface where it binds to plasminogen [55]. Alpha-enolase is known to be present in the tail of mature sperm and is associated with post-translational modification during sperm maturation. It was recently identified as a potential biomarker of bull fertility [56].

Lastly, proteins representing the CCT complex (TCP1, CCT2, CCT4, CCT5, CCT7, CCT8) and PMSC6 of the 26S proteasome, all of which were expected to be cytosolic in location were identified as more abundant in the non-adhered cell populations. Interestingly, these proteins were also identified in a recent study in our laboratory examining the plasma membrane fraction of bovine sperm, implying that they are indeed associated with the plasma membrane [57]. The CCT complex is present on the surface of capacitated spermatozoa playing a role in binding to the zona pellucida during fertilization [58], and the membrane proteasome of mammalian sperm is required for the acrosome

reaction and fertilization [59].

While the proteomic analysis was limited to two biological replicates, we were able to compare and confirm some of the results with gene expression data utilising four biological replicates, and with immunohistochemistry data of independent testis tissue sections. We identified a group of ten candidate genes from the non-adhered cell fraction which showed a cell localisation pattern akin to undifferentiated spermatogonia in human testis tissue images at the Human Protein Atlas (www.proteinatlas.org). Higher gene expression levels observed in the non-adhered cell populations for *DDX6*, *NAP1L4* and *TKTL1* correlated with the results of the proteomic analysis where DDX6 and NAP1L4 were identified only in the non-adhered cell fraction, and TKTL1 showed higher abundance in the non-adhered fraction. These three genes are known to be associated with spermatogonial cell function: *DDX6* codes for an ATP-dependent RNA helicase that has been found highly expressed in mammalian spermatogonia [60], *NAP1L4* encodes a nucleosome assembly chaperone protein that has been shown to be required for the incorporation of the testis-specific H3t histone variant into nucleosomes [61] and the *TKTL1* gene product was identified as a germ cell biomarker in a proteomics study of human seminal plasma [62].

Protein expression analysis of the differentially expressed genes DDX6, NAP1L4 and TKTL1 confirmed expression in spermatogonia. DDX6 and NAP1L4 were found to be expressed in early spermatogonia identified by DBA-lectin binding, indicating expression in an undifferentiated subset of spermatogonia. In comparison, probable ATP-dependent RNA helicase (DDX4) is expressed throughout the germ lineage [7, 8]. UCHL1 is an established marker for bovine undifferentiated spermatogonia [9], which includes but is not limited to DBA-lectin binding spermatogonia [12]. NAP1L4 in particular showed an expression profile more similar to DBA-lecting binding, and hence might be closer to a putative SSC marker than other established spermatogonial markers in the bovine. Further analysis will confirm expression patterns of DDX6 and NAP1L4 in bovine spermatogonia, and the utility of the new candidates when compared to established markers. From our data, further candidates for characterisation of the non-adhered cell population, which show enrichment for undifferentiated spermatogonia, are NSF, TGM2, ATIC, PSMC6, MDH2, ACLY, PHGDH, ENO1, IQGAP1, EHD2, ATP6V1A, ATP6V1B1, TLN1, FSCN1 and CAP1 as well as proteins of the CCT complex.

5. Concluding Remarks

This study represents the most comprehensive proteomic profile of bovine testicular cells with 1,387 proteins identified at a 1% false discovery rate. Furthermore, 39% of the proteins identified are associated with cellular membranes, primarily the plasma membrane. We have identified a number of potential surface markers that warrant further investigation. Fourteen proteins were assessed by qRT-PCR including the four known markers (DDX4, GATA4, UCHL1 and VIM). Of the ten candidate markers, three (DDX6, NAP1L4 and TKTL1) are also differentially expressed at the RNA level, and for those proteins expression in spermatogonia was confirmed by immunohistochemistry. Proteome maps such as this one provide the foundation for future studies of spermatogenesis and germ cell biology.

6. Supplementary material

Supplementary data and information is available at: http://www.jiomics.com/index.php/jio/rt/suppFiles/136/0

Supplemental Table 1 – Primers used for qRT-PCR.
Supplemental Table 2 – List of proteins identified in bovine testis isolates using nanoflow HPLC-MS/MS.
Supplemental Table 3 – List of proteins identified in non-adhered cell population of bovine testis isolates.
Supplemental Table 4 – List of proteins identified in adhered cell population of bovine testis isolates.
Supplemental Table 5 – List of proteins that appeared to be more abundant (based on protein score) in non-adhered cell population of bovine testis isolates. Proteins that were excluded from the final list (based on spectral count) are indicated in red italics.
Supplemental Table 6 – List of proteins that appeared to be more abundant (based on protein score) in adhered cell population of bovine testis isolates. Proteins that were excluded from the final list (based on spectral count) are indicated in red italics.
Supplemental Table 7 – List of proteins that appeared to be more abundant (based on protein score and spectral count) in non-adhered cell population of bovine testis isolates.
Supplemental Table 8 – List of proteins that appeared to be more abundant (based on protein score and spectral count) in adhered cell population of bovine testis isolates.

Acknowledgements

The authors wish to thank Miss Keren Byrne for assistance with bioinformatic analysis. Dr Shelly Hope and Dr Gene Wijffels provided helpful feedback and advice on the manuscript. Mass spectrometry equipment was provided by the Molecular & Cellular Proteomics Mass Spectrometry Facility at The University of Queensland. We wish to thank the staff at Highchester Abattoirs, Gleneagle, Queensland for their cooperation in obtaining bovine testes at slaughter.

References

[1] C. Dores, W. Alpaugh, I. Dobrinski, Cell Tissue Res, 349 (2012) 691-702. doi: 10.1007/s00441-012-1457-x

[2] R.L. Brinster, J.W. Zimmermann, Proc Natl Acad Sci U S A, 91 (1994) 11298-11302.

[3] P.M. Aponte, M.P. van Bragt, D.G. de Rooij, A.M. van Pelt,

APMIS, 113 (2005) 727-742. doi: 10.1111/j.1600-0463.2005.apm_302.x

[4] J.M. Oatley, R.L. Brinster, Methods Enzymol, 419 (2006) 259-282. doi: 10.1016/S0076-6879(06)19011-4

[5] K. von Kopylow, C. Kirchhoff, D. Jezek, W. Schulze, C. Feig, M. Primig, V. Steinkraus, A.N. Spiess, Hum Reprod, 25 (2010) 1104-1112. doi: 10.1093/humrep/deq053

[6] F. Izadyar, J. Wong, C. Maki, J. Pacchiarotti, T. Ramos, K. Howerton, C. Yuen, S. Greilach, H.H. Zhao, M. Chow, Y.C. Chow, J. Rao, J. Barritt, N. Bar-Chama, A. Copperman, Hum Reprod, 26 (2011) 1296-1306. doi: 10.1093/humrep/der026

[7] T. Noce, S. Okamoto-Ito, N. Tsunekawa, Cell Struct Funct, 26 (2001) 131-136.

[8] E. Raz, Genome Biol, 1 (2000) REVIEWS1017. doi: 10.1186/gb-2000-1-3-reviews1017

[9] R. Rathi, A. Honaramooz, W. Zeng, S. Schlatt, I. Dobrinski, Reproduction, 130 (2005) 923-929. doi: 10.1530/rep.1.00912

[10] F. Izadyar, G.T. Spierenberg, L.B. Creemers, K. den Ouden, D.G. de Rooij, Reproduction, 124 (2002) 85-94.

[11] C. Ertl, K.H. Wrobel, Histochemistry, 97 (1992) 161-171.

[12] M. Herrid, R.J. Davey, J.R. Hill, Cell Tissue Res, 330 (2007) 321 -329. doi: 10.1007/s00441-007-0445-z

[13] T. Imai, Y. Kawai, Y. Tadokoro, M. Yamamoto, Y. Nishimune, K. Yomogida, Mol Cell Endocrinol, 214 (2004) 107-115. doi: 10.1016/j.mce.2003.10.065

[14] K. Steger, M. Schimmel, K.H. Wrobel, Arch Histol Cytol, 57 (1994) 17-28.

[15] K.H. Wrobel, D. Bickel, R. Kujat, Acta Anat (Basel), 153 (1995) 263-272.

[16] E. Guillaume, B. Evrard, E. Com, E. Moertz, B. Jegou, C. Pineau, Mol Reprod Dev, 60 (2001) 439-445. doi: 10.1002/mrd.1108

[17] X. Guo, W. Ying, J. Wan, Z. Hu, X. Qian, H. Zhang, F. He, Electrophoresis, 22 (2001) 3067-3075. doi: 10.1002/1522-2683(200108)22:14<3067::AID-ELPS3067>3.0.CO;2-V

[18] H. Dihazi, G.H. Dihazi, J. Nolte, S. Meyer, O. Jahn, G.A. Muller, W. Engel, J Proteome Res, 8 (2009) 5497-5510. doi: 10.1021/pr900565b

[19] H. Dihazi, G.H. Dihazi, O. Jahn, S. Meyer, J. Nolte, A.R. Asif, G.A. Mueller, W. Engel, J Proteome Res, 10 (2011) 1962-1973. doi: 10.1021/pr1012015

[20] L. Harkness, H. Christiansen, J. Nehlin, T. Barington, J.S. Andersen, M. Kassem, Stem Cell Res, 1 (2008) 219-227. doi: 10.1016/j.scr.2008.06.001

[21] B.K. Han, J.G. Jung, J. Nam, J.K. Moon, J.N. Kim, S.I. Lee, J.K. Kim, H. Kim, J.Y. Han, J Androl, 30 (2009) 690-702. doi: 10.2164/jandrol.108.007401

[22] G. Loppion, R. Lavigne, C. Pineau, P. Auvray, P. Sourdaine, Comp Biochem Physiol Part D Genomics Proteomics, 5 (2010) 157-164. doi: 10.1016/j.cbd.2010.03.004

[23] S. Chocu, P. Calvel, A.D. Rolland, C. Pineau, Syst Biol Reprod Med, 58 (2012) 179-190. doi: 10.3109/19396368.2012.691943

[24] A.R. Bellve, J.C. Cavicchia, C.F. Millette, D.A. O'Brien, Y.M. Bhatnagar, M. Dym, J Cell Biol, 74 (1977) 68-85.

[25] M. Nagano, M.R. Avarbock, E.B. Leonida, C.J. Brinster, R.L. Brinster, Tissue Cell, 30 (1998) 389-397. doi: S0040-8166(98)80053-0 [pii]

[26] S. Conrad, M. Renninger, J. Hennenlotter, T. Wiesner, L. Just, M. Bonin, W. Aicher, H.J. Buhring, U. Mattheus, A. Mack, H.J. Wagner, S. Minger, M. Matzkies, M. Reppel, J. Hescheler, K.D. Sievert, A. Stenzl, T. Skutella, Nature, 456 (2008) 344-349. doi: 10.1038/nature07404

[27] J.R. Wisniewski, A. Zougman, N. Nagaraj, M. Mann, Nat Methods, 6 (2009) 359-362. doi: 10.1038/nmeth.1322

[28] W.H. Tang, I.V. Shilov, S.L. Seymour, J Proteome Res, 7 (2008) 3661-3667. doi: 10.1021/pr070492f

[29] Z. Du, X. Zhou, Y. Ling, Z. Zhang, Z. Su, Nucleic Acids Res, 38 (2010) W64-70. doi: 10.1093/nar/gkq310

[30] F. Supek, M. Bosnjak, N. Skunca, T. Smuc, PLoS One, 6 (2011) e21800. doi: 10.1371/journal.pone.0021800

[31] P.D. Thomas, A. Kejariwal, M.J. Campbell, H. Mi, K. Diemer, N. Guo, I. Ladunga, B. Ulitsky-Lazareva, A. Muruganujan, S. Rabkin, J.A. Vandergriff, O. Doremieux, Nucleic Acids Res, 31 (2003) 334-341.

[32] W. Huang da, B.T. Sherman, R.A. Lempicki, Nucleic Acids Res, 37 (2009) 1-13. doi: 10.1093/nar/gkn923

[33] W. Huang da, B.T. Sherman, R.A. Lempicki, Nat Protoc, 4 (2009) 44-57. doi: 10.1038/nprot.2008.211

[34] D. Binns, E. Dimmer, R. Huntley, D. Barrell, C. O'Donovan, R. Apweiler, Bioinformatics, 25 (2009) 3045-3046. doi: 10.1093/bioinformatics/btp536

[35] A. Krogh, B. Larsson, G. von Heijne, E.L. Sonnhammer, J Mol Biol, 305 (2001) 567-580. doi: 10.1006/jmbi.2000.4315

[36] T.N. Petersen, S. Brunak, G. von Heijne, H. Nielsen, Nat Methods, 8 (2011) 785-786. doi: 10.1038/nmeth.1701

[37] J.D. Bendtsen, L.J. Jensen, N. Blom, G. Von Heijne, S. Brunak, Protein Eng Des Sel, 17 (2004) 349-356. doi: 10.1093/protein/gzh037

[38] S.Y. Huang, J.H. Lin, Y.H. Chen, C.K. Chuang, E.C. Lin, M.C. Huang, H.F. Sunny Sun, W.C. Lee, Proteomics, 5 (2005) 4205-4212. doi: 10.1002/pmic.200401284

[39] Y.F. Zhu, Y.G. Cui, X.J. Guo, L. Wang, Y. Bi, Y.Q. Hu, X. Zhao, Q. Liu, R. Huo, M. Lin, Z.M. Zhou, J.H. Sha, J Proteome Res, 5 (2006) 2217-2225. doi: 10.1021/pr0600733

[40] X. Guo, P. Zhang, R. Huo, Z. Zhou, J. Sha, Proteomics Clin Appl, 2 (2008) 1651-1657. doi: 10.1002/prca.200780120

[41] E. Com, B. Evrard, P. Roepstorff, F. Aubry, C. Pineau, Mol Cell Proteomics, 2 (2003) 248-261. doi: 10.1074/mcp.M300010-MCP200

[42] C.N. Tomes, G.A. De Blas, M.A. Michaut, E.V. Farre, O. Cherhitin, P.E. Visconti, L.S. Mayorga, Mol Hum Reprod, 11 (2005) 43-51. doi: 10.1093/molehr/gah126

[43] A. Kumar, H. Gao, J. Xu, J. Reuben, D. Yu, K. Mehta, PLoS One, 6 (2011) e20701. doi: 10.1371/journal.pone.0020701

[44] C.A. Gaudry, E. Verderio, R.A. Jones, C. Smith, M. Griffin, Exp Cell Res, 252 (1999) 104-113. doi: 10.1006/excr.1999.4633

[45] C.A. Gaudry, E. Verderio, D. Aeschlimann, A. Cox, C. Smith, M. Griffin, J Biol Chem, 274 (1999) 30707-30714.

[46] W.Y. Lui, D.D. Mruk, C.Y. Cheng, J Cell Physiol, 202 (2005) 49-66. doi: 10.1002/jcp.20098

[47] J. Liu, X. He, Y. Qi, X. Tian, S.J. Monkley, D.R. Critchley, S.A. Corbett, S.F. Lowry, A.M. Graham, S. Li, Mol Cell Biol, 31 (2011) 3366-3377. doi: 10.1128/MCB.01403-10

[48] U.C. Sharma, D. M Vu, Journal of Stem Cell Research & Therapy, 02 (2012) 123. doi: 10.4172/2157-7633.1000123

[49] S. Benjamin, H. Weidberg, D. Rapaport, O. Pekar, M. Nudelman, D. Segal, K. Hirschberg, S. Katzav, M. Ehrlich, M. Horowitz, Biochem J, 439 (2011) 433-442. doi: 10.1042/BJ20111010

[50] N. Pashai, H. Hao, A. All, S. Gupta, R. Chaerkady, A. De Los

Angeles, J.D. Gearhart, C.L. Kerr, PLoS One, 7 (2012) e39088. doi: 10.1371/journal.pone.0039088

[51] J.C. Adams, Curr Opin Cell Biol, 16 (2004) 590-596. doi: 10.1016/j.ceb.2004.07.009

[52] B. Tubb, D.J. Mulholland, W. Vogl, Z.J. Lan, C. Niederberger, A. Cooney, J. Bryan, Exp Cell Res, 275 (2002) 92-109. doi: 10.1006/excr.2002.5486

[53] M.A. Baker, G. Reeves, L. Hetherington, R.J. Aitken, Proteomics, 10 (2010) 482-495. doi: 10.1002/pmic.200900574

[54] M. Bajpai, G. Gupta, S.K. Jain, B.S. Setty, Andrologia, 30 (1998) 311-315.

[55] S. Moscato, F. Pratesi, A. Sabbatini, D. Chimenti, M. Scavuzzo, R. Passatino, S. Bombardieri, A. Giallongo, P. Migliorini, Eur J Immunol, 30 (2000) 3575-3584.

[56] Y.J. Park, W.S. Kwon, S.A. Oh, M.G. Pang, J Proteome Res, 11 (2012) 4162-4168. doi: 10.1021/pr300248s

[57] K. Byrne, T. Leahy, R. McCulloch, M.L. Colgrave, M.K. Holland, Proteomics, 12 (2012) 3559-3579. doi: 10.1002/pmic.201200133

[58] M.D. Dun, N.D. Smith, M.A. Baker, M. Lin, R.J. Aitken, B. Nixon, J Biol Chem, 286 (2011) 36875-36887. doi: 10.1074/jbc.M110.188888

[59] R. Sanchez, M. Deppe, M. Schulz, P. Bravo, J. Villegas, P. Morales, J. Risopatron, Andrologia, 43 (2011) 114-120. doi: 10.1111/j.1439-0272.2009.01031.x

[60] K. Matsumoto, O.Y. Kwon, H. Kim, Y. Akao, Developmental dynamics : an official publication of the American Association of Anatomists, 233 (2005) 1149-1156. doi: 10.1002/dvdy.20429

[61] H. Tachiwana, A. Osakabe, H. Kimura, H. Kurumizaka, Nucleic Acids Res, 36 (2008) 2208-2218. doi: 10.1093/nar/gkn060

[62] A.D. Rolland, R. Lavigne, C. Dauly, P. Calvel, C. Kervarrec, T. Freour, B. Evrard, N. Rioux-Leclercq, J. Auger, C. Pineau, Hum Reprod, 28 (2013) 199-209. doi: 10.1093/humrep/des360