The Physiology and Reproductive Potential of Sperm

Kateřina Komrsková

HABILITATION THESIS

Department of Zoology

Faculty of Science, Charles University

January 2021

CONTENTS

PROLOGUE

At the time of writing this habitation thesis, I find myself mid-career. At this stage, I would like to reflect upon my contributions to the scientific community and then identify areas of possible research to further advance the understanding and application of reproductive biology.

I consider myself lucky and humbled that I have had the opportunity to work with and learn from several eminent biologists, to gain inspiration and to ultimately publish with them. My first mentor and now esteemed colleague was Prof. Harry Moore. I was accepted as a postgraduate student into his laboratory at the University of Sheffield, UK, and he significantly broadened my knowledge about mammalian germ cells. I completed my doctoral thesis on the dynamics of cytoskeletal proteins in mammalian sperm during their post-testicular maturation and acrosome reaction. I learned to interpret and publish data to contribute to increasing knowledge in the field. We had what seemed like endless entertainment discovering details about the incredible sperm of a wild wood mouse (*Apodemus sylvaticus*), which Harry's cat brought him as an offering one Spring morning. I recall when Harry brought the mouse to the laboratory that its morphology was strikingly different to that of a standard laboratory mouse. When in season, wood mouse males possess large testes that account for an incredible 4% of their total body weight. To our further amusement, the sperm was morphologically unusual possessing an exceptionally long apical hook that enabled the sperm to form clusters, which we started calling *trains*. I remember Harry looking through the microscope and telling me: *"Kat, this is something, let's fully focus on it."* By this approach he taught me to follow my scientific instincts, to capitalize on opportunities as they presented themselves, and to distinguish the more significant from the less significant. This passion for wood mice persisted, and my knowledge of their reproductive behaviour was further extended by working with Doc. Pavel Stopka, who is an expert on the reproductive strategies of field mice (*Apodemus*). It was after my postdoctoral training in Prof. Michael Russel's laboratory in the Immunology Department, Buffalo University, USA, that I met Pavel. We shared an excitement about wood mouse biology, and it was a natural step to merge our biological studies and start working together. At this time, I also transferred from the Department of Cell Biology at Charles University to the Department of Zoology, of which Pavel became the Head. To my pleasure I found an excellent team in my new department with a happy

and relaxed environment and a great respect for each other and for students. These are qualities I value the most among colleagues, and I am proud of being part of this community.

The humble wood mouse seemed to become my destiny and a red ribbon in my scientific research. It was due to the phenotypic behaviour of wood mouse sperm prior to fertilization (which will be discussed below), that I met Prof. Peter Johnson from University of Liverpool, UK. We established a productive collaboration targeting the genomic basis of wood mouse reproductive traits. This initial meeting took place in the "Cafe Louvre" in Prague, when Peter told me about a protein that captivated his interest with the boring name CD46 otherwise known as MCP (membrane cofactor protein). He told me about a weird phenotype of the CD46 knock out mouse, how it was supposed to be infertile and how it turned out to be hyperfertile with its sperm possessing self-sacrificing fertilizing potential. My reply was: "*This sounds exactly like in the wood mouse*", and so the seeds of a decade of scientific research were sown. Naturally, our discussion was of a much deeper scientific nature, but for the sake of brevity I have simplified the narrative. Doc. Pavel Stopka and researchers from his laboratory became part of this project which resulted in several publications.

Peter and I had an inspiring time working on the CD46 protein until he decided to retire from the biological field and dedicate himself to the world of competitive sailing. I fondly remember collaborative research we had on CD46, and my current work revolves around the discoveries we initially made while looking deeper into molecular interactions and protein-protein network formation prior to sperm-egg binding and membrane fusion. Recently I also turned my focus to oocytes when it became clear to me that working solely with sperm was giving a one-sided and therefore incomplete picture. This was especially significant when considering the formation of the protein-protein interactive network. The machinery required for gamete interactions is established during sperm and oocyte maturation, so a complete understanding of the process of fertilization can only be reached by merging our knowledge of both male and female fertilization strategies, ultimately resulting in the conception of new life. Studying the proteins involved in membrane fusion became the focus of my recent scientific research.

The main goal of my research is the translation of basic science into practical applications. This involves selecting novel markers and designing innovative tools and techniques for infertility diagnostics. My interest in the wood mouse has not totally

disappeared and I have established collaboration with Prof. Eduardo Roldan, MNCN-CSIC Madrid, to research the kinetic patterns of sperm trains in clades of field mice. I also maintain an ongoing project with Pavel Stopka on the chemical communication of gametes.

I also have significant ongoing research projects with eminent scientists in the field. In summary, there is ongoing fruitful research with Prof. Klaus Steger, University of Giessen, on classification of novel epigenetic markers connected to human infertility, as well as research addressing interactive networks between the nucleoskeleton and the cytoskeleton in sperm. Recently I have had the opportunity to work with Prof. Kate Loveland of Monash University, a leading scientist in the field of testicular cancer and testis development, and our collaboration is addressing importin subunit localization in sperm, with the potential to develop new knowledge of importins as molecular scaffolds during spermatogenesis. In a productive collaboration with Prof. Jiri Neuzil, Institute of Biotechnology and Griffith University, I have been drawn further into cancer research via work on transgenic mouse lines as indispensable tools to compare mitochondrial behavior between healthy and malignant cells *in vivo*.

There are many more short- or long-term projects which have delivered or promise to deliver significant results. Regretably, I cannot acknowledge all of them in detail in this thesis. Before proceeding further, I would like to thank all my past mentors and colleagues with whom I have worked over the years, as well as with those whom I am currently collaborating with the prospect of further exciting discoveries.

I would particularly like to thank Harry Moore for years of scientific discussions, support and friendship; to Mike Russel for introducing me to the Mucosal Immunology field and being so accommodating overseas; to Peter Johnson for all the knowledge he shared and for our discussions large and small; to Jana Peknicova for believing in me and supporting me; to Pavel Stopka for showing me the importance of independent research and for continued collaboration; to Zuzana Bosakova for her analytical approach to biological data and their kinetic interpretation by her wonderful father Antonin Tockstein; to Jana Antalikova and Jana Jankovicova for our joint talks about eggs and fruitful collaboration; to Agnieszka Paradowska for wonderful collaboration and being my soul mate; to Jitka Zurmanova for being my colleague and true friend; to Jiri Neuzil for new inspiring scientific challenges, collaboration, mutual human understanding and friendly discussions; to Kate Loveland for her valuable comments and helpful feedback on this

habitation thesis. I am also thankful for all the inspiration of my team members and publication co-authors. It has been and will always be my pleasure to work with each of them. It has been a particular pleasure to share and discuss everyday science with Michaela Frolikova, Natasa Sebkova and Pavla Postlerova, who give me ever so needed support. I am thankful to all my faculty colleagues for the inspiring academic environment at my *Alma Mater*.

I am also thankful to my friends and family who have always helped me: to my mother for babysitting my children so that I could work; to both my parents for all their love and simply for everything; to Timothy Hort, farther of my children, for all the challenges that made me stronger and independent, for English humour and for proofreading of nearly all my scientific work, for which I am truly grateful; to my brother Tomas for being always cheerful, supportive and caring; to my partner Duncan Lloyd for loving me so much and taking care of me in both good and bad times; and to my dearest children Kitty and Toby for loving me so purely and giving me all the happiness in the world, the true purpose of life.

AIM OF THE HABILITATION THESIS

The aim of this habilitation thesis is to summarize and connect my key publications into a cohesive narrative, that will take readers on an expedition into the life of sperm before it becomes worthy of the egg. My research to date has revealed but a small piece of a great puzzle which is, how the gametes complete their ultimate task of fertilization to begin a new life. I aim to deliver a thesis that would be understandable to a wide scientific audience, including students and colleagues who work outside the field of reproduction. For those interested, the detailed research can be found in the published papers, therefore, I will summarize here the crucial points of selected publications as a retrospective, with key facts that in early times seemed to be so clear and monochromatic, and only later attained new dimensions and the beautiful colours of a rainbow when integrated into a larger body of work.

INTRODUCTION TO SELECTED PUBLICATIONS

The selected publications represent my dedication over nearly two decades to a better understanding of mammalian sperm behaviour prior to fertilization. Sperm are unique, fascinating and surprising cells, whose sole task is to fertilize the egg. However, to fulfil this task, they go through an extraordinarily complex journey. They are even willing to cooperate (Moore et al., 2002), to beat the competitive sperm from other males in the promiscuous community of wood mouse and other mouse species. These rodent sperm differ in presence (*Mus* sp.) or absence (*Apodemus* sp.) of a specific protein called CD46 based on the regulation of *Cd46* gene expression (Johnson et al., 2007). The CD46 protein is involved in the stabilization of the crucial organelle of the sperm called acrosome, used for chemical drilling through the egg vestments, including the *cumulus oophorus* and *zona pellucida*. The competition among the sperm to reach and fertilize the ovum is also seen during the complex molecular changes referred to as capacitation; this involves membrane destabilization in response to cholesterol efflux, inner layer phospholipid exposure, specific protein tyrosine phosphorylation and changes in the sperm motility pattern from progressive to hyperactivated.

This event of sperm maturation is also represented by the relocation of specific proteins such as Izumo1, which is the key mediator of sperm fusion and which is required

for sperm fusion with the egg plasma membrane. Our study (Sebkova et al., 2014) monitored Izumo1 dynamic movements during the acrosome reaction, when the sperm head undergoes preparation for fusion with the egg membrane. We compared sperm of *Mus* and *Apodemus* species and discovered that the speed of Izumo1 relocation positively correlates with promiscuous mating behaviour of the species. When the sperm competition is fierce the acrosome reaction is accompanied by significantly faster Izumo1 distribution from the apical part of the sperm head to the fusogenic equatorial segment and finally over the whole sperm head. Moreover, we have recently shown that other proteins, such as CD46 and β1 integrin participate in these transitions; they interact and move together to the sperm equatorial segment where mammalian sperm-egg membrane fusion occurs, but only when the acrosome reaction is triggered (Frolikova et al., 2016). Based on this highly significant discovery, we proposed CD46 and β1 integrin subunit as new diagnostic markers for selecting the best sperm for assisted reproduction treatment. The fundamental mechanisms by which protein rearrangements mediate sperm head compartment remodelling during and after the acrosome reaction require cytoskeletal proteins such as actin (Dvorakova et al., 2005; Frolikova et al., 2016), spectrin, and tubulin (Dvorakova et al., 2005). These proteins build the skeleton of every cell type, serving central roles in cell division, cell-cell communication, and intracellular transport.

When cell communication is disturbed, pathological events can be triggered. Substances that may cause such disruption include environmental pollutants, which are present in elevated concentrations in nature as the result of human non-biodegradable waste and man-made chemical products. These pollutants are present in drinking water and are linked with declining animal and human fertility. We studied multiple factors including estrogenic hormones that could have a negative impact on sperm quality and fertilizing potential (Sebkova et al., 2012). We found evidence that sperm capacitate faster in the presence of endocrine disruptors, which results in premature loss of their fertilizing potential, and it is alarming how much these disruptors diminish the ability of spermatozoa to undergo processes required to fertilize an egg. Besides environmental pollutants, a parasitic disease such as Toxoplasmosis can also compromise male fertility. Our work has contributed to the evidence that *Toxoplasma gondii* could modify the epigenetic information, which is passed to the early developing embryo by the sperm (Dvorakova-Hortova et al., 2014). It is becoming more widely accepted worldwide that endocrine disruptors and infections are newly discovered contributors to idiopathic

infertility in men, and we have shown that specific epigenetic aberrations in the sperm epigenome can be transferred to the egg and influence early embryogenesis (Vieweg M and Dvorakova-Hortova K et al., 2015). Therefore, better infertility diagnostics and preselection of the best sperm for *in vitro* fertilization are highly desirable for improving clinical management of fertility.

Our recent discovery (Frolikova et al., 2016) demonstrates that the CD46 protein could serve as a new diagnostic marker to detect sperm impairment. There is currently an urgent need for new technologies that would be used not only as reliable and rapid diagnostic tools but would also allow technicians to select and separate high quality sperm for further use in assisted reproductive techniques. We developed a unique technology (patent applications: EP20152484.0, EP20152470.9 and PV 2020-223, PV 2020-224) based on the knowledge that the CD46 protein is not surface-exposed in healthy sperm but it is detectable only if the sperm head is damaged. This allows us to diagnose, select and remove a population of spermatozoa with premature partial or total loss of the sperm acrosome, cells which may have an increase in DNA damage and exhibit decreased progressive motility. Our capacity to separate "good" sperm from "bad" based on CD46 profiling could increase the embryo quality and consequently improve pregnancy rates.

CD46 was also proposed to form a "tetraspanin-like" protein network on sperm (Frolikova et al., 2016), similar to that already well characterised in oocytes. CD46 behaviour in sperm is similar to that of CD81 and CD151, other newly described tetraspanins in sperm, which exhibit a similar expression profile and function during sperm maturation and acrosome reaction. We described not only their localization in the sperm head but also their redistribution and spatial conformation in the sperm membrane prior to sperm-egg membrane fusion (Jankovicova et al., 2016; Jankovicova et al., 2020). The dynamic distribution of CD81 was also addressed in early embryogenesis in the bovine model, and CD81 was demonstrated to be released into the perivitelline space as a part of membrane-bound extracellular macrovesicles similarly to CD9, where it could mediate intercellular blastomere communication (Jankovicova et al., 2016).

Tetraspanins are not solitary proteins; they function in tandem. Therefore, based on recent findings, it was logical to target cross-talk between two major tetraspanins, CD9 and CD81, as a potential mediator of sperm-egg interactions (Frolikova et al., 2018). These two proteins were shown to exhibit species-specific expression and relocation patterns during human, mouse, and boar sperm maturation events prior to fertilization,

including their relocation dynamics during acrosomal exocytosis. Based on structural modelling, it was proposed that both homologous and heterologous interactive networks of CD9 and CD81 form, both in sperm, and when gamete membrane fusion occurs (Frolikova et al., 2018).

In order to present a more comprehensive picture of protein-protein interactions and communication prior to fertilization, we aimed to clarify the subcellular localization of individual heterodimers within specific sperm compartments. Integrins are transmembrane cell receptors involved in intracellular signalling and cell adhesion, which both represent two crucial processes integral to successful fertilization. We discovered the existence of α6β4, α3β1 and α6β1 complexes in distinct membrane regions overlaying specialised microdomain structures in the mouse sperm head. We contributed to the knowledge that the different protein composition of these individual membrane rafts allows each to perform a specialized role, based on their involvement in sperm-epithelium and sperm-egg membrane interactions (Frolikova et al., 2019).

SELECTED PUBLICATIONS TO BE DISCUSSED

I. Sperm competition is reflected by acrosome reaction

1. Moore H, **Dvorakova K**, Jenkins N, Breed W (2002) Exceptional sperm cooperation in the wood mouse. *Nature,* 418, 174-177.
2. Johnson PM, Clift LE, Andrlikova P, Jursova M, Flanagan BF, Cummerson JA, Stopka, P, **Dvorakova-Hortova K** (2007) Rapid sperm acrosome reaction in the absence of acrosomal CD46 expression in promiscuous field mice (*Apodemus*). *Reproduction*, 134, 739-747.
3. Sebkova N, Ded L, Vesela K, **Dvorakova-Hortova K** (2014) Progress of sperm IZUMO1 relocation during spontaneous acrosome reaction. *Reproduction*, 145 (3), 255- 63.

II. Sperm maturation involves protein dynamics prior to fertilization

4. **Dvorakova K,** Moore H, Sebkova N, Palecek J (2005) Cytoskeleton localization in the sperm head prior to fertilization. *Reproduction*, 130, 61-69.
5. Frolikova M, Sebkova N, Ded L, **Dvorakova-Hortova K** (2016) Characterization of CD46 and β1 integrin dynamics during sperm acrosome reaction. *Sci Rep.*, 6: 33714.
6. Jankovicova J, Frolikova M, Sebkova N, Simon M, Cupperova P, Lipcseyova D, Michalkova K, Horovska L, Sedlacek R, Stopka P, Antalikova J, **Dvorakova-Hortova K** (2016) Characterization of tetraspanin protein CD81 in mouse spermatozoa and bovine gametes *Reproduction*, 152(6), 785-793.
7. Frolikova M, Manaskova-Postlerova P, Cerny J, Jankovicova J, Simonik O, Pohlova A, Secova P, Antalikova J, **Dvorakova-Hortova K** (2018) CD9 and CD81 Interaction and their Structure Modelling in Sperm Prior to Fertilization. *Int J Mol Sci*., 19(4).
8. Frolikova M, Valaskova E, Cerny J, Lumeau A, Sebkova N, Palenikova V, Sanches-Hernandez N, Pohlova A, Manaskova-Postlerova P, **Dvorakova-Hortova K** (2019) Addressing the compartmentalization of specific integrin heterodimers in mouse sperm. *Int J Mol Sci.,* 20(5) pii: E1004.
9. Jankovicova J, Frolikova M, Palenikova V, Valaskova E, Cerny J, Secova P, Bartokova M, Horovska L, Manaskova-Postlerova P, Antalikova J, **Komrskova K** (2020) Expression and distribution of CD151 as a partner of alpha6 integrin in male germ cells. *Sci Rep.,* 10(1):4374.

III. Sperm reproductive fitness is modified by pathological interventions

10. Sebkova N, Cerna M, Ded L, Peknicova J, **Dvorakova-Hortova K** (2012) The slower the better: how sperm capacitation and acrosome reaction is modified in the presence of estrogens. *Reproduction*, 143, 297-307.
11. Vieweg M and **Dvorakova-Hortova K**, Dudkova B, Waliszewski P, Otte M, Oels B, Hajimohammad A, Turley H, Schorsch M, Schuppe HC, Weidner W, Steger K, Paradowska-Dogan A (2015) The methylation analysis of histone H4K12ac associated promoters in sperm of healthy donors and subfertile patients. *Clinical Epigenetics,* 7:31.
12. **Dvorakova-Hortova K**, Sidlova A, Ded L, Hladovcova D, Vieweg M, Steger K, Stopka P, Paradowska-Dogan A (2014) *Toxoplasma gondii* decreases the reproductive fitness in mice. *PLoS One,* 9 (6): e96770.

GENERAL DISCUSSION

I. Sperm competition is reflected by acrosome reaction

In less than two decades, sperm competition has progressed to being studied more from molecular perspective. This change was not just due to the revolution in genomic and proteomic methods. New traits were discovered of relevance to the function and evolution of molecular processes, novel physiological roles of sperm specific proteins, seminal fluid composition and immunity were identified as key players in sperm competition and sperm utilization, and quantification of trade-offs has been revisited (Moore et al., 2002; Parker and Pizzari, 2010; McDonough et al, 2016; Wigby et al., 2019). Amongst small mammals, multiple matings resulting in sperm competition and mixed paternity in littermates are believed to be widespread (Birkhead and Moller, 1996; Bryja and Stopka, 2005), and the energy allocated by males into reproductive fitness and sperm quality under circumstances of promiscuity is enormous. One very curious example of sperm competition we introduced is in the wood mouse (*Apodemus sylvaticus*) (Moore et al., 2002), in which males are engaged in competition to mate polygynously with promiscuous females (Tew and Macdonald, 1994). Due to the unusual falciform shape of the sperm head that is dominated by a long apical hook, the sperm are morphologically equipped to hook to one another to create so called sperm "trains", which are formed *in vivo* by dozens of sperm within the female reproductive tract (Moore et al, 2002). Similar formations were later described in deer mice (*Peromyscus sp.*) (Fisher and Hoekstra, 2010). Surprisingly, in wood mouse the sperm train formations were twice as fast as individual sperm (Moore at al., 2002) and provided functional sperm competition between individual males in a competitive mating environment, in contrast to in monogamous species (Fisher and Hoekstra, 2010; Fisher et al., 2016). However, whatever mating strategies are in play, only one sperm can fertilize the egg and the question remains to be answered: how does sperm selection occur in the context of the sperm train?

It had been postulated that, in mammals, only capacitated sperm are able to undergo the acrosome reaction. In order for sperm to fertilize the egg, the acrosome reaction is triggered by the egg extracellular matrix called the *zona pellucida* and the release of acrosome contents provides the tool required for sperm to pass through the zona (Yanagimachi 1994). In the competitive sperm environment of wood mice, the acrosome reaction seems to have another role, and its premature (*zona pellucida*-independent)

spontaneous onset in the majority of sperm results in sperm train disintegration (Moore et al., 2002; Johnson et al, 2007; Clift et al., 2009). By sperm releasing their acrosomal vesicle content, their reproductive success is compromised (Moore et al., 2002); giving up reproductive potential was previously described as reproductive cooperation and sperm altruistic behaviours (Triver 1971; Baker and Bellis, 1987; Kura and Nakashima 2000; Moore et al., 2002; Lehman and Keller, 2006; Immler, 2008; Pizzari and Foster, 2008). We could only speculate whether behaviour such as altruism could be adopted from organisms to describe the behaviour of cells. Higginson and Pitnick (2011) interpreted sperm heteromorphism and conjugation not as cooperation, but rather as traits selected at the level of the male, much like other ejaculatory traits such as accessory gland proteins and ejaculate size. On the other hand, they noted that sperm are often considered to be individuals, in part because of their unique genetic identities produced as a result of synapsis during meiosis, and in part due to their unique ecology, being ejected away from the soma to continue their existence in a foreign environment (Higginson and Pitnick, 2011). Either way, this provides everlasting fruitful discussions and excitement in the field of sperm competition. However, at the end of the day, whatever way we decide to call it, the speed and percentage of sperm which undergo the spontaneous acrosome reaction in field mice (*Apodemus* sp.) positively correlates with a level of individual species promiscuity, from promiscuous *A. agrarius, A. sylvaticus* and *A. flavicolis* to the facultatively monogamous *A. microps (uralensis)* in which sperm pre-fertilizing behaviour is similar to house mouse (*Mus m. musculus* and *Mus m. domesticus*) or BALB/c, traits defined on behavioural level (Stopka and Macdonald 1998, 1999) as well as by the genetic relatedness of offspring in one litter (Bryja and Stopka, 2005).

The cytoskeleton protein actin and electron dense material are each associated with sperm train formation (Moore et al., 2002), but the force that triggers the onset of acrosome reaction and therefore the sperm train disintegration lies in gene expression. This was found due to the unexpected discovery that *Cd46* gene disruption in inbred laboratory mice led to elevated spontaneous acrosome reaction compared to the wild-type gene (Inoue et al. 2003). CD46 (membrane cofactor protein) is membrane-associated glycoprotein that is present in human cells, including sperm, and in cells of other mammals. In sperm, CD46 is present as an unusual lower molecular weight hypo-glycosylated isoform which localized to the acrosomal cap that only becomes surface-exposed after acrosome reaction of sperm (Riley et al., 2002, Cummerson et al., 2006).

Differences in *Cd46* gene expression between sperm of wild field mice were later revealed (Johnson et al., 2007; Clift at al., 2009), and we demonstrated that wild-caught *A. sylvaticus* exhibit a more rapid acrosome reaction than house mice (*Mus musculus)*. It was shown that *A. sylvaticus* fail to express any CD46 protein, in both the testis and epididymis, and we extended these observations to also document that additional species within the *Apodemus* genus behave similarly to *A. sylvaticus*. Collectively, these findings led to the theory that CD46 may play a role in acrosomal membrane stabilization (Inoue et al., 2003; Johnson et al., 2007; Clift at al., 2009), and we proposed it serves a role in stabilizing the whole acrosome vesicle and is of central importance to mating strategies and sexual selection forces (Figure 1).

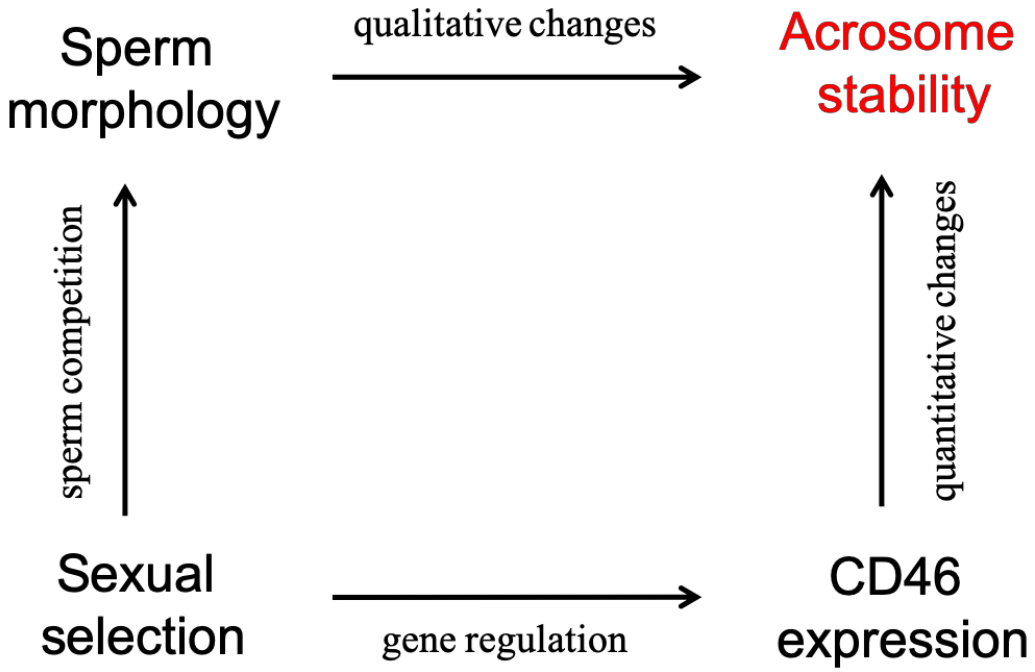

Figure 1. *Graphical representation of the acrosome biogenesis and its function, reflecting the evolution of sperm traits influenced by developmental processes of sperm morphogenesis, showing the central role of CD46.*

A new perspective on the spontaneous acrosome reaction was brought later by studying mouse fertilization (Jin et al., 2011), when and the complex physiology and functionality of this process was ascertained. Acrosome-reacted mouse sperm obtained from the perivitelline space were capable of fertilizing *zona pellucida*-intact eggs and

yielding new progeny (Inoue et al., 2011b). This break-through finding brought a new perspective to the importance of the sperm spontaneous acrosome reaction because it became obvious that rather than being a disadvantage, it represents a time-related advantage in the promiscuous competitive mating environment of field mice.

In the light of this discovery, the well-established phenomenon of protein relocation during acrosome reaction was studied. It was documented that after a successfully completed acrosome reaction, the protein composition of the sperm head equatorial segment and post-acrosomal region is modified, including the sperm primary fusion protein Izumo1 which relocates from the apical acrosome into the sperm fusion region called equatorial segment (Inoue et al., 2005; Sosnik et al. 2009; Inoue et al., 2011a; Satouh et al., 2012). An important research approach has been to study the spontaneous acrosome reaction, defined as the acrosome reaction triggered in the absence of *zona pellucida* or *cumulus oophorus* cells (Wolf et al., 1992). It was important to ask whether sperm undergoing a spontaneous acrosome reaction are capable of Izumo1 protein relocation, and whether the speed of its redistribution is governed by species promiscuity. In both field mice and house mice, after the spontaneous acrosome reaction Izumo1 relocalized towards the equatorial segment of the sperm head, and the standardized indicator of successful capacitation, signalling pathways leading to protein tyrosine phosphorylation, was activated (Sebkova et al., 2014). Moreover, we provided evidence that the speed of Izumo1 relocation positively correlates with the level of species promiscuity and negatively with the acrosome stability, by comparing *A. sylvaticus*, *A. microps (uralensis)* and laboratory BALB/c mouse sperm. This finding delivered another piece into the mosaic of spontaneous acrosome reaction physiology and brought yet another proof that these sperm represent functional competitors, maintaining their undisputable ability to fertilize the egg.

II. Sperm maturation involves protein dynamics prior to fertilization

Sperm can be regarded as DNA storage, the only purpose of which, is to deliver the DNA to the egg in order to generate progeny. However, mammalian sperm on its journey is maturing from a "pubertal boy" to an "adult man" and this transformation that takes place in the female reproductive tract is called capacitation. This maturation process entails several crucial changes in the capacity for sperm motility, but also involves alterations in membrane fluidity and sperm head restructuring. The acrosome reaction is a signalling-rich remodelling event involving phosphorylation on protein tyrosine residues; it occurs only at the end of capacitation as the plasma and outer acrosomal membranes fuse together and the lytic contents of acrosome are released (Yanamagichi, 1994, Visconti et al., 1999). Sperm functions such as motility may be subjected to greater evolutionary adaptation which might have resulted in the large morphological differences. Although the shape and relative size of the sperm head may vary considerably, basic organelles such as the apical acrosome, equatorial segment and post- acrosomal region are invariably present and these structures can be identified readily in spermatozoa of most mammals (Dvorakova et al., 2005). This may indicate that sperm are mechanistically linked, despite species differences and crucial sperm head functions, such as capacitation, the acrosome reaction and sperm–egg fusion, are conserved. The morphology of the mammalian sperm head can vary significantly in shapes and features (Yanagimachi 1981, Eddy and O'Brien 1994). The head shape is formed during spermiogenesis, with the cytoskeleton of the sperm head and perinuclear theca contributing to shaping the nucleus (Oko 1998); more dynamic proteins of the cortical cytoskeleton are also involved. Besides serving classical morphological functions, cytoskeletal proteins are also involved in cell signalling processes. For example, in sperm actin polymerization an important regulatory pathway is associated with tyrosine phosphorylation and Ca^{2+} channel activation (Brener et al., 2003, Seligman et al. 2004; Breitbart et al., 2005; Finkelstein et al 2013; Finkelstein at al., 2020). The dynamic relocation of cytoskeletal proteins during the acrosome reaction is not random progresses from the apical part of the mammalian sperm head to the equatorial segment (Dvorakova et al., 2005). This process of cytoskeletal proteins movement seems significant, as despite the great variety of sperm head morphology, the process directionality remains conserved and evidence was provided that showed cytoskeletal proteins are important during the arosome reaction to reorganize membrane

domains into fusogenic regions (Inoue et al., 2005; Sosnik et al. 2009; Inoue et al., 2011a; Satouh et al., 2012; Frolikova et al, 2016).

Although the function of cytoskeletal proteins, mainly actin, is well known during the acrosome reaction, it remained unclear for a long-time which proteins participate in directing the dynamics of its organization. We have already discussed in the previous chapter that CD46 is present on the acrosomal membranes and that it is not surface-exposed until the acrosome reaction is completed (Inoue et al., 2003). Data was also provided that indicated CD46 could be responsible for stabilization of the acrosomal membrane and consequently the whole acrosomal region (Johnson et al., 2007; Clift et al., 2009). Two alternative mechanisms were proposed for actin arrangement in sperm during the acrosome reaction: one involving CD46, and the other involving integrins. Integrins possess actin-binding capacity through the beta subunit (Barraud-Lange et a., 2007); due to their presence on sperm, they could therefore influence actin reassembly. As a further possibility, CD46 and integrins in one unified manner could modify actin remodelling. The interaction between CD46 and β1 integrins is well known in somatic cells, including those of humans (Lozahic et al., 2000), but it was not until our study (Frolikova at al., 2016) that evidence of the partnership of these proteins in sperm was provided. Our data provided strong evidence that CD46 and the β1 integrin subunit are binding partners and thus may play an important role in directing the acrosome reaction onset via interactions with the actin cytoskeleton. In support of this theory, we demonstrated that both proteins relocate from the apical acrosomal cap into the equatorial segment, and later translocate across the whole sperm head during the acrosome reaction. However, the timing of protein dynamic relocation may differ on the basis of their membrane localization (Frolikova et al., 2016).

It remains to be determined whether CD46 activity is dependent on calcium signalling (Kallstrom et al., 1998) which is of particular relevance to the acrosome integrity. The acrosome reaction onset is known to involve actin reorganization following protein kinase signalling and could be triggered, as in a somatic cell, directly upon CD46 stimulation (Wong et al., 1997; Zaffran et al. 2001) or by specific CD46 binding to β1 integrins and subsequent indirect associations via the integrin tail anchor into the surrounding cortical cytoskeleton (Lozahic et al., 2000; Kurita-Taniguchi et al., 2002; Rezcallah et al., 2005). Yet again, this brought the CD46 role prior to sperm-egg fusion to scientific attention, and based on experimental evidence it was hypothesized that α6β1

integrin localized to the apical hook of the intact mouse sperm head may play a role in the mouse sperm train assembly (Frolikova et al., 2016).

In the previously discussed publication (Frolikova et al., 2016) we proposed that CD46 creates an extensive protein network in sperm which is similar to a tetraspanin web. Tetraspanins are Cluster of Differentiation (CD) molecules and members of this superfamily, including CD9, CD81, CD151, CD63 are crucial in the contex of gamete interaction and fusion (Rubinstein et al., 2006; Barraud-Lange and Boucheix 2013) or play a role in gamete maturation, fertilization, and embryo implantation (Machtinger et al., 2016). These proteins were initially regarded as egg-specific, with CD9 expression on the egg plasma membrane shown to be indispensable for sperm–egg fusion in mice (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al*.,* 2000). *Cd81* and *Cd9* double knockout female mice were later revealed to be completely infertile, with microinjection of CD81 mRNA partially compensating for the absence of CD9 in *Cd9*-deficient oocytes (Rubinstein et al., 2006). These data suggest that CD81 functions in a tetraspanin web formation during egg fertilization in a manner similar or complementary to CD9 (Ohnami et al., 2012).

As a majority of oocyte research is carried out using mouse as a model, we extended knowledge of its possible function by examining the CD81 protein expression profile in maturing bovine oocytes and during early embryogenesis (Jakovicova et al., 2016). CD81 was identified on the bovine oocyte plasma membrane, organized in membrane-derived vesicles. It accumulates in the perivitelline space of *in vitro*-produced zygotes and in four-cell bovine embryos (Jankovicova et al., 2016), so our observation was in accord with the reported release of CD9-positive vesicles derived from murine oocytes prior to fertilization (Miyado *et al.* 2008). The presence of CD9 and CD81 in the extracellular matrix of oocyte and in exosomes (Ohnami et al., 2012) suggests to mediate intercellular communication between cells and tissues of murine (Machtinger *et al.* 2016) and bovine model (Jankovicova et al., 2016). Consequently, similar to knowledge about CD9 network in mouse sperm (Ito et al., 2010) we addressed protein expression of CD81 in male germ cells and investigated an existence of CD81 tetraspanin web within sperm head membranes. Our data suggest that CD81 protein is located on the plasma membrane covering the apical acrosome both in bull-ejaculated sperm and in mouse epididymal sperm. An interesting point of difference was the finding that, during sperm maturation CD81 was lost from the surface of the acrosome-reacted spermatozoa in bull, while in

contrast mouse sperm exhibited CD81 protein relocalization during the acrosome reaction first across the equatorial segment and then across the whole sperm head. These published results highlighted conserved aspects of CD81 expression between cattle and mice, which together with mouse-specific traits in sperm CD81 behaviour, highlights certain species-specific mechanisms of fertilization that should beconsidered (Jankovicova et al., 2016).

The story of integrin protein networks in sperm continues. My team has provided additional highly detailed information about the compartmentalization of proteins into sperm head-specific domains which reflects their functionality during sperm-egg interactions (Frolikova et al., 2019). The laminin-binding integrins, α3β1, α6β1 and α6β4, are usually co-expressed in cells, and they are major receptors used by epithelial cells to bind laminins. The α3 and α6 subunits are able to cooperate and promote distinct function and signalling outcomes when bound to the β1 subunit (Yazlovitskaya et al., 2018). However, to dynamically interact, they need to occupy a similar or the same membrane rafts within individual sperm compartments (Figure 2a, b). Each of these integrin pairs interact with the actin cytoskeleton, but the different heterodimer, α6β4, is known to bind mainly to intermediate filaments and provide mechanical and structural cell stability (Kierszenbaum et al., 2007; Göb et al., 2010; Guyonnet et al., 2012). We observed α6β4 integrin surrounding the nucleus and speculate that this heterodimer may provide nuclear anchoring to other cellular components and enhancing its stability (Figure 2c) (Frolikova et al., 2019). The integrins are usually found in the cells with adhesive activity, and in relevance to sperm-egg binding and membrane fusion, the previously reported organization of integrins into nanoclusters (Spiess et al., 2018) may be appropriate to consider during gamete interaction.

The sperm head membrane protein network is the key element that facilitates gamete fusion and interesting results came from structural modelling of interactions potentially occurring in cis (within a single membrane) and trans (between the interacting membranes of sperm and egg) orientations. In most recent publication (Frolikova et al., 2019), we predicted that during the acrosome reaction, as membrane fluidity increases, the α3 and α6 integrin subunits could interchange between the β1 subunit to modulate cytoskeletal function during membrane fusion (Figures 2d). Moreover, active and inactive forms of integrins are known to occupy different nanoclusters within membrane microdomains and converting the inactive form into the active one results in signalling activity. The inner acrosomal membrane which becomes surface-exposed after the

acrosomal exocytosis is also rich in integrins and it merges in the equatorial segment with the plasma membrane covering the post-acrosomal region. Based on super-resolution micrographs data, it seems that α3β1and α6β4 integrins pairs are predominantly present on the sperm head plasma membrane and inner acrosomal membrane which will be in first contact with the oolema (Figure 2a, c). It is important to note, that α3 integrin subunit is also localised to the oolema (Spiess et al., 2018) and based on a modelling of activated states of α/β integrin heterodimers and a probable arrangement of integrin heterodimers in the open state the trans-interactions between α3 (oolema) and β4 (sperm membranes) integrin subunits show favourable and stable trans position compared to the cis one (Figure 2e). The data could be interpreted that the newly established interaction between proximal domains of α3/β4 integrin trans-conformation could facilitate the fusion. The structural modelling, which we included in our recent publications in collaboration with the Laboratory of Structural Bioinformatics of Proteins (Institute of Biotechnology of the Czech Academy of Sciences) provides valuable interpretation of experimental data and has indicated novel future perspectives.

Our work contributed to identification of membrane compartmentalization and an understanding of the complexity of membranes overlaying specialized microdomain structures of the sperm head. This level of precise mapping is well beyond that previously documented, and it has been linked with a specific set of signalling molecules to help identify the mechanisms that mediate processes essential to fertilization. The different protein composition of each domain is predicted to correspond with the specialized role of these individual membrane rafts, facilitating “in” and “out” signalling events during sperm maturation, the acrosome reaction, as well as the sperm-uterine epithelium and sperm-egg interactions (Frolikova et al., 2019).

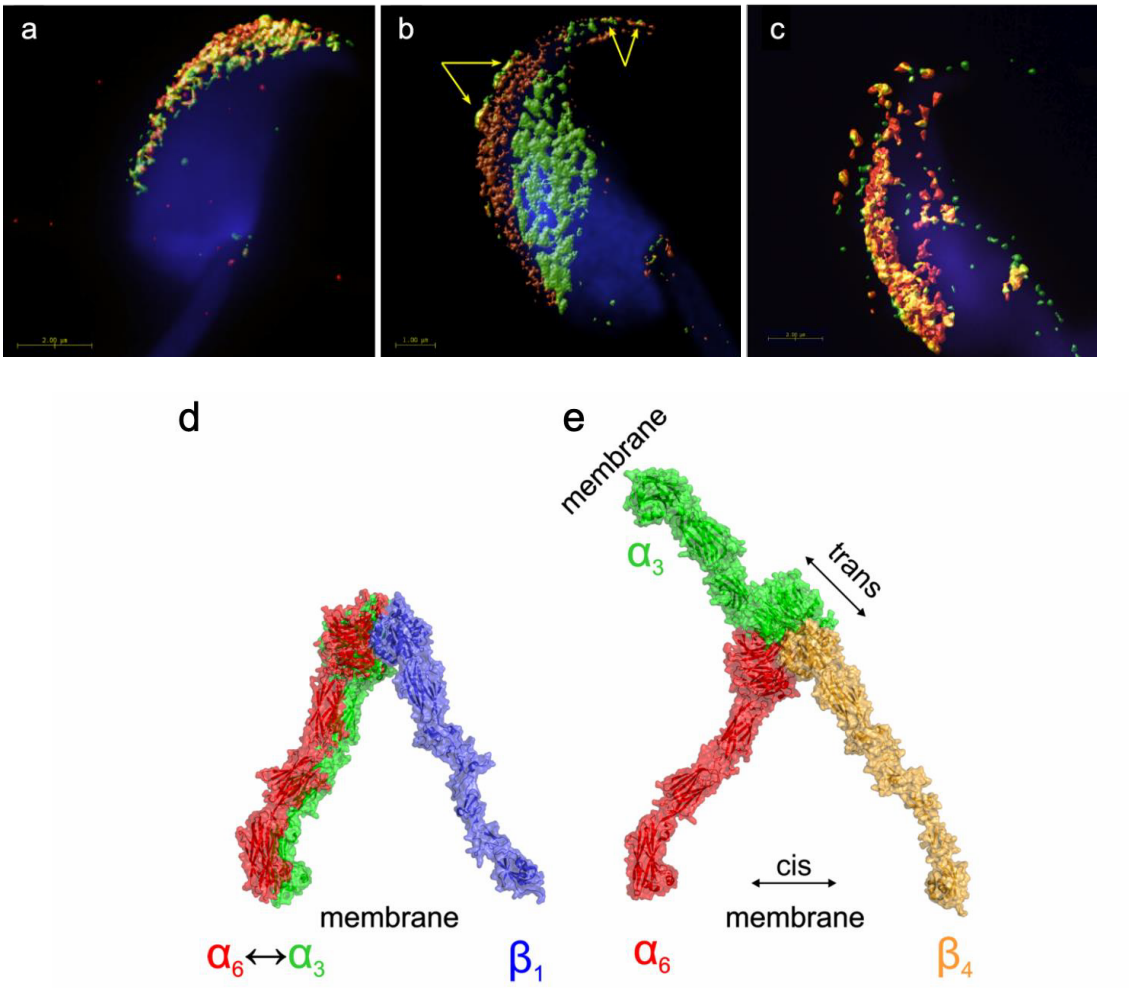

Figure 2. *Presence of α3β1* ***(a)****, α6β1* ***(b)*** *and α6β4* ***(c)*** *integrin heterodimers in mouse sperm visualized by indirect immunofluorescence and captured by super-resolution microscopy. Colocalization maps (yellow a-c, highlighted by arrows, b) are produced based on Pearson's correlation coefficient. α3, α6 (green, a,b), β1 (red, a,b); α6 (red, c), β4 (green, c), nucleus (blue), scale bar represents 1 μm (a) and 2 μm (b,c). Model of activated states of α/β integrin heterodimers and a probable arrangement of integrin heterodimers in the open state* ***(d,e)****. The modelling suggests that while the α3β1, α6β1* ***(d)****, and α6β4* ***(e)*** *integrins are involved in a cis interaction adopting the expected conformation with the membrane proximal domains separated, the orientation of the α3β4 N-terminal domains would lead to a complex with α and β subunits pointing in nearly opposite directions* ***(e)*** *supporting the possible trans interaction. Adapted from Frolikova et al., 2019.*

The next logical step towards a deeper understanding of interactive protein networks in male germ cells called for addressing the interactions between integrins and tetraspanins, some of which are well described in somatic cells. In our previous study, we identified both α6β1 and α6β4 integrin heterodimers on sperm (Frolikova et al., 2019). However, the α6 subunit preferentially creates the α6β4 heterodimer in the presence of both β4 and β1 subunits (Kajiji et al., 1989; Hemler et al., 1989; Simon-Assmann et al., 1994), such as α6β4 integrin heterodimer and its interaction with CD151 in hemidesmosomes and in cancer cells (Lotus et al., 2000; Sadej et al., 2014), where CD151 plays a role in integrin trafficking and subcellular distribution (Liu et al., 2007; Winterwood et al., 2006). Moreover, in hemidesmosomes, CD151 strengthens the adhesion complex of α3β1 with α6β4 and plectin (Molder et al., 2019), which in sperm surrounds the nucleus (Kierszenbaum et al., 2007). CD151 and α6β4, but not α3β1 integrin share the same location in the membrane overlaying the equatorial segment, a region which defines the cytosolic space above the sperm nucleus. Based on this, we hypothesized that CD151 could be involved via α6β4 and plectin in equatorial domain stabilization during the acrosome reaction preceding sperm-egg membrane fusion (Jankovicova et al., 2020). It is also of relevance that the β4 integrin subunit is structurally different from the other known β subunits as a result of its unusually large cytoplasmic domain, and this can directly affect cytoskeleton organization (Spinardi et al., 1993; Mainiero et al., 1995; Hynes et al., 2002) including Rac1 protein and induce its activation (Colburn et al., 2017), Rac1 was recently shown to play a key role in capacitation and the acrosome reaction in guinea pig sperm (Ramirez-Ramírez et al., 2019), participating in actin remodelling in the apical acrosome region during these events. We proposed that relocation of CD46 into the equatorial segment of the sperm (Frolíková et al., 2016) can be facilitated through direct interaction with β1 integrin and indirectly via α3, α6 integrin subunit interacting with CD151 (Lozahic et al., 2000; Kazarov et al., 2002).

Even though the primary CD151 location is indisputably in the sperm head equatorial region, it is interesting to consider the dynamic behaviour of this protein during the acrosome reaction (Jankovicova et al., 2020). We detected CD151 in the anterior inner acrosomal membrane, which defines the acroplaxome, overlaying the nucleus (Jankovicova et al., 2020). One can speculate that, due to massive relocation of many crucial proteins involved in sperm-egg membrane recognition and fusion, the CD151 tetraspanin network could also be actively remodelled during the acrosome reaction from

the posterior part of the acrosome equatorial segment to its apical part. CD151 is a member of the tetraspanin superfamily that possesses specific biological characteristics described in somatic cells. Our finding that this protein is expressed in male germ cells during spermatogenesis and remains localized in sperm during epididymal transport and ejaculation opens up a wide range of further possible investigations (Jankovicova et al., 2020). We have shown that CD151 can actively interact with the α6 integrin subunit in mouse sperm and based on structural modelling we proposed that the α6 integrin domain interacts with the CD151 protein in its extended conformation of this interaction (Jankovicova et al., 2020). It is interesting to note that CD151 protein localization was consistent between three mammalian species (mouse, bull and human), despite the previously reported species-specific differences for other sperm proteins including other tetraspanins, which highlights the conserved nature of CD151 protein prior to fertilization.

III. Sperm reproductive fitness is modified by pathological interventions

Infertility in the human population has become a major concern in many developed countries. According to the World Health Organization (WHO, 2010) up to 15% of couples in reproductive age suffer from infertility related problems, and male infertility is the primary or a contributing factor in 40% - 50 % of the cases (Kumar and Singh, 2015). As many studies now show, globally increasing environmental pollution, including pollutants with oestrogenic activity, can lead to decreased sperm quality and reproductive fitness, even in at extremely low concentrations (ng/ml) (Storgaard et al., 2005; Sebkova et al., 2012; Dostalova et al., 2017). Oestrogens used to be considered as predominantly female hormones, but they are also present in males where their main source of endogenous production lies in the testes. This male reproductive organ synthesises an enzyme called aromatase, which is responsible for the irreversible conversion of testosterone into oestrogens. Due to this fact, relatively high levels of oestrogens are present at the site of sperm production, and they are even higher than serum levels in females (Hess et al., 2013; Dostalova et al., 2017).

Oestrogens were not considered important in male reproduction, until it was shown that a gene disruption of the oestrogen receptor leads to decreased fertility in male mice (Lubahn et al., 1993). Oestrogen receptors are present on sperm of many species including human and mouse (Durkee et al., 1998, Aquila et al. 2004; Solakidi et al. 2005; Rago et al., 2006; Sebkova et al., 2012; Dostalova et al., 2017) and the importance of oestrogen signalling in male fertility is indicated by the adverse effects of certain oestrogen-like compounds which can interact with oestrogen receptors and cause pathologies. Our results filled in an interesting piece of the puzzle of how sperm maturation, measured by percentage of positive sperm head protein tyrosine phosphorylation, in the presence of oestrogens was increased, however the ability of these exposed sperm to undergo the acrosome reaction was significantly lowered in mouse (Sebkova et al., 2012), correlating with results from studies of human sperm (Baldi et al. 2000, Vigil et al. 2008). The progress of sperm maturation, called capacitation, is measured by an increase of protein tyrosine phosphorylation as a reliable indicator of crucial signalling pathway activity (Visconti et al., 1995a; Visconti et al., 1995b; Visconti et al., 1999; Diekman et al., 2002). Our study provided an evidence that oestrogens significantly stimulate the mouse sperm capacitation progress in a concentration-

dependent manner. Both natural and synthetical oestrogens, which represent a part ofthe environmental pollution, significantly elevated sperm head protein tyrosine phosphorylation, compared to the sperm capacitated without oestrogens. On the other hand, a presence of oestrogens resulted in the decreased number of acrosome-reacted sperm after the induced acrosome reaction, which is in contrast with physiological fertilization. It is alarming that premature sperm maturation did not result in natural ability of sperm to undergo the acrosome reaction, and ability to fertilize the egg, but instead blocked it, ultimately reducing the fertilization potential of these sperm (Sebkova et al, 2012).

Further data suggest that exogenous estrogenic compounds are even able to developmentally reprogram the sperm epigenome, with potential negative consequences for human health and male reproductive potential (Mirbahai et al., 2014; Zatecka et al., 2014). The epigenetic state of mature sperm is represented by histone modification and retention, protamine incorporation into the chromatin, DNA methylation, and range of RNAs which are delivered to the oocyte by sperm and thus influence embryo development and/or health of the offspring. Altered histone modifications, DNA methylation, improper histone to protamine replacement and small non-coding RNAs in sperm are factors important for male fertility and early embryogenesis and can influence the offsprings' health. For an example, the enrichment of histone 3 acetylated on lysine 9 (H3K9ac) was found in promoters of genes that play an important role in male fertility and its different genome distribution was noticed in infertile patients compared to fertile men (Steilmann et al., 2011). During spermiogenesis, there is global reprogramming when most of the DNA methylation marks are erased and most histones are replaced by protamines. Yet it is now established that epigenetic inheritance is transmitted via the male germ line (Soubry et al., 2014), including through regions of DNA which escape the global reprograming providing a basis for trans-generational epigenetic inheritance (Stouder at al., 2010). Moreover, aberrant methylation of promoters for specific genes (e.g. DAZL) and imprinted loci, are strongly associated with various forms of male infertility and sperm defects (Pacheco et al., 2011; Navarro-Costa et al., 2010). In addition, not all histones are replaced by protamines and some parts of DNA remain histone-associated in sperm. The most important amongst these are histone 2A and B (H2A and H2B), histone 3 (H3), histone 4 (H4), and the testes variant (tH2B) (Gatewood et al., 1990). It has been shown that 1% of rodent sperm DNA and up to 15% of human sperm DNA remains

associated with histones (Oliva et al., 2006), therefore certain histone marks are transmitted to the oocyte upon fertilization (Hammoud et al., 2009; Arpanahi et al., 2009; Vieweg et al., 2015). Our earlier study showed that histone H4 acetylated at lysine 12 (H4K12ac) is enriched in regulatory elements (CTCF binding sites) and in promoters of genes involved in developmental processes (Paradowska et al., 2012). H4K12ac-associated genes revealed a correlation with genes expressed in 8-cell embryo and in the blastocyst (Paradowska et al., 2012). Furthermore, we have delivered evidence that chromatin immunoprecipitation (ChIP) with anti-H4K12ac antibody revealed aberrant histone acetylation within developmentally important gene promoters in sub-fertile men (Vieweg et al., 2015) and it was suggested that histone acetylation could represent an epigenetic mark that is transmitted from sperm to oocyte and involved in the regulation of gene expression in the early embryo. Our studies (Paradowska et al., 2012; Vieweg et al., 2015) showed that aberrant histone acetylation, within developmentally important gene promoters in infertile/sub-fertile men may reflect insufficient sperm chromatin compaction and affect transfer of epigenetic information to the oocyte. In promoter of *NSD1* (nuclear receptor-binding SET domain protein 1, methyltransferase) gene a significant H4K12ac binding site depletion and significant decrease of DNA methylation in men with idiopathic infertility was observed (Vieweg et al., 2015). Interestingly, mutation of this gene causes Sotos syndrome which is slightly increased in children conceived by intracytoplasmic sperm injection (Nagai et al., 2003).

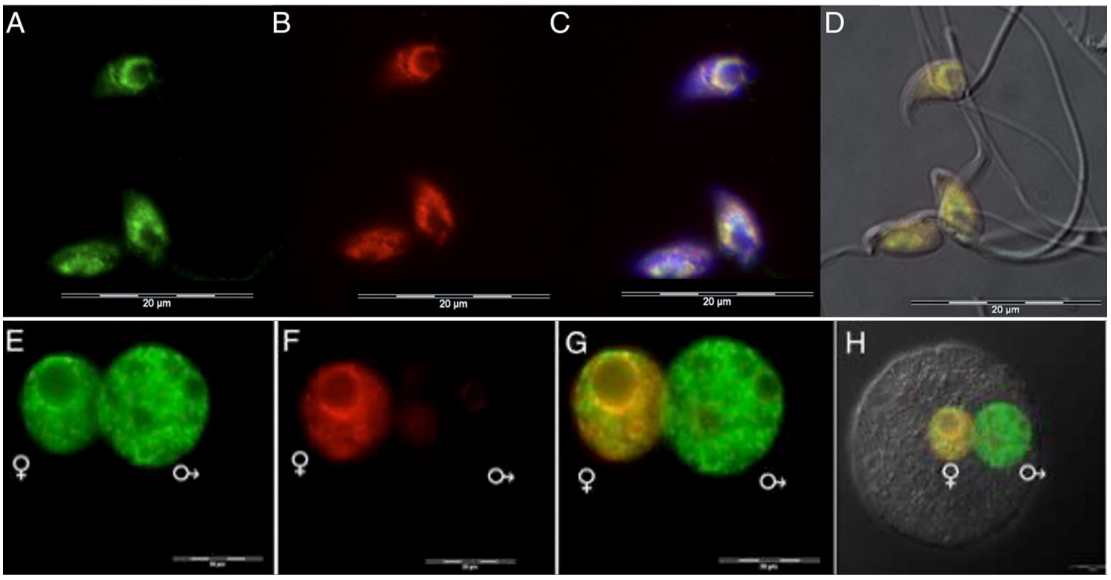

Figure 3. *Immunofluorescent labelling of H4K12ac and 5-methylcytosine (5mC) in mouse sperm nuclei shows that these epigenetic marks occupy the same compartment of*

the sperm nucleus, but during pronuclei fusion thepaternal pronucleus displays a strong acetylation (H4K12ac), but it is not methylated. Double stained spermatozoa with anti-H4K12ac antibody (green) (A), anti-5mC antibody (red) (B), merged with DAPI (C), and merged with DIC (D). Double stained mouse embryos pronucleus stage PN5 with antibodies anti-H4K12ac (green) (E), anti-5mC (red) (F), merged (G), merged with DIC (H). Adapted from Vieweg M *et al., 2015.*

DNA methylation is potent marker of epigenetic changes, and it was interesting to investigate if and how *Toxoplasma gondii* parasitic infection could modify its host's reproductive parameters through the epigenetic route, such as by altering the methylation of specific genes regulating spermatogenesis (Dvorakova-Hortova et al., 2014). Co-evolution of pathogens and eukaryotic cells enables the pathogen to utilize host cells to survive, replicate and escape the immune system (Angrisano et al., 2010). Pathogens can even modify the host cell immunological reaction by manipulating the epigenetic status of immune cells, which can lead to the development of a chronic infection (Paschos et al., 2010). Beside viruses and bacteria, micro-parasites, such as protozoa, as well as macro-parasites such as helminths and environmental factors (as discussed above) can trigger pathological changes in the epigenome of infected host cells. They can hyper or hypo-methylate specific key gene promoters including those responsible for ongoing spermatogenesis (Trasler et al., 2009, Rajender 2011). However, certain patho-epigenetic changes are reversible or avoidable if well understood, which is of major therapeutic importance (Minarovits et al., 2009).

Our results from quantification of testicular global DNA methylation revealed a significantly elevated DNA methylation in the *T. gondii* infected group of mice and gave the first indication that *T. gondii* is able to modify its host epigenome (Dvorakova-Hortova et al., 2014). DNA methylation representing an epigenetic modification of cytosine residues within CpG islands, which is associated with modulation of specific gene expression, was further tested for three genes (*Hsp1, Crem and Creb1*). These genes were carefully selected based on the data collected from the functional study which showed a diminished number of leptotene primary spermatocytes and spermatids, but in contrary the number of Sertoli cells and the tubule diameter were elevated in the *T. gondii* infected mice (Dvorakova-Hortova et al., 2014). We suspected that this drop in the germ cell number compared to the number of Sertoli cells is possibly caused by increased

apoptosis that is triggered by a decreased gonadotropin level. Elevated apoptosis of male germ cells in *T. gondii* infected mice was previously reported (Yang et al., 2006) and another study has shown (Marathe et al., 1995) that specific immunoneutralization of luteinising hormone (LH) causes apoptotic cell death of meiotic and post-meiotic germ cells in rat testis. In correlation with decreased quality of standard sperm parameters (motility, viability concentration) and morphometric data of testicular spermatogenesis, we also detected decreased of LH level supporting the hypothesis of ongoing pathological alterations in the spermatogenesis process in *T. gondii* infected males (Dvorakova-Hortova et al., 2014).

Decreased sperm motility in *T. gondii* infected individuals was reported (Zhou et al., 2003; Terpsidis et al., 2009). The epigenetically regulated *Hspa1* gene, which codes a testis specific heat shock cognate protein HSC70t expression, is required by post-meiotic germ cells for the assembly and function of protein complexes involved in energy production (Eddy et al., 2002). Therefore, the reported elevated Hspa1 gene methylation (Dvorakova-Hortova et al., 2014) may result in a lowered protein expression contributing to compromised sperm motility reported in *T. gondii* affected humans (Zhou et al., 2003) and rats (Terpsidis et al., 2009).

The CREM and CREB proteins are cyclically expressed at high levels during spermatogenesis in germinal and somatic Sertoli cells and correlate with the fluctuations in cAMP signalling, which is induced by the pituitary-gonadotropin hormones LH and FSH both during sexual maturation of testis and during the 12-day cycles of spermatogenesis that occur in adult testis (Walker et al., 1996). We suspected that our data reporting on decreased LH level in *T. gondii* infected mice (Dvorakova-Hortova et al., 2014) could possibly trigger misbalanced CREM and CREB protein expression via modification of cAMP signalling. However, the epigenetic modification of *Crem* or *Creb1* genes can also lead to abnormal protein transcription, resulting in modified signalling pathways, including of gonadotropins. A reduction of spermatogenesis and a decrease of sperm motility and quality was reported in correlation with abnormal *Crem* methylation (Nanassy and Carell., 2011). Moreover, it was shown that CREB is required to produce a Sertoli cell-derived factor that is critical for germ cell survival (Scobey et al., 2001) and increased apoptosis of meiotic and post-meiotic male germ cells is increased in the case of *Creb314*-deficient mice (Adham et al., 2005). CREM activators are expressed in post-meiotic haploid germ cells and are essential for spermatid maturation

(Behr et al., 2001). Our data showed the statistically significant decrease of *Crem* DNA methylation in one CpG position in the *T. gondii* group compared to the control group (Dvorakova-Hortova et al., 2014) . However, it remains to be clarified, whether the detected decreased methylation in *Crem* CpG in the specific position could lead to an abnormally increased expression of CREM protein and consequently to a disturbance of the spermatogenesis and spermatid maturation observed in immunohistochemical analysis of the *T. gondii* infected mouse testis (Dvorakova- Hortova et al., 2014). CREB proteins have the ability responses to various stress conditions and help maintain cellular homeostasis (Hai et al., 2001; Persengiev and Green, 2003). For this reason, their dynamic action during *T. gondii* infection may be suspected. In Sertoli cells, the level of CREB protein fluctuates regularly during the cycle of the seminiferous epithelium, and it is dependent on specific cell association during spermatogenesis (Don et al., 2002). CREB activity is regulated by phosphorylation in seminiferous tubules of the adult testis according to the spermatogenic cycle and can rapidly respond to the current state of the organism (Kim et al., 2009).

Our pilot study generated the first evidence that the overall sperm fitness of *T. gondii*-infected males is decreased in several tested parameters, but these mice were still fertile, indicating these effects could be compensated. A large-scale experiment with a greater number of animals and generations will be required to draw further conclusions. The communication between somatic and male germinal cells is crucial for their proliferation, differentiation and maturation and its regulation happens on several levels, including hormonal and epigenetic. Any disturbance of this fragile system is reflected in the abnormal phenotype, such as of testis morphology or fertility status to give few examples. Whether the reported changes in *T. gondii* infected mice are permanent or reversible, as well as whether they reflect a universal mechanism for *T. gondii* modification of its mammalian hosts, remains to be identified. These findings demonstrate a direct relation between *T. gondii* infection and the decrease of male reproductive fitness in mice, which may contribute to an increase of idiopathic infertility in humans. Based on the current knowledge, it seems fair to stress that understanding of the role of various epigenetic marks in sperm will lead to better diagnoses, and treatment of male infertility with potential to decrease the risk of developing diseases and thus be beneficial for the health and well-being of the next generations.

CONCLUSIONS AND FUTURE PERSPECTIVES

Recently, I have been considerably involved in translational research, as I have always envisioned putting the knowledge gained from scientific research into practical applications. I am investing my energy into the development of new diagnostic tools for identification of sperm parameters of relevance to fertilization capacity, and for the selection of sperm with intact and damaged acrosome to be used in Centres of Assisted Reproduction. Iam humbled to have the strong support of an investor and his team, with whom we are stepping into the wild waters of patent applications, regulations, and clinical trials. I would like to contribute to artificial fertilization by sperm selection in alliance with nature. Hand in hand on this chosen path I am recently focusing on spermatogenesis and sperm parameters assessment after pathological challenges including cancer.

My involvement in cancer research has developed due utilization of transgenic mouse strain C57BL/6^{Su9DsRed2}, expressing red fluorescent protein in mitochondria, which evolved into an invaluable tool for understanding cancer cell behaviour *in vivo*. Using this mouse strain, it was proven that intact mitochondria with its mtDNA payload are transferred into a developing tumour from the host animal, leading to normalization of mitochondrial respiration. In this manner, functional evidence was provided of an essential role of oxidative phosphorylation in cancer (Dong et al., *eLife*, 2017). Moreover, using the same model, it was later shown that pyrimidine biosynthesis dependent on respiration-linked dihydroorotate dehydrogenase (DHODH) is required to overcome cell-cycle arrest, while mitochondrial ATP generation is dispensable for tumorigenesis (Bajzlikova et al., *Cell Metab*, 2019). The translational potential of this publication lies ahead; it builds on the discovery that DHODH-driven pyrimidine biosynthesis is an essential pathway linking respiration to tumorigenesis, and points to inhibitors of DHODH as potential anti-cancer agents. The most recent achievement in this exciting collaboration brought me to examine the wound-healing activity of mesenchymal stem cells enhanced by platelets, which upon activation, transfer respiratory-competent mitochondria into mesenchymal stem cells via dynamin-dependent clathrin-mediated endocytosis. By combining *in vitro* and *in vivo* experiments including the C57BL/6^{Su9DsRed2} mouse strain, it was demonstrated that platelet-derived mitochondria promote the pro-angiogenic activity of mesenchymal stem cells via their capacity to enable metabolic remodelling (Levoux et al., *Cell Metab*, accepted).

All this experience provided me with important knowledge and the urge to initiate my own cancer research in the field of reproduction by including the topic of onco-fertility in cancer survivals. Currently, with my colleagues, medical doctors and financial support from the Czech Health Research Council, I am addressing the testicular germ cell tumour (TGCT) related sperm and testis germ cell pathologies from both experimental and clinical angles. TGCT represents the most common solid tumours and a relatively frequent cause of cancer-related death in young adult men of 18 - 35 years of age, and they present a significant burden in terms of morbidity and mortality in the population of men of reproductive age. The ultimate goal is to deliver novel diagnostic strategies for the evaluation of samples from TGCT patients prior to surgery and after the therapeutic intervention, in order to be able to monitor and compare the quality of fertility parameters and to recommend which cryopreserved semen should be used in programmes of assisted reproduction as the first choice. Thus, the outputs of the project should considerably improve the patients' chances of successful conception.

I find deep satisfaction in combining basic and applied science, because I feel like the trust and financial support given to us as scientists by the public can pay off and help the community at large to progress. I would like to carry on and contribute to increase the chance of healthy progeny with novel diagnostic and selection tools designed for assisted reproduction as well as to contribute with targeted diagnostics and possibly treatment for cancer patients.

REFERENCES

Personal publications

Bajzikova M, Kovarova J, Coelho AR, Boukalova S, Oh S, Rohlenova K, Svec D, Hubackova S, Endaya B, Judasova K, Bezawork-Geleta A, Kluckova K, Chatre L, Zobalova R, Novakova A, Vanova K, Ezrova Z, Maghzal GJ, Magalhaes Novais S, Olsinova M, Krobova L, An YJ, Davidova E, Nahacka Z, Sobol M, Cunha-Oliveira T, Sandoval-Acuña C, Strnad H, Zhang T, Huynh T, Serafim TL, Hozak P, Sardao VA, Koopman WJH, Ricchetti M, Oliveira PJ, Kolar F, Kubista M, Truksa J, Dvorakova-Hortova K, Pacak K, Gurlich R, Stocker R, Zhou Y, Berridge MV, Park S, Dong L, Rohlena J, Neuzil J (2019) Reactivation of Dihydroorotate Dehydrogenase-Driven Pyrimidine Biosynthesis Restores Tumor Growth of Respiration-Deficient Cancer Cells. *Cell Metab* 29(2), 399-416.

Clift LE, Andrlikova P, Frolikova M, Stopka P, Bryja J, Flanagan BF, Johnson PM, Dvorakova-Hortova K (2009) Absence of spermatozoal CD46 protein expression and associated rapid acrosome reaction rate in striped field mice (Apodemus agrarius). *Reprod Biol Endocrinol* 7: 29.

Dong LF, Kovarova J, Bajzikova M, Bezawork-Geleta A, Svec D, Endaya B, Schaphibulkij K, Coelho A, Sebkova N, Ruzickova A, Tan AS, Kluckova K, Judasova K, Zamecnikova K, Rychtarcikova Z, Gopalan V, Sobol M, Yan B, Pattnaik B, Bhatraju N, Truksa J, Stopka P, Lam A, Sedlacek R, Oliveira P, Kubista M, Agarwal A, Dvorakova-Hortova K, Rohlena J, Berridge MV,Neuzil J (2017) Horizontal transfer of whole mitochondria recovers tumorigenic potential in mtDNA-deficient cells. *eLife* 6, e22187.

Dostalova P, Zatecka E, Dvorakova-Hortova K (2017) Of Oestrogens and Sperm: A Review of the Roles of Oestrogens and Oestrogen Receptors in Male Reproduction. *Int J Mol Sci* 18(5).

Dvoráková K, Moore HD, Sebková N, Palecek J (2005) Cytoskeleton localization in the sperm head prior to fertilization. *Reproduction* 130(1), 61-9.

Dvorakova-Hortova K, Sidlova A, Ded L, Hladovcova D, Vieweg M, Steger K, Stopka P, Paradowska-Dogan A (2014) Toxoplasma gondii decreases the reproductive fitness in mice. *PLoS One*, 9 (6), e96770.

Frolikova M, Sebkova N, Ded L, Dvorakova-Hortova K (2016) Characterization of CD46 and β1 integrin dynamics during sperm acrosome reaction. *Sci Rep* 6, 33714.

Frolikova M, Manaskova-Postlerova P, Cerny J, Jankovicova J, Simonik O, Pohlova A, Secova P, Antalikova J, Dvorakova-Hortova K (2018) CD9 and CD81 Interactions and Their Structural Modelling in Sperm Prior to Fertilization. *Int J Mol Sci*. 19;19(4):1236.

Frolikova M, Valaskova E, Cerny J, Lumeau A, Sebkova N, Palenikova V, Sanches-Hernandez N, Pohlova A, Manaskova-Postlerova P, Dvorakova-Hortova K (2019) Addressing the Compartmentalization of Specific Integrin Heterodimers in Mouse Sperm. *Int J Mol Sci* 26;20(5):1004.

Jankovicova J, Frolikova M, Sebkova N, Simon M, Cupperova P, Lipcseyova D, Michalkova K, Horovska L, Sedlacek R, Stopka P, Antalikova J, Dvorakova-Hortova K (2016)Characterization of tetraspanin protein CD81 in mouse spermatozoa and bovine gametes. *Reproduction* 152(6), 785-793.

Jankovicova J, Frolikova M, Palenikova V, Valaskova E, Cerny J, Secova P, Bartokova M, Horovska L, Manaskova-Postlerova P, Antalikova J, Komrskova K (2020) Expression and distribution of CD151 as a partner of alpha6 integrin in male germ cells. *Sci Rep* 10, 4374.

Johnson PM, Clift LE, Andrlikova P, Jursova M, Flanagan BF, Cummerson JA, Stopka P, Dvorakova-Hortova K (2007) Rapid sperm acrosome reaction in the absence of acrosomal CD46 expression in promiscuous field mice (Apodemus). *Reproduction* 134(6), 739-747.

Levoux J, Prola A, Lafuste P, Gervais M, Chevallier N, Koumaiha Z, Kefi K, Braud L, Schmitt A, Yacia A, Schirmann A, Ben Larbi S, Komrskova K, Rohlena J, Relaix F, Neuzil J, Rodriguez AM Platelets enhance pro-angiogenic activity of mesenchymal stem cells via mitochondrial transfer and metabolic remodeling. *Cell Metab*, Dec 24:S1550-4131(20)30661-6. Epub ahead of print.

Moore H, Dvoráková K, Jenkins N, Breed W (2002) Exceptional sperm cooperation in the wood mouse. *Nature* 418(6894), 174-7.

Paradowska AS, Miller D, Spiess AN, Vieweg M, Cerna M, Dvorakova-Hortova K, Bartkuhn M, Schuppe HC, Weidner W, Steger K (2012) Genome wide identification of promoter binding sites for H4K12ac in human sperm and its relevance for early embryonic development. *Epigenetics* 7(9), 1057-1070.

Sebkova N, Cerna M, Ded L, Peknicova J, Dvorakova-Hortova K (2012) The slower the better: how sperm capacitation and acrosome reaction is modified in the presence of estrogens. *Reproduction* 143(3), 297-307.

Sebkova N, Ded L, Vesela K, Dvorakova-Hortova K (2013) Progress of sperm IZUMO1 relocation during spontaneous acrosome reaction. *Reproduction* 147(2), 231-40.

Vieweg M, Dvorakova-Hortova K, Dudkova B, Waliszewski P, Otte M, Oels B, Hajimohammad A, Turley H, Schorsch M, Schuppe HC, Weidner W, Steger K, Paradowska-Dogan A (2015) Methylation analysis of histone H4K12ac-associated promoters in sperm of healthy donors and subfertile patients. *Clin Epigenetics* 7(1): 31.

External references

Adham IM, Eck TJ, Mierau K, Müller N, Sallam MA, Paprotta I, Schubert S, Hoyer-Fender S, Engel W (2005) Reduction of spermatogenesis but not fertility in Creb3l4-deficient mice. *Mol Cell Biol* 25(17), 7657-7664.

Adham IM, Eck TJ, Mierau K, Müller N, Sallam MA, Paprotta I, Schubert S, Hoyer-Fender S, Engel W (2010) LPS-induced IL-8 activation in human intestinal epithelial cells is accompanied by specific histone H3 acetylation and methylation changes. *BMC Microbiol* 10, 172.

Aquila S, Sisci D, Gentile M, Middea E, Catalano S, Carpino A, Rago V, Andò S (2004) Estrogen receptor (ER)alpha and ER beta are both expressed in human ejaculated spermatozoa: evidence of their direct interaction with phosphatidylinositol-3-OH kinase/Akt pathway. *J Clin Endocrinol Metab* 89(3), 1443-1451.

Arpanahi A, Brinkworth M, Iles D, Krawetz SA, Paradowska A, Platts AE, Saida M, Steger K, Tedder P, Miller D (2009) Endonuclease-sensitive regions of human spermatozoal chromatin are highly enriched in promoter and CTCF binding sequences. *Genome Res* 19(8), 1338-1349.

Baldi E, Luconi M, Bonaccorsi L, Forti G (2002) Signal transduction pathways in human spermatozoa. *J Reprod Immunol* 53(1-2), 121-131.

Baker RR, Bellis MA (1987) 'Kamikaze' sperm in mammals? *Animal Behaviour* 36, 936–938.

Barraud-Lange V, Naud-Barriant N, Saffar L, Gattegno L, Ducot B, Drillet AS, Bomsel M, Wolf JP, Ziyyat A (2007) Alpha6beta1 integrin expressed by sperm is determinant in mouse fertilization. *BMC Dev Biol* 7, 102.

Barraud-Lange V, Boucheix C (2013) The Role of Tetraspanin Complexes in Egg-Sperm Fusion. In: Berditchevski F., Rubinstein E. (eds) Tetraspanins. Proteins and Cell Regulation. *Springer* vol 9, Dordrecht, 203–231.

Behr R, Weinbauer GF (2001) cAMP response element modulator (CREM): an essential factor for spermatogenesis in primates? *Int J Androl* 24(3), 126-135.

Birkhead TR, Moller AP (1996) Sperm Competition and Sexual Selection. *Academic*, London, ISBN 9780121005436

Breitbart H, Cohen G, Rubinstein S (2005) Role of actin cytoskeleton in mammalian sperm capacitation and the acrosome reaction. *Reproduction* 129(3), 263-268.

Bryja J, Stopka P (2005) Facultative promiscuity in a presumably monogamous mouse *Apodemus microps*. *Acta Theriol* 50, 189–196.

Colburn ZT, Jones JC (2017) α(6)β(4) Integrin Regulates the Collective Migration of Epithelial Cells. *Am J Respir Cell Mol Biol* 56(4), 443-452.

Cummerson JA, Flanagan BF, Spiller DG, Johnson PM (2006) The complement regulatory proteins CD55 (decay accelerating factor) and CD59 are expressed on the inner acrosomal membrane of human spermatozoa as well as CD46 (membrane cofactor protein). *Immunology* 118(3), 333-342.

Don J, Stelzer G (2002) The expanding family of CREB/CREM transcription factors that are involved with spermatogenesis. *Mol Cell Endocrinol* 187(1-2), 115-124.

Durkee TJ, Mueller M, Zinaman M (1998) Identification of estrogen receptor protein and messenger ribonucleic acid in human spermatozoa. *Am J Obstet Gynecol* 178(6), 1288-1297.

Eddy EM (2002) Male germ cell gene expression. *Recent Prog Horm Res* 57, 103-128.

Finkelstein M, Etkovitz N, Breitbart H (2020) Ca(2+) signaling in mammalian spermatozoa. *Mol Cell Endocrinol* 516, 110953.

Finkelstein M, Megnagi B, Ickowicz D, Breitbart H.(2013) Regulation of sperm motility by PIP2(4,5) and actin polymerization. *Dev Biol* 381(1), 62-72.

Fisher HS, Hoekstra HE (2010) Competition drives cooperation among closely related sperm of deer mice. *Nature* 463(7282), 801-803.

Fisher H, Jacobs-Palmer E, Lassance JM (2016) The genetic basis and fitness consequences of sperm midpiece size in deer mice. *Nat Commun* 7,13652.

Gatewood JM, Cook GR, Balhorn R, Schmid CW, Bradbury EM (1990) Isolation of four core histones from human sperm chromatin representing a minor subset of somatic histones. *J Biol Chem* 265(33), 20662-20666.

Göb E, Schmitt J, Benavente R, Alsheimer M (2010) Mammalian sperm head formation involves different polarization of two novel LINC complexes. *PLoS One* 5(8), e12072.

Guyonnet B, Zabet-Moghaddam M, SanFrancisco S, Cornwall GA (2012) Isolation and proteomic characterization of the mouse sperm acrosomal matrix. *Mol Cell Proteomics* 11(9), 758-774.

Hai T, Hartman MG (2001) The molecular biology and nomenclature of the activating transcription factor/cAMP responsive element binding family of transcription factors: activating transcription factor proteins and homeostasis. *Gene* 273(1), 1-11.

Hammoud SS, Nix DA, Zhang H, Purwar J, Carrell DT, Cairns BR (2009) Distinctive chromatin in human sperm packages genes for embryo development. *Nature* 460(7254), 473-478.

Hemler ME, Crouse C, Sonnenberg A (1989) Association of the VLA alpha 6 subunit with a novel protein. A possible alternative to the common VLA beta 1 subunit on certain cell lines. *J Biol Chem* 264(11), 6529-6535.

Hess RA (2003) Estrogen in the adult male reproductive tract: a review. *Reprod Biol Endocrinol* 1, 52.

Higginson DM, Pitnick S (2011) Evolution of intra-ejaculate sperm interactions: do sperm cooperate? *Biol Rev Camb Philos Soc* 86(1), 249-270.

Hynes RO (2002) Integrins: bidirectional, allosteric signaling machines. *Cell* 110(6), 673-687.

Immler S (2008) Sperm competition and sperm cooperation: the potential role of diploid and haploid expression. *Reproduction* 135(3), 275-283.

Inoue N, Ikawa M, Isotani A, Okabe M (2005) The immunoglobulin superfamily protein Izumo is required for sperm to fuse with eggs. *Nature* 434(7030), 234-238.

Inoue N, Ikawa M, Nakanishi T, Matsumoto M, Nomura M, Seya T, Okabe M (2003) Disruption of mouse CD46 causes an accelerated spontaneous acrosome reaction in sperm. *Mol Cell Biol* 23(7), 2614-2622.

Inoue N, Ikawa M, Okabe M (2011) The mechanism of sperm-egg interaction and the involvement of IZUMO1 in fusion. *Asian J Androl* 13(1), 81-87.

Inoue N, Satouh Y, Ikawa M, Okabe M, Yanagimachi R (2011) Acrosome-reacted mouse spermatozoa recovered from the perivitelline space can fertilize other eggs. *Proc Natl Acad Sci USA* 108(50), 20008-20011.

Ito C, Yamatoya K, Yoshida K, Maekawa M, Miyado K, Toshimori K (2010) Tetraspanin family protein CD9 in the mouse sperm: unique localization, appearance, behavior and fate during fertilization. *Cell Tissue Res* 340(3), 583-594.

Jin M, Fujiwara E, Kakiuchi Y, Okabe M, Satouh Y, Baba SA, Chiba K, Hirohashi N (2011) Most fertilizing mouse spermatozoa begin their acrosome reaction before contact with the zona pellucida during in vitro fertilization. *Proc Natl Acad Sci USA* 108(12), 4892-4896.

Kaji K, Oda S, Shikano T, Ohnuki T, Uematsu Y, Sakagami J, Tada N, Miyazaki S, Kudo A (2000) The gamete fusion process is defective in eggs of Cd9-deficient mice. *Nat Genet* 24(3), 279-282.

Kajiji S, Tamura RN, Quaranta V (1989) A novel integrin (alpha E beta 4) from human epithelial cells suggests a fourth family of integrin adhesion receptors. *Embo j* 8(3), 673-680.

Kallstrom H, Islam M, Berggren PO, Jonsson AB (1998) Cell signalling by the type IV pili of pathogenic Neisseria. *J. Biol. Chem*. 273, 21777–21782.

Kazarov AR, Yang X, Stipp CS, Sehgal B, Hemler ME (2002) An extracellular site on tetraspanin CD151 determines alpha 3 and alpha 6 integrin-dependent cellular morphology. *J Cell Biol* 158(7), 1299-1309.

Kierszenbaum AL, Rivkin E, Tres LL (2007) Molecular biology of sperm head shaping. *Soc Reprod Fertil Suppl* 65, 33-43.

Kim JS, Song MS, Seo HS, Yang M, Kim SH, Kim JC, Kim H, Saito TR, Shin T, Moon C (2009) Immunohistochemical analysis of cAMP response element-binding protein in mouse testis during postnatal development and spermatogenesis. *Histochem Cell Biol* 131(4), 501-507.

Kumar N, Singh AK (2015) Trends of male factor infertility, an important cause of infertility: A review of literature. *J Hum Reprod Sci*8(4), 191-196.

Kura T, Nakashima Y (2000) Conditions for the evolution of soldier sperm classes. *Evolution* 54(1), 72-80.

Kurita-Taniguchi M, Hazeki K, Murabayashi N, Fukui A, Tsuji S, Matsumoto M, Toyoshima K, Seya T (2002). Molecular assembly of CD46 with CD9, alpha3-beta1 integrin and protein tyrosine phosphatase SHP-1 in human macrophages through differentiation by GM-CSF. *Mol Immunol* 38(9), 689-700.

Le Naour F, Rubinstein E, Jasmin C, Prenant M, Boucheix C (2000) Severely reduced female fertility in CD9-deficient mice. *Science* 287(5451), 319-321.

Lehmann L, Keller L (2006) The evolution of cooperation and altruism--a general framework and a classification of models. *J Evol Biol* 19(5), 1365-1376.

Liu L, He B, Liu WM, Zhou D, Cox JV, Zhang XA (2007) Tetraspanin CD151 promotes cell migration by regulating integrin trafficking. *J Biol Chem* 282(43), 31631-31642.

Lozahic S, Christiansen D, Manié S, Gerlier D, Billard M, Boucheix C, Rubinstein E (2000) CD46 (membrane cofactor protein) associates with multiple beta1 integrins and tetraspans. *Eur J Immunol* 30(3), 900-907.

Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O (1993) Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. *Proc. Natl. Acad. Sci. USA* 90, 11162–11166.

Machtinger R, Laurent LC, Baccarelli AA (2016) Extracellular vesicles: roles in gamete maturation, fertilization and embryo implantation. *Hum Reprod Update* 22(2), 182-193.

Mainiero F, Pepe A, Wary KK, Spinardi L, Mohammadi M, Schlessinger J, Giancotti FG (1995) Signal transduction by the alpha 6 beta 4 integrin: distinct beta 4 subunit sites mediate recruitment of Shc/Grb2 and association with the cytoskeleton of hemidesmosomes. *Embo j* 14(18), 4470-4481.

Marathe CK, Shetty J, Dighe RR (1995) Selective immunoneutralization of luteinizing hormone results in the apoptotic cell death of pachytene spermatocytes and spermatids in the rat testis. *Endocrine* 3(10), 705-709.

McDonough CE, Whittington E, Pitnick S, Dorus S (2016) Proteomics of reproductive systems: Towards a molecular understanding of postmating, prezygotic reproductive barriers. *J Proteomics* 135, 26-37.

Minárovits J. (2009) Microbe-induced epigenetic alterations in host cells: the coming era of patho-epigenetics of microbial infections. A review. Acta *Microbiol Immunol Hung* 56(1), 1-19.

Miyado K, Yamada G, Yamada S, Hasuwa H, Nakamura Y, Ryu F, Suzuki K, Kosai K, Inoue K, Ogura A, Okabe M, Mekada E (2000) Requirement of CD9 on the egg plasma membrane for fertilization. *Science* 287(5451), 321-324.

Nagai T, Matsumoto N, Kurotaki N, Harada N, Niikawa N, Ogata T, Imaizumi K, Kurosawa K, Kondoh T, Ohashi H, Tsukahara M, Makita Y, Sugimoto T, Sonoda T, Yokoyama T, Uetake K, Sakazume S, Fukushima Y, Naritomi K (2003) Sotos syndrome and haploinsufficiency of NSD1: clinical features of intragenic mutations and submicroscopic deletions. *J Med Genet* 40(4), 285-289.

Nanassy L, Carrell DT (2011) Abnormal methylation of the promoter of CREM is broadly associated with male factor infertility and poor sperm quality but is improved in sperm selected by density gradient centrifugation. *Fertil Steril* 95(7), 2310-2314.

Navarro-Costa P, Nogueira P, Carvalho M, Leal F, Cordeiro I, Calhaz-Jorge C, Gonçalves J, Plancha CE (2010) Incorrect DNA methylation of the DAZL promoter CpG island associates with defective human sperm. *Hum Reprod* 25(10), 2647-2654.

Ohnami N, Nakamura A, Miyado M, Sato M, Kawano N, Yoshida K, Harada Y, Takezawa Y, Kanai S, Ono C, Takahashi Y, Kimura K, Shida T, Miyado K, Umezawa A (2012) CD81 and CD9 work independently as extracellular components upon fusion of sperm and oocyte. *Biol Open* 1(7), 640-647.

Oliva, R. (2006). Protamines and male infertility. *Hum Reprod Update* 12(4), 417-435.

Parker GA, Pizzari T (2010) Sperm competition and ejaculate economics. *Biol Rev Camb Philos Soc* 85(4), 897-934.

Paschos K, Allday MJ (2010) Epigenetic reprogramming of host genes in viral and microbial pathogenesis. *Trends Microbiol* 18(10), 439-447.

Persengiev SP, Green MR (2003) The role of ATF/CREB family members in cell growth, survival and apoptosis. *Apoptosis* 8(3), 225-228.

Pizzari T, Foster KR (2008) Sperm sociality: cooperation, altruism, and spite. *PLoS Biol* 6(5): e130.

Rago V, Siciliano L, Aquila S, Carpino A (2006) Detection of estrogen receptors ER-alpha and ER-beta in human ejaculated immature spermatozoa with excess residual cytoplasm. *Reprod Biol Endocrinol* 4: 36.

Rajender S, Avery K, Agarwal A (2011) Epigenetics, spermatogenesis and male infertility. *Mutat Res* 727(3), 62-71.

Ramírez-Ramírez D, Salgado-Lucio ML, Roa-Espitia AL, Fierro R, González-Márquez H, Cordero-Martínez J, Hernández-González EO (2020). Rac1 is necessary for capacitation and acrosome reaction in guinea pig spermatozoa. *J Cell Biochem* 121(4), 2864-2876.

Rezcallah MS, Hodges K, Gill DB, Atkinson JP, Wang B, Cleary PP (2005) Engagement of CD46 and alpha5beta1 integrin by group A streptococci is required for efficient invasion of epithelial cells. *Cell Microbiol* 7(5), 645-653.

Riley RC, Kemper C, Leung M, Atkinson JP (2002) Characterization of human membrane cofactor protein (MCP; CD46) on spermatozoa. *Mol Reprod Dev* 62(4), 534-546.

Rubinstein E, Ziyyat A, Prenant M, Wrobel E, Wolf JP, Levy S, Le Naour F, Boucheix C (2006) Reduced fertility of female mice lacking CD81. *Dev Biol* 290(2), 351-358.

Rubinstein E, Ziyyat A, Wolf JP, Le Naour F, Boucheix C (2006) The molecular players of sperm-egg fusion in mammals. *Semin Cell Dev Biol* 17(2), 254-263.

Sadej R, Grudowska A, Turczyk L, Kordek R, Romanska HM (2014) CD151 in cancer progression and metastasis: a complex scenario. *Lab Invest* 94(1), 41-51.

Satouh Y, Inoue N, Ikawa M, Okabe M (2012) Visualization of the moment of mouse sperm-egg fusion and dynamic localization of IZUMO1. *J Cell Sci* 125(Pt 21), 4985-4990.

Scobey M, Bertera S, Somers JP, Watkins SC, Zeleznik AJ (2001) Delivery of a cyclic adenosine 39,59-monophosphate response element-binding protein (creb) mutant to seminiferous tubules results in impaired spermatogenesis. *Endocrinology* 142, 948–954.

Simon-Assmann P, Duclos B, Orian-Rousseau V, Arnold C, Mathelin C, Engvall E, Kedinger M (1994) Differential expression of laminin isoforms and alpha 6-beta 4 integrin subunits in the developing human and mouse intestine. *Dev Dyn* 201(1), 71-85.

Solakidi S, Psarra AM, Nikolaropoulos S, Sekeris CE (2005) Estrogen receptors alpha and beta (ERalpha and ERbeta) and androgen receptor (AR) in human sperm: localization of ERbeta and AR in mitochondria of the midpiece. *Hum Reprod* 20(12), 3481-3487.

Sosnik J, Miranda PV, Spiridonov NA, Yoon SY, Fissore RA, Johnson GR, Visconti PE (2009) Tssk6 is required for Izumo relocalization and gamete fusion in the mouse. *J Cell Sci* 122(Pt 15), 2741-2749.

Soubry A, Hoyo C, Jirtle RL, Murphy SK (2014) A paternal environmental legacy: evidence for epigenetic inheritance through the male germ line. *Bioessays* 36(4), 359-371.

Spiess M, Hernandez-Varas P, Oddone A, Olofsson H, Blom H, Waithe D, Lock JG, Lakadamyali M, Strömblad S (2018) Active and inactive β1 integrins segregate into distinct nanoclusters in focal adhesions. *J Cell Biol* 217(6), 1929-1940.

Spinardi L, Ren YL, Sanders R, Giancotti F (1993) The beta 4 subunit cytoplasmic domain mediates the interaction of alpha 6 beta 4 integrin with the cytoskeleton of hemidesmosomes. *Mol Biol Cell* 4(9), 871-884.

Steilmann C, Paradowska A, Bartkuhn M, Vieweg M, Schuppe HC, Bergmann M, Kliesch S, Weidner W, Steger K (2011) Presence of histone H3 acetylated at lysine 9 in male germ cells and its distribution pattern in the genome of human spermatozoa. *Reprod Fertil Dev*. 23(8), 997-1011.

Sterk LM, Geuijen CA, Oomen LC, Calafat J, Janssen H, Sonnenberg A (2000) The tetraspan molecule CD151, a novel constituent of hemidesmosomes, associates with the integrin alpha6beta4 and may regulate the spatial organization of hemidesmosomes. *J Cell Biol* 149(4), 969-982.

Stopka P, Macdonald DW (1998) Signal interchange during mating in the wood mouse (*Apodemus sylvaticus*): the concept of active and passive signalling. *Behaviour* 135, 231–249.

Stopka P, Macdonald DWn(1999) The market effect in the wood mouse *Apodemus sylvaticus*: selling information on reproductive status. *Ethology* 105, 969–982.

Storgaard L, Bonde JP, Olsen J (2006) Male reproductive disorders in humans and prenatal indicators of estrogen exposure. A review of published epidemiological studies. *Reprod Toxicol* 21(1), 4-15.

Stouder C, Paoloni-Giacobino A (2010) Transgenerational effects of the endocrine disruptor vinclozolin on the methylation pattern of imprinted genes in the mouse sperm. *Reproduction* 139(2), 373-379.

Tavarna T, Phillips PL, Wu XJ, Reyes L (2020) Fetal growth restriction is a host specific response to infection with an impaired spiral artery remodeling-inducing strain of Porphyromonas gingivalis. *Sci Rep* 10(1), 14606.

Te Molder L, Juksar J, Harkes R, Wang W, Kreft M, Sonnenberg A (2019) Tetraspanin CD151 and integrin α3β1 contribute to the stabilization of integrin α6β4-containing cell-matrix adhesions. *J Cell Sci* 132(19).

Terpsidis KI, Papazahariadou MG, Taitzoglou IA, Papaioannou NG, Georgiadis MP (2009) Toxoplasma gondii: reproductive parameters in experimentally infected male rats. *Exp Parasitol* 121(3), 238-241.

Tew T, Macdonald D (1994) Dynamics of space use and male vigour amongst wood mice, *Apodemus sylvaticus*, in the cereal ecosystem. *Behav Ecol Sociobiol* 34, 337–345.

Trivers RL (1971) The evolution of reciprocal altruism. *The Quarterly Review of Biology* 46, 35–57.

Vigil P, Toro A, Godoy A (2008) Physiological action of oestradiol on the acrosome reaction in human spermatozoa. *Andrologia* 40(3), 146-151.

Visconti PE, Bailey JL, Moore GD, Pan D, Olds-Clarke P, Kopf GS (1995a) Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. *Development* 121, 1129–1137.

Visconti PE, Moore GD, Bailey JL, Leclerc P, Connors SA, Pan D, Olds-Clarke P, Kopf GS (1995b) Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. *Development* 121(4), 1139-1150.

Visconti PE, Ning X, Fornés MW, Alvarez JG, Stein P, Connors SA, Kopf GS (1999) Cholesterol efflux-mediated signal transduction in mammalian sperm: cholesterol release signals an increase in protein tyrosine phosphorylation during mouse sperm capacitation. *Dev Biol* 214(2), 429-443.

Visconti PE, Westbrook VA, Chertihin O, Demarco I, Sleight S, Diekman AB (2002) Novel signaling pathways involved in sperm acquisition of fertilizing capacity. *J Reprod Immunol* 53(1-2), 133-150.

Walker WH, Habener JF (1996) Role of transcription factors CREB and CREM in cAMP-regulated transcription during spermatogenesis. *Trends Endocrinol Metab* 7(4), 133-138.

Wigby S, Suarez SS, Lazzaro BP, Pizzari T, Wolfner MF (2019) Sperm success and immunity. *Curr Top Dev Biol* 135, 287-313.

Winterwood NE, Varzavand A, Meland MN, Ashman LK, Stipp CS (2006) A critical role for tetraspanin CD151 in alpha3beta1 and alpha6beta4 integrin-dependent tumor cell functions on laminin-5. *Mol Biol Cell* 17(6), 2707-2721.

Wolf DE, McKinnon CA, Leyton L, Loveland KL, Saling PM (1992) Protein dynamics in sperm membranes: implications for sperm function during gamete interaction. *Mol Reprod Dev* 33(2), 228-234.

World Health Organization (WHO) (2010) Laboratory manual for the examination and processing of human semen. 5th edition, ISBN: 978 92 4 154778 9.

Wong TC, Yant S, Harder BJ, Korte-Sarfaty J, Hirano A (1997) The cytoplasmic domains of complement regulatory protein CD46 interact with multiple kinases in macrophages. *J Leukoc Biol* 62(6), 892-900.

Yanagimachi R (1994) Mammalian fertilization. *Physiology of Reproduction*, 189–317.

Yazlovitskaya EM, Viquez OM, Tu T, De Arcangelis A, Georges-Labouesse E, Sonnenberg A, Pozzi A, Zent R (2019) The laminin binding α3 and α6 integrins cooperate to promote epithelial cell adhesion and growth. *Matrix Biol* 77, 101-116.

Yang R, Hou Y, Zhao J, Liu Z, Zhang Q (2006) Effect of Toxoplasma Infection on the Apoptosis of Sperm in Mice. *Chinese Journal of Zoonoses*, 2006–11.

Zaffran Y, Destaing O, Roux A, Ory S, Nheu T, Jurdic P, Rabourdin-Combe C, Astier AL (2001) CD46/CD3 costimulation induces morphological changes of human T cells and activation of Vav, Rac, and extracellular signal-regulated kinase mitogen-activated protein kinase. *J Immunol* 167(12), 6780-6785.

Zatecka E, Ded L, Elzeinova F, Kubatova A, Dorosh A, Margaryan H, Dostalova P, Korenkova V, Hoskova K, Peknicova J (2014) Effect of zearalenone on reproductive parameters and expression of selected testicular genes in mice. *Reprod Toxicol* 45, 20-30.

Zhou Y, Lu Y, Hu Y (2003) Experimental study of influence of Toxoplasma tachyzoites on human sperm motility parameters in vitro. *Chinese Journal of Zoonoses*, 2003–04.