The Sodium Pump Regulates Sperm and Sertoli Cell Function

by

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A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY

GRADUATE PROGRAM IN VETERINARY MEDICAL SCIENCES

CALGARY, ALBERTA

AUGUST, 2016

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Abstract

Abnormalities in sperm function at the submicroscopic level (not detectable during routine semen evaluation) influence bull fertility and therefore the efficiency of cattle production. New knowledge regarding the molecular basis of spermatogenesis and sperm function will enable us to develop evidence-based approaches for improving fertility. The overall aim of this thesis was to investigate role of Na/K-ATPase (the sodium pump) isoforms in sperm function, Sertoli cell function and male fertility. In fresh bovine sperm, I identified two distinct pools (raft and non-raft) of the testis-specific isoform of Na/K-ATPase (ATP1A4) in the plasma membrane. The raft pool of ATP1A4 interacted with caveolin-1 and EGFR, whereas the non-raft pool of ATP1A4 interacted with EGFR, Src and ERK1/2 in capacitated sperm. In addition, a comprehensive analysis revealed that the ATP1A4 interactome differed between raft and non-raft fractions of capacitated sperm. Specifically, ATP1A4 interacted and co-localised with plakoglobin (member of β-catenin family of proteins involved in cell adhesion) in the equatorial segment of capacitated sperm; this suggests a potential role for these proteins in sperm-oolemma fusion. During investigation of ATP1A4 involvement in lipid rafts, I determined that ATP1A4 content and activity were increased during capacitation, perhaps due to translation of ATP1A4 mRNA in mitochondrial or mitochondrial-type ribosomes. In frozen-thawed sperm, content and activity of ATP1A4 was greater in high- versus low-fertility bulls and significantly correlated with fertility. Additionally, ATP1A4-induced ROS, calcium, actin polymerization and tyrosine phosphorylation were also involved in regulating post-thaw sperm function in these bulls. My results also demonstrated that prepubertal rat Sertoli cells expressed ATP1A1 (the ubiquitous isoform of Na/K-ATPase) and that ATP1A1-ouabain interaction regulated formation (modulation of claudin 11 and connexin 43 expression) and function (transepithelial electric
resistance) of Sertoli cell junctional complexes through Src-EGFR-ERK1/2- CREB pathway in a
dose-dependent manner. Overall, results demonstrated that isoforms of Na/K-ATPase have
unique roles in controlling several aspects of sperm and Sertoli cell physiology, acting through
its well-established enzyme activity and signaling functions. Consequently, isoforms of Na/K-
ATPase are potential candidates for reversible male contraception and a biomarker for male
fertility.
Acknowledgements

I thank my supervisor Dr. Jacob Thundathil for giving me the opportunity to work on this project and for his guidance, support and mentorship throughout my doctoral degree. The belief that he had in me and in my bench skills helped me to learn a lot as a researcher over the past years. I really appreciate his open-door policy and to the endless paper/thesis editing sessions.

I sincerely thank my supervisory committee members Drs. Frans van der Hoorn, Ina Dobrinski and John Kastelic for their valuable guidance and critical insights on the project. Special thanks to Dr. Claudia Klein for use of her laboratory for Sertoli cell culture experiments and for troubleshooting tips with PCR.

I thank UCVM for their entrance scholarship, Alberta Children’s Hospital Research Institute for their graduate student trainee scholarship and also UCVM and University of Calgary for numerous travel awards. I thank the financial support of Natural Sciences and Engineering Research Council and Alberta Livestock and Meat Agency for funding my project.

I acknowledge the technical support of Laurent Brechenmacher for mass spectrometry data analysis. I am also thankful to Tom Kroetsch for his advice on fertility evaluation of dairy bulls, Grace Kwong for her help with statistics and Laurie Kennedy for her expertise with flow cytometry.

I thank Alta Genetics Inc. for providing fresh semen samples for the entire duration of my study and Semex Alliance Inc. for their contribution of frozen-thawed semen samples from dairy bulls. I thank current members of Dr. Thundathil lab (Alysha Dance, Mina Ojaghi, Chinju Johnson and Guilherme Rizzoto) for their support and friendship. If not for these people, working in the lab wouldn’t have been easy. I thank Mr. Doug Nickel for helping me procure to semen samples from Alta Genetics and for his advice on laboratory techniques. I appreciate all
the help that I got from the Reproduction and Regenerative Medicine group for their generous
collection of antibodies and use of their microscopes. Finally, I would like to thank all of my
friends in Calgary and the rest of my family in India for being there in my ups and downs.
Dedication

To my boys

Rio – Nothing in this world can be compared to the toothless smile of yours

Vijay – For your patience and acceptance, for all the calls and coffees

To my parents

Late Dad – I know you are still watching me

Mum – For sending me back to pursue my goals and for coming to Canada
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<tbody>
<tr>
<td>ADAM</td>
<td>A disintegrin and metalloprotease domain</td>
</tr>
<tr>
<td>AI</td>
<td>Artificial insemination</td>
</tr>
<tr>
<td>AKAP</td>
<td>A kinase anchoring protein</td>
</tr>
<tr>
<td>Akt/PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>ATP1A4</td>
<td>$\alpha$ subunit of testis-specific isoform of Na/K-ATPase</td>
</tr>
<tr>
<td>ALH</td>
<td>Amplitude of lateral head displacement</td>
</tr>
<tr>
<td>ATP1B3</td>
<td>$\beta$ subunit of Na/K-ATPase</td>
</tr>
<tr>
<td>ATP1A1</td>
<td>$\alpha$ subunit of ubiquitous isoform of Na/K-ATPase</td>
</tr>
<tr>
<td>AMH</td>
<td>Anti-Müllerian hormone</td>
</tr>
<tr>
<td>AK1</td>
<td>Adenylate kinase 1</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BBSE</td>
<td>Bull Breeding Soundness Evaluation</td>
</tr>
<tr>
<td>BSP</td>
<td>Binder of sperm protein</td>
</tr>
<tr>
<td>BS$^3$</td>
<td>Bissulfosuccinimidyl suberate</td>
</tr>
<tr>
<td>BTB</td>
<td>Blood-testis barrier</td>
</tr>
<tr>
<td>CHAPS</td>
<td>(3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
</tr>
<tr>
<td>CID</td>
<td>Collision induced dissociation</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CASA</td>
<td>Computer assisted sperm analyser</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>CBM</td>
<td>Caveolin binding motif</td>
</tr>
<tr>
<td>CCT5 and 8</td>
<td>T-complex protein 1 subunits ε and θ</td>
</tr>
<tr>
<td>CP</td>
<td>Chloramphenicol</td>
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<tr>
<td>CV</td>
<td>Coefficient of variation</td>
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<td>CSD</td>
<td>Caveolin scaffolding domain</td>
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<td>CCT/TRiC</td>
<td>Chaperonin containing TCPI-ring complex</td>
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<td>DAPI</td>
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<td>DAG</td>
<td>Diacyl glycerol</td>
</tr>
<tr>
<td>dbcAMP</td>
<td>Dibutyryl cAMP</td>
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<td>DCFDA</td>
<td>2’,7’ –dichlorofluorescin diacetate</td>
</tr>
<tr>
<td>DPSS</td>
<td>Diode pumped solid state laser</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DRM</td>
<td>Detergent resistant membrane</td>
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<tr>
<td>DS</td>
<td>Desmosomes</td>
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<td>EGF and EGFR</td>
<td>Epidermal growth factor and receptor</td>
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<tr>
<td>ERK1/2</td>
<td>Extracellular signal-regulated protein kinases 1 and 2</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
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<td>ECM</td>
<td>Extracellular matrix</td>
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<td>ELSPBP1</td>
<td>Epididymal sperm-binding protein E12</td>
</tr>
<tr>
<td>ES</td>
<td>Ectoplasmic specialization</td>
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<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ES</td>
<td>Equatorial segment</td>
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<td>Description</td>
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<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>FAA</td>
<td>Fertility associated antigen</td>
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<tr>
<td>FSC</td>
<td>Forward scatter</td>
</tr>
<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>GJ</td>
<td>Gap junctions</td>
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<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<td>β-Glycerophosphate</td>
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<td>GATA4</td>
<td>GATA (consensus sequence) binding protein 4</td>
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<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
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<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
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<td>HSP</td>
<td>Heat shock proteins</td>
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<td>HF</td>
<td>High-fertility bulls</td>
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<td>Inositol triphosphate</td>
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<td>IBMX</td>
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<tr>
<td>JAM</td>
<td>Junctional adhesion molecule</td>
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<tr>
<td>KH₂PO₄</td>
<td>Potassium dihydrogen phosphate</td>
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xviii
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<tr>
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<td>KLF17b</td>
<td>Kruppel like factor 17 member b</td>
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<td>Knock out</td>
</tr>
<tr>
<td>LIN</td>
<td>Linearity</td>
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<td>Liquid chromatography</td>
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<tr>
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<td>LDH</td>
<td>Lactate dehydrogenase</td>
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<tr>
<td>MES</td>
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<td>MFI</td>
<td>Median fluorescence intensity</td>
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<td>MDCK</td>
<td>Madin-Darby canine kidney</td>
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<td>mtIF2 and IF3</td>
<td>Mitochondria initiation factor 2 and 3</td>
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<td>Macrophage migration inhibitory factor</td>
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<td>MAPK</td>
<td>Mitogen activated protein kinases</td>
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<td>Na$_2$Co$_3$</td>
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<td>NRR</td>
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<td>Platelet derived growth factor</td>
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<td>Plasma membrane Ca$^{2+}$ ATPase</td>
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</tr>
<tr>
<td>PTK</td>
<td>Protein tyrosine kinase</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PA</td>
<td>phosphatidic acid</td>
</tr>
<tr>
<td>PC</td>
<td>phosphatidyl choline</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PLC$\zeta$</td>
<td>Phospholipase zeta</td>
</tr>
<tr>
<td>PH-20</td>
<td>Sperm adhesion molecule 1</td>
</tr>
<tr>
<td>PLD</td>
<td>Phospholipase D</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol 3,4,5-triphosphate</td>
</tr>
<tr>
<td>P&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Inorganic phosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein phosphatase 2</td>
</tr>
<tr>
<td>PS</td>
<td>Posterior acrosome</td>
</tr>
<tr>
<td>PAWP</td>
<td>Postacrosomal WW binding protein</td>
</tr>
<tr>
<td>PG</td>
<td>Plakoglobin</td>
</tr>
<tr>
<td>PV</td>
<td>Perivitelline space</td>
</tr>
<tr>
<td>PT</td>
<td>Perinuclear theca</td>
</tr>
<tr>
<td>Q-TOF</td>
<td>Quadrupole-time of flight</td>
</tr>
<tr>
<td>Rho A</td>
<td>Ras homolog gene family member A</td>
</tr>
<tr>
<td>RPE</td>
<td>Retinal pigment epithelium</td>
</tr>
<tr>
<td>RNP</td>
<td>Ribonucleoprotein particles</td>
</tr>
<tr>
<td>RBP</td>
<td>RNA binding proteins</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation buffer</td>
</tr>
<tr>
<td>Ras</td>
<td>Rat sarcoma protein</td>
</tr>
<tr>
<td>Raf</td>
<td>Rapidly accelerated fibrosarcoma</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>Sp-TALP</td>
<td>Tyrode Albumin Lactate Pyruvate medium for sperm capacitation</td>
</tr>
<tr>
<td>Sos</td>
<td>Son of sevenless</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>STI</td>
<td>Soybean trypsin inhibitor</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Src</td>
<td>Sarcoma</td>
</tr>
<tr>
<td>StAR</td>
<td>Steroidogenic acute regulatory protein</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter</td>
</tr>
<tr>
<td>SNPs</td>
<td>Single nucleotide polymorphisms</td>
</tr>
<tr>
<td>tRNA&lt;sub&gt;Met&lt;/sub&gt;</td>
<td>Methionine loaded tRNA</td>
</tr>
<tr>
<td>TALPH</td>
<td>Tyrode Albumin Lactate Pyruvate Hepes medium</td>
</tr>
<tr>
<td>TJ</td>
<td>Tight junctions</td>
</tr>
<tr>
<td>TER</td>
<td>Transepithelial electrical resistance</td>
</tr>
<tr>
<td>TIMP2</td>
<td>Type-2 tissue inhibitor of metalloproteinases</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane domain</td>
</tr>
<tr>
<td>tACE</td>
<td>Testis-specific isoform of angiotensin converting enzyme</td>
</tr>
<tr>
<td>TTBS</td>
<td>Tween-20 Tris buffered saline</td>
</tr>
<tr>
<td>VCL</td>
<td>Curvilinear velocity</td>
</tr>
<tr>
<td>VASA/DDX4</td>
<td>DEAD-box helicase 4</td>
</tr>
<tr>
<td>WT-1</td>
<td>Wilms tumour protein 1</td>
</tr>
<tr>
<td>WT&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Wild type</td>
</tr>
<tr>
<td>YWHA</td>
<td>14-3-3 phospho-serine/phospho-threonine binding proteins</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Zona occludens 1</td>
</tr>
<tr>
<td>ZP</td>
<td>Zona pellucida</td>
</tr>
<tr>
<td>2D</td>
<td>Two dimension</td>
</tr>
</tbody>
</table>
3D       Three dimension
3β-HSD   3 beta-hydroxysteroid dehydrogenase
Chapter One: Introduction

World population is expected to reach nearly ~10 billion by 2050, necessitating increased efficiency of global food production. With ~35 million beef cows in American beef herds, a modest 3% increase in reproductive rate would yield ~1 million more beef calves annually (Senger PL 2012). Similarly, a 3% increase in pregnancy rates in American dairy herds would yield an additional 15 million litres of milk per year. Therefore, improving reproductive efficiency of beef and dairy cattle is of utmost importance for meeting increasing global demand for animal proteins. In that regard, bull fertility is particularly critical, as one bull can breed thousands of females by artificial insemination (AI) or 20 to 30 females via natural service per breeding season. Bull breeding soundness evaluation (BBSE) identifies bulls with semen that is grossly abnormal. Notwithstanding, semen samples classified as satisfactory based on these traditional approaches differ in fertility; therefore, perhaps there are submicroscopic differences in sperm characteristics affecting fertility. Therefore, a better understanding on the molecular regulation of sperm and testis function promotes development of novel, evidence-based approaches to managing male fertility.

The overall aim of this project was to understand the role of Na/K-ATPase in regulation of sperm function, Sertoli cell function and male fertility. Recently the α4 isoform of Na/K-ATPase (ATP1A4) has received considerable attention due to its testis-specific expression in post-meiotic germ cells and mature sperm and its regulation of sperm motility and capacitation. We previously demonstrated that incubation of bovine sperm with ouabain (a specific ligand for Na/K-ATPase) induced capacitation through signaling involving kinases. However, mechanisms by which Na/K-ATPase orchestrates and activates various signaling molecules in this process
remain unknown. In somatic cells, Na/K-ATPase signaling involves lipid rafts; furthermore, the importance of lipid rafts in regulation of sperm function was also reported (Thaler et al. 2006, Bou Khalil et al. 2006, Gadella et al. 2008). Perhaps Na/K-ATPase orchestrates and activates several molecules in lipid rafts of the sperm plasma membrane during capacitation. In addition, downstream events associated with Na/K-ATPase signaling (including increases in ROS, intracellular calcium, protein tyrosine phosphorylation and actin polymerisation) have been linked to fertility of frozen-thawed sperm. Therefore, it is likely that ATP1A4 content and activity are related to post-thaw sperm function and fertility. Apart from sperm, ATP1A1, the ubiquitous isoform of Na/K-ATPase, has been involved in regulation of epithelial cell tight junctions. Since Sertoli cells are the only epithelial cells responsible for germ cell development and maturation in the testis, perhaps ATP1A1 is involved in formation and function of the blood testis barrier (BTB) and thus male fertility. Therefore, the thesis project was undertaken to investigate the role of Na/K-ATPase isoforms in events leading to sperm capacitation, male fertility and Sertoli cell junctional complexes. A review of literature relevant for this research is provided in this chapter.

1.1 Review of literature

1.1.1 Bull breeding soundness evaluation and current challenges in sire selection:
Dairy and beef industries strive to achieve high pregnancy rates from genetically superior bulls. Therefore, fertility is more important than production traits; in that regard, estimated relative importance of reproductive traits to growth and carcass traits are in the ratio of 4:2:1, respectively (Schiefelbein 1998). Bull fertility is particularly critical, with ~80% of bulls deemed satisfactory based on a traditional BBSE. The standards for BBSE established by the Society for
Theriogenology (www.therio.org) are intended to assess the likelihood of a bull establishing pregnancy in >25 healthy, cycling females in a 65-70 d breeding season. A bull that is healthy and sound, with adequate scrotal circumference, >30% progressive motile sperm, >70% morphologically normal sperm, and <20% sperm head defects, is designated as a satisfactory potential breeder. Despite being classified as satisfactory, there is typically 10-20% variation in fertility among bulls (natural mating or AI), due to submicroscopic/molecular differences in their sperm (Larson & Miller 2000). Bulls with reduced sperm fertility can cause substantial economic losses due to delayed conception, prolonged calving seasons, reduced calf weaning weights, and increased number of breeding females that are culled due to failure to become pregnant or delayed pregnancy establishment. Although subfertility of bulls may not be evident when used in a multiple-sire or low breeding pressure system, such bulls typically result in reduced fertility when they are used for single-sire mating or AI (Kastelic & Thundathil 2008, Kasimanickam et al. 2012). Therefore, bull effects are paramount. For example, a modest 1% increase in the reproductive rate would generate up to three times more return on investments (Hansen 2006). BBSE eliminates bulls that are grossly abnormal (due to general health, physical characteristics or semen characteristics) and conventional frozen semen analysis eliminates semen samples that do not meet criteria established by the Society of Theriogenology (Barth 1993). Regardless, the subjective nature and lack of precision in conventional semen analysis suggests that acceptable semen may be erroneously rejected, and concurrently, semen of unacceptable quality may be used for inseminations (Christensen et al. 2005). Therefore, bull or semen selection could be aided by complementing traditional BBSE and frozen semen evaluation using novel laboratory assays focused on molecular sperm function (sperm capacitation; see below), which may improve bull fertility predictions.
1.1.2 Sperm capacitation

Ejaculated sperm must undergo changes in the female reproductive tract to achieve fertilizing ability; this includes a series of physiological and biochemical modifications termed capacitation. Biochemical changes include an efflux of cholesterol from the plasma membrane leading to an increase in membrane fluidity, hyperpolarization of the plasma membrane (Hernandez-Gonzalez et al. 2006), changes in protein phosphorylation and protein kinase activity (Baldi et al. 2000, Visconti 2009), increase in bicarbonate (HCO$_3^-$), Ca$^{2+}$ and cyclic adenosine monophosphate (cAMP) concentration and intracellular pH. Several molecules are required for successful capacitation to occur; these include HCO$_3^-$, serum albumin (BSA) and Ca$^{2+}$. The co-transporter Na$^+$/HCO$_3^-$ facilitates entry of HCO$_3^-$ into sperm (Demarco et al. 2003) and physiological increase in HCO$_3^-$ concentration activate scramblase enzyme, leading to a rapid collapse of the asymmetry of the sperm plasma membrane (Gadella & Harrison 2000), thereby increasing availability of cholesterol to external acceptors (Salicioni et al. 2007). This increase in the HCO$_3^-$ concentration also increases intracellular pH, activates a unique soluble adenylyl cyclase which in turn increases cAMP and cAMP-dependent PKA activation during capacitation. Bicarbonate, in combination with BSA, also increases membrane hyperpolarization due to enhanced K$^+$ permeability (Martinez-Lopez et al. 2009). Downstream of bicarbonate, PKA activation modulates the response of calcium channels such as CatSper, which changes intracellular Ca$^{2+}$ concentrations. Furthermore, PKA phosphorylates several proteins on serine and threonine residues, thereby activating (directly or indirectly) several protein kinases and/or inhibiting protein phosphatases, which culminate in increased phosphorylation of tyrosine residues (Bajpai et al. 2003, Visconti et al. 2011). Due to capacitation-associated changes
mentioned above, motility pattern of sperm changes from a linear progressive motion (swimming in a relatively straight line) to a localised, non-progressive motion (hyperactivation) that facilitates sperm-oocyte contact. Binding of sperm to an oocyte initiates fusion of the sperm plasma membrane and outer acrosome membrane that allows release of acrosomal enzymes from sperm (acrosome reaction) which digest the zona pellucida of the oocyte. Sperm now enters the perivitelline space (between the zona pellucida and oocyte plasma membrane/oolemma) and fuses with the oolemma, leading to successful fertilization. Since sperm DNA is generally considered transcriptionally inactive, sperm functions (motility, capacitation and sperm-oocyte interaction) are regulated by sperm proteins and their post-translational modifications without additional protein synthesis. In that regard, sperm proteomics has received considerable attention for identification of markers for submicroscopic differences in sperm function.

1.1.3 Comparative physiology of mammalian sperm capacitation

In mammals, regulators of capacitation such as calcium, bicarbonate and BSA have different effects among species. For example, HCO$_3^-$, BSA and Ca$^{2+}$ are necessary for the capacitation of mouse sperm (Visconti et al. 1995) while BSA is not necessary for the capacitation of boar (Tardif et al. 2003) and ram sperm (Patricia Grasa et al. 2006). Visconti & Kopf (1998) suggested a cooperative effect of Ca$^{2+}$ and HCO$_3^-$ by an increase in cAMP levels and subsequent phosphorylation of different proteins. Increasing amounts of extracellular Ca$^{2+}$ alone increases tyrosine phosphorylation in mouse (Visconti et al. 1995) and human sperm (Lecerc et al. 1998). When exposed to a variety of substrates, mouse, human and bovine sperm capacitation correlate with an increase in protein tyrosine phosphorylation (Viscontil et al. 1995, Galantino-Homer
et al. 1997, Carrera et al. 1996). Glucose inhibits heparin-induced bovine capacitation in vitro by a mechanism involving cAMP metabolism and a reduction of intracellular pH (Parrish et al. 1994). Paradoxically, glucose is beneficial for capacitation in other species. BSA, present in the capacitation media (e.g., mouse, hamster, cattle, and human), is believed to function during in vitro capacitation as a sink for the removal of cholesterol from the sperm plasma membrane. In vitro, the average time required to complete the capacitation process, judged by the acquisition of fertilizing potential varies among species, for example, 2 h in mouse, 4 – 5 h in bovine and rabbit, 1 h in human (Austin 1985) which correlate with sperm cholesterol/phospholipid ratios. Lesser the cholesterol/phospholipid ratio is associated with lesser time to complete capacitation (Davis 1981). Essential role of ROS as modulators of capacitation is recognised in human (Herrero et al. 2006), mouse (Herrero et al. 2003), bovine (O’Flaherty et al. 2006) and boar (Funahashi 2002) sperm.

1.1.4 Sperm proteomics – the key to identify biomarkers of fertility

Many sperm components, including lipids (Brinsko et al. 2007), ions such as calcium (Collin et al. 2000), proteins (Bellin et al. 1998, Parent et al. 1999), and nucleic acids (Lalancette et al. 2008) were upregulated or downregulated (depending on fertility status) in various mammalian species. Since sperm functions are regulated by proteins present in mature sperm, understanding the role of individual sperm proteins could lead to identification of novel biomarkers of fertility. For example, content of P25b, a bovine sperm membrane antigen, was lower in semen from subfertile bulls than in high fertility bulls (Parent et al. 1999). A 30-kDa heparin-binding protein (fertility-associated antigen, FAA), was differentially expressed in sperm membranes of beef bulls with varying fertility (Bellin et al. 1998a). Furthermore, Sutovsky (Sutvosky et al. 2015)
described positive and negative protein biomarkers of fertility. Negative fertility markers included proteins exclusively associated with certain types of sperm defects, whereas positive biomarkers were more abundant in morphologically and functionally normal sperm, except that they may either be upregulated or downregulated. One of the negative protein biomarkers of sperm quality is ubiquitin, which has been assessed in numerous species including humans (Sutovsky et al. 2001), horses (Sutovsky et al. 2003), cattle (Sutovsky et al. 2002), and pigs (Kuster et al. 2004), and is correlated with infertility and indications of poor sperm quality, including primary and total morphological defects (Purdy 2008). Since the bovine AI industry uses elite bulls, such as high- (HF) and low-fertility (LF) bulls (that are 3% above and below the breed average for fertility, respectively), upregulation or downregulation of specific proteins may contribute to differences in fertility among these bulls and enable identification of biomarkers of fertility. Using a 2D-gel electrophoresis-mass spectrometry approach, D’Amours (D’Amours et al. 2010) reported that T-complex protein 1 subunits ε and θ (CCT5 and CCT8), epididymal sperm-binding protein E12 (ELSPBP1), proteasome subunit α type-6, and binder of sperm 1 (BSP1) were highly expressed in the LF group, whereas adenylate kinase isoenzyme 1 (AK1) and phosphatidylethanolamine-binding protein 1 (PEBP1) were highly expressed in the HF group. In a similar approach (Peddinti et al. 2008), HF bull sperm had upregulated expression of pyruvate kinase, COX3, ATP5B, casein kinase, AKAP4, EGF and PDGF signaling pathways, whereas integrin and DNA damage check point regulation pathways were significant hits identified in LF bull sperm. Comparing normal versus abnormal sperm induced by elevated testicular temperature, Newton (Newton et al. 2009) demonstrated differential expression of several sperm proteins in morphologically abnormal sperm, including ATP1A4, as the molecular basis for impaired function.
1.1.5 Na/K-ATPase structure and isoforms

It is well established that Na/K-ATPase is a plasma membrane protein with two fundamental roles in regulation of cell function. First, it is responsible for maintaining Na\(^+\) and K\(^+\) gradients across the plasma membrane of most mammalian cells. In that regard, this enzyme contributes to maintenance of cell volume and pH, resting membrane potential, osmotic balance, and generation of a Na\(^+\) gradient for coupled transmembrane ion transport (Skou and Esmann 1992; Sweadner 1989). Secondly, it is the receptor for cardiotonic steroids such as ouabain (specific inhibitor of Na/K-ATPase); in that regard, interaction of ouabain with Na/K-ATPase initiates signaling critical for regulation of various cell functions. The functional Na/K-ATPase consists of two subunits, the α subunit (110 kDa) and the β subunit (35-60 kDa, depending on glycosylation; (Blanco & Mercer 1998). The α polypeptide is the catalytic unit responsible for ionic translocation as well as ouabain-dependent signaling events (Jorgensen et al. 2003), whereas the β subunit is essential for the enzyme’s activity, as well as folding and localisation in the membrane (Geering 1991). There are four α isoforms (α1, α2, α3, and α4) and three β isoforms (β1, β2, and β3) in mammalian tissues (Blanco & Mercer 1998, Mobasher et al. 2000). The α1 and β1 isoforms are expressed in almost every cell (function as housekeeping Na/K-ATPase), whereas other α polypeptides have a more restricted expression, with specific roles (Mobasher et al. 2000).

1.1.6 Ouabain – inhibitor of Na/K-ATPase enzyme activity

Digitalis extract, whose principle component is ouabain, have been used for treatment of cardiac diseases for centuries. A similar endogeneous compound with digitalis like reactivity was identified from the adrenal gland (Laredo et al. 1994) and hypothalamus (Dorell et al. 2005).
This endogeneous hormone has a specific binding site on the extracellular loops (TM1-TM2, TM5-TM6, and TM7-TM8) of α subunit of Na/K-ATPase, facilitating a conformational change in the enzyme which prevents K⁺ ion binding and its transport (Burns et al. 1996). Dissimilarities in the amino acid sequences between different species and isoforms underlie the different sensitivity of Na/K-ATPase to cardiotonic steroids (Blanco et al. 1999, Geering 2005). As an example, aminoacids 111 to 122 in the extracellular loop between TM1-TM2 form the most important part of the putative ouabain binding site. In rodents, α1 isoform has a low affinity for ouabain due to presence of charged amino acids between TM1-TM2 which is not present in the highly sensitive α2 and α3 isoforms. However in case of humans, little difference exists among the highly sensitive α1, α2, and α3 isoforms in terms of their ouabain affinity. With regard to the α4 isoform in rats and mouse, ouabain affinity is in the nM range (Woo et al. 1999) and this isoform is well conserved among species (Li and Langhans 2015). In the reproductive tract, ouabain like reactivity was detected in bovine vaginal fluid (Daniel et al. 2010) and therefore we inferred that the interaction of ouabain with Na/K-ATPase may be involved in regulation of sperm functions.

1.1.7 Expression and localisation of Na/K-ATPase subunits in the male reproductive tract

ATP1A4 has received considerable attention in recent years due to its sperm-specific expression along with the ubiquitous α1 isoform (ATP1A1; Blanco et al. 2000). The α1 and α4 subunits are co-expressed in sperm with the β1 and β3 isoforms; the α4 isoform associates with both β subunits equally, with similar kinetic properties (Arystarkhova & Sweadner 1997). In addition to α1, α4, β1, and β3 isoforms, the α3 and β2 subunits were also present in bovine sperm (Hickey & Buhr 2011). The α4 isoform has high affinity for Na⁺ but low affinity for K⁺, and very high
sensitivity to ouabain, in contrast to other isoforms (Woo et al. 1999), with two-thirds of total Na/K-ATPase activity of sperm attributed to ATP1A4 (Wagoner et al. 2005). Expression of α4 isoform peaked in mature testes in rats (Woo et al. 2000, Wagoner et al. 2005) and humans (Hlivko et al. 2006), whereas expression of the α1 isoform was constant throughout spermatogenesis. Within the flagellum, α4 expression was restricted to the mid-piece (rat) and principal piece (human; (Woo et al. 2000, Hlivko et al. 2006), whereas α1 was present throughout the flagellum (Wagoner et al. 2005). In our studies with fresh bovine sperm, α4 was restricted to the head (Thundathil et al. 2006). In the Sertoli cell line 93RS2, isolated from 15-d-old rats, α4 mRNA was detected, although no evidence of the α4 protein was reported (Konrad et al. 2011). However, there is contradictory evidence regarding this observation in Sertoli cells. In that regard, Lucas (Lucas et al. 2012) did not detect ATP1A4 protein from primary Sertoli cell cultures obtained from 16-d-old rats, whereas McDermott (McDermott et al. 2012) used GFP instead of the ATP1A4 gene, downstream of the ATP1A4 promoter, and reported that α4 expression was not detected in Sertoli cells from 7- or 18-d-old or adult mice.

1.1.8 Ion transport-dependent functions of Na/K-ATPase in sperm

An isoform of Na/K-ATPase (ATP1A4) dedicated to sperm function suggests that this protein has a specific role in sperm physiology. Consequently, it was no surprise that sperm from ATP1A4 KO mice displayed severe reduction of total motility due to a characteristic bend in the sperm tail and cell membrane depolarization (Jimenez et al. 2011a). Simultaneously, over-expression of ATP1A4 resulted in plasma membrane hyperpolarization, higher progressive motility and enhanced hyperactivation, implicating the role of ATP1A4 in sperm motility under both noncapacitating and capacitating conditions (Jimenez et al. 2011b). In addition, Jimenez
Jimenez et al. 2012 also reported that ATP1A4 activity was upregulated at the plasma membrane during sperm capacitation. The enzyme activity of ATP1A4 influences sperm motility due to its indirect role in regulation of pH, membrane potential and intracellular calcium release. ATP1A4 is not primarily responsible for controlling membrane potential; presumably this protein is linked to other K\(^+\) channels which are involved in depolarization and hyperpolarization. Consequently, ouabain inhibition of ATP1A4 caused sperm membrane depolarization (Jimenez et al. 2010). In addition to its role in Na\(^+\) and K\(^+\) transport, ATP1A4 indirectly regulates sperm pH by coupling to Na/H-exchanger (NHE), a flagellar protein, that uses the Na\(^+\) gradient established by the Na/K-ATPase to remove H\(^+\) from the cell in exchange for Na\(^+\) (Counillon & Pouyssegur 2000). Therefore, inhibition of Na/K-ATPase eliminates the Na\(^+\) gradient used by the Na/H-exchanger to move H\(^+\) out of the cell. Loss of NHE activity may lead to acidification of the intracellular compartment, which suppresses movement of dynein and reduces flagellar movement (Woo et al. 2002). Furthermore, ATP1A4 is also functionally linked to sperm calcium regulation via a Na/Ca-exchanger (NCX), which uses the secondary Na\(^+\) influx generated by Na/K-ATPase for calcium efflux. Inhibition of Na/K-ATPase increases intracellular Na\(^+\) concentration which disrupts calcium efflux by NCX, thereby increasing intracellular calcium concentrations (Jimenez et al. 2010a). Accordingly, sperm expresses NCX in the mid-piece of the flagellum (Krasznai et al. 2006, Bedu-Addo et al. 2008), where ATP1A4 is most abundant in rat sperm (Woo et al. 2000, Wagoner et al. 2005, Sanchez et al. 2006).

### 1.1.9 Signaling function of Na/K-ATPase in somatic cells

In addition to pumping ions, Na/K-ATPase functions as a classical receptor, inducing ouabain-mediated signaling pathways involved in regulation of various physiological processes (Xie
Binding of ouabain to Na/K-ATPase causes conformational changes in the enzyme and allows interactions with neighbouring membrane proteins resulting in activation of Src and transactivation of the epidermal growth factor receptor (EGFR). Transactivation of EGFR has a central role in relaying Na/K-ATPase-ouabain signaling to downstream pathways, including activation of the mitogen activated protein kinase (MAPK) cascade, phospholipase C (PLC) and protein kinase C (PKC) isozymes, generation of second messengers (e.g. Ca\(^{2+}\)) from intracellular stores and reactive oxygen species (ROS) from mitochondria (Ullrich & Schlessinger 1990, Haas et al. 2000, Liu et al. 2000). After ouabain interaction, a signalplex is formed involving Na/K-ATPase, Src and PLC leading to activation of PLC and increased hydrolysis of phosphatidylinositol-4, 5-bisphosphate (PIP\(_2\)), generating inositol 1, 4, 5-triphosphate (IP\(_3\)) and diacylglycerol (DAG). In addition, IP\(_3\) can activate IP\(_3\)R, which in turn releases intracellular Ca\(^{2+}\) (Yuan et al. 2005). Furthermore, DAG activates protein kinase C (PKC) and PKC in turn cross talks with Raf in promoting phosphorylation of ERK1/2 (Mohammadi et al. 2001).

1.1.10 Signaling function of Na/K-ATPase in sperm

Based on known signaling roles of Na/K-ATPase in somatic cells, it is clear that sequelae of Na/K-ATPase signaling events in somatic cells resemble some events associated with sperm capacitation, namely increase in intracellular Na\(^{+}\) and Ca\(^{2+}\) concentrations, generation of ROS, and activation of ERK1/2. Therefore, we hypothesized that Na/K-ATPase is involved as a signaling molecule during sperm capacitation and demonstrated that PKA, RTK and Src kinases were involved in this process (Newton et al. 2010). Under capacitating conditions, ouabain induced tyrosine phosphorylation and an acrosome reaction in a dose-dependent manner in fresh bovine sperm (Thundathil et al. 2006). In addition, a recent study implicated the ERK pathway in
this process (Anpalakan 2010). Our previous study demonstrated that pre-incubation of bovine sperm with a PKA inhibitor (H89) inhibited ouabain-induced tyrosine phosphorylation. Perhaps generation of ROS and increase in intracellular calcium during ouabain signaling activates PKA, leading to tyrosine phosphorylation. In addition, activated PKA interacts with PKC and activates phospholipase D (PLD), which subsequently hydrolyses phosphatidyl choline (PC) to phosphatidic acid (PA), mediating polymerisation of globular (G)-actin to filamentous (F)-actin. Actin polymerisation is involved in capacitation and acrosome reaction in bovine sperm (Yagi & Paranko 1995, Cohen et al. 2004). Based on our knowledge of Na/K-ATPase-ouabain interaction in sperm and in somatic cells, we proposed a model depicting downstream pathways potentially regulated by ouabain (Figure 1.1; (Thundathil et al. 2012). An intriguing question that remains to be answered from all of these studies is: How is Na/K-ATPase able to activate several signaling pathways? Perhaps the receptor could be preassembled with its signaling partners in specific domains of the plasma membrane known as lipid rafts.
Figure 1.1 A proposed model for bovine sperm capacitation mediated by the interaction of ATP1A4 with its ligand ouabain (adapted from Thundathil et al. 2012)

1.1.11 Role of lipid rafts in somatic cell signaling events

Membrane lipids are not homogenously distributed in the membrane bilayer; this lipid heterogeneity gives rise to formation of lipid rafts which are defined as highly dynamic, sterol and sphingolipid-enriched domains that maintain a certain degree of rigidity and are less fluid than the surrounding bilayer. Due to their particular composition, lipid rafts are characterised by their resistance to extraction with non-ionic detergents (e.g. Triton X-100) at low temperature (4 °C); therefore, they are often termed detergent-resistant membranes (DRM). Subsets of lipid rafts are designated caveolae, flask-shaped membrane invaginations marked by the protein caveolin.

Studies in somatic cells identified three possible mechanisms by which receptors initiate signal transduction in rafts (Simons & Toomre 2000). First, receptors that are associated at steady state with lipid rafts could be activated through ligand binding. Second, receptors with weak raft affinity could oligomerize on ligand binding, leading to increased residency time in rafts. Finally, activated receptors could recruit additional proteins that bind to proteins in neighbouring rafts, leading to raft coalescence. Since cholesterol is a major component of lipid rafts and sterol efflux is one of the events during capacitation, studies so far have examined effects of cholesterol efflux on stability (dissociation/coalescence) and distribution of lipid rafts during sperm capacitation. However, none of the studies were able to attribute signal initiation arising in rafts leading to capacitation. Taken together, it is likely that a steady pool of ATP1A4 resides in rafts and is activated in response to ouabain, leading to signal transduction that culminates in sperm capacitation.

1.1.12 Role of lipid rafts during sperm capacitation

Ejaculated bovine sperm plasma membrane display significantly reduced cholesterol and raft localisation of proteins compared to epididymal sperm. For example, bull cauda epididymal sperm have proteins associated to rafts (P25b and AK1) and nonrafts domains (aldose reductase and MIF; Girououard et al. 2008). When the localization of these proteins is evaluated in post-ejaculated sperm, P25b and AK1 proteins were excluded from the raft domains. Whereas AK1 is displaced to nonrafts domains as early as 15 minutes after ejaculation, P25b completes its
migration after 30 minutes. These changes were accompanied by a decrease in the cholesterol content in ejaculated compared with cauda epididymal sperm (Caballero et al. 2011).

Nonetheless, membrane domains analogous to lipid rafts observed in somatic cells have also been detected in sperm of all mammalian species (Cross 2004, Shadan et al. 2004, Sleight et al. 2005, Bou Khalil et al. 2006, Nixon & Aitken 2009). Furthermore, there seems to be various raft subtypes in sperm (Asano et al. 2009). Uniform localisation of rafts was identified in non-capacitated sperm, but rafts were restricted to the anterior acrosome in capacitated sperm, suggesting that lipid rafts serve as platforms for spatial constraint of molecules involved in zona-pellucida binding (Shadan et al. 2004, Bou Khalil et al. 2006, Nixon & Aitken 2009).

Additionally, isolated pig sperm rafts were able to bind with high affinity and specificity to oocytes of homologous species, similar to mechanisms employed by intact sperm and anterior head plasma membranes (van Gestel et al. 2007). In subsequent proteomic studies, sperm-egg receptors (IZUMO, ADAM, basigin, ACE, hexokinase, sperm adhesion molecule 1, ZP3 receptor, arylsulfatase A) and chaperones were identified in lipid rafts. Although these studies demonstrated a role for lipid raft proteins in sperm-oocyte interactions, involvement of raft proteins in signaling events leading to sperm capacitation has not been reported.

1.1.13 Na/K-ATPase and regulation of post-thaw sperm function

Since ATP1A4 regulates various sperm functions, including motility and capacitation, content and activity of this protein or its association with lipids in the plasma membrane influence sperm function and fertility of frozen-thawed sperm. In the bovine artificial insemination (AI) industry, semen is collected from bulls that are deemed satisfactory breeders (passed a standard BBSE) and subsequently their frozen-thawed semen is used to impregnate females. It is well known that
semen cryopreservation procedures (including dilution, cooling, freezing, and thawing) affect several sperm structures and functions. One of the primary sites of cryopreservation-induced damage is the sperm plasma membrane (Bailey et al. 2000). Sperm head and tail membrane contain several ATPases that tightly regulate movement of several ions responsible for motility and capacitation in sperm (Zhao & Buhr 1996). There is increasing evidence that there is reordering of membrane lipids during cooling and rewarming, thereby disturbing the lipid-lipid and lipid-protein interactions in the membrane (Hammerstedt et al. 1990). Furthermore, ATP1A4 is localised on the bovine sperm head and is extremely sensitive to its lipid environment; therefore, enzyme function is reduced in sperm that have undergone cryopreservation (Zhao & Buhr 1996). As mentioned in Section 1.1.7, some of the downstream effectors of Na/K-ATPase signaling (e.g., ROS generation, intracellular calcium release, and phosphorylation of ERK1/2) are involved in regulating several sperm functions leading to successful fertilization. Therefore it is reasonable to assume that ATP1A4 regulates post-thaw sperm function and fertility through these effectors of ATP1A4 signaling.

In sperm, controlled production of ROS is a physiological process and functions as an important second messenger in signaling during sperm capacitation. Despite the beneficial role of ROS in sperm functions, an imbalance between ROS production and antioxidant defense is detrimental to sperm physiology and can damage all cellular components including lipids, proteins and DNA (Bansal & Bilaspuri 2010). Sperm are highly susceptible to oxidative attack due to their high content of PUFA (polyunsaturated fatty acids; mainly localised to phospholipids of the sperm plasma membrane) and low levels of antioxidant defense systems (Bansal & Bilaspuri 2010). An attack by ROS usually initiates a lipid peroxidation cascade leading to a loss of membrane fluidity and integrity, which are responsible for subsequent fusion
events leading to successful fertilization (Lovercamp 2009). Apart from membrane effects, ROS also decreases mitochondrial membrane potential (Lopes et al. 1998, Sanocka & Kurpisz 2004), and increases chromatin fragmentation by causing single and double DNA breaks (Hughes et al. 1996, Kodama et al. 1997), thereby interfering with sperm fertilizing ability and embryo development (Aitken & Krausz 2001). Cryopreservation also increases sperm sensitivity to ROS attack by decreasing superoxide dismutase and glutathione peroxidase activity, two major enzymes involved in antioxidant defense in bovine sperm (Bilodeau et al. 2000). Presumably due to these adverse effects, increased production of ROS was linked to poor fertility of frozen-thawed semen (Simoes et al. 2013, Del Olmo et al. 2014).

Apart from ROS production, yet another downstream process that occurs due to Na/K-ATPase-ouabain interaction is polymerisation of G-actin to F-actin. Cryopreservation causes extreme temperature fluctuations and osmotic stress in sperm, resulting in sublethal and lethal damage to cells. In sperm, the major cytoskeletal network is present in the sperm head, especially surrounding the nucleus (perinuclear theca) and an intact cytoskeleton is required for maintaining normal cell shape, coordinated motility (Hall 1998) and appropriate cell volume regulation after freezing and thawing. However, in response to osmotic stress, the actin network undergoes reorganization via rapid activation of MAPK cascade, a phenomenon described from yeast to mammals (de Nadal et al. 2002). Furthermore, β-dystrobrevin, a perinuclear protein, was reduced in cryopreserved sperm due to depolymerization of F-actin network (Felipe-Perez et al. 2012) compared to fresh sperm. Aside from cell volume regulation, actin polymerisation (conversion to G-actin to F-actin) and depolymerisation (conversion of F-actin back to G-actin) are crucial for capacitation and acrosome reaction, respectively (Brener et al. 2003, Breitbart et al. 2005).
Apart from ultra-low temperatures that occur during freezing, events such as increase in intracellular calcium concentration could depolymerise actin filaments (Hall et al. 1993). Cryopreserved sperm had greater intracellular calcium concentration compared to fresh sperm (Bailey & Buhr 1993). Restructured membranes and distorted lipid-protein associations were believed to favour calcium ion influx (Lemma 2011) and frozen-thawed sperm fail to regulate normal internal calcium concentration (Plummer & Watson 1985). Such abnormal concentrations of calcium would disrupt normal capacitation and/or the acrosome reaction and consequently viability of sperm in the female reproductive tract, which would severely compromise fertilizing potential of post-thaw sperm (Valcarcel et al. 1994). Accordingly, frozen-thawed sperm from HF bulls contain lower Ca\(^{2+}\) concentrations than their LF counterparts (Collin et al. 2000, Peris 2008). However, the molecular basis of generation/differential regulation of these molecules (ROS, F-actin and calcium) remains unknown. Consequently comparing sperm from bulls with varying levels of fertility, such as HF and LF bulls, may identify these submicroscopic differences (for example, up- or down-regulation of specific proteins, specifically ATP1A4 content and activity) and yield molecular markers of fertility.

1.1.14 Role of Na/K-ATPase in formation and function of epithelial cell tight junctions (TJs)

Tight junctions are unique structures, present in polarised epithelial and endothelial cells that regulate flows of ions and solutes and whose functions are regulated by several signaling and molecular mechanisms. Several studies reported that the ubiquitous isoform of Na/K-ATPase (\(\alpha_1\) isoform, ATP1A1) modulates TJ development, permeability and polarity in mammals, drosophila and zebrafish. Pumping activity of various subunits of Na/K-ATPase has a role in organization and permeability of TJs. Exogenous expression of the \(\beta\) subunit of Na/K-ATPase
and E-cadherin allowed MSV-MDCK cells to display epithelial phenotype with functional TJs, suggesting that these two proteins function synergistically in TJ assembly and function (Rajasekaran et al. 2001). Following Na/K-ATPase inhibition, RhoA GTPase, which has been implicated in regulation of TJs through actin formation, was also inhibited, whereas overexpression of RhoA GTPase significantly reduced the effect of Na/K-ATPase inhibition on TJ assembly, thereby indicating that RhoA is a key molecule functionally coupled with Na/K-ATPase (Rajasekaran et al. 2001). Therefore, function of Na/K-ATPase is necessary, not only for formation of TJs, but also to maintain their function and structure. Inhibition of Na/K-ATPase function in retinal pigment and in pancreatic polarised cell lines demonstrated that membrane contact points were reduced between cells and increased permeability to both ionic and non-ionic molecules (Rajasekaran & Rajasekaran 2003, Rajasekaran et al. 2007). In addition, inhibition of Na/K-ATPase reduced protein phosphatase 2 (PP2A) activity, which increased phosphorylation of occludin and TJ permeability (Rajasekaran et al. 2007).

Since Sertoli cells, the supporting cells of the testis, form junctional complexes during puberty, it is likely that Na/K-ATPase isoforms are involved in a role similar to that detected in non-testicular epithelial cells. However, unlike in somatic epithelial cells where the α1 isoform is present, Sertoli cell line 93RS2 has α4, the sperm-specific isoform. Three independent studies led by Scheiner-Bobis demonstrated that low doses of ouabain activated c-Src-Ras-ERK1/2-CREB pathway similar to non-classical testosterone signaling (Konrad et al. 2011). Furthermore, this pathway increased expression of steroidogenic acute regulator protein (StAR), a key enzyme involved in steroidogenesis (Dietze et al. 2013) and claudin 1 and 11 which are involved in BTB maintenance (Dietze et al. 2015). In all these studies, only the mRNA corresponding to ATP1A4 was detected, but there was no conclusive demonstration of ATP1A4 protein in Sertoli cell lines.
Furthermore, there was no consensus among studies regarding the existence of this isoform in Sertoli cells. Therefore, the final objective of my thesis was to resolve controversies regarding the existence of Na/K-ATPase isoforms in Sertoli cells and subsequently to investigate the role of these isoforms in formation and function of Sertoli cell junctions.

1.2 Aims, hypotheses, objectives and outcomes

The overall goal of this thesis was to investigate roles of ATP1A4 and ATP1A1 isoforms of Na/K-ATPase in sperm and Sertoli cell function, respectively. Specific hypotheses, objectives and key outcomes of these studies are summarised below:

In Chapter 2, I used a candidate protein approach to investigate relative distribution of a cohort of known signaling molecules (Src, EGFR, ERK1/2, and caveolin-1) in raft and non-raft membrane fractions and their activation during ouabain induced sperm capacitation.

Hypothesis: Studies described under Chapters 2 and 3 are based on the hypothesis that raft and non-raft pools of ATP1A4 exist in sperm membrane, which activate specific signaling pathways involved in bovine sperm capacitation.

Objectives:

a) To determine relative content of ATP1A4, and total and phosphorylated forms of a cohort of signaling molecules (Src, EGFR, ERK1/2, and caveolin-1) in raft and non-raft membrane fractions prepared from uncapacitated and capacitated sperm.

b) To identify ATP1A4 interaction with caveolin-1, Src, EGFR, and ERK1/2 in rafts and non-raft membrane fractions during sperm capacitation.
Outcomes:

a) I identified raft- and non-raft pools of ATP1A4 in bovine sperm plasma membrane.

b) The ATP1A4-caveolin-1-EGFR pathway was restricted to rafts, whereas the ATP1A4-Src-EGFR-ERK1/2 pathway occurred in non-rafts, indicating that these two distinct pools of ATP1A4 were involved in activation of specific signaling pathways leading to bovine sperm capacitation.

In Chapter 3, I used a discovery-based approach to identify novel signaling partners for ATP1A4 in raft and non-raft membrane fractions.

Objectives:

a) To identify novel signaling partners for ATP1A4 in rafts and non-raft membrane fractions during capacitation by immunoprecipitation mass spectrometry approach.

Outcomes:

a) Comprehensive proteomic analysis revealed that each of the interacting partners of ATP1A4 were distributed in the non-raft, raft- or both membrane fractions (64, 18 and 18%, respectively) of capacitated sperm.

b) Gene ontology revealed that the ATP1A4 interactome comprised of proteins involved in sperm-oocyte interactions, metabolism, chaperones, proteases, and functioned as cytoskeletal and adaptor proteins.

c) ATP1A4 co-localised with plakoglobin in the equatorial segment of the sperm head during capacitation.
Studies described under **Chapter 2** consistently demonstrated a capacitation-associated increase in content of raft- and non-raft pools of ATP1A4. Therefore, in **Chapter 4**, I investigated mechanisms leading to capacitation-associated increase in ATP1A4 content.

**Hypothesis:** ATP1A4 synthesis during sperm capacitation occurs through translation of mRNA in mitochondrial or mitochondrial-type ribosomes.

**Objectives:**

a) To demonstrate the increase in the content of functionally active ATP1A4 molecules during sperm capacitation.

b) To evaluate roles of transcription and translation in ATP1A4 synthesis during capacitation.

**Outcomes:**

a) Increase in ATP1A4 content during capacitation was attributed to mitochondrial translation of ATP1A4 mRNA present in ejaculated sperm, rather than due to gene transcription or protein translocation.

**Manuscript:** Cell and Tissue Research (CTR-16-0178) – under peer review

In **Chapter 5**, I developed a flow cytometry-based assay for quantification of ATP1A4 content and validated a colorimetric assay for quantification of ATP1A4 enzyme activity in bull sperm.

In **Chapter 6**, I investigated the relationship between ATP1A4 content and enzyme activity to field fertility of dairy bulls.

**Hypothesis:** Dairy bulls designated as satisfactory based on a standard BBSE differ in their content and activity of ATP1A4, modulating sperm function and fertility.
Objectives:

a) To compare ATP1A4 content, activity and measure sperm functional parameters such as ROS, calcium, actin polymerization (indicated by F-actin content) and tyrosine phosphorylation in low-fertility (LF) vs high-fertility (HF) bull sperm.

b) To compare ATP1A4-mediated regulation of ROS, calcium, actin polymerization and tyrosine phosphorylation in LF and HF bull sperm.

c) To determine associations among fertility, ATP1A4 content, activity, and ATP1A4 induced ROS, calcium, and actin polymerization in LF and HF bulls.

Outcomes:

a) Frozen-thawed sperm from HF bulls had increased ATP1A4 content and activity compared to LF bulls.

b) Exposure of sperm to ouabain augmented the post-thaw increase in tyrosine phosphorylation, ROS production, and F-actin content in HF bulls, whereas the increase in intracellular calcium was low compared to LF bull sperm.

c) Content and activity of ATP1A4, ATP1A4 induced ROS production, and contents of F-actin and calcium were significantly correlated with fertility.

In Chapter 7, I evaluated prepubertal rat Sertoli cell primary cultures for expression of Na/K-ATPase isoforms and Na/K-ATPase-mediated regulation of junctional proteins

Hypothesis: Na/K-ATPase regulates formation and function of junctional complexes between Sertoli cells.
Objectives:

a) To establish Sertoli cell cultures in Matrigel-coated bicameral units and measure transepithelial electric resistance (TER).

b) To characterise Na/K-ATPase isoforms in prepubertal rat Sertoli cell cultures.

c) To assess dose-dependent effects of ouabain on TER and the content and localisation patterns of junctional proteins.

d) To identify ouabain-mediated signaling pathways involved in regulation of junctional molecules in Sertoli cells.

Outcomes:

a) Prepubertal Sertoli cell cultures predominantly expressed the ubiquitous isoform (α1) of Na/K-ATPase (ATP1A1).

b) A non-inhibitory dose (50 nM) of ouabain (specific inhibitor/ligand of ATP1A1) increased TER, claudin 11 and connexin 43 expression, whereas an inhibitory dose (1 mM) decreased TER and expression of junctional molecules.

c) Na/K-ATPase-ouabain interaction regulated formation and function of Sertoli cell tight and gap junctions through the Src-EGFR-ERK1/2-CREB pathway.

Manuscript: Biology of Reproduction (BIOLREPROD/2016/141267) – under peer review
Chapter Two: **Testis-specific isoform of Na/K-ATPase (ATP1A4) regulates bovine sperm capacitation through raft and non-raft mediated signaling**

2.1 Abstract

Sperm plasma membrane consists of highly dynamic lipid microdomains (rafts), which harbour a cohort of signaling proteins involved in the regulation of sperm capacitation. Testis-specific isoform of Na/K-ATPase (ATP1A4) is a highly abundant protein present in the plasma membrane of bovine sperm head. We previously demonstrated that incubation of bovine sperm with ouabain induces tyrosine phosphorylation of several sperm proteins and capacitation. The aim of this study was to investigate the role of membrane rafts in ATP1A4 signaling during bovine sperm capacitation. Rafts and non-raft fractions were prepared from uncapacitated and capacitated sperm and assessed for signaling functions of ATP1A4. There was a comparative increase in phosphorylation of signaling molecules both in rafts (caveolin-1) and non-rafts (EGFR and ERK1/2) during capacitation. Although Src was activated in both membrane fractions, the non-raft fraction had higher content of p-Src. Subsequent immunoprecipitation studies suggested that interaction of ATP1A4 with Src, EGFR and ERK1/2 occurred in the non-raft fraction whereas interaction with caveolin-1 and EGFR were evident in the raft fraction of ouabain-capacitated sperm. Overall, we inferred that the ATP1A4-caveolin-1-EGFR pathway was restricted to rafts, whereas the ATP1A4-Src-EGFR-ERK1/2 pathway occurred in non-rafts, indicating that these two distinct pools of ATP1A4 were involved in specific signaling events leading to bovine sperm capacitation.
2.2 Introduction

Ejaculated sperm must remain in the female reproductive tract for a species-dependent interval to attain fertilizing ability; these maturational changes are collectively known as sperm capacitation (Yanagimachi 1994). Numerous physiological events have been implicated in capacitation, including a sterol efflux and a subsequent increase in membrane fluidity, membrane hyperpolarization, generation of reactive oxygen species (ROS), increased concentrations of calcium and cAMP, protein tyrosine phosphorylation, elevated intracellular pH, remodelling of actin, and hyperactivated motility (Salicioni et al. 2007). These capacitation-associated changes were attributed to changes in the intracellular ionic environment and initiation of several signaling cascades. However, specific roles of individual sperm proteins in regulation of sperm capacitation are not well characterized. Although ATP1A4, is involved in regulation of sperm motility (Jimenez et al. 2011a, Jimenez et al. 2011b) and capacitation (Thundathil et al. 2006, Newton et al. 2010), underlying molecular mechanisms have not been clearly elucidated.

Functional Na/K-ATPase, a heterodimer of α and β subunits, is responsible for maintenance of Na\(^+\) and K\(^+\) gradients between the cell and its environment. In mammalian cells, Na/K-ATPase is expressed as various isozymes, depending on the association of the four isoforms of the α subunit (α1, α2, α3, and α4) and three β isoforms (β1, β2, and β3) which are specific to various cell types and appear to regulate specific functions, depending on the physiological needs of the cell (Blanco & Mercer 1998, Mobasheri et al. 2000). In sperm, α1 and α4 subunits are co-expressed with the β1 and β3 isoforms, although the α4 isoform associates with both β subunits equally, with similar kinetic properties (Arystarkhova & Sweadner 1997). Moreover, 75% of total Na/K-ATPase activity of sperm is attributed to the α4 isoform (ATP1A4), whereas the remainder (25%) is contributed by the ubiquitous α1 isoform (Wagoner
et al. 2005). Testis-specific expression of ATP1A4 suggests this protein is critical for regulation of sperm function. In that regard, knockout of ATP1A4 severely reduced total sperm motility, due to a characteristic bend in the sperm tail and cell membrane depolarization (Jimenez et al. 2011a). Furthermore, over-expression of ATP1A4 resulted in plasma membrane hyperpolarization, higher progressive motility and hyperactivation, implicating the ATP1A4 isoform in sperm motility (Jimenez et al. 2011).

In addition to its role as an ion transporter, Na/K-ATPase binds cardiac glycosides such as ouabain, which inhibits its enzyme activity (Schwartz et al. 1988, Aperia 2007) and induces conformational changes in this protein, leading to initiation of several downstream signaling pathways critical for various cellular functions (Li & Xie 2009). Majority of Na/K-ATPase resides in specialized microdomains of membrane phospholipid bilayer called lipid rafts or caveolae, which may facilitate its signaling function, due to proximity to other signaling molecules within these microdomains (Liu et al. 2003, Liang et al. 2007). Caveolae are a subpopulation of rafts made up of ~25 kDa protein named caveolin (Patel & Insel 2009, Lajoie & Nabi 2010). Rafts and caveolae are enriched in cholesterol and sphingolipids, which maintain a certain degree of rigidity and are less fluidic compared to the remainder (non-raft) of the plasma membrane. Domains analogous to membrane rafts in somatic cells have been detected in mammalian sperm (Cross 2004, Shadan et al. 2004, Sleight et al. 2005, Bou Khalil et al. 2006, Nixon & Aitken 2009); apparently, there are several raft subtypes in sperm (Asano et al. 2009). Although previous studies demonstrated a role for lipid raft proteins in sperm-oocyte interaction (van Gestel et al. 2005, Bou Khalil et al. 2006, van Gestel et al. 2007), involvement of raft proteins in signaling events leading to sperm capacitation has apparently not been reported.
We previously reported that incubation of bovine sperm with ouabain induced tyrosine phosphorylation and capacitation through a mechanism involving receptor tyrosine kinases, non-receptor tyrosine kinases, protein kinases A and C (PKA and PKC; (Newton et al. 2010). In addition, another study in bovine sperm implicated the ERK1/2 pathway in this process (Anpalakan 2010). However, mechanisms by which Na/K-ATPase orchestrates these signaling pathways remain unknown. We hypothesized that raft and non-raft pools of ATP1A4 exist in sperm membrane, which activate specific signaling pathways involved in bovine sperm capacitation. The overall objective of this study was to investigate involvement of lipid rafts in ATP1A4 signaling during bovine sperm capacitation.

2.3 Materials and methods

2.3.1 Semen processing and capacitation

Fresh semen was collected (artificial vagina), from mature Holstein bulls, immediately diluted 1:1 with TALPH (Galantino Homer et al. 1997) and maintained at 35 °C (in a thermos) during transportation to the laboratory. Semen was subjected to a percoll wash on a two-layer percoll gradient (45% – 90%) by centrifugation (700 × g, 30 min, 25 °C). The resulting sperm pellet was re-suspended in Sp-TALP and washed to remove percoll (380 × g, 10 min, 25 °C). Concentration of the resulting sperm pellet was determined using a hemocytometer and adjusted to 200 × 10^6 sperm/ml with Sp-TALP. Stock solutions of ouabain (100 µM) and heparin (1 mg/ml) were prepared in Sp-TALP and stored at 4 °C. Working solutions of ouabain (50 nM) and heparin (10 µg/ml) were prepared in complete Sp-TALP medium (Sp-TALP containing 1 mM pyruvate, 25 mM NaHCO₃, 2 mM Ca²⁺) on the day of use. A combination of heparin, IBMX and dbcAMP was included as one of the study groups, as it is a known combination of agents that induces
capacitation in bovine sperm. This group served as the positive control of capacitation and also helped to detect similarities or differences with regard to the ATP1A4 signaling function under various capacitating conditions. Stock solutions of IBMX (10 mM in dimethyl sulfoxide) and dbcAMP (100 mM in Milli-Q water) were prepared and stored at –20 °C. This experimental design included two incubation controls: fresh uncapacitated sperm (designated as control 0 h) and sperm incubated in Sp-TALP for 4 h (control 4 h). The ouabain and heparin (combination of heparin + IBMX + dbcAMP) groups were also incubated in the Sp-TALP medium for 4 h at 39 °C under high humidity. After confirming capacitation status by evaluating changes in sperm motility patterns and phosphotyrosine content (described below), these sperm preparations were used for isolation of raft and non-raft membrane fractions (described below).

2.3.2 Isolation of raft and non-raft membrane fractions

Nondetergent-based lysis buffer was used for preparation of raft and non-raft membrane fractions from uncapacitated and capacitated samples, as described (Liu & Askari 2006). Nondetergent lysis buffer consisted of 0.5 M Na₂CO₃ (pH 11), 1 mM EDTA, 1 mM Na₃VO₄, 1 mM NaF, and a cocktail of protease inhibitors (Roche, Laval, QC, Canada). Sperm (1 x 10⁹) were lysed in pre-chilled (4 °C) lysis buffer followed by homogenization using a sonicator, and sperm preparations were maintained on ice for 30 min. After pelleting by centrifugation (1000 x g, 5 min), the supernatant was mixed with an equal volume of 80% sucrose (w/v) in MBS buffer (25 mM MES, 150 mM NaCl, pH 6.5). This suspension was placed at the bottom of the tube and overlaid with 35% sucrose, with 5% sucrose added on the top. Thereafter, samples were centrifuged at 110,000 x g for 12 h at 4 °C (SW 41 Ti rotor, Beckman Coulter Inc., Brea, CA, USA). The raft fraction was floating as an opalescent band at the 30% – 5% sucrose interface,
whereas non-raft fraction sedimented at the bottom of the tube. Both raft and non-raft fractions were ultra-centrifuged again in a TLA 100.3 rotor (Beckman Coulter Inc.) for 1 h at 189,000 x g to remove the contaminating sucrose and the pelleted fractions were used for subsequent analyses.

2.3.3 ATP1A4 content, distribution of raft and non-raft markers, fatty acid profiles, and morphology of membrane vesicles in raft- and non-raft membrane fractions prepared from uncapacitated sperm

2.3.3.1 ATP1A4 content and distribution of raft and non-raft markers in the membrane fractions
Raft and non-raft protein preparations were electrophoresced on SDS-PAGE gels and immunoblotted with ATP1A4, caveolin-1, GM-1 and α-tubulin antibodies (detailed in Section 2.3.7 of this chapter).

2.3.3.2 Fatty acid analysis
Fatty acid analysis of raft and non-raft fractions was performed at Lipid Analytical Laboratories, University of Guelph, Guelph, ON, Canada. Total lipids were extracted from both raft and non-raft fractions using the Bligh and Dyer method, with diheptadecanoyl phosphatidyl choline as the internal standard (BLIGH & DYER 1959). Fatty acid methyl esters were prepared using boron trichloride in methanol and heating the methylation tubes in a boiling water bath. The resulting fatty acid methyl esters were analyzed on a Varian 3400 gas-liquid chromatograph (Varian, Palo Alto, CA, USA) with a 60-m DB-23 capillary column (0.32 mm internal diameter).
2.3.3.3 Transmission electron microscopy

Pellets from raft and non-raft fractions were resuspended in PBS on ice and aliquots from both fractions were placed on a dish and formvar/carbon coated 300-mesh copper grids were allowed to float on the droplets for 5 min. Subsequently, grids were stained with 1% phosphotungstic acid for 5 min and then examined with a transmission electron microscopy (Tecnai F20, FEI, Hillsboro, OR, USA; Microscopy and Imaging Facility, University of Calgary).

2.3.4 Confirmation of capacitation status of sperm based on sperm motility patterns and phosphoprotein content

Sperm motility was evaluated with a computer assisted sperm analyzer (CASA; Sperm Vision, Minitube, Ingersoll, ON, Canada). An aliquot of the sample was loaded into a prewarmed (37 °C) Leija slide and seven fields per sample was analysed using the bovine sperm motility program. Hyperactivation during capacitation was defined by the following kinematic parameters: Amplitude of lateral head displacement (ALH) > 7 µm, linearity (LIN) < 0.65 and curvilinear velocity (VCL) > 80 µm. For evaluation of tyrosine phosphorylation, sperm were concentrated (10,000 × g, 3 min, RT) and the pellet washed (10,000 × g, 5 min) in 1 ml of PBS containing 0.2 mM Na₂VO₃. The pellet was boiled in sample buffer for 5 min at 100 °C and the supernatant used for SDS-PAGE and immunoblotting (explained in Section 2.3.7).

2.3.5 Capacitation associated changes in the total protein and cholesterol content in raft and non-raft sperm membrane fractions

Total protein concentration in both fractions were quantified using a DC protein assay
(Bio-Rad, Mississauga, ON, Canada) and cholesterol was estimated using an Amplex Red cholesterol kit (Invitrogen, Burlington, ON, Canada) as per the manufacturer’s instructions. Briefly, cholesterol and its esters present in the raft and non-raft fractions were hydrolysed by cholesterol esterase and subsequently oxidised by cholesterol oxidase to yield \( \text{H}_2\text{O}_2 \). In the presence of horseradish peroxidase, the amplex red reagent reacts with \( \text{H}_2\text{O}_2 \) to form resorufin which was detected spectrofluorometrically at excitation and emission wavelengths of 585 and 571 nm, respectively.

2.3.6 Immunoprecipitation

Pelleted raft and non-raft fractions isolated from uncapacitated and capacitated sperm were lysed with ice-cold lysis buffer containing 150 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1% Triton X-100, 60 mM octylglucoside, 1X protease inhibitor cocktail, 1 mM NaF, 1 mM \( \text{Na}_3\text{VO}_4 \) for 30 min on ice. The supernatant obtained by high-speed centrifugation (16,000 \( g \) for 15 min at 4 °C) was precleared with protein A beads for 30 min at 4 °C on a rocker. Precleared supernatants from raft and non-raft fractions of uncapacitated and capacitated groups were incubated with protein-A beads conjugated with ATP1A4 antibody (3 \( \mu \text{g/ml} \)) for 12 h at 4 °C on a rocker. After incubation, beads containing the antibody-antigen and its interacting partners of interest were pelleted by slow speed centrifugation (500 \( g \) for 1 min at 4°C). Beads were washed 3X with PBS containing 0.1% Tween-20 and proteins bound to the collected beads were eluted by boiling in sample buffer at 95 °C for 5 min. The eluted supernatant was subjected to SDS-PAGE and probed with appropriate antibodies (as indicated in Section 2.3.7).
2.3.7 Immunoblotting

Sperm proteins were separated on 10% SDS-PAGE gels and electrotransferred to nitrocellulose membranes. After blocking with 5% (w/v) skim milk in Tris buffered saline containing Tween-20 (TTBS) for 1 h, membranes were incubated with the respective antibodies overnight at 4 °C. We used the following antibodies: GM-1 (1:500); phosphotyrosine (1:10,000; Millipore, Billerica, MA, USA); p-caveolin-1 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA); p-Src (1:2500; Abcam, Toronto, ON, Canada); p-ERK1/2 (1:500; Cell Signaling Technology, Boston, MA, USA); p-EGFR (1:500; Cell Signaling Technology); affinity-purified ATP1A4 (1:10,000; custom made at the University of Calgary); caveolin-1 (1:1000; Cell Signaling Technology); Src (1:2500; Millipore); ERK1/2 (1:2500; Sigma Aldrich, Oakville, ON, Canada) and EGFR (1:1000; Santa Cruz Biotechnology). Membranes were washed three times in TTBS for 10 min and subsequently incubated with either HRP conjugated goat anti-rabbit or anti-mouse IgG for 1 h at RT. For immunoprecipitation studies, protein A HRP secondary antibody (1:10,000; Millipore), which binds to the intact IgG molecule and not to the denatured heavy and light chains of the antibody was used. Following washing, immunoreactive bands were detected using chemiluminescence. Membranes were stripped and probed with antibodies corresponding to total proteins or β-tubulin to ensure equal protein loading.

2.3.8 Statistical analyses

Data were analyzed with GraphPad software (GraphPad Software Inc, La Jolla, CA, USA). Results are shown as mean ± SEM of the number of samples indicated in each case. Results from the raft and non-raft fatty acid analysis (uncapacitated sperm), total protein, total cholesterol (uncapacitated and capacitated sperm), and phosphorylation of signaling molecules from raft and
non-raft fractions were analysed using repeated-measures ANOVA, followed by Tukey’s multiple comparison post-hoc test. Differences in sperm kinematic parameters among the control and capacitated groups were analysed by one-way ANOVA, followed by Tukey’s post-hoc test. For all analyses, P < 0.05 was considered significant.

2.4 Results

2.4.1 ATP1A4 content, distribution of raft and non-raft markers, fatty acid profile, and morphology of membrane vesicles in the raft- and non-raft membrane fractions prepared from uncapacitated sperm

Using a non-detergent method, we determined that both ATP1A4 and caveolin-1 (Figure 2.1A; upper and bottom panels, respectively) were distributed in both raft and non-raft fractions. Caveolin-1 was traditionally considered a raft marker, but due to its presence in the non-raft fraction, another raft marker, ganglioside M1 (GM-1; Figure 2.1B) was used to confirm purity of the fractions. Results demonstrated that this protein was predominantly present in the raft fraction; multiple bands indicated the presence of several GM-1 binding proteins in sperm. A non-raft marker (α-tubulin; Figure 2.1C) was detected mostly in the non-raft fraction, thereby confirming effective separation of raft and non-raft membrane fractions. Consistent with properties of lipid rafts (Simons & Toomre 2000), the content of saturated fatty acids was higher in the raft fraction, in contrast to the content of polyunsaturated fatty acids, which was higher in non-raft fractions (Figure 2.1D). Morphologically, the raft fraction had significantly larger vesicles with a mean diameter of 68.40 ± 21.41 nm (range: 10 – 110) in comparison to the non-raft fraction, which had smaller vesicles (mean diameter 23.89 ± 12.46 nm; range: 9 - 66). In
addition, vesicle morphology appeared to be relatively homogeneous in the raft fraction compared to the non-raft fraction, which had vesicles of varying sizes (Figure 2.1E and F).

Figure 2.1 ATP1A4 content, distribution of raft and non-raft markers, fatty acid profiles, and morphology of membrane vesicles in raft- and non-raft membrane fractions prepared from uncapacitated sperm. (A) Exposure of sperm to a non-detergent (sodium bicarbonate)
extraction buffer and subsequent immunoblotting of raft and non-raft fractions with anti-caveolin-1 (lower panel) and anti-ATP1A4 antibody (upper panel). The image was consolidated by removing the middle lane of the western blot (indicated by dividing line). (B) Distribution of GM1 (raft marker) binding proteins (indicated by black solid arrows) in raft and non-raft membrane fractions of uncapacitated bovine sperm. (C) Distribution of α-tubulin (non-raft marker) in raft and non-raft fractions of uncapacitated bovine sperm. (D) Fatty acid profile was characterised using gas liquid chromatography and results were shown as percentage fatty acids. (E) Raft and (F) non-raft fractions were negatively stained and visualised using transmission electron microscopy. All data were expressed as mean ± SEM (n = 5). SFA and PUFA denote saturated fatty acids and polyunsaturated fatty acids, respectively. a–cValues without a common letter differed (P < 0.05).

2.4.2 Characterization of capacitation status of sperm based on sperm motility patterns and phosphoprotein content

The percentage of total or progressively motile sperm was not different between ouabain- and heparin-capacitated groups. However, percentage of sperm undergoing hyperactivation, as evidenced by increased ALH and VCL and decreased linearity (Figure 2.2A) were higher in these groups compared to the incubation control at 4 h. A cohort of sperm proteins were also tyrosine phosphorylated in these capacitated groups compared to the incubation control 4 h (Figure 2.2B).
Figure 2.2

A

![Bar charts showing different parameters with control, Ouabain, and Heparin treatments.](image)
Figure 2.2. Characterization of sperm capacitation status based on sperm motility patterns and phosphoprotein content. (A) Motility parameters (total and progressive motility) were recorded using CASA and hyperactivation was monitored by three kinematic values including ALH, VCL, and LIN under control and capacitating conditions (ouabain, heparin) for 4 h. (B) For evaluation of tyrosine phosphorylation, total sperm homogenates were immunoblotted with anti-phosphotyrosine antibody (upper panel; indicated by black solid arrows) and reprobed with β-tubulin (lower panel) for equal protein loading. Data shown were expressed as mean ± SEM (n = 5). a,bValues without a common letter differed (P < 0.05).

2.4.3 Capacitation associated changes in the content of total protein, cholesterol in raft and non-raft sperm membrane fractions

Overall, cholesterol content of raft membrane fractions from all the groups were higher than that of non-raft membrane fractions. Although treatments did not affect cholesterol content of raft
fractions, cholesterol content of non-raft fractions from control 4 h and capacitated groups were lower than that of control 0 h (uncapacitated group; Figure 2.3A). Total protein content of raft fractions was lower than that of corresponding non-raft fractions among the groups (Figure 2.3B). Overall, there was no effect of capacitation on total protein content in these fractions.

**Figure 2.3**

(A) Total cholesterol and (B) total protein were quantified using commercial kits and expressed as units/10⁹ sperm from uncapacitated and various capacitated groups. All data were expressed as mean ± SEM (n = 5). a-bValues without a common letter differed (P < 0.05).
2.4.4 Signaling function of ATP1A4 under ouabain-induced capacitating conditions

Content of phosphorylated caveolin-1 (p-cav-1) was significantly higher in the raft fraction compared to the non-raft fractions of capacitated sperm (Figure 2.4). In contrast, content of p-ERK1/2 and p-EGFR were higher in non-rafts in comparison to raft membrane fractions of capacitated sperm (Figure 2.5 and Figure 2.6). Moreover, phosphorylated forms of these molecules were higher in ouabain- versus heparin-capacitated sperm. Although p-Src was detected in raft and non-raft fractions, ouabain-capacitated sperm had higher p-Src content in relation to its heparin counterpart in the raft fraction. However, content of p-Src was higher in non-raft fractions prepared from these capacitated groups compared to raft fraction (Figure 2.7).

Although raft-and non-raft preparations from control 4 h sperm had phosphorylated signaling molecules, their content was lower than that of ouabain- and heparin-capacitated sperm. Since there was differences in the content of unphosphorylated forms of signaling molecules across the treatment groups, content of each phosphorylated signaling molecules were normalized to its total (unphosphorylated) protein content.
Figure 2.4. Effect of capacitating conditions on caveolin-1 phosphorylation in raft and non-raft membrane fractions. (A) Representative western blot showing activation (phosphorylation) of caveolin-1 under ouabain- or heparin-induced capacitating conditions (consolidated images indicated by dividing lines; left panel). Membranes were stripped and probed with their respective antibodies for total protein to normalise the data (right panel). (B) Relative pixel intensity was calculated by normalising pixel intensity of phosphorylated protein to the pixel value of their corresponding total protein. Data shown were expressed as mean ± SEM (n = 3). a-f Values without a common letter differed (P < 0.05).
Figure 2.5

(A) Representative western blot showing activation (phosphorylation) of ERK1/2 under ouabain- or heparin-induced capacitating conditions (consolidated images indicated by dividing lines; left panel). Membranes were stripped and probed with their respective antibodies for total protein to normalise the data (right panel). (B) The relative pixel intensity was calculated by normalising the pixel intensity of phosphorylated protein to the pixel value of their corresponding total protein. Average pixel intensity of the three replicates from the raft fraction of all the groups were represented by 0. Mean pixel intensity of the non-raft fraction from control 0 h, control 4 h, ouabain 4 h and heparin 4 h were indicated by 0.7, 1.5, 2.3 and 1.8,
respectively. Data shown were expressed as mean ± SEM (n = 3). *Values without a common superscript differed (P < 0.05).

**Figure 2.6**

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<tr>
<th>A</th>
<th>p-EGFR</th>
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<td><strong>Non-raft</strong></td>
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**Figure 2.6** **Effect of capacitating conditions on EGFR phosphorylation in non-raft membrane fractions.** (A) Representative western blot showing activation (phosphorylation) of EGFR under ouabain- or heparin-induced capacitating conditions (consolidated images indicated by dividing lines; left panel). Membranes were stripped and probed with their respective antibodies for total protein to normalise the data (right panel). (B) Relative pixel intensity was calculated by normalising pixel intensity of phosphorylated protein to the pixel value of their corresponding total protein. Average pixel intensity of the three replicates from the raft and non-raft fractions of control groups, raft fraction of ouabain 4 h and heparin 4 h were represented by
Mean pixel intensity of the non-raft fraction of ouabain 4 h and heparin 4 h groups were 1.9 and 1.4, respectively. Data shown were expressed as mean ± SEM (n = 3).\textsuperscript{a-c}Values without a common superscript differed (P < 0.05).

**Figure 2.7**

**A**

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<td>Non-raft</td>
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**B**

Figure 2.7 Effect of capacitating conditions on Src phosphorylation in raft and non-raft membrane fractions. (A) Representative western blot showing activation (phosphorylation) of Src under ouabain- or heparin-induced capacitating conditions (consolidated images indicated by dividing lines; left panel). Membranes were stripped and probed with their respective antibodies for total protein to normalise data (right panel). (B) Relative pixel intensity was calculated by normalising pixel intensity of phosphorylated protein to the pixel value of their corresponding
total protein. Data shown were expressed as mean ± SEM (n = 3). Values without a common letter differed (P < 0.05).

2.4.5 Interaction of ATP1A4 with signaling molecules in the raft and non-raft membrane fractions during capacitation

Using immunoprecipitation, we identified that ATP1A4 interacted with caveolin-1 only in the raft fraction. Moreover, caveolin-1 content in the immunoprecipitate from the raft fraction of ouabain-capacitated sperm was higher compared to the rest of the groups [heparin 4 h and controls (0 h and 4 h)]. Interaction of EGFR with ATP1A4 was detected in both membrane fractions of ouabain capacitated sperm, with minimal interaction in other groups. In contrast, interaction of ATP1A4 with Src and ERK1/2 was evident only in the non-raft fraction of ouabain capacitated group. Although ATP1A4 interacted with ERK 1/2 in the non-raft fraction, the content of this protein was higher in the immunoprecipitate from the 4 h incubation control and capacitated groups compared to control 0 h (Figure 2.8).
Figure 2.8 Interaction of ATP1A4 with signaling molecules in raft and non-raft membrane fractions during capacitation. Raft and non-raft fractions were solubilized and immunoprecipitated with the ATP1A4 antibody-protein A bead slurry for 12 h and immunoblotted with the antibodies against unphosphorylated forms of caveolin-1, EGFR, Src and ERK1/2, as described.

2.5 Discussion

Involvement of Na/K-ATPase in regulation of signaling molecules in somatic cells has been well documented. However no information is available on raft and non-raft localisation of ATP1A4 in sperm and its consequences for initiation of downstream signaling pathways leading to capacitation. In this study, using a combination of membrane fractionation and immunoprecipitation techniques, we demonstrated that interaction of ouabain with ATP1A4
regulated signaling pathways localised to the raft and non-raft sperm membrane fractions. In that regard, caveolin-1 phosphorylation was higher in the raft fraction, whereas ERK1/2 and EGFR phosphorylation were higher in the non-raft fraction of sperm capacitated with ouabain. A minor Src population was activated in the raft fraction, whereas the majority was in the non-raft fraction of ouabain capacitated sperm. Subsequent immunoprecipitation studies demonstrated that ATP1A4 interacted with caveolin-1 in the raft fraction. However, an interaction of ATP1A4 with Src, EGFR and ERK1/2 was more evident in the non-raft fraction. Overall, we inferred that ATP1A4 regulated sperm capacitation through both raft- and non-raft mediated signaling.

To investigate raft-mediated ATP1A4 signaling, the first step was to extract raft and non-raft sperm membrane fractions. Since Na/K-ATPase in sperm and in other cell types was soluble in ice-cold Triton X-100 (Wang et al. 2004, Cross 2004), we used a non-detergent (sodium carbonate) based approach to separate the raft and non-raft domains and their biochemical profile was characterised as reported (Asano et al. 2009). Quite surprisingly, caveolin-1 was detected in the non-raft fraction, even though it is a traditional resident protein of caveolae. In addition, to serve as a structural protein for caveolae formation, caveolin-1 participates in a multitude of signaling processes through its scaffolding domain, facilitating movement of other proteins to and from the membrane (Collins et al. 2012, Byrne et al. 2012). Therefore, localization of this protein outside of lipid rafts suggested a potential role for this protein in cell signaling outside of lipid rafts (Head & Insel 2007, Boscher & Nabi 2012). Due to uncertainty of caveolin-1 localisation in both fractions, GM-1 (raft marker) and α-tubulin (non-raft marker) were additionally used to confirm purity of the membrane fractions, as they are classical markers of sperm raft and non-raft membrane fractions, respectively (Cross et al. 2004, Asano et al. 2009).
The greater content of SFA in the raft fractions was consistent with a more rigid raft fraction, whereas higher content of PUFA in non-raft fractions was consistent with a more fluid lipid environment (non-raft) in the plasma membrane. Subsequent ultrastructural analysis demonstrated that rafts had bigger membrane vesicles and that migration in a sucrose gradient was based on their typical lipid composition, and not on their size or morphology (Asano et al. 2009). In fresh uncapacitated sperm (control 0 h), the cholesterol content varied significantly, with greater amounts in the raft fraction and comparatively lower amounts in the non-raft fraction. In capacitated sperm, the decrease in the cholesterol content was more pronounced in the non-raft fraction, consistent with the global cholesterol efflux that occurs during capacitation. The primary function of rafts is signal transduction; therefore, they contain only a very limited subset of proteins (Simons & Toomre 2000), consistent with the lower protein content in these fractions in comparison to non-raft fractions of uncapacitated and capacitated sperm.

During capacitation, sperm undergo numerous biochemical changes, including initiation of signaling pathways before participating in fertilization. Studies have localised p-Src to various regions of capacitated sperm (human; Mitchell et al. 2008; Varano et al. 2008; mouse: Baker et al. 2006; bull: Etkovitz et al. 2009). Likewise, EGFR is involved in acrosomal exocytosis and actin remodelling in bovine sperm (Lax et al. 1994, Etkovitz et al. 2009) and MAPK pathway is involved in regulation of sperm capacitation (pig: Awda & Buhr 2010; mouse: Nixon et al. 2010; human: de Lamirande and Gagnon 2002; Thundathil et al. 2003). Lipid rafts act as a signaling hub in sperm and in other cell types, as co-localization of multiple proteins within a restricted space is more conducive to initiate a specific signaling process than random distribution of molecules in the plasma membrane (Liu et al. 2003). However, non-raft domains also contain multiple proteins that could have critical roles in signal transduction upon interaction with
receptors, signaling molecules and ligands through protein-protein interactions (Simons & Ikonen 1997). For example, in cardiac myocytes and smooth muscle cells, Na/K-ATPase signaling was not only limited to the raft pool but also occurred in the non-raft portion of the plasma membrane (Liu et al. 2011). Likewise, differential distribution of molecules have been reported for GliPr1L1 (Glioma pathogenesis-related 1-like protein 1 (Caballero et al. 2012) and adenylate kinase 1 (Girouard et al. 2008) among raft and non-raft domains in bovine epididymal sperm.

We detected differences in the distribution of phosphorylated signaling molecules in raft and non-raft fractions during ouabain-mediated sperm capacitation through immunoblotting studies. In somatic cells where ATP1A1 predominates, there was a time-dependent increase in phosphorylation of signaling molecules that was noticed in the raft fraction after 5 to 15 min of ouabain exposure, suggesting that rafts act as a signaling centre for ATP1A1-ouabain signaling. Consistent with this, caveolin-1 and a minor Src population were phosphorylated in the raft fraction of ouabain capacitated sperm, whereas the remainder of molecules (EGFR, ERK1/2) including Src were predominantly phosphorylated in the non-raft fraction. Overall, ATP1A4 signaling in sperm had some similarities to ATP1A1 signaling in somatic cells (raft signaling). However, the majority of signaling events that occurred during sperm capacitation were mediated through non-rafts, suggesting that ATP1A4 may exhibit different roles depending on the cell type and neighbouring proteins in the cell. Protein tyrosine phosphorylation is a crucial event during capacitation, which is maintained by a balanced act between kinases and phosphatases in sperm (Signorelli et al. 2012). Since capacitation in bovine sperm occurs over a 4 h interval, it is possible that active phosphorylation and dephosphorylation events could change involvement of downstream signaling molecules that was recorded at the end of the capacitation
period. Furthermore, signaling molecules could move out of the rafts and be recruited into rafts from other regions of the plasma membrane, as in the case of EGF receptors which rapidly move out of rafts (Mineo et al. 1999), whereas insulin receptors are recruited into rafts upon ligand binding (Vainio et al. 2002). This dynamic movement of molecules between various compartments of the plasma membrane could add to the complexity of interpreting raft and non-raft signaling pathways mediated by ATP1A4 during capacitation.

Protein recruitment to caveolae/rafts and subsequent interaction with caveolin is believed to be mediated via a ∼20 amino acid N-terminal region on the caveolin molecule known as the caveolin scaffolding domain (CSD) and an aromatic-rich caveolin binding motif (CBM) on the associated protein. The ubiquitous ATP1A1 isoform directly interacts with caveolin-1 mediated through putative CBM located at the N- and C-terminus (Cai et al. 2008, Byrne et al. 2012). Although ATP1A4 shares only 80% homology with the ATP1A1 subunit, perhaps this sperm specific isoform could interact directly with caveolin-1 through its CBM domain in the same manner as ATP1A1-caveolin-1 interaction, or through regions independent of a typical CBM. Studies have highlighted that atypical CBM could also facilitate caveolin interactions, for example, binding of the DNA-binding protein inhibitor, ID-1, to caveolin-1 occurs via a helix-loop-helix domain, a region devoid of CBM (Zhang et al. 2007). Additionally, the kinase domain of EGFR has a CBM, which mediates its interaction with caveolin-1 (Couet et al. 1997) consistent with EGFR interaction in the raft fraction.

The interaction between ATP1A4 and caveolin-1 could recruit and activate other CBM containing molecules such as PLC and PKC which are involved in calcium release and F-actin formation, respectively. In somatic cells, PLC increases hydrolysis of PIP2, generating IP3 which can activate IP3R, and release intracellular Ca^{2+} (Yuan et al. 2005). In addition, activation of
PLC also generates DAG, which activates PKC. PKC subsequently hydrolyses phosphatidylcholine (PC) to phosphatidic acid (PA), mediating polymerisation of G-actin to F-actin. Increase in intracellular calcium and F-actin formation are hallmark features of sperm undergoing capacitation.

In somatic cells, the ubiquitous ATP1A1 directly interacts with Src through specific domain-domain interactions and keeps Src in an inactivated state (Tian et al. 2006). Binding of ouabain with ATP1A1 causes conformational changes in ATP1A1 which activates Src. Activated Src could trigger phosphorylation of EGFR, which is capable of recruiting adaptor proteins such as growth factor receptor-bound protein 2 (Grb2), son of sevenless (SOS) and Rat sarcoma protein (Ras), resulting in assembly and activation of Ras/Raf/ERK 1/2 cascade. Consistent with Src interaction in the non-raft fraction, we confirmed that EGFR and ERK1/2 interaction also occurred in the same fraction. Even though the presence of adaptor proteins wasn't investigated in this study, others have reported that Shc, Grb2, Ras, Raf are present in sperm (de Lamirande & Gagnon 2002). Perhaps EGFR acts through these proteins to initiate the MAPK cascade during sperm capacitation. That EGFR could activate ERK1/2 pathway outside of rafts suggested that these membrane domains may not be absolutely critical for activating this pathway. Activation of ERK1/2 induces tyrosine phosphorylation in sperm, yet another characteristic feature of capacitation, via PTK (O'Flaherty et al. 2006). A proposed model regarding potential raft and non-raft mediated ATP1A4 signaling pathways are shown (Figure 2.9). Based on immunoprecipitation studies, it is reasonable to assume that the increased interaction between ATP1A4 and signaling molecules (total) in presence of ouabain could have led to a corresponding increase in the phosphoprotein content of these signaling molecules, as evident from immunoblotting studies.
In conclusion, we inferred that the raft and the non-raft pools of ATP1A4 may regulate distinct downstream signaling events, which are necessary to support capacitation and fertilizing ability of bovine sperm. During spermatogenesis, sperm acquires specific proteins, for example, testis-specific lactate dehydrogenase (LDH-C4), testis-specific GAPDH, testis specific angiotensin converting enzyme (tACE), which are not present in any other cell of the body, perhaps interacting partners of ATP1A4 are different from their somatic cell counterparts. Therefore, future studies are aimed at investigating the ATP1A4 interactome in the raft and non-raft fraction by immunoprecipitation- mass spectrometry-based approaches.
Figure 2.9 A hypothetical model for ATP1A4-mediated raft- and non-raft signaling pathways during bovine sperm capacitation. This model is based on current knowledge regarding the role of ATP1A1 as a signaling molecule in somatic cells, and available data on involvement of ATP1A4 in bovine sperm capacitation. The two (raft and non-raft) pools of ATP1A4 could trigger distinct downstream pathways during sperm capacitation. In the raft, ouabain signaling involves ATP1A4-caveolin-1-EGFR complex which could bind and activate PLC, thereby increasing hydrolysis of PIP$_2$, generating IP$_3$ and DAG, which in turn activates PKC. IP$_3$ binds to IP$_3$R causing an increase in intracellular calcium, whereas PKC mediates polymerisation of G-actin to F-actin through other mediator proteins. Within non-raft, ATP1A4 signaling activates ERK1/2, leading to protein tyrosine kinase (PTK) mediated tyrosine phosphorylation of proteins. Increase in F-actin, intracellular calcium and protein tyrosine phosphorylation contribute to capacitation-associated changes in sperm.
Chapter Three: Characterization of the testis-specific isoform of Na/K-ATPase (ATP1A4) interactome in raft and non-raft membrane fractions from capacitated bovine sperm

3.1 Abstract

Sperm plasma membrane consists of highly dynamic lipid microdomains (rafts), which harbour a cohort of signaling proteins involved in regulation of sperm capacitation. We previously reported that incubation of bovine sperm with ouabain induced sperm capacitation through signaling mechanisms involving kinases and that two distinct pools of ATP1A4 were localised in raft and non-raft fractions of the sperm plasma membrane. The objective of the present study was to investigate the ATP1A4 interactome in membrane rafts and non-rafs isolated from capacitated bovine sperm. Rafts and non-raft fractions were prepared from uncapacitated and capacitated bovine sperm using a non-detergent based approach. Proteomic analysis using a gel-based LC-MS/MS approach identified that the non-raft interactome comprised of hexokinase, plakophilin-1, 14-3-3 protein, cathepsin D, and heat shock protein. A disintegrin and metalloprotease (ADAM) and annexin A2 were exclusive to the raft fraction, whereas actin and plakoglobin were identified in both raft and non-raft fractions of ouabain capacitated sperm. Based on gene ontology, these differentially interacted proteins are putatively involved in sperm-oocyte interaction, metabolism, protease activity and act as adaptor and cytoskeletal proteins. Significant differences in plakoglobin-, hexokinase-, and actin- interaction with ATP1A4 across treatments were subsequently validated using immunoblotting and immunocytochemical studies. Overall, we identified novel proteins that interacted with ATP1A4 and provided new insights regarding their potential functions in mammalian fertilization.
3.2 Introduction

Ejaculated sperm must remain in the female reproductive tract for a species-dependent interval to attain fertilizing ability; these maturational changes are collectively known as sperm capacitation (Yanagimachi 1994). Capacitation-associated changes including hyperactivated motility, tyrosine phosphorylation and other physiological events are attributed to changes in the intracellular ionic environment and initiation of several signaling cascades. However, specific roles of individual sperm proteins in regulation of sperm capacitation are not well characterized.

ATP1A4 serves two functions in sperm, including a classical function as an ion transporter/enzyme and a non-classical function as a signaling molecule. Most of the signaling function has been attributed to Na/K-ATPase localised in raft microdomains, due to its proximity to other signaling molecules within the raft microenvironment (Liu et al. 2003, Liang et al. 2007). However, non-raft domains also contain multiple proteins that could have critical roles in signal transduction upon interaction with receptors, signaling molecules and ligands through protein-protein interactions (Simons & Ikonen 1997). In that regard, a recent study identified that the signaling function of Na/K-ATPase was not only limited to the raft pool and that the non-raft domain contained various signaling molecules, including Src, EGFR, PI3K, ERK1/2 and Akt (Liu et al. 2011). Sperm plasma membrane is also enriched in rafts and contains a cohort of molecules that could be involved in sperm-oocyte interaction (Cross 2004, Shadan et al. 2004, Sleight et al. 2005, Bou Khalil et al. 2006, Nixon & Aitken 2009). Asano (Asano et al. 2009) identified non-raft domains in sperm, but nothing is known about their functional importance in sperm capacitation or in subsequent steps leading to fertilization. Furthermore ouabain is secreted from the bovine adrenal gland and is present in bovine vaginal fluid (Daniel et al. 2010).
Therefore, the presence of ATP1A4 in sperm and ouabain in the female reproductive tract suggests that this protein has a specific role in sperm physiology.

Based on studies in somatic cells, Na/K-ATPase is capable of forming multiple protein-protein interactions mediated via ATP1A1. In the previous chapter, we highlighted that there are two distinct pools (raft and non-raft) of ATP1A4 which trigger specific downstream signaling pathways. The process of sperm development is a unique phenomenon and probably one of the most complex differentiation processes in higher eukaryotes. During spermatogenesis, sperm acquire several proteins or their isoforms that are unique and cell-specific to meet functional demands of the cell, including lactate dehydrogenase (LDH-C4), sperm adhesion molecule 1 (PH-20), and testis-specific isoform of Angiotensin Converting Enzyme (t-ACE). Therefore, we hypothesized that ATP1A4 interacts with novel sperm proteins during capacitation. To test this hypothesis, we compared (immunoprecipitation-mass spectrometry) the interactome profile of ATP1A4 between raft- and non-raft membrane fractions prepared from capacitated sperm and proposed a potential role for ATP1A4 in sperm-oocyte interaction.

3.3 Materials and methods

3.3.1 Semen collection, preparation of reagents, preparation of raft and non-raft fractions from uncapacitated and capacitated sperm

Please refer to Chapter 2, Sections 2.3.1 and 2.3.2 for detailed experimental procedures.

3.3.2 Immunoprecipitation and SDS-PAGE

Raft and non-raft fractions isolated from uncapacitated and capacitated sperm were pelleted and lysed with ice-cold lysis buffer containing 150 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1% Triton
X-100, 60 mM octylglucoside, 1X protease inhibitor cocktail, 1 mM NaF, 1 mM Na₃Vo₄ for 30 min on ice. The supernatant that was obtained by high-speed centrifugation (16,000 g for 15 min at 4 °C) was precleared with protein A beads for 30 min at 4 °C on a rocker. Simultaneously, ATP1A4 antibody (3 µg/ml) was incubated with the protein-A bead slurry for 1 h on a rocker at 4 °C before the cross-linking procedure following the manufacturer’s instructions (Thermo Fisher Scientific). Unbound antibody was washed off the beads and incubated with conjugation buffer (20 mM Na₃PO₄, pH 7.4) containing BS³ for 30 min at RT. The reaction was terminated by quenching with 1 M Tris, pH 7.5 for 15 min at RT. Precleared supernatant from raft and non-raft fractions of control and capacitated groups were incubated with the cross-linked antibody-protein A bead slurry for 12 h at 4 °C on a rocker. At the end of the incubation, the beads containing the antibody-antigen and its interacting partners of interest were pelleted by slow speed centrifugation (500 g for 1 min at 4 °C). Beads were washed three times with PBS containing 0.1% Tween-20 and proteins bound to the collected beads were eluted by adding 100 mM glycine pH 2.5 to the beads with gentle rocking at RT for 10 min. This elution was done twice and combined supernatants were neutralized with 1 M Tris pH 10. The eluted supernatant was subjected to SDS-PAGE and stained with Coomassie Brilliant Blue G-250.

3.3.3 Protein digestion

All steps, including in-gel trypsin digestion, LC-MS/MS analysis and database search for protein identification in the raft and non-raft fractions were performed at the mass spectrometric facility, SAMS centre, University of Calgary. Coomassie-stained protein gel bands were cut and sliced into small pieces (~1 mm³). These gel pieces were resuspended in 50 mM ammonium bicarbonate/ acetonitrile (50:50, v/v) before being reduced with 10 mM dithiothreitol for 30 min
at 56 °C and alkylated with iodoacetamide for 30 min in the dark at room temperature. Trypsin digestion was performed for 16 h at 37 °C. After digestion, the supernatant (tryptic peptides) were transferred into a new tube containing 5 µl of acidifying solution (acetonitrile/water/10% trifluoroacetic acid (60:30:10, v/v). Samples were then lyophilized and resuspended in 10 µl of 1% formic acid in water.

3.3.4 LC-MS/MS analysis

Tryptic peptides were analyzed by liquid chromatography (LC; Agilent 1260 Infinity chip cube interface) tandem mass spectrometry (MS/MS) on an Agilent 6550 iFunnel quadrupole (Q)-time-of-flight (TOF) mass spectrometer. The LC and the Q-TOF were both controlled by MassHunter (version B.05.00). The capillary pump used for loading the sample was run using A1 (97% water, 2.9% acetonitrile, 0.1% formic acid) and B1 (90% acetonitrile, 9.9% water; 0.1% formic acid) solutions whereas the nanopump used to generate the gradient for peptide elution, was run with A1 (97% water, 2.9% acetonitrile, 0.1% formic acid) and B1 (97% acetonitrile, 2.9% water; 0.1% formic acid) solutions. Tryptic peptides (1 µl) were loaded onto a C18 trap column of an Agilent chip operating in enrichment mode at a flow rate of 2.5 µl/min and 3% B1. Once the sample loaded on the enrichment column, the chip valve was switched from enrichment to analysis mode and elution of the peptides was performed using a 25 min linear gradient from 3 to 50% B1 generated by the nanopump operated at 0.3 µl/min. Peptides were electrosprayed into the Q-TOF which was operated in positive auto MS/MS mode. The precursor ions with a m/z comprised between 275 and 1700 were acquired at a scan rate of 250 ms/spectrum and the 10 most abundant precursors for each cycle having a charge higher than 1, an intensity of at least 1000 counts and a peptidic isotopic model fragmented by collision
induced dissociation (CID). Fragment ions having an m/z between 50 and 1700 were acquired at a scan rate of 333.3 ms/spectrum.

3.3.5 Database search

The generated LC/MS-MS data files (*.d file) were imported into Agilent MassHunter qualitative analysis software (version B.05.00) and converted into a Mascot Generic Format (MGF) file using default parameters. MGF file was used to search the UniProt database using Mascot algorithm (Matrix Sciences; version 2.4). Search parameters for MS data included *Bos taurus* taxonomy, trypsin as enzyme, a maximum number of missed cleavage of 1, cysteine carbamidomethylation as fixed modification, methionine oxidation as variable modification and a mass error tolerance of 0.2 Da for both the MS and MS/MS data. Only peptides with a Mascot expectation value of < 0.05 and Mascot ion score of 23 were kept for further analysis.

3.3.6 Criteria for protein identification

Scaffold 4 (Proteome Software Inc., Portland, OR, USA) was used to validate identified peptides and proteins. Peptide identifications were accepted if they could be established at >95% probability as specified by the Peptide Prophet algorithm. Protein identifications were accepted if they could be established at >95% probability and contained at least two identified peptides. Common contaminants (such as keratins) and other non-specifically eluted proteins were manually excluded from subsequent analyses.
3.3.7 Western blotting

Immunoprecipitated elute from the raft and non-raft fractions of control and capacitated sperm were separated on 10% PAGE gels and electrotransferred to nitrocellulose membranes. After blocking with 3% (w/v) skim milk in TTBS for 1 h, the membrane was incubated with hexokinase (1:2000; Novus Biologicals, ON, Canada), plakoglobin (1:100; Sigma Aldrich, Oakville, ON, Canada), actin (1:500; Santa Cruz Biotechnology, TX, USA), phosphotyrosine (1:10,000; Millipore) and β-tubulin (1:10,000; Sigma) antibodies overnight at 4 °C. Membranes were washed three times in TTBS for 10 min and subsequently incubated with respective HRP conjugated goat anti-rabbit and anti-mouse IgG (1:5,000) for 1 h at RT. Following washing, immunoreactive bands were detected using chemiluminescence.

3.3.8 Colocalisation of plakoglobin and PLCζ with ATP1A4 in capacitated sperm

For immunostaining, uncapacitated and capacitated sperm were adhered onto poly-l-lysine coated slides and subsequently fixed with 2.5% PFA for 15 min, permeabilized with 0.2% Tween-20 for 10 min, followed by washing in PBS and incubation with 10% serum for blocking depending on the source of the secondary antibodies. Co-incubation of ATP1A4 (1:100) with either plakoglobin (1:20) or PLCζ (1:20; Santa Cruz Biotechnology) antibodies were performed in 1% serum overnight at 4 °C. Following washing in PBS, chicken anti-rabbit alexa 488 (ATP1A4), goat anti-mouse Cy3 (plakoglobin), and donkey anti-goat Cy3 (PLCζ) secondary antibodies were added at 1:200 for 1 h at RT. Cells were washed in PBS and mounted with Vectashield (Vector laboratories) containing DAPI and stored at –20 °C pending further imaging.
3.3.9 Flow cytometric analysis of F-actin in sperm

Staining of F-actin filaments were performed based on the manufacturer’s instructions (Thermo Fisher Scientific). Briefly, at the end of capacitation, control and ouabain treated sperm were washed with PBS and 1 µl of Fixable Live and Dead Cell Stain (Thermo Fisher Scientific) was added for 30 min at RT. Subsequently, sperm were fixed with 4% PFA for 10 min, permeabilized with 0.1% Triton X-100 for 5 min followed by washing in PBS and incubated in PBS containing 1% BSA and FITC-phalloidin (0.33 µM; Thermo Fisher Scientific) for 1 h. Cells were washed and data were acquired using an Attune Acoustic Focusing Cytometer (BD Biosciences, Mississauga, ON, Canada). The excitation source was a 488 nm laser for FITC and a 405 nm laser for the fixable live and dead (violet) dye. Voltage settings used were as follows: FSC – 1250, SSC – 1650, FITC – 1500, violet – 1650. Subsequently, detector 1 (emission range of 450 ± 20 nm) was used for detecting violet fluorescence (viability) and detector 2 (emission range of 530 ± 15 nm) was used for detecting green (FITC-phalloidin) fluorescence. A total of 20 x 10³ events were recorded in the form of a scatter plot and histogram.

3.3.10 Statistical analysis

Results are shown as mean ± SEM of the number of samples indicated in each case. Proteomic analysis was done on raft and non-raft fractions isolated from control and ouabain capacitated sperm obtained from three bulls. Since expression levels of certain proteins were zero, fold change was calculated as the difference between the log2 values of spectral counts from control and ouabain capacitated sperm. Significant differences in fold change values were analysed by Student’s t-test. All data were analyzed with commercial statistical software (GraphPad Software Inc., La Jolla, CA, USA) and P < 0.05 was considered significant.
3.4 Results

3.4.1 Identification of ATP1A4 interacting partners by mass spectrometry

Hyperactivated motility and tyrosine phosphorylation, hallmark features of capacitated sperm, were confirmed as detailed in Chapter 2. The ATP1A4 interactomes from the raft and non-raft fractions of uncapacitated and capacitated sperm were compared using LC-MS/MS analysis. Raw data regarding protein identities, and their probability scores and peptide sequences (>95% confidence level) in raft and non-raft fractions were obtained from Mascot (Table 3.1 and Table 3.2). Scaffold subsequently identified four (raft) and seven (non-raft) differentially interacted proteins between control and ouabain capacitated sperm, based on a peptide threshold set at 95% and a minimum of two peptides (Table 3.3). Based on gene ontology, raft ATP1A4 interactome comprised of ADAM (endopeptidase), annexin A2 (calcium dependent phospholipid binding protein), plakoglobin (cell-cell adhesion) and actin (cytoskeletal component). These four functional classes of proteins predominantly interacted and contributed equally (25% each) to the ATP1A4 interactome (Figure 3.1A). Non-raft ATP1A4 interactome consisted of plakoglobin, plakophilin (cell-cell adhesion), heat shock protein (chaperone), hexokinase (metabolic enzyme), 14-3-3 ζ/δ (adaptor protein), and cathepsin D (protease). Proteins involved in cell-cell adhesion were the predominant category (28%) that immunoprecipitated with ATP1A4 in the non-raft fraction. Compared with raft fraction, adaptor protein, chaperone, metabolic enzyme, cytoskeletal component, and protease were additional categories that were noticed in the non-raft fraction (Figure 3.1B). These new categories of interacting partners contributed equally (14.29%) to the non-raft interactome (Figure 3.1B). Overall, 64% of differentially interacted proteins were exclusive to the non-raft fraction, 18% were identifiable only in the raft fraction and the remainder (18%) was common to both fractions.
Table 3.1: Data from Mascot showing the identity of proteins, their probability scores (protein score and expect score) and peptide sequences (>95% confidence) in the raft fraction.

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<th>Protein name</th>
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<th>Peptide sequences</th>
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Table 3.2: Data from Mascot showing the identity of proteins, their probability scores (protein score and expect score) and peptide sequences (>95% confidence) in the non-raft fraction.

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Table 3.3: Spectral counts of differentially interacted proteins (control vs ouabain-capacitated sperm) in raft and non-raft membrane fractions

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<th>Fold change (Log2)</th>
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<td>(non-raft interactome)</td>
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</tr>
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3.4.2 Validation of mass spectrometry data for selected candidate proteins

Although the list of interacting partners represents high confident proteins, validation was performed on significant protein hits using their respective antibodies. Non-raft protein, hexokinase and raft proteins, plakoglobin and actin that interacted with ATP1A4 were chosen for validating mass spectrometry data, based on their significant interaction in ouabain capacitated sperm. Based on spectral counts, plakoglobin was equally distributed between raft and non-raft fractions. However, immunoblotting on raft and non-raft immunoprecipitates indicated that plakoglobin was restricted to the raft fraction (Figure 3.2A). Plakoglobin was localised to the equatorial segment of uncapacitated sperm and capacitated sperm. Although the ATP1A4 signal was predominantly localised to the anterior acrosome in control sperm, it become pronounced in the equatorial segment, and postacrosome region in capacitated sperm (Figure 3.2C). Presence of hexokinase was more evident in the non-raft fraction compared to the raft fraction of ouabain capacitated sperm and no interaction was apparent in the control group, consistent with their spectral counts (Figure 3.2A). Increased amount of actin was detected in the raft fraction as compared to the non-raft fraction of ouabain capacitated sperm, consistent with the spectral counts (Figure 3.2A). We also confirmed the formation of F-actin in control and capacitated sperm by flow cytometry. Relative content of F-actin (based on intensity of FITC-phalloidin fluorescence) was higher in ouabain-capacitated sperm, compared to uncapacitated sperm (Figure 3.2B), consistent with results of immunoblotting.
Figure 3.1 Potential functions of differentially interacted proteins (control vs ouabain-capacitated sperm) in raft and non-raft membrane fractions. (A) and (B) correspond to the percentage contribution of each protein and its function that differentially interacted with ATP1A4 in the rafts and non-rafts, respectively.

Figure 3.2

<table>
<thead>
<tr>
<th></th>
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<tr>
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<tr>
<td>Non-Raft</td>
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<td>kDa 103</td>
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<tr>
<td>kDa 82</td>
<td>IB: Hexokinase</td>
<td>IB: Plakoglobin</td>
</tr>
<tr>
<td>kDa 42</td>
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</table>
B

Unstained sperm
Control 4 h sperm
Ouabain 4 h sperm

C

ATP1A4  Plakoglobin  Nucleus  Merged

Control 4 h

Ouabain 4 h
Figure 3.2 Validation of selected ATP1A4 interactomes and ATP1A4-plakoglobin interaction during sperm capacitation. (A) Raft and non-raft membrane fractions were solubilized and immunoprecipitated with the crosslinked ATP1A4 antibody-protein A bead slurry for 12 h and immunoblotted against the unphosphorylated forms of the antibodies as described. (B) Capacitated sperm were labelled with FITC-phalloidin and levels of fluorescence were recorded (flow cytometer) and a representative fluorescence intensity histogram is shown. (C) Representative images of ATP1A4 (green), plakoglobin (red), nuclei (blue) and merged plakoglobin, ATP1A4 and DAPI staining in control and ouabain capacitated sperm.

3.5 Discussion

We applied a proteomic approach to investigate profiles of ATP1A4 interacting partners in the raft and non-raft membrane fractions from uncapacitated and capacitated sperm. Non-raft interactome was comprised of hexokinase, plakophilin-1, 14-3-3 protein, cathepsin D, and heat shock proteins, whereas ADAM and annexin A2 were exclusive to the raft fraction. Actin and plakoglobin were identified in both raft and non-raft fractions of ouabain capacitated sperm. These differentially interacted proteins were putatively involved in sperm-oocyte interaction, metabolism, protease activity and act as adaptor and cytoskeletal proteins. Subsequent validation on plakoglobin indicated that this protein was localised to the rafts and to the equatorial segment of ouabain capacitated sperm. Interaction of ATP1A4 with hexokinase was more evident in the non-raft fraction whereas interaction with actin was noticed in the raft fraction and highest amount of F-actin fluorescence was recorded in ouabain capacitated sperm.

Label-free methodologies, e.g. spectral count, have gained acceptance as a semi-quantitative approach to measure protein abundance in proteomic studies and had strong linear correlation with relative protein abundance with a dynamic range more than two orders of
magnitude (Liu et al. 2004, Lundgren et al. 2010). Spectra count is defined as the total number of spectra/peptide sequences (recognized by intensity versus mass to charge ratio plot of aminoacids detected in a peptide sequence) identified for a particular protein. Using this approach, relative protein quantification is achieved by comparing the number of identified MS/MS spectra for the same protein across experimental groups. This is possible because an increase in protein abundance typically results in an increase in the number of its proteolytic peptides, which results in an increase in protein sequence coverage, the number of identified unique peptides, and the number of identified total MS/MS spectra (spectral count) for each protein (Washburn et al. 2001). Therefore, we used spectral count as a reliable index for relative protein quantification to assess fold changes in ATP1A4 interacting partners in raft and non-raft fractions isolated from control and ouabain capacitated sperm. In the previous chapter (Chapter 2) we identified signaling molecules that interacted with ATP1A4 using a candidate molecule approach, however, none of them were identified by mass spectrometry. A possible reason is that all of the candidate molecules were either kinases or substrates of kinases which either undergo autophosphorylation (transfer of highly charged PO₄⁻ groups from ATP) or act as downstream targets. Generally charged peptides are poorly ionised by electrospray MS unless phospho-protein enrichment strategies were followed (Mann et al. 2002). Other factors to consider include efficient extraction of the peptides and preparation of the sample for MS while trying to minimize the presence of salts or detergents may also adversely affect ionization of kinases (Baldwin 2004). Sometimes, peptides may arise from proteolysis but simply not be detected, or it may have an unexpected mass due to one or more modifications, or the residues may indeed be present in the sequence but the proteolysis may not proceed as anticipated. Since mass spectrometry is a multi-step complex procedure, the information derived will depend on the
nature and complexity of the sample, the actual mass spectrometry technology and the particular search engine that was used to identify the peptide hits (Baldwin 2004). All these factors could have affected the overall results and contributed to the absence of the candidate proteins (kinases) that interacted with ATP1A4 as discussed in the previous chapter.

During fertilization, capacitated and acrosome reacted sperm penetrate the zona pellucida, enter the perivitelline space and is followed by an adhesion step where an initial contact is made between sperm and egg plasma membrane. This step is required for sperm-egg fusion, which is initiated by fusion between the equatorial segment (ES) of the sperm head and the microvilli present on the oolemma (plasma membrane of oocyte; (Yanagimachi 1994). Oocyte integrins and their respective sperm ligands (ADAM family of proteins), IZUMO and CD9 are well-characterised proteins in mediating the above-mentioned process (Mitamura et al. 1992, Inoue et al. 2005).

In epithelial cells, the process of cell-cell adhesion is facilitated by E-cadherin, where their extracellular domain forms homo and heterophilic bonds with cadherins on adjacent cells; these bonds are further strengthened by interactions between the cytoplasmic domain of E-cadherin and actin cytoskeleton (Yamada et al. 2005). The γ-catenin (plakoglobin) binds to the cytoplasmic domain of E-cadherin, whereas α-catenin binds to γ-catenin (Piepenhagen & Nelson 1993). It has been demonstrated that E-cadherin and catenin proteins are expressed in sperm and oocytes (Rufas et al. 2000, De Vries et al. 2004) where they are specifically localised to the ES of the sperm head and microvillar region on oolemma (Takezawa et al. 2011). If gamete interaction involves mechanisms that are similar to epithelial cell adhesion, it is likely that these proteins are involved in events leading to sperm-oocyte adhesion and fusion considering their strategic location in sperm and in oocytes. Mass spectrometry revealed that plakoglobin was
localized to both the raft and non-raft fractions but subsequent validation on ouabain capacitated sperm indicated that this protein interacted only in rafts, suggesting the importance of the raft microdomain in gamete interaction. Generally, proteome analysis involves detection of peptide sequences which could be unique to a particular protein or shared across proteins. It is possible that the mass spectrometric identification of peptide sequences were not specific to plakoglobin in the non-raft fraction. Therefore, when we used a specific antibody against plakoglobin we weren’t able to identify this protein in the non-raft fraction. Plakoglobin and ATP1A4 were colocalised in the equatorial segment in ouabain capacitated sperm. Since plakoglobin belongs to the catenin family, it is reasonable to assume that ATP1A4-plakoglobin interaction would be involved in gamete fusion, leading to fertilization.

Apart from facilitating physical attachment during sperm-oocyte fusion process, interaction of ATP1A4 with its binding partners may have key functional roles in oocyte activation following gamete fusion. During sperm-oocyte fusion, a soluble oocyte activation factor (SOAF) is released into the oocyte cytoplasm from sperm, which elicits calcium oscillations leading to oocyte activation. Two potential sperm proteins, PLCζ and post acrosomal WW binding protein (PAWP) are considered as SOAF candidates. The IP3-IP3R mediated calcium release in oocytes is dependent upon a sperm-based PLCζ released during fusion events (Saunders et al. 2002). In somatic cells, ATP1A1-ouabain interaction induces PLCγ phosphorylation and calcium release via activation of IP3 receptors. In sperm, the PLC isoform (PLCζ) was localised to the equatorial segment (Cooney et al. 2010). ATP1A4 colocalised with PLCζ at the ES and postacrosome region in ouabain capacitated sperm (Figure 3.3) and that ATP1A4-plakoglobin-E-cadherin interaction could facilitate entry of activated PLCζ into the oocyte following gamete fusion.
The adhesion process is also facilitated by the trans-dimerization of the integral proteins via interactions between their extracellular domains (Hartsock & Nelson 2008). In that regard, the N-glycan mediated interaction of the β-subunit of Na/K-ATPase could contribute to cell adhesion by binding to its respective subunit in neighbouring cells. Since β1, and β3 subunits are demonstrated in sperm, it is likely that it interacts with its corresponding subunit that is expressed in the oolemma (De Sousa et al. 1997), thereby facilitating cell-cell adhesion independent of the E-cadherin pathway. A proposed model involving plakoglobin, α and β subunits of ATP1A4, E-cadherin and PLCζ is shown in Figure 3.4. Further research is required to elucidate the physiological relevance of this interaction.

Heat shock proteins (HSP; chaperones) have well established roles in de novo folding of proteins, assembly of multiprotein complexes, prevention of protein aggregation (Hendrick & Hartl 1993, Neuer et al. 2000) and delivery of proteins (protein trafficking) to their respective intracellular domain. In that regard, HSP 70 binds directly with Na/K-ATPase and facilitates its movement to the apical domain from cytoskeletal attachments following renal cell injury (Riordan et al. 2006). In sperm, perhaps redistribution of ATP1A4 from the anterior acrosome in uncapacitated sperm to the equatorial segment and postacrosomal region during capacitation is mediated by HSPs.

The 14-3-3 (YWHA) family of proteins act as adaptor proteins and appear to regulate diverse physiological events such as cytoskeletal rearrangements, metabolism, cell cycle control, apoptosis, stress response, and gene expression (Aitken 2006). The 14-3-3 proteins regulate intracellular signaling pathways via their interactions with molecules containing phosphoserine and phosphothreonine residues such as PKC, Raf-1 kinases and phosphatidylinositol 3 kinase [PI3-kinase; (Robinson et al. 1994, Li et al. 1995)]. In opossum kidney (OK) cells, 14-3-3
protein interacts with the phosphorylated Na/K-ATPase α-subunit (ser 18) and forms an anchor for subsequent binding of PI3K to the N-terminus of the phosphorylated α-subunit (Efendiev et al. 2005). In our study, PI3K was identified as a significant hit, but didn’t show up as a differentially interacting protein due to the subsequent threshold cut-offs applied for peptide and protein identification. In sperm, PI3K catalyses formation of PIP₃ (phosphatidylinositol 3,4,5-triphosphate) from PIP₂ which acts as a cofactor for PLD (phospholipase D) activation and in turn mediates F-actin formation from G-actin (Etkovitz et al. 2007). We used a phalloidin probe to stabilize F-actin levels in sperm and determined their content by flow cytometry. Content of F-actin was higher in ouabain capacitated sperm, demonstrated by movement of the histogram along the X axis to the right. It has been documented that F-actin creates a scaffold between the plasma membrane and outer acrosomal membrane of the sperm, immobilizing molecules like PLC during capacitation (Brener et al. 2003, Breitbart et al. 2005).

In addition to the above described 14-3-3 protein-ATP1A4-PI3K-PLD mechanism, other pathways may also contribute to capacitation-associated F-actin polymerization. The Rho family of GTPases (an upstream activator of PLD) in bull sperm may be an additional link between Na/K-ATPase and actin polymerization (Ducummon & Berger 2006). Annexins are a group of calcium-dependent phospholipid binding proteins with high affinity towards PIP₂ and similar to YWHA family, they act as a linker between Na/K-ATPase and actin (Kimura et al. 2007, Skrahina et al. 2008). In pig kidney outer medulla, annexin A2 tetramers and subunits of Na/K-ATPase were part of a large multiprotein complex residing in rafts located at the interface between two adjacent renal cells (Liu et al. 2011). In conclusion, it is likely that F-actin formation occurs during ouabain-induced capacitation through pathways involving 14-3-3, RhoA GTPase and annexin proteins.
Identification of an interaction between ATP1A4 and proteases (cathepsin D) was intriguing. We observed a high intensity of ATP1A4 fluorescence in the ES and postacrosomal region of the sperm head compared to anterior acrosomal region after capacitation. Although the exact mechanism behind this observation remains unknown, one possibility is translocation of ATP1A4 from the anterior acrosomal region to the ES and postacrosomal region. To accomplish this, the protein must be dissociated from its cytoskeletal anchorage. The protease calpain is able to hydrolyze a variety of proteins such as spectrin and ankyrin (Suzuki & Sorimachi 1998), which are capable of binding with ATP1A4 and securing it to the actin cytoskeleton. In sperm, calpain relocated from cytoplasm to plasma membrane where it could interact with spectrin, leading to acrosome reaction (Bastian et al. 2010). Similar proteolytic mechanisms could operate during capacitation-associated ATP1A4 redistribution via cathepsins.

Oxidative phosphorylation in mitochondria provides the most efficient generation of ATP in sperm, yet major consumers of ATP are the dynein ATPases, which are located throughout the flagellum. Instead of ATP diffusing throughout the entire flagellum, there could be compartmentalised glycolysis occurring in sperm (Travis et al. 2001). Accordingly, Naz et al. 1996 identified that hexokinase is localised to the acrosome in human sperm. In HeLa cells, triose-phosphate isomerase, a member of the glycolytic pathway interacts with phosphorylated cofilin (an actin binding protein), which in turn binds with the cytoplasmic domain of Na/K-ATPase (Jung et al. 2002). It is likely that ATP1A4, which is localised to the sperm head could interact with hexokinase through cofilin or other actin binding proteins. Hexokinase catalyses the first step in the glycolysis pathway and could provide the necessary ATP to ATP1A4 for carrying out its enzymatic activity.
In conclusion, this study represented the first comprehensive report on ATP1A4 interactome from capacitated bovine sperm. Although the relevance of the interaction of these proteins with ATP1A4 in mammalian fertilization is yet to be investigated, this ATP1A4 interactome provided critical insights into potential mechanisms by which ATP1A4 influences gamete interaction, leading to successful fertilization.

Figure 3.3

**Figure 3.3 Colocalisation of ATP1A4-PLCζ during sperm capacitation.** Representative images of ATP1A4 (green), PLCζ (red), nuclei (blue) and merged ATP1A4, PLCζ and DAPI staining in ouabain capacitated sperm.
Figure 3.4 A hypothetical model depicting the involvement of plakoglobin, α and β subunits of ATP1A4, E-cadherin and PLCζ during sperm-oocyte fusion. Complementary E-cadherin molecules on the sperm and oocyte could bind to each other; these interactions could be further strengthened by binding of the cytoplasmic domain of E-cadherin (EC) to plakoglobin (PG) –α-catenin (αC) –actin network. ATP1A4 could bind to an anchor protein, ankyrin (AN), which in turn interacts with the actin cytoskeleton and indirectly links ATP1A4 to plakoglobin-E-cadherin complex. This interaction could facilitate entry of sperm-derived PLCζ (indicated by broken arrows) which results in increase of intracellular calcium and resumption of meiosis in the oocyte. In addition, ATP1A4 could be involved in sperm-oocyte interactions by N-glycan motifs-mediated binding of β-subunits of Na/K-ATPase (ATP1B1/ATP1B3) present in gametes. PV: perivitelline space; ZP: zona pellucida; and ES: equatorial segment.
Chapter Four: Content of testis-specific isoform of Na/K-ATPase (ATP1A4) is increased during bovine sperm capacitation through translation in mitochondrial ribosomes

4.1 Preamble

The entire chapter has been submitted to Cell and Tissue Research and is currently under peer-review

Title: Content of testis-specific isoform of Na/K-ATPase (ATP1A4) is increased during bovine sperm capacitation through translation in mitochondrial ribosomes

Gayathri D Rajamanickam, John P Kastelic and Jacob C Thundathil

G.D. performed 100% of the experiments, analysed the data and wrote the manuscript. J. T. supervised the project. J.T and J.K contributed to critical thinking and reviewed the manuscript.

4.2 Abstract

Capacitation comprises a series of structural and functional modifications of sperm that confer fertilizing ability. We previously reported that the testis-specific isoform of Na/K-ATPase (ATP1A4) regulated bovine sperm capacitation through signaling mechanisms involving kinases. During subsequent investigations to elucidate mechanisms by which ATP1A4 regulates sperm capacitation, we observed that ATP1A4 was localised in both raft and non-raft fractions of the sperm plasma membrane and its total content was increased in both membrane fractions during capacitation. Objective of the present study was to investigate mechanism(s) of capacitation-associated increase in the content of ATP1A4. Despite the widely accepted dogma of transcriptional/translational quiescence, incubation of sperm with either ouabain (specific ligand for ATP1A4) or heparin increased ATP1A4 content in raft and non-raft sperm membrane
fractions, total sperm protein extracts (immunoblotting) and fixed sperm (flow cytometry), with a concurrent increase in Na/K-ATPase enzyme activity. This capacitation-associated increase in ATP1A4 content was partially decreased by chloramphenicol (mitochondrial translation inhibitor) but not affected by actinomycin D (transcription inhibitor). To demonstrate de novo ATP1A4 synthesis, we evaluated incorporation of bodipy conjugated lysine in this protein during capacitation. A partial decrease in bodipy-lysine incorporation occurred in ATP1A4 from sperm capacitated in the presence of chloramphenicol. Therefore, increased ATP1A4 during capacitation was attributed to mitochondrial translation of ATP1A4 mRNA present in ejaculated sperm, rather than gene transcription. To our knowledge, this is the first report demonstrating ATP1A4 synthesis during bovine sperm capacitation.

4.3 Introduction

In somatic cells, Na/K-ATPase is located in two distinct compartments (raft and non-raft) in the plasma membrane. The raft pool of the enzyme was involved in relaying the ouabain signal to intracellular compartments via activation of various protein kinases, thereby initiating pathways critical for numerous cellular functions (Li & Xie 2009). In our investigation on involvement of lipid rafts in ATP1A4 signaling during bovine sperm capacitation (Chapter 2), total content of ATP1A4 was increased in both raft and non-raft membrane fractions of capacitated sperm compared to uncapacitated sperm. During spermatogenesis, somatic histones are replaced by sperm-specific protamines, resulting in a highly stable chromatin condensation, and a transcriptionally inactive sperm genome. A long-standing premise in sperm biology is that mature sperm cannot synthesise new proteins (with few exceptions). Therefore, increased
ATP1A4 content during capacitation challenges this existing dogma and merits further investigation.

Translation of sperm RNAs are required for successful completion of capacitation, or to replace degraded proteins. For example, AKAP3 protein concentrations decreased (degradation) during capacitation (Hillman et al. 2013), and the 12S rRNA, a component of the 28S small subunit of the 55S mitochondrial ribosome, were identified in the polysome fractions. Since polysomes are indicative of active translation, it is possible that AKAP3 was likely translated by the sperm mitochondrial ribosome (Gur and Breitbart 2006). The c-myc protein has a short half-life (10 to 30 min) in somatic cells (Dani et al. 1985); however, the presence of c-myc protein in mature sperm indicates the potential for translation by c-myc mRNA (Kumar et al. 1993). Since cytoplasmic ribosomal components are absent in sperm, studies have questioned the possibility of de novo translation during capacitation. Interestingly, mitochondrial translation inhibitors such as chloramphenicol inhibited synthesis of new proteins, suggesting the role of mitochondrial ribosomes as a potential location for atypical sperm RNA translation in sperm. Consequently, we examined mechanisms responsible for the capacitation-dependent increase in ATP1A4 content in bovine sperm.

4.4 Materials and methods

4.4.1 Semen collection and preparation of reagents

Please refer to Chapter 2 Section 2.3.1 for detailed experimental procedures. In addition, stock solutions of actinomycin D (5 mg/ml in acetone) were stored at −20 °C and a working solution (1 mg/ml) were prepared in Sp-TALP. Chloramphenicol stock solution (10 mg/ml in Milli-Q water) was prepared and a working solution (100 µg/ml) was prepared in Sp-TALP.
4.4.2 Sperm preparations for in vitro capacitation and assessment of motility and tyrosine phosphorylation

Sperm were capacitated in various concentrations and volumes, depending on the specific experimental objective (1 x 10⁹ sperm for isolation of lipid rafts; 100 x 10⁶ sperm for Na/K-ATPase enzyme activity; 10 x 10⁶ sperm for Na/K-ATPase expression and transcription and translational inhibition). Capacitation was induced with either ouabain (50 nM) or with heparin (10 µg/ml) containing IBMX (100 µM) and dbcAMP (1 mM) and incubated for 4 h at 39 °C in a humidified incubator with 5% CO₂. The latter combination was included as one of the study groups, as these conditions consistently capacitate bovine sperm. Transcription and mitochondrial translation were inhibited with 100 µg/ml of actinomycin D and 100 µg/ml of chloramphenicol. These inhibitors were co-incubated with either ouabain or heparin for 4 or 10 h, respectively. In addition, a vehicle control (acetone alone) was included as a group in the actinomycin D study to rule out possible effects mediated by acetone on ATP1A4 transcription. Longer incubation times of chloramphenicol could be attributed to the reduced penetration of the inhibitor/drug in the midst of membrane perturbations (drastic changes in membrane fluidity) during capacitation as well as phylogenetic differences in the sensitivity of the eukaryotic mitochondrial system to the antibiotic as compared to prokaryotes (Ott et al. 2016).

4.4.3 Isolation of raft and non-raft fractions from bovine sperm

Please refer to Chapter 2 Section 2.3.2 for detailed experimental methodology related to raft and non-raft isolation from uncapacitated and capacitated sperm.
4.4.4 Immunoblotting

Sperm proteins were separated on 10% PAGE gels and electrotransferred to nitrocellulose membranes. After blocking with 5% (w/v) skim milk in TTBS for 1 h, membranes were incubated with the following antibodies: Na/K-ATPase α4 (1:10,000; custom-made antibody at University of Calgary, Canada), and bodipy (1:2500; Invitrogen, Burlington, ON, Canada) overnight at 4 °C. Membranes were washed (3X) in TTBS for 10 min and subsequently incubated with HRP conjugated goat anti-rabbit IgG for 1 h at RT. Following washing, immunoreactive bands were detected using chemiluminescence. Membranes were stripped and probed with β-tubulin antibody (1:10,000; Sigma Aldrich, Oakville, ON, Canada) to ensure equal protein loading.

4.4.5 Flow cytometry

After the capacitation period, heparin-capacitated sperm was treated with protamine sulphate (20 µg/ml) to detach agglutinated sperm and facilitate single-cell suspension. All groups were subsequently washed in PBS to remove capacitation-associated reagents. Then, 1 µl of Fixable Live and Dead Cell Stain (Thermo Fisher Scientific) was added to the sperm suspension and incubated for 30 min at RT. Sperm were fixed with 2.5% PFA for 15 min and blocked with 5% chicken serum (Sigma Aldrich) for 45 min at RT. Anti-ATP1A4 antibody (7 µg/ml) was added and incubated overnight at 4 °C. Alexa-conjugated anti-rabbit secondary antibody (Invitrogen) was added (1:1000) for 1 h at RT and data acquired using a BD LSR II cytometer (BD Biosciences, Mississauga, ON, Canada). The excitation source was a diode pumped solid state (DPSS) 488 nm laser. Voltage settings (log scale) used were as follows: FSC – 165, SSC – 165, FITC – 588, violet – 438. Compensation was applied to minimise overlap of the violet
fluorescence being detected in the green channel. Subsequently, Detector 1 (emission range of 450 ± 25 nm) was used for detecting violet fluorescence (viability status), whereas Detector 2 (emission range of 530 ± 15 nm) was used for detecting green (ATP1A4) fluorescence. A total of 20 x 10³ events were recorded for uncapacitated and capacitated sperm samples in the form of a scatter plot and histogram. The resulting flow cytometric data were analysed by computing relative median fluorescence intensity (MFI) of each sample.

4.4.6 Na/K-ATPase enzyme activity
The ATP1A4 activity was determined as described (Spokas & Spur 2001). Extracted sperm membrane proteins were incubated (30 min at 39 °C, pH 7.4) in a reaction mixture containing 100 mM NaCl, 15 mM KCl, 5 mM MgCl₂, 50 mM Tris-HCl, and 4 mM Tris-ATP, with and without 1 mM ouabain. The Na/K-ATPase enzyme hydrolyses ATP to form ADP and Pᵢ (inorganic phosphate); this reaction was stopped with 10% sodium dodecyl sulphate (SDS) which denatures the enzyme. Ammonium molybdate/ascorbic acid solutions (0.1 and 10%, respectively) were added for color development (30 min at RT). The inorganic phosphate that is released couples with ammonium molybdate to form a phosphomolybdate complex, which is reduced to a deep blue color (molybdenum blue) in the presence of a reducing agent (ascorbic acid) and quantified in a Genesys UV-Vis spectrophotometer at 870 nm (Thermo Fisher Scientific). The Na/K-ATPase activity was calculated as the difference in the Pᵢ concentration in the presence or absence of 1 mM ouabain. Enzyme activity (Pᵢ concentration) was normalised to total protein concentration to express total activity of Na/K-ATPase (pmoles of Pᵢ/μg of protein/min). However, ATP1A4 comprised 75% of the total Na/K-ATPase content (based on
immunoblotting) in bull sperm, similar to that reported for rat sperm (Wagoner et al. 2005). Therefore, in this thesis, total Na/K-ATPase activity was presented as ATP1A4 activity.

4.4.7 Isolation of sperm RNA and real-time PCR

Percoll-washed sperm were used for capacitation and subsequent RNA isolation to prevent contamination with somatic cells and leukocytes. Total RNA from sperm was isolated using TRIzol reagent (Thermo Fisher Scientific), following the manufacturers protocol. The final RNA pellet was washed with 1 ml of 75% ethanol, air dried and re-suspended in 10 µl of nuclease-free water (Ambion, Burlington, ON, Canada). The RNA samples were treated with DNase using the protocol in the DNase kit (Thermo Fisher Scientific). Concentrations of RNA were measured using a NanoDrop UV/Vis spectrometer (Thermo Scientific, Wilmington, DE, USA) at 260 nm and samples stored at -80 °C until used for subsequent analysis. Sperm RNA (25 ng) was reverse-transcribed using the high capacity cDNA reverse transcription kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. Results pertaining to Figure 4.6A were based on the intron-spanning primers for the targeted transcripts, designed using the NCBI primer designing tool (Table 4.1). Presence of full-length ATP1A4 mRNA was demonstrated by designing primer sequences that spanned the entire length of the mRNA (Table 4.2).

The PCR amplifications were performed in a 25-µl reaction volume using StepOnePlus Real-Time PCR system (Applied Biosystems, Burlington, ON, Canada) and AmpliTaq fast DNA polymerase (Fast SYBR Green master mix, Applied Biosystems) with 10 pmol of each primer and 25 ng of cDNA. The PCR conditions were 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, primer-dependent annealing temperature for 30 s, then 72 °C for 2 min, followed by a final extension at 72 °C for 10 min. In addition, a negative control (without cDNA) and no
template control were included to ensure specific amplification. The PCR products were purified using E.Z.N.A cycle pure kit (Omega Bio-Tek Inc, Norcross, GA, USA) and both strands were sequenced (DNA sequencing lab, University of Calgary, Calgary, AB, Canada) and their identity confirmed with NCBI BLAST. The amount of ATP1A4 was normalised against GAPDH for each sample and relative expression was calculated using the 2−ΔΔCT (threshold cycle) method.
### Table 4.1: Primer sequences used for detecting ATP1A4 mRNA in bovine sperm

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank accession #</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Atp1a4</em></td>
<td>NM_001144103.2</td>
<td>GTTGCTAGGGGTCTGCTTC</td>
<td>GCCCCACGAAACAGAGTTA</td>
<td>108 bp</td>
</tr>
<tr>
<td><em>Gapdh</em></td>
<td>NM_001034034.2</td>
<td>CAGGTTGTCTCCTCGACTT</td>
<td>TGTCGTACCAGGAAATGAGCTT</td>
<td>109 bp</td>
</tr>
</tbody>
</table>

### Table 4.2: Primer sequences used for detecting full-length ATP1A4 mRNA in bovine sperm

<table>
<thead>
<tr>
<th>Location on the GenBank accession #</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP1A4 gene 800 – 1200 bp</td>
<td>TTTGGGCTCTCGCTTATCTTGG</td>
<td>TGTCAGGGTCAGGCATCATGTG</td>
<td>123</td>
</tr>
<tr>
<td>ATP1A4 gene 1 – 200 bp</td>
<td>TCCAAACCCAGGACCTACAA</td>
<td>ATTTGTGATCATCCATCATGACTTC</td>
<td>116</td>
</tr>
<tr>
<td>ATP1A4 gene 1610 – 1760 bp</td>
<td>ACGCCTTCAAAACGCCTA</td>
<td>TTCAAGAAGCAGAACCCTAGCA</td>
<td>70</td>
</tr>
<tr>
<td>ATP1A4 gene 2600 – 3000 bp</td>
<td>TTCGTCAGCATCGTGATCGT</td>
<td>TCTTGTTCATGCCCTGCTG</td>
<td>91</td>
</tr>
</tbody>
</table>
4.4.8 Detection of protein synthesis by fluorescent amino acid incorporation during capacitation

Sperm were prepared for in vitro capacitation (as detailed above) in the presence or absence of chloramphenicol under ouabain-induced capacitating conditions. Cells were partially permeabilized with 0.02% Triton X-100 for 30 s and incubated with a charged lysine transfer RNA labelled with the fluorophore BODIPY-FL (FlouroTect Greenlys in vitro translation system, Promega, Madison, WI, USA), which incorporates into lysine residues during protein translation. Sperm were pelleted and lysed with a buffer containing 250 mM sucrose, 50 mM imidazole, 1 mM EDTA, pH 7.4 with 0.1% sodium deoxycholate, and protease inhibitor tablets for 45 min on ice. The ATP1A4 antibody was coupled to protein A beads (GE Life Sciences, Mississauga, ON, Canada) for 1 h on a rocker at 4 °C and subsequently incubated with capacitated total-cell lysates from control and chloramphenicol-treated groups for 4 h and eluted at 70 °C for 2 min. The immunoprecipitated eluate was electrophoresed under standard conditions (see Section 4.4.4).

4.4.9 Statistical analyses

Results are shown as mean ± SEM, with the number of samples indicated. Median fluorescence intensity values from the flow cytometric analyses were tested using Mann-Whitney U test. Data from Na/K-ATPase enzyme activity, PCR, detergent soluble and insoluble ATP1A4 expression and sperm kinematic parameters were analysed by one-way ANOVA, followed by a post-hoc Tukey’s test. Results of ATP1A4 content from raft and non-raft fractions, translation and transcription inhibition experiments were analysed using repeated-measures ANOVA (with main effects of groups (raft and non-raft) and treatments (incubation conditions), followed by Tukey’s
multiple comparison post-hoc test. All data were analyzed with commercial statistical software (GraphPad Software Inc., La Jolla, CA, USA) and P < 0.05 was considered significant.

4.5 Results

4.5.1 Content of ATP1A4 increased in raft and non-raft membrane fractions during capacitation

Since Na/K-ATPase mediates cell signaling through its association with lipid rafts and non-rafts in the plasma membrane, we evaluated relative distribution of ATP1A4 molecules in both raft and non-raft sperm membrane fractions during capacitation. Ouabain and heparin (4 h) capacitating conditions induced significant increase in ATP1A4 content in both raft and non-raft fractions compared to control samples (control 4 h and 0 h; Figure 4.1A and B).

Figure 4.1
Figure 4.1. ATP1A4 content in membrane fractions under ouabain- or heparin-induced capacitating conditions. (A) Bovine sperm (1000 x 10^6 cells) were capacitated for 4 h and membrane (raft and non-raft) fractions were immunoblotted for ATP1A4. The image was consolidated by removing the middle lane of the western blot (indicated by dividing line). (B) Mean pixel intensity of ATP1A4 was calculated and recorded as arbitrary units. Values without a common letter differed (P < 0.05)

4.5.2 Content and activity of ATP1A4 is increased during capacitation in detergent soluble sperm protein extracts

In the absence of a suitable loading control for raft and non-raft membrane preparations, total sperm extracts were used to determine whether content and activity of ATP1A4 increased during capacitation. Consistent with our membrane data, ATP1A4 content was higher in the group capacitated by ouabain or heparin, compared to control groups (control 4 h and 0 h) in detergent-soluble lysates (Figure 4.2A and B). To determine if increase in total protein levels were associated with an increase in function (enzyme activity), activity was measured in both uncapacitated and capacitated sperm. Similar to the increase in the ATP1A4 content, enzyme activity was higher in the ouabain-capacitated group compared to all other groups (Figure 4.2C).
Figure 4.2. Content of ATP1A4 and activity in detergent-soluble sperm protein extracts prepared from ouabain or heparin capacitated sperm. (A) Bovine sperm were capacitated for 4 h and detergent-soluble sperm cell homogenates (extracted under sodium deoxycholate conditions) were immunoblotted to determine ATP1A4 content. The pellet that remained after detergent extraction was boiled in sample buffer to determine content of β-tubulin. (B) Relative pixel intensity of detergent-soluble ATP1A4 content was calculated by normalizing mean pixel intensity of ATP1A4 to β-tubulin. (C) Enzyme activity of ATP1A4 was recorded as hydrolysis of ATP to inorganic phosphate (P_i) in the presence of 1 mM ouabain and normalised to total protein content (pmoles/µg of protein/min) from uncapacitated and capacitated groups and their incubation control. Data were expressed as mean ± SEM (n = 5). a-dValues without a common letter differed (P < 0.05).
4.5.3 Capacitation-associated increase in content of ATP1A4 was not due to translocation of ATP1A4 from subcellular compartments

To ascertain that an increase in membrane ATP1A4 content was not due to translocation from other subcellular compartments, sperm proteins were extracted from the insoluble sperm pellet left after detergent solubilisation (detailed above) from capacitated sperm [ouabain and heparin (4 h)] and its incubation controls (control 4 h and 0 h; Figure 4.3A and B) with a radioimmunoprecipitation (RIPA) assay buffer. The RIPA buffer contained 1% Triton X-100, 0.1% SDS and 0.5% sodium deoxycholate which should extract cytoskeletal, nuclear and mitochondrial-associated proteins. Demonstration of similar content of ATP1A4 among all four groups excluded the possibility of protein translocation to the plasma membrane from other sperm compartments.

Figure 4.3

Figure 4.3 Analysis of ATP1A4 levels in detergent-insoluble extracts from ouabain and heparin capacitated sperm. (A) Representative western blot data on sperm insoluble protein extracts, prepared solubilizing the pellet left after sodium deoxycholate extraction in RIPA buffer and subsequently probed with ATP1A4 antibody. (B) Relative pixel intensity of detergent-
insoluble ATP1A4 content was calculated by normalizing mean pixel intensity of ATP1A4 to β-tubulin. Data shown were expressed as mean ± SEM (n = 5).

4.5.4 Confirmation of capacitation-associated increase in content of ATP1A4 by flow cytometry

We used flow cytometry to confirm capacitation-associated increase in ATP1A4 content. Gate settings, distinctions between dead and live sperm population and their corresponding dot plots were shown in the flow cytometry profile (Figure 4.4A). Ouabain-capacitated sperm had increased ATP1A4, as evidenced by the histogram shifted to the right (right shift) on the FITC log scale on the x-axis (Figure 4.4B) and also increased relative median fluorescence intensity values compared to heparin or controls (Table 4.3).
Figure 4.4 Analysis of ATP1A4 content in fixed sperm (flow cytometry) under ouabain- or heparin-induced capacitating conditions. (A) Capacitated sperm were labelled with ATP1A4 antibody and levels of fluorescence recorded; P1 represented the total cell population analysed; P2 represented dead cells; P3 denoted viable cells; P4 corresponded to ATP1A4 fluorescence from the viable population; Q1 & Q3 dot plots corresponded to viable sperm that had low and high ATP1A4 fluorescence respectively; and Q2 & Q4 corresponded to dead sperm with low and high ATP1A4 fluorescence, respectively. (B) Representative fluorescence intensity histogram from capacitated and uncapacitated sperm.
Table 4.3: Relative median fluorescence intensity (flow cytometry) values from uncapacitated and capacitated sperm immunostained with ATP1A4 antibody

<table>
<thead>
<tr>
<th>Group</th>
<th>Relative MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 0 h</td>
<td>1591&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control 4 h</td>
<td>3298&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ouabain 4 h</td>
<td>4732&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Heparin 4 h</td>
<td>4310&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>abcd</sup> Values without a common superscript differed (p < 0.05)

4.5.5 Capacitation of bull sperm in presence of actinomycin D did not prevent capacitation associated increase in ATP1A4 content

Roles of transcription/translation machinery to increase ATP1A4 content during capacitation were investigated. Capacitation of sperm with ouabain or heparin in presence of actinomycin D (inhibitor of gene transcription) did not affect capacitation-associated increase in content of ATP1A4 compared to the respective control (incubation in control 4 h; Figure 4.5A and B).

**Figure 4.5**

![Figure 4.5](image-url)
Figure 4.5. Effects of actinomycin D on ATP1A4 transcription during capacitation of bovine sperm. (A) Total sperm homogenates were prepared from sperm incubated with and without actinomycin D in presence of either ouabain or heparin for 4 h and immunoblotted with ATP1A4 antibody. (B) Content of ATP1A4 was normalized to β-tubulin and relative pixel intensity calculated. *a*dValues without a common letter differed (P < 0.05).

4.5.6 Capacitation of bull sperm in presence of chloramphenicol inhibited capacitation associated increase in ATP1A4 content

Because actinomycin D did not affect ATP1A4 content, we quantified ATP1A4 mRNA content and evaluated effects of chloramphenicol (inhibitor of mitochondrial translation) on ATP1A4 protein content during capacitation. Although ATP1A4 mRNA was present in bull sperm, there was no significant difference in the content of ATP1A4 mRNA between control (sperm incubated in control 4 h) and capacitated groups (ouabain and heparin (4 h); Figure 4.6A). However, capacitation of sperm [ouabain and heparin (4 h)] in the presence of chloramphenicol partially decreased ATP1A4 content, suggesting mitochondrial translation of ATP1A4 mRNA during capacitation (Figure 4.6B and C). Based on relative pixel intensity data, ouabain and heparin capacitated sperm had 24 and 12% increases, respectively, compared to incubation
control alone (Sp-TALP 4 h) in the absence of chloramphenicol. Similar patterns of decrease were noticed for ouabain (22%) and heparin (12.5%) capacitated sperm in the presence of chloramphenicol. In addition, presence of full-length ATP1A4 mRNA in bovine sperm was also demonstrated (Figure 4.7).

**Figure 4.6**

![Graph A](image)

**Figure 4.6 B**

![Graph B](image)
Figure 4.6 Effects of chloramphenicol on ATP1A4 translation during capacitation of bovine sperm. (A) Real-time PCR was used to quantify ATP1A4 mRNA content in uncapacitated and capacitated sperm and normalised to GAPDH levels. (B) To study effects of chloramphenicol on ATP1A4 protein content, sperm were incubated with and without chloramphenicol in presence of either ouabain or heparin for 10 h, and total sperm homogenates were probed for ATP14 content. (C) Pixel intensity for ATP1A4 was expressed for various treatment groups after normalizing values to that of β-tubulin from the respective groups. Data shown were expressed as mean ± SEM (n = 3) (D). a-cValues without a common letter differed (P < 0.05).
Figure 4.7 Screenshots of NCBI blast corresponding to the sequenced PCR products obtained from ATP1A4 mRNA in bull sperm
4.5.7 Detection of protein synthesis by fluorescent amino acid incorporation during capacitation

A conclusive demonstration for protein synthesis requires demonstration of amino acid incorporation during capacitation. Under ouabain-induced capacitating conditions, sperm incorporated the charged lysine transfer RNA tagged with bodipy, which was evident by an increase in the intensity of band at ~110 kDa and the presence of additional bands (~75, 50 and 37 kDa) in the total cell lysate. This incorporation was partially inhibited in the presence of chloramphenicol (Figure 4.8A). Reprobing this immunoblot with ATP1A4 antibody demonstrated that this 110 kDa band contained ATP1A4 (Figure 4.8A). Immunoprecipitation of total protein extracts (prepared from sperm capacitated with or without chloramphenicol) with ATP1A4 antibody, followed by immunoblotting with bodipy, further confirmed a partial decrease in the content of bodipy-tagged ATP1A4 in the group capacitated in presence of chloramphenicol (Figure 4.8B and C).

Figure 4.8

![Immunoblot images showing detection of protein synthesis](image)
Figure 4.8. Inhibition of ATP1A4 synthesis in the presence of chloramphenicol (CP) during capacitation. (A) Total sperm lysates were prepared from ouabain-capacitated sperm in the presence or absence of chloramphenicol, immunoblotted with bodipy, stripped and reprobed with ATP1A4 and β-tubulin antibodies. (B) Immunoprecipitation of total lysates with ATP1A4 antibody and subsequent immunoblotting with bodipy antibody. (C) Pixel intensity for ATP1A4 was expressed for various treatment groups after normalizing values to that of β-tubulin from the respective groups. Data shown were expressed as mean ± SEM (n = 3). *P < 0.05.

4.6 Discussion

To understand mechanisms by which ATP1A4 activates multiple signaling pathways during sperm capacitation, we investigated capacitation-associated changes in distribution of ATP1A4 in both raft and non-raft sperm membrane fractions. Results demonstrated that content of ATP1A4 increased in both raft and non-raft fractions from capacitated sperm in comparison to uncapacitated sperm. To confirm that ATP1A4 content increased in the plasma membrane, immunoblotting experiments were conducted using protein extracts (detergent-soluble and detergent insoluble) prepared from capacitated and uncapacitated sperm. Results of these studies also demonstrated a capacitation-associated increase in the content and activity of ATP1A4, which was not due to capacitation-associated relocation of ATP1A4 to sperm plasma membrane.
from other subcellular components. In addition, flow cytometry-based quantification of ATP1A4 from capacitated sperm also demonstrated capacitation-associated increase in the content of ATP1A4. Synthesis of ATP1A4 was further confirmed by demonstrating bodipy-labeled ATP1A4 in sperm protein extracts prepared from capacitated sperm, potentially through mitochondrial translation of ATP1A4 mRNA present in sperm. To our knowledge, this is the first report on capacitation-associated increase in the content of ATP1A4 and total activity of ATP1A4 in bovine sperm.

We first isolated raft and non-raft fractions from sperm plasma membrane using a non-detergent methodology (sodium carbonate), as the classical detergent (Triton X-100) approach solubilized Na/K-ATPase, leading to its release from lipid rafts to the non-raft fraction (Wang et al. 2004, Cross 2004). Despite using a similar sperm concentration (1000 x 10⁶ cells) across various treatment groups, the capacitation process increased the content of ATP1A4 in both raft and non-raft fractions in comparison to these fractions from uncapacitated sperm. However, these experiments lacked a loading control, as actin and β-tubulin are not suitable for this purpose (Thaler et al. 2006, Donatello et al. 2012). Therefore, to confirm an increase in ATP1A4, immunoblotting experiments were conducted using total sperm homogenates (detergent-soluble extracts) prepared from capacitated and uncapacitated sperm, where β-tubulin could be used as a loading control. Consistent with membrane fraction data, whole-sperm extracts also had increased ATP1A4 content during capacitation.

A previous study demonstrated an increase in plasma membrane ATP1A4 during rat sperm capacitation due to translocation of this protein to sperm membrane from other subcellular compartments (nucleus, cytoskeleton, cytosol, etc.; Jimenez et al. 2012). To rule out possible translocation of ATP1A4 from subcellular compartments to sperm membrane during sperm
capacitation, we compared ATP1A4 content in detergent-insoluble extracts of uncapacitated and capacitated sperm. If there was capacitation-associated redistribution of ATP1A4 to the sperm surface, there would have been decreased ATP1A4 in other subcellular compartments (detergent-insoluble extracts) and a concurrent increase in ATP1A4 in the membrane (detergent-soluble extracts) at the end of the capacitation period. However, our results excluded this possibility, as ATP1A4 content were similar in detergent-insoluble homogenates prepared from uncapacitated versus capacitated sperm. Alternatively, an increased content of ATP1A4 at the plasma membrane could be due to unmasking of epitopes during capacitation due to removal of steric hindrance factors that mask antibody binding sites (Villarroya & Scholler 1987, Boue et al. 1996). However, this possibility was unlikely, as we observed a concurrent increase in ATP1A4 enzyme activity along with increased content of ATP1A4. Antibodies also behave differently in fixed cells versus total cell extract, due to the accessibility of the epitope to the antibody and protein configuration (He & Fox 1996). Therefore, we used flow cytometry in fixed uncapacitated and capacitated cells to compare our results from immunoblotting experiments. Results from both these approaches were in complete agreement regarding the total increase in ATP1A4 during capacitation.

The DNA of mature sperm is generally considered transcriptionally inactive due to its association with protamines. However, sperm might still undergo transcription, as the histone-to-protamine conversion is not 100% complete and approximately 5-15% of sperm DNA is still packed with histones in mammalian sperm (Gatewood et al. 1987), leaving the possibility for gene transcription in sperm (Naz 1998). Furthermore, actinomycin D inhibited RNA (90-95%) and protein concentrations (75%) in bovine sperm (Premkumar & Bhargava 1972). Therefore, perhaps the ATP1A4 gene was transcribed and translated, leading to ATP1A4 protein synthesis.
during sperm capacitation. To test this hypothesis we capacitated sperm with or without actinomycin D. We demonstrated that ATP1A4 content was similar between sperm capacitated with or without actinomycin D. We believe that the effects of actinomycin D were specific to sperm because we used swim-up sperm populations for capacitation which were devoid of contaminating leukocytes, epithelial cells and bacteria. In addition, the inhibitory concentration of actinomycin D for RNA polymerase II (transcribes protein-coding genes) is > 1 µg/ml (Bensaude 2011) for somatic cells and the inhibitor has been reported to intercalate with little or no reversibility within the GC rich sequences within minutes of exposure. Moreover, it takes only ~ 10 – 20 min to transcribe a gene length of 20,000 bp in humans (Heyn et al. 2015). Considering the rapidity of actinomycin D action, average transcription time and the concentration (1 mg/ml) we used in our study, we assert that the observed inability of actinomycin D to block capacitation-associated increase in ATP1A4 was not due to impenetrability of the inhibitor, insufficient incubation time (4 h) or concentration. Therefore, we inferred that ATP1A4 gene transcription and subsequent translation of the sperm RNA was not the mechanism behind increased content of ATP1A4 during sperm capacitation. Alternatively, we considered the possibility that increased ATP1A4 content during capacitation was due to translation of mRNA, which had been transcribed during spermatogenesis.

During spermatogenesis, mRNAs are synthesised and stored due to a global cessation in transcription, which occurs several days before post-meiotic male germ cell differentiation (Braun 1998). The ability of sperm to store and protect these RNAs until fertilization and activation of the embryonic genome suggests a physiological function, despite translational quiescence. We investigated translation of ATP1A4 mRNA as a potential mechanism leading to increased content of ATP1A4 during capacitation. In that regard, we first quantified ATP1A4
mRNA in uncapacitated versus capacitated sperm. Similar content of ATP1A4 in uncapacitated and capacitated sperm supported our previous conclusion that transcription of ATP1A4 gene is not occurring during capacitation and the possibility of capacitation-associated translation of ATP1A4 mRNA as a potential mechanism behind the increased content in sperm. Although it has been generally assumed that there is a direct relationship between mRNA and protein levels, there was only a 40% correlation between mRNA and protein abundance in mammalian cells, presumably due to transcription and mRNA degradation (Tian et al. 2004, Mata et al. 2005, de Sousa Abreu et al. 2009, Vogel & Marcotte 2012). Since mRNAs can undergo several rounds of translation initiation, elongation and termination (Decker & Parker 2012), it is possible that regulation at the translational level (the proportion present in polysomes, and the speed of translation) may influence the correlation between mRNA and protein abundance (Brockmann et al. 2007, Maier et al. 2009). Therefore, the increased content of ATP1A4, despite the similar mRNA content, could have been affected by one or more of the above-mentioned steps.

Based on high-throughput technologies (e.g. RNA sequencing), ejaculated sperm contain a complex repertoire of transcripts and intact mRNAs (Das et al. 2013, Card et al. 2013, Sendler et al. 2013). Furthermore, the subcellular localisation of mRNA in sperm is documented (Kumar et al. 1993). It was suggested that de novo protein translation from sperm mRNA is essential to supplement proteins that were degraded or to support functional changes during capacitation (Gur & Breitbart 2006). This de novo protein production was also demonstrated in other mammals (Gur & Breitbart 2006), suggesting a widespread existence of this mechanism across species. Since sperm lack classical cytoplasmic machinery (Ostermeier et al. 2002, Gilbert et al. 2007), mitochondrial ribosomes seem to be responsible for protein synthesis during capacitation. A gold-standard assay to demonstrate de novo protein synthesis is incorporation of
radioactive labeled aminoacids ($^{35}$S Methionine and $^{35}$S Cysteine) into newly synthesised polypeptides. However, due to concerns regarding personnel safety and disposal limitations, we used an alternative approach which incorporated bodipy-labeled amino acids (lysine) to detect de novo ATP1A4 synthesis during capacitation (Doi et al. 2002). Bodipy-tagged lysine was incorporated in several proteins at ~110, 75, 50 and 37 kDa; however, in the presence of chloramphenicol (mitochondrial translation inhibitor), band intensities at ~110 and 37 kDa were partially inhibited, whereas bands at 75 and 50 kDa were completely abolished, suggesting that protein synthesis occurred during bovine sperm capacitation. To determine whether the 110 kDa band was comprised of ATP1A4, we stripped and reprobed the bodipy incorporated blot with ATP1A4 antibody. Using this system, there was ~22% decrease in ATP1A4 in total proteins extracted from sperm capacitated in presence of chloramphenicol. In a similar study, when rats sperm were treated with chloramphenicol under capacitating conditions, the content of ATP1A4 was reduced by 80% (Gur & Breitbart 2006). We speculated apparent differences in the percentage inhibition of ATP1A4 could be due to different conditions used for capacitating rat and bovine sperm, which in turn could have affected sensitivity of mitochondrial ribosomes to the inhibitor (chloramphenicol). In a study by Cunningham and Bridgers (Cunningham & Bridgers 1970), there was a 30% reduction in protein synthesis when chloramphenicol was added to brain mitochondrial population, whereas Bosman & Hemsworth (1970) reported a 60% reduction in mitochondrial protein synthesis in the cerebral cortex, indicating differential sensitivity of ribosomes to chloramphenicol among studies. To conclusively demonstrate protein synthesis, we immunoprecipitated ATP1A4 from total cell lysates and measured immunoreactivity of bodipy in the presence of chloramphenicol.
The exact mechanism by which nuclear-encoded ATP1A4 mRNA is translated by mitochondrial ribosomes remains unclear. We propose a possible mechanism by which protein synthesis could occur in sperm where translation of ATP1A4 transcripts could occur in mitochondrial-type ribosomes in localised regions within the sperm head (Figure 4.9). The mRNAs are targeted to subcellular compartments in diverse somatic cells that display compartmentalization and polarisation, e.g. neurons (Besse & Ephrussi 2008). Accordingly, it was proposed that the nucleus, perinuclear theca, and the post-acrosomal sheath could serve as mRNA repositories in sperm (Miller & Ostermeier 2006). We previously documented that the ATP1A4 protein is localised to the sperm head (Newton et al. 2010). Therefore, it is reasonable to speculate that localised translation of ATP1A4 mRNA could occur in the sperm head. Perhaps ATP1A4 mRNA could exist as a translationally inactive mature ribonuclear protein particle (RNP) due to its association with RNA binding proteins (RBP), motor proteins (kinesins and dyneins) and translational repressors. However, transport of this mRNA from the site of transcription to sperm head and its existence at this site in a translationally repressed form, awaiting appropriate translation-inducing stimuli, remains speculative.

It is noteworthy that only cytoplasmic ribosomes have been implicated in translation initiation in other cell types. However, mitochondrial or mitochondrial-type ribosomes may perform a similar function in sperm, as cytoplasmic machinery is lost during spermatogenesis (Toshimori 2009). Interestingly, extra-mitochondrial localisation of mitochondrial ribosomes, especially 16s rRNA, has been demonstrated in murine oocytes and zygotes (Ninomiya & Ichinose 2007) and sperm nucleus (Villegas et al. 2002) and functional competency of such mitochondrial ribosomes has been demonstrated in Drosophila embryos (Amikura et al. 2001, Amikura et al. 2005). All these studies supported our proposition that ribosomal RNAs and
proteins could form functional mitochondria-type ribosomes outside mitochondria. In the current model (Figure 4.9), we hypothesise that ATP1A4 mRNA, mitochondrial or mitochondrial-type ribosomes and translation initiation and elongation factors are present outside the mitochondria (perinuclear theca and post-acrosome regions in the head) in ejaculated sperm. However, the ATP1A4 mRNA is prevented from being translated due to the inhibitory action of RBPs and the unavailability of a functional 55 S mitochondrial ribosome in ejaculated sperm. Capacitation associated changes removes the inhibitory effect of RBPs and the binding of the mRNA and tRNA$^{\text{Met}}$ to the small ribosome subunit (28 S) are facilitated by the mitochondria initiation factors, mtIF3 and mtIF2, respectively. The latter step is markedly enhanced in the presence of GTP. These initial steps recruit the large ribosome subunit (39 S) to join the small ribosome subunit (28 S) and thereby a fully functional mitochondrial ribosome is assembled which stimulates dissociation of mtIF3 and mtIF2. The initiation phase is completed and translation can proceed with the elongation and termination phase leading to production of a functional protein.

Perhaps post-translational modifications of ATP1A4 are carried out by membrane-associated molecular chaperones and their ability to interact with proteins through hydrophobic and electrostatic interactions, ultimately guiding them to productive folding and transportation pathways. One of the most highly expressed molecular chaperone complexes in haploid germ cells is the chaperonin containing T-complex/TCPI-ring complex (CCT/TRiC; (Silver 1985) that acts as the folding machinery for 5 – 10% of newly synthesised proteins (Kubota et al. 1994). Mature human and rat sperm have an intact CCT/TRiC complex in the sperm head (Dun et al. 2012); perhaps this chaperone complex is conserved across species and regulates productive folding of nascent proteins to functional proteins and its transport to the sperm membrane.
In summary, we provided evidence that mitochondrial ribosome-associated translation of ATP1A4 mRNA accounted for the increased ATP1A4 content in bull sperm during capacitation. Furthermore, the ability of sperm to synthesise protein highlighted the importance of atypical, yet functional translation pathways to meet physiological demands during capacitation and opens up new areas of investigation in mammalian sperm biology.
Figure 4.9 Hypothetical schematic view of the possible mechanisms involved in translation of sperm mRNA during capacitation. During spermatogenesis, RBPs bind with pre-mRNA in the nucleus and are subsequently exported together to the cytoplasm of spermatocytes and early round spermatids. The RBP-mRNA complex further recruits motor proteins, which allows them to be transported along the cytoskeletal protein network to the developing sperm head. During the transport, RBPs block the association of the translation initiation machinery with the mRNA, thereby keeping the transcript inactive. The perinuclear theca (PT) and postacrosomal sheath (PS) in the sperm could serve as translation sites in the sperm head. B) The localised mRNA in the PT and PS are kept silent in the ejaculated sperm (uncapacitated) due to its association with RBPs and lack of a translation competent mitochondrial ribosome (55 S comprised of 39 S and 29 S subunits). C1) During capacitation, post-translational protein modifications such as phosphorylation, release the mRNA from the inhibitory effect of RBPs. C2) Translation initiation factors, mtIF3 and mtIF2 facilitate binding of the mRNA and tRNA\textsuperscript{Met}, respectively, to 28 S in the presence of GTP hydrolysis. C3) Molecular events described in C2 initiate binding of 39 S to 28 S-mRNA-tRNA complex and formation of a functional 55S mitochondrial or mitochondrial-type ribosome, followed by the dissociation of the mtIF3 and mtIF2-GDP complex from the 28 S. C4) Synthesis of ATP1A4 protein and its translocation to the plasma membrane. Dotted lines indicate sperm tail.
Chapter Five: **Development and validation of laboratory assays to determine content and activity of testis-specific isoform of Na/K-ATPase (ATP1A4) in bull sperm**

### 5.1 Abstract

Traditional BBSE eliminates bulls that are grossly abnormal; however, bulls classified as satisfactory potential breeders still vary in field fertility, implying submicroscopic differences in sperm characteristics. ATP1A4 is involved in regulation of sperm motility and capacitation in bulls, by virtue of its enzyme activity and signaling functions. The objective was to develop and validate laboratory assays to determine content and activity of ATP1A4 in frozen-thawed bull sperm. We developed a flow cytometry-based assay for quantification of ATP1A4 content and validated a colorimetric assay for determining ATP1A4 activity in bull sperm. Fixation with 2.5% PFA and use of fixable live and dead cell stain yielded adequate conditions for detection of ATP1A4 fluorescence in viable sperm by flow cytometry. Isolation of sperm membrane protein extracts in the presence of ionic detergent like sodium deoxycholate yielded maximum enzyme activity after 30 min of post incubation with substrate ATP. The enzyme assay was also validated and variability (intra- and inter-assay) and accuracy of the enzyme assay were < 20% and <120%, respectively. In future studies, content and activity of ATP1A4 will be determined from bulls with known fertility status to evaluate the association between these parameters and field fertility.
5.2 Introduction

Improving reproductive efficiency of cattle is important for meeting global demands for animal proteins. In that regard, bull fertility is particularly critical, as one bull can breed thousands of females by artificial insemination (AI), or 20 to 30 females under natural breeding situations. Traditional BBSE eliminates bulls that are grossly abnormal. However, bulls or their semen samples classified as satisfactory still differ in fertility, suggesting submicroscopic differences in the sperm characteristics (Larson & Miller 2000). Consequently, it would be of great benefit for the cattle industry to develop marker-based laboratory assays to complement a traditional BBSE and to better predict variations in bull fertility. As proteins already present in mature sperm (without additional protein synthesis) are responsible for sperm function, the content and activity of these sperm proteins may reflect molecular/submicroscopic differences and serve as biomarkers of fertility. Accordingly, Sutovsky (Sutovsky et al. 2015) identified ubiquitin in defective sperm (negative biomarker), whereas positive biomarkers are proteins that are up- or down-regulated in morphologically normal motile sperm. Examples of positive biomarker proteins include fertility-associated antigen (FAA; (Bellin et al. 1998b), osteopontin (OPN; (Cancel et al. 1997), lipocalin type-D prostaglandin (PGD) synthase (Gerena et al. 1998) and type-2 tissue inhibitor of metalloproteinases (TIMP-2; (McCauley et al. 2001).

A ubiquitous integral membrane protein, Na/K-ATPase is responsible for maintaining Na\(^+\) and K\(^+\) gradients across the plasma membrane of most mammalian cells. The \(\alpha\) subunit of Na/K-ATPase is the catalytic subunit responsible for the enzyme activity by binding to Na\(^+\), K\(^+\), ATP and ouabain (Jorgensen et al. 2003). It is noteworthy that inhibition of ATP1A4 with ouabain inhibits several motion characteristics in rat sperm, including total and progressive motility and several sperm kinematic parameters (Jimenez et al. 2010). However, in bull sperm,
ATP1A4 inhibition did not significantly decrease total sperm motility (Thundathil *et al.* 2006), suggesting species differences regarding the role of ATP1A4 in regulation of sperm function. Therefore developing assays to quantify ATP1A4 content and activity may enable us to develop novel diagnostic approaches for fertility predictions.

Although a radioactive and a colorimetric assay are available to quantify ATP1A4 activity in sperm samples (Zhao & Buhr 1996, Blanco & Mercer 1998), both approaches have limitations, including handling radioactive substrates and complex methods of membrane isolation from sperm which would be difficult in a commercial AI industry setting. Therefore there is a need to develop and validate an enzyme assay that circumvents these limitations. Validation of an enzyme assay includes all procedures that demonstrate an assay which is used for quantitative measurement of a particular analyte of interest (P$_i$ released from Na/K-ATPase) in a given biological sample (sperm) is reliable and reproducible for the intended use. Some of the fundamental parameters for validation include precision, accuracy and linearity.

Flow cytometry was preferred for quantification of ATP1A4 content, since it is widely accepted and used technique by AI industries. Flow cytometry analyzes cells based on their size and granularity and studies have correlated the flow cytometric detection of sperm proteins to the fertility status of breeding bulls (Lovercamp *et al.* 2007, Dogan *et al.* 2015).

Since the content and activity of ATP1A4 in sperm may regulate bull fertility, objectives of the current study were to: i) develop a flow cytometry-based assay for quantification of ATP1A4 content; and ii) develop and validate an enzyme assay for determining ATP1A4 activity. Our long-term goal is to use these assays for fertility predictions.
5.3 Materials and methods

5.3.1 Validation of Na/K-ATPase activity assay

5.3.1.1 Processing of frozen-thawed sperm bull sperm

The enzyme and flow cytometry assay were validated using frozen-thawed bull semen. First, frozen semen straws were thawed (60 s) in a water bath maintained at 37 °C. Then, semen was gently layered on top of 45% percoll in PBS and centrifuged (400 x g for 20 min at RT). Resulting sperm pellets were washed once again in PBS to remove percoll and number of sperm in the pellet was determined (hemocytometer).

5.3.1.2 Preparation of sperm membrane protein extracts

To extract sperm membrane proteins, 50 x 10⁶ sperm was re-suspended in 500 µl of extraction buffer containing 250 mM sucrose, 50 mM imidazole, 1 mM EDTA, pH 7.4 with either 0.1% sodium deoxycholate or 0.25% Triton X-100 or 0.5% CHAPS, and protease inhibitor tablets (Roche Diagnostics, Indianapolis, IN, USA) for 45 min on ice, with occasional vortexing. After incubation, samples were centrifuged (12 000 x g for 10 min at 4 °C) and the supernatant containing the total membrane proteins was kept on ice pending further analyses. Protein content was determined with spectrophotometry (Thermo Scientific™ Genesys 10S UV-Vis, Burlington, ON, Canada) using a protein assay kit (DC™ Protein Assay; Bio-Rad, Hercules, CA, USA).

5.3.1.3 Determination of ATP1A4 activity in sperm

Please refer to Chapter 4 Section 4.4.6 for detailed experimental procedures.
5.3.1.4 Validation parameters for ATP1A4 activity assay

The ATP1A4 activity assay was validated by determination of linearity, precision, and accuracy. Linear dependency of $\text{P}_i$ formation were determined for varying time points and protein concentrations. Precision was evaluated through repeatability and reproducibility. To assess reproducibility (inter-assay variability), frozen-thawed semen sample from the same bull was assayed three times over a period of 1 wk to determine inter-assay CV. Repeatability (intra-assay variability) was assessed with multiple replicates ($n = 5$) from the same bull on a single day in the same reaction to calculate the intra-assay CV. Accuracy was determined through spike recovery and dilutional linearity. Known amounts of $\text{KH}_2\text{PO}_4$ (positive control, releases inorganic phosphate when mixed with water) was spiked into the biological sample (extracted sperm protein) and $\text{P}_i$ concentration (recovery values) was compared to spiked samples in extraction buffer alone. Recovery of $\text{P}_i$ was evaluated at various dilutions of spiked samples.

5.3.2 Flow cytometric evaluation of ATP1A4 content in fresh sperm

Percoll-washed sperm ($5 \times 10^6$) were incubated with 1 µl of Fixable Live and Dead Cell Stain (Thermo Fisher Scientific) for 30 min at RT. The live and dead cell stain incorporates into primary amines on the surface of live cells, resulting in a dim fluorescence, whereas both surface and internal primary amines of proteins are exposed to the stain in dead cells, thereby resulting in brighter fluorescence. The remainder of the procedure follows exactly the same protocol explained in Chapter 4 Section 4.4.5.
5.3.3 Immunolocalization of ATP1A4 in sperm

For immunostaining, sperm were adhered onto poly-l-lysine coated slides and subsequently fixed with 2.5% PFA for 15 min (treatment 1), fixed with 2.5% PFA and permeabilized with 0.1% Triton X-100 for 5 min (treatment 2), or fixed and permeabilized with – 20 °C methanol for 5 min (treatment 3), followed by washing in PBS and incubation with 10% normal chicken serum for blocking. Anti-ATP1A4 antibody was incubated at 1:100 dilution in 1% chicken serum overnight at 4 °C. Following washing in PBS, chicken anti-rabbit alexa 488 antibody was added at 1:1000 for 1 h at RT. Cells were washed in PBS and mounted with Vectashield containing DAPI and stored at – 20 °C pending imaging.

5.3.4 Statistical analyses

Data were analysed with commercial software (GraphPad Software Inc., La Jolla, CA, USA). Data from effects of detergents on ATP1A4 activity were analysed by two-way ANOVA, followed by Tukey’s post-hoc test. For all statistical analyses, p < 0.05 was considered significant.

5.4 Results

5.4.1 Validation of ATP1A4 enzyme activity

Sperm proteins extracted with sodium deoxycholate had a significantly higher enzyme activity compared to extraction with either Triton X-100 or CHAPS (Figure 5.1A). Furthermore, there was an increasing linear trend in P_i concentrations over 15 and 30 min time points with various protein concentrations. At higher protein concentrations (total protein of 100 and 160 µg/µl), linearity decreased, especially at longer incubation times (45 and 60 min; Figure 5.1B). Inter-
assay and intra-assay CVs ranged from 11 to 19% and 1.9 to 2.8%, respectively (Table 5.1 and 5.2). Spike recovery was close to 100% for low-, medium- and high-spikes (Table 5.3) and ranged from 98 to 127% for linearly diluted samples (Table 5.4).

**Figure 5.1.** Validation of ATP1A4 enzyme activity in frozen-thawed bull sperm. (A) Effects of 0.1% sodium deoxycholate, 0.25% Triton X-100, and 0.5% CHAPS, pH 7.4 on activity of ATP1A4. Total protein concentrations ranging from 100 to 150 µg/µl were isolated using the above-mentioned conditions were assayed for ATP1A4 activity by hydrolysis of ATP for 30 min in the presence of 1 mM ouabain. B) Linear dependency of P_i formation on incubation time was determined using intervals ranging from 15 to 60 min, whereas linearity of P_i formation on
protein content was determined using protein concentrations ranging from 20 to 160 µg/µl (based on sperm concentration in commercial semen straws [~40 x 10⁶/ml] and thawing 2 to 10 straws). Data shown were expressed as mean ± SEM (n = 4). Values without a common letter differed (P < 0.05).

Table 5.1: Precision of the enzyme assay evaluated by inter-assay CV.

<table>
<thead>
<tr>
<th>Sample no</th>
<th>Mean (pmol/µg of total protein/min)</th>
<th>SD (pmol/µg of total protein/min)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bull 1</td>
<td>28.28</td>
<td>5.53</td>
<td>19.53</td>
</tr>
<tr>
<td>Bull 2</td>
<td>10.14</td>
<td>1.18</td>
<td>11.72</td>
</tr>
</tbody>
</table>

Table 5.2: Precision of the enzyme assay evaluated by inter-assay CV

<table>
<thead>
<tr>
<th>Sample no</th>
<th>Mean (pmol/µg of total protein/min)</th>
<th>SD (pmol/µg of total protein/min)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bull 1</td>
<td>14.17</td>
<td>0.40</td>
<td>2.82</td>
</tr>
<tr>
<td>Bull 2</td>
<td>6.56</td>
<td>0.12</td>
<td>1.91</td>
</tr>
</tbody>
</table>
Table 5.3: Accuracy of the enzyme assay evaluated through spike recovery

<table>
<thead>
<tr>
<th>Sample no</th>
<th>Spiking concentration (pmoles/µl)</th>
<th>Observed value</th>
<th>Expected value</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bull 1</td>
<td>Low (50 pmoles/ µl)</td>
<td>22.02</td>
<td>22.29</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>Medium (125pmoles/ µl)</td>
<td>48.84</td>
<td>50.76</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>High (200 pmoles/ µl)</td>
<td>77.57</td>
<td>78.53</td>
<td>101</td>
</tr>
</tbody>
</table>

Table 5.4: Accuracy of the enzyme assay evaluated through dilutional linearity

<table>
<thead>
<tr>
<th>Sample no</th>
<th>Dilution factor (DF)</th>
<th>Observed value</th>
<th>Expected value</th>
<th>Observed value x DF</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bull 1</td>
<td>Undiluted (800 pmoles)</td>
<td>349.98</td>
<td>349.98</td>
<td>349.98</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>400 pmoles (1:2)</td>
<td>189.67</td>
<td></td>
<td>379.94</td>
<td>108</td>
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<tr>
<td></td>
<td>200 pmoles (1:4)</td>
<td>98.45</td>
<td></td>
<td>393.8</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td>100 pmoles (1:8)</td>
<td>50.78</td>
<td></td>
<td>406.24</td>
<td>116</td>
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<tr>
<td></td>
<td>50 pmoles (1:16)</td>
<td>25.67</td>
<td></td>
<td>410.72</td>
<td>117</td>
</tr>
</tbody>
</table>
5.4.2 Validation of ATP1A4 content in frozen-thawed bovine sperm by flow cytometry

Frozen-thawed sperm were exposed to various fixation and permeabilization conditions. Uniform staining of the sperm head was obtained after fixation with 2.5% PFA. However, sperm fixed with 2.5% PFA and subsequently permeabilized with 0.1% Triton X-100 had decreased staining intensity of ATP1A4, whereas no signal was obtained when sperm were fixed and permeabilised with methanol (Figure 5.2A). Since ATP1A4 content in viable sperm is physiologically significant, we investigated various amounts (0.75, 1.0 and 1.5 µl) of live and dead cell stain, with 1 µl of dye sufficient to obtain distinct dead and live sperm populations (Figure 5.2B). Gate settings and a representative flow cytometric profile showing the threshold for distinguishing live and dead sperm population and for recording ATP1A4 fluorescence from viable sperm, along with their corresponding dot plots, are shown in Figure 5.2C.

Figure 5.2
Figure 5.2 Validation of flow cytometry to determine ATP1A4 content in frozen-thawed bull sperm. (A) Optimization of fixation and permeabilization conditions for detection of ATP1A4 in sperm by immunofluorescence. Uniform staining of the sperm head was apparent in sperm fixed with 2.5% PFA, whereas an overall reduction in the staining intensity was noticed in sperm fixed with a combination of 2.5% PFA and 0.1% Triton X-100. No staining was apparent when sperm were fixed and permeabilized with methanol or after incubation with only secondary antibody in the absence of ATP1A4 antibody. (B) Optimization of concentration of the fixable live and dead cell stain by flow cytometry. Populations P2 and P3 represented dead and live sperm, respectively. (C) Frozen-thawed sperm were labelled with anti-ATP1A4 antibody and levels of fluorescence recorded; P1 represented the gated cell population that was analysed; P2 represented dead cells; P3 denoted the ATP1A4 fluorescence from viable population; Q1 & Q2 corresponded to dead cells with low and high ATP1A4 fluorescence, respectively; and Q3 & Q4
dot plots corresponded to viable sperm cells that had low and high ATP1A4 fluorescence, respectively.

5.5 Discussion

In this study, we developed a flow cytometric assay for quantification of ATP1A4 and validated a colorimetric assay for determination of its activity in frozen-thawed beef bull semen. Chemicals and reagents used in an enzyme assay should keep the protein of interest stable and active. Therefore, the detergent used for extraction of proteins should be carefully chosen (de Lima Santos & Ciancaglini 2000). In that regard, we compared three categories of detergents, sodium deoxycholate (ionic), Triton X-100 (non-ionic) and CHAPS (zwitterionic) for solubilisation of ATP1A4 from sperm plasma membrane and assayed for the enzyme activity. Triton X-100 and CHAPS detergents yielded significantly lower enzyme activity compared to sodium deoxycholate from sperm homogenates. One possible reason is that Triton X-100 inactivates proteins irreversibly by disrupting the quaternary structure of Na/K-ATPase (Elesmann 1983), whereas deoxycholate activates proteins (Dahl & Hokin 1974). The traditional method for assaying Na/K-ATPase activity from sperm is either a radioactive approach for detecting inorganic phosphorus (P_i) released during catalysis of ATP (Blanco & Mercer 1998) or a colorimetric approach with a citrate/arsenite/acetic acid solution (Zhao & Buhr 1996). There are issues to both of these approaches; radioactive methodology requires special disposal methods and has safety concerns, whereas colorimetric methods use strong acids that promote non-enzymatic hydrolysis of phosphates (ERNSTER & LINDBERG 1956). Consequently, we adapted a colorimetric method (Spokas & Spur 2001) using an ascorbic acid/ammonium molybdate combination that can detect low concentrations (10 nmol of P_i) from membrane
preparations but does not promote non-enzymatic hydrolysis of phosphates. Use of strong acids (e.g. trichlorocetic or perchloric) results in precipitation of proteins that must be pelleted before colour development is determined (Chifflet et al. 1988). In the Spokas and Spur procedure, sodium dodecyl sulfate was used to terminate the reaction by rapidly denaturing the enzyme, leaving proteins in solution and not affecting subsequent product measurement. Inter- and intra-assay CVs of our enzyme assay were <20%, consistent with other practical enzyme assays (Reed et al. 2002). Recovery (detection) of P_i in the assay was between 100 and 117% and in accordance with a standard range of 90 to 120% (Wood et al. 2011). However, at higher total protein concentrations and prolonged incubation, there was a loss of linearity. Perhaps at higher enzyme concentrations, the rate of ATP hydrolysis by ATP1A4 gradually diminished with time, as ADP accumulated (Emmelot & Bos 1966). Therefore, we decided to assay ATP1A4 enzyme activity by indirectly measuring P_i release at 30 min post incubation.

Since procedures associated with semen processing could affect exposure of antigenic motifs, we optimised fixation and permeabilization conditions for detection of ATP1A4 antigen in sperm. We used a custom antiserum against a peptide sequence in the N-terminal (cytoplasmic) side of ATP1A4, which required permeabilization for the antibody to gain access to the intracellular epitope. Accordingly, we used either Triton X-100 or methanol to permeabilize cells which resulted in inadequate staining of ATP1A4 in sperm. Since ATP1A4 is membrane-bound, the non-ionic detergent Triton X-100 could disrupt protein association with the lipid bilayer; perhaps the antigenic site of ATP1A4 was sensitive to membrane perturbations, thereby reducing intensity of immunolabeling in sperm (Ariano et al. 1998). Furthermore, extraction with Triton X-100 significantly decreased enzyme activity. Overall, we concluded that Triton X-100 was unsuitable for detection of ATP1A4 antigen in sperm. In contrast, sperm fixed
with PFA had intense localisation of ATP1A4 in the sperm head, consistent with the known function of this enzyme. In some cells, PFA is known to increase membrane permeability (Amidzadeh et al. 2014). Perhaps partial permeabilization during fixation increased accessibility of the antibody to the ATP1A4 epitope, located on the cytoplasmic side. Flow cytometry was our preferred detection method, as it is commonly used under field conditions to assess various sperm functional parameters. Since sperm were fixed with PFA, standard dyes such as SYBR-14 or propidium iodide were inappropriate to distinguish the live and dead cell population by flow cytometry. Therefore, we optimised a fixable live and dead stain that selectively incorporated into the primary amines of proteins, thereby preserving the staining pattern following formaldehyde fixation. Overall, these assays were deemed effective and reliable. Therefore, using these assays, we subsequently investigated association among ATP1A4 content, activity and field fertility in dairy bulls (Chapter 6).
Chapter Six: **Testis-specific isoform of Na/K-ATPase (ATP1A4) regulates sperm function and fertility in dairy bulls through mechanisms involving reactive oxygen species, intracellular calcium and actin polymerization**

**6.1 Abstract**

Traditional BBSE eliminates bulls whose semen is grossly abnormal; however, bulls classified as satisfactory potential breeders still vary in field fertility, implying submicroscopic differences in sperm characteristics. ATP1A4 is involved in regulation of sperm motility and capacitation in bulls through well-established enzyme activity and signaling functions. The objective was to determine ATP1A4 content and activity and their relationship to post-thaw sperm function and field fertility, using semen samples from LF and HF Holstein bulls (n = 20 each) with known FERTSOL rates (measure of field fertility, based on 56-d nonreturn rates). Frozen-thawed sperm from HF bulls had higher ATP1A4 content and activity compared to LF bulls. Furthermore, post-thaw sperm from HF bulls showed an increase in tyrosine phosphorylation, ROS, F-actin content, and low intracellular calcium in comparison to LF bulls. Subsequent incubation of HF bull sperm with ouabain further augmented the post-thaw increase in tyrosine phosphorylation, ROS production, and F-actin content, whereas the increase in intracellular calcium was still low compared to LF bull sperm. Content and activity of ATP1A4, ATP1A4-induced ROS, F-actin, and calcium were significantly correlated with fertility. In conclusion, we inferred that ATP1A4 may regulate sperm function through mechanisms involving ROS, F-actin, calcium and tyrosine phosphorylation in frozen-thawed sperm of HF and LF bulls.
6.2 Introduction

Traditional BBSE is intended to eliminate bulls that are grossly abnormal. However, semen from bulls deemed satisfactory on a BBSE often differ in pregnancy rates by 20 – 25% (Larson & Miller 2000). Therefore, we inferred that there may be submicroscopic differences among sperm with apparently normal morphology. Consequently, there is a need to develop marker-based laboratory assays to complement traditional BBSE and better predict these variations in bull fertility. Sutovsky (Sutovsky et al. 2015) reported negative and positive biomarkers of fertility. Negative biomarkers (e.g. ubiquitin) were detected predominantly in defective sperm, whereas positive biomarkers (sperm proteins) were present in morphologically normal, motile sperm except that the marker may either be down- or up-regulated or post-translationally modified. The bovine artificial insemination (AI) industry uses elite bulls, including HF and LF bulls (3% above and below the breed average, respectively) for breeding purposes. Consequently, comparing sperm from bulls with varying levels of fertility may identify submicroscopic differences (for example, expression of specific proteins) in sperm and determine molecular markers of fertility.

Na/K-ATPase transduces the energy from ATP hydrolysis to catalyse the exchange of cytoplasmic Na⁺ for extracellular K⁺ (Kaplan 2002). In somatic cells, the ionic gradients generated by the Na/K-ATPase play a central role in maintaining cell volume and pH and resting membrane potential (Hoffmann & Simonsen 1989). Na/K-ATPase is an oligomer composed of two major polypeptides, the α and β subunits. Interestingly, four isoforms of the Na/K-ATPase α subunit, namely α1, α2, α3, and α4, are expressed in mammalian tissues. Although α1 is present in nearly every tissue and it is believed to maintain the basal ion gradients in the cells, the other α polypeptides are more restricted in their expression and appear to play tissue-specific roles.
(Blanco & Mercer 1998a, Mobasher et al. 2000b). In particular, the α4 isoform is found in the testis, where it is specifically expressed in the male germ cells after meiosis (Woo et al. 1999, Blanco et al. 2000). Moreover, α4 has a very high sensitivity to ouabain, a characteristic that most prominently distinguishes it from the α1 isoform (Blanco et al. 1999). The presence of a Na/K-ATPase catalytic subunit with particular attributes, such as those of α4, suggests that this polypeptide has a specific role in the physiology of sperm. In support of this, inhibition of α4 activity diminished total motility of spermatozoa (Woo et al. 1999, Sanchez et al. 2006).

Na/K-ATPase-ouabain interaction stimulates phosphorylation of downstream effectors such as Src, EGFR which in turn recruit adaptor proteins like Grb2, SOS resulting in activation of MAPK pathway and generation of second messengers such as calcium and reactive oxygen species (ROS) (Ullrich & Schlessinger 1990, Liu et al. 2000, Haas et al. 2000). Furthermore, downstream events associated with Na/K-ATPase signaling (increase in ROS, release of intracellular calcium and actin polymerization) resemble events during sperm capacitation.

Controlled release of ROS drives capacitation-associated changes in sperm through redox regulation of protein tyrosine phosphorylation and subsequent hyperactivation (de Lamirande & Gagnon 1998, Rivlin et al. 2004). Factors that trigger tyrosine phosphorylation of proteins can stimulate polymerization of actin monomers (G-actin) to actin polymers (F-actin). Bicarbonate activation of cAMP/PKA pathway and oxidants such as H₂O₂ induce tyrosine phosphorylation of gelsolin (an actin binding protein) which has a key role in F-actin formation (Liu et al. 2000, Brener et al. 2003, Shahar et al. 2014). Apart from F-actin formation, in response to cAMP, there is a pulsatile pattern of calcium release from intracellular stores (Aitken & Nixon 2013) which in turn stimulates gelsolin to assemble actin filaments (Ickowicz et al. 2012) in the sperm head. Furthermore, based on a computational approach, the capacitation network of boar sperm
contained three important nodes, calcium, ATP and actin polymerization that are linked to one another (Bernabo et al. 2011). Collectively, it appears that ROS, calcium and F-actin create a self-perpetuating cascade with one process stimulating another during capacitation and thereby affecting sperm fertility. In that regard, several studies have investigated the correlation of various sperm functional parameters, including ROS, intracellular calcium (individually and/or collectively) to fertility in frozen-thawed sperm (Collin et al. 2000, Peris 2008, Simoes et al. 2013, Del Olmo et al. 2014).

We previously reported that sperm from HF bulls were in transition to hyperactivation, whereas LF bull sperm had only forward progression (Shojaei et al. 2012). Since ATP1A4 is involved in regulation of sperm motility and capacitation, aims of the present study were to: i) investigate ATP1A4 content and activity and measure sperm functional parameters such as tyrosine phosphorylation, ROS, calcium, actin polymerization (indicated by F-actin content) which also serve as downstream effectors of ATP1A4 signaling in HF and LF bull sperm; ii) ATP1A4-mediated regulation of tyrosine phosphorylation, ROS, calcium and actin polymerization in HF and LF bull sperm; and iii) determine associations among fertility, ATP1A4 content and activity, ATP1A4 induced ROS, calcium, and F-actin in HF and LF bulls.

6.3 Materials and methods

6.3.1 Frozen semen production

The study reported was approved by the University of Calgary Institutional Animal Care and Use committee (protocol number M09078). Bull management, frozen semen production and fertility evaluations were done by Semex Alliance Inc. as detailed below. Forty Holstein bulls maintained under standard housing and feeding conditions at this AI center were used for this study. Based
on a standard BBSE, all bulls were classified as satisfactory breeders. Semen was collected, extended and cryopreserved in a standard Tris-egg yolk-glycerol extender (200 mM Tris, 66.7 mM Citric acid, 55.5 mM D-fructose, 594 mM glycerol, 1.10 Benzylpenicillin-G IU/L, 1.10 Streptomycin sulphate IU/L, 0.51 mM Lincomycin, 0.228 mM Spectinomycin, 200 mL/L Egg yolk). Approximately 15 x 10^6 sperm were loaded into 0.25 ml straws and batches with a post-thaw motility of atleast 25% motility and a rate of 3 were used for insemination in a minimum of 10 herds. The percentage of cows having not returned to estrus within 56 d after insemination (56-d non-return rate; NRR) was determined optimal due to a high correlation of 96% with both the 60 to 90-d NRR and 75-d NRR (Doormaal 1998). Estrus detection rate for Holstein herds in Ontario ranged between 39 – 42% in small herds (<50 cows) and from 39 - 54% in larger herds (>300 cows). Fertility was calculated based on 56-d NRR and was adjusted to include the month of insemination, age of cow at insemination, semen price, breed of service sire, insemination technician and herd management to produce the FERTSOL value. Bulls were classified as either high fertility (FERTSOL > +3.6; range: 3.6 to 6.7) or low-fertility (FERTSOL < -4.0; range: -4 to -19). Frozen semen from HF and LF bulls (n = 20 each) were provided by Semex Alliance Inc. and used for this study as described below.

6.3.2 Processing of frozen-thawed sperm from HF and LF bulls

Frozen straws of semen were thawed (60 s) in a water bath maintained at 37 °C. Briefly, sperm was gently layered on top of 45% percoll and centrifuged (400 x g for 20 min at RT). Pellets were washed once again in PBS to remove percoll and number of sperm in the pellet was determined using a hemocytometer. For experiments detailed in Results Sections 6.4.1 and 6.4.2,
samples were processed as mentioned above and immediately analyzed without further incubation (0 h). For experiments related to Results Section 6.4.3, the sperm sample was divided into two aliquots and incubated with and without 50 nM ouabain in Sp-TALP containing 1 mM pyruvate, 25 mM NaHCO₃, 2 mM Ca²⁺ at 39 °C in a humidified incubator with 5% CO₂ for 2 h.

6.3.3 Preparation of sperm crude membrane extracts
Sperm crude membrane extracts were prepared as described in Chapter 5, Section 5.3.1.2.

6.3.4 Enzyme activity and flow cytometric evaluation of ATP1A4 content in frozen thawed sperm of HF and LF bulls
Please refer to Chapter 4, Sections 4.4.5 and 4.4.6 for relevant experimental methodologies.

6.3.5 Measurement of intracellular ROS in HF and LF bull sperm
The intracellular ROS content in HF and LF bull sperm was measured using 2′,7′ – dichlorodihydrofluorescein diacetate (H₂-DCFDA, Sigma Aldrich) using flow cytometry. In this assay, H₂-DCFDA is a cell-permeable non-fluorescent probe which is de-esterified intracellularly and becomes highly fluorescent 2′,7′-dichlorofluorescin after oxidation. Intensity from 2′,7′-dichlorofluorescin increases as ROS production increases within the cell. Briefly, percoll-washed sperm (10 x 10⁶) was re-suspended in 1 ml PBS and incubated with H₂-DCFDA dye at a final concentration of 0.5 µM at 35 °C in the dark for 30 min before addition of propidium iodide (PI; final concentration of 24 µM) and immediately analysed.
6.3.6 Measurement of intracellular calcium in HF and LF bull sperm

Intracellular sperm calcium concentrations were measured as described (Harrison et al. 1993). When calcium is not bound, the cell permeable probe fluo-3 AM is non-fluorescent but on calcium ion binding, it becomes highly fluorescent. Percoll-washed sperm cells (10 x 10⁶) were loaded with the fluorescent probe fluo3-AM (Thermo Fisher Scientific) at a final concentration of 10 µM for 30 min at 35 °C in the dark in the presence of 0.02% pluronic acid. Thereafter, fluo-3 loaded sperm was resuspended in PBS before addition of PI and subsequently analysed by flow cytometry.

6.3.7 Flow cytometric evaluation of F-actin content in HF and LF bull sperm

Formation of F-actin in sperm was evaluated by use of FITC phalloidin (a form of phallatoxin; Thermo Fisher Scientific) which binds and stabilizes polymeric F-actin and is unable to bind to monomeric G-actin. Post-thaw semen was washed in percoll as explained above and 10 x 10⁶ percoll washed sperm was added to 1 µl of Fixable Live and Dead Cell Stain (Thermo Fisher Scientific) and incubated for 30 min at RT. Subsequently, sperm were fixed with 4% PFA for 20 min, washed, permeabilized with 0.1% Triton X-100 for 5 min and incubated with 0.33 µM FITC-phalloidin for 60 min at 37 °C. Thereafter, samples were washed in PBS and used for further analysis.

6.3.8 Flow cytometry settings for fluoroprobes used in the study

For assessment of viability, DCFDA (ROS) and Fluo-3 (calcium) probes which fluoresce green were combined with PI (red), whereas FITC-phalloidin (green) was combined with Fixable violet live and dead cell stain (violet). Data were acquired using an Attune Acoustic Focusing
Cytometer (BD Biosciences, Mississauga, ON, Canada). The excitation source was a 488 nm laser for FITC and PI dyes and a 405 nm laser for the fixable live and dead (violet) dye. Voltage settings used were as follows: FSC – 1250, SSC – 1650, FITC – 1500, PI – 2050, violet – 1650. Subsequently, detector 1 (emission range of 450 ± 20 nm) was used for detecting violet fluorescence (viability), detector 2 (emission range of 530 ± 15 nm) was used for detecting green (FITC-phalloidin/DCFDA/fluo-3 fluorescence and detector 3 (emission range of 570 ± 10 nm) was used for detecting PI (viability) fluorescence. A total of 20 x 10³ events were recorded in the form of a scatter plot and histogram. Resulting flow cytometric data were analysed by computing the relative median fluorescence intensity (MFI) of each sample.

6.3.9 Assessment of sperm tyrosine phosphorylation

For evaluation of tyrosine phosphorylation, 5 x 10⁶ sperm was concentrated (10,000 x g, 3 min, RT) and the pellet washed (10,000 x g, 5 min) in 1 ml of PBS containing 0.2 mM Na₂VO₃. The pellet was boiled in sample buffer for 5 min at 100 °C and the supernatant separated on 10% PAGE gels and electrotransferred to nitrocellulose membranes. After blocking with 5% (w/v) skim milk in TTBS for 1 h, the membrane was incubated with phosphotyrosine antibody (1:10,000; Millipore, Billerica, MA, USA) overnight at 4 °C. Membranes were washed (3X) in TTBS for 10 min and subsequently incubated with HRP conjugated goat anti-mouse IgG (1:5,000) for 1 h at RT. Following washing, immunoreactive bands were detected using chemiluminescence. Membranes were stripped and probed with β-tubulin antibody (1:10,000; Sigma Aldrich) to ensure equal protein loading.
6.3.10 Statistical analyses

Data were analysed with a statistical software program (STATA version 12, Statacorp LP, College Station, TX, USA). Wherever necessary, data were log-transformed before further analysis. Differences in the post-thaw ATP1A4 enzyme activity was analysed by student’s t-test. Median fluorescent values for ATP1A4 content, ROS, calcium and F-actin from flow cytometry between HF and LF bulls were analysed by Mann-Whitney U test. Spearman correlation coefficients were calculated to determine the nature of relationship between ATP1A4 content, activity, ATP1A4-induced ROS, calcium, F-actin and fertility. Multivariate regression analysis was performed to determine the influence of ATP1A4 content, activity, ATP1A4-induced ROS, calcium and F-actin content on bull fertility. For all statistical analyses, p < 0.05 was considered significant.

6.4 Results

6.4.1 ATP1A4 content and activity in HF and LF bull sperm

Since ATP1A4 is the major isoform of Na/K-ATPase in sperm, content of ATP1A4 in the post-thaw sperm of HF and LF bulls was determined by immunoblotting. The HF bull sperm had higher content of ATP1A4, detected as a single band at 110 kDa, compared to LF bull sperm (Figure 6.1A). Since content of ATP1A4 in viable sperm is physiologically relevant compared to ATP1A4 expression from both dead and live sperm, ATP1A4 fluorescence were quantified from viable sperm from HF and LF bulls using flow cytometry. The ATP1A4 fluorescence was higher in HF bulls, manifested by a higher relative median fluorescence and the HF histogram pushed more towards the right (right shift) on the FITC log scale (x-axis) compared to LF bulls. A representative histogram (Figure 6.1B) and bar graph (Figure 6.1C) illustrated overall median
fluorescence intensity from all HF and LF bulls used in this study. The enzyme activity was measured by hydrolysis of the substrate ATP resulting in the accumulation of inorganic phosphate (P_i) in the reaction mixture. Consistent with increased ATP1A4 content (as demonstrated through western blotting and flow cytometry), HF bulls had higher enzyme activity compared to LF bulls in their post-thaw sperm (Figure 6.1D).

**Figure 6.1**

![Image of western blot and flow cytometry graphs](image-url)
**Figure 6.1. Post-thaw content and activity of ATP1A4 from HF and LF bulls.** Representative immunoblots (A) of ATP1A4 content from a subpopulation of HF and LF bulls (n = 14 each) used in this experiment. Sperm pellet obtained after detergent extraction of ATP1A4 from HF and LF samples was boiled in sample buffer to probe for β-tubulin to ensure equal sample loading. (B) Sample histogram (from flow cytometry) of ATP1A4 content from a HF and LF bull. (C) Overall median fluorescence data for the ATP1A4 content from viable sperm of all LF and HF bulls (n = 20 each). (D) Bar graph of ATP1A4 activity (pmoles of Pᵢ/µg protein/min) from all LF and HF bulls (n = 20 each). Data shown were expressed as mean ± SEM. a,bValues without a common letter differed (P < 0.05).

### 6.4.2 Post-thaw sperm functional parameters in HF and LF bull sperm

Sperm functional parameters, including tyrosine phosphorylation, intracellular ROS, calcium and F-actin content were determined in frozen-thawed sperm of HF and LF bulls. Sperm proteins at ~200, 150, 100 and 35 kDa (indicated by black solid arrows) had a higher intensity of tyrosine phosphorylation in HF bulls (Figure 6.2A). Additionally, sperm from HF bulls had higher content of ROS compared to LF bulls, shortly after thawing and a representative histogram is shown in Figure 6.2B. Similarly, for actin polymerization post-thaw sperm from HF bulls had more F-actin than LF bulls which is evident in the histogram (Figure 6.2C). In contrast, a sample histogram obtained from post-thaw sperm of LF bulls had greater intracellular calcium concentration compared to HF bulls (Figure 6.2D). Bar graphs depicting the overall median fluorescence intensity for ROS, calcium and F-actin from all HF and LF bulls used in this study were shown in Figure 6.2B, C and D.
Figure 6.2

(A) Comparison of protein expression in HF and LF bulls. The blots show bands corresponding to phosphotyrosine and β-tubulin.

(B) Graph showing the relative levels of ROS (reactive oxygen species) in HF and LF groups. The graph indicates a statistically significant difference (*) between the two groups.
Figure 6.2. Post-thaw sperm functional parameters in HF and LF bulls. Representative immunoblot (A) of HF and LF total sperm proteins (n = 4 each); note differences in tyrosine phosphorylation (indicated by black solid arrows) in post-thaw sperm. Protein loading was assessed by β-tubulin. Representative histograms showing median fluorescence intensity values from one HF and one LF bull for their (B) ROS, (C) F-actin and (D) calcium content. Corresponding bar graphs depict overall median fluorescence values for ROS, F-actin and calcium content from all HF and LF bulls. Data shown were expressed as mean ± SEM (n = 20 each). * P < 0.05.
6.4.3 ATP1A4 induced changes in sperm functional parameters in HF and LF bull sperm

There was a higher content of ATP1A4 and higher enzyme activity with concurrent increase in tyrosine phosphorylation, ROS and F-actin content, along with low intracellular calcium in HF bulls. Since the signaling function of ATP1A1 is involved in regulation of ROS, calcium, F-actin formation and tyrosine phosphorylation in somatic cells, we investigated whether such a mechanism could operate in sperm via ATP1A4. In order to determine if higher ATP1A4 content in HF bulls was responsible for the above-mentioned changes in these functional parameters, sperm from HF and LF bulls were incubated with ouabain. Incubation of HF bull sperm with ouabain for 2 h showed higher content of tyrosine phosphoproteins, intracellular ROS and F-actin in relation to LF bulls (in a time-dependent manner). Sperm proteins isolated from ouabain treated HF bull sperm had higher intensity of tyrosine phosphorylation at 150, 100, 75 and 30 kDa (indicated by black solid arrows) compared to band intensities from ouabain-treated LF bull sperm (Figure 6.3A). High intracellular ROS and F-actin were manifested by an increasing trend line in HF bulls, with each point showing the difference between the median fluorescence intensity values obtained from control and ouabain treated groups (Figure 6.3B and C). However, trendlines for intracellular calcium were lower in HF bulls in contrast to the LF bulls (Figure 6.3D). In all end points assessed, HF and LF ouabain-treated sperm had increased ROS, F-actin, protein tyrosine phosphorylation and calcium compared to control sperm.
Figure 6.3

A

<table>
<thead>
<tr>
<th>HF bulls</th>
<th>LF bulls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 0 h</td>
<td>Ouabain 0 h</td>
</tr>
<tr>
<td>Control 2 h</td>
<td>Ouabain 2 h</td>
</tr>
</tbody>
</table>

- phosphotyrosine
- β-tubulin

B

![Graph showing ROS vs time](image)

- LF bulls
- HF bulls

C

![Graph showing F-actin vs time](image)

- HF bulls
- LF bulls
Figure 6.3. Assessment of ATP1A4-induced changes in sperm functional parameters in HF and LF bulls. Representative immunoblot (A) of sperm proteins (indicated by black arrows) derived from control and ouabain-treated groups of HF and LF bulls for 2 h, and probed with phosphotyrosine antibody (β-tubulin was used as a loading control). Patterns of intracellular (B) ROS generation (C) F-actin (D) intracellular calcium release in HF and LF bull sperm were represented by a trendline, with each dot/square representing normalised median fluorescence intensity values obtained from control and ouabain-treated groups at 0 h and 2 h, respectively. Data shown were expressed as mean ± SEM (n = 4). *, # P < 0.05.

6.4.4 Relationship between ATP1A4 content, activity, sperm functional parameters and fertility

The ATP1A4 content, determined through semi-quantitative densitometry (Western blotting) and flow cytometry, enzyme activity, ATP1A4-induced ROS and F-actin, were positively correlated to fertility (significant), whereas ATP1A4-induced calcium release had a significant negative relationship with fertility (Figure 6.4 and Table 6.1). Among independent variables, enzyme activity, ROS, F-actin were positively correlated, whereas calcium was negatively correlated to ATP1A4 content (both were significant; Table 6.1) Since these experimental variables were significantly correlated to each other, ATP1A4 content (flow cytometry) was chosen as the
single predictor variable to determine changes in fertility. The predictive value of the ATP1A4 content for fertility was determined using regression analysis and calculated as fertility = -37.58 + 5.02 ATP1A4 content. Based on regression equation, ATP1A4 content influenced fertility (p < 0.05; Figure 6.5).

**Figure 6.4**

A. \( r = 0.6, p = 0.001 \)

B. \( r = 0.72, p = 0.0002 \)

C. \( r = 0.42, p = 0.032 \)

D. \( r = 0.47, p = 0.007 \)

E. \( r = -0.52, p = 0.003 \)

F. \( r = 0.67, p = 0.000049 \)
Figure 6.4 Correlations of various predictor variables to fertility in Holstein bulls.
Association among ATP1A4 content (A: densitometry; B: flow cytometry); enzyme activity of ATP1A4 (C); ATP1A4-induced ROS (D), F-actin (E), and calcium (F), and fertility of dairy bulls, as demonstrated by correlation analysis.

Figure 6.5 Regression analysis for prediction of fertility in Holstein bulls
Table 6.1: Correlation of ATP1A4 content (semi-quantitative densitometry and flow cytometry values), enzyme activity, ROS, calcium and F-actin with fertility in HF and LF bulls

<table>
<thead>
<tr>
<th>Variables</th>
<th>ATP1A4 content (densitometry)</th>
<th>ATP1A4 content (flow cytometry)</th>
<th>Enzyme activity</th>
<th>ROS</th>
<th>Calcium</th>
<th>F-actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fertility</td>
<td>0.6081*</td>
<td>0.7294*</td>
<td>0.4208*</td>
<td>0.4790*</td>
<td>-0.5245*</td>
<td>0.6715*</td>
</tr>
<tr>
<td>ATP1A4 content (densitometry)</td>
<td>-</td>
<td>0.6603*</td>
<td>0.2231</td>
<td>0.3951*</td>
<td>-0.3987*</td>
<td>0.4392*</td>
</tr>
<tr>
<td>ATP1A4 content (flow cytometry)</td>
<td>-</td>
<td>-</td>
<td>0.4448*</td>
<td>0.3651*</td>
<td>-0.4920*</td>
<td>0.5595*</td>
</tr>
<tr>
<td>Enzyme activity</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.0178</td>
<td>-0.0897</td>
<td>0.4320*</td>
</tr>
<tr>
<td>ROS</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-0.3342</td>
<td>0.1745</td>
</tr>
<tr>
<td>Calcium</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-0.1294</td>
</tr>
<tr>
<td>F-actin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* P < 0.05
6.5 Discussion

Since mature sperm DNA is generally transcriptionally quiescent, sperm functions are regulated by proteins already present without additional protein synthesis with few exceptions. Therefore, sperm proteins may serve as molecular markers for variations in fertility of bulls or semen classified as satisfactory potential breeders. In this study, we evaluated ATP1A4 content and activity in frozen-thawed sperm from satisfactory potential breeders with varying levels of fertility, and investigated potential mechanisms by which this protein regulates fertility.

Content of ATP1A4 and enzyme activity were higher in HF bulls compared to LF bulls. A possible explanation for the difference in content could be attributed either to increased sensitivity to cryo-elution (leakage of sperm components) of ATP1A4 from the sperm membrane of LF sires during the process of freeze-thawing or these bulls inherently might have had a lower content of ATP1A4. Unfortunately, it was not possible to investigate the latter possibility, due to the lack of fresh semen from these bulls. Lateral phase separation of lipids and subsequent lateral reordering of membrane components followed by the loss of PUFA and cholesterol are common after freeze-thawing, which lead to leakage of many sperm components (cryo-elution). Therefore, it is possible that membrane and cytoplasmic bound proteins and enzymes (Lasso et al. 1994, Ollero et al. 1998, Lessard et al. 2000, Gadea et al. 2004) were cryo-eluted from sperm. Due to significant membrane alterations in LF bull sperm (Shojaei et al. 2012), coupled with the fact that Na/K-ATPase is extremely sensitive to its lipid environment (Duran et al. 2010), perhaps ATP1A4 is cryo-eluted from the surface in higher amounts, leading to the decreased content of ATP1A4 in frozen-thawed sperm from LF bulls. Cryo-elution of other sperm surface proteins such as superoxide dismutase and P25b, have been described for human and bovine sperm, respectively (Lasso et al. 1994, Lessard et al. 2000).
Furthermore, we demonstrate herein, apparently for the first time, a significant predictive value of ATP1A4 content to in vivo bull fertility. To determine relationships between sperm proteins and bull fertility, it is critical to use in vitro procedures with good accuracy, repeatability, and objectivity. In that regard, flow cytometry was our primary choice, whereas immunoblotting was a secondary tool to complement flow cytometry data. Both approaches used a custom antiserum against a peptide sequence in the N-terminal (cytoplasmic) side of ATP1A4. During the procedure, cells were fixed with paraformaldehyde, which could increase membrane permeability (Amidzadeh et al. 2014). Perhaps partial permeabilization during fixation increased accessibility of the antibody to the ATP1A4 epitope, located in the cytoplasmic side. Flow cytometry analysis of cells is based on their size; therefore, the presence of contaminants (e.g. egg yolk particles from extender) could affect outcome (Kennedy et al. 2011). However, in our study, we excluded all non-sperm events by appropriate gating, based on forward and side scatter properties. Furthermore, a viability dye was used to quantify relative content of ATP1A4 exclusively from viable sperm due to its functional significance. Even though both western blotting and flow cytometry yielded comparable results with regard to fertility, use of flow-cytometry should be more practical than immunoblotting, since the former has already been adapted to evaluate several other sperm functions in a commercial AI setting (Graham 2001, Hossain et al. 2011). Apart from inhibiting enzyme activity, binding of ouabain to Na/K-ATPase activates multiple downstream pathways, including increase in ROS, release of intracellular calcium, actin polymerisation, and tyrosine phosphorylation of proteins. All of the above-mentioned parameters of Na/K-ATPase-ouabain signaling are very similar to molecular events during capacitation. Consequently, these downstream effectors which also serve as sperm...
functional parameters were measured to determine if ATP1A4 content was related to fertility in HF and LF bulls.

In somatic cells where the ubiquitous isoform (ATP1A1) of Na/K-ATPase predominates, communication between ATP1A1 and ROS forms a positive feedback loop, with ROS activating the signaling function of ATP1A1, which in turn stimulates mitochondrial production of ROS (Xie et al. 1999). In sperm, generation of ROS is a normal physiological process and controlled concentrations of ROS function as important second messengers in signaling during sperm capacitation. However, imbalances between ROS generation and scavenging activity are deleterious to sperm function (Sharma & Agarwal 1996). Even though it is widely accepted that increased ROS was related to cryo-capacitation (Bailey et al. 2000) and HF sperm had low ROS concentration (Simoes et al. 2013, Del Olmo et al. 2014), the results from our flow cytometry study suggest otherwise. One caveat associated with all studies involving flow cytometric detection is the inability to establish a minimum threshold value for ROS levels; any value above the threshold will be categorised as higher ROS production, whereas values below the threshold will be classified as low in ROS generation (Zini & Sigman 2009). Due to the lack of a consensus value, comparisons can be made only within a study and not across studies, which use different protocols, fluorescent probes and different cytometers for analysis. We believe that in our study, HF and LF sperm might have undergone a phenomenon different from cryo-capacitation. This could be attributed to different sperm subpopulations; one subpopulation that has normal plasma membrane with a better cryotolerance and another subpopulation having capacitation-like changes (Farstad 2012). Perhaps there is a higher percentage of normal sperm with a healthy, viable plasma membrane compared to the cryo-capacitated sperm subpopulation in HF and LF groups that is responsible for a physiological response after thawing. Furthermore,
the higher level of ROS in HF sperm may actually correspond to the minimum threshold needed for successful capacitation. Calcium is a universal intracellular second messenger with dynamic roles in hyperactivation and sperm capacitation. In somatic cells such as cardiac myocytes, ouabain regulates intracellular calcium via the Na/K-ATPase (Kelly & Smith 1993). Furthermore, a direct interaction between the α subunit of Na/K-ATPase and IP₃R, an intracellular calcium store receptor has been identified in several studies (Miyakawa-Naito et al. 2003, Yuan et al. 2005). Ouabain-activated signalplex can recruit and activate PLC-γ1 and the activated PLC-γ1 can produce the ligand IP₃, which binds to IP₃R (Yuan et al. 2005) and opens intracellular calcium stores, leading to an increase in intracellular calcium concentration. Subsequently, clearance pumps, including the plasma membrane Ca²⁺-ATPase (PMCA) and sodium-calcium exchanger (NCX) function in order to remove the transient increase in cytosolic calcium concentration, thereby keeping intracellular concentrations in a low resting state (Wennemuth et al. 2003, Olson et al. 2011). The existence of PLC isoforms, presence of IP₃ ligand and IP₃R, PMCA, and NCX has already been confirmed in sperm by various studies (Walensky & Snyder 1995, Ho & Suarez 2001, Parrington et al. 2002, Wennemuth et al. 2003). Considering that sperm are equipped with the machinery needed for ouabain-induced calcium release, it is possible that low concentrations of ouabain would activate a similar pathway by acting on ATP1A4 during capacitation. Previous studies have indicated the cytosolic calcium concentrations were negatively correlated with fertility; HF sperm had low intracellular calcium compared to LF sperm (Collin et al. 2000, Peris 2008). Consistent with other studies, we also noticed that HF bull sperm had low intracellular calcium, despite a high ATP1A4 content. It is possible that calcium clearance mechanisms might have been activated in HF bull sperm, which could have removed the increase in intracellular
calcium induced by ouabain by the end of 2 h. In LF bulls, due to the presence of a defective plasma membrane (Shojaei et al. 2012), sperm could have suffered from inefficient calcium clearing mechanisms, which might have contributed to the increase in the calcium concentrations at the end of 2 h. In summary, it is noteworthy that calcium increased in both fertility groups in response to ouabain, albeit at a higher magnitude in LF sperm. Contrary to our results, (Thundathil et al. 2006) reported no increase in cytosolic calcium concentrations in fresh sperm after incubation with ouabain. It is likely that several variables, including the source of sperm (fresh versus frozen thawed), duration of calcium measurement (4 versus 2 h) and the fluoroprobes (Indo-1 AM versus fluo-3) could have contributed to the differences in results in the presence of ouabain. Nevertheless, a significant correlation between ATP1A4-induced calcium content and bull fertility was clearly evident in the present study.

Regulation of actin dynamics is pivotal for many sperm processes, including capacitation. Two putative actin-binding sites have been identified in the α-subunit of Na/K-ATPase and direct binding with actin filaments stimulate Na/K-ATPase activity (Cantiello 1995). Even though studies of F-actin are very limited in frozen-thawed sperm, somatic cells provide abundant clues regarding F-actin regulation in sperm. In epithelial cells, Na/K-ATPase was involved in assembling actin at the tight junctions by modulating the Rho GTPase activity (Rajasekaran et al. 2001, Rajasekaran & Rajasekaran 2003). In non-epithelial cells, targeted production of ROS modulates actin reorganisation through a process that involves the GTP binding protein Rho and the actin binding proteins cofilin (Carlier et al. 1999, Moldovan et al. 2000) or gelsolin (Shahar et al. 2014) Therefore, perhaps, increased ROS contributes to increase in actin polymerisation in HF bull sperm. In sperm, Rho protein is localised to the acrosome
(Baltierrez-Hoyos et al. 2012) and is involved in actin remodeling in capacitation of fresh sperm (Brener et al. 2003).

In the present study, there was a link between ATP1A4 content and activity with in vivo bull fertility through mechanisms involving ROS generation, calcium release and actin polymerization. These results may aid in development of novel laboratory assays for better prediction of fertility of bulls that are classified as satisfactory potential breeders based on traditional BBSE. This could prevent subfertile semen from entering the market, thereby improving the efficiency of cattle reproduction. Moreover, it is likely that single nucleotide polymorphisms (SNPs) may be associated with this phenotype. Identifying such genetic markers and its association with other production traits may enable genetic companies to utilize this information for genome-enhanced selection of bulls.
Chapter Seven: The ubiquitous isoform of Na/K-ATPase (ATP1A1) regulates junctional proteins, connexin 43 and claudin 11 via Src-EGFR-ERK1/2-CREB pathway in prepubertal rat Sertoli cells

7.1 Preamble

The entire chapter has been submitted to Biology of Reproduction and is currently under peer-review

Title: The ubiquitous isoform of Na/K-ATPase (ATP1A1) regulates junctional proteins, connexin 43 and claudin 11 via Src-EGFR-ERK1/2-CREB pathway in prepubertal rat Sertoli cells

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G.D. performed 100% of the experiments, analysed the data and wrote the manuscript. J. T. supervised the project. J.T and J.K contributed to critical thinking and reviewed the manuscript.

7.2 Abstract

Interaction of Na/K-ATPase with its ligand ouabain has been implicated in regulation of various biological processes. The objective was to investigate roles of Na/K-ATPase isoforms in formation and function of junctional complexes in Sertoli cells. Primary cultures of Sertoli cells were obtained by enzymatic digestion of prepubertal (20 d) rat testes and grown on Matrigel-coated dishes for 7 d. Sertoli cells predominantly expressed the ubiquitous isoform (ATP1A1) of Na/K-ATPase, confirmed by immunoblotting, PCR, immunofluorescence and mass spectrometry. Treatment of Sertoli cells with 50 nM ouabain increased transepithelial electrical resistance (TER) and expression of claudin 11 and connexin 43, whereas 1 mM ouabain had opposite effects. Involvement of Src-EGFR-ERK1/2-CREB pathway in ouabain-mediated
expression of claudin 11 and connexin 43 was evaluated. Incubation of Sertoli cells with 50 nM ouabain increased content of p-Src, p-EGFR, p-ERK1/2, and p-CREB; in contrast, 1 mM ouabain decreased phosphorylation of these signaling molecules. Pre-incubation of Sertoli cells with inhibitors of Src and MAPK pathways inhibited ouabain-induced effects on these signaling molecules, TER, and expression of claudin 11 and connexin 43. In conclusion, we inferred that ATP1A1 regulated Sertoli cell tight junctions and gap junctions through the Src-EGFR-ERK1/2-CREB pathway. Ouabain is an endogenous steroid; therefore, its interaction with ATP1A1 may be a critical signaling mechanism for regulation of Sertoli cell function and male fertility.

7.3 Introduction
Proliferation of Sertoli cells occurs during fetal and early neonatal life and continues until day 15 - 16 in rodents. Thereafter, Sertoli cells cease to proliferate and attain functional maturation (differentiation), characterized by alterations in gene expression, establishment of the blood-testis barrier (BTB) and sustained spermatogenesis (Sharpe et al. 2003, Tarulli et al. 2012). The BTB physically divides the seminiferous epithelium into two physiological compartments (basal and adluminal), which confers cell polarity, selectively permits transepithelial fluxes of ions and molecules, and protects germ cell antigens from the immune system, thereby providing a specialised microenvironment for development and differentiation of spermatocytes.
Ultrastructurally, BTB is comprised of specialised junctional complexes between adjacent Sertoli cells near the basement membrane. There are three types of Sertoli cell junctional complexes in the testis: tight junctions (TJs), gap junctions (GJs) and adherens junctions. However, tight junctions are the only known occluding junctions in mammalian epithelia and endothelia and act as the first line of defense against entry of small non-charged solutes, ions and macromolecules
via diffusion through the paracellular pathway (Schneeberger & Lynch 2004). Several transmembrane proteins have been localised to TJs, including claudins, occludin, and junctional adhesion molecule (JAM). Occludin null male mice were infertile due to disrupted spermatogenesis and few postmeiotic germ cells (Saitou et al. 2000). There are indications that JAMs are not essential for formation and maintenance of TJs. Several primary and established fibroblast cell lines have endogenous populations of JAM-A or JAM-C, but do not form TJs (Morris et al. 2006). However, claudins appeared to be major structural components of TJ strands and important for optimal establishment of tight junctions. Claudin 1 KO mice died from dehydration, due to loss of tight junctional barriers to water and macromolecules in the epidermis (Furuse et al. 2002). Furthermore, claudin 5 KO male mice had evidence of leakage into the seminiferous lumen when tracer element was injected into the interstitial space (Morrow et al. 2009). Despite the presence of seven claudins (claudin 1, 3, 5, 7, 8 and 11) in the testis, claudin 11 seemed to be functionally relevant, as its deficiency resulted in loss of epithelial phenotype of Sertoli cells (Mazaud-Guittot et al. 2010), followed by sterility due to lack of TJ strands and seminiferous tubules filled with clumps of nucleated cells that lacked normal spermatogenesis (Gow et al. 1999, McCabe et al. 2015).

Gap junctions are intercellular plasma membrane channels that provide a passage for ions and molecules between cells that are forming junctions. The GJs facilitate intercellular communication via non-covalent interaction of channels (coupled connexons) between two cells or communicate with the extracellular environment via hemichannels (uncoupled connexons) (Mruk & Cheng 2004, Li et al. 2009). Each connexon is comprised of six integral membrane subunits or connexions, which in association with other connexons, form a GJ plaque. Within seminiferous tubules, GJs are located in conjunction with occluding TJs (Eusebi et al. 1983).
between Sertoli cells at the site of BTB, and between Sertoli and specific stages of germ cells (McGinley et al. 1979, Mruk & Cheng 2004). Since GJs communicate signals between Sertoli and germ cells, they may have a crucial role in germ cell movement during spermatogenesis (Russell 1977). Several connexin members (connexin 26, 32, 33, 46, 50) have been identified in adult testis, yet connexin 43 seems to be best studied, as it is predominantly located in the basal compartment of the seminiferous tubules (Brehm & Steger 2005). Also, a connexin 43-targeted deletion in mice resulted in male infertility (Plum et al. 2000) most likely due to arrest of spermatogenesis and Sertoli-cell-only (SCO) syndrome, characterized by intratubular Sertoli cell clusters and abnormal SC cytoplasmic vacuoles (Brehm et al. 2007, Sridharan et al. 2007). Furthermore, disorganised spatial arrangements of claudin 11 and connexin 43 were noted in men with primary seminiferous tube failure, presumably due to loss of BTB function (Haverfield et al. 2013).

Ion transporters and ion channels interact with tight junction proteins (e.g., Na/K-ATPase). Structurally, the sodium pump is a heterodimer composed of noncovalently linked α and β subunits which transports 3 Na⁺ out and 2 K⁺ into cells by hydrolysis of ATP. The catalytic α subunit contains binding sites for Na⁺, K⁺, ATP and also acts as a receptor for cardiotonic steroids which specifically inhibit Na/K-ATPase. The β subunit is a glycosylated membrane protein required for normal enzyme activity (Noguchi et al. 1987, Horowitz et al. 1990). Four distinct α isoforms (α1, α2, α3, α4) and three β isoforms (β1, β2, β3) have been identified, with the α1 isoform having ubiquitous expression, whereas the α4 isoform was expressed only in spermatocytes and sperm. In Sertoli cell line 93RS2, a low ouabain concentration modulated expression of claudin 1 and claudin 11 mediated through the α4 isoform of Na/K-ATPase (Dietze et al. 2015). In addition to maintenance of ionic homeostasis, the pump
also regulates intracellular pH, cell volume and membrane potential. The ion transport function of Na/K-ATPase affects ionic composition of seminiferous tubular fluid (Rato et al. 2010) and intracellular pH of Sertoli cells (Oliveira et al. 2009). However, in addition to ion transport, many ionic pumps also have non-classical (signaling) functions. For example, low doses of ouabain modulated expression of connexin 32 (Larre et al. 2006) and regulated specific expression and distribution of claudin 1, 2 and 4 via ERK1/2 and c-Src during the polarised epithelial phenotype transformation in MDCK cells (Larre et al. 2010). However, the mechanism by which the pumping and signaling activity of other isoforms of Na/K-ATPase in the regulation of Sertoli cell tight junction and gap junctions are currently unknown. Therefore, the objective was to determine effects of Na/K-ATPase-ouabain interaction in formation and function of TJs and GJs in prepubertal rat Sertoli cells.

7.4 Materials and methods

7.4.1 Animals
Sprague-Dawley rats (20 d) were purchased from Charles River Laboratories (Quebec, QC, Canada) and housed at the Health Science Animal Resource Centre (University of Calgary, Calgary, AB, Canada). The study reported herein was approved by the University of Calgary Institutional Animal Care and Use committee (protocol number AC13-0147).

7.4.2 Chemicals and antibodies
Ouabain, Collagenase, Hyaluronidase, Trypsin, Soybean trypsin inhibitor, RIPA buffer, Insulin-Transferrin-Selenium (ITS) supplement, Epidermal growth factor (EGF), Bacitracin, and Gentamycin were purchased from Sigma Aldrich, ON, Canada. Vectashield was purchased from
Vector Laboratories, Burlingame, CA, USA. Transwell standing insert and Matrigel were purchased from BD Biosciences, MA, USA. Corning cell recovery solution was from Corning, NY, USA. Protease inhibitor tablets purchased from Roche Diagnostics, QC, Canada, four-well chamber slides, cDNA reverse transcription kit, nuclease free water, DNase, TRIzol and DMEM-F12 with HEPES were from Thermo Fisher Scientific, ON, Canada. Primary antibodies were purchased from the following companies: AMH, WT-1, GATA-4, Actin (Santa Cruz Biotechnology, TX, USA), ATP1A2 and claudin 11 (Thermo Fisher Scientific, ON, Canada), ATP1A3 and VASA (Abcam, MA, USA), connexin 43, p-EGFR, p-ERK1/2, p-CREB, EGFR (Cell Signaling Technology, MA, USA), ATP1A1, p-Src, Src, ERK1/2 (Millipore, MA, USA) and affinity-purified ATP1A4 was custom made at Pierce Antibodies. Secondary antibodies were purchased from Thermo Fisher Scientific, ON, Canada: chicken anti-rabbit Alexa 488 and goat anti-rabbit Alexa 555. Secondary antibodies for immunoblotting (goat anti-mouse and anti-rabbit IgG HRP conjugate) were purchased from Millipore, MA, USA.

7.4.3 Isolation and culture of prepubertal rat Sertoli cells

Testes from 20-d-old rats were decapsulated and cut into ~1 mm pieces, which were digested with 40 mg trypsin and 0.8 mg DNase in a shaking water bath at 110 oscillations/min for 30 min. Tubules along with interstitial cells were centrifuged at 800 x g for 2 min. Subsequently, they were digested with 1 M glycine, 2 mM EDTA, pH 7.4 containing 0.01% STI and 0.8 mg DNase for 10 min at RT to lyse interstitial cells. Intact tubules were washed by centrifugation at 800 x g for 2 min and re-suspended in 40 mg collagenase and 0.2 mg DNase and allowed to shake at 110 oscillations/min for 30 min. Digested tubules were washed three times (centrifugation at 800 x g for 2 min), and then re-suspended in media containing 40 mg hyaluronidase and 0.2 mg DNase
and shaken at 110 oscillations/min for 30 min. Cells were washed five times by centrifuging at 800 x g, re-suspended in DMEM-F12 media containing 10 µg/ml insulin, 5 µg/ml transferrin, 2.5 ng/ml EGF, 5 µg/ml bacitracin and 20 µg/ml gentamycin, and then seeded with varying densities (objective specific) on to Matrigel coated culture dishes/bicameral units in a humidified incubator at 35 °C with 95% air and 5% CO₂. Sertoli cells were cultured in the absence of serum or androgens as these are positive inducers of several junctional proteins in Sertoli cells. Therefore these chemicals were not added to Sertoli cell media during in vitro culture which could potentially mask the original effect induced by ouabain. A hypotonic treatment (20 mM Tris, pH 7.4 for 2.5 min; (Wagle et al. 1986)) was performed 48 h after culture to lyse contaminating germ cells. Purity of Sertoli cell cultures were determined by various markers namely, anti-Mullerian hormone (AMH), Wilms tumour (WT1), and GATA binding protein 4 (GATA4) by immunoblotting and microscopically by staining for WT1. Contamination of Leydig cells and germ cells in Sertoli cell cultures were identified by their immunoreactivity to 3β-HSD and VASA/DDX4 (3beta-hydroxysteroid dehydrogenase and DEAD-box helicase 4, markers for Leydig and germ cells, respectively).

7.4.4 Mass spectrometry on Sertoli cell extracts

Total Sertoli cell extracts were prepared as detailed in Section 7.4.8, electrophoresed under standard conditions and stained with Coomassie brilliant blue G-250. All steps, including in-gel trypsin digestion, LC-MS/MS analysis and database search for protein identification in the raft and non-raft fractions were performed at the mass spectrometric facility, SAMS centre, University of Calgary. Please refer to Chapter 3, Sections 3.3.3 and 3.3.4 for detailed procedures on protein digestion and LC-MS/MS analysis. The following parameters were used for NCBI
database search: *Rattus norvegicus* taxonomy, maximum of one missed trypsin cleavage, methionine oxidation as variable modification, cysteine carbamidomethylation as fixed modification, and a mass error tolerance of 20 ppm. All significant peptides had a MASCOT expectation value <0.05 and an ion score >28. Scaffold (version Scaffold_4.3.2, Proteome Software Inc., Portland, OR, USA) was used to validate MS/MS based peptide and protein identifications. Protein identifications were accepted if they could be established at >95% probability and contained at least two identified peptides.

### 7.4.5 Measurement of transepithelial electrical resistance (TER) of Sertoli cells

Matrigel-coated standing inserts were seeded with freshly isolated Sertoli cells at high density (1.2 × 10⁶ cells/cm²) to promote TJ assembly that was quantified using a Millicell electrical resistance system (Millipore), as described (Grima *et al.* 1998). Initially, TER was measured across the Sertoli cell epithelium 24 h after cells were plated (i.e. day 1), and then daily until day 7. The resulting values were multiplied by the surface area of the filter (1.1 cm²) and data presented as Ohm.cm². The net value of electrical resistance was calculated by subtracting the background resistance obtained by measurement of cell-free Matrigel-coated bicameral units. A functional TJ barrier was monitored by TER values and expression of TJ markers by PCR, immunoblotting and immunofluorescence. From this time point, Sertoli cells were either incubated with 50 nM (stimulates pump signaling) or 1 mM (inhibits pump activity) ouabain or control media to determine effects of Na/K-ATPase enzyme inhibition and signaling on TJ and GJ function. Viability of Sertoli cells incubated under various ouabain conditions was determined with a trypan blue dye exclusion test. For inhibitor based studies, Sertoli cells were preincubated with PP2 (Src kinase inhibitor), PD98059 (MAPK inhibitor) for 30 min and
extracts were probed for phosphorylation of signaling molecules, claudin 11 and connexin 43 expression and measurement of TER.

**7.4.6 Isolation of Sertoli cell RNA and quantification of junctional molecules by RT-qPCR**

Total RNA was extracted from Sertoli cells and testis with TRIzol reagent according to the manufacturer’s instructions. The final RNA pellet was washed with 1 ml of 75% ethanol, air dried and re-suspended in 30 µl of nuclease free water. The RNA samples were treated with DNase using the protocol in the DNase kit, RNA concentrations measured using the NanoDrop UV/Vis spectrometer (Thermo Scientific, Wilmington, DE, USA) at 260 nm, and samples stored at -80 °C until used for subsequent analysis. Sertoli cell and testis RNA (100 ng) was reverse-transcribed using a high-capacity cDNA reverse transcription kit according to the manufacturer’s instructions. Intron-spanning primers for targeted transcripts were designed using the primer-designing tool from NCBI (Table 7.1). All PCR amplifications were performed in a 25 µl reaction volume using StepOnePlus Real-Time PCR system (Applied Biosystems, Burlington, ON, Canada) and AmpliTaq fast DNA polymerase (Fast SYBR Green master mix, Applied Biosystems) with 10 pmol of each primer and 25 ng of cDNA. The PCR conditions were 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, primer-dependent annealing temperature for 30 s, then 72 °C for 2 min, followed by a final extension at 72 °C for 10 min. In addition, a negative control (without cDNA) and no template control were included to ensure specific amplification. Relative changes in gene expression was calculated using the $2^{-\Delta\Delta C_{T}}$ (threshold cycle) method.
Table 7.1: Primer sequences used for detecting transcripts of TJ and GJ molecules, and isoforms of Na/K-ATPase in prepubertal Sertoli cells

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank accession #</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cldn1</td>
<td>NM_031699.2</td>
<td>GCTGTGATCAGGGGCATAAT</td>
<td>CCTGGCAGAATTCACTACCTGG</td>
<td>136</td>
</tr>
<tr>
<td>Cldn3</td>
<td>NM_031700.2</td>
<td>CTCCGCTCCTCCACGAGCATCAG</td>
<td>CCCACTATGAGCCTCTCTGCA</td>
<td>78</td>
</tr>
<tr>
<td>Cldn4</td>
<td>NM_001012022.1</td>
<td>AGGCTCCAGCGAGGATAGAT</td>
<td>TCCACTGTGCTACCCAGAGCA</td>
<td>104</td>
</tr>
<tr>
<td>Cldn11</td>
<td>NM_053457.2</td>
<td>CCTCATCCCTCCCTGGTTACG</td>
<td>GAATAAGGAGCACCCACAGCC</td>
<td>173</td>
</tr>
<tr>
<td>Ocln</td>
<td>NM_031329.2</td>
<td>GCCGGATGAATTCAACCCCA</td>
<td>GCGATGCAGATCAGATGAC</td>
<td>195</td>
</tr>
<tr>
<td>Tjp1</td>
<td>NM_001106266.1</td>
<td>TCAAGTCGAGACAGCCAGTAGT</td>
<td>ATGCTGGGCTAAGAATCCC</td>
<td>96</td>
</tr>
<tr>
<td>Gjb1</td>
<td>NM_017251.2</td>
<td>CCAGGGAGGTGTGAATTAGG</td>
<td>CTCGGCCAATGAGCTGTAGGA</td>
<td>91</td>
</tr>
<tr>
<td>Gja5</td>
<td>NM_019280.1</td>
<td>AGGAGCAGAAGCGTCAGCAGC</td>
<td>ACCTGAGCCATGAGCGAGGA</td>
<td>113</td>
</tr>
<tr>
<td>Gja1</td>
<td>NM_012567.2</td>
<td>TTAAGTGAAGAGAGGTGCCCA</td>
<td>AGGCAGACTGTTCATCACCC</td>
<td>179</td>
</tr>
<tr>
<td>Atp1a1</td>
<td>NM_012504.1</td>
<td>TCCCTAAGCGTGCAATAGCG</td>
<td>CTCATCTCCATACGGAGCC</td>
<td>88</td>
</tr>
<tr>
<td>Atp1a4</td>
<td>NM_022848.3</td>
<td>CCGTGCATCCTTATCATCCAGTGAGA</td>
<td>AGTGAGCGCTGTCTCTTTTC</td>
<td>85</td>
</tr>
<tr>
<td>PcnA</td>
<td>NM_022381.</td>
<td>CCTGCTGGGACATCAGTTCG</td>
<td>TTTGGACATGCTGGTGAGTT</td>
<td>155</td>
</tr>
<tr>
<td>Wt1</td>
<td>NM_031534.2</td>
<td>TCCGGTGCAGCATCTGAACC</td>
<td>TTCTCACAGGTGTGCTTCCG</td>
<td>121</td>
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<tr>
<td>Gapdh</td>
<td>NM_017008.4</td>
<td>CAGGGCTGCTTCTGCTTTG</td>
<td>GATCTGGCTGTGGAGATGG</td>
<td>188</td>
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</tbody>
</table>
7.4.7 **Immunofluorescence**

For immunostaining, Sertoli cells were plated at a density of $0.05 \times 10^6$ cells/cm$^2$ in four-well chamber slides. Cells were fixed in 4% PFA for 15 min, permeabilized with 0.1% Triton X-100 for 15 min (5 min for claudin 11 and connexin 43), followed by incubation with 10% normal chicken serum or goat serum (depending on the source of the secondary antibody) for blocking. Primary antibodies were incubated overnight at 4 °C at the following dilutions: claudin 11 and connexin 43 (1:100), p-CREB (1:500), VASA (1:200), 3β-HSD (1:50), ATP1A1 (1:50). Following washing in PBS, secondary antibodies were added at the following dilutions: chicken anti-rabbit Alexa 488 (1:1000 for VASA and p-CREB; 1:200 for claudin 11, connexin 43 and ATP1A1) and goat anti-rabbit Alexa 555 (1:1000 for 3β-HSD) for 1 h at RT. Cells were washed in PBS and mounted with Vectashield containing DAPI and stored in – 20 °C until further imaging. Images were captured using a Zeiss AxioVert.A1 inverted fluorescence microscope (Carl Zeiss microscope GmbH, Germany) using the Axiocam ICc 5 camera system and representative 20X and 40X images were shown.

7.4.8 **Preparation of Sertoli cell lysates and immunoblotting**

Sertoli cells ($0.5 \times 10^6$ cells/cm$^2$) attached to Matrigel-coated dishes were first recovered by adding a cell recovery solution for 1 h at 4 °C. These cells were washed in PBS and centrifuged at 500 x g for 10 min at 4 °C. Pelleted Sertoli cells were subsequently lysed with RIPA buffer containing 1X protease inhibitor cocktail and phosphatase inhibitor cocktail which included 1 mM Na$_3$Vo$_4$, 1 mM NaF and 1 mM β-GP for 30 min at 4 °C. The supernatant (containing extracted proteins) was obtained by high-speed centrifugation (12,000 g for 5 min at 4 °C). Proteins were separated on 8% SDS-PAGE gel and electrotransferred to nitrocellulose
membranes. After blocking with 5% (w/v) skim milk in Tris-buffered saline containing TTBS for 1 h, membranes were incubated overnight in TTBS at 4 °C with the following antibodies: AMH (1:100), WT-1 (1:100), GATA-4 (1:100), ATP1A1 (1:1000), claudin 11 (1: 200), connexin 43 (1:1000), p-Src (1:1500), p-EGFR (1:1500), p-ERK1/2 (1:1500), p-CREB (1:1000), Src (1:1500), ERK1/2 (1:1500), and EGFR (1:1000). Membranes were washed (3X) in TTBS for 10 min and subsequently incubated with either HRP conjugated goat anti-rabbit or anti-mouse IgG for 1 h at RT. Following washing, immunoreactive bands were detected using chemiluminescence. Membranes were stripped and probed with anti-actin antibody (1:1000) for whole-cell homogenates and for phosphoproteins, antibodies corresponding to total proteins to ensure equal protein loading.

7.4.9 Statistical analysis

Data were analysed with commercial statistical software (GraphPad Software Inc., La Jolla, CA, USA). Data regarding the transcript levels of claudin 11, zona occludens-1 and TER values that were recorded from day 1 to day 6 of in vitro culture, TER values in Sertoli cells obtained after ouabain and inhibitor (PP2 and PD98059) exposure were analysed using one-way ANOVA followed by Tukey’s post-hoc test. Differences in viability, phosphorylated signaling molecules, claudin 11 and connexin 43 expression between control and ouabain treated groups were analysed using two-way ANOVA followed by Tukey’s post-hoc test. Fold changes in the expression of various TJ and GJ molecules subjected to ouabain treatment were analysed using two-way ANOVA followed by Sidak’s multiple comparison test. For all analyses, P < 0.05 was considered significant and results were reported as mean ± SEM.
7.5 Results

7.5.1 Characterisation of Sertoli cells from prepubertal rat testis

Our Sertoli cell culture system was validated (immunoblotting and immunofluorescence) using markers predominantly expressed in prepubertal rats, including AMH, WT1 and GATA4 (Figure 7.1A and B). Sertoli cell cultures were 90% pure (based on positive staining to WT1), with 6% cells designated as Leydig cells (positive for 3β-HSD) and the remaining 4% immunoreactive to VASA (presumptive germ cells; Figure 7.1C).
Figure 7.1 Characterisation of Sertoli cells from prepubertal rat testis. (A) Western blot of total Sertoli cell extracts documenting presence of Sertoli cell markers including AMH, WT1 (doublets identified by black solid arrows) and GATA4 (indicated by black solid arrows). (B) Staining pattern of WT1 in the nucleus (green), DAPI staining in the nucleus (blue) and merged image of WT1 and DAPI. This image was captured from Sertoli cells that were grown on non-matrigel conditions. (C) Purity of Sertoli cell cultures were evaluated by staining for 3β-HSD and VASA/DDX4 (markers for Leydig and germ cells, respectively) and DAPI for the nucleus.

7.5.2 Increase in claudin 11 expression indicates the formation of Sertoli cell TJs

Based on various criteria including TER, mRNA and protein profiles of claudin 11, prepubertal Sertoli cells grown at high density established TJs in vitro. A simplified diagram of TER assembly is shown in Figure 7.2A. The TER values started to increase on day 1 and peaked on day 4, consistent with formation of a complete TJ. The TER values decreased on day 5, but were maintained thereafter (Figure 7.2B). Concurrently, mRNA levels of claudin 11 and zona
occludens-1 reached maximum values by day 3, prior to establishment of a functional TJ barrier by day 4 (Figure 7.2C and E). Protein profiles of claudin 11 were consistent with its mRNA levels (Figure 7.2D). To confirm differentiation, Sertoli cells were assessed for transcript levels of proliferating cell nuclear antigen (PCNA) from days 1 to 5 of in vitro culture conditions. The mRNA levels of PCNA did not change significantly throughout culture (Figure 7.2F).

Localisation patterns of claudin 11 were disorganized and incomplete by day 3, with staining noticed in the form of dots and patches. Complete staining was apparent at interfaces between cells at day 4, consistent with the various TJ measurements mentioned above (Figure 7.3).

**Figure 7.2**

A. Diagram showing Transwell insert, Apical compartment, Sertoli cells, and Basal compartment.

B. Graph showing TER Ω cm² over days 1 to 6.

C. Bar graph showing ΔCT (claudin 11 - GAPDH) for days 2 to 5.

D. Western blot showing Claudin 11 and Actin bands for days 1 to 4.
**Figure 7.2 Formation of Sertoli cell junctional complexes in vitro.** (A) Schematic diagram of the TER assembly used to assess tightness of TJs in Sertoli cell monolayers. (B) TER recordings obtained from Sertoli cells grown *in vitro* for 7 d. (C), (D) and (E) Monitoring formation of junctional complexes by assessment of transcript and protein levels of claudin 11 mRNA and protein levels (immunoblotting), and zona occludens-1 (ZO-1) mRNA, respectively. (F) RT-qPCR values for proliferating cell nuclear antigen (PCNA) obtained from Sertoli cell cultures maintained *in vitro* for 5 d. Values represent mean ± SEM (n = 3 replicates). a–e Values without a common letter differed (P < 0.05).
Figure 7.3 Monitoring the formation of Sertoli cell junctional complexes *in vitro* by claudin 11 staining. Representative images showing the staining patterns of claudin 11 (green; left panel) and nucleus (blue; middle panel) and merged image of claudin 11 and DAPI (right panel) on days 2, 3, and 4 of *in vitro* Sertoli cell culture.

7.5.3 Detection of α1 and β3 subunits of Na/K-ATPase in Sertoli cells

The presence of α1 subunit in Sertoli cells was detected using RT-PCR and later confirmed by immunoblotting (Figure 7.4A and B). Staining of Sertoli cells with Na/K-ATPase α1 antibody revealed the presence of the protein at the membrane interface between adjacent Sertoli cells.
Figure 7.4C. However, the presence of sperm-specific α4 isoform in Sertoli cells was not confirmed by either of these approaches (Figure 7.4A and B). Immunoblotting of Sertoli cells with α4-specific antibody did not detect a 110 kDa band corresponding to the molecular weight of the protein, although several non-specific bands were apparent (Figure 7.4A). Subsequently, mass spectrometric identification confirmed the presence of only α1 and β3 subunits in Sertoli cell extract (Table 7.2).
Figure 7.4. Characterisation of Na/K-ATPase isoforms in rat Sertoli cells. (A) Total Sertoli cell extracts were probed with antibodies against the α1 isoform, pre-immune (PI) serum and custom-made antibody against the α4 isoform of Na/K-ATPase. Blocking peptide (BP) was used to confirm the specificity of antigen-antibody binding. Sperm was used as a positive control for detection of ATP1A4. (B) Real-time PCR detection of α1, α4 isoforms and Sertoli cell marker (WT1) transcripts in Sertoli cells. Testis was used as a positive control for detection of ATP1A4 mRNA respectively. (C) Immunofluorescent staining of Sertoli cells against ATP1A1 (green) and the nucleus (blue) and merged image of ATP1A1 and DAPI staining.
Table 7.2: Mass spectrometric detection of Na/K-ATPase isoforms in prepubertal rat Sertoli cells

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7.5.4 Dose-dependent regulation of ouabain on claudin 11 and connexin 43 expression and its effect on TER

To test whether the pump and signaling function of Na/K-ATPase are essential for TJ and GJ function in Sertoli cells, two distinct concentrations of the ligand ouabain were used. The higher dose (1 mM) inhibits ion transport activity, whereas the low non-inhibitory dose (50 nM) activates signaling pathways (Nguyen et al. 2007). Furthermore, since 1 mM ouabain interferes with cell viability, we monitored cell vitality with a trypan blue dye exclusion test. There was no significant difference in cell vitality between groups exposed to 50 nM or 1 mM ouabain (Figure 7.5A). Furthermore, tightness of junctional complexes was assessed by measurement of TER values. Incubation with a low concentration of ouabain resulted in increased TER values (reached significance after 72 h of treatment; Figure 7.5B). In contrast, a high ouabain concentration decreased TER. In addition, RT-PCR was used to ascertain whether changes in TER values were accompanied by changes in expression of the junctional molecules associated with TJs and GJs. Claudin 1, 3, 4, occludin, ZO-1, connexin 32, and 40 mRNA levels did not change significantly following ouabain treatment (Figure 7.5C). However, there were significant changes in the transcriptional and translational activities of claudin 11 and connexin 43 after 72 h of treatment with ouabain. Transcript levels of both claudin 11 and connexin 43 were increased when Sertoli cells were treated with 50 nM ouabain; however, there was a corresponding decrease in both molecules when cells were exposed to higher concentrations (1 mM) of ouabain (Figure 7.5C). Consistent with changes in mRNA expression, 50 nM ouabain induced an increase in claudin 11 and connexin 43 protein, whereas 1 mM ouabain had a decreasing effect (Figure 7.5D and E). In addition, effects of varying concentrations of ouabain on distribution of claudin 11 and connexin 43 expressions were also determined. Incubation of cells with the low
non-inhibitory concentration of ouabain increased staining intensity of claudin 11 expression at cell-cell junctions, whereas the inhibitory dose of ouabain led to a discontinuous staining pattern between cells, compared to control cells (Figure 7.6). Staining pattern similar to claudin 11 was noticed for connexin 43 under different ouabain conditions (data not shown).

**Figure 7.5**

![Graph A](image1.png)  
A  
Graph A shows the viability of cells treated with different doses of ouabain at various time points (24 h, 48 h, 72 h). The x-axis represents time (in hours), and the y-axis represents viability (%). The graph compares control cells with 50 nM and 1 mM ouabain.

![Graph B](image2.png)  
B  
Graph B illustrates the TER (Ω cm²) values for control, 50 nM, and 1 mM ouabain treatments. The x-axis represents the treatment groups, and the y-axis represents TER. The graph includes data points labeled with superscript letters a, b, and c.

![Graph C](image3.png)  
C  
Graph C displays the fold change in expression levels of various genes (Gja1, Cldn1, Cldn3, Cldn4, Cldn11, Odh, Tjp1) under control and ouabain conditions. The x-axis lists the genes, and the y-axis represents fold change. The bars indicate the expression levels with error bars for each condition.
Figure 7.5 Dose-dependent regulation of ouabain on expression of TJ and GJ proteins. (A) Assessment of viability in Sertoli cells incubated with various concentrations of ouabain for 24 – 72 h. (B) Recording of TER values (Sertoli cell monolayers) under both control and ouabain-treated conditions. (C) RT-qPCR based quantification of transcript levels of various members of the TJ and GJ family of proteins, normalised to GAPDH levels under varying conditions of ouabain exposure for 72 h. (D) Representative immunoblot of claudin 11 and connexin 43 expression obtained from analogous experimental conditions as described above (cropped image indicated by dividing lines). (E) Relative pixel intensity of these proteins was calculated by normalizing mean pixel intensity of claudin 11 and connexin 43 to actin. Values shown were expressed as mean ± SEM (n = 3 replicates). a-cValues without a common letter differed (P < 0.05).
Figure 7.6

**Figure 7.6** Dose-dependent regulation of ouabain on localisation patterns of claudin 11 in Sertoli cells. Representative images of claudin 11 (green), nucleus (blue) and merged image of claudin 11 and DAPI in Sertoli cell cultures after treatment with various ouabain concentrations for 72 h. Similar staining patterns were obtained for connexin 43 (data not shown).
7.5.5 Involvement of Src-EGFR-ERK1/2-CREB pathway in ouabain mediated regulation of claudin 11 and connexin 43 and its effect on TER

Increase in Src, EGFR, ERK1/2, CREB phosphorylation were apparent in Sertoli cells after 15 min of exposure to a low concentration of ouabain (50 nM), compared to control cells, as shown in Figure 7.7A-E. Even though treatment with a higher concentration (1 mM) of ouabain decreased phosphorylation of Src, ERK1/2, and CREB compared to the 50 nM ouabain group, phosphorylation was still higher compared to the control group, except for p-Src (Figure 7.7A-E). However, EGFR phosphorylation in the 1 mM ouabain treated group was not significantly different from other groups in the study. There were no differences in unphosphorylated forms of the above-mentioned signaling molecules under any of these conditions. Consistent with the p-CREB expression, staining was more intense in the nuclei of the low-dose ouabain-treated group compared to the control and high-dose ouabain groups (Figure 7.8). To confirm whether the Src-EGFR-ERK1/2-CREB pathway was central for ouabain-induced effects on junctional molecules, PP2 and PD98059, inhibitors that classically block Src and MAPK kinase activity, respectively, were used. Pre-incubation of cells with 10 µM PP2 completely abolished phosphorylation of EGFR, ERK1/2 and CREB (Figure 7.9A) whereas 25 µM PD98059 prevented phosphorylation of CREB, suggesting that Src and ERK1/2 were crucial to activate this pathway (Figure 7.10A). Expression of claudin 11 and connexin 43 were also decreased in the presence of inhibitors and further exposure to ouabain did not reverse inhibitory effects (Figure 7.9A). However, neither inhibitor affected expression of non-phosphorylated forms of Src and ERK1/2. Also, inhibition of Src and MAPK by PP2 and PD98059 decreased TER and subsequent stimulation with either 50 nM or 1 mM ouabain partially reversed effects of the inhibitors, indicating involvement of
ATPA1-Src-EGFR-ERK1/2-CREB pathway in ouabain-mediated regulation of TJ and GJ molecules (Figure 7.9B and 7.10B).
Figure 7.7. Involvement of Src-EGFR-ERK1/2-CREB pathway in ouabain-mediated regulation of TJ and GJ proteins. Total Sertoli cell homogenates were isolated from cells incubated in the absence or presence of 50 nM or 1 mM ouabain for 15 min and probed for various signaling molecules, including (A) Src, (B) EGFR, (C) ERK1/2 and (D) CREB (indicated by black solid arrows). Top panels of (A), (B), (C) and (D) indicates phosphorylated signaling molecules, whereas the bottom panels correspond to the unphosphorylated forms of these signaling molecules. Actin was used as a loading control for p-CREB. (E) Content of activated signaling molecules were normalised to their unphosphorylated forms and relative pixel intensity calculated. Data shown were expressed as mean ± SEM (n = 3 replicates). a–cValues without a common letter differed (P < 0.05).
Figure 7.8

Representative images of p-CREB staining in the nuclei (green), DAPI staining in the nucleus (blue) and merged image of p-CREB and DAPI in Sertoli cells that were incubated under various ouabain conditions.

**Figure 7.8 Dose-dependent regulation of ouabain on p-CREB staining in Sertoli cells.**
Figure 7.9

A

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Figure 7.9 Effect of Src inhibition on phosphorylation of signaling molecules, claudin 11 and connexin 43 expression and its effects on TER. (A) Representative immunoblot from total Sertoli cell extracts preincubated with PP2 for 30 min and subsequently stimulated with either 50 nM or 1 mM ouabain and probed with phosphorylated signaling molecules. Src was used as a loading control. (B) Measurement of TER in Sertoli cell monolayers that were preincubated with PP2 for 30 min and stimulated with 50 nM or 1 mM ouabain. Values are mean ± SEM (n = 2 replicates). a-c Values without a common letter differed (P < 0.05).
Figure 7.10 Effect of ERK1/2 inhibition on CREB phosphorylation and its effects on TER.

(A) Sample western blot of Sertoli cell homogenates preincubated with PD98059 for 30 min and probed for phosphorylation of ERK1/2 and CREB. (B) Measurement of TER in Sertoli cell monolayers that were preincubated with PD98059 for 30 min and stimulated with 50 nM or 1
mM ouabain. ERK1/2 was used as a loading control. Values are mean ± SEM (n = 2 replicates).

\(^{a-d}\) Values without a common letter differed (P < 0.05).

### 7.6 Discussion

Sertoli cells and their junctional complexes have key roles in spermatogenesis and male fertility. In this study, we provided evidence that the \(\alpha_1\) subunit of Na/K-ATPase (ATP1A1) was localised to junctional complexes in prepubertal Sertoli cells. We also demonstrated dose-dependent regulation of ouabain on ion transport and signaling functions of ATP1A1, which in turn regulated expression of two major junctional proteins, claudin 11 and connexin 43 in Sertoli cells via the Src-EGFR-ERK1/2-CREB pathway.

A functional TJ barrier was formed *in vitro* when Sertoli cells cultured at a high density on matrigel-coated transwell insert system after 3 d of culture. Similar results were obtained from other studies involving prepubertal Sertoli cell primary cultures (Janecki *et al.* 1991, Grima *et al.* 1998). It is interesting to note that somatic epithelial cells display TER values (200 – 2500 \(\Omega\text{cm}^2\)) under *in vitro* culture conditions. In the testis, BTB is constituted by coexisting TJs, GJs, basal ectoplasmic specialisation (basal ES), and desmosomes (DS) along with germ cells and peritubular myoid cells which contribute either directly or indirectly in determining the tightness of the barrier. Although the BTB formed by Sertoli cells represent one of the tightest barrier systems in the body, it is possible that the absence of germ cells or peritubular myoid cells from our culture could have contributed to the low TER values compared to somatic epithelial cells. Additionally, we don’t know whether our Matrigel (culture) conditions support the formation of other junctional complexes including basal ES and DS. Therefore it is possible that the absence of protein(s) contributing to other junctions could be a causative factor for the low TER values.
Nevertheless, the increase in TER readings under *in vitro* culture conditions were not attributed to increase in Sertoli cell number, but rather reflected maturational changes in Sertoli cells due to formation of TJ. This was further confirmed using RT-PCR for detecting changes in the proliferating cell nuclear antigen (PCNA), a marker used for assessment of post-natal Sertoli cell development (Jarvis *et al.* 2005). We also detected a steady increase in mRNA levels of claudin 11 and ZO-1 preceding establishment of a TJ barrier by day 4 in our Sertoli cell cultures. Claudin 11 and ZO-1 can be used as markers for monitoring establishment of TJ barriers *in vitro* (Chung *et al.* 1999, Wong *et al.* 2000) especially claudin 11, which is necessary for morphology of TJ strands, as their presence maintains Sertoli cells in a differentiated state due to contact inhibition (Morrow *et al.* 2010). Subsequent localisation of claudin 11 at the inter-Sertoli cell junctions was consistent with other studies (McCabe *et al.* 2015).

Various isoforms of Na/K-ATPase are expressed in a tissue-specific and developmental-specific manner. In that regard, ATP1A1 is expressed ubiquitously, ATP1A2 is detected in adipocytes, muscle, heart and brain, ATP1A3 is abundant in neurons, and finally ATP1A4 is a testis-specific isoform with a restricted expression in post-meiotic germ cells and sperm. It was reported that ATP1A4 mRNA was present in the 93RS2 Sertoli cell line, originally isolated from 15 d-old prepubertal rats (Konrad *et al.* 2011). However, in that study, there was no conclusive demonstration that the ATP1A4 protein was present. It is well known that Sertoli cells in rats proliferate until 15 – 16 d postnatally, after which no proliferative activity is detected. There are also differences with respect to reproductive hormone profiles in rats. For example, after postnatal day 15, inhibin negative feedback on FSH secretion declines (Rivier *et al.* 1988) and testosterone concentrations increase (Lee *et al.* 1975). It is well known that FSH and testosterone are regulators of Na/K-ATPase function and expression (Blok *et al.* 1999, Sasson *et al.* 2003).
Moreover, cell lines are genetically manipulated, which may alter their response to stimuli, even though they may appear phenotypically normal (Kaur & Dufour 2012). Therefore, it is possible that there is age- and hormone-dependent regulation on expression of ATP1A4 mRNA and protein in Sertoli cell lines. In total contrast, McDermott (McDermott et al. 2012) used GFP expression, downstream of the ATP1A4 promoter as a tool to identify localisation patterns of ATP1A4 protein and reported that the α4 protein was detected only in differentiated male germ cells, but not in Sertoli cells isolated from mice that were 7-, or 18-d old, or adults. Therefore, due to contradictory reports regarding the presence of Na/K-ATPase isoforms, we decided to use various approaches to detect ATP1A4 at the protein level using a custom-made rat ATP1A4 antibody for immunoblotting and mass spectrometry. Neither approach detected evidence for existence of ATP1A4, but rather provided evidence for ATP1A1 and ATP1B3 in prepubertal Sertoli cell extracts. Similarly, Lucas (Lucas et al. 2012) reported that Sertoli cell primary cultures from 15-d-old rats had ATP1A1 but not the sperm-specific ATP1A4. The staining pattern of ATP1A1 in our study was noticeable at cell-cell contacts, consistent with observations in other epithelial cell types. The β3 subunit is predominantly expressed in the testis (Arystarkhova & Sweadner 1997) and co-expression of α1 and β3 subunits in baculovirus infected Sf-9 cells formed α1β3 that exhibited enzymatic properties characteristic of Na/K-ATPase (Yu et al. 1997).

Apart from its classical role in ion transport, Na/K-ATPase isoforms also act as a receptor for its ligand ouabain. Various short- and long-term effects have been reported for Na/K-ATPase-ouabain interaction, including proliferation/viability (Abramowitz et al. 2003, Quintas et al. 2010), differentiation (Fedorova et al. 2009), apoptosis (Jiang et al. 2010) and contraction (Mohammadi et al. 2003). The above-mentioned effects of ouabain were concentration-
dependent, with the rat α1 isoform being resistant to the inhibitory action of ouabain. In that regard, a dose as high as $1 \times 10^{-3}$ M is needed to inhibit the pumping activity of ATP1A1, whereas non-inhibitory doses as low as $10^{-9}$ M activate the signaling function of ATP1A1. Similarly, 0.1 to 10 nM ouabain prevented cell death and promoted cell growth, whereas concentrations >$10^{-7}$ M prevented cell proliferation associated with extensive inhibition of Na/K-ATPase enzyme activity (Nguyen et al. 2007). Accordingly, when we subjected Sertoli cells to inhibitory doses of ouabain (1 mM), there were significant decreases in the mRNA and protein profiles of claudin 11 and connexin 43, whereas low non-inhibitory doses (50 nM) had the opposite effect. Likewise, in other epithelial cells, 50 nM ouabain increased expression of claudin 1, 2 and 4 and their localisation patterns to become more distributed towards cell-cell junctions (Larre et al. 2010). In another study, a similar concentration of ouabain increased gap junction communications after 1 h of treatment (Ponce et al. 2014). In contrast, 1 mM ouabain caused a time-dependent loss of connexin 43 expression in A7r5 smooth muscle cells (Martin et al. 2003).

As mentioned above, higher doses of ouabain could cause Na/K-ATPase pump inhibition and the mechanisms by which this pump inhibition affects TJ permeability could be multifaceted. This could involve biochemical changes, including an increase in intracellular calcium, secondary changes due to increase in intracellular sodium, inhibition of Rho GTPase activity which in turn affects actin polymerization, and loss of interaction between Na/K-ATPase and E-cadherin in recruitment of TJ proteins or mislocalisation of TJ proteins at the plasma membrane interface (Rajasekaran & Rajasekaran 2003). Due to these biochemical changes that occurred secondary to pump inhibition, perhaps downstream targets or pathways differed in the 1 mM versus 50 nM group. We believe that the altered intracellular environment or unidentified
downstream pathways/molecules, or perhaps a combination of both, could have perturbed the junctional complexes at the site of BTB and thereby contributed to the leakiness and a subsequent decrease in TER in the 1 mM ouabain treatment group.

Na/K-ATPase also interacts with membrane and cytosolic proteins, either directly or indirectly and activates several downstream signaling pathways (Pierre & Xie 2006). In general, ouabain-activated Na/K-ATPase signaling occurred at concentrations too low to inhibit the enzyme. In contrast, we determined that both inhibitory and non-inhibitory doses of ouabain activated Src, ERK1/2 and CREB in a dose-dependent manner except EGFR through the α1 isoform in Sertoli cells. There is considerable evidence that distinct and probably interconvertible pools of Na/K-ATPase (raft and non-raft) exist in cells with signaling and pumping functions, respectively. Perhaps both the signaling and pumping pool of ATP1A1 are activated in response to low concentrations of ouabain, promoting increased phosphorylation of signaling molecules. The pumping pool of the enzyme might be inhibited, whereas only the signaling pool is activated in response to high concentration of ouabain, consistent with moderate phosphorylation of signaling molecules in Sertoli cells.

In 93RS2 Sertoli cell line exposed to low (nM) concentrations of ouabain, the α4 isoform of Na/K-ATPase modulated expression of claudin 1 and claudin 11 involving the c-Src/c-Raf/ERK1/2/CREB pathway, similar to non-classical testosterone signaling (Dietze et al. 2015). The most proximal interaction in Na/K-ATPase signaling is activation of non-receptor tyrosine kinase Src. This ouabain induced c-Src phosphorylation was conserved among cardiac myocytes, LLC-PK1, and A7r5 cells, providing evidence that this signaling process is common. In Sertoli cells, testosterone induces both rapid and sustained (within 5 and 20 min, respectively) c-Src phosphorylation, followed by phosphorylation of EGFR through the non-classical signaling
pathway (Cheng et al. 2007). In the present study, there was a similar type of response with regard to Src activation when prepubertal Sertoli cells were exposed to non-inhibitory doses of ouabain. Although exposure of inhibitory doses of ouabain did not cause much change in activation of EGFR, it caused changes in Src, ERK1/2 and CREB phosphorylation. Higher concentrations of ouabain are toxic and interfere with cell viability. In order to ascertain that downregulation of the signaling molecules in the 1 mM ouabain treated group were not due to loss of viability of the Sertoli cells, we monitored vitality by trypan blue dye. We observed no gross morphological changes and in the percentage of cells that excluded the viability dye. It is possible that either the duration or the magnitude of Src, ERK1/2 and CREB activation and associated secondary changes within the cell due to pump inhibition could have altered expression of claudin 11 and connexin 43 in this ouabain treated group. Similarly, a high ouabain concentration (1 µM) activated the MAPK pathway (Lucas et al. 2012), whereas 1 mM ouabain caused moderate ERK1/2 phosphorylation in rat salivary epithelial cells (Plourde & Soltoff 2006).

A rapid and sustained level of phosphorylation of ERK1/2 and CREB (1 min to more than 12 h) was reported when Sertoli cells were treated with testosterone (Fix et al. 2004). Accordingly, we detected increased phosphorylation of CREB on ser 133 residue after exposure to ouabain. It is suggested that a sustained activation of CREB is needed to induce changes in transcription for CREB-mediated genes (Fix et al. 2004). The importance of CREB phosphorylation is also evident from studies involving over expression of mutant CREB isoform in Sertoli cells that is unable to be phosphorylated. In such a case, spermatogenesis was disrupted due to the presence of apoptotic spermatocytes and subsequent loss of spermatids (Scobey et al. 2001). Once phosphorylated on Ser 133, CREB can induce the transcription of several genes
including claudin 11 (Morrow et al. 2010) and connexin 43 (Salameh et al. 2009). The GATA/NF-Y region of claudin 11 promoter is induced by the transcription factor complex comprising of GATA4/NF-YA/CREB (Morrow et al. 2010) in Sertoli cells, whereas the association of CREB with ATF/AP-1 complex induces transcription of connexin 43 in cardiomyocytes (Salameh et al. 2009). As the promoter regions of claudin 11 and connexin 43 contain binding sites for CREB, it is reasonable to assume that the increase in mRNA and protein expression of these junctional molecules after ouabain exposure might have occurred through this pathway. In several studies, the presence of transcription factors such as AP-1, CREB were markedly enhanced in the nucleus after activation of the MAPK cascade (Markou et al. 2004). The physiological relevance of ouabain-mediated increase in claudin 11 and connexin 43 expression were reflected in TER values with a low concentration of ouabain contributing to increased TER, although high concentrations decreased TER values. Perhaps effects of ouabain on TER values in the present study were a direct effect of the increase in TJ or GJ molecules, or they could have been an indirect effect of phosphorylated signaling molecules on TJ and GJ structures.

To assess if the above-mentioned signaling molecules were central to ouabain-mediated regulation of claudin 11 and connexin 43, Src inhibitor (PP2) was used which completely abolished phosphorylation of EGFR, ERK1/2, and CREB; therefore, we inferred that activation of Src was important to phosphorylate other signaling molecules involved in this pathway. In addition, MAPK inhibitor (PD98059) prevented CREB phosphorylation, indicating that ERK1/2 activation occurred upstream of CREB phosphorylation. Furthermore, PP2 blocked expression of claudin 11 and connexin 43 in Sertoli cells and subsequent stimulation with either 50 nM or 1 mM ouabain did not reverse inhibitory effects of Src and MAPK on expression of TJ and GJ
molecules. In addition, both PP2 and PD98059 reduced TER values but subsequent stimulation with ouabain was able to partially reverse the inhibitory effects, suggesting a compensatory mechanism from other molecules that potentially contribute to TJs and GJs. Likewise, PP2 and PD98059 inhibited ouabain-induced effects on claudin 1 and claudin 4 expression (Larre et al. 2010), whereas inhibitory effects of these inhibitors on connexin 32 and 43 were reported in MDCK cells (Ponce et al. 2014).

We concluded that both ion pumping and signaling functions of ATP1A1-ouabain interaction worked together to modulate several properties associated with Sertoli cell-cell contacts. Furthermore, effects of low and high concentrations of ouabain were linked to activation of several signaling pathways that converged on ERK1/2 and CREB molecules (Figure 7.11). Since ouabain is an endogenous hormone, its interaction with the α1 isoform of Na/K-ATPase could have a very important role in maintenance of BTB and thus male fertility.
Figure 7.11. Schematic representation of signaling pathway mediated by ATP1A1-ouabain interaction (non-inhibitory dose) in prepubertal Sertoli cells. Ouabain activated Src-EGFR-ERK1/2-CREB pathway leading to expression of claudin 11 (CL11) and connexin 43 (CX43). Expression and localisation of these TJ (tight junction) and GJ (gap junction) proteins enhanced the functionality of junctional complexes, which in turn regulates the communication between Sertoli cells at the blood-testis barrier (TER) and between Sertoli cells and germ cells. The numbers 1 – 5 marked on germ cells correspond to spermatogonial stem cell, preleptotene spermatocyte, pachytene spermatocyte, round spermatid and elongating spermatid, respectively.
Ionic homeostasis is essential for sperm and Sertoli cell function. Specifically, active translocation of Na\(^+\) and K\(^+\) ions between the intracellular and extracellular environments is under the control of Na/K-ATPase (sodium pump). In this thesis, I investigated the role of the testis-specific isoform of Na/K-ATPase (ATP1A4) in sperm function and fertility, as well as the role of the ubiquitous isoform (ATP1A1) in Sertoli cell tight junctions and gap junctions.

Similar to their functions in somatic cells, lipid rafts also had key roles in mediating ATP1A4 signaling events during sperm capacitation. Traditionally, lipid rafts act as a signaling hub for initiation of downstream signaling events. Accordingly, in our previous work, we determined that ATP1A4 signaling involved PKA, RTK and Src kinases. Perhaps these signaling molecules were pre-assembled with ATP1A4 in sperm lipid rafts. Subsequently, in this thesis, I determined that ATP1A4 was present in raft and non-raft membrane fractions of the sperm plasma membrane. In addition, I demonstrated that ATP1A4-ouabain mediated activation (phosphorylation) of EGFR, ERK1/2 and Src were higher in the non-raft membrane fraction, whereas increase in p-caveolin-1, and p-Src occurred, albeit at a lower level, in the raft membrane fraction during capacitation. Subsequent immunoprecipitation experiments indicated that ATP1A4 interacted with Src, EGFR and ERK1/2 in the non-raft fraction, whereas caveolin-1 and EGFR interaction was evident in the raft fraction, suggesting that raft and non-raft pools of ATP1A4 stimulated different signaling pathways leading to capacitation. However, I did not investigate downstream effectors of raft and non-raft signaling mediated by ATP1A4 during capacitation. Based on studies in somatic cells, it is well understood that ATP1A1 is involved in regulation of intracellular ROS, calcium and actin polymerisation. As a logical extension of this
work, future studies should be focused on investigating the above-mentioned ATP1A4 downstream targets that could be easily detected with commercial fluoro-probes. All these downstream changes are important regulators of sperm capacitation and defects in capacitation associated signaling could contribute to idiopathic infertility in normospermic men. Understanding the molecular basis by which this protein functions in normal sperm could help us to develop novel diagnostic approaches to identify male infertility.

The above-mentioned signaling molecules were chosen based on reports from somatic cells in which the ubiquitous isoform (ATP1A1) predominates. However, sperm development is a unique phenomenon, among the most complex differentiation processes in higher eukaryotes. During spermatogenesis, sperm acquire several proteins (or their isoforms) that are unique and cell-specific in order to meet functional demands of the cell. Therefore, it is likely that interacting partners of ATP1A4 are different from their somatic cell counterparts, which motivated us to investigate the ATP1A4 interactome during sperm capacitation (using an immunoprecipitation-mass spectrometry approach). In that study, interacting partners of ATP1A4 differed between raft and non-raft pools of capacitated sperm. Overall, the interactome was suggestive of proteins known to be involved in metabolism, sperm-oocyte interactions, chaperones, proteases, cytoskeletal and adaptor proteins. Specifically, plakoglobin, a molecule involved in cellular adhesion and signaling, was localised with ATP1A4 in the equatorial segment during sperm capacitation. In epithelial cells, the process of cell-cell adhesion is facilitated by homo- or heterophilic bonds between E-cadherins on adjacent cells; these bonds are further strengthened by interactions between the cytoplasmic domain of E-cadherin and catenins, which are further linked to the actin cytoskeleton (Yamada et al. 2005). E-cadherin and catenin proteins are specifically localised to the equatorial segment of the sperm head and
microvillar region on the oolemma (Takezawa et al. 2011). If gamete interactions involve mechanisms that are similar to epithelial cell adhesion, it is likely that these proteins are involved in events leading to sperm-oocyte fusion, considering their strategic locations in sperm and in oocytes. Since plakoglobin belongs to the catenin family, it is reasonable to assume that ATP1A4-plakoglobin interaction would be involved in gamete fusion, leading to fertilization. Plakoglobin is also present in both adherens junctions and desmosomes between Sertoli cells and between Sertoli and germ cells (Mruk & Cheng 2011); therefore, it would be interesting to investigate the physiological relevance of Na/K-ATPase-plakoglobin interactions in testis in future studies.

Apart from their direct role in mediating sperm-oocyte adhesion and fusion, E-cadherin-catenin complex could also be involved indirectly in recruitment of PLCζ (sperm-borne oocyte activating factor; SOAF) to the membrane. In epithelial cells, E-cadherin-β-catenin complex recruits and activates PI3K leading to PIP3 accumulation which in turn brings PLC-γ1 to the plasma membrane (Xie & Bikle 2007). Based on the hypothetical model proposed in Chapter 3, perhaps ATP1A4-plakoglobin-PLCζ form a complex with ATP1A4 responsible for activation of PLCζ and that the E-cadherin-plakoglobin (catenin) complex helps to recruit the activated PLCζ to the sperm plasma membrane. Activated PLCζ enters the oocyte and causes intracellular calcicum release, thereby awakening the oocyte (meiotic resumption).

The adhesion process is also facilitated by the trans-dimerization of the integral proteins via interactions between their extracellular domains (Hartsock & Nelson 2008). In that regard, the N-glycan mediated interaction of the β-subunit of Na/K-ATPase could contribute to cell adhesion by binding to its respective subunit in neighbouring cells (Barwe et al. 2007). Since β1 and β3 subunits are demonstrated in sperm (Hickey & Buhr 2011), it is likely that it interacts
with its corresponding subunit (homotypic interaction) that is expressed in the oolemma, thereby facilitating sperm-oocyte adhesion independent of the E-cadherin pathway. To test this hypothesis, a straightforward experiment would involve staining the beta subunit (for example: β1) of acrosome-reacted sperm with a specific fluorochrome (e.g. green) and the beta subunit (β1) of oocyte with another fluorochrome (red) separately, incubate them and look for colocalisation patterns.

During the course of my investigation on ATP1A4 involvement in raft and non-raft signaling, I determined that both content and activity of ATP1A4 increased during sperm capacitation; furthermore, this increase was not due to protein translocation from other subcellular compartments, nor was it due to transcriptional activity. However, the capacitation-associated ATP1A4 increase (monitored by incorporation of fluorescently labelled lysine) was partially sensitive to the mitochondrial translation inhibitor, chloramphenicol, indicative of ATP1A4 mRNA being translated on mitochondrial ribosomes or mitochondrial-like ribosomes during capacitation. In future studies, it would be worthwhile to investigate ATP1A4 mRNA turnover and degradation after capacitation (not investigated in the current thesis work).

Functionally intact and stable sperm mRNAs can be delivered to the oocyte during fertilization and are important during the critical window of activation of the embryonic genome and its epigenetic regulation (Sendler et al. 2013). Perhaps this sperm-specific mRNA persists in the oocyte until activation of the embryonic genome and has critical roles in embryo development which remains to be investigated.

The functional significance of the increase in ATP1A4 protein is that it could either be used by sperm to meet physiological demands during capacitation, or possibly serve a secondary function (e.g. sperm-oocyte interaction and oocyte activation). Before fertilization, mammalian
oocytes are arrested in metaphase II (MII) stage, but they are activated after sperm-oocyte fusion due to a transient increase in intracellular calcium levels (Stricker 1999, Runft et al. 2002). The fertilizing sperm delivers the sperm-borne oocyte-activating factor (SOAF) required for initiating intracellular calcium release through the phosphoinositide signaling pathway, where PIP₂ is cleaved by PLC into IP₃ and DAG. Subsequently, IP₃ triggers release of calcium from endoplasmic reticulum (ER) by binding to IP₃ receptors located on the ER membrane (Xu et al. 1994). Two potential sperm proteins, PLCζ and post acrosomal WW binding protein (PAWP) are considered as SOAF candidates. In that regard, microinjection of recombinant PAWP (rPAWP) or rPLCζ into mouse oocytes induced a high rate of meiotic resumption and pronuclear formation. Furthermore, corresponding antibodies against PAWP or PLCζ or their competitive peptides blocked sperm-induced fertilization, suggesting that either PAWP or PLCζ could be the genuine oocyte activating factor (Wu et al. 2007), Saunders et al. 2002). Although certain steps of oocyte activation are defined, it is still unclear which sperm factor(s) act upstream of intracellular calcium release to initiate activation. We propose that ATP1A4 could act upstream of PLCζ via its redistribution to the equatorial segment (site of sperm-oocyte interaction) and post-acrosome region during capacitation. This possibility can be tested by microinjecting sperm obtained from ATP1A4 KO mice (already developed by Blanco et al. 2011) and recording the intracellular calcium levels in comparison to WT mice. If ATP1A4 works upstream and activates PLCζ, we expect to see a decrease or a disturbance in the dynamics of intracellular calcium release in oocytes. Before testing the ATP1A4-PLCζ hypothesis on intracellular calcium release, it is imperative to test whether the ATP1A4 signal persists in the equatorial segment and postacrosome region in acrosome-reacted sperm.
Sperm RNAs are reflective of past events during spermatogenesis, whereas sperm protein composition provides information regarding cell function; therefore, both are tightly linked to fertility. In the bovine AI industry, semen is collected from bulls that are deemed satisfactory breeders (passed a standard bull breeding soundness evaluation) and subsequently their frozen-thawed semen is used to impregnate females. Despite being deemed sound for breeding, these bulls often differ in their pregnancy rates by 20 – 25% (Larson & Miller 2000) possibly due to submicroscopic differences in sperm characteristics. Similarly, in cases of idiopathic infertility in humans, routine semen evaluation methods do not consistently provide an etiological basis for male infertility. However, methods to detect submicroscopic differences in sperm are emerging in animals and humans, providing clues about underlying mechanisms of differences in fertility/infertility. Consequently, comparing sperm from bulls with varying levels of fertility may identify these submicroscopic differences (for example, up- or down-regulation of specific proteins or RNA) and help to identify molecular markers of fertility, which may provide insights regarding diagnosis of idiopathic infertility in humans. Although approaches for biomarker discovery and development are steadily increasing, translation of biomarkers from research to clinical applications is still slow. In order to address this, I tested ATP1A4 content, its correlation with fertility and mechanisms by which this protein is involved in regulating fertility in a group of HF and LF bulls whose fertility rates were determined under controlled conditions (based on NRR). Since ATP1A4 is involved in regulating several aspects of motility and capacitation, it is likely that its content and activity are related to post-thaw sperm function and fertility. To address this objective, I developed an enzyme assay and a flow cytometry assay for quantification of ATP1A4 activity and content, respectively, in bull sperm. I also demonstrated that both content and activity of ATP1A4 were higher in HF bulls compared to LF bulls and
were positively correlated with fertility. Additionally, there was differential regulation of downstream effectors of ATP1A4 signaling (ROS, calcium, F-actin) in HF and LF bulls that were correlated to fertility, implying molecular/submicroscopic differences between HF and LF bulls in sperm characteristics. Most of the assays that I used for this objective were based on flow cytometry, a technique widely adapted at the field level by AI industries. I believe that the flow cytometric quantification of ATP1A4 content and other sperm functional parameters may contribute to development of novel laboratory assays for better prediction of fertility of bulls that are classified as satisfactory potential breeders based on traditional assessment. Finally, I also developed a regression equation by which fertility of unknown samples could be predicted based on their ATP1A4 content. However, further testing on a larger sample size is recommended to further validate ATP1A4 as a potential fertility marker. Moreover, it is likely that single nucleotide polymorphisms (SNPs) may be associated with this phenotype. Identifying such genetic markers and its association with other production traits may enable AI companies to utilize this information for bull selection.

In a series of studies (Konrad et al. 2011, Dietze et al. 2015) on the rat prepubertal Sertoli cell line 93RS2, the authors suggested that ATP1A4 mRNA, but not the ATP1A4 protein, was present in these cell lines. Quite interestingly, in other studies, there was no evidence of ATP1A4 mRNA or protein being present in Sertoli cells. Alternatively, there was evidence for ATP1A1 isoform in rat prepubertal Sertoli cell primary cultures. Since ATP1A4 expression is unique to the male reproductive tract, the final objective of my thesis was to resolve controversies regarding the existence of Na/K-ATPase isoforms and subsequently to investigate their role in the assembly and regulation of junctional complexes in Sertoli cells. Using Sertoli cell primary cultures obtained from prepubertal rats, I determined that ATP1A1 mRNA and protein were
present in these cultures. Similar to sperm, participation of some of the signaling molecules (Src, EGFR and ERK1/2) were identified in ATP1A1-ouabain interaction in Sertoli cells. Furthermore, I identified that ouabain-mediated ATP1A1 signaling regulated expression of claudin 11 and connexin 43 in Sertoli cells in a dose-dependent manner. In rats, it is well known that the mechanism of ouabain action on Na/K-ATPase is dependent upon concentration; a low dose (nM) activates the signaling pathway with no inhibition of the enzyme activity of Na/K-ATPase, whereas a high dose (mM) inhibits enzyme activity with no effect on the signaling pathway. Adding 50 nM (a low, non-inhibitory dose) of ouabain increased TER in Sertoli cells, increased phosphorylation of signaling molecules (Src, EGFR, ERK1/2, CREB) and subsequently claudin 11 and connexin 43 expression, whereas 1 mM (high, inhibitory dose) had opposite effects. Claudin 11 is the predominant member of the claudin family that comprise the tight junctions which are important constituents of BTB. Furthermore, connexin 43, yet another predominant member of the connexin family, is present in gap junctions, between Sertoli cells, and Sertoli and germ cells. In my thesis work, I cultured Sertoli cells on Matrigel coated substratum for a defined interval, after which cells assembles junctions that mimiced the BTB in vivo. Using this model, I believe that I have acquired information regarding the role of ATP1A1 on TJ and GJ assembly and regulation. However, a potential drawback is that Sertoli cells exists in two- dimensional (2D) architecture in cell culture systems, whereas these cells exist in a three-dimensional (3D) architecture in the tissue to perform specific functions. Even though the Sertoli cells form TJ and GJs, this barrier was not very tight, suggesting that it might not accurately reflect the biology of BTB in vivo. To mitigate this, the next logical step would be to attempt 3D culture of Sertoli cells where cells are embedded within a scaffold consisting of extracellular matrix (ECM) proteins and cultured on a thin layer of Matrigel within a transwell
system. Sertoli cells would eventually achieve a 3D conformation where they aggregate to form a TJ bearing tubular-like structure with germ cells at the centre (Gassei et al. 2010). Using this system, it would be interesting to study effects of ouabain on preleptotene/leptotene spermatocyte migration when they are co-cultured with Sertoli cells that exist in 3D confirmation. One of the potential clinical applications that I envision for this particular study is to manipulate the ATP1A1-ouabain interaction to develop novel approaches for male contraception. In that regard, the 3D coculture of Sertoli cells and germ cells would give us valuable information regarding the role of germ cells in ATP1A1-ouabain interaction. It is noteworthy that ATP1A1-ouabain interaction targets cell-cell interaction in the testis without interfering with the hypothalamus-pituitary-testis hormonal axis. Therefore, this interaction may have potential for development of reversible male contraception, with advantages over current hormonal methods that have undesirable side effects.

Fertility is an important trait in both humans and animals; understanding the molecular basis of sperm and Sertoli cell function will therefore advance knowledge in reproductive biology, with implications for developing novel approaches for regulating fertility.
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